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Bacteriochlorophyllide *c* C-8^2 and C-12^1 Methyltransferases Are Essential for Adaptation to Low Light in *Chlorobaculum tepidum*$^{\nabla}$

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Bacteriochlorophyll (BChl) *c* is the major photosynthetic pigment in the green sulfur bacterium *Chlorobaculum tepidum*, in which it forms protein-independent aggregates that function in light harvesting. BChls *c*, *d*, and *e* are found only in chlorosome-producing bacteria and are unique among chlorophylls because of methylations that occur at the C-8^2 and C-12^1 carbons. Two genes required for these methylation reactions were identified and designated *bchQ* (CT1777) and *bchR* (CT1320). BchQ and BchR are members of the radical *S*-adenosylmethionine (SAM) protein superfamily; each has sequence motifs to ligate a [4Fe-4S] cluster, and we propose that they catalyze the methyl group transfers. *bchQ*, *bchR*, and *bchQ bchR* mutants of *C. tepidum* were constructed and characterized. The *bchQ* mutant produced BChl *c* that was not methylated at C-8^2, the *bchR* mutant produced BChl *c* that was not methylated at C-12^1, and the double mutant produced [8-ethyl, 12-methyl]-BChl *c* that lacked methylation at both the C-8^2 and C-12^1 positions. Compared to the wild type, the Q_y absorption bands for BChl *c* in the mutant cells were narrower and blue shifted to various extents. All three mutants grew slower and had a lower cellular BChl *c* content than the wild type, an effect that was especially pronounced at low light intensities. These observations show that the C-8^2 and C-12^1 methylations of BChl *c* play important roles in the adaptation of *C. tepidum* to low light intensity. The data additionally suggest that these methylations also directly or indirectly affect the regulation of the BChl *c* biosynthetic pathway.

Green sulfur bacteria (GSB) are strictly anaerobic photoautotrophs and have been isolated from highly diverse environments worldwide, from the deep sea to hot springs (1, 21, 26, 35–37). They have evolved a large and highly sophisticated light-harvesting antenna structure, the chlorosome, which is built from self-aggregating bacteriochlorophylls (BChls) that require no protein scaffold for their assembly (7, 11, 13). Depending on the organism, these pigments can be BChl *c*, *d*, or *e*, and these BChls share common features that are not found in other types of chlorophylls (Chls). These "*Chlorobium*" Chls, as they were historically named (17, 18, 27), have a hydroxyl group at the C-3^1 position that can chelate the Mg atom of a neighboring BChl molecule. They also lack the C-13^2 methyl-carboxyl group found in all other Chls and BChls. This methyl-carboxyl group would interfere with the hydrogen-bonding network between the keto group of one molecule and the C-3^1 hydroxyl of a neighboring molecule in a BChl aggregate (19, 33). These modifications contribute to the formation of the large aggregates that make up the core of the chlorosome (13).

The green sulfur bacterium *Chlorobaculum tepidum*, formerly known as *Chlorobium tepidum*, is a moderate thermophile that was isolated from a sulfide-rich hot spring in New Zealand (37). *C. tepidum* makes three types of Chls: BChl *a* esterified with phytol (BChl a_P), Chl *a* esterified with Δ2,6-phytadienol (Chl a_{PD}), and BChl *c* esterified with farnesol (BChl c_F) (5, 14). BChl c_F comprises more than 97% of the total BChl/Chl content in the organism, and each of the chlorosomes in *C. tepidum* typically contains more than 200,000 BChl *c* molecules (24). The naturally occurring BChl c_F in *C. tepidum* is produced as a mixture of four homologs that carry different side chains at the C-8 and C-12 positions (Fig. 1). Radiolabeling experiments in *Chlorobium vibrioforme* showed that these different side chains arise from methylation using *S*-adenosylmethionine (SAM) as the methyl donor (20). The methylation level varies with light intensity: BChl *c* produced by cultures grown at lower light intensities is more highly methylated at the C-8^2 position than the BChl *c* produced by cells of the same organism grown at higher light intensities (2, 3, 20, 29). When *C. tepidum* is grown at a moderate light intensity (50 μE m^{-2} s^{-1}), the homolog distribution is 10% [E,M]-BChl c_F, 60% [E,E]-BChl c_F, 20% [Pr,E]-BChl c_F, and 5% [I,E]-BChl c_F (Fig. 1). 8-Neopentyl BChl *c* homologs have not been detected in *C. tepidum*; however, this bacterium does produce small amounts of BChl *c* that are esterified with alcohols other than farnesol (3).

The methylations at C-8^2 and C-12^1 are not essential for the formation of BChl *c* aggregates or for the assembly of chlorosomes. The filamentous anoxygenic phototroph *Chloroflexus aurantiacus* also synthesizes BChl *c* and assembles chlorosomes, but the BChl *c* produced by *Chloroflexus aurantiacus* is not methylated at the C-8^2 or C-12^1 carbons (23). Moreover, it has been shown that highly purified [E,M]-BChl c_F from *Chlorobium limicola* self-assembles in vitro in various organic solvents to form macroscopic, polymeric aggregates that are similar to those found in chlorosomes (32). Finally, experiments

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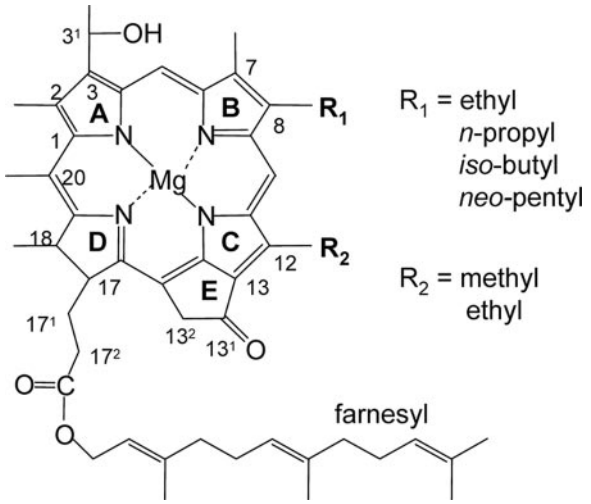

FIG. 1. Structure of BChl *c* molecules found in GSB. In *C. tepidum*, [N,E]-BChl *c* is normally not observed.

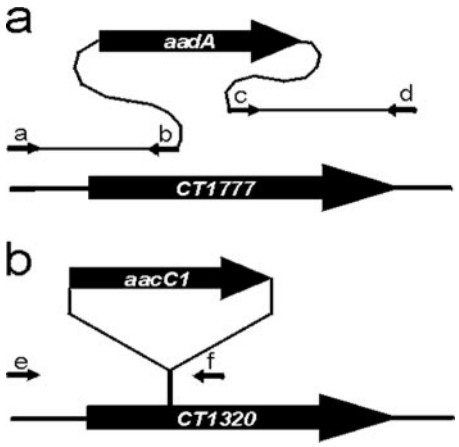

FIG. 2. Physical maps of the constructions used to inactivate the *bchQ* and *bchR* genes of *C. tepidum*. (a) Map of ORF CT1777 (*bchQ*) of *C. tepidum* and the primer binding positions for its inactivation by megaprimer PCR with the streptomycin-spectinomycin resistance-conferring gene *aadA*. (b) Map of *C. tepidum* ORF CT1320 (*bchR*) and primer binding positions for inactivation by insertion of *aacC1* into the coding sequence at a unique BstXI site. See Table 1 for primer sequences. a to f indicate the positions of oligonucleotide primers CT1777a to CT177d, CT1320F, and CT1320R, respectively (Table 1).

with synthetic BChl *e* analogs in organic solvents have also shown that the assembly of these molecules is not affected by varying the side chain at the C-8 position (28). Although the methyl groups in the C-8^2 and C-12^1 positions had been shown to originate from SAM (20), the enzymes responsible for these methylations had not been identified, and the functional roles of these modifications were likewise not well understood. In the studies reported here, we have identified two gene products that are required for the methylations that occur at the C-8^2 and C-12^1 positions of BChl *c* in *C. tepidum*, and we have named these genes *bchQ* and *bchR*, respectively. It is proposed that BchQ is a methyltransferase that adds either one or two methyl groups to the C-8^2 carbon of the ethyl side chain of BChl *c* and that BchR is a methyltransferase that adds a single methyl group to the methyl carbon at the C-12^1 position of BChl *c*. These methylations play a role in fine-tuning the structural arrangement of the BChl *c* aggregates in chlorosomes and therefore directly influence the absorption properties of chlorosomes. We additionally demonstrate that these modifications either directly or indirectly influence the light intensity-dependent regulation of the BChl *c* biosynthetic pathway in *C. tepidum.* We suggest that the methylated BChl *c* intermediates modulate the allosteric regulation of a key enzyme of the pathway.

MATERIALS AND METHODS

Construction of mutants. The plating strain WT2321 (38), derived from strain ATCC 49652 (37) of *C. tepidum*, was used in all experiments. Genes CT0072, CT1502, CT1697, CT1777, CT1903, and CT1959 were targeted for inactivation using the megaprimer PCR method as described previously (10, 12) (Fig. 2a). The primers employed are listed in Table 1, which also includes primer sequences used to amplify genes CT1777 and CT1320 from *C. tepidum* genomic DNA. To inactivate open reading frame (ORF) CT1320, the coding sequence was amplified by PCR, and by using EcoRI and PstI sites introduced via the PCR primers, the resulting amplicon was cloned into plasmid pUC19. The *aadA* gene from plasmid pSRA2 (12), encoding streptomycin and spectinomycin resistance, was subsequently inserted into the unique BstXI site within the coding sequence as shown in Fig. 2a. The megaprimer PCR products or linearized plasmid DNA were used to transform *C. tepidum* as previously described (10, 12). In order to isolate segregated mutants, transformations were plated onto selective plates containing 150 μg streptomycin ml^{-1} and 300 μg spectinomycin ml^{-1} for the mutations made with the *aadA* resistance marker or 100 μg gentamicin ml^{-1} for mutations made with the *aacC1* gene, which confers gentamicin resistance. Single mutants for CT1320 and CT1777 were obtained using the *aadA* marker, and the CT1777/CT1320 double mutant was constructed by transforming the spectinomycin-resistant/streptomycin-resistant CT1777 mutant strain with a construct in which CT1320 was interrupted with *aacC1* (Fig. 2b). The spectinomycin-resistant/streptomycin-resistant mutant CT1777 was weakly resistant to gentamicin, and therefore, the antibiotic concentration on the selective plates was increased to 400 μg gentamicin ml^{-1} in order to obtain fully segregated double mutants. The plates were cultivated anaerobically at 42°C for 5 to 10 days, and single colonies were transferred onto new selective plates. After four transfers, single colonies were used to start liquid cultures, and the transformants were checked for segregation by analytical PCR as previously described (9, 12).

A CT1777/CT1320 double mutant was used to construct a BChl *d*-producing strain lacking methylation at the C-8^2 and C-12^1 positions. A chloramphenicol and erythromycin resistance-conferring fragment from pRL409 was inserted into plasmid pCFT (22) at the unique KpnI site. This plasmid was linearized with restriction endonuclease AhdI and used to transform mutant strains. The transformants were plated onto selective plates with 5 μg erythromycin ml^{-1} in addition to the antibiotics described above. The cultures were grown as described above and were checked for segregation by analytical PCR.

Growth rate calculations. Cells of the *C. tepidum* wild type and of the *bchQ*, *bchR*, and *bchQ bchR* mutant strains were grown in a radial rotator in liquid medium at a temperature between 45°C and 46.5°C as described previously (10). Cultures were grown at light intensities of 8 μE m^{-2} s^{-1}, 20 μE m^{-2} s^{-1}, 100 μE m^{-2} s^{-1}, 150 μE m^{-2} s^{-1}, and 350 μE m^{-2} s^{-1}, provided by incandescent light bulbs. The optical density at 600 nm (OD_{600}) of the cultures was recorded by absorbance with a Genesys spectrophotometer (ThermoSpectronic, Rochester, NY) at intervals of 4 to 8 h depending on the light intensity. Growth rate measurements were performed in triplicate for each strain and light intensity.

Pigment analysis. Cultures of the *C. tepidum* wild type and of the *bchQ*, *bchR*, and *bchQ bchR* mutants were grown at different light intensities as described above. The cells from 1.0 ml of a liquid culture were pelleted by centrifugation, and the pigments were extracted with sonication with acetone-methanol (7:2, vol/vol) and then immediately subjected to analytical high-performance liquid chromatography (HPLC). Reverse-phase HPLC was used to resolve the peaks of the different species of BChl *c* as described previously (8). The resulting data were analyzed using ChemStation Rev. A.10.03 software (Agilent Technologies, Palo Alto, CA) and confirmed by HPLC-mass spectrometry on a Micromass Quattro II mass spectrometer equipped with a Shimadzu (Columbia, MD) LC10ADvp pump. The resulting data were analyzed with MassLynx software (version 3.5; Micromass, Ltd., Manchester, United Kingdom). Protein-to-BChl ratios were obtained by extracting the cells from 1 ml of pelleted culture with

TABLE 1. Primer sequences used for amplification of CT1777 and CT1320 genes from *C. tepidum* genomic DNA

Primer	Sequence[a]
CT1777a	5′-TGGACGATTGAAACAGCCTCC-3′
CT1777b	5′-GTTACCACCGCTGCGTTCGCCAACCACGATACAGTCTGC-3′
CT1777c	5′-CAAGGTAGTCGGCAAATAATGTGCGACATCACCTTCAGCG-3′
CT1777d	5′-GAACTCTTTTTTCCCTCATCCATTC-3′
CT1320F	5′-CAGGAATTCAATGCCAACCACCTC-3′
CT1320R	5′-CGGAAGCTCTTGCTGCAGACCGTC-3′
CT0072a	5′-AATCGTGCTGCGTGCTATGC-3′
CT0072b	5′-TACCACCGCTGCGTTCGGCTTTGAGCGAATCGG-3′
CT0072c	5′-CAAGGTAGTCGGCAAATAATGTCCCACAAGAAGACGCAGAACC-3′
CT0072d	5′-GCATCCACCAAGAACAATGAGG-3′
CT1502a	5′-CGTCGTCCTCTCTCGGTTCTTCT-3′
CT1502b	5′-TACCACCGCTGCGTTCGGAAAGGTCGGGAAATGC-3′
CT1502c	5′-CAAGGTAGTCGGCAAATAATGTCAACGCCGACGAAGCCTAC-3′
CT1502d	5′-TGCCGCCGCAAGTCTTTCCT-3′
CT1697a	5′-GCAAGCGGCTGGAAGAACTG-3′
CT1697b	5′-CACCGCTGCGTTCGGTCATAGACGAGATGGCAAC-3′
CT1697c	5′-AGGTAGTCGGCAAATAATGTCTGGAAGGAGAACGGCGTG-3′
CT1697d	5′-TCGGGTAGGGCATCGTGTCC-3′
CT1903a	5′-GGTGAAGCGTAACATCGGTGTG-3′
CT1903b	5′-GTTACCACCGCTGCGTTCGCACGAAATAGTCAACCTCGGG-3′
CT1903c	5′-CAAGGTAGTCGGCAAATAATGTCAAAATCCGCACCAAAACCAG-3′
CT1903d	5′-TTGTCGCCGCTTGAATGGG-3′
CT1959a	5′-CGAGCAGATGGTCAACAAAACC-3′
CT1959b	5′-GTTACCACCGCTGCGTTCAACGCACCGCCGATGTAG-3′
CT1959c	5′-CAAGGTAGTCGGCAAATAATGTCGACAAAGAGACACGCTCCG-3′
CT1959d	5′-CCGCTACGATGCCGTTTTTC-3′

[a] EcoRI and PstI sites are underlined.

methanol, determining the BChl *c* content by absorbance, and then measuring the protein content by using a P5656 protein assay kit (Sigma, St. Louis, MO) according to instructions provided by the manufacturer.

Chlorosome analysis. Batch cultures of cells (2 liters) were grown at 46°C at a light intensity of approximately 100 μE m^{-2} s^{-1} provided by incandescent light bulbs and harvested when the cultures reached an OD_{600} of >2. The chlorosomes were isolated as described previously (4, 11, 34). The in vivo extinction coefficient was determined as described previously (22) by using equivalent volume dilutions in methanol to determine the BChl *c* content and in buffer (10 mM potassium phosphate, 50 mM sodium chloride [pH 7.0]) to determine the in vivo molar extinction coefficient of each chlorosome preparation. Molar extinction coefficients were calculated using the molar extinction coefficients for BChl *c* in methanol as reported previously (31). The in vivo extinction coefficients were calculated using the molar extinction coefficient at 669 nm of BChl *c*, 69,737 M^{-1} cm^{-1} in methanol, for the wild-type chlorosomes by assuming 5% [E,M]-BChl *c*, 60% [E,E]-BChl *c*, 30% [Pr,E]-BChl *c*, and 5% [I,E]-BChl *c* homolog distribution; a molar extinction coefficient at 669 nm of 69,256 M^{-1} cm^{-1} and 5% [E,M]-BChl *c* and 95% [E,E]-BChl *c* homolog distribution for the chlorosomes from the *bchQ* mutant; a molar extinction coefficient at 669 nm of 68,834 M^{-1} cm^{-1} and a homolog distribution of 50% [E,M]-BChl *c*, 40% [Pr,M]-BChl *c*, and 10% [I,M]-BChl *c* for the chlorosomes from the *bchR* mutant; and a molar extinction coefficient at 669 nm of 68,112 M^{-1} cm^{-1} and a homolog distribution of 100% [E,M]-BChl c_F for chlorosomes from the *bchQ bchR* mutant. The approximate molar absorptivities of BChl *c* in the Q_y region (~750 nm) of chlorosomes were determined from three independent chlorosome preparations and in triplicate for each sample. The ratio of pigments to total chlorosome protein was obtained by extracting an aliquot (10 μl) of a chlorosome preparation in acetone (1 ml) overnight at −20°C. To determine the BChl *c* content, chlorosomes were diluted in methanol (1:20, vol/vol), and the absorbance was measured at 669 nm. The minor contribution of BChl *a* to the absorption was ignored since greater than 95% of the BChl in chlorosomes from *C. tepidum* was BChl *c* (Table 2), and carotenoids absorb only at wavelengths shorter than 550 nm. The protein content was determined for whole cells as described above by using a P5656 protein assay kit (Sigma, St. Louis, MO) as recommended by the manufacturer.

TABLE 2. Pigment contents in isolated chlorosomes

Strain	Pigment content (μg pigment/mg of protein)		
	BChl *a*	BChl *c*	Carotenoids
Wild type	125 ± 15	2,394 ± 112	15 ± 0
bchQ	108 ± 4	2,580 ± 205	16 ± 2
bchR	113 ± 1	2,096 ± 27	17 ± 0
bchQ bchR	114 ± 5	1,965 ± 52	23 ± 1

Electron microscopy. For transmission electron microscopy, chlorosome preparations were adsorbed onto Formvar-coated copper grids, negatively stained with 1% (wt/vol) uranyl acetate, and observed on a JEOL 1200EXII electron microscope as described previously (4). To calculate the average dimensions of wild-type and mutant chlorosomes, micrographs of the negatively stained chlorosome preparations were recorded at three initial magnifications (×70,000, ×80,000, and ×100,000), and the lengths and diameters of individual chlorosomes were measured. A total of 100 ± 10 individual chlorosomes in 9 to 14 micrographs were measured to obtain an average length and width for each strain.

RESULTS

Identification of genes encoding BChl *c* methyltransferases in *C. tepidum*. The addition of a methyl group to an unactivated methyl carbon, such as the C-8^2 and C-12^1 positions of BChl *c*, is a chemically difficult reaction. Strictly anaerobic microorganisms cannot use molecular oxygen to activate such substrates, so these organisms presumably rely on radical chemistry to catalyze reactions of this type (5, 14). Mg-protoporphyrin IX monomethyl ester oxidative cyclase, predicted to be encoded by the *bchE* gene (CT1959) in *C. tepidum* (6), is the enzyme responsible for the introduction of a keto group at a methylene carbon of the propionate methyl ester side chain of the C ring of Mg-protoporphyrin IX monomethyl ester. BchE is additionally believed to catalyze the formation of the isocyclic ring of BChls in *Rhodobacter capsulatus*, and by analogy, it is also assumed to perform the same functions in *C. tepidum* (6). Analogously to the activation of the C-8^2 and C-12^1 carbons for methylation, these reactions require the activation of a methylene carbon of the C-13 propionate side chain through radical chemistry (25). Consistent with this possibility, Sofia et al. (30) previously identified BchE as being a member of the radical SAM superfamily of proteins, which notably includes several methyltransferases (16). The presence of six homologs of *bchE* (CT1959) in the *C. tepidum* genome therefore strongly suggested that some of these homologs might be involved in BChl *c* methylation.

ORFs CT0072, CT1320, CT1502, CT1697, CT1777, and CT1903 encode the homologs of BchE in *C. tepidum* (6). We were able to insertionally inactivate each of these six genes, but it was not possible to inactivate CT1959. This result is consistent with the expectation that CT1959 encodes BchE, an essential enzyme for BChl *a* and Chl *a* biosynthesis, since *C. tepidum* is obligately photoautotrophic (5, 14). Four of the six

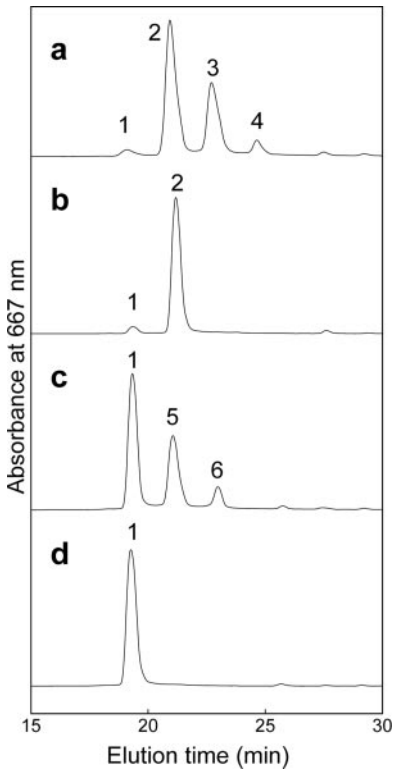

FIG. 3. A portion of the HPLC elution profiles of pigments extracted from *C. tepidum* wild-type and mutant cells recorded at 667 nm. (a) Wild-type *C. tepidum*. (b) *bchQ* mutant. (c) *bchR* mutant. (d) *bchQ bchR* mutant. Peak 1 is [E,M]-BChl *c*, peak 2 is [E,E]-BChl *c*, peak 3 is [Pr,E]-BChl *c*, peak 4 is [I,E]-BChl *c*, peak 5 is [Pr,M]-BChl *c*, and peak 6 is [I,M]-BChl *c*.

mutants (CT0072, CT1502, CT1903, and CT1697) produced BChl *c* that had the same methylation pattern as BChl *c* of the wild type (data not shown). However, CT1777 and CT1320, which will hereafter be referred to as *bchQ* and *bchR*, respectively, showed altered methylation patterns for BChl *c* that were consistent with the absence of the C-8^2 and C-12^1 methyltransferase activities, respectively (Fig. 3). It is formally possible that BchQ adds only the first methyl group at C-8^1 and that another enzyme performs the second methylation reaction. However, we believe that this is very unlikely, since, as noted above, none of the other tested genes encoding radical SAM proteins in the genome of *C. tepidum* had any effect on BChl *c* synthesis. Methylation of the C-8^2 position of the C-8 *n*-propyl side chain of [Pr,E]-BChl *c* to produce an *iso*-butyl side chain would likely require a chemical mechanism similar to that for the addition of the first methyl group. Complete or nearly complete genome sequence data are now publicly available for 10 strains of GSB. Database searches and sequence analyses show that each of these genomes encodes one or two genes with the greatest similarity to *bchQ* and one or two genes with the greatest similarity to *bchR* (data not shown).

Pigment analysis of mutants. The differently methylated species of BChl *c* were resolved by HPLC (8). Figure 3a shows the elution profile of a pigment extract from wild-type *C. tepidum* recorded at 667 nm, the absorbance maximum of BChl *c* in the HPLC solvent system used. The elution profile reveals four obvious peaks (Fig. 3a), which were identified by their absorption spectra and molecular masses (29): peak 1, [E,M]-BChl c_F ($M^+ = 792$); peak 2, [E,E]-BChl c_F ($M^+ = 806$); peak 3, [Pr,E]-BChl c_F ($M^+ = 820$); and peak 4, [I,E]-BChl c_F ($M^+ = 834$). Previous analyses have shown that the species [Pr,M]-BChl c_F and [I,M]-BChl c_F are present in very small amounts in the wild type and are not always resolved in this kind of analysis (20, 27). When the pigment extract of the *bchQ* mutant was analyzed by this method, only the [E,M]-BChl c_F and [E,E]-BChl c_F peaks were observed (Fig. 3b). This indicates that methylation occurs only at the C-12^1 carbon in this mutant. The HPLC elution profile of the pigment extract from the *bchR* mutant exhibited three peaks (Fig. 3c), the first of which corresponds to [E,M]-BChl c_F. Peaks 5 and 6 (Fig. 3c) had elution times similar to those of [E,E]-BChl c_F and [Pr,E]-BChl c_F, and their masses were 806 Da and 820 Da, respectively. However, given the relative distribution of peaks 1, 5, and 6 in the *bchR* mutant (Fig. 3c) compared with the wild type (Fig. 3a) and the identification of *bchQ* as a C-8^2 methyltransferase, *bchR* must encode the C-12^1 methyltransferase. This implies that peaks 5 and 6 are actually [Pr,M]-BChl c_F and [I,M]-BChl c_F, respectively. Consistent with this interpretation, the pigment extract from the mutant lacking functional copies of both *bchQ* and *bchR* exhibits only a single peak (Fig. 3d) with an elution time and mass (792 Da) corresponding to [E,M]-BChl c_F. Compared to the wild type, a larger amount of BChl *c* was esterified with alcohol tails other than farnesol, e.g., geranylgeraniol, in the *bchQ bchR* double mutant (data not shown).

Effects on the in vivo absorption properties. The absorption spectra of the mutant cells were different from those of the wild type and varied with the light intensity at which the cells were grown. Both the position of the absorbance maximum and the half-bandwidth (bandwidth at half-maximal absorbance) for the Q_y absorption peak of BChl *c* aggregates in the mutants were altered, and these effects were most pronounced when the cells were grown at a very low light intensity (8 μE m^{-2} s^{-1}) (Fig. 4). When wild-type *C. tepidum* was grown at different light intensities, the half-bandwidth increased from 45 nm when the cells are grown in very high light (~350 μE m^{-2} s^{-1}) to 57 nm in very low light (8 μE m^{-2} s^{-1}) (Fig. 5a). Although the magnitude of the increase was smaller, a similar effect was observed for the *bchQ* mutant. The half-bandwidth of the Q_y absorption peak of BChl *c* aggregates increased from 42 nm at high light intensity to 47 nm at low light intensity. The half-bandwidth of the Q_y absorption peak of BChl *c* aggregates was 36 nm for both the *bchR* mutant and the *bchQ bchR* double mutant, and the bandwidth did not change as a function of the light intensity in mutants lacking BchR (Fig. 5a).

Consistent with data from previous reports (2, 3), the in vivo absorbance maximum of the Q_y peak of BChl *c* aggregates of chlorosomes of wild-type cells was 747 nm at 100 μE m^{-2} s^{-1}, and when cells were grown at lower light intensities (8 μE m^{-2} s^{-1}), this absorption maximum increased by up to 6 nm (Fig. 4c) (2, 3, 29). Compared to the wild type and at all light intensities tested, the in vivo Q_y absorbance maxima of the BChl *c* aggregates of chlorosomes in intact mutant cells were shifted to shorter wavelengths (Fig. 4a, b, and c). The Q_y absorbance maxima of the BChl *c* aggregates in cells of the three mutants exhibited a progressive shift to longer wave-

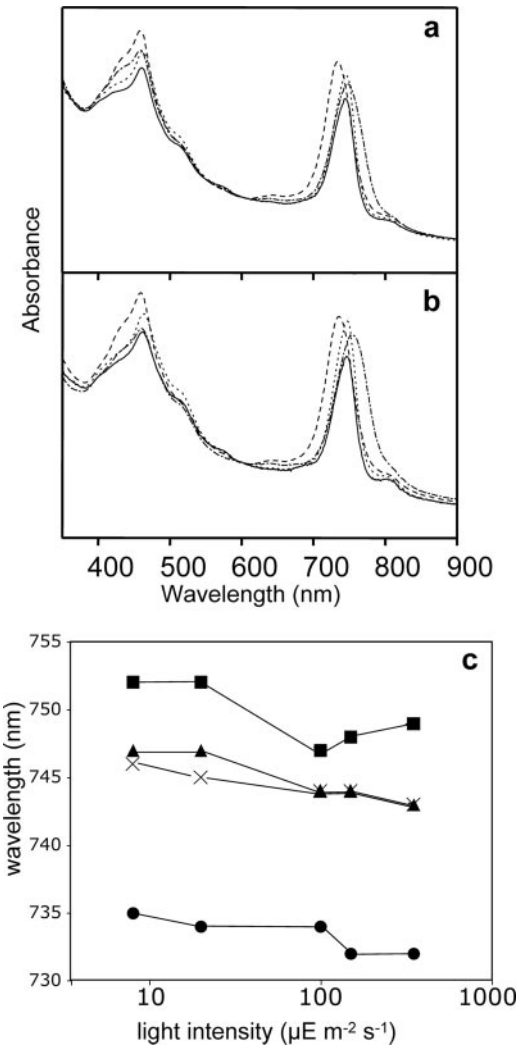

FIG. 4. Absorption spectra of whole cells of wild-type *C. tepidum* (dotted-dashed line) and *bchQ* (dashed line), *bchR* (dotted line), and *bchQ bchR* (solid line) mutants grown in liquid culture. (a) Absorption spectra of cultures grown at high light intensity (350 μE $m^{-2}s^{-1}$). The Q_y peak absorption maxima are 750 nm for the wild type, 731 nm for the *bchQ* mutant, and 744 nm for the *bchR* and *bchQ bchR* mutants. (b) Absorption spectra of cultures grown at low light intensity (8 μE m^{-2} s^{-1}). The Q_y absorption maxima are 756 nm for the wild type, 734 nm for the *bchQ* mutant, and 747 nm for the *bchR* and *bchQ bchR* mutants. Spectra were normalized at OD_{600}. (c) The Q_y absorption maxima of whole cells of the *C. tepidum* wild type (■) and the *bchQ* (●), *bchR* (▲), and *bchQ bchR* (×) mutants are plotted as a function of the growth light intensity.

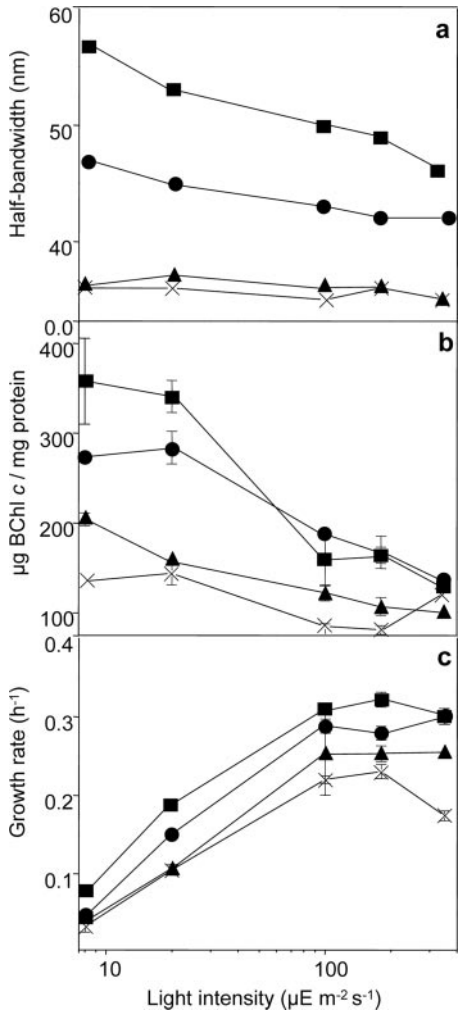

FIG. 5. Physiological characteristics of *C. tepidum* wild-type and mutant cell cultures at various light intensities. (a) Bandwidth at half-maximal absorption of the Q_y band of BChl *c* aggregates in whole cells as a function of growth light intensity. (b) Ratio of μg BChl *c* per mg cellular protein as a function of growth light intensity. (c) Growth rate of the wild-type and mutant strains as a function of light intensity. Symbols: ■, wild type; ●, *bchQ* mutant; ▲, *bchR* mutant; ×, *bchQ bchR* mutant.

lengths as the light intensity decreased, but the maximum shift observed, only ~3 nm, was smaller than that for the wild type. The Q_y absorbance maximum for the chlorosomes of the *bchQ* mutant showed the greatest shift of the three mutant strains: a blue shift of 15 nm relative to the wild type (λ_{max} of 732 nm at optimal light intensities). This value shifted to red by ~3 nm to 735 nm at low light intensity. When cells were grown at 100 μE m^{-2} s^{-1}, the Q_y absorbance maxima of the BChl *c* aggregates of chlorosomes in the *bchR* and *bchQ bchR* cells had absorbance maxima at 744 nm; this maximum shifted ~3 nm to 747 nm at low light intensity (Fig. 4c).

Effects on pigment content. In order to adapt to environments in which light may be limiting, wild-type *C. tepidum* cells increase their BChl *c* content as the light intensity decreases (3, 29) (Fig. 5b). Analyses of the ratios of pigment to total cellular protein for the wild-type and mutant strains revealed that the mutant lacking a functional *bchQ* gene had 26% less BChl *c* than the wild-type cells at low light intensities (Fig. 5b). The BChl *c* content of the *bchQ* mutant nevertheless still decreased with increasing light intensity and followed a trend similar to that observed in the wild-type cells. At saturating light intensities (>180 μE m^{-2} s^{-1}), the ratio of BChl *c* to cell protein was the same as that observed for the wild type. The *bchR* mutant cells had a much lower ratio of BChl *c* to cell protein than wild-type cells at all light intensities tested, and these cells exhibited only a slight decrease in BChl *c* content as the light intensity increased. The cells of the double mutant had a very

low ratio of BChl *c* to protein, which was only ~40% of that of wild-type cells at low light intensities; interestingly, the ratio of BChl *c* to cell protein remained constant and did not change as a function of light intensity in the cells of the *bchQ bchR* mutant (Fig. 5b).

The ratio of pigment to total protein was also determined for isolated chlorosomes of various strains. The ratios of BChl *c* to protein for chlorosomes of the *bchQ*, *bchR*, and *bchQ bchR* mutants were 108%, 87%, and 82%, respectively, of the value for wild-type chlorosomes. The ratios of BChl *a* to chlorosome protein were 86% of that of the wild type for *bchQ*, 90% for *bchR*, and 91% for the *bchQ bchR* double mutant (Table 2). The ratios of carotenoids to chlorosome protein of the wild type and the *bchQ* mutant were similar, but chlorosomes from the *bchR* mutant had 13% more carotenoids than protein. Chlorosomes from the *bchQ bchR* mutant contained 50% more carotenoids relative to protein than did wild-type chlorosomes.

Effects on growth rate. The *bchQ* mutant had a growth rate that was indistinguishable from that of the wild type when this strain was grown at light intensities of 100 μE m^{-2} s^{-1} and higher. However, when the *bchQ* mutant strain was grown at lower light intensities, the growth rate dropped to 60% of that of the wild type at the lowest light intensity tested (8 μE m^{-2} s^{-1}) (Fig. 5c). The *bchR* mutant and the *bchQ bchR* double mutant exhibited even more drastic growth phenotypes. The *bchR* mutant grew at 53% of the rate of the wild-type strain at a very low light intensity (8 μE m^{-2} s^{-1}) and could grow only at up to 85% of the growth rate of the wild type at optimal light intensities (100 μE m^{-2} s^{-1} and higher). The *bchQ bchR* double mutant could grow at a rate that was up to only 72% of that of the wild type at optimal light intensities, but the double mutant could grow only at 41% of the wild-type rate at a low light intensity (Fig. 5a).

Absorption properties of BChl *c* in isolated chlorosomes. The approximate molar absorption coefficients of the Q_y peaks (based upon BChl *c* content) for the aggregated BChl *c* in intact, isolated chlorosomes were 52,420 liters mol^{-1} cm^{-1} for the wild type, 39,417 liters mol^{-1} cm^{-1} for the *bchQ* mutant, 54,746 liters mol^{-1} cm^{-1} for the *bchR* mutant, and 66,719 liters mol^{-1} cm^{-1} for the *bchQ bchR* double mutant. (Fig. 6). The mutant chlorosomes had integrated molar absorptions of the Q_y peaks (670 nm to 820 nm) that were 80% of the wild-type chlorosomes for the *bchQ* mutant, 85% for the *bchR* mutant, and 96% for the *bchQ bchR* mutant. However, the most important change in the absorption properties of the wild-type chlorosomes versus the mutant chlorosomes is in the far-red region of the visible light spectrum above 740 nm, where the wild-type chlorosomes have integrated absorbances that are 39%, 26%, and 19% greater than those of the chlorosomes from the *bchQ*, *bchR*, and *bchQ bchR* mutants, respectively. Consistent with the results obtained from whole cells, we observed that the chlorosomes from the mutants lacking the ability to add a methyl group at the C-12^1 position had a half-bandwidth that was narrower than that of the wild-type chlorosomes. The wild type and chlorosomes from the *bchQ* mutant had half-bandwidths of 56 nm, while the chlorosomes from the *bchR* mutant had a half-bandwidth of 44 nm, and the chlorosomes from the *bchQ bchR* mutant had a half-bandwidth of only 39 nm.

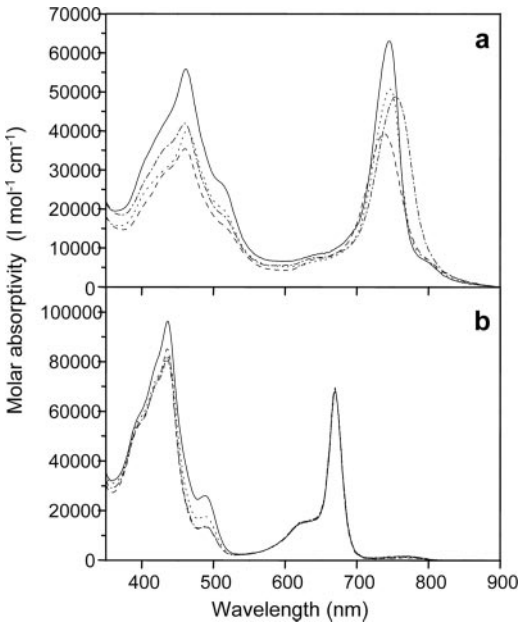

FIG. 6. Approximate molar absorptivity spectra plotted on the basis of BChl *c* content in isolated chlorosomes recorded in aqueous buffer (10 mM potassium phosphate, 50 mM sodium chloride [pH 7.0]) (a) and methanol (b). Chlorosomes were prepared from wild-type *C. tepidum* (dotted-dashed line) and the *bchQ* (dashed line), *bchR* (dotted line), and *bchQ bchR* (solid line) mutants. Since greater than 95% of the BChl in chlorosomes is BChl *c*, the spectra in the Q_y region (~750 nm) are essentially molar absorptivity plots. In the region below 550 nm, there are significant contributions to the absorbance from carotenoids and the Soret absorption band of BChl *a*.

Chlorosome size. Chlorosomes isolated from wild-type *C. tepidum* cells were shown by electron microscopy to have an average length of 142 ± 31 nm and an average width of 57 ± 13 nm. The wild-type chlorosomes are very uniform in size and shape (Fig. 7a); however, the average sizes of the chlorosomes differed greatly in the three mutant strains. Chlorosomes from the *bchQ* mutant had an average length of 129 ± 34 nm and an average width of 44 ± 13 nm (Fig. 7b). Chlorosomes from the *bchR* mutant had an average length of 83 ± 29 nm and an average width of 42 ± 17 nm (Fig. 7c). The chlorosomes from the *bchQ bchR* mutant were much smaller than those isolated from *bchQ* and *bchR* single mutants and had an average length of 64 ± 14 nm and an average width of 38 ± 10 nm (Fig. 7d). To validate the observation that the chlorosomes from the *bchQ* mutant appeared to be more irregular in shape than those of the wild type or the *bchR* and *bchQ bchR* mutants, the length-to-width ratio was calculated as a reflection of the overall shape and its variability. The wild-type chlorosomes had a length-to-width ratio of 2.6 ± 0.25, the *bchQ* chlorosomes had a ratio of 3.0 ± 0.88, the *bchR* chlorosomes had a ratio of 2.1 ± 0.24, and the *bchQ bchR* chlorosomes had a ratio of 1.8 ± 0.25. These data are consistent with the notion that chlorosomes of the *bchQ* mutant exhibit greater size variation than is observed in the wild type and the other mutants.

BChl *d* content of a *bchQ bchR bchU* mutant. The difference between BChl *d* and BChl *c* is the presence of a methyl group at the C-20 carbon in the latter. The presence of a C-20 methyl group changes the absorbance spectrum of BChl *c* by shifting

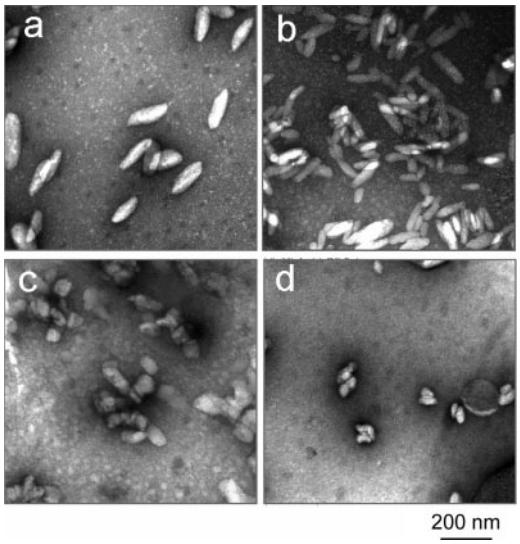

FIG. 7. Transmission electron micrographs of chlorosomes isolated from the wild type (a) and *bchQ* (b), *bchR* (c), and *bchQ bchR* (d) mutants. The bar indicates 200 nm.

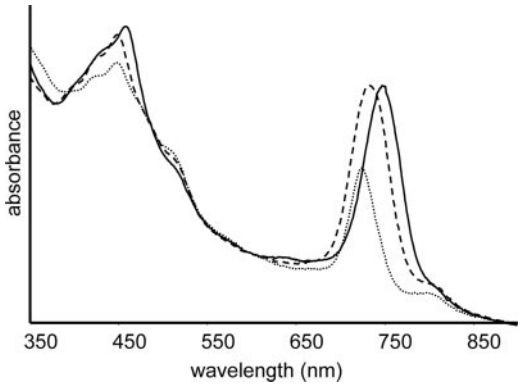

FIG. 8. Absorption spectra of whole cells of wild-type *C. tepidum* (solid line) and *bchU* (dashed line) and *bchQ bchR bchU* (dotted line) mutants grown in liquid medium. The absorption spectra were normalized for cell density at OD_{600}.

the Soret, Q_x, and Q_y absorption peaks to a longer wavelength. The enzyme encoded by the *bchU* gene is responsible for the addition of this C-20 methyl group (22). To produce a *C. tepidum* mutant strain that cannot modify bacteriochlorophyllide (BChlide) *d* by methylation, a triple mutant was constructed by inactivating the *bchQ*, *bchR*, and *bchU* genes. At equal cell densities, the integrated absorbance of the Q_y of the *bchQ bchR bchU* mutant is only 45% of that of the *bchQ bchR* mutant. This indicates that the amount of BChl *d* produced by this mutant is only ~20% of that found in the wild-type BChl *c*-producing strain (Fig. 8). This is ~20% lower than the amount of BChl *c* found in the *bchQ bchR* double mutant under the same conditions (see Fig. 5b and above) and is consistent with the lower BChl *d* content previously reported for the *bchU* mutant (22). Thus, a *C. tepidum* mutant that is unable to methylate BChl *c* at C-20, C-8, and C-12 produces much less BChl *c* than the wild type.

DISCUSSION

Since *C. aurantiacus* produces chlorosomes from BChl *c* that are not modified at either the C-8^2 or C-12^1 positions, the methylations that occur at these positions in GSB and other chloroflexi (e.g., *Chloronema* sp.) (15) are obviously not required for the assembly of BChl *c* aggregates or chlorosomes. With over 200,000 BChl *c* molecules per chlorosome (24) and up to 30% of the cellular carbon being present as BChl *c* (3), significant amounts of energy and cellular carbon go into the addition of these methyl groups to BChl *c*. Until the present study, it was obvious why these organisms would use such large amounts of cellular resources in methylating the BChl *c* molecule when they can produce a fully functional chlorosome without these modifications. Previous studies showed that an increase in the proportion of highly methylated BChl homologs was correlated with a red shift of the absorbance maximum of the Q_y peak (2, 20, 29), but no clues on what other effects, if any, these methylations might have on the chlorosomes were suggested.

Prior to the completion of the genome sequence of *C. tepidum*, no candidate genes had been found to be responsible for these methylations. The addition of a methyl group at any of the three possible C-8^2 positions or the C-12^1 position requires that the methyl carbons be activated in the absence of oxygen. This activation is likely possible only through radical intermediates, and radical SAM chemistry provided a reasonable mechanism by which these methylations could occur (16, 39). By searching among predicted ORFs in the genome sequence, we identified seven genes that encoded members of the BchE/P-methyltransferase family of the radical SAM proteins. We showed by gene inactivation and biochemical analyses that two of these radical SAM proteins are necessary for the methylations at the C-8^2 and C-12^1 positions, and we suggest that these proteins are in fact methyltransferases. Additionally, by constructing *C. tepidum* mutants that lack the ability to modify the C-8^2 and C-12^1 carbons of BChl *c*, we have been able to observe the effects that the absence of such modifications have on the physiology of the organism and on the chlorosomes themselves.

Mutants that lack one or both of the C-8^2 or C-12^1 methyltransferases have impaired growth relative to the wild type. The mutant phenotype is accentuated at low light intensities, and the deficiencies in growth rate can be attributed largely to the reduced amount of BChl *c* found in the mutant cells. Unlike natural strains of GSB (2, 20, 29), the *C. tepidum* mutant strains lacking the C-8^2 or C-12^1 methyltransferase functions are limited in their ability to modify their cellular content of BChl *c* in response to changes in light intensity (Fig. 5b). For example, the BChl *c* content of the *bchQ bchR* mutant strain, relative to the amount of total cell protein, is constant regardless of the light intensity and the growth rate. However, in wild-type *C. tepidum*, the amount of BChl *c* per cell increases by 280%, and the half-bandwidth of the Q_y absorbance peak broadens as the light intensity decreases. The *bchQ bchR* mutant, which lacks the ability to methylate BChl *c* and makes chlorosomes containing exclusively [E,M]-BChl *c*, has an absorption band with a significantly more narrow half-bandwidth than wild-type *C. tepidum*. This means that the range of avail-

able wavelengths of light in the far-red region that can be utilized by the mutant organism is narrower than that for the wild-type organism and that the absorption cross section is therefore smaller. Because the overall light-harvesting potential of the mutants at limiting light intensities is drastically reduced, each of the mutants grows more slowly than the wild type.

In addition to their reduced contents of BChl *c*, mutant cells also have altered in vivo absorption spectral properties. When we compared the in vivo absorption spectra of the mutant cultures with that of the wild type, we observed a shift in the absorption maxima of the Q_y peak in the mutants to a shorter wavelength. Such a blue shift of the absorbance maximum results in two conditions that could limit the growth rate. First, it could reduce the efficiency of excitation energy transfer from BChl *c* aggregates to the BChl *a*-CsmA complexes of the baseplate. Second, since the incandescent light used for growth is enriched in red and near-infrared wavelengths, a blue shift would significantly reduce the amount of light harvested by the mutant for growth. In natural environments, this would additionally produce a greater overlap of the chlorosome absorption spectrum with that of the Chl *a*-containing complexes of cyanobacteria, algae, and plants. The absorption spectrum of the *bchQ bchR* mutant cells is more similar to that of the *bchR* mutant (only lacking methylation at C-12^1) than to that of the *bchQ* mutant (lacking methylation at C-8^2). This observation suggests that BChl *c* aggregates comprised of mostly [E,E]-BChl *c* differ in structure from aggregates comprised of [E,M]-BChl *c*. This finding is consistent with in vitro studies of BChl *c* aggregation (28). This is also consistent with the observation that the chlorosomes of the *bchQ* mutant are more irregularly shaped than the smaller but more uniformly shaped chlorosomes of the *bchQ bchR* mutant (Fig. 7) It is possible that these properties are due to the absence of some BChl *c* with *S* stereochemistry at the C-3^1 chiral carbon; the amount of these stereoisomers increases as the degree of methylation at the C-8^2 position increases (13).

One of the possible roles for the C-8^2 and C-12^1 methylations of BChl *c* is that they might stabilize the formation of larger BChl aggregates. The larger aggregates could assemble a larger chlorosome containing a greater number of BChl *c* molecules, which would in turn allow an organism to increase its relative BChl *c* content. Another possibility is that these modifications of the BChl *c* molecule increase disorder in the BChl aggregates and allow a much higher packing density of BChl *c* in the chlorosomes without causing crystallization (33). While some evidence suggests that the absence of these physical interactions in the mutants would contribute to the reduced BChl *c* content in the cells, both cases would cause a dramatic change in the relative composition of the chlorosomes. If structural and composition changes were solely responsible for the BChl *c* content, the 30 to 60% decrease in the cellular BChl *c* content should also be observed in the ratio of BChl *c* to other chlorosome components such as protein. However, the ratio of BChl *c* to protein of the chlorosomes isolated from the double mutant was only 18% lower than that for the wild type (Table 2). The mutants lacking only one methyltransferase had ratios of BChl *c* to chlorosome protein even more similar to that of the wild type. While the BChl *c* packing within the chlorosomes might be affected by the absence of the C-8^2 and C-12^1 methylations, this result indicates that it does not solely account for the overall decrease in BChl *c* content in the cell.

The analysis of the chlorosomes of the mutants created in this study highlights a new problem, because there remains a 20% decrease in the BChl *c* content of the mutant cells with respect to that of the wild type, which is not accounted for by the chlorosome composition. The other factor that could explain this additional 20% decrease in BChl *c* content is a reduction in the rate of BChl *c* biosynthesis in the mutants. This decrease could be caused by an increased feedback inhibition of the BChl *c* pathway through an increase in the pool of [E,M]-BChlide *c* or by the accumulation of BChl *c* that has not been incorporated into the chlorosomes because of altered methylation. A preliminary analysis of the *bchQ bchR bchU* triple mutant indicates that it has greatly reduced BChl *d* content. This is consistent with the hypothesis that [E,M]-BChlide *d* possibly inhibits a key enzyme of BChl biosynthesis, perhaps the Mg-chelatase that catalyzes the first committed step in BChl biosynthesis (14). Further experimentation will be required to determine the cause of this reduced flow of intermediates through the BChl *c* biosynthetic pathway and the possible targets of such feedback (or activation) effects. Nevertheless, we conclude that GSB add methyl groups to the C-8^2, C-12^1, and C-20 positions of BChl *c*, *d*, and *e* in order to fine-tune the absorbance properties of their light-harvesting antenna, which enables these organisms to grow faster when light is limiting. Since GSB are almost always found in light-limited environments, these modifications are crucial to their survival and competitiveness in natural populations.

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