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# Visualizing the Perturbation of Cellular Cyclic di-GMP Levels in Bacterial Cells

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Supporting Information Placeholder

**ABSTRACT:** Cyclic di-GMP (c-di-GMP) has emerged as a prominent intracellular messenger that coordinates biofilm formation and pathogenicity in many bacterial species. Developing genetically encoded biosensors for c-di-GMP will help us understand how bacterial cells respond to environmental changes via the modulation of cellular c-di-GMP levels. Here we report the design of two genetically encoded c-di-GMP fluorescent biosensors with complementary dynamic ranges. By using the biosensors, we found that several compounds known to promote biofilm dispersal trigger a decline in c-di-GMP level in *E. coli* cells. In contrast, the cellular c-di-GMP levels were elevated when the bacterial cells are treated with subinhibitory concentrations of biofilm-promoting antibiotics. The biosensors also revealed that the *E. coli* cells engulfed by macrophages exhibit lower c-di-GMP levels, most likely as a response to the enormous pressures of survival during phagocytosis.

In recent years, the cyclic dinucleotide c-di-GMP has emerged as a prominent messenger that coordinates the cellular functions associated with bacterial biofilm formation and pathogenicity[1-3]. In bacterial cells, the concentration of c-di-GMP is controlled by a large number of diguanylate cyclases (DGCs) and c-di-GMP phosphodiesterases (PDEs) (**Fig. 1a**). C-di-GMP exerts its effect by binding to a diverse array of receptors that include enzymes, transcriptional factors, adaptor proteins and riboswitches[4]. It has become increasingly clear that the c-di-GMP signaling networks play central roles in bacterial biofilm formation and virulence gene expression in some of the clinically important pathogens such as *Vibrio cholerae*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*[5-8]

One of the most remarkable features of the c-di-GMP signaling networks is that they usually consist of a large number of DGCs and PDEs, with many of them containing putative sensory domains for perceiving environmental cues[9-12]. It is believed that the diverse array of sensory domains allows the bacterial cells to respond to environmental changes by modulating cellular c-di-GMP concentration through the DGC and PDE proteins. However, with a few exceptions, the vast majority of the environmental signals and associated c-di-GMP pathways remain to be unveiled. In this regard, developing biosensors that can report the changes in cellular c-di-GMP levels would facilitate the identification of the proteins and pathways involved in the response mechanisms that are essential for environmental adaptation.

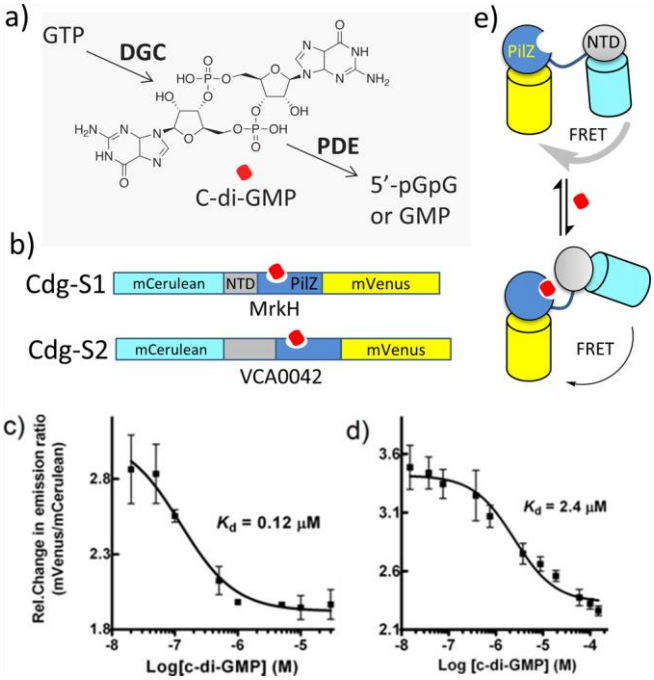


**Figure 1**. Design and *in vitro* characterization of c-di-GMP biosensors. a) Synthesis and degradation of c-di-GMP by DGCs and PDEs. b) Construction of the genetically encoded FRET-based biosensors for c-di-GMP by using MrkH and VCA0042. Both proteins contain a c-di-GMP binding PilZ domain and an N-terminal domain (NTD). c) and d) Fluorescence titration curves for cdg-S1 and cdg-S2 (Experimental conditions are included in online Supporting Information). e) Schematic illustration of the conformational change induced by c-di-GMP binding to cdg-S1 and cdg-S2.

We previously reported a fluorescent dye-labeled c-di-GMP biosensor for *in vitro* c-di-GMP detection by using the non-enzymatic EAL domain of the FimX protein[13,14]. Here we describe the design of two genetically encoded fluorescent biosensors for monitoring cellular c-di-GMP concentration by using two natural c-di-GMP binding proteins. The FRET (Förster resonance energy transfer)-based fluorescent biosensors were constructed by using MrkH and VCA0042, two c-di-GMP-

binding proteins from *K. pneumoniae* and *V. cholerae* respectively[6,15]. The two proteins share a common C-terminal PilZ domain for c-di-GMP binding but contain different N-terminal domains (NTD). The genes encoding the two proteins were cloned into pET28b and pUCP18-based expression vectors that harbor the mCerulean and mVenus genes to produce cdg-S1 and cdg-S2 (**Fig. 1b**), after optimization of the length of the linkers flanking the *mrkH* and *VCA0042* genes. mCerulean and mVenus were derived from the standard cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) to generate more stable fluorescent proteins with brighter fluorescence[16]. For *in vitro* characterization of the biosensors, cdg-S1 and cdg-S2 were produced by using the *E. coli* expression system and treated with the protein RocR to remove the associated c-di-GMP[17]. Upon full maturation of mCerulean and mVenus, fluorescence titrations were performed to show that the addition of c-di-GMP gradually reduces the relative changes in emission ratio (**Figs. 1c & 1d**), which implies a reduction in FRET efficiency. Based on the crystal structure of VCA0042[15], the reduced FRET efficiency is likely caused by a ligand-induced conformational change that affects the dipole-dipole orientation (or distance) between mCerulean and mVenus (**Fig. 1e**). The reduction of FRET efficiency upon c-di-GMP binding is also reminiscent of the biosensor developed from the protein YcgR[18]. Fitting of the titration data yielded the dissociation constants ($K_d$) of 0.12 μM and 2.4 μM for cdg-S1 and cdg-S2 respectively. Considering that the estimated concentrations of c-di-GMP in bacterial cells are in the range of 0.1 to 10 μM, the complementary dynamic ranges of the two biosensors will allow the reporting of cellular c-di-GMP levels at both the low and high ends of the concentration gradient. The presence of other nucleotides (e.g. cGMP, GXP, AXP, $NADP^+$ etc) at physiologically relevant concentrations did not interfere with the performance of the biosensors (see **Table S1**). The two biosensors are also insensitive to pH changes in the pH range of 6 to 9 (data not shown).

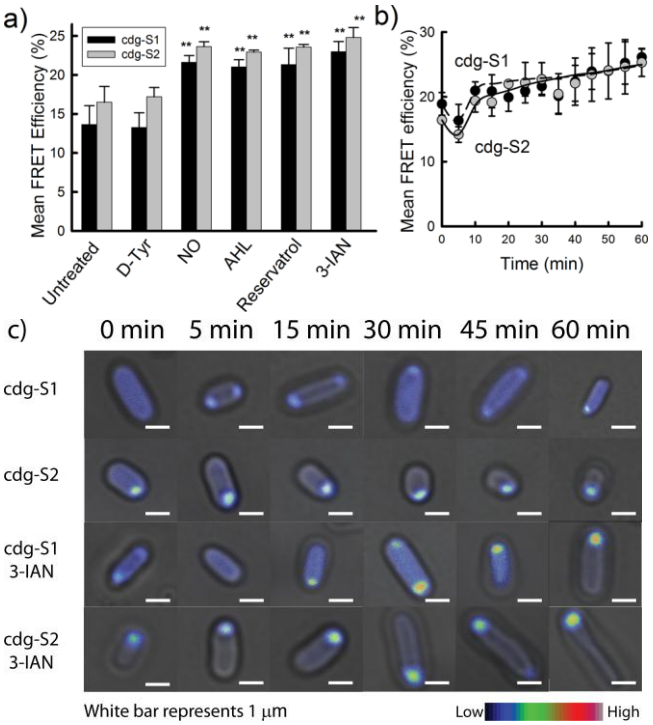


**Figure 2**. Perturbation of c-di-GMP levels in BL21 *E. coli* cells by biofilm-dispersing agents. a) Average FRET efficiencies for the *E. coli*-containing biosensors before and after the treatment with biofilm-dispersing agents for 30 min. FRET efficiency was measured by observing the change in donor emission (466 nm) upon acceptor photobleaching (see Supporting Information). Statistical significance is indicated by the asterisk (*, $P < 0.05$; **, $P < 0.01$). Changes in FRET efficiency are directly correlated to changes in the population of ligand binding biosensors and thus to c-di-GMP level. b) Time-dependent change of the FRET efficiencies for 3-IAN-treated cells. c) Representative images of YFP (λ430 nm excitation, λ528 nm emission) prior to acceptor photobleaching.

When cdg-S1 and cdg-S2 were expressed in the BL21 *E. coli* strain by isopropylthio-β-galactoside (IPTG) induction or constitutively in the uropathogenic *E. coli* strain UTI89, cyan and yellow fluorescence could be readily detected using a confocal microscope. Interestingly, in contrast to a diffuse distribution of the two biosensors in the *E. coli* UTI89 cells, most of the biosensors appear to be sequestered to the poles of the BL21 cells regardless of expression level. We found that the single-expressed mVenus or mCerulean also has a strong tendency to cluster at the poles in BL21 cells (**Fig. S1**), indicating that the polar localization could be due to the interaction between mVenus and unknown *E. coli* polar proteins. As we demonstrate below, similar results were obtained from the two *E. coli* strains regardless of the polarization of the biosensors.

A group of structurally diverse compounds are known to hinder the formation of robust bacterial biofilm or trigger the dispersal of biofilm[19-24]. It was also known that biofilm dispersal can be induced by sequestering cellular c-di-GMP by overexpressing c-di-GMP binding proteins, which indicates that a low c-di-GMP level could directly lead to biofilm dispersal[25]. By using the biosensors, we asked whether some of the biofilm-dispersing compounds (or dispersal factors) can cause a reduction in cellular c-di-GMP levels in planktonic *E. coli* cells. The answer to the question would yield insight into the biofilm-dispersing mechanism of these compounds. The compounds under investigation include one of the AHL (N-acylhomoserine lactones) autoinducers (N-hexanoyl-DL homoserine lactone), the plant auxin 3-indolyacetonitrile (3-IAN)[23], D-tyrosine[20], resveratrol[26] and the nitric oxide (NO) donor MAHMA-NONOate[21]. As shown in **Fig. 2, Fig. S2 and Table S2**, an increase of FRET efficiency was observed for four of the five compounds in both *E. coli* strains. D-tyrosine is the only compound that did not seem to perturb the FRET efficiency. A time-dependent study of 3-IAN showed an overall increase of FRET efficiency over time, indicating that the c-di-GMP level decreases gradually after an initial increase (**Figs. 2b, 2c & Figs. S2b, S2c**). In comparison, the control experiment showed that the FRET efficiencies did not change significantly for both *E. coli* strains during the one-hour observation window in the absence of dispersal factors (**Fig. 2c**). Note that cdg-S1 and cdg-S2 exhibit similar response dynamics albeit the different dynamics ranges (**Fig. 2b**), indicating that the cellular c-di-GMP concentration is likely in the range of 0.1 to 10 μM. Based on the standard curves obtained from *in vitro* titration data, we estimated that the compounds caused a drop of cellular c-di-GMP concentration from 0.8-2 μM to 0.2-0.5 μM. These results indicate that some of the biofilm-dispersing compounds may indeed induce biofilm dispersal by reducing cellular c-di-GMP concen-

tration. The observation that D-tyrosine did not perturb c-di-GMP level is in accordance with the view that D-amino acids trigger biofilm disassembly through the replacement of D-alanine in the biosynthesis of cell wall fibers, a process that does not involve the cytoplasmic c-di-GMP messenger[20]. The effect of NO on c-di-GMP level is probably exerted through the direct regulation of the DGC or PDE proteins[21,27]; while the detailed molecular mechanisms for the other dispersal factors remain to be determined.

In contrast to the dispersal factors, studies have shown that subinhibitory concentrations of aminoglycosides and cell wall-targeting antibiotics can promote biofilm formation[28-31]. Given the central role of c-di-GMP in biofilm formation, it was speculated that the biofilm-promoting effect of some of the antibiotics is exerted through c-di-GMP. The biosensors allowed us to test directly whether the treatment of subinhibitory concentrations of antibiotics can raise cellular c-di-GMP concentrations. As shown by **Fig. 3**, **Fig. S3** and **Table S3**, when the *E. coli* cells

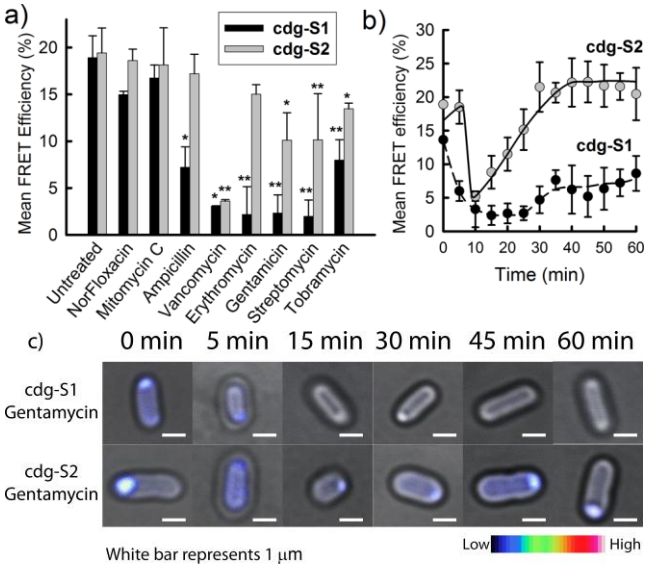


**Figure 3**. Perturbation of c-di-GMP levels in BL21 *E. coli* cells by subinhibitory concentration antibiotics. a) Average FRET efficiencies for the *E. coli*-containing biosensors before and after the treatment with antibiotics for 30 min. FRET efficiency was measured by observing the change in donor emission (466 nm) upon acceptor photobleaching (see Supporting Information). Statistical significance is indicated by the asterisk (*, $p < 0.05$; **, $P < 0.01$). Data were obtained by averaging the readings for multiple cells ($n > 10$). Changes in FRET ratio are directly correlated to changes in the population of ligand binding biosensors and thus to c-di-GMP level. b) Time-dependent change of the FRET efficiencies for gentamycin. c) Representative images of YFP (λ430 nm excitation, λ528 nm emission) prior to acceptor photobleaching.

were treated with the representative antibiotics at subinhibitory concentrations, significant decreases in FRET efficiency were observed for some of the antibiotics. Overall, the aminoglycoside antibiotics (tobramycin, gentamicin and streptomycin) and macrolide antibiotic (erythromycin) that target ribosome and the antibiotics (ampicillin, vancomycin) that target cell wall biosynthesis caused substantial reduction in FRET efficiency, indicating an increase in cellular c-di-GMP concentration. In contrast, the two antibiotics (mitomycin C, norfloxacin) that are not known to induce biofilm formation did not seem to change the c-di-GMP levels. The observations provide support for the view that subinhibitory concentrations of antibiotics trigger biofilm formation by raising the cellular c-di-GMP concentration. Notably, the role of c-di-GMP in aminoglycoside-induced biofilm formation was already unveiled by the discovery that aminoglycosides induce the up-regulation of YdeH, a DGC that produces c-di-GMP in *E. coli*[30]. A c-di-GMP specific PDE from *P. aeruginosa* has also been found to play a crucial role in tobramycin-inducible biofilm formation[29].

The time-dependent change of FRET efficiency during gentamycin treatment revealed the dynamics of the response to the antibiotic. The results show a rapid rise of c-di-GMP levels in the first 10 min of treatment before a gradual decreasing process **(Figs. 3b & S3b).** Noted that the antibiotic treatment reduced the FRET signal of cdg-S1 to very low levels and that the recovery of FRET signal is consistently much slower than cdg-S2. In the UT189 strain, the difference between the two biosensors is not as prominent **(Fig. S3b)**. The discrepancies between the two biosensors in the two *E. coli* strains are likely caused by the different dynamic ranges of the biosensors and higher c-di-GMP levels in the UT189 cells under antibiotic stresses. Based on the results, we estimated that the biofilm-promoting antibiotics can raise the cellular c-di-GMP concentration to as high as 5 - 10 μM in BL21 cells, from the basal level of 0.8-2 μM. The c-di-GMP levels in the UT189 cells could be even higher, with both the cdg-S1 and cdg-S2 saturated upon antibiotic treatment.

Lastly, c-di-GMP has been suggested to play a role in the *in vivo* survival of some intracellular pathogenic bacteria[32,33]. With the biosensor, we would like to know whether the c-di-GMP level changes when bacterial cells are challenged by host immune system. One of the most challenging environments for bacterial cells is inside the phagosome of macrophages during the phagocytosis process. During phagocytosis, the bacterial cells are under enormous stress in a low-pH environment that is inundated with a host of antimicrobial agents such as NO, reactive oxygen species (ROS) and antimicrobial peptides. By averaging the readings from multiple bacterial cells, we observed increased FRET efficiencies for both cdg-S1 and cdg-S2 when the *E. coli* cells were engulfed by RAW 264.7 macrophage cells **(Figs. 4 & S4)**. The increase in FRET efficiency upon engulfment can also be seen from the histogram of the free and engulfed cells **(Figs. 4c & S4c,** note that only the cdg-S2 results are shown**).** These observations indicate a reduction of c-di-GMP concentration in the engulfed *E. coli* cells, similar to the effect triggered by the biofilm-dispersing compounds (e.g. NO) discussed above. The reduced c-di-GMP level in the macrophage-engulfed cells is most likely part of the counter-attack strategy used by the bacterial cells to boost virulence expression to cope with the enormous pressure of survival. The view is further supported by the observation that a decrease in c-di-GMP level in the intracellular pathogen *Bordetella* is correlated with its ability to kill macrophages (Yang et al, unpublished results).

In summary, we have developed two genetically encoded FRET biosensors for monitoring the fluctuation of c-di-GMP levels in bacterial cells. The biosensors revealed a decline of c-di-GMP level when the cells were treated with biofilm-dispersing agents or in the hostile environment of macrophages. On the contrary, the biosensors reported elevated c-di-GMP levels in *E. coli* when the cells were treated with subinhibitory

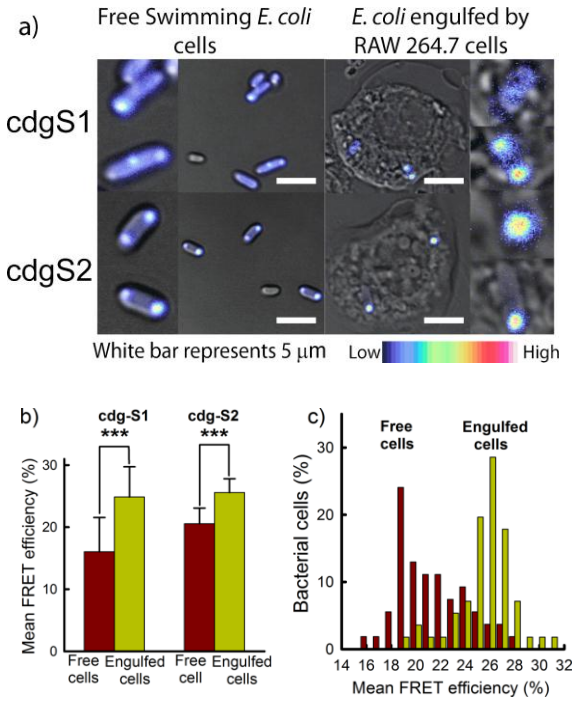


**Figure 4**. Perturbation of c-di-GMP level in the BL21 *E. coli* cells engulfed by macrophage. a) Representative images of YFP (λ430 nm excitation, λ528 nm emission) prior to acceptor photobleaching of the *E. coli* cells outside and inside of RAW264.7 macrophage cells. Side panels show the enlarged views of the bacterial cells. b) Average FRET efficiencies for the *E. coli* cells outside and inside of the macrophage. Statistical significance is indicated by the asterisk (***, $P < 0.001$). c) Histogram showing the FRET efficiency distribution for cdgS2 in the free and macrophage-engulfed *E. coli* cells. (Total number of cells are 50 (free) and 53 (engulfed))

concentrations of biofilm-promoting antibiotics. These observations are supportive of the view that high c-di-GMP level promotes sessility and biofilm formation while low c-di-GMP level promotes biofilm dispersal. The results establish the biosensors as valuable chemical biology tools and indicate a crucial role played by c-di-GMP in stress response and environmental adaptation. In conjunction with the studies on mutant strains, the biosensors will further help us identify the specific c-di-GMP signaling proteins and pathways involved in stress response in the future. By studying the biosensor-containing bacterial cells embedded in biofilm matrix, we will also be able to gain a better understanding of the roles of c-di-GMP in the highly dynamic and complex processes of biofilm formation and dispersal.

## ASSOCIATED CONTENT

**Supporting Information**. Experimental details, supporting figures and tables are included in Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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## TOC Graphics