Genetic Manipulation of Carotenoid Biosynthesis in the Green Sulfur Bacterium Chlorobium tepidum†

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Received 16 March 2004/Accepted 14 May 2004

The green sulfur bacterium Chlorobium tepidum is a strict anaerobe and an obligate photoautotroph. On the basis of sequence similarity with known enzymes or sequence motifs, nine open reading frames encoding putative enzymes of carotenoid biosynthesis were identified in the genome sequence of C. tepidum, and all nine genes were inactivated. Analysis of the carotenoid composition in the resulting mutants allowed the genes encoding the following six enzymes to be identified: phytoene synthase (crtB/CT1386), phytoene desaturase (crtP/CT0807), \( \xi \)-carotene desaturase (crtQ/CT1414), \( \gamma \)-carotene desaturase (crtU/CT0323), carotenoid \( 1',2' \)-hydratase (crtC/CT0301), and carotenoid cis-trans isomerase (crtH/CT0649). Three mutants (CT0180, CT1357, and CT1416 mutants) did not exhibit a discernible phenotype. The carotenoid biosynthetic pathway in \( C. \) tepidum is similar to that in cyanobacteria and plants by converting phytoene into lycopene using two plant-like desaturases (CrtP and CrtQ) and a plant-like cis-trans isomerase (CrtH) and thus differs from the pathway known in all other bacteria. In contrast to the situation in cyanobacteria and plants, the construction of a \( ctb \) mutant completely lacking carotenoids demonstrates that carotenoids are not essential for photosynthetic growth of green sulfur bacteria. However, the bacteriochlorophyll \( a \) contents of mutants lacking colored carotenoids (crtB, crtP, and crtQ mutants) were decreased from that of the wild type, and these mutants exhibited a significant growth rate defect under all light intensities tested. Therefore, colored carotenoids may have both structural and photoprotection roles in green sulfur bacteria. The ability to manipulate the carotenoid composition so dramatically in \( C. \) tepidum offers excellent possibilities for studying the roles of carotenoids in the light-harvesting chlorosome antenna and iron-sulfur-type (photosystem I-like) reaction center. The phylogeny of carotenogenic enzymes in green sulfur bacteria and green filamentous bacteria is also discussed.

Carotenoids are synthesized by all photosynthetic organisms and by many nonphotosynthetic microorganisms (7, 12). Carotenoids are also important nutritional supplements for an even wider range of organisms, including animals and humans. In photosynthetic organisms, carotenoids function in light harvesting, photoprotection, and in some systems, structure stabilization (18). The light-harvesting function involves absorption of blue light with wavelengths from 400 to 550 nm and transfer of excitation energy to chlorophylls (Chls) or bacteriochlorophylls (BChls) via singlet states (18). Photoprotection is conferred by carotenoids by their ability to quench (B)Chl triplet states and to scavenge singlet oxygen and harmful radicals. Finally, carotenoids appear to serve a structural role in assembly and stabilization of some pigment-protein complexes in purple bacteria and plants (46).

The first committed step in the biosynthesis of \( C_{40} \) carotenoids is the head-to-head condensation of two \( C_{20} \) geranylgeranyl diphosphate molecules by phytoene synthase to form phytoene (7, 12). Phytoene is then converted to all-trans-lycopene by one of two principal pathways: (i) desaturation and cis-trans isomerization by a single enzyme, CrtI (which may produce all-trans-neurosporene in some microorganisms), or (ii) desaturation to cis-\( \xi \)-carotene by CrtP, desaturation of cis-\( \xi \)-carotene to cis-lycopene by CrtQ, and finally cis-trans isomerization by CrtH to produce all-trans-lycopene (7, 12, 30). The CrtI-dependent pathway is found in fungi and most prokaryotes, whereas the CrtP/CrtQ/CrtH-dependent pathway until now has been found only in oxygenic photosynthetic organisms (cyanobacteria, algae, and plants). Cyclization, catalyzed by a lycopene cyclase, may then take place at one or both ends of lycopene. Neurosporene, lycopene, and their cyclization products, can be modified by additional enzymes in various organisms to produce a plethora of different carotenoids (7, 12, 52).

Green sulfur bacteria are obligately photoautotrophic and strictly anaerobic, and they form a distinct phylogenetic group (29, 44). These bacteria contain very large and unique antenna complexes, called chlorosomes, whose major pigment is BChl \( c \), \( d \), or \( e \) (8, 9). The major and characteristic carotenoids of green sulfur bacteria are chlorobactene (\( \phi \),\( \psi \)-carotene) and isorenieratene (\( \phi \),\( \psi \)-carotene), and these carotenoids are unusual by having aromatic \( \phi \) end groups (38, 39, 52). Aromatic carotenoids with \( \phi \) end groups are otherwise found only in some actinomycetes and a few sponges (Reniera spp.) (7, 12).

Because it is naturally transformable (19) and because its genome has been sequenced (15), Chlorobium tepidum (57)
has emerged as the principal model organism for studies of green sulfur bacteria. Much has already been learned about the biosynthesis of BChl c in this organism by bioinformatic and gene inactivation approaches (22–24), and one objective of the present study was to study the biosynthesis and functions of carotenoids in the same organism by similar approaches. The major carotenoid in C. tepidum is chlorobactene; minor carotenoids are γ-carotene, 1,2′,2′-dihydromchlorobactene, 1,2′,2′-dihydro-γ-carotene, OH-chlorobactene, and the larurate esters of both OH-chlorobactene glucoside and OH-γ-carotene glucoside (53). In C. tepidum, about 90% of the cellular carotenoids are located in the chlorosomes (20, 54). These carotenoids are not easily removed by detergent treatment of isolated chlorosomes (21). This observation suggests that the majority of the carotenoids are located in the chlorosome interior. The predominant carotenoids associated with isolated reaction center complexes are OH-carotenoid glucoside larurate esters; therefore, these carotenoids probably play a specific role in the reaction center (54).

By using a combination of genome analysis and gene inactivation in C. tepidum, we have elucidated the biosynthetic pathway leading to chlorobactene and OH-chlorobactene (Fig. 1). We have also established that the enzymes converting phytoene to lycopene are related to those in the lineage of oxygenic photosynthetic organisms (cyanobacteria, algae, and plants), which raises some interesting evolutionary issues. The observation that carotenoid biosynthesis can be completely eliminated in C. tepidum shows that, in principle, the remaining unidentified carotenogenic enzymes can also be identified by gene inactivation.

MATERIALS AND METHODS

Organism and growth conditions. The strain of C. tepidum used was the plating strain WT2321 (58) derived from strain TLS (ATCC 49652) (57). The genome of the latter strain has been sequenced (15). Cells were grown in liquid CL medium or on solid CP medium as previously described (19, 25). Cells were grown in liquid medium or on solid medium were incubated at 42°C under dim incandescent illumination (approximately 150 μmol of photons m−2 s−1) unless stated otherwise. Cells for pigment analysis were grown in 25-ml cultures to the early stationary phase before analysis. Growth rates were measured in 25-ml cultures at 47°C as previously described (22).

Construction of crt mutants. C. tepidum mutants were obtained by natural transformation and homologous recombination as previously described (19, 25). All DNA constructs used for transformation were made entirely in vitro either by megaprim PCR (CT0323, CT0807, CT1386, and CT1414) or by ligation of PCR products with a plasmid fragment (CT0180, CT0301, CT0649, CT1357, and CT4146) as described previously (25) using the primers listed in Table S1 in the supplemental material (Fig. 2). Transformants were analyzed for segregation by PCR as previously described (25) using the primers listed in Table S2 in the supplemental material. All primers used for PCR were derived from the genome sequence of C. tepidum (15) using MacVector software (version 7.0; Genetics Computer Group, Madison, Wis.). An aadA marker conferring spectinomycin and streptomycin resistance was used for all constructs and was obtained from plasmid pSR2A2 (26) in the case of megaprim PCR constructs and from plasmid pSR8A1 in the case of ligation constructs. Plasmid pSR8A1 (3,685 bp) was made as follows. A fragment containing aadA was generated by PCR using primers aadAFS (5′-GGTCCAGAACGTCGACCGAACGCA-3′) and aadABX (5′-GCGGGCGTCGCTACGCGAAATTG-3′) and plasmid pH4P51 (48) as the template (the Sall site in aadAFS and the XhoI site in aadABX are underlined). This fragment was digested with Sall and XhoI and cloned into pUC19 digested with Sall. A plasmid with the following configuration was selected and named pSR8A1: HindIII-SphI-PstI-Sall-aadA-XhoI-HindIII-SphI-PstI and the nucleotide sequence of aadA was confirmed by sequencing. From pSR8A1 containing aadA contains 1,038 bp.

Pigment analysis. The pigment composition of C. tepidum cells was analyzed as previously described (20) with minor modifications. Pelleted cells from a dense culture (1.0 ml) were extracted under dim light for no more than 20 s with 0.4 ml of acetonemethanol (7:2, vol/vol) using an ultrasonicator. The extracts were briefly centrifuged to pellet cell debris and further with a 0.45-μm-pore-size polytetrafluoroethylene syringe filter (Whatman, Maidstone, England). Extracts were analyzed by both HPLC-DAD (high-pressure liquid chromatography with diode-array detection) and HPLC-MS (HPLC with mass spectrometric detection). For analysis by HPLC-DAD, the extracts were injected onto the HPLC column immediately after extraction; for analysis by HPLC-MS, the extracts were dried under vacuum and redissolved in a small volume of acetonemethanol (7:2, vol/vol) before analysis. In either case, the pigment extract was supplemented with 0.1 volume of 1 M ammonium acetate prior to injection to improve peak resolution. The HPLC column was a Discovery C18 column (25 cm by 4.6 mm; 5-μm diameter beads) (Supelco, Bellefonte, Pa.). The flow rate was 1 ml/min−1, and the mobile phase was composed of solvent A (methanol-acetonitrile-water [42:33:25 by volume]) and solvent B (methanol-acetonitrile-ethyl acetate [50:20:30 by volume]) as follows: 30% solvent B at the time of injection, linear increase to 100% solvent B in 52 min, constant at 100% solvent B for 6 min, and return to 30% solvent B in 2 min. The HPLC-DAD system consisted of an Agilent 1100 series binary pump (model G1312A), vacuum degasser (model G1379A), manual injector (model G1328A), and diode-array detector (model G1315B), and the data acquired were analyzed with Chemstation for LC 3D software (version A.08.03; Agilent Technologies, Waldbronn, Germany). The HPLC-MS system consisted of a Micromass Quattro II mass spectrometer equipped with a Shimadzu LC/10ADop pump (Shimadzu, Columbia, Md.). Samples were analyzed using atmospheric pressure chemical ionization in negative ion mode with a corona needle potential of −4.0 kV, and the data were acquired with MassLynx software (version 3.5; Micromass, Ltd., Manchester, United Kingdom).

Pigments were identified by a combination of elution time, absorption spectrum, and molecular mass analysis and by comparison to the previously determined carotenoid composition of wild-type C. tepidum (53). The chlorophores used for identification (all-trans isomers) and absorption maxima were as follows: chlorobactene and γ-carotene, 436 (shoulder), 461, and 491 nm; lycopene, 447, 472, and 503 nm; neurosporene, 417, 440, and 469 nm; ω-carotene, 380, 401, and 425 nm; phytoene, 276 (shoulder), 287, and 298 nm; BChl c, 435 and 667 nm; and BChl a, 364, 602, and 770 nm. Absorption coefficients (in liters per gram per centimeter) used for quantification (22, 51) follow: for chlorobactene and γ-carotene, 265 at a wavelength of 490 nm; for glucoside derivatives of OH-chlorobactene and OH-γ-carotene, 198 at 490 nm; for glucoside larurate ester derivatives of OH-chlorobactene and OH-γ-carotene, 158 at 490 nm; for lycopene, 345 at 470 nm; for ω-carotene, 256 at 400 nm; for phytoene, 125 at 287 nm; for BChl c, 86 at 667 nm; and for BChl a, 60 at 770 nm. Retention times and masses of individual pigments are given below (see Table 1).

Screening for carotenoid biosynthesis. Protein sequences were aligned and analyzed with ClustalX (version 1.83) (http://www.ibgc.u-strasbg.fr/BioInfo/ClustalX/) (55), MacVector (version 7.0; Genetics Computer Group), and TreeView (version 1.6.6) (http://taxonomy.zoology.gla.ac.uk/rod/treemview.html) (45). Unrooted phylogenetic trees for presentation were constructed with PAUP (version 4.0; Sinauer Associates, Sunderland, Mass.).

RESULTS

Identification of genes involved in carotenoid biosynthesis. On the basis of sequence similarity with enzymes known to be involved in carotenoid biosynthesis in other organisms, genes encoding the following proteins were tentatively identified in the C. tepidum genome sequence (15): one CrtB-type phytoene synthase (CT1386), two CrtP/CrtQ-type plant or cyanobacterial phytoene desaturases (CT0807 and CT1414), two proteins related to CrtH-type plant or cyanobacterial carotenoid cis/trans isomerases (CT0180 and CT0649), one CrtC-type purple bacterial carotenoid hydratase (CT0301), and one CrtU-type β-carotene desaturase (CT0323). Except for ctc/CT0301, which is located immediately upstream of the petC and petB genes that encode the Rieske iron-sulfur protein and the associated cytochrome b, none of the genes encoding these proteins clustered with any other genes obviously related to pho-
A putative glycosyl transferase \((CT1416)\) was identified immediately upstream of chlorosome protein CsmH \((CT1417)\) and downstream of \(crtQ/CT1414\); because of this gene organization, \(CT1416\) was hypothesized to encode an \(\text{OH}-\text{carotenoid glucosyltransferase}\). No homolog of any known lycopene cyclase was found. A putative flavoprotein \((CT1357)\) with little similarity to any proteins in the databases was hypothesized to be a possible lycopene cyclase or carotenoid

FIG. 1. Proposed pathway of carotenoid biosynthesis in \(C.\ tepidum\). The HPLC peak numbers are shown in parentheses after the chemical names. All genes shown in this scheme have been inactivated. Geranylgeranyl-PP, geranylgeranyl diphosphate.

FIG. 2. Maps illustrating the DNA constructs used to inactivate genes. The small labeled arrows indicate the binding sites of the primers used to generate PCR products. The gene-inactivating constructs were made by fusion of the PCR products with a fragment containing the \(aadA\) antibiotic resistance marker by megaprimer PCR or by ligation. See text and reference 25 for details.
TABLE 1. Pigment composition of wild-type and mutant strains of C. tepidum

<table>
<thead>
<tr>
<th>Pigment (peak no.)</th>
<th>Retention time (min)*</th>
<th>Mass (m/z)</th>
<th>Wild type</th>
<th>CT1386 (crtB)</th>
<th>CT0807 (crtP)</th>
<th>CT1414 (crtQ)</th>
<th>CT0323 (crtU)</th>
<th>CT0301 (crtC)</th>
<th>CT0649 (crtH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytoene (1)</td>
<td>54.7, 55.1</td>
<td>544.5</td>
<td>1</td>
<td>0</td>
<td>81</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ζ-Carotene (2)</td>
<td>52.5, 52.9</td>
<td>540.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lycopene (3)</td>
<td>49.9, 50.2</td>
<td>536.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>γ-Carotene (4)</td>
<td>51.6, 51.7</td>
<td>536.4</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>41</td>
<td>4</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Chlorobactene (5)</td>
<td>48.0, 48.2</td>
<td>532.4</td>
<td>47</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>45</td>
<td>4</td>
</tr>
<tr>
<td>1’, 2’-Dihydro-γ-carotene (6)</td>
<td>53.6, 53.8</td>
<td>538.5</td>
<td>tr</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>1’, 2’-Dihydrochlorobactene (7)</td>
<td>50.2, 50.4</td>
<td>532.4</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
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</tr>
<tr>
<td>OH-γ-Carotene (8)</td>
<td>41.4</td>
<td>554.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OH-Chlorobactene (9)</td>
<td>36.6</td>
<td>550.4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OH-γ-Carotene glucoside laurate (10)</td>
<td>50.9</td>
<td>898.7</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OH-Chlorobactene glucoside laurate (11)</td>
<td>47.6</td>
<td>894.6</td>
<td>−2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total carotenoids</td>
<td></td>
<td></td>
<td></td>
<td>58</td>
<td>0</td>
<td>81</td>
<td>65</td>
<td>55</td>
<td>52</td>
</tr>
<tr>
<td>BChl a (12)</td>
<td>34.9</td>
<td>910.5</td>
<td>19</td>
<td>13</td>
<td>11</td>
<td>13</td>
<td>18</td>
<td>16</td>
<td>17</td>
</tr>
</tbody>
</table>

* Retention times are from Fig. 4 and 5. More than one retention time is due to the presence of both cis and trans isomers.

* Each value represents the average of at least two measurements on the same cell culture (for values higher than 4, the standard deviation did not exceed 26% of the average). tr, trace.

* The total carotenoid amounts are shown in boldface type for emphasis. The amount of total carotenoid may be higher than the sum of the amounts of the listed carotenoids because not all carotenoid species are listed. See text for details.

1’,2’-saturase. All of the putative desaturases and isomerases (CT0180, CT0323, CT0649, CT0807, CT1357, and CT1414) contain a conserved, N-terminal flavin-binding motif: (A/V/L/I)(V/L/I)(U/F)(G/A)G(V/L/I)(G/A)G(L/I)(A/S/T)XEX5GG. This motif is characteristic of all known carotenoid desaturases and cis-trans isomerases (36, 50).

The incomplete genome sequence of Chloroflexus aurantius was also surveyed using data available from the websites of the Joint Genome Institute (Walnut Creek, Calif.) (http://www.jgi.doe.gov) and the National Center for Biotechnology Information (Bethesda, Md.) (http://www.ncbi.nlm.nih.gov). Full-length homologs of one CrtB-type phytoene synthase, one CrtI-type phytoene desaturase (ORF2 in Fig. 3C), one CrtO-type carotenoid ketolase (ORF1 in Table S3 in the supplemental material), and one CrtY-type lycopene cyclase were found. In addition, truncated homologs of one CrtC-type carotenoid desaturase (ORF3 in Fig. 3C) and one CrtP-type carotenoid desaturase (ORF4 in Table S3 in the supplemental material) were also found. (For detailed sequence information and accession numbers, see Table S3 in the supplemental material.)

**Phylogenetic analysis of carotenogenic enzymes.** Figure 3 shows unrooted phylogenetic trees for most of the carotenogenic enzymes identified in *C. tepidum* and *Chloroflexus aurantius*. The trees show that the sequences from *C. tepidum* (including that of CrtC; data not shown) are not closely related to the sequences from *Chloroflexus aurantius* or any other organism. A CrtU desaturase produces aromatic carotenoids in the actinomycetes *Mycobacterium aurum* (56), *Streptomyces griseus* (36), and *Brevibacterium linens* (35) and in *C. tepidum* (this work; see below). Surprisingly, CrtU homologs were also identified in four cyanobacteria (Fig. 3B). Cyanobacteria do not produce aromatic carotenoids, and the function of the CrtU homolog in these bacteria is not known. Interestingly, the CrtU sequences from *C. tepidum* and the cyanobacteria contain a Rieske iron-sulfur domain of 116 amino acids in the middle of the sequence; this domain is not found in the CrtU sequences from actinomycetes. This suggests that the CrtU enzymes in *C. tepidum* and cyanobacteria may have a common evolutionary origin. The CT0180 protein in *C. tepidum* has one homolog with unknown function in each of the eight cyanobacteria whose genomes have been completely sequenced (Fig. 3C) (based on information available from the Cyanobase database [Kazusa DNA Research Institute, Kisarazu, Japan] [http://www.kazusa.or.jp/cyano]); however, no close homologs occur in any other organism.

**Pigment analysis of the wild type and crt mutants of C. tepidum.** The levels of BChl c in cells in the wild type and all mutants were very similar as judged from the absorption spectrum of intact cells (data not shown). In addition, no significant changes were observed in the absorption properties of BChl c in intact cells of the mutants. Thus, the absence of carotenoids did not have an effect on BChl c biosynthesis or on the aggregation of BChl c in chlorosomes. The only effect detected on pigmentation other than carotenoids was observed in the *crtB*, *crtP*, and *crtQ* mutants; the BChl a level of each of these mutants was about 65% of that of the wild type (Table 1). These three *crt* mutants lack a carotenoid chromophore absorbing in the same wavelength range as the carotenoids of the wild type and the other mutants.

A detailed analysis of the carotenoid composition of cells of the wild type and mutants is shown in Fig. 4 and 5 and in Table 1. The major carotenoid in the wild type was chlorobactene, in addition to smaller amounts of phytoene, γ-carotene, 1’,2’-dihydro-γ-carotene, 1’,2’-dihydrochlorobactene, OH-chlorobactene, OH-chlorobactene glucoside, OH-chlorobactene glucoside laurate, and OH-γ-carotene glucoside laurate. A trace amount of neurosporene was also found, but lycopene and ζ-carotene were not detected.

The CT1386 mutant completely lacked carotenoids, and the CT1386 gene was thus identified as *crtB* encoding phytoene synthase. Similarly, the CT0807 mutant lacked carotenoids except for phytoene; therefore, the CT0807 gene encodes a phy-
toene desaturase. Because the major carotenoid in the CT1414 mutant was /H9256-carotene, it was logical to identify CT0807 as crtP, encoding a phytoene desaturase that produces /H9256-carotene and to identify CT1414 as crtQ, encoding a /H9256-carotene desaturase that produces lycopene. The CT1414 mutant also accumulated small amounts of phytoene and a compound that was tentatively identified as 1,2-dihydro/H9256-carotene.

The dominant carotenoid in a cell extract of the CT0649 mutant was all-trans-lycopene (eluting at 50.2 min; absorption maxima of 474, 472, and 503 nm; Fig. 4). Another component eluting at 49.9 min had the same mass as lycopene and absorption maxima of 445, 467, and 499 nm and was tentatively identified as a cis isomer of lycopene. Small amounts of phytoene, chlorobactene, /H9253-carotene, and 1,2-dihydrolycopene were also found. Evidence of OH-lycopene or derivatives of OH-lycopene was not found. These observations strongly suggest that CT0649 encodes a CrtH-type lycopene cis-trans isomerase if one considers the following. Desaturation of /H9256-carotene by CrtQ produces a cis-lycopene and most likely takes place in the cytoplasmic membrane (7, 12). A mutant lacking crtH may therefore be expected to accumulate cis-lycopene, because all known lycopene cyclases act only on all-trans-lycopene (30). Although cis-lycopene is relatively stable by itself, it is readily isomerized to all-trans-lycopene when exposed to light and a photosensitizing molecule like BCHls.

However, most of the cis-lycopene produced in the CT0649 mutant is probably translocated to the chlorosome without being isomerized prior to translocation. Once in the chlorosome, this cis-lycopene is photoisomerized to all-trans-lycopene due to photosensitization by BCHl c but is then unavailable for further enzymatic modification. Although it was not explicitly shown that lycopene in the CT0649 mutant accumulated in the chlorosomes, it was shown that phytoene in the CT0807 mutant predominantly accumulated in the chlorosomes (data not shown), thus suggesting that most carotenoids in all crt mutants are correctly translocated to the chlorosomes.

A small proportion of the cis-lycopene produced in the CT0649 mutant is probably photoisomerized in the cytoplasmic membrane before being translocated to the chlorosome and thus would be available to lycopene cyclase and other enzymes. This probably accounts for the small amounts of chlorobactene and /H9253-carotene found in the CT0649 mutant.

The CT0301 mutant lacked OH-chlorobactene and OH-/H9253-carotene and their derivatives; therefore, the CT0301 gene was identified as crtC, which encodes a carotenoid 1',2'-hydratase. The CT0323 mutant lacked chlorobactene and all its derivatives and accumulated only γ-carotene and its derivatives. The CT0323 gene was thus identified as crtU encoding γ-carotene desaturase. No changes in the carotenoid composition of the CT0180, CT1357, and CT1416 mutants were detected, which

![Diagram](http://jb.asm.org/)
means that these three genes probably do not function in carotenoid biosynthesis.

Upon careful analysis of the HPLC traces, an unidentified component named F (for flexirubin-like) with an elution time of 49.3 min and absorption peaks at 285, 325, and 453 nm was found (Fig. 6). This component F was found in the CT1386, CT0807, CT1414, CT0323, and CT0649 mutants but was not unambiguously identified in the CT0301 and CT0180 mutants or in the wild type due to masking by carotenoids. However, previous analyses using different HPLC systems suggest that component F is also present in the wild type (N.-U. Frigaard,

FIG. 4. HPLC chromatograms of cell extracts of the wild-type and mutant strains of *C. tepidum* with detection wavelengths of 270 nm (---), 400 nm (———), and 490 nm (———). The amount of extract injected corresponded to about 10 μg of BChl c. Peaks are identified in Table 1. Other peaks are as follows: peak 13, OH-y-carotene glucoside (35.6 min); peak 14, OH-chlorobactene glucoside (30.3 min); peak 15, chlorobiumquinone 7 (44.8 min); peak 16, 1'-hydroxymenaquinone 7 (44.5 min); peak 17, menaquinone 7 (52.5 min); peak 18, 1,2-dihydro-β-carotene (54.2 min); and peak 19, 1,2-dihydrolycopene (51.8 min).

FIG. 5. HPLC chromatograms of cell extracts of the wild-type and mutant strains of *C. tepidum* with a detection wavelength of 490 nm. See the legend to Fig. 4 and text for details.

FIG. 6. Absorption spectrum of an unidentified component (the F component) found in *C. tepidum*. The absorption spectrum was obtained from the HPLC detector at the time of elution. There were three peaks at 285, 325, and 453 nm.
unpublished data). The absorption band around 453 nm resembles that of a conjugated system of nine double bonds, and due to the lack of fine structure, the spectral data suggest the presence of at least one carbonyl group (51). Component F is not likely to be a carotenoid, because it was present in the CT1386 mutant (which lacks phytoene synthase) and because no homolog of the CT1386 gene that could encode an alternative carotene synthase is present in C. tepidum. The absorption spectrum and chromatographic properties of component F resemble those of the flexirubin-type pigments found in some Cytophaga, Flavobacterium, and Flexibacter species (1, 51), to which C. tepidum is distantly related on the basis of 16S rRNA and whole-genome phylogeny (15). Flexirubin-type pigments are not derived from isoprenoid precursors and consist of a polyenoic acid chromophore terminated by a hydroxyphenyl group and esterified with an alkyl-substituted phenol group of the resorcinol type. The function of these pigments is not known. If component F has a specific absorption coefficient of about 200 liters g\(^{-1}\) cm\(^{-1}\) at a wavelength of 453 nm, cell extracts of C. tepidum contain roughly 1 mg of component F per g of BCHl c. Analyses show that component F is present in isolated chlorosomes (data not shown).

Inhibition of lycopene cyclization in C. tepidum. The gene encoding lycopene cyclase in C. tepidum was not identified in this study. Nevertheless, it was observed that the addition of the herbicide 2-(4-methylphenoxy)triethylamine hydrochloride (MPTA) to the growth medium of C. tepidum inhibited lycopene cyclization (data not shown). MPTA has been shown to inhibit lycopene cyclization in both microbes and plants (7). Lycopene constituted about 80% of the total carotenoids in cultures of wild-type C. tepidum containing 3 \(\mu\)M MPTA and about 90% of the carotenoids in cultures containing 100 \(\mu\)M MPTA (the remaining carotenoid mostly being chlorobactene). The growth rate and coloration of wild-type C. tepidum cultures were not significantly affected by MPTA concentrations between 3 and 100 \(\mu\)M. MPTA also inhibited lycopene cyclization in a bchK mutant of C. tepidum. This mutant does not contain BCHl c but contains various carotenoids in amounts similar to those in the wild type (22). When the bchK mutant was grown in the presence of 3 \(\mu\)M MPTA, about 85% of the total carotenoid pool was lycopene. In contrast to the wild type, the bchK mutant grew more slowly when the MPTA concentration was 3 \(\mu\)M or higher and grew very little when the MPTA concentration was 100 \(\mu\)M. Nicotine has also been shown to inhibit lycopene cyclization in C. tepidum (53). Isolation of C. tepidum mutants resistant to these chemical inhibitors of lycopene cyclization may help in identifying the genetic basis of lycopene cyclization.

Effects of crt mutations on the growth of C. tepidum. No effects on cell morphology in the crt mutants were observed by light microscopy (data not shown). This contrasts with observations of Chlorobium phaeobacteroides, which forms filaments when cells are treated with the carotenoid biosynthesis inhibitor 2-hydroxybiphenyl (4, 5). Thus, filament formation is probably due to secondary effects of the inhibitor on cell morphology and division. Chlorosomes are apparently formed normally in the C. tepidum mutants, since chlorosomes similar to wild-type chlorosomes were isolated from the crtB and crtP mutants (data not shown). Chlorosomes from the crtP mutant contained most of the phytoene present in the cells, demonstrating that this carotenoid precursor is translocated to the chlorosomes, as are most of the carotenoids in the wild type.

Figure 7 shows the growth rates of the wild type and various crt mutants of C. tepidum at three different light intensities. The growth rates of four mutants (crtU, crtH, CT0180, and CT1416 mutants) showed no significant deviation from the wild-type growth rate at any light intensity (data for the CT0180 and CT1416 mutants not shown). The growth rate of the crtC mutant differed from the wild-type growth rate only at the lowest light intensity (10 \(\mu\)mol of photons m\(^{-2}\) s\(^{-1}\), limiting for growth in all strains), when it grew only about 60% as fast as the wild type. The growth rates of the three other mutants (crtB, crtP, and crtQ mutants) were significantly lower than the wild-type growth rate at all light intensities. At the lowest light intensity, the growth rates of the crtP and crtQ mutants were about 40% that of the wild type, and the growth rate of the crtB mutant was only about 25% that of the wild type. At increasing light intensities, the growth rates of the crtB, crtP, and crtQ mutants were higher but did not reach the wild-type growth rate, even at the highest light intensity examined (588 \(\mu\)mol of photons m\(^{-2}\) s\(^{-1}\)), when these mutants grew about 65% as fast as the wild type.

DISCUSSION

Carotenoid biosynthesis in green sulfur bacteria. On the basis of the mutational studies in C. tepidum in this work, we propose the carotenoid biosynthetic pathway shown in Fig. 1. The intermediates and enzymes leading to lycopene are identical to those in cyanobacteria and plants (7, 12). A CrtU-like enzyme carries out the desaturation of \(\gamma\)-carotene to chlorobactene; this enzyme is related to but distinct from the enzyme found in actinomycetes. A CrtC enzyme, similar to that in purple bacteria, hydrates both \(\gamma\)-carotene and chlorobactene. Since hydrated species of lycopene or earlier intermediates in the pathway were not found in the wild type or any mutant,
CrtC apparently cannot efficiently hydrate these species. There appears to be little or no feedback inhibition or obligatory multienzyme complex formation in this pathway, since the genetic inactivation of one enzyme did not significantly affect the apparent activity of the remaining enzymes or the total carotenoid content (Table 1).

Four enzymes are still unidentified in the proposed pathway in Fig. 1. These unidentified enzymes include the glucosyltransferase and a laurate transferase that are required to produce the OH-carotenoid glucoside laurate esters (53). It is noteworthy that the enzymes that form the glucoside laurate esters seem to have higher affinity for γ-carotene than chlorobacterium, since the ratio of chlorobacterium to γ-carotene ranges from 10 to 20, whereas the ratio of OH-chlorobacterium glucoside laurate to OH-γ-carotene glucoside laurate ranges from 1 to 2.

Another enzymatic activity not yet completely understood is the carotenoid 1,2-saturase, which produces 1',2'-dihydrochlo-ro bacterium and 1',2'-dihydro-γ-carotene (53). In C. tepidum, 1',2'-dihydrochlorobacterium can be a major carotenoid species under certain growth conditions, such as prolonged incubation (N.-U. Frigaard, unpublished data) or low light intensities (10). The only other organism in which 1,2-dihydrocarotenoids have been detected is Blastochloris (formerly Rhodopseudomonas) viridis (41). The major carotenoid in this organism is 1',2'-dihydroneusporone, and 1,2-dihydrocarotenoids may constitute up to 94% of the total carotenoids. Insufficient DNA sequences are currently available from the photosynthesis gene cluster of Blastochloris viridis to allow a detailed comparison with the C. tepidum sequences.

Two paralogous genes (bchP/CT2256 and bchO/CT1232) in C. tepidum encode putative geranylgeraniol reductases related to the BchP enzyme involved in BChl a biosynthesis in purple bacteria (15, 23). The bchP and bchO genes have been insertionally inactivated in C. tepidum to produce two independent mutants (A. Gomez Maqueo Chew, N.-U. Frigaard, and D. A. Bryant, unpublished data). Both the bchP and bchO mutants produce altered BChl a and Chl a species that are consistent with the products of these genes functioning in reduction of the geranylgeranyl tail of (B)Chls. Additionally, the bchP and bchO mutants are deficient in 1',2'-dihydrochlorobacterium. Thus, it appears that BchP and BchO, in some as yet poorly understood but interdependent manner, are responsible for the saturation of the isoprenoid moiety of both BChl a, Chl a, and carotenoids in C. tepidum. Since it is not simple to distinguish between the phenotypic effects of modified (B)Chls and modified carotenoids in the bchP and bchO mutants, these mutants were not further characterized in this work. Further studies with these mutants to resolve these issues are in progress.

The only other important carotenogenic enzyme not yet identified in C. tepidum is lycopene cyclase. The genome does not encode any homolog of the three known types of lycopene cyclases (50). Interestingly, some cyanobacteria also lack an identifiable lycopene cyclase. Given the similarity of many of the carotenogenic enzymes and other proteins involved in photosynthesis between C. tepidum and cyanobacteria (23), the possibility that C. tepidum and some cyanobacteria share a novel type of lycopene cyclase is intriguing. Since four cyanobacteria in Fig. 3B (Synechocystis sp. strain PCC 6803, Nostoc sp. strain PCC 7120, “Gloeobacter violaceus,” and Thi-
It is not yet clear what causes the decreased growth rates of the crtB, crtP, and crtQ mutants. It is also not clear whether the cause of the decreased growth rate under low light intensities is the same as the cause for the decreased growth rate under high light intensities. However, it is striking that the three mutants with decreased growth rates (crtB, crtP, and crtQ mutants) also have decreased BCHl a levels (Table 1). Although BCHl a is not the major light-harvesting pigment, it is essential for the transfer of excitation energy from the major light-harvesting pigment (BChl c) in the chlorosome to the reaction center. Thus, the decreased growth rates could be due to less efficient energy conservation because of decreased BCHl a levels. How the absence of certain carotenoids could cause a decrease in the BCHl a level is not known, but there may be structural or functional requirements for such carotenoids in the BCHl a-containing CsmA protein in the chlorosome baseplate (13, 43, 49) or in the reaction center (54) or both. Both spectroscopic analyses (3, 6, 42) and chemical analyses (13, 43) suggest that BCHl a and carotenoids are intimately associated in the chlorosome baseplate and that the CsmA protein probably binds to at least one carotenoid molecule. Other analyses also suggest a correlation between colored carotenoids, BCHl a, and CsmA. Chlorosomes isolated from C. phaeobacteroides and Chloroflexus aurantiacus grown in the presence of the phytoene desaturase inhibitor 2-hydroxybiphenyl are almost completely devoid of colored carotenoids, and the levels of BCHl a and CsmA in such cells are decreased by roughly 35 to 60% (4, 5, 17). Analogously, when crtI is inactivated in the purple bacterium Rhodobacter sphaeroides (which then accumulates colorless phytoene), formation of light-harvesting complex 2 is blocked, whereas formation of light-harvesting complex 1 is not (11, 37, 46).

Another possibility is that isolated triplets of BCHls accumulate in the crtB, crtP, and crtQ mutants and that these BCHl triplets cause damage to the photosynthetic apparatus. Quenching of BCHl triplets requires a carotenoid with a minimum of nine conjugated double bonds (18). Such carotenoids are absent only in the crtB, crtP, and crtQ mutants, and these mutants should thus accumulate increased levels of BCHl c and BCHl a excited triplet states. Both BCHl c and BCHl a triplets have been detected in both cells and isolated chlorosomes of green sulfur bacteria (3, 6, 42). BCHl triplets are known to be detrimental for photosynthetic organisms under aerobic conditions because of singlet O₂ formation (18). However, this cannot explain the growth deficiency of the crtB, crtP, and crtQ mutants, because they grow only under strictly anaerobic conditions. Nevertheless, accumulation of high levels of BCHl c and BCHl a triplets in C. tepidum may damage the cells in a yet unidentified manner.

Although carotenoids may certainly play a role in light harvesting in natural environments, it is unlikely that the growth defects observed here at low light intensity are due to reduced light harvesting in the mutants lacking colored carotenoids. The tungsten lamps used to provide the illumination in the growth experiments emit relatively little light with wavelengths shorter than 600 nm. In order to explore the roles of carotenoids in light harvesting, future studies will systematically study the growth behavior of the mutants under different light regimens.

**Evolution of carotenogenic enzymes.** It is not clear why two different biosynthetic pathways for lycopene exist in nature nor is it clear what advantages each may present over the other. The lycopene biosynthetic enzymes (CrtP, CrtQ, and CrtH) in green sulfur bacteria and cyanobacteria are obviously related, although very distantly (Fig. 3C and D), and they have thus far been found only in these two bacterial groups (photosynthetic eukaryotes aside). It is therefore likely that these enzymes originated in a common ancestor of modern-day green sulfur bacteria and cyanobacteria and were inherited vertically. This common ancestor probably contained an ancestral form of a type I photosynthetic reaction center (33), since the CrtP, CrtQ, and CrtH enzymes occur only in organisms containing type I reaction centers.

cis carotenoids appear to perform important functions in reaction centers (34). It has been suggested that all reaction centers contain 15-cis carotenoid isomers, which can be generated by thermal isomerization of the corresponding all-trans isomers (34). In addition, photosystem I from cyanobacteria contains 9-cis carotenoids, which are not found in type II reaction centers (27, 34). The presence or absence of 9-cis carotenoids in green sulfur bacterial reaction centers has not been conclusively demonstrated. Since 9-cis carotenoids are not easily formed by thermal isomerization, the enzymatic formation of cis carotenoids other than the 15-cis isomers may have provided a selective advantage to the ancestral type I reaction center. Because the CrtI-dependent biosynthetic pathway for lycopene does not produce cis-isomer intermediates, we propose that the evolution of the CrtP/CrtQ/CrtH-dependent pathway is linked to the postulated advantage of having particular cis carotenoids in the ancestral type I reaction centers.

On the basis of sequence similarity, CrtH and CrtI are distantly related (Fig. 3C), and CrtH probably evolved from the much more widespread CrtI (50). Analogously, on the basis of sequence similarity, CrtP, CrtQ, and CrtU are distantly related (50). CrtP and CrtQ probably arose by an ancient gene duplication event. Sandmann has proposed that CrtU originated in actinomycetes and that CrtP and CrtQ evolved from CrtU after horizontal transfer to cyanobacteria (50). However, given that the function of CrtU in green sulfur bacteria and actinomycetes is the same but that CrtP and CrtQ evolved from CrtU actinomycetes and that CrtP evolved from CrtU, we propose that the divergence of CrtP/CrtQ/CrtH occurred in an ancestor of modern-day green sulfur bacteria and cyanobacteria. We therefore propose that the divergence of CrtP/CrtQ and CrtU occurred in an ancestor of the green sulfur bacteria.

**ACKNOWLEDGMENTS**

This work was supported in part by grant DE-FG02-94ER20137 to D.A.B. from the U.S. Department of Energy.

We thank Francis X. (Buddy) Cunningham, Jr. (University of Maryland), for supplying the herbicide MPTA, Kazuhito Inoue (Kanagawa University, Kanagawa, Japan) for sharing unpublished DNA sequences from Blastochloris viridis, and Harry A. Frank (University of Connecticut) for discussions on carotenoids.

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