SUPPLEMENTARY INFORMATION FOR:

c-di-AMP hydrolysis by the phosphodiesterase AtaC promotes differentiation of multicellular bacteria

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EXTENDED EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

All strains used in this study are listed in Table S1. *Escherichia coli* strains were grown in LB medium under aerobic conditions at 37 °C. If required, LB was supplemented with 100 µg/ml ampicillin (Amp), 50 µg/ml kanamycin (Kan), 50 µg/ml apramycin (Apr) and/or 15 µg/ml chloramphenicol (Cam). If hygromycin B (Hyg) was used, LB agar was replaced by Nutrient Agar (NA; Roth) and LB was substituted by LBon (LB without salt) with addition of 16 µg/ml and 22 µg/ml Hyg, respectively. *S. venezuelae* strains (Table S1) were grown aerobically at 30 °C in liquid Maltose-Yeast Extract-Malt Extract (MYM) medium (1) supplemented with trace element solution (2) or on MYM agar. For growth analysis, 50 ml MYM were inoculated with spores at a final concentration of 10^6 CFU/ml and OD was measured at 578 nm. To study development, 12 µl of 10^5 CFU/µl *S. venezuelae* spores were spread as patches on MYM agar and bacteria were photographed using a Canon EOS 1300D (W) camera after 4 days of growth at 30 °C. For osmostress experiments, 10 µl of serially diluted of *S. venezuelae* spores (10^1 to

10^4 CFU/µl) were dropped on NA medium with or without 0.5 M NaCl, respectively. Plates were incubated at 30 °C and pictures were taken using a Canon EOS 1300D (W) camera. For *in vivo* interaction studies using Bacterial Adenylate Cyclase Two-Hybrid (BACTH) assays three corresponding single transformants of *E. coli* W3110 Δ*cya* were suspended in 1 ml of sterile phosphate buffered saline (PBS) and 3 µl of the resulting suspension was spotted on a plate containing MacConkey Base Agar (Difco) supplemented with Amp (100 µg/ml), Kan (50 µg/ml) and maltose (1%). The plate was photographed using a Canon EOS 1300D (W) camera after growth at 26 °C for 24 h.

Generation and complementation of *S. venezuelae* c-di-AMP mutants

Oligonucleotides and bacterial strains used for mutagenesis are listed Table S1.

Generation of vnz_27310 (ataC) deletion mutant and phage transduction. The *ataC* deletion was conducted using a modified Redirect PCR targeting protocol (3, 4). *E. coli* BW25113/pIJ790 cells induced for λ Red mediated recombination and containing cosmid PI1_F15 (with *ataC*) were transformed with PCR amplified *aac(3)IV-oriT* (*apr-oriT*) cassette extended with regions homologous to the *ataC* locus. After growth of transformants in presence of apramycin only, cosmids were isolated and re-transformed into *E. coli* W3110 with selection for Apr resistance. Correct integration of the Apr resistance cassette was verified by PCR using test primers annealing to the *ataC* flanking region (Table S1). PI1_F15 *ataC::apr* was transformed into strain ET12567/pUZ8002 and conjugated into wild type *S. venezuelae.* Bacteria were plated on SFM agar, incubated overnight at room temperature (RT), overlaid with 20 µl of 25 mg/ml nalidixic acid (Nal) and of 50 mg/ml Apr in 2 ml ddH_2O and incubated at 30 °C. *S. venezuelae* colonies were selected on NA containing Apr and Nal to remove *E. coli*. Kan^S and Apr^R mutants were confirmed by PCR.

The *ataC::apr* allele was transduced into a new wild type background via SV1 phage using a modified protocol from (5). Briefly, SV1 wild type phages were diluted and mixed with *S. venezuelae* Δ*ataC* spores. After overnight incubation at 30 °C, plates were soaked with LB at RT and phages were gathered and filtered using a 0.45 µm filter. 100 µl phages containing the *ataC::apr* allele were plated with wild type spores on MYM agar and incubated overnight at RT. For selection of desired transductants, plates were overlaid with 20 µl 50 mg/ml Apr in 2 ml ddH_2O. The *ataC* deletion was confirmed by PCR.

Generation of disA deletion mutant. The *disA* mutant was generated by transduction of the *disA::apr* allele (6) into the *S. venezuelae* wild type using SV1 phage.

Generation of $disA_{D86A}$ point mutation. The point mutation was introduced using a combination of modified Redirect PCR targeting and single stranded DNA recombineering protocols as described in (7).The Kan resistance cassette in cosmid SV-4-B12 (containing *disA*) was exchanged with *apr-oriT* extended with homologous regions to *neo*. The resulting cosmid was used to generate the *E. coli* strain HME68/SV-4-B12 *neo::apr-oriT* which was induced for λ Red recombination and electroporated with the mutagenic oligonucleotide disA_D86Achr_rev and oligo100 (8) and plated on MacConkey agar containing 1% galactose and Apr. Red, Apr^R clones were analyzed for the *disA::*$disA_{D86A}$ allele by PCR using a primer pair specific for the D86A mutation and sequencing. Purified cosmid was electroporated into ET12567/pUZ8002 and conjugated into wild type *S. venezuelae*. Single colonies were selected on NA medium containing Apr and Nal followed by growth on NA without antibiotics to achieve loss of the cosmid. Colonies sensitive to Apr were verified for the *disA* D86A mutation by sequencing.

Complementation of the deletion mutants. To complement the *disA* deletion, a DNA fragment containing the wild type allele with 528 bp upstream of the start codon (including *disA* promoter) was cloned into pIJ10770 or its derivative p3xFLAG which allow integration at the $attB_{\Phi BT1}$ site in the *S. venezuelae* chromosome. pIJ10770-*disA* and p3xFLAG-*disA*, respectively, were conjugated into *disA::apr* using *E. coli* ET12567/pUZ8002. After selection on NA medium containing Nal and Hyg, Hyg^R colonies were grown on MYM agar for spore stock generation.

The complementation of the *ataC* deletion mutant with the wild type allele was conducted similarly. Here, using an overlap PCR, a DNA fragment corresponding to 200 bp upstream of the start codon of *vnz_27305* was fused to full length *ataC* resulting in the construct *vnz_27305prom-ataC*. The fragment was cloned into pIJ10770 resulting in the plasmid pSVAL11 and introduced into the Δ*ataC* chromosome as described above. For complementation of Δ*ataC* with the D269N allele, pSVAL11 was used as template in a backbone PCR to amplify a circular construct with the primers D269N_backbone_f and D269N_backbone_r. The plasmid pIJ10770-*vnz_27305prom-ataC*$_{D269N}$ was generated, the mutation confirmed by sequencing and conjugated into Δ*ataC* as described above.

Construction of plasmids

Oligonucleotides used for cloning are listed in Table S1. *disA, vnz_31010*, *ataC* and *vnz_28055* were amplified from *S. venezuelae* genomic DNA (gDNA). D86A point mutation in *disA* was

introduced by following the four-primer/two-step PCR protocol (9). PCR products of all constructs were cloned into the pET15b vector. The *ataC*$_{D269N}$ construct was obtained using quick change site directed mutagenesis using the pET15b-*ataC* plasmid as a template. Codon optimized *ataC*$_{Spn}$ was synthesized *de novo* and cloned into pET15b via the NdeI and BamHI restriction sites by GenScript.

Full-length *cpeA* (*vnz_28055*) and *cpeB* (*vnz_28050*) excluding the respective stop codons were cloned into pUT18 and pKNT25 (Euromedex). The resulting constructs carry in-frame fusions of the sequences encoding the T18 and T25 fragments of *cyaA* from *Bordetella pertussis* to the 3' end of *cpeA* and *cpeB*, respectively. Expression of the fused genes is under control of the *lac* promoter(*lac*$_P$). To introduce *disA-FLAG* into pUT18-*vnz_28055,* the gene was PCR-amplified from the plasmid p3xFLAG-*disA* with introduction of homologous regions to *lac*$_P$ and pUT18-*vnz_28055.* Additionally, *lac*$_P$ was amplified from pUT18 with homologous regions to pUT18-*vnz_28055* and *disA-FLAG.* pUT18-*vnz_28055*, *lac*$_P$ and *disA-FLAG* were combined via Gibson Assembly (New England BioLabs) to generate the plasmid pUT18-*vnz_28055-disA-FLAG*. The resulting plasmid encodes the *cpeA-T18* fusion as well as *lac*$_P$-controlled *disA-FLAG* downstream of the T18 fragment. A quick change site directed mutagenesis using pUT18-*vnz_28055-disA-FLAG* and primers disA_D86A_fwd and disA_D86A_rev was performed to generate pUT18-*vnz_28055-disA*$_{D86A}$*-FLAG*.

Protein overexpression and purification

pET15b constructs were transformed into *E. coli* BL21 (DE3) pLysS. Rosetta (DE3) pET28-*disA*$_{Bsu}$ was directly used for overexpression. LB containing Amp and Cam (and 0.2% glucose in case of PDEs) was inoculated 1:100 with overnight cultures and grown with shaking at 37 °C. For DisA$_{Bsu}$ overexpression, Amp was replaced with 25 µg/ml Kan. Cultures were induced with a final concentration of 0.1-0.2 mM IPTG at OD_{578} between 0.5 and 0.7; cultures for PDE overexpression were supplemented with 0.35 mM $MnCl_2$ (10). Proteins were overexpressed overnight at 16 °C and shaking. Subsequently, cultures were pelleted and lysed using a FrenchPress. Strains expressing DisA variants and 6xHis-CpeA were lysed in DisA lysis buffer (20 mM Tris HCl, pH 8; 300 mM NaCl, 10% glycerol, 20 mM imidazole; 0.05% Triton X-100; 0.5 mM DTT; 5 mM $MgCl_2$) supplemented with cOmplete protease inhibitor cocktail tablets, EDTA-free (Roche). Strains expressing 6xHis-Vnz_31010, 6xHis-AtaC and 6xHis-AtaC$_{Spn}$ were lysed in PDE lysis buffer containing cOmplete protease inhibitor cocktail tablets, EDTA-free (similar to DisA lysis buffer but Tris HCl, pH 8 replaced by 20 mM Tris HCl 7.5; $MgCl_2$

replaced by 10 mM $MnCl_2$). Clarified lysate supernatants of 6xHis-tagged proteins were loaded on 0.5-1 ml 50% Ni-NTA SuperFlow (iba) overnight at 4 °C. Then, the matrix was washed with respective lysis buffers. DisA protein variants and 6xHis-CpeA were eluted with the following buffer: 50 mM Tris HCl, pH 8; 300 mM NaCl; 10% glycerol; 250 mM imidazole; 0.5 mM DTT; 5 mM $MgCl_2$. The PDE elution buffer was similar to DisA elution buffer but containing 50 mM Tris HCl, 7.5 instead of Tris HCl, pH 8 and 10 mM $MnCl_2$ instead of $MgCl_2$. Fractions containing eluted proteins (identified by Coomassie staining of 12% polyacrylamide gels) were pooled. Eluates of DisA variants and 6xHis-CpeA were dialyzed twice in DisA cyclase buffer (25 mM Tris HCl, pH 8; 250 mM NaCl, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10% glycerol (modified from (11), and tested PDEs were dialyzed twice in PDE buffer with 5-10% glycerol (20 mM Tris HCl, pH 7.5; 50 mM NaCl; 10 mM $MnCl_2$ (modified from (10) at 4 °C under stirring. Dialyzed proteins were stored at -20 °C until further use in diadenylate cyclase (DAC), differential radial capillary action of ligand (DRaCALA) or phosphodiesterase (PDE) assays.

For characterization of biophysical properties of 6xHis-AtaC and 6xHis-$AtaC_{D269N}$, Rosetta (DE3) cell pellets containing pET15b-*ataC* and pET15b-*ataC*$_{D269N}$ constructs, respectively, were resuspended in buffer A (20 mM HEPES, 300 mM NaCl, 20 mM imidazole, 10% glycerol, 0.5 mM $MnCl_2$, pH 7.5) and lyzed by sonication. After centrifugation, clear supernatant was loaded on Ni-NTA columns. The columns were washed with buffer A and proteins were eluted with buffer B (20 mM HEPES, 100 mM NaCl, 250 mM imidazole, 10% glycerol, 0.5 mM $MnCl_2$, pH 7.5). Protein elution fractions were concentrated prior to size exclusion chromatography on a HiLoad Superdex 200 column (GE Healthcare) equilibrated with buffer C (20 mM HEPES, 100 mM NaCl, 0.5 mM $MnCl_2$, pH 7.5). Pure proteins were concentrated, flash frozen in liquid nitrogen and stored at -80 °C.

c-di-AMP extraction and quantification

The nucleotide extraction protocol from (12) was adapted to *Streptomyces*. Wild type, Δ*disA* and Δ*ataC* strains were grown in 100 ml MYM. Beginning with 10 h, 5 ml samples for c-di-AMP extraction and two 1 ml samples for protein determination were taken every 2 h. c-di-AMP samples were centrifuged at 4000 rpm and 4 °C for 15 min using a swing rotor (Heraeus Megafuge 16R, Thermo Scientific), frozen in liquid nitrogen and stored at -80 °C. Protein samples were centrifuged at max. speed and stored at -20 °C.

For c-di-AMP extraction, samples were suspended in 800 µl Extraction mixture II (acetonitrile/methanol/water [2:2:1]), transferred into 2 ml screw cap tubes prefilled with 0.1

mm silica beads (Biozym), shock frozen for 15 s in liquid nitrogen and heated for 10 min at 95 °C. After cooling on ice, samples were disrupted using the BeadBlaster at 4 °C with 2 cycles at 6 m/s for 45 s and 2 min interval. Samples were cooled for 15 min on ice and centrifuged at max. speed and 4 °C for 15 min. Supernatants were transferred into a 2 ml reaction tubes. Remaining pellets were suspended in 600 µl Extraction mixture I (acetonitrile/methanol [1:1]), pulsed two times for 30 s at 6 m/s with a 60 s interval, incubated on ice and centrifuged as above. The extraction with 600 µl Extraction mixture I was repeated once. All supernatants (~2 ml) were combined and stored for protein precipitation for two days at -20 °C. Precipitated proteins were removed by centrifugation and the precipitation step was repeated. Finally, samples were air dried in a SpeedVac Plus SC110A connected to Refrigerated Vapor Trap RVT100 (Thermo Scientific) at low temperature settings and analyzed using LC-MS/MS as described in (12).

Samples for protein quantification were suspended in 800 µl 0.1 M NaOH, transferred into 2 ml screw cap tubes prefilled with 0.1 mm silica beads (Biozym) and heated for 10 min at 98 °C. Cell lysis was performed in BeatBlaster with 2 pulses for 30 s at 6 m/s and an interval of 2 min. Lysates were centrifuged at max. speed and 4 °C for 15 min. Supernatant was saved and the extraction step was repeated. Supernatants were combined and protein concentration was determined via Bradford using Roti-Quant.

For normalization of c-di-AMP concentration to the protein amount, following formula was used:

$$\frac{c\text{-}di\text{-}AMP\ [nM] \cdot 200\ \mu l}{cV\ [ml] \cdot c590\ [\frac{\mu g}{ml\ cells}]} = \frac{c\text{-}di\text{-}AMP\ [pmol]}{protein\ [mg]}$$

Isothermal Titration Calorimetry

ITC experiments were performed using a Malvern PEAQ-ITC system with 21 µM protein in ITC buffer (20 mM HEPES, pH = 7.5; 100 mM NaCl) in the cell. The respective nucleotides (210 µM) were titrated into the cell by 19 injections of 2 µl, spaced 150 s apart, at 25 °C. The data was analyzed using the MicroCal PEAQ-ITC analysis software provided with the instrument. All titrations were repeated to confirm robustness of the assay.

Size-exclusion chromatography (SEC) coupled static light scattering and analytical SEC

Determination of the molecular weight of AtaC was performed using a 24 ml Superdex S200 increase size-exclusion column (GE Healthcare Lifesciences) connected to a multi-angle laser light scattering and a refractive index monitor (WYATT miniDAWN TREOS, WYATT

Optilab T-rEX). Data were analyzed using the ASTRA software package provided with the instrument (Wyatt). Static light scattering data of CpeA samples were obtained using the same column connected to a ÄKTAmicro system (GE Healthcare Lifesciences) equipped with Malvern/Viscotek RI and RALS (right-angle laser scattering) devices (as described in (13)). Data evaluation was done using the OmniSEC software package. Analytical SEC runs were performed on an ÄKTAmicro system and a 24 ml Superdex S75 column.

Surface plasmon resonance

SPR experiments were conducted using a Biacore X100+ (GE Healthcare Lifesciences) at 25 °C. 2'-Biotin-16-c-di-AMP (BioLog) was immobilized on a SA-chip (GE Healthcare Lifesciences) in HPS-EP+ buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% v/v Surfactant P20) to a final RU of 150. The analyte (CpeA) was injected at concentrations of 1-100 µM (dimer concentration) in HPS-EP+ with a contact time of 120 s. Between every injection the chip was regenerated by 30 s injections of 6 M Urea and 2 M NaCl. The resulting sensorgrams were analysed by the Biacore evaluation software. Steady state report point for data evaluation was chosen 5 seconds prior to injection stop.

Heat resistance assay

Spore heat resistance was assessed as previously described (5). ~10^9 CFU/ml spores were shocked at 50 °C for 1 h. Spores were serially diluted for colony forming unit (CFU) determination. The ratio of CFU after heat shock versus CFU before the heat shock was used to calculate the survival rate for each strain.

Bioinformatic characterization of AtaC and its abundance in prokaryotes

A local PATRIC database was installed and used to determine the conservation of AtaC (PGF_00172869) across prokaryotes (Dataset S1). Duplicated entries with identical genome were removed (177 in total), but keeping the first entry. An in-house python script was used to extract taxonomic information for each of the AtaC homologues. Specifically, the accession number of each species was used to access NCBI taxonomy and the taxonomic information was integrated with the original PATRIC table. Only phyla with more than 5 genomes were kept, and entries with no taxonomic information were excluded from the analysis.

Table S1. Strains, plasmids and oligonucleotides used in this study

	Genotype or comments	**Source or reference**
Strains		
S. venezuelae		
NRRL B-65442	Wild type	(NCBI Reference Sequence: NZ_CP018074.1)
disA::apr	ATCC 10712 *SVEN_3211::aac(3)IV*; Apr^R	(6)
SVAL5	Δ*disA::apr, attB$_{\Phi BT1}$*::p3xFLAG-*disA*; Apr^R, Hyg^R	This study
SVAL8	*disA::disA$_{D86A}$*	This study
SVAL19	Δ*disA* (SV1-transduction); Apr^R	This study
SVAL20	Δ*vnz_27310::apr* (Redirect); Apr^R	This study
SVAL22	Δ*ataC::apr* (SV1-transduction from SVAL20); Apr^R	This study
SVAL24	Δ*disA::apr, attB$_{\Phi BT1}$*::pIJ10770-*disA*; Apr^R, Hyg^R	This study
SVAL26	Δ*ataC::apr, attB$_{\Phi BT1}$*::pIJ10770-*vnz_27305prom-ataC*; Apr^R, Hyg^R	This study
SVAL27	Δ*ataC::apr, attB$_{\Phi BT1}$*::pIJ10770-*vnz_27305prom-ataC$_{D269N}$*; Apr^R, Hyg^R	This study
E. coli		
W3110	K-12 derivative; *F-, λ- ,rpoS(Am), rph-1, Inv(rrnD-rrnE)*	(14)
W3110 Δ*cya*	W3110 derivative with deleted adenylate cyclase	(15)
ET12567/pUZ8002	*dam*, *dcm*, *hsd*; Kan^R, Cm^R	(16)
BW25113/pIJ790	(Δ(*araD-araB*)*567*, Δ*lacZ4787*(::*rrnB-4*), *lacIp-4000*($lacI^Q$), λ-, *rpoS369*(Am), *rph-1*, Δ(*rhaD-rhaB*)568, *hsdR514*; Cm^R	(17)

BL21 (DE3) pLysS	F– *ompT hsdS*(rB– mB–) *gal dcm* λ(DE3), Cm^R	Promega
HME68	W3110 Δ(*argF-lac*)*U169 galKtyr145UAG mutS<>cat*	(18)
Rosetta (DE3) pET28-$disA_{Bsu}$	Overexpression of *Bacillus subtilis* DisA; Kan^R, Cm^R	(19)
Rosetta 2 (DE3)	F^- *ompT* $hsdS_B(r_B^-\ m_B^-)$ *gal dcm* (DE3) pRARE2 (Cam^R)	Novagen
Plasmids		
pIJ773	Plasmid template for amplification of the *apr-oriT* cassette for 'Redirect' PCR-targeting; Apr^R	(3)
pIJ790	Modified λRED recombination plasmid [*oriR101*] [*repA101*(ts)] *araBp-gam-be-exo*; Cm^R	(3)
pIJ10770	pMS82 derivative; Hyg^R	(20)
pUZ8002	RP4 derivative with defective oriT; Kan^R	(16)
p3xFLAG	pIJ10770 derivative containing *3xFLAG* sequence downstream of MCS; Hyg^R	(21)
pGEX_6P_1	T7 expression vector (modified MCS); Amp^R	GE Healthcare
pET15b	T7 expression vector; Amp^R	Novagen
pKNT25	Low copy vector encoding the T25 fragment of *Bordetella pertussis cyaA* downstream of the MCS; Kan^R	Euromedex
pUT18	High copy vector encoding the T18 fragment of *B. pertussis cyaA* downstream of the MCS; Amp^R	Euromedex
pECAL1	pET15b-*disA*; Amp^R	This study
pECAL4	pET15b-$disA_{D86A}$; Amp^R	This study
pECAL12	pET15b-*ataC*; Amp^R	This study
pECAL13	pET15b-*vnz_31010*; Amp^R	This study

pECAL16	pET15b-*ataC*$_{Spn}$; Amp^R	This study
pECAL17	pET15b-*vnz_28055 (cpeA)*; Amp^R	This study
pECAL18	pKNT25-*vnz_28050 (cpeB)*; Kan^R	This study
pECAL19	pUT18-*vnz_28055 (cpeA)*; Amp^R	This study
pECAL20	pUT18-*vnz_28055-disA*-FLAG; Amp^R	This study
pECAL21	pUT18-*vnz_28055-disA*$_{D86A}$*-FLAG*; Amp^R	This study
pSVAL6	pIJ10770-*disA*; Hyg^R	This study
pSVAL11	pIJ10770-*vnz_27305prom-ataC* (200 bp upstream of *vnz_27305* start codon fused to *ataC*); Hyg^R	This study
pSVAL12	pIJ10770-*vnz_27305prom-ataC*$_{D269N}$ (pSVAL11 derivative); Hyg^R	This study
pSVNT-10	p3xFLAG-*disA*; Hyg^R	This study
pDD29	pET15b-*ataC*$_{D269N}$ (pECAL12 derivative); Amp^R	This study

Underlined nucleotides indicate restriction sites, nucleotides in bold represent introduced mutations and nucleotides in italics indicate sequences overlapping to other genes

Oligonucleotide	Sequence
Oligonucleotides used for generation of the chromosomal *disA* D86A point mutation, PCR verification and sequencing	
disA_D86Achr_rev	TCTTGGTGATGTCCTTGTCGAGGACGAGCGCGCC**CG**CGAGCTTGCAC AGCTCCCGCAGCCGCGTGGCGGC
disA_D86A_check_fwd	GGAGCTGTGCAAGCTCGCG
disA_fwd_NdeI	TATCATATGGTGGCAGCCAAGGAC
disA_rev_XhoI	TATCTCGAGCTAGACGTACCGCTCAAG
Oligonucleotides used for verification of *disA* deletion	
disA_test_f	GTGGTTCACTCACGCCGCATGAACGGTTC

disA_test_r	GGCACGTACCTGGTGGAGGCGAAGGTG
Oligonucleotides used for complementation of *disA* deletion with *disA-3xFLAG*	
3142_NdeI-for	GCTGCATATGGGCCGGCGGGTCG
3142_XhoI-rev	GCAGCCTCGAGGACGTACCGCTCAAGGATC
Oligonucleotides used for complementation of *disA* deletion with wild type *disA*	
3142_NdeI-for	GCTGCATATGGGCCGGCGGGTCG
disA_rev_XhoI	TATCTCGAGCTAGACGTACCGCTCAAG
Oligonucleotides used for *vnz_27310 (ataC)* deletion and PCR verification	
27310_fwd_Apra	CGAAGCGATCGCGGCCACCGCCGCGCCCACCCGCTGATG*ATTCCGGG GATCCGTCGACC*
27310_rev_Apra	GGTCGTGGGGGGGGAGGAGACGGGTGAGGAGTGGGCTCA*TGTAGGC TGGAGCTGCTTC*
27310_test_f	ACACCGTGCGGCAGACCC
27310_test_r	TTCCCGCAGTCCATGGTTCC
Oligonucleotides used for complementation of *ataC* deletion (sequences overlapping to putative promoter in italics)	
27305prom_f_NdeI	TATCATATGGGTGTCCCGGCTCGTCGAC
27305prom_r_OL_ataC	GCTGCACCAT*GGACTCCATCCTACGGGGCT*
ataC_f_OL_27305prom	*GATGGAGTCC*ATGGTGCAGCCGACCGCCGT
5409_rev_XhoI	TATACTCGAGTCAGGTGCGGACTTCGAG
Oligonucleotides used for generation of *pIJ10770-vnz_27305prom-ataC*$_{D269N}$	
D269N_backbone_f	CGGCGCTGTACGTCACGGCC**A**ACCACGGCATGGTCGACAT
D269N_backbone_r	ATGTCGACCATGCCGTGGT**T**GGCCGTGACGTACAGCGCCG
Oligonucleotides used for generation of pET15b overexpression constructs	

disA_fwd_NdeI	TATCATATGGTGGCAGCCAAGGAC
disA_rev_XhoI	TATCTCGAGCTAGACGTACCGCTCAAG
disA_D86A_fwd	GCAAGCTCG**CG**GGCGCGCTC
disA_D86A_rev	GAGCGCGCC**CG**CGAGCTTGC
5409_fwd_NdeI	TATCATATGATGGTGCAGCCGACCG
5409_rev_XhoI	TATACTCGAGTCAGGTGCGGACTTCGAG
6143_fwd_NdeI (for *vnz_31010*)	TATCATATGGTGATCGTCATCGCCCATGT
6143_rev_BamHI (for *vnz_31010*)	TATGGATCCTCAGACCGGCACGGTC
28055_fwd_NdeI	TAT CATATG GTGCCTGCTCCACGGATG
28055_rev_XhoI	TATA CTCGAG TCACTCCCGTCCGAGTATGG
Oligonucleotides used for generation of the pET15b-*ataC*$_{D269N}$ construct	
DD60AtaC(D269N)_fwd	GTACGTCACGGCC**A**ACCACGGCATGGTCGA
DD61AtaC(D269N)_rev	GGCCGTGACGTACAGCGCCGAGC
Oligonucleotides used for generation of the constructs for bacterial two hybrid interaction studies	
28050_NT25f_XbaI	TATTCTAGACGTGCATTCCGCTCTGTTCCT
28050_NT25r_KpnI	TATGGTACCCGCGCGCGGCCGGCCGGATCCT
28055_T18f_KpnI	TATGGTACCGCCTGCTCCACGGATGAGC
28055_T18r_EcoRI	TAGCAGAATTCGACTCCCGTCCGAGTATGGAGG
T18RCKC_disAFLAG_f	*cgacaagtag*TATGGTGCACTCTCAGTAC
T18RCKC_lacp_r	*gtgccagctg*TTACTTAGTTATATCGATTGGC
lacp_T18RCKC_f	*aactaagtaa*CAGCTGGCACGACAGGTTTC
lacp_disAFLAG_r	*ccttggctgc*CATAGCTGTTTCCTGTGTGAAATTGTTATC

disAFLAG_lacp_f	*aacagctatg*GCAGCCAAGGACGGGGCA
disAFLAG_T18RCKC_r	*gtgcaccata*CTACTTGTCGTCATCGTCCTTGTAGTCG
T18RCKC_testFLAG_f	gagcggacgttcgaagttct
T18RCKC_testFLAG_r	cggtcacagcttgtctgtaa

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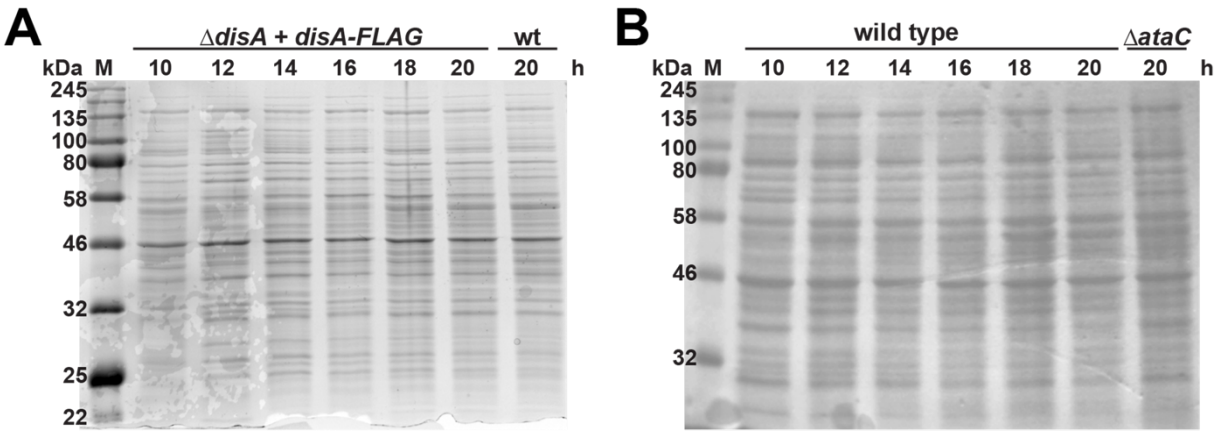


Figure S1. Loading controls for Western blot analysis of DisA-FLAG and AtaC expression patterns.

(A) C-terminally FLAG-tagged *disA* was introduced using the integrative pIJ10770 vector into Δ*disA* strain and expressed under the control of its native promoter from the $attB_{\Phi BT1}$ integration site.

(B) Wild type *S. venezuelae* was used for AtaC analysis.

Strains were grown in liquid MYM at 30 °C and samples were taken every 2 hours after indicated time of growth (numbers above the lanes). *S. venezuelae* wild type and Δ*ataC* samples harvested after 20 hours of growth served as negative controls in the relevant assays. Whole cell lysates were prepared as described in (21). 5 and 10 µg of whole cell proteins were used for DisA-FLAG and AtaC detection, respectively. For each Western blot analysis, the proteins were separated on two SDS polyacrylamide gels that were run in parallel in SDS buffer at 50 mA for 40-50 min. One gel was stained with Coomassie and used as loading control (Figure S1A, B). The other gel was used for Western blotting and detection of DisA-FLAG with anti-FLAG as primary antibody (Sigma) (Figure 1C) or AtaC with anti-AtaC antiserum as primary antibody (generated by Pineda GmbH using purified 6xHis-AtaC) (Figure 4B).

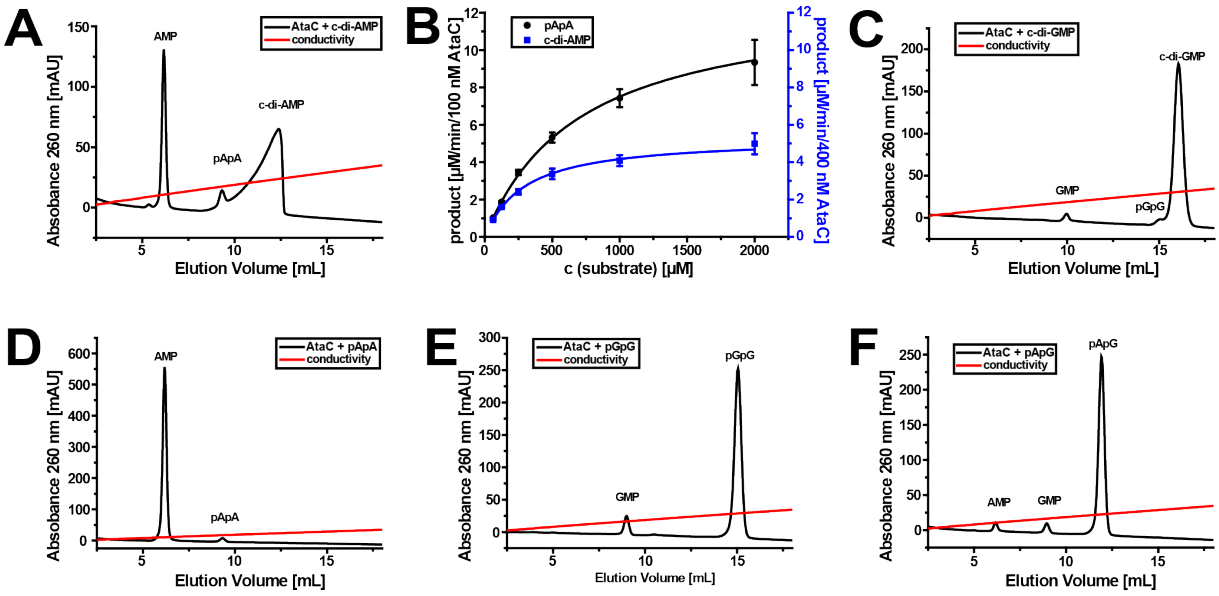


Figure S2. Hydrolysis activity of AtaC is specific for adenosine bases.

(A) Ion-exchange chromatography run on a Resource Q column of the reaction products after 1 h incubation from a 100 µl reaction containing 100 nM AtaC + 250 µM c-di-AMP.

(B) Michaelis-Menten kinetics of the reactions from 400 nM AtaC + c-di-AMP (62.5 – 2000 µM) and 100 nM AtaC + 5′-pApA (62.5 – 2000 µM) after 1 h of incubation at 37 °C. c-di-AMP, K_M= 285 ± 32 µM, k_{cat}= 0.2 s^{-1}; 5′-pApA, K_M= 698 ± 32 µM, k_{cat}= 2.1 s^{-1}. Shown are mean values of n=3 independent experiments and standard deviation.

(C) Ion-exchange chromatography run on a Resource Q column of the reaction products after 1 h incubation from a 100 µl reaction containing 100 nM AtaC + 250 µM c-di-GMP.

(D) Ion-exchange chromatography run on a Resource Q column of the reaction products after 1 h incubation from a 100 µl reaction containing 100 nM AtaC + 250 µM c-di-AMP.

(E) Ion-exchange chromatography run on a Resource Q column of the reaction products after 1 h incubation from a 100 µl reaction containing 100 nM AtaC + 250 µM 5′-pGpG.

(F) Ion-exchange chromatography run on a Resource Q column of the reaction products after 1 h incubation from a 100 µl reaction containing 100 nM AtaC + 250 µM 5′-pApG.

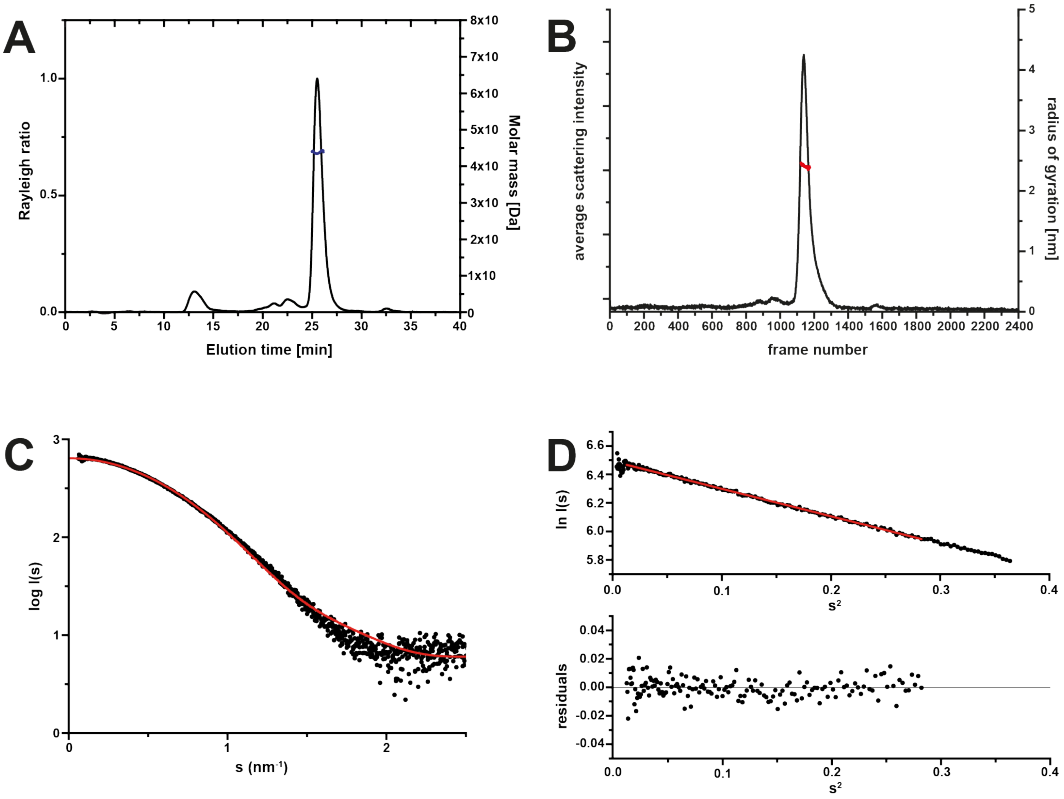


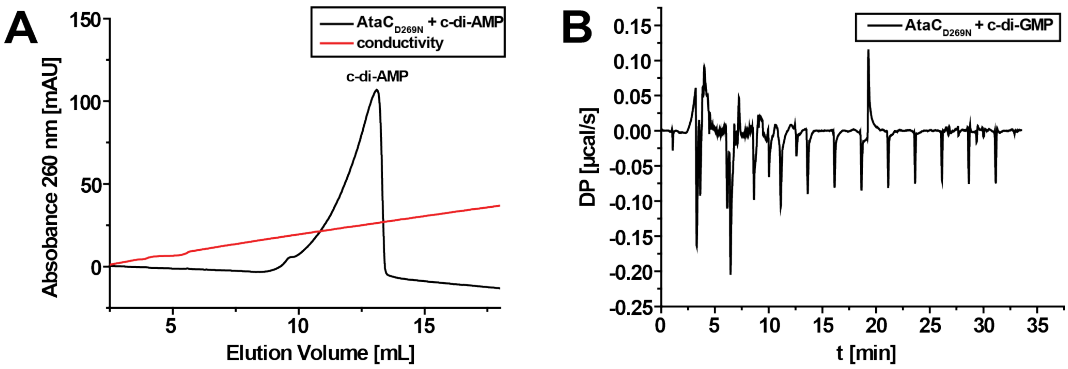
Figure S3. AtaC is a monomer in solution.

(A) Molecular weight determination of AtaC by SEC coupled multi-angle laser light scattering. The obtained molecular weight is 43.7 kDa and stable for the main protein peak at 25 ml.

(B) shows the relative scattering intensity of the sample during a size-exclusion coupled SAXS run at EMBL-P12 using a 24 ml Superdex increase S200 10/300 column (Intensity vs. frame No.). The respective estimated radius of gyration for each frame in the main peak is shown in red (right Y-axis).

(C) Measured SAXS curve of AtaC in 200 mM NaCl, 20 mM HEPES pH 7.5 and a theoretical scattering curve (red) of the model of AtaC using PhnA as template (obtained from HHpred/MODELLER (22), χ^2=3.6).

(D) Guinier plot ln I(s) vs. s² (top part) of the averaged buffer corrected scattering data (from B) and the respective residuals of the linear regression (R_G= 2.41 ± 0.1nm). The equally distributed errors of the linear regression (for s*R_G<1.3, Guinier approximation) indicates that the sample is not aggregating.



277

278 **Figure S4. AtaC$_{D269N}$ does not cleave c-di-AMP and does not bind c-di-GMP.**

279 (A) Ion-exchange chromatography run on a Resource Q column of the reaction products after

280 1 h incubation from a 100 µl reaction containing 1 µM AtaC$_{D269N}$ + 250 µM c-di-AMP.

281 (B) ITC measurement of 20 µM AtaC titrated with 140 µM c-di-GMP. No binding was

282 detected.

283

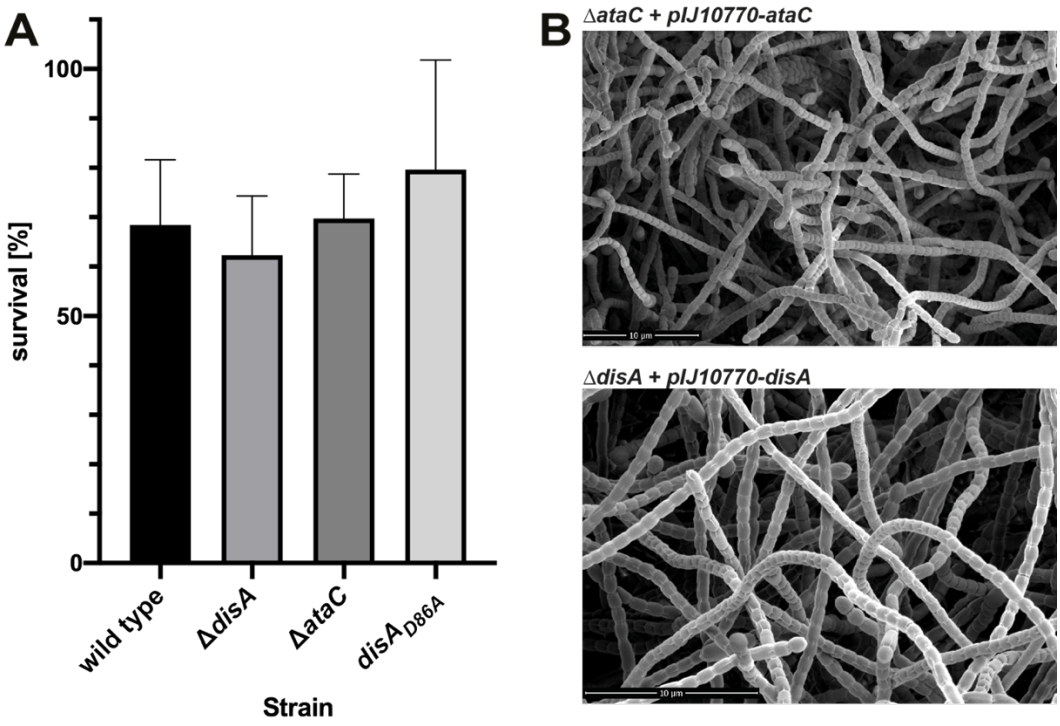


Figure S5. Viability of spores produced by c-di-AMP mutants and complementation of the Δ*ataC* and Δ*disA* strains with the wild type alleles.

(A) Deletion or inactivation of c-di-AMP metabolizing enzymes has no impact on spore viability of the respective mutants. Spores (~10^9 CFU/ml) were heat shocked for 1 h at 50 °C. Spores were serially diluted and colony forming units (CFU) were determined. The survival of each strain was assessed by the ratio CFU after the heat shock and CFU before the heat shock. Data are presented as the mean of CFU counts from at least three different dilutions ± standard deviation and show representative data of three independent experiments.

(B) Upper panel: scanning electron micrographs show that expression of *ataC* from the $attB_{\Phi BT1}$ site under the control of the native promoter from pIJ10770 complemented the delayed developmental phenotype of the *ataC* mutant. Lower panel: Expression of *disA* from pIJ10770 in the *disA* mutant did not alter the wild type phenotype of the mutant. For comparison see also Figure 5B in the main text. Cells were grown on MYM for 4 days at 30 °C.

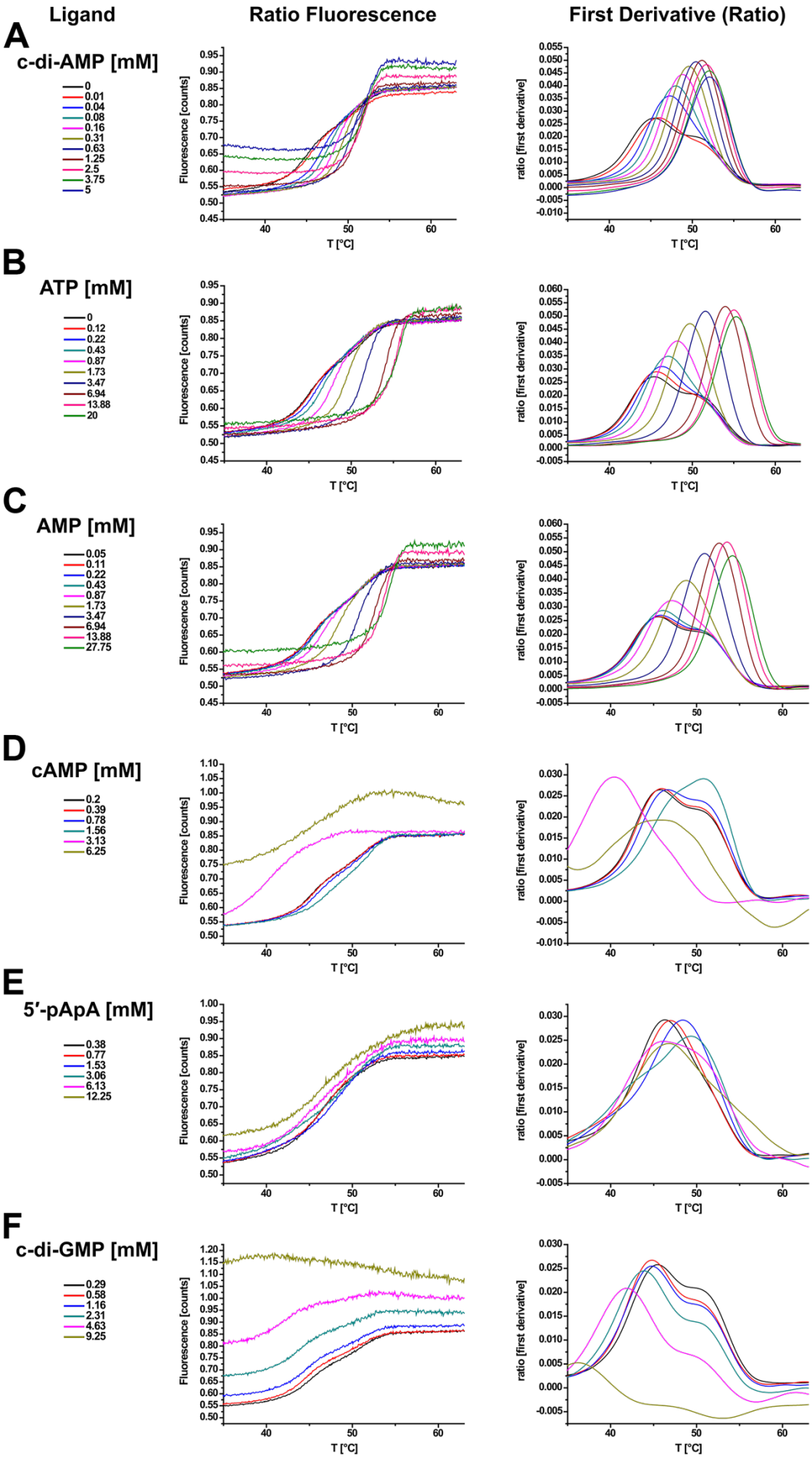

Ligand
Ratio Fluorescence
First Derivative (Ratio)
A
c-di-AMP [mM]
0
0.01
0.04
0.08
0.16
0.31
0.63
1.25
2.5
3.75
5
B
ATP [mM]
0
0.12
0.22
0.43
0.87
1.73
3.47
6.94
13.88
20
C
AMP [mM]
0.05
0.11
0.22
0.43
0.87
1.73
3.47
6.94
13.88
27.75
D
cAMP [mM]
0.2
0.39
0.78
1.56
3.13
6.25
E
5′-pApA [mM]
0.38
0.77
1.53
3.06
6.13
12.25
F
c-di-GMP [mM]
0.29
0.58
1.16
2.31
4.63
9.25
Fluorescence [counts]
ratio [first derivative]
T [°C]

Figure S6. CpeA preferentially binds c-di-AMP in nanoDSF.

nanoDSF thermal shift assays at a heating rate of 1.5 °C/min: concentration legend (left panel), raw data ratio fluorescence (middle panel), raw data first derivative of ratio (right panel).

(A) nanoDSF thermal shift assay of 20 µM CpeA with different concentrations of c-di-AMP (0 - 5 mM).

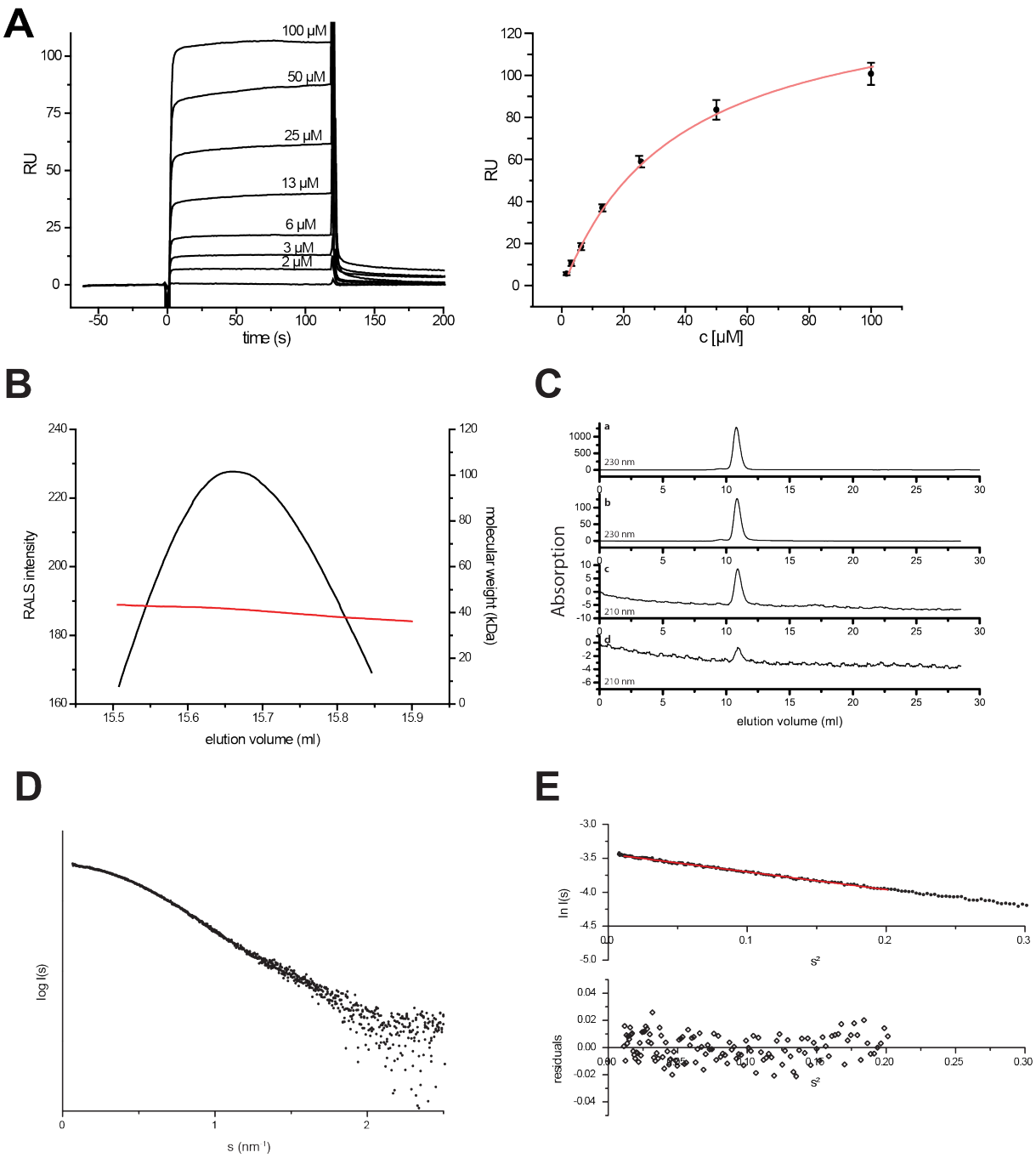
(B) nanoDSF thermal shift assay of 20 µM CpeA with different concentrations of ATP (0 - 20 mM).

(C) nanoDSF thermal shift assay of 20 µM CpeA with different concentrations of AMP (0.05 - 27.75 mM).

(D) nanoDSF thermal shift assay of 20 µM CpeA with different concentrations of cAMP (0.2 - 6.25 mM).

(E) nanoDSF thermal shift assay of 20 µM CpeA with different concentrations of 5′-pApA (0.38 - 12.25 mM).

(F) nanoDSF thermal shift assay of 20 µM CpeA with different concentrations of c-di-GMP (0.29 - 9.25 mM).



316

317 **Figure S7. CpeA binding of c-di-AMP and CpeA *in vitro* properties.**

318 (A) left panel: SPR sensorgram of biotin-c-di-AMP immobilized on a SA-chip with injections
319 of 1-100 µM dimeric CpeA. Right panel: Binding curve fit of the steady state response units
320 for the titration of CpeA (dimer) and 2'-biotin-C_{16}-c-di-AMP (one-site binding model, K_D =
321 37 ± 2.9 µM, R^2=0.997) determined by SPR at 25 °C.
322 (B) shows the (only) peak of a size-exclusion coupled light scattering experiment using a CpeA-
323 sample (5 mg/ml CpeA) on loaded onto a Superdex S200 increase 10/300 column equilibrated

in buffer 20 mM Tris HCl pH 8, 200 mM NaCl, 3% glycerol, 1 mM DTT and the respective molecular weight curve (red) for the peak. The average molecular weight for the peak fraction is $M_W^{SECRALS}$=40.2 kDa and stable for the whole peak region indicating that CpeA forms a dimer in solution.

(C) shows analytical size exclusion runs of different concentrations of CpeA (injected 100 µl of a) 5 mg/ml, b) 0.5 mg/ml, c) 0.03 mg/ml, and d) 0.005 mg/ml) loaded on a Superdex S75 10/300 column. The dimer peak at $V_{elution}$=10.8 ml is stable for all concentrations indicating no detectable dissociation in this concentration regime.

(D) shows the small-angle X-ray scattering curve of a 1.6 mg/ml CpeA sample (in 200 mM NaCl, 30 mM NaP_i pH 6.5, 5% v/v glycerol),

(E) the respective Guinier Plot ln(I) vs s^2 and residuals of the linear regression region (red dots). The molecular weight of CpeA determined from SAXS is Mw=39.1 kDa with a radius of gyration of R_G= 2.7 nm (extrapolated from a concentration series to c=0 mg/ml, the R_G for the curve shown in (C+D) is $R_G^{c=1.6mg/ml}$= 2.78 nm).

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