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Analyzing diguanylate cyclase activity *in vivo* using a heterologous *Escherichia coli* host

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Abstract

Bacterial biofilms are notorious for their deleterious effects on human health and industrial biofouling. Key processes in biofilm formation are regulated by the second messenger signal cyclic dimeric guanosine monophosphate (c-di-GMP): accumulation of c-di-GMP promotes biofilm formation while lowering c-di-GMP promotes motility. Complex networks of modular enzymes are involved in regulating c-di-GMP homeostasis. Understanding how these enzymes function in bacterial cells can help enlighten how bacteria use environmental cues to modulate c-di-GMP and cell physiology. In this protocol, we describe a workflow that utilized *Escherichia coli* as a heterologous host to allow the researcher to identify genes encoding potential c-di-GMP metabolizing proteins, express the gene of interest from an inducible plasmid, and directly detect changes in intracellular c-di-GMP using ultra performance liquid chromatography-tandem mass spectrometry.

Introduction

Cyclic diguanylate guanosine monophosphate (c-di-GMP) is an intracellular cyclic dinucleotide second messenger found nearly ubiquitously in microorganisms. As a second messenger, it transduces signals from the environment to the cell where it dictates cellular physiology. After years of research in different bacterial species, an invariable trend has emerged: low intracellular c-di-GMP tends to be associated with motile phenotypes while high intracellular c-di-GMP tends to be associated with surface adhered phenotypes, or specifically, biofilms. However, recent findings have also discovered that the physiological role of c-di-GMP signaling is diverse, as it regulates other aspects of cell physiology including virulence (Tischler and Camilli, 2005), cell cycle progression (Christen et al., 2010), secretion systems (Sloup et al., 2017), and DNA repair (Fernandez et al., 2018).

C-di-GMP is generated by cyclization of two GTP molecules by enzymes called diguanylate cyclases (DGC). DGC enzymes contain the enzymatic active site amino acid motif GGDEF where the tandem glycine residues are involved in GTP binding while the other residues participate in divalent metal coordination and catalysis (Chan et al., 2004). C-di-GMP is degraded by enzymes called phosphodiesterases (PDE). PDE enzymes can have one of two active site motifs that render different degradation products: 1) the EAL active site motif, which degrades c-di-GMP into the linear dinucleotide pGpG, or 2) the HD-GYP active site motif which completely degrades c-di-GMP into 2 GMP molecules (Romling et al., 2013). Recent work has characterized oligoribonuclease (Orn) as the primary pGpG processing enzyme that breaks pGpG into 2 molecules of GMP (Cohen et al., 2015; Orr et al., 2015).

Due to the conservation of enzymatic active site motifs in DGC and PDE domains, bioinformatic analysis has uncovered hundreds of species with at least 1 gene encoding an enzyme hypothetically capable of metabolizing c-di-GMP; however, only a fraction of these genes has been functionally assayed *in vivo* and *in vitro*. One robust approach to study these genes is to express them in a heterologous host that has naturally low levels of c-di-GMP. Here, we describe how to identify potential c-di-GMP synthesizing enzymes, over-express the protein in the heterologous host *Escherichia coli*, and measure changes in intracellular c-di-GMP using ultra-performance liquid chromatography and tandem mass spectrometry (UPLC-MS/MS).

Basic Protocol 1: Detection and quantification of intracellular c-di-GMP from DH5 alpha *E. coli* using UPLC-MS/MS.

Introduction.

Whole genome sequencing has amassed genome sequences from various microorganisms into the NCBI database RefSeq. Further analysis of these sequences specifically for genes with c-di-GMP-related domains led to the development of an online database categorizing the distribution of c-di-GMP-related domains among sequenced bacteria (https://www.ncbi.nlm.nih.gov/Complete_Genomes/c-di-GMP.html) (Amikam and Galperin, 2006). Using this database, one can identify genes that encode c-di-GMP metabolizing proteins as potential candidates for analysis. Further, there is sequence and domain information that can aid generation of hypotheses regarding protein function. Besides the active site amino acids that these domains are named for, other conserved amino acids are necessary for enzymatic activity (see Figure 1 in Bobrov et. al., 2012), and the presence or absence of these amino acids can be indicators of DGC or PDE activity. Alternatively, protein domain prediction software that is updated frequently, such as InterPro, can be used identify protein domains from a given primary amino acid sequence (Finn et al., 2017).

The following protocol describes the expression of proteins of interest in DH5α *E. coli*, extraction of nucleotides, and quantification of intracellular c-di-GMP using UPLC-MS/MS. As discussed below, this strain of *E. coli* is less sensitive to the toxic effects of c-di-GMP overproduction.

Materials.

- ***Over-expression of DGC enzymes in DH5α E. coli***
 - Luria broth or other rich, undefined media
 - Antibiotics at standard concentrations
 - Shaking incubator – 37 °C, 220 RPM
- ***Detection via UPLC-MS/MS***
 - Nucleotide extraction buffer (see recipe in Reagents and Solutions)
 - Mobile Phase A – Chromatography Solution (see Reagents and Solutions)

- Mobile Phase B – Chromatography Solution (see Reagents and Solutions)
- 1.5 mL Microcentrifuge tubes
- Spectrophotometer (Absorbance set at 595 nm)
- Table-top microcentrifuge
- Heated, vacuum centrifuge (For example, SpeedVac – Savant)
- Chemically synthesized c-di-GMP (Biolog) in UPLC-grade water.

Overexpression of DGC enzymes in DH5α *E. coli*—Use conventional cloning methods (Green and Sambrook, 2012) to generate inducible vector harboring gene of interest.

1. Streak out *E. coli* DH5α on LB agar plates + appropriate antibiotics for selection and incubate overnight at 37 degrees Celsius (°C).
2. Pick an isolated colony or colonies and inoculate 3mL of LB plus selection and grow at 37 °C for 16–18 hours with aeration (200 RPM).
3. Dilute the culture 1:100 in triplicate in 3 mL LB + selection. For induced cultures, add the appropriate inducer. We routinely use the P_{tac} promoter induced with 100 μM isopropyl-β-D-1-thiogalactopyranoside (IPTG). For uninduced cultures, add equal volume water.
4. Grow the culture at 37 °C, 220 RPM until the OD₆₀₀ reaches ~ .5 (~ 2–3 hours).
5. During incubation, make nucleotide extraction buffer.
 - a. 100 μL per sample
 - b. Place on ice or 4 °C
6. Remove two 1 mL aliquots from each culture and place in 1.5 mL microcentrifuge tubes.
7. Centrifuge samples at full speed for 30 seconds in a benchtop microcentrifuge to pellet bacteria.
8. During the spin, remove 500 μL of culture and add 500 μL LB to dilute the culture two-fold. Measure and record OD₆₀₀. Multiple dilutions may be necessary depending on the starting turbidity of the culture.
 - a. Additionally, bacteria can be enumerated by serial dilution in sterile buffer and plated on LB plates or cells can be lysed and total protein concentration quantified (see detailed information regarding bacterial quantification in *Commentary*).
9. Quickly remove the supernatant using a P1000 pipette and resuspend the pellet in 100 μL cold extraction buffer to lyse the cells and quench metabolism
10. Placed the quenched samples in –20 °C for 20 minutes.

11. After incubation, centrifuge samples in microcentrifuge for 10 minutes at $21,130 \times g$.
12. Remove supernatant and place in new tube. Remove the extraction buffer by evaporation in a heated vacuum centrifuge for 3–4 hours at full speed.
13. Resuspend dried samples in 100 μL UPLC-grade water. Samples can be analyzed immediately or stored in -80°C for up to one week.
 - a. It may be necessary to centrifuge resuspended samples to pellet insoluble material. Alternatively, samples can be filtered with a $.45 \mu\text{m}$ filter prior to analysis. Debris visible to the eye can occlude the inlet lines during chromatography, resulting in decreased signal.

Mass Spectrometry Analysis

1. Adjust settings on Acquity UPLC Column Manager and Waters Quattro Premier Tandem Quadrupole Mass Spectrometry:
 - a. Column and chromatography settings
 - i. Column – Waters BEH C18 $2.1 \times 50 \text{ mm}$
 - ii. Temperature – 50.0°C
 - iii. Flow rate for equilibration - $.3 \text{ mL/min}$
 - iv. Injection volume – $10 \mu\text{L}$
 - v. Gradient parameters:

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0.0	99	1
2.5	80	20
7.0	35	65
7.5	5	95
9.01	99	1

- b. Mass Spectrometer
 - i. Cone voltage – 50V
 - ii. Cone gas (nitrogen) flow – 50 L/h
 - iii. Capillary voltage – 1kV
 - iv. Collision energy – 30V
 - v. Collision gas (nitrogen) - 0.15 mL/min
 - vi. Source temperature – 110.0°C
 - vii. Desolvation tempature – 350.0°C

Reagents and Solutions

- UPLC and Mass Spectrometry Solutions
 - Nucleotide Extraction Buffer -
 - ◆ 40:40:20 Methanol:Acetonitrile:Water + .1N Formic acid
 - Methanol, for HPLC > 99.9% (Sigma Aldrich. Product #: 34680 –1L)
 - Acetonitrile, for HPLC, gradient grade > 99.9% (Sigma Aldrich. Product #: 34851 – 1L)
 - Formic Acid, 88% (JT Baker, Baker Analyzed A.C.S. Reagent. Product # 0128–01. 500mL)
 - HPLC Grade water (Sigma Aldrich. Product #: 270733–1L)
 - Mobile Phase A – Chromatography Solution
 - ◆ 10 mM Tributylamine
 - ◆ 15 mM Acetic acid
 - ◆ 97:3 Water:methanol
 - Mobile Phase B – Chromatography Solution
 - ◆ 100% Methanol

Understanding results.

- **Data from mass spectrometry**
 - The concentration reported by the mass spectrometer is nM of c-di-GMP in a 100 µL concentrated sample. To determine the concentration of the sample aliquot taken during sample collection, use the following dilution equation:
 - ◆
$$\frac{[\text{cyclic di - GMP}] \text{ nM} \times 100 \text{ } \mu\text{L}}{\text{Volume of aliquot (}\mu\text{L)}} = [\text{cyclic di - GMP}] \text{ nM}$$
- Normalization to cell number or protein concentration.
 - The concentration of c-di-GMP in the aliquoted sample may not be informative without normalization to the total amount of bacteria isolated for extraction. There are several reported methods that normalize raw c-di-GMP concentrations to the number of bacteria at the time of extraction. We describe two common methods below:
 - ◆ **Estimated intracellular concentration.** This approach estimates the intracellular concentration of c-di-GMP, which is useful for understanding kinetic parameters of the signaling pathway such as receptor binding. This value is determined by

dividing the total number of c-di-GMP molecules per the summation of total cell volume that was extracted.

1. Determine concentration of c-di-GMP in μM :
 - i. $[\text{c-di-GMP}]_{\text{nM}} * 1000 = [\text{c-di-GMP}]_{\mu\text{M}}$
2. Determine number of c-di-GMP molecules in μmoles :
 - i.
$$\frac{[\text{cyclic di-GMP}] \mu\text{moles}}{10^6 \mu\text{L}} * 100 \mu\text{L aliquot} = \mu\text{moles}$$
3. Determine number of CFU at time of extraction can be done using two methods:
 - a. Dilution plating followed by counting of CFUs. This approach can be problematic with bacterial cultures that form robust biofilms due to aggregate formation as discussed below.
 - b. Quantify total CFUs – OD₆₀₀ Method
 - i. Prior to the experiment, a growth curve should be generated that plots CFU (as measured by dilution plating) versus OD₆₀₀ for multiple time points.
 - ii. Estimate the best-fit mathematical function to generate an equation describing this curve using a program like GraphPad Prism.
 - iii. Use the measured OD₆₀₀ at the time of extraction to estimate CFU/mL of the culture.
 - iv. $\text{Sample volume (mL)} * \text{CFU/mL} = \text{Total CFU extracted}$
4. Determine total intracellular volume in sample
 - i. Estimated volume of bacterial cell (L) * total CFU
 1. Volume needs to be determined empirically using microscopy and image analysis software such as ImageJ or Fiji.

2. For reference, the average cell volume of *E. coli* grown to exponential phase was found to be 4.4×10^{-15} L (Volkmer and Heinemann, 2011).
5. Total intracellular c-di-GMP concentration:
 - a. Number of c-di-GMP molecules (μ moles) / total intracellular volume (L) = estimated intracellular c-di-GMP concentration μ M. Note this is an average concentration and does not consider any population heterogeneity.

Protein normalization.

- This approach involves saving an aliquot of sample for cell lysis and protein quantification using the Bradford Assay (BioRad). Methods of cell lysis should be optimized to ensure total cell lysis for the most accurate results. This method is advantageous if it is difficult to estimate the number of bacteria that were extracted (such as if aggregate formation has occurred) or if the individual cell volume is highly variable.
- Calculation of c-di-GMP concentration (μ moles/ μ g protein)
 - Use equations 1 and 2 to convert concentration into μ moles
 - Determine mass of protein (μ g) in sample aliquot and normalize c-di-GMP molecule number (μ moles) by μ g protein.

Commentary

Background information.

Bacteria produce the second messenger c-di-GMP in response to external or internal stimuli to control behavior. Enzymes called diguanylate cyclases synthesize c-di-GMP from two GTP molecules in response to certain stimuli. Perhaps the best studied behavior is the transition between a motile state to an adhered, biofilm state where high intracellular c-di-GMP promotes adhesion to biotic and abiotic surfaces. Using this protocol, researchers can determine the *in vivo* DGC activity using UPLC-MS/MS to measure changes in intracellular c-di-GMP.

One advantage to using a heterologous host such as DH5 α *E. coli* to test DGC activity is that it bypasses the need to express and purify the enzyme to test it *in vitro*. While *in vitro* activity assays yield important information, purification and functionality *in vitro* require laborious and time-consuming troubleshooting and optimization. Further, many DGC are modular and harbor N-terminal ligand sensing domains that regulate DGC activity (Romling et al., 2013; Amikam and Galperin, 2006). The N-terminal specific ligand may be missing *in vitro*, thus decreasing overall enzymatic activity.

While this protocol describes quantification of c-di-GMP after induction of a specific DGC, it can also be adapted to assess the effects of certain environmental conditions on intracellular c-di-GMP pools. Instead of inducing DGC containing proteins from a vector, one can grow bacteria in conditions of interest as well as control conditions and extract c-di-GMP as described in the protocol.

Critical parameters.

Pelleting and supernatant removal. It is important to *quickly* pellet the bacteria, remove the supernatants, and resuspend the pellet in extraction buffer. Using a vacuum hose connected to a biohazardous waste collection vessel will allow for the quick removal of supernatants without disrupting the pellet. If a vacuum apparatus is unavailable, then use a pipette to remove supernatants rather than dumping and process less samples at a time (~4–8 samples at a time).

Troubleshooting.

- **Low/undetectable c-di-GMP signal.** In some cases, net intracellular c-di-GMP levels are below the limit of detection (< 1.9 nM) despite overproduction of a certain DGC. Increasing the total culture volume or cell number at extraction may increase the yield into the range of the standard curve. This can be achieved by growing larger volumes and extracting using the ratio of 100 μ L extraction buffer per $\sim 1 \times 10^9$ CFU. Alternatively, cells could be grown to a higher cell density prior to extraction. It is worth noting, however, that some organisms regulate c-di-GMP through quorum sensing. For example, at high cell densities in the human pathogen *Vibrio cholerae*, net intracellular c-di-GMP concentrations are low compared to low cell density cultures (Waters et al., 2008). Lastly, the absence or presence of certain ligands in the growth media may decrease DGC activity. In these cases, it will likely be necessary to switch growth conditions to more biologically relevant conditions. For example, we have observed that for *V. cholerae* growth in minimal media significantly increases intracellular c-di-GMP compared to rich, undefined media (Koestler and Waters, 2013), and for a number of species that grow as a colony on solid agar plates rather than liquid cultures increased intracellular c-di-GMP.
- **High variation in technical samples.** Most error in this protocol comes from cell collection and sample incubation in extraction buffer. We recommend 2–3 technical replicates per biological replicate to ensure accuracy of data.

- **Cloning and plasmid stability issues.** In some cases, introducing and inducing expression of certain DGC genes from heterologous sources may cause plasmid instability and cytotoxicity in *E. coli*. For example, the T7 protein overexpression strain BL21 (DE3) was unable to harbor a multicopy plasmid encoding a gene coding for a DGC despite including pLysE, which decreases leaky expression from the T7 promoter (Ryjenkov et al., 2005). Further, certain DGC enzymes on multicopy plasmids demonstrated toxicity after induction in *E. coli* K12 strains (Sarenko et al., 2017). Our group and others have successfully overexpressed DGC enzymes without toxicity issues in the strain DH5 α (Ryjenkov et al., 2005). Thus, we recommend using DH5 α as the host for ectopic DGC expression as a starting point. However, high intracellular concentrations of c-di-GMP greater than 100 μ M will likely inhibit growth in all strains, and it may be necessary to optimize induction conditions.

Time considerations.

In total, the protocol from culture inoculation to data analysis will take 3–4 days. Overnight cultures should not exceed 24 hours growth prior to the experiment. For induction experiments, induction time and inducer concentrations should be determined empirically for each protein of interest. A sufficient starting point would be growing cultures into mid-log phase and inducing protein expression for 1–3 hours. C-di-GMP extractions will take ~40 minutes to 1 hour, depending on how many samples are processed at once. Similarly, the total time for UPLC-MS/MS analysis depends on sample number, with each sample taking 10 minutes per injection.

An example experiment would look similar to the outline below:

Day 1

- Streak out strain onto LB + selection

Day 2

- Start cultures from isolated colonies

Day 3

- Subculture overnights 1:100 and grow until mid-log phase (2–3 hours)
- Add inducer and induce for 1–3 hours
- Pellet cells and begin c-di-GMP extractions
- Evaporate solvent in Speed vac (time range: 2–3 hours to overnight)

Day 4

- Thaw and dilute c-di-GMP standards
- Prepare samples for UPLC-MS/MS analysis
- Mass spectrometric analysis
- Data analysis, quantification, and normalization.

- Days 2 and 3 can be combined into one to shorten the protocol to 2 days.

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