INTRODUCTION

Chlorosomes are the defining property of green bacteria and are the light-harvesting structures used for phototrophic growth of these bacteria (Blankenship and Matsuura, 2003; Frigaard and Bryant, 2006; Oostergetel et al., 2010; Bryant et al., 2012). Green bacteria include all known phototrophic members of the phylum Acidobacteria, some members of the Chloroflexi, and “Candidatus Chloracidobacterium thermophilum,” the only known phototrophic member of the phylum Acidobacteria (Bryant et al., 2007, 2012). Green sulfur bacteria (GSB) that are green in color produce chlorosomes containing either bacteriochlorophyll (BChl) d or BChl c and the carotenoid chlorobactene, but brown-colored GSB produce chlorosomes containing BChl e and usually the carotenoid isorenieratene (Chew and Bryant, 2007; Maresca et al., 2008; Liu and Bryant, 2012). A single chlorosome can contain up to ∼250,000 BChl c, d, or e molecules (Martinez-Planells et al., 2002; Montanio et al., 2003) which self-assemble into one of several different suprastructures (Ganapathy et al., 2009, 2012; Garcia Costas et al., 2011). A GSB cell contains ∼200 chlorosomes, and thus a green bacterial cell contains ∼50 million BChl molecules, which together account for ∼30% of the cellular carbon (Frigaard and Bryant, 2006). These enormous light-harvesting antennas allow green bacteria to grow at extremely low irradiances at which no other phototrophs may survive. Examples include GSB that grow at a depth of ∼110 meters in the Black Sea (Manske et al., 2005; Marschall et al., 2010) and a GSB that was isolated at a depth of ∼2200 m on the floor of the Pacific Ocean near a black smoker (Beatty et al., 2005).

The chlorophylls (Chls) found in chlorosomes were once commonly referred to as “Chlorobium” Chls, and they differ from other (bacterio)chlorophylls [(B)Chls] in several important ways (Chew and Bryant, 2007; Liu and Bryant, 2012). Firstly, although these molecules are commonly referred to as BChls, they are in fact chlorins and have properties more similar to Chl a than to those of bacteriochlorins, such as BChl a. Secondly, they carry a hydroxyl group at the chiral C-3’ carbon atom, and they lack the methylcarboxyl moiety found in all other types of (B)Chls at C-13’. These two properties allow BChl c, d, and e to self-aggregate in a protein-independent manner in the interior of the chlorosome (Ganapathy et al., 2009, 2012). Thirdly, these BChls can be methylated at any or all of three positions, C-8’, C-12’, and C-20, on the periphery of the tetrapyrrole macrocycle (Maresca et al., 2011).
Methylation on the C-20 methine bridge by the BchU methyltransferase converts bacteriochlorophyllide \( d \) into bacteriochlorophyllide \( c \), and causes a red-shift of about 15 nm in the absorption spectrum of both monomeric and aggregated BChls in chlorosomes (Maresca et al., 2004; Wada et al., 2006). This methylation also affects the supramolecular structures that can form inside the chlorosomes and other aspects of the physiology and metabolism of this organism (e.g., Tsukatani et al., 2004; Li et al., 2009). However, progress toward understanding the properties of BChl \( e \), and structural and functional properties of the photosynthetic apparatus of brown-colored GSB, has been markedly slower because of the absence of a tractable genetic system in such organisms. In this study we report the construction of a mutation in the \( bchU \) gene of the brown-colored GSB, \textit{Chlorobaculum limnaeum}, by conjugative transfer of a suicide plasmid from \textit{Escherichia coli}. The resulting mutant produces chlorosomes containing BChl \( f \), the BChl that has never been observed in natural GSB populations.

**MATERIALS AND METHODS**

**BACTERIAL STRAINS AND GROWTH CONDITIONS**

\textit{Chlorobaculum limnaeum} strain DSM 1677\(^T\) (Imhoff, 2003) was obtained from the culture collection of Dr. Johannes Imhoff and was maintained in liquid culture at room temperature in standard SL10 medium for GSB (Overmann and Pfennig, 1989). Although this strain had not previously been reported to grow on thiosulfate, it was found that the strain could grow well on the same medium used for cultivation of \textit{C. tepidum} (Wahlund and Madigan, 1995). \textit{C. limnaeum} cells were acclimated to growth in the presence of 50 \( \mu \)g kanamycin \( \text{ml}^{-1} \) by gradually increasing the concentration of kanamycin in the growth medium. Cells were grown at room temperature on CL medium (Frigaard and Bryant, 2001) at irradiances of 10–100 \( \mu \)mol photons \( \text{m}^{-2} \text{s}^{-1} \) provided by either tungsten or cool white fluorescent lamps as specified in the text.

Recombinant DNA procedures were performed using chemically competent cells of \textit{Escherichia coli} strain TOP10\(F^\prime\). Conjugation experiments were performed with \textit{E. coli} strain S17-1. \textit{E. coli} cells were grown in Luria–Bertani medium supplemented with 100 \( \mu \)g spectinomycin \( \text{ml}^{-1} \).

**CONJUGATIVE INACTIVATION OF THE \textit{bchU} GENE**

The genome sequence of \textit{C. limnaeum} has been determined and will be reported elsewhere (Vogl et al., in preparation). To introduce suicide plasmids into \textit{C. limnaeum} by conjugation from \textit{E. coli}, the mobilizable plasmid pCLCON1 was constructed as follows. The \textit{oriT}-containing region of pLO2 (Lenz et al., 1994) was amplified by PCR by using a proofreading DNA polymerase together with primers pLO2F and pLO2Rev, which included HindIII and EcoRI restriction sites, respectively (Table 1). The resulting PCR product and plasmid pSRA81 (Frigaard et al., 2007).
Table 1 | Oligonucleotide primers*.

| Primer          | Oligonucleotide sequence (5’ to 3’)                                                                 | Restriction enzyme |
|-----------------|----------------------------------------------------------------------------------------------------|--------------------|
| **PRIMER FOR PLASMID pCLCON** |                                                                                                     |                    |
| pLO2F           | CTCGAGGAGAACAGAAGCTTCCGCCTGTAAT                                                                  | HindIII            |
| pLO2Rev         | GGTTTAAGAGAAGCTTGACATATAGA                                                                      | EcoRI              |
| **PRIMERS FOR CLONING PART OF bchU AND VERIFICATION OF CONJUGANTS** |                                                                                                     |                    |
| CL_bchUF        | GACATGAGCACAGCTTGAACCTCTGTA                                                                     | HindIII            |
| CL_bchURrev     | GTAGAGAAGCTGCGAACATCACCAG                                                                     | PstI               |
| bchUtestF       | TGACGGCAACCCAGCATGTG                                                                       |                    |
| aadAtestRev     | ATCCACTGTTGCTTACGCC                                                                      |                    |

*Primers used to generate plasmid pCLCON and to amplify a part of bchU and to verify the insertion of the plasmid into the genome of *C. limnaeum*. Sequences in bold indicate the restriction sites introduced for cloning (indicated at the right).

2004b) were digested with HindIII and EcoRI. The resulting HindIII-EcoRI fragment from pSRA81 containing the *aadA* gene, and the digested PCR product from pLO2, were ligated, resulting in plasmid pCLCON. A fragment of the *bchU* gene (GenBank accession JX292262) was amplified by the polymerase chain reaction (PCR) using the following primers: CL_bchUF and CL_bchURrev (Table 1), and the resulting amplicon was cloned into the HindIII and PstI sites of plasmid pCLCON to produce pCLCON1. This plasmid was transformed into *E. coli* strain SI7-1, in which the genes required for conjugative transfer are integrated into the *E. coli* chromosomes (Simon et al., 1983).

Overnight cultures of the strain harboring pCLCON1 were diluted 1:10 and grown to OD550 nm ~0.6. The cells from a 1.5-ml aliquot of the culture were pelleted and washed three times with CL medium that did not contain sulfide and bicarbonate. The *E. coli* cell pellet was then transferred to an anoxic chamber (Coy Laboratory Products, Grass Lake, MI). *C. limnaeum* was kept on plates because the plating efficiency remained higher than when cells were maintained in liquid media. The mating mixture for conjugation was established as follows. About two inocula of the culture were pelleted and washed three times with a French press at 138 MPa. Chlorosomes were separated from membranes on continuous sucrose density gradients (10–53% linear gradients prepared in isolation buffer) by ultracentrifugation at 220,000 × g for 2 h. The chlorosomes were separated from membranes on continuous sucrose density gradients (10–53% linear gradients prepared in isolation buffer) by ultracentrifugation at 220,000 × g for 18 h at 4°C. The chlorosomes were washed twice with 4 volumes of phosphate buffer (10 mM potassium phosphate pH 7.2, 150 mM NaCl) and pelleted by ultracentrifugation at 220,000 × g for 1.5 h. The isolated chlorosomes were resuspended in 1–2 ml of phosphate buffer containing 1.0 mM PMSF and 2.0 mM DTT and stored at 4°C until further required.

Chlorosome proteins were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) using a Tris-Tricine buffer system (Schägger and von Jagow, 1987). The stacking gel was 3% monomer and 3.3% crosslinker and the resolving gel was 15% monomer and 3.3% crosslinker. Briefly, samples containing ~20 μg of BChl c were incubated at 56°C in 1× loading buffer (0.1 M Tris-HCl buffer, pH 6.8, 24% (v/v) glycerol, 1% (w/v) SDS, 2% (w/v) mercaptoethanol, 0.02% (w/v) Coomassie blue for about 2 min and electrophoresed for 16 h at constant voltage of 70V. Proteins were visualized through silver staining as previously described (Blum et al., 1987).

ABSORPTION AND FLUORESCENCE MEASUREMENTS

Room temperature absorbance spectra for whole cells were measured with a GENESYS 10 spectrophotometer (Thermo Fisher Scientific Corp., Waltham, MA), with a Cary 14 spectrometer modified for computerized data acquisition and operation by OLIS, Inc (Bogart, GA), or with a Lambda 950 UV/Vis/NIR spectrophotometer (Perkin Elmer Inc., Waltham, MA). Fluorescence spectra of isolated chlorosomes were measured using a customized PTI fluorometer (Photon Technology International Inc., Birmingham, NJ) consisting of a Xe excitation lamp, excitation monochromator, emission monochromator, signal chopper, lock-in amplifier, and avalanche photodiode detector. For fluorescence emission, the BChl c and BChl f chlorosomes were excited at their Soret bands, at 457 nm and 446 nm, respectively. Isolated chlorosomes were prepared for spectroscopy by dilution to a Qy band absorption of 0.1 with 20 mM Tris-HCl buffer, pH 8.0. When necessary, the chlorosomes were fully reduced by the addition of sodium dithionite to a final concentration of 25 mM, and subsequent incubation in the dark for 1 h at 4°C prior to measurements. Absorbance and fluorescence spectra of isolated chlorosomes were recorded at 77K by adding glycerol to the samples to a final concentration of 50% (v/v); the sample was then cooled with liquid nitrogen in an Optistat DN2 cryostat (Oxford Instruments, Oxfordshire, UK).
DYNAMIC LIGHT SCATTERING MEASUREMENTS

Dynamic light scattering (DLS) was used to compare the hydrodynamic diameter ($d_H$) of the chlorosomes of the WT and the bchU mutant. The measurements were performed with a ZetaSizer Nano ZS (Malvern Instruments Inc., UK) in dynamic light scattering mode. All measurements were made in a 1 cm plastic cuvette, at ambient temperature, with back angle scattering detection at 173° to incident beam. The absorbance of each chlorosome sample was adjusted to 0.5 at the $Q_\lambda$ maximum using 20 mM Tris-HCl buffer, pH 8.0, before measurements. This technique measures the diffusivity of the particle (D) and calculates $d_H$ of the particles using Einstein’s equation: $d_H = kT/3\pi\eta D$, where $k$ is the Boltzmann constant, $T$ is the absolute temperature in Kelvin, and $\eta$ is the viscosity of the solution. Because chlorosomes are not spherical, DLS estimates the diameter of a solvated hypothetical solid sphere having the same diffusion coefficient as the chlorosome.

PIGMENT ANALYSIS BY HPLC AND MASS SPECTROMETRY

Reversed-phase HPLC was used to resolve the peaks of the different homologs of BChl $e$ and BChl $f$ as previously described (Frigaard et al., 1997). Cells from 1.0 ml of liquid culture were pelleted by centrifugation, and the pigments were extracted by sonication with acetone-methanol (7:2 v/v). The pigment extracts were filtered, and 0.1 volume of 1.0 M ammonium acetate was added to the filtered samples immediately before injection onto the HPLC column. The resulting pigment extract was immediately subjected to analytical HPLC on an Agilent Series 1100 HPLC system (Agilent Technologies, Palo Alto, CA). Pigments were separated on a 25 cm by 4.6 mm Discovery 5$\mu$m C18 column (Supelco, Bellefonte, PA) attached to a 1,024-element diode array detector (Model G1315B, 1100 Series, Agilent Technologies, Palo Alto, CA). The resulting data were analyzed using ChemStation software (Agilent Technologies, Palo Alto, CA).

For mass analyses of BChls, pigments were separated by a reversed-phase HPLC system that was directly coupled with a tandem mass spectrometer for MS–MS analysis. The samples were demethylated to form the corresponding pheophytins by post-column introduction of formic acid into the eluent flow as previously described (Airs and Keely, 2000). The resulting data were analyzed with Masslynx software version 3.5 (Micromass, Ltd., Manchester UK). All mass spectrometric measurements were performed at the Mass Spectrometry Facility in The Huck Institutes for the Life Sciences at The Pennsylvania State University (University Park, PA).

GROWTH RATE MEASUREMENTS

*C. limnaeum* was grown at room temperature (22–25°C) on a rotating wheel that was uniformly illuminated from the front. Two different light sources were used. In some experiments, light was provided by tungsten lamps which are highly enriched in light at wavelengths longer than ~600 nm. In other experiments, light was provided by cool white fluorescent lamps, which are enriched in blue-green light with wavelengths shorter than 600 nm. Before growth rates were determined, cultures were acclimated to the light intensity and the light source to be used for the growth rate measurement. Inocula from the starter cultures were diluted into fresh medium at OD$_{650\text{nm}} = 0.05$, and the OD$_{650\text{nm}}$ was then monitored until the culture reached early stationary phase. In cultures grown at high irradiance, the transient appearance of polysulfide and elemental sulfur globules interfered with the light scattering measurements for OD$_{650\text{nm}}$ values in the range of 0.3–0.7. Nevertheless, it was still possible to estimate the growth rate from OD$_{650\text{nm}}$ measurements with reasonable accuracy. Under low irradiance conditions, polysulfide and sulfur globules did not interfere with the growth rate determination.

RESULTS

CONSTRUCTION AND VERIFICATION OF A bchU MUTANT OF *C. limnaeum*

As described in the “Materials and Methods,” plasmid pCLCON1 was introduced by conjugation from *E. coli* strain S17-1 into *C. limnaeum*. After selection on plates containing kanamycin, streptomycin, and spectinomycin, brownish green transconjugant colonies arose after about 10 days. Selected colonies were subjected to three rounds of restreaking on selective media, and individual transconjugant colonies were screened by PCR amplification of a fragment spanning the *bchU* gene and the inserted plasmid (see Figures 2A,B). The specific amplification of a ~1500 bp fragment using PCR primers bchUtestF and aadAtestRev (Table 1) from the desired transconjugants (Figure 2B), but not from the WT strain, demonstrated that the *bchU* gene had been insertionally inactivated by a single-crossover, homologous recombination event as illustrated in Figure 2. It was not possible to obtain a PCR product spanning the entire inserted plasmid; however, as will be described below, the phenotype of the resulting mutant showed that no functional copies of the *bchU* gene remained because BChl $e$ was no longer detectable in the transconjugant cells.

As shown in Figure 3, the resulting *bchU* mutant strain was greenish brown in color and was easily distinguishable from the WT, which was reddish brown in color. Figure 4 shows a comparison of the whole-cell absorption spectra for the WT and the *bchU* mutant. The WT cells had absorption maxima at 453, 522, and 722 nm, while the *bchU* mutant had absorption maxima at 448, 506, and 706 nm. As expected for the loss of the C-20 methyl group, the Q$_\lambda$ absorption band of the BChl aggregates in the chlorosomes was blue-shifted by ~16 nm.

Figure 5A shows a portion of the elution profile for pigments extracted from WT *C. limnaeum* cells. Four BChl peaks were observed with elution times between 20 and 25 min, and all four had the same absorption spectra, with maxima at 471 and 656 nm (Figure 5C), which showed that these peaks are methylation homologs of BChl $e$. Mass spectroscopic analyses showed that these peaks corresponded to BChl $e$ esterified with farnesol. Furthermore, it could be concluded from the mass spectroscopic data that all of the homologs were methylated at the C-12$^1$ position. Thus, the four peaks, which had *m/z* values of 820, 834, 848, and 862, corresponded to [8-Et, 12-Et]-BChl $e_1$, [8-Pr, 12-Et]-BChl $e_1$, [8-iso, 12-Et]-BChl $e_1$, and [8-neo, 12-Et]-BChl $e_1$.

Figure 5B shows a portion of the elution profile of pigments extracted from cells of the *bchU* mutant. Although the relative proportions of the homologs were slightly different from those of the WT, four peaks were again observed. However, the elution
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FIGURE 2 | (A) Scheme showing the general features and the arrangement in suicide plasmid pCLCON1 and how a single cross-over recombination event between the gene-internal fragment of bchU (bchU′) leads to insertional inactivation of the chromosomal bchU gene. (B) Agarose gel electrophoresis of PCR products to demonstrate insertional inactivation of the bchU gene. Lanes: M, size markers with sizes in bp indicated; lane 1, the template DNA was from a bchU mutant; lane 2, template DNA from the WT strain. The ~1500 bp amplicon resulting from primers flanking bchU and the aadA (bchUtestF and aadAtestRev; see Table 1) gene (small arrows in panel A) in lane 1 shows that the bchU gene has been insertional inactivated.

FIGURE 3 | Cultures of C. limnaeum 1677T WT (tube WT) and the bchU mutant (tube M).

Isolation and Characterization of Chlorosomes from the bchU mutant

Chlorosomes were isolated from the WT and bchU mutant cells, and their properties were compared. As shown in Figure 6, chlorosomes from the WT and the bchU mutant had similar average hydrodynamic diameters (mean diameters, 83 and 73 nm, respectively) as estimated by light scattering. The polypeptide complement of chlorosomes from WT C. limnaeum differs from that of C. tepidum (Figure 7), but the polypeptide compositions of chlorosomes from the WT and bchU mutant strains were essentially indistinguishable (compare Figure 7, lanes 2 and 3). Thus, inactivation of the bchU gene and replacement of BChl e by BChl f did not alter the polypeptide composition of the chlorosome envelope in the bchU mutant.

Figure 8 shows a comparison of the room temperature (Figure 8A) and low temperature (Figure 8B) absorption spectra for chlorosomes from the WT and the bchU mutant. Except for the baseplate absorption arising from BChl a at ~795 nm, the absorption maxima for chlorosomes from the bchU mutant are shifted to the blue. The 77 K absorption spectra provide sufficient peak resolution to show that the absorption of the BChl a-containing baseplate is unchanged between the WT and methyl group (Note: Because of the low yield, it was not possible to determine the mass of the compound eluting at 22.5 min). The combination of these results indicated that these four peaks correspond to [8-Et, 12-Et]-BChl f, [8-Pr, 12-Et]-BChl f, [8-Iso, 12-Et]-BChl f, and probably [8-Neo, 12-Et]-BChl f.

Times were shifted about 1 min earlier for each, which indicates that these compounds were less hydrophobic than BChl e. The absorption spectra of these four peaks were identical and had absorption maxima at 461 and 641 nm, which are the expected values for BChl f (Figure 5D). Mass spectroscopic analyses confirmed this identification and showed that the m/z values for these homologs were 14 mass units smaller than the corresponding peaks for the WT, which is consistent with the loss of one
mutant. This indicates that the BChl e and BChl f oligomers transfer energy to the same type of acceptor in the chlorosome. Otherwise, the spectra of the mutant are very similar in shape to those for WT chlorosomes. WT chlorosomes had absorption maxima at 467, 528, 721, and 795 nm at room temperature (Figure 8A), while chlorosomes from the bchU mutant had absorption maxima at 446, 508, 705, and 794 nm. Similar absorption maxima were observed for the two samples at low temperature (Figure 8B). The half-band width of the Qy absorption band was very slightly narrower for the chlorosomes from the bchU mutant (51 nm) than for those from the WT (55 nm).

The fluorescence emission spectra for chlorosomes isolated from the WT and the bchU mutant of C. limnaeum, under oxidizing and reducing conditions, are presented in Figures 9 and 10, respectively. Two emission peaks, with maxima at about 749 and 817 nm, are observed for reduced chlorosomes from the WT, and similar to C. tepidum, energy transfer from the BChl e aggregates to the BChl a associated with the baseplate was severely attenuated under oxidizing conditions (Figure 9A). For reduced chlorosomes from the bchU mutant, two emission peaks were also observed at room temperature, with maxima at about 737 and 820 nm (Figure 10A). Surprisingly, under oxidizing conditions, most of the fluorescence emission occurred at ~662 nm.
which presumably arises from BChl f monomers, and a weaker secondary emission occurred at about 721 nm, but very little if any energy transfer to the BChl a of the baseplate occurred (Figure 10A). This behavior is different from that observed in chlorosomes of other GSB, which typically do not exhibit any significant fluorescence from pigment monomers. Figure 10B shows the fluorescence emission spectra of oxidized and reduced chlorosomes for the bchU mutant at 77 K. In addition to the emission from pigment monomers at 655 nm, the main chlorosome fluorescence band was resolved into two components that were centered at 704 and 747 nm. The 747 nm component is consistent with fluorescence emission from BChl f oligomers, and the 704 nm component is consistent with emission from a low energy vibrational state of monomeric BChl f (Figure 10B).

GROWTH RATE DETERMINATIONS FOR THE WT AND bchU STRAINS OF C. limnaeum

Because photoautotrophs depend directly on efficient light harvesting for growth, the growth rates of the WT and bchU mutant of C. limnaeum were measured to compare the light-harvesting properties of chlorosomes containing BChl e and BChl f. As shown in Figure 11, when the WT and bchU mutant were grown at high irradiance (100 μmol photons m⁻² s⁻¹) with either tungsten light (Figures 11A,B) or cool white fluorescent light (Figures 11C,D), the two strains had virtually identical growth rates. Both strains grew faster with light provided by a tungsten source than with light from a cool white fluorescent source (~14-h doubling times vs. ~21-h doubling times). Each strain obviously grew much more slowly at low light intensity (10 μmol photons m⁻² s⁻¹), but at low light intensity the WT type strain grew about 40% faster than the bchU mutant, and this difference was independent of the type of light source used (Figures 11B,D). These results are consistent with the results discussed above, which showed that poorer energy transfer to the chlorosome baseplate and much poorer overall energy transfer efficiency occurs in chlorosomes of the bchU mutant.

DISCUSSION

Brown-colored GSB, those that assemble chlorosomes containing BChl e and usually isorenieratene as principal pigments, are typically found at extremely low irradiance values, which exclude the growth of most if not all other phototrophs, in anoxic layers with low redox potential (Vila and Abella, 1994; van Gemerden and Mas, 1995). In stratified freshwater lakes, these organisms typically occur at and/or below the chemocline (Vila and Abella, 2001; Tonolla et al., 2003), and in some exceptional cases, as in the Black Sea (Manske et al., 2005; Marschall et al., 2010), the photon fluxes where these organisms grow and persist can approach the extreme limit of one to ten photons absorbed per BChl molecule per day. That any organism is capable of growth under such extremely energy-limited conditions implicitly means that it must have a remarkably efficient light-harvesting system.

Progress in studying the light-harvesting apparatus of brown-colored GSB, however, has been severely limited because of the absence of a tractable genetic system for any of these organisms.
Over a period of ∼10 years, members of the Bryant laboratory systematically searched for an organism that provided the requisite properties required for the development of a genetic system: a reasonable growth rate on solid medium, a high plating efficiency, appropriate sensitivity to antibiotics, and the ability to take up DNA by natural transformation, electroporation, or conjugation and to recombine this DNA with chromosomal sequences. Several strains were tested and discarded over the years because none had the right combination of properties and none proved to be reliably transformable. Because of its phylogenetic relationship to *C. tepidum*, and because of its ability to use thiosulfate as an electron donor, *C. limnaeum* strain DSM 1677^T^ was eventually selected and tested for the development of a genetic system. As shown by this study, it is possible to produce transconjugants with suicide plasmids introduced by conjugal transfer from *E. coli* into this strain. We will publish the genome sequence of this organism elsewhere (Vogl et al., in preparation).

In *C. tepidum*, BchU is thought to catalyze the penultimate step in BChl *c* synthesis. The methylation reaction BchU catalyzes probably takes place immediately prior to the addition of the farnesyl tail to bacteriochlorophyllide *d* (Wada et al., 2006; Liu and Bryant, 2012). Because the enzymes that convert the 7-methyl group to the 7-formyl group of BChl *e* have not yet been identified, the order of the reactions leading from chlorophyllide *a* to bacteriochlorophyllide *e* are not yet known. Only BChl *f* was detected in the *bchU* mutant of *C. limnaeum*, and no other intermediates in the synthesis of BChl *e* were detected. Further studies will be required to elucidate the complete biosynthetic pathway for BChl *e*.

Inactivation of the *bchU* gene of *C. tepidum* produced several effects on chlorosomes (Maresca et al., 2004). Firstly, the absence of the C-20 methyl group caused a blue-shift of the Qy absorption of the BChl *d* aggregates in chlorosomes. Secondly, the half-bandwidth of the Qy absorption decreased, possibly because of a change in the supramolecular structure of the BChl aggregates (Ganapathy et al., 2009, 2012). Finally, the BChl of the cells of the *bchU* mutant was lower than that of wild-type cells grown at the same light intensity. The combination of these factors caused the mutant to grow significantly slower than the WT under all light conditions tested (Maresca et al., 2004). Some of these same effects may occur in the *bchU* mutant of *C. limnaeum*, although the effects of the mutation on the half-bandwidth and BChl content of the cells appear to be smaller than in *C. tepidum*.

At least thirteen major types of Chls and BChls are known to occur in bacteria: Chl *a*, Chl *b*, Chl *d*, Chl *f*, 8^1^-hydroxy-Chl...
a, 3,8-divinyl Chl a, 3,8-divinyl-Chl b; and BChl a, b, c, d, e, and f (Chew and Bryant, 2007; Liu and Bryant, 2012). Given the diversity of chemical structures and corresponding absorption properties represented by these molecules, it was a paradox why BChl f, which differs from BChl e only by the absence of a single methyl group at the C-20 methine carbon, had not been detected in any natural sample (Tamiaki et al., 2011). Although there is some overlap between the absorption of aggregated BChl f and Chl a, the absorption properties of chlorosomes containing BChl f do not overlap extensively with the absorption of other Chls except Chl d (Miyashita et al., 1996; Li et al., 2012) and Chl f (Chen et al., 2010; Li et al., 2012), which are rare Chls produced by only a few cyanobacteria. However, red light does not penetrate sufficiently deeply into most stratified water columns to reach the anoxic water layers where GSB can obtain the reduced sulfur compounds they require as electron donors for photoautotrophic growth (Vila and Abella, 1994, 2001). Finally, BChl f does not appear to have sufficiently unique absorption properties to define a natural light niche that would allow these organisms to outcompete other types of phototrophic bacteria (see Stomp et al., 2007). Thus, BChl f may not occur naturally because anoxic environments where sulfide concentrations are high, redox potentials are low, and appropriate irradiance characteristics occur to define a unique light niche are either uncommon or non-existent.

The results from this study suggest several other possible reasons why BChl f has not been found in nature. Firstly, the aggregated forms of BChl f in chlorosomes are only slightly red-shifted relative to Chl a, and because all oxygenic photosynthetic organisms produce Chl a, GSB living deep in the anoxic layers of stratified lakes and producing BChl f would receive light that would be strongly filtered by the Chl a associated with cyanobacteria and algae in the oxic layer of stratified systems. Moreover, carotenoids associated with light-harvesting proteins in eukaryotic algae and prokaryotes would additionally filter some blue light that might otherwise be absorbed by BChl f. Secondly, there is a very large energy gap, \( \sim 90 \) nm, between the BChl f aggregates in chlorosomes and the BChl a associated with CsmA in the baseplate. Although the transfer is downhill energy-wise, this gap may be too large to allow efficient energy transfer (this will be explicitly explored in future studies). This conclusion is supported by initial inspection of the...
fluorescence emission spectra for the fully reduced chlorosomes. Energy transfer appears to be less efficient in the chlorosomes of the $bchU$ mutant than in those of the WT, because the amplitude of the emission from the BChl $a$ associated with the CsmA baseplate is lower for the mutant than the WT at equal absorption. The molar extinction coefficient for BChls $e$ and $f$ are expected to be similar and should not significantly factor into this difference because of the high degree of structural similarity for the two pigments. Finally, similar to previous observations for other chlorosomes (Wang et al., 1990; Blankenship et al., 1993; Frigaard et al., 1997; Garcia Costas et al., 2011), energy transfer in both the WT and the $bchU$ mutant was extremely sensitive to the oxidation state of the chlorosomes. The presence of even low amounts of oxygen could be sufficient to cause significant quenching of energy transfer in these organisms. Whatever the actual cause(s), the growth rate studies presented here demonstrate convincingly that cells synthesizing BChl $f$ could not compete effectively with WT cells producing BChl $e$ for their chlorosomes in any natural, light-limited environment.

Furthermore, any mutant that did arise by inactivation of the $bchU$ gene would quickly be eliminated from the natural community, because those cells would be unable to compete effectively with cells producing BChl $e$ for the light energy required for growth.

CONCLUSION

We report here the construction of a $bchU$ mutant in C. limnaeum, the first targeted mutation constructed in a brown-colored GSB. This mutant produced chlorosomes containing aggregates of BChl $f$, a pigment that had not previously been reported to occur in any natural system. The $bchU$ mutant grew much slower than the WT at low irradiance values. Energy transfer from the BChl $f$ aggregates to the BChl $a$ in the chlorosome baseplates was less efficient than in chlorosomes containing BChl $e$ aggregates. It appears that energy transfer is less efficient in the chlorosomes of the $bchU$ mutant, but the causes of that inefficiency are currently under investigation and will be the subject of further studies. Ongoing static and time-resolved spectroscopic
studies will hopefully provide a more complete explanation for the poor light-harvesting properties of BCHl f. Whatever the reason(s), it is obvious that bacteria producing this pigment could not compete well with BCHl c-producing strains in natural light-limited environments and would be quickly eliminated from any natural population in which they aroze because BCHl f does not allow the cells to occupy a unique light niche.

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