

Enzymatic Production of c-di-GMP Using a Thermophilic Diguanylate Cyclase

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Abstract

C-di-GMP has emerged as a prevalent bacterial messenger that controls a multitude of bacterial behaviors. Having access to milligram or gram quantities of c-di-GMP is essential for the biochemical and structural characterization of enzymes and effectors involved in c-di-GMP signaling. Although c-di-GMP can be synthesized using chemical methods, diguanylate cyclases (DGC)-based enzymatic synthesis is the most efficient method of preparing c-di-GMP today. Many DGCs are not suitable for c-di-GMP production because of poor protein stability and the presence of a c-di-GMP-binding inhibitory site (I-site) in most DGCs. We have identified and engineered a thermophilic DGC for efficient production of c-di-GMP for characterizing c-di-GMP signaling proteins and riboswitches. Importantly, residue replacement in the inhibitory I-site of the thermophilic DGC drastically relieved product inhibition to enable the production of hundreds of milligrams of c-di-GMP using 5–10 mg of this robust biocatalyst.

Key words c-di-GMP, Diguanylate cyclase, Thermophilic enzyme, *Thermotoga maritima*

1 Introduction

C-di-GMP (3', 5'-cyclic diguanylate) is an intracellular messenger that can be found in many environmental and pathogenic bacteria [1–3]. Accumulating evidence suggests that c-di-GMP plays an active role in the chronic and acute infections caused by many human and plant pathogenic bacteria. There is strong interest in the microbiology community to identify and characterize the c-di-GMP signaling enzymes and effectors involved in virulence expression and biofilm formation. C-di-GMP has also attracted much attention in the aftermath of the findings that c-di-GMP can bind to specific receptors in human cell and modulate host cellular response by inhibiting basal and growth factor-induced proliferation of human carcinoma cells [4–6]. Hence, the use of c-di-GMP and its structural analogs as vaccine adjuvant or immunomodulatory molecule with immunoprophylactic properties is being actively explored [4].

Having access to milligrams or gram quantities of c-di-GMP is essential for biochemical and structural characterization of the proteins and riboswitches involved in c-di-GMP signaling. C-di-GMP can be synthesized chemically or enzymatically. While the chemical synthesis approach is time-consuming and environmentally unsustainable because of the multistep nature of the synthetic process [7–12], enzymatic production of c-di-GMP by using diguanylate cyclases (DGC) only involves a single step that condenses two GTP molecules into c-di-GMP [13]. However, the synthesis of c-di-GMP by using WspR and many other mesophilic DGC proteins suffers from poor yield as a result of poor thermostability and strong product inhibition [14]. In the search for a more robust and efficient DGC for c-di-GMP production, we identified a thermophilic DGC (TM1788) from *Thermotoga maritima* after assessing the possibility of producing soluble protein. The 241 residue-containing TM1788 is predicted to contain a single GGDEF domain (88–241 aa) and a membrane-embedded N-terminal segment (1–86). A gene construct (referred to as tDGC) that encodes the stand-alone GGDEF domain (82–241 amino acids) was synthesized with codon-optimized for *E. coli* overexpression. The recombinant tDGC protein was soluble and enzymatically active, but the yield of c-di-GMP production using this enzyme is low due to strong product inhibition. The inhibition by c-di-GMP was substantially alleviated by replacing a single residue (Arg158) in the I-site with Ala [15, 16]. This highly efficient tDGC_{R158A} mutant (tDGCm) has been used in our lab for the production of c-di-GMP or ³²P-labelled c-di-GMP for biochemical and structural studies [14, 17–22]. Solution and X-ray crystallography studies revealed that while tDGC exists as a dimer in solution with c-di-GMP bound in the I-site at the dimerization interface (Fig. 1a), tDGCm exists as a c-di-GMP-free monomer in solution (Fig. 1b) [23]. In contrast to mesophilic enzymes that usually exhibit poor thermostability under in vitro conditions, tDGCm retained 90% of its enzymatic activity after 24 h when incubated at 45 °C and 10 h at 55 °C (Fig. 1c). The tDGCm protein can be immobilized in sol-gel blocks or particles to further extend its shelf-life at room temperature [16]. The sol-gel immobilized tDGCm can be stored at room temperature for up to 6 months and used as a convenient chemical catalyst for c-di-GMP production. In this protocol, we describe the procedures used in our lab for the expression and purification of tDGCm and enzymatic preparation of c-di-GMP in solution. Although we consider tDGCm as the most efficient enzyme currently available for the large-scale production of c-di-GMP given its thermostability and high enzymatic activity, the readers should be aware that several other mesophilic DGCs were assessed and optimized for the synthesis of c-di-GMP in other research labs [24–26].

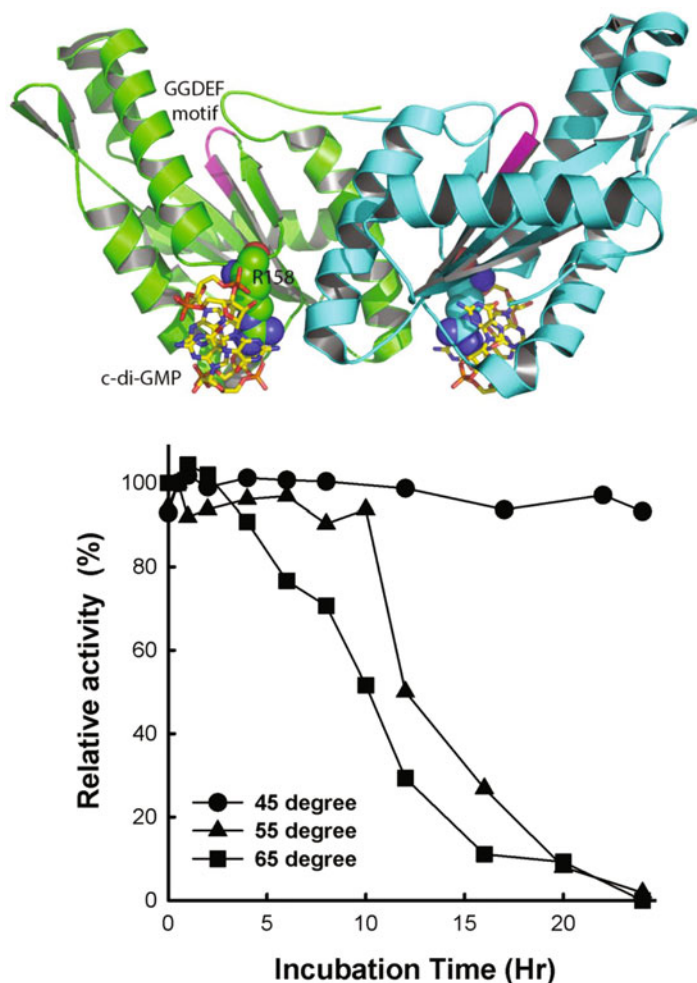


Fig. 1 Structure and thermostability of tDGC. (a) Crystal structure of the dimeric tDGC [23]. The conserved GGDEF motifs are highlighted in pink. The dimeric c-di-GMP molecules bound at the I-sites are shown in stick representation. The residue R158 from the RXXD motif for binding c-di-GMP in the I-site is shown in sphere representation. Replacement of R158 is crucial for relieving product inhibition and increasing c-di-GMP production. (b) Crystal structure of the monomeric tDGC_{R158A} mutant (tDGCm). (c) Thermostability test suggests that tDGCm maintains its enzymatic activity for over 24 h at temperatures lower than 45 °C in our reaction buffer

2 Materials

2.1 Production and Purification of tDGCm

1. Overexpression plasmid pET-tDGCm. The overexpression plasmid is derived from the pET28b(+) vector and harbors a codon-optimized *tDGCm* gene for *E. coli* overexpression. The *tDGCm* gene was cloned into the pET28b(+) vector using the NdeI and XhoI sites to produce N-terminal (His)₆-tagged tDGCm. The overexpression plasmid can be obtained from the Liang lab at Nanyang Technological University (E-mail: zxliang@ntu.edu.sg).

2. BL21(DE3) *E. coli* strain.
3. Luria–Bertani medium: 10 g of bacto tryptone, 5 g of yeast extract, and 10 g of NaCl in a 2 l conical flask. Add double distilled H₂O (ddH₂O) or nanopure H₂O to make up the final volume of 1 l. Autoclave the liquid medium and store at room temperature or in a cold room (for storage more than 1 day) until use.
4. 1 M Isopropyl β -D-1-thiogalactopyranoside (IPTG) stock solution filtered using 0.2 μ m filter disc.
5. Lysis buffer: 50 mM Tris–HCl (ultrapure), 300 mM NaCl, 5% glycerol, 1% β -mercaptoethanol, and 1 mM phenylmethyl sulfonyl fluoride (PMSF). Mix and adjust pH to 8.0 with HCl. Make up the final volume to 1 l using ddH₂O and filter the buffer using a 0.2 μ m filter disc.
6. Wash buffers: W1: 50 ml Lysis buffer that contains 20 mM imidazole (Molecular biology grade >99%) and W2: 20 ml Lysis buffer that contains 50 mM imidazole.
7. Elution buffer: 20 mM Tris–HCl, 200 mM NaCl, 5% glycerol with 200 mM, 300 mM, or 500 mM of imidazole, respectively. Adjust the pH of the three buffers to 8.0 with HCl. Make up the final volume to 1 l using distilled water and filter using a 0.2 μ m filter disc.
8. Gel filtration buffer: 50 mM Tris–HCl, 300 mM NaCl, 5% glycerol, and 1 M dithiothreitol (DTT). Mix and adjust pH to 8.0 with HCl. Make up the final volume to 1 l using distilled water and filter using a 0.2 μ m filter disc.
9. Disposable PD-10 Desalting Column (14.5 \times 50 mm), with 8 ml of Sephadex™ G-25 resin beads (GE Healthcare).
10. 15% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE).
11. Bradford dye reagent or other assay kits for protein concentration measurement.
12. Quartz or disposable cuvettes for UV-Vis spectrophotometer.
13. UV-Vis spectrophotometer (SHIMADZU UV-1600).
14. ÄKTA Fast protein purification system (GE Healthcare) (Optional).
15. HiLoad 16/600 Superdex 75 pg gel-filtration size-exclusion column (Optional).
16. Amicon concentrator (10 kDa, 15 ml) from Millipore.
17. Sonicator. SonicsVibra cell VCX500—Tip diameter 1/2" (13 mm) with threaded end and replaceable tip (volume 10–250 ml).
18. Beckman centrifuge and rotors JA10 rotor (Max speed: 17,700 $\times g$ or 10,000 rpm); JA25.50 rotor (75,600 $\times g$ or 25,000 rpm). Refrigeration is needed for both rotors.

19. Shaking incubator.
20. 0.2 and 0.45 μm Minisart syringe filters.
21. Ni^{2+} -nitrilotriacetic acid (NTA) resin (5 ml, Qiagen).
22. Liquid nitrogen.

2.2 Enzymatic Synthesis of c-di-GMP

1. Reaction buffer: 50 mM Tris-HCl, 300 mM NaCl, and 20 mM MgCl_2 . Dissolve the buffer salts in 190 ml ddH₂O, adjust pH to 8.0 using HCl, make up the final volume to 200 ml with ddH₂O and filter the buffer using a 0.2 μm filter disc.
2. Analytical high performance liquid chromatography (HPLC) system (LC1200, Agilent Technologies) equipped with a 150 \times 4.6 mm reverse phase C18 column.
3. HPLC solvent: mix 20 ml of triethyl ammonium bicarbonate (20 mM) buffer and 90 ml methanol (HPLC grade) in 890 ml distilled water to make a 1 l solution. Adjust pH to 7.0 using acetic acid and filter using a 0.2 μm filter disc.
4. GTP stock prepared in situ: Dissolve 1 g GTP powder (sealed and kept in -20°C freezer) in 1.9 ml distilled water to obtain 1 M stock solution.
5. Water bath set to 45 and 95 $^\circ\text{C}$.
6. Timer.
7. Stirring plate.

2.3 Purification and Quantification of c-di- GMP

1. Preparative HPLC LC-8A equipped with a fraction collector.
2. Preparative HPLC column (Phenomenex, Jupiter RP-C18 300A, 250 \times 21.2 mm, 5 μm).
3. Rotary vacuum evaporator (EYELA Rotary evaporator-N-1000, with KIF LAB-vacuum controller and EYELA Digital Waterbath SB-1000).
4. Lyophilizer and lyophilizer flask (Labconco).
5. Methanol (HPLC grade).
6. HPLC buffer: Mix 20 ml of triethyl ammonium bicarbonate (20 mM) buffer and 90 ml methanol in 890 ml distilled water to make 1 l solution. Adjust pH to 7.0 using acetic acid and filter the solvent using a 0.2 μm filter disc.
7. Tris-HCl buffer, 5 mM Tris, pH 7.0.
8. UV-Vis spectrophotometer (Shimadzu UV-1600).

3 Methods

3.1 Expression and Purification of tDGCm

1. The tDGC_{R158A} mutant (tDGCm) gene was cloned into the expression vector pET28b(+) to yield the plasmid pET-

tDGCm for the expression of N-terminal (His)₆-tagged recombinant protein. The plasmid pET-tDGCm can be transformed into *E. coli* BL21 (DE3) or other *E. coli* expression strains by standard electroporation methods. Store the *E. coli* cells as 20% glycerol stocks in a -80°C freezer.

2. For protein expression, inoculate 2 ml of autoclaved LB medium (Kanamycin 50 $\mu\text{g}/\text{ml}$) with the frozen BL21(DE3) cells with an inoculation loop or toothpick. Grow the cells overnight at 37°C in a shaking incubator.
3. Inoculate 1 l autoclaved LB medium (Kanamycin 50 $\mu\text{g}/\text{ml}$) with 2 ml of the overnight culture in a 2 l conical flask and grow the cells at 37°C (180 rpm) till they reach an optical density ($\text{OD}_{600\text{nm}}$) of 0.8 (3.5–4 h). Cool down the culture broth to 16°C by adjusting the temperature of the incubator. After the temperature reaches 16°C in about 40 min, induce protein expression using 0.8 mM IPTG and continue the cultivation at 16°C (180 rpm) overnight (*see* **Note 1**).
4. Harvest the cells by centrifugation using a JA10 rotor at $11,300 \times g$ (8000 rpm) for 8 min. Resuspend the cells in 20 ml of lysis buffer and lyse the cells using a sonicator (settings—02 s ON and 01 s OFF at 40% amp for 15 min) (*see* **Note 2**). Separate the supernatant from cell debris by centrifugation using a JA25.50 rotor at $48,200 \times g$ (20,000 rpm) for 30 min and filter the supernatant using a 0.45 μm minisart[®] syringe filter (cellulose acetate).
5. Wash 5 ml of Ni^{+2} -NTA resin slurry with the lysis buffer in a 10 ml syringe without the plunger. We use a porous filter-disc recycled from a PD-10 column to prevent the resin from falling out. Wash the resin with the lysis buffer a few times to remove ethanol from the resin storage buffer. Allow 5 min during each wash cycle for the resin to settle down. Mix the filtrate obtained above with the resin in the syringe and incubate the mixture at 4°C for 30 min.
6. Wash the resin with 50 ml of buffer W1 and then with 20 ml of buffer W2. Elute the recombinant protein using a step-gradient with a series of elution buffers containing 200 mM (10 ml), 300 mM (5 ml) and 500 mM (5 ml) imidazole, respectively. Check the content of the eluted fractions using PAGE gel electrophoresis (15% SDS-PAGE gel). Pool the fractions that contain more than 90% recombinant protein.
7. Remove the excess imidazole from the pooled protein fractions using either a PD-10 desalting column (*see* the instruction from GE Healthcare about the use of the PD-10 column) or a Superdex column (*see* **Note 3**). After desalting, concentrate the protein using a 10 kDa Amicon concentrator at $4000 \times g$ to a final concentration of 5–10 mg/ml.

8. To determine the final protein concentration, add 200 μ l of Bradford reagent to 800 μ l of ddH₂O in a 1 ml cuvette and mix well. Add 1 μ l of the protein solution, mix and incubate for 5 min. Measure the absorbance at 595 nm. Calculate the concentration using a BSA (bovine serum albumin) standard curve.
9. Divide the protein solvent into 200 μ l aliquots and freeze the protein samples using liquid nitrogen and store the samples in a -80°C freezer (*see* **Note 4**).

3.2 Enzymatic Production of c-di- GMP

1. Before setting up the enzymatic reaction, prepare the analytical HPLC system by connecting and equilibrating the HPLC column (*see* **Note 5**). Set the wavelength of the UV detector of the HPLC system to 254 nm.
2. Thaw 5 mg of the frozen tDGCm protein sample and transfer the protein solution into 30 ml reaction buffer in a 50 ml Falcon tube. Add 22 μ l of freshly prepared 1 M GTP stock solution to the solution to a final concentration of 0.75–0.8 mM GTP (*see* **Note 6**). Mix the solution gently using a spatula and close the lid and incubate at 45°C in a water bath (*see* **Note 7**).
3. It normally takes 40–50 min for more than 90% of GTP to be converted to c-di-GMP at 45°C (*see* **Note 8**). The conversion of GTP to c-di-GMP can be conveniently monitored by using an analytical HPLC. Monitoring the progress of the enzymatic reaction is strongly recommended if the users have access to an analytical HPLC. We recommend checking the turnover of GTP 45 min after adding GTP by analyzing 2 μ l reaction mixture (isocratic gradient). The turnover of GTP can be estimated by comparing the area of the GTP peak at time zero (before adding enzyme) and any time afterward.
4. When the turnover of GTP reaches 90%, add the next batch of GTP (22 μ l of 1 M GTP solution) to the same reaction mixture and continue to incubate the reaction mixture at 45°C for another 50 min. Repeat the addition of GTP and enzymatic reaction till the turnover of GTP becomes low (<50% after 45 min incubation). Typically, 8–12 batches of GTP can be added in a period of ~10 h before the turnover at 45 min drops below 50%.
5. Following the enzymatic reactions, heat the reaction mixture at 95°C in a water bath for 5 min to denature and precipitate the enzyme. Centrifuge at $6000 \times g$ and filter the supernatant using a 0.45 μm filter disc. Keep the filtrate in a -20°C freezer before the purification process.

3.3 Purification of c-di-GMP Using Preparative HPLC

1. Set up the preparative HPLC system by preparing the solvent and equilibrating the HPLC column (isocratic gradient) (*see Notes 9 and 10*). Set the wavelength of the detector to 254 nm and set up the fraction collector according to the size of the collecting tubes.
2. Take out the frozen supernatant from the freezer. Inject 5 ml of the supernatant through the manual sample loading loop (*see Note 11*). Start the run and collect the fractions that contain c-di-GMP. A typical chromatogram from one of our HPLC runs is shown in Fig. 2. The peak of the remaining GTP appears at 7 min and the peak of c-di-GMP appears at 18 min. A small peak that represents the linear dinucleotide intermediate can also be seen on the chromatogram.
3. Collect and pool the fractions containing c-di-GMP. The volume of each fraction is 2 ml and usually a total of 7–9 fractions are to be collected for each run. Repeat the HPLC separation for the rest of the supernatant by loading 5 ml each time. At the

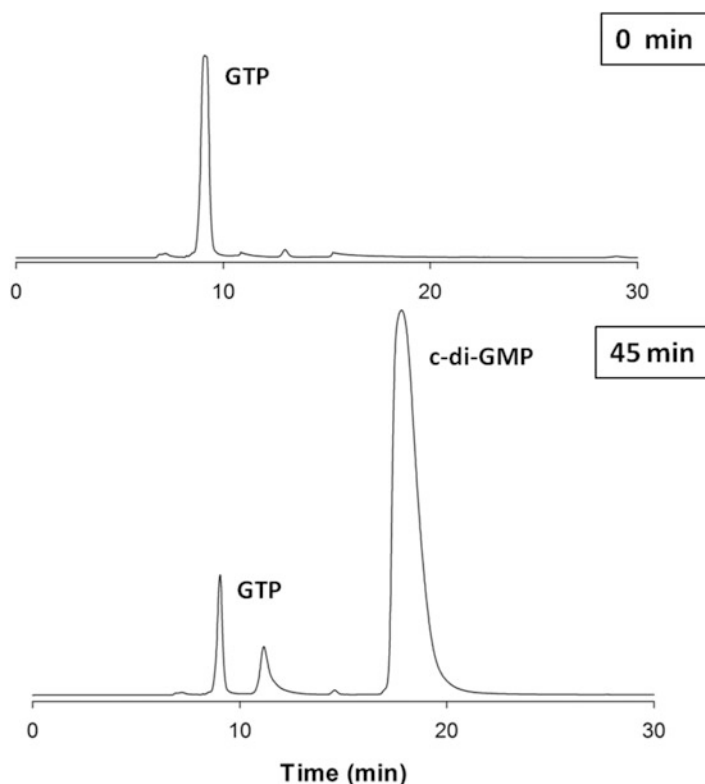


Fig. 2 Preparative HPLC chromatogram showing the turnover of GTP to c-di-GMP. The small peak at 11 min represents a linear dinucleotide intermediate that is often observed during c-di-GMP production

end of the last run, wash the chromatographic column with methanol for 10 min before disconnecting it.

4. We use a two-step procedure to remove the organic solvent and water from the HPLC fractions that contain c-di-GMP. First remove the organic solvent from the pooled fractions using a rotary evaporator with the temperature of the water bath set at 35 °C. The rotary evaporator should be monitored constantly to avoid bumping (*see* **Note 12**). After the removal of organic solvent, transfer the solution to 50 ml falcon tubes. Use a lyophilizer or freeze-dryer to remove H₂O to yield white powder. For researchers who have access to a GeneVac evaporator, the organic solvent and H₂O can be conveniently removed in one step.
5. After solvent removal, dissolve the powder in either a small volume of ddH₂O or 5 mM Tris buffer (pH 7.0) for quantification and storage.
6. Measure the concentration of the c-di-GMP solution using a UV-Vis spectrophotometer according to Beer's law. Dilution of the stock c-di-GMP is required for accurate measurement of the absorbance. The concentration of c-di-GMP can be calculated using the absorbance at 254 nm and extinction coefficient (26,100 M⁻¹ cm⁻¹). For example, if the diluted c-di-GMP solution (dilution factor = 500) has an Abs₂₅₄ of 0.85 Au, then the concentration of the stock c-di-GMP solution will be $(0.85 / (26,100 \text{ M}^{-1} \text{ cm}^{-1} \times 1 \text{ cm})) \times 500 = 16.3 \text{ mM}$. The c-di-GMP solution can be stored in -20 °C or -80 °C freezer (*see* **Note 13**).

4 Notes

1. Under our expression conditions, tDGCm is expressed as soluble protein in reasonably high yield (12–15 mg per liter culture). We usually only observe a small fraction (<5%) of tDGCm in inclusion body.
2. If only smaller sonication probes are available, the culture can be split into smaller volumes and sonicated separately. Alternatively, a French press or microfluidizer can be used for lysing the *E. coli* cells. Our lab uses all three methods and we do not observe significant difference in the activity of tDGCm using different methods of cell lysis.
3. For protein produced by 1 l culture broth, we found that the use of a prepacked desalting PD10 column is the fastest way of removing imidazole. However, for the preparation of more than 10 mg protein, we use an AKTA FPLC system and a

size-exclusion Superdex 75 HR 16/60 column (Amersham Biosciences) for desalting and removing imidazole. The buffer we use for size-exclusion chromatography contains 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5% glycerol, and 1 mM DTT.

4. We found that the tDGCm protein stored (at -80°C) for a long period of time (>3 months) is not as efficient as freshly prepared protein in c-di-GMP production. For best result, we recommend the use of freshly prepared enzyme or enzyme that has been stored for a short period of time (<2 weeks).
5. For analytical HPLC column equilibration, connect the buffer bottles (Buffer A: 20 mM TEAB + 9% methanol and Buffer B: 100% methanol) to the two pumps—A and B respectively. Connect the column to the adapters of the HPLC machine and wash the column with 100% methanol using a flow-rate of 1 ml/min for 10 min. Then equilibrate the column with Buffer A using a flow-rate of 1 ml/min for 20 min.
6. GTP should be kept dry in a sealed container in a -20°C freezer. Due to hydrolysis of GTP, it is always a good practice to prepare fresh GTP solution in situ for the enzymatic reaction. The production yield decreases after GTP reached ca. 0.8 mM, suggesting inhibition of enzymatic activity at high substrate concentration. Hence, it is important to keep the GTP concentration below 0.8 mM.
7. When setting up the enzymatic reaction, it is important to gently stir the solution with a spatula for 5–10 s to disperse the enzyme molecules.
8. Conditions that include pH, salt and temperature were screened to for the enzymatic reaction. The enzyme tDGCm is most efficient at pH 7.5 and at relatively high salt conditions (250–300 mM NaCl). Thermostability analysis showed that the activity of the enzyme drops slowly with time at 45 and 55°C (Fig. 1c).
9. It is recommended to use a guard column to protect the HPLC column.
10. For preparative HPLC column equilibration, follow the same steps and use the same buffers as detailed for the analytical HPLC in **Note 5**, but change the flow-rate of the column to 10 ml/min.
11. As determined by the sample-loading loop, the maximum volume of the concentrated supernatant that can be loaded onto our preparative HPLC column is 5 ml. The users can load more sample with a longer sample-loading loop as long as the separation is not compromised. Alternatively, if the users want to reduce the number of HPLC runs, they can reduce the

total volume of the reaction mixture by lyophilization and then redissolve the powder in a small volume of ddH₂O.

12. Bumping occurs when liquid is heated very rapidly and large vapor bubbles are formed to push the liquid out of the container. This rapid expulsion of boiling liquid could pose a serious hazard to researchers.
13. The procedures described here allow us to readily synthesize hundreds of milligrams of c-di-GMP with standard equipment from biochemistry laboratories. For example, with 10 mg of enzyme, we were able to obtain more than 800 mg of c-di-GMP by adding multiple batches of substrate within a period of several hours. There should not be any difficulty to produce c-di-GMP on the scales of multi-grams with more enzyme and longer reaction time.

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References

1. Galperin MY (2004) Bacterial signal transduction network in a genomic perspective. *Environ Microbiol* 6:552–567
2. Romling U, Gomelsky M, Galperin MY (2005) C-di-GMP: the dawning of a novel bacterial signalling system. *Mol Microbiol* 57 (3):629–639
3. Römling U, Galperin MY, Gomelsky M (2013) Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol Mol Biol Rev* 77(1):1–52
4. Chandra D, Quispe-Tintaya W, Jahangir A, Asafu-Adjei D, Ramos I, Sintim HO, Zhou J, Hayakawa Y, Karaolis DK, Gravekamp C (2014) STING ligand c-di-GMP improves cancer vaccination against metastatic breast cancer. *Cancer Immunol Res* 2(9):901–910
5. Karaolis DKR, Chengb K, Lipskyc M, Elnabawia A, Catalanod J, Hyodoe M, Hayakawae Y, Raufman J-P (2005) 3',5'-Cyclic diguanylic acid (c-di-GMP) inhibits basal and growth factor-stimulated human colon cancer cell proliferation. *Biochem Biophys Res Commun* 329:40–45
6. Karaolis DKR, Newstead MW, Zeng X, Hyodo M, Hayakawa Y, Bhan U, Liang H, Standiford TJ (2007) Cyclic Di-GMP stimulates protective innate immunity in bacterial *Pneumonia*. *Infect Immun* 75(10):4942–4950
7. Hayakawa Y, Nagata R, Hirata A, Hyodo M, Kawai R (2003) A facile synthesis of cyclic bis (3'→5')diguanylic acid. *Tetrahedron* 59:6465–6471
8. Ross P, Mayer R, Weinhouse H, Amikam D, Huggirat Y, Benziman M, de Vroom E, Fiddler A, de Paus P, Sliedregt LA (1990) The cyclic diguanylic acid regulatory system of cellulose synthesis in *Acetobacter xylinum*. Chemical synthesis and biological activity of cyclic nucleotide dimer, trimer, and phosphothioate derivatives. *J Biol Chem* 265:18933–18943
9. Zhang Z, Gaffney BL, Jones RA (2004) c-di-GMP displays a monovalent metal ion-dependent polymorphism. *J Am Chem Soc* 126(51):16700–16701
10. Yan H, Aguilar AL (2007) Synthesis of 3',5'-cyclic diguanylic acid (cdiGMP) using 1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl as a protecting group for 2'-hydroxy functions of ribonucleosides nucleosides. *Nucleosides Nucleotides Nucleic Acids* 26:189–204
11. Kiburu I, Shurer A, Yan L, Sintim HO (2008) A simple solid-phase synthesis of the ubiquitous bacterial signaling molecule, c-di-GMP and analogues. *Mol Biosyst* 4:518–520

12. Clivio P, Coantic-Castex S, Guillaume D (2013) (3'-5')-Cyclic dinucleotides: synthetic strategies and biological potential. *Chem Rev* 113(10):7354–7401
13. Chan C, Paul R, Samoray D, Amiot NC, Giese B, Jenal U, Schirmer T (2004) Structural basis of activity and allosteric control of diguanylate cyclase. *Proc Natl Acad Sci U S A* 101(49):17084–17089
14. Rao F, Yang Y, Qi Y, Liang Z-X (2008) Catalytic mechanism of c-di-GMP specific phosphodiesterase: a study of the EAL domain-containing RocR from *Pseudomonas aeruginosa*. *J Bacteriol* 190:3622–3631
15. Rao F, Pasunooti S, Ng Y, Zhuo W, Lim L, Liu W, Liang Z-X (2009) Enzymatic synthesis of c-di-GMP using a thermophilic diguanylate cyclase. *Anal Biochem* 389:138–142
16. Pasunooti S, Surya W, Tan SN, Liang ZX (2010) Sol-gel immobilization of a thermophilic diguanylate cyclase for enzymatic production of cyclic-di-GMP. *J Mol Catal B: Enzym* 67(1-2):98–103
17. Chen MW, Kotaka M, Vonnrhein C, Bricogne G, Rao F, Chuah ML, Svergun D, Schneider G, Liang ZX, Lescar J (2012) Structural insights into the regulatory mechanism of the response regulator RocR from *Pseudomonas aeruginosa* in cyclic Di-GMP signaling. *J Bacteriol* 194(18):4837–4846
18. Qi Y, Chuah MLC, Dong X, Xie K, Luo Z, Tang K, Liang Z-X (2011) Binding of C-di-GMP in the non-catalytic EAL domain of FimX induces a long-range conformational change. *J Biol Chem* 286:2910–2917
19. Qi Y, Rao F, Liang Z-X (2009) A flavin-binding PAS domain regulates c-di-GMP synthesis in AxDGC2 from *Acetobacter xyli- num*. *Biochemistry* 48:10275–10285
20. Qi Y, Xu L, Dong X, Yau YH, Ho CL, Koh SL, Shochat SG, Chou SH, Tang K, Liang ZX (2012) Functional divergence of FimX in PilZ binding and type IV pilus regulation. *J Bacteriol* 194(21):5922–5931
21. Rao F, See RY, Zhang DW, Toh DC, Ji Q, Liang ZX (2010) YybT Is a signaling protein that contains a cyclic dinucleotide phosphodiesterase domain and a GGDEF domain with ATPase activity. *J Biol Chem* 285(1):473–482
22. Xu L, Venkataramani P, Ding Y, Liu Y, Deng Y, Yong GL, Xin L, Ye R, Zhang L, Yang L, Liang Z-X (2016) A Cyclic di-GMP-binding adaptor protein interacts with histidine kinase to regulate two-component signaling. *J Biol Chem* 291(31):16112–16123
23. Deepthi A, Liew CW, Liang ZX, Swaminathan K, Lescar J (2014) Structure of a diguanylate cyclase from *thermotoga maritima*: insights into activation, feedback inhibition and thermostability. *PLoS One* 9(10):e110912
24. Spehr V, Warrass R, Höcherl K, Ilg T (2011) Large-scale production of the immunomodulator c-di-GMP from GMP and ATP by an enzymatic cascade. *Appl Biochem Biotechnol* 165(3):761–775
25. Zahringer F, Massa C, Schirmer T (2011) Efficient enzymatic production of the bacterial second messenger c-di-GMP by the diguanylate cyclase YdeH from *E. coli*. *Appl Biochem Biotechnol* 163(1):71–79
26. Christen M, Christen B, Folcher M, Schauerte A, Jenal U (2005) Identification and characterization of a cyclic di-GMP-specific phosphodiesterase and its allosteric control by GTP. *J Biol Chem* 280(35):30829–30837

c-di-GMP Signaling

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