A chemical proteomics approach to identify c-di-GMP binding proteins in *Pseudomonas aeruginosa*

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Abstract

In many bacteria, high levels of the ubiquitous second messenger c-di-GMP have been demonstrated to suppress motility and to promote the establishment of surface-adherent biofilm communities. While molecular mechanisms underlying the synthesis and degradation of c-di-GMP have been comprehensively characterized, little is known about how c-di-GMP mediates its regulatory effects. In this study, we have established a chemical proteomics approach to identify c-di-GMP interacting proteins in the opportunistic pathogen *Pseudomonas aeruginosa.* A functionalized c-di-GMP analog, 2'-aminohexylcarbamoyl-c-di-GMP (2'-AHC-c-di-GMP), was chemically synthesized and following its immobilization used to perform affinity pull down experiments. Enriched proteins were subsequently identified by high-resolution mass spectrometry. 2'-AHC-c-di-GMP was also employed in surface plasmon resonance studies to evaluate and quantify the interaction of c-di-GMP with its potential target molecules *in vitro*. The biochemical tools presented here may serve the identification of novel classes of c-di-GMP effectors and thus contribute to a better characterization and understanding of the complex c-di-GMP signaling network.

Keywords

c-di-GMP-affinity pull down; surface plasmon resonance; chemical proteomics

1. Introduction

Secondary messengers enable the integration of various environmental cues into fine-tuned regulatory pathways that control bacterial behavior on the single-cell level. In many bacteria, the second messenger bis-(3'-5')-cyclic di-GMP[2] (c-di-GMP) is a key player in the decision between motile planktonic and sessile biofilm-associated lifestyles (Hengge, 2009; Jenal and Malone, 2006; Römling and Amikam, 2006). The intracellular c-di-GMP level is tightly controlled by the opposing activities of two classes of enzymes, diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) (Kulasakara et al., 2006; Ryan et al., 2006; Ryjenkov et al., 2005; Schmidt et al., 2005; Simm et al., 2004; Tal et al., 1998). C-di-GMP is synthesized from two GTP molecules by DGCs containing a conserved GGDEF domain. A common feature of most DGCs is an allosteric feedback inhibition, as binding of c-di-GMP to an I-site motif within the GGDEF domain inhibits enzymatic activity (Chan et al., 2004; Christen et al., 2006). The break down of c-di-GMP to pGpG or two molecules GMP is mediated by PDEs harboring an EAL or, less common, a HD-GYP domain.

Bioinformatic analyses revealed that GGDEF, EAL and HD-GYP domains are widely distributed in the bacterial kingdom and that they are often fused to transmembrane and signal input domains (Galperin et al., 2001; Seshasayee et al., 2010). It furthermore became evident, that many bacteria possess multiple homologs of GGDEF, EAL and HD-GYP domain proteins which in part seem to be degenerate and rather have adopted a regulatory than an enzymatic role.

Despite extensive research, many details of the c-di-GMP signaling system are still unknown, e.g. the nature of the signals that can affect DGC and PDE activity or how the c-di-GMP level is translated into an adaptive cellular response. So far, only few effectors that relay the c-di-GMP signal have been identified. Predicted and known classes of c-di-GMP binding

proteins include PilZ domain proteins, proteins with degenerate GGDEF and EAL domains and few examples of transcriptional regulators that do not possess a common domain organization or c-di-GMP binding motif (Hengge, 2009; Hickman and Harwood, 2008; Krasteva et al., 2010; Leduc and Roberts, 2009; Schirmer and Jenal, 2009). It was furthermore demonstrated that c-di-GMP is able to bind to untranslated regions of different mRNAs thereby affecting gene expression via riboswitches (Lee et al., 2010; Sudarsan et al., 2008). Overall, considering the multitude of c-di-GMP metabolizing enzymes and the fact that a wide range of cellular functions is regulated by c-di-GMP, the identification of further classes of c-di-GMP effectors is to be expected. However, the identification of c-di-GMP binding proteins by extensive bioinformatic studies seems to have come to an end and the search for new output targets has largely been hampered by the limited disposability of pure c-di-GMP.

In this study, we present novel biochemical tools enabling the identification and characterization of c-di-GMP binding proteins. Functionalized 2'-aminohexylcarbamoyl-c-di-GMP (2'-AHC-c-di-GMP) covalently coupled to sepharose beads was used to enrich components of the complex c-di-GMP signaling network from *Pseudomonas aeruginosa*. Proteins isolated by affinity chromatography were subsequently analyzed and identified by mass spectrometry based peptide sequencing. Furthermore, we used 2'-AHC-c-di-GMP to establish highly sensitive c-di-GMP binding studies via surface plasmon resonance assays to reveal the affinity of c-di-GMP to its target molecule. If broadly used, the methods presented here are expected to significantly contribute to our understanding of how the c-di-GMP signal is communicated to downstream elements and thus confers to the switch to a biofilm mode of growth.

2. Materials and methods

2.1. Bacterial strains, media and plasmids

P. aeruginosa strain PA01 was cultured in Luria-Bertani (LB) medium at 37 °C with shaking. *Escherichia coli* strain DH5α was used for all cloning steps and strain BL21 (DE3) for protein overexpression. The *E. coli* strains were grown in LB medium with shaking, supplemented with 100 μg/ml ampicillin if required.

To construct plasmids for protein overexpression, respective genes were PCR amplified from PA01 genomic DNA using Pfu Polymerase and gene specific primers. PCR products were digested using appropriate restriction enzymes and cloned into the expression vector pET21a+ (Novagen) or pASK-IBA7+ (IBA). The detailed procedure of plasmid construction is available on request. Obtained constructs were verified by sequencing before being transformed into chemically competent *E. coli* BL21 (DE3). An *E. coli* strain overexpressing the diguanylate cyclase PleD*-His_6 was kindly provided by Urs Jenal (University of Basel, Switzerland) and an *E. coli* strain overexpressing His_6-RpoS by Vittorio Venturi (International Center for Genetic Engineering and Biotechnology, Trieste, Italy). The PA01 PA4396 transposon mutant from the Tn5 *lux* transposon library (Lewenza et al., 2005) was kindly provided by Robert E. Hancock (University of British Columbia, Canada).

2.2. Immobilization of c-di-GMP on Sepharose 6B

Preparation of 2'-AHC-c-di-GMP affinity resin was performed as described previously for the immobilization of cAMP analogs (Bertinetti et al., 2009) with some modifications: A total of ~12.5 ml settled gel of N-hydroxysuccinimide-(NHS)-activated Sepharose 4FF (GE Healthcare), 13.75 μmol 2'-AHC-c-di-GMP, 15.13 μmol ethyldiisopropylamine and 10 μmol cGMP as internal standard were suspended in 15 ml absolute DMSO in a sealed 40 ml

polypropylene tube and placed in a thermomixer at 300 rpm at 25 °C. Progress of immobilization was monitored by analytical HPLC using a 6% acetonitrile / 20 mM triethylammonium formate buffer, pH 6.8, as eluent. The HPLC-system consisted of a L 6200 pump, a L 4000 variable wavelength UV-detector, and a D 2500 GPC integrator (all Merck-Hitachi). The stationary phase was YMC ODS-A 120-10 (YMC, Kyoto, Japan) in a 250 x 4.6 mm stainless steel column. The coupling reaction was proceeded for 18 h and any unreacted NHS-groups were blocked by the addition of 250 µl ethanolamine and additional incubation for 1 h. After filtration and multiple washing steps (2 x 25 ml 20% ethanol, 2 x 25 ml water, 2 x 25 ml 30 mM NaH_2PO_4, pH 7) the affinity resin was stored in 30 mM NaH_2PO_4, 1% NaN_3, pH 7, at 4 °C. In order to quantify coupling efficiency, HPLC analysis during coupling was employed to determine the concentration of free 2'-AHC-c-di-GMP in the supernatant of the reaction mixture. Defined concentrations of 2'-AHC-c-di-GMP were used as external standards. By determining and calculating the decrease of the area under the curve value (AUC) for the 2'-AHC-c-di-GMP peak, the final ligand density was concluded to be ~1 µmol per ml of settled resin.

In a control experiment, 2'-AHC-c-di-GMP was incubated with 1.1 equivalents ethyldiisopropylamine in DMSO without affinity resin at 25 °C for 18 h. After this period less than 2% degradation of 2'-AHC-c-di-GMP was detected by analytical HPLC.

A control affinity resin was prepared by incubating NHS-activated Sepharose 4FF with 250 µl ethanolamine in DMSO at 25 °C for 2 h.

2.3. Affinity pull down protocol

Main cultures (500 ml) of *P. aeruginosa* PA01 were inoculated with a preculture and grown to stationary phase at 37 °C. Cells were harvested by centrifugation for 15 min at 4 °C and 6000 ×*g*, the pellet was washed once with ice-cold phosphate buffered saline (PBS), pH 7.4,

followed by a second centrifugation step. The bacteria (3.5 - 4.5 g wet weight) were resuspended in 15 ml ice-cold PBS containing 1 mM dithiothreitol (DTT), protease inhibitors (Complete mini, EDTA free, Roche), Benzonase Nuclease (Novagen) and 10 mM ATP, 1 mM GTP, 0.25 mM cGMP and 0.25 mM cAMP in order to reduce nonspecific binding.

The cells were lysed by passage through a French Pressure cell (at about 16000 psi, SLM-Aminco) and after a centrifugation step at 4 °C, at 37500 ×*g* for 45 min, the supernatant was mixed with 200 µl sepharose slurry, which has been pre-equilibrated with PBS buffer. Binding of proteins to the affinity matrix was allowed to progress for 2 h at 4 °C under gentle rotation in a batch format. Subsequently, the resin was washed five times with 10 ml ice-cold PBS, before transferring the resin into a small reaction tube. After a final washing step with 1 ml ice-cold PBS, c-di-GMP binding proteins were eluted by incubating the resin with 100 µM c-di-GMP in PBS for 40 min at 4 °C under gentle rotation. All eluates were finally precipitated with chloroform / methanol according to Wessel and Fluegge (Wessel and Flügge, 1984) and subjected to SDS-PAGE analysis.

Hydrolytic stability of 2'-AHC-c-di-GMP sepharose under affinity pull down conditions was analyzed indirectly using the mother compound 2'-AHC-c-di-GMP which can be monitored with analytical HPLC. In brief, 0.2 µmol 2'-AHC-c-di-GMP were added to 15 ml cell lysate and reacted for 2 h at 4 °C under gentle rotation. Proteins were denatured for 5 min at 95 °C and after centrifugation the supernatant was analyzed by HPLC (see above) using 4% acetonitrile / 20 mM triethylammonium formate buffer, pH 6.8, as eluent.

2.4. Sample preparation for LC-MS/MS analysis

Coomassie stained gel slices were excised and destained in a solution containing 30% acetonitrile and 50 mM NH_4HCO_3. Destained gel slices were reduced for 30 min at 56 °C with 20 mM DTT in 50 mM NH_4HCO_3 and subsequently alkylated with 20 mM

iodoacetamide in 50 mM NH_4HCO_3. Gel slices were cut in small pieces, washed twice in 50 mM NH_4HCO_3 and then dried after dehydration in acetonitrile. For all procedures the volume of the solutions was higher than 20 times the gel volume. Dried samples were reswollen in a solution containing 50 mM NH_4HCO_3, 10% acetonitrile and sequencing grade modified trypsin (Promega) in a ratio of about 1:20. Digestion of the protein was performed at 37 °C overnight. Peptides were subsequently eluted with H_2O, 1% trifluoroacetic acid and two times 30% acetonitrile containing 0.1% trifluoroacetic acid and dried in a vacuum centrifuge (Jouan RC 1010). For LC-MS/MS the dried peptides were desalted using ZipTip RP18 Tips (Millipore), evaporated again and resolubilized in 3% acetonitrile with 0.5% formic acid and centrifuged for 20 min at 100000 ×*g* at 20 °C.

2.5. LC-MS/MS analysis and database searching

LC-MS/MS was performed on a Dionex Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) connected to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were flushed onto a C_{18} precolumn (Acclaim PepMap 100, 75 µm x 2 cm, Dionex) with a flow rate of 15 µl/min and washed at constant flow for 3 min. Peptides were then separated on an analytical column (Acclaim PepMap RSLC, 75 µm x 25 cm, Dionex) with UPLC buffer A (0.1% formic acid in water) and UPLC buffer B (0.1% formic acid in acetonitrile) via linear B gradients from 0 – 25% over 60 min followed by a linear gradient from 25 – 50% over 30 min at a flow rate of 300 nl/min. Data-dependent acquisition of MS and MS/MS data was under control of Xcalibur ™ software (V2.1). Doubly and triply charged peptide ions were automatically selected and fragmented with m/z dependent collision energy settings. The raw data files were processed using the Mascot Deamon (V2.32). Database searches were carried out with a local Mascot server (V2.2) in a PA01 database (NCBI) using the given settings (enzyme, trypsin; maximum

missed cleavages, 1; fixed modification: carbamidomethyl (Cys), and variable oxidation (Met); peptide tolerance, 5 ppm; MS/MS tolerance, 0.4 Da). Scaffold (version Scaffold_3_00_01, Proteome Software Inc.) was used to visualize and validate MS/MS based peptide and protein identifications. This software package uses statistical algorithms to calculate the probability that each search result is correct. Details of the applied Peptide Prophet algorithm and Protein Prophet algorithm have been published (Keller et al., 2002; Nesvizhskii et al., 2003). Proteins were only accepted as identified when at least 1 unique peptide showed 95% confidence and the total protein confidence was at least 75%.

2.6. Protein overexpression and purification

E. coli BL21 (DE3) cells were grown to an OD_{600} of 0.5-0.7 before inducing protein expression by adding 0.1-0.5 mM isopropyl 1-thio-β-D-galactopyranoside IPTG (for pET21 based overexpression) or 200 ng/ml anhydrotetracycline (for pASK-IBA based overexpression).

After harvesting cells by centrifugation, the pellets were resuspended in suitable lysis buffers containing 1 mg/ml lysozyme, protease inhibitors (Complete mini, EDTA free, Roche) and Benzonase Nuclease (Novagen). Cells were lysed by passage through a French pressure cell at least twice and then centrifuged at 37500 ×*g* for 45 min in order to remove unbroken cells. Proteins containing a C-terminal His_6-tag were purified by affinity chromatography using nickel-nitrilotriacetic acid resin (Quiagen) following standard protocols. Proteins containing an N-terminal StrepII-tag were purified using Strep-Tactin as affinity resin and eluted as recommended by the supplier (IBA). Eluted fractions were examined for the presence and purity of recombinant protein by SDS-PAGE analysis. Preparations of pure protein (> 90%) were pooled and dialyzed for 16 h at 4 °C. Protein concentrations were determined by using a Bradford assay (Bio-Rad protein assay).

2.7. Dot blot c-di-GMP binding assay

Enrichment of c-di-GMP binding proteins by affinity pull downs was evaluated with a fluorescent based dot blot assay using 2'-O-(6-[Fluoresceinyl]aminohexylcarbamoyl)-c-di-GMP (2'-Fluo-AHC-c-di-GMP, excitation wavelength λ_{exc} = 494 nm, emission wavelength λ_{em} = 517 nm; Biolog LSI, Bremen, Germany). The purified PilZ domain protein PA3353 was used as a positive control and purified RpoS, an alternative sigma factor, served as a negative control.

Prior to dot blot analysis, all samples were adjusted to the same total protein concentration by dilution with PBS buffer. Of each sample, 3 µl where spotted on a Immobilon-nitrocellulose membrane (0.45 µm, Millipore) and the dots were allowed to dry. After repeating this step, dried membranes were blocked in Tris-buffered saline containing 0.2% (v/v) Tween 20 (TBS-T) and 5% (w/v) skim milk powder for 1 h at room temperature. Blocked membranes were incubated with 2'-Fluo-AHC-c-di-GMP (1 µM in TBS-T containing 5% (w/v) skim milk powder) for 1 h at room temperature, before washing twice with TBS-T for 5 min. Dot blots were scanned with a FLA-9000 reader (Fujifilm) using the LPB filter and SYBR Green I settings.

2.8. C-di-GMP binding studies using surface plasmon resonance

All experiments were performed on a Biacore 3000 instrument (GE Healthcare, Biacore) at 20 °C. To generate a c-di-GMP sensor chip, 2'-AHC-c-di-GMP was dissolved in 150 mM NaCl, 20 mM MOPS, pH 7 (buffer A) in an ultrasonic bath and by heating at 50 °C. The concentration of the analog solution was determined via the respective extinction coefficient (ε = 23700 l/(mol·cm)) (Hayakawa et al., 2003). For covalent coupling of 2'-AHC-c-di-GMP, a carboxymethylated sensor chip surface (CM5, research grade, Biacore GE Healthcare) was

activated with NHS/EDC for 10 min according to the instructions of the manufacturer (amine coupling kit, Biacore GE Healthcare). The c-di-GMP analog (1 mM in 100 mM borate running buffer, pH 8.5) was injected for 7 min at a flow rate of 10 µl/min. Deactivation of the surface was performed using 1 M ethanolamine-HCl, pH 8.5 for 10 min. A reference cell was activated accordingly and deactivated subsequently without ligand immobilization.

Binding analysis was performed in buffer A containing 0.005% (v/v) surfactant P20, 1 mg/ml CM dextran and 1 mM DTT. For some experiments, proteins were preincubated with a ten-fold concentration of free c-di-GMP prior to injection. Association and dissociation were monitored for 9 min and 3 min, respectively (flow rate 10 µl/min). Nonspecific binding was excluded by subtracting blank runs performed on the reference cell. Resulting binding signals were plotted using the Biacore 3000 evaluation software, version 4.0.1 (Biacore GE Healthcare).

Protein/nucleotide interactions were monitored as solution competition experiments (Moll et al., 2006; Zimmermann et al., 2002) in buffer A plus 0.005% (v/v) surfactant P20. In brief, proteins (15 nM – 1 µM) were preincubated with varying amounts of free c-di-GMP using at least eleven different concentrations and injected over the 2'-AHC-c-di-GMP surface. The association was monitored for 3 min and at the end of the association phase the binding signal was collected. After subtracting the reference cell, the resulting binding signals were plotted against the logarithm of the free c-di-GMP concentration and EC_{50} values were calculated from the dose response curve using GraphPad Prism 5.01 (GraphPad Software, San Diego, USA).

After each binding experiment the sensor surfaces were regenerated by two injections of 3 M guanidinium HCl and one injection of 0.05% SDS and the drifting baseline was stabilized by one injection of 1 M NaCl.

2.9. Enzymatic assays

Diguanylate cyclase (DGC) assays were carried out as previously described (Christen et al., 2005; Paul et al., 2004) with the following modifications. DGC-reactions in a total volume of 50 µl were performed at 30 °C in buffer containing 10 µM of purified protein, 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM $MgCl_2$ and [α-^{32}P]GTP (3.33 pmol, 3000 Ci/mmol, Hartmann analytic). At several time points, 5 µl aliquots were removed and mixed with an equal volume of 0.5 M EDTA, pH 8.0, to stop the enzymatic reaction. Product formation was analyzed with thin layer chromatography by spotting 1 µl on Polygram® CEL 300 polyethyleneimine cellulose plates (Machery-Nagel) and developing the plates in 1:1.5 (v/v) saturated NH_4SO_4 and 1.5 M KH_2PO_4, pH 3.6, for 60 min. Dried plates were exposed to a film for at least 1 h and qualitatively analyzed using a phosphor imager. In a control reaction, [^{32}P]c-di-GMP was enzymatically synthesized by the DGC PleD*.

3. Results

3.1. Coupling of c-di-GMP to sepharose beads

For the utilization of immobilized c-di-GMP as a bait for its cellular effector molecules, we coupled a functionalized c-di-GMP analog, 2'-AHC-c-di-GMP, to NHS-activated sepharose beads. This novel c-di-GMP analog (Biolog LSI, Bremen, Germany) has a 6-aminohexylcarbamoyl group attached to the 2' alcohol group of one ribose ring (Fig. 1) and can be easily immobilized via its primary amino group. The coupling reaction proceeded with very high efficiency (91%) and degradation of 2'-AHC-c-di-GMP under the chosen reaction conditions was below 2% (data not shown). HPLC analysis during the coupling reaction indicated a final ligand density of ~1 μmol 2'-AHC-c-di-GMP per 1 ml settled resin.

3.2. Affinity pull downs with PA01 cell lysates

The 2'-AHC-c-di-GMP sepharose served as an enrichment tool to isolate c-di-GMP binding proteins from *P. aeruginosa* strain PA01 by affinity chromatography. NHS-activated sepharose transformed with ethanolamine was used as a negative control in order to identify proteins that bind to the sepharose matrix nonspecifically. Freshly prepared cell lysates of PA01 in the stationary phase were incubated with the affinity resins and following extensive washing steps, the enriched proteins were eluted by the addition of free c-di-GMP. Proteins eluted in three independent pull down experiments were analyzed following SDS-PAGE by automated peptide sequencing (LC-MS/MS) and inspection of the derived result lists was supported by the Scaffold software program. The comparison of the results of all three pull downs revealed a large overlap in the control bead-proteome as well as in the c-di-GMP interactome: 193 out of 393 proteins bound to the control resin, and 320 out of 509 proteins

bound to the c-di-GMP resin, in at least two of the three performed experiments (Fig. 2A and B).

In addition to the peptide identification of the eluted protein fractions by mass spectrometry, we spotted the c-di-GMP sepharose eluates and their respective controls onto a nitrocellulose membrane and following blocking of nonspecific binding, incubated the eluates with c-di-GMP coupled to a fluorescent dye (2'-Fluo-AHC-c-di-GMP, Fig. 1). In all three eluates of the c-di-GMP resin, an overall enhanced binding of fluorescent c-di-GMP could be detected as compared to the controls (Fig. 3). These results clearly suggest an enrichment of c-di-GMP binding proteins in the eluates of the c-di-GMP affinity matrix that has been subjected to PA01 cell lysates.

We next categorized the proteins, enriched by the c-di-GMP and the control resin according to their function as assigned by the *Pseudomonas* Genome Database (www.pseudomonas.com) (Winsor et al., 2005). In Fig. 4, those functional categories are depicted to which at least 5% of the c-di-GMP or control proteins (which were detected in at least in two of the three pull down experiments) could be assigned to. Approximately 79% of all proteins isolated by the control sepharose could be grouped into these nine main categories, whereas the c-di-GMP sepharose pull down showed a broader distribution and only 69% of the identified proteins belong to the depicted categories. Remarkably, a higher relative number of proteins of the functional categories translation, transcription and transcriptional regulators was isolated by the control sepharose, whereas the relative proportion of hypothetical proteins, proteins involved in chemotaxis, transport of small molecules and in energy metabolism was found to be increased in the c-di-GMP sepharose pull down. The latter group includes proteins binding ATP and NADH, thus small molecules with nucleotide structures.

3.3. PA01 proteins specifically enriched by the c-di-GMP sepharose

Overall, 128 distinct putative c-di-GMP binding proteins were identified in *P. aeruginosa* PA01 lysates in at least two of the three c-di-GMP pull down experiments but in none of the control (Table S1). Another 29 proteins were identified three times on the c-di-GMP sepharose and only once on the control resin (Table S2).

Previously described or predicted c-di-GMP binding proteins in *P. aeruginosa* include seven PilZ domain proteins (there are 8 PilZ domains in *P. aeruginosa*, however, one (PA2960) did not seem to bind c-di-GMP *in vitro* (Merighi et al., 2007)), the membrane associated PelD protein (Lee et al., 2007), the transcriptional regulator FleQ (Hickman and Harwood, 2008), 23 GGDEF/GGDEF-EAL domain proteins with an I-site c-di-GMP binding motif, and the catalytically inert GGDEF-EAL hybrid protein FimX (Navarro et al., 2009). We searched our list for these c-di-GMP targets and found FleQ and six of the seven c-di-GMP binding PilZ domain proteins. The PilZ domain protein Alg44 was not identified in our pull down experiment, nor was PelD. Both proteins were described as membrane-bound proteins (Lee et al., 2007; Merighi et al., 2007).

Seven proteins containing the c-di-GMP binding motif of the I-site were identified, among them the response regulator WspR, a characterized DGC known to be prone to allosteric feedback inhibition (De et al., 2008; Hickman et al., 2005), the probable two component response regulator PA4396, and the hypothetical proteins PA2771, PA3311, PA5487, PA2072 and PA3177. Additionally, two proteins with a degenerated GGDEF domain, BifA and PA1433, were detected, as well as the HD-GYP domain protein PA4781. Despite the identification of FimX, which harbors a catalytically inert GGDEF-EAL hybrid domain, in all three c-di-GMP pull down experiments, FimX is not listed in Table S1 or S2, due to the fact that this protein was also identified in all three control pull downs. Nevertheless, manual

inspection of the mass spectrometry data revealed that there was a clear enrichment of unique FimX peptides in the c-di-GMP pull down (≥ 18) as compared to the control (≤ 3).

Apart from the previously described c-di-GMP binding proteins, we identified several proteins harboring putative nucleotide binding sites such as GTP- and ATPases, ATP-binding components of ABC transporters or proteins binding the dinucleotide NAD or FAD. Among the remaining identified proteins, 29 are hypothetical proteins and a very large fraction (24 proteins) is involved in flagellum- or pili-mediated chemotaxis.

3.4. Surface plasmon resonance binding studies with c-di-GMP

To approve c-di-GMP binding to purified proteins *in vitro*, we applied surface plasmon resonance (SPR) with the aim to explore the interaction between an analyte in the mobile phase and a ligand immobilized on a sensor surface. The resulting SPR signal (plotted as RU (resonance units), 1000 RU corresponds to 1 ng/mm^2) (Stenberg et al., 1991) is caused by mass changes upon complex formation on the sensor surface.

We successfully coupled 2'-AHC-c-di-GMP via its terminal linker-amino group to a sensor chip and analyzed the purified proteins at 20 °C using a Biacore 3000 instrument. In order to verify the surface functionality of our sensor chip, we started our interaction analysis with PA3353, a PilZ domain protein for which c-di-GMP binding has been demonstrated previously (Merighi et al., 2007). Fig. 5A shows the association and dissociation of PA3353-His_6 to the high density 2'-AHC-c-di-GMP sensor surface with increasing binding signals upon rising protein concentrations (2-500 nM). In a solution competition assay, a constant concentration of PA3353-His_6 (15 nM and 5 nM, respectively) together with increasing amounts of free c-di-GMP (50 pM-10 μM) was added to the sensor chip. By plotting the SPR signal (for each c-di-GMP concentration) at the end of the association phase against the

c-di-GMP concentration, an apparent EC_{50} (half maximal effective concentration) value was determined to be 262 ± 66 nM for PA3353-His_6 (Fig. 5B).

Next, we examined PA4396 which harbors a degenerate GGDEF domain (the conserved sequence motif is changed to DEQHF) with an intact I-site motif. We expressed and purified PA4396 with an N-terminal Strep-tag and monitored its DGC activity. As expected, Strep-PA4396 was not able to synthesize c-di-GMP from [α-^{32}P]GTP (data not shown). The protein was then tested for its ability to interact with the 2'-AHC-c-di-GMP sensor surface. Strep-PA4396 (100 nM) interacted specifically with immobilized 2'-AHC-c-di-GMP since this binding could be completely competed with free c-di-GMP in solution. The EC_{50} value was determined to be 59 ± 19 µM (Fig. 5C) in the SPR solution competition assay. This verifies that the degenerate GGDEF domain protein PA4396 is a new c-di-GMP binding protein, however, the cellular function of this interaction is still unknown. A PA01 PA4396 transposon mutant (Lewenza et al., 2005) displayed normal swimming, swarming and twitching motility and was able to attach to the walls of 96-well plates in our crystal violet biofilm assays comparable to the respective wild-type (data not shown).

The affinity chromatography performed on PA01 revealed also a group of chemotaxis proteins as putative c-di-GMP effectors. Since flagellar driven motility is known as a trait controlled by c-di-GMP, we expressed and purified PA3348, which was repeatedly isolated in the pull down experiments. PA3348 does not harbor any known c-di-GMP binding motif and has been shown to be the chemotaxis methyltransferase CheR1 (Schmidt et al., 2011). The protein was expressed and purified with a C-terminal His_6-tag and c-di-GMP binding was tested with SPR. Injection of CheR1-His_6 resulted in a SPR signal – however, coinjection of CheR1-His_6 with free c-di-GMP did not result in significant signal reduction. Even when various different parameters, cofactors and detergents (protein concentration up to 1 µM, buffer composition, pH value, *S*-adenosylmethionine, cGMP, cAMP, $MgCl_2$, CM dextran,

DTT) were applied, the SPR signals were identical. Furthermore, binding to the c-di-GMP sensor surface could not be competed with a wide range of c-di-GMP concentration (100 nM – 10 µM), indicating nonspecific binding of CheR1-His_6 to the sensor surface (Fig. 6A). To further address this question, binding experiments were done in presence of 100 nM c-di-GMP, cAMP and cGMP (Fig. 6B). Injection of CheR1-His_6 to the sensor surface resulted in a low SPR signal in comparison to the employed protein concentration (1 µM). More importantly, binding of CheR1 could neither be competed with c-di-GMP, nor with cAMP and cGMP. Thus, although repeatedly isolated by affinity chromatography, we were not able to validate CheR1 as a c-di-GMP binding protein.

4. Discussion

C-di-GMP is an important intracellular signaling molecule that is involved in the regulation of surface attachment, aggregation and biofilm formation in a wide range of bacteria. Due to the medical and biotechnological relevance of biofilms, much effort has been undertaken to understand the role of c-di-GMP in the establishment and maintenance of bacterial biofilms. Thus far, research has mainly focused on cellular processes responsible for the synthesis and degradation of c-di-GMP, whereas we are only at the beginning to understand the role of c-di-GMP effectors and downstream signaling pathways.

In this study, we aimed at the identification of c-di-GMP binding proteins by using an enrichment tool that particularly targets the c-di-GMP effector sub-proteome. Chemically modified c-di-GMP was immobilized on a solid support and used for the isolation of c-di-GMP binding proteins out of complex protein mixtures in *P. aeruginosa*. No c-di-GMP affinity resin has been described so far, although other cyclic nucleotide affinity beads have been previously used for the isolation of their respective interactomes (Bertinetti et al., 2009; Hanke et al., 2011; Scholten et al., 2006). The use of synthetic small molecules to purify and/or identify novel binding partners is commonly termed chemical proteomics (Rix and Superti-Furga, 2009) and involves high-resolution mass spectrometry which assures unambiguous identifications of the affinity purified small molecule binding proteins.

The main obstruction for the development of c-di-GMP affinity beads was the lack of ample amounts of this cyclic di-nucleotide needed for the synthesis of c-di-GMP resins. However, recent advances in the synthesis and modification of c-di-GMP now enable novel approaches to study c-di-GMP signaling pathways. In this study, we used the functionalized 2'-AHC-c-di-GMP to perform and optimize a coupling reaction with NHS-activated sepharose. With an overall efficiency of 91%, 2'-AHC-c-di-GMP was successfully immobilized on the

sepharose beads. Especially when small ligands are immobilized, a critical step is to retain binding activity of the molecule. In c-di-GMP, the N7 and O6 of the guanyl ring act as main hydrogen bond acceptors and in addition, H-bonds to the phosphate groups of the c-di-GMP backbone have been shown to mediate interactions between c-di-GMP and amino acids of the target protein (Benach et al., 2007; Chan et al., 2004; De et al., 2008; Ko et al., 2010; Navarro et al., 2009; Wassmann et al., 2007). In the 2'-AHC-c-di-GMP sepharose, c-di-GMP is linked to the sepharose matrix via a linker coupled to the 2'-hydroxyl group of one ribose ring (Fig.1) – a position which is less involved in c-di-GMP/protein interactions. Therefore, the immobilized c-di-GMP is expected to still be able to bind its cellular targets.

Another critical point is the stability of the c-di-GMP affinity resin during the incubation with crude cell extracts that contain phosphodiesterases which potentially degrade the immobilized c-di-GMP. To reduce enzymatic activity, all pull down experiments were conducted at 4 °C. In a model reaction using uncoupled 2'-AHC-c-di-GMP (see materials and methods), only a moderate degradation of 20-25% was detected under those conditions.

Our final list of protein candidates that directly or indirectly interacted with c-di-GMP included PilZ domain proteins, proteins with I-site motifs and degenerate GGDEF/EAL domains, but also proteins without any known c-di-GMP binding motif (Table S1 and S2). These lists are not only largely impacted by the definition of the inclusion and exclusion criteria based on protein identification scores and the reference proteome, but the listed c-di-GMP interacting proteins are also those of a certain time point (here: stationary phase of bacterial growth) and a certain subcellular localization (here: mainly cytoplasmic fraction). Thus, differences in culture conditions and cell extract preparations, the use of altered parameters defining the identification of the proteins and an alternative control bead-proteome, are expected to originate in the identification of additional c-di-GMP effector candidates.

A second task of this study was the generation of a robust and sensitive c-di-GMP binding assay in order to verify c-di-GMP binding *in vitro*. We therefore established a SPR based assay by using a 2'-AHC-c-di-GMP sensor chip to detect c-di-GMP/protein interactions. 2'-AHC-c-di-GMP can easily be immobilized via the terminal amino group of its 2'-AHC-linker using primary amine coupling. Advantages of the SPR technique are the low sample consumption, the high sensitivity, and that binding reaction can be followed in real time. In a solution competition assay, it furthermore enables the determination of EC_{50} (half-maximal effective concentration) values for unmodified ligands reflecting the potency of a ligand to interact with the target. This value can then be used to estimate whether the affinity is in the range of biologically effective c-di-GMP concentrations (<50 nM to a few μM) (Hengge, 2009).

SPR solution competition experiments for the PilZ domain protein PA3353 revealed an EC_{50} value of approximately 260 nM, which is in agreement with the sub-micromolar K_d values observed for other PilZ domains (Hengge, 2009) and thus demonstrates the applicability of our method. We also examined the putative response regulator PA4396, which is included in our list of putative c-di-GMP binding proteins and harbors a degenerate GGDEF domain with the c-di-GMP binding I-site motif. Purified strep-PA4396 was able to bind to the 2'-AHC-c-di-GMP sensor surface, however, in SPR competition experiments, the EC_{50} value was determined to be approximately 60 μM, which is slightly higher than any known affinity of a c-di-GMP binding site (Hengge, 2009). The *in vitro* binding assays might be impeded by the use of improperly folded proteins, the absence of important co-factors or interaction partners and the lack of post-translational modifications. Further investigations should reveal whether e.g. phosphorylation of the receiver domain of PA4396 might enhance the affinity of this response regulator to its ligand and should specify the cellular role of PA4396 and its involvement in the c-di-GMP signaling network.

A third protein from the list of putative c-di-GMP effectors, the chemotaxis methyltransferase CheR1 (PA3348), was tested in SPR for c-di-GMP binding interactions. CheR1 has no common c-di-GMP binding motif, but the repeated identification in the pull downs in PA01, and the correlation to flagellum-mediated motility renders CheR1 an interesting c-di-GMP binding candidate. Furthermore the large number of proteins involved in chemotaxis that were enriched on the c-di-GMP sepharose, argues for a role in c-di-GMP signaling. However, SPR binding studies revealed a nonspecific binding mode of CheR1-His_6 to c-di-GMP as the binding event could not be efficiently competed with up to 10 μM free c-di-GMP. Further investigations should reveal whether the absence of important co-factors or interaction partners *in vitro* might be responsible for the lack of specific binding or whether CheR1 itself is not binding c-di-GMP but stably interacts with a c-di-GMP effector protein.

Taken together, in this study we have developed a 2'-AHC-c-di-GMP affinity resin as a valuable novel tool for the isolation of c-di-GMP binding proteins. In general, affinity chromatography was shown to enrich for proteins exhibiting an enhanced binding of fluorescent c-di-GMP (2'-Fluo-AHC-c-di-GMP). Enriched proteins in the model organism *P. aeruginosa* harbored known but also new c-di-GMP binding motifs. Furthermore, the applicability of the 2'-AHC-c-di-GMP sensor chip for SPR binding studies was demonstrated. The use of chemical proteomics for the discovery of novel c-di-GMP binding motifs also in other bacterial species will give further insights into the complex c-di-GMP signaling network and might uncover novel targets for the development of alternative strategies to counteract chronic biofilm-associated infections.

Acknowledgments

We thank Urs Jenal (University of Basel), Vittorio Venturi (International Center for Genetic Engineering and Biotechnology, Trieste) and Robert E. W. Hancock (University of British Columbia) for kindly providing strains and PA01 transposon mutants. We thank Kirsten Minkhart for support with the MS analysis and Thortsen Johl (Helmholtz Center for Infection Research) for assistance with the bioinformatic analyses. We also wish to thank Michaela Hansch and Nadja Arens (University of Kassel) for technical assistance and Stephan Drewianka (Biaffin) for helpful discussions regarding SPR studies.

This work was supported by the Helmholtz-Gemeinschaft and the German Research Foundation (DFG) (SFB900). Both funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. FWH and BZ are supported by the grant “Affinity Proteome” from EU (222635) and FWH’s group is a member in the EU FP6 Proteome Binders consortium. The commercial companies Biolog Life Science Institute and Biaffin contributed to reagents (Biolog) and analysis tools (Biaffin GmbH & Co KG).

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[2]Abbreviations used: c-di-GMP, cyclic diguanylic acid; DGC, diguanylate cyclase; PDE, phosphodiesterase; pGpG, linear diguanylic acid; 2'-AHC-c-di-GMP, 2'-aminohexylcarbamoyl-c-di-GMP; NHS, N-hydroxysuccinimide; 2'-Fluo-AHC-c-di-GMP, 2'-O-(6-[Fluoresceinyl]-aminohexylcarbamoyl)-c-di-GMP; SPR, surface plasmon resonance; RU, resonance units; EC_{50}, halfmaximal effective concentration

Figure Legends

Fig. 1. Chemical structure of c-di-GMP and its functionalized analogs. In 2'-AHC-c-di-GMP, the 2'OH group of one ribose ring is substituted with a 6-aminohexylcarbamoyl group. In 2'-Fluo-AHC-c-di-GMP, 5-carboxyfluorescein has been attached to the 2'OH group of one ribose ring via an aminohexylcarbamoyl spacer.

Fig. 2. Comparisons of proteins that were pulled down in *P. aeruginosa* PA01 lysates using the c-di-GMP affinity resin (A) and control resin (B) in three different experiments.

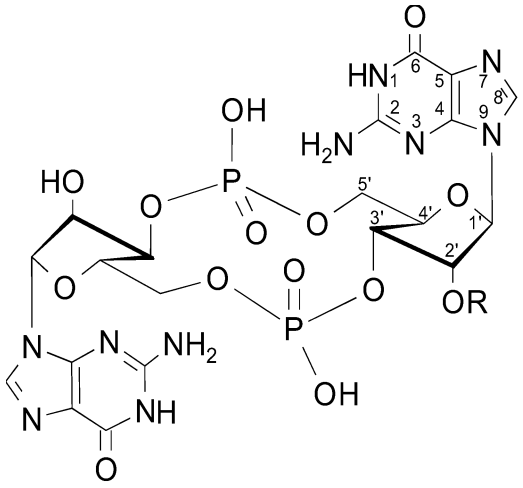
Fig. 3. Dot Blot c-di-GMP binding assay. Eluates of the c-di-GMP sepharose and control sepharose, respectively, were tested for the enrichment of c-di-GMP binding proteins by incubation with 1 μM 2'-Fluo-AHC-c-di-GMP on a nitrocellulose membrane. Purified PA3353-His_6 and His_6-RpoS were used as a positive and negative control, respectively. Representative data from one pull down out of three are shown.

Fig. 4. Classification of the pulled down proteins in PA01 according to PseudoCAP function. Only those proteins were classified which were identified in at least two of three performed pull downs using the c-di-GMP and control resin, respectively. Values represent the percentage of proteins of the c-di-GMP (gray bars) and control (black) pull down experiments that belong to a distinct PseudoCap function. Only those groups with at least 5% of the c-di-GMP or control interactome are shown.

Fig. 5. C-di-GMP binding analysis by SPR with purified proteins and a 2'-AHC-c-di-GMP sensor chip. (A) PA3353-His_6 binds to 2'-AHC-c-di-GMP in a concentration dependent

manner (protein concentration range 2-500 nM). (B) Solution competition assays of PA3353-His_6 using protein concentrations of 15 nM (dashed line) and 5 nM (continuous line) revealed an EC_{50} value of 262 ± 66 nM. Normalized data are shown. First data points are in brackets, indicating SPR signals of protein incubated without c-di-GMP. (C) For Strep-PA4396 an EC_{50} value of 59 ± 19 μM was determined using 100 nM protein in the competition experiments (n=2, continuous and dashed line, respectively). Normalized data are shown. First data points are in brackets, indicating SPR signals of protein incubated without c-di-GMP.

Fig. 6. C-di-GMP binding analysis by SPR with purified CheR1-His_6 and a 2'-AHC-c-di-GMP sensor chip. (A) Binding analysis of purified CheR1-His_6 demonstrates that CheR1 (1 μM, black line) binds to the sensor surface, but that binding cannot be competed with free c-di-GMP (e.g. 10 μM, gray line). (B) To analyze whether binding to the sensor chip is unspecific, binding experiments using 1 μM CheR1-His_6 (black line) were done in presence of 100 nM c-di-GMP (light gray), cAMP (dark gray) or cGMP (gray line). Binding was not competed by any of the cyclic nucleotides indicating an unspecific mode of binding.



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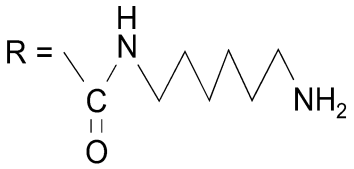
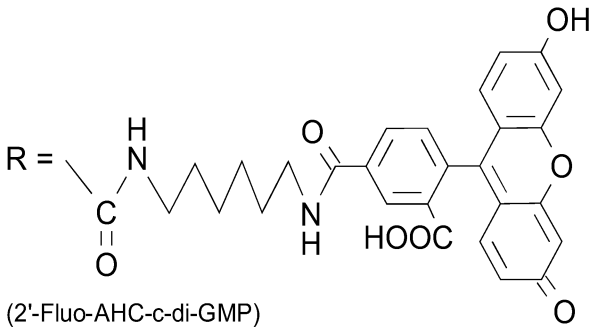
(c-di-GMP)

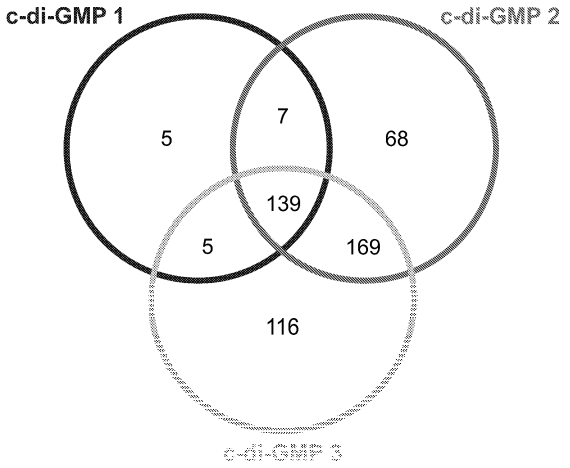


(2'-AHC-c-di-GMP)



(2'-Fluo-AHC-c-di-GMP)

A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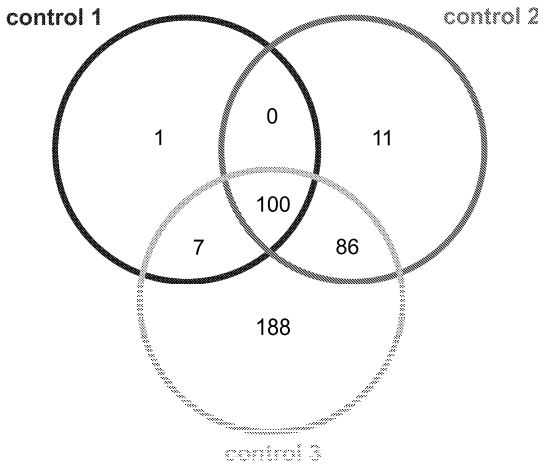
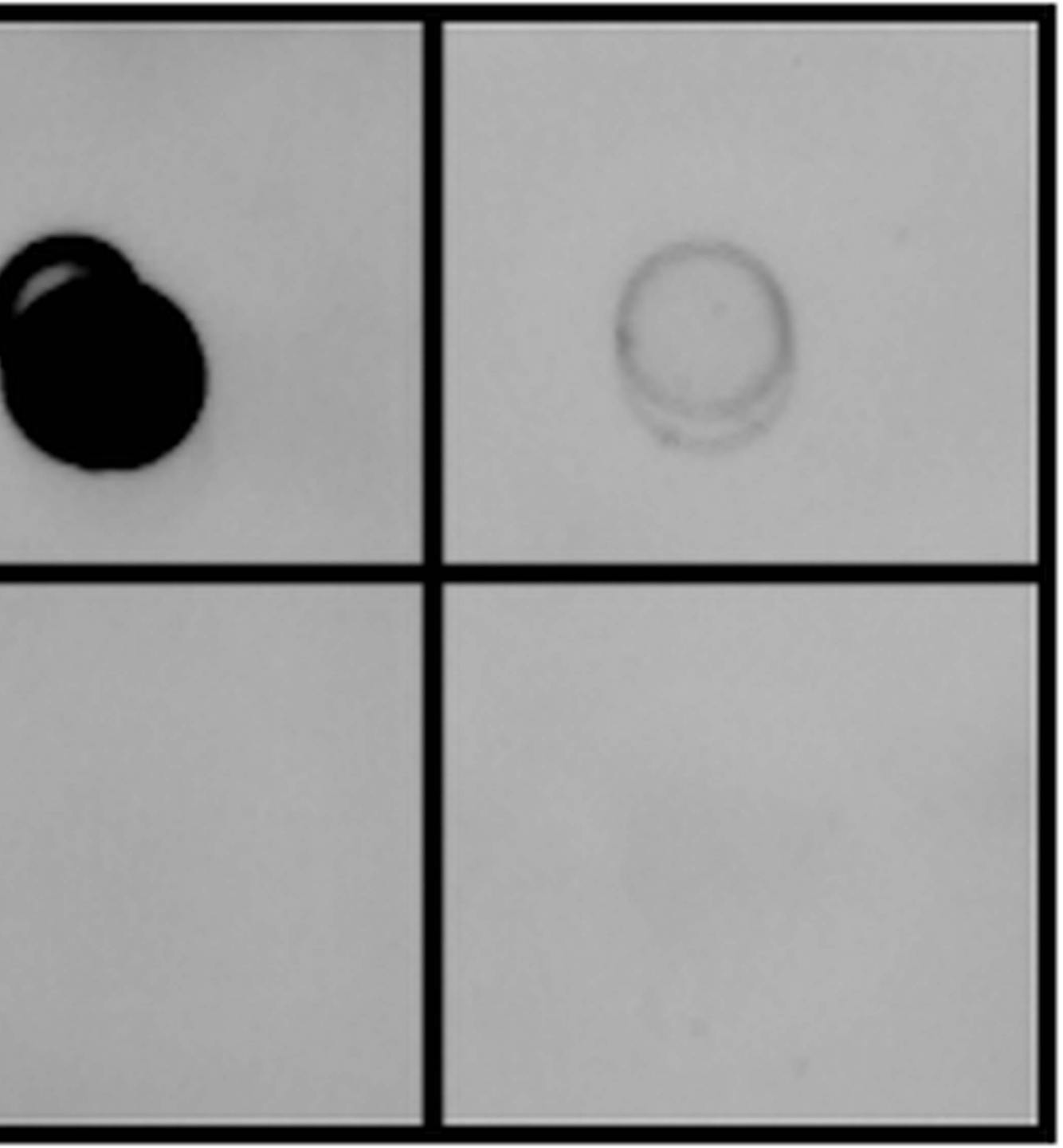
c-di-GMP 2
c-di-GMP 3
5
7
68
139
5
169
116

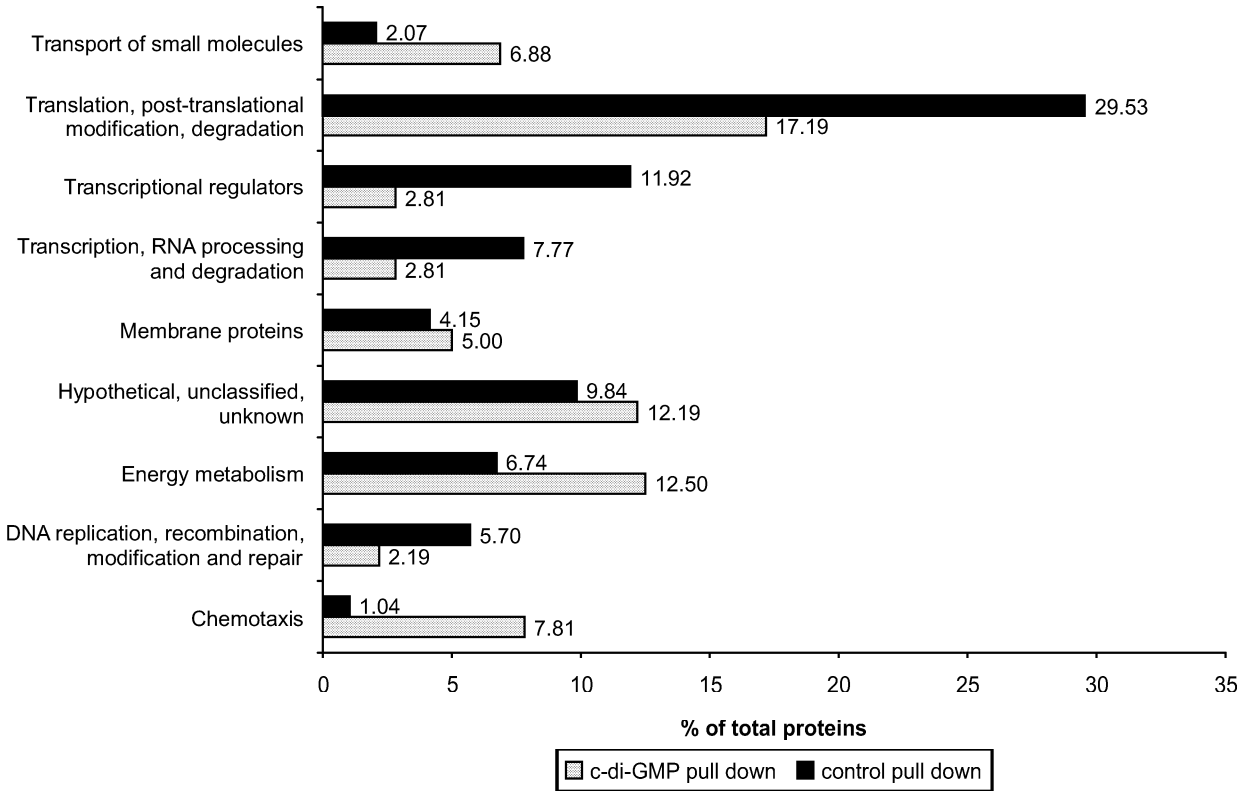
B

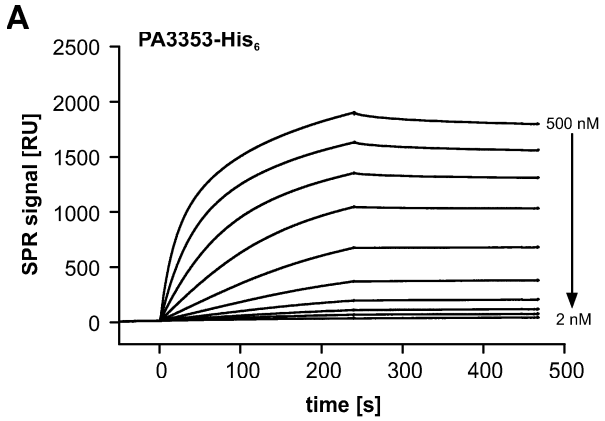

control 1
control 2
control 3
1
0
11
100
7
86
188

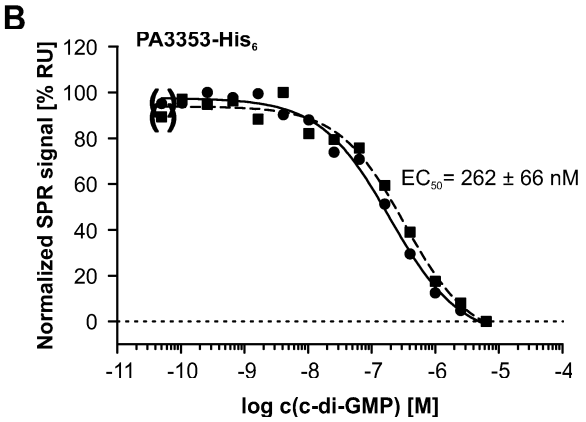


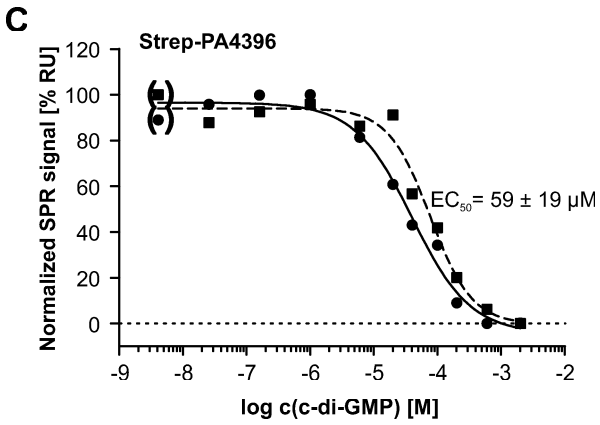
Eluate
c-di-GMP

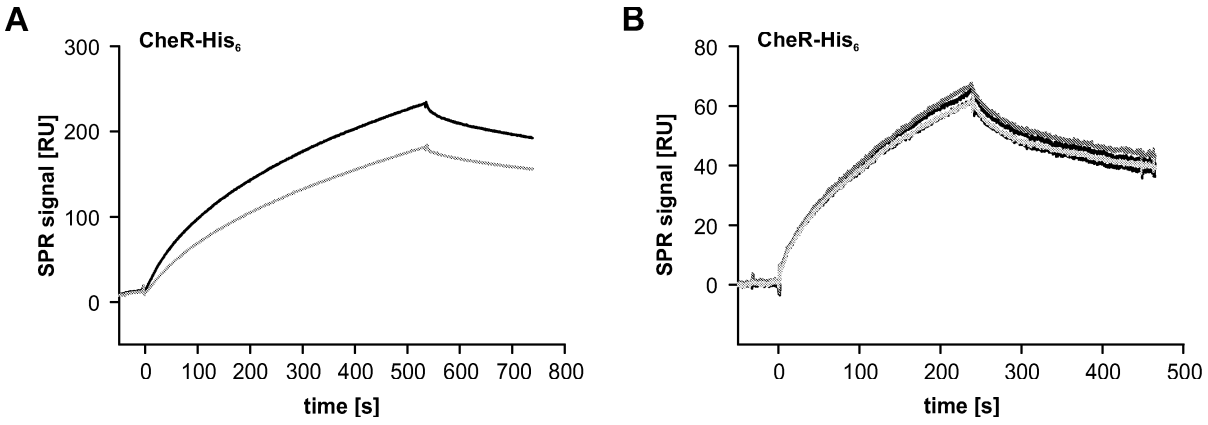
Eluate
control S


Transport of small molecules
2.07
6.88
Translation, post-translational modification, degradation
29.53
17.19
Transcriptional regulators
11.92
2.81
Transcription, RNA processing and degradation
7.77
2.81
Membrane proteins
4.15
5.00
Hypothetical, unclassified, unknown
9.84
12.19
Energy metabolism
6.74
12.50
DNA replication, recombination, modification and repair
5.70
2.19
Chemotaxis
1.04
7.81
0
5
10
15
20
25
30
35
% of total proteins
c-di-GMP pull down
control pull down


A
PA3353-His6
SPR signal [RU]
2500
2000
1500
1000
500
0
500 nM
2 nM
0
100
200
300
400
500
time [s]


B
PA3353-His6
Normalized SPR signal [% RU]
120
100
80
60
40
20
0
EC50= 262 ± 66 nM
-11
-10
-9
-8
-7
-6
-5
-4
log c(c-di-GMP) [M]


C
Strep-PA4396
Normalized SPR signal [% RU]
120
100
80
60
40
20
0
EC50= 59 ± 19 µM
-9
-8
-7
-6
-5
-4
-3
-2
log c(c-di-GMP) [M]


A
CheR-His6
SPR signal [RU]
300
200
100
0
0 100 200 300 400 500 600 700 800
time [s]
B
CheR-His6
SPR signal [RU]
80
60
40
20
0
0 100 200 300 400 500
time [s]

Supplementary data

Table S1. Proteins that were identified in at least two of the three c-di-GMP pull down experiments but in none of the control.

PA number[a]	(alternate) gene name	description	comment
PA0002	dnaN	DNA polymerase III, beta chain	
PA0008	glyS	glycyl-tRNA synthetase beta chain	
PA0012		hypothetical protein	PilZ domain[a]
PA0174		conserved hypothetical protein	Che2 system
PA0175	cheR2	probable chemotaxis protein methyltransferase	
PA0176	aer2	aerotaxis transducer Aer2	
PA0180		probable chemotaxis transducer	
PA0372		probable zinc protease	
PA0412	pilK	methyltransferase PilK	pil-chp system
PA0473	psfA	probable glutathione S-transferase	
PA0766	mucD	serine protease MucD precursor	
PA0769		hypothetical protein	
PA0783	putP	sodium/proline symporter PutP	
PA0963	aspS, syd	aspartyl-tRNA synthetase	
PA0972	tolB	TolB protein	
PA1179	phoP	two-component response regulator PhoP	
PA1339		amino acid ABC transporter ATP binding protein	
PA1344	yvaG	probable short-chain dehydrogenase	
PA1433		conserved hypothetical protein	degenerate GGDEF domain, degenerate EAL domain [4]
PA1442	fliL	conserved hypothetical protein	
PA1458	cheA	probable two-component sensor	
PA1481	ccmG, dsbE	cytochrome C biogenesis protein CcmG	
PA1550		hypothetical protein	
PA1561	aer	aerotaxis receptor Aer	
PA1608		probable chemotaxis transducer	
PA1641		hypothetical protein	
PA1681	aroC	chorismate synthase	
PA1803	lon, lopA, muc, deg, capR	Lon protease	
PA2014	liuB, gnyB	methylcrotonyl-CoA carboxylase, beta-subunit	
PA2072		conserved hypothetical protein	GGDEF domain with I-site motif, EAL domain[b]
PA2231	pslA	PslA	
PA2247	bkdA1	2-oxoisovalerate dehydrogenase (alpha subunit)	
PA2249	bkdB	branched-chain alpha-keto acid dehydrogenase (lipoamide component)	
PA2250	lpdV	lipoamide dehydrogenase-Val	
PA2290	gcd	glucose dehydrogenase	
PA2554		probable short-chain dehydrogenase	
PA2573		probable chemotaxis transducer	

PA number[a]	(alternate) gene name	description	comment
PA2587	pqsH	probable FAD-dependent monooxygenase	
PA2612	serS	seryl-tRNA synthetase	
PA2637	nuoA	NADH dehydrogenase I chain A	
PA2641	nuoF	NADH dehydrogenase I chain F	
PA2643	nuoH	NADH dehydrogenase I chain H	
PA2644	nuoI	NADH Dehydrogenase I chain I	
PA2649	nuoN	NADH dehydrogenase I chain N	
PA2652		probable chemotaxis transducer	
PA2654		probable chemotaxis transducer	
PA2707		hypothetical protein	
PA2771		conserved hypothetical protein	GGDEF domain[b] with I-site motif
PA2867		probable chemotaxis transducer	
PA2920		probable chemotaxis transducer	
PA2989		hypothetical protein	PilZ domain[b]
PA2997	nqrC	Na+-translocating NADH:ubiquinone oxidoreductase subunit Nrq3	
PA2999	nqrA	Na+-translocating NADH:ubiquinone oxidoreductase subunit Nrq1	
PA3141	wbpM	nucleotide sugar epimerase/dehydratase WbpM	
PA3158	wbpB	probable oxidoreductase WpbB	
PA3177		hypothetical protein	GGDEF domain[b] with I-site motif
PA3187	gltK	probable ATP-binding component of ABC transporter	
PA3311		conserved hypothetical protein	GGDEF domain, EAL domain[b]
PA3328		probable FAD-dependent monooxygenase	
PA3341		probable transcriptional regulator	
PA3348	cheR1	probable chemotaxis protein methyltransferase	
PA3414		hypothetical protein	
PA3478	rhlB	rhamnosyltransferase chain B	
PA3602	yerD	conserved hypothetical protein	
PA3617	recA	RecA protein	
PA3641		probable amino acid permease	
PA3674		hypothetical protein	
PA3690		probable metal-transporting P-type ATPase	
PA3700	lysS	lysyl-tRNA synthetase	
PA3702	wspR	probable two-component response regulator	GGDEF domain[b] with I-site motif
PA3801	yfgM	conserved hypothetical protein	
PA3804		hypothetical protein	
PA3816	cysE	O-acetylserine synthase	
PA3820	secF	secretion protein SecF	
PA3821	secD	secretion protein SecD	
PA3848		hypothetical protein	
PA3925		probable acyl-CoA thiolase	
PA3930	cioA	cyanide insensitive terminal oxidase	
PA4044	dxs	1-deoxyxylulose-5-phosphate synthase	
PA4129		hypothetical protein	
PA4206	mexH	probable Resistance-Nodulation-Cell Division (RND) efflux membrane fusion protein precursor	
PA4218		probable transporter	
PA4222	pchI	probable ATP-binding component of ABC transporter	
PA4290		probable chemotaxis transducer	
PA4307	pctC	chemotactic transducer PctC	

PA number[a]	(alternate) gene name	description	comment
PA4310	pctB	chemotactic transducer PctB	
PA4324		hypothetical protein	PilZ domain[b]
PA4362		hypothetical protein	
PA4367	bifA	BifA	degenerate GGDEF domain, EAL domain [4]
PA4396		probable two-component response regulator	degenrate GGDEF domain with I-site motif [4]
PA4399	yvqK	conserved hypothetical protein	
PA4423	yraM	conserved hypothetical protein	
PA4441		hypothetical protein	
PA4454	yrbD	conserved hypothetical protein	
PA4460	yhbN	conserved hypothetical protein	
PA4481	mreB, rodY, envB	rod shape-determining protein MreB	
PA4491	pufY, yfaA	conserved hypothetical protein	
PA4520		probable chemotaxis transducer	
PA4556	pilE	type 4 fimbrial biogenesis protein PilE	
PA4571		probable cytochrome c	
PA4606	cstA	conserved hypothetical protein	
PA4633		probable chemotaxis transducer	
PA4639		hypothetical protein	
PA4704		hypothetical protein	
PA4717		conserved hypothetical protein	
PA4743	rbfA	ribosome-binding factor A	
PA4747	secG	secretion protein SecG	
PA4751	ftsH, tolZ, mrsC, hflB	cell division protein FtsH	
PA4760	dnaJ	DnaJ protein	
PA4771	lldD, lctD	L-lactate dehydrogenase	
PA4781		probable two-component response regulator	HD-GYP domain [43]
PA4812	fdnG, fdhG	formate dehydrogenase-O, major subunit	
PA4839	speA	arginine decarboxylase (ADC)	
PA4907	ydfG	probable short-chain dehydrogenase	
PA4915		probable chemotaxis transducer	
PA5005		probable carbamoyl transferase	
PA5040	pilQ	Type 4 fimbrial biogenesis outer membrane protein PilQ precursor	
PA5042	pilO	type 4 fimbrial biogenesis protein PilO	
PA5043	pilN	type 4 fimbrial biogenesis protein PilN	
PA5072		probable chemotaxis transducer	
PA5077	mdoH	periplasmic glucans biosynthesis protein MdoH	
PA5170	arcD	arginine/ornithine antiporter	
PA5200	ompR	two-component response regulator OmpR	
PA5243	hemB	delta-aminolevulinic acid dehydratase	
PA5322	algC, pmm	phosphomannomutase AlgC	
PA5487		hypothetical protein	GGDEF domain[b] with I-site motif
PA5490	cc4	cytochrome c4 precursor	
PA5553	atpC, papG, uncC	ATP synthase epsilon chain	

[a] Proteins are listed according to ascending PA numbers.

[b] As predicted by Pfam.

Table S2. Proteins that were identified in all three c-di-GMP pull down experiments but only once in the control.

PA number[a]	(alternate) gene name	description	comment
PA0366		probable aldehyde dehydrogenase	
PA0413	chpA, pilL	component of chemotactic signal transduction system	
PA0425	mexA	Resistance-Nodulation-Cell Division (RND) multidrug efflux membrane fusion protein MexA precursor	
PA0537		conserved hypothetical protein	
PA0973	oprL, excC pal	Peptidoglycan associated lipoprotein OprL precursor	
PA1097	fleQ	transcriptional regulator FleQ	binds c-di-GMP [15]
PA1528	zipA	cell division protein ZipA	
PA2633		hypothetical protein	
PA2638	nuoB	NADH dehydrogenase I chain B	
PA2788		probable chemotaxis transducer	
PA2799		hypothetical protein	PilZ domain[b]
PA2953		electron transfer flavoprotein-ubiquinone oxidoreductase	
PA3150	wbpG	LPS biosynthesis protein WbpG	
PA3353		hypothetical protein	PilZ domain[b]
PA3458		probable transcriptional regulator	
PA3822	yajC	conserved hypothetical protein	
PA4309	pctA	chemotactic transducer PctA	
PA4430		probable cytochrome b	
PA4431		probable iron-sulfur protein	
PA4489		conserved hypothetical protein	
PA4550	fimU	type 4 fimbrial biogenesis protein FimU	
PA4563	rpsT	30S ribosomal protein S20	
PA4608		hypothetical protein	PilZ domain[b]
PA4640	mqoB	malate:quinone oxidoreductase	
PA4941	hflC	protease subunit HflC	
PA4958		hypothetical protein	
PA5304	dadA	D-amino acid dehydrogenase, small subunit	
PA5528		hypothetical protein	
PA5557	atpH, papE, uncH	ATP synthase delta chain	

[a] Proteins are listed according to ascending PA numbers.

[b] As predicted by Pfam.