Engineered Biosynthesis of Bacteriochlorophyll g_F in *Rhodobacter sphaeroides*

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ABSTRACT

Engineering photosynthetic bacteria to utilize a heterologous reaction center that contains a different (bacterio)chlorophyll could improve solar energy conversion efficiency by allowing cells to absorb a broader range of the solar spectrum. One promising candidate is the homodimeric type I reaction center from *Heliobacterium modesticaldum*. It is the simplest known reaction center and uses bacteriochlorophyll (BChl) *g*, which absorbs in the near-infrared region of the spectrum. Like the more common BChls *a* and *b*, BChl *g* is a true bacteriochlorin. It carries characteristic C3-vinyl and C8-ethylidene groups, the latter shared with BChl *b*. The purple phototrophic bacterium *Rhodobacter* (*Rba.*) *sphaeroides* was chosen as the platform into which the engineered production of BChl g_F, where F is farnesyl, was attempted. Using a strain of *Rba. sphaeroides* that produces BChl b_P, where P is phytyl, rather than the native BChl a_P, we deleted *bchF*, a gene that encodes an enzyme responsible for the hydration of the C3-vinyl group of a precursor of BChls. This led to the production of BChl g_P. Next, the *crtE* gene was deleted, thereby producing BChl *g* carrying a THF (tetrahydrofarnesol) moiety. Additionally, the $bchG^{Rs}$ gene from *Rba. sphaeroides* was replaced with $bchG^{Hm}$ from *Hba. modesticaldum*. To prevent reduction of the tail, *bchP* was deleted, which yielded BChl g_F. The construction of a strain producing BChl g_F validates the biosynthetic pathway established for its synthesis and satisfies a precondition for assembling the simplest reaction center in a heterologous organism, namely the biosynthesis of its native pigment, BChl g_F.

1. INTRODUCTION

Heliobacteria are an intriguing genus of chlorophototrophic bacteria, the first species of which, *Heliobacterium (Hba.) chlorum*, was isolated in 1983 by Gest and Favinger [1]. These strict anaerobes are obligate heterotrophs; they can grow either photoheterotrophically under anoxic conditions with light and organic substrates [2] or chemotrophically by fermentation of pyruvate in the dark [3]. Phylogenetic analysis of 16S rRNA [4] and their ability to form endospores [5] places heliobacteria in the phylum *Firmicutes*, making them the only Gram-positive bacterial chlorophototrophs discovered to date [6]. They are found mainly in terrestrial environments, particularly in rice paddies, where they play a role in the process of dinitrogen fixation. This makes a symbiotic relationship with rice plants possible, with the plant providing organic compounds to the bacteria [7]. N_2 fixation requires highly reducing ferredoxins, which can be generated by phototrophy in heliobacteria [8].

Heliobacteria contain the simplest known photosystem, a homodimeric type I reaction center (RC), located in the cytoplasmic membrane [8]. The heliobacterial RC (HbRC) has only a rudimentary peripheral antenna system consisting of two PshX apoproteins, four BChl *g* molecules, and two carotenoids; the core antenna system associated with the PshA homodimer has fewer total pigments (~60) compared to Photosystem I (PSI) of cyanobacteria (~100) and plants (~200), which are both heterodimeric type I RCs [9–11]. They are the only chlorophototrophic bacteria to use bacteriochlorophyll (BChl) *g* [12,13] that under light and oxic condition converts to a form of chlorophyll (Chl) *a* [14–16]. The use of BChl *g* allows heliobacteria to harvest light in the near-infrared region of the spectrum, which provides an advantage in soil environments because these wavelengths penetrate to greater depth. It has been suggested that introducing recombinant RCs with longer wavelength (B)Chls into

chlorophototrophic hosts could yield engineered organisms capable of harvesting broad ranges of the solar spectrum, and thus increasing photosynthetic efficiency [17].

BChl *g*, along with BChls *a* and *b*, are 'true' BChls. These pigments differ from chlorins such as Chl *a*, which are ubiquitous in oxygenic phototrophs, and "BChls" that are found in the chlorosomes of green chlorophototrophic bacteria [18], in that rings B and D of the tetrapyrrole macrocycle of bacteriochlorins are reduced, while the B ring of chlorins remains unsaturated (**Fig. 1**) [19,20]. The major structural differences between the bacteriochlorin macrocycles are the C3 and C8 substituents. BChls *a* and *b* carry an acetyl group at C3, while BChl *g* has a vinyl group. BChls *b* and *g* contain an ethylidene substituent at C8, while BChl *a* contains an ethyl group, the latter being common to the majority of Chls found in nature [19,20].

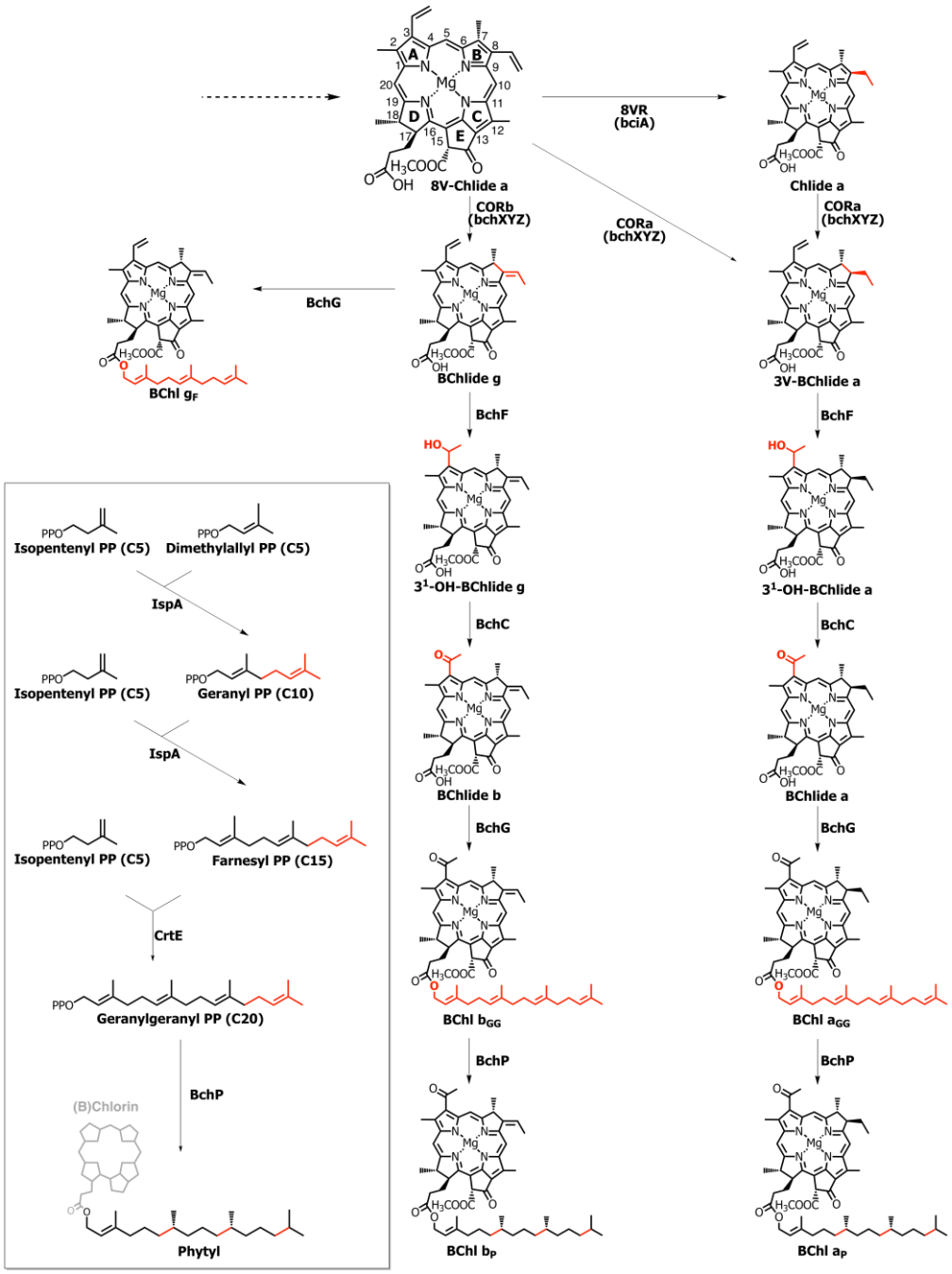

Figure 1

Pathways for the biosynthesis of BChl *a*, *b* and *g* from the universal precursor 8V-Chlide *a* (IUPAC numbered) and early isoprenoid biosynthesis leading to the attachment of a geranylgeranyl pyrophosphate (GGPP) moiety to a BChlide, prior to its reduction (inset).

During the synthesis of BChl *a*, the C8-vinyl side chain of the universal precursor to all (B)Chls, 8-vinyl chlorophyllide (8V-Chlide), is converted to an ethyl group by an 8-vinyl-Chlide *a* reductase (8VR) to produce Chlide *a*, the direct precursor to Chl *a* (**Fig. 1**) [21]. In the following first committed step of bacteriochlorin biosynthesis, Chlide *a* oxidoreductase (COR), encoded by the *bchXYZ* genes [22], reduces the C7=C8 bond of Chlide *a*, producing 3-vinyl-bacteriochlorophyllide *a* (3V-BChlide *a*) [23]. The C3 group then undergoes sequential hydration and dehydrogenation reactions, catalyzed by the gene products of *bchF* and *bchC*, respectively, yielding BChlide *a* [24,25]. Finally, addition and reduction of a hydrophobic isoprenoid alcohol results in mature BChl *a* [26]. It has recently been demonstrated that the COR enzyme from BChl *a*-utilizing organisms (COR*a*) surprisingly has an additional 8VR activity [27,28]; disruption of an 8VR encoding gene, (*bciA*) in these organisms does not perturb accumulation of BChl *a* carrying an ethyl group at C8 [29]. Organisms synthesizing BChls *b* or *g* lack a conventional 8VR (BciA or BciB), and the COR enzyme found in these strains (COR*b*) lacks a secondary 8VR activity. COR*b* converts 8V-Chlide *a* to BChlide *g*; in heliobacteria this pigment is directly esterified with a isoprenoid alcohol to yield BChl *g*, while BChl *b* is formed via modifications at C3 and C17 shared in common with BChl *a* [27,30–32].

Esterification of BChls with hydrophobic isoprenoid alcohol chains specifies their localization in the membrane. Isoprenoids are a group of metabolites that play an indispensable role in basic functions (cell-wall and membrane biosynthesis, biosynthesis of carotenoids, etc.); they derive from the common C_5-precursor units: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (**Fig. 1 inset**) [33]. The addition of IPP units to a single DMAPP results in the production of isoprene intermediates of differing length, including C_{15} farnesyl diphosphate (FPP), the moiety carried by BChl *g* in heliobacteria (BChl g_F, where F is

farnesyl), and C_{20} geranygeranyl diphosphate (GGPP), carried by BChls *a* and *b* in some purple bacteria, which is later triply saturated to produce the phytyl moiety (BChl a_P/BChl b_P, where P is phytyl) [34,35].

The purple chlorophototrophic bacterium *Rhodobacter* (*Rba*.) *sphaeroides* is a BChl a_P-producing model organism widely used to study photosynthesis and pigment biosynthesis [36]. Its versatile metabolism allows chemotrophic growth in the dark, thereby permitting manipulation of genes involved in photosynthesis [37]. The native pathway for the production of BChl a_P has previously been diverted towards the production of BChl b_P by removal of 8VR activity: deletion of *bciA* and replacement of the native *bchXYZ* genes with those encoding COR*b* from *Blastochloris viridis* [38]. In this study we have used this BChl *b*-producing mutant as a platform to engineer a strain of *Rba. sphaeroides* that can synthesize BChl g_F. To accomplish this, the native isoprenoid biosynthetic pathway was also modified to promote the esterification of BChlide *g* with the correct alcohol moiety. Our results overcome a major hurdle, the biosynthesis of BChl g_F, as a necessary precondition for the production of a simple type I RC in a heterologous system.

2. MATERIALS & METHODS

2.1. Growth of described strains

All strains and plasmids used in the present study are listed in **Table S1**. Liquid cultures of *Rba. sphaeroides* were grown microoxically in the dark in a rotary shaker or phototrophically under illumination (90 μmol photons·m^{-2}·s^{-1}) at 34 °C in M22+ medium [39] supplemented with 0.1% casamino acids. Growth on solid M22+ medium was performed in the dark at 34°C.

Liquid cultures of *Hba. modesticaldum* were grown anoxically under illumination (200 μmol photons·m^{-2}·s^{-1} supplied by a home-built 25x25 cm panel containing 85 light-emitting diodes that emit with maximum output at 780 nm and a half-width of 26 nm (Part # L780-04AU, Marubeni America Corporation)) in PYE medium [40]. An oxygen reporter dye, resazurin, was added to a final concentration of 0.001% (w/v). All other manipulations for cultivation of this organism were performed under anoxic conditions.

Escherichia coli strains Stellar (Clontech) and S17-1 [41] transformed with plasmid pK18*mobsacB* were grown in a rotary shaker at 37 °C in LB medium supplemented with 30 μg kanamycin ml^{-1}.

2.2.Construction of mutants of Rba. sphaeroides

Construction of deletion mutants was performed using the allelic-exchange vector pK18*mobsacB* [42]. Sequences upstream and downstream of the relevant gene were amplified with their respective primers (**Table S2**). The upstream and downstream PCR products were fused by overlap extension PCR, and the resulting product was ligated with In-Fusion HD cloning kit (Clontech) into pK18*mobsacB* previously digested with *Eco*RI and *Hin*dIII. After DNA-sequence verification, a selected cloned fragment was conjugated into *Rba. sphaeroides*

from *E. coli* S17-1, and transconjugants in which the plasmid had integrated into the genome by homologous recombination were selected on M22+ medium supplemented with kanamycin. Transconjugants that had undergone a second recombination event were then selected on M22+ supplemented with 10% (w/v) sucrose, lacking kanamycin. Sucrose-resistant and kanamycin-sensitive colonies had excised the allelic-exchange vector through the second recombination event [43]. The deletion of genes was confirmed by colony PCR using the relevant CheckF-F and CheckF-R primers (**Fig. S1**).

The replacement of the endogenous *bchG (bchG^{Rs})* with the orthologous gene from *Hba. modesticaldum* (*bchG^{Hm}*) was performed as follows. Sequences upstream and downstream of *bchG^{Rs}* were amplified with UpG-F and UpG-R, and DownG-F and DownG-R. The *bchG^{Hm}* gene was amplified with the primers BchG-F and BchG-R containing homology regions with the upstream and downstream sequences of *bchG^{Rs}*. The upstream and downstream fragments of *bchG^{Rs}*, and the *bchG^{Hm}* fragment were mixed and fused by overlap extension PCR, and a pK18*mobsacB* construct harboring this fusion product was constructed, verified by DNA sequencing, and introduced into *Rba. sphaeroides* as described above. Replacement of the native *bchG* with *bchG^{Hm}* was confirmed by sequencing using the CheckG-F and CheckG-R primers.

2.3.Extraction of pigments

After cells had been washed in 50 mM Tris-HCl pH 8.0 and pelleted, pigments were extracted under anoxic conditions by adding 7:2 (v/v) acetone/methanol (10 pellet volumes); the cell pellets were resuspended by vortex-mixing for 30 s, and the resulting suspension was incubated on ice for 30 min. The extracts were clarified by centrifugation (15000*g* for 5 min at 4

°C), and the supernatants were filtered with a 0.22-μm PVDF membrane filter and immediately analyzed.

2.4.Analysis of pigments by HPLC

BChl extracts were separated by reversed-phase HPLC on a Supelco Discovery HS C18 (5 μm particle size, 120 Å pore size, 250 × 4.6 mm) on an Agilent 1100 HPLC system using a method modified from that of Addlesee et al., (1996) [44]. Solvents A and B were 64:16:20 (v/v/v) methanol/acetone/H_2O and 80:20 (v/v) methanol/acetone, respectively. Pigments were eluted at 1 ml·min^{-1} at RT with a linear gradient of 50%–100% solvent B over 10 min, followed by further elution with 100% solvent B for 25 min. To detect species of BChls *a*, *b* and *g*, absorbance changes between 350 and 900 nm were collected at 0.5 s intervals, allowing data to be extracted at 770 nm, 795 nm and 752 nm, respectively.

2.5.Characterization of Bacteriochlorophylls by Liquid Chromatography – Mass Spectrometry (LC-MS)

An Agilent 1200 HPLC system utilizing a 6410 QQQ mass spectrometer was used in the detection and characterization of BChl molecules extracted from *Hba. modesticaldum* and *Rba. sphaeroides*. Data were collected and analyzed with Agilent technologies MassHunter software version B.03.01.

Pigments were separated using a Zorbax Rapid Resolution HT Extend C-18 column (4.6 × 5.0 mm; 1.8 μm particle size) using a method modified from above. The column was equilibrated with 50% solvent A (80:20 methanol/aqueous 500 mM ammonium acetate) and 50% solvent B (80:20 methanol/acetone). After injection, an isocratic gradient of 50% solvent B lasted for 2 min

before a 50-92% gradient of solvent B was applied over 0.5 min. A 92-94% gradient of solvent B was applied from 2.5 min to 11 min. To ensure that no analytes contaminated the subsequent injection, the column was washed with 100% solvent B from 12 min to 13 min and then returned to 50% solvent B over 0.5 min. The column was re-equilibrated for 3.5 min. A flow rate of 0.5 $ml \cdot min^{-1}$ was used throughout the entirety of the method. In-line absorbance was monitored as detailed in the previous section. Isotopic distributions of mass/charge (*m/z*) ratios were assigned using an electrospray ionization M2S scan method run in positive mode. All m/z values reported in the text correspond to the M+H adduct of the chlorophyll pigment being detected. The scan window was from 700 *m/z* to 1000 *m/z* with a scan time of 500 ms. The fragmentation voltage applied was 90 V.

3. RESULTS

3.1. Deletion of bchF in a BChl b-producing strain of Rba. sphaeroides leads to the production of an analog of BChl g

To re-direct pigment production towards BChl g_F in a previously constructed strain (Δ*bciA*/*bchXYZ*^{Bv}) of *Rba. sphaeroides* that produces BChl *b*, the hydration of the C3-vinyl group of BChl *g* had to be blocked (**Fig. 1**). This reaction is performed by BchF hydratase. Thus, the *bchF* gene was deleted in the both the WT and Δ*bciA*/*bchXYZ*^{Bv} strains. The resulting strains were confirmed by PCR performed with purified genomic DNA and primers CheckF-F and CheckF-R (**Table S2**) (**Fig. S1**). After culturing the strains under microoxic conditions in the dark, pigments were extracted from pelleted cells under anoxic conditions and analyzed by HPLC and LC-MS (**Fig. 2 and Fig. S2**).

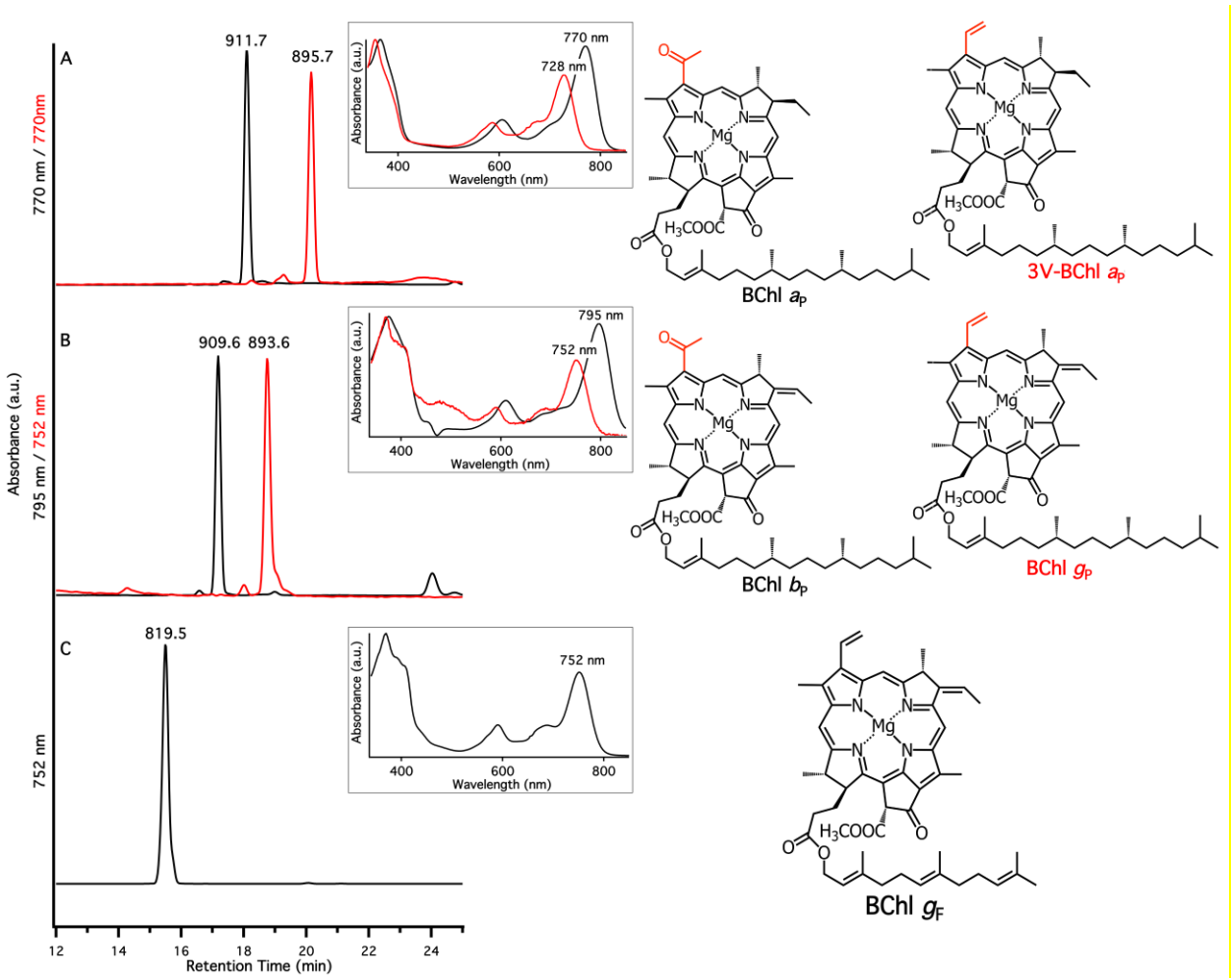

Figure 2

Analysis of pigments extracted from *Rba. sphaeroides* strains lacking *bchF*.

Reversed phase-HPLC elution profiles of samples from (A) WT (black line) and Δ*bchF* (red line), (B) Δ*bciA*/*bchXYZ*^{Bv} (black line) and Δ*bchF*/Δ*bciA*/*bchXYZ*^{Bv} (red line), (C) authentic BChl g_F from *Hba. modesticaldum*. Monitoring wavelengths for parent (black) and mutant (red) samples are indicated on the y-axis. The *m/z* ratio of the most abundant peak in each sample is labeled (see **Fig. S2** for mass spectra), the absorption spectra of these peaks are shown (inset), and the chemical structures of these pigments are displayed (right).

Deletion of *bchF* in the WT leads to the production of a pigment (**Fig. 2A**) displaying a longer retention time than BChl a_P, absorbing maximally at 728 nm. MS analysis (**Fig. S2B**) showed that this compound has a mass corresponding to 3V-BChl a_P, confirming that hydration of the C3-vinyl group was blocked, but that this precursor can be esterified with phytol. Additionally, when *bchF* was deleted in the Δ*bciA*/*bchXYZ*^{Bv} background, we observed the presence of a pigment (**Fig. 2B**) absorbing maximally at 752 nm with a longer retention time than those of BChl *a* (**Fig. 2A**) and authentic BChl *g* (**Fig. 2C**). The absorption spectrum of this pigment matches that of BChl *g* (**Fig. 2B, inset**), but characterization of this pigment by MS analysis (**Fig. S2D**) indicated that it had an *m/z* 893.6, corresponding to BChl *g* esterified with phytol (BChl g_P). These results suggest that BChl g_P, carrying a longer tail than authentic BChl g_F found in *Hba. Modesticaldum*, is produced in this heterologous system, possibly due to the abundance of the C_{20} isoprenoid GGPP (**Fig. 2C, Fig. S2E**). It follows that this analog would be more hydrophobic than the authentic pigment, and thus exhibit a longer retention time on reversed-phase HPLC.

3.2.Deletion of crtE does not completely abolish production of C_{20} GGPP

To engineer the production of BChls carrying the shorter C_{15} alcohol moiety in *Rba. sphaeroides*, the native isoprenoid pathway was modified. In *Hba. modesticaldum* bacteriochlorophyllide (BChlide) *g* is esterified with farnesol to make BChl g_F, but in the majority of chlorophototrophs, CrtE adds a further IPP unit to FPP, yielding the C_{20} molecule geranylgeranyl diphosphate (GGPP), which is the precursor to all C_{40} carotenoids (**Fig. 1 inset**). In *Rba. sphaeroides*, BChlide *a* is esterified with geranylgeraniol, and the resulting compound is then sequentially reduced three times to produce BChl *a* with a phytyl tail (**Fig. 1**). Therefore,

crtE was deleted in both the WT and Δ*bchF*/Δ*bciA*/*bchXYZ*^{Bv} backgrounds, and the resulting strains were confirmed as described above. Due to its inability to synthesize native C_{40} carotenoids, the Δ*crtE* strain was visibly blue-colored, but this strain was able to grow both chemotrophically and phototrophically. In contrast, the Δ*crtE*/Δ*bchF*/Δ*bciA*/*bchXYZ*^{Bv} strain was unable to grow phototrophically. After culturing the strains under microoxic conditions in the dark, their pigments were extracted under anoxic conditions from the pellets and were analyzed by HPLC and LC-MS (**Fig. 3 and Fig. S3**).

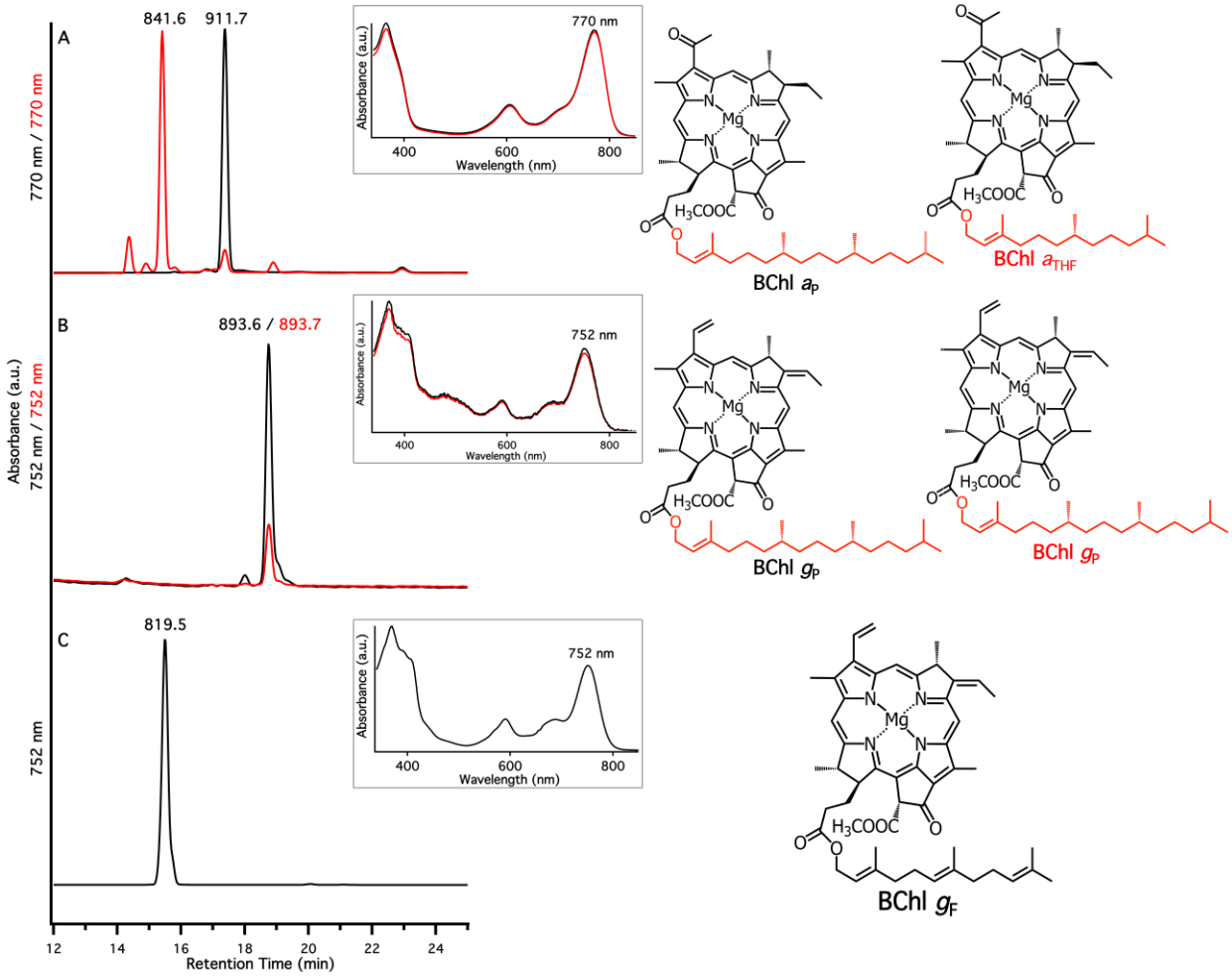

Figure 3

Analysis of pigments extracted from *Rba. sphaeroides* strains lacking *crtE*.

Reversed phase-HPLC elution profiles of samples from (A) WT (black line) and Δ*crtE* (red line), (B) Δ*bchF*/Δ*bciA*/*bchXYZ*^{Bv} (black line) and Δ*crtE*/Δ*bchF*/Δ*bciA*/*bchXYZ*^{Bv} (red line), (C) authentic BChl g_F from *Hba. modesticaldum*. Monitoring wavelengths for parent (black) and mutant (red) samples are indicated on the y-axis. The *m/z* ratio of the most abundant peak in each sample is labeled (see **Fig. S3** for mass spectra), the absorption spectra of these peaks are shown (inset), and the chemical structures of these pigments are displayed (right).

The extract from Δ*crtE* contained a major pigment absorbing maximally at 770 nm, but with a shorter retention time (15.7 min) than that of authentic BChl a_P (**Fig. 3A**). This pigment had an *m/z* of 841, corresponding to BChlide *a* carrying a tetrahydrofarnesol (THF) tail (BChl a_{THF}). This compound is esterified with a C_{15} alcohol and we predict that bonds between C6-C7 and C10-C11 are saturated (**Fig. S3D**), indicating that the native BchP protein is able to reduce this shorter alcohol moiety. Surprisingly, this strain accumulated a small amount of BChl a_P, as well as another pigment with a shorter retention time (14.7 min) than the major pigment in this extract (**Fig. 3A**). This pigment had an m/z of 837.4, corresponding to BChlide *a* carrying a farnesyl tail. Interestingly, analysis of Δ*crtE*/Δ*bchF*/Δ*bciA*/*bchXYZ*^{Bv} showed only the presence of BChl g_P (**Fig. 3B, Fig S3D**). The native photosynthetic apparatus does not assemble in this strain, and consequently the pigments accumulate only at a low level. These results suggest that the native *Rba. sphaeroides* BChl synthase (BchG) has a strong preference for the addition of a C_{20} moiety, even when its availability is presumably dwarfed by that of the C_{15} substrate, as should be the case when *crtE* is absent [45]. These results demonstrate that modification of the isoprenoid pathway in *Rba. sphaeroides* to make farnesylated pigments is possible, but that an alternative pathway that produces GGPP (C_{20}) in this background still exists.

3.3.Replacement of the Rba. sphaeroides bchG gene with its ortholog from Hba. modesticaldum

Hba. modesticaldum lacks CrtE, thus GGPP is unlikely to accumulate in this strain, preventing it from synthesizing C_{40} carotenoids. Its native BchG, which shares only 39% identity with that of *Rba. sphaeroides*, may therefore have much greater specificity for esterifying BChlide *g* with FPP. To accomplish the esterification of BChls with a farnesyl tail in *Rba. sphaeroides*, we replaced $bchG^{Rs}$ from *Rba. sphaeroides* with $bchG^{Hm}$ from *Hba. modesticaldum* at the original locus in the chromosome. This replacement was performed in both the WT and Δ*crtE*/Δ*bchF*/Δ*bciA*/$bchXYZ^{Bv}$ strains; the resulting strains were cultured, and their pigments were extracted and analyzed (**Fig. 4, Fig. S4**).

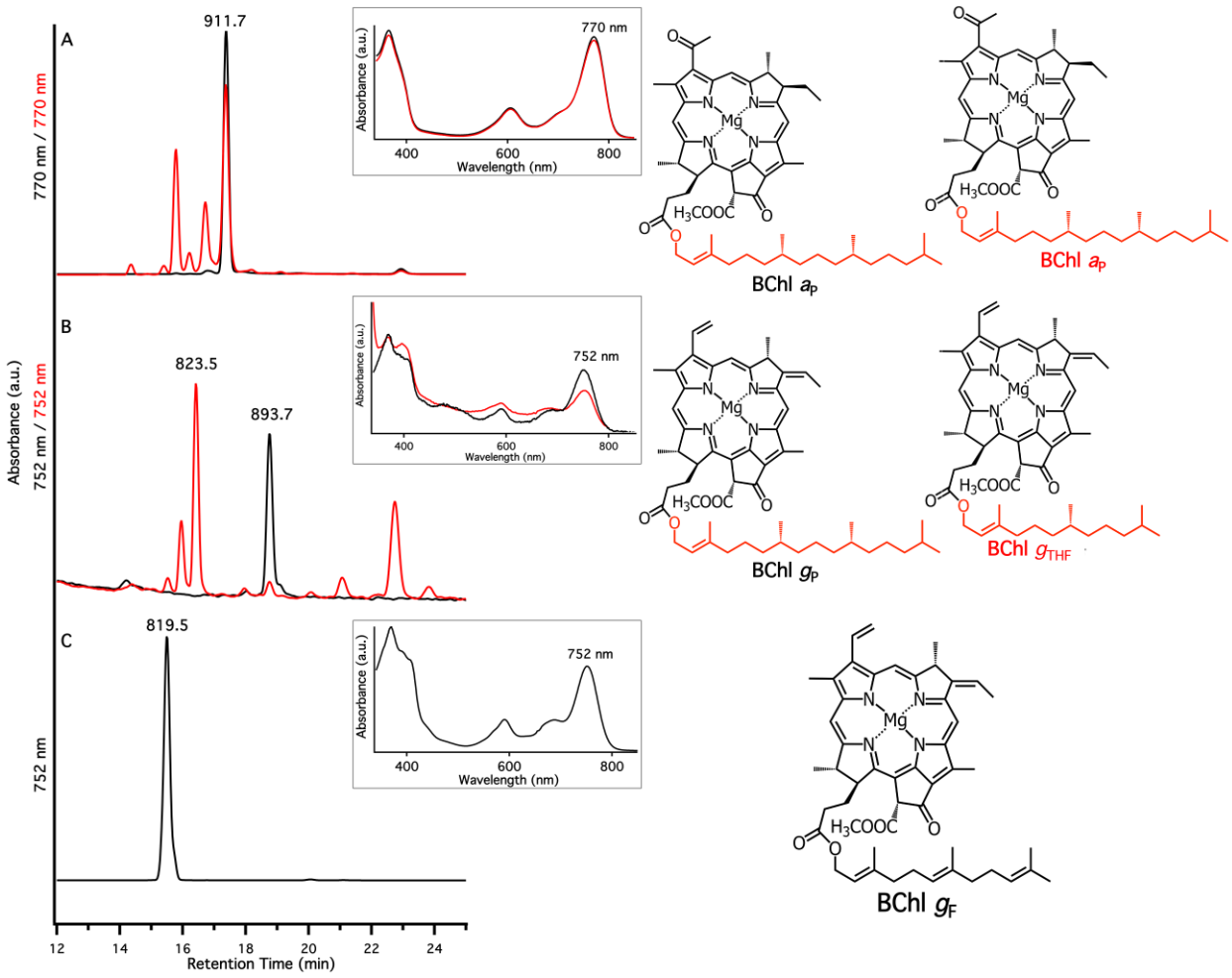

Figure 4

Analysis of pigments extracted from strains of *Rba. sphaeroides* in which the native *bchG* is replaced with *bchG* from *Hba. modesticaldum.*

Reversed phase-HPLC elution profiles of samples from (A) WT (black line) and $bchG^{Hm}$ (red line), (B) Δ*crtE*/Δ*bchF*/Δ*bciA*/$bchXYZ^{Bv}$ (black line) and Δ*crtE*/Δ*bchF*/Δ*bciA*/$bchXYZ^{Bv}$/$bchG^{Hm}$ (red line), (C) authentic BChl g_F from *Hba. modesticaldum*. Monitoring wavelengths for parent (black) and mutant (red) samples are indicated on the y-axis. The *m/z* ratio of the most abundant peak in each sample is labeled (see **Fig. S4** for mass spectra), the absorption spectra of these peaks are shown (inset), and the chemical structures of these pigments are displayed (right).

When the *bchG*^{Hm} gene was introduced into a WT background, the resulting strain synthesized the same major pigment as the WT strain, BChl a_P, although the amount produced was only ~15% that of the WT (**Fig. 4A, Fig. S4B**). It also produced three minor pigments with shorter retention times that were also detected in the WT (**Fig. 4A**); these are BChl *a* esterified with geranylgeraniol (BChl a_{GG}), dihydrogeranylgeraniol (BChl a_{DHGG}), and tetrahydrogeranylgeraniol (BChl a_{THGG}), the substrate and intermediates in the reaction catalyzed by BchP. The extract from the Δ*crtE*/Δ*bchF*/Δ*bciA*/*bchXYZ*^{Bv}/*bchG*^{Hm} mutant showed the presence of a pigment with a shorter retention time than BChl g_P, absorbing maximally at 752 nm (**Fig. 4B**). Its identity was confirmed by MS to be BChlide *g* carrying THF (BChl g_{THF}; *m/z* 823.5) (**Fig. S4D**), again indicating that the native BchP protein is able to reduce this shorter alcohol moiety. It also produced a minor pigment at 23 min with a longer retention time that was assign to bacteriopheophytin *g* with a THF tail. These results demonstrate that BchG from *Hba. modesticaldum* preferentially esterifies BChlides with C_{15} rather than C_{20} moieties, but can use the latter substrate when it is available. BchG^{Hm} is also able to esterify BChlides with a BChl *a*-like macrocycle carrying an acetyl group at C3.

3.4.Deletion of bchP is necessary for the production of authentic BChl g_F in Rba. sphaeroides

Hba. modesticaldum produces BChl g_F, and its genome does not contain an ortholog of *bchP*. Because the *Rba. sphaeroides* mutant Δ*crtE*/Δ*bchF*/Δ*bciA*/*bchXYZ*^{Bv}/*bchG*^{Hm}, in which the native *bchP* gene product is functional, accumulated BChl g_{THF}, it seems likely that BchP is responsible for this modification. To avoid the reduction of the alcohol tail, *bchP* was deleted in

the WT and Δ*crtE*/Δ*bchF*/Δ*bciA*/*bchXYZ*^{Bv}/*bchG*^{Hm} strains, and the pigments from the resulting strains were analyzed (**Fig. 5, Fig. S5**).

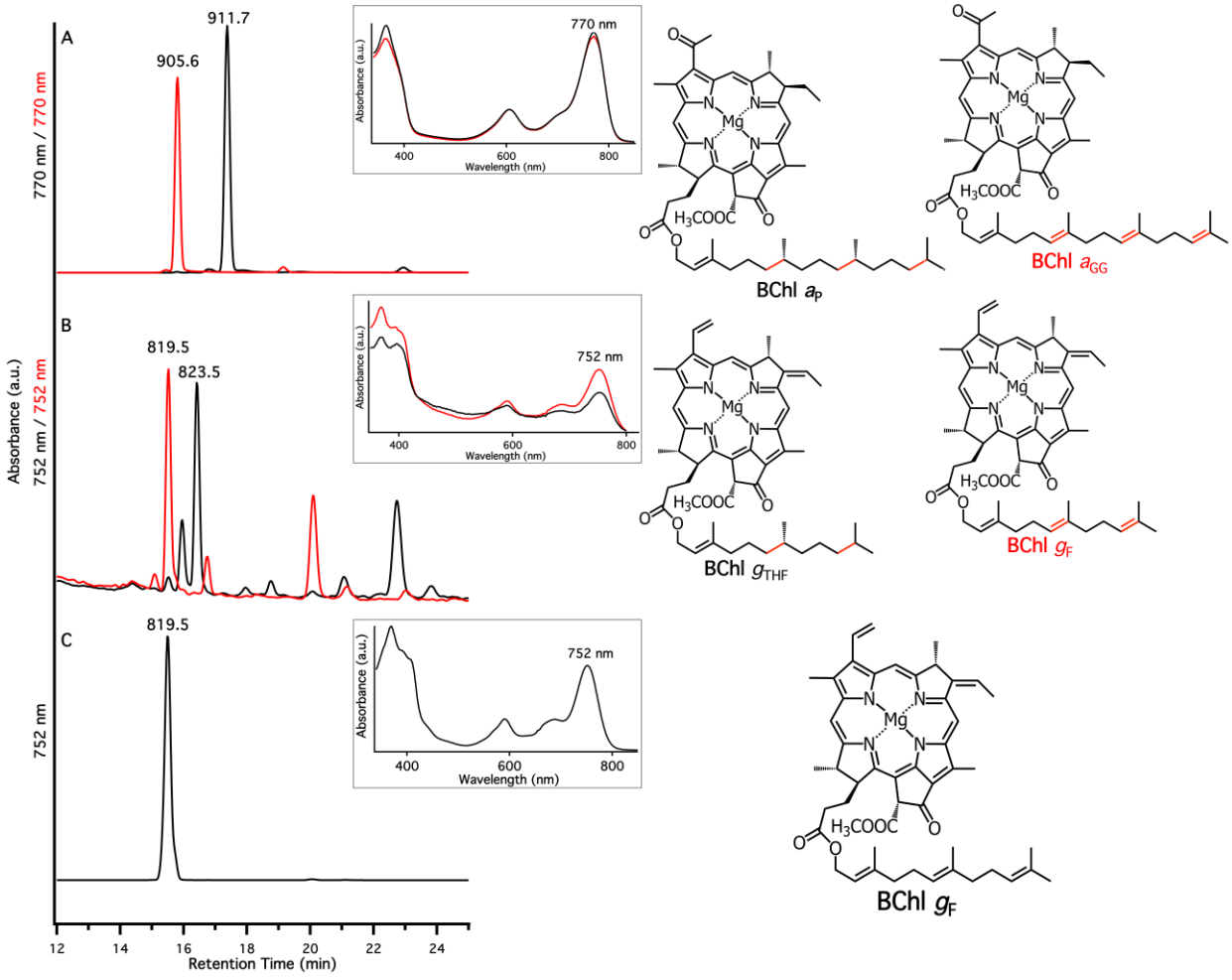

Figure 5

Analysis of pigments from *Rba. sphaeroides* strains lacking *bchP*.

Reversed phase-HPLC elution profiles of samples from (A) WT (black line) and Δ*bchP* (red line), (B) Δ*crtE*/Δ*bchF*/Δ*bciA*/*bchXYZ*^{Bv}/*bchG*^{Hm} (black line) and Δ*bchP*/Δ*crtE*/Δ*bchF*/Δ*bciA*/*bchXYZ*^{Bv}/*bchG*^{Hm} (red line), (C) authentic BChl g_F from *Hba. modesticaldum*. Monitoring wavelengths for parent (black) and mutant (red) samples are

indicated on the y-axis. The *m/z* ratio of the most abundant peak in each sample is labeled (see Fig. S5 for mass spectra), the absorption spectra of these peaks are shown (inset), and the chemical structures of these pigments are displayed (right).

As expected, deletion of *bchP* in the WT led to the presence of a single major pigment with a shorter retention time than BChl a_P, absorbing maximally at 770 nm (**Fig. 5A**); MS analysis showed this pigment had an *m/z* of 905.6, which corresponds to BChl a_{GG} (**Fig. S5B**). The Δ*bchP*/Δ*crtE*/Δ*bchF*/Δ*bciA*/*bchXYZ*^{Bv}/*bchG*^{Hm} strain accumulated a pigment with a retention time and absorption spectrum identical to that of BChl g_F extracted from *Hba. modesticaldum* (**Fig. 5B inset**). The *m/z* of the pigment, 819.5, confirmed its identity as BChl g_F (**Fig. S5D**). A minor pigment is also visible at 20 min with a longer retention time and was assign to bacteriopheophytin *g* with a farnesyl tail. These results indicate that *bchP* is able to reduce shorter farnesyl tails, as well as more common C_{20} geranylgeraniol tails, and deletion of this gene in Δ*crtE*/Δ*bchF*/Δ*bciA*/*bchXYZ*^{Bv}/*bchG*^{Hm} leads to the production of authentic BChl g_F, achieved by manipulation of both the native BChl and isoprenoid biosynthetic pathways. Another interesting observation is the presence of a small amount of 8^1-OH-Chl a_F in the extract from the BChl g_F-producing strain (**Fig. S6**). As has been previously described in the literature, conversion of BChl g_F to 8^1-OH Chl *a* on exposure to light and oxygen has been demonstrated [14,16], although every effort to prevent this was taken during the performance of the described experiments.

4. DISCUSSION

In the present study, we have engineered a model purple chlorophototrophic bacterium to produce the major photopigment used by heliobacteria, BChl g_F. This required the deletion and replacement of native genes involved in the biosynthesis of both BChlide *a* and isoprenoids, beginning with the deletion of *bchF*. This gene encodes an enzyme responsible for the hydration of the C3-vinyl group of a precursor of BChls that will be later oxidized to an acetyl moiety by BchC [46,47] (**Fig. 1**). The deletion of *bchF* in *Rba. sphaeroides* led to the absence of any BChl a_P and the accumulation of 3-vinyl-BChl a_P (Fig. 2A, Fig. S2B). The same deletion in the *Rba. sphaeroides* mutant that produces BChl b_P rather than BChl a_P (Δ*bciA*/*bchXYZ*^{Bv}) led to the production of an analogue of BChl g_F, BChl g_P (**Fig. 2B, Fig. S2D**), confirming that BChlide *g* is a precursor to BChl *b* [32]. Interestingly, *in vitro* assays have demonstrated that BChl synthase (BchG) is unable to esterify Chlide *a*, while Chl synthase (ChlG) is unable to esterify BChlide *a* [48], and when the *Rba. sphaeroides* BChl biosynthesis pathway is blocked at Chlide *a*, native BchG^{Rs} is unable to synthesize Chl *a* [49]. These studies demonstrate a strict substrate specificity of (B)Chl synthase enzymes, yet we observe that BchG^{Rs} is able to esterify a BChlide carrying a vinyl group at C3, as found on the macrocycle of Chlide *a*. These observations suggest that the activity of this enzyme is primarily determined by whether the C7=C8 bond has been reduced by COR.

Deletion of *bchF* resulted in the production of BChl g_P, but synthesis of authentic BChl g_F requires esterification with a C_{15}, rather than C_{20}, alcohol moiety. FPP is converted to GGPP by GGPP synthase, encoded by *crtE*; thus, deletion of *crtE* should lead to the accumulation of the desired C_{15} precursor. In the WT this deletion leads to the production of a significant amount of BChl a_{THF} and a small amount (~20% of total) of BChl a_P (**Fig. 3 and Fig S3B**). This result

indicates that there is an alternative pathway to make GGPP, but that the route is less efficient than that catalyzed by CrtE. It has been suggested previously that IspA, catalyzing two sequential additions of C_5 IPP to DMAPP to produce FPP, may be able to ligate an additional C_5 unit resulting in the formation of GGPP, albeit at reduced efficiency [50]. Surprisingly, the *crtE* mutant, which produces mainly BChl a_{THF}, is still able to grow phototrophically, and the ratio of BChl a_{THF}:BChl a_P does not change under these conditions. This indicates that the presence of a shorter farnesyl tail on most BChls does not significantly hamper the light-dependent growth of this organism.

Oddly, our results showed that the deletion of *crtE* in the BChl g_P-producing mutant still resulted in the production of BChl g_P (**Fig. 3B and Fig. S3D**). This indicated that the small amount of GGPP made is preferentially used to esterify BChlide *g*, which is much less abundant in the mutant compared to BChlide *a* in the WT. This demonstrates that BchG from *Rba. sphaeroides* has a strong bias for the addition of GGPP over FPP. However, by replacing the $bchG^{Rs}$ gene with that from *Hba. modesticaldum* in the WT, we observe a significant decrease in the accumulation of BChl a_P (**Fig. 4A**), suggesting that $BchG^{Hm}$ did not function efficiently in the heterologous platform, possibly due to its preference for a C_{15} substrate (or because of reduced availability of the preferred substrate). Additionally, we discovered that this *bchG* replacement in the mutant producing BChl g_P led to the production of BChl g_{THF} (**Fig. 4B and Fig. S4D**).

This result implies that the enzyme responsible for the reduction of the geranylgeranyl moiety in *Rba. sphaeroides* can similarly reduce a farnesyl moiety. Interestingly, a recent publication identified Chl a_F in Photosystem II (PSII) from a thermophilic cyanobacterium *Thermosynechococcus elongatus,* in which the major photopigment is Chl a_P [51]. This could

indicate that the ortholog of BchP in cyanobacteria and plants, ChlP, may be unable to reduce farnesyl moieties, in contrast to the phototrophic bacterial enzyme. Deletion of *bchP* in the Δ*crtE*/Δ*bchF*/Δ*bciA*/*bchXYZ*^{Bv}/*bchG*^{Hm} led to the production of BChl g_F (**Fig. 5B and Fig. S5D**). This last result confirms that BchP is responsible for the reduction of the double bonds of the farnesyl moiety and additionally establishes that we are able to make the identical pigment in *Rba. sphaeroides* as that produced by *Hba. modesticaldum*.

As with the previously published mutant producing BChl *b* [38], the strain we have constructed that synthesizes BChl g_F is not able to grow phototrophically. No proteins of the light-harvesting complexes or RCs were seen in spectra of the whole cells (**Fig. S7)**, possibly due to steric hindrance of the C-8 and C-3 side chains of non-native pigments with host apoproteins. It might be possible to engineer or select for strains producing a modified RC and antenna polypeptides that permit the binding of these pigments, assembling novel light-harvesting complexes tuned to capture different wavelengths of light. Ultimately, this mutant has been constructed as the platform in which to assemble the type I HbRC. This complex, the simplest RC discovered thus far in terms of proteins and cofactors, is composed of a homodimer of PshA, the core protein containing the electron transfer pathway (11 transmembrane helices), and two copies of a newly discovered PshX polypeptide (1 transmembrane helix), presumed to act as an antenna binding protein. They collectively coordinate 60 tetrapyrrole pigments: 54 BChl g_F, 4 BChl g_F' (the C13^2 epimer of BChl g_F), 2 8^1-OH-Chl a_F, and 2 C_{30} carotenoids (4,4′-diaponeurosporene) [11].

Rba. sphaeroides is an ideal host in which to assemble heterologous membrane proteins due to its many-fold increase of the membrane surface when cells are grown in light and/or lowered oxygen tension [52]. The primary electron donor in the HbRC is a special pair of BChl

g_F' [53,54]. 8^1-OH-Chl a_F acts as the primary electron acceptor, and it has been demonstrated that BChl g_F isomerizes to 8^1-OH-Chl a_F in the presence of oxygen and light [14]. Preliminary data on our samples show the presence of some 8^1-OH-Chl a_F (**Fig. S6**) either due to spontaneous hydration/oxidation of BChl g_F or an unknown hydratase in *Rba. sphaeroides* that may be related to an as-yet unidentified hydratase in *Hba. modesticaldum* that could convert BChl g_F to 8^1-OH-Chl a_F. Further studies will be carried out to understand this observation. In addition to the BChls and Chls, two C_{30} carotenoid molecules are present in the HbRC. To determine whether they are necessary for the assembly of the HbRC, we will need to engineer the production of 4,4′-diaponeurosporene in *Rba. sphaeroides* [55].

5. CONCLUSION

Despite the fact that photosynthesis is one of the most important biological processes on Earth and has evolved over billions of years, improvements in efficiency can still be made. In this study, we have modified the biosynthetic pathways of BChl a_P and isoprenoids to make BChl g_F, the major BChl synthesized by heliobacteria, which absorbs in the near-infrared. The next task is to use this strain as a platform in which to assemble the simple HbRC. This work provides new opportunities to understand the coordinate roles of pigment biosynthesis and assembly of complex pigment-binding proteins in chlorophototrophs.

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