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Characterization of Genes Involved in Chromatic Acclimation in the Cyanobacterium Synechococcus sp. A 15-62

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Characterization of genes involved in chromatic acclimation

in the cyanobacterium *Synechococcus sp*. A 15-62.

An Honors Thesis

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The Department of Biology

University of New Orleans

In Partial Fulfillment

of the Requirements for the Degree of

Bachelor of Science with University High Honors

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Honors in Biology

By

SUMAN POKHREL

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Acknowledgements

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Abstract

Synechococcus, a genus of photosynthetic cyanobacteria, is the second most abundant oxygenic microorganism in the marine environment that contributes significantly to the ocean's primary productivity (Humily et al. 2013; Shukla et al. 2012). They are capable of utilizing available light of different wavelengths in the visible spectrum to perform photosynthesis and fix carbon dioxide and thus inhabit a wide range of light niches in the ocean along horizontal (coast vs offshore) and vertical gradients (depth) (Humily et al. 2013). A gene encoding a putative lyase isomerase, *mpeQ*, is present in phycoerythrin-II encoding operon that is expressed constitutively and a gene encoding putative lyase, *mpeW*, is present in CA-4 genomic island whose expression is regulated by ambient light color were identified and characterized in *Synechococcus sp*. A15-62, a strain having a blue light specialist phenotype in its basal state. The amino acid sequence of the proteins encoded by *mpeW* and *mpeQ* are similar to other characterized lyases and these genes are conserved in cyanobacteria strains containing the CA4-B genomic island, which controls CA4 (Humily et al. 2013). The MpeW and MpeQ proteins were produced in *E. coli* and co-expressed with recombinant HT-MpeA and phycoerythrobilin (PEB) synthesis machinery. Site directed mutants of the HT-MpeA protein (Cys75Ala, Cys83Ala, Cys140Ala) were used to investigate the site for bilin attachment. The recombinant protein co-expression experiments of MpeQ and MpeW demonstrated that MpeQ attaches phycoerythrobilin (PEB) to cysteine-83 site on α-phycoerythrin II and isomerizes it to phycourobilin (PUB) and MpeW attaches phycoerythrobilin (PEB) to the same site.

Keywords: *Synechococcus*, phycoerythrin, lyase, lyase isomerase, phycoerythrobilin, phycourobilin, phycobilisomes.

1. Introduction

1.1 *Cyanobacteria: Background*

Cyanobacteria, also referred as blue-green algae, are one of the largest sub-groups of gram-negative bacteria constituting a global biomass of 3×10^{14} g C (Gracia et al. 2003). They are oxygenic photosynthetic prokaryotes inhabiting a diverse range of habitats and are among the most significant micro-organisms on the planet. There are carbon isotopic data consistent with the presence of Rubisco-mediated CO_2 fixation in stromatolites which provides geological and molecular evidence that cyanobacteria existed 3.5 billion years ago (Schopf 2014). Cyanobacteria are credited with the creation of the oxygen rich atmosphere on earth that exists today. Autotrophic eukaryotes that evolved later in the time scale incorporated these cyanobacteria in their cells, referred as chloroplasts, in order to obtain ability to perform oxygenic photosynthesis (McFadden 1999). Cyanobacteria contain different types of chlorophylls and accessory pigments that allow them to harvest available light of different wavelengths in the visible spectrum enabling them to thrive in different light niches. In addition, some species are also able to fix atmospheric nitrogen into a reduced form that is indispensable for biological processes. Their ability to fix atmospheric CO_2 using different wavelengths of light and atmospheric N_2 provide them competitive advantage over other organisms making them extremely successful life-forms (Oliver and Ganf 2000). These robust physiological strategies employed by cyanobacteria for survival elicit interest in the scientific community for further research. In addition to interesting physiology, they also contribute significantly to the earth's primary productivity making them an important subject of study from ecological perspective (Flombaum 2013). Cyanobacteria are also known to produce bioactive compounds with anti-microbial or anti-cancer activities, cosmetic agents and biofuels stimulating commercial interests (Zhou and Li 2010; Majee et al. 2017).

1.2 *Phycobilisome (PBS): Structure, function and assembly*

Cyanobacteria harvest light for photosynthesis using phycobilisomes (PBS), mega-dalton protein complexes that sit on the cytoplasmic surface of the thylakoid membrane. Phycobiliproteins (PBP) are the major proteins in cyanobacteria and make up to half of the total protein synthesized by cells (Glazer 1989). Photons are absorbed by PBP and the energy is transferred unidirectionally to the reaction centers in photosystem–II (Glazer 1985). Phycobilisomes are comprised of rods and a core containing phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (AP); all of these PBPs are interconnected by linker polypeptides (see **Fig. 1**). PBP have covalently attached bilin chromophores that impart characteristic spectroscopic properties to those proteins and enable absorption of photons of different wavelengths in the visible spectrum. These bilins are attached to the specific cysteine residues in apo-PBP by the reaction catalyzed by phycobilin lyases (Six et al. 2007).
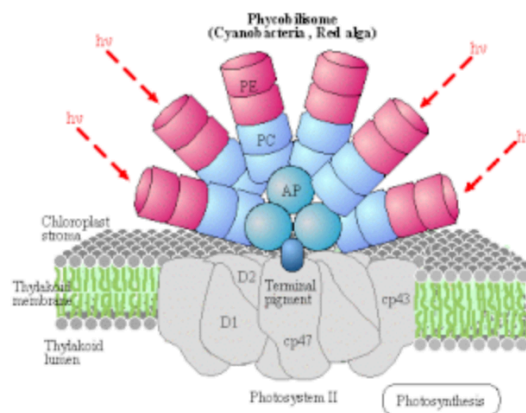
Fig. 1. Phycobilisome (taken from de Marsac 1994)**:** The structure of PBS in cyanobacteria and its location on the thylakoid membrane is shown. It is situated in the cytoplasmic side of the thylakoid membrane and is composed of core made up of allophycocyanin (AP) and rods radiating from the core that is made up of phycocyanin (PC) in the proximal end and phycoerythrin (PE) in the distal end. The photons are absorbed and are transferred unidirectionally to Photosystem-II.

Allophycocyanin, present in the core of the PBS, has covalently bound phycocyanobilin (PCB) and absorbs light in the red-light region (λ_{max} = 650-655nm) (Yoshikawa and Belay 2008). Phycocyanin (PC) on the other hand is present in the proximal part of the rod and also has bound PCB and absorbs light in orange and red-light region (λ_{max} = 615-640nm) (Siegel and Gerth 2008). Phycoerythrin (PE) is present in the distal part of the rod and has attached phycoerythrobilin (PEB) and its isomer, phycourobilin (PUB) (Six et al. 2007). PE absorbs light in blue-green region in the visible spectrum (λ_{max} = 495-575nm) (Humily et al. 2013). Cyanobacterial phycobilisomes may also have an additional type of phycobiliprotein called phycoerythrocyanin bound to phycoviolobilin (PVB) which absorbs photons in yellow region (λ_{max} = 575nm) (Parbel et al. 1997).

Each type of PBP is composed of two subunits: α and β polypeptides in equimolar quantities (Glazer 1989). Bilin is bound to the specific cysteine residues of apo-α and apo-β by the action of phycobilin lyases (see **Fig. 2**). The bilin bound subunits, holo-α and holo-β, come together to give ($\alpha\beta$) monomers. Three of these monomers are associated to give disc shaped ($\alpha\beta$)$_3$ trimers and two of these trimers assemble in face to face manner to give rise to hexamers ($\alpha\beta$)$_6$ (see **Fig. 2**) (Glazer 1989; Wang et al. 2014). These hexameric discs are linked to one another by the help of linker proteins and forms rods and core of phycobilisome. The linker protein may or may not have attached phycobilins (Wilbanks and Glazer 1993). The phycobiliproteins, within phycobilisomes, are assembled in such a way to create an energy transfer cascade such that lower wavelength (higher energy absorbing) bilins transfer their excitation energy to other bilins which absorb at higher wavelength (lower energy) until energy reaches the core of the PBS. (Shively et al. 2009). This structure of the PBS ensures the unidirectional flow of energy to PS-II reaction centers.

apo-α ⟷ apo-β

Lyase   Lyase

holo-α   holo-β

α β
Monomer
(αβ)

Trimer
(αβ)$_3$

L

Hexamer
(αβ)$_6$
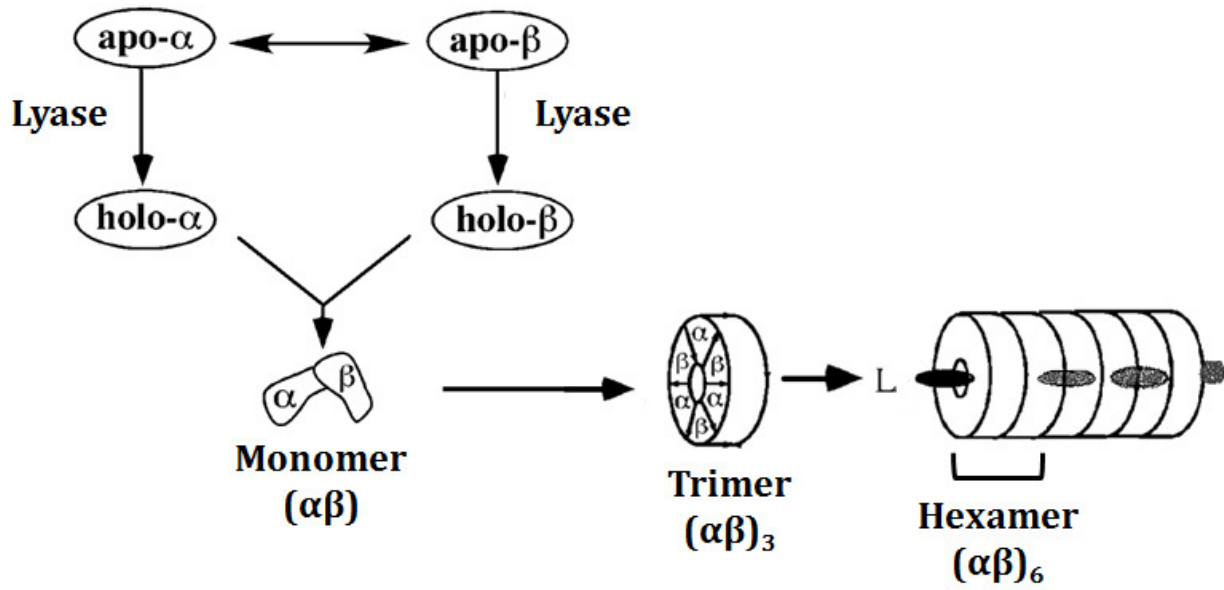
Fig. 2. Phycobilisome assembly in cyanobacteria (taken from Schluchter et. al 2010)**:** Bilin is bound to apo α and β peptides via lyase mediated reactions. Holo subunits form (αβ) monomers and three of these monomers give trimers, (αβ)$_3$. Two trimers associate to form hexamers, (αβ)$_6$. Hexameric discs are linked to each other to form core and rods of phycobilisome (Schluchter 2010).

1.2.1   *Phycoerythrin (PE): Structure and types*

Phycoerythrin (PE) is located in the distal part of the rods of phycobilisome. Marine species of cyanobacteria contain two types of PE: PE-I and PE-II whereas the freshwater species of cyanobacteria contain only one type of PE (Ong and Glazer 1991). PE-II lies towards the end of the rod whereas PE-I is sub-terminal in position. In this thesis, phycoerythrin from the marine strain of *Synechococcus sp.* A15-62 was studied. PE-I and PE-II are composed of α and β subunits, referred as CpeA/CpeB and MpeA/MpeB, respectively (Wilbanks and Glazer 1993). Each sub-unit has two to three cysteine residues where the phycobilins PEB or PUB are attached by the enzymatic reaction catalyzed by phycobilin lyases (see **Table 1**).

In this thesis, I am presenting my work on α subunit of PE-II, MpeA, of *Synechococcus sp.* A 15-62 and the bilins bound to different cysteine residues in different light conditions and the lyases responsible for the attachment process during phycobilisome biosynthesis were investigated.

PBP	Protein	Cysteine Position	Bilin attached in GL	Bilin attached in BL
PEII	MpeA	75	PUB	PUB
		83	**PEB**	**PUB**
		140	**PEB**	**PUB**
PEII	MpeB	50, 61	PUB	PUB
		82	PEB	PEB
		165	PEB	PEB
PEI	CpeA	82	PEB	PEB
		139	**PEB**	**PUB**
PEI	CpeB	50, 61	PUB	PUB
		82	PEB	PEB
		159	PEB	PEB

Table 1. Bilin binding sites in PE (taken from Shukla et al. 2012)**:** Table shows bilin binding sites and types of bilins bound in different subunits of PE in different light conditions in *Synechococcus sp.* RS 9916.

1.3 *Phycobilins: Types and biosynthetic pathway*

Phycobilins (or bilins) are linear tetrapyrroles that form biological pigments. Cyanobacterial PBS contain PBP bound with four types of isomeric bilins: phycocyanobilin (PCB), phycoviolobilin (PVB), phycoerythrobilin (PEB) and phycourobilin (PUB) (Ong and Glazer 1991). They differ from one another in terms of the length of their conjugated double bond system which enables them to absorb photons of varying wavelength (see **Fig. 3**). Free bilins are not very fluorescent on their own but when bound to phycobiliproteins they are held in a stretched confirmation, enhancing their fluorescent properties giving brilliant coloration (Glazer 1989; Scheer and Zhao 2008). Phycocyanobilin is a blue colored chromophore and absorbs light in red-light region (λ_{max} = 650-655nm) when bound to allophycocyanin and in orange and red-light region (λ_{max} = 615-640nm) when bound to phycocyanin. Phycoviolobilin is a violet colored bilin that binds to phycoerythrocyanin and absorbs light in yellow region (λ_{max} = 575nm). PE contains PEB and PUB that are pink and orange in color and absorb light in green (λ_{max} = 560nm) and blue (λ_{max} = 490nm) light regions, respectively.
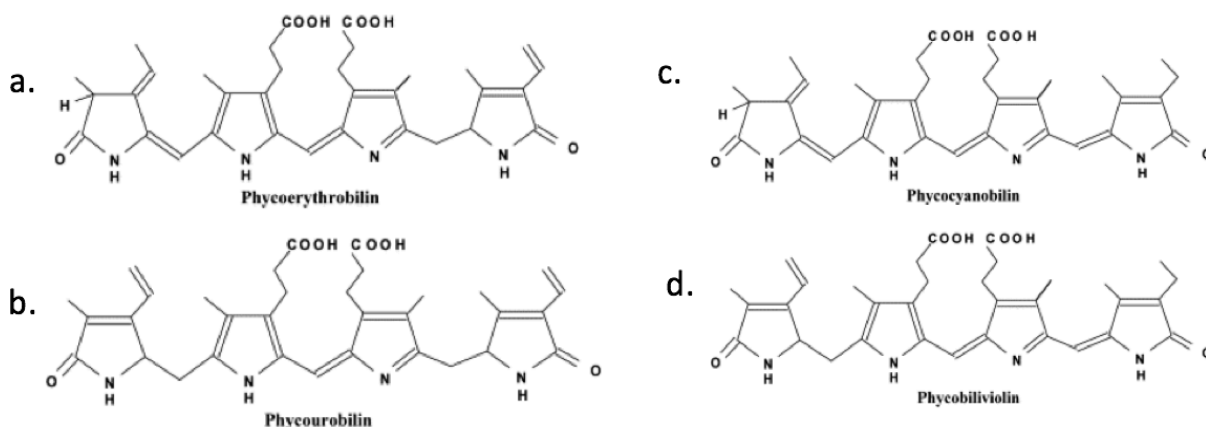
Fig. 3. Structure of bilins: Structure of bilins found attached to the phycobiliproteins in cyanobacteria is shown. Chemically, bilins are linear tetrapyrroles and they differ from one another in terms of length of conjugated double bonds.

These bilins are bound to the specific cysteine residues on phycobiliproteins via a single thioether linkage (at C-3' position on bilin) or double thioether linkage (at C-3' and C-18' position on bilin) by the action of phycobilin lyases (see **Fig. 4**), but sometimes, mainly for linker proteins, the addition can be autocatalytic (Biswas et al. 2011; Fairchild and Glazer 1994).

3^1- Cys - PΦB $3^1(,18^1$-Di)- Cys - PEB
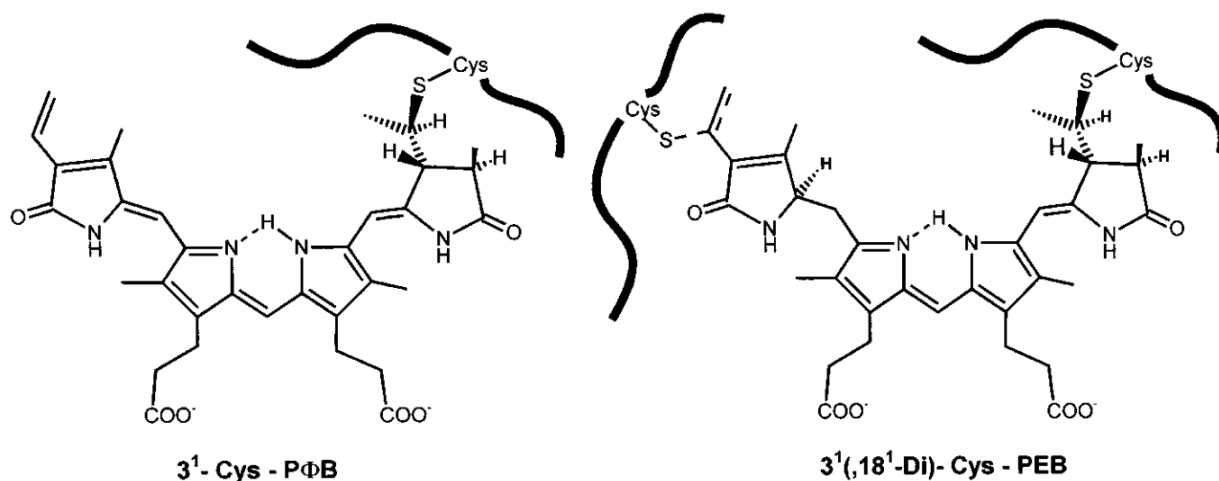
Fig. 4. Bilins bound to phycobiliproteins (taken from Storf and Parbel 2001)**:** The binding of bilins to phycobiliproteins is shown. Bilins are bound to the specific cysteine residues on phycobiliproteins via single thioether linkage at C-3' position on the bilin (left) or sometimes there is attachment via two thioether linkages at C-3' and C-18' position on the bilin.

Bilin biosynthesis in cyanobacteria starts off with oxidative cleavage of heme in presence of Heme Oxygenase (HOI) to give biliverdin IXα, a precursor molecule for all types of cyanobacterial bilins (Dammeyer and Frankenberg 2008). Biliverdin IXα is then converted to phycocyanobilin and phycoerythrobilin by the action of ferredoxin-dependent bilin reductases (FDBR) (see **Fig. 5**) (Frankenberg et al. 2001).
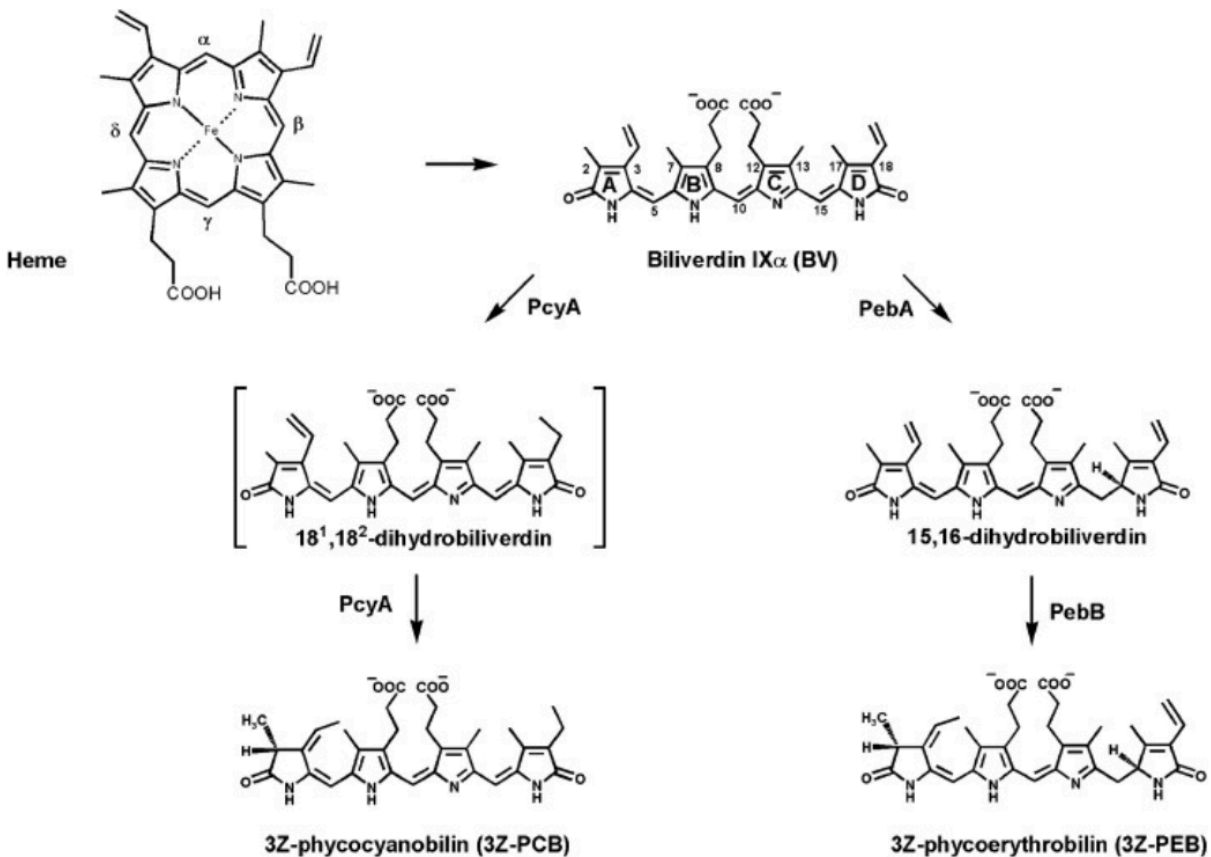
Fig. 5. Bilin biosynthesis (taken from Dammeyer et al. 2007)**:** Biosynthesis of PEB and PCB from heme is shown. Heme is first converted into biliverdin IXα via oxidative cleavage by heme oxygenase (HOI) and is subsequently converted to bilins by ferredoxin-dependent bilin reductases (FDBR).

In this thesis, the bilins I am studying are PEB and PUB. PEB in cyanobacteria is synthesized via a consecutive two-step two electron reduction process of biliverdin IXα by ferredoxin-dependent bilin reductases, PebA and PebB (see **Fig. 6**). In the first step, biliverdin IXα is converted into 15, 16- dihydrobiliverdin by adding two electrons. 15, 16- dihydrobiliverdin is further reduced into PEB by PebB enzyme by adding two more electrons (Dammeyer et al. 2007). In some organisms, PEB can be synthesized in one step. Myovirus infecting *Procholorococcus* encode a PebS enzyme which can perform a 4-electron reduction to synthesize PEB from

biliverdin IXα in a single step (Dammeyer et al. 2008). The HOI and PebS enzymes can be used

in cells to generate PEB from endogenous heme in *E. coli*.
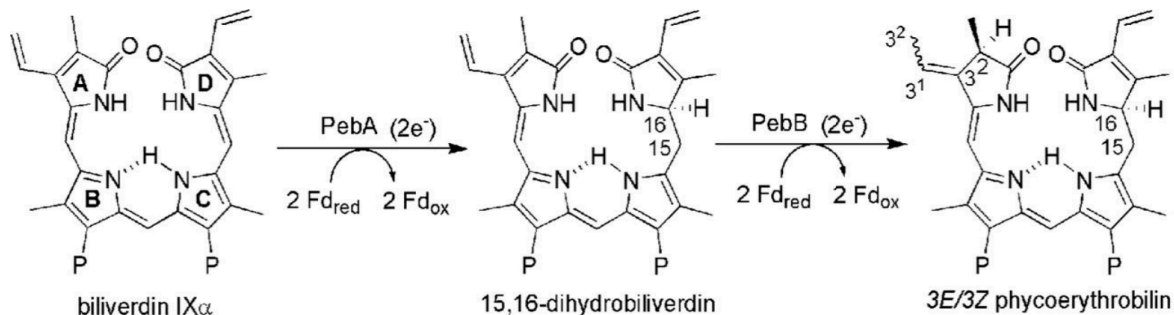
Fig. 6. PEB biosynthesis in cyanobacteria (taken from Biswas 2011)**:** Biosynthesis of PEB from
biliverdin IXα is shown. 15, 16- dihydrobiliverdin is synthesized in the first step by addition of
two electrons by PebA which is subsequently reduced to PEB by addition of two more electrons
by PebB.

Cyanobacteria however don't have enzymes to synthesize PUB directly from biliverdin

IXα or other heme derivatives. Recent studies have shown that PUB in cyanobacteria is formed by

a special category of bilin lyase which also isomerizes the bilin during the attachment process.

During this process, the lyase/isomerase attaches PEB to phycobiliproteins and Δ4→Δ2

isomerization takes place to form PUB (Blot et al. 2009).

1.4 *Chromatic acclimation: Introduction and types*

Chromatic acclimation can be defined as the ability of the photosynthetic organisms to adapt to the changes in ambient light color. Cyanobacteria are able to remodel their light absorbing machinery, phycobilisomes, in order to utilize available light to carry out optimum level of photosynthesis (Kehoe 2010). Cyanobacteria exhibit chromatic acclimation that can generally be divided into four types: Type I, II, III, IV. In Type I chromatic acclimation, the strains of cyanobacteria are not able to chromatically acclimate, and they don't change their PC or PE content in response to light. Strains exhibiting Type II chromatic acclimation accumulate PE under green light but not under red light, and PC is produced constitutively (Hirose et al. 2015). Type II chromatic acclimation is unidirectional, and this type of chromatic acclimation can be found in *Geminocystis sp.* strains (Hirose et al. 2015). Type III chromatic acclimation is bidirectional and can be found in freshwater cyanobacteria like *Fremyella diplosiphon* (Kehoe and Gutu 2006). During Type III chromatic acclimation, the cyanobacterial strains accumulate PC in red light and accumulate PE in green light (See **Fig. 9**) (Kehoe and Gutu 2006).
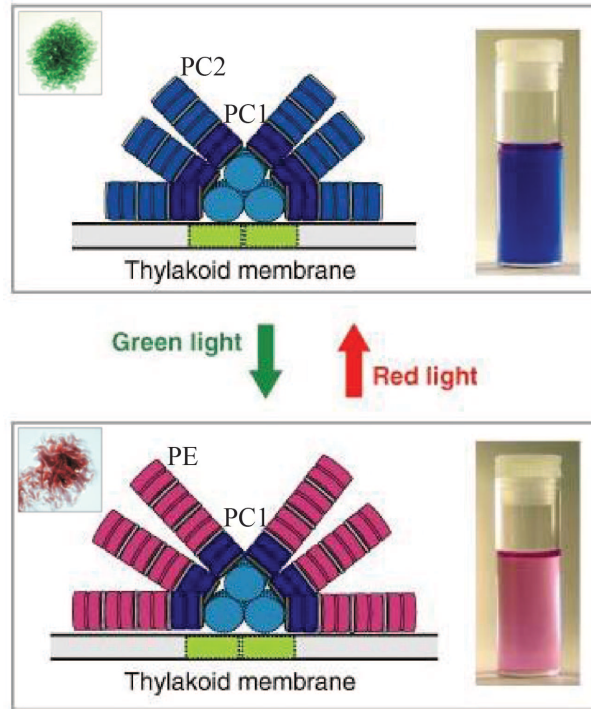
Fig. 7. Type III chromatic acclimation in *F. diplosiphon* (taken from Kehoe and Gutu 2006)**:** *F. diplosiphon* accumulate PC in red light and accumulate PE in green light giving blue and pink pigmentation respectively.

During Type IV chromatic acclimation, the strains of cyanobacteria adjust the ratio of phycobilins bound to their PBP in response to the change in ambient light color. *Syncechococcus sp.* A 15-62, the strain studied in this thesis, undergoes Type IV chromatic acclimation.

1.4.1   *Chromatic acclimation Type 4 (CA 4) in Syncechococcus sp.:*

Synechococcus strains undergo Type 4 chromatic acclimation (CA4) where they adjust their phycobilin content in response to the changes in light conditions (shift from green light to blue light or vice-versa) in order to achieve optimum photosynthetic activity (see **Fig. 8**) (Kehoe 2010). During CA4, there are three sites of differential chromophore attachment to PBP, specifically on CpeA (Cys139) and MpeA (Cys83 and Cys140) (Shukla et al. 2012).

12
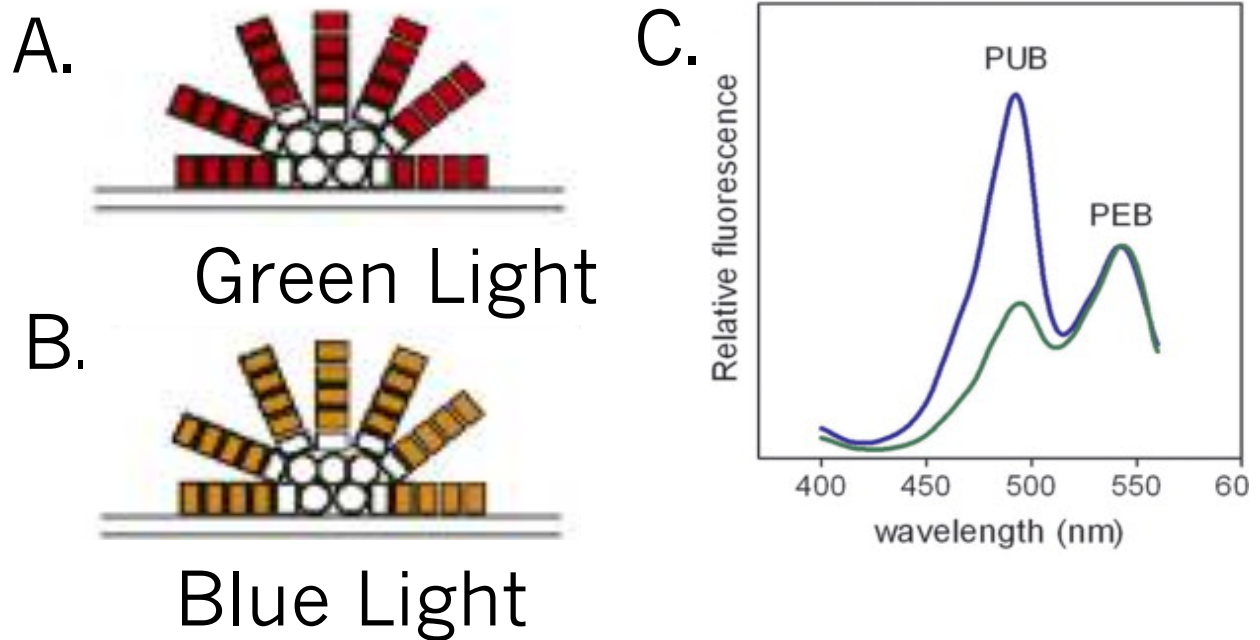
Fig. 8. CA 4 in *Synechococcus sp.* (taken from Kehoe and Gutu 2006)**:** PE in phycobilisomes have more **A**. PEB in green light and **B**. PUB in blue light. **C**. Relative fluorescence of whole cells at 490nm and 545nm showing more PUB in blue light and more PEB in green light.

A specific gene island (CA4 island) with two distinct configurations (A and B) containing genes for two transcriptional regulators (*fciA* and *fciB*), a phycobilin lyase gene (*mpeZ* or *mpeW*) and an unknown gene (*unk10*) were identified in the strains of *Synechococcus* demonstrating CA4 through whole genome analysis of the strains (see **Fig. 9**) (Humily et al. 2013).
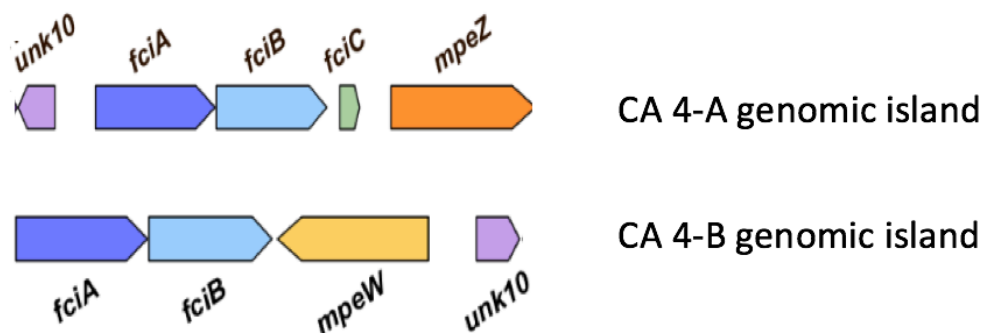
Fig. 9. Two types of CA4 genomic islands in *Synechococcus sp.* (taken from Humily et al. 2013)**:** CA4-A genomic island with two transcriptional regulators (*fciA* and *fciB*), a phycobilin lyase gene (*mpeZ*) and an unknown gene (*unk10*) and CA4-B genomic island with two transcriptional regulators (*fciA* and *fciB*), a phycobilin lyase gene (*mpeW*) and an unknown gene (*unk10*).

In the CA4-A genomic island, the phycobilin lyase/isomerase gene present is *mpeZ* which has already been characterized in *Synechococcus* sp. RS 9916. The *mpeZ* gene was shown to encode a lyase isomerase, MpeZ, that is expressed more under blue light conditions and attaches PEB to the Cys83 residue on MpeA and isomerizes it to PUB (Shukla et al. 2012). These strains contain a gene in the PEII encoding operon, *mpeY*, closely related phylogenetically to *mpeZ* (Six et al. 2007). The *mpeY gene i*s expressed constitutively, and the enzyme it encodes attaches PEB to the Cys83 residue on MpeA (J. E. Sanfilippo et al. unpublished). MpeY attaches PEB to Cys83 site on MpeA in green light. In BL, MpeZ is produced, outcompetes MpeY, and attaches PUB to the C83 on MpeA during phycobilisome biosynthesis (J. E. Sanfilippo et al. unpublished).

Similarly, the putative phycobilin lyase gene, *mpeW*, is present in the CA4-B island. *mpeW*, an uncharacterized relative of *mpeZ*, is observed to be expressed more in green light than in blue light (Humily et al. 2013). These CA4-B strains have a gene, *mpeQ*, in the PEII encoding operon in the same location as *mpeY* in the strains with CA4-A island, and the *mpeY* and *mpeQ*

genes were found to be very similar. In this thesis, the proteins encoded by *mpeW* and *mpeQ* were biochemically characterized and their role in attachment of phycobilins in MpeA was investigated.

During the process of CA4, there are two sites of differential chromophore attachment on MpeA (see **Table 2**). Different bilins are attached to those cysteine residues under different light conditions. Attachment at Cys-83 on MpeA is catalyzed by different members of MpeY, MpeZ and MpeW sub-family of lyases. The contribution of this thesis was to characterize the role of MpeQ and MpeW from the CA4-B strain *Synechococcus* sp. A15-62.

Cysteine Position	Bilin attachment in GL		Bilin attachment in BL	
	CA 4-A	CA 4-B	CA 4-A	CA 4-B
75	PUB	?	PUB	?
83	PEB (MpeY)	PEB (MpeW)	PUB (MpeZ)	PUB (MpeQ)
140	PEB	?	PUB	?

Table 2. Bilin attachment sites in MpeA of *Synechococcus sp.* RS9916 and A15-62.: Bilin attachment sites in MpeA of strains demonstrating CA4-A and CA4-B and bilins attached to those sites in different light conditions is shown. Lyases responsible for this attachment are given in parentheses.

1.5 *Phycobilin lyases: Types and function*

The addition of bilins to the apo-phycobiliproteins are catalyzed by a class of enzymes called phycobilin lyases. The bilins are attached to specific cysteine residues in the phycobiliproteins where they are held in a stretched conformation making the holo-phycobiliproteins highly fluorescent (Scheer and Zhao 2008). PBP lyases act like protective carriers that momentarily bind the bilin chromophores and transfer it to the apo-phycobiliproteins (Zhao et al. 2017). In addition to ligating the bilins to the phycobiliproteins, phycobilin lyases can also be responsible for isomerization of bilins or removal of bilins from the phycobiliproteins (Shukla et al. 2012; Grossman 1999). Bilin lyases are categorized into three types: CpcEF type, CpcSU type and CpcT type (Schluchter et al. 2010). These phycobilin lyases differ from one another in terms of the primary amino acid sequences and have different tertiary structures (Kronfel et al. 2013) and bind bilins to different phycobilisome subunits. However, in some cases the addition of bilins to phycobiliproteins can be autocatalytic where the phycobiliproteins can catalyze the addition of bilin to itself (Zhao et al. 2005; Biswas et al. 2010).

E/F-type lyases are known to attach phycocyanobilin (PCB) or phycoerythrobilin (PEB) to the Cys-84 site of α-subunits of phycocyanins (PCs), phycoerythrins and phycoerythrocyanin (PEC) (Fairchild and Glazer 1994). Some E/F-type lyases are also known to have the dual function of attaching a bilin and isomerizing it during the process of attachment. The MpeZ protein from *Synechococcus* sp. RS 9916 was found to attach PEB to α-PE II and isomerize it to PUB (see **Fig. 10**) (Shukla et al. 2012).
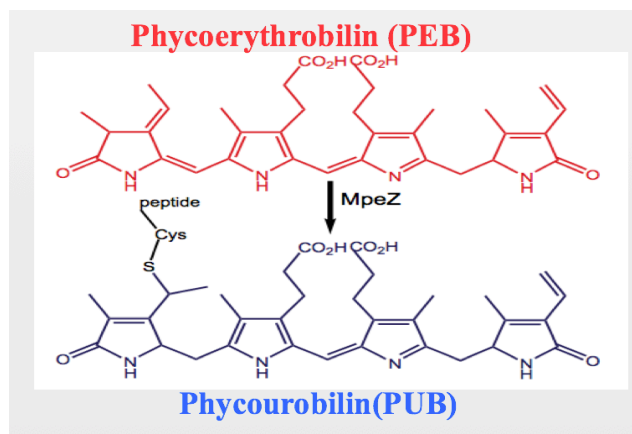
Fig. 10. Lyase isomerase function of a bilin lyase (taken from Shukla et al. 2012)**:** Lyase isomerase function of MpeZ is shown. MpeZ attaches PEB to MpeA and isomerizes it to PUB.

Also, some lyases of this type are also known to take part in PBP degradation. The NblB protein detaches chromophores from PBP (Grossman 1999) under nitrogen deprivation conditions. E/F-type lyases assume an α-solenoid structure for their tertiary structure (Zhao et al. 2017).

S/U-type lyases are responsible for attaching bilins to the Cys-81 site of allophycocyanins and the Cys-84 site of β-subunits of PBP (Shen et al 2004; Zhao et al. 2007 Saunee et al. 2008). This type of lyase assumes a 10 stranded-β-barrel in their tertiary structure and closely resemble the fatty acid binding proteins (Kronfel at al. 2013). T-type lyases, which adopt a β-barrel tertiary structure, catalyze the ligation of bilins to the Cys-155 site of β-subunits in PBPs (Zhou et al. 2014). T-type lyases attach bilin to Cys-155 with the C_3^1 chiral carbon in the *S* stereochemistry unlike S/U type and E/F type bilin lyase that attach bilins to Cys with *R* stereochemistry (Shen et al. 2006). In some cases, the addition of bilins to the PBP can be autocatalytic where the protein subunit might itself catalyze the addition of bilin to itself. This phenomenon of autocatalytic ligation has been recorded in large core membrane linker protein of PBP designated as ApcE which contains a PCB (Zhao et. al 2005; Biswas et al., 2010).

1.5.1   *MpeQ, MpeW, MpeY, and MpeZ subfamily: Structure and function*

A subfamily including MpeQ, MpeW, MpeY, and MpeZ belongs to the E/F-type lyase family; this sub-family includes lyases responsible for attachment of bilins to the α-PE II (MpeA) in PBS (Shukla et al. 2012; Sanfilippo et al unpublished; T. Grébert, L. Garczarek, D. Kehoe, and F. Partensky, unpublished). Members of this QWYZ family are characterized by the presence of an α/α-superhelix fold and Armadillo repeat motifs (see **Fig. 11**) (Shukla et al. 2012). Two enzymes belonging to this family, MpeY and MpeZ, have been recently characterized in *Synechococcus* sp. RS 9916. MpeY was expressed constitutively and responsible for attachment of PEB to central Cys-83 site on MpeA in green light condition whereas MpeZ expression increased 35-fold in blue light condition and it was shown to attach PUB to the same site (J. E. Sanfilippo et al. unpublished; Shukla et al. 2012; J. E. Sanfilippo et al. 2016). In this thesis, two lyases belonging to this sub-family of lyases, MpeQ and MpeW, from *Synechococcus* sp. A15-62 are biochemically characterized.
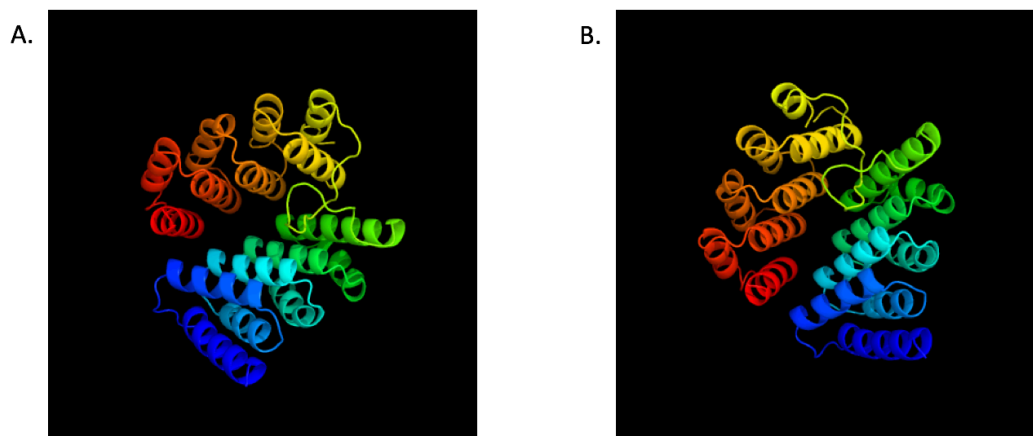
Fig. 11. Phyre² predicted structures: Phyre² predicted structures of **A**. MpeW and **B**. MpeQ are shown. These lyases have α/α-superhelix fold and Armadillo repeat motifs (Shukla et al. 2012).

1.6 *Purpose of work:*

Synechococcus sp. A15-62 possesses the CA4-B genomic island containing phycobilin lyase gene, *mpeW*, an uncharacterized relative of *mpeZ*. The amino acid sequence of protein encoded by *mpeW* and *mpeZ* were found to be 53% similar when these genes were translated and aligned using ClustalW program. The analysis of the mutant cells with disrupted *mpeW* showed that there is less PEB attached to phycobilisomes in green light as compared to the wild type (T. Grébert, L. Garczarek, D. Kehoe, and F. Partensky, unpublished). These observations have led us to hypothesize that *mpeW* encodes a lyase that attaches PEB to MpeA. These CA4-B strains also have a gene, *mpeQ,* in the PEII encoding operon in the same location as *mpeY* in the strains with the CA4-A island (Humily et al. 2013). *mpeQ* and *mpeY* were found to be 77% similar when these putative lyase genes were translated and the amino acid sequence was aligned using ClustalW program. The analysis of the mutant cells with disrupted *mpeQ* showed that there is less PUB attached to the phycobilisomes in blue light (T. Grébert, L. Garczarek, D. Kehoe, and F. Partensky, unpublished). Based on these observations we hypothesize that *mpeQ* encodes a lyase/isomerase that attaches PEB to MpeA and isomerizes it to PUB, and *mpeW* encodes for a lyase that attaches PEB to the same site.

The goal of this project is to perform the biochemical characterization of putative lyases/isomerases MpeW and MpeQ, members of QWYZ enzyme family of lyases, from *Synechococcus* sp. A 15-62 using a multiplasmid heterologous expression system in *E. coli* to produce PBP substrates, enzyme machinery responsible for synthesis of phycobilins and the putative lyases/isomerases.

2. Materials and Methods

2.1 *Construction of inducible expression plasmids:*

Table 3 lists the plasmids used in this study. The recombinant plasmids constructed for the purpose of our experiments were sequenced at the W. M. Keck Conservation and Molecular Genetics Laboratory (University of New Orleans) to confirm sequences. Each gene was amplified by PCR from *Synechococcus* RS 9916 and *Synechococcus* A 15-62 DNA. The primers used for PCR are listed in **Table 4**. Some of the expression plasmids used in this study were previously described.

Table 3. Expression plasmids used in this study

Plasmid name	Recombinant proteins produced	Parent vector	Antibiotic	Reference
pMpeA	*Synechococcus* sp. A 15-62 HT-MpeA	pCOLA Duet	Km	This work
pMpeA	*Synechococcus* sp. RS 9916 HT-MpeA	pCOLA Duet	Km	Shukla et al. 2012
pMpeB	*Synechococcus* sp. RS 9916 HT-MpeB	pCOLA Duet	Km	Shukla et al. 2012
pCpeA	*Synechococcus* sp. RS 9916 HT-CpeA	pCOLA Duet	Km	Shukla et al. 2012
pMpeA:C83A	*Synechococcus* sp. RS 9916 HT-MpeA (Cys83 mutated to Ala)	pCOLADuet	Km	Shukla et al. 2012
pMpeA:C75,140A	*Synechococcus* sp. RS 9916 HT-MpeA (Cys75,140 mutated to Ala)	pCOLADuet	Km	Shukla et al. 2012
pMpeQ	*Synechococcus* sp. A 15-62 Nus-MpeQ	pET-44b	Ap	This work
pMpeW	*Synechococcus* sp. A 15-62 Nus-MpeW	pET-44b	Ap	This work
pPcyA	*Synechocystis* sp. PCC 6803 Ho1 and *Synechococcus* sp. PCC 7002 HT-PcyA	pACYCDuet-1	Cm	Biswas et al. 2010
pPebS	Myovirus Ho1 and NT-PebS	pACYCDuet-1	Cm	Kronfel 2017

Table 4. Primers used in this study

Primer	Sequence (5' to 3')	Use
A15-62 Nus MpeQ;F (SacI)	GTAGGAGCTCGCCGAGCGATTCGACAA	Amplification of A15-62 *mpeQ*
A15-62 Nus MpeQ; R (EcoRI)	GCCGGAATTCTTATGACAACTGTTTAAGGA	Amplification of A15-62 *mpeQ*
A15-62 HT MpeA;F (BamHI)	GTAGGGATCCGAGTCCGTTATCACC	Amplification of A15-62 *mpeA*
A15-62 HT MpeA; R (EcoRI)	GTAGGAATTCTCAGCCGAGGGAGTTG	Amplification of A15-62 *mpeA*
A15-62 Nus MpeW;F (BamHI)	GTAGGGATCCGGTGCCAAGTTCAC	Amplification of A15-62 *mpeW*
A15-62 Nus MpeW; R (HindIII)	GCCGAAGCTTGCCTATAGTTCACGTAAGAC	Amplification of A15-62 *mpeW*

2.1.1 *MpeA expression construct:*

The *mpeA* gene was amplified from *Synechococcus sp.* A 15-62 chromosomal DNA using PCR using primers mpeAF and mpeAR (See **Table 4**) and cloned into pCOLA Duet (Novagen, Madison, WI) by digesting with BamHI and EcoRI. The resultant plasmid was called pMpeA. The construct results in the production of amino-terminal hexa-histidine-tagged MpeA which has a molecular mass of 19.2 kDa (See **Fig. 12**).

mpeA = 498 bp   pCOLA = 3719 bp
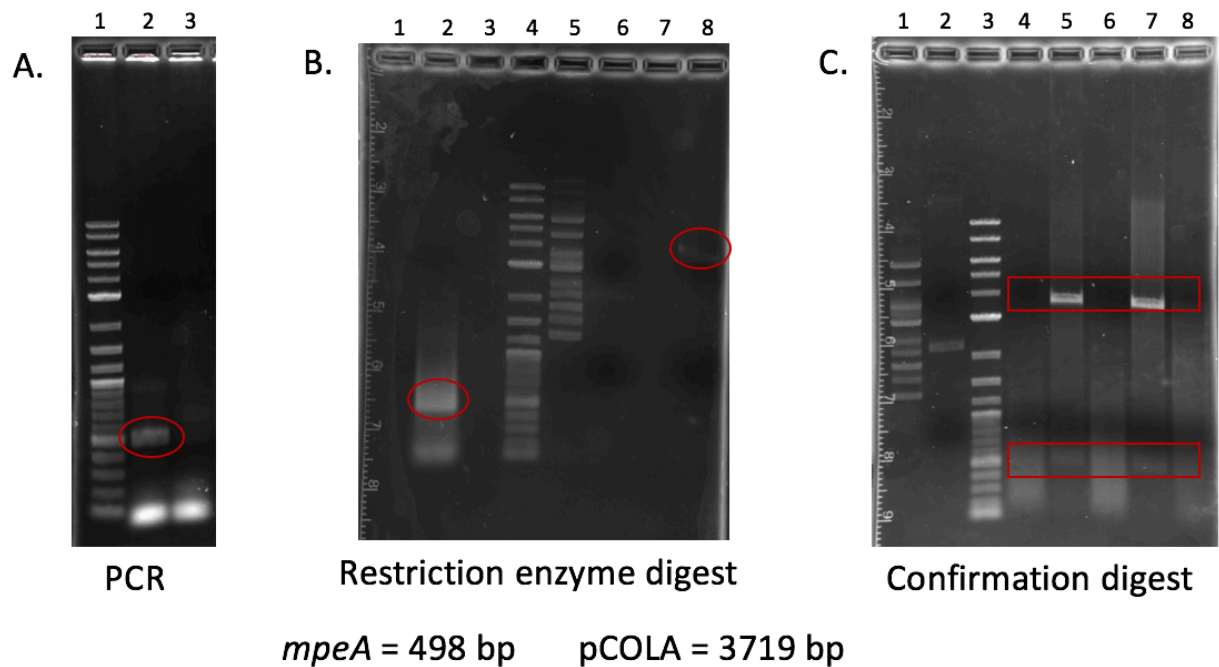
Fig. 12. Cloning *mpeA* from *Synechococcus sp.* A 15-62 into pCOLA DUET. *mpeA* gene was cloned using standard cloning technique. **(A)** PCR amplification of *mpeA* gene from *Synechococcus sp.* A 15-62 chromosomal DNA using mpeAF and mpeAR (See **Table 4**) primers is shown. The size of *mpeA* gene (Lane 2) is about 498bp. **(B)** Restriction enzyme digest of PCR product (Lane 2) and empty pCOLA vector (Lane 8) using BamHI and EcoRI is shown. **(C)** Restriction enzyme digest of potential HT-MpeA/pCOLA clones (Lanes 4-8) using BamHI and EcoRI is shown. Potential clones in Lane 5 and Lane 7 only seemed to have *mpeA* insert.
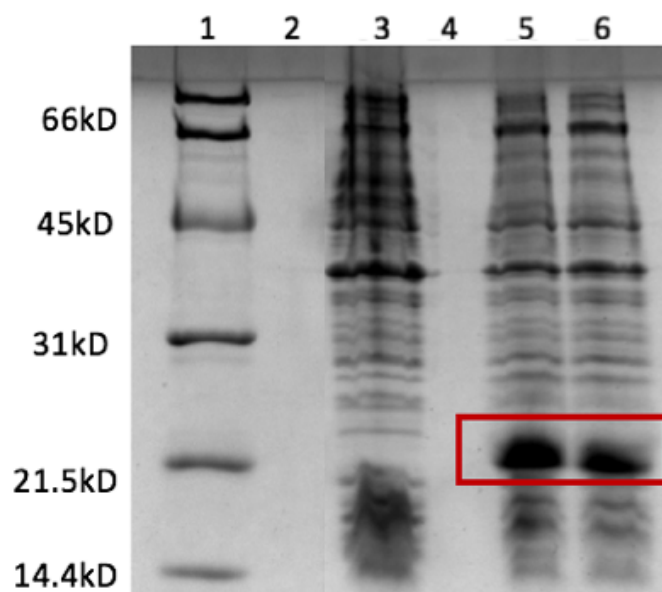
Fig. 13. SDS-PAGE analysis of whole cells of *E. coli* BL21 [DE3] expressing MpeA. A quick expression experiment was done to check if the potential clones produced proteins. The whole cell SDS samples of the potential clones with HT-MpeA/pCOLA after quick expression experiment in lanes 5 and 6 have HT-MpeA protein with molecular mass of 19.2 kDa. Lane 3 has proteins from *E. coli* BL21 [DE3] whole cells with empty pCOLA vector.

2.1.2 *MpeW expression construct:*

The *mpeW* gene was amplified from *Synechococcus sp*. A 15-62 chromosomal DNA by PCR using primers mpeWF and mpeWR (See **Table 3**) and cloned into pET-44b (Novagen, Madison, WI) by digesting with BamHI and HindIII. The resultant plasmid was called pMpeW. The construct results in the production of amino terminal NusA-tagged MpeW which has a molecular mass of 106.9 kDa (see **Fig. 15**). The MpeW protein was NusA-tagged in order to increase its solubility in *E. coli* as studies have shown the NusA tag increases solubility of the tagged recombinant protein (De Marco et al. 2004).

mpeW = 1194 bp   pET-44b = 7311 bp

Fig. 14. Cloning *mpeW* from *Synechococcus sp*. A 15-62 into pET-44b. *mpeW* gene was cloned using standard cloning technique. **(A)** PCR amplification of *mpeW* gene from *Synechococcus sp*. A 15-62 chromosomal DNA using mpeWF and mpeWR (See **Table 4**) primers is shown. The size of *mpeW* gene (Lane 3 and 4) is about 1194bp. **(B)** Restriction enzyme digest of PCR product (Lane 2) and empty pET-44b vector (Lane 8) using BamHI and HindIII is shown. **(C)** Restriction enzyme digest of potential Nus-MpeW/pET-44b clones (Lanes 4-8) using BamHI and HindIII is shown. All the potential clones (Lane 4-8) seemed to have *mpeW* insert.

Fig. 15. SDS-PAGE analysis of whole cells of *E. coli* BL21 [DE3] expressing MpeW. Quick expression experiment was done to check if the potential clones produced proteins. The whole cell SDS samples of the potential clones after quick expression experiment in lanes 3, 4 and 5 have Nus-MpeW proteins with molecular mass of 106.9 kDa. Lane 2 has proteins from *E. coli* BL21 [DE3] whole cells with empty pET-44b vector and NusA protein tag with molecular mass of 67.8 kDA can be observed.

2.1.3 *MpeQ expression construct:*

The *mpeQ* gene was amplified from *Synechococcus sp*. A 15-62 chromosomal DNA using PCR using primers mpeQF and mpeQR (See **Table 3**) and cloned into pET-44b (Novagen, Madison, WI) by digesting with SacI and EcoRI. The resultant plasmid was called pMpeQ. The construct results in the production of amino terminal Nus-tagged MpeQ with a molecular mass of 106.1 kDa (see **Fig. 17**). The MpeQ protein was NusA-tagged in order to increase its solubility in *E. coli*.
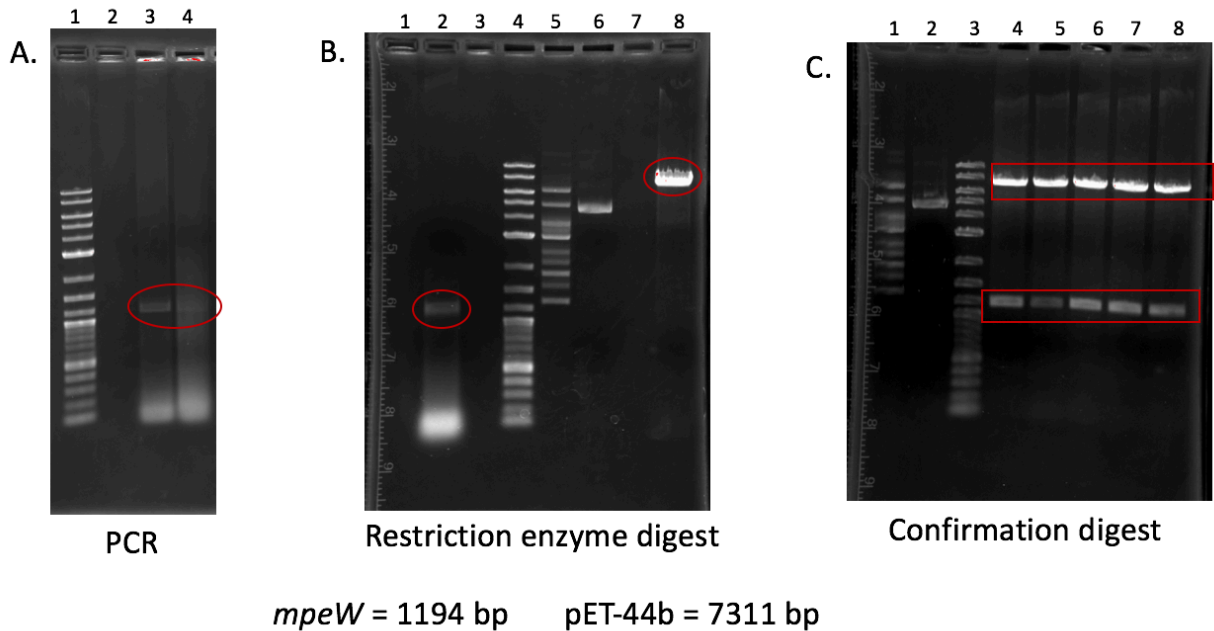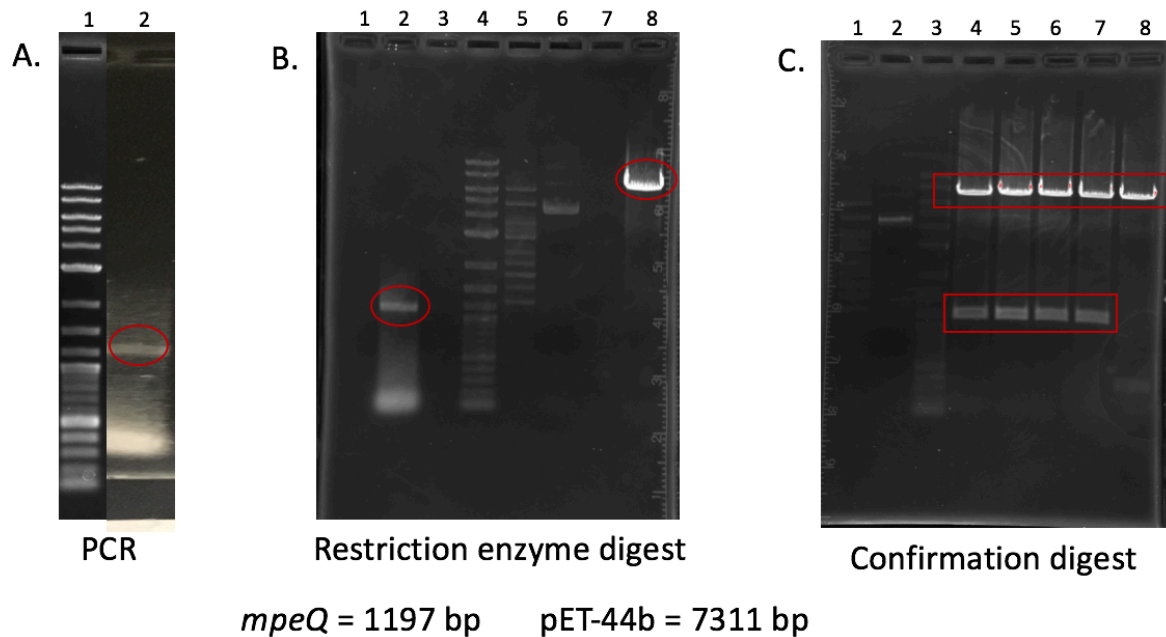

Fig. 16. Cloning *mpeQ* from *Synechococcus sp*. A 15-62 into pET-44b. *mpeQ* gene was cloned using standard cloning technique. **(A)** PCR amplification of *mpeQ* gene from *Synechococcus sp*. A 15-62 chromosomal DNA using mpeQF and mpeQR (See **Table 4**) primers is shown. The size of *mpeQ* gene (Lane 2) is about 1197bp. **(B)** Restriction enzyme digest of PCR product (Lane 2) and empty pET-44b vector (Lane 8) using SacI and EcoRI is shown. **(C)** Restriction enzyme digest of potential Nus-MpeQ/pET-44b clones (Lanes 4-8) using SacI and EcoRI is shown. The potential clones in Lane 4, 5, 6 and 7 only seemed to have *mpeQ* insert.
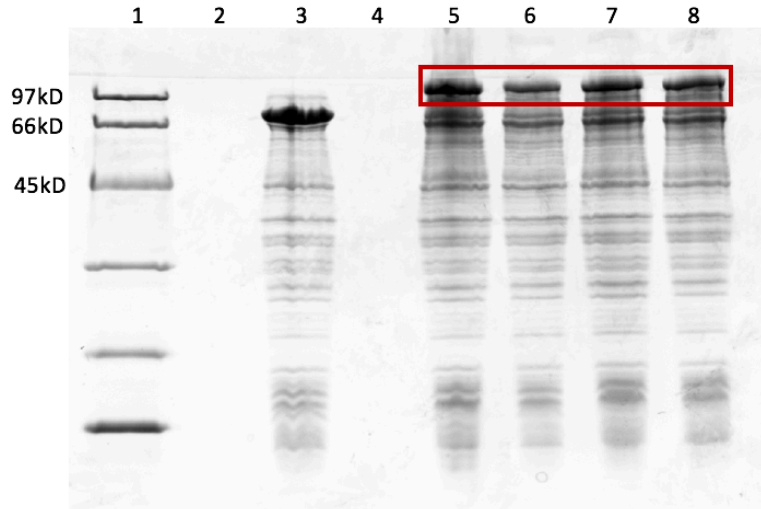
Fig. 17. SDS-PAGE analysis of whole cells of *E. coli* BL21 [DE3] expressing MpeQ. A quick expression experiment was performed to check if the potential clones produced proteins. The whole cell SDS samples of the potential clones after quick expression experiment in lanes 5, 6, 7 and 8 have Nus-MpeQ proteins with molecular mass of 106.1 kDa. Lane 3 has p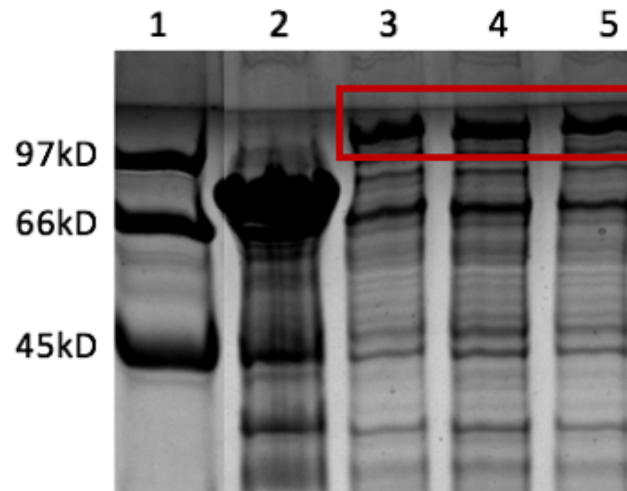roteins from *E. coli* BL21 [DE3] whole cells with empty pET-44b vector and NusA protein tag with molecular mass of 67.8 kDA can be observed.

2.2 *Recombinant protein expression and purification:*

Recombinant expression plasmids were co-transformed into *E. coli* BL21 [DE3] cells, and colonies were selected on Luria Bertani (LB) plates with the appropriate antibiotics (see **Table 4**) at the following concentrations: ampicillin (Ap: 100 µg ml^{-1}), chloramphenicol (Cm: 34 µg ml^{-1}), kanamycin (Km: 50 µg ml^{-1}), spectinomycin (Sp: 100 µg ml^{-1}). A 50-ml overnight starter culture was added to 1 L of LB medium with the appropriate antibiotics. This culture was shaken at 37°C for 2 h until the optical density reached 0.6 (O. D$_{600 nm}$ = 0.6) and was induced by the addition of 1 mM isopropyl β-D thiogalactoside (IPTG). The induced cultures expressing PEB biosynthesis pathway proteins were placed at 18°C for approximately 24h at 220 rpm. Cells were harvested by centrifugation at 11,000 x g for 10 min, and pellets were stored at -20°C.

Cell pellets were resuspended in approximately 10 ml of Buffer O (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mMKCl), 0.1x complete mini protease inhibitor cocktail and 0.01 mg·mL^{-1} lysozyme; exact volumes used were based on the relative mass of the pellets. The cells were lysed by passage through a French pressure cell press at 138 MPa three times. The lysed cell suspension was centrifuged for 20 min at 13,000 x g to remove inclusion bodies and cell debris. Hexa-histidine tagged recombinant proteins were purified by passing the supernatant over nickel-nitrilotriacetic acid- Superflow-affinity column (Qiagen, Inc., Chatsworth, CA) containing 6 ml of resin, and proteins were purified as previously described (Shen et al. 2006). The recombinant protein(s) were dialyzed with buffer O containing 10 mM 2-mercaptoethanol overnight at 4 °C to remove imidazole.

2.3 *Protein analysis by gel-electrophoresis:*

Protein samples were separated by polyacrylamide gel electrophoresis (PAGE, 15% w/v), in the presence of sodium dodecyl sulfate (SDS), and fluorescence from bilins linked to proteins was detected with excitation at 488 nm or 532nm using BIO-RAD ChemiDoc™ MP Imaging System (Universal Hood III).

2.4 *Fluorescence Emission and Absorbance Spectra of Purified Proteins:*

Perkin Elmer LS55 fluorescence spectrophotometer (Waltham, MA) with slit widths set at 10 nm was used to obtain the fluorescence emission spectra. The excitation wavelength was set to 440 nm to detect PUB and 490 nm to detect PEB. The chromophorylated samples were diluted before obtaining their fluorescence spectra whereas negative control samples with no obvious chromophore attached were not diluted. Absorbance spectra were acquired using a lambda 35, dual-beam UV-Vis spectrophotometer (Perkin Elmer, Waltham, MA).

2.5 *HPLC Separation of recombinant proteins:*

Purified recombinant HT-MpeA was further purified by collecting fractions eluting from a C4 (BioBAsic-4; Thermo Fisher Scientific, Waltham, MA) column and digested with trypsin as described previously (Biswas et al. 2011).

2.6 *Analysis of tryptic peptides by liquid chromatographic, ultraviolet-visible absorption spectroscopy/tandem mass spectrometry:*

Dried protein samples were reconstituted with 40 uL of LCMS grade water (Thermo Fisher Scientific); 15 uL of this solution was combined with 15 uL of 0.1% formic acid in a low volume

autosampler vial insert. Peptides were analyzed on an Orbitrap Lumos Fusion mass spectrometer (Thermo Fisher) with an Agilent 1100 Capillary HPLC as its inlet. HPLC solvents were (A) 0.1% v/v formic acid in water and (B) 5% v/v water, 0.1% v/v formic acid in acetonitrile. The column was a 0.3 mm inner diameter, 100 mm long Zorbax SB300 C18 (Agilent) and it was held at 55 °C throughout the analysis. The gradient was:

Table 5. HPLC solvent gradient

Time (min)	Solv. B %	Flow (uL/min)
0	0.5	7
4.01	0.5	7
10	20	7
40	45	7
43	95	7
48	95	7
49	0.5	7

UV-VIS spectra were recorded from 200-750 nm by an Agilent 1315D diode array detector placed between the column and the mass spectrometer source. Individual chromatogram traces for 490, 550, 620, and 280 nm were recorded simultaneously. The time delay between UV-VIS detection and mass spectrometric detection was measured to be 0.71 minutes.

The mass spectrometer was operated in positive ion, electrospray ionization mode with data dependent MS-MS and a 2.0 sec cycle time. MS1 scans were recorded from m/z 250-1500 at 120,000 resolving power; precursors were selected with a 1.2 m/z isolation window using the quadrupole mass filter. The precursor ions were fragmented in HCD mode at 35% relative

collision energy and analyzed in the Orbitrap at 50,000 resolving power. All mass spectra were calibrated internally with fluoranthrene ions generated by the Easy-IC source.   The   tandem mass spectra were processed using Thermo Proteome Discover 2.1 software; a simplified protein database consisting of only the 40 proteins expected to be part of the phycobilisome was used to speed up the analysis. Bilin-containing peptides were confirmed by manual inspection of their associated MS1, MS2, and UV-VIS spectra.

3. Results

3.1 *Analysis of genes from Synechococcus sp. A 15-62 involved in PE-II biosynthesis:*

The genes involved in PE-II biosynthesis in *Synechococcus sp.* A 15-62 are located in the PE-II encoding operon and CA4-B genomic island. PE-II encoding operon and CA-4 B genomic island are shown in the diagram below (see **Fig. 18**). The genes of unknown function, *unk9* and *unk10,* are indicated by black color; the genes encoding PE-II subunit, *mpeB and mpeA*, are indicated in red; the gene encoding a linker protein, *mpeC*, is indicated in brown; putative lyase genes, *mpeQ, mpeU and mpeW,* are represented in blue; and the transcriptional regulators, *fciA* and *fciB*, are indicated in green.
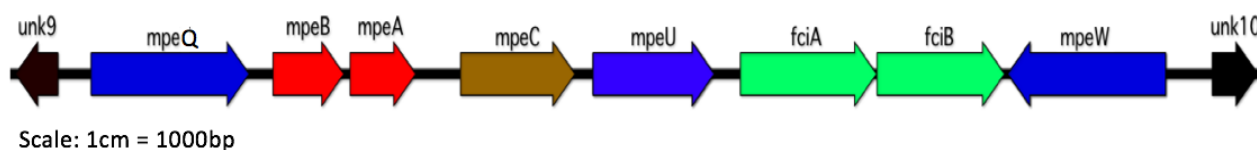
Fig. 18. PE-II specific genes in *Synechococcus sp.* A 15-62. Genes involved in PE-II biosynthesis are shown. The genes of unknown function are indicated by black color; the genes encoding PE-II subunit are indicated in red; the gene encoding a linker protein is indicated in brown; putative lyase genes are represented in blue; and the transcriptional regulators are indicated in green.

3.1.1 *The mpeA gene:*

The *mpeA* gene is 498 base pairs and encodes for the α subunit of PE-II and is expressed constitutively. This gene lies in the PE-II encoding operon and downstream of *mpeB* that encodes for the β subunit (see **Fig. 18**). The protein encoded by the *mpeA* gene from *Synechococcus sp.* A 15-62 containing the CA4-B genomic island, and that encoded by the *mpeA* gene from *Synechococcus sp.* RS 9916, strain having CA4-A genomic island, were very similar. The amino acid sequence of the protein encoded by *mpeA* genes from these two strains were found to be 96% similar when aligned using ClustalW program with 152 conserved residues and 7 similar residues

(see **Fig. 19**). Based on these findings, we hypothesized that the lyases from QWYZ enzyme sub-family from one strain should also be active on the MpeA from another species. Recombinant protein co-expression experiments demonstrated that MpeQ and MpeW from *Synechococcus sp*. A 15-62 were active on MpeA from *Synechococcus sp*. RS 9916 (see **Fig. 24**; **Fig. 29**). As MpeQ and MpeW from *Synechococcus sp*. A 15-62 were active on MpeA from *Synechococcus sp*. RS 9916, existing site directed mutants of MpeA from *Synechococcus sp*. RS 9916 (see **Table 3**) were used to investigate the site for bilin attachment.
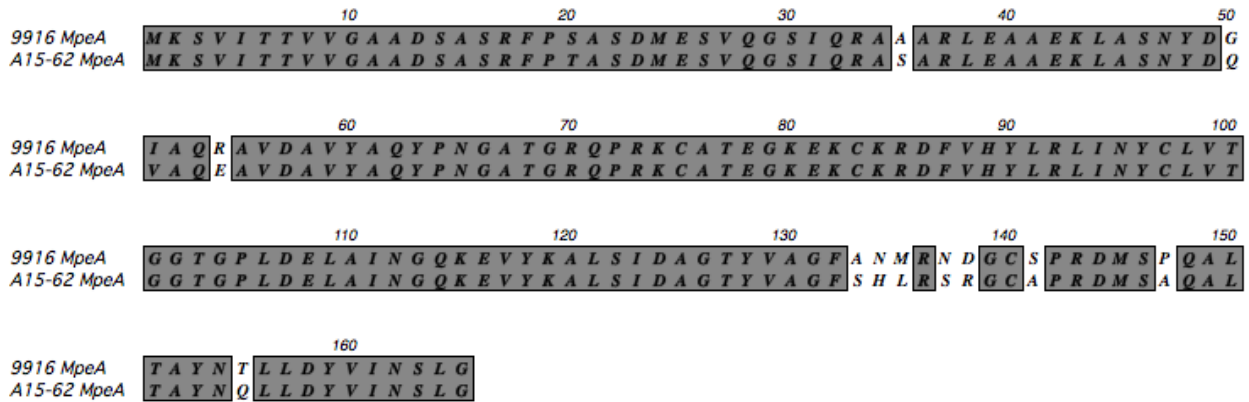
Fig. 19. Amino acid sequence alignment of RS 9916 MpeA and A 15-62 MpeA using ClustalW. Highlighted sections represent the conserved residues in the sequence.

3.1.2   *The mpeW gene:*

The *mpeW* gene is a 1194 base pair long putative bilin lyase gene that lies in the CA4-B genomic island downstream of two transcriptional regulators (*fciA* and *fciB;* see **Fig. 18**). This gene was found to be regulated by those transcriptional regulators and was overexpressed in green light condition (T. Grébert, L. Garczarek, D. Kehoe, and F. Partensky, unpublished). *mpeW* from *Synechococcus sp*. A15-62, a strain possessing CA4-B genomic island, was found to be very similar to the known lyase gene *mpeZ* from *Synechococcus sp*. RS9916, a strain possessing CA4-A genomic island (Shukla et al. 2012). The amino acid sequences of proteins encoded by *mpeW*

and *mpeZ* showed similarity of 53% (140 conserved residues and 86 similar residues) (see **Fig. 20**). The amino acid sequence of MpeW also showed 58% similarity (157 conserved residues and 79 similar residues) with MpeY, a characterized PEB lyase from *Synechococcus sp.* RS9916 (J. E. Sanfilippo et al. unpublished) (see **Fig. 20**). These similarities were indicative of the potential bilin lyase activity of the protein encoded by *mpeW*.

Fig. 20. Amino acid sequence alignment of RS 9916 MpeZ, RS 9916 MpeY and A 15-62 MpeW using ClustalW. Highlighted sections represent the conserved residues in the sequence.

3.1.3 *The mpeQ gene:*

A 1197 base pair long putative bilin lyase isomerase gene, *mpeQ,* lies upstream of the *mpeA* and *mpeB* gene encoding α- and β- subunits of PE-II respectively (see **Fig. 18**). This gene is present in the PE-II encoding operon and was found to be expressed constitutively (T. Grébert, L. Garczarek, D. Kehoe, and F. Partensky, unpublished). The *mpeQ* gene from *Synechococcus sp.* A15-62, a strain with basal level blue light phenotype, was found to be very similar to the known lyase gene *mpeY* from *Synechococcus sp*. RS9916, a strain with basal level green light phenotype (J. E. Sanfilippo et al. unpublished). The amino acid sequence of proteins encoded by *mpeQ* and *mpeY* showed similarity of 77% (236 conserved residues and 72 similar residues) (see **Fig. 21**). The amino acid sequence of MpeQ also showed 66% similarity (211 conserved residues and 63 similar residues) with MpeZ, a characterized PEB lyase isomerase from *Synechococcus sp*. RS9916 (Shukla et al. 2012) (see **Fig. 21**). This similarity suggested that the protein encoded by *mpeQ* might be a bilin lyase.

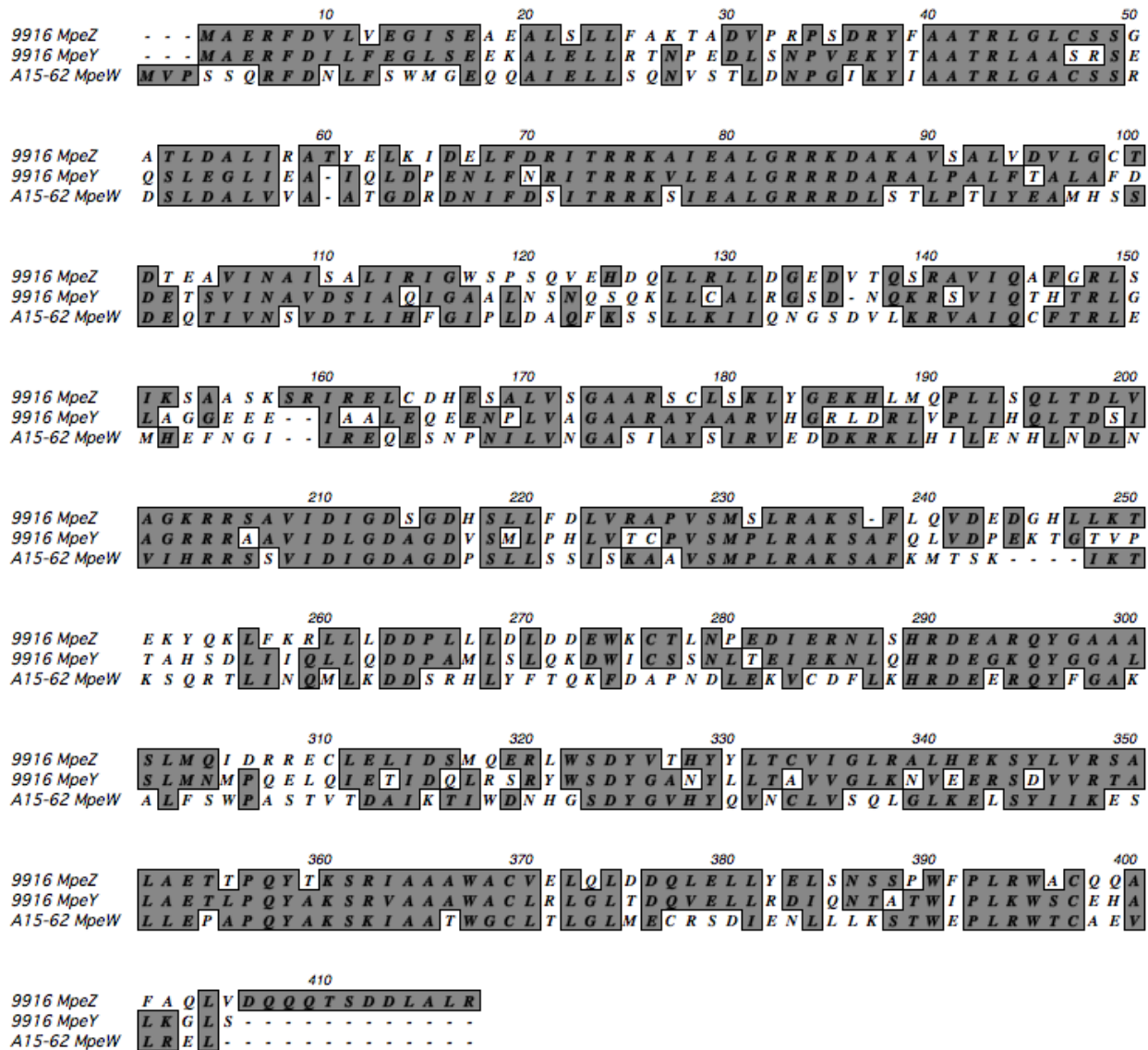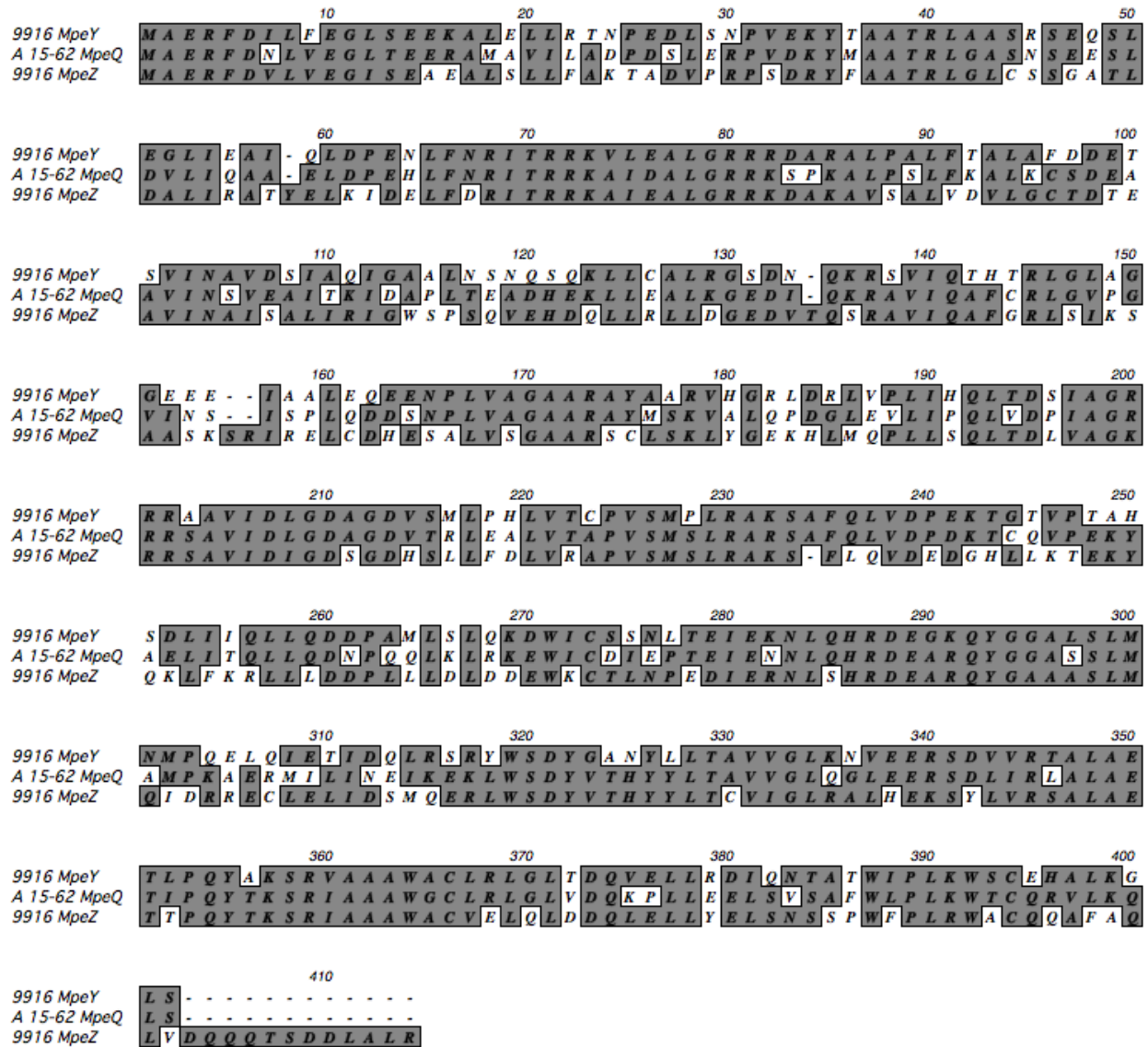**Fig. 21. Sequence alignment of RS 9916 MpeZ, RS 9916 MpeY and A 15-62 MpeQ using ClustalW**. Highlighted sections represent the conserved residues in the sequence.

3.2 *Analysis of Synechococcus sp. A15-62 whole cells of the knock out mutants:*

The putative lyase genes from *Synechococcus sp.* A15-62 were knocked out and the phenotypes of the mutant cells in different light conditions compared to the wild type cells were observed using the fluorescence spectroscopy. Relative fluorescence emission set at 585nm of the wild type whole cells normalized for PEB emission (545nm) showed more PUB emission (490nm) than PEB in blue light condition and more PEB emission than PUB in green light (Grébert T. 2017). The insertion mutant of *mpeQ* showed similar phenotype as the wild type in green light but the PUB emission was drastically decreased in blue light condition indicative of missing PUB molecules bound to the phycobilisomes (Grébert T. 2017). Similarly, the insertion mutant of *mpeW* showed similar phenotype as the wild type in blue light but the PEB emission was drastically decreased in green light condition suggesting fewer PEB molecules bound to the phycobilisomes (Grébert T. 2017). These results suggested potential role of the protein encoded by *mpeQ* in PUB addition to the phycobilisomes in blue light and that of *mpeW* in PEB addition to the phycobilisomes in green light. Amino acid sequence similarity of the proteins encoded by the *mpeQ* and *mpeW* genes with the known lyase genes combined with the results from knock out mutants of these genes called for further investigation of MpeQ and MpeW as PEB lyase isomerase and PEB lyase respectively.

3.3 *Analysis of recombinant protein activity:*

The recombinant proteins were purified and analyzed using SDS-PAGE, absorbance spectroscopy, fluorescence spectroscopy and mass spectroscopy.

3.3.1   *Analysis of recombinant PE subunits co-expressed with MpeQ and PEB:*

Heterologous co-expression of MpeQ with the six-histidine–tagged (HT) potential protein substrates in *E. coli* in order to demonstrate its bilin lyase isomerase activity. The *E. coli* cells also contained a plasmid which allows expression of HO1 and PebS enzymes that are required to produce PEB, a bilin substrate for MpeQ. HT-MpeA expressed from the *E. coli* cells co-expressing MpeQ was purified and investigated using spectroscopy. Absorbance spectra of the purified MpeA protein showed the absorbance maxima at 492 nm which is a characteristic feature for PUB bound to phycobiliproteins (see **Fig. 22**). HT-MpeA from the *E. coli* cells not expressing MpeQ was used as a negative control and did not show any absorbance (see **Fig. 22**). This result indicated that HT-MpeA is unable to bind bilins auto-catalytically in the absence of bilin lyases. HT-MpeB and HT-CpeA were also investigated as potential substrates for MpeQ. Neither HT-MpeB nor HT-CpeA purified after co-expression with MpeQ showed any absorbance suggesting that MpeQ acts specifically on MpeA (see **Fig. 22**). HT-CpeA and HT-MpeB from *Synechococcus sp.* RS9916 were used as these PE subunits are highly conserved between strains of *Synechococcus*.
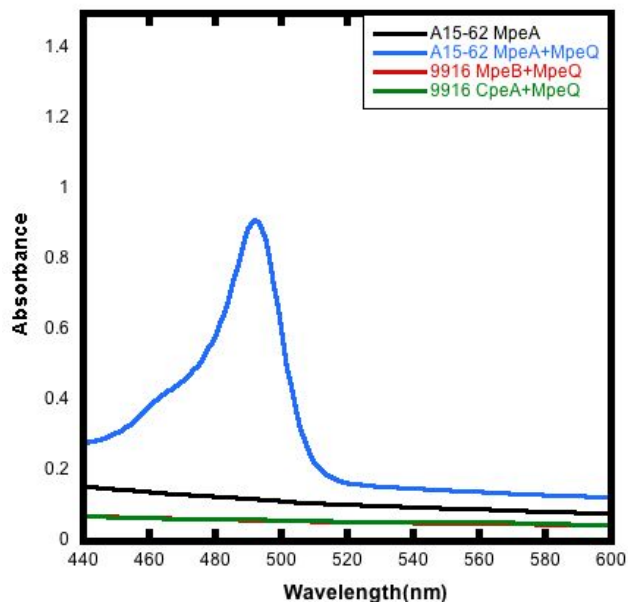
Fig. 22. Absorption spectra for purified PE subunits co-expressed with MpeQ.

The fluorescence spectra of HT-MpeA co-expressed with MpeQ excited at 440 nm revealed fluorescence emission maxima at 501 nm which is typical for a phycobiliprotein with bound PUB. The fluorescence spectra had no emission maxima when excited at 490 nm confirming the bilin bound to the HT-MpeA is PUB and not PEB (see **Fig. 23**). There was no fluorescence emission for HT-MpeB co-expressed with MpeQ nor for HT-MpeA expressed by itself when excited at both 440 nm and 490 nm indicating the absence of bound bilin (see **Fig. 23**). HT-CpeA co-expressed with MpeQ showed a small peak at 570 nm when excited at 490 nm due to weak non-enzymatic addition of PEB (see **Fig. 23**). HT-CpeA has been shown to have weak autocatalytic PEB addition even in the absence of bilin lyases.
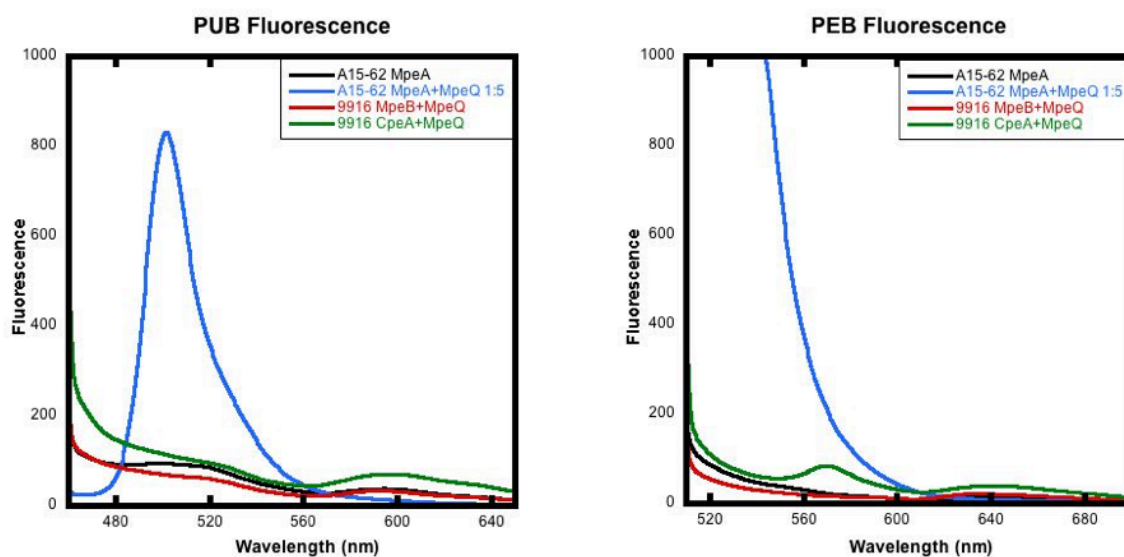
Fig. 23. Fluorescence emission spectra of the purified PE subunits co-expressed with MpeQ. HT-MpeA purified after co-expression with MpeQ was very fluorescent and therefore was diluted 5 times relative to other purified proteins.

Zinc enhanced fluorescence of proteins after SDS-PAGE was used to establish the identity of bilin and the protein with bound bilin. Bivalent zinc ions chelate the protein-bound bilins holding them in rigid conformation and hence the fluorescence properties of bilins is enhanced. SDS-PAGE resolves proteins based on their size and zinc ions help to visualize the bilin bound protein by enhancing bilin fluorescence when illuminated with specific wavelengths of light (488 nm excites PUB strongly and PEB weakly and 532 nm excites PEB strongly and PEB weakly).
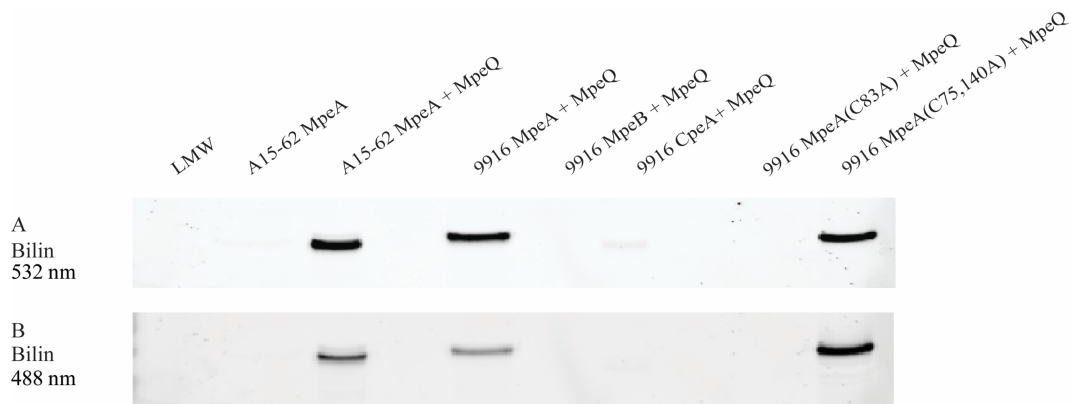
Fig. 24. Zinc enhanced bilin fluorescence of purified PE subunits from MpeQ recombinant co-expressions after purification and separation by SDS-PAGE. Proteins after co-expression with MpeQ were resolved by SDS-PAGE and the Zinc stained SDS-PAGE was illuminated with **(A)** 532nm to detect PEB bound proteins. **(B)** 488nm light to detect PUB bound proteins.

Zinc enhanced fluorescence of SDS-PAGE of the purified HT-MpeA co-expressed with MpeQ showed fluorescence when illuminated with light of 488 nm indicative of PUB bound protein (see **Fig. 24**). As expected, MpeQ was also active on HT-MpeA from *Synechococcus* sp. RS9916 as the phycoerythrin subunits are highly conserved between these strains (see **Fig. 19**; **Fig. 24**). However, HT-MpeA from the *E. coli* cells not expressing MpeQ did not show any fluorescent properties (see **Fig. 24**). HT-MpeB and HT-CpeA were also studied as the potential substrate for MpeQ. HT-MpeB and HT-CpeA purified after co-expressions with MpeQ had no fluorescent properties, suggesting that MpeQ exhibits lyase isomerase activity exclusively on MpeA (see **Fig. 24**). The faint band present for HT-CpeA is due to weak non-enzymatic chromophorylation of HT-CpeA (see **Fig. 24**). The results of zinc enhanced fluorescence in the SDS-PAGE were consistent with the results from absorbance and fluorescence spectroscopy and demonstrated that MpeQ attaches PUB to MpeA.

The three differential cysteine residues (75,83 and 140) on MpeA which are potential targeted sites of attachment by MpeQ were investigated using site directed mutants. There are only these three cysteines at positions 75, 83, and 140 capable of binding bilins within MpeA. These cysteine residues were changed to alanine (Shukla et al 2012). Different combinations of mutated HT-MpeA was co-expressed with MpeQ in the *E. coli* cells producing PEB. Purified HT-MpeA (C83A) showed no fluorescence in zinc enhanced SDS-PAGE whereas HT-MpeA (C75A, C140A) exhibited fluorescence when illuminated at 488 nm (see **Fig. 24)**. This result was consistent with the result from fluorescence spectroscopy where HT-MpeA (C83A) co-expressed with MpeQ showed no fluorescence emission whereas HT-MpeA (C75A, C140A) exhibited fluorescence emission at 501 nm when excited at 440 nm similar to that of wild type recombinant HT-MpeA (see **Fig. 25**).
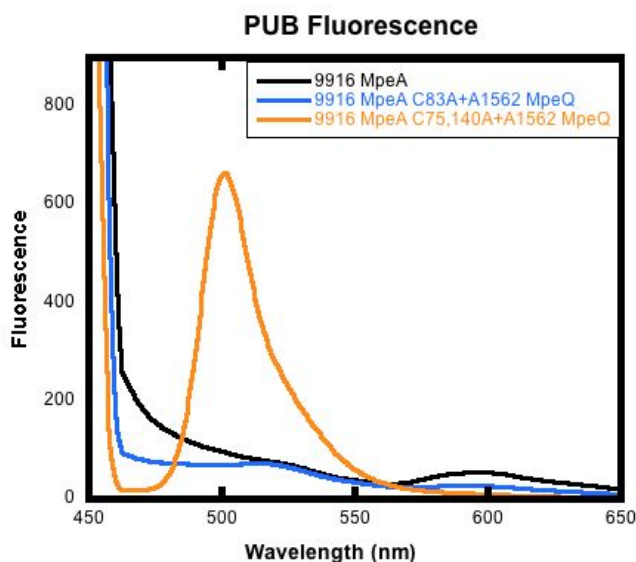
Fig. 25. Fluorescence emission spectra of the purified HT-MpeA mutants co-expressed with MpeQ.

In addition, mass spectroscopic analysis of the peptides containing Cys83 obtained from tryptic digests of recombinant HT-MpeA co-expressed with MpeQ was performed to confirm the

site and type of bound bilin. MS/UV-Vis absorption spectra of the peptides had fragments with

m/z of 504.7524^{2+} and 336.8371^{3+} at 490 nm corresponding to C*(PUB)KR+O (see **Fig. 26**). No

bilins were detected in Cys75 (C*ATEGK or KC*ATEGK) Cys140 (GC*APR) fragments. This

result confirms that MpeQ adds PUB to Cys 83 site on MpeA.
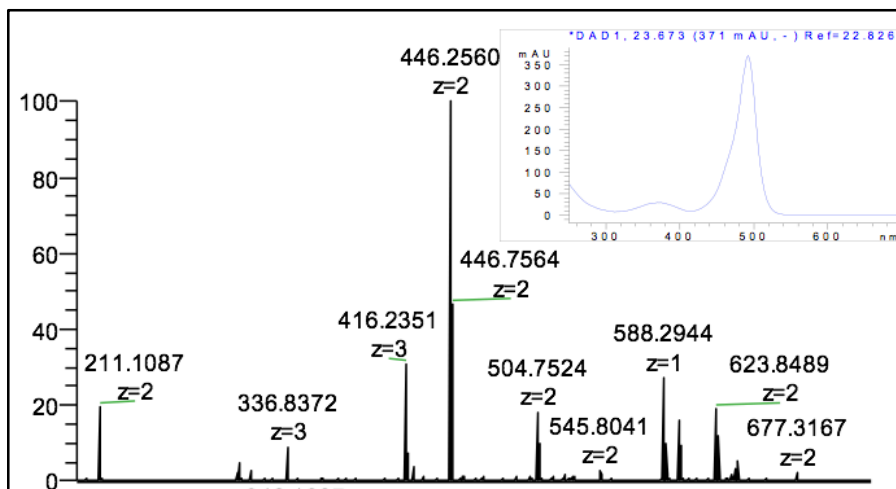
Fig. 26. MS/UV-Vis Analysis of peptides containing Cys 83 obtained from tryptic digest of recombinant HT-MpeA co-expressed with MpeQ. MS/UV-Vis absorption spectra (inset) of the peptides obtained from tryptic digest of recombinant HT-MpeA co-expressed with MpeQ had fragments with m/z of 504.7524^{2+} and 336.8371^{3+} at 490 nm corresponding to C*(PUB)KR+O confirming PUB addition to Cys 83 site on MpeA.

Together, these data provide strong evidence that PUB is bound to the Cysteine 83

residue in MpeA when co-expressed with MpeQ, proving that it is a lyase/isomerase which is

responsible for attachment of PUB at that position, assisting cells in acclimating for blue light

capture.

3.3.2 *Analysis of recombinant PE subunits co-expressed with MpeW and PEB:*

Recombinant MpeW was co-expressed with the six-histidine–tagged (HT) potential phycobiliprotein subunits substrates in *E. coli* in order to demonstrate its bilin lyase activity as discussed earlier. HT-MpeA expressed from the *E. coli* cells co-expressing MpeW was purified and investigated using spectroscopy. Absorbance spectra of the purified HT-MpeA protein showed an absorbance maxima at 546 nm which is typical of PEB-bound phycobiliproteins (see **Fig. 27**). However, there was a small shoulder at 498 nm indicating the presence of some PUB bound MpeA. This shoulder is seen in many PEB-bound proteins (e.g. CpeA chromophorylated by the PEB lyase CpeY; Biswas et al., 2011). HT-MpeA from the *E. coli* cells not expressing MpeW was used as a negative control, and it did not show any absorbance (see **Fig. 27**). This result indicates that HT-MpeA is unable to bind bilins auto-catalytically in the absence of bilin lyases and was consistent with the data presented above. HT-MpeB and HT-CpeA were also investigated as potential substrates for MpeW. HT-MpeB and HT-CpeA purified after co-expression with MpeW did not show any absorbance suggesting that MpeW, like MpeQ, also acts specifically on MpeA (see **Fig. 22; Fig. 27**).
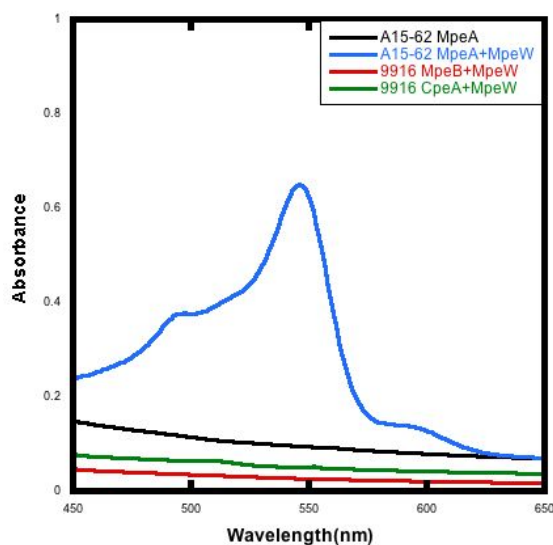
Fig. 27. Absorption spectra for purified PE subunits co-expressed with MpeW.

The fluorescence spectra of HT-MpeA co-expressed with MpeW excited at 490 nm revealed fluorescence emission maxima at 560 nm which is typical for a phycobiliproteins with bound PEB. The fluorescence spectra had no emission maxima when excited at 440 nm confirming the bilin bound to the HT-MpeA is PEB and not PUB (see **Fig. 28**). There was no fluorescence emission for HT-MpeB co-expressed with MpeW nor for HT-MpeA (with no lyase) when excited at both 440 nm and 490 nm indicating the absence of bound bilin (see **Fig. 28**). HT-CpeA co-expressed with MpeW showed a small peak at about 570nm when excited at 490 nm due to weak autocatalytic addition of PEB (see **Fig. 28**). This autocatalytic addition of PEB to HT-CpeA was also seen when HT-CpeA was co-expressed with MpeQ (see **Fig. 23**).
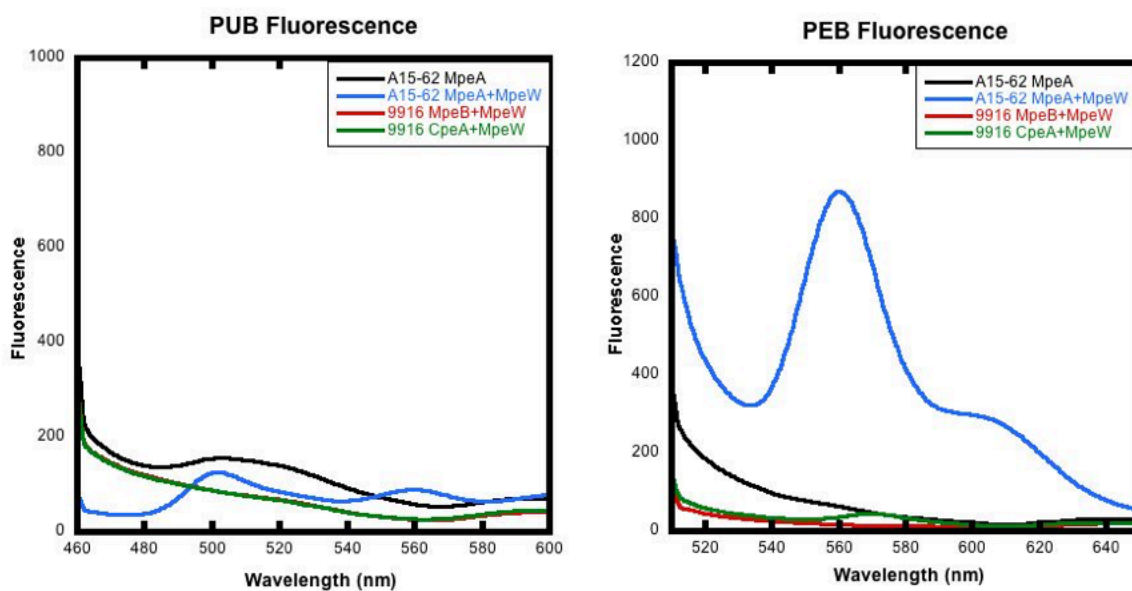
Fig. 28. Fluorescence emission spectra of the purified PE subunits co-expressed with MpeW.

Zinc enhanced SDS-PAGE of the purified HT-MpeA co-expressed with MpeW showed fluorescence when illuminated with light of 532 nm indicative of PEB-bound protein (see **Fig. 29**). MpeW, like MpeQ, was active on HT-MpeA from *Synechococcus* sp. RS9916 as the phycoerythrin subunits are highly conserved between these strains (see **Fig. 19**; **Fig. 29**). However, HT-MpeA

from the *E. coli* cells not expressing MpeW did not show any fluorescent properties (see **Fig. 29**).

HT-MpeB and HT-CpeA were also studied as possible substrates for MpeW. HT-MpeB and HT-CpeA purified after co-expressions with MpeW had no fluorescent properties, suggesting that MpeW exhibits lyase isomerase activity solely on MpeA (see **Fig. 29**). The faint band is present for HT-CpeA due to weak non-enzymatic chromophorylation of HT-CpeA (see **Fig. 29**). The results of zinc enhanced SDS PAGE was consistent with the results from absorbance and fluorescence spectroscopy.
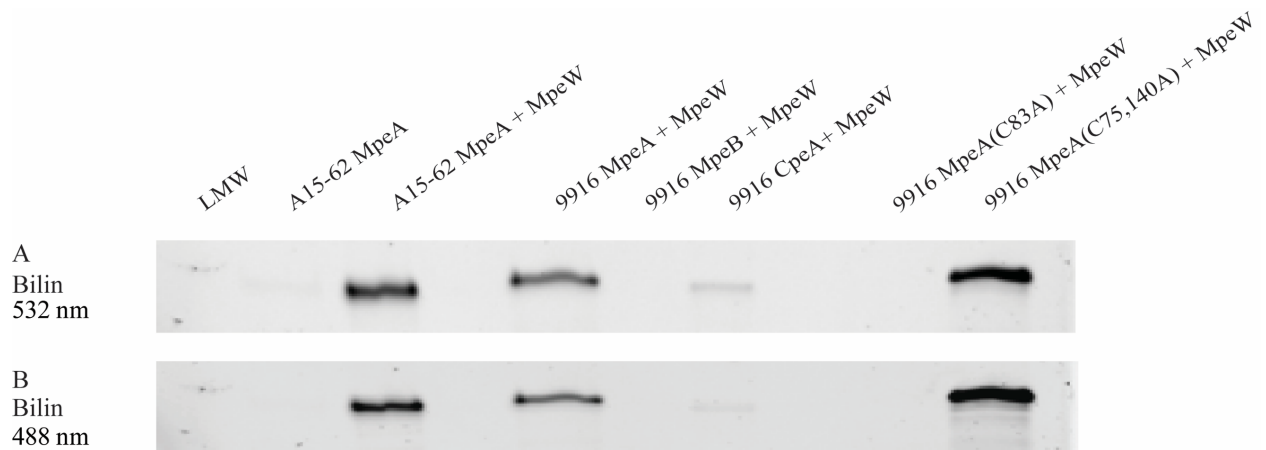
Fig. 29. Zinc enhanced bilin fluorescence of purified PE subunits from MpeW recombinant co-expressions after purification and separation by SDS-PAGE. Proteins after co-expression with MpeW were resolved by SDS-PAGE and the Zinc stained SDS-PAGE was illuminated with **(A)** 532nm to detect PEB bound proteins. **(B)** 488nm light to detect PUB bound proteins.

Different combinations of site directed mutants of HT-MpeA (from *Synechococcus sp.* RS9916) was co-expressed with MpeW in the *E. coli* cells producing PEB. Purified HT-MpeA (C83A) showed no fluorescence in zinc enhanced SDS-PAGE whereas HT-MpeA (C75A, C140A) exhibited fluorescence when illuminated at 532 nm (see **Fig. 29**). The result was consistent with the result from fluorescence spectroscopy where HT-MpeA (C83A) co-expressed with MpeW

showed no fluorescence emission whereas HT-MpeA (C75A, C140A) exhibited fluorescence emission at 560 nm when excited at 490 nm similar to that of wild type recombinant HT-MpeA (see **Fig. 30**). A small shoulder at 602 nm was observed in fluorescence emission for HT-MpeA and HT-MpeA (C75A, C140A) co-expressed with MpeW (see **Fig. 27**, **Fig. 30**). This might be due to the addition of 15, 16- dihydrobiliverdin, an intermediate in PEB synthesis, formed by 2-electron reduction of biliverdin IXα or 2-electron oxidation product of PEB.
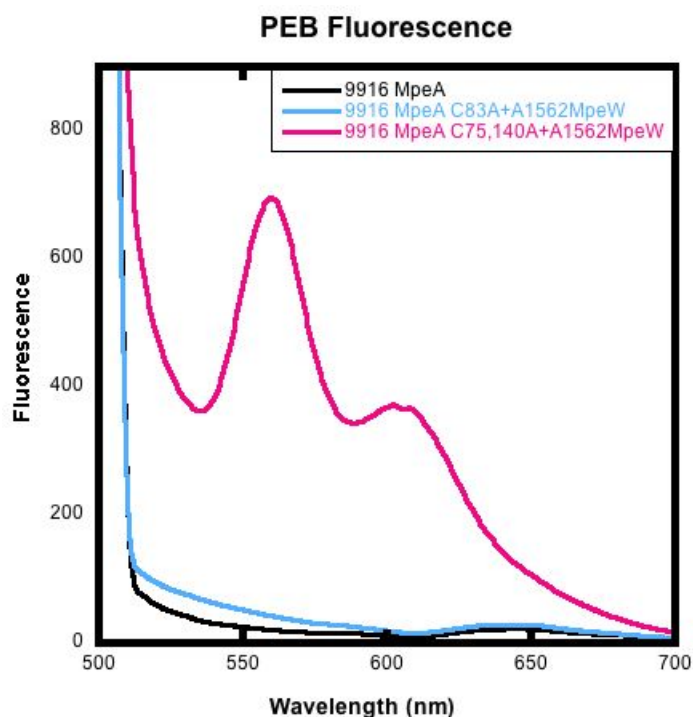
Fig. 30. Fluorescence emission spectra of the purified HT-MpeA mutants co-expressed with MpeW.

The data from spectroscopic analysis and Zinc enhanced SDS-PAGE provide strong evidence that MpeW is responsible for PEB attachment to the Cysteine 83 residue on MpeA assisting cells for capturing more green light.

4. Discussion

The research presented in this study primarily focus on investigation of MpeQ as the lyase isomerase that adds PUB specifically to the cysteine 83 residue on α subunit of PE-II in blue light condition and MpeW as the lyase that adds PEB to the same site in green light. PUB is the accessory pigment with absorption maxima in the blue light region, and hence it must be bound to the phycobiliproteins in PBS to enable the organism to absorb blue light and transfer it to the photosynthetic reaction centers. Similarly, the accessory pigment with absorption maxima in the green light region is the PEB, and this accessory pigment should be bound to the phycobiliproteins subunits to provide ability to the PBS to absorb green light. Therefore, MpeQ adds PUB to the Cys-83 residue on α-subunit of PE-II during the biosynthesis of PBS and help these strains adapt to the blue light and MpeW adds PEB to the same site during the biosynthesis of PBS and enable these strains to adapt to green light.

The study of *mpeQ* knock-out mutants suggested that there is a missing PUB in blue light as compared to the wild type whereas the phenotype in green light was identical to the wild type. The difference in phenotype of the wild type and mutant blue light suggested that MpeQ was required for addition of PUB to MpeA in blue light condition. However, the similarity of the phenotypes in wild type green light versus mutant green light indicates that there is no effect of MpeQ in adding bilins to MpeA in green light conditions (Grébert, T. 2017). The results from the co-expression of recombinant proteins in *E. coli* also confirm lyase isomerase activity of recombinant MpeQ specific to Cys-83 site on MpeA.

The investigation of the cells with disrupted *mpeW* showed that a PEB molecule is missing in the phycobilisomes in green light as compared to the wild type cells whereas the relative fluorescence profile of the mutant demonstrated reiteration to that of wild type (Grébert, T. 2017). This difference in the relative fluorescence in the mutant and wild type in green light clearly indicates that MpeW is the lyase responsible for attachment of PEB to Cys-83 site on MpeA in green light condition. There is very little expression of *mpeW* in blue light conditions which suggests that it has no role in adding bilins in blue light conditions (Grébert, T. 2017). The lyase activity of MpeW specific to Cys-83 site on MpeA is also established using the recombinant proteins co-expression experiments in *E. coli.*

Based on all the evidence, it is understood that the *mpeQ* gene is expressed constitutively and MpeQ, a lyase isomerase adds PUB to the Cys-83 site on MpeA to maintain the basal blue light phenotype. The *mpeW* gene, present in the CA4-B genomic island of this organism, which encodes for a PEB lyase is induced under green light and likely outcompetes MpeQ to add PEB to the same site. This competition between these lyases would provide these strains with a basal blue light specialist phenotype to tune their ability of their PBS to acclimate to the change in ratio of blue and green light to perform optimum photosynthetic activity.

This result also has implications in understanding the evolution of functional heterogeneity within a family of lyase. MpeQ and MpeW described in this study and MpeZ and MpeY described previously (Shukla et al. 2012; J. E. Sanfilippo et al. unpublished) belong to the same family of QWYZ sub-family of lyases that are specific to the Cys-83 site of α-subunit of PE-II. Despite the relatively high level of sequence similarity between these two proteins, they exhibit a drastic

change in function from one being a lyase to the other being a lyase isomerase. This functional heterogeneity results in a large phenotypic difference in the organisms possessing them in terms of ability to absorb different wavelengths of light. As a consequence, these organisms are able to thrive in different light niches and hence are very successful in the marine habitat.

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