Molecular mechanism of PilZ adaptor protein mediated cyclic di-GMP signaling

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NANYANG
TECHNOLOGICAL
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**MOLECULAR MECHANISM OF PILZ
ADAPTOR PROTEIN MEDIATED CYCLIC
DI-GMP SIGNALING**

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SCHOOL OF BIOLOGICAL SCIENCES

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ABBREVIATIONS

c-di-GMP/cyclic di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
DGC	Diguanylate cyclase
PDE	Phosphodiesterase
GTP	Guanosine Triphosphate
GMP	Guanosine monophosphate
5'-pGpG	5'-phosphoguanylyl-(3'-5')- guanosine
I-site	Inhibitory site
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
<i>E. coli</i>	<i>Escherichia coli</i>
T4P	Type IV pili
EPS	Extracellular polymeric substances
CW	Clockwise
CCW	Counter clockwise
K _d	Dissociation constant
PCR	Polymerase Chain Reaction
Co-IP	Co-immunoprecipitation
LB	Lysogeny broth
6×His tag	Hexa-histidine tag
HA tag	Hemagglutinin tag (YPYDVPDYA)
OD ₆₀₀	Optical density at 600 nm
cGMP	Cyclic guanosine monophosphate
c-di-AMP/cyclic di-AMP	Cyclic di-adenosine monophosphate
Ado-Met/SAM	S-adenosyl methionine
PBS	Phosphate buffered saline
IPTG	Isopropyl β-D-1-thiogalactopyranoside
HRP	Horseradish peroxidase
ITC	Isothermal titration calorimetry
3D-SIM	Three dimensional structured illumination microscopy
YFP	Yellow fluorescence protein
GFP	Green fluorescence protein
BSA	Bovine serum albumin
MCP	Methyl-accepting chemotaxis protein
TCEP	Tris(2-carboxyethyl)phosphine
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SSN	Sequence Similarity Network
HPLC	High performance liquid chromatography

PUBLICATIONS INCLUDED IN THIS THESIS

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ABSTRACT

The universal bacterial second messenger molecule cyclic di-GMP interacts with a wide variety of receptors to regulate cellular processes. Free-standing PilZ domain proteins are the most prevalent cyclic di-GMP effectors, and elucidating their functions is essential for understanding the role of cyclic di-GMP in many bacteria. In this study, we found that the single-domain PilZ protein PA4608 (named as MapZ-methyltransferase associated PilZ) from the opportunistic pathogen *Pseudomonas aeruginosa* directly interacts with a chemotaxis methyltransferase CheR1 in the presence of cyclic di-GMP. We demonstrated that cyclic di-GMP inhibits the methylation of chemoreceptor by CheR1 through MapZ. We found that cyclic di-GMP and the pole-localized MapZ control the frequency of swimming direction reversal and persistence of runs through flagellar motor to mediate swimming motility and surface attachment during biofilm formation. Furthermore, we found that elevated cellular cyclic di-GMP levels suppress flagellar motor switching through MapZ to cause decreases in swimming reversal frequency, which is controlled by a pool of cyclic di-GMP governed by a specific set of cyclic di-GMP phosphodiesterases (PDEs), with the three PDEs DipA, RbdA and NbdA exerting the greatest control on flagellar motor switching.

To understand the molecular mechanism in cyclic di-GMP mediated protein regulation, we solved the co-crystal structure of the PilZ adaptor protein MapZ in complex with cyclic di-GMP and its protein target CheR1. The co-crystal structure, together with the structure of free CheR1, revealed that the binding of cyclic di-GMP induces dramatic structural changes in MapZ that are crucial for CheR1 binding. Importantly, restructuring and repositioning of two C-terminal helices enable MapZ to disrupt the active site of CheR1 by dislodging a structural domain. The crystallographic observations are reinforced by protein-protein binding and single cell-based flagellar motor switching analysis. Our studies further suggest that the regulation of chemotaxis by cyclic 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. Collectively, the studies unveil a novel cyclic di-GMP regulatory mechanism to control flagellar motor switching through the PilZ adaptor protein and advance our understanding of the molecular mechanism of cyclic di-GMP signaling.

CHAPTER 1. Introduction

1.1. The second messenger cyclic di-GMP

Bis-(3'-5')-cyclic dimeric guanosine monophosphate, known as c-di-GMP or cyclic di-GMP, is a universal second messenger in bacteria, which was first discovered in 1987 as the cellulose synthase activator in *Acetobacter xylinum* (3). Although forming higher oligomers, cyclic di-GMP appears as monomeric or dimer forms under physiological conditions (4,5). Playing key roles in intracellular signal transduction, cyclic di-GMP regulates cellular functions including virulence, biofilm formation, motility, cell cycle and other processes. Importantly, cyclic di-GMP can be captured as a unique bacterial molecule by mammalian immune systems, which makes it to be a potential vaccine adjuvant (4).

1.1.1. Metabolism of cyclic di-GMP

Cyclic di-GMP is synthesized from guanosine triphosphate (GTP) and degraded to guanosine monophosphate (GMP) via the intermediate 5'-phosphoguanylyl-(3'-5')-guanosine (5'-pGpG) (Figure 1.1). Diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) are identified to contribute to its synthesis and hydrolyzation respectively (4). Both biochemical and genetic studies have suggested that GGDEF domain is the catalytic domain in DGCs, being part of the substrate GTP binding site, and EAL and HD-GYP are two functional domains in PDEs (4,6-8).

Diguanylate cyclases

The highly conserved motif GG[D/E]EF in DGC is the active domain and responsible for substrate binding specifically. GGDEF domains function as homodimers to form an active site at the dimer interface. Two GTP molecules bind to GGDEF domains respectively and form the intermolecular phosphoester bond. This is supported by the crystal structure of PleD, a GGDEF domain containing protein in *Caulobacter crescentus* (9,10). In the phosphoester bond formation, Mg^{2+} and Mn^{2+} cations are indispensable. Previous studies have suggested that in GG[D/E]EF motif, the first two Gly residues work for GTP binding, the fourth Glu residue is essential for metal ion coordination while the third Asp/Glu residue is required for both catalysis and metal

coordination. One of the common GGDEF domain activation regulation mechanism is phosphorylation. In the pathogenic spirochete *Borrelia burgdorferi*, the DGC Rrp1 is activated while its REC domain is phosphorylated *in vitro* (11). Another popular regulation mechanism is a feedback inhibition occurring at a conserved inhibitory site (I-site) containing an RxxD motif (where x is any residue) (12). I-site locates five amino acids upstream of the GGDEF motif. Dimeric cyclic di-GMP binds to the I-site and formed a product feedback inhibition to PleD as shown in its crystal structure (4,10,13). For these GGDEF domain DGCs lacking I-sites, they may carry out inhibition through some other ways. Cyclic di-GMP can bind to the active site in XCC4471, a DGC in plant pathogen *Xanthomonas campestris*, suggesting a noncompetitive inhibition regulation mechanism here (14).

Phosphodiesterases

Previous genetic and biochemical studies have suggested that EAL domains are associated with PDE activity. It is noted that some EAL domain PDEs are catalytically inactive and only retain the cyclic di-GMP binding ability, maybe serving as receptors. The purified PDEs in *Gluconacetobacter xylinus* are able to degrade cyclic di-GMP into 5'-pGpG (linear dimeric GMP), which is also required Mg^{2+} and Mn^{2+} cations as GGDEF domains, and which can be inhibited by Ca^{2+} (15). The EAL domain protein YahA in *E. coli* also showed cyclic di-GMP specific hydrolysis activity. Due to the low hydrolysis rate of EAL domains in degrading 5'-pGpG, some other enzymes may function for its degradation *in vivo* (16). Although some EAL domains function as monomers, majority of EAL domain PDEs form dimers for enzymatic activation *in vivo* (4). Cyclic di-GMP binds to EAL domain PDEs with help of two metal cations, and the conserved Glu residue is responsible for metal binding (4,17).

Besides EAL domains, another family possessing hydrolytic activities, HD-GYP domain, also works in cyclic di-GMP degradation. HD-GYP domain is part of the large HD family of hydrolytic enzymes and was predicted to have PDE activity from genetic evidence of linkage with GGDEF domains (18,19). Similar to EAL domains, some HD-GYP domain PDEs also serve as cyclic di-GMP receptors instead due to the deficiency of catalytic activity. The first structure of HD-GYP domain protein, the non-functional PDE Bd1817 in *Bdellovibrio bacteriovorus* was solved in 2011 (20). And the only structure of functional HD-GYP domain protein PmGH from *Persephonella*

marina was solved recently, indicating a novel trinuclear iron catalytic core. The conserved residues serve to bind to either irons or ligand (21).

Figure 1.1 Metabolism and physiology of cyclic di-GMP. Cyclic di-GMP is synthesized by DGCs with conserved GGDEF motifs and is degraded by PDEs with conserved EAL or HD-GYP motifs. After binding to receptors, c-di-GMP can regulate multiple cellular processes.

1.1.2. Physiological roles of cyclic di-GMP

As the universal second messenger, cyclic di-GMP regulates a wide range of cellular activities, including motility, virulence, cell cycle, biofilm and more, at transcriptional, translation and post-translational levels.

Regulation of bacterial motility

Lifestyles of majority of bacteria cells have a transition between sessility and motility, in which cyclic di-GMP plays an essential role. For these flagellated bacteria, they rotate flagella with different frequencies and directions to swim in liquid or attach on some abiotic or biotic surfaces. In *E. coli*, YcgR, a cyclic di-GMP receptor, interacts with the

FliG and FliM flagellar rotor proteins to regulate the rotation bias. High cyclic di-GMP concentration can strengthen the interaction to maintain the flagellar rotation direction in counter-clockwise (CCW), which bundle the flagella to propel the cell forward. This “backstop brake” mechanism is found in some other bacteria species also (4,22,23). Cyclic di-GMP also controls other flagellar motility, like swarming motility in *P. aeruginosa* through YcgR homologue FlgZ by interacting with MotCD (24). In *P. aeruginosa* PA14, the DGC SadC and PDE BifA control cyclic di-GMP level to mediate swarming motility through a chemotaxis-like-pathway (25-27). Besides the regulation through flagella motor, cyclic di-GMP also controls the type IV pili (T4P) biogenesis and twitching motility in response to environmental factors in *P. aeruginosa* requiring a response regulator protein FimX, containing GGDEF-EAL domain (28).

In addition to the fast flagellar behaviour regulation, cyclic di-GMP is found to control some motility genes expression through some of its receptors working as transcriptional factors. One example is the FleQ protein in *Pseudomonas*, in which FleQ controls polysaccharide *pel* biosynthesis genes expression. In *Xanthomonas*, the cyclic di-GMP binding Clp proteins loss ability to bind DNA, resulting lower expression of flagellin *fliC* gene (29-31). VspT in *Vibrio cholerae* is also found to suppress flagellar genes expression (32).

Regulation of biofilm formation

Biofilm is a bacterial community embedded within an extracellular matrix containing exopolysaccharides, extracellular DNA and proteins. It usually involves several stages, including attachment, microcolony formation, maturation and dispersion. In *P. aeruginosa*, the mucoid colonies isolated from cystic fibrosis patients’ lungs are related to increased cyclic di-GMP level and biofilm formation (4). Biofilm formation protects bacteria from treatment of multiple drugs and antibiotics.

The biosynthesis of cellulose, known as a common component of bacterial biofilms, was the first process found to be regulated by cyclic di-GMP (3). Cyclic di-GMP binds to the C-terminal PilZ domain in *G. xylinus* cellulose synthases, supporting an allosteric regulatory mechanism (33). The synthesis of alginate, Pel and Psl polysaccharides is found to be regulated by cyclic di-GMP positively in *P. aeruginosa*. The *wsp* chemosensory pathway modulates cyclic di-GMP levels to control biofilm formation. Deletion of *wspF* affects the expression level of at least 560 genes and increases the

expression of *pel* and *psl* operons. The WspF phenotypes depend on a response regulator protein WspR, which contains a GGDEF domain to be a functional DGC (34). FleQ is a cyclic di-GMP receptor and represses the transcription of *pel* and *psl* and this repression is relieved by high cyclic di-GMP level. Besides, FleQ also can activate the expression of flagella biosynthesis genes (4,35). In addition, the cyclic di-GMP receptor PelD, DGC RoeA, SadC and PDE BifA are all found to regulate Pel synthesis at the posttranslational level (4).

In addition to biofilm formation, the dispersion is also a critical process during the biofilm maintenance. In *P. aeruginosa*, the activation of a DGC, NicD, leads to the phosphorylation of BdlA, a chemotaxis transducer protein, which in turn stimulates the disperse-specific PDE, DipA. And the dispersed cells are found with distinct virulence from planktonic and biofilm cells (36-39).

1.1.3. Effectors of cyclic di-GMP

Different from cyclic di-GMP synthesis and degradation enzymes characterized by GGDEF, EAL and HD-GYP domain proteins, identifying functions of cyclic di-GMP receptors and effectors from sequencing study are more difficult. The diverse classes of cyclic di-GMP effectors mostly have no sequence or structural similarity, increasing the difficulty to study their functions. The predicted cyclic di-GMP receptors include PilZ domain proteins, degenerate GGDEF, EAL and HD-GYP domain proteins, transcription factors and riboswitches (Figure 1.1) (4,40-42).

Besides PilZ domain proteins, serving as the typical family of cyclic di-GMP receptors with the conserved cyclic di-GMP binding motifs, some other cyclic di-GMP receptor proteins are found in past few years. Although some GGDEF, EAL and HD-GYP domain proteins have lost their catalytical activity, their product-inhibiting I-site can still bind to cyclic di-GMP, which makes them to potentially serve as cyclic di-GMP receptors. Some have been studied and found to regulate different cellular processes. One example is a response regulator PopA in *C. crescentus*. Cyclic di-GMP binds to the conserved I-site in the GGDEF domain of PopA, which sequesters PopA to recruit the machinery responsible for CtrA degradation during cell cycle progression (43). Another example is PelD in *P. aeruginosa*, mediating the production of exopolysaccharide to regulate biofilm formation (44). The structure study suggested that cyclic di-GMP can bind to the RXXD motif in its degenerated GGDEF domain (45). A transmembrane

protein LapD in *Pseudomonas fluorescens*, binds to cyclic di-GMP with its enzymatically inactive EAL domain to promote the surface localization of LapA to control the surface attachment in biofilm formation (46,47). The degenerated C-terminal EAL domain in FimX from *P. aeruginosa* also serves as cyclic di-GMP receptor to regulate T4P formation and related motility (4,28).

In past few years, some new types of cyclic di-GMP binding proteins have been discovered but none of them are predicted from sequence analysis. FleQ in *P. aeruginosa* works to activate genes expression in flagella biosynthesis and repress gene transcription in EPS biosynthesis. Cyclic di-GMP can bind to FleQ to relieve this repression, which indicates FleQ as a novel type of cyclic di-GMP transcriptional regulator (48,49). Another cyclic di-GMP receptor family is riboswitches (50,51). Riboswitches are noncoding RNA segments with specific secondary structures. After binding to its small molecule ligand, its structures change, resulting in monitoring cellular environment and controlling gene expression (4,52).

Figure 1.2 Currently known cyclic di-GMP effectors in *P. aeruginosa* PAO1 (28,49,53-59).

1.2. PilZ domain proteins

First predicted as cyclic di-GMP receptors by Amikam and Galperin in 2006, PilZ domain proteins appear to have highest cyclic di-GMP binding affinities among its

receptors (4,60). Named after the PilZ protein PA2960 in *P. aeruginosa*, the PilZ domain protein is one of the most widespread family of cyclic di-GMP binding receptors. Bioinformatics, biochemical and structural studies have confirmed that two conserved motifs, RxxxR and (D/N)x(S/A)xxG, work for cyclic di-GMP binding. The solved structure of PilZ domain proteins indicate that the RxxxR motif forms a flexible loop at N-terminal to wrap cyclic di-GMP and the two Arg residues form hydrogen bonds with cyclic di-GMP. In addition, PilZ domain proteins form an eight-stranded antiparallel β -barrel of 68-74 residues and the (D/N)x(S/A)xxG motif locates on the surface of barrels to bind to cyclic di-GMP (4,61-64). Typically, cyclic di-GMP binding induces conformational changes of PilZ domain proteins, likely required for downstream signaling. Cyclic di-GMP is able to bind PilZ domain proteins with different forms, for example, monomer to PlzD (VCA0042) while intercalated dimer to PA4608 (63,65). Apart from the canonical cyclic di-GMP binding PilZ domain proteins, non-canonical PilZ domain proteins lacking cyclic di-GMP-binding capability have also been discovered, ironically including the eponymous PilZ (66-68). Bacterial genomes encode a large number of PilZ domain proteins that feature different protein length and domain composition (60). Among all PilZ domain proteins, almost half of them are stand-alone PilZ domain proteins consisting of the sole PilZ domain of 90~130 residues while others also contain other functional domains in addition to PilZ domains (69-72). As PilZ proteins usually share low sequence similarity, it is challenging to deduce the function of functionally unknown PilZ proteins, especially those stand-alone PilZ domain proteins, solely based on the sequence-function relationship.

1.2.1. Single-domain PilZ proteins

Single-domain PilZ protein is the largest group of PilZ proteins and only contains the β -barrel core, an N-terminal loop and a short C-terminal motif which is more variable among different proteins. Previous studies have suggested that these PilZ proteins can regulate a wide range of physiological processes including T4P biogenesis, motility, biofilm formation and virulence. However, it is more difficult to study the functions of these single-domain PilZ proteins because there are no clues compared to multi-domain PilZ proteins.

Although the eponymous PilZ cannot bind cyclic di-GMP, it was found to regulate T4P formation and twitching motility together with a cyclic di-GMP binding protein FimX,

which contains the degenerate GGDEF and EAL domains (73,74). Although no evidence suggests the direct interaction between PilZ and FimX in *P. aeruginosa* due to the replacement of several key residues in FimX, their orthologs in *Xanthomonas axonopodis* pv. *citri* (*Xac*) can interact physically in the presence of cyclic di-GMP to form a ternary complex (74-78). The structure studies of XAC1133 and XC1028, PilZ orthologs in *Xanthomonas axonopodis* pv. *citri* (*Xac*) and *Xanthomonas campestris* pv. *campestris* (*Xcc*), show that their different N-terminal β -barrel arrangement and lack of RxxxR and (D/N)x(S/A)xxG motifs result the cyclic di-GMP binding deficiency (77,79). In addition, XAC1133 -XAC2398 (FimX ortholog) complex was found to bind to an ATPase PilB to regulate T4P polymerization and biogenesis. Interestingly, the ternary complex formation is not affected by the presence of cyclic di-GMP.

Besides the pilus-mediated twitching motility, some flagellum-mediated bacterial motility including swarming, swimming and chemotaxis were also found to be regulated by PilZ domain proteins. One example is the PilZ domain proteins DgrA and DgrB in *Caulobacter crescentus* (80). Overexpression of the two PilZ domain proteins can block swarming motility. Cyclic di-GMP is essential for this regulation since DgrA mutants of cyclic di-GMP binding residues could not control motility. In addition, DgrA and DgrB regulate motility not by controlling flagellar gene expression and assembly, but through suppressing the expression level of the protein FliL, required for flagellar rotation.

HapZ, single-domain PilZ protein in *P. aeruginosa*, was reported recently to directly bind to histidine kinase SagS to block the phosphotransfer to its downstream partner HptB in a cyclic di-GMP dependent manner (69). As SagS is a two-component signaling protein that regulates the motile-sessile transition and biofilm, HapZ is suggested to regulate surface attachment and biofilm formation through regulating gene expression level. The NMR structure of another single-domain PilZ protein PA4608 with dimeric cyclic di-GMP in *P. aeruginosa* has been solved (62,63). The conformational changes with cyclic di-GMP binding may prepare it for downstream regulation, although its function was unknown by that time. In this case, study of its function and regulatory mechanism is my Ph.D. project target and its crystal structure with its binding partner will also be discussed in following chapters.

In addition to the regulation on bacterial motility, the PilZ domain proteins also regulate virulence in *Xanthomonas campestris* pv. *campestris* (*Xcc*) (81). In the *Xcc* strain 8004, there are four PilZ domain protein, in which XC0965, XC2249 and XC3221 are single-domain PilZ proteins. Mutation of these PilZ domain proteins affected motility or extracellular enzyme production to some extent, but not biofilm formation. Importantly, XC0965, XC2249 and XC3221 mutants all showed significant deficiency on virulence. Interestingly, although with the cyclic di-GMP binding ability, XC2249 can form a protein complex with a HD-GYP domain protein RpfG and two GGDEF domain proteins in a cyclic di-GMP independent manner and the protein complex interacts with two pilus motor proteins PilU and PilT to modulate pilus function to regulate motility (82,83).

The single-domain PilZ proteins PXO_02374 and PXO_02715 were also found to regulate virulence and motility in *Xanthomonas oryzae* pv. *oryzae* strain PXO99 (84,85). The PilZ domain protein PXO_02374 can bind to cyclic di-GMP to regulate virulence and sliding motility, but not exopolysaccharide production and biofilm formation. It can regulate the type III secretion system related *hrp* gene expression. Although lack of the cyclic di-GMP binding ability with *in vitro* detection, PXO_02715 can interact with a cyclic di-GMP receptor, FimX like protein Filp, containing degenerated GGDEF and EAL domain for cyclic di-GMP binding, to control the virulence via regulation of *hrp* gene expression in type III secretion system.

In *Vibrio cholerae*, there are five PilZ domain protein, in which PlzA is the only stand-alone PilZ domain protein (65,86). PlzA is essential for bacterial survival for the failure in preparation of *plzA* deletion strain. However, overexpression of *plzA* has no effect on bacterial motility, biofilm formation or virulence. Its function still remains unknown.

1.2.2. Di-domain and Multi-domain PilZ proteins

Besides these stand-alone PilZ domain proteins, some PilZ domain proteins possess an additional C- or N-terminal domain to the PilZ domain, which usually feature longer protein sequences and are considered as di-domain and multi-domain PilZ proteins to regulate multiple physiological processes including motility, biofilm formation, virulence, gene transcription, exopolysaccharides and alginate synthesis. YcgR_N domain is by far the most abundant domain found in the di-domain PilZ proteins (70-72). In addition to the YcgR_N domain, some other domains including phosphoreceiver (REC)

domain, DNA-binding domain, GGDEF domain and a second PilZ domain can also be found in di-domain PilZ proteins (69-72). The multi-domain PilZ proteins contain a variety of protein domains, with the most common domains predicted to be glycosyltransferase, GGDEF, EAL, methyl-accepting chemoreceptor and other sensory protein domains (58,69-72,87-90). The biological functions of the di-domain and multi-domain PilZ proteins seem to be easier to study based on domain annotation and characterized homologs compared to the stand-alone single PilZ domain proteins.

YcgR, a di-domain PilZ protein in *E. coli*, is known as a “backstop brake” to control flagella-mediated bacterial motility including swimming, swarming and chemotaxis, but not through controlling flagellar genes expression (22,33,91). YcgR can interact with flagellar motor proteins FliG and FliM and the stator protein MotA to inhibit the torque generation and reduce motility by decreasing flagellar rotational speed and altering flagellar rotational direction in a cyclic di-GMP dependent manner. Previous studies also suggest four DGCs and one PDE YhjH in *E. coli* to control cellular cyclic di-GMP levels for motility regulation. Similar to YcgR, the PilZ domain protein YpfA in *Bacillus subtilis* also suppresses motility with binding to the flagellar motor protein MotA (92). Importantly, the regulation is also under control of a specific PDE YuxH. Moreover, YpfA is also suggested to have a mild effect on biofilm formation.

The di-domain PilZ protein FlgZ in *Pseudomonas* also contains a weak conserved YcgR domain at N-terminus and its PilZ domain shows homology with a similar domain in DgrA in *Caulobacter* (93). FlgZ is known to regulate bacterial motility including both swarming and swimming, and biofilm formation under the regulation of cyclic di-GMP level controlled by a DGC WspR and a PDE BifA (24,93). In *Pseudomonas*, there are two sets of stator complexes in control of polar flagellum, MotAB preventing swarming motility and MotCD promoting swarming motility (24,94). Different from YcgR, FlgZ directly interacts with MotC instead of MotA to prevent the engagement of MotCD with the rotor to finally control swarming motility with control of cyclic di-GMP levels. Importantly, the polar cellular localization of FlgZ is also dependent on MotCD and cyclic di-GMP level.

The only di-domain PilZ protein in *Xanthomonas campestris* pv. *campestris* (*Xcc*) is XC2317 containing an additional YcgR_N (81). However, unlike other single PilZ domain proteins in *Xcc*, mutation of XC2317 does not have any significant effect on

bacterial motility or virulence. The only effect observed is that mutation of *XC2317* affects production of extracellular enzymes, in which case the function of *XC2317* still remains unclear.

The di-domain PilZ protein PXO_00049 also contains an N-terminal YcgR domain and a C-terminal cyclic di-GMP binding PilZ domain (95). Similar to PXO_02374, PXO_00049 can regulate bacterial sliding motility and virulence, but not exopolysaccharides production and biofilm formation. It also affects the *hrp* gene expression in type III secretion system to control bacterial virulence in rice.

Another four PilZ domain proteins in *Vibrio cholerae* have been studied together with PlzA, in which PlzD and PlzE are di-domain PilZ proteins and PlzB and PlzC are multi-domain PilZ proteins (65,86). Same as PlzA, the failure in preparation of *plzE* deletion strain suggests PlzE is also essential for bacterial survival, but its function still remains unknown as its overexpression did not affect virulence, motility or biofilm formation. Interestingly, different from the other four PilZ domain proteins containing only one PilZ domain, PlzC contains two PilZ domains. The *in vitro* binding assay shows that only PlzC and PlzD specifically bind cyclic di-GMP, with similar affinities. PlzB and PlzC show positive regulation on motility and PlzD may work as a negative motility regulator in a cyclic di-GMP independent manner. Furthermore, PlzB and PlzC also regulate biofilm formation for the reduced biofilm in their deletion strains. In addition, PlzB, PlzC and PlzD are all required for intestinal colonization to affect virulence and pathogenicity. Importantly, the di-domain PilZ protein PlzD (VCA0042) contains an YcgR_N domain and a PilZ domain, whose crystal structure has been solved (65). Binding of monomeric cyclic di-GMP to the PilZ domain in PlzD, which forms dimer *in vitro*, induces conformational changes according to the solved crystal structure of PlzD-cyclic-di-GMP complex. Interestingly, the structure of PilZ domain protein PP4397 in *Pseudomonas putida* is also studied (64). Similar to PlzD, it also has the YcgR_N domain and PilZ domain. And it can bind two intercalated cyclic di-GMP molecules and this binding induces PP4397 to transit from dimer to monomer. These findings support the hypothesis that PilZ domain proteins exhibit heterogeneity in cyclic di-GMP binding stoichiometry, quaternary structures and finally the biophysical function.

PlzA (BB0733) is the sole PilZ domain protein in *Borrelia burgdorferi* (96,97). It can bind cyclic di-GMP to its conserved cyclic di-GMP binding RxxxR and D/NxSxxG motifs with high affinity (K_d of 1.25 μ M). Previous studies also suggest that PlzA can

Figure 1.3 Structures of PilZ domain proteins. (A) FimX_{EAL}-PilZ-c-di-GMP complex (PDB: 4FOU). (B) PA4608-c-di-GMP complex (PDB: 2L74). (C) PlzD-c-di-GMP complex (PDB: 2RDE). (D) PP4397-c-di-GMP complex (PDB: 3KYF). (E) MrkH-c-di-GMP complex (PDB: 5EJL). (F) PilZ domain of XCC6012 (PDB: 3RQA). (G) CsaA (PDB: 4I86). (H) Alg44-c-di-GMP complex (PDB: 4RT0). PilZ domain is shown in tan, FimX_{EAL} domain is shown in light blue, YcgR_N domain is shown in dark grey and cyclic di-GMP is shown in sticks.

suppress bacterial swarming motility and virulence in the mice infection experiments. PlzA, working as a transcription regulator, can modulate the production of a global regulator protein RpoS through BosR to build a connection between the two-component signal transduction systems Hk1-Rrp1 and Hk2-Rrp2 to regulate virulence (98). Furthermore, PlzA also regulates *rpoS* expression independently of Rrp1, supporting the multi-function feature of PlzA.

MrkH is a novel PilZ-domain containing transcriptional regulator to regulate biofilm formation in the non-motile nosocomial pathogen *Klebsiella pneumoniae* (99,100). Upon binding of cyclic di-GMP, MrkH can activate the *mrkA* promoter to initiate the expression of *mrkABCD* operon encoding type III fimbriae components, which significantly mediates cell attachment and biofilm formation. The role of cyclic di-GMP is essential in the transcription activation because the mutation of cyclic di-GMP binding residues totally abolishes the function of MrkH. Suggested by the crystal structure, MrkH contains a PilZ-like fold N-terminal domain and a PilZ domain at C-terminus (101,102). MrkH can interact with two intercalated cyclic di-GMP molecules similar to PP4397. Collaborated with the N-domain, the PilZ domain can bind not only cyclic di-GMP but also DNA, providing a novel sight in PilZ domain protein functions.

Shared the identical sequence with the single PilZ domain protein XC2249, XCC6012 from *Xcc* strain 17 is a unique PilZ-like protein and exhibits a novel tetrameric structure (103). As XC2249, XCC6012 also showed effect on virulence. It does not contain the complete RxxR and D/NxSxxG motif but only has a partial SxxG, resulting the failure in detection of cyclic di-GMP binding *in vitro*. Although the monomer shares the canon PilZ structure as other PilZ domain proteins and shows weak cyclic di-GMP binding affinity, four XCC6012 proteins can self-assemble into a tetramer to form a parallel four-stranded coiled-coil, which is further enclosed by two sets of helices. This new PilZ domain quaternary structure further supports the heterogeneity and complexity of cyclic di-GMP regulation.

Besides these di-domain PilZ proteins, some multi-domain PilZ proteins also regulate motility, biofilm and virulence. TDE0214 is a multi-domain PilZ protein in *Treponema denticola* (104). Importantly, TDE0214 contains a PilZ domain with two conserved cyclic di-GMP binding motifs and binds to cyclic di-GMP at high affinity. *TDE0214* deletion mutant showed suppressed swimming motility, less biofilm formation and

reduced invasiveness in mouse skin model, all indicating its various regulation on motility, biofilm formation and virulence of the spirochete.

A novel cyclic di-GMP binding family protein was found to broaden our understanding of PilZ protein and cyclic di-GMP regulation in chemotaxis activity, which is an essential flagellum-regulated motility (90,105). Tlp1 in *Azospirillum brasilense* is a type of aerotaxis and chemotaxis receptor protein containing a C-terminal PilZ domain for cyclic di-GMP binding. Upon cyclic di-GMP binding, bacterial swimming speed is enhanced and swimming reversal frequency is suppressed, resulting persistent motility tendency and aerotaxis responses. The finding of chemotaxis receptors proteins with cyclic di-GMP binding PilZ domains provides direct and solid evidences for cyclic di-GMP regulation in chemotaxis motility.

Besides YcgR, another common PilZ domain protein in most enterobacteria is BcsA, containing a PilZ domain at C-terminus with a cellulose synthase subunit (106,107). Cellulose is the essential exopolysaccharide in biofilm matrix. The binding of cyclic di-GMP to BcsA generates conformational changes to allow UDP-glucose to access the catalytic site during cellulose synthesis process (108). Furthermore, accumulated cellulose works in cooperation with YcgR to block bacterial motility and mediate lifestyle transition from motility to sessility under high cellular cyclic di-GMP level generated by deletion of a key PDE YhjH in *Salmonella*. Homolog of BcsA, CseA is the first verified cyclic di-GMP receptor protein and component of cellulose synthase complex (88,109,110). The crystal structure of its PilZ domain has been solved, indicating two more residues Lys573 and Arg643 in the recognition of cyclic di-GMP besides the RxxxR motif. Furthermore, the structure of the crystalline cellulose synthase AcsA containing PilZ domain is solved within the terminal complex with AcsB in *Gluconacetobacter hansenii* (111). Like BcsA, AcsA also has a catalytic domain in an intracellular loop and a PilZ domain at C-terminus. Importantly, the crystal structure of AcsAB shares similar structure containing conserved catalytic and membrane translocation components with the BcsAB synthase in the non-crystalline cellulose producing bacterium *Rhodobacter sphaeroides*.

Alg44 is a unique PilZ domain protein for extracellular polysaccharide alginate biosynthesis in *P. aeruginosa* (87). Alg44 contains a central transmembrane domain, an N-terminal PilZ domain working for cyclic di-GMP binding, and a C-terminal

membrane-fusion-proteins (MFPs) like periplasmic domain, possible for downstream interaction (58,112). Working together with the major glycosyltransferase Alg8, Alg44 is essential for alginate production by controlling polymerization of the polysaccharide. Its crystal structure further indicates that the intercalated dimeric cyclic di-GMP binding induces the homodimerization of PilZ domain folding in Alg44 (89). And the cyclic di-GMP binding is found to be critical for its alginate synthesis function, further supporting the key role of cyclic di-GMP in signaling regulation in bacteria.

1.3. Objectives

Significant progress has been made in understanding the role of cyclic di-GMP in bacterial signaling regulation during the past 30 years. As the largest family of cyclic di-GMP binding proteins, the single-domain PilZ proteins, have been implied in regulating bacterial motility, virulence and polysaccharide production. However the physiological function and mechanism of the single-domain PilZ proteins remain largely unknown. My Ph.D. research project focuses unravelling the function of the unknown single-domain PilZ protein, PA4608 in *P. aeruginosa*, which is a common human pathogen and potentially lethal for immunocompromised patients.

My first objective is to uncover the physiological role played by PA4608 in *P. aeruginosa*. In this regard, the identification of its binding partner, a chemotaxis methyltransferase CheR1, led to the discovery that cyclic di-GMP regulates chemotaxis, bacterial motility and biofilm formation through the adaptor protein PA4608. My second objective is to identify the PDEs which control the pool of cyclic di-GMP sensed by PA4608. We screened flagellar rotational pattern of the unknown function PDEs mutant strains and found three PDE, RbdA, NbdA and DipA, which are the major PDEs controlling the cyclic di-GMP pool sensed by PA4608. My third objective is to understand how cyclic di-GMP mediates the interaction between PA4608 and CheR1 at the molecular level. We crystalized the complex of PA4608-CheR1 with dimeric cyclic di-GMP and determined the crystal structure of the ternary complex. The structural observations were verified by isothermal titration calorimetry and cell tethering motility assay. For the first time, the data provide detailed structural insights into how the single-domain PilZ proteins act as cyclic di-GMP binding adaptor.

CHAPTER 2. Cyclic di-GMP regulates flagellar motor switching through the PilZ protein MapZ in *Pseudomonas aeruginosa*

2.1. Introduction

Cyclic di-GMP is a widespread second messenger that controls a multitude of cellular functions and bacterial behaviours (113-115). In human pathogenic bacteria such as *P. aeruginosa*, *Salmonella enterica* and *Vibrio cholerae*, cyclic di-GMP plays a prominent role in regulating the motile-sessile transition and formation of antibiotic-resistant biofilm communities. Cyclic di-GMP exerts its control on cellular functions by binding to a variety of protein and riboswitch targets (116,117). Among the known cyclic di-GMP binding proteins, stand-alone single-domain PilZ proteins are widespread and by far the most prevalent ones (60,70,107,118-121). 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.

As an opportunistic pathogen, *P. aeruginosa* is notorious for its ability to cause several acute and chronic infections in immunocompromised individuals. In addition to its intrinsic resistance to multiple antibiotics, the formation of structured bacterial communities on abiotic and biotic surfaces, known as biofilms, also enhances the embedded bacteria resistance to harsh environment, drugs, and physical treatments. *P. aeruginosa* harbors a highly complex cyclic di-GMP signaling network that plays a central role in biofilm formation and dispersal (122,123). The cyclic di-GMP network of *P. aeruginosa* consists of more than 30 DGCs and PDEs and a dozen of cyclic di-GMP binding effector proteins.

In *P. aeruginosa*, there are seven cyclic di-GMP binding PilZ domain proteins, including multi-domain protein Alg44, di-domain proteins FlgZ and PA2989, and single-domain proteins PA0012, PA4324, HapZ and PA4608. By binding to cyclic di-GMP, the PilZ domain proteins regulate multiple bacterial cellular processes. Alg44 was reported to be involved in bacterial alginate biosynthesis (58). FlgZ interacted with MotC/D stator to mediate bacterial swimming motility as well as biofilm formation (124). In addition, as a single-domain PilZ protein, HapZ was found to regulate the two-

component signaling and gene expression by interaction with a histidine kinase SagS (69).

To further understand the roles of cyclic di-GMP and PilZ proteins in *P. aeruginosa*, here we studied the unknown function single-domain PilZ protein PA4608, which interacts with a chemotaxis methyltransferase CheR1 in *P. aeruginosa* to regulate bacterial motility by controlling flagellar output. It may unveil a potential widespread mechanism by which cyclic di-GMP regulates bacterial motility through PilZ adaptors. Furthermore, we have known that the flagellar motility is critical for the immune system response to *P. aeruginosa* (125). This regulation of PilZ adaptor through chemotaxis pathway by cyclic di-GMP may provide us a potential drug target for *P. aeruginosa* infections.

2.2. Material and methods

2.2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1 and Table S2. The DNA fragments encoding full length or truncated proteins were amplified by PCR from *P. aeruginosa* PAO1 genomic DNA and by using primers designed based on the published *P. aeruginosa* PAO1 genome sequence (126). The primers used for PCR and molecular cloning are summarized in Table S3. Plasmids were constructed by standard molecular cloning technique unless further instruction. The MapZ^{R13A} was generated using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies). Some of the *P. aeruginosa* PAO1 mutant strains used in this study were obtained from the PA two-allele *Pseudomonas aeruginosa* PAO1 transposon mutant library (127).

2.2.2. Preparation of the *P. aeruginosa* mapZ^{R13A} mutant

The preparation of the *P. aeruginosa* mapZ^{R13A} mutant was done by Prof. Xu Linghui's Lab, China (1). The mutant strain was created using the allelic exchange vector pK18GT-MapZ^{R13A} as described before with modification (128). Briefly, a DNA fragment of 1,216 bp containing the mapZ^{R13A} gene construct was amplified with *P. aeruginosa* PAO1 genomic DNA as template by overlap extension PCR. The primers used are listed in Table S3, with primers 1 and 2 for upstream fragment amplification, primers 3 and 4 for downstream amplification, and primers 1 and 4 for full-length gene

amplification. The PCR product was cloned into the vector pK18GT at BamHI and HindIII restriction sites to generate the suicide plasmid pK18GT-MapZ^{R13A}. The suicide plasmid was transformed into *E. coli* DH5 α for the subsequent conjugation with PAO1 by triparental mating with the helper plasmid pRK2013. The trans-conjugants were selected on *Pseudomonas* isolation agar (PIA) plates (QingDao Hope Biotechnology) containing 100 μ g/ml gentamycin. Colonies resulting from the first crossover events were streaked onto LB agar plate supplemented with 10% sucrose, and sucrose sensitive colonies were selected as positive colonies. Colonies that contained the correct mutation were identified by sequencing of the DNA fragment amplified using primers 5 and 6.

2.2.3. Bacterial two-hybrid screening using *P. aeruginosa* genomic DNA libraries

The bacterial two-hybrid screening was done by Prof. Xu Linghui's Lab, China (1). The construction of the *P. aeruginosa* genomic DNA libraries used for the two-hybrid assays has been described previously (69). The plasmid pBT-MapZ was used as the bait to probe the genomic DNA libraries using the BacterioMatch II Two-Hybrid system (Stratagene). The plasmids extracted from the cells harboring the library fragments were co-transformed into the provided *E. coli* reporter strain with the bait plasmid by electroporation. The co-transformed cells were grown on M9⁺ His-deficient medium that contained 5 mM 3-amino-1,2,4-triazole (3-AT) for 48 to 72 hours at 30 °C. Colonies that grew on these plates were selected as positive colonies. Positive colonies were picked and re-streaked on M9⁺ His-deficient medium containing 5 mM 3-AT and 12.5 μ g/ml streptomycin. The colonies that grew on the double selection medium were cultured in liquid medium, and used for colony PCR with pTRG forward and reverse primer pairs to amplify the insert from the library plasmid. For the specific two-hybrid binding assay, the bait and prey plasmids were co-transformed, and the co-transformed mixtures were plated onto selective and non-selective media and incubated at 37 °C overnight. Normal growth on the selective screening medium was considered as an indicator of positive protein-protein interaction.

2.2.4. Co-immunoprecipitation (Co-IP) assay

The plasmids pUCP18-6 $\times$ His-CheR1 and pUCP18-HA-MapZ/6 $\times$ His-CheR1 were constructed and transformed into *P. aeruginosa* PAO1 strain. The strain harboring pUCP18-6 $\times$ His-CheR1 expressed the protein 6 $\times$ His-CheR1 while the strain harboring

pUCP18-HA-MapZ/6×His-CheR1 produced both HA-MapZ and 6×His-CheR1. The strains were cultured in LB medium that contained 300 µg/ml carbenicillin with shaking at 37 °C for overnight. The strains were subsequently subcultured to an optical density at 600 nm (OD₆₀₀) of 1.0 at 37 °C. Cells were harvested by centrifugation at 11,325 g for 10 min at 4 °C and lysed by sonication on ice with ice-cold cell lysis buffer [Phosphate-buffered saline (PBS) (pH7.4) (1st base), 1% (v/v) NonidetTM P 40 (NP-40) (Sigma-Aldrich), 10% (v/v) glycerol and protease inhibitor cocktail (Roche)]. The total cell lysates were clarified by centrifugation at 75,600 g for 1 h at 4 °C, and the supernatant was filtered by 0.45 µm Minisart[®] high flow syringe filters (Sartorius). Lysates were subsequently immunoprecipitated with the EZviewTM Red Anti-HA Affinity Gel (Sigma-Aldrich). Each IP sample containing 1 ml of lysate and 20 µl of the prewashed beads with different concentrations of nucleotides (cyclic di-GMP, GMP, cGMP or c-di-AMP) was incubated for 1 h at 4 °C with rotation. The beads were collected by centrifugation, and washed six times with lysis buffer at 4 °C. The immunoprecipitated proteins were diluted with 2×loading dye and subjected to SDS-poly-acrylamide gel electrophoresis (PAGE) and Western Blotting with THETM His Tag Antibody [HRP] (GenScript) and HA-tag Antibody [HRP] (GenScript).

2.2.5. Expression and purification of recombinant proteins

The plasmids pET28b-MapZ, pET28b-MapZ^{R13A} and pET28b-CheR1 were constructed and transformed into *E.coli* BL21 (DE3) cell line and single colony of each on LB agar plates containing 50 µg/ml Kanamycin was inoculated into 20 ml LB medium containing 50 µg/ml Kanamycin. The cultures were grown at 37 °C with shaking for 16 h. The overnight cultures were used to inoculate 2 liters of fresh LB medium supplemented with 50 µg/ml Kanamycin. When the culture reached an OD₆₀₀ of 0.6 to 0.8, the temperature was reduced from 37 °C to 16 °C, and protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (0.3 mM for CheR1 and 0.4 mM for MapZ and MapZ^{R13A}). After 20 h, cells were harvested by centrifugation and pellets were stored at -80 °C till next step. Cells were resuspended in PBS buffer (pH7.4) that contained 1 mM Dithiothreitol (DTT) and protease inhibitor (Roche) and lysed by French Press at 1,200 Bar. After centrifugation at 75,600 g for 1 h at 4 °C, supernatant was filtered by 0.45 µm filter and incubated with Ni-NTA Agarose Beads (QIAGEN). After 1 h incubation at 4 °C with rotation, the beads were sequentially washed with 10 mM and 20 mM imidazole-containing PBS buffer (pH7.4). The

recombinant protein was eluted using PBS buffer that contained 400 mM Imidazole. The fractions containing the recombinant proteins were desalted using PD-10 Desalting Columns (GE Healthcare) and eluted using PBS buffer (pH7.4). The proteins were concentrated by Amicon concentrators (5 kDa cutoff for MapZ and MapZ^{R13A} and 10 kDa cutoff for CheR1; Sartorius) and their concentrations were determined before the proteins were flash-frozen and stored at -80 °C.

2.2.6. Isothermal titration calorimetry (ITC)

The ITC assay was done with help from Dr. Tang Xuhua, Singapore (1). ITC measurements were performed at 22 °C using MicroCal VP-ITC (MicroCal Inc.). Protein samples were dialyzed into a buffer containing 20 mM Tris (pH8.0) and 100 mM NaCl. A sample syringe with stirring speed of 290 rpm was used to inject cyclic di-GMP (1.18 mM) into a cell containing 50 µM protein. The titration comprised 29 injections of 10 µl each, separated by 240 seconds equilibration time. The datasets were analyzed using the Origin 7.0 program, fitted to a single-site binding model.

2.2.7. In vitro methylation assay

Methylation of PctA by CheR1 was examined by performing in vitro methylation assay with 6×His-tagged recombinant proteins with [³H-methyl]-S-adenosylmethionine {[³H]Ado-Met}. Membrane fractions containing PctA and cyclic di-GMP were prepared as described previously (129,130). The reaction mixture consisting of 50 µl of membrane fractions (2 mg/ml), 0.1 µM 6×His-CheR1, 50 mM NaH₂PO₄ (pH8.0), 300 mM NaCl was preincubated at 30 °C for 10 min. The reaction was initiated by adding 0.625 µM [³H]Ado-Met (specific activity 15 Ci/mmol; PerkinElmer) and incubated at 30 °C for 30 minutes. The reaction was stopped by adding 2×loading dye. Methylation of PctA was visualized by running 12% SDS-PAGE and autoradiography. The effects of MapZ and MapZ^{R13A} (0.1 µM), cyclic di-GMP and other nucleotides on PctA methylation were examined by incubating the proteins and nucleotides in the reaction mixture accordingly.

2.2.8. Cellular localization by superresolution three-dimensional structured illumination microscopy (3D-SIM)

The 3D-SIM localization assay was done in collaboration with Prof. Yang Liang's lab, Singapore (1). The YFP-MapZ or YFP-MapZ^{R13A} gene-containing pUCP18-based

plasmids were constructed and transformed into the PA14 and mutant strains, which were cultured overnight. PA14 and mutant cells that expressed the YFP-MapZ or YFP-MapZ^{R13A} fusion protein were immobilized using 1% agarose and placed onto the glass slide for microscopy imaging. Cell images were captured using the LSM 780 ELYRA PS.1 superresolution structured illumination system (Carl Zeiss) with a 100× plan-apochromatic oil immersion objective lens (numerical aperture, 1.46). YFP was excited using a 488 nm optically pumped semiconductor laser (OPSL) line for observation and data collection. The acquired images were further processed with the Zen 2011 software (Carl Zeiss).

2.2.9. Cellular localization by antibody-based immunofluorescence assay

The IF assay was done in collaboration with Prof. Xu Linghui's lab, China (1). The pUCP18-based plasmid that expresses the HA-tagged MapZ was constructed and transformed into PA14 and mutant strains. Cells expressing HA-tagged MapZ in the mid-exponential phase were subjected to immunofluorescence staining as described previously with modification (131). Briefly, cells were fixed with 4% (w/v) paraformaldehyde, permeabilized with 0.5% (v/v) Triton X-100 in PBS (PBST) solution, blocked with 1% BSA-containing PBST and then incubated with the primary antibody rabbit antibody specific for HA tag (1:1000; Abcam) and then with an antibody specific for rabbit IgG Alexa Fluor 488 (1:2000; Abcam). The stained cells were mounted on adhesion microscope slides (CITOGLAS[®] REF188105) and image was taken using a Leica TCS SP2 confocal microscope.

2.2.10. Agarose plug chemotaxis assay

The assay was performed using a procedure similar to a previously published protocol (132). Cells were grown at 30 °C in Murashige and Skoog (MS) medium [10 mM Na₂HPO₄, 20.6 mM KH₂PO₄, 25 mM NH₄NO₃, 0.8 mM MgSO₄, and 1.0 ml of trace elements solution (63 mM FeCl₃, 3.3 mM CoCl₂, 4 mM CaCl₂, 1.2 mM Na₂MoO₄, 1.6 mM H₃BO₃, 0.7 mM ZnSO₄, 0.7 mM CuSO₄, 0.7 mM MnSO₄)] containing 10 mM succinate until the cultures reached an OD₆₀₀ of 0.8. Cells were harvested by centrifugation and washed twice and grown in fresh MS medium until the cultures reached an OD₆₀₀ of 0.8. Four aliquots (100 µl each) of melted low melting point agarose [2% (w/v); Sigma-Aldrich] containing 10 mM L-serine (Sigma-Aldrich) were transferred to the petri dish to form small agarose plugs. As negative controls, three

agarose plugs containing MS medium were plated on the left side of the petri dish. After the solidification of the agarose, 10 ml of bacterial suspension was added around the agarose plugs and photos were taken after 10 min to visualize the zone or ring formed by the bacterial cells around the agarose plugs.

2.2.11. Swimming trajectory analysis

The swimming trajectory assay was done in collaboration with Prof. Chiam Keng-Hwee's Lab, Singapore (1). Free swimming *P. aeruginosa* cells with their intact flagellum were grown to exponential phase (OD_{600} of 1.0) in LB medium, diluted to 1:500 in 0.9% NaCl containing 3% Ficoll (Sigma-Aldrich) before being loaded in cell chambers with a channel height of 20 μ m and observed in the middle of the chamber depth (away from the coverslip). Cells were visualized under 40 $\times$ objective using an inverted microscope (Nikon TE2000-U) and videos of bacteria were taken at 25 frames per second (fps) using a CMOS camera (Thorlabs DCC1645). Image with cell outlines were obtained using ImageJ (NIH) and bacterial swimming trajectories were obtained using particle segmentation and tracking algorithms as described previously (133,134) and implemented through the software TrackMate plugin in Fiji (135). Briefly, the cell outlines in each image of the time-lapsed video were segmented and identified based on the difference of Gaussians approach (133). The segmented cell outlines were then tracked by linking the individual cell outlines from frame to frame based on the linear assignment problem (LAP) method (134). The velocities and directional persistence of individual swimming cells were then obtained from the trajectories from the time-lapsed videos.

The directional persistence of individual swimming cells was calculated as follows: The mean squared displacement of a tracked cell at any point in time with two-dimensional coordinates x and y , was denoted as r^2 , where $r^2 = x^2 + y^2$. The movement of each cell was described with $r^2 \propto t^Y$, where t was the time that has passed since the start of the cell tracking and Y was denoted as a cell movement persistence factor. When $Y=1$, then $r^2 \propto t$, and the displacement of the cell was proportional to the square root of the elapsed time. In this case, the cell was said to exhibit a random swimming behaviour. When $Y=2$, then $r^2 \propto t^2$, where the displacement of the cell was proportional to the elapsed time and the cell exhibited directed swimming persistence. Hence, particle movement persistence can be quantified using Y .

2.2.12. Cell tethering assay for the analysis of the flagellar rotation

The cell tethering assay was done in collaboration with Prof. Chiam Keng-Hwee's Lab, Singapore (1). Single colonies were used to inoculate 10 ml of LB medium that was kept at 37 °C with shaking at 250 rpm overnight. Cultures were diluted to an OD₆₀₀ of 0.2 using M9 media and grown at 37 °C with shaking at 250 rpm until the cells reached the late exponential growth phase at an OD₆₀₀ of 0.8. Cell cultures were then diluted 10-fold before the microfluidic experiments. Cell tethering assays were carried out using a microfluidic stagnation flow device pre-coated with flagellar antibodies (136). 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 nontethered cells were washed away using M9 media. Cells were visualized using an inverted microscope (Nikon TE2000-U) under 40× objective. Videos of tethered bacteria were taken at 40 fps for 1 to 5 min using a CMOS camera (Thorlabs DCC1645). L-serine (10 mM in M9 medium) [0.5% (w/v)] was used as the chemoattractant in the analysis of flagellar rotation in chemical gradient. The M9 blank and L-serine solution were rapidly and completely gated to expose the cells to a temporally precise chemoattractant signal. The gating of the chemoattractant was performed through flow deflection between the chemo-attractant and buffer inlets (136). Flow deflection ensured that cells tethered in the stagnation flow chamber were shielded from the L-serine prior to chemotaxis experiments. The rotation of the cells were monitored and recorded following the reported procedure (137). The time spent by each cell in CW, CCW or pause phases was tallied in 20 s intervals. Image processing of the tethered cells were carried out as previously described (138) with videos of the tethered cells converted to grey scale and the contrast of the cells adjusted (saturated pixels, 0.4; histogram stack) using ImageJ (NIH). 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 bacterial tethering analysis program (BTAP) in MATLAB.

2.2.13. Flow-cell biofilm formation analysis

The biofilm assay was done in collaboration with Prof. Yang Liang's Lab, Singapore (1). The mini-Tn7-eGFP-Gmr cassette was inserted into *P. aeruginosa* strains to tag them with green fluorescent protein (GFP). Biofilms were grown in flow chambers with

individual channel dimensions of 1 mm × 4 mm × 40 mm. The flow chambers were inoculated by injecting 350 µl of overnight culture diluted to an OD₆₀₀ of 0.01 into each flow channel using a small syringe. After inoculation, the flow channels were left without flow for 1 h, after which medium flow was started using a Watson Marlow 205S peristaltic pump. The mean flow velocity in the flow chambers was 0.2 mm s⁻¹. All microscopic observations and image acquisitions were done with a Zeiss LSM780 confocal laser scanning microscope (CLSM). Data were analyzed to generate the simulated 3D images using the IMARIS software package (Bitplane AG) (139). The same software was used to quantify the biomass or biovolume.

2.2.14. Statistical analysis

The 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.

2.3. Results

2.3.1. PA4608 interacts with the chemotaxis methyltransferase CheR1

Emerging evidences suggest that the single-domain PilZ proteins function as adaptor proteins to enable cyclic di-GMP to control its protein targets indirectly (118,119,140). So we hypothesized that, as a single-domain PilZ protein, PA4608 works as a functional adaptor protein after interacting with some other proteins. Based on this, we carried out a bacterial two hybrid screening assay using PA4608 as bait protein to probe the potential binding partners in the built genomic DNA libraries in *P. aeruginosa* PAO1 (69). The libraries were prepared using the original and two engineered pTRG vectors to maximize the coverage of the open reading frames (ORFs) in genome. Despite these false positives containing shifted ORFs, most colonies from screening results encoded parts of the gene *PA3348*, encoding a chemotaxis methyltransferase in *P. aeruginosa* PAO1 (www.pseudomonas.com; (129)). Then we performed the specific two hybrid assay with the full length PA3348 (Figure 2.1A). The robust growth of colonies on selective medium plate compared to controls also suggested the interaction between PA4608 and PA3348. PA3348, known as CheR1, contains a conserved CheR-type MCP

methyltransferase domain for S-adenosyl-L-methionine (SAM) binding (Figure 2.1B). Here we renamed PA4608 as MapZ, standing for methyltransferase-associated PilZ protein.

2.3.2. Cyclic di-GMP enhances the interaction between MapZ and CheR1

To validate the interaction between MapZ and CheR1 in the presence of cyclic di-GMP, we performed the co-immunoprecipitation (Co-IP) assay using cell lysate containing expressed proteins. In this assay, the N-terminal 6×His-tagged CheR1 and N-terminal HA-tagged MapZ were expressed in *P. aeruginosa* PAO1 cells. The results showed that CheR1 was co-immunoprecipitated with MapZ as shown in Figure 2.1C. This further supported the protein-protein interaction. In addition, the interaction was enhanced specifically by cyclic di-GMP, but not other nucleotides including GMP, cGMP or cyclic di-AMP, and this effect was dose-dependent within 0-50 μ M, which was consistent with the binding affinity of MapZ for cyclic di-GMP measured by ITC (K_d of $8.8 \pm 1.2 \mu$ M) (Figure S1) and fluorescence resonance energy transfer (FRET) titration (K_d of $6.1 \pm 0.5 \mu$ M) (141).

Figure 2.1 Identification of CheR1 as the binding partner of PA4608 (MapZ). (A) Bacterial two hybrid assay of PA4608 and CheR1. pTRG- Δ N12 encoded PA3348 lacking the N-terminal 12 residues and LGF2-Gallp pair was positive control. This assay was done in collaboration with Prof. Xu Linghui's lab, China (1). (B) Domain organization of CheR1. (C) Co-IP results using cell lysate of PAO1 harbouring either

pUCP18-6×His-CheR1 (lane 1) or pUCP18-HA-MapZ/6×His-CheR1 (lane 2). The 6×His-CheR1 and HA-MapZ were immunoprecipitated by anti-HA beads and detected by antibodies specific for 6×His and HA tags. (D) Co-IP results in the presence of GMP, cGMP, cyclic di-AMP and cyclic di-GMP. Data in figures are representative of two independent experiments.

2.3.3. Cyclic di-GMP binding MapZ inhibits the CheR1 catalyzed methylation of the chemoreceptor PctA

Based on the validated protein interaction, we hypothesized that MapZ, binding to cyclic di-GMP, could be involved in the CheR1 catalyzed methylation of chemotaxis receptors. Previous studies have suggested that CheR1 methylates one chemoreceptor PctA, which responds to amino acids in *P. aeruginosa* PAO1 (129). Here we performed an *in vitro* methylation assay to study the role of MapZ and cyclic di-GMP on CheR1 catalyzed methylation of PctA. The PctA containing membrane fractions were incubated with CheR1, MapZ as well as nucleotides in the presence of substrate, [³H]-Ado-Met. We found that CheR1 methylated PctA as shown in Figure 2.2A, lane 2. Furthermore, addition of cyclic di-GMP could inhibit this methylation in a dose-dependent manner. The methylation was under detectable level in this method at cyclic di-GMP concentration of 10 μM (Figure 2.2A, lane 4-8). Meanwhile, the addition of GMP, cGMP and cyclic di-AMP could not inhibit the methylation as cyclic di-GMP, suggesting that the effect of cyclic di-GMP was specific (Figure 2.2B). In addition, we also prepared a MapZ^{R13A} mutant, which is incapable of binding to cyclic di-GMP according to the NMR structure of MapZ with dimeric cyclic di-GMP (Figure 2.2C; Figure S1). Applying MapZ^{R13A} to the methylation assay, we found that cyclic di-GMP could not inhibit CheR1 catalyzed methylation of PctA (Figure 2.2D).

The methylation assay results suggested that MapZ could inhibit the methylation function of CheR1 to chemoreceptor PctA, in which cyclic di-GMP binding was essential. This further implied that cyclic di-GMP could potentially regulate PctA and CheR1 involved chemotaxis pathway through its adaptor protein MapZ by regulating methylation level of chemoreceptor.

2.3.4. MapZ is localized at cell poles, depending on the presence of cyclic di-GMP, CheR1 and PctA

PctA was reported to be localized to cell poles in *P. aeruginosa* with other chemotactic proteins (142). Here we supposed that if MapZ could interact with CheR1 *in vivo*, it may localize to the cell poles near chemosensory complex as well. To investigate the cellular localization of MapZ, we tagged MapZ with YFP tag for imaging. We expressed the YFP-MapZ in PA14 instead of PAO1 because of the potential loss of biosensor encoding plasmid (143).

Figure 2.2 Effect of MapZ and cyclic di-GMP on the CheR1-catalyzed methylation of the chemoreceptor PctA. (A) Methylation assay results using [³H]-Ado-Met and membrane fractions containing PctA as substrates, in the presence of increasing concentrations of cyclic di-GMP. (B) Methylation assay results in the presence of GMP, cGMP, cyclic di-AMP and cyclic di-GMP. (C) Solution NMR structure of MapZ binding to dimeric cyclic di-GMP (PDB code: 2L74). The conserved residue Arg13 for cyclic di-GMP binding was shown in sticks and the N-terminal loop was shown in magenta. (D) Methylation assay results using MapZ^{R13A} instead. Data in figures are representative of two independent experiments.

The superresolution microscopy, 3D-SIM, was used for imaging to gain higher lateral and axial resolution. As shown in Figure 2.3A, YFP-MapZ showed a bipolar distribution in PA14 cells. Instead, distribution along cell membrane or in cytoplasm was observed in the *cheR1::Tn* and *pctA::Tn* cells respectively, suggesting that CheR1 and PctA were both critical for the localization of MapZ. Furthermore, the polar localization of MapZ also disappeared using YFP-MapZ^{R13A} instead (Figure 2.3B), indicating that cyclic di-GMP was essential for polar distribution of MapZ as well.

We also performed the protein cellular localization experiment using an immunofluorescence (IF) imaging method with HA tagged MapZ to exclude the potential effect of large YFP tag. From the IF images we also observed the bipolar localization of MapZ. In addition, the fluorescence signals dispersed in the whole cell instead of locating at cell poles in the *cheR1::Tn* cells.

In conclusion, the cellular localization experiment results suggested that CheR1, PctA and cyclic di-GMP were all essential for the polar localization of MapZ, further supporting the *in vivo* CheR1-MapZ interaction. It also indicated that MapZ could be likely involved in the chemotaxis signaling pathway colocalizing with other chemotactic protein.

Figure 2.3 Cellular localization of MapZ in wild type and mutant cells. (A) Cellular localization of YFP-MapZ in *P. aeruginosa* PA14 cells by super-resolution 3D-SIM fluorescence microscopy (Scale bars, 1 μm). (B) Cellular localization of YFP-MapZ^{R13A} in *P. aeruginosa* PA14 cells by super-resolution 3D-SIM fluorescence microscopy (Scale bars, 1 μm). This assay was done in collaboration with Prof. Yang Liang's lab, Singapore (1). (C) Cellular localization of HA-MapZ in *P. aeruginosa* PA14 cells by immunofluorescence (IF) imaging. Data in figures are representative of two independent experiments. This assay was done in collaboration with Prof. Xu Linghui's lab, China (1).

2.3.5. MapZ decreases the frequency of swimming direction reversal

Since results above implied that MapZ may be involved in the chemotactic pathway, we carried out the agarose plug chemotaxis assay to examine this and results were shown in Figure S2. PAO1 and Δ *mapZ* cells were observed to gather around agarose plug containing chemoattractant of L-serine, sensed by PctA (144), indicating the normal chemotaxis activity. In contrast, the Δ *cheR1* cells did not form a visible zone around the

agarose plug, suggesting that most mutant cells have lost their chemotaxis response to chemoattractant. For the *mapZ*⁺ mutant, which is PAO1 harbouring a plasmid overexpressing *mapZ*, most cells were not chemotactic for the low cell density around agarose like *ΔcheR1*, indicating the loss of chemotaxis swimming ability. Interestingly, the *mapZ*⁺ cells also tended to form clumps and the insoluble aggregation were also observed in the liquid culture.

P. aeruginosa cells navigate stochastically by alternating periods of forward runs, backward runs and turns (145). Agarose plug chemotaxis assays could mask the real motility due to the heterogeneity of cell population. To further investigate how MapZ affect bacterial chemotaxis swimming motility, we studied the swimming trajectory of individual cells analysed by microscopy. Most PAO1 cells made runs ending in a sharp turn or reversal and ran in a different direction subsequently (Figure 2.4A). Reversal is often followed by a change of swimming direction with acute turn angle. This “run-reverse-turn” pattern was in agreement with the swimming behaviour of *Pseudomonas* species reported before, with the sharp turn or reversal caused by a change in the flagellar rotational direction and turns associated with diffusional cell realignment (Figure 2.4B) (129,146). In contrast to PAO1 cells, *ΔcheR1* cells only swam in a single direction and lacked the ability to reverse. Similar swimming pattern was also observed for the majority of *mapZ*⁺ cells. Meanwhile, the swimming trajectories of *ΔmapZ* and *mapZ*^{R13A} cells were notably heterogeneous, with some cells exhibiting similar swimming behaviour to PAO1 with runs and reversals, but with others losing the ability to reverse. Apart from the differences in direction switching, the mutants did not exhibit significant differences in swimming velocities (Figure 2.4C). In addition, the persistence of run, which correlated with the frequency of direction reversal, showed that *mapZ*⁺ and *ΔcheR1* cells had longer runs than PAO1, *ΔmapZ* and *mapZ*^{R13A} (Figure 2.4D), which was consistent with the swimming trajectories. The swimming trajectories and analysis among strains suggested that MapZ regulated swimming motility mainly by affecting the direction reversal frequency. Because frequent changes in swimming direction are a hallmark of cells that undergo chemotaxis dependent random walk (147), the observations suggest that MapZ could be an important motility regulator that contributes to such the random walk swimming pattern.

2.3.6. MapZ controls flagellar output by suppressing motor switching

Considering that monotrichous *P. aeruginosa* cells reverse swimming direction by switching the direction of flagellar rotation, we next studied whether MapZ controlled flagellar motor switching. To directly observe flagellar rotation, we performed a cell tethering assay (145) in which single bacterium is attached to a coverslip using a flagellar protein-specific antibody (Figure 2.5A).

Figure 2.4 Effect of MapZ on the frequency of direction reversal in swimming. (A) Swimming trajectories of *P. aeruginosa* PAO1 wild type and mutant cells. Data in figures are representative of two independent experiments. (B) Illustration of the "run-reverse-turn" swimming mechanism used by *P. aeruginosa* cells. (C) Average swimming speed of PAO1 wild type and mutant cells. (D) Swimming persistence of PAO1 wild type and mutant cells. Data are means $\pm$ SD ($n = 30$ to 36 cells) from two independent experiments (two-tailed t test, $*P < 0.05$). This assay was done in collaboration with Prof. Chiam Keng-Hwee's lab, Singapore (1).

The rotation of cell body was recorded using microscope to measure the rotational speed and the frequency of switching between the clockwise (CW) rotation, counter clockwise (CCW) rotation and pause phases (145,148). We found that the differences of flagellar rotational speed among PAO1 and mutants were not significant (Figure 2.5B), suggesting that MapZ did not affect flagellar rotational speed. This is in agreement with the similar swimming velocities observed for the strains (Figure 2.4C). All five strains paused intermittently and the duration of the pausing phase (less than 1 s) did not show significant differences either (Figure 2.5C). Because the duration of the pause determines the turning angle of *P. aeruginosa* cells in diffusion-driven rotation (145), this observation suggested that MapZ does not control swimming motility by affecting the diffusional rotation based direction change. A large percentage of $\Delta cheR1$ and $mapZ^+$ cells spent considerably greater amount of time in the CCW rotation mode (Figure 2.5C), which was consistent with the longer runs in their swimming trajectories.

Further analysis of the switching frequencies by comparing the amount of time the cells undergo CW and CCW rotation revealed notable differences among the strains. PAO1 cells switched direction frequently and spent about equal amount of time in CW and CCW rotation (Figure 2.5D). In contrast, the $\Delta cheR1$ cells underwent either CW (42%) or CCW (58%) rotation without switching the rotation direction. This finding was consistent with the unidirectional swimming trajectory of the $\Delta cheR1$ cells we (Figure 2.4A) and others (129) observed. Meanwhile, $\Delta mapZ$ cells were heterogeneous in rotational switching, with 18% of the cells rotating in single direction and 82% of the cells switching direction stochastically. The $mapZ^+$ cells also exhibited heterogeneity, but unlike the $\Delta mapZ$ cells, the majority of $mapZ^+$ cells (62%) rotated in one direction and 38% of the cells switched direction occasionally. The single chromosomal mutation ($mapZ^{R13A}$) that abolished cyclic di-GMP binding of MapZ also exhibited heterogeneity in direction switching similar to that of $\Delta mapZ$, strongly suggesting that cyclic di-GMP binding was essential for maintaining the unbiased flagellar motor switching.

To further study how MapZ affected flagellar rotation in a chemoattractant gradient, we also performed time-dependent cell tethering assay in an L-serine gradient. We found that the addition of L-serine to PAO1 cells triggered a rotational bias toward CW or CCW rotation in the first 20 seconds (Figure 2.5E). Such rotational bias was agreement with the longer and persistent runs needed to deviate from a random walk behaviour. The rotational bias almost disappeared by the end of 60s with the dissipation of the

Figure 2.5 Effect of MapZ on flagellar motor switching in cell tethering assay. (A) Schematic illustration of cell tethering assay. Flagellum was tethered by antibodies and cell body rotation was monitored using microscope. (B) Average flagellar rotational speed of PAO1 wild type and mutant cells. Data are means $\pm$ SD (n = 19 to 33 cells) from two independent experiments (two-tailed *t* test, *P* > 0.05 in all cases). (C) Average

duration time of flagellar rotation in CW, CCW or pause of PAO1 wild type and mutant cells. Data are means $\pm$ SD (n = 19 to 33 cells) from two independent experiments. Differences of the pause duration among strains are not statistically significant (two-tailed t test, $P > 0.05$ in all cases). Differences of the CCW duration among wild type, *ΔcheR1* and *mapZ*⁺ mutants were statistically significant (two-tailed t test, * $P < 0.05$). (D) Bar graphs showing the percentage of time spent in CW rotation of PAO1 wild type and mutant cells. The percentage of cells exhibiting bidirectional rotation (CW and CCW) and unidirectional rotation (CW or CCW) was summarized below bar graphs. Data are means $\pm$ SD (n = 19 to 33 cells) from two independent experiments. (E) Bar graphs showing flagellar rotation during chemotactic response to L-serine within 60 seconds. Data are means $\pm$ SD (n = 30 to 50 cells) from two independent experiments. This assay was done in collaboration with Prof. Chiam Keng-Hwee's lab, Singapore (1).

attractant. Majority of the *ΔmapZ* and *mapZ*^{R13A} cells already exhibited rotational bias before adding L-serine, and the addition of L-serine induced even greater rotational bias in the first 20s, with about one third of the cells undergoing single directional rotation only. Meanwhile, the serine gradient had little effect on the rotation switching of *mapZ*⁺ cells because of the strong existing rotational bias before the addition of chemoattractant.

Together, the cell tethering assay results suggested that cyclic di-GMP and MapZ were likely to contribute to chemotaxis and bacterial motility by controlling the frequency of flagellar motor switching.

2.3.7. MapZ promotes irreversible surface attachment and biofilm formation

Bacterial motility and chemotaxis are critical for biofilm formation in *P. aeruginosa* (129,149,150). Previous study has suggested that CheR1 affected cell attachment in biofilm formation (129). As potential regulator of CheR1, we studied flow cell biofilm formation of *P. aeruginosa* PAO1 and mutant cells to investigate role of MapZ in biofilm formation (151). PAO1 cells formed typical mushroom shaped biofilm in flow cells after the five days growth, whereas the *ΔmapZ* mutant formed a fragile and thin biofilm with substantially reduced biomass (Figure 2.6). The *ΔmapZ* strain also seemed to be less efficient in surface attachment, as suggested by the lower surface coverage at the end of day three (Figure 2.6). In contrast, the *mapZ*⁺ strain exhibited enhanced surface attachment at day three and formed a much thicker biofilm at day five. Neither deletion nor overexpression of *mapZ* seemed to noticeably affect the formation of the mushroom structures, which suggested that MapZ regulated biofilm formation mainly at the surface attachment stage but not maturation.

Figure 2.6 Effect of MapZ on surface attachment and early stage of biofilm formation. (A) Flow cells biofilm images of PAO1 wild type and mutant cells monitored by confocal microscope at the end of day 1, 3 and 5 (Scale bars, 20 μm). (B) Quantification of the biovolume or biomass using Imaris 8.2.0 at the end of day 1, 3 and 5. For each strain, data (means $\pm$ SD) were calculated from the amalgamated data obtained from four biofilm growth channels (two-tailed t test, * $P < 0.05$, ** $P < 0.001$). Data in figures are representative of two independent experiments. This assay was done in collaboration with Prof. Yang Liang's lab, Singapore (1).

2.4. Discussion

As the main family of cyclic di-GMP receptors, unveiling functions of PilZ domain proteins is essential for understanding cyclic di-GMP regulation in *P. aeruginosa* and many other bacterial species. Our study suggested that the *P. aeruginosa* PilZ domain protein MapZ could regulate bacterial motility by directly interacting with a methyltransferase CheR1. The dependence on cyclic di-GMP in the MapZ-CheR1 interaction indicated that cyclic di-GMP could control the motility of *P. aeruginosa* by modulating the methylation of chemoreceptors. Our results further revealed that cyclic

di-GMP and MapZ regulated bacterial motility by controlling the switching frequency, but not the speed, of flagellar rotation. The mechanism here supported by our study is distinct from currently known cyclic di-GMP dependent regulatory mechanisms of controlling bacterial motility. Moreover, our results showed that the inhibition of CheR1 by cyclic di-GMP binding MapZ correlated with enhanced surface attachment, which suggested a mechanism by which cyclic di-GMP regulated the early stage of biofilm formation.

Although a common bacterial behaviour regulated by cyclic di-GMP is flagellum-dependent motility, the mechanism by which cyclic di-GMP controls flagellar output in *P. aeruginosa* remains to be fully understood. One characterized mechanism involves FleQ, a cyclic di-GMP responsive transcriptional regulator in *P. aeruginosa*. FleQ activates the expression of flagellar biosynthetic genes and regulates bacterial motility. It can also suppress the transcription of exopolysaccharide genes required for biofilm formation and this repression can be relieved by cyclic di-GMP (48,152). In *P. aeruginosa*, cyclic di-GMP suppressed swarming motility through a di-domain PilZ protein FlgZ by binding to the MotCD (24). FlgZ is also found to regulate swimming motility and biofilm formation in other *Pseudomonas* species (124). Apart from *P. aeruginosa*, cyclic di-GMP controls flagellar function in several other bacterial species. The best-known mechanism is the YcgR-dependent “backstop brake” mechanism in *E. coli*, whereby the binding of cyclic di-GMP to YcgR promotes the binding of YcgR to flagellar switching complex to reduce swimming motility (71,91,153). A similar mechanism operates in *Caulobacter crescentus* with increased concentrations of cyclic di-GMP promoting the interaction between the cyclic di-GMP binding DgrA and the motor protein FliL to block motility (80). In addition to the mechanism that involves the use of a motor brake, cyclic di-GMP also regulates flagellum-dependent motility through chemotactic pathways. In *Azospirillum brasilense*, cyclic di-GMP binds directly to the chemoreceptor Tlp1 through the C-terminal PilZ domain to promote persistent motility (90). In *Borrelia burgdorferi*, cyclic di-GMP binds to the PilZ protein PlzA to regulate the flagellum-dependent flexing motility, putatively by interacting with an unidentified chemotactic protein (154). The MapZ-dependent mechanism in *P. aeruginosa* in this project is different from all these mechanisms discussed above, as featured by the involvement of a trans-acting PilZ binding adaptor and the regulation of the methylation of chemoreceptors.

In *P. aeruginosa* PAO1, there are four chemotaxis gene clusters and a total of 26 chemoreceptor genes in the genome. Two of the four gene clusters (Che and Che2) are involved in the regulation of chemotaxis and flagellum-dependent motility (155,156). The Che system essential for chemotaxis includes homologs of the *E. coli* chemotactic proteins CheA, CheB, CheW, CheY and CheZ. Although *pctA* and *cheR1* are not localized within the *che* gene cluster, PctA and CheR1 are believed to function as part of the Che chemotactic system (157). The Che2 system consists of two chemoreceptors (McpA and McpB), CheA2, CheB2, CheW2, CheY2, CheR2, CheD and plays a role in chemotaxis only when the cells enter stationary growth (157,158). For *E. coli*, a cell reorients in a concentration gradient by resetting the CheA autophosphorylation level after each linear run (159). Such an “adaptation mechanism” relies on the reversible methylation of chemoreceptors catalyzed by the methyltransferase CheR and methylesterase CheB. Methylation of the chemoreceptors decreases the affinity of the receptor for attractants and stimulates the auto-kinase activity of CheA to phosphorylate CheY. In general, while the methylesterase CheB is regulated by CheA through phosphorylation, the methyltransferase CheR is believed to be constitutively active and its activity is not regulated. In *P. aeruginosa* and some other *Pseudomonas* species, CheR1 is essential for flagellum mediated chemotaxis because of its role in methylating chemoreceptors (129,160,161). Our results suggest that CheR1, and the methylation status of its cognate chemoreceptor, can be regulated by cyclic di-GMP through the adaptor protein MapZ. A relatively simple chemotactic system, such as the one in *E. coli*, may be sufficient for the cells to navigate in a chemoattractant gradient. It is conceivable that a bacterium with more complex lifestyles such as *P. aeruginosa* could benefit from having a cyclic di-GMP regulatory mechanism integrated into the chemotactic system to fine-tune flagellar output. Moreover, the polar localization of MapZ, for which CheR1, PctA and cyclic di-GMP are essential, further supports the regulation of MapZ in the chemotaxis signaling pathway. Although the overexpressed YFP-labelled MapZ and MapZ^{R13A} achieved localization under super-resolution microscopy, we still believe that with the help of fast developed microscopy technology, more detailed cellular localization information of MapZ and related chemosensory proteins could be obtained using the genomic labelled proteins.

Unlike *E. coli* cells that swim using a “run-tumble-run” mechanism (162), monotrichous *Pseudomonas* cells follow a “run-reverse-turn” mechanism (145). *P. aeruginosa* cells

move forward (“run”) or backward (“reversal”) when the flagellum undergoes CCW or CW rotation. In addition to reversal, the cells can also change swimming direction when flagellum pauses to allow the cell body to undergo diffusional reorientation (“turn”). The frequency of flagellar motor switching determines the duration or persistence of the runs. Suppression of motor switching generates more persistent runs, which is crucial for cells to shift from random walk to directional swimming. Our cell tethering assays showed that the flagellar motor of PAO1 has a high switching frequency in the absence of chemo-attractant and the levels of MapZ/cyclic di-GMP can substantially impact the switching frequency. Our data supports a model whereby cyclic di-GMP promotes the formation of the CheR1/MapZ complex near the chemoreceptor array at the pole (Figure 2.7A). When triggered by environmental cues, a rise of cyclic di-GMP concentration promotes the formation of CheR1/MapZ complex to inhibit the methyltransferase activity of CheR1. Inhibition of CheR1 reduces the methylation of PctA to suppress the auto-kinase activity of CheA. The immediate effect of suppressing the auto-kinase activity of CheA is unclear as the signaling proteins downstream of CheA are not yet known. In *E. coli*, low CheA activity results in an increase in the number of unphosphorylated CheY molecules; and the dissociation of unphosphorylated CheY from the flagellar motor induces a CW to CCW motor switching. In *P. aeruginosa*, the role of CheY in motor switching is most likely to be different as the knockout of *cheY* did not prevent the flagellum from rotating in both directions (145). We propose that the decreased auto-kinase activity of CheA at high cyclic di-GMP concentration leads to the dephosphorylation of another protein (protein “X” in Figure 2.7A). We further postulate that the dephosphorylated X has lower affinity for flagellar motor proteins, and that dissociation of dephosphorylated X from flagellar motor prevents motor switching. A lower frequency of flagellar direction switching causes persistent unidirectional rotation and directional swimming. These results further indicate that the maintenance of a relatively low level of cyclic di-GMP near the chemoreceptor array is important for the random walk behaviour in uniform solution, and that generating a pool of higher cyclic di-GMP concentration will be crucial for effective chemotactic response.

Figure 2.7 A model for the regulation of flagellar motor switching by cyclic di-GMP and MapZ. (A) In cells with low cyclic di-GMP cellular concentration, CheR1 methylates the chemotaxis receptor MCP to stimulate the auto-kinase activity of CheA and increase the phosphorylated level of X. Binding of phosphorylated X to the flagellar motor enables the flagellum switching between CW and CCW. In cells with high cyclic di-GMP cellular concentration, MapZ binds to CheR1 to block the methylation of PctA to lower auto-kinase activity of CheA. This decreases the population of phosphorylated X and impedes flagellar motor switching. “P” stands for phosphoryl group and “M” stands for methyl group. (B) Time courses of chemotactic responses with low and high cellular cyclic di-GMP concentration. Addition of chemoattractant results in a fast decrease in CheA kinase activity which is followed by a slow CheR1 dependent adaptation mechanism. The heterogeneity in cellular cyclic di-GMP concentrations gives rise to a range of chemotactic responses.

Heterogeneity in cellular cyclic di-GMP concentration was proposed to be required for generating a diverse range of motility usually observed in a *P. aeruginosa* cell population (143). The diversity in bacterial motility may be crucial for the success and survival of *P. aeruginosa* as it allows the cell population to survey the surroundings more efficiently. The cyclic di-GMP effector responsible for generating such diversity or heterogeneity in bacterial motility was unknown. The current study indicates that the cyclic di-GMP binding MapZ could be crucial for generating heterogeneity in bacterial motility given the great heterogeneity in swimming trajectory and flagellar direction-switching exhibited by MapZ-deficient cells. As illustrated by the two hypothetical chemotactic response curves (Figure 2.7B), cyclic di-GMP concentration influences MCP methylation and adaptation time; namely, the time required to restore the CheA auto-kinase activity to pre-stimulus amounts. As a result, heterogeneity in cellular cyclic di-GMP concentration can give rise to a wide range of chemotactic responses, with cells with high cyclic di-GMP concentration exhibiting less frequent direction switching and more persistent runs than the cells with lower cyclic di-GMP concentration. As cyclic di-GMP networks usually include many sensory domain containing proteins, incorporation of a cyclic di-GMP binding effector in the chemotactic machinery would allow the sensing of additional environmental or metabolic inputs for fine-tuning flagellar output and generating heterogeneity. Bacterial species differ in the number of signaling proteins and complexity of signaling network used for sensing environmental stimuli (163). Given the advantage of having such a cyclic di-GMP dependent system embedded in the chemotactic system to fine-tune flagellar output, a similar mechanism may also be used in other non-*Pseudomonas* species to modulate chemoreceptor methylation and bacterial motility.

CHAPTER 3. A specific set of PDEs control the cyclic di-GMP pool sensed by MapZ

3.1. Introduction

As monotrichous bacteria, *P. aeruginosa* can swim in liquid and swarm across solid surfaces using its single polar flagellum. The flagellum-dependent motility of *P. aeruginosa* plays an active role in establishing acute and chronic infections. Flagellum-dependent swimming motility enables *P. aeruginosa* to rapidly disseminate from the initial infection site of burn patients to cause the potentially life-threatening systemic infections (164). The flagellum, which enables *P. aeruginosa* cells to navigate through mucus and interact with epithelial membranes, may also be crucial for the establishment of respiratory tract infection (165,166). Moreover, formation of surface-associated biofilm is a hallmark of chronic infection involving *P. aeruginosa* (167). A temporal control of flagellum-dependent motility is crucial for establishing robust *P. aeruginosa* biofilm microcolonies on surfaces (149,168,169). There is a correlation between the loss of flagellum-dependent motility and the persistence of *P. aeruginosa* in chronically infected patients, as indicated by the observation that many *P. aeruginosa* strains isolated from the airway of cystic fibrosis patients lack flagella. Lastly, phagocytosis is one of the primary ways by which the host controls *P. aeruginosa* and other bacterium-induced pneumonia during lung infection. Flagellar motility can be recognized by phagocytes to start engulfment, and be considered as a key pathogen-associated molecular pattern (170). Loss of motility in chronic *P. aeruginosa* infections may enable *P. aeruginosa* to evade assimilation and ingestion by phagocytes both *in vitro* and *in vivo*.

In the last decade, the second messenger cyclic di-GMP has emerged as a prominent regulator of flagellum-dependent motility. It is increasingly clear that *P. aeruginosa* has evolved different cyclic di-GMP dependent mechanisms and signaling pathways to regulate flagellum-dependent motility. In *P. aeruginosa*, there are 17 GGDEF domain proteins, five EAL domain proteins, three HD-GYP domain protein and 16 proteins containing both GGDEF and EAL domains (19,171). Some were suggested to regulate flagellum-dependent swimming and swarming motility (25,123,172,173). In addition, a few cyclic-di-GMP binding effectors that mediate flagellar function on either

transcriptional or post-translational levels were identified. The cyclic di-GMP binding transcriptional regulator FleQ allows cyclic di-GMP to regulate the biosynthesis of flagellar motor complex (48,152). On the protein level, the cyclic di-GMP binding FlgZ interacts with the stator protein MotC to suppresses swarming motility at high cyclic di-GMP levels, putatively by affecting the torque output of the flagellar motor (174). And our study also indicates that the PilZ adaptor protein MapZ interacts with the chemotaxis methyltransferase CheR1 at elevated cyclic di-GMP levels to suppress flagellar motor switching. One challenge in current field is to identify the DGCs, PDEs and effectors constituting the seemingly insulated cyclic di-GMP signaling pathways to understand the molecular mechanism of signaling specificity.

Although our previous studies already implicated cyclic di-GMP in the regulation of flagellar motor switching in *P. aeruginosa*, it has not been demonstrated directly how changes of cellular cyclic di-GMP concentration impact flagellar motor switching, and which cyclic di-GMP metabolizing enzymes are involved in the regulation of flagellar motor switching. In this study, we demonstrate that elevated cellular cyclic di-GMP concentration suppresses flagellar motor switching through the MapZ adaptor protein. Our study further reveals that MapZ responds to a pool of cyclic di-GMP governed by a specific set of PDEs, including three membrane-anchored PDEs. In summary, the results suggest that *P. aeruginosa* has evolved a group of cyclic di-GMP metabolizing enzymes and binding effector to specifically control flagellar motor switching.

3.2. Material and methods

3.2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains, plasmids and growth conditions were used as described in chapter 2.2.1.

3.2.2. Preparation of the *P. aeruginosa rbdA*, *nbdA* and *dipA* mutants

The *P. aeruginosa rbdA*, *nbdA* and *dipA* mutants were prepared as described in chapter 2.2.2 with modification. Briefly, DNA fragments of 1,518 bp containing the *rbdA-E585A&L586A&L587A* gene, 1,593 bp containing the *nbdA-E551A&L553A* gene and 1,455 bp containing the *dipA-E675A&L677A* gene were amplified from *P. aeruginosa* PAO1 genomic DNA by overlap extension PCR. The PCR products were cloned into

the vector pK18GT and the suicide plasmids were transformed into *E. coli* DH5 α for the subsequent conjugation with PAO1 or *mapZ*^{R13A} by triparental mating with *E. coli* containing helper plasmid pRK600. Colonies containing the correct mutation were identified by sequencing of the DNA fragment amplified using primers listed in Table S3.

3.2.3. Cell tethering assay for the analysis of the flagellar rotation

The cell tethering assay was performed as described in chapter 2.2.12.

3.2.4. Swimming trajectory analysis

The swimming trajectory analysis was performed as described in chapter 2.2.11.

3.2.5. Statistical analysis

The statistical analysis was done as described in chapter 2.2.11.

3.2.6. Cellular c-di-GMP level measurement using HPLC

The overall c-di-GMP concentrations in mPAO1 and mutant strains were measured as described before with modification (175-177). Briefly, overnight cultures were diluted in LB medium and grown to OD₆₀₀ of 1.0. 10 ml of cultures were obtained and washed twice by ice-cold PBS. Cells were resuspended in 500 μ l ice-cold PBS and incubated at 100 °C for 5 min. Ice-cold 100% ethanol was added to a final concentration of 65% and supernatant was removed after centrifugation. Extraction was repeated twice for each sample and supernatant was dried and resuspended in 100 μ l water. 80 μ l of each sample was analyzed by high performance liquid chromatography (HPLC) at 253 nm using a reverse-phase C₁₈ Targa column (4.6 $\times$ 150 mm; 5 μ m) and a flow rate of 1.0 ml/min in solvents (Solvent A: 10 mM ammonium acetate in water; Solvent B: 10 mM ammonium acetate in methanol). The following gradient was used to elute c-di-GMP within 10 min: 1% Solvent B and 99% Solvent A. Standard curve was prepared using c-di-GMP purchased from Sigma-Aldrich. For protein quantification, 1 ml of cultures were pelleted and resuspended in 1 ml of 0.1 M NaOH by heating for 15 min at 95 °C and subsequently performing standard Bradford assay (Biorad). Measurements were repeated in triplicates and final c-di-GMP levels were normalized to total cellular protein levels.

3.2.7. C-di-GMP level measurement in reporter strains

The overall c-di-GMP concentration in mPAO1 and mutants strains were measured as described before with modification (178-180). Briefly, the plasmid pCdrA::gfp encoding the fluorescence-based reporter for c-di-GMP was transformed into mPAO1 and transposon mutant strains by electroporation. Overnight cultures were diluted to OD₆₀₀ of 0.01 in LB medium and 200 µl of diluted cultures were transferred into 96-well plate. A TECAN Infinite 200 PRO plate reader was used to measure c-di-GMP concentration in planktonic cells based on *cdrA::gfp* fluorescence (excitation 485 nm, emission 530 nm, excitation bandwidth 9 nm and emission bandwidth 20 nm; manual gain of 80), and growth was monitored by quantifying the absorbance at OD₆₀₀. Measurements were recorded at 15 min intervals. The relative fluorescence of GFP at OD₆₀₀ of 1.0 was used as a measure of c-di-GMP level per cell.

3.3. Results

3.3.1. High cellular cyclic di-GMP level suppresses flagellar motor switching and rotational speed

To study the effect of high cyclic di-GMP level on flagellum-dependent motility regulation, we employ the cell tethered assay using *P. aeruginosa* PAO1 strain with an overexpression plasmid carrying a gene encoding the highly active DGC protein YedQ. Overexpression of *yedQ* artificially raised the cellular cyclic di-GMP concentration by 21-fold using the well-established HPLC method (Figure 3.1A). Our cell tethered assays showed that, while the polar flagellum of PAO1 cells undergoes bi-directional rotation that is characterized by frequent directional switching of the flagellar motor, the flagellum of most PAO1/pYedQ cells exhibited either clockwise (CW) or counter-clockwise (CCW) unidirectional rotation (Figure 3.1B). In addition, the motor speed is significantly lower for the PAO1/pYedQ cells comparing to PAO1 cells (Figure 3.1C). The observations suggest that elevated global cyclic di-GMP level in *P. aeruginosa* suppresses both switching frequency and speed of flagellar rotation.

Figure 3.1 Effect of high cellular cyclic di-GMP level on flagellar motor switching and rotational speed. (A) Relative overall c-di-GMP concentration normalized to total protein level of PAO1, PAO1/pYedQ and *mapZ*^{R13A}/pYedQ cells measured by HPLC. Data are carried out in triplicates. (B) Bar graphs showing the percentage time spent in CW rotation of PAO1, PAO1/pYedQ and *mapZ*^{R13A}/pYedQ cells. (C) Average flagellar rotational speed of PAO1, PAO1/pYedQ and *mapZ*^{R13A}/pYedQ cells. Data (n = 12 to 37 cells) are representative of two independent experiments (two-tailed *t* test, **P* < 0.05). This cell tethering assay was done in collaboration with Prof. Chiam Keng-Hwee's lab, Singapore.

3.3.2. Cyclic di-GMP suppresses flagellar motor switching through the adaptor protein MapZ

As discussed in Chapter 2, we have discovered that the cyclic di-GMP binding PilZ protein MapZ suppresses flagellar motor switching by inhibiting the chemotaxis methyltransferase CheR1. To test whether high cellular cyclic di-GMP concentrations suppress flagellar motor switching through MapZ, we prepared the mutant strain *mapZ*^{R13A}/pYedQ with high cyclic di-GMP level compared to wild type (Figure 3.1A) by overexpressing YedQ in the *mapZ*^{R13A} mutant strain in which the expressed

MapZ^{R13A} mutant protein loses the ability to bind cyclic di-GMP. In contrast to the suppressed motor switching observed for the PAO1/pYedQ strain, the mutant strain *mapZ^{R13A}/pYedQ* exhibited bidirectional flagellar motoring switching that is similar to PAO1 (Figure 3.1B). This suggests that the effect of high cellular cyclic di-GMP on flagellar motor switching is exerted through MapZ. Meanwhile, the flagellum of the *mapZ^{R13A}/pYedQ* mutant exhibited comparable rotational speed as the PAO1/pYedQ mutant (Figure 3.1C), suggesting that MapZ is dispensable in the regulation of flagellar motor rotational speed.

3.3.3. Distinct effects of phosphodiesterases (PDEs) on flagellar motor switching

The cellular levels of cyclic di-GMP in *P. aeruginosa* are maintained by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs). It remains unknown which ones of the enzymes are involved in the regulation of flagellar motor switching. In this study, we examined a comprehensive set of *pde* mutant strains to identifying the PDEs that regulate flagellar motor switching. We focus on the *pde* mutants here because we previously found that flagellar motor switching seems to be very sensitive to the increase, but not the decrease, of cellular cyclic di-GMP level, presumably due to the already low cellular cyclic di-GMP concentration in planktonic *P. aeruginosa* cells. Here we monitored the motor switching for the transposon mutant strains (*pde*::Tn) of all the 22 enzymatically active PDEs by cell tethering assay. As shown in Figure 3.2A, most of the *pde* mutants exhibited similar pattern as the PAO1 strain, with the motor switching between CW and CCW rotation. The most notable differences were observed for the mutants, *rbdA*::Tn, *nbdA*::Tn and *dipA*::Tn. The majority of the cells for the three mutant strains underwent unidirectional or highly biased CW/CCW rotation, reflecting very low frequencies of flagellar motor switching. Meanwhile, we measured the flagellar rotational speed for all the *pde* mutants. None of the mutants exhibited significantly reduced flagellar rotational speed (Figure S3B), in contrast to the decreased rotational speed for the *yedQ* overexpression strain. These observations suggest that the PDEs in *P. aeruginosa* are non-equivalent and only a specific subset of the PDEs are involved in the regulation of flagellar motor switching.

Figure 3.2 Effects of phosphodiesterases (PDEs) on flagellar motor switching. (A) Bar graphs showing the percentage time spent in CW rotation of *P. aeruginosa* PAO1 PDE transposon mutant strains in cell tethering assay. Data (n = 21 to 28 cells) are representative of two independent experiments. This cell tethering assay was done in collaboration with Prof. Chiam Keng-Hwee's lab, Singapore. (B) Relative c-di-GMP concentration of PAO1 and mutant strains measured at OD₆₀₀ of 1.0. Data are carried out in triplicates.

To study how the inactivation of the *pde* genes perturbs cellular cyclic di-GMP levels to impact flagellar motor output, we measured the relative cyclic di-GMP levels of *pde::Tn* strains using *cdrA* promoter-fused green fluorescent protein (GFP). We found that most mutant strains did not exhibit significantly higher cyclic di-GMP levels than PAO1 (Figure 3.2B). The strains *rbdA::Tn* and *dipA::Tn* are the only two strains that exhibited significantly high cyclic di-GMP concentration. Interestingly, the *nbdA::Tn* mutant seems to have a similar cellular cyclic di-GMP level as PAO1. Collectively, the results suggest that RbdA and DipA are the two major PDEs in *P. aeruginosa* governing global cyclic di-GMP concentration and motor switching. The observation that deletion of *nbdA* did not perturb global cyclic di-GMP level but still caused a severe suppression in flagellar motor switching suggests that the cyclic di-GMP pool controlling motor switching could also be regulated at a sub-cellular level.

3.3.4. The PDE activities of RbdA, NbdA and DipA are essential for suppressing flagellar motor switching

The three proteins RbdA, NbdA and DipA were reported to regulate bacterial motility and biofilm formation or dispersal (39,181,182). All three proteins contain enzymatically active EAL domains and are likely to control cellular processes mainly by degrading cyclic di-GMP (Figure 3.3A) (183,184). To confirm that the suppression of flagellar motor switching observed for the transposon mutants is indeed due to the abolishment of PDE activity, chromosomal mutations were introduced to replace the conserved and catalytically active E(A/V)L motif of RbdA, NbdA and DipA with an AAA motif. Cell tethering assays showed the PDE defective mutant strains *rbdA*^{PDE*}, *nbdA*^{PDE*}, and *dipA*^{PDE*} also exhibited suppressed flagellar motor switching as their transposon mutants (Figure 3.3B). Hence, the results support that the suppression of motor switching is due to the abolishment of PDE activity.

To find out whether the three PDEs also control flagellar motor switching through MapZ, we introduced another chromosomal mutation into the *mapZ* gene of the *rbdA*^{PDE*}, *nbdA*^{PDE*}, and *dipA*^{PDE*} strains to replace the essential c-di-GMP binding residue Arg13 with Ala. This generated three double mutant strains *rbdA*^{PDE*}&*mapZ*^{R13A}, *nbdA*^{PDE*}&*mapZ*^{R13A} and *dipA*^{PDE*}&*mapZ*^{R13A} that harbour the defective PDE and MapZ lacking c-di-GMP binding ability. Remarkably, cell tethering assays showed that the three double mutant strains exhibited bidirectional flagellar motor switching that is

similar to the PAO1 strain. The results suggest that MapZ is essential for suppression of flagellar motor switching, and that MapZ is a downstream c-di-GMP binding effector for RbdA, NbdA and DipA in *P. aeruginosa*.

Figure 3.3 Effects of RbdA, NbdA and DipA on flagellar motor switching. (A) Domain organization of RbdA, NbdA and DipA. (B) Bar graphs showing the percentage time spent in CW rotation of *P. aeruginosa* PAO1 *rbdA*, *nbdA* and *dipA* mutant strains in cell tethering assay. Data (n = 29 to 34 cells) are representative of two independent experiments. This cell tethering assay was done in collaboration with Prof. Chiam Keng-Hwee’s lab, Singapore.

3.3.5. RbdA, NbdA and DipA control swimming reversal frequency to mediate swimming motility

Given the effect of *rbdA*, *nbdA* and *dipA* deletion on flagellar motor switching, we also examined the swimming trajectories of individual mutant cell to study how three PDEs mediate swimming motility (Figure 3.4). As reported previously, PAO1 cells ran ending in a sharp turn or reversal to a different direction, showing the typical “run-reverse-turn”

trajectories (185). In the *yedQ* overexpression mutant, the cells exhibited almost no turn or reverse and only ran in single direction, which is in agreement with the unidirectional

A

B

C

Figure 3.4 Effects of RbdA, NbdA and DipA on swimming motility. (A) Swimming trajectories of *P. aeruginosa* PAO1 and mutant cells. Data in figures are representative of two independent experiments. (B) Swimming persistence of *P. aeruginosa* PAO1 and mutant cells. (C) Average swimming speed of *P. aeruginosa* PAO1 and mutant strains. Data are means $\pm$ SD ($n = 12$ to 37 cells) from two independent experiments (two-tailed t test, $*P < 0.05$). This assay was done in collaboration with Prof. Chiam Keng-Hwee's lab, Singapore.

rotation of flagellum observed for PAO1/pYedQ. The three *rbdA*^{PDE*}, *nbdA*^{PDE*}, and *dipA*^{PDE*} strains also exhibited little reversal in their swimming trajectories, which is also in accordance with the highly suppressed flagellar motor switching for the three strains. In the contrast, three mutant strains *rbdA*^{PDE*}&*mapZ*^{R13A}, *nbdA*^{PDE*}&*mapZ*^{R13A} and *dipA*^{PDE*}&*mapZ*^{R13A} exhibit normal directional reversal, once again reinforcing the pivotal role of MapZ in controlling swimming motility. From the swimming trajectories, we calculated the persistence of runs, which is inversely proportional to the frequency of direction reversal. Expectedly, the PAO1/pYedQ, *rbdA*^{PDE*}, *nbdA*^{PDE*}, and *dipA*^{PDE*} have greater persistence than PAO1 and the double mutants. This suggests that suppression of motor switching can generate more persistent runs to prefer a directional swimming mode. On the other hand, the average swimming speed extracted from the trajectory video did not show any significant differences among PAO1 and the *pde* mutants, once again suggesting that the three PDEs may not control motor speed.

3.4. Discussion

Adjustment of flagellum-dependent motility according to the surroundings is crucial for the survival and pathogenicity of *P. aeruginosa*. Here we further studied the regulation of cyclic di-GMP on flagellum-dependent motility through MapZ by observing the rotary motion generated by flagellar motor. Our results suggest that a rise of cellular cyclic di-GMP has a profound impact on flagellar output, which includes the stator-generated torque and motor switching frequency, and finally determines the swimming speed and the frequency of directional reversal respectively for monotrichous *P. aeruginosa*. Our results further suggest that elevated cyclic di-GMP level suppresses flagellar motor switching through the MapZ mediated chemotaxis signaling pathway, in which three PDEs, including RbdA, NbdA and DipA, play critical role. This mechanism allows cyclic di-GMP to fine-tune flagellum-dependent motility and chemotactic responses in solution and on surfaces.

MapZ was identified as one of the conserved nonessential genes which could be evolutionarily important for the lifestyle of *P. aeruginosa* as a successful environmental bacterium and opportunistic pathogen (186). The MapZ mediated mechanism is distinct from the best-known YcgR-dependent “backstop brake” mechanism used by cyclic di-GMP to control swimming motility in *E. coli* and other bacteria (71,91,153), or another similar mechanism whereby cyclic di-GMP promotes the interaction between the cyclic

di-GMP binding DgrA and the motor protein FliL to impede motility in *Caulobacter crescentus* (118). The mechanism, as characterized by a trans-acting PilZ adaptor protein, is also distinct from the mechanism that cyclic di-GMP binds directly to the chemoreceptor Tlp1 through an integrated PilZ domain to control motility in *Azospirillum brasilense* (187,188).

Figure 3.5 Proposed model for the regulation of flagellar motor switching of cyclic di-GMP and MapZ through RbdA, NbdA and DipA. Under the specific signal stimulation, the membrane-anchored PDEs RbdA, NbdA and DipA could be activated to regulate the cyclic di-GMP level for MapZ. Upon binding of cyclic di-GMP, MapZ inhibits the methylation of CheR1 to MCP to block the phosphorylation in downstream pathway and finally to control flagellar output. “P” stands for phosphoryl group and “M” stands for methyl group.

of nitric oxide (NO)-induced biofilm dispersal (189). DipA has been proposed to play a role in flagellum-driven motility and chemotaxis in *P. aeruginosa*. While still producing flagella, $\Delta dipA$ cells displayed reduced swimming and swarming motility and decreased chemotactic response compared to the parental strain (190,191). DipA was also known to control Psl production and play an important role in biofilm formation and dispersal. While some of the phenotypic changes caused by the $\Delta rbdA$, $\Delta nbdA$ and $\Delta dipA$ mutation are likely due to the impact on flagellar motor switching, the observations suggest that not all the phenotypic changes are caused by the suppression of flagellar motor switching. It suggests that MapZ is not the only downstream effector for the three PDEs, and it is most likely that the pool of cyclic di-GMP governed by the three PDEs are also sensed by other cyclic di-GMP binding effectors to control production of EPS, rhamnolipids and other cellular functions.

Given the presence of more than 20 PDEs in *P. aeruginosa* cells, the observation that only $\Delta rbdA$, $\Delta nbdA$ and $\Delta dipA$ had the most noticeable effect on flagellar motor switching raises the interesting question that how the signaling specificity between MapZ and the three PDEs is achieved. As indicated in Chapter 2, MapZ localizes at cell poles through the interaction with CheR1 and the chemoreceptor array consisting of methyl-accepting chemotaxis proteins (MCPs). DipA is also known to have a polar localization, and was suggested to interact directly or indirectly with the chemotaxis kinase CheA (143). Both DipA and RbdA interact with the chemotaxis transducer BdlA and other proteins to form a multiprotein complex (150,191,192). Although the cellular localization of NbdA is unknown at this moment, the observation that NbdA enzymatically inactivation severely suppressed flagellar motor switching without raising global cyclic di-GMP level strongly supported the idea that NbdA is likely to act locally to control the cyclic di-GMP pool sensed by MapZ. Hence, we propose a model as shown in Figure 3.5 where MapZ is located near these PDEs and the chemotactic complex at cell poles to rapidly respond to a sub-cellular cyclic di-GMP concentration change. Similar models that involve the co-localization between cyclic di-GMP metabolizing enzymes and binding effector have been invoked to rationalize observations made on other cyclic di-GMP signaling networks (193,194). Altogether, our results suggest that a subset of PDEs control the cyclic di-GMP pool sensed by MapZ to control flagellar motor rotation to regulate motility through chemotaxis pathway.

CHAPTER 4. Structural basis for the MapZ mediated cyclic di-GMP regulation of methyltransferase CheR1

4.1. Introduction

Cyclic di-GMP is known to regulate multiple cellular processes including antibiotic-resistant biofilm formation and bacterial motility through different families of receptors in human pathogen *P. aeruginosa* (113-115). As the most prevalent family of cyclic di-GMP receptors, PilZ domain proteins have been studied for several years. Importantly, the progress has been achieved that the solution NMR structure of MapZ has been solved (62,63). Binding of dimeric cyclic di-GMP to its β -barrels and N-terminal loop causes conformational changes, especially for its C-terminal α -helix, and forms a highly negative charged surface. Since previous studies have indicated that cyclic di-GMP controls its real protein targets through PilZ domain proteins as adaptors, the structural changes may provide the interaction basis for downstream signaling. However, structural evidence remains scarce for understanding the detailed molecular mechanism of how cyclic di-GMP regulates the activity of its protein targets through the PilZ adaptor proteins. The key question of how specificity in cyclic di-GMP signaling, which is crucial for preventing crosstalk among different pathways, through the PilZ adaptor proteins also remains to be answered.

As discussed in Chapter 2, we have discovered that cyclic di-GMP controls the enzymatic activity of the methyltransferase CheR1 in *P. aeruginosa* through the PilZ adaptor protein MapZ. High cellular cyclic di-GMP levels lead to the formation of the CheR1/MapZ/cyclic di-GMP complex and subsequently inhibits the methyltransferase activity of CheR1. Inhibition of CheR1 results in a decrease in the methylation level of MCPs, which ultimately lead to the suppression of flagellar motor switching and decrease of swimming reversal frequency. Because cellular cyclic 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 interaction between CheR1 and MapZ is highly specific and dependent on cyclic di-GMP, the structural basis for the cyclic-di-GMP mediated specific protein-protein interaction is unknown. To date, structures of several PilZ domain proteins have been solved and well-

studied. Canonical PilZ domain possesses a PilZ fold, which is characterized by the presence of the antiparallel β -barrel core with the conserved (D/N)x(S/A)xxG motif on surface for cyclic di-GMP binding. Another RxxxR binding motif always forms a flexible loop at N-terminus (62,63,78,101). Although several complex structures containing non-c-di-GMP-binding PilZ domain proteins and its partners have been solved (76,78,85), the structural information of cyclic di-GMP binding PilZ domain proteins, especially single domain PilZ proteins, along with its binding partners is limited. In this chapter we report the crystal structures of the free CheR1 and the CheR1/MapZ/c-di-GMP 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.

4.2. Material and methods

4.2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains, plasmids and growth conditions were used as described in chapter 2.2.1.

4.2.2. Preparation of MapZ mutant constructs and overexpression strains

For the recombinant proteins used for crystallization, the PCR fragments encoding genes *cheR1* and *mapZ* were cloned into pACYCDuet-1 expression vector (Novagen), which provides a hexa-histidine tag at the N-terminus of the recombinant CheR1 and MapZ, respectively. The DNA fragments of encoding *mapZ* and mutants including D93A, H97A, R99A, R100A, and L104A were synthesized by Integrated DNA Technologies Company. Synthesized DNA fragments were digested and ligated into the pET28b and pUCP18 vectors providing a HA tag at the N-terminus of proteins. The constructions were verified by sequencing and transformed into *E.coli* BL21 (DE3) and *P. aeruginosa* PAO1 strain respectively for further analysis.

4.2.3. Expression and purification of recombinant proteins

For the proteins used for crystallization, the plasmid pACYCDuet-1-6xHis-MapZ/6xHis-CheR1 was transformed into *E.coli* BL21 (DE3) cell line and protein expression was induced by 0.2 mM IPTG at 16 °C overnight. Cells were harvested by centrifugation and the pellet was resuspended and homogenized five times using

EmulsiFlex-C3 (Avestin). The lysate was centrifuged at 40,000 rpm for 1 h to remove insoluble cell debris. The supernatant was applied onto Ni-NTA affinity column, sequentially washed and eluted by gradient increase of imidazole concentration in buffer. The pooled fractions were treated with TEV protease and dialyzed against the buffer containing 20 mM Tris (pH7.5), 100 mM NaCl. After removal of TEV 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.

Proteins used for ITC assay were prepared as described in chapter 2.2.5.

4.2.4. Crystallization and data collection

The crystal structure study was done in collaboration with Prof. Gao Yong-Gui's Lab, Singapore (2). The initial crystal hint of the isolated CheR1 was achieved at 20 °C by mixing 1.0 µl of protein with 1.0 µl of reservoir containing 1.5 M sodium phosphate monobasic/potassium phosphate dibasic (pH5.0). For further optimization, the crystallization condition was supplemented with 1% (v/v) glycerol and 3% (v/v) Dextran sulfate sodium salt (Mr 5,000). The crystals were flash frozen (100 K) in the reservoir solution supplemented with 30% (v/v) glycerol. The native data set (total 3600 images with 0.2 oscillations) was collected at PXIII, SLS.

For the CheR1-MapZ complex, the molar ratio between two proteins was determined to be 1:1 by analytical gel filtration. The CheR1-MapZ complexed with cyclic di-GMP 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 containing 0.1 mM BIS-TRIS pH 5.5, 19% PEG3350, 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) PEG3350. The data set (total 2000 images with 0.1 oscillations) for MapZ-CheR1 complex was collected at SLS.

4.2.5. Data processing, structure determination and refinement

The crystal structure study was done in collaboration with Prof. Gao Yong-Gui's Lab, Singapore (2). All data were processed using the XDS package (195). The structure of MapZ/CheR/c-di-GMP ternary complex was determined by molecular replacement with Phenix combining Phaser (196), using *BsCheR* (PDB ID: 5FTW) and MapZ (PDB ID:

2L74) as search models. The structure of apo CheR1 was determined by molecular replacement (Phaser/Phenix) (196) using the CheR1 model from our ternary complex as a search model. The models were improved by manual model building with coot (197), and refined using Phenix (196). The crystallographic statistic details of the structures are listed in Table S4, and all structural figures were generated with Pymol (Delano Scientific).

4.2.6. Isothermal titration calorimetry (ITC)

ITC measurements were performed at 25 °C using MicroCal ITC200 (MicroCal Inc.). Protein samples were dialyzed into a buffer containing 20 mM Tris (pH8.0), 100 mM NaCl and 1 mM TCEP (Sigma-Aldrich). 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 and mutants in the presence of c-di-GMP (250-300 µM). The titration comprised 19 injections of 2 µl each, separated by 150 s equilibration time. The datasets were analysed using Microcal PEAQ-ITC Analysis Software fitted to a single-site binding model.

4.2.7. Western blotting for checking protein expression level

Single colonies of *P. aeruginosa* PAO1, *mapZ* and mutants overexpression strains were used to inoculate 10 ml of LB medium that was kept at 37 °C with shaking at 250 rpm overnight. 1 ml of culture were centrifuged at 11,325g 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 with THETM HA-tag Antibody [HRP] (GenScript) and *E. coli* RNA Polymerase beta Monoclonal Antibody (loading control) (Neoclone).

4.2.8. Cell tethering assay for the analysis of the flagellar rotation

The cell tethering assay was performed as described in chapter 2.2.12.

4.2.9. Statistical analysis

The statistical analysis was done as described in chapter 2.2.11.

4.2.10. Genome mining and phylogenetic relationship analysis

The bioinformatic study was done by Dr. Cheang Qing Wei and Prof. Liang Zhao-Xun, Singapore (2). The homologs of MapZ were identified by mining PilZ protein sequences

deposited in The InterPro database (<http://www.ebi.ac.uk/interpro/>) (198). A total of 12,000 sequences were submitted to the EFI/EST sequence similarity networking (SSN) server (http://efi.igb.illinois.edu/efi-est/tutorial_startscreen.php) (199) for analysis. A cluster that contains 1,023 sequences, including that of MapZ, was identified by the SSN Server as potential MapZ homologs. The 1,023 sequences were curated manually to ensure that they all contain the C-terminal helices and key residues for CheR1 binding.

4.3. Results

4.3.1. Determination of crystal structure of CheR1/MapZ/c-di-GMP ternary complex

To study the molecular mechanism for MapZ-CheR1 interaction, we crystallized the protein complex together with cyclic di-GMP and solved its structure at 2.3 Å resolution as shown in Figure 4.1A and Figure S5, with the crystallographic statistic details listed in Table S4. The atomic coordinates and structure factors have been deposited in Protein Data Bank (PDB) with accession codes 5Y4R and 5Y4S for the CheR1/MapZ/c-di-GMP complex and the isolated CheR1, respectively. Difference Fourier map (Fo-Fc) clearly shows the ligand cyclic di-GMP (Figure S6). Cyclic di-GMP binding MapZ is composed of a β -barrel core consisting of eight antiparallel β -strands, an N-terminal loop and two C-terminal α helices (Figure 4.1B) as reported before in other PilZ proteins (61,72,200-202). Cyclic di-GMP is observed as an intercalated dimer in the electron density map and is held in position by residues stemmed from the N-terminal loop and the strands β 4, β 5, β 7 and β 8. The methyltransferase CheR1 is composed of two domains as shown in Figure 4.1C. 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. 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 (203). 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. The β -subdomain, which is inserted between α 7 and α 10, is unique to chemotaxis methyltransferases (Figure S7)(204-206). The N- and C-terminal domains are connected by a flexible domain linker (70-80 aa), 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 $\alpha 1$ and $\alpha 2$ 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 CheR1/MapZ/c-di-GMP ternary complex.

Figure 4.1 Crystal structure of MapZ/c-di-GMP/CheR1 ternary complex. (A) Overall view of the ternary complex. MapZ and CheR1 are shown in salmon and green respectively. The intercalated c-di-GMP dimer is bound by MapZ and shown as sticks, with carbon, oxygen, and nitrogen atoms colored cyan, red, and blue respectively. (B) An enlarged view of c-di-GMP bound MapZ. The view is rotated by 90 ° along the vertical axis in panel A. (C) Cartoon diagram of CheR1. The N-terminal domain, domain linker, C-terminal domain and β -subdomain are colored red, blue, light blue and limon, respectively. The secondary structures for protein are numbered in sequential order from N- to C-terminus. This study was done in collaboration with Prof. Gao Yong-Gui's lab, Singapore (2).

4.3.2. Specific interactions between MapZ and CheR1

In the ternary complex, MapZ makes direct contacts with CheR1 through its C-terminal motifs, the $\beta 6$ - $\beta 7$ loop and a small segment of the N-terminal loop. The C-terminal motifs of MapZ encompasses the two helices $\alpha 1$ (92-106aa), $\alpha 2$ (109-121 aa) and the adjacent loops. The helix $\alpha 1$ seems to play a central role in mediating the MapZ-CheR1 interaction by situating in the central cleft of CheR1, with several highly specific polar interactions formed between $\alpha 1$ and CheR1. In particular, Arg⁹⁹ interacts with Asp⁶⁷ and Thr⁷⁰ of CheR1 through salt bridge and hydrogen bonds; and Arg¹⁰⁰ forms salt bridge with Asp¹⁴⁴ (Figure 4.2A). The side chains of Asp⁹³, Ser⁹⁶, His⁹⁷ and Asn¹⁰⁵, and the main chain O of Leu¹⁰⁴, also interact with various CheR1 residues through hydrogen bonds. The side chain of several residues from $\alpha 1$, including Leu⁹², Ile⁹⁵, Leu¹⁰¹, Leu¹⁰⁴, as well as Arg⁹⁹, Arg¹⁰⁰ and Glu¹⁰³, interact with various CheR1 residues through hydrophobic forces. Meanwhile, $\alpha 2$ seems to interact with CheR1 mainly through hydrophobic interaction using the residues Leu¹¹², Val¹¹⁶, Ala¹¹⁷, Leu¹¹⁹ and Val¹²⁰ (Figure 4.2B). The only noticeable direct polar interaction that involves $\alpha 2$ seems to be the hydrogen bond formed between Glu¹⁰⁹ ($\alpha 2$) and Ser¹⁴⁶ (CheR1). Apart from the residues in $\alpha 1$ and $\alpha 2$, the main chain groups of Leu⁹² and Ile⁹⁰ from the loop prior to $\alpha 1$ form three hydrogen bonds with Val²² and Gly²⁰ of CheR1 (Figure 4.2C); whereas His¹²³ and Asp¹²⁴ from the loop downstream of $\alpha 2$ interact with Thr¹⁸ and Arg⁶² of CheR1 through hydrogen bond and salt bridge respectively.

In addition to the residues from the C-terminal motifs, several residues from other non-C-terminal regions of MapZ are also involved in direct binding of CheR1. Asp⁶⁶ and Val⁶⁷ from the $\beta 6$ - $\beta 7$ loop interact with Arg⁷⁸, Ser²⁵⁰ and Ile²²¹ of CheR1 through hydrogen bond and hydrophobic interaction; whereas Phe¹⁶ and Asp¹⁷ from the N-terminal loop interact with CheR1 residues through hydrophobic and hydrogen interactions respectively (Figure 4.2C).

4.3.3. 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 ($K_d = 28.6 \pm 17.7$ nM). Cyclic di-GMP is essential for MapZ-CheR1 interaction because no binding could be detected without cyclic di-GMP. To evaluate the contribution of the aforementioned residues of $\alpha 1$ and $\alpha 2$ to CheR1

Figure 4.2 Specific interactions that stabilize the MapZ/c-di-GMP/CheR1 ternary complex. (A) Specific interactions involving residues from the helix $\alpha 1$ of MapZ. The helix $\alpha 1$ (92-106aa) is positioned in the central cleft of CheR1 to form extensive contacts, including salt bridges for Arg⁹⁹-Asp⁶⁷, Arg¹⁰⁰-Asp¹⁴⁴; hydrogen bonds for His⁹⁷-Tyr²²², Arg⁹⁹-Thr⁷⁰, Leu¹⁰⁴-Asn²⁰⁰, Asn¹⁰⁵-Tyr²²²; and hydrophobic interactions for Leu¹⁰⁴-Tyr²²²&Phe²²³ (the former residues in the pairs are from MapZ, same as below). (B) Specific interactions involving the helix $\alpha 2$ and adjacent loops of MapZ. The residues in $\alpha 2$ and the adjacent loops in MapZ mainly interact with the N-terminal domain ($\alpha 1$ and $\alpha 4$) of CheR1. (C) Specific interactions involving residues from the non-C-terminal regions of MapZ. The N-terminal $\beta 1$ - $\beta 2$ loop and the $\beta 6$ - $\beta 7$ loop in MapZ contact the C-terminal domain of CheR1; whereas the loop residues Ile⁹⁰ and Leu⁹² between $\beta 8$ and $\alpha 1$ engage in hydrogen-bonding interactions with the N-terminal loop ($\alpha 1$ - $\alpha 2$) residues Gly²⁰ and Val²² in CheR1. For clarity, the residues Asp⁹³ and Ser⁹⁶ from the $\alpha 1$ of MapZ interacting with Lys²⁷ and Arg³⁴ of CheR1 is shown in this panel, instead of in panel A. Note that residues involved the above interactions are shown as sticks, with oxygen and nitrogen atom colored red and blue, respectively. Hydrogen bonds and salt bridge are shown as dashed lines. The critical interactive residues are underlined. This study was done in collaboration with Prof. Gao Yong-Gui's lab, Singapore (2).

Figure 4.3 Assessment of the contribution of the residues from helices $\alpha 1$ and $\alpha 2$ to protein-protein interaction by binding affinity measurement. (A) Binding isotherm (top panels) and data-fitting curves (bottom panels) obtained from isotherm titration calorimetry (ITC) assay. Solution containing CheR1 was titrated into solution containing MapZ (or a mutant) and saturating c-di-GMP (250 μ M). The experimental

conditions are described in the experimental procedure section. The binding measurements were performed in two independent experiments and the representative data set is presented here. (B) Apparent dissociation constant (K_d) values obtained by fitting the isotherm data to a 1:1 binding model.

binding, we measured the binding affinity of several MapZ mutants for CheR1. The results (Figure 4.3) show that Arg⁹⁹ is the most crucial residue as the substitution of Arg⁹⁹ by Ala reduced the binding affinity to below detection limit, consistent with the structural observation that the residue Arg⁹⁹ engages in multiple interactions with CheR1 residues (Figure 4.2A). The substitution of the other $\alpha 1$ 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 $\alpha 2$ helix (MapZ ^{$\Delta\alpha 2$}) decreased the K_d by 14.6-fold. These observations support the relevance of the crystal structure and reinforce the central role of $\alpha 1$ in mediating the MapZ-CheR1 interaction.

4.3.4. 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 mutation in MapZ impacts flagellar motor switching in *P. aeruginosa* cells by cell tethering assay. 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* as shown in Figure 2.5D. Most of the MapZ overexpression cells cannot switching motor direction and exhibit unidirectional flagellar rotation. The suppression is attributed to the ability of MapZ to inhibit CheR1 and decrease MCP methylation at high cyclic di-GMP levels. Hence, it was expected that overexpression of a defective MapZ mutant that lacks key residues for cyclic 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^{H97A}, MapZ^{R110A}, MapZ^{L104A}, and MapZ ^{$\Delta\alpha 2$} exhibited less suppression than MapZ to various extent (Figure 4.4A). Overexpression of MapZ^{R13A}, which is defective in cyclic di-GMP binding, also exhibited much weaker suppression effect. As supported by Western Blotting assays, the different suppressing effect

observed for the mutant proteins is not caused by reduction in protein expression level (Figure 4.4B and Figure S8). Taken together, the results provide strong support for the physiological relevance of the CheR1/MapZ/c-di-GMP ternary structure and establish the pivotal role of the C-terminal motifs in mediating MapZ-CheR1 interaction and suppressing flagellar motor switching.

4.3.5. Structural rearrangement in MapZ induced by the binding of cyclic di-GMP and CheR1

The structures of MapZ with and without cyclic di-GMP binding were determined previously by NMR spectroscopy (63,207). It was noted that MapZ undergoes large structural rearrangements with the binding of cyclic di-GMP. Comparing the ternary complex structure with the NMR structures (PDB: 1YWU) (207) reveals how the structural rearrangement induced by cyclic di-GMP binding prepares MapZ for CheR1 binding. Upon the binding of cyclic di-GMP, the N-terminal loop becomes organized by wrapping itself around the intercalated cyclic di-GMP dimer (Figure 4.5A). Without the c-di-GMP-induced reorganization, the N-terminal loop would hinder the binding of CheR1 sterically. In the C-terminal region, one of the most notable changes is that cyclic di-GMP binding results in the straightening of the initially bent $\alpha 2$ and concomitant dissociation of $\alpha 2$ from the β -barrel core. The restructuring and displacement of $\alpha 2$ is crucial for setting up MapZ for the interaction with CheR1.

Unexpectedly, the cyclic di-GMP binding mode in the ternary complex differs to some extent from that in the c-di-GMP/MapZ binary complex (PDB: 2L74) (63). The N-terminal loop adopts a rather dissimilar conformation, as characterized by the different position and orientation of several loop residues (e.g. Glu⁷, Phe¹¹ and His¹²) in the ternary complex (Figure 4.5B). Also noticeably, the helix $\alpha 1$ from the C-terminus is tilted towards the β -barrel core by ~ 2 Å and $\alpha 2$ is pushed towards $\alpha 1$ by as much as 12 Å in the ternary complex. While the residues Arg⁸, Arg⁹, Arg¹⁰, Arg¹³, Asp³⁵ and Trp⁷⁷ are seen to interact with cyclic di-GMP directly in both the binary and ternary complexes, three additional residues (Glu⁷, Phe¹¹ and Arg⁷⁹) are directly involved in the binding of cyclic di-GMP only in the ternary complex (Figure 4.5C&D).

Figure 4.4 Residues from helices $\alpha 1$ and $\alpha 2$ are important for the function of MapZ in suppressing flagellar motor switching. (A) Bar graphs showing the percentage time that *P. aeruginosa* PAO1 and overexpression mutant strains underwent clockwise (CW) rotation. The sample sizes are 32, 30, 30, 28, 27, 25, 27 and 31 cells for PAO1, *mapZ*⁺, *mapZ*^{R13A+}, *mapZ*^{D93A+}, *mapZ*^{H97A+}, *mapZ*^{R99A+}, *mapZ*^{R100A+}, and *mapZ*^{L104A+} respectively. Data are the representative of two independent experiments. This study was done in collaboration with Prof. Gao Yong-Gui's lab, Singapore (2). (B) Protein expression levels of MapZ and MapZ mutants in *P. aeruginosa* by Western-Blotting using HA-specific antibody. The RNA polymerase beta subunit (RNAP- β) served as loading control and was visualized using RNAP- β specific antibody. Blots are representative of two independent experiments.

4.3.6. Binding of MapZ to CheR1 results in disruption of the Ado-Met-binding pocket and inhibition of methyltransferase activity

We also determined the crystal structure of free CheR1 protein to find out how the binding of MapZ impacts the structure of CheR1. Crystals of the free CheR1 belong to the tetragonal space group $I4_1$, with 10 copies of the molecule (chains A-J) in an asymmetric unit. Superposition of these crystallographic independent chains B-J onto chain A gives an average root mean square deviation (RMSD) of 0.76 Å over main-chain atoms, indicating almost identical conformations and a limited influence of crystal

packing on the individual chain. The structure was refined to 3.4 Å resolution, and the simulated-annealing 2Fo-Fc composite omit map clearly demonstrates the two domains and the domain linker that are nicely modelled (Figure S9). Comparison of the structures of the free CheR1 and the ternary complex shows that while the C-terminal domains are almost superimposable, the N-terminal domain adopts a very different conformation in the two structures (Figure 4.5E). The spatial positioning of the N-terminal domain relative to the C-terminal domain is also different from that of *BsCheR* (29) (Figure S10), despite the fact that the structure of the N-terminal domains are highly similar (Figure S11). The N-terminal domain and the domain linker of CheR1 have relatively higher B-factors (Figure S12), suggesting that they are intrinsically flexible. The most important implication from the comparison of the structures of free CheR1 and the ternary complex is that the binding of MapZ induces a relocation of the N-terminal domain by a translocation of more than 10 Å and a rotation of ~105°, as well as a noticeable conformational change in the domain linker (Figure 4.5E).

Based on the studies on other chemotaxis methyltransferases (205,206), the C-terminal domain and the domain linker are likely involved in the binding of the cofactor S-adenosyl methionine (Ado-Met). In the free CheR1 structure, an Ado-Met-binding pocket can be readily identified by overlaying the structure with that of the S-adenosyl-L-homocysteine (SAH)-bound *BsCheR* (PDB: 5FTW, Figure 4.5F). Two highly conserved catalytic residues (Asp¹⁴⁴ and Tyr²²²) that are essential for Ado-Met binding can also be identified. In the ternary complex, the Ado-Met binding pocket is completely occupied by MapZ upon the dislodging of the N-terminal domain and conformational changes in the domain linker. The Ado-Met binding pocket is filled by residues from the α1 of MapZ, with Asp¹⁴⁴ and Tyr²²² engaging in multiple interactions with residues from α1 (Figure 4.2A&Figure 4.5F). These observations strongly suggest that the C-terminal motifs of MapZ, particularly α1, has evolved to interact specifically with the conserved residues in the CheR1 active site to inhibit its enzymatic activity.

4.3.7. MapZ-mediated regulation of chemotaxis by cyclic di-GMP is conserved in many proteobacteria

Together with our previous study, the current study firmly establishes the regulation of chemotaxis by cyclic di-GMP through the MapZ mediated mechanism in *P. aeruginosa*.

Figure 4.5 Conformational changes in MapZ and CheR1 induced by c-di-GMP binding and protein complex formation. (A) Superimposition of the structures of CheR/c-di-GMP/MapZ complex and free MapZ (grey, PDB: 1YWU). The c-di-GMP binding pocket and CheR1 are represented by the cyan and green surfaces. The N-terminal loop and C-terminal helices ($\alpha 1$ and $\alpha 2$) of the free MapZ adopt conformations that prohibit the binding of CheR1 because of steric hindrance. (B) Superimposition of

CheR-bound MapZ (salmon) and CheR1-free MapZ (grey, PDB: 2L74) in the presence of c-di-GMP. Structures are aligned according to the β -barrel cores. The residue Phe¹¹ and c-di-GMP in both structures are shown in sticks. In the case of CheR1 binding, carbon, oxygen, and nitrogen atoms in c-di-GMP are colored cyan, red, and blue, respectively. Phe¹¹ interacts with $\alpha 2$ in the two structures. Upon CheR1 binding, conformational changes in $\alpha 2$ cause rearrangement of the N-terminal loop of MapZ, through their interactions. Given the N-terminal loop interacting with c-di-GMP, such a rearrangement would thus cause deformation of c-di-GMP binding pocket. Upon CheR1 binding, the conformational changes of $\alpha 2$ helix, residue Phe¹¹ and c-di-GMP are indicated by arrows. (C) Coordination of dimeric c-di-GMP in the ternary complex. Three additional residues (Glu⁷, Phe¹¹ and Arg⁷⁹) are involved in the binding of c-di-GMP in the ternary complex. (D) Coordination of dimeric c-di-GMP in the binary complex based on the NMR structure (PDB: 2L74). His¹² is involved in binding c-di-GMP in the binary complex, but not in the ternary complex. Hydrogen bonds are shown as dashed lines. (E) Superimposition of the structures of free CheR1 and CheR1 from the ternary complex. The most notable differences are seen in the position of the N-terminal domain and the conformation adopted by the domain linker. (F) Superimposition of the structures of MapZ-bound CheR1 and S-Adenosyl-L-homocysteine (SAH)-bound BsCheR (PDB: 5FTW). The two methyltransferase (BsCheR in grey) structures are aligned according to their C-terminal domains. SAH is shown as stick with carbon, oxygen, nitrogen, and sulfur atom colored yellow, red, blue, and brown respectively. In the enlarged view of the cofactor-binding site, it can be seen that Arg¹⁰⁰ and Leu¹⁰⁴ from the $\alpha 1$ of MapZ occupy the SAH binding site to prevent the binding of the cofactor Ado-Met. The highly conserved catalytic residue Asp¹⁴⁴/Asp¹³⁰ (CheR1/BsCheR) that interacts with Arg¹⁰⁰ (MapZ) is also highlighted as sticks. This study was done in collaboration with Prof. Gao Yong-Gui's lab, Singapore (2).

We further asked whether this regulatory mechanism is conserved in the genus of *Pseudomonas* and other non-*Pseudomonas* species by searching MapZ encoded by 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 (198). From this collection, we identified 1,063 potential function orthologs/homologs of MapZ according to the following two criteria. First, the proteins share high sequence similarity (>60 similarity) with MapZ. Second, the proteins must contain two highly similar C-terminal helices with the conserved CheR1-binding residues. As shown by the sequence logo (Figure 4.6A), the critical CheR1-interacting residues are highly conserved in the putative orthologs/homologs. 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 gamma-proteobacteria (Figure 4.6B). The bioinformatics analysis suggests that the MapZ mediated regulation of chemotaxis motility by cyclic di-GMP could be conserved in many proteobacteria.

Figure 4.6 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 DxSxxG) and the C-terminal $\alpha 1$ and $\alpha 2$ helices are highlighted by the bars. The residues from $\alpha 1$ and $\alpha 2$ that are involved in direct binding of CheR1 are highlighted by the 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 phylum of proteobacteria; while a small number of homologs belong to unclassified species whose sequences were obtained by metagenomics projects. This study was done by Dr. Cheang Qing Wei and Prof. Liang Zhao-Xun, Singapore (2).

4.4. Discussion

To establish the structural basis for the regulation of the methyltransferase CheR1 by cyclic di-GMP through the PilZ protein MapZ, we solved the crystal structure of the complex, supported by both the affinity binding and cell tethering assay data. Cyclic di-GMP binding restructures the C-terminal α helices in MapZ, setting it up for interaction with CheR1. MapZ contacts directly with CheR1 through its C-terminal motifs, the β 6- β 7 loop and a small segment of the N-terminal loop. The cyclic di-GMP binding MapZ occupies the Ado-Met binding pocket, resulting the N-terminal domain relocation and rotation as well as domain linker conformational changes in CheR1. Furthermore, the bioinformatics analysis also suggested that the established regulation mechanism of chemotaxis by cyclic di-GMP through MapZ is conserved in many proteobacteria. Together, the structural data further support the model whereby MapZ, at high cellular cyclic di-GMP concentrations, undergoes dramatic conformational changes and occupies the CheR1 active site to inhibit the enzyme activity (Figure 4.7). Inhibition of CheR1 decreases the methylation level of MCPs and ultimately results in the suppression of flagellar motor switching.

The cyclic di-GMP regulation of flagellum based chemotaxis motility has been studied and some PilZ domain proteins have been implicated in the regulation of flagellum motility (154). With the analysis of the MapZ structure in the crystal complex, it should be noted that although Glu⁷ and Phe¹¹ are conserved in most single-domain PilZ proteins, their functions remain unknown previously. Our structure suggested that the two residues are conserved because they play a direct role in cyclic di-GMP binding. On the contrary, His¹², which is not a conserved residue and seen to form a hydrogen bond with cyclic di-GMP in the binary complex, does not participate in cyclic di-GMP binding in the ternary complex. The discrepancies in cyclic di-GMP binding between the binary (PDB: 1YWU) and ternary complexes we solved here highlighted the malleability of the cyclic di-GMP binding pocket and called for caution when interpreting the structures of PilZ proteins determined in the absence of their protein partners. Furthermore, MapZ only interacts with CheR1, but not the other three chemotaxis methyltransferases in *P. aeruginosa* (160,208). Sequences alignment of the four methyltransferases suggests that this is likely because of that some of the MapZ binding residues in CheR1 are absent in the other three CheR proteins (Figure S13).

Figure 4.7 A model that depicts the cyclic di-GMP regulated methylation of MCP through the PilZ adaptor protein MapZ.

From the analysis of taxonomic and phylogenetic relationship, a small percentage of the orthologs/homologs were encoded by genes from unclassified bacteria species uncovered by metagenomics projects. PlzA, one of the PilZ proteins from the pathogenic bacterium *Vibrio cholera* (119) 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. Cholera* by binding to a chemotactic methyltransferase to regulate chemotaxis. Furthermore, for the MapZ homolog/ortholog containing bacterial species whose genome has been sequenced, we could identify genes encoding chemotaxis methyltransferases that are highly similar to CheR1. 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. While the variation in sequence and structure may reflect the diverse protein partners that the PilZ proteins interact with, 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. Altogether, the MapZ mediated cyclic di-GMP regulatory mechanism of fine-tuning chemotactic response and bacterial motility is potentially widely distributed in many proteobacteria.

REFERENCES

1. 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. *Science signaling* **9**, ra102
2. Yan, X.-F., Xin, L., Yen, J. T., Zeng, Y., Jin, S., Cheang, Q. W., Fong, R. A. C. Y., Chiam, K.-H., Liang, Z.-X., and Gao, Y.-G. (2017) Structural Analyses Unravel the Molecular Mechanism of Cyclic di-GMP Regulation of Bacterial Chemotaxis via a PilZ Adaptor Protein. *Journal of Biological Chemistry*
3. Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., Mayer, R., Braun, S., de Vroom, E., van der Marel, G. A., van Boom, J. H., and Benziman, M. (1987) Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. *Nature* **325**, 279-281
4. Romling, U., Galperin, M. Y., and Gomelsky, M. (2013) Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiology and molecular biology reviews : MMBR* **77**, 1-52
5. Gentner, M., Allan, M. G., Zaehring, F., Schirmer, T., and Grzesiek, S. (2012) Oligomer formation of the bacterial second messenger c-di-GMP: reaction rates and equilibrium constants indicate a monomeric state at physiological concentrations. *Journal of the American Chemical Society* **134**, 1019-1029
6. Ausmees, N., Mayer, R., Weinhouse, H., Volman, G., Amikam, D., Benziman, M., and Lindberg, M. (2001) Genetic data indicate that proteins containing the GGDEF domain possess diguanylate cyclase activity. *FEMS Microbiol Lett* **204**, 163-167
7. Simm, R., Morr, M., Kader, A., Nimtz, M., and Romling, U. (2004) GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Molecular microbiology* **53**, 1123-1134
8. Tal, R., Wong, H. C., Calhoon, R., Gelfand, D., Fear, A. L., Volman, G., Mayer, R., Ross, P., Amikam, D., Weinhouse, H., Cohen, A., Sapir, S., Ohana, P., and Benziman, M. (1998) Three *cdg* operons control cellular turnover of cyclic di-GMP in *Acetobacter xylinum*: genetic organization and occurrence of conserved domains in isoenzymes. *Journal of bacteriology* **180**, 4416-4425
9. Wassmann, P., Chan, C., Paul, R., Beck, A., Heerklotz, H., Jenal, U., and Schirmer, T. (2007) Structure of BeF₃⁻-modified response regulator PleD: implications for diguanylate cyclase activation, catalysis, and feedback inhibition. *Structure (London, England : 1993)* **15**, 915-927
10. Chan, C., Paul, R., Samoray, D., Amiot, N. C., Giese, B., Jenal, U., and Schirmer, T. (2004) Structural basis of activity and allosteric control of diguanylate cyclase. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 17084-17089
11. Ryjenkov, D. A., Tarutina, M., Moskvina, O. V., and Gomelsky, M. (2005) Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. *Journal of bacteriology* **187**, 1792-1798
12. Paul, R., Abel, S., Wassmann, P., Beck, A., Heerklotz, H., and Jenal, U. (2007) Activation of the diguanylate cyclase PleD by phosphorylation-mediated dimerization. *The Journal of biological chemistry* **282**, 29170-29177
13. Christen, B., Christen, M., Paul, R., Schmid, F., Folcher, M., Jenoe, P., Meuwly, M., and Jenal, U. (2006) Allosteric control of cyclic di-GMP signaling. *The Journal of biological chemistry* **281**, 32015-32024
14. Yang, C. Y., Chin, K. H., Chuah, M. L., Liang, Z. X., Wang, A. H., and Chou, S. H. (2011) The structure and inhibition of a GGDEF diguanylate cyclase complexed with (c-di-

- GMP)(2) at the active site. *Acta crystallographica. Section D, Biological crystallography* **67**, 997-1008
15. Ross, P., Aloni, Y., Weinhouse, H., Michaeli, D., Weinberger-Ohana, P., Mayer, R., and Benziman, M. (1986) Control of cellulose synthesis *Acetobacter xylinum*. A unique guanyl oligonucleotide is the immediate activator of the cellulose synthase. *Carbohydrate Research* **149**, 101-117
 16. Schmidt, A. J., Ryjenkov, D. A., and Gomelsky, M. (2005) The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. *Journal of bacteriology* **187**, 4774-4781
 17. Tchigvintsev, A., Xu, X., Singer, A., Chang, C., Brown, G., Proudfoot, M., Cui, H., Flick, R., Anderson, W. F., Joachimiak, A., Galperin, M. Y., Savchenko, A., and Yakunin, A. F. (2010) Structural insight into the mechanism of c-di-GMP hydrolysis by EAL domain phosphodiesterases. *J Mol Biol* **402**, 524-538
 18. Sultan, S. Z., Pitzer, J. E., Boquoi, T., Hobbs, G., Miller, M. R., and Motaleb, M. A. (2011) Analysis of the HD-GYP domain cyclic dimeric GMP phosphodiesterase reveals a role in motility and the enzootic life cycle of *Borrelia burgdorferi*. *Infection and immunity* **79**, 3273-3283
 19. Ryan, R. P., Lucey, J., O'Donovan, K., McCarthy, Y., Yang, L., Tolker-Nielsen, T., and Dow, J. M. (2009) HD-GYP domain proteins regulate biofilm formation and virulence in *Pseudomonas aeruginosa*. *Environmental microbiology* **11**, 1126-1136
 20. Lovering, A. L., Capeness, M. J., Lambert, C., Hobley, L., and Sockett, R. E. (2011) The structure of an unconventional HD-GYP protein from *Bdellovibrio* reveals the roles of conserved residues in this class of cyclic-di-GMP phosphodiesterases. *MBio* **2**
 21. Bellini, D., Caly, D. L., McCarthy, Y., Bumann, M., An, S. Q., Dow, J. M., Ryan, R. P., and Walsh, M. A. (2014) Crystal structure of an HD-GYP domain cyclic-di-GMP phosphodiesterase reveals an enzyme with a novel trinuclear catalytic iron centre. *Mol Microbiol* **91**, 26-38
 22. Paul, K., Nieto, V., Carlquist, W. C., Blair, D. F., and Harshey, R. M. (2010) The c-di-GMP binding protein YcgR controls flagellar motor direction and speed to affect chemotaxis by a "backstop brake" mechanism. *Mol Cell* **38**, 128-139
 23. Fang, X., and Gomelsky, M. (2010) A post-translational, c-di-GMP-dependent mechanism regulating flagellar motility. *Molecular microbiology* **76**, 1295-1305
 24. Baker, A. E., Diepold, A., Kuchma, S. L., Scott, J. E., Ha, D. G., Orazi, G., Armitage, J. P., and O'Toole, G. A. (2016) PilZ Domain Protein FlgZ Mediates Cyclic Di-GMP-Dependent Swarming Motility Control in *Pseudomonas aeruginosa*. *J Bacteriol* **198**, 1837-1846
 25. Merritt, J. H., Ha, D. G., Cowles, K. N., Lu, W., Morales, D. K., Rabinowitz, J., Gitai, Z., and O'Toole, G. A. (2010) Specific control of *Pseudomonas aeruginosa* surface-associated behaviors by two c-di-GMP diguanylate cyclases. *MBio* **1**
 26. Caiazza, N. C., Merritt, J. H., Brothers, K. M., and O'Toole, G. A. (2007) Inverse regulation of biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. *Journal of bacteriology* **189**, 3603-3612
 27. Merritt, J. H., Brothers, K. M., Kuchma, S. L., and O'Toole, G. A. (2007) SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. *Journal of bacteriology* **189**, 8154-8164
 28. Huang, B., Whitchurch, C. B., and Mattick, J. S. (2003) FimX, a multidomain protein connecting environmental signals to twitching motility in *Pseudomonas aeruginosa*. *Journal of bacteriology* **185**, 7068-7076
 29. Tao, F., He, Y. W., Wu, D. H., Swarup, S., and Zhang, L. H. (2010) The cyclic nucleotide monophosphate domain of *Xanthomonas campestris* global regulator Clp defines a new class of cyclic di-GMP effectors. *Journal of bacteriology* **192**, 1020-1029

30. Lee, M. C., Weng, S. F., and Tseng, Y. H. (2003) Flagellin gene *fliC* of *Xanthomonas campestris* is upregulated by transcription factor Clp. *Biochemical and biophysical research communications* **307**, 647-652
31. Leduc, J. L., and Roberts, G. P. (2009) Cyclic di-GMP allosterically inhibits the CRP-like protein (Clp) of *Xanthomonas axonopodis* pv. *citri*. *Journal of bacteriology* **191**, 7121-7122
32. Krasteva, P. V., Fong, J. C., Shikuma, N. J., Beyhan, S., Navarro, M. V., Yildiz, F. H., and Sondermann, H. (2010) *Vibrio cholerae* VpsT regulates matrix production and motility by directly sensing cyclic di-GMP. *Science* **327**, 866-868
33. Ryjenkov, D. A., Simm, R., Romling, U., and Gomelsky, M. (2006) The PilZ domain is a receptor for the second messenger c-di-GMP: the PilZ domain protein YcgR controls motility in enterobacteria. *J Biol Chem* **281**, 30310-30314
34. Hickman, J. W., Tifrea, D. F., and Harwood, C. S. (2005) A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 14422-14427
35. Hickman, J. W., and Harwood, C. S. (2008) Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. *Molecular microbiology* **69**, 376-389
36. Basu Roy, A., and Sauer, K. (2014) Diguanylate cyclase NicD-based signalling mechanism of nutrient-induced dispersion by *Pseudomonas aeruginosa*. *Molecular microbiology* **94**, 771-793
37. Morgan, R., Kohn, S., Hwang, S. H., Hassett, D. J., and Sauer, K. (2006) BdlA, a chemotaxis regulator essential for biofilm dispersion in *Pseudomonas aeruginosa*. *Journal of bacteriology* **188**, 7335-7343
38. Li, Y., Petrova, O. E., Su, S., Lau, G. W., Panmanee, W., Na, R., Hassett, D. J., Davies, D. G., and Sauer, K. (2014) BdlA, DipA and induced dispersion contribute to acute virulence and chronic persistence of *Pseudomonas aeruginosa*. *PLoS pathogens* **10**, e1004168
39. Roy, A. B., Petrova, O. E., and Sauer, K. (2012) The Phosphodiesterase DipA (PA5017) Is Essential for *Pseudomonas aeruginosa* Biofilm Dispersion. *Journal of bacteriology* **194**, 2904-2915
40. Sondermann, H., Shikuma, N. J., and Yildiz, F. H. (2012) You've come a long way: c-di-GMP signaling. *Current opinion in microbiology* **15**, 140-146
41. Krasteva, P. V., Giglio, K. M., and Sondermann, H. (2012) Sensing the messenger: the diverse ways that bacteria signal through c-di-GMP. *Protein science : a publication of the Protein Society* **21**, 929-948
42. 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 in microbiology* **20**, 235-242
43. Duerig, A., Abel, S., Folcher, M., Nicollier, M., Schwede, T., Amiot, N., Giese, B., and Jenal, U. (2009) Second messenger-mediated spatiotemporal control of protein degradation regulates bacterial cell cycle progression. *Genes & development* **23**, 93-104
44. Lee, V. T., Matewish, J. M., Kessler, J. L., Hyodo, M., Hayakawa, Y., and Lory, S. (2007) A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. *Molecular Microbiology* **65**, 1474-1484
45. Whitney, J. C., Colvin, K. M., Marmont, L. S., Robinson, H., Parsek, M. R., and Howell, P. L. (2012) Structure of the Cytoplasmic Region of PelD, a Degenerate Diguanylate Cyclase Receptor That Regulates Exopolysaccharide Production in *Pseudomonas aeruginosa*. *The Journal of Biological Chemistry* **287**, 23582-23593

46. Newell, P. D., Monds, R. D., and O'Toole, G. A. (2009) LapD is a bis-(3',5')-cyclic dimeric GMP-binding protein that regulates surface attachment by *Pseudomonas fluorescens* Pf0-1. *Proc Natl Acad Sci U S A* **106**, 3461-3466
47. Newell, P. D., Yoshioka, S., Hvorecny, K. L., Monds, R. D., and O'Toole, G. A. (2011) Systematic analysis of diguanylate cyclases that promote biofilm formation by *Pseudomonas fluorescens* Pf0-1. *J Bacteriol* **193**, 4685-4698
48. Baraquet, C., Murakami, K., Parsek, M. R., and Harwood, C. S. (2012) The FleQ protein from *Pseudomonas aeruginosa* functions as both a repressor and an activator to control gene expression from the *pel* operon promoter in response to c-di-GMP. *Nucleic acids research* **40**, 7207-7218
49. Hickman, J. W., and Harwood, C. S. (2008) Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. *Mol Microbiol* **69**, 376-389
50. Sudarsan, N., Lee, E. R., Weinberg, Z., Moy, R. H., Kim, J. N., Link, K. H., and Breaker, R. R. (2008) Riboswitches in eubacteria sense the second messenger cyclic di-GMP. *Science* **321**, 411-413
51. Lee, E. R., Baker, J. L., Weinberg, Z., Sudarsan, N., and Breaker, R. R. (2010) An allosteric self-splicing ribozyme triggered by a bacterial second messenger. *Science* **329**, 845-848
52. Barrick, J. E., and Breaker, R. R. (2007) The distributions, mechanisms, and structures of metabolite-binding riboswitches. *Genome Biology* **8**, R239-R239
53. An, S.-q., Caly, D. L., McCarthy, Y., Murdoch, S. L., Ward, J., Febrer, M., Dow, J. M., and Ryan, R. P. (2014) Novel Cyclic di-GMP Effectors of the YajQ Protein Family Control Bacterial Virulence. *PLoS pathogens* **10**, e1004429
54. Chambers, J. R., Liao, J., Schurr, M. J., and Sauer, K. (2014) BrlR from *Pseudomonas aeruginosa* is a c-di-GMP-responsive transcription factor. *Molecular microbiology* **92**, 471-487
55. Wolfe, A. J., and Visick, K. L. (2008) Get the Message Out: Cyclic-Di-GMP Regulates Multiple Levels of Flagellum-Based Motility. *Journal of bacteriology* **190**, 463-475
56. Valentini, M., and Filloux, A. (2016) Biofilms and Cyclic di-GMP (c-di-GMP) Signaling: Lessons from *Pseudomonas aeruginosa* and Other Bacteria. *The Journal of biological chemistry* **291**, 12547-12555
57. McCarthy, R. R., Mazon-Moya, M. J., Moscoso, J. A., Hao, Y., Lam, J. S., Bordi, C., Mostowy, S., and Filloux, A. (2017) Cyclic-di-GMP regulates lipopolysaccharide modification and contributes to *Pseudomonas aeruginosa* immune evasion. *Nature microbiology* **2**, 17027
58. Merighi, M., Lee, V. T., Hyodo, M., Hayakawa, Y., and Lory, S. (2007) The second messenger bis-(3'-5')-cyclic-GMP and its PilZ domain-containing receptor Alg44 are required for alginate biosynthesis in *Pseudomonas aeruginosa*. *Mol Microbiol* **65**, 876-895
59. Düvel, J., Bense, S., Möller, S., Bertinetti, D., Schwede, F., Morr, M., Eckweiler, D., Genieser, H.-G., Jänsch, L., Herberg, F. W., Frank, R., and Häussler, S. (2016) Application of Synthetic Peptide Arrays To Uncover Cyclic Di-GMP Binding Motifs. *Journal of bacteriology* **198**, 138-146
60. Amikam, D., and Galperin, M. Y. (2006) PilZ domain is part of the bacterial c-di-GMP binding protein. *Bioinformatics* **22**, 3-6
61. 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
62. Ramelot, T. A., Yee, A., Cort, J. R., Semesi, A., Arrowsmith, C. H., and Kennedy, M. A. (2007) NMR structure and binding studies confirm that PA4608 from *Pseudomonas aeruginosa* is a PilZ domain and a c-di-GMP binding protein. *Proteins* **66**, 266-271

63. Habazettl, J., Allan, M. G., Jenal, U., and Grzesiek, S. (2011) Solution structure of the PilZ domain protein PA4608 complex with cyclic di-GMP identifies charge clustering as molecular readout. *J Biol Chem* **286**, 14304-14314
64. 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
65. Benach, J., Swaminathan, S. S., Tamayo, R., Handelman, S. K., Foltá-Stogniew, E., Ramos, J. E., Forouhar, F., Neely, H., Seetharaman, J., Camilli, A., and Hunt, J. F. (2007) The structural basis of cyclic diguanylate signal transduction by PilZ domains. *The EMBO journal* **26**, 5153-5166
66. Guzzo, C. R., Salinas, R. K., Andrade, M. O., and Farah, C. S. (2009) PILZ Protein Structure and Interactions with PILB and the FIMX EAL Domain: Implications for Control of Type IV Pilus Biogenesis. *J. Mol. Biol.* **393**, 848-866
67. Li, T. N., Chin, K. H., Liu, J. H., Wang, A. H. J., and Chou, S. H. (2009) XC 1028 from *Xanthamanas campestris* adopts a PilZ domain-like structure without a c-di-GMP switch. *Proteins* **75**, 282-288
68. Qi, Y., Xu, L., Dong, X., Yau, Y. H., Ho, C. L., Koh, S. L., Shochat, S. G., Chou, S. H., Tang, K., and Liang, Z. X. (2012) Functional divergence of FimX in PilZ binding and type IV pilus regulation. *Journal of bacteriology* **194**, 5922-5931
69. Xu, L., Venkataramani, P., Ding, Y., Liu, Y., Deng, Y., Yong, 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
70. Ryjenkov, D. A., Simm, R., Romling, 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
71. Paul, K., Nieto, V., Carlquist, W. C., Blair, D. F., and Harshey, R. M. (2010) The c-di-GMP Binding Protein YcgR Controls Flagellar Motor Direction and Speed to Affect Chemotaxis by a "Backstop Brake" Mechanism. *Mol. Cell.* **38**, 128-139
72. Ko, J., Ryu, K.-S., Kim, H., Shin, J.-S., Lee, J.-O., Cheong, C., and Choi, B.-S. (2011) Structure of PP4397 Reveals the Molecular Basis for Different c-di-GMP Binding Modes by Pilz Domain Proteins. *Journal of Molecular Biology* **398**, 97-110
73. Alm, R. A., Boder, A. J., Free, P. D., and Mattick, J. S. (1996) Identification of a novel gene, pilZ, essential for type 4 fimbrial biogenesis in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **178**, 46-53
74. Qi, Y., Xu, L., Dong, X., Yau, Y. H., Ho, C. L., Koh, S. L., Shochat, S. G., Chou, S. H., Tang, K., and Liang, Z. X. (2012) Functional divergence of FimX in PilZ binding and type IV pilus regulation. *J Bacteriol* **194**, 5922-5931
75. Bian, X., Huang, F., Stewart, F. A., Xia, L., Zhang, Y., and Muller, R. (2012) Direct cloning, genetic engineering, and heterologous expression of the syringolin biosynthetic gene cluster in *E. coli* through Red/ET recombineering. *Chembiochem* **13**, 1946-1952
76. Chin, K. H., Kuo, W. T., Yu, Y. J., Liao, Y. T., Yang, M. T., and Chou, S. H. (2012) Structural polymorphism of c-di-GMP bound to an EAL domain and in complex with a type II PilZ-domain protein. *Acta Crystallogr D Biol Crystallogr* **68**, 1380-1392
77. Guzzo, C. R., Salinas, R. K., Andrade, M. O., and Farah, C. S. (2009) PILZ protein structure and interactions with PILB and the FIMX EAL domain: implications for control of type IV pilus biogenesis. *J Mol Biol* **393**, 848-866
78. Guzzo, C. R., Dunger, G., Salinas, R. K., and Farah, C. S. (2013) Structure of the PilZ-FimXEAL-c-di-GMP Complex Responsible for the Regulation of Bacterial Type IV Pilus Biogenesis. *J Mol Biol* **425**, 2174-2197

79. Li, T. N., Chin, K. H., Liu, J. H., Wang, A. H., and Chou, S. H. (2009) XC1028 from *Xanthomonas campestris* adopts a PilZ domain-like structure without a c-di-GMP switch. *Proteins* **75**, 282-288
80. Christen, M., Christen, B., Allan, M. G., Folcher, M., Jenö, 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
81. 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*. *Molecular plant pathology* **9**, 819-824
82. Ryan, R. P. (2013) Cyclic di-GMP signalling and the regulation of bacterial virulence. *Microbiology* **159**, 1286-1297
83. Ryan, R. P., McCarthy, Y., Andrade, M., Farah, C. S., Armitage, J. P., and Dow, J. M. (2010) Cell-cell signal-dependent dynamic interactions between HD-GYP and GGDEF domain proteins mediate virulence in *Xanthomonas campestris*. *Proc Natl Acad Sci U S A* **107**, 5989-5994
84. Yang, F., Tian, F., Chen, H., Hutchins, W., Yang, C.-H., and He, C. (2015) The *Xanthomonas oryzae* pv. *oryzae* PilZ Domain Proteins Function Differentially in Cyclic di-GMP Binding and Regulation of Virulence and Motility. *Applied and Environmental Microbiology* **81**, 4358-4367
85. Yang, F., Tian, F., Li, X., Fan, S., Chen, H., Wu, M., Yang, C. H., and He, C. (2014) The degenerate EAL-GGDEF domain protein Filp functions as a cyclic di-GMP receptor and specifically interacts with the PilZ-domain protein PXO_02715 to regulate virulence in *Xanthomonas oryzae* pv. *oryzae*. *Mol Plant Microbe Interact* **27**, 578-589
86. Pratt, J. T., Tamayo, R., Tischler, 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
87. Remminghorst, U., and Rehm, B. H. (2006) Alg44, a unique protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. *FEBS Lett* **580**, 3883-3888
88. 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
89. Whitney, J. C., Whitfield, G. B., Marmont, L. S., Yip, P., Neculai, A. M., Lobsanov, Y. D., Robinson, H., Ohman, D. E., and Howell, P. L. (2015) Dimeric c-di-GMP is required for post-translational regulation of alginate production in *Pseudomonas aeruginosa*. *J Biol Chem* **290**, 12451-12462
90. Russell, M. H., Bible, A. N., Fang, X., Gooding, J. R., Campagna, S. R., Gomelsky, M., and Alexandre, G. (2013) Integration of the second messenger c-di-GMP into the chemotactic signaling pathway. *MBio* **4**, e00001-00013
91. Boehm, A., Kaiser, M., Li, H., Spangler, C., Kasper, C. A., Ackermann, M., Kaever, V., Sourjik, V., Roth, V., and Jenal, U. (2010) Second messenger-mediated adjustment of bacterial swimming velocity. *Cell* **141**, 107-116
92. Chen, Y., Chai, Y., Guo, J. H., and Losick, R. (2012) Evidence for cyclic Di-GMP-mediated signaling in *Bacillus subtilis*. *J Bacteriol* **194**, 5080-5090
93. Martinez-Granero, F., Navazo, A., Barahona, E., Redondo-Nieto, M., Gonzalez de Heredia, E., Baena, I., Martin-Martin, I., Rivilla, R., and Martin, M. (2014) Identification of flgZ as a flagellar gene encoding a PilZ domain protein that regulates swimming motility and biofilm formation in *Pseudomonas*. *PLoS One* **9**, e87608
94. Kuchma, S. L., Delalez, N. J., Filkins, L. M., Snavely, E. A., Armitage, J. P., and O'Toole, G. A. (2015) Cyclic di-GMP-mediated repression of swarming motility by *Pseudomonas aeruginosa* PA14 requires the MotAB stator. *J Bacteriol* **197**, 420-430

95. Yang, F., Tian, F., Chen, H., Hutchins, W., Yang, C. H., and He, C. (2015) The *Xanthomonas oryzae* pv. *oryzae* PilZ Domain Proteins Function Differentially in Cyclic di-GMP Binding and Regulation of Virulence and Motility. *Appl Environ Microbiol* **81**, 4358-4367
96. Freedman, J. C., Rogers, E. A., Kostick, J. L., Zhang, H., Iyer, R., Schwartz, I., and Marconi, R. T. (2010) Identification and molecular characterization of a cyclic-di-GMP effector protein, PlzA (BB0733): additional evidence for the existence of a functional cyclic-di-GMP regulatory network in the Lyme disease spirochete, *Borrelia burgdorferi*. *FEMS Immunol Med Microbiol* **58**, 285-294
97. Pitzer, J. E., Sultan, S. Z., Hayakawa, Y., Hobbs, G., Miller, M. R., and Motaleb, M. A. (2011) Analysis of the *Borrelia burgdorferi* cyclic-di-GMP-binding protein PlzA reveals a role in motility and virulence. *Infect Immun* **79**, 1815-1825
98. He, M., Zhang, J. J., Ye, M., Lou, Y., and Yang, X. F. (2014) Cyclic Di-GMP receptor PlzA controls virulence gene expression through RpoS in *Borrelia burgdorferi*. *Infect Immun* **82**, 445-452
99. Wilksch, J. J., Yang, J., Clements, A., Gabbe, J. L., Short, K. R., Cao, H., Cavaliere, R., James, C. E., Whitchurch, C. B., Schembri, M. A., Chuah, M. L., Liang, Z. X., Wijburg, O. L., Jenney, A. W., Lithgow, T., and Strugnell, R. A. (2011) MrkH, a novel c-di-GMP-dependent transcriptional activator, controls *Klebsiella pneumoniae* biofilm formation by regulating type 3 fimbriae expression. *PLoS Pathog* **7**, e1002204
100. Yang, J., Wilksch, J. J., Tan, J. W., Hocking, D. M., Webb, C. T., Lithgow, T., Robins-Browne, R. M., and Strugnell, R. A. (2013) Transcriptional activation of the mrkA promoter of the *Klebsiella pneumoniae* type 3 fimbrial operon by the c-di-GMP-dependent MrkH protein. *PLoS One* **8**, e79038
101. Schumacher, M. A., and Zeng, W. (2016) Structures of the activator of *K. pneumoniae* biofilm formation, MrkH, indicates PilZ domains involved in c-di-GMP and DNA binding. *Proc Natl Acad Sci U S A* **113**, 10067-10072
102. Wang, F., He, Q., Su, K., Gao, F., Huang, Y., Lin, Z., Zhu, D., and Gu, L. (2016) The PilZ domain of MrkH represents a novel DNA binding motif. *Protein & Cell* **7**, 766-772
103. Li, T. N., Chin, K. H., Fung, K. M., Yang, M. T., Wang, A. H., and Chou, S. H. (2011) A novel tetrameric PilZ domain structure from xanthomonads. *PLoS One* **6**, e22036
104. Bian, J., Liu, X., Cheng, Y.-Q., and Li, C. (2013) Inactivation of Cyclic Di-GMP Binding Protein TDE0214 Affects the Motility, Biofilm Formation, and Virulence of *Treponema denticola*. *Journal of Bacteriology* **195**, 3897-3905
105. O'Neal, L., Ryu, M. H., Gomelsky, M., and Alexandre, G. (2017) Optogenetic Manipulation of Cyclic Di-GMP (c-di-GMP) Levels Reveals the Role of c-di-GMP in Regulating Aerotaxis Receptor Activity in *Azospirillum brasilense*. *J Bacteriol* **199**
106. Zorraquino, V., García, B., Latasa, C., Echeverz, M., Toledo-Arana, A., Valle, J., Lasa, I., and Solano, C. (2013) Coordinated Cyclic-Di-GMP Repression of *Salmonella* Motility through YcgR and Cellulose. *Journal of Bacteriology* **195**, 417-428
107. Pultz, I. 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
108. Morgan, J. L., Strumillo, J., and Zimmer, J. (2013) Crystallographic snapshot of cellulose synthesis and membrane translocation. *Nature* **493**, 181-186
109. Weinhouse, H., Sapir, S., Amikam, D., Shilo, Y., Volman, G., Ohana, P., and Benziman, M. (1997) c-di-GMP-binding protein, a new factor regulating cellulose synthesis in *Acetobacter xylinum*. *FEBS Lett* **416**, 207-211
110. Amikam, D., and Benziman, M. (1989) Cyclic diguanylic acid and cellulose synthesis in *Agrobacterium tumefaciens*. *Journal of Bacteriology* **171**, 6649-6655

111. Du, J., Vepachedu, V., Cho, S. H., Kumar, M., and Nixon, B. T. (2016) Structure of the Cellulose Synthase Complex of *Gluconacetobacter hansenii* at 23.4 Å Resolution. *PLoS One* **11**, e0155886
112. Oglesby, L. L., Jain, S., and Ohman, D. E. (2008) Membrane topology and roles of *Pseudomonas aeruginosa* Alg8 and Alg44 in alginate polymerization. *Microbiology* **154**, 1605-1615
113. 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
114. Jenal, U., Reinders, A., and Lori, C. (2017) Cyclic di-GMP: second messenger extraordinaire. *Nature reviews. Microbiology* **15**, 271-284
115. 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
116. 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
117. 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
118. Christen, M., Christen, B., Allan, M. G., Folcher, M., Jenö, 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*. *Proceedings of the National Academy of Sciences* **104**, 4112-4117
119. Pratt, J. T., Tamayo, R., Tischler, 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
120. 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*. *Molecular Plant Pathology* **9**, 819-824
121. Pitzer, J. E., Sultan, S. Z., Hayakawa, Y., Hobbs, G., Miller, M. R., and Motaleb, M. A. (2011) Analysis of the *Borrelia burgdorferi* Cyclic-di-GMP-Binding Protein PlzA Reveals a Role in Motility and Virulence. *Infect. Immun.* **79**, 1815-1825
122. Kulasakara, H., Lee, V., Brencic, A., Liberati, N., Urbach, J., Miyata, S., Lee, D. G., Neely, A. N., Hyodo, M., Hayakawa, Y., Ausubel, F. M., and Lory, S. (2006) Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. *Proc. Natl. Acad. Sci. USA* **103**, 2839-2844
123. Ha, D.-G., Richman, M. E., and O'Toole, G. A. (2014) Deletion Mutant Library for Investigation of Functional Outputs of Cyclic Diguanylate Metabolism in *Pseudomonas aeruginosa* PA14. *Applied and Environmental Microbiology* **80**, 3384-3393
124. Martínez-Granero, F., Navazo, A., Barahona, E., Redondo-Nieto, M., González de Heredia, E., Baena, I., Martín-Martín, I., Rivilla, R., and Martín, M. (2014) Identification of flgZ as a Flagellar Gene Encoding a PilZ Domain Protein That Regulates Swimming Motility and Biofilm Formation in *Pseudomonas*. *PloS one* **9**, e87608
125. Patankar, Y. R., Lovewell, R. R., Poynter, M. E., Jyot, J., Kazmierczak, B. I., and Berwin, B. (2013) Flagellar Motility Is a Key Determinant of the Magnitude of the Inflammasome Response to *Pseudomonas aeruginosa*. *Infection and Immunity* **81**, 2043-2052
126. Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrenner, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S., and Olson, M. V. (2000) Complete

- genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**, 959-964
127. Jacobs, M. A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C., Levy, R., Chun-Rong, L., Guenther, D., Bovee, D., Olson, M. V., and Manoil, C. (2003) Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences* **100**, 14339-14344
 128. An, S., Wu, J., and Zhang, L. H. (2010) Modulation of *Pseudomonas aeruginosa* biofilm dispersal by a cyclic-Di-GMP phosphodiesterase with a putative hypoxia-sensing domain. *Appl. Environ. Microbiol.* **76**, 8160-8173
 129. Schmidt, J., Musken, M., Becker, T., Magnowska, Z., Bertinetti, D., Moller, S., Zimmermann, B., Herberg, F. W., Jansch, L., and Haussler, S. (2011) The *Pseudomonas aeruginosa* chemotaxis methyltransferase CheR1 impacts on bacterial surface sampling. *PloS one* **6**, e18184
 130. Rao, F., Pasunooti, S., Ng, Y., Zhuo, 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
 131. Ivanov, I. E., Boyd, C. D., Newell, P. D., Schwartz, M. E., Turnbull, L., Johnson, M. S., Whitchurch, C. B., O'Toole, G. A., and Camesano, T. A. (2012) Atomic force and super-resolution microscopy support a role for LapA as a cell-surface biofilm adhesin of *Pseudomonas fluorescens*. *Res. Microbiol.* **163**, 685-691
 132. Yu, H. S., and Alam, M. (1997) An agarose-in-plug bridge method to study chemotaxis in the Archaeon *Halobacterium salinarum*. *FEMS microbiology letters* **156**, 265-269
 133. Lowe, D. G. Distinctive Image Features from Scale-Invariant Keypoints. *Inter. J. Comp. Vision* **60**, 91-110
 134. Jaqaman, K., Loerke, D., Mettlen, M., Kuwata, H., Grinstein, S., Schmid, S. L., and Danuser, G. (2008) Robust single-particle tracking in live-cell time-lapse sequences. *Nat Meth* **5**, 695-702
 135. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012) Fiji: an open-source platform for biological-image analysis. *Nat Meth* **9**, 676-682
 136. Alicia, T. G. G., Yang, C., Wang, Z., and Nguyen, N.-T. (2016) Combinational concentration gradient confinement through stagnation flow. *Lab on a Chip* **16**, 368-376
 137. 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
 138. 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
 139. Tolker-Nielsen, T., and Sternberg, C. (2011) Growing and Analyzing Biofilms in Flow Chambers. in *Curr. Protoc. Microbiol.* pp 21:21B.22.21-21B.22.17.
 140. Xu, L., Venkataramani, P., Ding, Y., Liu, Y., Deng, Y., Yong, 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
 141. Christen, M., Kulasekara, H. D., Christen, B., Kulasekara, B. R., Hoffman, L. R., and Miller, S. I. (2010) Asymmetrical Distribution of the Second Messenger c-di-GMP upon Bacterial Cell Division. *Science* **328**, 1295-1297

142. Güvener, Z. T., Tifrea, D. F., and Harwood, C. S. (2006) Two different *Pseudomonas aeruginosa* chemosensory signal transduction complexes localize to cell poles and form and remould in stationary phase. *Molecular microbiology* **61**, 106-118
143. Kulasekara, B. R., Kamischke, C., Kulasekara, H. D., Christen, M., Wiggins, P. A., and Miller, S. I. (2013) c-di-GMP heterogeneity is generated by the chemotaxis machinery to regulate flagellar motility. *eLife* **2**, e01402
144. Rico-Jimenez, M., Munoz-Martinez, F., Garcia-Fontana, C., Fernandez, M., Morel, B., Ortega, A., Ramos, J. L., and Krell, T. (2013) Paralogous chemoreceptors mediate chemotaxis towards protein amino acids and the non-protein amino acid gamma-aminobutyrate (GABA). *Molecular microbiology* **88**, 1230-1243
145. Qian, C., Wong, C. C., Swarup, S., and Chiam, K.-H. (2013) Bacterial Tethering Analysis Reveals a "Run-Reverse-Turn" Mechanism for *Pseudomonas* Species Motility. *App. Environ. Microbiol.* **79**, 4734-4743
146. Duffy, K. J., and Ford, R. M. (1997) Turn angle and run time distributions characterize swimming behavior for *Pseudomonas putida*. *J. Bacteriol.* **179**, 1428-1430
147. Larsen, S. H., Reader, R. W., Kort, E. N., Tso, W.-W., and Adler, J. (1974) Change in direction of flagellar rotation is the basis of the chemotactic response in *Escherichia coli*. *Nature* **249**, 74-77
148. Lapidus, I. R., Welch, M., and Eisenbach, M. (1988) Pausing of flagellar rotation is a component of bacterial motility and chemotaxis. *J. Bacteriol.* **170**, 3627-3632
149. Caiazza, N. C., Merritt, J. H., Brothers, K. M., and O'Toole, G. A. (2007) Inverse Regulation of Biofilm Formation and Swarming Motility by *Pseudomonas aeruginosa* PA14. *J. Bacteriol.* **189**, 3603-3612
150. Morgan, R., Kohn, S., Hwang, S.-H., Hassett, D. J., and Sauer, K. (2006) BdlA, a Chemotaxis Regulator Essential for Biofilm Dispersion in *Pseudomonas aeruginosa*. *J. Bacteriol.* **188**, 7335-7343
151. Chua, S. L., Liu, Y., Yam, J. K. H., Chen, Y., Vejborg, R. M., Tan, B. G. C., Kjelleberg, S., Tolker-Nielsen, T., Givskov, M., and Yang, L. (2014) Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles. *Nat. Commun.* **5**
152. Hickman, J. W., and Harwood, C. S. (2008) Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. *Mol. Microbiol.* **69**
153. Fang, X., and Gomelsky, M. (2010) A post-translational, c-di-GMP-dependent mechanism regulating flagellar motility. *Mol. Microbiol.* **76**, 1295-1305
154. Sultan, S. Z., Pitzer, J. E., Boquoi, T., Hobbs, G., Miller, M. R., and Motaleb, M. A. (2011) Analysis of the HD-GYP Domain Cyclic Dimeric GMP Phosphodiesterase Reveals a Role in Motility and the Enzootic Life Cycle of *Borrelia burgdorferi*. *Infection and immunity* **79**, 3273-3283
155. Kim, H. E., Shitashiro, M., Kuroda, A., Takiguchi, N., Ohtake, H., and Kato, J. (2006) Identification and characterization of the chemotactic transducer in *Pseudomonas aeruginosa* PAO1 for positive chemotaxis to trichloroethylene. *J. Bacteriol.* **188**, 6700-6702
156. Sampedro, I., Parales, R. E., Krell, T., and Hill, J. E. (2014) *Pseudomonas* chemotaxis. *FEMS Microbiology Reviews* **39**, 17-46
157. Guvener, Z. T., Tifrea, D. F., and Harwood, C. S. (2006) Two different *Pseudomonas aeruginosa* chemosensory signal transduction complexes localize to cell poles and form and remould in stationary phase. *Mol Microbiol* **61**, 106-118
158. Schuster, M., Hawkins, A. C., Harwood, C. S., and Greenberg, E. P. (2004) The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. *Mol Microbiol* **51**, 973-985
159. Vladimirov, N., and Sourjik, V. (2009) Chemotaxis: how bacteria use memory. *Biological chemistry* **390**, 1097-1104

160. Garcia-Fontana, C., Reyes-Darias, J. A., Munoz-Martinez, F., Alfonso, C., Morel, B., Ramos, J. L., and Krell, T. (2013) High specificity in CheR methyltransferase function: CheR2 of *Pseudomonas putida* is essential for chemotaxis, whereas CheR1 is involved in biofilm formation. *The Journal of biological chemistry* **288**, 18987-18999
161. Garcia-Fontana, C., Corral Lugo, A., and Krell, T. (2014) Specificity of the CheR2 methyltransferase in *Pseudomonas aeruginosa* is directed by a C-terminal pentapeptide in the McpB chemoreceptor. *Science signaling* **7**, ra34
162. Wadhams, G. H., and Armitage, J. P. (2004) Making sense of it all: Bacterial chemotaxis. *Nat. Rev. Mol. Cell. Biol.* **5**, 1024-1037
163. Galperin, M. Y. (2004) Bacterial signal transduction network in a genomic perspective. *Environ. Microbiol.* **6**, 552-567
164. Lyczak, J. B., Cannon, C. L., and Pier, G. B. (2000) Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist¹*Address for correspondence: Channing Laboratory, 181 Longwood Avenue, Boston, MA 02115, USA. *Microbes and Infection* **2**, 1051-1060
165. Feldman, M., Bryan, R., Rajan, S., Scheffler, L., Brunnert, S., Tang, H., and Prince, A. (1998) Role of Flagella in Pathogenesis of *Pseudomonas aeruginosa* Pulmonary Infection. *Infection and immunity* **66**, 43-51
166. Sadikot, R. T., Blackwell, T. S., Christman, J. W., and Prince, A. S. (2005) Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. *American journal of respiratory and critical care medicine* **171**, 1209-1223
167. Costerton, J. W. (2001) Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. *Trends in Microbiology* **9**, 50-52
168. George A. O'Toole, R. K. (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* **30**, 295-304
169. Klausen, M., Heydorn, A., Ragas, P., Lambertsen, L., Aaes-Jorgensen, A., Molin, S., and Tolker-Nielsen, T. (2003) Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol. Microbiol.* **48**, 1511-1524
170. Lovewell, R. R., Hayes, S. M., O'Toole, G. A., and Berwin, B. (2014) *Pseudomonas aeruginosa* flagellar motility activates the phagocyte PI3K/Akt pathway to induce phagocytic engulfment. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **306**, L698-L707
171. Kulasakara, H., Lee, V., Brencic, A., Liberati, N., Urbach, J., Miyata, S., Lee, D. G., Neely, A. N., Hyodo, M., Hayakawa, Y., Ausubel, F. M., and Lory, S. (2006) Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 2839-2844
172. Kuchma, S. L., Brothers, K. M., Merritt, J. H., Liberati, N. T., Ausubel, F. M., and O'Toole, G. A. (2007) BifA, a Cyclic-Di-GMP Phosphodiesterase, Inversely Regulates Biofilm Formation and Swarming Motility by *Pseudomonas aeruginosa* PA14. *J. Bacteriol.* **189**, 8165-8178
173. Merritt, J. H., Brothers, K. M., Kuchma, S. L., and O'Toole, G. A. (2007) SadC Reciprocally Influences Biofilm Formation and Swarming Motility via Modulation of Exopolysaccharide Production and Flagellar Function. *J. Bacteriol.* **189**, 8154-8164
174. Baker, A. E., Diepold, A., Kuchma, S. L., Scott, J. E., Ha, D. G., Orazi, G., and Armitage, J. P. (2016) PilZ Domain Protein FlgZ Mediates Cyclic Di-GMP-Dependent Swarming Motility Control in *Pseudomonas aeruginosa*. **198**, 1837-1846
175. Petrova, O. E., and Sauer, K. (2017) High-performance liquid chromatography (HPLC)-based detection and quantitation of cellular c-di-GMP. *Methods in molecular biology (Clifton, N.J.)* **1657**, 33-43

176. Roy, A. B., Petrova, O. E., and Sauer, K. (2013) Extraction and Quantification of Cyclic Di-GMP from *P. aeruginosa*. *Bio-protocol* **3**, e828
177. Spangler, C., Böhm, A., Jenal, U., Seifert, R., and Kaefer, V. (2010) A liquid chromatography-coupled tandem mass spectrometry method for quantitation of cyclic di-guanosine monophosphate. *Journal of microbiological methods* **81**, 226-231
178. Fong, J., Yuan, M., Jakobsen, T. H., Mortensen, K. T., Delos Santos, M. M. S., Chua, S. L., Yang, L., Tan, C. H., Nielsen, T. E., and Givskov, M. (2017) Disulfide Bond-Containing Ajoene Analogues As Novel Quorum Sensing Inhibitors of *Pseudomonas aeruginosa*. *Journal of Medicinal Chemistry* **60**, 215-227
179. Nair, H. A., Periasamy, S., Yang, L., Kjelleberg, S., and Rice, S. A. (2017) Real Time, Spatial, and Temporal Mapping of the Distribution of c-di-GMP during Biofilm Development. *The Journal of biological chemistry* **292**, 477-487
180. Rybtke, M. T., Borlee, B. R., Murakami, K., Irie, Y., Hentzer, M., Nielsen, T. E., Givskov, M., Parsek, M. R., and Tolker-Nielsen, T. (2012) Fluorescence-Based Reporter for Gauging Cyclic Di-GMP Levels in *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology* **78**, 5060-5069
181. An, S., Wu, J. e., and Zhang, L.-H. (2010) Modulation of *Pseudomonas aeruginosa* Biofilm Dispersal by a Cyclic-Di-GMP Phosphodiesterase with a Putative Hypoxia-Sensing Domain. *Applied and Environmental Microbiology* **76**, 8160-8173
182. Li, Y., Heine, S., Entian, M., Sauer, K., and Frankenberg-Dinkel, N. (2013) NO-Induced Biofilm Dispersion in *Pseudomonas aeruginosa* Is Mediated by an MHYT Domain-Coupled Phosphodiesterase. *Journal of bacteriology* **195**, 3531-3542
183. Rao, F., Yang, Y., Qi, Y., and Liang, Z. X. (2008) Catalytic mechanism of c-di-GMP specific phosphodiesterase: a study of the EAL domain-containing RocR from *Pseudomonas aeruginosa*. *J. Bacteriol.* **190**, 3622-3631
184. Chen, M. W., Kotaka, M., Vornrhein, C., Bricogne, G., Rao, F., Chuah, M. L., Svergun, D., Schneider, G., Liang, Z. X., and Lescar, J. (2012) Structural insights into the regulatory mechanism of the response regulator RocR from *Pseudomonas aeruginosa* in cyclic Di-GMP signaling. *J Bacteriol* **194**, 4837-4846
185. Xu, L., Xin, L., Zeng, Y., Yam, J. K. H., 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. *Science signaling* **9**, ra102-ra102
186. Dötsch, A., Klawonn, F., Jarek, M., Scharfe, M., Blöcker, H., and Häussler, S. (2010) Evolutionary conservation of essential and highly expressed genes in *Pseudomonas aeruginosa*. *BMC Genomics* **11**, 234
187. Russell, M. H., Bible, A. N., Fang, X., Gooding, J. R., Campagna, S. R., Gomelsky, M., and Alexandre, G. (2013) Integration of the Second Messenger c-di-GMP into the Chemotactic Signaling Pathway. *mBio* **4**
188. O'Neal, L., Ryu, M.-H., Gomelsky, M., and Alexandre, G. (2017) Optogenetic Manipulation of Cyclic Di-GMP (c-di-GMP) Levels Reveals the Role of c-di-GMP in Regulating Aerotaxis Receptor Activity in *Azospirillum brasilense*. *Journal of Bacteriology* **199**
189. Li, Y., Heine, S., Entian, M., Sauer, K., and Frankenberg-Dinkel, N. (2013) NO-induced biofilm dispersion in *Pseudomonas aeruginosa* is mediated by an MHYT domain-coupled phosphodiesterase. *J. Bacteriol.* **195**, 3531-3542
190. Roy, A. B., Petrova, O. E., and Sauer, K. (2012) The phosphodiesterase DipA (PA5017) is essential for *Pseudomonas aeruginosa* biofilm dispersion. *J Bacteriol* **194**, 2904-2915
191. Li, Y., Petrova, O. E., Su, S., Lau, G. W., Panmanee, W., Na, R., Hassett, D. J., Davies, D. G., and Sauer, K. (2014) BdlA, DipA and Induced Dispersion Contribute to Acute

- Virulence and Chronic Persistence of *Pseudomonas aeruginosa*. *PLOS Pathogens* **10**, e1004168
192. Petrova, O. E., and Sauer, K. (2012) Dispersion by *Pseudomonas aeruginosa* requires an unusual posttranslational modification of BdlA. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 16690-16695
 193. Ozaki, S., Schalch-Moser, A., Zumthor, L., Manfredi, P., Ebbensgaard, A., Schirmer, T., and Jenal, U. (2014) Activation and polar sequestration of PopA, a c-di-GMP effector protein involved in *Caulobacter crescentus* cell cycle control. *Mol. Microbiol.* **94**, 580-594
 194. Dahlstrom, K. M., and O'Toole, G. A. (2017) A Symphony of Cyclases: Specificity in Diguanylate Cyclase Signaling. *Annu Rev Microbiol*
 195. Kabsch, W. (2010) XDS. *Acta crystallographica. Section D, Biological crystallography* **66**, 125-132
 196. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., 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., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallographica Section D: Biological Crystallography* **66**, 213-221
 197. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta crystallographica. Section D, Biological crystallography* **66**, 486-501
 198. Hunter, S., Apweiler, R., Attwood, T. K., Bairoch, A., Bateman, A., Binns, D., Bork, P., Das, U., Daugherty, L., Duquenne, L., Finn, R. D., Gough, J., Haft, D., Hulo, N., Kahn, D., Kelly, E., Laugraud, A., Letunic, I., Lonsdale, D., Lopez, R., Madera, M., Maslen, J., McAnulla, C., McDowall, J., Mistry, J., Mitchell, A., Mulder, N., Natale, D., Orengo, C., Quinn, A. F., Selengut, J. D., Sigrist, C. J. A., Thimma, M., Thomas, P. D., Valentin, F., Wilson, D., Wu, C. H., and Yeats, C. (2009) InterPro: the integrative protein signature database. *Nucleic Acids Research* **37**, D211-D215
 199. 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
 200. Whitney, J. C., Whitfield, G. B., Marmont, L. S., Yip, P., Neculai, A. M., Lobsanov, Y. D., Robinson, H., Ohman, D. E., and Howell, P. L. (2015) Dimeric c-di-GMP is Required for Post-translational Regulation of Alginate Production in *Pseudomonas aeruginosa*. *J. Biol. Chem.*
 201. 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* **4**, 02463-02461
 202. Schumacher, M. A., and Zeng, W. (2016) Structures of the activator of *K. pneumonia* biofilm formation, MrkH, indicates PilZ domains involved in c-di-GMP and DNA binding. *Proc. Natl. Acad. Sci. USA* **113**, 10067-10072
 203. Boriack-Sjodin, P. A., and Swinger, K. K. (2016) Protein Methyltransferases: A Distinct, Diverse, and Dynamic Family of Enzymes. *Biochemistry* **55**, 1557-1569
 204. Djordjevic, S., Goudreau, P. N., Xu, Q., Stock, A. M., and West, A. H. (1998) Structural basis for methylesterase CheB regulation by a phosphorylation-activated domain. *Proc. Natl. Acad. Sci. USA* **95**, 1381-1386
 205. Batra, M., Sharma, R., Malik, A., Dhindwal, S., Kumar, P., and Tomar, S. (2016) Crystal structure of pentapeptide-independent chemotaxis receptor methyltransferase (CheR) reveals idiosyncratic structural determinants for receptor recognition. *J. Struct. Biol.* **196**, 364-374

206. Djordjevic, S., and Stock, A. M. (1997) Crystal structure of the chemotaxis receptor methyltransferase CheR suggests a conserved structural motif for binding S-adenosylmethionine. *Structure* **5**, 545-558
207. Ramelot, T. A., Yee, A., Cort, J. R., Semesi, A., Arrowsmith, C. H., and Kennedy, M. A. (2007) NMR structure and binding studies confirm that PA4608 from *Pseudomonas aeruginosa* is a PilZ domain and a c-di-GMP binding protein. *Proteins: Structure, Function, and Bioinformatics* **66**, 266-271
208. Ferrandez, A., Hawkins, A. C., Summerfield, D. T., and Harwood, C. S. (2002) Cluster II che genes from *Pseudomonas aeruginosa* are required for an optimal chemotactic response. *Journal of Bacteriology* **184**, 4374-4383
209. Matsuyama, B. Y., Krasteva, P. V., Baraquet, C., Harwood, C. S., Sondermann, H., and Navarro, M. V. A. S. (2016) Mechanistic insights into c-di-GMP-dependent control of the biofilm regulator FleQ from *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences* **113**, E209-E218
210. Paul, R., Abel, S., Wassmann, P., Beck, A., Heerklotz, H., and Jenal, U. (2007) Activation of the diguanylate cyclase pleD by phosphorylation-mediated dimerization. *J. Biol. Chem.* **282**, 29170-29177
211. Wolfe, A. J., Conley, M. P., and Berg, H. C. (1988) Acetyladenylate plays a role in controlling the direction of flagellar rotation. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 6711-6715
212. Chen, Y., Yuan, M., Mohanty, A., Yam, J. K., Liu, Y., Chua, S. L., Nielsen, T. E., Tolker-Nielsen, T., Givskov, M., Cao, B., and Yang, L. (2015) Multiple diguanylate cyclase-coordinated regulation of pyoverdine synthesis in *Pseudomonas aeruginosa*. *Environmental microbiology reports* **7**, 498-507
213. Rybtke, M. T., Borlee, B. R., Murakami, K., Irie, Y., Hentzer, M., Nielsen, T. E., Givskov, M., Parsek, M. R., and Tolker-Nielsen, T. (2012) Fluorescence-based reporter for gauging cyclic di-GMP levels in *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **78**, 5060-5069

APPENDIX

Figure S1 Measurement of the c-di-GMP binding affinity for MapZ and MapZ^{R13A} mutant by ITC. (A) Titration of c-di-GMP into the MapZ solution yielded a K_d of $8.8 \pm 1.2 \mu\text{M}$ (mean $\pm$ SD) using a single-site binding model. The raw binding isotherm and data-fitting curve are shown in the top and bottom panels respectively. (B) Titration of c-di-GMP into the MapZ^{R13A} solution showed that R13A mutation completely abolished c-di-GMP binding, as evidenced by the similar endothermic profiles observed from the titration of c-di-GMP into buffer in panel C. (C). Control experiment showed the titration of c-di-GMP into assay buffer. Similar isotherms have been documented previously for the dilution of c-di-GMP solution and signals are attributed to a change of the oligomeric form of c-di-GMP during the titration (or dilution) process (209,210). Data in figures are representative of three independent experiments. This study was done with help from Dr. Tang Xuhua, Singapore (1).

Figure S2 *P. aeruginosa* PAO1 and mutants responding to L-serine in the agar plug chemotaxis assay. For each plate, the three agar plugs that do not contain L-serine are located on the left and the four agar plugs that contain L-serine include the one in the middle and three on the right. Photograph was taken after 10 min after adding the liquid medium containing the bacterial cells to the petri dish. The white particles observed in the *mapZ*⁺ strain were caused by cell aggregation. Data in figures are representative of three independent experiments.

Figure S3 Average flagellar rotational speed of *P. aeruginosa* PAO1 and mutants in cell tethering assay. Data are means $\pm$ SD (n = 12 to 37 cells) from two independent experiments (two-tailed *t* test, **P* < 0.05). This study was done in collaboration with Prof. Chiam Keng-Hwee's lab, Singapore.

Figure S4 Controls for the ITC between CheR1 and MapZ wild type and mutants. CheR1 was titrated to the MapZ and mutants without c-di-GMP.

Figure S5 Stereoview showing MapZ-CheR1 complex fitted to the 2Fo-Fc electron-density map. The 2Fo-Fc composite omit map (blue mesh) was calculated with 3,000 K simulated annealing using Phenix (196) and was contoured at 1σ . The simulated-annealing omit map supports the overall arrangement of MapZ-CheR1 in complex with c-di-GMP. The MapZ (green), CheR1 (salmon), and c-di-GMP (stick model) were represented as that in the main text, respectively. This study was done in collaboration with Prof. Gao Yong-Gui's lab, Singapore (2).

Figure S6 The overall structure of CheR1 complexed with c-di-GMP bound MapZ (A) and the Fo-Fc omit map of c-di-GMP (B). After the removal of c-di-GMP from the complex model, refinement of the ligand-omitted complex was carried out using Phenix (196). The omit map, shown as green mesh, is contoured at 1σ . This study was done in collaboration with Prof. Gao Yong-Gui's lab, Singapore (2).

Figure S7 Sequence alignment of the present CheR1 with other CheR proteins from various organisms. Amino acid sequences of CheR from *Pseudomonas aeruginosa* (O87131), *Clostridium acetobutylicum* (Q97GZ4), *Campylobacter jejuni* (Q0P9X6), *Thermotoga maritima* (Q9WYT5), *Bacillus subtilis* (P31105), *Salmonella typhimurium* (P07801), *Shigella flexneri* (Q0T3N6), *Yersinia pestis* (Q8D0P3), *Photobacterium luminescens* (Q7N5T2) and *Escherichia coli* (P07364) are aligned using Clustal Omega and manually adjusted in Jalview (Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. (2011 October 11) *Molecular systems biology* 7 :539, PMID: 21988835). Conserved residues are color-coded (D,E in purple; R,K in red; N,Q,S,T in green; L, I, V, F, Y, W, M, C in blue, P in yellow and G in brown). The secondary structure elements shown above the sequence are from the isolated CheR1 in this study. Asterisk indicates the critical residues involved in SAM/SAH recognition (Asp144) and catalysis (Tyr222), while arrowheads indicate conserved Glycine embedded in the β -subdomain. The conserved motifs are boxed in dashed line: box1 for recognition motif of the methylation site (especially two conserved, positively charged residues); box2 for β -subdomain (PDCheR classes: involved in downstream protein interaction; PICheR classes: unknown); box3 for conserved catalytic motif. This study was done in collaboration with Prof. Gao Yong-Gui's lab, Singapore (2).

Figure S8 Total protein quantification of MapZ and mutants overexpression strains. Cell pellet for 1 ml overnight culture of each strain was dissolved in 1 ml 0.1 M NaOH. After boiling for 10 min, the solutions were clarified by centrifugation. Ten microliter of supernatant for each sample was used for standard Bradford Assay (Bio-Rad). Data were obtained by averaging data of four replicates from two independent experiments.

Figure S9 Stereoview showing CheR1 fitted to the 2Fo-Fc electron-density map. The 2Fo-Fc composite omit map was calculated with 3,000 K simulated annealing using Phenix (196) and was contoured at 1 σ . The simulated-annealing omit map clearly shows the N-terminal and C-terminal domains, as well as the domain linker, in support of our overall arrangement of apo CheR1 modelled at 3.4 Å. This study was done in collaboration with Prof. Gao Yong-Gui's lab, Singapore (2).

Figure S10 Comparison of the structures of CheR1 and BsCheR. The apo CheR1 (magenta, the present study), *BsCheR* (grey, PDB:5FTW), and MapZ-bound CheR1 (green, the present complex) are aligned by using their C-terminal domains. The ligand SAH in *BsCheR*, is shown in sticks and atoms are color-coded (oxygen in red, nitrogen in blue and carbon in yellow). The C-terminal domains of all structures superposed quite well, but the N-terminal domains adopt different orientations in relating to their C-terminal domains, demonstrating the flexibility of the domain linker and the effect of MapZ binding. This study was done in collaboration with Prof. Gao Yong-Gui's lab, Singapore (2).

Figure S11 Structural comparison of the N-terminal domains of apo CheR1, MapZ-bound CheR1 and SAH-bound *BsCheR*. (A) Superposition of the N-terminal domains of MapZ-bound CheR (green) and apo CheR (magenta). (B) Superposition of the N-terminal domains of MapZ-bound CheR (green) with *BsCheR* (grey, PDB: 5FTW). The structural arrangement of the N-terminal domain in the present CheR1 resembles that of *BsCheR*, and the two domains were superimposed with a Z-score of 8.1 Å and a RMSD of 2.4 Å for C α chain, respectively. This study was done in collaboration with Prof. Gao Yong-Gui's lab, Singapore (2).

Table S1 List of bacterial strains used in this study.

Strains	Relevant characteristics	Source
BacterioMatch II reporter strain	Host strain used for library screening and detection of protein-protein interactions in BacterioMatch II two-hybrid system	Stratagene Catalog #200195
<i>E. coli</i>		
<i>E. coli</i> DH5α	Cells for cloning	Invitrogen
<i>E. coli</i> TOP10	Cells for cloning	Invitrogen
<i>E. coli</i> BL21(DE3)	Cells for high level expression of heterologous proteins in <i>E. coli</i>	Stratagene
<i>E. coli</i> HCB721	Cells for expression of <i>P. aeruginosa</i> MCP pctA	(211)
<i>P. aeruginosa</i> PAO1		
PAO1	Wild type strain from the Washington Genome Center PAO1 mutant library	(127)
<i>ΔcheR1</i>	PA3348 transposon mutant PW6640 from the Washington Genome Center PAO1 mutant library	(127)
<i>ΔmapZ</i>	PA4608 transposon mutant PW8764 from the Washington Genome Center PAO1 mutant library	(127)
<i>mapZ</i> ⁺	PA4608 overexpression strain, mPAO1 strain containing pUCP18-HA-MapZ, Car ^r	This study
<i>mapZ</i> ^{R13A}	PA4608-R13A mutation strain, mPAO1 strain containing R13A mutation of PA4608 in genomic DNA	(1)
<i>mapZ</i> ^{R13A+}	PA4608-R13A overexpression strain, mPAO1 strain containing pUCP18-HA-MapZ ^{R13A} , Car ^r	This study
<i>mapZ</i> ^{D93A+}	PA4608-D93A overexpression strain, mPAO1 strain containing pUCP18-HA-MapZ ^{D93A} , Car ^r	This study
<i>MapZ</i> ^{H97A+}	PA4608-H97A overexpression strain, mPAO1 strain containing pUCP18-HA-MapZ ^{H97A} , Car ^r	This study
<i>mapZ</i> ^{R99A+}	PA4608-R99A overexpression strain, mPAO1 strain containing pUCP18-HA-MapZ ^{R99A} , Car ^r	This study
<i>mapZ</i> ^{R100A+}	PA4608-R100A overexpression strain, mPAO1 strain containing pUCP18-HA-MapZ ^{R100A} , Car ^r	This study
<i>mapZ</i> ^{L04A+}	PA4608-L104A overexpression strain, mPAO1 strain containing pUCP18-HA-MapZ ^{L104A} , Car ^r	This study
<i>PA0285::Tn</i>	PA0285 transposon mutant PW1521 from the Washington Genome Center PAO1 mutant library	(127)

<i>PA0575::Tn</i>	PA0575 transposon mutant PW2060 from the Washington Genome Center PAO1 mutant library	(127)
<i>rbdA::Tn</i>	PA0861 transposon mutant PW2570 from the Washington Genome Center PAO1 mutant library	(127)
<i>PA1181::Tn</i>	PA1181 transposon mutant PW3133 from the Washington Genome Center PAO1 mutant library	(127)
<i>mucR::Tn</i>	PA1727 transposon mutant PW4043 from the Washington Genome Center PAO1 mutant library	(127)
<i>PA2072::Tn</i>	PA2072 transposon mutant PW4568 from the Washington Genome Center PAO1 mutant library	(127)
<i>PA2567::Tn</i>	PA2567 transposon mutant PW5309 from the Washington Genome Center PAO1 mutant library	(127)
<i>PA3258::Tn</i>	PA3258 transposon mutant PW6466 from the Washington Genome Center PAO1 mutant library	(127)
<i>nbdA::Tn</i>	PA3311 transposon mutant PW6568 from the Washington Genome Center PAO1 mutant library	(127)
<i>bifA::Tn</i>	PA4367 transposon mutant PW8372 from the Washington Genome Center PAO1 mutant library	(127)
<i>morA::Tn</i>	PA4601 transposon mutant PW8754 from the Washington Genome Center PAO1 mutant library	(127)
<i>dipA::Tn</i>	PA5017 transposon mutant PW9424 from the Washington Genome Center PAO1 mutant library	(127)
<i>PA5295::Tn</i>	PA5295 transposon mutant PW9918 from the Washington Genome Center PAO1 mutant library	(127)
<i>PA5442::Tn</i>	PA5442 transposon mutant PW10193 from the Washington Genome Center PAO1 mutant library	(127)
<i>PA2133::Tn</i>	PA2133 transposon mutant PW4665 from the Washington Genome Center PAO1 mutant library	(127)
<i>PA2200::Tn</i>	PA2200 transposon mutant PW4748 from the Washington Genome Center PAO1 mutant library	(127)
<i>arr::Tn</i>	PA2818 transposon mutant PW5718 from the Washington Genome Center PAO1 mutant library	(127)

<i>PA3825::Tn</i>	PA3825 transposon mutant PW7454 from the Washington Genome Center PAO1 mutant library	(127)
<i>rocR::Tn</i>	PA3947 transposon mutant PW7674 from the Washington Genome Center PAO1 mutant library	(127)
<i>PA2572::Tn</i>	PA2572 transposon mutant PW5317 from the Washington Genome Center PAO1 mutant library	(127)
<i>PA4108::Tn</i>	PA4108 transposon mutant PW7949 from the Washington Genome Center PAO1 mutant library	(127)
<i>PA4781::Tn</i>	PA4781 transposon mutant PW9032 from the Washington Genome Center PAO1 mutant library	(127)
<i>rbdA^{PDE*}</i>	mPAO1 strain containing <i>E585A&L586A&L587A</i> mutation of PA0861 in genomic DNA	This study
<i>nbdA^{PDE*}</i>	mPAO1 strain containing <i>E551A&L553A</i> mutation of PA3311 in genomic DNA	This study
<i>dipA^{PDE*}</i>	mPAO1 strain containing <i>E675A&L677A</i> mutation of PA5017 in genomic DNA	This study
<i>rbdA^{PDE*}&mapZ^{R13A}</i>	<i>mapZ^{R13A}</i> strain containing <i>E585A&L586A&L587A</i> mutation of PA0861 in genomic DNA	This study
<i>nbdA^{PDE*}&mapZ^{R13A}</i>	<i>mapZ^{R13A}</i> strain containing <i>E551A&L553A</i> mutation of PA3311 in genomic DNA	This study
<i>dipA^{PDE*}&mapZ^{R13A}</i>	<i>mapZ^{R13A}</i> strain containing <i>E675A&L677A</i> mutation of PA5017 in genomic DNA	This study
PAO1/pYedQ	mPAO1 strain containing pBBR1MCS-5-yedQ, Gm ^r	This study
<i>mapZ^{R13A}</i> /pYedQ	<i>mapZ^{R13A}</i> strain containing pBBR1MCS-5-yedQ, Gm ^r	This study
<i>P. aeruginosa</i> PA14		
PA14	Wild type strain	Dr. Wei-Hui Wu
<i>mapZ::Tn</i>	PA4608 transposon mutant	Dr. Wei-Hui Wu
<i>cheRI::Tn</i>	PA3348 transposon mutant	Dr. Wei-Hui Wu
<i>pctA::Tn</i>	PA4309 transposon mutant	Dr. Wei-Hui Wu

^a Amp^r, ampicillin resistance; Car^r, carbenicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamycin resistance; Kan^r, kanamycin resistance; Tet^r, tetracycline resistance;.

^b The antibiotic concentrations used were as follows: for *E.coli*, ampicillin at 100µg/ml, chloramphenicol at 12.5µg/ml, gentamycin at 20 µg/ml, kanamycin at 50µg/ml, tetracycline at 25µg/ml; and for *P. aeruginosa*, carbenicillin at 300µg/ml, gentamycin at 40 µg/ml.

Table S2 List of plasmids used in this study.

Plasmids	Relevant characteristics	Source
pBT	Empty bait plasmid used for detection of protein-protein interactions in BacterioMatch II two-hybrid system, Cm ^r	Agilent Technologies Catalog # 240067
pBT-LGF2	Positive interaction control plasmid in BacterioMatch II two-hybrid system, encoding dimerization domain of Gal4 transcriptional activator protein, Cm ^r	Agilent Technologies Catalog # 240065
pBT-MapZ	A DNA fragment encoding full length of PA4608 cloned into pBT vector EcoRI/BamHI, Cm ^r	(1)
pBT-YFP-MapZ	YFP-PA4608 gene cloned into pBT vector for preparation of R13A mutation of PA4608, EcoRI/BamHI, Cm ^r	This study
pTRG	Empty prey plasmid used for detection of protein-protein interactions in BacterioMatch II two-hybrid system, Tetr ^r	Agilent Technologies Catalog #240066
pTRG-Gal11 ^P	Positive interaction control plasmid in BacterioMatch II two-hybrid system, encoding a domain of mutant form of Gal11 protein, Tetr ^r	Agilent Technologies Catalog # 240065
pTRGA	A pTRG-derived mutation was made by insertion of the nucleotides 'A' between the end of C-terminal of RNAP-alpha(992bp) and BamHI site(993bp) of vector pTRG, Tetr ^r	(1)
pTRGAC	A pTRG-derived mutation was made by insertion of the nucleotides 'AC' between the end of C-terminal of RNAP-alpha(992bp) and BamHI site(993bp) of vector pTRG, Tetr ^r	(1)
pTRG-CheR1	A DNA fragment encoding full length of PA3348 cloned into pBT vector EcoRI/BamHI, Tetr ^r	(1)
pET28b(+)	Plasmid for overexpression of proteins with N and C terminal 6×His tags, Kan ^r	Novagen
pET28b-MapZ	PA4608 gene with stop codon cloned into pET28b(+) NdeI/HindIII, Kan ^r	This study
pET28b-MapZ ^{R13A}	PA4608_R13A gene with stop codon cloned into pET28b(+) NdeI/HindIII, Kan ^r	This study
pET28b-MapZ ^{D93A}	PA4608_D93A gene with stop codon cloned into pET28b(+) NdeI/HindIII, Kan ^r	This study

pET28b-MapZ ^{H97A}	PA4608_H97A gene with stop codon cloned into pET28b(+) NdeI/HindIII, Kan ^r	This study
pET28b-MapZ ^{R99A}	PA4608_R99A gene with stop codon cloned into pET28b(+) NdeI/HindIII, Kan ^r	This study
pET28b-MapZ ^{R100A}	PA4608_R100A gene with stop codon cloned into pET28b(+) NdeI/HindIII, Kan ^r	This study
pET28b-MapZ ^{L104A}	PA4608_L104A gene with stop codon cloned into pET28b(+) NdeI/HindIII, Kan ^r	This study
pET28b-MapZ ^{Δα2}	PA4608_Δα2 gene with stop codon cloned into pET28b(+) NdeI/HindIII, Kan ^r	This study
pET28b-CheR1	PA3348 gene with stop codon cloned into pET28b(+) NdeI/HindIII, Kan ^r	This study
pUCP18	<i>E.coli-P. aeruginosa</i> shuttle expression vector with P _{lac} , Amp ^r /Car ^r	This study
pUCP18-MapZ	MapZ gene cloned into pUCP18 vector, Amp ^r /Car ^r	This study
pUCP18-HA-MapZ	MapZ gene fused with N-terminal Ribosomal binding site and HA tag cloned into pUCP18 vector, Amp ^r /Car ^r	This study
pUCP18-HA-MapZ ^{R13A}	PA4608_R13A gene with stop codon cloned into pUCP18-HA vector, Amp ^r /Car ^r	This study
pUCP18-HA-MapZ ^{D93A}	PA4608_D93A gene with stop codon cloned into pUCP18-HA vector, Amp ^r /Car ^r	This study
pUCP18-HA-MapZ ^{H97A}	PA4608_H97A gene with stop codon cloned into pUCP18-HA vector, Amp ^r /Car ^r	This study
pUCP18-HA-MapZ ^{R99A}	PA4608_R99A gene with stop codon cloned into pUCP18-HA vector, Amp ^r /Car ^r	This study
pUCP18-HA-MapZ ^{R100A}	PA4608_R100A gene with stop codon cloned into pUCP18-HA vector, Amp ^r /Car ^r	This study
pUCP18-HA-MapZ ^{L104A}	PA4608_L104A gene with stop codon cloned into pUCP18-HA vector, Amp ^r /Car ^r	This study
pUCP18-YFP-MapZ	YFP-PA4608 gene cloned into pUCP18 vector, BamHI/EcoRI, Amp ^r /Car ^r	This study
pUCP18-YFP-MapZ ^{R13A}	YFP-PA4608 ^{R13A} gene cloned into pUCP18 vector, BamHI/EcoRI, Amp ^r /Car ^r	This study

pUCP18-6xHis-CheR1	PA3348 gene fused with N-terminal Ribosomal binding site and 6×His tag cloned into pUCP18 vector, Amp ^r /Car ^r	This study
pUCP18-6xHis-CheR1-HA-MapZ	PA4608 and PA3348 gene fused with N-terminal Ribosomal binding site, and HA and 6×His tag respectively cloned into pUCP18 vector, Amp ^r /Car ^r	This study
pUC57-YFP-MapZ	YFP-PA4608 gene cloned into pUC57 vector, BamHI/EcoRI, Amp ^r	Bio Basic Inc.
pHSe5-pctA	PA4309 gene with stop codon cloned into pHSe5 vector, inducible with IPTG, Amp ^r	(129)
pK18GT	pK18mobsacB with the Gmr cassette inserted at NcoI site, Gm ^r	(128)
pK18GT-mapZ ^{R13A}	pK18GT containing mapZ ^{R13A} gene for generation of mapZ ^{R13A} point mutant, Gm ^r	This study
pK18GT- <i>rbdAAAA</i>	pK18GT containing <i>E585A&L586A&L587A</i> mutation of PA0861 gene, Gm ^r	This study
pK18GT- <i>nbdAAAA</i>	pK18GT containing <i>E551A&L553A</i> mutation of PA3311 gene, Gm ^r	This study
pK18GT- <i>dipAAAA</i>	pK18GT containing <i>E675A&L677A</i> mutation of PA5017 gene, Gm ^r	This study
pRK2013	Helper vector for triparental mating used in conjugations, Kan ^r	(128)
RK600	Helper vector for triparental mating used in conjugations, Cm ^r	(212,213)
pBBR1MCS-5-yedQ	pBBR1MCS-5 containing <i>yedQ</i> gene, Gm ^r	(212)
pCdrA::gfp	pUCP22Not-P _{cdrA} -RBS-CDS-RNaseIII-gfp(Mut3)-T ₀ -T ₁ , Amp ^r /Gm ^r	(213)
pACYCDuet	Plasmid for overexpression of proteins with N and C terminal 6×His tags, Chl ^r	Novagen
pACYCDuet-MapZ	PA3348 gene with stop codon cloned into pACYCDuet vector BamHI/HindIII, Chl ^r	(2)
pACYCDuet-CheR1	PA4608 gene with stop codon cloned into pACYCDuet vector BamHI/XhoI, Chl ^r	(2)

^a Amp^r, ampicillin resistance; Car^r, carbenicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamycin resistance; Kan^r, kanamycin resistance; Tetr^r, tetracycline resistance;.

^b The antibiotic concentrations used were as follows: for *E.coli*, ampicillin at 100µg/ml, chloramphenicol at 12.5µg/ml, gentamycin at 20 µg/ml, kanamycin at 50µg/ml, tetracycline at 25µg/ml; and for *P. aeruginosa*, carbenicillin at 300µg/ml, gentamycin at 40 µg/ml.

Table S3 List of primers used in this study.

Primers	Sequence (5' - 3')	Application
pTRGFA	AAACCAGAGGCGGCC A GGATC CGCGGCCGCAAGAATTCAGTC	For insert mutation of pTRGA
PTRGRT	TGCCGGCGCGGATCC T GGCCG CCTCTGGTTTCTCTTCTTTC	
pTRGFAC	AAACCAGAGGCGGCC AC GGAT CCGCGGCCGCAAGAATTCAGTC	For insert mutation of pTRGAC
pTRGRGT	TGCCGGCGCGGATCC GT GGCC GCCTCTGGTTTCTCTTCTTTC	
4608EcoRIF	CCG GAATTC ATGAGTGACCAGC ACGACGAACG	For construction of pBT-MapZ and pUCP18-HA-MapZ and mutants
4608SmaIF	TCC CCCGGG GCAGCAGCGGCGC TAGCATGAGTGACCAGCACGAC GAACGTCGACGTTTCCAC	
4608BamHIR	CGC GGATCC TCAGTCGTCGTGGG CGCTGACCA	
4608NdeIF	GATC CATATG AGTGACCAGCAC GACGAACGTCGACGTTTCCACC GG	For construction of pET28b-MapZ and mutants
4608HindIIIR	GATC AAGCTT TCAGTCGTCGTG GGCGCTGACCAGCAGGGC	
4608A2BamHI & HindIIIR	GATC GAATTCAAGCTT CTACTTG GCCCGGTAGATGATGCCCGG	For construction of pET28b-MapZ ^{Δα2}
4608R13AF	CGACGAACGTCGACGTTTCCAC GCG ATCGCCTTCGAC	For site-directed mutagenesis of MapZ ^{R13A}
4608R13AR	GCAGCTGCAAAGGTG CGCTAG CGGAAGCTGCGGCTG	
4608BamHIF	CGC GGATCC GGAAAACCTGTAT TTTCAGGGCAGTGACCAGCACG ACGAACG	For construction of pACYCDuet-MapZ
4608HindIIIR2	CCC AAGCTT TCAGTCGTCGTGG GCGCTG	
3348FBamHI& NdeI	GATC GGATCCCATATG GTGTCCG CAGCTAATGCGGATTTCGAGTT G	For construction of pTRG-CheR1 and pET28b-CheR1
3348REcoRI&H indIII	GATC GAATTCAAGCTT CTACTTG GCCCGGTAGATGATGCCCGG	
pUCP18His3348 F	CCG GAATTC CGTGCTCGTGCTA ATAATTTTGTTTAACTTTAAGAA GGAG	For construction of pUCP18-His-CheR1 and pUCP18-His- CheR1-HA-MapZ
pUCP18His3348 R	CGC GGATCCCTCGAG TTACTAC TTGGCCCGGTAGAT	
pUCP18YFP460 8F	GATC GAATTC ATGGTGAGCAAG GGCGAGGAGCTGTTC	For construction of pUCP18-YFP- MapZ, pUCP18- YFP-MapZ ^{R13A} and pBT-YFP-MapZ
pUCP18YFP460 8R	GATC GGATCC TCAGTCGTCGTG GGCGCTGACCAGC	

3348BamHIF	CGC GGATCC GGAAAACCTGTAT TTTCAGGGCTCGGCAGCTAATG CGGATTT	For construction of pACYCDuet-CheR1
3348SalIR	ACGC GTCGAG CTACTTGGCCCCG GTAGATGA	
1-4608_UpF	CGC GGATCC GTCCTTGGCTTCCAC GCCGAGCA	For the construction of plasmid pK18Gm-MapZ ^{R13A}
2-4608_UpR	TCGAAGGCGATGGCGTGGAAACG TCGACG	
3-4608_DownF	GTCGACGTTTCCACGCCATCGCCT TCGAC	
4-4608_DownR	CCC AAGCTT ACCGCCTCCGACCTG GCGTTGAT	
5-4608_F	CGTCGCATCCTTGCTCGCGAATAT C	For sequencing of strain <i>mapZ</i> ^{R13A}
6-4608_R	CCTCGACCAGAACCGCCTGGCGA TG	
1-0861_UpF	agctcggtaccgggGAACGGGCCAG TCACCCTGGCC	For the construction of plasmid pK18GT- rbdAAAA
2-0861_UpR	GCGCAG GGCGGGCG GACGTGC AAGCCCTCCTCCGCACCTTC	
3-0861_DownF	CACGTC GGCGGGCG CTGCGCCT GCGCGACGAGGGCGGG	
4-0861_DownR	cgacggccagtccaCACCTGGACCATG GCGCGATC	
5-0861_F	CAGGCAAGGCCTCGGGAGTTCTG C	For sequencing of strain <i>rbdA</i> ^{PDE*}
6-0861_R	GGATAGCTTCCCGCCTCCTGCAAG	
7-0861F650seq	CGCCAGCGTGCTCATCCTCCTGG	
8-0861F1350seq	GGGCGGCGACGAGTTCGGCATCC	
9-0861AAAF	GGGCTTGACGTC GGCGGGCG	
10-0861AAAR	CAGCTCGTGACCGGAAACCCG	
1-3311_UpF	agctcggtaccgggGCGATGCGCTCGGT GGCCAC	For the construction of plasmid pK18GT- nbdAAAA
2-3311_UpR	GCGCAC GGCTGGGG CAACTGG TGTACCTCGCCGTCGGCG	
3-3311_DownF	CAGTTG GGCGAGCC GTGCGCTG GCGCCATCCGACCCAG	
4-3311_DownR	cgacggccagtccaCTCCACGCCCTCGG CGACGAC	
5-3311_F	GCAACTCCTGAAACGTAACGG	For sequencing of strain <i>nbdA</i> ^{PDE*}
6-3311_R	CTCAAGGATCTCGATAACGC	
7-3311F500seq	GTACATGACCGCCCCGCCGCGC	

8-3311F1100seq	GAGAATTGTCGCCTGAGAGGC	
9-3311AAAF	GTACACCAGTTG GCCGCAGCC	
10-3311AAAR	CCGTTGCAGATCCTCGCCCCGC	
1-5017BamHI_UpF	CG <u>GGATCC</u> GCAAGAACCACGAGC CTTACTG	For the construction of plasmid pK18GT-dipAAAA
2-5017_UpR	CAG <u>GGCGGCGGC</u> CAGGCCGAGC AACTGGCCGCTG	
3-5017_DownF	CTG <u>GCCGCGGCC</u> CTGCGCTGGCA GCATCCGGAAA	
4-5017HindIII_DownR	CCC <u>AAGCTT</u> GAAGCCTGGGCGAT CAGTGCAGG	
5-5017_F	GCAGACCTCCGACCGTCCAGC	
6-5017_R	GGCGTGGGCAGCTCGCTTTTC	
7-5017F600seq	CACCCAGGCCGCCTGCGAAGC	For sequencing of strain <i>dipA</i> ^{PDE*}
8-5017F1260seq	CCGCAGCCGCCGCAAGAACCAC	
9-5017AAAF	GTTGCTCGGCCTG <u>GCCGCGGCC</u>	
10-5017AAAR	CGCGGTCAGCGGGCGGCCGTTG	
Pk18-F	TGCTTCCGGCTCGTATGTTG	For sequencing of plasmid pK18GT-PDE-AAA
Pk18-R	GCGAAAGGGGGATGTGCTG	

^a agctcggtaccgagg and cgacggccagtggca are adapters from suicide vector pK18GT sequences adjacent to BamHI and HindIII restriction digestion sites respectively.

^b Restriction sites are colored red and underlined. Mutation bases are colored red and bold.

Table S4 Summary of crystallographic data and refinement statistics.

Data collection		
	<i>Apo</i>CheR	CheR/MapZ/c-di-GMP
Space group	I4 ₁	P2 ₁ 2 ₁ 2 ₁
PDBID	5Y4S	5Y4R
Wavelength (Å)	0.9779	0.9537
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	279.13, 279.13, 138.58	88.44, 98.82, 110.66
α , β , γ (°)	90, 90, 90	90, 90, 90
Molecules/ASU	10	2
Resolution (Å) ^a	50-3.41 (3.61-3.41)	50-2.30 (2.44-2.30)
CC1/2	99.8 (53.1)	99.8 (56.8)
<i>I</i> / <i>σ</i> (<i>I</i>)	13.82 (1.42)	12.64 (1.43)
Completeness (%) ^a	99.9 (99.8)	99.8 (99.0)
Redundancy ^a	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
R _{work} (R _{free}) (%)	25.7 (29.5)	19.4 (23.9)
No. atoms		
Protein	20244	6230
Ligand/ion	-	C2E, 184; SO4, 60
Water	-	163
B-factors (Å ²)		
Protein	246.3	59.4
Ligand/ion	-	60.7
Water	-	50.9
R.m.s. deviations		
Bond lengths (Å)	0.004	0.011
Bond angles (°)	1.19	1.44
Ramachandran plot	90.0 (0.56)	95.0 (0.26)
% favored (outlier)		

^a Values for the highest-resolution shell are in parentheses.

^b This study was done in collaboration with Prof. Gao Yong-Gui's lab, Singapore (2).

AUTHOR'S PUBLICATIONS

(* Equal contribution)

1. **Xin, Lingyi***, Y. Zeng.*, S. Sheng, R. A. C. Y. Fong, J. K. Yam, L. Yang, L. H. Xu, K. H. Chiam and Z. X. Liang (2018). "Specific Control of Flagellar Motor Switching by C-di-GMP Phosphodiesterases in *Pseudomonas Aeruginosa*." (Manuscript in preparation).
2. Yan, X.*, **Xin Lingyi ***, J. T. Yen, Y. Zeng, S. Jin, Q. W. Cheang, R. A. C. Y. Fong, K. H. Chiam, Z. X. Liang and Y. G. Gao (2018). "Structural Analyses Unravel the Molecular Mechanism of Cyclic di-GMP Regulation of Bacterial Chemotaxis via a PilZ Adaptor Protein." *Journal of Biological Chemistry* **293**(1): 100-111.
3. Liu, C., C.W. Liew, Y.H. Wong, S. T. Tan, W. H. POH, S. M. S. Manimekalai, S. Rajan, **Xin Lingyi**, Z. X. Liang, G. Gruber, S. A. Rice and J. Lesca (2018). "Insights into biofilm dispersal regulation from the crystal structure of the PAS-GGDEF-EAL region of RbdA from *Pseudomonas aeruginosa*." *Journal of Bacteriology* **200**(3): e00515-17.
4. Xu, L.*, **Xin Lingyi ***, Y. Zeng, J. K. Yam, Y. Ding, P. Venkataramani, Q. W. Cheang, X. Yang, X. Tang, L. H. Zhang, K. H. Chiam, L. Yang and Z. X. Liang (2016). "A cyclic di-GMP-binding adaptor protein interacts with a chemotaxis methyltransferase to control flagellar motor switching." *Science Signaling* **9**(450): ra102.
5. Xu, L., P. Venkataramani, Y. Ding, Y. Liu, Y. Deng, G. L. Yong, **Xin Lingyi**, R. Ye, L. Zhang, L. Yang and Z. X. Liang (2016). "A Cyclic di-GMP-binding Adaptor Protein Interacts with Histidine Kinase to Regulate Two-component Signaling." *Journal of Biological Chemistry* **291**(31): 16112-16123.

CONFERENCE TALK

American Society for Microbiology (ASM) Conference on *Pseudomonas* 2015.
Washington D.C., USA; 8th-12th September 2015

Awarded the Travel Grant from ASM to deliver an oral presentation on "Role of a Cyclic Di-GMP Binding Protein in Chemotaxis Regulation".