Second messenger molecules are crucial components of environmental signaling systems to integrate multiple inputs and elicit physiological responses. Among various kinds of second messengers, cyclic nucleotides cAMP and cyclic di-GMP (c-di-GMP) play pivotal roles in bacterial environmental responses. However, how these signaling systems are interconnected for a concerted regulation of cellular physiology remains elusive. In a thermophilic cyanobacterium Thermosynechococcus vulcanus strain RKN, incident light color is sensed by cyanobacteriochrome photoreceptors to transduce the light information to the levels of c-di-GMP, which induces cellular aggregation probably via cellulose synthase activation. Herein, we identified that Tlr0485, which is composed of a cGMP-specific phosphodiesterases, adenylate cyclases, and FhlA (GAF) domain and an HD-GYP domain, is a cAMP-activated c-di-GMP phosphodiesterase. We also show biochemical evidence that the two class-III nucleotide cyclases, Cya1 and Cya2, are both adenylate cyclases to produce cAMP in T. vulcanus. The prevalence of cAMP-activated c-di-GMP phosphodiesterase genes in cyanobacterial genomes suggests that the direct crosstalk between cAMP and c-di-GMP signaling systems may be crucial for cyanobacterial environmental responses.

Key Words: cAMP; cyanobacteria; cyclic di-GMP; second messenger; sessility; signal transduction; Thermosynechococcus

Introduction

Second messenger molecules are capital components of environmental signaling systems since it can integrate many sensory inputs, amplify the signal, and control various downstream effectors and responses (Agostoni and Montgomery, 2014). Nucleotide second messengers such as cAMP, cGMP, c-di-GMP, and (p)ppGpp have been found to play important roles in signaling in all domains of life, including bacteria (Gomelsky, 2011; Kalia et al., 2013). cAMP is the first discovered second messenger involved in various functions, such as eukaryotic signal transduction and bacterial catabolite repression (McDonough and Rodriguez, 2012). cAMP is generated from ATP by adenylate cyclases (ACs) and cAMP-specific phosphodiesterases (cAMP-PDEs) catalyze its hydrolytic degradation to AMP. c-di-GMP was discovered as an allosteric activator of cellulose synthase in 1987 (Ross et al., 1987). Since then, c-di-GMP has been extensively investigated to be identified as a critical regulator involved in biofilm formation, motility, cell cycle progression, and virulence (Jenal et al., 2017; Römling et al., 2013). The GGDEF domain is responsible for diguanylate cyclase (DGC) activity to synthesize c-di-GMP from two...
GTP molecules (Paul et al., 2004), whereas EAL and HD-GYP domains exhibit c-di-GMP PDE activity to degrade c-di-GMP into two GMP molecules via intermediate, pGpG (Christen et al., 2005; Dow et al., 2006). The coordination of synthesis and breakdown of the second messengers controls their intracellular levels and governs the cellular physiology.

cAMP- and c-di-GMP-related genes are also found in almost all of the cyanobacterial species except for marine picocyanobacteria (Agostoni and Montgomery, 2014; Ohmori and Okamoto, 2004). cAMP is involved in cyanobacterial phototactic motility, respiration, mat formation, and inorganic carbon responses (Bhaya et al., 2006; Ohmori et al., 1993; Selim et al., 2018; Terauchi and Ohmori, 1999). c-di-GMP signaling has been intensively investigated in a thermophilic cyanobacterium Thermosynechococcus vulcanus strain RKN (equivalent to NIES-2134). T. vulcanus, a very close relative of Thermosynechococcus elongatus strain BP-1 (equivalent to NIES-2133) (Nakamura et al., 2002), shows cellulose-dependent cell aggregation under blue light and at a low temperature of 31°C (Enomoto et al., 2014; Kawano et al., 2011; Maeda et al., 2018). The cell aggregation is regulated by c-di-GMP levels according to ambient light color by the three cyanobacteriochrome-type photoreceptors: SesA, a blue light-activated DGC; SesB, a teal light- and GTP-activated c-di-GMP PDE; and SesC, a bifunctional protein with DGC activity induced by blue light and c-di-GMP PDE activity induced by green light (Enomoto et al., 2014, 2015). Tlr1612 protein is crucial for repressing cell aggregation under green light conditions, and it functions downstream of SesA/B/C light sensor proteins in the c-di-GMP signaling (Enomoto et al., 2018).

To express the full-length Tlr0485 of T. elongatus strain BP-1 (equivalent to NIES-2133) as the N-terminally His-tagged protein in Escherichia coli C41 (DE3), the protein-encoding DNA was cloned into pET28a (Novagen) using the In-Fusion system (TaKaRa). The protein purification and SDS-PAGE, followed by Coomassie Brilliant Blue staining were performed as described (Enomoto et

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**Fig. 1.** Biochemical characterization of Tlr0485.

A. Domain composition of Tlr0485 deduced by SMART (http://smart.embl-heidelberg.de). GAF, cGMP phosphodiesterase/adenylate cyclase/FhlA; HD-GYP, phosphodiesterase domain. B. CBB-stained SDS-PAGE gels of the purified Tlr0485 protein (the calculated molecular mass: 63.3 kDa) expressed in and purified from an E. coli expression system. C. HPLC chromatograms revealing the nucleotide composition of the c-di-GMP PDE (c-di-GMP → pGpG) assay solution. Reaction mixtures including 100 µM c-di-GMP were incubated for 10 min at 45°C in the absence (left panel) or presence (right panel) of 10 µM cAMP. Nucleotides were detected at 254 nm. The internal standard NAD (final concentration, 50 µM) was added into each sample just before injection. D. The cAMP concentration dependence curve for the PDE activity of Tlr0485. The reaction velocity is calculated from the amount of the reaction product, pGpG. Shown data are mean ± standard deviations of three independent experiments.
Tlr0485 is a cAMP-activated c-di-GMP phosphodiesterase in a cyanobacterium Thermosynechococcus (al., 2012) (Fig. 1B). We measured the c-di-GMP-specific PDE activity of the purified Tlr0485 protein. The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol, and 100 µM c-di-GMP (Biolog). Each reaction was initiated by addition of the purified Tlr0485 protein to a pre-warmed reaction mixture and incubated at 45°C. Each reaction was stopped by addition of EDTA (final concentration, 10 mM) and immediately heated at 98°C for 3 min to denature the protein, followed by centrifugation at 20,400 × g for 5 min. Each supernatant was subjected to a reversed-phase HPLC through a C18 column (150 mm × 6 mm I.D., DAI SOPAK SP-120-5-ODS-AP, Daiso) to separate nucleotides. Samples (20 µl) were injected and eluted at 1.4 ml min⁻¹ using buffer A (100 mM potassium phosphate, 4 mM tetrabutylammonium hydrogen sulfate [pH 6.0]) and buffer B (75% buffer A, 25% methanol) and the gradient protocol: 0.0, 0; 2.5, 0; 5.0, 30; 14.0, 40; 25.0, 100; 32.0, 100; 33.0, 50; and 34.0, 0 (the first of each set of values is the time (min) and the second in each set is the percentage of buffer B) (Enomoto et al., 2015).

When incubated with c-di-GMP, the Tlr0485 protein produced pGpG (5′-phosphoguanayl-(3′,5′)-guanosine) from c-di-GMP (Fig. 1C). When incubated with 100 µM cAMP or cGMP, the protein did not produce AMP or GMP, suggesting that Tlr0485 is a c-di-GMP-specific PDE.

The GAF domain is known to be one of the largest superfamilies that bind small molecules, such as amino acids, nucleotides, heme, and open-chain tetrapyrrrole chromophore (Aravind and Ponting, 1997; Chen et al., 2018; Fushimi and Narikawa, 2019). The GAF domain of Tlr0485 shows a weak similarity to known cyclic mononucleotide binding GAF domains (Supplementary Figs.

Fig. 2. Biochemical characterization of Cya1 and Cya2. A. Domain compositions of Cya1 (Tll2410) and Cya2 (Tll2280) deduced by SMART (http://smart.embl-heidelberg.de). FHA, Forkhead associated domain; CYCc, type-III cyclase domain; TM, transmembrane region; CHASE2, cyclase/histidine kinase-associated sensing extracellular 2. B. CBB-stained SDS-PAGE gels of the purified Cya1 protein (the calculated molecular mass: 44.6kDa) and the Cya2ΔN protein (the calculated molecular mass: 27.8kDa) expressed in and purified from an E. coli expression system. C, D. HPLC chromatograms revealing the nucleotide composition of the cAMP production (ATP → cAMP) assay solution including the Cya1 protein (C) or the Cya2ΔN protein (D). Reaction mixtures, including 100 µM ATP and 10 mM MnCl₂, were incubated for 0 min (left panel) and 60 min (right panel) at 45°C. Nucleotides were detected at 254 nm. The internal standard NAD (final concentration, 50 µM) was added into each sample just before injection.
S1 and S2). To test whether the GAF domain of Tlr0485 binds cyclic mononucleotides, various nucleotides were added to the c-di-GMP PDE reaction mixtures. Although the addition of 100 μM ATP, ADP, AMP, GTP, GDP, GMP, and cGMP did not affect the PDE activity of Tlr0485, the addition of 100 μM cAMP greatly increased the PDE activity (Fig. 1C), indicating that Tlr0485 is a cAMP-activated c-di-GMP phosphodiesterase.

We measured the kinetic parameters of the c-di-GMP PDE activity of Tlr0485 in the presence of the activator cAMP (2 μM). The $K_m$ for c-di-GMP was 6.76 ± 1.97 (μM) and $k_{cat}$ was 0.28 ± 0.04 (s⁻¹) (n = 3) (Supplementary Fig. S3). The GAF domain of Tlr0485 likely works as a regulatory domain by specifically binding cAMP. To assure that the binding of cAMP by the GAF domain is physiologically relevant, we measured the effective concentration (EC50) of cAMP for activation of c-di-GMP PDE. The obtained EC50 value for cAMP was 0.54 ± 0.16 (μM) (Fig. 1D), which was lower than that of the known cAMP receptor protein of Synechocystis, SyCRP ($K_s = 3$ μM) (Yoshimura et al., 2000). These results led us to expect that the c-di-GMP-specific PDE activity of Tlr0485 is regulated by cAMP signaling in vivo.

There was no knowledge about cAMP signaling in Thermosynechococcus. Among six classes of ACs, which are classified based on their amino acid sequences, cyanobacterial adenylate cyclases are classified into class III, as is the case with all known eukaryotic adenylate cyclases (Ohmori and Okamoto, 2004). T. elongatus genome harbors two class-III nucleotides cyclases (Nakamura et al., 2002), TII2410 and TII2280 (Fig. 2A), which are homologs of Cya1 and Cya2 of a mesophilic cyanobacterium Synechocystis sp. PCC 6803, respectively. In Synechocystis, Cya1 is an adenylate cyclase (Masuda and Ono, 2004; Terauchi and Ohmori, 1999), whereas Cya2 is a guanylate cyclase to produce cGMP (Rauch et al., 2008). We purified the full-length TII2410 (Cya1) protein and the catalytic region of TII2280 (Cya2) protein (Cya2ΔN, 426–654 a.a.). Both proteins are of T. vulcanus and expressed as the N-terminally His-tagged proteins in E. coli (Fig. 2B). For the purification of Cya1, the used concentration of NaCl was 1 M instead of 100 mM and dialysis was omitted because the protein tended to aggregate probably due to a weak hydrophobic stretch (Ohmori and Okamoto, 2004).

We measured cAMP-producing activities of the purified Cya1 and Cya2ΔN proteins. Nucleotides were separated by reversed-phase HPLC using the same set-up as the c-di-GMP PDE assay, except for the modified gradient protocol: 0.0, 0; 2.5, 0; 5.0, 30; 14.0, 40; 45.0, 100; 47.0, 100; 48.0, 50; and 49.0, 0 (the first of each set of values is the time (min) and the second in each set is the percentage of buffer B). Because we could not perform dialysis of the purified Cya1 protein, the reaction mixture for Cya1 contained 20 mM HEPES-NaOH (pH 7.5), 1 M NaCl, 10% (w/v) glycerol, ~200 mM imidazole and 10 mM MgCl₂ or 10 mM MnCl₂. Each reaction was initiated by addition of the substrate ATP (final concentration, 100 μM) to a pre-warmed reaction mixture.

When incubated with ATP and Mg²⁺, Cya2ΔN but not Cya1 produced CAMP. Because previous reports showed that Mn²⁺ instead of Mg²⁺ dramatically increased the adenylate cyclase activity of Synechocystis Cya1 (Masuda and Ono, 2004), we used Mn²⁺ instead of Mg²⁺ in the reaction mixtures of Cya1 and Cya2ΔN. When assayed with 10 mM MnCl₂ instead of 10 mM MgCl₂, 50 mM Tris–HCl (pH 7.5) was used instead of pH 8.0 to prevent oxidative precipitation of manganese. Cya1 then produced cAMP from ATP and Cya2ΔN showed the higher activity of producing cAMP (Figs. 2C and D). The concomitant production of ADP in the Cya1 preparation may be due to the contaminated proteins from E. coli or somehow artificial activity of Cya1 because of the absence of the Mn²⁺ activation. In the literature (Yang and Epstein, 1983), Cya from E. coli was equally activated by Mg²⁺ and Mn²⁺, in contrast with our results. Both Cya1 and Cya2ΔN did not produce cGMP when incubated with GTP. Taken together, these results showed that both Cya1 and Cya2 are adenylate cyclases in T. vulcanus. This is consistent with the protein alignment of the class-III cyclases (Supplementary Fig. S4), which suggests that both Cya1 and Cya2 harbor the conserved amino acids crucial for the selectivity of ATP as a substrate instead of GTP (Ryu et al., 2010). The presence of adenylate cyclases strongly suggests that cAMP signaling is operating in T. vulcanus.

c-di-GMP induces T. vulcanus cell aggregation at a relatively low temperature of 31°C (Enomoto et al., 2015). Sequence analysis revealed that Tlr0485 is highly conserved between T. elongatus and T. vulcanus; the HD-GYP domain is identical, and the GAF domain has a single amino acid substitution (R167T in T. vulcanus), which should be located in a peripheral region (Bellini et al., 2014) and another single substitution is located at the N-terminal region (S28A in T. vulcanus). This suggests that Tlr0485 of T. vulcanus retains biochemical properties similar to that of T. elongatus. The T. vulcanus strain of tlr0485 gene disruption mutant showed no phenotype in cell aggregation at 31°C under irradiation with blue/red light (an inducing condition) or with green/red light (a repressing condition), although tlr0485 was expressed under these conditions (Enomoto et al., 2018). This result suggests that
cAMP signaling is not stimulated and Tlr0485 is not activated under the experimental conditions. Future work will explore environmental signals that activate Cya1 or Cya2 to induce cAMP signaling.

Here, we have biochemically identified that Tlr0485 is a cAMP-activated c-di-GMP phosphodiesterase, which may work as the mediator of the direct crosstalk between cAMP and c-di-GMP signaling pathways in *T. vulcanus*. In our signaling model (Fig. 3), unknown environmental signals activate cAMP production of Cya1 or Cya2. cAMP binds to the GAF domain of Tlr0485 and activates the c-di-GMP-specific PDE activity. Tlr0485 decrease c-di-GMP levels, resulting in the inhibition of cell aggregation, which may be induced by SesA/B/C. In other words, certain signal(s) may suppress the cell aggregation via cAMP/Tlr0485 even under conditions of blue light and low temperature. The canonical target(s) of cAMP signaling in *T. vulcanus* has to be addressed. In *Synechocystis* sp. PCC 6803, blue light activates Cya1 via an unknown photoreceptor (Terauchi and Ohmori, 2004), and the produced cAMP binds to CRP. The cAMP-bound CRP activates the transcription of pili genes, resulting in the induction of pili-dependent twitching motility (Terauchi and Ohmori, 1999; Yoshimura et al., 2002). CAMP may also activate motility in *T. vulcanus* because CRP and pili genes are present. cAMP-dependent repression of c-di-GMP-induced cell aggregation by Tlr0485 might be advantageous for efficient motility, because cellulose and extracellular polymeric substances are crucial for cell aggregation, but may hinder motility. Indeed c-di-GMP represses motility in *Synechocystis* via an unknown mechanism (Savakis et al., 2012). Tlr0485 may work as the central component for the coordinated regulation of cAMP-induced motility and c-di-GMP-induced cell aggregation. The direct connection via Tlr0485 will result in a much faster response than transcriptional regulation-based networks, which is indeed one of the advantages of second messenger signaling.

Tlr0485 homologs with the same domain organization are present in various cyanobacteria, such as *Synechococcus elongatus* PCC 7942, *Trichodesmium erythraeum* IMS101, and *Arthrospira platensis* NIES-39 (Figs. S1 and S2). Tlr0485 homologs are also identified in bacteria including PmGH, of which the structure has already been determined (Bellini et al., 2014) (eggNOG database accession: ENOG4105C4Z). Whereas the GAF domain of PmGH falls into the clade of the known cNMP-binding domains, those of cyanobacterial Tlr0485 homologs constitute the distinct clade in the phylogenetic tree (Fig. S2). These results suggest that the GAF domains of cyanobacterial Tlr0485 homologs form a novel cNMP-binding group. Recently, *Bdellovibrio bacteriovorus* Bd1971 was found to be a cAMP/cGMP-activated c-di-GMP phosphodiesterase with a cNMP domain-EAL domain architecture (Cadby et al., 2019). Homologous proteins of Bd1971 are also present in various cyanobacteria, e.g. Strh0482 in *Synechocystis*, which was previously suggested to be cAMP-binding protein (Ochoa de Alda and Houmard, 2000). These results suggest that the direct repression of c-di-GMP signaling by cAMP signal may be generally crucial for cyanobacteria.

Recently more reports are identifying the interrelation between cAMP and c-di-GMP signaling systems. In *Vibrio cholera* and *Klebsiella pneumoniae*, cAMP represses c-di-GMP signaling via transcriptional regulation of multiple genes encoding c-di-GMP synthesizing/degrading enzymes (Fong and Yildiz, 2008; Lin et al., 2016). In *Pseudomonas aeruginosa*, cAMP activates the transcriptional regulator Vfr, resulting in up-regulation (Luo et al., 2015) or down-regulation of c-di-GMP signaling (Almblad et al., 2019). Interestingly a cAMP-binding GAF domain is harnessed to activate the downstream GGDEF domain to induce c-di-GMP production in *Leptospira* (da Costa Vasconcelos et al., 2017). It is crucial to reveal how cAMP and c-di-GMP signaling are regulated concertedly for a deeper understanding of complicated bacterial signaling networks that control broad-spectrum environmental responses.

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**Author Contributions**

G.E., R.N., and M.I. designed research; A.K. and Y.O. performed research; A.K., G.E., R.N., and M.I. analyzed data; and G.E. and M.I. wrote the paper.

**Supplementary Materials**

Supplementary figures are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).
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