PHYCOBILISOME ABUNDANCE REGULATOR PSOR AND ITS CONSERVATION AND FUNCTION WITHIN CYANOBACTERIA

By

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ABSTRACT

Phycobilisomes (PBSs) are accessory light harvesting antennae that bind to the thylakoid membranes in cyanobacteria and help shuttle light energy to the photosystems for photosynthesis. These large protein complexes are modified during a process called chromatic acclimation (CA). During CA, cyanobacteria perceive and respond to changes in their light environment to maximize productivity and minimize damage caused by excess light. During a routine color mutant screening, a blue-green mutant was found and determined to have a transposon within the phycobilisome abundance regulator gene, *psoR*, in *Fremyella diplosiphon*. The lack of PsoR disrupted the organism's ability to change pigmentation/PBSs under different light conditions. Along with phenotypic characterization, bioinformatic studies were performed to uncover the putative function of *psoR*, and phylogenetic analysis conducted to determine if this gene was found in all PBS-containing organisms or unique to certain species. PsoR was found to have homology to β-CASP proteins, which are involved in nucleic acid processing, which suggests PsoR may be involved in regulating CA-related genes. The resulting phylogenetic tree showed homologs present throughout the cyanobacteria phylum, but noticeably absent in Gloeobacter, an ancient clade of cyanobacteria that lacks thylakoid membranes and has atypical PBSs. Overall, these results suggest that PsoR is highly conserved amongst cyanobacteria and could play a role in the regulation of PBSs and CA in cyanobacteria.

To my parents, for supporting and encouraging me
in everything I strive for in life.

To my sister, for all of the late night laughs we've shared
and the hairbrained schemes we come up with.

And

To my friends, who have stuck by me all of these years,
providing endless moral support and laughs when I needed it the most.

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CHAPTER ONE

The Role of Chromatic Acclimation and Pigmentation in Cyanobacteria

Cyanobacteria are photosynthetic prokaryotes that have been around for at least 2.4 billion years (Blankenship, 2010; Demoulin et al., 2019; Sánchez-Baracaldo et al., 2022). These organisms have been implicated in the Great Oxygenation Event when a rise in atmospheric oxygen caused a mass extinction of anaerobic organisms (Lyons et al., 2014; Schirrmeister et al., 2015; Guéguen & Maréchal, 2021). Cyanobacteria are also ancestors to chloroplasts in plants and green algae (Raven & Allen, 2003; Pinevich, 2020). Presently, cyanobacteria play important roles in the global carbon cycle, with marine cyanobacteria contributing a significant amount to carbon sequestration and oxygen production (Zwirglmaier et al., 2008; Zehr et al., 2011; Flombaum et al., 2013). Estimates show that cyanobacteria also may be responsible for up to 50% of nitrogen fixation in the global nitrogen cycle (Karl et al., 1997; Hutchins & Capone, 2022; Schvarcz et al., 2022).

As oxygenic phototrophs, cyanobacteria represent excellent experimental models for examining how photosynthetic organisms change in dynamic environments. Key features that allow cyanobacteria to serve as exemplary research model organisms include that facts that they are the precursors of chloroplasts, that many species of cyanobacteria are easy to cultivate, and that many molecular tools are available for research in numerous strains.

One critical aspect of a cyanobacterium's environment is light availability, including light quality and intensity. Light energy is captured and transformed into chemical energy through photosynthesis. Both light wavelength and intensity impact photosynthetic energy production and cyanobacterial metabolism (Montgomery, 2016). In natural environments, light availability can vary greatly due to cloud and foliage coverage, time of day, and water depth (for both marine and freshwater species). To respond to this variability and optimize survival, cyanobacteria have evolved a form of photomorphogenesis called chromatic acclimation (CA) (Montgomery, 2017; Ho et al., 2017; Sanfilippo et al., 2019; Montgomery, 2022). CA is a mechanism by which cyanobacteria tune their pigmentation, metabolism, and physiology in response to their light environment to maximize production and minimize light-associated damage caused by exposure to excessive light. In particular, the light harvesting antenna complex called the phycobilisome (PBS) undergoes remodeling to support maximizing light absorption,

including light wavelength and quantity (Gutu & Kehoe, 2012; Adir et al., 2006). The shape and size of cyanobacterial cells and filaments also undergo light-dependent changes for several cyanobacterial species (Bennet & Bogorad, 1973; Singh & Montgomery, 2011; Singh & Montgomery, 2015; Montgomery, 2016).

PBSs are found in cyanobacteria and red algae, and are complex structures made of dozens to hundreds of phycobiliproteins (PBPs) depending on the species (Grossman & Byaha, 1995; Adir et al., 2019). The overall PBS structure is a collection of cylinders that are bound to one another using linker proteins, and core-membrane linker proteins attach the core of the PBSs to the thylakoid membrane, which transfers energy to the chlorophylls in the reaction centers of photosystem I (PSI) and photosystem II (PSII) (Figure 1.1) (Hourmand et al., 1988). The number of cylinders that make up the core varies from two, three, and five cylinders depending on the species (Adir et al., 2006; Gutu & Kehoe, 2012). Each cylinder is made up of three discs, in which one disc contains three heterodimers of α and β allophycocyanin (AP) subunits. The core then has rods that are attached extending away from the thylakoid membrane. The number of rods also varies among species from six rods up to 14 rods per core (Zhang et al., 2017). Each rod is made up of stacked discs, with each disc made up of three heterodimers of α and β subunits of PBPs, similar to the core. Like the core, the rods are held together by rod linker proteins, with core-rod linker proteins tethering the rods to the core of the PBS (Federspiel et al., 1990). The type of PBP that makes up the rods, particularly the outer portion of rods, can change depending on the light conditions in which cells are grown (Figure 1.1) (Kehoe & Gutu, 2006). Light quality (or color) and intensity can cause changes in PBSs, including PBP content or rod length (Tandeau de Marsac, 1977). Certain cyanobacteria have PBSs with longer rods in low light or shorter rods in high light (Chenu et al., 2017). All of the PBPs and regulatory elements are encoded by a network of genes that control the production of PBPs in response to light cues or other environmental factors such as nutrient availability (Singh et al., 2003; Tamary et al., 2012; Collier & Grossman, 1994; Nagarajan et al., 2019; Six et al., 2007).

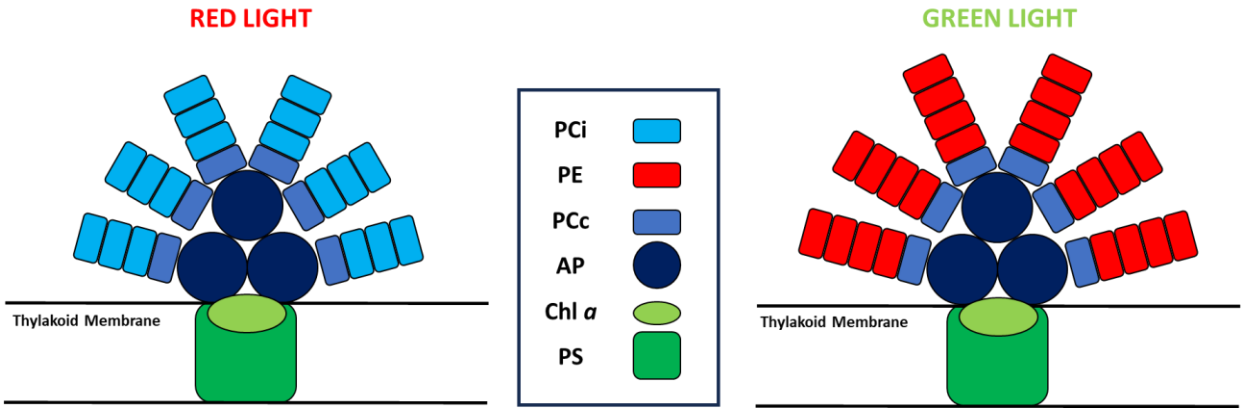


Figure 1.1. Cartoon schematic of phycobilisome (PBS) structures under red versus green light conditions. The structure of the PBS assists in the absorption and transfer of light energy to the chlorophyll *a* (chl *a*) reaction centers in the photosystems (PS). The rods, that consist of either PC or PE, radiate out from the AP core. Linker proteins (not depicted) are involved in binding the structure together and aid in energy transfer.

The tuning of PBP content in PBSs by light quality occurs in the aforementioned CA process. There are various types of CA, which are categorized based on the different modifications the PBSs undergo and the wavelengths of light that regulate the acclimation process (Gutu & Kehoe, 2012; Montgomery, 2017). The most prominent and studied CA type is complementary chromatic acclimation (CCA), otherwise known as type 3 CA. *Fremyella diplosiphon*, a filamentous freshwater cyanobacterium that is also known as *Tolypothrix* PCC 7601, is a typical model organism studied that exhibits CCA (Haney & Kehoe, 2019). CCA predominantly responds to red and green wavelengths of light (Figure 1.1). Under red light, the rods contain PBPs called phycocyanin (PC) which is blue-green in color and best absorbs light in the red (610-625 nm) range of light. The rods are also typically shorter compared to under green light; and, in red light cells take on a round shape and filaments are shorter. Under green light, the rods contain PBPs called phycoerythrin (PE) which is red in color and best absorbs light in the green (490-570nm) light range, cells are more rod-like in shape, and filaments are longer. In type 1 CA, it was originally believed that the PBPs remain unchanged under different light conditions, however recent research has revealed that the core-rod linker protein is switched out in response to red and green light (Tandeau de Marsac, 1977; Kondo et al., 2007; Sanfilippo et al., 2019). In type 2 CA, PE levels increase under green light conditions and decrease under red light

conditions, whereas PC remains unchanged under varying light conditions (Hirose et al., 2010). New forms of CA have been discovered in recent years. Type 4 CA involves a change in the chromophore bound to PE in response to green and blue light (Everroad et al., 2006; Sanfilippo et al., 2016). Another form of CA called FaRLiP (Far-Red Light Photoacclimation) uses red-shifted PBSs and photosystems (PSs) that allow cyanobacteria with this CA type to grow in far-red light-enriched conditions (Gan et al., 2014; Zhao et a., 2015).

The CCA gene network involves the Rca pathway, a complex phosphorelay system that upregulates and downregulates the expression of PBS-related operons in response to external light cues (Kehoe & Grossman, 1996; Terauchi et al., 2004). Under red light conditions, RcaE is the phytochrome-class photoreceptor with a histidine-kinase domain that auto-phosphorylates (Hirose et al., 2013). The phosphate is passed on to the response regulator RcaF, which then passes the phosphate to RcaC, a DNA-binding response regulator that binds to the *cpc2* operon, activating its gene expression. The *cpc2* operon contains the genes encoding the α and β subunits of PC2, the inducible form of PC (Conley et al., 1988; Oelmüller et al., 1988). The *cpc2* operon also contains the gene *pcyA,* which encodes the oxidoreductase that produces phycocyanobilin, the chromophore that binds to PC. The cpc2 operon also encodes the linker proteins that hold the PC rods together (Alvey et al., 2007). RcaC also binds to the L box of the *cpeCDESTR* (*cpeC*) operon, preventing its transcription under red light. The *cpeC* operon encodes the PE linker proteins and CpeR (Cobley et al., 2002; Federspiel & Grossman, 1990). CpeR is the activator that induces expression of the operon *pebAB* encoding two oxidoreductases that produce phycoerythrobilin, the chromophore that binds to PE, and the operon *cpeBA,* which transcribes both the α and β PE subunits (Alvey et al., 2003; Mazel et al., 1986). Under green light, RcaE is not phosphorylated, which leaves RcaF and RcaC in their unphosphorylated forms. Without RcaC being phosphorylated, the *cpc2* operon is not transcribed and transcription of the *cpeC* operon occurs.

F. diplosiphon has one of the largest sensory networks among prokaryotes, with 305 two-component system proteins and 27 phytochrome-family proteins predicted within its 10,065 protein-coding sequences (Yerrapragada et al., 2015). Given that

PBPs make up a significant amount of the dry cell weight in *F. diplosiphon* and given their significant role in CCA and photosynthesis, the regulation of PBS structure and abundance is vital (Aoki et al., 2021). Despite over 50 years of research into CCA, several questions remain unanswered regarding the gene network that regulates PBS structure and abundance. Recently, one of our lab's spontaneous *F. diplosiphon* mutants was found to have a transposon inserted within the *psoR* (Phycobilisome abundance regulator) gene and to have a disrupted CCA response under red vs green light conditions. Wildtype has a brick-red pigmentation when grown under green light, and green pigmentation when grown under red light. Under both red and green lights, the cultures of this mutant are blue-green in color. The *psoR* gene had previously been implicated in the CCA mechanism when a *psoR* deletion mutant showed a similar color phenotype (Cobley et al., 2008). This deletion mutant also had an overproduction of PBSs, which gave *psoR* its name. Overexpression of *psoR* from a shuttle plasmid in *F. diplosiphon* led to a 10-20-fold decrease in the PBS/chl *a* ratio compared to the parental strain (Cobley et al., 2008). Not much else was known about *psoR* or its specific role in CCA within cyanobacteria. In this thesis, I showcase the current work on determining the role *psoR* plays in CCA and in cyanobacteria through characterizing the phenotype of a *psoR* mutant, revealing the widespread presence of PsoR within the cyanobacteria phylum, exploring homology of PsoR to the β-CASP family of exo- and endoribonucleases, and examining its effect on the expression levels of CCA-related genes.

CHAPTER TWO

Homologs of Phycobilisome Abundance Regulator PsoR Are Widespread across Cyanobacteria

This chapter contains previously published work:

Layer, A.; Montgomery, B.L. Homologs of Phycobilisome Abundance Regulator PsoR Are Widespread across Cyanobacteria. Microbiol. Res. 2022, 13, 167–182.

2.1 Abstract

During chromatic acclimation (CA), cyanobacteria undergo shifts in their physiology and metabolism in response to changes in their light environment. Various forms of CA, which involve the tuning of light-harvesting accessory complexes known as phycobilisomes (PBS) in response to distinct wavelengths of light, have been recognized. Recently, a negative regulator of PBS abundance, PsoR, about which little was known, was identified. We used sequence analyses and bioinformatics to predict the role of PsoR in cyanobacteria and PBS regulation and to examine its presence in a diverse range of cyanobacteria. PsoR has sequence similarities to the β-CASP family of proteins involved in DNA and RNA processing. PsoR is a putative nuclease widespread across cyanobacteria, of which over 700 homologs have been observed. Promoter analysis suggested that *psoR* is co-transcribed with upstream gene *tcpA*. Multiple transcription factors involved in global gene regulation and stress responses were predicted to bind to the *psoR-tcpA* promoter. The predicted protein–protein interactions with PsoR homologs included proteins involved in DNA and RNA metabolism, as well as a phycocyanin-associated protein predicted to interact with PsoR from *Fremyella diplosiphon* (FdPsoR). The widespread presence of PsoR homologs in cyanobacteria and their ties to DNA- and RNA-metabolizing proteins indicated a potentially unique role for PsoR in CA and PBS abundance regulation.

2.2 Introduction

Cyanobacteria are oxygenic phototrophs that are responsible for 20–30% of global carbon fixation and the majority of global nitrogen fixation (Zwirglmaier et al., 2008; Zehr, 2011). These prokaryotes serve as model organisms for studying photosynthesis and photoacclimation because of their fast growth and the tools available for the genetic manipulation of many species. In addition to research on the mechanisms governing their growth and fitness in natural contexts, there has been ongoing research into their use as a microbial chassis in the biotech industry for the production of biofuels, biopharmaceuticals, food supplements, bioremediation, and biofertilizers (Santos-Merino et al., 2019; Hays & Ducat, 2015). Due to the growing interest in this ancient phylogeny of bacteria, it is important to fully understand the genes and mechanisms that control their photosynthetic potential, productivity, and

adaptive responses.

Cyanobacteria have evolved to survive in a wide range of ecological niches, including freshwater and marine environments, as well as in arid deserts, arctic tundra, and hot springs (Jungblut et al., 2005; Martinez et al., 2019; Samolov et al., 2020). The dynamic conditions in these environments present various challenges for cyanobacteria, including nutrient limitations and fluctuating light levels, which impact organismal growth and fitness. To deal with variable photo-environments, cyanobacteria have evolved a mechanism called chromatic acclimation (CA), which allows them to perceive and respond to a given light condition and adjust their physiology and metabolism accordingly to maximize productivity while minimizing the photodamage caused by excess light absorption (Montgomery, 2016; Ho et al., 2017; Sanfilippo et al., 2019).

A major cellular change that occurs during CA includes alterations to the pigment profile of phycobilisomes (PBSs), which are accessory light-harvesting antennae that capture and transfer light energy to chlorophyll-containing photosystems for photosynthesis. PBSs are made up of phycobiliproteins that form a core with outward-radiating rods (Gutu & Kehoe, 2012; Adir et al., 2006). The PBS core is attached via a core-member linker to chlorophyll-containing reaction centers embedded in thylakoid membranes (Zhao et al., 2005). In addition to altering the rod pigments during CA in response to changes in the prevalence of red light (RL) vs. green light (GL) and the light intensity, PBSs undergo restructuring and degradation under high-stress conditions, such as in high light and with nutrient limitations (Bennett & Bogorad, 1973; Singh et al., 2003; Tamary et al., 2012; Collier & Grossman, 1994; Nagarajan et al., 2019; Six et al., 2007). As PBSs are light-harvesting accessory complexes, their structure changes during CA and in response to stresses, which has implications for pigment coupling and energy transfer rates (Chenu et al., 2017; Kolodny et al., 2021), and ultimately, the overall photosynthetic potential and efficiency.

Three main types of CA have been discussed in the literature for nearly 50 years. Distinct CA types are largely categorized by whether and how RL-absorbing phycocyanin (PC) and GL-absorbing phycoerythrin (PE) phycobiliproteins are synthesized and incorporated into the rods of PBSs in response to the availability of external light (Gutu & Kehoe, 2012; Montgomery, 2017). Type I CA occurs when

cyanobacteria do not alter the levels of PC or PE under different light conditions. In cyanobacteria that exhibit type II CA, PC levels remain unchanged, whereas PE levels are tuned in response to light cues, where PE is induced under conditions rich in GL wavelengths. In cyanobacteria with type III CA, which is also known as complementary chromatic acclimation (CCA), PE accumulates under GL, whereas inducible PC (PCi) differentially accumulates in the outer portion of PBS rods under RL (Tandeau de Marsac, 1977). Recently, additional forms of CA have been discovered in cyanobacteria that respond to other types of light, including type IV CA, in which organisms respond to blue and green wavelengths of light (Everroad et al., 2006) and far-red light photoacclimation (FaRLiP), which results in cyanobacterial tuning of PBSs in response to RL and far-red (FR) light (Gan et al., 2014; Zhao et al., 2015).

CCA, which is the form of CA that has been studied the most extensively in freshwater filamentous cyanobacterium *Fremyella diplosiphon* (Bennett & Bogorad, 1973), is largely under the control of the photoreceptor RcaE (Kehoe & Grossman, 1996). RcaE works through two response regulators, RcaF and RcaC, to exert transcriptional regulation of the pigment content of PBSs in response to dynamic light signals (Kehoe & Grossman, 1997; Cobley & Miranda, 1983; Terauchi et al., 2004). RcaC is a DNA-binding transcriptional regulator that directly controls the expression of phycobiliprotein-encoding genes (Li et al., 2008; Li & Kehoe, 2005; Bezy & Kehoe, 2010). Under RL conditions, RcaC binds to and upregulates the *cpc2* operon, which encodes PCi proteins and upregulates *pcyA*, which in turn encodes an oxidoreductase that produces phycocyanobilin. The latter is the bilin that attaches to PC (Li et al., 2008; Alvey et al., 2007). In RL, RcaC also represses the *cpe* operon, which produces PE proteins, and the *pebAB* operon, which encodes two oxidoreductases that produce phycoerythrobilin, which is the bilin that attaches to PE (Li et al., 2008; Bezy & Kehoe, 2010). Under GL conditions, the repression of PE synthesis genes is relieved, resulting in PE accumulation (Gutu & Kehoe, 2012). In addition to RcaE, PE levels are also regulated by the Cgi system, a GL-dependent regulatory system (Bezy et al., 2011).

An *F. diplosiphon* mutant with a spontaneous transposon insertion within the phycobilisome abundance regulator gene, *psoR*, displays disrupted pigmentation and growth (Cobley et al., 2008). Here, we describe bioinformatic analyses that were

conducted to explore the presence of PsoR across cyanobacteria, as well as an assessment of conserved residues and putative interacting partners, with the aim of understanding PsoR's potential function. Sequence and phylogenetic analyses determined that PsoR was widespread throughout the cyanobacterial phylum but absent both outside of cyanobacteria and in PBS-containing red algae. The results of the analyses of conserved protein sequences and amino residues suggested that PsoR functions as a β-CASP domain-containing ribonuclease within the metallo-β-lactamase fold superfamily, which may have direct implications for regulating pigment synthesis in vivo. Additionally, putative PsoR-interacting proteins further supported a potential role for the protein in regulating PBS abundance in response to external light cues and/or stress.

2.3 Results

2.3.1 PsoR Is a Putative β-CASP Domain-Containing Ribonuclease

Using the Phyre2 server to predict the structure of *F.diplosiphon* PsoR (FdPsoR), we found matches with β-CASP domain-containing proteins within the metallo-β-lactamase fold superfamily (Table 2.1). Based on the solved structures of known β-CASP proteins, Phyre2 was able to predict the overall structure of PsoR with 82% of residues modeled at >90% confidence (Figure 2.1). The β-lactamase superfamily contains proteins that act on a wide range of substrates, including DNA and RNA (Daiyasu et al., 2001). β-CASP domain-containing proteins are a subgroup that typically displays endo- and exonuclease activity on DNA and RNA substrates. These proteins also play a role in DNA repair and pre-mRNA maturation (Callebaut et al., 2002). Although both DNA- and RNA-processing β-CASP nucleases have been found in eukaryotes, only β-CASP homologs acting on RNA have been found in bacteria and archaea to date (Dominski et al., 2013). The enzymes in this family include the *Saccharomyces cerevisiae* 3'-processing endonuclease Ysh1 and the cleavage and polyadenylation specificity factor MTH1203 from *Methanothermobacter thermautotrophicus* (Mandel et al., 2006; Silva et al., 2011).

The β-lactamase and β-CASP domain regions of FdPsoR span residues 20–430. A C-terminal region follows from residue 431 to residue 554, and the protein contains a small n-terminal region comprising residues 1 to 19. β-CASP enzymes in prokaryotes

typically function as dimers, with the C-terminal regions involved in dimerization (Dominski et al., 2013; Silva et al., 2011; Mathy et al., 2010; Phung et al., 2013). Considering the 123-amino-acid C-terminal region of FdPsoR, this region may serve to dimerize or facilitate the interaction of PsoR with other proteins in vivo, although this C-terminal region did not share sequence or structural similarities with putative interaction domains of other β-CASP proteins.

Table 2.1. Phyre2 top hits for the PsoR sequence. The PsoR amino acid sequence from *Fremyella diplosiphon* was submitted to the Phyre2 Protein Fold Recognition Server. Multiple hits were retrieved with similarities to the Ysh1 superfamily of proteins. ID = identity.

PDB ID	Organism	Description	Alignment Coverage	Confidence	Percent ID
c2ycbA_	*Methanothermobacter thermautotrophicus* str. Delta H	Hydrolase; archaeal β-CASP protein with n-terminal2 kh domains	15-430	100.0	22%
c2xr1B_	*Methanosarcina mazei*	Hydrolase; dimeric archaeal cleavage and polyadenylation specificity2 factor with n-terminal kh domains (kh-cpsf)	15-430	100.0	18%
c2xr1A_	*Methanosarcina mazei*	Hydrolase; dimeric archaeal cleavage and polyadenylation specificity2 factor with n-terminal kh domains (kh-cpsf)	18-430	100.0	16%
c3af5A_	*Pyrococcus horikoshii* OT3	Hydrolase; archaeal cpsf subunit	15-430	100.0	20%
d2i7xa1	*Saccharomyces cerevisiae*	Family: β-CASP RNA-metabolizing hydrolases	19-430	100.0	14%
c2i7xA_	*Saccharomyces cerevisiae*	RNA-binding protein, protein binding	19-430	100.0	14%
d2dkfa1	*Thermus thermophilus*	Family: β-CASP RNA-metabolizing hydrolases	20-430	100.0	23%
c5habB_	*Methanolobus psychrophilus* R15	Hydrolase: mpy-rnase j (mutant h84a), an archaeal rnase j2	15-430	100.0	16%
c5a0tA_	*Streptomyces coelicolor* A3(2)	Hydrolase: catalysis and 5' end sensing by ribonuclease rnase j of the2 metallo-beta-lactamase family	16-431	100.0	17%
d2i7ta1	"Human (*Homo sapiens*); *Saccharomyces cerevisiae*	Family: beta-CASP RNA-metabolizing hydrolases	18-431	100.0	24%

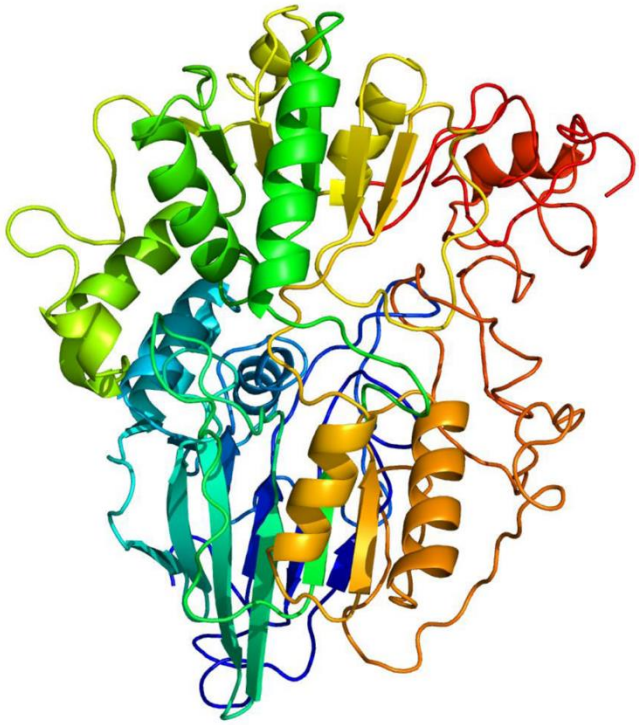


Figure 2.1. Phyre2 modeling of the *Fremyella diplosiphon* PsoR protein. The structure of FdPsoR was modeled on the β-CASP containing enzyme MTH1203 from *Methanothermobacter thermautotrophicus* [46, 47]; 82% of the FdPsoR residues were modeled at > 90% confidence. The protein is rainbow-colored: red (N-terminus) to blue (C-terminus).

The β-CASP family of proteins has seven conserved residues that are involved in substrate binding and hydrolysis (Callebaut et al., 2002). FdPsoR contains all seven conserved residues associated with enzymatic activity (Figure 2.3). The motifs contain aspartic acids, glutamic acids, and histidines, which are involved in binding metal ions, particularly zinc and magnesium (Table 2.2) (Ryan et al., 2004).

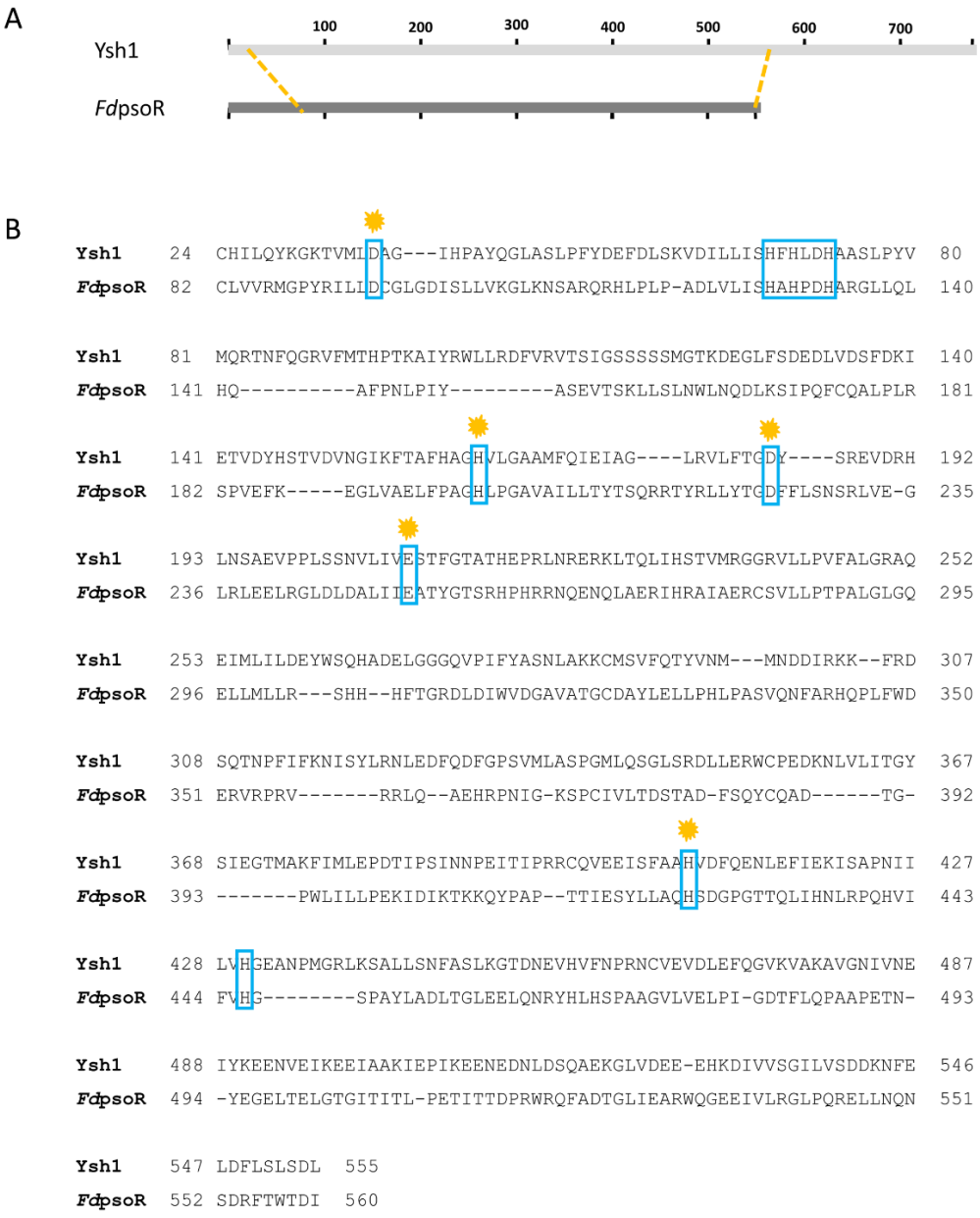


Figure 2.3. Sequence alignment of *Fremyella diplosiphon* PsoR with mRNA endonuclease Ysh1 from *Saccharomyces cerevisiae*. Amino acid sequence alignment with FdPsoR and the mRNA endonuclease Ysh1 from *S. cerevisiae* using the NCBI Protein BLAST program: (A) Schematic showing the overall location of homology between FdPsoR and Ysh1. Orange dashed lines indicate where homology begins and ends. (B) Amino acid alignment of homologous regions between Ysh1 and FdPsoR. Blue boxes indicate conserved residues found in the seven motifs present in β-CASP proteins. Orange marks indicate areas where point mutations in Ysh1 resulted in a loss of endo-nuclease activity (Ryan et al., 2004).

Table 2.2. The seven conserved motifs of β-CASP proteins present in FdPsoR and Ysh1. Motifs 1–4 are found in the Metallo-β-lactamase superfamily of proteins involved in the hydrolysis of different substrates. In addition to these four motifs are motifs A, B, and C found in β-CASP domain-containing proteins involved in nucleolytic activity, using DNA and RNA as substrates. The motifs are involved in binding metals, such as zinc and magnesium, to form the catalytic reaction center of the protein where cleavage occurs.

B-CASP Motif	Ysh1 Residue	*F*dPsoR Residue
Motif 1	D37	D47
Motif 2	H73	H85
Motif 3	H163	H156
Motif 4	D184	D175
Motif A	E209	E203
Motif B	H408	H376
Motif C	H430	H398

2.3.2 PsoR Is Widespread Throughout the Cyanobacteria Phylum

A PSI-BLAST search resulted in the identification of 798 homologs in the cyanobacteria phylum. Species containing a PsoR homolog had only one copy. No homologs were found outside the cyanobacteria. PsoR homologs were noticeably absent from red algae, another group of photosynthetic organisms containing PBSs.

Homologs were found in all orders of cyanobacteria except *Gloeobacter*, the oldest extant group of cyanobacteria, which lacks thylakoid membranes and has atypical PBS structures (Krogmann et al., 2007; Guglielmi et al., 1981). A PsoR phylogenetic tree was generated and rooted using an unrelated β-CASP protein found in *Gloeobacter* (Figure 2.4a). PsoR homologs are generally grouped based on order and family clades, in which PsoR from *F. diplosiphon* is grouped with other homologs found in *Nostocales* species. The overall tree structure resembles that of a previous phylogenetic tree based on cyanobacterial genomes (Shih et al., 2013), suggesting that PsoR was vertically inherited. The majority of homologs (742 of 799) were the closest in size to FdPsoR, and the entire group ranged from 28 to 228 amino acids. These homologs contained one to three conserved motifs of β-CASP proteins. The shortest homologs in this group were not grouped in a single clade but were interspersed among the larger homologs. Despite their small size, these homologs were retained in the analysis because they may represent truncated forms of PsoR. Fifty-seven homologs

ranging in size from 114 to 306 amino acids, grouped in the phylogenetic tree closest to the outgroup, and were found predominantly in *Oscillatoriales* (Figure 2.4b).

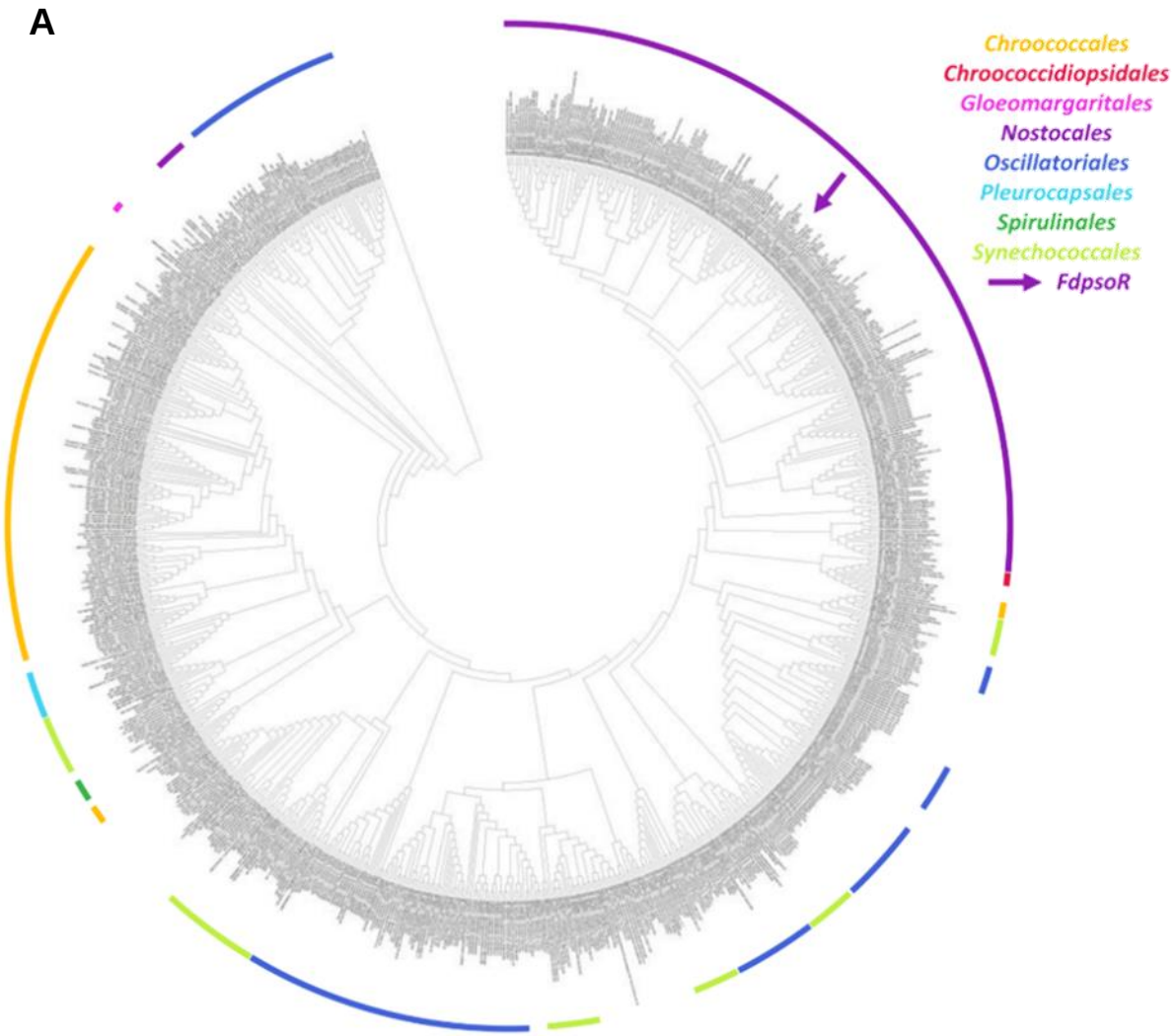
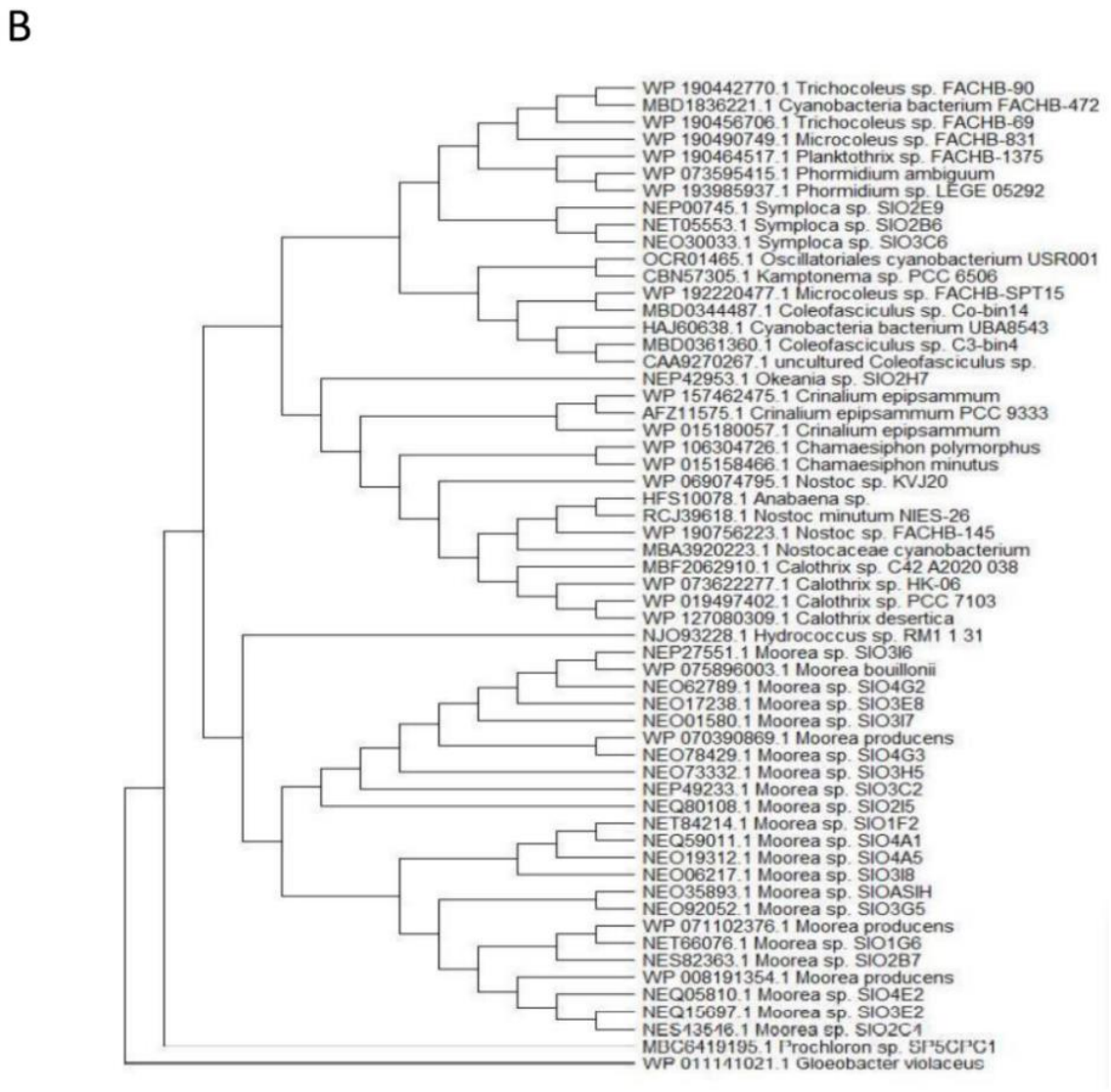


Figure 2.4. Phylogenetic tree of PsoR within cyanobacteria. Protein sequences of PsoR homologs from cyanobacteria were identified using FdPsoR as the query sequence with the PSI-BLAST (Position-Specific Iterated BLAST) algorithm on the NCBI website (Altschul et al., 1997). The identified PsoR homologs were aligned through the MEGA-7 program using MUSCLE (Edgar, 2004). (A) A phylogenetic tree was created using the maximum likelihood method and Jones-Taylor-Thornton matrix-based model in MEGA-X (Kumar et al., 2018). The likelihood log was -746644.20. An unrelated β-CASP protein from *Gloeobacter violaceus* (Gene ID: WP_011141021.1) was used to root the phylogenetic tree. Colors indicate clusters of Cyanobacterial orders. (B) Phylogenetic tree of the PsoR homolog branch closest to the *Gloeobacter violaceus* outgroup.

Figure 2.4. (cont'd)



2.3.3 Promoter Analysis for psoR *in* Fremyella diplosiphon

To determine the locations of potential promoter regions for *psoR* from *F. diplosiphon* and of possible transcriptional regulatory elements, a sequence-based promoter analysis was performed. Using the BPROM program from the Softberry website, three putative promoter regions and several transcription factor binding sites were located. Two of these promoter regions were upstream of a gene named *tcpA*, which is found upstream of *psoR*, while the third promoter region was located within *tcpA* itself (Figure 2.5). In addition to the -10 and -35 promoter regions, 13 transcription

factor binding sites were found (Table 2.3). All but one of the transcription factor binding sites were predicted to colocalize with one of the three promoter sites, and Ihf was predicted to bind two promoter regions; *ihf* and *fis* encode global regulators that transcriptionally control hundreds of genes in response to environmental stimuli (Monteiro et al., 2020). Another transcription factor binding site for OmpR was identified. Of note, the response regulator RcaC, which is involved in the regulation of CCA, is a member of the OmpR/PhoB family of DNA-binding proteins (Bezy & Kehoe, 2010). *lexA* encodes a transcriptional repressor involved in salt stress responses (Takashima et al., 2020).

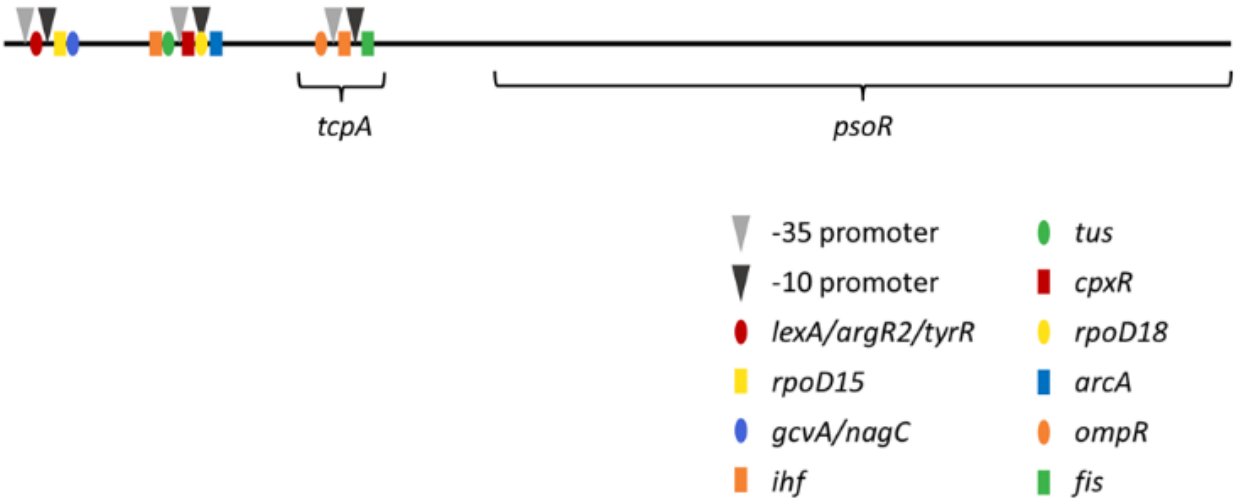


Figure 2.5. Schematic of potential promoter and transcription factor binding sites upstream of *psoR*. Predicted promoter and transcription factor binding sites for *psoR* shown with upstream gene *tcpA*. The BPROM program (Solovyev & Salamov, 2011) was used to analyze the potential promoter and transcription factor binding sites associated with the *F. diplosiphon tcpA-psoR* genomic region. The sequence analyzed was retrieved from the NCBI database (accession ID: DQ286230.1). -10 and -35 promoter sites are indicated by arrowheads, while the predicted transcription factor binding sites are indicated by color-coded symbols.

Table 2.3. List of potential promoter sites and transcription factor binding sites upstream of *psoR* in *Fremyella diplosiphon*. The upstream region of *psoR* contained three predicted promoter regions. Potential transcription factor binding sites were also found, with binding sequences and locations indicated. Bp = base pairs; TF = transcription factor. Base pairs indicate distance from the start of the *psoR* gene.

Promoter	-10 Promoter		-35 Promoter		Oligonucleotides from known TF binding sites		
bp	Sequence	bp	Sequence	bp	Name	Sequence	bp
84	CGATATACT	69	TTGTAT	45	*lexA*	TATATAAA	55
					argR2	ATATAAAT	56
					lexA	ATAAATAA	58
					tyrR	TAAATAAA	59
					rpoD15	TAAGGTTA	83
					gcvA	TTATATTT	92
					nagC	ATATTTTA	94
402	AAATATATT	387	TTACTA	364	*ihf*	AAATAAAA	343
					tus	CATTAGTA	351
					cpxR	TAAAAAGA	376
					rpoD18	AAATATAT	387
					arcA	TTAATTAA	404
818	TTTTATGAT	803	TTGTTT	779	*ompR*	TCATATTT	759
					ihf	ACAAAAAA	791
					fis	ACAATTAT	816

2.3.4 Protein–Protein Interactions of FdPsoR and Synechocystis PsoR Homologs

To predict whether FdPsoR and its homologs interact with other proteins *in vivo*, the STRING program was used to predict protein–protein interactions (Szklarczyk et al., 2021). For FdPsoR, 10 potential interactions were determined (Figure 2.6). The majority of the proteins predicted to interact with FdPsoR were involved in DNA and RNA metabolism (Table 2.4). Of note, given the potential role of PsoR in regulating PBS abundance, one of the proteins identified as a potential interacting partner for FdPsoR was EKF03351.1, a phycocyanin-associated protein.

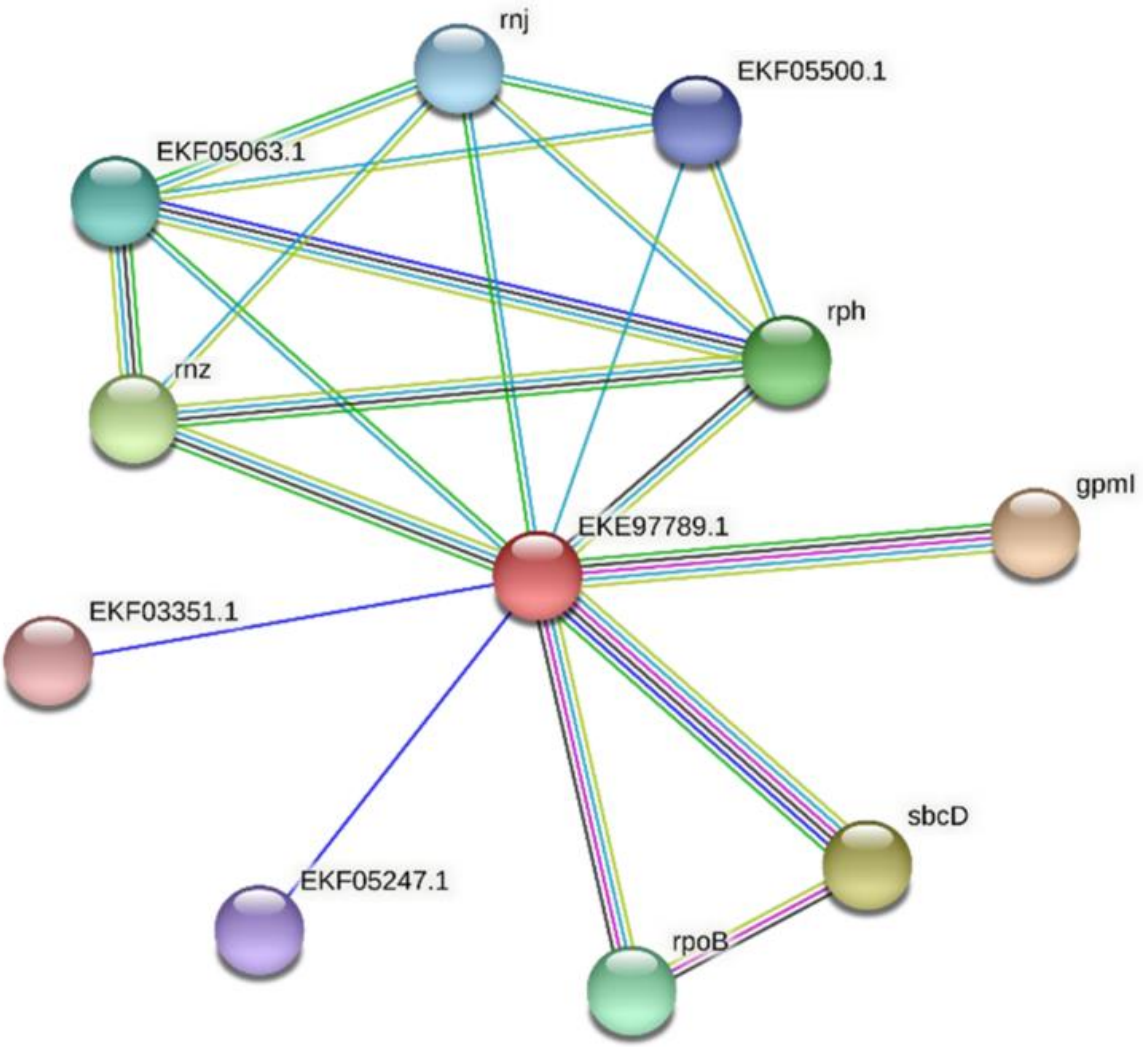


Figure 2.6. Protein–protein interaction network for FdPsoR. A protein–protein interaction network was generated using STRING (Szklarczyk et al., 2021). The PsoR protein sequence (GenBank ID: EKE97789.1) from *F. diplosiphon* was used as a query. Edges represent functional associations, and differently colored lines representing different types of interactions: green line, neighborhood of genes; blue line, co-occurrence across species; purple line, experimental evidence; yellow-green line, text mining; light blue line, database documentation; and black line, co-expression data.

Table 2.4. Proteins predicted to interact with FdPsoR using the STRING program.

Predicted interaction partner	Description	Confidence score
GpmI	Catalyzes the interconversion of 2-phosphoglycerate and 3-phosphoglycerate.	0.871
SbcD	SbcCD cleaves DNA hairpin structures. Subunit D. The complex acts as a 3'->5' double strand exonuclease that can open hairpins. It also has 5' single-strand endonuclease activity.	0.749
Rnz	Zinc phosphodiesterase displays some tRNA 3'-processing endonuclease activity. It is probably involved in tRNA maturation by removing a 3'-trailer from precursor tRNA.	0.727
Rph	Phosphorolytic 3'-5' exoribonuclease plays an important role in tRNA 3'-end maturation.	0.689
RpoB	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates.	0.688
EKF05063.1	Ribonuclease E/G	0.680
Rnj	An RNase that has 5'-3' exonuclease and possibly endonuclease activity. Involved in maturation of rRNA and, in some organisms, mRNA maturation and/or decay.	0.678
EKF05500.1	Ribonuclease T(2) family protein	0.666
EKF05247.1	Uncharacterized protein	0.656
EKF03351.1	Phycocyanin-associated protein	0.654

Two homologs of FdPsoR, Sll0514 from *Synechocystis* sp. PCC 6803 and ACA99583.1 from *Synechocystis* sp. PCC 7002 were also submitted to STRING. Nine proteins were predicted to interact with Sll0514 (Figure 2.7, Table 2.5), and 10 proteins were predicted to interact with ACA99583.1 (Figure 2.8, Table 2.6). All three PsoR homologs were predicted to interact with Gpm, the 2,3-bisphosphoglycerate-independent phosphoglycerate mutase involved in glucose metabolism, and the components of SbcC/D, a heterodimer that cleaves DNA hairpins (Nukui et al., 2007; Connelly et al., 1998). Both *Synechocystis* homologs were predicted to interact with PolA, which is DNA polymerase I (Patel et al., 2001).

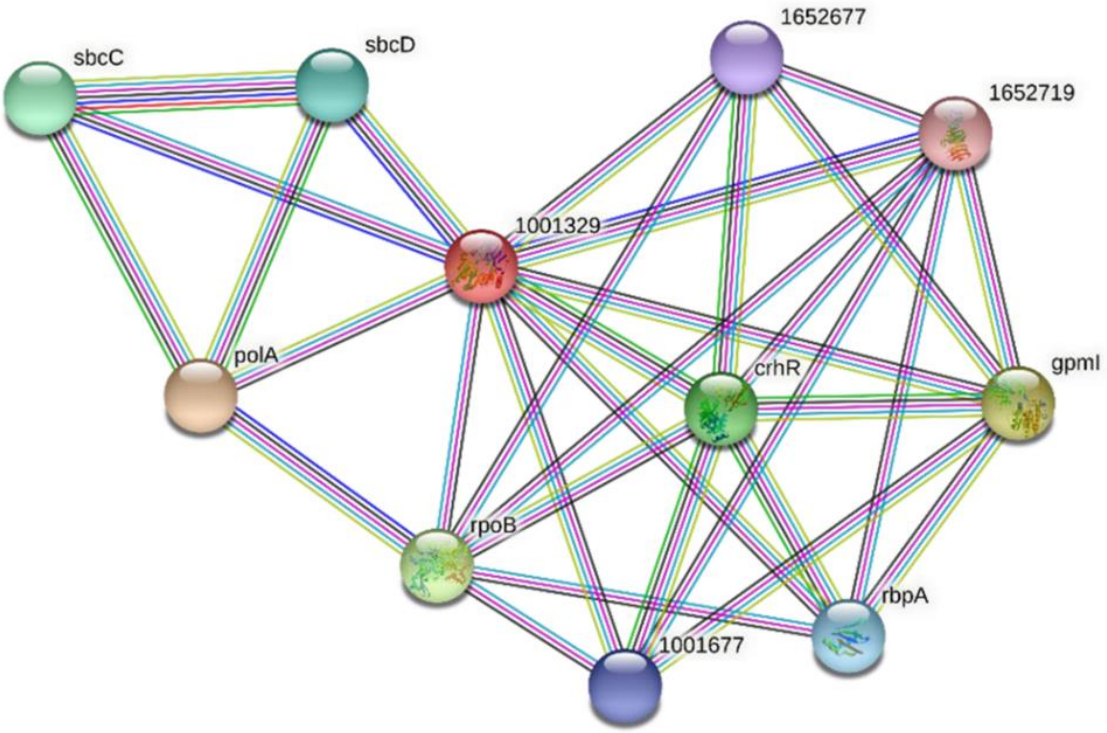


Figure 2.7. Protein–protein interaction network for PsoR homolog Sll0514 in *Synechocystis* sp. PCC 6803. A protein–protein interaction network was generated using STRING [42]. The Sll0514 protein sequence (GenBank ID: sll0514) from *Synechocystis* sp. PCC 6803 was used as a query. Edges represent functional associations with different color lines representing different types of interactions: red line, fusion of genes; green line, neighborhood of genes; blue line, co-occurrence across species; purple line, experimental evidence; yellow-green line, text mining; light blue line, database documentation; and black line, co-expression data.

Table 2.5. Proteins predicted to interact with Sll0514 from *Synechocystis* sp. PCC 6803 using the STRING program.

Predicted interaction partner	Description	Confidence score
PolA	DNA polymerase I. In addition to polymerase activity, this DNA polymerase exhibits 3'-5' and 5'-3' exonuclease activity.	0.937
GpmI	2,3-bisphosphoglycerate-independent phosphoglycerate mutase. Catalyzes the interconversion of 2-phosphoglycerate and 3-phosphoglycerate.	0.900
RpoB	DNA-directed RNA polymerase subunit beta. DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates.	0.736
CrhR	RNA helicase CrhR. An ATP-dependent bidirectional RNA helicase with RNA-dependent ATPase activity.	0.700
SbcC	Nuclease SbcCD subunit C. SbcCD cleaves DNA hairpin structures.	0.691
RbpA	Putative RNA-binding protein	0.674
1001677	Slr0193; RNA-binding protein	0.674
1652677	Ssr1480; RNA-binding protein	0.674
1652719	sSlr1410; uncharacterized WD repeat-containing protein	0.658

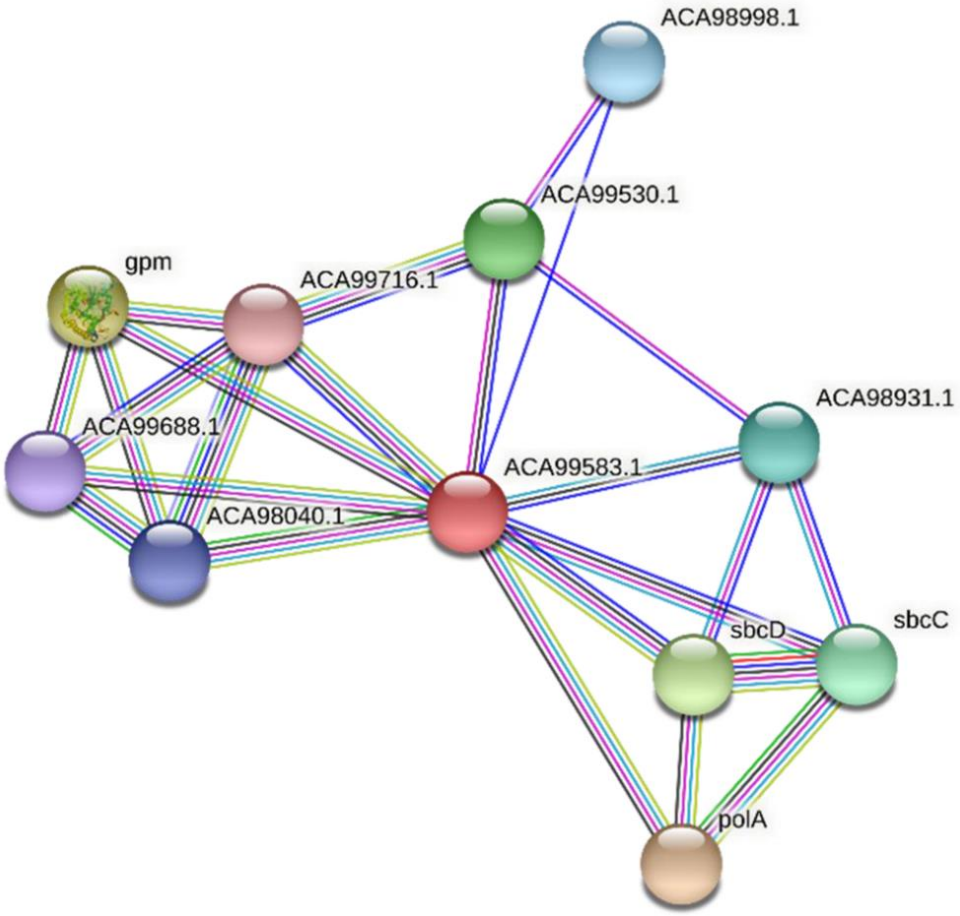


Figure 2.8. Protein–protein interaction network for PsoR homolog ACA99583.1 in *Synechocystis* sp. PCC 7002. A protein–protein interaction network was generated using STRING [42]. The ACA99583.1 protein sequence (GenBank ID: ACA99583.1) from *Synechocystis* sp. PCC 7002 was used as a query. Edges represent functional associations with different color lines representing different types of interactions: red line, fusion of genes; green line, neighborhood of genes; blue line, co-occurrence across species; purple line, experimental evidence; yellow-green line, text mining; light blue line, database documentation; and black line, co-expression data.

Table 2.6. Proteins predicted to interact with ACA99583.1 from *Synechocystis* sp. PCC 7002 using the STRING program.

Predicted interaction partner	Description	Confidence score
PolA	DNA polymerase I. In addition to polymerase activity, this DNA polymerase exhibits 5'-3' exonuclease activity.	0.932
Gpm	2,3-bisphosphoglycerate-independent phosphoglycerate mutase. Catalyzes the interconversion of 2-phosphoglycerate and 3-phosphoglycerate.	0.870
SbcD	Nuclease SbcCD subunit D. SbcCD cleaves DNA hairpin structures.	0.757
ACA99530.1	Uncharacterized protein	0.751
SbcC	Nuclease SbcCD subunit C. SbcCD cleaves DNA hairpin structures.	0.740
ACA98931.1	FHA-domain protein	0.728
ACA98998.1	SH3b domain-containing protein	0.721
ACA98040.1	WD-repeat protein	0.718
ACA99688.1	TPR-repeat containing protein	0.711
ACA99716.1	Serine/threonine kinase	0.681

2.4 Discussion

Considering the critical role of light energy in photosynthesis, mechanisms that allow photosynthetic organisms, such as cyanobacteria, to perceive and respond to fluctuating light environments are vital for organisms to survive and thrive. In cyanobacteria, CCA involves a network of photoreceptors, effectors, and gene operons to fine-tune pigmentation, cell morphology, filament morphology, and metabolisms in response primarily to RL and GL. The *psoR* gene has been reported to encode a protein that negatively regulates PBS abundance (Cobley et al., 2008). Although significant insights have been gained into the regulation of structural genes needed for PBS synthesis, particularly in response to light cues during CA, the regulation of PBS abundance has not been extensively examined in cyanobacteria at the mechanistic level. To understand the roles of PsoR in CCA and cyanobacteria, we assessed its potential structure and function and searched for homologs across the cyanobacterial kingdom to determine when this gene may have arisen in these organisms.

The putative function of PsoR was investigated using the Phyre2 program. The protein encoded by *psoR* contains a predicted β-CASP domain, including a collection of conserved motifs/residues involved in the processing of DNA and RNA substrates. The β-CASP family of enzymes belongs to the metallo-β-lactamase superfamily, which

includes proteins that process a wide variety of substrates in organisms. β-CASP proteins are found in all three domains of life, and they play a predominant role in pre-mRNA processing, although some β-CASP enzymes are involved in DNA repair (Callebaut et al., 2002; Dominski et al., 2013; Condon & Gilet, 2011). The conserved motifs of PsoR are involved in metal binding, typically zinc ions, and in stabilizing nucleic acid substrates for cleavage. Because of its β-CASP domain and the fact that all prokaryotic β-CASP enzymes identified thus far have been ribonucleases, the role of PsoR in cyanobacteria may be the regulation of mRNA; PsoR may specifically regulate genes involved in PBS abundance. Other β-CASP proteins tend to function as dimers or within larger protein complexes, usually binding through the C-terminal region of the β-CASP domain or a separate C-terminal region (Dominski et al., 2013; Silva et al., 2011; Mathy et al., 2010; Phung et al., 2013). With the presence of a 123 residue C-terminal region in FdPsoR, PsoR may also function as a dimer in vivo or may work through interactions with other proteins.

Homologs of PsoR from *F. diplosiphon* were found across the Cyanobacteria phylum, and only Gloeobacter lacked a homolog. Gloeobacter are an ancient clade of cyanobacteria that lack thylakoid membranes and have atypical PBSs. Instead of rods grouped around a hemidiscoidal core, Gloeobacter PBSs are grouped in bundles of parallel rod-shaped structures attached to cytoplasmic membranes (Krogmann et al., 2007; Guglielmi et al., 1981). *Gloeobacter* also lacks the genes *psbY, psbZ,* and *psb27*, which encode subunits of photosystem II (Guglielmi et al., 1981; Yokono et al., 2008). Homologs of PsoR were not found in red algae or other organisms, which suggests that PsoR homologs arose sometime after branching off from Gloeobacter, perhaps around the time that more complex PBSs evolved. Although red algae have PBSs similar to those in cyanobacteria, the lack of PsoR homologs may indicate a different enzyme or system for regulating PBS abundance in these distinct organisms.

The range of sizes found among PsoR homologs is notable. Because the smallest homologs did not form a distinct branch but were found to be intermingled with homologs in sizes closer to FdPsoR, perhaps these shorter homologs are truncated forms of PsoR. Whether they retain the functionality of PsoR needs to be explored experimentally. The 57 homologs that formed their own branch closest to the

Gloeobacter outgroup contained the longest homologs found in our analysis, at as much as 306 amino acids (Figure 2.4b). Predominantly of the *Oscillatoriales* order, they could be extended forms of PsoR.

Three predicted promoters and multiple transcription factor binding sites preceded the *psoR* gene; yet, one of the two regions also upstream of the neighboring *tcpA* gene may be a more likely transcription start site (Figure 2.5). Previous work established that TcpA, or tetracontapeptide A, is a 40 amino acid peptide encoded by a gene that appears to be co-transcribed with *psoR* and may also play a role in PBS abundance regulation (Cobley et al., 2008). Because *tcpA* was found upstream of *psoR* in every cyanobacterial genome searched by Cobley et al. (Cobley et al., 2008), the presence of these two promoter regions upstream of *tcpA* provides further evidence that the two genes are co-transcribed in cyanobacteria where they are both found, and they may even work together in some way to regulate PBS abundance in these bacteria. The presence of a promoter region within *tcpA* may indicate differential transcription, allowing the transcription of *psoR* without *tcpA*.

The predicted protein–protein interactions indicated interactions with protein partners involved in gene and metabolism regulation. Because FdPsoR is a putative β-CASP nuclease, it is possible that FdPsoR interacts with other proteins involved in regulating CCA and metabolism in response to environmental stimuli. Of note, a PC-associated protein was among the proteins predicted to interact with FdPsoR.

Light fluctuations in terrestrial and aquatic environments occur frequently, in which light quality and quantity are altered by factors such as cloud coverage and shading by other objects and organisms. The ability to perceive and respond to changing light conditions is important for cyanobacteria to maximize productivity and minimize damage caused by excess light in these dynamic conditions. Because of the important role PBSs play in CA and photosynthesis, understanding how PBSs undergo remodeling or how their abundance is tuned to external cues could extend our understanding of how cyanobacteria survive and thrive in fluctuating light environments. Although significant insights into the light quality-dependent regulation of pigment content have been obtained during 50 years of research on CCA, many aspects of PBS regulation remain to be elucidated. Here, PsoR has been reported to be widespread

across cyanobacteria, where it likely plays a critical role in cellular PBS regulation. Understanding how PsoR functions as a β-CASP protein and its role in CCA and cyanobacteria can help lead to a better understanding of how photosynthetic organisms fine-tune their photosynthetic machinery in response to changes in their light environment. This knowledge may contribute to improvements in using cyanobacteria for industrial and pharmaceutical purposes.

2.5 Materials and Methods

2.5.1 Sequence Analysis and Protein Modeling

The 554 amino acid sequence of PsoR from *F. diplosiphon* was obtained from the National Center for Biotechnology Information (NCBI) database (GenBank ID: EKE97789.1) and analyzed using the Phyre2 Protein Fold Recognition Server (Kelley et al., 2015) to predict protein structure, including a search for conserved domains, sequence features, and prediction(s) of putative functions.

2.5.2 Sequence Homology and Phylogeny

The FdPsoR protein sequence was run through the position-specific iterated BLAST (PSI-BLAST) algorithm on the NCBI website (Altschul et al., 1997). Three successive iterations were performed to refine the results. Multiple sequence alignments of the obtained sequences were generated through the MEGA-7 program using MUSCLE (Edgar, 2004). A phylogenetic tree was created using the maximum likelihood method and the Jones-Taylor-Thornton matrix-based model in MEGA-X (Kumar et al., 2018). The likelihood log was-746644.20. An unrelated β-CASP protein from *Gloeobacter violaceus* (Gene ID: WP_011141021.1) was used to root the phylogenetic tree.

2.5.3 Promoter Analyses

The BPROM program (Solovyev & Salamov, 2011) was used to analyze the potential promoters and transcription factor binding sites associated with the *F. diplosiphon psoR* gene sequence as well as the upstream and downstream areas flanking it. The analyzed sequence was retrieved from the NCBI database (accession ID: DQ286230.1).

2.5.4 Predicted Protein–Protein Interactions

We used the STRING program to search for potential proteins that may interact with *psoR* from *F. diplosiphon* (FdPsoR) and its homologs (Szklarczyk et al., 2021). The FdPsoR protein sequence (GenBank ID: EKE97789.1) and homologs from *Synechocystis* sp. PCC 6803 (GenBank ID: sll0514) and *Synechocystis* sp. PCC 7002 (GenBank ID: ACA99583.1) were used.

2.6 Acknowledgments

I am grateful to members of the Montgomery Laboratory for assistance with experiments and discussion of the project.

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CHAPTER THREE

Characterization of phycobilisome abundance regulator *psoR* mutant in *Fremyella diplosiphon*

3.1 Abstract

Cyanobacteria perceive and respond to their light environment using a mechanism called complementary chromatic acclimation (CCA), that allows them to maximize photosynthetic productivity while minimizing damage caused by excessive light. CCA involves regulation of pigmentation and of cellular and filament morphology in cyanobacteria. Despite decades of research on CCA, genes are still being discovered that fit within the network of genes involved in cellular responses to changes in the external light environment. A *psoR*::IS701 mutant found through routine color mutant screening retains its blue-green pigmentation and lacks the ability to switch color under different light conditions that is exhibited by wild-type *Fremyella diplosiphon* cells. We characterized this *psoR*::IS701 mutant using growth curves, whole cell spectral scans, and pigment extraction and quantification. Altered levels of phycobiliproteins (PBP) were found in the *psoR*::IS701 mutant compared to wild-type, which supported the measured growth rates and spectral scans under red, green, and white light. As the phenotype was unlike that found in red or green mutants, which have a clear lack of one PBP or another, imbalance of PBPs in a *psoR*::IS701 mutant under all light conditions shows potential for better understanding the network of genes involved in CCA.

3.2 Introduction

Cyanobacteria are photosynthetic prokaryotes that are widespread across the globe, found in nearly every niche present (Jungblut et al., 2005; Martinez et al., 2019; Samolov et al., 2020). These oxygenic phototrophs utilize the light energy in their environment to fuel photosynthesis. Because both light quality and quantity regularly fluctuate over timespans of hours to seasons in natural environments, cyanobacteria have evolved mechanisms to balance the light energy they absorb to maximize productivity while minimizing photodamage due to excess light (Montgomery, 2016; Ho et al., 2017; Sanfilippo et al., 2019). One of these mechanisms is chromatic acclimation (CA), in which cyanobacteria modulate their physiology and metabolism to fine-tune light energy absorption. Various types of CA are used by different species of cyanobacteria, including complementary chromatic acclimation (CCA, also known as type III CA) (Ho et al., 2017; Montgomery, 2016; Sanfilippo et al., 2019).

CCA primarily responds to changes in red and green wavelengths of light (Tandeau de Marsac, 1977; Bennett & Bogorad, 1973). When a change in light quality or quantity is perceived by cells, accessory light harvesting complexes called phycobilisomes (PBSs) undergo remodeling to tweak light absorption to match the predominant wavelengths or intensity of light. PBSs are multi-protein antennas that are attached via linker proteins to the photosystems in the thylakoid membranes, shuttling light energy via resonance energy transfer from the tips of PBS rods into the core and then on to the chlorophyll-containing reaction centers (Gutu & Kehoe, 2012; Adir et al., 2006; Zhao et al., 2005). The typical core of the PBS in CCA-capable cyanobacteria is made up of red-light absorbing allophycocyanin (AP) in the shape of 2, 3, or 5 stacked cylinders depending on the species; the core is linked to the photosystems by a core-membrane linker protein (Zhao et al., 2005). From the core, rods radiate out that are comprised of phycocyanin (PC) and phycoerythrin (PE) phycobiliproteins (PBP). The proximal region of the rods is made up of constitutive PC (PCc) under both RL and GL conditions. It is the most distal portion of PBS rods that predominantly changes under remodeling during CCA. Under RL, the outer portions of rods are made up of inducible phycocyanin (PCi), a pigment best suited to capture light in the 620 nm range, and under GL, the outer portion of PBS rods is made up of PE, a pigment best suited to capture light in the 560 nm range (Grossman, 2003).

Fremyella diplosiphon, also known as *Tolypothrix* PCC 7601 (Haney & Kehoe, 2019), is a model organism most often used to study organismal changes involved in CCA (Bennett & Bogorad, 1973). Changes in pigmentation and morphology are under the control of the Rca phosphorelay system (Kehoe & Grossman, 1996). RcaE is a photoreceptor that undergoes red-light activated auto-phosphorylation, while under GL it remains unphosphorylated, and this is the start of the Rca signaling cascade (Terauchi et al., 2004). RcaF and RcaC are response regulators that function downstream of RcaE. In red light, the phosphorylated form of RcaC upregulates the *cpc2* operon, which contains the genes for the PC subunits and linker proteins, and blocks transcription of the *cpe* operon, which contains the genes for the PE subunits and linker proteins (Kehoe & Grossman, 1997; Li et al., 2008). Under GL, RcaC is not phosphorylated and does not interact with either the *cpc* or *cpe* operon. Under these conditions, expression

of the *cpe* operon can occur to support PE synthesis. There is a second system involved in PE regulation called the Cgi system, or GL-dependent regulatory system, that also contributes to PE synthesis (Bezy et al., 2011). Because pigmentation is a major aspect of CCA, mutants that display disrupted color changes have been used for over 40 years to identify key components of the CCA regulatory gene network or structural components contributing to CCA (Cobley & Miranda, 1983; Montgomery, 2011).

A spontaneous *F. diplosiphon* mutant with transposon *IS701* inserted within the phycobilisome abundance regulator gene, *psoR*, was isolated in a routine color mutant screen from another mutant named MRGL2. While MRGL2 has an unknown mutation causing a slight shift in coloration compared to wildtype, it still undergoes pigmentation changes during CA, shifting to red under green light and green under red light. In my thesis work, I set out to characterize the blue-green *psoR*::*IS701* mutant, quantifying the changes in growth and pigmentation under both RL and GL.

3.3 Results

3.3.1 Spontaneous transposon insertion in psoR *gene reveals CCA pigmentation and growth defects*

Through a color mutant screen, we isolated a new mutant that was blue-green in color and that did not change its pigmentation when switched between red, green, and white light (Figure 3.1a & 3.1b). This blue-green mutant was isolated from a previous mutant called MRGL2. MRGL2 was isolated after heat-shock treatment and displays similar pigmentation changes to wild-type. The wild-type strain of F. diplosiphon is a shortened-filament strain that displays wild-type pigmentation under RL and GL (Cobley et al., 1993) PCR and DNA sequencing of the *psoR* gene revealed a transposon, *IS701*, inserted within the *psoR* gene (Figure 3.1c & 3.1d). Thus, the blue-green mutant is hereafter referred to as *psoR*::IS701.

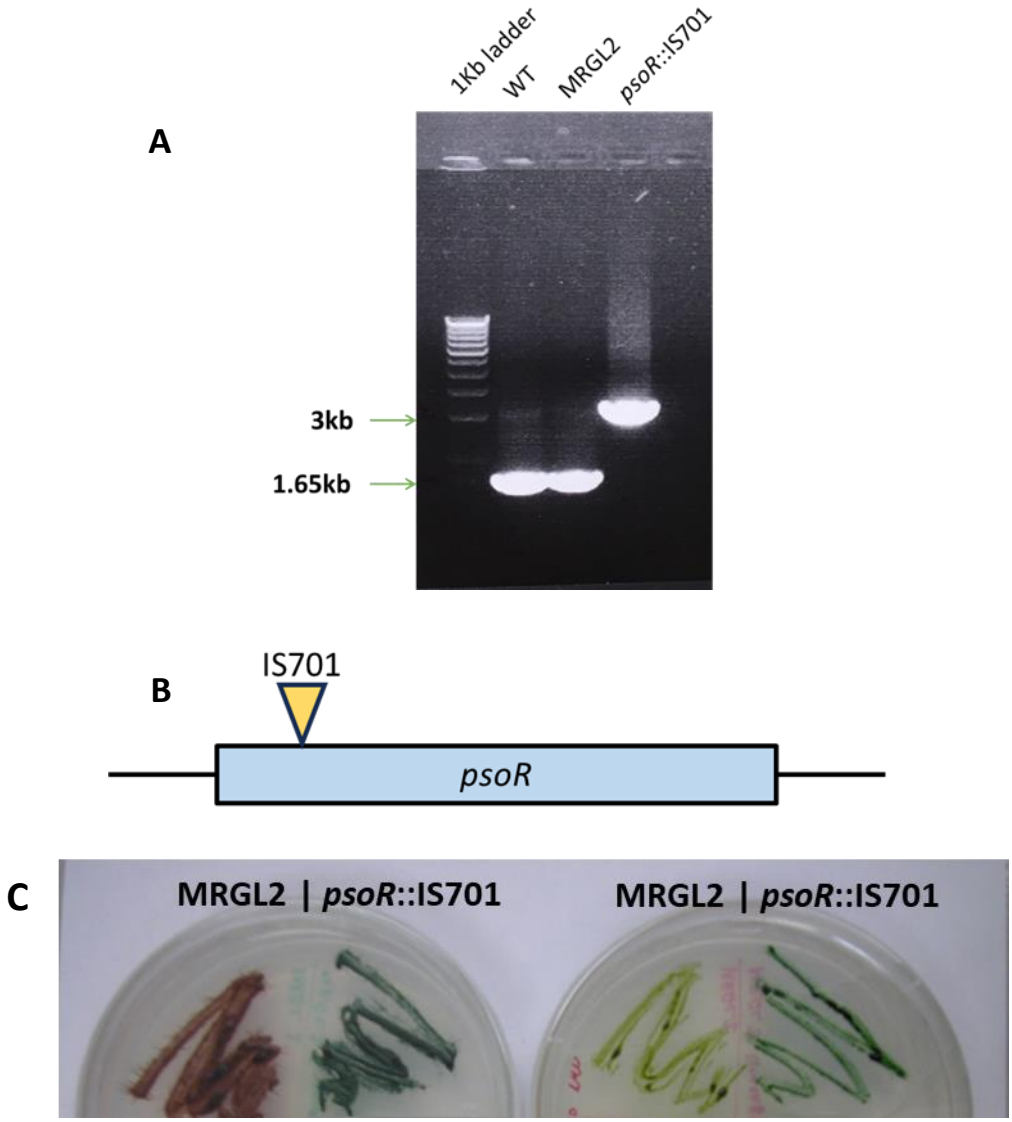

Figure 3.1. IS701 transposon insertion in the *psoR* gene in *Fremyella diplosiphon*. A) Gel electrophoresis of PCR amplifying the *psoR* gene region in WT, MRGL2, and *psoR*::IS701 mutant. B) Cartoon depiction of where the IS701 transposon is inserted in the *psoR* gene in *psoR*::IS701. C) Panel C shows the WT, MRGL2 and *psoR*::IS701 strains under green light (left plate) compared to red light (right plate); image taken by Melissa Whitaker. D) Panel D shows the WT, MRGL2, and *psoR*::IS701 strains grown in the white light multi-cultivator.

Figure 3.1. (cont'd)

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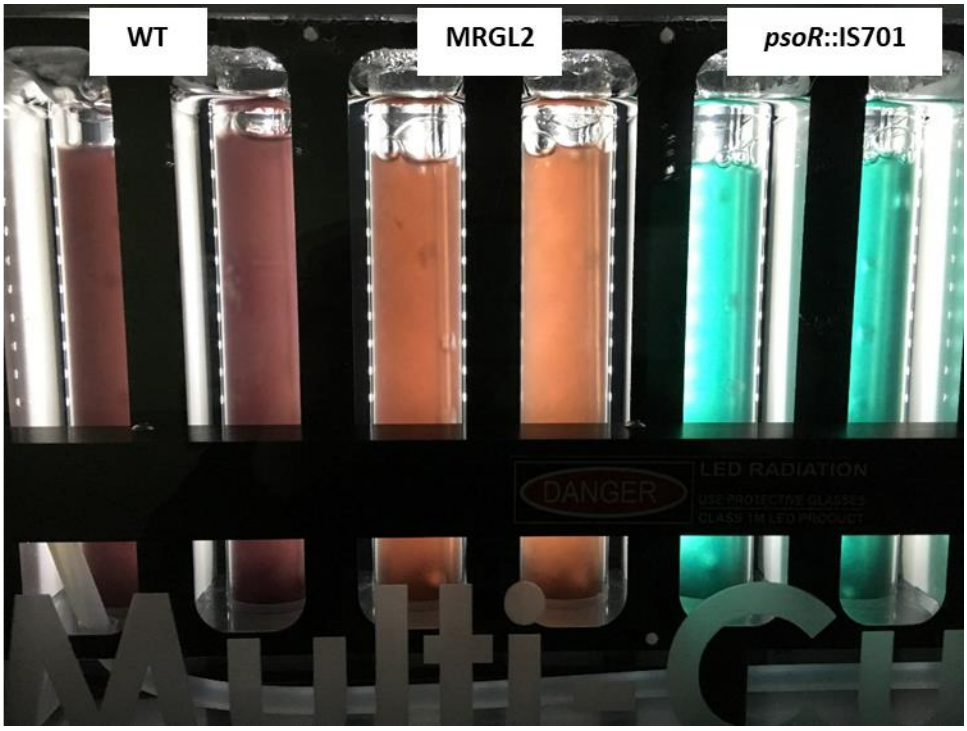


To assess how the observed lack of pigmentation change in *psoR*::IS701 under red and green light affected growth, we grew *psoR*::IS701, parental strain MRGL2, and the wild-type (WT) strain under RL and GL for 14 days (Figure 3.2a & 3.2b). Under RL, *psoR*::IS701 had a slightly higher growth rate compared to WT and MRGL2, while under GL *psoR*::IS701 lagged behind. The strains were grown in a green-enriched white light (WL) bioreactor exhibiting higher light intensity to see how they would grow under mixed light and higher intensity conditions (Figure 3.2c). Of note, both *psoR*::IS701 and MRGL2 lagged behind WT in bioreactor growth conditions.

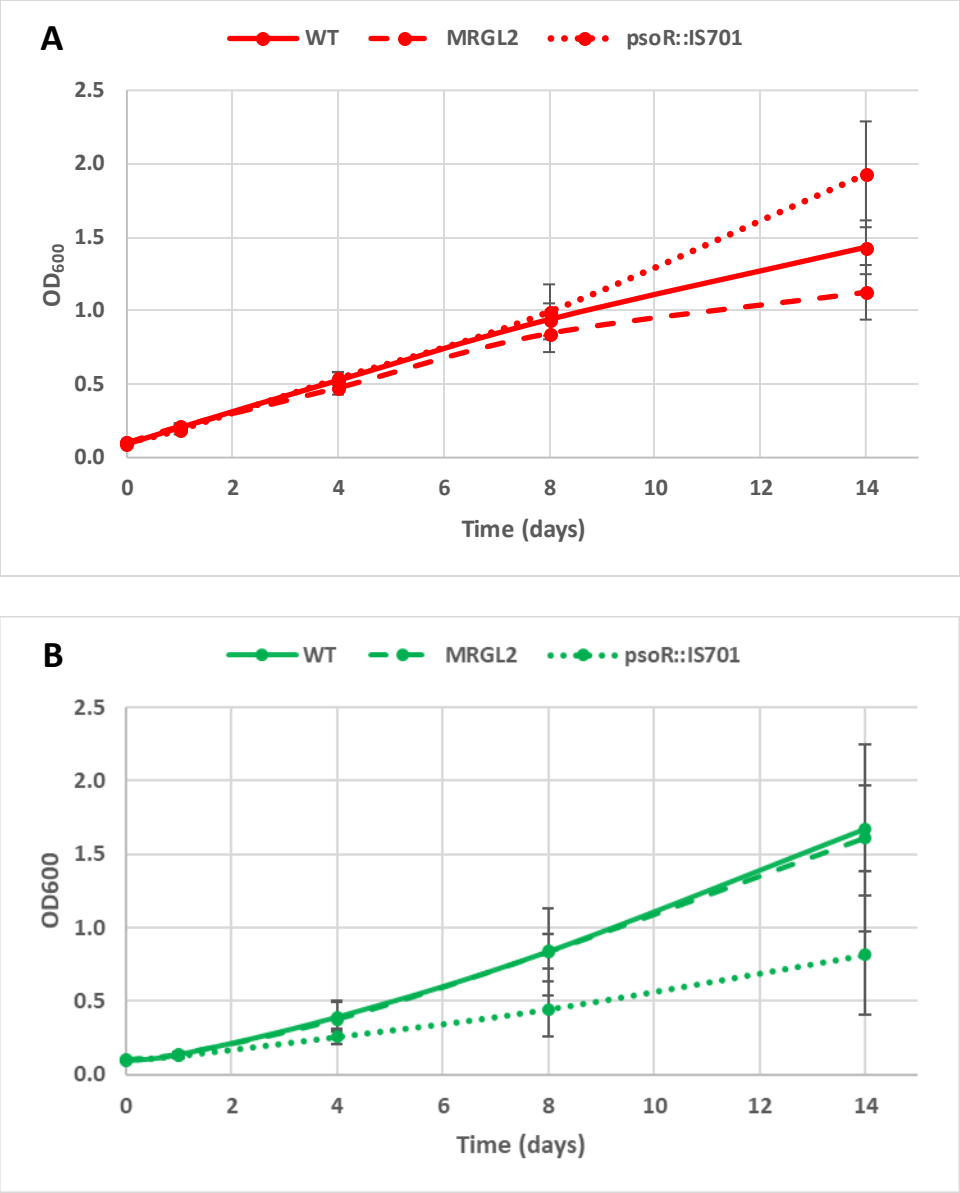
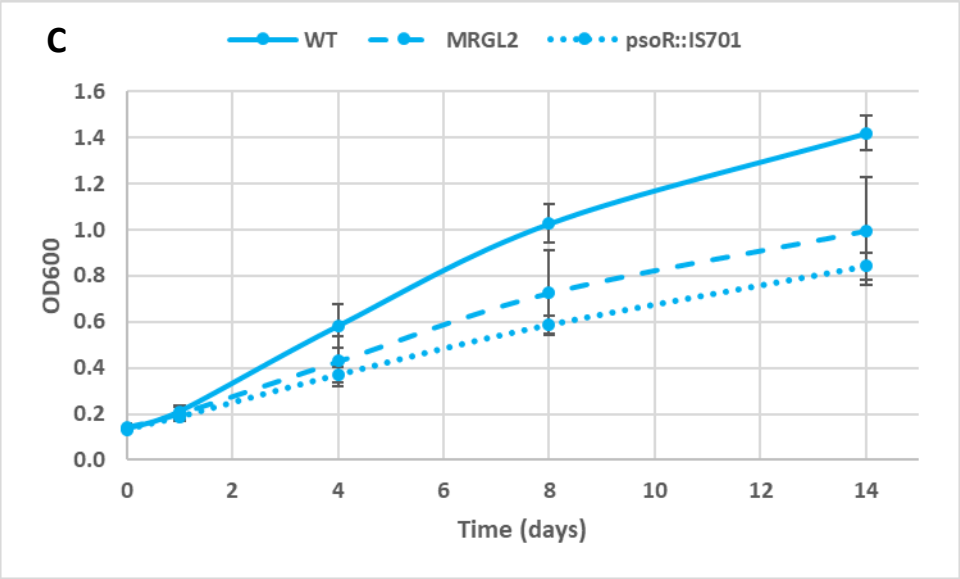


Figure 3.2. Growth curves of WT, MRGL2, and *psoR*::IS701 under a) red, b) green, and c) white light conditions. 4-6 biological replicates per strain, with standard deviations indicated by the error bars at each time point.

Figure 3.2. (cont'd)



To check if observed differences in growth for the *psoR*::IS701 strain relative to MRGL2 and WT were potentially due to absorption of available light by accumulation of distinct pigments, spectral scans were performed for all three strains under RL, GL, and white light (Figure 3.3). WT and MRGL2 tended to follow a similar pattern in light absorption under red, green, and white light. The *psoR*::IS701 strain exhibited a pattern similar to WT and MRGL2 under GL, but it diverged under RL and white light. Under RL, *psoR*::IS701 had a higher peak at 625nm, which is indicative of more red-light absorption and correlates to higher PC accumulation. Under white light, *psoR*::IS701 had a strong PC peak (i.e., peak at 625nm) while WT and MRGL2 lacked a corresponding PC peak.

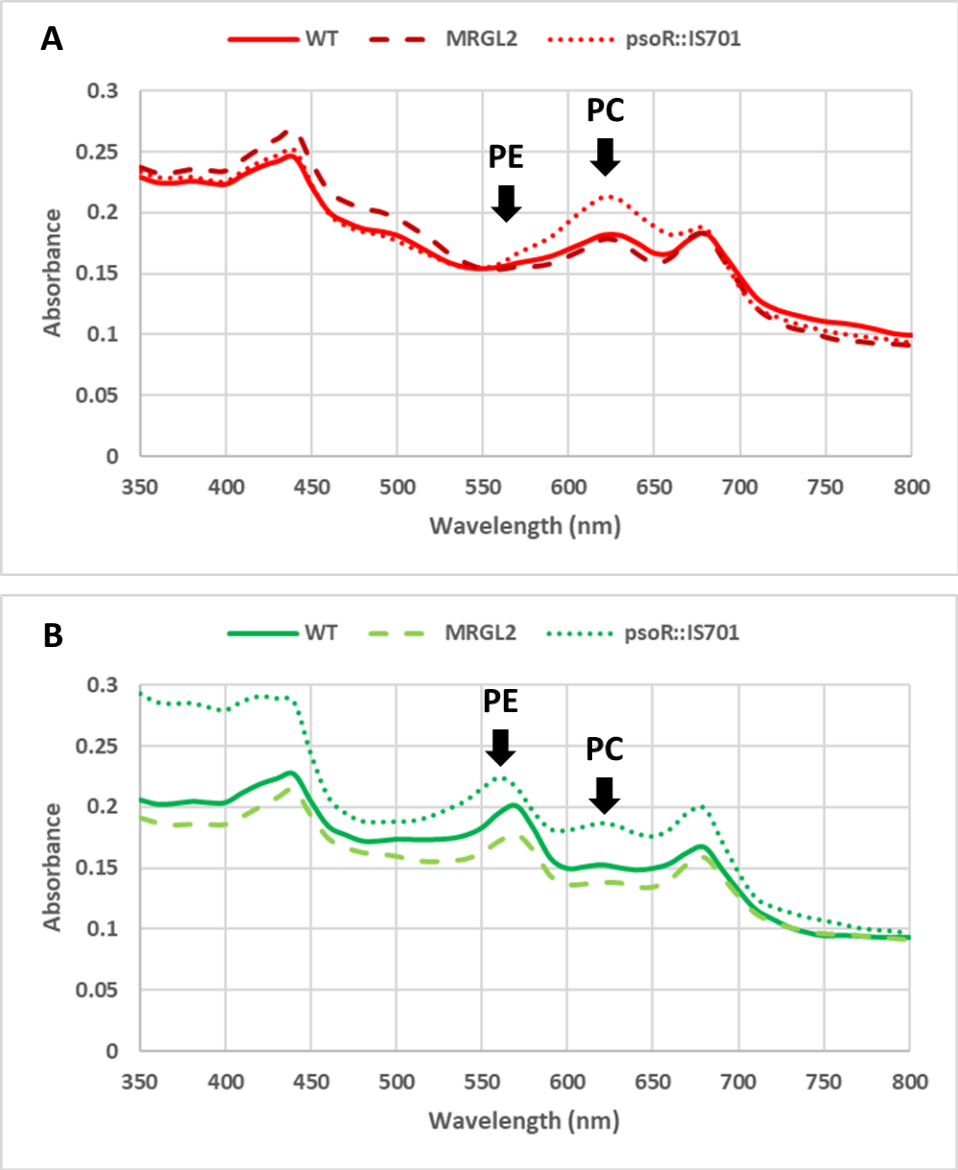
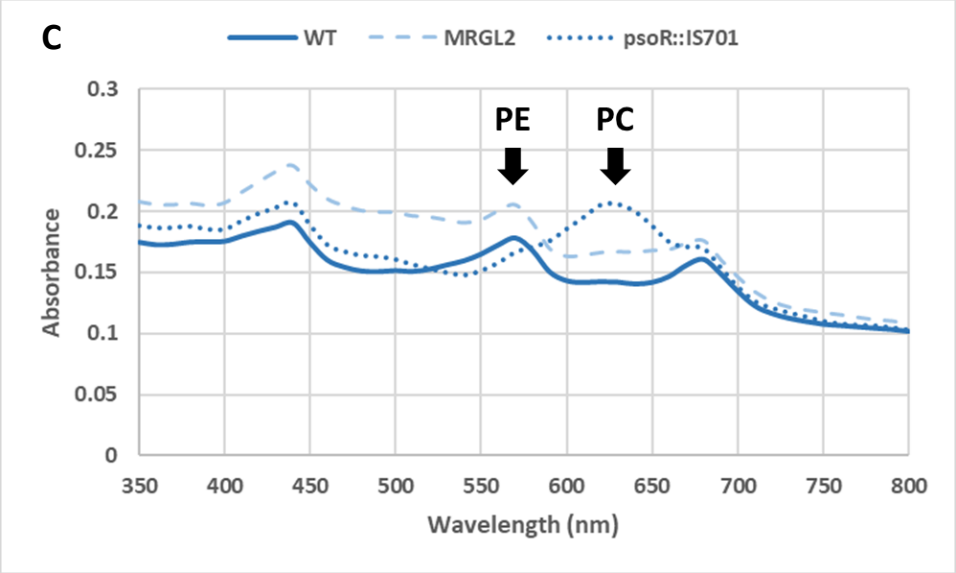


Figure 3.3. Spectral scans of WT, MRGL2, and *psoR*::IS701 under a) red, b) green, and c) white light conditions. 4-6 biological replicates per strain, with a representative replicate chosen for comparison. Black arrows indicate maximum absorption peaks for PE and PC.

Figure 3.3. (cont'd)



3.3.2 The psoR::IS701 *mutant shows altered phycobiliprotein levels*

Whole cells were harvested during the growth phase and pigments were extracted to quantify the chlorophyll *a*, carotenoids, and PBPs accumulated. Post hoc Tukey test was performed for pairwise comparisons between all three strains under RL, GL, and white light. Chlorophyll *a* levels (chl *a*) were similar under RL and GL conditions for all three strains, while WT had significantly higher chl *a* content compared to MRGL2 and *psoR*::IS701 under white light (Figure 3.4a). MRGL2 contained higher levels of carotenoids compared to WT and *psoR*::IS701 under RL and GL, whereas it was significantly higher than WT under white light (Figure 3.4b). The *psoR*::IS701 mutant had carotenoid levels similar to WT under RL and GL but contained significantly more than WT under white light (Figure 3.4b).

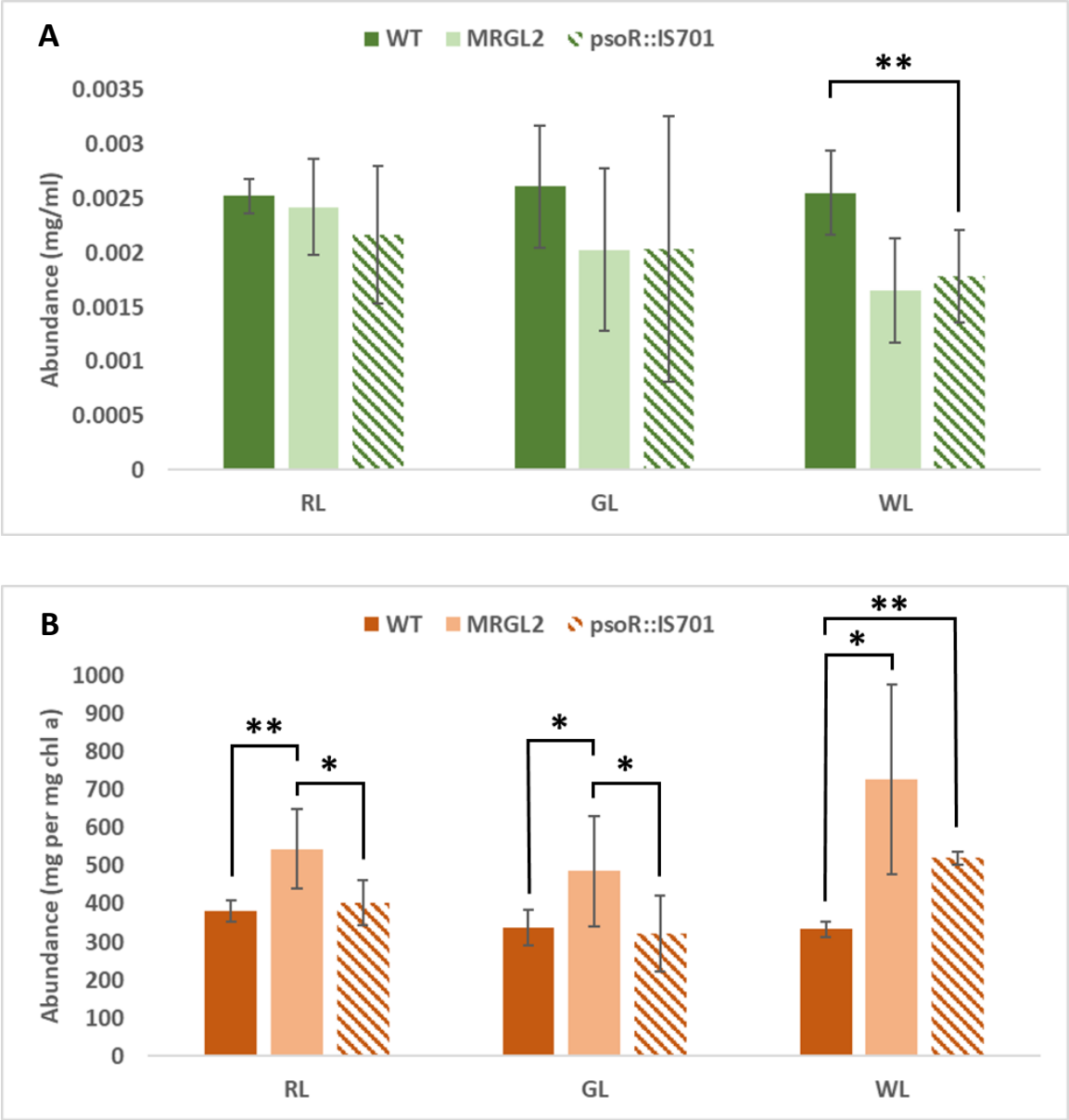


Figure 3.4. Quantification and pairwise comparison using post-hoc Tukey test of a) chlorophyll *a* and b) carotenoids under red, green, and white light. Error bars represent standard deviations between 4-6 biological replicates per strain. Statistical significance is marked by * and ** for $p<0.05$ and $p<0.01$, respectively.

As for phycobiliproteins, WT and MRGL2 had similar levels of PE, PC, and AP under all light conditions. In contrast, *psoR*::IS701 contained significantly more PE than WT under RL, but significantly less under GL and white light (Figure 3.5A). MRGL2 was not significantly different from WT or *psoR*::IS701 under red and green light. Under white light, the *psoR*::IS701 mutant contained approx. 4 times less PE than both WT

and MRGL2. PC abundance for *psoR*::IS701 was greater than WT and MRGL2 under both RL and GL conditions, while they were statistically similar under white light (Figure 3.5B). Lastly, AP levels were higher for the *psoR*::IS701 mutant under RL compared to the other two strains, although potential errors in isolating the PBPs caused a large variance in the AP quantities measured (Figure 3.5C).

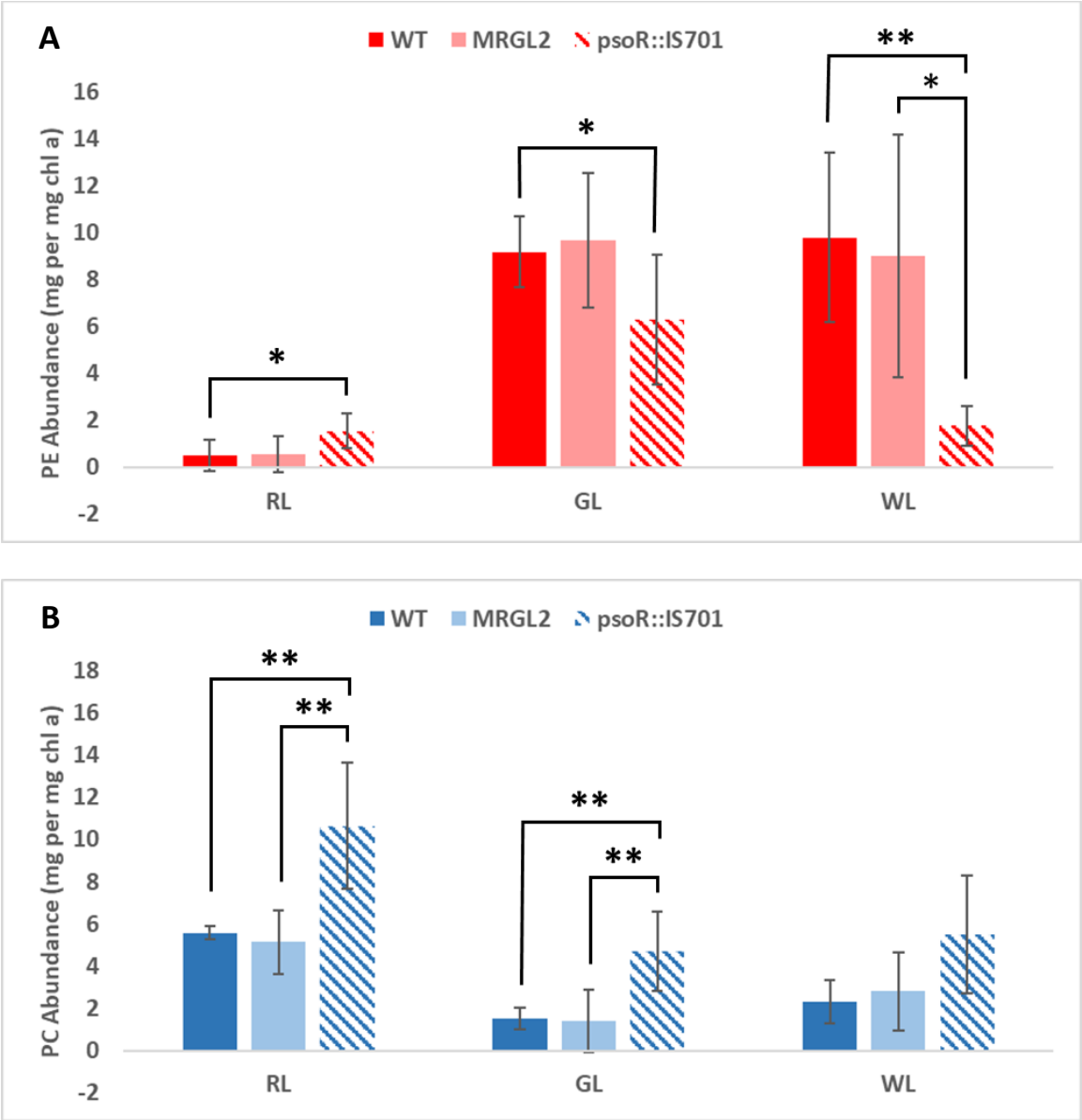
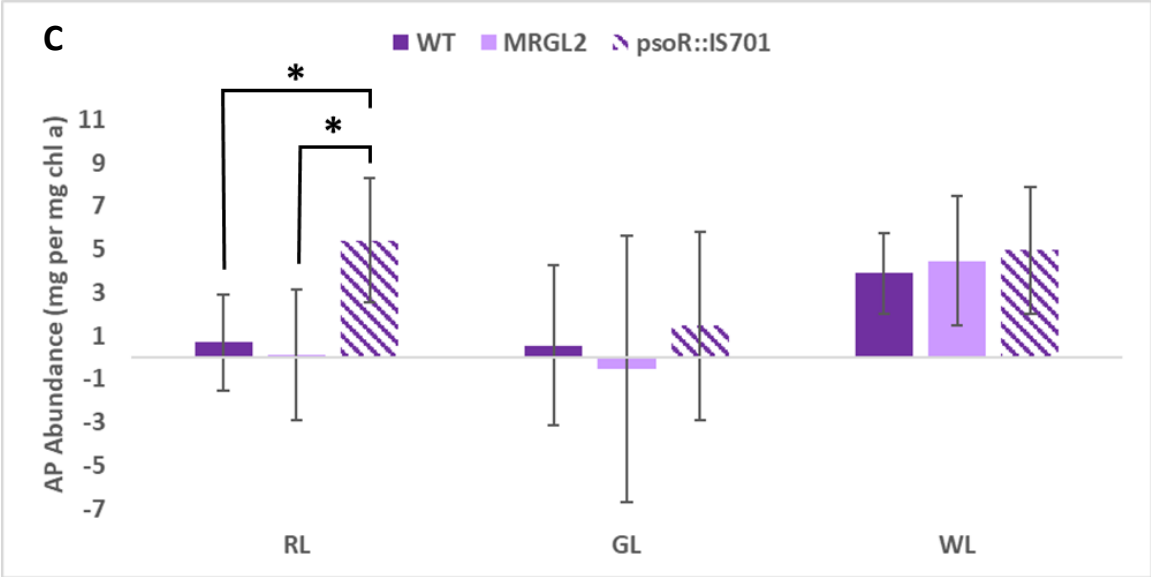


Figure 3.5. Quantification and pairwise comparison using post-hoc Tukey test of a) PE, b) PC, and c) AP PBPs under red, green, and white light. Error bars represent standard deviations between 4-6 biological replicates per strain. Statistical significance is marked by * and ** for $p<0.05$ and $p<0.01$, respectively.

Figure 3.5. (cont'd)



The PBP ratios such as PC/AP and PE/AP can predict the length of the PBS rods compared to the PBS core (Table 3.1). As expected for WT, the PC/AP ratio was highest under RL and the PE/AP ratio was highest under GL. MRGL2 had confounding ratios, likely due to the AP quantification error. The *psoR*::IS701 mutant had a similar pattern for its PBP ratios as WT, with PC/AP ratios higher under RL and PE/AP ratios higher under GL.

Table 3.1. PBP ratios of WT, MRGL2, and *psoR*::IS701 under red, green, and white light.

Strain	Light Condition	PC/PE	PC/AP	PE/AP
WT	RL	11.26	7.95	0.71
	GL	0.16	2.72	16.57
	WL	0.23	0.59	2.52
MRGL2	RL	9.21	35.21	3.82
	GL	0.14	-2.56	-18.00
	WL	0.31	0.63	2.01
***psoR*::IS701**	RL	6.96	1.97	0.28
	GL	0.75	3.21	4.29
	WL	3.15	1.11	0.35

3.4 Discussion

Pigmentation in photosynthetic organisms affects the type and amount of light cells absorb for photosynthesis, and cyanobacteria have evolved mechanisms to tweak their pigmentation to maximize production and minimize damage caused by excessive light. One acclimation mechanism, CCA, responds primarily to red and green wavelengths of light and changes the levels of phycobiliprotein pigments PC and PE according to the external light environment. A blue-green color mutant of *F. diplosiphon* found in a routine mutant color screening was found to have an IS701 transposon inserted in the *psoR* gene, a gene found in most cyanobacterial species and reportedly a negative regulator of PBS abundance (Layer & Montgomery, 2022; Cobley et al., 2008). This *psoR*::IS701 mutant does not change its color when switched between red and green light conditions, which led to the investigation of its growth rate and pigment accumulation in this work.

The *psoR*::IS701 mutant has altered levels of PBP compared to its parent strain MRGL2 and to WT. The higher accumulation of PC in the *psoR*::IS701 strain explains the higher peak at 625 nm in the whole cell spectral scan, as PC has its maximum absorption rate at 625 nm. PE accumulation is lower in the *psoR*::IS701 mutant, but is not completely wiped out, so along with PC and AP, the *psoR* gene appears to alter the accumulation of PBPs in *F. diplosiphon*. Whether the change in pigmentation is due to changes in the production or degradation rates of the PBPs is yet to be fully determined.

Chapter 2 showed that the *psoR* gene is found in every clade of Cyanobacteria with the exception of *Gloeobacter*, one of the earliest groups of cyanobacteria, which lacks thylakoids and has atypical PBSs (Layer & Montgomery, 2022). PsoR was also found to be homologous to β-CASP proteins, which belong to the metallo-β-lactamase superfamily of proteins involved in processing numerous substrates. β-CASP proteins have endo-and exonuclease activity, with seven conserved motifs involved in the active site, all of which present in PsoR. Given its putative function as nucleic acid-processing enzyme and the physical characteristics of the *psoR*::IS701 mutant, PsoR may play a role in the transcription of CCA genes.

With the important role of light in photosynthesis, cyanobacteria have evolved mechanisms such as CCA to maximize light absorption for production as well as

minimize excess light absorption that can cause damage through generation of reactive oxygen species. CCA uses a network of genes to perceive and respond to light availability, and our understanding of it grows as we investigate color mutants such as *psoR*::IS701. *F. diplosiphon* has 305 two-component system proteins and 27 different phytochromes predicted within its 10,000 coding sequences, so there is still much to learn about how cyanobacteria, and *F. diplosiphon* in particular, respond to light (Haney & Kehoe, 2019). Since PsoR is a putative negative regulator of PBS abundance and a potential β-CASP enzyme, future work should include investigating what substrates PsoR acts on and where it fits within the CCA network.

3.5 Materials and Methods

3.5.1 Strains & Culture Conditions

F. diplosiphon SF33, a shortened-filament strain that displays wild-type (WT) pigmentation under RL and GL, was used as WT (Cobley et al., 1993). One mutant strain, MRGL2 was used and is described in this study. Strains were grown in BG-11 medium buffered with 20 mM HEPES at pH 8.0 (hereafter BG-11/HEPES) at 28°C. Liquid cultures were grown with shaking at 175 rpm. Plated cultures were maintained on BG-11/HEPES solidified with 1.5% (w/v) agar.

3.5.2 Growth Curves and Spectral Scans

Starter cultures grown under white light (WL) at 10^{-15} µmol m^{-2} s^{-1} in exponential phase were diluted to an OD_{750} of ~0.1 and transferred to indicated light conditions to start growth experiments. Cell density was measured by determining optical density at 750 nm (OD_{750}) at two- or three-day intervals across 14 days. At least three biological replicates of each strain were grown under RL or GL conditions at 10-15 µmol m^{-2} s^{-1}. For WL growth experiments, the strains were grown in the MC 1000-OD Multi-cultivator from PSI (Photon Systems Instruments) at 30 µmol m^{-2} s^{-1}.

Whole-cell spectral scans were obtained using a SpectraMax M2 microplate reader (Molecular Devices). Cells were adjusted to an OD_{800} of ~0.1 and measurements taken between 350 nm an 800 nm.

3.5.3 Phycobiliprotein Harvesting and Quantification

Phycobiliproteins, carotenoids, and chlorophyll *a* were harvested and quantified as previously described (Bordowitz and Montgomery, 2008; Singh & Montgomery, 2011).

3.5.4 Statistical Analysis

Statistical significance between the levels of chlorophyll *a*, carotenoids, and PBPs between strains was determined using single-factor ANOVA and the Post-hoc Tukey test (https://astatsa.com/OneWay_Anova_with_TukeyHSD/). Analyses were performed with 95% and 99% confidence intervals ($p < 0.05$ and $p < 0.01$, respectively).

CHAPTER FOUR

Conclusion

The work presented in this thesis focused on characterizing the pigmentation phenotype of a *psoR*::IS701 transposon mutant in *Fremyella diplosiphon*, and to determine if PsoR was present within all PBS-containing organisms or conserved within the Cyanobacteria phylum. Previous work on *psoR* revealed it to be a putative negative regulator of phycobilisomes (PBSs), with a mutated *psoR* causing an increase in PBSs and a decrease in phycoerythrin (PE) levels under green light (Cobley et al., 2008). Through bioinformatics, we discovered that *psoR* homologs are found throughout the Cyanobacteria phylum, but not within red algae, which also contain PBS, indicating that this gene evolved in cyanobacteria, has been conserved, and likely plays an important role in chromatic acclimation (CA) in cyanobacteria (Layer & Montgomery, 2022).

A blue-green mutant found in our lab during a routine color screen of *F. diplosiphon* was found to have a IS701 transposon inserted in the *psoR* (Phycobilisome abundance regulator) gene (Figure 3.1A & 3.1B). The *psoR*::IS701 mutant exhibited altered levels of PBPs compared to the WT strain and to its parent strain, MRGL2, indicating the involvement of PsoR in the regulation of PBP accumulation and/or PBS synthesis (Figure 3.5 & Table 3.1). The *psoR*::IS701 mutant displayed higher accumulation of phycocyanin (PC) and lower levels of phycoerythrin (PE) compared to WT and the parent strain, suggesting that PsoR influences the production or degradation of PBPs. The spectral scans of the *psoR*::IS701 mutant further supported these findings, showing a distinct peak at 625 nm due to the higher PC levels (Figure 3.3).

The *psoR* gene is widespread across the cyanobacterial kingdom, with homologs found in all clades except for *Gloeobacter* (Figure 2.4), the oldest clade of cyanobacteria, which lacks thylakoid membranes and has atypical PBS structures. The putative function of PsoR as a β-CASP protein suggests its involvement in nucleic acid processing, potentially regulating gene expression related to PBS abundance and CA regulation (Table 2.1, Figures 2.1 & 2.2). The protein-protein interaction predictions of three PsoR homologs point to PsoR being involved with various DNA and RNA metabolism enzymes, further suggesting that PsoR plays a role in nucleic acid processing (Tables 2.4, 2.5, & 2.6). The presence of PsoR homologs across all but the oldest of cyanobacteria clades, along with its putative function as a nucleic acid

processing enzyme, suggests that it has a highly conserved role in regulating PBS abundance and may play a broader role in cyanobacterial gene regulation.

Understanding the precise mechanisms by which PsoR functions and its interactions with other proteins involved in CCA and metabolism can provide insights into the broader network of gene regulation in response to light cues. Future work on this gene should include generating a complete knockout (*ΔpsoR)* of the *psoR* gene in *F. diplosiphon* and other cyanobacteria, particularly ones with different types of CA to see if *psoR* functions in a similar manner in different species. RNA sequencing of a *ΔpsoR* mutant compared to WT under red and green light conditions could uncover a plethora of genes affected by PsoR. Furthermore, investigating the substrates that PsoR acts upon, in particular determining if it acts on DNA and/or RNA, could further expand our understanding of its position within the CCA network and would contribute to a comprehensive understanding of how cyanobacteria fine tune their photosynthetic machinery in dynamic light environments.

In conclusion, the research presented here highlights the significance of investigating the roles of PsoR and the broader CCA network in cyanobacteria. This work deepens our understanding of how photosynthetic organisms, such as cyanobacteria, adapt to varying light environments, and provides a foundation for future studies aiming to uncover the intricacies of light perception and response in these organisms. Insight into the regulation of PBS structures and abundance is not only fundamental to basic research but also has practical implications. Cyanobacteria have potential applications in various industries, including biofuel and biopharmaceutical production, and as beneficial food additives and biofertilizers. Enhancing our understanding of how cyanobacteria respond to fluctuating light conditions can aid in optimizing their performance in these applications. Ultimately, this knowledge may contribute to the development of more efficient and sustainable applications of cyanobacteria in biotechnology and environmental engineering.

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