Quantitative determination of cyclic-di-GMP concentrations in nucleotide extracts of bacteria by Matrix Assisted Laser Desorption Ionization – Time of Flight mass spectrometry

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Running title: c-di-GMP measurement by MALDI-TOF analysis

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Abstract

The physiological response to small molecules (secondary messengers) is the outcome of a delicate equilibrium between biosynthesis and degradation of the signal. Cyclic di-GMP (c-di-GMP) is a novel secondary messenger present in many bacteria. It has a complex cellular metabolism whereby usually more than one enzyme synthesizing and degrading c-di-GMP is encoded by a bacterial genome. In order to assess the in vivo conditions of c-di-GMP signaling we developed a HPLC - mass spectrometry based method to detect c-di-GMP with high sensitivity and to quantify the c-di-GMP concentration in the bacterial cell as described here in detail. We successfully used the methodology to determine and compare the c-di-GMP concentrations in bacterial species like *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Vibrio cholerae*. Furtheron, we describe the use of the methodology to assess the change in c-di-GMP concentration during growth phase and the contribution of an individual GGDEF domain protein to the overall cellular c-di-GMP concentration.

Keywords: MALDI-TOF, c-di-GMP, quantification, HPLC

Introductory statements

Signalling by secondary messenger molecules is used in all kingdoms of life. In bacteria, the unorthodox nucleotide cyclic (5'→3')-diguanosine monophosphate (c-di-GMP) is an important signalling molecule involved in the regulation of major physiological transitions, such as the transition from motile to sessile cells [1]. Bioinformatics analysis revealed that the genes encoding c-di-GMP metabolising proteins are widely distributed in eubacteria whereby the pathway is especially predominant in the Gram-negative proteobacteria [2].

Biochemical analysis determined the enzymatic function of protein domains participating in c-di-GMP metabolism; the GGDEF domain has di-guanylate cyclase function [3], while the unrelated EAL and HD-GYD domains work as c-di-GMP specific phosphodiesterases [4; 5]. However, the *in vitro* quantitative analysis of protein activity does not shed light on the *in vivo* impact of c-di-GMP metabolizing enzymes, since the number of c-di-GMP producing and degrading proteins encoded by a single genome can go up to almost 100. Thereby, it is commonly agreed that local and/or global dynamic adjustment of the c-di-GMP concentration is a factor for the observed signal specificity of the physiological response [1]. In addition, while all biochemically characterized proteins involved in c-di-GMP metabolism have predicted cytoplasmic location, the majority of the c-di-GMP metabolizing enzymes are membrane proteins where *in vitro* activity is more difficult to analyse.

Following nucleotide extraction the amount of c-di-GMP exceeding the pico-mol range can be conveniently determined after separation of the extract by reversed phase-HPLC chromatography [6]. If at all, such amounts of c-di-GMP are commonly only observed after overexpression of di-guanylate cyclases, c-di-GMP synthesizing enzymes. Relative changes in the concentration of c-di-GMP can be determined by 2D-thin layer chromatography of nucleotide extracts [7; 8]. However, this methodology only gives relative values and requires specific culture conditions for radioactive labelling to detect the nucleotide.

Determination of the c-di-GMP concentration in bacterial cells and analysis of the contribution of c-di-GMP metabolizing enzymes to the overall c-di-GMP pool is a prerequisite for the understanding of the relation between the c-di-GMP regulatory network and the physiological output. In this manuscript, we describe in detail the development of a HPLC-MALDI-TOF based method to detect and quantify down to fmol amounts of c-di-GMP.

Materials and methods

Bacterial cultures

Bacteria were pre-cultured on Luria Bertani (LB) medium agar plates at 37ºC over night. From the pre-culture, bacteria were streaked on LB without salt plates if not otherwise stated.

Nucleotide extraction and sample preparation

Bacteria from a quarter of an agar plate (approximately 50 mg of wet weight) were resuspended in 300µl ice-cold 0.19% formaldehyde in pre-weighted tubes. Alternatively, ice-cold formaldehyde was added to a final concentration of 0.19% to liquid cultures. The bacterial suspension was put on ice for 10 min and subsequently pelleted by centrifugation at 5000xg for 15 minutes. The supernatant was decanted and the bacterial pellet was resuspended in 300µl deionized water. The bacterial suspension was heated to 95°C for 10 minutes. Nucleotides were extracted by mixing the cell lysate with 700µl 99% ethanol. Cell debris was spun down and the supernatant was saved. Extraction was repeated by re-suspending the bacterial debris in 1 ml 70% ethanol followed by pelleting the cell debris. Nucleotide extracts were combined, frozen at -80°C for 1h and subsequently lyophilized. The precipitate was re-suspended in 500µl HPLC grade water, centrifuged and the supernatant passed through a 0.22µm filter. The volume of the sterile filtered nucleotide extract was determined and the volume/mass ratio was calculated. Supernatant equivalent to 10 mg cells was adjusted to 0.1 M Triethylamine, pH 6.0 (HPLC-buffer A), vortexed and centrifuged for 5 minutes.

Isolation of c-di-GMP by ion-pair chromatography

An ÄKTA BASIC system with the Unicorn v. 4.00.16 software was used for HPLC analysis. Nucleotides were separated by ion-pair chromatography using a Hypersil ODS C18 column

with the dimensions 4.5x250 mm and 5 µm particles together with a 4x10 mm pre-column with 5 µm diameter particles. The mobile phase was: Buffer A: 0.1M Triethylamine/acetic acid, pH 6.0 and buffer B: 80% acetonitrile mixed with 20% Buffer A. The flow rate of the mobile phase was 1ml/min. The gradient was divided into 3 segments. Segment 1: 0% to 4% B in one column volume; segment 2: 4% to 5.6% B in four column volumes and segment 3 up to 10% buffer B in 1 column volume. Fractions of 1 ml were collected during the second and third segment, frozen at -80ºC and lyophilized. The lyophilized samples were dissolved in 10µl HPLC grade water and stored at -20ºC until further analysis. In a sample spiked with 200 pmol c-di-GMP the peak appeared after 21 minutes corresponding to fraction 15.

Sample application

Samples were applied on an Anchorchip^{TM} var/384 plate (Bruker Daltoniks, Leipzig, Germany) by the fast evaporation method [9]. The matrix (a-cyano-4-hydroxycinnamic acid) was dissolved in 99% acetone/water solution at a concentration of 20 mg/ml. A small amount of matrix was applied on the mixed anchorchip plate by sucking 0.5µl matrix into the pipette and carefully touching the tip of the pipette to the plate. Thereby, the liquid was spotted onto the plate by capillary forces, without forcing the liquid out of the tip. The matrix crystallises quickly and evenly on the plate. The matrix crystals were used as seed for the crystallisation of the sample by pipetting 0.5µl sample on the matrix spot and letting the liquid evaporate. After crystallisation, the sample was washed two times by pipetting a drop of 10 µl HPLC-grade water on top of the sample spot which was carefully removed after 20 seconds.

Matrix assisted laser desorption ionization - time of flight

MALDI mass spectra were recorded with a Bruker Reflex IV MALDI-TOF mass spectrometer (Bruker-Daltoniks, Leipzig, Germany) using a N_2 laser with 337 nm wavelength

and 3 ns pulse width. Pressures in the source and reflector region were required to be below $3x10^{-7}$ and 10^{-7} mbar respectively for appropriate sensitivity and reproducibility. For spectrum acquisition, the instrument was operated in reflector mode and the polarity was set to detection of negative ions. The matrix deflection option was set to 300 Da and the mass range was set to 300 – 1000 Da. Accelerating voltage was 20 kV and the grid voltage was set to 16.4 kV. The pulsed ion extraction time was set to a delay of 200 ns. The spectra were evaluated using the FlexAnalysis software (Bruker Daltoniks).

Construction of the c-di-GMP standard curve

The standard curve was constructed by analysing samples containing a pre-determined amount of c-di-GMP and a constant amount of the internal standard cyclic di-adenosine-monophosphate c-di-AMP (1μM). C-di-GMP and c-di-AMP used in the construction of the standard curve were chemically synthesized as described [10][11]. The MS-spectrum of c-di-GMP has distinct peaks at a mass to charge ratio of 689, 711 and 727 Da, which corresponds to $[\text{c-di-GMP} - H^+]^-$, $[\text{c-di-GMP} - 2H^+ + Na^+]^-$ and $[\text{c-di-GMP} - 2H^+ + K^+]^-$ respectively. The c-di-AMP gives a spectrum with characteristic peaks at mass to charge ratio 657, 679 and 695 Da corresponding to $[\text{c-di-AMP} - H^+]^-$, $[\text{c-di-AMP} - 2H^+ + Na^+]^-$ and $[\text{c-di-AMP} - 2H^+ + K^+]^-$ respectively. The area of the peaks in the MS-spectrum corresponding to c-di-GMP and the area corresponding to the internal standard c-di-AMP were calculated and the ratio between the area of the c-di-GMP and c-di-AMP peaks was determined. By plotting the ratio of c-di-GMP over c-di-AMP against the c-di-GMP concentration a logarithmic-linear curve was obtained.

Since the bacterial samples were prepared by pooling fractions, they contain other molecules apart from c-di-GMP or the internal standard. These other molecules absorb energy during the ion extraction and may influence the spectra of a given sample. In order to mimic the

conditions of the bacterial samples, the samples for the standard curve were prepared by pooling fractions that did not contain c-di-GMP (or c-di-AMP). Variable concentrations of c-di-GMP were added to these pooled fractions and the internal standard was added at a fixed concentration of 1μM.

Sample analysis by MALDI-TOF MS

The fractions obtained from the HPLC separation were analysed by MALDI-TOF MS to pinpoint the fractions containing c-di-GMP. The fractions containing c-di-GMP and one fraction before and after the c-di-GMP containing fractions were pooled. The internal standard was added and the sample was analysed by MALDI-TOF MS. The value obtained was used to determine the c-di-GMP concentration by inserting the value into the equation for the standard curve.

Results

MALDI-TOF analysis is a methodology that ionizes molecules precipitated on a template by laser desorption. The time of flight to a detector in an electric field can be used to determine the molecular weight of the substance consequently MALDI-TOF analysis is mainly used to determine the molecular weight of molecules. By definition, small molecules fly better than larger ones, whereby the lower limit of detection is determined by the noise created by the molecules from the matrix that co-crystallize with the sample on the template. Our goal was to set up a quantitative assay for the measurement of the small molecule cyclic di-nucleotide c-di-GMP.

Detection limit for c-di-GMP by MALDI-TOF

First, we determined the detection limit for c-di-GMP. We consistently detected 1fmol c-di-GMP in the applied sample, which corresponds to 2 nM sample concentration (data not shown). The limit of detection was defined as the c-di-GMP concentration with a signal to noise ratio of at least 3 in the mass spectrum. In this context, the application of the sample to the mixed anchorchip plate was optimized. Conventionally, the sample is mixed with the matrix preparation and applied to the mixed anchorchip plate. We found this application procedure to result in analyte-matrix heterogeneity leading to difficulties in acquiring signals, poor reproducibility and reduced sensitivity. However, the fast evaporation method [9], which provides the sample on a pre-crystallized matrix, yielded reproducible results with high sensitivity.

Standard curve for c-di-GMP

The intensity of the signal obtained by MALDI-TOF analysis is relative and depends e.g. on the purity of the sample. Therefore a standard curve for c-di-GMP was created using c-di-

AMP as an internal standard. C-di-AMP was chosen since it is also a purin-based cyclic di-nucleotide of similar size, which was expected to have similar desorption and ionisation characteristics as c-di-GMP. Fig. 1 shows the spectra for c-di-GMP and c-di-AMP in the same sample. The spectra for c-di-GMP and c-di-AMP do not overlap.

The presence of other compounds absorbs energy during ion extraction and consequently influences the intensity of the signal of c-di-GMP. We found that the c-di-GMP/c-di-AMP signal with a given c-di-AMP concentration is linear with c-di-GMP concentrations varying in ratios from approx. 0.1 to 1. Therefore standard curves were produced for two c-di-AMP concentrations, 1 μM and 0.1 μM (fig. 2). The limit of quantification was defined by the linear range of the standard curve. Accordingly, for the standard curve with 0.1 μM c-di-AMP as internal control, the lower and upper limits of quantification were 5 nM and 50 nM c-di-GMP, respectively. Using 1μM c-di-AMP as internal control, the corresponding values were 20 nM and 200 nM c-di-GMP. In practise, appropriate sample dilutions adjust the c-di-GMP concentration to fit into the linear range of the standard curve, eliminating the upper limit of quantification.

Statistical analysis

The equation describing the ratio of c-di-GMP over c-di-AMP as a function of c-di-GMP concentration using 1μM internal control $y = 0.003x + 0.071$ has a correlation coefficient $r = 0.975$ and a standard error of the estimate $S_{y,x} = 0.04040$. Analysis of variance (ANOVA) gave a F-value of 372.9, 1 degree of freedom (d.f.) for the regression and 19 d.f. for the residuals (1,19). This value indicates that the relationship between x and y is statistically significant, $p = 0.001$. The corresponding equation and statistics for the standard curve with 0.1μM internal control is $y = 0.02x+0.23$ with $r = 0.977$, $S_{y,x} = 0.08303$. $F(1,13) = 267.3$

indicates statistically significant relationship between x an y, p = 0.001. The confidence bands for the respective standard curves are shown in fig 2b and 2d.

Determination of c-di-GMP concentration

After separation of nucleotide extracts from 10 mg cells by HPLC, the fractions expected to contain c-di-GMP were lyophilized and dissolved in 10 µl water. The prescence/abscence of c-di-GMP was determined for each fraction by MALDI-TOF analysis. One µl of each fraction containing c-di-GMP were pooled, c-di-AMP was added and the volume was adjusted to 10 µl. The ratio of the areas of the c-di-GMP peaks and the c-di-AMP peaks was determined from the results of the MALDI-TOF analysis. By introducing the value into the equation for the standard curve the concentration of the pooled fractions sample was determined in M. The amount of c-di-GMP per mg cells is estimated by n= 10*C*V, where C is the concentration (M), n is the amount (mol) and V is the volume of the pooled sample (L).

To determine the recovery rate, *S. typhimurium* UMR1 cells were grown under conditions, where low intrinsic c-di-GMP concentrations were expected (at 37°C for 20h on agar plates). Before resuspension of the bacteria in 300µl ice-cold 0.19% formaldehyde, the sample was spiked with 100 fmol c-di-GMP/mg cells, an amount of c-di-GMP close to the detection limit. The recovery rate was determined to be 85% ± 11% after substraction of the c-di-GMP concentration from the un-spiked samples.

c-di-GMP concentration in different bacterial species

It was previously demonstrated by MS/MS analysis that the c-di-GMP produced by *Salmonella typhimurium* is structurally identical to a synthetic c-di-GMP standard [6]. To demonstrate the use of the novel methodology, we determined the amount of c-di-GMP in

different bacteria (fig. 3). In one mg of bacterial cells, pico-mol amounts of c-di-GMP were found. However, the amounts of c-di-GMP varied significantly between bacterial species. Under similar growth conditions, c-di-GMP concentrations were 10 times higher in *Vibrio cholerae* than in *Salmonella typhimurium* (fig. 3a). Similarily, under other growth conditions, *V. cholerae* showed two times more c-di-GMP than *Pseudomonas aeruginosa* and over 20 times more c-di-GMP than *Escherichia coli* (fig. 3b).

c-di-GMP concentrations are higher in Salmonella typhimurium expressing a multicellular behaviour

To estimate the amount of c-di-GMP in biofilm forming cells versus cells not forming the biofilm, we compared the c-di-GMP concentrations in wild type *Salmonella typhimurium* (strain UMR1) and an isogenic mutant (strain MAE52) with constitutive expression of multicellular behaviour [12]. C-di-GMP concentrations were 2.5-fold higher in the mutant with constitutive expression of multicellular behavior compared to the wild type which does not express multicellular behaviour under the given growth conditions (fig. 4a). These values are consistent with the hypothesis of elevated c-di-GMP concentrations in biofilm-forming cells [13].

C-di-GMP concentrations during growth phase

C-di-GMP concentrations were determined during the growth phase in strain UMR1 expressing multicellular behaviour in the late growth phase [12]. Since in this model bacteria are grown on agar plates, harvesting of a sufficient amount of bacteria from an agar lawn was possible only after 10 h. C-di-GMP concentrations were found to be maintained at the same concentration after 10 and 16 h of growth, while a three-fold decline was observed after 24 h of growth (fig. 4b and [14]). Since multicellular behaviour is predominantly visible after 24 h

of growth, this finding suggests that c-di-GMP signalling mainly takes place during the establishment of multicellular behaviour, but seems to decrease once the behaviour is expressed.

Discussion

Conventionally biochemical analysis is used to characterize enzyme activity. In this way also the characteristics of GGDEF and EAL/HD-GYP domain proteins have been determined [3; 4]. In vivo, the overall activity of a c-di-GMP metabolising enzyme might not only be determined by the level of protein expression at a certain growth condition, but also by complex allosteric modulation of the enzymatic activity by a range of signals and/or metabolic products. Therefore, determination of enzymatic activities in vitro has only limited value for the in vivo impact of a c-di-GMP metabolizing protein on c-di-GMP concentrations. Overexpression of a c-di-GMP producing enzyme (di-guanylate cyclase) and subsequent determination of the c-di-GMP concentration *in vivo* provides information about the potential of the protein to produce c-di-GMP at a given growth condition, whereby the c-di-GMP concentration can be measured directly by HPLC analysis. Systematic studies found that the changes in c-di-GMP concentrations, by overexpression of individual c-di-GMP metabolising proteins, did not always correlate with the observed (c-di-GMP dependent) phenotype [15{Kader, 2006 #397, whereby the c-di-GMP concentration can be measured directly by HPLC analysis. However, systematic studies found that the changes in c-di-GMP concentrations, by overexpressing of individual c-di-GMP metabolising proteins, did not always correlate with the observed (c-di-GMP dependent) phenotype {Kulesekara, 2006 #489]. This finding indicates the predicted existence of localized c-di-GMP pools [1]. Limitations in the analysis come up when the amounts of c-di-GMP are below the detection limit of the HPLC and when the overexpression of the enzyme overrides control mechanisms. In addition, the in vivo activity of c-di-GMP dependent phosphodiesterases cannot be assessed with this approach.

The above described limitations can be overcome by the methodology described in this manuscript. We present the development of a method to quantify c-di-GMP with a detection

limit of 1 fmol. This level of sensitivity allows the determination of c-di-GMP concentrations in bacterial cells at any growth condition. Consequently, the use of this method in combination with genetic studies in bacteria will improve our understanding of the c-di-GMP regulatory network in all organisms where this regulatory network exists. In addition, similar quantification methods can be set up for other bacterial secondary messengers as the newly identified cyclic di-AMP [16].

One has to mention a remaining technical problem is the variable quality of the sample spot. Uneven matrix crystallisation and equal spreading of matrix on the template are the most frequently experienced experimental pitfalls. Methodologically there is a certain loss of sample due to the multistep process. Therefore the values obtained might be slight underestimates of the actual cellular concentrations.

Despite of these pitfalls, this methodology has been proven to be highly valuable in comparing the levels of c-di-GMP concentration in different bacterial species (this manuscript), the change in c-di-GMP concentration during the growth phase ([14] and this manuscript) and the in vivo impact of c-di-GMP specific phosphodiesterases [17]. Interestingly, in different bacteria, the amount of c-di-GMP in the cell correlated grossly with the number of potential c-di-GMP metabolizing enzymes. *V. cholerae*, which showed the highest c-di-GMP concentration under different growth conditions, has 62 potential c-di-GMP metabolizing proteins, while *P. aeruginosa* has 41, *E. coli* 29 and *S. typhimurium* 20 [2].

The methodology also allows the estimation of the amount of c-di-GMP molecules in one cell. Considering the weight of a bacterial cell to be 0.66 pg [18], *S. typhimurium* strain UMR1 has 233±37 molecules c-di-GMP/cell at 28ºC after 10 hs of growth, 255±55 after 16hs and 86±5 after 24 hs. Using another approach for calculation, namely the assessment that 1mg dry weight cells is associated with 4µl (4mg) water [19] and assuming that one bacterial cell has the dimensions 0.5µm x 0.5µm x 2µm, the calculation results in 217±35, 237±51 and

80±5 molecules c-di-GMP/cell. The estimated numbers are significantly higher than the previously estimated 8 molecules c-di-GMP/cell in wild type *Salmonella typhimurium* [18]. However, the calculated numbers are probably low enough to explain an apparently local c-di-GMP effect by binding constants of localized c-di-GMP receptors while c-di-GMP is freely diffusible in the cell..

In summary, determination of the amounts of the secondary messenger c-di-GMP in vivo will give novel insights into the mechanisms of c-di-GMP signalling.

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Figure legends

Fig. 1. MALDI-spectrum showing c-di-GMP peaks and c-di-AMP peaks. Mass spectrum displaying the characteristic patterns of c-di-GMP and c-di-AMP isotope peaks in negative ionization mode. Peaks at 657.4 m/z, 679.4 m/z and 695.4 m/z corresponds to $[(\text{c-di-AMP})\text{-}H^+]^-$, $[(\text{c-di-AMP})\text{-}2H^+\text{+}Na^+]^-$ and $[(\text{c-di-AMP})\text{-}2H^+\text{+}K^+]^-$. Peaks at 689.4 m/z, 711.4 m/z and 727.4 m/z corresponds to $[(\text{c-di-GMP})\text{-}H^+]^-$, $[(\text{c-di-GMP})\text{-}2H^+\text{+}Na^+]^-$ and $[(\text{c-di-GMP})\text{-}2H^+\text{+}K^+]^-$. a.i, arbitrary intensity.

The mass-spectrum was retrieved from a sample containing 0.6μM of the analyte, c-di-GMP and 1μM of the internal standard, c-di-AMP.

Fig. 2. Calibration curves used for c-di-GMP quantification by MALDI-TOF mass spectrometry. Calibration curves were constructed by using an internal standard (c-di-AMP) at a fixed concentration whereas the concentration of c-di-GMP was varied. The areas of the isotope peaks for c-di-GMP as well as c-di-AMP were calculated and the ratio between the c-di-GMP peak area over c-di-AMP peak area was determined for each sample. The Ratio (c-di-GMP/c-di-AMP) was plotted against the c-di-GMP concentration. For quantification of high c-di-GMP concentrations the internal standard c-di-AMP was used at a concentration of 1μM and the c-di-GMP concentration spanned from 20 to 10 000 nM c-di-GMP (A). The linear part of the curve is shown in B spanning from 20-200 nM c-di-GMP with a R^2-value >0.95. At lower c-di-GMP concentrations the internal standard c-di-AMP was used at a concentration of 0.1μM and the c-di-GMP concentration spanned from 5 to 1000 nM (C). The linear part of the curve (D) spans from 5 to 50 nM c-di-GMP and has a R^2-value>0.95. In fig 2B and 2D, the solid line represent the trend line for the standard curve, whereas the dashed lines represent the 95 % upper and lower confidence bands.

Fig. 3. Comparison of c-di-GMP amounts in different bacteria. (A) Comparison of intracellular c-di-GMP concentrations in *S. typhimurium* strain UMR1 and *V. cholerae* strain C6707. C-di-GMP was extracted from bacteria grown for 16 – 18 hs on LB without salt agar plates at 37°C. (B) Bacteria were grown in batch culture containing LB-medium at 37°C until OD_{600} 1.5 – 2 (*E. coli* MG1655 [20] and *V. cholerae* C6707, respectively) and for 48h (*P. aeruginosa* WT20265 [21]). Error bars represent the standard deviation.

Fig. 4. Quantification of the intracellular c-di-GMP concentration in *S. typhimurium.* (A) Comparison of the c-di-GMP concentrations in *S. typhimurium strain* UMR1 and its isogenic mutant MAE52 upregulated in multicellular behaviour. UMR1 displays a highly regulated temperature dependent biofilm expression, while biofilm formation is repressed at temperatures >30ºC [22]. MAE52 carries a point-mutation that allows biofilm formation above 30ºC [12]. The increased c-di-GMP levels in MAE52 corresponds to the higher biofilm expression in MAE52 compared to UMR1 on LB without salt agar plates at 37ºC after 16h growth [12]. (B) Intracellular c-di-GMP concentration after incubation of *S. typhimurium* strain UMR1 on LB without salt agar plates at 28ºC for 10 h, 16 h and 24 h. Error bars represent the standard deviation.

Fig 1.

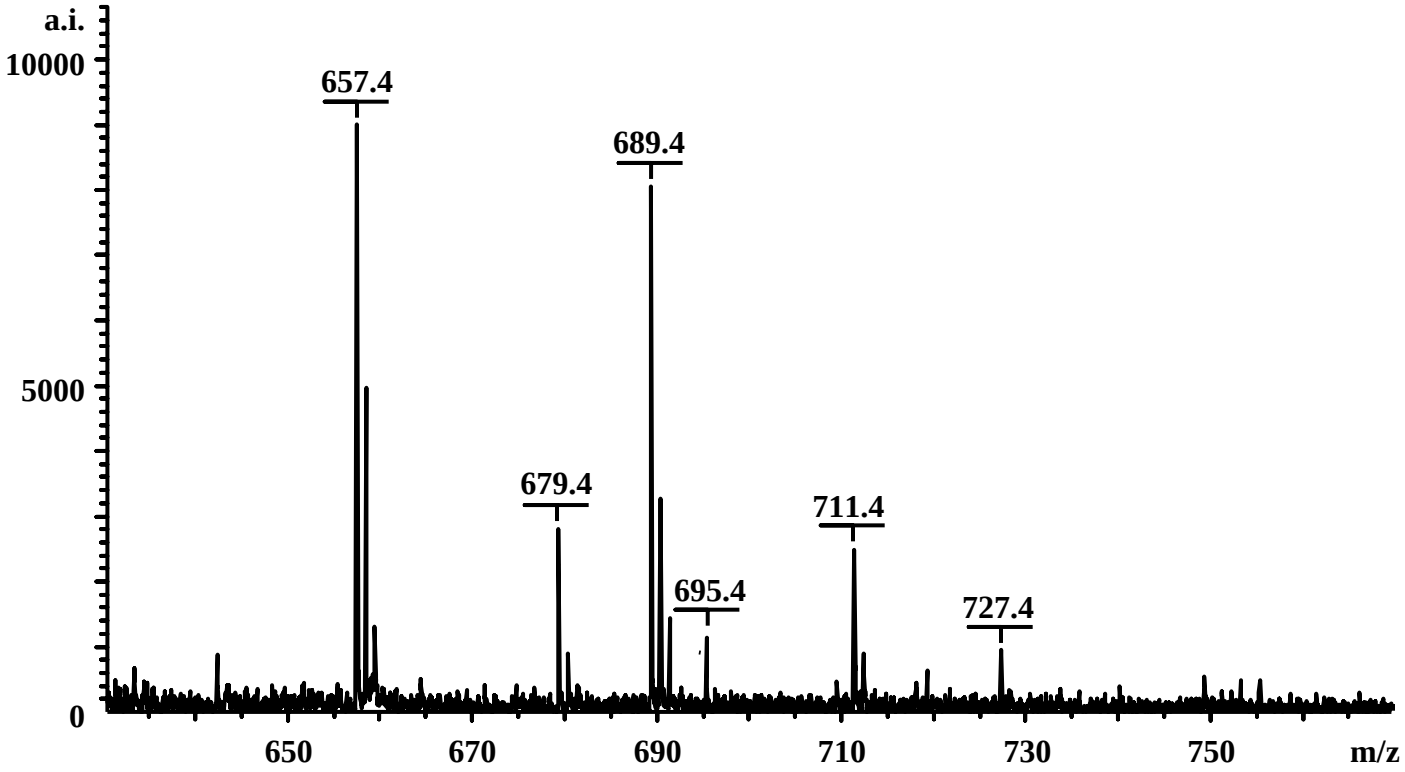

a.i.
10000
5000
0
657.4
689.4
679.4
711.4
695.4
727.4
650
670
690
710
730
750
m/z

Fig 1.

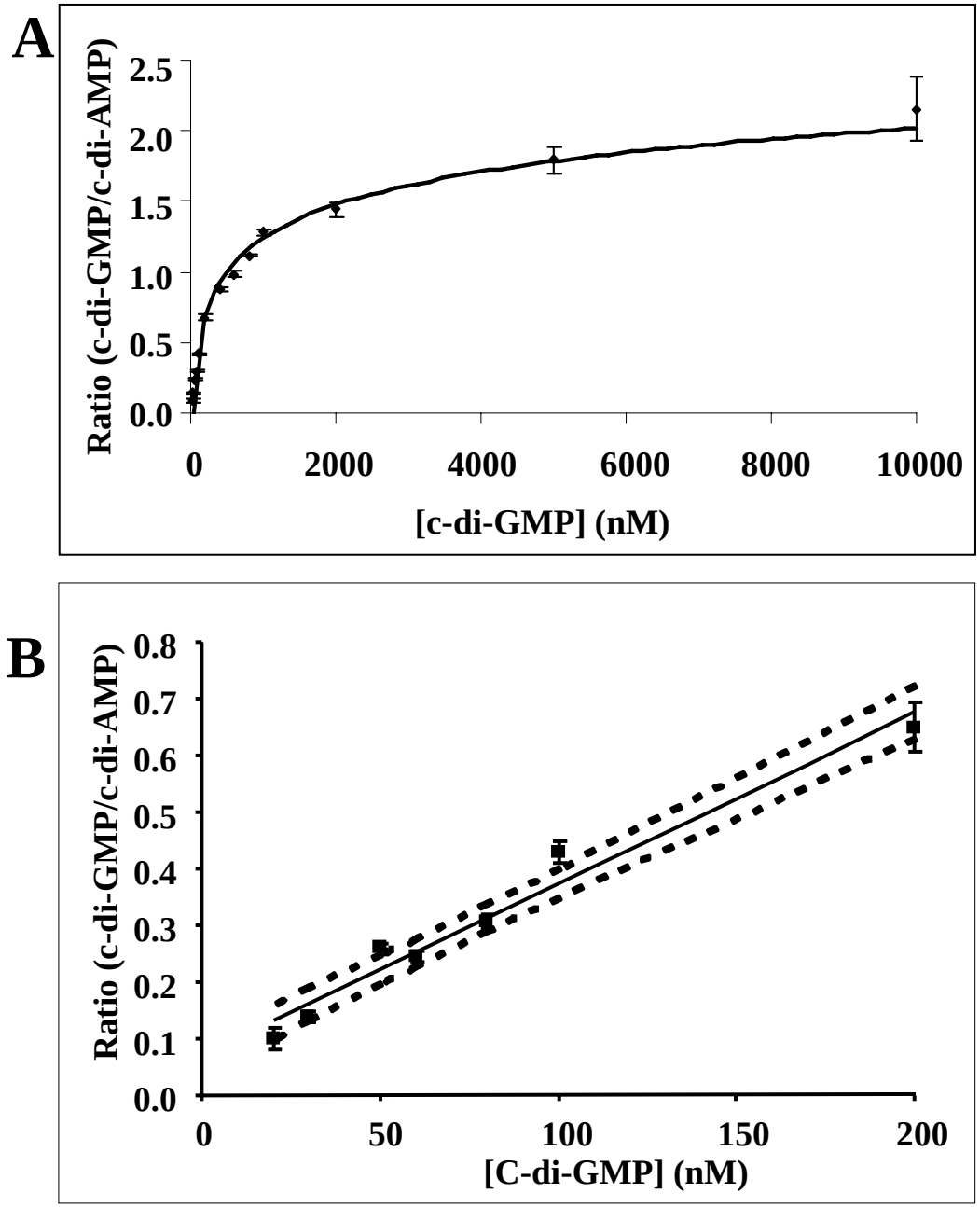
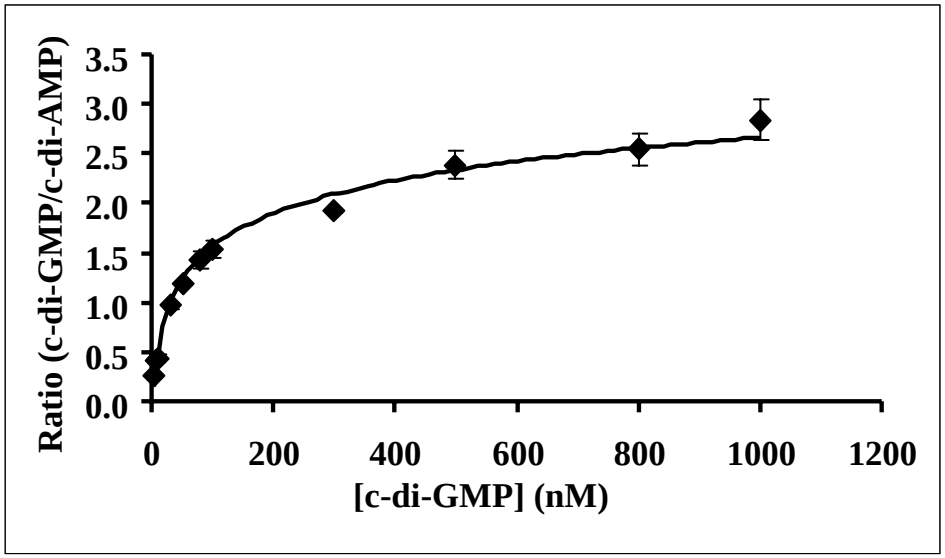

A
Ratio (c-di-GMP/c-di-AMP)
2.5
2.0
1.5
1.0
0.5
0.0
0
2000
4000
6000
8000
10000
[c-di-GMP] (nM)
B
Ratio (c-di-GMP/c-di-AMP)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
50
100
150
200
[C-di-GMP] (nM)

Fig 2.

C


Ratio (c-di-GMP/c-di-AMP)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
200
400
600
800
1000
1200
[c-di-GMP] (nM)

D

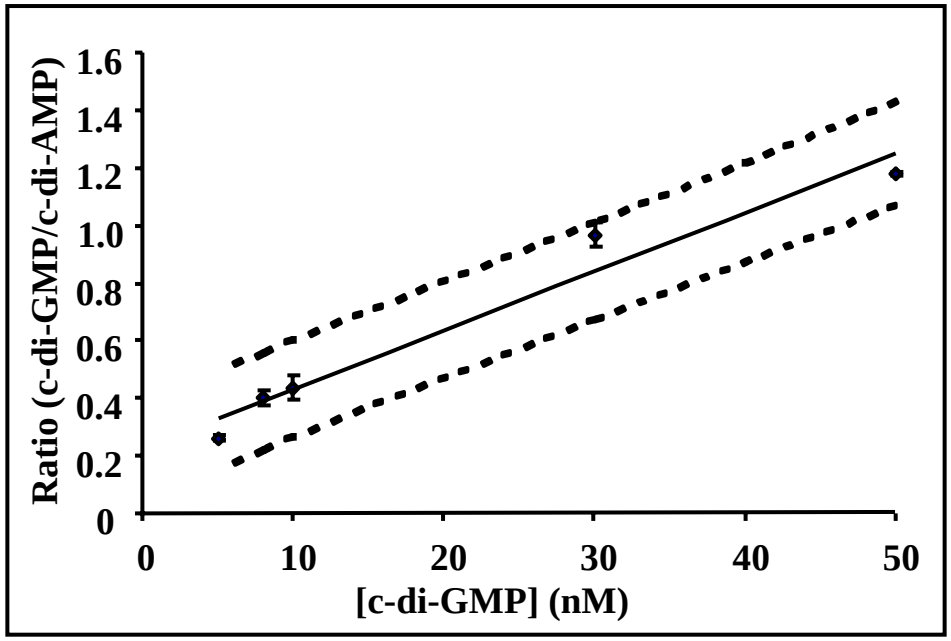

Ratio (c-di-GMP/c-di-AMP)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
[c-di-GMP] (nM)

Fig 2.

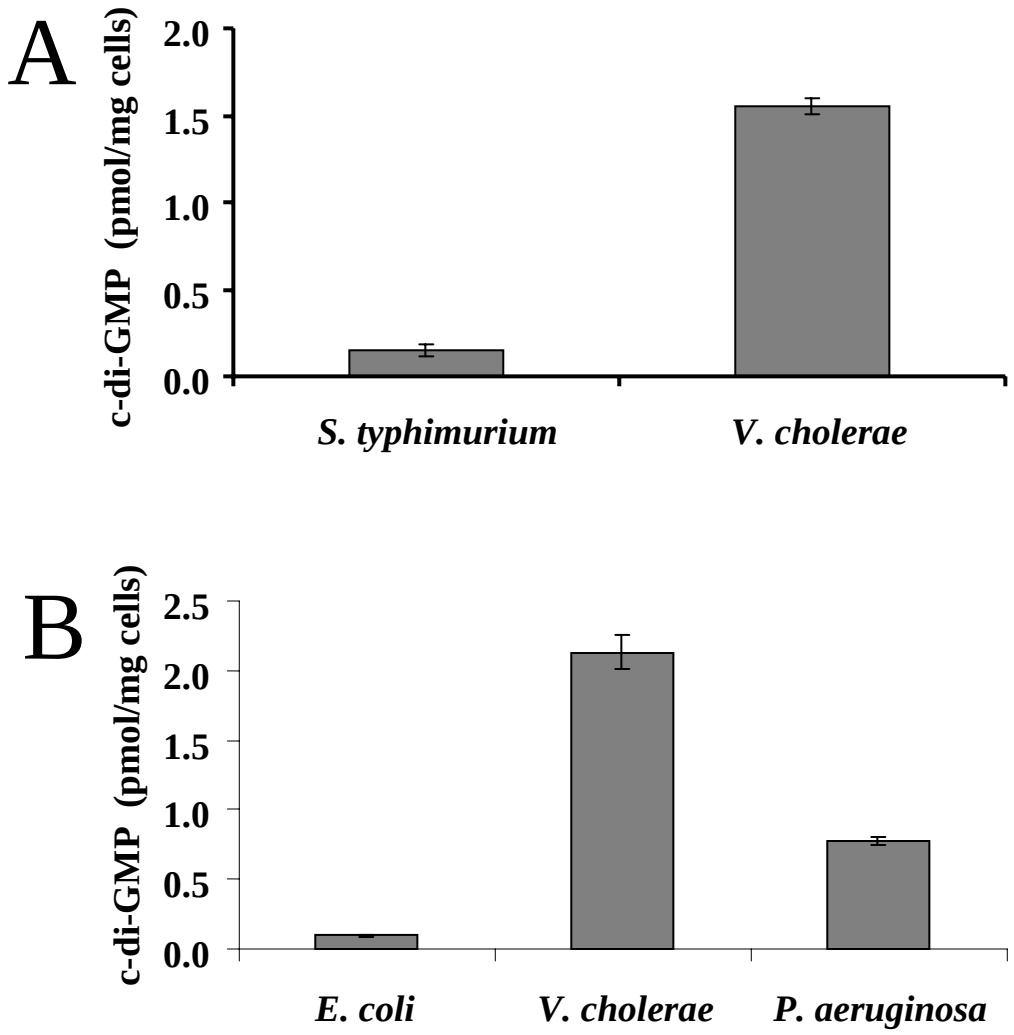

A
c-di-GMP (pmol/mg cells)
2.0
1.5
1.0
0.5
0.0
S. typhimurium
V. cholerae
B
c-di-GMP (pmol/mg cells)
2.5
2.0
1.5
1.0
0.5
0.0
E. coli
V. cholerae
P. aeruginosa

Fig 3.

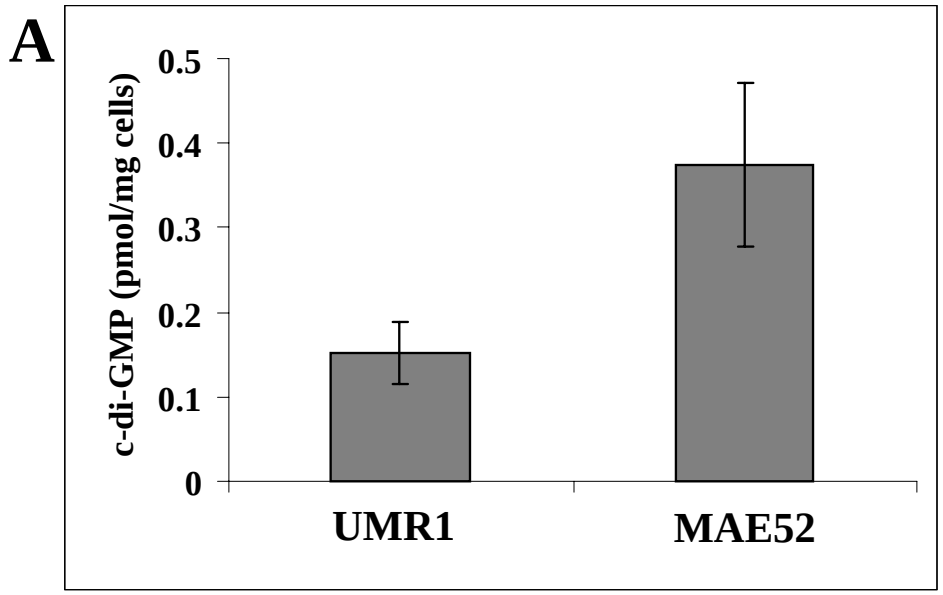


Fig 4.

B

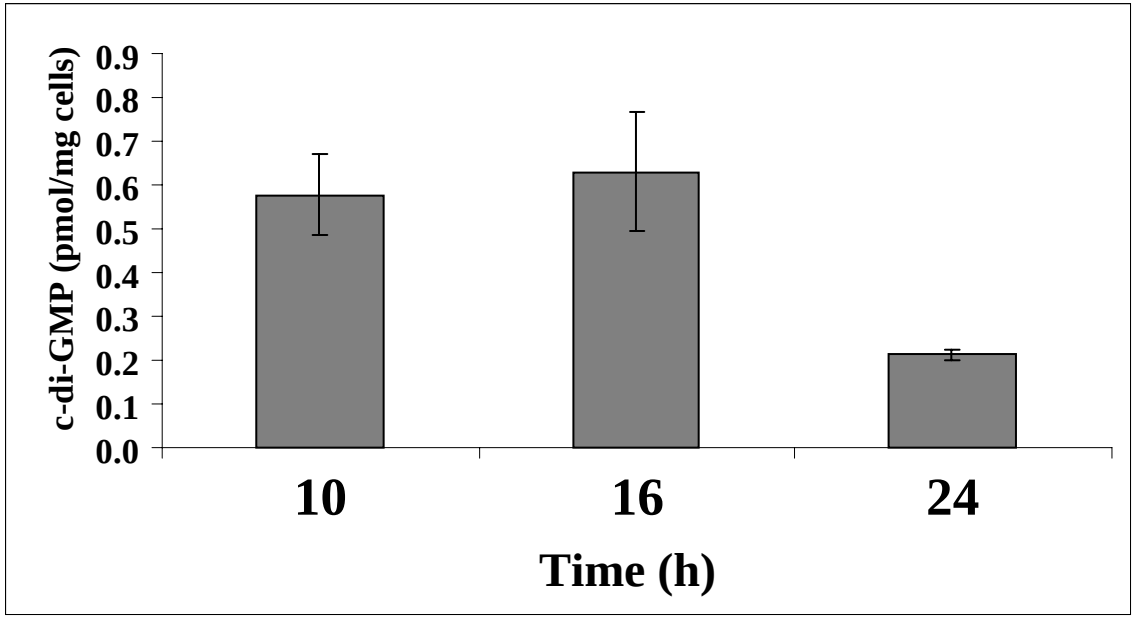


Fig 4.