CpeF is the Bilin Lyase that Ligates the Doubly Linked Phycoerythrobilin on Phycoerythrin in the Cyanobacterium Fremyella Diplosiphon

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CpeF is the bilin lyase that ligates the doubly linked phycoerythrobilin on β-phycoerythrin in the cyanobacterium *Fremyella diplosiphon*

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**Running title:** CpeF is a bilin lyase for β-phycoerythrin

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**ABSTRACT**

Phycoerythrin (PE) is a green light-absorbing protein present in the light-harvesting complex of cyanobacteria and red algae. The spectral characteristics of PE are due to its prosthetic groups, or phycoerythrobilins (PEB), that are covalently attached to the protein chain by specific bilin lyases. Only two PE lyases have been identified and characterized so far, and the other bilin lyases are unknown. Here, using *in silico* analyses, marker-less deletion, biochemical assays with purified and recombinant proteins, and site-directed mutagenesis, we examined the role of a putative lyase-encoding gene, *cpeF*, in the cyanobacterium *Fremyella diplosiphon*. Analyzing the phenotype of the *cpeF* deletion, we found that *cpeF* is required for proper PE biogenesis, specifically for ligation of the doubly linked PEB to Cys-48/Cys-59 residues of the CpeB subunit of PE. We also show that in a heterologous host, CpeF can attach PEB to Cys-48/Cys-59 of CpeB, but only in the presence of the chaperone-like protein CpeZ. Additionally, we report that CpeF likely ligates the A ring of PEB to Cys-48 prior to the attachment of the D ring to Cys-59. We conclude that CpeF is the bilin lyase responsible for attachment of the doubly ligated PEB to Cys-48/Cys-59 of CpeB and together with other specific bilin lyases contributes to the post-translational modification and assembly of PE into mature light-harvesting complexes.

Cyanobacteria, often referred to as “blue-green algae,” are photosynthetic prokaryotes capable of absorbing light energy from their environment using a light-harvesting complex called the phycobilisome (PBS; see Fig. 1A). The PBS is comprised of homologous, pigmented phycobiliproteins which are composed of α- and β-subunits in heterohexameric forms (αβ)₆, assembled in disk-like stacked structures with the aid of specific linker proteins. In the freshwater cyanobacterium *Fremyella diplosiphon*
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(Tolypothrix sp. PCC 7601), the PBS consists of an allophycocyanin (AP; \( \lambda_{\text{max}} = 650-655 \) nm) core and both phycocyanin (PC; \( \lambda_{\text{max}} = 615-640 \) nm) and phycoerythrin (PE; \( \lambda_{\text{max}} = 495-575 \) nm) rods, with PE being core-distal (1).

Many cyanobacteria are capable of altering their PBS rod compositions in the presence of a changing photo-environment in a process called chromatic acclimation or CA (2). *F. diplosiphon* is classified as a Type III member (CA3) in which the distal portions of the PBS rods are made of PE under green light conditions, but under red light conditions they are made of PC (3). This is a photo-reversible process that is regulated both at transcriptional and post-transcriptional levels (2,4) to control the expression of PE and PC subunits, lyases, chromophore biosynthesis enzymes and linker proteins necessary for the assembly of the appropriate PBS (2). The AP core and the rod-proximal portions remain unaltered under both light conditions and are vital for the final energy transfer to the photosynthetic complexes embedded in the thylakoid membrane.

The brilliant color and light absorbing properties of phycobiliproteins are due to the presence of tetrapyrrole phycobilins or bilin chromophores which are covalently attached by thioether linkages to highly conserved cysteine residues (5). The two bilins found in *F. diplosiphon*, which differ in double bond conjugation, are phycocyanobilin (PCB; \( \lambda_{\text{max}} = 620-650 \) nm) attached to AP and PC, and phycocerythrobilin (PEB; \( \lambda_{\text{max}} = 540-565 \) nm) attached to PE (6). PCB and PEB are derived from heme (hence the name of linear tetrapyrroles with rings A to D; see Fig. S1A) which is first converted to biliverdin-IX\( \alpha \) then reduced by ferredoxin-dependent bilin reductases within cyanobacteria (7). Chromoprotein ligation to phycobiliprotein α- and β-subunits occurs post-translationally through bilin lyase catalyzed reactions. Each bilin attachment site in the AP and PC subunits is chromophorylated by a specific bilin lyase (8). PE in *F. diplosiphon* contains five covalently attached PEB chromophores for efficient photosynthetic light capture, two on CpeA (α-subunit of PE) at Cys-82 and Cys-139, and three on CpeB (β-subunit of PE) one each at Cys-80, Cys-165, and one that is doubly attached at Cys-48/Cys-59 (see Fig. S1A).

There are three major families of bilin lyses that have been characterized thus far in cyanobacteria: the CpcS/CpcU-type, the CpcT-type, and the CpcE/CpcF-type (9). Members of the CpcS/CpcU family of lyases have an antiparallel β-barrel structure (10), and are involved in the ligation of PEB to PCB to the Cys-82-equivalent on β-PC, β-PE, α-AP, and β-AP subunits (8,11-13). The CpcT family of lyases are related to CpcS/CpcU lyases (13), and have high chromophorylation site specificity for the Cys-153 equivalent position on β-PC (9,14). The crystal structure of CpcT from *Nostoc (Anabaena)* sp. PCC 7120 (15) as well as the homolog ΦCpeT from the P-HM1 cyanophage (16) were recently elucidated, and are similar to CpcS/CpcU lyases; both CpcT and ΦCpeT proteins form a homodimer with a β-barrel fold. The third family of lyases is the CpeE/CpeF-type (E/F), which is unrelated in sequence and structure to the other lyase families. Members of this E/F family that have been characterized are generally involved in ligating chromophores to the α-subunits of phycobiliproteins (17-21). Some members of this lyase family have the ability to perform chaperone-like functions (21,22) while others have been shown to function as bilin lyase/isomerases (23-25). All E/F-type lyases contain multiple HEAT-repeat motifs that facilitate protein-protein interactions (23,25,26). The crystal structure of CpeE/CpeF from *Nostoc* sp. PCC 7120 was determined, and the structure is primarily alphahelical consisting of crescent-shaped elongated super-helices or solenoids (27).

It has been suggested that the five PEB binding sites on PE in *F. diplosiphon* are chromophorylated by a minimum of five bilin lyses (28,29). Thus far, only two PE lyses have been characterized, the CpcS/CpcU-type homolog CpeS (attaches PEB to CpeB-Cys-80 (29)) and the E/F-type homolog CpeY (attaches PEB to CpeA-Cys-82 with the aid of the CpeZ chaperone (29,30)). The other bilin lyases for the remaining Cys sites are unknown, although it is hypothesized to include lyses from each of the three lyase families (28), i.e. CpeT (14,31), CpeU (11,31), and CpeF (31,32). Here we characterize the functional role of CpeF, a member of the E/F-type lyase family in *F. diplosiphon*, by using a cpeF deletion mutant and recombinant protein studies and find that it is involved in the chromophorylation of
CpeB at position Cys-48/Cys-59, and we show that attachment at ring A of PEB to Cys-48 likely occurs prior to ring D ligation at Cys-59. This is the first report of an E/F-type lyase being responsible for chromophore ligation to a β-subunit of a phycobiliprotein.

RESULTS

In silico analysis of CpeF from F. diplosiphon

In F. diplosiphon, the 912 bp cpeF gene is located approximately 400 bp downstream from the pebAB operon (Fig. S1B), whose transcript abundance increases in cells grown under green light (33) and encodes the ferredoxin-dependent bilin reductases PebA and PebB. These two proteins, in addition to heme oxygenase (encoded elsewhere in the genome by hol), are responsible for producing PEB from cellular heme (7).

To gain insight into the potential role of CpeF, we analyzed its sequence (32.4 kDa predicted, residues 11-313 from GenBank Accession Number EKF00796.1) by BLASTp 2.3.1 (34,35), and found high sequence similarity to: phycobiliprotein lyases (41-74% similarity), phycocerythrin II biosynthesis protein MpeU from cyanobacteria (43-54% similarity), glycosyl transferases family 2 (37-87% similarity), and hypothetical proteins. CpeF showed high similarity to MpeV and MpeU from the marine Synechococcus sp. WH 8020 (54% and 50% similar, respectively), both of which were originally identified within the phycocerythrin II operon and are members of the E/F family of lyases (32,36,37). CpeF was 36% similar to CpeE and 28% similar to CpeF from Synechococcus sp. PCC 7002, the first E/F-type bilin lyase to be characterized (20). CpeF was also 30% similar to MpeZ, an E/F-type lyase/isomerase characterized from Synechococcus sp. RS 9916 responsible for attaching PEB to Cys-83 of PEII α-subunit and subsequently isomerizing it to different type of bilin called phycourobilin (PUB, λ_{max}=490-495 nm) (25). We further analyzed the CpeF sequence by Phyre² modeling program (38) to predict the secondary and 3D structures. Using PDB ID: 5N3U as a template (27), Phyre² predicted that the CpeF protein has an α-solenoid type structure (Fig. S1C), consistent with the structure of the only E/F-type lyase crystallized to date, CpeE/CpeF from Nostoc sp. PCC 7120 (27). 74% of CpeF’s secondary structure was predicted with very high confidence to form α-helices, and only 9% of the protein was disordered. The protein CpeF appears to have six HEAT-repeat domains present (39) which are featured in all members of the E/F lyase family (23,25,26). For the predicted 3D structure (Fig. S1C), 243 residues of CpeF (303 residues total; 80%) were modeled with 99.89% confidence by the template PDB ID: 5N3U (27).

After taking into account multiple factors, including its location in the F. diplosiphon genome, the six HEAT-repeat motifs within the sequence, the amino acid homology to other known E/F-type lyases, and the predicted secondary and 3D structure, we hypothesized that cpeF encodes an E/F-type bilin lyase that is responsible for attaching PEB to one or both subunits of PE.

Phenotypes and whole cell analyses of wild type Fremyella diplosiphon and ΔcpeF

To determine the function of cpeF in cyanobacteria, we generated a markerless deletion (40-42) of cpeF in F. diplosiphon (ΔcpeF; Fig. S1B) and observed that these cells manifested a PE-deficient phenotype (Fig. S2). This phenotype was readily observable for cells grown in green light, which normally induces the red-colored PE in wild type (WT; hence the brick-red colored cultures; Fig. S2A). In the ΔcpeF cells, green light failed to induce a high level of PE (hence the green colored cultures; Fig. S2A). Whole cell absorbance spectra confirmed that ΔcpeF mutant had reduced levels of PE when compared to WT (Fig. S2B). We also found that the ΔcpeF mutant grew slower than WT in green light (Fig. S2C), but in red light, there was no difference in growth between the mutant and WT cells (data not shown). These results indicate that deleting cpeF has an adverse effect on the expression of PE in F. diplosiphon during growth in green light.

Analyses of PBS assembly in ΔcpeF vs WT cells

In order to determine the effect of deleting cpeF on PBS composition, PBS from WT and ΔcpeF were purified using sucrose density gradients and analyzed using absorbance and fluorescence emission spectroscopy. Following ultracentrifugation, two colored fractions were observed in the WT sucrose density gradient: a minor “top” fraction consisting of small amounts of dissociated phycobiliproteins and a major
“bottom” purple fraction containing fully assembled PBS (Fig. 1A). The absorption spectra of the WT complete PBS showed a large amount of PE (λ_{max}=568 nm) in comparison to the amount of PC (λ_{max}=615 nm) and AP (λ_{max}=649 nm; Fig. 1B and Table S1). When excited at 490 nm, WT PBS emit strongly at 669 nm with a small shoulder at 582 nm, indicating a transfer of energy from PE to PC to AP with some emission from PE (582 nm; Fig. 1B and Table S1).

In the ΔcpeF mutant, three fractions were collected from the sucrose gradients (which purified PBS of various sizes and composition) and labeled as top, middle, and bottom with the bulk of the PBS migrating to the middle and bottom fractions (Fig. 1A). As in WT, the “top” fractions consisted of dissociated proteins and were not analyzed further due to the small amounts present. The migration of the ΔcpeF PBS to the middle fraction indicates a smaller molecular mass for the PBS compared to those in the bottom fractions. Nevertheless, both the middle and bottom fractions isolated from ΔcpeF were blue in color, indicating a reduction in PE and a predominance of PC and AP. The absorbance spectra confirmed severely reduced levels of PE within the PBS (Fig. 1B). However, when exciting the PBS at 490 nm, energy was transferred from PE to PC to AP and exhibited fluorescence emission at 673 nm, indicating the presence of some PE within both middle and bottom fractions of the PBS, albeit less than that found in WT (Fig. 1B and Table S1). The maximal absorbance by PE within the PBS from the mutant cells in both middle and bottom fractions was at 577 nm (shoulder peak in Fig. 1B), which is red-shifted when compared to WT peak (λ_{max}=568 nm; Fig. 1B).

PBS samples from WT and ΔcpeF were further analyzed by SDS-PAGE for protein and bilin content. Covalent bilin attachment to proteins was examined by fluorescence emission of the gel after incubation with ZnSO₄ with excitation at 532 nm (Fig. 1D), which detects both PEB and PCB, or at 635 nm (Fig. 1E), which detects PCB only (43). The protein stain indicated that in comparison to WT, both CpeA and CpeB levels were reduced in the ΔcpeF mutant PBS samples, with CpeB being less abundant than CpeA (Fig. 1C). PEB fluorescence was reduced as measured by Zn-enhanced fluorescence when excited at 532 nm (Fig. 1D), with CpeB fluorescing less than CpeA. This reduced Zn-enhanced fluorescence may be attributed to a reduced amount of PE produced in the mutant. Western blot analysis confirmed the presence of both CpeA and CpeB in the ΔcpeF samples, but the quantities of both proteins were drastically reduced compared to WT (Fig. 1, panels F and G). The relative amount of PC also apparently increased in the ΔcpeF mutant in comparison to WT (Fig. 1, panels C, E, and H). Together, these data show that when the putative lyase-encoding gene cpeF was deleted, PE synthesis was severely reduced and normal levels of PE being assembled into the PBS was hindered, and the PE absorption peak was significantly red-shifted.

**Analyses of PE in ΔcpeF vs WT cells**

To determine how PE alone (i.e. free and assembled in PBS) was being affected in the ΔcpeF mutant, total PE was isolated from WT and ΔcpeF cells and subsequently analyzed using spectroscopy and SDS-PAGE (Fig. 2). WT PE had a maximum absorbance at 563 nm with a shoulder at 545 nm and the ΔcpeF PE had a maximum absorbance at 565 nm which was lower relative to the shoulder at 544 nm, but both PE samples had a maximum fluorescence at 573 nm when excited at 490 nm (Fig. 2A). The small absorbance peak at 625 nm present in the mutant sample was caused from PC/AP contamination (data not shown). Zn-enhanced fluorescence (excitation at 532 nm) of WT PE showed a high fluorescence signal for both CpeB and CpeA (Fig. 2C). However, in the ΔcpeF sample, CpeB protein levels and Zn-enhanced fluorescence of CpeB were severely reduced when compared to CpeA (Fig. 2, panels B and C). Western blot analyses using both Anti-CpeA and Anti-CpeB antibodies (Fig. 2, panels D and E, respectively) confirmed that CpeB levels in the mutant PE sample were severely reduced when compared to CpeA.

To identify potential changes in the post-translational modifications of PE, we used LC-MS/MS to analyze PE samples purified from both WT and ΔcpeF after trypsin digestion (Table 1). As expected, there were five peptides (see Table S5 in (30)) containing PEB detected from the WT PE sample; two from CpeA and three from CpeB (Table 1). Peptides were separated by HPLC and ligated chromophores detected using visible
absorbance in line with the mass spectrometer. The non-chromophorylated peptides containing the five Cys sites were also detected but in very low amounts within the peptide mixtures (see Fig. S2 in (30)). In contrast, PE purified from the ΔcpeF mutant only had four observable peaks with bound PEB (Table 1). The m/z 1200.06 peak, corresponding to the (M+4H)^4+ ion of CpeB-Cys-48/Cys-59 peptide with the doubly ligated PEB, was not detected (Fig. 3A). Generally, a peptide with such a large mass-to-charge ratio is more difficult to detect, especially given its size and the low abundance of CpeB in the sample. However, the same CpeB peptide was detected without a bilin (Table 1 and Fig. 3A; m/z 1404.32^35; PEB MW=587 Da). The unmodified version of this peptide (no bound PEB) was modified by 2-mercaptoethanol (BME) present in the buffer during the protein purification and digestion processes (Fig. 3A). Interestingly, the CpeB-Cys-80 peptide mixtures from the ΔcpeF mutant PE sample had PEB, PCB, or PUB attached, as identified by their UV-Vis spectra (Fig. 3, panels B-H; PEB, λ_{max}=540-565 nm; PCB, λ_{max}=620-650 nm; PUB, λ_{max}=490-495 nm). However, the ratio of peptides with PUB to peptides with PCB attached was 3:2 within the peptide mixture, and very low amounts of PUB-bound peptides were detected. Based on these observations we conclude that in vivo, cpeF is required for Cys-48/Cys-59 PEB chromophorylation on CpeB. Abrogation of this chromophorylation induces loss of CpeB and overall PE reduction in the cell. PEB addition at the Cys-48/Cys-59 sites may also enhance PEB addition at subsequent sites such as Cys-80 in cyanobacteria.

**Recombinant CpeF protein coexpressions**

To better define the role of CpeF in PE chromophorylation, we examined its role in a heterologous system where both CpeF and CpeB were coexpressed in *E. coli* that were engineered to produce PEB (30,44). Hexahistidine-tagged (HT-) CpeB was purified using Ni-NTA affinity chromatography after cell lysis and analyzed using fluorescence emission spectroscopy and SDS-PAGE (Fig. 4). Recombinant CpeF was coproduced with HT-CpeB and the PEB synthesis enzymes PebS/Hol (45) to detect any potential lyase activity. This expression combination resulted in very low HT-CpeB solubility (Fig. 4B) with no detectable CpeB fluorescence (Fig. 4, panels A and C). It has been previously observed that very little soluble CpeB accumulates within *E. coli* when it is expressed without a PEB chromophore post-translationally attached to Cys-80 (29). Therefore, we used the lyase CpeS (CpeB-Cys-80 lyase (29)) to enhance the solubility of HT-CpeB both alone and in the presence of CpeF. In these experiments, CpeF was expressed after all others using a sequential induction system to chromophorylate Cys-80 prior to CpeF expression (see Experimental Procedures). Isolated HT-CpeB showed an increase in fluorescence (Fig. 4A) both in the presence and absence of CpeF due to the covalent attachment of PEB to Cys-80 verified by Zn-enhanced fluorescence (Fig. 4C); however, the solubility of HT-CpeB remained very low (Fig. 4B) suggesting that even partially chromophorylated CpeB does not accumulate in an *E. coli* system very efficiently.

To further increase the amount of soluble HT-CpeB and potentially boost the lyase activity of CpeF, we asked if the chaperone-like protein CpeZ could enhance these processes (29,30). Coexpressing HT-CpeB, the lyase CpeS, the chaperone-like protein CpeZ, and PebS/Hol, with subsequent expression of CpeF, dramatically increased both HT-CpeB protein solubility (Fig. 4B) and PEB fluorescence (Fig. 4, panels A and C) when compared to the same coexpressions without CpeS. When HT-CpeB was coproduced with CpeS, CpeZ, and PebS/Hol, a fluorescent product was detected but the signal was less intense than when CpeF was present. Since CpeS is known to attach PEB to Cys-80 of CpeB (29) and CpeZ acts as a chaperone only (30), we reasoned that CpeF was directly involved in PEB attachment to one or both of the remaining Cys sites in CpeB (Cys-165 and Cys-48/Cys-59).

To further examine PEB attachment on CpeB at these cysteine sites, the recombinant protein samples were digested with trypsin and analyzed by MALDI-MS. As predicted, any sample including the lyase CpeS had a PEB attached to Cys-80 (m/z 1250.71^111; Table S2). When HT-CpeB was expressed alone with CpeF or with CpeF and CpeZ, no PEB chromophore was detected on any HT-CpeB peptide by MS (Table S2). However, when HT-CpeB was expressed with CpeZ, CpeS and CpeF, a PEB chromophore
was detected on Cys-48/Cys-59 (m/z 4627.4^{1+}) as well as Cys-80 (m/z 1250.7^{1+}; Table S2 and Fig. 5). The peptide with m/z 4627.4^{1+} was detected in low quantities (Fig. 5, arrow 3^*); however, its detection by MS indicated that when recombinant CpeB was partially chromophorylated by CpeS and in the presence of the chaperone-like protein CpeZ, then CpeF functioned as a lyase by attaching one PEB to CpeB at Cys-48/Cys-59.

Given the previous observations that recombinant phycobiliprotein production is enhanced when both α- and β-subunits are coexpressed (13, 14), we sought to test if coexpression with CpeA would improve the yield of soluble CpeB. For this we made a new construct, HT-CpeBA, and analyzed CpeB as before. To account for chromophorylation of CpeA-Cys-82, we also coexpressed the CpeY lyase (29). Although CpeA was not His-tagged, small amounts co-purified with HT-CpeB, its natural interacting partner, in these expressions. When compared to the previous coexpressions, a greater amount of soluble HT-CpeB protein accumulated in the presence of CpeA and PebS/HoI (Fig. S3B), and very little non-enzymatic PEB addition occurred (Fig. S3C). No fluorescence was observed when the sample was excited at 490 nm (Fig. S3A). The control experiments with CpeF or CpeZ alone in the presence of HT-CpeBA and PebS/HoI resulted in low soluble protein yields and little to no fluorescence (Fig. S3, panels A-C) as seen previously when CpeA was not present (see Fig. 4). However, when both CpeF and CpeZ were expressed together with HT-CpeBA and PebS/HoI, a slightly fluorescent product was produced indicating the presence of a covalently bound PEB (Fig. S3, panels A-C). Furthermore, increasing the number of covalently bound PEB molecules to either CpeB (by CpeS (29)) or CpeA (by CpeY (29)) helped increase the accumulation (Fig. S3E) and the fluorescence (Fig. S3, panels D and F) of CpeB.

To determine which Cys residues had a PEB covalently bound, all fluorescent protein samples were digested with trypsin and analyzed by LC-MS/MS. As expected, in every sample that had CpeS expressed, a PEB was covalently bound to CpeB at Cys-80 (m/z 417.54^{3+}; Table 2). In the control sample where only CpeS was expressed, no other Cys residues were chromophorylated in the peptide mixtures (Table 2 and Fig. 6A). Also, as expected, in every sample that had CpeY expressed, a PEB was bound to CpeA at Cys-82 (m/z 468.23^{2+}) even though only small amounts co-purified with HT-CpeB. When CpeF was expressed alone with HT-CpeBA, no fluorescence was detected and therefore was not analyzed by MS. However, when both CpeF and CpeZ were expressed together, a small amount of fluorescence was detected, and MS revealed that a very small amount of CpeB-Cys-48/Cys-59 peptides were chromophorylated with PEB (Table 2 and Fig. 6B). This chromophorylated peptide was detected in every sample where CpeF was coexpressed (Table 2). The amount of chromophorylated CpeB-Cys-48/Cys-59 peptides appeared to increase as more proteins were expressed in the system, such as the lyases CpeS and CpeY and the chaperone-like protein CpeZ (Table 2 and Fig. 6, panels C and D). Overall, these results indicate that CpeF acts as a bilin lyase responsible for attaching PEB to CpeB at Cys-48/Cys-59, and this activity is increased in the presence of the CpeZ chaperone and when either CpeA (if present) or CpeB is partially chromophorylated.

Analyses of CpeF mechanism of PEB attachment using site-directed variants

Crystal structures of PE from various species of cyanobacteria reveal that the doubly bound bilin to CpeB is attached to the Cys-48 and Cys-59 equivalent residues at the A ring and D ring of the bilin, respectively (46-48); however, the order of PEB attachment to these sites has not been examined. To identify the order of this attachment and, thus, gain insight into the mechanism of CpeF PEB ligation, site-specific variants within CpeB were produced (C48S and C59S) in our HT-CpeBA construct. Recombinant CpeF was coproduced in E. coli with HT-CpeBA, C48S, or C59S variants with the CpeZ chaperone and the PEB synthesis enzymes PebS/HoI (45). His-tagged proteins were purified and analyzed by fluorescence emission spectroscopy, SDS-PAGE, and immunoblotting (Fig. 7). All three coexpressions produced fluorescent products due to the attachment of PEB to CpeB (Fig. 7, panels A and B); however, the fluorescence produced from the mutant variants was reduced (Fig. 7, panels A and B) when compared to the WT HT-
CpeBA. The amount of soluble HT-CpeB in all samples (Fig. 7, panels A and B) was relatively similar as measured by Western blot analyses (Fig. 7C). We quantitated the PEB fluorescence (Fig. 7B) and the amount of soluble CpeB protein band intensities (Fig. 7C) from four independent replicates. Fig. 7D represents how much CpeB was chromophorylated in each protein sample (e.g. bilin content:protein amount) after normalization (see Experimental Procedures). The average bilin content:CpeB ratio (n=4) of C59S variant was 46% of WT while the C48S variant was 24% of WT, indicating the C48S variant had a more severe reduction of chromophorylation which was significantly different from that of WT (P=0.0277; Fig. 7D). The chromophore conformation in the binding pocket of CpeB is such that Cys-48 will ligate to ring A followed by ligation at ring D to Cys-59 (46). Together, these data indicate that when either Cys-48 or Cys-59 are mutated to Ser, PEB addition to CpeB is still possible; however, there is a more severe reduction in binding to the C48S variant, indicating this is likely the first site of attachment for PEB followed by thioether formation with ring D at Cys-59.

**DISCUSSION**

This study investigates the bilin lyase function of CpeF. The deletion of *cpeF* caused a reduced PE phenotype, indicating that this protein plays a role in PE biosynthesis and/or stability. The remaining and minor PE amount that was incorporated into the PBS of the Δ*cpeF* mutant had a 9 nm red-shifted absorbance when compared to WT PE. This red shift might be the result of the presence of a non-covalently attached PEB within the bilin-binding pocket of PE. This non-covalent PEB would have an extra double bond in conjugation (11,18,19,49). Each bilin within the structure of the PBS contributes uniquely to the overall absorbance spectrum (1,50). Another possibility is the complete absence of PEB from one or more Cys sites, which may also cause a shift in the absorbance spectrum as previously demonstrated in other lyase mutants when bilins were missing from phycobiliprotein subunits (11,17,18). CpeF’s role as a bilin lyase is consistent with this phenotype. The absence of PEB at CpeB-Cys-48/Cys-59 in our Δ*cpeF* mutant is likely the cause of the decrease in the peak at 565 nm relative to the shoulder in the absorbance spectrum for the PE.

An unexpected result was that the MS analysis of PE purified from Δ*cpeF* mutant cells also showed that PCB (Fig. 3H) and PUB (Fig. 3D) were covalently attached to CpeB peptides at Cys-80 (in addition to PEB). This Cys-80 binding site is normally chromophorylated by CpeS (29). Since atypical bilins are covalently attaching to this site in the Δ*cpeF* mutant, it is possible that CpeF assists in the activity of CpeS *in vivo* to facilitate the interaction between CpeS, PEB, CpeZ and/or CpeB, however no interactions between CpeF and CpeS proteins were detected in pull-down assays (data not shown). Another possibility is that in cyanobacteria, CpeF acts before CpeS in PEB ligation and that without CpeF, less efficient PEB ligation at Cys-80 by CpeS occurs. Bilins naturally have a high affinity for their binding pockets in phycobiliproteins. Non-enzymatic bilin addition has been seen in PE subunits, where PEB was ligated at very slow reaction rates and in some cases, resulted in the attachment of oxidized or isomerized bilin adducts (51). Since these analyses were performed on PE purified from whole cells, it is possible that these CpeB variants are not incorporated into mature PBS. Phycobiliproteins with large defects in folding or chromophorylation are prevented from assembling into PBS (52), and these partially chromophorylated subunits may be targeted for turnover (step 2B in the model in Fig. 5 from (30)).

The lack of bilins on phycobiliproteins (due to a mutation of the Cys ligation site of the phycobiliprotein or in the gene encoding the bilin lyases) affects stability, turnover rates of the protein, the assembly of phycobiliproteins into the PBS, and energy transfer efficiency (1,50,52,53). Once apo-phycobiliproteins are chromophorylated, they form monomers (αβ), trimers (αβ)₃ and then hexamers (αβ)₆ which are assembled into the PBS. If PE is missing one or more PEB chromophores, this could result in instability of the PE subunits leading to protein degradation, preventing proper assembly into the PBS, thereby decreasing PBS light capturing and energy transfer efficiency (1,53,54). In the absence of bilin lyases and bilin ligation, there is a marked decrease in phycobiliprotein content (11,14,17,55-57),
consistent with our results when cpeF was deleted in *F. diplosiphon*.

Our heterologous expression system in *E. coli* demonstrated that recombinant CpeF was able to attach PEB to Cys-48/Cys-59 on CpeB only after CpeB was already chromophorylated by CpeS on the central Cys-80 site and stabilized by CpeZ (29). This sequential induction system was useful for detecting the lyase function of CpeF. CpeZ showed a chaperone-like function and helped stabilize the conformation of recombinant CpeB (30). It is possible that the stabilized conformation of CpeB is necessary for CpeF to recognize the Cys-48/Cys-59 site for the attachment of PEB. It is likely that an ordered ligation of each PEB to PE subunits is necessary *in vivo* to ensure that holo-PE is properly synthesized in cyanobacteria. Biswas *et al.* suggested that addition to the central bilin at CpeB-Cys-80 occurs first to produce the appropriate conformational stability for CpeB which allows the other lyses to act on the other two sites at Cys-165 and Cys-48/Cys-59 (29). However, Zhao *et al.* investigated the order of bilin addition in the homologous protein CpcB in *Nostoc* PCC 7120 (58), and demonstrated that the primary addition of a bilin to the CpcB-Cys-155 site allowed for the subsequent chromophorylation of the CpcB-Cys-84 site, whereas the inverse scenario inhibited the addition of the second bilin (58). CpeB from *F. diplosiphon* is more complex than CpcB in terms of bilin-binding sites (CpcB has two, CpeB has three). Further experimentation is required to determine the actual sequence of PEB addition to CpeB.

The mechanism of E/F-type lyases is believed to be complex and has been investigated using the heterodimeric CpeE/CpeF and PecE/PecF (21,22,27). Both subunits are necessary to efficiently attach a bilin to the corresponding α-subunit. For example, PecE first binds PecA and is able to slowly attach PCB, which is accelerated if PecE and PecA form a complex prior to the addition of PCB, indicating that protein-protein interaction is the rate-limiting step (22). When the isomerizing subunit PecF is bound to the complex, the PCB on PecA is isomerized to phycoviolobilin (22). In *F. diplosiphon*, CpeF and CpeB interaction may be efficiently facilitated by CpeZ, which stabilizes CpeB’s conformation. Ligation of ring A of PEB to Cys-48 and then ring D to Cys-59 likely occurs because the two proximal Cys residues are oriented appropriately in relation to PEB by CpeF. The site-specific variants showed that while single thioether linkages to either ring A (C59S) or ring D (C48S) were possible, the amount of PEB attached when only attachment via ring D was possible was about half that seen when only attachment at ring A was possible. This suggests a mechanism whereby ring A ligates first followed by ring D; however, another interpretation is that ligation at ring D is more contingent on ligation at ring A than the reverse scenario. If CpeB is not stabilized or does not adopt the appropriate structure, the Cys binding site(s) may not be accessible for CpeF to recognize and bind to CpeB. The modeled structure of CpeF (Fig. S1C) is most similar to the crystal structure for CpeE (27); we did not investigate whether CpeF is active as a monomer or a dimer, but binding of the PEB might occur in the groove of CpeF (red arrow in Fig. S1C).

In conclusion, we have shown that CpeF plays a role in the biosynthesis of PE in cyanobacteria by functioning as the bilin lyase that attaches PEB to Cys-48/Cys-59 of CpeB. This double ligation mechanism likely occurs by the attachment of the ring A to Cys-48 first and then the ring D is ligated to Cys-59. This study provides insight into the complicated process of PE biosynthesis in cyanobacteria and the mechanism of how this CpeF lyase facilitates the double ligation of PEB to CpeB.

**EXPERIMENTAL PROCEDURES**

**Generation of cpeF deletion strain and culture conditions**

SF33 (59,60), a shortened filament mutant of *Freymella diplosiphon* UTEX 481 (also called *Tolypothrix* sp. PCC 7601) was used as wild type and was cultured as previously described (61). To avoid polar effects on neighboring genes, the cpeF knockout mutant was generated as a clean deletion as previously described (40-42) with a few alterations. The cpeF deletion construct made by fusing two DNA fragments corresponding to the upstream and downstream regions of cpeF was cloned in the NcoI site of pJCF276 suicide plasmid (41) and used to transform *F. diplosiphon* as described previously (30). The site of *cpeF* deletion was designed to leave a short chimeric 28-long amino acid polypeptide. The primers used to clone the *cpeF* deletion construct are provided...
in Table S3. Clean deletion of cpeF gene was confirmed by PCR polymorphism with primers flanking the region of interest.

Growth curves were generated from 2 independent replicates of cells grown in ~15 μmol m⁻² s⁻¹ of green light monitored by absorbance at 750 nm every 24 h for 12 days.

**Genome and sequence analysis**

The nucleotide sequence for cpeF from *F. diplosiphon* UTEX 481 was retrieved from the GenBank database (GenBank Accession Number AGCR01000053.1; locus tag: FDUTEX481_08608) (62). Amino acid sequences (CpeF (residues 11-313), GenBank Accession Number EKF00796.1) were analyzed using the ClustalW alignment tool from MacVector software V. 12.7.5 (MacVector Inc., Apex, NC), BLASTp 2.3.1 from NCBI (34,35), and Phyre² prediction system (38).

**Construction of expression plasmids**

Plasmids used in this study are listed in Table S4. Some expression constructs were previously described, but the newly produced constructs were sequenced at the W. M. Keck Conservation and Molecular Genetics Laboratory (University of New Orleans). Each gene was amplified by PCR from *F. diplosiphon* chromosomal DNA using the primers listed in Table S3. Each resulting amplicon was cloned into a Duet-1 vector (Novagen/EMD Millipore Corp., Darmstadt, Germany) or pBAD/Myc-His A vector (Thermo Fisher Scientific, Waltham, MA) as listed in Table S4 after digestion with restriction enzymes (whose recognition sites were embedded into the primers; see Table S3).

The pCpeBA plasmid was used as a template to produce the site-specific variants pCpeB(C48S)CpeA and pCpeB(C59S)CpeA using standard methods as previously described (29) and using the primers listed in Table S3.

**Heterologous expression and purification of recombinant proteins**

Recombinant proteins were expressed and purified from *E. coli* BL21 (DE3) cells as previously described (29). For cells containing both Duet-1 vectors (Novagen/EMD) and pBAD/Myc-His A vector (Thermo Fisher Scientific), cultures were sequentially induced first with 1 mM isopropyl l-thio-β-D-galactopyranoside (for expression of Duet-1 vectors) at 18 °C for 2-3 h, then with 0.2 mg ml⁻¹ L-arabinose (for expression of the pBAD/Myc-His A vector) at 18 °C for an additional 16 h before being harvested by centrifugation. After purification, total protein concentrations were quantified using the Quick Start Bradford Protein Assay kit (Bio-Rad).

**Isolation of cyanobacterial proteins**

All protocols to isolate and purify *F. diplosiphon* proteins including PBS and PE were previously described (30). Total protein concentrations were quantified using the Quick Start Bradford Protein Assay kit (Bio-Rad).

**Tryptic digestion and mass spectrometry analysis**

Tryptic digestion of protein samples containing HT-CpeB expressed from the plasmids pCpeB2 and pCpeBZ was conducted as described earlier (29). All other digestions were conducted using the altered protocol as previously described (30). After digestion, samples were analyzed as described using MALDI MS and tandem MALDI MS/MS (29) or LC-MS/MS analysis using a Waters Synapt HDMS Quadrupole-TOF mass spectrometer (25,30).

**Protein analysis by spectroscopy and gel-electrophoresis**

Spectroscopy and gel-electrophoresis were conducted as previously described (30). Protein purification was verified using SDS-PAGE. Protein band intensities and Zn-enhanced bilin fluorescence band intensities were quantified and analyzed using Quantity One 1-D Analysis Software and Image Lab Software V5.2.1 (Bio-Rad). Ratios of the fluorescence from PEB:CpeB intensities from four independent replicates were analyzed by a 2-way ANOVA (α=0.05) with experimental replicate and strain (or mutant) as factors. The WT ratio was set to 1 and the mutants were represented relative to this value.

**Immunoblotting analysis**

Immunoblotting analysis was performed as described (29) using the following primary antisera generated in rabbits: Anti-CpeA (1:5,000 dilution (29)), Anti-CpeB (1:20,000 dilution), Anti-CpeA (1:5,000 dilution (11)) and Anti-CpcS.
(1:5,000 (13)). Anti-CpeB antibodies were generated in rabbits against holo-CpeB purified from *F. diplosiphon* as described (30) (YenZym Antibodies, San Francisco, CA). Protein band intensities were quantified and analyzed using Quantity One 1-D Analysis Software and Image Lab Software V5.2.1 (Bio-Rad).

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The nucleotide sequence for *cpeF* from *F. diplosiphon* UTEX 481 can be accessed through the GenBank database under GenBank Accession Number AGCR01000053.1; locus tag: FDUTEX481_08608. The amino acid sequence of this protein (CpeF; residues 11-313) can be accessed through the GenBank database under GenBank Accession Number EKF00796.1.

**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article.
REFERENCES
CpeF is a bilin lyase for β-phycoerythrin


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**FOOTNOTES**
This research was supported by National Science Foundation Grants to D.M.K. (MCB-1029414 and MCB-1818187) and to W.M.S. (MCB-1244339 and MCB-0843664) and by the Performance and Accountability fellowship to C.M.K. from the University of New Orleans Graduate School.

The abbreviations used are: AP, allophycocyanin; BME, 2-mercaptoethanol; CA3, Type III chromatic acclimation; EICs, extracted ion chromatograms; HT-, hexahistidine-tagged; MW, molecular weight; Ni-NTA, nickel-nitritriacetic acid; NT-, non-tagged; PBS, phycobilisome(s); PC, phyocyanin; PCB, phycocyanobilin; PE, phycoerythrin; PEB, phycoerythrobilin; PUB, phycourobilin.

**TABLES**

Table 1: Observed LC-MS/MS peaks of tryptic digested PE peptides

<table>
<thead>
<tr>
<th>Strain</th>
<th>α-82 (m/z)+1</th>
<th>α-139 (m/z)+1</th>
<th>β-80 (m/z)+1</th>
<th>β-165 (m/z)+1</th>
<th>β-48/59 (m/z)+1</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>936</td>
<td>1361</td>
<td>1251</td>
<td>2105</td>
<td>4797</td>
</tr>
<tr>
<td>ΔcpeF</td>
<td>936</td>
<td>1361</td>
<td>1251</td>
<td>2105</td>
<td>4210b</td>
</tr>
</tbody>
</table>

a Peptide mixtures contained peptides with PEB, PCB, or PUB bound at that specific Cys residue.
b Only the peptide without bound PEB (m/z 4210; PEB MW=587 Da) was observed.

Table 2: Observed LC-MS/MS peaks of tryptic digested recombinant HT-CpeBA peptides

<table>
<thead>
<tr>
<th>Samplea</th>
<th>α-82</th>
<th>α-139</th>
<th>β-80</th>
<th>β-165</th>
<th>β-48/59 (% PEB)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA+S+PEB</td>
<td>ND</td>
<td>unmod</td>
<td>PEB</td>
<td>unmod</td>
<td>unmod (0)</td>
</tr>
<tr>
<td>BA+SF+PEB</td>
<td>unmod</td>
<td>unmod</td>
<td>PEB</td>
<td>unmod</td>
<td>PEB (14.1)</td>
</tr>
<tr>
<td>BA+F+Z+PEB</td>
<td>unmod</td>
<td>unmod</td>
<td>PEB</td>
<td>ND</td>
<td>PEB (12.5)</td>
</tr>
<tr>
<td>BA+F+YZ+PEB</td>
<td>PEB</td>
<td>unmod</td>
<td>unmod</td>
<td>unmod</td>
<td>PEB (2)</td>
</tr>
<tr>
<td>BA+SF+Z+PEB</td>
<td>unmod</td>
<td>unmod</td>
<td>PEB</td>
<td>unmod</td>
<td>PEB (18.1)</td>
</tr>
<tr>
<td>BA+SF+YZ+PEB</td>
<td>PEB</td>
<td>unmod</td>
<td>PEB</td>
<td>unmod</td>
<td>PEB (35.6)</td>
</tr>
</tbody>
</table>

“ND” represents peptides that were not detected.
“unmod” represents unmodified peptides that lack a PEB chromophore.
a See Fig. S3 for sample descriptions.
b Values in parentheses represent the estimated percentage of β-48/59 peptides chromophorylated with PEB relative to non-chromophorylated β-48/59 peptides based upon the integrated intensities for the various charge states and missed cleavages or non-enzymatic modifications (e.g. addition of BME, oxidation of methionine, internal disulfide bond).
**Fig. 1: Analyses of PBS purified from WT and ΔcpeF**

WT PBS structure is illustrated above the sucrose gradient (63). (A) Sucrose gradients of WT (left) and ΔcpeF (right) cellular extracts from cells grown in green light. Fractions are labeled “top,” “middle,” and “bottom.” (B) Absorbance (solid lines) and fluorescence emission (dashed lines; excitation set at 490 nm) spectra of PBS fractions purified from WT (bottom fraction, black lines) and ΔcpeF (middle fraction, cyan lines; bottom fraction, blue lines) *F. diplosiphon* cells grown in green light. (C) The Coomassie-stained SDS-polyacrylamide gel for PBS purified from WT (bottom fraction) and ΔcpeF (middle and bottom fractions). The gel was loaded with 5 μg total protein per sample. (D) and (E) The Zn-enhanced fluorescence of the gel in part C excited at (D) 532 nm and (E) 635 nm. Lane “Std” indicates the molecular weight standard. (F) through (H) Western blot analyses of purified PBS samples from WT (bottom fraction) and ΔcpeF (bottom and middle fractions) using (F) Anti-CpeA, (G) Anti-CpeB, or (H) Anti-CpcA antibodies. The gel was loaded with 5 μg total protein per sample. These results are representative of three independent replicates.
CpeF is a bilin lyase for \( \beta \)-phycoerythrin

Fig. 2: Analyses of PE purified from WT and \( \Delta cpeF \)

(A) Absorbance (solid lines) and fluorescence emission (dashed lines, excitation set at 490 nm) spectra of PE purified from WT (black lines) and \( \Delta cpeF \) (cyan lines) \( F. \) diplosiphon cells grown in green light. (B) The Coomassie-stained SDS-polyacrylamide gel for PE purified from WT and \( \Delta cpeF \). Lane “Std” indicates the molecular weight standard. The gel was loaded with 10 \( \mu l \) of each sample. (C) The Zn-enhanced fluorescence of the gel in part B excited at 532 nm. (D) and (E) The Western blot analyses of PE purified from WT and \( \Delta cpeF \) using (D) Anti-CpeA and (E) Anti-CpeB antibodies. These results are representative of three independent replicates.
CpeF is a bilin lyase for β-phycoerythrin

Fig. 3: Extracted ion chromatograms and LC-MS spectra for tryptic digested peptides from ΔcpeF PE

(A) Combined extracted ion chromatograms (EICs) for the peptides RLDAVNAIASNASC\textsubscript{48}MVSDAVAGMIC\textsubscript{59}ENQGLIQAGGNCYPR at \textit{m/z} 1200.06 ((M+4H)\textsuperscript{4+}, PEB modified, blue line) and \textit{m/z} 1404.32 ((M+3H)\textsuperscript{3+}, unmodified + 1 BME, red line) of CpeB isolated from ΔcpeF cells grown in green light. Numbers 1-3 indicate three versions of the unmodified peptide observed (1 BME on Cys 48, 59, or 71).

(B) EICs for the peptides MAAC\textsubscript{80}LR at \textit{m/z} 625.81 ((M+2H)\textsuperscript{2+}, bilin modified, blue line) and \textit{m/z} 332.67 ((M+2H)\textsuperscript{2+}, unmodified, red line and asterisk) of CpeB isolated from ΔcpeF cells grown in green light. Numbers 1-5 indicate the MAAC\textsubscript{80}LR peptides that have a bilin bound.

(C) MS of unmodified MAAC\textsubscript{80}LR.

(D) through (H) MS for the 5 modified versions of the peptide MAAC\textsubscript{80}LR containing Cys-80 as specified in panel B. Inset graphs are the UV-Vis absorbance spectra for the peaks at the retention times that are numbered with a + charge. The type of bilin bound to Cys-80 is indicated per panel. These results are representative of two independent replicates.
**Fig. 4: Recombinant CpeF coexpressions with HT-CpeB**

(A) Fluorescence emission (excitation set at 490 nm) spectra of purified HT-CpeB obtained from *E. coli* cells expressing pPebS in addition to pCpeB2 and pCpeS (B+S+PEB; blue); pCpeB2 and pHT-CpeF2 (B+F+PEB; grey); pCpeB2, pCpeS and pHT-CpeF2 (B+S+F+PEB; orange); pCpeBZ and pCpeS (BZ+S+PEB; cyan); pCpeBZ and pHT-CpeF2 (BZ+F+PEB; magenta); or pCpeBZ, pCpeS, and pHT-CpeF2 (BZ+S+F+PEB; red). (B) The Coomassie-stained SDS-polyacrylamide gel for purified HT-CpeB samples from panel A. Lane “Std” indicates the molecular weight standard. (C) The Zn-enhanced fluorescence of the gel in panel B excited at 532 nm. These results are representative of three independent replicates.
Fig. 5: MALDI-MS spectrum of recombinant HT-CpeB peptides
MALDI-MS spectrum of the peptide mixture resulting from the trypsin digestion of HT-CpeB purified from *E. coli* cells expressing pCpeBZ, pCpeS, pHT-CpeF2, and pPebS (BZ+S+F+PEB sample). Arrows bearing numbers indicate peaks that were identified as tryptic peptides containing a Cys residue without PEB bound: Cys-80 (arrow 1, *m/z* 664.4\(^{+}\)), Cys-165 (arrow 2, *m/z* 1518.8\(^{+}\)) and Cys-48/Cys-59 (arrow 3, *m/z* 4040\(^{+}\)). Arrows bearing numbers with asterisks indicate peaks that were identified as tryptic peptides containing a PEB chromophore. Attachment was found to occur at Cys-80 (arrow 1*, *m/z* 1250.7\(^{+}\)) and double attachment at Cys-48/Cys-59 (arrow 3*, *m/z* 4627.4\(^{+}\)). No attachment to Cys-165 was detected. Inset graph is a close-up view of the *m/z* 4500-4900 range. These results are representative of two independent replicates.
Fig. 6: Extracted ion chromatograms for tryptic digested peptides from HT-CpeBA coexpressions
Combined extracted ion chromatograms for unmodified Cys-48/Cys-59 peptides (red) and PEB cross-linked versions of Cys-48/Cys-59 peptides (blue, and indicated with arrows) from purified HT-CpeBA obtained from E. coli cells expressing pCpeBA and pNT-PebS in addition to (A) pCpeS (BA+S+PEB), (B) pCpeF2 and pNT-CpeZ3 (BA+F+Z+PEB), (C) pCpeSF2 and pNT-CpeZ3 (BA+SF+Z+PEB), or (D) pCpeSF2 and pNT-CpeYZ (BA+SF+YZ+PEB). These results are representative of two independent replicates.
Fig. 7: Recombinant CpeF coexpressions with HT-CpeB variants show the order of PEB ring A vs ring D ligation

(A) Fluorescence emission (excitation set at 490 nm) spectra of purified HT-CpeBA variants obtained from *E. coli* cells expressing pCpeF2, pNT-CpeZ3, and pNT-PebS in addition to pCpeBA (BA+F+Z+PEB; magenta); pCpeB(C48S)CpeA (B(C48S)A+F+Z+PEB; purple); or pCpeB(C59S)CpeA (B(C59S)A+F+Z+PEB; cyan). All protein samples were diluted to 0.75 µg µl⁻¹ prior to obtaining the fluorescence spectra. (B) The Zn-stained SDS-polyacylamide gel for purified HT-CpeBA variant samples from panel A. Lane “Std” indicates the molecular weight standard with size markers shown at left. The gel was loaded with 2 µg total protein per lane. (C) Western blot analysis of purified HT-CpeBA variant samples using Anti-CpeB antibodies. The gel was loaded with 2 µg total protein per sample. The asterisk indicates degraded CpeB; this degradation product was not observed in panel B because it did not have PEB covalently attached. (D) Scatter plot representing the normalized ratio of Zn fluorescence from PEB (panel B):CpeB intensities as obtained from the Western blot in panel C for the HT-CpeB samples. Error bars represent the standard deviation of four independent replicates (mean values are indicated with a horizontal line for each sample set). The average BA+F+Z+PEB ratio was set to 1, and the mutants are represented relative to this value in panel D. Normalized fluorescence data were analyzed by a 2-way ANOVA (α=0.05) with experimental replicate and strain (or mutant) as factors. Fluorescence of the strains were significantly different (*P*=0.0292). Of note, B(C48S)A+F+Z+PEB showed significantly lower fluorescence compared to BA+F+Z+PEB (Tukey’s Honestly, *P*=0.0277, noted with asterisk). Strain B(C59S)A+F+Z+PEB was intermediate between B(C48S)A+F+Z+PEB and BA+F+Z+PEB (*P*=0.0941 vs B(C48S)A+F+Z+PEB and *P*=0.6054 vs BA+F+Z+PEB).