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Cloning, sequencing and functional assignment of the chlorophyll biosynthesis gene, *chlP*, of *Synechocystis* sp. PCC 6803

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Abstract A gene from the cyanobacterium *Synechocystis* sp. PCC 6803 has been cloned and sequenced, and subsequently used to partially complement a *bchP* mutant of the purple photosynthetic bacterium *Rhodobacter sphaeroides*. This mutant is blocked in the terminal hydrogenation steps of bchl*a* biosynthesis and possesses only bchl esterified with geranylgeraniol. It also has a reduced cellular level of the light-harvesting LH2 complex, and the 850 nm absorbance maximum of LH2 is red-shifted by approximately 6 nm. Upon heterologous expression of the *Synechocystis bchP* homologue, not only are hydrogenated forms of bchl*a*GG detectable, but the level of LH2 is increased and the red-shift reversed by several nm. We conclude that this gene, which we term *chlP*, encodes the enzyme catalysing the stepwise hydrogenation of geranylgeraniol to phytol during chl*a* biosynthesis.

Key words: Photosynthesis; (Bacterio)chlorophyll; *Synechocystis*; *Rhodobacter*; Hydrogenase; Phytol

1. Introduction

The biosynthesis of chlorophyll (chl) and bacteriochlorophyll (bchl) molecules is essential for the formation of photosynthetic complexes. The final stage in the synthesis of chl*a* and bchl*a* is the esterification of the tetrapyrrole moiety, chlorophyllide or bacteriochlorophyllide respectively, with an alcohol. The commonest of these esterifying alcohols is the C_{20} isoprenoid, phytol. However, principally as a result of kinetic studies analysing the accumulation and loss of chlorophyll esters, it has become apparent that phytol is often not the initial esterifying alcohol. Rather, the tetrapyrrole is esterified with an activated form of the biosynthetic precursor of phytol, geranylgeranyl pyrophosphate (GGPP), which subsequently undergoes successive reductions of three of its four C-C double bonds to generate the final pigment (Fig. 1). This is probably true of bchl*a* biosynthesis [1,2]. In plants, it seems that this pathway [3–5], and the direct phytylation route involving the attachment of phytyl pyrophosphate (PPP) to chlorophyllide, may exist side by side, with different pathways predominating at different stages of development or with different substrate availabilities [6–8]. A GGPP reductase catalysing the conversion of free GGPP to PPP has been located in the chloroplast envelope and has been shown to produce more PPP than is required for the competing reactions of tocopherol and phylloquinone synthesis [6]. The hydrogenase catalysing the stepwise reduction of the GG side-chain of chl*a* to P has been shown to be located in the thylakoid membrane [6], as has the enzyme activity, termed chlorophyll synthetase, which esterifies chlorophyllide with either GGPP or PPP [6,9,10].

A number of steps in the magnesium branch of tetrapyrrole biosynthesis have been elucidated through overexpression of the relevant genes in *E. coli*. This was reported for the methyltransferase gene, *bchM*, of *Rhodobacter capsulatus* [11] and *R. sphaeroides* [12], and subsequently it was shown that the genes *bchH*, *I*, and *D* encode subunits of magnesium chelatase, using the same approach [13]. More recently, this has been extended to the assignment of the function of *chlH*, *I* and *D* in the chl*a*-containing cyanobacterium *Synechocystis* sp. PCC 6803 [14]. All of these studies built upon earlier work on bchl biosynthesis initiated in purple photosynthetic bacteria, such as the assignments of gene function from the construction of bchl-deficient mutants by insertional mutagenesis (for example [11,15]). Such mutants are also useful as recipients for DNA from organisms other than *Rhodobacter* so that gene assignments can be made in these other organisms. For example, fragments of DNA from *Synechocystis* may restore pigment synthesis to bchl-deficient mutants of *Rhodobacter*. Such a complementation approach, which relies on functional homology rather than sequence homology, was used by Suzuki and Bauer [16] to isolate the gene encoding the light-driven protochlorophyllide reductase from *Synechocystis* by complementation of *bchN*, *bchB* and *bchL* mutants of *R. capsulatus*. In this study, we report the cloning and sequencing of a gene from *Synechocystis*, and its subsequent use to partially complement a *bchP* mutant of *R. sphaeroides*. This mutant is blocked in the terminal hydrogenation steps of bchl*a* biosynthesis and possesses only bchl esterified with GG. Also, the cellular level of the light-harvesting LH2 complex is significantly reduced and its B850 absorbance maximum is red-shifted by approximately 6 nm. Upon heterologous expression of the *Synechocystis bchP* homologue, not only are hydrogenated forms of bchl*a*GG detectable, but the level of LH2 is increased and the red shift reversed by several nm. We conclude that this gene, which we term *chlP*, encodes the enzyme catalysing the stepwise hydrogenation of GG to P during chl*a* biosynthesis. This may occur either at the activated, pyrophosphate level or following chlorophyllide esterification.

2. Materials and methods

2.1. Isolation of a bchP homologue from Synechocystis

The degenerate oligonucleotides 5′-GGGAATTCC(AGCT)-

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Abbreviations: bchl, bacteriochlorophyll; chl, chlorophyll; GG, geranylgeraniol; GGPP, geranylgeranyl pyrophosphate; P, phytol; PPP, phytyl pyrophosphate; DHGG, dihydro-GG; THGG, tetrahydro-GG; LH1, light-harvesting complex 1; LH2, light-harvesting complex 2; HPLC, high-pressure liquid chromatography; ORF, open reading frame

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GA(CT)TT(CT)TA(CT)GG(AGCT)TGGGT(AGCT)TT-3′ and 5′-GGTCTAGAGC(AG)(AT)A(AG)TA(AGT)AT(AGCT)CC(CT)TC-(AGCT)CC-3′ were designed according to the regions of derived amino acid similarity between orf391 (*bchP*) of the *R. capsulatus* photosynthetic gene cluster (J.E. Hearst, EMBL sequence submission, accession number Z11165; [2]) and an expressed sequence tag from *Arabidopsis thaliana* identified via its homology to *bchP*. These were used to amplify a 284 bp fragment from genomic DNA of *Synechocystis* sp. PCC 6803 by PCR. This was sequenced and the deduced amino acid sequence was found to display significant similarity to each of the original sequences.

A *Synechocystis* sp. PCC 6803 genomic DNA library constructed in vector Lambda GEM-11 (Promega) was plated out in *E. coli* strain KW251 (Promega). The library was screened using the amplified *Synechocystis* fragment, and a genomic clone of approximately 20 kb was isolated. Restriction digestion and probing of Southern blots were used to identify a *Xho*I-*Hin*dIII fragment of approximately 3.5 kb which contained the previously amplified sequence. This fragment was subcloned into pBluescript II SK+ (Stratagene), for sequencing.

2.2. DNA sequencing

This was carried out on an ABI 373A sequencer using an ABI DyeDeoxy reagent kit (Perkin Elmer).

2.3. Complementation of a R. sphaeroides mutant

The oligonucleotides 5′-TTTCTCTAGAGGAGACGACCAT-ATGGTATTACGGGTAGCAGTCGTTG-3′ and 5′-GTTGAAGC-TTGGATCCTTAAGGGGCTAAAGCGTTACCCCG-3′ were used to amplify the *chlP* gene by PCR, and introduce *Xba*I and *Hin*dIII sites which enabled cloning into pRKEB, yielding plasmid pEBchlP. Also, the start codon (underlined) was mutated from TTG to ATG because ATG is the most frequent start codon in both *E. coli* and *R. sphaeroides*. Plasmid pRKEB consists of the *Eco*RI-*Bam*III fragment of pRKEK [17], containing the strong *puf* operon promoter, cloned into pRK415 [18]. Both pRKEB and pEBchlP were transformed into *E. coli* strain S17-1 [19], permitting conjugative transfer of each of the plasmids into the *bchP* transposon Tn*5* mutant T6G5 of *R. sphaeroides* strain NCIB 8253 (Addlesee and Hunter, unpublished). *R. sphaeroides* strains were grown in M22+ medium [20]. For *R. sphaeroides*, antibiotic concentrations were neomycin 20 μg ml and tetracycline 1 μg/ml

2.4. Pigment extractions and high pressure liquid chromatography

Pigments were extracted from dark, semi-aerobically grown cultures of *R. sphaeroides* using acetone/methanol 7:2 (v/v). Bchl*a* esters were separated by HPLC on a Merck LiChrospher 100 RP-18 column (250 x 4 mm, i.d.). The flow rate was 1 ml/min with an initial composition of 64% methanol, 16% acetone, 20% water, changing over the course of 10 min to 80% methanol, 20% acetone. This buffer composition was maintained for 15 min, during which time the bchl*a* esters were eluted. Elution of bchls was monitored using a Waters 996 photodiode array detector, scanning from 350 to 800 nm every 2 seconds. The chromatograms at 365 nm were derived from the accumulated absorbance scans using the Millenium software [Waters]. Using this system, bchl*a*GG eluted at approximately 18.4 min, bchl*a*DHGG at approximately 19 min, bchl*a*THGG at approximately 19.8 min, and bchl*a*P at approximately 20.7 min.

2.5. Whole cell absorbance spectroscopy

Absorbance spectra of dark, semi-aerobically grown cultures at identical cell densities were obtained on a Guided Wave Model 260 spectrophotometer.

3. Results and discussion

3.1. Sequence of the chlP gene

Fig. 2A shows the sequence of the *Synechocystis* DNA isolated and sequenced in this study. It contains an ORF of 1224 bp which is preceded by a putative ribosome binding sequence (AGGAG). The start codon is TTG, a feature which is shared with the *frxC* (*chlL*) gene of the same organism [21]. The predicted product of this gene has 37% identity with the product of the *R. capsulatus bchP* gene (Fig. 2B). We have therefore designated this *Synechocystis* gene *chlP*. The predicted protein has a molecular weight of 44 869.

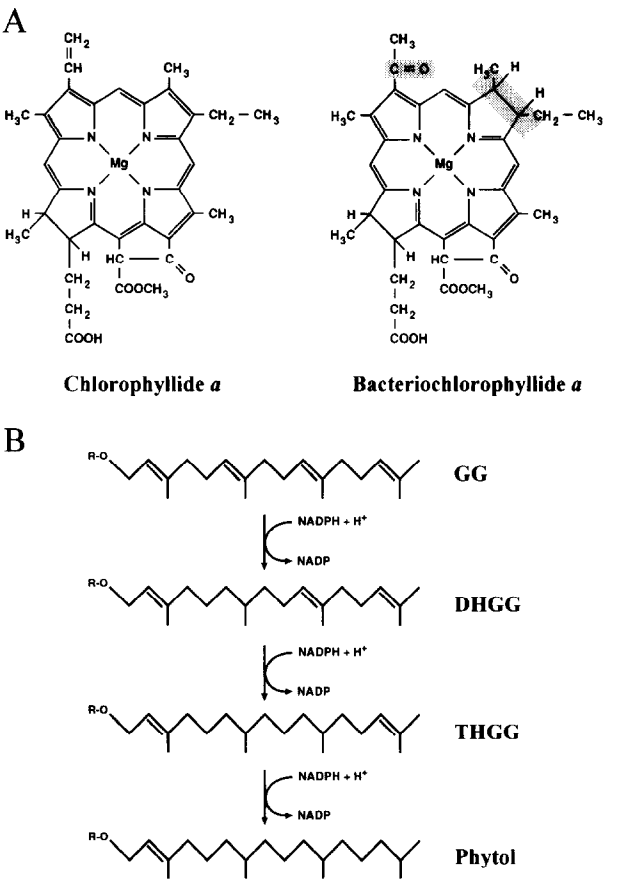

Fig. 1. (A) Structures of chlorophyllide *a* and bacteriochlorophyllide *a*. The reduced B ring and the hydrated 2-vinyl substituent of bacteriochlorophyllide are shaded. (B) The terminal hydrogenation steps of (bacterio)chlorophyll biosynthesis. R represents either chlorophyllide, bacteriochlorophyllide or a diphosphate group.

3.2. Expression of the chlP gene in a R. sphaeroides bchP mutant, and analysis of the resulting phenotype

The PCR-amplified *chlP* gene was cloned into pRKEB, generating plasmid pEBchlP. When this was introduced into the *R. sphaeroides bchP* mutant T6G5, the bchl*a* ester composition, as determined by HPLC analysis of acetone/methanol extracts of whole cells, was markedly altered (Fig. 3). In the presence of pRKEB alone, the composition was unchanged from that in the absence of an expression vector, the only detectable bchl*a* ester being bchl*a*GG. In contrast, plasmid pEBchlP partially restored the pigment composition to wild type, with significant levels of bchl*a*P (approx. 5.8%) being present. A similar proportion of a pigment identified as the second partially hydrogenated intermediate, bchl*a*THGG, was also detected. The principal esters present, however, were identified as bchl*a*GG and the first partially hydrogenated intermediate, bchl*a*DHGG, with the proportion of the latter pigment being slightly greater.

Whole cell absorption spectra of the bchl*a*GG-containing mutant T6G5, with or without pRKEB present (Fig. 4A), show lowered cellular levels of LH2 in comparison with the wild type (Fig. 4C). Furthermore, the B850 peak is red-shifted by approximately 6 nm. Such effects are not unexpected, since

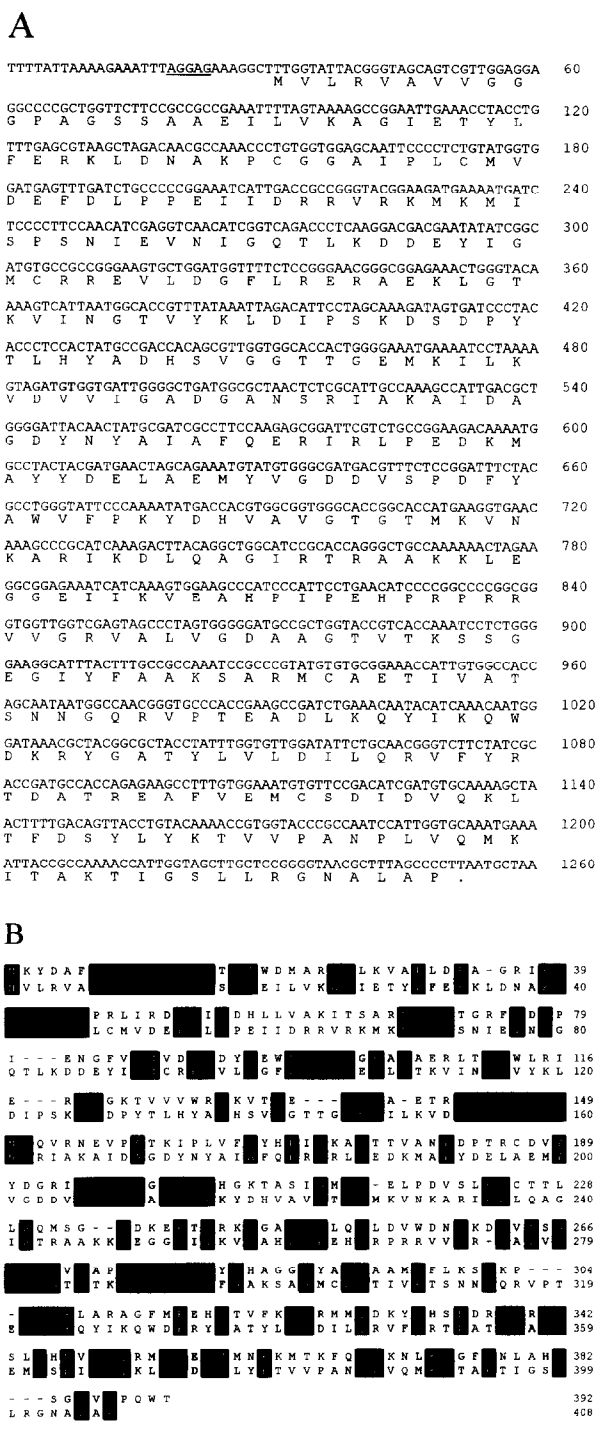

Fig. 2. (A) DNA sequence of the *Synechocystis chlP* gene, and the deduced protein sequence. A putative ribosome binding sequence is underlined. The sequence reported in this paper has been assigned the accession number X97972. (B) Protein sequence alignment between *R. capsulatus* BchP (top) and *Synechocystis* ChlP (bottom). Alignments were performed using the Clustal V method in the Lasergene software (DNAStar).

the phytol moiety of the bchl constitutes 30% of the molecular weight of the molecule, and it also greatly influences the properties of the chlorophyll molecule as a result of the increase in hydrophobicity (lipophilicity) brought about by the attachment of GG, followed by the reduction of the alcohol sidechain. The phytol group plays a crucial role in the stability and function of pigment-protein complexes, and it is known that the assembly process cannot be completed without the attachment of GG [22]. The crystallography carried out on the bacterial reaction centre and light-harvesting complexes has provided an abundance of structural information [23,24], so that it is possible to examine the conformation adopted by each phytol chain in atomic detail. In the case of the reaction centre, it is clear that in the 'active' branch

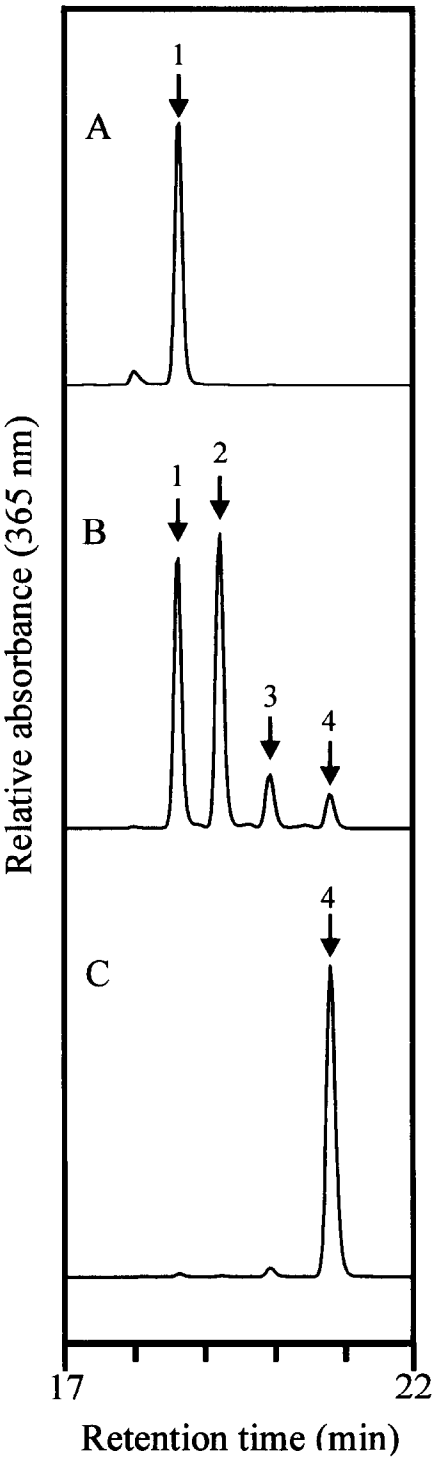

Fig. 3. HPLC traces of acetone/methanol extracts from (A) *R. sphaeroides bchP* mutant T6G5[pRKEB], (B) T6G5[pEBchlP], and (C) wild type *R. sphaeroides*. Labelled peaks are (1) bchl*a*GG, (2) bchl*a*DHGG, (3) bchl*a*THGG and (4) bchl*a*P.

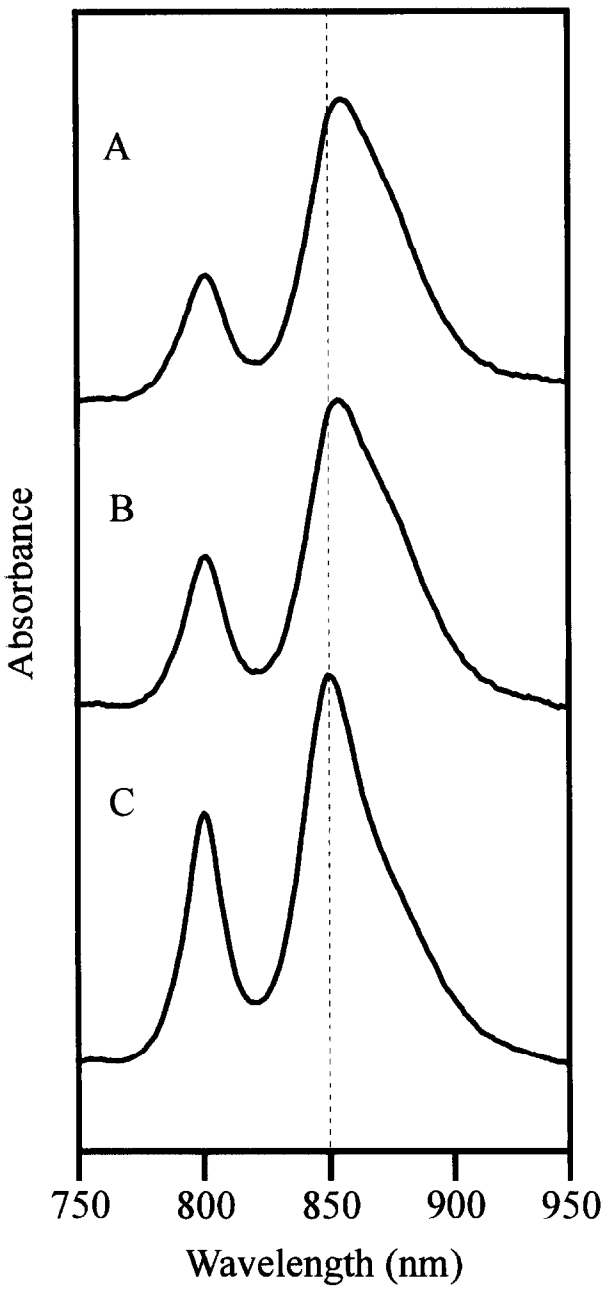

Fig. 4. Whole cell absorption spectra of (A) *R. sphaeroides bchP* mutant T6G5[pRKEB], (B) T6G5[pEBchlP], and (C) wild type *R. sphaeroides*.

the phytols are far more tightly packed than in the inactive branch. In LH2, it appears that the close intertwining of the phytol tails of the B800 and B850 bchls imparts a significant amount of stability to the complex. The tails also play a major role in controlling the orientation of the transition dipoles of the tetrapyrrole rings, which is crucial for fast energy transfer.

Some of the red shift seen in mutant T6G5 could arise from the enhanced contribution from the LH1 complex, which forms a shoulder on the main LH2 peak at approximately 875 nm. The separate effects of this alteration in bchl pigment on LH2 and LH1 have not been analysed so far. The heterologous expression of *Synechocystis chlP* in mutant T6G5 had a significant impact on the composition of the pigment-protein complexes: with *Synechocystis chlP* present, there is a greater abundance of LH2, and the red shift of mutant T6G5 with respect to the wild type is decreased by approximately 2 nm (Fig. 4B).

3.3. Concluding remarks

We have shown that *Synechocystis* possesses an ORF displaying significant similarity at the amino acid level with the *bchP* locus of *R. capsulatus*, which is known to catalyse the terminal hydrogenation steps of bchl biosynthesis [2]. We have further demonstrated that this ORF, which we have termed *chlP*, is capable of at least partially complementing a *bchP* mutant of *R. sphaeroides*. This mutant normally only accumulates bchl*a*GG. Upon heterologous expression of *chlP* the partially hydrogenated intermediate, bchl*a*DHGG, was detected in abundance, as well as small but significant quantities of bchl*a*THGG and phytylated pigment. A shift towards normality is also demonstrated by whole cell absorption spectra. We conclude, therefore, that *chlP* encodes a hydrogenase enzyme which, in its native environment, is responsible for catalysing the terminal hydrogenation stage of chl*a* biosynthesis, and that this stage may proceed in the same manner, via two partially hydrogenated intermediates, as that of bchl*a* biosynthesis. This, therefore, constitutes a functional assignment of the *chlP* gene of *Synechocystis*.

A three-step hydrogenation mechanism has been previously proposed, principally on the basis of kinetic evidence, for the terminal stages of both chl*a* and chl*b* biosynthesis in higher plants [3–5] (see Fig. 1B). However, the enzyme activity of oat seedlings thought to be responsible for the esterification of chlorophyllide *a*, termed chlorophyll synthetase, has been shown to accept PPP as a substrate in vitro [9]. Furthermore, an enzyme catalysing the conversion of free GGPP to PPP is present in chloroplasts [6]. It is possible, therefore, that the *Synechocystis* enzyme cloned in this study is involved in the hydrogenation of free GGPP, rather than that already incorporated in a chl molecule. It is interesting to note that the bchl precursor, bacteriochlorophyllide, is a very poor substrate for chlorophyll synthetase in vitro [25]. This specificity is due to the hydrogenated nature of the B ring of bacteriochlorophyllide preventing its binding to chlorophyll synthetase (see Fig. 1A). The fact that the ChlP enzyme does display considerable sequence homology to BchP of *R. capsulatus* and *R. sphaeroides* (H.A. Addlesee and C.N. Hunter, unpublished results), which is encoded by a gene present within the photosynthetic gene cluster amongst the other bchl biosynthesis genes, is strongly supportive of the view that the cyanobacterial enzyme we have identified is that which normally catalyses the hydrogenation steps in vivo, whether the hydrogenation occurs before or after esterification, or perhaps both.

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