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Detection of cyclic di-AMP using a competitive ELISA with a unique pneumococcal cyclic di-AMP binding protein

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Abstract

Cyclic di-AMP (c-di-AMP) is a signaling molecule that has been shown to play important roles in bacterial physiology and infections. Currently, c-di-AMP detection and quantification relies mostly on the use of high-performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS). In this study, a competitive enzyme-linked immunosorbent assay (ELISA) for the quantification of c-di-AMP was developed, which utilizes a novel pneumococcal c-di-AMP binding protein (CabP) and a newly commercialized c-di-AMP derivative. With this new method, c-di-AMP concentrations in biological samples can be quickly and accurately quantified. Furthermore, this assay is much more efficient than current methods as it requires less overall cost and training while processing many samples at once. Therefore, this assay can be extensively used in research into c-di-AMP signaling.

Keywords

c-di-AMP; ELISA; c-di-AMP binding protein; Detection

1. Introduction

Cyclic nucleotides have been well studied over the past few decades and are integral to signal transduction in both eukaryotic and prokaryotic organisms (Kalia *et al.*, 2012, Romling *et al.*, 2013). However, cyclic di-AMP (c-di-AMP) was only recently discovered and the roles of c-di-AMP in various organisms have just started to be recognized (Corrigan & Grundling, 2013, Kalia *et al.*, 2012, Witte *et al.*, 2008). Currently, c-di-AMP is linked to bacterial infections, biofilm formation, potassium uptake, cell wall homeostasis, and resistance to antibiotics (Bai *et al.*, 2013, Bai *et al.*, 2014, Barker *et al.*, 2013, Corrigan *et al.*, 2011, Corrigan *et al.*, 2013, Griffiths & O'Neill, 2012, Luo & Helmann, 2012, Mehne *et*

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Competing interests statement

The authors declare no competing financial interests.

al., 2013, Pozzi *et al.*, 2012, Schwartz *et al.*, 2012, Yamamoto *et al.*, 2012, Yang *et al.*, 2014, Ye *et al.*, 2014). Additionally, c-di-AMP produced by *Listeria monocytogenes* has been shown to activate a host type I interferon response (Woodward *et al.*, 2010) mediated by host effector proteins STING and DDX41 (Bowie, 2012, Burdette *et al.*, 2011, Jin *et al.*, 2011, Parvatiyar *et al.*, 2012, Sauer *et al.*, 2011). This widespread influence has made the study of c-di-AMP a quickly developing area of research for the foreseeable future.

Enzymatically, c-di-AMP is synthesized from two molecules of ATP, which is catalyzed by diadenylate cyclase (DAC). This enzyme was first discovered through a structural study of DisA in *Bacillus subtilis* and *Thermotoga maritima* (Witte *et al.*, 2008). DAC selectively converts either ATP or ADP to c-di-AMP, and does not utilize GTP, ITP, CTP, or UTP as its substrate (Bai *et al.*, 2012, Witte *et al.*, 2008). In *B. subtilis*, DisA is named after DNA integrity scanning protein A and it has three interdependent domains: an N-terminal DAC domain (known as DUF147), a linker domain, and an RuvA-like C-terminal DNA-binding helix-hairpin-helix (HhH) domain. Furthermore, two additional proteins with DAC domains, CdaA (also referred to as YbbP) and CdaS (also referred to as YojJ), were also found to produce c-di-AMP in *B. subtilis* (Mehne *et al.*, 2013, Romling, 2008). It has been noticed that the DAC domain is widespread in bacteria and archaea (Corrigan & Grundling, 2013, Romling, 2008). In addition to *B. subtilis* and *T. maritima*, the presence of functional DAC has also been reported in *L. monocytogenes* (Woodward *et al.*, 2010), *Staphylococcus aureus* (Corrigan *et al.*, 2011), *Streptococcus pyogenes* (Kamegaya *et al.*, 2011), *Mycobacterium tuberculosis* (Bai *et al.*, 2012), *Mycobacterium smegmatis* (Zhang & He, 2013), *Chlamydia trachomatis* (Barker *et al.*, 2013), and *Streptococcus pneumoniae* (Bai *et al.*, 2013). However, little is known about the signaling pathways mediated by c-di-AMP.

In order to extensively investigate c-di-AMP mediated signaling pathways and the biological roles of c-di-AMP, it is essential to establish a simple and sensitive assay to determine the levels of this dinucleotide. Recently, a c-di-AMP binding protein (CabP) in *S. pneumoniae* was identified using c-di-AMP affinity chromatography with 2'-AHC-c-di-AMP-agarose resin (BioLog) (Bai *et al.*, 2014). We have demonstrated that CabP directly interacts with a potassium transporter and plays a role in pneumococcal potassium uptake. The physical interaction between the two proteins can be impaired by elevated bacterial c-di-AMP. Additionally, CabP displays a high affinity for c-di-AMP, and the c-di-AMP binding site in this protein is extremely specific. The interaction between CabP and c-di-AMP could not be interrupted by an excessive amount (1,000-fold) of c-di-GMP, cAMP, ATP, ADP, AMP, pApA, NAD⁺, NADH, NADP⁺, and NADPH (Bai *et al.*, 2014). This protein was therefore utilized as the initial coating protein to establish a competitive enzyme-linked immunosorbent assay (ELISA) for the detection of c-di-AMP. Additionally, biotin-labeled c-di-AMP (B-c-di-AMP) has recently become commercially available, such as "2'-[biotin]-AHC-c-di-AMP" (BioLog). The advent of these developments has allowed for the creation of a novel assay utilizing a competitive-ELISA technique to quantify c-di-AMP levels in biological samples, as we have reported (Bai *et al.*, 2013, Bai *et al.*, 2014, Yang *et al.*, 2014). The principle of this assay is using B-c-di-AMP to compete with c-di-AMP in a sample or standard for binding with CabP. Therefore, higher c-di-AMP concentration presents in a sample will be reflected by less B-c-di-AMP that can be detected. Here we

provide a detailed protocol for evaluating the concentrations of c-di-AMP by measuring c-di-AMP bound to pneumococcal CabP in a competitive ELISA assay (Fig. 1). This assay has been optimized and is shown to have an extreme specificity (Fig. 2) and high sensitivity (Fig. 3) for c-di-AMP, making it ideal to fill the need in the field of c-di-AMP research.

2. Materials and Methods

2.1. Reagents and materials

Both c-di-AMP and B-c-di-AMP were purchased from BioLog and were resuspended as 5 mM stocks. Aliquots were stored at -20°C until use.

The following buffers and solutions were used in sample preparation and the assay. The coating buffer contains 50 mM Na_2CO_3 and 50 mM NaHCO_3 with a final pH of 9.6. Phosphate buffered saline (PBS, pH 7.4) contains 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 137 mM NaCl, and 2.7 mM KCl. PBST wash buffer consists of 0.05 % (v/v) Tween 20 in PBS (pH 7.4). Citric acid buffer contains 0.1 M citric acid titrated with 0.2 M Na_2HPO_4 to a final pH of 5.0. Substrate buffer is prepared by dissolving a tablet of o-phenylenediamine dihydrochloride (OPD, 10 mg/tablet; Sigma) and 20 μl H_2O_2 in 20 ml citric acid buffer, which is mixed thoroughly at room temperature and used immediately. All buffers and solutions were stored at 4°C until use unless otherwise specified.

2.2. Preparation of c-di-AMP samples from bacteria

To create bacterial samples for evaluating this assay, *Escherichia coli* was grown in 10 ml of Luria-Bertani (LB) media to the expected growth phase. Testing conditions vary based on c-di-AMP levels present in the bacterium being studied and various testing scenarios. The cell suspension to be collected is processed by centrifuge for 2 min at 13,000 rpm. The resulting bacterial pellet is then washed three times with 1 ml of PBS (pH 7.4). Once washed, the pellet was then resuspended in 0.5 ml of 50 mM Tris-HCl (pH8.0) and further disrupted using a sonic dismembrator (Model 100, Fischer Scientific) for periods of 10 sec with a 10 sec interval on ice. Samples were then heated at 95°C for 10 min and subsequently centrifuged for 10 min at 13,000 rpm. Supernatants were collected and used immediately or stored at -20°C until use. The sample preparation methods for *S. pneumoniae* and *M. tuberculosis* has been reported earlier (Bai *et al.*, 2013, Yang *et al.*, 2014). In all these studies, no more than 10 ml of bacterial culture was needed for three repeats in the assay of bacterial c-di-AMP.

2.3. Procedure of the assay

Recombinant CabP was purified as described previously (Bai *et al.*, 2014) and was diluted to 10 $\mu\text{g}/\text{ml}$ or 50 $\mu\text{g}/\text{ml}$ in coating buffer and coated at 100 $\mu\text{l}/\text{well}$ in a Nunc MaxiSorp plate (Thermo Scientific). The plate was sealed with plastic wrap and incubated overnight (14-18 h) at 4°C . The coating buffer was decanted from the wells, and the plate was washed three times with 200 μl PBST wash buffer. After washing, the wells were blocked with 100 μl of 1% BSA in PBS for 1 h at room temperature. The blocking solution was then decanted from the wells, and the plate was washed 3 times with 200 μl PBST wash buffer. Samples, controls, and calibrator dilutions were prepared in sample diluent containing 25 nM of B-c-

di-AMP. An aliquot of 100 μ l of this prepared sample, control, or calibrator was directly added to the 96-well plate and incubated for 2 h at room temperature. After washing the wells 3 times each with 200 μ l PBST wash buffer, 100 μ l of 1:10,000 HRP-conjugated streptavidin (Thermo Scientific) was added. The plate was then incubated for 1 h at room temperature. After washing with PBST buffer for 3 times, 100 μ l of substrate buffer was added to each well and incubated for 30 min at room temperature. The colorimetric reaction was then stopped with 100 μ l of 2 M H₂SO₄. The absorbance of each well was determined at 490 nm using a plate reader.

2.4. Analysis of data collected

The use of a standard curve and proper calibrator wells is important to obtain reproducible results. First the average values of the blank calibrator wells were subtracted from those of the samples and the standards. At least 6 standard dilutions were used to cover low (10 nM), medium (25-75 nM), and high (200 nM) concentrations of c-di-AMP. A standard curve was generated by plotting the concentrations of the standards using a log₂ scale (x axis) against the readings of optical density at 490 nm (OD₄₉₀) (y axis) and then fitted with a second-order polynomial curve as shown in Fig. 4. A standard curve was repeated if the correlation coefficient was < 0.90. The c-di-AMP concentration of each sample was determined by taking the absorbance readings at OD₄₉₀, which was corrected from control wells, and calculated with the equation generated from the standard curve. The amount of c-di-AMP in the samples was subsequently corrected for dilution factors and normalized against either the bacterial numbers or the OD readings of the culture.

3. Results and Discussion

3.1. Detection of c-di-AMP in the samples

To evaluate the specificity of this assay, we prepared different *E. coli* samples to detect c-di-AMP. Previously, it has been reported that no c-di-AMP existed in the lysates of *E. coli* BL21 (DE3) harboring the empty vector pET28a+ (Novagen) or pET28b+ (Novagen) (Corrigan *et al.*, 2011, Mehne *et al.*, 2013). In contrast, c-di-AMP has previously been detected in the lysate of *E. coli* expressing *S. aureus* *dacA*, *B. subtilis* *cdaA* or *cdaS* using LC-MS (Corrigan *et al.*, 2011, Mehne *et al.*, 2013). In this study, *E. coli* W3110 was grown in 10 ml LB broth overnight to an OD₆₀₀ of 1.0. *E. coli* BL21 (DE3) harboring either the empty pET28a+ vector (Novagen) or the plasmid expressing *M. tuberculosis* *disA* (also referred to as *dacA*) (Bai *et al.*, 2012) were grown in 10 ml LB broth to an OD₆₀₀ of 0.5 and then induced at room temperature for 3 hours with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation, and each bacterial pellet was resuspended into 0.5 ml 50 mM Tris-HCl (pH 8.0). Samples were then prepared and c-di-AMP was detected following the established protocol. Our results displayed that the *E. coli* W3110 and BL21 (DE3) strains did not produce c-di-AMP, which is consistent with the observations described in the other reports (Corrigan *et al.*, 2011, Mehne *et al.*, 2013). In contrast, the *E. coli* strain expressing *disA* exhibited high levels of c-di-AMP (Fig. 5), which is consistent with the result that was previously reported and analyzed using LC-MS (Mehne *et al.*, 2013). This indicates that the novel assay can be used to directly quantify

c-di-AMP levels from a biological sample. These findings also suggest that this assay is highly specific for c-di-AMP rather than other molecules present in the *E. coli* lysate.

3.2. Comparison of the assay with other current available methods

As the study of c-di-AMP evolves, simple and sensitive methods for the detection of this molecule in biological samples will be needed. Currently, very few methods for quantifying c-di-AMP have been reported and they rely primarily on high-performance liquid chromatography (HPLC) (Bai *et al.*, 2012, Oppenheimer-Shaanan *et al.*, 2011), liquid chromatography–mass spectrometry (LC-MS) (Barker *et al.*, 2013, Burhenne & Kaefer, 2013, Corrigan *et al.*, 2011, Mehne *et al.*, 2013), or complex formation between c-di-AMP and coralyne (Zhou *et al.*, 2014). HPLC and LC-MS both have a relatively short analysis time for single samples, strong resolving power, reproducibility, and acceptable sensitivity of results. However, training and equipment costs for each technique make these methods expensive and inaccessible for many laboratories. Additionally, these current techniques either utilize large volumes of culture (Oppenheimer-Shaanan *et al.*, 2011), or need [^{13}C , ^{15}N]-c-di-AMP to generate internal standards (Burhenne & Kaefer, 2013, Corrigan *et al.*, 2011, Mehne *et al.*, 2013). Because of limitations with the current methods, we developed the ELISA that is inexpensive, reproducible, and easily performed. Moreover, each 96-well plate has the capacity to measure up to 80 distinct samples, or 40 samples in duplicate, on the same plate under identical condition. This is a massive advantage over current quantification methods that rely on individually measured samples. Similar detection methods have been developed for the study of cyclic AMP (cAMP), which propelled the field forward as evident by the myriad of commercially available cAMP ELISA-based detection kits. The analogous development of the ELISA-based c-di-AMP detection assay would similarly make quantification of c-di-AMP in biological samples assessable to broader users.

3.3. Limitation of the assay

This method has a detection limit of approximately 10 nM. It is simple to determine c-di-AMP levels within bacteria, as detailed in the Methods and Results sections. The c-di-AMP levels vary from different bacteria and their growth stages. For *S. pneumoniae* and *M. tuberculosis*, by collecting bacteria from 1 to 10 ml of culture at their end logarithmic phase and preparing samples in 0.5 ml Tris-HCl, the c-di-AMP levels were ranging from 25 to 100 nM. However, it is more complicated in measuring c-di-AMP levels secreted by bacteria, either in culture media or in host cells. c-di-AMP levels in culture media may be undetectable directly for some bacteria; such samples might need to be concentrated prior to detection. Moreover, components in media may affect the assay, thus chemically defined media (CDM) are preferable for analysis of c-di-AMP secretion using this assay. As an example, Sauton's broth was used for determination of secreted c-di-AMP by *M. tuberculosis* (Yang *et al.*, 2014).

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Abbreviations

CDM	chemically defined medium
c-di-AMP	cyclic di-AMP
B-c-di-AMP	biotin-labeled c-di-AMP
DAC	diadenylate cyclase
ELISA	enzyme-linked immunosorbent assay
HhH	helix-hairpin-helix
HPLC	high-performance liquid chromatography
LC-MS	liquid chromatography-mass spectrometry

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Highlights

- ☐ We have established a simple assay to determine bacterial c-di-AMP levels
- ☐ The assay is highly specific to c-di-AMP
- ☐ The assay is easy to perform
- ☐ Many samples can be processed in one assay

Fig. 1.

Diagrammatic representation of the competitive ELISA for detection of c-di-AMP. Symbols are defined where appropriate. The first part of the assay relies on the coating of wells with CabP protein followed by a standard blocking step with BSA. A mixture of biotin-labeled and unlabeled c-di-AMP in standards or samples is then added to create the competitive condition in each well. Finally, the bound B-c-di-AMP is detected by incubation with HRP-conjugated streptavidin and followed by reaction with an OPD substrate.

Fig. 2.

Specificity of the ELISA assay. (A) CabP binds c-di-AMP specifically. Using coating concentrations of 10 and 50 µg/ml, CabP and BSA were coated into each well similarly. Subsequently, a titration of B-c-di-AMP ranging from 0 nM to 1,000 nM was performed in order to optimize efficiency and lower overall use of B-c-di-AMP. Our results from this titration indicate that a concentration of >1.5 nM was required for proper detected. (B) c-di-AMP binding site in CabP is specific for c-di-AMP. Using a 10 µg/ml coat of CabP and a standard 25 nM B-cdi-AMP tracer, 200 nM of various closely related nucleotides were used to challenge the performance of this assay. The only nucleotide that significantly reduced the signal was c-di-AMP, indicating that this assay is specific for c-di-AMP and not the other nucleotides tested. Data shown are the means of two independent experiments. The error bars denote the standard deviation (SD). Data set labeled “Control” represents repeats without any nucleotide challenge.

Fig. 3. Sensitivity of CabP with titration of B-c-di-AMP and c-di-AMP standards. Using standard coating conditions, B-c-di-AMP was 2-fold serially diluted from 100 nM and c-di-AMP was titrated 2-fold up to 1,000 nM. This representation provides photographic evidence for the optimization of B-c-di-AMP in the assay.

Fig. 4.

Example of a standard curve. The curve was generated using standard conditions and performing a 1:2 dilution scheme from 200 nM down to 6.25 nM c-di-AMP in 25 nM B-c-di-AMP sample diluent media. Data shown are the means of absorbance of duplicate wells. The error bars denote SD.

Fig. 5.

Detection of intracellular c-di-AMP in *E. coli* strains. *E. coli* W3110, BL21 with empty vector pET28a+ or the plasmid expressing *M. tuberculosis disA*. The samples were processed by following the protocol described in the Experimental section. Data shown are the means of three independent experiments. The error bars denote the SD. Abbreviations: n.d. indicates not detectable.