Cyclic Diguanylate Is a Ubiquitous Signaling Molecule in Bacteria: Insights into Biochemistry of the GGDEF Protein Domain†

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Proteins containing GGDEF domains are encoded in the majority of sequenced bacterial genomes. In several species, these proteins have been implicated in biosynthesis of exopolysaccharides, formation of biofilms, establishment of a sessile lifestyle, surface motility, and regulation of gene expression. However, biochemical activities of only a few GGDEF domain proteins have been tested. These proteins were shown to be involved in either synthesis or hydrolysis of cyclic-bis(3′→5′) dimeric GMP (c-di-GMP) or in hydrolysis of cyclic AMP. To investigate specificity of the GGDEF domains in Bacteria, six GGDEF domain-encoding genes from randomly chosen representatives of diverse branches of the bacterial phylogenetic tree, i.e., Thermotoga, Deinococcus-Thermus, Cyanobacteria, spirochetes, and α and γ divisions of the Proteobacteria, were cloned and overexpressed. All recombinant proteins were purified and found to possess diguanylate cyclase (DGC) activity involved in c-di-GMP synthesis. The individual GGDEF domains from two proteins were overexpressed, purified, and shown to possess a low level of DGC activity. The oligomeric states of full-length proteins and individual GGDEF domains were similar. This suggests that GGDEF domains are sufficient to encode DGC activity; however, enzymatic activity is highly regulated by the adjacent sensory protein domains. It is shown that DGC activity of the GGDEF domain protein Rpa1 from Borrelia burgdorferi is strictly dependent on phosphorylation status of its input receiver domain. This study establishes that majority of GGDEF domain proteins are c-di-GMP specific, that c-di-GMP synthesis is a widely-spread phenomenon in Bacteria, and that it is highly regulated.

The approximately 170-amino-acid-long protein domain GGDEF (http://www.sanger.ac.uk/Software/Pfam), also referred to as domain of unknown function 1 (designated DUF1) (http://smart.embl-heidelberg.de), is a conserved domain in Bacteria. However, its function has yet to be properly characterized. The domain name originates from the amino acid motif GGDEF (Gly-Gly-Asp-Glu-Phe). This domain is present in most sequenced genomes from all branches of the phylogenetic tree of Bacteria. This implies that GGDEF domain-containing proteins play important roles in Bacteria; however, such roles remain largely unknown. The presence of GGDEF domains in the genomes of representatives of the deepest branches of the bacterial tree, e.g., Aquifex and Thermotoga, suggests their ancient evolutionary origin (10, 11). It is evident that proper elucidation of structure-function relationships of the GGDEF domain and GGDEF domain-containing proteins will improve our understanding of the physiology, metabolism, and behavior of bacteria and will therefore enhance our ability to manipulate them.

The limited, yet rapidly accumulating, genetic and biochemical evidence suggests that the GGDEF domain is involved in the synthesis and hydrolysis of cyclic diguanylate, or cyclic-bis(3′→5′) dimeric GMP (c-di-GMP) (reviewed in references 8 and 13). This compound was originally identified as an allo-steric activator of cellulose synthase in Gluconacetobacter xylinus, formerly known as Acetobacter xylinum (20, 22). Three paralogous diguanylate cyclases, (DGC) from G. xylinus involved in c-di-GMP synthesis were found to contain two conserved protein domains, GGDEF and EAL (http://www.sanger.ac.uk/Software/Pfam), where EAL is also known as DUF2 (http://smart.embl-heidelberg.de). A DGC was shown to catalyze the synthesis of c-di-GMP from two molecules of GTP via two distinct pyrophosphate-releasing steps with a linear dinucleotide, pppGpG, as an intermediate (20). Interestingly, enzymes involved in the original step in c-di-GMP hydrolysis, phosphodiesterases A (PDE-A), purified from G. xylinus also contain GGDEF and EAL domains. PDE-A cleave a single phosphodiester bond in the cyclic structure, yielding a linear dimer GpGp (l-di-GMP), which is further converted to 5′-GMP by different PDE (20, 21).

Pei and Grishin noticed low yet significant sequence similarity between GGDEF domains and catalytic domains of mammalian adenylyl cyclases. They predicted that the GGDEF domain may possess DGC activity (18). Up to date, biochemical activities of only a few GGDEF domain-containing proteins have been tested directly, all originating from the proteobacterial species. These proteins include PleD from Caulobacter crescentus (17) and Dos from Escherichia coli (23), as well as the G. xylinus proteins mentioned above. PleD functions as DGC (17), whereas Dos possesses cyclic AMP (cAMP)-dependent PDE activity (Fig. 1A) (23).

There is a growing body of evidence linking additional GGDEF domain proteins to c-di-GMP synthesis. For example, when overexpressed in rhizobia, some proteobacterial GGDEF domain proteins were able to activate cellulose syn-
from Thermotoga maritima Bactozol kit (Molecular Research Center, Cincinnati, Ohio). Genomic DNA of B. burgdorferi (shown in parentheses):

http://www.sanger.ac.uk/Software/Pfam; http://smart.embl-heidelberg.de).

main of response regulators from two-component systems (http://www.cosyesrs.dnu).

c-di-GMP; PAS, ubiquitous signal sensor domain; REC, receiver do-
cyclic GMP-specific PDE; EAL, PDE domain probably specific to

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E. coli sp. PCC6803, and ing proteins.

Genomic DNA from Vibrio cholerae, which is anticipated to be c-di-GMP dependent (2). Overexpression of the GGDEF domain-containing proteins AdrA from Salmonella enterica serovar Typhimurium (24) and VCA0956 from Vibrio cholerae (29) resulted in increased intracellular levels of c-di-GMP, thus implying that these pro-
teins possess DGC activity. However, in these instances, alter-
native explanations, i.e., indirect effects of the GGDEF pro-
teins, cannot be excluded.

Our study addressed the following questions concerning GGDEF domain proteins. How specific are GGDEF domain proteins to synthesis or hydrolysis of c-di-GMP? Are other substrates, cAMP in particular, involved to an appreciable degree? How widespread is c-di-GMP synthesis in Bacteria? Is the GGDEF domain sufficient for DGC activity? What role do domains adjacent to GGDEF play in enzymatic activity? To answer these questions, we cloned several genes encoding GGDEF domain proteins from various bacteria and assayed for their enzymatic activities.

MATERIALS AND METHODS

Construction of plasmids for overexpression of the GGDEF domain-containing proteins. Genomic DNA from Rhodobacter sphaeroides 2.4.1, Synechocystis sp. PCC6803, and E. coli DH5α was purified from bacterial cells using the Bacterial kit (Molecular Research Center, Cincinnati, Ohio). Genomic DNA from Thermotoga maritima DSM3109, Deinococcus radiodurans R1, and Boreiella burgdorferi B31 was purchased from the American Type Culture Collection. The genes encoding GGDEF domain proteins from these bacteria were PCR ampli-
fied with Pfu HololStart DNA polymerase (Stratagene) and the following primers (shown in parentheses): R. sphaeroides RSP5313 (F3513-EcoRI, 5′-CGGGATCAGT

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E. coli DH5α or into vector pETDuet1 (Invitrogen).

PCR fragments were gel purified with the Gel Recovery kit (Zymo Research), digested with the indicated restriction enzymes, and cloned into vector pMAL-c2x (New England Biolabs) in strain E. coli DH5α or into vector pET23a (In-

vitroneg, Carlsbad, Calif.) in strain E. coli BL21(DE3) containing either pLYsE or pLYsE6 (Invitrogen).

Protein overexpression and purification. The constructed plasmids were used for overexpression of proteins and protein domains as fusions to maltose binding protein (MBP). GGDEF domain protein overexpression was performed essentially according to the manufacturer’s instructions (pMAL expression system; New England Biolabs). Briefly, IPTG (isopropyl-β-D-thiogalactopyranoside; final concentration, 0.2 mM) was added to exponentially growing E. coli DH5α cells containing the appropriate plasmids (at an optical density [A600] of 0.6 to 0.8). After 2 h of induction, cells were collected by centrifugation. Cell pellets were resuspended in a buffer containing 200 mM NaCl, 0.5 mM EDTA, 5 mM MgCl2, 20 mM Tris-HCl (pH 7.6), and 5% glycerol that also contains protease inhibitors (phenylmethylsulfonyl fluoride and P4645; Sigma, St. Louis, Mo.) at the concentrations specified by the manufacturer. Cell suspensions were passed through a French pressure minicell (Spectronic Instruments), followed by brief sonication (Sonifier 250; Branson). Crude cell extracts were centrifuged at 15,000 × g for 45 min. Soluble protein fractions were collected and mixed with the preequilibrated amylose resin (New England Biolabs) for 1 h at 4°C. MBP-

protein fusions were eluted with maltose and dialyzed against the cyclase assay buffer in Slide-A-Lyzer dialysis cassettes (Pierce) according to the instructions of the manufacturer. Protein purity was assessed by capillary electrophoresis (Bio-
analyzer; Agilent Technologies) or sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentration was measured with the BCA protein assay kit (Pierce). Proteins requiring further purification were subjected to gel filtra-
tion by fast protein liquid chromatography (FPLC).

Enzymatic assays. The assay buffer and reaction conditions were essentially as described elsewhere (22). A standard reaction mixture (total volume, 0.6 ml) contained 5 μM enzyme in 50 mM Tris-HCl (pH 7.6), 10 mM MgCl2, 0.5 mM EDTA, and 50 mM NaCl. The reaction was started by the addition of 4.5 μl of substrate (final concentration, 150 μM) to the prewarmed reaction mixture and was carried out for 0, 3, 5, 15, 30, or 60 min. Aliquots (each, 100 μl) were withdrawn at the indicated time points and immediately placed in a boiling water bath for 3 min, followed by centrifugation at 15,000 × g for 2 min. The supernatant was filtered through a 0.22-μm-pore-size filter and analyzed by high-

pressure liquid chromatography (HPLC). Activities of RSP5313, Slr1143, and Rpl1 were assayed at 30°C; YeaP and DRB0044 were assayed at 37°C, and TM1163 was assayed at 37 and 50°C.

HPLC Samples (each, 20 μl) were injected into the 1.5- by 4.6-cm Supelcosil LC-18-T column (Supelco) and separated by reversed-phase HPLC (Summit HPLC system; Dionex). The following buffers were used in the gradient program: buffer A (100 mM KH2PO4, 4 mM tetrabutyl ammonium hydrogen sulfate [pH 5.9]) and buffer B (75% buffer A, 25% methanol). The following protocol was used for separation (the values are times in minutes and percentage of buffer B used): 0.0, 0.2, 0.5, 0.1, 0.5, 1.0, 2.5, 3.0, 5.0, 7.0, 10.0, 11.0, 10.0, 12.0, 10.0, 15.0, 20.0, 22.0, 20.0, 23.0, 0.0 at a flow rate of 0.7 ml min−1. Nucleotides were detected at a wavelength of 254 nm. Equal amounts of nicotine ADP (final concentration, 15 μM) were added to each sample prior to injection for quantification purposes.

FPLC. Purification of proteins and determination of their oligomeric state was done with a Superdex 200 10/300 GL gel filtration column (Amersham Bio-

FIG. 1. (A) Domain organization of the GGDEF domain proteins whose biochemical activities have been tested prior to this study. (B) Phylogenetic tree of Bacteria showing proteins investigated in this study. GAF, signal sensor domain often found in phototrophs and cyclic GMP-specific PDE; EAL, PDE domain probably specific to c-di-GMP, PAS, ubiquitous signal sensor domain; REC, receiver do-

n. Equal amounts of nicotine ADP (final concentration, 15 μM) were added to each sample prior to injection for quantification purposes.
which often functions as a small ligand binding sensory domain (7, 16), suggesting that it encodes a functional protein. In addition, the RSP3513 protein contains a ubiquitous domain-encoding gene with the strain with a tighter control activity turned out to be strain dependent. As is shown below, we successfully overexpressed several GGDEF domain proteins in strain E. coli DH5α. The reason for strain-specific toxicity is unknown at present. Our results must be considered as a warning against using BL21-derived strains for cloning the GGDEF domain-encoding genes.

**R. sphaeroides RSP3513 encodes DGC.** The intact RSP3513 gene was cloned into the pMAL-c2x vector with E. coli DH5α, overexpressed as an amino-terminal MBP fusion, and purified to homogeneity by affinity and gel filtration chromatography (Fig. 2A). Activity of the purified protein was tested with the following nucleotides: ATP, ADP, AMP, GTP, GDP, GMP, CTP, TTP, c-di-GMP, cAMP, and cGMP. GTP was found to be the sole substrate for the RSP3513 protein. GTP was quantitatively converted to c-di-GMP (Fig. 2B). Several lines of evidence show that the product of RPS3513 is c-di-GMP. (i) The retention time of the reaction product on the HPLC column is identical to that of the chemically synthesized c-di-GMP (data not shown). (ii) Treatment of the reaction product with the previously described c-di-GMP-specific PDE-A from G. xylinus, PdeA1 (26), resulted in the same outcome. (iii) The molecular mass of the reaction product was determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry. The lower panel corresponds to the product of the HPLC fraction, with retention time of 19 min (see Fig. 2A, bottom).

![FIG. 2. R. sphaeroides DgcA protein (RSP3513).](image)

(A) Protein overexpression and purification (protein chip, Bioanalyzer; Agilent Technologies). Lane M, molecular mass markers in kilodaltons; lane 1, crude extract of E. coli cells prior to induction of expression of the MBP::DgcA fusion protein; lane 2, crude extract of E. coli cells after 2-h induction with IPTG; lane 3, pure protein after affinity chromatography and gel filtration. (B) Enzymatic activity of DgcA. Substrate (top; 0 min) and products (bottom; 60 min) of the reaction, separated by reversed-phase HPLC. Nicotine ADP was used as an internal control for quantification purposes. (C) Mass spectroscopy analysis of the product synthesized by MBP::DgcA. The lower panel corresponds to the product of the HPLC fraction, with retention time of 19 min (see Fig. 2A, bottom).
of the product of RSP3513 reaction measured by mass spectrometry, 689.32 Da, was found to be identical to the molecular mass of the chemically synthesized c-di-GMP, 689.34 (Fig. 2C).

This establishes RSP3513 as a DGC, which is hereby designated DgcA. This suggests that *R. sphaeroides* synthesizes c-di-GMP under a variety of growth conditions. The role of this compound in *R. sphaeroides* is currently under investigation.

**DGC activity is ubiquitous in the bacterial world.** Based on the presence of GGDEF domain-encoding proteins in almost all sequenced bacterial genomes, it has been predicted that c-di-GMP is ubiquitous in bacteria (10, 11). However, it is unknown whether the GGDEF domain proteins from species outside of the proteobacterial branch are specific to c-di-GMP or cAMP, as both compounds have been associated with the GGDEF domain (Fig. 1A). It is possible that some GGDEF domain proteins have mixed specificities, which is characteristic of a group of mammalian PDE (31). It is also possible that a different type of nucleotide is used as a substrate for GGDEF domain proteins.

To investigate these possibilities, we tested proteins containing GGDEF domains from representatives of diverse branches of the phylogenetic tree of *Bacteria*. The following proteins were chosen: TM1163 from the hyperthermophilic marine bacterium *T. maritima* (branch Thermotogae), DRB0044 from the radiation- and desiccation-resistant soil bacterium *D. radio-durans* (Deinococcus/Thermus), Slr1143 from the phototrophic *Synechocystis* sp. (Cyanobacteria), Rrp1 (BB0419) from the intracellular parasite *B. burgdorferi* (spirochetes), and YeaP from the intestine inhabitant *E. coli* (γ subdivision of the Proteobacteria) (Fig. 1B). These proteins were chosen essentially at random. The anticipated ease of protein purification, i.e., relatively small size and lack of transmembrane domains, were used as sole criteria. The domain architecture of most chosen proteins turned out to be similar to that of *R. sphaeroides* DgcA, i.e., GAF plus GGDEF. The *B. burgdorferi* Rrp1 protein had a different domain structure, i.e., REC plus GGDEF (Fig. 1B), where REC is a receiver domain characteristic of response regulators of bacterial two-component systems (http://smart.embl-heidelberg.de). REC is also known as the CheY domain (http://www.sanger.ac.uk/Software/Pfam). None of the functions of these proteins was known prior to this study.

All selected genes were amplified from genomic DNA from their respective species and overexpressed in *E. coli* DH5α as fusions to the MBP protein. The recombinant proteins were purified by affinity chromatography and gel filtration, where needed, and assayed for biochemical activities with various nucleotides (Fig. 3A and data not shown). Four of five proteins (TM1163, DRB0044, Slr1143, and YeaP) used GTP as a substrate and showed DGC activity similar to that of the *R. sphaeroides* DgcA protein (Fig. 3B and data not shown).

The purified recombinant Rrp1 protein (Fig. 4A) showed no activity, even on prolonged incubation (Fig. 4B, top right). This prompted us to investigate Rrp1 further. Considering the domain architecture of Rrp1, we predicted that its activity could depend on the phosphorylation status of its REC domain. To test this hypothesis, we incubated Rrp1 with acetyl phosphate, a small-molecule phosphate donor. Acetyl phosphate specifically phosphorylates the acceptor aspartyl residues of response regulators, which are phosphorylated in vivo by histidine protein kinases (14). It is worth noting that in the *B. burgdorferi* genome (GenBank accession number AE001146), a gene encoding histidine protein kinase, *hpk1* (BB0420), is located immediately upstream of the *rrp1* gene. Furthermore, *hpk1* and *rrp1* appear to be cotranslated. This suggests that *hpk1* is likely to encode the cognate kinase of Rrp1.

The Rrp1 protein, which was incubated with acetyl phosphate and subsequently dialyzed, showed DGC activity. Preincubations with higher concentrations of acetyl phosphate resulted in higher levels of DGC activity (Fig. 4B and data not shown). These observations allow us to suggest that the DGC activity of Rrp1 depends on the phosphorylation status of its REC domain. Similar to other DGC proteins, Rrp1 was highly specific to GTP as a substrate.

The fact that six arbitrary chosen GGDEF domain proteins from diverse branches of *Bacteria* possess DGC activity establishes that synthesis of c-di-GMP is a widespread phenomenon and that c-di-GMP is ubiquitous in bacteria. The presence of DGC activity in *T. maritima*, which belongs to one of the deepest branches of the bacterial phylogenetic tree, suggests that c-di-GMP is an ancient molecule that apparently evolved at the dawn of bacterial evolution. This is supported by the presence of several GGDEF domain proteins in *Aquifex aeolicus*, a representative from yet another early diverging branch of bacteria (Fig. 1B). Our data strongly suggest that majority of GGDEF domain proteins encoded in bacterial genomes are involved in synthesis and/or hydrolysis of c-di-GMP, not cAMP.

No full-length GGDEF domains are encoded in the currently sequenced genomes of *Archaeeae*. We identified one protein, *Methanopyrus kandleri* MK0296, and one protein fragment, *Haloferax volcanii* Q9C4S7, that contain sequences of apparently truncated GGDEF domains, i.e., 144 and 84 amino acids, respectively. Whether or not these proteins are functional is unknown. It is possible that the archaeal GGDEF-like domains have been acquired via interkingdom gene transfer from *Bacteria*. However, we could not predict this with certainty, based on the G+C content or the gene neighborhood of the MK0296 gene (http://www.tigr.org).

Six putative GGDEF domain-containing proteins are predicted in the unfinished genome of a eukaryote, mosquito *Anopheles gambiae* (http://smart.embl-heidelberg.de). Some of these genes, e.g., ENSANGG00000000049 and ENSAN GP00000001831, are predicted to be composed of more than one exon, i.e., they have a eukaryotic gene structure (http://wwwensembl.org/Anopheles_gambiae). Interestingly, these proteins are very similar to the GGDEF domain proteins of the *Proteobacteria*, e.g., ENSANGG00000023215 from mosquito is 65% identical over the entire protein length to the putative Na+/Ca2+ antiporter from *Desulfovibrio desulfuricans*. Such level of similarity is unprecedented for an apparently nonessential protein. It is possible that the mosquito genes represent a recent case of interkingdom gene transfer from *Bacteria*. Alternatively, the GGDEF domain-encoding sequences may have originated from a contaminant genomic DNA of a bacterial symbiont or parasite of mosquito and genome sequence misassembly. In summary, functionality of the GGDEF domain proteins and the presence of c-di-GMP in *Archeaeeae* and *Eukarya* are highly questionable.

**Enzymatic activity of the GGDEF domains.** We investigated enzymatic activities of individual GGDEF domains. To this
end, we cloned and overexpressed sequences corresponding to GGDEF domains from two proteins, *R. sphaeroides* DgcA and *Synechocystis* sp. Slr1143. The MBP::GGDEF fusion proteins were purified and assayed with various nucleotides. Low amounts of c-di-GMP were formed upon incubation of each of these proteins with GTP (Fig. 3 and data not shown). DGC activity of the GGDEF domains of DgcA and Slr1143 was significantly, i.e., approximately 2 orders of magnitude, lower than those of the corresponding full-length proteins. Both isolated GGDEF domains possessed somewhat higher phosphatase, i.e., GTPase, activity that converted GTP into GDP (Fig. 3B).

That DGC activity is an intrinsic property of the GGDEF domain is shown here for the first time. Why is such activity low compared to that of full-length proteins? We hypothesized that either conformation or the oligomeric state of individual GGDEF domains prevents expression of high-level DGC activity. The latter possibility seemed reasonable given that adenyl cyclases, which share sequence similarity with the GGDEF domains (18), function as dimers (27). This prompted us to compare oligomeric states of the full-length proteins and individual GGDEF domains.

**Oligomeric state of DGCs.** All six full-length DGC proteins analyzed in this study were found to exist primarily as dimers and/or trimers (Fig. 3C, top; Fig. 4C, top; and data not shown). The individual GGDEF domains from DgcA and Slr1143 also formed dimers and trimers (Fig. 3C, bottom, and data not shown). Therefore, GGDEF domains have intrinsic propensity to dimerization. This suggests that low DGC activity of individual GGDEF domains did not result from an improper oligomeric state.

It is possible that, although dimers and trimers are formed by individual GGDEF domains, they are folded improperly. We explored this issue further by using the Slr1143 protein. Slr1143 contains a well-defined CC motif (http://smart.embl-heidelberg.de) between the GAF and GGDEF domains. Hydrophobic interactions between two CC motifs are known to promote protein dimerization. We reasoned that presence of the CC motifs would impose proper folding constraints on the CC-plus-GGDEF protein fragment, thus ensuring that the fold of the GGDEF domain resembles its fold in the full-length protein. We cloned and overexpressed the DNA fragment corresponding to the CC plus GGDEF portions of Slr1143 and purified the MBP::(CC + GGDEF) fusion (Fig. 3A, middle). The oligomerization state of the fusion protein was found to be identical to that of the individual GGDEF domain or full-length Slr1143 (Fig. 3C, middle). However, DGC activity of
this construct remained low and similar to that of the single GGDEF domain (Fig. 3B, middle).

From these studies, we conclude that the ability of GGDEF domains to convert GTP into c-di-GMP, although intrinsic to the GGDEF domain, is strongly affected by neighboring protein domains or possibly by interacting proteins. In case of Rrp1, the phosphorylated REC domain is apparently required for high-level DGC activity. The nonphosphorylated REC domain either does not promote DGC activity or inhibits it. In the cases of five other proteins, the presence of the GAF domains is sufficient for expression of the DGC activity in vitro. We suggest that activities of these five proteins in vivo are further modulated via ligands anticipated to bind to the GAF domains. At present, the identities of these ligands, if any, are unknown. However, neither cAMP nor cGMP, both of which bind to some GAF domains (33), stimulated DGC activities of the tested proteins, and neither did c-di-GMP, thus excluding the possibility of positive autoregulation by the reaction product (data not shown).

Strong dependence on activating stimuli provides a possible answer to the question of how bacteria can maintain dozens of GGDEF domain proteins yet have very low levels of intracellular c-di-GMP (24, 29). Most DGC in cells are likely to be inactive or almost inactive, unless a specific environmental or intracellular signal activates them.

c-di-GMP as an underappreciated ubiquitous signaling molecule in Bacteria. Architectures of GGDEF domain-containing proteins encoded by bacterial genomes indicate that they sense various environmental and intracellular signals, e.g., oxygen, light, small ligands, and membrane-derived signals (10, 11). Mutations in the GGDEF domain proteins or overexpression of such proteins affect exopolysaccharide synthesis in various proteobacterial species, including G. xylinus (20), V. cholerae (5, 19), Pseudomonas aeruginosa (9), Pseudomonas fluorescens (25), Agrobacterium tumefaciens (2), E. coli, and S. enterica (24, 32). In some of these species, this further affects exopolysaccharide-dependent formation of biofilms. In C. crescentus, flagellum ejection, which is required for the switch from motile to sessile lifestyle, is impaired in the GGDEF domain protein PleD mutation (1). In Synechocystis sp. PCC 6803 (30), P. aeruginosa (12), and Vibrio parahaemolyticus (4), mutations in the GGDEF domain proteins impair surface motility. In V. cholerae and Synechococcus elongatus, the proteins anticipated to be involved in c-di-GMP turnover affect gene expression (28, 29). Apparently, the GGDEF domain proteins integrate various environmental and intracellular stimuli into changes in c-di-GMP levels, which in turn control bacterial life, primarily life on surfaces. However, the full range of functions and targets of c-di-GMP has yet to be revealed. For example, in this work we observed strong toxicity of c-di-GMP to E. coli BL21. The target(s) of c-di-GMP action resulting in toxicity has yet to be identified.

Conclusions. We provided biochemical evidence that one of the most ubiquitous protein domains encoded in bacterial genomes, GGDEF, is primarily associated with c-di-GMP, not cAMP. We reported that diverse species of Bacteria are capable of c-di-GMP synthesis. We showed that DGC activity of GGDEF domain proteins is apparently highly regulated by the adjacent sensory domains. When taken together with the physiological and behavioral data on the GGDEF proteins cited above, our work establishes c-di-GMP as a ubiquitous signaling molecule in Bacteria that apparently functions as a second messenger. Among the best characterized and widely distributed second messengers are cyclic mononucleotides, cAMP and cyclic GMP (3, 6). The time has come for c-di-GMP to join the ranks of these structurally related but currently much better understood second messengers.

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