Specific Gene \textit{bciD} for C7-Methyl Oxidation in Bacteriochlorophyll \textit{e} Biosynthesis of Brown-Colored Green Sulfur Bacteria

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Abstract

The gene named \textit{bciD}, which encodes the enzyme involved in \textit{C7}-formylation in bacteriochlorophyll \textit{e} biosynthesis, was found and investigated by insertional inactivation in the brown-colored green sulfur bacterium \textit{Chlorobaculum limnaeum} (previously called \textit{Chlorobium phaeobacteroides}). The \textit{bciD} mutant cells were green in color, and accumulated bacteriochlorophyll \textit{c} homologs bearing the 7-methyl group, compared to \textit{C7}-formylated \textit{BChl} \textit{e} homologs in the wild type. \textit{BChl-c} homolog compositions in the mutant were further different from those in \textit{Chlorobaculum tepidum} which originally produced \textit{BChl-c}: (3\textsuperscript{\textit{S}})-8-isobutyl-12-ethyl-\textit{BChl c} was unusually predominant.

Introduction

Chlorophyll(\textit{Chl})s and bacteriochlorophyll(\textit{BChl})s are key pigments for the initial stage of photosynthetic processes, harvesting sunlight, transferring excited energy, and performing charge separation. Each (\textit{BChl}) pigment has a distinctive absorption character in ultraviolet, visible, and near-infrared regions which is dependent on the molecular structure \cite{1}. Photosynthetic organisms live under various light conditions and use one or two (\textit{BChl})s species for efficient absorption of habitat- or niche-specific light. All oxygenic photosynthetic organisms commonly possess \textit{Chl a} (or 8-vinyl-\textit{Chl a} in the case of \textit{Prochlorococcus}). In addition to \textit{Chl a}, plants and green algae have \textit{Chl b}, and some cyanobacteria possess \textit{Chl d} \cite{2} or \textit{Chl f} \cite{3}. \textit{Chls b, d, and f} have different absorption properties from \textit{Chl a} (\textit{Q}, and \textit{Soret maxima} = 660.8 and 429.6 nm in diethyl ether) due to the presence of formyl group at the \textit{C7}, \textit{C3}, and \textit{C2} positions of chlorin ring, respectively (\textit{Q}, and \textit{Soret maxima for; \textit{Chl b}, 642.2/453.0 nm; \textit{Chl d}, 685.8/ 445.8 nm; \textit{Chl f}, 694.5/439.5 nm \textit{[4]}). Among \textit{Chls b, d, and f}, a gene encoding enzyme for formylation at \textit{C7} of \textit{Chl b} has already been identified as chlorophyllide (\textit{Chlide}) \textit{a} oxygenase gene, \textit{CAO} \textit{[5]}. It was observed that the mutation of \textit{CAO} gene in the green algae \textit{Chlamydomonas reinhardtii} synthesized \textit{no Chlb}, but exclusively \textit{Chl a}, and a recombinant \textit{CAO} enzyme converted the methyl group at \textit{C7} of \textit{Chlide a} to the \textit{7-formyl group of Chlide b} via a hydroxymethyl group by two successive monoxygenations \textit{in vitro} \textit{[6]}. In contrast, the genes for formylation in \textit{Chls d} and \textit{f} have not yet been found. Elucidation of the genes encoding the oxidation to a formyl group is significant to understand the evolution of \textit{BChl} pigment biosynthesis as well as the photosynthetic mechanism.

Among \textit{BChl} molecules found in anoxygenic photosynthetic bacteria in natural environments, \textit{BChl e} is the only formylated pigment at the \textit{C7} position (see Fig. 1). \textit{BChls e} and \textit{d} as well as \textit{BChl e} are found in green sulfur bacteria (GSB) \textit{[1,7]}. These pigments self-aggregate to form characteristic extra-membrane antenna systems, called chlorosomes. In a chlorosome, a large amount of \textit{BChl e}, \textit{d}, or \textit{e} molecules give highly-ordered and protein-free suprastructures, and permit efficient absorption of light and rapid migration of excitation energy. The biosynthesis of \textit{BChls} \textit{e} and \textit{d} has been investigated using \textit{Chlorobaculum (Cha.) tepidum}, a genetically tractable model organism of GSB \textit{[8,9]}. Following the recent discovery of an enzyme for removal of the \textit{13\textsuperscript{2}}-methoxy-carbonyl group from \textit{Chlide a}, called \textit{BciC} \textit{[10]}, all the genes encoding \textit{BChls e} and \textit{d} biosynthetic enzymes were revealed. However, the second stage of biosynthesis of \textit{BChl e} has been unidentified, i.e., an enzyme producing a formyl group at the \textit{C7} position of bacteriochlorophyllide (\textit{BChlide}) \textit{e} (Fig. 1) was not
found, as was also true in the cases of Chls d and f mentioned above.

Very recently, the mutagenesis of one species of the GSB Cha. limnaeum [phylogenetically renamed from Chlorobium (Chl.) phaeobacteroides [11]] which synthesized BChl e, was achieved based on a double cross-over event between homologous regions by natural transformation [12,13], or a single cross-over event by the E. coli conjugation system [14]. In these studies, a mutant deleting C20-methyltransferase gene (bchE) for BChl e synthesis was constructed, and analysis of its pigment compositions showed accumulation of C20-unsubstituted BChl f, whose name and structure were proposed, based on BChl e which was discovered in 1975 [15], but the BChl f has never been observed in nature [16]. In the mutant cells, BChl f self-aggregates were formed chlorosomes and could transfer their harvested light energy to BChl a associated with baseplate proteins as an initial energy acceptor in a chlorosome. Therefore, BChl f was assigned as a photosynthetically active pigment in the bacterial cells. The mutagenesis of Cha. limnaeum opens the door for the discovery of genes for BChl e biosynthesis.

In this study, we describe the identification of a gene denoted bciD which encodes an enzyme requisite for the C7-methyl oxidation (formylation) in BChl e synthesis. The bciD gene was initially identified by performing phylogenetic profiling analysis of genomic DNA sequence data among BChl e producing strains in GSB. To verify that BciD played a role in BChl e biosynthesis, a null mutant of Cha. limnaeum was constructed by deleting the bciD gene. This mutant was unable to synthesize BChl e, instead accumulating BChl c. By characterizing the BciD compositions, we show that BciD is essential for the oxidation to a 7-formyl group in BChl e biosynthesis, and discuss the enzymatic function of BciD and its role in biosynthetic pathway. This work was preliminarily reported by our group [17,18], and independently, a similar result was appeared in the oral presentation by Prof. Bryant [19].

Materials and Methods

Bacterial Strains and Culture Conditions

The strain RK-j-1 [13] of Cha. limnaeum was used as a parent wild type strain. Cha. tepidum WT2321 [20] was used as a control having BChl e homologs for HPLC analyses. These bacteria were anaerobically grown in a 30 mL or 1 L screw-capped bottle with liquid CL medium, or on a solid CP plate [21]. The growth temperature was adjusted to 30℃ for Cha. limnaeum or 45℃ for Cha. tepidum. E. coli DH5α was grown in LB medium containing 100 μg/mL of ampicillin or 20 μg/mL of streptomycin.

Plasmid Constructions

The draft genome sequence of Cha. limnaeum strain RK-j-1 has been determined and will be reported elsewhere (J. Harada et al., in preparation). Complete DNA sequences of the bciD gene and its flanking genes were deposited in the GenBank (Accession No. AB762294). To construct the Cha. limnaeum mutant lacking BciD (Fig. 2A), pTAbciDSm plasmid was used as the template for PCR with the primer set aadA-F (CTGTTCCGGTGTAAGCTGT) and aadA-R (CTGCGGCTTTAAGGATTGT). While, a 1.99-kbp DNA fragment containing the bciD gene was amplified from the genomic DNA of Cha. limnaeum by PCR using bciD-F (TCACTGTATATGGTGCTGGGTA) and bciD-R (GTTAAGCACCATTGCCGAA) primers. The PCR product containing bciD was cloned into the TA-cloning site of T-Vector pTA2 (TOYOBO, Japan), yielding pTA2-bciD plasmid. To amplify the DNA fragment from pTA2-bciD without the inner portion of bciD, the plasmid was used as the template for PCR with primers bciD-inf-F (TCTGTCGAGCCGACTCCGCGGAGGATCATGTC) and bciD-inf-R (TTAGACCGAAACGTGTTAACCCTCAGCTTGGC). Underlined sequences of these primers were designed to overlap with partial DNA sequences of the above-mentioned aadA-F and aadA-R primers for the following In-Fusion cloning. The resulting PCR product and the PCR fragment containing the aadA gene were ligated using an In-Fusion® HD Cloning Kit (Clontech, USA), creating pTAbciDSm.

Transformation of Cha. limnaeum

Natural transformation using the Cha. limnaeum RK-j-1 strain was performed as previously described [13]. About 0.1 mg of EsoRI-digested pTAbciDSm was used for the transformation. CP plates, containing both 100 μg/mL of streptomycin and 150 μg/mL of spectinomycin, were used for selection of transformants.

PCR analysis was carried out to monitor segregation of wild type and mutant alleles using bciD-comF (CATCAGAGGGGCGCAAATAGA) and -R (CTTGCGCCGAGAGGATTAT) primers. A DNA molecular weight marker, λ/Sty digest (TAKARA, Japan), was used for molecular mass estimations of PCR products. DNA sequence analyses of the PCR fragments
Results

Identification of Candidate Gene for C7-Methyl Oxidation in BChl e Biosynthesis

To identify candidate genes encoding enzymes specifically involved in BChl e biosynthesis, we first performed Chlorobium-specific BLAST analysis, a program equipped by the Joint Genome Institute [JGI: http://genome.jgi.doe.gov/pages/blast.jsf?db=chloroh], using the CAO gene of *Chlamydomonas reinhardtii* as a query [5]. The results showed no hit of a similar gene of CAO among genomes of eleven GSB species containing three brown-colored BChl e-producing strains, *Chl. phaeobacteroides* BS1, *Chl. phaeobacteroides* DSM266, and *Pelodictyon phaeolathiforme* BU-1.

Then, we used a computer-aided gene discovery program, Correlation Coefficient Calculation Tool (CCCT) [23], to find candidate genes for C7-methyl oxidase in BChl e biosynthesis. CCCT is based on a comparative analysis of whole gene sets between different organisms, which allows for the identification of genes involved in a particular function in a certain class of organisms [23,24]. In this study, we made two assumptions: (1) the above three strains producing BChl e must have a specific enzyme(s) responsible for BChl e synthesis; (2) the enzyme(s) would not be found in any other GSB that possesses BChl c or d and filamentous anoxygenic phototrophic bacteria containing BChl c. The program was used with gene sets from 9 species whose genome sequences have been determined (see Table S1). Here, ORFs of *Chl. phaeobacteroides* BS1 were used as query sequences. In the output data, all ORFs of strain BS1 were ranked according to their correlation coefficients, such that ORFs specifically conserved in the three strains of BChl e-producing bacteria were expected to show higher values. In the top genes of the output data (Table S2), Cphamn1_0270 in BS1 strain, Ppha_2747 in BU-1 strain and Cpha266_0196 in DSM266 strain had a higher similarity with one another, and were annotated as radical S-adenosyl-L-methionine (SAM) enzymes. The gene was found only in the three brown-colored bacteria but not in the other 6 bacteria. The conversion from a methyl to formyl group at the C7 position is suggested to involve in radical reactions [25]. Further, the radical SAM gene in each genome of these strains is interestingly present in the very similar gene cluster containing the brown-colored GSB specific genes for cyclization of γ-carotene, *cruB* [26], and C3-vinyl hydratase (*BchF*)-like gene (here called *bchF2*) (see Fig. 2A). Therefore, we choose this radical SAM gene as a good candidate for a gene coding C7 formylation (methyl oxidation) enzyme. This proposal was supported by another bioinformatic analysis [27]. The radical SAM gene was designated *bciD*.

Construction of bciD Mutant of Cba. limnaeum RK-j-1

We next tested whether *bciD* encodes the enzyme oxidizing the 7-methyl to formyl group involved in BChl e biosynthesis. Mutational analysis is effective in investigating the function of an unknown gene, but the above three BChl e-producing strains could not be utilized for such genetic studies. Therefore, we used the brown-colored BChl e-producing GSB *Cba. limnaeum* RK-j-1 whose mutagenesis method by natural transformation was recently established [13]. For this study, we determined the genomic DNA of *Cba. limnaeum* mutant.

(A) Schematic map of genes arrangement around *aadA1*. The *mycin, was inserted in bciD*.

![Figure 2. Construction of Cba. limnaeum bciD gene inactivated mutant.](image-url)

Figure 2. Construction of *Cba. limnaeum bciD* gene inactivated mutant. (A) Schematic map of genes arrangement around bciD gene in the genome of *Cba. limnaeum* RK-j-1, and its insertional inactivation. The *aadA1* gene, conferring resistance to streptomycin and spectinomycin, was inserted in *bciD*. Arrows represent the primers bciD-F (i), bciD-R (ii), bciD-inf-F (iii), bciD-inf-R (iv), bciD-comf-F (v), and bciD-comf-R (vi). (B) PCR confirmation of gene interruption. The bciD gene was amplified from genomic DNA extracted from the wild type (lanes 1 and 2) and a mutant (lanes 3 and 4) of *Cba. limnaeum*, using above bciD-comf-F and -R primers. The products in lanes 2 and 4 were then digested by restriction enzyme EcoRV, and the fragments yielded from wild type and the mutant were 1.36 and 0.78, and 2.22 kbp, respectively. Lane M, molecular size marker (the sizes of bands are indicated at left). doi:10.1371/journal.pone.0060026.g002

were determined using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA).

Determination of Compositions of BChl c Homologs

Pigments of the *Cba. limnaeum* mutant strain were extracted and analyzed as follows. A mixture of acetone and methanol (9:1, v/v) was added to the harvested cells and mixed using a vibrator. A mixture of diethyl ether and petroleum ether (1:1, v/v) and then acetonitrile:acetone: H2O (65:15:20, v/v/v) was added to the harvested cells and mixed using a vibrator. The mixture was allowed to settle, and the ether layer was collected and evaporated to dryness under a stream of N₂ gas, and the residues were dissolved in a small amount of high performance liquid chromatography (HPLC) eluent, described below. Liquid chromatography mass spectrometry (LCMS) analysis was performed using a Shimadzu LCMS-2010EV system (Shimadzu, Japan) comprising a liquid chromatograph (SCL-10Avp system controller, LC-10ADvp pump, and SPD-M10Avp photodiode-array detector) and a quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) probe. Reverse-phase HPLC was performed under the following conditions: column, Cosmosil 5C₁₈-AR-II (4.6 φ × 150 mm, Nacalai Tesque, Japan); eluent, acetonitrile:acetone: H2O (65:15:20, v/v/v); flow rate, 1.0 mL/min; detection wavelength, 415, 435 and 465 nm. APCI-MS spectra were measured as follows: resolution, ±0.15 Da; capillary temperature, 250°C; APCI vaporizer temperature, 400°C; ionization voltage, 4.5 kV; sheath gas flow, 2.5 L/min; drying gas pressure, 0.02 MPa.
sequence of _Chlamydomonas reinhardtii_ RK-j-1 strain, and performed a BLAST search analysis using BciD amino acid sequence as the query. The result showed that the gene cluster conserved among the above three strains as well as the _bciD_ gene, was also observed in the genome of the RK-j-1 strain. Thus, the _bciD_ gene of this strain was inactivated by insertion of the _aadA_ streptomycin and spectinomycin resistance cassette, as shown in Fig. 2A. To confirm the mutation, PCR analysis was performed using primers _bciD-comf-F_ and _-R_, and then an amplified DNA fragment containing _bciD_ gene and its flanking genes was digested with a restriction enzyme EcoRV. Agarose gel electrophoresis analysis (Fig. 2B) showed EcoRV-treated 1.36- and 0.78-kbp DNA fragment bands of wild type (lane 2) and while non-digested 2.22-kbp DNA band of the _bciD_ mutant (lane 4), because a large part of the _bciD_ gene containing EcoRV site was replaced with the _aadA_ cassette. This result indicated that the _aadA_ gene was correctly introduced into the _bciD_ gene in the mutant strain. We also confirmed this mutation by DNA sequence analysis of the PCR products. These results indicated that the _bciD_ mutant was completely segregated.

**Absorption Properties of _bciD_ Mutant of _Chlamydomonas reinhardtii_**

Unlike the brown-color wild type cells of _Chlamydomonas reinhardtii_, the _bciD_ mutant cells showed green-color similar to GSB species containing BChl _e_ or _d_ (Figure S1). Figure 3A shows the UV-Vis-NIR absorption spectra of whole cells of the wild type (broken line) and the mutant (solid line). The _Q_ and _Soret_ bands of the mutant were observed at 746.0 and 457.5 nm, respectively. These absorption bands displayed maxima different to those of the wild type, especially, the _Q_ peak of the _bciD_ mutant, red shifted by 32 nm relative to the wild type. These changes in absorption spectra by the mutation were reminiscent of GSB producing BChl _e_ ( _Q_ peak = 745–755 nm) [24,28]. Figure 3B shows the absorption spectra of pigments extracted from the wild type (broken line) and _bciD_ mutant (solid line) of _Chlamydomonas reinhardtii_, together with the spectrum from _Chlorella tepidum_ cells (dotted line) as control for BChl _e_ in a mixture of acetone and methanol. The spectrum of the mutant was quite different from that of the wild type containing BChl _e_, but was identical to _Chlorella tepidum_ containing BChl _e_ with the exception of the carotenoid region (~450–500 nm). These results strongly indicated that the _bciD_ mutant cells accumulated BChl _e_ as its dominant composite pigment.

**Determination of Compositions of BChl c Homologs in _Chlamydomonas reinhardtii_ bciD Mutant**

Pigments extracted from the wild type and mutant cells of _Chlamydomonas reinhardtii_ as well as _Chlorella tepidum_ cells were analyzed by APCILCMS (Fig. 4 and Table 1). The elution profiles of the pigments from _Chlorella tepidum_ contained seven types of BChl _c_ (see Fig. 4A) possessing an _R_ or _S_ stereo-configuration at the C31-asymmetric position, and different degrees of methylation at the C62 and C121 positions: (3’_R_)-8-ethyl-12-ethyl-[R,E,M]BChl _e_ (peak 1), (3’_R_)-8-ethyl-12-ethyl-[R,E,E]BChl _e_ (peak 2), (3’_S_)-8-ethyl-12-ethyl-[S,E,E]BChl _e_ (peak 3), (3’_R_)-8-propyl-12-ethyl-[R,P,E]BChl _e_ (peak 4), (3’_S_)-8-propyl-12-ethyl-[S,P,E]BChl _e_ (peak 5), (3’_R_)-8-isobutyl-12-ethyl-[R,E,E]BChl _e_ (peak 6), and (3’_S_)-8-isobutyl-12-ethyl-[S,E,E]BChl _e_ (peak 7). In HPLC profiles of the _bciD_ mutant cells of RK-j-1, six peaks, 2 to 7, corresponding to the above BChl _e_ in _Chlorella tepidum_ were detected as _R_[E,E]BChl _e_, _S_[E,E]BChl _e_, _R_[P,E]BChl _e_, _S_[P,E]BChl _e_, _R_[I,E]BChl _e_, and _S_[I,E]BChl _e_ (Fig. 4B). These six components exhibited the same retention times, mass numbers, and fragmentation patterns in APCILCMS as those of the authentic BChls _e_ in _Chlorella tepidum_ (summarized in Table S3), indicating that the _bciD_ mutant accumulated BChl _e_ homologs. However, the compositional pattern of BChl _e_ homologs in the mutant cells were greatly different from that in _Chlorella tepidum_; the former synthesized a significant amount of C31S epimers and more highly C82-methylated BChl _e_ pigments than the latter. The _bciD_ mutant cells contained _S_[I,E]BChl _e_ (peak 7) as a major BChl species and also showed comparable amounts of two epimers, _R_- and _S_[P,E]BChl _e_ (peaks 4 and 5); in contrast, _Chlorella tepidum_ cells synthesized _R_[E,E]BChl _e_ (peak 2) as its main pigment and a trace amount of _S_[I,E]BChl _e_. The _bciD_ mutant cells gave no detectable amount of BChl _e_ homologs, _R_[S,E,E]_–, _R_[S,P,E]_–, _R_[S,I,E]BChl _e_, that were seen in the wild type of _Chlamydomonas reinhardtii_ as peaks 9/9, 10/11, and 12/13, respectively (Fig. 4C). The pigment analysis indicated that the _bciD_ gene was responsible for the C7 formylation (methyl oxidation) in the BChl _e_ biosynthetic pathway.

**Discussion**

The _bciD_ gene-inactivated mutant of _Chlamydomonas reinhardtii_ was constructed and its pigments were analyzed. This mutant cells did not synthesize BChl _e_ pigments, but accumulated BChl _e_ species which were found in _Chlorella tepidum_ cells producing BChl _e_.
homologs, although the composition of BChl ε homologs was largely different between both cells. The ratio of C3\(^1\)R and S epimers of BChl species in this mutant was 33/67 (S-rich), while those in *Cba. tepidum* cells showed R-rich 92/8 as well as in *Cba. limnaeum* wild type, 77/23 (Table 1). The homolog composition of ethyl, propyl, and isobutyl groups at the C8 position in *bciD* mutant showed “14/41/45” and was different from its wild type “11/45/14”.

Since the epimer and homolog compositions of the C20-unsubstituted BChl f pigments in the *bchU* mutant of *Cba. limnaeum* were almost the same as those of BChl ε in the wild type cells [13], the present formation of S-rich epimers and isobutyl-rich homologs were ascribable to the alteration of the C7 substituent (7-methyl to formyl group) by the mutation.

It was reported that C3-vinyl hydrolases, BchF and BchV converted the vinyl group at C3 position to the 1-hydroxyethyl group with R and S configurations, respectively [29]. The enzymatic activity of the BchV in *Cba. limnaeum* over the BchF might increase for the hydration of a C7-methyl substrate, in comparison with that of a C7-formyl derivative. Methylation at the C8 position was catalyzed by a methylase BchQ to afford propyl and isobutyl groups from the 8-ethyl group [30]. The BchQ enzyme would have a higher activity against C7-methyl substrates than C7-formyl ones. Moreover, the alteration of pigment compositions of the *beID* mutant should be attributable to the activity of BChl synthase for BChl ε. It is known that BChl ε synthase (BchK) [31], as well as Bch a and ε synthases (BchG and ChlG, respectively), esterified the C17-propionate of (B)Chlides with a long aliphatic chain and recognized a cyclic tetrapyrrole structure of each (B)Chl species, showing a strict substrate specificity [32]. In *Cba. limnaeum* cells, the BChl ε synthase would recognize the 7-formyl group of BChlide ε, while in the case of *beID* mutant, this enzyme seemed to catalyze faraday-esterification of S[LE]BChlide ε possessing a bulky substituent and lacking an electron-withdrawing group at the B ring more favorably than any other species. The mutational study indicates that BciD is involved in the oxidation of the 7-methyl group in BChl ε biosynthesis, but the reaction mechanism of this enzyme is unclear at present. Based on the CAO-catalyzed oxidation in the biosynthesis from Chl a to b [6], it is considered that the 7-methyl group of BChlide ε is converted to the 7-formyl group of BChlide ε by two-step monoxygenation (Fig. 1). The accumulation of BChl ε species in the *beID* mutant shows that BciD is necessary for the first-step of oxygenation ([ii] in Fig. 1), but it is unclear whether BciD catalyzes the second-step of oxidase reaction ([iii] in Fig. 1). No detection of 7\(^1\)-hydroxy-BChl ε in the present LCMS also supports the participation of BciD in the first oxidation. The CAO-mediated two-step reactions required radical species and oxygen molecule [25]. The BciD protein belongs to the radical SAM family and can initiate radical reactions. Since GSB including *Cba. limnaeum* are strictly anaerobes, water molecule will be used for a BciD-mediated reaction as an oxygen source. In BChl biosynthesis, a BchE enzyme catalyzes to add an oxo-group at the C13\(^1\) position using water under anaerobic growth conditions, and successively forming the E ring of protochlorophyllide (see Fig. 1) [33,34]. To clarify the enzymatic properties of BciD, further molecular genetics and biochemical studies including its expression in BChl ε-producing GSB cells and in vitro oxidation by purified BciD protein are required.

Table 1. Compositions of BChl species in *Cba. limnaeum* wild type and *bciD* mutant, and *Cba. tepidum*.

| Strains                  | BCN (%) | Total (%) | Total (%) |
|-------------------------|---------|-----------|-----------|
|                         | R[ε,M]  | R[ε,E]   | S[ε,E]   | R[ε,P]  | S[ε,P]  | R[ε,E]  | S[ε,E]  | 3\(^1\)R/S\(^1\)S | 8-E/P/I   |
| *Cba. tepidum* wild type (BChl c) | 1.8     | 64.8     | 0.1      | 25.6    | 5.5     | 0.1     | 2.1     | 92.3/7.7       | 66.7/31.1/2.2 |
| *Cba. limnaeum* bciD mutant (BChl c) | 0.0     | 12.6     | 1.9      | 19.6    | 20.9    | 1.2     | 43.8    | 33.4/66.6      | 14.5/40.5/45.0 |
| *Cba. limnaeum* wild type (BChl ε) | 0.0     | 36.1     | 4.6      | 39.9    | 5.3     | 1.1     | 13.0    | 77.1/22.9      | 40.7/45.2/14.1 |

\(^a\)Ratio of 3\(^1\)R and 3\(^1\)S configurations of BChl species.  
\(^b\)Ratio of ethyl (E), propyl (P), and isobutyl groups (I) at C8 of BChl species.  

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Figure 4. HPLC elution profiles of extracted pigments from GSB. (A) From *Cba. tepidum* as control for BChl c, recorded at 435 nm. (B) From *Cba. limnaeum* wild type recorded at 435 nm. (C) From *Cba. limnaeum* wild type at 465 nm.

| Strains                  | BCN (%) | Total (%) |
|-------------------------|---------|-----------|
| *Cba. limnaeum* bciD mutant (BChl c) | 0.0     | 12.6     | 1.9      | 19.6    | 20.9    | 1.2     | 43.8    | 33.4/66.6      | 14.5/40.5/45.0 |
| *Cba. limnaeum* wild type (BChl ε) | 0.0     | 36.1     | 4.6      | 39.9    | 5.3     | 1.1     | 13.0    | 77.1/22.9      | 40.7/45.2/14.1 |

\(^a\)Ratio of 3\(^1\)R and 3\(^1\)S configurations of BChl species.  
\(^b\)Ratio of ethyl (E), propyl (P), and isobutyl groups (I) at C8 of BChl species.  

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The BciD enzymes were highly conserved among BChl e-possessing GSB (identity > 86%, similarity > 93%) using *Cba. limnaeum* BciD as query as mentioned above. We found the paralogs of *bciD* in various organisms by performing a BLAST analysis using the *bciD* of *Cba. limnaeum* as query. The paralogs belonging to the radical SAM family and were distributed in photosynthetic (Fig. S2) and non-photosynthetic bacteria, but the identities of overall proteins were low (<30%). Such *bciD* paralogs were found in genomes of some purple bacteria including *Rhodopseudomonas* and *Rhodo<sub>ater*</sub> (Table S1). These paralogs among *Rba. capsulatus* and *Acaryochloris marina* showed high identities (identity/similarity = 26/45%), and *BciD* as query) as mentioned above. We found the paralogs of *bciD* were also present: *Synechococcus PCC7335* (S7335_2575, 30/48%), *Synechocystis PCC6803* substrain PCC-N (s10785, 26/49%), and Chl d-producing *Acaryochloris marina* MBI11017 (AM1_2229, 24/44%). It should be reconfirmed that no *bciD* paralogs were seen in genomes of GSB and filamentous anoxygenic photosynthetic bacteria producing BChls e and/or d. Functional analyses for these paralogs will be useful to understand the evolutions of *BciD* involved in (B)Chl pigment biosynthesis as well as radical SAM enzymes.

**Supporting Information**

**Figure S1** A photograph of liquid cultures of wild type (left) and *bciD* mutant (right) grown under phototrophic conditions. (DOC)

**Figure S2** Phylogenetic analysis of *bciD* paralogs among photosynthetic bacteria. A neighbor-joining tree was constructed with translated sequences of *bciD* paralogs that showed over 1e-10 of the BLASTP e-value. Bootstrap values for each clade were obtained by 1500 replications, and indicated. The accession numbers of sequences to construct the tree are as follows: *Acaryochloris marina* MBI11017, YP_001516556; *Acaryochloris* sp. CCMEE 5410, ZP_09251378; *Chl. phaeobacteroides* BS1, YP_001958720; *Chl. phaeobacteroides* DSM266, YP_910687; *Pelidun<sub>ys* ph<sub>aeo</sub>clathratiforme* BU-1, YP_000219518; *P<sub>h</sub>ro<sub>chlorococcus* marinus* MIT9301, YP_001091538; *Rba. capsulatus* SB 1003, YP_003578374; *Rhodomonas vir<sub>ae</sub> ATCC17100, YP_004013831; *Rps. sphaeroides* ATCC17025, YP_00116690; *Rps. palust<sub>ris* CGA009, NP_947556; *Rps. palust<sub>r* TIE-1, YP_001991868; *Rps.* palust<sub>r* Bsa53, YP_782106; *Rubrivivax gelatines<sub>us* HI144, YP_005437366; *Synechococcus elongatus* PCC6301, YP_171412; *Synechococcus elongatus* PCC7942, YP_399856; *Synechococcus* sp. CC9605, YP_381421; *Synechococcus* sp. PCC7002, YP_00173926; *Synechococcus* sp. PCC7335, ZP_05036141; *Synechococcus* sp. WH5701, ZP_01004318; *Synechococcus* sp. WH 7805, ZP_01123862; *Synechocystis* sp. PCC6803, NP_442645. (DOC)

**Table S1** GSB whose genome sequences were used for CCCT analysis. (DOC)

**Table S2** Top 5 genes specifically conserved among brown-colored GSB, that was calculated by CCCT. (DOC)

**Table S3** APC-mass spectrometric data of BChl c and e homologs found in the full-growth cells of the wild type and *bchU* mutant of *Cba. limnaeum*. (DOC)

**Author Contributions**

Edited the manuscript: HT, JH TM SS VT MY MN AT. Performed the mutational analyses: JH. Contributed spectroscopic analysis of the bchU mutant cells and its extracted pigments: JH TM. Determined the compositions of BChl c homologs in the mutant: TM. Analyzed genomic DNA sequence of *Cba limnaeum* KK-j-1: JH YT. Performed comparative genome analysis by CCCT program using GSB genomic data: SS MY. Conceived and designed the experiments: JH MN AT HT. Analyzed the data: JH. Wrote the paper: JH HT.

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