Bacterial Rotary Export ATPases are Allosterically Regulated by the Nucleotide Second Messenger Cyclic-di-GMP

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Running title: Cyclic-di-GMP Binding to Bacterial Export ATPases

Keywords: cyclic di-GMP (c-di-GMP); ATPase; bacterial signal transduction; Pseudomonas; type III secretion system (T3SS); flagellum; second messenger

Background: AAA+ ATPase proteins play integral roles in the export apparatus of many bacterial organelles.

Results: The second messenger cyclic-di-GMP binds specifically to multiple export ATPases at a highly conserved binding site.

Conclusion: Cyclic-di-GMP binding is central to the function of many different bacterial export complexes.

Significance: This profoundly affects our understanding of numerous important bacterial organelles, including flagella, type-III and type-VI secretion systems.

ABSTRACT

The widespread second messenger molecule cyclic-di-GMP (cdG) regulates the transition from motile and virulent lifestyles to sessile, biofilm-forming ones in a wide range of bacteria. Many pathogenic and commensal bacterial-host interactions are known to be controlled by cdG signalling. While the biochemistry of cyclic-dinucleotide metabolism is well understood, much remains to be discovered about the downstream signalling pathways that induce bacterial responses upon cdG binding. As part of our ongoing research into the role of cdG signalling in plant-associated Pseudomonas species, we carried out an affinity-capture screen for cdG binding proteins in the model organism P. fluorescens SBW25.

The flagella export AAA+ ATPase FliI was identified as a result of this screen, and subsequently shown to bind specifically to the cdG molecule, with a $K_D$ in the low µM range. The interaction between FliI and cdG appears to be very widespread. In addition to FliI homologs from diverse bacterial species, high-affinity binding was also observed for the type-III secretion system homolog HrcN and the type-VI ATPase ClpB2. Addition of cdG was shown to inhibit FliI and HrcN ATPase activity in vitro. Finally, a combination of site-specific mutagenesis, mass spectrometry and in silico analysis was used to predict that cdG binds to FliI in a pocket of highly conserved residues at the interface between two FliI subunits. Our results suggest a novel, fundamental role for cdG in controlling the function of multiple important bacterial export pathways, through direct allosteric control of export ATPase proteins.

Pseudomonas fluorescens is a widespread soil bacterium that forms commensal relationships with plant species. Members of the P. fluorescens species group non-specifically colonise the rhizosphere and phyllosphere of many plants, and promote plant growth as well as providing potent antifungal and other biocontrol capabilities (1-3). The related bacterium Pseudomonas syringae is a Gram negative phytopathogen and is responsible for numerous important plant diseases. P. syringae produces a large number of species-specific phytoxins and type-III-secreted effector molecules that subvert plant defences (4,5), and infects host plants by migration through open stomata and wounds on the plant surface. Two of the most important organelles for efficient host
colonisation by both commensal and pathogenic Pseudomonas sp. are the flagellum and the type III secretion system (T3SS). Flagella-mediated motility is critical during the initial stages both of infection and benign plant colonisation, and is required to move through the soil towards plant roots, to colonise plant surfaces and to migrate into the apoplastic space (6). Type III secretion systems, needle-like structures that inject effector proteins into plant cells, play a critical role in P. syringae virulence (4) and have also been shown to be important for rhizosphere colonisation by P. fluorescens (6).

Assembly of the bacterial flagellum is tightly regulated and proceeds via the export of extracellular subunits through the central pore of the extending complex (7-9). The AAA+ ATPase FliI, together with FliH and FliJ, forms the soluble component of the flagellar export apparatus (8,10,11). FliI and FliH form a heterotrimer (FliH$_2$-FliI) in vivo, and along with FliJ deliver export substrates from the cytoplasm to the flagellum export gate. There, FliI forms a hexameric ring and is anchored to the export gate by FliJ and FliH (12). While the majority of the energy required for flagella formation is provided by the proton motive force, FliI ATPase activity is required for efficient flagella formation, and plays a role in the initiation of protein export (13,14). The secretion apparatus of flagella and T3SS share a conserved core architecture, with many proteins in common including the protein export apparatus (9,13).

Investigations into the signalling pathways that control interactions between pathogenic and commensal Pseudomonas sp. and their host plants have highlighted a central role for the bacterial second messenger cyclic-di-GMP (cdG) (15-21). CdG is a ubiquitous regulator of bacterial behaviour, controlling the transition between motility and sessility, and chronic and virulent lifestyles in a wide range of bacteria. Recently, cdG has emerged as a crucial factor in the signalling pathways of most bacterial species, determining when, where and how bacteria form biofilms, progress through the cell cycle, or regulate different aspects of motility and virulence (22). Broadly speaking, cdG production is associated with community behaviour phenotypes such as biofilm formation and surface attachment. Conversely, low cdG levels are connected to unicellular, motile and virulent lifestyles (22). CdG affects cell phenotypes by regulating the expression, production and activity of different phenotypic output pathways. These outputs are controlled by cdG binding to effectors that function at transcriptional (23), translational (24), and post-translational, allosteric levels (25,26). Individual phenotypic outputs may be controlled at multiple regulatory stages. For example, the expression of multiple flagella genes are controlled by the cdG-binding transcriptional regulator FleQ (23,27). Flagella function is also allosterically controlled by cdG, with binding to the basal-body associated proteins YcgR and FlgZ leading to reduced flagella rotation speed in E. coli and P. putida respectively (28,29).

CdG mediated signalling pathways are typically highly complex. Numerous species, including the pseudomonads, contain dozens of metabolic enzymes and display diverse cdG-triggered phenotypes (22). While the synthesis and degradation of cdG by GGDEF, EAL and HD-GYP proteins is fairly well-understood (30-33), much remains to be discovered about the effector proteins that bind the dinucleotide molecule and elicit downstream responses in the cell. While several predictable cdG binding folds are known, for example the PilZ (34) and degenerate GGDEF and EAL domains (35-37), cdG is a promiscuous molecule and binds to a diverse range of protein folds. In many cases, these cdG binding motifs are impossible to bioinformatically predict in advance (27,38-41). This, combined with the complexity of cdG-signalling and the diverse array of interconnected, cdG-associated phenotypes (22,42,43) suggests that a great many cdG binding proteins still await discovery.

Recent investigations by several research groups have made effective use of biochemical and spectrometric techniques for the isolation and identification of cdG binding proteins (44-46). These studies have both increased our understanding of the phenotypes and cellular functions controlled by this second messenger, and substantially expanded the number of recognised binding motifs and protein domains (40,42,43). To better understand the role of cdG in the interactions between plant-associated Pseudomonas species and their hosts, we used a cdG-capture compound assay (45) to screen for cdG binding proteins in the model P. fluorescens strain SBW25 (47). Among the
proteins we identified in this screen was the flagella export ATPase FliI (PFLU4436). Subsequent biochemical analysis showed that SBW25 FliI specifically binds to cdG with a $K_D$ in the low μM range. FliI-cdG binding was not confined to P. fluorescens, but was also seen for FliI homologs from several other bacterial species, as well as the closely-related T3SS export ATPase HrcN from P. syringae (PSPT01400), and the significantly more divergent T6SS secretion ATPase ClpB2 (PFLU6025).

In vitro addition of cdG induced a marked, concentration-dependent inhibition of ATPase activity for both FliI and HrcN. However, the association between ATPase activity and cdG binding is not absolute – mutation of critical active site residues in both FliI and HrcN abolishes ATP hydrolysis, while dinucleotide binding is unaffected. To further probe the relationship between FliI and cdG, a combination of mass spectrometry and in silico analysis was used to predict the FliI dinucleotide binding site. These results suggest that cdG may bind in a pocket of highly conserved residues at the interface between two domains of the FliI hexameric ring. Our results suggest a fundamental new role for the signalling molecule cdG, in the structure and function of bacterial export pathways.

EXPERIMENTAL PROCEDURES

**Strains and growth conditions** - Strains and plasmids are listed in Table 2. Primers are listed in Table 3. Unless otherwise stated all P. fluorescens strains were grown at 28°C and E. coli at 37°C in lysogenic broth (LB) (48), solidified with 1.5% (w/v) agar where appropriate. For protein overexpression, terrific broth (TB) was used. Kanamycin was used at 50 μg/ml, carbenicillin at 100 μg/ml and chloramphenicol at 30 μg/ml. For inducible plasmids, IPTG was added to a final concentration 0.5 mM as appropriate.

**Molecular biology procedures** - Cloning was carried out in accordance with standard molecular biology techniques. The pETNde-M-11-fliI, hrcN and clpB2 purification vectors were produced by ligating PCR fragments (amplified with primers A-M from appropriate genomic DNA) between the NdeI and EcoRI sites of plasmid pETNdeM-11 (49) as appropriate. Strand overlap extension (50) was used to produce Walker A and Walker B mutants in FliI and HrcN using primers N-U, before cloning into expression vectors as appropriate.

**CdG-capture compound experiments** - Experiments were performed as described by Nesper et al. (45). P. fluorescens cells were grown in M9 0.4% (w/v) pyruvate medium ± 0.4% (w/v) CAS-amino acids to stationary phase and to mid-logarithmic phase, LB to stationary phase and KB to logarithmic phase. Cells were collected by centrifugation for 5 min at 5,000g. The pellet was resuspended in lysis buffer (6.7 mM MES, 6.7 mM HEPES pH 7.5, 200 mM NaCl, 6.7 mM Na-acetate and 10 mM β-mercaptoethanol) with protease inhibitors and DNase I (Roche). Cells were lysed using a French press (3 x 20,000 psi) and lysates were centrifuged at 100,000 x g for 1 hour. The supernatant was then used to identify soluble cdG binding proteins. 600 μg of the soluble protein mixture was used and was mixed with 20 μL of capture buffer (100 mM HEPES pH 7.5, 250 mM Na-acetate, 50 mM Mg-acetate, 50% (v/v) glycerol), plus 12.5 μL of 10 mM cdG for the control samples. Volumes were adjusted to 100 μL with water and the reactions were then incubated for 2 hours at 4°C, before UV irradiation for 4 minutes using a caproBox (Caprotec Bioanalytics GmbH, Berlin). Magnetic streptavidin beads (50 μL) were added with 25 μL of 5x wash buffer (250 mM Tris pH 7.5, 5 M NaCl, 0.1% (w/v) n-octyl-β-glucopyranoside), and the samples were incubated for 45 minutes at 4°C on a rotary wheel. The beads were collected with a magnet and the samples were washed six times with 200μL of 1x wash buffer. The beads were resuspended in 20 μL sample buffer, incubated for 10 minutes at 95°C, and separated for 10 minutes on a pre-cast 12% (w/v) SDS acrylamide gel at 100 V. Protein bands were then excised, and sent for mass spectrometric analysis. The same protocol was followed for the competition experiment (Figure 1C). Similarly to the controls, 1 mM of each nucleotide was added to a protein mixture of 10 μM and pre-incubated for 1 hour before the addition of the capture compound (10 μM).

**Protein Purification - E. coli BL21-(DE3) pLysS** - pLysS overexpression cultures were inoculated from overnight cultures in a 1:100 ratio and grown at 37°C to an OD$_{600}$ of 0.4, before protein expression was induced overnight with 0.5 mM IPTG at 18°C. Cells were then lysed by French press (3 x 20,000 psi).
psi), centrifuged, and proteins purified from the supernatant by NTA-Ni chromatography. 1 ml HiTrap chelating HP columns (Amersham) were equilibrated with 10 volumes of washing buffer (20 mM HEPES pH 7.5, 250 mM NaCl, 2 mM MgCl₂, and 2.5% (v/v) glycerol pH 7.5) and loaded with cell lysate. Following protein immobilization, the column was washed with 10 volumes of buffer containing 50mM imidazole, before proteins were eluted using 500mM imidazole buffer in a single step elution.

**Differential Radial Capillary of Ligand Assay (DRACALA)** - The method was performed as described by Roelofs et al. (51). Purified PldD* (52) was used to synthesise radiolabelled cdG from [γ³²-P]-GTP. The assays were conducted using increasing concentrations of purified FliI proteins mixed with 4nM radiolabelled cdG in each case. Samples were incubated for 2 minutes at room temperature with γ³²-P-cdG in reaction buffer (25 mM Tris, 250 mM NaCl, 10 mM MgCl₂, 5 mM β-mercaptoethanol pH 7.5). 5μL of each sample were then spotted on nitrocellulose, samples were dried and results visualised using a phosphorimager screen. In some cases 2'-Fluo-AHC-c-diGMP was used (BioLog 009) as an alternative to γ³²-P-cdG, at a concentration of 0.6 μM. The results were visualised using a charge-coupled device (CCD) camera. For the competition experiments, 1 mM of each nucleotide was mixed with 10 μM of FliI₁₋₁₈ and incubated for 30 minutes before the addition of the fluorescent cdG.

**Surface plasmon resonance ( SPR)** - SPR experiments were conducted at 25°C with a Biacore T200 system (GE Healthcare) using a Streptavidin SA sensor chip (GE healthcare), which has four flow cells each containing SA pre-immobilized to a carboxymethylated dextran matrix. Flow cell one (FC1) and flow cell three (FC3) were kept blank to use for reference subtraction. The chip was first washed three times with 1 M NaCl, 50 mM NaOH to remove any unconjugated streptavidin. 100 nM biotinylated cdG (BioLog B098) was immobilised on FC2 and FC4 of the streptavidin chip at a 50 RU immobilisation level with a flow rate of 5 μL/min. Soluble proteins at the required concentrations were prepared in SPR buffer (10 mM HEPES, 150 mM NaCl, 0.1% (v/v) Tween 20, 2 mM MgCl₂) by adjusting the pH for the different proteins. For FliH₉₃, FliH₉₅ mutants, HrcN₉₃, HrcN₉₅ mutants, and ClpB2, the optimal pH was 6.5 whereas for FliI₇₃ the optimal pH was 7.5 and for FliI₃₁₋₁₈ it was 5.5. Samples were injected with a flow rate of 5 μL/min over the reference and cdG cells for either 60, 90, or 120 seconds depending on their saturation level followed by buffer flow for either 60 or 90 seconds. The chip was washed at the end of each cycle with 1 M NaCl. Replicates for each protein concentration were included as appropriate. In certain cases (e.g. FliI₇₃), protein precipitation at higher concentrations prevented the acquisition of a saturated binding curve. In these cases, a representative dataset is presented from at least three independent repetitions. All sensorgrams were analysed using Biacore T200 BiaEvaluation software version 1.0 (GE Healthcare). Data were then plotted using Microsoft Excel and GraphPad Prism.

**Linked Pyruvate Kinase / Lactate Dehydrogenase ( PK/LDH) ATPase activity assay** - ATPase activity was measured indirectly by monitoring NADH oxidation. The reaction buffer consisted of 50 mM Tris-Cl (pH 8.0), 2 mM MgCl₂, 1 mM DTT and 10 mM KCl. Each reaction contained 5 mM NADH in 10mM NaOH, 80 mM phosphoenolpyruvic acid, 1.5 μL PK/LDH (Sigma) and appropriate concentrations of FliI/HrcN and cdG, and was initiated by the addition of ATP. Enzyme kinetics were determined by measuring A₃₄₀ nm at 1 minute intervals. Kinetic parameters were calculated by plotting the specific activity of the enzyme (nmol ATP hydrolysed/min/mg of protein) versus ATP concentration and by fitting the non-linear enzyme kinetics model (Hill equation) in GraphPad Prism.

**Mass spectrometry of cross-linked FliI** - 10 μM FliI₉₃ protein was incubated with 10 μM cdG-capture compound (Caprotec) and cross linked in a UV Stratalinker on ice for 4 minutes. Cross-linked sample was then separated from non-cross-linked using magnetic beads as described for the capture compound screen (above). Cross-linked FliI-cdG, and a non-cross-linked control sample were then run into an SDS gel and FliI bands excised for protein identification. Samples were analyzed by Nano-LC-MS/MS on an Orbitrap Fusion™ (Thermo Scientific, Hemel Hempstead, UK). The sample was separated on a PepMap™ 100 C18 LC Column (C18, 2 μm, 500 x 0.75 mm, Thermo) using a
gradient of 0.75% (v/v) min\(^{-1}\) acetonitrile from 6% to 40% (v/v) in water/0.1% (v/v) formic acid at a flow rate of 0.3 µl min\(^{-1}\) and infused directly into the mass spectrometer. The mass spectrometer was run in positive ion mode, with no quad isolation, at 120K resolution over the mass range 350-1800 (m/z) for the precursor scans (Orbitrap). One microscan of 50 ms with an AGC target of 2e\(^5\) was used. MS2 threshold was set to 1.5e\(^4\) and precursors fragmented by both CID and HCD with CE=30 and an isolation window of 1.6 Da (quadrupole) using the automatic maximum speed option with ion injection for all available parallelizable time. Dynamic exclusion was set to 1 count and 30 s. Recalibrated peaklists were generated using MaxQuant 1.5.2.8 (www.MaxQuant.org) and the database search was performed with the merged HCD and CID peaklists using Mascot 2.4 (Matrixscience, London, UK). The search was performed with a precursor tolerance of 6 ppm and a fragment tolerance of 0.6 Da on a partial E. coli database, to which the expected FliI\(_{His}\) protein sequence was added. The enzyme was set to trypsin/P with a maximum of 2 allowed missed cleavages. Carbamidomethyl (C) was set as fixed modification, and oxidation (M) and acetylation (Protein N-term) were used as variable modifications. The Mascot search results were imported into Scaffold 4.4.1.1 (www.proteomsoftware.com) using identification probabilities of 99% and 95% for proteins and peptides.

**Homology model production** - A homology model of a Pseudomonas fluorescens FliI monomer was created by the Phyre2 server (http://www.sbg.bio.ic.ac.uk/phyre2)(53) using the crystal structure of FliI from Salmonella enterica as a template (PDB accession code 2DPY; 63% amino acid sequence identity)(54). FliI is predicted to be structurally homologous to the α and β subunits of F\(_1\)-ATPase and the latter forms a hexameric ring of alternating α and β subunits around a single copy of a γ subunit. Using the Secondary Structure Matching algorithm (55) within the program COOT (56), a model of a FliI hexamer was generated by superposing six copies of the monomer onto each of the α and β subunits of the bovine F\(_1\)-ATPase α, β, γ complex (PDB accession code 2JIZ) (57). All structural figures were prepared using CCP4mg (58).

**Mass Spectrometry-Peak Shift Analysis (MS-PSA)** - MS-PSA analysis of the sample containing the treated protein FliI\(_{His}\) (i.e. cross-linked to cdG) was performed as described previously [51]. We further improved the method to specifically search for spectra relations (i.e. modified vs unmodified peptide) between spectra from two different samples. Accordingly, we found many related peptides between the pure (untreated) FliI\(_{His}\) sample and the treated sample (FliI\(_{His}\) cross-linked to cdG), particularly for the peptide NVLLLMDSLTR. The following MS-PSA parameters were used for both analyses: num=8 pmr=0.001 t1=5 t2=100 fmr=0.5 mnds=2 clusterq=True signif=1 tol=1 outl=0.1 nmfp=20 mofp=9 mnspg=2 csf=2 peakfreq=2 nummods=1 pwss=4 cp=3 qual1=0.2 qual2=0.2 qual1p2=0.5 mrms=150 maxdev=1.

**RESULTS**

*The flagellar ATPase FliI binds specifically to cdG* - As part of our ongoing efforts to define the cdG regulon of *P. fluorescens* SBW25, we carried out a series of screening experiments for cdG binding proteins using a cdG-capture compound assay (45), Caprotec). These experiments identified homologs of confirmed binding proteins, including FleQ (23) and WspR (59), as well as several uncharacterised PilZ, GGDEF and EAL-domains and numerous proteins for which no previous experimental or predicted link to cdG signalling had been made. Among these previously-unidentified cdG targets, the flagellar export protein FliI was identified in screens conducted under a number of different experimental conditions (KB log phase, LB stat. phase and M9 Pyruvate + CAS-amino acids). In addition to the suppression of flagellar gene expression (23) and flagellum rotation (29,60), FliI-cdG binding suggests a central, previously unsuspected role for cdG in the regulation of flagellum protein export and assembly. Consequently we selected FliI for further biochemical analysis. First, to confirm that SBW25 FliI binds to cdG in vitro the full-length, his-tagged protein (FliI\(_{His}\)) was purified (Figure 1A) and nucleotide binding tested using the DRaCALA binding assay (44). FliI\(_{His}\) bound strongly to \(^{32}\)P cdG, but did not bind to \(^{32}\)P GTP even at far higher protein concentrations (Figure 1B). To further test the specificity of FliI-cdG binding, competitive DRaCALA and capture compound experiments were performed (Figure 1C). A variety of
nucleotides (cyclic di-GMP, ADP, ATP, NADH, cAMP, cGMP, cyclic di-AMP) were added in excess to compete the 2′-Fluo-AHC-c-diGMP and capture compound respectively. In both experiments, binding was abolished only with the addition of cdG, strongly suggesting that cdG binding by FliI is specific. Our initial attempts to define the biochemical parameters of cdG binding to FliI\textsubscript{His} used isothermal titration calorimetry (ITC). This technique showed tight, concentration-dependent cdG binding with a $K_D$ of approximately 10 µM (data not shown). However, we were unable to refine the ITC protocol sufficiently to produce publishable data. Consequently, we turned to surface plasmon resonance (SPR) to examine FliI\textsubscript{His} binding to biotinylated cdG. In this experiment, FliI\textsubscript{His} bound to the cdG chip in a concentration dependent manner with a $K_D$ of 2.4 ± 0.2 µM (Figure 2A, 2B, Table 1).

Much of the structural and functional analysis of FliI to date has been conducted in *Salmonella enterica*, whose purified FliI homolog is unstable in vitro unless the N-terminus of the protein is truncated (61). This modification affects FliI multimerisation, with the truncated allele unable to form hexameric complexes in vitro (62). To examine the effects of removing the FliI N-terminus on protein-ligand interactions, a truncated FliI allele missing the first 18 residues (FliI\textsubscript{∆1-18}) was purified and analysed alongside full-length FliI\textsubscript{His}. The FliI\textsubscript{∆1-18} allele bound tightly and specifically to cdG in both DrACALA and SPR experiments, with a dissociation constant of 0.75 ± 0.03 µM (Figure 1B, 2C, 2D). The binding constants for both full length and truncated FliI fall comfortably within the affinity range of previously characterised cdG binding proteins (between low nM and 10-15 µM (63)), indicating that FliI-cdG binding is likely to occur in *P. fluorescens* under physiologically relevant conditions.

FliI homologs from diverse bacterial species and the export ATPases of type-III and type-VI secretion systems bind cdG at physiological concentrations - Flagella-driven motility, and hence FliI mediated export, is ubiquitous among Gram negative bacteria. To investigate whether cdG-binding to FliI is similarly widespread, full-length FliI homologs from several bacterial species were cloned, expressed and purified, then tested for cdG binding using SPR. FliI homologs were selected from human and plant pathogens, as well as commensal and symbiotic plant-growth-promoting organisms. The tested FliI homologs included representatives from the α- and γ-proteobacterial classes, and both monotrichous and polyflagellated bacteria. Concentration-dependent cdG binding was detected for full-length FliI alleles from the phytopathogen *P. syringae pv. tomato* (*Pto*) DC3000 (FliI\textsubscript{Pto}), the human pathogen *Salmonella enterica* serovar Typhimurium (FliI\textsubscript{SCT}), and the nitrogen-fixing symbiont *Sinorhizobium meliloti* (FliI\textsubscript{Sm}) (Figure 3, Table 1). Despite a reasonably high degree of *fliI* amino acid sequence divergence (SBW25 and *S. meliloti* share only 35.4% identity), and significant differences in flagella regulation and cdG signalling between the tested species, all four FliI homologs bound to the dinucleotide molecule with affinities well within the expected physiological range of intracellular cdG concentrations.

The export apparatus of the bacterial flagellum is closely related to that of the type-III secretion system (T3SS), with both complexes sharing a common ancestor (8). Furthermore, cdG has been associated with the control of T3SS function in the opportunistic pathogen *Pseudomonas aeruginosa* (64,65) although the mechanism of this regulation is currently unclear. In light of this, our data for FliI-cdG binding implicate the T3SS export ATPase HrcN as a further potential cdG-binding target. To test this, we purified the full-length, his-tagged protein from *Pto* DC3000 (HrcN\textsubscript{His}) and examined cdG binding using SPR. As predicted, HrcN\textsubscript{His} also bound strongly to cdG, with a dissociation constant of 3.2 ± 0.2 µM (Figure 4A, 4B). The type-VI secretion system (T6SS) export ATPase (ClpB2) is far more distantly related to FliI, both in terms of primary sequence and the organisation of the ATPase subunits within the type-VI secretion complex (66). Nonetheless, as ClpB2 is a rotary ATPase and type-VI secretion is known to be under reciprocal, cdG-linked control with type-III secretion (65), full-length ClpB2 (ClpB2\textsubscript{His}) was purified and tested for cdG binding. To our surprise, ClpB2 also displayed strong, concentration dependent binding to the cdG with a physiologically relevant binding affinity of 9.5 ± 0.5 µM (Figure 4C, 4D). These data strongly suggest that binding to the cdG second messenger
is a widespread characteristic across diverse rotary ATPase export proteins.

**Addition of cdG inhibits FliI and HrcN ATPase activity** - The established model for cdG function associates increased dinucleotide levels with reduced motility and virulence (22). This has been shown to be the case for both *P. fluorescens* (67) and *P. syringae* (17,18,21). Consequently we hypothesised that cdG binding may negatively affect the ATP-dependent export activity of FliI and/or HrcN. To examine the effect of cdG binding on FliI/HrcN ATPase activity, pyruvate kinase/lactate dehydrogenase-linked ATPase activity assays were conducted for the full-length protein alleles FliI<sub>His</sub> and HrcN<sub>His</sub>. Purified FliI<sub>His</sub> metabolised ATP with a *K<sub>m</sub>* of 0.48 ± 0.03 µM and a *V<sub>max</sub>* of 1262 ± 54.46 nm ATP/min/mg. Addition of 50 µM cdG led to a noticeable drop in *V<sub>max</sub>* to 867.2 ± 51.65 nm ATP/min/mg (Figure 5A). The *IC<sub>50</sub>* of cdG for FliI<sub>His</sub> (36.7 ± 1.13 µM) was then determined by increasing cdG levels while maintaining a constant ATP concentration in the reaction (Figure 5B). Similar cdG inhibitory activity was seen for FliI<sub>Δ1-18</sub>, which metabolised ATP with a *K<sub>m</sub>* of 0.48 ± 0.03 µM and a *V<sub>max</sub>* of 691.5 ± 41.90 nm ATP/min/mg without cdG. Upon addition of 50 µM cdG, *V<sub>max</sub>* dropped to 375.4 ± 35.34 nm ATP/min/mg (Figure 5C, 5D). Addition of GTP had no effect on cdG binding when included in DRaCALA assays (Figure 1C). These results indicate that cdG binding and ATPase activity may be uncoupled.

**Identifying the site of cdG-capture compound binding in FliI** - To further investigate the site of cdG binding on FliI, we constructed a homology model for SBW25 FliI based on the crystal structure of its *Salmonella* homolog (54) (Figure 7). The location of ATP, and the conserved residues across the six cdG-binding ATPases in this study were then mapped onto the model and a predicted FliI hexameric complex was produced (Figure 7A). Next, purified FliI<sub>His</sub> was incubated and UV cross-linked to the cdG-capture compound. Following tryptic digestion and mass spectrometry, mass-shifted peptides were identified using Mass Spectrometry Peak-Shift Analysis (MS-PSA), a recently developed analysis method for the identification of unexpected/unknown peptide modifications (70) (Figure S1). Two MS-PSA analyses were performed, treated (i.e. cross-linked to cdG) vs untreated FliI<sub>His</sub> (S1A) and treated FliI<sub>His</sub> alone (S1B). In the treated sample we expected to identify both pure FliI<sub>His</sub> and FliI<sub>His</sub> with bound cdG. Accordingly, many spectra relations corresponding to modified and unmodified peptides were identified (Figure S1). Importantly, the most densely-modified peptide following cdG-capture compound crosslinking comprised residues 259-269 (NVLLLMDSLTR, Figure S1). We identified 36 spectra relations where the lighter peptide was Mascot annotated NVLLLMDSLTR, and the heavier not-annotated partner carried a modification >150Da. By only comparing spectra between the treated and untreated sample we
identified 52 corresponding NVLLLMDSLTR spectra relations. The NVLLLMDSLTR peptide represents the central strand of a β-sheet at the core of the SBW25 FliI homology model, plus short loops at either end (Figure 7B, in green). The C-terminus of the β-strand also contains the conserved aspartate (D265) of the Walker B motif (Figure 7B, in pink). Interestingly, the end of the capture compound cross-linked peptide emerges close to a cluster of highly conserved residues that could form a pocket at the interface between two FliI subunits in our model (Figure 7C, 7D, in red). As well as several glycine and proline residues, this conserved pocket contains two arginines (R170 and R337) from one subunit and a glutamate (E208) from the second. Both arginine and glutamate are highly important for dinucleotide binding in all previously characterised cdG binding proteins (40,42).

To confirm the importance of these residues to cdG binding, specific amino acid substitutions were produced (R170H, E208Q, and R337H) in FliIΔ1-18. The solubility of the resulting FliIΔ1-18 alleles was confirmed by gel filtration (data not shown), then cdG binding and ATPase activity were tested. All three substitutions showed seriously compromised cdG binding, with KD values that were too high to be accurately determined (Figure 8A, Table 1) and a complete abolition of ATPase activity (Figure 8B). Finally, we detected no cdG binding for the hexameric ATPase protein NtrC from Azotobacter vinelandii, which shares a tertiary structure fold with the export ATPases but does not have the residues of the proposed binding site (Figure 8C).

Together, these results strongly indicate that the binding indeed occurs at the proposed site shown in Figure 7D.

DISCUSSION

Here we show that the second messenger cdG binds to the bacterial flagellum export ATPase FliI. This cyclic dinucleotide binding is apparently widespread, with FliI homologs from multiple different bacterial species showing strong, concentration-dependent binding activity upon the addition of cdG. Excitingly, cdG binding at physiologically relevant (low micromolar) affinities was also determined for the closely-related type III secretion exporter HrcN from Pto DC3000, and the much more distantly-related P. fluorescens Type VI ATPase ClpB2. Our findings implicate cdG in the direct, allosteric regulation of both flagellar protein export and type III/type VI-mediated virulence for a range of pathogenic, commensal and beneficial bacterial species.

The ATPase activity of both FliI and HrcN is suppressed by the addition of cdG. In this respect, the relationship between cdG and the export ATPase proteins is reminiscent of the transcriptional motility regulators FleQ (27) and FlrA (71). These proteins both contain AAA+ ATPase domains and bind cdG close to the Walker A motif of the protein. However, there appear to be important differences between the binding characteristics of FleQ/FlrA and the export ATPase proteins described here. In FleQ, cdG interacts with the Walker A site of the protein, leading to competitive inhibition of ATPase activity (27). Similarly, cdG binding to an arginine residue (R176) downstream of the Walker A motif of FlrA inhibits binding to its target promoter sequence (71). In the case of the export ATPase proteins, we were able to uncouple cdG binding from ATPase activity.

Our FliI/HrcN K181A mutants bound strongly to cdG, but displayed no ATPase activity, while the G176A mutant retained substantial ATPase activity, but showed severely compromised cdG binding. Furthermore, the FlrA R176 residue is conserved in FleQ, but not in FliI/HrcN/ClpB2 (Figure 9, in purple), again suggesting a distinct cdG binding mechanism.

Based on our biochemical and mutagenesis data for FliI/HrcN, mass spectrometric analysis of cdG cross-linked FliI, and in silico modelling of hexameric FliI, we identified an intriguing potential site for cdG binding to the rotary export ATPases. This site, at the interface between two protein monomers, contains highly conserved glutamate and arginine residues known to be required for cdG binding (42). The putative binding pocket also contains both a proline and two glycines, which may play a role in maintaining the structure of the binding pocket. Mutagenesis and subsequent in vitro binding tests confirmed that the conserved glutamate and arginine residues were indeed required for cdG binding. Although loss of binding was accompanied by loss of ATPase activity in each case, this result is perhaps unsurprising given that the mutations made were at the FliI dimerisation interface. Structural determination of FliI and HrcN in complex with cdG is underway.
and should allow us to determine exactly where and how cdG interacts with export ATPase complexes.

In general, cdG represses the production and function of flagella and type III secretion systems and promotes type VI secretion, although the relationship between cdG and these different pathways in *Pseudomonas* sp. is both highly complex and not fully understood. Flagella gene expression in *Pseudomonas* sp. is controlled by cdG through FlqE, which binds to numerous flagellar loci and whose inactivation by cdG binding (or deletion) abolishes flagella production (23). Flagella rotation is also likely to be under cdG control in *P. fluorescens* and *P. syringae*, as both contain close homologs to the *P. putida* rotation controller FlgZ (28). CdG has also been shown to control swarming motility in *P. aeruginosa* by switching between two different stator complexes; MotAB and MotCD (60). Other cdG-regulated pathways, such as pili synthesis (37) and EPS production (23,72) also have indirect impacts on both flagella deployment and motility (73,74).

CdG is also known to control the level/activity of the *Pseudomonas aeruginosa* type III and type VI secretion systems (64,65), although here the regulatory pathways are less well-understood. Moscoso et al. (65) show that cdG mediates the switch between production of type III and type VI secretion pathways, and that this switch requires the sRNAs RsmY and RsmZ, linking cdG signalling to the small translational regulatory protein RsmA. Translation of both flagella and type III mRNAs are controlled by RsmA in *P. aeruginosa* (75), which is itself involved in a complex regulatory network involving downstream cdG signalling (76,77). A role for cdG in the allosteric suppression of FliI/HrcN export *in vivo* is entirely consistent with the wider literature for both flagellar motility and type III secretion. Certainly, our biochemical data strongly suggests that increased intracellular cdG levels would suppress the ATPase activity, and hence might be expected to suppress the export activity of these proteins. However, whether suppression of ATPase activity represents the actual *in vivo* function of export ATPase-cdG binding is currently uncertain.

Recently, Minamino *et al.* (14) showed that only residual FliI ATPase activity is actually required for flagellum production in *Salmonella*, with the majority of the energy for protein export provided by the proton motive force. FliI ATPase activity is still required for effective export to occur, but is thought to play a gatekeeper role, where it provides the basal body with the energy required to initiate protein export (13,14). If, as Minamino *et al.* suggest, reduced FliI ATPase activity upon cdG binding does not necessarily translate into reduced flagellar protein export, then what else might be the role of cdG? Our results show that cyclic dinucleotide binding is both widespread and highly conserved among export ATPases. Furthermore, the binding affinities we observed for cdG are sufficiently high that dinucleotide binding should occur frequently under ‘normal’ environmental conditions.

We propose that FliI-cdG binding may play a more fundamental role in controlling flagella function and assembly. Specifically, a basal level of FliI-cdG binding may be required for the initiation of FliI export, via the promotion of multimerisation, imposition of rotational asymmetry to the FliI hexamer (78) or another undefined mechanism. In support of this hypothesis, basal levels of cdG have been shown to be required for flagella synthesis in both *Salmonella enterica* (79) and *Caulobacter crescentus* (80). In both cases, deletion of all GGDEF domain-containing proteins, and hence cdG, from the cell resulted in a loss of flagella-driven motility. In *Salmonella*, cdG removal led to increased expression of flagella basal-body genes, but a severe defect in the export of FliC (79). Similarly for *C. crescentus*, the production of basal body proteins was unaffected by cdG removal, while class III and class IV gene expression was severely reduced (80). In this case, reduced flagella-gene expression could be explained by anti-σ factor-induced feedback upon the loss of flagellar export (81). Whether such a mechanism also applies to the ATPases of type III and type VI systems is unclear at this stage. While the cdG-null strain of *S. enterica* showed a loss of virulence consistent with loss of T3SS function (79), more evidence is required to confidently propose a model for the relationship between cdG and HrcN/ClpB2 function. Research is ongoing to determine the exact nature of the relationship between cdG and the rotary ATPase proteins, and the impact of cdG-ATPase binding on motility and virulence in bacterial species.
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Author contributions: ET: conceived and designed the study, conducted most of the experimental work, produced figures 1-6 and 8, analysed data and contributed to writing the manuscript. CEMS: contributed to the SPR work and relevant data analysis. DML: produced figure 7, including modelling, figure preparation and data analysis. TW: conducted the in silico MS-PSA analysis and produced Figure S1. RHL: purified proteins and provided technical assistance and support to this study. JGM: conceived and designed the study, produced figure 9, and wrote the manuscript.

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FOOTNOTES
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Abbreviations used in the text: CCD, Charge Coupled Device; SPR, Surface Plasmon Resonance; cdG, cyclic-di-GMP; ITC, Isothermal Titration Calorimetry; DRaCALA, Differential Radial Capillary of Ligand Assay; PK/LDH, Pyruvate Kinase/Lactate Dehydrogenase; GTP, Guanidine Triphosphate; ATP, Adenosine Triphosphate; ADP, Adenosine Diphosphate; NADH, Nicotinamide Adenine Dinucleotide; cGMP, Cyclic Guanidine Monophosphate; cAMP, Cyclic Adenosine Monophosphate; cdA; cyclic-di-AMP

FIGURE LEGENDS

FIGURE 1A: Coomassie stained SDS-PAGE gel showing purified FliI\textit{His} fractions eluted with 500mM imidazole. 1B: Differential radial capillary action of ligand assay (DRaCALA) for \textsuperscript{32}P-cdG and \textsuperscript{32}P-GTP binding to increasing concentrations of full length FliI (FliI\textit{His}). Positive (10 \textmu M PleD*) and negative (10 \textmu M BSA) binding controls are included, as well as N-terminal truncated FliI (10 \textmu M FliI\textsc{\textsubscript{∆1-18}}). 1C: DRaCALA competition experiment performed on FliI\textsc{\textsubscript{∆1-18}}. A variety of nucleotides were included in the reaction to test the specificity of cdG binding. SDS-PAGE gel showing protein bound to the capture compound after pre-incubation with different nucleotides.

FIGURE 2A: SPR sensorgrams showing affinity measurements for FliI\textit{His} binding to biotinylated cdG. A range of FliI\textit{His} concentrations was used (0.312, 0.625, 1.25, 2.5, 5, 10, 20 and 40 \textmu M) and concentration replicates were included as appropriate together with buffer only and BSA controls. The protein binding and dissociation phases for all sensorgrams are shown. 2B: Affinity fit for FliI\textit{His} – cdG binding. The binding response for each concentration was recorded 4s before the end of the injection and the \textit{K}_D values for FliI\textit{His} binding to cdG (2.4 \pm 0.2 \textmu M) were calculated using the BiaEvaluation software and confirmed by GraphPad Prism. 2C: SPR sensorgrams showing affinity measurements for FliI\textsc{\textsubscript{Δ1-18}} binding to biotinylated cdG. A range of protein concentrations was used (0.078, 0.156, 0.3125, 0.625, 1.25, 2.5, 5 and 10 \textmu M) and concentration replicates were included as appropriate together with buffer only and BSA controls. The protein binding and dissociation phases for all sensorgrams are shown. 2D: Affinity fit for FliI\textsc{\textsubscript{Δ1-18}} – cdG binding. Binding responses were measured 4s before the end of the injection and the \textit{K}_D values for FliI\textsc{\textsubscript{Δ1-18}} binding to cdG (0.8 \pm 0.03 \textmu M) was calculated using the BiaEvaluation software and confirmed by GraphPad Prism.

FIGURE 3A-B: SPR sensorgrams and resulting affinity fit for FliI\textit{Pto} binding to biotinylated cdG. 3C-D: SPR sensorgrams and affinity fit for FliI\textsc{\textsubscript{LcT}} binding to biotinylated cdG. In all three cases, a range of protein concentrations was used (0.625, 1.25, 2.5, 5 and 10 \textmu M) and concentration replicates were included as appropriate together with buffer only and BSA controls. The protein binding and dissociation phases for all sensorgrams are shown. For the affinity fits, binding responses were measured 4s before the end of the injection and \textit{K}_D values for each protein calculated using the BiaEvaluation software and confirmed by GraphPad Prism (see Table 1).

FIGURE 4A-B: SPR sensorgram and resulting affinity fit for HrcN (Type III export ATPase) binding to biotinylated cdG. 4C-D: SPR sensorgram and resulting affinity fit for ClpB2 (Type VI export ATPase) binding to biotinylated cdG. In both cases a range of protein concentrations was used (0.625, 1.25, 2.5, 5 and 10 \textmu M) and concentration replicates were included as appropriate together with buffer only and BSA controls. The protein binding and dissociation phases for all sensorgrams are shown. For the affinity fits,
binding responses were measured 4s before the end of the injection and $K_D$ values for each protein calculated using the BiaEvaluation software and confirmed by GraphPad (see Table 1).

**FIGURE 5A:** ATPase activity for FliI<sub>His</sub> ± 50 µM cdG. FliI<sub>His</sub> specific activity (nmol ATP hydrolysed/min/mg protein) is shown for increasing ATP concentrations. Addition of cdG causes a decrease of the $V_{max}$ of FliI<sub>His</sub> ATPase activity. **5B:** IC50 curve for FliI<sub>His</sub> ATPase activity upon addition of increasing cdG concentrations. A constant concentration of ATP (1 mM) was included alongside 1 µg FliI<sub>His</sub> protein. **5C-D:** ATPase activity ± 50 µM cdG, and IC50 curve upon addition of increasing cdG concentrations, for HrcN. All parameters remain the same as in 5A. The IC50 curve also includes results for GTP titration showing no ATPase inhibition.

**FIGURE 6A:** Affinity fit for cdG binding to different FliI alleles (FliI<sub>His</sub>, ∆1-18, K181A, D265A, and G176A). Sensorgrams obtained using biotinylated cdG (Figure 1D, 2B) were used to calculate the $K_D$ values for FliI binding to cdG (see Table 1). At each protein concentration, the responses were recorded 4s before the end of the injection. **6B:** ATPase activity for different FliI alleles. Protein specific activity in each case (nmol ATP hydrolysed/min/mg protein) is shown for increasing ATP concentrations.

**FIGURE 7A:** Homology model of the predicted hexameric form of SBW25 FliI, based on the crystal structure of FliI from *S. typhimurium* (PDB code: 2DPY). Conserved residues between the six cdG-binding proteins tested in this study are marked in red (on the grey and cyan coloured subunits only), and ADP (stick model; taken from template structure) is shown bound at the interfaces between the individual FliI subunits. **7B:** Close-up of the interface between two FliI subunits, showing the NVLLLMDSLTR peptide implicated in cdG-capture compound binding (circled, in green) and the conserved Walker B aspartate (D265) in pink. **7C:** Locations of conserved residues between the six cdG-binding proteins tested in this study (red). **7D:** Close-up of the proposed cdG binding pocket (circled). Conserved residues suggested to form the cdG binding site are labelled.

**FIGURE 8A:** Affinity fit for cdG binding to different FliI alleles (FliI<sub>∆1-18</sub>, FliI<sub>∆1-18</sub> R170H, FliI<sub>∆1-18</sub> E208Q, FliI<sub>∆1-18</sub> R337H). **8B:** ATPase activity for different FliI alleles. Protein specific activity in each case (nmol ATP hydrolysed/min/mg protein) is shown for increasing ATP concentrations. **8C:** DRAcALa binding assay for 32P-cdG to 10 µM NtrC (*Azotobacter vinelandii*). Positive (10µM BldD<sup>*</sup>) and negative binding controls (NC) were included as appropriate.

**FIGURE 9:** Clustal alignment of conserved residues between FliI, HrcN and ClpB2 proteins in this study. Identities between all six residues are marked with a ‘*’, similarities across all six with ‘.’ or ‘:’. The mutated FliI Walker A/B residues (see Figure 6) are marked in red, the capture compound-binding NVLLLMDSLTR peptide is marked in blue, the position of the conserved cdG binding arginine in FlrA (R176) is marked in purple and the conserved residues of the proposed cdG binding site are marked in green (see Figure 7).
**TABLE 1: Binding affinity data for tested FliI, HrcN and ClpB2 variants**

Dissociation constant ($K_D$) values for the ATPase proteins analysed in this study. Binding to FliI<sub>SeT</sub> was concentration dependent but did not saturate in the kinetic experiment.

| Protein                  | $K_D$ (µM)  |
|--------------------------|-------------|
| FliI<sub>His</sub> (full-length) | 2.4 ± 0.2   |
| FliI<sub>Δ1-18</sub> (N-term. truncated) | 0.8 ± 0.03 |
| FliI<sub>Δ1-18</sub> R170H | Not determined |
| FliI<sub>Δ1-18</sub> E208Q | Not determined |
| FliI<sub>Δ1-18</sub> R337H | Not determined |
| FliI<sub>Pto</sub> (Pto DC3000) | 7.6 ± 0.8   |
| FliI<sub>SeT</sub> (S. enterica) | Not determined |
| FliI<sub>Sm</sub> (S. meliloti) | 3.2 ± 0.7   |
| HrcN<sub>His</sub> | 3.2 ± 0.2   |
| ClpB2<sub>His</sub> | 9.5 ± 0.5   |
| FliI G176A (Walker A mutant) | 11.0 ± 1.1  |
| FliI K181A (Walker A mutant) | 2.2 ± 0.2   |
| HrcN K181A (Walker A mutant) | 3.8 ± 0.4   |
| FliI D265A (Walker B mutant) | 4.5 ± 0.2   |
## TABLE 2: Strains and Plasmids used in this study

| Strains                  | Description                                                                 | Reference          |
|--------------------------|-----------------------------------------------------------------------------|--------------------|
| *E. coli* BL21-(DE3)     | Sm^R, K12 recF143 lacI^a lacZ^Δ.M15, xylA                                      | Novagen            |
| *E. coli* DH5α           | endA1, hsdR17(rK-mK^+), supE44, recA1, gyrA (Na^R), relA1, Δ(lacIZYA-argF)U169, deoR, Φ80dlacΔ(lacZ)M15 | (82)               |
| SBW25                    | Environmental *P. fluorescens* isolate                                       | (83)               |
| *Pto* DC3000             | Rif^R derivative of *P. syringae* pv. Tomato NCPPB 1106                      | (84)               |
| *Salmonella enterica* pv. | Strain LT2                                                                   | (85)               |
| *Typhimurium*            |                                                                              |                    |
| *Sinorhizobium meliloti* | Strain 1020                                                                  | (86)               |
| **Plasmids**             |                                                                              |                    |
| pETNdeM-11               | Km^R, purification vector, N-terminal His_6-tag                             | (49)               |
| pETNdeM-11-overexpression vectors | Various overexpression vectors for *fliI* alleles, *hrcN*, and *clpB2* ligated between the Ndel and EcoRI sites of pETNdeM-11 | This study         |
### TABLE 3: Primers

| Name | Gene Target | Modification | Sequence (5’-3’)          |
|------|-------------|--------------|---------------------------|
| A    | PFLU4436 (fliI) | None         | TTACTTCATATGCACCTGACGACCACG |
| B    | PFLU4436 (fliI) | None         | ATATTCATATGGTACGCCAGGCGGCGG |
| C    | PFLU4436 (fliI) | Δ1-18        | TTACTTCATATGCACCTGACGACCACG |
| D    | PSPTO1961 (fliI) | None         | TTACTTCATATGACCGGTCCGTCG |
| E    | PSPTO1961 (fliI) | None         | AAATCAATGGTACGACCACGACCACG |
| F    | STY2180 (fliI) | None         | TTACTTCATATGACCGGTCCGTCG |
| G    | STY2180 (fliI) | None         | AAATCAATGGTACGACCACGACCACG |
| H    | SMc03025 (fliI) | None         | TTACTTCATATGACCGGTCCGTCG |
| I    | SMc03026 (fliI) | None         | AAATCAATGGTACGACCACGACCACG |
| J    | PSPTO1400 (hrcN) | None         | TTACTTCATATGACCGGTCCGTCG |
| K    | PSPTO1400 (hrcN) | None         | AAATCAATGGTACGACCACGACCACG |
| L    | PFLU6025 (clpB2) | None         | TTACTTCATATGACCGGTCCGTCG |
| M    | PFLU6025 (clpB2) | None         | AAATCAATGGTACGACCACGACCACG |
| N    | PFLU4436 (fliI) | G176A        | CCTTTCATATGACCGGTCCGTCG |
| O    | PFLU4436 (fliI) | G176A        | CCTTTCATATGACCGGTCCGTCG |
| P    | PFLU4436 (fliI) | K181A        | GCGGTGTAGCGGTCCGTCG |
| Q    | PFLU4436 (fliI) | K181A        | GCGGTGTAGCGGTCCGTCG |
| R    | PFLU4436 (fliI) | D265A        | GCACCCCGCCACAGGCACCACG |
| S    | PFLU4436 (fliI) | D265A        | GCACCCCGCCACAGGCACCACG |
| T    | PSPTO1400 (hrcN) | K181A        | GCGGTGTAGCGGTCCGTCG |
| U    | PSPTO1400 (hrcN) | K181A        | GCGGTGTAGCGGTCCGTCG |
FIGURE 1

A

B

C
FIGURE 2

A

B

C

D

Response (RU) vs. Time (seconds)

Response (RU) vs. Protein concentration (µM)

Increasing concentration

Protein concentration (µM)
FIGURE 4

A
HbcN
(Type III secretion system ATPase)

B
Response (RU)

Time (seconds)

C
CipB2
(Type VI secretion system ATPase)

D
Response (RU)

Time (seconds)

Protein concentration (μM)

Protein concentration (μM)
FIGURE 5

A  

Specific Activity  (nmol ATP hydrolysed min⁻¹ mg⁻¹)

0.0  0.5  1.0  1.5  2.0  2.5  3.0
[ATP]mM

FliI full length
+ 50μM cdG

B  

Specific Activity  (nmol ATP hydrolysed min⁻¹ mg⁻¹)

0.0  0.5  1.0  1.5  2.0  2.5
log [cyclic di-GMP]μM

C  

Specific Activity  (nmol ATP hydrolysed min⁻¹ mg⁻¹)

0.0  0.5  1.0  1.5  2.0  2.5
[ATP]mM

HrcN full length
+ 50μM cdG

D  

Specific Activity  (nmol ATP hydrolysed min⁻¹ mg⁻¹)

-1  0  1  2
log [cyclic di-GMP, GTP]μM

GTP
CdG
FIGURE 6

A

Response (RU)

0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0

Protein concentration (μM)

FliI Δ1-18
FliI
K181A
D265A
G176A

B

Specific Activity
(nmol ATP hydrolysed/min/mg)

0.0 0.5 1.0 1.5 2.0

[ATP] mM

FliI
Δ1-18
G176A
D265A
K181A
Bacterial Rotary Export ATPases are Allosterically Regulated by the Nucleotide
Second Messenger Cyclic-di-GMP
Eleftheria Trampari, Clare E. M. Stevenson, Richard H. Little, Thomas Wilhelm, David M.
Lawson and Jacob G. Malone

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