Identification of an 8-vinyl reductase involved in bacteriochlorophyll biosynthesis in *Rhodobacter sphaeroides* and evidence for the existence of a third distinct class of the enzyme

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Page Heading: Rsp_3070 is an 8-vinyl reductase in *Rhodobacter sphaeroides*

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Keywords: bacteriochlorophyll biosynthesis, chlorophyll, photosynthesis, *Rhodobacter sphaeroides*, *Synechocystis* sp. PCC6803, 8-vinyl reductase

Abbreviations used: *R*., *Rhodobacter*; Chl, chlorophyll; BChl, bacteriochlorophyll; 8V, C8-vinyl; 8E, C8-ethyl; Pchlide, protochlorophyllide; *A*., *Arabidopsis*; Chlide, chlorophyllide; *C*., *Chlorobaculum*; *Synechocystis*, *Synechocystis* sp. PCC6803

SUMMARY

The purple phototrophic bacterium *Rhodobacter* (*R.*) *sphaeroides* utilises bacteriochlorophyll *a* for light harvesting and photochemistry. The synthesis of this photopigment includes the reduction of a vinyl group at the C8 position to an ethyl group, catalysed by a C8-vinyl reductase. An active form of this enzyme has not been identified in *R. sphaeroides* but its genome contains two candidate ORFs similar to those reported to encode C8-vinyl reductases in the closely related *R. capsulatus* (*bchJ*), and in plants and green sulphur bacteria (rsp_3070). To determine which gene encodes the active enzyme, knock-out mutants in both genes were constructed. Surprisingly, mutants in which one or both genes were deleted still retained the ability to synthesise C8-ethyl bacteriochlorophyll. These genes were subsequently expressed in a cyanobacterial mutant that cannot synthesise C8-ethyl chlorophyll *a*. *R. sphaeroides* rsp_3070 was able to restore synthesis of the WT C8-ethyl chlorophyll *a* in the mutant, while *bchJ* did not. These results demonstrate that Rsp_3070 is a functional C8-vinyl reductase and that *R. sphaeroides* utilises at least two enzymes to catalyse this reaction, indicating the existence of a third class, while there remains no direct evidence for the activity of BchJ as a C8-vinyl reductase.

INTRODUCTION

The process of photosynthesis, the conversion of solar energy into chemical potential energy, is dependent on light-absorbing (bacterio)chlorophyll ((B)Chl) pigments. The antenna complexes of plants and phototrophic bacteria contain a variety of light-harvesting (B)Chl molecules. Structural modifications to the tetrapyrrole macrocycle are responsible for the specific absorption and energy-transfer features of the light-harvesting apparatus, influencing both pigment-pigment and pigment-protein interactions within the antenna complexes [1-3].

In the (B)Chl precursor 8-vinyl- (8V) protochlorophyllide (Pchlide) there are two vinyl groups, at the C3 and C8 positions. With the exception of some *Prochlorococcus* species [4], the (B)Chls utilised for photosynthesis typically carry an ethyl group in the C8 (8E) position of the macrocycle (Figure 1). Many 8E- and 8V-Chl precursors have been detected in plants and algae, which cannot be accounted for by the stepwise operation of a linear biosynthetic pathway [5-7]. The stage at which the 8V reductase (EC 1.3.1.75) operates has not been firmly established, although it is generally accepted that this reaction occurs when 8V-Pchlide is converted to 8E-Pchlide [8,9]. Additional processing of the precursor to generate BChl includes the conversion of the C3-vinyl group to acetyl, whereas in Chl biosynthesis it is retained.

Most of the genes involved in BChl biosynthesis in the purple non-sulphur bacterium *R. capsulatus* are located within a 46 kb region of the chromosome [10,11]. Studies on *R. capsulatus*, utilising interposon-directed mutagenesis targeted to the 'photosynthesis gene cluster', revealed that disruption of ORF213 (termed *bchJ*) resulted in the excretion of the 8V form of Pchlide [8], and that disruption of *bchJ* along with the gene encoding the BchL subunit of the Pchlide reductase led to an increased ratio of 8V- to 8E-Pchlide [12]. BchJ was therefore assigned as a subunit of the 8V reductase.

The enzyme responsible for 8V reduction in *Arabidopsis* (*A.*) *thaliana* was identified through a mutation in the AT5G18660 gene, unrelated to *bchJ*, which resulted in the accumulation of 8V- rather than 8E-Chls [13,14]. Recombinant AT5G18660, produced in *E. coli*, was shown to reduce 8V-chlorophyllide (Chlide) to the 8E form, demonstrating 8V reductase activity [13]. Subsequently, mutations in orthologues of this gene in the green sulphur bacterium *Chlorobaculum* (*C.*) *tepidum* (CT1063) [15] and rice (Os03g22780) [16] also resulted in mutants producing only 8V-(B)Chls. In the *C. tepidum* study, deletion of a *bchJ* orthologue (CT2014) did not result in the accumulation of 8V-(B)Chls, suggesting that,

in this organism, the reduction of the C8 group is mediated by CT1063 (named BciA), not BchJ.

The genomes of many cyanobacteria, including *Synechocystis* sp. PCC6803 (*Synechocystis*) do not contain orthologues of either the *A. thaliana* 8V reductase or *bchJ*, even though they utilise 8E-Chl *a* for energy trapping. Therefore, it was hypothesised that many cyanobacteria contain a reductase that is unrelated to the enzyme in plants and green sulphur bacteria. In 2008 two groups reported that mutants in the *Synechocystis* slr1923 ORF were unable to grow under high light conditions and did not synthesise Chl reduced at the C8 position [17,18]. An orthologue of this gene, Ctha_1208 from the green sulphur bacterium *Chloroherpeton thalassium,* was shown to complement the *C. tepidum* ΔCT1063 mutant and recover normal (8E) (B)Chl production, demonstrating 8V reductase activity of this second class of the enzyme, renamed as BciB [19].

The genome of the purple non-sulphur bacterium *R. sphaeroides* contains orthologues of both *bchJ* and *bciA* (rsp_3070) (Supplementary Figure 1), but no orthologue of *bciB*. The rsp_3070 ORF is found on chromosome 2 whereas *bchJ* is located in the photosynthesis gene cluster, the 40.7 kb stretch of ORFs on chromosome 1 containing all the currently known genes responsible for BChl and carotenoid biosynthesis in this organism, as well as many of those encoding photosystem subunits [20,21]. In this study, the candidate 8V reductase genes from *R. sphaeroides* were expressed in a *Synechocystis* Δslr1923 mutant and the transconjugant strains characterised. The mutant expressing *bchJ* was unable to restore 8E-Chl *a* production and could not grow under high light conditions. The mutant expressing rsp_3070 was able to grow at a rate similar to that of the WT strain under high light conditions and pigment analysis demonstrated that 8E-Chl production had been restored, confirming that Rsp_3070 is a BciA-type 8V reductase. Single (Δrsp_3070 and Δ*bchJ*) and double (Δrsp_3070/Δ*bchJ*) deletion mutants of *R. sphaeroides* were also constructed and their BChl *a* contents were analysed. Unexpectedly, none of the mutants accumulated 8V-BChl *a*, instead synthesising the fully reduced 8E- form. These findings provide evidence for the existence of at least one 8V reductase additional to Rsp_3070 in *R. sphaeroides* and a third distinct class of the enzyme.

EXPERIMENTAL

Growth conditions

R. sphaeroides strains were either grown semi-aerobically in the dark in a rotary shaker at 34°C or anaerobically under illumination at room temperature in liquid M22+ medium [22] supplemented with 0.1% casaminoacids.

Synechocystis strains were grown photoautotrophically in a rotary shaker under normal light conditions (50 μmol photons·m^{-2}·s^{-1}) at 30°C in liquid BG-11 medium [23] supplemented with 10 mM TES pH 8.2.

E. coli strains JM109 [24] and S17-1 [25] transformed with pK18*mobsacB* plasmids were grown in a rotary shaker at 37°C in LB medium supplemented with 30 μg·ml^{-1} kanamycin. All strains and plasmids used in this study are listed in Supplementary Table 1.

Construction of in-frame deletion mutants of *R. sphaeroides*

R. sphaeroides bchJ and rsp_3070 were deleted using the allelic exchange vector pK18*mobsacB* [26]. The sequences up- and downstream of the genes were amplified with the relevant UpF and UpD, and DownF and DownR primers, respectively (a list of the sequences of all of the primers used in this study can be found in Supplementary Table 2). The up- and downstream PCR products were digested with the relevant restriction enzymes and ligated into cut pK18*mobsacB*. Sequenced clones were conjugated into *R. sphaeroides* from *E. coli* S17-1 and transconjugants in which the clone 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% sucrose and lacking kanamycin. Sucrose-resistant, kanamycin-sensitive colonies had excised the allelic exchange vector through the second recombination event [27]. The deletion of the desired gene was confirmed by colony PCR using relevant CheckF and CheckR primers.

Construction of deletion mutant of *Synechocystis*

Replacement of the central portion of the *Synechocystis* slr1923 ORF with an erythromycin resistance cassette was achieved using a modification of the megaprimer mutagenesis method [28]. Sequences of approximately 450 bp up- and downstream of slr1923 were amplified using the slr1923-UpF and UpR, and DownF and DownR primers, respectively, to generate primary megaprimers. The upstream reverse and downstream forward primers contained overhang sequences able to amplify the erythromycin resistance cassette from the pERY plasmid [29]. The upstream megaprimer, along with the Ery^RR primer, and the downstream megaprimer with the Ery^RF primer were used to amplify large overlapping portions of the resistance cassette. These resulting secondary megaprimers were then used for overlap extension PCR to generate the final mutagenesis fragment. This fragment was transformed into WT *Synechocystis* and transformants were selected on BG-11 agar plates containing 5 $\mu g \cdot ml^{-1}$ erythromycin and fully segregated by incrementally doubling the concentration of antibiotic to 40 $\mu g \cdot ml^{-1}$. Construction of a fully segregated strain was confirmed by colony PCR using the UpF and DownR primers used to generate the primary megaprimers.

Construction of *Synechocystis* mutants containing *R. sphaeroides* genes

BchJ and rsp_3070 were amplified from WT *R. sphaeroides* genomic DNA using the relevant PDF and PDR primers, digested and cloned into the *Nde*I/*Bgl*II sites of pPD-FLAG [30] containing the *Synechocystis psbAII* promoter and flanking sequences for homologous recombination that allow the insertion of genes into the *Synechocystis* genome in place of the *psbAII* gene. *PsbAII* is one of three ORFs encoding the D1 protein of photosystem II and deletion of one copy of the gene does not affect photosynthetic capability [31]. The resulting sequenced plasmids were transformed into the Δslr1923 *Synechocystis* strain and transformants were selected on BG-11 agar plates containing 10 $\mu g \cdot ml^{-1}$ neomycin and fully segregated by incrementally doubling the concentration of antibiotic to 80 $\mu g \cdot ml^{-1}$. Construction of fully segregated strains was confirmed by colony PCR using the psbAIICheckF and psbAIICheckR primers.

Purification of Pchlide

Pchlide was purified from the growth medium of the *R. capsulatus* ZY5 mutant [32] by the method described in Heyes *et al*. [33]. This strain has a mutation in *bchL*, encoding one of the three subunits required for the light-independent reduction of Pchlide, resulting in the accumulation of the pigment.

Extraction of pigments

Pigments were extracted from cell pellets after washing in 20 mM HEPES pH 7.2 or from clarified growth medium by adding 9 volumes of 0.2% ammonia in methanol, vortexing for 30 s and incubating on ice for 20 min. The extracts were clarified by centrifugation and the supernatants immediately analysed on an Agilent 1200 HPLC system.

Analysis of pigments by HPLC

Pchlide species were separated on a YMC30 C30 reverse-phase column (3 µm particle size, 250 x 4.6 mm) using a method modified from that of Kruk and Myśliwa-Kurdziel [34]. 30:70 (v/v) methanol:500 mM ammonium acetate and methanol were used as solvents A and B respectively. Pigments were eluted at 1 ml·min^{-1} at 40°C on a linear gradient of 82-98% solvent B over 40 min, increasing to 100% to wash the column. Elution of Pchlide species were monitored by checking absorbance at 632 nm.

(B)Chl extracts were separated on a Phenomenex Aqua C18 reverse-phase column (5 µm particle size, 125 Å pore size, 250 x 4.6 mm) using a method modified from that of van Heukelem *et al*. [35]. 80:20 (v/v) methanol:500 mM ammonium acetate and 80:20 (v/v) methanol:acetone were used as solvents A and B respectively. Pigments were eluted at 1 ml·min^{-1} at 40°C on a linear gradient of 92-94% solvent B over 25 min, increasing to 100% to wash the column. Elution of Chl *a* and BChl *a* species were monitored by checking absorbance at 665 nm and 770 nm, respectively.

Purification of (B)Chls for analysis by mass spectrometry

Pigments were extracted from cultures in MS grade solvents as described above. (B)Chls were separated from their precursors and carotenoids by solid phase extraction on DSC-18 reverse-phase columns (Supelco) using a method derived from an HPLC protocol by Borrego and Garcia-Gil [36]. 70:30 (v/v) methanol:1 M ammonium acetate and 50:30:20 (v/v/v) methanol:ethyl acetate:acetonitrile were used as solvents A and B respectively. After equilibration of the column with 0.5 ml 50:50 (v/v) A:B, the clarified extract was loaded and allowed to enter the column by gravity flow. The column was washed with 1 ml 50:50 (v/v) A:B to elute (B)Chl precursors and (B)Chls were eluted into dark glass vials by the addition of 0.5 ml 20:80 (v/v) A:B. The vials were immediately flushed with nitrogen, sealed and stored at -20°C.

Quantification of Chl in *Synechocystis* strains

Cultures standardised by OD_{750} were pelleted in mid-exponential phase and Chls were extracted by addition of an excess of 100% methanol. Chl contents were calculated from spectrophotometric data [37].

Mass spectrometry

Samples were diluted either 10- or 50-fold with LC-grade methanol immediately before analysis. Mass spectra were acquired in positive polarity/profile mode during infusion at 3 µl min^{-1} into a standard electrospray ionization source on a Maxis UHR-TOF instrument (Bruker Daltonics, Bremen, Germany). Default parameters provided by the manufacturer were used except that the transfer time was adjusted to 60 µs to enhance sensitivity around 900 m/z. Product ion (MS/MS) spectra were generated by collision-induced dissociation at 30 eV.

RESULTS

Deletion of *bchJ* and/or rsp_3070 in *R. sphaeroides* does not lead to the accumulation of 8V-BChl

In order to determine whether deletions of the *R. sphaeroides bchJ* and rsp_3070 result in the accumulation of 8V-BChl, strains lacking one or both of these genes were constructed. PCR analysis of genomic DNA confirmed the removal of these genes (Supplementary Figure 2). While all of these strains were viable when grown semi-aerobically in the dark, both the Δ*bchJ* and Δrsp_3070/Δ*bchJ* strains were unable to grow phototrophically, whereas the WT and Δrsp_3070 strains displayed similar doubling times under both high and low light (Table 1).

Previously isolated *bchJ* mutants of *R. capsulatus* and *C. tepidum* have been reported to excrete 8V-Pchlide into the growth medium [8,15]. To ascertain whether a mutation in either this gene or rsp_3070 in *R. sphaeroides* resulted in the same phenotype the WT and three mutant strains described above were grown semi-aerobically in the dark and pigments were extracted from the growth medium and analysed by HPLC (Figure 2). Neither WT nor Δrsp_3070 excreted Pchlide into the medium, whereas the traces from both the Δ*bchJ* and Δrsp_3070/Δ*bchJ* strains displayed single peaks, both with a retention time of 46.6 min. A purified Pchlide standard, containing both 8V and 8E forms, displayed peaks with retention times of 45.5 min and 46.6 min. Analysis of the absorbance profiles of these peaks indicates that the pigment with a retention time of 45.5 min is Pchlide reduced at the C8 position, with the 8V form eluting over 1 min later. Therefore, consistent with results from studies on other photosynthetic bacteria, the deletion of *bchJ* in *R. sphaeroides* leads to the accumulation and excretion of 8V-Pchlide.

BChl pigments from these strains, along with those from the parent WT, were extracted from pellets of semi-aerobically grown cultures and analysed by HPLC (Figure 3). The retention times and absorbance profiles of the single BChl peak from each strain were identical, therefore analysis of these pigments by MS was conducted to confirm their identities. MS analysis indicated the presence of three BChl species in each of the WT, Δrsp_3070, Δ*bchJ* and Δrsp_3070/Δ*bchJ* strains: 8E-BChl (m/z 910.55), oxidised 8E-BChl (m/z 926.54) and oxidised BChl lacking two hydrogen atoms (m/z 924.52) (Figure 4A). The oxidised pigments arise from the preparative procedure used. To find out if the non-reduced group arises from the presence of an 8V moiety, further characterisation was performed using tandem MS (MS/MS) analysis (Figure 4B-D). This analysis shows that the BChl (m/z 924.52) in each of the four samples is the tetrahydrogeranylgeranyl (THGG) form of the pigment, lacking one reduction on the alcohol tail moiety (Figure 1). Thus, the loss of two hydrogen atoms does not arise from a failure to convert the 8V to the 8E group on the macrocycle. These results demonstrate that deletion of one or both of *bchJ* and rsp_3070 does not lead to the accumulation of 8V-BChl, and that the 8V form of the pigment is not found at any detectable level in any of these strains.

Complementation of the *Synechocystis* Δslr1923 mutant with the *R. sphaeroides bchJ* and rsp_3070 genes

Disruption of the slr1923 ORF in *Synechocystis* results in the accumulation of 8V-Chl and this mutant is unable to grow under high light conditions [17,18]. A similar mutant in which the central portion of the slr1923 was replaced with an ampicillin resistance cassette was constructed and PCR analysis of genomic DNA extracted from this strain confirmed the complete segregation of the mutant allele (Supplementary Figure 3). In order to test whether expression of *bchJ* or rsp_3070 could reinstate 8E-Chl production in this mutant background the *R. sphaeroides* genes were inserted in place of the *psbAII* gene (Figure 5).

When grown under normal light conditions the complemented *Synechocystis* strains, along with the WT and Δslr1923 mutant, were viable but the Δslr1923 mutant was unable to grow under high light, consistent with the previously published results [17,18]. Complementation with rsp_3070, but not *bchJ*, restored growth under high light (Figure 6). Growth rates of the mutants cultured in triplicate in liquid medium under different light conditions were compared with those of the WT. The doubling times of the WT, Δslr1923 and Δslr1923::rsp_3070 strains were similar under normal light (50 μmol photons·m^{-2}·s^{-1}), while the growth of the Δslr1923::*bchJ* strain was approximately 1.5 fold slower. Under high light (250 μmol photons·m^{-2}·s^{-1}), Δslr1923 and Δslr1923::*bchJ* were unable to grow, as on solid medium, whereas WT and Δslr1923::rsp_3070 had comparable growth rates (Table 2).

Whole cell absorption spectra show that both Δslr1923 and Δslr1923::*bchJ* have red-shifted absorption maxima in the Soret region, consistent with whole cell spectra of *Synechocystis* mutants lacking a functional 8V reductase [17,18] (Figure 7). In addition these strains had a decreased Chl content (~ 680 nm) and increased phycobiliprotein content (~ 630 nm) with respect to the WT. In contrast, the spectrum of Δslr1923::rsp_3070 is almost identical to the WT. Chl contents were calculated from the spectra in Figure 7 and show that the Δslr1923 mutant has ~50% of the Chl levels found in the WT and in Δslr1923::rsp_3070, while Δslr1923::*bchJ* has only ~30% WT levels. Ito *et al*., [17] suggested that the increase in the ratio of phycobiliprotein to Chl associated with the Δslr1923 mutation may arise from reduced flux down the biosynthetic pathway dedicated to Chl production and increased flow down the haem branch. This may then lead to an accumulation of phycobilins, synthesised by oxidative opening of the haem ring.

Chls from these strains grown in liquid medium under normal light were extracted and analysed by HPLC (Figure 8). The Chl extracted from the Δslr1923 mutant had a retention time 0.5 min earlier than that of the WT. Analysis of the absorbance profiles of these peaks demonstrate that the Soret band maximum from Δslr1923 is red shifted by 11 nm relative to that from the WT spectrum, indicating that it is the 8V form of the pigment [17,18]. The retention time and absorbance profile of the Chl peak from Δslr1923::*bchJ* were identical to those of Δslr1923, while those from Δslr1923::rsp_3070 were identical to the WT. Therefore, *R. sphaeroides bchJ* is unable to reinstate 8V reductase activity in *Synechocystis* while rsp_3070 successfully recovers the WT status, with respect to 8E-Chl synthesis.

MS/MS analysis of Chls extracted from pelleted cultures (spectra not shown) confirmed that the Δslr1923 and Δslr1923::*bchJ* strains synthesised only 8V-Chl *a* (Table 2). Under normal light conditions the 8V-Chl contents of the WT and Δslr1923::rsp_3070 strains were 11.8% (± 4.4) and 13.4% (± 1.5), respectively, whereas at high light 8V-Chl was undetectable in each strain, indicating that Rsp_3070 fully restores 8V reductase activity to the Δslr1923 mutant.

DISCUSSION

Interposon-based mutagenesis of the photosynthesis gene cluster of *R. capsulatus* originally led to the assignment of *bchJ* as a gene encoding a component of the 8V reductase in purple non-sulphur bacteria [8]. The authors observed excretion of Pchlide by the mutant, demonstrated by various analyses to be the 8V form of the pigment. This interposon disruption mutant, along with an isolated deletion mutant, was able to synthesise BChl, although at reduced levels, and was viable photosynthetically. When a gene encoding a subunit of the light-independent Pchlide reductase (*bchL*) was disrupted the resulting mutant accumulated Pchlide, 68.2% of which was in the 8E form, but in a *bchL-bchJ* double mutant only 23.6% of the Pchlide was reduced at the C8 position [12]. These results indicated that, although BchJ alters the ratio of 8V:8E Pchlide it is not absolutely required for the reduction of the C8 group, leading the authors to hypothesise that BchJ was one of at least two functional 8V reductases in *R. capsulatus*. Subsequently, a gene, *AT5G18660*, encoding a functional 8V reductase was first identified in *A. thaliana* [13]. The product of this gene, encoding the only 8V reductase in *A. thaliana*, displayed no similarity to *bchJ* or to slr1923, but orthologues of both *AT5G18660* and *bchJ* have been identified in the green sulphur bacterium *C. tepidum* [15]. Mutational analysis in *C. tepidum* enabled assignment of the *A. thaliana* orthologue (*bciA*) as an 8V reductase; however, the mutant in the *bchJ* orthologue excreted 8V Pchlide, similar to the *bchJ* mutant in *R. capsulatus*, but produced 8E-(B)Chls, although, in the case of the *C. tepidum* BChl c_F, at a reduced amount. The authors therefore suggested the possibility that BchJ is able to act as a substrate chaperone or substrate delivery

protein, and the absence of this protein leads to a disruption of normal flux of intermediates through the (B)Chl biosynthetic pathway.

Subsequently, BchJ has been shown to bind to both the substrate (protoporphyrin IX) and product (magnesium protoporphyrin IX) of the magnesium chelatase reaction, the first committed step of (B)Chl biosynthesis [38]. BchJ was also shown to interact with the BchH subunit of the magnesium chelatase, and was able to shift the equilibrium of the chelation reaction towards the product. These results led to the hypothesis that, in anoxygenic photosynthetic organisms, BchJ acts as a chaperone of magnesium protoporphyrin IX to the methyltransferase, the next enzyme in the (B)Chl biosynthetic pathway, mimicking the role of Gun4 in plants and cyanobacteria [39,40].

The results in the present study, along with those previously published, do not provide any direct evidence for the activity of BchJ as an 8V reductase. Unlike previously described *bchJ* mutants, neither the *R. sphaeroides* Δ*bchJ* nor the Δrsp_3070/Δ*bchJ* mutant can photosynthesise, yet both strains are able to produce fully reduced BChl *a* when grown in the dark. The inability to grow when illuminated may possibly indicate that *R. sphaeroides* is more susceptible than other organisms to phototoxic attack resulting from the illumination of cultures accumulating 8V-Pchlide.

To date, the oxygenic phototrophic organisms in which functional 8V reductases have been identified rely on a single class of the enzyme. The plant reductases have all been the BciA-type, whereas 8E-Chl-utilising cyanobacteria encode either the BciA or BciB-type reductase. Interestingly, the genomes of *A. thaliana* and rice contain ORFs similar to *bciB*, although the encoded proteins do not appear to function as 8V reductases. This is possibly explained by horizontal transfer of one class of the gene and the progenitor of the chloroplast providing the other, *bciB* becoming redundant as plant species evolved. The mutant plants and cyanobacteria lacking a functional 8V reductase, although demonstrating altered absorption spectra, are able to grow phototrophically, as with the *Prochlorococcus* species that utilise the 8V forms of Chl *a* and Chl *b* to capture light unused by other marine phototrophs [41].

Much like cyanobacteria, the green sulphur bacteria can utilise BciA and BciB-type enzymes (e.g. *C. tepidum* and *Chloroherpeton thalassium*, respectively). As with *A. thaliana* and rice, some of these photosynthetic bacteria appear to have multiple 8V reductases, either copies of each type (e.g. *Prosthecochloris aestuarii*) or more than one copy of *bciB* (e.g. *Chlorobium phaeobacteroides*) [19]. This potential utilisation of unrelated enzymes to catalyse a single reaction, and proteins encoded by a gene present in more than one copy, would not be uncommon in photosynthesis. The magnesium protoporphyrin monomethyl ester cyclase and Pchlide reductase enzymes exist in two distinct classes in oxygenic phototrophs, each employing different reaction mechanisms [42]. The photosystem II D1 protein is also encoded by multiple copies of the same gene [31]. From the data presented in this study, it appears that purple phototrophs such as *R. sphaeroides* can utilise two different functional enzymes to produce an 8E photopigment.

This employment of multiple 8V reductases, along with data indicating that 8V-BChl is not found in detectable amounts in *R. sphaeroides* under any growth conditions, suggests that this bacterium, possibly along with some of the green sulphur species, may use this mechanism to ensure that fully reduced (B)Chls are utilised for light harvesting and photochemistry. In contrast, *Synechocystis* has been shown to tolerate 8V-Chl *a* as a photopigment under moderate and low light, with the WT strain utilising a mixture of 8E- and 8V-Chl *a* under these conditions. The related *Prochlorococcus* species that contain pigments that are not reduced at the C8 position use this feature to their advantage by harnessing wavelengths of light unused by other marine oxygenic phototrophs. Currently there are no known species of anoxygenic phototroph employing the same tactic to garner

energy from light not used by other (B)Chl-containing species. The photosystems of the mutant plant and *Synechocystis* strains display reduced efficiency of energy transfer from LHCII and phycobilisome antenna complexes, respectively, to photosystem complexes and are less stable when 8E-Chl is replaced by 8V-Chl [18]. The reduction in photosystem stability is explained by the disruption of the fit between the photosystem Chl binding sites and the 8V pigments, increasing the degradation rate of the continuously recycled complexes above the rate at which they can be synthesised, leading to photodamage under strong illumination. Indeed, it has been hypothesised that the 8V-Chl-containing photosystems of *Prochlorococcus* had to evolve to increase their stability before the species could become tolerant of high light intensities [43]. Some anoxygenic phototrophs may therefore employ multiple 8V reductases to limit the disruption to photosystem assembly, as the penalties of using 8E pigments may be more severe when BChls, rather than Chls, are utilised.

An *in vitro* study of the recombinant enzyme from *A. thaliana* indicated that the 8V form of Chlide *a* is the preferred substrate for reduction, with no conversion of the 8V forms of magnesium protoporphyrin IX, Pchlide, Chlide *b*, Chl *a* and Chl *b* [44], while the related enzyme from rice, which was not tested on magnesium protoporphyrin IX or Pchlide, could convert both Chlide *a* and Chl *a* to their 8E forms [16]. However, the recombinant *C. tepidum* enzyme, related to both plant proteins, has been shown to catalyse the reduction of 8V Pchlide under similar experimental conditions [15]. There is currently no *in vitro* assay described for a BciB enzyme, but it may be that these proteins preferentially catalyse the reduction of a substrate different to those of the BciA enzymes. It is conceivable, therefore, that utilisation of multiple 8V reductases acting on substrates at different stages of the (B)Chl biosynthetic pathway can increase the efficiency of production of fully reduced (B)Chls in a biological system. The missing 8V reductase in *R. sphaeroides* may utilise a substrate exclusive to the BChl pathway (e.g. (B)Chlide), and this enzyme would therefore be redundant in Chl-producing organisms.

The *Roseiflexus* species of green non-sulphur bacteria, which utilise 8E BChl *a* for their photochemistry [45] and therefore contain an 8V reductase, do not contain ORFs resembling *bciA* or *bciB* [17]. It is possible that the *Roseiflexus* enzyme and the second 8V reductase in *R. sphaeroides* are related proteins. Identification of a third class of this enzyme could lead to a complete picture of 8V reduction in all photosynthetic organisms.

AUTHOR CONTRIBUTIONS

Daniel Canniffe, Philip Jackson and Sarah Hollingshead performed the experiments. Daniel Canniffe, Mark Dickman and Neil Hunter designed the experiments. Daniel Canniffe, Philip Jackson and Neil Hunter wrote the manuscript.

ACKNOWLEDGEMENTS

Neil Hunter, Daniel Canniffe, Philip Jackson and Mark Dickman were supported by a grant (BB/G021546/1) from the Biotechnology and Biological Sciences Research Council (UK). Sarah Hollingshead was supported by a doctoral studentship from the University of Sheffield.

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TABLE LEGENDS

Table 1. Photosynthetic growth rates of studied strains of *R. sphaeroides* under different light regimes.

A dash (—) indicates data could not be measured.

Table 2. Growth rates and 8V-Chl *a* content, from MS/MS analysis, of studied strains of *Synechocystis* under different light regimes.

A dash (—) indicates data could not be measured.

FIGURE LEGENDS

Figure 1. Chemical structures of (B)Chls *a* and their alcohol moieties.

(A) IUPAC numbered chemical structure of Chl *a* with a vinyl group at the C8 position (circled) and (B) chemical structure of BChl *a* with an ethyl group at the C8 position (circled), having been reduced by an 8V reductase. X denotes the attached alcohol moiety. The structural differences between Chl *a* and BChl *a* are indicated by asterisks. (C) Alcohol moieties of (bacterio)chlorins, denoted by R. The circled bonds are those sequentially reduced by the gene product of *chlP*/*bchP*. GGPP, geranylgeranyl pyrophosphate; DHGG, dihydrogeranylgeranyl pyrophosphate; THGG, tetrahyrogeranylgeranyl pyrophosphate.

Figure 2. HPLC analysis of Pchlide extracted from growth medium of *R. sphaeroides* strains lacking *bchJ*

HPLC elution profiles of extracts from growth medium of (A) WT, (B) Δrsp_3070, (C) Δ*bchJ* and (D) Δrsp3070/Δ*bchJ*, and (E) a sample of purified Pchlide from *R. capsulatus* ZY5. Retention times of 45.5 and 46.6 min, and Soret absorption maxima at 434 and 441 nm (inset), are indicative of 8E-Pchlide and 8V-Pchlide, respectively, in the HPLC solvents.

Figure 3. Analysis of BChls extracted from *R. sphaeroides* strains

Typical HPLC elution profile (A) and absorption spectrum (B) of BChls extracted from described strains of *R. sphaeroides*. The retention times and spectra of WT and mutant BChls were identical in each case.

Figure 4. Typical mass spectra of a BChl extract from described *R. sphaeroides* strains.

(A) Mass spectrum showing radical cations for BChl, oxidised BChl (BChl + O) and oxidised, dehydrogenated BChl (BChl + O – 2H). The latter two species could either be 8V/phytyl- or 8E/tetrahydrogeranylgeranyl-BChl since the two forms would be isobaric. (B) Product ion (MS/MS) spectrum for BChl (m/z 910.55). The product ion at m/z 632.24 is generated by the neutral loss of the phytyl moiety ($C_{20}H_{38}$, 278.31 Da). (C) Product ion (MS/MS) spectrum for oxidised, dehydrogenated BChl (m/z 924.52). The product ion at m/z 648.24 is generated by the neutral loss of the tetrahydrogeranylgeranyl moiety ($C_{20}H_{36}$, 276.28 Da). Therefore this relatively low abundance species represents BChl with an incompletely processed aliphatic tail. On the macrocycle, the 8V group has been converted to 8E by an 8V reductase. (D) Product ion (MS/MS) spectrum for oxidised BChl (m/z 926.54). The product ion at m/z 648.24 is generated by the neutral loss of the phytyl moiety ($C_{20}H_{38}$, 278.31 Da).

Figure 5. Construction of *Synechocystis* mutants designed to express genes encoding putative *R. sphaeroides* 8V reductases.

(A) Diagram depicting the replacement of the native *psbAII* gene with a gene of interest by homologous recombination via pPD-FLAG constructs. (B) Isolation of fully segregated complementation mutants of *Synechocystis* confirmed by colony PCR amplifying the *psbAII* locus in (1) WT, (2) Δslr1923, (3) Δslr1923::rsp_3070 and (4) Δslr1923::*bchJ*.

Figure 6. Agar plates of *Synechocystis* strains grown at different light intensities.

The sections on the agar plates contain the following strains: (1) WT, (2) Δslr1923, (3) Δslr1923::*bchJ* and (4) Δslr1923::rsp_3070. The plates were incubated under (A) 50 µmol photons·m^{-2}·s^{-1} and (B) 250 µmol photons·m^{-2}·s^{-1}.

Figure 7. Cell spectra and Chl content of *Synechocystis* strains

Whole-cell absorbance spectra of WT (solid black), Δslr1923 (solid grey), Δslr1923::rsp_3070 (dotted black) and Δslr1923::*bchJ* (dashed black) strains of *Synechocystis* grown phototrophically under normal light (50 µM photons·m^{-2}·s^{-}1). Spectra were recorded with samples standardised by OD_{750} and normalized to light scattering at 750 nm. The Chl content of each strain was determined from three biological replicates of these spectra.

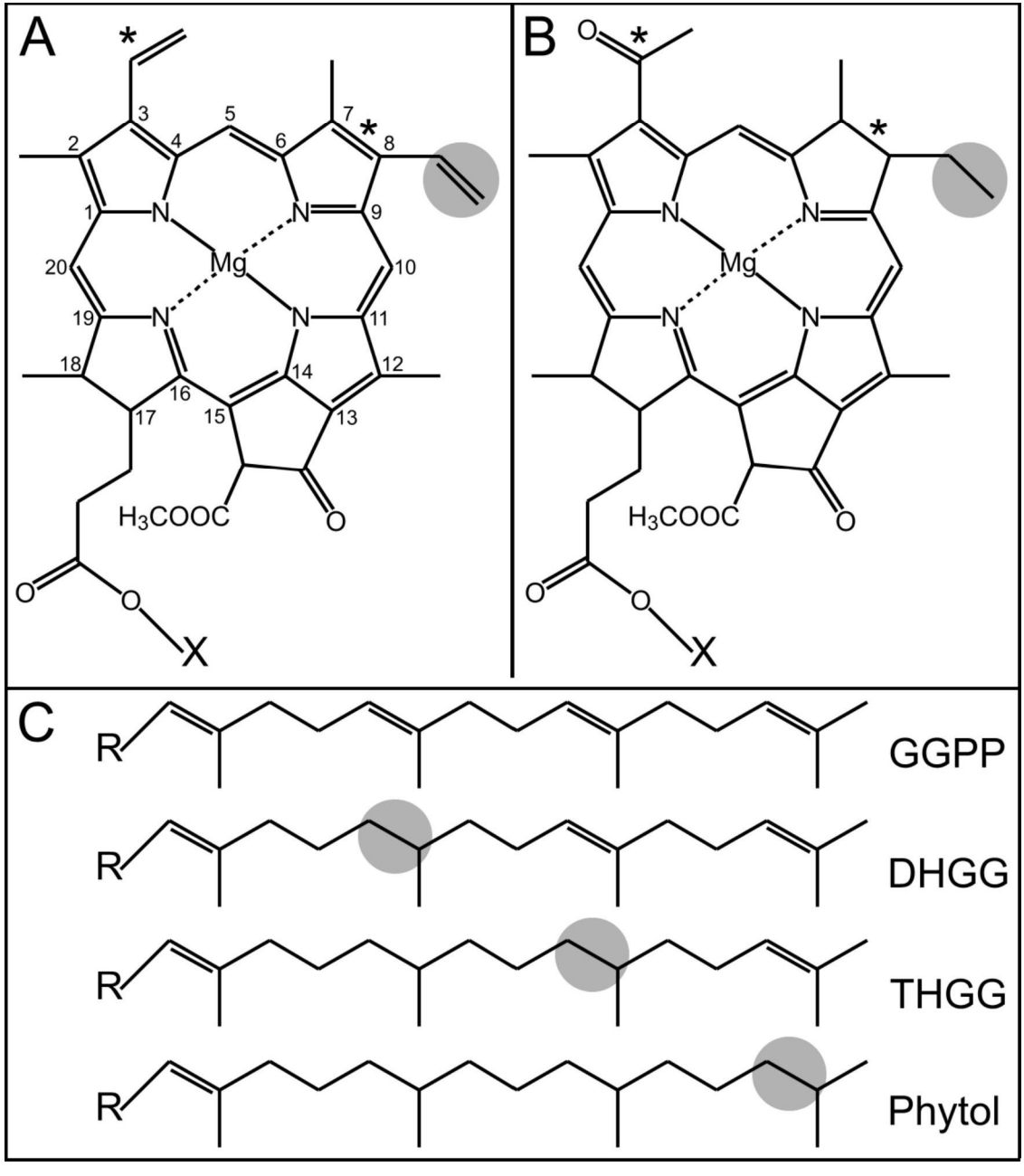
Figure 8. HPLC analysis of Chls extracted from *Synechocystis* strains

HPLC elution profiles and absorption spectra (inset) of Chls extracted from (A) WT, (B) Δslr1923, (C) Δslr1923::rsp_3070 and (D) Δslr1923::*bchJ*. Retention times of 22.7 and 22.2 min, and Soret absorption maxima at 431 and 442 nm are indicative of 8E-Chl *a* and 8V-Chl *a*, respectively, in the HPLC solvents.

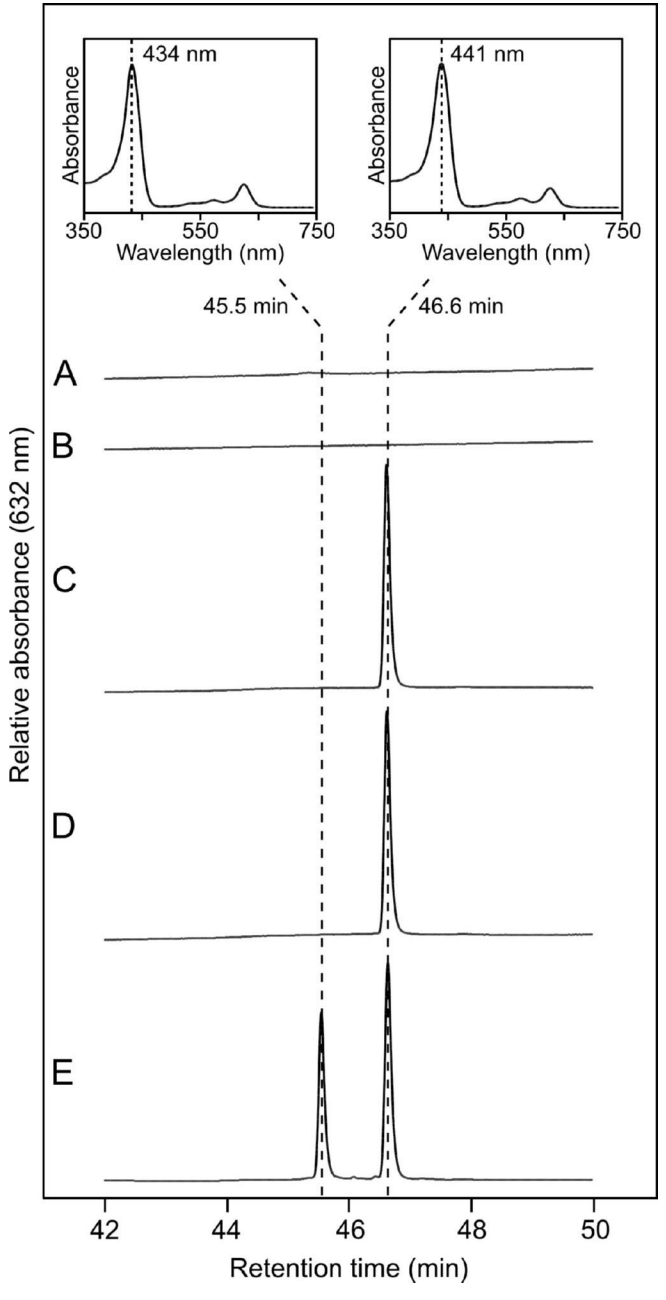
RELATED PAPERS IN PRESS OR UNDER EDITORIAL CONSIDERATION
N/A

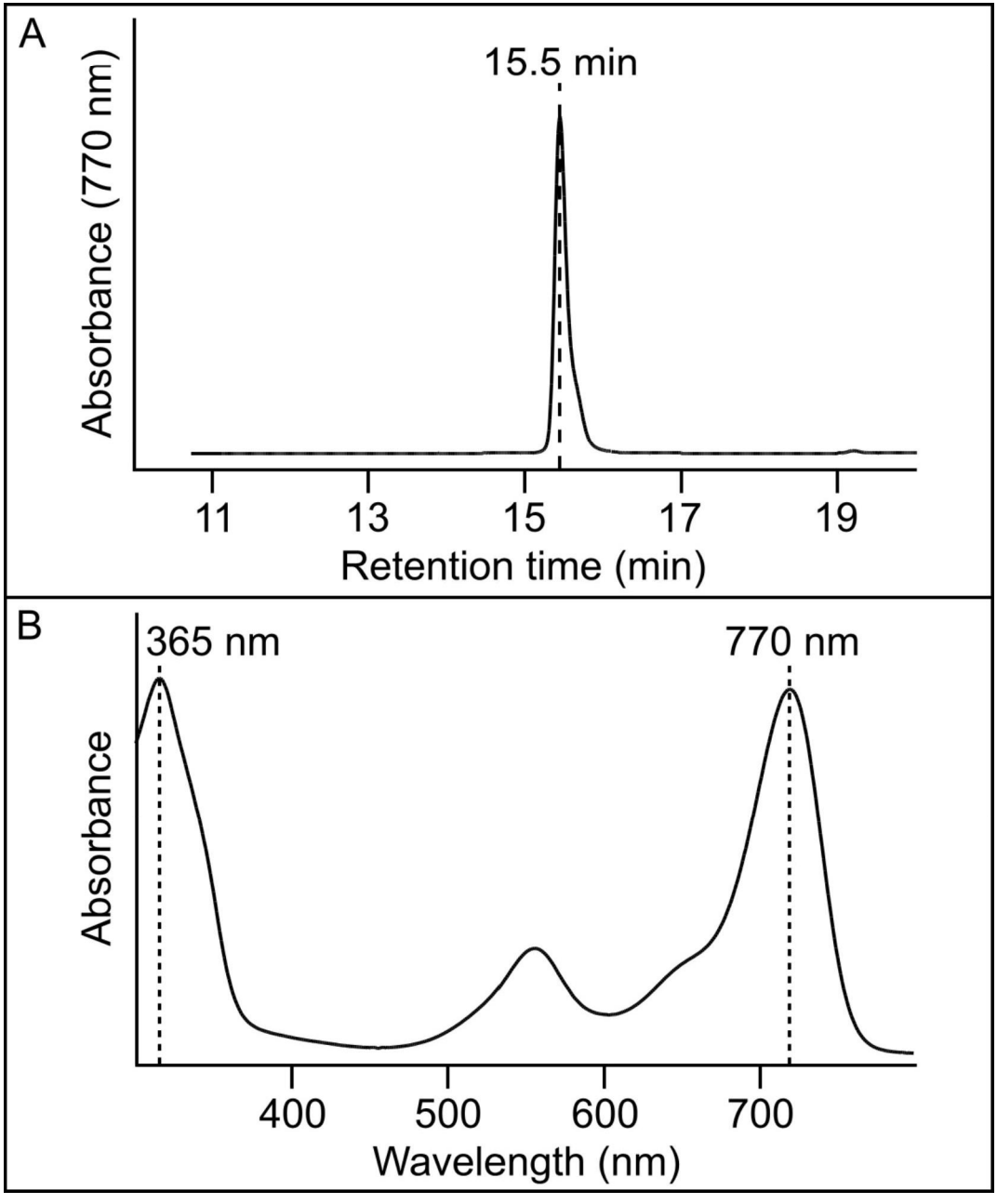
EVIDENCE OF APPROVAL OF PERSONAL COMMUNICATIONS
N/A

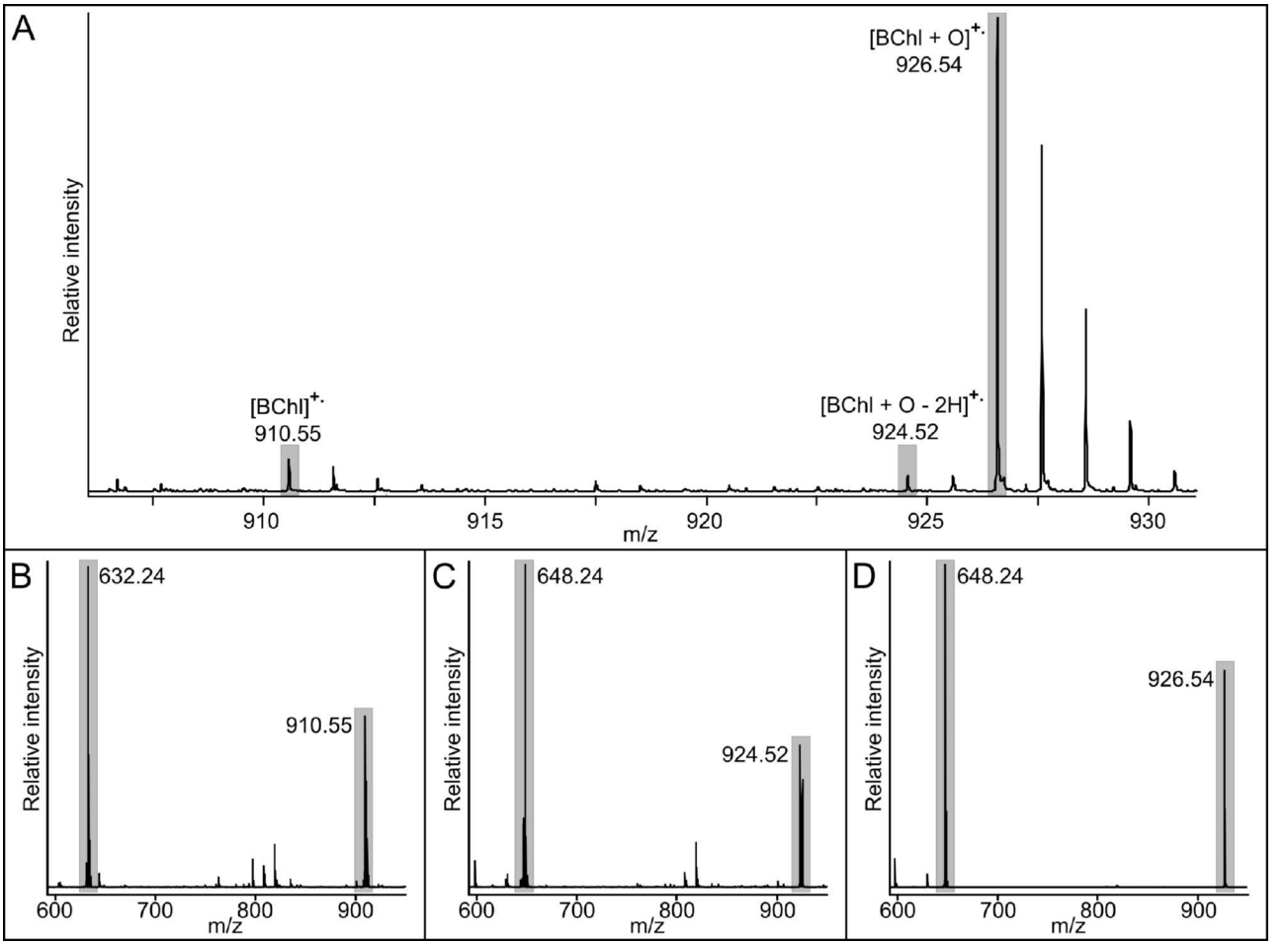
EVIDENCE OF SUBMISSION OF NUCLEIC ACID OR PROTEIN SEQUENCES TO AN APPROPRIATE DATA BANK
N/A

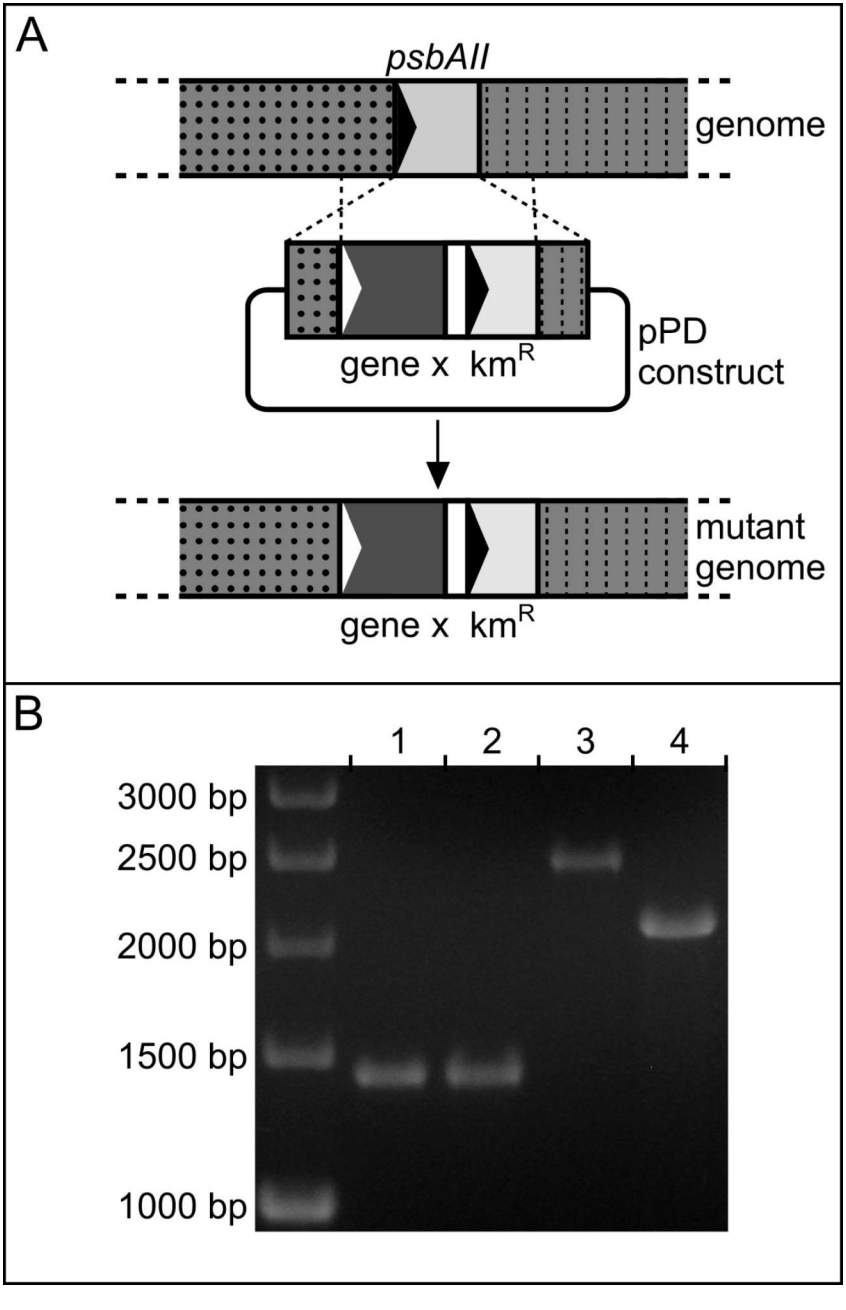

A
B
C
Mg
N
O
X
R
GGPP
DHGG
THGG
Phytol

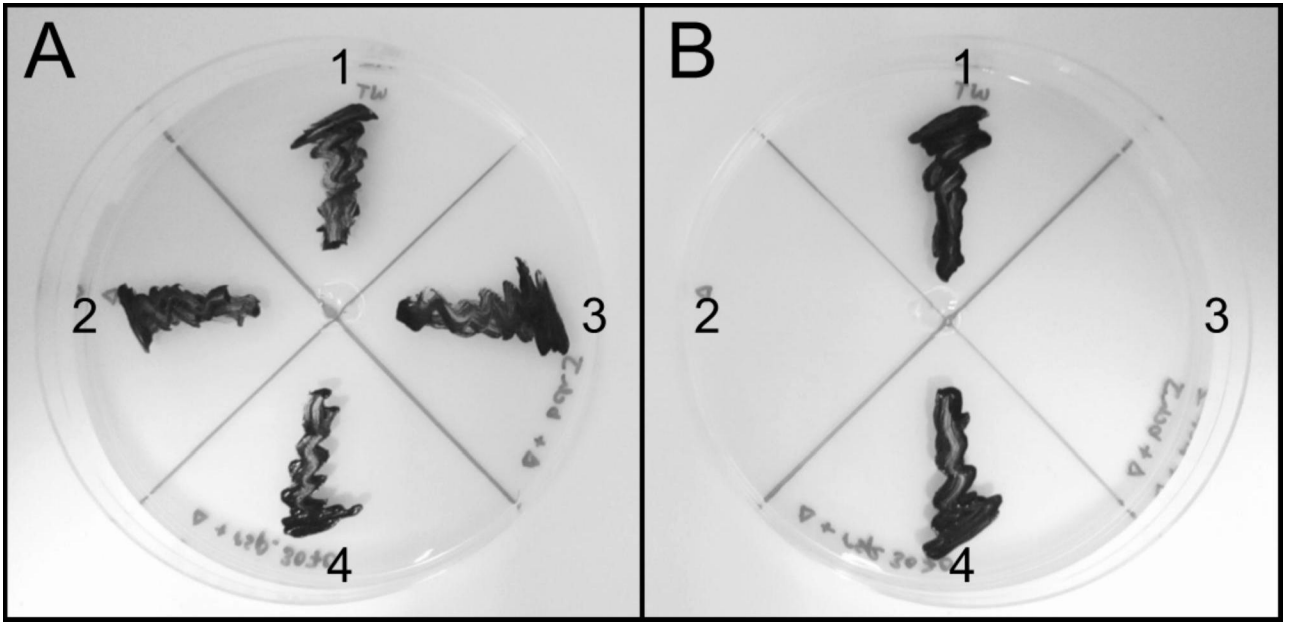
	Doubling time (h)	
Strain	10 µmol photons·m^{-2}·s^{-1}	90 µmol photons·m^{-2}·s^{-1}
WT	17.3 (± 1.4)	5.9 (± 0.2)
Δrsp_3070	15.2 (± 3.5)	6.2 (± 0.1)
ΔbchJ	—	—
Δrsp_3070/Δ*bchJ*	—	—


434 nm
Absorbance
350
550
750
Wavelength (nm)
441 nm
Absorbance
350
550
750
Wavelength (nm)
45.5 min
46.6 min
A
B
C
D
E
Relative absorbance (632 nm)
42
44
46
48
50
Retention time (min)

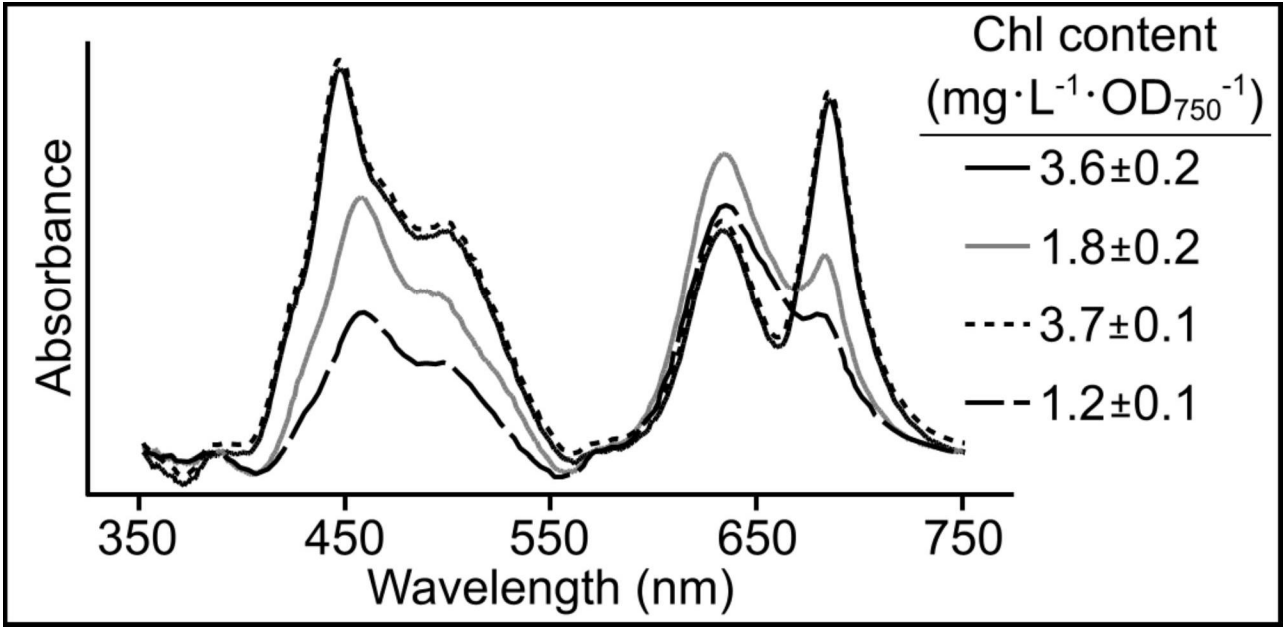

A
15.5 min
Absorbance (770 nm)
11
13
15
17
19
Retention time (min)
B
365 nm
770 nm
Absorbance
400
500
600
700
Wavelength (nm)

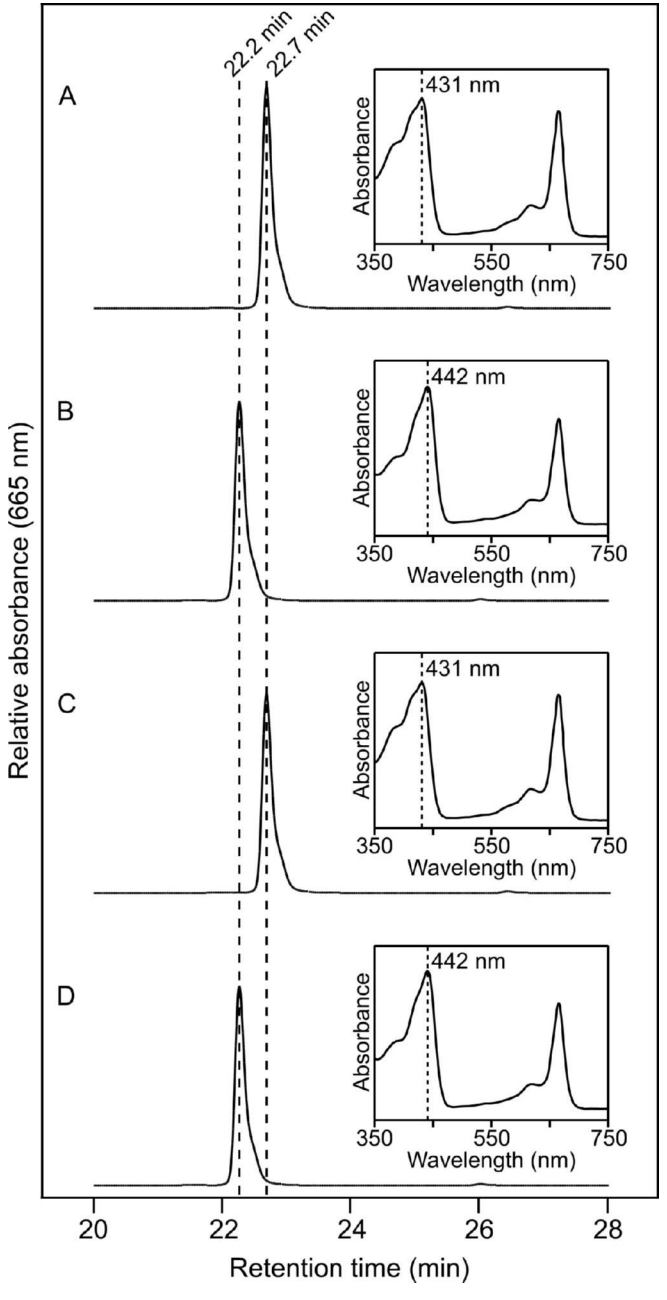

A
Relative intensity
[BChl + O]+·
926.54
[BChl]+·
910.55
[BChl + O - 2H]+·
924.52
910
915
920
925
930
m/z
B
632.24
910.55
Relative intensity
600
700
800
900
m/z
C
648.24
924.52
Relative intensity
600
700
800
900
m/z
D
648.24
926.54
Relative intensity
600
700
800
900
m/z


A
psbAII
genome
pPD
construct
gene x km^R
mutant
genome
gene x km^R
B
1
2
3
4
3000 bp
2500 bp
2000 bp
1500 bp
1000 bp


A
1
2
3
4
B
1
2
3
4

	50 µmol photons·m^{-2}·s^{-1}		250 µmol photons·m^{-2}·s^{-1}	
Strain	Doubling Time (h)	8-vinyl Chl *a* (%)	Doubling Time (h)	8-vinyl Chl *a* (%)
WT	16.9 (± 1.2)	11.8 (± 4.4)	13.7 (± 1.9)	0
Δslr1923	18.5 (± 0.4)	100	—	—
Δslr1923::rsp_3070	15.2 (± 2.0)	13.4 (± 1.5)	13.9 (± 1.4)	0
Δslr1923::*bchJ*	24.0 (± 0.9)	100	—	—


Chl content
(mg·L-1·OD750-1)
3.6±0.2
1.8±0.2
3.7±0.1
1.2±0.1
Absorbance
350
450
550
650
750
Wavelength (nm)


22.2 min
22.7 min
A
B
C
D
431 nm
442 nm
Absorbance
350
550
750
Wavelength (nm)
Relative absorbance (665 nm)
20
22
24
26
28
Retention time (min)