Video Article

Capture Compound Mass Spectrometry - A Powerful Tool to Identify Novel c-di-GMP Effector Proteins

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Abstract

Considerable progress has been made during the last decade towards the identification and characterization of enzymes involved in the synthesis (diguanylate cyclases) and degradation (phosphodiesterases) of the second messenger c-di-GMP. In contrast, little information is available regarding the molecular mechanisms and cellular components through which this signaling molecule regulates a diverse range of cellular processes. Most of the known effector proteins belong to the PilZ family or are degenerated diguanylate cyclases or phosphodiesterases that have given up on catalysis and have adopted effector function. Thus, to better define the cellular c-di-GMP network in a wide range of bacteria experimental methods are required to identify and validate novel effectors for which reliable *in silico* predictions fail.

We have recently developed a novel Capture Compound Mass Spectrometry (CCMS) based technology as a powerful tool to biochemically identify and characterize c-di-GMP binding proteins. This technique has previously been reported to be applicable to a wide range of organisms[1]. Here we give a detailed description of the protocol that we utilize to probe such signaling components. As an example, we use *Pseudomonas aeruginosa*, an opportunistic pathogen in which c-di-GMP plays a critical role in virulence and biofilm control. CCMS identified 74% (38/51) of the known or predicted components of the c-di-GMP network. This study explains the CCMS procedure in detail, and establishes it as a powerful and versatile tool to identify novel components involved in small molecule signaling.

Video Link

The video component of this article can be found at https://www.jove.com/video/51404/

Introduction

c-di-GMP is a key second messenger used by most bacteria to control various aspects of their growth and behavior. For instance, c-di-GMP regulates cell cycle progression, motility and the expression of exopolysaccharides and surface adhesins[2-4]. Through the coordination of such processes c-di-GMP promotes biofilm formation, a process which is associated with chronic infections of a range of pathogenic bacteria[5]. c-di-GMP is synthetized by enzymes called diguanylate cyclases (DGCs) that harbor a catalytic GGDEF domain[4]. Some DGCs possess an inhibitory site that down regulates the cyclase activity upon c-di-GMP binding. The degradation of c-di-GMP is catalyzed by two distinct classes of phosphodiesterases (PDEs) harboring either a catalytic EAL or HD-GYP domain[6,7].

The majority of the known effector proteins that directly bind c-di-GMP belong to one of only three classes of proteins: catalytically inactive GGDEF or EAL domains and PilZ domains, small molecular switches that undergo conformational changes upon c-di-GMP binding[8]. DGCs, PDEs and PilZ proteins are well characterized and their domains can be predicted *in silico* relatively safely. A particular interest is now focused on the identification of new classes of c-di-GMP effectors. Some c-di-GMP effectors with different binding motifs were described recently such as the CRP/FNR protein family Bcam1349 in *Burkholderia cenocepacia* or the transcriptional regulator FleQ in *P. aeruginosa*[9,10]. In addition, c-di-GMP-specific riboswitches were recently identified and shown to control gene expression in a c-di-GMP-dependent manner[11]. The c-di-GMP binding motifs of different effectors are only poorly conserved making bioinformatic predictions of such proteins difficult. To address this issue, we developed a biochemical method, which is based on the use of a c-di-GMP specific Capture Compound combined with mass spectrometry [1,12,13].

We have recently engineered a novel trivalent c-di-GMP Capture Compound (cdG-CC, **Figure 1**)[1]. This chemical scaffold is composed of: 1) a c-di-GMP moiety used as bait to capture c-di-GMP binding proteins, 2) a UV-photoactivatable reactive group used to cross link the cdG-CC to the bound proteins and 3) a biotin to isolate the captured proteins using streptavidin-coated magnetic beads. The cdG-CC can be used to directly and specifically capture c-di-GMP effectors from complex mixture of macromolecules as cell lysates. Capture Compound based and chemical proteomics based approaches have previously been reported to be applicable to a wide range of organisms, *e.g. Caulobacter crescentus*, *Salmonella enterica* serovar *typhimurium* and *P. aeruginosa*[1,14].

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In this methodological paper, we provide an in depth description of the CCMS procedure using extracts of *P. aeruginosa* as an example. This study establishes CCMS as a powerful and versatile tool to biochemically identify novel components involved in small molecule signaling.

Protocol

1. Lysate Preparation

1. Grow *P. aeruginosa* cells in LB to the desired OD.
   NOTE: For guidance: use ≈ 100 ml culture/sample for stationary phase cultures and ≈ 500 ml cultur /sample for log phase cultures (OD_{600nm} = 0.5).
2. Pellet by centrifugation for 20 min at 5,000 x *g*.
3. Resuspend 0.5-1 g of pellet in 1 ml lysis buffer (6.7 mM MES, 6.7 mM HEPES, 200 mM NaCl, 6.7 mM KAc, DDT 1 mM, pH 7.5) and add protease inhibitor (complete mini, EDTA-free) as well as DNaseI.
4. Lyse the cells by 3 passages through a French pressure cell, at 20,000 psi (see Materials List).
5. Ultra-centrifuge the cell lysate at 100,000 x *g* for 1 hr at 4 °C.
6. Save the supernatant (go to step 2).
7. Wash the pellet with 1 ml 1x lysis buffer by pipetting up and down.
8. Ultracentrifuge at 100,000 x *g* for 1 hr at 4 °C.
9. Flash-freeze the pellet in liquid nitrogen and store at -20 °C until used for the capture of membrane proteins (see step 3).

2. Removal of Free c-di-GMP and Other Nucleotides (Soluble Fraction Only)

1. Wash a PD10 desalting column (see Materials List) with 10 ml of cold lysis buffer.
2. Pour the supernatant (≈ 1 ml) onto the PD10 in order to remove nucleotides.
3. Elute with 4 ml cold lysis buffer (500 µl steps).
4. Select the most concentrated fractions as determined by Bradford assay, and pool them.

3. Pellet Resuspension and Solubilization (Membrane Fraction Only)

1. Resuspend the pellet in 500 to 1,000 µl of 1x capture buffer (without detergent) (see **Table 1**).
   NOTE: The pellet is difficult to resuspend. It is advised to first pipette up and down with a pipette to roughly resuspend the pellet, then to use a syringe 27 G to well homogenize the solution.
2. Add 1% (w/v) n-dodecyl-β-D-maltopyranoside (DDM).
3. Incubate at 4 °C for at least 2 hr (or O/N) on a rotating wheel.
4. Ultracentrifuge at 100,000 x *g* for 1 hr at 4 °C.
5. Harvest the supernatant.

4. Protein Concentration Measurement

1. Measure the protein concentration by Bradford assay (by BCA assay for the membrane fraction).
2. Set the total protein concentration to 10 mg/ml.

5. Capture

1. Mix 300 µg of protein with 1 mM of GDP, GTP, ATP, CTP, and 20 µl of 5x capture buffer (100 mM HEPES, 250 mM KAc, 50 mM MgAc, 5% glycerol, pH 7.5) (**Table 1**). Adjust the reaction volume to 100 µl with H_2O.
   NOTE: the total volume includes the volume of the cdG-CC and c-di-GMP in case of the competition control (see step 5.3 and **Table 2**).
   NOTE: all experiments were performed in 200 µl 12-tube PCR strips (Thermo Scientific).
   NOTE: for the membrane fraction, make sure to always keep the DDM concentration above the critical micelle concentration (0.01% w/v) until the protein solubilization step in 8 M Urea (Step 7.1).
2. Incubate at 4 °C for 30 min on a rotating wheel.
3. Add 10 µM cdG-CC (final concentration).
   NOTE: include a control without cdG-CC (referred as “bead control”), and a control supplemented with 1 mM c-di-GMP (“competition control”) (see **Table 2**).
   NOTE: the cdG-CC concentration can be adjusted from 1 to 10 µM.
4. Incubate at 4 °C for at least 2 hr (O/N for the membrane fraction) on a rotating wheel, in the dark.
5. After a short spin, cross-link by activation of the reactive moiety of the cdG-CC with UV light for 4 min, using a CaproBox (see Materials List, λ = 310 nm, Irradiance ≥10 mW/cm², distance from the source = 2 cm). NOTE: remove the lid of the strips prior cross-linking.
6. Add 25 µl 5x wash buffer (5 M NaCl, 250 mM Tris, pH 7.5) and 50 µl of well resuspended streptavidin magnetic beads, gently homogenize.
7. Incubate at 4 °C for 1 hr on a rotating wheel.

6. Washing Steps

NOTE: (Magnet: see Materials List). Start with a capture of the magnetic beads in the PCR strip lid, with the magnet. Then replace the PCR strip by a new one containing the next washing solution. Remove the magnet and resuspend the beads, and incubate 2 min. Spin down and replace the lid by a fresh lid.

1. Washing steps (soluble fraction only)
    1. Wash 6 times in 200 µl 1x washing buffer.
    2. Wash once in 200 µl HPLC grade H_2O.
    3. Wash 6 times in 200 µl 80% acetonitrile.
    4. Wash 2 times in 200 µl HPLC grade H_2O.
2. Washing steps (membrane fraction only)
    1. Wash 5 times in 200 µl 1x washing buffer + 0.1% DDM.
    2. Wash 2 times in 200 µl 1x washing buffer + 0.05% DDM.
    3. Wash once in 200 µl 1x washing buffer + 0.025% DDM.
    4. Wash once in 200 µl 1x washing buffer + 0.0125% DDM.
    5. Wash 3 times in 200 µl 100 mM ABC (ammonium bicarbonate, NH_4CO_3) + 2 M Urea.

7. MS Sample Preparation

1. Resuspend the beads (directly in the lid) in 20 µl 100 mM ABC (100 mM ABC + 8 M Urea for the membrane fraction) and transfer into 1.5 ml tubes.
2. Membrane fraction only: incubate at 60 °C for 5 min, shaking at 500 rpm.
3. Add 0.5 µl 200 mM TCEP (tris(2-carboxyethyl)phosphine) and incubate at 60 °C for 1 hr, shaking at 500 rpm. Cool down to 25 °C.
4. Add 0.5 µl of freshly prepared 400 mM iodoacetamide and incubate at 25 °C for 30 min, shaking at 500 rpm and in the dark.
5. Add 0.5 µl 0.5 M *N*-acetyl-cysteine and incubate at 25 °C for 10 min, shaking at 500 rpm.
6. Membrane fraction only: add 1 µl Lys-C and incubate at 37 °C, O/N.
7. Add 2 µg trypsin and incubate O/N at 37°C, shaking at 500 rpm (wrap in Parafilm to prevent drying).
   NOTE: the samples can be stored at -20 °C at this stage.
   NOTE: membrane fraction only: add 100 mM ABC to adjust the urea concentration to <2 M before addition of trypsin.
8. Briefly spin down the tubes and collect beads with the magnet.
9. Transfer the supernatant into a new 1.5 ml tube (repeat this step if beads are left).
10. Add 5 µl 5% TFA (trifluoroacetic acid) + 1 µl 2 M HCl (15 µl 5% TFA+ 5 µl 2 M HCl for the membrane fraction).
11. Condition C18 MicroSpin columns (The Nest Group, MA, USA) with 150 µl acetonitrile (spin 20 sec at 2,400 rpm).
12. Equilibrate the C18 columns 2 times with 150 µl 0.1% TFA (spin 20 sec, 2,400 rpm).
13. Load the sample and spin 2 min, 2,000 rpm.
14. Reload the flow-through onto the column and repeat the spinning step (2 min, 2,000 rpm).
15. Wash 3 times with 150 µl 0.1% TFA, 5% acetonitrile (20 sec, 2,400 rpm).
16. Take a new tube and elute twice with 150 µl 0.1% TFA, 50% acetonitrile (2 min, 2,000 rpm).
17. Dry the peptides in a speed-vac.
18. Resuspend in 40 µl 98% H_2O, 2% acetonitrile, 0.15% formic acid.
19. Sonicate 20 sec (pulse cycle 0.5, amplitude 100%; see Materials List) and spin down 5 sec, 12,000 rpm (benchtop centrifuge). Vortex 10 sec, and spin down 5 sec, 12,000 rpm. Transfer in a HPLC vial for LC-MS/MS analysis.
20. Freeze at -20 °C.
    NOTE: the samples can be stored at -20 °C at this stage.

8. LC-MS/MS Analysis

1. Run a nano-LC (nano-LC systems) equipped with a RP-HPLC column (75 µm x 37 cm) packed with C18 resin (Magic C18 AQ 3 µm) using a linear gradient from 95% solvent A (0.15% formic acid, 2% acetonitrile) and 5% solvent B (98% acetonitrile, 0.15% formic acid) to 35% solvent B over 60 min at a flow rate of 0.2 µl/min.
2. Analyze the peptides using LC-MS/MS (dual pressure LTQ-Orbitrap Velos mass spectrometer, connected to an electrospray ion source).
   NOTE: The data acquisition mode was set to obtain one high resolution MS scan in the FT part of the mass spectrometer at a resolution of 60,000 FWHM followed by MS/MS scans in the linear ion trap of the 20 most intense ions. To increase the efficiency of MS/MS attempts, the charged state screening modus was enabled to exclude unassigned and singly charges ions. Collusion induced dissociation was triggered when the precursor exceeded 100 ion counts. The dynamic exclusion duration was set to 30 sec. The ion accumulation time was set to 300 msec (MS) and 50 msec (MS/MS).

9. Database Search

1. Download the *P. aeruginosa* NCBI-database via the NCBI homepage (http://www.ncbi.nlm.nih.gov/).
2. Convert the MS raw spectra into mascot generic files (mgf) using the MassMatrix conversion tool (http://www.massmatrix.net/mm-cgi/downloads.py).
3. Search this mgf file using MASCOT version 2.3 against the *P. aeruginosa* NCBI-database containing forward and reverse-decoy protein entries.

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4. Perform an *in silico* trypsin digestion after lysine and arginine (unless followed by proline) tolerating two missed cleavages in fully tryptic peptides.
5. Set the database search parameters to allow oxidized methionines (+15.99491 Da) as variable modifications and carboxyamidomethylation (+57.021464 Da) of cysteine residues as fixed modification. For MASCOT searches using high-resolution scans, set the precursor mass tolerance to 15 p.p.m. and set the fragment mass tolerance to 0.6 Da. Finally, set the protein FDR to 1%.
6. Import the Mascot searches of *P. aeruginosa* CCMS experiments into Scaffold (Proteomesoftware, Version 3), set the parameters to obtain a protein FDR close to 1%, and extract the total spectral counts.
7. For the representative results presented in this paper, we used a paired T test to compare the experiment with the competition control, and only considered hits with a *p* value below 0.1, and a spectral count ratio above 2 (experiment spectral counts/competition control spectral counts).
8. Data can be exported in any spreadsheet software for further analysis.

10. Label-free Quantification

1. Import the raw files into Progenesis LC-MS software (Nonlinear Dynamics, Version 4.0).
2. Perform LC-MS alignment and feature detection in default settings.
3. Export the data in mgf format from Progenesis LC-MS.
4. Search the MS/MS spectra using the MASCOT against the NCBI *P. aeruginosa* database containing forward and reverse-decoy protein entries.
5. Import the database search results into Progenesis LC-MS and map the peptides identifications to MS1 features.
6. For data evaluation (calculation of significance levels, fold-change ratios) the ProteinSQAnalysis script of SafeQuant was used (threshold: *q* value below 0.1, spectral count ratio above 2).

Representative Results

To identify novel c-di-GMP effectors in *P. aeruginosa* we systematically used CCMS to analyze the soluble and membrane fractions of *P. aeruginosa* strain PAO1 from a log phase culture (OD_{600} = 0.5). Here we summarize and discuss representative results of this fishing expedition. Four independent biological replicas were used. For each experiment two different cdG-CC concentrations were used (5 µM and 10 µM). To probe for specificity, experiments were carried out in the presence or absence of 1 mM c-di-GMP as competitor and, finally, with a bead control (*i.e.* without cdG-CC) (**Table 1**).

When following the method described in detail above (**Figure 2**) we produced a list of captured proteins in a Scaffold format. Likely contaminants were removed. This included ribosomal proteins, streptavidin, trypsin, serum albumin, keratin and other human proteins. The protein identification false discovery rate (FDR) was set to 1% by using the Scaffold software, and the data exported to excel. The accession number provided by Scaffold can be converted into locus numbers using the VLOOKUP function in Excel linked to a list of the *P. aeruginosa* locus numbers. At this stage, the hit list comprises 768 proteins for the soluble fraction and 433 proteins for the membrane fraction. However, most proteins are not significantly enriched in the capture experiment. Thus, proteins that are likely captured non-specifically (positive in the bead control or only in the presence of c-di-GMP competitor) were removed. We calculated a spectral count ratio between the capture experiment and the competition control and only considered proteins with a ratio larger than two. In addition we employed a paired t-test on spectral counts to provide a significance measure between the capture experiment and the competition control, and set a permissive threshold of 0.1. Lastly, we considered only robust hits with at least four peptides identified in the four experiments for the 2 capture compound concentrations taken altogether. These criteria should be adjusted according to the needs and using the verified and predicted c-di-GMP binding proteins as standard to set the threshold. After sorting, the list was decreased to 76 hits for the soluble fraction, and 133 proteins for the membrane fraction. This included 13 soluble and 21 membrane proteins from *P. aeruginosa* that are known or predicted to bind c-di-GMP (**Table 2**). The other 63 soluble and 112 membrane proteins are new putative c-di-GMP binding proteins that do not contain one of the known c-di-GMP binding domains. These hits have now to be validated by testing their specific binding to c-di-GMP.

In a previous screen we fished GlyA2 (PA2444), GlyA3 (PA4602) and Gsp69 (PA1127)[1]. These 3 proteins were cloned, overexpressed, and purified from *E. coli* and could be validated to bind c-di-GMP in UV-cross linking experiments using ^{33}P labeled c-di-GMP[15]. The K_ds were determined to 1.0, 2.0 and 6.9 µM respectively, indicating that indeed novel effectors can be identified by using CCMS.

In addition to this representative example, we used CCMS with extracts of cells harvested from different growth conditions and with various intracellular c-di-GMP concentrations (n = 24). Overall we captured 74% (38/51) of the known or predicted *P. aeruginosa* PAO1 c-di-GMP signaling components (24/32 soluble proteins, 14/19 membrane proteins). Given that at least nine of these genes were shown to be transcribed under specific conditions (oxidative stress, quorum sensing, biofilms)[16] and that some may not bind c-di-GMP at all, this degree of coverage might be close to saturation. This together with the observation that most of these components were captured with high specificity (**Table 2**) strongly argues that this technique is effective and powerful.

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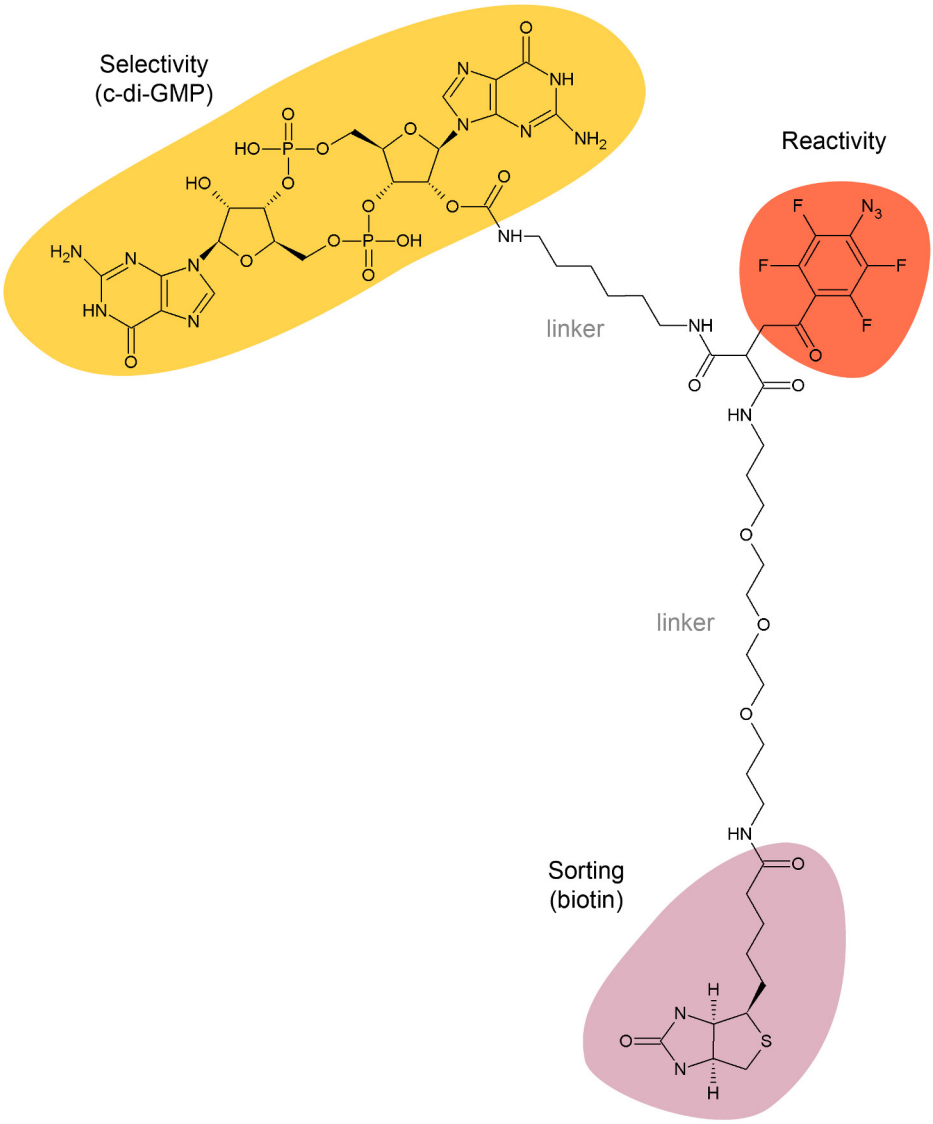

Figure 1: Chemical structure of the c-di-GMP Capture Compound.

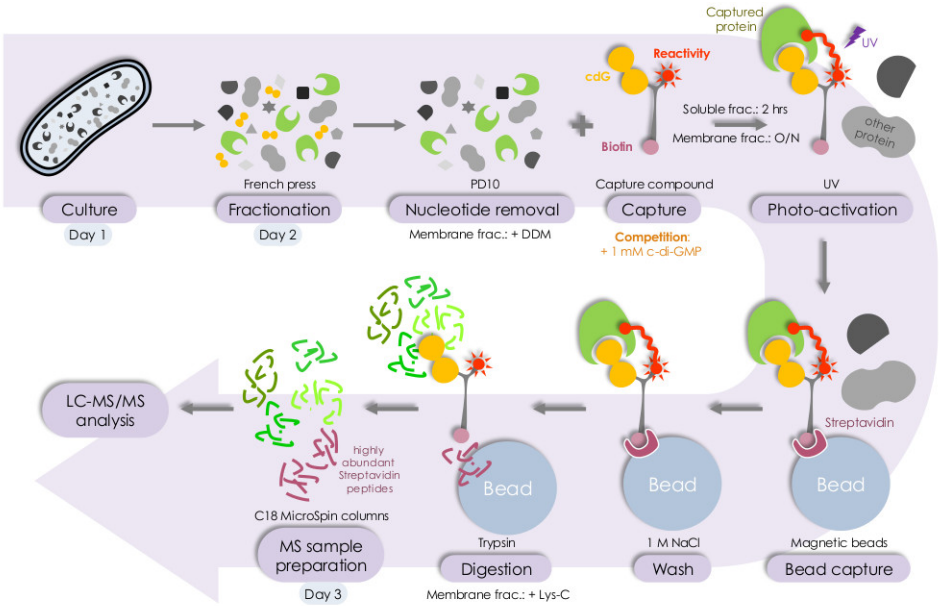

Figure 2: CCMS workflow summary. After mechanical lysis the free nucleotides are removed using a PD10 exclusion column. Proteins from the soluble or membrane fractions are incubated with the cdG-CC and the mixture is exposed to UV irradiation to cross-link captured proteins. Steps of harsh washing are carried with compounds bound to streptavidin coated magnetic beads. On-bead tryptic digestion provides peptides, which are then separated from the beads and protonated for their mass spectrometry identification. Please click here to view a larger version of the figure.

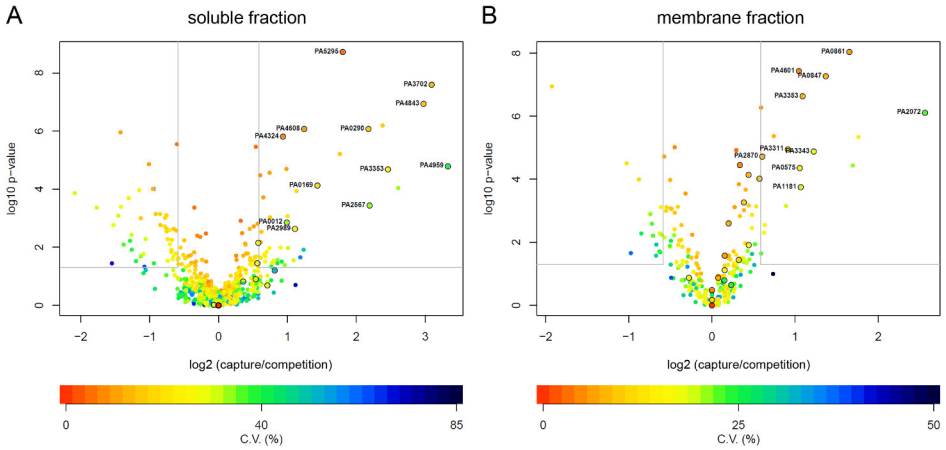

Figure 3: Volcanoplots of *P. aeruginosa* proteins significantly enriched by CCMS. Following LC-MS/MS analysis and label-free quantification, proteins were sorted as described in the text. Log2-intensity ratio of detected peptide between the capture and competition experiments were calculated and plotted versus values derived from significance analysis (modified t-statistic, empirical Bayes method[17]). Proteins within the significance thresholds for p-values <0.05 and intensity ratios >1.5-fold are indicated in a grey box. The 4 replicates for the soluble fraction (**A**) and the membrane fraction (**B**) were performed in the presence of 10 µM c-di-GMP-CC, and the competition experiment with 1 mM c-di-GMP. The circled dots correspond to known c-di-GMP binding proteins. Please click here to view a larger version of the figure.

Capture:	Buffer	Chemical	Source	Concentration
	Bacterial Lysis Buffer 10x	MES	Sigma	67 mM
	pH 7.5	HEPES	Sigma	67 mM
		NaCl	Merck	2 M
		Na Acetate	Merck	67 mM
		DTT	Fluka	10 mM
		DNaseI	Roche	20 U/ml

		Complete Protease Inhibitor Cocktail	Roche	1 tab / 10 ml
	Capture Buffer 5x	HEPES	Sigma	100 mM
		KAc	Sigma	250 mM
		MgAc (anhydrous)	Sigma	50 mM
		Glycerol	Sigma	50% (V/V)
		GDP, GTP, ATP, CTP	sigma	1 mM each
	Wash buffer 5x	Tris-HCl	Merck	1 M
	(for the soluble fraction)	EDTA	Sigma	0.5 M
	pH 7.5	NaCl	Sigma	5 M
		n-octyl-β-D-glucopyranoside	Anagrade (Affymetrix)	42.5 µM
	Wash buffer 5x	Tris-HCl	Merck	1 M
	(for the membrane fraction)	EDTA	Sigma	0.5 M
	pH 7.5	NaCl	Sigma	5 M
Other chemicals:		Ammonium Bicarbonate (ABC)	Fluka	
		Urea	Applichem	
MS sample preparation:	**Buffer**	**Chemical**	**Source**	**Concentration**
	C18 Buffer A	TFA	Pierce	0.1% (V/V)
		H_2O HPLC grade		99.9% (V/V)
	C18 Buffer B	TFA	Pierce	0.1% (V/V)
		Acetonitrile	Biosolve	49.9% (V/V)
		H_2O HPLC grade		50% (V/V)
	C18 Buffer C	TFA	Pierce	0.1% (V/V)
		Acetonitrile	Biosolve	5% (V/V)
		H_2O HPLC grade		94.9% (V/V)
	LC Buffer A	Formic acid	Sigma	0.15% (V/V)
		Acetonitrile	Biosolve	2%
		H_2O HPLC grade		97.85%
Other chemicals:		tris(2-carboxyethyl)phosphine (TCEP)	Sigma	
		iodoacetamide (IAA)	Sigma	
		N-acetyl-cysteine	Sigma	
		Endoproteinase Lys-C	Wako	
		Trypsin	Promega	

Table 1: Buffers composition. Summary of the buffers composition, chemicals and suppliers. The c-di-GMP-capture compound, c-di-GMP (for the competition control), streptavidin coated magnetic beads, capture buffer, and washing buffer are included in the caproKit.

	Bead control	**Capture experiment**	**Competition control**
Protein extract (10 mg/ml)	30 µl	30 µl	30 µl
c-di-GMP (10 mM)	0 µl	0 µl	10 µl
Nucleotides (10 mM of each nucleotide)	10 µl	10 µl	10 µl
Capture buffer 5x	20 µl	20 µl	20 µl
H_2O	42 µl	32 µl	22 µl
30 min incubation			

c-di-GMP~CC (stock 100 µM)	0 µl	5-10 µl	5-10 µl
Final concentrations:	**Bead control**	**Capture experiment**	**Competition control**
c-di-GMP~CC (µM)	0 µM	5-10 µM	5-10 µM
Competitor c-di-GMP (µM)	0 µM	0 µM	1,000 µM

Table 2: Capture reaction mix. Summary of the reaction mix for the bead control (*i.e.* without capture compound), the capture experiment (with the c-di-GMP-CC), and the competition control which contains a large excess of c-di-GMP. The c-di-GMP-CC final concentration can be adjusted, and is typically set between 5 and 10 µM.

Protein name	**Locus ID**	**Domain architecture**	**capture experiment / competition experiment** [1]							
a) Soluble fraction			cdG-CC = 5 µM				cdG-CC = 10 µM			
-	PA4843	REC-REC-GGEEF*	14/0	14/0	13/0	11/0	14/0	12/0	14/0	14/0
WspR	PA3702	REC-GGEEF*	9/0	9/0	10/0	9/0	11/0	10/0	11/0	11/0
-	PA2567	GAF-SPTRF-EAL	8/0	4/0	9/0	0/0	7/0	3/0	8/0	8/0
-	PA3353	PilZ	11/0	12/0	13/0	12/0	12/0	10/0	11/0	12/0
-	PA0290	PAS-GGDEF	5/0	3/0	6/0	5/0	8/0	5/0	6/0	6/0
-	PA5295	GDDEF-EAL	3/0	3/0	3/0	1/0	6/0	6/0	5/0	4/0
FimX	PA4959	PAS-GDSIF-EVL	23/1	21/0	21/0	11/0	24/3	23/2	22/0	20/0
-	PA4608	PilZ	3/0	3/0	3/0	0/0	3/0	2/0	3/0	3/0
-	PA0012	PilZ	3/0	2/0	2/0	2/0	2/0	2/0	4/0	2/0
-	PA2989	PilZ	1/0	1/0	2/0	1/0	1/0	2/0	3/0	3/0
-	PA4324	PilZ	2/0	2/0	1/0	1/0	2/0	2/0	1/0	2/0
-	PA3177	GGEEF	2/0	1/0	3/0	0/0	1/0	1/0	3/0	1/0
-	PA4396	REC-DEQHF	0/0	1/0	4/0	0/0	1/0	0/0	5/0	1/0
-	PA0169	GGEEF*	3/0	2/0	6/0	7/0	7/1	6/2	9/1	7/1
-	PA2799	PilZ	1/0	0/0	2/0	0/0	0/0	0/0	3/0	1/0
-	PA5017	PAS-GAF-PAS-ASNEF-EAL	1/0	2/0	1/0	0/0	1/0	3/2	0/0	0/0
-	PA5487	GGEEF*	0/0	0/0	0/0	1/0	1/0	0/0	1/0	1/0
b) Membrane fraction			cdG-CC = 5 µM				cdG-CC = 10 µM			
-	PA2072	CHASE4-TM-PAS-GGDEF-EAL	13/1	25/0	27/0	19/0	36/0	36/0	31/0	23/0
-	PA0861	TM-PAS-GGDEF-ELL	6/1	14/0	13/0	10/0	17/0	18/0	13/0	8/0
-	PA3353	PilZ	6/0	10/0	9/0	7/0	10/0	10/0	6/0	5/0
-	PA3343	5TM-GGDEF	3/0	7/0	7/0	4/0	12/0	10/0	7/0	7/0
-	PA1181	MASE1-PAS-PAS-PAS-PAS-	3/0	6/0	9/0	3/0	12/0	12/0	5/0	2/0

		GGDEF-ELL								
-	PA0847	TM-CHASE4-HAMP-PAS-GGDEF	0/0	4/0	4/0	1/0	15/0	13/0	8/0	6/0
-	PA0575	PBPb-TM-PAS-PAS-PAS-PAS-GGDEF-EAL	1/0	7/0	6/0	3/0	10/0	10/0	6/0	2/0
yfiN	PA1120	2TM-HAMP-GGDEF	2/0	4/0	3/0	3/0	5/0	4/0	3/0	1/0
-	PA0290	PAS-GGDEF	1/0	4/0	3/0	2/0	1/0	5/0	1/0	2/0
-	PA4929	7TMR:DISMED2-GGDEF	2/0	3/0	2/0	2/0	2/0	2/0	2/0	1/0
morA	PA4601	TM-TM-PAS-PAS-PAS-PAS-GGDEF-EAL	3/0	7/0	7/3	5/0	9/0	10/0	5/0	4/0
-	PA1851	5TM-GGDEF	1/0	2/0	1/0	2/0	4/0	3/0	1/0	1/0
-	PA2870	TM-GGDEF	0/0	0/0	1/0	0/0	4/0	4/0	4/0	2/0
-	PA3311	TM-MHYT-MHYT-MHYT-AGDEF-EAL	1/1	5/0	7/1	4/0	8/0	8/0	5/1	3/0
bifA	PA4367	TM-GGDQF-EAL	1/0	2/0	1/0	2/0	1/0	2/0	2/1	1/0
-	PA4608	PilZ	0/0	1/0	1/0	0/0	3/0	3/0	2/0	2/0
-	PA4332	5TM-GGEEF	1/0	3/0	2/0	1/0	1/0	1/0	2/0	0/0
-	PA0012	PilZ	1/0	1/0	1/0	1/0	1/0	1/0	1/0	0/0
-	PA2989	PilZ	4/0	8/0	7/0	7/0	5/2	11/2	7/2	8/3
-	PA1433	HAMP-RGGEF-KVL	0/0	0/0	0/0	0/0	1/0	1/0	1/0	1/0
-	PA4843	REC-REC-GGEEF	0/0	0/0	1/1	1/0	0/0	1/0	1/0	0/0

* = GGDEF domain containing an I site

[1] number of spectral counts of identified peptides

Table 3: *P. aeruginosa* known c-di-GMP signaling components specifically captured. Identified proteins were first sorted as described in the text. Proteins are identified with to their name and locus number, and we indicate their architecture predicted with the NCBI Conserved Domain Database online tool (n = 4, cdG-CC = 5 µM or 10 µM) in order to show the capture specificity and the reproducibility of the method.

Discussion

Special care should be taken at several steps of the protocol. The protein concentration is a critical parameter with a concentration of 10 mg/ml being difficult to reach when cells are grown under specific growth conditions (*e.g.* biofilms or small colony variants). Thus, the pellet resuspension should be performed in a low volume of lysis buffer. Protein concentrations can be decreased to 8 mg/ml. Compared to the method published by Nesper *et al.*[1], we added various nucleotides to the capture reaction to minimize non-specific capturing of nucleotide binding

proteins. Although the addition of nucleotides improved the specificity, it may at the same time prevent the capture of proteins that bind different nucleotides at the same site. For example the effector FleQ, which was recently shown to bind ATP and c-di-GMP[18] was fished specifically in the absence of ATP in our previous experiment[1], but not anymore in the data presented here in the presence of an excess of ATP.

The cdG-CC should be carefully protected from light. Although ambient light contains only a small fraction of UV, it is recommended to keep the capture compound stock wrapped in aluminum foil, as well as the capture mix prior to activation by UV irradiation. The washing steps that follow can be very stringent to increase the specificity, as the captured proteins are covalently bound to the cdG-CC. Regarding the LC-MS/MS analysis, the experiments should be carried in a clean keratin free environment. Moreover, HPLC compatible buffers should be used, especially after the washing steps. The candidate list typically comprises between 300 and 800 proteins (for a quadruplicate), with low variations between the replicates (see **Table 2** as an example).

Some parameters like the protein and the cdG-CC concentration might need to be optimized depending on the organisms analyzed. Since low abundant proteins or proteins expressed only under specific conditions can easily be missed, care should be taken regarding the culture conditions employed. This problem can be overcome by comparing the hit list with a global protein ATLAS collected for the same culture conditions. Finally, the optimization of the detergent can be challenging, as it needs to be optimized with respect to its ability to solubilize membrane proteins and also needs to be MS compatible.

One needs to keep in mind that the c-di-GMP molecule of the CC is chemically modified, as it is linked via the 2'OH group of one ribose to the rest of the scaffold. This modification could alter its ability to bind to some effectors thereby providing false-negatives. In this context it is noteworthy that we never captured proteins that harbor an EAL domain but lack a GGDEF domain in *P. aeruginosa*, although EAL proteins were captured in other species, like *Caulobacter crescentus*. This could be due to a poor access or a low affinity of the cdG-CC to the binding site, or to the degradation of the cdG-CC by EAL proteins. In contrast, many nucleotide-binding proteins were captured with a relatively low specificity and, to a large degree, are probably false positives. Further validation of the specific binding to c-di-GMP using techniques such as DRaCALA[20], UV cross-linking[15], differential scanning fluorimetry (DSF)[21], Microscale thermophoresis (MST)[22], isothermal calorimetry (ITC)[23-24]… is thus necessary.

It is also possible that a fraction of the c-di-GMP moiety of the capture compound is degraded by phosphodiesterases from the cell lysate. This is one of the reasons why the procedure has to be carried out at 4 °C, therefore limiting the phosphodiesterases activity before cross-linking.

The procedure can be adapted to many bacterial species, and has been successfully used for 3 different bacterial species with very minor modifications[1]. Capture Compound based technology can reduce false-positives by using thorough washing (*e.g.* 1 M salt, high detergent concentration, 2 M urea in case of membrane proteins, 80% acetonitrile), as compared to other techniques that do not rely on covalent binding. Given that validation of candidates can be a tedious and time-consuming process, this is a major advantage to alternative methods like chemical proteomics based approaches[14].

This illustrated video method establishes CCMS as a powerful and versatile tool to identify and characterize novel components involved in small molecule signaling. In the future, similar Capture Compounds harboring other selectivity groups could be used to capture proteins involved in small molecule signaling, such as the novel c-di-AMP effectors.

Disclosures

The authors have nothing to disclose.

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