Characterization of cyanobacterial allophycocyanins absorbing far-red light

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Abstract:

Phycobiliproteins (PBPs) are pigment proteins that comprise phycobilisomes (PBS), major light-harvesting antenna complexes of cyanobacteria and red algae. PBS core substructures are made up of allophycocyanins (APs), a subfamily of PBPs. Five paralogous AP subunits are encoded by the Far-Red Light Photoacclimation (FaRLiP) gene cluster, which is transcriptionally activated in cells grown in far-red light (FRL; λ = 700 to 800 nm). FaRLiP gene expression enables some terrestrial cyanobacteria to remodel their PBS and photosystems and perform oxygenic photosynthesis in far-red light (FRL). Paralogous AP genes encoding a putative, FRL-absorbing AP (FRL-AP) are also found in an operon associated with improved low-light growth (LL; <50 μmol photons m^{-2} s^{-1}) in some thermophilic *Synechococcus* spp., a phenomenon termed Low-Light Photoacclimation (LoLiP). In this study, *apc* genes from FaRLiP and LoLiP gene clusters were heterologously expressed individually and in combinations in *Escherichia coli*. The resulting novel FRL-APs were characterized and identified as major contributors to the FRL absorbance observed in whole-cells after FaRLiP and potentially in LoLiP. Post-translational modifications of native FRL-APs from FaRLiP cyanobacterium, *Leptolyngbya* sp. strain JSC-1, were analyzed by mass spectrometry. The PBP complexes made in two FaRLiP organisms were compared, revealing strain-specific diversity in the FaRLiP responses of cyanobacteria. Through analyses of native and recombinant proteins, we improved our understanding of how different cyanobacterial strains utilize specialized APs to acclimate to FRL and LL. We discuss some insights into structural changes that may allow these APs to absorb longer light wavelengths than their visible-light-absorbing paralogs.

Introduction

Phycobiliproteins (PBPs) are brilliantly colored, water-soluble proteins that bind linear tetrapyrroles known as bilins (Glazer 1989; Ledermann et al. 2017; Bryant and Canniffe 2018). In most cyanobacteria and red algae, PBPs are assembled into megacomplexes, phycobilisomes (PBS), which harvest visible light (λ = 400 to 700 nm) for oxygenic photosynthesis (Bryant et al. 1979; Chang et al. 2015; Zhang et al. 2017; Bryant and Canniffe 2018; Ma et al. 2020). PBS are composed of peripheral rods radiating from a multi-cylinder, core subcomplex that is organized and anchored to the thylakoid membrane by a specialized linker-PBP, ApcE, also known as the core-membrane linker (L_{cm}) (Bryant 1988, 1991; Capuano et al. 1993; Sidler 1994; Chang et al. 2015; Bryant and Canniffe 2018). The PBS core comprises two linker proteins (ApcE and ApcC) and various allophycocyanin (AP) subunits, which bind the bilin chromophore phycocyanobilin (PCB) and usually absorb maximally at ~650 nm. Trimeric AP, $(\alpha\beta)_3$, forms toroid-shaped heterohexameric complexes, comprising three $(\alpha\beta)$ heterodimers (note that these heterodimers are usually described as AP monomers or protomers) (Bryant et al. 1976; Brejc et al. 1995). Stacks of these trimers form the core cylinders of PBS (Bryant et al. 1976, 1979; Brejc et al. 1995; Singh et al. 2015; Chang et al. 2015; Zhang et al. 2017; Bryant and Canniffe 2018; Ma et al. 2020).

The peripheral rods of PBS contain similar stacks of trimeric $(\alpha\beta)_3$ or hexameric $(\alpha\beta)_6$ toroids of phycocyanin (PC; λ_{max} ~620 nm; Bryant et al. 1979; Schirmer et al. 1985, 1986, 1987; Zhang et al. 2017) and in some cases phycoerythrin (PE; λ_{max} ~540 nm; Bryant et al. 1979; Kumar et al. 2016; Zhang et al. 2017; Ma et al. 2020) or phycoerythrocyanin (λ_{max} ~575 nm; Bryant et al. 1976; Glazer 1989; Dürring et al. 1990; Bryant and Canniffe 2018). The varied spectroscopic properties of PBPs result either from the presence of different isomeric bilins or from different bilin-bilin and protein-bilin interactions (Glazer 1989; Xu et al. 2016; Miao et al. 2016). The funnel-like organization of PBPs in PBS, from higher-to-lower energy-absorbing and emitting complexes from the periphery to the core, facilitates efficient transfer of excitation energy from the peripheral rods to the core, and subsequently to accessory chlorophylls of the photosystems (Glazer 1989; Dong et al. 2009; Liu et al. 2013; Ho et al. 2019). In a

process known as complementary chromatic acclimation, some cyanobacteria are able to alter the PBP or bilin content of their PBPs/PBS in response to different light conditions, thereby adjusting their absorbance to allow more efficient light harvesting (Kehoe and Gutu 2006; Shukla et al. 2012; Ho et al. 2017a,b,c).

Some terrestrial cyanobacteria undergo a different photoacclimation process, Far-Red Light Photoacclimation (FaRLiP), in response to far-red light (FRL) (Gan et al. 2014; Ho et al. 2017a,b,c). When exposed to FRL (λ = 700 to 800 nm), these cyanobacteria produce specialized, paralogous APs that comprise the cores of PBS or smaller, bicylindrical core (BC) complexes, which enable cells to absorb FRL (Ho et al. 2017b,c; Bryant et al. 2020). Similar paralogous AP proteins have also been implicated in improving the ability of *Synechococcus* spp. ecotypes to grow in low light in microbial mats through a process called Low-Light Photoacclimation (LoLiP) (Nowack et al. 2015; Olsen et al. 2015; Ho et al. 2017c). Cyanobacteria that perform FaRLiP can overcome visible-light limitation by utilizing FRL, which constitutes a significant portion of total solar irradiance reaching the surface of Earth. FRL penetrates more deeply than visible light in environments where light is often strongly filtered by chlorophyll *a* or physically scattered (Gan and Bryant 2015), as occurs in cyanobacterial mats (Ohkubo and Miyashita 2017), shaded areas beneath plants, soils (Zhang et al. 2019), beachrock (Trampe and Kühl 2016), and caves (Behrendt et al. 2020). FaRLiP strains of cyanobacteria possess a highly conserved cluster of 20 genes that encode most of the core subunits of the two photosystems and PBS (Gan et al. 2014, 2015). When FaRLiP strains are grown in FRL, they synthesize two new Chls, Chls *f* and *d*, which replace about 8% and 1% of Chl *a* molecules, respectively. Additionally, core subunits of the PBS and photosystem core complexes are replaced with paralogous proteins from the FaRLiP cluster, which enables the organism to utilize FRL for oxygenic photosynthesis (Gan et al. 2014; Li et al. 2016; Ho et al. 2017a,b,c; Herrera-Salgado et al. 2018; Bryant et al. 2020; Gisriel et al. 2020).

Low-light (LL) ecotypes of Type A *Synechococcus* isolated from ~1 mm within the upper green photic layer of a microbial mat in Mushroom Spring, Yellowstone National Park, were discovered to have an operon containing a putative Chl-binding protein (IsiX) and a pair of AP genes, *apcD4* and *apcB3,* that

are not present in high-light (HL) ecotypes that grow above them in the mat (Becraft et al. 2015; Olsen et al. 2015). According to phylogenetic analysis, the LoLiP AP (ApcD4-ApcB3) is similar to FaRLiP APs (Gan et al 2015), and we predicted that this protein would absorb FRL. Furthermore, LL-adapted *Synechococcus* ecotypes exhibit improved growth at low irradiance and far-red fluorescence emission that was absent in HL ecotypes (Nowack et al. 2015). This fluorescence could be the result of ApcD4-ApcB3 being expressed and incorporated into the photosynthetic apparatus of cells, which was termed low-light photoacclimation (LoLiP) because this phenotype was evident in LL- but not HL-grown cells (Olsen et al. 2015, Ho et al. 2017c).

An important goal of this study was to compare the FaRLiP responses of two cyanobacteria after short-term and long-term growth in FRL in order to determine whether the responses were similar or different. Additionally, we sought to understand better the properties of individual FRL-APs produced in cyanobacteria that can perform FaRliP or LoLiP. The FaRLiP gene cluster encodes three α-type AP subunits (ApcD5, ApcD2, and ApcD3), one β-type AP subunit (ApcB2), and an L_{cm} subunit (ApcE2); all are paralogs of proteins that are expressed when cells grow in visible light (**Fig. S1**) (Gan et al. 2014; Bryant et al. 2020). ApcE2 has two linker repeat (REP) domains and an N-terminal PCB-binding domain that is similar to α-subunit of PBPs, (Gan et al. 2014, 2015). This domain has previously been shown to absorb FRL by binding PCB noncovalently (Miao et al. 2016). The other four genes are presumed to encode three heterodimeric (αβ) APs: ApcD5-ApcB2, ApcD2-ApcB2 and ApcD3-ApcB2, which have the potential to form trimeric or mixed trimeric complexes as occurs in the PBS cores of other cyanobacteria and red algae (Chang et al. 2015; Zhang et al. 2017; Bryant and Canniffe 2018; Ma et al. 2020). In order to characterize these paralogous APs individually, combinations of α-type and β-type genes from *Leptolyngbya* JSC-1 were heterologously expressed in *Escherichia coli* with a polyhistidine [His]$_{10}$-tag on the α-type subunits. These proteins were co-expressed with the genes for PCB biosynthesis (*ho1*, *pcyA*) and a bilin lyase (*cpcS*) to catalyze the covalent attachment of PCB to the apo-PBPs. Similarly, ApcD4-ApcB3 from *Synechococcus* A1463 was heterologously produced in *E. coli* to facilitate spectroscopic characterization and demonstrate that it is a FRL-absorbing AP (FRL-AP).

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Materials and Methods

Strain information and growth conditions

Detailed information about the FaRLiP strains and the growth media used in this study were previously reported in Gan *et al.* (2014) for *Leptolyngbya* sp. strain JSC-1 (hereafter *Leptolyngbya* JSC-1) and in Ho et al. (2017a) for *Synechococcus* sp. PCC 7335 (hereafter *Synechococcus* 7335). In order to grow these cyanobacteria in FRL, a growth chamber was constructed with a combination of red (GamColor 250) and green (GamColor 650) plastic filters and LED light panels with output centered at 720 nm (L720-06AU, http://www.epitex.com, Marubeni, Santa Clara, CA). Two lamps with soft white halogen bulbs were placed outside of the box to provide additional FRL illumination through the plastic filters. This apparatus delivered approximately ~65 μmol photons $m^{-2} s^{-1}$ of continuous FRL light (λ = 700-800 nm) to the cells. The light intensity was monitored with a solar radiation meter (Apogee Instruments model MP-200, Logan, UT). The cultures were maintained for up to 18 months in FRL by serially subculturing into fresh growth medium once per month. Cultures were incubated at 35 °C, stirred with magnetic stir bars, and gently sparged with a mixture of air and 1% (v/v) CO_2 (Shen et al. 2019). Conditions for growth of cells in red light (RL) have been described previously (Gan et al. 2014; Ho et al. 2017a).

The low-light-adapted *Synechococcus* spp. strain A1463 (hereafter *Synechococcus* A1463) was isolated from Mushroom Spring, Yellowstone National Park, under permit #YELL-SCI-0129 held by David M. Ward (Montana State University) and administered under the authority of Yellowstone National Park. as described (Allewalt et al. 2006; Becraft et al. 2015). This strain was grown in B-HEPES medium (Dubbs and Bryant 1991) at 60 °C with slow sparging with 5% CO_2 (v/v) balanced with nitrogen and no stirring. Unless otherwise stated, cultures received approximately 40 μmol photons $m^{-2} s^{-1}$ of continuous white light provided by a soft white halogen bulb.

E. coli α-Select Silver Efficiency competent cells (Bioline, Memphis, TN) were used for routine cloning and preparation of plasmids in this study. *E. coli* strain BL21 (DE3) (New England Biolabs,

Ipswich, MA) was used for heterologous protein production. *E. coli* cells were grown in Luria-Bertani (LB) medium (pH = 7.0) supplemented with antibiotics as appropriate at 30 °C with shaking at 200 rpm.

Isolation of PBP complexes

The isolation of PBS, core complexes, and other PBP complexes was carried out as described by Ho *et al.* (2017b) with one modification. To improve the separation of multiple PBP complexes, the sucrose step gradients in this study were prepared with 2 mL 0.50 M sucrose, 3 mL 0.75 M sucrose, 4 mL 0.85 M sucrose, 4 mL 1.00 M sucrose, 3 mL 1.20 M, 3 mL 1.30 M sucrose, and 1 mL 1.50 M sucrose. All sucrose solutions were prepared in 1.50 M K-phosphate buffer (pH = 7.0) before adjusting to the final K-phosphate concentration to 0.75 M.

Mass spectrometric analysis of native PBPs

Sucrose gradient fractions containing FRL-absorbing PBPs from *Leptolyngbya* JSC-1 were pooled, dialyzed against Buffer 20 (20 mM NaCl, 20 mM K-phosphate, pH 7.0), and passed through an anion exchange column packed with diethylaminoethyl (DEAE) cellulose (Whatman DE52; MilliporeSigma, St. Louis, MO) pre-equilibrated with Buffer 20. A light-blue-colored fraction that eluted with 150 mM NaCl was collected and desalted with a PD-10 column containing Sephadex G-25 (GE Healthcare, Marlborough, MA). The column had been pre-equilibrated with 16 mg mL^{-1} ammonium bicarbonate, and proteins were eluted with 16 mg mL^{-1} ammonium bicarbonate. The protein solution was concentrated to ~100 µg mL^{-1} with an Amicon Ultrafiltration cartridge (3000 nominal molecular weight limit; Burlington, MA) by centrifugation at 8600 × *g*. After concentration, 2-mercaptoethanol was added to a final concentration of 1 mM, and formic acid was added to 0.1% (v/v) before the sample was passed through a 0.2-µm single-step polyvinylidene difluoride filter and subjected to high-performance liquid chromatography (HPLC) with electrospray-ionization mass spectrometric detection (LC-ESI-MS).

The LC-ESI-MS was performed on a Vanquish Flex liquid chromatography system (ThermoFisher Scientific, Waltham, MA) interfaced with a Q Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA). An aliquot of mixed PBPs (6 µL) was loaded on a BioBasic-4 column (50 mm

× 2.1 mm, 5-µm particle size, 300 Å pore size; ThermoFisher Scientific, Waltham, MA) and separated using the following gradient of solvent B in solvent A: 0 min to 1 min 5% B, 1 min to 3 min 5% to 50% B, 3 min to 13 min 50% to 70% B, and 13 min to 18 min 70% B. The composition of Solvent B was 33% isopropanol, 67% acetonitrile, 0.1% formic acid. Solvent A was 0.1% formic acid in water. The flow rate was 0.200 mL min^{-1}, and the column was maintained at 40 °C.

The mass spectrometer acquired full-scan, positive-ion mass spectra over the range 400 to 2000 m/z with the following parameters: in-source collision-induced dissociation, 10 eV; 2 microscans at resolution 140,000; automatic gain control target 5×10^6, and a maximum injection time of 300 ms. The heated electrospray ion source parameters were as follows: spray voltage 3.5 kV, capillary temperature 300 °C, S-lens Radio Frequency level 50. The sheath and auxiliary gas flows were adjusted based on the HPLC flow rate at 15 and 12 arbitrary units, respectively. The resulting mass spectra were interpreted manually using XCalibur Qual Browser software (Thermo Fisher Scientific, Waltham, MA), and protein molecular masses were calculated using ESIprot 1.0 (Winkler 2010).

In-solution trypsin digestion and peptide mass fingerprinting

To identify proteins in fractions from sucrose gradients, protein solutions containing PBPs were subjected to in-solution digestion with trypsin as described by Ho *et al.* (2017b) and by following protocols recommended by the Penn State Proteomics and Mass Spectroscopy Core Facility. The subsequent LC-MS-MS analyses were performed on a Thermo LTQ Orbitrap Velos ETD mass spectrometer equipped with a Dionex UltiMate 3000 Nano-flow 2D LC system (Thermo Fisher Scientific, Waltham, MA). Proteins were identified by database searches against the total proteomes of *Leptolyngybya* JSC-1 and *Synechococcus* 7335.

DNA constructs for heterologous production of PBPs in E. coli

The pAQ1-Ex (Xu et al. 2011) expression vector was used for heterologous production in *E. coli* of nearly all of the individual PBP subunits and PBP subunit combinations described: ApcA1-ApcB1, ApcD5-ApcB2, ApcD2-ApcB2, ApcD4-ApcB3, ApcD5, ApcD2, ApcD3, ApcD4, ApcB2, ApcB3,

ApcD5-ApcB1, ApcD2-ApcB1, ApcD3-ApcB1 and ApcD4-ApcB1, the genes for which were inserted between the NdeI and BamHI restriction sites either by restriction digestion and ligation or by Gibson assembly (Gibson et al. 2009). Whenever two subunits were produced in pAQ1-Ex, the corresponding genes were inserted into the plasmid as an operon. In order to express genes that do not naturally occur adjacent to one another in the chromosome (e.g., *apcD5-apcB1*, *apcD2-apcB1*, *apcD3-apcB1*, *apcD4-apcB1*, and *apcD2-apcB2*) in pAQ1-Ex with an N-terminal [His]$_{10}$-tag added to the α-type subunit, the corresponding gene was amplified with a 5′ overhang complementary to the non-coding region upstream of the desired β subunit gene. The β subunit gene was likewise amplified with a 3′ overhang complementary to the 5′ end of the α subunit gene. These complementary DNA fragments were assembled into one fragment by PCR and inserted into pAQ1Ex by Gibson assembly (Gibson et al. 2009). For co-expression of *apcD3* and *apcB2*, the pCDFDuet-1 vector was used with *apcD3* inserted by restriction digestion and ligation between BamHI and SalI in the first multiple-cloning site, and *apcB2* inserted between NdeI and XhoI in the second. The genes were amplified from *Leptolyngbya* JSC-1 or *Synechococcus* A1463 using Q5 High-Fidelity polymerase Master Mix (NEB #M0492S; New England Biolabs, Ipswich, MA) and the oligonucleotide primers listed in **Table S1**. For chromophorylation of all subunits, the *cpcS* bilin lyase gene from *Thermosynechococcus elongatus* was co-expressed using plasmid p*Te*CpcS (Kronfel et al. 2013), as well as genes for PCB biosynthesis in *E. coli* (*ho1*, *pcyA*) encoded on plasmid pPcyA (Biswas et al. 2010).

PBP production in E. coli

Plasmids pPcyA and pTeCpcS were transformed into electrocompetent *E. coli* strain BL21 (DE3) by electroporation to create a platform strain (BL21-PBP) for the expression of PBPs. Tubes containing 50 μL of BL21-PBP cells in 10% glycerol were stored at –80 °C and thawed on ice prior to transformation with a PBP-encoding plasmid. Cells were transformed by electroporation, diluted with 1 mL LB medium, and incubated at 37 °C for 20 min. Cells were plated on LB agar containing antibiotics as appropriate in the following concentrations: 25 μg chloramphenicol mL^{-1}, 50 μg spectinomycin mL^{-1}, 100 μg

kanamycin mL^{-1} and 200 μg carbenicillin mL^{-1}. The genotypes of individual colonies were verified by colony PCR and DNA sequencing before inoculation of 75 mL starter cultures of LB medium containing antibiotics. These starter cultures were shaken overnight at 200 rpm at 30 °C before being used to inoculate 1.0 L of LB medium containing antibiotics. Cultures were grown at 30 °C until OD$_{600}$ = 0.7 was reached, at which point isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 1 mM to induce gene expression from pPcyA and pTeCpcS. The flasks were shaken rapidly at 30 °C for 4 h, and the temperature was then decreased to 18 °C, and the cultures were shaken overnight or until the culture turned greenish-blue. Blue-colored *E. coli* cells were pelleted by centrifugation at 9,184 × *g* and stored at –20 °C until needed.

Recombinant protein purification

Cell pellets were resuspended in Buffer 100 (100 mM NaCl, 100 mM K-phosphate, pH = 7.0) and disrupted by three passages through a chilled French pressure cell at 138 MPa. The lysates were centrifuged at 90,000 × *g* for 30 min to pellet unbroken cells and larger debris. The supernatants containing recombinant proteins were purified by immobilized metal affinity chromatography (IMAC) on columns packed with ~0.75 mL Ni^{2+}-nitrilotriacetate affinity resin (Goldbio, St. Louis, MO) that had been pre-equilibrated with Buffer 100. After the entire lysate had been passed through the resin three times, the column was washed with five column volumes of each of the following buffers: Buffer A1 (20 mM Tris–HCl, pH 8.0, 50 mM NaCl, 50 mM KCl 5% v/v glycerol); Buffer B (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 500 mM KCl); and Buffer A2 (20 mM Tris–HCl, pH 8.0, 50 mM NaCl, 50 mM KCl) adapted from Cai et al. (2001), with a final wash in Buffer 100. Tagged proteins (and subunits associated with them) were eluted from the column with Buffer 100 containing 200 mM imidazole, and proteins were immediately passed through a PD-10 column (Sephadex G-25; GE Healthcare, Marlborough, MA) pre-equilibrated with Buffer 100 and eluted with Buffer 100 to remove imidazole. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was performed as previously described (Ho et al. 2017b). Gels were first soaked in 25 mM ZnSO$_4$ to visualize proteins carrying

covalently bound phycocyanobilin chromophores (Berkelman and Lagarias 1986) and were subsequently stained with Coomassie blue R-250.

Absorbance and fluorescence spectroscopy

Absorbance spectra were acquired with a Cary 14 spectrophotometer modified for on-line data acquisition (On-line Systems, Inc.; Bogart, GA). Low-temperature (77 K) fluorescence emission spectra were collected for each protein using an SLM 8000C spectrofluorometer, modified for digital data acquisition by On-line Systems, Inc. (Bogart, GA). The excitation wavelength was set to 590 nm in all cases in order to excite PBPs preferentially. Prior to measuring fluorescence emission spectra at 77 K, each sample was diluted 1:1 in 1.5 M K-phosphate buffer (pH = 7.0).

Modeling of phycocyanobilin

Phycocyanobilin (PCB) bound to ApcA1 was modeled in Chimera using the crystal structure of allophycocyanin from *Pyropia yezoensis* (PDB 1KN1:A) (Liu et al. 1999). PCB bound to ApcD1 was modeled in Chimera using the crystal structure of allophycocyanin-B from *Synechocystis* sp. PCC 6803 (PDB 4PO5:A) (Peng et al. 2014). The "Adjust Torsions" tool in Chimera was used on the model of PCB bound to ApcD1 to create a hypothetical, planar PCB molecule.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed on isolated, negatively stained PBP complexes as described previously (Ho et al. 2017b).

Results

PBP complexes in Leptolyngbya JSC-1 cells after long-term acclimation to far-red light

The PBP complexes present in cells of the FaRLiP strain, *Leptolyngbya* JSC-1, after growth for two months in FRL were compared to those present after 18 months of continuous growth in FRL, which was achieved by serially subculturing the cells. Additionally, the PBP complexes of *Synechococcus* 7335 after

18 months of continuous growth in FRL were compared to those of *Leptolyngbya* JSC-1 and to previously published descriptions of PBP complexes isolated from *Synechococcus* 7335 cells that had been acclimated to FRL for much shorter time periods (1 to 21 days; see Ho et al. 2017b).

Multiple types of PBP complexes were present in *Leptolyngbya* JSC-1 after growth in FRL for both 2 and 18 months. Four fractions containing PBPs (compare **Figs. 1** and **2,** fractions F1 to F4), were obtained from both types of cells after fractionation on sucrose gradients. The absorbance spectra of the four fractions showed no obvious absorbance due to PE, but fractions F4 and especially F2 contained significant amounts of PC absorbing maximally at ~628 nm. Although all of the fractions contained FRL-AP absorbing maximally at 708 nm, fractions F1 and F3 predominantly contained FRL-AP as judged from the absorbance spectra. Based upon the PBP absorbance spectra, fluorescence emission spectra, and TEM analysis of negatively stained preparations of the fractions (see **Fig. 1** and **2** for examples), the four fractions were identified as bicylindrical cores comprised primarily of FRL-AP (BC; F1); dissociated peripheral rods composed primarily of PC (F2); core substructures comprised primarily of FRL-AP, most of which were pentacylindrical with few or no peripheral rods (F3); and PBS with pentacylindrical cores comprised primarily of FRL-AP and some PC in peripheral rods (F4). Interestingly, even after cells were grown in FRL for 18 months, fraction F4 exhibited substantial fluorescence emission at 681 nm, the emission wavelength typical of intact PBS from cells grown in visible light (see data for red-light PBS in **Fig. S2**; Gan et al. 2014; Ho et al. 2017b, 2020). This emission primarily arises from the terminal emitters in those PBS, namely ApcE1 and ApcD1 (Glazer and Bryant 1975; Zhao et al. 1992; Sidler, 1994; Dong et al. 2009; Ho et al. 2017b, 2020). ApcE1 from *Leptolyngbya* JSC-1 has four REP domains and is predicted to assemble PBS with "pentacylindrical" core substructures (Glauser et al. 1992; Sidler, 1994; Gan et al. 2014), and the presence of PBS with pentacylindrical cores in F4 was confirmed by TEM (**Fig. 1** and **2**). The fluorescence emission at 681 nm from ApcE1 was most intense in the largest PBS complexes from *Leptolyngbya* JSC-1 after two months of FRL growth (**Fig. 1,** F4). After 18 months of growth in FRL, the 681-nm emission from ApcE1 in F4 was still present; however, the emission amplitude was lower than that from ApcE2 (724 nm) (**Fig. 2,** F4).

To evaluate the PBP contents of the four fractions collected from a sucrose gradient like that shown in **Fig. 2**, the proteins of the four fractions were digested with trypsin and subjected to peptide fingerprinting by MS/MS analysis. The resulting lists of identified proteins were manually examined to identify PBPs and linker proteins associated with PBS and other PBP complexes. As shown in **Table 1**, Fraction 1 (F1) contained all of the polypeptides expected for BCs (ApcD5, ApcD2, ApcD3, ApcB2, ApcF, ApcE2, and ApcC; Ho et al. 2017b). Fewer peptides were detected for PBPs normally produced in visible light, including ApcA1, ApcB1, and ApcE1, suggesting that these proteins were probably present in lower amounts. Consistent with the absorption and fluorescence emission data for Fraction 2 (F2), the proteomic analysis showed that CpcA1, CpcA2, CpcB1, and CpcB2 were present in this fraction. The detection of CpcC1 and CpcC2 strongly suggests that this fraction contains PC complexes similar to peripheral rods of PBS (Bryant and Caniffe 2018). All of the AP subunits of BCs that were present in F1 were also found in this fraction; these probably represent cross-contamination with BCs from F1, which were not separated very effectively on the sucrose gradients (**Figs. 1** and **2**).

Repeated proteomic analyses of F3 surprisingly showed that the major PBPs in this fraction are the same as those in the BCs in F1 (i.e., ApcD5, ApcD2, ApcD3, ApcB2, ApcF, ApcE2, and ApcC), but the complexes containing FRL-AP subunits in F3 are clearly larger than BCs (F1). The fluorescence emission spectra from F3 also had a small band at 681 nm that is similar or identical to the emission maximum for ApcE1 (Ho et al., 2019). The peptide analysis for F3 in **Table 1** shows that some ApcE1 and other AP subunits produced by cells grown in visible light are present in this fraction, although this could be due to cross-contamination with complexes from fraction F4. Pentacylindrical cores substructures were frequently observed in this fraction by TEM (**Figs. 1** and **2**). Further comments about this fraction will follow in the Discussion.

Fraction 4 (F4) had similar attributes to those of F3. TEM of this fraction clearly showed the presence of PBS with short peripheral rods and pentacylindrical cores (**Figs. 1** and **2**). As observed for F3, the fluorescence emission spectrum showed maxima at both 681 nm and 724 nm, consistent with the presence of two terminal emitters, ApcE1 and ApcE2, respectively. The emission amplitude from ApcE1

at 681 nm was smaller than that from ApcE2 at 724 nm. Proteomic analyses showed that ApcE1 was present, probably in lower amounts than ApcE2, along with PC subunits and AP subunits that are also produced by cells in visible light. These observations are consistent with the presence of PBS with pentacylindrical cores and short peripheral rods that were observed in this fraction by electron microscopy. Similar to F3, the predominant proteins in this fraction based on peptide analyses are FRL-APs. Implications of these observations will also be deferred until the Discussion.

PBP complexes in Synechococcus *7335 cells after long-term acclimation to far-red light*

After 18 months of continuous growth in FRL, *Synechococcus* 7335 cells only contained two types of PBP complexes: FRL-absorbing BCs as previously characterized (**Fig. 3,** F1; Ho et al. 2017b), and a minor fraction of peripheral rod-like complexes primarily composed of PC but also containing a small amount of PE (**Fig. 3,** F2). Such *Synechococcus* 7335 cells no longer contained any visible-light-type PBS, which were previously shown to be retained for at least 21 days after a transfer of cells from red light into FRL (Ho et al. 2017b, 2019). Neither fraction exhibited fluorescence emission at 682 nm characteristic of ApcE1 (Ho et al. 2017b, 2019). Fraction 2 was enriched in PE and PC (see **Table 2,** F2) and contained the CpcL, CpcC1 and CpcC2 linker polypeptides as well as CpeC and CpeE, which is consistent with this fraction containing complexes similar to peripheral rods of PBS. The presence of CpcL may explain the relatively red-shifted emission maximum at 664 nm in addition to the emission maximum at 650 nm that is more typical of unassembled PC (**Fig. 3,** F2) (Glazer, 1976; de Lorimier et al. 1990; Liu et al. 2019). The ~664 nm fluorescence emission maximum is similar to that reported for rod complexes containing PC and CpcL in *Synechocystis* sp. PCC 6803 (Watanabe et al. 2014; Liu et al. 2019).

Mass spectrometry of PBPs from Leptolyngbya *JSC-1 cells grown in far-red light*

The AP subunits produced by cyanobacteria after growth in FRL contain multiple, highly conserved cysteine residues in addition to the uniquely conserved cysteine at position 78 in α subunits and position 81 in ApcB2 that binds a single PCB chromophore covalently to the protein (Gan et al. 2015; Herrera-

Salgado et al. 2018; Bryant et al. 2020). LC-ESI-MS was used to obtain masses of intact PBPs produced by *Leptolyngbya* JSC-1 cells grown in FRL. The resulting masses were consistent with the calculated masses of each subunit from their gene sequences, when the usual post-translational modifications for the paralogous subunits synthesized by cells grown in visible light were included: cleavage of the N-terminal methionine residue and the presence of a single, covalently bound PCB chromophore on α-AP subunits, and methylation of asparagine-71 and the presence of a single, covalently bound PCB chromophore on the β-AP subunit (**Table 3**) (Shen et al. 2008; Schluchter et al. 2010). We were able to identify PCB-bearing peptides in trypsin digests that included Cys78 for ApcD5, ApcD2 and Cys81 for ApcB2, establishing that PCB was attached at the canonical chromophore binding residue (data not shown). When expressed recombinantly, C78A variants of ApcD3 and ApcD2 were unpigmented and exhibited no zinc-induced fluorescence after SDS PAGE, indicating that no covalently bound chromophore was present and again establishing that Cys78 was the site of PCB attachment (**Fig. S5**). Thus, we conclude that the additional conserved cysteine residues in FRL-AP subunits do not bind additional PCB chromophores covalently.

Characterization of heterologously produced APs associated with FaRLiP

With the exception of *apcE2,* the genes encoding AP subunits from the FaRLiP gene cluster in *Leptolyngbya* JSC-1 were heterologously expressed in *E. coli,* both individually and in combinations of α-type and β-type subunits. As a control, the *apcA1* and *apcB1* genes, encoding the principal AP of *Leptolyngbya* JSC-1 cells grown in visible light, were also co-expressed in *E. coli.* The 650-nm absorbance maximum and 660-nm fluorescence emission maximum of recombinant ApcA1-ApcB1 matched the values commonly reported in the literature for AP (**Fig. S3**) (Glazer 1989; MacColl 2004).

Absorbance and fluorescence emission spectra of the FRL-AP subunits and FRL-APs were acquired from IMAC-purified protein samples (**Figs. 4** and **5**, respectively). The recombinant ApcD2 and ApcB2 subunits had absorbance maxima at 614 nm, although the latter had a substantial shoulder at about 675 nm. ApcD5 had an absorbance maximum at 682 nm and a shoulder at 660 nm, and ApcD3 had an

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absorption maximum that was the most red-shifted of these four subunits at 694 nm with a shoulder at 660 nm. Correspondingly, ApcD5 and ApcD3 also had the most red-shifted fluorescence emission maxima at 710 nm and 718 nm, respectively (**Fig. 4b**). Considering the shapes of these spectra, the scattering evident from the increase in the baseline at shorter wavelengths, and the tendency of PBPs to form oligomers (Bryant et al. 1976; Maccoll et al. 1981; Cai et al. 2001), it is possible that some of the individual subunits form dimers and possibly other oligomers or aggregates.

Fig. 5 shows the absorbance and fluorescence emission spectra of three recombinantly produced FRL-AP complexes, ApcD5-ApcB2, ApcD2-ApcB2 and ApcD3-ApcB2. The ApcB2 subunit copurified with the $[His]_{10}$-tagged α-type subunit in each case, as evidenced by the differences between the spectral properties of the individual subunits (**Fig. 4**) and the co-expressed subunit combinations (**Fig. 5**), and by SDS-PAGE (**Fig. S4**). The spectra of these recombinantly produced complexes differ considerably, but all three show some absorbance at wavelengths greater than 700 nm. ApcD2-ApcB2 has a large absorbance feature at 613 nm and a very small absorbance band at 705 nm. ApcD5-ApcB2, the AP composed of the two most abundant proteins in BCs (Ho et al. 2017b), resembles the absorbance of BCs, but with the amplitudes of the two absorbance features at 613 and 707 nm reversed compared to BCs. The FRL absorbance of ApcD5-ApcB2 was previously identified at 712 nm when the corresponding genes from FaRLiP organism *Chroococcidiopsis thermalis* sp. PCC7203 were heterologously expressed in *E. coli* (Xu et al. 2016). Finally, ApcD3-ApcB2 has absorbance maxima at 660 and 701 nm but a shoulder at about 620 nm was also observed. Interestingly, this protein had the most red-shifted emission maximum (735 nm), which is consistent with the previous suggestion that it might be one of the terminal emitters in the BCs (Ho et al. 2017b; Herrera-Salgado et al. 2018).

To test whether the β-type subunit, ApcB2, plays an essential role in producing FRL absorbance, operons encoding *apcD5-apcB1*, *apcD2-apcB1* and *apcD3-apcB1* from *Leptolyngbya* JSC-1 were created via PCR using primers listed in **Table S1,** and these operons were expressed in *E. coli* using the pAQ1-Ex plasmid. Interestingly, the absorbance bands of the resulting proteins occur at similar positions to those when ApcB2 was the partner subunit, but the amplitudes of the bands differed (compare **Figs. 5** and **6**).

Each of the variants produced with ApcB1 had strong absorbance bands at about 615 nm, but all additionally exhibited FRL absorbance (692 to 704 nm) after affinity purification (**Fig. 6a**), and each variant had a long-wavelength fluorescence emission maximum at 712 to 720 nm (**Fig. 6b**). The presence of shorter wavelength fluorescence emission maxima between 640 and 700 nm suggested that there may have been some homodimeric alpha-subunit complexes present as well as complexes containing only ApcB1, or that energy transfer in these recombinant proteins was less efficient than when ApcB2 was the partner protein in heterodimers.

Characteristics of the FRL-AP expressed in low-light-adapted Synechococcus *A1463*

The paralogous AP genes *apcD4* and *apcB3* from the LoLiP operon in *Synechococcus* A1463 were predicted to encode a FRL-AP because of their phylogenetic similarity to the FRL-AP subunits of FaRLiP strains (Gan et al. 2015). To test if this hypothesis was correct, these proteins were expressed individually and jointly in *E. coli* and the resulting proteins were characterized spectroscopically (**Fig. 7**). ApcB3 had an absorbance maximum at 615 nm, and ApcD4 had an absorbance maximum at 678 nm and a shoulder at ~625 nm (**Fig. 7a**). Purified, recombinant ApcD4-ApcB3 had a narrow, intense absorbance band with a maximum at 708 nm, and a broader, less intense absorbance band with a maximum at 618 nm. The fluorescence emission spectrum of this protein at 77 K was very narrow and had a maximum at 718 nm (λ_{ex} = 590 nm) (**Fig. 7b**). To test whether the paralogous β-type subunit ApcB3 was necessary for FRL absorbance, an operon of genes *apcD4* and *apcB1* from *Synechococcus* A1463 was created via PCR using the primers listed in **Table S1** and expressed in *E. coli*. The resulting protein, ApcD4-ApcB1, had absorbance maxima at 615 nm and ~702 nm and a fluorescence emission maximum at 712 nm (**Fig. 6**). As hypothesized, *apcD4* and *apcB3* from LL-adapted *Synechococcus* A1463 encode a FRL-AP.

Discussion

Phycobiliprotein complexes produced during far-red light photoacclimation

The FRL-PBP complexes of FaRLiP strains *Leptolyngbya* JSC-1 and *Synechococcus* 7335 exemplify the diversity and progression of FaRLiP responses after transfer of two different cyanobacteria to FRL. These organisms employ the FRL-APs produced from the FaRLiP gene cluster in somewhat different ways. *Leptolyngbya* JSC-1 maintains PBS complexes (having both core-cylinders and peripheral PC rods) in FRL much longer than *Synechococcus* 7335 and incorporates the FRL-APs into both BCs and PBS (**Figs. 1** and **2**). In *Synechococcus* 7335 the FRL-APs are almost exclusively assembled into BCs (**Fig. 3**) and the PBS that continue to persist for at least 21 days in FRL are identical to those made in visible light, and thus have no FRL absorbance (Ho et al. 2017b). Based upon published accounts and consistent with phylogenetic relationships, another characterized FaRLiP strain, *H. hongdechloris* (which is phylogenetically similar to many *Leptolyngbya* spp. strains), seems to be more similar to *Synechococcus* 7335 than *Leptolyngbya* JSC-1 (Li et al. 2016; Chen et al. 2019). It should be noted that *Synechococcus* 7335 is likely to be a natural short-chain mutant of a filamentous strain that is similar to *Leptolyngbya* spp.

The appearance of FRL-APs in PBP complexes of cells undergoing FaRLiP is a slow and gradual process (Ho et al. 2017b), and complete dilution of PBS complexes produced during growth in visible light after a transfer to FRL apparently takes months. Cells of *Leptolyngbya* JSC-1 grown in FRL for 18 months have a higher ratio of FRL-AP absorbance (~712 nm) to visible light PBP absorbance (~620-650 nm) in their PBP complexes (**Fig. 2**) compared to complexes isolated from cells after two months in FRL (**Fig. 1**; also see Ho et al. 2017b). This is also reflected in the fluorescence emission from the ApcE2 at 724 nm relative to ApcE1 (681 nm). These spectral shifts imply a gradual increase in the percentage of FRL-APs in the FRL-PBP complexes of *Leptolyngbya* JSC-1 over the course of months, but note that small amounts of ApcE1 and other visible-light AP paralogs are apparently synthesized even after prolonged periods in FRL. This result is consistent with results from transcription profiling, which showed that PBP genes normally expressed in visible light continued to be expressed in FRL; however,

transcript levels were much lower than in visible light (Gan et al., 2014). This differs from *Synechococcus* 7335, which eventually stops producing ApcE1 and PBS altogether (**Fig. 3, Table 2**).

Each of the AP subunits encoded in the FaRLiP gene cluster was detected in each of the FRL-absorbing PBP complexes separated on sucrose gradients (**Figs. 1, 2** and **3, Tables 1** and **2**). This includes the bicylindrical cores found in F1, the pentacylindrical cores of PBS found in F4, and a fraction (F3) that we have tentatively designated as pentacylindrical cores lacking peripheral rods. If these assignments are correct, then it follows that alternative structures can be formed from the same set of polypeptides but possibly using different subunit stoichiometries. A model to account for this is shown in **Fig. 8**. Each copy of ApcE2 provides two linking REP domains, allowing the assembly of a BC with two copies of ApcE2 and two copies of ApcF (see Ho et al. 2017b). A scaffold for a pentacylindrical core requires eight REP domains (Glauser et al. 1992; Sidler 1994), which could be provided by four copies of ApcE2 and four copies of ApcF in a core assembly (**Fig. 8**). The presence of a complex larger than BC but containing the same subunits in *Leptolyngbya* JSC-1strongly suggests that the PBPs encoded by the FaRLiP gene cluster of this organism can form more than one type of core complex.

Electrophoretic analyses have suggested that ApcD5 is the most abundant α-type AP subunit in FRL-PBP complexes in *Synechococcus* 7335 (Ho et al. 2017b; Herrera-Salgado et al. 2018). The exact stoichiometries of ApcD2 and ApcD3 are unknown for *Leptolyngbya* JSC-1, but ApcD5 also appears to be the most abundant α-type FRL-AP subunit in this cyanobacterium. It is also unknown if these subunits form specific subassemblies or are just randomly inserted in the core, although the latter could result in less efficient energy transfer to the photosystem chlorophylls. All attempts to subfractionate PBP complexes containing FRL-APs chromatographically, even in the presence of chaotropes such as urea, were unsuccessful. This indicates that either the complexes are far more stable than AP complexes found in the cores of typical hemidiscoidal PBS, or that all subcomplexes have a similar composition.

It is possible for FRL-AP trimers in a core complex to be formed from mixtures of ApcD5, ApcD2 and/or ApcD3, or $[(\alpha_{D5/D2/D3})\text{-}(\beta_{B2})]_3$, as well as the FaRLiP L_{cm} (ApcE2) and ApcF (**Fig. 8**). Proteomic analyses have revealed that even after 18 months, ApcE1 (**Table 1,** F4) and the major AP

subunits produced in visible light (ApcA1 and ApcB1; **Table 1,** F1-4, **Table 2,** F1) are also present in the FRL-PBP complexes in each organism. This adds considerable complexity to the possible composition of any given AP trimer produced in FRL: $[(\alpha_{A1/D1/D5/D2/D3})-(\beta_{B1/B2})]_3$. The possibility of a complex containing both ApcE1 and ApcE2 cannot be ruled out at this time, although the differences between F3 and F4 in *Leptolyngbya* JSC-1 suggest that ApcE1 mostly occurs in PBS but that ApcE2 occurs in both core complexes and PBS (**Figs. 1 and 2, Table 1**). Presently, it is not known how these various subunits are organized in the core complexes made in FRL. The presence of nearly every AP subunit in each sucrose fraction containing FRL-AP (**Tables 1** and **2**), and the demonstrated ability of recombinant FRL α-AP subunits to form complexes with ApcB1 (**Fig. 6**), is indicative of diversity in the composition of FRL-AP trimers and cores, even after prolonged growth in FRL. This diversity could be functionally relevant to enhancing light harvesting under non-optimal, FRL-enriched conditions by inhomogeneously broadening the absorbance properties of the PBP antenna complexes.

Post-translational modifications of FRL-AP subunits

The α and β subunits of APs each typically carry a single PCB chromophore that is covalently attached by a thioether linkage to a conserved cysteine residue (cysteine 81) (Sidler, 1994; Bryant and Caniffe, 2018). The α-type AP subunits encoded in the FaRLiP gene cluster (i.e., ApcD5, ApcD2 and ApcD3) and the LoLiP α-type subunit (ApcD4) have additional, conserved cysteine residues and therefore have the potential to bind multiple chromophores covalently (Gan et al. 2014, 2015). Previous studies concluded that ApcE2 (Miao et al. 2016) and in some cases ApcD3 (Herrera-Salgado et al. 2018) can bind PCB non-covalently, but it was not known with certainty how many chromophores are covalently bound by other FRL-AP subunits. The intact masses of ApcD5, ApcD2, ApcD3 and ApcB2 from *Leptolyngbya* JSC-1 were acquired by LC-ESI-MS, and the data demonstrate that each subunit (except ApcE2, not characterized in this study) binds a single PCB chromophore. Furthermore, peptide fingerprinting and expression of mutant variants showed that the PCB chromophore was bound at the canonical cysteine-78/81 position (**Fig. S5**, **Table 3**). The additional cysteine residues in FRL-APs do not bind PCB, but a

recent study showed that they could potentially play a role in chlorophyll *d* synthesis (Bryant et al. 2020). The mass data also indicate that the FaRLiP AP subunits from *Leptolyngbya* JSC-1 have the same post-translational modifications as AP subunits produced in visible light: cleavage of the N-terminal methionine residue in α-AP subunits, and methylation of the γ-amide nitrogen of asparagine-71 in all β-type PBP subunits (Shen et al. 2008; Schluchter et al. 2010).

The masses obtained for the intact FRL-AP subunits from LC-ESI-MS do not inform one about possible non-covalently bound chromophores associated with these proteins, but retention of FRL absorbance after affinity purification rules out loose, non-covalent binding of phycocyanobilin. Xu et al. (2017) observed a loss of FRL absorbance in heterologously expressed ApcD5-ApcB2 from *C. thermalis* sp. PCC 7203 after IMAC purification. In this study rapid removal of imidazole with a desalting column equilibrated with buffer 100 (100 mM NaCl, 100 mM potassium phosphate pH = 7.0) resulted in a purified protein that retained the absorbance features of the preceding clarified lysate. A buffer with higher salt concentration (0.75 M potassium phosphate pH = 7.0) was used for acquisition of fluorescence emission spectra. From these results we conclude that the each FRL-AP subunit covalently binds one molecule of PCB through a thioether linkage to cysteine-78/81, except in cases where this residue is alanine or serine (see Gan et al. 2015; Herrera-Salgado et al. 2018). Importantly, these data rule out the possibility that the long-wavelength absorbance properties of most FRL-APs result from additional or chemically modified chromophores or from unique post-translational modification of the apoproteins.

Recombinant FRL-APs

Heterologous coexpression of genes encoding various combinations of AP subunits from *Leptolyngbya* JSC-1 were carried out with an N-terminal deca-histidine ([His]$_{10}$) tag on the α-AP subunit (or on the β-AP subunit when it was the only subunit expressed). Each combination of α-AP and β-AP subunits tested led to copurification upon IMAC, which suggests that these various subunit combinations form very stable complexes (**Fig. S4**). When only a single gene was expressed and the corresponding protein purified, the doubled or broadened absorbance band(s) of ApcD5, ApcD3 and ApcB2 suggest the

formation of homodimers or other oligomers (**Fig. 4**). The α-β AP subunit combinations consistently had longer wavelength absorbance and fluorescence emission maxima than the individual subunits, which is consistent with the formation of (α-β) heterodimers and/or (αβ)$_3$ trimers for each FaRLiP AP (**Fig. 5**). Because each FaRLiP (αβ) AP combination exhibited FRL absorbance, all α-type subunits are thus potential contributors to the FRL absorbance of the PBP complexes produced during FaRLiP (Gan et al. 2014; Ho et al. 2017b).

The absorbance and fluorescence emission spectra of each FRL-AP are informative regarding the roles of these proteins in PBP core complexes produced during FaRLiP. For example, recombinant ApcD3-ApcB2 has the longest-wavelength fluorescence emission maximum of any of the FRL-APs at ~735 nm (**Fig. 5**), suggesting that it is potentially a terminal emitter in FaRLiP PBP core complexes, analogous to allophycocyanin-B (AP-B; ApcD1-ApcB1) in typical hemidiscoidal PBS (Glazer and Bryant 1975; Bryant, 1991; Zhao et al. 1992; Dong et al. 2009; Liu et al. 2013). ApcD3-ApcB2 also has a visible light absorbance band at ~660 nm, contributing to the visible absorbance present in various sucrose fractions (~650 nm; **Figs. 1, 2** and **3**), along with paralogous visible-light APs present in the same fractions (e.g., ApcA1, ApcB1, ApcE1; **Tables 1** and **2**). The remaining FaRLiP APs, ApcD5-ApcB2 and ApcD2-ApcB2, are thought to be the primary components of the core cylinders in FRL-PBP complexes, analogous to ApcA1-ApcB1 in hemidiscoidal PBS produced in visible light (**Fig. S3**). The potential association of the β-type subunit ApcB1 with various α-type subunits from the FaRLiP cluster in these complexes could further expand the range of wavelengths that could be absorbed (**Fig. 6**). In particular, the FRL absorbance of recombinant ApcD2-ApcB1 (**Fig. 6**) is even more pronounced than that of recombinant ApcD2-ApcB2 (**Fig. 5**). Subunits that can associate with ApcB1 and absorb beyond 650 nm could gradually alter the transition from visible light-absorbing complexes to FRL-absorbing complexes for an organism undergoing FaRLiP or provide an intermediate state for light-harvesting in environments receiving a mixture of RL and FRL. Due to difficulties in separating these highly similar, paralogous APs directly from FaRLiP cyanobacteria, characterization of the heterologously expressed FRL-APs from *E.*

coli has been useful for assigning their putative roles, even if their organization is more complex in native FaRLiP complexes.

FRL-AP putatively produced during Low-Light Photoacclimation

A second type of FRL-absorbing AP was discovered in low-light-adapted *Synechococcus* sp. ecotypes isolated from Mushroom Spring, Yellowstone National Park. These organisms, which grow beneath other cyanobacteria and anoxygenic phototrophs in the microbial mat (Becraft et al. 2015, Tank et al. 2017), have limited access to visible light and should benefit from harvesting FRL for photosynthesis. As with the AP paralogs found in FaRLiP cyanobacteria, the α-AP subunit (ApcD4) and β-AP subunit (ApcB3) were heterologously expressed separately and together in *E. coli*. The absorbance spectrum acquired from affinity-purified ApcD4-ApcB3 showed that, as predicted by sequence homology (Gan et al. 2015), it is an FRL-AP with a narrow absorbance band with a maximum at 708 nm and maximal fluorescence-emission at 718 nm (λ_{ex} = 590 nm) (**Fig. 7**). This FRL-AP is the likely source of the far-red fluorescence-emission feature (λ_{max} ~720 nm) observed in the whole-cell fluorescence emission spectra at 77 K of LL-adapted *Synechococcus* (λ_{ex} = 590 nm) (Nowack et al. 2015).

Predicted structural changes in FRL-APs

The absence of unique post-translational modifications on FRL-AP subunits indicates that their long-wavelength absorbance is a consequence of unique bilin-protein interactions that do not occur in other APs. In the crystal structure of AP-B (ApcD1-ApcB1), a terminal emitter with its maximal absorbance red-shifted 20 nm compared to bulk AP (ApcA1-ApcB1), the phycocyanobilin chromophore on the α-subunit is bound such that three of the four pyrrole rings (B, C, and D) are nearly coplanar (PDB 4PO5: A, C, E) (Peng et al. 2014). This contrasts with the PCB molecule bound by ApcA1, in which only the B and C rings are coplanar, and the A ring is bent out-of-plane at an acute angle (**Fig. 9**). The increased planarity of the chromophore in ApcD1 effectively extends the π-conjugation system of the chromophore, which presumably lowers the energy required for excitation and results in a 20-nm red-shift in the

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absorbance of ApcD-ApcB1 compared to ApcA1-ApcB1 (Peng et al. 2014). A further flattening and stretching of the chromophore, resulting in a coplanar arrangement of the A, B, C and D pyrrole rings of the α subunits of FRL-AP could thus be responsible for most of the additional 40-nm bathochromic shift in absorbance of FRL-APs with covalently bound PCB chromophores.

The paralogous FRL-specific β subunits, ApcB2 in FaRLiP and ApcB3 in LoLiP strains, are also potential factors affecting the absorbance of FRL by these variant APs. ApcB2 and ApcB3 presumably diverged from ApcB1 to improve FRL absorption. Co-expression of FRL-specific α-AP subunits with ApcB1 produced in visible light challenges the simplest version of this idea. All combinations of FRL α-AP subunits with ApcB1 still absorbed beyond 650 nm, and in all cases except ApcD5-ApcB1, were still capable of absorbing FRL (**Fig. 6**). However, the absorbance properties of these ApcB1-associated proteins are different from APs incorporating ApcB2, which may be very significant to cells in natural environments. It is also possible that ApcB2 has higher affinity for FRL α-AP subunits than ApcB1 and produces more stable trimers when it is present. ApcB2 might further have specific assembly capabilities that make it preferable for forming the PBP complexes employed in FRL-grown cells. Finally, it could be advantageous to have the genes encoding the α-type and β-type subunits on the same FRL operon, which could then be regulated independently from the genes encoding PBPs produced in visible light, the transcripts for which are strongly depleted in cells grown in FRL (Gan et al. 2014; Zhao et al. 2015; Ho and Bryant, 2019). Thus, from a biological perspective, there are several possible reasons for the divergence of ApcB2 and ApcB3 from ApcB1. However, from the perspective of the relationship between structure and absorbance, the proteins primarily responsible for FRL absorbance are the paralogous α-AP subunits: ApcD5, ApcD2, and ApcD3 in FaRLiP and ApcD4 in LoLiP.

Conclusions

In this study on paralogous APs and the complexes produced from them in cells grown in FRL and low light, we have further defined their contributions to Far-Red Light Photoacclimation (FaRLiP) and potentially to Low-Light Photoacclimation (LoLiP). As shown by comparisons of complexes from

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Leptolyngbya JSC-1 and *Synechococcus* 7335 with each other and to results from previous studies (Gan et al. 2014; Ho et al. 2017b), we found that the PBP complexes produced during FaRLiP differ considerably between the two strains compared here and over time. We have also gained a better understanding of the proteins themselves and have determined that their FRL absorbance results from protein-bilin interactions rather than novel post-translational modifications or unusual chromophores. With the exception of ApcE2 and ApcD3 in some strains (Miao et al. 2016; Herrera-Salgado et al. 2018), we show that these paralogous AP proteins covalently bind a single phycocyanobilin chromophore despite the presence of additional, highly conserved cysteine residues. In addition to their biological importance in expanding light harvesting for oxygenic photosynthesis, these FRL-absorbing proteins are a promising platform for the development of FRL-emitting fluorophores for biomedical and biotechnological applications.

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Conflict of Interest Statement

The authors declare that this research was conducted in the absence of any commercial, financial, or non-financial relationships that could be construed as a potential conflict of interest.

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Figure Legends

Fig. 1 Sucrose density-gradient separation of PBP complexes from *Leptolyngbya* JSC-1 cells grown for two months in FRL. PBP complexes were separated into four distinct fractions: F1, F2, F3 and F4. The absorbance spectrum (solid lines) and low-temperature (77 K) fluorescence emission spectrum (λ_{ex} = 590 nm; dashed lines) is shown for each fraction: F1, black lines; F2, red lines, F3 in blue lines; and F4, green lines. Fluorescence emission occurs at 650 nm for PC, at 681 nm from ApcD1/ApcE1 and at 724 nm from ApcD3/ApcE2. Both absorbance and fluorescence emission spectra have been normalized at the maximum amplitude for each spectrum. The three images at the right show selected electron micrographs of representative complexes from fractions F1, F3 and F4; F2 was not examined. To the right of the electron micrographs is a drawing representing the predominant PBP complex from each fraction, with cyan indicating allophycocyanin (AP) and blue indicating phycocyanin (PC). The size bar below the electron micrographs indicates 100 nm. Note that although the largest PBS complexes in F4 (solid green line) have absorbance at 708 nm, the fluorescence emission amplitude is greater at 681 nm than at 724 nm (dashed green line).

Fig. 2 Separation of PBP complexes by sucrose density-gradient centrifugation from cells of *Leptolyngbya* JSC-1 grown continuously in FRL for 18 months. PBP complexes were separated into four distinct fractions: F1, F2, F3 and F4. The absorbance spectrum (solid lines) and low-temperature (77 K) fluorescence emission spectrum (λ_{ex} = 590 nm; dashed lines) is shown for each fraction: F1, black lines, F2, red lines; F3, blue lines; and F4, green lines. The three images at the right show electron micrographs of representative complexes from fractions F1, F3 and F4; no images were made from F2. To the right of the electron micrographs is a drawing representing the predominant PBP complex from each fraction, with cyan indicating allophycocyanin (AP) and blue indicating phycocyanin (PC). The size bar below the electron micrographs indicates 100 nm. Note that although the largest PBS complexes in F4 (solid green

line) have greatest absorbance at 708 nm, the fluorescence emission amplitude at 681 nm is still substantial but less than that at 724 nm (dashed green line).

Fig. 3 Separation of PBP complexes by sucrose density-gradient centrifugation from *Synechococcus* 7335 cells grown for 18 months in FRL. PBP complexes were separated into two fractions: F1 and F2. The absorbance spectrum (solid line) and low-temperature (77 K) fluorescence emission spectrum (λ_{ex} = 590 nm; dashed line) is shown for each fraction: F1, red lines; F2, blue lines. To the right of the spectra are drawings representing the major PBP complexes in each fraction. Cyan indicates allophycocyanin (AP), blue indicates phycocyanin (PC), and red indicates phycoerythrin (PE).

Fig. 4 Spectral properties of FaRLiP AP subunits from *Leptolyngbya* JSC-1 after heterologous expression in *E. coli* and purification by IMAC. **a** The absorbance spectra of individual subunits: ApcD5, green; ApcD2, blue; ApcD3, magenta; and ApcB2, red. **b** The 77-K fluorescence emission spectrum of each subunit (λ_{ex} = 590 nm).

Fig. 5 Spectral properties of FRL-APs from *Leptolyngbya* JSC-1 after heterologous expression in *E. coli* and purification by IMAC. The absorbance spectrum (**a**) and 77-K fluorescence emission spectrum (λ_{ex} = 590 nm) (**b**) of each FRL-AP heterodimer combination: ApcD5-ApcB2, green lines; ApcD2-ApcB2, blue lines; and ApcD3-ApcB2, magenta lines.

Fig. 6 Absorbance (**a**) and low-temperature (77 K) fluorescence emission (**b**) (λ_{ex} = 590 nm) spectra resulting from heterologous expression and purification of recombinant FRL α-APs co-expressed in *E. coli* with ApcB1 (the β-AP normally present in cells grown in visible light) from either *Leptolyngbya* JSC-1 (ApcD5-ApcB1, purple lines; ApcD2-ApcB1, blue lines; ApcD3-ApcB1, green lines) or *Synechococcus* A1463 (ApcD4-ApcB1, red lines). The absorbance or fluorescence emission of β-AP (ApcB1 from *Leptolyngbya* JSC-1 in black lines) is prominent in each spectrum.

Fig. 7 Absorbance spectra (solid lines) and low-temperature (77 K) fluorescence emission spectra ($\lambda_{ex} =$ 590 nm; dashed lines) of LoLiP AP subunits ApcD4 and ApcB3 **(a)**, and the LoLiP AP ApcD4-ApcB3 **(b)** from *Synechococcus* A1463 after heterologous expression in *E. coli* and IMAC purification. The black lines show spectra for ApcD4-ApcB3, the orange lines show spectra for ApcD4, and the purple lines show the spectra for ApcB3.

Fig. 8 Models of a putative pentacylindrical core-complex (PCC) in *Leptolyngbya* JSC-1 after FRL growth. In *Leptolyngbya* JSC-1, FRL-PBP complexes larger than BC are rich in ApcE2 peptides (**Table 1**) and fluorescence emission from ApcE2 (**Figs. 1** and **2,** F3). These complexes were visualized by TEM and identified as PCC (**Figs. 1** and **2,** F3). Four copies of ApcE2, which has two REP domains, would be necessary to assemble a PCC (Glauser et al. 1992; Sidler, 1994; Gan et al. 2014). In this model, the two membrane-facing cylinders each contain two copies of ApcE2 in separate AP trimers. The α-AP subunits could be any of ApcD5, ApcD2, ApcD3 or ApcA1, although ApcD3 is most likely present in the same core cylinders as ApcE2 due to its extremely long-wavelength fluorescence emission and potential role as a terminal emitter in FRL-PBP complexes (**Fig. 5**). The β-AP subunits could be either ApcB2 or ApcB1. If a complex like this carried peripheral rods it would be present in the lowest gradient fraction (**Figs. 1** and **2,** F4).

Fig. 9 a Absorbance of recombinant ApcA1-ApcB1 (green), ApcD1-ApcB1 (pink) and ApcD4-ApcB3 (dark red) from *Synechococcus* A1463. These three spectra are representative of AP and AP-B produced by cyanobacteria in visible light (Glazer and Bryant, 1975) and FRL-AP produced by cyanobacteria in FaRLiP (Gan et al. 2014, Ho et al. 2017b) and LoLiP (this study). **b** Models showing the linear tetrapyrrole phycocyanobilin (PCB) from the crystal structures of ApcA1 in green (PDB 1KN1: A) (Liu et al. 1999) and ApcD1 in red (PDB 4PO5: A, C, E) (Peng et al. 2014). A molecule of PCB in a completely planar, hypothetical conformation is depicted in red, which is suggested to represent the conformation adopted by PCB in the α-type subunits of FRL-APs. The four pyrrole rings of the PCB

molecules are labelled A, B, C, and D with the thioether linkage to the protein occurring at the $C3^1$ position of ring A.

Table 1 Identification of peptides from sucrose density-dependent separation of PBP complexes in *Leptolyngbya* **JSC-1 after 18 months of growth in FRL.** Fraction 1 (F1), Fraction 2 (F2), Fraction 3 (F3) and Fraction 4 (F4) correspond to those shown in **Fig. 2**. PEAKS DB search engine (PEAKS Studio 8.5, Bioinformatics Solutions Inc.) was used to generate scores for each protein; a higher score indicates higher statistical significance of protein identification. The protein scores (-10lgP) are calculated as the weighted sum of the -10lgP scores of their supporting peptides; a peptide -10lgP score is based on the p-value as a measure of the statistical significance of each peptide-spectrum match.

JSC-1 F1			JSC-1 F2		
Protein	Score	#Unique Peptides	Protein	Score	#Unique Peptides
ApcD5	296.19	116	CpcA1	244.24	12
ApcB2	261.47	50	CpcA2	237.43	6
ApcF	258.07	55	ApcB2	216.22	31
ApcE2	257.32	95	CpcB1	214.46	38
ApcD3	234.02	55	ApcE2	214.21	40
ApcA1	221.97	30	ApcD5	200.17	34
ApcD2	184.37	25	ApcF	194.78	20
ApcB1	171.94	13	ApcA1	193.46	12
ApcC	122.44	11	CpcB2	170.53	15
CpcB2	120.95	8	ApcD3	164.45	11
ApcE1	115.22	10	ApcB1	145.83	5
CpcB1	97.14	6	CpeA	140.07	8
CpcA1	76.42	7	CpcC2	134.27	10
			ApcC	133.52	9
			CpeB	127.93	5
			ApcE1	126.29	3
			CpcC1	122.81	8
			ApcD2	93.13	7

JSC-1 F3				JSC-1 F4		
Protein	Score	#Unique Peptides		Protein	Score	#Unique Peptides
ApcD5	378.38	149		ApcD5	289.66	145
ApcB2	326.60	67		ApcE2	249.77	99
ApcE2	309.76	113		ApcB2	247.46	52
ApcF	306.12	57		ApcF	233.49	51
ApcA1	296.27	51		ApcA1	227.75	39
ApcD3	273.18	42		ApcD3	197.39	43
ApcB1	204.83	16		ApcD2	173.75	21
ApcD2	145.98	9		ApcB1	166.14	12
ApcE1	129.50	3		ApcE1	136.70	14
CpcC1	96.69	3		CpcB1	127.71	10
CpcC2	70.52	2		CpcB2	126.65	11
CpcB2	67.29	3		ApcC	107.46	6
ApcC	62.92	2		CpcA1/A2	97.36	13
				CpcC1	96.84	5

Table 2   Identification of peptides from sucrose density-gradient-dependent separation of PBP complexes in *Synechococcus* 7335 after 18 months of growth in FRL. Fraction 1 (F1) and Fraction 2 (F2) correspond to the fractions shown in **Fig. 3**. Scores for each protein were generated with Proteome Discoverer 1.3 with the SEQUEST search algorithm; a higher score indicates more accurate protein identification.

7335 F1			7335 F2		
Protein	Score	#Unique Peptides	Protein	Score	#Unique Peptides
ApcB2	1049.48	17	CpeA	424.44	14
ApcE2	466.28	35	CpcB2	254.38	12
ApcD5	395.59	11	CpcA2	237.24	8
ApcF	296.67	11	ApcB2	220.86	11
CpeA	230.04	15	CpcA1	208.36	8
CpcA2	229.24	9	CpcB1	189.98	5
ApcD2	193.79	8	CpeB	112.3	9
CpcB2	175.85	8	CpcC2	106.36	6
ApcB1	119.23	7	CpcL	62.48	5
CpcA1	106.47	9	ApcD5	52.52	5
CpcB1	73.12	4	ApcE2	34.78	7
ApcD3	59.36	5	CpcD	24.92	5
CpeB	38.09	5	ApcF	23.56	5
CpcD	14.53	3	CpeC	14.75	4
ApcA1	11.59	3	ApcB1	13.74	3
ApcD4	7.26	2	CpcC1	13.09	4
CpeE	7.07	2	ApcD2	11.97	3
ApcB3	5.52	2	CpeE	6.72	2

Table 3 Intact masses of PBP subunits from *Leptolyngbya* JSC-1 cells grown in FRL. Intact masses of PBPs detected by LC-ESI-MS from a mixed sample of FR-PBPs isolated from FRL-grown *Leptolyngbya* JSC-1 and separated by anion-exchange chromatography as described in the Methods. The intact masses of ApcF and ApcB1, β-type AP subunits produced in cells grown in visible light, were also detected in the sample and are included in the table. The observed masses were obtained from ion traces with ESIprot (Winkler 2010). Da indicates Daltons, $1 \times$ PCB indicates the designated subunit binds one PCB (586.7 Da) per polypeptide, $+CH_3$ indicates methylation, most likely of the conserved N71/72 residue in β-AP (Klotz et al. 1986; Shen et al. 2008), and –Methionine indicates loss and presumed recycling of the N-terminal Met residue in α-type AP subunits.

Protein Name	Observed Mass (Da)	Theoretical Mass (Da)	Post-Translational Modifications
ApcF	19376.245	19377.714	$1 \times$ PCB, $+CH_3$
ApcD5	18289.823	18291.714	$1 \times$ PCB, –Methionine
ApcD2	18245.844	18247.706	$1 \times$ PCB, –Methionine
ApcD3	19673.458	19674.351	$1 \times$ PCB, –Methionine
ApcB2	18111.491	18112.476	$1 \times$ PCB, $+CH_3$
ApcB1	17871.889	17872.266	$1 \times$ PCB, $+CH_3$