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<th>Visualizing the perturbation of cellular cyclic di-GMP levels in bacterial cells</th>
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<td>Ho, Chun Loong; Chong, Kavin Shi Jie; Oppong, Jamila Akosua; Chuah, Mary Lay-Cheng; Tan, Suet Mien; Liang, Zhao-Xun</td>
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In recent years, the cyclic dinucleotide 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 inhibit lower c-di-GMP levels. Here we describe the design of two genetically encoded fluorescent biosensors for c-di-GMP by using non-enzymatic EAL domain of the FimX protein. 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 biosensors were constructed by using MrkH and VCA0042, two c-di-GMP.
binding proteins from K. pneumoniae and V. cholerae respectively. 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 cdgS1 and cdgS2 (Fig. 1b), after optimization of the length of the linkers flanking the mHK 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.

For in vitro characterization of the biosensors, cdgS1 and cdgS2 were produced by using the E. coli expression system and treated with the protein RocR to remove the associated c-di-GMP. 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, 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. Fitting of the titration data yielded the dissociation constants (Kd) of 0.12 μM and 2.4 μM for cdgS1 and cdgS2, respectively. Considering that the estimated concentrations of c-di-GMP in bacterial cells are in the range of 0.1 to 10 μM, the dynamic range 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).

When cdgS1 and cdgS2 were expressed in the BL21 E. coli strain by isopropylthio-β-galactoside (IPTG) induction or constitutively in the uropathogenic E. coli strain UT189, 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 UT189 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. 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. 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-indolylacetonitrile (3-IAN), D-tryosine, resveratrol and the nitric oxide (NO) donor MAHMA-NONOate. As shown in Fig. 2, Fig. S2 and Table S2, an increase in FRET efficiency was observed for four of the five compounds in both E. coli strains. D-tryosine 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 cdgS1 and cdgS2 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-
It was observed that the increase in cellular c-di-GMP concentration did not perturb c-di-GMP level in accordance with the view that Damino acids trigger biofilm disassembly through the replacement of D-lysine in the biosynthesis of cell wall fibers, a process that does not involve the cytoplasmic c-di-GMP messenger. The effect of NO on c-di-GMP level is probably exerted through the direct regulation of the DGC or PDE proteins; 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. 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 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. A c-di-GMP-specific PDE from P. aeruginosa has also been found to play a crucial role in tobramycin-inducible biofilm formation.

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 cdgS1 to very low levels and that the recovery of FRET signal is consistently much slower than cdgS2. 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 cdgS1 and cdgS2 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. With the biosensor, we would like to know whether the c-di-GMP level changes when bacterial cells are engulfed 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 cdgS1 and cdgS2 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 cdgS2 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
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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REFERENCES
