THE ROLE OF THE SECOND MESSENGER CYCLIC DI-GMP IN LIGHT-DEPENDENT RESPONSES IN CYANOBACTERIA

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ABSTRACT

THE ROLE OF THE SECOND MESSENGER CYCLIC DI-GMP IN LIGHT-DEPENDENT RESPONSES IN CYANOBACTERIA

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Second messengers are intracellular substances regulated by specific external stimuli globally known as first messengers. Cells rely on second messengers to generate rapid responses to environmental changes and the importance of their roles is becoming increasingly realized in cellular signaling research. Cyanobacteria are photooxygenic bacteria that inhabit most of Earth's environments. The ability of cyanobacteria to survive in ecologically diverse habitats is due to their capacity to adapt and respond to environmental changes. Chapter 1 will review known second messenger-controlled physiological processes in cyanobacteria. Second messengers used in these systems include the element calcium (Ca^{2+}), the nucleotide-based guanosine tetraphosphate or pentaphosphate (ppGpp or pppGpp, represented as (p)ppGpp), the cyclic adenosine 3′,5′-monophosphate (cAMP), the c-di-GMP, the cyclic guanosine 3′,5′-monophosphate (cGMP), and the cyclic dimeric AMP (c-di-AMP), and the gaseous nitric oxide (NO). The discussion will focus on processes central to cyanobacteria such as nitrogen fixation, light perception, photosynthesis-related processes, and gliding motility. Chapter 2 will confirm that many putative c-di-GMP synthesis or degradation domains are found in genes that also harbor light-responsive signal input domains, suggesting that light is an important signal for altering c-di-GMP homeostasis. I will also compare intracellular levels of c-di-GMP in *Synechocystis* sp. PCC 6803 and *Fremyella diplosiphon* under different light qualities, confirming that light is an important factor for regulating this second messenger *in vivo*. Chapter 3 will demonstrate that biofilm formation, cellular aggregation or flocculation, and cellular

buoyancy are under the control of c-di-GMP in *Synechocystis* sp. PCC 6803 and *F. diplosiphon*. In this chapter, I present data on *Synechocystis* and *F. diplosiphon* transformed with a plasmid for constitutive expression of genes encoding diguanylate cylase (DGC) and phosphodiesterase (PDE) proteins from *Vibrio cholerae* and *Escherichia coli*, respectively. Engineering of these strains allowed modulation of intracellular c-di-GMP levels. Chapter 4 will confirm the role of c-di-GMP in *F. diplosiphon* in regulating pigments. I show that c-di-GMP is involved in light signalling networks and correctly tunes the pigments of cells under changes to ambient light. In chapter 5, I conclude by addressing future research trajectories needed to better understand the signaling networks and cross talk in the signaling pathways of these molecules in cyanobacteria. Second messengers have significant potential to be adapted as technological tools and possible novel and practical applications are highlighted based on current understanding of these molecules and the signaling networks that they control.

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CHAPTER ONE

Survival Strategies in the Aquatic and Terrestrial World: The Impact of Second Messengers on Cyanobacterial Processes

This chapter contains previously published work:

Agostoni M and Montgomery BL (2014). Survival strategies in the aquatic and terrestrial world: The impact of second messengers on cyanobacterial processes. Life (Basel) 4(4):745-769

1.1 INTRODUCTION

Microorganisms need to cope with variations of the external environment and rely on signaling molecules to translate these changes into intracellular responses and to adapt to the new condition. After cells sense an external stimulus (i.e., first messenger), a second messenger will be synthesized or degraded to rapidly amplify the first messenger signal and initiate physiological changes. Proteins involved in the synthesis and degradation of second messengers are generally constitutively present in the cell to support rapid activation. First messenger-induced fluctuations of second messengers are propagated in cells through binding to DNA, RNA, or proteins/protein complexes. The ligand-effector complex will then trigger a signal cascade involving specific receptors, outputs and feedback processes. This signal cascade is common for all known second messengers (Fig. 1.1).

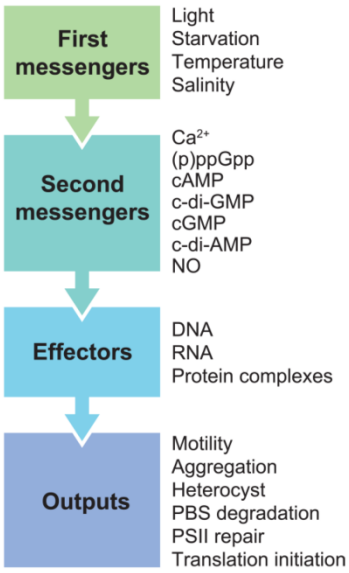

Figure 1.1. Second messengers in cyanobacteria. Specific second messengers are regulated by an external stimulus and will bind specific effectors. In turn, the effector will initiate a signal cascade, which leads to organism-specific outputs (examples shown for cyanobacteria). This mechanism is common for all known second messengers.

There is a rich variation of second messengers in prokaryotic organisms, from cyclic nucleotides to gases. In cyanobacteria, the most intensely studied second messengers are calcium (Ca^{2+}), guanosine tetraphosphate or pentaphosphate (ppGpp or pppGpp; hereafter (p)ppGpp), and cyclic adenosine 3′,5′-monophosphate (cAMP). Lesser studied second messengers in cyanobacteria are cyclic dimeric GMP (c-di-GMP), cyclic guanosine 3′,5′-monophosphate (cGMP) and nitric oxide (NO). Finally, cyclic dimeric AMP (c-di-AMP) remains to be characterized in cyanobacteria. All of these second messengers are commonly studied in pathogenic bacteria. Calcium has been considered for its ability to influence cell structure and differentiation, motility, and gene expression in pathogenic bacteria (1). The molecule (p)ppGpp is mainly studied in these bacteria for is involvement under cell starvation stress (2). Cyclic AMP has been implicated in sugar metabolism (3), motility (4) and virulence (5) in pathogenic bacterial systems. Cyclic di-GMP impacts several processes in bacterial cells including transcription, RNA turnover, biofilm formation, protein synthesis, motility, and virulence (6). NO serves as an important factor in host-pathogen interactions (7). Finally, the most recently discovered nucleotide second messengers are c-di-AMP, which impacts ion transport, membrane lipid homeostasis, DNA integrity (8), and cGMP, which has been implicated in the control of cyst formation (9).

Cyanobacteria are an ancient and distinct group of gram-negative photoautotrophic bacteria for which there have been limited investigations into the roles of most of these second messengers. Cyanobacteria are one of the most abundant photoautotrophic organisms in oceans (10) and are able to fix both carbon and nitrogen, thereby playing key roles in global carbon and nitrogen cycles. Elucidating the processes controlling cyanobacterial adaption to aquatic habitats is critical for understanding their roles as primary producers controlling global carbon and nitrogen cycles. Cyanobacteria exhibit extraordinary diversity in terms of genome plasticity,

morphological characteristics, ecological niches, and physiological properties (11-13). These organisms rely on complex signal transduction systems, which reflect their ecophysiologies and abilities to colonize a wide range of habitats. Cyanobacteria possess a much larger repertoire of two-component proteins compared to other bacteria (14), and they rely heavily on cyclic nucleotide signaling proteins (15-17).

In recent years there has been a growing interest in utilizing cyanobacteria as systems for the production of valuable bioindustrial compounds from sugars to biofuels (18, 19). A number of natural physiological processes of cyanobacterial systems could be regulated to improve their use as bioproduction platforms. Sugar metabolism, motility, and biofilm production are just some examples of physiological processes under the control of second messengers. Regulatable control of these processes could lead to improvements in the efficiency of growing photosynthetic bacteria in partially or fully enclosed photobioreactors.

This chapter is designed to highlight the major advances in knowledge about the second messengers Ca^{2+}, (p)ppGpp, cAMP, c-di-GMP, cGMP, c-di-AMP, and nitric oxide and their roles in cyanobacteria. Compared to other bacteria, the major contribution of these second messengers in cyanobacteria is to regulating key processes such as nitrogen fixation, the perception of a variety of light qualities, photosynthesis-related processes, and gliding motility. This chapter aims to emphasize continuing areas of needed investigation for these signaling molecules and to address useful applications of knowledge about the signaling pathways to practical biotechnological interventions.

1.2 SECOND MESSENGERS IN CYANOBACTERIA

Studies on second messengers in cyanobacteria started ~40 years ago and have demonstrated that these molecules can influence several physiological processes. Genetic studies on the functional roles of second messengers have highlighted the involvement of these molecules in controlling physiological processes and biochemical studies have described complex interactions between second messengers with DNA, RNA, proteins, and protein complexes.

1.2.1 Calcium, Ca^{2+}

One of the most intensely studied second messengers in cyanobacteria is the ion/element Ca^{2+}. A role for calcium as a second messenger in stimulus–response coupling has been correlated frequently with a variety of environmental stresses such as heat and cold (20), oxygen stress (21), and osmotic stress (22). It can impact a number of physiological responses, including motility, nitrogen fixation, and responses to stress (1). Calcium must be tightly regulated to create a concentration gradient utilized by the cells to transfer information to downstream processes. Internal calcium levels can be increased by an influx of Ca^{2+} present in the external medium, or by releasing intracellular stores of bound Ca^{2+} from Ca^{2+}-binding proteins (Fig. 1.2).

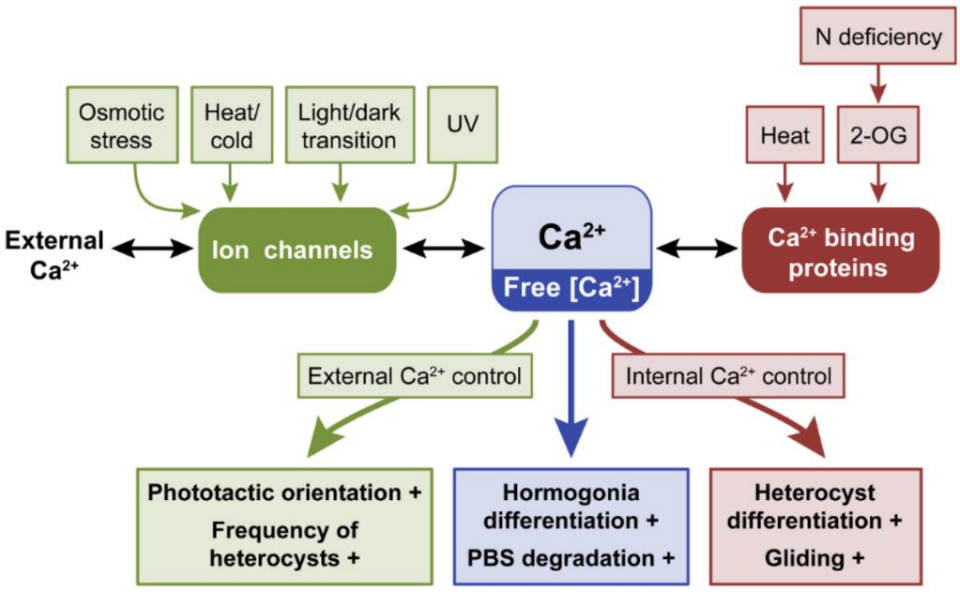

Figure 1.2. External signals controlling free intracellular Ca2+ levels and phenotypes or processes that are controlled by Ca^{2+} in cyanobacteria. Levels of free intracellular Ca^{2+} are regulated externally by influx(es) of extracellular Ca^{2+} through ion channels or internally by release of Ca^{2+} from Ca^{2+}-binding proteins. Green arrow from free [Ca^{2+}] indicates increased Ca^{2+} levels from external sources lead to noted phenotypes, whereas red arrow indicates that increased Ca^{2+} levels from internal sources lead to the noted phenotypes. Purple arrow indicates that referenced studies did not demonstrated whether the noted phenotypes are under external or internal control. +, indicates process promoted by increased cellular Ca^{2+} levels; -, indicates process inhibited by increased intracellular Ca^{2+} levels; no symbol, a positive or negative impact of increased intracellular Ca^{2+} levels was not indicated.

1.2.2. Calcium Controls Motility

Calcium is involved in behavioral responses in prokaryotes, including directional motility in cyanobacteria (1, 23-25). The first reported study to investigate the effects of Ca^{2+} as a second messenger in cyanobacteria showed that gliding was attributed to an increase in cellular Ca^{2+} concentration (26). A study of hormogonia, which are motile filaments of cells, confirmed that Ca^{2+} was critical to promote the differentiation of hormogonia and sustain motility (27). In

Synechocystis sp. PCC 6803 (hereafter *Synechocystis*), depletion of free Ca^{2+} resulted in diminished photoorientation and gliding speed (28). Based on Ca^{2+} chelation and calcium ionophore studies, the authors concluded that phototactic orientation was likely caused by the uptake of extracellular Ca^{2+}, whereas gliding motility was dependent on internal Ca^{2+} mobilization or release from Ca^{2+}-binding proteins (28). Using genetic and biochemical approaches, proteins containing Ca^{2+}-binding domains were discovered (29-31). A key step to understand gliding motility in cyanobacteria was to isolate surface fibrils. Fibrils in cyanobacteria are composed of a single type of protein called oscillin, which contains multiple Ca^{2+}-binding motifs (29). *P. uncinatum* filaments that did not accumulate oscillin were not able to glide (29). Motility in *Synechococcus* sp. WH8102 also depended on the protein SwmA (a homolog of oscillin). A mutant lacking *swmA* could still rotate but no longer exhibited swimming motility (30). Calcium was also shown to promote swimming in *Synechococcus* strain WH8113 (31).

1.2.3. The Role Of Ca^{2+} In Heterocyst Differentiation

Calcium is required for the activity of the nitrogen-fixation enzyme nitrogenase *in vivo* and is purported to have a role in protecting nitrogenase from oxygen-dependent inactivation (32-35). Moreover, the frequency of heterocysts found in a filament varies with different Ca^{2+} concentrations in the growth medium, which indicates a Ca^{2+}-regulated mechanism for determining heterocyst abundance and placement (35). These early experiments provided evidence that Ca^{2+} was involved in nitrogen fixation.

The Ca^{2+}-binding photoprotein aequorin can be used to measure intracellular levels of Ca^{2+} (36). Determining the levels of free cytosolic Ca^{2+} is fundamental for establishing the role of Ca^{2+} as second messenger. Expression of an exogenous aequorin in *Anabaena* sp. PCC7120 (hereafter *Anabaena*) resulted in the detection of a distinct Ca^{2+} transient after nitrogen deprivation (37). Alteration of the amplitude or duration of the Ca^{2+} transient using pharmacological treatments arrested heterocyst differentiation at an early stage (37). Thus, proper regulation of the timing and amplitude of the transient promotes heterocyst differentiation (37). Notably, an increase of Ca^{2+} in the cell after nitrogen deficiency originated from an intracellular source of Ca^{2+} (37).

Cyanobacteria can regulate Ca^{2+} homeostasis by using mechanosensitive ion channels and through protein-Ca^{2+} complexes that may serve in Ca^{2+} storage (Fig. 1.2). The gene *hetR* in *Anabaena* encodes a calcium-stimulated protease essential for vegetative cells to differentiate into heterocysts (38). In vegetative cells, intracellular free Ca^{2+} levels are ten times lower than in mature heterocysts (39). Overexpression of *ccbP*, which encodes a Ca^{2+}-binding protein that is localized in vegetative cells, suppressed heterocyst formation, whereas a *ccbP* mutant exhibited multiple contiguous heterocysts (39). Thus, accumulation of CcbP in vegetative cells may contribute to sequestration of Ca^{2+} in these cells and its absence in heterocysts likely allows the accumulation of Ca^{2+} and associated induction of *hetR* and heterocysts differentiation (39).

Nitrogen deficiency is also an important signal mediated by Ca^{2+} in the unicellular, non-diazotrophic cyanobacterium *Synechococcus elongatus* PCC 7942 (40). Cells under nitrogen deficiency produce 2-oxoglutarate (2-OG), an important biological compound involved in the carbon-nitrogen status signal. The anion 2-OG can trigger Ca^{2+} accumulation transiently in *S. elongatus* as an increase in 2-OG occurs in cells before observed changes in intracellular Ca^{2+} levels (40). In response to nitrogen starvation, *S. elongatus* can degrade phycobilisomes to

recycle nitrogen-rich amino acids through activating two transcriptional regulators, NtcA and NblR (41, 42). Under nitrogen deficiency, an increased level of intracellular Ca^{2+} can induce NtcA (a member of the cAMP-receptor transcriptional regulator protein family) which in turn regulates the expression of genes involved in the process of phycobiliprotein degradation (40).

1.2.4. Responses To Temperature Stress And Other Stresses Are Mediated By Ca^{2+}

Calcium is also an important second messenger for cells to regulate responses to stresses such as temperature shock, osmotic stress, and light-to-dark transitions (20, 22, 43). The mechanosensitive ion channel protein MscL that is found in the plasma membrane of *Synechocystis* is involved in Ca^{2+} homeostasis regulation as mutant analyses demonstrated that this protein regulates an influx of Ca^{2+} (44). In *Anabaena*, heat shock at 44 °C resulted in an induction of intracellular Ca^{2+} levels that had a higher amplitude when Ca^{2+} was present in the external medium; similarly, a cold shock at 10 °C induced an increased magnitude of intracellular Ca^{2+} accumulation in cells only at higher external Ca^{2+} levels (20). The use of inhibitors or pharmacological agents indicated that the source of Ca^{2+} in the heat shock-induced elevation of cellular Ca^{2+} levels is both from Ca^{2+} in the external medium and internal stores, whereas the cold-shock induced elevation of Ca^{2+} levels results primarily from the external medium (20).

Calcium is also induced by other stresses, including salinity, osmotic stress, light-to-dark transitions, and UV irradiation (22, 43, 45). Intracellular levels of Ca^{2+} increased when *Anabaena* was exposed to salt or osmotic stresses. The source of increased intracellular Ca^{2+} levels was external as inhibiting calcium channels or the use of Ca^{2+}-free medium eliminated this response

(22). Detecting changes in external light availability are critical for photosynthetic organisms such as cyanobacteria. An increase of internal Ca^{2+} levels was observed in heterocysts in *Anabaena* when cells were exposed to UV irradiation (45). A Ca^{2+} transient also occurred during a light-to-dark transition in *Anabaena* (43). The observed elevation of intracellular Ca^{2+} levels was not associated with a specific photoreceptor, but more likely occurs in response to changes in the redox state of components of the photosynthetic electron transport chain (43). Biochemical assays indicated that the source of the Ca^{2+} was external during the response to UV irradiation and light-to-dark transitions (43, 45). Together, the rapid Ca^{2+} transients that occur in cyanobacteria under temperature, osmotic, nitrogen and light stresses provide evidence that Ca^{2+} signaling is involved in early responses to these environmental stimuli.

1.2.5 Guanosine-3', 5'-(bis) pyrophosphate, (p)ppGpp

Guanosine-3', 5'-(bis) pyrophosphate, (p)ppGpp, was the first second messenger characterized in cyanobacteria 40 years ago. This molecule is involved in the stringent response, during which alterations in metabolism and gene expression occur due to limitation in the availability of amino acids or nutrition stress (46). During the stringent response, resources are diverted away from growth towards amino acid synthesis to support survival until unfavorable conditions improve. During this process, ppGpp or pppGpp is synthesized from ATP and GTP/GDP by a (p)ppGpp synthetase, RelA (47), and can be degraded to GTP and pyrophosphate by a (p)ppGpp hydrolase, SpoT (47) (Fig. 1.3). (p)ppGpp inhibits translation initiation to limit excessive protein synthesis during nutritional deficiencies (46). Recently, more details of the regulation of (p)ppGpp and downstream processes emerged. In *E. coli* (p)ppGpp binds the β' subunit of RNA polymerase

and decreases the half-life of rRNA, resulting in decreased transcription (48). (p)ppGpp also binds the β' subunit of the plastid RNA polymerase in chloroplasts of plants(49), suggesting that (p)ppGpp likely binds the β' subunit of the RNA polymerase in cyanobacteria, which are widely recognized as the progenitor of plastids (50).

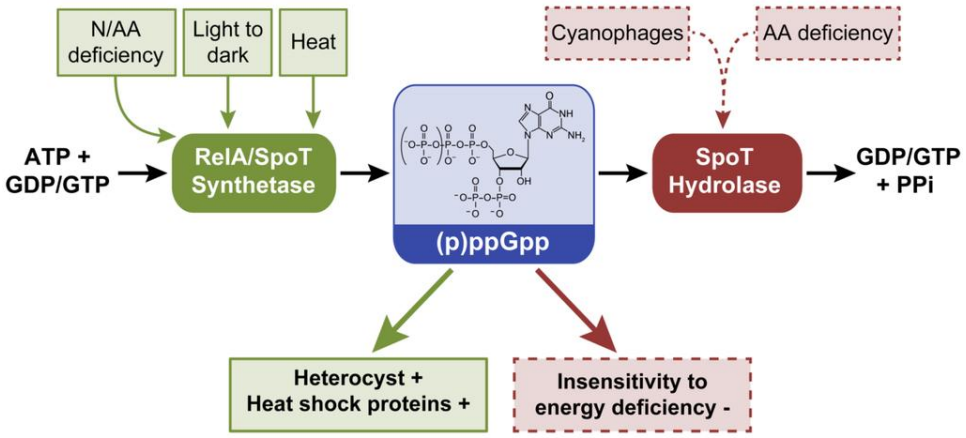

Figure 1.3. External factors controlling intracellular levels of (p)ppGpp and phenotypes or processes that are controlled by (p)ppGpp in cyanobacteria. (p)ppGpp is synthesized from GDP or GTP by RelA or SpoT proteins and degraded to GDP or GTP by SpoT. Dash lines denote hypothetical or suggested role of cyanophages in controlling internal (p)ppGpp levels. Green arrow indicates that increased synthesis of (p)ppGpp leads to noted phenotypes, whereas red arrow indicates that degradation of (p)ppGpp leads to noted phenotypes. +, indicates process promoted by increased (p)ppGpp synthesis (green) or increased (p)ppGpp degradation (red).

The (p)ppGpp homeostasis enzymes RelA/SpoT (PF04607) are present as bifunctional gene homologs, sometimes referred to as RSH (Rel Spo homologs) genes (46), in each of the 83 finished genomes present in the Integrated Microbial Genomes (IMG) database (51) suggesting a widespread utilization of (p)ppGpp as a signaling molecule in cyanobacteria (Table 1). *Cyanobium gracile* PCC 6307, *Cyanothece* sp. BH68 ATCC 51142, and *Nodularia spumigena* CCY9414 possess two copies of the RSH genes annotated as RelA/SpoT (Supplemental material http://www.mdpi.com/2075-1729/4/4/745#stats).

Anabaena possesses a single *relA*/*spoT* homolog (*all1549* or Ana-RSH) that regulates intracellular levels of (p)ppGpp in response to amino acid deprivation (52). Ana-RSH was determined to be essential as it could not be deleted from the genome (52). Ana-RSH is maintained at a basal level in *Anabaena* under non-stressful conditions and appears to be regulated at the enzymatic rather than at a transcriptional level under amino acid deprivation (52). As noted above, nitrogen deprivation leads to transient increases in (p)ppGpp levels in some cyanobacteria (53, 54); thus, the role of Ana-RSH during nitrogen deprivation and heterocysts development was explored. Although Ana-RSH was enriched in vegetative cells relative to heterocysts, the regulation of Ana-RSH or (p)ppGpp were not correlated with heterocyst formation and nitrogen fixation (52). A more recent study, however, reported up-regulation of Ana-RSH under nitrogen deprivation and confirmed enrichment in vegetative cells (55). These authors also were not able to delete Ana-RSH from the genome, but isolated a non-functional insertion mutant of Ana-RSH that exhibited reduced (p)ppGpp levels in response to amino acid starvation and failed to form heterocysts in nitrogen-limited conditions (55).

Table 1.1. Total number of genomes with specific pfam. Present in the finished genomes (n=83) represented in the IMG database[a].

Second messenger	Pfam	Function	Number of genomes
(p)ppGpp	04607	(p)ppGpp synthesis and degradation	83
cAMP	00211	Adenylate and guanylate cyclase	65
c-di-GMP	00990	Diguanylate cyclase	61
	00563	Diguanylate phosphodiesterase	60
Nitric oxide	00394	Nitrite reductase	27
	07731	Nitrite reductase	49
	07732	Nitrite reductase	47
	13442	Nitrite reductase	80
	00115	Nitric oxide reductase	83
c-di-AMP	02457	Diadenylyl cyclase	83

[a] IMG, Integrated Microbial Genomes database(51).

1.2.6. The Role of (p)ppGpp In Cyanobacterial Cells

The sources of carbon and energy are usually one and the same in heterotrophic organisms; however, in phototrophic organisms the source of energy, i.e., light, differs from the source of carbon. Initial studies of (p)ppGpp in phototrophic organisms compared the effects of variations in light to nutrient starvation. The cyanobacterium *Anacystis nidulans* responds to a reduction of ambient light, which is equivalent to an energy reduction, with reduced growth and an associated decrease in RNA synthesis and a transient increase in GTP levels. The levels of GTP decreased as (p)ppGpp began to accumulate (56). Notably, amino acid deprivation induced similar responses in *A. nidulans* (57). Nitrogen deprivation also transiently increased intracellular levels of (p)ppGpp in *Anabaena cylindrical* (53) and in *A. nidulans* (54). Under nitrogen starvation in *A. nidulans*, the regulation of (p)ppGpp is primarily due to (p)ppGpp synthesis rather than decreased degradation of (p)ppGpp (54). In the cyanobacterium *Synechococcus* sp. PCC 6301, light-to-dark shifts and temperature stress led to accumulation of (p)ppGpp with an associated accumulation of heat shock proteins (58). The effects of these two environmental factors could be separated, as ppGpp levels also increased when this species was exposed to 47 °C in the dark (58). Thus, although the source of energy and carbon differ in cyanobacteria, a limitation of either results in a role of (p)ppGpp in transducing the environmental change.

1.2.7. Cyanophage And (p)ppGpp

A fascinating aspect of (p)ppGpp regulation in cyanobacteria was observed when *A. nidulans* cells were infected with the AS-1 cyanophage (a cyanomyovirus). Cyanophage-infected *A.*

nidulans cells failed to exhibit significantly increased intracellular (p)ppGpp levels under amino acid or energy deficiency as did uninfected cells, suggesting that infected cells did not perceive starvation (59). One way for a cyanomyovirus to maintain low levels of (p)ppGpp is to express the protein MazG in the host; MazG is a protein found in all cyanomyovirus isolates (60). In *E. coli*, MazG can hydrolyse (p)ppGpp (61), and if this is also true for cyanobacteria, MazG could allow the cyanomyoviruses to induce host cells to maintain basal levels of (p)ppGpp, which would support a normal cellular growth rate, where the physiological state is expected to be optimal for production of progeny phage (62).

1.2.8. Cyclic adenosine 3′,5′-monophosphate, cAMP

Cyclic AMP is synthesized by adenylate cyclase (AC) (class III nucleotidyl cyclases family, Pfam: Pfam00211) proteins using ATP as a substrate and hydrolyzed to AMP by cAMP-specific phosphodiesterases (PDE) (Fig. 1.4). Cyclic AMP is a widespread molecule in cyanobacteria. In an analysis of cyanobacterial genomes, only the picocyanobacteria *Prochlorococcus* and *Synechococcus* were reported to lack cAMP receptors, which can bind cAMP and serve as transcriptional regulators that impact diverse responses(17). Species lacking cAMP receptors likely lost them during the course of evolution to adapt to new environments (17). In an assessment of 83 finished cyanobacterial genomes present in the IMG database, AC genes homologous to the class III AC family (Pfam00211) were also found to be widespread, expect in *Prochlorococcus* and *Synechococcus* (Table 1 and Supplement material http://www.mdpi.com/2075-1729/4/4/745#stats).

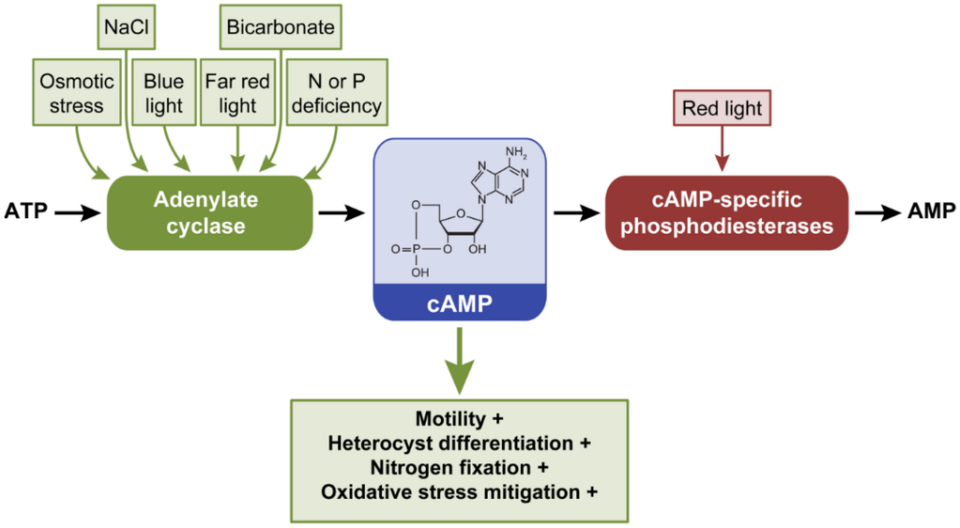

Figure 1.4. External factors controlling intracellular levels of cAMP and phenotypes or processes that are controlled by cAMP in cyanobacteria. Cyclic AMP is synthesized from ATP by adenylate cyclases (AC) and degraded to AMP by cAMP-specific phosphodiesterases. +, indicates process promoted by increased cAMP levels.

1.2.9. Cyclic AMP Regulates Motility Under Blue, Red, And Far Red Light

Cyclic AMP was initially recognized for its importance in controlling motility. In the filamentous cyanobacterium *Spirulina platensis*, external cAMP supplementation stimulated gliding motility and algal mat formation (63). A *Synechocystis* mutant lacking the AC gene *cya1* exhibited lower intracellular levels of cAMP and did not exhibit movement on agar plates under standard white light growth conditions (64). Further studies showed the blue light significantly and rapidly increased intracellular cAMP levels in *Synechocystis* compared to red or far red light (65). Motility was enhanced by blue light in a fluence- and Cya1-dependent manner (65), indicating that a blue-light dependent photoreceptor was involved in the regulation of Cya1 during this process.

Intracellular levels of cAMP are regulated by light in several cyanobacteria. Blue light serves as a crucial signal for cAMP regulation in *Microcoleus chthonoplastes* PCC 7420. A protein encoding a blue light-responsive LOV (light, oxygen, voltage) domain with an associated AC domain supported an increased synthesis of cAMP *in vitro* upon exposure to blue light (66). Whereas blue light increases cAMP levels in *Synechocystis*, *Anabaena cylindrical* exhibits reversible red light-mediated reductions in cAMP levels and far-red light-induced increases in cAMP content (67). Similarly, in *Anabaena*, red light decreased intracellular levels of cAMP, and far-red light levels increased cellular cAMP content (68). Together, these findings suggest photoreceptor regulation of cAMP levels. Indeed, a mutant lacking a functional *aphC* gene, which encodes a phytochrome histidine kinase-like protein, did not exhibit FR-induced accumulation of cAMP (68). In its photoregulation of intracellular cAMP levels, AphC purportedly phosphorylates and activates the AC CyaC, thereby inducing cAMP synthesis (68).

1.2.10. cAMP-dependent Transcriptional Regulation of Motility

In *Synechocystis*, the cAMP-receptor protein SYCRP1 is required for motility and appears to impact pili biogenesis (69). Non-motile *sycrp1* mutant cells showed a drastic change in the pili phenotype as the length and number of pili were extremely reduced. Additional insights into the signaling pathway that controls motility emerged when it was demonstrated that several SYCRP1-regulated genes were under the control of Hfq, a RNA-binding protein (70). Notably, a mutant lacking *hfq* lost motility which was correlated with the absence of pili on the cell surface. When SYCRP1 is activated it controls cell surface proteins CccS and CccP (71), both of which

were also identified as targets of Hfq (69). Similar to Δ*sycrp1* and Δ*hfq* mutants, a *Synechocystis* mutant lacking *cccS* showed an absence of thick pili (71).

1.2.11. Cyclic AMP As Nutrient Deficiency Signal

Not only does cAMP control motility, but it is also an important signal for environmental nutrient deficiencies (72). Nitrate and phosphate deficiencies resulted in transient increases in cAMP levels in *Anabaena flos-aquae* (72). In *Anabaena variabilis*, an increased intracellular cAMP concentration was observed under nitrogen starvation that coincided with early heterocyst development (73). Notably, cAMP can bind to AnCrpA, which in turn binds the 5' upstream region of *nifB*, a nitrogen fixation-related gene, impacting its expression and that of other genes related to nitrogen fixation and heterocyst differentiation in the presence of nitrate (74).

1.2.12. The Role Of cAMP Under Other Stresses

An additional link between NaCl-induced cAMP signaling and heterocyst formation was also suggested (75). NaCl, preferential to KCl or LiCl, transiently increased intracellular cAMP levels and induced expression of genes related to heterocyst formation (75). Cyclic AMP levels transiently increase during rehydration following dessication in *Anabaena* (76). AC CyaC is important for this cellular response as a *cyaC* mutant is disrupted in cAMP accumulation and associated regulation of recovery from desiccation during rehydration (76). This mutant exhibited impairments in oxygen evolution, increased ROS levels and increased respiration compared to wild-type cells (76). Respiration in cyanobacteria can yield CO_2, which equilibrates

to bicarbonate in solution. Of note, bicarbonate causes a structural change in a cyanobacterial CyaC enzyme that has been shown to stimulate its AC activity and thereby result in increased cAMP synthesis (77). A bicarbonate-stimulated induction of cAMP accumulation during rehydration could serve a protective role, as cAMP treatment of the *cyaC* mutant mitigated oxidative stress and growth impairments (76).

1.2.13. Cyclic guanosine 3′,5′-monophosphate, cGMP

Cyclic GMP (cGMP) is a fairly recently confirmed second messenger in bacteria (78), although it has been well characterized in eukaryotic systems. Cyclic GMP is synthesized from GTP by guanylyl cyclases (GC), which are homologous to the class III adenylate cyclases. Class III AC and GC enzymes share similar catalytic domains and are thought to have evolved from a common ancestor (79). Cyclic GMP is hydrolyzed to GMP by cGMP-specific phosphodiesterases (PDE) (Fig. 1.5). Cyanobacteria contain higher levels of cGMP compared to other bacteria(80). To date the only confirmed bacterial GC reported is the protein Cya2 from *Synechocystis* (81). Mutational analysis of *cya2* indicated that the encoded protein contributes to intracellular cGMP levels, but does not impact cAMP levels (81). Cya2 has higher specific activity for synthesizing cGMP from GTP than for the production of cAMP from ATP (82). This cGMP specificity has been attributed to faster turnover of GTP than ATP by Cya2 rather than preferential affinity for GTP (82). The only cyanobacterial phosphodiesterase (PDE) known to degrade cGMP is encoded by the gene *slr2100* in *Synechocystis* (83). This cGMP-specific PDE is required for the adaptation of the cells to UV-B radiation. Intracellular cGMP levels decreased

in wild-type after exposure to UV-B radiation, but not in the *slr2100* mutant. The *slr2100* mutant exhibited reduced transcripts of genes encoding components essential for PSII repair (83).

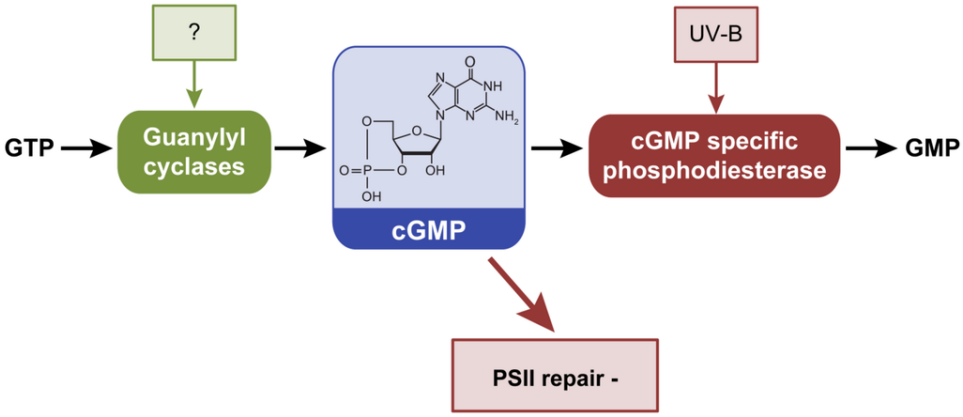

Figure 1.5. External factors controlling intracellular levels of cGMP and phenotypes or processes that are controlled by cGMP in cyanobacteria. Cyclic GMP is synthesized by guanylyl cyclases and degraded by cGMP-specific phosphodiesterases. +, indicates process promoted by increased cGMP degradation.

1.2.14. Cyclic dimeric guanosine 3′,5′-monophosphate, c-di-GMP

Cyclic di-GMP is synthesized from two GTP molecules by diguanylate cylase (DGC) (Pfam 00990) activity, whereas c-di-GMP-specific PDE (Pfam 00563 and 01966) proteins degrade the molecule into pGpG or GMP (Fig. 1.6). Proteins that can impact the synthesis or degradation of c-di-GMP and c-di-GMP-based signaling exist in a range of cyanobacterial species, yet are largely underexplored in these systems relative to their characterization in pathogenic bacteria (16). Many cyanobacterial proteins contain light-responsive domains linked to domains that can impact c-di-GMP synthesis or degradation, suggesting that light is an important signal for altering c-di-GMP homeostasis and associated development, physiology and metabolism in cyanobacteria (16). Among the species present in the CyanoBase database

(http://genome.kazusa.or.jp/cyanobase), the only species found to lack c-di-GMP signaling systems were *Prochlorococcus* and some strains of *Synechococcus* (16), similar to a report for cAMP (17). DGC and PDE domain sequences (i.e., Pfam00990 and Pfam00563) were used to identify conserved c-di-GMP domains in the 83 finished genomes in IMG (Table 1). These new results confirmed that only *Prochlorococcus* and some strains of *Synechococcus* lack c-di-GMP domains, with the exception of UCYN-A, an uncultured unicellular cyanobacterium associated with a eukaryotic cell (84) (Supplemental material http://www.mdpi.com/2075-1729/4/4/745#stats). Similarly to cAMP receptors (17), species adapted to stable habitats may have lost containing c-di-GMP-modulating proteins (16). To date, the gene *all2874* in *Anabaena*, the phytochrome-like protein Cph2 in *Synechocystis* and the cyanobacteriochrome SesA in *Thermosynechococcus elongatus* are the only reported cyanobacterial proteins with a functional DGC activity characterized *in vitro* (85-87). The gene *all2874* exhibited decreased heterocyst differentiation and reduced vegetative cell size under relative high light intensity Cph2 has three GAF domains, two DGCs and one PDE domain. GAF domains bind a light-absorbing chromophore in phytochrome family proteins (88, 89). Cph2 has been studied for its involvement in inhibiting phototaxis toward blue light in *Synechocystis* (85). Although wild-type *Synechocystis* cells did not move toward blue light, mutants lacking Cph2 showed phototaxis toward the light source. Covalent binding of a tetrapyrrole to conserved cysteine residues has been shown for two of the GAF domains of Cph2, as has light-induced photoconversion (85, 90). The DGC SesA possesses one GAF domain that can sense green and blue light(86). SesA is responsible for cell aggregation under blue light at relatively low temperature (86). Together, these results suggest that light can serve as a signal for regulating c-di-GMP levels in these two species.

Recently, we demonstrated that intracellular levels of c-di-GMP are regulated by light in some cyanobacteria *in vivo* (16). Levels of c-di-GMP were higher under blue light than other qualities of light in *Synechocystis*, whereas c-di-GMP levels were lower under blue light and higher under white and red light in the chromatically-acclimating *Fremyella diplosiphon*. Intracellular c-di-GMP levels in *F. diplosiphon* were overall higher than those measured in *Synechocystis*. These data confirmed that light is an important first messenger for regulating this second messenger in cyanobacteria.

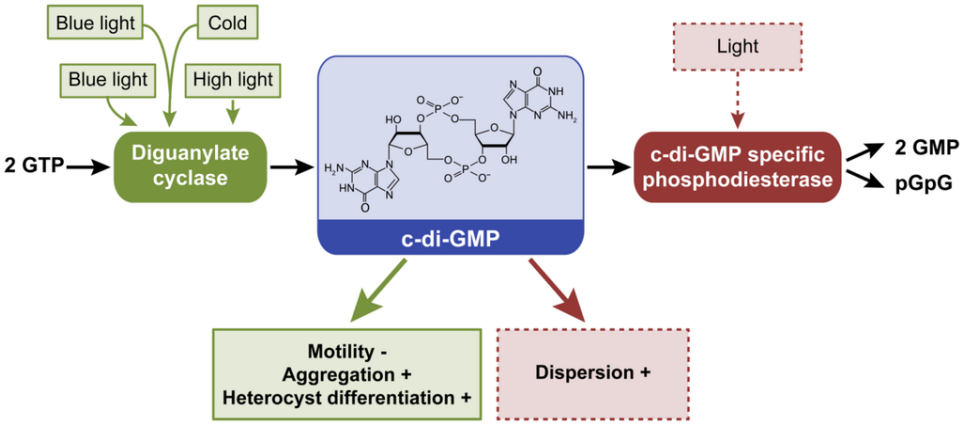

Figure 1.6. External factors controlling intracellular levels of c-di-GMP and phenotypes or processes that are controlled by c-di-GMP in cyanobacteria. Cyclic di-GMP is synthesized from two GTP by diguanylate cyclases and degraded to two GMP or pGpG by phosphodiesterases. Dashed lines denote hypothetical or suggested roles of light in activating PDEs; these are proposed as PDEs are often associated with photoreceptors in cyanobacteria (16) and this class of proteins induces motility and represses dispersion in several pathogenic bacteria (6). Green arrow indicates that increased c-di-GMP synthesis promotes the noted phenotypes, whereas a red arrow indicates that increased degradation of c-di-GMP is associated with the noted phenotypes. +, indicates process promoted by altered c-di-GMP levels; -, indicates process inhibited by increased altered c-di-GMP levels.

1.2.15. Nitric oxide, NO

Cyanobacteria are proposed to have contributed to the rise of NO in the atmosphere due to the production of ozone from the photolysis of O_2, the latter of which is generated as a by-product of oxygenic photosynthesis (91). NO is able to neutralize ozone, scavenge reactive oxygen species and mediate their potentially damaging effects (91). NO is an intermediate of denitrification produced through reduction of nitrite by nitrite reductase by bacteria; NO can be reduced to nitrous oxide by nitric oxide reductase (Fig. 1.7). Although exogenous NO can be a toxic gas, at low concentrations NO can be used as signaling molecule involved in the regulation of diverse biochemical and physiological processes.

A limited number of cyanobacteria have been shown to produce NO when grown in nitrate-containing media (92). However, there are limited insights into the *in vivo* role(s) of NO as a second messenger in cyanobacteria. Increasing concentrations of NO were produced and released at higher cell densities in the cyanobacterium *Microcystis aeruginosa* (93). Increasing the nitrogen-to-phosphorous ratio of the growth medium also supported higher levels of NO accumulation in this organism (93). NO accumulation appears to be primarily due to the activity of nitrate reductase, rather than NO synthase in *Microcystis aeruginosa* (93). In the 83 finished cyanobacterial genomes, nitrite reductase (Pfam13442, Pfam00394, Pfam07731, or Pfam07732) homologs are largely present in cyanobacteria (Table 1; Supplemental material http://www.mdpi.com/2075-1729/4/4/745#stats). The nitric oxide reductase (Pfam00115) is present in all finished cyanobacterial genome (Table 1; Supplemental material http://www.mdpi.com/2075-1729/4/4/745#stats).

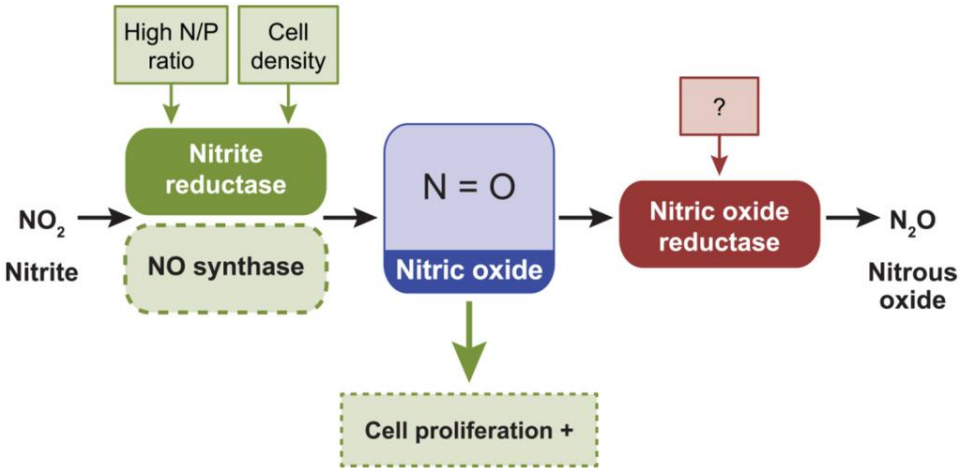

Figure 1.7. External factors controlling intracellular levels of NO and phenotypes or processes that are controlled by NO in cyanobacteria. NO is synthesized from nitrite (NO_2) during denitrification by nitrite reductase (and/or NO synthase) and reduced to nitrous oxide (N_2O) by nitric oxide reductase. Dashed lines denote hypothetical roles of NO synthase in controlling NO concentration and indicates that cell proliferation could be induced (+) by high intracellular levels of NO (93).

1.2.16. New Second Messenger-dependent Phenotypes in Cyanobacteria

The DisA_N domain shows diadenylyl cyclase (DAC) activity and synthesizes c-di-AMP from two molecules of ATP; c-di-AMP is degraded to pApA by c-di-AMP specific PDE enzymes (8) (Fig. 1.8). All cyanobacteria with a finished genome in IMG possess at least one DAC (PF02457) (Table 1.1). *Cyanothece* sp. PCC 7424, *Cyanothece* sp. PCC 7822, *Gloeobacter kilaueensis* JS1, *Gloeobacter violaceus* PCC 7421, and *Synechococcus* sp. PCC 7002 instead possess two DAC genes (Supplemental material http://www.mdpi.com/2075-1729/4/4/745#stats). The presence of DAC in sequenced cyanobacterial genomes suggests an important role for c-di-AMP in these organisms. Surprisingly, DAC are orphan proteins; they are not associated with other sensing domains. It has been suggested that c-di-AMP functions under

osmotic stress in cyanobacteria based on assessment of regulons of riboswitches involved in binding c-di-AMP that include targets implicated in the transport and synthesis of osmoprotectants (94). Cyclic di-AMP could also control the synthesis of c-di-GMP in some organisms, thereby potentially unveiling additional new roles of this widespread nucleotide (94).

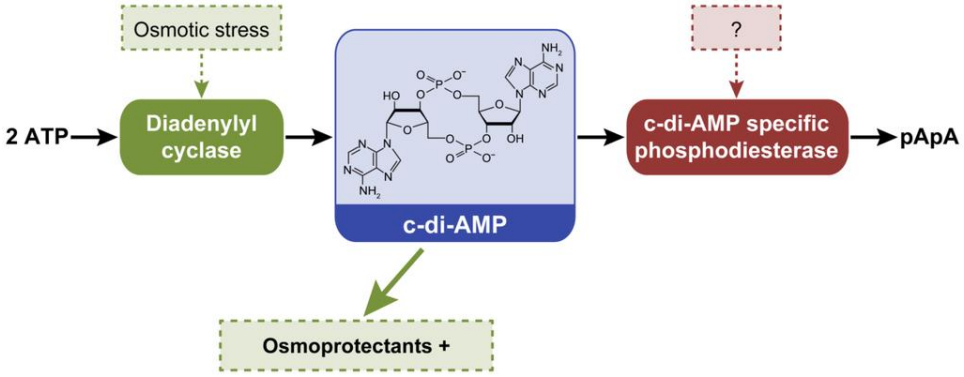

Figure 1.8. Suggested external factors controlling intracellular levels of c-di-AMP and phenotypes or processes that are proposed to be controlled by c-di-AMP in cyanobacteria. Cyclic di-AMP is synthesized from two ATP by diadenylyl cyclases and degraded to two AMP by a putative phosphodiesterase. +, indicates process promoted by increased c-di-AMP synthesis.

Recently the hybrid cyclic dinucleotide c-AMP-GMP was discovered in *Vibrio cholerae* (95). The protein DncV can produce c-AMP-GMP from ATP and GTP. To date, the physiological function of c-AMP-GMP remains unsubstantiated. Using the program BLAST, the best matching sequences from the 83 finished genomes present in the IMG database were the genes *Nos7107_0246* from *Nostoc* sp. PCC 7107 and *Glo7428_5202* from *Gloeocapsa* sp. PCC 7428. The statistically significant E-values for these genes were $9e^{-11}$ and $9e^{-10}$, respectively, with identities around 27% for both.

1.2.17. Cross Talk in Second Messenger Signaling

Interactions between signaling pathways creates a flexible signaling network that allows an organism to finely tune its responses to complex, and perhaps interacting, external stimuli. There are reported examples of regulatory cross talk between second messenger signaling pathways in cyanobacteria. For example, a partially purified adenylate cyclase was activated by Ca^{2+} in *Anabaena* sp. ATCC 29151(96). As cyanobacteria have been demonstrated to possess both cAMP and cGMP, a potential for cross talk between their regulatory networks emerges in such organisms. Indeed, in a cGMP PDE mutant that exhibits elevated cGMP levels, transcript levels were increased for AC gene *cya1*, guanylyl cyclase gene *cya2*, and a cAMP receptor protein-encoding gene (83). These results provide evidence for crosstalk between some second messenger signaling pathways in cyanobacterial systems.

1.3. CHALLENGES FOR THE FUTURE

1.3.1. The Complexity of Second Messengers

Second messengers are widespread in the genomes of cyanobacteria and a single molecule can regulate several phenotypes. For instance, Ca^{2+} can control gliding motility, heterocyst differentiation, and degradation of phycobilisomes (Fig. 1.2). (p)ppGpp regulates ribosomal RNA accumulation, heat shock proteins, and heterocyst differentiation (Fig. 1.3). Cyclic AMP is involved in photoprotection, heterocyst differentiation, photoheterotrophic growth, and nitrogen and phosphorous uptake (Fig. 1.4). In addition to a single second messenger controlling multiple phenotypes, multiple genes may encode proteins that regulate the synthesis or degradation of a

single second messenger molecule. One extreme case is apparent with the c-di-GMP signaling system in bacteria. Among cyanobacteria possessing c-di-GMP proteins, there is an average of 20 enzymes that synthesize or degrade c-di-GMP for each species in which they are found (16). There are several mechanisms by which c-di-GMP signaling specificity is achieved in systems exhibiting such complexity. One mechanism involves regulating the timing of accumulation of c-di-GMP relative to the presence of c-di-GMP receptors or effectors in cells. For instance, expression of c-di-GMP homeostasis enzymes could occur under environmental conditions which support accumulation of c-di-GMP specific targets. This has been demonstrated in *E. coli* for c-di-GMP in the control of biofilm formation during stationary phase (97). Alternatively, in light of evidence that c-di-GMP enzymes are constitutively expressed in many species, a different mechanism could explain signaling specificity. In this instance, temporal or spatial sequestration of individual c-di-GMP components could be used to control signaling, in which case c-di-GMP molecules would target co-localized receptors. The use of c-di-GMP sensors has demonstrated distinct patterns of spatial localization of c-di-GMP in some bacterial cells (98). Individual second messenger homeostasis enzymes also could distinctly effect second messenger pools and thereby control distinct phenotypes in the organism. Such signaling is referred to as high-specificity signaling and has been reported for distinct c-di-GMP synthesis enzymes in *Vibrio cholera* (99). Also, by controlling the presence of receptors of a second messenger that have differences in binding affinities, the activation of distinct receptors could be achieved at different intracellular second messenger concentrations, thereby allowing specificity of control of distinct phenotypes (100). Although compartmentalization of several second messengers has been well demonstrated in eukaryotic cells (101), signaling specificity in cyanobacterial systems has not been well investigated. Technologies already in use in pathogenic bacteria, such as

fluorescence resonance energy transfer (FRET) (98) or single fluorescent protein-based indicators (102) could be used to monitor c-di-GMP or cAMP concentrations to permit visualization of asymmetrical distributions of these second messengers in cyanobacteria.

CHAPTER TWO

The occurrence of cyclic di-GMP modulating output domains in cyanobacteria: An illuminating perspective

This chapter contains previously published work:

Agostoni M, Koestler BJ, Waters CM, Williams BL, Montgomery BL (2013). Occurrence of cyclic di-GMP-modulating output domains in cyanobacteria: an illuminating perspective. mBio 4(4)

2.1 INTRODUCTION

Photosynthetic microorganisms in the water column are subjected to environmental fluctuations in light, temperature, nutrient availability, and predation, which they respond to through physiological and morphological adaptations (103-105). Microorganisms evolved molecular mechanisms that allow them to rapidly modulate cellular behavior by monitoring environmental fluctuations using perception systems based on sensing environmental signals, initiating signal cascades, and responding to biophysical inputs, all of which involve specific receptors and feedback processes (106). Second messengers are signaling molecules fundamental for responses to external signals; rapid turnover of these molecules is energetically inexpensive and enables rapid phenotypic changes (107).

Bis-(3'-5')-cyclic dimeric guanosine monophosphate (cyclic di-GMP or c-di-GMP) is a ubiquitous bacterial second-messenger molecule synthesized and degraded by proteins that contain GGDEF, EAL or HD-GYP domains. GGDEF domain (Pfam 00990) proteins exhibit diguanylate cylase (DGC) activity, resulting in the synthesis of c-di-GMP from two GTP molecules (108). EAL domains (Pfam 00563) are associated with phosphodiesterase (PDE) activity that degrades c-di-GMP to the linear molecule 5’-pGpG (108, 109). HD-GYP domains (Pfam 01966) also possess PDE activity but completely degrade c-di-GMP into two GMP molecules (110). In bacteria, the intracellular concentrations of the second messenger c-di-GMP are regulated in response to a variety of environmental stimuli. Cyclic di-GMP plays critical roles in regulating numerous cellular processes in various bacteria including transcription, RNA turnover, biofilm formation, protein synthesis, motility, virulence, bacterial predation, and altering activities of proteins or protein complexes (111-116).

To date, the regulation of GGDEF and EAL domain-containing proteins has been studied in a limited number of cyanobacterial systems (85, 87, 111). Cyanobacteria are one of the most abundant photoautotrophic organisms in aquatic environments; they are able to fix both carbon and nitrogen under aerobic conditions and thus play a key role in regulating global carbon and nitrogen cycles. The phylum Cyanobacteria represents a highly diverse group of Gram-negative bacteria that are unicellular, filamentous, or colonial. They are present in the majority of biotopes on Earth, being found in marine, freshwater, and soil environments. They have also colonized deserts (117), polar waters (118), and geothermal environments (119). They are the progenitors of chloroplasts in eukaryotic photosynthetic organisms (120), and since they are the only bacteria capable of oxygenic photosynthesis, cyanobacteria also played a critical role in the initial rise of oxygen in the atmosphere around 2.3 billion years ago (121).

Recent studies have provided evidence that light can regulate c-di-GMP levels in cyanobacteria (85, 122). Light is an important stimulus for both non-photosynthetic and photosynthetic microorganisms, where light quality, intensity, and duration are used to control a variety of cellular processes. Light is sensed by specialized photosensory proteins that are activated by bound organic cofactors or chromophores, thereby transmitting photobiological signals to a downstream output domain (78, 123, 124). In bacteria, light-responsive domains are often linked to an output domain that impacts c-di-GMP homeostasis, thereby impacting bacterial physiology (125). These proteins likely function similar to the blue-light-sensing PDE from *Klebsiella pneumonia* (125, 126); i.e., light induces a conformational change in the photosensory domain that results in alteration in the linked GGDEF, EAL and/or HD-GYP domain that impacts c-di-GMP modulating activity. In this study, we assessed the occurrence of c-di-GMP-modulating domain-containing proteins in sequenced genomes of cyanobacteria that occupy diverse

environmental habitats. We examined the abundance of proteins containing c-di-GMP modulating domains in association with photoreceptor domains, and we propose that the widespread occurrence of these proteins indicates an important role in the environmental regulation of c-di-GMP levels in cyanobacteria. In addition, we document examples of the acquisition of new c-di-GMP signaling proteins by horizontal gene transfer (HGT) of c-di-GMP domain-containing genes and discuss which factors could have led to these events. Additionally, we quantified intracellular concentrations of c-di-GMP in *Fremyella diplosiphon* and *Synechocystis sp*. PCC6803 (hereafter *Synechocystis*) under white, blue, red, and green light. These two species showed different concentrations of c-di-GMP under different light qualities, validating the importance of light in regulating c-di-GMP signaling in cyanobacteria.

2.2 RESULTS

2.2.1 Light-responsive domains associated with c-di-GMP in cyanobacteria

Analyses of sequenced cyanobacterial genomes showed that 20 out of 37 cyanobacteria (54%) in CyanoBase possessed proteins containing c-di-GMP regulatory domains (Table 2.1; see also Supplemental material http://mbio.asm.org/content/4/4/e00451-13.full#sec-14). Putative light-responsive domains were common in cyanobacterial DGCs and PDEs (Table 2.1, Fig. 2.1, Supplemental material http://mbio.asm.org/content/4/4/e00451-13.full#sec-14 and (6)); of the 398 c-di-GMP-containing proteins, 131 (33%) were associated with light-responsive domains (Table 1). Notably, only two genes (6%) with an EAL-only domain (i.e. gene with only the EAL domain without the GGDEF domain as opposed to a hybrid protein that contains both GGDEF

and EAL domains) were associated with photoreceptors domains. Likewise, nine HD-GYP domains (35%) were associated with photoreceptor domains (Table 1).

Table 2.1. Numbers of c-di-GMP domain-containing proteins. And subsets containing photoreceptors domains in sequenced cyanobacterial genomes.

NAME	c-di-GMP domains					Photoreceptors associated with c-di-GMP		
	GGDEF	GGDEF and EAL	EAL	HD-GYP	TOTAL DOMAINS	GAF	PYP-like	LOV
Acaryochloris marina MBIC11017 6,503,724 bp[c]	28 (7[a])	26 (23)	6 (0)	0 (0)	60 (13[b])	8	0	0
Arthrospira platensis NIES-39 6,788,435 bp	21 (38)	13 (38)	4 (0)	0 (0)	38 (34)	7	2	4
Cyanothece sp. PCC 7424 5,942,652 bp	19 (26)	14 (36)	0 (0)	2 (0)	35 (29)	6	3	1
Cyanothece sp. ATCC 51142 4,934,271bp	12 (50)	18 (56)	3 (33)	1 (0)	34 (50)	3	13	1
Synechocystis sp. PCC 6803 3,573,471 bp	13 (46)	9 (33)	5 (0)	2 (0)	29 (31)	7	1	1
Cyanothece sp. PCC 8801 4,679,413 bp	13 (69)	10 (20)	2 (50)	1 (0)	26 (46)	10	2	0
Nostoc punctiforme ATCC 29133 8,234,322 bp	11 (55)	10 (70)	1(0)	2 (0)	24 (54)	2	10	1
Cyanothece sp. PCC 7425 5,374,574 bp	7 (14)	12 (42)	2 (0)	1 (100)	22 (32)	5	2	0
Synechococcus elongatus PCC 6301 2,696,255 bp	9 (56)	8 (63)	1 (0)	2 (100)	20 (60)	8	2	2

Table 2.1 (cont'd)

Synechococcus elongatus PCC 7942 2,695,903 bp	9 (56)	8 (63)	1 (0)	2 (100)	20 (60)	8	1	3
Anabaena variabilis ATCC 29413 6,365,727 bp	8 (0)	6 (50)	2 (0)	2 (0)	18 (18)	2	0	1
Anabaena sp. PCC 7120 6,413,771 bp	8 (13)	6 (33)	1 (0)	2 (0)	17 (17)	2	0	1
Synechococcus sp. PCC 7002 3,008,047 bp	7 (14)	6 (33)	2 (0)	0 (0)	15 (20)	0	2	1
Thermosynechococcus elongates BP-1 2,593,857 bp	5 (20)	5 (60)	1 (0)	1 (100)	12 (42)	5	0	0
Synechococcus sp. JA-2-3B'a(2-13) 3,046,682bp	4 (25)	0 (0)	0 (0)	3 (33)	7 (29)	2	0	0
Synechococcus sp. JA-3-3Ab 2,932,766 bp	3 (0)	0 (0)	1 (0)	2 (50)	6 (17)	1	0	0
Synechococcus sp. CC9311 2,606,748 bp	1 (0)	3 (0)	1 (0)	0 (0)	5 (0)	0	0	0
Trichodesmium erythraeum IMS101 7,750,108 bp	1 (0)	3 (0)	0 (0)	1 (100)	5 (20)	1	0	0
Microcystis aeruginosa NIES-843 5,842,795 bp	1 (0)	1 (0)	0 (0)	1 (0)	3 (0)	0	0	0
Gloeobacter violaceus PCC 7421 4,659,019 bp	1 (0)	0 (0)	0 (0)	1 (0)	2 (0)	0	0	0
TOTAL	181 (31)	158 (40)	33 (6)	26(35)	398 (33)	77	38	16
Without c-di-GMP domains								
Prochlorococcus marinus MED4								
Prochlorococcus marinus MIT9313								
Prochlorococcus marinus SS120								
Prochlorococcus marinus str. AS9601								

Table 2.1 (cont'd)

Prochlorococcus marinus str. MIT 9211
Prochlorococcus marinus str. MIT 9215
Prochlorococcus marinus str. MIT 9301
Prochlorococcus marinus str. MIT 9303
Prochlorococcus marinus str. MIT 9312
Prochlorococcus marinus str. MIT 9515
Prochlorococcus marinus str. NATL1A
Prochlorococcus marinus str. NATL2A
Synechococcus sp. CC9605
Synechococcus sp. CC9902
Synechococcus sp. RCC307
Synechococcus sp. WH 7803
Synechococcus sp. WH8102

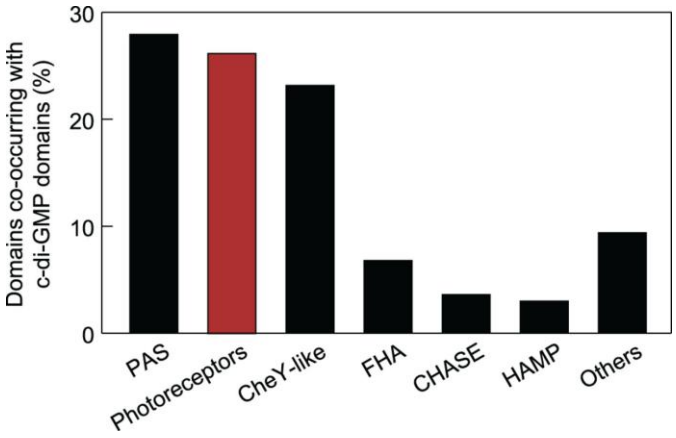

Figure 2.1. Partner domain occurrence for c-di-GMP domains, **in percentages**. The most common domain associated with c-di-GMP domains was PAS (28%), followed by photoreceptors (26%) and CheY-like domains (23%). PAS, Per-Arnt-Sim domain; CheY-like, receiver domain; FHA, forkhead associated; CHASE, cyclases/histidine kinases, associated sensory extracellular; HAMP, histidine kinases, adenylate cyclases, methyl-accepting proteins, and phosphatases.

The number of c-di-GMP domains in cyanobacteria could be dependent on genome size. However, an alternate explanation is that the habitat characteristics could have influenced the

number of c-di-GMP domains in cyanobacteria. To distinguish between these two possibilities, we compared the size of the genome and the number of genes with the total number of c-di-GMP related domains. Although the size of genome and the total number of genes were positively correlated with the number of c-di-GMP domains (Fig. 2.2A and 2.2B), they only explained 43% and 61% of the total variance, respectively. These correlations indicate that the number of c-di-GMP domains are not simply correlated with genome size, but may also be determined by bacterial adaptation. For instance, *Trichodesmium erythraeum* IMS101 and *Microcystis aeruginosa* NIES-843 have relatively large genome sizes (Table 1) with only five and three c-di-GMP domains respectively. In contrast, the genome sizes of *Synechocystis* and *Cyanothece sp*. ATCC 51142 are half that of *T. erythraeum* or *M. aeruginosa* genomes (Table 1) but contain 29 and 35 c-di-GMP domains, respectively. *T. erythraeum* inhabits tropical and subtropical oceans known to be nutrient-poor and relatively stable waters (127) and *M. aeruginosa* inhabits low-nutrient lakes (128). On the other hand, *Synechocystis* is adapted to dynamic environments as it can grow heterotrophically in the dark (129) and its metabolism is controlled largely by numerous circadian clock genes (130); *Cyanothece sp*. ATCC 51142 was isolated from intertidal waters in Texas subjected to a range of harsh conditions (131). These examples strongly suggest that c-di-GMP domains are determined by bacterial adaptation, and that this can occur independent of genome size.

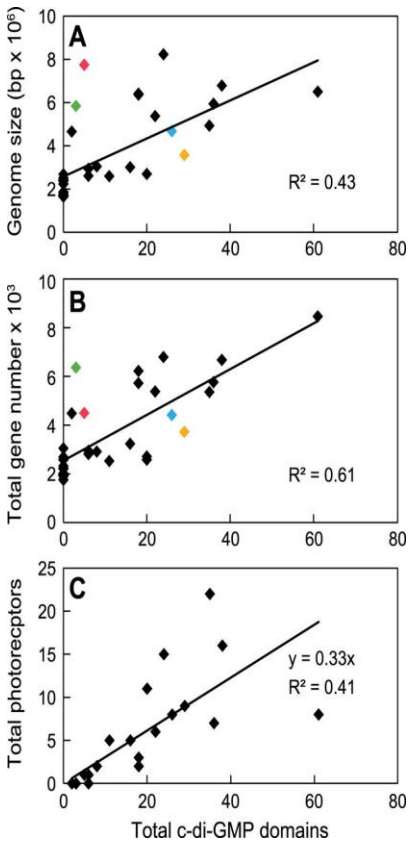

Figure 2.2. Correlation between genome size (A), total number of domains (B), and total number of photoreceptor domains (C) associated with c-di-GMP modulating domains. Each dot represents every species present in Table 1, for a total of 37 species. Colored dots represent individual species: red, *Trichodesmium erythraeum*; green, *Microcystis aeruginosa*; orange, Synechocystis sp. strain PCC6803; and blue, *Cyanothece* sp. strain ATCC 51142.

The total number of c-di-GMP domains associated with photoreceptor domains in cyanobacteria (33%; Fig. 2.2C and Table 2.1) are in contrast with the total c-di-GMP-domain containing proteins associated with photoreceptors from all bacteria (8%; (6)). In bacterial genomes, the most common photoreceptor domain associated with c-di-GMP domains is the BLUF (sensor of blue light using FAD) domain (40%), followed by the LOV (light-oxygen-voltage) domain, a sub-family of the PAS domain family (21%), and the phytochrome-characteristic GAF domain (8%) (132), used by cyanobacteria to sense UV-A, blue, green and/or red light (133-136). Our

analyses showed different results in that c-di-GMP domains in cyanobacteria were mostly associated with the GAF domain (58%), the PYP-like domain (related to PYP of xanthopsin photoreceptors) (36%), and less frequently in association with the LOV domain (6%) (Table 1). A number of LOV-domain containing proteins have already been shown to be associated with regulating c-di-GMP levels or with c-di-GMP signaling in cyanobacteria (122, 137, 138). We did not identify any BLUF domain associated with DGCs or PDEs in the available cyanobacterial genomes, however this finding is not unexpected based on the low abundance of BLUF domains in cyanobacterial genomes (139).

Some DGC proteins possess an allosteric inhibition site (I-site) proximal to the GGDEF active site (140). This c-di-GMP-dependent allosteric site, characterized by a RXXD motif, is important to control DGC activity; when levels of c-di-GMP are high, the second messenger can bind the RXXD motif thereby repressing the DGC activity. Among all the GGDEFs associated with photoreceptor domains in cyanobacteria, the allosteric site was found in 94% of GGDEF-only proteins and 81% of hybrid proteins (Supplemental material http://mbio.asm.org/content/4/4/e00451-13.full#sec-14). These values are slightly higher than all the GGDEFs unassociated with photoreceptor domains in cyanobacteria, where the I-site was found in 82% of GGDEF-only proteins and 63% of hybrid proteins (Supplemental material http://mbio.asm.org/content/4/4/e00451-13.full#sec-14). The frequency of the RXXD motif in cyanobacteria is different from other bacteria where only half of the DGCs possessed an allosteric site (141). The high occurrence of GGDEF proteins containing the I-site suggests that cyanobacteria tightly control c-di-GMP synthesis when DGCs are associated with photoreceptors.

2.2.2 Cyclic di-GMP domains: vertical and horizontal gene transfer

Cyclic di-GMP domains are widespread in bacteria, thus it has been suggested that c-di-GMP is an ancient second messenger (142). However, an alternate explanation which is not mutually exclusive is that cyanobacteria acquired c-di-GMP associated domains through HGT events. We examined whether the presence of c-di-GMP domains was a result of vertical transfer events or if HGT events regularly occurred in cyanobacteria. Genes can be considered acquired via HGT events when phylogenetic analysis of genes shows clustering of distantly related species. We performed this analysis for the conserved EAL-only proteins by generating a phylogenetic tree of 15 non-cyanobacterial EAL domain proteins and 24 conserved cyanobacterial EAL-only proteins.

The phylogenetic analysis of multiple conserved EAL domain sequences showed two distinctive clades (Fig. 2.3 and Supplemental material http://mbio.asm.org/content/4/4/e00451-13.full#sec-14). One clade was composed of only cyanobacteria EAL-only domains, with the exception of a single non-cyanobacterial sequence, and the other was composed of a mix of non-cyanobacterial and cyanobacterial EAL-only domains. The only species having genes widely distributed in both clades was *Acaryochloris marina,* a unique cyanobacterium that uses a distinctive light-acclimation method based on chlorophyll *d*, a far-red/infrared absorbing chlorophyll (143). It would be interesting to explore why this species possesses such a great diversification of EAL domains. In addition, the gene *A28LD_0392* from *Idiomarina* sp. A28L, a Gram-negative, aerobic, flagellar gammaproteobacterium present in a wide range of aquatic saline habitats (144), was the only non-cyanobacterial gene clustered together within the cyanobacteria-predominant clade. This gene exhibited high amino acid sequence identity to *Synechocystis slr6110* (E value

10^{-110}). Notedly, the gene *slr6110* is not incorporated in the genomic DNA but found on the *Synechocystis* plasmid pSYSX and itself could have been acquired via a HGT event. When we compared the sequences between *slr6110* and *A28LD_0392*, we observed two long conserved regions and one small deletion not present in the other genes, suggesting a shared evolutionary history unique to these two sequences (Fig. 2.4).

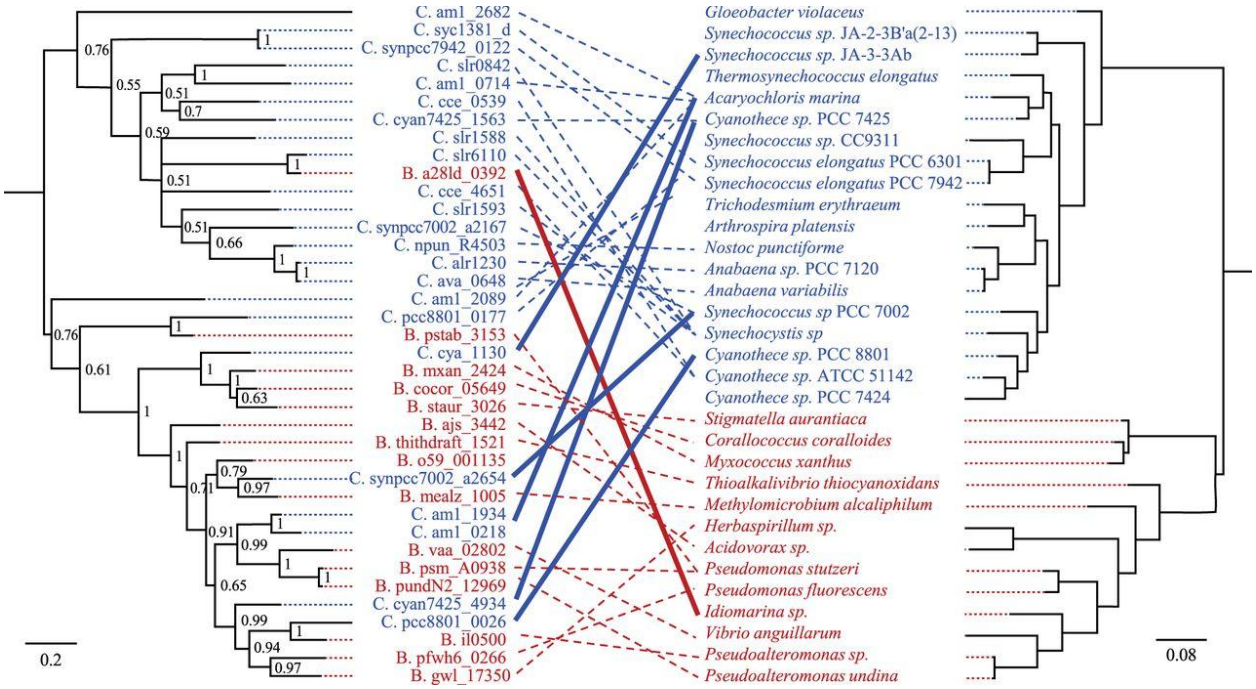

Figure 2.3. Phylogenetic analysis based on multiple presumably functionally conserved EAL-only sequences (left). (Right) Phylogenetic analysis based on 16S genes of the species representing the EAL-only genes. Dotted lines link species to their genes that were presumably acquired vertically. Continuous lines link species to their genes that were presumably acquired horizontally. Blue, cyanobacterial species; red, bacterial species.

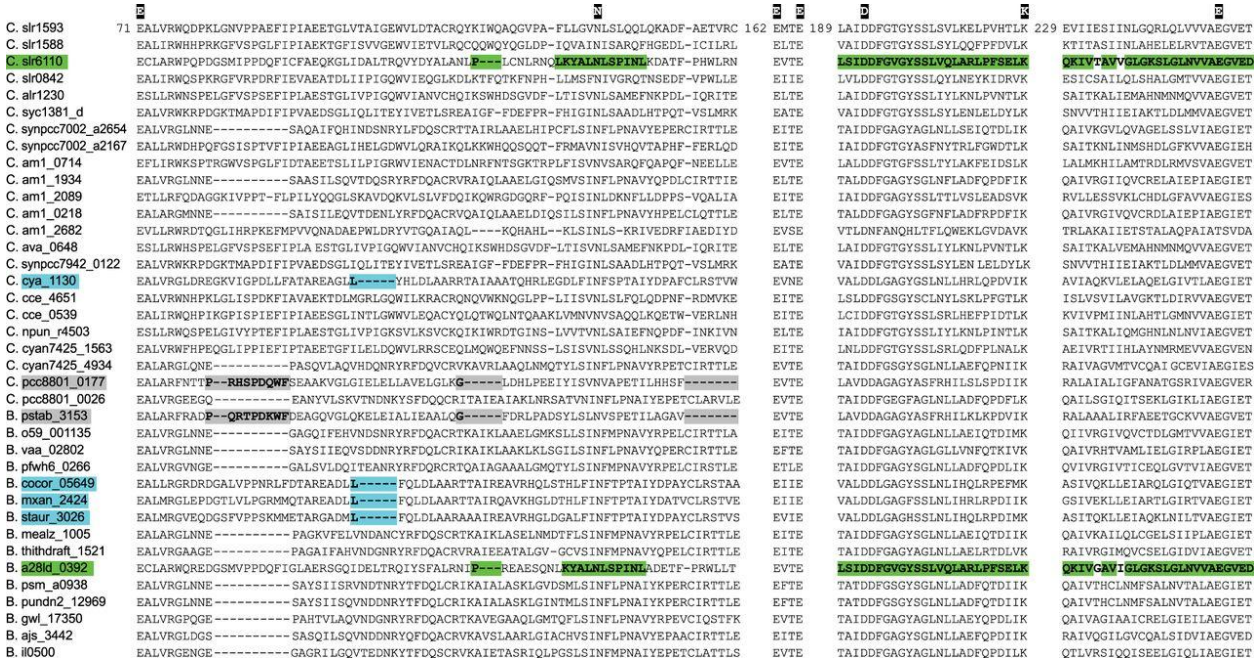

Figure 2.4. Alignment of EAL-only domains showing insertion and deletion regions. Gene names starting with a "C" denote cyanobacterial genes. Genes starting with a "B" denote bacterial genes. Blue backgrounds show deletion regions present in the genes *cya_1130*, *cocor_05649*, *mxam_2424*, and *staur_3026* that are not present in other EAL domains. Gray backgrounds show insertion and deletion regions present in the genes *pcc8801_0177* and *pstab_3153* that are not present in other EAL domains. Green backgrounds show conserved regions between *a28ld_0392* and *slr6110* genes. Amino acid residues critical for activity are indicated with black backgrounds.

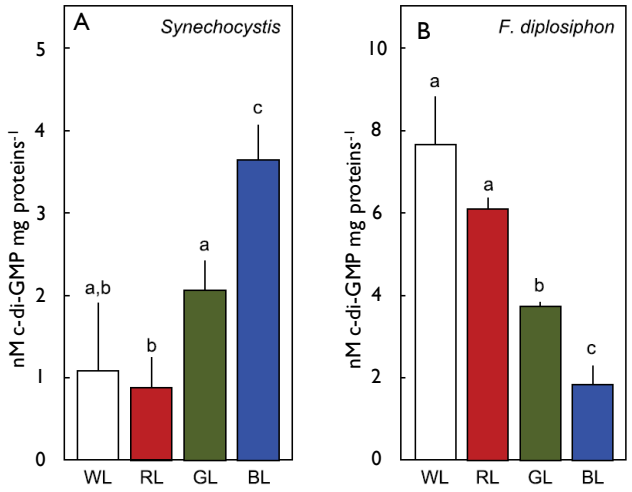

Figure 2.5. Effect of light quality on intracellular concentration of c-di-GMP normalized to total proteins. Cyclic di-GMP concentration (in nmol c-di-GMP μg protein−1) in F. diplosiphon (A) and Synechocystis (B). The colors of the bars indicate color of light under which cells were grown. WL, white light; RL, red light; GL, green light; BL, blue light. Means with different letters are significantly different ($P < 0.05$). Vertical bars represent standard errors.

After performing the phylogenetic analyses, we searched for rare genetic events like indels (insertions and deletions) in our alignment that could be used to link closely related genes despite disparate evolutionary history for the remainder of the genome, indicative of HGT events. The EAL domains encoded by *cya_1130*, *cocor_05649*, *mxam_2424*, and *staur_3026* exhibited a conserved deletion of 5 amino acids (Fig. 2.4). The genes *cocor_05649*, *mxam_2424*, and *staur_3026* are from a group of bacteria called myxobacteria and were closely related to the cyanobacterial gene *cya_1130* with an E value < 10^{-78}. However, the genomes of these four species possessed a G+C content value very similar with each other, constraining the use of G+C content analysis between the four species. Also, the cyanobacterial gene *pcc8801_0177* and the gene *pstab_3153* from *Pseudomonas stutzeri* possessed insertions and deletions of amino acids

that were not present in the other EAL domains (Fig. 2.4). The presence of these indels led us to hypothesize that the EAL domains from *cya_1130*, *cocor_05649*, *mxam_2424*, and *staur_3026* genes and the genes *pcc8801_0177* and *pstab_3153* were closely related based on independent phylogenetic analyses, which exclude sequence alignment gaps. In addition, *pcc8801_0177* had a G+C content of 53.1% while the remainder of the *Cyanothece sp*. PCC 8801 genome had a G+C content of 39.8%, suggesting that *pcc8801_0177* was also another HGT event. These rare genetic events indeed confirmed the predictions based on the phylogenetic analyses that those EAL domains were acquired through HGT. Thus, our results suggest that a c-di-GMP signaling system evolved early in the phylum Cyanobacteria, confirming the ancient origin of these signaling systems, but HGT events enriched the number of these domains in cyanobacterial genomes.

2.2.3 Comparison of the intracellular levels of c-di-GMP between cyanobacterial species under different light qualities.

In cyanobacteria, approximately 30% of c-di-GMP domains are associated with photoreceptor domains (Fig. 2.2C and Table 2.1). This finding suggests that sensing of light by photoreceptor domains may impact cellular c-di-GMP levels through modulation of enzymatically active DGCs and PDEs. Therefore, we quantified the levels of intracellular c-di-GMP of two cyanobacterial species, the filamentous freshwater cyanobacterium *F. diplosiphon* and the single cell model cyanobacterium *Synechocystis*, exposed to different light qualities. These two species are adapted to specific environmental niches and we suggest that these cyanobacteria would differently regulate intracellular levels of c-di-GMP under diverse light conditions. Notably, *F.*

diplosiphon, a cyanobacterium able to change its pigmentation to absorb available red or green wavelengths (145), showed higher intracellular levels of c-di-GMP under white light and red light, than under green light and blue light ($p<0.05$) (Fig. 2.5A). *Synechocystis* inhabits freshwater lakes and has been intensively studied for its capability to sense and respond to blue light. For instance, *Synechocystis* can grow heterotrophically in the dark, but requires blue light for a few minutes each day (129). Also, *Synechocystis* can use blue light in a photoprotective mechanism to quench excess energy through a soluble carotenoid binding protein (146). Finally, *Synechocystis* moves away from the light source if exposed to blue light. This negative phototaxis response is controlled by the second messengers cyclic AMP (65) and c-di-GMP (85). *Synechocystis* showed higher levels of c-di-GMP under blue light ($p<0.05$), than under white light, green light, and red light. Red light exposure resulted in lower c-di-GMP levels than did green light ($p<0.05$) (Fig. 2.5B). Notably, the intracellular c-di-GMP was lower in *Synechocystis* than in *F. diplosiphon* in all light conditions except blue light. These results suggest that different cyanobacteria respond differently to light conditions, likely due to adaptation to specific environmental niches.

2.3 DISCUSSION

2.3.1 Occurrence of c-di-GMP domains

Light sensing is critical for cyanobacteria to respond to different light spectra. The high number of GGDEF-only domains associated with photoreceptor domains indicates that light predominately controls synthesis rather than degradation of c-di-GMP. Cyanobacteria differ

from other bacteria regarding the number of photoreceptor domains associated with c-di-GMP domains. We demonstrated that many proteins containing c-di-GMP regulatory domains were found to be associated with blue and red light-dependent photoreceptor domains. The most common photoreceptor associated with c-di-GMP in non-cyanobacteria was the BLUF domain (139), whereas in cyanobacteria such associations in the genomes of sequenced cyanobacteria were rare. Rather in cyanobacteria, the GAF domain in association with c-di-GMP domains was more common. These results were in accordance with the general distribution of the photoreceptor types among the bacterial groups (139). Although the majority of cyanobacteria contain more GAF domains than PYP-like domains associated with c-di-GMP genes, *Synechococcus sp*. PCC 7002 that inhabits mud areas, *Nostoc punctiforme* ATCC 29133 that inhabits soil, and *Cyanothece sp.* ATCC 51142 that was isolated from a warm intertidal area (147-149) possess more PYP-like domains than GAF domains associated with c-di-GMP genes. Since *Nostoc sp.* and *Cyanothece sp*. were found in microbial mats in their natural habitats (150, 151), we speculate that the PYP-like domains functionally linked to c-di-GMP domains could control biofilm formation in cyanobacteria as suggested in Gomelsky and Hoff (125), whereas the GAF domain could be an important sensor for photosensory behavior (85). This phenomenon, known as c-di-GMP signaling specificity, has been observed in other bacterial systems (152). Furthermore, the PYP blue light sensor did not affect the phototactic motility in the purple nonsulfur bacterium *Rhodospirillum centenum* in response to blue light (153). Nonetheless, it has been shown that in a heterotrophic deep sea bacterium, *Idiomarina loihiensis*, a homologue of the PYP domain regulated biofilm formation (154). On the other hand, the GAF domain associated with c-di-GMP domains was involved in photosensory behavior in *Synechocystis* in response to blue light (85). The production of biofilms in cyanobacteria is a

mechanism used to withstand harsh conditions (155), and the PYP-like blue light sensor may be an important receptor to regulate biofilm formation for species that are exposed more frequently to high irradiance.

2.3.2 Evolutionary history of c-di-GMP in cyanobacteria

To understand the evolutionary history of c-di-GMP in cyanobacteria we decided to explore whether there was a correlation with organisms from a specific habitat and the presence of c-di-GMP domains. To address this question, we decided to compare the habitat characteristics of *Prochlorococcus* and *Synechococcus* strains lacking c-di-GMP domains with related strains that possessed them (Table 1). The presence of domains associated with c-di-GMP homeostasis in these two genera correlated with the presence of cAMP receptor proteins in cyanobacteria (17). The only species lacking genes for cAMP receptors inhabited marine oligotrophic environments, whereas species that possess cAMP receptor inhabited both marine and fresh water (17). Notably, the only genera lacking cAMP receptors were the picocyanobacteria *Prochlorococcus* and *Synechococcus* (with the exception of the species *G. violaceus*). It has been suggested that species such as *Prochlorococcus* that have adapted to stable habitats lost cAMP receptor proteins (17). *Prochlorococcus*, therefore, lost flexibility due to a lack of selection to maintain the receptors or as an adaptation in exchange for efficiency in stable conditions (156). In addition to cAMP receptor proteins (17), our analyses suggest that species growing under frequent environmental fluctuations cope with habitat changes by using second messengers to respond to external signals. *Synechococcus* strains containing c-di-GMP modulating-domains inhabit both marine and freshwater habitats and are found in nutrient rich (eutrophic) waters (Supplemental

material http://mbio.asm.org/content/4/4/e00451-13.full#sec-14). *Prochlorococcus* and *Synechococcus* strains lacking c-di-GMP regulatory domains inhabit marine low nutrient (oligotrophic) habitats (147), with the exception of CC9902 that is found in a coastal habitat (Supplemental material http://mbio.asm.org/content/4/4/e00451-13.full#sec-14). Currently there are no sequenced freshwater *Synechococcus* strains that lack these domains. Hence, the presence of c-di-GMP signaling systems may reflect the environmental characteristics of the habitats of picocyanobacteria *Prochlorococcus* and *Synechococcus.*

We also addressed the evolutionary origin of c-di-GMP signaling in cyanobacteria. *G. violaceus* and *Thermosynechococcus elongatus* BP-1 are considered the most divergent species relative to extant cyanobacteria (157, 158) . *G. violaceus* possesses two degenerate c-di-GMP modulating domains and *T. elongatus* has 11 domains predicted to be associated with c-di-GMP homeostasis or signaling, half of which are associated with GAF domains (Table 1). Because the synthesis and degradation of c-di-GMP is a widespread phenomenon in cyanobacteria (Table 1; (106)), it is tempting to speculate that a c-di-GMP signaling system with light controlled c-di-GMP homeostasis evolved early with the primordial cyanobacteria. Alternatively, it is also possible that these domains were spread via HGT, although multiple HGT events from one or few donors are less likely. We subsequently performed phylogenetic analyses of conserved EAL-only domain. The phylogenetic analyses of multiple conserved EAL domain sequences identified two clades in EAL-only domains: one clade predominantly containing cyanobacterial c-di-GMP domains, the other containing both non-cyanobacterial and cyanobacterial c-di-GMP domains. The structure of these phylogenetic trees suggests that one clade of domains evolved vertically, whereas many of the EAL domains from the other clade arose as a result of HGT events from within the phylum Cyanobacteria.

Although the functional roles of GGDEF, EAL, and HD-GYP proteins in cyanobacteria are only beginning to emerge, we believe that the abundance of these classes of proteins in a range of cyanobacteria that occupy diverse environmental habitats supports an important role for these proteins and the regulation of c-di-GMP levels in cyanobacteria. We also provided evidences that different cyanobacterial species possess different levels of intracellular c-di-GMP under different light qualities (Fig. 2.5). *F. diplosiphon* showed twice the concentration of c-di-GMP under green light compared to *Synechocystis*, and concentration of c-di-GMP was almost six times higher in *F. diplosiphon* under red light than *Synechocystis*. It is not surprising to see high levels of c-di-GMP in *F. diplosiphon* as this species belongs to the order *Nostocales*. Species belonging to the order *Nostocales* were associated with thrombolitic microbial mats (159) and could produce large amounts of biofilm, a cellular process regulated by c-di-GMP. Since *F. diplosiphon* may be evolved to sense red and green light by changing its pigmentation and morphology to absorb available wavelengths in the prevailing light quality under different depths in the water column (145), c-di-GMP may play a role in regulating cell morphology and buoyancy. On the other hand, in *Synechocystis* blue light can regulate several important processes from heterotrophic growth, to photoprotection, to phototaxis. This species showed high levels of c-di-GMP under blue light (Fig. 2.5B). Since under white light, levels were lower than under blue light, we can speculate that intracellular levels of c-di-GMP were overall repressed when cells were exposed to wavelengths other than blue light. Our results are in congruence with Savakis *et al.* (85), who suggested that *Synechocystis* had higher c-di-GMP levels under blue light. Indeed, the DGC Cph2 has been studied for its involvement in inhibiting phototaxis toward blue light (85). Biofilm formation in *Synechocystis* is a less common process that is not usually visible under laboratory conditions. Indeed, Schatz *et al.* (2012) suggested that for the species

Synechococcus elongatus PCC 7942, biofilm development is self-repressed (160). Lower levels of c-di-GMP under red, green, white lights could therefore be associated with the repression of biofilm formation.

2.4 MATERIAL AND METHODS

2.4.1. c-di-GMP domain-containing proteins

For the identification of GGDEF, EAL, or HD-GYP domains, we searched the database http://genome.kazusa.or.jp/cyanobase (hereafter referred to as CyanoBase), which contains genome sequences of cultured cyanobacteria (161). Conserved regions were identified manually by assessing the presence of eight amino acid residues known to be critical for activity in the GGDEF domain (162, 163); seven in the EAL domain (164); and 11 in the HD-GYP domain (165). The RXXD allosteric site, which is a motif found 5-12 amino acids before the GGDEF sequence in DGC proteins, was identified manually.

2.4.2 Light-dependent receptors

Proteins containing blue and red light-dependent signaling domains associated with c-di-GMP regulatory domains were identified using gene information from CyanoBase. This option was not available for the LOV domains. Thus, to assess the potentiality of photochemical activity, LOV domains were individually screened to discriminate them from similar PAS domains with different biochemical activities. The chromophore-binding cysteine residue and the presence of

20 conserved amino acids was verified in order to identify functionally conserved LOV domains (166).

2.4.3. Genome comparisons

Phylogenetic analyses of multiple conserved EAL domain sequences were performed using TOPALi v2.5 (167). Multiple alignments of amino acid were generated using MUSCLE. BLASTP similarity searches were used to identify sample of homologs to produce a tree composed by cyanobacterial and non-cyanobacterial EAL domains. EAL domains were selected as those homologs that possess either an E value of less than 10^{-80} or greater than 59% amino acid identity. In addition, because we were focused on general patterns of evolution, rather than an exhaustive description of all EAL domain evolution, we limited our taxon sampling to no more than three homologs from each cyanobacterial species for further analyses. Phylogenetic trees were inferred using Bayesian inference in the software package MrBayes, using the Whelan and Goldman model with gamma-distributed rates for the EAL domains, and the Hasegawa, Kishino and Yano model with gamma distributed rates for 16S ribosomal RNA sequence data. MrBayes was run with two independent runs of 1,000,000 generations of 3 heated chains and one cold chain, with a burn-in of 25% of trees that were sampled every 10 generations. Convergence was assessed as maximum potential scale reduction factor (PSRF) values of 1.001 (EAL tree) and 1.002 (16s tree). Posterior probabilities which range from 0 to 1.0 were used to evaluate the statistical confidence of a particular cluster of sequences. Likelihood log of -11774.17 for 16S tree; likelihood log of -5974.33 for EAL-only tree.

2.4.4. Cyclic di-GMP quantification and culture conditions

F. diplosiphon strain SF33, a shortened-filament mutant strain that displays WT pigmentation (168), and *Synechocystis* were maintained axenically and grown in BG-11 (169) containing 20 mM HEPES at pH 8.0 (hereafter BG-11/HEPES). Liquid cultures were adapted to white light (red light enriched; Philips F32T8/TL741/ALTO) at 35 µmol photons m^{-2} s^{-1} in glass flasks, and maintained at an optical density at 750 nm (OD_{750}) and 730 nm (OD_{730}) of 0.6 for *F. diplosiphon* and *Synechocystis*, respectively. Flasks were maintained with mixing at 150 rpm. Cells adapted to white light were then transferred in glass flasks to white, red (LED wholesalers, 2506RD), green (λmax at 530 nm; Geneva Scientific LLC), and blue light (LED wholesalers, 2506BU) at 35 µmol photons m^{-2} s^{-1}. Since growth rate was different under different light qualities for *Synechocystis,* flasks were inoculated at an OD_{730} of 0.1 for cells transferred to white light and red light, 0.4 for flasks under blue light, and 0.3 for flasks under green light. For *F. diplosiphon*, white light, green light and red light flasks were inoculated at an OD_{750} of 0.1 and an OD_{750} of 0.4 for flasks under blue light. Protein and c-di-GMP quantification was performed after five days at OD_{750} and OD_{730} of 0.6±0.1.

Cyclic di-GMP was determined as described in (152). In brief, 1.5 ml of cells were centrifuged and resuspended in 150 µl of ice-cold extraction solvent containing 40% acetonitrile, 40% methanol, 0.1N formic acid and mixed for 30 s by vortexing, followed by incubation for 30 min at -20 ºC. The cell suspension was then centrifuged at 17000 g for 5 min at 4 ºC. The supernatant was transferred to a new 1.5 ml tube and stored at -80 ºC until analysis. Before quantification, the supernatants were concentrated using a vacuum manifold and resuspended in an equal volume of Milli-Q purified water and then filtered through a 0.45 µm filter unit (TITAN syringe

filter PVDF). Each sample was analyzed employing liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The samples were compared to chemically synthesized c-di-GMP (Biolog) concentration standards ranging from 250 nM to 1.9 nM to determine the c-di-GMP concentration. Cyclic di-GMP was normalized to total soluble proteins. Soluble proteins were extracted by lysing a parallel cell sample with CelLytic B Bacterial Cell Lysis/Extraction reagent (Sigma). Cells were then mixed with an equal volume of <106 μm glass beads (Sigma) on a vortex with 10 repeated cycles of 1 min agitation with cooling intervals of 1 min. During the process cells were maintained in a cold room or in ice water bath. The cell extract was collected after initial centrifugation at 1000 *g* for 1 min at 4ºC and further centrifuged at 17000 *g* for 30 min at 4ºC. The protein concentration was determined using a Pierce BCA protein assay kit.

The statistically significant effects of light conditions on c-di-GMP levels were determined using one way analysis of variance (ANOVA) with Fisher post-hoc test using OpenStat statistical software (version 10.01.08; W. G. Miller http://www.Statprograms4U.com). Statistical analyses were performed utilizing 95% confidence intervals ($p < 0.05$).

CHAPTER THREE

Regulation of biofilm formation and cellular buoyancy through modulating intracellular cyclic di-GMP levels in engineered cyanobacteria

3.1 INTRODUCTION

Cyanobacteria evolved mechanisms to monitor and rapidly adapt to environmental changes, including using second messengers to regulate physiology or metabolism (170). When an external input or first messenger is perceived, a specific second messenger can initiate a physiological change by amplifying the biological input signal to a downstream output effector. One of the most common second messengers in bacteria is cyclic dimeric (3′→5′) GMP (cyclic di-GMP or c-di-GMP) (6). Cyclic di-GMP is synthesized by diguanylate cylase (DGC) (108), and hydrolysed by phosphodiesterases (PDE) (109, 110). DGCs activity contain the GGDEF domain (Pfam 00990) (108), whereas PDEs contain either EAL or HD-GYP domains (Pfam 00563 and Pfam 01966, respectively) (109, 110). Cyclic di-GMP is implicated in the transition between motile and sessile lifestyles in bacteria, but also controls numerous other cellular processes including transcription, RNA turnover, protein synthesis, virulence, bacterial predation (111-116).

Cyanobacteria are capable of controlling or altering their lifestyle from motile to sessile in order to withstand harsh or dynamic conditions (171). The lifestyle change from a motile to sessile state is associated with biofilm formation in numerous bacteria and it is controlled by c-di-GMP (6). Despite significant studies of c-di-GMP signalling in pathogenic bacteria (6), little is known about proteins that impact the synthesis or degradation of c-di-GMP and associated c-di-GMP-based signalling systems in cyanobacterial species (170). The phylum Cyanobacteria represents a highly diverse group of Gram-negative bacteria capable of oxygenic photosynthesis, many of which are able to fix both carbon and nitrogen under aerobic conditions. *In vitro* and mutant analyses of genes encoding c-di-GMP homeostasis proteins have been associated with a limited

number of cyanobacterial phenotypes (85-87). A mutant with a disrupted DGC exhibits altered control of heterocyst development under high-light conditions in the filamentous cyanobacterium *Anabaena sp*. strain PCC 7120 (87). Mutational analysis of the gene *cph2*, which encodes a protein containing both DGC and PDE domains, indicated that Cph2 is involved in inhibiting phototaxis in a blue light-dependent manner in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*; (85)). Mutation of the gene *sesA*, encoding a protein that contains a DGC domain, resulted in cell aggregation under blue light (BL) at relatively low temperature in *Thermosynechococcus elongatus* (86). Previously, we quantified intracellular levels of c-di-GMP in cyanobacteria and demonstrated that levels of c-di-GMP in *Synechocystis* and *Fremyella diplosiphon* are distinctly regulated under different light qualities (16). This study confirmed that light is an important environmental factor that controls intracellular concentrations of c-di-GMP in cyanobacteria.

There is a growing practical interest in understanding the molecular bases of biofilm formation in pathogenic bacteria. Despite the environmental and industrial applications of regulating biofilm formation in cyanobacteria (172), information on the mechanisms that trigger such a transition in cyanobacterial lifestyle is scarce. Recent studies have provided important initial insight into the factors that lead to cellular aggregation or biofilm formation in cyanobacteria (160, 173-176). For instance, the gene cluster encoding enzymes involved in exopolysaccharides (EPS) production in *Synechocystis* are involved in cell-cell aggregation and biofilm formation, is involved in regulating light-associated oxidative stress (173). EPS also mitigates salt and metal stresses in *Synechocystis* (174). It is also important to note that biofilm formation is self-repressed in wild-type *Synechococcus elongatus* PCC 7942 cells, a phenomenon that is likely to prevent aggregation and maximize light absorption (160).

In this study, we used the model unicellular cyanobacterium *Synechocystis* and the chromatically-acclimating filamentous cyanobacterium *F. diplosiphon*. *Synechocystis* and *F. diplosiphon* represent good case studies to assess cyanobacterial phenotypes under the control of different levels of c-di-GMP as *Synechocystis* maintains low levels of c-di-GMP (16), whereas *F. diplosiphon* maintains high levels of c-di-GMP in controlled-environment growth chamber experiments (16). Based on our prior study in which we demonstrated that BL increased c-di-GMP levels in *Synechocystis* more than other wavelengths of light (16), we hypothesized that BL is capable of inducing biofilm formation and aggregation in *Synechocystis*. In *F. diplosiphon*, we hypothesized that low c-di-GMP levels seen under green light (GL) and BL (16) may be associated with increased cellular buoyancy. To further assess the role of c-di-GMP in regulating biofilm formation and aggregation, we constitutively expressed a DGC or PDE (from *Vibrio cholerae* and *Escherichia coli*, respectively) in *Synechocystis* and *F. diplosiphon*. We demonstrate in both cyanobacterial species that high levels of c-di-GMP led to biofilm formation and cellular deposition while low intracellular levels of c-di-GMP led to increased cellular buoyancy. In addition to providing insight into the role of c-di-GMP in the control of native phenotypes in these organisms, these results establish that control of the intracellular concentrations of c-di-GMP in cyanobacteria has practical applications in biotechnology, such as exogenous control of buoyancy and biofilm formation in partially or fully enclosed photobioreactors or in treatment of metal-containing wastewaters.

3.2 RESULTS

3.2.1 Cyclic di-GMP promotes biofilm formation and cellular aggregation in *Synechocystis*.

Previously, we showed that c-di-GMP levels in *Synechocystis* are three times higher under BL compared to white light (WL) and red light (RL), and almost twice as much when compared to cells under GL (16). We hypothesized that BL-grown *Synechocystis* cells exhibiting high c-di-GMP levels could produce biofilm and cells could aggregate since c-di-GMP plays a key role in promoting biofilm formation and aggregation in other bacteria (6). When maintained under BL, *Synechocystis* indeed produced biofilm in an adhesive layer on the bottom of flasks and it was easily perceived by eye (Fig. 3.1a). By comparison, under RL, GL, and WL cells did not adhere to the surface of glass flasks (Fig. 3.1a). Although a different rate of cell growth could affect the quantification of biofilm formation simply based on higher cell density, growth in BL was slower – the doubling time for cells growing under BL was twice as much as under GL, and four times that of cells under RL (Fig. 3.2) – ruling out this possibility.

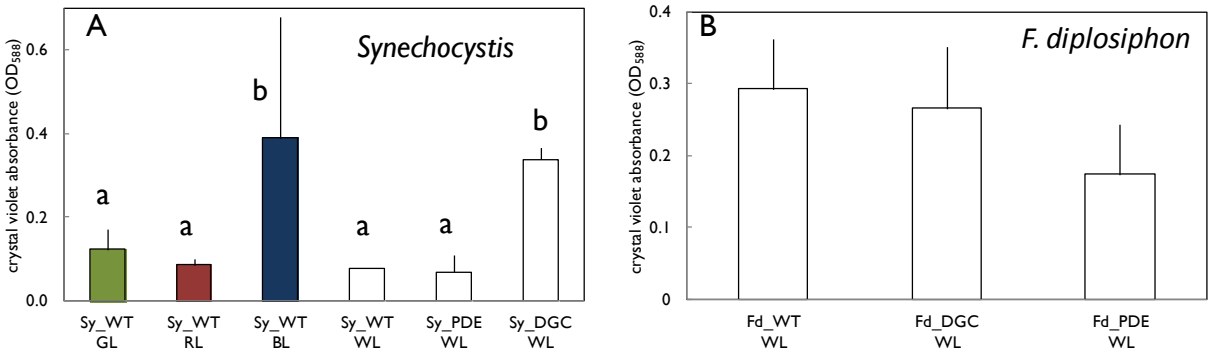

Figure 3.1. Quantification of crystal violet associated with biofilm production on glass flasks after staining. The colors of the bars indicate color of light under which cells were maintained. A) Biofilm formation ability of *Synechocystis* and its overexpression strains with DGC from *Vibrio cholerae* (Sy_DGC) and PDE from *Escherichia coli* (Sy_PDE) under white light (WL), red light (RL), or blue light (BL). B) Biofilm formation ability of *F. diplosiphon* and its overexpression strains with DGC from *V. cholerae* (Fd_DGC) and PDE from E.coli (Fd_PDE) under WL. Means with different letters are significantly different ($P<0.05$). Vertical bars represent standard deviations.

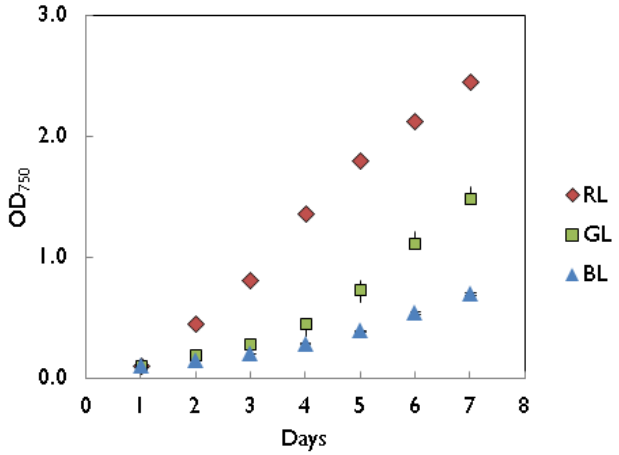

Figure 3.2. Growth curve of *Synechocystis.* It was measured as optical density at 750 nm (OD_{750}) in 24 hour intervals for 7 days under red light (RL), green light (GL), and blue light (BL). Vertical bars represent standard deviations. RL, red light; GL, green light; BL, blue light.

BL not only promoted biofilm formation in *Synechocystis*, but also induced cellular aggregation or reduced buoyancy. Under BL, 75% of the total cells sank to the bottom of tubes into which cells had been dispensed over 48 hours (Fig. 3.3a). Cells did not sink under RL or GL, resulting in maintenance of a homogenous density throughout the tube (Fig. 3.3a). Although in WL c-di-GMP levels were intermediate between c-di-GMP levels in RL and GL (16), 50% of *Synechocystis* cells sank to the bottom of the tube under WL (Fig. 3.3a), suggesting that biofilm formation is not just under the control of the c-di-GMP network in *Synechocystis*.

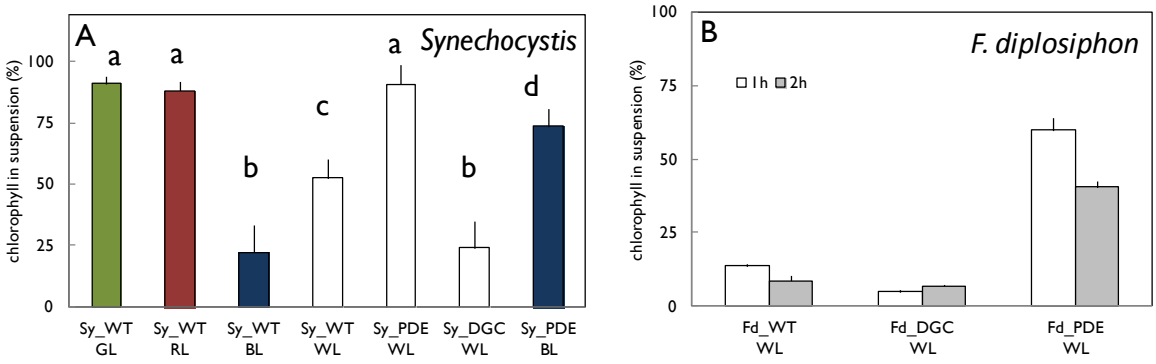

Figure 3.3. Quantification of chlorophyll in suspension (in percentage) for cyanobacterial cultures in borosilicate glass tubes. Cells were adapted for 5 days at 28 °C in 35 µmol m^{-2} s^{-1} white light (WL). A) *Synechocystis* and its DGC (Sy_DGC) and PDE (Sy_PDE) overexpression strains under WL, red light (RL), or blue light (BL) after 48 hours. B) *F. diplosiphon* and its DGC (Fd_DGC) and PDE (Fd_PDE) overexpression strains under WL after one (white bar) and two (grey bar) hours. Means with different letters are significantly different ($P<0.05$). Vertical bars represent standard deviations. PDE, phosphodiesterase strain; DGC, diguanylate cylase strain.

After demonstrating that BL is a specific first messenger that can trigger biofilm formation and aggregation, whereas other light quality signals do not, we tested whether c-di-GMP is sufficient to induce biofilm formation independently of BL by modulating intracellular c-di-GMP

concentrations in strains using a heterologous DGC and PDE. We constructed WT *Synechocystis* strains (hereafter *Sy*_WT) carrying a plasmid for constitutive overexpression of an exogenous DGC from *V. cholerae* (hereafter *Sy*_DGC) or an exogenous PDE from *E. coli* (hereafter *Sy*_PDE). The VC1067 DGC from *V. cholerae* has been shown to synthesize high levels of c-di-GMP (99). The YhjH PDE from *E. coli* has been previously shown to counteract c-di-GMP-dependent cellulose biosynthesis in *Salmonella enteric* serovar Typhimurium (177). We tested the capability of these two enzymes to modulate c-di-GMP levels in *Synechocystis* under WL. We confirmed that the genes were expressed at the transcriptional level (Fig. 3.4). *Sy*_PDE showed 66% lower levels of c-di-GMP than *Sy*_WT ($p<0.05$), whereas *Sy*_DGC exhibited 2.5 fold higher c-di-GMP content in the cells than *Sy*_WT ($p<0.05$) (Fig. 3.5a).

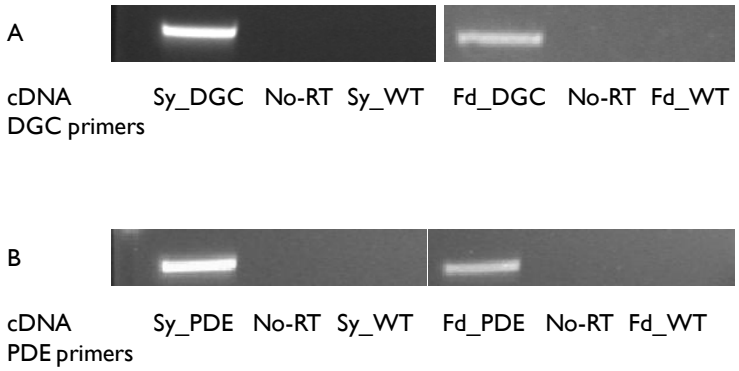

Figure 3.4. Reverse transcription PCR (RT-PCR) analysis of the expression of DGC (A) and PDE (B) genes in *Synechocystis* and *F. diplosiphon* . *Synechocystis* DGC (Sy_DGC), *Synechocystis* PDE (Sy_PDE), *F. diplosiphon* DGC (Fd_DGC), and *F. diplosiphon* PDE (FD_PDE) overexpression strains. No RT, control reaction with no reverse transcriptase enzyme.

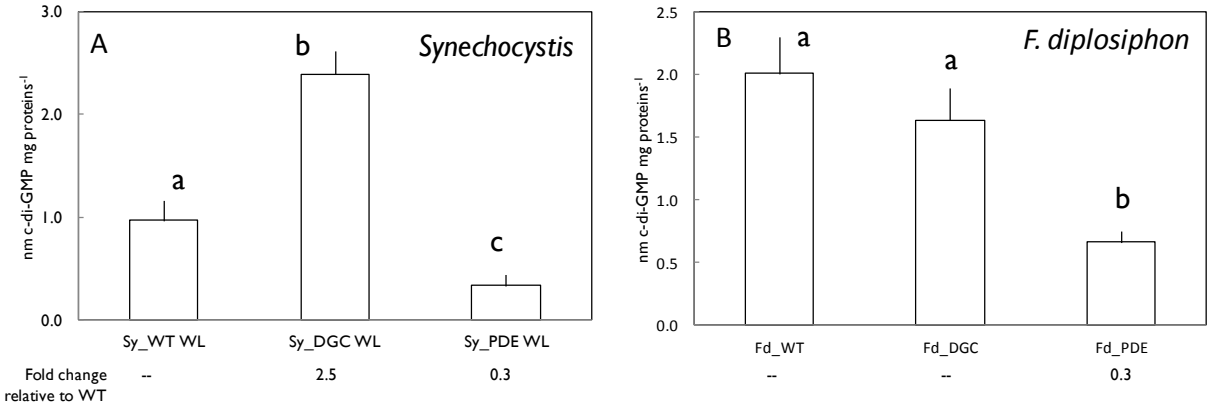

Figure 3.5. Effect of exogenous diguanylate cyclase (DGC) and phosphodiesterase (PDE) on the intracellular concentration of c-di-GMP. Normalized to total proteins in *Synechocystis* (A) and *F. diplosiphon* (B). Means with different letters are significantly different (P<0.05). Vertical bars represent standard deviations. *Synechocystis* wild type (Sy_WT), *Synechocystis* DGC (Sy_DGC), *Synechocystis* PDE (Sy_PDE), *F. diplosiphon* wild type (Fd_WT), *F. diplosiphon* DGC (Fd_DGC), and *F. diplosiphon* PDE (FD_PDE) strains. Numbers below bars represent fold change in c-di-GMP levels relative to cognate WT parental strain.

After confirmation that our enzymes were expressed and active, we next tested whether our engineered strains exhibited biofilm formation or aggregation under WL condition. *Sy*_PDE cells did not produce biofilm similar to *Sy*_WT ($p>0.05$) (Fig. 3.1a), but were homogenously distributed through a vertical tube and homogenously floated 48 hours after transfer, which was significantly different from *Sy*_WT exposed to WL ($p<0.05$) (Fig. 3.3a). In fact, *Sy*_PDE cells exposed to WL behaved more similarly to *Sy*_WT exposed to RL and GL in which the levels of c-di-GMP are low ($p>0.05$). *Sy*_DGC, which exhibits high levels of c-di-GMP (Fig. 3.4a), produced biofilm under WL in contrast to *Sy*_WT ($p<0.05$) (Fig. 3.1a), and around 75% of *Sy*_DGC cells sank to the bottom of the glass tubes under WL. This aggregation of *Sy*_DGC exposed to WL is significantly different from *Sy*_WT exposed to WL, yet is more similar to the

aggregation of *Sy*_WT under BL in which the concentration of c-di-GMP is naturally high ($p>0.05$) (Fig. 3.3a).

To confirm that c-di-GMP is responsible for the observed cellular aggregation in *Synechocystis*, we also tested whether constitutive expression of a PDE in *Synechocystis* exposed to BL will alter cellular aggregation. In this condition, *Sy*_WT cells have naturally high levels of c-di-GMP and aggregate. If c-di-GMP is responsible for this phenotype under BL, we expect expression of a PDE will decrease aggregation. Indeed, under BL, only 25% of *Sy*_PDE cells sank compared to *Sy*_WT for which around 80% of cells sank ($p<0.05$) (Fig. 3.3a). These data taken together demonstrated that high intracellular levels of c-di-GMP promote biofilm formation and aggregation in *Synechocystis*, whereas low levels of c-di-GMP drive floating in suspension.

3.2.2. Low c-di-GMP promotes floating in *Fremyella diplosiphon*

Having shown that c-di-GMP impacted biofilm formation and aggregation in a unicellular cyanobacterium, we also wanted to test whether the DGC and PDE enzymes could modulate c-di-GMP levels, and biofilm formation and aggregation, in a filamentous cyanobacterium. We chose the filamentous cyanobacterium *F. diplosiphon* that is capable of acclimating to changes in external light wavelengths in the process known as complementary chromatic adaptation (145). *Synechocystis* and *F. diplosiphon* exhibited different internal concentrations of c-di-GMP and different responses to light (16); overall *F. diplosiphon* had a higher intracellular concentration of c-di-GMP compared to *Synechocystis* (16) and almost four times more c-di-GMP under WL than under BL (16). In contrast to *Synechocystis*, BL in *F. diplosiphon* caused the lowest levels of c-di-GMP (16). We first verified transcription of the exogenous DGC and PDE genes (Fig.

3.4), and next compared the intracellular levels of c-di-GMP between the WT strain (hereafter *Fd*_WT), the DGC expressing strain (hereafter *Fd*_DGC), and the PDE expressing strain (hereafter *Fd*_PDE). In contrast to *Synechocystis*, c-di-GMP levels in *Fd*_DGC were lower than in *Fd*_WT (Fig. 3.5b). Similar to *Synechocystis*, *Fd*_PDE had lower c-di-GMP content than *Fd*_WT under WL (Fig. 3.5b). Since *Fd*_PDE did not show high levels of c-di-GMP, we did not expect to see physiological differences in biofilm formation or aggregation compared to *Fd*_WT. Indeed, *F. diplosiphon* did not show significant differences in biofilm formation among *Fd*_WT, and *Fd*_PDE, and *Fd*_DGC strains ($p>0.05$) under the condition we tested, although *Fd*_PDE had a lower mean (Fig. 3.1b). This result suggests that other factors besides c-di-GMP control biofilm formation of *F. diplosiphon* in these conditions. WT *F. diplosiphon* cells, which have naturally high levels of c-di-GMP, rapidly sink to the bottom of vertical glass tubes within an hour after transfer under WL (Fig. 3.3b). However, reduction of c-di-GMP levels by expression of the PDE inhibited aggregation under WL as only 40 % and 58% of *Fd*_PDE cells deposited in glass tubes and after one or two hours, respectively (Fig. 3.3b).

3.2.3 Physiological analyses in cyanobacterial strains with high and low levels of c-di-GMP

High levels of c-di-GMP have been shown to inhibit growth of some bacteria (142). We grew *Synechocystis* and *F. diplosiphon* strains with different intracellular c-di-GMP levels under WL for 6 days, but did not see a significant difference in growth rate in *Synechocystis* and *F. diplosiphon* among the strains with distinct c-di-GMP levels (Fig. 3.6). In *F. diplosiphon* there were no differences between *Fd*_WT and *Fd*_PDE with distinct c-di-GMP levels (Fig. 3.6b). Although *Fd*_DGC grew similarly to *Fd*_PDE at low concentration of antibiotic (5 μg/ml of

kanamycin) used to maintain the plasmid, when *Fd*_DGC was exposed to higher concentrations of kanamycin the growth was impaired (Fig. 3.6b).

The levels of photosynthesis-associated pigments, i.e. phycobiliprotein phycocyanin (PC), total chlorophyll *a* (chl*a*) and carotenoids levels were similar in *Sy*_WT, *Sy*_PDE, and *Sy*_DGC (Fig. 7a and 7c). Although in *F. diplosiphon* the levels of allophycocyanin (AP), phycoerythrin (PE), PC, and carotenoids were similar between *Fd*_WT, *Fd*_PDE, and *Fd*_DGC strains, *Fd*_PDE exhibited higher levels of chl *a* compared to *Fd*_WT and *Fd*_DGC (Fig. 3.7b and 3.7d). The chl*a* levels in *Fd*_PDE were 1.4 fold higher than in *Fd*_WT (Fig. 3.7d).

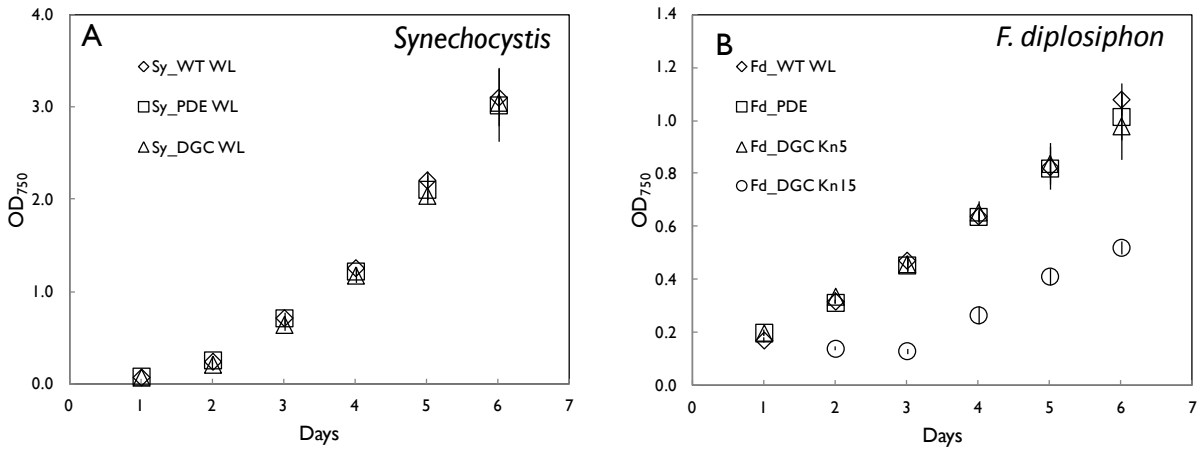

Figure 3.6. Growth curves of *Synechocystis* (A) and *F. diplosiphon* (B) under white light (WL). Cells were growing at 28 °C in 35 µmol m-2 s-1 WL. Vertical bars represent standard deviations. WT, wild type strain; PDE, phosphodiesterase strain; DGC, diguanylate cylase strain.

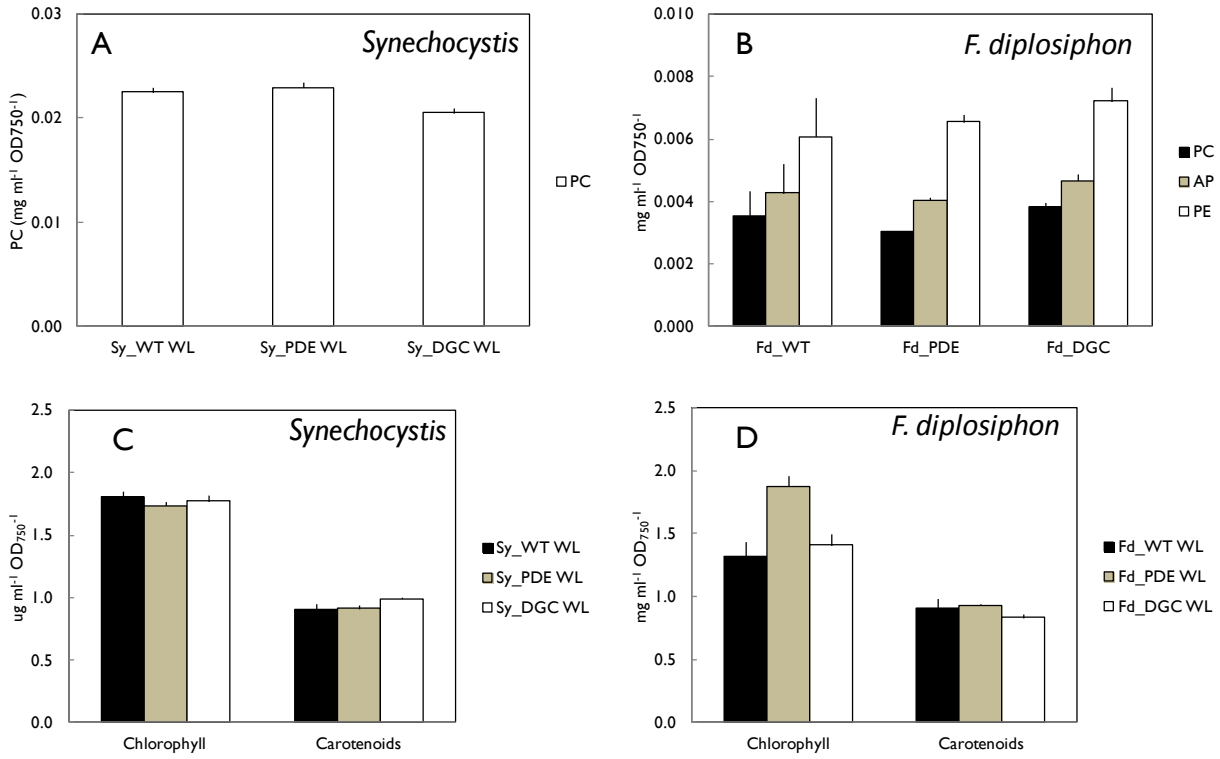

Figure 3.7. Photosynthetic pigment levels in *Synechocystis* and *F. diplosiphon* strains with differing levels of second messenger c-di-GMP. Quantification of phycocyanin (PC) (A), total chlorophyll and carotenoids (C) in *Synechocystis*. Quantification of phycobiliproteins, including phycocyanin (PC), allophycocyanin (AP), and phycoerythrin (PE) (B), and total chlorophyll and carotenoids (D) in *F. diplosiphon*.

3.3 DISCUSSSION

The distinct differences in intracellular c-di-GMP concentrations and morphological differences make *Synechocystis* and *F. diplosiphon* particularly interesting models to study the role of c-di-GMP in the cyanobacteria. These two distinct cyanobacteria exhibited different responses of c-di-GMP signalling to light inputs (16). The filamentous *F. diplosiphon* belongs to the order *Nostocales*, which are commonly associated with microbial mats in nature (159) , whereas biofilm formation has not been commonly seen for *Synechocystis* in laboratory experiments even

though *Synechocystis* is naturally found in biofilms (178, 179). Consistent with this observation *Synechocystis* had low levels of c-di-GMP under controlled-environment growth conditions (16). High irradiance of BL may cause photodamage in cyanobacteria (180), and BL may induce an increase of intracellular c-di-GMP levels as a photoprotective mechanism in *Synechocystis*. It has been shown that strong BL could not cause photoinhibition when cyanobacteria cells were embedded in a biofilm (181). We hypothesized that the naturally high levels of c-di-GMP in cells exposed to BL could induce biofilm formation, and indeed we found that BL specifically induced biofilm formation in *Synechocystis*. This result suggests that *Synechocystis* could induce biofilm formation as a protective mechanism against BL by increasing cellular c-di-GMP. In addition to providing protection from BL, biofilms can support the scavenging of reactive oxygen species from the surrounding environment (182), and reduce oxidative stress through altering the structural integrity or surface electrical charge of microbial community structure (183, 184).

We confirmed that biofilm formation is a c-di-GMP dependent process in *Synechocystis* as overexpressing a gene encoding a DGC enzyme induced biofilm formation and aggregation in WL whereas overexpressing a PDE gene inhibited these phenotypes. Notably, in the cyanobacterium *Thermosynechococcus vulcanus* RKN, cell aggregation is associated with cellulose accumulation (185), and cellulose production in some bacteria has been shown to be under the control of c-di-GMP (186). Alternatively, cell deposition also could be caused by pili impairment (187, 188).

We also tested whether an exogenous DGC or PDE could be used to modulate intracellular concentrations of c-di-GMP in filamentous cyanobacteria. Contrary to *Synechocystis*, the filamentous cyanobacterium *F. diplosiphon* contains higher levels of c-di-GMP when adapted to

WL (Agostoni et al., 2013). Although the DGC gene in *F. diplosiphon* was transcribed we did not see c-di-GMP changes in *Fd*_DGC. High levels of c-di-GMP can inhibit growth of some bacteria (142). We speculate that heterologous expression of a DGC in *F. diplosiphon* was regulated at post transcriptional level. When antibiotic concentration was increased, growth rate drastically decreased suggesting that this protein may be toxic in *F. diplosiphon*. *Fd*_WT, *Fd*_DGC, and *Fd*_PDE strains produced a statistically similar amount of biofilm even with different intracellular concentrations of c-di-GMP, suggesting that other regulatory inputs are more important to drive biofilm formation in this organism. With regard to cellular deposition, *F. diplosiphon* cells tend to sink in less than one hour when not shaken. *Fd*_PDE cells, with lower intracellular levels of c-di-GMP, were able to float longer (i.e., for at least two hours). For filamentous cyanobacteria, we hypothesize that c-di-GMP could be a useful tool to control buoyancy. Different concentrations of c-di-GMP did not vary the levels of pigments in *Synechocystis*, however Chl *a* changed in *F. diplosiphon* with low c-di-GMP levels. Since light plays a fundamental role *in F. diplosiphon* pigmentation and morphology (189, 190), future analyses are needed to examine the effect of c-di-GMP in light-dependent responses in this cyanobacterium.

Controlling exogenous DGC or PDE enzyme levels in cyanobacteria is a useful tool for practical biotechnological applications. Cyanobacteria can be used to purify aquatic systems contaminated with heavy metals (191), and increasing biofilm formation by inducing DGCs could help EPS-producing cyanobacteria to increase metal uptake capability because negatively charged biofilms (192) sequester positively charged heavy metal ions (191). In addition, one constraint of mass production of biofuels or other desired bioproducts from aquatic photosynthetic microorganisms is the efficient harvesting of cells (193). To decrease the costs of biomass production in

photosynthetic microorganisms and reduce environmental trade-offs, efficient harvesting techniques need to be developed (193). Flocculation is one technique to harvest cyanobacteria in laboratory-scale cultivation systems and is a process in which dispersed cells are induced to form large aggregates (194); it has also been proposed that flocculation is very cost-effective method to harvest cells (195). Flotation is another technique that can be used to harvest microorganisms. This process involves the induction of cells to float on a liquid surface through altering cellular buoyancy (193). In this study, we found that exogenous DGC and PDE enzymes in both unicellular and filamentous cyanobacteria can be used to regulate physiological processes such as flocculation and cellular buoyancy. Although we constitutively overexpressed DGC- and PDE-encoding genes, such genes could be used under the control of an inducible promoter such as a light-switchable promoter (196). Such engineering could be used to replace conventional chemical inducers to contain water management issues or promote energy efficient biomass harvesting.

3.4 METHODS

3.4.1 Culture conditions

Axenic cultures of *Synechocystis* and *F. diplosiphon* were grown at 28 °C in BG-11 (169) containing 20 mM HEPES at pH 8.0 with the indicated antibiotic. *F. diplosiphon* strain SF33, a shortened-filament mutant strain that displays wild-type pigmentation (168), was used as wild type (WT) strain. Cultures (30 ml) in 250 ml glass flasks were adapted to fluorescent white light (WL; Philips F32T8/TL741/ALTO) at 35 µmol m^{-2} s^{-1} with shaking at 150 rpm. Cultures of

Escherichia coli were grown at 37 °C in Luria–Bertani (LB) broth with the appropriate antibiotic. LB was solidified with 1.5% (w/v) Bacto-agar for growth on solid medium.

3.4.2. Plasmid construction

To increase intracellular levels of c-di-GMP both in *Synechocystis* and *F. diplosiphon*, the diguanylate cyclase VC1067 from *V. cholerae* was used as DGC. This DGC when induced in *V. cholerae* resulted in the synthesis of relatively high levels of c-di-GMP (99). To decrease intracellular levels of c-di-GMP both in *Synechocystis* and *F. diplosiphon*, the phosphodiesterase YhjH from *E. coli* was used as PDE. This PDE was demonstrated to hydrolyze c-di-GMP when expressed *in vivo* in pathogen *Salmonella enterica* serovar Typhimurium (177). In *Synechocystis*, genes encoding DGC and PDE enzymes were constitutively overexpressed using the plasmid pRL1342 (C. Peter Wolk, GenBank: AF403427.1) under the control of the *ftsQ* promoter (GenBank: BAA17497.1) The WT strain carried an empty pRL1342 plasmid as negative control. Promoters were added to the DGC- or PDE-encoding genes by overlap PCR using primers indicated in Table 3.1. The genes were amplified from genomic DNA using PrimeSTAR Max DNA polymerase (Takara Bio, Inc.) with primers that created terminal *Xho*I (5′) and *BamH*I (3′) restriction sites (Table 3.1). The pRL1342 plasmid and the promoter-gene fusion containing the *ftsQ* promoter were cut with *BamH*I and *Xho*I and the restricted products combined using Takara's DNA Ligation Kit, Mighty Mix. The ligation mixture was transformed into chemically competent *E. coli* DH5α (LifeTechnologies, Inc.) and transformants were selected with 50 μg ml^{-1} (w/v) chloramphenicol. The DNA sequences of plasmids were confirmed by Sanger sequencing. The resulting plasmids then were introduced into *Synechocystis* by triparental

mating using the broad-host-range *E. coli* helper strain J53 (RP4). The two *E. coli* strains were washed twice with LB without antibiotic and 5 ml of *Synechocystis* was pelleted and resuspended in 1 ml of BG-11. Each *E. coli* strain (100 µl of each) and *Synechocystis* (1 ml) were mixed and spotted on nitrocellulose membrane on BG-11 plates and incubated at 28 ºC for one day (197, 198). The following day, the nitrocellulose membrane was transferred to BG-11 plates with 25 µg ml^{-1} (w/v) erythromicin. Putative transformant colonies were selected after two weeks.

In *F. diplosiphon*, the *apcA* promoter was used to constitutively overexpress the DGC and PDE genes. Overlap PCR was conducted as described above for *Synechocystis* using indicated primers (Table 3.1). The genes were amplified from genomic DNA using PrimeSTAR Max DNA polymerase (Takara Bio, Inc.), creating two terminal *BamH*I restriction sites (Table 3.1). The restricted PCR product was inserted in the *BamH*I site of the pPL2.7 plasmid (199) using Takara's DNA Ligation Kit, Mighty Mix. The resulting plasmids were introduced in *F. diplosiphon* by triparental mating as previously described (200). Colonies were selected after two weeks on BG-11 plates with 25 µg ml^{-1} (w/v) kanamacyin. The WT strain carried an empty pPL2.7 as a negative control.

To verify the expression of these exogenous genes in both *Synechocystis* and *F. diplosiphon*, we isolated RNA from cells growing under white light (WL) in exponential phase as described previously (200). RT-PCR was performed using GoTaq Green master mix (Promega) with primers specific for DGC gene *VC1067* and PDE gene *yhjH* (for sequences see Table 3.1). Cycling parameters for *VC1067*were 95 °C for 2 min, 29 cycles of 95 °C for 30 s, 51 °C for 30 s, and 72 °C for 1 min. Cycling parameters for *yhjH* were 95 °C for 2 min, 29 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min.

Table 3.1. Primers used in this study.

	Forward primer (5' - 3')[a]	Reverse primer (5' - 3')
Synechocystis		
P_{FtsQ} for *Sy*_PDE	CACG**CTCGAG**ACCGTTGAAAGTAC	AACCTGCCTTATCATGGGAGTTCAATAACC
*Sy*_PDE	GGTTATTGAACTCCCATGATAAGGCAGGTT	GTT**GGATCC**TTATAGCGCCAGAAC
P_{FtsQ} for *Sy*_DGC	CATA**CTCGAG**TTACTGGCGAACCGC	TCGTAAATACCCCCATTTGGTTTAACCTCCTTTAAA
*Sy*_DGC	TTTAAAGGAGGTTAAACCAAATGGGGGTATTTACGA	CGC**GGATCC**TTAAACGATGTGTAATCC
Fremyella diplosiphon		
P_{apcA} for *Fd*_PDE	CGC**GGATCC**CGTAAATCTGAAAAG	AACCTGCCTTATCATTAATGGATTCCTCCA
*Fd*_PDE	TGGAGGAATCCATTAATGATAAGGCAGGTT	GTA**GGATCC**TTATAGCGCCAGAAC
P_{apcA} for *Fd*_DGC	CACGCTCGAGACCGTTGAAAGTAC	CGTAAATACCCCCATTAATGGATTCCTCCA
*Fd*_DGC	TGGAGGAATCCATTAATGGGGGTATTTACG	GCG**GGATCC**TTAAACGATGTGTAA
RT-PCR		
*Sy*_PDE	GGTTATTGAACTCCCATGATAAGGCAGGTT	GTTGGATCCTTATAGCGCCAGAAC
*Fd*_PDE	TGGAGGAATCCATTAATGATAAGGCAGGTT	GTAGGATCCTTATAGCGCCAGAAC
*Sy_Vc*DGC/*Fd*_DGC	CGATATTCTGGTGGCGATAGAG	CTGCCAATCATGGTGTCTTCTA

[a] Bold text indicates sequence of restriction site.

3.4.3. Physiological analyses

In *Synechocystis*, chlorophyll *a* (chl*a*) concentration was determined by extraction in 90% methanol and phycocyanin (PC) content was calculated from wavelength-dependent, whole-cell absorbance as described (176). For *F. diplosiphon*, chl*a* and phycobiliproteins (PBPs) were extracted and quantified as previously described (201). Cyclic di-GMP was extracted and levels quantified as described (16). The growth rate was estimated by optical density at 750 nm (OD_{750}).

3.4.4. Biofilm assays

Fifteen ml of *Synechocystis* and *F. diplosiphon* cultures in 250 ml glass flasks at an initial OD_{750} of 0.5 were maintained without shaking for 14 days. After removing the non-adherent cells by aspiration of medium, 0.5% (w/v) crystal violet was added for two minutes to stain the adherent cells. After removal of the stain, flasks were washed with 15 ml of 1X PBS at least three times. For biofilm quantification, the biofilm-associated crystal violet was resuspended in 10 ml of 95% ethanol for 30 min, and the OD_{588} of the resulting suspension was measured as an estimate of biofilm formation.

3.4.5. Deposition assays

Two ml of *Synechocystis* and *F. diplosiphon* cultures with starting OD_{750} of 0.5 were added to 13×100 mm borosilicate glass tubes (Baxter T1290-4) and maintained without shaking under WL, RL (2506RD; LED Wholesalers), GL (Geneva Scientific LLC), or BL (2506BU; LED

Wholesalers) for two days for *Synechocystis* and for one or two hours for *F. diplosiphon*. The deposition or sinking of cellular aggregates was measured by quantifying chl*a* content at the surface (160). Briefly, 1 ml of cells was taken from the surface without disturbing the cells deposited. A reference tube was maintained under the same conditions but mixed before collecting 1 ml of cells. The chl*a* content measured for the aliquot of cells collected from this reference tube served as 100% chl*a* reference.

3.4.6. Statistics

The statistical significance of the effect of particular light conditions on c-di-GMP levels was determined via one way analysis of variance (ANOVA) with Fisher post-hoc test using OpenStat statistical software (version 10.01.08; W. G. Miller http://www.Statprograms4U.com). Statistical analyses were performed utilizing 95% confidence intervals ($p < 0.05$).

CHAPTER FOUR

The role of endogenous regulation of c-di-GMP in controlling photosynthetic pigments in the cyanobacterium *Fremyella diplosiphon*

4.1 INTRODUCTION

A response to light signals is among the most important adaptive responses in photosynthetic organisms. Second messengers can be utilized to generate rapid responses to environmental changes. In cyanobacteria, photooxygenic gram-negative bacteria that inhabit most of Earth's environments, second messengers are widely regulated to respond to light changes in aquatic and terrestrial environments (170). For instance, light-to-dark transitions are associated with rapid elevation of intracellular Ca^{2+} levels (43) and with accumulation of (p)ppGpp (58). (p)ppGpp is also increased during reduction of ambient light (56). UV photoprotective mechanisms in cyanobacteria are regulated by the second messengers cGMP and Ca^{2+} (45, 83). Cyclic AMP is regulated under different light qualities, and in turn can regulate motility (65-68). To date in cyanobacteria, all the confirmed phenotypes under the control of the second messenger c-di-GMP are light dependent. *In vivo* levels of c-di-GMP are modulated by light quality in cyanobacteria (16). Phototaxis, a process in which cells move towards or away from light, is regulated by c-di-GMP under blue light in *Synechocystis* (85). Blue light and relatively cold temperature also induced cellular aggregation in *Thermosynechococcus elongatus*, which is under the control of c-di-GMP (86). Under relatively high light c-di-GMP regulates heterocyst differentiation (specialized cells able to fix atmospheric nitrogen) in *Anabaena* (87).

Cyclic di-GMP is modulated in the cells by two classes of enzymes: diguanylate cylase (DGC) which synthesizes c-di-GMP (108) and phosphodiesterase (PDE), which degrade c-di-GMP (109, 110). DGCs contain the GGDEF domain (Pfam 00990) (108), whereas PDEs contain either EAL or HD-GYP domains (Pfam 00563 and Pfam 01966, respectively). The majority of c-di-GMP proteins are multi-domain proteins composed of c-di-GMP and sensory domains. These

sensory domains likely regulate the activity of c-di-GMP domains (202). DGC and PDE domains can also be present in the same 'hybrid' protein (141).

Previously we showed that *F. diplosiphon* displayed different intracellular levels of c-di-GMP under different light qualities (16). In Chapter 3, we showed that Chl*a* is upregulated in *F. diplosiphon* expressing a heterologous PDE that results in lower intracellular c-di-GMP levels compared to the WT strain. We hypothesized that this phenotype are under the control of native c-di-GMP proteins composed of c-di-GMP domains and a sensory light domain. There are two hybrid c-di-GMP genes associated with photoreceptors in the *F. diplosiphon* draft genome present in the Integrated Microbial Genomes (IMG) database (51). *F. diplosiphon* evolved to sense available wavelengths of visible light that prevail at different depths in the water column (203). Since light plays a fundamental role in *F. diplosiphon* pigmentation and morphology, we examined the effect of c-di-GMP on light-dependent physiological responses in *F. diplosiphon*.

4.2 RESULTS

There are 20 c-di-GMP domains annotated in the genome of the filamentous cyanobacterium *F. diplosiphon* (Table 4.1). Two of these c-di-GMP genes were associated with the GAF (cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA) photoreceptor domain. GAF domains can perceive different light qualities and intensities. The gene 2501542931 (hereafter *cdgA*) is composed of a GAF domain, a PAS domain, a DGC domain, and a PDE domain (Fig.4.1). The gene 2501545761 (hereafter *cdgE*) is composed of a FHA domain, a GAF domain, a DGC domain, and a PDE domain (Fig.4.1). The DGC and PDE domains in both genes have eight amino acid residues known to be critical for activity in functional DGC domains (162, 163), and

seven characteristic of functional PDE domains (164) (data not shown). On the other hand, the GAF domains did not show high identity based on amino acid sequence with known conserved GAF domains (204-206) (data not shown).

Table 4.2. **List of c-di-GMP genes in *F. diplosiphon* and relative co-domains occurring with c-di-GMP domains.**

Gene ID	**Domains**
GGDEF only	
2501539817	PAS, CHASE2, GGDEF
2501541100	Trans_reg_C, Hpt, GGDEF, Response_reg
2501542176	PAS, GGDEF, Response_reg
2501539560	GGDEF
2501546243	GGDEF, Response_reg
2501543484	PAS, GGDEF
2501540849	GGDEF, Response_reg
2501546246	GGDEF
GGDEF+EAL	
2501545863	EAL, GGDEF
2501545271	EAL, CHASE, GGDEF
2501542931 (***cdgA***)	EAL, PAS, GAF, GGDEF
2501546300	EAL, GGDEF, Response_reg
2501545761 (***cdgE***)	FHA, EAL, GAF, GGDEF
EAL only	
2501541878	EAL, Response_reg
2501547151	EAL, Peptidase_C14
HD-GYP	
2501539982	HD, 7TMR-HDED, 7TM-7TMR_HD
2501541282	HD, 7TMR-HDED, 7TM-7TMR_HD
2501540456	HD, AAA_33
2501543416	HD, Ppx-GppA

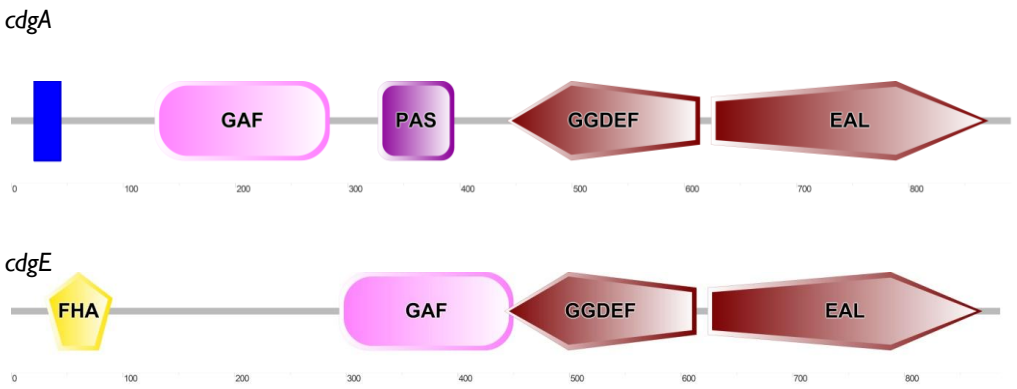

Figure 4.1. **Structural domains of the *cdgA* and *cdgE* genes**. GAF, photoreceptor domain; PAS, signal sensor; GGDEF, DGC domain; EAL, PDE domain; FHA, forkhead-associated domain.

We decided to analyze differences in pigmentation under low light (10 µmol m^{-2} s^{-1}) and under relatively high light (60 µmol m^{-2} s^{-1}) conditions. The growth rate between the WT and the Δ*cdgA* mutant did not vary under low light intensity or under relatice high light (Fig, 4.2). The Δ*cdgE* mutant grew similar to WT under high light (Fig. 4.2)

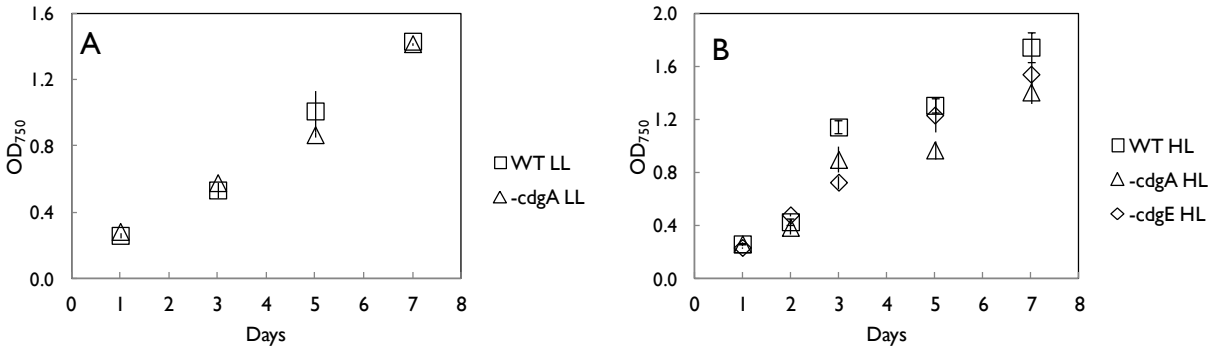

Figure 4.2. *F. diplosiphon* growth curve under low light (A) and high light (B). Cells were grown at 28 °C in 10 µmol m^{-2} s^{-1} WL for the low light condition and 60 µmol m^{-2} s^{-1} WL for the high light condition Vertical bars represent standard deviations. WT, wild-type strain; -cdgA, mutant lacking the *cdgA* gene; -cdgE, mutant lacking the *cdgE* gene.

The total Chl*a* level was similar between the WT and the Δ*cdgA* mutant, with the exception of the 5th day where Chl*a* in Δ*cdgA* was higher than in WT (Fig, 4.3A). Total carotenoids did not show differences among WT and Δ*cdgA* under low light with the exception of the 7th day where the Δ*cdgA* mutant had lower carotenoids (Fig. 4.3C). Under high light WT, Δ*cdgA* and Δ*cdgE* showed stronger differences compared to the condition of low light. Total Chl*a* was lower during the entire growth in the Δ*cdgA* mutant compared to WT (Fig. 4.3B). The Δ*cdgE* mutant had similar levels of Chl*a* to WT for the exception of the last day (Fig. 4.3B). The WT strain adapted to low light and transferred to high light conditions showed an increase of carotenoids after the second day confirming adaptation to the new condition (Fig. 4.3D). However, the Δ*cdgA* mutant showed a slower response compared to WT when exposed to high light (Fig. 4.3D). In general, carotenoids were lower than WT for the entire growth. The Δ*cdgE* mutant had similar levels of carotenoids compared to WT with the exception of the second day (Fig. 4.3D).

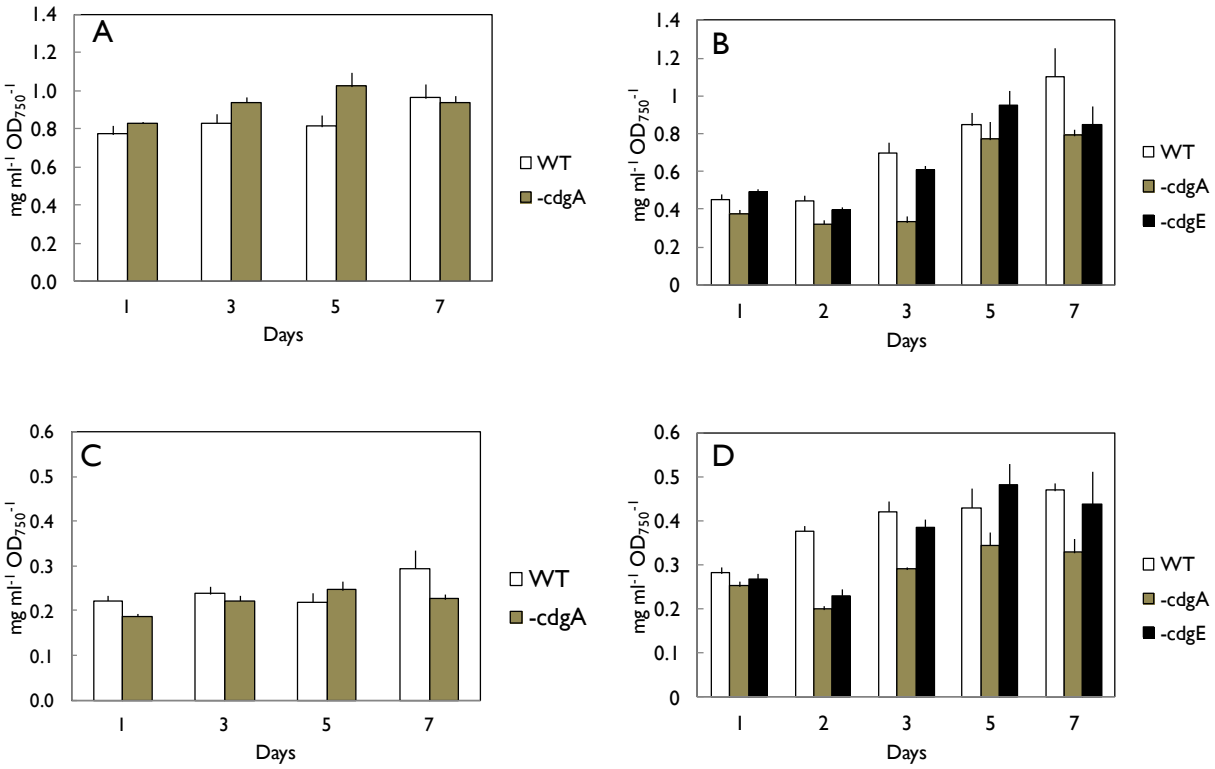

Figure 4.3. Photosynthetic pigment levels in *F. diplosiphon* strains. Quantification of total chl*a* under low light (A) and high light (B). Total carotenoids under low light (C) and high light (D). Vertical bars represent standard deviations. WT, wild-type strain; -cdgA, mutant lacking the *cdgA* gene; -cdgE, mutant lacking the *cdgE* gene.

Production of reactive oxygen species (ROS) is induced when pigments are not tuned to the external environment in photosynthetic organisms creating imbalance of photosynthetic electron transport (207). Under stressful light conditions, protection from ROS and reduction of chlorophyll content are enhanced (208, 209). Under low light conditions (with cells adapted to low light) ROS levels were low and similar in WT and Δ*cdgA* (Fig. 4.4A). However, under high light the Δ*cdgA* mutant showed higher production of ROS compared to WT both on the first and the second day (Fig. 4.4B). TheΔ*cdgE* mutant showed lower ROS levels compared to WT (Fig. 4.4B).

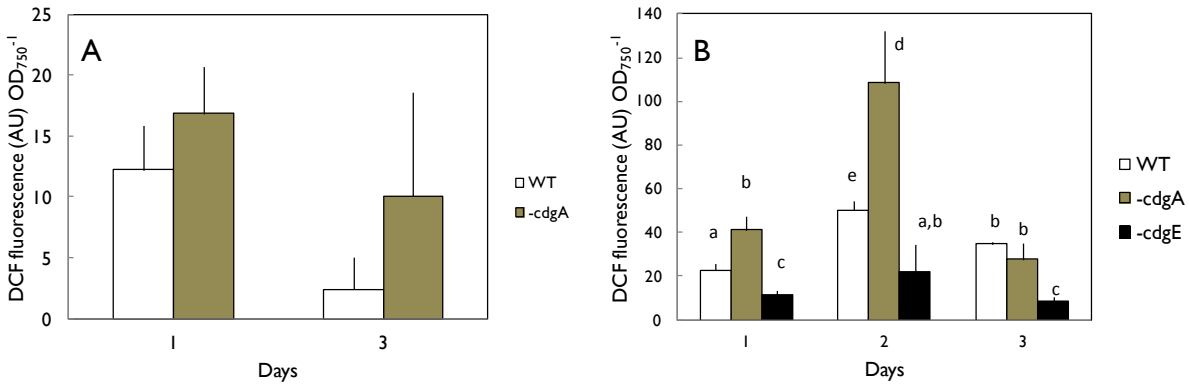

Figure 4.4.. **Reactive oxygen species (ROS) in *F. diplosiphon* under low light (A) and high light (B) regimes.** Sampling was normalized to OD_{750} of 0.2. Fluorescence values were obtained by subtracting fluorescence from growth medium mixed with DCFH-DA dye and fluorescence from samples containing only cells and lacking DCFH-DA dye. Bars represent averages (±standard deviation) from three independent biological replicates. Means with different letters are significantly different ($p < 0.05$). WT, wild-type strain; -cdgA, mutant lacking the *cdgA* gene; -cdgE, mutant lacking the *cdgE* gene.

The sensory domain could control the balance of the two enzymatic activities in hybrid c-di-GMP proteins (such as CdgA) where the DGC and PDE domains possess conserved amino acid residues (141). To understand the activity of the protein under the conditions in which we conducted the experiments, *in vivo* quantification of c-di-GMP was crucial. Cyclic di-GMP levels were similar in WT and Δ*cdgA* under low light (Fig. 4.5A). On the other hand, the intracellular level of c-di-GMP was higher in Δ*cdgA* and Δ*cdgE* than WT when low-light adapted cultures were exposed to high light (Fig.4.5B). This result confirmed that the CdgA and CdgE proteins have PDE activity under high light conditions, yet resulted in different phenotypes.

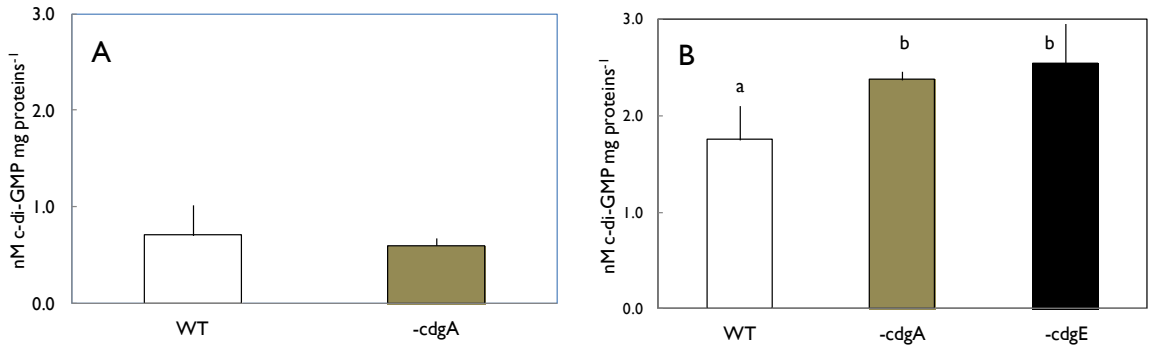

Figure 4.5. Effect of low light (A) and high light (B) on the intracellular concentration of c-di-GMP normalized to total proteins in *F. diplosiphon*. Means with different letters are significantly different (P<0.05). Vertical bars represent standard deviations. WT, wild-type strain; -cdgA, mutant lacking the *cdgA* gene; -cdgE, mutant lacking the *cdgE* gene.

4.3 DISCUSSION

In this study, we demonstrated that c-di-GMP is involved in light signaling networks by tuning the pigments of cells under changes to ambient light. Total chl*a* decreased in the three strains when low-light adapted cells were exposed to high light. The WT and Δ*cdgE* strains increased the total chl*a* levels after the third day likely to adapt to higher cell density in the flasks, whereas Δ*cdgA* increased the total chl*a* after the 4th day. The total carotenoids were overall higher under high light than under low light in WT, Δ*cdgA*, and Δ*cdgE*. However, after the first day the WT increased the amount of carotenoids under high light, whereas the Δ*cdgA* and Δ*cdgE* mutants did not. The higher generation of ROS in Δ*cdgA* also confirmed that the mutant was not acclimating to the new light condition as fast as WT or Δ*cdgE*. Taken together, these results indicate that the Δ*cdgA* and Δ*cdgE* mutants cannot correctly balance pigments under stressful high light growth. In specific, Δ*cdgA* is more affected under this condition. Although both mutants had higher

amount of c-di-GMP compared to WT, the Δ*cdgA* mutant showed a distinct phenotype compared to the Δ*cdgE* mutant.

In a previous study (Chapter 3), we confirmed differences in total Chl*a* when we artificially modulated levels of c-di-GMP in *F. diplosiphon*. These results confirmed that c-di-GMP can regulate pigment composition in *F. diplosiphon* under high light and that this second messenger is important for the cells to adapt to changes in light conditions.

4.4 FUTURE DIRECTIONS

Our understanding of the mechanisms of c-di-GMP signaling in *F. diplosiphon* is currently limited. To date, a detailed analysis of the composition of carotenoids in *F. diplosiphon* is unknown. The carotenoid composition in the Δ*cdgA* mutant could be compared to WT to determine potential differences in carotenoids profiles in these distinct strains. In addition, the Δ*cdgA* mutant showed similar Chl*a* levels to WT on day 5 under high light. This observation could be a result of c-di-GMP controlling pigments tuning only at early stages of adaptation.

4.4 METHODS

4.4.1 Culture conditions

F. diplosiphon was grown axenically and maintained at 28 °C in BG-11 (169) containing 20 mM HEPES at pH 8.0 with the indicated antibiotic when needed.The shortened-filament mutant strain SF-33, which displays wild-type (WT) pigmentation (168), was used as WT. Cells were

adapted to low white light (WL; Philips F32T8/TL741/ALTO) at 10 µmol m^{-2} s^{-1} with shaking at 175 rpm for at least 7 days. The flasks were then transferred to low WL (as control) and high WL condition of 60 µmol m^{-2} s^{-1}.

Cultures of *Escherichia coli* were grown at 37 °C in Luria–Bertani (LB) broth with the appropriate antibiotic. LB was solidified with 1.5% (w/v) Bacto-agar for growth on solid medium.

4.4.2 Plasmids construction

To study the effects of the c-di-GMP protein CdgA (Gene ID IMG 2501542931) and CdgE (Gene ID IMG 2501545761), *cdgA* and a *cdgE* knockout mutant were produced by introducing the suicide vector pJCF276 (210) containing homologous flanking regions to remove the two genes. Two kb upstream and downstream of the two genes were amplified from genomic DNA using PrimeSTAR Max DNA polymerase (Takara Bio, Inc.), creating two terminal *BspH*I restriction sites (table 1). The restricted PCR product was inserted in the *BspH*I site of the pJCF276 plasmid using Takara's DNA Ligation Kit, Mighty Mix. The resulting plasmids were introduced in *F. diplosiphon* by triparental mating as previously described (200). Colonies were selected after two weeks on BG-11 plates with 10 µg ml^{-1} (w/v) neomycin and successively on 5% sucrose. The absence of *cdgA* was confirmed by PCR.

Table 4.1. **Primers used in this study.**

	Forward primer (5' - 3')[a]	Reverse primer (5' - 3')
Δ*cdgA* Upstream	GAA**TCATGA**GGTGACCATGCT GAGC	CCAATGACAACTTTATAATTAAT AACCTGATTTAATATATAAC
Δ*cdgA* Downstream	GTTATATATTAAATCAGGTTAT TAATTATAAAGTTGTCATTGG	CGC**TCATGA**AAGAAGGCTGGGT G
Δ*cdgE* Upstream	TAT**CCATGG**CCATCCTGGTGA AACT	GACAATGTGAGGAAAAGACAATC CAAAATTTAAATAG
Δ*cdgE* Downstream	CTATTTAAATTTTGGATTGTCT TTTCCTCACATTGTC	CAT**CCATGG**ACATACCACCTGCT CTG

[a] Bold text indicates sequence of restriction site.

4.4.3 Pigments analyses

Chlorophyll *a* (chl*a*) and phycobiliproteins (PBPs) were extracted and quantified as described (201) on day one, three, five, and seven. Cyclic di-GMP was extracted and levels quantified as described (16). The growth rate was estimated by optical density at 750 nm (OD_{750}) at day one, three, five, and seven. Reactive oxygen species (ROS) were estimated as described in Appendice B at day one, two, and three.

4.4.4 Bioinformatics analyses

The available draft genome present in the Integrated Microbial Genomes (IMG; IMG Submission ID 551) system during the preparation of this manuscript was used to assess the presence of indicated conserved c-di-GMP domains in *F. diplosiphon*.

4.4.5 Statistics

The statistical significance of the effect of particular light conditions on c-di-GMP levels was determined via one way analysis of variance (ANOVA) with Fisher post-hoc test using OpenStat statistical software (version 10.01.08; W. G. Miller http://www.Statprograms4U.com). Statistical analyses were performed utilizing 95% confidence intervals ($p < 0.05$). The c-di-GMP domains and the GAF domain were aligned with MUSCLE (211).

CHAPTER FIVE

Conclusion

CONCLUDING REMARKS

To date, second messengers have been shown to play key roles in controlling fundamental and perhaps underappreciated aspects of photosynthetic-related processes. Heterocyst differentiation is regulated by a complex signaling network involving cAMP, Ca^{2+}, and (p)ppGpp. Phototaxis is regulated by both cAMP and c-di-GMP, whereas UV photoprotective mechanisms are regulated by cAMP and cGMP. Second messengers in cyanobacteria also function in pathways mediating cellular responses to oxidative stress, nutrient imbalances, and temperature variations in the environment. In the future, new studies are anticipated to shed light on additional phenotypes that are under control of second messenger molecules. The regulation of c-di-GMP and light sensing may offer niche differentiation, reduce competition, and allow a variety of phytoplankton to move using pilus-based motility. Defining genes and phenotypes controlled by c-di-GMP, elucidating the mechanisms for regulating the intracellular concentration of c-di-GMP in the cell, and characterizing the regulatory pathways impacting c-di-GMP levels may help us to better understand light signaling networks in cyanobacteria. In addition, elucidation of the roles of uncharacterized second messengers, including c-di-AMP, is anticipated to provide additional insights into the complex biological networks and physiological responses regulated by second messengers. Such additional knowledge gained about second messenger signaling pathways may support the development of new tools for biotechnological, optogenic, and therapeutic applications.

5.1. Second Messengers and Practical Application in Biotechnology or Therapeutics

Cyanobacteria have a realistic potential to generate high-value bioindustrial products using partially or fully enclosed bioreactors (212, 213). Biofuel, ethanol, isobutanol, alkanes, biodiesel, hydrogen, sugars, and medical products are just a few examples of compounds that cyanobacteria can produce (reviewed by (18, 19)). Engineering cyanobacteria for efficient growth and harvesting is a priority to decrease the costs and reduce environmental trade-offs. Additional insights will provide a better understanding into the roles of second messengers in regulating specific aspects of cyanobacterial growth, including the impact of environmental factors and the ability to induce floating or biofilm formation. Introducing exogenous enzymes that could regulate intracellular levels of second messengers under the control of inducible promoters could be an attractive tool for regulated growth of these organisms. For instance, in partially or fully enclosed bioreactors cells could be induced to aggregate and deposit in the bottom or to float to the surface by expressing exogenous enzymes to promote energy-efficient harvesting of biomass. In addition, the induction of biofilm formation could be applied in environments contaminated by positively charged heavy metal ions, as biofilms include exopolysaccharides which have been considered useful for metal biosorption (191).

Second messengers that are not synthesized by eukaryotes have enormous engineering potential for use in mammalian cell therapeutics. Many potential applications are possible, including controlling the levels of second messengers through light-dependent mechanisms by associating second messenger homeostasis domains with a photoreceptor (214). Proteins containing cAMP and cGMP synthesizing domains associated with photoreceptors have been engineered for potential optogenetic applications (214-218); yet, as cAMP and cGMP are present in mammals these two second messengers may not be optimal for cell-based therapeutics. However, there may be potential for the development and use of light-activatable, nucleotide second messenger-

degrading enzymes to regulate levels of these molecules *in vivo* for therapeutic purposes. Cyclic di-GMP or c-di-AMP are particularly interesting therapeutic targets as they are not present in mammalian cells and therefore are not expected to interfere with or alter native physiological processes (219). Cyclic di-GMP and c-di-AMP can directly induce a STING protein-dependent response, which leads to the production of cytokines essential for the induction of an innate immunity response to bacterial infection (220). Furthermore, cyanobacterial photoreceptor domains such as the GAF domain can absorb a wide range of light wavelengths (204); including wavelengths that represent regions of low light absorption in mammalian tissues (221). Thus, there is significant potential for the development of ontogenetic tools based on the large number of putative light-responsive c-di-GMP homeostasis proteins found in cyanobacteria (16).

APPENDICES

APPENDIX A

Analyses of c-di-AMP and c-di-GMP under osmotic and ionic stress in cyanobacteria.

INTRODUCTION

Osmotic stress is induced by the addition of organic solutes to the medium, which are not permeable to the bacterial cell. These organic solutes affect the water potential without producing ionic stress. Although salt stress is often referred to as osmotic stress (222), the ions Na^+ and Cl^- are actively transported outside the cells under hypersaline conditions or transported inside the cell under hyposaline conditions (223). In bacteria, osmotic stress causes accumulation of osmolytes in the cytoplasm (224), which will lead to enlargement of cells in volume (224), with a decrease of diffusion of macromolecules (225), ending with an impairment of growth rate (226-228).

It has been proposed that cyanobacteria, prokaryotic organisms capable of oxygenic photosynthesis, that are adapted to osmotic or salt stress have a lower amount of protein per cell resulting in reduction of growth rate (229). In cyanobacteria osmotic stress and salt stress control different sets of genes (230, 231). In the cyanobacteria *Synechococcus* R-2 and *Synechocystis* sp. PCC 6803 (here after *Synechocystis*) osmotic stress decreased the cytoplasmic volume and inactivated photosynthetic activity (232-234). In *Synechocystis* and *Nostoc commune* sorbitol inhibited the energy transfer from phycobilisomes to chlorophyll in photosystems (235, 236). Salt stress does not decrease the cytoplasmic volume as effectively as osmotic stress (230), but inactivates photosynthetic machinery thereby blocking the transport of electrons from water to photosystem II (232, 233). However, under salt stress photosystem I activity increased in *Spirulina platensis* (237). Both cellular morphology and photosynthetic pigment accumulation were impaired in *F. diplosiphon* under salt stress (238, 239).

In cyanobacteria, the second messenger Ca^{2+} is involved in sensing environmental osmotic and salt changes (22). Recently, assessment of regulons of riboswitches involved in binding the second messenger c-di-AMP suggested a function of c-di-AMP under osmotic stress in cyanobacteria (94). Cyclic di-AMP is a relatively newly discovered cyclic dinucleotide (240, 241). In the Gram-positive bacterium *Bacillus subtilis*, c-di-AMP promotes progression of sporulation and c-di-AMP levels decrease in response to DNA lesions (242). In another Gram-positive bacterium *Staphylococcus aureus* c-di-AMP is involved in controlling cell size and envelope stress (243). More recently, it has been proposed that c-di-AMP is also an essential signal molecule required for cell wall homeostasis in *Bacillus subtilis* (244). It has also been shown that c-di-AMP is critical for growth, cell wall homeostasis, and infection in the Gram-positive bacterium *Listeria monocytogenes* (245).

Cyclic di-AMP is synthesized by diadenylyl cyclase (DAC; PF02457) from two molecules of ATP and degraded by specific phosphodiesterase (PDE) enzymes into pApA (8). All cyanobacteria possess at least one DAC, with some exceptions carrying two DACs (170). To date, two PDEs have been discovered in bacteria: one containing a DHH-DHHA1 domain (8, 240) and probably not present in cyanobacteria; the other with a domain architecture similar to the 7TMR-HD protein family (246). The 7TM_7TMR_HD is more common than DHH-DHHA1 domain-containing PDEs in bacteria and it is also present in cyanobacteria (232, 246). In this study, we investigated the activity of DAC and PDE enzymes in *Synechocystis* and we assessed the c-di-AMP modulating activity in *Synechocystis*, *F. diplosiphon*, *Synechococcus elongatus* PCC 7942 (hereafter *Synechococcus*), and *Anabaena* sp. PCC 7120 (hereafter *Anabaena*) under osmotic and ionic stresses. In addition, since c-di-AMP could also control the synthesis of the

second messenger c-di-GMP (94) we assessed the concentration of c-di-GMP in these four species under osmotic and ionic stress.

RESULTS

Bioinformatics analysis

All cyanobacteria contain one copy of DAC, with the exception of *Cyanothece* sp. PCC 7424, *Cyanothece* sp. PCC 7822, *Gloeobacter kilaueensis* JS1, *Gloeobacter violaceus* PCC 7421 which carry two copies (170). Notably, the DACs from cyanobacteria are lacking additional domains, in contrast with DACs from other bacteria (247). The genus *Gloeobacter* which represents the primordial cyanobacteria (248) possess DACs suggesting that c-di-AMP signaling was present early during the evolution of cyanobacteria. Phylogenetic analysis based on amino acid sequence of DAC in cyanobacteria (Fig. AA.1) suggested that DACs have been vertically transferred as similar typology tree from phylogenetic diversity of cyanobacterial genomes is apparent (249). Interestingly, one of the two copies of DAC in cyanobacterial species having two DACs is extremely divergent from that of all the other DACs from cyanobacterial species with just one copy (Fig. AA.1).

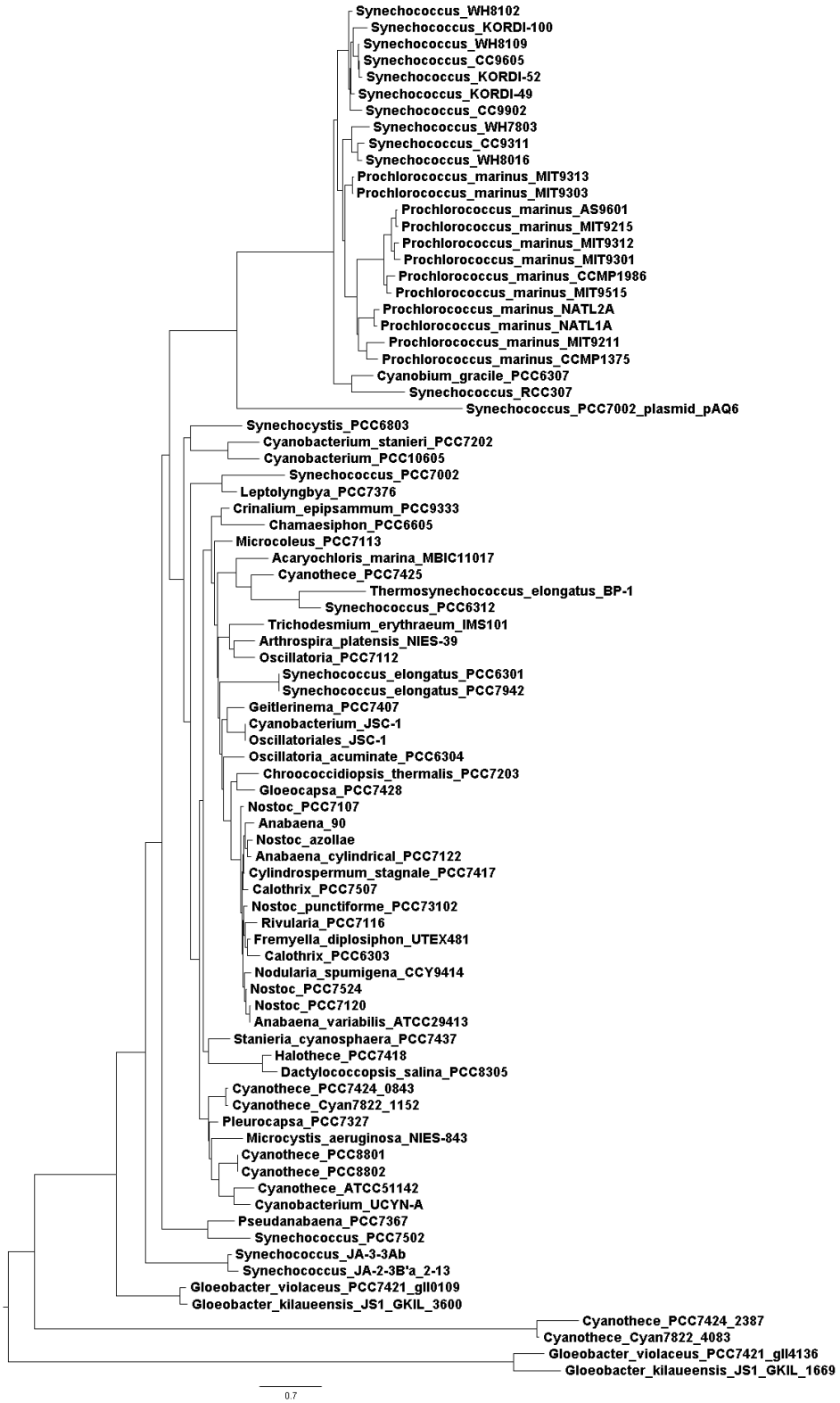

Figure AA.1. **Phylogenetic analysis based on multiple presumably functionally conserved DAC sequences.** For the species with two DACs, the gene identification number was added.

The genes diaminopimelate decarboxylase (DAPDC) and undecaprenyl pyrophosphate synthase (UPP) are always downstream and upstream, respectively, from the DAC gene in cyanobacteria. It appears that the grouping of these three genes is a unique feature of cyanobacteria. In the species with two DACs, only one DAC is found in this gene arrangement. DAPDC catalyzes the last step in the biosynthesis of lysine, a peptidoglycan component (250), while the UPP is critical as lipid carrier for peptidoglycan synthesis(251). The DAPDC (*sll0504*) in *Synechocystis* is upregulated under salt stress (252). The gene encoding UPP has been well conserved during evolution and in *E. coli* temperature-sensitive mutants showed abnormal swollen cell shapes at nonpermissive temperatures (253). In *Synechocystis*, there is a gap of 42 bases between the DAPDC (*sll0504*) and DAC (*sll0505*), while the DAC is overlapping with the UPP gene (*sll0506*). We decided to verify whether the three genes were co-transcribed. RT-PCR showed that the three genes were transcribed together (Fig. AA.2) suggesting that they may be united by a coherent functional theme.

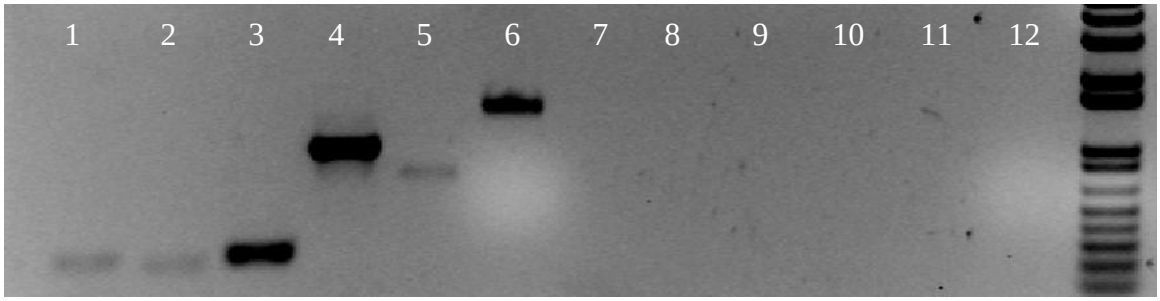

Figure AA.2. **RT-PCR of Diaminopimelate Decarboxylase (DAPDC; *sll0504*), DAC (*sll0505*), and Undecaprenyl Pyrophosphate Synthase (UPP; *sll0506*) in *Synechocystis***. Column 1, DAPDC; Column 2, DAC; Column 3, UPP; Column 4, DAPDC and DAC; Column 5, DAC and UPP; column 6, DAPDC and UPP. Column 7, DAPDC without RT reaction; Column 8, DAC without RT reaction; Column 9, UPP without RT reaction; Column 10, DAPDC and DAC without RT reaction; Column 11, DAC and UPP without RT reaction; column 12, DAPDC and UPP without RT reaction.

Overexpression of native DAC and PDE in Synechocystis

In *Synechocystis* there is only one DAC (PF02457, *sll0505*) and one PDE (belonging to the 7TM_7TMR_HD family; *slr0104*). In many bacteria c-di-AMP is essential for survival (247). Similarly, we were not able to produce a mutant lacking *sll0505* in *Synechocystis* (data not shown). We then decided to overexpress the DAC and PDE native enzymes in *Synechocystis* to confirm their activity *in vivo*. Quantitative RT-PCR showed increased accumulation of the mRNA for DAC and PDE genes in the DAC and PDE overexpression strains, respectively (Fig. AA.3). The DAC strain had a 40-fold increased level of the DAC mRNA compared to WT (Fig. AA.3A), whereas the PDE strain had a 200 fold increase of compared to WT (Fig. AA.3B). Enzymes responsible for the synthesis and degradation of second messengers are generally constitutively present in the cell for rapid activation. For this reason, we quantified changes of DAC and PDE mRNA levels in WT under osmotic and ionic stresses. After 24 hours of osmotic or ionic stress, levels of DAC and PDE mRNA decreased compared to conditions of no-stress (Fig. AA.3). The DAC mRNA levels decreased 0.37-fold and 0.66-fold under sorbitol and salt stress, respectively (Fig. AA.3A). The PDE mRNA levels decreased 0.55-fold and 0.66-fold under sorbitol and salt stress, respectively (Fig. AA.3B).

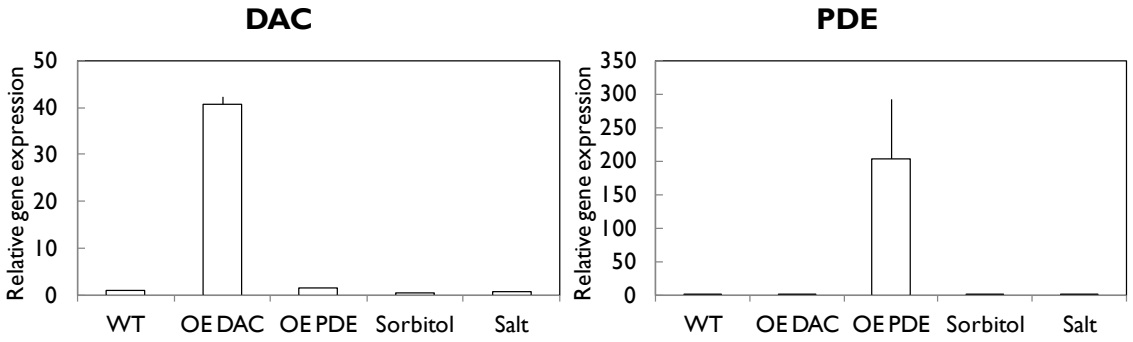

Figure AA.3. **Quantitative reverse transcriptase PCR (qRT-PCR) analysis of the expression of DAC (A) and PDE (B) genes.** In *Synechocystis* under 15 µmol m^{-2} s^{-1} white light. Wild-type (WT), overexpression DAC (OE DAC), overexpression PDE (OE PDE), WT under sorbitol stress (Sorbitol), WT under salt stress (Salt). Vertical bars represent standard deviations.

Quantification of c-di-AMP levels of the DAC and PDE overexpression strains confirmed that these two native enzymes could modulate levels of c-di-AMP in the cells (Fig. AA.4). The levels of c-di-AMP in the DAC overexpression strain were 1.7 fold higher ($p<0.05$) than the in WT strain. The levels of c-di-AMP in the PDE overexpression strain were half as much as inWT strain but not significantly different. Although it has been suggested that c-di-AMP could control the synthesis of the second messenger c-di-GMP (94), there was no difference in c-di-GMP levels between WT, DAC, and PDE strains (data not shown).

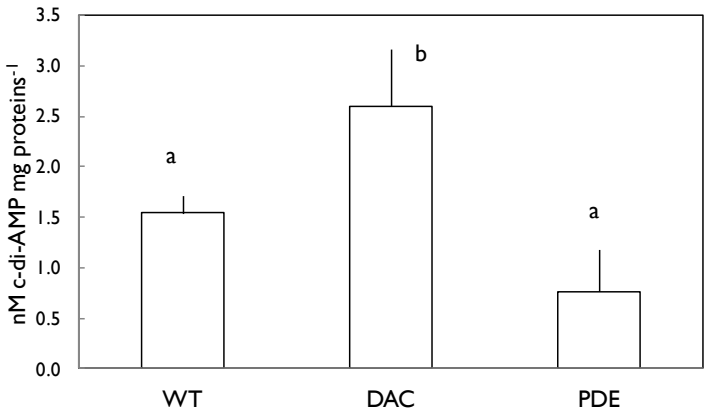

Figure AA.4. **Cyclic di-AMP levels in Synechocystis.** Under 15 µmol m^{-2} s^{-1} white light. WT, wild-type strain; DAC, strain overexpressing the native DAC (*sll0505*); PDE, strain overexpressing the native PDE (*slr0104*). Means with different letters are significantly different ($P<0.05$). Vertical bars represent standard deviations.

Previously, it has been observed that c-di-AMP homeostasis is fundamental for optimal growth (254). Our overexpressing strains which showed intracellular c-di-AMP differences *in vivo* compared to the WT also showed a slower growth rate compared to the WT (Fig. AA.5A). These growth differences may be due to WT growing homogenously in the medium, whereas DAC and PDE strains formed longer or short aggregates, respectively, in the flasks (Fig. AA.5B).

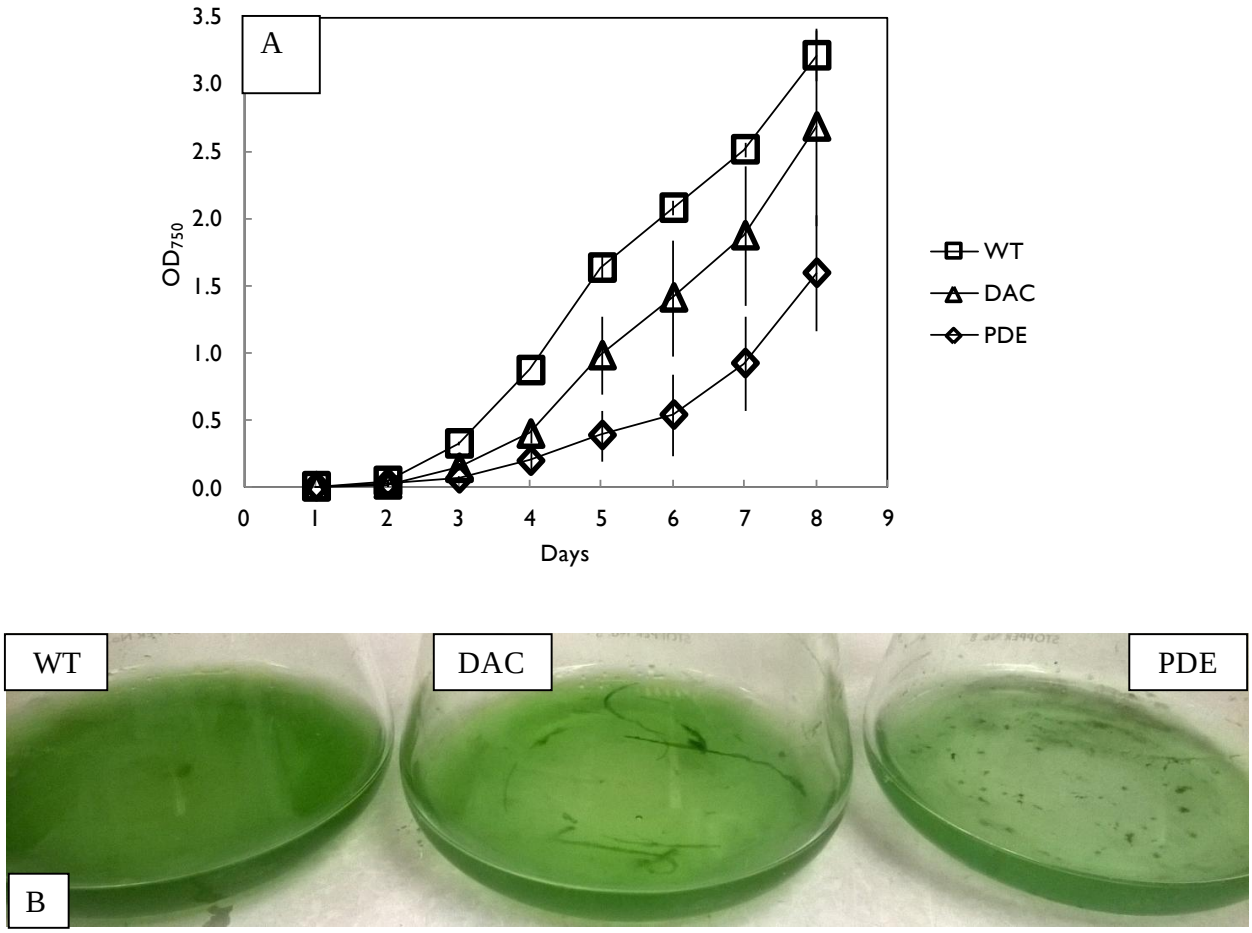

Figure AA.5. (**A) Growth curve.** Of *Synechocystis* wild-type (WT), strain overexpressing diadenylyl cyclase (DAC), and strain overexpressing phosphodiesterase (PDE) under 35 µmol m^{-2} s^{-1} white light.. (B) Picture of WT, DAC, and PDE strains. WT was homogenous during growth; however DAC and PDE displayed anomalous aggregation. Vertical bars represent standard deviations.

Osmotic and ionic stresses.

It has been suggested that in cyanobacteria c-di-AMP could be critical for synthesis of osmoprotectants (94). We exposed four different species of cyanobacteria to sorbitol or salt stress for 24 hours. Two unicellular cyanobacteria, *Synechocystis* and *Synechococcus*, and two filamentous cyanobacteria *Anabaena* and *F. diplosiphon*. In *Synechocystis*, qRT-PCR showed a

0.66-fold decrease in DAC mRNA levels and a 0.51-fold decrease in PDE mRNA levels under osmotic stress (Fig. AA.3). There was also a decrease in mRNA accumulation of the DAC and PDE genes under ionic stress, 0.33-fold for DAC and 0.31-fold for PDE (Fig. AA.3). In *Synechocystis* c-di-AMP levels were 3 fold higher under sorbitol stress. However, under our conditions there were no differences in intracellular levels of c-di-AMP in *Synechococcus*. In the filamentous cyanobacterium *F. diplosiphon* c-di-AMP levels increased 2 fold under sorbitol stress. In *Anabaena* sorbitol did not result in an increase in c-di-AMP under our conditions (Fig. AA.6A). These analyses confirmed that under our conditions c-di-AMP is important during osmotic stress at least in *Synechocystis* and *F. diplosiphon*.

We also investigated whether c-di-AMP levels varied in these four cyanobacterial species under salt stress (fig. AA.6A). Under salt stress c-di-AMP levels were overall lower than under no-stress conditions.

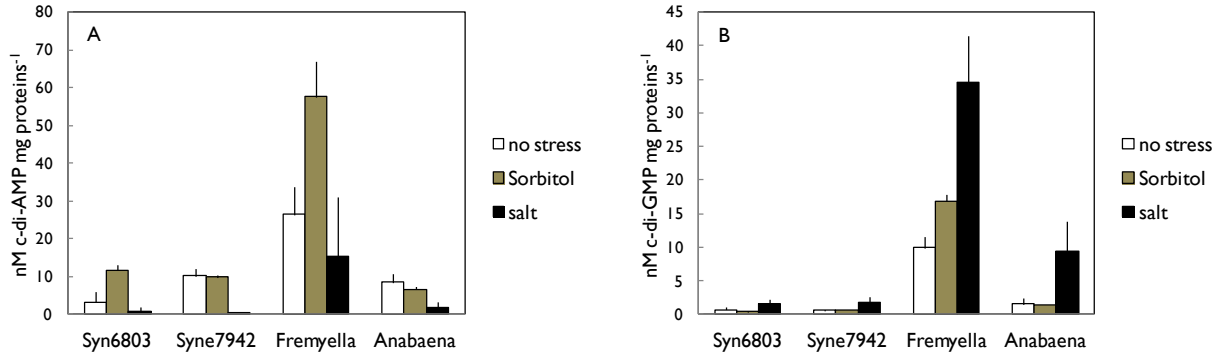

Figure AA.6. **Cyclic di-AMP (A) and cyclic di-GMP (B) levels under sorbitol and salt stress.** Cyclic di-AMP and cyclic di-GMP levels normalized to total proteins. Vertical bars represent standard deviations.

In cyanobacteria, biofilm formation is a protective mechanism against salt stress (174). We previously demonstrated that induction of biofilm formation is under the control of c-di-GMP (Chapter 3). As a consequence, we quantified c-di-GMP levels under salt stress. Not

surprisingly, c-di-GMP levels increased in these cyanobacteria after 24 hours of salt stress (Fig. AA.6B) in all four species. In contrast to stress caused by salt, under sorbitol stress the levels of c-di-GMP did not vary in *Synechocystis*, *Synechococcus*, and *Anabaena* (Fig. AA.6B). Cyclic di-GMP increased under sorbitol stress in *F. diplosiphon* (Fig. AA.6B). Taken together, these analyses suggested that c-di-AMP responded to osmotic stress, whereas c-di-GMP responded to ionic stress.

DISCUSSION

The majority of cyanobacterial species have one encoded DAC with the exception of *Cyanothece* sp. PCC 7424, *Cyanothece* sp. PCC 7822, *Gloeobacter kilaueensis* JS1, *Gloeobacter violaceus* PCC 7421. In all cyanobacteria, the DAC gene is surrounded by the Diaminopimelate Decarboxylase and the Undecaprenyl Pyrophosphate Synthase genes downstream and upstream, respectively. In the species with two encoded DAC genes, one of the two DACs does not have the Diaminopimelate Decarboxylase and the Undecaprenyl Pyrophosphate Synthase genes downstream and upstream. These isolated DACs also outgrouped from the rest of the DACs with the Diaminopimelate Decarboxylase and the Undecaprenyl Pyrophosphate Synthase genes downstream and upstream, suggesting that they have significant genomic differences. Since the genus *Gloeobacter*, which is considered primordial group of cyanobacteria (248), possesses two DAC enzymes, in contrast with the majority of cyanobacterial species that have only one DAC enzyme, we speculate that the primordial cyanobacteria (genus Gloeobacter) possessed two DAC enzymes, but during evolution the second DAC was lost. Since the DAC, Diaminopimelate Decarboxylase and the Undecaprenyl Pyrophosphate Synthase cassette is

extremely conserved among the cyanobacteria, these three genes together likely play a critical role in regulating cell wall synthesis.

Quantitative RT-PCR analysis of DAC and PDE in *Synechocystis* under our conditions did not show regulation of mRNA accumulation of these genes. We were able to strongly increase transcript accumulation of DAC and PDE using overexpression plasmids (Fig. 3). In addition, both under osmotic and ionic stress there was an overall decrease of transcript levels. Prior studies (232, 255) showed very little differences in transcripts in *Synechocystis* after 20 minutes exposure to sorbitol and salt both for DAC and PDE (see also (256)). If we assume that accumulated transcripts levels in the overexpression strains reflects the same pattern of accumulation at the protein level, c-di-AMP is not controlled at the transcriptional level, but more likely at a translational or post-translational level.

Higher or lower levels of c-di-AMP are detrimental to normal growth in *Bacillus subtilis*, *Listeria monocytogenes,* and *Staphylococcus aureus* (243, 245, 254). In *B. subtilis* the differences in growth rates between WT and a strain with strong accumulation of c-di-AMP are probably due to aberrant cell morphologies (254). In *L. monocytogenes*, c-di-AMP is critical for bacterial replication (245). A mutation of the *S. aureus* phosphodiesterase GdpP results in increased peptidoglycan cross-linking severing growth (243). In this study, both *Synechocystis* strains with overexpression of DAC and PDE grew slower than WT, confirming that the correct homeostatic levels of c-di-AMP are critical for the fitness of the cyanobacterium *Synechocystis*. Indeed, the DAC and PDE strains formed longer and short aggregates, respectively, compare to WT which grew homogenously in the medium.

Sorbitol was an important factor in the regulation of c-di-AMP homeostasis, whereas salt was a critical factor in the regulatation c-di-GMP homeostasis. Under osmotic stress, bacteria respond

with enlargement of cells in volume (224). Change in shape of c-di-AMP mutants was observed in several bacteria (243, 245, 254). We propose that cell morphology could be affected in cyanobacteria as well in the overexpression strains DAC and PDE in *Synechocystis*. In the natural habitat, an increase of biofilm is associated with salt stress tolerance (257, 258). Cells induce biofilm formation as protective mechanism for salt stress (174).

CONCLUSION

Intensification of osmotic and ionic stresses is correlated with evaporation and precipitation fluxes (259). Osmotic and ionic stresses are becoming more common in natural ecosystems (232, 260, 261) and identifying the mechanisms by which cyanobacteria can tolerate osmotic and ionic stresses is critical. Species able to maintain osmotic equilibrium under these conditions will most probably be the predominant organisms for cyanobacterial mass cultivation (222). There is still a lack of knowledge on how cyanobacteria specifically sense osmotic and ionic stresses. Determining the molecular mechanisms of c-di-AMP and c-di-GMP signaling networks during cyanobacterial adaptation is necessary to understand how cyanobacteria survive in stressful and fluctuating environments and ensure biomass and product yields under osmotic and ionic stresses to improve applications and fundamental research in solving environmental problems.

METHODS

Plasmid construction in Synechocystis.

Levels of c-di-AMP in *Synechocystis* were artificially increased by overexpressing the native DAC protein Sll0505 and artificially decreased by overexpressing the native PDE protein Slr0104. DAC and PDE enzymes were constitutively overexpressed using the plasmid pRL1342 (C. Peter Wolk, GenBank: AF403427.1) under the *apcE slr0335* promoter. Promoters were added to the DAC- or PDE-encoding genes by overlap PCR using primers indicated in Table AA.1. The plasmid was inserted in *Synechocystis* has described in Chapter 3. Table 1 shows primers used for ligation.

Culture conditions

Axenic cultures of *Synechocystis*, *F. diplosiphon*, *Synechococcus*, and *Anabaena* were grown at 28 °C in BG-11 (169) containing 20 mM HEPES at pH 8.0 with the indicated antibiotic. *F. diplosiphon* strain SF33, a shortened-filament mutant strain that displays WT pigmentation (168), was used as the WT strain. Cultures (25 ml) in 250 ml glass flasks were adapted to fluorescent white light (WL; Philips F32T8/TL741/ALTO) at 15 µmol m^{-2} s^{-1} with shaking at 175 rpm for at least a week.

Growth rate of the WT, DAC, and PDE strains was estimated by optical density at 750 nm (OD_{750}) every day under WL (Philips F32T8/TL741/ALTO) at 35 µmol m^{-2} s^{-1} with shaking at 175 rpm.

Osmotic stress and c-di-AMP / c-di-GMP quantification.

Cells were grown to an optical density (OD_{750}) of 1 and transferred to new 250 ml flasks with 0.2 M sorbitol for the osmotic stress, or 0,2 M NaCl for the ionic stress at concentration. For *Synechocystis* the concentration was 0.6 M. As control, BG-11 was added without sorbitol or NaCl. Cells were maintained under osmotic stress for 24 hours. After 24 hours c-di-AMP and c-di-GMP were quantified as described in (16).

Quantitative reverse transcriptase PCR (qRT-PCR) and RT-PCR in Synechocystis.

For RNA extraction, *Synechocystis* cells from a 10 ml aliquot of culture were collected 24 hours after the osmotic stress or after subculturing. RNA was isolated as described (262). cDNA synthesis was performed as described in Appendix B. Table AA.1 shows primers used for qRT-CPR and RT-PCR.

Table AA.1. **Primers used in this study**.

	Forward primer (5' - 3')[a]	Reverse primer (5' - 3')
sll0504	ACCGGATGAACGACGAAATTA	TAGACAATCCTGGCGCAATAG
sll0505	GGAGTCGCCATTGACAGTAA	TCCTCGGAAACGACAATACAA
sll0506	CCGGATTTGGACCAGCA	TCCTTTAATTCCCGCCGTAG
slr0104	CGCCCAACTCAAACAAGAAAG	GTTGCTGCTCCAGGGTAAA
OEsll0505 _prapcE	CGC**GCTCGA**GTTAAAACTGCA TTATCAG	CTGTCAATGGCGACTCCCCGATT GAGGAAA
OEsll0505	TTTCCTCAATCGGGGAGTCGC CATTGACAG	CGC**GGATCC**TCATTTTTTGTCGTT
OEslr0104 _prapcE	CGC**GCTCGA**GTTAAAACTGCA TTATCAG	GGCAAAAATTGCTTTCATTGGAT TTCATTATCTCCC
OE slr0104	GGGAGATAATGAAATCCAATG AAAGCAATTTTTGCC	CTC**GGATCC**CTAAAATCTGGTGG TG

[a] Bold text indicates sequence of restriction site.

Genome comparisons.

Phylogenetic analyses of multiple conserved DAC domain sequences (PF02457) from the 83 finished cyanobacterial genomes present in the IMG database were performed using SeaView4 software (263). Multiple alignments of amino acid sequences were generated using MUSCLE (211). Phylogenetic trees were inferred using maximum likelihood-based method 100 bootstraps, and the Jones-Taylor-Thornton model (264). The likelihood log was -23144.7.

Statistics

The statistical significance of c-di-AMP and c-di-GMP levels was determined via one way analysis of variance (ANOVA) with Fisher post-hoc test using OpenStat statistical software (version 10.01.08; W. G. Miller http://www.Statprograms4U.com). Statistical analyses were performed utilizing 95% confidence intervals ($p < 0.05$).

APPENDIX B

Fluctuating light and phycobilisome flexibility in the cyanobacterium *Fremyella diplosiphon* using a competition approach

INTRODUCTION

The dynamic environments in which photosynthetic microorganisms live create selective pressures that contribute to niche partitioning in the water column (265). Natural selection drives niche partition or the differential use of resources or adaptation to environmental factors in the environment. Light is an important factor that regulates vertical niche differentiation; photosynthetic microorganisms receive higher irradiance and different predominant wavelengths near the surface than cells growing in deeper waters. Higher irradiance near the surface can potentially lead to photoinhibition or production of reactive oxygen species (ROS) that can damage the organism (266). In order to compete with other photosynthetic organisms, cells need to optimize photosynthesis and minimize photo-oxidative damage that could lead to cellular damage (267). In addition to vertical gradient differences in the water column, cells are also exposed to fluctuating light (FL) conditions. In aquatic systems, irradiance can change suddenly due to shifting meteorological conditions, a canopy of surrounding vegetation, or due to refraction and reflection of light in surface waters (268).

There is growing evidence that photosynthetic organisms are particularly sensitive to rapid changes in light, temperature, and nutrient availability. Recent studies have highlighted the importance of examining organismal phenotypes under a variety of different conditions (269-271) with a growing interest in the function and structural characteristics of the photosynthetic apparatus under different light regimes, including those more similar to natural environments (272-274). In these studies, some mutants showed no apparent phenotypes under standard growth chamber experiments, yet exhibited emergent mutant phenotypes under fluctuating environmental conditions which mimic more natural conditions (269, 270). Controlled

experiments with microalgae have been carried out in conditions ranging from natural environments to bioreactors with intermittent light regimes (275). It appears that the dark period during the intermittent light regime is an important factor for maintaining cellular metabolism (275). Intermittent light regimes can be separated into three major categories: low, medium, and high frequency fluctuations (276). Low frequency fluctuations in natural contexts are processes that control diurnal and seasonal variations (277); medium frequency cycles range from seconds to minutes associated with waves at the water surface (278); high frequency cycles considered shorter than 100 ms (279) can be caused by refraction and reflection in the surface waters (278). To cope with dynamic environments photosynthetic organisms regulate cell shape (105), photoprotective mechanisms (280), and acclimation or reconfiguration of the photosynthetic light-harvesting apparatus, such as alterations in phycobiliprotein pigment composition (265) and chlorophyll content (281).

Cyanobacteria are one of the most abundant oxygenic phototrophs in the ocean; many are able to fix both carbon and nitrogen under aerobic conditions and thus play a key role in the global carbon and nitrogen cycles (282). In most cyanobacteria, light is captured mainly by phycobilisomes (PBSs), protein complexes attached to the stromal surface of thylakoid membranes and associated with the chlorophyll-containing photosystems (283). The major role of PBS complexes is to transfer light energy to the chlorophylls of the reaction centers during photosynthesis (284). PBSs can be tuned to maximize light absorption in response to changes in the external environment. Through a process called state transitions, cyanobacteria redistribute PBSs between photosystem II (PSII) and photosystem I (PSI) to maximize absorption of light energy under low-light stress or to protect cells from excess of high light intensities. (285-288).

There is also evidence that PBSs can generate potentially deleterious ROS when the PBS size and composition is not tuned to the external environment (289, 290).

There is growing interest in the utilization of cyanobacteria for chemical and biomass production (18, 19). Recent studies were focused on reducing the PBSs size in cyanobacteria to increase biomass accumulation (291-293). *Synechocystis sp*. PCC 6803 (hereafter *Synechocystis*) mutants either with truncated or lacking PBSs grew poorly under standard carbon-sufficient lab growth conditions (292-295). By comparison, these mutants exhibited increased productivity in carbon-limited and high light conditions (292, 294, 295). It is important to note that these studies were conducted with the single cell cyanobacterium *Synechocystis* and cells were grown under continuous light conditions. Furthermore, studies to assess truncated antenna strains have to date been conducted with cyanobacteria that contain only phycocyanin (PC) in the PBS rods and not strains that exhibit greater flexibility of PBS structure and size such as *Fremyella diplosiphon*. *F. diplosiphon* is a freshwater filamentous cyanobacterium that has been extensively studied for its ability to adapt to variations in light quality and intensity by changing the size and pigment composition of its PBSs. *F. diplosiphon* changes pigmentation to maximize absorption of prevailing wavelengths of available light (145). In response to green light (GL), red-colored, GL-absorbing phycoerythrin (PE) is synthesized; conversely under red light (RL), green-colored, RL-absorbing phycocyanin (PC) is predominant. PC and PE are phycobiliproteins within the rods of PBSs that can capture and efficiently transfer light energy to the photosynthetic reaction centers within the thylakoid membranes, thereby enabling *F. diplosiphon* to maximize photosynthesis and growth under different external photoenvironments (296). This colorful phenotypic plasticity is an acclimation response historically called complementary chromatic adaptation (CCA) (145).

We used a competition approach to examine the fitness costs of modulating antenna size and composition in *F. diplosiphon* wild-type (WT) with defined PBS structures and a mutant lacking PBSs (FdCh1) (297) under standard or FL growth conditions. For the FL regime, we chose medium frequency fluctuations, i.e., cycles that range from seconds to minutes (278), as they are the most common type of fluctuations to which algae and cyanobacteria are exposed in natural habitats (278) (Fig. AB.1). CpcF encodes a subunit of a heterodimeric lyase enzyme that catalyzes the attachment of the chromophore to the α subunit of PC (298, 299). Thus, the CpcF-deficient FdCh1 strain is incapable of producing PBSs (297), which results in an inability to optimize absorption of available RL and GL. When the size and protein composition of the light-harvesting complexes are not tuned to the external environment in photosynthetic organisms, imbalances in electron transport can alter the cellular redox state and result in cellular damage (300-302). In direct competitions of the WT pigmentation strain of *F. diplosiphon* and the mutant FdCh1 lacking PBSs, we demonstrated that low antennae mutants can perform well relative to WT cells with a larger and light-tunable antenna under FL regimes. We also investigated the molecular bases of the observed differential growth to determine the biochemical and biophysical bases of comparative fitness of distinct strains under defined growth conditions.

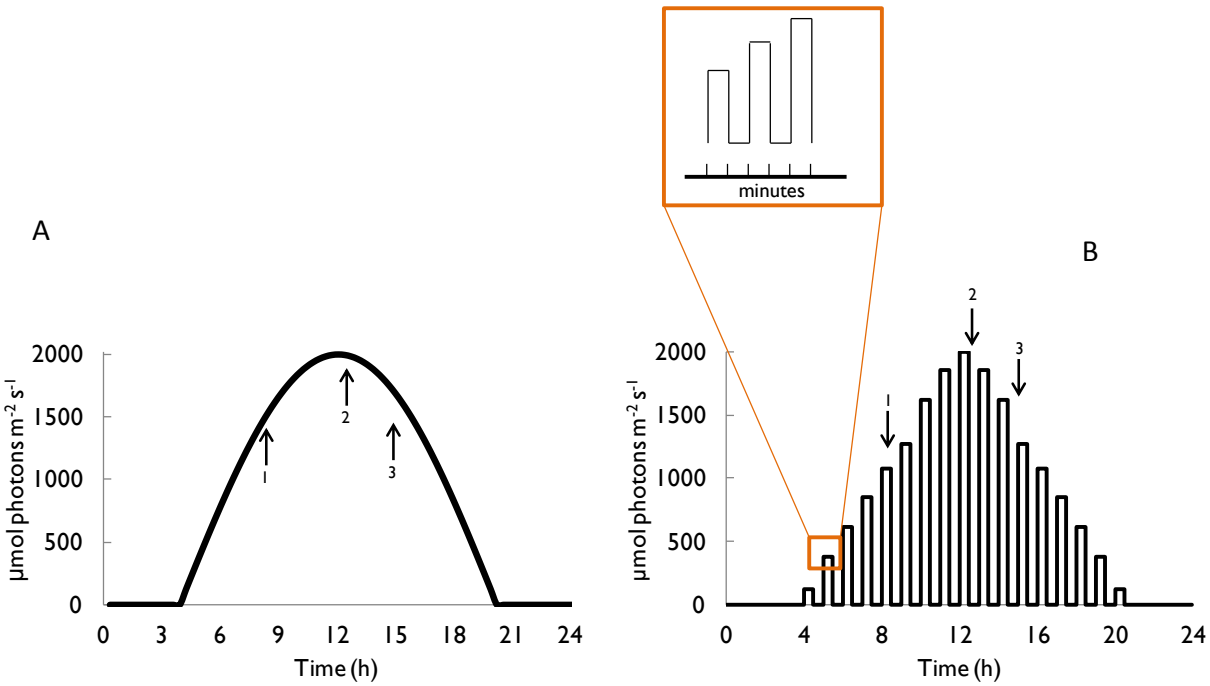

Figure AB.1. **Competition experiment growth conditions. (**A) continuous light; (B) fluctuating light regimes. Arrows indicate time of cell aliquots collected for (1) pigment extraction and quantification, qPCR, and qRT-PCR; (2) ROS measurements; (3) qRT-PCR.

RESULTS

We examined competitive fitness under high light stress of the WT strain that exhibits qualitative and quantitative flexibility of PBS relative to the FdCh1mutant that lacks PBSs. DNA samples of mixed cultures at OD_{750} of 0.1 were collected every 3 or 4 days to quantify relative cell density of the population. After 7 days, the WT started to outcompete the FdCh1 mutant under continuous light (CL) regimes. By 28 days, 91% (±3) of the population consisted of WT cells under CL conditions (Fig. AB.2A). By comparison, under fluctuating light (FL) regimes FdCh1 initially outnumbered WT and the WT composed 52% (±4) of the total population after 28 days (Fig. AB.2B), demonstrating that FdCh1 competed well against WT under fluctuating light conditions.

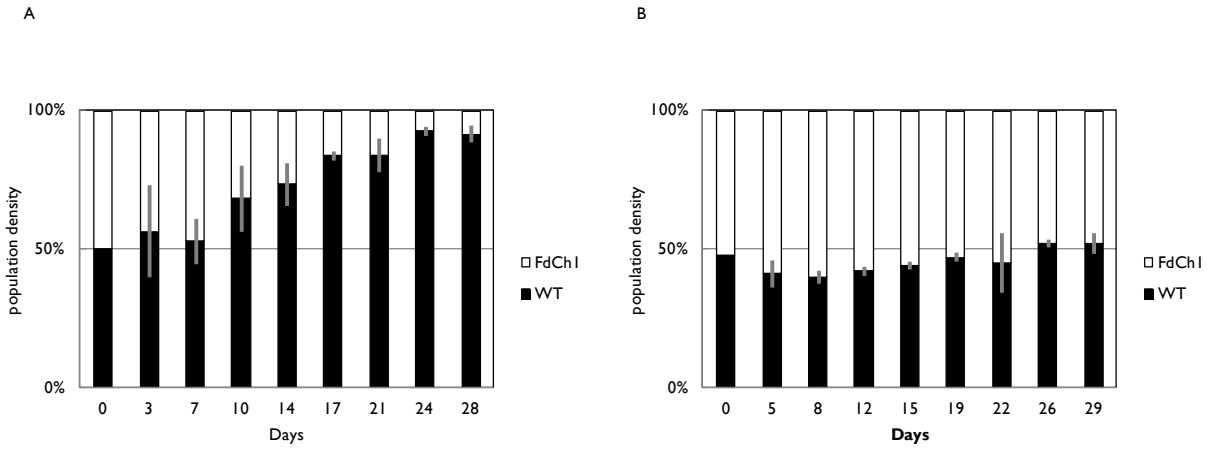

Figure AB.2. **Direct competition of wild-type (WT) and FdCh1 strains of *Fremyella diplosiphon* under continuous light (A) and fluctuating light (B) regimes**. The competition experiments were initiated with the two strains at the same initial density. Samples were collected every 3 or 4 days to quantify relative cell density of the population. Data points represent averages from three independent biological replicates. Vertical bars represent the standard deviation.

Physiological analyses

To gain insight into the mechanisms underlying the observed performance differences of the strains in the competition experiments, we analyzed physiological characteristics of WT and FdCh1 mutant grown in monoculture under CL and FL regimes to assess physiological differences that may be associated with the differential competitiveness of the strains under the distinct regimes.

Cells grew faster under CL regimes.

We investigated whether the growth of WT and the FdCh1 mutant were similar in monoculture under the two different light regimes, or whether the strains grew faster under one regime

compared to the other. For both strains, the growth rate was higher under CL regime compared to FL ($p<0.05$) (Fig. AB.3). Notably, the FdCh1 mutant (doubling time 0.48 d-1) grew faster than the WT (doubling time 0.4 d-1) ($p<0.05$) in monoculture (doubling time 0.48 d-1) than the WT under CL (doubling time 0.4 d-1) ($p<0.05$). Under FL, WT grew faster (doubling time 0.24 d^{-1}) than FdCh1 (doubling time 0.18 d^{-1}) ($p<0.05$) (Fig. AB.3). These findings confirm that FL resulted in decreased growth in both strains. As the grow rates of the strains in monoculture did not reflect the differences in fitness observed in the competition experiments, other parameters apart from growth rate may have impacted the WT, leading to its impaired fitness relative to FdCh1 in FL light compared to its performance in CL regimes.

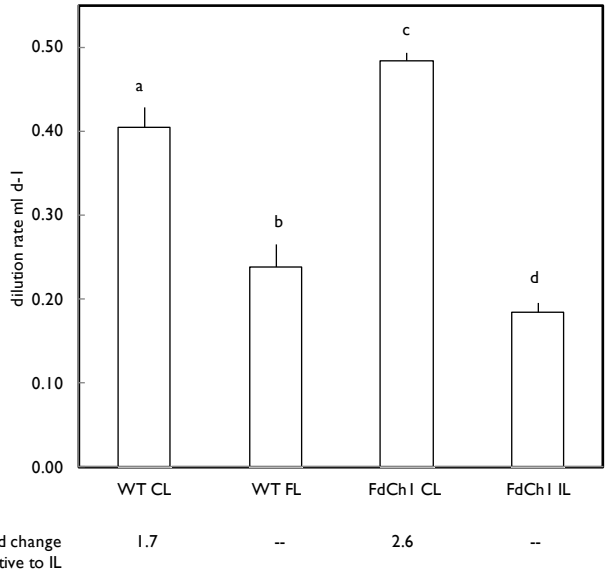

Figure AB.3. **Growth rate of monocultures of wild-type (WT) and FdCh1 strains of *Fremyella diplosiphon* under continuous light (CL) and fluctuating light (FL) regimes.** The growth rate was calculated as the rate of flow of medium over the volume of culture in the bioreactor per day. Bars represent averages (±standard deviation) from three independent biological replicates. Means with different letters are significantly different ($p < 0.05$).

WT produces longer PBSs under FL.

To investigate the differential regulation of photosynthetic pigment levels in WT compared to FdCh1, we quantified pigment composition under CL and FL regimes. Total Chl *a* per ml of culture was higher under FL (0.51 ml^{-1}) than under CL (0.38 ml^{-1}) in WT ($p<0.05$), but not significantly different under CL compared to FL in FdCh1 (Fig. AB.4A). Overall, WT contained more Chl *a* per ml than the FdCh1 mutant ($p<0.05$) (Fig. AB.4A). Carotenoids levels followed a similar trend. Carotenoids were slightly higher under FL than CL in both strains and overall higher in the WT than in FdCh1 (but not significantly in any case) (Fig. AB.4B). There was no significant difference in the PC to allophycocyanin (AP) ratio in WT under the two light regimes. However, the PE/AP and PE/PC ratios increased significantly under FL (1.76 and 2.98 respectively) compared to CL regime (1.13 and 1.82 respectively), suggesting acclimation of the PBS in WT under FL compared to CL. These increased PBP ratios were a function of higher levels of PE, which composes the majority of the PBSs under GL-enriched white light conditions (145) (Fig. AB.4C).

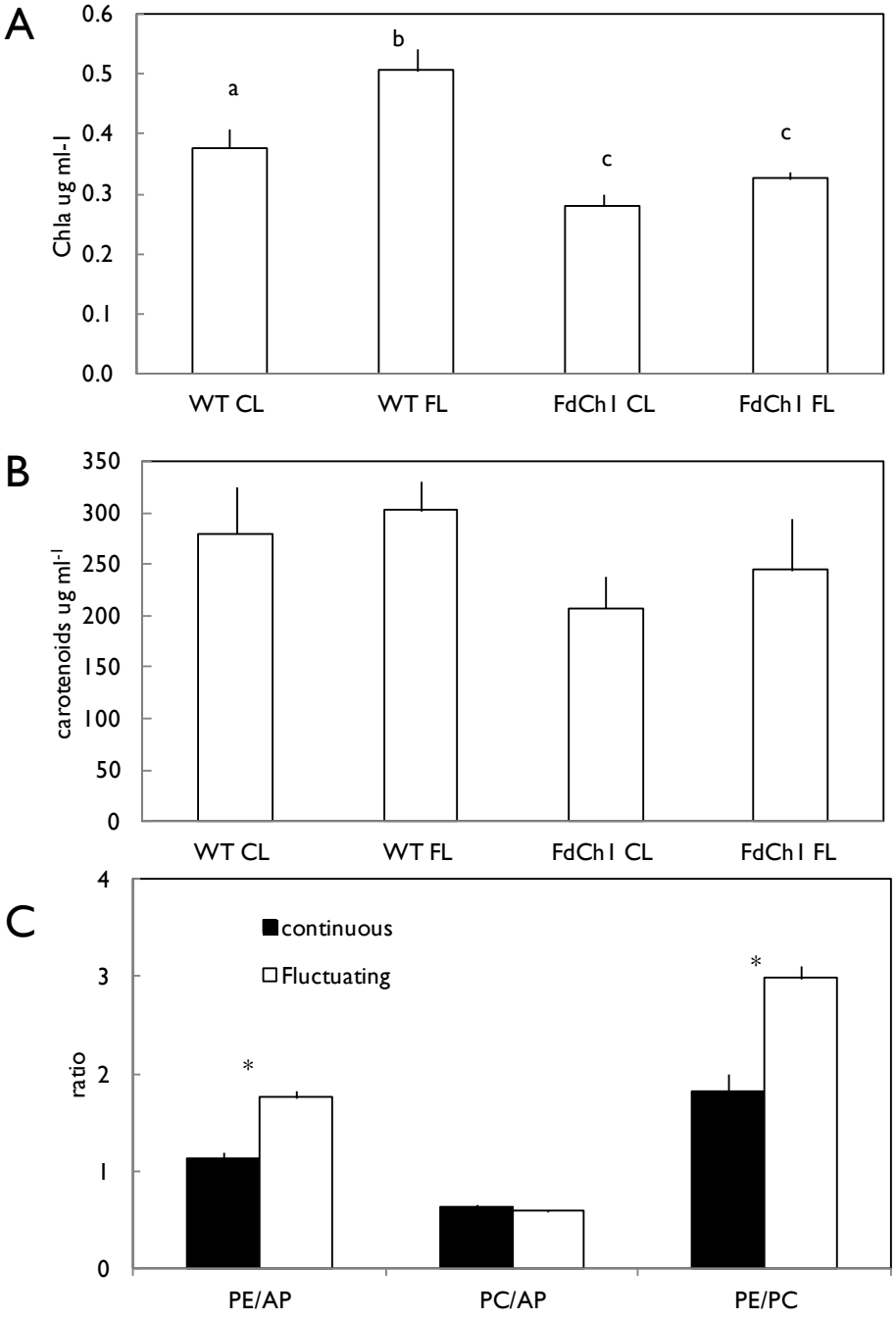

Figure AB.4. **Photosynthetic pigment levels were performed for monocultures of wild-type (WT) and FdCh1 strains of *Fremyella diplosiphon* under continuous light (CL) and fluctuating light (FL) regimes**. Total chlorophyll *a* (Chl*a*) (A), carotenoids (B), and PBSs (C). Pigment extractions and quantifications were carried out three hours before the maximum peak of light. PE, phycoerythrin; PC, phycocyanin; AP, allophycocyanin. Bars represent averages (±standard deviation) from three independent biological replicates. Means with different letters are significantly different ($p < 0.05$).

Reactive oxygen species (ROS) were higher in WT under FL regimes.

PBSs can be a source of ROS when cyanobacteria are exposed to light at intensities that exceed the capacity for photochemistry (289). Intracellular levels of ROS were quantified under CL and FL regimes using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). The total ROS production in WT was higher ($p<0.05$) under FL (0.86 AU) than under CL (0.26 AU) and relative to the FdCh1 mutant under FL conditions (0.31 AU) on the first day (Fig. AB.5). ROS levels in the FdCh1 mutant were similar under FL and CL conditions. ROS levels decreased in WT under FL over the course of the experiment from 0.86 AU to 0.67 AU the third day, and to 0.53 AU the seventh day, suggesting that acclimatory processes were induced. By comparison, the FdCh1 mutant did not exhibit a significant increase in ROS levels under FL during the time course of the experiment, although on the first day ROS levels were 50% lower under FL than under CL (however $p>0.05$).

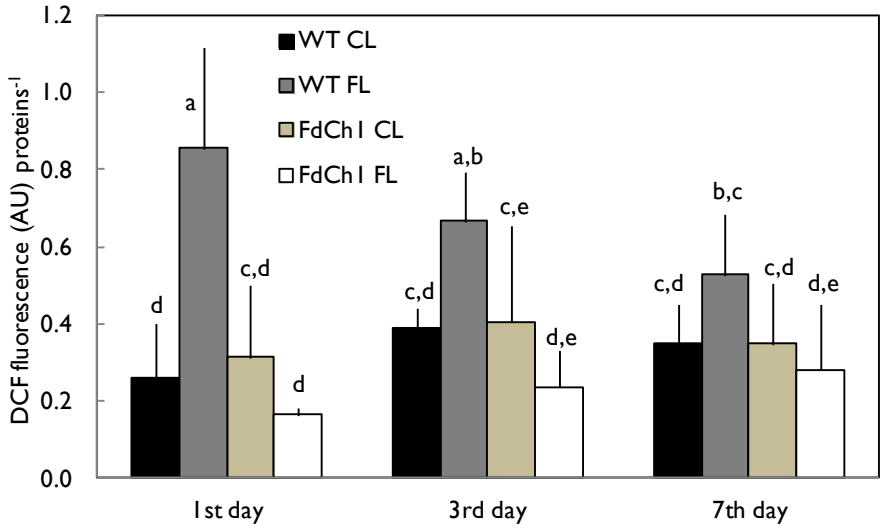

Figure AB.5. **Reactive oxygen species (ROS) of monocultures of wild-type (WT) and FdCh1 strains of *Fremyella diplosiphon* under continuous light (CL) and fluctuating light (FL) regimes**. Sampling was carried out one hour after the maximum peak of light. ROS were normalized to total proteins. Fluorescence values were obtained

by subtracting fluorescence from growth medium mixed with DCFH-DA dye and fluorescence from samples containing only cells and lacking DCFH-DA dye. Bars represent averages (±standard deviation) from three independent biological replicates. Means with different letters are significantly different ($p < 0.05$).

Gene expression.

To investigate the transcriptional responses of cells under FL vs. CL, we analyzed the change in gene expression of a number of genes, including photoinhibition and photosystem marker genes, at the mRNA level by performing quantitative real-time reverse transcriptase PCR (qRT-PCR) (Fig.AB.6). Two full-length copies of the gene encoding the orange carotenoid-binding protein (OCP), denoted *ocp1* and *ocp2*, are annotated in the genome of *F. diplosiphon.* OCP is a carotenoid-bound photoactive protein essential for triggering a photoprotective mechanism in which absorbed light is dissipated as heat (280). Levels of mRNA for *ocp1* were higher in the WT than in FdCh1 under both CL and FL ($p<0.05$). In the FdCh1 mutant, the relative gene expression of *ocp1* was down-regulated in FL compared to CL (Fig. AB.6A). The *ocp2* gene showed a difference in expression compared to *ocp1*. The level of *ocp2* transcripts was higher before the maximum peak of light during the daily cycle than after the peak for both strains under all light conditions, whereas *ocp1* levels did not differ greatly before and after the maximum peak of light.

There are two copies of the gene encoding the D1subunit of PSII annotated in the genome of *F. diplosiphon*, referred here as D1.1 and D1.2. The relative gene expression of D1.1 in WT showed high levels of expression in CL after the peak, but no significant differences in expression under FL (Fig. Ab.6C). By comparison, the expression of D1.1 increased under FL compared to CL both before and after the peak in the FdCh1 ($p<0.05$) (Fig. Ab.6C). The expression of D1.2 was

overall higher before the maximum peak in CL in both strains (Fig. Ab.6D). FdCh1exhibited a threefold higher expression of D1.2 before the peak compared with other conditions. There was no apparent impact of FL on the expression of D1.2 in either strain (Fig. Ab.6D). A highly conserved core subunit of PSI, encoded by *psaD*, exhibited a threefold greater accumulation of mRNA in FdCh1 than in WT under CL after the maximum peak (Fig. Ab.6E). Notably, the levels of *psaD* mRNA were significantly higher in FdCh1 than in WT under all conditions tested. The relative expression of the flavodiiron protein-encoding gene *flv1B*, which is essential for protecting cells against oxidative stress (303), was not significantly different between WT and FdCh1 under CL. However, there was a decrease in *flv1B* mRNA levels in WT under FL compared to CL that was not observed in FdCh1 (Fig. AB.6F).

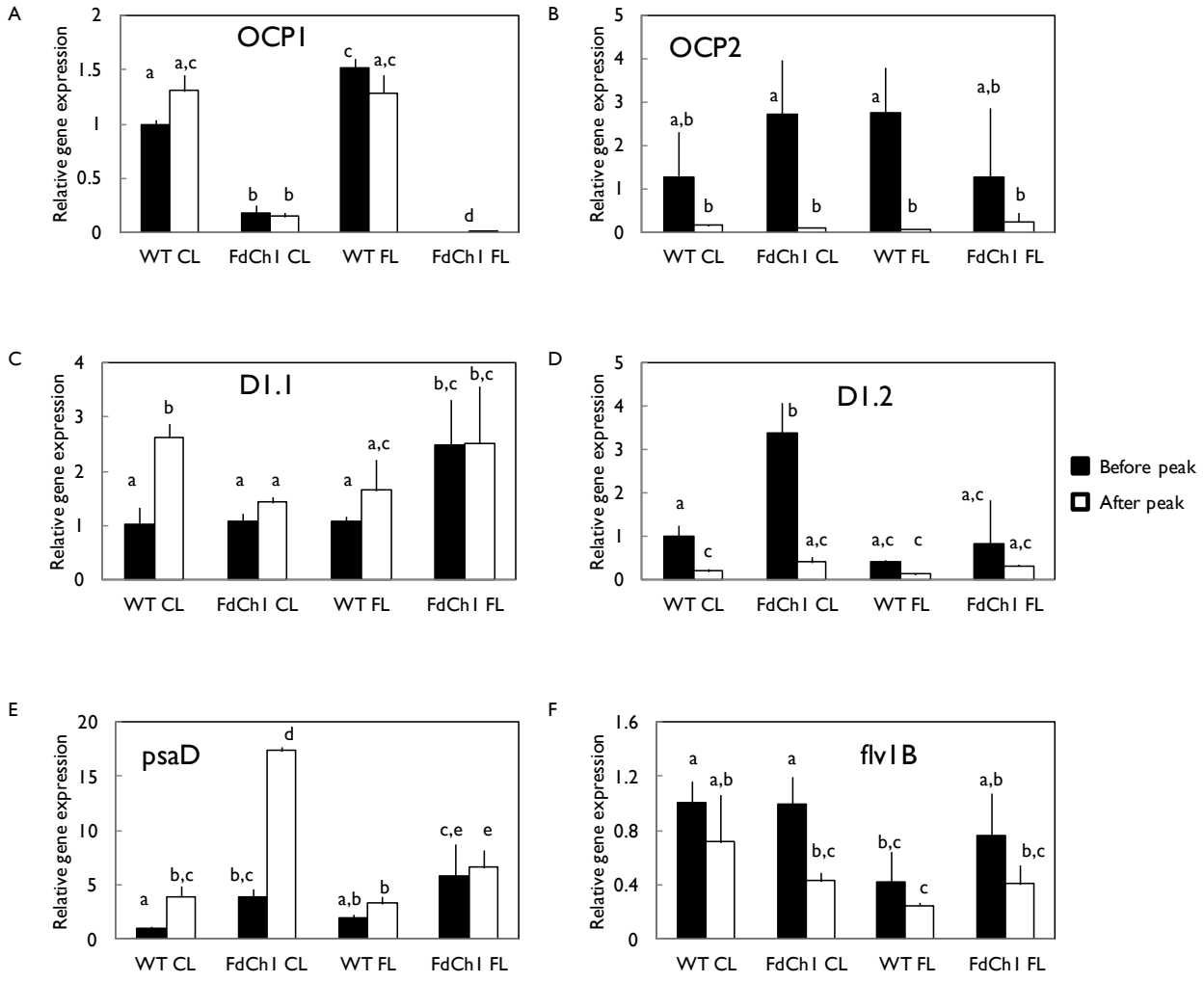

Figure AB.6. **Effect of continuous light (CL) and fluctuating light (FL) regimes on photoinhibition and photosystem marker genes for monocultures of wild-type (WT) and FdCh1 strains of *Fremyella diplosiphon***. Expression of the *ocp1* (A), *ocp2* (B), D1.1 (C), D1.2 (D), *psaD* (E), and *flv1b* (F) genes three hours before (black bars) and after (white bars) the maximum peak of light. Transcript levels were normalized to the house keeping *orf10B* and are shown as relative to gene expression at WT CL regime. Bars represent averages (±standard deviation) from three independent biological replicates. Means with different letters are significantly different ($p < 0.05$).

Sequenced cyanobacterial species lacking PBSs domains.

PBSs are widespread in cyanobacteria, contribute to their ability to adapt to a wide range of environments and thus are presumed to be largely adaptive in natural contexts. Cyanobacterial species lacking PBSs are found in natural habitats but appear to be rare. To investigate which species carried conserved PBS domains, we used the IMG database containing 202 available cyanobacterial genomes and Pfam 00502 (http://pfam.sanger.ac.uk/search) was used to identify conserved PBSs domains. As previously shown by Lea-Smith et *al*. (294), some strains of *Prochlorococcus marinus*, some strains of *Prochlorococcus sp*. and the cyanobacterium UCYN-A lacked genes for PBS domains altogether. Other strains of *P. marinus* and *Prochlorococcus sp*. only possessed genes for PE as previously described (304). These strains lack genes for the core components of PBSs, including AP and thus are not thought to assemble functional PBS, but to carry orphan PBP genes. In addition to the species lacking PBS described by Lea-Smith et *al*. (294), we found *Caenarcanum bioreactoricola*, some *Gastranaerophilaceae* strains, *Obscuribacter phosphatis*, and *Scytonema hofmanni* PCC 7110 lacking PBSs (Table AB.1).

Table AB.1. **Cyanobacterial species lacking PBSs**.

Name	Ecosystem	PBP genes[b]	Sequencing Status	Details
Caenarcanum bioreactoricola	sludge blanket reactor	Absent	Draft	Fermentation with ethanol and lactate
Cyanobacterium sp. UCYN-A[a]	Marine	Absent	Final	Lacking some genes for PS II
Gastranaerophilaceae MH_37	Host-associated	Absent	Draft	Digestive system
Gastranaerophilaceae Zag_1	Host-associated	Absent	Draft	Digestive system
Gastranaerophilaceae Zag_111	Host-associated	Absent	Draft	Digestive system
Gastranaerophilus phascolarctosicola	Host-associated	Absent	Draft	Digestive system
Obscuribacter phosphatis	Wastewater Treatment Plant	Absent	Draft	Found in the dark
Prochlorococcus marinus AS9601[a]	Mediterranean Sea	Absent	Final	High-light adapted

Table AB.1 (cont'd)

Prochlorococcus marinus MIT 9301[a]	Sargasso Sea	Absent	Final	Marine
Prochlorococcus marinus MIT 9312[a]	Gulf Stream of North Atlantic Ocean	Absent	Final	Low light adapted
Prochlorococcus marinus MIT 9313[a]	Gulf Stream of North Atlantic Ocean	Absent	Final	Low light adapted
Prochlorococcus marinus pastoris CCMP 1986[a]	Mediterranean Sea	Absent	Final	High-light adapted
Prochlorococcus sp. W10	Pacific Ocean	Absent	Draft	Marine
Prochlorococcus sp. W11	Pacific Ocean	Absent	Draft	Marine
Prochlorococcus sp. W12	Pacific Ocean	Absent	Draft	Marine
Prochlorococcus sp. W2	Pacific Ocean	Absent	Draft	Marine
Prochlorococcus sp. W3	Pacific Ocean	Absent	Draft	Marine
Prochlorococcus sp. W4	Pacific Ocean	Absent	Draft	Marine
Prochlorococcus sp. W5	Pacific Ocean	Absent	Draft	Marine
Prochlorococcus sp. W9	Pacific Ocean	Absent	Draft	Marine
Prochlorococcus marinus CCMP 1375[a]	Atlantic Ocean	PE	Final	Very low light adapted
Prochlorococcus marinus MIT 9211[a]	Equatorial Pacific	PE	Final	Marine
Prochlorococcus marinus MIT 9215[a]	Equatorial Pacific	PE	Final	Marine
Prochlorococcus marinus MIT 9303[a]	Sargasso Sea	PE	Final	Marine
Prochlorococcus marinus MIT 9515[a]	Equatorial Pacific	PE	Final	Marine
Prochlorococcus marinus MIT9202[a]	South Pacific Ocean	PE	Final	Marine
Prochlorococcus marinus NATL1A[a]	North Atlantic Ocean	PE	Final	Marine
Prochlorococcus marinus NATL2A[a]	North Atlantic Ocean	PE	Final	Marine
Prochlorococcus sp. W6	Pacific Ocean	PE	Draft	Marine
Prochlorococcus sp. W7	Pacific Ocean	PE	Draft	Marine
Prochlorococcus sp. W8	Pacific Ocean	PE	Draft	Marine
Scytonema hofmanni PCC 7110	Bermuda	PE	Draft	limestone cave

Table AB.1 (cont'd)

[a] Described in (294)

[b] PBP genes, genes encoding orphan phycobiliproteins.

DISCUSSION

In this study, we explored the fitness costs of maintaining the flexibility of PBSs under defined growth conditions in *F. diplosiphon*, a cyanobacterium capable of acclimating to changes in light quality and intensity by adjusting pigment composition of its PBSs to maximize absorption of the prevailing light spectrum (145) under different light intensities (189, 305). Here, we compared WT *F. diplosiphon* and a mutant lacking PBSs, i.e., FdCh1. Whereas WT is able to adapt its PBS content to variations of light quality and intensity, FdCh1 lacking PBSs does not. Therefore, these two strains were a good case study to assess the costs and value of maintaining flexible PBSs under stressful conditions. We grew the two strains under continuous light and fluctuating light conditions which mimic more natural conditions (Fig. 1). A few studies have examined the beneficial aspects of CCA in organisms possessing the capability of adapting pigment composition to different light qualities relative to species that lack CCA (306, 307). These experiments demonstrated that species capable of CCA exhibit a competitive advantage in fluctuating environments compared to species lacking CCA. The broad implications of such studies and an ability to definitely attribute the observed fitness differences to the presence or absence of CCA are limited by potential complexities that arise from comparing relative fitness of completely different species. In the studies we described here, by competing a mutant lacking

CCA against the WT, we explored the potential adaptive significance of CCA that is associated with a defined genetic basis in a single species.

Our competition experiments showed that the phenotypic flexibility of WT *F. diplosiphon* cells with a larger and light-tunable antenna resulted in an impairment in growth under fluctuating light conditions relative to a PBS-deficient strain. We suggest two potential explanations. First, we hypothesized that there are energy costs for WT cells during acclimation to changing light conditions; energy is used to reconfigure PBSs and photosynthetic efficiency is transiently reduced. Prior studies with *F. diplosiphon* demonstrated a reduced light use efficiency during acclimation of PBS content to new light conditions (296), suggesting that during acclimation less energy is produced to support growth. By comparison, FdCh1, which lacks an ability to respond to these continuous changes, would not experience the energy cost for reconfiguring PBSs and an associated change in light use efficiency. WT initially exhibits a reduction in its abundance in the population under FL conditions that is not apparent for FdCh1 (Fig. 2B). This is in agreement with the idea that WT experiences a period of acclimation under FL which includes using energy to promote a transcriptional response to adjust the pigment composition of its PBSs rather than for active growth. Alternatively, the light fluctuations we imposed under FL may have been too rapid for WT to adequately adjust its PBSs to either the dark or the light phase, leading to ROS formation.

PBS pigment content, rod length and overall abundance are controlled in response to light intensity and quality (308). This response is critical for cellular protection as PBSs can generate damage in the cell when the PBS size and composition is not tuned to the external environment (289, 290). The accumulation of high levels of PE under FL (Fig. 4C) suggests that WT produces longer rods to increase light absorption under the FL conditions, conditions under which the cells

receive less overall light compared to CL. Cyanobacterial cells growing in light-limited conditions exhibit longer PBS rods than cells growing in excess light (308). However, in response to the dark-to-light transitions imposed under FL regimes, the observed higher PBS pigment levels together with Chl*a* could be a source of ROS, which may negatively contribute to the growth of WT. We confirmed that WT produced higher ROS under FL regimes, before starting to acclimate to growth under FL (Fig. 5). By contrast, FdCh1 did not exhibit a significant change in ROS levels over the course of the experiments. Comparing WT and FdCh1 responses suggests that longer PBSs are the source of ROS generated under FL and may be associated with the differential competitive growth responses observed under CL compared to FL.

Short-term photosynthetic acclimation to fluctuating environmental conditions can occur for instance as a result of gene expression changes or enzyme modifications occurring within organisms (309). Relative gene expression of photoinhibition and photosystem marker genes at the mRNA level was performed before and after the "middle of day" maximum peak of 2000 µmol photons m^{-2} s^{-1}, for both CL and for FL. Relative gene expression before the peak could inform on the status of the cells before reaching the highest light stress; on the other hand, after the peak, gene expression could give insight into the condition of the cells under this intense stress.

The *ocp1* gene encodes a photoactive protein (OCP) essential for triggering a photoprotective mechanism by physically interacting with PBSs to dissipate excess absorbed light energy (280). The *ocp1* gene was down-regulated in FdCh1 compared to WT (Fig. 6A). This finding suggested that relative abundance or lack of PBSs may regulate expression of the *ocp1* gene by a negative feedback mechanism. In WT, the levels of *ocp1* transcripts were higher when cells were exposed

to higher stress, both after the maximum peak in CL and overall in FL regime. This denoted a cellular response to higher stress experienced under these conditions.

Elevated levels of D1 transcripts may indicate increased turnover rate of the D1 protein (310). Expression of the D1.1 gene was upregulated during FL regimes in the FdCh1strain, whereas the levels in WT were significantly lower under the same regime (Fig. 6C). Low expression of D1.1 during FL regimes in WT could suggest that WT was subjected to oxidative stress under these conditions. Similar to some other strains, including *Synechococcus* sp. PCC 7942 (311), *F. diplosiphon* contains two D1 genes. In *Synechococcus* sp. PCC 7942, expression of the D1.1 variant increases under low light; whereas expression of D1.2 variant increases under highlight or stress conditions (311). This differential regulation of these two D1 variants tunes fitness of cells to the prevailing light intensity (311). In accordance with Vinyard et al. (311), also in *F. diplosiphon* D1.2 transcripts were higher compared to D1.1 transcripts under highlight and stressful conditions (data not shown).

The gene *flv1B* encodes a protein known for protecting against oxidative stress (303). WT showed lower levels of accumulation of the *flv1B* transcript under FL regimes (Fig. 6F). If the level of *flv1B* transcripts reflects the same pattern of accumulation at the protein level, the pool of Flv1B available for associating with the reaction center complexes could be low under FL regimes, suggesting that Flv1B -mediated photoprotection may be impaired under FL compared to CL. Hence, low expression of genes associated with limiting photodamage or inducing photoprotection in WT under FL regimes suggest that WT may have been subjected to oxidative stress under FL regimes.

The apparently beneficial mutation in FdCh1 under FL regimes was in agreement with studies that showed that in the model cyanobacterium *Synechocystis*, the absence of PBSs increased

productivity in carbon-limited and high light conditions (292, 294, 295). It was not possible to compare these studies directly with our results as we grew cells in different environmental conditions, where cells were exposed to sinusoidal (fluctuating or continuous) light regimes. In nature, species lacking PBS are present in habitats exposed to strong light conditions (Table 2). Not surprisingly some species which were host-associated or were heterotrophic lack PBSs (Table 2). Lea-Smith et al. (294) showed that some strains of *P. marinus*, which typically inhabit tropical open ocean, lack PBSs. These authors suggested that the absence of PBSs in nature can be advantageous either to decrease light absorption under strong light conditions or for maintaining a small size for nutrient uptake. As some strains of *P. marinus* that lack PBSs are adapted to low light (Table 2), a combination of the explanations might be more likely.

CONCLUSION

In this study, we demonstrated a competitive advantage for strains lacking PBSs and PBS-associated cellular damage under FL. Eliminating an entire antenna system can lead to increased fitness under certain environmental conditions. This implies that there are fitness tradeoffs to having a large antenna system, even in a cyanobacterial species that exhibit great qualitative and quantitative flexibility of PBS such as *F. diplosiphon*. It may be possible to exploit these tradeoffs to optimize the efficiency of cyanobacterial energy capture to improve culture productivity.

METHODS

Strains and culture conditions

F. diplosiphon strain SF33, a shortened-filament mutant strain that displays WT pigmentation (168), was used as WT. The mutant FdCh1, which is deficient in PBSs, was isolated from the WT after heat-shock treatment (297). Axenic cultures were grown in BG-11 (169) containing 20 mM HEPES at pH 8.0 (hereafter BG-11/HEPES). Liquid cultures were adapted to white light for 14 days at 45 µmol photons m^{-2} s^{-1} in glass flasks and used to inoculate 210 ml cultures (i.e., water column height of 10 cm to avoid shading effects (294)) at an optical density at 750 nm (OD_{750}) of 0.1 in bioreactors. Bioreactors were maintained with mixing at 200 rpm and with a steady flow of filtered air. Experiments were conductedusing environmental photobioreactor (ePBR) units described by Lucker et al. (312). The total volume of medium in each bioreactor was 210 ml, imposing a gradient of light intensities down the 10 cm depth of the column. All irradiance values given correspond to incident light on the surface of the water column.

Competition experiment growth conditions

For the competition experiments, WT and FdCh1 were both added to ePBRs in equal concentration for a final OD_{750} of 0.1. Cultures were maintained at 26 °C with a 18:6 light:dark cycle at two different sinusoidal light regimes with maximal irradiance peak of 2000 µmol photons m^{-2} s^{-1}, provided by a white high power LED (Seoul P7 LED). Cells were either irradiated with continuous light (CL) or with a well-defined, square-wave intermittent pattern with cyclic periods of 1 min light followed by1 min dark (FL) (Fig. 1). The experiments were

conducted using semi-continuous growth; the cells were diluted to OD_{750} of 0.1 during the dark cycle once a day. For each light condition, at least three biological replicates were used.

Quantification of strain abundance

For competition experiments, the relative abundance of each strain was determined by quantitative PCR (qPCR). The FdCh1 strain possesses an insertion of one copy of the insertion sequence element *IS701*, which disrupts the c*pcF* gene (297). Specific primers were designed to amplify the *cpcF* region from either the WT or FdCh1 genomic DNA (Table 1). Genomic DNA was extracted using a ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research, USA) from cell aliquots collected every 3-4 days. The first DNA sample was collected immediately following inoculation of cells to confirm that the two strains were added in equal amounts. We verified that all primer sets had similar amplification efficiency and that each amplified only one PCR amplicon using melting curve analyses. In all samples, the cycle threshold (Ct) values of three technical replicates had a standard deviation <0.3, confirming the accuracy of the data. Each set of primers was compared to a reference gene *orf10B* (313). To convert the Ct values into a measure of the relative abundance of each strain, the total ΔCt for each sample was calculated as [total ΔCt = ΔCt gene of interest WT + ΔCt gene of interest FdCh1] and was set to 100% population density. ΔCt for each sample was calculated as [ΔCt gene of interest = 2^ (Ct reference gene ORF10B – Ct gene of interest)]. For comparison, therefore, the density of each strain present in the sample was calculated as [density for each strain = ΔCt gene of interest / total ΔCt. To analyze the relative transcript levels, Fast qPCR was performed using 1 × Fast SYBR Green master mix (Applied Biosystems, Inc.) on an ABI 7500 Fast Real-time PCR system

(Applied Biosystems, Inc.). Cycling parameters were 95 °C for 20 s, 40 cycles of 95 °C for 3 s, and 60 °C for 30 s, followed by the default dissociation cycle for melting curve analysis. To confirm the results, on the last day cells were counted using both confocal laser scanning microscope and colony forming unit (CFU) quantification after plating on BG-11/HEPES and growing under 20 µmol photons m^{-2} s^{-1} of RL at 28 °C. Colonies of WT and FdCh1, which were distinguishable by color, were visible on plates for counting by eye after one week.

Physiological analyses

Pigment extraction and detection of reactive oxygen species (ROS). Physiological analyses were performed for monocultures of WT or FdCh1 strains adapted to continuous light or fluctuating light as described above. For each condition, at least three biological replicates were used. The growth rate was calculated as the rate of flow of medium over the volume of culture in the bioreactor (210 ml) per day. Pigments, including chlorophyll *a* (Chl*a*) and phycobiliproteins (PBPs), were extracted and quantified as described (201), while carotenoids were extracted and quantified as described (189). Pigment extraction and quantification were carried out three hours before the maximum peak of light.

The detection of ROS was performed as described in Singh et al. (314) with modifications. Briefly, 5 ml of cells taken from cultures one hour after the maximum peak of light on the first, third, and seventh day postinoculation were incubated with 10 µM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; EMD chemicals) for 1 h at room temperature in the dark. Cells were pelleted and 4 ml of the initial 5 ml of medium were removed. The cell pellet was resuspended in the remaining 1 ml of medium with DCFH-DA and ROS levels

determined by detecting ROS-dependent generation of the fluorescent DCF molecule at 520 nm with excitation at 485 nm by using a SpectraMax M2 microplate reader (Molecular Devices).

Quantitative reverse transcriptase PCR (qRT-PCR)

For RNA extraction, *F. diplosiphon* cells from a 50 ml aliquot of culture were collected 48 hours after the transfer of cells to the reactors. Cultures were sampled three hours before and three hours after the maximum peak of light and RNA isolated as described (262). Total RNA extracted was treated with a TURBO DNA-free kit (Ambion, Austin, TX). cDNA synthesis was performed with 0.5 μg of total RNA using the Reverse Transcription System (Promega Corporation, Madison, WI) following the manufacturer's protocol. We verified that all primer sets (Table AB.1) had similar amplification efficiency by diluting *F. diplosiphon* cDNA and PCR product containing the gene fragments (1:1; 1:10; 1:100; 1:1000; 1:10,000).

Table AB.2. **Primers used.** To quantify cell abundance or concentration for each strain by qPCR and used for amplification of *ocp1*, *ocp2*, D1.1, D1.2, *psaD*, *flv1b*, and *orf10B* genes by qRT-PCR.

Genes	Forward	Reverse
cpcF WT	AACTACACAGCCAGATCTTGGC[a]	CCTCTGGGAACCAATGCCATTCA
cpcF FdCh1	AACTACACAGCCAGATCTTGGC[a]	AAATTTGACTGTTGGTGCGGCTGG
ocp1	GATTGTGGGTAGGGAGAACATC	CTCCTTCTGCTGGTTCAGATAC
ocp2	AACATAGCTGCTGGTGAAGAA	CTTCACCTG TGCGATCAATTTC
D1.1	TCGGCAGTGGGAATTATCTTAC	GGAACCTTGACCCAAAGAGTAG
D1.2	CTAGCATTCTCTGCACCAGTAG	CCAGAGATACCCAAAGGCATAC
psaD	CTATCACCTGGACTAGCCCTAA	AGT TGACCACCCAAAGCTATAC
flv1b	CGCCAAGTAGAAACAGCATTAG	GGTAAGTTCAATCAGCGCATAAC
orf10B	AGAACTACAGCGTCAGCTTAAT	CTGCTTCGCTTTCAGCATTT

[a] Forward primer for qPCR of full length *cpcF* or deleted *cpcF* region is identical both for WT and FdCh1.

Bioinformatics analyses

A total of 202 available cyanobacterial genomes present in the Integrated Microbial Genomes (IMG) system during the preparation of this manuscript were used to assess the presence of indicated conserved domains.

Statistical analysis

The statistical significance of effects of light conditions on *F. diplosiphon* were determined using one- or two-way analysis of variance (ANOVA) with Fisher post-hoc test using OpenStat statistical software (version 10.01.08; W. G. Miller http://www.Statprograms4U.com). Statistical analyses were performed utilizing 95% confidence intervals ($p < 0.05$).

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