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Effects of Modified Phycobilin Biosynthesis in the Cyanobacterium Synechococcus sp. Strain PCC 7002

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The pathway for phycocyanobilin biosynthesis in Synechococcus sp. strain PCC 7002 comprises two enzymes: heme oxygenase and phycocyanobilin synthase (PcyA). The phycobilin content of cells can be modified by overexpressing genes encoding alternative enzymes for biliverdin reduction. Overexpression of the pebAB and HY2 genes, encoding alternative ferredoxin-dependent biliverdin reductases, caused unique effects due to the overproduction of phycocyanobilin and phytochromobilin, respectively. Colonies overexpressing pebAB became reddish brown and visually resembled strains that naturally produce phycoerythrin. This was almost exclusively due to the replacement of phycocyanobilin by phycoerythrobilin on the phycocyanin α-subunit. This phenotype was unstable, and such strains rapidly reverted to the wild-type appearance, presumably due to strong selective pressure to inactivate pebAB expression. Overproduction of phytochromobilin, synthesized by the Arabidopsis thaliana HY2 product, was tolerated much better. Cells overexpressing HY2 were only slightly less pigmented and blue-green than the wild type. Although the pcyA gene could not be inactivated in the wild type, pcyA was easily inactivated when cells expressed HY2. These results indicate that phytochromobilin can functionally substitute for phycocyanobilin in Synechococcus sp. strain PCC 7002. Although functional phycobilisomes were assembled in this strain, the overall phycobiliprotein content of cells was lower, the efficiency of energy transfer by these phycobilisomes was lower than for wild-type phycobilisomes, and the absorption cross-section of the cells was reduced relative to that of the wild type because of an increased spectral overlap of the modified phycobiliproteins with chlorophyll a. As a result, the strain producing phycobiliproteins carrying phytochromobilin grew much more slowly at low light intensity.

Most cyanobacteria employ light-harvesting antennae known as phycobilisomes (PBS) to collect light that is not efficiently absorbed by chlorophyll (Chl) for photosynthesis. PBS are multisubunit, supramolecular structures composed of both pigmented phycobiliproteins (PBPs) and usually nonpigmented linker proteins (14). Four different linear tetrapyrrrole chromophores (bilins), phycocyanobilin (PCB), phycorythrobilin (PBR), phycoviolobilin (PVB), and phycourobilin (PUB), can naturally be bound to cyanobacterial PBPs (28). These four bilins are isomers that differ only in the number of conjugated double bonds that form the chromophore, and all are derived from a common biosynthetic precursor, biliverdin IXα (2–4, 8, 19, 49). Biliverdin IXα is synthesized from heme by oxidative cleavage of the α-methylene bridge of heme by the enzyme heme oxygenase (11). PCB:ferredoxin oxidoreductase, PcyA, uses four electrons from reduced ferredoxin to synthesize PCB by regio-specific reduction of the exo vinyl group of ring D and the endo vinyl group of ring A of biliverdin IXα (19). The reactions leading to the conversion of heme into biliverdin IXα, PCB, and PEB are shown in Fig. 1. PEB is synthesized in a similar manner from biliverdin IXα by the sequential actions of two reductases, PheA and PheB, or the more recently discovered cyanoviral enzyme PheS, which catalyzes the same reactions performed jointly by PheA and PheB (Fig. 1) (12, 19). The

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to produce an isomerizing or nonisomerizing lyase, which then dictates the conjugation state, and thus the absorption properties, of an attached chromophore (16).

Phytochromobilin (P\(_{\text{B}}\)), the chromophore of higher-plant photosensory proteins known as phytochromes, is similar in structure to PCB, but no cyanobacterium has been shown to synthesize this bilin naturally. P\(_{\text{B}}\) biosynthesis from biliverdin in plants, does not occur naturally in cyanobacteria.

Properly assembled PBPs are important to cyanobacterial light harvesting for growth. Because of the variety of possible chromophores that may be found in a particular cell type, it has largely been assumed that the bilin lyases responsible for the attachment of chromophores to their cognate apoprotein must exhibit a high degree of specificity in the recognition of their appropriate bilin chromophore and its acceptor apoprotein. PBP lyases have been most completely defined in the cyanobacterium Synechococcus sp. PCC 7002 (34). The CpcE/CpcF heterodimeric lyase is solely responsible for the attachment of PCB to CpcA (51). The CpcT attaches PCB to the Cys153 site on CpcB (35), while the CpcS/CpcU heterodimer attaches PCB to the remaining site on CpcB (Cys82) and to the single chromophore-bind- ing sites on ApcA, ApcB, ApcD, and ApcF (7, 33, 36). Cyanobacterial species that also produce PE are thought to have additional PEB-specific lyases that catalyze chromophore attachment to these proteins, but these lyases have remained largely uncharacterized (23, 46, 50).

The studies here report the effects of genetic alterations of chromophore content on PBPs through the overexpression of HY2 and pebAB and the insertional inactivation of pcyA in a cyanobacterial strain that naturally possesses only pcyA as its sole ferrodoxin-dependent biliverdin reductase.

**MATERIALS AND METHODS**

**Cyanobacterial growth conditions.** Medium A (medium A containing 12 mM sodium nitrate) was routinely used for growth of both wild-type (WT) and mutant strains of Synechococcus sp. strain PCC 7002 (40). Unless otherwise specified, cells were grown under standard conditions, which are 38°C, 250 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\) and with sparging with air supplemented with 1% (vol/vol) \(\text{CO}_2\). A glycerol-tolerant strain of Synechococcus sp. strain PCC 7002 was generated through the continuous growth of the wild-type strain in liquid A medium containing 10 mM glycerol and was used as a background for the generation of the pentAB and HY2 overexpression strains (20). Before, during, and after transformations, cells were cultivated at approximately 200 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\) of cool white fluorescent light.

**Construction of bilin biosynthesis mutants.** To inactivate the pcyA gene of Synechococcus sp. strain PCC 7002, regions of approximately 600 bp immediately upstream and downstream of the pcyA gene were amplified by PCR with primers pcyAR1F (5'-GCTGTCCTACGCGGTCG-3'; Nsi site underlined) and pcyAR2R (5'-GGACCAAGAGCGACGTTAG-3'; Nco site underlined) for the upstream sequence and pcyAR2F (5'-ATTACGCGTCGTCGTACGACAAGGA-3'; Nco site underlined) and sal-aadr (5'-GGAGCGTCTGATAGCGCACTTATTCC-3'; SphI site underlined) for the downstream sequence. These fragments were digested with NcoI and ligated together. The resulting product was digested with SphI and NsiI and ligated into pJEM-72II (+) (Promega Corp, Madison, WI) to make pJEM-PCyAR1R2. The sal-aadr cassette from pSRA81 (20) was amplified by PCR with primers pcyAR1F and pcyAR2R to create a 2.3-kb linear DNA fragment for natural transformation into Synechococcus sp. strain PCC 7002. The primers pcyAR1F and pcyAR2R were also used to assess segregation of the pcyA and pcyA::aadA alleles at the pcyA locus.

To introduce pcyAB genes into plasmid pAQ1 of Synechococcus sp. strain PCC 7002, the pcyAB genes from Synechococcus sp. strain PCC 7335 were amplified by PCR with primers 7335pebAF (5'-GCTGTCCTACGCGGTCG-3'; Nsi site underlined) and 7335pebBR (5'-ATATGAGACGCTTATGCTCGAAAGATGCTTATACTTATT-3'; Nco site underlined) and by cloning into pAQ1cpcEx that had also been digested with XbaI and SalI. The resulting product was digested with XbaI and SalI and cloned into this modified pAQ1cpcEx vector (47), which was cut with NcoI and PstI.

**Transformation of Synechococcus sp. strain PCC 7002.** To inactivate the pebAB genes from Synechococcus sp. strain PCC 7335 were amplified by PCR with primers 7335pebAF (5'-GCTGTCCTACGCGGTCG-3'; Nsi site underlined) and 7335pebBR (5'-ATATGAGACGCTTATGCTCGAAAGATGCTTATACTTATT-3'; Nco site underlined) and by cloning into pAQ1cpcEx that had also been digested with XbaI and SalI. The resulting product was digested with XbaI and SalI and cloned into this modified pAQ1cpcEx vector (47). This plasmid allows for the congenic cloning and introduction of foreign genes, the expression of which is driven by the cpcA promoter of Synechocystis sp. strain PCC 6803, into the endogenous pcyA plasmid of Synechococcus sp. strain PCC 7002 by homologous recombination.

**Construction of foreign gene expression vectors.** The pcyA gene was also expressed on endogenous plasmid pAQ1 under the control of the Synechocystis sp. strain PCC 6803 pcyB promoter; however, due to the presence of the most conservative restriction sites (in particular Ndel, NcoI, and BamHI) in the HY2 gene itself, the pAQ1cpcEx vector was modified to facilitate the cloning of this gene. First, the gentamicin cassette from pMS266 (5) was amplified by using primers gentF (5'-ATATGAGACGCTTATGCTCGAAAGATGCTTATACTTATT-3'; Nco site underlined) and gentR (5'-GGACCAAGAGCGACGTTAG-3'; Nsi site underlined) for the upstream sequence and pcyAR2F (5'-GGACCAAGAGCGACGTTAG-3'; Nco site underlined) and sal-aadr (5'-GGAGCGTCTGATAGCGCACTTATTCC-3'; SphI site underlined) for the downstream sequence. The resulting amplicon was digested with NcoI and SalI and cloned into similarly digested pJEM-PCyAR1R2. The resulting plasmid was used as a template in a PCR using primers pcyAR1F and pcyAR2R to create a 2.3-kb linear DNA fragment for natural transformation of a glycerol-adapted strain of Synechocystis sp. strain PCC 6802. The primers pcyAR1F and pcyAR2R were also used to assess segregation of the pcyA and pcyA::aadA alleles at the pcyA locus.

During, and after transformations, cells were cultivated at approximately 200 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\) of cool white fluorescent light.
Spectroscopic and compositional analyses of bilin biosynthesis strains and their PBS. Absorption spectra of whole cells of *Synechococcus* sp. strain PCC 7002 and its isolated PBS were recorded with a GENESYS 10 spectrophotometer (ThermoElectronic, Rochester, NY).

Chl a was extracted using 100% methanol, and concentrations were determined according to previously described methods (27, 29). The relative PBP contents of cells were determined using heat-induced bleaching at 65°C as described previously (32, 48). Because the PBB chromophore has a more red-shifted absorption than the PCB chromophore, the method was modified slightly; the peak absorption maxima differences between the PBPs of the two strains were compared.

PBSs were isolated as described previously (35), and their protein contents were analyzed by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS). Proteins separated on a 14% (wt/vol) polyacrylamide gel were first stained with a 100 mM ZnCl2 solution to enhance the fluorescence of the bilins (6), which were visualized using a Typhoon 8600 variable mode imager (GE Healthcare Life Sciences, Pittsburgh, PA). Total proteins were then stained with Coomassie blue and imaged with an Epson Perfection V750 flatbed scanner. His-tagged preparations of *Synechocystis* sp. strain PCC 6803 holo-CpcA, chromophorylated with either PCB or PEB, were used for comparisons in these experiments. These recombinant proteins were heterologously expressed in *Escherichia coli* BL21(DE3) cotransformed with pBS414v (43) and either pPeA (7) for PCB-CpcA or pTDho1pebS (12) for PEB-CpcA and were isolated by metal chelation chromatography as described previously (43).

Fluorescence measurements of whole cells and isolated PBS were recorded with an SLM 8000C spectrofluorometer that was modernized for computerized data acquisition by On-Line Systems, Inc. (Bogart, GA). Whole-cell fluorescence at 77 K was measured by resuspending cells in 50 mM HEPES-NaOH buffer, pH 7.0, containing 60% (vol/vol) glycerol, and the mixtures were frozen in liquid nitrogen as described previously (35).

PBPs were analyzed by a high-performance liquid chromatography (HPLC) method similar to that previously described (41). Prior to HPLC separation, PBP samples were dialyzed against 10 mM sodium phosphate (pH 7.0), 1.0 mM 2-mercaptoethanol. The dialyzed protein solutions were diluted 1:1 (vol/vol) with 6 M guanidinium hydrochloride, pH 5.2, and centrifuged for 5 min prior to injection onto a C8 reversed-phase HPLC column (RP-304; 4.5 mm by 350 mm; Bio-Rad, Richmond, CA) that had previously been equilibrated with 65% trifluoroacetic acid (TFA; 0.1%) in water (buffer A), and 35% 2:1 acetonitrile-water (buffer B) linear gradient to 65% buffer A–35% buffer B; 37 to 45 min, linear gradient to 100% buffer B; 45 to 55 min, linear gradient to 65% buffer A–35% buffer B. HPLC was performed using Waters model 510 pumps and a model 600 automated gradient controller (Waters Chromatography Division, Milford, MA). Data were acquired using a Waters 299b photodiode array detector; spectra were collected from 220 to 700 nm at 1-s intervals.

RESULTS

Attempted insertional inactivation of *pcyA* in wild-type *Synechococcus* sp. strain PCC 7002. In order to gain a better understanding of the dual role of PCB in both light harvesting and light sensing in *Synechococcus* sp. strain PCC 7002, an attempt was made to produce a *pcyA* null mutant by insertional inactivation of the gene. As for other mutations that could potentially affect the photosynthetic capacity of this cyanobacterium, constructions to inactivate the *pcyA* gene were transformed into a glycerol-tolerant strain that could grow photomixotrophically on glycerol (20). Similar conditions were previously employed to produce mutants lacking PC and AF in this cyanobacterium (9). Colonies from the resulting transformation with the *pcyA::aadA* construct were initially indistinguishable from wild-type *Synechococcus* sp. strain PCC 7002 (Fig. 2A). However, upon several rounds of streaking and selection, the majority of the colonies were yellow-green (chlorotic), which was consistent with the inability to synthesize PBPs (Fig. 2B). Despite continued careful selection of colonies lacking the dark, blue-green pigmentation of the wild type, colonies continued sectoring. This suggested that wild-type alleles of *pcyA* continued to persist in the transformed cells. PCR analyses confirmed the presence of both the *pcyA* and *pcyA::aadA* alleles within the population (Fig. 2C), and full segregation of the *pcyA* and *pcyA::aadA* alleles was never observed. These observations suggested that under standard growth conditions the *pcyA* gene of *Synechococcus* sp. strain PCC 7002 is essential.

Attachment of PEB to CpcA in *E. coli* and *Synechococcus* sp. strain PCC 7002. Heterologous production of holo-PCB-CpcA in *E. coli* has previously been demonstrated (43). Holo-PEB-CpcA can also be produced when *pebS* is provided as an alternative ferredoxin-dependent biliverdin reductase and coexpressed with *cpcA* and the *cpcE*cpeF lyase in *E. coli*. This observation prompted a study to determine if this phenomenon could be replicated in a cyanobacterium, in which chromophory-
loration might be more tightly controlled because of the potential to cause deleterious effects on the photosynthetic capacities of cells. For this experiment, pebA and pebB, encoding 15,16-dihydrobili-
verdin:ferredoxin oxidoreductase and phycoerythrobilin:ferre-
doxin oxidoreductase, respectively, from the chromatically accli-
matizing cyanobacterium *Synechococcus* sp. strain PCC 7335 were placed under the control of the strong *cpcBA* promoter of *Synechocystis* sp. strain PCC 6803 and introduced into a neutral site on the endogenous *pAQ1* plasmid of *Synechococcus* sp. strain PCC 7002 (47). This construct was transformed into a glycerol-tolerant strain, which was grown mixotrophically with glycerol. As for the transformants containing the *pcyA-aadA* construct, transformants possessing the *pebAB* construct were initially indistinguishable from the wild type. However, after several successive rounds of selection and streaking, colonies began to show regions with a brownish to brick red color. By careful selection of colonies, it was eventually possible to iso-
late nearly uniformly reddish-brown colonies that resembled the colonies of cyanobacterial strains that naturally produce PE. Despite numerous attempts to grow this strain under vari-
ous green light intensities, which might favor cells with in-
creased green absorption, complete elimination of colonies with the blue-green coloration of the wild type was never completely achieved (Fig. 3A). The absorption spectrum of a typical transformant strain (Fig. 3B) revealed an additional absorption peak at about 556 nm (the absorption maximum of PE) and an apparent decrease in the relative absorption by PC at ~635 nm.

To assess the PBP composition of cells overproducing PebA and PebB, intact PBS were isolated. In order to have sufficient cell material for analysis, liquid cultures were started with a relatively high inoculum and were grown for a relatively short period (~40 h). Smaller starter inocula and/or longer incuba-
tion times tended to favor the appearance of revertants that resembled wild-type cells and lacked brown pigmentation. This might have been due to the accumulation of mutations that inactivated the expression of *pebA* and *pebB*, which would allow more rapidly growing, wild-type-like cells to predomi-
nate. Despite the instability of the genotype/phenotype, by keep-
ing the inocula large and the incubation times minimal, it was possible to grow cells that retained most of the phenotype that was visible on plates (Fig. 3A).

In order to examine the distribution of PEB among the major PBPs isolated from PBS purified from the *pebA pebB* and wild-type strains, PBPs were separated by analytical SDS-
PAGE alongside aliquots of purified recombinant His6-CpcA carrying PCB or PEB. The proteins were detected by both zinc-enhanced fluorescence and by staining with Coomassie blue (Fig. 4A). Using excitation lasers at 532 nm and 633 nm, the zinc-enhanced fluorescence was imaged on a Typhoon 8600 variable mode imager. The results were compared to those for proteins with known chromophore contents to assess qualitatively which polypeptides carried PEB chromophores in the PBS isolated from cells overproducing PebA and PebB. CpcA was significantly more fluorescent when scanned with the 532-nm laser than was the case for CpcA from the wild-
type strain, and the intensity of its fluorescence was similar to that of the recombinant His6-CpcA-PEB protein standard. These same PBP samples were subsequently analyzed by HPLC in order to confirm these results and to determine if PEB was incorporated into any of the other PBS components. When the absorbance profiles of the major PBP components of PBS isolated from the *pebA pebB* overexpression strain (Fig. 4C) were compared to those from the wild-type strain (Fig. 4B), only CpcA exhibited a major difference in absorption. A very small amount of PEB was also bound to the ApcA sub-
unit, but little or no PEB was incorporated into CpcB (i.e., the PC β-subunit). These results confirmed that PEB was mostly attached to CpcA and that very little PEB was incorporated into other PBP subunits that could be assembled into PBS.

**pcyA could be inactivated in a strain expressing HY2.** When the *Arabidopsis thaliana* HY2 gene was introduced into plasmid *pAQ1* of *Synechococcus* sp. strain PCC 7002 under the control of the very strong *cpcBA* promoter of *Synechocystis* sp. strain PCC 6803 (47), the transformed strain appeared slightly less blue, and thus slightly greener, than the WT (data not shown). An examination of the absorption spectra for this strain revealed that the absorption maximum for PC was red-
shifted by approximately 10 nm (Fig. 5). Absorption shifts of a similar magnitude, about 10 nm to a shorter wavelength, have typically been observed when phytochromes have been recon-
stituted with PCB instead of PΦB (15). Because of the very high expression levels of HY2 expected when driven by the *cpcBA* promoter, and the structural similarity of the resulting PΦB chromophore to the native PCB chromophore, it seemed possible that the large proportion of the PBPs in these cells might carry PΦB and not PCB chromophores. Previously, it was demonstrated that *pcyA* can largely complement a HY2-
deficient strain of *A. thaliana*; this indicates that the two chro-
mophores are nearly interchangeable in *A. thaliana* (24). Be-
cause this strain carried functional copies of both *pcyA* and

![FIG. 3. Overexpression of *pebA* and *pebB* in *Synechococcus* sp. strain PCC 7002. (A) Colonies of *Synechococcus* sp. strain PCC 7002 transformed with the *pebAB* overexpression cassette mostly exhibit a reddish-brown phenotype resembling strains that naturally synthesize PE. A sector that has reverted to the wild-type color phenotype can be seen (arrow). (B) Whole-cell absorption spectra of wild-type *PE*. A sector that has reverted to the wild-type color phenotype can be transformed with the *pebA* and *pebB* strain *PCC 7002* (solid line) and the strain overexpressing *pebAB* (dashed line).](image-url)
HY2, it was possible that sufficient PCB was available to allow the selective assembly of some PBPs with PCB, as was seen for the strain overexpressing pebA and pebB.

In order to determine if PΦB could serve as the only bilin chromophore for Synechococcus sp. strain PCC 7002, cells overproducing HY2 were transformed with the same pcyA::aadA construct previously used unsuccessfully in the attempt to inactivate pcyA in the wild type. In contrast to the previous results, the transformant colonies in which pcyA was inactivated in the HY2 overexpression background did not develop a chlorotic appearance, and segregation of the mutant and wild-type alleles was rapidly achieved (Fig. 6B). The absorption spectrum of the resulting strain was similar to that of the background strain (Fig. 5 and 6A). However, because no wild-type alleles of pcyA could be detected by PCR, it was likely that all of the PBPs produced in this strain carried PΦB rather than PCB chromophores.

Properties of cells and PBS derived from a pcyA mutant expressing HY2. In order to examine the effects of global changes in PBS comprised of PBPs in which PCB was completely replaced by PΦB chromophores, low-temperature (77 K) fluorescence emission spectra of cells were measured. Spectra for both the wild type and the strain overexpressing HY2 in a pcyA deletion background (ΔpcyA::aadA + HY2) were recorded with the excitation wavelength set to 590 nm, to excite mainly the PBPs (Fig. 7). For the wild type, the observed maxima at ~655 nm, ~670 nm, and ~688 nm represented emission from PC, AP, and the terminal emitters ApcD and ApcB.
ApcE, respectively (13, 37). As expected, based on the absorption profile of the \( \Delta \text{pcyA::aadA} + \text{HY2 strain} \), the replacement of all PCB chromophores with P\( \text{B} \) caused a red shift in the fluorescence emission of the PBPs in this strain. Fluorescence emission peaks for PC, AP, and ApcD/ApcE each shifted 12 to 14 nm, with the new maxima occurring at 665 nm, 681 nm, and 700 nm, respectively. Although the fluorescence intensity was lower for the mutant strain, the relative intensities of the three emission bands were similar for the mutant strain. This observation suggested that the relative amounts of PC, AP, and terminal emitters had not significantly changed in the PBS of the mutant strain.

The results described above for the \( \Delta \text{pcyA::aadA} + \text{HY2 strain} \) were further corroborated by analyses of the PBS from the mutant strain (Fig. 8). Zinc and Coomassie staining of the gel showed generally similar chromophore contents and similar relative abundances of the PBS components in the two strains (Fig. 8A and B). Although similar amounts of the CpcA and ApcB proteins were loaded on the gel, as evidenced by the Coomassie staining, the CpcB band from the \( \Delta \text{pcyA::aadA} + \text{HY2 strain} \) was much less focused and showed less signal when the same gel was zinc stained and visualized by fluorescence (Fig. 8A and B). This suggested that a portion of the CpcB subunit might be assembled into PBS with only a single P\( \text{B} \) chromophore. As expected, the absorbance and fluorescence emission maxima of the PBS isolated from the \( \Delta \text{pcyA::aadA} + \text{HY2 strain} \) were red-shifted relative to those of wild-type PBS (Fig. 8C and D). Wild-type PBS had an absorption maximum at 630 nm, while PBS from the \( \Delta \text{pcyA::aadA} + \text{HY2 strain} \) had an absorption maximum at 640 nm (Fig. 8C). The 77 K fluorescence emission of WT PBS exhibited maxima at 655 nm from PC and 683 nm from the terminal emitters ApcD and ApcE. The 77 K fluorescence emission spectrum of WT PBS exhibited maxima at 655 nm from PC and 683 nm from the terminal emitters ApcD and ApcE. The 77 K fluorescence emission spectrum of the PBS isolated from the \( \Delta \text{pcyA::aadA} + \text{HY2 strain} \) had red-shifted emission maxima at 668 nm and 694 nm. When PBS samples of equal maximal absorption were compared, the PBS from the \( \Delta \text{pcyA::aadA} + \text{HY2 strain} \) showed much less fluorescence emission from the terminal emitters. This result suggested that energy transfer to the terminal emitters was much less efficient in PBS produced from proteins carrying P\( \text{B} \) chromophores (Fig. 8D). Lastly, the absorption spectra of the PBS samples denatured in acidic urea were examined (Fig. 8E). As expected due to the larger number of conjugated double bonds in the P\( \text{B} \) chromophore, the absorption maximum of the denatured PBS was red-shifted relative to the absorption spectra of the native PBS.
PBS from the ΔpcyA::aadA + HY2 strain was red-shifted ~10 nm, from 664 nm for the WT to 674 nm for the mutant strain.

Comparisons of the PBP and Chl contents of the wild type and the ΔpcyA::aadA + HY2 strain revealed that this strain contained about 40% less PBPs than the wild type, while the Chl contents of the two strains remained similar (data not shown). Taken together, the fluorescence emission properties of isolated PBS indicated not only that the PBPs are less abundant in the ΔpcyA::aadA + HY2 strain, but also that they are less effective in transferring absorbed light energy to the terminal acceptors of the PBS. Thus, the ΔpcyA::aadA + HY2 strain should exhibit growth defects due to impaired light harvesting.

The apparent requirement for either PCB or PnP chromophores by Synechococcus sp. strain PCC 7002 allowed for the cultivation and further characterization of this strain in the absence of the antibiotics normally used to select for strains harboring the recombinant DNA constructs. Growth rate measurements were conducted to determine the effects of the altered bilin content on the light-harvesting capabilities of this strain. Growth rates were determined under standard growth conditions for Synechococcus sp. strain PCC 7002 (38°C, 1% [vol/vol] CO2 in air, nitrate as N-source) at three different light intensities: 50, 200, and 500 μmol photons m−2 s−1 (Fig. 9).

When light was limiting (50 μmol photons m−2 s−1), the doubling time for the ΔpcyA::aadA + HY2 strain was more than 2-fold longer than that of the wild type. At an intensity slightly less than saturating (200 μmol photons m−2 s−1), the doubling time for the ΔpcyA::aadA + HY2 strain was about 50% longer than the wild type. At a suprasaturating light intensity (500 μmol photons m−2 s−1), the growth rates for the two strains were virtually indistinguishable. These data showed that the ΔpcyA::aadA + HY2 strain was significantly impaired in light harvesting in comparison to the wild type but that the photosynthetic reaction centers were unaffected.

**DISCUSSION**

The inability to inactivate the pcyA gene in the genome of Synechococcus sp. strain PCC 7002 suggested that PcyA, and by extension its product, PCB, are normally required for cellular viability. This observation was surprising for several reasons. Previous studies had shown that it is possible to produce strains in which the genes for cpeBA and apcAB are deleted and the double mutant does not accumulate detectable levels of PBPs (9). In those studies, however, the apo-PBPs were not synthesized, and it remains a possibility that the additional back-selection pressure for retention of the capacity to synthesize the antennae prevented complete segregation of the pcyA and pcyA::aadA alleles in the experiments described here. An alternative explanation for these observations might be that there are other proteins that require PCB chromophores that are essential when cells are grown in continuous illumination. Candidates for such proteins include phytochrome and cyanobacteriochrome-like proteins encoded in the Synechococcus sp. strain PCC 7002 genome. While several of these are homologous to proteins characterized in Synecochystis sp. PCC 6803, neither their potential cognate chromophore nor the phenotype of mutants lacking the products of these genes has been investigated.

The requirement for PCB for purposes other than the chromophore for PBPs is further supported by the recent sequencing of the genome of the cyanobacterium UCYN-A, a marine cyanobacterium with a highly reduced genome (44). Although this genome no longer encodes any PBPs, it nevertheless has retained the pcyA gene and several genes for sensory proteins that may function as photoreceptors.

High-level expression of pebAB in the absence of PE apoproteins or associated linker polypeptides produced cells with a whole-cell absorption spectrum that resembled that of a PE-producing cyanobacterium (Fig. 3). Strains with this phenotype were extremely unstable, however, and could only be maintained by carefully choosing the most reddish-brown colonies, and even with this precaution, apparent phenotypic revertants (to blue-green coloration) were common (Fig. 3A). When cells were cultivated for more than about 2 days, liquid cultures rapidly reverted to wild-type pigmentation and absorption. One explanation for this is that hyperexpression of pebA and pebB might limit the production of PCB. Because PcyA and PebA utilize the same substrate, biliverdin IXα, severe overexpression of pcyA could limit the availability of PCB, which, as discussed above, appears to be required for viability when cells are grown in continuous light. Although
PCB in principle should still be produced, high levels of PEB might also competitively inhibit reactions in which PCB is the natural substrate. In vitro experiments using CpcA with its cognate CpcEF lyase have previously demonstrated that PEB can function as a competitive inhibitor for PCB addition (17). If PCB is required to produce a functional phytochrome-like cognate CpcEF lyase have previously demonstrated that PEB was not determined in the studies reported here. Attempts to use the CpcS/CpcU and CpcT lyases to introduce PEB into other PBP subunits in heterologous expression systems have resulted in much lower levels of addition than those seen for CpcE/CpcF/CpcA (A. Biswas and W. M. Schluchter, unpublished observations). This suggests that PBP lyases other than CpcE/CpcF are capable of distinguishing PCB from PEB.

PΦB, the chromophore associated with phytochromes of plants, has a double bond at the 15–16 position and only differs from PCB by the presence of an additional double bond at the C-18 position. Because of the structural similarity to the native PCB, it was expected that overexpression of PΦB synthase (HY2) would be much less detrimental to the cell than overexpression of pebA and pebB. Expression of HY2 was sufficient to overcome the apparent growth defect that prohibits the inactivation of pcyA in a wild-type background, indicating that PΦB is an acceptable substitute chromophore for PCB. Except for the lower growth rate of this strain at limiting light intensities, the replacement of PCB by PΦB seemed to produce no additional deleterious effects. This was reflected by the fact that the mutant and wild-type strains had nearly identical growth rates when the strains were grown at high light intensity (Fig. 9). Although not directly addressed in this study, it is also important to note that light sensing is likely altered in this strain. Phytochrome-like photosensory proteins that normally utilize PCB or PVB should bind PΦB or phytoviolobilin (the Δ5-to-Δ2 double-bond isomer of PΦB), although those that bind biliverdin are probably unaffected, because the cells can still synthesize biliverdin.

Previous studies on the PCs of red algae and marine *Synechococcus* sp. strains have shown that at least four alternative PCs occur (8, 38, 39). The red alga *Porphyridium cruentum* synthesizes an R-PC in which the β-155 Cys carries a PEB chromophore but the α-84 and β-84 positions carry PCB chromophores (22). In *Synechococcus* sp. WH8103, the β-84 position carries a PCB chromophore but the α-84 and β-155 positions carry PEB chromophores (31). The α-84 position of *Synechococcus* sp. WH8501 PC carries a PUB chromophore, while the two β-subunit cysteines carry PCB chromophores (42). Finally, there is a trichromatic type of R-PC found in *Synechococcus* sp. WH8102 that contains PUB on α-84, PEB at β-153, and PCB at β-82 (8). Interestingly, the configuration of one PEB on CpcA and two PCBs on CpcB, which occurs when pebAB is aberrantly expressed in *Synechococcus* sp. strain PCC 7002, is the same configuration reported to occur naturally on R-PC-III of *Synechococcus* sp. WH7805 (30). PCs exhibit the highest diversity in chromophore contents among PBPs. Examples are known that can bind each of the four naturally occurring cyanobacterial chromophores: PCB, PEB, PVB, and PUB (38). The β-84 position of CpcB invariably carries PCB, however. These observations, as well as data showing that PE subunits can accommodate PUB chromophores in place of PEB chromophores in strains exhibiting type IV chromatic acclimation (16), demonstrate that at least some PBP subunits can readily accommodate alternative chromophores.

Given that cyanobacteria have evolved to use four different bilin chromophores for light harvesting and that a single amino acid substitution can cause PcyA to lose its ability to catalyze the reduction of the C-18 double bond and thus produce PΦB (45), it is surprising that no naturally occurring PBPs with PΦB chromophores have yet been found in cyanobacteria. The results reported here suggest two reasons why this might be the case. First, PBPs with PΦB chromophores have absorption properties that overlap more strongly with Chl a, so PBPs carrying PΦB chromophores produce less additional absorption cross-section (other than an increase in total chromophore number) for the two photosystems. Additionally, the intrinsic spectroscopic properties of PΦB may not be well suited for energy transfer. This is suggested by the lower apparent quantum yield of fluorescence and the apparently less-efficient energy coupling of major PBPs to the terminal emitters in PBS from the *pcyA* mutant strain overproducing HY2. Further characterization of purified PBPs carrying PΦB will be required to determine whether energy transfer is inefficient because of altered chromophore-protein interactions or whether some other property of PΦB limits the energy transfer efficiency of this chromophore.

In conclusion, this study shows that PBPs with unique properties can be generated rather easily by rather simple changes in the bilin biosynthetic pathway. Although it has long been recognized that gene duplication and divergence are important aspects of PBP diversification and evolution (1, 21, 38), an equally important component leading to the emergence of new light-absorbing molecules has been the duplication and divergence within the ferredoxin-dependent biliverdin reductase family (PcyA, PebS, PebA, PebB, and HY2) as well as the emergence of isomerizing bilin lyases (e.g., PecE/PecF). This study shows that modifications in bilin synthesis might be sufficiently well tolerated under some light conditions to allow genetic changes to occur that could ultimately be selected and fixed in populations. It is likely that bilin synthetases and bilin lyases have played very important roles in the evolution of proteins with new absorption properties. Thanks to recombinant methodologies, combinations of bilins and PBP not yet found or even possible in nature can be produced, characterized, and applied to the study of specific biological problems.

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