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CpeT is the phycoerythrobilin lyase for Cys-165 on β-phycoerythrin from *Fremyella diplosiphon* and the chaperone-like protein CpeZ greatly improves its activity

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Abstract:

Bilin lyases are enzymes which ligate linear tetrapyrrole chromophores to specific cysteine residues on light harvesting proteins present in cyanobacteria and red algae. The lyases responsible for chromophorylating the light harvesting protein phycoerythrin (PE) have not been fully characterized. In this study, we explore the role of CpeT, a putative bilin lyase, in the biosynthesis of PE in the cyanobacterium *Fremyella diplosiphon*. Recombinant protein studies show that CpeT alone can bind phycoerythrobilin (PEB), but CpeZ, a chaperone-like protein, is needed in order to correctly and efficiently attach PEB to the β-subunit of PE. MS analyses of the recombinant β-subunit of PE coexpressed with CpeT and CpeZ show that PEB is attached at Cys-165. Purified phycobilisomes from a *cpeT* knockout mutant and wild type (WT) samples from *F. diplosiphon* were analyzed and compared. The *cpeT* mutant contained much less PE and more phycocyanin than WT cells grown under green light, conditions which should maximize the production of PE. In addition, Northern blot analyses showed that the *cpeCDESTR* operon mRNAs were upregulated while the *cpeBcpeA* mRNAs were downregulated in the *cpeT* mutant strain when compared with WT, suggesting that CpeT may also play a direct or indirect regulatory role in transcription of these operons or their mRNA stability, in addition to its role as a PEB lyase for Cys-165 on β-PE.

Keywords: bilin lyase, chaperone, cyanobacteria, phycoerythrobilin, phycobilisome, post-translational modification

1 Introduction

Cyanobacteria are known for their characteristic colors due to their production of different pigments such as chlorophyll, carotenoids and phycobiliproteins (PBP). They maximize

light harvesting for photosynthesis using megadalton protein complexes known as phycobilisomes (PBS). PBS are composed of PBP, which are heterodimeric protomers (αβ) that assemble into trimers and hexamers with the help of linker proteins [1]. The freshwater cyanobacterium *Fremyella diplosiphon* UTEX 481 (*Tolypothrix* sp. PCC 7601) used in this study has a PBS that is composed of rods containing phycocyanin [PC, absorbance maximum (λ_{max}) ~620 nm] and phycoerythrin (PE, λ_{max} ~560 nm) which protrude from a core consisting of rods that contain allophycocyanin (λ_{max} ~650 nm)[2, 3]. Each of these PBP is composed of α and β subunits which contain at least one covalently ligated linear tetrapyrrole or bilin. In *F. diplosiphon*, PC and AP contain exclusively phycocyanobilin (PCB) while PE contains only phycoerythrobilin (PEB) [2, 3]. The structure of the PBS from the red alga *Grifithsia pacifica* was solved and shows the complex nature of the interactions between different proteins as well as the complex arrangement of bilins, which play a major role in the photosynthetic function of the PBS [4].

Some cyanobacteria can change their pigmentation to adapt to different light conditions using a process called chromatic acclimation [3]. *F. diplosiphon* undergoes Type-III chromatic acclimation (CA3), during which the PBP content of the PBS is changed under different light conditions. In *F. diplosiphon*, the distal rods of the PBS are composed of mostly PE under green light (GL) conditions while under red light (RL) conditions the rods are composed mostly of PC [5, 6]. Chromophore ligation to Cys residues on PBP is catalyzed by bilin lyases, enzymes which ensure that the right bilin is ligated to the correct cysteine (Cys) residue with the appropriate stereochemistry [7, 8]. There are three main families of bilin lyases; the CpcE/F family, the CpcS/U family and the CpcT family [7-11]. These families of lyases differ in structure, functionality and substrate specificity. Structurally, CpcE/F-type lyases are distinctly different from CpcS/U- and CpcT-type lyases. The crystal structure of CpcE/F was elucidated and shows that these enzymes have an α-solenoid structure formed by a series of roughly parallel layers of alpha helices that together are shaped like twisted crescents [12]. The structures of several CpcS/U-type lyases have been solved and form an 8-stranded anti-parallel, β-barrel structure with an α-helix [13, 14]. CpcT-type lyases are similar in structure to CpcS/U-type lyases and consist of a calyx-shaped β-barrel fold [15, 16]. CpcT-type lyases attach bilins to the β-subunit of PC at the Cys-159-equivalent position [10, 17]. The CpcT-family of lyases seems to be specific for one of the sites on β-subunits where the ligated bilin is in the *S* configuration at the chiral $C3^1$ rather than the more common *R* configuration found in bilins attached by CpcE/F- or CpcS/U-type family members [10].

In *F. diplosiphon*, not all of the lyases responsible for the chromophorylation of PE have been characterized. PE has six conserved Cys residues which act as ligation sites for five PEBs and include Cys-82 and Cys-139 on the α-subunit of PE (called CpeA); Cys-48/59, Cys-80, and Cys-165 on the β-subunit of PE (called CpeB) [18]. The PE lyases that have previously been characterized in *F. diplosiphon* include CpeY, CpeS, and CpeF, and these lyases require the chaperone-like protein CpeZ for optimal activity [19, 20]. CpeF, CpeY, and CpeZ are part of the CpcE/F lyase family while CpeS is in the CpcS/U family of lyases [20]. CpeS and CpeF were characterized as lyases that were responsible for the attachment of PEB to Cys-80 and Cys-48/59, respectively in *F. diplosiphon* CpeB [19, 20]. CpeY is the lyase responsible for the attachment of PEB to Cys-82 of CpeA in *F. diplosiphon;* however, the yield of *F. diplosiphon* CpeA-PEB was higher when CpeY and CpeZ were expressed together [19]. The improved chromophorylation efficiency when CpeZ was present prompted more thorough studies of CpeZ's function. CpeZ is a chaperone-like protein which helps facilitate CpeF, CpeS, and CpeY

lyase activity by assisting with the folding and stability of PE subunits, especially *F. diplosiphon* CpeB [21].

The gene *cpeT* is paralogous to *cpcT* and is found in cyanobacteria that synthesize PE [10, 22, 23]. The similarities between *cpeT and cpcT* motivated us to characterize CpeT to complete the biosynthetic pathway characterization of the important fluorescent protein PE. The fluorescent nature of PE is a result of the attachment of bilins to the protein by lyases. Recently, the Φ*cpeT* gene in the cyanophage P-HM1 was found to encode a putative PBP lyase related to cyanobacterial CpcT-type lyases. The crystal structure of ΦCpeT showed that it had a similar fold and structure as CpcT from *Nostoc* sp. PCC 7120 [16]. Biochemical studies showed that the ΦCpeT specifically bound PEB *in vitro*, however, it was unable to attach the bilin to host PBP β-subunit [16]. The binding of the bilin to ΦCpeT is significant because it was previously believed that the configuration of the lyase-bound bilin determined the final configuration of the newly formed stereocenter at $C3^1$ of PEB at the Cys-site of attachment as either the *R* or *S* isomer [14]. However, in this case, the bound ligand of ΦCpeT was determined to be 3(Z)-PEB as opposed to the expected preferred 3(E)-isomer that the *S*-configuration suggests [15].

Here we examine the role and function of CpeT in the biosynthesis of PE in *F. diplosiphon* using heterologous multiplasmid coexpression experiments in *E. coli* and gene deletion experiments. Purified PBS of a *cpeT* knockout mutant (*cpeT*$^-$)and wild type (WT) samples from *F. diplosiphon* were analyzed and compared. These data, along with the analysis of the recombinant protein activity, show that CpeT is the bilin lyase responsible for the ligation of PEB to Cys-165 on CpeB and its activity requires the chaperone-like protein, CpeZ, in order to efficiently ligate PEB to this position in *E. coli*. In addition, the *cpeT*$^-$ deletion mutant had higher *cpeC* operon transcript levels and lower *cpeBA* transcript abundance, suggesting CpeT may also play a direct or indirect regulatory role on the expression of these operons, in addition to its role as a PEB lyase for Cys-165 on β-PE.

2 Materials and Methods

2.1 *Cyanobacterial mutant strains and growth conditions*

F. diplosiphon was cultured as previously described [24, 25]. To create *cpeT* null mutant strains, *cpeT* fragments were PCR amplified from *F. diplosiphon* genomic DNA. Fusion PCR using *cpeT* primers listed in Table S1 was performed and the *cpeT* fragments were cut with restriction enzymes that cleaved within the primer sequences and ligated into pJCF276 cut with EcoRI and NcoI. The clean deletion constructs of *cpeT* were confirmed by sequencing. *F. diplosiphon* was transformed by triparental mating and *cpeT* null mutant strains were selected as previously described [26, 27]. All parent *E. coli* strains used for triparental mating were DH5α MCR except 803, which carried the RP4 conjugative plasmid [28]. Growth curves were generated from 2 independent replicates of cells monitored at 750 nm every 24 h for 12 days. Variances were expressed as standard errors of the means.

2.2 *Genome and sequence analysis*

F. diplosiphon gene sequences were retrieved from GenBank [29]. Amino acid sequences were analyzed using the ClustalW alignment tool from MacVector software V. 12.7.5 (MacVector Inc., Apex, NC), BLASTp 2.3.1 from NCBI [30, 31], and the $Phyre^2$ prediction system [32].

2.3 *RNA purification and Northern blot analyses*

RNA blot analysis was performed as previously described [24]. At least three independent experiments were carried out for each cell type. Probes used for examining mRNA levels were prepared by PCR amplification using the primers listed in Table S1. Blots were imaged and quantified as previously described [33]. Mann-Whitney U statistical test for significance was performed using SyStat13.

2.4 *Construction of expression plasmids*

Plasmids used in this study are listed in Table S2. Some expression constructs were previously described, but the newly produced constructs were sequenced for confirmation. Each gene was amplified by PCR from *F. diplosiphon* chromosomal DNA using the primers listed in Table S1. Each resulting amplicon was cloned into Duet vectors (Novagen, Madison, WI) as listed in Table S2 after digestion with restriction enzymes (engineered into the primers; see Table S1). pCpeT2 (see Table S2) was used only to generate pCpeT3. HT-CpeT was soluble, but its solubility increased when fused to NusA.

2.5 *Heterologous expression and purification of recombinant proteins*

Recombinant proteins were expressed and purified from *E. coli* BL21 (DE3) cells as previously described [19]. Cells containing the Duet-1 vectors (Novagen/EMD Millipore Corp., Darmstadt, Germany) were grown to an OD_{600nm} of 0.6 at 37°C and induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside, after which the cells were allowed to grow at 18°C for an additional 24 h before being harvested by centrifugation. Cells were stored at -20 °C until ready for purification and analysis.

The cells were resuspended in Buffer O (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM KCl), 0.1x complete mini protease inhibitor cocktail (Roche, Indianapolis, IN) and 0.01 mg ml^{-1} lysozyme in volumes based on the relative mass of the pellets. After a 30-min incubation period on ice, the sample was homogenized and passed three times through a French Pressure Cell Press at 18,000 psi, while keeping the sample chilled on ice. The sample was then centrifuged at 17,000 RCF for 20 min at 4℃ and separated into a pellet and supernatant sample. The supernatant sample was passed through Ni-NTA column to purify the hexahistidine-tagged (HT-) protein as described [10]. This protein was dialyzed overnight in Buffer O and concentrated by ultrafiltration through an Amicon YM10 (Novagen/EMD Millipore Corp., Darmstadt, Germany) and analyzed by absorbance and fluorescence spectroscopy (described below).

2.6 *Isolation of cyanobacterial proteins*

PBS were isolated from *F. diplosiphon* as described [34, 35]. When cells reached an OD between 0.6-0.9 at 750 nm, cells were harvested by centrifugation at 8,000x*g* for 10 min. The cells were washed with and then resuspended in 0.65 M NaH_2PO_4/ K_2HPO_4 buffer, pH 7.5, 0.1x complete mini protease inhibitor cocktail, and 0.01 mg ml^{-1} lysozyme before being passed through a French Pressure Cell Press at 18,000 psi three times. Intact PBS extracted from the sucrose gradients were initially analyzed with absorbance and fluorescence emission spectroscopy (described below) before long term storage in the dark at -20°C.

2.7 *Protein analysis by spectroscopy and gel-electrophoresis*

Fluorescence emission and absorbance spectra were acquired on a Perkin Elmer LS55 fluorescence spectrometer (Waltham, MA) and a Lambda 35, dual-beam UV/Vis spectrometer

(Perkin Elmer) as previously described [13]. For fluorescence emission measurements, excitation wavelength was set to excite PEB at 490 nm.

Polyacrylamide gel electrophoresis (PAGE, 15% w/v) with sodium dodecyl sulfate (SDS) was used to analyze polypeptide samples acquired after purification of proteins as described [10]. Samples were diluted 1:1 in 2x Laemmli Sample Buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01 % bromophenol blue, 5% BME; Bio-Rad) and analyzed. Proteins were loaded with equal amounts determined by Quick Start Bradford Protein Assay kit (Bio-Rad). The samples were then visualized as previously described using zn-enhanced fluorescence to detect covalently bound bilin [36]. Zn-enhanced fluorescence of covalently attached bilins was visualized using ChemiDoc XRS (Bio-Rad) with excitation at 532 nm (detects PEB, and PCB) or at 635 nm (detects PCB). Proteins in the gels were then stained by incubation in Coomassie brilliant blue G-250 overnight and destained in 10% methanol and 10% acetic acid.

2.8 *Tryptic digestion and mass spectrometry*

Tryptic digestion of proteins was conducted as previously described [19, 20, 37]. Tryptic-digested samples were analyzed using LC-MS/MS on an Orbitrap Lumos Fusion mass spectrometer (Thermo Fisher) with an Agilent 1100 Capillary HPLC as its inlet. LC-MS/MS analysis was performed as previously described [19, 34]. The tandem mass spectra were processed using Thermo Proteome Discover 2.1 software. A simplified protein database consisting of about 40 proteins expected to be part of the PBS was used to speed up the analysis. Bilin-containing peptides were confirmed by manual inspection of their associated MS1, MS2, and UV-VIS spectra. Extracted ion chromatograms (EICs) for quantitative estimates were made using ±4 ppm window from the predicted *m/z* and integrations were made assuming equal ionizability for the modified and unmodified forms of the peptides after a 5-point boxcar smoothing of the EIC. All integrations were performed with XCalibur 4.0 software's default algorithms.

3 Results

3.1 *Whole cell analyses of wild type and cpeT- deletion mutant*

CpeT from *F. diplosiphon* is a putative bilin lyase which is 50-60% similar in amino acid sequence to the known lyase CpcT from *Synechococcus* PCC 7002 and *Anabaena* PCC 7120 but only 27.1% similar to the cyanophage HM1 CpeT (see Fig. S1A). Based on CpcT's role of adding PCB to Cys-153 on β-PC, the high sequence similarity of CpcT to CpeT (Fig. S1B), similarity in predicted structure (Fig. S1C), and its genomic context in the *cpeCDSTR* operon [22], we hypothesized that CpeT is the PEB lyase for the Cys-165 position on β-PE [10]. In order to characterize the function of CpeT, a *cpeT-* clean deletion mutant was generated (Fig. S2). When grown under GL conditions to maximize PE production, the phenotype of *cpeT-* cells looked green in color while WT cells were reddish brown in color (Fig. 1A). The phenotype of the *cpeT-* mutant was very dramatic and indicated that very low levels of PE were accumulating under GL conditions. Absorbance spectra of the whole cell samples of the WT and *cpeT-* mutant were taken and compared (Fig. 1C). The resulting comparison showed that the mutant sample contained much less PE than the WT sample (λ_{max} ~560 nm). This low PE phenotype may be attributed to CpeT playing a role as a bilin lyase in PE synthesis. Under RL conditions, both WT and mutant cells had similar phenotypes and were green in color and had similar profiles when compared using absorbance spectroscopy (Fig. 1D, F). Analysis of the growth curves for mutant

and WT cells also showed a severe reduction of growth rate in the *cpeT*- mutant grown under GL conditions when compared with WT cells (Fig. 1B). No growth rate differences were observed when comparing the cells grown under RL conditions (Fig. 1E).

3.2 *Analysis of effects of cpeT deletion on adjacent genes*

The effects of *cpeT* deletion on its adjacent genes were examined by monitoring RNA accumulation in cells grown under GL conditions. *cpeT* is located in the *cpeCDSTR* operon (Fig.2A) [22]. The deletion of *cpeT* was confirmed by the absence of *cpeT* mRNA in the null strain (Fig. S2). RNA blot autoradiographs of WT and *cpeT*- deletion mutants confirmed that *cpeT* deletion was successful in the mutant sample (Fig. 2B). An increase in transcript abundance from the *cpeCDSTR* operon, which was measured by a ~10-fold increase of *cpeR* mRNA abundance (1029% of WT), in the mutant sample was observed (Fig. 2B and D). Since there was an increase in the *cpeCDSTR* operon, the expression of *cpeBA*, genes encoding the PE subunits, was examined (Fig. 2C). Unexpectedly, the *cpeBcpeA* mRNA levels were downregulated between ~6 and ~3-fold in the mutant strain when compared with WT (Fig. 2D; 16% and 34% of WT levels, respectively). The reason for the difference seen between *cpeB* and *cpeA* levels is not known, but may be due to some variability in hybridization of the probes. However *cpeB* and *cpeA* levels in the *cpeT*- mutant were significantly lower than those seen in WT cells.

3.3 *Biochemical characterization of purified PBS isolated from WT and cpeT- mutant*

Whole PBS were isolated and purified from wild-type (WT) and *cpeT*- deletion mutant strains of *F. diplosiphon* to characterize the function of CpeT. Whole PBS were isolated and purified using a sucrose gradients and analyzed by absorbance and fluorescence spectroscopy (Fig. S3). The PBS of both samples were collected from the main, colored band at the bottom of the gradients (Fig. S3 inset). The WT PBS were purple in color, which indicated high PE levels, while the *cpeT*- mutant PBS were blue in color, which indicated a reduction in PE levels. Absorbance spectroscopy confirmed that WT PBS contained more PE (λ_{max}=568 nm) than PC (λ_{max}=615 nm) and allophycocyanin (λ_{max}=649 nm; Fig. S3). When excited at 490 nm (which preferentially excites PE), WT PBS fluoresce at 669 nm with a small shoulder at 582 nm, indicating a transfer of energy from PE to PC to allophycocyanin (emission at 669 nm) with some energy fluorescing from PE (582 nm; Fig. S3). Analysis of the *cpeT*- mutant PBS also confirmed reduced levels of PE while PC levels were increased (Fig. S3). When excited at 490 nm, the *cpeT*- mutant PBS showed some energy transfer from PE to PC with a broad PC peak indicating that the energy transfer from PE to PC was inefficient, likely a result of the decreased PE (Fig. S3).

3.4 *Characterization of the bilin lyase CpeT using recombinant protein coexpression system*

The bilin lyase activity of CpeT was studied using a heterologous multi-plasmid coexpression system in *E. coli.* All coexpressions were done in the presence of PEB synthesis enzymes (PebS/HO1*)*[38]. Recombinant NT non-hexahistidine-tagged (NT)-Nus-CpeT (pCpeT3; see Table S2) was coexpressed with recombinant hexahistidine-tagged (HT)-CpeB and NT-CpeA (hereafter, labeled as CpeBA) and PebS/HO1 to determine if CpeT demonstrated any lyase activity. CpeB and CpeA were coexpressed together and used in these expressions because previous research has shown that co-expressing these subunits increases the solubility of CpeB [10, 39]. After expression, HT-proteins were purified using Ni-NTA affinity chromatography and analyzed by fluorescence emission spectroscopy and SDS-PAGE (Fig. 3). Purified HT-CpeB

from coexpressions containing CpeBA, NT-CpeT, and PEB (together, hereafter labeled as CpeBAT) were analyzed by SDS-PAGE and fluorescence spectroscopy. The fluorescence emission spectra of this coexpression resulted in very little fluorescence emission at 565 nm expected for correct PEB ligation (Fig. 3A). SDS-PAGE analysis of the sample revealed that there was a small amount of chromophorylation of CpeB when the gel was excited at 532 nm (Fig. 3C). This amount of chromophorylation was comparable to the autocatalytic attachment of a bilin to HT-CpeB which can be seen in the negative control coexpression containing CpeBA and PEB (Fig. 3) and the coexpression containing CpeBA, NT-CpeZ, and PEB (Fig. 3). CpeZ is unable to attach bilin to a PE substrate by itself [19].

Since CpeZ is a chaperone-like protein which increases the solubility of CpeB allowing lyases to interact/attach bilins [19, 21], NT-CpeZ was coexpressed with NT-Nus-CpeT (pCpeT3) and CpeBA (referred to as CpeBATZ) to increase CpeB solubility and to study the possible effects that CpeZ may have on CpeT lyase activity. The HT-CpeB purified from the coexpression was analyzed using fluorescence emission spectroscopy and SDS-PAGE (Fig. 3). When CpeBA was coexpressed with both CpeT and CpeZ, there was a fluorescence emission peak at 565 nm after excitation at 490 nm (Fig. 3A). This fluorescence emission corresponds to that expected for PEB bound to protein. When this sample was analyzed using zn-enhanced fluorescence, there was an observed fluorescent product when the gel was excited at 532 nm corresponding to PEB (Fig. 3B). The fluorescent product on the gel was expected to be HT-CpeB based on its size of ~21 kDa (Fig. 3B). A small amount of non-enzymic addition of PEB to CpeB was observed in the CpeBA alone, CpeBA plus CpeZ and the CpeBA and CpeT (Fig. 3B). These results suggest that the CpeZ chaperone-like protein is required to facilitate folding/stabilization of CpeB, allowing CpeT to function as a lyase. Purified HT-CpeT was capable of binding PEB tightly with an emission peak at ~610 nm and shows bilin fluorescence after SDS-PAGE, indicating tight binding (Fig. 4), but it was unable to attach it to HT-CpeB as evidenced by the low zn-enhanced fluorescence observed for HT-CpeB (Fig 4C).

The samples from the CpeBAT and CpeBATZ coexpressions were digested with trypsin and analyzed by LC-MS/MS to determine which Cys- sites, if any, had a covalently-bound PEB as shown in Table 1. Although the sample containing CpeBAT showed little fluorescence (Fig. 3A), LC-MS/MS analysis revealed that there were peptides with masses which corresponded to Cys-165 of CpeB, an unmodified peptide and a peptide which was modified with PEB (Fig. S4 and Table 1). The ratio of unmodified:modified at this position was 2:1, suggesting an inefficient PEB modification of this position by CpeT in the absence of CpeZ and a binding of PEB that is not in its native fluorescent (stretched) conformation (Fig. S4). The control experiment with CpeBA, CpeZ and PebS/HoI resulted in low soluble protein yields and little to no fluorescence and were not analyzed further (Fig. 3) [20]. LC-MS/MS analysis of the CpeBAZT sample showed that Cys-165 of CpeB (*m/z* 702.32^{3+}) contained a bound PEB (Fig. 5A and Table 1), which was the expected site of lyase activity for CpeT. The other Cys sites on CpeA (Cys-82 *m/z* 441.19^{2+}, Cys-139 *m/z* 517.25^{2+}) and CpeB (Cys-48/59 *m/z* 1092.52^{4+}, Cys-80 *m/z* 664.3^{2+}) were detected but in their unmodified form (Table 1). These results indicate that recombinant CpeT functions as a bilin lyase responsible for attaching PEB to Cys-165 on CpeB, and that the presence of CpeZ increases the efficiency of this ligation.

4 Discussion and conclusions

The study of *F. diplosiphon* CpeT and its function as a bilin lyase was investigated through the studies reported here to understand the importance and role of CpeT in the overall complex pattern of PBS biogenesis in cyanobacteria. CpeT's role in PE biosynthesis was not well characterized previously. One recent study showed red algal CpeT was capable of ligating PEB to CpeB, but the activity levels were very low and the biochemical characterization of the enzyme and product was incomplete [40]. CpeT is a bilin lyase that shares 50-60 % similarity in amino acid sequence to CpcT proteins, which attach PCB to Cys-153 of the PC β-subunit (Fig S1) [10, 15, 32]. The crystal structures of CpcT and ΦCpeT have recently been elucidated and suggest a possible mechanism for the T-type lyases [15, 16]. Structurally, CpcT forms a dimer and adopts a calyx-shaped β-barrel fold; ΦCpeT appears to be a smaller, more compact version of the same structure [15, 16]. One difference between the two proteins is that CpcT binds and attaches PCB to PC while ΦCpeT binds PEB. Gasper et al. showed that although the phage protein could bind PEB, it was unable to transfer PEB to the host PBP β-subunit. They showed that ΦCpeT loaded with PEB could transfer bilin to the native CpeS lyase, allowing it to ligate PEB to CpeB [15, 16]. The ΦCpeT with bound PEB had a fluorescence emission at 607 nm, similar to what we observed with *F. diplosiphon* CpeT (Fig. 4).

In vivo studies were used to demonstrate loss of function effects from deleting the *cpeT* gene. Initial analyses of *cpeT* mutants made earlier by Cobley and coworkers also showed lower PE protein levels overall [41, 42]. Previous studies of different bilin lyases from *F. diplosiphon* have shown that the deletion of genes encoding them have affected both PE and PC biosynthesis in cyanobacteria [20] [21]. In these studies, PE lyase mutants (specifically *cpeF*$^-$ *and cpeY*$^-$) were generated and grown in different light conditions causing changes in the phenotypes and PE/PC content of the cells. The same result was seen here in the *cpeT*$^-$ mutant. The *cpeT*$^-$ mutant PBSs were blue in color while the PBSs from WT cells were pink. These observations agree with previous studies in which a blue phenotype in PBSs corresponds with high PC content while a pink phenotype indicates a high PE content [6]. PBSs from *cpeT*$^-$ deletion mutants grown in GL showed reduced PE content with an increase in PC content, suggesting that CpeT is involved in PE biosynthesis. The phenotype of increased PC levels has been observed previously in *cpeF* [20], *cpeY*, and *cpeZ* [21] mutants where there were also reduced PE phenotypes and can be attributed to an increase in the relative amount of PC produced to compensate for the loss of PE. In addition to these phenotypic changes, there was an observed reduction in doubling time. This was likely a result of the inefficient light harvesting or PBS instability caused by the reduced PE levels in the mutant, similar to the phenotype previously observed in *cpeF* [20], *cpeY* and *cpeZ* [21]mutants.

The reduced PE levels observed in the *cpeT*$^-$ deletion mutants may also indicate a role of CpeT in the regulation of the biosynthesis of PE. The effects of deleting *cpeT* on the genes adjacent to it in the *cpeCDESTR* operon (hereafter called *cpeC* operon) were measured by monitoring RNA accumulation in cells grown under GL conditions (Fig. 2B). These cells showed a 10-fold increase in the operon transcript, which includes *cpeR*, a gene encoding an activator required for the expression of the PE operon (*cpeBA*) and also of the *pebAB* operon (encoding enzymes required for PEB synthesis in *F. diplosiphon*) [22, 24, 43]. The expression of *cpeR* plays a major role in the regulation of CA3 in *F. diplosiphon*. CA3 regulation involves either the *rca* (regulator of chromatic acclimation) or *cgi* (control of GL induction) systems [6, 44]. Both systems control the expression of the *cpeC* operon in GL and RL [45]. The activation of the *cpeC* operon leads to an increase in CpeR, which activates the *cpeBA* and *pebAB* operons, ultimately increasing PE content. The reason for the increase in the *cpeC* operon RNA levels

could be a result of increased transcription, decreased transcription termination, or increased transcript stability. We did not find any rho-independent transcription termination sites within the deleted portion of *cpeT*, however, Bezy et al., found that there is a transcript of the length (~4100 nt) that suggests there is a termination site within *cpeT* [25]. Therefore, it is possible that the increased transcripts containing *cpeR* are due to greater readthrough to the end of *cpeR* because the *cpeT*$^{-}$ deletion mutant removed this potential termination site. Although the RNA levels for *cpeR* are increased in the *cpeT*$^{-}$ mutant, it was surprising that the amount of *cpeB* and *cpeA* mRNA accumulation decreased significantly but not in equal amounts, given they are co-transcribed. Recent studies have shown that genes within same operon may show different transcript stoichiometry due to differential mRNA decay [46]. These results suggest that *cpeT* or its protein product could play some type of regulatory role in the transcription or transcript stability of PE genes, and more research is needed for further understanding of this result. This lower *cpeBA* transcript phenotype in a *cpeT* deletion mutant was also noted previously [42]. It is important to mention that other lyase mutants (e.g. *cpeY*$^{-}$ and *cpeF*$^{-}$) have higher residual, partially chromophorylated PE subunits present than was observed in the *cpeT*$^{-}$ mutant, suggesting that the low *cpeBA* transcripts found in the *cpeT*$^{-}$ mutant may be a particular phenomenon related to CpeT's function [20] [21].

Recombinant protein coexpression studies in *E. coli* confirmed that CpeT was able to bind PEB (Fig. 4) but was unable to efficiently attach the bilin to recombinant CpeB with the appropriate conformation that established a highly fluorescent product (Fig. 3). The ΦCpeT was completely unable to ligate PEB to its substrate, but was able to efficiently bind PEB [16]. Recombinant CpeZ increased the chromophorylation by CpeB-specific lyases CpeS and CpeF and helped renature CpeB after chemical denaturation [21]. Therefore, we hypothesized that its chaperone-like activity on CpeB may increase solubility of the apo-subunit to facilitate CpeT lyase activity. Indeed, CpeT, in the presence of CpeZ, had a much higher chromophorylation of PEB to CpeB at Cys-165 than CpeT alone. Interestingly we did not see a defect of PEB attachment at Cys-165 in the analyses of a *cpeZ* mutant, suggesting CpeZ is not absolutely required for CpeT activity inside cyanobacteria [21]. These results confirm CpeT's role as the bilin lyase responsible for the attachment of PEB to Cys-165 of CpeB and reaffirm the role of CpeZ as a chaperone-like protein for CpeB, aiding in the activity of lyases for CpeB. Now, with the characterization of CpeT from *F. diplosiphon*, the bilin lyase enzymes required for the post-translational modification of CpeB have fully been characterized [19-21].

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Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.

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Abbreviations: The abbreviations used are: BME, 2-mercaptoethanol; CA3, Type III chromatic acclimation; EICs, extracted ion chromatograms; GL, green light; HT-, hexahistidine-tagged; MS, mass spectroscopy; MW, molecular weight; Ni- NTA, nickel-nitrilotriacetic acid; NT-, non-hexahistidine-tagged; Nus, NusA-tagged; PAGE, polyacrylamide gel electrophoresis; PBP, phycobiliprotein; PBS, phycobilisome(s); PC, phycocyanin; PCB, phycocyanobilin; PE, phycoerythrin; PEB, phycoerythrobilin; RL, red light; SDS, sodium dodecyl sulfate; WT, wild-type

References

[1] A.N. Glazer, Light guides: directional energy transfer in a photosynthetic antenna, J. Biol. Chem., 264 (1989) 1-4.
[2] A. Gutu, D.M. Kehoe, Emerging Perspectives on the Mechanisms, Regulation, and Distribution of Light Color Acclimation in Cyanobacteria, Molec. Plant, 5 (2012) 1-13.
[3] N. Tandeau de Marsac, Occurrence and nature of chromatic adaptation in cyanobacteria, J. Bacteriol., 130 (1977) 82-91.
[4] J. Zhang, J. Ma, D. Liu, S. Qin, S. Sun, J. Zhao, S.-F. Sui, Structure of phycobilisome from the red alga *Griffithsia pacifica*, Nature, 551 (2017) 57-63.
[5] J.E. Sanfilippo, L. Garczarek, F. Partensky, D.M. Kehoe, Chromatic acclimation in cyanobacteria: A diverse and widespread process for optimizing photosynthesis, Ann. Rev. Microbiol., 73 (2019) 407-433.
[6] D.M. Kehoe, A. Gutu, Responding to color: The regulation of complementary chromatic adaptation, Ann. Rev. Plant Biol., 57 (2006) 127 - 150.
[7] W.M. Schluchter, G. Shen, R.M. Alvey, A. Biswas, N.A. Saunée, S.R. Williams, C.A. Miller, D.A. Bryant, Phycobiliprotein biosynthesis in cyanobacteria: Structure and function of enzymes involved in post-translational modification, in: P.C. Hallenbeck (Ed.) Advances in Experimental Medicine & Biology, Springer, NY, NY, 2010.
[8] H. Scheer, K.H. Zhao, Biliprotein maturation: the chromophore attachment, Molec. Microbiol., 68 (2008) 263-276.
[9] C.D. Fairchild, J. Zhao, J. Zhou, S.E. Colson, D.A. Bryant, A.N. Glazer, Phycocyanin α subunit phycocyanobilin lyase, Proc. Natl. Acad. Sciences, U. S. A., 89 (1992) 7017-7021.
[10] G. Shen, N.A. Saunee, S.R. Williams, E.F. Gallo, W.M. Schluchter, D.A. Bryant, Identification and characterization of a new class of bilin lyase: the *cpcT* gene encodes a bilin lyase

responsible for attachment of phycocyanobilin to Cys-153 on the beta subunit of phycocyanin in *Synechococcus* sp. PCC 7002, J. Biol.Chem., 281 (2006) 17768 - 17778.
[11] A. Bretaudeau, F. Coste, F. Humily, L. Garczarek, G. Le Corguillé, C. Six, M. Ratin, O. Collin, W.M. Schluchter, F. Partensky, CyanoLyase: a database of phycobilin lyase sequences, motifs and functions, Nucleic Acids Res., 41 (2013) D396-D401.
[12] C. Zhao, A. Höppner, Q.-Z. Xu, W. Gärtner, H. Scheer, M. Zhou, K.-H. Zhao, Structures and enzymatic mechanisms of phycobiliprotein lyases CpcE/F and PecE/F, Proc. Natl. Acad. Sciences, U. S. A., 114 (2017) 13170-13175.
[13] C.M. Kronfel, A.P. Kuzin, F. Forouhar, A. Biswas, M. Su, S. Lew, J. Seetharaman, R. Xiao, J.K. Everett, L.-C. Ma, T.B. Acton, G.T. Montelione, J.F. Hunt, C.E.C. Paul, T.M. Dragomani, M.N. Boutaghou, R.B. Cole, C. Riml, R.M. Alvey, D.A. Bryant, W.M. Schluchter, Structural and Biochemical Characterization of the Bilin Lyase CpcS from *Thermosynechococcus elongatus*, Biochemistry, 52 (2013) 8663-8676.
[14] K. Overkamp, R. Gasper, K. Klaus, C. Herrmann, E. Hofmann, N. Frankenberg-Dinkel, Insights into the Biosynthesis and Assembly of Cryptophycean Phycobiliproteins, J. Biol. Chem., 289 (2014) 26691-26707.
[15] W. Zhou, W.-L. Ding, X.-L. Zeng, L.-L. Dong, B. Zhao, M. Zhou, H. Scheer, K.-H. Zhao, X. Yang, Structure and Mechanism of the Phycobiliprotein Lyase CpcT, J. Biol.Chem., 289 (2014) 26677-26689.
[16] R. Gasper, J. Schwach, J. Hartmann, A. Holtkamp, J. Wiethaus, N. Riedel, E. Hofmann, N. Frankenberg-Dinkel, Distinct Features of Cyanophage-encoded T-type Phycobiliprotein Lyase ΦCpeT: The role of auxiliary metabolic genes, J. Biol. Chem., 292 (2017) 3089-3098.
[17] K.H. Zhao, J. Zhang, J.M. Tu, S. Boehm, M. Ploescher, L. Eichacker, C. Bubenzer, H. Scheer, X. Wang, M. Zhou, Lyase activities of CpcS- and CpcT-like proteins from *Nostoc* PCC7120 and sequential reconstitution of binding sites of phycoerythrocyanin and phycocyanin beta-subunits, J. Biol.Chem., 282 (2007) 34093-34103.
[18] W. Sidler, B. Kumpf, W. Rüdiger, H. Zuber, The complete amino-acid sequence of C-phycoerythrin from the cyanobacterium *Fremyella diplosiphon*, Biological Chemistry Hoppe-Seyler, 367 (1986) 627-642.
[19] A. Biswas, M.N. Boutaghou, R.M. Alvey, C.M. Kronfel, R.B. Cole, D.A. Bryant, W.M. Schluchter, Characterization of the Activities of the CpeY, CpeZ, and CpeS Bilin Lyases in Phycoerythrin Biosynthesis in *Fremyella diplosiphon* Strain UTEX 481, J. Biol. Chem., 286 (2011) 35509-35521.
[20] C.M. Kronfel, C.V. Hernandez, J.P. Frick, L.S. Hernandez, A. Gutu, J.A. Karty, M.N. Boutaghou, D.M. Kehoe, R.B. Cole, W.M. Schluchter, CpeF is the bilin lyase that ligates the doubly linked phycoerythrobilin on beta-phycoerythrin in the cyanobacterium *Fremyella diplosiphon*, J. Biol. Chem., 294 (2019) 3987-3999.
[21] C.M. Kronfel, A. Biswas, J.P. Frick, A. Gutu, T. Blensdorf, J.A. Karty, D.M. Kehoe, W.M. Schluchter, The roles of the chaperone-like protein CpeZ and the phycoerythrobilin lyase CpeY in phycoerythrin biogenesis, Biochim. Biophys. Acta. Bioenergetics, 1860 (2019) 549-561.
[22] J.G. Cobley, A.C. Clark, S. Weerasurya, F.A. Queseda, J.Y. Xiao, N. Bandrapali, I. D'Silva, M. Thounaojam, J.F. Oda, T. Sumiyoshi, M.H. Chu, CpeR is an activator required for expression of the phycoerythrin operon (*cpeBA*) in the cyanobacterium *Fremyella diplosiphon* and is encoded in the phycoerythrin linker- polypeptide operon (*cpeCDESTR*), Molec. Microbiol., 44 (2002) 1517-1531.

[23] C. Six, J.-C. Thomas, L. Garczarek, M. Ostrowski, A. Dufresne, N. Blot, D. Scanlan, F. Partensky, Diversity and evolution of phycobilisomes in marine *Synechococcus* spp.: a comparative genomics study, Genome Biol., 8 (2007) R259.
[24] L.O. Seib, D.M. Kehoe, A Turquoise Mutant Genetically Separates Expression of Genes Encoding Phycoerythrin and Its Associated Linker Peptides, J. Bacteriol., 184 (2002) 962-970.
[25] R.P. Bezy, L. Wiltbank, D.M. Kehoe, Light-dependent attenuation of phycoerythrin gene expression reveals convergent evolution of green light sensing in cyanobacteria, Proc. Natl. Acad. Sciences, U. S. A., 108 (2011) 18542-18547.
[26] D. Kehoe, A. Grossman, Similarity of a Chromatic Adaptation Sensor to Phytochrome and Ethylene Receptors, Science, 273 (1996) 1409-1412.
[27] D.M. Kehoe, A.R. Grossman, New classes of mutants in complementary chromatic adaptation provide evidence for a novel four-step phosphorelay system, J. Bacteriol., 179 (1997) 3914-3921.
[28] C.J. Kuhlemeier, A.A. Thomas, A. van der Ende, R.W. van Leen, W.E. Borrias, C.A. van den Hondel, G.A. van Arkel, A host-vector system for gene cloning in the cyanobacterium *Anacystis nidulans* R2, Plasmid, 10 (1983) 156-163.
[29] S. Yerrapragada, A. Shukla, K. Hallsworth-Pepin, K. Choi, A. Wollam, S. Clifton, X. Qin, D. Muzny, S. Raghuraman, H. Ashki, A. Uzman, S.K. Highlander, B.G. Fryszczyn, G.E. Fox, M.R. Tirumalai, Y. Liu, S. Kim, D.M. Kehoe, G.M. Weinstock, Extreme Sensory Complexity Encoded in the 10-Megabase Draft Genome Sequence of the Chromatically Acclimating Cyanobacterium *Tolypothrix* sp. PCC 7601, Genome Announc., 3 (2015).
[30] T.L.M. Stephen F. Altschul, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, David J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res., 25 (1997) 3389-3402.
[31] J.C.W. Stephen F. Altschul, E. Michael Gertz, Richa Agarwala, Aleksandr Morgulis, Alejandro A. Schäffer, Yi-Kuo Yu, Protein database searches using compositionally adjusted substitution matrices, FEBS J., 272 (2005) 5101-5109.
[32] L.A. Kelley, S. Mezulis, C.M. Yates, M.N. Wass, M.J.E. Sternberg, The Phyre2 web portal for protein modeling, prediction and analysis, Nat. Protocols, 10 (2015) 845-858.
[33] A. Gutu, R.M. Alvey, S. Bashour, D. Zingg, D.M. Kehoe, Sulfate-driven elemental sparing is regulated at the transcriptional and posttranscriptional levels in a filamentous cyanobacterium, J. Bacteriol., 193 (2011) 1449-1460.
[34] A. Shukla, A. Biswas, N. Blot, F. Partensky, J.A. Karty, L.A. Hammad, L. Garczarek, A. Gutu, W.M. Schluchter, D.M. Kehoe, Phycoerythrin-specific bilin lyase-isomerase controls blue-green chromatic acclimation in marine *Synechococcus*, Proc. Natl. Acad. Sciences, U. S. A., 109 (2012) 20136-20141.
[35] J.L. Collier, A.R. Grossman, Chlorosis induced by nutrient deprivation in *Synechococcus* sp. strain PCC 7942: not all bleaching is the same, Journal of Bacteriology, 174 (1992) 4718-4726.
[36] S. Raps, Differentiation between phycobiliprotein and colorless linker polypeptides by fluorescence in the presence of of ZnSO4, Plant Physiol., 92 (1990) 358-362.
[37] D.M. Arciero, D.A. Bryant, A.N. Glazer, *In vitro* attachment of bilins to apophycocyanin I: specific covalent adduct formation at cysteinyl residues involved in phycocyanobilin binding in C-phycocyanin, J. Biol. Chem., 263 (1988) 18343-18349.
[38] T. Dammeyer, S.C. Bagby, M.B. Sullivan, S.W. Chisholm, N. Frankenberg-Dinkel, Efficient phage-mediated pigment biosynthesis in oceanic cyanobacteria, Current Biol., 18 (2008) 442-448.
[39] N.A. Saunée, S.R. Williams, D.A. Bryant, W.M. Schluchter, Biogenesis of phycobiliproteins. II. CpcS-I and CpcU comprise the heterodimeric bilin lyase that attaches phycocyanobilin to Cys-

82 of beta -phycocyanin and Cys-81 of allophycocyanin subunits in *Synechococcus* sp. PCC 7002, J. Biol. Chem., 283 (2008) 7513-7522.
[40] X. Cao, X. Zang, Z. Liu, Y. Jin, D. Sun, Y. Guo, Z. Wang, F. Zhang, J. Lin, Molecular cloning of the *cpeT* gene encoding a bilin lyase responsible for attachment of phycoerythrobilin to Cys-158 on the β-subunit of phycoerythrin in *Gracilariopsis lemaneiformis*, J. Appl. Phycol., 31 (2019) 3331-3340.
[41] P.S. Idumalla, CpeS and CpeT play a role in the regulation of phycoerythrin expression in *F. diplosiphon* Chemistry Department, University of San Francisco, 2003.
[42] T. Sumiyoshi, Transcription of the *cpeBA* and *cpeCDE* operons in wild type and green mutant strains of the cyanobacterium, *Fremyella diplosiphon*, Chemistry Department, University of San Francisco, 2003.
[43] R.M. Alvey, J.A. Karty, E. Roos, J.P. Reilly, D.M. Kehoe, Lesions in phycoerythrin chromophore biosynthesis in *Fremyella diplosiphon* reveal coordinated light regulation of apoprotein and pigment biosynthetic enzyme gene expression, Plant Cell 15(10) (2003) 2448-2463.
[44] J.E. Sanfilippo, L. Garczarek, F. Partensky, D.M. Kehoe, Chromatic Acclimation in Cyanobacteria: A Diverse and Widespread Process for Optimizing Photosynthesis, Ann. Rev. Microbiol. 73(1) (2019) 407-433.
[45] B.L. Montgomery, Seeing new light: recent insights into the occurrence and regulation of chromatic acclimation in cyanobacteria, Curr. Opin. Plant Biol. 37 (2017) 18-23.
[46] D. Dar, R. Sorek, Extensive reshaping of bacterial operons by programmed mRNA decay, PLOS Genetics 14(4) (2018) e1007354.

Figures:

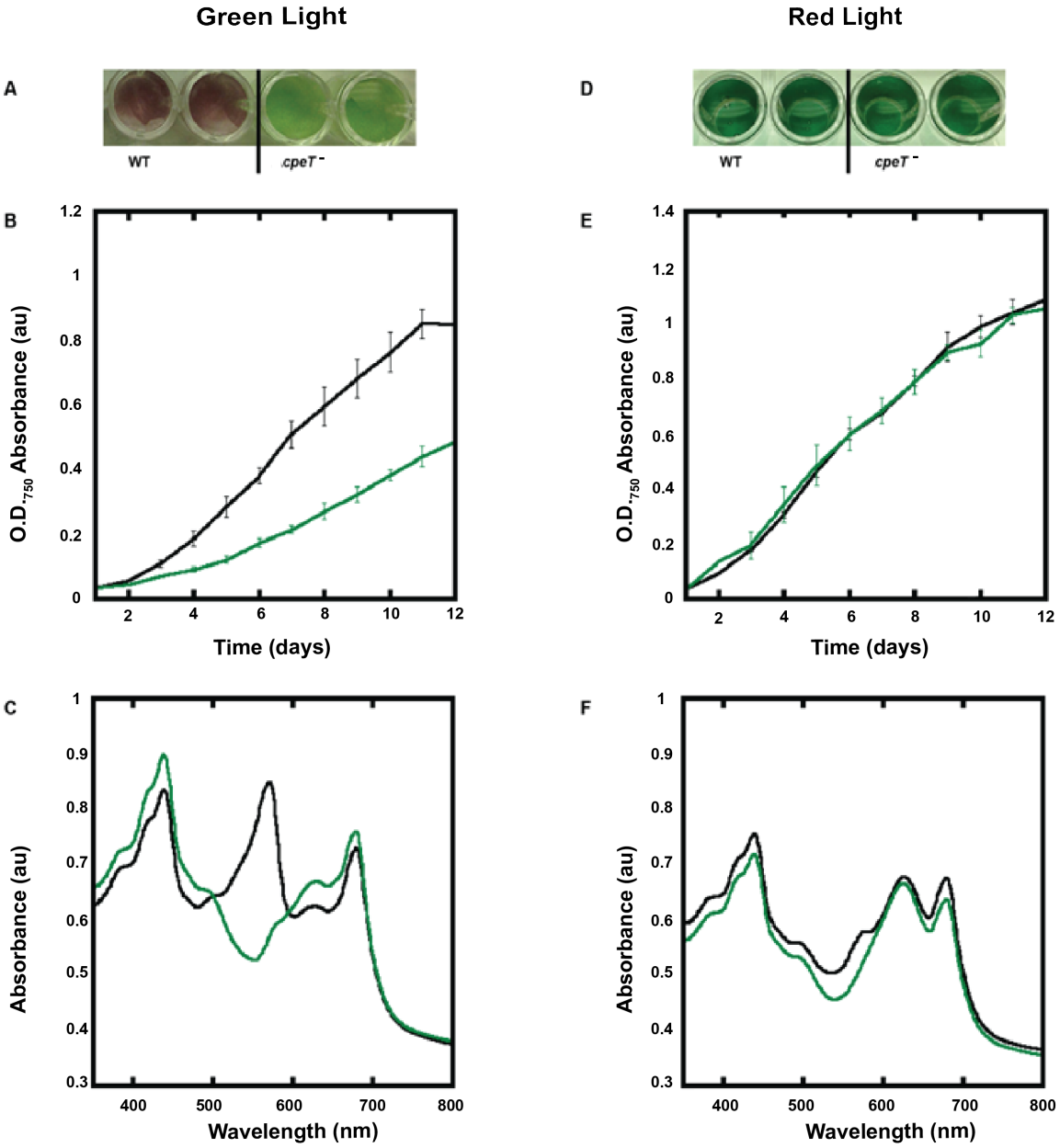

Figure 1. Whole cell phenotype and absorbance spectra of WT and *cpeT*- deletion mutant
Whole-cell color phenotype of WT and *cpeT*- deletion mutant grown under GL conditions (A) and red-light conditions (D). Panels B-F show the differences between WT (black line) and *cpeT*- *(*green line) whole cell absorbance spectra. GL severely affects *cpeT*- growth (B) while there is no growth defect under RL (E). Whole-cell absorption spectra of *cpeT*- deletion mutant cells grown in GL show that cell PE accumulation is nearly undetectable (C) while PC accumulation under RL conditions are comparable to WT cells. Cells were grown under 15 µmol photons m^{-2} s^{-1} GL or RL. Chlorophyll peaks are present at 430 nm and 680 nm; PE peak is at 565 nm; and PC peak is at 620 nm. Error bars represent standard errors of the means. These results are representative of two independent replicates.

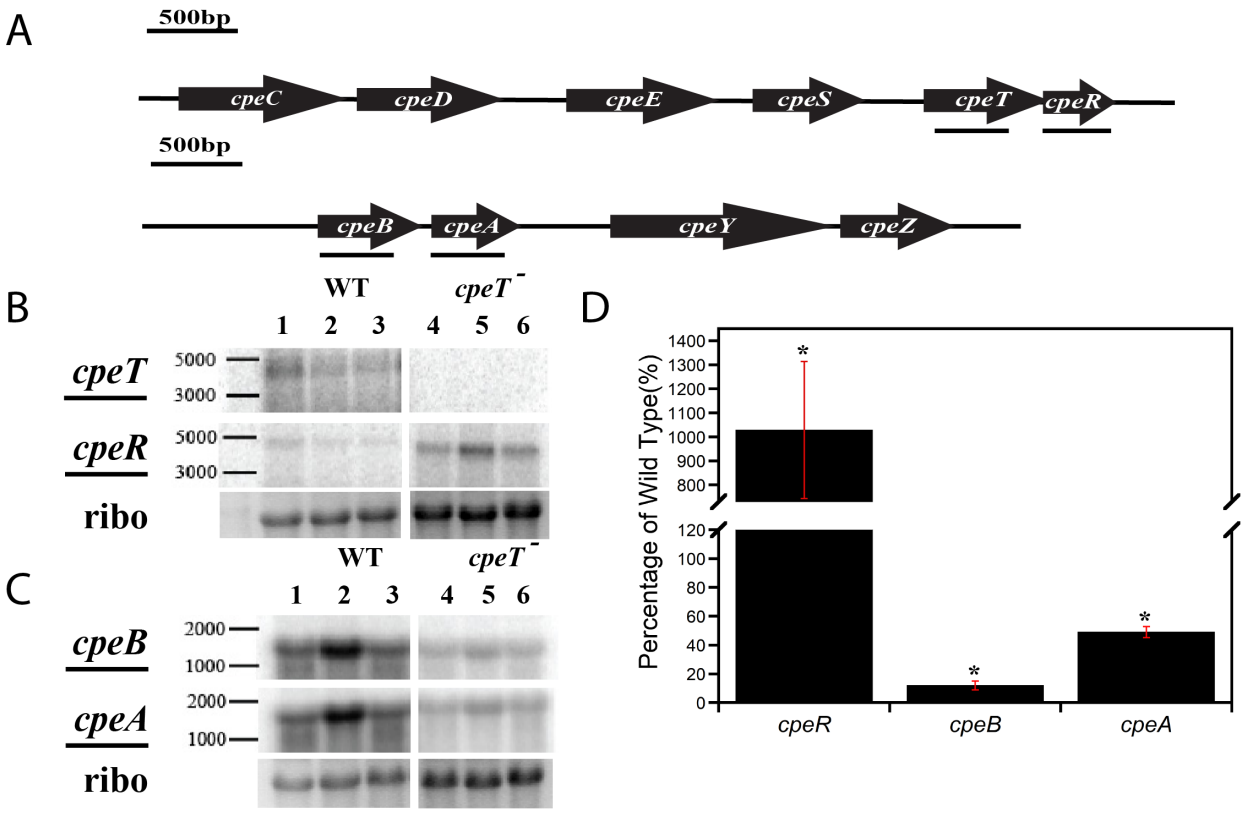

Figure 2. RNA gel blot analyses of *cpeCDSTR* and *cpeBA* operons in GL-grown WT and *cpeT* deletion mutant

(A) Genomic locations of *cpeT* and *cpeR* (located within the 3' end of the *cpeCDESTR* operon) and *cpeBA*. Lines underneath *cpeT*, *cpeR*, *cpeA*, and *cpeB* represent binding sites for 200 bp probes generated by PCR using primers in Table S1. RNA blot autoradiographs for (B) *cpeT* and *cpeR* and (C) *cpeB* and *cpeA* in WT and *cpeT*$^-$ mutants with loading controls for 16S ribosomal bands (ribo). Molecular mass markers in nucleotides are shown to the left. The WT *cpeCDESTR* transcript is ~4900 nucleotides and the *cpeBA* transcript is 1450 nucleotides. (D) Mean values from the three technical replicates (shown in B and C) for *cpeR*, *cpeB* and *cpeA* RNA levels in the *cpeT*$^-$ mutant expressed as a percentage of the WT GL value, which was set to 100%. All measurements were normalized using relative ribosomal RNA intensity values before calculation of the means. Standard errors are shown, with p-values (Mann-Whitney U) indicated as $* p < 0.05$.

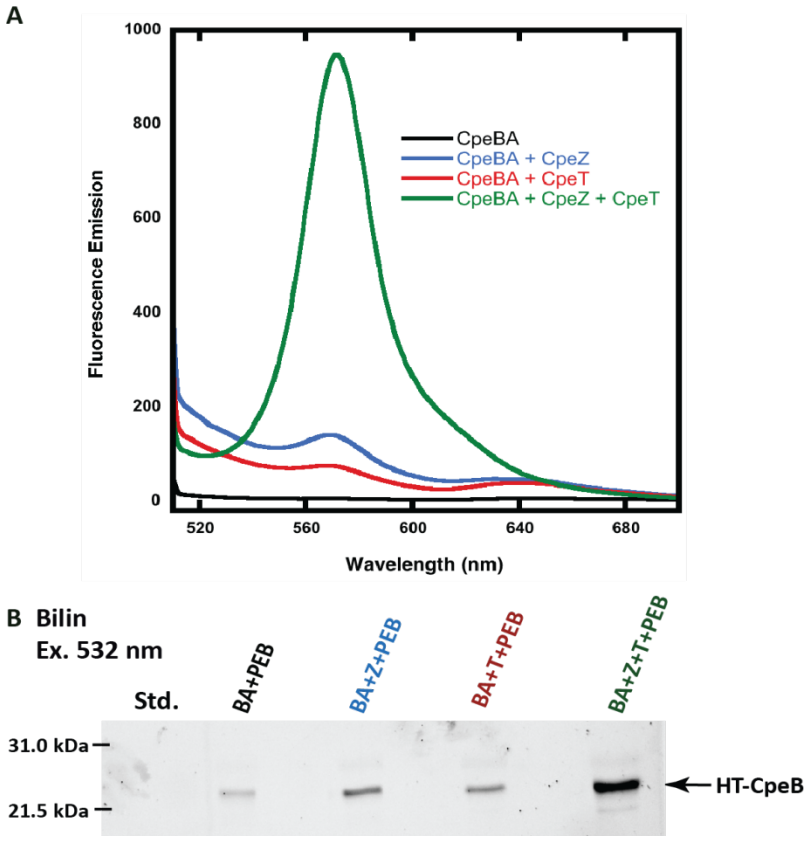

Figure 3. Recombinant CpeT coexpressions with CpeBA
(A) Fluorescence emission (excitation set at 490 nm) spectra of purified CpeBA coexpressed with PEB (black; pCpeB/CpeA; see Table S2), CpeZ (blue; pCpeZ; see Table S2), CpeT (red; (pCpeT3; see Table S2), and both CpeZ and CpeT (green); (B) SDS-PAGE analysis of the coexpressions, zn-enhanced bilin fluorescence analysis with excitation at 532 nm. Std denotes molecular weight standard with sizes indicated on the left. All coexpressions were completed with the presence of enzymes required for PEB synthesis (pPebS; see Table S2). These results are representative of three independent replicates.

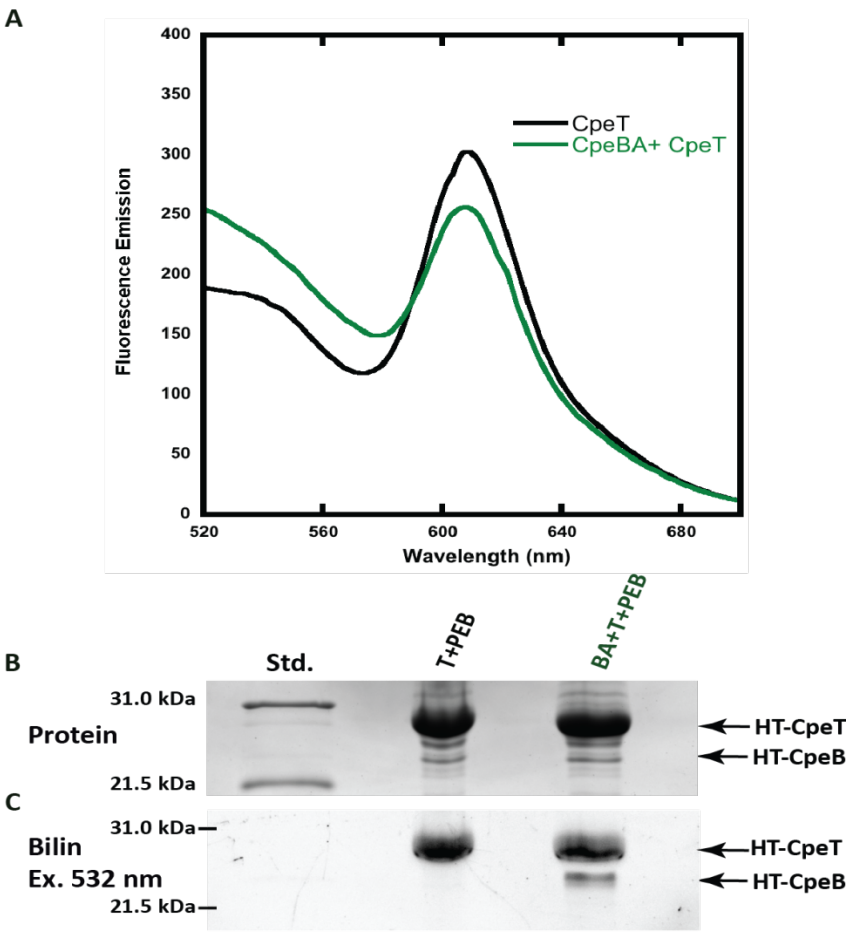

Figure 4: Recombinant HT-CpeT binds PEB.
HT-CpeT (pCpeT1; see Table S2) was expressed with PEB synthesis enzymes with and without CpeBA. (A) Fluorescence emission spectra (excitation set at 490 nm) of purified HT-CpeT coexpressed with PEB (black) or CpeBA, HT-CpeT and PEB (green). (B) SDS-PAGE analysis of the coexpressions stained with Coomassie Blue. (C) Zn-enhanced bilin fluorescence of the same gel shown in panel B with excitation at 532 nm. The location of the HT-CpeT (MW=25 kDa) and HT-CpeB (MW=23 kDa) are indicated with arrows at the right. These results are representative of three independent replicates.

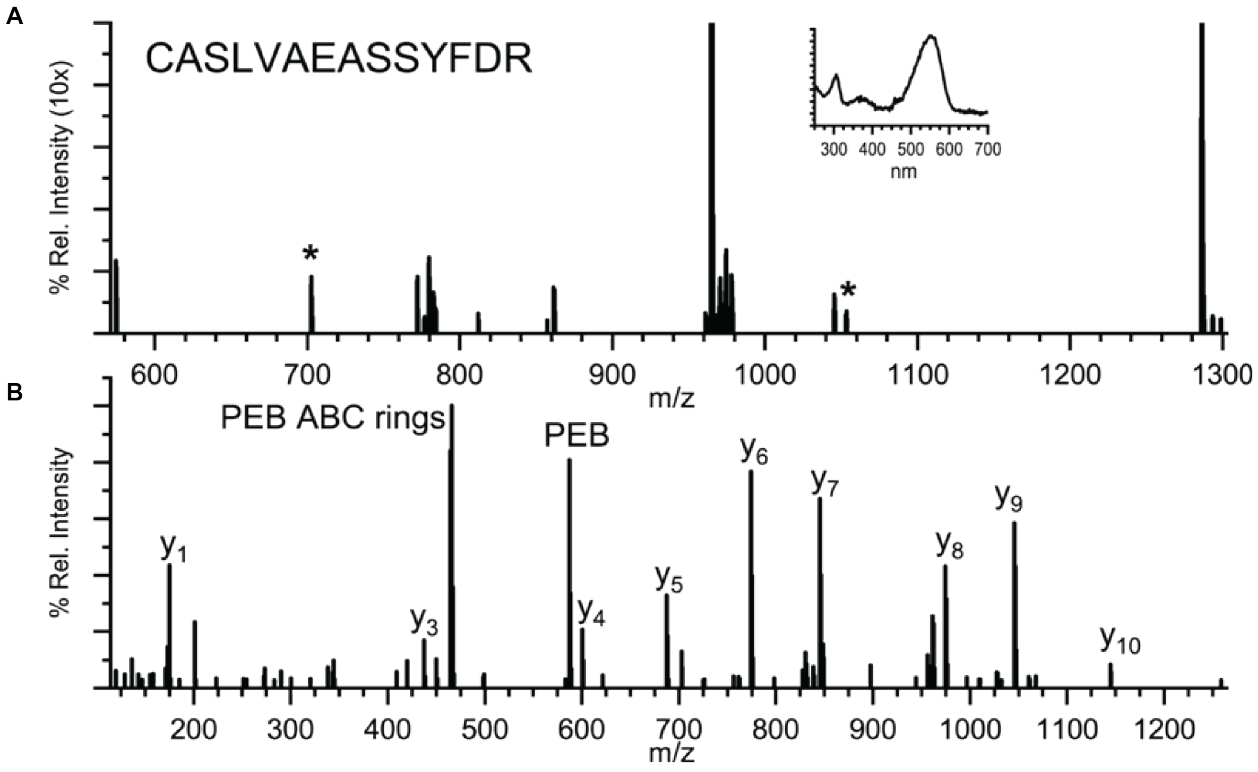

Figure 5. MS/MS data of recombinant CpeBA expressed with CpeT, CpeZ and PEB synthesis enzymes

(A) MS^1 data from 28.3 min peak in CpeBAZT sample. Asterisks indicate $(M+2H)^{2+}$ and $M+3H)^{3+}$ of peptide C_{165}ASLVAEASSYFDR from CpeB (vertical scale expanded 10-fold). (Inset) shows UV-VIS spectrum observed @ 28.3 minutes indicating presence of PEB on the peptide. (B) MS2 data from *m/z* 702.3284 @ 28.3 min; y ions are labeled, all masses are <3 ppm from predicted vales. These results are representative of two independent replicates.

Table 1: Observed LC-MS/MS peaks of trypsin digested recombinant HT-CpeBA peptides

Sample[^]	α-82	α-139	β-80	β-165	β-48/59
CpeBA+ CpeT (BAT)	ND[*]	ND	unmod	2:1 Unmod:PEB	ND
CpeBA+ CpeT + CpeZ (BATZ)	unmod	unmod	unmod	PEB	unmod

[^]Both coexpressions were completed with the presence of enzymes required for PEB synthesis (pPebS; see Table S2).

[*] ND: not detected; These peptides were not observed (modified or unmodified)