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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 (phosphodiersterases) 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 phosphodiersterases 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 organisms1. 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

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TITLE:
Capture Compound Mass Spectrometry - a powerful tool to identify novel c-di-GMP effector proteins

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SHORT ABSTRACT:
The ubiquitous second messenger c-di-GMP controls growth and behavior of many bacteria. We have developed a novel Capture Compound Mass Spectrometry based technology to biochemically identify and characterize c-di-GMP binding proteins in virtually any bacterial species.

LONG 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 (phosphodiersterases) 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 phosphodiersterases 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.

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 phosphodiersterases (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-photoactivable 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].

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

1.1. Grow *P. aeruginosa* cells in LB to the desired OD.

Note: For guidance: use ≈ 100mL culture / sample for stationary phase cultures and ≈ 500mL culture / sample for log phase cultures (OD_{600nm} = 0.5)

1.2. Pellet by centrifugation for 20 minutes at 5000 x *g.*

1.3. Resuspend 0.5 - 1g of pellet in 1 mL lysis buffer (6.7 mM MES, 6.7 mM HEPES, 200 mM NaCl, 6.7 mM KAc, DDT 1mM, pH 7.5) and add protease inhibitor (complete mini, EDTA-free) as well as DNaseI.

1.4. Lyse the cells by 3 passages through a French pressure cell, at 20,000 psi (see Materials Table).

1.5. Ultra-centrifuge the cell lysate at 100,000 x *g* for 1hr at 4°C.

1.6. Save the supernatant (go to step 2).

1.7. Wash the pellet with 1 mL 1 x lysis buffer by pipetting up and down

1.8. Ultracentrifuge at 100,000 x *g* for 1hr at 4°C

1.9. 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)

2.1. Wash a PD10 desalting column (see Materials Table) with 10mL of cold lysis buffer.

2.2. Pour the supernatant (≈ 1 mL) onto the PD10 in order to remove nucleotides.

2.3. Elute with 4 mL cold lysis buffer (500 µL steps).

2.4. Select the most concentrated fractions as determined by Bradford assay, and pool them.

3. Pellet resuspension and solubilization (membrane fraction only)

3.1. Resuspend the pellet in 500 to 1 000 µL of 1 x 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 27G to well homogenize the solution.

3.2. Add 1% (w/v) n-dodecyl-β-D-maltopyranoside (DDM).

3.3. Incubate at 4°C for at least 2 h (or O/N) on a rotating wheel.

3.4. Ultracentrifuge at 100 000 x *g* for 1hr at 4°C.

3.5. Harvest the supernatant.

4. Protein concentration measurement

4.1. Measure the protein concentration by Bradford assay (by BCA assay for the membrane fraction)

4.2. Set the total protein concentration to 10 mg/mL.

5. Capture

5.1. Mix 300 µg of protein with 1 mM of GDP, GTP, ATP, CTP, and 20 µL of 5 x 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 8M Urea (Step 7.1).

5.2. Incubate at 4°C for 30 min on a rotating wheel.

5.3. 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.

5.4. Incubate at 4°C for at least 2 h (O/N for the membrane fraction) on a rotating wheel, in the dark.

5.5. 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 table, λ = 310 nm, Irradiance ≥ 10 mW/cm², distance from the source = 2 cm). Note: remove the lid of the strips prior cross-linking.

5.6. 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.

5.7. Incubate at 4°C for 1 h on a rotating wheel.

6. Washing steps

Note: all the washing steps are performed thanks to a neodymium magnet (caproMag, see Materials Table). 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.

6.1. Washing steps (soluble fraction only)

6.1.1 Wash 6 times in 200 µL 1 x washing buffer. Wash once in 200 µL HPLC grade H_2O.

6.1.2 Wash 6 times in 200 µL 80% acetonitrile. Wash 2 times in 200 µL HPLC grade H_2O.

6.2. Washing steps (membrane fraction only)

6.2.1 Wash 5 times in 200 µL 1 x washing buffer + 0.1% DDM. Wash 2 times in 200 µL 1 x washing buffer + 0.05% DDM

6.2.3 Wash once in 200 µL 1 x washing buffer + 0.025% DDM. Wash once in 200 µL 1 x washing buffer + 0.0125% DDM. Wash 3 times in 200 µL 100 mM ABC (ammonium bicarbonate, NH_4CO_3) + 2 M Urea

7. MS sample preparation

7.1. 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.

7.2. Membrane fraction only: incubate at 60°C for 5 min, shaking at 500 rpm

7.3. Add 0.5 µL 200 mM TCEP (*tris*(2-carboxyethyl)phosphine) and incubate at 60°C for 1 h, shaking at 500 rpm. Cool down to 25°C.

7.4. Add 0.5 µL of freshly prepared 400 mM iodacetamide and incubate at 25°C for 30 min, shaking at 500 rpm and in the dark.

7.5. Add 0.5 µL 0.5 M N-acetyl-cysteine and incubate at 25°C for 10 min, shaking at 500 rpm.

7.6. Membrane fraction only: add 1 µL Lys-C and incubate at 37°C, O/N

7.7. 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.

7.8. Briefly spin down the tubes and collect beads with the magnet.

7.9. Transfer the supernatant into a new 1.5mL tube (repeat this step if beads are left).

7.10. 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).

7.11. Condition C18 MicroSpin columns (The Nest Group, MA USA) with 150 µL acetonitrile (spin 20 sec at 2400 rpm).

7.12. Equilibrate the C18 columns 2 times with 150 µL 0.1% TFA (spin 20 sec, 2400 rpm).

7.13. Load the sample and spin 2 min, 2000 rpm.

7.14. Reload the flowthrough onto the column and repeat the spinning step (2 min, 2000 rpm).

7.15. Wash 3 times with 150 µL 0.1% TFA, 5% acetonitrile (20 sec, 2400 rpm).

7.16. Take a new tube and elute twice with 150 µL 0.1% TFA, 50% acetonitrile (2 min, 2000 rpm).

7.17. Dry the peptides in a speed-vac.

7.18. Resuspend in 40 µL 98% H_2O, 2% acetonitrile, 0.15% formic acid.

7.19. Sonicate 20 sec (pulse cycle 0.5, amplitude 100%; see Materials Table) 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.

7.20. Freeze at -20°C.
Note: the samples can be stored at -20°C at this stage.

8. LC-MS/MS analysis

8.1 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 minutes at a flow rate of 0.2 µL/min.

8.2 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 30s. The ion accumulation time was set to 300ms (MS) and 50ms (MS/MS).

9. Database search

9.1 Download the *P. aeruginosa* NCBI-database via the NCBI homepage (http://www.ncbi.nlm.nih.gov/).

9.2 Convert the MS raw spectra into mascot generic files (mgf) using the MassMatrix conversion tool (http://www.massmatrix.net/mm-cgi/downloads.py)

9.3 Search this mgf file using MASCOT version 2.3 against the *P. aeruginosa* NCBI-database containing forward and reverse-decoy protein entries.

9.4 Perform an *in silico* trypsin digestion after lysine and arginine (unless followed by proline) tolerating two missed cleavages in fully tryptic peptides.

9.5 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%.

9.6 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.

9.7 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).

9.8 Data can be exported in any spreadsheet software for further analysis.

10. Label-free quantification

10.1 Import the raw files into Progenesis LC-MS software (Nonlinear Dynamics, Version 4.0).

10.2 Perform LC-MS alignment and feature detection in default settings.

10.3 Export the data in mgf format from Progenesis LC-MS.

10.4 Search the MS/MS spectra using the MASCOT against the NCBI *P. aeruginosa* database containing forward and reverse-decoy protein entries.

10.5 Import the database search results into Progenesis LC-MS and map the peptides identifications to MS1 features.

10.6 For data evaluation (calculation of significance levels, fold-change ratios) the ProteinSQAnalysis script of SafeQuant was used (https://github.com/eahrne/SafeQuant). Use the same threshold than previously: *q* value below 0.01, and a 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 which 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 labelled 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.

FIGURE LEGENDS:

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.

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.

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.

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.

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 (http://www.ncbi.nlm.nih.gov/Structure/cdd/docs/cdd_search.html). Spectral counts are indicated for each experiment (*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 phosphodiersterases from the cell lysate. This is one of the reasons why the procedure has to be carried out at 4°C, therefore limiting the phosphodiersterases 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.

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DISCLOSURES:

The authors have nothing to disclose.

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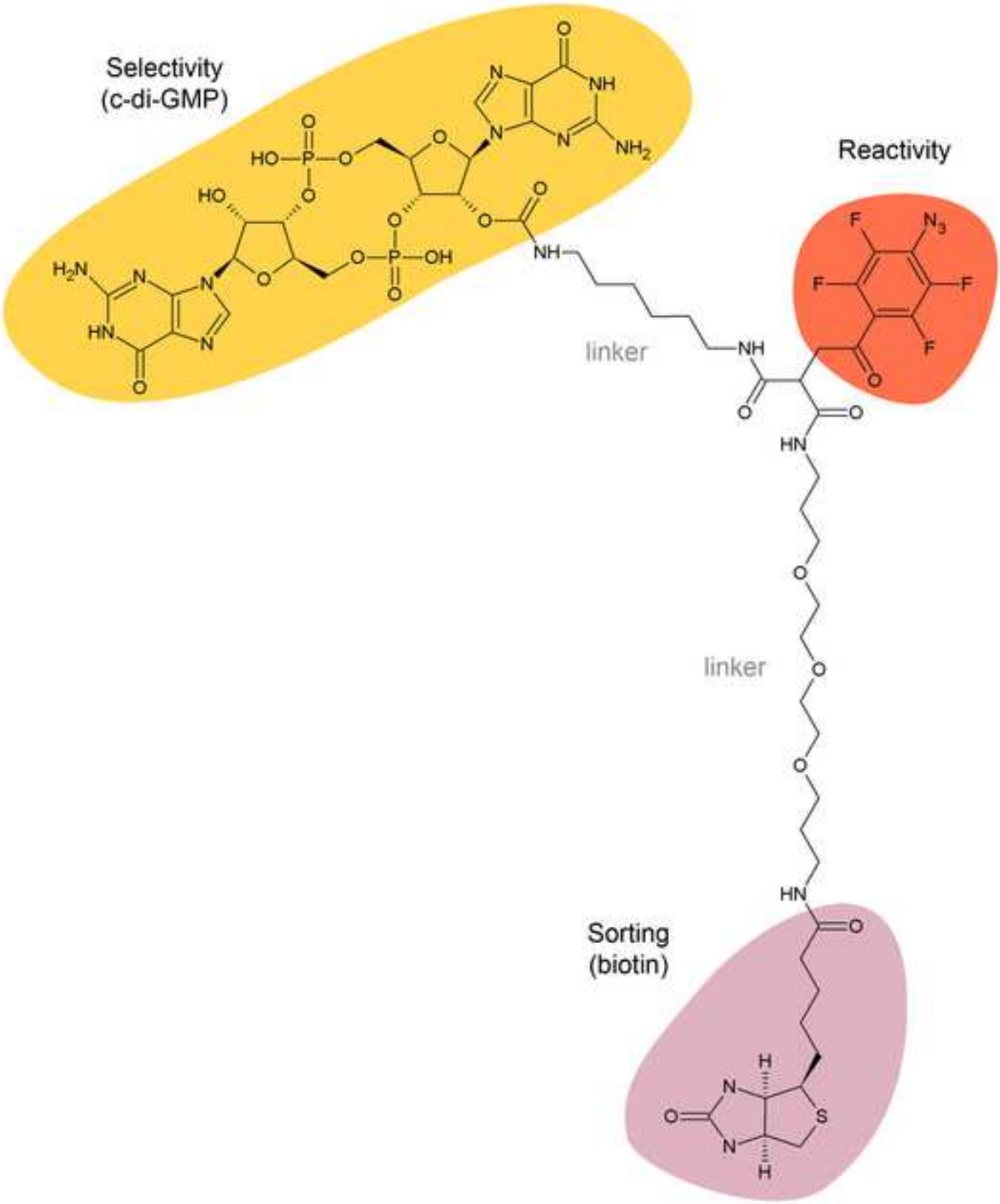

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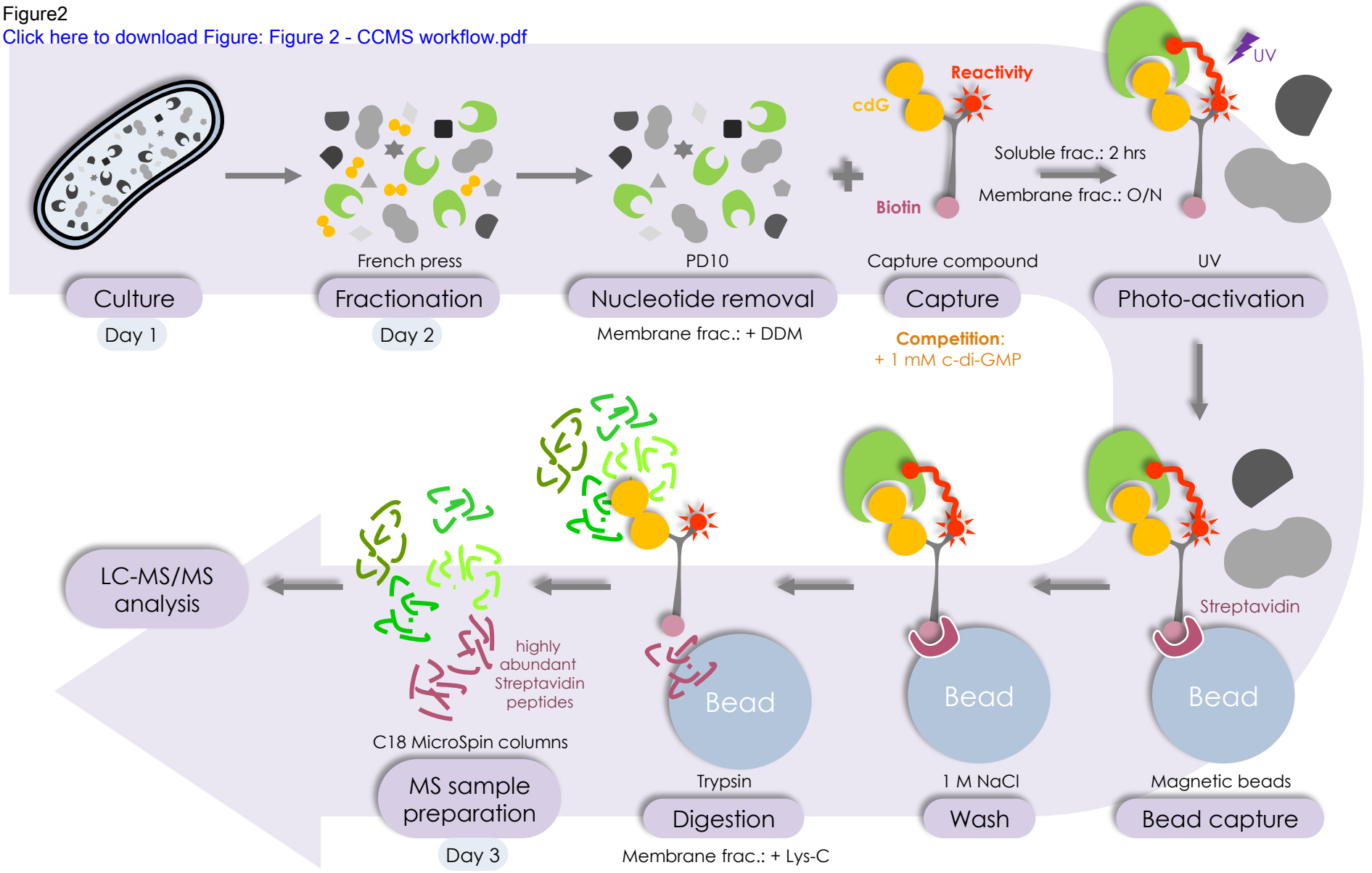

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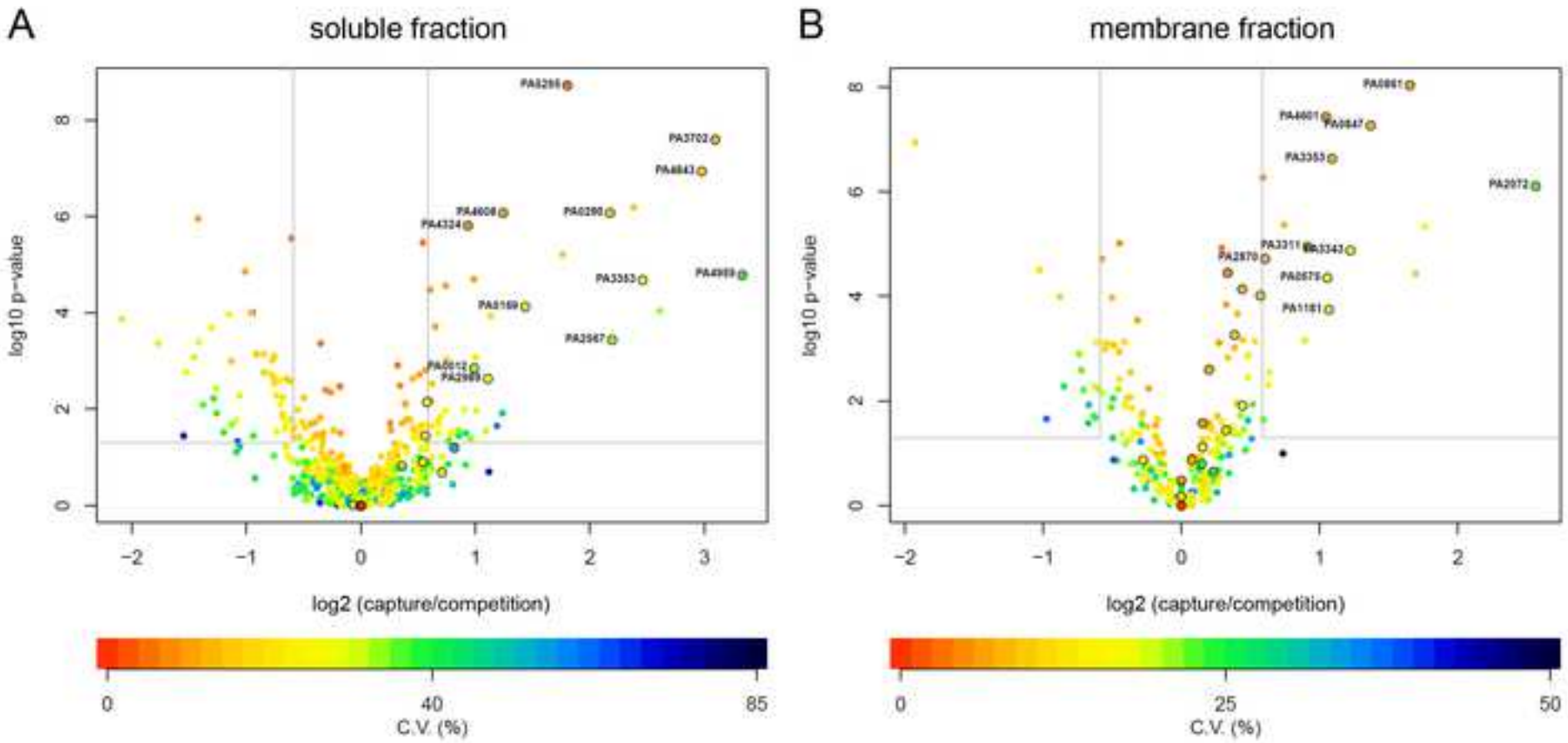

Table1

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	Buffer	Chemical
Capture:	**Bacterial Lysis Buffer 10X**	MES
	pH7.5	HEPES
		NaCl
		Na Acetate
		DTT
		DNaseI
		Complete Protease Inhibitor Cocktail
	Capture Buffer 5X	HEPES
		KAc
		MgAc (anhydrous)
		Glycerol
		GDP, GTP, ATP, CTP
	Wash buffer 5X	Tris-HCl
	(for the soluble fraction)	EDTA
	pH7.5	NaCl
		n-octyl-β-D-glucopyranoside
	Wash buffer 5X	Tris-HCl
	(for the membrane fraction)	EDTA
	pH7.5	NaCl
Other chemicals:		Ammonium Bicarbonate (ABC)
		Urea

	Buffer	Chemical
MS sample preparation:	**C18 Buffer A**	TFA
		H2O HPLC grade
	C18 Buffer B	TFA
		Acetonitrile
		H2O HPLC grade
	C18 Buffer C	TFA
		Acetonitrile
		H2O HPLC grade
	LC Buffer A	Formic acid
		Acetonitrile
		H2O HPLC grade
Other chemicals:		tris(2-carboxyethyl)phosphine (TCEP)
		iodacetamide (IAA)
		N-acetyl-cysteine
		Endoproteinase Lys-C
		Trypsin

Source	Concentration
Sigma	67 mM
Sigma	67 mM
Merck	2 M
Merck	67 mM
Fluka	10 mM
Roche	20 U / mL
Roche	1 tab / 10 mL
Sigma	100 mM
Sigma	250 mM
Sigma	50 mM
Sigma	50% (V/V)
sigma	1 mM each
Merck	1 M
Sigma	0.5M
Sigma	5 M
Anagrade (Affymetrix)	42.5 µM
Merck	1 M
Sigma	0.5M
Sigma	5 M
Fluka	
Applichem	

Source	Concentration
Pierce	0.1% (V/V)
	99.9% (V/V)
Pierce	0.1% (V/V)
Biosolve	49.9% (V/V)
	50% (V/V)
Pierce	0.1% (V/V)
Biosolve	5% (V/V)
	94.9% (V/V)
Sigma	0.15% (V/V)
Biosolve	2%
	97.85%
Sigma	
Sigma	
Sigma	
Wako	
Promega	

Table2

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	Bead control	***Capture experiment***
Protein extract (10 mg/mL)	30 µL	30 µL
c-di-GMP (10 mM)	0 µL	0 µL
Nucleotides (10 mM of each nucleotide)	10 µL	10 µL
Capture buffer 5x	20 µL	20 µL
H_2O	42 µL	32 µL
	30 min incubation	
c-di-GMP~CC (stock 100 µM)	0 µL	5-10 µL

Final concentrations:	***Bead control***	***Capture experiment***
c-di-GMP~CC (µM)	0 µM	5-10 µM
Competitor c-di-GMP (µM)	0 µM	0 µM

Competition control
30 µL
10 µL
10 µL
20 µL
22 µL

5-10 µL

Competition control
5-10 µM
1000 µM

Tablel3

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Protein name	Locus ID	Domain architecture	capture experiment / competit				
a) Soluble fraction			cdG-CC = 5 µM				c
-	PA4843	REC-REC-GGEEF*	14/0	14/0	13/0	11/0	14/0
WspR	PA3702	REC-GGEEF*	9/0	9/0	10/0	9/0	11/0
-	PA2567	GAF-SPTRF-EAL	8/0	4/0	9/0	0/0	7/0
-	PA3353	PilZ	11/0	12/0	13/0	12/0	12/0
-	PA0290	PAS-GGDEF	5/0	3/0	6/0	5/0	8/0
-	PA5295	GDDEF-EAL	3/0	3/0	3/0	1/0	6/0
FimX	PA4959	PAS-GDSIF-EVL	23/1	21/0	21/0	11/0	24/3
-	PA4608	PilZ	3/0	3/0	3/0	0/0	3/0
-	PA0012	PilZ	3/0	2/0	2/0	2/0	2/0
-	PA2989	PilZ	1/0	1/0	2/0	1/0	1/0
-	PA4324	PilZ	2/0	2/0	1/0	1/0	2/0
-	PA3177	GGEEF	2/0	1/0	3/0	0/0	1/0
-	PA4396	REC-DEQHF	0/0	1/0	4/0	0/0	1/0
-	PA0169	GGEEF*	3/0	2/0	6/0	7/0	7/1
-	PA2799	PilZ	1/0	0/0	2/0	0/0	0/0
-	PA5017	PAS-GAF-PAS-ASNEF-EAL	1/0	2/0	1/0	0/0	1/0
-	PA5487	GGEEF*	0/0	0/0	0/0	1/0	1/0
b) Membrane fraction			cdG-CC = 5 µM				c
-	PA2072	CHASE4-TM-PAS-GGDEF-EAL	13/1	25/0	27/0	19/0	36/0
-	PA0861	TM-PAS-GGDEF-ELL	6/1	14/0	13/0	10/0	17/0
-	PA3353	PilZ	6/0	10/0	9/0	7/0	10/0
-	PA3343	5TM-GGDEF	3/0	7/0	7/0	4/0	12/0
-	PA1181	MASE1-PAS-PAS-PAS-PAS-GGDEF-ELL	3/0	6/0	9/0	3/0	12/0
-	PA0847	TM-CHASE4-HAMP-PAS-GGDEF	0/0	4/0	4/0	1/0	15/0
-	PA0575	PBPb-TM-PAS-PAS-PAS-PAS-GGDEF-EAL	1/0	7/0	6/0	3/0	10/0
yfiN	PA1120	2TM-HAMP-GGDEF	2/0	4/0	3/0	3/0	5/0
-	PA0290	PAS-GGDEF	1/0	4/0	3/0	2/0	1/0
-	PA4929	7TMR:DISMED2-7TMR:DISMED2-GGDEF	2/0	4/0	2/0	2/0	2/0
morA	PA4601	TM-TM-PAS-PAS-PAS-PAS-GGDEF-EAL	3/0	7/0	7/3	5/0	9/0
-	PA1851	5TM-GGDEF	1/0	2/0	1/0	2/0	4/0
-	PA2870	TM-GGDEF	0/0	0/0	1/0	0/0	4/0
-	PA3311	TM-MHYT-MHYT-MHYT-AGDEF-EAL	1/1	5/0	7/1	4/0	8/0
bifA	PA4367	TM-GGDQF-EAL	1/0	2/0	1/0	2/0	1/0
-	PA4608	PilZ	0/0	1/0	1/0	0/0	3/0
-	PA4332	5TM-GGEEF	1/0	3/0	2/0	1/0	1/0
-	PA0012	PilZ	1/0	1/0	1/0	1/0	1/0
-	PA2989	PilZ	4/0	8/0	7/0	7/0	5/2
-	PA1433	HAMP-RGGEF-KVL	0/0	0/0	0/0	0/0	1/0
-	PA4843	REC-REC-GGEEF	0/0	0/0	1/1	1/0	0/0

* = GGDEF domain containing an I site

[1] number of spectral counts of identified peptides

ion experiment [1]

:dG-CC = 10 µM		
12/0	14/0	14/0
10/0	11/0	11/0
3/0	8/0	8/0
10/0	11/0	12/0
5/0	6/0	6/0
6/0	5/0	4/0
23/2	22/0	20/0
2/0	3/0	3/0
2/0	4/0	2/0
2/0	3/0	3/0
2/0	1/0	2/0
1/0	3/0	1/0
0/0	5/0	1/0
6/2	9/1	7/1
0/0	3/0	1/0
3/2	0/0	0/0
0/0	1/0	1/0
:dG-CC = 10 µM		
36/0	31/0	23/0
18/0	13/0	8/0
10/0	6/0	5/0
10/0	7/0	7/0
12/0	5/0	2/0
13/0	8/0	6/0
10/0	6/0	2/0
4/0	3/0	1/0
5/0	1/0	2/0
2/0	2/0	1/0
10/0	5/0	4/0
3/0	1/0	1/0
4/0	4/0	2/0
8/0	5/1	3/0
2/0	2/1	1/0
3/0	2/0	2/0
1/0	2/0	0/0
1/0	1/0	0/0
11/2	7/2	8/3
1/0	1/0	1/0
1/0	1/0	0/0

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number
caproBox	caprotec bioanalytics	1-5010-001 (220 V)
caproMag	caprotec bioanalytics	included in the CCMS Starter Kit
c-di-GMP caproKit	caprotec bioanalytics	upon request
Disposable PD-10 Desalting Columns	GE Healthcare	17-0851-01
12-tube PCR strips	Thermo Scientific	AB-1114
UIS250v sonicator with VialTweeter	Hielscher ultrasound technology	UIS250v and VialTweeter
Miniature French Pressure Cell	Thermo Electron Corporation	FA-003

Comments/Description
UV lamps coupled to a cooling 96-plate cooling block, for the photoactivation
For easy handling of magnetic particles without pipetting
The kit contains the c-di-GMP-capture compound, c-di-GMP (for the competition control), streptavidin coated magnetic beads, capture buffer, and washing buffer

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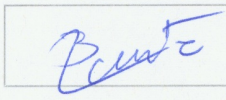
Signature:  Date: 11/07/2013

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Reviewers' comments:

Reviewer #1:

This JoVE manuscript from Urs Jenal's group is a timely description of a Capture Compound Mass Spectrometry (CCMS) method to identify proteins that bind to c-di-GMP. This is a very useful method for the field in helping to better understand these complex systems. I also think this method would be ideal for JoVE as it seems to involve a lot of steps, and it will be beneficial to actually see these performed. My only comment is that

1. The authors set up the rationale for this method as an approach to identify novel c-di-GMP binding domains. I was thus disappointed when it appears most (all?) of the domains belong to the **PilZ and DGC/PDE classes** that are easy to identify.
    - In the representative results described in this paper, only the known components of the c-di-GMP network are shown, as it indicates that the approach works. Apart from FleQ, all the known c-di-GMP related proteins of *Pseudomonas aeruginosa* contain GGDEF, EAL, HD-GYP or PilZ domains. All the novel putative effectors fished don't harbor one of these domains and belong to new c-di-GMP effectors classes. They first have to be validated for binding and then will be published in a separate research article. Even though the names of the putative novel effectors are not mentioned in this article they are shown in figure 3 as un-circled dots.
2. For example, **FleQ** was not identified using this approach. This is not to say that I do not think the method is valuable, but I think the authors need to address this point. As c-di-GMP has been shown to bind as dimers and in both an extended and closed structure to proteins, I wonder if this CCMS method excludes or inhibits the formation of some of these forms. A discussion of how the tagging impacts c-di-GMP structure would be warranted.
    - Actually, we fished FleQ, but it was not specifically enriched in the capture experiment compared to the c-di-GMP competition experiment (64 spectral count versus 49 respectively), as there was an excess of ATP present. Recently it was shown that c-di-GMP competes with ATP to bind to FleQ (Baraquet and Harwood 2013: "Cyclic diguanosine monophosphate represses bacterial flagella synthesis by interacting with the Walker A motif of the enhancer-binding protein FleQ"; PNAS 110:18478). In the initially performed CCMS experiments, when no ATP was present, FleQ was fished specifically (28 spectral count for the capture experiment, 0 for the competition experiment; Nesper *et al* 2012). The disadvantage of this experimental setup was that we fished many unspecific ATP binders. This point is now addressed in the discussion part (p.10, line 412). We agree with the reviewer that this should be mentioned in the article and included the following text (page 10, line 419): "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 here presented data in the presence of an excess of ATP."

Reviewer #2:
The manuscript by Laventie *et al*. presents a detailed description of the CCMS pulldown procedure applied for identification of c-di-GMP-binding proteins from cell extracts. The authors provide representative data for *Pseudomonas aeruginosa* showing that 74% of the predictable components of the c-di-GMP network in this bacterial species were captured using this technique.

Altogether, this manuscript describes a useful tool for enrichment of putative c-di-GMP binding proteins. The visualization of the CCMS-approach will be certainly very helpful for successful application of this method. I have only a few questions/suggestions as detailed below.

In agreement with our currently limited knowledge about the nature of c-di-GMP-sensors, the authors emphasize the need for an approach allowing the identification of in silico non-predictable c-di-GMP-effectors.

1. However, all c-di-GMP binding proteins enriched using the CCMS-method and shown in table 2 contain the well-defined **GGDEF-, EAL-, or PilZ-domains**. The authors do not mention whether they were indeed able to identify and verify a non-predictable novel c-di-GMP-binding protein using this method.
    - Compare also comment to reviewer 1, point 1. Only the known components of the c-di-GMP network are shown, as the new putative effectors will be published elsewhere.
    - To show that we indeed can identify and verify novel effectors, we included in the manuscript (new paragraph added in the representative results) 3 examples of CCMS hits with non-predictable novel c-di-GMP-binding domains. We included now the following text in the representative result part (page 9, line 355): "The other 63 soluble and 112 membrane proteins are new putative c-di-GMP binding proteins which 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 33P labelled c-di-GMP15. 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."

2. Moreover, *Pseudomonas aeruginosa* encodes for a known c-di-GMP-binding transcription factor **FleQ**, belonging to the non-predictable class of c-di-GMP-responsive proteins (Hickman JW, Harwood CS, 2008). FleQ was successfully captured in the initial analysis using the CCMS-technique (Nesper *et al.*, 2012) but is not listed in the representative results in the manuscript by Laventie *et al*. Could the authors comment on that? Is that possible that changes in the protocol were rather disadvantageous?
    - Compare also comment to reviewer 1, point 2.With the protocol that we describe here, we actually fished FleQ, but it was never specifically enriched in the capture experiment compared to the c-di-GMP competition experiment, and therefore was below our threshold of selection. The recently published paper Baraquet and Harwood (2013) "Cyclic diguanosine monophosphate represses bacterial flagella synthesis by interacting with the Walker A motif of the enhancer-binding protein FleQ" in PNAS, the authors showed that c-di-GMP probably competes with ATP to bind FleQ.

- In case of FleQ the addition of ATP was therefore rather disadvantageous, on the other hand less ATP binding proteins were fished, decreasing the list of proteins to test for specific binding.
- We included the following text in this article (page 10, line 419): "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 here presented data in the presence of an excess of ATP."

3. The protocol is written in a quite clear and understandable way but a **table** containing the required materials (including source for reproducibility) and buffer compositions would be very helpful for the user. It would be also useful to mention, that the capture buffer, the washing buffer and the magnetic beads are included in the c-di-GMP caproKit.
    - The table containing the required materials has been updated, and a new table 1 with the buffers compositions and the chemicals sources has been added. The "JoVE_Materials" materials tables provide a list of specific materials required.
4. It might be more user-friendly to describe the treatment of the **soluble fraction and membrane fraction in two separate sections** since it is confusing to follow the different handling of the two samples.
    - The steps 2 and 6.1 are soluble fraction specific, and the steps 3 and 6.2 are membrane fraction specific. The intermediate steps 4 and 5 are common, and the step 7 almost identical. We chose not to separate the soluble fraction and membrane fraction in two sections in order to save space as the manuscript gets to long otherwise.
5. The **logic of highlighting of critical steps** is not very clear and not consistent e.g. headlines 1-7 are highlighted whereas 8-10 are not. Some notes are also highlighted as being critical, although they are not (e.g. note following 7.7 "the samples can be stored at -20°C at this stage").
    - Highlighted text corresponds to the sections that will be featured in the video, and is limited by JoVE to 2.75 pages of protocol text. As this protocol is 5.5 pages long, we chose to focus on the wet lab part that is more specific to this method and will probably benefit more from a video compared to the *in silico* analysis, although they have equal importance. Highlighting has now been slightly adjusted to re-focus on critical steps.
6. The authors discuss the major drawbacks of the CCMS method but do not mention whether pronounced **losses of the compound** are expected in the sample due to 1) the c-di-GMP degradation activity of EAL-domain containing phosphodiesterases in the cell lysate and 2) the hydrophobic nature (with affinity towards the cell membrane itself) of the scaffold tail of the capture compound.
    - The degradation of the c-di-GMP moiety of a fraction of the capture compound by phosphodiersterases may occur. This is one of the reasons why the procedure has to be carried out at 4°C and the buffer does not contain Mg, which PDEs need for activity, therefore limiting the phosphodiersterases activity. After cross linking, the captured protein cannot be released from the capture compound even if the c-di-GMP moiety is degraded (as they are covalently bound to the reactive moiety).

- This point is now integrated in the discussion section (page 11, line 445) and reads: "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."
- Regarding the hydrophobic nature of the scaffold tail of the capture compound, we do not expect problems for the soluble fraction as all the membranes and membrane associated components are removed by centrifugation at 100 000 *g*. For the membrane fraction there might be a loss, as it could in principle integrate into the mixed detergent/membrane micelles. On the other hand this seems not to happen under the experimental setup we are using, relatively high amounts of Capture compound are added, as we fish almost all of the known proteins.

7. In addition, it might be noteworthy that after the on-beads digest, Strep**tavidin-derived peptides** are present in a very high abundancy, thus possibly masking some potential peptide-spectra of desired proteins. For completion, the workflow **Figure 2** should also include the highly pronounced presence of Streptavidin peptides.
    - Indeed Streptavidin-derived peptides are highly abundance. We included that now in Figure 2. It might also mask some peptide-spectra of fished proteins. However we don't think that this is a problem, as there are always several experiments performed and analyzed together and it is unlikely that always the same peptides are masked. But this may explain why some hits appear only in 2 or 3 of the performed experiments and not in all 4.

Specific comments on writing:
Introduction (end of 2nd sentence): it is more appropriate to **cite** ?Hengge, R. 2009. Principles of cyclic-di-GMP signaling. Nature Rev. Microbiol. 7:263-273" instead of Hengge, R. Cyclic-di-GMP reaches out into bacterial RNA world, see Reference no 2" in this context

- We replaced the citation.

Protocol 1.1: Could the authors mention which **culture volume** they used for e.g. log-phase and stat. phase culture to get the desired pellet size/total protein amount.

- Culture volume now integrated in the protocol section (1.1)

Protocol 1.4: Could the authors specify which **French press** cell they used to be able to French press the volume as little as 1 ml without losing the material.

- The French press cell model is now mentioned in the protocol section 1.4 (page3, line 96) (Miniature French Pressure Cell, suitable for volumes from 1 to 4 mL, Cat. N° FA-003, Thermo Electron Corporation)

Protocol 1.9: refer to step 3 at the end of the sentence

- We changed it.

Protocol 2: It is confusing that the authors first remove nucleotides using a PD10 column and then add nucleotides in step 5. Could the authors explain why the **removal of nucleotides** is necessary at all?

- The PD10 desalting column allows us to remove first all small molecules of the cell lysate including free c-di-GMP, which could compete with binding to the c-di-GMP capture compound. It is especially required when users are using this method with cells

containing elevated c-di-GMP concentrations (*e.g.* engineered strains overexpression a diguanylate cyclase, or *Pseudomonas aeruginosa* small colony variants). By adding a selection of nucleotide the overall specificity of the capture reaction can then be improved.

- We changed the text in the protocol section 2 (page 3, line 110) to: "Removal of free c-di-GMP and other nucleotides (soluble fraction only)"

Protocol 3.1: the **composition of the capture buffer** should be mentioned here (or in separate table as suggested above) and not in the later step 5.1

- A table (Table 1) with the buffers compositions and the chemicals providers has been added.

Protocol 5.1: refer to table 1 at the end of 1st note and to step 7.1 at the end of 3rd note

- done

Protocol 5.3: refer to table 1 at the end of 1st note

- done

References: **References** no 2, 3, 4, 5, 9, and 10 do not specify the journal in which the cited work has been published.

- References were adapted.

Reviewer #3:
In this manuscript, the authors describes how to use a capture compound technology to identify candidate proteins that bind c-di-GMP. The manuscript is well-written and presented and the procedures easy to follow.

Comment

1. This type of technology could potentially be used with other selectivity determinants. Are there examples in the literature for other usages of the a capture compound technology?
    - Indeed there are examples of other applications of similar capture compounds. Caprotec, the company selling the c-di-GMP capture compound, also sells capture compound with other selectivity groups, such as GDP, S-adenosyl-(L)-homocysteine (SAH) or staurosporine. These past years, several studies using the CCMS technology were published; here is a selection:

i. Luo, Y., Fischer, J.J., Baessler, O.Y., Schrey, A.K., Ungewiss, J., Glinski, M., Sefkow, M., Dreger, M., and Koester, H. (2010). **GDP-capture compound**--a novel tool for the profiling of GTPases in pro- and eukaryotes by capture compound mass spectrometry (CCMS). Journal of Proteomics 73, 815-819.
ii. Lenz, T., Poot, P., Weinhold, E., and Dreger, M. (2012). Profiling of methyltransferases and other **S-Adenosyl-L-homocysteine**-binding proteins by Capture Compound mass spectrometry. Methods Mol Biol 803, 97-125.
iii. Fischer, J.J., Graebner Baessler, O.Y., Dalhoff, C., Michaelis, S., Schrey, A.K., Ungewiss, J., Andrich, K., Jeske, D., Kroll, F., Glinski, M., et al. (2010). Comprehensive identification of **staurosporine**-binding kinases in the hepatocyte cell line HepG2 using Capture Compound Mass Spectrometry (CCMS). Journal of proteome research 9, 806-817.

Reviewer #4:

Major Concerns:

1. I am concerned about a relatively sloppy statistics pertaining to the assessment of performance of the c-di-GMP CCMS method. This information is necessary to researchers who want to choose a method of detecting c-di-GMP-binding proteins.
    - One on the major challenge with the CCMS data is to define a threshold to decide which candidates are real c-di-GMP receptors. In our experience, the selection of the best candidates depends on the specificity of the capture that can be expressed by the ratio of detected spectral counts between the capture and competition experiments. Known c-di-GMP binding proteins are always within this category and putative novel once that are already validated also belong to this class. We therefore think that this threshold should be adjusted according to the specificity and significance of the known c-di-GMP related proteins for each experiments and each species investigated
    - In addition, we performed a modified t-test (empirical Bayes method, reference 17) on the spectral counts to provide p-values that represents the enrichment significance; that we believe is a statistical strategy widely used in proteomic studies.
2. What exactly does "74% of the known or predicted c-di-GMP-binding proteins" mean?
    - In *Pseudomonas aeruginosa* PAO1 there are 51 proteins related to c-di-GMP. They are predicted diguanylate cycles (DGCs), phosphodiesterases (PDEs), PilZ proteins, plus FleQ that doesn't belong to the previous categories. 38 of them were captured by CCMS under the different conditions tested. In principle it could be that the 13 missing ones are not expressed under the tested conditions or that they do not bind c-di-GMP at all (see also point 3). Meaning that **at least** 74 % of the proteins can be identified.
    -
3. How many of these bind c-di-GMP (as verified by other techniques), and what is the basis of predictions for unverified proteins?
    - See also point 2. At least the 38 identified are expected to bind c-di-GMP.
    - The prediction was originally based on bioinformatics (apart for FleQ). Regarding the DGCs and PDEs, they can harbor conserved or degenerated motifs (GGDEF, EAL, HD-GYP), and can be enzymatically active or not. Some may only synthetize or degrade c-di-GMP without being c-di-GMP receptors, but this information is not available for most of them. At least 16 DGCs and PDEs were shown to be active enzymes *in vitro* or *in vivo*, without information about their capacity to bind c-di-GMP. The DGCs harboring an "I-site" (site that down regulates the DGC activity upon c-di-GMP binding) are likely to bind c-di-GMP (as a negative feedback control). Affinity values are only available for 5 of the 8 PilZ proteins (summarized in Christen 2010, Science) and FleQ (Baraquet 2013, PNAS).
4. Is the percentage of false positives detected by the c-di-GMP CCMS known?
    - We don't know what the fraction of false positive is. We will learn it over time, but it is first necessary to validate several CCMS hits to be able to state if they are real c-di-GMP receptors or false positives.
5. Is the percentage of false negatives known, i.e., how many of the known c-di-GMP-binding proteins have been detected by MS but not detected by c-di-GMP CCMS?

- To be able to calculate this percentage, it could be considered to create a proteome atlas of *Pseudomonas aeruginosa* prepared in the same conditions that the CCMS samples. We did not create such an atlas, and are thus unable to calculate the percentage of false negatives.

Minor Concerns:
Page 2 "Most of these compounds?": The accuracy of this sentence is questionable. Many c-di-GMP-binding proteins have emerged from genetic analysis. Delete.

- Sentence deleted.

Page 3; 2.1: What is PD10 column? Manufacturer?

- PD10 columns are desalting columns. We changed the text (page 3, line 112) to: "Wash a PD10 desalting column (GE Healthcare) with 10mL of cold lysis buffer"

Page 4, 5.3: Do you mean add cdG-CC to 10 µM, final concentration?

- To clarify this step, it is now mentioned that it correspond to 10µM final concentration (page 4, line 157), and we added a reference to the Table 2 which details the capture reaction mix.

Page 4, 5.5: Need details about UV light: flux (intensity); wavelength; distance from the source.

- Details added in the text (page 4, line 166) (λ = 310 nm, Irradiance $\geq$ 10 mW/cm^2, distance from the source = 2 cm).

Page 6, 7.19: Sonication parameters? Brief spin parameters?

- The sonication and centrifugation parameters have been added to the text (page 6, line 253) ("Sonicate 20 sec (pulse cycle 0.5, amplitude 100%, UIS250v sonicator with VialTweeter, Hielscher ultrasound technology), and spin down 5 sec, 12 000 rpm (benchtop centrifuge).

Page 8 last paragraph; Page 9 first paragraph; Table 2:
If Table 2 contains "at least four peptides identified in the four individual experiments" and proteins not detected with empty beads (i.e., "n/0" in four replicates), then why proteins having "0/0" are listed?

- We agree with the reviewer that the sentence was misleading. We changed the text in the manuscript (page 9, line 349) to: "Lastly, we considered only robust hits with at least four peptides identified in the four experiments for the 2 capture compound concentrations taken altogether"

Page 9 (last sentence of Representative Results): The statement "this technique is most effective and powerful" needs better statistics (see above) about performance of this method and its comparison to performance of other methods of identifying c-di-GMP-binding proteins.

- With this sentence, we were not intending to compare the efficiency with other techniques.. Beside, only the paper by Düvel *et al*. (2012) provides a comparable method of screening of c-di-GMP effectors. However as this paper only provides a list of selected hits (and not the raw MS data), it was not possible to accurately compare the 2 methods. As far as we can compare, for the same sample (log phase *Pseudomonas aeruginosa*

culture, soluble fraction), Düvel *et al* co-immunoprecipitated 14 proteins that are known or predicted to bind c-di-GMP, while we captured 13.

- We corrected this sentence to moderate this statement (page 9, line 370) to: "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."

Page 10 Discussion:

- Why "a concentration of 10 mg/mL being difficult to reach in some cases"?

- For liquid cultures it is relatively easy to reach proteins concentration of 10 to 15 mg/mL. But it can be more challenging to obtain enough biomass to reach a suitable amount of protein when growing cells under special conditions or using special strains (*e.g.* biofilms, small colony variants, or cells collected from infected mice).
- The text was adjusted and reads now (page 10, line 413): "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)"

- Why "various nucleotides [were added] to the capture reaction to minimize non-specific capturing of nucleotide binding proteins"? Shouldn't only non-labeled c-di-GMP be added? If this is not so, explain and provide details.

- The nucleotide mix (1mM GDP, GTP, ATP, CTP) were added in both the capture and the competition experiment, while the unmodified c-di-GMP was added only in the competition experiment. In our previous CCMS paper Nesper et al., 2012, we were not using this excess of free nucleotides, and noticed that we fished a large proportion of nucleotide binding proteins. Although some of these hit can be real c-di-GMP effectors, we feared to fish many aspecific proteins. As the c-di-GMP binding validation is a long and tedious procedure, we opted for this additional step that efficiently decrease the amount of (hopefully aspecific) nucleotide binding proteins fished. The downside is to loose c-di-GMP effectors such as FleQ, which possess a unique binding site for both ATP and c-di-GMP.
- The following text was added (page 10, line 419) (compare also Reviewer 1 and 2): "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 here presented data in the presence of an excess of ATP."

- Provide details regarding light sensitivity: "cdG-CC should be carefully protected from light". What light environment is safe?

- Details provided in the discussion section and reads as follows (page 10, line 425): "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 previous to the activation by UV irradiation."

- Poor detection of EAL domain proteins is explained by "low affinity of cdG-CC for the active site of these proteins". Poor access to the binding site, or degradation of the cdG-CC by EAL domain phosphodiesterases seem more plausible.

- The text has been adjusted as suggested (page 11, line 445).