JOURNAL OF BACTERIOLOGY, Aug. 2004, p. 5210–5220 Vol. 186, No. 16
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.16.5210–5220.2004
Copyright © 2004, American Society for Microbiology. All Rights Reserved.

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

Niels-Ulrik Frigaard,[1]* Julia A. Maresca,[1] Colleen E. Yunker,[1] A. Daniel Jones,[2] and Donald A. Bryant[1]

Department of Biochemistry and Molecular Biology[1] and Department of Chemistry,[2] The Pennsylvania State University, University Park, Pennsylvania 16802

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*), ζ-carotene desaturase (*crtQ*/*CT1414*), γ-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 *crtB* 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*-ζ-carotene by CrtP, desaturation of *cis*-ζ-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 (ϕ,ψ-carotene) and isorenieratene (ϕ,ϕ-carotene), and these carotenoids are unusual by having aromatic ϕ end groups (38, 39, 52). Aromatic carotenoids with ϕ 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)

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, 232 South Frear Building, The Pennsylvania State University, University Park, PA 16802. Phone: (814) 863-7405. Fax: (814) 863-7024. E-mail: nxf10@psu.edu.

† Supplemental material for this article may be found at http://jb.asm.org/.

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′-dihydrochlorobactene, 1′,2′-dihydro-γ-carotene, OH-chlorobactene, and the laurate 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 laurate 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 in liquid medium or on solid medium were incubated at 42°C under 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 megaprimer PCR (*CT0323*, *CT0807*, *CT1386*, and *CT1414*) or by ligation of PCR products with a plasmid fragment (*CT0180*, *CT0301*, *CT0649*, *CT1357*, and *CT1416*) 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 pSRA2 (26) in the case of megaprimer PCR constructs and from plasmid pSRA81 in the case of ligation constructs. Plasmid pSRA81 (3,685 bp) was made as follows. A fragment containing *aadA* was generated by PCR using primers aadAFS (5′-GGTCCAGAAC<u>GTCGAC</u>CGAACGCA-3′) and aadABX (5′-GCGGCGTCGG<u>CTCGAG</u>CGAATTG-3′) and plasmid pHP45Ω (48) as the template (the SalI site in aadAFS and the XhoI site in aadABX are underlined). This fragment was digested with SalI and XhoI and cloned into pUC19 digested with SalI. A plasmid with the following configuration was selected and named pSRA81: HindIII-SphI-PstI-SalI-*aadA*-XbaI-BamHI-AvaI-KpnI-EcoRI (*aadA* is transcribed from left to right in this orientation). The EcoRI-PstI fragment from pSRA81 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 acetone-methanol (7:2, vol/vol) using an ultrasonicator. The extracts were briefly centrifuged to pellet cell debris and filtered 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 acetone-methanol (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 C_{18} 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 LC10ADvp 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 acquired were analyzed 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 chromophores 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; ζ-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 laurate ester derivatives of OH-chlorobactene and OH-γ-carotene, 158 at 490 nm; for lycopene, 345 at 470 nm; for ζ-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).

Sequence analysis and phylogenetic trees. Protein sequences were aligned and analyzed with ClustalX (version 1.83) (http://www-igbmc.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/treeview.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 *crtC*/*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-

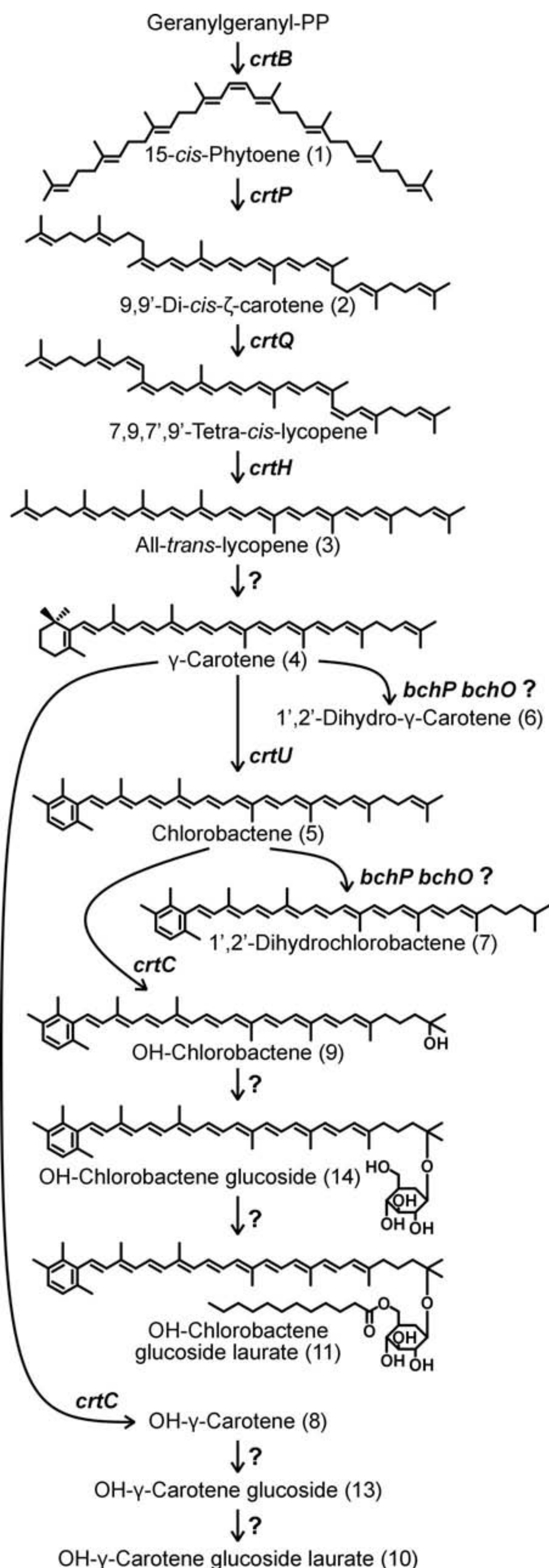

FIG. 1. Proposed pathway of carotenoid biosynthesis in *C. tepidum*. The HPLC peak numbers are shown in parentheses after the chemical names. All genes shown in this scheme have been inactivated. Geranylgeranyl-PP, geranylgeranyl diphosphate.

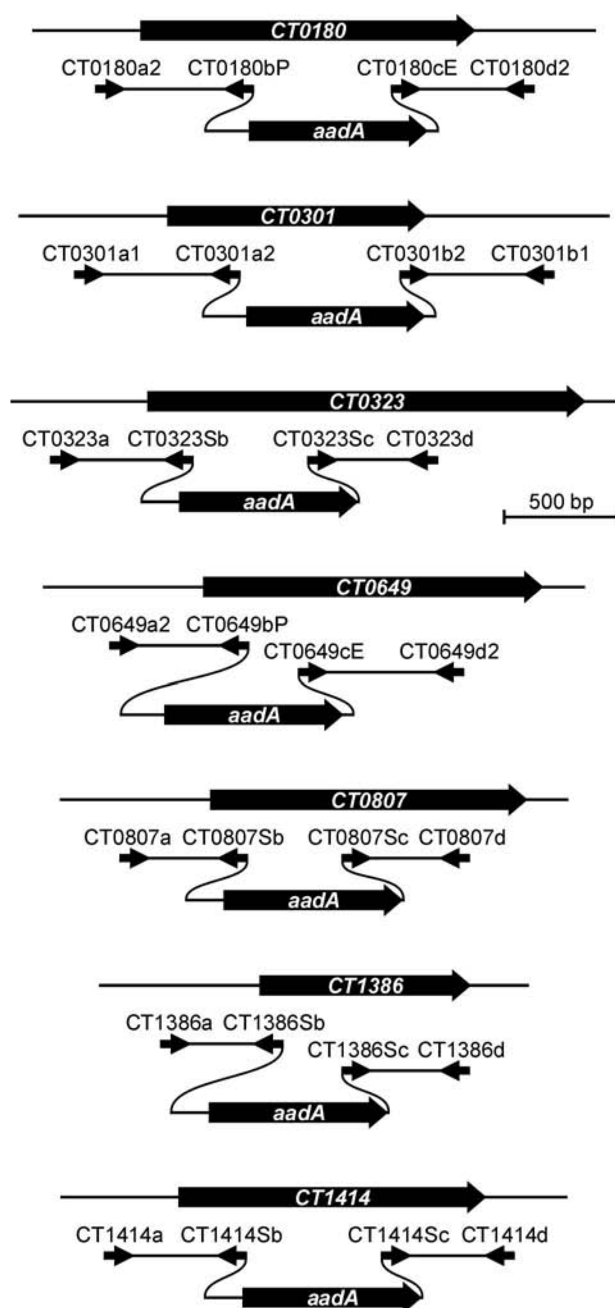

FIG. 2. Maps illustrating the DNA constructs used to inactivate genes. The small labeled arrows indicate the binding sites of the primers used to generate PCR products. The gene-inactivating constructs were made by fusion of the PCR products with a fragment containing the *aadA* antibiotic resistance marker by megaprimer PCR or by ligation. See text and reference 25 for details.

tosynthesis. 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 OH-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

TABLE 1. Pigment composition of wild-type and mutant strains of *C. tepidum*

Pigment (peak no.)	Retention time (min)[a]	Mass (*m/z*)	Pigment content (mg/g of BChl *c*)[b]						
			Wild type	*CT1386* (*crtB*)	*CT0807* (*crtP*)	*CT1414* (*crtQ*)	*CT0323* (*crtU*)	*CT0301* (*crtC*)	*CT0649* (*crtH*)
Phytoene (1)	54.7, 55.1	544.5	1	0	81	7	1	1	1
ζ-Carotene (2)	52.5, 52.9	540.5	0	0	0	48	0	0	0
Lycopene (3)	49.9, 50.2	536.4	0	0	0	0	0	0	21
γ-Carotene (4)	51.6, 51.7	536.4	3	0	0	0	41	4	~5
Chlorobactene (5)	48.0, 48.2	532.4	47	0	0	0	0	45	4
1′, 2′-Dihydro-γ-carotene (6)	53.6, 53.8	538.5	tr	0	0	0	5	tr	tr
1′, 2′-Dihydrochlorobactene (7)	50.2, 50.4	532.4	3	0	0	0	0	2	tr
OH-γ-Carotene (8)	41.4	554.4	0	0	0	0	tr	0	0
OH-Chlorobactene (9)	36.6	550.4	1	0	0	0	0	0	0
OH-γ-Carotene glucoside laurate (10)	50.9	898.7	3	0	0	0	7	0	0
OH-Chlorobactene glucoside laurate (11)	47.6	894.6	~2	0	0	0	0	0	0
Total carotenoid[c]			**58**	**0**	**81**	**65**	**55**	**52**	**39**
BChl *a* (12)	34.9	910.5	19	11	13	13	18	16	17

[a] Retention times are from Fig. 4 and 5. More than one retention time is due to the presence of both *cis* and *trans* isomers.
[b] 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 12% of the average; for values of 4 or lower, the standard deviation did not exceed 26% of the average). tr, trace.
[c] 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/I)(I/F)G(G/A)G(V/L/I)(G/A)G(L/I)(A/S/T)X_4LX_8(V/L)XEX_5GG. This motif is characteristic of all known carotenoid desaturases and *cis-trans* isomerases (36, 50).

The incomplete genome sequence of *Chloroflexus aurantiacus* 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 CrtI-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 aurantiacus*. 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 aurantiacus* 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-

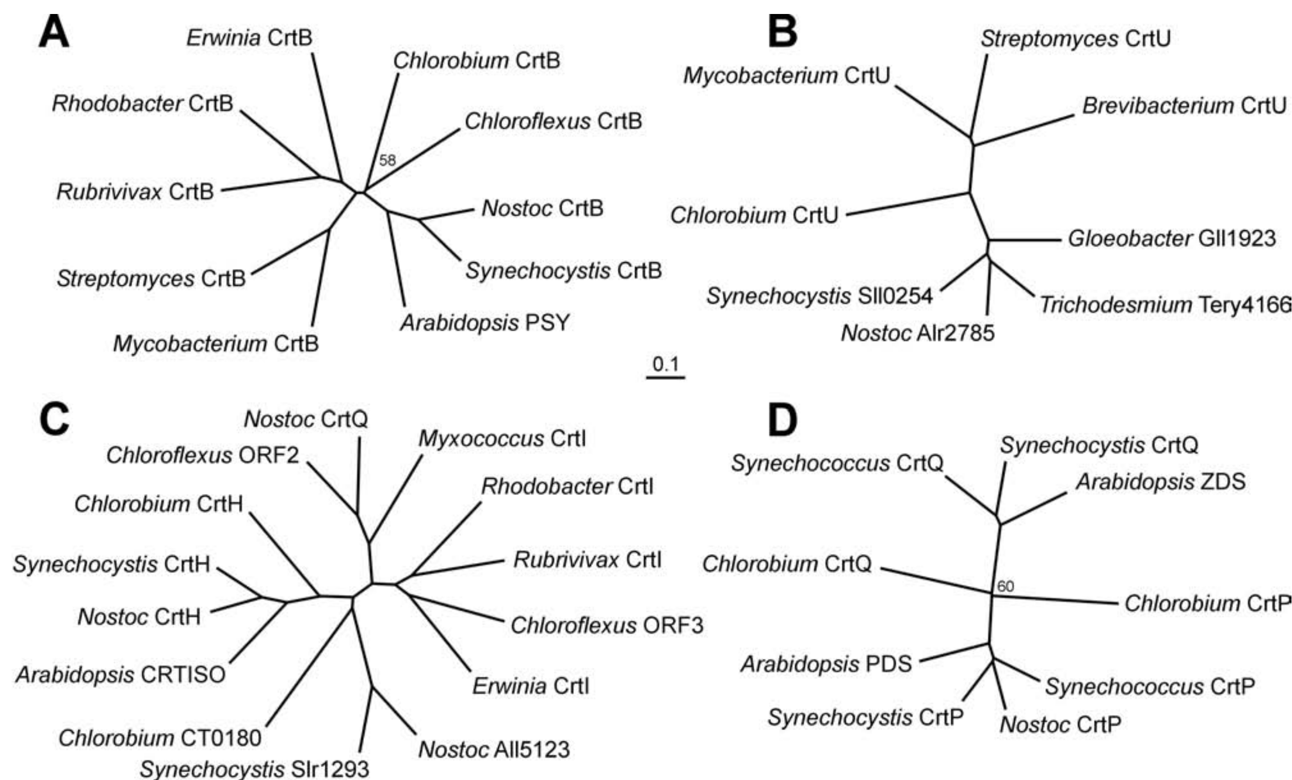

FIG. 3. Unrooted neighbor-joining phylogenetic trees of different enzymes and proteins. (A) CrtB-type phytoene synthases; (B) CrtU-type β-carotene and γ-carotene desaturases; (C) CrtI-type phytoene desaturases, CrtH-type *cis-trans* isomerases, and related proteins; and (D) CrtP- and CrtQ-type desaturases. Bootstrap values were calculated on the basis of 1,000 replicates; only values less than 70% are shown. The sequences used were obtained from the following organisms: *Arabidopsis thaliana*, *Rubrivivax gelatinosus*, *Brevibacterium linens*, *Chlorobium tepidum*, *Chloroflexus aurantiacus*, *Erwinia herbicola*, "*Gloeobacter violaceus*" strain PCC 7421, *Mycobacterium aurum*, *Myxococcus xanthus*, *Nostoc* (formerly *Anabaena*) sp. strain PCC 7120, *Rhodobacter sphaeroides*, *Streptomyces griseus*, *Synechococcus* sp. strain PCC 7942, *Synechocystis* sp. strain PCC 6803, and *Trichodesmium erythraeum*. For further information on the protein sequences, see Table S3 in the supplemental material.

toene desaturase. Because the major carotenoid in the *CT1414* mutant was ζ-carotene, it was logical to identify *CT0807* as *crtP*, encoding a phytoene desaturase that produces ζ-carotene and to identify *CT1414* as *crtQ*, encoding a ζ-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-ζ-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, γ-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 ζ-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 (30). 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 γ-carotene found in the *CT0649* mutant.

The *CT0301* mutant lacked OH-chlorobactene and OH-γ-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

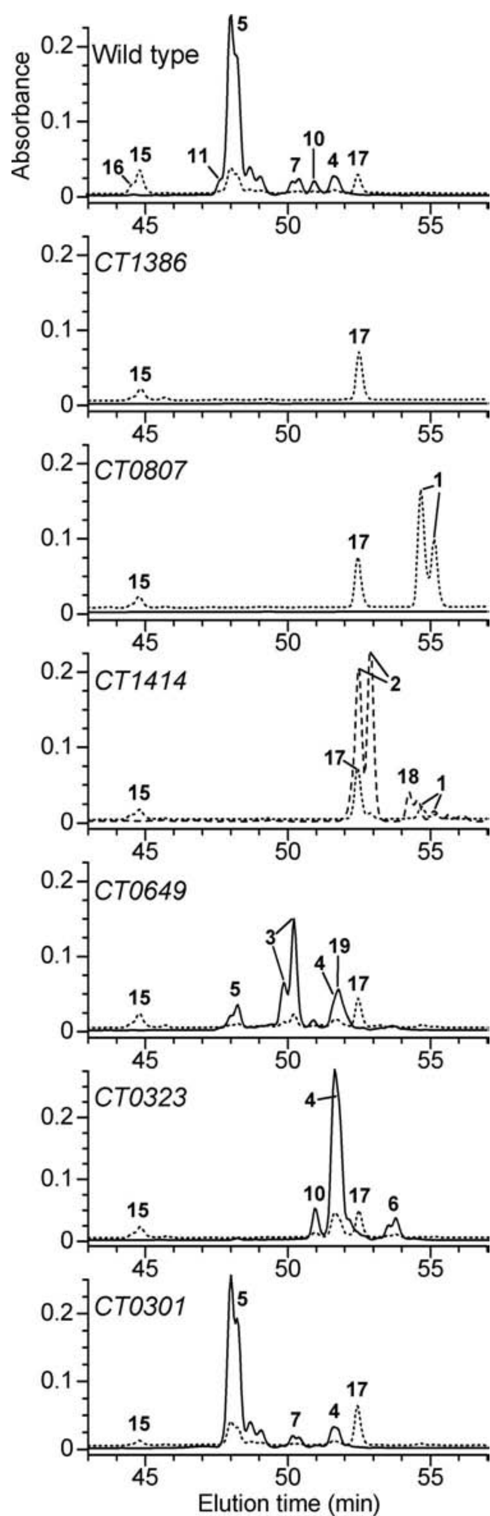

FIG. 4. HPLC chromatograms of cell extracts of the wild-type and mutant strains of *C. tepidum* with detection wavelengths of 270 nm (- - - -), 400 nm (— — —), and 490 nm (———). The amount of extract injected corresponded to about 10 μg of BChl *c*. Peaks are identified in Table 1. Other peaks are as follows: peak 13, OH-γ-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-ζ-carotene (54.2 min); and peak 19, 1,2-dihydrolycopene (51.8 min).

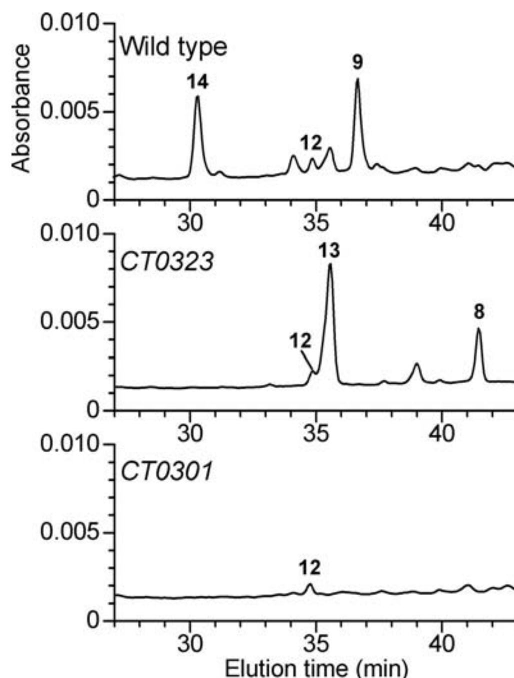

FIG. 5. HPLC chromatograms of cell extracts of the wild-type and mutant strains of *C. tepidum* with a detection wavelength of 490 nm. See the legend to Fig. 4 and text for details.

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,

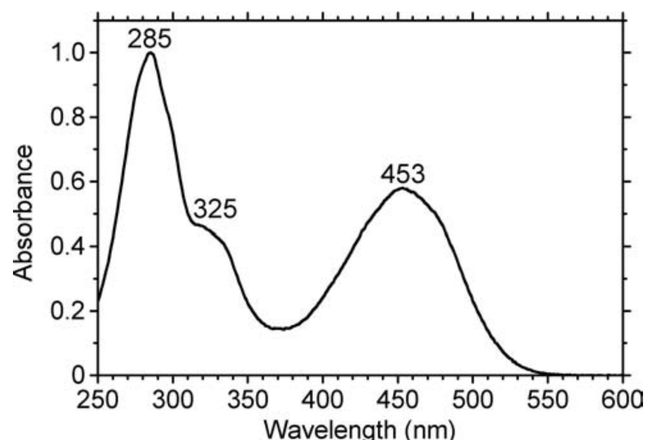

FIG. 6. Absorption spectrum of an unidentified component (the F component) found in *C. tepidum*. The absorption spectrum was obtained from the HPLC detector at the time of elution. There were three peaks at 285, 325, and 453 nm.

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 μM MPTA and about 90% of the carotenoids in cultures containing 100 μM 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 μM. 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 μM 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 μM or higher and grew very little when the MPTA concentration was 100 μM. 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.

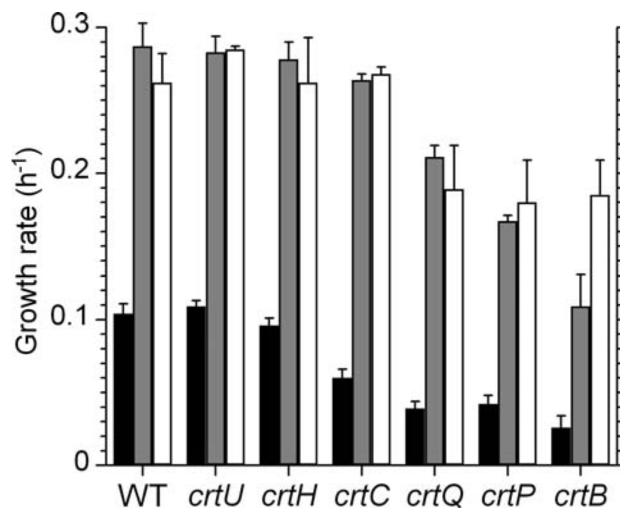

FIG. 7. Growth rates of the wild-type and mutant strains of *C. tepidum* at various light intensities. The light intensities used were 10 (black bars), 76 (shaded bars), and 588 (white bars) μmol of photons m^{-2} s^{-1}. The data are the means ± standard deviations (error bars) from at least four separate experiments. WT, 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 μmol 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 μmol 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 γ-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 γ-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 chlorobactene, since the ratio of chlorobactene to γ-carotene ranges from 10 to 20, whereas the ratio of OH-chlorobactene 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′-dihydrochlorobactene and 1′,2′-dihydro-γ-carotene (53). In *C. tepidum*, 1′,2′-dihydrochlorobactene 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′-dihydroneurosporene, 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′-dihydrochlorobactene. 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 *Trichodesmium erythraeum*) also lack an identifiable lycopene cyclase, the possibility that the CrtU homologs of these cyanobacteria could function as lycopene cyclases is interesting. Consistent with this suggestion, other cyanobacteria that have an identifiable lycopene cyclase (for example, three *Prochlorococcus marinus* strains and *Synechococcus* sp. strain WH8102) lack homologs of CrtU (based on information available from the CyanoBase database).

Carotenoid biosynthesis in green filamentous bacteria. The only organisms other than green sulfur bacteria that contain chlorosomes and BChl *c* are the green filamentous bacteria, represented by *Chloroflexus aurantiacus* (28, 32). However, the biosynthetic pathway leading to lycopene and its cyclization products in *Chloroflexus aurantiacus* appears to be different from the pathway in *C. tepidum*. The portion of the *Chloroflexus aurantiacus* genome that has been sequenced contains one or possibly two genes encoding homologs of CrtI-type carotene desaturases and possibly one gene encoding a homolog of a CrtP-type desaturase, but it does not encode a homolog of the carotenoid *cis-trans* isomerase. *Chloroflexus aurantiacus* may thus employ a classical bacterial CrtI-dependent phytoene desaturation (see introduction). Alternatively, *Chloroflexus aurantiacus* may employ a hybrid pathway like *Nostoc* sp. strain PCC 7120, which uses a CrtP-like phytoene desaturase that produces ζ-carotene and a ζ-carotene desaturase related to CrtI proteins (sometimes denoted CrtQ or CrtQa [Fig. 3C]) (2, 40, 50). The major carotenoids in *Chloroflexus aurantiacus*, β-carotene and γ-carotene, are apparently formed by a classical bacterial lycopene cyclase of the CrtY family. Under oxic conditions, carotenoids that contain 4-oxo-β end groups, including echinenone (4-oxo-β-carotene) and myxobactone (4-oxo-OH-γ-carotene glucoside) (14, 31, 47), are probably formed by a CrtO ketolase similar to those in cyanobacteria (16). (The GenBank accession numbers of all the *Chloroflexus aurantiacus* protein sequences mentioned in this paragraph are listed in Table S3 in the supplemental material.)

Effect of carotenoid composition on growth of *C. tepidum*. The growth rates of two mutants with altered carotenoid composition (*crtU* and *crtH* mutants) were not significantly different from those of the wild type at any light intensity (Fig. 7). Thus, the absence of aromatic carotenoids (chlorobactene and its derivatives) in the *crtU* mutant was not a significant disadvantage under the growth conditions tested. Likewise, elimination of most of the cyclic carotenoids and the accumulation of lycopene in the *crtH* mutant did not significantly affect the growth of this strain. The absence of hydroxylated carotenoids and their derivatives (glucosides and fatty acid glucoside esters) in the *crtC* mutant appeared to cause a growth deficiency only at limiting light intensity (10 μmol of photons m^{-2} s^{-1}). This could be due to reduced carotenoid level or efficiency of the reaction centers, because most of the hydroxylated carotenoids and their derivatives are associated with the reaction centers (54). In contrast, growth was impaired in the *crtB*, *crtP*, and *crtQ* mutants under all light intensities examined (Fig. 7). These three mutants share the common property that they lack colored carotenoids, i.e., carotenoids with a chromophore consisting of nine or more conjugated double bonds. The wild type and all *crt* mutants with no growth rate phenotype have colored carotenoids (see chemical structures in Fig. 1).

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 excited triplet states 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 O_2 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 CrtU proteins from green sulfur bacteria and cyanobacteria are more similar structurally, it seems unlikely that CrtU has been exchanged horizontally between actinomycetes 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.

REFERENCES

1. **Achenbach, H., W. Kohl, and H. Reichenbach.** 1978. The flexirubin-type pigments—a novel class of natural pigments from gliding bacteria. Rev. Latinoam. Quim. **9:**111–124.
2. **Albrecht, M., H. Linden, and G. Sandmann.** 1996. Biochemical characterization of purified ζ-carotene desaturase from *Anabaena* PCC 7120 after expression in *Escherichia coli*. Eur. J. Biochem. **236:**115–120.

3. **Arellano, J. B., T. B. Melø, C. M. Borrego, J. Garcia-Gil, and K. R. Naqvi.** 2000. Nanosecond laser photolysis studies of chlorosomes and artificial aggregates containing bacteriochlorophyll *e*: evidence for the proximity of carotenoids and bacteriochlorophyll *a* in chlorosomes from *Chlorobium phaeobacteroides* strain CL1401. Photochem. Photobiol. **72:**669–675.
4. **Arellano, J. B., J. Psencik, C. M. Borrego, Y.-Z. Ma, R. Guyoneaud, J. Garcia-Gil, and T. Gillbro.** 2000. Effect of carotenoid biosynthesis inhibition on the chlorosome organization in *Chlorobium phaeobacteroides* strain CL1401. Photochem. Photobiol. **71:**715–723.
5. **Arellano, J. B., C. M. Borrego, A. Martínez-Planells, and L. J. Garcia-Gil.** 2001. Effect of carotenoid deficiency on cells and chlorosomes of *Chlorobium phaeobacteroides*. Arch. Microbiol. **175:**226–233.
6. **Arellano, J. B., T. B. Melø, C. M. Borrego, and K. R. Naqvi.** 2002. Bacteriochlorophyll *e* monomers, but not aggregates, sensitize singlet oxygen: implications for a self-photoprotection mechanism in chlorosomes. Photochem. Photobiol. **76:**373–380.
7. **Armstrong, G.** 1999. Carotenoid genetics and biochemistry, p. 321–352. *In* D. E. Cane (ed.), Isoprenoids including carotenoids and steroids, vol. 2. Elsevier, Amsterdam, The Netherlands.
8. **Blankenship, R. E., J. M. Olson, and M. Miller.** 1995. Antenna complexes from green photosynthetic bacteria, p. 399–435. *In* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (ed.), Anoxygenic photosynthetic bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
9. **Blankenship, R. E., and K. Matsuura.** 2003. Antenna complexes from green photosynthetic bacteria, p. 195–217. *In* B. R. Green and W. W. Parson (ed.), Light-harvesting antennas in photosynthesis. Kluwer Academic Publishers, Dordrecht, The Netherlands.
10. **Borrego, C. M., P. D. Gerola, M. Miller, and R. P. Cox.** 1999. Light intensity effects on pigment composition and organisation in the green sulfur bacterium *Chlorobium tepidum*. Photosynth. Res. **59:**159–166.
11. **Brand, M., and G. Drews.** 1997. The role of pigments in the assembly of photosynthetic complexes in *Rhodobacter capsulatus*. J. Basic Microbiol. **37:**235–244.
12. **Britton, G.** 1998. Overview of carotenoid biosynthesis, p. 13–147. *In* G. Britton, S. Liaaen-Jensen, and H. Pfander (ed.), Carotenoids: biosynthesis and metabolism, vol. 3. Birkhäuser, Basel, Switzerland.
13. **Bryant, D. A., E. V. Vassilieva, N.-U. Frigaard, and H. Li.** 2002. Selective protein extraction from *Chlorobium tepidum* chlorosomes using detergents. Evidence that CsmA forms multimers and binds bacteriochlorophyll *a*. Biochemistry **41:**14403–14411.
14. **Castenholz, R. W.** 1989. Genus *Chloroflexus* Pierson and Castenholz 1974a, 7^{AL}, p. 1698–1702. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. Williams & Wilkins, Baltimore, Md.
15. **Eisen, J. A., K. E. Nelson, I. T. Paulsen, J. F. Heidelberg, M. Wu, R. J. Dodson, R. Deboy, M. L. Gwinn, W. C. Nelson, D. H. Haft, E. K. Hickey, J. D. Peterson, A. S. Durkin, J. L. Kolonay, F. Yang, I. Holt, L. A. Umayam, T. Mason, M. Brenner, T. P. Shea, D. Parksey, W. C. Nierman, T. V. Feldblyum, C. L. Hansen, M. B. Craven, D. Radune, J. Vamathevan, H. Khouri, O. White, T. M. Gruber, K. A. Ketchum, J. C. Venter, H. Tettelin, D. A. Bryant, and C. M. Fraser.** 2002. The complete genome sequence of *Chlorobium tepidum* TLS, a photosynthetic, anaerobic green-sulfur bacterium. Proc. Natl. Acad. Sci. USA **99:**9509–9514.
16. **Fernández-González, B., G. Sandmann, and A. Vioque.** 1997. A new type of asymmetrically acting β-carotene ketolase is required for the synthesis of echinenone in the cyanobacterium *Synechocystis* sp. PCC 6803. J. Biol. Chem. **272:**9728–9733.
17. **Foidl, M., J. R. Golecki, and J. Oelze.** 1997. Phototrophic growth and chlorosome formation in *Chloroflexus aurantiacus* under conditions of carotenoid deficiency. Photosynth. Res. **54:**219–226.
18. **Frank, H. A., and R. J. Cogdell.** 1996. Carotenoids in photosynthesis. Photochem. Photobiol. **63:**257–264.
19. **Frigaard, N.-U., and D. A. Bryant.** 2001. Chromosomal gene inactivation in the green sulfur bacterium *Chlorobium tepidum* by natural transformation. Appl. Environ. Microbiol. **67:**2538–2544.
20. **Frigaard, N.-U., S. Takaichi, M. Hirota, K. Shimada, and K. Matsuura.** 1997. Quinones in chlorosomes of green sulfur bacteria and their role in the redox-dependent fluorescence studied in chlorosome-like bacteriochlorophyll *c* aggregates. Arch. Microbiol. **167:**343–349.
21. **Frigaard, N.-U., K. Matsuura, M. Hirota, M. Miller, and R. P. Cox.** 1998. Studies of the location and function of isoprenoid quinones in chlorosomes from green sulfur bacteria. Photosynth. Res. **58:**81–90.
22. **Frigaard, N.-U., G. D. Voigt, and D. A. Bryant.** 2002. *Chlorobium tepidum* mutant lacking bacteriochlorophyll *c* made by inactivation of the *bchK* gene, encoding bacteriochlorophyll *c* synthase. J. Bacteriol. **184:**3368–3376.
23. **Frigaard, N.-U., A. Gomez Maqueo Chew, H. Li, J. A. Maresca, and D. A. Bryant.** 2003. *Chlorobium tepidum*: insights into the structure, physiology, and metabolism of a green sulfur bacterium derived from the complete genome sequence. Photosynth. Res. **78:**93–117.
24. **Frigaard, N.-U., A. Gomez Maqueo Chew, J. A. Maresca, and D. A. Bryant.** Bacteriochlorophyll biosynthesis in green bacteria. *In* B. Grimm, R. Porra, W. Rüdiger, and H. Scheer (ed.), Biochemistry and biophysics of chlorophyll, in press. Kluwer Academic Press, Dordrecht, The Netherlands.
25. **Frigaard, N.-U., Y. Sakuragi, and D. A. Bryant.** 2004. Gene inactivation in the cyanobacterium *Synechococcus* sp. PCC 7002 and the green sulfur bacterium *Chlorobium tepidum* using *in vitro*-made DNA constructs and natural transformation. Methods Mol. Biol. **274:**325–340.
26. **Frigaard, N.-U., H. Li, K. J. Milks, and D. A. Bryant.** 2004. Nine mutants of *Chlorobium tepidum* each unable to synthesize a different chlorosome protein still assemble functional chlorosomes. J. Bacteriol. **186:**646–653.
27. **Fromme, P., P. Jordan, and N. Krauss.** 2001. Structure of photosystem I. Biochim. Biophys. Acta **1507:**5–31.
28. **Garrity, G. M., and J. G. Holt.** 2001. Phylum BVI. *Chloroflexi* phy. nov., p. 427–446. *In* D. R. Boone and R. W. Castenholz (ed.), Bergey's manual of systematic bacteriology, 2nd ed., vol. 1. Springer-Verlag, New York, N.Y.
29. **Garrity, G. M., and J. G. Holt.** 2001. Phylum BXI. *Chlorobi* phy. nov., p. 601–623. *In* D. R. Boone and R. W. Castenholz (ed.), Bergey's manual of systematic bacteriology, 2nd ed., vol. 1. Springer-Verlag, New York, N.Y.
30. **Giuliano, G., L. Giliberto, and C. Rosati.** 2002. Carotenoid isomerase: a tale of light and isomers. Trends Plant Sci. **7:**427–429.
31. **Halfen, L. N., B. K. Pierson, and G. W. Francis.** 1972. Carotenoids of a gliding organism containing bacteriochlorophylls. Arch. Mikrobiol. **82:**240–246.
32. **Hanada, S., and B. K. Pierson.** 2002. The family *Chloroflexaceae*, release 3.11. *In* M. Dworkin (ed.), The prokaryotes: an evolving electronic resource for the microbiological community, 3rd ed. Springer-Verlag, New York, N.Y. [On-line.] http://link.springer-ny.com/link/service/books/10125/.
33. **Heathcote, P.** 2001. Type I photosynthetic reaction centers. Biochim. Biophys. Acta **1507:**1–2.
34. **Koyama, Y., and R. Fujii.** 1999. *cis-trans* carotenoids in photosynthesis: configurations, excited-state properties and physiological functions, p. 161–188. *In* H. A. Frank, A. J. Young, G. Britton, and R. J. Cogdell (ed.), The photochemistry of carotenoids. Kluwer Academic Publishers, Dordrecht, The Netherlands.
35. **Krubasik, P., and G. Sandmann.** 2000. A carotenogenic gene cluster from *Brevibacterium linens* with novel lycopene cyclase genes involved in the synthesis of aromatic carotenoids. Mol. Gen. Genet. **263:**423–432.
36. **Krügel, H., P. Krubasik, K. Weber, H. P. Saluz, and G. Sandmann.** 1999. Functional analysis of genes from *Streptomyces griseus* involved in the synthesis of isorenieratene, a carotenoid with aromatic end groups, revealed a novel type of carotenoid desaturase. Biochim. Biophys. Acta **1439:**57–64.
37. **Lang, H. P., and C. N. Hunter.** 1994. The relationship between carotenoid biosynthesis and the assembly of the light-harvesting LH2 complex in *Rhodobacter sphaeroides*. Biochem. J. **298:**197–205.
38. **Liaaen-Jensen, S., E. Hegge, and L. M. Jackman.** 1964. Bacterial carotenoids. XVII. The carotenoids of photosynthetic green bacteria. Acta Chem. Scand. **18:**1703–1718.
39. **Liaaen-Jensen, S.** 1965. Bacterial carotenoids. XVIII. Aryl-carotenoids from *Phaeobium*. Acta Chem. Scand. **19:**1025–1030.
40. **Linden, H., N. Misawa, T. Saito, and G. Sandmann.** 1994. A novel carotenoid biosynthesis gene coding for ζ-carotene desaturase: functional expression, sequence and phylogenetic origin. Plant Mol. Physiol. **24:**369–379.
41. **Malhotra, H. C., G. Britton, and T. W. Goodwin.** 1970. A novel series of 1,2-dihydro carotenoids. Int. Z. Vitaminforsch. **40:**315–322.
42. **Melø, T. B., N.-U. Frigaard, K. Matsuura, and K. R. Naqvi.** 2000. Electronic energy transfer involving carotenoid pigments in chlorosomes of two green bacteria: *Chlorobium tepidum* and *Chloroflexus aurantiacus*. Spectrochim. Acta Part A **56:**2001–2010.
43. **Montaño, G. A., H.-M. Wu, S. Lin, D. C. Brune, and R. E. Blankenship.** 2003. Isolation and characterization of the B795 light-harvesting baseplate from the chlorosomes of *Chloroflexus aurantiacus*. Biochemistry **42:**10246–10251.
44. **Overmann, J.** 2000. The family *Chlorobiaceae*, release 3.1. *In* M. Dworkin (ed.), The prokaryotes: an evolving electronic resource for the microbiological community, 3rd ed. Springer-Verlag, New York, N.Y. [On-line.] http://link.springer-ny.com/link/service/books/10125/.
45. **Page, R. D. M.** 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12:**357–358.
46. **Paulsen, H.** 1999. Carotenoids and the assembly of light-harvesting complexes, p. 123–135. *In* H. A. Frank, A. J. Young, G. Britton, and R. J. Cogdell (ed.), The photochemistry of carotenoids. Kluwer Academic Publishers, Dordrecht, The Netherlands.
47. **Pierson, B. K., and R. W. Castenholz.** 1974. Studies of pigments and growth in *Chloroflexus aurantiacus*, a phototrophic filamentous bacterium. Arch. Microbiol. **100:**283–305.
48. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
49. **Sakuragi, Y., N.-U. Frigaard, K. Shimada, and K. Matsuura.** 1999. Association of bacteriochlorophyll *a* with the CsmA protein in chlorosomes of the photosynthetic green filamentous bacterium *Chloroflexus aurantiacus*. Biochim. Biophys. Acta **1413:**172–180.
50. **Sandmann, G.** 2002. Molecular evolution of carotenoid biosynthesis from bacteria to plants. Physiol. Plant **116:**431–440.
51. **Schmidt, K., A. Connor, and G. Britton.** 1994. Analysis of pigments: caro-

tenoids and related polyenes, p. 403–461. *In* M. Goodfellow and A. G. O'Donnell (ed.), Chemical methods in prokaryotic systematics. John Wiley and Sons, Chichester, England.

52. **Takaichi, S.** 1999. Carotenoids and carotenogenesis in anoxygenic photosynthetic bacteria, p. 39–69. *In* H. A. Frank, A. J. Young, G. Britton, and R. J. Cogdell (ed.), The photochemistry of carotenoids. Kluwer Academic Publishers, Dordrecht, The Netherlands.
53. **Takaichi, S., Z.-Y. Wang, M. Umetsu, T. Nozawa, K. Shimada, and M. T. Madigan.** 1997. New carotenoids from the thermophilic green sulfur bacterium *Chlorobium tepidum*: 1′,2′-dihydro-γ-carotene, 1′,2′-dihydrochlorobactene, and OH-chlorobactene glucoside ester, and the carotenoid composition of different strains. Arch. Microbiol. **168:**270–276.
54. **Takaichi, S., and H. Oh-oka.** 1999. Pigment composition in the reaction center complex from the thermophilic green sulfur bacterium, *Chlorobium tepidum*: carotenoid glucoside esters, menaquinone and chlorophylls. Plant Cell Physiol. **40:**691–694.
55. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **24:**4876–4882.
56. **Viveiros, M., P. Krubasik, G. Sandmann, and M. Houssaini-Iraqui.** 2000. Structural and functional analysis of the gene cluster encoding carotenoid biosynthesis in *Mycobacterium aurum* A+. FEMS Microbiol. Lett. **187:**95–101.
57. **Wahlund, T. M., C. R. Woese, R. W. Castenholz, and M. T. Madigan.** 1991. A thermophilic green sulfur bacterium from New Zealand hot springs, *Chlorobium tepidum* sp. nov. Arch. Microbiol. **156:**81–90.
58. **Wahlund, T. M., and M. T. Madigan.** 1995. Genetic transfer by conjugation in the thermophilic green sulfur bacterium *Chlorobium tepidum*. J. Bacteriol. **177:**2583–2588.

SUPPLEMENTAL TABLE 1. Primers used for generating gene inactivation constructs

Gene	Primer name	Primer sequence [a]
CT0180	CT0180a2	5’-TGAAGTGGGTTGACGATAATGGC-3’
	CT0180bP	5’-ATGAAGGA**CTGCAG**AAAGGCGGAGT-3’
	CT0180cE	5’-CACGAAGCCC**GAATTC**TGGTTTG-3’
	CT0180d2	5’-AGTCATTGGAGAGCAGGATTTTCA-3’
CT0301	CT0301a1	5’-TCTTCAGGGGTTGTTGCTTTTATG-3’
	CT0301a2	5’-CGATTCCACCAT**CTGCAG**AGGCG-3’
	CT0301b1	5’-TGTCAGCGGCTTCATCCCA-3’
	CT0301b2	5’-GCAATTTTTTACGG**GAATTC**GTGTCTG-3’
CT0323	CT0323a	5’-GCTCCTTCAGCAGTTCACCCAC-3’
	CT0323Sb	5’-GTTACCACCGCTGCGTTCAATCACCACCGCTTTTTAGG-3’
	CT0323Sc	5’-CAAGGTAGTCGGCAAATAATGTGGCGATGCGTTACTTCCATTTC-3’
	CT0323d	5’-TCCTCAGCGGTGACAACCAC-3’
CT0649	CT0649a2	5’-TTTGAACAACCACGCCTTGC-3’
	CT0649bP	5’-TTGCGTCGTCA**CTGCAG**AAACCGTA-3’
	CT0649cE	5’-GCTGGGTCGCTC**GAATTC**GTGATACAC-3’
	CT0649d2	5’-CGGTATTCATACTGCTCCCACTG-3’
CT0807	CT0807a	5’-TGTCGTTGAATCACCCACTGAAG-3’
	CT0807Sb	5’-GTTACCACCGCTGCGTTCAGAAACCTTGCCGCCATAGAT-3’
	CT0807Sc	5’-CAAGGTAGTCGGCAAATAATGTACCGCATTCCCGATTCGT-3’
	CT0807d	5’-GAACGCTTTGGTCAACCCG-3’
CT1357	CT1357a1	5’-TCGAGGCATTGAGACAGGCTG-3’
	CT1357a2	5’-TGGATGTGA**CTGCAG**AGACGGGTT-3’
	CT1357b1	5’-TAGCGAGTGCTCCCAGTATGCC-3’
	CT1357b2	5’-AGGAT**GAATTC**AGCCCTGCCC-3’
CT1386	CT1386a	5’-CGGGCTGTTTCTTTTCCTGG-3’
	CT1386Sb	5’-GTTACCACCGCTGCGTTCTGGCGGCAGTAGTCGTAGG-3’
	CT1386Sc	5’-CAAGGTAGTCGGCAAATAATGTGGTTACAGCAACAAGGAGGCAC-3’
	CT1386d	5’-TGAGCGAAACTGCGGGTGAC-3’
CT1414	CT1414a	5’-CGCTGATGAACCAGATGCCC-3’
	CT1414Sb	5’-GTTACCACCGCTGCGTTCATGACTGCTTGCCGTCCGAC-3’
	CT1414Sc	5’-CAAGGTAGTCGGCAAATAATGTCGATACCGACAACCTGATGTTCTC-3’
	CT1414d	5’-CCTATGTGTTCGCCCTGCTTTC-3’
CT1416	CT1416a1	5’-CTCGGACTTCATCCCCAACAC-3’
	CT1416a2	5’-TCGGAG**CTGCAG**ACGACAAC-3’
	CT1416b1	5’-AGTTGATGGTGGTGGTGACCC-3’
	CT1416b2	5’-ATCATTCTGC**GAATTC**ATTTTCTCC-3’

[a] Italicized sequences are homologous to *C. tepidum* genomic DNA, underlined sequences are homologous to regions on the pSRA2 plasmid that flank the *aadA* marker gene, and sequences in bold indicate a restriction site (either *Eco*RI or *Pst*I).

SUPPLEMENTAL TABLE 2. Primers used for PCR analysis of *C. tepidum* wild type and mutants

Gene	Primers	Size of amplified fragment (bp)	
		Wild type	Mutant
CT0180	CT0180a2 and CT0180d2	1936	2344
CT0301	CT0301a1 and CT0301b1	2127	2421
CT0323	CT0323a and CT0323d	1725	2152
CT0649	CT0649a2 and CT0649d2	1574	2347
CT0807	CT0807a and CT0807d	1539	2060
CT1357	CT1357a1 and CT1357b1	1776	2403
CT1386	CT1386a and CT1386d	1485	2051
CT1414	CT1414a and CT1414d	1818	2162
CT1416	CT1416a1 and CT1416b1	1482	2408

SUPPLEMENTAL TABLE 3. Accession numbers for protein sequences used for phylogenetic analysis

Organism	Protein	GenBank acc. no.	CyanoBase acc. no.
Arabidopsis thaliana	CRTISO	AAF63149 (less 62 N-term. residues)	-
	PDS	Q07356 (less 75 N-term. residues)	-
	PSY	AAM62787 (less 70 N-term. residues)	-
	ZDS	NP_187138 (less 34 N-term. residues)	-
Brevibacterium linens	CrtU	AAF65586	-
Chlorobium tepidum	CrtB	AAM72615	CT1386
	CrtC	AAM71547	CT0301
	CrtH	AAM71888	CT0649
	CrtP	AAM72043	CT0807
	CrtQ	AAM72642	CT1414
	CrtU	AAM71569	CT0323
	CT0180	AAM71428	CT0180
Chloroflexus aurantiacus	CrtB	ZP_00020562 (Chlo3603)	-
	CrtI homolog (ORF2)	ZP_00019255 (Chlo2255)	-
	CrtI homolog, truncated (ORF3)	Fusion of 150 amino acid residues derived from JGI contig 0123 and ZP_00020402 (Chlo3434)	-
	CrtO homolog (ORF1)	Fusion of ZP_00017980 (less two C-terminal residues) and ZP_00017981 (less 19 N-terminal residues)	-
	CrtP homolog, truncated (ORF4)	ZP_00019755 (Chlo2762)	-
	CrtY	ZP_00019083 (Chlo2082)	-
Erwinia herbicola Eho13	CrtB	AAA21264	-
	CrtI	AAA21263	-
Gloeobacter violaceus PCC 7421	CrtU homolog	BAC89864	Gll1923
	CT0180 homolog	BAC90815	Gll2874
Mycobacterium aurum	CrtB	CAB94795	-
	CrtU	CAB94798	-
Myxococcus xanthus	CrtI	Q02861	-

Nostoc sp. PCC 7120	CrtB	BAB73532	Alr1833
	CrtH	BAB73763	Alr2064
	CrtP	BAB73531	Alr1832
	CrtQ (CrtQa)	BAB78339	All7255
	CrtU homolog	BAB74484	Alr2785
	CT0180 homolog	BAB76822	All5123
Rhodobacter sphaeroides	CrtB	T50746	-
	CrtC	CAB38742	-
	CrtI	CAB38739	-
Rubrivivax gelatinosus	CrtB	AAB87738	-
	CrtI	AAO93135	-
Streptomyces griseus	CrtB	P54977	-
	CrtU	AAG28703	-
Synechococcus sp. PCC 7942	CrtL	Q55276	-
	CrtP	CAA39004	-
	CrtQ	CAB65434	-
Synechocystis sp. PCC 6803	CrtB	BAA17848	Slr1255
	CrtH	NP_442727	Sll0033
	CrtO	BAA10561	Slr0088
	CrtP	BAA17847	Slr1254
	CrtQ	BAA18400	Slr0940
	CrtU homolog	BAA17934	Sll0254
	CT0180 homolog	BAA16840	Slr1293
Trichodesmium erythraeum	CrtU homolog	ZP_00074839 (Tery4166)	-