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Characterization of cpeY and cpeZ mutants in *Fremyella* diplosiphon strain UTEX 481

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Characterization of *cpeY* and *cpeZ* mutants in *Fremyella diplosiphon* strain UTEX 481

A Thesis

Submitted to the Graduate Faculty of the
University of New Orleans
in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biological Sciences

by

Christina M. Kronfel

B.S. University of New Orleans, 2010

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Table of Abbreviations

Abbreviation	Designation
AP	allophycocyanin
BV	biliverdin IX α
CA(3 or 4)	type III or IV chromatic acclimation
Cgi	control of green light induction
DHBV	15,16-dihydrobiliverdin
<i>E. coli</i>	<i>Escherichia coli</i>
FNR	ferredoxin:NADP ⁺ oxidoreductase
GFP	green fluorescent protein
HT	hexa-histidine tagged
NADP ⁺ /H	nicotinamide adenine dinucleotide phosphate
PAGE	polyacrylamide gel electrophoresis
PBP	phycobiliprotein(s)
PBS	phycobilisome(s)
PC	phycocyanin
PCB	phycocyanobilin
PCC	Pasteur culture collection
PCR	polymerase chain reaction
PDB	protein data bank
PE	phycoerythrin
PEB	phycoerythrobilin
PEC	phycoerythrocyanin
PUB	phycourobilin
PVB	phycoviolobilin
Rca	regulator of chromatic acclimation
SDS	sodium dodecylsulfate
sp.	species
WT	wild type

Table of Nomenclature

Nomenclature	Designation
<i>cpeY</i>	Genes are represented with italics
CpeY	Proteins are represented with first letter capitalization
$\Delta cpeY/\Delta cpeZ$	Mutants with <i>cpeY</i> or <i>cpeZ</i> gene deleted
α -AP/ β -AP or ApcA/ApcB	Alpha or beta subunits of allophycocyanin
α -PC/ β -PC or CpcA/CpcB	Alpha or beta subunits of phycocyanin
α -PE/ β -PE or CpeA/CpeB	Alpha or beta subunits of phycoerythrin
α -PEC/ β -PEC or PecA/PecB	Alpha or beta subunits of phycoerythrocyanin
α -82	Amino acid position 82 of the alpha subunit

Abstract

Phycoerythrin (PE) present on the outer phycobilisome (PBS) rods in *Fremyella diplosiphon* contains covalently attached phycoerythrobilin (PEB) chromophores for efficient photosynthetic light capture. Chromophore ligation on phycobiliprotein subunits occurs through bilin lyase catalyzed reactions. The *cpeY* and *cpeZ* genes in *F. diplosiphon* were shown to attach PEB on alpha-82 of PE. To better understand the individual functions of *cpeY* and *cpeZ* in native cyanobacteria, we characterized PBS and PE purified from *cpeY* and *cpeZ* deletion mutants and compared them with wild type (WT). Both *cpeY* and *cpeZ* mutants generated much less PE than WT as well as assembling much less PE into the PBS. PE purified from *cpeY* mutant had phycocyanobilin on alpha-PE in place of PEB. The mutation of *cpeZ* affected the biosynthesis and accumulation of beta-PE with a red-shifted absorbance compared to WT PE. CpeY was shown to function as a bilin lyase, and CpeZ possibly functions as a chaperone.

Keywords: Phycobilisome, Phycobiliprotein, Allophycocyanin, Phycocyanin, Phycoerythrin, Phycocyanobilin, Phycoerythrobilin

1. Introduction

1.1 Cyanobacteria background

Cyanobacteria are Gram-negative prokaryotic organisms that perform photosynthesis for energy production. Cyanobacteria, often referred to as “blue-green algae,” have inhabited the earth for a long time with fossil records dating back 3.5 billion years ago [1]. They are believed to be responsible for converting earth’s early reducing atmosphere to its oxidizing atmosphere, thus providing a tolerable environment for other aerobic organisms [2]. There are over 1500 species of cyanobacteria, and as of 2010, the genome sequences of 39 different species of cyanobacteria have been completed and are available in searchable databases [3]. Cyanobacteria inhabit a large number of diverse environments including aquatic and terrestrial habitats as well as extreme climates [4]. Cyanobacteria are also able to perform nitrogen fixation which is vital for the synthesis of many biological compounds [4, 5], thus making them ideal model systems for studying fundamental processes such as nitrogen fixation and photosynthesis.

The amazing versatility of cyanobacteria has attracted huge scientific interest in recent years, especially in the field of engineering cyanobacteria for the production of renewable fuels in hopes of replacing crop-based biofuels [6]. As photosynthetic organisms, cyanobacteria are capable of converting solar energy to chemical energy with high efficiency. They are capable of tolerating high carbon dioxide levels, and naturally produce chemicals as byproducts during cellular processes. Engineering cyanobacteria to optimize these processes, especially photosynthesis, for the production of biofuels can be highly beneficial for the economy. Therefore, understanding the first step of photosynthesis, the light harvesting step, in cyanobacteria is crucial in furthering biofuel production technology.

1.2 Phycobilisome

1.2.1 Phycobilisome structure and function

Phycobilisomes (PBS) are the light harvesting complexes present in classical cyanobacteria, red algae, and cryptophytes [7, 8]. PBS consist of two structural domains: a core which connects to the photosynthetic thylakoid membrane and rods which radiate from the core (see Fig. 1). PBS are macromolecular complexes ranging in molecular weight from 5×10^6 Da to 20×10^6 Da [9]. The PBS core and rods consist of homologous, light harvesting pigmented phycobiliproteins (PBP) which are composed of α and β subunits in heterohexameric form $(\alpha\beta)_6$. Generally, the α subunit has a molecular weight between 15 and 20 kDa, and the β subunit has a molecular weight between 17 and 22 kDa [7]. PBP α and β subunits form monomers $(\alpha\beta)$ by hydrophobic, polar and electrostatic interactions. They further form trimers $(\alpha\beta)_3$ and hexamers $(\alpha\beta)_6$ which are interconnected by nonpigmented, structural linker proteins to form the large structure of the PBS (see Fig. 1 and Fig. 2). Linker proteins connecting rods to other rods are generally 20 to 40 kDa in size while linker proteins connecting the rod to the core are approximately 27 kDa [7]. The smallest linker proteins are located in the allophycocyanin core (approximately 10 kDa) and in the most distal rods to the core (approximately 9 kDa) [7]. The entire PBS complex is connected to the cytosolic side of the thylakoid membrane through a large linker protein ApcE (approximately 75 to 120 kDa). ApcE is often referred to as the “anchor” protein, and facilitates energy transfer from the PBS to the chlorophyll-*a* reaction centers of Photosystems I and II [10]. PBS from many cyanobacterial species also have non-structural proteins associated with them such as ferredoxin:NADP⁺ oxidoreductase (FNR) which oxidizes ferredoxin using NADP⁺ and H⁺ to form oxidized ferredoxin and NADPH, a vital reducing agent in cellular metabolic processes, during the photosynthetic process (see Fig. 1) [9, 11].

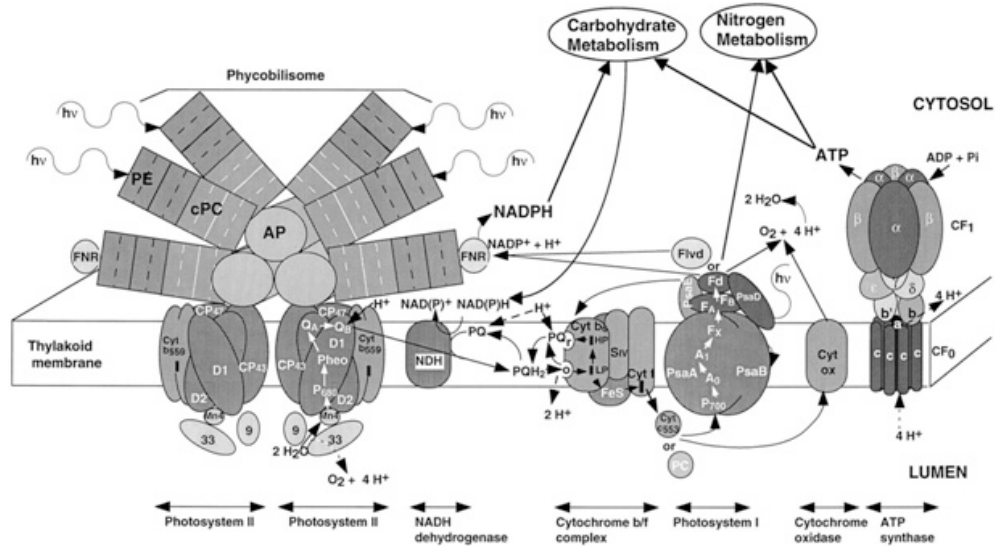


Fig. 1 Phycobilisome structure and photosynthesis

The phycobilisome, the light harvesting complex in cyanobacteria, consists of phycoerythrin outer rods, phycocyanin inner rods, an allophycocyanin core, and linker proteins. Light energy is harvested by the outer rods and transferred down the inner rods to the core of the PBS where it is then transferred to the chlorophyll reaction centers of Photosystem II. The macromolecular complex sits on the cytosolic surface of the thylakoid membrane where photosynthesis occurs and ATP energy is generated [12].

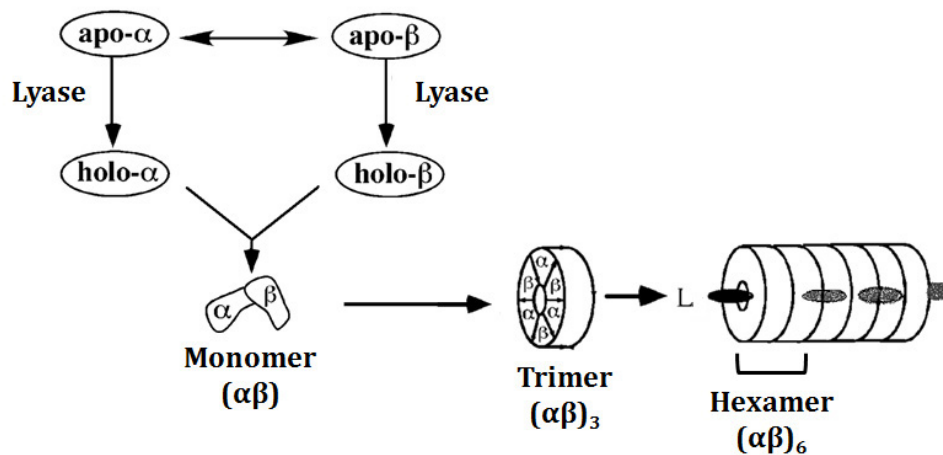


Fig. 2 Phycobiliprotein assembly

When the appropriate lyases are present, apo-α and apo-β subunits of phycobiliproteins are fully chromophorylated, forming holo proteins. Holo-α and holo-β form monomers (αβ) and further form trimers (αβ)₃ and hexamers (αβ)₆ building the rods and core of the phycobilisome complex which are held together by linker proteins (L).

1.2.2 Phycobiliproteins

In cyanobacteria, there are four major classes of PBP assigned based on absorbance properties and sequence homologies: bluish green colored allophycocyanin (AP, $\lambda_{\max} = 650\text{-}655$ nm), blue colored phycocyanin (PC, $\lambda_{\max} = 615\text{-}640$ nm), red colored phycoerythrin (PE, $\lambda_{\max} = 495\text{-}575$ nm) and purple colored phycoerythrocyanin (PEC, $\lambda_{\max} = 575$ nm) [13]. Phylogenetic analyses and amino acid sequence alignments suggest that PBP have evolved from a single common ancestral gene [14]. PBP crystal structures have been ascertained from a number of species and are available in the Protein Data Bank (PDB) repository. All PBP are comprised of α helices with loop linkages as seen in Fig. 3. The core of the PBS is composed of AP which is approximately 110 kDa in its trimeric form. In cyanobacteria with simple PBS, the rods of the PBS are solely composed of PC which is approximately 230 kDa in its hexameric form. In more complex PBS-containing species, the rods distal to the core also contain PE which is approximately 250 kDa in its hexameric form [15]. Instead of PE in the distal rods, some species produce PEC which has a molecular weight of approximately 225 kDa in its hexameric form.

PC and PE are further subdivided into classes based on slight variations in maximum absorbance. PC and PE are given the prefixes C- or R-, originally representing the taxa from which that PBP was first isolated. For example, C-PC ($\lambda_{\max} = 620$ nm) and C-PE ($\lambda_{\max} = 565$ nm) were isolated from blue-green algae Cyanophyceae, and R-PC ($\lambda_{\max}: 617 > 555$ nm) and R-PE ($\lambda_{\max}: 567 > 538 > 498$ nm) were isolated from the red algae Rhodophyceae [16, 17]. PE can also be classified as B-PE ($\lambda_{\max}: 545 > 563 > 498$ nm) which was originally isolated from the red algae Bangioideae [16, 17]. R-PC, R-PE, and B-PE have multiple absorbance peaks due to the presence of two or three types of linear tetrapyrrole molecules called phycobilins.

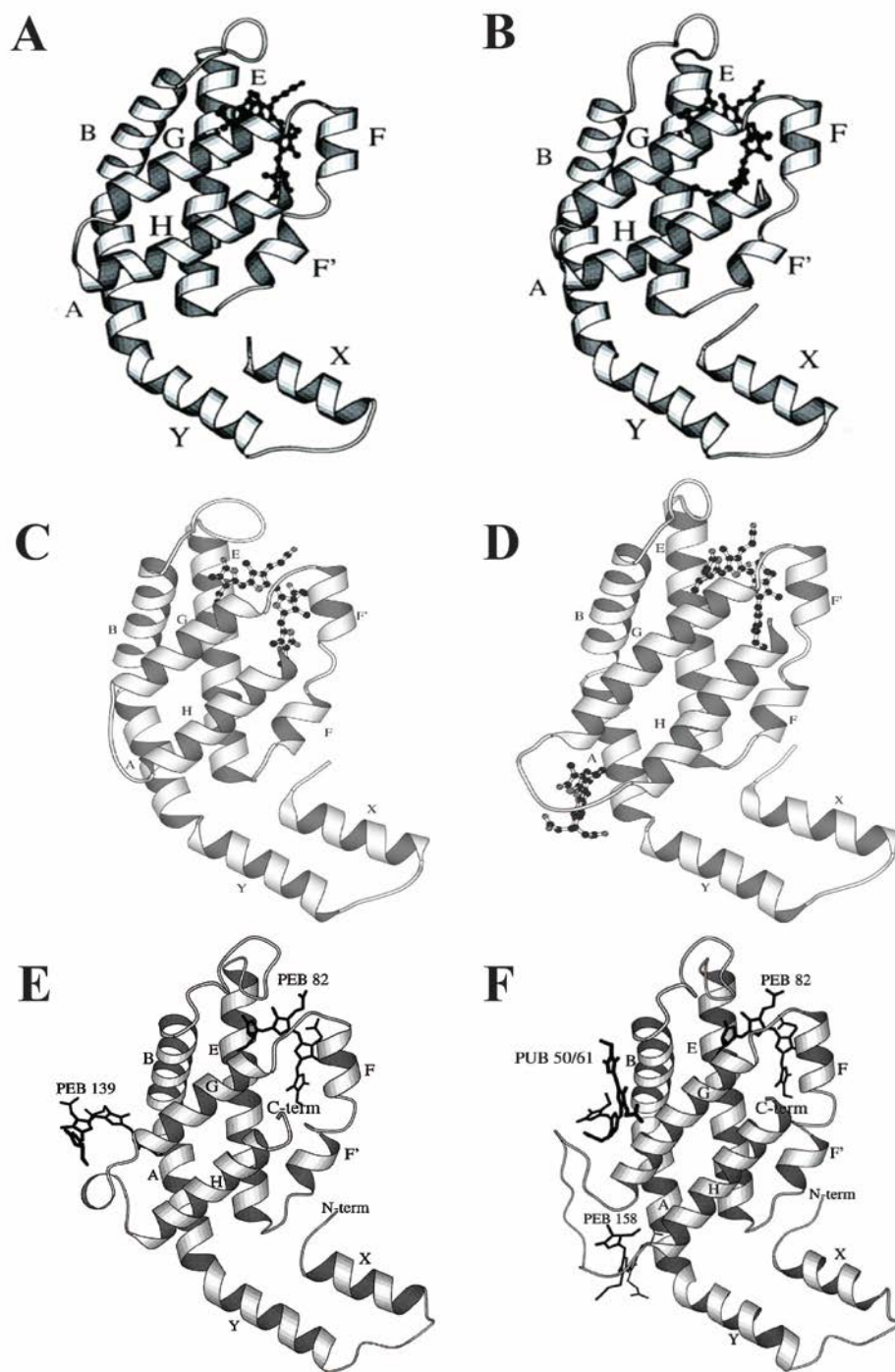


Fig. 3 Crystal structures of PBP α and β subunits

Ribbon diagram of: **A.** α subunit and **B.** β subunit of AP from *Spirulina platensis*; Protein Data Bank (PDB) ID: 1ALL [18]. **C.** α subunit and **D.** β subunit of C-PC from *Spirulina platensis*; PDB ID: 1HA7 [19]. **E.** α subunit and **F.** β subunit of R-PE from *Griffithsia monilis*; PDB ID: 1B8D [20]. Chromophores are shown as ball and stick figures or thick lines.

1.2.3 Bilin chromophores

PBP exhibit brilliant color and light absorbing properties due to the presence of covalently bound phycobilins or bilin chromophores which are covalently attached by thioether linkages to highly conserved cysteine residues (see Fig. 4) [9]. There are four different types of isomeric bilin chromophores, each having a molecular weight of 587 Da; blue colored phycocyanobilin (PCB; λ_{\max} = 620-650 nm), red colored phycoerythrobilin (PEB; λ_{\max} = 540-565 nm), purple colored phycoviolobilin (PVB; λ_{\max} = 568 nm) and yellow colored phycourobilin (PUB; λ_{\max} = 490 nm) [15], present in cyanobacteria. All four bilins are similar in structure, but differ in double bond conjugation which causes the difference in spectral properties. In general, the longer the conjugated double bond system, the longer wavelengths of light the bilin can absorb.

The most abundant bilins in cyanobacteria are PEB and PCB which are derived from heme (see Fig. 5), and reduced by ferredoxin-dependent bilin reductases. In the presence of heme oxygenase (HoI), heme is cleaved into the linear tetrapyrrole biliverdin IX α (BV). 3Z-Phycocyanobilin: ferredoxin oxidoreductase (PcyA) reduces BV in a single four electron reduction step to form the blue bilin PCB which has an absorbance maximum of 620-650 nm (see Fig. 5) [21]. To form PEB in cyanobacteria, BV is first reduced by 15,16-dihydrobiliverdin:ferredoxin oxidoreductase (PebA) at the 15,16-double bond of BV to form 15,16-dihydrobiliverdin (DHBV). DHBV is then reduced at the A ring diene structure by phycoerythrobilin:ferredoxin oxidoreductase (PebB) forming the red colored bilin PEB which has an absorbance maximum of 540-656 nm (see Fig. 5) [21].

PEB can also be produced by phycoerythrobilin synthase (PebS) which is a homolog of PebA originally found in a *Prochlorococcus* cyanophage called myovirus P-SSM2 [22]. PebS is

capable of reducing BV directly to PEB in a single four electron reduction step; whereas PebA and PebB perform two separate two electron reduction steps to form PEB (see Fig. 5).

Recombinant PebS has been successfully used to recreate the PEB biosynthesis pathway inside *E. coli* [22], a very useful tool in forming PEB in laboratory settings [23]. The other two bilins PVB and PUB are created through an alternative pathway by lyase/isomerase enzymes (see Introduction 1.3.1 for further details).

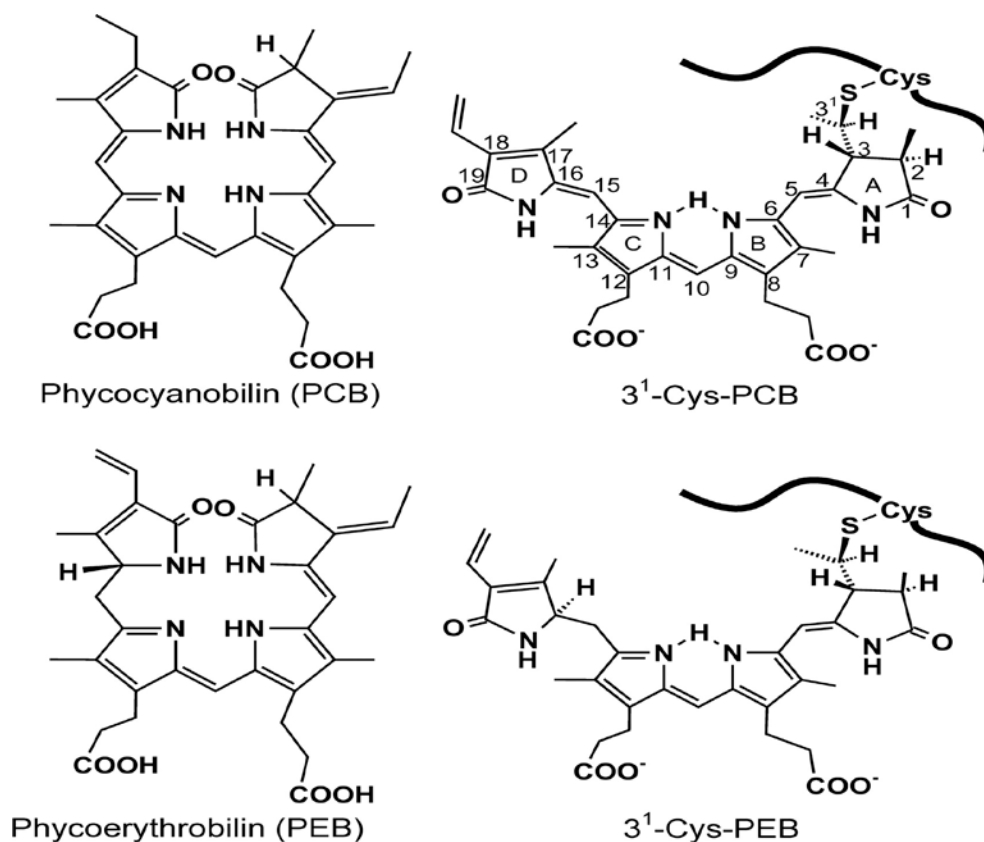


Fig. 4 Structures of free and bound PEB and PCB

Free PEB and PCB (**left**) become bound (**right**) to specific cysteine residues of PBP by thioether linkages to the A ring at carbon-3¹. The pyrrole rings are labeled A-D [24].

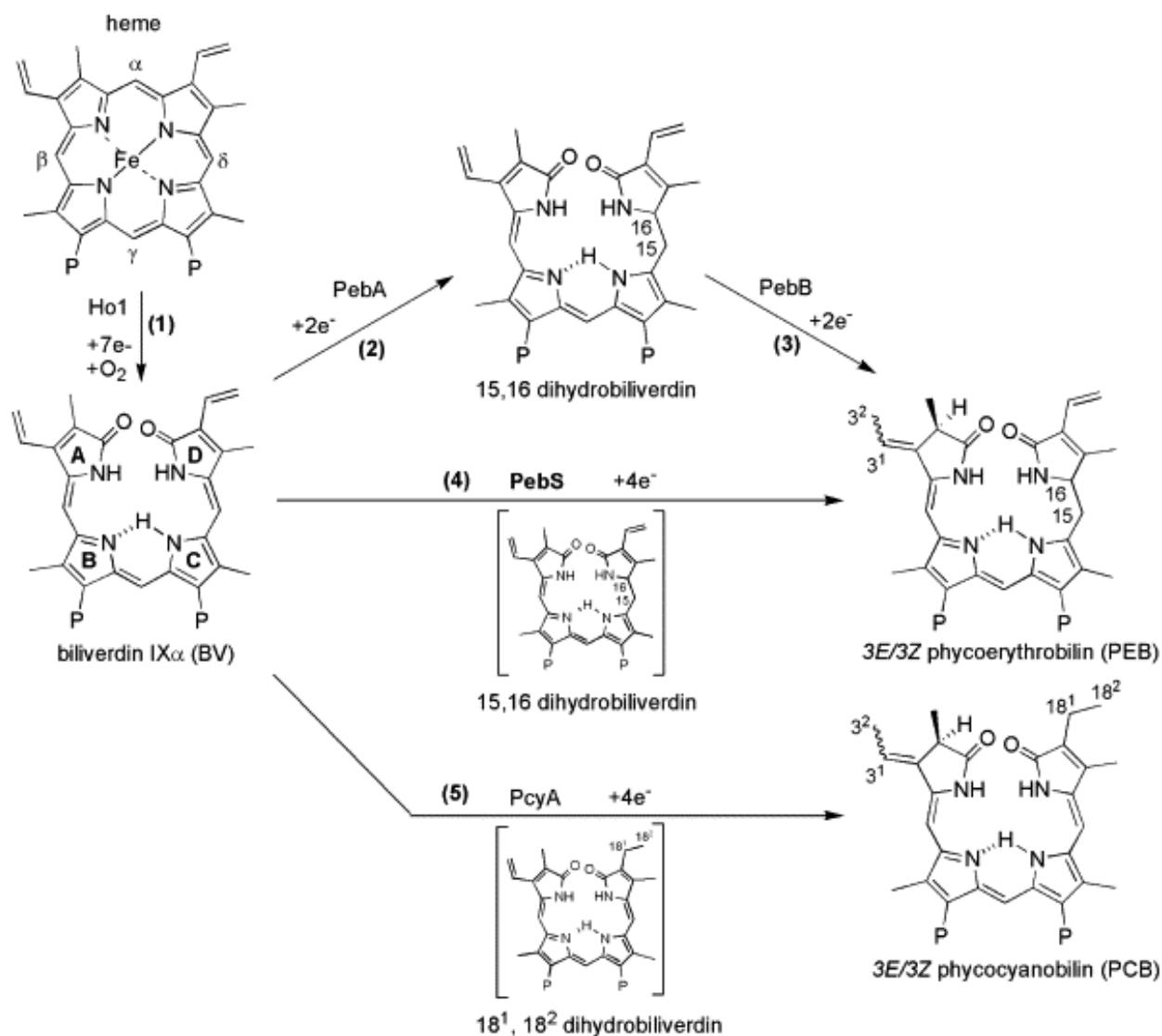


Fig. 5 Bilin biosynthesis

The two major bilin chromophores PCB and PEB are derived from heme which is cleaved open by heme oxygenase forming biliverdin IX_α (BV). 3Z-Phycocyanobilin: ferredoxin oxidoreductase (PcyA) reduces BV to the blue bilin PCB ($A_{\text{max}} = 620\text{-}650\text{ nm}$). The red bilin PEB ($A_{\text{max}} = 540\text{-}565\text{ nm}$) can be produced in two ways. In cyanobacteria, BV is first reduced by 15,16-dihydrobiliverdin:ferredoxin oxidoreductase (PebA) forming 15,16-dihydrobiliverdin (DHBV) which is then reduced by phycoerythrobilin:ferredoxin oxidoreductase (PebB) forming PEB. Alternatively, in the presence of the myovirus protein phycoerythrobilin synthase (PebS), BV is reduced in one step to form PEB [25].

Phycobilins are covalently attached to PBP at the carbon (C) 3¹ position of the bilin A ring; however, at some Cys positions, PEB and PUB are doubly attached at both the C3¹ of the A ring and the C18¹ of the D ring (see Fig. 6). Double attachment of phycobilins to PBP has been identified in a number of different species. In the red algae *Griffithsia monilis*, one PUB is doubly attached to the β -50/61 positions of PE (see Fig. 3 F) [20]. PE from *Fremyella diplosiphon* UTEX 481 (*Calothrix* sp. PCC 7601) described here contains five PEB molecules at cysteines α -82, α -139, β -48/59, β -80 and β -165 with one PEB doubly attached to the β -48/59 positions [26]. When bilins are covalently attached to their PBP substrate, they are bound in a rigid conformation (see Fig. 4) thus enhancing the fluorescent properties. These fluorescent properties facilitate efficient light harvesting and energy transfer from the PBS to the photosynthetic thylakoid membrane. Light is absorbed by the bilins of the PE outer rods and then transferred to the bilins of the PC inner rods. The energy is then absorbed by the AP core where it is then transferred to the chlorophyll reaction centers in Photosystems I and II in the thylakoid membrane (see Fig. 1). PBP do not exhibit the necessary fluorescent properties unless these bilins are covalently bound. The ligation of phycobilins to specific Cys residues on all PBP is generally catalyzed by bilin lyases, with the exception of ApcE linker protein which is auto-catalytically chromophorylated [10].

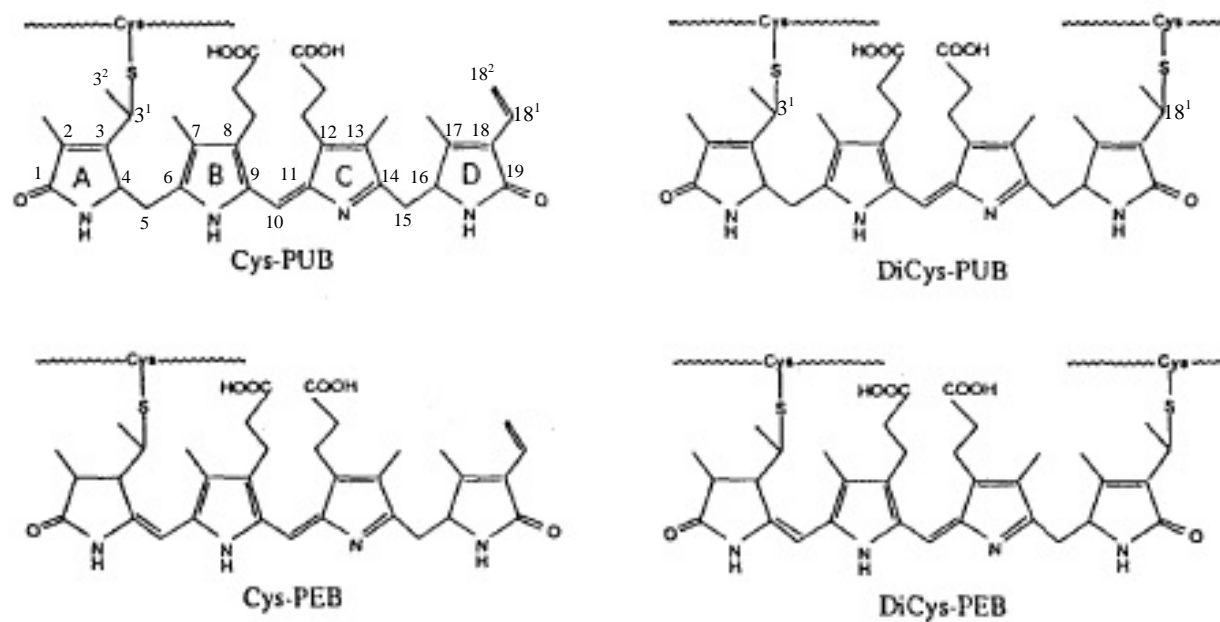


Fig. 6 PEB and PUB singly versus doubly attachment to PBP

Singly attached PEB or PUB at the carbon-3¹ position of the A ring (**left**) versus doubly attached PEB and PUB at the carbon-3¹ position of the A ring and the carbon-18¹ position of the D ring (**right**) to specific cysteine residues of PBP by thioether linkages [15].

1.3 Bilin lyases

1.3.1 *CpcEF* type lyase

Bilin lyases are the enzymes responsible for ligating bilins to their respective PBP substrates at specific Cys residues. There are four major classes of bilin lyases that have been characterized based on sequence similarity: CpcEF type, CpcSU type, CpcT type and the autocatalytic type. The four classes differ from each other in primary amino acid sequence and in the attachment of different bilin chromophores [27].

The first lyase to be characterized is the CpcEF type involved in ligating PCB to the α subunit of PC [28-32]. Unlike other lyases, members of the CpcEF lyase family can both attach and remove bilins [31]. CpcEF is a heterodimer composed of CpcE and CpcF in a 1:1 ratio. The E- and F- subunits may have evolved from a single gene based on sequence similarity. In cyanobacteria, the genes encoding the PBS polypeptides, including PBP, linker polypeptides, and lyases, are generally clustered on the genome [7]. The *cpcEF* genes are located downstream of the structural genes encoding PC, *cpcBA* in *Synechococcus* sp. PCC 7002 [28] and in *Anabaena* sp. PCC 7120 [33]. Although no crystal structure of any CpcEF type lyases have been solved, Phyre² analyses [34] of a CpcEF type lyase MpeZ from marine *Synechococcus* sp. RS9916 [35] suggests that the structure is primarily alpha-helical. All CpcEF type lyases contain 5-6 HEAT-repeat motifs that facilitate protein-protein interactions [36, 37]. CpcEF type lyases have been identified and characterized in other species.

PecE and PecF belong to the CpcEF lyase family and share 47% sequence similarity with CpcE and CpcF, respectively [38]. PecEF functions as a lyase/isomerase by attaching PCB and isomerizing it to PVB at Cys-84 of α -PEC (see Fig. 7) [38, 39]. The motif (NHCQGN) responsible for the isomerase activity of the enzyme is present in the F-subunit PecF [40]. PecEF

catalyzes a double bond shift of ($\Delta 4 \rightarrow \Delta 2$) in the A ring of PCB, thus forming PVB (see Fig. 7) [41]. PecEF is found within three *Anabaena* species including *Anabaena (Nostoc)* sp. PCC 7120 [38], as well as within *Mastigocladus laminosus* [39], and *Microcoleus chthonoplastes* [42]. RpcG (fusion of RpcE/RpcF) from the marine cyanobacteria *Synechococcus* sp. strain WH8102 is another homolog of CpcEF. RpcG functions in the same manner as PecEF by attaching PEB and isomerizing it to PUB at Cys-84 of the alpha subunit of R-PC (RpcA; see Fig. 7) [43]. The proposed isomerase function motif (NHCQGN) is found within the C-terminus of the protein [43]. However, the lyase/isomerase MpeZ from marine *Synechococcus* sp. RS9916 (another CpcEF type lyase) does not contain this motif (see Introduction 1.4 for more details) [35].

Another homolog of CpcEF, whose real function has yet to be fully characterized, is CpeYZ from *Fremyella diplosiphon* [44]. Kahn *et al.* isolated a transposon mutant in the *cpeY* gene in Fd33 (a short filament mutant strain of *F. diplosiphon* UTEX 481) [44]. Mutating *cpeY* resulted in a green phenotype when cells were grown in green light (wild type cells were brown), and purified PBS contained 46% of the PE levels present in the wild type strain. The *cpeY* and *cpeZ* genes are cotranscribed on the same polycistronic mRNA, and mutating *cpeY* affected the transcription of *cpeZ*, also known as a polar effect. Complementation studies revealed that the wild type phenotype was restored in the *cpeY* mutant only in the presence of native *cpeBA* and *cpeYZ* or *cpeYZ* alone. These results suggested both *cpeY* and *cpeZ* might function in PE biosynthesis, and that CpeYZ possibly functions as a bilin lyase for either α or β PE (CpeA and CpeB, respectively) [44].

CpeYZ from *F. diplosiphon*, characterized in this study, was previously analyzed in our lab using an *in vivo* heterologous plasmid coexpression system in *E. coli* [23]. Recombinant CpeY showed lyase activity on Cys-82 of α -PE and CpeZ enhanced this activity by 40%,

although CpeZ did not show lyase activity alone. There was also no evidence of a stable interaction between recombinant CpeY and CpeZ using pulldown assays [23]. CpeY is a much larger protein (429 amino acids) than the typical members of the CpcEF lyase family (approximately 200 amino acids). Sequence alignments show that *cpeY* might have resulted from a fusion of ORFs encoded by a *cpcE* and *cpcF*-like gene. This possibly explains the significant lyase activity of CpeY in the absence of CpeZ. CpeY is most closely related to CpcE from *Synechocystis* sp. PCC 6803 (22% identical and 32% similar). CpeZ (205 amino acids) is also closely related to CpcE (25% identical and 38% similar), whereas CpeZ and CpcF (from *Synechocystis* sp. PCC 6803) are 23% identical and 37% similar [23]. The *cpeY* and *cpeZ* genes are also present downstream of the structural genes encoding the α and β subunits of PE, *cpeBA*. The similarities in gene locus, sequence, and protein activity indicate that CpeYZ from *F. diplosiphon* are homologs of CpcEF bilin lyase.

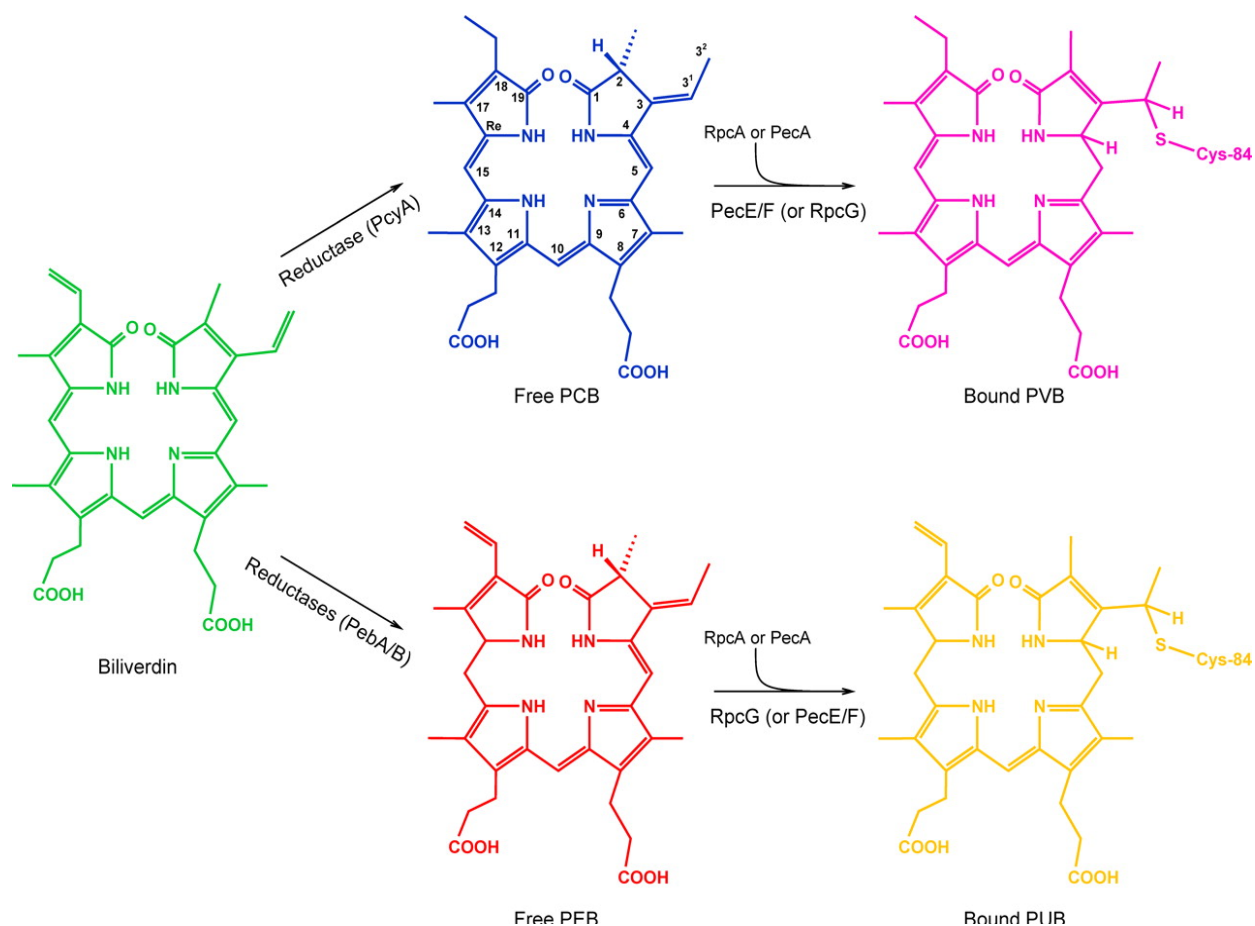


Fig. 7 PVB and PUB structures and biosynthesis

In certain species of cyanobacteria, free PCB is isomerized to PVB and attached to PecA by the lyase/isomerise PecE/F (RpcG is also capable of isomerising and attaching PVB to RpcA in heterologous systems). Free PEB is isomerized to PUB and attached to RpcA by the lyase/isomerise RpcG (PecE/F is also capable of isomerising and attaching PUB to PecA in heterologous systems) [43].

1.3.2 CpcSU type lyase

The second type of bilin lyase is the CpcSU type which is unrelated in sequence to the CpcEF type lyase. This lyase has high binding site specificity but low chromophore and PBP substrate specificity. Members of the CpcSU family of lyases are involved in the ligation of PEB or PCB to the Cys-82 equivalent on β -PC, β -PE, α -AP, and β -AP [24, 45-47]. There are three CpcS subfamilies or clades denoted CpcS-I, CpcS-II, and CpcS-III which vary slightly in their sequences [45]. The CpcSU type lyase from *Synechococcus* sp. PCC 7002 was purified as a heterodimer composed of CpcS-I and CpcU in a 1:1 ratio. The CpcS and CpcU proteins share a high level of sequence similarity to each other (~53%) [45]. Biochemical analysis showed that CpcSU was the lyase responsible for ligating PCB to Cys-82 of β -PC as well as ligating PCB to Cys-81 of α -AP and of β -AP in *Synechococcus* sp. PCC 7002 [45, 47].

Another homolog of CpcSU is CpcS-III from *Thermosynechococcus elongatus* which is highly related in sequence to CpcS-I (46% identity, 60.6% similarity) and CpcU (29.9% identity, 44.2% similarity) from *Synechococcus* sp. PCC 7002 [48]. Biochemical analysis showed that CpcS-III functioned as a bilin lyase, and was able to ligate cognate and non-cognate bilins to all AP subunits and to β -PC at Cys-82 [48, 49]. In 2007, Jon Hunt's group solved the crystal structure of CpcS-III from *T. elongatus* strain BP-1 as part of a structural genomics initiative and entered it into the PDB repository as Ycf58 with the PDB ID: 3BDR [50]. This is the first bilin lyase whose crystal structure has been solved. CpcS-III crystallized as a homodimer, and is comprised of a 10-stranded antiparallel β -barrel with two α -helices (see Fig. 8). Since CpcS-III is highly similar in sequence to CpcS-I and CpcU from *Synechococcus* sp. PCC 7002 and other CpcSU type lyases [49], it is likely that this β -barrel structure may be the structure of other CpcSU type lyases.

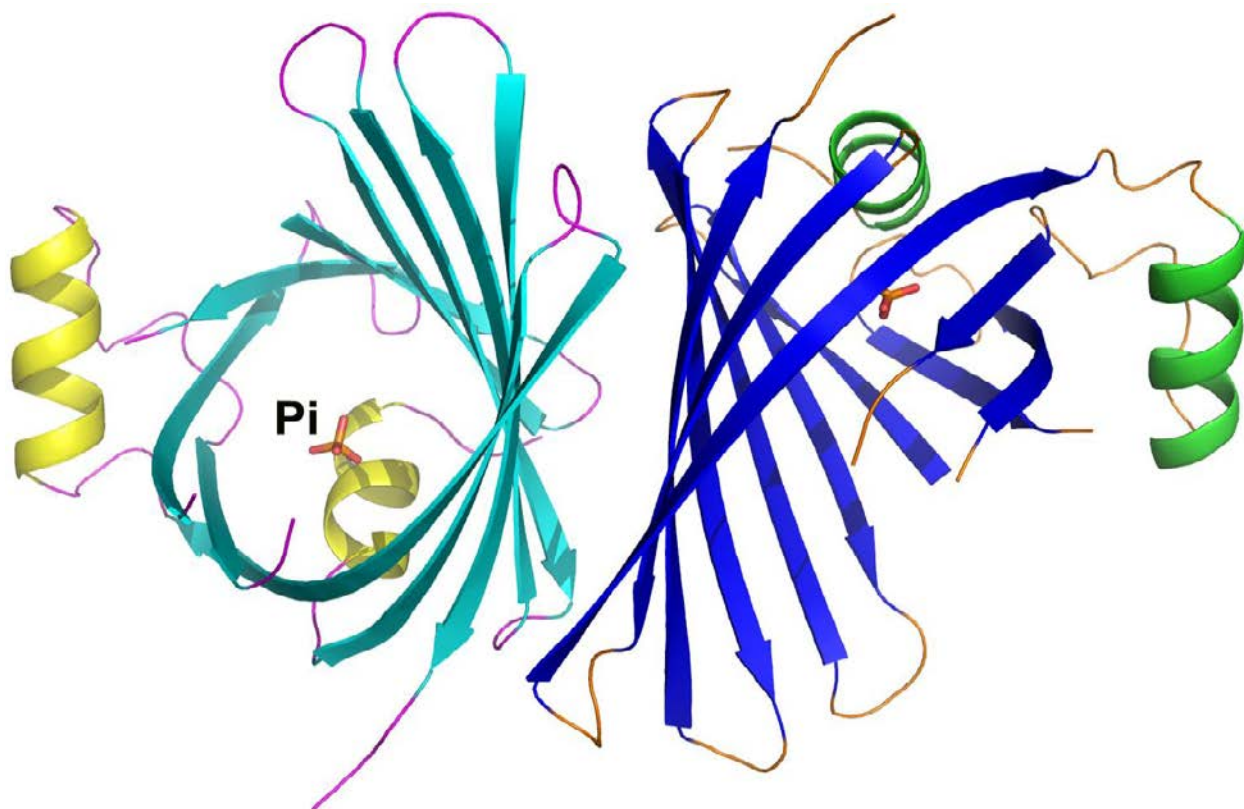


Fig. 8 Crystal structure of CpcS-III from *Thermosynechococcus elongatus* BP-1

The structure of CpcS-III crystallized as a homodimer (PDB ID: 3BDR). This protein has a 10-stranded antiparallel β -barrel with two α -helices. A phosphate ion co-crystallized with each subunit in the structure [49, 50].

1.3.3 *CpcT* type lyase

The third type of bilin lyase is the CpcT type lyase. CpcT type lyases have a very low sequence homology to CpcSU type lyases, but Phyre² analyses suggest they are β -barrel type structures (Schluchter *et al.* unpublished). CpcT from *Synechococcus* sp. PCC 7002 was shown to ligate PCB on Cys-153 of β -PC [51]. Mutating *cpcT* in *Synechococcus* sp. PCC 7002 resulted in a 40% reduction of PC levels when compared to wild type cells. SDS-PAGE analysis of pure PBS from *cpcT* mutant cells showed that β -PC was slightly smaller in molecular weight in comparison to wild type β -PC due to the absence of one PCB chromophore (587 Da). *In vitro* analysis confirmed that CpcT was a lyase responsible for ligating PCB to Cys-153 of β -PC [51]. The protein CpcT1 from *Nostoc* sp. PCC 7120 is homologous to the CpcT type bilin lyase and was shown to ligate PCB on Cys-155 of β -PC and of β -PEC [52]. CpcT type lyases have high chromophorylation site specificity with a moderate PBP specificity [27].

1.3.4 Autocatalytic type lyase

The fourth type of lyase is the autocatalytic lyase where ApcE linker protein can ligate PCB autocatalytically without the aid of a separate enzyme [10, 46]. ApcE is the only known PBP capable of chromophore autoligation. ApcE is a large linker protein (approximately 75 to 120 kDa) which is responsible for connecting the entire PBS complex to the cytosolic side of the thylakoid membrane. ApcE contains a chromophore binding site at Cys-195 and PCB attachment to this site is conducted through the N-terminal portion of ApcE (240 amino acids). ApcE is often referred to as the “anchor” protein, and facilitates energy transfer from the PBS to the chlorophyll-*a* reaction centers of Photosystems I and II [10].

1.4 Chromatic acclimation and *Fremyella diplosiphon*

Cyanobacteria have evolved to alter their PBS composition in response to environmental stimuli. In the presence of low light intensities, more PBS are synthesized and the outer rods are extended to efficiently capture more light [53-55]. Certain cyanobacteria which contain PE are also capable of altering their PBS protein composition in response to light quality, such as red light versus green light wavelengths, in a process called chromatic acclimation (CA) [56-58]. During CA, the rods distal to the core are altered, a modification that is necessary for many cyanobacteria to readily capture the most abundant wavelengths of light available in the environment. Cyanobacteria are classified into four groups (I-IV) based on their pigmentation alterations in different light qualities.

Group I cyanobacteria are incapable of performing CA when the wavelength of light is altered. Such species include *Synechococcus* sp. PCC 7424, *Nostoc* sp. PCC 7121, and *Gloeobacter* sp. PCC 7421 [17]. Group II cyanobacteria, such as *Synechocystis* sp. PCC 6701 [59], can alter their PE levels in red and green light. When grown in green light, cells produce PBS with an extra distal PE rod disc when compared to cells grown in red light [56]. Both PE abundance and *cpeBA* mRNA levels are altered during changes in light quality [60]. Group III cyanobacteria, such as *F. diplosiphon*, can alter both PE and PC levels with changes in light quality to efficiently capture the wavelengths of light available in the environment [57]. The rods proximal to the core are composed of PC1 (constitutive PC) under both red and green light conditions. Under red light conditions, PC production is induced, and the rods distal to the core are assembled with PC2 (inducible PC). Under green light conditions, PC2 production is inhibited, and PE synthesis and assembly into the rods distal from the core are induced (see Fig. 9). The genes for PC1 and PC2 are encoded on 2 different operons (*cpcB1A1* and *cpcB2A2*,

respectively). The PC1 and PC2 proteins differ in amino acid sequence mainly in residues on the surface of the β -subunits where hexameric interactions occur [61]. AP core protein levels are unaffected during CA in *F. diplosiphon* [62, 63].

The last group, Group IV cyanobacteria can alter the bilin chromophore content on the outermost rods of the PBS as opposed to altering PBP content. Some strains of marine *Synechococcus* species, which possess two forms of PE (PEI and PEII), alter PEB/PUB ratios on PEI and PEII during shifts from blue light to green light or white light [64]. In marine *Synechococcus* sp. RS9916, chromophorylation is affected on the α subunits of PEI (CpeA) and PEII (MpeA) by one or more bilin lyases/isomerases during type IV chromatic acclimation (CA4) [35, 65]. Under green light conditions, PEB molecules are bound to Cys-139 of CpeA and to Cys-83 and Cys-140 of MpeA. However, under blue light conditions, PUB molecules are bound to all three Cys positions in place of PEB. In marine *Synechococcus* sp. RS9916, the protein MpeZ was characterized as a lyase/isomerase responsible for ligating PEB to Cys-83 of MpeA and isomerizing it to PUB during CA4 [35]. The lyase/isomerase enzymes responsible for chromophorylating Cys-140 of MpeA and Cys-139 of CpeA during CA4 are still unknown.

Fremyella diplosiphon, used in this study, is a freshwater, filamentous cyanobacterium possessing both PC and PE in the rods of the PBS. This species is capable of performing type III chromatic acclimation (CA3, see Fig. 9) and as such, is a model organism for studying this biological process. Pigmentation alteration in CA3 is controlled at the RNA level in *F. diplosiphon* where transcription levels of the *cpcB2A2* (encoding α and β PC2) and *cpeBA* (encoding α and β PE) operons as well as *cpcHID* (encoding PC linker proteins) and *cpeCDEST* operons (first three genes encoding PE linker proteins) are affected [66].

Mechanisms for this transcriptional regulation during CA3 have been studied in *F. diplosiphon*, which is regulated by two signal transduction pathways (see Fig. 10).

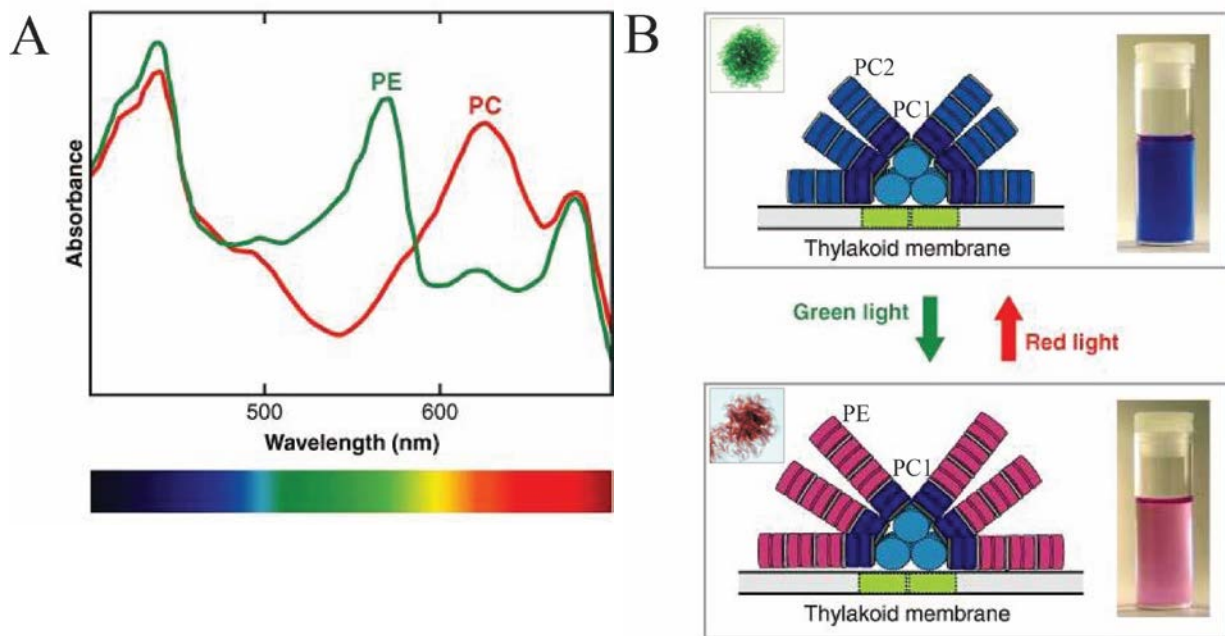


Fig. 9 Type III chromatic acclimation in *F. diplosiphon*

A: Whole cell absorbance spectra of *F. diplosiphon* cells grown in green and red light with PE absorbing around 570 nm (green light) and PC absorbing around 620 nm (red light). The remaining peaks in the blue and red regions represent absorption by chlorophyll *a* and carotenoids. **B:** In red light, PE is replaced by inducible PC (PC2, blue colored) on the outer rods of the PBS, resulting in green colored cells. In green light, PC2 is replaced by PE (red colored) on the outer rods of the PBS, resulting in brown colored cells [67]. Constitutive PC (PC1) is unaltered and present under both light conditions.

The first signal transduction pathway is the Cgi (control of green light induction) system, which represses *cpeCDESTR* expression in red light (see Fig. 10) [68]. Under green light conditions, *cpeCDESTR* expression is activated, and CpeR further activates expression of *cpeBA* and *pebAB* (genes encoding PebA and PebB) by binding specific promoter sequences (the N-box) [69]. The second and more widely studied signal transduction pathway is the Rca (regulator of chromatic acclimation) system which uses a phosphorelay system to activate *cpcB2A2* and *pcyA* expression and repress *cpeCDESTR* expression in red light (see Fig. 10) [70]. Red and green light are sensed by the photosensory receptor protein RcaE, which has an N-terminal chromophore-binding GAF domain with a PCB chromophore covalently attached to Cys-248, and a C-terminal histidine kinase domain which is light-regulated [71, 72]. Under red light conditions, the bilin undergoes photoisomerization which triggers a proton transfer from the bilin to Glu-217 of RcaE [72]. The protonation states of the bilin and Glu-217 causes a structural change within RcaE which activates its kinase activity, thereby phosphorylating and activating a downstream CA response regulator RcaF [72, 73]. RcaF further phosphorylates and activates a second CA response regulator RcaC which is a transcription factor that binds specific promoter sequences (the L-box) of *pcyA* and *cpcB2A2*, thus inducing expression (see Fig. 10) [74]. Under green light conditions, the PCB chromophore attached to RcaE undergoes photoisomerization favoring a protonated state of the bilin allowing for the absorbance of green light [72]. RcaE kinase activity is deactivated by the protonated state of the PCB chromophore, and Rca-controlled expression of *cpcB2A2* is reduced due to the dephosphorylated states of the Rca proteins (see Fig. 10) [75]. There are many steps in the mechanism of CA regulation that are still unknown and continuing to be studied.

The diagram illustrates the Cgi System, which regulates gene expression through a series of protein interactions. At the top left, RcaE is shown as a blue protein with a green box labeled 'H~P' and a blue box labeled 'ATP'. A curved arrow points from RcaE to RcaF, which is a blue oval labeled 'P~D'. A dashed arrow labeled 'P~' points to RcaF. Below RcaF, a curved arrow points to RcaC, which is a blue protein with a red box labeled 'D~P' and a green box labeled 'H~P'. A dashed arrow labeled 'P~' points to RcaC. RcaC is shown interacting with a DNA sequence. The DNA sequence is a horizontal line with several boxes labeled 'L box', 'cpeC', 'D', 'E', 'S', 'T', and 'R'. A thick black line with a perpendicular bar (a repressor binding site) is shown binding to the 'L box' region. Below the main DNA sequence, there are two smaller DNA sequences. The first one has boxes labeled 'L box', 'pcyA', 'cpcB2', 'A2', 'H2', 'I2', and 'D2'. The second one has boxes labeled 'N box', 'cpeB', 'A', 'pebA', and 'B'. Arrows indicate the flow of information from RcaE to RcaF to RcaC, and from RcaC to the DNA sequences.

The diagram illustrates the Cgi System, a regulatory mechanism in bacteria. It shows the following components and processes:

- RcaE:** A protein complex (orange, grey, green, and blue subunits) that binds to the DNA at the **L box** (purple box) and the **pcyA** gene (red arrow).
- RcaF:** A protein (blue oval) that binds to the DNA at the **L box** and the **cpeC** gene (red arrow).
- RcaC:** A protein complex (blue, red, green, and blue subunits) that binds to the DNA at the **L box** and the **cpcB2** gene (red arrow).
- CpeR:** A protein (green oval) that binds to the DNA at the **N box** (green box) and the **cpeB** gene (red arrow).
- Genetic Organization:** The DNA sequence includes several genes: *pcyA*, *cpcB2*, *A2*, *H2*, *I2*, *D2*, *cpeC*, *D*, *E*, *S*, *T*, and *R*.
- Regulation:** The RcaE, RcaF, and RcaC complexes are shown binding to the DNA at the **L box** and the **pcyA** gene. The CpeR protein is shown binding to the DNA at the **N box** and the **cpeB** gene.
- Phosphorylation:** The RcaF protein is shown with a phosphate group (P-OH) and a phosphorylated state (P~).

Under red light conditions, *pcyA* and *cpcB2A2* expression is activated by the Rca signal transduction system, while *cpeCDEST*R and *cpeBA* expression is repressed by both Rca and Cgi systems. Under green light conditions, Rca-controlled expression of *cpcB2A2* is reduced due to the dephosphorylated states of the Rca proteins, and the expression of *cpeCDEST*R and *cpeBA* operons are activated possibly by the Cgi signal transduction system (indicated by the dashed line) [62].

1.5 Phycobiliprotein applications in biotechnology

Fluorescent molecules or fluorophores, such as PBP, are widely used in many biological applications. C-PC, R-PE, and B-PE have been used as a natural protein dye in food products [76] such as chewing gum, dietary products, and candy, as well as in cosmetics [77]. PBP have also been used in pharmaceuticals to stimulate the immune defense system, and they possess antioxidant, anti-inflammatory, anti-viral, anti-cancer, and cholesterol-lowering effects [78]. In addition, PBP have been used in textiles and printing dyes [79].

PBP have unique spectral properties and produce high fluorescent quantum yields, making them a valuable resource for biotechnology. Holo-PBP, most commonly AP, R-PE and B-PE, have been engineered as fluorescent probes, which are often used in histochemistry, fluorescence microscopy, flow cytometry, fluorescence activated cell sorting, and fluorescence immunoassays [15, 80, 81]. Other fluorophores are available in place of PBP, such as the widely used green fluorescent protein (GFP) and its derivatives. However, PBP are more advantageous due to their broader absorbance spectral range (490-650 nm), high fluorescent quantum yields, and stability under a wide range of biological pH (4.5-8.0) [81]. The continued study of these valuable proteins is beneficial to the advancement of this biotechnology.

1.6 Purpose of this work

The study conducted by Kahn *et al.* of the transposon mutant in the *cpeY* gene in *F. diplosiphon* suggested that *cpeY* and *cpeZ* may function in PE biosynthesis [44]. The resulting polar effects from the transposon mutant make the determination of the exact roles of CpeY and CpeZ in cyanobacteria difficult. In our lab, we characterized recombinant CpeY as a specific bilin lyase for attaching PEB to Cys-82 of α -PE using a heterologous plasmid coexpression system in *E. coli*, site-directed mutagenesis, and mass spectrometry (see Fig. 11) [23]. Recombinant CpeZ enhanced this activity by 40%, but CpeZ alone was unable to ligate PEB to α -PE, suggesting a chaperone-like function. Pulldown assay analysis suggests that recombinant CpeY and CpeZ do not form a stable interaction with each other. Neither CpeY, CpeZ nor CpeYZ were able to ligate PEB to β -PE [23]. The function of CpeZ is still unclear, and CpeY may function differently within native cyanobacteria. In this thesis, I describe a detailed characterization of single, clean deletion mutants of *cpeY* and *cpeZ* genes in *Fremyella diplosiphon* strain UTEX 481 using biochemical and molecular approaches.

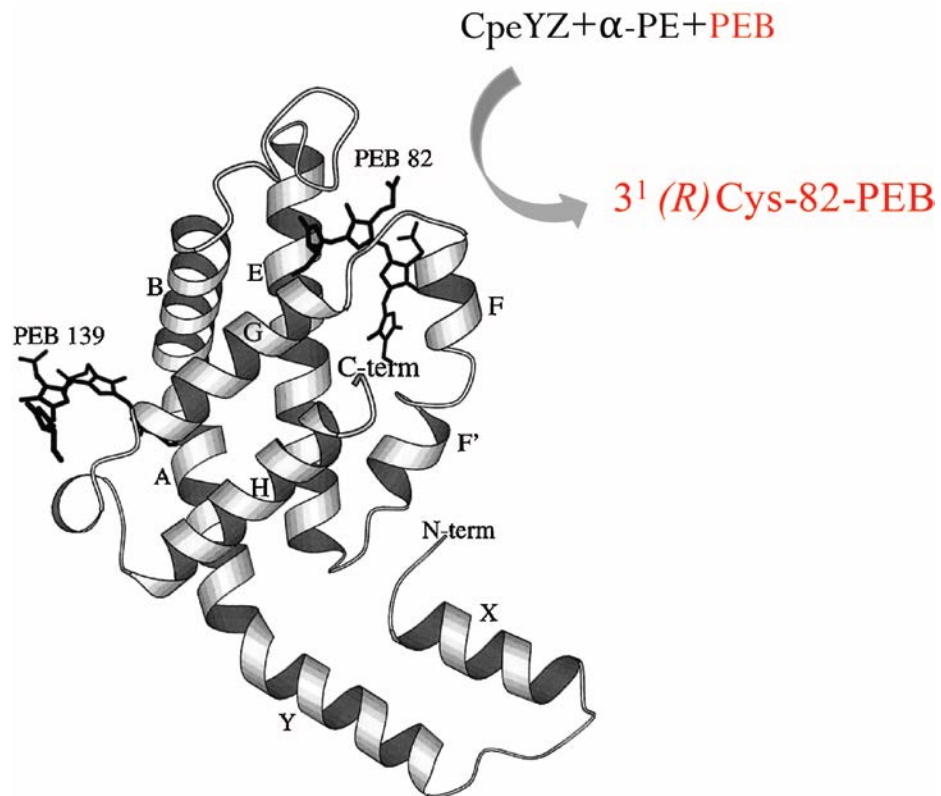


Fig. 11 Crystal structure of α -PE

In the presence of the lyase CpeYZ, α -PE is chromophorylated with PEB at Cys-82 as represented in the ribbon diagram above. PEB molecules are bound to the subunit at both Cys-139 and Cys-82. PDB ID: 1B8D [20]

2. Materials and Methods

2.1 Construction of expression vectors

All expression plasmids used in this study were previously described and are listed below in Table 1.

Table 1: Plasmids used in this study

Plasmid name	Recombinant proteins produced ^a	Parent vector	Antibiotic ^b	Reference
pCpeA	HT-CpeA from <i>F. diplosiphon</i>	pETDuet-1	Ap	[23]
pCpcEF	CpcE and CpcF from <i>Synechocystis</i> sp. PCC 6803	pBS414v	Sp	[46]
pPcyA	PcyA from <i>Synechococcus</i> sp. PCC 7002 and Ho1 from <i>Synechocystis</i> sp. PCC 6803	pACYCDuet-1	Cm	[46]
pPebS	Ho1 and PebS from Myovirus	pACYCDuet-1	Cm	[25]

^a Proteins produced as Hexa-histidine-tagged fusions are indicated as HT-

^b Antibiotic resistance used to select for the presence of the plasmid (Ap: ampicillin; Cm: chloramphenicol; Sp: spectinomycin)

2.2 *In-vivo* heterologous expression and purification of recombinant proteins

Recombinant plasmids were co-transformed into BL21 (DE3) competent *E. coli* cells (Novagen, Darmstadt, Germany), and colonies were selected on LB plates in the presence of the appropriate combination of antibiotics (see Table 1). Cells were grown in liquid LB media with the appropriate combinations of antibiotics at the following concentrations: ampicillin (Ap: 100 $\mu\text{g}\cdot\text{mL}^{-1}$), chloramphenicol (Cm: 34 $\mu\text{g}\cdot\text{mL}^{-1}$), spectinomycin (Sp: 100 $\mu\text{g}\cdot\text{mL}^{-1}$) at 37°C for 2 to 4 hours at 220 rpm. When the optical density (OD) at 600 nm reached 0.6, 1 mM Isopropyl- β -D-thiogalactoside (IPTG; Gold Biotechnology, St. Louis, MO) was added to induce T7 RNA polymerase expression from Duet vectors. The induced cultures expressing PEB synthesis

proteins were placed at 18°C for approximately 16 hours at 220 rpm. Cultures expressing PCB synthesis proteins were induced at 30°C for only 3 hours. Cells were harvested at 11,000 x g for 10 min in a Sorvall RC 5C Plus centrifuge (Kendro Laboratory Products, Newtown, CT). Cell pellets were stored at -20°C for later use.

Cell pellets were resuspended in Buffer O (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM KCl), 0.1X complete mini protease inhibitor cocktail (Roche, Indianapolis, IN), and 0.1 mg·ml⁻¹ lysozyme. After incubating for 30 min on ice, cells were passed through a French Pressure Cell Press at 12,000 psi three times to ensure cell lysis. Cellular debris, inclusion bodies, and unbroken cells were removed via centrifugation at 20,000 x g for 20 min in a Sorvall RC 5B Plus centrifuge (Kendro Laboratory Products) with the supernatant containing soluble proteins.

Soluble, hexa-histidine tagged (HT) proteins were passed through a high density nickel-nitrilotriacetic acid (Ni-NTA) agarose affinity column (Gold-Bio, St. Louis, MO) three times to ensure binding. The protein-bound slurry was washed with one column volume of Buffer A1 (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM KCl, 20 mM imidazole, 5% glycerol), Buffer B (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.5 M KCl), then Buffer A2 (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM KCl, 30 mM imidazole). HT proteins were then eluted from the Ni-NTA column with a small volume of Buffer C (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM KCl, 200 mM imidazole). Imidazole introduced during elution was removed by dialysis against Buffer O and 10 mM 2-mercaptoethanol (β-Me) overnight at 4°C. The next day, the dialysis buffer was exchanged with fresh buffer and allowed to stir for another 2 hours. Diluted recombinant proteins were concentrated by ultrafiltration through an Amicon YM10 (Millipore, Billerica, MA) for further analysis. Recombinant proteins were stored on ice at 4°C in the dark.

2.3 Purification of phycobiliproteins from cyanobacteria

2.3.1 Growth of *Fremyella diplosiphon*

The *Fremyella diplosiphon* cells were grown in 50 ml CytoOne T25 filter cap TC flasks (USA Scientific, Ocala, FL) in Blue Green (BG-11) medium [82] with 10 mM HEPES, pH 8.0 (B-HEPES). The cultures were grown on a Thermolyne Bigger Bill orbital shaker (Barnstead Thermolyne, Dubuque, IA) at 100 rpm, and illuminated with approximately 10-20 $\mu\text{E}/\text{m}^2/\text{s}$ cool white light (enriched in green light) or soft white light (enriched in red light). The cell density was monitored with UV-Vis spectrophotometry at 750 nm. Cells were also streaked on B-HEPES agar plates containing 10 $\mu\text{g}\cdot\text{mL}^{-1}$ carbendazim, an antifungal agent, under the same conditions as liquid cultures.

The *F. diplosiphon* knockout mutants in *cpeY* or *cpeZ* were generated as clean deletions that have no polarity effects (generously provided by David Kehoe and Andrian Gutu of Indiana University). These deletions were verified by polymerase chain reaction (PCR) and Southern blot analyses (data not shown).

2.3.2 Phycobilisome (PBS) purification

Phycobilisomes from wild type and mutant *F. diplosiphon* were separated following the protocol described by Glazer [83] with a few minor alterations. Unless specified, all steps were performed at room temperature. Cyanobacterial cells were collected via centrifugation at 20,000 x g for 30 min at 4°C. The cells were washed in 20-30 mL 0.75 M sodium/potassium (Na/K) phosphate, pH 7.0 and subjected to centrifugation. The cells were resuspended in 5 to 15 mL 0.75 M Na/K phosphate, pH 7.0, 0.1X protease inhibitor cocktail, and 0.02% lysozyme. After 30 min incubation on ice, the cells were lysed using a French Press Cell Press (see Materials and

Methods 2.2). Triton X-100 was immediately added to 2% from a 20% stock (v/v) solution, and the solution was allowed to gently stir in the dark for 20 min. The samples were subjected to centrifugation for 10 min at 21,000 x g. Three layers were detected after centrifugation: unbroken cells and debris pelleted, phycobiliproteins collected in the central aqueous layer, and chlorophyll and lipids fractionated in the top layer. Additional chlorophyll extractions were performed when necessary. Phycobiliproteins were collected from the aqueous layer and 1.5 mL was loaded onto sucrose density gradients in 14 x 89 mm Ultra-Clear Centrifuge Tubes (Beckman, Palo Alto, CA). All of the sucrose solutions were prepared in 0.75 M Na/K phosphate, pH 7.0, 1 mM sodium azide, and the following concentrations were poured into the centrifuge tubes from bottom to top: 2 M (0.75 mL), 1 M (2.5 mL), 0.75 M (2.75 mL), 0.5 M (2.25 mL), and 0.25 M (2.0 mL). The sucrose gradients were balanced in a SW 41 Ti swinging bucket rotor and subjected to ultracentrifugation at 151,000 x g for 18 hrs at 4°C using a Beckman L-60 ultracentrifuge. Intact phycobilisomes were recovered from the 0.75 M sucrose layer for wild type extracts and 0.5 M sucrose layer for the mutant extracts and further analyzed by absorbance and fluorescence spectroscopy (see Materials and Methods 2.4.2). Pure PBS samples were diluted to approximately 0.5-1.0 OD (at λ_{\max}) with 0.75 M Na/K phosphate, pH 7.0 prior to obtaining their absorbance spectra. PBS samples from wild type *F. diplosiphon* had a λ_{\max} at OD_{565 nm}, and *F. diplosiphon* mutants PBS samples had a λ_{\max} at OD_{620 nm}. Quantification of PBP were calculated using the following equations [84].

$$\text{PC (mg} \cdot \text{mL}^{-1}) = (\text{OD}_{620 \text{ nm}} - 0.7 \times \text{OD}_{650 \text{ nm}}) / 7.38$$

$$\text{AP (mg} \cdot \text{mL}^{-1}) = (\text{OD}_{650 \text{ nm}} - 0.19 \times \text{OD}_{620 \text{ nm}}) / 5.65$$

$$\text{PE (mg} \cdot \text{mL}^{-1}) = (\text{OD}_{565 \text{ nm}} - 2.8[\text{PC}] - 1.34[\text{AP}]) / 12.7$$

PBS samples were precipitated from sucrose prior to analysis on SDS-PAGE using 25% trichloroacetic acid (TCA) and incubation on ice for 30 min in the dark. The samples were subjected to centrifugation at 21,000 x g for 5 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 100 mM Tris-HCl, pH 8.8. Excess Tris-HCl was added to neutralize residual acid when necessary.

2.3.3 *Phycoerythrin (PE) purification*

PE purification from *F. diplosiphon* wild type, *cpeY* mutant, and *cpeZ* mutant was performed following the protocol described by Glazer [85] with minor alterations. The entire procedure was conducted in dim light and at 4°C when possible. *F. diplosiphon* cells were collected via centrifugation at 20,000 x g for 30 min. The cells were resuspended in 5 to 15 mL of 1 M sodium acetate (NaOAc, to dissociate proteins within the PBS), pH 5.0, 0.1X protease inhibitor cocktail, and 0.1 mg·mL⁻¹ lysozyme. The samples were broken by passage through the French Pressure cell (see Materials and Methods 2.2) and subjected to ultracentrifugation at 77,000 x g for one hour at 4°C using a Beckman L-60 ultracentrifuge (SW 41 Ti swinging bucket rotor) to pellet unbroken cells and thylakoid membranes.

The supernatant was brought to 35% saturation with solid ammonium sulfate ((NH₄)₂SO₄) and allowed to stir slowly for 90 min. The sample was subjected to centrifugation at 17,000 x g for 15 min. The PE-containing pellet was resuspended in a small volume (approximately 2 to 4 mL) of 0.1 M NaOAc, pH 5.0 and dialyzed overnight against the same buffer plus 10 mM β-Me. The next day, the dialysis buffer was exchanged with fresh buffer and allowed to stir for an additional 2 hours.

Dialyzed samples were then passed through a Sephadex G-100 size exclusion column (1.5 x 27 cm) preequilibrated with 0.1 M NaOAc, pH 5.0. Fractions were analyzed for high PE content using absorbance and fluorescence emission spectroscopy (see Materials and Methods 2.4.2). PE-containing fractions were collected and brought to 35% saturation with $(\text{NH}_4)_2\text{SO}_4$. Samples were stirred for an hour and subjected to centrifugation at 17,000 x g for 15 min. The PE-containing pellet was resuspended in a small volume (approximate 3 mL) of 5 mM potassium phosphate, pH 7.0 and dialyzed overnight against the same buffer plus 10 mM β -Me. The next day, the dialysis buffer was exchanged with fresh buffer and allowed to stir for another 2 hours.

Dialyzed samples were applied to a column (2.5 x 18 cm) of DEAE-Cellulose DE-52 (Whatmann), preequilibrated with 5 mM potassium phosphate, pH 7.0. After washing with 1 volume of starting buffer, the column was developed with a 550 ml linear 5-200 mM potassium phosphate, pH 7.0, gradient. Fractions were analyzed using absorbance and fluorescence emission spectroscopy to detect PE purity. Pure PE fractions were collected, brought to 70% saturation with $(\text{NH}_4)_2\text{SO}_4$, and allowed to stir for 30 min. Samples were then subjected to centrifugation at 17,000 x g for 15 min, resuspended in small volumes of 100 mM sodium phosphate, 1 mM sodium azide, pH 7.0, and dialyzed against the same buffer plus 10 mM β -Me overnight. Once all the $(\text{NH}_4)_2\text{SO}_4$ was sufficiently removed, pure PE samples were available for further analysis. For long-term storage, pure PE samples were stored in 70% $(\text{NH}_4)_2\text{SO}_4$ in a low phosphate buffer containing 1 mM sodium azide, in the dark at 4°C.

2.4 Protein and bilin analysis

2.4.1 Polyacrylamide gel electrophoresis

Polypeptides were diluted 1:1 in 2X Laemmli Sample Buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue, 5% β -Me; Bio-Rad, Hercules, CA), and were resolved by polyacrylamide gel electrophoresis (PAGE; 14% Tris-HCl w/v) in the presence of sodium dodecyl sulfate (SDS). SDS samples of PBS were only boiled for 3 min. Other polypeptide samples were boiled for 5 min prior to loading. Electrophoresis running buffer (1X) contained 25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3 [86]. Gels were run for 13 min at 100 V then 55 min at 200 V. Time varied between 45-60 min for the 200 V stage during electrophoresis to ensure appropriate polypeptide separation. The gels were then soaked in 10 mM zinc sulfate (ZnSO_4) for 10 min to visualize peptide linked bilins [87]. Zn-enhanced fluorescence of covalently attached bilins was visualized using FX imaging system (Bio-Rad) with excitation at 532 nm (detects PEB and PCB) and/or 635 nm (detects PCB only). Proteins in the gels were then stained by incubation in Coomassie brilliant blue G-250 overnight and destained in 10% methanol and 10% acetic acid.

2.4.2 Fluorescence emission and absorbance spectroscopy

Fluorescence emission and excitation spectra were recorded with a Perkin Elmer LS55 fluorescence spectrophotometer (Waltham, MA) with slit widths of 10 nm. The excitation wavelength was set at 490 nm for proteins containing PEB and at 590 nm for those with PCB. Chromophorylated samples were diluted to ~ 0.05 OD (at λ_{max}) prior to obtaining their fluorescence emission spectra to accommodate the sensitivity range of the machine. Samples produced in the absence of a lyase, which had very little or no chromophore attached, were

measured without dilution because they had an absorbance less than 0.05. Absorbance spectra were acquired using a lambda 35, dual-beam UV/Vis spectrometer (Perkin Elmer) with slit widths set to 0.5 nm. Chromophorylated samples were diluted to approximately 0.5-1.0 OD (at λ_{max}) prior to obtaining their absorbance spectra to accommodate the sensitivity range of the machine.

2.4.3 Western blotting

Western blots were performed to identify cyanobacteria proteins as previously described [88, 89] with few minor alterations. Two 14% Tris-HCl SDS-PAGE gels were loaded identically with pre-stained low molecular weight standards (Bio-Rad). One gel was stained and analyzed (see Materials and Methods 2.4.1) and the other was used for Western blot detection. For the protein transfer, a piece of PVDF transfer membrane was cut to the size of the gel and pre-wet in 100% methanol for 10 seconds, washed in ddH₂O 2 x 2.5 min and then equilibrated with cold Western transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% (v/v) methanol, 0.1% SDS) for at least 10 min. Four pieces of Whatman paper (3 mm) were cut slightly larger than the gel, and soaked in cold Western transfer buffer. The Western blot transfer was set up as follows: two pieces of Whatman paper, the SDS gel, the PVDF membrane, and two pieces of Whatman paper. Air bubbles were removed to prevent disruption during the transfer. The entire assembly was placed in a cassette and then into the electrode central core assembly module. The entire apparatus was put into a SDS-PAGE buffer tank with cold Western transfer buffer and a frozen cooling unit. The entire tank was surrounded by ice. Proteins were transferred at 100 volts for 1 hour.

Membranes were removed and placed in blocking buffer (TBST (20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% (v/v) Tween-20) and 5% (w/v) non-fat dried milk) for one hour at room temperature with mild agitation, and then washed with TBST. Membranes were then submerged in 40 ml TBST and primary polyclonal rabbit antibodies at the following dilutions: Anti B-PE 1:1,000 (against holo-B-PE purified from the red algae, *Porphyridium cruentum*; NOVUS Biologicals, Littleton, CO) and Anti FDCpeA 1:5,000 (against recombinant α -PE from *Fremyella diplosiphon* purified from *E. coli*; YenZym Antibodies, San Francisco, CA). The membrane incubated with the primary antibodies for an hour at room temperature with mild agitation. The antibody solution was removed and saved with 5% (w/v) bovine serum albumin for a maximum of 3 uses. The membrane was immediately washed with 100 mL of TBST for 20 seconds twice to remove any unbound antibodies from the containers. The membrane was then washed in 100 mL TBST five times for 3 minutes each wash with high agitation. The secondary antibodies (goat-anti rabbit, horse radish peroxidase-conjugated antibodies, Bio-Rad) were added to 1:10,000 dilution and incubated with the membrane for 45 minutes at room temperature with mild agitation. The secondary antibody solution was removed and the membrane was washed in the same manner as before. Membranes remained in TBST until ready for chemiluminescence detection. Luminal/enhancer and peroxide reagents (Bio-Rad) for enhanced chemiluminescence were mixed in a 1:1 ratio and incubated with the membrane for 3 minutes. Chemiluminescence was detected by the Chemi-Doc System (Bio-Rad). Membranes were stored in TBST at 4°C.

Membranes were stripped using 100 mM β -Me, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7. Membranes were submerged in the stripping buffer for 30 min at 70°C then washed in TBST 2 x 10 min. Membranes incubated with blocking buffer for one hour at room temperature with mild agitation before being probed with different primary antibodies.

2.4.4 Tryptic digestion and mass spectrometry

Tryptic digestion of proteins were conducted following the protocol described earlier [90]. Purified protein was exhaustively dialyzed against 2 mM sodium phosphate, pH 7.0 with 10 mM β -Me and then concentrated by ultrafiltration through an Amicon YM10 (Millipore). Concentrated samples were titrated to pH 2.0 with 1N HCl. The solution incubated for 45 min in the dark at room temperature for complete unfolding of protein. Trypsin was added to 2% (w/w) from a 5 mg/ml stock solution in 1 mM HCl. Ammonium bicarbonate was added to 0.1 M and the mixture was titrated to pH 7.5 with 1N NaOH. The digested mixture incubated at 30°C for 2h in the dark. An additional aliquot of trypsin was added (2% w/w) and incubated for another 2h. The reaction was quenched by adding 30% (v/v) glacial acetic acid. The mixture was passed through a C8 Sep-Pak cartridge for peptide purification (Waters Corporation, Milford, MA). The eluted sample was partially vacuum-dried and stored at -20°C for MALDI-Mass spectrometry (MS) analysis which was performed by our collaborators Richard Cole and Mohamed Boutaghou of the University of New Orleans as previously described [23].

3. Results

3.1 Phenotypes and whole cell analysis of wild type *F. diplosiphon* versus mutants

CpeY and CpeZ from *F. diplosiphon* were previously characterized using a heterologous coexpression system in *E. coli* as a CpcEF type lyase [23]. CpeY showed lyase activity on α -82 of PE, and CpeZ enhanced this activity by 40%, although CpeZ alone did not show any PEB ligation activity on α -PE or β -PE.

To better understand the functions of these proteins within native cyanobacteria, *cpeZ* and *cpeY* genes were knocked out in *F. diplosiphon* as clean deletions (generously provided by David Kehoe and Andrian Gutu of Indiana University) for further analysis. These deletions were generated to minimize polar effects. All cells were grown in green-enriched light, conditions which induce maximal PE production. Mutant strains were confirmed using PCR and Southern blot analysis (data not shown). Wild type (hereafter referred to as WT) cells grown in green-enriched light were characteristically brown in color as a result of producing both the red PE and the blue PC proteins within the rods of the phycobilisome (PBS) (see Fig. 12). Both the *cpeY* and *cpeZ* mutants ($\Delta cpeY$ and $\Delta cpeZ$, respectively) displayed phenotypes that differed from WT. Both $\Delta cpeY$ and $\Delta cpeZ$ mutants grown in green-enriched light were green in color, indicating a reduction of PE produced (see Fig. 12). The whole cells were analyzed by absorbance spectroscopy as shown in Fig. 13. The absorbance spectrum for WT (see Fig. 13, solid line) shows a large amount of PE ($\lambda_{\text{max}} = 560\text{-}570\text{ nm}$), whereas the spectrum for the $\Delta cpeY$ mutant (see Fig. 13, dotted line) indicates no evidence of PE. Very little PE was detected in the $\Delta cpeZ$ mutant also (see Fig. 13, dashed line). From this observation, we can conclude *cpeY* and *cpeZ* are two genes involved in the induction, biosynthesis, or stability of PE.

A



B



Fig. 12 Phenotypes of WT versus $\Delta cpeY$ and $\Delta cpeZ$ mutant cells grown in green-enriched light

A. Liquid cultures of wild type *F. diplosiphon* (FD WT, brown in color), $\Delta cpeY$ mutant (FD $\Delta cpeY$, green in color), and $\Delta cpeZ$ mutant (FD $\Delta cpeZ$, green in color) cells. Cells were grown in green light in B-HEPES liquid media. **B.** B-HEPES agar plates of wild type *F. diplosiphon* (WT, brown in color), $\Delta cpeY$ mutant (green in color), and $\Delta cpeZ$ mutant (green in color) cells. Cells were grown in green-enriched light on B-HEPES agar plates containing $10 \mu\text{g}\cdot\text{mL}^{-1}$ of the antifungal agent carbendazim.

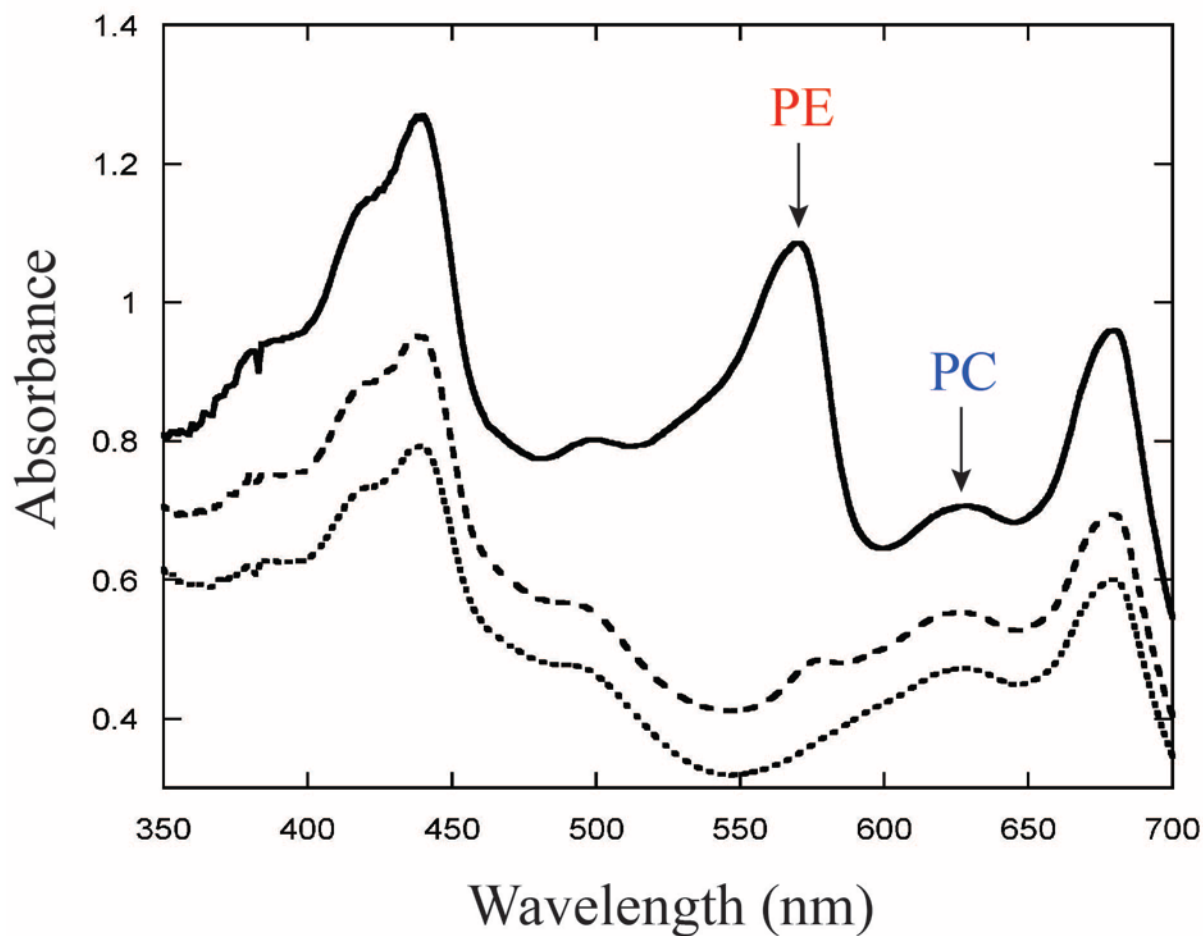


Fig. 13 Whole cell spectra of WT versus $\Delta cpeY$ and $\Delta cpeZ$ mutant cells

This figure represents the whole cell absorbance spectrum of WT (solid line), $\Delta cpeY$ mutant (dotted line) and $\Delta cpeZ$ (dashed line) from *F. diplosiphon* grown under green-enriched light. The peaks at 560 nm represents PE, 620 nm represent PC, and 440/680 nm represent chlorophyll *a*.

3.2 Analysis of phycobilisomes purified from *F. diplosiphon* WT versus mutants

3.2.1 Sucrose density gradient ultracentrifugation

Phycobilisomes (PBS) are macromolecular complexes ranging in molecular weight from 5×10^6 Da to 20×10^6 Da depending on the cyanobacterial species and phycobiliprotein (PBP) composition, the latter of which can be altered by a number of factors [9]. PBS from WT and mutant *F. diplosiphon* cells grown in green-enriched light were purified using sucrose density gradients. The bulk of pure PBS from WT migrated to the 0.75 M sucrose layer (fraction 2), whereas the bulk of pure PBS from both the $\Delta cpeY$ and $\Delta cpeZ$ mutants migrated to the 0.50 M sucrose layer (fraction 1) (see Fig. 14). Fractions 3 and 4 contained dissociated PBP. Proteins in the 0.50 M sucrose layer are lower in molecular weight and less dense than the proteins in the 0.75 M sucrose layer. Larger and denser protein complexes migrate further within the gradient. These results indicate that the PBS from the mutants are smaller in size and less dense due to the reduced PE levels associated with the PBS. The PBS extracted from WT were purple in color due to the presence of both PC and PE in the rods. PBS isolated from the $\Delta cpeY$ and $\Delta cpeZ$ mutants were blue in color since PE levels were reduced (see Fig. 14).

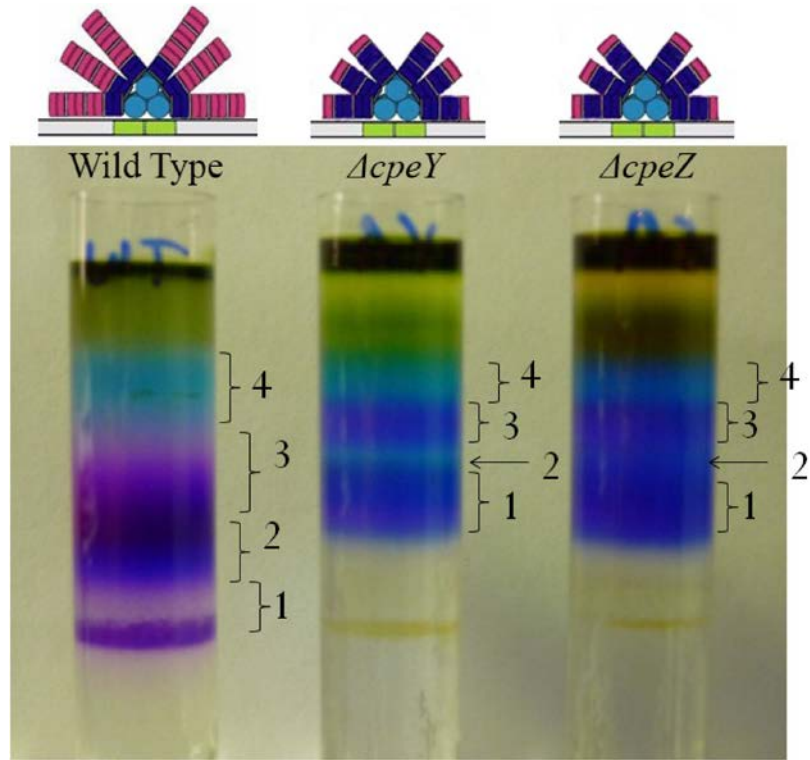


Fig. 14 PBS sucrose density gradients

This figure shows the sucrose density gradients for wild type, $\Delta cpeY$ mutant, and $\Delta cpeZ$ mutant samples. Four PBS fractions were collected from each sample (numbered 1 through 4). The PBS pictures above the gradients represent the suspected PBS generated from those cells.

3.2.2 Absorbance and fluorescence emission spectroscopy analysis

All four fractions were extracted from the sucrose gradients and analyzed by absorbance and fluorescence emission spectroscopy. The bulk of intact PBS from *F. diplosiphon* WT was isolated from fraction 2, and the bulk of intact PBS from both $\Delta cpeY$ and $\Delta cpeZ$ were isolated from fraction 1; therefore, these fractions were used for further analysis. The absorbance spectrum for WT PBS (see Fig. 15 A, black line) shows a large amount of PE ($\lambda_{\text{max}} = 568$ nm) production in comparison to PC ($\lambda_{\text{max}} = 616$ nm) and to AP ($\lambda_{\text{max}} = 650$ nm). When excited at 490 nm, a wavelength that excites PE preferentially, the fluorescence emission peak around 646 nm indicates most of the energy is being transferred to PC (see Fig. 15 B, black line). Excitation at 490 nm directly excites PE thus resulting in the peak around 580 nm (see Table 2). Both $\Delta cpeY$ and $\Delta cpeZ$ mutants have very little PE incorporated into the PBS (see Fig. 15 A, blue and red lines, respectively) when compared to WT, but some energy transfer from PE to PC is observed (see Fig. 15 B, blue and red lines, respectively). PE in PBS isolated from both $\Delta cpeY$ and $\Delta cpeZ$ mutants has a maximum absorbance at 578 and 577, respectively (see Fig. 15 A and Table 2), indicating a slight shift in the spectrum when compared to WT ($\lambda_{\text{max}} = 568$ nm). One possible explanation for this shift is the presence of a PCB chromophore on either subunit of PE. Another possibility is the presence of a non-covalently attached PEB chromophore in the bilin-binding pocket of one of the PE subunits. Both situations would result in a slightly red shifted absorbance spectrum of the protein due to the presence of an extra double bond within the bilin.

PE, PC, and AP levels were quantified as previously described (see Material and Methods 2.3.2) and are listed in Table 3. These quantitative data indicate both mutants have reduced levels of PE production and incorporation into the PBS when compared to WT. Both PC and AP levels were also increased when compared to WT PC and AP levels (see Table 3). This

result is most likely attributed to the compensation of light harvesting proteins in the absence of the distal rod PE protein synthesis.

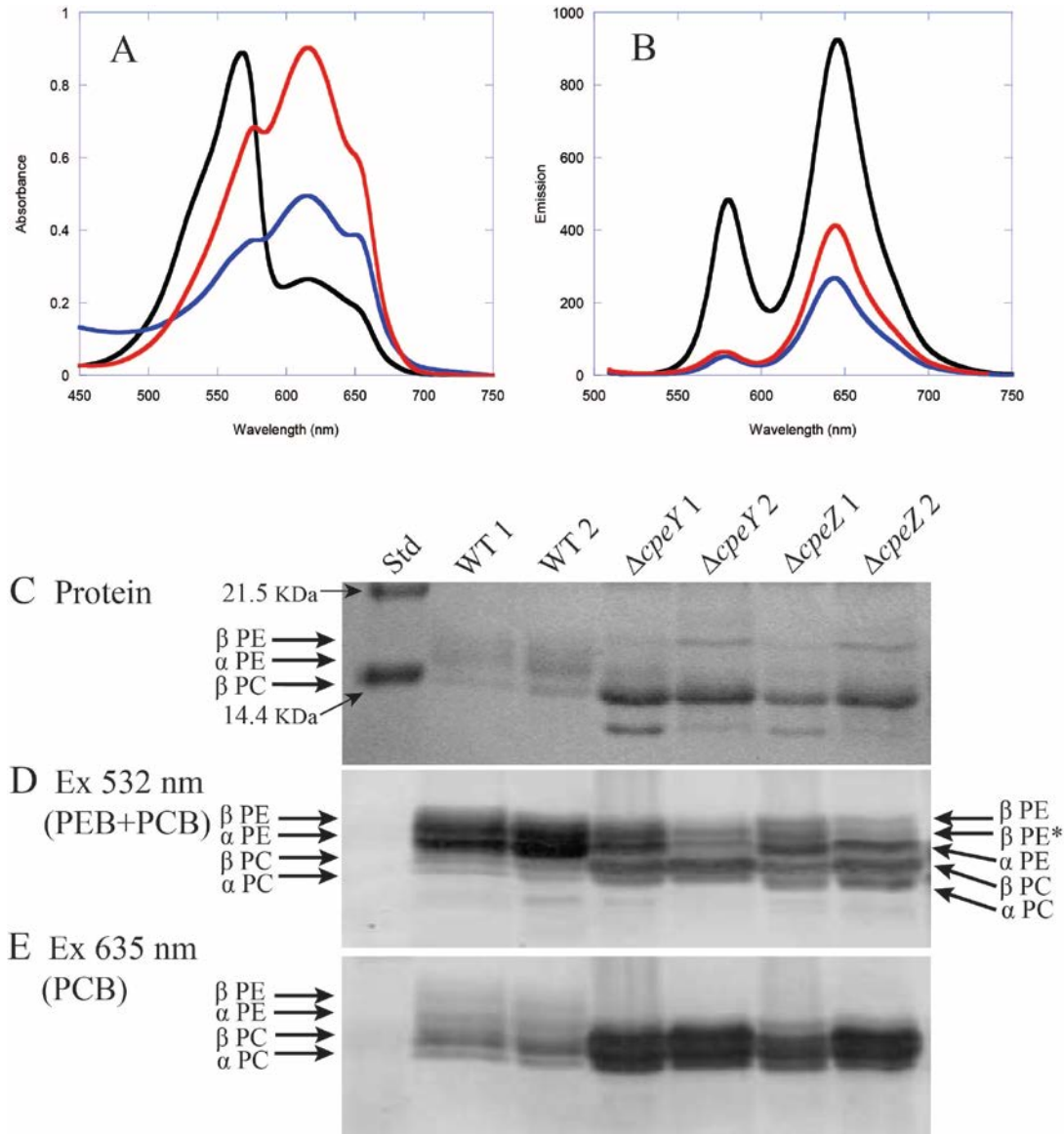


Fig. 15 Analysis of PBS purified from WT, $\Delta cpeY$, and $\Delta cpeZ$

A. Absorbance spectra of PBS samples purified from WT fraction 2 (black line), $\Delta cpeY$ fraction 1 (blue line), and $\Delta cpeZ$ fraction 1 (red line). **B.** Fluorescence emission (with excitation at 490 nm) spectra of PBS samples purified from WT fraction 2 (black line), $\Delta cpeY$ fraction 1 (blue line), and $\Delta cpeZ$ fraction 1 (red line). **C.** The Coomassie-stained SDS polyacrylamide gel for PBS purified from WT 1 and 2, $\Delta cpeY$ 1 and 2, and $\Delta cpeZ$ 1 and 2. Numbers 1 and 2 indicate the fractions extracted from the sucrose density gradients. **D and E.** The zinc enhanced fluorescence of the gel in part “C” excited at 532 nm “D” and 635 nm “E.” Lane “Std” indicates the molecular weight standard.

Table 2: Spectral properties of PBS purified from WT, $\Delta cpeY$, and $\Delta cpeZ$

Strain [*]	PE λ_{\max} (nm)	PC λ_{\max} (nm)	AP λ_{\max} (nm)	PE Fluorescence Emission λ_{\max} (nm)	PC Fluorescence Emission λ_{\max} (nm)
WT 2	568	616	650	580	646
$\Delta cpeY$ 1	578	615	650	579	644
$\Delta cpeZ$ 1	577	616	650	578	644

* Numbers 1 and 2 indicate the fractions extracted from the sucrose density gradients (see Fig. 14).

Table 3: Quantification of PBP in PBS samples purified from WT, $\Delta cpeY$, and $\Delta cpeZ$

Strain [*]	[PE] ¹	[PC] ¹	[AP] ¹	[PE/AP] ¹	[PC/AP] ¹	[PE/PC] ¹
WT 2	0.138	0.039	0.058	2.373	0.679	3.494
$\Delta cpeY$ 1	0.120	0.154	0.360	0.333	0.427	0.780
$\Delta cpeZ$ 1	0.090	0.203	0.274	0.331	0.742	0.446

* Numbers 1 and 2 indicate the fractions extracted from the sucrose density gradients (see Fig. 14)

¹ PBP concentrations are in mg·mL⁻¹ (see Materials and Methods 2.3.2)

3.2.3 SDS-PAGE and Western blot analysis

PBS samples from WT and mutant *F. diplosiphon* were further analyzed by SDS-PAGE to visualize PBP levels. The bilin attachment to proteins was examined by zinc staining of the gel to enhance covalently-bound bilin fluorescence as shown in Fig. 15 D and E; protein levels were shown by subsequent staining of the same gel with Coomassie Blue (see Fig. 15 C). PBP samples were loaded to approximately 1 μ g of AP (unaffected during PBP composition alteration during CA). When compared to WT, both $\Delta cpeY$ and $\Delta cpeZ$ mutants have drastically reduced levels of β -PE as well as reduced α -PE levels (see Fig. 15 C). The zinc stained gel was visualized after excitation at 532 nm to observe both PEB and PCB (see Fig. 15 D) or after excitation at 635 nm to observe the presence of PCB bound PBP (see Fig. 15 E). In both $\Delta cpeY$ and $\Delta cpeZ$ mutants, PE levels are reduced while PC levels are increased when compared to WT samples which have more PE associated into the PBS (see Fig. 15 D and E). In the $\Delta cpeZ$ mutant PBS sample, there appears to be two β -PE bands when separated by SDS-PAGE, both containing PEB as shown when excited at 532 nm (see Fig. 15 D, labeled β PE and β PE*). The extra β -PE band (β -PE*) appears to be slightly smaller in molecular weight than the actual size of β -PE as seen in WT (see Fig. 15 D). One possible explanation is that β -PE* is missing one or two PEB (587 Da each). Thus, in native cyanobacteria, when CpeY or CpeZ are absent from cells, PE levels are dramatically lower and PC production and association into the PBS appears to be increased to compensate.

To determine the presence of PE subunits in PBS samples purified from WT, $\Delta cpeY$ and $\Delta cpeZ$, PBS were subjected to SDS-PAGE and then transferred to a PVDF membrane and probed with two kinds of primary antibodies. The membrane was first probed with Anti-B-PE antibodies which detect both α and β subunits of PE (data not shown). Both α and β PE were

detected in WT, $\Delta cpeY$ 1 and $\Delta cpeZ$ 1 PBS samples (data not shown). The PVDF membrane was stripped and reprobed with Anti-FDCpeA antibodies which detect only α -PE from *F. diplosiphon* as seen in Fig. 16. When comparing the band intensity of α -PE, $\Delta cpeY$ 1 and $\Delta cpeZ$ 1 samples appear less intense when compared to WT despite all samples being loaded with approximately 1 μ g of AP. To fully analyze the effect of the lack of CpeY and CpeZ on PE synthesis or stability, PE was purified from WT and mutant strains of *F. diplosiphon* and analyzed further.

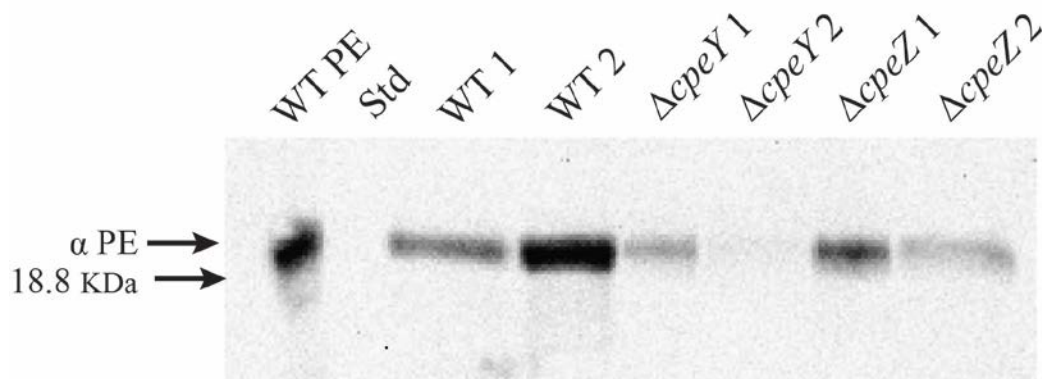


Fig. 16 Western blot analysis of PBS purified from WT, $\Delta cpeY$, and $\Delta cpeZ$

Western blot of PBS samples blotted with primary antibodies against FD CpeA (α -PE from *F. diplosiphon*). Numbers 1 and 2 indicate the fractions extracted from the sucrose density gradients. Lane “Std” indicates the molecular weight standard. “WT PE” was loaded as a control.

3.3 Biochemical characterization of PE purified from $\Delta cpeY$ mutant

PE was purified from both WT and $\Delta cpeY$ mutant *F. diplosiphon* cells (approximately 4 L) using gel filtration chromatography and ion exchange chromatography as described earlier (see Materials and Methods 2.3.3). These proteins were purified from whole cells and represent the entire pool of PE synthesized. This includes PE assembled into PBS and PE that may not be assembled into PBS. The purified PE was characterized using absorbance and fluorescence emission spectroscopy. PE from WT cells had an absorbance maximum at 563 nm with a shoulder at 545 nm, and was highly fluorescent with an emission maximum at 574 nm (see Fig. 17 A and Table 4). The yield of PE obtained from the $\Delta cpeY$ mutant was drastically less than that of WT. The major absorbance peak was at 566 nm with two shoulders at 599 nm and 616 nm, which might be due to the presence of some PCB (see Fig. 17 B, solid line and Table 4). For obtaining the fluorescence emission spectrum, the sample was excited at 490 nm initially to observe the presence of PEB (dashed line; emission maximum at 573 nm) and at 590 nm to observe the presence of PCB (dotted line; emission maximum at 635 nm) (see Fig. 17 B and Table 4). The presence of PCB might be due to contamination of the PE preparation with AP or PC.

For further analysis, the PE from both WT and $\Delta cpeY$ mutant were separated by SDS-PAGE. The bilin addition on both α and β subunits of PE were observed by zinc staining the gel to enhance bilin fluorescence. Both α and β subunits of WT PE contain exclusively PEB as expected (see Fig. 17 C and D, lane 1). Zinc staining of PE from the $\Delta cpeY$ mutant indicates the β -PE appears like the WT both in apparent molecular weight and PEB fluorescence, but the α -PE may have some PEB replaced with PCB (see Fig. 17 C and D, lanes 2-4). The PE purified from the $\Delta cpeY$ mutant is also slightly contaminated with AP. However, the main effect of the $\Delta cpeY$

appears to be on the α -PE subunit. In the absence of CpeY, it appears another lyase may be attaching PCB, presumably at Cys α -82.

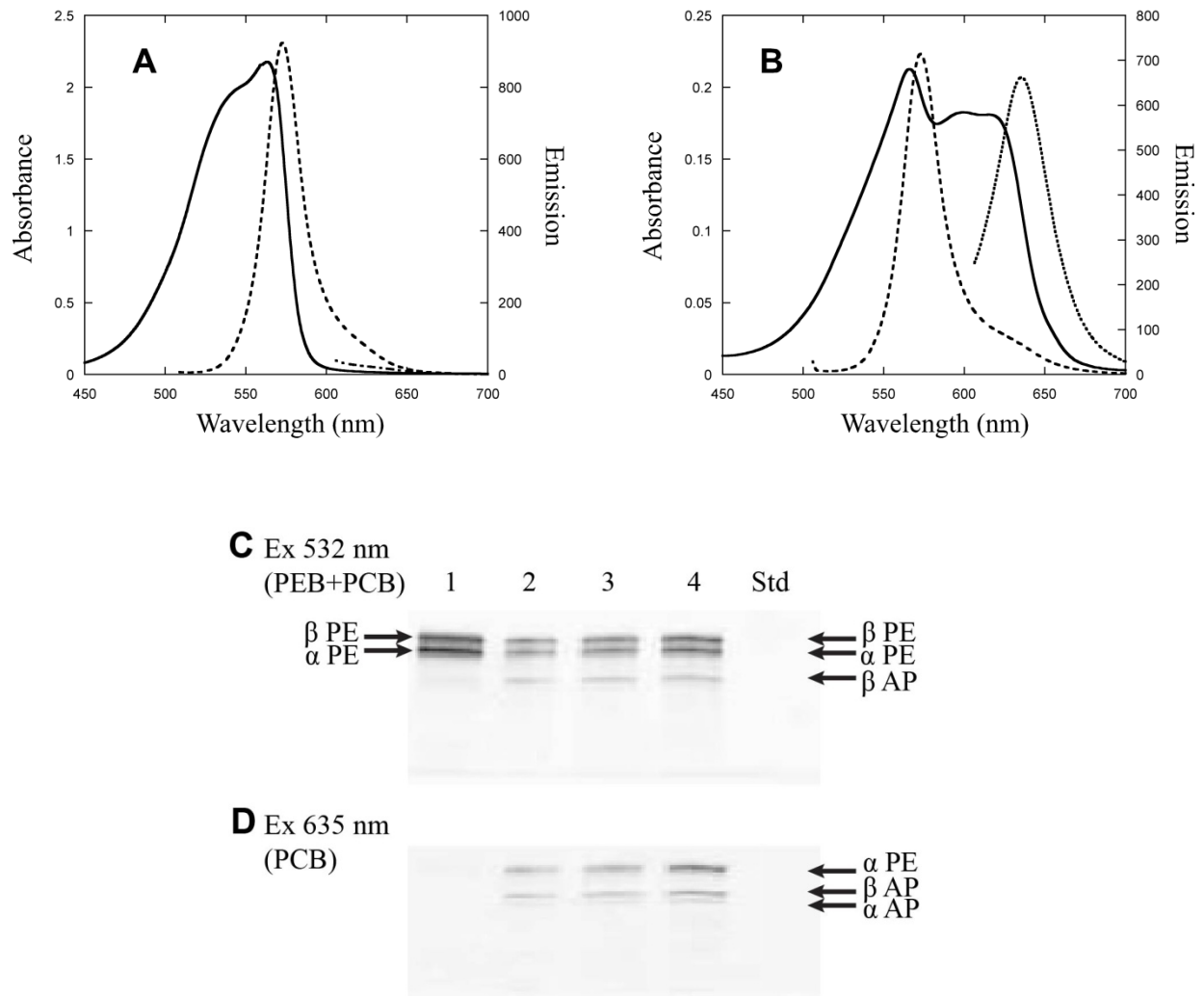


Fig. 17 Analysis of PE purified from WT and $\Delta cpeY$ mutant

A. Absorbance (solid line) and fluorescence emission (excited at 490 nm, dashed line; excited at 590 nm, dashed and dotted line) spectra of PE purified from WT *F. diplosiphon*. **B.** Absorbance (solid line) and fluorescence emission (excited at 490 nm, dashed line; excited at 590 nm, dotted line) spectra of PE purified from the $\Delta cpeY$ mutant. **C and D.** The zinc stained SDS polyacrylamide gel for PE purified from WT (lane 1) and $\Delta cpeY$ mutant (lanes 2-4) excited at 532 nm “C” and 635 nm “D.” Lane “Std” indicates the molecular weight standard.

Table 4: Spectral properties of PE purified from WT, $\Delta cpeY$, and $\Delta cpeZ$

Strain	λ_{max} (nm)	Fluorescence Emission λ_{max} (nm) excited at 490 nm	Fluorescence Emission λ_{max} (nm) excited at 590 nm
WT	545 (sh), 563	573	ND
$\Delta cpeY$	566, 599 (sh), 616 (sh)	573	635
$\Delta cpeZ$	542 (sh), 572	573	ND

“ND” represents data that were not determined

“sh” indicates shoulder

3.4 *Synechocystis* sp. PCC 6803 CpcEF lyase activity of PCB and PEB ligation to CpeA

Since the α -PE and α -PC subunits are similar in amino acid sequence and structure, and CpeY is homologous to a fusion of CpcE and CpcF bilin lyase which is specific for α -PC at Cys-82, we hypothesized that the CpcEF lyase may be able to attach PCB to CpeA. This CpcEF lyase is expressed in red light and green light since some PC is produced in both light conditions. To test our hypothesis, we used an *in vivo* heterologous coexpression system to express CpcEF lyase, hexa-histidine-tagged CpeA (HT-CpeA), and HoI/PcyA in *E. coli*. The plasmids pCpeA, pCpcEF, pPcyA were coexpressed in *E. coli* (see Table 1), and the purified HT-CpeA protein obtained was characterized using absorbance and fluorescence emission spectroscopy. *E. coli* cells containing pCpeA/pPcyA had no significant absorbance or fluorescence emission spectrum (data not shown) whereas cells containing pCpeA/pCpcEF/pPcyA was shown to have some absorbance at 625 nm and fluorescence emission at 640 nm, corresponding to native PCB bound phycobiliproteins (see Fig. 18 A). The purified HT-CpeA samples were analyzed by SDS-PAGE (see Fig. 18 B and C). The bilin addition to HT-CpeA was examined by zinc staining of the gel to enhance bilin fluorescence as shown in Fig. 18 C; protein content was shown by subsequent staining of the same gel with Coomassie Blue (see Fig. 18 B). Cells containing pCpeA, pCpcEF and pPcyA showed Zn-enhanced fluorescent protein (see Fig. 18 C). No fluorescent protein was detected in cells without CpcEF lyase (data not shown). The conclusion of this experiment is that CpcEF can attach PCB to CpeA, albeit not as efficiently as for its native substrate CpcA [31]. It is likely that in the absence of the CpeY lyase within the $\Delta cpeY$ mutant, CpcEF can ligate PCB to α -PE in *F. diplosiphon*.

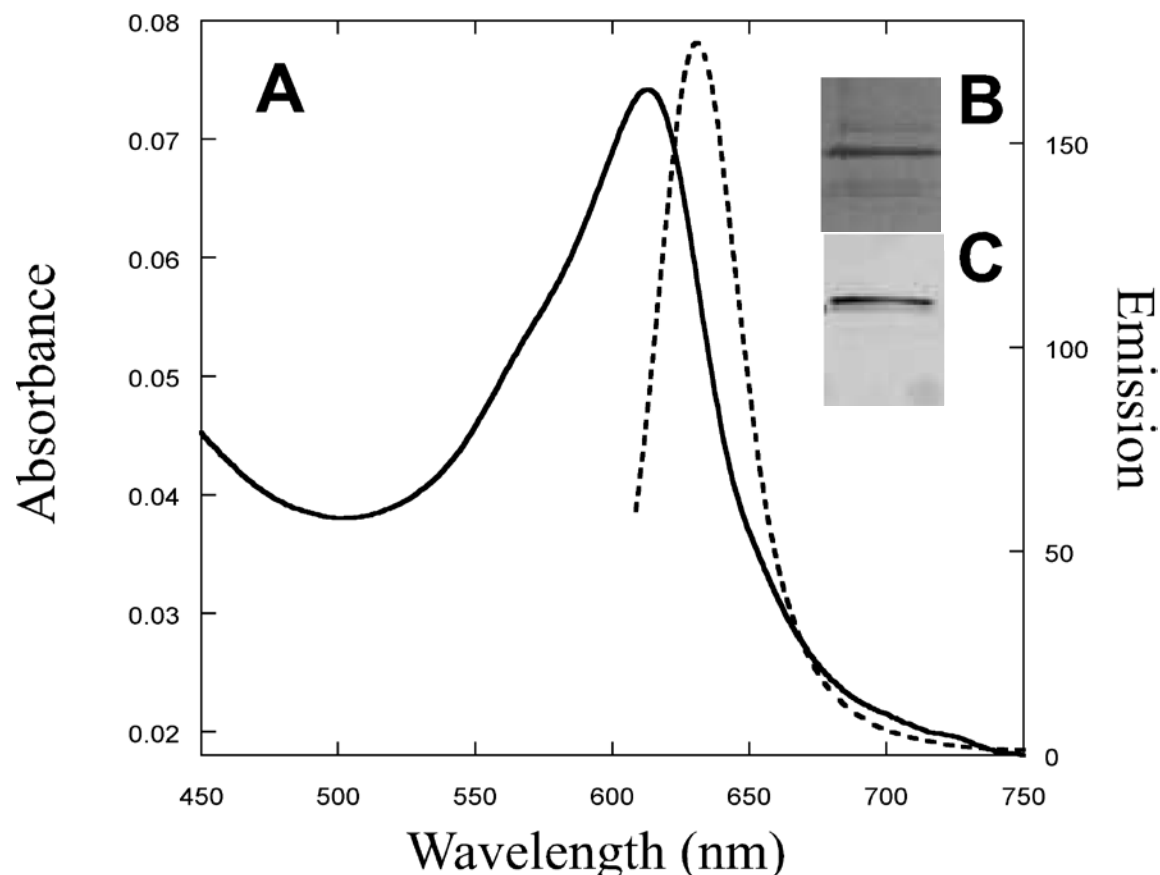


Fig. 18 PCB ligation on CpeA catalyzed by *Synechocystis* sp. PCC 6803 CpcEF

A. Absorbance (solid line) and fluorescence emission (dotted line) spectrum from purified HT-CpeA obtained by coexpressing pCpeA, pCpcEF, and pPcyA. **B.** The Coomassie-stained SDS polyacrylamide gel for purified HT-CpeA (CpeA-PCB). **C.** The zinc enhanced fluorescence of the gel pictured in part “B.”

3.5 Biochemical characterization of PE purified from $\Delta cpeZ$ mutant

PE was purified from the $\Delta cpeZ$ mutant of *F. diplosiphon*, and was characterized using absorbance and fluorescence spectroscopy. PE obtained from the $\Delta cpeZ$ mutant had an absorbance maximum of 572 nm with a shoulder at 542 nm (see Fig. 19 A, solid line), which is a little red shifted when compared to WT (see Fig. 17 A, solid line and Table 2). This may be caused by the presence of PCB, or the presence of PEB which is not covalently bound to the PE substrate. For obtaining the fluorescence emission spectrum, the sample was excited at 490 nm initially to observe the presence of PEB (dashed line; emission maximum at 573 nm) and 590 nm for PCB (dotted line; no emission from PCB attachment was observed) (see Fig. 19 A).

The pure PE samples were separated by SDS-PAGE. The bilin addition to both PE subunits was examined by zinc staining of the gel to enhance bilin fluorescence as shown in Fig. 19 C and D; protein content was shown by subsequent staining with Coomassie Blue (see Fig. 19 B). Protein staining of pure PE isolated from $\Delta cpeZ$ mutant shows that β -PE levels are drastically reduced and ultimately non-detectable (see Fig. 19 B, lanes 3-5) when compared to WT PE (see Fig. 19 B, lanes 1 and 2). Zinc staining of PE isolated from the $\Delta cpeZ$ mutant shows that α -PE contains only PEB but very little chromophorylated β -PE is detected (see Fig. 19 C and D, lanes 3-5). In WT, both α and β subunits of PE contain exclusively PEB (see Fig. 19 C and D, lanes 1 and 2); however, excitation of the SDS-polyacrylamide gel at 635 nm appears to excite PEB even though excitation at this wavelength excites PCB preferentially. Since no PCB was detected on mutant PE by fluorescence spectroscopy (Fig. 19 A, dotted line) and PEB was fluorescing in WT PE samples when excited at 635 nm (Fig. 19 D, lanes 1 and 2), the presence of PCB on PE purified from $\Delta cpeZ$ mutant is unlikely.

One interesting result was that both α and β subunits of PE obtained from $\Delta cpeZ$ mutant showed a slight molecular weight shift when compared to WT PE subunits. One possible explanation for this shift is that both α -PE and β -PE are missing one or two PEB (578 Da each). The main effect of deleting *cpeZ* appears to be the biosynthesis of the β subunit of PE, resulting in drastically reduced protein levels. Recombinant protein analysis showed CpeZ does not act as a bilin lyase alone but it does enhance the bilin lyase activity of CpeY [23]. It is likely that CpeZ is functioning as a chaperone, possibly for both α -PE and β -PE in native cyanobacteria. It may also interact or enhance activity of β -subunit lyases. These proteins were purified from whole cells and represent the total pool of PE synthesized. Some of this pool may not be incorporated into PBS as efficiently, especially if chromophores are missing. Stability and turnover for subunits without chromophores has been affected in other lyase mutants [28, 38, 45, 51, 91, 92]. Thus, in the absence of CpeZ, the main effect appears to be that β -PE is not accumulating to the same degree as the α -PE subunit, presumably because bilin attachment to the Cys residues of PE is not occurring.

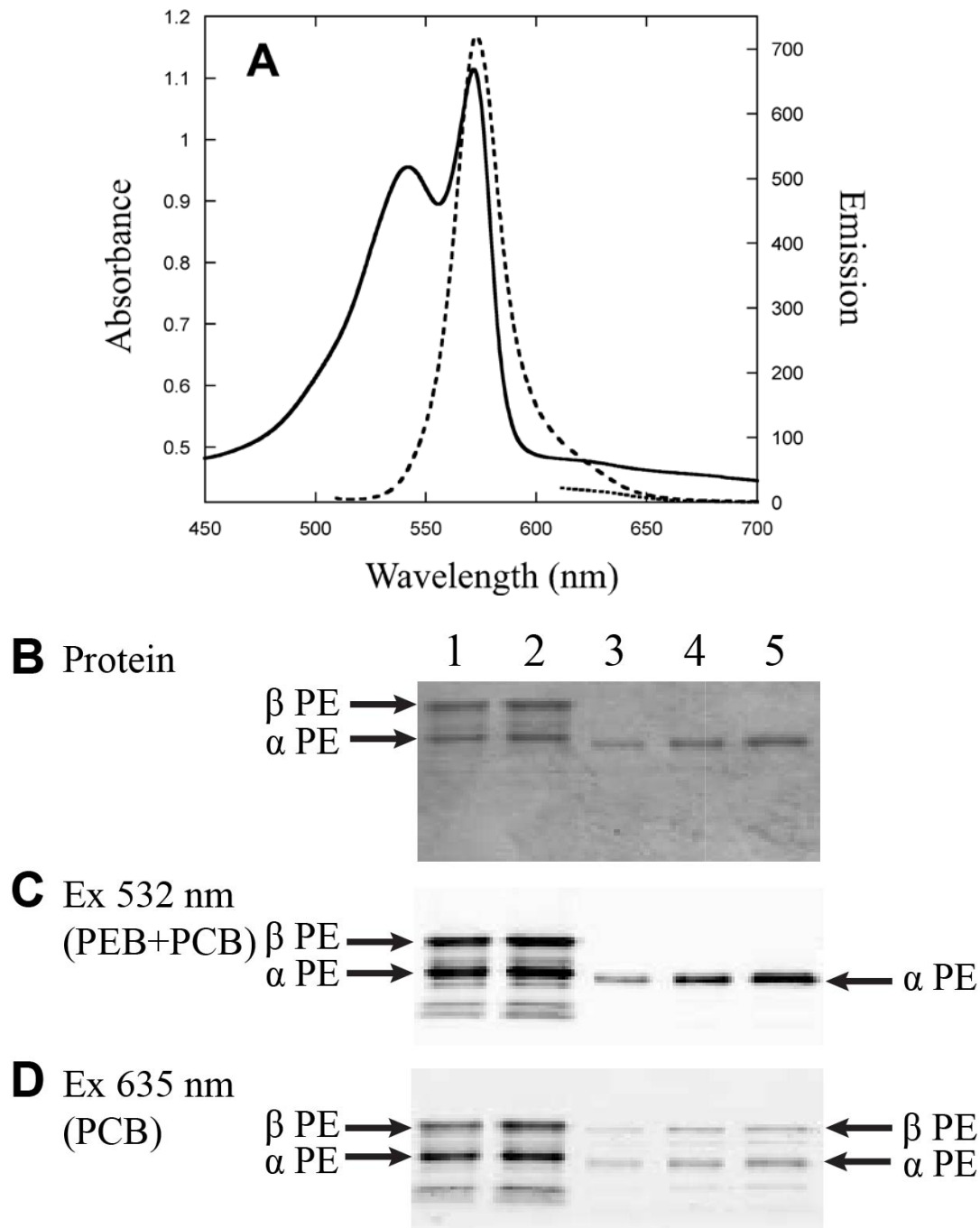


Fig. 19 Analysis of PE purified from WT and $\Delta cpeZ$ mutant

A. Absorbance and fluorescence emission spectra of PE purified from the $\Delta cpeZ$ mutant; solid line is absorbance, dashed line is the fluorescence emission excited at 490 nm, and dotted line is the fluorescence emission excited at 590 nm. **B.** The Coomassie-stained SDS polyacrylamide gel for PE purified from WT (lanes 1 and 2) and $\Delta cpeZ$ mutant (lanes 3-5); **C and D.** The Zinc enhanced fluorescence for the gel pictured in “B” excited at 532 nm “C” and 635 nm “D.”

3.6 Western blot analysis of PE purified from WT, $\Delta cpeY$ and $\Delta cpeZ$

PE samples purified from WT, $\Delta cpeY$ and $\Delta cpeZ$ were subjected to SDS-PAGE and then transferred to a PVDF membrane and probed with Anti-B-PE antibodies which detect both α and β subunits of PE as seen in Fig. 20. Both α and β PE were detected in WT PE. In $\Delta cpeY$ or $\Delta cpeZ$ mutants, β -PE was not detected; only α -PE was detected for both mutant samples. Figure 20 also shows that α -PE from $\Delta cpeY$ (denoted as α -PE*) is lower in molecular weight than α -PE from WT. In the absence of CpeY in *F. diplosiphon*, β -PE accumulation is reduced, and α -PE is lower in molecular weight most likely due to the absence of a PEB (587 Da) at Cys-82 of α -PE. In the absence of CpeZ in *F. diplosiphon*, β -PE biosynthesis and accumulation are also reduced.

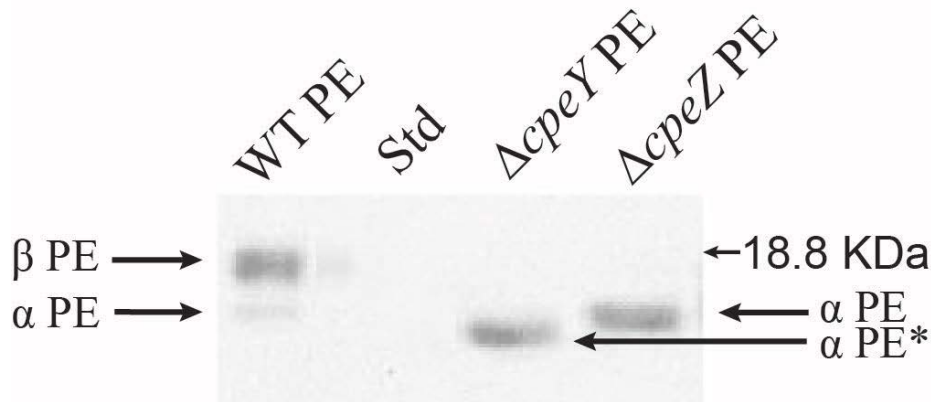


Fig. 20 Western blot analysis of PE purified from WT, $\Delta cpeY$, and $\Delta cpeZ$

Western blot analysis of PE purified from WT, $\Delta cpeY$, and $\Delta cpeZ$ blotted with primary antibodies against B-PE, which recognizes both α and β subunits of PE. Lane "Std" indicates the molecular weight standard. "WT PE" was loaded as a control.

3.7 Mass spectrometry analysis of PE purified from $\Delta cpeY$ and $\Delta cpeZ$ mutants

PE purified from WT, *cpeY* mutant, and *cpeZ* mutant was digested with trypsin, purified using a C8 Sep-Pak cartridge, and characterized using MALDI-mass spectrometry (MS). MALDI-MS analysis was performed by our collaborators Richard Cole and Mohamed Boutaghou of the University of New Orleans. Sequences of expected tryptic peptides containing Cys residues with bound PEB can be found in Table 5. PE extracted from WT resulted in five major peaks that were identified as peptides containing a PEB bilin; 2 from α -PE and 3 from β -PE (see Table 6). PEB attachment occurred at Cys-82 (m/z 935) and Cys-139 (m/z 1089) from α -PE. PEB attachment also occurred at Cys-80 (m/z 1250), Cys-165 (m/z 2104) and double attachment to Cys-48/Cys-59 (m/z 4641) from β -PE. PE from both $\Delta cpeY$ and $\Delta cpeZ$ only had PEB attachment to α -PE at both Cys-82 and Cys-139 (see Table 6). Neither chromophorylated nor non-chromophorylated β -PE peptides were detected in either mutant. These results confirm our previous results that in the absence of CpeY and CpeZ in *F. diplosiphon*, β -PE levels are reduced.

Table 5: Expected sequence and average mass per charge (m/z) of tryptic digested PE peptides

Cys Position	Avg. m/z	Avg. m/z +PEB (587)	Start ¹	End ¹	Sequence ²
α-82	349	936	82	84	(K) <u>C</u> AR(D)
α-139	504	1091	138	142	(R)G <u>C</u> APR(D)
β-80	665 (681)*	1252 (1268)*	77	82	(R)MAA <u>C</u> LR(D)
β-165	1520	2107	165	178	(R) <u>C</u> ASLVAEASSYFDR(V)
β-48/59	4044 (4060)* (4076)**	4631 (4647)* (4663)**	36	75	(R)LDAVNAIASNAS <u>C</u> MVSDAVA GMIC <u>E</u> NQGLIQAGGNCYPNR(R)

*represent m/z values of peptides with an oxidized methionine (+16) indicated in parentheses

** represent m/z values of peptides with 2 methionine oxidations (+32) indicated in parentheses

¹ represents the amino acid position

² Cys residues available for potential bilin binding are underlined

(Generated by MS-Digest in ProteinProspector 5.10.9 Basic from UCSF)

Table 6: Observed MALDI-MS peaks of tryptic digested PE peptides

Strain	α -82 m/z	α -139 m/z	β -80 m/z	β -165 m/z	β -48/59 m/z
WT	936	1090	1251	2104	4641
$\Delta cpeY$	936	1090	ND	ND	ND
$\Delta cpeZ$	935	1090	ND	ND	ND

“ND” represents peaks that were not detected in MS results

4. Discussion

Recombinant CpeY from *Fremyella diplosiphon* has been characterized as a CpcEF type lyase which attaches PEB to Cys-82 of α -PE, and CpeZ enhanced this activity by 40% [23]. Recombinant CpeZ did not interact with CpeY, and it did not act as a lyase alone on either α -PE or β -PE. Kahn *et al.* isolated a transposon mutant in the *cpeY* gene in *F. diplosiphon* [44], resulting in a reduced PE phenotype. Complementation studies revealed that only when both *cpeY* and *cpeZ* were added was the WT phenotype restored. However, the function of CpeZ was never fully analyzed. This study analyzes the role of both CpeY and CpeZ from clean deletion mutants in *F. diplosiphon*.

Just as Kahn *et al.* observed reduced PE levels in the *cpeY* mutant, we observed a reduced PE phenotype in our $\Delta cpeY$ mutant cells. PBS purified from the $\Delta cpeY$ mutant showed a slight red-shifted absorbance spectrum believed to be caused by the presence of PCB on α -PE. PCB has a longer conjugated double bond system than PEB which can cause the red-shifted absorbance spectrum. Our analysis of PE confirmed that α -PE did have PCB attached to it, most likely at Cys-82, in the absence of the CpeY lyase. We demonstrated that recombinant CpcEF lyase, which naturally has a higher affinity for PCB [32], was able to ligate PCB on α -PE albeit in lower amounts than it normally shows for its cognate substrate α -PC. We propose that in native cyanobacteria, endogenous CpcEF can also possibly ligate PCB to α -PE in the absence of CpeY. Jung *et al.* observed similar results from insertion mutants in the genes encoding the PecEF lyase/isomerase (homologs of CpcEF) generated and isolated from *Nostoc (Anabaena)* sp. PCC 7120 [38]. When both *pecE* and *pecF* were mutated, PEC levels were severely reduced, and the natural PVB covalently bound at α -84 was replaced with PCB by endogenous CpcEF lyase [38].

Another possibility for the red-shifted absorbance spectrum for the PE purified from the mutants is the presence of non-covalently attached PEB to PE. Shen *et al.* used mass spectrometry to detect non-covalent association of PCB with Cys-82 of β -PC in the absence of CpcS-I in mutants from *Synechococcus* sp. PCC 7002 [45]. Non-covalent binding of chromophores in the bilin-binding pocket of PBP results in a shift of the absorbance spectrum due to the presence of an extra double bond, as observed in our $\Delta cpeY$ mutant as well as our $\Delta cpeZ$ mutant analysis of purified PBS and PE.

PE purified from $\Delta cpeY$ mutant showed α -PE had a slightly lower molecular weight (α -PE*) on SDS-PAGE and detected by Western blotting when compared to WT α -PE. It is likely that the cause of this shift resulted from the absence of a PEB (587 Da) bilin on α -PE due to the deletion of the lyase CpeY. Bilin lyase mutants in other species of cyanobacteria have resulted in similar observations of molecular weight shifts due to the absence of a covalently-bound bilin to PBP. Molecular weight shifts in PC subunits as well as reduced amounts of PC produced were observed in *cpcE/cpcF* lyase mutants [28, 29, 92, 93], *cpcT* lyase mutants [51], and *cpcS/cpcU* lyase mutants [45]. Due to these previous studies in lyase mutants, it is expected that when a bilin is missing on a PBP subunit, a shift in molecular weight should be detected on SDS-PAGE, as observed in our analysis of PE purified from $\Delta cpeY$ mutant. Similar results were observed in PE isolated from the $\Delta cpeZ$ mutant. Both α and β subunits of PE had lower molecular weights when compared to PE purified from WT cells. PBS from $\Delta cpeZ$ mutant had an extra β -PE band denoted as β -PE*, which had a slightly smaller molecular weight than the normal β -PE. In the PBS samples purified from the *cpeY* transposon mutant isolated by Kahn *et al.*, both β -PE* and β -PE (same molecular weight as WT) subunits were present in roughly equal amounts [44].

Our MALDI-MS analysis of PE purified from both $\Delta cpeY$ and $\Delta cpeZ$ mutants showed that some amount of α -PE was chromophorylated at both Cys-82 and Cys-139 with PEB or PCB or both (since both PEB and PCB are the same mass, they cannot be differentiated by MALDI-MS). Bilins naturally have a high affinity for the bilin binding pockets in PBP. Non-enzymatic bilin addition has been seen in *F. diplosiphon* [94], where PEB was covalently attached to PE at very slow reaction rates. PCB was also shown to slowly become covalently attached to PC by rearrangement of PCB in the binding pocket in the absence of a lyase [45]. Biswas *et al.* demonstrated that recombinant CpeS lyase, whose natural substrate is β -PE at Cys-80 in *F. diplosiphon*, was able to covalently attach PEB to both Cys-82 and Cys-139 of α -PE at a slow rate and not very efficiently [23]. The small amounts of chromophorylated PE detected in our PE samples purified from $\Delta cpeY$ and $\Delta cpeZ$ mutants could have been generated from a slow covalent (non-enzymatic) reaction between the chromophore and apo-PE, or by the endogenous CpeS lyase present in *F. diplosiphon*.

Toole *et al.* generated site-directed mutants in *Synechocystis* sp. PCC 6701 by changing the bilin-binding residues from Cys to Ala in both subunits of PC [95]. These mutant analyses revealed that in the absence of proper chromophorylation, α -PC and β -PC heterodimer interaction strength is reduced. Formation and stability of PC is decreased and turnover rates are increased, most likely due to degradation pathway activity. Attachment of the central bilin (Cys-82 equivalent) facilitates stability of the subunits, thus allowing for proper heterodimer formation as well as hexamer formation and assembly into the PBS [95]. Biswas *et al.* also demonstrated that chromophorylation at this central position (Cys-82 of α -PE in *F. diplosiphon*) increased recombinant CpeA solubility and stability in *E. coli* [23]. We have seen that in the absence of CpeY or CpeZ in *F. diplosiphon*, PEB chromophorylation on PE is affected resulting in reduced

levels of PE. Both $\Delta cpeY$ and $\Delta cpeZ$ mutants generated PE with non-detectable β -PE in Western blots and mass spectrometry. These proteins were purified from whole cells and represent the total pool of PE synthesized. Our PE samples include PE that was not incorporated into the PBS as efficiently, especially if chromophores are missing. Therefore, due to proteolytic degradation, β -PE may not be accumulating in large enough amounts to be visualized after purification of total PE from cells. However, β -PE was detected in PBS samples at lower concentrations when compared to WT. Therefore, during our PE purification process from whole cells, the β subunit is likely lost due to its lack of PEB and lower solubility. In order to properly test the levels of β -PE, whole cells and intact PBS from mutants and WT should be analyzed by Western blot using highly specific primary antibodies against β -PE. Antibodies generated against apo-CpeB are not very specific (data not shown).

The lack of bilins on PBP in the absence of bilin lyases not only affects stability and turnover rates of the protein, but also the assembly of PBP into the PBS and energy transfer efficiency. Once apo-PBP are chromophorylated, they form monomers, trimers and then hexamers which are assembled into the PBS (see Fig. 2). The peripheral bilins of the disc-shaped trimer (the energy donors which absorb at shorter wavelengths) capture the light and transfer the energy to the bilins in the center of the disc-shaped trimer (the energy acceptors) [96]. These energy acceptors are bilins bound to the Cys-82 equivalent of both α and β subunits of PBP with the β -82 equivalent being the terminal energy acceptor in PE (Cys-80 in *F. diplosiphon*, see Fig. 21) [13]. If PE was missing one or more PEB chromophores, this could result in instability of the PE subunits leading to protein degradation, preventing proper assembly into the PBS, thereby decreasing PBS light capturing and energy transfer efficiency [13, 97, 98]. In the absence of bilin

lyases and proper bilin ligation, there is a marked decrease in PBP content [28, 38, 45, 51, 91, 92], and this was described in this thesis when *cpeY* and *cpeZ* were deleted in *F. diplosiphon*.

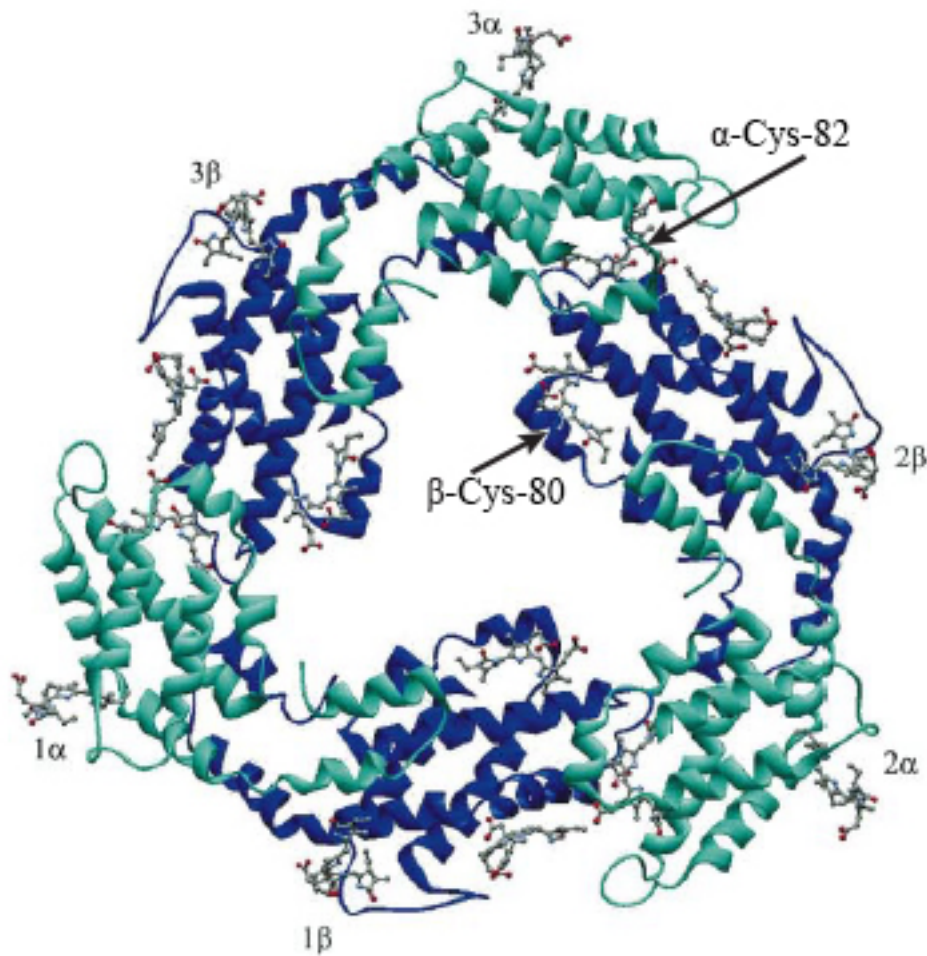


Fig. 21 Crystal structure of PE as a trimer

Ribbon diagram of R-PE trimer from *Gracilaria chilensis* [99]. α (blue-green colored) and β (dark blue colored) subunits of R-PE are labeled as “1-3 α ” and “1-3 β ,” respectively. Arrows are pointing to the energy acceptor PEB chromophores of α -PE (α -Cys-82) and β -PE (β -Cys-80) normally found in *F. diplosiphon*.

Recombinant protein studies of CpeY show this protein functions as a bilin lyase responsible for attaching PEB to α -82 of PE in *F. diplosiphon* [23]. Our analysis of $\Delta cpeY$ mutant confirmed that CpeY is a lyase for α -PE due to the loss of a PEB bilin as seen in the Western blot analysis of PE purified from the mutant. Recombinant protein studies of CpeZ showed that CpeZ does not function as a lyase alone but increases the lyase activity of CpeY [23]. Heterologous coexpression of recombinant *cpeZ* with *cpeB* and the genes to synthesize PEB resulted in no PEB addition to CpeB [23]. However, our $\Delta cpeZ$ mutant analysis shows that deleting *cpeZ* mainly affects β -PE in terms of proper chromophorylation and PE synthesis and accumulation. This suggests that CpeZ may play a role in PE biosynthesis as a chaperone. Böhm *et al.* provided the first evidence for a chaperone-like function of a lyase subunit [100]. The α subunit of PEC is able to autoligate PCB with very low efficiency, resulting in poor spectral properties. Incubation with PecE improved the spectral properties by inducing a conformational change in the PBP for a more stable and fluorescent product [100]. This reaction does not require ATP or GTP like other chaperones do; thus, PecE behaves as a chaperone-like protein. Performing an experiment such as this with α and β PE, PEB and CpeZ from *F. diplosiphon* may further provide evidence for the chaperone-like activity of CpeZ as suggested by our $\Delta cpeZ$ mutant results.

Another possibility is that CpeZ may interact with PE linker proteins as a chaperone, facilitating assembly. Linker proteins have structural roles in connecting PBP trimers together to form hexamers and connecting one type of PBP to another type. Linker proteins also have functional roles for efficient energy transfer in the PBS. Whitaker *et al.* isolated a mutant with a PE-deficient phenotype in *F. diplosiphon* which was shown to have a transposon in the *cpeE* gene encoding a PE rod-linker protein [101]. Mutating *cpeE* resulted in the reduction of PE

accumulation as seen in our $\Delta cpeZ$ mutant. It is possible that CpeZ may play a role in PE assembly with linkers also. There are many genes in *F. diplosiphon* that are involved in the synthesis and assembly of PE such as genes encoding linker proteins (CpeC, CpeD, and CpeE) [102], bilin lyases (CpeY, CpeS, and putative lyases, CpeF, CpeT and CpeU) [23, 49], CA regulatory proteins (Rca and Cgi regulatory pathway proteins) [62], and transcription factors (CpeR activator) [69]. Performing recombinant heterologous coexpressions in *E. coli* and protein-protein interaction assays with CpeZ and other *F. diplosiphon* proteins involved in PE biosynthesis and assembly may shed some light on the specific function of the chaperone-like function of CpeZ.

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