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# The *bchU* Gene of *Chlorobium tepidum* Encodes the C-20 Methyltransferase in Bacteriochlorophyll *c* Biosynthesis

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**Bacteriochlorophylls (BChls) *c* and *d*, two of the major light-harvesting pigments in photosynthetic green sulfur bacteria, differ only by the presence of a methyl group at the C-20 methine bridge position in BChl *c*. A gene potentially encoding the C-20 methyltransferase, *bchU*, was identified by comparative analysis of the *Chlorobium tepidum* and *Chloroflexus aurantiacus* genome sequences. Homologs of this gene were amplified and sequenced from *Chlorobium phaeobacteroides* strain 1549, *Chlorobium vibrioforme* strain 8327*d*, and *C. vibrioforme* strain 8327*c*, which produce BChls *e*, *d*, and *c*, respectively. A single nucleotide insertion in the *bchU* gene of *C. vibrioforme* strain 8327*d* was found to cause a premature, in-frame stop codon and thus the formation of a truncated, nonfunctional gene product. The spontaneous mutant of this strain that produces BChl *c* (strain 8327*c*) has a second frameshift mutation that restores the correct reading frame in *bchU*. The *bchU* gene was inactivated in *C. tepidum*, a BChl *c*-producing species, and the resulting mutant produced only BChl *d*. Growth rate measurements showed that BChl *c*- and *d*-producing strains of the same organism (*C. tepidum* or *C. vibrioforme*) have similar growth rates at high and intermediate light intensities but that strains producing BChl *c* grow faster than those with BChl *d* at low light intensities. Thus, the *bchU* gene encodes the C-20 methyltransferase for BChl *c* biosynthesis in *Chlorobium* species, and methylation at the C-20 position to produce BChl *c* rather than BChl *d* confers a significant competitive advantage to green sulfur bacteria living at limiting red and near-infrared light intensities.**

The light-harvesting antenna system of green sulfur bacteria, the chlorosome, is uniquely adapted to low-light environments (6, 7). With up to 215,000 molecules of bacteriochlorophyll (BChl) per chlorosome and up to 250 chlorosomes per cell (24), some green sulfur bacteria are able to harvest light energy and grow under light fluxes as low as 3 nmol of photons $m^{-2}$ $s^{-1}$ (28). For a single BChl molecule, this light intensity corresponds to a single absorption event in approximately 5 to 6 h. Green sulfur bacteria produce BChl *c*, *d*, or *e* as a primary antenna pigment, in addition to small amounts of BChl *a*, which is associated with the CsmA chlorosome base plate protein, the FmoA antenna protein, and the reaction center. These bacteria also synthesize Chl *a*, which serves as the primary electron acceptor in the reaction center (23). BChls *c*, *d*, and *e* differ in their methylation patterns: BChl *e* has a formyl group rather than a methyl group at the C-7 position and a methyl group at the C-20 position (19). BChls *c* and *d* both carry methyl groups at the C-7 position, but only BChl *c* has a methyl group at the C-20 position (20, 29) (Fig. 1).

Green sulfur bacteria are obligate anaerobes that are found below the chemocline in meromictic lakes or in sulfide-rich waters and sediments. These environments are usually light limited, with typical light intensities ranging from 0.1 to 10 μmol of photons $m^{-2}$ $s^{-1}$ (18, 38, 39). In nature, brown-colored BChl *e*-containing organisms are frequently found at lower light intensities than those containing BChl *c* or *d* (21, 25, 28, 41). Studies of a BChl *d*-producing strain of *Chlorobium vibrioforme* which spontaneously began to synthesize BChl *c* when grown at a low light intensity suggested that BChl *c* may be a more effective antenna molecule than BChl *d* at a low light intensity (8, 10, 31). These studies showed that for *C. vibrioforme* strain 8327*d*, the addition of the C-20 methyl group to BChl *d* shifts the absorption maximum of the $Q_y$ peak to the red, from 732 to 751 nm in whole-cell spectra and from 658 to 667.5 nm in methanol extracts (10) (Table 1).

The recently completed sequencing of the genome of *Chlorobium tepidum*, a BChl *c*-producing green sulfur bacterium, has provided the basis for investigating the genes involved in BChl and chlorosome biosynthesis (12, 16). Because *C. tepidum* is naturally transformable and performs homologous recombination, any nonessential gene can in principle be inactivated by inserting an antibiotic resistance cartridge into the coding sequence of the gene to be inactivated (14). Additionally, because a *bchK* mutant that completely lacks BChl *c* and yet is able to grow photoautotrophically has been constructed, the gene encoding any enzyme in the BChl *c* biosynthetic pathway can in principle be inactivated (15). These characteristics make *C. tepidum* a convenient model organism for elucidating the steps of the BChl *c* biosynthetic pathway. In this paper, we demonstrate that the product of the gene *CT0028*,

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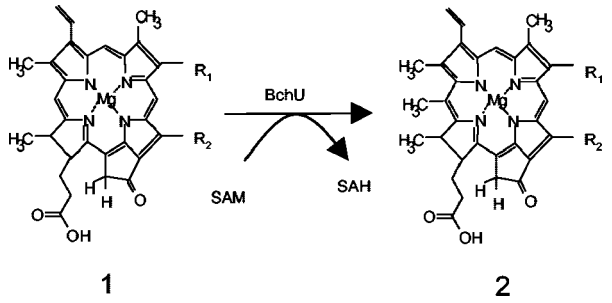


FIG. 1. Proposed reaction catalyzed by the *bchU* gene product. It should be noted that the substrate specificity of the BchU methyltransferase has not yet been established, so other related substrates may be methylated by an analogous reaction. Compounds: 1, [3-vinyl, 8-ethyl, 12-methyl] bacteriochlorophyllide *d*; 2, [3-vinyl, 8-ethyl, 12-methyl] bacteriochlorophyllide *c*; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine. $R_1$, ethyl, *n*-propyl, *iso*-butyl, or *neo*-pentyl; $R_2$, methyl or ethyl.

now denoted *bchU*, is responsible for methylation of the C-20 methine bridge in BChl *c* and probably also in BChl *e* from brown-colored *Chlorobium* species. We also demonstrate that this C-20 methylation confers a competitive advantage to green sulfur bacteria growing at low light intensities.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The strain of *C. tepidum* used was the plating strain WT2321 (43), derived from strain ATCC 49652 (44). *Chlorobium phaeobacteroides* strain 1549 is a BChl *e*-containing green sulfur bacterium that grows readily on plates containing thiosulfate as an electron donor. A BChl *c*-producing strain, *C. vibrioforme* 8327*c*, was previously isolated after serial subculturing of strain 8327*d*, a BChl *d*-producing strain, at a light intensity of 0.3 μmol of photons $m^{-2}$ $s^{-1}$ over a period of 5 months (10). All strains were grown anaerobically in liquid CL medium or solid CP medium as described by Frigaard and Bryant (14). Culture transfers and growth on agar plates were performed in a Coy (Grass Lake, Mich.) anaerobic chamber with an atmosphere of $CO_2$ (10%) and $H_2$ (5%) balanced with $N_2$. In the anaerobic chamber, *C. tepidum* was grown at 40°C and *C. vibrioforme* and *C. phaeobacteroides* were grown at ~30°C. Growth rate experiments with wild-type and mutant strains of *C. tepidum* were carried out at 46°C at light intensities of 707, 580, 146, 30, and 8 μmol of photons $m^{-2}$ $s^{-1}$ as previously described (15). *C. tepidum* transformants were selected on CP plates supplemented with 300 μg of spectinomycin $ml^{-1}$ and 150 μg of streptomycin $ml^{-1}$.

Routine recombinant DNA procedures were performed in electrocompetent *Escherichia coli* DH5α grown in Luria-Bertani broth. To select for *E. coli* with plasmid-conferred antibiotic resistance, Luria-Bertani broth was supplemented with 100 μg of ampicillin $ml^{-1}$, 100 μg of spectinomycin $ml^{-1}$, and 50 μg of streptomycin $ml^{-1}$.

**Inactivation of *CT0028*.** A 1.1-kb fragment of *C. tepidum* open reading frame *CT0028* was amplified from genomic DNA by using forward primer CT0028-FE and reverse primer CT0028-BP, which contain an EcoRI recognition site and a PstI recognition site, respectively (Table 2). Plasmid pCFT was obtained by digesting this PCR product with EcoRI and PstI and cloning it into the multiple cloning site of pUC19 digested with the same enzymes. Plasmid pCFT-X was made by excising the *aadA* gene, which encodes resistance to spectinomycin and streptomycin, from pSRA2 (17) by digestion with KpnI. This fragment was then inserted between internal KpnI sites within the coding sequence of the *CT0028* gene.

Plasmid pCFT-X was linearized with the restriction endonuclease AhdI, purified by gel electrophoresis, and used to transform wild-type *C. tepidum* after purification of the digested plasmid DNA by use of a QIAquick gel extraction kit (Qiagen, Valencia, Calif.). The transformation of *C. tepidum* with plasmid pCFT-X was performed as previously described (14). An aliquot (100 μl) of a dense culture of wild-type *C. tepidum* was centrifuged and the pellet was resuspended in sterile water (15 μl) containing 1.5 μg of plasmid DNA. The transformation mixture was incubated on nonselective CP plates for 10 h. The cells were then transferred to CP plates supplemented with spectinomycin and streptomycin to select for transformants. Genomic DNA from wild-type *C. tepidum* and two different transformant strains was extracted, and the *CT0028* gene was amplified with primers CT0028-F1 and CT0028-B1 (Table 2).

**Preparation of genomic DNA.** Genomic DNA from all *Chlorobium* species was extracted from 1.5 ml of cell culture according to a protocol described by Bickley and Owen (5).

**Amplification and sequencing of *CT0028* (*bchU*) genes.** Degenerate oligonucleotide primers based on the sequence of the *CT0028* (*bchU*) gene of *C. tepidum* (12) were designed with MacVector, version 7.1.1, software (Accelrys, Madison, Wis.). The *bchU* genes were amplified from *C. tepidum*, the *C. tepidum CT0028* (*bchU*) mutant strain, *C. vibrioforme* strain 8327*d*, *C. vibrioforme* strain 8327*c*, and *C. phaeobacteroides* strain 1549 by use of *Taq* polymerase and the primer pair CT0028-FD and CT0028-BD (Table 2). The PCR program had the following conditions: 4 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C; and then 10 min at 72°C. PCR products to be sequenced were incubated overnight in a solution of 2.5 M NaCl and 20% (wt/vol) polyethylene glycol (molecular weight, 8,000) and then were precipitated with ethanol and resuspended in filter-sterilized water. Sequencing of these products was performed with an ABI 3100 automated sequencer at the Nucleic Acid Facility at The Pennsylvania State University, using the same primer pair. PCR products were also cloned into pUC19, and the inserts were sequenced by using M13 forward and reverse primers.

**Growth rate measurements.** *C. tepidum* was grown at 46°C, and *C. vibrioforme* was grown at 31°C. Cultures were grown on a rotating wheel that was uniformly illuminated from the front. Before growth rates were measured, cultures were grown to late exponential phase at the light intensity at which the growth rate was to be measured. Cultures were then diluted to an optical density at 600 nm ($OD_{600}$) of 0.1, and the OD was monitored until the culture reached stationary phase. Because of the transient appearance of polysulfide and elemental sulfur granules, which affect light scattering in the tubes, measurements of $OD_{600}$ that were below ~0.8 were not used when determining the growth rate.

**Pigment and protein analysis.** Whole-cell spectra were recorded on a Cary-14 spectrophotometer modernized for computerized data acquisition (On-Line Instruments, Inc., Bogart, Ga.). A dense cell suspension was diluted 10-fold in cell-harvesting buffer (10 mM $KH_2PO_4$, 50 mM NaCl, pH 7.0) and mixed, and the absorption spectrum of the resulting suspension was recorded from 350 to 900 nm.

Pigments were extracted from 1-ml aliquots of cell suspension from late-exponential phase cultures by sonication in acetone-methanol (7:2 [vol/vol]). Cell debris was removed by centrifugation. The absorption spectrum of the pigment mixture was recorded after diluting the sample at least 1:10 in methanol, and the absorption spectrum of the extracts was recorded from 350 to 900 nm. Samples intended for high-performance liquid chromatography (HPLC) analysis were

TABLE 1. Absorbance properties of major BChl species in *C. tepidum* and *C. vibrioforme*

| Strain (BChl species) | Wavelength (nm) of maximum absorbance | | | |
|---|---|---|---|---|
| | Soret (in acetone) | $Q_y$ (in acetone) | Soret (in vivo)[a] | $Q_y$ (in vivo)[a] |
| *C. tepidum* (BChl *c*) | 435 | 667 | 460 | 751 |
| *C. tepidum bchU* (BChl *d*) | 428 | 655 | 451 | 736 |
| *C. vibrioforme* 8327*c* (BChl *c*) | 435 | 667 | 462 | 750 |
| *C. vibrioforme* 8327*d* (BChl *d*) | 428 | 656 | 446 | 733 |

[a] In vivo refers to the value for intact cells.

TABLE 2. Sequences of oligonucleotides used for cloning and sequencing of *bchU*

| Primer name | Sequence (5′–3′) |
|---|---|
| CT0028 FD | CAC AGG GCC AAC GAG CTG GTC TT(T/C) AA(A/G) |
| CT0028 BD | CAC AGC CTG AAC GAG CAG GTG (A/G)TC (A/G)TA |
| CT0028 FE[a] | CGG GGT GAA TTC GGA CAG GCT GGA TAA C |
| CT0028 BP[b] | GCC GAG CAC CCT GCA GGG CAT TCC |
| CT0028 F1 | TGA GCA ACA ATGACC TCC TGA ACT A |
| CT0028 B1 | ACA GCC TGA ACG AGC AGG TG |

[a] The underlined sequence is the EcoRI recognition site used for cloning.
[b] The underlined sequence is the PstI recognition site used for cloning.

filtered, and 0.1 volume of 1 M ammonium acetate was added to the filtrate immediately before injection into the HPLC column. The pigments were separated on a 25-cm by 4.6-mm Discovery 5-μm $C_{18}$ column (Supelco, Bellefonte, Pa.) attached to a 1,024-element diode array detector (model G1315B, 1100 series; Agilent Technologies, Palo Alto, Calif.) controlled with Agilent ChemStation software for HPLC. The solvent system was described previously (15). BChl *c* extracted from wild-type *C. tepidum* served as a standard. Elution of the pigments was monitored at 667 and 656 nm, the absorbance maxima for BChl *c* and BChl *d*, respectively; the absorption spectrum of the column eluate from 200 to 900 nm was recorded at 2-s intervals and stored.

The total cell protein concentration was determined from 1-ml aliquots of cells grown to mid-exponential phase ($OD_{600}$ of ~1.0). Pigments were extracted from pelleted cells by sonication in 1.0 ml of methanol, and the membranes and proteins were removed by centrifugation. The BChl content was determined by measuring the absorbance of the supernatant at 669 nm for wild-type cells and at 656 nm for BChl *d*-containing cells. The protein concentration in the pellet was determined by a modified Lowry protocol (40), with bovine serum albumin (2.0 mg $ml^{-1}$) (Pierce, Rockford, Ill.) used as a standard.

**Chlorosome preparation and analysis.** Cultures (2 liters) of the *C. tepidum* wild type and the *bchU* mutant were grown at ~150 μmol of photons $m^{-2}$ $s^{-1}$. Chlorosomes were isolated and purified from these cultures according to a previously described procedure (11, 40) and were stored at −80°C until used. For the comparison of molar extinction coefficients, chlorosomes were thawed on ice in the dark. Wild-type chlorosomes were diluted 1:10,000 in cell-harvesting buffer (10 mM $KH_2PO_4$, 50 mM NaCl, pH 7.0) or 1:100 in acetone-methanol (7:2 [vol/vol]) and then further diluted 1:100 in 100% methanol. Chlorosomes from the *bchU* mutant were diluted 1:5,000 in cell-harvesting buffer or 1:100 in acetone-methanol (7:2 [vol/vol]) and then further diluted 1:50 in 100% methanol. The absorption spectra of the resulting solutions, from 350 to 900 nm, were scanned immediately. The concentration of BChl *c* or *d* was determined from the absorbance maxima of the methanol extracts, using the following extinction coefficients: for BChl *c*, $\varepsilon_{667} = 86.0$ liters $g^{-1}$ $cm^{-1}$; for BChl *d*, $\varepsilon_{656} = 82.3$ liters $g^{-1}$ $cm^{-1}$ (33). Extinction coefficient units were converted to liters per mole per centimeter, assuming that 33% of the BChl was [8-propyl, 12-ethyl] BChl and 67% was [8-ethyl, 12-ethyl] BChl, giving molar extinction coefficients of 69,717 liters $mol^{-1}$ $cm^{-1}$ for BChl *c* and 66,000 liters $mol^{-1}$ $cm^{-1}$ for BChl *d*. The calculated concentrations were then used to calculate the molar extinction coefficients of the BChls in the chlorosomes.

**Competition experiments.** The BChl *c*- and *d*-containing strains of *C. tepidum* were mixed in CL medium with no antibiotic supplements to produce cultures in which the BChl *c*-containing strain represented 10, 20, 50, 80, and 90% of the total cells. The cultures were incubated on a rotating wheel at 46°C at 146 and 8 μmol of photons $m^{-2}$ $s^{-1}$. Cultures grown at the lower light intensity were incubated for 36 h, diluted 1:20, and incubated again for 18 h; this incubation time and light intensity correspond to approximately 12 generations. Cultures grown at the higher light intensity were incubated for 24 h, diluted 1:20, and incubated for another 24 h; this incubation time and light intensity correspond to a total of approximately 20 generations for the wild type. Because the BChl contents of the two strains were similar, the ratio of the two strains in the culture at the end of the experiment could be determined by measuring the ratio of BChl *c* to BChl *d*. Aliquots (1.0 ml) were removed from each culture before the experiment began and at the end of the experiment. Pigments were extracted from the cells by sonication in 0.4 ml of acetone-methanol (7:2 [vol/vol]), and the absorption spectrum of the extracts was measured from 350 to 900 nm. The ratio of BChl *c* to BChl *d* in each sample was calculated by using the equations $A_{656} = \varepsilon_{c,\,656} \times c_c + \varepsilon_{d,\,656} \times c_d$ and $A_{667} = \varepsilon_{c,\,667} \times c_c + \varepsilon_{d,\,667} \times c_d$, where $A$ is the value of the absorbance, ε is the extinction coefficient, and $c$ is the concentration. Extinction coefficients for the absorption maxima of the pigments in methanol were the same as those described above; $\varepsilon_{c,\,656}$ and $\varepsilon_{d,\,667}$ were calculated to be 52.4 and 21.9 liters $g^{-1}$ $cm^{-1}$, respectively.

**Nucleotide sequence accession numbers.** The sequences of the PCR-amplified *bchU* genes of *C. vibrioforme* strain 8327*d*, *C. vibrioforme* strain 8327*c*, and *C. phaeobacteroides* strain 1549 have been deposited in GenBank under accession numbers AY452765, AY452766, and AY452767.

## RESULTS

**Identification of genes potentially encoding BChl *c* C-20 methyltransferase.** The *C. tepidum* genome contains many methyltransferase genes, and since most of the genes involved in BChl biosynthesis are not organized in a large photosynthesis gene cluster or even as operons, the identification of the BChl *c* C-20 methyltransferase was not straightforward. During the biosynthesis of cobalamin, CbiL catalyzes the methylation of the C-20 methine bridge of cobalt-precorrin 2 (30, 32). *C. tepidum* appears to carry all of the genes that are necessary for the synthesis of cobalamin (12), including a gene (*CT0388*) encoding a protein with a strong sequence similarity to the CbiL proteins of other bacteria. In addition to this gene, the genome carries *CT1763*, encoding a protein that is weakly similar to CbiL and that was considered to be a possible candidate for the C-20 methyltransferase in BChl *c* biosynthesis. This *CT1763* gene, which encodes a protein with similarity to both CbiL and uroporphyrin-III C-methyltransferase, was insertionally inactivated, and a fully segregated mutant strain was obtained. However, this mutant still produced BChl *c* and had no apparent pigmentation differences or other phenotypic differences from the wild-type strain (data not shown).

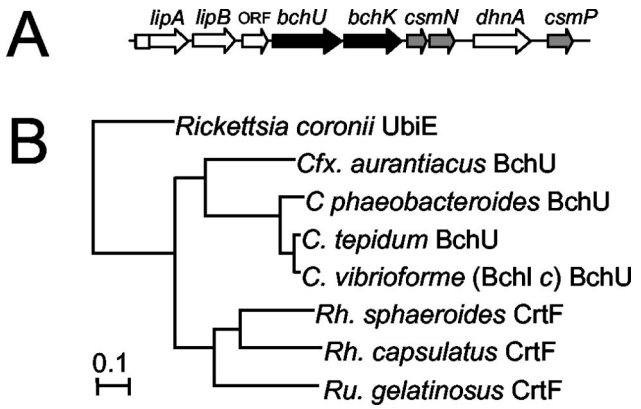


FIG. 2. Methyltransferases involved in biosynthesis of bacteriochlorophylls and carotenoids. (A) Gene organization of an apparent photosynthesis-related operon in *Chloroflexus aurantiacus*. Genes in black are required for BChl *c* biosynthesis, and genes in gray encode chlorosome envelope proteins. (B) Phylogenetic relationships between translated sequences of BchU (encoding the C-20 methyltransferase) in green bacteria and CrtF (encoding neurosporene *O*-methyltransferases) in purple bacteria. Amino acid sequences were aligned by using the ClustalW program and the phylogenetic tree was generated with MacVector, version 7.1.1. UbiE, an *O*-methyltransferase involved in ubiquinone biosynthesis in the nonphotosynthetic plant pathogen *Rickettsia coronii*, was used as the outgroup sequence.

Genes whose products are involved in the same biosynthetic pathway, whose products are found in the same cellular compartment, or whose products are coregulated are often clustered together in operons on bacterial genomes. As noted above, although such clustering is not pronounced in the *C. tepidum* genome, analyses of the chlorophyll biosynthesis genes in the green filamentous bacterium *Chloroflexus aurantiacus* showed more evidence for clustering (16). In *Chloroflexus aurantiacus*, BChl biosynthesis and chlorosome production are strongly induced when cells are shifted from oxic to anoxic conditions in light (13, 27). Interestingly, genes encoding several chlorosome envelope proteins are clustered together with *bchK*, the BChl *c* synthase, and a gene encoding a methyltransferase that had been annotated as *crtF* (26, 45) (Fig. 2). The *crtF* gene encodes an *O*-methyltransferase in the spheroidenone biosynthetic pathway of purple bacteria (1, 2). The genome of *C. tepidum* contains a gene, *CT0028*, which encodes a

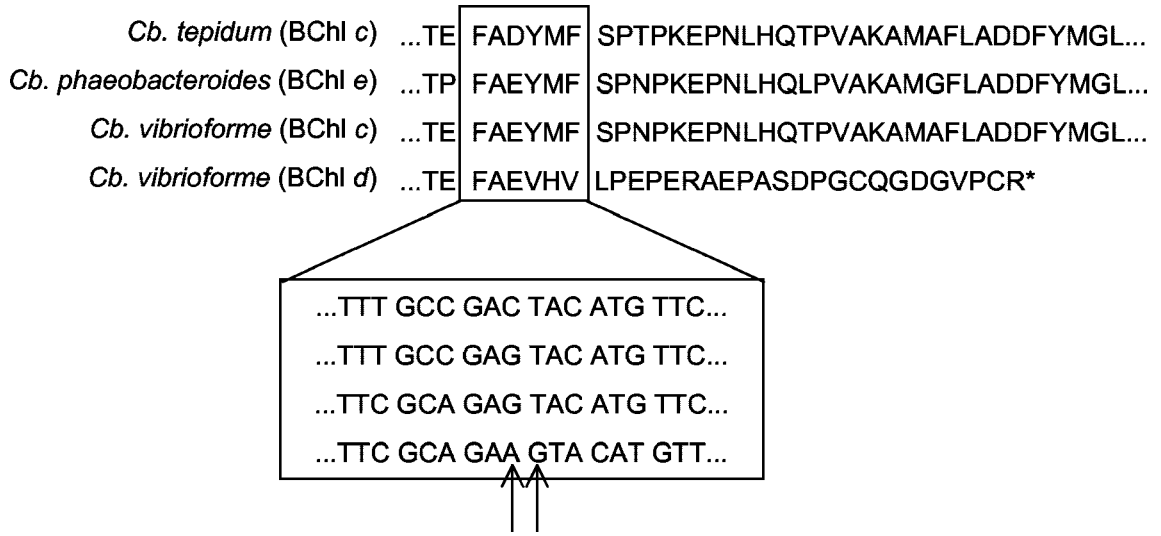


FIG. 3. Amino acid and nucleotide sequence alignments of a portion of the BchU sequences from BChl *c*-, *d*-, and *e*-producing species. The type of BChl produced by each species is indicated in parentheses. Arrows indicate the insertion of a single nucleotide in the BchU gene of *C. vibrioforme* 8327*d*, which causes a frameshift mutation that results in a premature stop codon (*). The deletion of an adenine residue in the same region in the BChl *c*-producing revertant *C. vibrioforme* 8327*c* returns the gene to the correct reading frame.

methyltransferase with very strong sequence similarity to the *Chloroflexus aurantiacus* "CrtF" protein. However, since neither *Chloroflexus aurantiacus* nor *C. tepidum* synthesizes O-methylated carotenoids (35, 36, 37), it was clear that the *Chloroflexus aurantiacus* gene annotated as *crtF* had been misidentified. The presence of this methyltransferase gene adjacent to *bchK*, known to be involved in BChl *c* biosynthesis and clustered with genes encoding structural components of the chlorosome, strongly suggested that this gene could encode a methyltransferase involved in BChl *c* biosynthesis.

Broch-Due and Ormerod (10) found that prolonged growth of *C. vibrioforme* strain 8327*d* at a low light intensity led to gain-of-function mutants which had acquired the ability to methylate BChl *d* and thus to produce BChl *c*. Similar results were later reported by Smith and coworkers (8, 22), and most recently, by Tamiaki and coworkers (31). These results clearly establish that the wild-type strain of *C. vibrioforme*, strain 8327*d*, carries the gene for the BChl *c* C-20 methyltransferase but that this gene is somehow inactivated. The apparently low frequency at which the gain-of-function mutants appeared in the various studies of three groups over a period of 25 years suggests that the gene encoding the methyltransferase was mutated to an inactive form but that the functionality of this gene could be restored by a secondary mutation. By sequencing of the methyltransferase genes from several strains, including *C. vibrioforme* strains 8327*d* and 8327*c*, this prediction was directly tested.

**Sequence analysis of *bchU* and *crtF* genes.** Degenerate primers were designed based on regions conserved between *crtF* in *Rhodobacter capsulatus*, *bchU* in *Chloroflexus aurantiacus*, and *CT0028* in *C. tepidum* (Table 2). These primers were used to amplify and sequence *CT0028* homologs from *C. tepidum*, *C. vibrioforme* strains 8327*d* and 8327*c*, and *C. phaeobacteroides* strain 1549. Using the translated sequences of these homologs, as well as the sequences of previously published *crtF* genes, we created a phylogenetic tree comparing the amino acid sequences of CT0028 homologs with CrtF homologs. The CT0028 sequences from green sulfur bacteria and *Chloroflexus aurantiacus* clearly clustered in a group that is well separated from the CrtF sequences of purple bacteria (Fig. 2B).

The nucleotide sequences of these genes showed that *C. vibrioforme* 8327*d* has a single nucleotide insertion 260 nucleotides downstream from the translation start site of the gene. This frameshift insertion resulted in an in-frame stop codon 78 nucleotides downstream of the mutation (Fig. 3), the result of which would be a peptide of irrelevant sequence that is only 87 amino acids long, or less than one-fourth of the length of the 338-amino-acid protein of the wild type. The BChl *c*-producing strain of *C. vibrioforme* (strain 8327*c*) has a deletion of an A residue in the same region, returning the gene to the correct reading frame. These results provide a molecular explanation for the observed shift in BChl type in *C. vibrioforme* strain 8327*d* cells cultured under low-light-intensity selection conditions.

**Construction and verification of a *CT0028* mutant of *C. tepidum*.** Wild-type *C. tepidum* was transformed with linearized plasmid pCFT-X, which contained the *CT0028* gene interrupted with the *aadA* antibiotic resistance marker (Fig. 4A). Green transformant colonies appeared on selective plates containing spectinomycin and streptomycin within 5 days. Segregation of the mutant and wild-type alleles was confirmed by PCR using primers CT0028F1 and CT0028B1 to amplify the *bchU* locus from genomic DNAs extracted from the wild-type and mutant strains (Fig. 4B). The product amplified from wild-type *C. tepidum* cells was 1.0 kb, while the product amplified from the *CT0028* mutant cells was 1.9 kb; this result confirmed that the *CT0028* gene had been insertionally inactivated with the *aadA* gene. No 1.0-kb fragment corresponding to the wild-type *CT0028* allele was amplified from the mutant strain. Together, these results indicate that the segregation of the *CT0028* and *CT0028*::*aadA* alleles was complete and that the *CT0028* gene is not essential under the growth conditions employed.

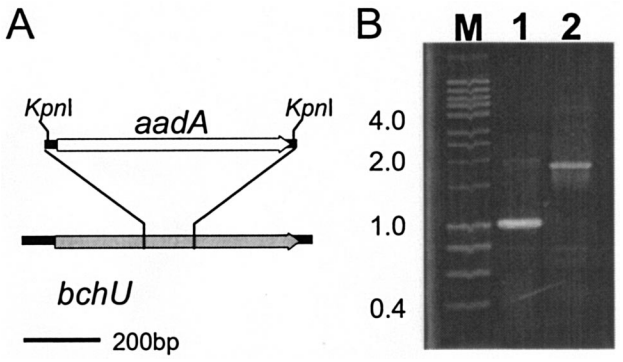


FIG. 4. Insertional inactivation of the *bchU* gene in *C. tepidum*. (A) The *aadA* gene, conferring resistance to streptomycin and spectinomycin, was inserted in *bchU* between KpnI sites that were 231 bp apart. (B) PCR confirmation of gene interruption. The *bchU* gene was amplified from genomic DNA extracted from the wild type (lane 1) and a mutant culture (lane 2) of *C. tepidum*, using primers CT0028 F1 and CT0028 B1. The fragment amplified from wild-type *C. tepidum* is 1.0 kbp; that amplified from the mutant is 1.9 kbp. Lane M, size markers; the sizes of bands (in kilobases) are indicated at the left.

**Pigment analysis of *C. tepidum CT0028* (*bchU*) mutant.** Pigments extracted with acetone-methanol from both the wild-type and *CT0028* mutant strains of *C. tepidum* were separated in a $C_{18}$ reverse-phase HPLC column. The elution profiles of the two strains demonstrated a similar distribution of BChl homologs, indicating that methylation at the C-8 and C-12 positions was not affected by the *CT0028* mutation (Fig. 5A). All BChl *c* homologs in the wild type had identical absorption spectra, with a maximum at 667 nm, whereas the major BChl species in the *CT0028* mutant had absorption spectra identical to that of BChl *d*, with a maximum at 657 nm. The BChl *d* homologs eluted approximately 1.5 min earlier than their BChl *c* counterparts; this indicates that the BChl *c* homologs are slightly more hydrophobic, as expected. Mass spectrometry of the BChl pigments showed that each of the four major BChl homologs produced by the mutant was 14 mass units lighter than the corresponding BChl *c* compound in the wild type. This change in mass indicates the loss of a single methyl group from each BChl homolog in the *CT0028* mutant (Fig. 5). These results confirm that the product of *CT0028* is the BChl *c* C-20 methyltransferase; accordingly, this gene has been renamed *bchU*.

The BChl *c*- or BChl *d*-to-protein ratio was determined for cells of the wild-type and *bchU* mutant strains of *C. tepidum* that had been grown to an $OD_{600}$ of ~1.0 at high (707 μmol of photons $m^{-2}$ $s^{-1}$) and low (10 μmol of photons $m^{-2}$ $s^{-1}$) light intensities. As previously reported for *C. tepidum* (9), the cellular content of antenna BChl increased as the growth light intensity decreased. The antenna BChl/protein ratios (wt/wt) of the two strains were identical (0.058 ± 0.007) for cells grown at a high light intensity. However, at a low light intensity, the antenna BChl/protein ratio of the wild type (0.174 ± 0.013) was 21% larger than that of the *bchU* mutant strain (0.143 ± 0.007). The BChl *c*/BChl *a* and BChl *d*/BChl *a* ratios were also determined by an HPLC analysis of pigments extracted from the same cells. These measurements showed that the ratio of BChl *c* to BChl *a* (54.2:1) in the wild-type cells grown at a high light intensity was almost identical to the ratio of BChl *d* to BChl *a* (53.6:1) in the *bchU* mutant. However, at a low light intensity, the ratio of BChl *c* to BChl *a* in the wild-type cells was 57:1 and the ratio of BChl *d* to BChl *a* in the *bchU* mutant cells was 41:1, a 28% decrease.

**Analysis of molar extinction coefficients of BChls *c* and *d*.** The absorption spectrum of BChl *c* in methanol has maxima at 667 and 435 nm, while that of BChl *d* has maxima at 654 and 428 nm (33). When the spectra of the pigments in organic solvents were adjusted to reflect their molar absorptivities (Fig. 6A), it was clear that BChl *c* has a higher absorbance level in both the blue and red regions of the spectrum. An integration of the absorption spectra from 350 to 800 nm showed that BChl *c* has about a 15% higher overall absorbance in this wavelength range than that of BChl *d*. When the absorption spectra in aqueous buffer of chlorosomes isolated from the wild type and the *bchU* mutant were plotted to reflect their respective molar absorptivities (Fig. 6B), several differences were observed for the chlorosomes containing the two BChl species. In the case of *C. tepidum*, the half-bandwidth of the $Q_y$ peak of chlorosomes containing BChl *c* was 59 nm, whereas that of chlorosomes containing BChl *d* was 43 nm. The Soret band of chlorosomes containing BChl *c* was 6% higher than that of chlorosomes containing BChl *d*, although this band was not detectably broader. In addition, BChl *c* absorbed slightly more than BChl *d* in the region between 550 and 650 nm. However, an integration of the spectra in the wavelength range from 650 to 850 nm showed that chlorosomes containing BChl *c* have a 25% higher absorption than chlorosomes containing

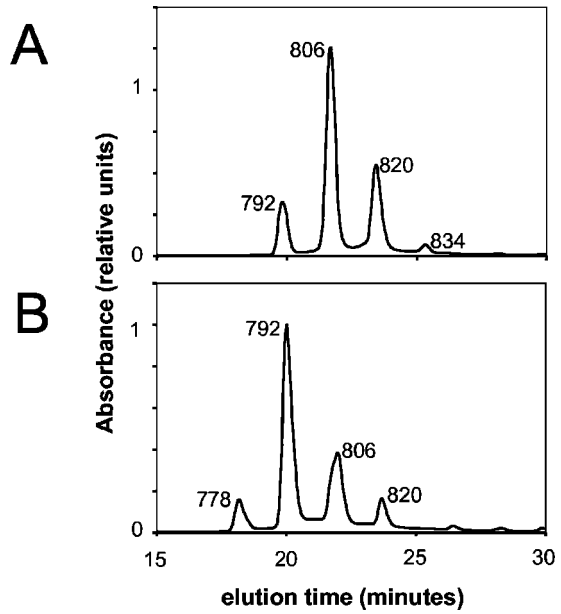


FIG. 5. Elution profiles of BChl *c* from wild-type *C. tepidum* (A) and BChl *d* from the *bchU* mutant (B). The numbers beside each peak indicate molecular masses (in daltons) of the compounds eluting in each peak, as determined by mass spectrometry. The four peaks in the upper panel correspond to [8-ethyl, 12-methyl] BChl *c* (792 Da), [8-ethyl, 12-ethyl] BChl *c* (806 Da), [8-propyl, 12-ethyl] BChl *c* (820 Da), and [8-isobutyl, 12-ethyl] BChl *c* (834 Da). Each BChl *d* peak is 14 mass units lighter than its corresponding BChl *c* counterpart; this difference reflects the loss of one methyl group ($-CH_3$) at the C-20 position.

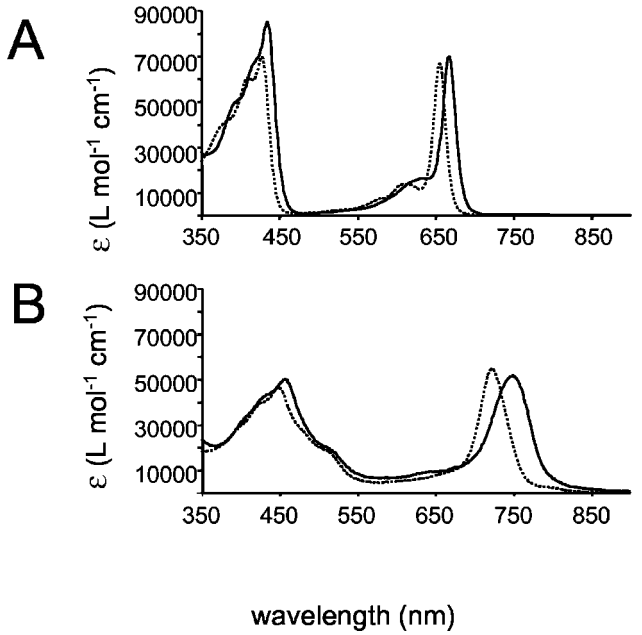


FIG. 6. Absorption profiles for BChls *c* and *d*. (A) Absorption spectra of BChl *c* (solid line) and BChl *d* (dashed line) in methanol from chlorosomes from *C. tepidum*. Pigments were separated on a reverse-phase $C_{18}$ HPLC column; spectra of the eluates were recorded with an online diode array detector. Spectra have been normalized to the molar extinction coefficients of the $Q_y$ peaks. (B) Molar extinction coefficients of BChl *c* (solid line)- and *d* (dashed line)-containing chlorosomes in cell-harvesting buffer (10 mM $KH_2PO_4$, 50 mM NaCl, pH 7.0).

BChl *d*. When these spectra were integrated in the wavelength range from 350 to 800 nm (Fig. 6B), these spectral differences resulted in about a 16% higher overall absorbance by BChl *c*-containing chlorosomes than by BChl *d*-containing chlorosomes.

**Growth characteristics of BChl *c* and BChl *d* strains.** Growth rate measurements of both BChl *c*- and *d*-producing strains of *C. tepidum* and *C. vibrioforme* were performed at nearly optimal growth temperatures and several light intensities. The growth rates and doubling times determined from these experiments are summarized in Table 3. At saturating light intensities, the BChl *c* and BChl *d* strains of both organisms grew at nearly identical rates (Table 3; Fig. 7). At limiting light intensities, the BChl *c*-producing strain of *C. vibrioforme* grew 30% faster than the BChl *d*-producing strain, while for *C. tepidum* the wild-type strain grew 50% faster than the *bchU* mutant. These results are similar to those previously reported for *C. vibrioforme* (10). The BChl *d*-producing strain of *C. vibrioforme* was shown to grow faster than the BChl *c* isolate at a very high light intensity (10); however, when *C. tepidum* was grown at 580 μmol of photons $m^{-2}$ $s^{-1}$, an inhibitory light intensity for the wild-type strain, the BChl *d*-producing strain still grew more slowly than the wild type.

TABLE 3. Growth rates of *C. tepidum* and *C. vibrioforme* under various light conditions

| Light intensity (μmol of photons $m^{-2}$ $s^{-1}$) | Strain | Growth rate ($h^{-1}$)[a] | Doubling time (h) | Fraction of wild-type growth rate |
|---|---|---|---|---|
| 8 | *C. tepidum* wild type | 0.095 | 6.9 | 1 |
| | *C. tepidum bchU* | 0.063 | 10.7 | 0.66 |
| 146 | *C. tepidum* wild type | 0.295 | 2.3 | 1 |
| | *C. tepidum bchU* | 0.283 | 2.5 | 0.96 |
| 580 | *C. tepidum* wild type | 0.239 | 2.9 | 1 |
| | *C. tepidum bchU* | 0.221 | 3.1 | 0.92 |
| 2 | *C. vibrioforme* 8327*c* | 0.045 | 15.5 | 1 |
| | *C. vibrioforme* 8327*d* | 0.034 | 20.7 | 0.76 |
| 70 | *C. vibrioforme* 8327*c* | 0.210 | 3.31 | 1 |
| | *C. vibrioforme* 8327*d* | 0.207 | 3.35 | 0.97 |

[a] The growth rates reported are the averages of two separate experiments in which each rate was determined in triplicate. The standard errors were <4% for all experiments.

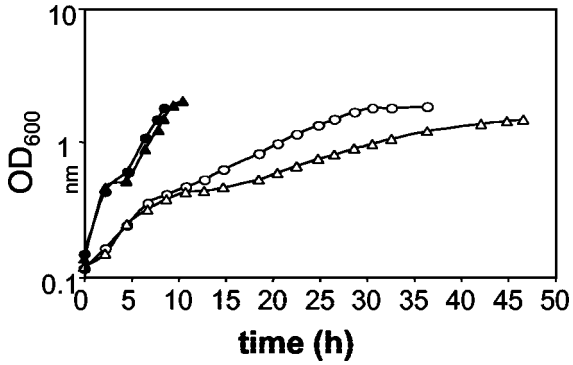


FIG. 7. Growth rate measurements of the wild type (circles) and a *bchU* mutant (triangles) of *C. tepidum* at 146 μmol of photons $m^{-2}$ $s^{-1}$ (closed symbols) and 8 μmol of photons $m^{-2}$ $s^{-1}$ (open symbols). Culture conditions are described in the text. For the determination of growth rates, the first point in the growth curve and the first point of the linear portion of the curve were used. The nonlinear "bulge" areas of the curves are due to the transient appearance of polysulfide and sulfur globules in the growth medium.

**Competition between BChl *c*- and *d*- containing strains.** On the basis of the determined growth rates of the two strains, the BChl *c* strain would be expected to grow to a much higher density than the BChl *d* strain within 12 generations if a mixed culture were grown at a low light intensity but not if it were grown at a saturating light intensity. To test this prediction, we mixed the wild type and the *bchU* mutant strain of *C. tepidum* at selected ratios and allowed them to grow at 146 or 8 μmol of photons $m^{-2}$ $s^{-1}$. As expected, the ratio of BChl *c* to BChl *d* in mixed cultures grown at a high light intensity remained essentially unchanged over 20 generations (Fig. 8B), indicating that the two strains were equally able to harvest and transfer light energy under those conditions. Under the conditions of these experiments, the growth rate difference between the two strains must have been <0.5%. However, in the mixed cultures grown at 8 μmol of photons $m^{-2}$ $s^{-1}$, the proportion of BChl *c* in the culture after 12 generations was much higher than it had been in the inoculum at the onset of the growth experiment (Fig. 8A). In those cultures in which 80 or 90% of the initial inoculum had been the BChl *c*-producing strain, >99% of the pigment in the culture was BChl *c* after 12 generations at a low light intensity. In the cultures in which the BChl *c* strain constituted between 10 and 50% of the initial inoculum, between 67 and 95% of the pigment in the culture was BChl *c* after 12 generations (Fig. 8A). Similar results were obtained

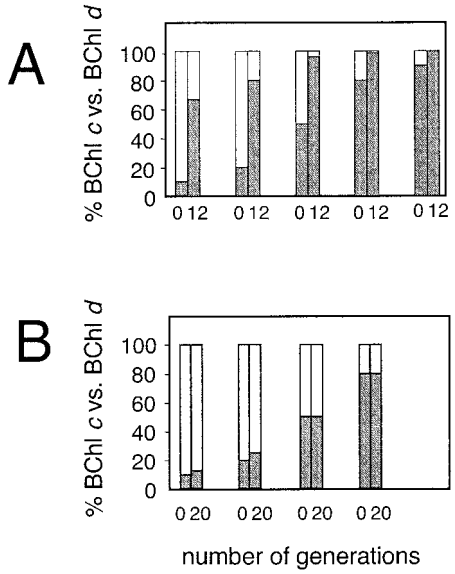


FIG. 8. Direct growth competition between BChl *c*- and *d*-producing strains of *C. tepidum*. Relative concentrations of BChls *c* (dark boxes) and *d* (light boxes), reported as fractions of the total BChl c plus BChl *d* concentration, were calculated as described in Materials and Methods. The percentages of BChl *c* plus BChl *d* composition at 0 generations and after 12 generations of growth at 8 μmol of photons $m^{-2}$ $s^{-1}$ (A) or 20 generations of growth at 146 μmol of photons $m^{-2}$ $s^{-1}$ (B) are indicated for each culture.

with mixed cultures of *C. vibrioforme* 8327*c* and 8327*d* (data not shown). The results obtained for these competition growth experiments could be very accurately predicted solely on the basis of the growth rates of the individual strains measured at the light intensities employed. This indicates that the results are solely due to the growth rate differences that result from the differences in the BChl contents in the chlorosomes of the two strains.

## DISCUSSION

The results presented here clearly establish that the *CT0028* gene, now denoted *bchU*, of *C. tepidum* encodes the BChl *c* C-20 methyltransferase. This gene was amplified and sequenced from three species (*C. tepidum*, *C. vibrioforme*, and *C. phaeobacteroides*) that methylate the C-20 position of their major BChl. In a BChl *d*-producing strain of *C. vibrioforme*, the gene is present but inactive because of a single nucleotide insertion which changes the reading frame of the gene and gives rise to a premature, in-frame stop codon that results in a truncated and inactive polypeptide. A secondary frameshift mutation has reactivated this gene in *C. vibrioforme* 8327*c*, which is a gain-of-function mutant derived from the wild-type parental strain. Finally, when the same gene was insertionally inactivated in *C. tepidum*, which produces BChl *c*, the resulting mutant strain only produced BChl *d*.

As reported previously, the C-20 methyl group is derived from *S*-adenosylmethionine (22). In the cobalamin biosynthetic pathway of *Salmonella enterica* serovar Typhimurium, the C-20 methyltransferase, CbiL, only methylates substrates that have cobalt inserted into the corrin ring and may require this metal for catalysis of the reaction (32). Although BchU and CbiL are not very similar in sequence, both enzymes could have a similar catalytic mechanism. BchU, like CbiL, probably transfers a methyl group directly from *S*-adenosylmethionine to the C-20 methine carbon under anaerobic conditions. However, at present it is not known when this methylation occurs in the context of the BChl *c* biosynthetic pathway.

The distribution of BChl *d* homologs in the *bchU* mutant of *C. tepidum* is virtually identical to the distribution of BChl *c* homologs in the wild type (Fig. 4), which suggests that the absence of methylation at the C-20 position does not affect the activities of the C-8 and C-12 methyltransferases in the *bchU* mutant (BchQ and BchR, respectively [16, 17]). Additionally, in *C. tepidum* strains in which the C-8 and C-12 methyltransferases have been inactivated (A. Gomez Maqueo Chew, N.-U. Frigaard, and D. A. Bryant, unpublished data), all BChl *c* homologs are methylated at the C-20 position. These observations suggest either that the C-20 methylation occurs before the other methylations or that BchU can methylate a wide range of substrates. Although it appears that all biosynthetic intermediates in various mutants that are defective for BChl *c* biosynthesis are methylated at the C-20 position (A. Gomez Maqueo Chew, N.-U. Frigaard, and D. A. Bryant, unpublished results), further studies with the purified BchU enzyme will be required to establish the range of substrates that can be methylated by this enzyme. In the purple photosynthetic bacterium *R. capsulatus*, CrtF has broad substrate specificity (3); the ability to methylate a broad range of substrates might be characteristic of the CrtF and BchU families of methyltransferases.

Only one green sulfur bacterium, *Chlorobium limicola* UdG6040, has been reported to accumulate both BChls *c* and *d* (4, 34). In this organism, the ratio of BChl *d* to BChl *c* increases when the culture grows very rapidly; this observation suggests that the C-20 methyltransferase in this organism may not be very efficient and that its activity may be rate-limiting. This observation also suggests that C-20 methylation occurs before the esterifying long-chain alcohol "tail" is added and that BchU is not able to methylate BChl *d*. However, if BChl *d* is rapidly imported into chlorosomes, it might not be available to BchU, since this molecule would be sequestered within the chlorosome envelope.

*Chlorobium* sp. strains that were unable to methylate the C-20 position of their antenna BChls grew significantly more slowly at low light intensities. The *bchU* mutant of *C. tepidum* grew as fast as the wild type (>99% of the rate of the wild type) at saturating light intensities, but it had a growth rate that was only 66% of that of the wild type when the light intensity was reduced to 8 μmol of photons $m^{-2}$ $s^{-1}$. When BChl *c*- and *d*-producing strains of *C. tepidum* were grown in direct competition, the ratio of BChl *c* to BChl *d* did not change when the cultures were grown at a high light intensity; this indicates that the two strains had identical growth rates under these conditions. However, at a low light intensity (8 μmol of photons $m^{-2}$ $s^{-1}$), BChl *c* predominated after only 12 generations of growth, even in cultures that initially had a BChl *c*/BChl *d* ratio of 1:10. Very similar results were obtained for BChl *c*- and *d*-producing strains of *C. vibrioforme* (data not shown).

These results explain why, as has been previously reported, *C. vibrioforme* is able to revert to producing BChl *c*. The cultures from which revertants were isolated had been grown at a

low light intensity for periods of several months to several years (10, 22, 31), thus applying significant selective pressure. Any organism that deleted a single nucleotide in the vicinity of the original insertion could produce BChl *c* and outcompete the BChl *d*-producing strain under the culture conditions employed. Interestingly, the BChl *d* strain of *C. vibrioforme* 8327 was reported to grow faster at high light intensities than the revertant BChl *c* strain (10), but the same was not true of the *C. tepidum* strains grown at a photoinhibitory light intensity (580 μmol of photons $m^{-2}$ $s^{-1}$). The *bchU* mutant grew at ~92% of the rate of the wild type under these high-light-intensity, photoinhibitory conditions (data not shown).

There appear to be two reasons that wild-type *C. tepidum* grows faster at low light intensities than does the *bchU* mutant. Firstly, BChl *c* has enhanced absorption relative to BChl *d* throughout the physiologically relevant wavelength range of 350 to 850 nm (Fig. 6). The $Q_y$ absorption peak of chlorosomes containing BChl *c* is significantly broader than that of chlorosomes containing BChl *d*; moreover, the Soret absorption and the absorption in the remainder of the visible region of BChl *c* are also higher than those for BChl *d* (Fig. 6B). Both in organic solvents and isolated chlorosomes, these small differences add up to a 16% difference in total absorption. However, the largest differences occur in the absorbance region from 650 to 850 nm, which includes the $Q_y$ absorption band (Fig. 6B). Since the growth experiments were performed with incandescent illumination that had relatively little intensity in the blue region of the spectrum, it can easily be seen that the large difference in $Q_y$ absorption would provide a strong selective advantage. Secondly, although the cellular content of antenna BChl was identical for the wild-type and *bchU* mutant strains of *C. tepidum* grown at a high light intensity, the cellular content of BChl *d* in the *bchU* mutant was significantly smaller (21%) than the content of BChl *c* in the wild type when cells were grown at a low light intensity. In combination, the enhanced absorption per BChl molecule and the larger number of antenna molecules per cell permit the wild type to grow ~50% faster than the *bchU* mutant at low light intensities.

The fact that isogenic BChl *d* and *c* strains have nearly identical growth rates at intermediate and high light intensities leads to the question of why, if BChl *c* is a more effective light-harvesting molecule over a wider range of light intensities, does BChl *d* exist at all? One possible explanation for this may be related to the observation that *C. tepidum* does not grow well at very high light intensities (>300 μmol of photons $m^{-2}$ $s^{-1}$) (15); this observation suggests that at high light intensities chlorosomes are unable to deliver harvested light energy efficiently to the reaction centers. Under such high-light-intensity conditions, a less efficient light-harvesting antenna might be more useful. If this were the case, BChl *d*-containing strains would grow faster than BChl *c*-containing strains at high light intensities; this was observed for *C. vibrioforme* (10) but not for *C. tepidum* in the studies reported here.

Field studies have consistently shown that the majority of green-colored green sulfur bacterial species found in nature produce BChl *d* (39); however, the majority of cultured species produce BChl *c*. This difference may reflect the culture conditions employed during initial enrichments: if green sulfur bacteria are enriched for and isolated under low-light-intensity conditions with an incandescent light source, then the BChl *c*-producing species will probably be strongly selected.

Field studies have also consistently shown a pattern of vertical stratification of green sulfur bacterial species (21, 25, 28, 41), with BChl *d*-containing species closer to the top of the water column, BChl *e*-containing brown species closer to the bottom, and BChl *c*-containing species in between. The predominance of BChl *e*-containing species at the lowest depth is probably due to the enhanced absorption of BChl *e* in the blue-green wavelength region of the visible spectrum, the wavelength range of light that penetrates most deeply in aquatic environments (41, 42). In a study of 41 lakes, Vila and Abella (41) found that green-colored (BChl *c*- or BChl *d*-containing) green sulfur bacteria predominated when red and near-infrared wavelengths were represented in the light reaching the metalimnion. As noted above, BChl *c* absorbs approximately 16% more than BChl *d* in the wavelength range from 350 to 850 nm; however, in the red and near-infrared region, this difference increases to 25%. Thus, BChl *c* would be even more advantageous in such natural environments. Vila and Abella (41) additionally reported that brown-colored, BChl *e*-containing green sulfur bacteria predominated in environments in which green-yellow light was available and longer wavelengths were absent. Since the proportion of red and near-infrared light decreases with increasing depth, the brown-colored green sulfur bacteria will usually be found at the lowest depth in the water column.

Although the availability of light is an important factor in determining which types of green sulfur bacteria will develop in a given environment, it is not the only important factor. The stratification of differently pigmented species can also reflect the geochemistry of the system in which these organisms live. The lower region of the euphotic zone in freshwater lakes often has a significantly lower redox potential than its upper part, and this low-light, high-sulfide region is usually dominated by the BChl *e*-containing green sulfur bacteria. Since the green species are more commonly found in low-light environments with slightly higher redox potentials (42), it may sometimes be that in a region in which the light conditions alone would favor either BChl *e*- or *c*- producing strains, the chemistry of the water favors the brown-colored, BChl *e*-containing strains, which out-compete the green bacteria and thus predominate.

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