Structural analyses unravel the molecular mechanism of cyclic di-GMP regulation of bacterial chemotaxis via a PilZ adaptor protein

Received for publication, September 1, 2017, and in revised form, November 10, 2017. Published, Papers in Press, November 16, 2017, DOI 10.1074/jbc.M117.815704

© Xin-Fu Yan‡§, Lingyi Xin‡, Jackie Tan Yen‡§, Yukai Zeng‡, Shengyang Jin‡§, Qing Wei Cheang‡, Rachel Andrea Chea Yuen Fong§, Keng-Hwee Chiam§, Zhao-Xun Liao‡, and Yong-Gui Gao‡§§

From the ‡School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Singapore, §NTU Institute of Structural Biology, Nanyang Technological University, 59 Nanyang Drive, Singapore 639798, Singapore, ¶Bioinformatics Institute (A*STAR), 30 Biopolis Street, Number 07-01, S138671 Singapore, Singapore, and ‡§Institute of Molecular and Cell Biology, A*STAR (Agency for Science, Technology and Research), 61 Biopolis Drive, Singapore 138673, Singapore

Edited by John M. Denu

The bacterial second messenger cyclic di-GMP (c-di-GMP) has emerged as a prominent mediator of bacterial physiology, motility, and pathogenicity. c-di-GMP often regulates the function of its protein targets through a unique mechanism that involves a discrete PilZ adaptor protein. However, the molecular mechanism for PilZ protein–mediated protein regulation is unclear. Here, we present the structure of the PilZ adaptor protein MapZ cocrystallized in complex with c-di-GMP and its protein target CheR1, a chemotaxis-regulating methyltransferase in Pseudomonas aeruginosa. This cocrystal structure, together with the structure of free CheR1, revealed that the binding of c-di-GMP induces dramatic structural changes in MapZ that are crucial for CheR1 binding. Importantly, we found that restructuring and repositioning of two C-terminal helices enable MapZ to disrupt the CheR1 active site by dislodging a structural domain. The crystallographic observations are reinforced by protein–protein binding and single cell–based flagellar motor switching analyses. Our studies further suggest that the regulation of chemotaxis by c-di-GMP through MapZ orthologs/homologs is widespread in proteobacteria and that the use of allosterically regulated C-terminal motifs could be a common mechanism for PilZ adaptor proteins. Together, the findings provide detailed structural insights into how c-di-GMP controls the activity of an enzyme target indirectly through a PilZ adaptor protein.

c-di-GMP.§ is a widespread second messenger that controls a multitude of cellular functions and bacterial behaviors (1–3). In human pathogenic bacteria such as Pseudomonas aeruginosa, Salmonella enterica, and Vibrio cholerae, c-di-GMP plays a prominent role regulating the motile–sessile transition and formation of antibiotic-resistant biofilm communities. c-di-GMP exerts its control on cellular functions by binding to a variety of protein and riboswitch targets (4, 5). Among the known c-di-GMP–binding proteins, discrete single-domain PilZ proteins are widespread and by far the most prevalent (6–12). As the single-domain PilZ proteins only contain 80–130 amino acids and do not exhibit enzymatic or other activities, their physiological roles often remain elusive. Emerging evidence suggests that the single-domain PilZ proteins function as adaptor proteins to enable c-di-GMP to control its protein targets indirectly (8, 9, 13, 14). However, the detailed molecular mechanism of how c-di-GMP regulates the activity of its protein targets through the PilZ adaptor proteins remains unknown. How specificity in c-di-GMP signaling, which is crucial for preventing cross-talk among different pathways, is achieved through the PilZ adaptor proteins also remains a key question.

We recently discovered that c-di-GMP controls the enzymatic activity of the methyltransferase CheR1 in P. aeruginosa through the PilZ adaptor protein MapZ (14). High cellular c-di-GMP concentrations lead to the formation of the MapZ–c-di-GMP–CheR1 ternary complex and subsequently inhibit the methyltransferase activity of CheR1. Inhibition of CheR1 results in a decrease in the methylation level of methyl-accepting chemoreceptor proteins (MCPs), which ultimately leads to the suppression of flagellar motor switching and a decrease of swimming reversal frequency. Because cellular c-di-GMP concentration fluctuates with metabolic and extracellular stimuli, P. aeruginosa can fine-tune its chemotactic response and motility in different environments using the MapZ-dependent regulatory mechanism. Although it is known that the protein–protein interaction between CheR1 and MapZ is highly specific and dependent on c-di-GMP, the structural basis for the c-di-GMP–mediated specific protein–protein interaction is unknown. Here, we report the crystal structures of the free CheR1 and the MapZ–c-di-GMP–CheR1 ternary complex along with other experimental data to support the physiological relevance of the structural observations. The results reveal an
exquisite allosteric mechanism that is potentially conserved in many PilZ adaptor proteins.

Results and discussion

Overall structure of the MapZ–c-di-GMP–CheR1 ternary complex

We determined the crystal structure of CheR1 in complex with MapZ and c-di-GMP at 2.3-Å resolution (Fig. 1A and Fig. S1) with the crystallographic statistics listed in Table 1. The difference Fourier map (F_o – F_c) clearly shows the ligand c-di-GMP (Fig. S2). The c-di-GMP–bound MapZ is composed of a β-barrel core consisting of eight antiparallel β-strands, an N-terminal loop, and two C-terminal α-helices as seen in other PilZ proteins (15–19) (Fig. 1B). Observed as an intercalated dimer in the electron density map, c-di-GMP is held in position by residues stemming from the N-terminal loop and strands β4, β5, β7, and β8. The methyltransferase CheR1 is composed of two domains (Fig. 1C). The N-terminal domain of CheR1 is a relatively small domain consisting of four perpendicularly packed α-helices, whereas the C-terminal domain contains a large α/β fold core and a small β-subdomain (Fig. 1C). The core of the C-terminal domain adopts a Rossmann fold consisting of a mixed seven-stranded β-sheet flanked by α-helices. The architecture of the C-terminal domain core is similar to those of other class I methyltransferases (20). In addition to the α/β fold core, the C-terminal domain also contains a β-subdomain consisting of two short helices and three antiparallel strands (Fig. 1C). The β-subdomain, which is inserted between α7 and α10, is unique to chemotaxis methyltransferases (21–23) (Fig. S3). The N- and C-terminal domains are connected by a flexible domain linker (aa 70–80) with a cleft formed between the two domains. In the ternary complex structure, the β-barrel core of MapZ sits on the top of CheR1 with the two C-terminal helices α1 and α2 wedged into the central cleft of CheR1, resulting in extensive contacts between the two proteins that are likely to account for the formation of a stable MapZ–c-di-GMP–CheR1 ternary complex.

Specific interactions between MapZ and CheR1

In the ternary complex, MapZ makes direct contacts with CheR1 through its C-terminal motifs, the β6–β7 loop, and a small segment of the N-terminal loop. The C-terminal motifs of MapZ encompass the two helices α1 (aa 92–106) and α2 (aa 109–121) and the adjacent loops. Helix α1 seems to play a cen-
**PilZ adaptor-mediated c-di-GMP signaling**

Table 1

| Summary of crystallographic data and refinement statistics | ApoCheR1 | MapZ–c-di-GMP–CheR1 |
|-----------------------------------------------------------|----------|---------------------|
| **Data collection** | | |
| Space group | 14 | P2_1 2_1 2_1 |
| Protein Data Bank code | 5745 | 574R |
| Wavelength (Å) | 0.9779 | 0.9537 |
| Cell dimensions | | |
| a, b, c (Å) | 279.13, 279.13, 138.58 | 88.44, 98.82, 110.66 |
| a, β, γ (°) | 90, 90, 90 | 90, 90, 90 |
| Molecules/ASU | 10 | 2 |
| **Resolution (Å)** | | |
| Resolution (Å)* | 50–3.41 (3.61–3.41) | 50–2.30 (2.44–2.30) |
| CC1/2 | 99.8 (53.1) | 99.8 (56.8) |
| Completeness (%)* | 99.9 (99.8) | 98.9 (99.0) |
| Redundancy* | 14.5 (14.7) | 6.75 (6.91) |
| **Refinement** | | |
| Resolution (Å) | 49.42–3.41 | 48.29–2.30 |
| No. reflections | 143,720 | 83,410 |
| Rwork (Rfree) (%) | 25.7 (29.5) | 19.4 (23.9) |
| No. atoms | | |
| Protein | 20,244 | 6,230 |
| Ligand/ion | | |
| c-di-GMP, 184 | | |
| Water | | |
| B-Factors (Å²) | 246.3 | 59.4 |
| Proein | 20,244 | 6,230 |
| Ligand/ion | | |
| c-di-GMP, 184 | | |
| Water | | |
| r.m.s. deviations | | |
| Bond lengths (Å) | 0.004 | 0.011 |
| Bond angles (°) | 1.19 | 1.44 |
| Ramachandran plot | | |
| Favorable (outlier) (%) | 90.0 (0.56) | 95.0 (0.26) |

*Values for the highest-resolution shell are in parentheses.

---

**Binding affinity measurements reinforce the key role of the MapZ C-terminal residues in binding CheR1**

The extensive contacts between the two proteins are consistent with the high thermodynamic binding affinity ($K_d = 28.6 \pm 17.7 \text{ nM}$) between the two proteins. c-di-GMP is essential for MapZ-CheR1 interaction because no binding could be detected in the absence of c-di-GMP. To evaluate the contribution of the aforementioned residues of α1 and α2 to CheR1 binding, we measured the binding affinity of several MapZ mutants for CheR1. The results (Fig. 3, A and B) show that Arg99 is the most crucial residue as the substitution of Arg99 by Ala reduced the binding affinity to below the detection limit, consistent with the structural observation that the residue Arg99 engages in multiple interactions with CheR1 residues (Fig. 2A). The substitution of other α1 residues, His97, Arg100, and Leu104, by Ala reduced the $K_d$ by 7.1-, 6-, and 2.4-fold, respectively. Replacement of Asp93, which positions His97 for interacting with CheR1 residues, caused a 10.4-fold decrease in $K_d$. In comparison, removal of the entire α2 helix (MapZ(Δα2)) decreased the $K_d$ by 14.6-fold. These observations support the relevance of the crystal structure and reinforce the central role of α1 in mediating the MapZ-CheR1 interaction.

**The C-terminal binding residues are important for the function of MapZ in suppressing flagellar motor switching**

To corroborate the structural and binding data and further validate the importance of the two C-terminal helices for the *in vivo* function of MapZ, we investigated how mutations in MapZ impact flagellar motor switching in *P. aeruginosa* cells by cell tethering assay (14, 24). We previously observed that the overexpression of MapZ in *P. aeruginosa* cells dramatically suppressed flagellar motor switching, which is similar to the effect caused by the deletion of cheR1 (14). Most of the MapZ-overexpressing cells cannot switch motor direction and exhibit unidirectional flagellar rotation (14). The suppression is attributed to the ability of MapZ to inhibit CheR1 and decrease MCP methylation at high c-di-GMP levels. Hence, it was expected that overexpression of defective MapZ mutants that lack key residues for c-di-GMP or CheR1 binding would exhibit reduced suppression on flagellar motor switching. Indeed, the cell tethering assays showed that overexpression of MapZ mutants that lack binding residues for CheR1 suppressed motor switching to a lesser extent than MapZ. MapZ(R99A) did not seem to suppress motor switching at all, whereas MapZ(D93A), MapZ(R99A), MapZ(L104A), and MapZ(R13A) exhibited less suppression than MapZ to various extents (Fig. 4A and Movies S1–S8). Overexpression of MapZ(R13A), which is defective in c-di-GMP binding (14), also exhibited a much weaker suppression effect. As supported by Western blotting assays, the different suppression effects observed for the mutant proteins are not caused by a reduction in protein expression level (Fig. 4B and Fig. S4). Taken together, the results provide strong support for the physiological relevance of the MapZ–c-di-GMP–CheR1 ternary structure and establish the pivotal role of the C-terminal motifs in mediating MapZ-CheR1 interaction and suppressing flagellar motor switching.

---

**Fig. S4**

The residues in the central cleft of CheR1 with several highly specific polar interactions formed between α1 and CheR1. In particular, Arg99 interacts with Asp66 and Val67 from the residues also involved in direct binding of CheR1. Asp66 and Val67 from the β6–β7 loop interact with Arg78, Ser250, and Ile221 of CheR1 through hydrogen bond and hydrophobic interactions, whereas Phe16 and Asp17 from the N-terminal loop interact with CheR1 residues through hydrophobic and hydrogen bond interactions, respectively (Fig. 2C).
PiZ adaptor-mediated c-di-GMP signaling

Structural rearrangement in MapZ induced by the binding of c-di-GMP and CheR1

The structures of MapZ with and without c-di-GMP bound were determined previously by NMR spectroscopy (25, 26). It was noted that MapZ undergoes large structural rearrangements upon the binding of c-di-GMP. Comparing the ternary complex structure with the NMR structures (Protein Data Bank code 1YWU (26)) reveals how the structural rearrangement induced by c-di-GMP binding prepares MapZ for CheR1 binding. Upon the binding of c-di-GMP, the N-terminal loop becomes organized by wrapping itself around the intercalated c-di-GMP dimer (Fig. 5A). Without the c-di-GMP–induced reorganization, the N-terminal loop would hinder the binding of CheR1 sterically (Fig. 5A). In the C-terminal region, one of the most notable changes is that the binding of c-di-GMP results in the straightening of the initially bent α2 and concomitant dissociation of α2 from the β-barrel core (Fig. 5A). The restructuring and displacement of α2 is crucial for setting up MapZ for the interaction with CheR1.

Unexpectedly, the c-di-GMP binding mode in the ternary complex differs to some extent from that in the c-di-GMP–MapZ binary complex (Protein Data Bank code 2L74) (25). The N-terminal loop adopts a rather dissimilar conformation as characterized by the different positions and orientations of several loop residues (e.g. Glu7, Phe11, and His12) in the ternary complex (Fig. 5B). Also, noticeably, the helix α1 from the C terminus is tilted toward the β-barrel core by ~2 Å, and α2 is pushed toward α1 by as much as 12 Å in the ternary complex. Although residues Arg8, Arg9, Arg10, Arg13, Asp35, and Trp77 are seen to interact with c-di-GMP directly in both the binary and ternary complexes, three additional residues (Glu7, Phe11, and Arg79) are directly involved in the binding of c-di-GMP only in the ternary complex (Fig. 5, C and D). It should be noted that although Glu7 and Phe11 are conserved in most single-domain PiZ proteins, their functions remain unknown. The above observations suggest that the two residues are conserved because they play a direct role in c-di-GMP binding. On the contrary, His12, which is not a conserved residue and seen to form a hydrogen bond with c-di-GMP in the binary complex, does not participate in c-di-GMP binding in the ternary complex. The discrepancies in c-di-GMP binding between the binary and ternary complexes highlight the malleability of the c-di-GMP–binding pocket and call for caution when interpreting the structures of PiZ proteins determined in the absence of their protein partners.

Binding of MapZ to CheR1 resulting in disruption of the S-adenosylmethionine (AdoMet)-binding pocket and inhibition of methyltransferase activity

We also determined the crystal structure of free CheR1 protein to determine how the binding of MapZ impacts the structure of CheR1. Crystals of free CheR1 belong to the tetragonal space group I41, with 10 copies of the molecule ( chains A–J) in an asymmetric unit. Superposition of crystallographic indepen-
**PilZ adaptor-mediated c-di-GMP signaling**

Figs. 2 and see 5. MapZ (or a mutant) and saturating c-di-GMP (250 μM) were loaded into bacterial strains and incubated at 30°C for 1 h. Cell extracts were prepared and equal amounts of protein were separated by Western blotting using HA-specific antibody. Blots are representative of two independent experiments. 

**MapZ-mediated regulation of chemotaxis by c-di-GMP is conserved in many proteobacteria**

Based on the studies on other chemotaxis methyltransferases (22, 23), the C-terminal domain and the domain linker are likely involved in the binding of the cofactor AdoMet. In the free CheR1 structure, an AdoMet-binding pocket can be readily identified by overlaying the structure with that of the S-adenosyl-L-homocysteine (SAH)-bound BsCheR (Protein Data Bank code 5FTW; Fig. 5F). Two highly conserved catalytic residues (Asp144 and Tyr222) that are essential for AdoMet binding can also be identified. In the ternary complex, the AdoMet-binding pocket is completely occupied by MapZ upon dislodging of the N-terminal domain and conformational changes in the domain linker. The AdoMet-binding pocket is filled by residues from the α1 of MapZ with Asp144 and Tyr222 engaging in multiple interactions with residues from α1 (Figs. 2A and 5F). These observations strongly suggest that the C-terminal motifs of MapZ, particularly α1, have evolved to interact specifically with the conserved residues in the CheR1 active site to inhibit its enzymatic activity. MapZ only interacts with CheR1 but not the other three chemotaxis methyltransferases of *P. aeruginosa* (27, 28). Sequence alignment of the four methyltransferases suggests that this is likely because some of the MapZ-binding residues in CheR1 are absent in the other three CheR proteins (Fig. 5).

**Figure 3. Assessment of the contribution of the residues from helices α1 and α2 to protein-protein interaction by binding affinity measurement.** A, binding isotherm (top panels) and data-fitting curves (bottom panels) obtained from ITC assay. Solution containing CheR1 was titrated into solution containing MapZ (or a mutant) and saturating c-di-GMP (250 μM). The experimental conditions are described under “Experimental procedures.” The binding measurements were performed in two independent experiments, and the representative data set is presented here. DP, differential power. B, apparent dissociation constant (Kd) values obtained by fitting the isotherm data to a 1:1 binding model.

**Figure 4. Residues from helices α1 and α2 are important for the function of MapZ in suppressing flagellar motor switching.** A, bar graphs showing the percentage of time that *P. aeruginosa* PA01 and overexpression mutant strains underwent clockwise (CW) rotation. The sample sizes are 32, 30, 28, 27, 25, 27, and 31 cells for PA01, mapZ*, mapZ(R13A)*, mapZ(D93A)*, mapZ(H97A)*, mapZ(R99A)*, mapZ(R100A)*, and mapZ(L104A)*, respectively. The corresponding videos for representative rotating cells can be found in Movies S1–S8. Data are representative of two independent experiments. B, protein expression levels of MapZ and MapZ mutants in PA01 (L104A) and protein expression levels of MapZ and MapZ mutants in PA01 (L104A) were visualized using RNAP-β-specific antibody. Blots are representative of two independent experiments.
bacterial genomes. We first identified and collected the sequences of a total of 11,781 single-domain PilZ proteins from the InterPro protein database with most sequences belonging to the IPR009875 family (29). From this collection, we identified 1,063 potential functional orthologs/homologs of MapZ according to the following two criteria. First, the proteins...
must share high sequence similarity (>60% similarity) with MapZ. Second, the proteins must contain two highly similar C-terminal helices with the CheR1-binding residues conserved. As shown by the sequence logo (Fig. 6A), the critical CheR1-interacting residues are highly conserved in the putative orthologs/homologs.

A taxonomic and phylogenetic relationship analysis for MapZ and the 1,063 proteins suggests that most MapZ orthologs/homologs come from *Pseudomonas*, *Vibrio*, and other proteobacteria, in particular γ-proteobacteria (Fig. 6B). A small percentage of the orthologs/homologs were encoded by genes from unclassified bacterial species uncovered by metagenomics projects. PlzA, one of the PilZ proteins from the pathogenic bacterium *V. cholerae* (9) with unknown cellular function was also identified as an ortholog/homolog of MapZ. This hints that PlzA is likely to play a similar role in *V. cholerae* by binding to a chemotactic methyltransferase to regulate chemotaxis. Furthermore, for the MapZ homolog/ortholog-containing bacterial species whose genomes have been sequenced, we could identify genes encoding chemotaxis methyltransferases that are highly similar to CheR1. Altogether, the bioinformatics analysis suggests that the MapZ-mediated mechanism for fine-tuning chemotactic response and bacterial motility is not a unique mechanism restricted to *P. aeruginosa* but a widely distributed mechanism adapted by many proteobacteria.

In summary, the studies described here establish the structural basis for the regulation of the methyltransferase CheR1 by c-di-GMP through the PilZ protein MapZ. The data support a model whereby MapZ, at high cellular c-di-GMP concentrations, undergoes dramatic conformational changes and occupies the CheR1 active site to inhibit the enzyme activity (Fig. 7). Inhibition of CheR1 decreases the methylation level of MCPs and ultimately results in the suppression of flagellar motor switching. Further analysis of the sequences of about 12,000 single-domain PilZ proteins encoded by sequenced bacterial genomes revealed that most of them contain C-terminal motifs that vary in sequence length and secondary structure. Although the variation in sequence and structure may reflect the diverse protein partners with which the PilZ proteins interact, the mechanism of allosteric regulation of C-terminal motifs for controlling protein-protein interaction observed in MapZ could be a common theme for the PilZ adaptor proteins.

**Experimental procedures**

**Materials**

All bacterial strains, plasmids, and primers used in this study are listed in Tables S1 and S2.

**Protein expression and purification**

For the recombinant proteins used for crystallization, the CheR1- and MapZ-encoding genes PA3348 and PA4608 were amplified by polymerase chain reaction (PCR) using the genomic DNA of *P. aeruginosa* PAO1 as template. The PCR fragments were cloned into pACYC-Duet-1 expression vector (Novagen), which provides a hexahistidine tag at the N terminus of recombinant CheR1 and MapZ, respectively. In brief, the expression plasmid carrying cheR1 or mapZ gene was transformed into *Escherichia coli* BL21/DE3 strain using a heat-shock method. Protein expression was induced by 0.2 mM isopropyl β-D-thiogalactoside at 16 °C overnight. Cells were harvested by centrifugation, and the pellet was resuspended and homogenized five times using an EmulsiFlex-C3 (Avestin). The lysate was centrifuged at 40,000 rpm for 1 h to remove insoluble cell debris. The supernatant was applied to a nickel-nitrilotriacetic acid affinity column, sequentially washed, and eluted by a gradient of increasing imidazole concentration in buffer. The pooled fractions were treated with tobacco etch virus protease and dialyzed against a buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl. After removal of tobacco etch virus protease and uncleaved protein by the second affinity column, the protein samples were further purified by size-exclusion chromatography. The target protein was concentrated to ~15 mg/ml for subsequent crystallization and biochemical analysis. The mutant MapZ proteins were generated using a QuikChange kit (Stratagene) and prepared following the aforementioned procedure except the final buffer contained 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine hydrochloride (Sigma).

**Crystallization and data collection**

The initial crystal of the isolated CheR1 was achieved at 20 °C by mixing 1.0 μl of protein with 1.0 μl of reservoir solution containing 1.5 M sodium phosphate monobasic/potassium phosphate dibasic, pH 5.0. For further optimization, the crystallization condition was supplemented with 1% (v/v) glycerol and 3% (v/v) dextran sulfate sodium salt (M_r 5,000). The crystals were flash frozen (100 K) in the reservoir solution supplemented with 30% (v/v) glycerol. The native data set (total of 3,600 images with 0.2 oscillations) was collected at PXIII, Swiss Light Source.

For the MapZ–c-di-GMP–CheR1 complex, the molar ratio between the two proteins was determined to be 1:1 by analytical gel filtration. The MapZ–c-di-GMP–CheR1 complex was prepared by incubating 0.3 mM CheR1 with 0.3 mM MapZ and 1 mM c-di-GMP (Sigma-Aldrich). Crystals of this ternary complex were grown at 20 °C by mixing 2.0 μl of protein with 1.0 μl of reservoir solution containing 0.1 mM Bis-Tris, pH 5.5, 19% PEG 3350, and 0.2 M lithium sulfate. The crystals were flash frozen (100 K) in the reservoir solution with a final concentration of 40% (v/v) PEG 3350. The data set (total of 2,000 images with 0.1 oscillations) for MapZ–c-di-GMP–CheR1 complex was collected at the Swiss Light Source.

**Data processing, structure determination, and refinement**

All data were processed using the XDS package (30). The structure of MapZ–c-di-GMP–CheR1 ternary complex was determined by molecular replacement with Phenix combined with Phaser (31) using *Bs*CheR (Protein Data Bank code 5FTW) and MapZ (Protein Data Bank code 2L74) as search models. The structure of apoCheR1 was determined by molecular replacement (Phaser/Phenix) (31) using the CheR1 model from our ternary complex as a search model. The models were improved by manual model building with Coot (32) and refined using Phenix (31). Crystallographic statistics of the structures are listed in Table 1, and all structural figures were generated with PyMOL (Schrödinger, LLC).
Figure 6. MapZ homologs are widespread in proteobacteria. A, sequence log for MapZ and its 1,063 functional homologs. The two essential c-di-GMP–binding motifs (RXXXR and DsxxG) and the C-terminal α1 and α2 helices are highlighted by bars. The residues from α1 and α2 that are involved in direct binding of CheR1 are highlighted by red residue numbers. B, phylogenetic tree analysis of MapZ homologs. The clades for the two major Pseudomonadales and Vibrionales orders are colored. Most of the remaining homologs belong to other orders from the Proteobacteria phylum, but a small number of homologs belong to unclassified species whose sequences were obtained by metagenomics projects. A FASTA file that contains the 1,063 sequences can be found in the supporting information.
Preparation of MapZ overexpression mutant strains and Western blotting assay

The DNA fragments of MapZ mutants, including MapZ(D93A), MapZ(H97A), MapZ(R99A), MapZ(R100A), and MapZ(L104A) were synthesized by Integrated DNA Technologies, Inc. Synthesized DNA fragments were digested and ligated into the pUCP18 overexpression vector (Novagen) (33). The constructs were verified by sequencing and transformed into P. aeruginosa mPAO1 strain following the established procedure (34). Single colonies of overexpression strains were used to inoculate 10 ml of LB medium that was kept at 37 °C with shaking overnight. One milliliter of culture was centrifuged at 11,325 g for 10 min at 4 °C. Cells were resuspended with 100 μl of 2× loading dye and heated at 95 °C for 10 min. Samples were analyzed by SDS-PAGE and Western blotting using THETM HA tag antibody (HRP) (GenScript) and RNA polymerase β-subunit (RNAP-β) (loading control) antibody (a kind gift from Dr. Wang).

Binding affinity measurement by isothermal titration calorimetry (ITC)

ITC measurements were performed at 25 °C using a MicroCal ITC200 (MicroCal Inc.). Protein samples were dialyzed into a buffer containing 20 mM Tris- HCl, pH 8.0, 100 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine hydrochloride. A sample syringe with stirring speed of 800 rpm was used to titrate the CheR1 (250 – 320 μM) into a cell containing 25 – 30 μM MapZ or MapZ mutant in the presence of c-di-GMP (250 μM), which was prepared enzymatically as described previously (35, 36). The titration comprised 19 injections of 2 μl each separated by 150-s equilibration time. The isothermal data were analyzed using the Origin 7.0 program, fitting to a single-site binding model.

Cell tethering assay for the analysis of the flagellar rotation

Single colonies of P. aeruginosa PAO1 MapZ and mutants overexpression strains were used to inoculate 10 ml of LB broth (BD Biosciences) that was kept at 37 °C with shaking at 250 rpm overnight. Cultures were diluted to an A600 of 0.2 using M9 medium and grown at 37 °C with shaking at 250 rpm until the cells reached the late-exponential growth phase at A600 of 0.8. Cell cultures were then diluted 10-fold before the microfluidic experiments. Cell tethering assays were performed by using a microfluidic stagnation flow device precoated with flagellar antibodies (37). Flagella were sheared off by passing the bacterial cells through a 34-gauge blunt-end needle four times. Cells were loaded into the cell chamber of the flow device, and non-tethered cells were washed away using M9 medium. Cells were
visualized using an inverted microscope (Nikon TE2000-U) under a 40× objective. Videos of tethered bacteria were taken at 40 frames/s for 1–5 min using a complementary metal-oxide semiconductor camera (Thorlabs DCC1645). The rotation of the cells was monitored and recorded using a procedure reported previously (38). The time spent by each cell in clockwise, counterclockwise, or pause phases was tallied in 20-s intervals. Image processing of the tethered cells was carried out as described previously (39) with videos of the tethered cells converted to grayscale and the contrast of the cells adjusted (saturated pixels, 0.4; histogram stack) using NIH ImageJ. Images of the cells were binarized to isolate them from the background for every frame in the video. The center of mass coordinates of the rotating cell bodies of each cell were measured from the obtained binarized image stack and imported into our in-house analysis program BTAP (bacterial tethering analysis program) in MATLAB. Student’s two-tailed t test was used to test the statistical significance of the data. The normality assumption was considered to be sound because, by the central limit theorem, sample means of moderately large samples are well-approximated by a normal distribution.

**Genome mining and phylogenetic relationship analysis**

Single-domain PilZ proteins were identified by mining PilZ protein sequences deposited in the InterPro database (http://www.ebi.ac.uk/interpro/) (29). A total of 11,781 sequences of single-domain PilZ proteins were then submitted to the EFI/EST sequence similarity network server. The FASTA file containing the aligned sequences is used to verify whether they contain the crucial C-terminal helices and key residues for CheR1 binding.

For the generation of the sequence logo, the 1,024 protein sequences contained in a FASTA file were first aligned using ClustalW. The FASTA file containing the aligned sequences is included in the supporting information and can be visualized using the free software UGENE and MEGA. ClustalW was also used to generate the phylogenetic tree file (.ph file) to allow visualization of the tree using the web-based tool interactive Tree Of Life (iTOL) (41).

**Author contributions**—Z.-X. L. and Y.-G. G. conceived and oversaw the design and implementation of the research project. X.-F. Y., J. T. Y., and S. J. crystallized the proteins and solved the structures. L. X., and R. A. C. Y. F. performed gene cloning, protein purification, and binding assays and prepared mutant strains. Z.-X. L. and Q. W. C. identified and analyzed the MapZ homologs by database mining. Y. Z. and K.-H. C. performed the cell tethering assay and data analysis. Y.-G. G. and Z.-X. L. wrote the manuscript with input from the other authors.

---

References

1. Römling, U., Galperin, M. Y., and Gomelsky, M. (2013) Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol. Mol. Biol. Rev.* 77, 1–52 CrossRef Medline

2. Jenal, U., Reinders, A., and Lori, C. (2017) Cyclic di-GMP: second messenger extraordinary. *Nat. Rev. Microbiol.* 15, 271–284 CrossRef Medline

3. Liang, Z. X. (2015) The expanding roles of c-di-GMP in the biosynthesis of exopolysaccharides and secondary metabolites. *Nat. Prod. Rep.* 32, 663–683 CrossRef Medline

4. Ryan, R. P., Tolker-Nielsen, T., and Dow, J. M. (2012) When the PilZ don’t work: effectors for cyclic di-GMP action in bacteria. *Trends Microbiol.* 20, 235–242 CrossRef Medline

5. Nelson, J. W., Sudarsan, N., Furukawa, K., Weinberg, Z., Wang, J. X., and Breaker, R. R. (2013) Riboswitches in eubacteria sense the second messenger c-di-AMP. *Nat. Chem. Biol.* 9, 834–839 CrossRef Medline

6. Amikam, D., and Galperin, M. Y. (2006) PilZ domain is part of the bacterial c-di-GMP binding protein. *Bioinformatics* 22, 3–6 CrossRef Medline

7. Ryjenkov, D. A., Simm, R., Römling, U., and Gomelsky, M. (2006) The PilZ domain is a receptor for the second messenger c-di-GMP: the PilZ protein YcgR controls motility in enterobacteria. *J. Biol. Chem.* 281, 30310–30314 CrossRef Medline

8. Christen, M., Christen, B., Allan, M. G., Folcher, M., Jeno, P., Grzesiek, S., and Jenal, U. (2007) DgrA is a member of a new family of cyclic diguanosine monophosphate receptors and controls flagellar motor function in *Caulobacter crescentus*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 4112–4117 CrossRef Medline

9. Pratt, J. T., Tamayo, R., Tischier, A. D., and Camilli, A. (2007) PilZ domain proteins bind cyclic diguanylate and regulate diverse processes in *Vibrio cholerae*. *J. Biol. Chem.* 282, 12860–12870 CrossRef Medline

10. McCarthy, Y., Ryan, R. P., O’Donovan, K., He, Y. Q., Jiang, B. L., Feng, J. X., Tang, J. L., and Dow, J. M. (2008) The role of PilZ domain proteins in the virulence of *Xanthomonas campestris pv. campestris*. *Mol. Plant Pathol.* 9, 819–824 CrossRef Medline

11. Pitzer, J. E., Sultan, S. Z., Hayakawa, Y., Hobbs, G., Miller, M. R., and Motaleb, M. A. (2011) Analysis of the *Boreillia burgdorferi* cyclic-di-GMP-binding protein PilZA reveals a role in motility and virulence. *Infect. Immun.* 79, 1815–1825 CrossRef Medline

12. Pultz, J. S., Christen, M., Kulasekara, H. D., Kennard, A., Kulasekara, B., and Miller, S. I. (2012) The response threshold of *Salmonella* PilZ domain proteins is determined by their binding affinities for c-di-GMP. *Mol. Microbiol.* 86, 1424–1440 CrossRef Medline

13. Xu, L., Venkataramani, P., Ding, Y., Liu, Y., Deng, Y., Gong, G. L., Xin, L., Ye, R., Zhang, L., Yang, L., and Liang, Z.-X. (2016) A cyclic di-GMP-binding adaptor protein interacts with histidine kinase to regulate two-component signaling. *J. Biol. Chem.* 291, 16112–16123 CrossRef Medline

14. Xu, L., Xin, L., Zeng, Y., Yam, J. K. H., Ding, Y., Venkataramani, P., Cheong, Q. W., Yang, X., Tang, X., Zhang, L.-H., Chiam, K.-H., Yang, L., and Liang, Z.-X. (2016) A cyclic di-GMP-binding adaptor protein interacts with a chemotaxis methyltransferase to control flagellar motor switching. *Sci. Signal.* 9, ra102 CrossRef Medline

15. Whitney, J. C., Whitfield, G. B., Marmor, L. S., Yip, P., Neculai, A. M., Lobosanov, Y. D., Robinson, H., Ohman, D. E., and Howell, P. L. (2015) Diminutive c-di-GMP is required for post-translational regulation of alginate production in *Pseudomonas aeruginosa*. *J. Biol. Chem.* 290, 12451–12462 CrossRef Medline

16. Ko, J., Ryu, K.-S., Kim, H., Shin, J.-S., Lee, J.-O., Cheong, C., and Choi, B.-S. (2010) Structure of PP4397 reveals the molecular basis for different c-di-GMP binding modes by PilZ domain proteins. *J. Mol. Biol.* 398, 97–110 CrossRef Medline

17. Shin, J. S., Ryu, K. S., Ko, J., Lee, A., and Choi, B. S. (2011) Structural characterization reveals that a PilZ domain protein undergoes substantial conformational change upon binding to cyclic dimeric guanosine monophosphate. *Protein Sci.* 20, 270–277 CrossRef Medline

18. Fujiwara, T., Komoda, K., Sakurai, N., Tajima, K., Tanaka, I., and Yao, M. (2013) The c-di-GMP recognition mechanism of the PilZ domain of bacterial cellulose synthase subunit A. *Biochem. Biophys. Res. Commun.* 431, 802–807 CrossRef Medline

---

5 Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party-hosted site.
29. Hunter, S., Apweiler, R., Attwood, T. K., Bairoch, A., Bateman, A., Binns, D., Bork, P., Das, U., Daugherty, L., Duqueen, L., Finn, R. D., Gough, J., Haft, D., Hulo, N., Kahn, D., et al. (2009) InterPro: the integrative protein signature database. *Nucleic Acids Res.* **37**, D211–D215 [CrossRef Medline]

30. Kabasch, W. (2010) XDS. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 12 [CrossRef Medline]

31. Adams, P. D., Afonine, P. V., Binkowski, F., Badiane, N., Bouyé, C., Carroll, A. J., Chen, Y. L., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., et al. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 213–221 [CrossRef Medline]

32. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 486–501 [CrossRef Medline]

33. Xu, L., Xin, L., Zeng, Y., Yam, J. K., Ding, Y., Venkataramani, P., Cheang, Q. W., Yang, X., Tang, X., Zhang, L. H., Chiam, K. H., Yang, L., and Liang, Z. X. (2016) A cyclic di-GMP-binding adaptor protein interacts with a chemotaxis methyltransferase to control flagellar motor switching. *Sci. Signal.* **9**, ra102 [CrossRef Medline]

34. Choi, K. H., Kumar, A., and Schweizer, H. P. (2006) A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. *J. Microbiol. Methods* **64**, 391–397 [CrossRef Medline]

35. Venkataramani, P., and Liang, Z. X. (2017) Enzymatic production of c-di-GMP using a thermophilic diguanylate cyclase. *Methods Mol. Biol.* **1657**, 11–22 [CrossRef Medline]

36. Rao, F., Patunooti, S., Ng, Y., Zhao, W., Lim, L., Liu, W., and Liang, Z.-X. (2009) Enzymatic synthesis of c-di-GMP using a thermophilic diguanylate cyclase. *Anal. Biochem.* **389**, 138–142 [CrossRef Medline]

37. Alicia, T. G., Yang, C., Wang, Z., and Nguyen, N. T. (2016) Combinational concentration gradient confinement through stagnation flow. *Lab Chip* **16**, 368–376 [CrossRef Medline]

38. Wang, C. J., Bergmann, A., Lin, B., Kim, K., and Levchenko, A. (2012) Diverse sensitivity thresholds in dynamic signaling responses by social amoebae. *Sci. Signal.* **5**, ra17 [CrossRef Medline]

39. Long, Z., Olliver, A., Brambilla, E., Sclavi, B., Lagomarsino, M. C., and Dorfman, K. D. (2014) Measuring bacterial adaptation dynamics at the single-cell level using a microfluidic chemostat and time-lapse fluorescence microscopy. *Analyst* **139**, 5254–5262 [CrossRef Medline]

40. Gerlt, J. A., Bouvier, J. T., Davidson, D. B., Imker, H. J., Sadkhin, B., Slater, D. R., and Whalen, K. L. (2015) Enzyme Function Initiative-Enzyme Similarity Tool (EFI-EST): a web tool for generating protein sequence similarity networks. *Biochim. Biophys. Acta* **1854**, 1019–1037 [CrossRef Medline]

41. Letunic, I., and Bork, P. (2007) Interactive Tree Of Life (iTOl): an online tool for phylogenetic tree display and annotation. *Bioinformatics* **23**, 127–128 [CrossRef Medline]