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Thiazole Orange-Induced c-di-GMP Quadruplex Formation Facilitates a Simple Fluorescent Detection of This Ubiquitous Biofilm Regulating Molecule

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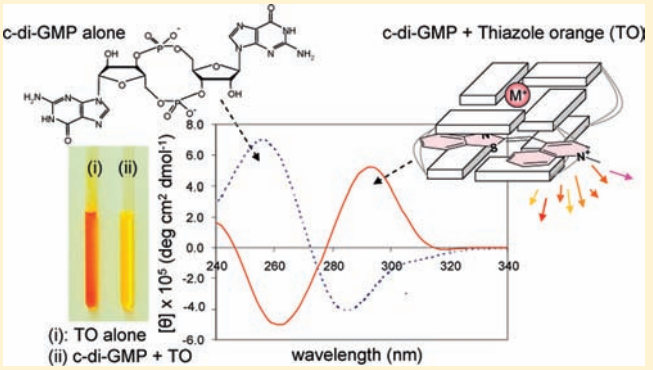
Supporting Information

ABSTRACT: Recently, there has been an explosion of research activities in the cyclic dinucleotides field. Cyclic dinucleotides, such as c-di-GMP and c-di-AMP, have been shown to regulate bacterial virulence and biofilm formation. c-di-GMP can exist in different aggregate forms, and it has been demonstrated that the polymorphism of c-di-GMP is influenced by the nature of cation that is present in solution. In previous work, polymorphism of c-di-GMP could only be demonstrated at hundreds of micromolar concentrations of the dinucleotide, and it has been a matter of debate if polymorphism of c-di-GMP exists under in vivo conditions. In this Article, we demonstrate that c-di-GMP can form G-quadruplexes at low micromolar concentrations when aromatic molecules such as thiazole orange template the quadruplex formation. We then use this property of aromatic molecule-induced G-quadruplex formation of c-di-GMP to design a thiazole orange-based fluorescent detection of this important signaling molecule. We determine, using this thiazole orange assay on a crude bacterial cell lysate, that WspR D70E (a constitutively activated diguanylate cyclase) is functional in vivo when overexpressed in *E. Coli*. The intracellular concentration of c-di-GMP in an *E. Coli* cell that is overexpressed with WspR D70E is very high and can reach 2.92 mM.



INTRODUCTION

Over 70% of hospital infections are caused by biofilm forming bacteria; yet there are no drugs currently in clinical use that inhibit biofilm formation in bacteria. In the past decade, there has been an explosion of research in quorum sensing (QS)[1] and 3′,5′-cyclic diguanylic acid (c-di-GMP) signaling[2] in bacteria. It is now well appreciated that these two processes regulate both virulence factors production and biofilm formation in a variety of bacteria, including those of clinical relevance. Investigations that unravel the molecular details of these two important transduction processes in bacteria would lead to the identification of novel drug targets for the development of next-generation anti-infectives, which are not bacteriostatic but rather target bacterial virulence and biofilm formation in bacteria.[3] Molecules that target bacterial pathogenesis rather than killing bacteria are expected to provide less evolutionary pressure for bacteria to develop resistance mechanisms.

C-di-GMP, a secondary messenger present uniquely and ubiquitously in bacteria, plays a central role in bacterial biofilm formation and regulation of virulence-related factors in diverse bacteria.[4] Biofilm formation encases the bacteria with a polysaccharide and proteinaceous matrix, resulting in enhanced resistance to chemical and physical stress, and the clinical manifestation is bacterial resistance to conventional antibiotics. Therefore, suppressing biofilms synthesis should make bacteria more susceptible to current antibiotics. C-di-GMP signaling initiates with synthesis by diguanylate cyclases (DGCs) and terminates when degraded by phosphodiesterase (PDEs). Investigations into all sequenced bacterial genomes reveal that all genomes have at least one DGC or PDE, suggesting the evolutionary importance of this signaling pathway in bacteria.[2] Despite the central role that c-di-GMP plays in bacteria, very little information is known about the actual macromolecular targets that c-di-GMP regulates. Only the metabolism proteins of c-di-GMP (DGCs and PDEs) as well as a handful of adaptor proteins[5] and riboswitches[6] have been characterized. Most of the adaptor proteins that bind to c-di-GMP do not have enzymatic properties on their own, but the effector proteins that these adaptor proteins regulate are largely unknown. Additionally, the environmental cues that regulate the metabolism of c-di-GMP are not well characterized. The dearth of detailed information regarding c-di-GMP signaling is probably due to a variety of factors, one of which is the lack of specific chemical probes to readily detect

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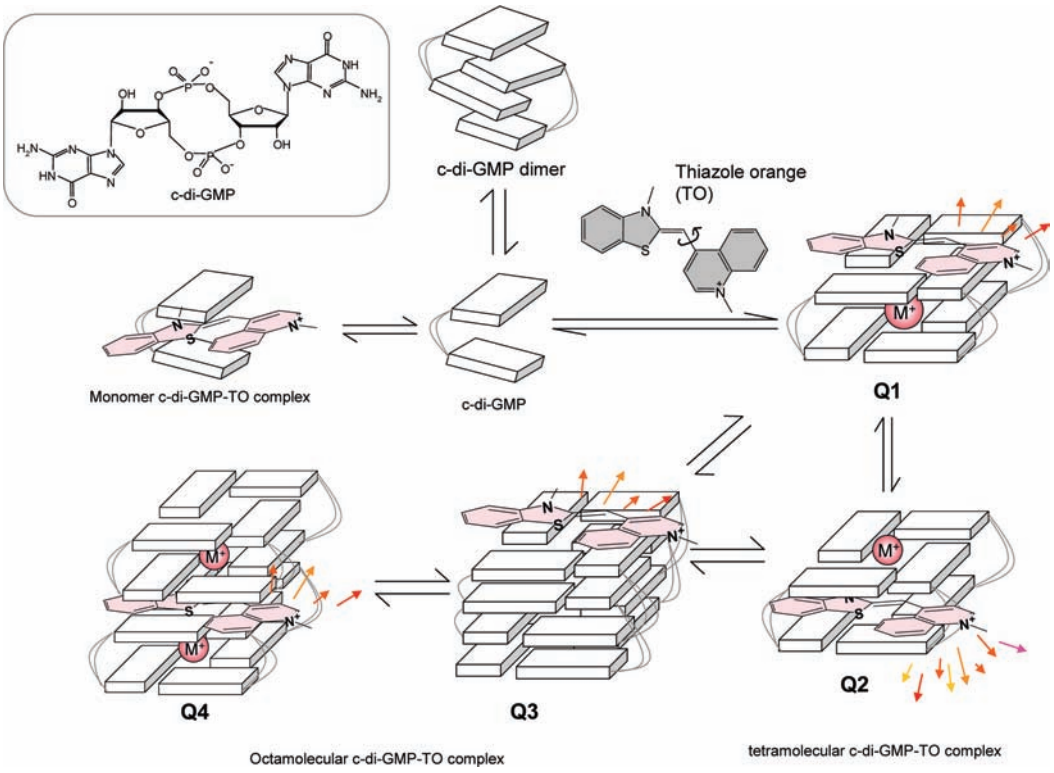

Figure 1. TO is predicted to interact with c-di-GMP to give a 1:1, 1:4, or 1:8 complexes in the presence of monovalent cations. In scenarios whereby TO is intercalated between the two planes of the G-tetrad (such as Q2), it is hypothesized that the fluorescence of TO will be enhanced.

c-di-GMP. Currently, c-di-GMP is detected in vitro and in vivo via a tandem HPLC-MS protocol or the use of recombinant fluorescent proteins, respectively.[7] Bacterial cells or culture media may contain myriads of other nucleotides or other compounds that might coelute with c-di-GMP.[8] Therefore, good resolving HPLC columns as well as sensitive MS instruments are needed to detect c-di-GMP in crude cell lysates via the tandem HPLC-MS protocol. The use of recombinant proteins to detect c-di-GMP is more appropriate for in vivo applications. Because this approach involves a transformation step, c-di-GMP cannot be detected in bacteria that do not transform or do not express the recombinant fluorescent protein very well. The aforementioned setbacks associated with current c-di-GMP detection assay argue for the development of new detection platforms for this signaling molecule. A detection method that uses simple shelf-stable reagents without the need for sophisticated instrumentation or separation step will complement the current two sate-of-the-art detection methods. Herein, we describe a simple and specific fluorescent detection of c-di-GMP using thiazole orange (TO, 1-methyl-4-[(3-methyl-2(3*H*)-benzothiazolylidene)-methyl]quinolinium *p*-tosylate). TO is not generally fluorescent in the presence of simple nucleotides such as GTP, GMP, or cGMP. We demonstrate that c-di-GMP is an exception to this rule and can be detected in vitro with this dye in a selective fashion without the need for prior HPLC purification.

RESULTS AND DISCUSSION

Thiazole orange (TO) is a known fluorescent intercalator of nucleic acids and can be used to detect different DNA and RNA structures.[9] TO has a low fluorescence quantum yield in aqueous solution ($\Phi f = 0.0002$).[10] TO can interact with polynucleotides, such as duplex DNA, via association with the minor groove, intercalation between base pairs, or association with the negatively charged phosphate backbone.[9a,11] Out of these three modes of TO association with polynucleic acids, intercalation and minor groove binding can lead to fluorescence enhancement but not TO association with the phosphate backbone. Upon TO confinement in nucleic acid cavities, the nonradiative decay channel is blocked due to the molecular confinement. The quantum yield of TO in confined cavities subsequently increases to up to 0.4.[10] It therefore follows that nucleotides such as GTP and cGMP, which do not have the ability to confine TO, cannot be detected with TO. C-di-GMP, on the other hand, has been shown to form aggregates such as tetramolecular quadruplexes and octamolecular complexes in solutions containing monovalent cations such as K^+.[12] We rationalized that we could use this unique property of c-di-GMP to form aggregates, for specific detection in the presence of other guanine containing nucleotides that do not generally form aggregates at micromolar concentrations in water. Specifically, we hypothesized that in the presence of potassium, sodium, or ammonium cations, c-di-GMP would either form a 4:1 complex with TO (see Q1 or Q2 in Figure 1) or an 8:1 complex with TO (see Q3 or Q4 in Figure 1), and as the TO in these complexes would be restricted to freely rotate, the nonradiative channel in excited TO would be closed and the quantum yield of the confined TO would increase appreciably for it to become fluorescent. Because the fluorescence enhancement in TO is derived from restricted rotation in the molecule, it is reasonable to assume that complexes Q2 and Q4 would be more fluorescent than Q1 and Q3 because the TO in Q2 or Q4 is embedded between two G-tetrad planes, whereas in Q1 or Q3 only one G-tetrad plane π-stacks with TO (Figure 1).

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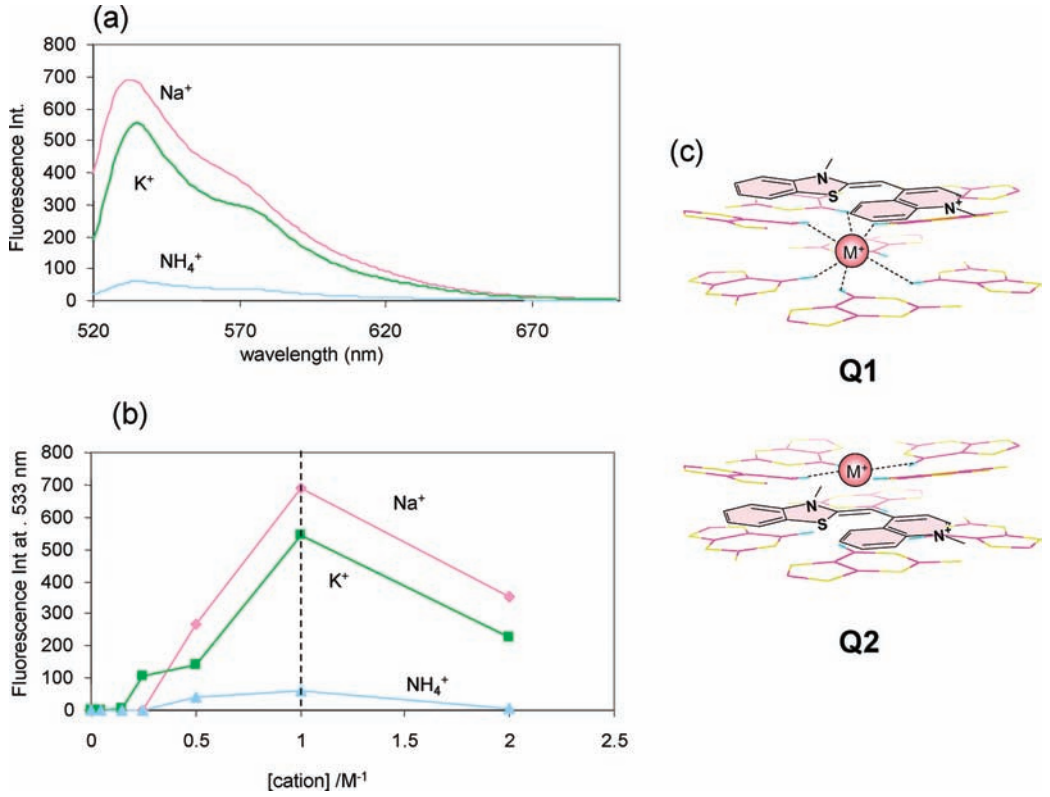

Figure 2. In the presence of monovalent cations, c-di-GMP interacts with TO and enhances the fluorescence of TO. The enhancement of TO fluorescence was dependent on the nature and concentration of the cation. [c-di-GMP] = 20 μM, [thiazole orange] = 30 μM. Ex. 507 nm, em. 518−700 nm. Buffer: 10 mM Tris-HCl (pH 7.5) containing (a) 1 M of NaCl, KCl, or NH_4OAc. (b) Fluorescence of TO−c-di-GMP complex in the presence of different salt concentrations (0 mM, 50 mM, 150 mM, 250 mM, 500 mM, 1 M, or 2 M). (c) Q1 and Q2 are two possible modes of TO interaction with c-di-GMP in the presence of a templating cation.

Based on our proposal in Figure 1, among alkali metals, Li^+ that do not promote G-quadruplex formation[12,13] would not be ideal for our detection scheme. In the c-di-GMP octamolecular complex, the G-tetrads interlock each other. It can therefore be argued that there is more space between the G-tetrad planes in c-di-GMP tetramolecular complexes than in c-di-GMP octamolecular complexes. Consequently, intercalation into tetramolecular complexes might be easier than in the octamolecular complex. Jones has shown that potassium promotes formation of the c-di-GMP octamolecular complexes, whereas the propensity for c-di-GMP to form octamolecular complexes is not high for sodium.[12] Although it is known that potassium forms more stable G-quadruplexes,[14] we rationalized (on the basis of our working model that TO would preferentially bind to c-di-GMP tetramolecular complexes) that sodium might be the cation of choice for c-di-GMP detection by TO. In agreement with this working model, the detection of c-di-GMP with TO was most sensitive in the presence of Na^+ as compared to in the presence of K^+ or NH_4^+ cation (see Figure 2).

The TO fluorescence in the TO−c-di-GMP complex was dependent on the concentration of the monovalent cation. As the concentration of the cation increased (up to 1 M), the fluorescence intensity also increased (see Figure 2b). This was expected as higher cation concentration would promote more of the higher aggregate of c-di-GMP, which is required for TO sequestration and fluorescence enhancement. Beyond 1 M cation concentration, the fluorescence intensity of TO decreased. This could be attributed to several reasons, such as the formation of higher order aggregates of c-di-GMP or TO, which are less fluorescent.

In a 1:4 TO−c-di-GMP complex, it is reasonable to assume that it is unlikely that both TO and the monovalent cations would reside between the planes of the G-tetrad. Therefore, the likely scenario is for the metal to reside between the G-tetrad planes, whereas TO interacts with the G-tetrad plane via $\pi-\pi$ interaction (Q1, end-stacking mode, Figure 2)[15] or whereby TO intercalates between the two c-di-GMP G-tetrads and the monovalent cation resides within the plane of the G-tetrad (Q2, Figure 2).[16] UV analysis of c-di-GMP interaction with TO revealed that when c-di-GMP was added to TO, there was a red shift in the TO absorption spectrum (Figure 3). This is an indication of a $\pi-\pi$ interaction between the guanine bases of c-di-GMP and TO.[17]

The detection of c-di-GMP with TO is specific. In the presence of increasing c-di-GMP concentration, the fluorescence intensity of TO increased (see Figure 4a). The detection limit for this assay is 5 μM. In line with expectation, a mixture containing several nucleotides such as GTP, cGMP, ATP, etc. (see Figure 4b) could not be detected with TO, whereas c-di-GMP or a mixture of several nucleotides plus c-di-GMP could be detected with TO. Importantly, the fluorescent signal of the sample that contained only c-di-GMP and that which contained several other nucleotides and c-di-GMP were similar (see Figure 4b, compare lines a and c). At high concentrations of both c-di-GMP and TO (300 μM), there was no need for a spectrophotometer to observe complex formation (see

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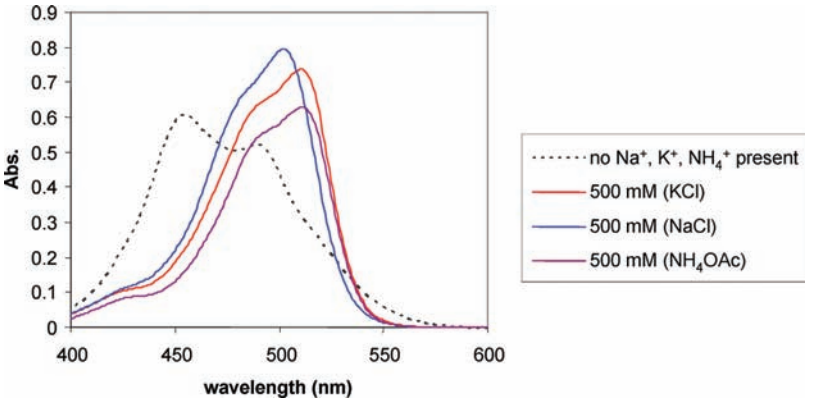

Figure 3. UV spectra of TO + c-di-GMP in buffer. Addition of monovalent cations to TO causes a red shift in the absorption spectrum. Buffer: 10 mM Tris-HCl (pH 7.5) containing 500 mM of one of these salts; NaCl, KCl, and NH_4OAc or no salt was added. [c-di-GMP] = 70 μM, [TO] = 30 μM.

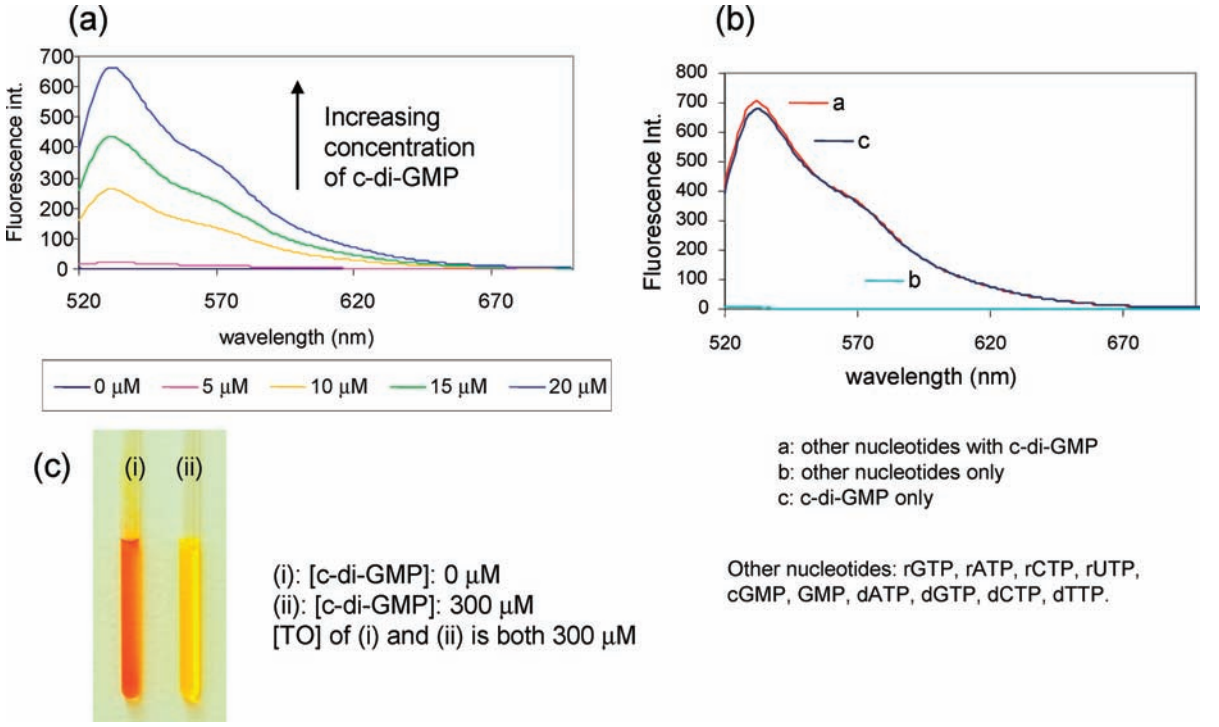

Figure 4. (a) Dose−response for TO−c-di-GMP interaction. [TO] = 30 μM, buffer: 10 mM Tris-HCl (pH 7.5) containing 1 M NaCl. Ex. 507 nm, em. 518−700 nm. (b) Selectivity of this detection system. [c-di-GMP] = 20 μM, each [nucleotide] = 20 μM, buffer: 10 mM Tris-HCl (pH 7.5) containing 1 M NaCl, [TO] = 30 μM. (c) Visible difference between the presence and absence of c-di-GMP.

Figure 4c). c-di-AMP has also been shown to be a signaling molecule in bacteria.[26] Incubation of TO with c-di-AMP did not lead to fluorescence enhancement of TO (see Supporting Information, Figure S1). c-di-AMP does not form quadruplex, and as we believe that the fluorescent enhancement of TO by c-di-GMP is via intercalation between the G-tetrad plane, the c-di-AMP result is not surprising and in fact augments our hypothesis.

Having established that TO can detect c-di-GMP in a concentration-dependent manner, we proceeded to gain some insights into the aggregation state of the TO−c-di-GMP complex. Circular dichroism (CD) is a powerful tool for identifying the aggregation state of nucleic acids.[12,18] Although CD cannot give a detailed molecular structure of G-quadruplex (tetramolecular or octamolecular complexes), it can be used to qualitatively determine if a G-quadruplex is present in solution. Jones has shown that a positive CD peak at around 300 nm is indicative of tetramolecular or octamolecular complex formation by c-di-GMP.[12] This CD signature, which indicates the presence of c-di-GMP tetramolecular or octamolecular complexes, was corroborated with NMR studies. Jones' study used high concentrations of c-di-GMP (hundreds of micromolar). The physiological concentration of c-di-GMP ranges from high nanomolar to tens of micromolar, so it is a matter of serious debate whether the polymorphism of c-di-GMP is indeed relevant under in vivo conditions. We therefore sought to establish if c-di-GMP can form tetramolecular and octamolecular complexes at low micromolar ranges (0−100 μM). Between 0 and 100 μM of c-di-GMP,

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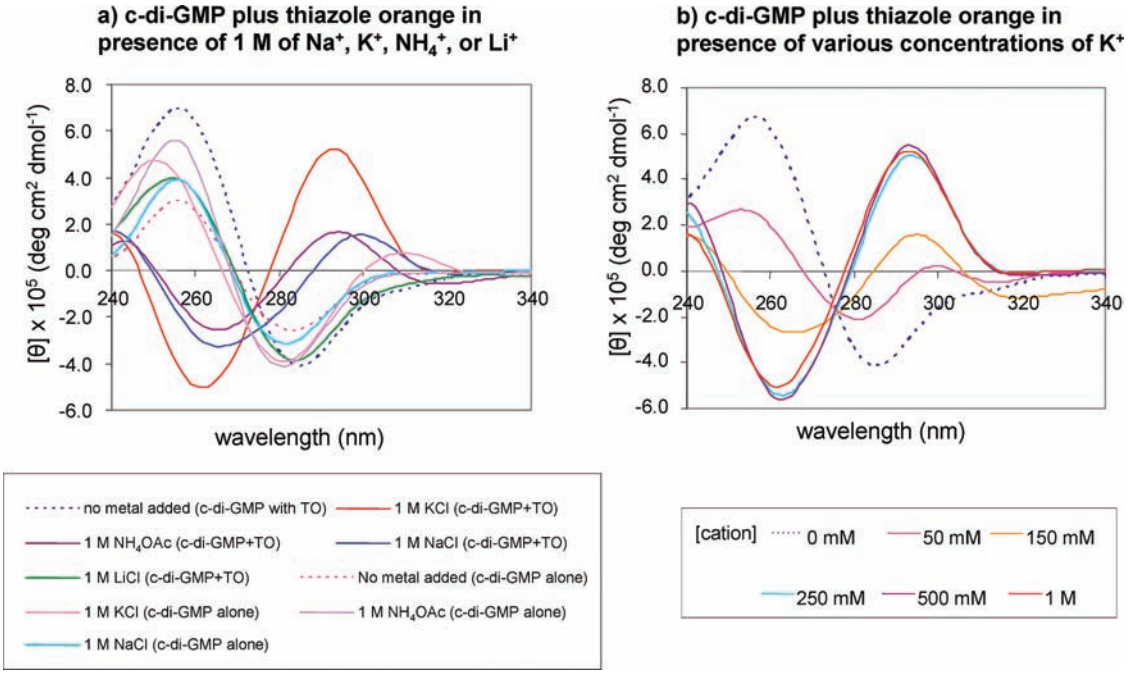

Figure 5. CD spectra of c-di-GMP alone and c-di-GMP-TO complexes. [c-di-GMP] = 70 μM, [TO] = 30 μM. Buffer: 10 mM Tris-HCl (pH 7.5) containing (a) 1 M salt (NaCl, KCl, NH_4OAc, and LiCl) or no metal added, (b) KCl at various concentrations, 0, 50, 150, 250, 500 mM, 1 M, was added to the buffer.

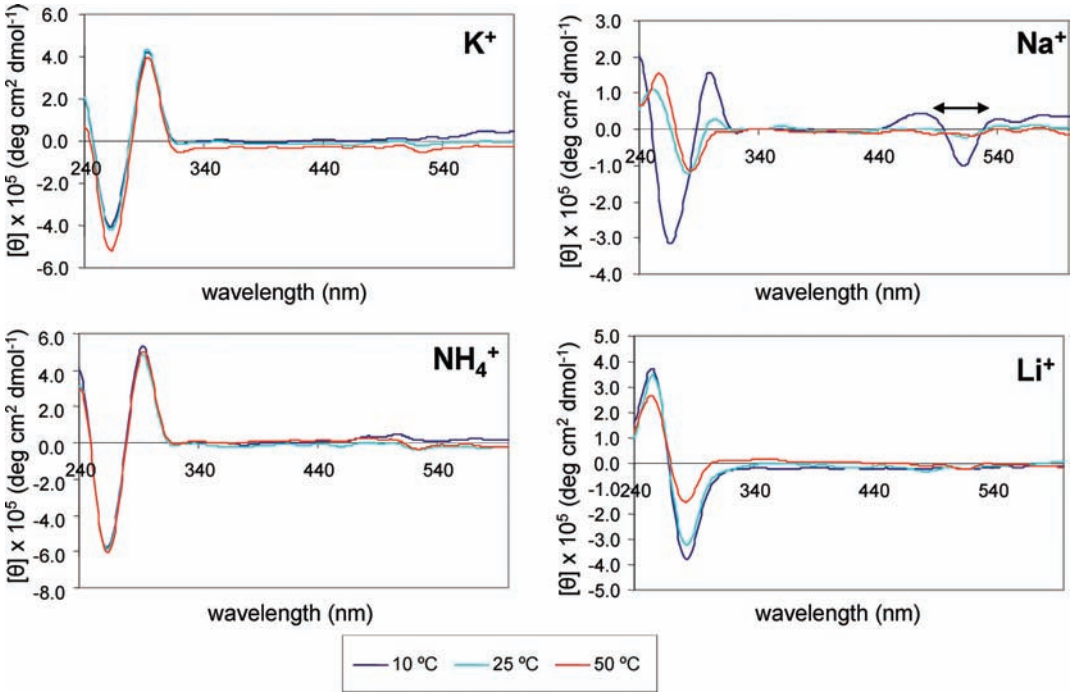

Figure 6. CD of TO−c-di-GMP complex in the presence of various monovalent cations at different temperatures. Buffer: 10 mM Tris-HCl (pH 7.5) containing 1 M salt (NaCl, KCl, NH_4OAc, and LiCl). [c-di-GMP] = 70 μM, [TO] = 30 μM. The temperatures used were 10, 25, and 50 °C.

the amount of c-di-GMP tetraplexes or octaplexes formed was minimal. In this concentration range, only potassium promoted some G-quadruplex formation (see Figure 5a, light pink solid line: 1 M KCl). However, upon the addition of TO to c-di-GMP in buffers containing monovalent cations Na^+, K^+, NH_4^+, but not Li^+, a strong positive CD peak around 300 nm appeared in the CD spectra (see Figure 5a, blue, red, purple, or green solid lines). It is noteworthy, that although G-quadruplexes formed by c-di-GMP have been previously observed in solution, the concentrations of c-di-GMP that was required to form G-quadruplexes were several hundreds of micromolar and were not physiologically relevant. Here, we show that in the presence of aromatic molecules such as TO, c-di-GMP can form stable G-quadruplexes at low micromolar concentrations.

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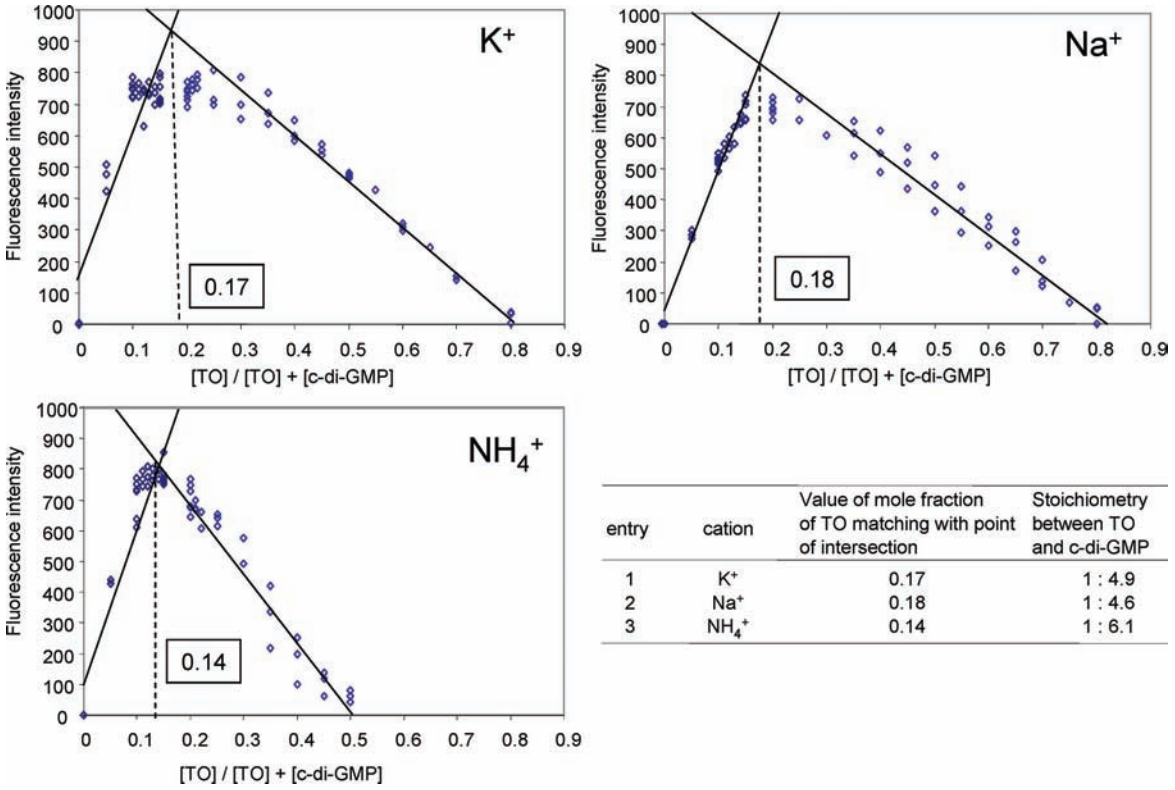

entry	cation	Value of mole fraction of TO matching with point of intersection	Stoichiometry between TO and c-di-GMP
1	K^+	0.17	1 : 4.9
2	Na^+	0.18	1 : 4.6
3	NH_4^+	0.14	1 : 6.1

Figure 7. Job plot. Total [TO] + [c-di-GMP] was fixed at 50 μM. The experiment was done in triplicate, and all of the triplicate data points are plotted on the graphs. Buffer: 10 mM Tris-HCl (pH 7.5) containing 1 M salt (NaCl, KCl, and NH_4OAc). Ex. 507 nm, em. 533 nm.

Interestingly, although TO fluorescence was highest when sodium was the templating cation, the CD intensity was highest when potassium was the templating cation (compare Figures 2a and 5a). Also, the maximum of the CD spectrum when sodium was the templating cation is slightly red-shifted as compared to those of potassium and ammonium (Figure 5a, compare the blue line (NaCl) with the red (KCl) or purple (NH_4OAc) lines). Surprisingly, the TO−c-di-GMP G-quadruplexes in buffers containing excess potassium or ammonium cations were stable even at 50 °C, whereas in the presence of excess sodium, the c-di-GMP G-quadruplex structure collapsed at 50 °C (see Figure 6). The lower stability of the c-di-GMP/TO/Na^+ complex, as compared to c-di-GMP/TO/K^+ or c-di-GMP/TO/NH_4^+ complexes, seems to be at odds with our observation of fluorescence enhancement, which we have attributed to the confinement of TO within the G-quadruplex planes. If TO confinement as well as the orientation of confinement both contribute to fluorescence enhancement in TO, then the stability of the complex alone cannot be used to predict which complex is going to be the most fluorescent. Indeed, both CD and UV data (Figures 3, 5, and 6) reveal that the orientation of TO in the c-di-GMP/TO complex, in the presence of different metal salts, is different. TO is achiral and therefore is not expected to have a CD spectrum. However, upon binding to chiral molecules such as nucleic acids, TO can exhibit induced CD. A CD band centered around 490−500 nm has been suggested as providing circumstantial evidence of intercalation of cyanine dyes into polynucleotides.[10a,19] We observed a small induced CD between 490 and 540 nm (the region where TO absorbs) only in the presence of sodium cation but not in the presence of potassium or ammonium cations (Figure 6). Without additional experimental evidence, it is dangerous to speculate the origin of this difference. We are however tempted to hypothesize that because Na^+ is a smaller cation, as compared to K^+ or NH_4^+ (size of Na^+ = 0.95 Å, K^+ = 1.33 Å, and NH_4^+ = 1.48 Å), Na^+ can nicely fit into the cavity in the plane of the G-tetrad to form structures such as Q2 (see Figures 1 and 2). On the other hand, K^+ and NH_4^+, being bigger than Na^+, prefer to sit between two G-tetrad planes to give other complexes such as Q1, Q3, or Q4 (see Figure 1). As these complexes are different, it is not surprising that the induced CD in TO in these different complexes also differ.

To determine the stoichiometry between TO and c-di-GMP, we performed a Job plot (Figure 7). The Job plot data indicate that the stoichiometry of TO to c-di-GMP in the presence of sodium or potassium or ammonium is 1:4.6, 1:4.9, and 1:6.1, respectively. From these data, one can conclude that the major TO−c-di-GMP aggregate species in buffers containing excess sodium or potassium is likely to be a tetramolecular complex, whereas more of higher order aggregates are present when ammonium acts as the templating cation. As the fluorescent intensity of TO decreases when the concentration of higher aggregates of TO−c-di-GMP increases (compare Figures 2 and 7), we are tempted to speculate that the aggregate, which is responsible for TO fluorescence enhancement, is the TO−c-di-GMP G-quadruplex form (see Figure 1, Q1 and Q2). Further work is however warranted to confirm this hypothesis.

As mentioned earlier in this Article, the main aim of this project was to find a simple shelf-stable reagent for detecting c-di-GMP, although the mechanistic insights that have been gained from this study regarding the interactions of c-di-GMP and TO are also interesting and important for the basic understanding of how different nucleic acid structures interact with dye molecules.

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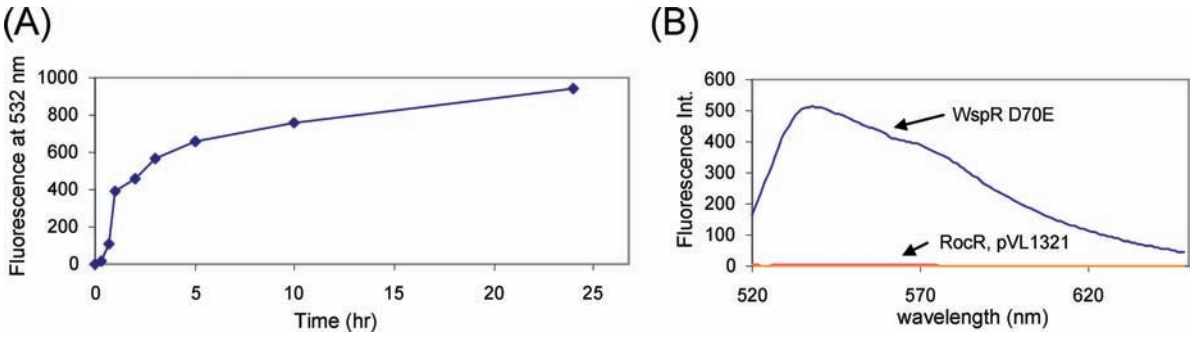

Figure 8. (A) Enzymatic conversion of GTP into c-di-GMP by WspR D70E (DGC). Enzymatic reaction conditions: [WspR] = 1 μM, buffer, 10 mM Tris-HCl (pH 8.0) containing 100 mM NaCl, 5 mM $MgCl_2$, reaction temperature was 37 °C. Detection conditions: [TO] = 30 μM, buffer, 10 mM Tris-HCl (pH 7.5) containing 1 M NaCl. (B) Fluorescence detection of c-di-GMP in *E. Coli* cell lysate (see Experimental Section for details). Detection conditions: [TO] = 30 μM, buffer, 10 mM Tris-HCl (pH 7.5) containing 1 M NaCl. In line with expectation, bacteria that overexpressed phosphordiesterase, RocR, or which contained an "empty" plasmid (pVL1321) did not produce detectable c-di-GMP.

Our group is interested in developing assays to quickly assay the activity of c-di-GMP metabolism proteins. Previously, others have done this particular assay using radiolabeled inorganic phosphate, which is not convenient to use due to safety reasons[20] or by HPLC detection of whole cell extracts.[21] Toward developing a measurement system that does not rely on radioactivity or separative methods, we have used our TO detection assay to assay the conversion of GTP into c-di-GMP by the diguanylate cyclase, WspR (see Figure 8A). To make meaningful conclusions from such experiments, it is always necessary to determine if the overexpressed protein is functional/active inside bacterial cells. Using our simple TO detection assay, we have managed to determine that WspR D70E (a constitutively activated DGC[22] that is overexpressed in *E. Coli*) is functional in vivo (see Figure 8B). The overexpression of WspR D70E in *E. Coli* resulted in the production of 8.79×10^5 c-di-GMP molecules per cell. The typical *E. Coli* cell has the dimension of 2 μm × 0.5 μm × 0.5 μm and a corresponding volume of 0.5×10^{-15} L, which implies that there is 2.92 mM c-di-GMP inside the *E. Coli* cell (see the Supporting Information for the details). It is of note that our TO detection of c-di-GMP was performed on crude cell lysate, without any prior separation step. We also used HPLC purification, followed by MS verification of the identity of c-di-GMP, to quantify intracellular c-di-GMP concentrations of the *E. Coli* (see the Supporting Information). The HPLC/MS quantification method gave an estimated c-di-GMP concentration of 3.43 mM inside the *E. Coli* cell, remarkably similar to our estimate of 2.92 mM using our TO assay. The estimate of 2.92 mM c-di-GMP inside *E. Coli* is significantly higher (1000×) than the basal concentration of c-di-GMP in a variety of bacteria (which is in the single digit micromolar range).[8,23] This goes to show that DGCs, such as WspR D70E, can synthesize c-di-GMP in bacteria in an uncontrolled fashion and flush the cells with high concentrations of c-di-GMP.

CONCLUSION

It has now been over two decades since Benziman published his seminal paper, which showed that c-di-GMP regulates cellulose production in bacteria.[24] Since then, it has been subsequently shown that c-di-GMP is a master regulator of several processes in bacteria and that unraveling the molecular details of c-di-GMP signaling will no doubt reveal important targets for antibiotic therapy. In pursuing this goal, it is imperative that we understand factors that affect c-di-GMP polymorphism and metabolism as well as identify the key players in this interesting signaling cascade. Fluorescent tools that detect bioanalytes[25] have played important roles in the advancement of biology, and it is expected that specific tools that can be used to characterize c-di-GMP will find broad utility in the c-di-GMP field. In this Article, we show that TO can discriminate c-di-GMP from other small nucleotides. This discovery could become useful for in vitro assays whereby other macromolecules such as DNA and RNA, which also bind to TO, can be separated from the nucleotides via a simple filtration process. Our TO detection assay for c-di-GMP is a nice complement of the tandem HPLC/MS method as it does not require a separation/purification step. We envisage potential future applications such as high-throughput assays to investigate c-di-GMP binding molecules. Importantly, this work also reveals that the polymorphism of c-di-GMP can be remarkably influenced by the presence of aromatic molecules, which form higher aggregates with c-di-GMP. Our fluorescence data reveal that even at low micromolar concentrations (less than 10 μM), c-di-GMP can enhance the fluorescence of TO. As it is likely that this enhancement of TO fluorescence by c-di-GMP occurs via tetramolecular complex formation, this work provides important evidence that the polymorphism of c-di-GMP is relevant at physiological conditions. Aggregation of c-di-GMP by aromatic molecules is bound to affect the intracellular concentrations of monomeric and dimeric c-di-GMP concentrations, and one can speculate that aromatic molecules-induced polymorphism of c-di-GMP could also be an important regulatory mechanism in bacterial biofilm formation.

EXPERIMENTAL SECTION

General Methods for Optical Measurements. Absorbance spectra were obtained on a JASCO V-630 spectrophotometer with 1 cm path length cuvette. Fluorescence studies were performed on a Varian Cary Eclipse fluorescence spectrophotometer with 1 cm path length cuvette. CD experiments were performed by a JASCO J-81 spectropolarimeter with 1 cm path length cuvette. The concentration of a stock solution of c-di-GMP and TO was determined by measuring the absorbance at 260 nm for c-di-GMP and 501 nm for TO and using 21 600 and 63 000 M^{-1} cm^{-1} as molar extinction coefficients for c-di-GMP and TO, respectively. TO was purchased from Sigma-Aldrich. rGTP, rATP, rCTP, and rUTP were purchased from Promega. dATP, dGTP, dCTP, and dTTP were purchased from New England Biolab. cGMP (guanosine 3′,5′-cyclic mono phosphate) was purchased from

CALBIOCHEM. GMP (guanosine-5′-monophosphate) was purchased from Amersco.

General Preparation of Sample before Measurements Assay. c-di-GMP, water, buffer solution (pH 7.5), and salt solutions were mixed, heated to 95 °C and kept at 95 °C for 5 min, and then cooled back to room temperature and kept at room temperature for 15 min. TO was then added to the mixture and incubated in the refrigerator at 4 °C overnight (about 12 h).

Measurement of Fluorescence. The instrument settings were chosen as follows: λ_{ex} = 508 nm (slit 5 nm), λ_{em} = 518−700 nm (slit 5 nm). The measurements were carried out at 10 °C.

Measurement of Thiazole Orange UV Spectra. The instrument settings were chosen as follows: wavelength = 400−600 nm, temperature was 10 °C. The concentration of c-di-GMP was 70 μM, TO was 30 μM, and buffer was 10 mM Tris-HCl (pH 7.5) containing 0 mM or 1 M metal (NaCl, KCl, NH_4OAc, and LiCl).

Circular Dichroism Experiments (CD). The concentration of c-di-GMP was 70 μM, TO was 30 μM, and buffer was 10 mM Tris-HCl (pH 7.5) containing 0 mM, 50 mM, 150 mM, 250 mM, 500 mM, or 1 M metal (NaCl, KCl, NH_4OAc, and LiCl). The measurement was performed at 10 °C. For stability study, 10, 25, and 50 °C were used as analysis temperatures. Data pitch, 1 nm; scan speed, 50 nm/min; response, 8 s; bandwidth, 1 nm.

Preparation of the Cell Lysate. *E. Coli* BL21(DE3) was used for production of WspR(D70E), RocR from pVL1321 (a pET vector derivative). Strains were grown up at 30 °C in Luria−Bertani (LB) medium (3 L) with shaking, and IPTG induction was performed for 6 h. Final OD (600 nm) was 2.8 for BL21(DE3) pVL1321-*wspR*, 6.8 for BL21(DE3) pVL1321-*rocR*, and 6.0 BL21(DE3) pVL1321 vector control cells. From plating of serial dilution of the culture, each OD (600 nm) unit is equivalent to 1×10^9 colony forming units (CFU). Cell were pelleted by centrifugation and resuspended to a final volume of 40 mL of 10 mM Tris-HCl (pH 8.0) containing 100 mM NaCl. 100 μL of 25 mg/mL Lysozyme was added to each resuspension. The resuspension was sonicated (20 s, three times) to disrupt membranes and allow lysozyme to reach cell walls. 60% perchloric acid (final concentration was 12%) was added to the resuspension to precipitate cellular macromolecules. The resuspension was incubated for 10 min on ice and neutralized by 3 M KOH containing 0.4 M Tris and 2 M KCl. The resuspension was centrifuged, and the collected supernatant was then filtered by a 0.2 μm filter and 3 kD exclusion columns.

■ ASSOCIATED CONTENT

Supporting Information. Additional figures and calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

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