

# The Characterization of a Cyclic-Di-GMP (c-Di-GMP) Pathway Leads to a New Tool for Studying c-Di-GMP Metabolic Genes

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Second messengers play an integral role in regulating a wide variety of pathways in response to triggers from the extracellular milieu. The intracellular second messenger cyclic diguanylate (c-di-GMP) has been shown to regulate microbial adaptation to extracellular environments in myriad ways. Primarily studied in Gram-negative bacteria thus far, c-di-GMP regulates motility, biofilm formation, virulence, and more (reviewed in references 1 to 3). Specifically, elevated c-di-GMP favors a sessile lifestyle, often stimulating biofilm formation. Conversely, reduced c-di-GMP promotes biofilm dispersal and motility, and low c-di-GMP can activate virulence pathways in numerous bacterial pathogens. In comparison, little is known about the functions of c-di-GMP in Gram-positive bacteria. Determining how and under what circumstances bacteria regulate c-di-GMP levels, and the outcomes of c-di-GMP modulation, is important for understanding the role of c-di-GMP in bacterial cell signaling and adaptation to changing extracellular cues. Yet a full appreciation of c-di-GMP signaling pathways is often hindered by the fact that many bacterial genomes encode multiple, sometimes several dozen, c-di-GMP synthase (called diguanylate cyclase, or DGC) and hydrolase (called phosphodiesterase, or PDE) enzymes. There are many examples of single-gene mutations that result in detectable changes in some aspect of bacterial physiology (4–10), but often such mutations do not result in observable phenotypic alterations. Lack of gene expression, lack of enzyme activity, redundancy among c-di-GMP metabolic enzymes, and compensatory effects of other DGC and/or PDE enzymes under the conditions tested are some of the reasons that a mutation in a single DGC or PDE gene may not cause detectable changes in phenotype. In the current issue of the *Journal of Bacteriology*, Gao et al. present a systematic analysis of the principal c-di-GMP signaling components of the model Gram-positive bacterium *Bacillus subtilis* (11). Taking advantage of the relatively simple c-di-GMP signaling network of *B. subtilis*, the authors describe a mutant *B. subtilis* strain that may be used to analyze putative DGC enzymes for activity using an easily assayed motility phenotype as a readout.

In the highlighted study (11), Gao et al. aimed to fully examine the *in vitro* and *in vivo* functions of putative c-di-GMP signaling components of *B. subtilis*, including four putative DGCs (identified based on the presence of a GGDEF diguanylate cyclase domain), two predicted PDEs (identified based on the presence of an EAL c-di-GMP phosphodiesterase domain), one protein with tandem GGDEF and EAL domains, and a putative c-di-GMP receptor containing a PilZ domain. Recently, Chen et al. used genetic approaches to implicate one PDE, YuxH (renamed PdeH by Gao et al.) and the PilZ domain c-di-GMP receptor YpfA (renamed DgrA by Gao et al.) in regulation of *B. subtilis* motility and biofilm formation, indicating that these processes are controlled by c-di-GMP (12). In that work, deletion of a single PDE gene, *pdeH*, led to a significant reduction of *B. subtilis* motility. The motility defect

was complemented by the expression in *trans* of wild-type *pdeH*, but not an allele of *pdeH* expected to encode an inactive PDE, suggesting that the decreased motility was specifically due to increased c-di-GMP as a result of the loss of PdeH enzymatic activity (12).

In the current study (11), Gao et al. built on that work by providing evidence that the reduced motility of a *B. subtilis* *pdeH* mutant is due to increased intracellular c-di-GMP. Whereas c-di-GMP was undetectable in the wild-type parent by liquid chromatography followed by tandem mass spectrometry (LC-MS-MS) (at least under the growth conditions used), c-di-GMP was measurable in the *pdeH* mutant, indicating that loss of PdeH activity leads to accumulation of c-di-GMP. In addition, the authors show that three of the five GGDEF domain proteins and one of the three EAL domain proteins of *B. subtilis* are enzymatically active *in vitro* and *in vivo*. Overexpressing any of the three DGCs that were active *in vitro* in *B. subtilis* drove c-di-GMP to detectable levels. Interestingly, mutation of the GGDEF domain-encoding genes, individually or in combination, had no effect on motility of *B. subtilis*, despite encoding functional DGCs. These results corroborate the findings of Chen et al. (12). The lack of phenotype associated with these mutations could be because the encoded DGCs regulate pathways other than motility. Alternatively, c-di-GMP already may be adequately low under the growth conditions used to allow maximal motility. Thus, further reduction of c-di-GMP by inactivation of DGCs would thus have no discernible effect.

Having established the set of enzymes capable of modulating intracellular c-di-GMP levels, the authors then investigated the mechanisms by which c-di-GMP could be sensed in the cell. They focused on a putative c-di-GMP receptor protein containing a PilZ domain, DgrA. Previously, it was shown that deletion of *dgrA* restores wild-type levels of motility to a *pdeH* mutant, suggesting that DgrA mediates the effect of increased c-di-GMP on motility (12). Further analysis revealed that DgrA interacts with the MotA motor protein (12), consistent with previous reports in other bacteria that c-di-GMP can act through PilZ domain receptors to block motility via interaction with MotA or FliG (13–15). Gao et al. used size exclusion chromatography and isothermal titration calorimetry to show that DgrA directly binds c-di-GMP *in vitro*. DgrA is the only PilZ domain protein predicted in *B. subtilis*, but it is likely that

Published ahead of print 16 August 2013

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doi:10.1128/JB.00925-13

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this bacterium produces other c-di-GMP receptors. The authors note that one EAL domain protein, YdaK, does not encode a functional PDE but binds c-di-GMP with moderate affinity. While YdaK has no measurable effect on motility, it may control other c-di-GMP-regulated processes that remain to be elucidated in *B. subtilis*. No putative c-di-GMP riboswitches from the classes identified thus far are predicted to be encoded in the *B. subtilis* genome (16, 17). Other c-di-GMP receptors that exist in *B. subtilis* clearly must be identified experimentally.

The Gao et al. study culminates with the development of a so-called “c-di-GMP null strain” that could serve as a useful tool for assessing the activity of putative DGC enzymes from other organisms. The c-di-GMP null strain has deletions of each of the functional DGCs and PDE, therefore lacking the ability to synthesize or degrade c-di-GMP, and demonstrates wild-type motility. The strain additionally overexpresses the *dgrA* gene and thus produces a c-di-GMP sensor capable of inhibiting motility in response to c-di-GMP. As proof of principle, genes encoding characterized or predicted DGCs from other bacterial species were expressed in the c-di-GMP null strain, and the effect of motility was assessed. Two of the three heterologous DGCs tested, DccA from *Clostridium difficile* (18) and DcpA from *Vibrio vulnificus* (19), inhibited the motility of the *B. subtilis* c-di-GMP null strain, consistent with the ability of DccA and DcpA to synthesize c-di-GMP. The third, putative DGC tested, Atu1297 from *Agrobacterium tumefaciens*, did not affect motility. Atu1297 is an orthologue of PleD from *Caulobacter crescentus* and is similarly a response regulator consisting of two phosphoreceiver (REC) domains and a GGDEF domain. Phosphorylation of the PleD REC domain positively regulates the activity of the GGDEF domain (20, 21); if such activating signals are required for DGC activity, this could explain the lack of motility inhibition in the c-di-GMP null strain. Nonetheless, the c-di-GMP null strain could be used as a heterologous host for assaying the activity of many DGCs that do not require specific activating signals. Moreover, if activating signals and/or partner proteins are known or suspected, the ease with which *B. subtilis* can be genetically manipulated could allow introduction of those components as well. Finally, it may be possible to modify the *B. subtilis* host strain to also examine the functions of putative PDEs or regulators of DGC activity. Mutating *pdeH* and leaving one or more of the DGC genes intact would allow the *B. subtilis* strain to produce c-di-GMP, resulting in reduced motility (11, 12). Enzymatic activity of putative PDEs from other organisms could be observed as partial or full restoration of motility.

Other groups have expressed genes encoding putative c-di-GMP metabolic enzymes in heterologous bacterial hosts to gauge enzymatic activity. For example, the function of each of the 37 GGDEF and EAL domain proteins from *C. difficile* was assessed by overexpression of the corresponding *C. difficile* genes in *Vibrio cholerae*, in which c-di-GMP has well-defined roles in regulating motility and biofilm formation (22). The *B. subtilis* c-di-GMP null strain offers several advantages for studying c-di-GMP metabolic genes. First, many other species carry genes that encode a (sometimes much) larger number of c-di-GMP metabolic enzymes, making studying them in the native host challenging. In *B. subtilis*, the deletion of all of the relatively small number of native, functional DGCs and PDEs eliminates the possibility that the endogenous c-di-GMP signaling pathway will interfere with detection of

activity of the targeted gene. Second, *B. subtilis* is extremely easy to manipulate genetically, so exogenous DNA can be readily introduced. Finally, *B. subtilis* is not a pathogen and so can be handled in biosafety level 1 (BSL-1) laboratories without specialized containment.

Through a systematic analysis of the core components of the c-di-GMP signaling system of *B. subtilis*, Gao et al. clearly demonstrate a correlation between c-di-GMP levels and motility of *B. subtilis* and describe one of the most well-defined c-di-GMP signaling pathways identified thus far. One remaining piece of the puzzle, in *B. subtilis* and in many other bacterial species, is the identification of the extracellular signals that influence intracellular c-di-GMP and, consequently, processes controlled by this second messenger.

## ACKNOWLEDGMENTS

R.T. is supported by NIH awards U54-AI057157 and R01-AI107029.

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