The PilZ Domain Is a Receptor for the Second Messenger c-di-GMP

**THE PILZ DOMAIN PROTEIN YcgR CONTROLS MOTILITY IN ENTEROBACTERIA**

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The ubiquitous bacterial second messenger c-di-GMP controls exopolysaccharide synthesis, flagella- and pili-based motility, gene expression, and interactions of bacteria with eukaryotic hosts. With the exception of bacterial cellulose synthases, the identities of c-di-GMP receptors and end targets have remained unknown. Recently, Amikam and Galperin (Amikam, D., and Galperin, M. (2006) Bioinformatics 22, 3–6) hypothesized that the PilZ domains present in the BcsA subunits of bacterial cellulose synthases function in c-di-GMP binding. This hypothesis has been tested here using the *Escherichia coli* PilZ domain protein YcgR, its individual PilZ domain and the PilZ domain from *Gluconacetobacter xylinus* BcsA. YcgR was purified and found to bind c-di-GMP tightly and specifically, $K_d$ 0.84 μM. Individual PilZ domains from YcgR and BcsA also bound c-di-GMP, albeit with lesser affinity, indicating that PilZ is sufficient for binding. The site-directed mutagenesis performed on YcgR implicated the most conserved residues in the PilZ domain directly in c-di-GMP binding. It is suggested that c-di-GMP binding to PilZ brings about conformational changes in the protein that stabilize the bound ligand and initiate the downstream signal transduction cascade. While the identity of the downstream partner(s) of YcgR remains unknown, it is shown that YcgR regulates flagellum-based motility in a c-di-GMP-dependent manner. The inactivation of ycgR improves swimming and swarming motility of the poorly motile yhjH mutants of *Salmonella enterica* serovar Typhimurium UMR1. Therefore, biochemical and genetic evidence presented here establishes PilZ as a long sought after c-di-GMP-binding domain and YcgR as a c-di-GMP receptor affecting motility in enterobacteria.

Cyclic dimeric GMP, c-di-GMP, has emerged as a second messenger specific to the domain of Bacteria. c-di-GMP controls a variety of cellular processes, mainly biogenesis and function of extracellular components, flagella and pili, and exopolysaccharide synthesis. This makes c-di-GMP a key regulator in bacterial transition from a motile, single-cellular to a sessile, often multicellular lifestyle (1).

c-di-GMP turnover is regulated through the action of diguanylate cyclases and c-di-GMP-specific phosphodiesterases. The cyclase activity is encoded in the GGDEF protein domains (also known as DUF1) (2, 3), while phosphodiesterase activity is encoded in the EAL (DUF2) (4–6) and HD-GYP domains (7). Bacteria, especially from the proteobacterial branch, contain numerous enzymes involved in c-di-GMP turnover to monitor various environmental and intracellular inputs and adjust c-di-GMP levels in the precise manner. Despite significant progress in elucidating the enzymology of c-di-GMP turnover and in uncovering c-di-GMP-dependent processes, the molecular mechanisms of c-di-GMP action remain essentially unknown (1). This represents the main impediment to our understanding of c-di-GMP-dependent signaling.

The only end target of c-di-GMP action that was verified in vitro is the membrane-associated cellulose synthase from *Gluconacetobacter xylinus*. This enzyme consists of the catalytic and regulatory subunits, BcsA and BcsB, which are sufficient for the catalysis in vitro. Benziman and colleagues (8–10) showed that c-di-GMP acts as an allosteric activator of BcsAB. Recently, Amikam and Galperin (11), based on the bioinformatics analysis, put forth a hypothesis that the C-terminal domain of BcsA is involved in c-di-GMP binding. This domain shares similarity with the described earlier single-domain PilZ protein from *Pseudomonas aeruginosa*, which gave rise to the name “PilZ domain” (Pfam: PF07238 (12)). The *P. aeruginosa* PilZ protein is involved in pili formation and twitching motility (13). Amikam and Galperin (11) noticed that some PilZ domains are localized C-terminally of the GGDEF-EAL and HD-GYP modules, which suggested their association with c-di-GMP. Several experimentally studied proteins involved in polysaccharide synthesis, motility, and cell differentiation, *i.e.* the processes known to be regulated by c-di-GMP, contain PilZ domains (14–16). This further strengthened the prediction that PilZ is involved in c-di-GMP binding.

Is the PilZ domain indeed the long sought after c-di-GMP receptor? Here we tested this hypothesis directly using the PilZ domain protein YcgR from *Escherichia coli* as well as its individually expressed PilZ domain and the PilZ domain from *G. xylinus* BcsA. YcgR is one of two PilZ domain proteins present in *E. coli*, the second one being BcsA-Ec. YcgR was originally described as a novel member of the *E. coli* flagellum machinery, because in a Δhns background lacking the H-NS nucleoid protein, the ycgR null mutation resulted in improved motility (17).

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—The following wild-type strains were used in this work: *Salmonella enterica* serovar Typhimurium...
UMR1 (18) and E. coli TOB1 (19). All strains used in this study are described in Table S1 in the online supplemental material. Knock-out mutants were constructed by a one-step gene inactivation described by Datsenko and Wanner (20). In general, the entire open reading frame except 40 bp at the beginning and end was replaced with the chloramphenicol resistance cassette. The Typhimurium yjiH ycgR double mutant MAE471 was created from the derivative of the yjiH mutant MAE420 by phage transduction using P22 HT105/1 int-201 (21). All mutants were colony purified twice and verified by PCR.

E. coli MG1655 was used for PCR amplification of the ycgR gene and the PilZ domain of YcgR, PilZ_YcgR, used for protein purification. G. xylinus was used for PCR-amplification of the fragment corresponding to the PilZ domain of BcsA1, PilZ_BcsA. The amplified DNA fragments were cloned into the overexpression vector pET-23a to generate C-terminal His6 fusions, which were expressed in BL21(DE3)pLysS (EMD Biosciences, San Diego, CA).

Site-directed mutagenesis was performed on the ycgR template using mutant primers and the QuikChange protocol (Stratagene, La Jolla, CA). For complementation analysis, the wild-type and mutant ycgR constructs were cloned into vector pBAD30 (22). All primers used in this study are shown in Table S2 in the online supplemental material.

**Protein Purification**—The YcgR and PilZ_BcsA were purified as soluble C-terminal His6-tag fusions using nickel affinity chromatography according to the manufacturer’s protocol (EMD Biosciences). Proteins were additionally purified with size exclusion chromatography, where necessary. PilZ_YcgR, which proved to be insoluble, was purified from inclusion bodies as described in the supplemental material. All proteins were purified to ≈90% purity as assessed by capillary electrophoresis (2100 Bioanalyzer, Agilent Technologies, Wilmington, DE). Protein concentrations were measured using a BCA protein assay kit (Pierce).

**Chromatography**—Purification of proteins and determination of their oligomeric states was done using a Superdex 200 10/300 GL gel filtration column in the fast protein liquid chromatography system (Amersham Biosciences) as described earlier (3). Nucleotides were separated and analyzed using the reversed phase HPLC system (Summit HPLC System, Dionex, Sunnyvale, CA), 15 × 4.6 cm Supercosil LC-18-T column (Sigma). The buffers and gradient program were described earlier (3). Proteins and nucleotides were monitored by A280.

**Equilibrium Dialysis**—The following buffer was used in the equilibrium dialysis experiments: 300 mM NaCl, 0.5 mM EDTA, 10% glycerol, 50 mM sodium phosphate, pH 7.4. Protein-nucleotide binding was examined by equilibrium dialysis in Dispo-Biodialyzer cassettes (The Nest Group, Southborough, MA). c-di-GMP or other tested nucleotides (concentrations from 2 to 50 μM) were injected into one cell of a cassette, while analyzed proteins were injected into the opposite cell, which is separated by a dialysis membrane with a 5-kDa molecular weight cutoff. The proteins were used at the following concentrations: YcgR and its site-directed mutants, 10 ± 2 μM; PilZ_YcgR, 10 μM; and PilZ_BcsA, 30 μM. The cassettes were maintained for 25 h at room temperature under moderate agitation to reach equilibrium. The samples (45 μl) from both cassette cells were taken, boiled for 4 min (to denature the protein, where present), and centrifuged, and supernatants were filtrated through a 0.22-μm microfilter. The nucleotide concentrations were quantified by measuring peak areas of the eluted nucleotides from the HPLC column. Binding constants were calculated by the GraphPad Prism software, version 4.03 (GraphPad Software, San Diego, CA) using nonlinear regression model.

**Motility Assays**—For swimming motility, 0.3% swim agar plates were inoculated with 5 μl of overnight culture resuspended in water (A660 was 5). After 6 h the diameter of the swimming zone was measured. For swarming motility, 0.5% swarm agar plates were used. The zones formed by the swarming bacteria were measured after 5 h.

**RESULTS**

**E. coli YcgR Is a c-di-GMP-binding Protein**—To test whether the E. coli PilZ domain protein YcgR (Fig. 1A) binds c-di-GMP, we overexpressed it as a C-terminal His-tag fusion and purified it using nickel column affinity and gel filtration chromatography (Fig. 2A). We tested the ability of YcgR to bind various nucleotides, including c-di-GMP, using equilibrium dialysis. YcgR bound c-di-GMP with a Kd of 0.84 ± 0.16 μM (Fig. 2B). This value is similar to or somewhat lower than the estimated intracellular c-di-GMP levels, believed to be in the 1–10 μM range in the proteobacterial species (2, 10, 23). Therefore, YcgR must be responsive to physiologically relevant changes in c-di-GMP levels. We detected no binding of other nucleotides, including cGMP, cAMP, or GMP (data not shown), which suggests that YcgR is highly c-di-GMP-specific.

The binding capacity of YcgR, Bmax, calculated based on the equilibrium dialysis experiments was 1.99 ± 0.11 mol c-di-GMP (mol protein)−1 (Fig. 2B). To further investigate the protein-c-di-GMP ratio, we incubated YcgR with the excess of c-di-GMP and subjected the mixture to size exclusion chromatography. The recovered YcgR protein contained bound c-di-GMP and the protein: c-di-GMP ratio was found to be 1:2.09 ± 0.11, which nicely corroborates the value obtained by equilibrium dialysis. Interestingly, the shape of the equilibrium dialysis curve is most consistent with YcgR containing a single binding site. Since c-di-GMP has been reported to exist in aqueous solutions primarily as a self-intercalated dimer (24), and a c-di-GMP dimer was observed in the allosteric site, I-site, of the Caulobacter crescentus diguanylate cyclase PleD (25), we suggest that one molecule of YcgR binds one c-di-GMP dimer at a single binding site.

YcgR is composed of two domains: PilZN (designated “YcgR” in the Pfam database, Pfam: PF07317) of unknown function and PilZ (Fig. 1A). To directly assess the role of the PilZ domain in c-di-GMP binding, we mutated several conserved residues of this domain. These were chosen based on analysis of the multiple sequence alignment of 691 PilZ domains (Fig. 1A). Only a small number of conserved motifs and residues are present in the PilZ domains, among which are the 154RxxR and 156E/D/ NxxGG motifs, where x is any residue, and z is a small hydro-
phobic residue (numbering based on YcgR) (Fig. 1A). Examination of the x-ray structure of the PilZ domain protein VCA0042, solved in the absence of c-di-GMP, reveals that these two motifs are located (i) in the immediate proximity of each other and (ii) close to the PilZNR domain, where PilZNR is a domain of unknown function unrelated to PilZN (11) (Fig. 1B). Since arginines were found to form hydrogen bonds with O6 and N7 of the guanyls of c-di-GMP in the I-site of PleD (25), we hypothesized that the conserved arginines of the PilZ domains, 114RxxxR, are directly involved in c-di-GMP binding.

To test the role of Arg118 in c-di-GMP binding, we generated by site-directed mutagenesis a 118R3D substitution (Fig. 1, A and B). The purified mutant YcgR118R3D protein was tested by equilibrium dialysis and found to be completely impaired in c-di-GMP binding (data not shown). This is consistent with our prediction that Arg118 is directly involved in c-di-GMP binding.

Furthermore, this confirms that YcgR has only one c-di-GMP-binding site. Ser147 is also highly conserved in PilZ domains (Fig. 1, A and B); however, its role in c-di-GMP binding is less obvious. We introduced the 147S3A mutation and found that it actually somewhat improved affinity to c-di-GMP, i.e. $K_d = 1.45 \mu M$. This is consistent with the involvement of the 145D/Nz5x5G motif in c-di-GMP binding but shows that Ser147 is not critical for binding. A replacement of two conserved glycines to alanines, 149GG3AA, resulted in the insoluble mutant protein. Apparently the loop formed by these glycines is essential for protein structural integrity. We did not pursue the in vitro analysis of the 149GG3AA mutant any further.

c-di-GMP Binding to Single PilZ Domains—The PilZ domain arrangement present in the E. coli YcgR is conserved in many bacteria. However, PilZ domains also exist as standalone, single-domain proteins or in association with domains different from PilZN. We wondered whether the PilZ domain in itself is sufficient for c-di-GMP binding. We overexpressed the PilZ domain from YcgR, PilZ_YcgR, as a C-terminal His-tag fusion. Upon overexpression, PilZ_YcgR formed inclusion bodies. We purified the denatured protein by nickel affinity chromatography followed by its renaturation. The soluble, renatured PilZ_YcgR bound c-di-GMP, however, less efficiently than the full-length protein ($K_d = 1.45 \mu M$). This establishes that an individual PilZ domain is sufficient for c-di-GMP binding.
To investigate whether PilZ domains from other sources could bind c-di-GMP, we overexpressed and purified the C terminus of G. xylinus BcsA containing the PilZ domain, PilZ_BcsA (Fig. 1A). We found that the purified PilZ_BcsA bound c-di-GMP; however, the binding affinity ($K_d$ 30 $\mu M$) was significantly worse than that reported for BcsBA (10). Taken together with the inferior c-di-GMP binding by PilZ_YcgR compared with binding by the full-length YcgR, this indicates that protein regions outside of the PilZ domains are involved in ligand binding.

What could be the role of these regions? One possibility is that c-di-GMP binding involves protein di-/oligomerization, which would be lacking in the stand-alone domains. To explore this possibility, we examined oligomeric states of YcgR, PilZ_YcgR, and PilZ_BcsA using size exclusion chromatography. We found that all proteins exist as monomers (Fig. 2C and data not shown). We further explored whether the oligomeric state of YcgR changes upon c-di-GMP binding and found that it did not (Fig. 2C). This implies that di-/oligomerization of PilZ domain-containing proteins is not important for c-di-GMP binding.

Another possibility is that c-di-GMP binding changes conformation of not only PilZ but also that of the N-terminal PilZN domain of YcgR. We noticed that in VCA0042, the conserved motifs implicated in c-di-GMP binding are localized near the PilZN-PilZ domain-domain interface (Fig. 1B). Therefore, one can envision that c-di-GMP binding to PilZ would also bring PilZN closer to PilZ and engage certain residues of PilZN in c-di-GMP binding. We tested this possibility by comparing the apparent molecular masses of the ligand-free and c-di-GMP-loaded YcgR. We observed that YcgR-c-di-GMP complex consistently migrated as a smaller, more compact, protein (apparent molecular mass 32.1 kDa) compared with the ligand-free YcgR (32.1 kDa) despite the fact that it actually is heavier by the mass of the c-di-GMP dimer (1.4 kDa) (Fig. 2C). Therefore, it does appear that c-di-GMP binding brings the two domains of YcgR closer. Therefore, PilZN may provide additional contacts to stabilize the bound c-di-GMP ligand and improve binding affinity.

YcgR Regulates Motility in Enterobacteria in the c-di-GMP-dependent Manner—To identify what c-di-GMP-dependent phenotypes are mediated by YcgR, we knocked out the ycgR genes in the wild-type E. coli isolate TOB1 and in Typhimurium UMR1, where ycgR is STM1798. We assayed for several phenotypes known to be c-di-GMP-dependent, i.e., rdar morphotype (rough, dry, and rugose colony) (26), adhesion to an abiotic surface and biofilm formation, as well as swimming and swarm ing motility. We detected no differences in any of the known c-di-GMP-associated phenotypes between the ycgR null mutants of Typhimurium or E. coli and their wild-type strains (data not shown). The lack of an observable phenotype(s) probably indicates that our assays were done under suboptimal conditions. For example, we could readily miss the ycgR knockout phenotype(s), if intracellular c-di-GMP levels were low and YcgR was inactive in the first place.

The yhjH mutation is expected to result in elevated c-di-GMP levels because YhjH is a c-di-GMP phosphodiesterase that strongly affects swimming motility in E. coli and Typhimurium (17, 23, 27, 28). We therefore tested the effect of the ycgR knockout in the yhjH mutant background. The Typhimurium yhjH mutant MAE420 displayed over 60% reduction in swimming motility as compared with the wild-type UMR1 (Fig. 3A), which is similar to what was observed in E. coli (17). The inactivation of the ycgR gene in the yhjH background greatly improved swimming motility, i.e., the motility of the yhjH ycgR double mutant MAE471 was restored to 80% of the wild type UMR1. This suggests that YcgR requires c-di-GMP to manifest its effect on swimming. Complementation of the Typhimurium yhjH ycgR double mutant MAE471 with the wild-type ycgR gene either from E. coli K12 or from Typhimurium brought back the swimming motility defect. The same was true, when we introduced the $^{147}S \rightarrow A$ mutant of YcgR, which is not impaired in c-di-GMP binding. However, when we introduced the $^{118}R \rightarrow D$ mutant defective in c-di-GMP binding, it had no effect on swimming, and neither did the $^{149}GG \rightarrow AA$ mutant (Fig. 3A). From these observations we conclude that YcgR regulates swimming motility in the c-di-GMP-dependent manner, i.e. it functions as a true c-di-GMP receptor in vivo.

The swimming motility is also dramatically decreased in the Typhimurium yhjH mutant MAE420 compared with the wild-type UMR1 (Fig. 3B). Consistent with the results from the swimming motility experiments, the swimming motility was partially restored in the yhjH ycgR double mutant MAE471. Furthermore, introduction of the wild-type and mutant ycgR genes into MAE471 resulted in the similar outcomes as those described for the swimming motility (compare Fig. 3, A and B).

When we assayed the Typhimurium yhjH ycgR double mutant, the yhjH mutant, and the wild type for additional c-di-GMP-dependent phenotypes, i.e., rdar morphotype, abiotic surface adherence, and biofilm formation, we found these strains

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indistinguishable. This suggests that YcgR is specific to c-di-GMP-dependent regulation of the flagellum-based motility.

**DISCUSSION**

Until recently, our understanding of the molecular mechanisms through which c-di-GMP operates has remained at the level of 1980s, when Benziman and co-workers (8, 9) showed that c-di-GMP works as an allosteric activator of cellulose synthase BcsAB in *G. xylinus*. The hypothesis of Amikam and Galperin (11) that the PilZ domain present in the C termini of the BcsA subunits of bacterial cellulose synthases is involved in c-di-GMP binding was a long awaited breakthrough. In this study, we tested this hypothesis both in vitro and in vivo. We presented evidence that the *E. coli* PilZ domain protein YcgR binds c-di-GMP in vitro tightly and specifically, with the affinity that would allow it to respond to changes in intracellular c-di-GMP levels. We also showed that YcgR requires c-di-GMP in vivo for regulation of the flagellum-based motility. YcgR is dedicated to regulation of motility and not any other known c-di-GMP-dependent phenotypes. The PilZN-PilZ domain arrangement present in YcgR is conserved in the proteobacteria beyond Enterobacteriaceae (genera *Burkholderia*, *Chromobacterium*, *Dechloromonas*, *Nitrosomonas*, *Ralstonia*, *Thiobacillus*). Therefore, the function of YcgR in motility control is likely to be conserved.

The PilZ domains of YcgR and BcsA in themselves are sufficient for c-di-GMP binding. However, the binding affinities are inferior compared with the full-length proteins. We suggest that the nearby domains (e.g. PilZNR in VCA0042 and PilZN in YcgR) are involved in stabilizing the bound c-di-GMP and that improves affinity. This interpretation is based on the observation that the c-di-GMP binding site is apparently located right at the junction between PilZNR and PilZ in VCA0042 (PilZN and PilZ in YcgR) and that c-di-GMP binding promotes a more compact conformation in YcgR compared with the ligand-free protein. We predict that this conformational change represents a switch from the inactive to the active, c-di-GMP-bound YcgR, which initiates the downstream signal transduction cascade.

Most enterobacteria contain only two PilZ domain proteins, YcgR and BcsA. Whether these proteins mediate all c-di-GMP-dependent effects or PilZ-independent pathways exist remains to be determined.

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