Rhodobacter sphaeroides mutants overexpressing chlorophyllide a oxidoreductase of Blastochloris viridis elucidate functions of enzymes in late bacteriochlorophyll biosynthetic pathways

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In previous studies we have demonstrated that chlorophyllide a oxidoreductases (CORs) from bacteriochlorophyll (BChl) a-producing Rhodobacter species and BChl b-producing Blastochloris viridis show distinct substrate recognition and different catalytic hydrogenation reactions, and that these two types of CORs therefore cause committed steps for BChls a and b biosynthesis. In this study, COR genes from B. viridis were incorporated and overexpressed in a series of R. sphaeroides mutants. We found that the following two factors are essential in making R. sphaeroides produce BChl b: the loss of functions of both intrinsic COR and 8-vinyl reductase (BciA) in the host R. sphaeroides strain; and expression of the BchYZ catalytic components of COR from B. viridis, not the complete set of COR (BchXYZ), in the host strain. In addition, we incorporated bchYZ of B. viridis into the R. sphaeroides mutant lacking BchJ and BciA, resulting in the strain accumulating both BChl a and BChl b. This is the first example of an anoxygenic photosynthetic bacterium producing BChls a and b together. The results suggest that BchJ enhances activity of the intrinsic COR. The physiological significance of BchJ in pigment biosynthetic pathways will be discussed.

Chlorophyllous pigments are essential for photosynthetic organisms to harvest light energy and drive photochemical reaction centers (RCs). Phototrophic species in the phylum Proteobacteria (so-called purple bacteria) produce either bacteriochlorophyll (BChl) a or BChl b, depending on species, and utilize these pigments for photochemistry1,2. The difference in chemical structures between BChls a and b occurs at the C8 position, an ethyl group on BChl a and an ethylidene group on BChl b (Fig. 1). The characteristic C8-ethylidene group on BChl b provides the extension of the π-conjugated system on the parental bacteriochlorin ring, and gives rise to the red shift in the longest wavelength absorption band (Qy band) of BChl b with respect to that of BChl a. Consequently, BChl b is the sole natural pigment that can efficiently absorb light energy of near-infrared wavelength (> 800 nm) in the monomeric state, although BChl a has the Qy band slightly overlapping the near-infrared light region. The pigment is useful for developing artificial light-harvesting systems, such as dye-sensitized solar cells and photodynamic therapy, which are in demand to utilize uncaptured photons in longer wavelength.

Purple bacteria capture sunlight energy by light-harvesting proteins, so-called LH1 and LH2 complexes (some species have only LH1 complexes), and transfer the light energy into the type-II RC, where conversion of light energy into chemical potential energy occurs3. The RC and LH1 complexes bind BChl a or BChl b, depending on...
species, and form a supercomplex in the cytoplasmic membrane. When BChl $a$ ($\lambda_{max} = 770$ nm in monomer) is incorporated into LH1 proteins, an absorption band of LH1 holoproteins usually occurs at $< 900$ nm in *Rhodobacter* species\(^8\). On the other hand, LH1 complexes binding BChl $b$ ($\lambda_{max} = 795$ nm in monomer) show a significantly red-shifted absorption band at $> 1000$ nm in *Blastochloris viridis*\(^9\). Although the difference in $\lambda_{max}$ wavelength in the monomeric state is 25 nm between BChls $a$ and $b$, the difference between Q$_y$ bands of LH1 complexes binding these two pigments is 140 nm.

Model organisms in the genus *Rhodobacter* (e.g., *R. capsulatus* and *R. sphaeroides*) are genetically amenable, have versatile ways of growing, and produce BChl pigments even when grown under dark microoxic conditions. Biosynthetic pathways for BChl $a$ are well established in the *Rhodobacter* species\(^8\). On the other hand, *B. viridis* can be grown only under light anoxic conditions, and is not genetically amenable. Study of the BChl $b$-containing RC complex of *B. viridis* has not progressed very far since the crystal structure of the RC was solved at the atomic level by Deisenhofer et al.\(^8\), for which they were awarded the Nobel Prize in 1988. The biosynthetic step to form the characteristic C8-ethylidene group on BChl $a$ and BChl $b$ has remained unknown until our *in vitro* enzymatic assays revealed the enzyme responsible for the ethylidene formation. Canniffe and Hunter later investigated the enzymatic activity by *in vivo* complementation experiments\(^9\).

Biosynthetic pathways for BChl $a$ and BChl $b$ are branched at the step catalyzed by chlorophyllide $a$ oxidoreductase (COR)$^9$. COR, a nitrogenase-like enzyme, is composed of three subunits: BchX is an electron-donating component, and BchY and BchZ form a heterotetramer (BchYZ) and work as a catalytic component. We have demonstrated that COR of the BChl $a$-producing bacterium *R. capsulatus* (a-COR) has dual functions: the 8-vinyl reduction of 8-vinylchlorophyllide (8V-Chlide) $a$ and the C7=C8 double bond reduction of resultant chlorophyllide (Chlide) $a$, forming 3-vinyl-bacteriochlorophyllide (3V-BChlide) $a$ as a product (Fig. 1$^{9,11,12}$). We have also revealed that, in contrast, COR from BChl $b$-producing *B. viridis* (b-COR) recognizes only 8V-Chlide $a$, not Chlide $a$, as its substrate and catalyzes the direct formation of 3V-BChlide $b$ ($= $ BChlide $g$) possessing the C-8 ethylidene group (Fig. 1$^9$). It is noteworthy that the two types of CORs are well conserved in their amino acid sequences, up to 89% similarities. The plasticity of the nitrogenase-like enzyme not only causes the committed pathways for biosynthesis of BChls $a$ and $b$, but also show a unique example of subtle amino acid substitutions in enzyme(s) that results in profound changes between the energetics of photosystems with BChl $a$ and BChl $b$.

Reduction of the 8-vinyl group of chlorophyll intermediates is performed by 8-vinyl reductase, also called divinyl reductase (DVR)$^{10,13-15}$. DVR is divided into two types: one is plant-type BciA using NADPH as electron donors, and the other is cyanobacterial-type BciB using ferredoxin as electron donors\(^13\). In addition, as mentioned above, COR of *R. capsulatus* can work like DVR, i.e., it has the 8-vinyl-reduction ability\(^9,12\). Until the function of BciA was revealed in 2007\(^14\), BchJ had been considered to be DVR\(^15\). Indeed, a bchJ-deletion mutant of *R. capsulatus* accumulated a large amount of an intermediate pigment, 8-vinyl-protochlorophyllide (8V-PChlide) $a$\(^16\). BchJ is known to be involved in BChl biosynthesis, although how is still unclear.

In this study, we introduced and overexpressed BchYZ of *B. viridis* in a series of *R. sphaeroides* mutant strains. The mutant of *R. sphaeroides* lacking functions of intrinsic BciA and COR and overexpressing extrinsic BchYZ of *B. viridis* produced BChl $b$ under dark microoxic conditions. We also constructed the *R. sphaeroides* mutant lacking BciA and BchJ and overexpressing BchYZ of *B. viridis*, resulting in the strain producing both BChl $a$ and BChl $b$. The proposed function of BchJ will be discussed.

**Results and discussion**

**Construction and pigment analysis of the platform *R. sphaeroides* mutant strains.** The wild-type strain of *R. sphaeroides* was used as a host strain to construct the single mutants, $\Delta$bchZ and $\Delta$bchJ (Fig. S1). The $\Delta$bcia/$\Delta$bchZ and $\Delta$bcia/$\Delta$bchJ mutants of *R. sphaeroides* were constructed in the same manner using the $\Delta$bcia mutant\(^12\) as a host strain. Analytical PCR experiments confirmed that the bchZ or bchJ allele was completely segregated in each mutant strain (Figs. S1CD,
MONITORED AT 435 nm. (C) IN-LINE ABSORPTION SPECTRUMS OF PEAKS 1-4 SHOWN IN STANDARD MIXTURES CONTAINING CHLIDE MONITORED AT 435 nm. (B, TRACE SCIENTIFIC (TRACE I). MAINLY PRODUCE BCHL (HPLC) ELUTION PROFILE OF THE AUTHENTIC BCHL

FIGURE 2 | REVERSE-PHASE HPLC-MS ANALYSIS OF PIGMENTS EXTRACTED FROM THE WILD-TYPE, ΔBCHZ, AND ΔBCIA/BCHZ STRAINS. (A, TRACES I-III) HPLC ELUTION PROFILES OF HYDROPHOBIC PIGMENTS FROM THE WILD-TYPE, ΔBCHZ, AND ΔBCIA/BCHZ STRAINS, RESPECTIVELY, MONITORED AT 770 nm. (A, INSET) IN-LINE ABSORPTION SPECTRUM OF THE ELUTION PEAK AT 10.5 MIN SHOWN IN TRACE I. MINOR ELUTION PEAKS SHOWN WITH ASTERISKS IN FIG. 2A ARE BCHL A ESTERIFIED WITH UNREDUCED (GERANYLGERANYL, DIHYDROGERANYLGERANYL, AND TETRAHYDROGERANYLGERANYL) TAILS AT THE C17 POSITION, ACCORDING TO THE PREVIOUS STUDY17. (B, TRACES I, II, AND III) HPLC ELUTION PROFILES OF HYDROPHILIC PIGMENTS EXTRACTED FROM ΔBCHZ AND ΔBCIA/BCHZ MUTANTS, RESPECTIVELY, MONITORED AT 435 nm. (B, TRACE III) HPLC ELUTION PROFILE OF HYDROPHILIC STANDARD MIXTURES CONTAINING CHLIDE A (PEAK 3) AND 8V-CHLIDE A (PEAK 4), MONITORED AT 435 nm. (C) IN-LINE ABSORPTION SPECTRA OF PEAKS 1-4 SHOWN IN FIG. 2B. (D) IN-LINE MASS SPECTRA OF PEAKS 1-4 SHOWN IN FIG. 2B.

Details of the analytical PCR are described in Supplementary Information.

FIGURE 2A SHOWS HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ELUTION PROFILE OF THE AUTHENTIC BCHL A EXTRACTED FROM THE R. SPHAEROIDES WILD-TYPE STRAIN (TRACE I). R. SPHAEROIDES IS KNOWN TO MAINLY PRODUCE BCHL A ESTERIFIED WITH PHYTOL AS A HYDROCARBON TAIL (TRACE I, PEAK AT 10.5 MIN), ALTHOUGH IT CONTAINS TRACE AMOUNTS OF BCHL A ESTERIFIED WITH UNREDUCED (GERANYLGERANYL, DIHYDROGERANYLGERANYL, AND TETRAHYDROGERANYLGERANYL) TAILS (TRACE I, ASTERISKS)17. IN-LINE ABSORPTION SPECTRUM OF THE ELUTION PEAK AT 10.5 MIN REPRESENTS A TYPICAL BCHL A ABSORPTION SPECTRUM WITH \( \lambda_{\text{max}} \) AT 770 nm (FIG. 2A, INSET). PIGMENTS OF R. SPHAEROIDES MUTANT STRAINS GROWN UNDER DARK MICROOXIC CONDITIONS WERE ALSO EXTRACTED AND ANALYZED BY HPLC. THE ΔBCHZ AND ΔBCIA/BCHZ MUTANTS DID NOT PRODUCE ANY HYDROPHOBIC BCHL COMPOUND (FIG. 2A, TRACES II AND III), BUT ACCUMULATED HYDROPHILIC CHLIDE-LIKE PIGMENTS (FIG. 2B, TRACES I AND II). THE CHLIDE-LIKE COMPONENT IN THE ΔBCHZ MUTANT (FIG. 2B, PEAK 1) ELUTED AT THE SAME TIME AS THE STANDARD CHLIDE A DID (PEAK 3). IN-LINE ABSORPTION AND MASS SPECTRA OF THE PIGMENT (FIGS. 2C, TRACES I) WERE IDENTICAL TO THOSE OF THE STANDARD CHLIDE A (FIGS. 2C, TRACE 3). THIS INDICATES THAT THE ΔBCHZ MUTANT LACKS THE FUNCTION OF COR AND THEREFORE ACCUMULATES CHLIDE A, AN INTERMEDIATE PIGMENT IN BIOSYNTHETIC PATHWAYS FOR BCHL A (SEE FIG. 1). THE CHLIDE-LIKE HYDROPHILIC COMPONENT FROM THE ΔBCIA/BCHZ MUTANT (FIG. 2B, PEAK 2) ELUTED 3-MIN LATER THAN CHLIDE A (PEAK 3) BUT AT THE SAME TIME AS THE STANDARD 8V-CHLIDE A (PEAK 4). THE IN-LINE ABSORPTION SPECTRUM OF PEAK 2 (FIG. 2C, TRACE 2) WAS ALMOST IDENTICAL TO THAT OF THE STANDARD 8V-CHLIDE A (FIG. 2C, TRACE 4). ALSO, THE IN-LINE MASS SPECTRUM OF PEAK 2 (FIG. 2D, TRACE 2) WAS ALMOST IDENTICAL TO THAT OF THE STANDARD 8V-CHLIDE A (FIG. 2D, TRACE 4). THESE INDICATE THAT THE ΔBCIA/BCHZ MUTANT ACCUMULATES 8V-CHLIDE A, A PRECURSOR FOR CHLIDE A (FIG. 1).

IN VITRO COR ACTIVITY ASSAYS USING HETEROLOGOUS BCHX AND BCHYZ. COR IS COMPOSED OF THREE SUBUNITS, BCHX, BCHY, AND BCHZ. BCHX IS AN ELECTRON-DONATING COMPONENT, AND BCHYZ WORKS AS A CATALYTIC COMPONENT11. IN THE PREVIOUS STUDY, WE CONSTRUCTED PLASMIDS TO OVEREXPRESS BCHX AND BCHYZ COMPONENTS IN E. COLI. IN THIS STUDY, WE SEPARATELY PURIFIED BCHX AND BCHYZ OF R. CAPSULATUS (A-X AND A-YZ) AND THOSE OF B. VIRIDIS (B-X AND B-YZ), AND ASSAYED COR ACTIVITIES IN VITRO IN THE HETEROLOGOUS COMBINATION. COR ACTIVITIES WERE ASSAYED BY ABSORPTION CHANGES IN 80% ACETONE EXTRACTS, ACCORDING TO OUR PREVIOUS STUDIES9,11. THE HETEROLOGOUS COMBINATION OF B-X AND A-YZ WAS MIXED WITH CHLIDE A (FIG. 3A). AFTER 60-MIN INCUBATION, THE SUBSTRATE PEAK FROM CHLIDE A AT 666 nm DECREASED, CONCOMITANTLY WITH THE APPEARANCE OF A NEW PEAK FROM THE ASSAY PRODUCT OF 3V-BCHLIDE A AT 732 nm (FIG. 3A). WHEN THE B-X AND A-YZ COMPONENTS WERE MIXED WITH 8V-CHLIDE A, THE SAME PHENOMENON WAS OBSERVED (FIG. 3B). THESE RESULTS ARE ALMOST IDENTICAL TO THE PREVIOUS RESULTS OF ASSAYS USING ALL BCHXYZ COMPONENTS FROM A BCHL A-PRODUCING BACTERIUM8. AS ANOTHER COMBINATION, A-X AND B-YZ COMPONENTS WERE MIXED WITH CHLIDE A, THEN NO PRODUCT PEAK WAS OBSERVED IN THE REGION OF 700-750 nm (FIG. 3C). BUT THE ASSAY MIXTURE OF A-X AND B-YZ WITH 8V-CHLIDE A SHOWED A NEW PEAK OF THE ASSAY PRODUCT OF 3V-BCHLIDE B (= BCHLIDE G) AT 762 nm (FIG. 3D). THESE ALSO SUPPORT THE RESULTS IN THE PREVIOUS STUDY USING ALL BCHXYZ COMPONENTS FROM B. VIRIDIS FOR THE ASSAY9. THESE ASSAY RESULTS CLEARLY INDICATE THAT BCHX IS ABLE TO transferring ELECTRONS TO HETEROLOGOUS BCHYZ COMPONENTS TO FORM ACTIVE CORs, AND THAT THE PATTERN OF CATALYTIC ACTIVITIES OF A- AND B-TYPE CORs IS BCHYZ-DEPENDENT.

R. SPHAEROIDES RECOMBINANT STRAINS OVEREXpressing BCHYZ OF B. VIRIDIS. TAKING THE RESULTS OF THE IN VITRO ENZYMATIC ASSAYS USING HETEROLOGOUS COR COMPONENTS INTO CONSIDERATION, WE INTRODUCED ONLY THE BCHYZ GENES OF B. VIRIDIS INTO THE WILD-TYPE AND MUTANT STRAINS OF R. SPHAEROIDES. THE PLASMID pJ7-BvYZ-Gm CARRYING THE BCHYZ GENES OF B. VIRIDIS WAS INTEGRATED INTO THE WILD-TYPE, ΔBCHZ, AND ΔBCIA/BCHZ STRAINS OF R. SPHAEROIDES, RESULTING IN STRAINS NAMED WT+BvYZ, ΔBCHZ+BvYZ, AND ΔBCIA/BCHZ+BvYZ, RESPECTIVELY. AFTER CONJUGATION, TRANSCONJUGANT COLONIES ON GENTAMYCIN- SENSITIVE PLATES WERE RE-STREAKED ON SELECTION PLATES TWO TIMES, THEN A SINGLE COLONY WAS PICKED UP AND GROWN IN LIQUID MEDIUM. THE CULTURES GROWN UNDER DARK MICROOXIC CONDITIONS WERE HARVESTED, AND PIGMENTS WERE EXTRACTED AND ANALYZED BY HPLC.
The BchYZ proteins are overexpressed. The COR still dominantly works in the mutant strain even when from wild type did (Fig. 2A, trace same elution time (Fig. 4A, trace 1)).

The pigment extracted from the WT+BvYZ strain showed the same elution time (Fig. 4A, trace i) as the authentic BChl a extracted from wild type did (Fig. 2A, trace i), indicating that the intrinsic a-COR still dominantly works in the mutant strain even when B. viridis BchYZ proteins are overexpressed. The AbchZ+BvYZ strain did not produce any hydrophobic BChl compound (Fig. 4A, trace ii), again even though B. viridis BchYZ components were overexpressed. This suggests that the intrinsic BciA of R. sphaeroides dominantly reacts with 8V-Chlide a, and therefore 8V-Chlide a, a suitable substrate for the B. viridis BChlYZ, is not available any longer. The suggestion can be proved by making the ΔbciA/bchZ+BvYZ mutant. Figure 4A, trace iii, shows that the BChl component extracted from the ΔbciA/bchZ+BvYZ strain had an elution time at about 10 min, 0.5-min earlier than that of BChl a. The authentic BChl b extracted from B. viridis showed the same retention time (Fig. 4A, trace iv). The BChl components eluting at 10 min from the ΔbciA/bchZ+BvYZ mutant and B. viridis were collected by preparative HPLC and electronic absorption spectra were measured. The absorption spectra of the collected pigments were identical and both showed λmax at 797 nm (Figs. 4A, insets), clearly indicating that the ΔbciA/bchZ+BvYZ strain produced BChl b. These results indicate that the loss of DVR and the replacement of intrinsic BchYZ catalytic components by BchYZ of BChl b-producing bacteria are required in order to make R. sphaeroides produce BChl b: i.e., exchanging all of the BchXYZ subunits is not necessary. The average amount of BChl b molecules produced in the ΔbciA/bchZ+BvYZ mutant was ~30 mg per 1-L culture. The R. sphaeroides mutant can grow and synthesize BChl b even under dark microoxic conditions, and it could be a good platform for industrial production of BChl b.

Although the ΔbchZ+BvYZ strain produced no hydrophobic BChl compound, it accumulated hydrophilic Chlide-like pigments (Fig. 4B). The HPLC elution profile of hydrophilic Chlide-like pigments extracted from the ΔbchZ+BvYZ mutant showed one major elution peak and three minor peaks (Fig. 4B). In-line absorption spectra of peaks 1 and 2 were identical, and both showed the Qy absorption band at 659 nm and the Soret band at 429 nm (Fig. 4C, traces 1 and 2), 7-nm and 4-nm blue-shifted from those of the standard Chlide a, respectively (Fig. 2C, trace 3). Also, the pigments eluted as peaks 1 and 2 had a mass of 633.4 (Fig. 4D, traces 1 and 2), 18 mass units larger than the mass of Chlide a (615.3, Fig. 2D, trace 3). These results, together with the presence of a fragment 615.4 mass peak (Fig. 4D, traces 1 and 2), and with the fact that peaks 1 and 2 eluted earlier than the standard Chlide a (Fig. 2B, peak 3), indicate that these two elution peaks are ascribable to 3-(1-hydroxyethyl)-Chlide a with 3′R- and 3′S-configurations. In-line absorption spectrum of the minor peak 3 in Fig. 4B showed the Soret band at 408 nm (Fig. 4C, trace 3), about 20-nm blue-shifted from those of peaks 1 and 2 (traces 1 and 2). The clear appearance of Qy bands at 500 nm was observed in the absorption spectrum of peak 3 (Fig. 4C, trace 3). The pigment eluting as peak 3 gave m/z = 611.4 as its parent mass peak (Fig. 4D, trace 3), which is 22 mass units smaller than that of 3-(1-hydroxyethyl)-Chlide a (Fig. 4D, traces 1 and 2). These suggest that the pigment eluting as peak 3 is 3-(1-hydroxyethyl)-phosphor-bide a lacking the central magnesium. In-line absorption and mass spectra as well as elution time of peak 4 (Figs. 4CD, traces 4, and Fig. 4B) were almost identical to those of the standard Chlide a (Figs. 2CD, traces 3, and Fig. 2B, peak 3), indicating that the pigment eluting as peak 4 was Chlide a.

The results indicate that the ΔbchZ+BvYZ mutant mainly accumulated 3-(1-hydroxyethyl)-Chlide a. On the other hand, Canniffe and Hunter recently constructed a similar mutant using a different method, homologous gene recombination, and reported different results. The mutant of R. sphaeroides they made, in which all the intrinsic bchXYZ genes were deleted and then the exogenous bchXYZ genes of B. viridis were incorporated into the genome, produced BChl a. However, our previous study has clearly indicated that the suitable substrate for the B. viridis COR proteins is 8V-Chlide a, not Chlide a, and the present study also supports the idea that BchYZ
components of *B. viridis* do not react with Chlide a (Figs. 3 and 4). Amino acid sequences of α-COR and β-COR proteins have very high similarities*. There is a possibility that their mutant could have second mutations in the amino acid sequence that changes the hydogenation mode of CORs, from 1,4-addition to 1,2-addition. This could happen by the change of even a few amino acid residues. Incorporated exogenous *bchXYZ* genes in the genome of their *R. sphaeroides* strain were confirmed to achieve its reaction is 8V-Chlide a, and concluded that BchJ is not DVR14. Thus, the function of BchJ in BChl biosynthesis has been enigmatic. Here, we investigated the function of BchJ by using the overexpression system of exogenous COR in *R. sphaeroides*. We first made the deletion mutant of the *bchJ* gene of *R. sphaeroides*. HPLC elution profiles of pigments extracted from the *ΔbchJ* mutant demonstrated that this mutant produced a small amount of a hydrophilic BChl pigment (Fig. 6A, trace i) and a hydrophilic pigment (Fig. 6B, trace i). Compared to elution profiles of the standards of BChl a (Fig. 2A, trace i) and 8V-PChlide a (Fig. 6B, peak 4), the two pigments from the *ΔbchJ* mutant of *R. sphaeroides* are ascribable to BChl a and 8V-PChlide a, respectively, as shown in the *ΔbchJ* mutants previously reported14,15. We next constructed the *ΔbchJ/bciA* double mutant, which showed the same phenotype on *R. capsulatus* 800 and 850 nm antenna complexes1. On the other hand, *B. viridis* does not have LH2 but has only RC-LH1 complex (1015 nm). Membrane suspensions from aerobically-grown *AbciA/ΔbchZ* ByVYZ cultures showed an absorption band at about 900 nm, but did not show any absorption peak over the 900-nm wavelength region (Fig. 5B). This implies that the large blue-shifted absorption band of the *B. viridis* RC-LH1 complex is mainly caused by polypeptide environments surrounding pigment cofactors, as well as by change of the embedded pigment (from BChl a to BChl b). Note that the *AbciA/ΔbchZ* ByVYZ mutant was not able to grow under light anoxic conditions.

**Function of BchJ as an enhancer for the COR activity**. BchJ has long been considered to be involved in BChl a biosynthesis, but its function is still uncertain. Previous study demonstrated that *ΔbchJ* mutant of the purple bacterium *R. capsulatus* accumulated 8V-PChlide a16, and therefore BchJ was first thought to work as DVR. Later, Chew and Bryant showed that BchJ mutant of the green sulfur bacterium *Chlorobaculum tepidum* also accumulated 8V-PChlide a in the spent medium, but their detailed analysis found that the mutant still produced a small amount of normal (8-ethylated) BChl a, and concluded that BchJ is not DVR14. Thus, the function of BchJ in BChl biosynthesis has been enigmatic. Here, we investigated the function of BchJ by using the overexpression system of exogenous COR in *R. sphaeroides*. We first made the deletion mutant of the *bchJ* gene of *R. sphaeroides*. HPLC elution profiles of pigments extracted from the *ΔbchJ* mutant demonstrated that this mutant produced a small amount of a hydrophilic BChl pigment (Fig. 6A, trace i) and a hydrophilic pigment (Fig. 6B, trace i). Compared to elution profiles of the standards of BChl a (Fig. 2A, trace i) and 8V-PChlide a (Fig. 6B, peak 4), the two pigments from the *ΔbchJ* mutant of *R. sphaeroides* are ascribable to BChl a and 8V-PChlide a, respectively, as shown in the *ΔbchJ* mutants previously reported14,15. We next constructed the *ΔbchJ/bciA* double mutant, which showed the same phenotype on pigment compositions (Figs. 6A, traces ii) as that of the single *ΔbchJ* mutant (Figs. 6A, traces i). This suggests that the intrinsic a-COR present in the double mutant works as DVR, as previously reported14, instead of the deleted BciA.

The plasmid pJ7-ByVYZ-Gm carrying the *bchYZ* genes of *B. viridis* was transformed into the *ΔbchJ/bciA* and *ΔbchJ/bciA* mutant strains of *R. sphaeroides*, resulting in strains termed *ΔbchJ/bciA+ByVYZ* and *ΔbciA+ByVYZ*, respectively. HPLC elution profile of the *ΔbchJ/bciA+ByVYZ* mutant exhibited two elution peaks of hydrophilic BChl pigments (Fig. 6A, trace iii). The two pigments (peaks 1 and 2 in Fig. 6A) had the same elution times as those of the BChl b
**Figure 5** | Electronic absorption spectra of *R. sphaeroides*, *B. viridis*, and the ΔbcfA/bchZ+ BvYZ mutant. (A) Electronic absorption spectra of *R. sphaeroides* cells grown under light anoxic (black solid line) and dark microoxic conditions (black dashed line) and *B. viridis* cells grown under light anoxic conditions (red line). (B) Electronic absorption spectrum of membrane suspensions (black lines) and whole cells (gray lines) of the ΔbcfA/bchZ+ BvYZ strain grown under dark microoxic conditions. Cells and membrane suspensions were suspened in 10 mM Tris-HCl buffer, pH 7.5.

**Figure 6** | Reverse-phase HPLC analysis of pigments extracted from the ΔbchJ-relevant strains of *R. sphaeroides*. (A, traces i-iv) HPLC elution profiles of hydrophilic pigments extracted from the ΔbchJ, ΔbchJ/bciA, ΔbchJ/bciA+ BvYZ, ΔbciA+ BvYZ mutant strains, respectively, monitored at 770 nm (solid line) and at 797 nm (dashed line). In-line absorption spectra of peaks 1 (left inset) and peak 2 (right inset) were measured with a Shimadzu photodiode-array spectrophotometer detector (SPD-M20A) equipped in a Shimadzu HPLC system. Note that the detection limit of the spectrophotometer detector for the long wavelength is 800 nm. (B, traces i-iii) HPLC elution profiles of hydrophilic pigments extracted from the ΔbchJ and ΔbchJ/bciA mutant strains and of a mixture of standards PChl d (peak 3) and 8V-PChl d (peak 4), respectively, monitored at 435 nm. (Fig. 4A, trace iv) and BChl a standards (Fig. 2A, trace i), respectively. In-line absorption spectra of peaks 1 and 2 showed λ_{max} at 797 nm and 770 nm, respectively (Fig. 6A, insets). These results indicate that the ΔbchJ/bciA+ BvYZ mutant produces both BChls a and b. This is the first example of a mutant of anoxygenic photosynthetic bacteria having both BChls a and b.

The ΔbciA+ BvYZ mutant lacking BciA accumulated only BChl a and did not show the elution peak of BChl b (Fig. 6A, trace iv), although 8V-Chl d a could be potentially available as the substrate for the overexpressed *B. viridis* BchYZ in the mutant, indicating that the activity of the intrinsic BchYZ is much faster than that of the overexpressed *B. viridis* BchYZ in the mutant. On the other hand, the activities of the intrinsic and extrinsic BchYZ seemed to be almost equal in the ΔbchJ/bciA+ BvYZ mutant lacking BchJ. These results from the two mutants suggest that BchJ in *R. sphaeroides* facilitates the catalytic activity of only the intrinsic a-COR. Further investigation is still needed to learn whether the exact function of BchJ is as a substrate carrier, a scaffold protein to form tertiary complexes, or a chaperon for pigment biosynthesis proteins. Because the amounts of BChls a and b produced in the ΔbchJ/bciA+ BvYZ mutant were almost the same (Fig. 6A, trace iii), both intrinsic a-COR and exogenous b-COR catalytic components were likely to be almost equally functional. This implies that BchJ may form a tertiary complex with pigment substrates and pigment biosynthesis enzymes. Therefore, in the ΔbchJ background, the *B. viridis* BchYZ proteins could access pigment substrates to a degree equal to a-COR (*R. sphaeroides* BchYZ).

Sawicki and Willows suggested that BchJ might play a role as a porphyrin carrier working at the steps between BChl d (magnesium chelatase) and BchM (Mg-protoporphyrin IX monomethyl esterase) in the early stages of BChl a biosynthesis of *R. capsulatus*. In addition, preliminary experiments have shown that BchJ enhances the catalytic activity of only the intrinsic BchYZ in the mutant, indicating that the activity of the intrinsic BchYZ is much faster than that of the overexpressed *B. viridis* BchYZ in the mutant. On the other hand, the activities of the intrinsic and extrinsic BchYZ seemed to be almost equal in the ΔbchJ/bciA+ BvYZ mutant lacking BchJ. These results from the two mutants suggest that BchJ in *R. sphaeroides* facilitates the catalytic activity of only the intrinsic a-COR. Further investigation is still needed to learn whether the exact function of BchJ is as a substrate carrier, a scaffold protein to form tertiary complexes, or a chaperon for pigment biosynthesis proteins. Because the amounts of BChls a and b produced in the ΔbchJ/bciA+ BvYZ mutant were almost the same (Fig. 6A, trace iii), both intrinsic a-COR and exogenous b-COR catalytic components were likely to be almost equally functional. This implies that BchJ may form a tertiary complex with pigment substrates and pigment biosynthesis enzymes. Therefore, in the ΔbchJ background, the *B. viridis* BchYZ proteins could access pigment substrates to a degree equal to a-COR (*R. sphaeroides* BchYZ).

**Methods**

**Construction of the ΔbchZ and ΔbcfA/bchZ mutants of *R. sphaeroides***. The wild-type strain 7001 and the ΔbchZ mutant of *R. sphaeroides*, constructed in a previous study, were used as host strains to construct the ΔbchZ and ΔbcfA/bchZ mutants, respectively. The plasmid pJSC-bchZ-Sm used for the insertional inactivation of *bchZ* was constructed as follows.

The *aadA* gene, conferring resistance to streptomycin and spectinomycin, was amplified from plasmid pHP45 by PCR using a primer set, aadA-F (Fig. 5A, shown as primer i) and aadA-R (primer ii). Primer positions and sequences are presented in Fig. S1 and Table S1, respectively. The ΔbchZ gene and a portion of ΔbchY were amplified from the genomic DNA of *R. sphaeroides* by PCR using bcZh-F (primer iii) and bcZh-Z (primer iv) primers. The PCR reactions were performed with KOD-plus DNA polymerase (TOYOBO, Osaka, Japan). The DNA fragment
with primers, bchJ-inf-FI (primer F) and bchJ-inf-RI (primer v). The DNA fragment and the above-mentioned PCR product of the aadA gene were ligated with an In-Fusion HD cloning kit (Clontech, USA), yielding pTA-bchZ-Sm (Fig. S1A). The DNA fragment containing the partial bchJ gene was amplified from the plasmid pTA-bchZ-Sm by PCR using primers bchZ-inf-FII (primer vii) and bchZ-inf-RII (primer viii), and sub-cloned into the Smal restriction sites of the pSC vector29 by the In-Fusion technique, producing pSC-bchZ-Sm. The plasmid pSC is a chloramphenicol-resistant suicide vector and has the sacb gene encoding an enzyme to express the expression of sacb in the presence of sucrose is lethal for most of the Gram-negative bacteria30.

The plasmid pSC-bchZ-Sm was transformed into the mobilizing E. coli strain S17-1 λ pir2. By conjugation method with the E. coli S17-1 strain2, pSC-bchZ-Sm was transferred into the wild-type strain and ΔbciA mutant of R. sphaeroides. Colonies grown in liquid PYS medium grown in the presence of 5% sucrose, 50 μg/mL streptomycin, and 10 μg/mL rifampicin were selected as double-crossover candidates, and the chromosomal insertion into bchZ by the adaA gene was confirmed by analytical PCR using bchZ-comI-F (Figs. S1A, primer ix) and bchZ-comF-R (primer x) primers (see Supplementary text for details of the analytical PCR experiments). The obtained ΔbciA and ΔbciA/bchZ mutants were grown under dark microoxic conditions in the PYS medium31 at 30°C under light anoxic conditions. Cells were harvested by centrifugation, and pigments were extracted with acetone/ methanol (1:1, vol/vol) and filtered with a PVDF 0.22-μm membrane filter. To analyze hydrophobic BChl-type pigments, reverse-phase HPLC measurements were performed using an octadecylated silica gel column (Cosmosil SC5-AR-14 4.6 × 150 mm, 5 μm, Nacalai Tesque, Kyoto, Japan) and the mobile phase of methanol: water = 95 : 5 with the flow rate of 1.0 mL/min. The HPLC-MS system for the analysis of hydrophilic Chloride-type pigments consisted of an octadecyl–polar group–silica gel column (Inertis ODS-3 3.0 × 150 mm, 5 μm, GL Sciences Inc., Tokyo), a photodiode-array spectrophotometer detector (SPD-M20A; Shimadzu, Kyoto) and a LCMS-2010EV quadrupole mass spectrometer equipped with an electrospray ionization (ESI) probe (Shimadzu). The mobile phase was methanol : aqueous 50 mM ammonium acetate (pH 5.25) = 70 : 30 (v/v), and the flow rate was isocratic at 0.5 mL/min. The ESI-MS spectra of the following pigments were as follows: capillary temperature, 230°C; sheath gas (N2) pressure, 0.1 MPa; and spray voltage, 15 kV (positive-ion ESI).

For the HPLC-MS analysis of hydrophilic PChlide-type pigments, the following setting was applied: a polymeric octadecylated silica gel column (Inertis ODS-P 3.0 × 150 mm, 5 μm, GL Sciences Inc.); eluent, methanol : acetonitrile : aqueous 50 mM ammonium acetate (pH 5.25) = 60 : 20 : 20 (v/v); flow rate, 0.75 mL/min.

Electron absorption spectroscopy measurements of cells and membrane suspensions. Cells of R. sphaeroides grown under light anoxic and dark microoxic conditions and V. carteri grown under light anoxic conditions were harvested and suspended in 20 mM Tris-HCl (pH 7.5). Cultures of the ΔbciA/bchZ + ΔbchZ mutant grown under dark microoxic conditions were harvested by centrifugation at 9,000 × g for 20 min, resuspended in 20 mM Tris-HCl (pH 7.5), and disrupted by passing three times through a French Press at 100 MPa. Unbroken cells were removed by centrifugation at 10,000 × g for 15 min, and the supernatant was used as membrane suspensions. Electron absorption spectra were measured using a Shimadzu UV-1800 spectrophotometer (Kyoto).

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**Author contributions**

Y.T. and H.T. designed the research. Y.T., J.H. and J.N. cloned genes and made inactivation of enzymes in late bacteriochlorophyll biosynthetic pathways. Y.T. and T.M. prepared substrates for the assay and identified assay products and pigment accumulated in the mutants by LC-MS. Y.T., Y.F. and H.T. analyzed the data and wrote the manuscript.

**Additional information**

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