Tlr1612 is the major repressor of cell aggregation in the light-color-dependent c-di-GMP signaling network of *Thermosynechococcus vulcanus*

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Cyclic diguanylate (c-di-GMP) is a bacterial second messenger involved in sessile/motile lifestyle transitions. We previously reported that c-di-GMP is a crucial inducer of cell aggregation of the cyanobacterium *Thermosynechococcus vulcanus*. The three cooperating cyanobacteriochrome photoreceptors (SesA/B/C) regulate cell aggregation in a light color–dependent manner by synthesizing/degrading c-di-GMP. Although a variety of c-di-GMP signaling proteins are encoded in cyanobacterial genomes, how c-di-GMP signaling networks are organized remains elusive. Here we experimentally demonstrate that the cellulose synthase Tl10007, which is essential for cell aggregation, binds c-di-GMP although the affinity is low ($K_d = 63.9 \pm 5.1 \mu M$). We also show that SesA—the main trigger of cell aggregation—is subject to strict product feedback inhibition ($IC_{50} = 1.07 \pm 0.13 \mu M$). These results suggest that SesA-produced c-di-GMP may not directly bind to Tl10007. We therefore systematically analyzed all 10 of the genes encoding proteins containing a c-di-GMP synthesis/degradation domain. We identified Tlr1612, harboring both domains, as the major repressor of cell aggregation under the repressing teal-green light irradiation. *tlr1612* acts downstream of *sesA* and is not regulated transcriptionally by light color, suggesting that Tlr1612 may be involved in c-di-GMP amplification in the signaling cascade. Post-transcriptional control is likely crucial for the light-regulated c-di-GMP signaling.

Cyclic diguanylate, or bis-(3′-5′)-cyclic diguanylic acid (c-di-GMP) is a second messenger that is most commonly found in bacteria. C-di-GMP was originally identified as an allosteric activator of cellulose synthesis in *Komagataeibacter xylinus*. Subsequent studies revealed that c-di-GMP generally induces sessile, multicellular lifestyles such as biofilms, and that it represses the motile, planktonic lifestyle in various bacteria. C-di-GMP is also involved in cell-cycle progression and the expression of virulence genes, indicating that c-di-GMP orchestrates various cellular responses to effect drastic changes in bacterial physiology. C-di-GMP is synthesized by the diguanylate cyclase (DGC) activity of GGDEF domains and degraded by the c-di-GMP–specific phosphodiesterase (PDE) activity of EAL and HD-GYP domains. C-di-GMP binds to and regulates various effectors such as proteins containing a PilZ domain or a MshEN domain, transcription factors, and riboswitches. Notably, bacterial genomes often contain multiple genes encoding DGCs and c-di-GMP PDEs that encode GGDEF/EAL/HD-GYP domain proteins, which contrasts with the number of genes that govern other second messengers such as cAMP. This suggests that a complex c-di-GMP–based signaling network operates in diverse bacterial cells.

Multiple genes encoding DGCs and c-di-GMP PDEs are also found in the genomes of cyanobacteria, which are photoautotrophic organisms that perform oxygenic photosynthesis. The prevalence of the c-di-GMP genes suggests that c-di-GMP has a crucial function in cyanobacterial physiology, the roles of c-di-GMP remains elusive. Several studies have reported that c-di-GMP regulates phototactic motility,
biofilm formation, and cellular buoyancy20 of the cyanobacterium Synechocystis. However, no cellular target for c-di-GMP has yet been identified in cyanobacteria; thus the functions of c-di-GMP remain enigmatic.

We previously reported that c-di-GMP is a crucial inducer of cell aggregation of the thermophilic cyanobacterium Thermosynechococcus vulcanus21. The three cyano bacteriochrome-type photoreceptors SesA, SesB, and SesC, perceive light of the blue-to-green window and regulate cell aggregation. SesA is a blue light–activated DGC and acts as the major trigger of cell aggregation upon blue-light irradiation22. SesB exhibits PDE activity that is upregulated by teal light23 and GTP, and SesB represses cell aggregation under teal-green-light irradiation. SesC is a bifunctional photoreceptor, the DGC activity of which is induced by blue light and PDE activity is induced by green light, thereby enhancing the sensitivity of cells to incident light color. Thus, the coordinated action of SesA/B/C enables induction of cell aggregation specifically under blue light but not under teal-green light, and this is mediated by c-di-GMP signaling24.

In addition, the cellulose synthase Tll0007 has been identified as an essential component for cell aggregation24, with its PilZ domain being necessary for cell aggregation22. Thus, it is very likely that c-di-GMP activates the cellulose synthase function of Tll0007 similarly to its homolog BcsA proteins25,26, resulting in induction of the cellulose-dependent cell aggregation of T. vulcanus. However, the binding of c-di-GMP to the PilZ domain of Tll0007 lacks experimental verification. Moreover, the Thermosynechococcus genome contains a total of 10 genes encoding DGCs and c-di-GMP PDEs (Fig. S1). Although this complexity is much less than that of other cyanobacteria (e.g., 28 genes in Synechocystis), the relationship and interdependence of the c-di-GMP DGC and PDE proteins remain to be explored in T. vulcanus.

Here, we investigated the binding of c-di-GMP to Tll0007 and the kinetics of c-di-GMP production by SesA to assess the action of c-di-GMP in the signaling cascade. Furthermore, we performed systematic analyses of all the genes encoding DGCs and c-di-GMP PDEs to gain a clearer understanding of global c-di-GMP signaling network with respect to the regulation of cell aggregation. This study fosters a better understanding of the sophisticated light-regulated c-di-GMP signaling system in cyanobacteria. Various cyanobacteria are found in a sessile multicellular community in nature and thermophilic cyanobacteria Thermosynechococcus have been isolated from the top layer of microbial mats in hot springs25–27. The understanding of the molecular mechanisms underpinning cell aggregation and multicellular lifestyles will lead to integration of our knowledge of molecular physiology and ecology of cyanobacteria.

**Results**

The cellulose synthase Tll0007 binds c-di-GMP via its PilZ domain. We previously reported that both c-di-GMP and the PilZ domain of Tll0007 are essential for inducing cell aggregation of T. vulcanus at 31 °C21,22. However, experimental verification of c-di-GMP binding by the PilZ domain of Tll0007 was lacking. To assess c-di-GMP binding to Tll0007, in the present study we designed c-di-GMP biosensor proteins using a fluorescence resonance energy transfer (FRET)-based system by concatenating two fluorescent proteins (mCyPet and mYPet, which are monomerized variants of CFP and YFP, respectively) with the PilZ domain of Tll0007 in between (Fig. 1A), according to28–30. For each purified biosensor protein, the addition of c-di-GMP resulted in a corresponding increase in FRET efficiency, whereas addition of GTP did not (Fig. 1B). The c-di-GMP concentration–response curve revealed a dissociation constant (K_d) of 63.9 ± 5.1 µM at 31 °C (Fig. 1C). Curve fitting revealed a Hill coefficient of 0.90 ± 0.14, indicating non-cooperative c-di-GMP binding.

The 601RXXXXR605 motif in PilZ domains is necessary for binding c-di-GMP30–32. We thus created the mutant R605A and assessed the binding of c-di-GMP. R605A lacked the corresponding increase in FRET efficiency upon c-di-GMP addition (Fig. 1C), indicating that c-di-GMP binding depends on Arg605 in the RXXXX motif of Tll0007. R605A had somewhat higher FRET efficiency than the unmodified wild-type (WT) PilZ domain in the absence of added nucleotides, suggesting that R605A exists in a locked conformation that is slightly activated. These results suggested that Tll0007 binds c-di-GMP via its PilZ domain.

The DGC activity of SesA exhibits product feedback inhibition. SesA is the major trigger of cell aggregation in T. vulcanus by producing c-di-GMP under blue-light irradiation31,32. The DGC activities of many GGDEF proteins are negatively regulated by their product, i.e., c-di-GMP33. To assess any potential product feedback inhibition of SesA, we measured the initial rate of the DGC reaction in the presence of various concentrations of c-di-GMP using a pyrophosphate assay kit, which monitors production of pyrophosphate—the byproduct of the DGC reaction. The purified protein of SesA was pre-irradiated with blue light to retain it in its active form during the assay. In the absence of c-di-GMP, WT SesA showed high initial pyrophosphate production activity, followed by a gradual decrease in production with increasing c-di-GMP concentration, which eventually stopped after ~100 s (at approximately 5 µM c-di-GMP) (Fig. 2A). Conversely, in the presence of 10 µM c-di-GMP, the DGC activity of WT SesA was strongly inhibited. These results indicated that the DGC activity of SesA is subject to product feedback inhibition.

The concentration–response curve for the DGC activity in the presence of c-di-GMP indicated an IC50 value (concentration for half-maximal inhibition) of 1.07 ± 0.13 µM at 31 °C (Fig. 2B). This affinity of c-di-GMP for the product inhibition site of SesA is comparable to values reported for other DGCs such as DgcA (IC50 = 0.96 µM) and PdeD (IC50 = 5.8 µM)33. The DGC activity of SesA in the absence of c-di-GMP was 20.5 ± 2.1 µmol pyrophosphate µmol protein −1 min −1; i.e., a turnover rate (k_cat) ~10 min −1. Curve fitting also indicated a Hill coefficient of 3.0 ± 0.3, implying high positive cooperativity of c-di-GMP binding to SesA.

Upon product feedback inhibition of DGCs, c-di-GMP binds to the allosteric product inhibition site (I-site), namely the RXXD motif34, which is located near the active-site within the GGDEF domain35–36. We used the SesA mutant R676A (within 68RXRX motif) to assess product inhibition. This mutant showed high pyrophosphate production activity regardless of the presence of 10 µM c-di-GMP, with the activity being sustained during the assay for 600 s (Fig. 2A). In fact, R676A maintained its high DGC activity even in the presence of 100 µM c-di-GMP.
Figure 1. FRET-based biosensor assay for assessment of c-di-GMP binding to the PilZ domain of Tll0007. (A) Scheme illustrating how binding of c-di-GMP to a target protein domain (here, Tll0007-PilZ) leads to a change in FRET efficiency between the attached mCyPet (CFP) and mYPet (YFP). (B) Fluorescence emission spectra of the biosensor protein in the presence of the indicated concentrations of c-di-GMP. A representative of three independent experiments is shown. (C) Nucleotide concentration-response curve for FRET efficiency (YFP emission at 527 nm/CFP emission at 475 nm; F527/F475) for c-di-GMP (left) and GTP (right). Data represents the mean ± SD of at least three independent experiments. Black symbols, WT Tll0007; red symbols, R605A mutant.

Figure 2. Pyrophosphate assay for assessing product feedback inhibition of SesA. (A) Mutant R676A or WT SesA (0.2 µM) was incubated at 31 ºC in the absence (solid line) or presence of 10 µM c-di-GMP (broken line), and pyrophosphate production was measured by continuously monitoring absorbance at 360 nm. Representative results of three independent experiments are presented. Black lines, WT; grey lines, R676A. (B) C-di-GMP concentration-response curve for the initial velocity of the DGC reaction of SesA (µM PPI/µM protein−1 min−1). A dose–response curve was fit for WT in KaleidaGraph software. PPI, pyrophosphate. Data represent the mean ± SD of three independent experiments. Solid circles, WT; open squares, R676A.
All the 10 genes were transcribed to a greater degree at 31 °C than at 45 °C, suggesting that a relatively low temperature leads to activation of the whole c-di-GMP signaling network. None of the genes were differentially expressed between the cells under blue light or teal-green light. We previously reported that blue light, whereas the WT and other mutants did not aggregate. Under teal-green light, Δtlr1612 cells underwent strong aggregation that was comparable with the aggregation observed under blue light, whereas the WT and other mutants did not aggregate. ΔsesB cells also underwent some aggregation under teal-green light, indicating that no other DGC besides SesA is essential for cell aggregation. Under teal-green light, Δtlr1612 cells underwent strong aggregation that was comparable with the aggregation observed under blue light, whereas the WT and other mutants did not aggregate. ΔsesB cells also underwent some aggregation under teal-green light, although the aggregation index value was much lower (~20%)[21]. These results suggested that Tlr1612 is the primary protein that represses the aggregation of cells irradiated with teal-green-light.

### Table 1. Results for the real-time qPCR analysis of 10 genes encoding DGCs and PDEs

| Gene    | Relative expression (vs. irradiation with white light at 45 °C) | Blue light at 31 °C | Teal-green light at 31 °C |
|---------|---------------------------------------------------------------|---------------------|---------------------------|
| sesA    | 5.4 ± 4.7                                                    | 6.5 ± 3.3           |                           |
| sesB    | 2.5 ± 0.7                                                    | 2.1 ± 0.3           |                           |
| sesC    | 4.9 ± 2.8                                                    | 7.9 ± 3.9           |                           |
| tlr1210 | 45 ± 43                                                      | 89 ± 65             |                           |
| tlr1158 | 3.2 ± 1.4                                                    | 6.1 ± 4.7           |                           |
| tll1049 | 4.2 ± 0.6                                                    | 4.5 ± 1.8           |                           |
| tll1859 | 4.1 ± 1.3                                                    | 6.0 ± 1.3           |                           |
| tll0627 | 4.1 ± 1.5                                                    | 6.2 ± 4.3           |                           |
| tlr1612 | 5.6 ± 3.5                                                    | 5.9 ± 2.7           |                           |
| tlr0485 | 7.0 ± 3.1                                                    | 7.9 ± 3.1           |                           |

Involvement of other DGC and PDEs in the regulation of cell aggregation. We created gene-disruption mutants of the remaining seven genes (tlr1210, tlr1158, tll1049, tll1859, tll0627, tlr1612, and tlr0485) of T. vulcanus and analyzed the aggregation of cells irradiated with blue or teal-green light irradiation at 31 °C (Fig. 3). The phenotypes of the other three mutants (sesA, sesB, and sesC) have already been reported[11]. Under blue light, WT cells underwent strong cell aggregation whereas Δtlr1210 mutant cells did not aggregate, in agreement with our previous reports[22,24]. None of the seven gene-disruption mutants displayed altered cell aggregation, indicating that no other DGC besides SesA is essential for cell aggregation. Under teal-green light, Δtlr1612 cells underwent strong aggregation that was comparable with the aggregation observed under blue light, whereas the WT and other mutants did not aggregate. ΔsesB cells also underwent some aggregation under teal-green light, although the aggregation index value was much lower (~20%)[21]. These results suggested that Tlr1612 is the primary protein that represses the aggregation of cells irradiated with teal-green-light.

Tlr1612 is epistatic to SesA for the regulation of cell aggregation. Δtlr1612 cells underwent strong aggregation under blue or teal-green light (Fig. 3), whereas ΔsesA cells showed little aggregation under either...
We therefore examined the possible epistasis of sesA and tlr1612 by creating the ΔsesA/Δtlr1612 double mutant. ΔsesA/Δtlr1612 cells underwent strong aggregation under both light conditions, similar to Δtlr1612 (Fig. 4), indicating that tlr1612 is epistatic to SesA for the regulation of cell aggregation.

Discussion

We investigated the c-di-GMP signaling network that regulates light-dependent cell aggregation in T. vulcanus. The cooperating cyanobacteriochrome photoreceptors SesA/B/C perceive blue-to-green light as an input to regulate c-di-GMP signaling21. However, it has remained elusive how other c-di-GMP signaling proteins function. The current study has refined our previous work and deepened our understanding of the sophisticated light-dependent c-di-GMP signaling network.

Tll0007 is the first cyanobacterial c-di-GMP receptor to be experimentally validated, corroborating the existence of c-di-GMP signaling in cyanobacteria. The affinity of the PilZ domain of Tll0007 for c-di-GMP (Kd = 63.9 ± 5.1 µM) (Fig. 1) was relatively low compared with other c-di-GMP receptors such as the Vibrio cholerae PlzD (Kd = 0.2 µM) and VpsT (Kd = 3.2 µM) and Pseudomonas aeruginosa Alg44 (Kd = 12.7 µM)12,28,41. Using our FRET biosensor assay, the full-length YcgR of Escherichia coli yielded an affinity of 0.42 ± 0.08 µM (Fig. S4), which is consistent with a previously reported value (Kd = 0.84 ± 0.16 µM)9, underscoring the reliability of our assay. We cannot exclude the possibility that the affinity of full-length Tll0007 for c-di-GMP is higher in vivo. However, Salmonella Typhimurium BcsA, a homolog of Tll0007, was found to have higher affinity (Kd = 8.4 µM) than Tll0007 even with a similar construct and assay28. These results strongly suggest that a relatively high cellular concentration of c-di-GMP may be necessary to activate Tll0007.

The Hill coefficient for Tll0007 was 0.90 ± 0.14 (Fig. 1), which contrasts with other PilZ domains that generally show positive cooperativity for c-di-GMP binding2,3,42. PilZ domains usually bind the c-di-GMP dimer2,31,32,43, although binding of the c-di-GMP monomer has also been reported42. The lack of cooperativity of c-di-GMP binding to Tll0007 suggests that Tll0007 can bind the c-di-GMP monomer and does not specifically recognize the c-di-GMP dimer. Notably, the binding of the c-di-GMP dimer to the PilZ domain protein Alg44 could induce
Figure 4. Epistasis analysis of sesA and tlr1612. Aggregation index values for WT and gene-disrupted mutants are shown. Cells were cultured at 31 °C for 48 h under blue light (filled bar) or teal-green light (open bar). Data represent the mean ± SD of at least three biological replicates. The data for WT and Δtlr1612 are duplicated from Fig. 3 for comparison.

Notably, disruption of all the three photoreceptors (sesA/B/C) still allowed some cell aggregation (the aggregation index was 40–70% for all the tested wavelengths31). This observation implies that certain levels of c-di-GMP are maintained during normal phototrophic growth owing to production by one or more DGCs(s), even though the possibly strong PDE activity of Tlr1612 is also functioning. There are many indications that multiple DGCs and PDEs are often spatially differentiated in prokaryotic cells, in addition to the well-known temporal regulations47. The possible PDE activity of Tlr1612 may also serve as an insulator for other, potentially unrelated, effects of c-di-GMP signaling, which are not shown in Fig. 5. To reconcile the ambiguity of our model, the biochemical properties of Tlr1612 and of other DGC proteins must at least be ascertained. A somewhat similar two-step c-di-GMP signaling system has been reported in Salmonella, wherein the “first” c-di-GMP pool activates the transcription of the gene encoding the DGC protein AdrA, which produces higher levels of the functional output, whereas binding of the c-di-GMP monomer could not, indicating that a specific form of c-di-GMP is necessary for protein activation31. The high cooperativity that has been demonstrated for most PilZ domains appears to reflect the preference for binding the c-di-GMP dimer. Conversely, TII0007 might be activated by the c-di-GMP monomer.

SesA is the major trigger of cell aggregation for T. vulcanus31, and we found that SesA is subject to the product feedback inhibition (Fig. 2). The high cooperativity of product inhibition permits SesA to respond in an all-or-none fashion over a narrow range of c-di-GMP concentration44. The DGC activity of SesA (kcat ~10 min⁻¹) is relatively high compared with known DGCs such as TM1788(R158A) (kcat ~2.6 min⁻¹)37 and YdeH/DgcZ (kcat ~1.6 min⁻¹)40, and comparable with Cph2 (kcat ~10.6 min⁻¹)18. Such high DGC activity is likely important for SesA to rapidly produce sufficient c-di-GMP for the induction of cell aggregation upon blue-light exposure. Notably, Synechocystis cells over-expressing SesA (R676A) were inviable under white-light irradiation, whereas these cells grew normally when irradiated with both green and red light. This finding suggests that the high and unlimited DGC activity of SesA (R676A), when activated by blue light, is toxic to the Synechocystis cells, probably owing to exhaustion of GTP substrate and/or overactivation of c-di-GMP signaling. Thus, it is logical that product feedback inhibition is necessary to control the high DGC activity of SesA. Product feedback inhibition is assumed to be common for GGDEF domain proteins and has been intensively investigated in vitro33–35,46. However, the physiological importance of DGC product inhibition remains an unresolved issue.

Our study identified Tlr1612 as the crucial protein to repress cell aggregation under teal-green light (Fig. 3B). The expression of tlr1612 did not differ between the blue-light and teal-green-light conditions (Table 1), excluding the possibility that transcriptional regulation underlies the activation of tlr1612. However, tlr1612 was found to be epistatic to sesA (Fig. 4), indicating that Tlr1612 acts downstream of SesA in the c-di-GMP signaling network. Furthermore, our biochemical studies demonstrated that SesA-produced c-di-GMP levels barely exceeded ~5 μM owing to product inhibition (Fig. 2), with this level being far lower than the Kd value for binding to TII0007 (Fig. 1). However, no other single DGC was responsible for cell aggregation under blue light, whereas tlr1612 was essential for suppression of cell aggregation under teal-green light (Fig. 3, and see31 for ΔsesB and ΔsesC). Although the predicted Tlr1612 protein harbors both GGDEF and EAL domains, it probably serves as the major PDE that suppresses cell aggregation under teal-green light and likely under blue light as well, according to our gene expression analysis. Nevertheless, SesA was able to trigger cell aggregation under blue light, indicative of a certain mechanism(s) that leads to an upshift of c-di-GMP level sufficient for the low-affinity site of TII0007. At present, it is not known whether such an upshift could be achieved by SesA-dependent downregulation of Tlr1612 PDE, SesA-dependent upregulation of Tlr1612 DGC, or SesA-dependent upregulation of two or more yet unidentified DGCs including SesC (as upregulation of any single DGC except SesA was excluded by our gene expression analysis. Nevertheless, SesA was able to trigger cell aggregation under blue light, indicative of a certain mechanism(s) that leads to an upshift of c-di-GMP level sufficient for the low-affinity site of TII0007. At present, it is not known whether such an upshift could be achieved by SesA-dependent downregulation of Tlr1612 PDE, SesA-dependent upregulation of Tlr1612 DGC, or SesA-dependent upregulation of two or more yet unidentified DGCs including SesC (as upregulation of any single DGC except SesA was excluded by our gene disruption experiments). These scenarios are summarized in Fig. 5.

Notably, disruption of all the three photoreceptors (sesA/B/C) still allowed some cell aggregation (the aggregation index was 40–70% for all the tested wavelengths31). This observation implies that certain levels of c-di-GMP are maintained during normal phototrophic growth owing to production by one or more DGCs(s), even though the possibly strong PDE activity of Tlr1612 is also functioning. There are many indications that multiple DGCs and PDEs are often spatially differentiated in prokaryotic cells, in addition to the well-known temporal regulations47. The possible PDE activity of Tlr1612 may also serve as an insulator for other, potentially unrelated, effects of c-di-GMP signaling, which are not shown in Fig. 5. To reconcile the ambiguity of our model, the biochemical properties of Tlr1612 and of other DGC proteins must at least be ascertained. A somewhat similar two-step c-di-GMP signaling system has been reported in Salmonella, wherein the “first” c-di-GMP pool activates the transcription of the gene encoding the DGC protein AdrA, which produces higher levels of the
“second” c-di-GMP pool that directly activates the cellulose synthase activity\textsuperscript{48,49}. Such transcriptional regulation is unlikely in \textit{Thermosynechococcus} as no gene exhibited a difference in transcript levels between the cell aggregation-ON and -OFF conditions (Table 1). Thus, post-transcriptional control will surely be of importance in the c-di-GMP signaling network of \textit{Thermosynechococcus}, as also proposed for \textit{E. coli}\textsuperscript{50}.

Besides SesA/B/C and Tlr1612, six other genes encode GGDEF/EAL/HD-GYP domain proteins in \textit{Thermosynechococcus}, although their functions remain completely unknown. Disruption of each of these six genes did not affect cell aggregation at 31 °C (Fig. 3), suggesting that these genes may have redundant roles in cell aggregation or be involved in other cellular responses—potentially regulated by c-di-GMP. All of the c-di-GMP signaling genes, especially \textit{tlr1210}, were expressed to a greater degree at 31 °C compared with 45 °C (Table 1), suggesting that the overall c-di-GMP signaling governs various cellular responses especially at relatively low temperatures. A candidate for a c-di-GMP–regulated cellular response is phototaxis; indeed, c-di-GMP regulates phototaxis in the cyanobacterium \textit{Synechocystis}\textsuperscript{18,19}. The PilB ATPase, which drives the extension of type IV pili\textsuperscript{51,52}, is a likely universal c-di-GMP–binding protein\textsuperscript{10}. Future work will address how each GGDEF/EAL/HD-GYP protein employs c-di-GMP to orchestrate cellular responses in cyanobacteria. The protein thermostability and the relatively small number of c-di-GMP genes of \textit{Thermosynechococcus} suggest the potential for further detailed characterization of every protein in the sophisticated c-di-GMP signaling network.

**Methods**

**Construction of plasmids and mutants.** Primers used are listed in Table S1. Plasmids were constructed using the In-Fusion System (TaKaRa). For constructing FRET biosensors, fluorescent proteins were cloned from pCyPet-His and pYPet-His (Addgene). We created monomerized variants of CyPet (mCyPet) and YPet (mYPet) by introducing A206K\textsuperscript{29,53}. The DNA encoding the chimeric protein consisting of mCyPet, TII0007-PilZ (or the full-length YcgR of \textit{E. coli}), and mYPet was cloned into pET28V, in which the protease recognition site in the

![Figure 5. Refined model for light wavelength-dependent c-di-GMP signaling for Thermosynechococcus vulcanus cell aggregation. Blue light regulates the activities of SesA/B/C concertedly to increase c-di-GMP. A relatively low c-di-GMP concentration is maintained via strict product feedback inhibition of the DGC activity of the major trigger, SesA. The c-di-GMP signal may lead to an increased cellular concentration of c-di-GMP possibly via post-transcriptional regulation of Tlr1612 and/or other DGC/PDE proteins. At higher concentrations, c-di-GMP binds to and activates the cellulose synthase TII0007, leading to cell aggregation.](image-url)
original pET28a plasmid (Novagen), for removal of the N-terminal His tag, was replaced with one for tobacco etch virus (TEV) protease. Site-directed mutagenesis was performed using PrimeSTAR Max Basal Mutagenesis kit reagents (TaKaRa). The vector pTCH2031V was used to overexpress SesA in *Synechocystis* sp. PCC 6803. The protease recognition site for removal of the N-terminal His tag was replaced with one for TEV protease, which could be used for future purification. The *E. coli* *rrnB* terminator sequence was inserted after the C-terminus of the protein-coding region incorporated into pTCH2031V.

For disruption of *tlr1210*, *tlr1158*, *tlr11049*, *tlr1859*, *tlr0627*, and *tlr1612* in *T. vulcanus*, a spectinomycin/spectromycin-resistance cassette was inserted at the start codon of each gene. For disruption of *tlr0485*, the majority of the open reading frame was replaced by a chloramphenicol resistance-cassette.

**Protein purification.** *E. coli* and *Synechocystis* protein-expressing cells were harvested by centrifugation at 4450 × *g* for 10 min, suspended in 50 mM Hepes-NaOH (pH 7.5) containing 300 mM NaCl, 10% (wt/vol) glycerol, 30 mM imidazole, and 0.5 mM Tris·(2-carboxyethyl)phosphine, and then frozen at −80 °C. After thawing, cells were subjected to three rounds of disruption using a French press (5501-M; Ohtake) at 1,500 kg·cm⁻². Each cyanobacterial cell homogenate was centrifuged at 12,000 × *g* for 10 min and then at 194,100 × *g* for 30 min. Each *E. coli* homogenate was centrifuged at 194,100 × *g* for 30 min. Each supernatant was filtered through a 0.8-μm cellulose acetate filter and then loaded onto a nickel-affinity His-Trap chelating column (GE Healthcare). Proteins were eluted with a linear gradient of 30–430 mM imidazole in 20 mM Hepes·NaOH (pH 7.5), 300 mM NaCl, 10% (wt/vol) glycerol, and 0.5 mM Tris·(2-carboxyethyl)phosphine. Ethylenediaminetetraacetic acid (1 mM) was added to the pooled peak fractions, which were then dialyzed against 20 mM Hepes·NaOH (pH 7.5), 300 mM NaCl, 10% (wt/vol) glycerol, and 1 mM diethyloctylamine (DTT). For purification of FRET-based biosensor proteins, the reducing agents (Tris·(2-carboxyethyl)phosphine and DTT) were omitted.

**Fluorescence spectroscopy.** Fluorescence spectra were recorded at 31 °C using a RF-5300PC spectrophotometer (Shimadzu, excitation at 425 nm, 5 nm excitation and 3 nm emission slit widths, emission scan from 450 to 600 nm) using 50 nM of an individual protein in 20 mM Hepes·NaOH (pH 7.5), 100 mM NaCl, and 10% (wt/vol) glycerol. Binding affinity was determined by adding increasing concentrations of c-di-GMP (10 nM to 1 mM) or GTP (1 mM) to 0.2 μM SesA and monitoring at 360 nm with a UV-2600PC spectrophotometer using EnzChek pyrophosphate assay kit reagents (Invitrogen). The kit includes inorganic pyrophosphatase, which catalyzes conversion of inorganic pyrophosphate into 2 equivalents of inorganic phosphate. In the presence of inorganic phosphate, the substrate 2-amino-6-mercapto-7-methylpurine ribonucleoside was enzymatically converted to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine, which absorbs 360-nm light. Each reaction contained 50 mM Tris·HCl, pH 7.5, 20 mM MgCl₂, and 0.2 μM SesA in the presence of a varying amount of c-di-GMP. The reaction was initiated by the addition of 100 μM GTP, incubated at 31 °C, and monitored for 600 s.

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**Cyanobacterial strains and culture conditions.** The *T. vulcanus* strain RKN (equivalent to National Institute for Environmental Studies 2134) that shows positive phototaxis was cultured at 45 °C in BG11 medium. Culture density was monitored at 730 nm. Transformations of *T. vulcanus* were performed according to. The antibiotic concentration in BG11 medium for selection of transformants was 5 μg·mL⁻¹ chloramphenicol, 80 μg·mL⁻¹ kanamycin, or 10 μg·mL⁻¹ spectinomycin plus 5 μg·mL⁻¹ streptomycin. PCR was used to confirm the complete segregation of the mutant allele (i.e., the WT loci were replaced with the mutant loci in all of the multiple copies of the cyanobacterial chromosome).

**Real-time qPCR assay.** Total RNA was extracted from *T. vulcanus* cells using the Qiagen RNeasy RNA purification kit with RNA Protect Bacteria Reagent (Qiagen). Cells were grown (1) under irradiation of a white-light fluorescent lamp (35 μmol photon·m⁻²·s⁻¹) at 45 °C (standard condition), (2) under blue light (λ_max = 448 nm; 5 μmol photon·m⁻²·s⁻¹; Valore Corp.) with photosynthetic red light (λ_max = 634 nm; 30 μmol photon·m⁻²·s⁻¹; Valore Corp.) at 31 °C (cell aggregation-ON condition), or (3) under red light (λ_max = 507 nm; 5 μmol photon·m⁻²·s⁻¹; Valore Corp.) with the red light at 31 °C (cell aggregation-OFF condition) for 48 h. RNA integrity was checked by agarose gel electrophoresis. cDNA was synthesized via reverse transcription of RNA (0.8 μg) from each sample using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa). Real-time qPCR was performed on a Takara Thermal Cycler Dice. Each 20-μL reaction contained 1 μL of cDNA, 300 nM of each primer (Table S2), and 10 μL THUNDERBIRD SYBR qPCR Mix (Toyobo). Samples were denatured initially by heating at 95 °C for 1 min, followed by a 40-cycle amplification and quantification program (95 °C for 15 s and 60 °C for 30 s). Melting-curve analysis was conducted to ensure amplification of a single product. The amplification efficiency of each primer pair was determined by running three-fold serial dilutions (seven dilution series). A standard curve was generated by plotting the cycle threshold (Ct) value determined using the second derivative maximum method against the log of the dilution factor. Gene expression was normalized to that of *rnpB*.

**Cell aggregation assay.** Cultures of *T. vulcanus* WT and its disrupted mutants grown at 45 °C (OD₇₅₀ 0.5–2) were diluted to yield a culture OD₇₅₀ of 0.2. These samples were then incubated at 31 °C for 48 h under photosynthetic red light along with blue or green light. The results of the cellulose-dependent cell-aggregation assay, described as follows, were reported as an aggregation index (%). Briefly, after the irradiation period, cell suspensions were thoroughly mixed and aliquots transferred to cuvettes. The samples were held at room temperature in the dark for 30 min, during which time most of the aggregated cells precipitated.
Then, the OD$_{730}$ of each sample was measured (denoted OD$_{NA}$, i.e., OD$_{730}$ of non-aggregated cells remaining in the culture medium). Next, cellulase (12.5 U mL$^{-1}$; Worthington Biochemical) was added to each cuvette sample, which was then incubated for 30 min at 37°C to completely disperse the aggregated cells. The OD$_{730}$ of each sample (denoted OD$_{total}$) was then measured. The aggregation index (%) was defined as ((OD$_{total}$ – OD$_{NA}$)/OD$_{total}$) × 100.

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Acknowledgements
We thank Drs Yu Kanesaki and Hirofumi Yoshikawa (Genome Research Center, NODAI Research Institute, Tokyo University of Agriculture) for providing the nucleic acid sequences of the *T. vulcanus* genome. This work was supported by a grant-in-aid for Young Scientists (B) (JSPS KAKENHI grant No. 17K15244) from the Japan Society for the Promotion of Science (GE) and by Core Research for Evolutional Science and Technology, Japan Science and Technology Agency (YO and MI).

Author Contributions
G.E. and M.I. conceived and designed the study. G.E., Y.O. and M.I. acquired, analyzed, and interpreted the data. G.E. and M.I. wrote the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-23628-4.

Competing Interests: The authors declare no competing interests.

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