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Identification and characterization of enzymes involved in the biosynthesis of different phycobiliproteins in cyanobacteria

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A Thesis

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By
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Table A. Table for abbreviations

Abbreviations	***Designations***
PBS	Phycobilisome
PBP(s)	Phycobiliprotein(s)
PCR	Polymerase chain reactions
E. coli	*Escherichia coli*.
AP	Allophycocyanin
PC	Phycocyanin
PE	Phycoerythrin
PCB	Phycyanobilin
PEB	Phycoerythrobilin
PUB	Phycourobilin
PVB	Phycobiliviolin
PFB	Phytochromobilin
GFP	Green fluorescent protein
FB	Fluorescent protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
PCC	Pasteur culture collection
HPLC	High pressure liquid chromatography
IEF	Isoelectric focusing
CA	Chromatic Acclimation

Abstract

A multi-plasmid, co-expression system was used to recreate the biosynthetic pathway for phycobiliproteins from the cyanobacterium Synechococcus sp. PCC 7002 in *E. coli*. This system efficiently produced chromophorylated allophycocyanin (ApcA/ApcB), α-phycocyanin, and β-phycocyanin. This system was used to demonstrate that CpcS-I and CpcU proteins are both required attaching PCB to allophycocyanin subunits ApcD ($\alpha^{AP\text{-}B}$) and ApcF (β^{18}). The N-terminal, AP-like domain of ApcE (L_{CM}^{99}) was produced in soluble form and shown to have intrinsic bilin lyase activity. In addition, this system was used to chromophorylated CpcA from *Synechococystis sp.* PCC 6803 with a non-cognate bilin; PEB with the aid of CpcEF type bilin lyase. However, the CpcSU type lyase displays much higher specificity for PCB (the native bilin in these species) than PEB.

Next, using a heterologous, co-expression system in *E. coli*, the PEB ligation activity of putative lyase subunits CpeY, CpeZ, and CpeS was tested on the CpeA and CpeB subunits from *F. diplosiphon*. CpeY/CpeZ was found to ligate PEB on CpeA, although CpeY alone had only 60% chromophorylation activity compared to CpeYZ together. Studies with site-directed variants of CpeA (C82S and C139S), revealed that CpeY/CpeZ attached PEB at Cys-82 on HT-CpeA. The CpeS bilin lyase ligated PEB at both Cys-82 and Cys-139 of CpeA, but the yield of attached PEB at Cys 82 was much lower than observed with CpeY or CpeY/CpeZ. However, CpeS efficiently attached PEB to Cys-82 of CpeB. Purified PE from *cpeY* deletion mutants in *F. diplosiphon* was found to have PCB added on α-PE instead of PEB, which was likely performed by CpcEF *in vivo*. However, a *cpeZ* knock-out mutant is affected in chromophorylation of both α and β subunits of PE with a red-shifted absorbance compared to wild type PE probably due to missing PEB on PE subunits.

Next a new type of bilin lyase isomerase for PEII (α subunit) named MpeZ from *Synechococcus sp.* RS 9916, was analyzed using the *E. coli* heterologous coexpression system. MpeZ acted as bilin lyase/isomerase chromophorylating α-PEII (MpeA) with PUB on Cys 83.

Keywords: Phycobiliprotein, Allophycocyanin, Phycocyanin, Phycoerythrin, Phycocyanobilin, Phycoerythrobilin, Phycourobilin.

1.0. INTRODUCTION

1.1 *Cyanobacteria: Background and History*

Cyanobacteria are fascinating photosynthetic, gram-negative prokaryotic organisms with immense biological importance. They are known to be the world's oldest oxygen-evolving organisms, and are found in fossils dating back more than 3.5 billion years old (Schopf 1983; Bengston 1994). These oxygenic photosynthetic organisms created a wonderful oxygen-rich atmosphere that we can breathe in today (Bengston 1994). Through endosymbiosis, cyanobacteria also contributed to the origin of plants and other oxygen-evolving organisms such as red, green, and cryptophyte algae (Bengston 1994; Sidler 1994). In addition, they can fix N_2 and survive in extreme environments (down to -60^{o}C). This makes them ideal model systems for studying fundamental processes such as nitrogen fixation and photosynthesis. In addition, cyanobacteria produce an array of bioactive compounds, some of which could become novel antimicrobial agents, anti-cancer drugs, UV protectants etc**.** (Gerwick, Mrozek et al. 1989; Eggen and Georg 2002; Mohammed and Vermaas 2004). The amazing versatility of cyanobacteria has attracted huge scientific interest in recent years especially in the field of engineering cyanobacterial for the production of renewable fuels or biofuels (Zhou and Li 2010). The genome sequences of 35 different species of cyanobacteria have been completed and are available in searchable databases. Having these genome sequences allows one to identify potential enzymes involved in phycobiliprotein biosynthesis (Yoshikawa, Adachi et al. 2000; Lluisma, Karmacharya et al. 2001; Pomati, Burns et al. 2004) based on similarity to previously characterized enzymes.

1.2. *Phycobilisome: Structure and Function:*

The light reactions in all photosynthetic organisms like cyanobacteria, and red-algae, begin with the absorption of photons by protein (antenna) complexes called phycobilisome (PBS) (Glazer 1989; Bryant, Stirewalt et al. 1991; Grant and Conti 1996) (Fig. 1). PBS were first purified from the cyanobacterial species; *Anacystis nidulans* (Gantt and Lipschultz 1972; Evans and Allen 1973) and are composed of brilliantly colored proteins known as phycobiliproteins (PBPs). PBS are present on the cytoplasmic surface of the thylakoid membrane and transfer energy to the membrane photosystem II complexes (Fig. 1) (Glazer 1985; Grossman, Bhaya et al. 2001) and Fig. 2. The PBP are highly water-soluble. The chomophores (eg. Chlorophyll) of all other photosynthetic complexes are extractable by organic solvents, but those of PBPs are not because they are covalently attached to polypeptides (Glazer 1988). The brilliant fluorescent colors of PBPs did not go unnoticed by early investigators. Sorby in 1877 (Sorby 1877) made a comment "*It would be difficult to find another series of coloring matters of greater beauty or with such remarkable and instructive chemical and physical peculiarities*"

PBP can make up 40-50% of the total proteins in cyanobacteria (Glazer 1989). Each PBP is mainly composed of two different polypeptides known as α and β in a 1:1 molar ratio. The α subunit has a molecular weight between 10-19 kDa and β subunit has between 14-21 kDa (Bennett and Bogorad 1971; Glazer and Cohen-Bazire 1971; P. and Killilea 1971; Gantt and Zuber 1974; Gysi and Zuber 1974). Each α and β subunit has at least one but up to three covalently attached bilin chromophore(s)s, which contributes to each PBP's unique spectroscopic properties allowing absorption of light energy in the visible range between 450-655 nm, chromophores. The linker polypeptides are colorless with exception of the γ –subunit linker. Found in marine *Synechococcus sp*. (and red-algae) this γ-subunit has a covalently attached bilin

(PUB) (Glazer and Hixson 1977; Klotz and Glazer 1985; Ong 1988). The phycobiliproteins are isolated from cyanobacteria as trimeric $(\alpha\beta)_3$ or as hexameric $(\alpha\beta)_6$ complexes or are isolated as dimeric $(\alpha\beta)_2$ or monomeric $(\alpha\beta)$ forms. Certain phycoerythrins (PEs) are isolated as assemblies with the composition $(\alpha\beta)_6\gamma$ where the γ subunit has a molecular weight of 30,000 Da**.** Specific linker proteins (L_{CM}) mediate the association of one hexameric disc to another and modulate the spectroscopic properties of the phycobiliproteins (Zhao, Ping et al. 2005), promoting unidirectional energy flow to photosynthetic reaction centers (Glazer, 1985). The linkers are usually non chromophorylated except in some marine cyanobacterial species (WH 8020, WH 8102, RS9916 etc.) they have one chromophore attached (Six, Thomas et al. 2005). Phycobiliprotein trimers are disc-shaped with a thickness of ~ 30°A and a diameter of ~ 120°A and two trimers associated into hexamric assemblies in a face to face manner. Spectroscopic data and electron microscopic analyses indicate that these complexes share common structural features with those within phycobilisomes (Glazer 1989). There are four different classes of phycobiliproteins; phycocyanin (PC, λ_{max}=615-640nm), allophycocyanin (AP, λ_{max}= 650-655), phycoerythrin (PE, λ_{max}= 495-575 nm) and phycoerythrocyanin (PEC, λ_{max}= 575 nm) (Ong and Glazer 1991).

A single PBS is generally composed of a central core (containing AP) and 6 to 8 radiating rods (containing PC) (Fig. 1). Some cyanobacterial species contain PE on the end of the rods adjacent to PC for more efficient light capture (Glazer and Hixson 1977) . In this thesis two phycoerythrin- containing cyanobacteria were studied; fresh-water-grown cyanobacteria *F. diplosiphon UTEX 481* (also known as *Tolypothrix sp.* PCC 7601 and marine cyanobacterial species *Synechococcus sp*. RS 9916. *F. diplosiphon* contains PE and PC. The PE contained in the rods of *F. diplosiphon* have red colored bilins, designated as phycoerythrobilin (PEB)

(described in detail later) having an absorbance maximum at 560 nm and fluorescence emission maximum at 572 nm (Fairchild and Glazer 1994) .

Marine *Synechococcus* strains contain two types of PE designated as PEI and PEII, which have different protein compositions and different chromophores (between five to six chromophores) (Ong and Glazer 1991) (See Fig 2). PE(II) and PE(I) exist in a weight ratio of 2-4:1, respectively. The energy absorbed by PE(II) gets transferred to PE(I). Their PE subunits contain a different group of bilin isomer the yellowish-orange colored; phycourobilin (PUB) (described later) as well as PEB. Because PUB absorbs light efficiently at 495 nm the phycobilisome in these species are more efficient in capturing blue light (BL), the main wavelength of light penetrating deep in the ocean (Ong, Glazer et al. 1984).

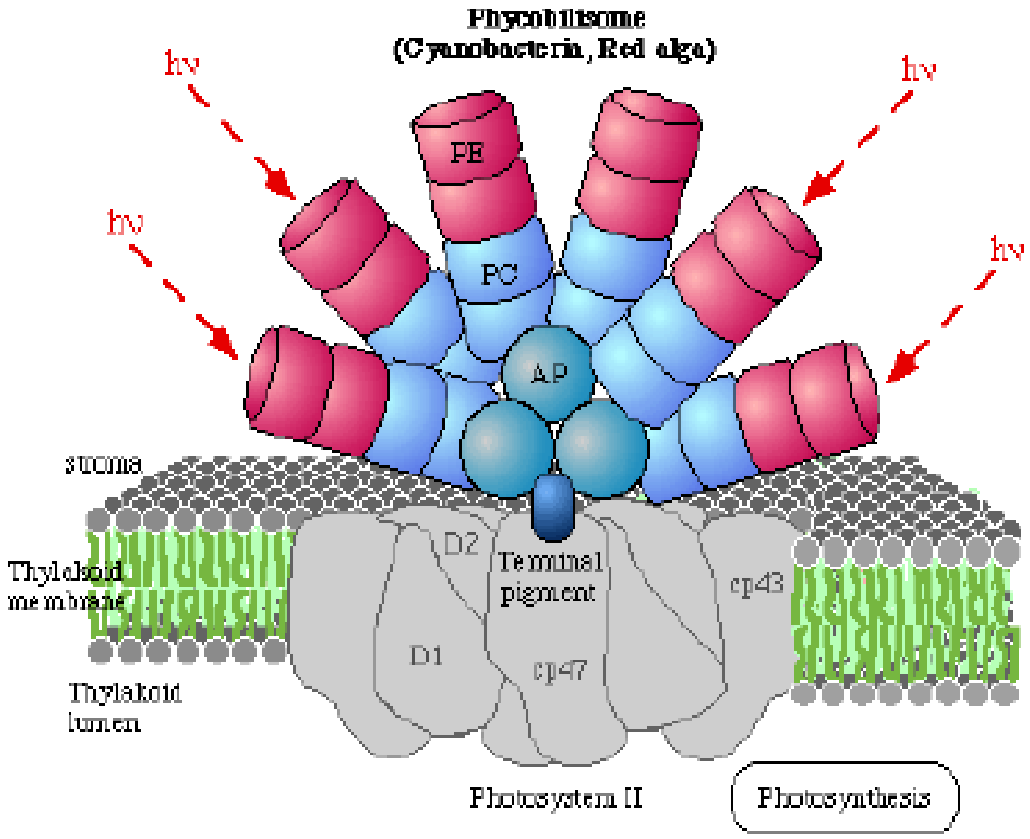


Fig. 1. Phycobilisome structure in cyanobacteria: The two photosystem (PS) shown separately; PSI and PS II. On the outer membrane of PSII contain the donut shaped phybilisome. Each phycobilisome consist of central code formed of Allophycocyanin (AP) and the radiating rods formed of Phycocyanin (PC) and in marine cyanobacteria the rods also contain the phycoerythrin (PE). The light energy shown in red arrow are first absorbed by PE, transferred to PC then to AP, finally reached the chloroplast of PSII.This figure is a modified version as drawn by N. Tandeau de Marsac (Tandeau de Marsac 1994)

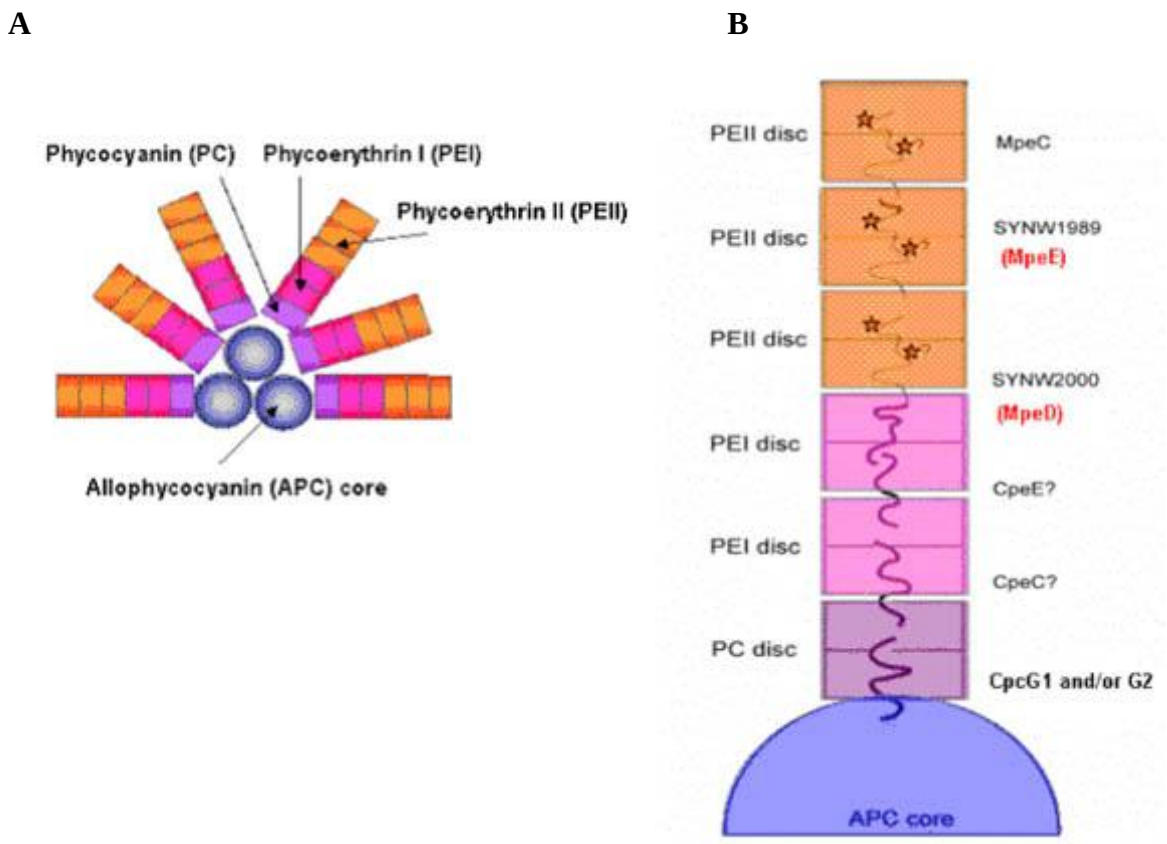
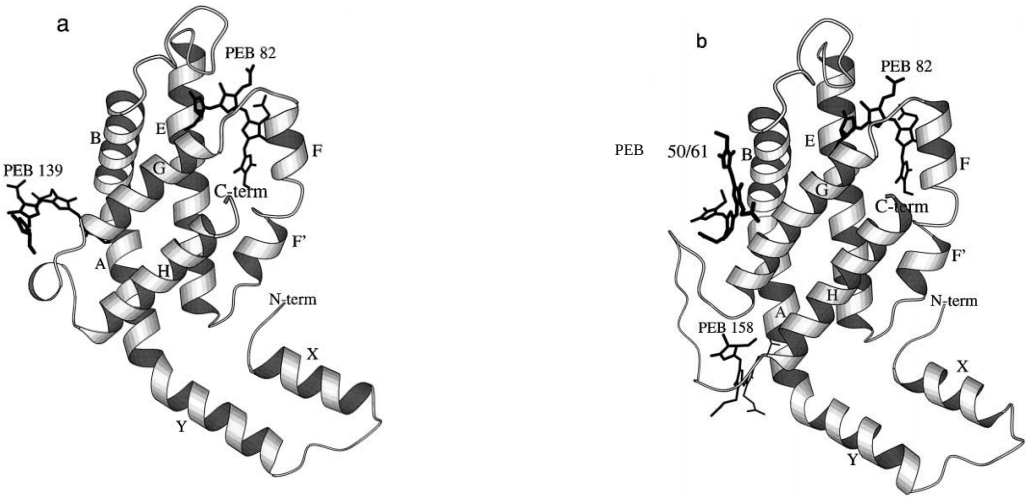


Fig. 2. Phycobilisome structure in marine cyanobacteria. A. Represent phycobilisome in marine species consisting of core composed of Allophycocyanin, rods made of Phycocyanin at the interior and exterior composed of Phycoerythrin (PEI and PEII). B. A diagramatic representation of the phocobilisome rod proteins showing the PC, PEI and PEII along with all the linkers (Six, Thomas et al. 2007)

1.3. *Structural elucidation for phycobiliproteins:*

The three dimensional crystal structures of all the phycobiliproteins; PE, APC and PC are shown in Fig. 3 (Brejc, Ficner et al. 1995; Ritter, Hiller et al. 1999; Padyana, Bhat et al. 2001). Each polypeptide α and β has eight α- helices (X, Y, A, B. E, F, G and H) with small loops along with various chromophores attached at specific cysteine residues (See Table 1). Each chromophore can act as a light energy acceptor or donor (Fig. 3) (Schirmer, Bode et al. 1985; Sidler 1994) . The PC, APC and PE subunits have a globin- like structure and possess closely-related counterparts to the myoglobin α-helices (A-H) (Schirmer, Bode et al. 1985). All PBPs have very closely related structures, and all contain the central bilin chromophore Cys-84 or equivalent position on both α and β subunit. In the case of phycocyanin the bilin attached at β-84 acts as a terminal energy acceptor whereas the α-84 and β-155 chromophore act as energy donors. In the 3-D spatial arrangement of phycocyanin the PCB-β-84 is located in the center of the trimeric disc, whereas both α-84 and β-155 are situated at the periphery (Schirmer, Huber et al. 1986). PE is similar in crystal structure that of PC, but each PE subunit contains more bilin chromophores (5-6) compared to that of PC (Table 1) (Ong and Glazer 1991; Fairchild and Glazer 1994). The extra sets of bilins are located at the periphery of the disc and function as efficient light capturing machinery for those cyanobacterial species growing at low light intensities (Ong and Glazer 1988) (See Table 1 for details).

1


a
PEB 82
B
E
F
PEB 139
G
C-term
F'
A
H
N-term
X
Y
b
PEB 82
PEB
50/61
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C-term
F'
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N-term
A
PEB 158
X
Y

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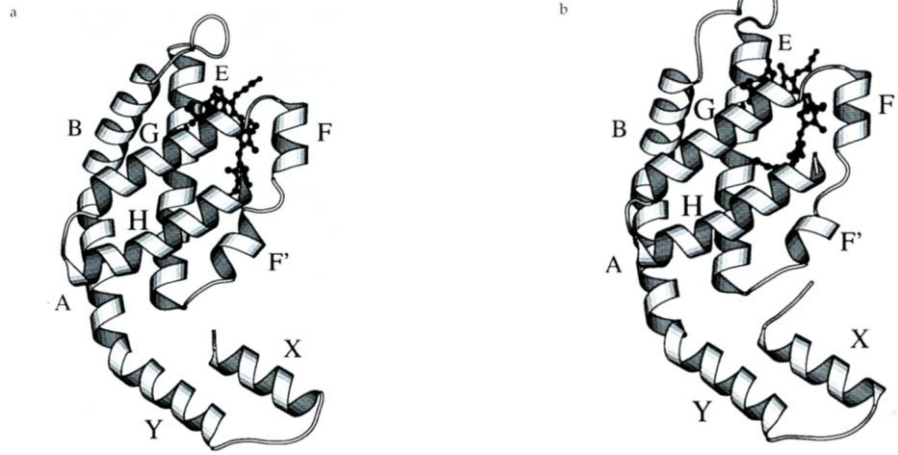
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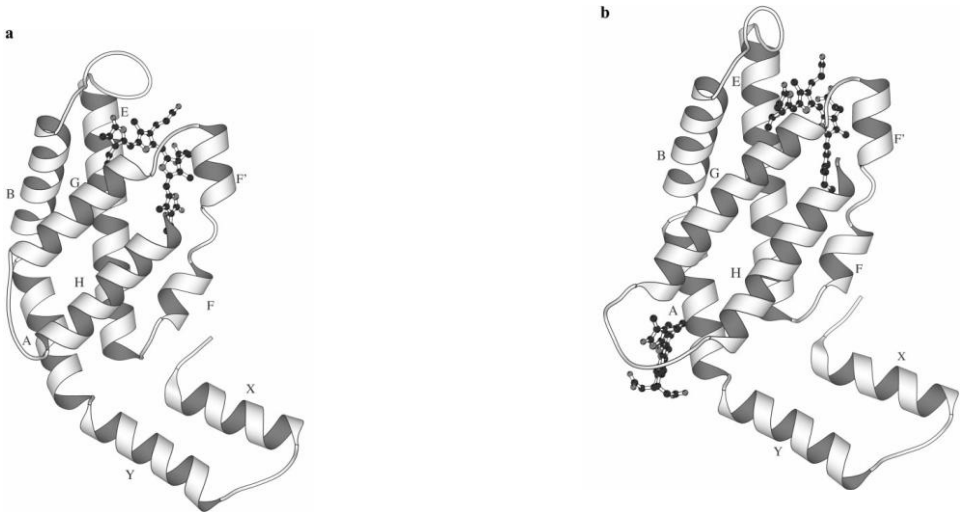
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Fig. 3. Represents ribbon structure of Phycobiliproteins (a) the α-subunit and (b) the β-subunit. 1a and 1b represents PE subunits, 2a and 2b are AP subunits. 3a and 3b represent PC subunits. The chromphores denoted by the amino acid residue numbers (Brejc, Ficner et al. 1995; Ritter, Hiller et al. 1999; Padyana, Bhat et al. 2001).

Table 1. Lists of the potential lyase/ isomerase that are involved in attaching different bilin in *Synechococcus sp.* PCC 7002, *F. diplosiphon* PCC 7601, and *Synechococcus sp.* RS 9916. It also includes the one have been published (in bold) and the rest of them are proposed as being involved.

Organism	PBP name	Gene name	Attachment sites-Bilin[a]	Candidate Lyase[b]
			Synechococcus	***sp.* PCC 7002**
Syn. PCC 7002	α^{PC}	*cpcA*	Cys-84-PCB	**CpcE/CpcF (Fairchild, Zhao et al. 1992; Zhou, Gasparich et al. 1992)**
Syn. PCC 7002	β^{PC}	*cpcB*	Cys-82-PCB Cys-153-PCB	**CpcS-I/CpcU (Saunée, Williams et al. 2008; Shen, Schluchter et al. 2008)** **CpcT (Shen, Saunee et al. 2006; Biswas, Vasquez et al. 2010)**
Syn. PCC 7002	α^{AP}	*apcA*	Cys-81-PCB	**CpcS-I/CpcU (Shen, Saunee et al. 2006; Biswas, Vasquez et al. 2010)**
Syn. PCC 7002	β^{AP}	*apcB*	Cys-81-PCB	**CpcS-I/CpcU (Shen, Saunee et al. 2006; Biswas, Vasquez et al. 2010)**
Syn. PCC 7002	β^{18}	*apcF*	Cys-81-PCB	**CpcS-I/CpcU (Biswas, Vasquez et al. 2010)**
Syn. PCC 7002	α^{AP-B}	*apcD*	Cys-81-PCB	**CpcS-I/CpcU (Biswas, Vasquez et al. 2010)**
Syn. PCC 7002	L_{CM}^{99}	*apcE*	Cys-186	**Autocatalytic (Zhao et al., 2005; Biswas, Vasquez et al. 2010)**
		Fremyella	***diplosiphon***	**UTEX 481**
Fd PCC 7601	α^{PE}	*cpeA*	Cys-82-PEB Cys-139-PEB	**CpeY/CpeZ (Biswas et al. Manuscript in preparation)** *?*
Fd PCC 7601	β^{AP}	*cpeB*	Cys-48,59-PEB Cys-80-PEB Cys-165-PEB	*?* **CpeS (Zhao, Su et al. 2007); Biswas et al. Manuscript in preparation)** *?CpeT*
			Synechococcus	***sp.* RS 9916 (GL)**
Syn. RS9916	α^{RPC}	*rpcA*	Cys-84-PUB	***RpcG (Blot, Wu et al. 2009)***
Syn. RS9916	β^{RPC}	*rpcB*	Cys-82-PCB Cys-155-PEB	*?CpcS* *?RpcT*
Syn. RS9916	α^{PE-I}	*cpeA*	Cys-83-PEB Cys-140-PEB	**CpeS (Zhao, Su et al. 2007 or CpeY/CpeZ** *?*
Syn. RS9916	β^{PE-I}	*cpeB*	Cys-50, 61-PEB Cys82-PEB Cys159-PEB	*?* *CpeS* *CpeT*
Syn. RS9916	α^{PE-II}	*mpeA*	Cys-75-PUB Cys-83-PEB Cys-140-PEB	*?* ***Described in this thesis*** *?*
Syn. RS9916	β^{PE-II}	*mpeB*	Cys-50,61-PUB Cys-82-PEB Cys-159-PEB	*?* *CpeU or CpeS* *CpeT*
Syn. RS9916	γLR	*mpeC*	Cys-49-PUB	*?*
			Synechococcus	***sp.* RS 9916 (BL)**
Syn. RS9916	α^{RPC}	*rpcA*	Cys-84-PUB	***RpcG (Blot, Wu et al. 2009)***
Syn. RS9916	β^{RPC}	*rpcB*	Cys-82-PCB Cys-155-PEB	*?CpcS* *?RpcT*
Syn. RS9916	α^{PE-I}	*cpeA*	Cys-83-PUB Cys-140-PEB	**CpeS (Zhao, Su et al. 2007) or CpeY/CpeZ** *?*
Syn. RS9916	β^{PE-I}	*cpeB*	Cys-50, 61-PEB Cys82-PEB Cys159-PEB	*?* *CpeS or CpeU* *CpeT*
Syn. RS9916	α^{PE-II}	*mpeA*	Cys-75-PUB Cys-83-PUB Cys-140-PUB	*CpeS or CpeU* ***Described in this thesis*** *?*
Syn. RS9916	β^{PE-II}	*mpeB*	Cys-50,61-PUB Cys-82-PEB Cys-159-PEB	*?* *CpeU or CpeS* *CpeT*
Syn. RS9916	γLR	*mpeC*	Cys-49-PUB	*?*

[a]Terminal acceptor bilin in rod proteins is underlined (Ong and Glazer, 1991).
[b]In bold, confirmed by experiment and citation is in parentheses; in italics, suggested by analogous position and other experiments from other systems, but not yet confirmed; ?- candidate less clear from experimental data on paralagous proteins
GL represents grown in Green light , BL represents grown in Blue Light

1.4. *Application of fluorescent proteins (FB):*

The major fluorescent protein complex in cyanobacteria is known as PBS (Described earlier). The presence of covalently attached tetrapyrrole pigments (or chromophores) on phycobiliproteins makes them highly fluorescent. There are several unique features compared to other flourophores like flourescein, tyrosine, tryptophan etc. and Green Flourescence proteins (GFP) and its derivatives which make cyanobacterial phycobiliproteins ideal candidates for various biological applications; they have high quantum yields (~ 0.65-0.98) (See Table 2), wide range of absorbance spectra (490-650 nm), they are stable at a wide range of biological pH (4.5-8.0), the fluorescence property of phycobiliproteins are free of interference from biological molecules, large Strokes shift provide greater signal to noise ratio compared to other small flourophores, and they are stable to photobleaching. Three major phycobiliproteins; Allophycocyanin (AP), R-phycoerythrin (R-PE), and B-phycoerythrin (B-PE) currently serve as fluorescent tags with several biological applications in flow cytometry, histochemistry, fluorescence activated cell sorting, and detection of reactive oxygen species etc.

1.4.1. *Application of Cyanobacterial phycobiliproteins as fluorescent tags:*

Cyanobacterial proteins require three components to be fluorescent, for the use as a fluorescent tags; namely phycobiliprotein subunits, the lyases which attach the chromophore and the chromophore (bilin) itself. The apo-protein chains of phycobiliprotein subunits contain amino and carboxyl groups that can form bonds to other molecules (Glazer and Stryer 1984; Glazer 1994; Sun, Wang et al. 2003). Oi et al. (Oi, Glazer et al. 1982) conjugated phycobiliproteins to immunoglobulins, protein A and avidin to develop fluorescent probes. These conjugates have been widely used in histochemistry, fluorescence microscopy, flow cytometry, fluorescence-

activated cell sorting and fluorescence immunoassays (Glazer and Stryer 1984; Glazer 1994; Sun, Wang et al. 2003). Phycobiliproteins can exist as hexamers ($\alpha_6\beta_6$) and trimers ($\alpha_3\beta_3$) or monomers ($\alpha\beta$). Hexamers tend to have higher molar extinction coefficients (Edwards, Hauer et al. 1997; Thoren, Connell et al. 2006) and greater quantum yields compared to monomers (Glazer and Stryer 1984; Glazer 1994; Sun, Wang et al. 2003), whereas denatured forms of phycobiliproteins have lower molar extinction coefficient values and almost no fluorescence (Fukui, Saito et al. 2004; Kupka, Jhang et al. 2009).

Back in the 1980s, phycoerythrin (PE) became one of the most widely used PBP in different biological applications mainly, as a fluorescent tag. Glazer et al. isolated R-PE as $\alpha_6\beta_6$ hexamers (Oi, Glazer et al. 1982; Glazer and Stryer 1984) with a fluorescence quantum yield of 81-90% (Oi, Glazer et al. 1982) (See Table 2). Phycoerythrin-immonoglobin, phycoerythrin-protein A, and phycoerythrin-avidin conjugates were made, (Oi, Glazer et al. 1982), and these bind specifically to beads containing covalently attached target molecules which renders them highly fluorescent. Femptomole (10^{-15} mole) quantities of phycoerythrin conjugates can be detected because of high extinction coefficient (ε_M= 2.4 X 10^6 cm^{-1} M^{-1} for 2.4 X 10^5 daltons) and high fluorescence quantum yield (Q= 0.8) of the PBP moiety. These conjugates are used for fluorescence-activated cell sorting and analyses, fluorescence microscopy, and fluorescence immunoassays. In 1983, Glazer and Stryer (Glazer and Stryer 1983) developed fluorescent tandem phycobiliprotein conjugates with a very large Stokes shift by covalently attaching PE to AP. The efficiency of energy transfer from PE to AP in this disulphide-linked conjugate was 90%. One of its distinctive features is the wide separation between the intense absorption maximum of phycoerythrin at 545 nm and the fluorescence emission maximum of allophycocyanin at 660 nm. This tandem conjugate was found to have more advantages than

APC or PE alone in fluorescence-activated cell sorting and analysis, fluorescence microscopy, and fluorescence immunoassays due to the large Stokes shift.

Oi et al. also isolated AP and PC, but it was a mixture of hexamers, trimers, and monomers with lower quantum yields (68 % for AP and 50% for PC) (Oi, Glazer et al. 1982). PC trimers can be stabilized by chemical cross-linking of polypeptide chains (Fukui, Saito et al. 2004; Sun, Wang et al. 2006). These stabilized PC trimers have similar spectral properties as native PC can be used in fluorescent probes different from other PBPs. Also complete phycobilisomes from *Arthospira platensis* composed of PC and AP have been chemically stabilized, combined to streptavidin and used as a fluorescent probes in flow-cytometry (Telford, Moss et al. 2001).

Another important use of PBP is in Flourescence immunoassay technique (FIT), a process used for the identification of various proteins or enzymes in diseased cells. Phycobiliproteins from different cyanobacteria and red-algae act as a valuable source for flourscent tag in this immunoassay technique. The phycobiliproteins isolated from various cyanobacterial and algal species possess certain chrateristics which make them ideal probes for the use in FIT: red shifted excitation and emission spectra causing less interference with biomolecules, a large Strokes shift, so that interferences from Rayleigh and Raman scatter and other fluorescing components is less significant, stability toward naturally occurring biological substances to be quenched, high solubility in an aqueous environment decreasing nonspecific binding effect, and high fluorescence quantum yield independent of pH (O'Donnel and Suffin 1979; Soini and Hemmila 1979).

Table. 2. Comparison of physical data between various flourophores and fluorescent proteins

Fluorescent Molecules	***Excitation maxima***	***Emission maxima***	***Relative quantum yield (θ_f)***	***Extinction coefficient (ε) ($M^{-1}cm^{-1}$)***	***Brightness $B = \varepsilon * \theta_f$***
GFP	484	510	0.6	53,100	37,100
YFP	512	529	0.54	45,000	24,300
CFP	439	476	0.15	20,000	3,000
B-PE	546,565	575	0.98	2,410,000	2361800
R-PE	480, 546, 565	578	0.82	1,960,000	1607200
APC	650	660	0.68	700000	476000
Flourescein	495	519	0.79	92300	72917
Tyrosine	428	455	0.14	1490	209
Tryptophan	280	295	0.12	5500	660

1.4.2. *Application of Cyanobacterial phycobiliproteins as commercial commodities:*

In addition to their use as fluorescent tags, PBP also have other uses. It might to helpful to review the medical and biotechnological research industries involved in using phycobiliproteins as biological tools. Phycocyanin from cyanobacteria has several pharmaceutical applications such as to stimulate the immune defense system and possess antioxidant, anti-inflammatory, anti-viral, anti-cancer, and cholesterol-lowering effects (Jensen, Ginsberg et al. 2001).

Several Biotechnology companies sell Cyanobacterial phycobiliprotein products (as summarized in Table 3):

Table: 3. Commercially used products from Cyanobacterial Phycobiliproteins:

Name of company	***Types of Phycobiliproteins***	***Products***	***Uses***
Cyanotech	R-PE,APC, and Cross linked APC, and C-phycocyanin	Fluorescent tags, markers	Flow cytometry, fluorescence immunoassay, food and cosmetic coloring
Prozyme	R-PC, C-PC, APC and Cross-linked APC, R-PE, B-PE and Y-PE (fluorescence emission towards yellow)	Fluorescence tags, markers	Multicolor fluorescence applications, fluorescence resonance energy transfer (FRET)
Dojindo	B-PE, R-PE and allophycocyanin	labeling kits	Immunoblotting and Immunostaining
Flogen	R-PC, R-PE, B-PE and APC	Fluorescence tags,	high sensitivity direct fluorescence detection in flow cytometry, fluorescence *in situ* hybridization, fluorescence activated cell sorting (FACS), receptor binding in fluorescence resonance energy transfer (FRET), fluorescence immunoassays, fluorescence microscopy, multi-color immunofluorescence and other imaging techniques
Martex Bioscience Corporation	R-PE, B-PE, APC, and R-PC	PBXL-1, PBXL-3 and P3L named SensiLightTM dyes	Flow cytometry, fluorescence immunoassay

1.5. ***Application of Green fluorescent proteins (GFP):***

Although cyanobacterial fluorescent proteins have varied usage, their utility as FB have been over-shadowed by the ground-breaking discovery of Green fluorescent protein (GFP) which earned the Noble prize in chemistry in 2008 (Shimomura, Chalfie et al. 2008) .

GFP was discovered by Shimomura et al. (Shimomura, Johnson et al. 1962) as a companion protein to aequorin, the famous bioluminescent protein from jellyfish *Aequorea victoria*. It is a protein composed of 238 amino acid (Prasher, McCann et al. 1985) residue with a molecular weight of 29.6 kDa exhibiting green fluorescence when exposed to blue light (Prendergast and Mann 1978; Tsein 1998). In a footnote to Shimomura's account of aequorin purification, they noted "*A protein giving solutions that look slightly greenish in sunlight though only yellowish under tungsten lights, and exhibiting a very bright, greenish fluorescence in the ultraviolet of Mineralite, has also been isolated from squeezates*"(Shimomura, Johnson et al. 1962).

GFP was first crystallized in 1974 (Morrise, O et al. 1974) but it took 22 years to solve the X-ray crystal structure (Ormo, Cubitt et al. 1996). It consists of 11 β-barrel strands, threaded by a α-helix running up the axis of cylinder. Residues 65-67 (Ser-Tyr-Gly) in the GFP sequence spontaneously form a fluorescent chromophore *p*-hydroxylbenzylideneimidazolinone (Shimomura 1979; Cody, Prasher et al. 1993), which is attached to the α-helix and which provides its fluorescent properties (Ormo, Cubitt et al. 1996). The crystal structure gave researchers insight as to how the amino acid residues in the GFP molecule interact with each other towards to contribute to its physical properties.

The chromophore formation occurs via a stepwise chemical reaction; first, GFP folds in a nearly native conformation, and then the imidazolinone is formed by nucleophilic attack (cyclilization) of the amide of Gly-67 on the carbonyl of residue Ser 65, followed by dehydration.

Finally, the presence of molecular oxygen dehydrogenates the α-β bond of the Tyr 66 residue creating a conjugated bond with its aromatic group and with imidazolinone (Heim, Prasher et al. 1994; Cubitt AB, Onno et al. 1995). At this stage the mature form of GFP has absorbance and fluorescent properties. So, unlike most cyanobacterial fluorescent proteins, GFP fluorescence is autocatalytic.

For the purpose of biotechnology applications scientists have mutated certain amino acid residues (replacing the bulky residues with the smaller ones) (Cubitt AB and Biol 1997; Patterson, Knobel et al. 1997; Ward 1997), which leads to the production of more soluble GFP (See Fig. 4). Currently, GFP is one the most widely used fluorescence protein with a wide array of biotechnology applications (Tsein 1998).

One of the major uses of GFP, involves fusing the gene for GFP in frame with a protein of interest in any cell to create a fluorescent fusion protein. In an ideal situation, if the fused protein maintains its original function and localization, it will now fluoresce. GFP localization has been accomplished in all major cellular organelles such as the mitochrondria (Perozzo, Ward et al. 1988; Murray and Kirschner 1989; DeGiorgi, Brini et al. 1996), the nucleus (Perozzo, Ward et al. 1988; Lim, Kimata et al. 1995; Hanakam, Albrecht et al. 1996), and the endoplasmic reticulum (Miyawaki, Llopis et al. 1997; Presley, Cole et al. 1997; Subramanian and Meyer 1997) etc.

The discovery of GFP and its derivatives (mutated versions) has revolutionized the use of flouresence microscopy techniques in different biological disciplines (Ormo, Cubitt et al. 1996). Compared to most small fluorescent molecules such as fluorescein isothiocyanate (FITC), which is strongly phototoxic, GFP is usually not harmful when illuminated in live cells (Tsein 1998). This triggered the development of highly automated live cell fluorescence microscopy systems,

which can be used to observe cells over time expressing one or more proteins tagged with FP (Sekar and Periasamy 2003).

Another powerful application of GFP is to express in a small set of specific cells, allowing researchers to optically detect specific types of cells *in vitro* or even *in vivo* (Chudakov, Lukyanov et al. 2005), especially in detecting any diseased cell lines. Other interesting applications of FBs involve using GFPs as sensors of neuron membrane potential (Baker, Mutoh et al. 2008), tracking of receptors on cell membranes, (Desnik, Nicoll et al. 2005) viral entry and the infection process (Lakadamyali, Rust et al. 2003; Joo and Wang 2008) etc**.**

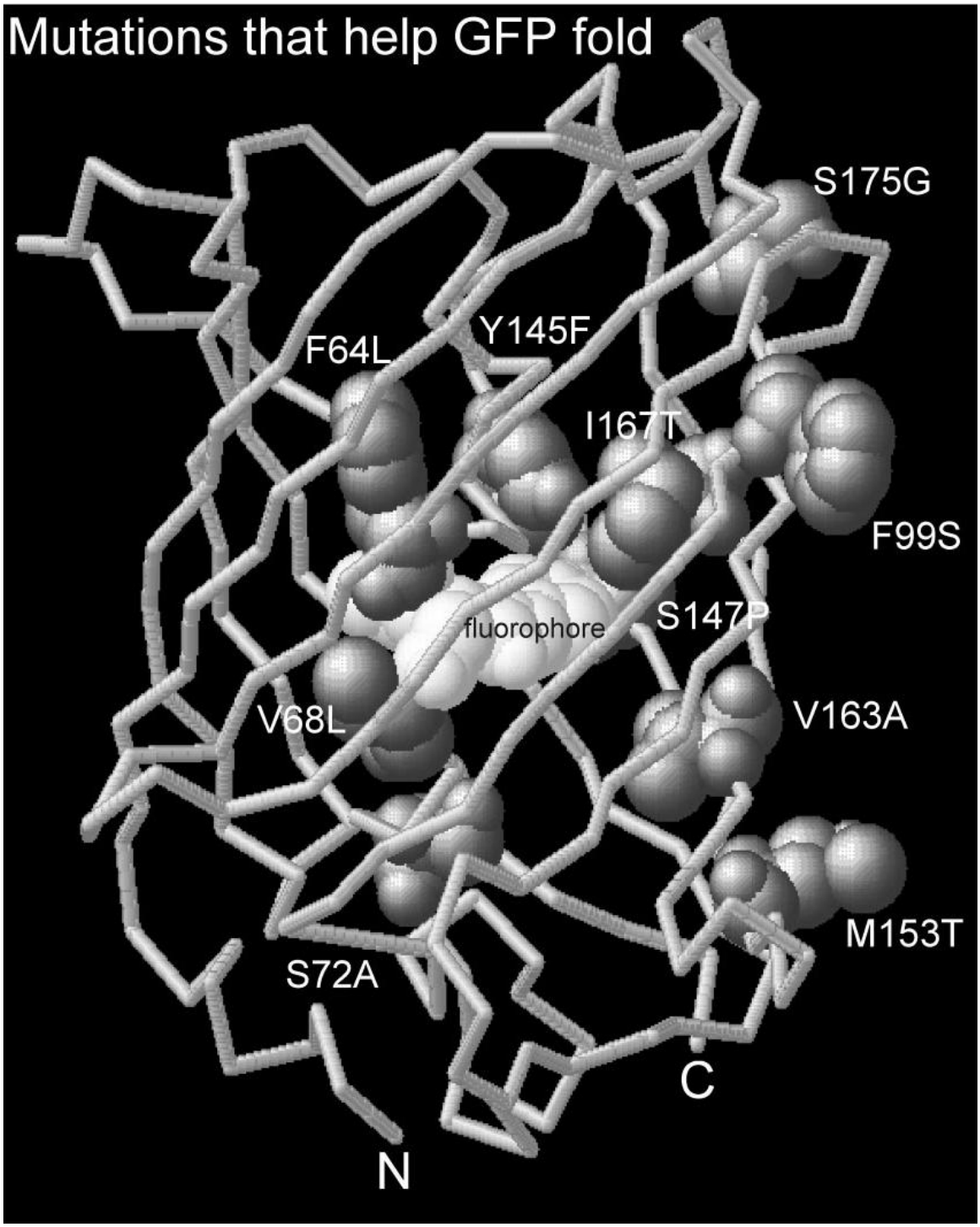


Fig. 4. Location within the GFP crystal structure (Ormo, Cubitt et al. 1996) of the most important sites that improve folding at 37°C**.** The amino acids shown in space-filling representation are the wild-type residues that are replaced by mutation.

1.6. ***Bilin: Types and Biosynthetic pathway:***

Bilins are biological pigments with a linear arrangement of four pyrrole rings (tetrapyrrole). There are four isomeric bilins found in the phycobiliproteins of cyanobacteria: phycocyanobilin (PCB- blue colored), phycoerthrobilin (PEB-red-colored), phycobiliviolin also called phycoviolobilin (PVB, purple-colored) and phycourobilin (PUB, yellowish orange-colored). These bilins are attached through thioether bonds to cysteine residues on the phycobiliproteins (Fig. 4) (Zuber 1987; Glazer 1988; Lagarias, Klotz et al. 1988). Most chromophore addition to the apoprotein cysteine residues are by a single thiother bond at the C-3^1 position of the bilin, but a second thioether linkage to another cys residue is present in some PEs where a (Fig. 4) PEB or PUB is bound at C-3^1 and at C-18^1 (Ficner and Huber 1993) (Fairchild and Glazer 1994). There are also some exceptions where binding occurs to C-3^2 of the C-3 side chain; for example, biliverdin (BV) is bound via C-3^2 in bacterial phytochromes (Lamparter 2004; Wagner, Brunzelle et al. 2005) and so is doubly bound 15, 16-dihydrobiliverdin (DBV) in the cryptophyte biliproteins (Beale 1993; Wemmer, Wedemayer et al. 1993).

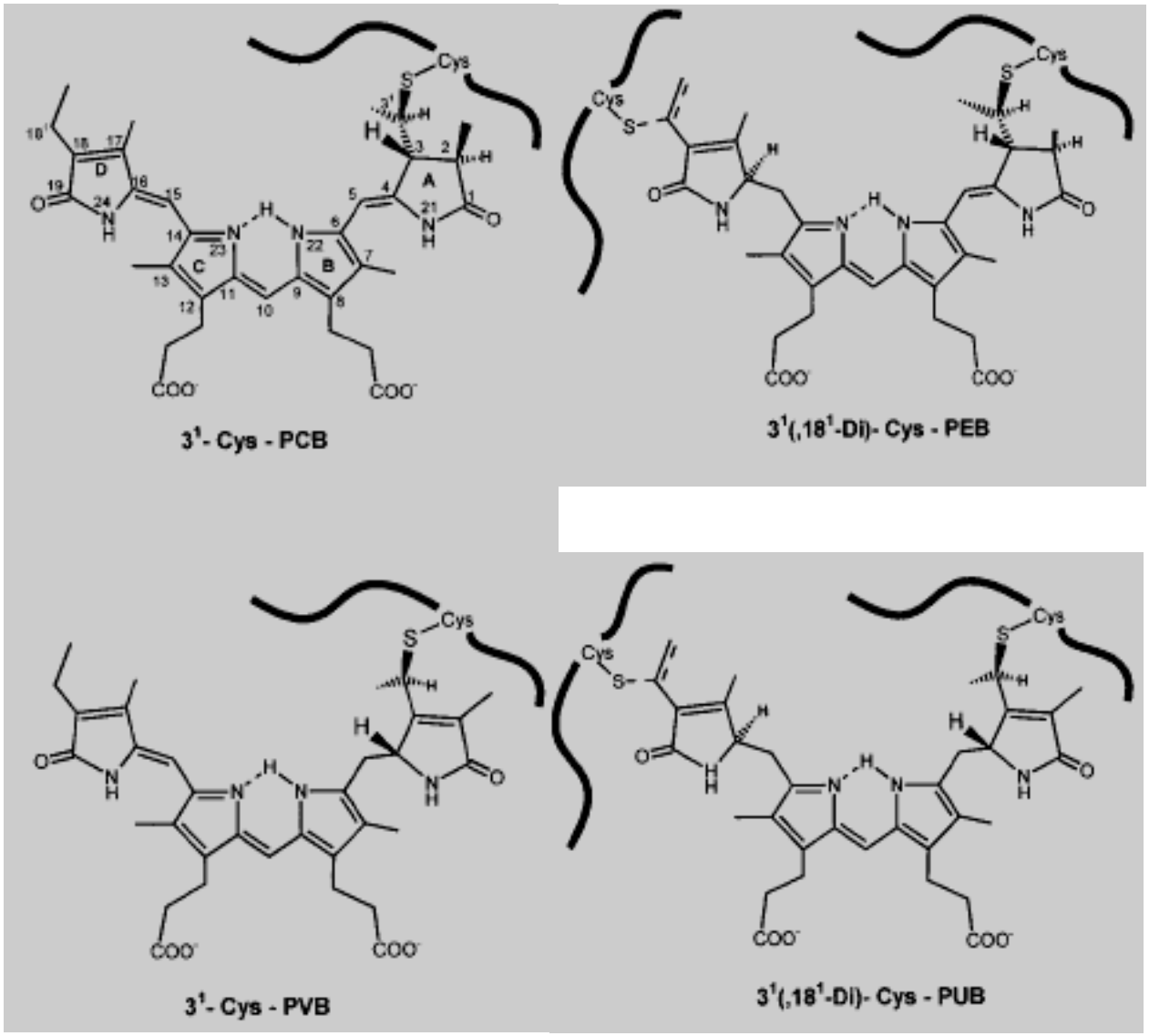


Fig. 5. **Structures of bound bilins**. (Top row) Type 1 chromophores with a single bond between C-2 and C-3; bottom row: type 2 chromophores with a Δ2,3-double bond. There is always a 3^1-linkage present, for some chromophores an optional second linkage (18^1) is indicated . The figure is modified from (Storf, Parbel et al. 2001) .

The phycobiliproteins in *Synechocystis sp*. PCC 6803 and *Synechococcus sp*. PCC 7002 have only PCB attached, whereas the PBP of *F. diplosiphon* have PCB and PEB (Fairchild and Glazer 1994). The PBPs of *Synechococcus sp.* WH8020, WH8102, or RS 9916 have PCB, PEB, and PUB attached (Lagarias, Klotz et al. 1988; Wilbanks and Glazer 1993; Six, Thomas et al. 2007). Among the different types of phycobiliproteins, AP exclusively contains PCB (Glazer 1985). PC mostly contains PCB (Glazer 1985) with exceptions such as in certain marine cyanobacterial species like *Synechococcus sp*. RS 9916 (known as R-PC instead of just PC) where it carries PCB, PEB, and PUB (Blot, Wu et al. 2009). PE may also contain PEB and/or PUB (Alberte, Wood et al. 1984; Kahn, Mazel et al. 1997; Six, Thomas et al. 2005).

The biosynthetic pathways for all bilins start with heme, also called protoheme (Beale 1999). The heme molecule undergoes an oxidative cleavage by the enzyme heme oxygenase (encoded by *ho1* gene) (Cornejo, Willows et al. 1998) to form the common precursor molecule for all bilins known as biliverdin IXα (BV) (See Fig. 6). The molecular mechanism of heme degradation may proceed via three independent steps involving attack by molecular oxygen, followed by elimination of carbon monoxide and formation of iron-biliverdin (Brown and Troxler 1982). The overall heme degradation pathway occurs via two intermediates: α-hydroxyheme and verdoheme, however, the redox stoichiometry for the overall HO1 reaction remains unclear (Sakamoto, Sugishima et al. 2002). BV then undergoes further reduction by highly specific ferredoxin- dependent bilin reductases (FDBRs) (Frankenberg, Mukougawa et al. 2001). These enzymes lack organic or metal cofactors (Frankenberg and Lagarias 2003; Dammeyer, Bagby et al. 2008) and are comprised of several members, each targeting specific double bonds in the tetrapyrrole (Ponkratov, Friedrich et al. 2004) with each electron coming from the FeS protein, ferredoxin. Phycocyanobilin: ferredoxin oxidoreductase (PcyA), which belongs to the family of

FDBRs, catalyzes the four-electron reduction of BV to PCB (See Fig 6); (Dammeyer, Homann et al. 2008)**.** FDBRs are found exclusively in oxygenic phytosynthetic organisms. This enzyme family can be distinguished from the NADPH-dependent biliverdin reducatses BVR (Kapitulnik and Maines 2009) and BvdR (Schluchter and Glazer 1997) by their ferredoxin-dependency and their double bond reduction regiospecificity (Frankenberg, Mukougawa et al. 2001; Frankenberg and Lagarias 2003). The latter property is responsible for the large diversity of their bilin products which absorb light throughout the visible and near- IR spectral regions (Tu, Gunn et al. 2004). PcyA mediates two, two-electron reductions at both vinyl groups of BV (Fig. 6) (Storf, Parbel et al. 2001; Frankenberg and Lagarias 2003; Dammeyer and Frankenberg-Dinkel 2006). In this reaction it converts BV to PCB through a visible (greenish-colored) semi-reduced intermediate 18^1, 18^2- dihydrobiliverdin (DHBV) (Frankenberg and Lagarias 2003). The DHBV undergoes further two-electron reduction forming PCB, which is evident from its native blue color and absorbance maximum at 665 nm (Glazer 1988) (See Fig. 6).

To form PEB there are two consecutive two-electron reduction steps catalyzed by two enzymes; PebA and PebB, belonging to the FDBRs family of radical enzymes (Dammeyer and Frankenberg-Dinkel 2006; Dammeyer, Michaelsen et al. 2007). BV reduction is first catalyzed by 15, 16- DHBV: ferredoxin oxidoreductase (PebA) and yields 15, 16- DHBV by reducing the C-15 methine bridge of BV. The 15, 16 DHBV undergoes further reduction by PEB: ferredoxin oxidoreductase (PebB) on the A-ring 2, 3 3^1,3^2- diene system to form PEB. PebA lacks the metal ion cofactors, and the reaction most likely proceeds via radical intermediates. Interestingly it was observed that DHBV bound to PebA can be re-oxidized to BV by molecular oxygen (Dammeyer and Frankenberg-Dinkel 2006). Reactive oxygen species (ROS) like peroxyradicals are known to reoxidize albumin bound bilirubin (BR) to BV (Stocker, Glazer et al. 1987) Recently a new

enzyme called phycoerythrobilin synthase (PebS) was discovered in the sequencing of a genome of a myovirus that infects a type of cyanobacteria called *Procholorococcus (*cyanophage PSSM-2) (Dammeyer, Bagby et al. 2008; Dammeyer, Homann et al. 2008). PebS was shown to catalyze a four-electron reduction of BV to PEB.

The four bilins found in cyanobacteria fall into two groups based upon their structure, reactivities, and abundance. PCB and PEB are the most abundant in PBP and can be cleaved from PBP producing a Δ 3, 3′ ethylidene group (see Fig. 6). The biosynthetic pathways for these two bilins have been characterized and were previously described**.**

The second group of bilins includes PUB and PVB. These bilins cannot be cleaved directly from PBP and contain a vinyl group at C3, so they should be added or produced via a different mechanism. The pathway for PVB is known; it is produced by a bilin lyase/ isomerase composed of PecE and PecF. This enzyme attaches PCB to the α subunit of PEC and then performs Δ4→Δ2 isomerization to form PVB (see Fig. 7) (Jung, Chan et al. 1995; Zhao, Deng et al. 2000; Storf, Parbel et al. 2001; Tooley and Glazer 2002; Zhao, Wu et al. 2002).

Recently the biosynthetic pathway of PUB of R-PC-V was elucidated. It is produced by a bilin lyase/isomerase composed of RpcG (Blot, Wu et al. 2009), where this enzyme attaches PEB to the α subunit of R-PC-V and then performs Δ4→Δ2 isomerization to form PUB. Part of this thesis project will focus on characterizing a new type of bilin lyase/isomerase specific for PEII subunits.

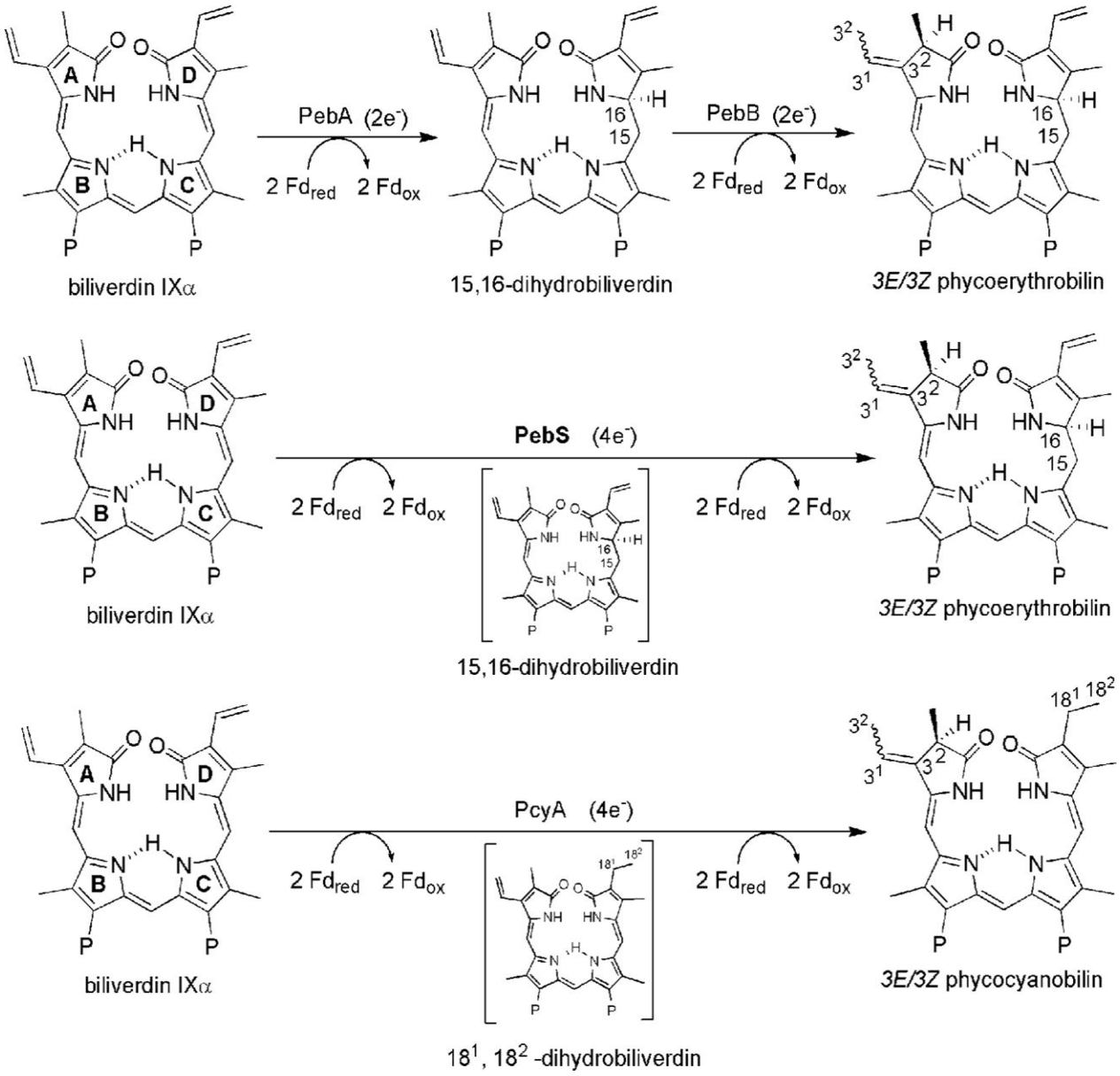


Fig. 6. Biosynthesis of PEB and PCB: PEB biosynthesis proceeds via two different pathways. PebA and PebB catalyze consecutive two-electron reductions of BV and 15, 16-DHBV to yield PEB. PebS catalyzes the four-electron reduction of BV to PEB via the two –electron intermediate 15, 16-DHBV. PcyA catalyzes a four-electron reduction of bilidervin IXα to PCB via the intermediate 18^1,18^2-DHBV. The electrons for all reactions come from reduced [2Fe-2S] ferredoxin (Fd_{red}). The carbons of the respective reduction sites are numbered. Fd_{ox} , oxidized ferredoxin; P, propionate side chain (Dammeyer, Homann et al. 2008).

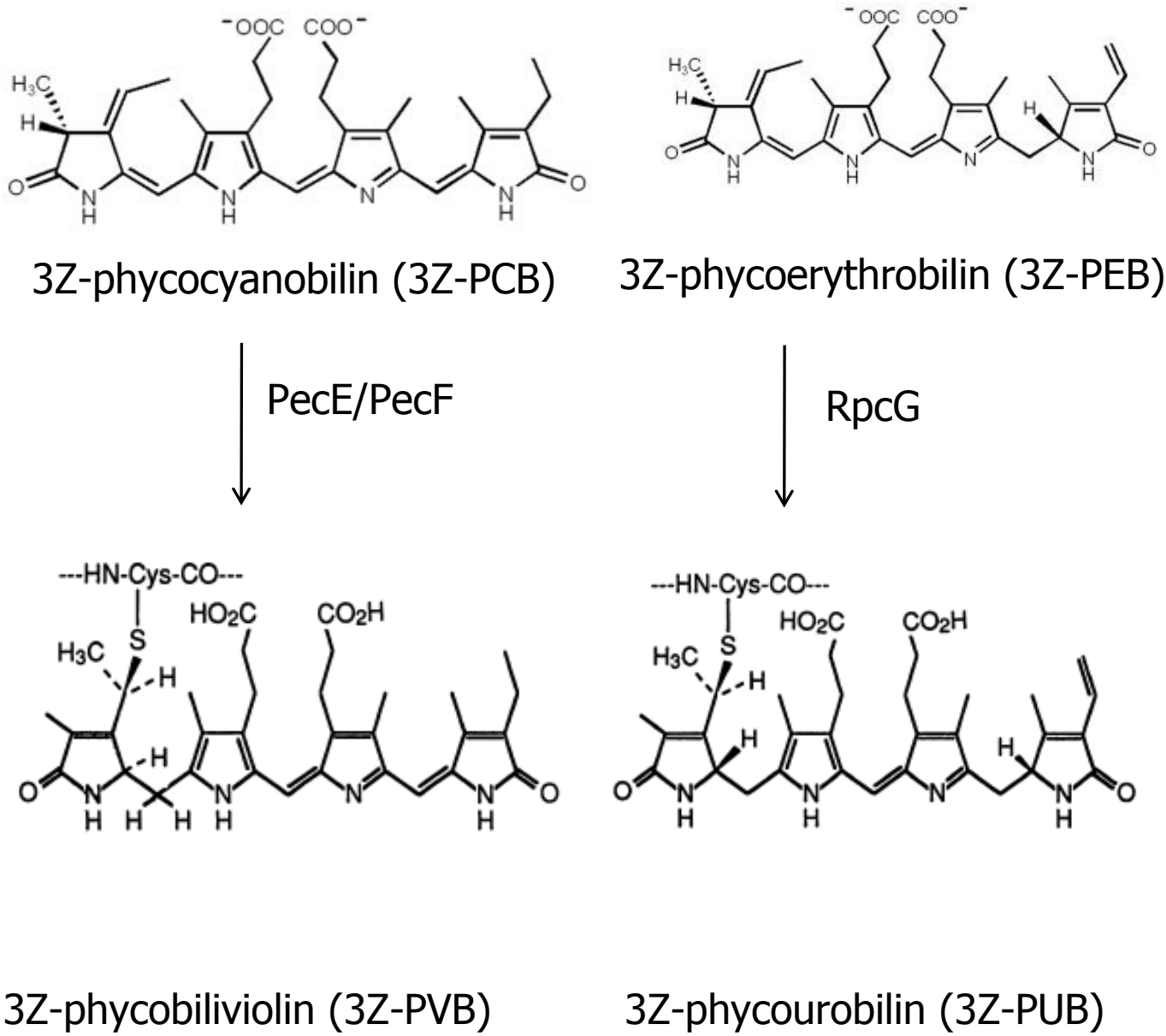


Fig. 7. Biosynthesis of PUB and PVB: The PVB biosynthesis pathway proceeds via lyase/isomerase activity. PecE/PecF acts as a lyase/isomerase converting PCB to PVB by isomerizing on ring A. Similiarly PEB to PUB isomerization proceed by same mechanism only there are more than one lyase/isomase available (Storf, Parbel et al. 2001; Blot, Wu et al. 2009).

1.7. *Bilin addition to phycobiliproteins:*

When any one of the four known cyanobacterial bilins get attached to the PBP in the correct manner associating with the PBP amino acids residues so that it is held in stretched conformation, the PBP becomes highly fluorescent (Scheer and Zhao 2008). The bilin ligation is aided by a category of enzymes designated as bilin lyases. Various published data show that different cyanobacterial bilin lyase enzymes are involved in bilin addition, isomerization and/ or detachment of bilin chromophores to the cysteine residues of PBP (Arciero, Bryant et al. 1988; Fairchild, Zhao et al. 1992; Zhou, Gasparich et al. 1992; Fairchild and Glazer 1994; Dolganov and Grossman 1999; Zhao, Deng et al. 2000; Zhao, Su et al. 2007). Four major classes of cyanobacterial bilin lyases are known: CpcEF type, CpcSU type, CpcT type and the autocatalytic type. Each of these categories of bilin lyases are unrelated in their primary amino acid sequences and are involved in attachment of different bilin chromophores to specific cysteine residues on PBP increasing the light capturing ability for the photosystem.

N.B. *For the purpose of simplicity while reviewing this thesis; if the protein name is designated Cpc, it means they are located or involved in various function in PC subunits; Apc means involvement in APC subunits, and Cpe meaning involment in PE, with only a few exceptions.*

1.7.1 *E/F-type lyase:*

The first bilin lyase to be characterized was a heterodimer composed of CpcE and CpcF (1:1) that is responsible for attachment of PCB to α – PC (also called CpcA) (Fairchild, Zhao et al. 1992; Fairchild and Glazer 1994). These two genes were first identified because they are encoded downstream of the operon encoding PC structural genes *cpcBACD.* The phycocyanin rods in *cpcF* mutants had apo- α-PC but normal levels of chromophorylated β-subunit of PC (CpcB) suggesting the CpcEF type lyases are specific for PCB attachment to the α subunit of PC

(Zhou, Gasparich et al. 1992; Zhao, Deng et al. 2000). This heterodimeric lyase can catalyze both the forward and reverse (releasing) reaction (Fairchild, Zhao et al. 1992). It also catalyzes the addition of PEB to apo-α-PC (CpcA) *in vitro* (Fairchild, Zhao et al. 1992) and *in vivo* (Alvey, Biswas et al. 2011).

For the purpose of generating constructs with phycobiliproteins for use as fluorescent probes in living cells, Tooley et al. recreated the entire fluorescent holo α-PC in *E .coli* by coexpressing *cpcA, cpcE, cpcF* (PC subunits and bilin lyase) from one plasmid and the bilin biosynthetic genes; *ho1* and *pcyA* from another plasmid (Fairchild, Zhao et al. 1992; Fairchild and Glazer 1994; Glazer and Wedemayer 1995). The product yield was shown to have 33% of the produced apo-CpcA converted to holo-CpcA.

PecE/PecF (from *Nostoc sp.* PCC 7120 and *Mastigocladus laminosus*) belongs to the CpcE/F family because they share 47 % sequence similarity to CpcE and CpcF, respectively. PEC- α subunit (PecA) contains the photoactive PVB chromophore at Cys-84, and PecE/F from these organisms not only attaches PCB to this site but also simultaneously isomerizes it to PVB (Jung, Chan et al. 1995; Storf, Parbel et al. 2001; Tooley and Glazer 2002; Zhao, Wu et al. 2002). Zhao et al. (Zhao, Deng et al. 2000) performed the *in vitro* reactions by adding recombinant PecE and PecF from *E. coli* to apo- α –PEC and saw formation of highly fluorescent holo-PecA based on absorbance and fluorescence spectra. When apo-α-PEC was incubated without PecE and PecF, the bilin adduct formed was mesobiliverdin instead of PVB which suggested that PecE and PecF proteins were necessary for the addition of PCB and then its subsequent isomerization to PVB. Tooley at al. (Tooley and Glazer 2002) recreated the pathway of α PEC biosynthesis in *E.coli* by heterologous co expression of two plasmids; one containing all essential genes for PCB biosynthesis (*ho1X* and *pcyA*) and another plasmid containing *pecA* (apo- α –PEC) and *pecE* and

pecF (bilin lyase/isomerase). The holo-α-PEC was purified and its spectral properties showed it had the same characteristics as native α-PEC.

There are other bilin lyases present related to CpcE/F in cyanobacteria are yet to be characterized. One member of the CpcE/F type bilin lyase family is found only in cyanobacterial species containing PE on their PBS rods (Glazer 1989) is CpeY and CpeZ. These putative bilin lyases are emcoded within the operon for PE rod structure proteins CpeBA (CpeBAZY). Kahn et al. showed that transposon insertional within *cpeY* in *F. diplosiphon* resulted in 46% less phycoerythrin (PE) being made (Kahn, Mazel et al. 1997), suggesting the *cpeYZ* gene might be a putative bilin lyase involved in the attachment of phycoerythrobilin to either the α or β subunits. Part of this thesis will include a detailed study on CpeYZ lyase activity. The CpcEF family of bilin lyase was found to be specific for the cysteines residues on the α subunits of PC or PC (central bilin). However there are many other Cys containing bilins for which no enzyme had been identified until 2004 (Shen, Saunee et al. 2004).

1.7.2 *SU type lyase:*

More recently, several studies have characterized the bilin attachment pathway on β subunits of PC, which possess two bilin ligation sites Cys- 82 and Cys-153.

In *F. diplosiphon* the PE linker polypeptide operon (CpeC CpeD and CpeF) encodes the genes: *cpeCDFSTR.* The *cpeS* and *cpeT* genes were found in the genomes of other organisms containing phycobiliproteins, but were absent in species lacking PE. There was no direct evidence that the *cpeS* and *cpeT* genes were transcriptional regulators as they had no sequence similarity to such DNA binding proteins. The Schluchter and Bryant labs showed that there are 2 paralogue genes to F. diplosiphon in *Synechococcus sp*. PCC 7002, which were called CpcS-I/CpcU. They

showed that CpcS and CpcU form a heterodimer (1:1), and that they catalyze the attachment of PCB to Cys-84 of β-phycocyanin (CpcB) and to α and β subunits of AP (Shen, Saunee et al. 2004; Saunée, Williams et al. 2008; Scheer and Zhao 2008; Shen, Schluchter et al. 2008). Zhao et al. showed that the bilin lyase genes they called *cpeS2* and *cpeS1* from *Nostoc sp.* PCC 7120 are homologous to the cyanobacterial lyase CpcS (Zhao, Su et al. 2007). CpcS-I a single subunit bilin lyase was described as a nearly "Universal bilin lyase" (Zhao, Su et al. 2007) (See Table 2) since it was found to be involved in PCB attachment to Cys-84 of β-phycocyanin (CpcB), to the AP subunits, and PEB attachment to Cys-82 of α and and Cys-80 β subunits of C-phycoerythrin from *F. diplosiphon in vitro* (Zhao, Su et al. 2007).

1.7.3. ***T-type lyase****:*

The third type of cyanobacterial bilin lyase is known as the CpcT-type, and is unrelated to CpcEF and CpcS type. They shared sequence similarity to the CpeT bilin lyase from *F. diplosiphon*. Shen et al. showed that the single subunit CpcT type bilin lyase present in *Synechococcus sp.* PCC 7002 can attach PCB to β-PC at Cys-153 (Shen, Saunee et al. 2006). Inactivating the *cpcT* gene in *Synechococcus sp.* PCC 7002 resulted in cyanobacteria with 40% less PC than wild type, smaller PBS and PBPs with more red –shifted absorbance and fluorescence spectra. Recombinant CpcT was shown to catalyze the regiospecific PCB ligation to Cys-155 on CpcB from *Synechococcus sp.* PCC 7002 (Shen, Saunee et al. 2006). The chiral carbon at C_3^1 carbon attached to Cys-153 has *S* stereochemistry (Shen, Saunee et al. 2006), whereas the S/U type and E/F type bilin lyase attach bilins to Cys with *R* stereochemistry. The protein CpcT1 from another cyanobacterium *Anabena sp*. PCC7120 is homologous to the CpcT type bilin lyase and was shown to attach PCB to Cys-155 of β subunit of both PC (CpcB) and PEC (PecB) (Zhao, Zhang et al. 2007).

1.7.4 *Autocatalytic lyase:*

For the last known family of bilin lyases, the lyase reaction is catalyzed by the biliprotein itself. For example, plants, cyanobacteria and other bacteria have phytochromes, which are switchable photoreceptors, responsive to red and far-red light sources, phytochrome-like proteins, or other cyanochromes. These proteins contain a bilin chromophore, which is autoligated without the aid of separate enzymes. (Wu and Lagarias 1996; Wu and Lagarias 2000; Montgomery and Lagarias 2002; Lamparter 2004; Zhao, Ping et al. 2005; Rockwell, Njuguna et al. 2008). The only other known example of a PBP that is capable of auto ligation is the AP- like domain of the large core membrane linker protein designated L_{CM} or ApcE which contains a PCB. It was shown to have a red-shifted absorbance maximum at ~665 nm, and played a role in accepting the energy from the chromophores in the core of the PBS and transferring it to the reaction centers (Capuano, Braux et al. 1991; Gindt, Zhou et al. 1994; Sidler 1994; Ajlani and Vernotte 1998). Autocatalytic addition of PCB was reported to occur for a truncation product of ApcE in 4 M urea (Zhao, Ping et al. 2005). Addition of detergents can eliminate that requirement for bilin lyases for some phycobiliproteins (Zhao, Ping et al. 2005), and so it is possible that the urea present in the reaction mixture with ApcE may have had the same effect as a detergent. ApcE autocatalytic lyase activity using an *in vivo* coexpression system in *E. coli* is described in this thesis.

1.8. *Other* post-translational modifications to phycobiliproteins:

While chromophore addition represent one type of posttranslational modification that all PBPs undergo, a second type of modification is a methylation reaction that occurs specifically only on the β subunits of most phycobiliproteins and is catalyzed by an S-adenosylmethionine-dependent methytranferase designated CpcM (Swanson and Glazer 1990; Miller, Leonard et al.

2008; Shen, Leonard et al. 2008). The methytransferase reaction produces a highly conserved γ-N-methylasparagine residue at the β-72 position of almost all β-subunits isolated from cyanobacteria, red algae, and cryptomonads (Klotz, Leary et al. 1986; Klotz and Glazer 1987; Rümbeli, Suter et al. 1987; Ducret, Sidler et al. 1994; Saunée, Williams et al. 2008). This modification is thought to change the environment of the chromophore at position β-82 to minimize the rates of nonradiative energy loss within PBS (Thomas, Bricker et al. 1993; Thomas, McMahon et al. 1994). However, characterization of *cpcM* mutants also showed that these strains contain very high levels of reactive oxygen species (Shen, Leonard et al. 2008) (Schirmer, Huber et al. 1986). *In vitro* studies of CpcM suggested that the enzyme probably methylates β subunits after chromophorylation but prior to trimer assembly in PBPs (Miller, Leonard et al. 2008)

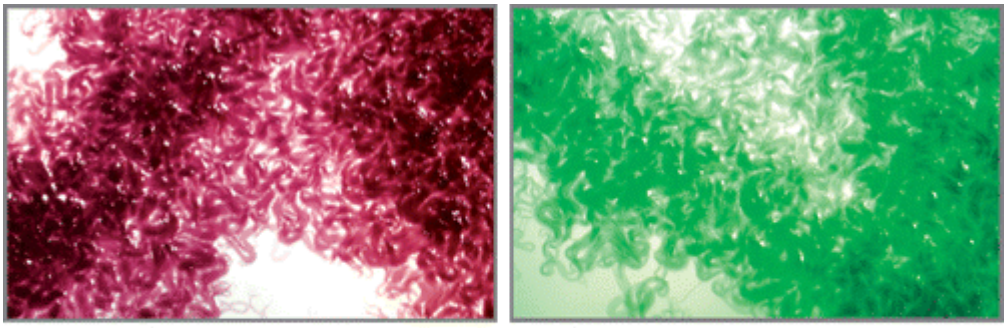
1.9. *Chromatic acclimation:*

Certain cyanobacterial species have the ability to undergo changes in their phycobilisome protein based on the light condition which is known as complementary chromatic acclimation (CCA). *F. diplosiphon* and *Synechococcus sp*. RS 9916 used in this study can undergo CCA. The term CCA was first coined by two scientists; Engelman and Gaidukov (Engelmann 1883; Engelmann 1902; Gaiducov 1902; Gaiducov 1903), but they have different ideas for CCA. Engleman proposed CCA as variations in the pigment distributions in various alge correlated to changes in the light sources. Although subsequent studies did not support this idea (Crossett, Drew et al. 1965; Ramus 1983; Ramus. J. and van der Meer 1983; Saffo 1987), this led to a modification proposed by Guidukov in 1902 that cyanobacterium *Oscillatoria sancta* changed their protein composition having a blue phenotype when grown in red light and red phenotype

when grown in green or white light. There are two major kinds of CCA: Type III and Type IV. Type III CCA found in *F. diplosiphon* was described as color change due to degradation of one rod protein and replacing it by different protein with different chromophores (Fig. 8) (Boresch 1922). PC accumulates (precisely inducible PC) in red light and PE accumulates in green light (Fig. 8). Thus Type III CCA can only occur in cyanobacterial species that make both PC and PE, although not all such species are capable of this process (Tandeau de Marsac 1977). Different studies over time showed that there are many additional cellular responses affected by shifts between green and red lights, including changes in cell and filament architecture (Bogorad, Gendel et al. 1982; Montgomery and Lagarias 2002; Whitaker, Pattanaik et al. 2011; Bennett and Bogorad 1973), cell differentiation states (Lazaroff and Schiff 1962; Damerval, Guglielmi et al. 1991) and the abundance of many RNAs and proteins that do not encode PBS components (Gendel, Ohad et al. 1979; Stowe-Evans, Ford et al. 2004).

The changes in the ratio of PC to PE in *F. diplosiphon* during CCA (Fig. 8) are typical of Type III species and lead to the synthesis of PBS with absorption characteristics that are optimized to capture the most abundant wavelength(s) of ambient light in the green-to-red region of the spectrum. This is because PE most efficiently absorbs green light, whereas PC absorbs red light most effectively (Fig. 8). There are two proposed signal transduction model system involved in this Type III CCA regulation- one is the regulation of complementary chromatic acclimation (Rca) system (Sobczyk, Schyns et al. 1993; Terauchi, Montgomery et al. 2004) and the second pathway suported by photobiology experiment is known as the Cgi (control of green light induction) (Sidler 1994). The Rca system affects the CCA regulation by decreasing the level of *cpeCDE* and *cpeBA* when exposed to red light while increasing gene expression when exposed to green light (Alvey, Karty et al. 2003; Li and Kehoe 2005).

Some marine *Synechococcus* sp (like RS 9916) strains which contains two type of PE (PEI and PEII) in their PBS rods, carry out the type IV chromatic acclimation. It occurs when the organism undergoes a shift from blue to green (or white) light. The main difference between Type III and Type IV is that in Type IV CA the overall content of both PEI and PEII remains same the only the ratios of the chromophores PUB:PEB changes. Everroad et al. proposed that the Type IV CA might be due to involvement of several bilin lyases and/ or lyase-isomerases which are being regulated by light quality (Everroad, Six et al. 2006). Fig. 10 depicts an excellent model system for Type IV CCA. In this thesis one of the lyase/isomerases known as MpeZ for the PEII α-subunit was characterized.



Kehoe DM, Gutu A. 2006.
Annu. Rev. Plant Biol. 57:127–50

Fig. 8. The color phenotypes of *F. diplosiphon* filaments grown on agar plates and fully acclimated to green light (left) and red light (right). The accumulation of different pigmented proteins into phycobilisome (PBS) rods renders the cells brick red or blue green (Kehoe and Gutu 2006)

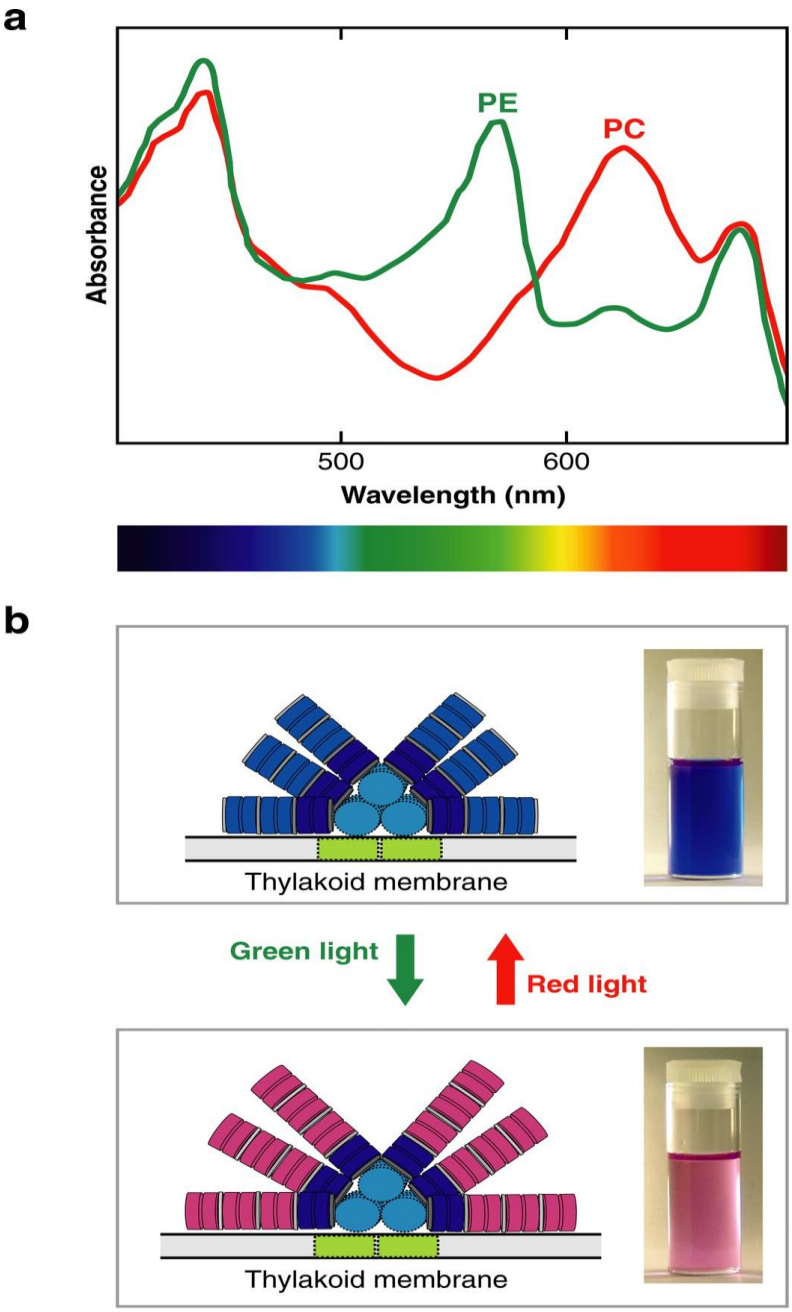


Fig. 9. (a) Whole-cell absorbance spectra of *F. diplosiphon* cells grown in green and red light. The phycoerythrin (PE) and phycocyanin (PC) absorption peaks are indicated**.** The remaining peaks in the blue and red regions represent absorption by chlorophyll *a* and carotenoids. (b) Red and green light induced structural changes in an *F. diplosiphon* phycobilicome (PBS) and the corresponding extracted phycobiliproteins. The water-soluble PBS associates with photosystem II reaction centers (green rectangles) and consists of a tricylindrical core (light blue) and the outwardly oriented rods. The PBS structural unit is a hexamer (disc containing of stacked pairs of cylindrical trimers when viewed on ends), allophycocyanin (AP) (light blue) in the core, and constitutive PC (dark blue), inducible PC (medium blue), and / or PE (pink) in the rods. Linker proteins (gray) serve as scaffolds. Green rectangles represent photosystem II reaction centers. This model was derived using ultrastructural and biochemical data (Bryant, Gugliemi et al. 1979; Rosinski, Hainfeld et al. 1981; Glazer 1982; Beguin, Guglielmi et al. 1985)

Kehoe DM, Gutu A. 2006.
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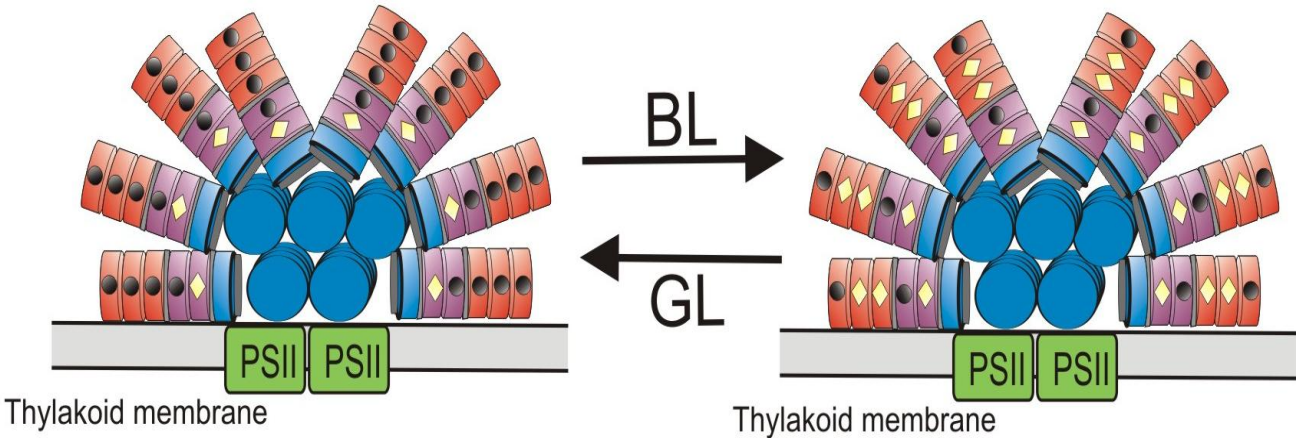

Fig. 10. Proposed models of PBS structure for the different *Synechococcus* pigment types and subtypes. PBS cores are generally composed of three cylinders, but in some chromatic aclimaters possessing an extended L_{CM}, it is likely composed of two additional half cylinders. The composition of the rods and core and indicated with colored symbols. In case of Type IV CA the ratios of PEB:PUB in case of both PEI and PEII varies (Six, Thomas et al. 2007).
The figure is modified from (Six, Thomas et al. 2007)

1.10. *Purpose of this work:*

The overall purpose of this thesis is to characterize the biochemical pathways involved in the formation of the brilliantly colored phycobiliproteins in various cyanobacterial speices and to demonstrate how they can be used for biotechnological applications. Here various lyase and/ or lyase/isomerase present in different species of cyanobacteria were characterized in detail.

The first goal of the research work presented here was to develop an *in vivo* multiplasmid heterologous expression system in *E.coli* by co-expressing three categories of genes: genes encoding the phycobiliproteins substrate, genes for biosynthetic enzymes of the prosthetic groups, and the enzymes which catalyze the ligation of the prosthetic groups.

This work included is identify the bilin lyases involved in ligating PCB on the less abundant allophycocyanin subunits ApcD and ApcF in *Synechococcus sp*. PCC 7002 and also elucidate intrinsic bilin lyase activity of N-terminal, allophycocyanin AP-like domain of ApcE (L_{cm}^{99}).

The second goal of this work was to create unique phycobiliproteins in *E.coli* cells by chromophorylating with unnatural occurring bilins, which may have potential biotechnological applications.

The third goal of this work was to identify and characterize the bilin lyases for PE subunits in *Fremyella diplosiphon.*

The fourth goal of this work was to elucidate the biochemical pathway for the less studied bilin; phycourobilin (PUB) by identifying and characterizing the bilin lyase/ isomerase for PE II subunits responsible for Type IV CA in the marine cyanobacterial species, *Synechococcus sp*. RS 9916.

The last goal of this thesis project was to characterize a new CpcS type lyase named as Ter 13 or TECpcS from *Thermosynechococcus elongatus* (Described in Appendix).

2.0. MATERIALS AND METHODS

2.1 ***Construction of expression vectors:***

Plasmids used in this study are listed in Table 4. Some of the expression vectors used in this study were previously described (Tooley, Cai et al. 2001; Shen, Saunee et al. 2006; Miller, Leonard et al. 2008) or made by our collaborators in the Bryant lab (Pennsylvania State University) or in the Kehoe lab (Indiana University) . All expression constructs newly produced for this study were sequenced at the W. M. Keck Conservation and Molecular Genetics laboratory (University of New Orleans) to confirm that no mutations had been introduced during PCR amplification and cloning.

Table 4: Summary of plasmids used for the experimental studies described in this thesis

Plasmid Name	*Recombinant proteins produced*[a]	*Parent vector*	*Anti-biotic*[b]	*Reference*
pApcAB	*Synechococcus* sp. PCC 7002 HT-ApcA and ApcB	pET100	Ap	Shen et al., 2006
pApcDB	*Synechococcus* sp. PCC 7002 HT-ApcD and ApcB	pET100	Ap	Miller et al., 2008
pApcD	*Synechococcus* sp. PCC 7002 HT-ApcD	pET100	Ap	Miller et al., 2008
pApcF	*Synechococcus* sp. PCC 7002 HT-ApcF	pET100	Ap	Miller et al., 2008
pPcyA	*Synechocystis* sp. PCC 6803 HO1 and *Synechococcus* sp. PCC 7002 HT-PcyA	pACYC Duet	Cm	Biswas et al., 2010
pCpcUS	*Synechococcus* sp. PCC 7002 CpcU and CpcS coexpressed on one mRNA	pCOLA Duet	Km	Biswas et al., 2010
pCpcU	*Synechococcus* sp. PCC 7002 CpcU	pCOLA Duet	Km	Biswas et al., 2010
pCpcS	*Synechococcus* sp. PCC 7002 CpcS	pCOLA Duet	Km	Biswas et al., 2010
pCpcT	*Synechococcus* sp. PCC 7002 CpcT	pCOLA Duet	Km	Biswas et al., 2010
pGST-ApcE	*Synechococcus* sp. PCC 7002 GST-ApcE (1-228 amino acids)	pGEX-2T	Ap	Biswas et al., 2010
pCpcBA	*Synechocystis sp.* PCC 6803 HT-CpcB and CpcA	pCDF Duet	Sp	Biswas et al., 2010
pCpcB	*Synechocystis sp.* PCC 6803 HT-CpcB	pBS150v	Sp	Biswas et al., 2010
pBS414v	*Synechocystis* sp. PCC 6803 HT-CpcA, CpcE and CpcF	pBS350v	Sp	Tooley et al., 2001
pBS415v	*Synechocystis* sp. PCC 6803 CpcE and CpcF	pBS350v	Sp	Biswas et al., 2010
pBS405v	*Synechocystis* sp. PCC 6803 HT-CpcA	pBS350v	Sp	Tooley et al., 2001
pAT101	*Synechocystis* sp. PCC 6803 HO1 and PcyA	pBS350v	Km	Tooley et al., 2001
pMpeA	*Synechococcus* sp. RC9916 HT-MpeA	pCOLA Duet	Km	Thesis
pCpeA	*Synechococcus* sp. RC9916 HT-CpeA	pCOLA Duet	Km	Thesis
pMpeZ	*Synechococcus* sp. RC9916 MpeZ	pCDF Duet	Sp	Thesis
pMpeA:C83A	*Synechococcus* sp. RC9916 HT-MpeA (Cys 83 mutated to Ala)	pCOLA Duet	Km	Thesis
pMpeA: C75A	*Synechococcus* sp. RC9916 HT-MpeA (Cys 75 mutated to Ala)	pCOLA Duet	Km	Thesis
pMpeA:C140A	*Synechococcus* sp. RC9916 HT-MpeA (Cys 140 mutated to Ala)	pCOLA Duet	Km	Thesis
pMpeA:C75A,C140A	*Synechococcus* sp. RC9916 HT-MpeA (Cys 74 and 140 mutated to Ala)	pCOLA Duet	Km	Thesis
pMpeA:C83A,C140A	*Synechococcus* sp. RC9916 HT-MpeA (Cys83 and 140 mutated to Ala)	pCOLA Duet	Km	Thesis
pMpeA:C83A,C75A,C140A	*Synechococcus* sp. RC9916 HT-MpeA (Cys83, 75 and 140 mutated to Ala)	pCOLA Duet	Km	Thesis
pMpeB	*Synechococcus* sp. RC9916 HT-MpeB	pCOLA Duet	Km	Thesis

Table 4 continued

P'CpeA pPebS	*Synechococcus* sp. RC9916 HT-CpeA Myovirus HO1 and HT-PebS	pCOLA Duet pACYC Duet	Km Cm	Thesis Dammeyer et al., 2008
pCpeA	*Fremyella diplosiphon*, HT-CpeA	pET Duet	Ap	Thesis
pCpeA:C82S	*Fremyella diplosiphon*, HT-CpeA (Cys 82 mutated to Ser)	pET Duet	Ap	Thesis
pCpeA:C139S	*Fremyella diplosiphon*, HT-CpeA (Cys 139 mutated to Ser)	pET Duet	Ap	Thesis
pCpeA:C82,139S	*Fremyella diplosiphon*, HT-CpeA (Cys 82 and 139 mutated to Ser)	pET Duet	Ap	Thesis
pCpeB	*Fremyella diplosiphon*, HT-CpeB	pET Duet	Ap	Thesis
pCpeB:C80S	*Fremyella diplosiphon*, HT-CpeB (Cys 80 mutated to Ser)	pET Duet	Ap	Thesis
pCpeB:C165S	*Fremyella diplosiphon*, HT-CpeB (Cys 165 mutated to Ser)	pET Duet	Ap	Thesis
pCpeB:C48,59S	*Fremyella diplosiphon*, HT-CpeB (Cys 48 and 59 mutated to Ser)	pET Duet	Ap	Thesis
pCpeZ	*Fremyella diplosiphon*, HT-CpeZ	pCOLA Duet	Km	Thesis
pCpeY	*Fremyella diplosiphon*, CpeY	pCOLA Duet	Km	Thesis
pCpeYZ	*Fremyella diplosiphon*, HT-CpeZ and CpeY coezpressed as one mRNA	pCOLA Duet	Km	Thesis
pCpeS	*Fremyella diplosiphon*, HT-CpeS	pCOLA Duet	Km	Thesis
pTECpcS pHy2	*Thermosynechococcuselongatus* CpcS *Synechocystis* sp. PCC 6803 HO1 and *Arabadopsis thaliana* HT-Hy2	pCOLA Duet pACYC Duet	Km Cm	Thesis Thesis

[a] Proteins that would be produced as fusions are indicated as either HT- or GST-

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

2.1.1. *cpcS-I and cpcU expression construct:*

The *cpcS-I* and *cpcU* genes were cloned in the pCOLA Duet vector (Novagen, Madison, WI) to generate plasmid pCpcUS, from which these genes would be cotranscribed to produce HT-CpcU and CpcS (see Table 4). The *cpcS-I* gene (SYNPCC7002_A1822) was amplified by PCR from *Synechococcus* sp. strain PCC 7002 chromosomal DNA using primers cpcSF and cpcSR (Refer to table 5) and cloned into pCOLA Duet after digestion with PstI and SalI (restriction sites in primers are underlined, and the forward primer contains a ribosome binding site) to create plasmid pCpcS. The *cpcU* gene (SYNPCC7002_A2053) was amplified by PCR from *Synechococcus* sp. strain PCC 7002 chromosomal DNA using cpcUF and cpcUR (Refer to table 5) and cloned into the pCOLA Duet plasmid using the restriction enzymes BamHI and EcoRI (restriction sites underlined in primers) to create the pCpcU plasmid. The *cpcU* gene was subcloned into the pCpcS plasmid using BamHI and EcoRI to create plasmid pCpcUS (See Fig. 11). The CpcU protein will have an amino terminal with 6-Histidine tag.

2.1.2. *cpcT expression construct:*

The *cpcT* gene (SYNPCC7002_A1822) was amplified from *Synechococcus* sp. PCC 7002 chromosomal DNA using primers cpcTF and cpcTR (Refer to table 5) and cloned into pCOLA Duet after digestion with NdeI and EcoRV (restriction sites are underlined in primers) to create plasmid pCpcT (See Fig. 12).

2.1.3. *pcyA/ho1 expression constructs:*

The heme oxygenase 1 gene, *ho1* (sll1184) was amplified by PCR from the chromosomal DNA of *Synechocystis* sp. PCC 6803 using hox1F and hox1R (Refer to table 5) and cloned into pACYC

Duet (Novagen, Madison, WI) using NdeI and EcoRV. The 3Z-phycocyanobilin:ferredoxin oxidoreductase gene, *pcyA* (SYNPCC7002_A2228) was amplified by PCR from chromosomal DNA of *Synechococcus* sp. strain PCC 7002 using pcyAF and pcyAR (Refer to table 5). The gene was cloned into the pACYC Duet vector containing *ho1* using EcoRI and SalI, and the resultant plasmid was named pPcyA**.** In *E. coli* the expression of these two gene products results in the production of PCB from heme (Frankenberg, Mukougawa et al. 2001; Frankenberg and Lagarias 2003; Shen, Saunee et al. 2006). (See Fig. 13).

2.1.4. *cpcBA and cpcB expression constructs:*

The *Synechocystis* sp. strain PCC 6803 *cpcBA* genes (encoding the β and α subunits of PC, respectively; sll1577 and sll1578) were amplified from an existing plasmid called *cpcBA*/pBS150v described in (Miller, Leonard et al. 2008). CpcB can be produced with a N-terminal hexa-histidine tag from this construction. The pBS150vNcoF primer, which anneals to the pBS150v vector sequence and the cpcAR primer (Refer to table 5), which is complementary to the 3' end of the cpcA gene, were used to amplify the product using the PCR. The product was cloned into the pCDF Duet vector (Novagen, Madison, WI) using the NcoI and HindIII sites (restriction sites in primers are underlined). This expression clone results in the production of histidine-tagged CpcB and non-tagged CpcA. Another clone just expressing HT-CpcB was created as follows. The *cpcB* gene from *Synechocystis* sp. PCC 6803 was amplified by PCR using the 6803cpcB.1 forward primer and the 6803cpcB.4 reverse primer. This *cpcB* gene was cloned into the NdeI and EcoRI sites of pBS150v to create pCpcB (see Table 5). (Fig. 14).

2.1.5. *apcE expression construct:*

The 5' end of the *apcE* gene, which encodes the AP-like domain encompassing amino acids 1-228 of the L_{CM}^{99} (SYNPCC7002_A2009), was amplified by PCR from *Synechococcus* sp. strain PCC 7002 DNA using the primers apcEF and apcER (Refer to table 5). This gene was cloned into the SmaI and EcoRI sites (underlined in primers) of pGEX-2T producing plasmid pGST-ApcE, which encodes a fusion protein consisting of GST at the N-terminus fused to the first 228 amino acids of ApcE (Vector map not shown).

2.1.6. *CpcEF expression construct:*

The plasmid pBS414v (Tooley, Cai et al. 2001) containing *Synechocystis* sp. strain PCC 6803 HT-*cpcA* along with *cpcE* and *cpcF* was digested with NcoI and EcoRI, the 5' overhangs were blunt ended using DNA Polymerase I and dNTPs, and this was self-ligated to create the plasmid pBS415v containing the lyase genes *cpcE* and *cpcF* (Refer to table 5). This expression construct was tested, and it expressed active CpcEF (vector map not shown).

2.1.7. *cpeA expression construct:*

The *cpeA* gene was PCR amplified from *Fremyella diplosiphon* PCC 7601 chromosomal DNA using primers cpeAF and cpeAR (See Table 5) and cloned into pET Duet (Novagen, Madison, WI) by digesting with BamHI and EcoRI (restriction enzyme sites are underlined in primers), the resultant plasmid was called pCpeA. The construct results in the production of the production of His-tagged CpeA (See Fig. 15)

2.1.8. *cpeZ and cpeY expression construct:*

The *cpeZ and cpeY* genes from *Fremyella diplosiphon* PCC 7601 were cloned in the pCOLA Duet vector (Novagen, Madison, WI) to generate plasmid pCpeZY, from which these genes would be cotranscribed in *E.coli* to produce HT-CpeZ and CpeY (see Table 4). The *cpeZ* gene was amplified by PCR from *Calotrix* sp. PCC 7601 chromosomal DNA using primers cpeZF and cpeZR (See Table 5) and cloned into pCOLA Duet after digestion with BamHI and ECoRI (restriction sites in primers are underlined, and the forward primer contains a ribosome binding site) to create plasmid pCpeZ. The *cpeY* gene was also amplified by PCR from *Fremyella diplosiphon* PCC 7601 chromosomal DNA using cpeYF and cpeYR (See Table 5) and cloned into the pCOLA Duet plasmid using the restriction enzymes NdeI and BglII (restriction sites underlined in primers) to create the pCpeY plasmid. The *cpeY* gene was subcloned into the pCpeZ plasmid using NdeI and BglII to create plasmid pCpcZY (See Fig. 16).

2.1.9. *cpeB expression construct:*

The *cpeB* gene was amplified by PCR from *Fremyella diplosiphon* PCC 7601 chromosomal DNA using primers cpeBF and cpeBR (See Table 5) and cloned into pET Duet after digesting with EcoRI and HindIII (restriction sites are underlined in primers) to create plasmid pCpeB. The construct results in the production of the production of His-tagged CpeB (See Fig. 17).

2.1.10. *cpeS expression construct:*

The *cpeS* gene was amplified from *Fremyella diplosiphon* PCC 7601 chromosomal DNA using primers cpeSF and cpeSR (See Table 5) and cloned into PCR 2.1 vector. The cpeS cloned in

PCR 2.1 vector was then sub cloned into pCOLA Duet after digestion with NdeI and XhoI (restriction sites are underlined in primers) to create plasmid pCpeS (See Fig. 18).

2.1.11. *pebS/ho1 expression constructs:*

The pPebS plasmid was a generous gift from Dr. Nicole Frankenberg-Dinkel, and it contained the *hox1* (heme oxygenase) and *pebS* (Phycoerythrobilin synthase) genes from a myovirus which infects *Prochloroccoccus (Dammeyer, Bagby et al. 2008)*. This plasmid resulted in the production of phycoerythobilin (PEB) from heme in *E.coli.* (See Fig. 19)

2.1.12. *CpeA and CpeB site-directed mutants construct:*

The pCpeA plasmid (described earlier in "Materials and Methods") was used as a template for generating different CpeA mutants. We used the Transformer™ Site-Directed Mutagenesis Kit from Clontech Laboratories, Inc. to create three different mutants; CpeA (C82S), CpeA (C139S) and CpeA (C82, 139S). The primers used are CpeA (C82S), CpeA (C139S), and CpeA (BamHI del) (See Table 5). There CpeB mutant was created uning the primers CpeB (C80S), CpeB (C165S), and CpeB (C48S/C59S), using same method as in case of CpeA (See Table 5).

Table 5: ***Primer sequences used in the study:***

Primer	Sequence (5' to 3')	Use
7002 pcyA;F (EcoRI)	CAGAATTCCATGACTGCCCCTGCAACCAAGC	Amplification of PCC 7002 pcyA
7002 pcyA;R(SalI)	AAGTCGACGATCTAGGCTGGAATATCAAACAGCACC	Amplification of PCC 7002 pcyA
7002 cpcU;F (BamHI)	AGGGGATCCTATGGATATCAATGCCTTTATC	Amplification of PCC 7002 cpcU
7002 cpcU;R (EcoRI)	GCCGAATTCTTAGTTACTGGCTTCAGCGGTTAC	Amplification of PCC 7002 cpcU
7002 cpcS;F (PstI)	TCCCTGCAGAAGGAGATTTCGATATGCAAAGCTTTGC	Amplification of PCC 7002 cpcs
7002 cpcS;R (SalI)	ACGGTCGACCTACCAACCGCTAATAGCGTAAAG	Amplification of PCC 7002 cpcs
7002 cpcT;F(NdeI)	CTCGCTTACATATGTCCCACTCTACCGATGCCCATAC	Amplification of PCC 7002 cpcT
7002 cpcT;R(XhoI)	TTCTCGAGTTAATGGGGTTGAACTTCCCCAGAGAAAT	Amplification of PCC 7002 cpcT
7002 apcE;F(SmaI)	AAACCCGGGAATGACGATTAAGGCCAGCGGTGG	Amplification of PCC 7002 apcE
7002 apcE;R (EcoRI)	AGAATTCACTGCATTTCGTGATTAACACATC	Amplification of PCC 7002 apcE
6803 cpcB;F (NcoI)	AACCATGGAGATCAGTAACAATAACTCTAGGG	Amplification of PCC 6803 cpcBA
6803 cpcA:R (HindIII)	ACTAAGCTTAATTAGCTGAAGGGCG	Amplification of PCC 6803 cpcBA
6803 cpcB;F	CAAGTAGGAGATTAATCATATGTTCGACGTA	Amplification of PCC 6803 cpcB
6803 cpcB;R	AGAATTCCTAGGCTACGGCAGCAGCGGCG	Amplification of PCC 6803 cpcB
6803 cpcE;F (KpnI)	AAGGTACCCGTCGACAAGGACCTTCATATG	Amplification of PCC 6803 cpcEF
6803 cpcF;R (XhoI)	AACTCGAGGTCTCCGGATCCTAGAAGACTA	Amplification of PCC 6803 cpcEF
Fd cpeB ;F(BamHI)	AAGGATCCGATGCTTGATGCTTTTTCTAGAGC	Amplification of Fd cpeB
Fd cpeB;R (EcoRI)	CCGAATTCTTAGCTCAAAGCAGAGATTACGCG	Amplification of Fd cpeB
Fd cpeS;F (NdeI)	CAAATAGCTAAAACATATGGAAACCAAAGTGTTG	Amplification of Fd cpeS
Fd cpeS;R (XhoI)	AACTGCAGCTAGGCACCAGTGTTTATG	Amplification of Fd cpeS
Fd cpeZ;F (BamHI)	CCGGATCCGATGCCGACAACAGAAGAACTATTCCAA	Amplification of Fd cpeZ
Fd cpeZ;R(EcoRI)	CCGAATTCTTATTTTTCTCCCCGCTGAAACTT	Amplification of Fd cpeZ
Fd cpeY;F (NdeI)	ACAAGGAGCTTGCATATGGATAAGCGCTTTTTT	Amplification of Fd cpeY

Table 5: continued

Fd cpeY;R (XhoI)	AACTCGAGGGCTGTGATTTCTTGATTTTTCAGGGT	Amplification of Fd cpeY
Fd cpeA;F (BamHI)	AAGGATCCGATGAAATCAGTTGTTACCACCGT	Amplification of Fd cpeA
Fd cpeA;R (EcoRI)	AAGAATTCCTAGGAGAGAGAGTTAATAGCGTA	Amplification of Fd cpeA
Fd cpeF;F (NdeI)	AATTTGTGCATATGAGTCAATCACTCAACTCAGAA	Amplification of Fd cpeF
Fd cpeF; R (XhoI)	AACTCGAGTTACCAATCATCTTCTTCGGATTG	Amplification of Fd cpeF
Fd CpeA (C82S)	5'-CCTTCAAAGCTAAGTCCGCTCGTGACATC-3'	FdCpeA mutation
Fd CpeA (C139S)	5'-CGTAACCGTGGTTCTGCACCTCGTGATATG-3'	Fd CpeA mutation
pETDuet(XhoI del)	5'-ACGTCGGTACCCTCCAGTCTGGTAAAGAA ACCGCTG-3'	Fd CpeA or Fd CpeB mutation
Fd CpeB (C80S)	5'-CGTATGGCTGCCTCCTTACGCGATGCA-3'	Fd CpeB mutation
Fd CpeB (C165S)	5'-GTTGAAGATCGTTCCGCTAGCTTAGTT-3'	Fd CpeB mutation
FdCpeB (C48, 59S)	5'-GCTAGCTCCATGGTTTCTGATGCGTAGC TGGAATGATCTCCGAAAACCAAGGT-3	Fd CpeB mutation
Duet UP2 primer	ATTGTACACGGCCGCATAATC	Sequencing
dUET DOWN primer	GATTATGCGGCCGTGTACAA	Sequencing
T7 TERMINATOR	GCTAGTTATTGCTCAGCGG	Sequencing
pET upstream primer	ATGCGTCCGGCGTAGAGG	Sequencing
pACYC DUETUP1	GGATCTCGACGCTCTCCCT	Sequencing

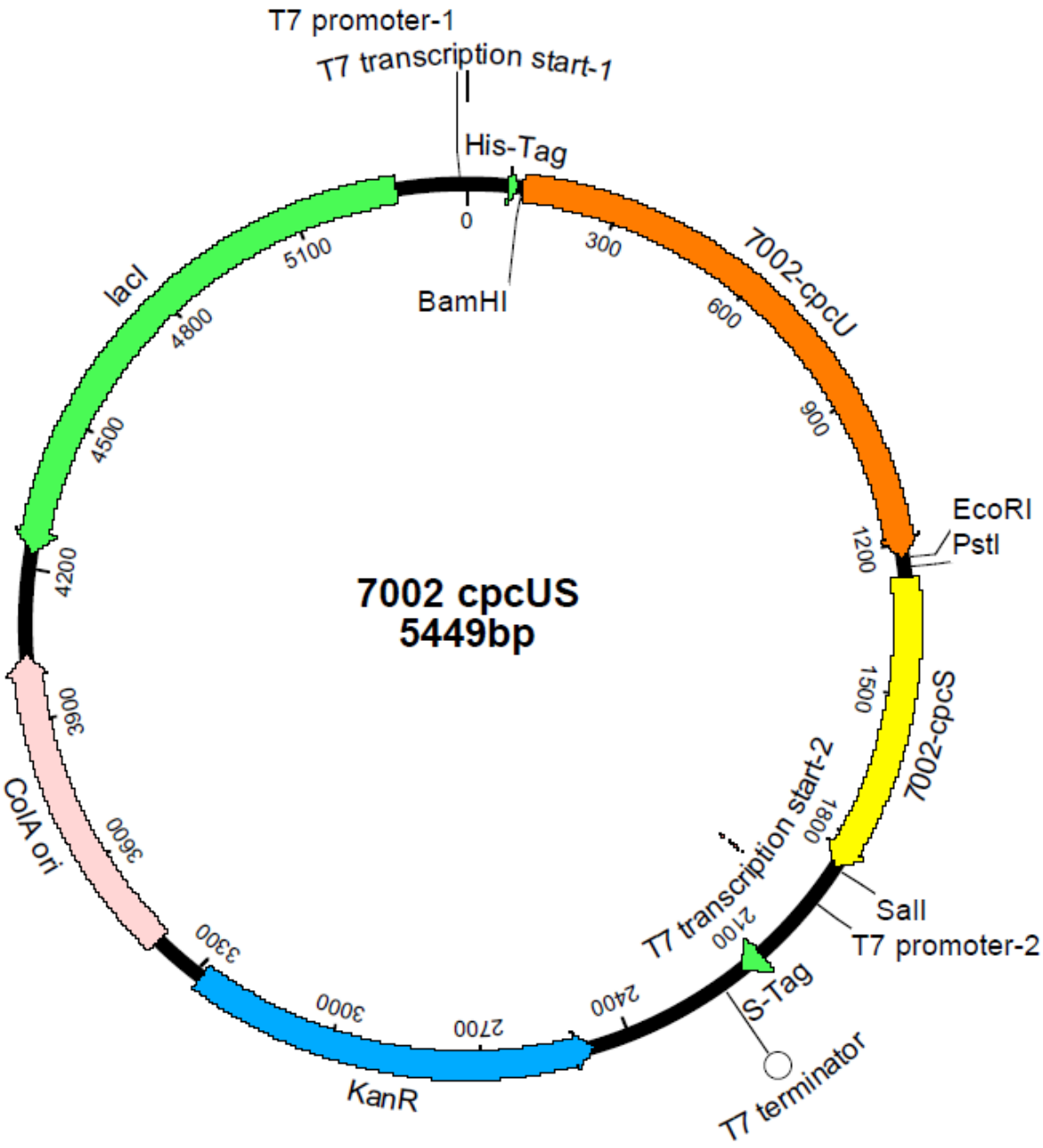

Fig. 11. Plasmid map of *Synechococcus sp.* PCC 7002 *cpcUS* cloned in pCOLA Duet, the features are described above.

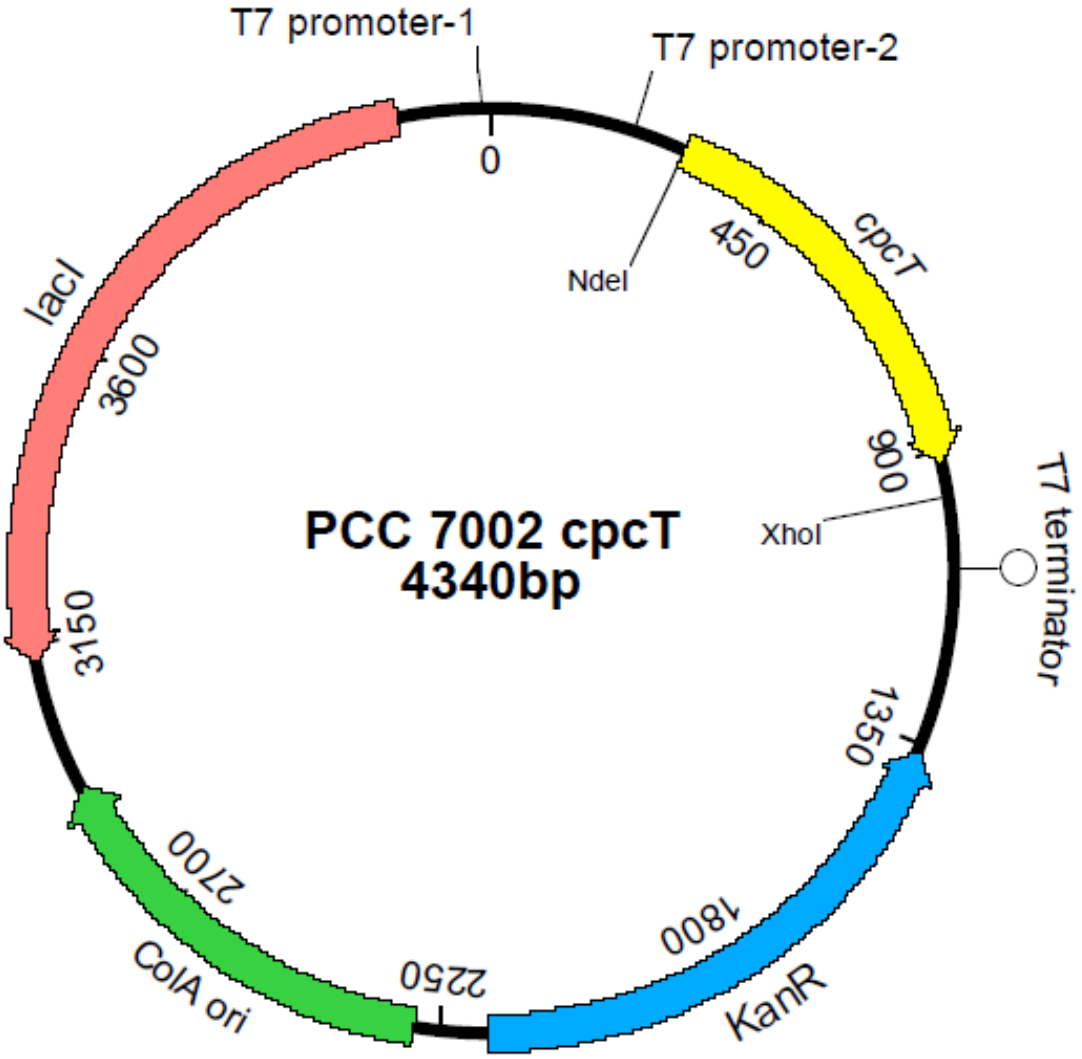

Fig. 12. Vector map of *Synechococcus sp* PCC 7002 CpcT construct, the features are described in the map itself

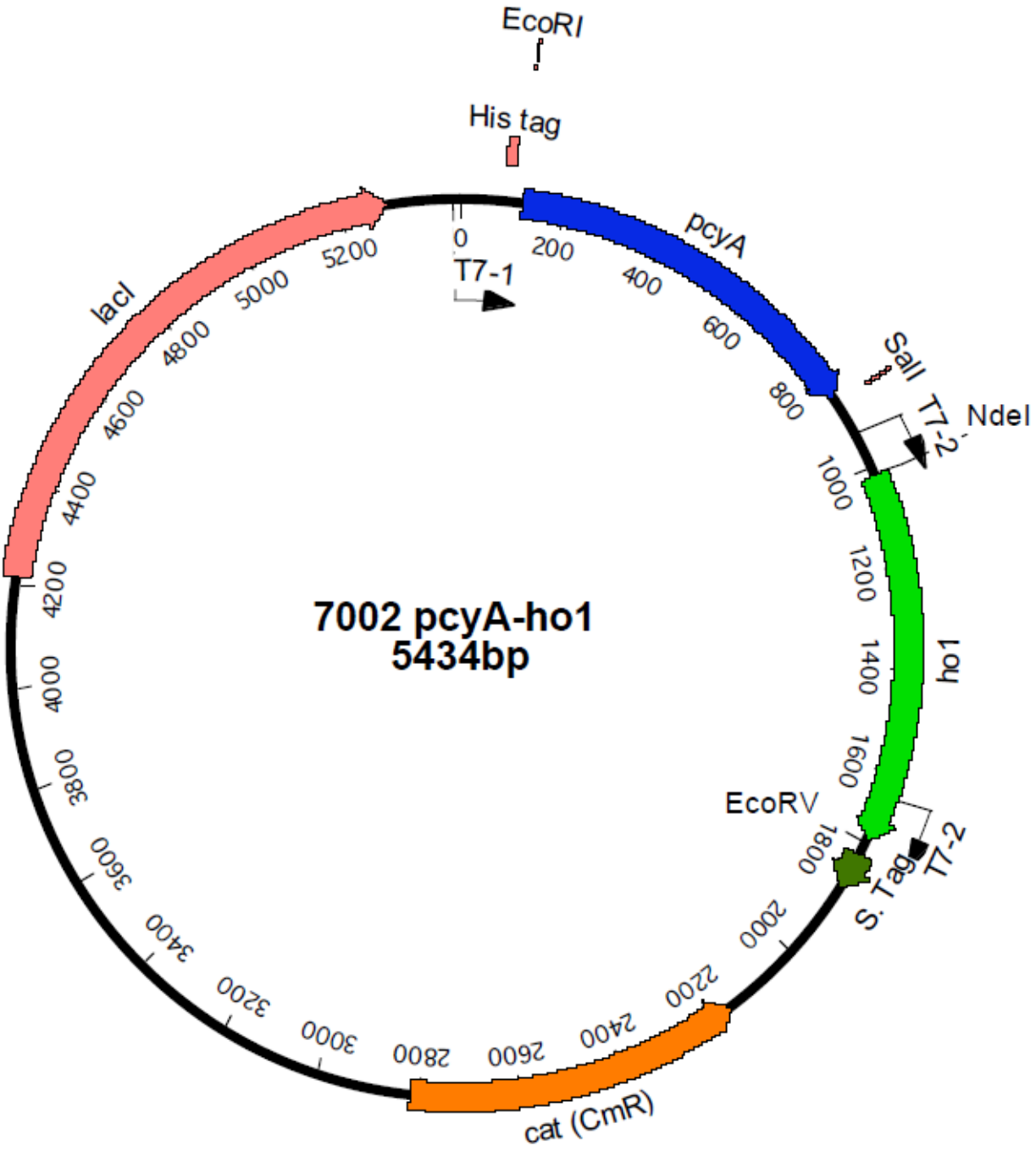

Fig. 13. Plasmid map representing pcyA (from PCC 7002) /ho1 (PCC 6803) cloned in pACYC Duet vector.

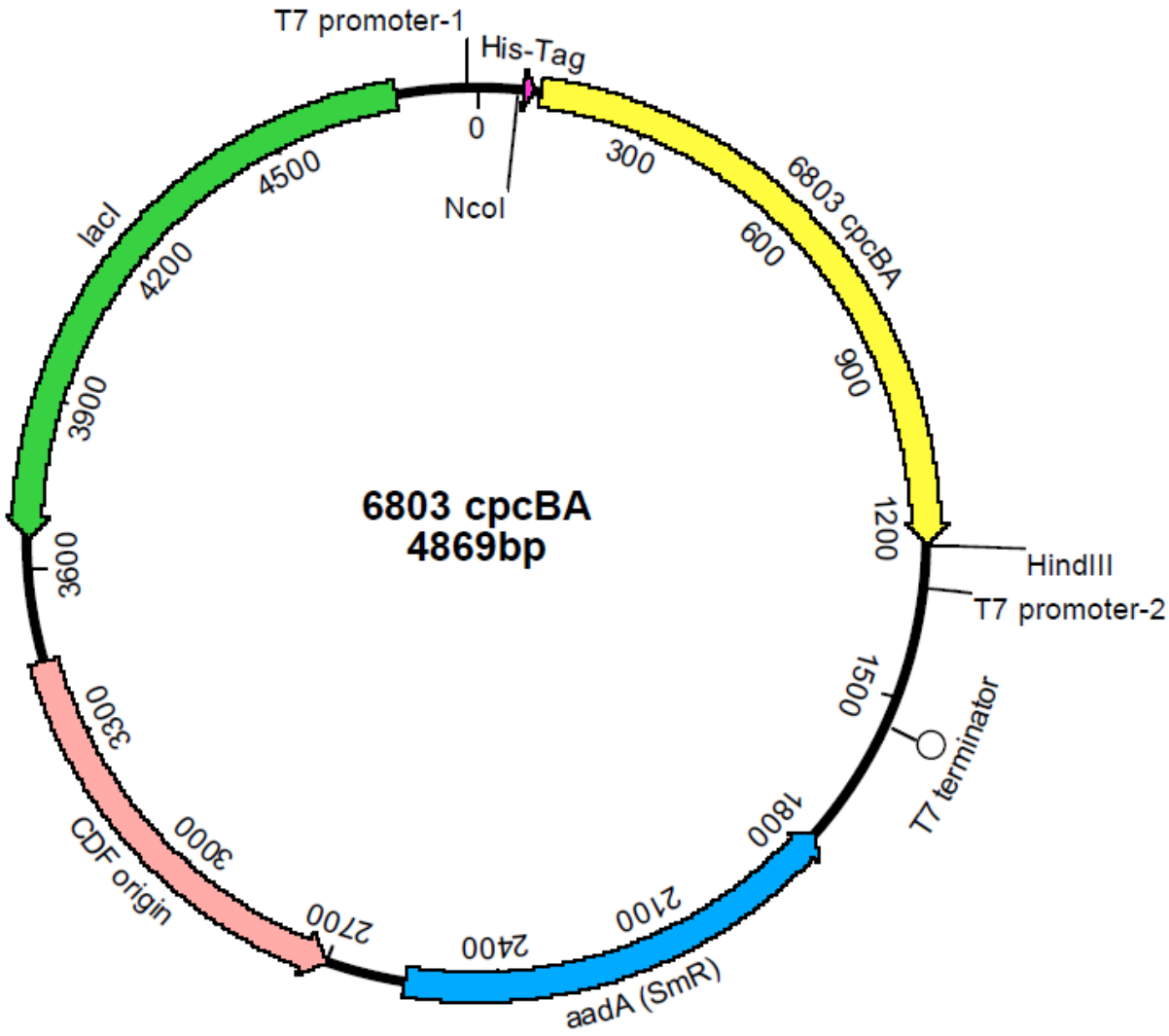

Fig. 14. Vector map of *Synechocystis sp.* PCC 6803 CpcBA in pCDF Duet

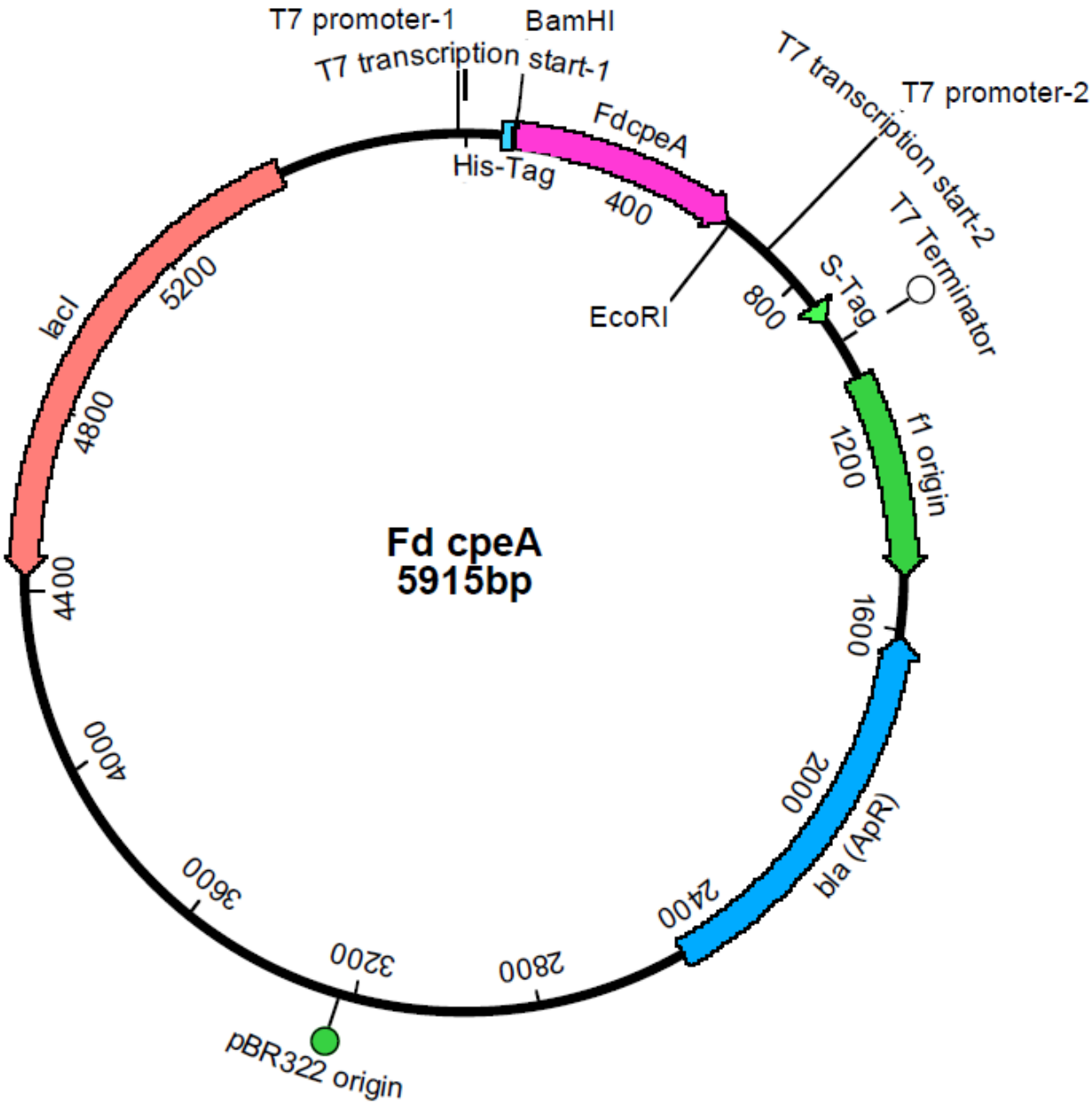

Fig. 15. Vector map of HT-CpeA from *F. diplosiphon* UTEX 481 cloned in pET Duet vector.

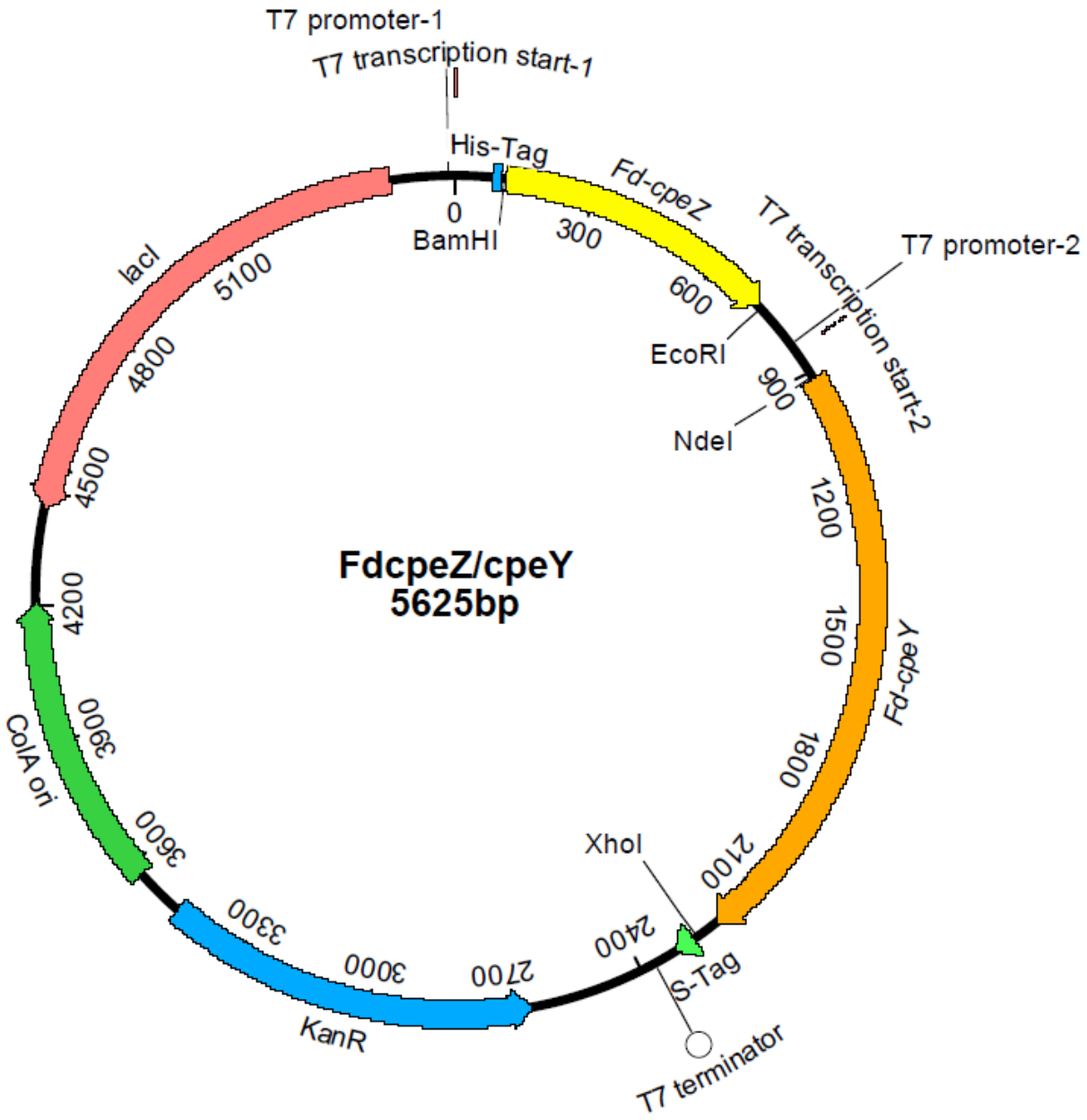

Fig. 16. Vector map of *F. diplosiphon* UTEX 481 *cpeZY* cloned in pCOLA Duet.

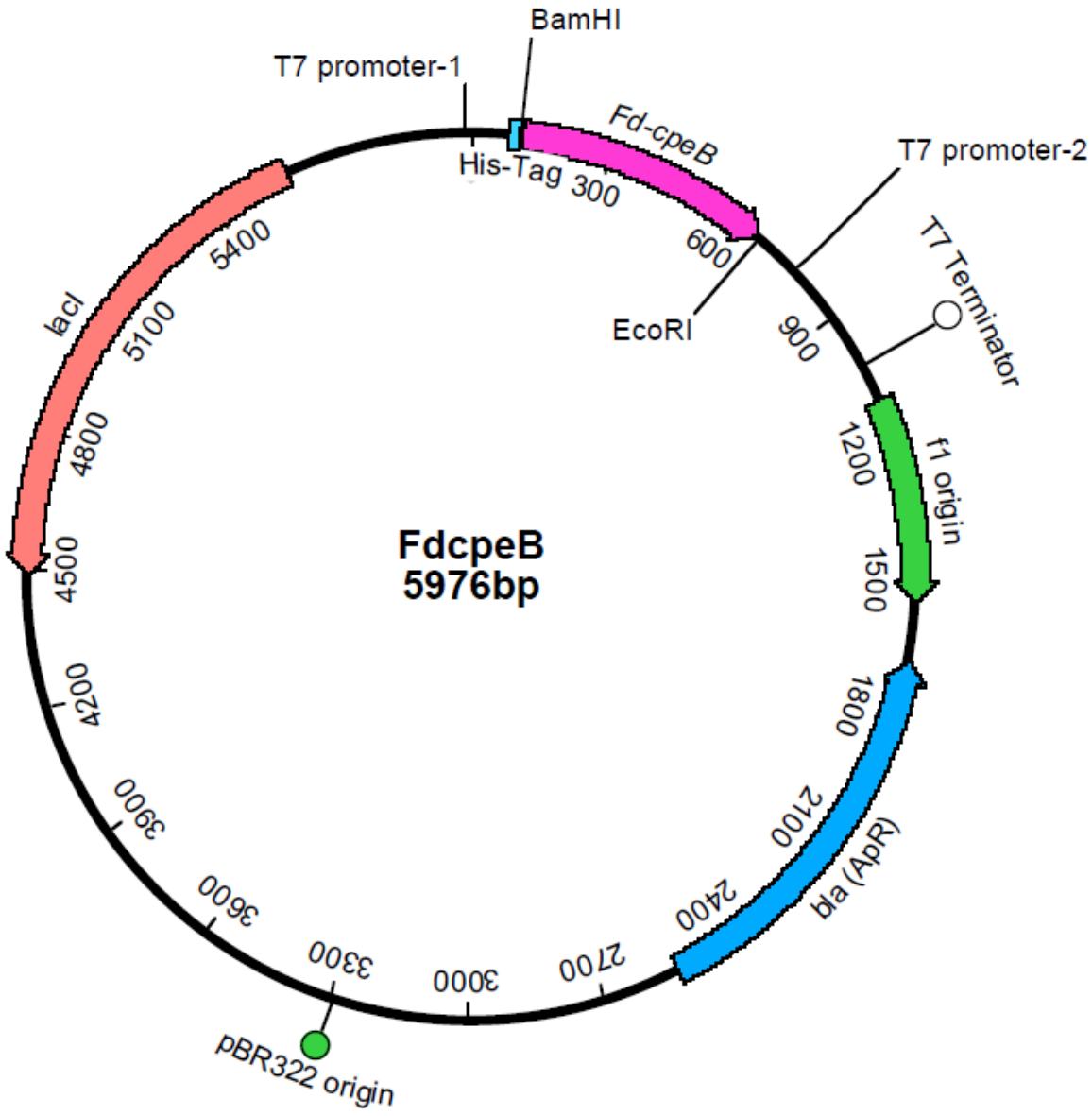

Fig. 17. Vector map of *F. diplosiphon* UETX 481 *cpeB* cloned in pET Duet.

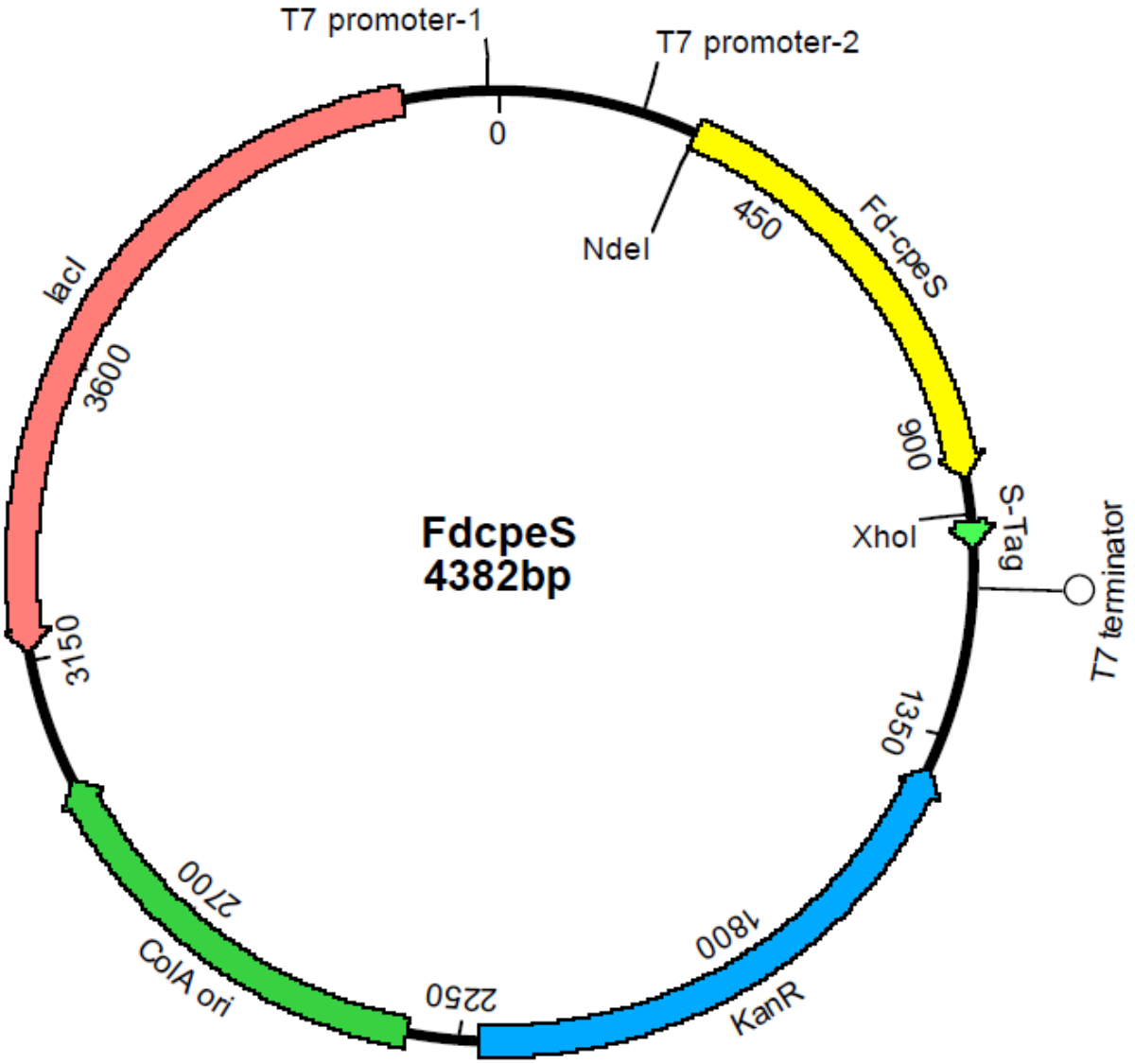

Fig .18. Vector map of *F. diplosiphon* UETX 481 *cpeS* cloned in pCOLA Duet.

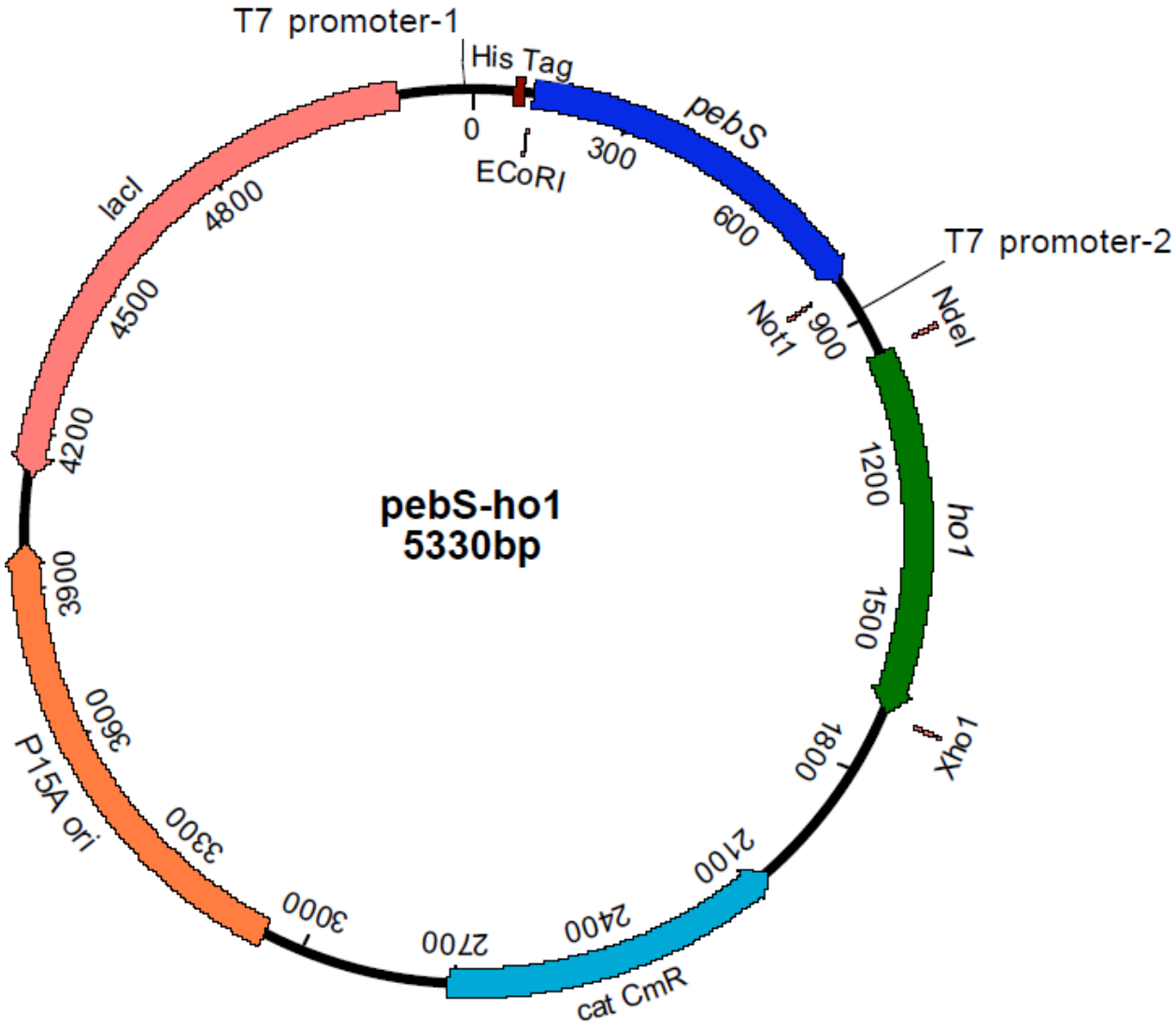

Fig. 19. Vector map of pebS (from *Prochlorococcus marinus* MED4)/ho1 cloned in pACYC Duet.

2.2. ***In-vivo heterologous expression and purification of recombinant proteins*****:** Expression plasmids were co-transformed into *E. coli* BL21 *DE3* cells, and colonies were selected on Luria Bertani (LB) plates in the presence of the appropriate combination of antibiotics (see Table 1) at the following concentrations: ampicillin (Ap: 100 μg ml^{-1}), chloramphenicol (Cm: 34 μg ml^{-1}), kanamycin (Km: 50 μg ml^{-1}), spectinomycin (Sp: 100 μg ml^{-1}). To produce PCB using the pPcyA expression plasmid, a 50-ml overnight starter culture was added to 1 L of LB medium with the appropriate combination of antibiotics. This culture was shaken at 37°C for 4 h until the optical density reached $OD_{600\ nm}$ = 0.6. Production of T7 RNA polymerase was induced by the addition of 0.5 mM isopropyl β-D thiogalactoside (IPTG). Cells were incubated with shaking at 225 rpm at 30°C for another 4 h before they were harvested by centrifugation at 10,000 × *g* for 10 min. Cells were incubated at 30° C after induction with IPTG to limit the production of inclusion bodies (data not shown). Cell pellets were stored at -20 °C until required**.**

E. coli cells containing recombinant proteins were thawed and resuspended in Buffer O (50 mM Tris-HCl, 150 mM NaCl, pH 8.0) at 2.5 ml/g (wet weight) and lysed by three passages through a chilled French pressure cell at 138 MPA**.** The lysed cell suspension was centrifuged for 20 min at 13,000 × *g* to remove inclusion bodies, cell debris and unbroken cells. To purify hexa-histidine-tagged recombinant proteins, the supernatant was passed over nickel-nitrilotriacetic acid-Superflow-affinity column (Qiagen, Inc., Chatsworth, CA) containing 5 ml of resin, and proteins were purified as previously described (Shen, Saunee et al. 2006). The recombinant protein(s) were dialyzed with buffer O containing 10 mM 2-mercaptoethanol overnight at 4 °C to remove the imidazole. For purification of GST-ApcE, cells were broken as described (Miller, Leonard et al. 2008) except that protease inhibitor cocktail tablets were added ("Complete Mini" purchased from

Roche, Mannheim, Germany). The clarified extract was passed over a 5-ml glutathione agarose column (Sigma) as described previously (Miller, Leonard et al. 2008).

The expression conditions were little modified in case of chromophorylation study with PEB (Biswas, Vasquez et al. 2010); to produce PEB using the pPebS expression plasmid, a 50-ml overnight starter culture was added to 1 L of LB medium with the appropriate combination of antibiotics. This culture was shaken at 37°C for 4 h until the optical density reached $OD_{600\ nm}$ = 0.6. Production of T7 RNA polymerase was induced by the addition of 1 mM isopropyl β-D thiogalactoside (IPTG). Cells were incubated with shaking at 190 rpm at 18°C for another 16 h before they were harvested by centrifugation at 10,000 × *g* for 10 min. Cells were incubated at 18° C after induction with IPTG to limit the production of inclusion bodies (data not shown). Cell pellets were stored at -20 °C until required**.**

To produce holo-MpeA using the pPebS with and without pMpeZ expression plasmid, a single colony was inoculated into a 200-ml overnight culture in LB medium with the appropriate combination of antibiotics. This culture was shaken at 20 °C at 180 rpm for 30-48 h until the optical density reached $OD_{600\ nm}$ ~ 0.6. Production of T7 RNA polymerase was induced by the addition of 1 mM isopropyl β-D thiogalactoside (IPTG). Cells were incubated with shaking at 180 rpm at 20 °C for another 48 h before they were harvested by centrifugation at 10,000 × *g* for 10 min. Cell pellets were immediately processed for protein purification in dark, following the procedure described earlier in “Methods and Materials”.

2.3. *Fluorescence emission and absorbance spectra:*

Fluorescence emission and excitation spectra were recorded with a Perkin Elmer LS55 fluorescence spectrophotometer (Waltham, MA) with slit widths set at 10 nm (excitation and emission). For recombinant phycobiliproteins, the excitation wavelength was set depending on the type of bilin bring used for *in vivo* assays; (PCB at 590 nm, PEB at 490 nm and PUB at 440 nm). The chromophorylated samples were diluted to ~0.05 OD (at λ_{max}) prior to obtaining their fluorescence spectra whereas negative control samples (*e. g.*, no lyase addition) with no obvious chromophore attached were not diluted (their OD was generally less than 0.05). Absorbance spectra were acquired using a lambda 35, dual-beam UV-Vis spectrophotometer (Perkin Elmer, Waltham, MA). For calculating the fluorescence relative intensity the sample was diluted to 0.05 OD_{560nm}. The fluorescence emission peak for wild type co-expression sample was set to 100%, and relative emission peak was calculated accordingly using same diluted sample (based on the equal protein concentration).

2.4. *Protein and bilin analysis:*

Polypeptides were resolved by polyacrylamide gel electrophoresis (PAGE; 15% w/v) in the presence of sodium dodecyl sulfate (SDS), and polypeptides were visualized by staining with Coomassie blue as described (Saunée, Williams et al. 2008). To visualize PCB linked to proteins, gels were soaked in 100 mM $ZnSO_4$ for approximately 5 min (Berkelman and Lagarias 1986; Berkelman and Lagarias 1986; Raps 1990). Zinc-enhanced fluorescence of bilins was visualized using an FX imaging system (BioRad, Hercules, CA) with excitation at 550 nm (whereas in the case of phycobiliprotein subunits having PCB or PEB, but 488 nm in case of PUB). In order to calculate % chromophorylation of each protein, BioRad's Quantity One™ software was used to

determine the relative abundance of each polypeptide. (PcyA and CpcU were His-tagged but the expression levels of HT-CpcU were very low, and the amount of this protein relative to the others was very small.) This abundance percentage was multiplied by the extinction coefficient of the protein at 280 nm (based upon Trp and Tyr content) to obtain the contribution of each protein to the absorbance at 280 nm. A modified extinction coefficient for the total protein concentration was used as shown below for purified HT-ApcA/ApcB.

$\varepsilon_{280\ nm}^{total} = (\%\text{HT-ApcA}*\varepsilon_{280\ nm}^{\text{HT-ApcA}} + \%\text{ApcB}*\varepsilon_{280\ nm}^{\text{ApcB}} + \%\text{HT-PcyA}*\varepsilon_{280\ nm}^{\text{HT-PcyA}})$

Equation:1

$\varepsilon_{280}^{\text{HT-ApcA/ApcB}} = (\%\text{ HT-ApcA} + \%\text{ ApcB})* \varepsilon_{280\,nm}^{total}$ Equation 2.

The PCB concentration was calculated by denaturing the recombinant protein in 8 M urea, pH 2 and using $\varepsilon_{663\,nm} = 35.4\ mM^{-1}\ cm^{-1}$ (Glazer 1988). The concentration of the bilin was divided by the concentration of the PBP to give % chromophorylation (see Table 2). Holo-HT-CpcA concentration was determined using $\varepsilon_{625\,nm} = 127.6\ mM^{-1}\ cm^{-1}$ (Tooley, Cai et al. 2001). The yield of chromophorylated PBP (expressed as mg of PBP l^{-1} *E. coli* culture) was estimated by determining the concentration of PCB bound to protein (obtained in 8 M urea, pH 2); this value was multiplied by the molecular weight of the PBP subunit(s) and the total volume of protein solution purified from the cells grown in 1 l of culture medium.

The % chromophorylation of CpeA and CpeB was calculated as described earlier (Biswas, Vasquez et al. 2010) with minor changes. BioRad's Quantity One™ software was used to determine the relative abundance of each polypeptide because PebS was also His-tagged. This abundance percentage was multiplied by the extinction coefficient of the protein at 280 nm (based upon Trp and Tyr content; mentioned below) to obtain the contribution of the protein to the

absorbance at 280 nm. A modified extinction coefficient for the total protein concentration was used as shown below for purified HT-CpeA or HT-CpeB (represented by X).

$\varepsilon_{280\ nm}^{total} = (\%HT\text{-}X * \varepsilon_{280\ nm}^{HT\text{-}X} + \%PebS * \varepsilon_{280\ nm}^{PebS})$ Equation 3.

$\varepsilon_{280}^{HT\text{-}X} = (\%\ HT\text{-}X + \%\ PebS) * \varepsilon_{280\ nm}^{total}$ Equation 4

The PEB-peptide concentration (attached to HT-CpeA or HT-CpeB) was calculated by denaturing the recombinant protein in 8 M urea, pH 2 and using $\varepsilon_{550\ nm} = 53.4\ mM^{-1}\ cm^{-1}$ (Glazer 1988). The concentration of the bilin was divided by the concentration of the PBP to give % chromophorylation (see result).

For comparing the PCB yield obtained in expression cells containing pPcyA and pAT101, 50 ml overnight cultures were transferred to 1 l of culture medium at 37 °C and grown until the $OD_{600\ nm}$ reached 0.6. IPTG (0.5 mM) was then added, and the cultures were incubated for an additional 4-h period at 30°C. Cells were harvested and homogenized with 35 ml of acetone (Hu, Lee et al. 2006). The supernatant was vacuum dried to remove the acetone, and the dried pellet was dissolved in methanol (1.0 ml). The total PCB from each construct was diluted 100-fold in methanol/ 5% HCl and the concentration was calculated using the relationship $\varepsilon_{680\ nm}^{PCB} = 37.9\ mM^{-1}\ cm^{-1}$ (Cole, Chapman et al. 1967). The PCB concentration was multiplied by the total volume in MeOH and by the molecular mass of PCB (587 g/mol) (Fu, Friedman et al. 1979; Fu, Friedman et al. 1979) to determine the grams of PCB produced per liter of culture.

2.5. ***Calculating fluorescence quantum yield*****:** Fluorescent quantum yields of the holo-CpeA was calculated as described (Parker and Rees 1960; Lakowicz 1983) using Perkin Elmer LS55

fluorescence spectrophotometer (Waltham, MA) with slit widths set at 10 nm (excitation and emission), relative to cresyl violet (in ethanol Φ_f= 0.59; Sigma Aldich). Samples were diluted to an absorbance of ~0.04-0.05 at $\lambda_{560\ nm}$ and fluorescence emission was acquired from 570-800 nm. Fluorescent quantum yield of the sample was calculating in comparison with the standard using the following equation

$$A_1\varepsilon_1/ A_2\varepsilon_2= \Phi_{f1}/ \Phi_{f2} \quad \text{Equation} \quad 5$$

Where A is the absorbance maxima, ε is the area of the fluorescence emission spectrum from 570 nm -800 nm, and Φ_f is fluorescence quantum yield.

2.6. ***Tryptic Digestion of Phycoerythrin:*** The partial holo-CpeA (pCpeA/pPebS,pCpeZY) and partial holo CpeB (pCpeB/pPebS/pCpeS) retrieved from the nickel-nitrilotriacetic acid-Superflow-affinity column was subjected to tryptic digestion following the protocol described in (Arciero, Bryant et al. 1988). In short, the purified holoprotein was exhaustively dialyzed against 2mM sodium phosphate buffer, pH 7.0, 1 mM 2-mercaptoethanol and then concentrated by ultrafiltration through an Amicon YM10 (Millipore, Billercia, MA). Concentrated sample was diluted 1:3 and titrated to pH 2.0 with 1N HCl. The solution was incubated for 45min in dark at room temperature for complete unfolding of protein. Trypsin was added to 2% (w/v) from a 5 mg/ml stock solution in 1 mM HCl. Ammonium bicarbonate was added in 0.1 M and the mixture was titrated to pH 7.5 with 1N NaOH. The digested mixture was incubated at 30 °C for 2h in dark. An additional aliquot of trypsin was added (2% w/v) and incubated for another 2h. The reaction was quenched by adding 30% v/v glacial acetic acid**.** The mixture was passed through a C-18 sep-pack cartridge. The eluted sample was vacuum dried and stored at -20°C for HPLC

separation as described (Shen, Saunee et al. 2006). The fraction peaks having absorbance maxima at 560 nm were collected, vaccum dried and stored at -20°C for Mass spectrometry analysis.

2.7. *Growth condition for Fremyella diplosiphon*: The *Fremyella diplosiphon* cells are grown in 250 ml conical flask in BG-11 medium (Allen 1968) with 10 mM HEPES (pH 8.0). The cultures are grown on a shaker (100 rpm) illuminated with ~ 50-70 $\mu E/m^2/x$ cool white light (encoded in green). The cell density was monitored with using UV-Vis spectrophotometer. The cells are harvested by centrifugation at 10,000 × *g* for 10 min, prior to proceeding with phycobilisome isolation.

2.8. *Separation of phycobilisome:* Phycobilisomes from *Fremyella diplosiphon* were separated following the protocol described by Glazer (Glazer 1988), with few minor changes. All steps were performed at room temperature. Cells were collected by continuous centrifugation and suspended at 1 g wet weight/ 15 ml in 0.75 M potassium phosphate buffer, pH 6.8, 1 % (w/v) Triton X-100. After stirring for 1 hr the extract was centrifuged for 20 min at 27,000 g, and the sedimented materials were discarded. Polyethylene glycol 6000 is added to the supernatant solution 15 % (w/v), the mixture was stirred for 1 hr and then centrifuged for 20 min at 27,000 g, and the supernatant wwas discarded. The sedimented material was suspended in 0.75 M potassium phosphate buffer, pH 6.8, 1 % (w/v) Triton X-100, 15 %(w/v) polyethylene glycol 6000, the mixture was centrifuged for 20 min at 27,000 g, and the supernatant was discarded. The purple sediment was suspended in 0.75 M potassium phosphate buffer, pH 6.8 centrifuged for 15 min at 27,000 g.

2.9. *Isolation of Phycoerythrin:* The phycoerythrin from *Fremyella diplosiphon* (both wild type and mutant) cells (grown in David Kehoe's lab) was purified following the protocol described by

Glazer (Glazer 1988) with minor changes. Cells (10 g wet weight) from both wide type and mutants were suspended in 25 ml of 1 M sodium acetate, pH 5.0. The slurry was broken by passing three times through a French pressure cells at 20,000 psi. The product was ultra centrifuged at 81,000 g for 1 h (rotor-Ti64) to remove the chlorophyll and other cellular membranes.

Solid ammonium sulphate was added to 35% of saturation in the case of wild type and to 45 % of saturation to the mutants extracts to the supernatants. The solution was allowed to stand for 90 min and then centrifuged at 16,000 g for 15 min. The pellet was dissolved in 15 ml of 100 mM sodium acetate, pH 5.0 and dialyzed overnight against 2 L of same buffer.

The dialyzed sample was passed through a column (4.0 X 24 cm) of Sephadex G-100 preequlibrated with the 100 mM acetate buffer. After each run the peaks tubes from the early eluting biliproteins peaks were pooled. The phycoerythrin containing solution was brought to 30 % of saturation and allowed to stand for 1 h prior to centrifugation. The pellets were resuspended in 5 mM potassium phosphate, pH 7.0, and then dialyzed overnight against 2 l of same buffer.

The dialyzed phycoerythrin- containing solution was applied to a column (2.2 X 24 cm) of DEAE-cellulose DE 52 (Watmann), preequilibrated with the 5 mM phosphate buffer. After elution with 1 volume of starting buffer, the column is developed with a 550 ml linear 5-200 mM potassium phosphate, pH 7.0, gradient. The peak tubes from the major phycoerythrin, were pooled, brought to 45 % saturation with $(NH_4)_2SO_4$, permitted to stand for 1 h prior to centrifugation. The pellet was suspended in a small volume of 100 mM sodium phosphate-1 mM sodium azide at pH 7.0 and dialyzed against the same buffer.

2.10. ***Isolation of PEI and PEII from Synechococcus sp. RS 9916:*** RS9916 species was used to study the bilin lyase isomerase for PE subunits. This is one of the cyanobacterial species which

can undergo Type IV chromatic acclimation. The cells were grown (David Kehoe's lab) in different light conditions (blue vs green) using LEDs to characterize the change in the chromophore composition in PEI and PEII. The cell pellets were used to isolate PEI and PEII proteins.

The separation was carried out following the protocol described earlier (Six, Thomas et al. 2007) with a few modifications. The cell pellets were dissolved in 0.75 M Na-K phosphate buffer (pH 7.0). The slurry was lysed by passing three times through a French pressure cell at 15,000-18,000 psi. The product was ultra centrifuged at 35,000 g for 3 h to remove the chlorophyll and other cellular debris. The supernatant was brought to 45 % saturation with $(NH_4)_2SO_4$, permitted to stand for 1 h prior to centrifugation. The pellet was suspended in a small volume of 5 mM sodium phosphate-1 mM sodium azide at pH 7.0 and dialyzed against the same buffer. The dialyzed samples were concentrated 10 fold (using Amicron concentrator with a MW cut off of 10kDa) and proceeded to next step of separation using IEF analysis.

The IEF (7 % acrylamide minigel containing ampholyte carrier pH 4 to 6.5, purchased from BioRad, Hercules, CA) gel separation were carried out using the BioRad protocol. The sample was diluted with equal amout of sample buffer (15 % glycerol) and loaded on a IEF gel. The cathode tank was filled with 20 mM NaOH and the anode one contained 20 mM orthrophosphoric acid. The PBPs were allowed to focus during approx. 2 h by increasing steps of 50 V every 10 min up to 350V.

The different color bands representing PEI and PEII were excised and then crushed in 10 mM Tricine buffer (pH 7.8) using a manual homogenizer. Acrylamide remnants were removed by centrifugation for 10 min at 14,000 x g. The extracted proteins were vaccuum dried and stored at -20°C until further use.

Separation of α and β subunits of PEI and PEII: The separation of the subunits were carried out following the protocol described by Swanson and Glazer (Swanson and Glazer 1990) using C4 reverse-phase HPLC (Waters). In brief the vacuum dried samples were dissolved in 300 ul of 65% TFA (0.1%) and 35% 2:1 acetronitril: isopropanol containing 0.1 % TFA. The samples were centrifuged for 10 mins to remove the undissolved proteins. Then the samples were separated on a C4 column using the running conditions described earlier (Swanson and Glazer 1990) . The α and β subunits of PEI and PEII peaks were collected based on the absorbance spectrum (490 and 550 nm). The separated subunits were vacuum dried and further analyzed by Mass spectrometry (Mass Spectometry facility at Indiana University).

3.0 RESULT

3.1. Chromophorylation efficiency and specificity of all bilin lyases from *Synechococcus* sp. strain PCC 7002

3.1.1. *Examination of Synechococcus sp. strain PCC 7002 PcyA activity in E. coli:*

The Duet vector system was used to clone genes required for the synthesis of PBP and to test the efficiency of producing large amounts of holo-PBP in *E. coli*. Firstly, the *ho1* gene from *Synechocystis* sp. strain PCC 6803 and the *pcyA* gene from *Synechococcus* sp. strain PCC 7002 were cloned into the pACYC Duet vector as described in the Materials and Methods to produce the pPcyA construct (see Table 4). The *Synechocystis* sp. strain PCC 6803 *ho1* gene was used because it was possible to achieve high levels of expression and activity in *E. coli* in several different expression vectors (data not shown). However, in order to improve the PCB production levels that were achieved using the *Synechocystis* sp. strain PCC 6803 *pcyA* gene employed by Tooley et al.,(Tooley, Cai et al. 2001) (pAT101; see Table 4), the pPcyA plasmid was tested**.** Although cells containing only the pAT101 plasmid were slightly blue in color (data not shown), the pPcyA expression cells produced large amounts of PCB and had a dull blue color (see Fig. 20). The PCB production per liter of *E. coli* cells for each plasmid combination was calculated, and the yield of PCB from pPcyA was 70.8 mg l^{-1} of cells, whereas for pAT101 the yield of PCB was 20.2 mg l^{-1} of cells. This new PCB expression system was next compared with the one previously developed by Tooley et al. to show that holo-CpcA could be formed in *E. coli* by cotransformation with pAT101 (encoding *Synechocystis* sp. strain PCC 6803 *ho1* and *pcyA*) and pBS414v (encoding *Synechocystis* sp. PCC 6803 *cpcA, cpcE, cpcF*) (Tooley, Cai et al. 2001). Under the same growth conditions, 48.1% of HT-CpcA was chromophorylated with PCB when

using plasmids pPcyA and pBS414v, whereas only 22.4% of HT-CpcA was chromophorylated when using the pAT101 plasmid from Tooley et al. that expresses *Synechocystis sp.* strain PCC 6803 *pcyA* (Tooley, Cai et al. 2001) (See Table 4). These observations indicated that expression levels and/or activity levels of *Synechococcus* sp. strain PCC 7002 PcyA are higher using pPcyA than those achieved with pAT101 carrying *Synechocystis* sp. strain PCC 6803 *pcyA***.** Yields of holo-HT-CpcA (calculated using the $\varepsilon_{625\ nm} = 127.6\ mM^{-1}\ cm^{-1}$ (Tooley, Cai et al. 2001) as described in Materials and Methods) using plasmid pPcyA were 3.2 mg of holo-HT-CpcA l^{-1} *E. coli cells*; see Fig. 21 for analyses of the HT-CpcA produced).

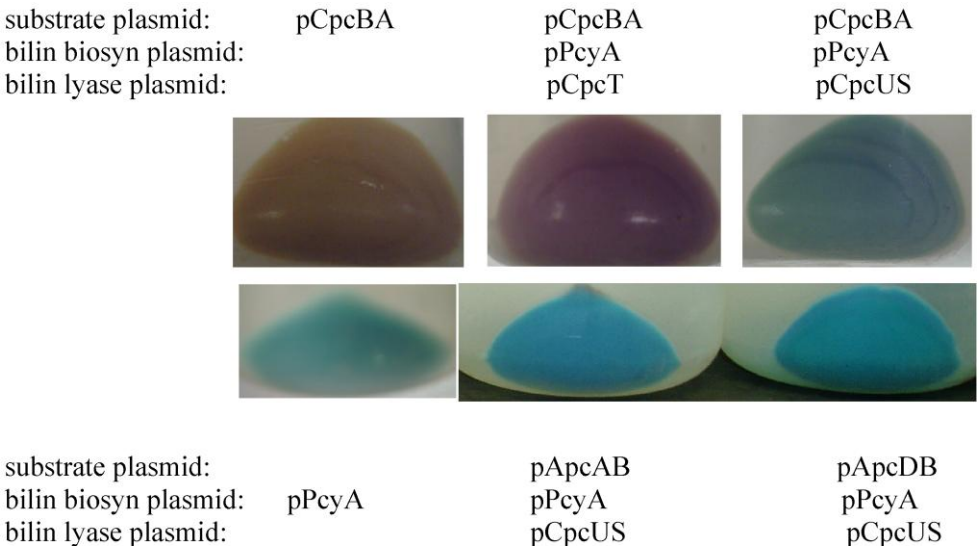

Fig. 20: Photographs of *E. coli* pellets after growth with the plasmids listed on the legend above or below each pellet.

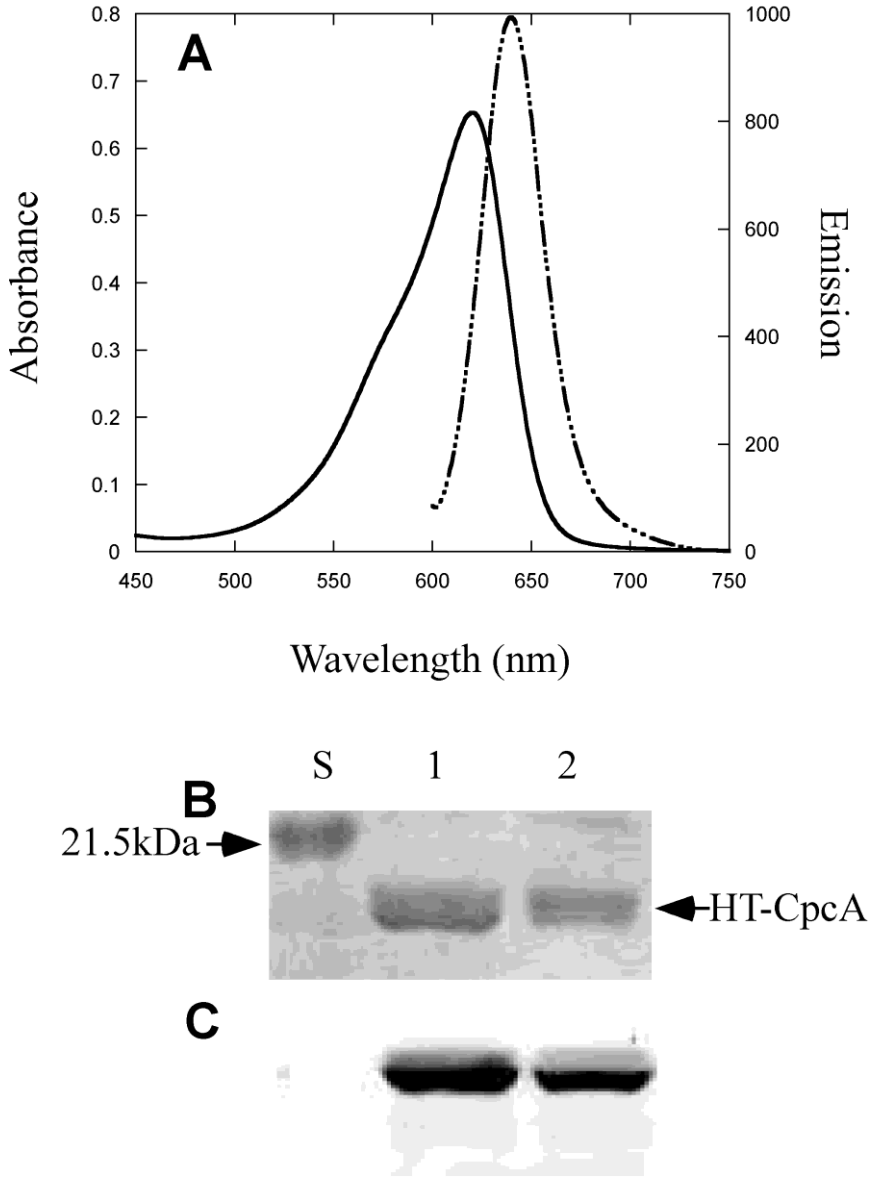

Fig. 21: Analyses of holo-HT-CpcA purified from *E. coli* cells. **A.** Absorbance (solid) and fluorescence emission (dashed dotted) spectra of HT-CpcA purified from cells containing pBS414v and pPcyA. **B.** Coomassie-stained SDS-polyacrylamide gel of HT-CpcA purified from cells containing pBS414v and pPcyA (lane 1) or pBS414v and pAT101 (lane 2). Molecular mass standards were loaded in the lane marked "S"; the position of the 21.5-kDa standard is indicated at the left. **C.** Zinc-enhanced bilin fluorescence of the gel in panel B.

3.1.2. *Development and use of a multi-plasmid system for expression of holo-AP:*

E. coli cells containing plasmids pPcyA and pApcAB produced almost no colored and fluorescent product (See Fig 21A; sample was not diluted prior to obtaining the fluorescence emission shown), but *E. coli* cell2 containing plasmids pPcyA, pApcAB, pCpcUS had a brilliant blue color (Fig. 20). HT-ApcAB purified from these cells had an absorbance maximum at 615 nm with a small shoulder at 653 nm (see Fig. 22A). This product had a fluorescence emission maximum at 634 nm, consistent with that of monomeric ($\alpha\beta$) holo-AP (see Fig. 22A; the sample shown was diluted to 0.05 OD_{615} prior to measuring the fluorescence emission). The ratio of the Vis:UV (615 nm:357 nm) maxima for HT-ApcA/ApcB was 2.53 (Table 6). Next, CpcS or CpcU alone was tested within this heterologous *E. coli* system to confirm the mutagenesis results and previous *in vitro* enzyme assay results (Shen, Saunee et al. 2006; Saunée, Williams et al. 2008). Cells transformed with either pCpcU or pCpcS, and thus expressing only one of the subunits of this heterodimeric lyase, were unable to produce a highly fluorescent product, similar to the results obtained in cells containing only pApcAB and pPcyA (Fig 22B; the sample was not diluted prior to obtaining the fluorescence emission). The HT-ApcA/ApcB samples purified from the different *E. coli* cells were analyzed by SDS-PAGE (Fig. 22C). The bilin content of each protein was examined by zinc-enhanced fluorescence of the gels as shown in Fig. 22D; protein content was examined after staining the same gel with Coomassie Blue (Fig. 22C). When no bilin lyase subunit was present, a small amount of covalent PCB addition to ApcB occurred (Fig. 22C, lane 2); this was similar to previous observations using *in vitro* reactions (Saunée, Williams et al. 2008). However, the yield of this covalent product was very low in this *in-vivo E. coli* system, and the absorption and fluorescence properties suggested that the bilin attached auto-catalytically to ApcB in the absence of a bilin lyase was not the biologically correct product (Arciero, Bryant et

al. 1988; Arciero, Dallas et al. 1988; Zhao, Zhu et al. 2004). When either CpcS or CpcU was present (Fig. 22D, lanes 3 and 4, respectively), the amount of PCB attached to ApcB was similar to that when no bilin lyase was present. However, when both CpcS and CpcU were present in cells (pCpcUS, pApcAB and pPcyA), bilin addition to both HT-ApcA and ApcB occurred. This was easily demonstrated by Zinc-enhanced fluorescence of the PCB attached to the proteins after SDS-PAGE (Fig. 22B, lane 1; note that 3-fold more protein was loaded in lanes 2 to 4 compared to lane 1 in order to visualize the small amount of fluorescence from PCB attached to ApcB in the absence of both CpcS and CpcU). A small amount of a proteolytic degradation of HT-ApcA was observed (lane 1, Fig. 22C). The amount of chromophorylated ApcB in the cells containing no CpcS or CpcU or in cells only containing one of the subunits was estimated to be only ~8% of the amount of ApcB chromophorylated in the presence of CpcSU. As described in the Materials and Methods, the total concentration of HT-ApcA/ApcB was calculated, and this value was compared to the PCB (linked to protein) concentration. On the basis of this calculation, approximately 72% of the HT-ApcA/ApcB polypeptides were chromophorylated**.** However, when the relative bilin fluorescence intensity of each polypeptide in Fig. 22C (lane 2) was evaluated, it was found that 100% of ApcB was chromophorylated while only ~40% of HT-ApcA was chromophorylated**.** At sufficiently high protein concentrations and appropriate ionic strength and pH conditions, AP forms trimers $(\alpha\beta)_3$ that have a characteristic red shift in the absorbance (λ_{653nm}) and fluorescence (λ_{663nm}) properties of the protein (Gysi and Zuber 1974; Gysi and Zuber 1979; MacColl, Csatorday et al. 1981; Beck and Sauer 1992). The linker protein ApcC, when present also has the effect of sharpening the absorbance spectrum of trimers as well (Scheer 2003). Although there is a small shoulder at ~650 nm in the absorbance spectrum (Fig 22A), the fluorescence emission spectrum showed no emission at 660 nm (Fig. 22A). After analysis of this sample by size-

exclusion HPLC, two peaks were observed for this the holo-HT-ApcA/ApcB**.** One had a molecular mass consistent with single α or β subunits while the other had a mass consistent with that of AP (αβ) protomers; no peak consistent with the elution properties for AP $(\alpha\beta)_3$ trimers was observed (data not shown). Because of the presence of a significant proportion of apo-subunits within the purified mixture, this was expected**.** Assuming a random association of chromophorylated and non-chromophorylated subunits with a chromophorylation rate of 40% for the α subunit and 100% for the β subunits, the probability of obtaining αβ monomers with both subunits chromophorylated is ~40% (1.0 * 0.40), and the probability of obtaining trimers in which all subunits are chromophorylated would be $((1.0)^3 * (0.4)^3)$ or ~6.4%.

The specificity of the other two known bilin lyases was also tested to see if either the CpcE/CpcF lyase or the CpcT lyase could attach PCB to HT-ApcA/ApcB in this *in-vivo* system. As judged by absorbance and fluorescence emission spectra (see Figure 23, panels A and B, respectively), no holo-AP was produced when either pCpcEF or pCpcT was introduced together with the pApcAB and pPcyA**.** In addition, only a small amount of PCB addition to ApcB was observed; this level was similar to that observed when no lyase was present (compare to Fig 22D, lane 2). Therefore, the specificity of the lyases for their PBP substrates was maintained within this *E. coli* system and was completely consistent with previous *in vitro* biochemical and mutational analyses (Fairchild, Zhao et al. 1992; Zhou, Gasparich et al. 1992; Fairchild and Glazer 1994; Shen, Saunee et al. 2006; Saunée, Williams et al. 2008; Shen, Schluchter et al. 2008).

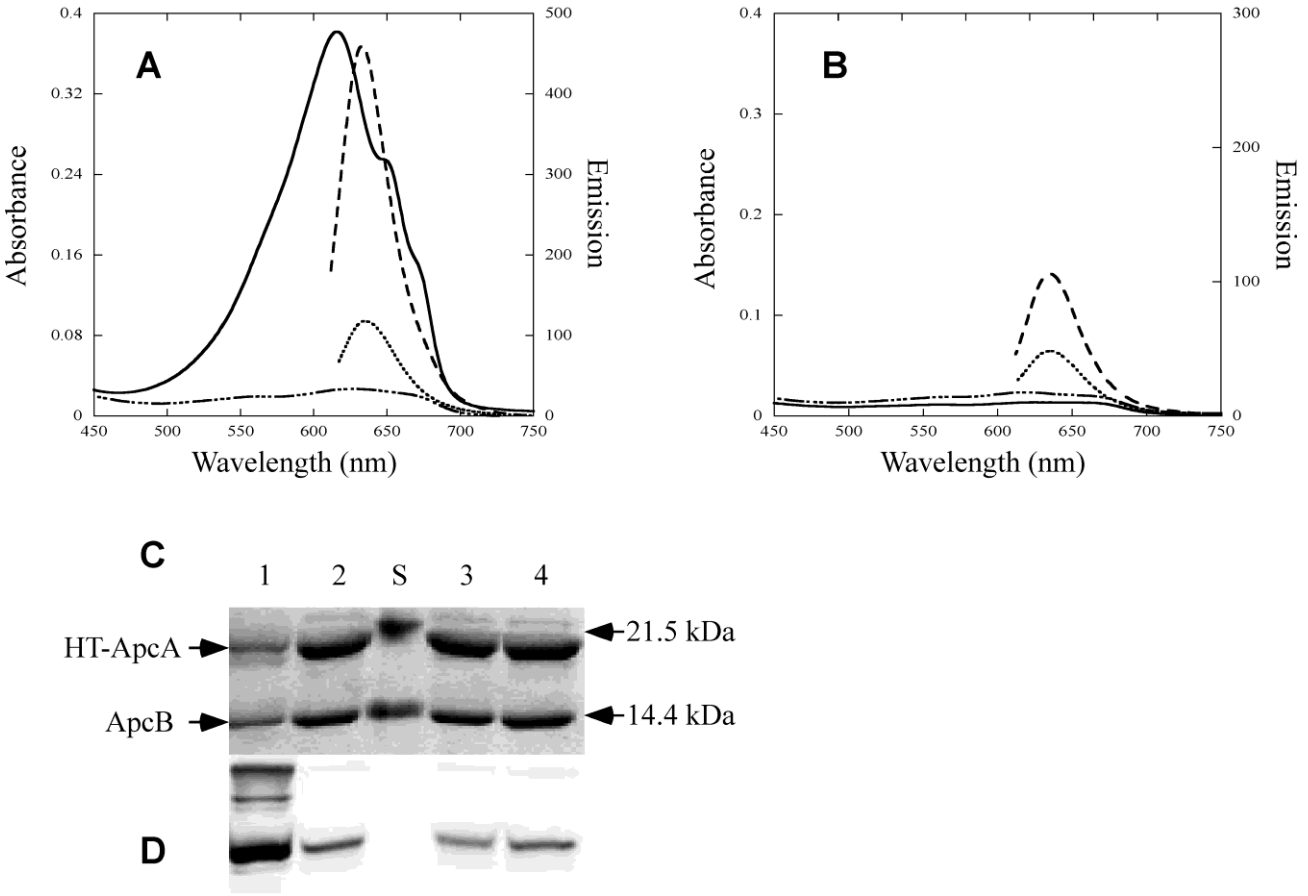

Fig. 22. **Analyses of major AP subunits ApcA and ApcB synthesized in *E. coli*. A.** Absorbance (solid line) and fluorescence emission (dashed line) spectra of HT-ApcA/ApcB purified from cells containing pApcAB, pPcyA with pCpcUS and absorbance (dashed dotted line), fluorescence (dotted line) without pCpcUS. **B.** Absorbance (solid line) and fluorescence emission (dashed line) spectra of HT-ApcA/ApcB purified from cells containing pApcAB, pPcyA and pCpcS; absorbance (dashed dotted line), fluorescence (dotted line) spectra of HT-ApcA/ApcB purified from cells containing pApcAB, pPcyA and pCpcU. In order to acquire the fluorescence emission spectra for the AP subunits produced in the presence of pCpcUS (dashed lines in panels A), the sample was diluted to $OD_{615\ nm}$ = 0.05 whereas the fluorescence emission spectra acquired from HT-ApcA/ApcB produced without CpcS-I/CpcU (panel A; dotted lines) or produced with pPcyA and pCpcS (Panel B, dashed line) or with pPcyA and pCpcU (Panel B, dotted line) were not diluted. **C.** Coomassie-stained SDS polyacrylamide gel containing HT-ApcA/ApcB purified from cells containing pApcAB, pPcyA with (lane 1) or without (lane 2) pCpcUS; HT-ApcA/ApcB purified from cells containing pApcAB, pPcyA, and either pCpcS (lane 3) or pCpcU (lane 4). Molecular mass standards are loaded in lane "S", and selected masses are indicated to the right. **D.** Zinc-enhanced fluorescence image of the gel pictured in panel C.

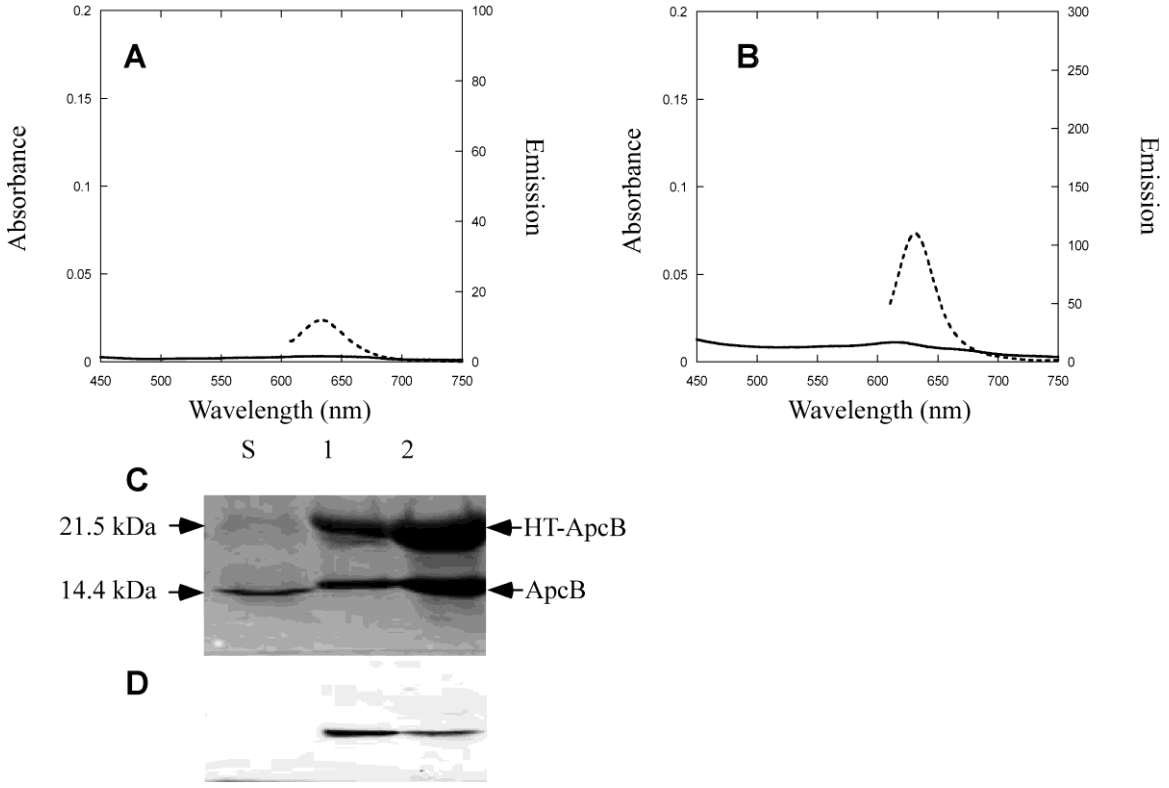

Fig. 23: **Analysis of HT-ApcAB purified from *E. coli* cells produced in the presence of CpcEF or CpcT. A.** Absorbance (solid) and fluorescence emission (dashed) spectra of HT-ApcAB purified from cells containing pApcAB, pBS415v (CpcEF), and pPcy**A. B.** Absorbance (solid) and fluorescence emission (dashed) spectra of HT-ApcAB purified from cells containing pApcAB, pCpcT, and pPcy**A. C.** Coomassie-stained SDS-polyacrylamide gel of HT-ApcA/ApcB purified from cells containing pApcAB, pCpcEF (lane 1), and pCpcT (lane 2) with pPcy**A.** Molecular mass standards were loaded in the lane marked "S"; the position of the 21.5-kDa mass standard is indicated. **D.** Zinc-enhanced bilin fluorescence of the gel in panel C.

3.1.3. *Chromophorylation Requirements for HT-ApcD:*

ApcD is a variant α-AP subunit ($\alpha^{AP\text{-}B}$) that pairs with β-AP (ApcB) forming AP-B which has an extremely red-shifted absorbance at 670 nm and is an important terminal emitter of the PBS involved in energy transfer to Photosystem I; two copies of ApcD are present per PBS (Glazer and Bryant 1975; Lundell and Glazer 1981; Fuglistaller, Mimuro et al. 1987; Maxson, Sauer et al. 1989; Zhao, Zhou et al. 1992; Ashby and Mullineaux 1999; Domg, Tang et al. 2009). An alignment of ApcD with other allophycocyanin subunits is shown in Fig. 24. The *apcD* gene was cloned together with *apcB* in order to produce HT-ApcD and ApcB, as this construct produced a more soluble recombinant apo-protein than a construct expressing *apcD* alone (L. Harrison, Jr. and W. M. Schluchter, unpublished data). *E. coli* cells containing plasmids pApcDB and pPcyA had a faint, dull blue color (data not shown), whereas cells containing plasmids pApcDB, pPcyA, and pCpcUS were a brilliant blue color (see Fig. 20). The absorbance and fluorescence emission spectra for HT-ApcD/ApcB after purification of the proteins by metal-affinity chromatography from cells containing these two plasmid combinations are shown in Fig. 25A**.** HT-ApcD/ApcB produced in the absence of the pCpcUS plasmid had very little absorbance or fluorescence, but HT-ApcD/ApcB produced in the presence of pCpcUS had absorbance peaks at 616 and 672 nm. When excited at 590 nm, this recombinantly produced HT-ApcD/ApcB had fluorescence emission maxima at 634 nm and 675 nm. When these proteins were separated by SDS-PAGE and evaluated by zinc-enhanced fluorescence, both subunits carried PCB chromophores (see Fig 25C, lane 1), but based upon quantitation of the fluorescence intensity (compare lane 1 in Figs. 25B and 25C), the chromophorylation level for HT-ApcD was only ~40% (with ApcB subunit at 100%). However, in the absence of pCpcUS, no bilin addition to HT-ApcD occurred, and very

little bilin addition to ApcB was detected (lane 2 in Figs. 25B and 25C). A degradation product of HT-ApcD is apparent in some preparations (indicated by a * in Fig. 25B).

In order to determine the absorbance spectrum of holo-HT-ApcD alone, HT-ApcD was produced in the presence of pPcyA and pCpcUS. As evidenced by the absorbance and fluorescence spectra shown in Fig. 25D and the SDS-PAGE and zinc-enhanced bilin fluorescence shown in Fig 25E and F, a small amount of chromophorylated product was obtained. This holo-HT-ApcD had an absorbance maximum at 642 nm and a fluorescence emission maximum at 653 nm.. This likely means that when holo-HT-ApcD associates with holo-ApcB, there are interactions between chromophores that red-shift the absorbance maximum to 672 nm. The 616 nm absorption maximum seen in Fig. 25A is probably due to holo-ApcB (paired with apo-HT-ApcD), because there is an excess of chromophorylated ApcB relative to holo-HT-ApcD (see Fig 25C). Supporting this idea, the fluorescence excitation spectrum of the holo-HT-ApcD/ApcB with emission at 675 nm, shows an excitation peak centered at 618 nm, indicating that some of the ApcB-PCB chromophore is transferring energy to ApcD (see Fig. 25G). An alternative hypothesis to explain the difference seen in the spectra of HT-ApcD produced with and without ApcB is that the folding of ApcD was adversely affected in the absence of ApcB, leading to the differences observed in the absorbance and fluorescence emission spectra**.**

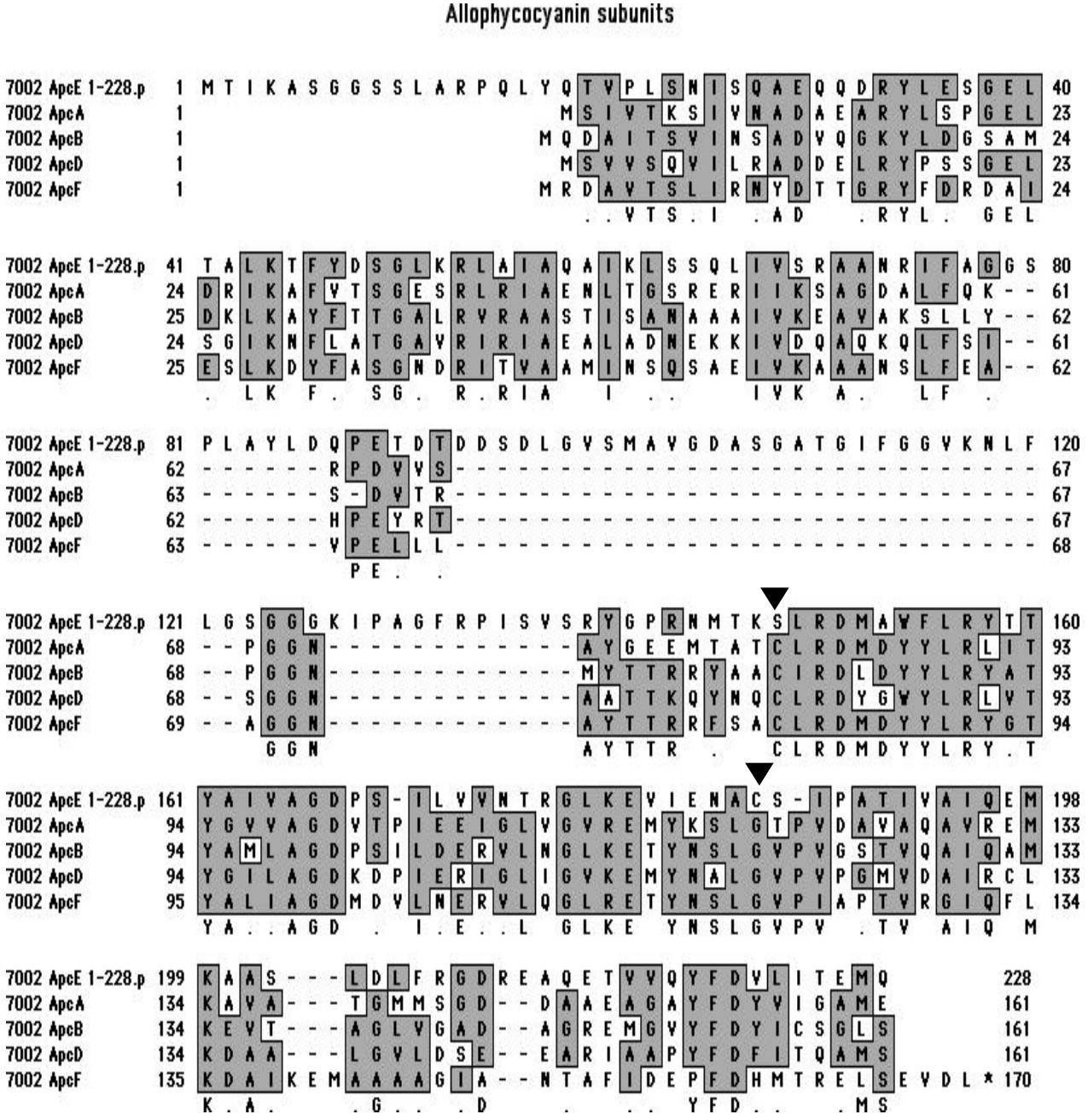

Fig. 24. Amino acid sequence aligment of *Synechococcus sp.* PCC 7002 ApcE (1-228 amino acid) with sequence of ApcA, ApcB, ApcD, and ApcF from *Synechococcus sp.* PCC 7002.
ApcB, ApcD, and ApcF from *Synechococcus sp.* PCC 7002.

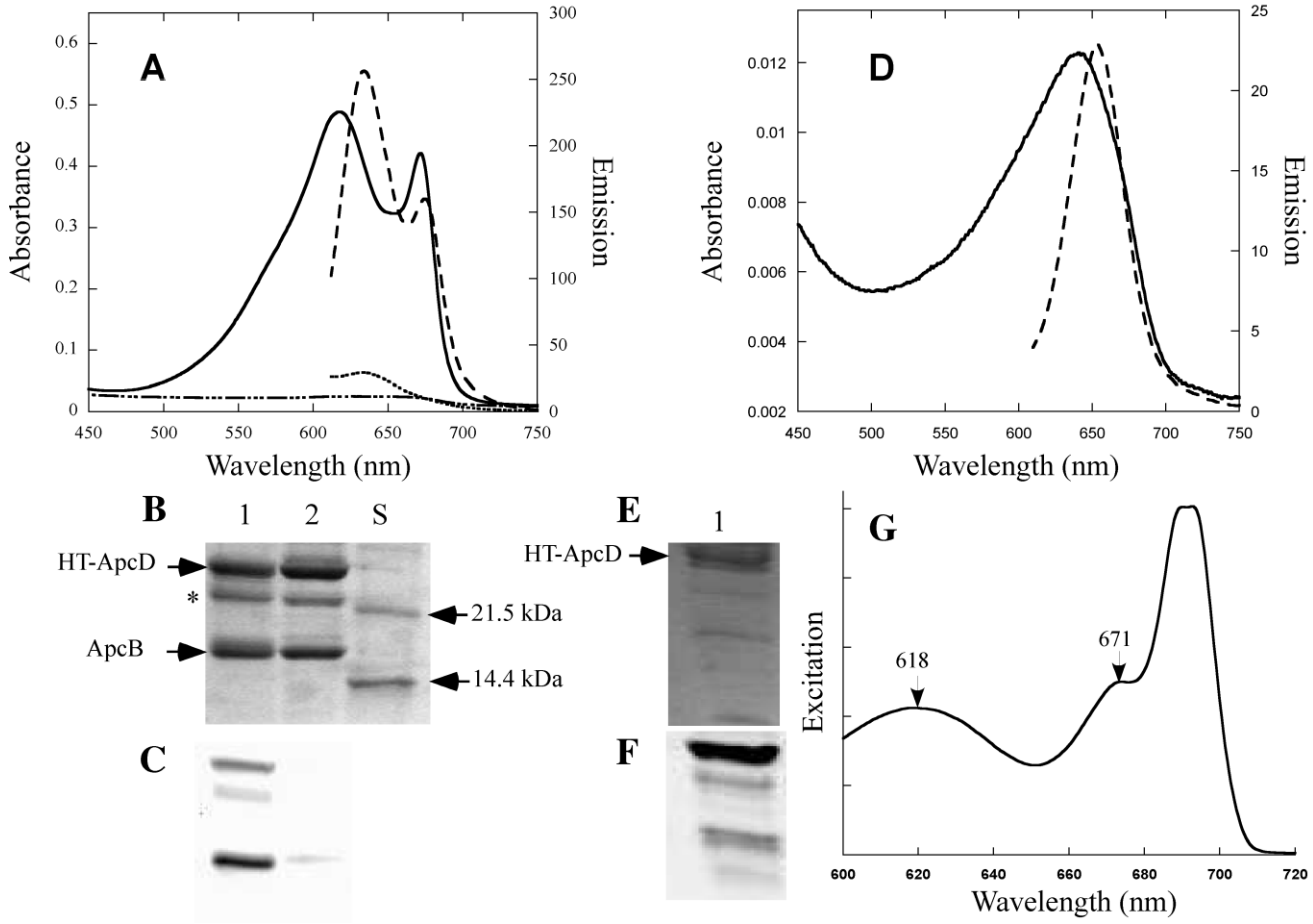

Fig. 25. **Analyses of AP-B *α*-subunits (ApcD) synthesized in *E. coli*. A.** Absorbance (solid lines) and fluorescence emission (dashed lines) spectra of HT-ApcD/ApcB purified from cells containing pApcDB, pPcyA with pCpcUS, and absorbance (dashed dotted line), fluorescence (dotted line) without pCpcUS are shown. **B.** Coomassie-stained SDS polyacrylamide gel containing HT-ApcD/ApcB purified from cells containing pApcDB, pPcyA with (lane 1) or without (lane 2) pCpcUS. Molecular mass standards are loaded in lane S, and selected masses are indicated to the right. **C.** Zinc-enhanced fluorescence from the bilins for the gel pictured in panel B. **D.** Absorbance (solid lines) and fluorescence emission (dashed lines) spectra of HT-ApcD purified from cells containing pApcD, pPcyA with pCpcUS. **E**. Coomassie-stained SDS polyacrylamide gel containing HT-ApcD purified from cells containing pApcD, pPcyA with (lane 1) pCpcUS. **F**. Zinc-enhanced fluorescence of the bilins for the gel in panel E. The * to the left of Panel E denotes the position of a proteolytic degradation product of HT-ApcD. **G.** Excitation spectrum of HT-ApcD/ApcB purified from cells containing pApcBD, pPcyA with pCpcUS. The emission wavelength was set at 676 nm. The excitation peak is marked with an arrow.

3.1.4. *Chromophorylation requirements for HT-ApcF:*

The requirements for synthesis of holo-HT-ApcF were examined next. ApcF is a variant β-AP subunit (also known as β^{18}) that partners with ApcE, a terminal emitter, and in *Synechococcus* sp. strain PCC 7002 influences energy transfer from the PBS to photosystem II (Zhao, Zhou et al. 1992; Gindt, Zhou et al. 1994; Ashby and Mullineaux 1999; Zhao, Shen et al. 2001). This His-tagged subunit was expressed in the presence of pPcyA or both pPcyA and pCpcUS. The absorbance and fluorescence spectra of purified HT-ApcF from cells with and without pCpcUS are shown in Fig. 26A**.** Holo-HT-ApcF was only produced in the presence of the CpcS-I/CpcU bilin lyase and had an absorbance maximum at 616 nm with a fluorescence emission at 637 nm. The protein purified from cells not expressing *cpcS-I* and *cpcU* had very little absorbance or fluorescence (Fig. 26A). This is also apparent if one compares the zinc-enhanced bilin fluorescence after SDS-PAGE of HT-ApcF purified from the two cell types (Fig. 26B and Fig. 26C, compare lanes 1 and 2). With this *in-vivo* system, 68% of HT-ApcF was estimated to be chromophorylated (see Table 6).

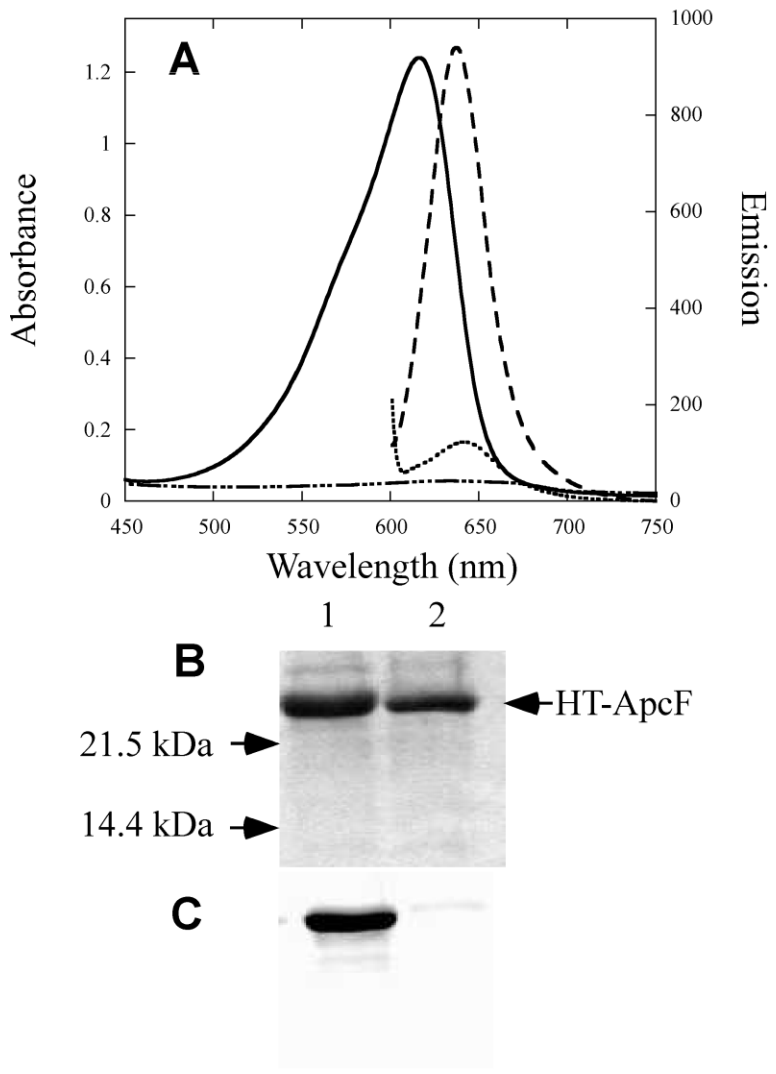

Fig. 26. Analyses of AP β[18]-subunit (ApcF) synthesized in *E. coli*. A. Absorbance (solid lines) and fluorescence emission (dashed lines) spectra of HT-ApcF purified from cells containing pApcF, pPcyA with pCpcUS, and absorbance (dashed dotted line), fluorescence (dotted line) without pCpcUS. **B.** Coomassie-stained SDS polyacrylamide gel containing HT-ApcF purified from cells containing pApcF, pPcyA with (lane 1) or without (lane 2) pCpcUS. The molecular masses of selected standards are shown at the left. **C.** Zinc-enhanced fluorescence of the bilins for the gel above in panel B.

3.1.5. *Chromophorylation requirements of ApcE:*

The AP-like domain of ApcE from *Synechococcus* sp. strain PCC 7002 contains a PCB at Cys-186 (see Fig. 27). The expression construct pGST-ApcE fused GST to amino acids 1-228 of the AP-like domain of ApcE (Table 4). The construct pGST-ApcE was transformed into *E. coli* cells with pPcyA, with or without pCpcUS, to determine whether the CpcS-I/CpcU bilin lyase was required for correct and efficient PCB addition at Cys-186. Fig.27A shows the absorbance and fluorescence spectra of the purified GST-ApcE from the two cell types. The ApcE domain alone is sufficient to attach PCB in the correct manner to produce a strongly red-shifted PCB product with an absorbance maximum at 662 nm and a fluorescence emission maximum at 675 nm. GST alone did not react with PCB (data not shown). GST-ApcE synthesized in cells containing pCpcUS also had absorbance and fluorescence properties similar to the native protein (Capuano, Braux et al. 1991; Gindt, Zhou et al. 1994; Zhao, Su et al. 2006), but given that the correct product can be formed in the absence of any additional enzyme, the CpcS-I/CpcU bilin lyase apparently is not required for bilin ligation to ApcE. The GST-ApcE proteins were then separated by SDS-PAGE and analyzed for the zinc-enhanced bilin fluorescence before staining for protein with Coomassie blue staining. It was necessary to lyse the cells in the presence of a mixture of protease inhibitors in order to avoid the formation of degradation products. The results of this purification are shown in Fig. 27B and 27C (and correspond to the spectra shown in Fig. 27A). Three polypeptides carried a bilin chromophore. One likely corresponded to the full-length fusion (GST-ApcE: predicted mass of 51,445 Da), whereas the other two polypeptides had estimated masses consistent with those of degradation products containing ApcE (amino acids 1-228) with an expected mass of 24.5 kDa. As judged by Zinc-enhanced fluorescence (Fig. 27C, lanes 1 and

2) and supporting the conclusion that ApcE has intrinsic bilin lyase activity, approximately equivalent PCB addition was observed whether pCpcUS was present or not.

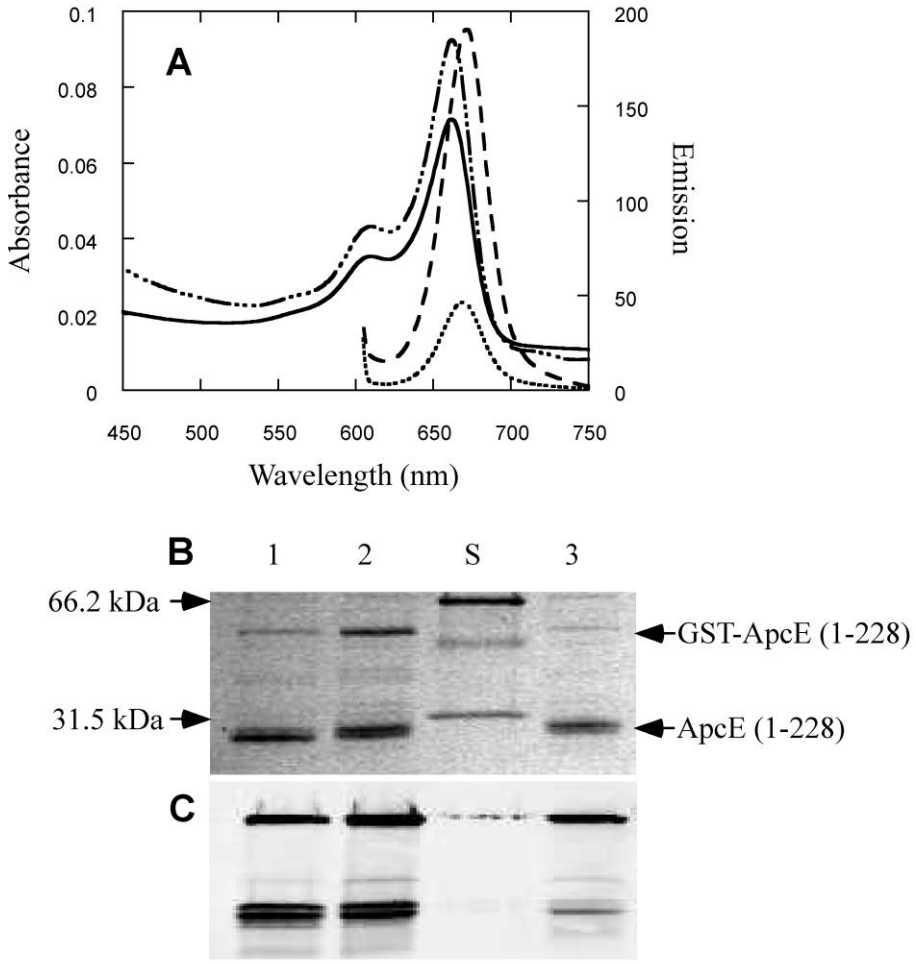

Fig. 27: Analyses of GST-ApcE purified from *E. coli* cells. A. Absorbance (solid line) and fluorescence emission (dashed line) spectra of GST-ApcE purified from *E. coli* cells containing pGST-ApcE, pPcyA and pCpcUS, and absorbance (dashed dotted line) and fluorescence (dotted line) spectra of GST-ApcE produced without pCpcUS. **B.** Coomassie-stained polyacrylamide gel of purified GST-ApcE from *E. coli* cells containing pGST-ApcE, pPcyA with (lane 1) or without pCpcUS (lane 2). Molecular mass standards are loaded in lane "S" and selected masses are indicated to the right. Identities of polypeptides are indicated to the left. **C.** Zinc-enhanced bilin fluorescence of the gel pictured in panel B.

3.1.6. *Creation of partially chromophorylated PBPs in E. coli:*

The efficiency of this *in-vivo* system in creating partially chromophorylated CpcB was examined in order to determine the order for post-translational modifications on CpcB (Miller, Leonard et al. 2008). The CpcS-I/CpcU lyase and the CpcT lyases were tested separately to confirm that each could attach PCB to CpcB in this system, and the efficiency of PCB addition to Cys-82 and Cys-153 in *E. coli* was estimated (see Table 6). Cells containing pCpcBA, pPcyA, and either pCpcUS or pCpcT produced strongly colored PBPs (See Fig. 20). The spectra of these two partially chromophorylated PCs are shown in Fig. 28 and 29. The PC produced by chromophorylation at Cys-82 of CpcB by CpcS-I/CpcU was deep blue (see Fig. 20) and had an absorbance maximum at 620 nm and a fluorescence emission maximum at 642 nm for the CpcSU-product at Cys-82 (See Fig. 28A, solid lines). The purple-colored PC (see Fig. 20) produced by chromophorylation of Cys-153 by CpcT had an absorbance maximum at 592 nm and a fluorescence emission maxima at 618.5 nm (see Fig. 29A and Table 6). SDS-PAGE analyses of these two PCs were also performed, and the Coomassie-stained gels of the purified proteins are shown in Figs. 28C and 28B, respectively. The zinc-enhanced bilin fluorescence analyses in Fig. 28D (lane 3) and 29C (lane 1) showed that CpcB was the subunit to which PCB had been added. As has been seen previously (Arciero, Bryant et al. 1988; Shen, Saunee et al. 2006; Zhao, Su et al. 2006; Saunée, Williams et al. 2008; Shen, Schluchter et al. 2008), no significant bilin addition is seen in the absence of the lyase. An estimated 37% of CpcB was chromophorylated by CpcS-I/CpcU, whereas CpcT only chromophorylated ~17% of the CpcB (Table 6). Thus, although both bilin lyases displayed activity toward CpcB in this *in-vivo E. coli* system, the chromophorylation levels were much lower than those observed with CpcS-I/CpcU for AP subunit substrates. To determine whether the presence of the CpcA subunit might be interfering with the chromophorylation by

CpcSU, *cpcB* was expressed from plasmid pCpcB construct (Table 4) together with pCpcUS and pPcyA**.** Although the yields of holo-HT-CpcB were much less than when co-expressed with CpcA (about 10-fold less), CpcSU was able to chromophorylate the HT-CpcB protein at a similar level. Comparing equal amounts of HT-CpcB loaded on SDS-PAGE (See Fig. 29C, lanes 3 and 4) and examining the bilin fluorescence (See Fig. 29D), the bilin fluorescence was roughly equal (within 10% as estimated by the Quantity One™ software). These observations indicated that CpcA did not interfere with chromophorylation of CpcB by CpcSU.

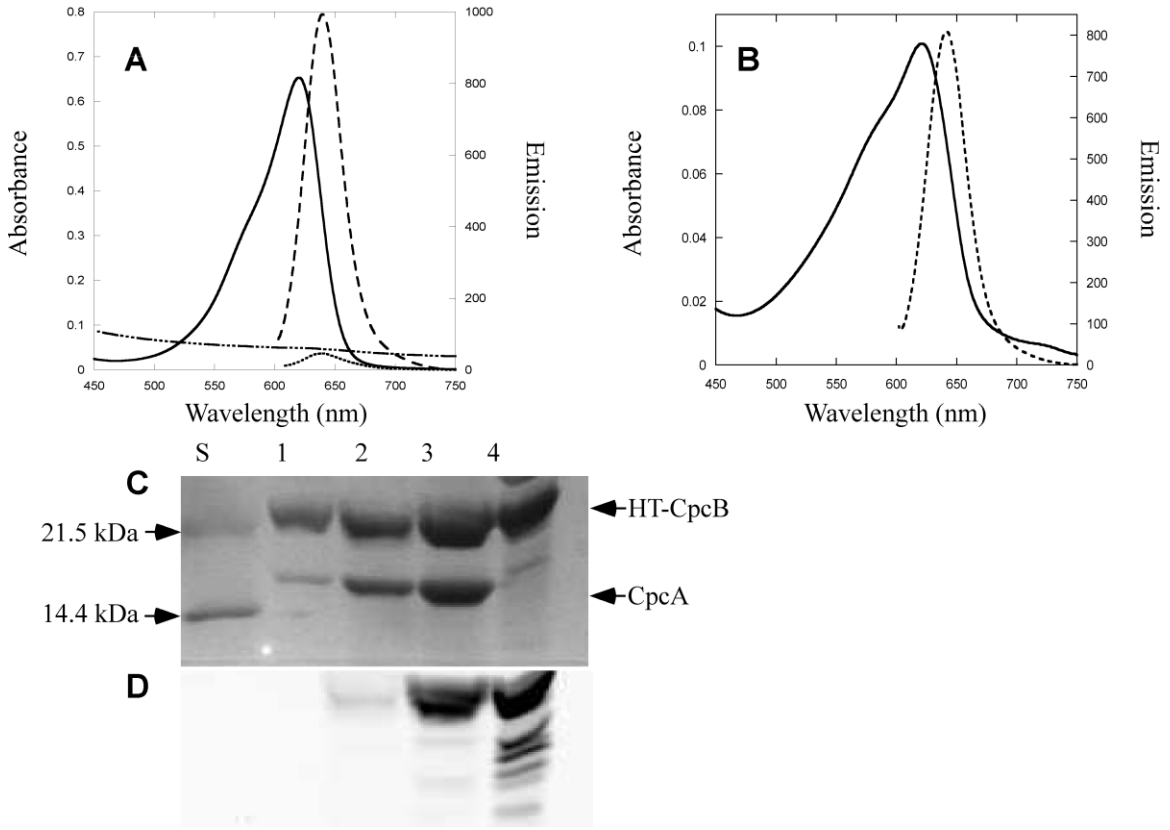

Fig 28. Analysis of HT-CpcB purified from *E. coli* cells chromophorylated by CpcS-I/CpcU at Cys-82. **A.** Absorbance (solid) and fluorescence emission (dashed) spectra of HT-CpcB purified from cells containing pCpcBA, pCpcUS, and pPcyA and absorbance (dashed dotted line), fluorescence (dotted line) without pCpcUS. **B.** Absorbance (solid) and fluorescence emission (dashed) spectra of HT-CpcB purified from cells containing pCpcB, pCpcUS, and pPcyA **C.** Coomassie-stained SDS-polyacrylamide gel of HT-CpcB/CpcA purified from cells containing pCpcBA alone (lane 1), from cells containing pCpcBA, and pPcyA (lane 2), from cells containing pCpcBA, pPcyA and pCpcUS (lane 3), and from cells containing pCpcB, pCpcUS and pPcyA (lane 4). Molecular mass standards were loaded in the lane marked "S"; the position of the 21.5-kDa mass standard is indicated. **D.** Zinc-enhanced bilin fluorescence of the gel in panel C.

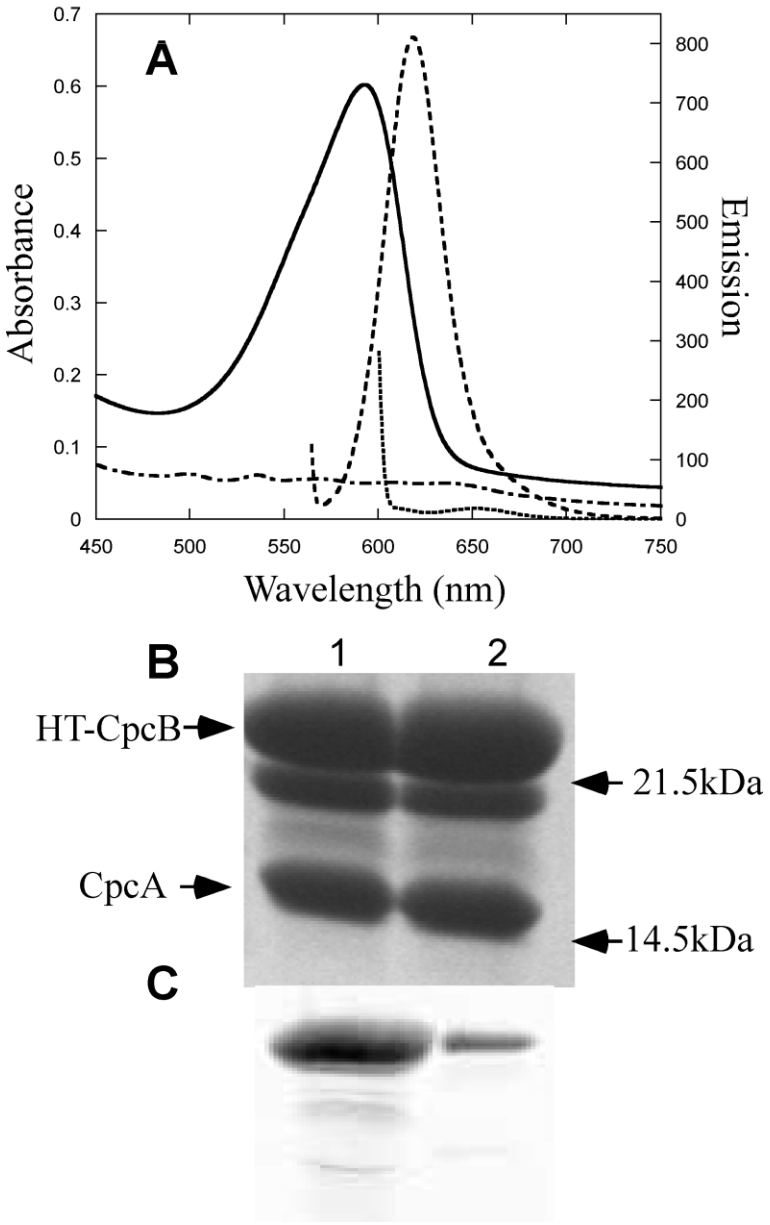

Fig. 29: Analysis of HT-CpcB purified from *E. coli* cells chromophorylated by CpcT at Cys-153. A. Absorbance (solid) and fluorescence emission (dashed) spectra of HT-CpcB purified from cells containing pCpcBA, pCpcT, and pPcyA and absorbance (dashed dotted line), fluorescence (dotted line) without pCpcT. **B.** Coomassie-stained SDS-polyacrylamide gel of HT- CpcB/CpcA purified from cells containing pCpcBA, pCpcT, and pPcyA (lane 1) or from cells containing pCpcBA and pPcyA (lane 2). Masses of the standards are indicated to the right. **C.** Zinc-enhanced bilin fluorescence of the gel in panel B.

Table 6: Properties of Recombinant Holo-PBPs for PC and AP subunits:

Holo-Recombinant PBP (Plasmids present)	λ_{max} *[nm]*	*Ratio Vis:UV*	*% Chromophorylation*[a]
HT-CpcA (pBS414v, pPcyA)	625/370	4.64	48.1
HT-CpcA (pBS414v, pAT101)	625/370	4.5	22.4
HT-ApcA/ApcB (pApcAB, pCpcUS, pPcyA)	615/357	2.53	71.9
HT-ApcD (pApcDB, pCpcUS, pPcyA)	672/370	2.21	ND[b]
HT-ApcD (pApcD, pCpcUS, pPcyA)	642/370	0.785	ND
ApcB (pApcDB, pCpcUS, pPcyA)	616/370	2.57	ND
HT-ApcF (pApcF, pCpcUS, pPcyA)	616/370	3.37	68.1
GST-ApcE (pGST-ApcE, pPcyA)	662/370	1.64	ND
HT-CpcB (pCpcBA, pCpcUS, pPcyA)	620/367.5	5.19	37.1
HT-CpcB (pCpcBA, pCpcT, pPcyA)	592/354	2.53	17.4

[a] % chromophorylation was estimated as described in materials and methods.

[b] Not determined due to difficulties with proteolysis or expression levels

3.2. Creation of Unique phycobiliproteins using PEB in *E.coli* for potential Biotechnological applications

3.2.1. *Creation of holo α-PC using PEB in E. coli:*

For this part of the project, the goal was to see if the different classes of PCB lyases were able to attach PEB to substrates using our *in vivo* heterologous system, as the ability to create phycobiliproteins with different chromophore content in this system would be a great biotechnological tool. PEb containing PBPs have higher quantum yield. First, the CpcE/CpcF lyase which normally attaches PCB to CpcA (α-PC) was used. The constructs we used had been used by Tooley et al. to show that holo-CpcA could be formed in *E. coli* using cotransformation with pAT101 (encoding *Synechocystis* sp. PCC 6803 *hox1* and *pcyA*) (Tooley, Cai et al. 2001). *E. coli* cells were transformed with the pPebS plasmid and either pBS414v (encoding *cpcA, cpcE* and *cpcF*) or pBS405v (encoding *cpcA*). Cells were grown as described, harvested, and exhibited a pink color as shown in Fig. 30D. The HT-CpcA was purified by metal affinity chromatography. Absorption spectra of the two HT-CpcA showed that only the one produced in the presence of CpcE and CpcF (pBS414v) contains significant PEB addition with an absorbance maximum at 560 nm (see Fig. 30A). The HT-CpcA produced in the presence of the bilin lyase CpcE/CpcF was highly fluorescent with a single emission peak at 567 nm whereas the HT-CpcA produced in cells with PEB but without the CpcE/CpcF bilin lyase (pBS405v) had two fluorescent emission peaks at 567 nm and 630 nm. Fairchild and Glazer previously performed *in vitro* reactions with apo-CpcA, PEB and with or without purified CpcE/CpcF; the fluorescence emission maxima for the CpcE/CpcF-mediated CpcA product was at 571 nm whereas the non-enzyme mediated CpcA product had a broad emission maxima in the range of 570-581 nm (Fairchild and Glazer 1994). Interesting enough the Quantum yield for holo-CpcA (PEB) was 0.98, whereas in the case of holo-CpcA (PCB) it was 0.85.

After separating the HT-CpcA proteins by SDS-PAGE and examining total protein content (Coomassie Blue staining, Fig. 30B) versus bilin-bound content (Zn-enhanced fluorescence in Fig. 30C), HT-CpcA contained PEB chromophore when purified from cells containing CpcEF (lane 1) whereas there was almost no PEB attached to the HT-CpcA purified from cells without CpcEF (lane 2). When the protein concentration of the HT-CpcA (OD_{280}) is divided by the PEB concentration of the sample (in 8 M urea, pH 2), 83.5% of HT-CpcA contains PEB (see Table 7). The chromophorylated product yield for holo CpcA (CpcA-PEB) was 11.7 mg L^{-1}.

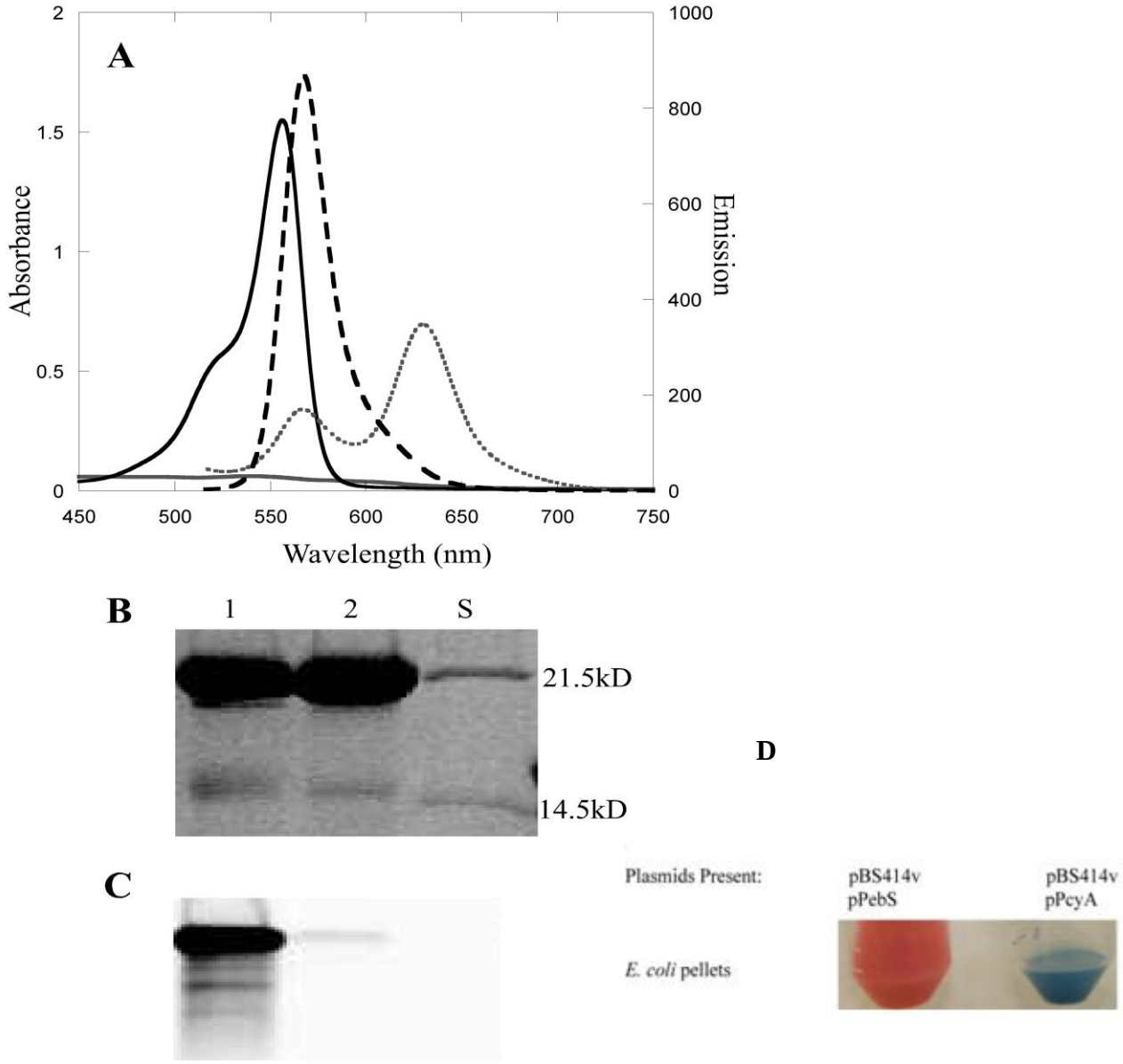

Fig. 30. Analyses of *Synechocystis* sp. PCC 6803 HT-CpcA purified from *E. coli* cells. A. The absorbance (solid) and fluorescence emission (dashed) spectra of HT-CpcA purified from *E. coli* cells containing PEB and either CpcA/CpcE/CpcF (black) or CpcA (gray). **B.** The Coomassie-stained SDS-polyacrylamide gel of purified HT-CpcA from cells containing pPebS and either CpcA/CpcE/CpcF (lane 1) or CpcA (lane 2). The molecular mass standards were loaded in lane "S" with masses indicated at right. **C.** The zinc-enhanced bilin fluorescence of the gel pictured above in panel B is shown. **D.** Represents the picture of pellets expressing pbs414v with pPebS and pPcyA.

3.2.2. CpcSU *ligation specificity for PEB on CpcB subunit in E. coli:* In order to examine how well the CpcS-I/CpcU lyase can attach PEB to CpcB, *E. coli* cells were transformed with pCpcBA, with or without pCpcUS and either the pPebS plasmids. Cells were grown as described and purified the HT-CpcBA produced HT-CpcBA purified from cells containing pPebS (PEB; see Fig. 31A) showed two absorbance peaks, the largest one at 607 nm and a smaller one at 572; the product had a fluorescence emission peak at 569 nm with a small shoulder at 610 nm. HT-CpcBA produced in the presence of pPebS and pCpcUS showed two absorbance peaks, the largest one at 559 nm and a smaller one at 606 nm; it had a sharp fluorescence emission peak at 568 nm. In the presence of the CpcS-I/CpcU bilin lyase, there is more covalent addition of PEB to CpcB as judged by the absorbance and fluorescence intensity as well as the comparison of the Coomassie-stained proteins with the zinc-enhanced bilin fluorescence (compare lanes 1 and 2 in Fig 31B and 31C). However, one of the products of the non-enzyme mediated addition of PEB to CpcB is red-shifted at 572 nm when compared to the CpcS-I/CpcU-mediated addition product at 559 nm. The second product with absorption at 607-610 nm present in both samples is a 15,16 dihydrobiliverdin adduct, an oxidized product that formed in *in vitro* PEB addition reactions with CpcB/CpcA and with α-phycoerythrin (CpeA) (Arciero, Dallas et al. 1988; Fairchild and Glazer 1994). In the *in vitro* PEB addition experiment with CpcB/CpcA, Arciero et al. demonstrated addition of PEB to Cys-84 on CpcA and to Cys-82 on CpcB (both PEB and 15,16 dihydrobiliverdin adducts were observed). In this *in vivo* heterologous system, addition to CpcB, not CpcA takes place, presumably at Cys-82. The CpcS-I/CpcU-mediated 559 nm absorption product is likely PEB attached to Cys-82 on CpcB in the stretched conformation as this product is also highly fluorescent with the fluorescence emission maxima at 568 nm near where one would expect for native PEB-containing phycobiliproteins. The CpcS-I/CpcU lyase showed less

addition of PEB (6% chromophorylation; see Table 7) to CpcB than it did for PCB (37% chromophorylation). The bilin at Cys-82 is the terminal energy acceptor within the PC trimer; having PEB attached here would have negative consequences for efficient energy transfer from PCB attached at Cys-153 on CpcB or from PCB attached to Cys-84 on CpcA**.** Energy absorbed by these other peripheral bilins is transferred to Cys-82 on CpcB and eventually to the AP core. Another explanation of the lower level of PEB chromophorylation to CpcB may be that the CpcS-I/CpcU lyase is not as active at the lower temperatures required for activity of PebS (12 hours at 18 °C), however the CpcE/CpcF lyase was able to achieve a high level of PEB chromophorylation of CpcA under those same conditions.

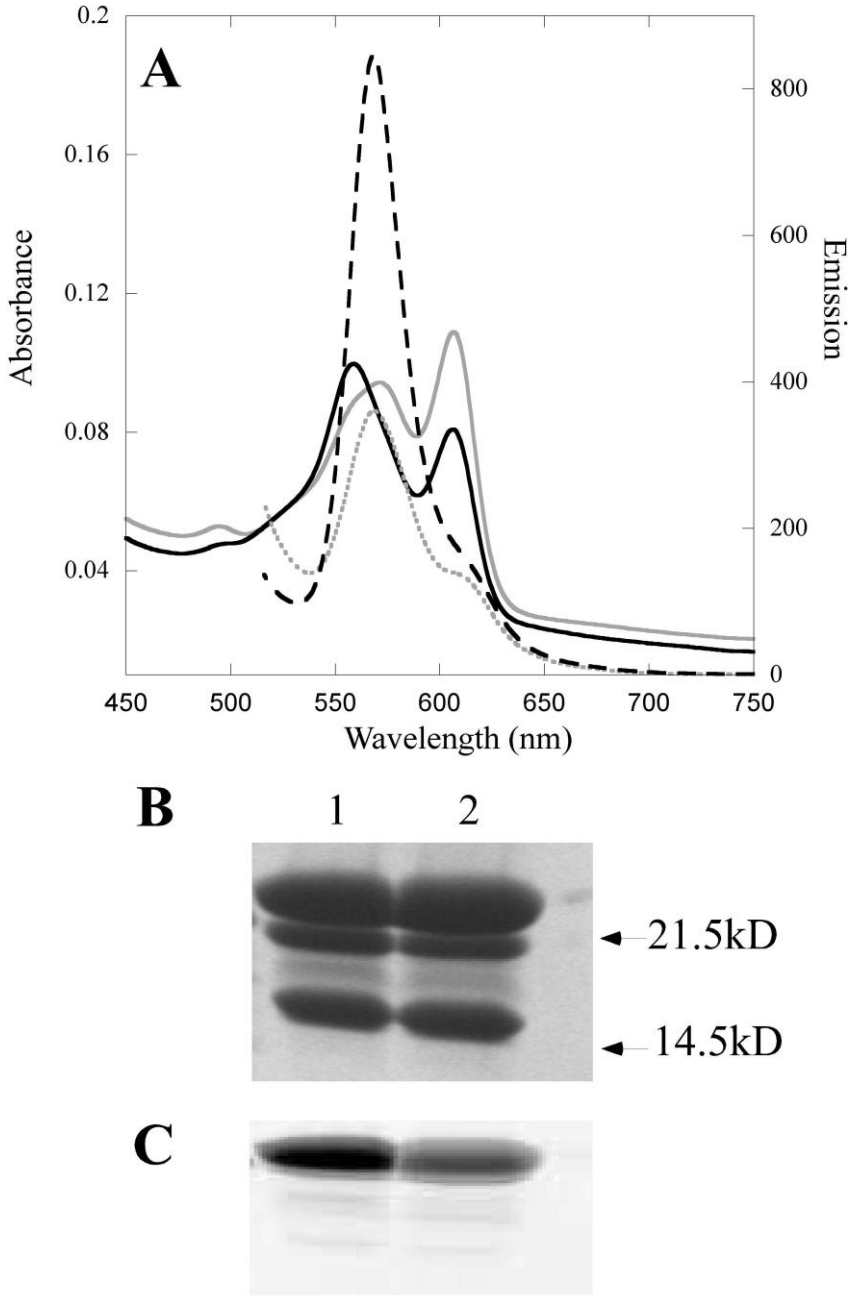

Fig 31. Analyses of *Synechocystis* sp. PCC 6803 HT-CpcB/CpcA purified from *E. coli* cells. **A.** Absorbance (solid) and fluorescence emission (dashed) spectra of HT-CpcB/CpcA purified from cells containing pCpcBA, pPebS, and with (black) or without (gray) pCpcUS. **B.**The Coomassie-stained SDS-polyacrylamide gel containing HT-CpcB/CpcA purified from cells containing pCpcBA, pPebS, and with (lane 1) our without (lane 2) pCpcUS. Molecular masses of standards are indicated at right. **C.** The zinc-enhanced bilin fluorescence of the gel above in panel B is shown here.

Table 7: Properties of Recombinant Holo-PBPs with non-cognate lyases

Holo-Recombinant PBP (Plasmids present)	λ_{max} *[nm]*	*Ratio Vis:UV*	*% Chromophorylation*[a]
HT-CpcA(pBS414, pPebS)	571	8.69	83.5
HT-CpcBA (pCpcBA, pCpcSU, pPebS)	572	0.707	6.06

[a] % chromophorylation was estimated as described in materials and methods.

[b] Not determined due to difficulties with proteolysis or expression levels

3.3. Characterization of CpeY, CpeZ and CpeS bilin lyases involved in phycoerythrin biosynthesis in *Fremyella diplosiphon* strain UTEX 481

3.3.1 ***Characterization of bilin lyase activity of CpeY and CpeZ with CpeA***. The *cpeY* and *cpeZ* genes occur downstream of the *cpeBA* genes encoding the β and α subunits of PE, respectively. Based upon their sequence similarity (~32 %), CpeY and CpeZ belong to the CpcE/CpcF family of bilin lyases (See Fig 32). Transposon mutants and complementation studies in *Fremyella diplosiphon* UTEX 481 suggested that these two proteins might be involved in PE biogenesis, but their specific roles were not elucidated (Kahn, Mazel et al. 1997). Recombinant CpeY and CpeZ from the cyanobacterium *F. diplosiphon* were soluble (data not shown). An *in vivo E. coli* heterologous co-expression system was used to test whether either of these genes encodes a bilin lyase. Constructs made for this study (thesis) are listed in Table 4.

E.coli cells containing only plasmids pCpeA and pPebS (*i. e.*, no lyase present) had no significant color (data not shown), but cells containing these two plasmids and pCpeYZ were bright pinkish-red in color (Fig. 33). Holo-HT-CpeA purified from these cells had an absorbance maximum at 560 nm (See Fig. 34A) and a very high fluorescence emission maximum at 574 nm (See Fig 34A; the sample was diluted 15-fold to 0.05 OD_{560} prior to obtaining the fluorescence spectrum), whereas the cells containing pCpeA and pPebS only did not have any significant absorbance or fluorescence emission (See Fig. 34A; the sample was not diluted prior to obtaining the fluorescence emission spectrum). CpeZ and CpeY were also tested individually to determine if the individual proteins could attach PEB to CpeA. The fluorescence emission spectrum obtained from the HT-CpeA purified from cells containing pCpeA, pPebS and pCpeY showed that only CpeY had significant activity by itself, but the amount of fluorescent product was lower than when both CpeY and CpeZ were present (See Fig. 34B). The relative yields of holo-CpeA produced when co-expressed with PebS along with either CpeY or CpeZ are given in Table 8.

Note that the HT-CpeA sample was not diluted prior to obtaining the fluorescence emission spectrum when HT-CpeA was co-expressed with CpeY; however, the HT-CpeA product produced with the other lyase subunit, CpeZ, was not fluorescent (See Fig. 34B, Table 8). The three HT-CpeA samples purified from *E. coli* cells were analyzed by SDS-PAGE (Fig. 34C). The bilin addition to HT-CpeA was examined by zinc staining of the gel to enhance bilin fluorescence (Fig. 34 D); protein content was shown by subsequent staining of the same gel with Coomassie Blue (Fig. 34C). The HT-CpeA purified from cells expressing both CpeY and CpeZ was highly fluorescent after Zn-staining (Fig. 34 D; lane 2), but HT-CpeA purified from cells containing no lyase subunit or with CpeZ alone was not fluorescent after Zn-staining. Thus, little or no ligation of PEB occurred in the absence of a lyase subunit or with CpeZ alone. (Fig. 34D; lanes 1 and 4, respectively). However, HT-CpeA purified from cells coexpressing CpeY produced a fluorescent product with a yield that was ~60% of of that achieved with both CpeY and CpeZ (Table 8), suggesting CpeZ enhances PEB ligation activity of CpeY. One of the interesting observations from this study was CpeA by itself when expressed in *E. coli* was seen to be insoluble. However, coexpressing it with either CpeY or CpeY/CpeZ lyase increased its solubility in *E. coli*. To confirm at HT-CpeA was getting expressed in all *E. coli* extract Western Blot analyses was performed using rabbit polyclonal anti α-PE antibodies; this showed HT-CpeA was present in inclusion bodies in all cells (Data not shown).

By comparing the protein concentration and the PEB concentration in the sample, it was estimated that 55% of the soluble HT-CpeA had been chromophorylated when both CpeY and CpeZ were coproduced with HT-CpeA. The total yield of HT-CpeA-PEB was 3.6 mg L^{-1} of *E. coli* culture when both CpeY and CpeZ were coexpressed. By comparison, when only CpeY was coproduced with HT-CpeA, only ~30 % of the protein carried a chromophore and the product

yield was 1.8 mg L^{-1} of culture. The fluorescence quantum yield for the partial holo-HT-CpeA was 0.72, which is quite high for PE subunits.

CpeYZ is a CpcEF type lyase based on the sequence alignment. The CpcE and CpcF proteins interact with each other (1:1) and copurify on a Ni-NTA column. However, in the case of CpeY and CpeZ no interaction of CpeY in a pull-down assay with HT-CpeZ was detected (Data not shown).

Fig. 32. Amino acid sequence alignment between CpeY from *Fremyella diplosiphon* and a fusion of CpcE with CpcF from *Synechococcus* sp. PCC 6803. The CpcE/CpcF proteins were combined to form one major protein. The software used was MacVector 9.0.

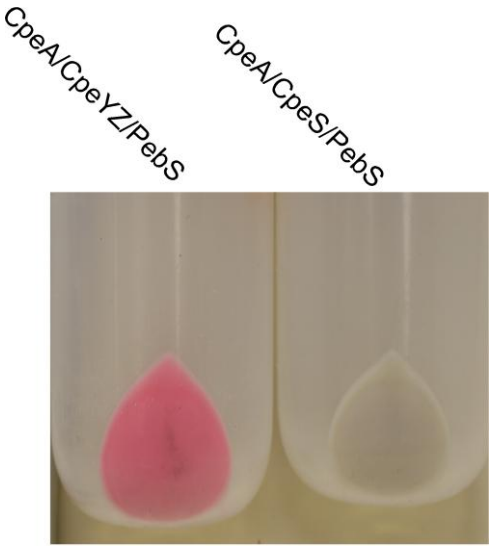

Fig. 33. Picture of the *E. coli* cell pellets from cells containing HT-CpeS, pPebS and with either pCpeYZ (left) or pCpeS (right).

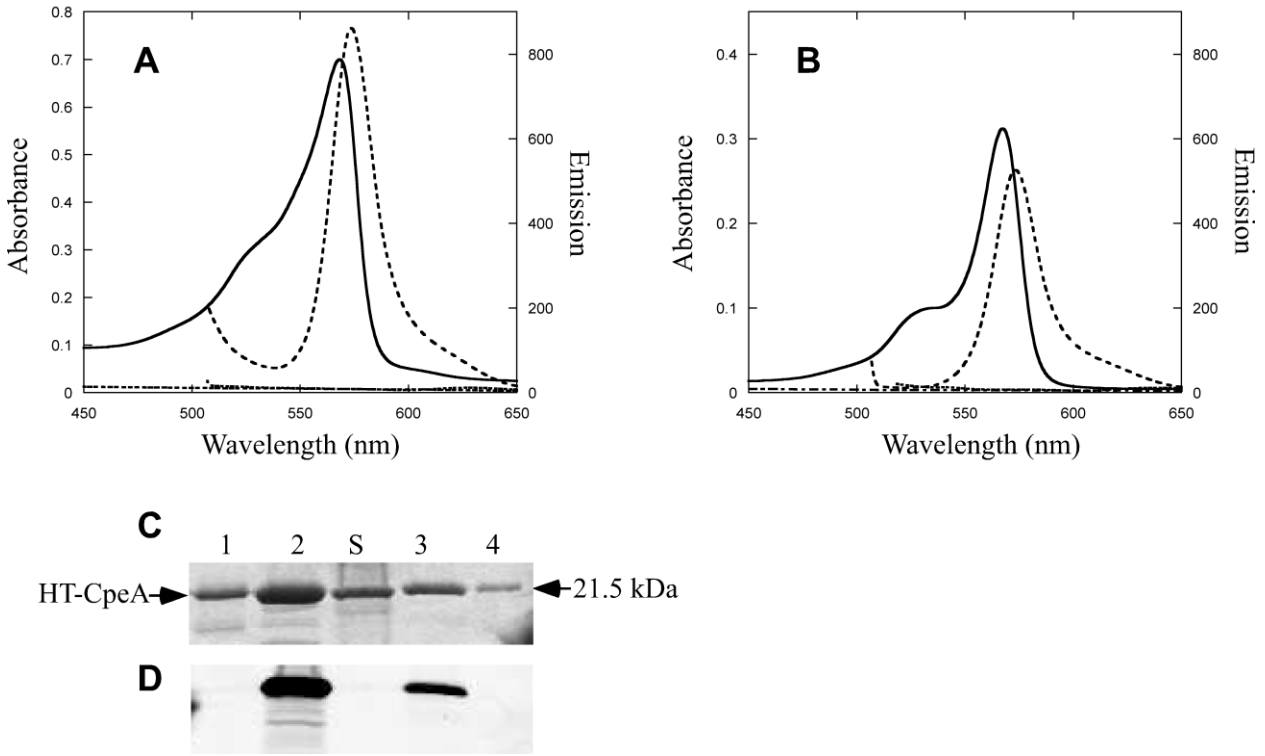

Fig. 34. **Analyses of HT-CpeA produced with CpeY and CpeZ in *E. coli*. A**. Absorbance (solid line) and fluorescence emission (dashed line) spectra of HT-CpeA purified from cells containing pCpeA, pPebS with pCpeYZ and absorbance (dashed dotted line), fluorescence (dotted line) without pCpeYZ are shown. **B**. Absorbance (solid line) and fluorescence emission (dashed line) spectra of HT-CpeA purified from cells containing pCpeA, pPebS with pCpeY and absorbance (dashed dotted line), fluorescence (dotted line) with pCpeZ are shown. In order to acquire the fluorescence emission spectra for the HT-CpeA produced in the presence of pCpeYZ and pCpeY (dashed lines in panels A and B) the samples were diluted to OD_{560nm} of 0.05; however, no dilution was performed on HT-CpeA produced in the absence of a lyase or in the presence of pCpeZ (dotted lines in panels A and B). **C**. This panel shows a Coomassie-stained SDS polyacrylamide gel containing HT-CpeA purified from cells containing pCpeA, pPebS with no lyase (lane 1) or with pCpeYZ (lane 2), or HT-CpeA purified from cells containing pCpeA, pPebS, and either pCpeY (lane 3) or pCpeZ (lane 4). Molecular mass standards are loaded in lane "S", and masses are indicated to the right. **D**. The zinc-enhanced fluorescence of the gel pictured in panel

3.3.2 *Analysis of which cysteine residues on α-PE is chromophorylated by the CpeY/CpeZ lyase-*

The holo-CpeA (α-PE subunit) isolated from *F. diplosiphon* carries two PEB chromophores at Cys-82 and Cys-139 (Fairchild and Glazer 1994). To test the site specificity of the CpeY/CpeZ bilin lyase, site-specific variants of CpeA (C82S, C139S and C82S/C139S) were produced in which cysteine residues were to change to serine. Each mutant gene was co-expressed with the CpeY/CpeZ lyase and the enzymes to synthesize PEB, and the HT-CpeA produced was purified. The results of the absorbance and fluorescence emission measurements on these proteins are shown in Figure 35 and Table 9. Only the C139S HT-CpeA variant was a substrate for PEB ligation by CpeY/CpeZ, and the product had an absorption maximum at 560 nm and a fluorescence emission maximum at 574 nm (Fig. 35A). These values were identical to those for HT-CpeA described above, and the results indicated that Cys82 is the residue that is chromophorylated with PEB by the CpeY/CpeZ lyase. The purified C82S HT-CpeA and C82S/C139S HT-CpeA variants produced in the presence of the CpeY/CpeZ lyase and PEB synthesis enzymes had no significant fluorescence emission (See Fig. 35A and Table 9). Similarly, no fluorescent products were observed when any of the variant proteins were produced in the absence of the lyase subunits (data not shown). The HT-CpeA variants produced in these experiments were also analyzed by SDS-PAGE. Bilin addition to each protein was examined by zinc-enhanced fluorescence of the gel (Fig. 35C). The purified C139S HT-CpeA variant was fluorescent due to the presence of covalently attached PEB (Fig. 35C, lane 2). After staining the same gel shown in Fig. 35C with Coomassie Blue (see Fig. 35 B), one can see that only when PEB has been ligated (lane 2, Fig. 35B) does the HT-CpeA accumulate in a soluble form in *E. coli*. From these experiments, it was concluded that the CpeYZ bilin lyase attaches PEB specifically to Cys-82 on CpeA.

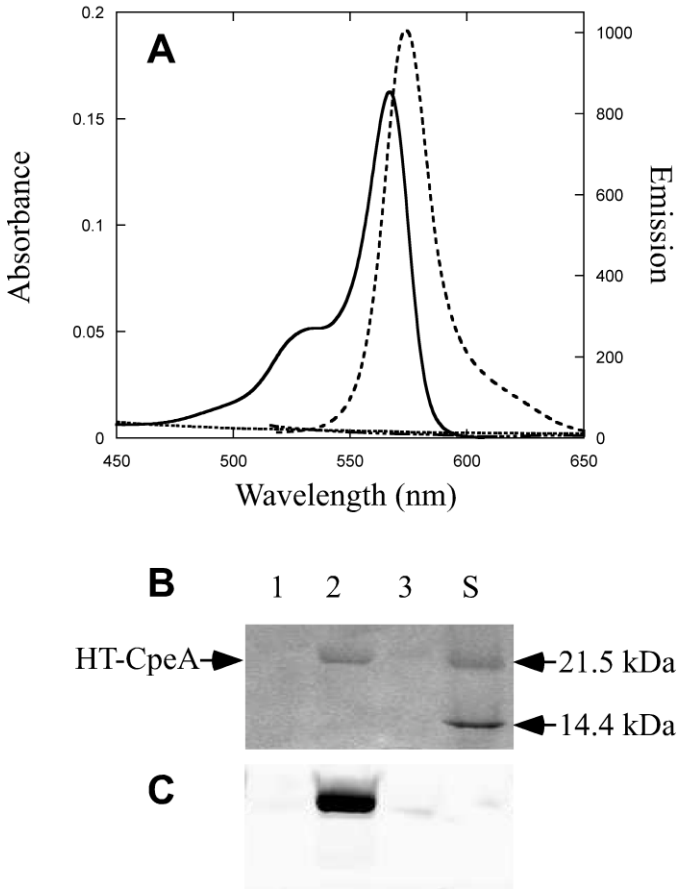

Fig. 35. **Analyses of the specific cysteine residue on HT-CpeA required for PEB addition by CpeYZ. A**. Absorbance (solid line) and fluorescence emission (dashed line) spectra of HT-CpeA(C139S) purified from cells containing CpeA(C139S), pPebS with pCpeYZ and the absorbance (dashed dotted line), fluorescence (dotted line) spectra from cells containing CpeA(C82S), pPebS with pCpeYZ are shown. In order to acquire the fluorescence emission spectra for the HTCpeA (C139S) produced in the presence of pCpeYZ (dashed lines in panel A), the sample was diluted to OD_{560nm} of 0.05; however, no dilution was performed on HT-CpeA(C82S) (dotted line in panel A). **B**. Coomassie-stained SDS polyacrylamide gel containing HTCpeA mutants purified from cells containing CpeA (C82S), pPebS, pCpeYZ with (lane 1) or (lane 2) CpeA (C139S), pPebS, pCpeYZ and (lane 3) purified from the cells containing CpeA (C82,139S), pPebS, pCpeYZ. Molecular mass standards are loaded in lane "S", and masses are indicated to the right. **C**. The zinc-enhanced fluorescence of the gel pictured in panel **B** is shown here.

The above result was also confirmed using Mass spectrometry. The tryptic peptide from CpeA containing PEB was identified (CAR) (Bhougatou and Cole, unpublished data).

3.3.3. ***Does CpeS also chromophorylate CpeA?*** - Zhao et al. (Zhao, Su et al. 2007) used a recombinant *E. coli in vivo* system to show that CpcS from *Nostoc* sp. PCC 7120 (formerly denoted CpeS; but this organism does not contain phycoerythrin) (Zhao, Su et al. 2006) as a " near universal lyase" that adds bilins to Cys-82 on most biliproteins, including the non-cognate substrate, CpeA, from *F. diplosiphon*. As shown in this thesis, the CpeY/CpeZ lyase attached PEB to the same Cys-82 of HT-CpeA. Thus, the activities of these two lyases were compared in our co-expression system to determine which one has the most activity on CpeA. The cognate *cpeS* gene from *F. diplosiphon* was cloned to create plasmid pCpeS (see Table 4). Fig. 36A shows the absorbance and fluorescence emission spectra of HT-CpeA purified from cells co-producing HT-CpeA, CpeS, and enzymes for PEB synthesis. The yield of chromophorylated HT-CpeA was much lower (see Fig. 36) when the lyase coproduced was CpeS rather than CpeY/CpeZ. However, the absorbance and fluorescence properties of the resulting HT-CpeA proteins were similar (see Table 9). The HT-CpeA produced in the presence of CpeS and PEB was analyzed by SDS-PAGE. The Zn-enhanced fluorescence in Fig. 36 C shows that PEB is covalently attached to the CpeA protein (Fig. 36B), but the amount of chromophorylation is only 1% (approx.) compared to CpeYZ.

Additionally, the site-specific variants of CpeA were also used to investigate the specificity of CpeS, and these results are shown in Fig. 37 and Table 8. Surprisingly, colored fluorescent products were obtained for both HT-CpeA (C82S) and HT-CpeA (C139S), which, suggested that CpeS could ligate PEB to both cysteines on CpeA, whereas no significant fluorescence emission was observed when CpeA mutants were co expressed in absence of the CpeS lyase (data not shown). The absorbance and fluorescence emission maxima for the two

variants were different; this suggested the different protein environments for the PEB in the two variants, affected its absorbance and emission properties (Table 8). In further support of this interpretation, two PEB-containing peptides were observed after tryptic digestion of wild-type HT-CpeA synthesized in the presence of PEB and CpeS (See Fig. 38).

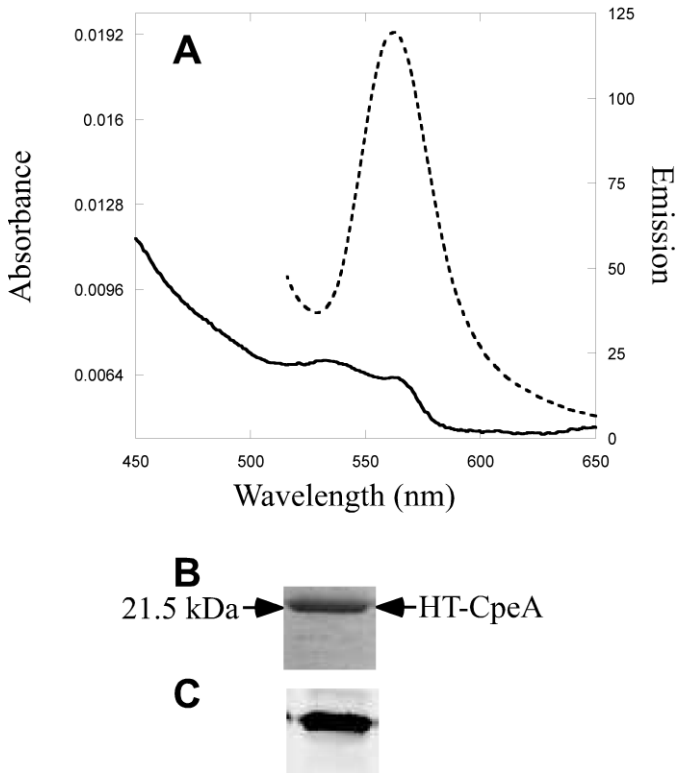

Fig. 36. **Analyses of HT-CpeA produced with CpeS in *E. coli* A**. Absorbance (solid line) and fluorescence emission (dashed line) spectra of HT-CpeA purified from cells containing pCpeA, pPebS with pCpeS **B**. Coomassie-stained SDS polyacrylamide gel containing HT-CpeA purified from cells containing pCpeA, pPebS, pCpeS. **C**. The zinc-enhanced fluorescence of the gel pictured in panel B is shown here.

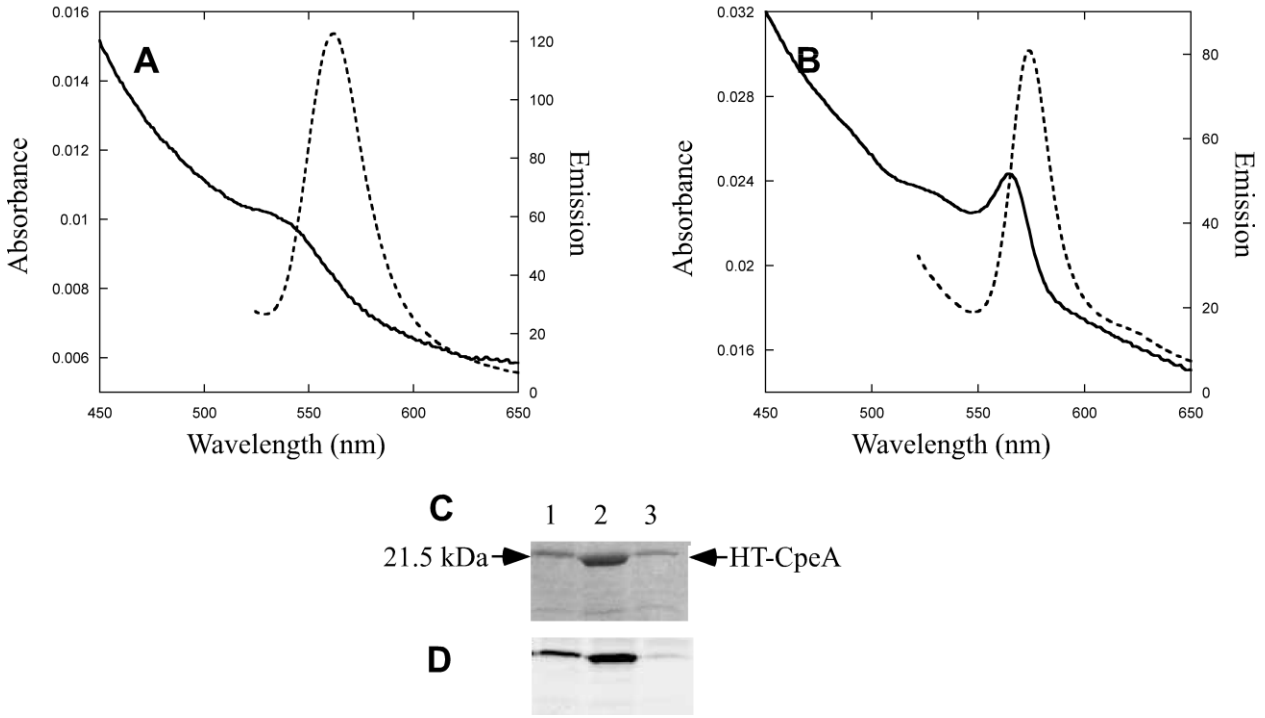

Fig. 37. **Analyses of the specific cysteine residue on HT-CpeA for PEB addition by CpeS**. **A**. Absorbance (solid line) and fluorescence emission spectra (dotted line) of HTCpeA (C82S) mutants purified from cells containing pCpeS and pPebS. **B**. Absorbance (solid line) and fluorescence emission spectra (dotted line) of HTCpeA (C139S) mutant purified from cells containing pCpeS and pPebS. **B**. Coomassie-stained SDS polyacrylamide gel containing HT-CpeA mutants purified from cells containing pPebS, pCpeS and either pCpeA (C82S) (lane 1), pCpeA(C139S) (lane2) or pCpeA (C82,139S) (lane 3) **C**. The zinc-enhanced fluorescence of the gel pictured in panel **B** is shown here.

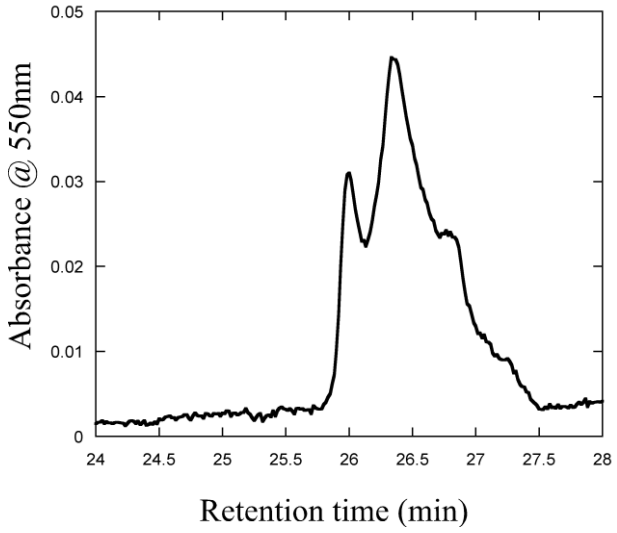

Fig. 38. Tryptic digest of partial holo HT-CpeA purified from cells containing pCpeA, pCpeS, pPebS. The chromatogram represents sample separated on a C_{18} RP-HPLC column.

3.3.4. *Comparison of the PEB ligation activity of CpeY/CpeZ and CpeS bilin lyases in CpeB-*

CpeB (β-PE) in *F. diplosiphon* has three PEB chromophores attached to four Cys residues: Cys-80 and Cys-165 carry singly linked PEBs and Cys-48 and Cys-59 carry a doubly linked PEB (Fairchild and Glazer 1994) (See Fig. 5). Two different sterioisomers occur in PE-β subunits ($C3^1$ and $C18^1$ of the bilin) (Schirmer, Huber et al. 1986). The R-isomer, the most common one is found at Cys-80 and Cys-48, 59, whereas the S-isomer is present the Cys-165 on CpeB. Using the in *vivo* co-expression system, HT-CpeB was coproduced with CpeS and enzymes for PEB synthesis. Fig. 39A shows the absorbance and fluorescence emission spectra of the resulting HT-CpeB product purified by metal-affinity chromatography. The absorbance maximum was 556 nm, and the fluorescence emission maximum was 572 nm. No significant chromophorylated HT-CpeB was synthesized in the absence of CpeS (Fig. 39A, dashed dotted line). The bilin content of each protein was examined by Zn-enhanced fluorescence staining of an SDS-polyacrylamide gel (Fig. 39 D) and the proteins were detected after staining the same gel with Coomassie Blue (Fig. 39C). PEB was covalently attached to CpeB only in the presence of CpeS (Fig. 39 D, lane 3). However, it was also clear that very little CpeB remained soluble unless an attached bilin (or lyase) was present (compare lanes 1, 2 and 3 in Fig. 39C). The HT-CpeB expression was confirmed by Western Blot using rabbit polyclonal anti β-PE, and was found to be present only in inclusion bodies.

The CpeY/CpeZ lyase was also coproduced with HT-CpeB; however, no absorbance or fluorescence emission was observed (Fig. 39B). When the purified protein from pCpeB/pCpeYZ/pPebS was separated on SDS-PAGE, no notable fluorescent band was observed (Fig. 39D, lane 1), indicating that CpeYZ was unable to ligate PEB on β-PE. CpeY and CpeZ

were also tested individually to see if each can chromophorylate CpeB alone, but no indication of PEB ligation was observed (Data not shown).

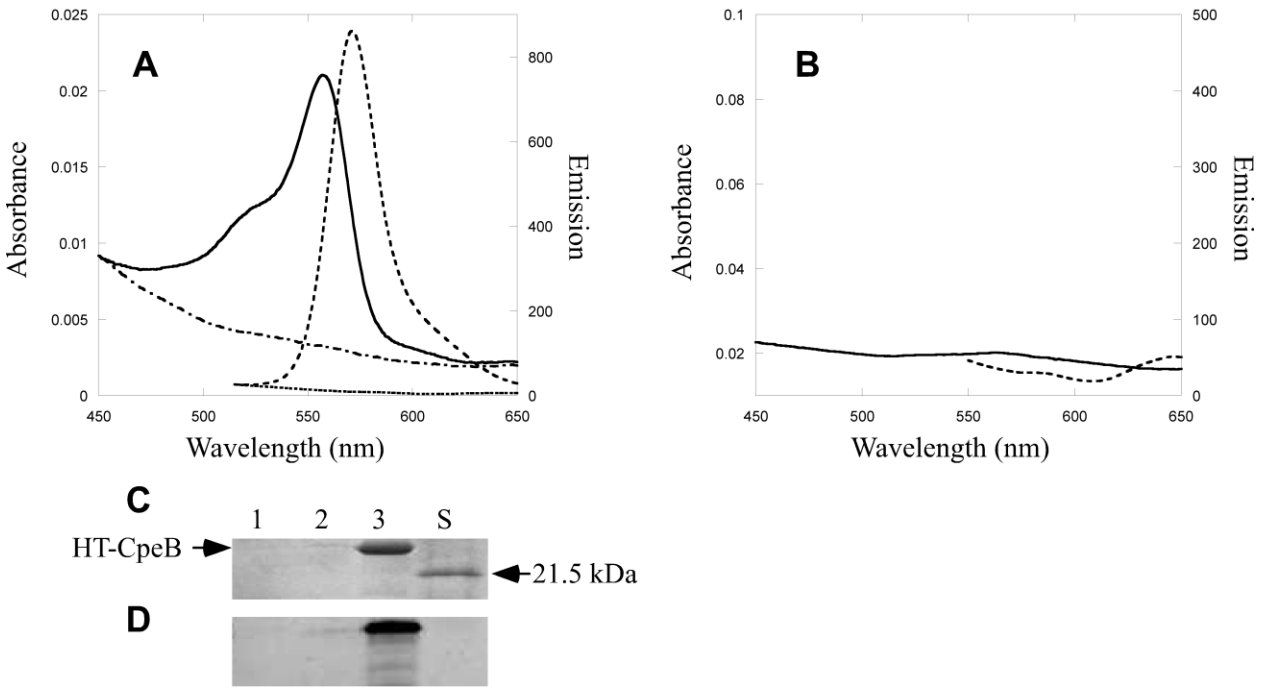

Fig. 39. **Analyses of the HT-CpeB (β-PE) produced in the presence of various lyases in *E. coli*. A**. Absorbance (solid line) and fluorescence emission (dashed line) spectra of HT-CpeB purified from cells containing pCpeB, pPebS with pCpeS and absorbance (dashed dotted line), fluorescence (dotted line) without pCpeS (no lyase) are shown. **B**. Absorbance (solid line) and fluorescence emission (dashed line) spectra of HT-CpeB purified from cells containing pCpeB, pPebS with pCpeYZ. In order to acquire the fluorescence emission spectra for the HT-CpeB produced in the presence of pCpeS (dashed lines in panel A), the sample was diluted to OD_{560nm} of 0.05; however, no dilution was performed on HT-CpeB produced in the absence of a lyase or with pCpeYZ (dotted lines in panels A and B). **C**. Coomassie-stained SDS polyacrylamide gel containing HT-CpeB purified from cells containing pCpeB, pPebS, and no lyase (lane 1) or from cells containing pCpeB pPebS, and pCpeYZ, (lane 2), or from cells containing pCpeB, pPebS, and pCpeS (lane 3). Molecular mass standards are loaded in lane "S", and masses are indicated to the right. **D**. The zinc-enhanced fluorescence of the gel pictured in panel C is shown here.

3.3.5. ***Analysis of specific Cys residue(s) of CpeB chromophorylated by CpeS in E. coli-*** To test the site specificity of the CpeS bilin lyase, site-specific variants of CpeB (C80S, C165S and C48S/C59S) were produced as substrates for CpeS involved in PEB chromophorylation. After coproduction of each site-specific variant with CpeS and the enzymes for PEB synthesis, the HT-CpeB product was purified, and the results of absorbance and fluorescence emission measurements are shown in Fig. 40. The C165S and C48S/C59S HT-CpeB variants had absorbance maxima at 560 nm and fluorescence emission maxima at 574 nm (Fig 40A and 5B), but the HT-CpeB (C80S) variant had no significant fluorescence emission (Fig. 40C). Control experiments without the CpeS lyase with all the CpeB variants were also performed, and in all cases no fluorescent product was observed (data not shown). The HT-CpeB produced in these experiments was also analyzed by SDS-PAGE. Bilin addition to each protein was examined by Zn-enhanced fluorescence of the gel (Fig. 40E). The HT-CpeB variants (C165S) and (C48S/C59S) exhibited fluorescence due to the presence of covalently attached PEB (Fig. 40D, lane 2 and 3), while the variant HT-CpeB (C80S) had no attached PEB (Fig. 40E, lane 1). After staining the same gel shown with Coomassie Blue (see Fig. 40E), one can see that only when PEB has been ligated (Fig. 40E, lane 2) does the HT-CpeB accumulate in a soluble form in *E. coli*. From these experiments, we concluded that the CpeS bilin lyase attaches PEB to Cys-80 of CpeB, but it does not play a significant role in PEB attachment to CpeA.

Comparing the total protein concentration and PEB content it was estimated the holo HT-CpeB (at Cys-80) was 66% chromophorylated when coproduced with CpeS. The total fluorescence product yield was 3.5 mg L^{-1} of culture. The fluorescence quantum yield in case of holo HT-CpeB (at Cys-80) was estimated to be ~0.89.

Maldi-Mass spectrometry(MS) and Tandem MS experiments on purified tryptic peptides from CpeS generated HT CpeB that Cys 80 is the addition site for CpeS on CpeB (tryptic peptide containing PEB was MAACLR) (Bhougatou, Cole unpublished data).

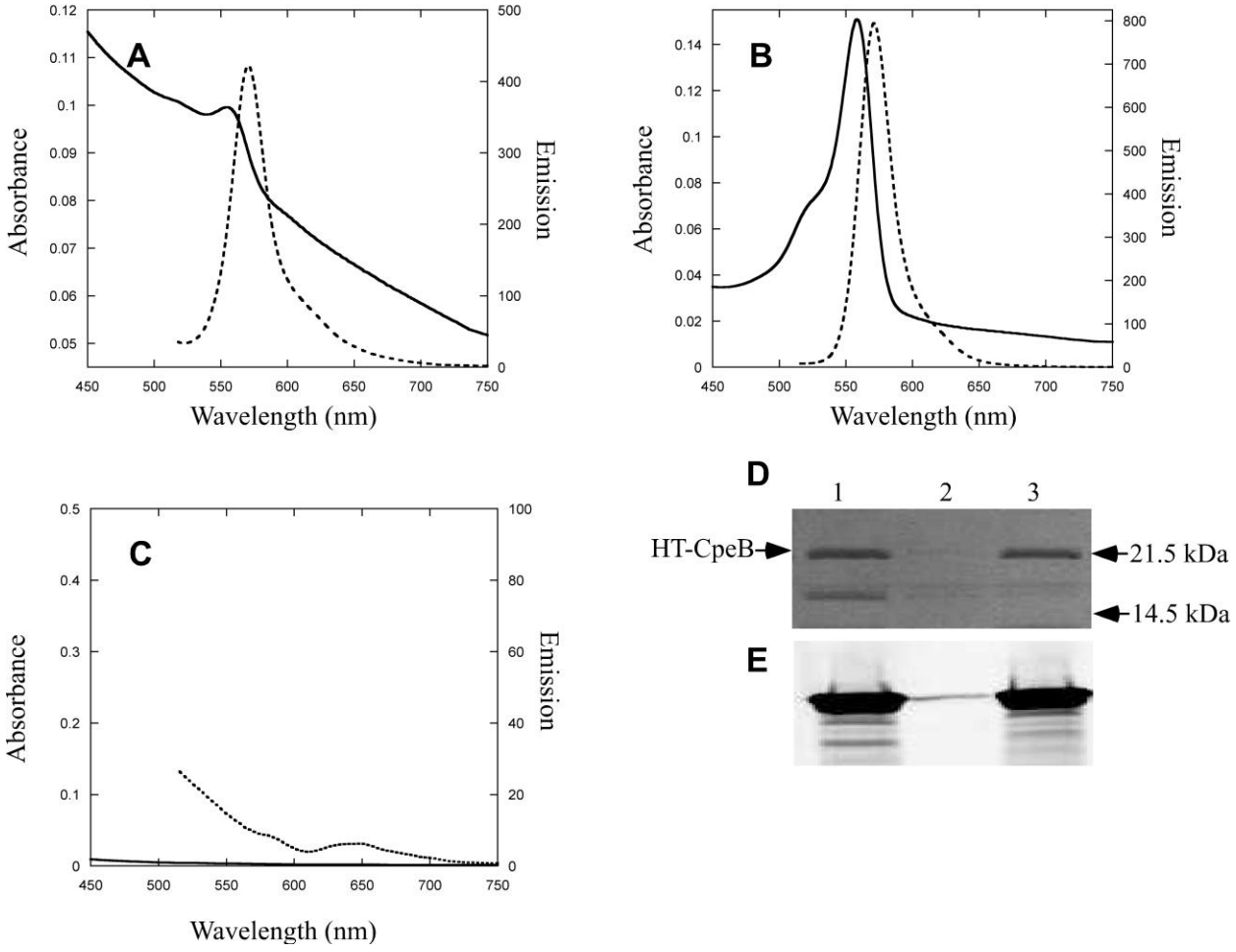

Fig. 40. Analyses of the specific cysteine residue on HT-CpeB required for PEB addition by CpeS: **A**. Absorbance (solid line) and fluorescence emission spectra (dotted line) of HT-CpeB obtained by coexpressing pCpeB(C165S), pCpeS, and pPebS. The samples have been diluted to OD of 0.05. **B**. Absorbance (solid black line) and fluorescence emission spectra (black dotted line) of HT-CpeB obtained by coexpressing pCpeB (C48S/C59S), pCpeS, and pPebS. **C**. Absorbance (solid line) and fluorescence emission spectra (dotted line) of HT-CpeB obtained by coexpressing pCpeB (C80S), pCpeS, pPebS. **D**. Coomassie-stained SDS polyacrylamide gel loaded with HT-CpeB purified from cells containing pCpeB (C48S/C59S), pPebS, and pCpeS (lane 1), pCpeB (C80S), pCpeS and pPebS (lane 2) or pCpeB (C165S), pPebS, pCpeS (lane 3). Molecular mass standards are loaded in lane "S", and masses are indicated to the right. **E**. The zinc-enhanced fluorescence of the gel pictured in panel D is shown here

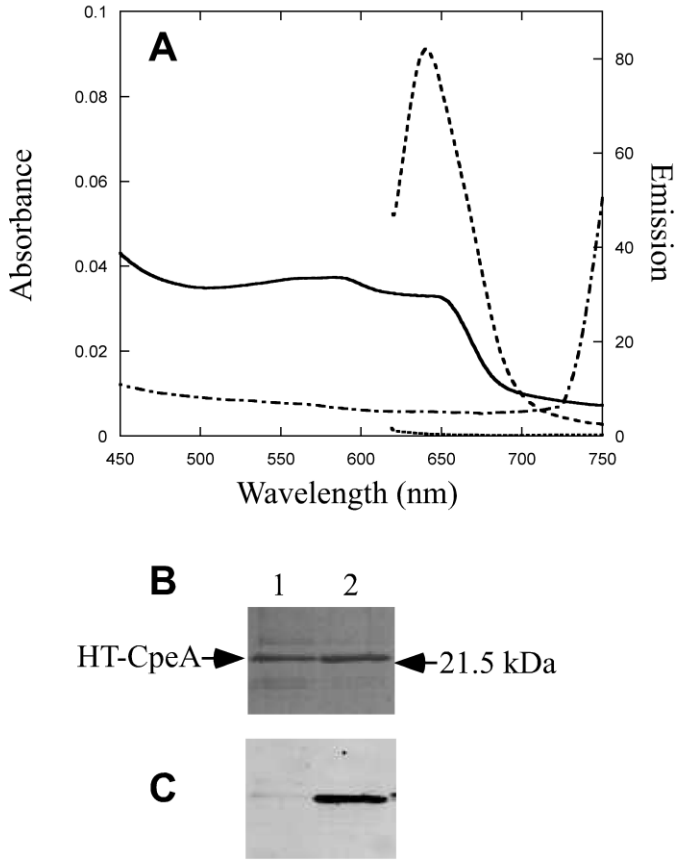

Fig. 41. Analyses of HT-CpeA-PCB produced in the presence of pPcyA and pCpeYZ A. Absorbance (solid line) and fluorescence emission (dashed line) spectra of HT-CpeA purified from cells containing pCpeA, pPcyA with pCpeYZ and absorbance (dashed dotted line), fluorescence (dotted line) without pCpeYZ are shown. **B**. Coomassie-stained SDS polyacrylamide gel containing HTCpeA purified from cells containing pCpeA, pPcyA (lane 1) and pCpeA, pPcyA, pCpeYZ (lane 2). Molecular mass standards are indicated to the right. **C**. The zinc-enhanced fluorescence of the gel pictured in panel B is shown here.

Table 8. **Comparison of spectral properties for various PE subunits produced with bilin lyases:**

Name of recombinant plasmids	λ_{max} *(nm)*($Q_{Vis/UV}$)	*Fluorescence Emission* λ_{max} *(nm)*
pCpeA + pCpeYZ[1]	566/410 (5.4)	573.5
pCpeA(C82S) + pCpeYZ[1]	NA	NA
pCpeA(C139S) + pCpeYZ[1]	566/410 (5.6)	574
pCpeA(C82, 139S) + pCpeYZ[1]	NA	NA
pCpeA + pCpeZ [1]	NA	NA
pCpeA + pCpeY [1]	566/410 (15.6)	573.5
pCpeA(C82S) + pCpeY [1]	NA	NA
pCpeA(C139S) + pCpeY [1]	566/410 (14.8)	574
pCpeA(C82,139S) + pCpeY[1]	NA	NA
pCpeA + pCpeS[1]	561/410 (0.315)	574
pCpeA(C82S) + pCpeS[1]	550/398 (0.6)	562
pCpeA(C139S) + pCpeS[1]	564/410 (0.6)	574
pCpeA(C82, 139S) + pCpeS[1]	NA	NA
pCpeB + pCpeS[1]	560/412 (5.2)	571
pCpeB(C80S) + pCpeS[1]	NA	NA
pCpeB(C165S) + pCpeS[1]	560/412 (5.4)	571
pCpeB(C48,59S) + pCpeS[1]	560/398 (5.3)	571
Native PE $(\alpha\beta)_6$ [3]	563/374 (9.5)	573

[1] Coexpressed with pPebS

[2] $Q_{Vis/UV}$ denoted the absorbance ratio of the visible and near- UV bands

2 Holo PE purified from *F. diplosiphon*.

Table 9. **Comparing Fluorescence Intensity for various recombinant holo α-PE:**

α-PE mutants *	% Fluorescence emission
CpeA+pCpeYZ	100
CpeA+pCpeY	60
CpeA+pCpeZ	0
CpeA+CpeS	0.8

* coexpressed with pPebS

3.4. *Mutants in cpeY and cpeZ genes are defective in phycoerythrin biosynthesis in Fremyella diplosiphon UTEX 481:*

To get a better idea of the roles of each lyase subunits "*in cyano*" a deletion mutant for *cpeY* and one for *cpeZ* was generated in *F.diplosiphon* in the Kehoe lab (Indiana University, Biology Department). This approach was taken to avoid polar effects that insertions of antibiotic resistant cartridges may cause. This was likely a problem in the transposon mutants generated by Kahn et al. (Kahn, Mazel et al. 1997) . Complete segregation of the knockouts was confirmed by PCR (data not shown). Both the wild type and the mutant cells were grown in white light. The wild type cells produced a large amount of PE, whereas as the *cpeY* and *cpeZ* mutant produced very little PE in comparison (See arrow in Fig. 42).

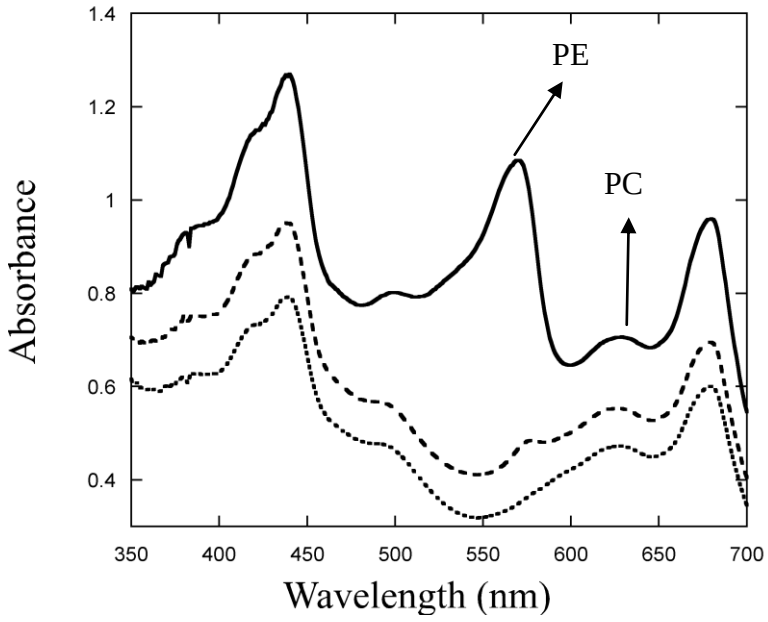

Fig. 42. Whole cell spectra from wild type and mutant cells. The spectra from whole cells grown under cool white light (which is enhanced green light) is shown. Cells from *Fremyella diplosiphon* wild type (solid line), the *cpeY* mutant (dotted line) and the *cpeZ* (dashed line) mutant are shown.

3.4.2. Chracterization of F. diplosphon cpeY mutant:

PE was purified from the wild type and the *cpeY* mutant and characterized using absorbance and fluorescence spectroscopy. Holo-PE from wild type cells had an absorbance maximum at 560 nm and a very high fluorescence emission maxima at 574 nm (See Fig. 43A the sample was diluted 15-fold to 0.05 OD_{560} prior to obtaining the fluorescence spectrum). The amount of PE obtained from the *cpeY* mutant was 20-fold less than in wild type, (See Fig. 43B; solid line; the *cpeY* mutant PE sample had to be concentrated 7-fold prior to obtaining the absorbance spectrum shown). The major absorbance peak was at 560 with a small peak between 620-630 nm, suggesting PCB may be present in this PE sample. For obtaining the fluorescence emission spectrum the sample was not diluted and excited at 490 nm initially to observe the presence of PEB (fluorescence emission at 574nm; Fig. 43B dashed line) and then the sample was excited at 590 nm for PCB (See Fig. 43B; dotted line). It is possible that the PCB absorbance could be due to contamination of AP or PC in the PE prep. To characterize this further, the PE from wild type and *cpeY* mutant were seperated on SDS-PAGE, the bilin addition on both α and β subunits of PE was observed by zinc staining the gel to enhance bilin fluorescence (described in Materials and Methods). The Zinc stained gel was visualized by excitation at 535 nm (which preferentially excites PEB over PCB) and at 635 nm which excites only PCB-containing phycobiliproteins (PBP) (See Fig. 43 C and D). Wild-type PE α and β subunits contain exclusively PEB as expected (See Fig. 43 C and D lane 1 and 2); the *cpeY* mutant has normal β PE but it has some PCB attached to the α-PE. No difference in migration was detected, indicating that both subunits contain covalently attached bilins (since lack of a bilin would shift the molecular weight by 0.5 kDa).

One explanation of the results of the cpeY mutant is that another lyase in the cells was able to ligate PCB to CpeA in the absence of CpeY. The most likely choice would be CpcEF, the lyase normally attaches PCB on CpcA. To examine if CpcEF can able to attach PCB on α-PE, heterologous coexpression system in *E. coli* was used.

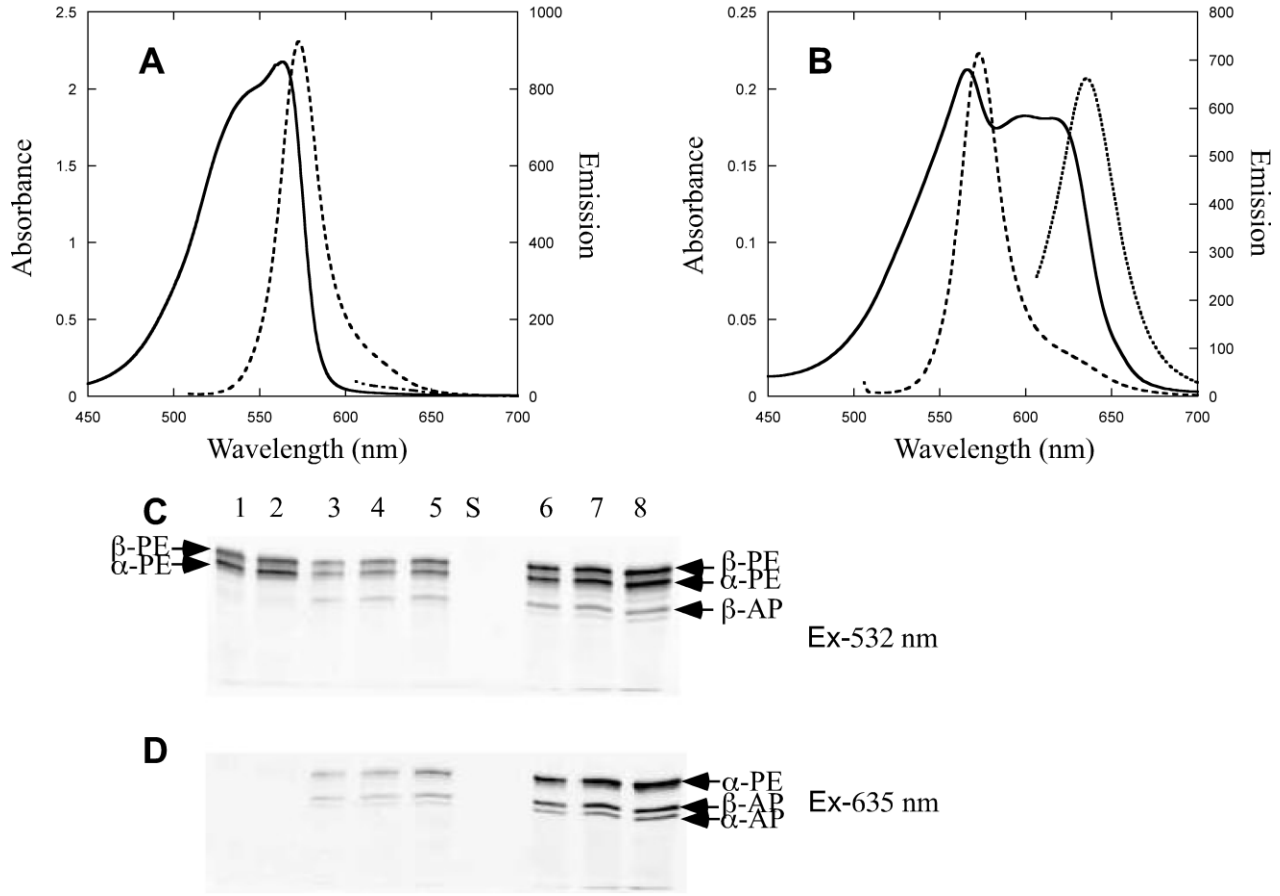

Fig. 43. Analysis of Phycoerythrin purified from wild type and the *cpeY* mutant cells: A. The absorbance (solid line) and florescence emission (dashed line) spectra of PE purified from wild type PCC 7601 is shown. The excitation wavelength used was 490 nm. **B**. The absorbance (solid line) and florescence emission spectra of PE purified from the *cpeY* mutant. The excitation wavelength used was 490 nm (dashed line) or 590 nm (dotted line). **C.** The Zn-stained SDS polyacrylamide gel shown with fluorescence excitation at 535 nm for purified PE from wild type (lane 1 and 2) lane 3-5 and lanes 6-8 represent the purified PE from the *cpeY* mutant. **D.** This is the same gel as above but with the fluorescence excitation at 635 nm to observe fluorescence from PCB.

3.4.3. PCC 6803 CpcEF lyase activity on PCB ligation on CpeA:

The pCpeA, pCpcEF, pPcyA were transformed into *E.coli* and was induced with IPTG for protein production. The purified HT-CpeA obtained (See Materials and Methods section) was characterized using absorbance and fluorescence emission spectrocopy. *E.coli* cells containing pCpeA/pPcyA has no significant absorbance or fluorescence emission spectra (data not shown), whereas cells containing pCpeA/pCpcEF/pPcyA was shown to have an absorbance and fluorescence emission maxima at 625 nm and 640 nm, respectively which corresponds to native PCB bound phycobiliproteins (See Fig. 44A; sample was concentrated 4 fold to obtain the spectrum).

The HT-CpeA samples purified from *E.coli* cells were analyzed by SDS-PAGE (Fig 44B and C). The bilin addition to CpeA was examined by zinc staining of the gel to enhance bilin fluorescence (described in Materials and Methods; Biswas et al.) as shown in Fig 44C; protein content was shown by subsequent staining of the same gel with Coomassie Blue (Fig. 44B). Cells containing pCpeA, pCpcEF and pPcyA showed Zn-enhanced fluorescent protein (Fig. 44C, lane 1; sample concentrated 4 fold) no fluorescent protein was seen in cells without CpcEF lyase (data not shown). CpcEF can ligate as bilin PCB on CpeA, suggesting this is how PCB is attached to CpeA in the *cpeY* mutant.

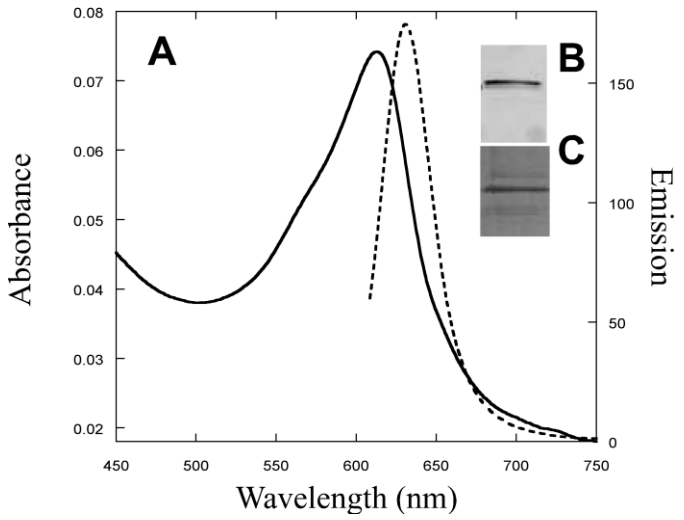

Fig. 44. PCB ligation on CpeA from *F. diplosiphon* PCC 7601 catalyzed by CpcEF from *Synechocystis sp.* PCC 6803: A. Represent the absorbance (solid line) and fluorescence emission (dotted line) spectrum from purified holo CpeA obtained by coexpressing pCpeA,pCpcEF,pPcyA. **B.** The Coomassie-stained SDS polyacrylamide gel for purified purified holo CpeA (CpeA-PCB). **C**. The Zinc enhanced fluorescence gel pictured for "B" was excited at 635 nm.

3.4.3. *Biochemical characterization of PE from the cpeZ mutant:*

Holo PE purified from the *cpeZ* mutant had an absorbance maximum of 560 nm (See Fig 45A solid line; sample was concentrated 7-fold). For obtaining the fluorescence emission spectrum, the sample was not diluted, excited at 490 nm initially to observe the presence of PEB (fluorescence emission at 574 nm; dashed line, Fig. 45A) and then at 590 nm to observe PCB (See Fig. 45B; dashed line and dotted line). No fluorescence emission from PCB attachment was observed (Data not shown).

PE purified from the *cpeZ* mutant was 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 45C and 45D; protein content was shown by subsequent staining with Coomassie Blue (Fig. 45B). Wild type PE showed that both α and β subunits contains exclusively PEB, as expected (See Fig. 45 C and D, lane 1 and 2). However, PE purified from the *cpeZ* mutant showed that while the α-PE contained PEB, there was very little chromophorylated β-PE present. In addition, we observed that both the α and β subunits of PE obtained from *cpeZ* mutant appeared to migrate faster in the SDS-polyacrylamide gel, suggesting their mass is smaller than those of the wild-type. Each subunit may be missing a ligated PEB chromophore (587 Da), which would account for the smaller mass observed. Samples have been sent to a Mass spectrometry facility (Indiana University) for analysis.

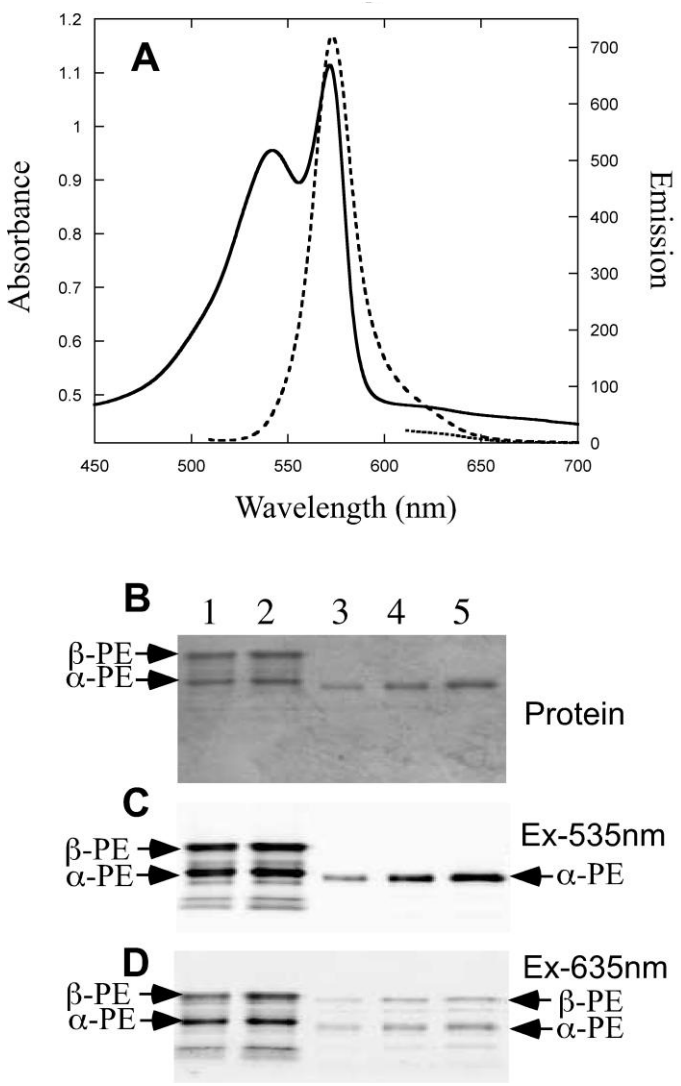

Fig 45. Analysis of PE purified from wild type and the *cpeZ* mutant cells. A. The absorbance (solid line) and florescence emission spectra of PE purified from the *cpeZ* mutant. The excitation wavelength used was 490 nm (dashed line) or 590 nm (dotted line). **B.** The Coomassie-stained SDS polyacrylamide gel for purified PE from wild type (lane 1 and 2) or the *cpeZ* mutant cells. Lanes 3-5 were loaded with different amounts of PE. **C.** The SDS polyacrylamide gel shown in panel B was incubated with Zn and then the fluorescence emitted after excitation at 535 nm is shown. **D.** This is the same gel as above but with the fluorescence excitation at 635 nm to observe fluorescence from PCB.

3.5. A *mpeZ* gene is involved in Type IV chromatic adaptation in marine *Synechococcus* cyanobacteria

Some of the marine *Synechococcus sp.* (WH 8020, WH 8103, and RS 9916) have a more complex phycobiliprotein structure (See Fig 19). They have two types of PE: PEI and PEII, each with multiple bilins (PEB or PUB) attached to the α and β subunits (See table 1). Some of these species (WH 8020, and RS 9916) can undergo Type IV CA replacing the chomophores. Everroad et al. (Everroad, Six et al. 2006) initially characterized the biochemical basis of Type IV CA in few *Synechococcus* species including in RS 9916. This group observed a change in chromophorylation on the α-PE-II subunit (MpeA). Under blue light, these sites are ligated with PUB, whereas under green light there was both PEB and PUB pigments attached. The phycobiliprotein themselves were same in both BL and GL. However, they were unable to identify the genes involved in the Type IV CA. Recently, Kehoe's group characterized the genes that are upregulated or down- regulated in *Synechococcus sp.* RS 9916 (Shukla et al. unpublished data) when shifted from GL to BL using a microarray. Based on the microarray data MpeZ was upregulated 3 fold in BL compared to green light. MpeZ shared sequence similarity to CpeY from *F. diplosiphon* (26%) (which is a lyase for α-PE, Biswas and Schluchter unpublished data) and 64% to CpeY from *Synechococcus sp* CC 9311 (Data not shown). However, MpeZ was not very similar to other E/F type lyase/ isomerase like RpcG from *Synechococcus sp.* RS 9916 (Blot, Wu et al. 2009) or *Nostoc sp.* PCC 7120 PecE/PecF (Zhao, Deng et al. 2000). These two E/F type lyase/ isomerases had a conserved 6- amino acid domain motif (NHCQGN) but this was not found in MpeZ (Blot, Wu et al. 2009) **.**

Based on this evidence we predicted that MpeZ might be a potential lyase for α-PEII (MpeA). I used a heterologous coexpression system in *E. coli* to test the MpeZ lyase/ isomerase activity on the PEII subunits.

3.5.1. *MpeZ is a novel phycoerythrin-II:phycoerythrobilin lyase-isomerase involved in type IV chromatic aclimation:*

Given the high differential expression level of *mpeZ* in blue relative to green light and its location in a gene cluster that is shared by all type IV chromatic adapters, it was hypothesized that the *mpeZ* gene could encode a PEII-PEB lyase-isomerase (Everroad, Six et al. 2006) . The Duet vector system was used to clone the *mpeZ* gene (pMpeZ, see Table 4), the *mpeA* gene (encoding the α-subunit of PE-II fused with a hexa-histidine tag; pMpeA, Table 4) and the bilin biosynthesis genes *ho1* (encoding heme oxygenase) and *pebS* (encoding phycoerythrobilin synthase; pPebS, Table 4). These plasmids were then co-transformed into *E. coli* BL21 DE3 cells. When only the pMpeA and pPebS plasmids were present (no MpeZ), there was very little soluble. HTMpeA produced, and this protein was non-fluorescent (Fig. 46A). However, when the pMpeZ plasmid was also present, the HT-MpeA produced had a slightly orange color with an absorbance maximum at 492 nm and a fluorescence emission maximum at 503 nm, indicative of PUB attachment. The purified HT-MpeA was separated on an SDS polyacrylamide gel, incubated in a Zn solution, which enhances the fluorescence of bilins attached to proteins as shown in Fig. 46C. Only the HT-MpeA made in the presence of the MpeZ protein contained a significant amount of covalently attached bilin that fluoresced after excitation of the gel at 488 nm (see lane 1 in Fig. 46C). After staining the gel with Coomassie Blue (Fig. 46B), it is apparent that there is less HT-MpeA purified in the absence of MpeZ (similar amounts of total (Blot, Wu et al. 2009)protein were loaded per lane; see lane 2, Fig. 46B); this is likely due to an increase in stability when the apo-protein is chromophorylated (Toole, Plank et al. 1998). Western blot was performed from the PEII purified from cells grown in both GL and BL using anti FD-CpeA, to confirm the presence of MpeA (data not shown), same way the recombinant partial holo MpeA was also confirmed

using western blot with the same antibody (data not shown). Although several phycocyanin lyase-isomerases have been described previously (Zhao, Deng et al. 2000; Blot, Wu et al. 2009), this is the first report of a phycobilin lyase-isomerase specific for a phycoerythrin.

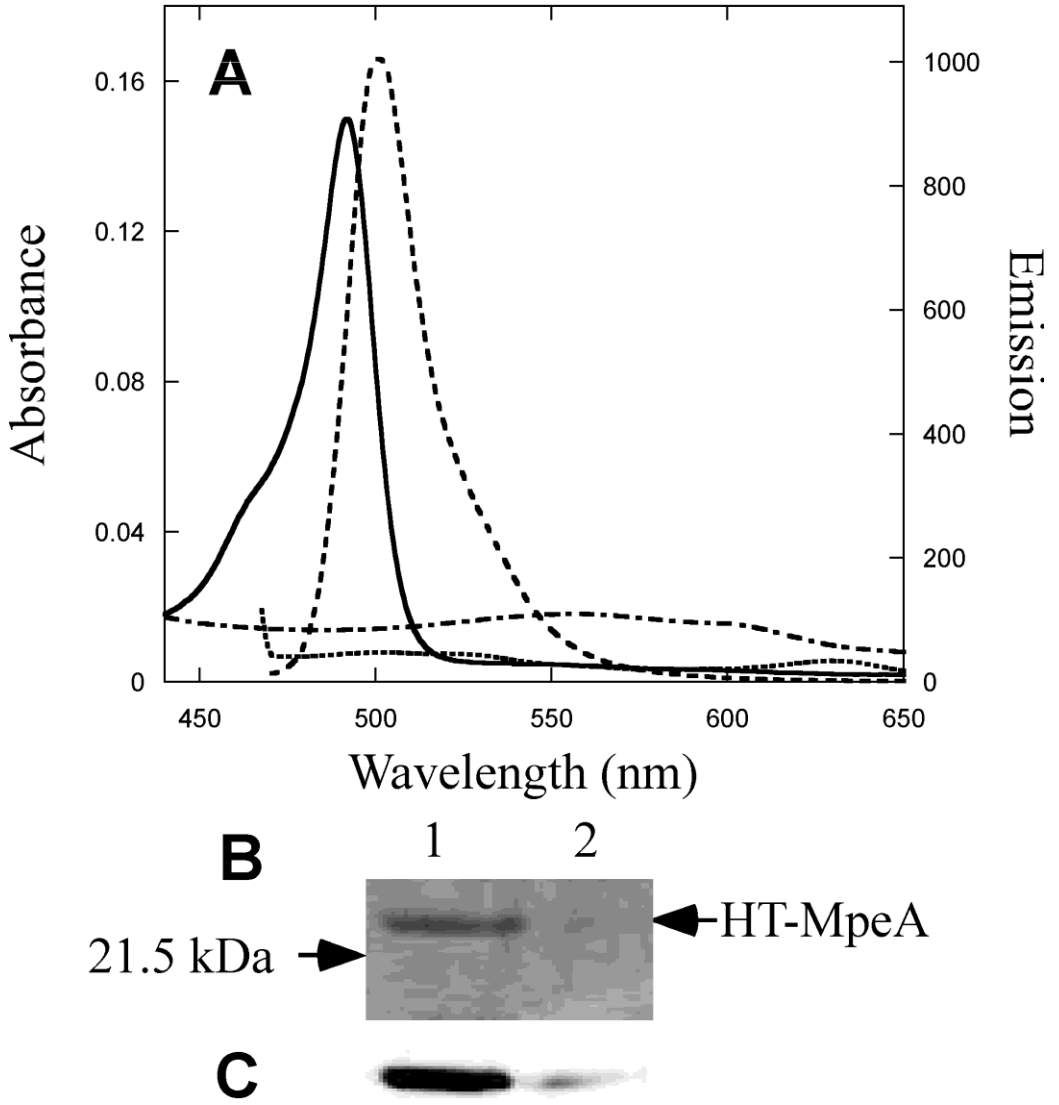

Fig. 46. **Analyses of MpeZ lyase for PEB addition to Phycoerythrin α subunit (PEII) in *E. coli*. A.** Absorbance (solid line) and fluorescence emission (dashed line) spectra of HT-MpeA purified from cells containing pMpeA, pPebS with pMpeZ and absorbance (dashed dotted line), fluorescence (dotted line) without pMpeZ are shown. In order to acquire the fluorescence emission spectra for the phycoerythrin α-subunit (PEII) produced in the presence of pMpeZ (black dashed lines in panels A), the sample was diluted to OD_{490nm} of 0.05; however, no dilution was performed on those produced in the absence of a lyase. **B.** Coomassie-stained SDS polyacrylamide gel containing HT-MpeA purified from cells containing pMpeA, pPebS with (lane 1) or without (lane 2) pMpeZ, Molecular mass standards are are indicated to the left with arrow heads. **C.** The zinc-enhanced fluorescence of the gel pictured in panel C is shown here.

3.2.2. *Specificity of MpeZ for cysteine residues on MpeA:*

In green light, MpeA has three Cys which contain bilins: PUB at Cys 75, PEB at Cys 83, and PEB at Cys 140 (Hammad, Shukla et al. 2011), whereas in blue light, PUB is located at all three positions. In order to determine which Cys was the preferred attachment site for MpeZ, site-directed mutants were created changing Cys to Ala at all three positions and in various combinations. These recombinant proteins were co-expressed in *E. coli* in the presence of PEB and MpeZ. The absorbance and fluorescence spectra for the purified HT-MpeA mutant proteins is shown in Fig. 47. The C75A, the C140A and the C75A/C140A MpeA proteins all contained PUB, as indicated by the strong absorbance at 492 nm and fluorescence emission at 503 nm (summarized in Table 10). When the C83A MpeA protein was purified, it had no absorbance or fluorescence emission (see Table 10); this indicates that MpeZ is a lyase isomerase specific for Cys-83 on MpeA. However, using our *in vivo* coexpression system in *E. coli* we were unable to show PUB chromophorylation on Cys-140 of MpeA.

Although there was no evidence that MpeB changes in GL-BL, it was also important to check whether MpeB was a potential substrate for the MpeZ lyase-isomerase. The pMpeB, pMpeZ and pPebS plasmids were co-transformed into *E. coli* and cultured as described. The HT-MpeB produced in the presence of the MpeZ protein and PEB was non-fluorescent, indicating that MpeB was not a potential substrate for MpeZ (see Table 10).

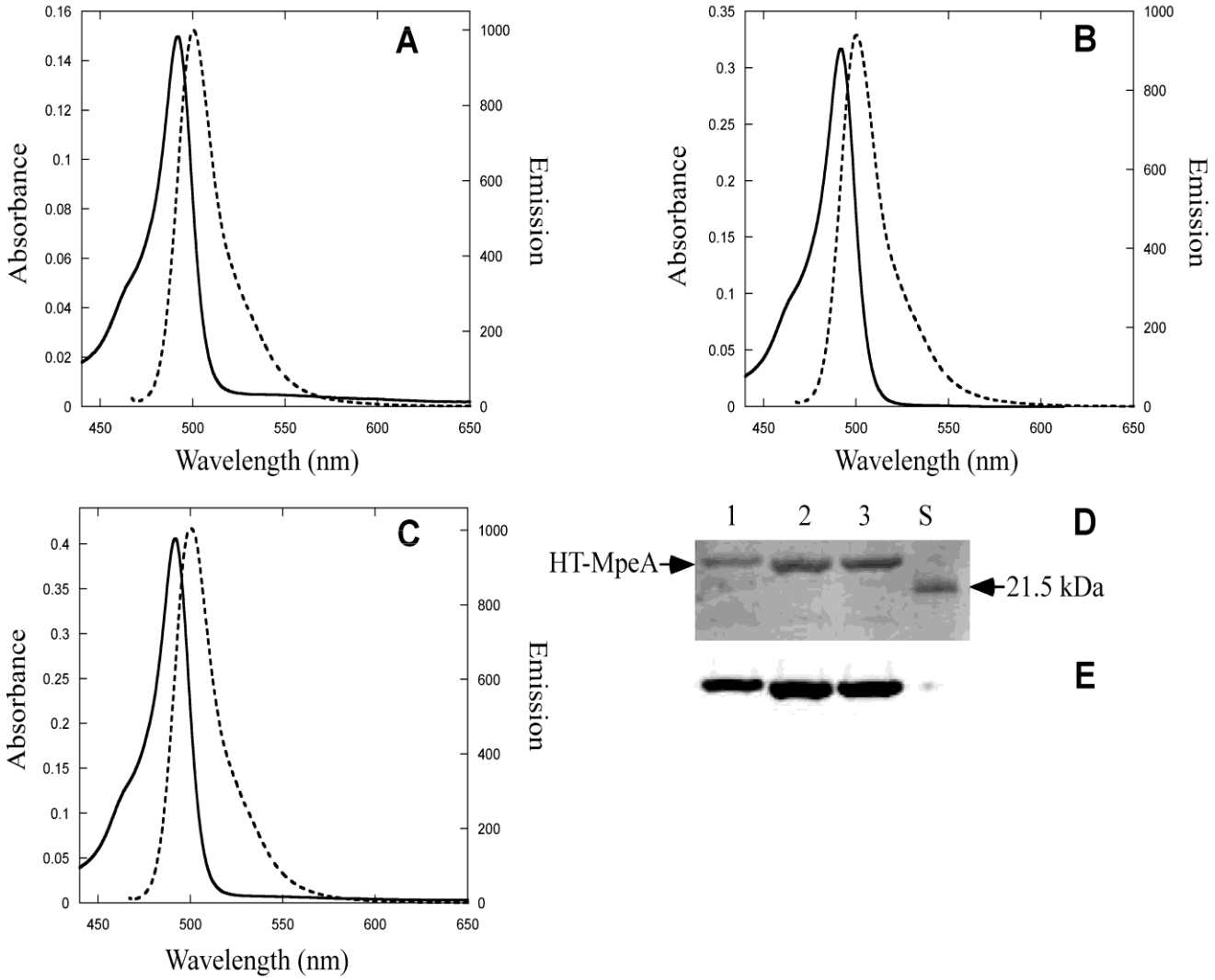

Fig. 47. **Site directed mutant analyses of specific cysteine residue for PEB addition to α-subunit PEII by MpeZ. A.** Absorbance (solid line) and fluorescence emission (dashed line) spectra of HT-MpeA (C75A) purified from cells containing MpeA(C75A), pPebS with pMpeZ is shown. **B.** Absorbance (solid line) and fluorescence emission (dashed line) spectra of HT-MpeA(C140A) purified from cells containing MpeA(C140A), pPebS and pMpeZ is shown. **C.** Absorbance (solid line) and fluorescence emission (dashed line) spectra of HT-MpeA(C75,140A) purified from cells containing MpeA(C75,140A), pPebS and pMpeZ is shown. In order to acquire the fluorescence emission spectra for the mutant holo MpeA The samples are diluted to OD_{490nm} of 0.05 (panel A, B and C; dashed lines). **D.** Coomassie-stained SDS polyacrylamide gel containing HT-MpeA mutants purified from cells containing MpeA (C75A), pPebS, pMpeZ with (lane 1) or (lane 2) CpeA (C140A), pPebS, pMpeZ and (lane 3) purified from the cells containing CpeA (C75,140A), pPebS, pMpeZ. Molecular mass standards are loaded in lane "S", and masses are indicated to the right. **E**. The zinc-enhanced fluorescence of the gel pictured in panel C is shown here.

Table 10: **Comparison of Fluorescence Emission for Recombinant PEII subunits**

Name of recombinant plasmid[a]	% Fluorescence emission[b]	Fluorescence Emission λ_{max}(nm)
pMpeA	100	503
pMpeA(C83A)	0	NA[c]
pMpeA(C75A)	106	503
pMpeA(C140A)	104	503
pMpeA(C75A,C140A)	108	503
pMpeA(C83A,C140A)	0	NA[c]
pMpeA(C75A, C83A,C140A)	0	NA[c]
pMpeB	0	NA[c]

[a] Coexpressed with pMpeZ and pPebS

[b] Fluorescence emission for the wild-type MpeA was set to 100% (pMpeA). All proteins were diluted to 0.05 OD at 492nm before determining the relative fluorescence emission.

[c] Not applicable

3.5.3. *Analyses of lyase activity on CpeA from RS 9916:*

Everroad el at suggested that α subunit of PEI (CpeA) may undergo changes in chromophore content during a shift from green light to blue light (Everroad, Six et al. 2006). So the next goal was to test MpeZ activity on CpeA, but no significant bilin ligation/isomerization was observed on MpeA (data not shown). Next, the CpeA clone was tested with two putative bilin lyases from other related species TE CpcS from *T. elongatus* (See appendix for description) and with CpeS from *F. diplosiphon* (Secton 3.3). These two have been shown to ligate PEB at Cys-82 or equivalent position in different PBP subunits. The purpose of this experiment was to verify that Synechococcus sp RS 9916 was soluble and a substrate in this *E. coli* system

The Duet vector system was used to clone the lyases TE *cpcS* and Fd *cpeS* (pTE CpcS and pCpeS; See Table 4), the *cpeA* gene (encoding the α-subunit of PE-I fused with a hexa-histidine tag; p'CpeA (9916), Table 4) and the bilin biosynthesis genes *ho1* (encoding heme oxygenase) and *pebS* (encoding phycoerythrobilin synthase; pPebS, Table 4). These plasmids

were then co-transformed into *E. coli* BL21 DE3 cells. When only pCpeA (9916) and pPebS were present (no potential lyase), there was almost no solubility (Fig. 49B, lane 2). HT-CpeA had no significant fluorescent or absorbance peaks (Fig 48 A). However, when pCpeA plasmid was expressed along with either pTE CpcS or pCpeS, some chromophorylaed CpeA was produced with an absorbance maximum of 561 nm and fluorescence emission of 570 nm, indicative of PEB attachment (Fig. 48A and Fig. 49 A). The purified HT-CpeA from all the experiments were separated on SDS-PAGE incubated with Zn solution, which enhances the fluorescence of bilins attached to the protein (See Fig. 48C and Fig 49C). Only HT-CpeA made in presence of bilin lyases TE CpcS or Fd CpeS contained covalently attached bilin that fluoresced on the gel (See lane 1 and 2 in Fig. 48C and lane 2 in Fig. 49C).

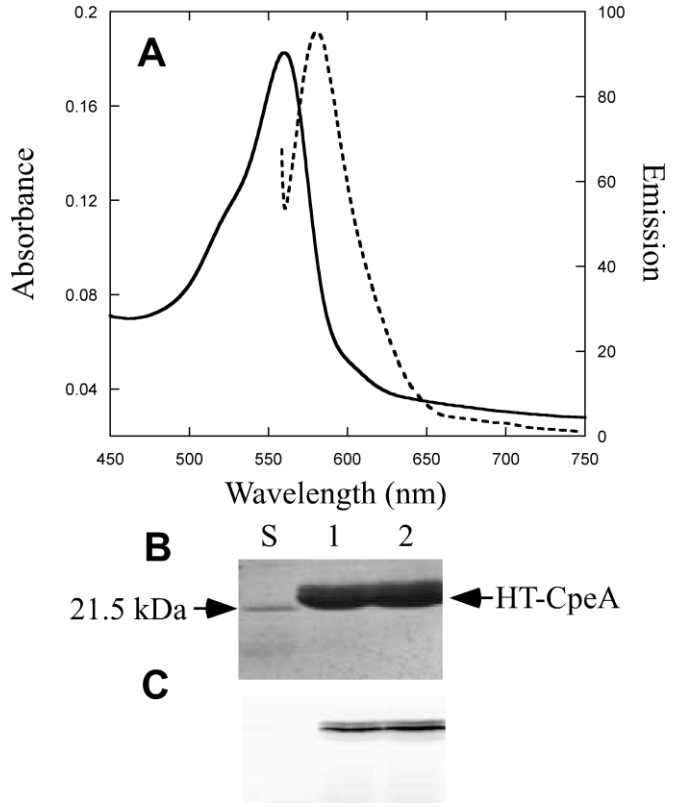

Fig. 48. **Analyzing TE CpcS lyase activity on RS 9916 CpeA chromophorylation.** A. Represent absorbance and fluorescence spectrum of holo CpeA purified from cells obtained by coexpressing CpeA/Ter13/PebS-Ho1, absorbance solid line and fluorescence dashed line. B. Coomassie-stained SDS polyacrylamide gel containing HT-CpeA purified from cells containing CpeA/ TECpcS/ PebS-Ho1 (lane 1 and lane 2) Molecular mass standards are loaded in lane "S", and masses are indicated to the right. **C**. The zinc-enhanced fluorescence of the gel pictured in panel B is shown here.

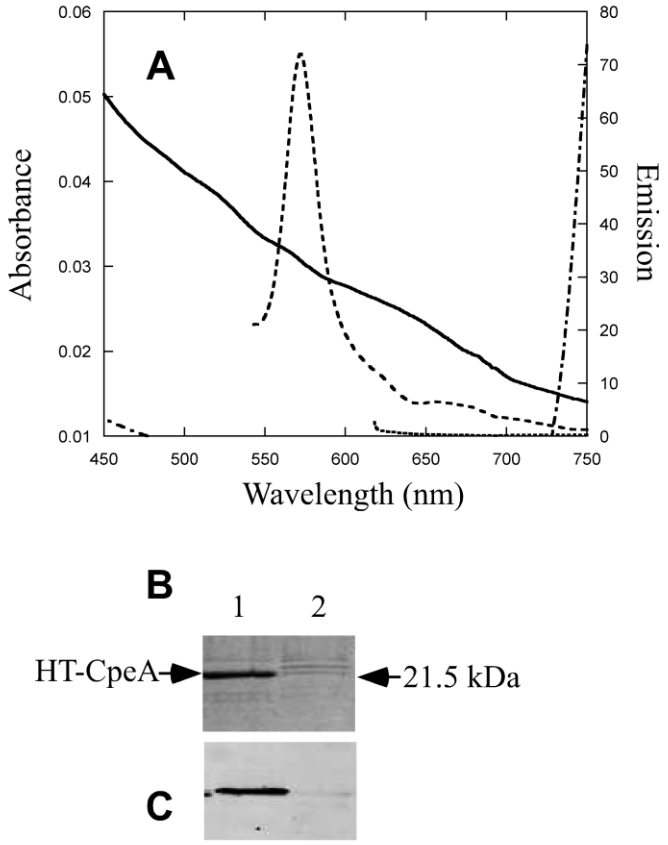

Fig. 49. Analyzing Fd CpeS lyase activity on RS 9916 CpeA chromophorylation. A. Represent absorbance and fluorescence spectrum of holo CpeA purified from cells obtained by coexpressing CpeA/PebS-Ho1 with and without CpeS, absorbance solid line and fluorescence dashed line obtained from cells containing CpeA/CpeS/PebS-Ho1. Absorbance dotted dashed line and fluorescence dotted line obtained from cells containing CpeA/PebS-Ho1. **B.** Coomassie-stained SDS polyacrylamide gel containing HT-CpeA purified from cells containing CpeA/ PebS-Ho1 with CpeS (lane 1) and without CpeS (lane 2) Molecular mass standards are indicated to the right. **C**. The zinc-enhanced fluorescence of the gel pictured in panel B is shown here.

4.0 DISCUSSIONS:

4.1. *Chromophorylation efficiency and specificity of all bilin lyases from Synechococcus sp. strain PCC 7002:*

In the studies reported here, an *in-vivo,* heterologous expression system was developed to determine the biosynthetic requirements for synthesis of holo-ApcD, ApcF and ApcE, to test the efficiency and specificity of various bilin lyases in chromophorylating substrates, and to produce large quantities of holo-PBPs that might be used as fluorescent probes. Others have used a similar approach to produce single subunits in *E. coli (Tooley, Cai et al. 2001; Tooley and Glazer 2002; Zhao, Su et al. 2007; Ge, Sun et al. 2009),* but the system described here produced the best yield of holo-PBPs in *E. coli* reported to date: 3 to 12 mg holo-PBP l^{-1} of *E. coli* culture (compared to 0.86-1.0 mg l^{-1} for AP subunits (Yang, Ge et al. 2008; Ge, Sun et al. 2009). When the system of Tooley et al. (pAT101 encoding *Synechocystis* sp. strain PCC 6803 *ho1* and *pcyA*) (Tooley, Cai et al. 2001) was directly compared with the system (pPcyA encoding *Synechocystis* sp. strain PCC 6803 *ho1* and *Synechococcus* sp. strain PCC 7002 *pcyA*) described here for the production of PCB, 3.5-fold more PCB was achieved in the same culture volume using pPcyA (compared to pAT101). This could be due to higher solubility, activity, and/or expression level of PcyA from *Synechococcus* sp. strain PCC 7002. It may also be due to a difference in plasmid copy number or the T7 promoter activity levels for the Duet plasmid pPcyA construct (pAT101 uses the *trc* (*trp-lac*) promoter). The *Synechocystis* sp. strain PCC 6803 heme oxygenase was used in both constructs, and the heme oxygenase enzyme produced by the Duet vector containing just *ho1* (parent to the pPcyA vector) was assayed *in vitro* and was very active (Y. M. Vasquez and W. M. Schluchter, unpublished results). It is possible that because *Synechococcus* sp. strain PCC 7002

grows optimally at nearly the same temperature as *E. coli* (38-40°C), its enzymes may be more stable and achieve higher activities when expressed in *E. coli* than those from *Synechocystis* sp. strain PCC 6803, which has a lower optimum growth temperature. In fact, it may be possible to improve the PCB production levels if the *ho1* gene of *Synechococcus* sp. strain PCC 7002 is used to replace the in *Synechocystis* sp. strain PCC 6803 *ho1* gene.

It was also possible to produce large quantities (5 to 12.4 mg of holo-AP subunit l^{-1} of *E. coli* culture) of holo-HT-ApcA/ApcB, holo-HT-ApcF, and holo-HTApcD/ApcB in *E. coli*. The CpcS-I/CpcU bilin lyase was required for PCB addition to all of these proteins, but it appears that holo-α-subunits (HT-ApcA and HT-ApcD) are produced in slightly lower quantities than their holo-β-subunit counterparts (see Figs. 25B and 25D). When produced in *E. coli*, AP subunits have previously been reported to have different solubility levels (Zhao, Su et al. 2007; Miller, Leonard et al. 2008; Yang, Ge et al. 2008; Ge, Sun et al. 2009; Zhang, Guan et al. 2009). Similarly, apo-HT-ApcF was not produced at very high levels unless ApcA was co-expressed with it (A. Fletcher and W. M. Schluchter, unpublished results). However, HT-ApcF was stabilized by chromophorylation, which permitted high levels of production to be achieved in the absence of ApcA; up to ~12 mg of this PBP were produced per liter of *E. coli* culture. However, HT-ApcD levels (produced alone) were low whether it was produced in the apo- or holo- form (Fig. 25D). Other studies have reported that holo-ApcB can be produced in *E. coli* (0.86 mg l^{-1}) (Ge, Sun et al. 2009) and in *Streptomyces* sp. (38 mg l^{-1}) (Hou, Qin et al. 2006) and that holo-ApcA can be produced in *E. coli* (1 g l^{-1}) (Yang, Ge et al. 2008). Although one report claimed that holo-ApcA could be produced without the need for any bilin lyase (Hu, Lee et al. 2006), the results presented here and other studies show that either the heterologous CpcS-I/CpcU-type bilin lyase or the CpcS-III single subunit-type bilin lyase (also called CpeS1) by Zhao et al. (Zhao, Su

et al. 2006) is required for maximal and correct addition of PCB to ApcA (Zhao, Su et al. 2006; Zhao, Su et al. 2007; Saunée, Williams et al. 2008; Ge, Sun et al. 2009). Analyses of bilin lyase mutants also strongly support the conclusion that bilin lyases are essential for AP biogenesis (Shen, Schluchter et al. 2008). However, small amounts of AP subunits could be detected by immunoblotting in a *cpcSUT* triple mutant (Shen, Schluchter et al. 2008). This suggests that some autocatalytic bilin addition may occur in the absence of bilin lyases in cyanobacteria. In the studies reported here, neither CpcE/CpcF nor CpcT attached PCB to ApcA or ApcB (data not shown).

The synthesis of holo-HT-ApcD/ApcB produced a product with a sharp, red-shifted absorption peak at 672 nm and a fluorescence emission maximum at 675 nm, both characteristic of native allophycocyanin B ($\alpha\beta$) (Glazer and Bryant 1975; Lundell and Glazer 1981). In studies by Zhao et al. (Zhao, Su et al. 2007) that showed that the single-subunit bilin lyase CpcS-III from *Nostoc* sp. PCC 7120 was required for bilin addition to ApcD, the absorbance spectrum of the product had a broad peak centered at 650 nm, possibly due to poor solubility. In the system described here, holo-HT-ApcD had an absorbance maximum at 642 nm. Coproducing ApcD with its partner subunit, ApcB, apparently improved its solubility, which resulted in a chromoprotein with red-shifted absorbance and fluorescence emission maxima very similar to those of native allophycocyanin B (Glazer and Bryant 1975; Lundell and Glazer 1981). ApcD may be particularly useful as a fluorescent tag due to its far-red absorbance and fluorescence emission.

For the production of chromophorylated AP and PC subunits, it appears that a major limitation may be the folding and/or conformation of the PBP subunit. For example, the chromophorylation rates by the CpcS-I/CpcU bilin lyase for ApcA/ApcB are much higher than those for CpcB, and the only difference is the PBP substrate. Because both substrates were

expressed as the α and β subunits, and because of the overall structural similarity of the two proteins (Schirmer, Huber et al. 1986; Brejc, Ficner et al. 1995), presumably there is little difference in the accessibility of the Cys-82 chromophorylation sites.

As first reported by Zhao et al (Zhao, Ping et al. 2005), the only PBP synthesized by *Synechococcus* sp. strain PCC 7002 that is capable of autocatalytic PCB attachment is ApcE. However, because of solubility problems with the truncation product of ApcE, Zhao et al. had to perform *in-vitro* bilin addition reactions with the PBP domain of ApcE (residues 1-240) in 4 M urea, which would not only affect the conformation of the protein substrate but also potentially of that of the PCB chromophore. The addition of detergents, such as Triton X-100, has been shown to affect chromophore conformation and to facilitate autocatalytic addition of bilins to PBPs in the absence of lyases (Zhao, Zhu et al. 2004). Therefore, a critical role for the bilin lyases is to bind the correct chromophore (in organisms producing more than one bilin) in the correct conformation to achieve the appropriate stereochemical attachment of the bilin to the apoprotein (Zhao, Zhu et al. 2004). At high bilin concentrations, that are probably never encountered inside cyanobacterial cells, autocatalytic attachment does occur *in vitro*, but the products of these reactions do not produce the naturally occurring holo-proteins (Arciero, Bryant et al. 1988; Fairchild and Glazer 1994; Scheer and Zhao 2008). For all of these reasons, it was important to show that a soluble form of ApcE, in the absence of detergents or urea, had an intrinsic bilin lyase activity.

The amino-terminal domain of ApcE, including amino acids 1-228, contains an AP-like domain that interacts with the AP-β-like subunit, ApcF, within an AP-trimer-like disc of the PBS core (Fig. 49). This domain probably has a largely alpha-helical, globin-like fold that is similar to

the structures of other AP and PC subunits (Brejc, Ficner et al. 1995). This AP-like domain of ApcE is interrupted by an insertion of 50 amino acid residues called the PB loop (Ajlani and Vernotte 1998), and this insertion is readily apparent when one examines an alignment of the amino-terminal domain with other AP subunits (Fig. 24). Because no other PBP subunit can efficiently autocatalytically ligate PCB to Cys residues, it is possible that this insertion may include residues that are important in the autocatalytic PCB ligation activity of ApcE. However, Ajlani and Vernotte showed that a deletion of this PB loop within ApcE did not affect its ability to attach a PCB chromophore (Ajlani and Vernotte 1998). Because the chromophore on ApcE is bound to the opposing helix of the binding pocket relative to every other biliprotein, the requirements for binding may be modified so that autocatalytic binding is possible. ApcE (residues 1-200) from *Synechococcus* sp. strain PCC 7002 and *Synechocystis* sp. strain PCC 6803 were aligned with the bilin lyase containing domains of Cph1 and Cph2, two cyanobacteriochromes (Wu and Lagarias 2000). One small motif that is conserved among these four proteins is the sequence DXXLE, corresponding to amino acids 32-36, in ApcE from *Synechococcus* sp. strain PCC 7002 and 185-189 in Cph1 or Slr0473 (see Fig. 50). The glutamic acid at the end of this region (underlined in the sequence above) is the one that was shown by mutagenesis to have a function in bilin binding in Cph1 (Wu and Lagarias 2000). The possibility that E36 in ApcE is also responsible for PCB binding will be tested in future studies.

To summarize this portion of work, it has now been completely defined how each PBP in *Synechococcus* sp. strain PCC 7002 is biosynthesized and has been established how one member of each of the four families of bilin lyases is responsible for PCB addition to the eight, discrete chromophore-binding sites on the seven different PBP subunits (Table 6) (Fairchild, Zhao et al. 1992; Fairchild and Glazer 1994; Shen, Saunee et al. 2006; Saunée, Williams et al. 2008; Shen,

Leonard et al. 2008). A heterologous, *in-vivo* expression system using PcyA from *Synechococcus* sp. strain PCC 7002 and Ho1 from *Synechocystis* sp. strain PCC 6803 appears to be very efficient and generates large amounts of PCB in *E. coli*. The use of a multi-plasmid system allows one to test biosynthetic requirements of uncharacterized PBPs from other organisms rapidly, to produce partially chromophorylated biliproteins for studies of the order of post-translational modifications in *in-vitro* reactions, and to generate holo-PBPs for use as fluorescent probes for bioimaging.

Allophycocyanin subunit and phytochromes

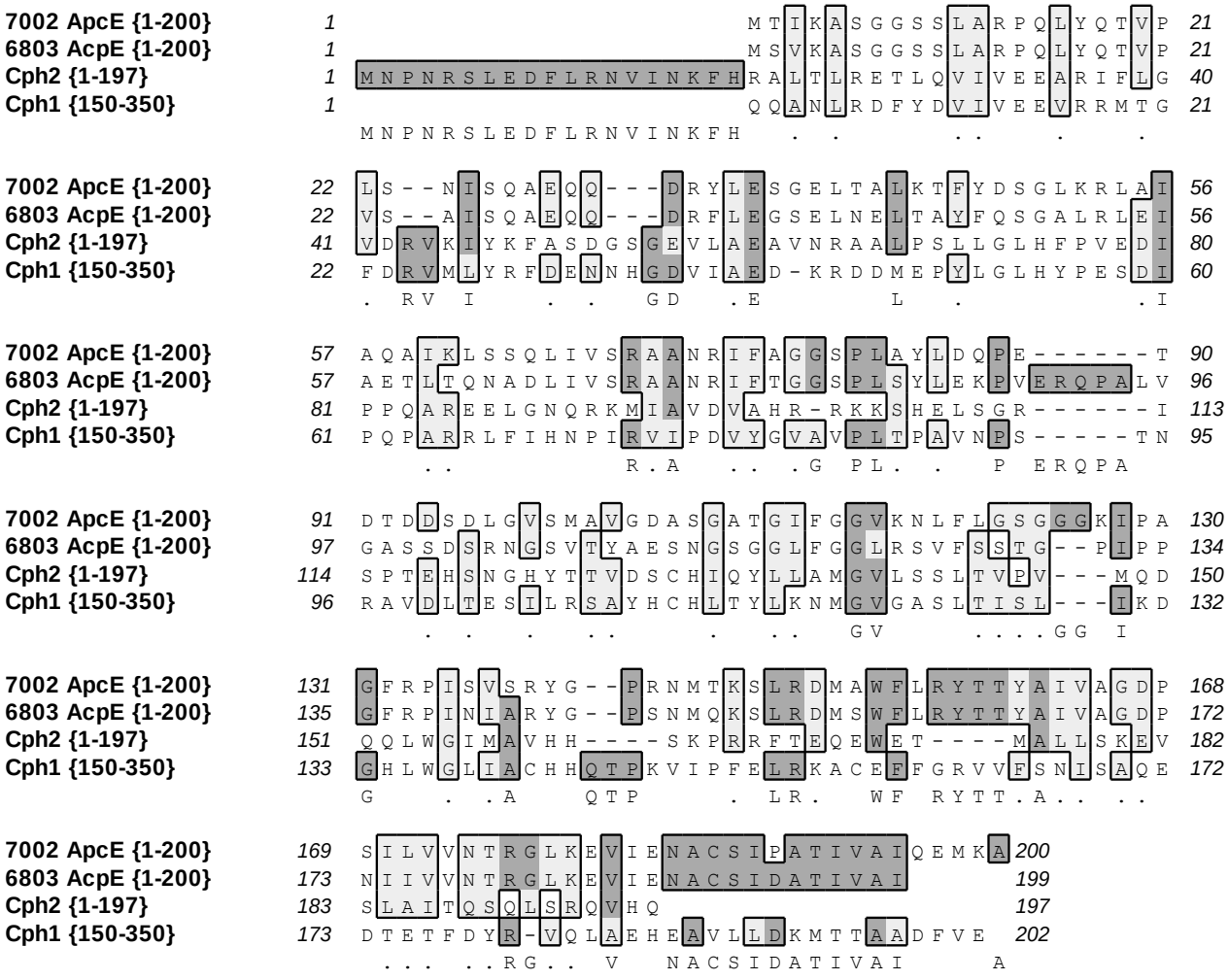

Fig. 50: Amino acid sequence alignment of *Synechococcus sp*. PCC 7002 ApcE (residues 1-200) with the sequences with *Synechocystis sp*. PCC 6803 ApcE (residues 1-200) and Cph2 and Cph1

4.2. *Creation of unique phycobiliproteins using PEB in E. coli for potential biotechnological applications:*

PBPs with PEB generally have higher quantum yields so the purpose of this project was to create unique phycobiliproteins using different bilin lyases and PEB in our *in vivo* heterologous system. Phycocyanin subunits are much more soluble in E. coli than PE subunits, and so CpcA and CpcB were tested for their ability to accept PEB in this system. The PebS enzyme (Dammeyer, Bagby et al. 2008), originally isolated from a phage that infects *Prochlorococcus* species produced larger amounts of PEB *in vivo* than did the construct containing *pebA* and *pebB* from *Synechococcus* WH8020 or from *Fremyella diplosiphon* (data not shown). The CpcE/CpcF lyase has been shown to favor PCB over PEB in terms of turnover rate (Fairchild and Glazer 1994), but in this system, CpcE/CpcF was extremely efficient in adding PEB to HT-CpcA. Greater levels of chromophorylation with PEB (83.5%) were achieved than with PCB (48.1%), but the expression cells containing pPebS were allowed to grow an additional 12-15 hours at 18°C (for optimal activity/folding of PebS) than cells containing pPcyA (30°C for 4 hr); this lower temperature may have been better for CpcE/CpcF folding, and the longer incubation time certainly allowed more time for PEB attachment. Therefore, this CpcEF class of bilin lyase is very suitable for creating unique phycobiliproteins (e.g. not found in nature) to be used as fluorescent labels. In fact CpcA was found to attach Phytochoromobilin (PφB) also (Alvey, Biswas et al. 2011). CpcA could also be recognized by PecEF, which ligated PCB and subsequently isomerized it to PVB (Alvey, Biswas et al. 2011).

The CpcS-I/CpcU and CpeS1 bilin lyases show broad PBP substrate specificity recognizing various AP subunits and β-PC as shown in the results presented here and in (Zhao, Su

et al. 2007; Saunée, Williams et al. 2008), but the CpcS-I/CpcU bilin lyase is quite specific for its bilin substrate, showing much lower activity when attaching PEB compared to the CpcE/CpcF bilin lyase. This makes sense when one thinks about the function of the Cys-82 on the β-subunit as the terminal energy acceptor within trimeric PC (See Fig. 3). In cyanobacterial cells that contain both PCB and PEB, a mistake by the CpcS or CpeS bilin lyase in attaching PEB at Cys-82 would mean that energy from PCB at β-Cys-153 or α-Cys-84 would not be transferred to PEB at β-Cys-82. Likewise, the intrinsic bilin lyase activity of ApcE also exhibited selectivity as much less activity was observed in the presence of PEB compared to PCB (data not shown). Therefore, three of the four known bilin lyase families (CpcS/U, CpcE/F, and autocatalytic ApcE) exhibit quite different specificities with respect to the bilin substrate. In the future, the CpcT bilin lyase will be tested for its ability to attach PEB to CpcB. Some of the *in vitro* preliminary data suggest CpcT can ligate PEB at Cys-153 on CpcB at very low levels compared to its activity with PCB (data not shown).

It was found that PEB is a better choice of bilin in the cyanobacterial family for real world applications since these have the highest quantum yield when ligated to CpcA (0.98), which is much greater than any other fluorophore available (See table 7). Also in this system CpcA was found to be the best choice of PBP subunit for creating fluorescent tag, because it is highly soluble, well characterized, has a very high yield, and can be ligated with numerous bilins (Alvey, Biswas et al. 2011).

In summary, unique phycobiliproteins not found naturally in *E. coli* systems have been successfully generated. This contruct may have potential biotechnological applications as an ideal fluorescent tag.

4.3. *Characterization of CpeY, CpeZ and CpeS bilin lyases involved in phycoerythrin biosynthesis in Fremyella diplosiphon strain UTEX 481*

This study compared the activities of three different proteins with similarity to bilin lyases on PE α and β subunit substrate derived from a filamentous cyanobacterium capable of Type III complementary chromatic adaptation, *F. diplosiphon*. Zhao et al. suggested that CpcS (also called CpeS1) from *Nostoc* sp. PCC 7120 had broad PBP substrate recognition and might attach all chromophores at position Cys-82 except for those of CpcA, PecA, and RpcA (Ritter, Hiller et al. 1999; Takano and Gusella 2002). However, because *Nostoc sp.* PCC 7120 does not synthesize PE or PEB, a more thorough examination of the substrate specificity of bilin lyases for PE subunits within one organism seemed necessary. The studies reported here showed that CpeY/CpeZ, and not CpeS, is the principal bilin lyase responsible for attachment of PEB at Cys-82 on CpeA. In fact, the PE produced in a *F. diplosiphon cpeY* mutant actually had PCB ligated at the Cys-82 position, and the likely candidate for this activity is CpcE and CpcF. CpeS performed poorly in attaching PCB to CpeA in this *E. coli* system (data not shown). Although CpeS could ligate PEB to Cys-82 on CpeA, a comparison of the yields obtained with CpeS and CpeY/CpeZ proteins in *E. coli* strongly suggested that the latter is more important in ligating PEB to CpeA *in vivo* (see Fig. 34). In addition, it was shown that CpeS could ligate PEB to Cys-139 of CpeA. However, based upon the very low levels of chromophorylation seen, it seems unlikely that this lyase is the one that catalyzes this reaction in cyanobacteria. Several other lyase candidates are currently being tested for PEB ligation at Cys-139.

At 429 amino acids, the CpeY protein is much larger than typical members of the E/F family. However, there are examples of larger lyases that belong to this family that have been

demonstrated to be involved in chromophore ligation and isomerization, such as RpcG, and it is likely these genes resulted from a fusion proteins (Blot, Wu et al. 2009). When the CpcE and CpcF sequences of *Synechocystis* sp. PCC 6803 were combined, the resulting CpcE/CpcF fusion protein aligned well with CpeY with 37% similarity (See Fig. 32). This could explain why CpeY has significant activity (60%) in the absence of CpeZ. Individual CpcE and CpcF subunits usually have low levels of ligation activity when assayed separately (Fairchild, Zhao et al. 1992; Fairchild and Glazer 1994). For example, compared to PecE/PecF together, PecE from *Mastigocladus laminosus* had only 10% PCB ligation activity on PecA (Bohm, Endres et al. 2007).

CpeZ (205 amino acids) is most similar to CpcE-like, HEAT-repeat proteins that are found in cyanobacteria and other bacteria that do not contain PBPs. All CpcE/CpcF-type bilin lyases contain 5-6 HEAT-repeat motifs; these motifs, which occur in many proteins from diverse eukaryotic organisms, are generally believed to facilitate protein-protein interactions (Andrade, Petosa et al. 2001; Takano and Gusella 2002). CpeZ increased the PEB ligation activity of CpeY, but no evidence for a stable interaction between CpeY and CpeZ was detected using pull-down assays (data not shown). Likewise, no demonstable interaction between HT-CpeA and either CpeY or CpeZ was observed (data not shown). The *cpeZ* mutant results suggest this protein has great importance for biosynthesis of CpeB and is less important for CpeA biosynthesis. The *cpeZ* cyanobacterial deletion mutant produces very little PE under green light. When we purified this PE, there was almost no chromophorylated CpeB present, but the CpeA produced in the mutant appeared normal. The CpeZ may play a chaperone-type role in assisting or regulating CpeA and CpeB's interaction with other bilin lyases (Collier and Grossman 1994; Baier, Lehmann et al. 2004; Bienert, Baier et al. 2006; Dines, Sendersky et al. 2007).

Similar to the results of Fairchild and Glazer (Fairchild and Glazer 1994) in which CpeA from *F. diplosiphon* was renatured from inclusion bodies, both CpeA (α-PE) and CpeB (β-PE) from *F. diplosiphon* were insoluble when expressed in apo-form in *E. coli* (data not shown). Co-expression of *cpeB* with *cpeA* did not increase the protein solubility as it often occurs with PC subunits (Biswas, Vasquez et al. 2010). However, chromophorylation at the Cys-82 equivalent position was obviously an important component in the solubility and accumulation of the folded proteins in *E. coli*. This observation suggests that chromophorylation at Cys-82 might be an important first step in PBP biosynthesis in cyanobacteria as well. Also, Zhao and Scheer (Scheer and Zhao 2008) looked at the order of chromophorylation in post-translational modification ; they suggested the T-type lyases first attach the bilin then the other lyases come into play. Here the observations were different; my results suggest that the S-type lyase needed to add the bilin on the central Cys residue, first in the order to make the subunits soluble, so that other lyases can function.

Bilin deletion mutants in PC (where Cys were mutated to Ala) in cyanobacteria showed lower stability *in vivo* (Toole, Plank et al. 1998). The absence of bilins at various positions reduces the strength of α/β interactions in the heterodimers, and the authors suggested that these mutants were diverted to a degradations pathway in cyanobateria (Anderson and Toole 1998; Toole, Plank et al. 1998).

Recently published data by Weithaus et al. (Wiethaus, Busch et al. 2010) showed that CpeS from *Prochlorococcus marinus* MED4 can ligate PEB on Cys-82 of β-PE, but this species is a little unusual in the sense it is devoid of regular phycobilisomes, and it lacks an α subunit; the function of β-PE in this species is unknown (Hess, Partensky et al. 1996; Steglich, Frankenberg-Dinkel et al. 2005). The CpeS bilin lyase in *F. diplosiphon* was found to be specific

in attachment of PEB to Cys-80 on β-PE. This is the first report to characterize the CpeS- type lyase from cyanobacteria containing PE in their phycobilisome rods. The CpeS described here is 42 % similar to that from *Prochlorococcus marinus* MED4. Also, CpeS activity was examined on CpeA as well. My results suggest that it is not the cognate lyase for CpeA, but it can act as a non-specific lyase on CpeA (as it had 1% of the activity of CpeY/CpeZ). The site-directed mutagenesis data showed that CpeS attached PEB to both Cys-82 and Cys-139 on CpeA (Fig. 43), whereas Zhao et al. showed that the *Nostoc* CpcS could chromophorylate only Cys-82 of CpeA. The data presented here tested the lyase for the CpeA and CpeB subunits all from *F. diplosiphon* whereas Zhao et al. (Zhao, Su et al. 2007) used a lyase known as CpcS from an organism (Nostoc) that does not produce PE. *F. diplosiphon* CpeS was also substrate specific, ligating only PEB, not PCB (data not shown).

Why are different lyases needed for alpha vs beta subunits of PBPs at Cys-82? The EF type lyase (CpcE/CpcF) was found to be specific for α-PC (Fairchild, Zhao et al. 1992), however some require the isomerizing activity of the bilin lyases that only EF types have, like PecE/PecF and RpcG (Zhao, Deng et al. 2000; Storf, Parbel et al. 2001; Blot, Wu et al. 2009). The α-subunits of PC and PE tend to have more varied chromophore content in cyanobacteria. In addition these chromophores are the ones that transfer energy to the terminal acceptor bilin present in Cys-82 on β-subunits. This suggests that there is more flexibility of chromophore content here and that seems to be provided by the E/F type lyases, some of which evolved the isomerase activity (One of them is described in Section 3.5).

The fluorescence quantum yield of HT- CpeA (Cys 82) and HT-CpeB (Cys 80) was 0.72 and 0.89 respectively, which is much higher compared to that of the best mutants of green fluorescent protein (GFP), which was 0.60 (Tsein 1998) (See Table 2). This property might be

useful in developing these as fluorescent tags. The disadvantage of using PBPs is the need to express the genes for lyases and bilin biosynthesis together; GFP is autocatalytic. The higher quantum yield and high chromophorylated product yield of CpeA might overcome the drawbacks of PBP compared to GFP and may be useful in cell biology and other biological discipline. Also CpeYZ was not as efficient in ligating the non-cognate bilin (PCB) (See Fig 44), whereas CpcEF can ligate both PCB and PEB efficiently (See section 3.2).

Finally there are lots of unresolved questions; left to be examined in the phycoerythrin biosynthesis, what the actual role of CpeZ? Is it a bilin lyase or does have other major role in PE biosynthesis/assembly? What specific bilin lyases can ligate PEB to the other cysteine residues like α-Cys-139, β-Cys-48, 59 and β-Cys-165?

4.4. *Mutants in cpeY and cpeZ genes are defective in phycoerythrin biosynthesis in Fremyella diplosiphon sp. UTEX 481*

My work showed that CpeY alone had 60% bilin binding activity compared to CpeY/CpeZ together (100%,. but, CpeZ alone had no significant activity (Section 3.3). Therefore to obtain more information about the roles of these two proteins, a reverse genetics approach was used. In both *cpeY* and *cpeZ* deletion mutants, a decrease in the growth rate in GL compared to the wild type was observed (data not shown), which corresponds with the earlier bilin lyases knock out studies (Swanson, Zhou et al. 1992; Shen, Saunee et al. 2006; Shen, Schluchter et al. 2008). This is due to very low PE content in GL, in these mutants, decreasing the amount of light absorbed for photosynthesis.

Khan et al. (Kahn, Mazel et al. 1997) suggested that CpeY and CpeZ are involved in the biogenesis of PE, but they could not determine which subunit was the likely target. Their

transposon mutants may have had polar effects. In this report the knockouts for *cpeY* and *cpeZ* were clean deletions, and the effect of each missing gene was examined.

It was observed that although CpeY is a specific lyase for α-PE (CpeA), the *cpeY* knockout mutant in cyanobacteria was only affected at on CpeA presumably at Cys-82. The small amount of PE made contained PCB at this position, not the normal PEB. Jung et al showed that in *pecE*/*pecF* knockouts, PCB was attached to PecA instead of PVB that is normally present (Jung, Chan et al. 1995). The possible explanation for this phenomenon observed in the *cpeY* mutant in *F. diplosiphon* might be that the CpcEF lyase that can ligate PCB on CpeA at very low levels (See Fig. 44), since this activity was observed in the *E. coli* system. Although CpeA in the *cpeY* mutants contains PCB, the amount of PE is much lower than seen in the wild type. Apo-CpcA is soluble and can be incorporated a low amounts into PBS in a *cpcE* lyase mutant, but perhaps missing PEB at Cys-82 on CpeA cannot fold properly, and hence it cannot accumulate. Further studies using Mass spectrometry will be performed to get a clearer picture of which site on each subunit of PE is affected in each mutant.

The real function of CpeZ is currently out of the scope of this thesis, but two different results were obtained in the case of *in vivo* coexpression experiments compared to the reverse genetics approach used here. In the case of *in vivo* coexpression experiments in *E. coli* no significant function was detected for CpeZ except that it increased the bilin ligation activity of CpeY on Cys-82 on CpeA (See Section 3.3). However, purified PE from the *cpeZ* mutant from *F. diplosiphon*, had a reduction of β-PE and probably loss of bilin on α-PE. These results suggested that CpeZ might have more of a function acting as a molecular chaperone during PE biosynthesis. Other lyase subunits have shown such an activity (Bohm, Endres et al. 2007) .

Bohm et al. (Bohm, Endres et al. 2007) mentioned that PecE acts as molecular chaperone, increasing the absorbance and fluorescence of PecA-PCB adducts. PecF is mostly needed for conversion from PCB to PVB. Their result coincides with our *cpeZ* mutant data, and CpeZ may act like PecE.

4.5. *The mpeZ is gene involved in Type IV chromatic adaptation in marine Synechococcus sp. RS 9916:*

When the *Synechococcus sp.* RS 9916 culture is shifted from GL to BL the PEB on Cys-83 and Cys-140 on MpeA get replaced with PUB (ASMS poster). During this shift, the only potential lyase/isomerase gene that gets upregulated is *mpeZ* (Shukla and Kehoe unpublished data). Using the hetrologous coexpression system in *E. coli* , the 414 amino acid MpeZ protein was shown to be a specific PEB lyase-isomerase for the PEII α subunit (MpeA), and that it was specific for Cys-83 of MpeA but not for Cys-140. One of the possible explanations for these results is that MpeA requires the central Cys-83 to be chromophorylated prior to ligating any bilin to other residues (like Cys-140). Once Cys-83 gets mutated and cannot act as a bilin ligation site, no bilin addition was observed in the recombinant system (data not shown). In this experiment, when the Cys-83 site directed MpeA recombinant protein was used as a substrate, no bilin addition by Mpe Z was observed (See Table 10). If ligation at Cys-83 is required for stability/folding followed by Cys-140 gets chromophorylation, then this would explain the data observed. Alternatively, it is possible that MpeZ interacts with and affects another lyase present in *Synechococcus sp.* RS 9916 (not present in our *E. coli* system) to isomerize PEB to PUB at Cys-140.

Earlier published data on the lyase-isomerase; PecE/PecF (Storf, Parbel et al. 2001) and RpcG (Blot, Wu et al. 2009), mentioned the presence of a six amino acid motif (NHCQGN) which is conserved in both PecF and RpcG (Zhao, Wu et al. 2005). These authors suggested that this motif might be responsible for isomerase activity. There is no indication that this motif is present in MpeZ. MpeZ has the most sequence similarity with CpeY (79% with CpeY from *F. diplosiphon*, CC 9311, and 68% with CpeY from WH 8102) (See Section 3.3). Although PecE/PecF and RpcG were designated as EF type lyase/ isomerases there is very little similiarity (16%) when each is compared to MpeZ. Everroad et al. (Everroad, Six et al. 2006) proposed that with the change of light conditions MpeA may change its chromophore content from 2:1, PEB: PUB; in GL to all PUB (0:3) in BL. This group also suggested that this Type IV CA could be controlled by only few lyase isomerase genes. In this report it was only possible to show that MpeZ is a lyase/isomerase enzyme for Type IV CA, ligating a PUB chromophore on Cys-83 of MpeA.

MpeA like other PBP subunits, is not soluble when expressed by itself, but once MpeA is chromophorylated with PUB at Cys-83, it accumulated in *E. coli* in a soluble form. This was also observed with CpeA and CpeB from *F. diplosiphon.*

Everroad et al also proposed that CpeA might be other subunit whose PUB content increases when shifted to BL, but MpeZ showed no activity on CpeA in the *E. coli* system. I was able to show that CpeA could be chromophorylated by other non-cognate lyases like TECpcS and *F. diplosiphon* CpeS, showing that CpeA from RS 9916 can serve as a substrate for other lyases in the *E. coli* system. Determination of cognate lyase or lyase/isomerase for CpeA is outside the scope of this thesis.

There are lots of remaining questions: Which lyase/isomerase gene is involved for Cys-140 on MpeA? Does CpeA also undergo Type IV CA, and if so what enzymes are involved? Samples for PEI and PEII purified in GL vs BL are currently being analyzed to try and determine whether chromophore content changes on each polypeptide in different light conditions.

5.0. Appendix

Characterization of bilin lyases have been done mostly on PC and APC subunits, but unfortunately there were no the X-ray crystal structures of the lyases are available. In 2007, Jon Hunt's group have solved the crystal structure of CpcS-III type lyase from *Thermosynechococcus elongatus* BPI (tl11696) as a part of a structural genomics project was made available in the protein database and named as Ycf58 (Kuzin, Su et al. 2007). In this thesis it is designated as TE-CpcS. The protein was crystallized as a dimer (See Fig. 51). The CpcS-III bilin lyase belongs to the lipocalin structural family of proteins. These proteins are composed of an 8-stranded anti-parallel, β-barrel structure (similar to GFP) with an α-helix; and can exist in different oligomeric states; monomers, homodimers, heterodimers, or tetramers, and they bind a diverse set of ligands including fatty acids, retinols, carotenoids, pheromones, prostaglandins, and biliverdin (Flower 1996; Bishop 2000; Hieber, Bugos et al. 2000; Newcomer and Ong 2000; Charron, Ouellet et al. 2005; Grzyb, Latowski et al. 2006). Kuzin et al. successfully co-crystallized TE CpcS along with biliverdin (Kuzin et al., unpublished results), but these crystals did not diffract well. For the bilin addition mechanism it seems as an initial step it needs to bind of the approproiate bilin before it was ligated to the apo-PBPs (Schluchter and Glazer 1999; Scheer and Zhao 2008). It was important to demonstrate that TE CpcS was a functional bilin lyase in *Thermosynechococcus sp.* contains AP and PC proteins with only PCB.

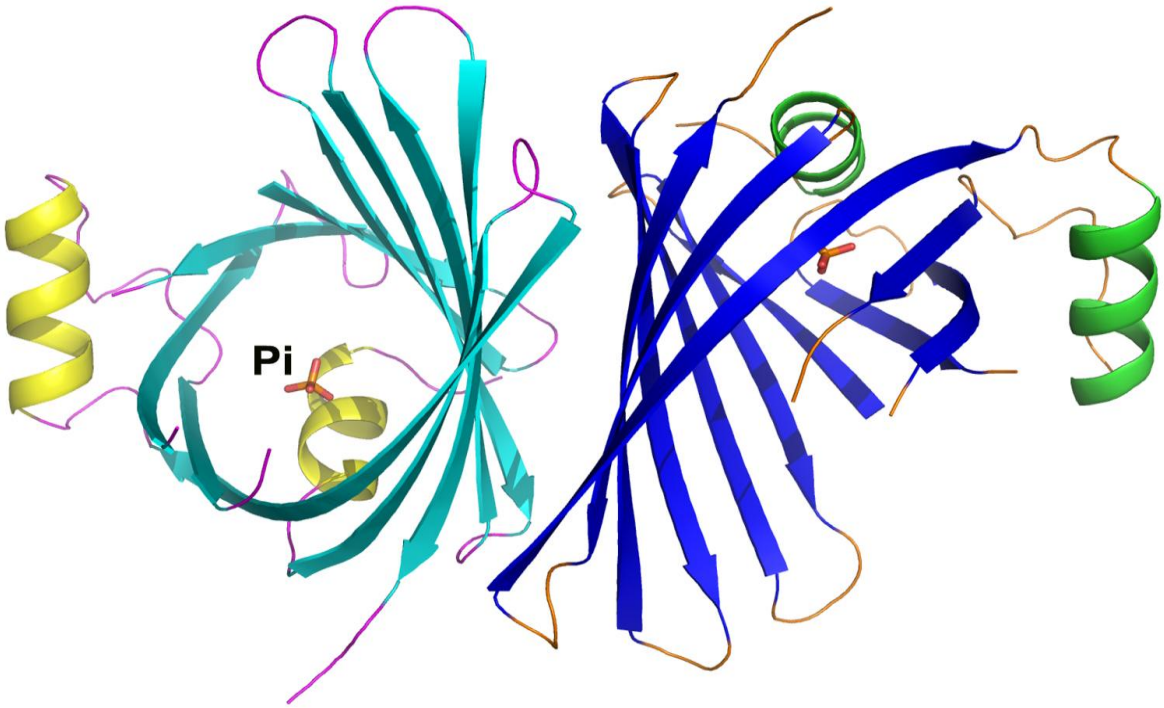

Fig. 51. Structure of Tlr 1699/ CpcS-III (Ter13) from *Thermosynechococcus elongates* BP-1 (PDB ID:3BDR). The structure of the homodimer that crystallized is shown here. The protein has an 8 stranded antiparallel β-barrel with an α-helix. A phosphate ion co-crystallized with the structure (Kuzin, Su et al. 2007).

5.1. ***Analysis of lyase activity of CpcS type lyase from Thermosynechococcus elongatus on phycocyanin subunit:***

The *cpcS* gene (*TE cpcS*) from *T. elongatus* is one of the first lyase whose crystal structure was resolved at 2.9 °A resolution (Fig 51) (Kuzin, Su et al. 2007). It shares sequence similarity with other known lyases CpcS (from *Nostoc sp*. PCC 7120), CpeS (from *F. diplosiphon*) and CpcSU (*Synechococcus sp*. PCC 7002). So it was important to test its lyase activity on various PBP subunits. Using the heterologous coexpression system in *E.coli the* purified protein obtained from the coexpression of cells containing pCpcBA/TECpcS/pPcyA (See Material and Methods) was analyzed using absorbance and fluorescence spectrum. Fig. 52 A shows the absorbance and flourescence spectra of PCB ligated CpcB at Cys 82. The blue solid line represents the absorbance spectrum and blue dotted line the fluorescence emission spectrum from the purified protein obtained by coexpressing with CpcBA/TECpcS/pPcyA. The black solid anddotted lines correspond to absorbance and fluorescence spectra, respectively for purified protein without TECpcS. To compare the lyase activity for TECpcS with the CpcS/CpcU lyase for chromophorylating CpcB at Cys 84, a coepxression was performed with CpcBA/CpcSU/pPcyA. In Fig. 52 A, the red solid line represents the absorbance spectrum and the dotted line represents the fluorescence emission spectrum for purified protein obtained by coexpressing pCpcBA/pCpcSU/pPcyA. The two spectra overlap (absorbance and fluorescence peaks from pCpcBA/TECpcS/pPcyA and pCpcBA/pCpcSU/pPcyA) and confirm that TECpcS acts a similar kind of lyase to CpcSU ligating PCB on β-PC (CpcB) (See Table 11).

All the purified HT-CpcBA proteins were separated by SDS-PAGE. Bilin addition was examined by Zinc enhanced fluorescence (Fig. 52C). In Fig. 52 C, lanes 1 and 2, (lane 3 also contains CpcBA purified from from coexpression of pCpcBA/TECpcS/pPcyA) show strong bilin

fluorescence indicating that both CpcSU and TE CpcS act as bilin lyase for CpcB (β-PC). The protein content was confirmed by staining the gel with Comassie blue stain (Fig. 52 B, lanes 1 and 2). This section concludes TE CpcS acts as an single subunit S-type lyase involved in bilin addition on Cys-84 of β-PC, and confirming that we now know that the first structure of a functional bilin lyase

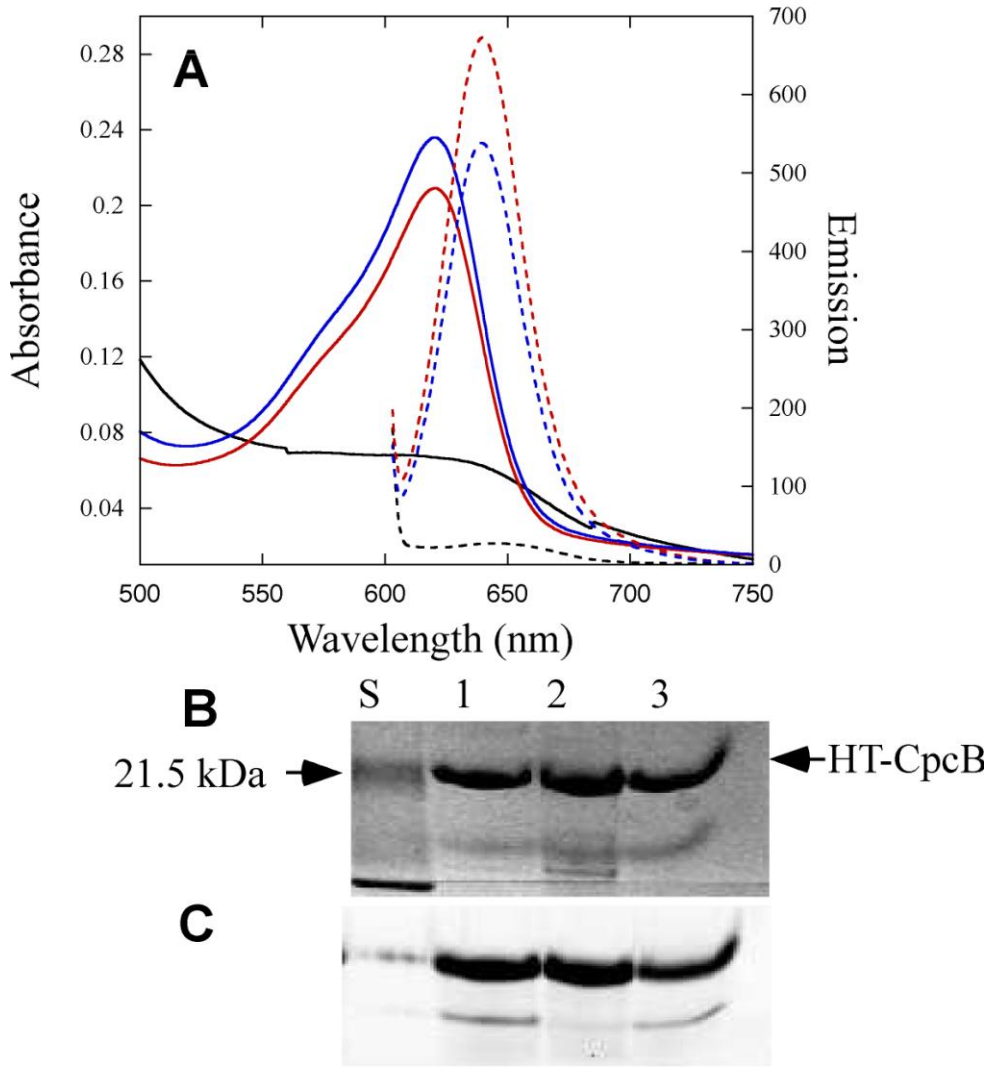

Fig. 52. Comparison of chromophorylation between CpcSU and TECpcS on β-PC. A. Abosrbance (solid) line fluorescence emission (dashed) spectra of HT-CpcBA purified from cells containing pCpcBA with TECpcS and pPcyA, absorbance (solid blue line) and fluorescence emission (blue dotted line) or pCpcBA with pCpcUS and pPcyA, absorbance (solid pink line) and fluorescence emission (pink dotted line) or pCpcBA with only pPcyA absorbance (black solid line) and fluorescence emission (black dotted line) **B.** Coomassie-stained SDS-polyacrylamide gel of HT-CpcBA purified from cells containing pCpcBA/TECpcS and pPcyA (lane 1), cells containing pCpcBA/pCpcUS and pPcyA (lane 2), lane 3 is same as lane only from different set of culture. Molecular mass standards were loaded in the lane marked "S"; the position of the 21.5-kDa mass standard is indicated. **C.** Zinc-enhanced bilin fluorescence of the gel in panel B.

5.2. *Analyzing lyase activity of TECpcS lyase from Thermosynechococcus elongatus on allophycocyanin subunit:*

The CpcSU type lyase chromophorylates all AP subunits at Cys-84 **(**Biswas, Vasquez et al. 2010). Here in this project the next project goal was to test TE CpcS bilin lyase activity on AP subunits (ApcA and ApcB). From a Biotechnology application stand point it was necessary to test TE CpcS lyase activity of different types of bilin substrate (PCB, PEB or PXB). The TE CpcS was tested to see if it could chromophorylate ApcAB with all three bilins; PCB, PEB, PfB. The pApcAB was coexpressed with TE CpcS and each of these three combinations (pPcyA/ho1 or pPebS/ho1 or phy2/ho1). The expressed proteins were purified using Metal affinity chromatography column. The purified proteins were analyzed using absorbance and fluorescence emission spectrum. In Fig. 53 A, B, and C the solid lines represent the absorbance, and the dotted lines represent fluorescence emission spectra (refer to the figure for color coding) of purified proteins from holo ApcAB ligated with PCB (blue) or PEB (red) or PfB (green). Each of the spectra show proper addition of PCB as predicted from earlier published data. This is the first report on PEB and PfB ligation on AP subunits (See Table 11). The purified proteins were separated on SDS-PAGE, stained with Zinc sulphate to confirm bilin addition on both α and β AP (ApcAB) was confirmed in Fig. 53E (lanes 1 through 3). For protein content the gel was stained with Comassie blue stain (Fig. 53 D). It was concluded that the newly identified novel CpcS type lyase; TECpcS had the ability to ligate PCB, PEB and PfB on both α and β AP (ApcAB). There was no significant bilin addition when no lyase was used in the all the three coexpressions (Data not shown).

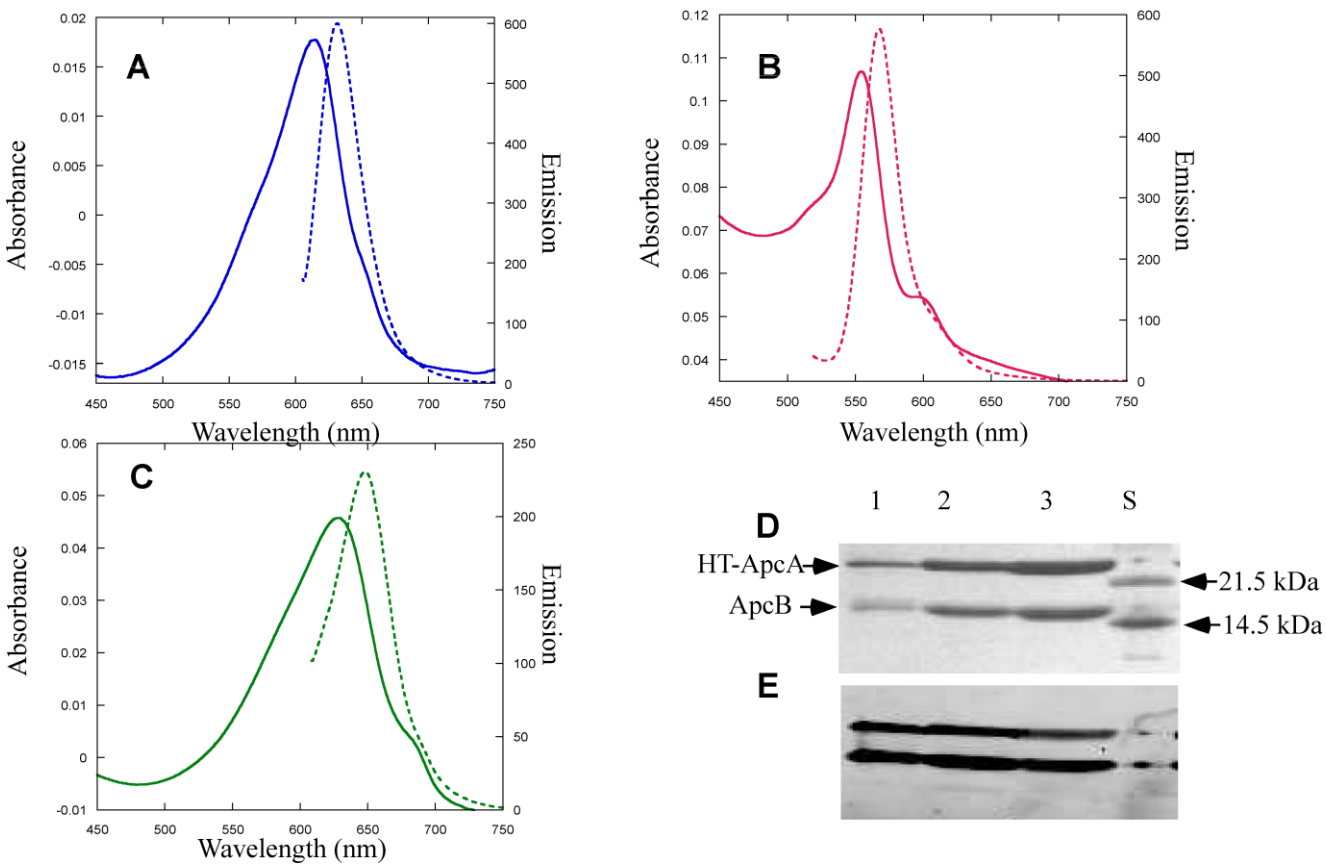

Fig. 53. Analysis of holo HT-ApcAB purified from *E.coli* cells chromophorylated by TECpcS. A. Abosrbance (solid blue) line fluorescence emission (blue dashed) spectra of HT-ApcAB purified from cells containing ApcAB/TECpcS and pPcyA, absorbance (blue-solid line) and fluorescence emission (bluedotted line). **B.** Absorbance (pink-solid) and fluorescence (pink-dotted) line from cells purified by coexpressing ApcAB/TECpcS with pPebS. **C.** Absorbance (green-solid) and fluorescence (green-dotted) line from cells purified by coexpressing ApcAB/TECpcS with pHy2. **D.** Coomassie-stained SDS-polyacrylamide gel of HT-ApcAB purified from cells containing ApcAB/TECpcS and pPcyA (lane 1), from cells containing ApcAB/TECpcS and pPebS (lane 2), from cells containing ApcAB/TECpcS, ho1/hy2 (lane 3). Lane S represent Molecular mass standards were indicated by arrows. **E.** Zinc-enhanced bilin fluorescence of the gel in panel D.

5.3. ***TE CpcS activity on AP α-like subunit ApcD:***

TE CpcS was coexpressed with ApcD with genes involved in formation of three bilins (PCB, PEB or PXB). The coexpressed cells were found to be colored (data not shown) indicative of covalent bilin ligation. The whole cells were purified using Ni-NTA column chromatography. The purified cells were characterized as described earlier using absorbance and fluorescence emission spectra. In Fig. 54 A, B, C, the solid line absorbance and dotted line represent the fluorescence emission spectra of holo ApcBD ligated with three different bilins PCB m(blue), PEB (red), PXB (green) (See Table 11). The purified proteins were separated on SDS-PAGE, stained with Zinc sulphate. Bilin ligation on ApcD was confirmed by fluorescent bands in Fig.54 E (lanes 1 through 3). For protein content the gel was futher stained with commassie stain (Fig. 54 D). In conclusion, TE CpcS is capable of attaching all there bilins on ApcD which is quite unusual since naturally ApcD only contains PCB and acts as a terminal energy acceptor. In case of ApcBD liagtion with PCB there is an energy transfer from the bilin on ApcB to ApcD going on which is evidence from the spectrum (See Fig 54A), which correspond to our earlier data (See Result Section 3.1), where very little enery transfer in case of ApcBD ligation with PEB (See Fig. 54B). However, in case of ApcBD ligation with PfB no energy transfer between the subunits was noticed. This might be an interesting observation where the AP subunits behave differently when non-cognate bilin are ligated in *E. coli* system.

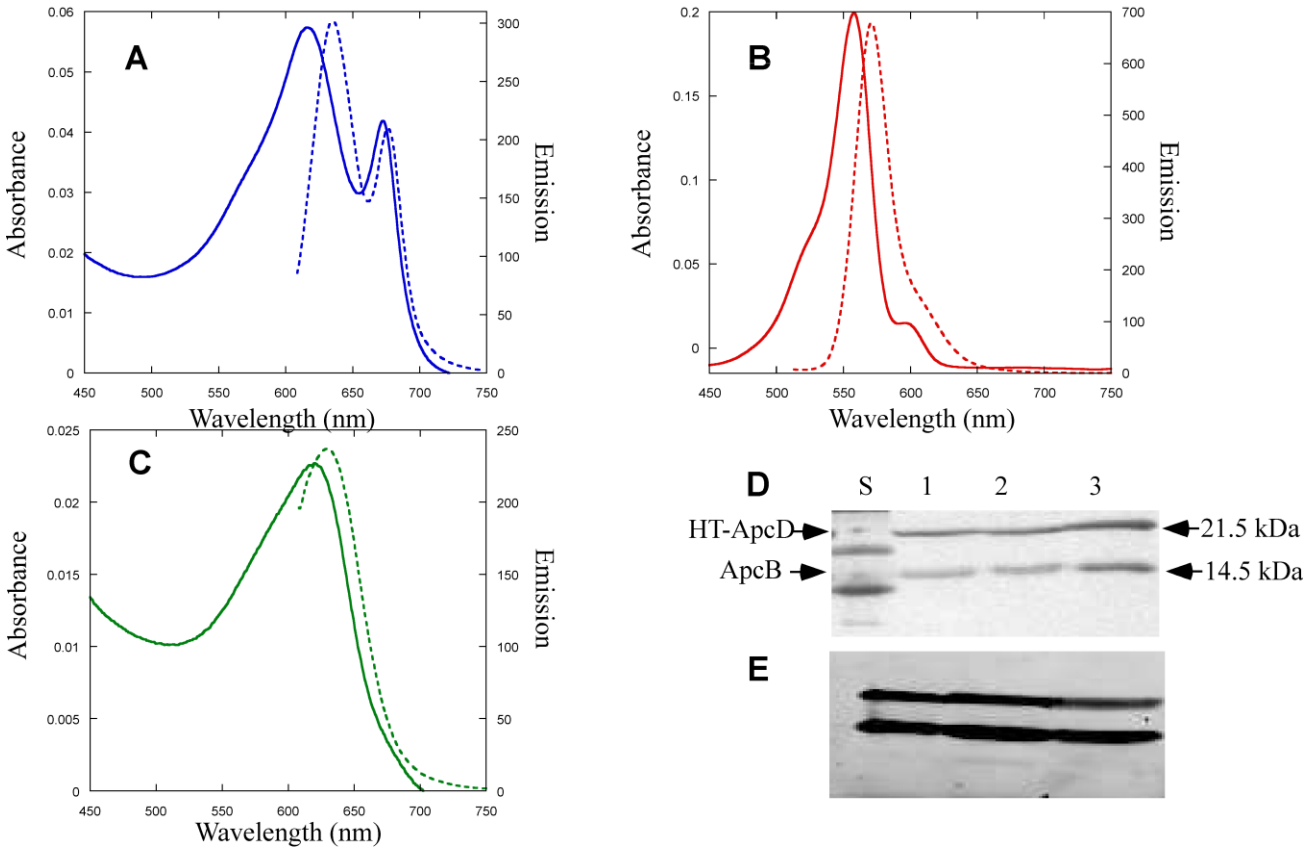

Fig. 54. Analysis of holo HT-ApcBD purified from *E.coli* cells chromophorylated by TECpcS. A. Absorbance (solid blue) line fluorescence emission (blue dashed) spectra of HT-ApcDB purified from cells containing ApcBD/TECpcS and pPcyA, absorbance (blue-solid line) and fluorescence emission (blue-dotted line). **B.** Absorbance (pink-solid and fluorescence (pink-dotted) line from cells purified by coexpressing ApcBD/TECpcS with pPebS. **C.** Absorbance (green-solid) and fluorescence (green-dotted) line from cells purified by coexpressing ApcDB/TECpcS with ho1/hy2. **D.** Coomassie-stained SDS-polyacrylamide gel of HT-ApcDB purified from cells containing ApcDB/TECpcS and pPcyA (lane 1), from cells containing ApcDB/TECpcS and pPebS (lane 2), from cells containing ApcDB/TECpcS, ho1/hy2 (lane 3). Lane S represent Molecular mass standards were indicated by arrows. **E.** Zinc-enhanced bilin fluorescence of the gel in panel D.

5.4. *TE CpcS activity on the less abundant AP β- like subunit ApcF:*

TE CpcS was coexpressed with ApcF with genes involved in formation of three bilins (PCB, PEB or PfB). The coexpressed cells were found to be colored (data not shown) indicative of covalent bilin ligation. The whole cells were purified using Metal affinity chromatography column chromatography. The purified cells were characterized as described earlier using absorbance and fluorescence emission spetra. In Fig. 55 A, B, the solid line represent absorbance and dotted line represent the fluorescence emission spectra of holo ApcF ligated with two different bilins PCB and PEB (See Table 11). The purified proteins were separated on SDS-PAGE, stained with Zinc sulphate to confirm bilin addition on less abundant β subunit of AP (ApcF) in Fig 55 E (lanes 1 through 3). For protein content the gel was futher stained with commassie stain (Fig. 55 D). In conclusion TE CpcS is capable of attaching all three bilins on ApcF which is quite unusual since naturally ApcF only contains PCB. When both PCB and PEB are present CpcS prefer attachment of the non-cognate PEB (Fig 55B). *Thermosynechococcus* PBPs only contain PCB, andit may be that there is little need to discriminate among bilins in this organism. *Synechococcus sp*. PCC 7002 may have evolutionary relatives that contain PEB, providing a greater need to discriminate, explaning why the CpcSU lyase is not as effective PEB attachment.

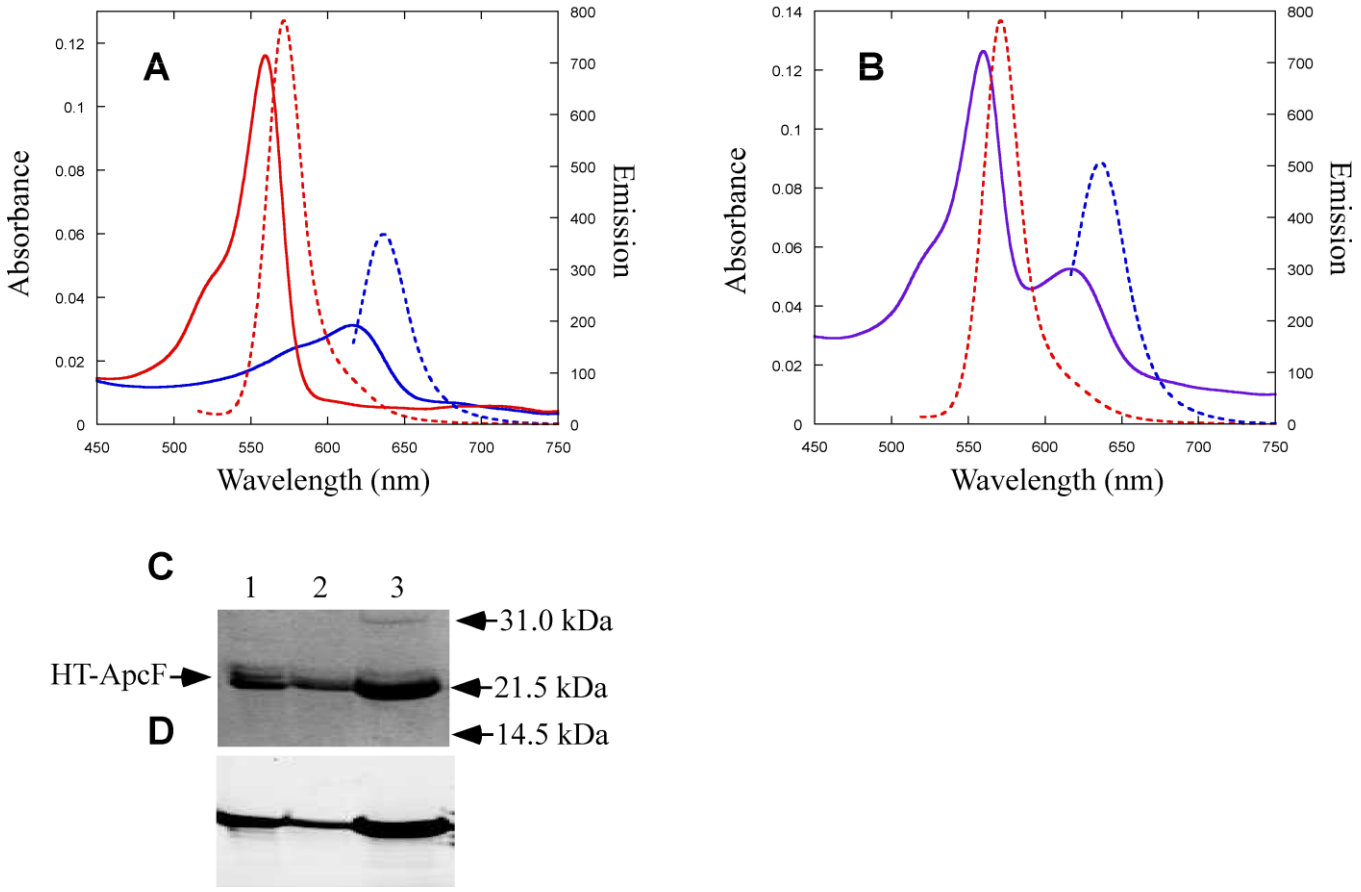

Fig. 55. Analysis of holo HT-ApcF purified from E.coli cells chromophorylated by TECpcS. **A.** Absorbance (solid) line fluorescence emission (dashed) spectra of HT-ApcF purified from cells containing ApcF/TECpcS and pPcyA, absorbance (blue-solid line) and fluorescence emission (blue-dotted line) or ApcF/TECpcS and pPebS, absorbance (pink-solid line) and fluorescence emission (pink-dotted line). **B.** Absorbance (solid) and fluorescence (dotted) line from cells purified by coexpressing ApcF/TECpcS with both pPcyA and pPebS, aborbance (solid-purple line) and flouresence emission (pink dotted) for PEB emission and blue-dotted for PCB emission.pPcyA. **C.** Coomassie-stained SDS-polyacrylamide gel of HT-ApcF purified from cells containing ApcF/TECpcS and pPcyA (lane 1), from cells containing ApcF/TECpcS and pPebS (lane 2), from cells containing ApcF/TECpcS, pPcyA and pPebS (lane 3). Molecular mass standards were indicated by arrows. **D.** Zinc-enhanced bilin fluorescence of the gel in panel C.

Conclusion: The overall conclusion of this small project is that we have characterized one new bilin lyase which shares 50-60 % sequence similarity with known CpcS type lyases. TE CpcS has the capacity to ligate variable bilins to all AP subunits. Since the X-ray crystal structure is available that may be helpful in using this lyase for designing various fluorescent tags for biotechnology applications and this project has proven that this homodimeric structure indeed functions as a bilin lyase.

Table 11: Spectral properties for PC and AP subunits chromophorylated with multiple bilins aided by TE CpcS :

Holo recombinant PBPs (Plasmid present)	λ_{max} *(nm)(Q* $_{Vis/UV}$*)*	*Fluorescence Emission* λ_{max} *(nm)*
HT-CpcB (pCpcBA + pPcyA[1])	629/394 (0.24)	644
HT-CpcB (pCpcBA + pPcyA[2])	628/393 (0.28)	644
HT-ApcA/ApcB (pApcAB+pPcyA[1])	614/392 (5.3)	632
HT-ApcA/ApcB (pApcAB+pPebS[1])	560/376 (8.3)	571
HT-ApcA/ApcB (pApcAB+pHy2 [1])	629/391 (4.8)	648
HT-ApcD (pApcDB+pPcyA [1])	672/370 (3.2)	672
HT-ApcD (pApcDB+pPebS[1])	572/371 (2.8)	571
HT-ApcD (pApcDB+pHy2 [1])	629/391 (2.4)	648
HT-ApcF (pApcF+pPebS[1])	560/376 (8.1)	572
HT-ApcF (pApcF+pPcyA [1])	615/393 (4.2)	632

[1]Coexpressed with TE CpcS
[2]Coexpressed with pCpcSU

Table 12 : List of the clones made which were made but not discussed in the result:

Plasmid Name	***Fragment description***	***Vector***
pMpeA1	*Synechococcus sp.* WH 8020 *mpeA*	pET 100
pMpeA2	*Synechococcus sp.* WH8020 *mpeA*	pET DUET
pMpeA3	*Synechococcus sp.* WH8020 *mpeA*	pGEX2T
pMpeA4	*Synechococcus sp.* WH8020 *mpeA*	pMAL C4x
pMpeB1	*Synechococcus sp.* WH 8020 *mpeB*	pET 100
pMpeB2	*Synechococcus sp.* WH8020 *mpeB*	pET DUET
pMpeB3	*Synechococcus sp.* WH8020 *mpeB*	pGEX2T
pMpeB4	*Synechococcus sp.* WH8020 *mpeB*	pMAL C4x
pMpeBA	*Synechococcus sp.* WH8020 *mpeB and mpeA*	pET Duet
pCpeBA	*Synechococcus sp.* WH8020 c*peB and cpeA*	pCOLA Duet
pCpeA1	*Synechococcus sp.* WH8020 c*peA*	pGEX2T
pCpeA2	*Synechococcus sp.* WH8020 c*peA*	pMAL C4x
pCpeB1	*Synechococcus sp.* WH8020 c*peB*	pGEX2T
pCpeB2	*Synechococcus sp.* WH8020 c*peB*	pMAL C4x
pMpeC	*Synechococcus sp.* WH8020 *mpeC*	pET 100
pMpeU1	*Synechococcus sp.* WH8020 *mpeU*	pET 100
pMpeU2	*Synechococcus sp.* WH8020 *mpeU*	pET Duet
pMpeU3	*Synechococcus sp.* WH8020 mpeU (fused to N-terminal His-tag)	pCDF Duet
pMpeV1	*Synechococcus sp.* WH8020 *mpeV*	pET 100
pMpeV2	*Synechococcus sp.* WH8020 *mpeV*	pET Duet
pPebAB1	*Synechococcus sp.* WH8020 *pebA* and *pebB*	pACYC Duet
pPEB1	*Synechococcus sp.* WH8020 *pebA* and *pebB and PCC 6803 ho1*	pACYC Duet
pPebAB2	*F. diplosiphon pebA* and *pebB*	pACYC Duet
pPEB2	*F. diplosiphon pebA* and *pebB and PCC 6803 ho1*	pACYC Duet

Table 12: continued

pCpeZY	*Synechococcus sp.* WH8020 *cpeA* and *cpeB*	pCOLA Duet
pMpeY	*Synechococcus sp.* WH8102 mpeY (fused to H-terminal His-tag)	pCDF Duet
pMpeYU	*Synechococcus sp.* WH8102 *mpeY* and *Synechococcus sp.* WH8020 *mpeU*	pCDF Duet
pMpeZ	*Synechococcus sp.* CC9311 *mpeZ*	pCDF Duet

Table. 13. Coexpression attempted with negative results:

***In vivo* (a) or *In vitro* reactions**	**PBP subunit (b)**	**Lyase**	**Chromophore**	**Notes**
In vivo	WH 8020 MpeA	WH 8020 MpeU	PEB	Negative result
In vivo	WH 8020 MpeA	WH 8102 MpeY	PEB	Negative result
In vivo	WH 8020 MpeA	WH 8020 MpeU/ WH 8102 MpeY	PEB	Negative result
In vivo	WH 8020 MpeB	WH 8020 MpeU	PEB	Negative result
In vivo	WH 8020 MpeB	WH 8102 MpeY	PEB	Negative result
In vivo	WH 8020 MpeB	WH 8020 MpeU/ WH 8102 MpeY	PEB	Negative result
In vivo	WH 8020 MpeA	WH 8102 CpeS	PEB	Negative result; only CpeS was found to be soluble
In vivo	WH 8020 MpeB	WH 8102 CpeS	PEB	Negative result; only CpeS was found to be soluble
In vivo	WH 8020 MpeC	WH 8020 MpeU/ WH 8102 MpeY	PEB	Negative result
In vivo	WH 8020 CpeA	WH 8020 CpeY/CpeZ	PEB	Negative result
In vivo	WH 8020 CpeB	WH 8020 CpeY/CpeZ	PEB	Negative result

In vivo	WH 8020 CpeBA	WH 8020 CpeY/CpeZ	PEB	Negative result
In vivo	WH 8020 MpeA	WH 8020 MpeV	PEB	Negative result
In vivo	WH 8020 MpeB	WH 8020 MpeV	PEB	Negative result
In vivo		WH 8020 MpeU/ WH 8102 MpeY	PEB	The *E. coli* cells had little purple color. The purified proteins have little fluorescence emission peak with a faint Zn-stain band on SDS-PAGE.
In vivo	WH 8020 MpeA	RS 9311 MpeZ	PEB	Negative result
In vivo	WH 8020 CpeA	WH 8102 CpeS	PEB	Negative result; only CpeS was found to be soluble
In vivo	WH 8020 CpeB	WH 8102 CpeS	PEB	Negative result; only CpeS was found to be soluble
In vitro	WH 8020 CpeA	WH 8102 CpeS	PEB	Negative result
In vitro	WH 8020 CpeB	WH 8102 CpeS	PEB	Negative result
In vivo	WH 8020 CpeA	WH 8020 CpeU	PEB	Negative result
In vivo	WH 8020 CpeB	WH 8020 CpeU	PEB	Negative result
In vitro	WH 8020 CpeA	WH 8020 CpeU	PEB	Showed a little fluorescence emission at 573 nm with a faint Zn stain band when separated on SDS-PAGE
In vitro	WH 8020 CpeB	WH 8020 CpeU	PEB	Showed a little fluorescence emission at 573 nm with a faint Zn stain band when separated on SDS-PAGE

In vivo	WH 8020 CpeA	WH 8020 CpeU/ WH 8102 CpeS	PEB	Negative result
In vivo	WH 8020 CpeB	WH 8020 CpeU/ WH 8102 CpeS	PEB	Negative result
In vitro	WH 8020 CpeA	WH 8020 CpeU / WH 8102 CpeS	PEB	Showed a little fluorescence emission at 573 nm with a faint Zn stain band when separated on SDS-PAGE
In vitro	WH 8020 CpeB	WH 8020 CpeU/ WH 8102 CpeS	PEB	Showed a little fluorescence emission at 573 nm with a faint Zn stain band when separated on SDS-PAGE

(a) *In vivo* here means the experiments was done by coexpressing all the necessary genes in the *E. coli* system.

(b) CpeA-represent α subunit of PEI; CpeB-represent β subunit of PEI; MpeA-represent α subunit of PEII;MpeB-represent β subunit of PEII

Note: All the combination reactions mentioned in the Table 12, were attempted using CpeA, CpeB, MpeA and MpeB with Histidine tag, GST tag, and Maltose tag (Refer to Table 11). The subunits cloned in pMAL vector formed inclusion bodies and was purified by arginine refolding. These were tried for *in vitro* reaction will all possible lyase combinations but with no positive outcome. The refolded protein can be found in Schluchter's lab at UNO in -20C freezer. For *in vitro* reaction was carried out following the procedure described earlier (Saunée, Williams et al. 2008), with minor changes. As a source for enzyme for making PEB, purified PebS was used, and the reactions were carried out for 1h 30 min in dark at room temperature.

Table 13: Oligonucleotide sequences used for clones in Appendix:

Name	***Sequence***	***Vector Cloned***
8020 PebA; F (NcoI)	AACCATGGTTGATTCATTTCTCAATGAGCT	pACYC Duet
8020 PebA; F (EcoRI)	TTGGAATCCTTATTTGTGAGAGGAGGAGGCGGG	pACYC Duet
8020 PebB; F (Sac)	AGAGCTCAAGGAGATAACAAATGACAAATCAAAGATTC	pACYC Duet
8020 PebB; R (PstI)	AAACTGCAGTTATAGATCAAAAAGCACAGTGTGG	pACYC Duet
8020 MpeA;F(PstI)	AAACTGCAGAAGGAGACAACTCATGAAGTCTGTTATCACC	pCOLA
8020 MpeA;R (SalI)	AAAGTCGACTCAACCCAGGGAGTTGATCA	pCOLA
8020 MpeB; F (BamHI)	CAGGATCCCATGCTCGACGCATTCTCCAGGAAGGC	pET Duet
8020 MpeB; R (EcoRI)	ATGAATTCAGATTCAGCTGATTGCGCTGATCACTG	pETDuet
8020 MpeA;F (SalI)	AAAGTCGACAAGGAGACAACTCATGAAGTCTGTTATCACC	pET Duet
8020 MpeA;R (HindIII)	AAAAAGCTTTCAACCAGGGAGTTGATCA	pET Duet
8020 MpeU;F (NdeI)	CTCGCTTACATATGACAGGAATAAATTCTCAAC	pET Duet
8020 MpeU;R(EcoRV)	AGATATCTTAGTGCTTCATTAGTTGATTCC	pET Duet
8020 MpeV;F (KpnI)	AAGGTACCAAGGAGACCTGCAATGTCTGATAGCAATC	pET Duet
8020 MpeV;R (XhoI)	TTCTCGAGATCTGTTTGCCGGAGTTTTTGAAT	pET Duet
8020 MpeB ;F	CACCATGCTCGACGCATTCTCCAGGAAGGCC	PET 100
8020 MpeB; R (EcoRI)	ATGAATTCAGATTCAGCTGATTGCGCTGATCACTG	PET 100
8020 MpeA;F	CACCATGAAGTCTGTTATCACCACCGTTGTC	PET 100
8020 MpeA;R (EcoRI)	GAGAATTCATATCAACCCAGGGAGTTGATCA	PET 100
8020 MpeC;F	CACCATGCTCGGAGCAGAAACAAGCCTGCAA	PET 100
8020 MpeC;R (HindIII)	CTAAGCTTCTAGAAGAAGATTCCAAATGGACGGAA	PET 100

Table 13: Continued

8020 MpeU;F	CACCATGACAGGAATAAATTCTCAACAAGAAGACATC	PET 100
8020 MpeU;R (BamHI)	TTGGATCCTTAGTGCTTCATTAGTTGATTCCTCGCG	PET 100
8020 MpeV;F	CACCATGTCTGATAGCAATCAAATTAAGAATTC	PET 100
8020 MpeV;R (HindIII)	TTAAGCTTTTAATCTGTTTGCCGGAGTTTTTGAAT	PET 100
8020 CpeZ;F (NdeI)	ATATTAGACATATGGATGCATGTGTATCAATGTCTG	pCOLA Duet
8020 CpeZ;R (BglII)	AAAGATCTATTAATTGCCAAAAAGGCT	pCOLA Duet
8020 CpeY;F (KpnI)	AAGGTACCAAGGAGATATAATGCTGATGAAGTCTGCAA	pCOLA Duet
8020 CpeY;R (XhoI)	TTCTCGAGTTATGAAGCTAACGCCATGCGAGC	pCOLA Duet
8020 PebB;F (KpnI)	AAGGTACCAAGGAGATATAATGACAAATCAAAGA	pACYC Duet
8020 PebB;R (XhoI)	TTCTCGAGTTATAGATCAAAAAGCAC	pACYC Duet
8020 Pebb; F2	ACAGACGAGGTCCACACCACT	pACYC Duet
8020 Pebb; R2	AGTGGTGTGGACCTCGTCTGT	pACYC Duet
8020 PebA; F2	CTCAAGGAACTGAATCAACGA	pACYC Duet
8020 PebA; R2	TCGTTGATTCAGTTCCTTGAG	pACYC Duet
8020 PebA;F (NcoI)	AACCATGGTTGATTCATTTCTCAATGAGCT	pACYC Duet
8020 PebA;R (EcoRI)	TTGGAATTCTTATTTGTGAGAGGAGGAGGCGGG	pACYC Duet
8020 Peb B;F (SacI)	AGAGCTCAAGGAGATAACAAATGACAAATCAAAGATTC	pACYC Duet
8020 Peb B;R (PstI)	AAACTGCAGTTATAGATCAAAAAGCACAGTGTGG	pACYC Duet
8020 MpeU-F2	ACTGCACAGCTTTTCAGATAGG	pCDF Duet
8020 MpeU-R2	CCTATCTGAAAAGCTGTGCAGT	pCDF Duet
8020 MpeV-F2	CAGAGACAACAGAAATGCAGATAG	pCDF Duet
8020 mpeV-R2	CTATCTGCATTTCTGTTGTCTCTG	pCDF Duet

Table 13: Continued

8102 CpeS; F (NCoI)	CTCCATGGGCACAATATTAAAAAGTATG	pCDF Duet
8102 CpeS; R (BamHI)	AAGGATCCTAGGCCTTGACTCGTCTGAC	pCDF Duet
8102 CpeT; F (NdeI)	CCTAGAGATACATATGAGAAATTATTCAGCG	pCOLA Duet
8102 CpeT; F (ECoRV)	ACGATATCTCAGGTTTTAAGTTCGAGCC	pCDF Duet
8020 CpeB;F (NcoI)	AAGGATCCGATGCTCGACGCATTCTCACGTTCG	pCOLA Duet
8020 CpeA;R (SalI)	AAAAGTCGACTCAAGAGAGAGCATTGATAACATA	pCOLA Duet
8020 CpeU;F(NdeI)	AACTGCCCATATGACAGCAACGATTGGACATT	pCDF Duet
8020 CpeU;R(EcoRV)	AAGATATCTTATAACCACGTTTCAGGTATG	pCDF Duet
8020 CpeT;F (KpnI)	AAGGTACCAAGAGAGTCAAGCATGACTGTGATGGAC	pCDF Duet
8020 CpeT;R (XhoI)	AACTCGAGTTACAAAGGATTGGTCTGAGCGAA	pCDF Duet
8020 MpeB;F (BamHI)	CAGGATCCATGCTCGACGCATTCTCCAGGAAGGC	pET Duet
8020 MpeA;F (BamHI)	CAGGATCCATGAAGTCTGTTATCACCACCGTTG	pGEX2T
8020 MpeA;R (EcoRI)	ATGAATTCTCAACCCAGGGAGTTGATCACGT	pGEX2T
8020 CpeA;F (BamHI)	CAGGATCCATGAAGTCCGTCGTGACAACC	pGEX2T
8020 CpeA;R (EcoRI)	ATGAATTCTCAAGAGAGAGCATTGATAA	pGEX2T
8020 CpeB;F (BamHI)	CAGGATCCATGCTCGACGCATTCTCACGTTCG	pGEX2T
8020 CpeB;R (EcoRI)	ATGAATTCTCAGGAAATAGCGCCGATGACG	pGEX2T
8102 MpeY;F (BamHI)	CAGGATCCCATGCAGAGCGTTTCGACAACTTGGT	pCDF DUET

Table 13: Continued

8102 MpeY;R (EcoRI)	ATGAATTCTAATCATGACAACTGTTTAAGTAC	pCDF DUET
8020 MpeU;F (BamHI)	AAGGATCCCATGACAGGAATAAATTCTCAACAA	pCDF DUET
8020 MpeU;R (EcoRI)	TTGAATTCTTAGTGCTTCATTAGTTGATTCCTC	pCDF DUET
8102 MpeY;F (BamHI)	AAGGATCCCATGGCAGAGCGTTTCGACAACTTG	pCDF DUET
8102 MpeY;R (EcoRI)	TTGAATTCTCATGACAACTGTTTAAGTACCTGC	pCDF DUET
8020 MpeA;F (EcoRI)	TCGAATTCATGCTCGACGCATTCTCCA	pMAL c4
8020 MpeA;R (BamHI)	TAGGATCCTCAGCTGATTGCGCTGATCAC	pMAL c4
8020 MpeB;F (EcoRI)	TCGAATTCATGCTCGACGCATTCTCCA	pMAL c4
8020 MpeB;R (BamHI)	TAGGACTCCTCAGCTGATTGCGCTGATC	pMAL c4
8020 CpeA;F (EcoRI)	AAGAATTCATGAAGTCCGTCGTGACAACCGTC	pMAL c4
8020 CpeA;R (BamHI)	TAGGACTCCTCAAGAGAGAGCATTGATAACATAAT	pMAL c4
8020 CpeB;F (EcoRI)	ATGAATTCATGCTCGACGCATTCTCACG	pMAL c4
8020 CpeB;R (BamHI)	ACGGACTCCTCAGGAAATAGCGCCGATGAC	pMAL c4
8020 MpeC; F (EcoRI)	TCGAATTCATGCTCGGAGCAGAAACAAGCCTGCAA	pMAL c4
8020 MpeC;R (BamHI)	TAGGATCCCTAGAAGAAGATTCCAAATGGACGGAA	pMAL c4
9311 MpeZ;F (EcoRI)	AAGAATTCGATGAGTGAAAATGATGCTTCTAAG	pCDF DUET
9311 MpeZ;R (HindIII)	CCAAGCTTTTAGTGCTGATAATCTAATTGCTGCTT	pCDF DUET
9311 MpeZ;F (NcoI)	AACCATGGAGGAAAATGATGCTTCTAAG	pCDF DUET

Table 13: Continued

8020 MpeV;F (BamHI)	TCGAGCTCGATGTCTGATAGCAATCAA	pCDF DUET
8020 MpeV;R (EcoRI)	GGGTCGACTTAATCTGTTTGCCGGAG	pCDF DUET
MalE BamHI	AAGGATCCATGAAAATCGAAGAAGGTAAA	
8020 MpeB;R (EcoRI)	CCGAATTCGCTGATTGCGCTGATCACTGC	
PebS;F (NcoI)	TACCATGGGCATGACTAAAAACCCAAGAAATA	pACYC Duet
PebS;R (EcoRI)	TCCCGAATTCTCATTTGTATGAAAAAAGGAAATC	pACYC Duet
8102 MpeA;F(BamHI)	AAGGATCCGATGAAGTCCGTCATCACCACCGTC	pET DUET
8102 MpeA;R(EcoRI)	AAGAATTCTCAGCCCAGGGAGTTGATAACGTA	pET DUET
8102 MpeU;F (BglII)	AAAGATCTCATGCCCACAGTGCTCCAGGATTTC	pCDF Duet
8102 MpeU;R (KpnI)	AAGGTACCTCACAAATTTCTGCTTAGTATGTT	pCDF Duet
8102 MpeU;F (EcoRI)	AAGAATTCGATGCCCACAGTGCTCCAGGA	pCOLA Duet
8102 MpeU;R (SalI)	AAGTCGACTCACAAATTTCTGCTTAGTATGTTGAT	pCOLA Duet
RS 9916 MpeY;F (BamHI)	AAGGATCCGGTGGCAGAAAACAGTAAGATGCCAGTG	pET Duet
RS 9916 MpeY;R (SalI)	AAGTCGACTTATGACAAGCCTTTAAGGGCGTGTTCGCA	pET Duet
RS 9916 MpeU; F (NdeI)	AGATATACATATGACCGAGCTTTCTGGAAACGCTCCA	pCDF Duet
RS 9916 MpeU; R (ECoRV)	AAGATATCTTATGAATTTATTTCTAGCTGGTTGAGTGC	pCDF Duet
9916 CpeB; F(BamHI)	TCT GGA TCC GAT GCT CGA CGC ATT CTC CCG TTC GG	pET Duet
9916 CpeB; R(SalI)	TGT GTC GAC TCA GGA GAC GGC TCC GAT CAC GCG	pET Duet

Table 13: Continued

9916 CpeS; F(NdeI)	TAT CGC TCA TAT GAA TAT TGA GCA ATT TGT TGC	pCOLA Duet
9916 CpeS; R(XhoI)	TGT CTC GAG TTA TGC GCT GAT TCT TTT GAC C	pCOLA Duet
9916 CpeU; F (NdeI)	TAT CGC TCA TAT GGC TGA TTT CTT CGA GGC C	pCOLA Duet
9916 CpeU; R (XhoI)	TGT CTC GAG TCA AAC ACG ATT TAG CTC CGG	pCOLA Duet
9916 CpeT; F (BglII)	TCC AGA TCT CAT TAT ACG GTT CGC AAA AAC G	pCOLA Duet
9916 CpeT; R (XhoI)	TGT CTC GAG TTA AGG ATC TCT ACT AGC CCA TTC	pCOLA Duet
PebS; F (EcoRV)	TTGATATCAATAAGGAGATATAATGACTAAAAACCCAAG	pACYC Duet
PebS; R (XhoI)	AACTCGAGTCATTTGTATGAAAAAAGGAAATCG	pACYC Duet

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