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Short Review

Control of light-dependent behaviour in cyanobacteria by the second messenger cyclic di-GMP

Gen Enomoto[1,2], Thomas Wallner[1], Annegret Wilde[1,*]

[1]Institute of Biology III, University of Freiburg, 79104 Freiburg, Germany
[2]Present address: Department of Engineering Science, Graduate School of Informatics and Engineering, The University of Electro-Communications, 1-5-1 Chofugaoka, Chofu, Tokyo 182-8585, Japan
*Corresponding author. Institute of Biology III, University of Freiburg, 79104 Freiburg, Germany. E-mail: Annegret.Wilde@biologie.uni-freiburg.de

Abstract

Nucleotide-derived signalling molecules control a wide range of cellular processes in all organisms. The bacteria-specific cyclic dinucleotide c-di-GMP plays a crucial role in regulating motility-to-sessility transitions, cell cycle progression, and virulence. Cyanobacteria are phototrophic prokaryotes that perform oxygenic photosynthesis and are widespread microorganisms that colonize almost all habitats on Earth. In contrast to photosynthetic processes that are well understood, the behavioural responses of cyanobacteria have rarely been studied in detail. Analyses of cyanobacterial genomes have revealed that they encode a large number of proteins that are potentially involved in the synthesis and degradation of c-di-GMP. Recent studies have demonstrated that c-di-GMP coordinates many different aspects of the cyanobacterial lifestyle, mostly in a light-dependent manner. In this review, we focus on the current knowledge of light-regulated c-di-GMP signalling systems in cyanobacteria. Specifically, we highlight the progress made in understanding the most prominent behavioural responses of the model cyanobacterial strains *Thermosynechococcus vulcanus* and *Synechocystis* sp. PCC 6803. We discuss why and how cyanobacteria extract crucial information from their light environment to regulate ecophysiologically important cellular responses. Finally, we emphasize the questions that remain to be addressed.

Keywords: second messenger signalling, cyanobacteria, c-di-GMP, light-dependent behaviour

Introduction

Cyanobacteria are obligate phototrophs and ancestors of the chloroplasts of algae and plants. It is not surprising that cyanobacteria are considered an excellent model for studying oxygenic photosynthesis and related cellular functions, such as light harvesting, energy and carbon metabolism, and its regulation. However, the ecology and behavioural responses of cyanobacteria have not been studied at a comparable molecular level. Nucleotide-based signalling molecules are known to control such responses in many bacteria at the transcriptional, translational, and post-translational levels. In cyanobacteria, studies on the role of prototypical second messengers cAMP, cGMP, and (p) ppGpp, as well as the later discovered cyclic dinucleotides c-di-GMP and c-di-AMP, have lagged behind. However, in the last 10 years, based on the availability of more sequencing data from cyanobacteria, it has become clear that these phototrophs encode a large variety of proteins that, based on their domains, are potentially involved in second messenger signalling. Notably, cyanobacteria are particularly rich in photoreceptors that contain output domains similar to those involved in c-di-GMP synthesis and degradation (Agostoni et al. 2013). For other bacteria, it is well established that intracellular c-di-GMP levels coordinate specific aspects of bacterial lifestyle, such as biofilm formation, aggregation, virulence, and motility (Jenal et al. 2017). High intracellular concentrations of c-di-GMP are usually associated with inhibition of motility and induction of biofilm formation. These behavioural processes have rarely been studied in cyanobacteria at the molecular level, for various reasons. On the one hand, most marine cyanobacteria that were intensively studied in their ecological context (e.g. marine *Prochlorococcus* and *Synechococcus* species) do not encode enzymes related to c-di-GMP metabolism. On the other hand, research on well-established and genetically tractable model cyanobacteria, which contain a large variety of such proteins, has focused on other general or unique characteristics of these strains, such as nitrogen fixation, photosynthesis, the circadian clock, and toxin production. Moreover, the most widespread cyanobacterial laboratory strains in research [e.g. *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803 (henceforth *Synechocystis*)] have lost many natural behavioural responses that are targeted by c-di-GMP due to the accumulation of mutations in key genes encoding components of cellular appendages such as type IV pili. In this review, we highlight the major advances in our understanding of the role of c-di-GMP in cyanobacterial behaviour, focusing on the two model cyanobacterial strains *Thermosynechococcus vulcanus* (*T. vulcanus*, recently also termed *Thermostichus vulcanus*) and *Synechocystis* (Fig. 1). It is noteworthy that most of the analysed behavioural responses require the participation of multiple cells, which Menon et al. (2021) refer to as 'collective behaviour'.

The physiological role of c-di-GMP in behavioural responses of cyanobacteria

C-di-GMP is a universal signalling molecule that is often involved in controlling developmental and lifestyle transitions in bacteria. Particularly noteworthy is its role in bacterial virulence and the switch between motile and adherent states. Although cyanobac-

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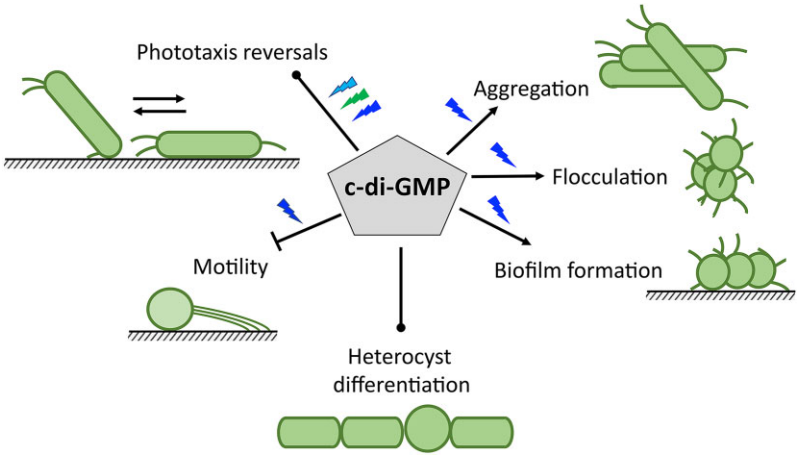

Figure 1. Regulation of cellular behaviour by c-di-GMP in cyanobacteria. Light, especially blue light, controls many c-di-GMP-dependent lifestyle decisions in cyanobacteria. However, other external factors might contribute to c-di-GMP-dependent regulation. In *Synechocystis* (coccoid bacteria), blue light leads to an overall increase in the cellular c-di-GMP concentration, which induces flocculation and biofilm formation and inhibits motility. A high c-di-GMP concentration leads to cellulose-dependent aggregation of *T. vulcanus* (a rod-shaped bacterium). C-di-GMP influences phototaxis reversals of *T. vulcanus* and heterocyst development in the filamentous cyanobacterium *Anabaena* sp. PCC 7120.

teria do not infect eukaryotic organisms, they have exceptionally versatile lifestyles. The phylum Cyanobacteria is defined by the ability to perform oxygenic photosynthesis. Apart from this unifying feature, cyanobacteria are highly diverse in their morphotypes, environmental requirements, developmental options, and gene content. There are unicellular and filamentous forms that can be part of complex phototrophic biofilms, form symbiotic associations, and survive in extreme environments such as deserts, the Arctic/Antarctic region, or hot springs. Owing to the phototrophic metabolism of cyanobacteria, most of these behavioural responses are directly or indirectly regulated by light. Therefore, it is expected that c-di-GMP metabolism is also controlled by light in cyanobacteria. The domains involved in c-di-GMP synthesis and degradation are GGDEF, EAL, and HD-GYP. Indeed, Agostoni et al. (2013) showed that cyanobacteria harbour a high percentage of c-di-GMP domains associated with photoreceptor-related domains. The association of photoreceptors with c-di-GMP domains has also been described in anoxygenic and non-photosynthetic bacteria, but relatively rarely. In contrast, in cyanobacteria, 26% of all c-di-GMP domains from 20 sequenced cyanobacteria have been shown to co-occur with predicted photoreceptor domains, and more than half of them were cGMP phosphodiesterase/adenylyl cylase/FhlA (GAF) domains (Agostoni et al. 2013). The GAF domains constitute the chromophore-binding domains of the large phytochrome photoreceptor family, including cyanobacteriochromes (CBCRs). CBCRs, which can absorb a great variety of wavelengths, are present only in cyanobacteria and are known to control light-dependent behaviour. In other bacteria, the blue light photoreceptor domains (LOV, BLUF, and PYP) are usually associated with c-di-GMP domains. However, red/far-red-sensing bacterial phytochromes are also known to signal via GGDEF and EAL domains in bacteria outside the cyanobacterial lineage (Gomelsky and Hoff 2011). Interestingly, a large group of cyanobacterial species does not contain any domains related to c-di-GMP metabolism. All *Prochlorococcus* and several *Synechococcus* strains belong to this group (Agostoni et al. 2013). These marine strains are adapted to stable oceanic habitats and have a significantly reduced genome (Partensky and Garczarek 2010). This might have led to the selective loss of genes related to light-dependent behavioural responses. Moreover, photoreceptor genes are rarely detected in these streamlined organisms.

Light-dependent changes of c-di-GMP concentration in different cyanobacteria and involved photoreceptors

Functional coupling of a photoreceptor domain with a c-di-GMP-related domain in cyanobacteria was first reported by Cao et al. (2010). They analysed two blue light-sensing proteins from *Synechococcus elongatus* PCC 7942. Both proteins contain a **L**ight, **O**xygen, or **V**oltage (LOV) domain that belongs to the PAS (**P**er-**A**RNT-**S**im) domain superfamily (Glantz et al. 2016, Matilla et al. 2022), in combination with a c-di-GMP-synthesizing GGDEF domain and a c-di-GMP-degrading EAL domain. Only one of them, the SL2 protein, showed a blue light-dependent increase in the phosphodiesterase activity of the EAL domain. However, no physiological function has been assigned to the *Synechococcus* LOV domain proteins.

Agostoni et al. (2013) first quantified the intracellular concentration of c-di-GMP in a few cyanobacterial species. For quantification, *Fremyella diplosiphon* [sometimes referred to as *Tolypothrix* (*Calothrix*) sp. PCC 7601] and *Synechocystis* cells were grown in liquid culture under white, blue, green, and red light illumination. In general, *F. diplosiphon* showed higher levels of c-di-GMP than *Synechocystis*, *Synechococcus elongatus* sp. PCC 7942, and *Anabaena* (*Nostoc*) sp. PCC 7120 (Agostoni et al. 2013, 2018). White and red light illumination of *F. diplosiphon* cells yielded the highest intracellular levels of the second messenger. Blue light irradiation of *Synechocystis* cells led to a 2-fold increase in c-di-GMP concentration (Wallner et al. 2020). This increase depends only on the activity of the phytochrome-like protein Cph2 (Wilde et al. 2002, Wallner et al. 2020). This photoreceptor contains a blue/green switchable CBCR at the C-terminus, which enhances the activity of the downstream GGDEF domain upon blue light irradiation (Savakis et al. 2012). A similar dependency on a single blue light photoreceptor that produces c-di-GMP was found in *T. vulcanus* (Enomoto et al. 2015). This cyanobacterium showed three times higher lev-

els of c-di-GMP under blue light illumination than under green, red, or white light conditions (Nakane et al. 2022). Inactivation of the blue/green-light-absorbing CBCR SesA abolished the increase in c-di-GMP upon blue light illumination.

Environmental stimuli other than light quality also change the c-di-GMP concentration in cyanobacterial cells. Shifting of *Anabaena* sp. PCC 7120 cells to nitrogen-free medium led to a 3-fold increase in c-di-GMP, reaching its peak after 3 h, followed by a slow decrease within 24 h. This pattern may indicate a role for c-di-GMP during heterocyst formation in this filamentous diazotrophic cyanobacterium (Huang et al. 2021). Such transitory alterations have also been observed in *Synechocystis* cells either grown in liquid media (planktonic lifestyle) or on solid surfaces (sessile lifestyle) (Oeser et al. 2021). Upon transfer of planktonic cells onto agar plates, the intracellular level of the second messenger increased within 10 min from a non-detectable level to a maximum. After 4 and 8 h, both planktonic and sessile cells contained similar levels of c-di-GMP.

The c-di-GMP-dependent behaviour of *Synechocystis*

Synechocystis is a model organism used to study phototaxis. This freshwater cyanobacterium was isolated from a local water sample at Berkley (California) in 1968 and colony movements were observed (Stanier et al. 1971). However, Stanier et al. (1971) described the motility of the initially isolated *Synechocystis* strain as 'sporadic and of limited extent' in contrast to several other cyanobacterial isolates, which were moving more rapidly (Stanier et al. 1971). The strain was then deposited into two culture collections. The strain deposited in the Pasteur Culture Collection (PCC) was described as motile by Rippka et al. (1979), whereas the strain from the American Type Culture Collection (ATCC) lost its motility. Cultivation in different laboratories worldwide has led to the emergence of further substrains with different phenotypic properties (e.g. Tichý et al. 2016, Zavřel et al. 2017). A non-motile ATCC strain originating from the so-called glucose-tolerant substrain (Williams et al. 1988) was sequenced in 1996 by Kaneko et al. (1996). The motile substrain, which originated from the PCC and was cultivated in the lab of S. Shestakov (Moscow University, Russia), was shown to be naturally transformable (Grigorieva and Shestakov 1982) and was sequenced 30 years later (Trautmann et al. 2012). Several laboratories that are interested in behavioural responses are working with motile *Synechocystis* PCC (sub)strains. Why is it important to know this in the context of this review? Many c-di-GMP-dependent characteristics related to community behaviour, such as aggregation and motility, depend on the presence of type IV pili. These cellular appendages are assembled on the surfaces of many bacteria, including cyanobacteria. Non-motile substrains of *Synechocystis*, such as the sequenced strain by Kaneko et al. (1996), harbour mutations in genes related to the structure and function of type IV pili (Trautmann et al. 2012). Therefore, many phenotypes which are potentially controlled by c-di-GMP cannot be studied in non-motile strains, or the responses are distorted in cells lacking type IV pili.

Proteins involved in c-di-GMP metabolism

Synechocystis encodes a large number of proteins related to the metabolism of c-di-GMP, which is comparable to that of *E. coli*. According to the online census of domains involved in c-di-GMP signalling (https://www.ncbi.nlm.nih.gov/Complete_Genomes/c-di-GMP.html; Römling et al. 2013, Roelofs et al. 2015, Chou and Galperin 2016, Wang et al. 2016), the *Synechocystis* genome encodes 13 proteins with a GGDEF domain, 4 proteins with an EAL domain, and 9 proteins with both GGDEF and EAL domains. In addition, two proteins with an HD-GYP domain have been identified (Römling et al. 2013). In total, there are 28 proteins potentially involved in c-di-GMP metabolism (Table 1). For comparison, *Nostoc punctiforme* PCC 73102, a filamentous cyanobacterial plant symbiont with a complex lifestyle, harbours 24 such proteins. For most *Synechocystis* c-di-GMP-related proteins, no specific function has been identified yet, with a few exceptions.

Role of the cyanobacterial phytochrome 2 (Cph2) in motility

The photoreceptor Cph2 (Fig. 2) is a multi-domain protein consisting of an N-terminal red/far-red-sensing phytochrome-like domain, a degenerated diguanylate cyclase domain (with the active-site motif changed to HGDGF), and an EAL domain followed by a blue/green-sensing CBCR domain that controls a downstream diguanylate cyclase (with the functional active-site motif GGEEF). The C-terminal CBCR-diguanylate cyclase domain is sufficient for *in vitro* blue light-dependent c-di-GMP synthesis, which is 2-fold higher under blue light than under green light (Savakis et al. 2012). In *Synechocystis* cells, the c-di-GMP content increased ~2-fold after incubation with blue light compared to green light. This increase was completely abolished in a Δ*cph2* mutant strain (Wallner et al. 2020). Deletion of *cph2* leads to a change in the phototactic behaviour of *Synechocystis*. Wild-type cells usually do not move towards blue light, suggesting that high c-di-GMP levels lead to inhibition of motility. This was confirmed by the artificial overexpression of a highly active diguanylate cyclase (Slr1143) (Ryjenkov et al. 2006) in *Synechocystis* cells, which led to the inhibition of motility under all light conditions (Angerer et al. 2017). A similar phenotype was observed for phototaxis under high-intensity red light (640 nm). In this setup, wild-type cells did not show colony migration on an agar plate, whereas inactivation of the *slr1143* gene led to positive phototaxis towards the red-light source (Angerer et al. 2017). However, under these conditions, no significant changes in cellular c-di-GMP content were measured. The c-di-GMP content in red-illuminated cells was comparable to that in the white-light control, suggesting that inhibition of motility under high-intensity red light was not due to an elevated c-di-GMP level, as was shown for blue light. Interestingly, it was shown that Slr1143 interacts with Cph2 via its EAL and GGDEF domains. Therefore, this diguanylate cyclase is now called Cph2-interacting protein 1 (Cip1) (Angerer et al. 2017). As both of these domains of Cph2 were shown to be active in *Synechocystis* cells (Savakis et al. 2012), integration of multiple signals by the Cph2-Cip1 complex may control pilus functions by locally acting c-di-GMP pools. There may be more Cph2 interaction partners that contribute to the complex phototactic behaviour of *Synechocystis*, which have yet to be discovered.

An uneven distribution of c-di-GMP may be required for the oriented movement of *Synechocystis* cells in a light vector. Such asymmetric synthesis or degradation of c-di-GMP can be achieved when the activity of the respective enzymes is controlled by locally acting photoreceptors. The cell polarity of the coccoid cyanobacterium *Synechocystis* is established by its lensing properties. When the cells are illuminated from one side, light is focused at the distal side of the cell, leading to a light spot that is approximately four times more intense than the light hitting the front of the cell (Schuergers et al. 2016). Therefore, it is feasible to speculate that c-di-GMP-controlling photoreceptors are locally

Table 1. List of proteins related to c-di-GMP metabolism in *Synechocystis*.

Gene product	Domain architecture
Proteins containing GGDEF and EAL domains	
Cph2 (Sll0821)	**GAF**-GAF-*GGDEF**-EAL-**GAF**-GGDEF
Slr0359	X-GAF-PAS-PAS-PAS-PAS-GGDEF-EAL
Slr2077	PBPb-GGDEF-EAL
Slr1305	CheY-PAS-PAS-GGDEF-EAL
Sll0267	X-CHASE-X-PAS-GAF-PAS-GGDEF-EAL
Slr1102	FHA-PAS-PAS-*GGDEF**-EAL
Slr1103	FHA-X-*GGDEF**-EAL
Slr1104	FHA-X-*GGDEF**-*EAL**
Slr1895	FHA-PAS-*GGDEF**-EAL
Proteins containing only GGDEF domains	
Slr1760	CheY-GGDEF
Slr1047	XXX-GAF-GGDEF
Slr0779	PAS-PAS-PAS-PAS-PAS-GGDEF
Slr1798	MASE1-GGDEF
Slr1657	XXX-GGDEF
Slr0829	TM-HAMP- PAS-PAS-PAS-PAS-GGDEF
Slr0687	CheY-GAF-GGDEF
Sll1673	CheY-PAS-GGDEF
Sll1687	X-PAS-PAS-PAS-PAS-GAF-*GGDEF**
Slr0302	PAS-PAS-PAS-GAF-*GGDEF**
Cip1 (Slr1143)	GAF-GGDEF
Sll0048	GAF-*GGDEF**
Sll1170	DUF1816-PAS-*GGDEF**
Proteins containing only EAL domains	
Slr1692	*EAL**
Slr1593	EAL
Slr0842	cNMP-EAL
Slr1588	CheY-EAL
Slr6110	CheY-*EAL**
Proteins containing a HD-GYP domain	
Sll1624	CheY-HD_GYP
Slr2100	CheY-HD_GYP

Domains in italics and with an asterisk contain degenerate motifs. GAF domains in bold are chromophorylated. PAS, Per-Arnt-Sim; FHA, forkhead associated domain; GAF, cyclic GMP/adenylyl cyclase/FhlA; CheY, CheY-like receiver domain; PBPb, bacterial periplasmic substrate-binding protein; CHASE, cyclases/histidine kinases associated sensory extracellular domain; DUF1816, domain of unknown function; TM, transmembrane domain; HAMP, histidine kinases/adenylate cyclases/methyl-accepting proteins/phosphatases domain; cNMP, cyclic nucleotide-monophosphate binding domain; MASE, membrane-associated sensor; X, undefined region.

activated because of the differences in light intensity in one cell. This can lead to asymmetric c-di-GMP concentrations, which depend on the position of the cell in a light beam. An excess of c-di-GMP in the cell (e.g. the strong c-di-GMP production after blue light illumination by Cph2) may prevent the formation of a localized signalling pool and thereby inhibit movement.

C-di-GMP-dependent transcriptional changes

Accumulation of second messengers can also lead to changes in gene expression, either by regulating transcription factors or by riboswitches. Römling et al. (2013) did not identify any potential c-di-GMP-dependent riboswitches in cyanobacteria. However, *Synechocystis* has two members of the CRP transcription factor family, which were shown to bind to cAMP and c-di-GMP in other bacteria (e.g. Fazli et al. 2011). In *Synechocystis*, the two CRP homologues, SyCRP1 and SyCRP2, are involved in the regulation of motility. SyCRP1, but not SyCRP2, has been shown to bind cAMP (Yoshimura et al. 2000), and both transcription factors can interact with each other (Song et al. 2018). To identify genes regulated by c-di-GMP, a microarray study was performed in cells with a blue light-induced high c-di-GMP content. In order to distinguish the results from potentially unrelated blue light-induced gene expression changes, the Δ*cph2* mutant strain served as a control. No change in the c-di-GMP concentration was observed in this control strain after blue light illumination (Wallner et al. 2020). Interestingly, most of the differentially expressed genes in this microarray study were genes with predicted or known function in motility, such as genes encoding minor pilins, chaperone usher pili, or components of chemotaxis operons. Moreover, there was considerable overlap with known SyCRP1-dependent genes, though the accumulation of the *sycrp1* mRNA itself was not changed upon blue light incubation (Wallner et al. 2020). In particular, two sets of minor pilin genes seem to be controlled by multiple factors, including c-di-GMP (Fig. 2). Minor pilins are potential subunits of the type IV pilus fibre, and they are defined by a cleavable N-terminal leader sequence similar to the major pilin. *Synechocystis* encodes a comparatively large number of potential 12 minor pilins. Two minor pilin operons (*pilA5–pilA6* and *pilA9–12*) are responsive to blue light-induced high c-di-GMP content in an opposite manner (Wallner et al. 2020). In cells grown under blue light, *pilA5–pilA6* mRNA levels declined, whereas more *pilA9–12* mRNA accumulated compared to control cells. Overexpression of the diguanylate cyclase Cip1 confirmed that it is indeed the increased c-di-GMP concentration that affects the expression of these operons in an opposite manner (Wallner et al. 2020). This inverse regulation suggests that these minor pilins have different functions. Indeed, PilA5 is essential for natural competence (Oeser et al. 2021). DNA uptake is known to be mediated by type IV pili in this cyanobac-

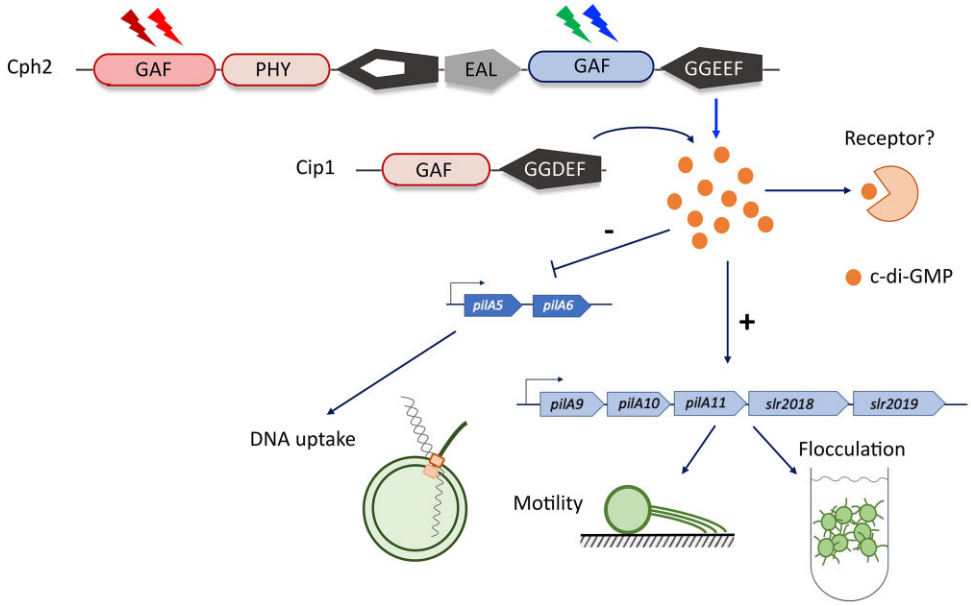

Figure 2. The role of c-di-GMP in *Synechocystis* behaviour. The C-terminal blue/green light photosensory module of Cph2 inhibits motility under blue light by generation of the second messenger c-di-GMP. The N-terminal red/far-red dependent phytochrome module of Cph2 harbours an active EAL domain and an inactive GGDEF domain. Cip1 is an active diguanylate cyclase that interacts with Cph2. C-di-GMP controls gene expression. The major targets are genes encoding minor pilins, which are potential components of type IV pili. Several of these minor pilins control different functions of type IV pili, including motility, flocculation, and natural competence.

terium (Yoshihara et al. 2001). However, other type IV pili functions, such as motility and aggregation, were not affected in a Δ*pilA5* mutant strain. In contrast, the minor pilins encoded in the operon *pilA9–12* are involved in type IV pilus-mediated cell aggregation (also called flocculation) and motility. Deletion of this operon led to a complete loss of these functions, whereas natural competence was not affected (Conradi et al. 2019, Wallner et al. 2020, Oeser et al. 2021). Moreover, it was shown that blue light induces flocculation and that the Δ*cph2* mutant strain, which does not synthesize c-di-GMP under blue light, shows a reduced aggregation score specifically under blue light conditions. It is not clear whether the lack of *pilA9–12* induction alone is responsible for this phenotype or whether c-di-GMP has more functions in motility and aggregation. Another mRNA that encodes the newly discovered minor pilin PilX1 (slr0226) responds to the blue light-dependent c-di-GMP increase, similar to the *pilA5–pilA6* operon (Wallner et al. 2020). However, this minor pilin, as well as the two other recently described PilX2 and PilX3 homologues, are important for flocculation but not for natural competence and phototaxis (Oeser et al. 2021). The functions of the other minor pilin genes (*pilA2*, *pilA4*, *pilA7*, and *pilA8*) have been poorly analysed so far, and their expression seems not to respond to c-di-GMP changes.

The accumulation of the *pilA9–12* and *pilA5–6* mRNAs is also influenced by the surface incubation of the cells (Oeser et al. 2021). The *pilA5–6* operon is upregulated in cells grown on an agar plate, whereas the *pilA9–12* operon is upregulated in planktonic cultures. These findings suggest that surface sensing regulates cellular c-di-GMP levels and thus controls the expression of these genes. However, the transcriptomic analysis was performed 4 and 8 h after acclimation to the surface, when the cellular c-di-GMP content decreased again to levels measured in planktonic cells (Oeser et al. 2021). Differences in local c-di-GMP concentrations at these time points in planktonic and plate cultures cannot be excluded.

Biofilm formation

In another approach to unravelling the function of c-di-GMP, Agostoni et al. (2016) engineered two *Synechocystis* mutants with artificial high and low levels of c-di-GMP. Expression of a diguanylate cyclase from *Vibrio cholerae* led to a 2.5-fold increase in the c-di-GMP concentration, which is comparable to the natural increase by blue light in the *Synechocystis* wild type. Overexpression of a phosphodiesterase from *E. coli* reduced the c-di-GMP level to one-third of the wild-type level under white light. These mutant strains were then used to study the involvement of c-di-GMP in biofilm formation, aggregation, and cellular buoyancy of *Synechocystis*. Notably, manipulating the c-di-GMP content did not affect the growth of the cultures (Agostoni et al. 2016). Biofilm formation can be quantified by measuring the absorbance of crystal violet on glass flasks. By quantifying chlorophyll in the cell suspension, they estimated whether the cells sank to the bottom of the glass tube or stayed in suspension and possibly aggregated (Agostoni et al. 2016). This assay can be compared to a so-called flocculation assay, which was used to evaluate the ability of the cells to aggregate (Conradi et al. 2019). High c-di-GMP concentrations, either artificially produced by a heterologously expressed diguanylate cyclase or by incubation with blue light, induced biofilm formation and increased cell aggregation and sinking compared with the wild type. Low c-di-GMP concentrations partly led to the opposite effect. Cells expressing the foreign phosphodiesterase to lower c-di-GMP levels do not form biofilms, similar to the wild-type control, suggesting that in wild-type cells, the c-di-GMP concentration is already low enough to repress biofilm formation. Moreover, these cells do not sink at all. Even additional blue light illumination, which should enhance the c-di-GMP content, was not sufficient to restore the sinking rate of the phosphodiesterase-expressing strain to wild-type levels (Agostoni et al. 2016).

In summary, increased c-di-GMP concentrations lead to biofilm formation, inhibition of motility, and increased aggregation of

Synechocystis cells, similar to what has been observed in other bacteria. Low c-di-GMP contents allow phototactic motility of *Synechocystis* and enhance buoyancy in the water column, preventing cell sedimentation. It seems that in the wild type, under laboratory conditions, the increase in the c-di-GMP concentration controlling this behaviour solely depends on the blue light activated diguanylate cyclase function of the C-terminal module of the photoreceptor Cph2. It remains largely unclear whether and how other diguanylate cyclases or phosphodiesterases contribute to these phenotypes. Ishikawa et al. (2020) studied the enzymatic characteristics of four *Synechocystis* diguanylate cyclases that contain multiple PAS domains. Only one of them, DgcA (Sll1687), showed high c-di-GMP synthesis activity, which was reduced dramatically when one to three PAS domains were removed. DgcB (Slr0302) and DgcC (Sll0799) showed very low activity, whereas DgcD (Slr0829) was inactive under the studied conditions. However, to get soluble proteins, the authors had to remove the transmembrane domains in DgcC and DgcD, which could also explain the very low activity *in vitro*. Surprisingly, none of the mutants had a phenotype in biofilm formation (motility, buoyancy, and aggregation were not studied). Only after the artificial overexpression of DgcA did the authors see an increase in biofilm formation, similar to the artificial overexpression of the *V. cholerae* diguanylate cyclase (Agostoni et al. 2016), supporting the finding that biofilm formation depends on the increase in cellular c-di-GMP concentration. Previously, it was shown that the addition of 0.5 M NaCl also induces biofilm formation in *Synechocystis* (Kera et al. 2018). Under these conditions, no further increase in the ability of the cells to form biofilms was detected after the overexpression of DgcA. Interestingly, Agostoni et al. (2018) showed that incubation of *Synechocystis* cells with 0.6 M NaCl led to an increase in the c-di-GMP level, suggesting that biofilm induction is controlled by salt-induced c-di-GMP signalling.

C-di-GMP dependent behaviour of *T. vulcanus*

Genes encoding proteins involved in c-di-GMP metabolism are enriched in terrestrial and freshwater cyanobacteria but absent in most marine species (Agostoni and Montgomery 2014). This can be attributed to the ability of many cyanobacteria to form microbial mats in terrestrial and freshwater environments. Thermophilic cyanobacteria of the genus *Thermosynechococcus* have been found in microbial mats in hot springs (Stolyar et al. 2014).

The light input module for c-di-GMP signalling in *T. vulcanus* comprises the three CBCR photoreceptors SesA, SesB, and SesC (Enomoto et al. 2015). A complete list of proteins potentially involved in c-di-GMP metabolism in *T. vulcanus* is provided in Table 2. SesA produces c-di-GMP under blue light (Enomoto et al. 2014), whereas SesB degrades c-di-GMP under teal light. SesC is a dual-function photoreceptor with diguanylate cyclase activity under blue light and c-di-GMP degradation activity under green light. The three proteins cooperatively function together to turn on c-di-GMP signalling under blue light and to repress it under teal-to-green light (Fig. 3). The cooperation of the multiple light-responsive c-di-GMP metabolizing proteins enables the highly colour-specific induction of c-di-GMP signalling (Enomoto et al. 2015). This specificity is beneficial for the cells in their ecological niche. By integrating three different light signals, cells can interpret the depth of their position in a microbial mat. The spectral quality of light penetrating a mat strongly changes with depth. In particular, blue light attenuates much more rapidly than green light through a cyanobacterial cell community (Enomoto and Ikeuchi 2020). Therefore, the light-dependent c-di-GMP signalling system of *T. vulcanus*, which measures the ratio between blue and green light, is perfectly suitable to depict and control dynamics in photosynthetic microbial mats in hot springs.

Cellular motility critically impacts the formation and dynamics of a multicellular community of bacteria. Generally, higher intracellular c-di-GMP contents lead to lower motility and the induction of biofilm formation in many bacteria (Römling et al. 2013). In motile cyanobacteria, light and light-dependent changes of c-di-GMP have a substantial effect on their phototactic responses (Wilde and Mullineaux 2017). *T. vulcanus* shows positive phototaxis towards green light, while they move in the opposite direction in the presence of additional blue light illumination (Nakane et al. 2022). The blue light-dependent induction of negative phototaxis requires SesA, which synthesizes c-di-GMP under these conditions. When the cells are subsequently illuminated with only green light again, they can switch back to positive phototaxis within a few minutes. This switch under green light after blue light illumination depends on SesC, which degrades c-di-GMP. Moreover, a spontaneous mutation in the EAL domain of the gene *tll1859* leads to constitutive negative phototaxis. These results suggest that a high c-di-GMP content induces negative phototaxis in *T. vulcanus* (Nakane et al. 2022). Since the direction of blue light was shown to be irrelevant for phototaxis switching, it was suggested that potential local activation of the c-di-GMP-producing SesA protein on one side of the cell is not crucial for the directional switch (Nakane et al. 2022).

In *T. vulcanus*, c-di-GMP induces negative phototaxis on a short-time scale and cell aggregation on a long-time scale. During phototaxis, when cells are illuminated with blue light, the cells not only move away from the light but also form microaggregates under the microscope (Nakane et al. 2022). These microcolonies are phototactic and move surprisingly well, similar to single cells. It is not known whether the formation of these small aggregates is the first step of macroscopically observable aggregation, which depends on the production of cellulose (see below). It is conceivable that this leads to biofilm formation and that prolonged blue light illumination will end up in non-motile cells, as shown for *Synechocystis* (Wilde et al. 2002). Measurements of the dynamics of intracellular c-di-GMP concentrations in response to light signals will help identify correlations with different cellular behaviours over time. It is of note that the Tlr1612 protein was identified as a crucial phosphodiesterase to repress c-di-GMP signalling under standard cultivation conditions, though the signalling input for the Tlr1612 protein is unknown (Enomoto et al. 2018). Tlr1612 may be a global phosphodiesterase that insulates local c-di-GMP signalling pathways and allows local specificity, as shown for other bacteria (Jenal et al. 2017).

In many bacteria, including cyanobacteria, there are multiple sets of genes encoding c-di-GMP metabolizing proteins, implying that various environmental signals are integrated into the c-di-GMP signalling network to govern the lifestyle of organisms (Hengge 2009). Biochemical characterization showed that the *T. vulcanus* protein Tlr0485 exhibits cAMP-activated c-di-GMP phosphodiesterase activity (Enomoto et al. 2020), suggesting that cAMP signalling is an input for the c-di-GMP-dependent network. Notably, exogenously added cAMP can stimulate the phototactic motility of *Synechocystis* and aggregate formation in *Arthrospira* (Ohmori et al. 1992, Bhaya et al. 2006). Although it is unclear whether cAMP universally affects the cellular physiology of cyanobacteria, one can assume that cAMP and c-di-GMP sig-

Table 2. List of proteins related to c-di-GMP metabolism in *T. vulcanus* NIES-2134.

Gene product	Domain architecture
Proteins containing GGDEF and EAL domains	
NIES2134_119 260/SesB	CheY-**GAF**-GGDEF∗-EAL
NIES2134_110 090/SesC	PAS-PAS-PAS-PAS-**GAF**-PAS-GGDEF-EAL
NIES2134_115 190/Tlr1612	PAS-PAS-GAF-PAS-PAS-PAS-GGDEF-EAL
NIES2134_106 140/Tll0627	FHA-PAS-PAS-GGDEF-EAL
NIES2134_117 830/Tll1859	PAS-PAS-GGDEF-EAL
Proteins containing only GGDEF domains	
NIES2134_109 940/SesA	CBS-CBS-CBS-CBS-PAS-**GAF**-GGDEF
NIES2134_102 920	PAS-PAS-PAS-PAS-PAS-PAS-GAF-GGDEF
NIES2134_113 480/Tlr1210	X-GGDEF
NIES2134_106 930/Tll1049	CheY-CheY-CheY-GGDEF
NIES2134_101 640/Tlr1158	X-GGDEF
Proteins containing a HD-GYP domain	
NIES2134_102 990/Tlr0485	GAF-HD_GYP

Domains in italics and with an asterisk contain degenerate motifs. GAF domains in bold are chromophorylated. PAS, Per-Arnt-Sim; GAF, cyclic GMP/adenylyl cyclase/FhlA; FHA, forkhead associated domain; CheY, CheY-like receiver domain; CBS, cystathionine beta synthase; PBPb, bacterial periplasmic substrate-binding protein; X, undefined region.

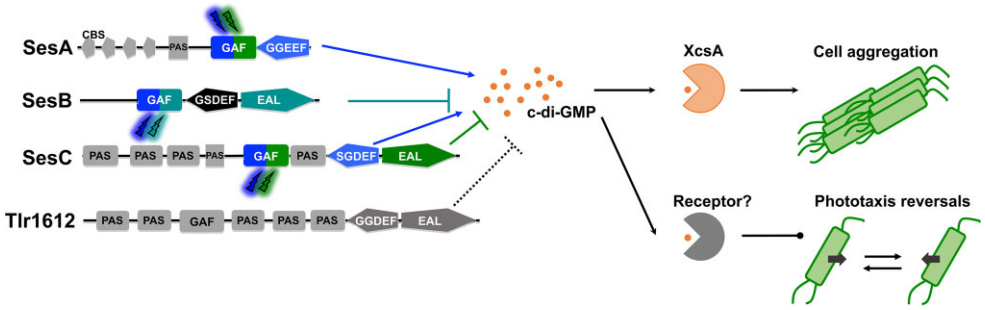

Figure 3. The role of c-di-GMP in *T. vulcanus* behaviour. Three CBCRs regulate c-di-GMP signalling. Blue light induces cyclic di-GMP formation, whereas teal and green light lead to c-di-GMP degradation. The cellulose synthase XcsA is activated by c-di-GMP and supports the aggregation of *T. vulcanus* cells. The effector for c-di-GMP-dependent phototaxis reversals is unknown.

nalling are coordinated in a cell to orchestrate lifestyle transitions to enable optimal life for surface growth.

C-di-GMP receptor proteins

The downstream targets of the c-di-GMP-based signalling cascade are poorly investigated in cyanobacteria. An exception is the formation of aggregates in *T. vulcanus* cultures. In a culture flask, blue light illumination leads to cellular aggregation (Enomoto et al. 2014). The cellulose synthase XscA binds to c-di-GMP via its PilZ domain (Enomoto et al. 2018) and is necessary for aggregate formation (Kawano et al. 2011). These studies imply that c-di-GMP activates the c-di-GMP receptor XcsA, which produces the extracellular cellulose covering the microcolony to form a stable aggregate (Fig. 3). However, to date, no potential c-di-GMP receptor protein involved in phototaxis has been identified. What is the nature of the c-di-GMP receptor that transduces c-di-GMP signals into a cellular response? *Synechocystis* does not encode a PilZ domain-containing protein or any other known c-di-GMP receptor homologue either, except for a number of GGDEF/EAL/HD-GYP domains, which in principle can bind c-di-GMP. Cyanobacteria utilize type IV pili for the movement on surfaces (Bhaya et al. 1999, Schuergers and Wilde 2015). As in heterotrophic bacteria, the motor protein PilB is responsible for the extension of type IV pili, whereas PilT is essential for their retraction (Craig et al. 2019). Cyanobacterial PilB proteins contain an MshEN domain (Wang et al. 2016), which has been described as a c-di-GMP-binding module in PilB proteins from other bacteria (Hendrick et al. 2017, Dye and Yang 2020). However, none of the five *Synechocystis* MshEN domains identified by Wang et al. (2016) contains the fully conserved tandem 24-residue c-di-GMP-binding motif. It is possible that the motor ATPase itself is controlled by c-di-GMP in *Synechocystis* and *T. vulcanus*. However, experimental validation of this hypothesis is still lacking for cyanobacterial strains. How the light-dependent modulation of c-di-GMP content regulates motility and phototaxis in cyanobacteria remains an outstanding question. Very recently, a new cyanobacteria-specific c-di-GMP receptor, CdgR, has been described that controls cell size in the filamentous strain *Anabaena* sp. PCC 7120 (Zeng et al. 2023). CdgR harbours a c-di-GMP binding site that has no similarity in sequence or structure to known motifs. There are two c-di-GMP binding pockets in CdgR, which are located at the dimer interface. Structural and mutational analyses revealed that dimerization of CdgR is essential for c-di-GMP binding, but dimerization does not depend on the binding of the second messenger as in other c-di-GMP receptor proteins (Zeng et al. 2023). In *Anabaena* sp. PCC 7120, c-di-GMP inhibits the interaction of CdgR with the transcription factor DevH, suggesting that changes in the intracellular c-di-GMP content alter the expression of genes belonging to the DevH regulon. DevH is an essential transcription factor for *Anabaena* sp. PCC 7120, but its targets need to be identified. *Synechocystis* encodes a homologue of this receptor (Slr1970), and Zeng et al. (2023) also showed c-di-GMP binding for this protein. CdgR is highly conserved in cyanobacteria, and *T. vulcanus* encodes a homologue of

this protein (NIES2134_110 700/Tlr0849) as well. The amino acids crucial for c-di-GMP binding are well conserved, and future work should reveal the function of this receptor in the behaviour of *Synechocystis*, *T. vulcanus*, and other cyanobacteria.

Role of blue light in lifestyle decisions

In this review, we have summarized the current knowledge on c-di-GMP signalling in cyanobacteria, focusing on light-dependent functions in two model unicellular cyanobacteria. It is striking that light, especially blue light, seems to be the dominant signal for cyanobacteria to change their lifestyle. In both cyanobacteria, *Synechocystis* and *T. vulcanus*, blue light enhances the diguanylate cyclase activity of CBCRs. Importantly, it is this blue light-dependent increase in the cellular c-di-GMP concentration that leads to changes in motility and aggregation, although both cyanobacteria have a multitude of other proteins involved in c-di-GMP metabolism. Moreover, *Synechococcus elongatus* PCC 7942 encodes a LOV-domain photoreceptor that shows blue light-dependent phosphodiesterase activity. Surprisingly, blue light is also an important signal for bacteria outside oxygenic phototrophs to make lifestyle decisions (Gomelsky and Hoff 2011). One prominent example is the function of the BLUF domain protein YcgF from *E. coli* (Tschowri et al. 2009). This protein harbours an inactive EAL at the C-terminus domain, which controls a transcription factor. The blue light-dependent interaction with YcgE induces a signal transduction chain that controls biofilm maturation. For more examples of blue light photoreceptors from non-phototrophs linked to c-di-GMP metabolism, see the excellent review by Gomelsky and Hoff (2011). Why these responses are mainly triggered by blue light remains an open question. Blue light has a general inhibitory effect on bacterial growth because several cofactors, such as flavin or heme, can absorb blue light and get damaged by strong exposure (Redmond and Gamlin 1999). It is noteworthy that blue light, which has a short wavelength, cannot easily penetrate the human or animal body. Therefore, the presence of blue light may also indicate being outside the host for pathogenic bacteria.

The quality of light in aquatic environments is affected by several factors, including water depth, water clarity, and the presence of suspended particles and photosynthetic microorganisms. Further, in microbial mats, light quality might also change with depth. Pigmented organisms can absorb light, thereby reducing the number of photons of a certain wavelength reaching deeper parts of a biofilm. A microbial mat close to a water surface will be subjected to UV and blue light. However, these harmful high-energy photons, together with red light, are absorbed rapidly by chlorophyll-containing organisms, whereas green/yellow and far-red light are only slightly attenuated. At 5 m below the water surface, red and far-red parts of the spectrum are completely missing due to absorption by the water column. Thus, light penetration through water and microbial mats is very different (Scholes et al. 2012). It has been suggested that *T. vulcanus* uses blue/teal-green CBCRs to sense the degree of shading in cyanobacterial mats and planktonic cultures (Enomoto and Ikeuchi 2020). However, the role of blue/green-sensing photoreceptors may be primarily significant for mats and cyanobacteria that lack phycoerythrin. *Thermosynechococcus vulcanus* can use green light as a reference for the blue monitoring light, as long as it is not absorbed by pigments such as phycoerythrin that absorb green light. In the microbial mats in the hot springs from which *T. vulcanus* was isolated, no phycoerythrin-containing cyanobacteria or red algae were detected on the top layer of the mats. *Fremyella diplosiphon* does contain phycoerythrin; thus, it absorbs blue, green, and red light. Interestingly, this strain produces more c-di-GMP under red and green light than under blue light (Agostoni et al. 2013). However, similar to *Synechocystis* which contains the highest c-di-GMP content under blue light, low c-di-GMP contents promote the sinking of *Fremyella* cells in a water column. It is possible that phycoerythrin-containing cyanobacteria use a different system to orient themselves in a water column or in photosynthetic mats.

To avoid the negative effects of light stress, most cyanobacterial species employ defence mechanisms, such as the production of UV-absorbing pigments, self-shading by aggregation/biofilm formation, or negative phototaxis. In response to light stress, blue light photoreceptors appear to be especially useful in detecting a harmful light environment. The aggregation of cells in water, which is regulated by blue light-dependent c-di-GMP signalling in *T. vulcanus* and *Synechocystis*, protects the cells from high light by self-shading. UV light is even more harmful to living organisms, and *Synechocystis* contains a UV-A photoreceptor that induces negative phototaxis away from the UV-A light source (Song et al. 2011). However, this signalling pathway depends on the CheY-like response regulator LsiR and is not known to involve second messengers. As UV light is rapidly attenuated in water, and red and far-red components of the spectrum are also completely extinguished at 5 m below the surface due to absorption by the water column (Scholes et al. 2012), high-energy blue photons seem to be ideal for monitoring the light environment deeper in a water column. Phototactic cells can detect harmful ultraviolet (UV) light on the surface of a microbial mat and respond accordingly.

It is of note that potentially light-independent c-di-GMP functions have also been found in cyanobacteria. Huang et al. (2021) demonstrated that at least one of the 16 genes that encode proteins involved in c-di-GMP metabolism in the filamentous cyanobacterium *Anabaena* sp. PCC 7120 plays a role in heterocyst development. This multi-domain protein (CdgSH) harbours a GAF domain that does not contain the amino acids important for chromophore binding in a typical photosensory GAF domain. Further, it has been shown that a phosphodiesterase (CdgS), which is part of a two-component system including the multi-domain histidine kinase CdgK, controls the cell size of this cyanobacterium (Sun et al. 2023). In both systems, the signal for the activation of c-di-GMP-related enzymes is unknown. Zeng et al. (2023) further proposed that the newly discovered c-di-GMP receptor, CdgR, functions together with the transcription factor DevH downstream of the CdgK–CdgS two-component system to control cell size and, most probably, essential functions in *Anabaena*. These studies revealed that c-di-GMP signalling might be involved in many more cyanobacterial responses than reflected in the current literature.

Concluding remarks

In recent years, several studies have uncovered that c-di-GMP is the master molecule governing the survival strategy of cyanobacteria through the control of many aspects of cellular physiology in an ecologically relevant context. Specifically, c-di-GMP is emerging as a crucial regulator of cyanobacterial collective behaviours, such as phototaxis and cell aggregation, processes that usually involve groups of cells. Since cyanobacteria are one of the founders of multicellularity (Hammerschmidt et al. 2020), c-di-GMP signalling systems could have been fundamental for the development of multicellular bacterial consortia. Future studies should provide a more comprehensive understanding of the c-di-GMP signalling network in cyanobacteria and will uncover the respective effectors and their downstream targets. These developments will be

crucial for comprehending the tremendous ecological and evolutionary success of cyanobacteria and their interaction with other phototrophic and non-phototrophic microorganisms in many different ecosystems.

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