Title: Complex structure of the *Bacillus subtilis* CdaA c-di-AMP cyclase domain and the phosphoglucomutase GlmM

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Abstract

Cyclic-di-adenosine monophosphate (c-di-AMP) is an important nucleotide signalling molecule, which plays a key role in osmotic regulation in bacteria. Cellular c-di-AMP levels are tightly regulated, as both high and low levels have a negative impact on bacterial growth. Here, we investigated how the activity of the main *Bacillus subtilis* c-di-AMP cyclase CdaA is regulated by the phosphoglucomutase GlmM. c-di-AMP is produced from two molecules of ATP by proteins containing a deadenylate cyclase (DAC) domain. CdaA is a membrane-linked cyclase with an N-terminal transmembrane domain followed by the cytoplasmic DAC domain. Here we show, using the soluble catalytic *B. subtilis* $CdaA_{CD}$ domain and purified full-length GlmM or the $GlmM_{F369}$ variant lacking the C-terminal flexible domain 4, that the cyclase and phosphoglucomutase form a stable complex *in vitro* and that GlmM is a potent cyclase inhibitor. We determined the crystal structure of the individual *B. subtilis* $CdaA_{CD}$ and GlmM proteins, both of which form dimers in the structures, and of the $CdaA_{CD}$:$GlmM_{F369}$ complex. In the complex structure, a $CdaA_{CD}$ dimer is bound to a $GlmM_{F369}$ dimer in such a manner that GlmM blocks the oligomerization of $CdaA_{CD}$ and formation of active head-to-head cyclase oligomers, thus providing molecular details on how GlmM acts as cyclase inhibitor. The function of a key amino acid residue in $CdaA_{CD}$ in complex formation was confirmed by mutagenesis analysis. As the amino acids at the $CdaA_{CD}$:GlmM interphase are conserved, we propose that the observed inhibition mechanism of CdaA by GlmM is conserved among Firmicutes.

Introduction

Nucleotide signalling molecules play important roles in helping bacteria to rapidly adapt to changing environmental conditions (1,2). One such signalling nucleotide, cyclic-di-adenosine monophosphate (c-di-AMP), which was discovered a little more than a decade ago (3), plays an important function in the osmotic regulation of bacteria by controlling potassium and osmolyte uptake (4-8). c-di-AMP also plays an important function in regulating cell size, either directly or indirectly through its function in osmotic regulation, cell-wall integrity and susceptibility to beta-lactam antibiotics, which target the synthesis of the peptidoglycan cell wall (9-12).

The function of c-di-AMP has been most extensively studied in a range of Firmicutes bacteria including the Gram-positive model organism *Bacillus subtilis* and Gram-positive bacterial pathogens such as *Staphylococcus aureus, Listeria monocytogenes* and several *Streptococcus* species (9,10,13-17). From these studies, it has become apparent that the cellular level of c-di-AMP needs to be tightly regulated as both an excess and a lack of c-di-AMP can negatively impact bacterial growth, physiology and virulence (17,18). To achieve the optimal level, a dynamic equilibrium must exist between the synthesis of c-di-AMP via diadenylate cyclases and its degradation into 5′-phosphadenylyl-adenosine (pApA) or two molecules of AMP by phosphodiesterases (18-20). As part of the current study, we investigated how the activity the *B. subtilis* c-di-AMP cyclase CdaA is regulated by GlmM, a phosphoglucomutase enzyme required for the synthesis of an essential peptidoglycan precursor.

c-di-AMP is formed from two molecules of ATP by enzymes containing a diadenylate cyclase (DAC) domain. These enzymes have been extensively characterized structurally as well as biochemically, but how their activity is regulated is an aspect that remains poorly understood. *B. subtilis* codes for three diadenylate cyclase enzymes (3,21-24). CdaA (also referred to as DacA in some bacteria) is a membrane-bound cyclase with three predicted N-terminal transmembrane helices and a cytoplasmic catalytic DAC domain. CdaA (DacA) is the "housekeeping" c-di-AMP cyclase in Firmicutes, as it is conserved and often the sole c-di-AMP cyclase in phylum (25,26). The two other *B. subtilis* c-di-AMP cyclases, DisA and CdaS, are soluble proteins, not as widely distributed among bacteria and have more specialized functions, with DisA involved in controlling DNA-repair processes during sporulation or spore outgrowth and CdaS also specifically expressed during the sporulation process (22,23,27). While there are no publications on the 3D-structures of the *B. subtilis* c-di-AMP cyclases, structures are available for the cytoplasmic enzymatic domains of the *L. monocytogenes* and *S. aureus* CdaA/DacA homologs (21,28), the DisA homolog from *Thermotoga maritima* (3) and the CdaS homolog from *B. cereus* (PDB 2FB5). These studies revealed that DAC domains have a mixed αß-fold, with highly conserved DGA and RHR amino acid motifs required for ligand binding (3,21,29). Formation of c-di-AMP requires a head-to-head conformation of two DAC domains. This was first demonstrated in the crystal structure of DisA, a protein which forms an octamer with four DAC domain dimers in the active head-to-head conformation (3). While also the *L. monocytogenes* and *S. aureus* CdaA/DacA catalytic

domains and the CdaS protein, where present as dimers and hexamers, respectively, they were in an inactive conformation. These proteins therefore either need to rearrange or more likely form higher oligomers in order to yield active enzymes with DAC domains in the head-to-head dimer conformation (24,28). Recently, another structure of the cytoplasmic catalytic domain of the *L. monocytogenes* CdaA enzyme (Δ100CdaA) has been reported with a c-di-AMP bound between two monomers, which based on the crystal cell packing, were arranged in an active dimer of dimer configuration (30). These finding are consistent with the idea that CdaA (DacA) enzymes will need to form higher oligomers to achieve an active enzyme configuration. Hence, factors influencing the ability of c-di-AMP cyclases to rearrange into an active conformation or to form higher oligomers will be able to regulate the activity of these enzymes.

The genetic arrangement and operon structure coding for the "housekeeping" c-di-AMP cyclase CdaA (DacA) is conserved in Firmicutes (29,31). Two genes, coding for the membrane-linked CdaA regulator CdaR (also named YbbR in some bacteria) and cytoplasmically-located peptidoglycan precursor synthesis enzyme GlmM, are found downstream and in an operon with *cdaA* (29,31). Through recent studies in *B. subtilis, L. monocytogenes, Lactococcus lactis* and *S. aureus,* it is has become apparent that these three genes are not only co-transcribed but that the encoded proteins also from a complex and that CdaR and GlmM can regulate the activity of the c-di-AMP cyclase CdaA (29,32,33). While CdaR has been reported to function as both an activator and repressor of CdaA activity depending on the growth conditions, GlmM has been shown to be a potent inhibitor of the cyclase activity (17,29,31,32,34). However, the molecular mechanisms on how the CdaA cyclase activity is regulated by these proteins are not yet known and this was further investigated as part of this study.

GlmM is a phosphoglucomutase enzyme catalysing the conversion of glucosamine-6-phosphate to glucosamine-1-phosphate, which is subsequently used to produce the essential peptidoglycan precursor UDP-N-acetyl-glucosamine (35). In *B. subtilis, L. monocytogenes* and *L. lactis* a protein-protein interaction between CdaA and GlmM has been detected using bacterial two-hybrid assays performed in *Escherichia coli* (29,31,33). In *B. subtilis* this interaction has been further confirmed by *in vivo* protein cross-linking and pulldown assays (29) and in *L. monocytogenes* by the co-elution of purified proteins (33). The first evidence that that GlmM serves as negative regulator of CdaA/DacA activity came from an *L. lactis* strain that produces a GlmM variant that is thought to form a stronger interaction with CdaA; this strain produces lower cellular c-di-AMP levels than the bacteria expressing wildtype GlmM (31). Furthermore, the activity of the soluble recombinant *S. aureus* DacA catalytic domain ($DacA_{CD}$) could be blocked almost completely by the addition of purified GlmM protein in *in vitro* assays and the recombinant proteins were shown to form a stable complex that could be purified via size-exclusion chromatography (28). Additional mass-spectrometry and small-angle X-ray scattering data (SAXS) analyses suggested that the complex is composed of a $DacA_{CD}$ dimer and a GlmM dimer (28). Crystal structures of the individual *S. aureus* $DacA_{CD}$ and GlmM dimers revealed that the *S. aureus* $DacA_{CD}$ protein assumed an "inactive" dimer conformation. GlmM had the typical four-domain fold of phosphoglucomutases with a flexible C-terminal domain 4 and the dimer was "M-shaped", characteristic for this class of enzymes (28). However, a high-resolution structure of the complex could not be obtained and only a model for the complex could be proposed by fitting the individual $DacA_{CD}$ and GlmM dimer structures into the SAXS envelope (28). Based on this, a model was proposed in which GlmM could potentially block the activity of the $DacA_{CD}$ cyclase by preventing the formation of higher oligomers.

Here, we set out to provide atomic resolution information on the CdaA:GlmM complex to gain insight into the molecular mechanism how GlmM can control the activity of the c-di-AMP cyclase enzyme. Using the purified *B. subtilis* CdaA catalytic domain ($CdaA_{CD}$) and purified full-length GlmM or the truncated $GlmM_{F369}$ variant lacking the flexible C-terminal domain 4, we show that the two proteins form a stable complex *in vitro* and that GlmM and $GlmM_{F369}$ are potent inhibits of the cyclase. Crystal structures of the *B. subtilis* $CdaA_{CD}$ cyclase, the GlmM phosphoglucomutase and the $CdaA_{CD}$:$GlmM_{F369}$ complex were obtained, revealing dimer conformations of the individual proteins as well as a dimer of dimer conformation in the complex structure. More importantly, from the complex structure the mechanism by which binding of GlmM inhibits the cyclase activity becomes apparent, that is by preventing the oligomerisation of CdaA and formation of active head-to-head cyclase oligomers.

140 **Results**

141 **The *B. subtilis* phosphoglucosamine GlmM interacts with and inhibits the activity of the c-di-AMP**
142 **cyclase $CdaA_{CD}$**

143 Using the purified *S. aureus* $DacA_{CD}$ catalytic domain and GlmM, it has been shown that the proteins
144 form a stable complex *in vitro* and that GlmM is a potent inhibitor of the c-di-AMP cyclase without
145 requiring any additional factors (28). To examine if this is also the case for the *B. sutbilis* proteins, the
146 full-length *B. subtilis* GlmM protein as well as the truncated $GlmM_{F369}$ variant were expressed and
147 purified along with the soluble catalytic domain of the *B. subtilis* c-di-AMP cyclase $CdaA_{CD}$. The
148 $GlmM_{F369}$ variant lacks the flexible C-terminal domain 4 and was constructed to aid subsequent
149 structural investigations. The proteins were expressed as His-tagged proteins in *E. coli* and purified
150 individually via Ni-NTA affinity chromatography followed by size exclusion chromatography (Fig. 1).
151 To test for a CdaA-GlmM interaction, lysates of strains producing $CdaA_{CD}$ and GlmM (Fig. 1A) or
152 $CdaA_{CD}$ and $GlmM_{F369}$ (Fig. 1B) were mixed prior to affinity and size-exclusion chromatography. The
153 elution profiles and analysis of the retention volumes revealed that $CdaA_{CD}$ formed a complex with
154 GlmM and with $GlmM_{F369}$ that eluted as a single, higher-mobility species compared to the individual
155 proteins (Fig. 1). The peak fractions of each complex were further analysed by SDS-PAGE, confirming
156 the presence of both proteins (Fig. 1, inserts). To determine if the *B. subtilis* GlmM protein impacts the
157 activity of $CdaA_{CD}$, *in vitro* cyclase activity assays were performed, and the conversion of ATP (spiked
158 with a small amount of α-^{32}P -labelled ATP) into c-di-AMP assessed. The purified *B. subtilis* $CdaA_{CD}$
159 protein was enzymatically active in the presence of the divalent metal ion Mn^{2+} but showed only limited
160 activity in the presence of Co^{2+} or Mg^{2+} (Fig. 2A) and after 4 h incubation approximately 50% of the
161 ATP substrate was converted to c-di-AMP (Fig. 2B). Addition of GlmM or $GlmM_{F369}$ at a 2:1 molar
162 ratio over $CdaA_{CD}$, led to a significant reduction in the conversion of ATP to c-di-AMP (Fig. 2C). Taken
163 together, these data show that the purified *B. subtilis* $CdaA_{CD}$:GlmM as well as $CdaA_{CD}$:$GlmM_{F369}$
164 proteins form a stable complex *in vitro* and that both the full-length and truncated GlmM variant, inhibit
165 the activity of the c-di-AMP cyclase $CdaA_{CD}$.
166

167 **Crystal structures of the *B. subtilis* $CdaA_{CD}$ and GlmM proteins**

168 To gain atomic level details of the $CdaA_{CD}$ and GlmM protein complex, we started off by determining
169 the crystal structures of the individual proteins. The tag-less *B. subtilis* $CdaA_{CD}$ protein was crystallised
170 and the structure solved at 2.8 Å (Table 1 and Fig. 3). The protein displayed the expected diadenylate
171 cyclase protein fold, with a central β-sheet made up of 6 antiparallel strands flanked by 5 helices (Fig.
172 3A). However, it lacked the seventh β strand that was seen in the structures of CdaA homologs of other
173 bacteria (21,28). In *B. subtilis* $CdaA_{CD}$ the residues corresponding to this β-strand are instead in a loop
174 that adapts a very similar confirmation to the β strand observed in other CdaA structures. Superposition
175 of the *B. subtilis* $CdaA_{CD}$ structure with the *L. monocytogenes* Δ100CdaA (PDB 4RV7) (21), and *S.*
176 *aureus* $DacA_{CD}$ (PDB 6GYW) (28) structures, all lacking the N-terminal transmembrane helices, gave
177 r.m.s.ds of 0.79 and 0.75, respectively, highlighting the overall structural similarities of these enzymes
178 (Fig. 3B). The *B. subtilis* $CdaA_{CD}$ structure was solved as a dimer in the asymmetric unit with hydrogen-
179 bonding interactions observed at the interaction interface (Fig 3C). Interactions were observed between
180 the side chains of amino acid residues Asn166, Thr172 and Leu174 (site 1) and residues Leu150, Lys153
181 and Met155 (site 2) (Fig 3C). Similar hydrogen-bonding interactions were also identified in the *S. aureus*
182 $DacA_{CD}$ and *L. monocytogenes* Δ100CdaA structures with amino acid residues in site 1 being absolutely
183 conserved (28,30) (Fig. S1). Analysis of the interface with PDBePISA (36) indicated a buried surface
184 of 1400 $Å^2$, which is similar to the value of 1460 $Å^2$ previously reported for the *S. aureus* $DacA_{CD}$
185 protein, indicative of a stable dimer formation. In this dimer confirmation, the active sites face opposite
186 directions and hence cannot be engaged in a catalytically active head-to-head conformation (Fig. 3A).
187 Taken together, these data indicate that the conformationally-inactive dimerization interface is
188 conserved among different CdaA homologs in Gram-positive bacteria and that the enzyme needs to
189 form higher oligomers for catalysis.
190 The His-tagged *B. subtilis* GlmM protein was crystallised and the structure solved by molecular
191 replacement using the *B. anthracis* GlmM structure (PDB 3PDK; (37)) as the search model (Table 1
192 and Fig. 3D). The *B. subtilis* GlmM protein displayed a four-domain architecture typical for
193 phosphoglucosamine mutase proteins (28,37) (Fig. 3D). Domains 1-3 are comprised of α-β mixed cores
194 linked via a flexible loop to domain 4, which displays a 3-stranded β sheet fold surrounded by two α-

195 helices (Fig. 3D). While one GlmM molecule was present in the asymmetric unit, the typical "M-
196 shaped" GlmM dimer arrangement was observed in the crystal cell packing (Fig. S2). Interactions were
197 formed between domains 1, leading to the formation of a large groove at the top of the dimer molecule,
198 mostly formed by domain 2 and the active site of each monomer subunit facing the opposite direction.
199 Two different structures were solved for the *B. subtilis* GlmM protein at 2.9 and 3.0 Å resolutions with
200 a superposition r.m.s.d. score of 0.29 (Table 1 and Fig. S2). One of the crystal structures was obtained
201 with a divalent cation bound to the catalytic serine residue, which during catalysis is thought to be
202 converted to a phosphoserine residue and the metal ion playing an important role during catalysis (Fig.
203 S2). The exact type of metal ion could not be deduced due to the limitation of the structural resolution.
204 However, we speculate that it is a magnesium ion, as magnesium was present in the crystallisation
205 conditions and this metal ion is usually also bound in fully active enzymes. Furthermore, when a
206 magnesium ion was modelled into the structure and analyzed using the program CheckMyMetal (38), a
207 better fit was observed as compared to zinc or calcium ions, which could also fill the density. In the
208 second structure, a phosphate molecule (PO_4) was bound to Arg419, located within a loop region in
209 domain 4 (Fig S2B) at a similar location as observed in the *B. anthracis* GlmM structure (37). The
210 superimposition of the *B. subtilis* GlmM structure with the *S. aureus* (6GYZ; (28)) and *B. anthracis*
211 (3PDK; (37)) GlmM structures, gave small r.m.s.d. values of 1.0261 and 1.0668, respectively (Fig. 3E),
212 indicating high similarity. However, the inter-residue distance between Arg419 in the phosphate binding
213 site in domain 4 and the catalytic Ser100 in domain 1 was 20.22 Å in the phosphate bound *B. subtilis*
214 GlmM structure compared to 18.4 Å in the *S. aureus* GlmM (PDB 6GYZ) or 15.18 Å in the *B. anthracis*
215 GlmM (PDB 3PDK) structures (Fig. 3F). This highlights the flexibility of domain 4 in GlmM enzymes
216 and also reveals that the *B. subtilis* GlmM protein was captured in most open state of the enzyme
217 reported so far in a crystal structure.
218
219 **Structure of the *B. subtilis* $CdaA_{CD}$:$GlmM_{F369}$ complex**
220 To understand how GlmM interacts and inhibits the CdaA, we next aimed to obtain the structure of the
221 complex. Any crystals obtained for the *B. subtilis* $CdaA_{CD}$:GlmM complex diffracted poorly. On the
222 other hand, diffracting crystals were obtained for the $CdaA_{CD}$:$GlmM_{F369}$ complex, in which the GlmM
223 protein lacks the flexible C-terminal domain 4. The crystals were obtained under two different
224 conditions (see experimental procedures section), and the structure of the $CdaA_{CD}$:$GlmM_{F369}$ complex
225 could be solved at 3.6 Å (Complex 1) and at 4.2 Å (Complex 2) by molecular replacement using the *B.*
226 *subtilis* $CdaA_{CD}$ and GlmM (dimer) structures as search models (Table 1 and Fig. 4, Fig. S3). While
227 obtained under two different conditions, complex 1 and complex 2 were nearly identical and overlapped
228 with an r.m.s.d of 0.22 Å (Fig. S4A). Furthermore, in both complex structures, three complex molecules
229 were obtained in the asymmetric unit and each complex was composed of a $GlmM_{F369}$ dimer and a
230 $CdaA_{CD}$ dimer in the inactive dimer configuration (Fig. 4 and Fig. S3). The three complexes obtained
231 in the asymmetric unit were almost identical to each other, as indicated by the superposition r.m.s.d. of
232 0.15 Å to 0.20 Å for complex 1 (Fig. S4B) and of 0.15 Å to 0.16 Å for complex 2 (Fig. S4C). Since the
233 obtained complex structures were basically identical, all further descriptions are based on the higher
234 resolution complex 1 structure. In the complex, a $CdaA_{CD}$ dimer was positioned in the large groove at
235 the top of the $GlmM_{F369}$ dimer and formed interactions with domain 2 of GlmM (Fig 4A-4C). The
236 complex was asymmetric, with one of the $CdaA_{CD}$ monomer, $CdaA_{CD}$(2) (shown in light blue in Fig. 4)
237 placed in the center of the $GlmM_{F369}$ groove and the other monomer, $CdaA_{CD}$(1) (shown in dark blue in
238 Fig. 4) projecting towards the solvent. Similarly, most of the interactions of the $GlmM_{F369}$ dimer with
239 the $CdaA_{CD}$ dimer were made by $GlmM_{F369}$(1) (shown in dark pink in Fig 4). PDBePISA analysis
240 revealed an average buried surface area of 996 $Å^2$ in the interface between $GlmM_{F369}$(1) and the $CdaA_{CD}$
241 dimer, which was stabilized by 4 hydrogen bond and 4 ionic bond interactions between $GlmM_{F369}$(1)
242 and $CdaA_{CD}$(1) and 5 hydrogen bond and 3 ionic bond interactions with $CdaA_{CD}$(2) (Table S1 and Fig.
243 S5). On the other hand, only an average 220.3 $Å^2$ surface area is occluded in $GlmM_{F369}$(2) (shown in
244 light pink in Fig. 4). Based on the PDBePISA analysis, $GlmM_{F369}$(2) only formed two hydrogen bond
245 interactions with the $CdaA_{CD}$(2) monomer but no interaction with $CdaA_{CD}$(1) (Table S1 and Fig. S5). A
246 more detailed analysis of the interface showed that several interactions are made between two α-helices
247 from domain 2 of $GlmM_{F369}$(1), α1 and α2, with the $CdaA_{CD}$(1) and $CdaA_{CD}$(2) monomers, respectively
248 (Fig 4C and 4D). The main interactions in the complex were formed between three residues, D151,
249 E154, and D194 of domain 2 in $GlmM_{F369}$(1) and residue R126 in each of the $CdaA_{CD}$ monomers. More

specifically, ionic bonds were formed between residue D195 in $GlmM_{F369}$(1) and residue R126 in $CdaA_{CD}$(2). In addition, salt bridges were formed between residues D151 and E154 in $GlmM_{F369}$(1) and residue R126 but this time from $CdaA_{CD}$(1) (Fig 4C and 4D, Table S2 and Fig. S5). The data suggest that residue R126 in $CdaA_{CD}$ is potentially one of the most critical residues for complex formation, as it contributes to a number of ionic as well as hydrogen-bond interactions and even though the complex is asymmetric, it contributes to interactions in both $CdaA_{CD}$ monomers.

$CdaA_{CD}$ cannot form active oligomers in complex with GlmM

To gain insight how GlmM inhibits the activity of the c-di-AMP cyclase, we inspected the location of the active sites of $CdaA_{CD}$ in the complex. The active site of DAC-domain enzymes is characterized by DGA and RHR motifs, corresponding to residues D_{171}GA and R_{203}HR in *B. subtilis* CdaA (Fig. 5; areas highlighted in yellow and green in the $CdaA_{CD}$ monomers). The active site in $CdaA_{CD}$(2) was completely occluded upon interaction with the $GlmM_{F369}$ dimer (Fig. 5, dark blue $CdaA_{CD}$ monomer with active site region highlighted in yellow) but the active site in $CdaA_{CD}$(1) appeared at least partially exposed (Fig. 5; light blue $CdaA_{CD}$ monomer with active site region highlighted in green). For $CdaA_{CD}$ to produce c-di-AMP active head-to-head dimers need to be formed (3). The crystal structure of the *L. monocytogenes* Δ100CdaA cyclase was recently determined with a c-di-AMP molecule bound in the catalytic site and an active head-to-head dimer conformation seen in the crystal packing (30). Using the *L. monocytogenes* Δ100CdaA structure (PDB 6HVL) as model, an active *B. subtilis* $CdaA_{CD}$ dimer was modelled and superimposed on $CdaA_{CD}$(1) in the complex structure (Fig. 5). Although the active site of the $CdaA_{CD}$(1) was exposed and accessible in the complex with $GlmM_{F369}$, in an active dimer conformation, parts of the second $CdaA_{CD}$ molecule would collide and overlap with $GlmM_{F369}$, highlighting that also $CdaA_{CD}$(1) cannot form active head-to-head oligomers in the complex (Fig. 5). Taken together, these data indicate that in the complex, the interaction of GlmM with $CdaA_{CD}$ will prevent the formation of functional diadenylate cyclase enzyme oligomers, which is essential for the formation of c-di-AMP. The crystal structure of the $CdaA_{CD}$:$GlmM_{F369}$ complex therefore provides insight on an atomic level on the catalytic inhibition of the diadenylate cyclase $CdaA_{CD}$ by the phosphoglucosamine enzyme GlmM.

Small angle X-ray scattering analysis of *B. subtilis* $CdaA_{CD}$:GlmM complex

To determine whether the full-length *B. subtilis* GlmM protein interacts with $CdaA_{CD}$ in a similar manner as observed for $GlmM_{F369}$, a structural characterisation of the $CdaA_{CD}$:GlmM complex was performed via small-angle X-ray scattering (SAXS). To this end, the individual purified *B. subtilis* $CdaA_{CD}$ and GlmM proteins as well as the purified $CdaA_{CD}$:GlmM complex were passed over an analytical size-exclusion column, followed by continuous automated SAXS data collection throughout the run (Fig. 6, Fig S6 and Table S2). For $CdaA_{CD}$ and GlmM, the reconstructed maps were consistent with the proteins forming dimers and the maps were a good fit for the *B. subtilis* $CdaA_{CD}$ dimer (Fig. 6A) and GlmM dimer (Fig. 6B) structures, respectively. The map reconstructed for the $CdaA_{CD}$:GlmM complex was bigger in volume and dimensions (V_c: 890.1, R_g: 44.65 Å and d_{max}: 161 Å) as compared to the individual maps calculated for GlmM (V_c: 625.8; R_g: 37.29 Å, d_{max}: 122 Å) and $CdaA_{CD}$ (V_c: 373.2, R_g: 26.94 Å, d_{max}: 88 Å), which is consistent with the formation of a complex. From the Guinier plot analysis, the molecular weight of the *B. subtilis* $CdaA_{CD}$:GlmM complex was calculated to be 130 kDa, which is consistent with the theoretical molecular weight of 144.46 kDa for a complex made of two $CdaA_{CD}$ and two GlmM molecules. To fit a $CdaA_{CD}$:GlmM dimer complex into the reconstructed map, a model of the complex with full-length GlmM was first produced, by superimposing the crystal structure of full-length GlmM onto the $CdaA_{CD}$:$GlmM_{F369}$ complex structure. The resulting complex model was subsequently fitted in the reconstituted SAXS envelope of the complex. A good fit of the $CdaA_{CD}$:GlmM dimer model complex into the reconstructed envelope was observed, however an elongated density on one side remained unoccupied (Fig. 6C). It is plausible that the flexible C-terminal domain 4 of the GlmM protein is responsible for this extra density. These data suggest that the full-length GlmM protein likely forms a dimer-of-dimer complex with the c-di-AMP cyclase $CdaA_{CD}$ and could assume a similar arrangement as observed for the $CdaA_{CD}$:$GlmM_{F369}$ complex.

303 **Arginine 126 in *B. subtilis* $CdaA_{CD}$ is essential for complex formation**
304 The complex structure highlighted key interactions between residues D194 and residues D151/E154 in
305 GlmM with residue R126 in each of the $CdaA_{CD}$ monomers (Fig. 5D). To confirm our structural
306 findings, a site-directed mutagenesis analysis was performed. To this end, D195A, D151A/E154A and
307 D151A/E154A/D191A alanine substitution GlmM variants were created. Furthermore, residue R126 in
308 $CdaA_{CD}$, which in both monomers makes contacts with GlmM, was mutated to an alanine. The different
309 alanine substitution variants were expressed and purified from *E. coli* and complex formation assessed
310 by size-exclusion chromatography. While our initial experiments using the GlmM single, double and
311 triple alanine substitution variants appeared not or only marginally to affect complex formation with
312 $CdaA_{CD}$ (Fig. S7), no complex-specific peak was observed when the interaction between the $CdaA_{CD}$-
313 R126A variant and GlmM was assessed. Instead, two peaks were observed for the $CdaA_{CD}$-
314 R126A:GlmM sample, one corresponding to the retention volume of GlmM and the another to $CdaA_{CD}$-
315 R126A (Fig. 7A). Analysis of the elution fractions from the $CdaA_{CD}$-R126A/GlmM sample by SDS-
316 PAGE and Coomassie staining showed that only a very small fraction of the $CdaA_{CD}$-R126A protein
317 co-eluted with GlmM (Fig. 7A). These data highlight that, consistent with the structural data, residue
318 R126 in $CdaA_{CD}$ plays a key role for the complex formation with GlmM. Based on these data, it can be
319 predicted that the cyclase activity of the $CdaA_{CD}$-R126A variant should now longer be inhibited by
320 GlmM. To test this experimentally, *in vitro* cyclase enzyme activity assays were performed. The
321 $CdaA_{CD}$-R126A variant was active, although the activity was reduced as compared to wild-type $CdaA_{CD}$
322 (Fig. 7B). Importantly and in contrast to wild-type $CdaA_{CD}$, the enzyme activity of this variant was no
323 longer inhibited by the addition of GlmM (Fig. 7B). These data show that residue Arg126 in *B. subtilis*
324 $CdaA_{CD}$ plays a critical role for complex formation and that GlmM can only inhibit the activity of the
325 c-di-AMP cyclase after the formation of a stable complex.
326
327 **Discussion**
328 In this study, we show that the *B. subtilis* GlmM and $CdaA_{CD}$ cyclase domain form a stable
329 dimer-of-dimer complex. GlmM acts through this protein-protein interaction as a potent inhibitor of the
330 c-di-AMP cyclase without requiring any additional factors. Based on the atomic-resolution complex
331 structure data, we suggest that GlmM inhibits the activity of $CdaA_{CD}$ by preventing the formation of
332 active head-to-head cyclase oligomers.
333 For CdaA to produce c-di-AMP, two monomers need to be arranged in an active head-to-head
334 conformation. As part of this study, we determined the structure of the *B. subtilis* $CdaA_{CD}$ protein and
335 show that it has the typical DAC domain fold. While the protein was also found as a dimer in the
336 structure, the dimer was in an inactive conformation, with the two active sites facing in opposite
337 directions. The interface creating the inactive dimer conformation is conserved among CdaA proteins.
338 The *L. monocytogenes* and *S. aureus* homologs, for which structures are available, were found in the
339 same inactive dimer conformation even though the proteins crystallized under different conditions and
340 were found in different space groups (21,28,30). This makes it less likely that a crystallographic
341 symmetry artefact is responsible for the observed inactive dimer configuration. In addition to the
342 inactive dimer configuration within an asymmetric unit, an active dimer conformation was observed in
343 the *L. monocytogenes* Δ100CdaA protein by inspecting adjacent symmetry units (30). However, no such
344 active dimer head-to-head conformations were identified for the *B. subtilis* $CdaA_{CD}$ protein across
345 different symmetry units in the current structure. While not further investigated as part of this study,
346 previous work on the *S. aureus* homolog indicated that the inactive dimer conformation is very stable,
347 and in order for the protein to produce an active enzyme, the protein needs to form higher-level
348 oligomers (28). Given the similarity in the interaction interface, this is likely also the case for the *B.*
349 *subtilis* $CdaA_{CD}$ enzyme and we would suggest that the *B. subtilis* $CdaA_{CD}$ dimer observed in the
350 structure is unlikely to rearrange into an active dimer conformation.
351 We also solved the structure of the *B. subtilis* GlmM enzyme. The protein assumed the typical
352 4-domain architecture previously reported for GlmM enzymes (28,37) and the "M shape" in the dimer
353 conformation, which in the case of the *B. sutbtilis* GlmM protein was formed across two adjacent
354 crystallographic units. The *B. subtilis* GlmM structure further highlighted the flexibility of the most C-
355 terminal domain 4, which was found in the most open conformation seen in any GlmM protein structure
356 up to date. The conformational flexibility of domain 4 is probably also a main factor why we were
357 unsuccessful in determining the structure of a complex between $CdaA_{CD}$ and full-length GlmM.

358 However, GlmM domain 4 is not required for the interaction with and inhibition of $CdaA_{CD}$, since a *B.*
359 *subtilis* GlmM variant lacking domain 4 formed a complex and inhibited the activity of *B. subtilis*
360 $CdaA_{CD}$. Futhermore, by using the *B. subtilis* $GlmMF_{F369}$ variant lacking the flexible domain 4, we were
361 able to obtain the structure of the $CdaA_{CD}$:$GlmM_{F369}$ complex, revealing for the first time structural
362 details at the atomic level for this complex, thereby identifying the amino acids important for the
363 interaction between the two proteins. Several electrostatic interactions were detected between
364 $CdaA_{CD}$:$GlmM_{F369}$: between the negatively charged residues D151, E154 and D195 in domain 2 of *B.*
365 *subtilis* $GlmM_{F369}$ with the positively charged residue R126 in $CdaA_{CD}$. We could further show that
366 replacing residue R126 in $CdaA_{CD}$ with an alanine abolished complex formation and the activity of the
367 $CdaA_{CD}$-R126 variant was no longer inhibited by GlmM. A direct protein-protein interaction between
368 CdaA and GlmM has now been reported for these proteins in several Firmicutes bacteria, and hence the
369 amino acids required for the interaction might be conserved. Indeed, a ConSurf (39) analysis using 250
370 CdaA protein sequences, showed that the residue corresponding to R126 in *B. subtilis* CdaA is conserved
371 between the different homologs (Fig. S8). Likewise, all the three negatively charged residues, D151,
372 E154 and D195 in GlmM, which mediate the primary electrostatic interactions with R126 of $CdaA_{CD}$,
373 are highly conserved (Fig. S8). In previous work, we have shown, that the *S. aureus* $DacA_{CD}$ (the $CdaA_{CD}$
374 homolog) does not interact with GlmM proteins from *E. coli* and *Pseudomonas aeruginosa,* two Gram-
375 negative bacteria (28). While negatively charged amino acids corresponding to residues E154 and D195
376 in *B. subtilis* GlmM are also found in the GlmM protein from the Gram-negative bacteria (D153 and
377 E194 in *E. coli* and D152 and E193 in *P. aeruginosa*), the amino acids at the equivalent position of
378 D151 in *B. subtilis* GlmM is an arginine residue (R150 in *E. coli* and R149 in *P. aeruginosa*), which
379 may hinder the complex formation between CdaA (DacA), and GlmM proteins of Gram-negative
380 bacteria (28).
381 In a previous work, we proposed a model whereby GlmM inhibits the activity of CdaA cyclase
382 activity by preventing the formation of active head-to-head oligomers (28). In the absence of an actual
383 atomic resolution structure of the complex, this model was based on SAXS envelope data and fitting
384 individual protein structures. The model predicted that the likely interaction site between CdaA and
385 GlmM proteins is domain 2 of GlmM (28). The structure of *B. subtilis* $CdaA_{CD}$:$GlmM_{F369}$ complex we
386 present here now provides experimental evidence for such a model and shows that GlmM indeed inhibits
387 the activity of the CdaA cyclase *in vitro* by preventing the formation of active head-to-head oligomers.
388 The level of c-di-AMP is regulated by a fine balance between the activities of the cyclase, which
389 synthesizes c-di-AMP, and the phosphodiesterases, which break it down. Interestingly, these two classes
390 of enzyme appear to be regulated very differently; whereas the activity of several phosphodiesterases
391 has been shown to be regulated by small molecules, cyclase activity appears to be regulated through
392 protein-protein interaction. For example, the stringent response alarmone (p)ppGpp has been shown to
393 inhibit the activity both GdpP and PgpH enzymes (11,20). Furthermore, binding of heme to the Per-
394 ARNT-Sim (40) signalling domain in GdpP (which is separate from its DHH/DHHA1 enzymatic
395 domain that is responsible for the degradation of c-di-AMP) was shown in *in vitro* enzyme assays to
396 lead to reduced phosphodiesterase activity (40). Interestingly, the ferrous form of heme bound to GdpP
397 could form a pentacoordinate complex with nitric oxide (NO), resulting in increased c-di-AMP
398 phosphodiesterase activity. Based on these data it has been suggested that GdpP is a heme or NO sensor,
399 resulting in decreased or increased activity respectively (41). The function consequence and impact of
400 (p)ppGpp, heme or NO binding to the phosphodiesterases on bacterial physiology has not yet been fully
401 investigated. However, from these data it is clear that the activity of the c-di-AMP phosphodiesterases
402 can be regulated by small molecule ligands.
403 On the other hand, several proteins have been found to interact with and regulate the activity of
404 c-di-AMP cyclases. The *B. subtilis* DisA protein is involved in monitoring the genomic stability
405 ensuring that damaged DNA is repaired before cells progress with the sporulation process or exit from
406 spores (27,42). DisA is encoded in a multi-gene operon and the gene immediately upstream of *disA*
407 codes for RadA (also referred to as SMS). *B. subtilis* RadA possesses 5' to 3' DNA helicase activity,
408 contributes to DNA repair and DNA transformation processes in *B. subtilis* and has been shown to
409 interact and negatively impact the activity of DisA (27). However, the mechanistic basis of how RadA
410 binding to DisA inhibits the cyclase is currently not known. There is now ample evidence that the
411 activity of the "house-keeping" membrane-linked c-di-AMP cyclase CdaA is impacted by two
412 interacting proteins, the membrane-linked regulator protein CdaR and the cytoplasmic

413 phosphoglucomutase enzyme GlmM (29,33). We have shown provided experimental evidence for the
414 mechanistic basis by which GlmM inhibits the activity of CdaA, that is by preventing the formation of
415 active higher-level oligomers. How CdaR regulates the cyclase activity of CdaA remains unclear.
416 Recent work on the homologous proteins in *L. monocytogenes* indicated that the interaction of CdaA
417 with CdaR takes place via the N-terminal transmembrane region of CdaA, GlmM has been shown to
418 interact directly with the cytoplasmic cyclase domain of CdaA in *S. aureus* (28,33). Here, we show that
419 this is also the case for the *B. subtilis* GlmM protein, which can bind without the requirement of any
420 additional factor to the catalytic $CdaA_{CD}$ domain. In future works, it will be interesting to determine the
421 structure of the full-length CdaA enzyme, which might provide further insight into how the enzyme
422 forms higher oligomers for activity as well as how it interacts with CdaR. Furthermore, it will be
423 interesting to further investigate the interaction between GlmM and CdaR with CdaA within bacterial
424 cells to determine if this interaction is dynamic and which stimuli will promote or prevent complex
425 formation to fine-tune the synthesis of c-di-AMP. Identifying how interacting proteins regulate the
426 activity of these important cyclases, will provide important insight how bacterial cells maintain proper
427 levels of c-di-AMP under different growth conditions and in different environments.
428
429 **Experimental procedures**
430 **Bacterial strains and plasmid construction**
431 All bacterial strains and primers used in this work are listed in Tables S3 and S4, respectively.
432 pET28b-derived plasmids were constructed for the overproduction of the C-terminal catalytic domain
433 of the *Bacillus subtilis* CdaA enzyme starting from amino acid Phe97 and referred to as $CdaA_{CD}$, GlmM
434 and the $GlmM_{F369}$ variant comprising residues Met1 to Phe369 but lacking the C-terminal domain 4. To
435 this end, the corresponding DNA fragments were amplified by PCR using *B. subtilis* strain 168
436 chromosomal DNA as template and primer pairs ANG2760/ANG2761 ($cdaA_{CD}$), ANG2762/ANG2763
437 (*glmM*) and ANG2762/ANG2764 ($glmM_{F369}$). The PCR products were purified, digested with
438 NheI/BamHI ($cdaA_{CD}$) or NcoI/XhoI (*glmM* and $glmM_{F369}$) and ligated with pET28b, which had been
439 cut with the same enzyme. $CdaA_{CD}$ was cloned in frame with an N-terminal thrombin cleavable 6-
440 histidine tag, while GlmM and $GlmM_{F369}$ were cloned in frame with a C-terminal 6-histidine tag and a
441 thrombin cleavage site was introduced in front of the His-tag as part of the primer sequence. The
442 resulting plasmids pET28b-*his-*$cdaA_{CD}$, pET28b-*glmM-his* and pET28b-$glmM_{F369}$*-his* were initially
443 recovered in *E. coli* XL1-Blue, yielding the strains ANG4583, ANG4584 and ANG4585 and
444 subsequently transformed for protein expression into strain *E. coli* BL21(DE3), yielding strains
445 ANG4597, ANG4598 and ANG4599, respectively. Plasmids pET28b-*his-*$cdaA_{CD}$*-R126*, pET28b-
446 *glmM-D194A-his,* pET28b-*glmM-D151A/E154A-his* were constructed for the expression of $CdaA_{CD}$
447 and GlmM alanine substitution variants. The plasmids were constructed by QuikChange mutagenesis
448 using pET28b-*his-*$cdaA_{CD}$ and primer pair ANG3373/ANG3374 or plasmid pET28b-*glmM-his* and
449 primer pairs ANG3381/ANG3382 and ANG3383/3384, respectively. The plasmids were initially
450 recovered in *E. coli* XL1-Blue, yielding strains ANG5933, ANG5937, ANG5938 and subsequently
451 introduced for protein expression into *E. coli* strain BL21(DE3) yielding strains ANG5940, ANG5944,
452 ANG5945. In addition, plasmid pET28b-*glmM-D151A/E154A/D194A-his* for expression of a GlmM
453 variant with a triple Asp151 (D151), *Glu154 (E154) and Asp195 (D195)* alanine substitution variant
454 was constructed by QuikChange mutagenesis using plasmid pET28b-*glmM-D194A-his* as template and
455 primer pair ANG3383/3384 to introduce the D151A and E154A mutations. Plasmid pET28b-*glmM-*
456 *D151A/E154A/D194A-his* was recovered in *E. coli* strain XL1-Blue, yielding strain ANG5939 and
457 subsequently introduced for protein expression into strain BL21(DE3) yielding strain ANG4946. The
458 sequences of all plasmid inserts were verified by fluorescent automated sequencing at Eurofins.
459
460 **Protein expression, purification and quantification**
461 Proteins $CdaA_{CD}$, GlmM and $GlmM_{F369}$ were expressed and purified from 1 L cultures as previously
462 described (28). Briefly, when bacterial cultures reached an OD_{600} of approximately 0.6, protein
463 expression was induced with 1 mM IPTG (final concentration) for 3 hours at 37˚C. Cells were harvested
464 by centrifugation, suspended in 20 mL of 50 mM Tris pH 7.5, 500 mM NaCl buffered and lysed using
465 a French Press system. Lysates were clarified by centrifugation and the supernatant loaded onto a gravity
466 flow column with 3 mL of Ni-NTA resin. Immobilized proteins were washed with 20 mL of 50 mM
467 Tris pH 7.5, 500 mM NaCl, 50 mM imidazole buffer and eluted in 5 x 1 ml fractions using 50 mM Tris

468 pH 7.5, 200 mM NaCl, 500 mM imidazole buffer. Fractions containing the proteins were pooled and
469 loaded onto a preparative Superdex 200 HiLoad 16/60 column equilibrated with 1 column volume of 30
470 mM Tris pH 7.5, 150 mM NaCl buffer. When appropriate, the purified proteins were concentrated using
471 10 mL 10 kDa cutoff Amicon concentrators for downstream applications. For the purification of the
472 $CdaA_{CD}$:GlmM and $CdaA_{CD}$:$GlmM_{F369}$ complexes or complexes of $CdaA_{CD}$ and GlmM alanine-
473 substitution variants, cell lysates of strains overproducing the respective proteins were mixed after the
474 French press step, then the same protein purification procedure steps as described above used for the
475 purification of individual proteins were performed. Protein concentrations were determined using the
476 BCA assay kit (Pierce™ BCA Protein Assay Kit). For each sample, the readings were taken in triplicates
477 and then averaged to obtain the protein concentration. Purified proteins were also separated on 12%
478 SDS PAGE gels and detected by Coomassie staining.
479
480 **Protein crystallisation, data processing and analysis**
481 For crystallisation, the histidine tag was removed from the purified *B. subtilis* $CdaA_{CD}$ protein. This was
482 done by incubating 10 mg purified protein with 20 U thrombin overnight at 4˚C with agitation. The
483 following day, the tag less $CdaA_{CD}$ was purified by size-exclusion chromatography as described above.
484 The $CdaA_{CD}$ protein was crystallized at a concentration of 4 mg/ml in 0.1M sodium cacodylate pH 6.5,
485 0.1M ammonium sulfate, 0.3 M sodium formate, 6% PEG 8000, 3% γ-PGA via the vapour diffusion
486 method. The crystal screens for *B. subtilis* GlmM (including the His tag) were set up at a concentration
487 of 10 mg/ml and protein crystals were obtained in two different conditions. The structure with bound
488 PO_4 (GlmM:PO_4) was obtained in the Morpheus screen containing 0.1 M buffer system 1 (Imidazole;
489 MES, pH 6.5), 0.09 M NPS (NaN03; Na_2HPO_4; $(NH_4)_2SO_4$) and 37.5% MPD_P1K_P3350 (75% MPD,
490 PEG 1K, PEG 3350) and the divalent-ion bound crystal structure (GlmM:metal ion) was obtained in
491 0.05 M Magnesium chloride hexahydrate, 0.1 M HEPES pH 7.5, 30% v/v Polyethylene glycol
492 monomethyl ether 550 buffer. The crystals for the $CdaA_{CD}$:$GlmM_{F369}$ complex were set up at a protein
493 concentration of 10 mg/ml and crystals were obtained in 0.12 M alcohols, 0.1 M buffer system
494 (Imidazole; MES, pH 6.5) and 30% GOL_P4K (60% glycerol, PEG 4K) (Complex 1) and 0.1 M
495 carboxylic acids, 0.1 M buffer system 1 (Imidazole; MES, pH 6.5) and 30% GOL_P4K (60% glycerol,
496 PEG 4K) (Complex 2). The crystals were fished and stored in liquid nitrogen to test for diffraction at
497 the I03 beamline at the Diamond Light Source (Harwell Campus, Didcot, UK). Data were reduced with
498 DIALS (43) and scaled and merged with AIMLESS (44). The structures of $CdaA_{CD}$ and GlmM were
499 solved by the molecular replacement method using the program MR-PHASER (45) in the Phenix suite
500 (46), using the *L. monocytogenes* CdaA structure (PDB 4RV7; (21)) and *B. anthracis* GlmM structure
501 (PDB 3PDK; (37)) as the search models, respectively. To solve the phase problem for the structure of
502 the $CdaA_{CD}$:$GlmM_{F369}$ complex, dimers of *B. subtilis* $CdaA_{CD}$ and GlmM (each) were used as the search
503 models using the MR-PHASER program in Phenix. The models were manually built using COOT (47)
504 followed by iterative cycles of structure refinement using the Phenix-Refine program (48). The final
505 refined structures were analysed using the PDBePISA server (36) to identify buried interface areas for
506 each protein. To search for conserved residues among the phylogenetically related homologs, a protein
507 BLAST search was performed using *B. subtilis* $CdaA_{CD}$ and GlmM amino acid sequences as query
508 sequences and a multiple sequence alignment (MSA) of the top 2500 homologs found in Firmicutes was
509 prepared. The MSA was then used to identify conserved residues among the homologs using the
510 ConSurf server (49).
511
512 **$CdaA_{CD}$ activity and inhibition assays**
513 To assess the activity of the *B. subtilis* $CdaA_{CD}$ enzymes, 20 µl enzyme reactions were set up in 100 mM
514 NaCl, 40 mM HEPES pH 7 buffer containing 10 mM $MnCl_2$ (or 10 mM $MgCl_2$ or 10 mM $CoCl_2$ for
515 metal dependent assays), 100 mM ATP spiked with a-P^{32}-labelled ATP (Perkin Elmer; using 0.4 µl of
516 a 3.3 µM, 250 µCi solution per 20 µl reaction) and 5 µM $CdaA_{CD}$ enzyme. The mixture was incubated
517 at 37˚C for 4 hours, followed by heat inactivation at 95˚C for 5 minutes. After centrifugation for 10
518 minutes at 21,000 x g, 1 µl of the mixture was deposited onto a polyethylenimine-modified cellulose
519 TLC plate (Millipore) and nucleotides separated by running the plate for 20 minutes using a 3.52 M
520 $(NH_4)_2SO_4$ and 1.5 M KH_2PO_4 buffer system mixed at a 1:1.5 v/v ratio. Radioactive signals for ATP and
521 the c-di-AMP reaction product were detected using a Typhoon FLA-700 phosphor imager. The bands
522 were quantified using the ImageQuant program and the obtained values used to calculate the percent

conversion of ATP to c-di-AMP. For the time course experiment, a 100 µl reaction mixture was prepared as described above and incubated at 37˚C. Ten µl aliquots were removed at the indicated time points and the enzyme reactions stopped by incubation the removed aliquots at 95˚C for 5 minutes. To assess the activity of $CdaA_{CD}$ in the presence of GlmM or $GlmM_{F369}$, the full length GlmM protein or C-terminally truncated GlmM variant were added to the reaction mixture at a 1:2 ($CdaA_{CD}$: GlmM or $CdaA_{CD}$: $GlmM_{F369}$) molar ratio and the reactions incubated at 37˚C for 4 hours, stopped and analysed as described above. The enzyme activity assays were performed in triplicates with two independently purified protein preparations.

SAXS sample preparation and analysis

For the SAXS analysis, purified $CdaA_{CD}$, GlmM and $CdaA_{CD}$:GlmM complex protein samples where purified a second time by size exclusion chromatography as described above and subsequently concentrated to 5 mg/ml for $CdaA_{CD}$ and 24 mg/ml each for GlmM and the $CdaA_{CD}$:GlmM complex. Next, 50 µl protein samples were loaded on a high pressure Shodex column (KW403: range 10 kDa to 700 kDa) fitted to an Agilent 1200 HPLC system at the B21 beamline at the Diamond Light Source (Didcot, UK). The size-exclusion column was equilibrated with 30 mM Tris pH 7.5, 150 mM NaCl buffer prior to loading the protein sample and the data were collected continuously throughout the protein elution. The analysis of the datasets was done via ScÅtter (50) using the scattering frames corresponding to the elution peaks. The ab-initio analysis of the SAXS data to reconstruct a low-resolution shape of the model was done using DAMMIF (51). The program performed 13 *ab-initio* runs to generate models from each run that were averaged to determine the most persistent three-dimensional shape of the protein. The cross-correlation Normalised Spatial Discrepancy (NSD) values were calculated using SUPCOMB from each of the 13 generated models. The mean NSD values calculated for each of the protein were: 0.591 ± 0.088 ($CdaA_{CD}$), 0.598 ± 0.014 (GlmM) and 0.661 ± 0.064 ($DacA_{CD}$:GlmM Complex). Chimera (52) was used to visualise the reconstructed SAXS maps. The crystal structures of $CdaA_{CD}$ and GlmM dimers were fitted in the respective SAXS envelopes in Chimera using the one-step fit function. For the $CdaA_{CD}$: GlmM complex data, a structural model of the $CdaA_{CD}$ full-length GlmM complex was generated based on the crystal structure of $CdaA_{CD}$: $GlmM_{F369}$ complex, which was then fitted into the SAXS envelope data using Chimera.

Data availability

The structure coordinates of the *B. subtilis* proteins were deposited in the Protein Database (https://www.rcsb.org), under PDB codes 6HUW ($CdaA_{CD}$), 7OJR (GlmM: PO_4 bound), 7OML (GlmM: metal bound) and 7OLH ($CdaA_{CD}$:$GlmM_{F369}$ Complex 1) and 7OJS ($CdaA_{CD}$:$GlmM_{F369}$ Complex 2). SAXS models were deposited in the SASBDB database, under the accession codes SASDL25 (Complex $CdaA_{CD}$:GlmM), SASDLZ4 (GlmM) and SASDLY4 ($CdaA_{CD}$).

Supporting Information

This article contains a supporting information file with the following additional references (36,49,50,53,54).

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Author contribution statement

Monisha Pathania: Conceptualization, Investigation, Data analysis, Visualization, Writing – original draft preparation. **Tommaso Tosi:** Conceptualization, Investigation, Data analysis, Writing – review & editing. **Charlotte Millsership:** Investigation, Data analysis, Writing – review & editing. **Fumiya Hoshiga:** Investigation, Data analysis, Writing – review & editing. **Rhodri M. L. Morgan:** Data analysis, Supervision, Writing – review & editing. **Paul S. Freemont:** Conceptualization, Funding acquisition, Supervision, Data analysis, Writing – review & editing. **Angelika Gründling:** Conceptualization, Funding acquisition, Data analysis, Supervision, Writing – original draft preparation.

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Conflict of Interest

The authors declare no conflicts of interest in regard to this manuscript.

589 **References**
590

591 1. Hengge, R., Gründling, A., Jenal, U., Ryan, R., and Yildiz, F. (2016) Bacterial Signal
592 Transduction by Cyclic Di-GMP and Other Nucleotide Second Messengers. *J Bacteriol* **198**,
593 15-26
594 2. Krasteva, P. V., and Sondermann, H. (2017) Versatile modes of cellular regulation via cyclic
595 dinucleotides. *Nat Chem Biol* **13**, 350-359
596 3. Witte, G., Hartung, S., Buttner, K., and Hopfner, K. P. (2008) Structural biochemistry of a
597 bacterial checkpoint protein reveals diadenylate cyclase activity regulated by DNA
598 recombination intermediates. *Mol Cell* **30**, 167-178
599 4. Bai, Y., Yang, J., Zarrella, T. M., Zhang, Y., Metzger, D. W., and Bai, G. (2014) Cyclic di-
600 AMP impairs potassium uptake mediated by a cyclic di-AMP binding protein in *Streptococcus*
601 *pneumoniae*. *J Bacteriol* **196**, 614-623
602 5. Whiteley, A. T., Garelis, N. E., Peterson, B. N., Choi, P. H., Tong, L., Woodward, J. J., and
603 Portnoy, D. A. (2017) c-di-AMP modulates *Listeria monocytogenes* central metabolism to
604 regulate growth, antibiotic resistance and osmoregulation. *Mol Microbiol* **104**, 212-233
605 6. Huynh, T. N., Choi, P. H., Sureka, K., Ledvina, H. E., Campillo, J., Tong, L., and Woodward,
606 J. J. (2016) Cyclic di-AMP targets the cystathionine beta-synthase domain of the osmolyte
607 transporter OpuC. *Mol Microbiol* **102**, 233-243
608 7. Corrigan, R. M., Campeotto, I., Jeganathan, T., Roelofs, K. G., Lee, V. T., and Gründling, A.
609 (2013) Systematic identification of conserved bacterial c-di-AMP receptor proteins. *Proc Natl*
610 *Acad Sci U S A* **110**, 9084-9089
611 8. Gundlach, J., Herzberg, C., Kaever, V., Gunka, K., Hoffmann, T., Weiss, M., Gibhardt, J.,
612 Thurmer, A., Hertel, D., Daniel, R., Bremer, E., Commichau, F. M., and Stülke, J. (2017)
613 Control of potassium homeostasis is an essential function of the second messenger cyclic di-
614 AMP in *Bacillus subtilis*. *Sci Signal* **10**
615 9. Corrigan, R. M., Abbott, J. C., Burhenne, H., Kaever, V., and Gründling, A. (2011) c-di-AMP
616 is a new second messenger in *Staphylococcus aureus* with a role in controlling cell size and
617 envelope stress. *PLoS Pathog* **7**, e1002217
618 10. Luo, Y., and Helmann, J. D. (2012) Analysis of the role of *Bacillus subtilis* sigma(M) in beta-
619 lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. *Mol*
620 *Microbiol* **83**, 623-639
621 11. Corrigan, R. M., Bowman, L., Willis, A. R., Kaever, V., and Gründling, A. (2015) Cross-talk
622 between two nucleotide-signaling pathways in *Staphylococcus aureus*. *J Biol Chem* **290**, 5826-
623 5839
624 12. Dengler, V., McCallum, N., Kiefer, P., Christen, P., Patrignani, A., Vorholt, J. A., Berger-Bächi,
625 B., and Senn, M. M. (2013) Mutation in the c-di-AMP cyclase *dacA* affects fitness and
626 resistance of methicillin resistant *Staphylococcus aureus*. *PLoS One* **8**, e73512
627 13. Woodward, J. J., Iavarone, A. T., and Portnoy, D. A. (2010) c-di-AMP secreted by intracellular
628 *Listeria monocytogenes* activates a host type I interferon response. *Science* **328**, 1703-1705
629 14. Witte, C. E., Whiteley, A. T., Burke, T. P., Sauer, J. D., Portnoy, D. A., and Woodward, J. J.
630 (2013) Cyclic di-AMP is critical for *Listeria monocytogenes* growth, cell wall homeostasis, and
631 establishment of infection. *mBio* **4**, e00282-00213
632 15. Oppenheimer-Shaanan, Y., Wexselblatt, E., Katzhendler, J., Yavin, E., and Ben-Yehuda, S.
633 (2011) c-di-AMP reports DNA integrity during sporulation in *Bacillus subtilis*. *EMBO Rep* **12**,
634 594-601
635 16. Devaux, L., Sleiman, D., Mazzuoli, M. V., Gominet, M., Lanotte, P., Trieu-Cuot, P., Kaminski,
636 P. A., and Firon, A. (2018) Cyclic di-AMP regulation of osmotic homeostasis is essential in
637 Group B *Streptococcus*. *PLoS Genet* **14**, e1007342
638 17. Mehne, F. M., Gunka, K., Eilers, H., Herzberg, C., Kaever, V., and Stülke, J. (2013) Cyclic di-
639 AMP homeostasis in *Bacillus subtilis*: both lack and high level accumulation of the nucleotide
640 are detrimental for cell growth. *J Biol Chem* **288**, 2004-2017
641 18. Commichau, F. M., Heidemann, J. L., Ficner, R., and Stülke, J. (2019) Making and Breaking of
642 an Essential Poison: the Cyclases and Phosphodiesterases That Produce and Degrade the
643 Essential Second Messenger Cyclic di-AMP in Bacteria. *J Bacteriol* **201**

644 19. Rao, F., See, R. Y., Zhang, D., Toh, D. C., Ji, Q., and Liang, Z. X. (2010) YybT is a signaling
645 protein that contains a cyclic dinucleotide phosphodiesterase domain and a GGDEF domain
646 with ATPase activity. *J Biol Chem* **285**, 473-482
647 20. Huynh, T. N., Luo, S., Pensinger, D., Sauer, J. D., Tong, L., and Woodward, J. J. (2015) An
648 HD-domain phosphodiesterase mediates cooperative hydrolysis of c-di-AMP to affect bacterial
649 growth and virulence. *Proc Natl Acad Sci U S A* **112**, E747-756
650 21. Rosenberg, J., Dickmanns, A., Neumann, P., Gunka, K., Arens, J., Kaever, V., Stülke, J., Ficner,
651 R., and Commichau, F. M. (2015) Structural and biochemical analysis of the essential
652 diadenylate cyclase CdaA from *Listeria monocytogenes*. *J Biol Chem* **290**, 6596-6606
653 22. Zheng, C., Ma, Y., Wang, X., Xie, Y., Ali, M. K., and He, J. (2015) Functional analysis of the
654 sporulation-specific diadenylate cyclase CdaS in *Bacillus thuringiensis*. *Front Microbiol* **6**, 908
655 23. Müller, M., Deimling, T., Hopfner, K. P., and Witte, G. (2015) Structural analysis of the
656 diadenylate cyclase reaction of DNA-integrity scanning protein A (DisA) and its inhibition by
657 3'-dATP. *Biochem J* **469**, 367-374
658 24. Mehne, F. M., Schroder-Tittmann, K., Eijlander, R. T., Herzberg, C., Hewitt, L., Kaever, V.,
659 Lewis, R. J., Kuipers, O. P., Tittmann, K., and Stülke, J. (2014) Control of the diadenylate
660 cyclase CdaS in *Bacillus subtilis*: an autoinhibitory domain limits cyclic di-AMP production. *J*
661 *Biol Chem* **289**, 21098-21107
662 25. Fahmi, T., Port, G. C., and Cho, K. H. (2017) c-di-AMP: An Essential Molecule in the Signaling
663 Pathways that Regulate the Viability and Virulence of Gram-Positive Bacteria. *Genes (Basel)* **8**
664 26. Corrigan, R. M., and Gründling, A. (2013) Cyclic di-AMP: another second messenger enters
665 the fray. *Nat Rev Microbiol* **11**, 513-524
666 27. Torres, R., Carrasco, B., Gandara, C., Baidya, A. K., Ben-Yehuda, S., and Alonso, J. C. (2019)
667 *Bacillus subtilis* DisA regulates RecA-mediated DNA strand exchange. *Nucleic Acids Res* **47**,
668 5141-5154
669 28. Tosi, T., Hoshiga, F., Millership, C., Singh, R., Eldrid, C., Patin, D., Mengin-Lecreulx, D.,
670 Thalassinos, K., Freemont, P., and Gründling, A. (2019) Inhibition of the *Staphylococcus*
671 *aureus* c-di-AMP cyclase DacA by direct interaction with the phosphoglucosamine mutase
672 GlmM. *PLoS Pathog* **15**, e1007537
673 29. Gundlach, J., Mehne, F. M., Herzberg, C., Kampf, J., Valerius, O., Kaever, V., and Stülke, J.
674 (2015) An essential poison: Synthesis and degradation of cyclic di-AMP in *Bacillus subtilis*. *J*
675 *Bacteriol* **197**, 3265-3274
676 30. Heidemann, J. L., Neumann, P., Dickmanns, A., and Ficner, R. (2019) Crystal structures of the
677 c-di-AMP-synthesizing enzyme CdaA. *J Biol Chem* **294**, 10463-10470
678 31. Zhu, Y., Pham, T. H., Nhiep, T. H., Vu, N. M., Marcellin, E., Chakrabortti, A., Wang, Y.,
679 Waanders, J., Lo, R., Huston, W. M., Bansal, N., Nielsen, L. K., Liang, Z. X., and Turner, M.
680 S. (2016) Cyclic-di-AMP synthesis by the diadenylate cyclase CdaA is modulated by the
681 peptidoglycan biosynthesis enzyme GlmM in *Lactococcus lactis*. *Mol Microbiol* **99**, 1015-1027
682 32. Pham, T. H., Liang, Z. X., Marcellin, E., and Turner, M. S. (2016) Replenishing the cyclic-di-
683 AMP pool: regulation of diadenylate cyclase activity in bacteria. *Curr Genet* **62**, 731-738
684 33. Gibhardt, J., Heidemann, J. L., Bremenkamp, R., Rosenberg, J., Seifert, R., Kaever, V., Ficner,
685 R., and Commichau, F. M. (2020) An extracytoplasmic protein and a moonlighting enzyme
686 modulate synthesis of c-di-AMP in *Listeria monocytogenes*. *Environ Microbiol* **22**, 2771-2791
687 34. Rismondo, J., Gibhardt, J., Rosenberg, J., Kaever, V., Halbedel, S., and Commichau, F. M.
688 (2016) Phenotypes Associated with the Essential Diadenylate Cyclase CdaA and Its Potential
689 Regulator CdaR in the Human Pathogen *Listeria monocytogenes*. *J Bacteriol* **198**, 416-426
690 35. Barreteau, H., Kovac, A., Boniface, A., Sova, M., Gobec, S., and Blanot, D. (2008) Cytoplasmic
691 steps of peptidoglycan biosynthesis. *FEMS Microbiol Rev* **32**, 168-207
692 36. Krissinel, E., and Henrick, K. (2007) Inference of macromolecular assemblies from crystalline
693 state. *J Mol Biol* **372**, 774-797
694 37. Mehra-Chaudhary, R., Mick, J., and Beamer, L. J. (2011) Crystal structure of *Bacillus anthracis*
695 phosphoglucosamine mutase, an enzyme in the peptidoglycan biosynthetic pathway. *J Bacteriol*
696 **193**, 4081-4087

38. Zheng, H., Cooper, D. R., Porebski, P. J., Shabalin, I. G., Handing, K. B., and Minor, W. (2017) CheckMyMetal: a macromolecular metal-binding validation tool. *Acta Crystallogr D Struct Biol* **73**, 223-233
39. Celniker, G., Nimrod, G., Ashkenazy, H., Glaser, F., Martz, E., Mayrose, I., Pupko, T., and Ben-Tal, N. (2013) ConSurf: Using Evolutionary Data to Raise Testable Hypotheses about Protein Function. *Isr J Chem* **53**, 199-206
40. Tan, E., Rao, F., Pasunooti, S., Pham, T. H., Soehano, I., Turner, M. S., Liew, C. W., Lescar, J., Pervushin, K., and Liang, Z. X. (2013) Solution structure of the PAS domain of a thermophilic YybT protein homolog reveals a potential ligand-binding site. *J Biol Chem* **288**, 11949-11959
41. Rao, F., Ji, Q., Soehano, I., and Liang, Z. X. (2011) Unusual heme-binding PAS domain from YybT family proteins. *J Bacteriol* **193**, 1543-1551
42. Raguse, M., Torres, R., Seco, E. M., Gandara, C., Ayora, S., Moeller, R., and Alonso, J. C. (2017) *Bacillus subtilis* DisA helps to circumvent replicative stress during spore revival. *DNA Repair (Amst)* **59**, 57-68
43. Winter, G., Waterman, D. G., Parkhurst, J. M., Brewster, A. S., Gildea, R. J., Gerstel, M., Fuentes-Montero, L., Vollmar, M., Michels-Clark, T., Young, I. D., Sauter, N. K., and Evans, G. (2018) DIALS: implementation and evaluation of a new integration package. *Acta Crystallogr D Struct Biol* **74**, 85-97
44. Evans, P. R., and Murshudov, G. N. (2013) How good are my data and what is the resolution? *Acta Crystallogr D* **69**, 1204-1214
45. Bunkoczi, G., Echols, N., McCoy, A. J., Oeffner, R. D., Adams, P. D., and Read, R. J. (2013) Phaser.MRage: automated molecular replacement. *Acta Crystallogr D Biol Crystallogr* **69**, 2276-2286
46. Liebschner, D., Afonine, P. V., Baker, M. L., Bunkoczi, G., Chen, V. B., Croll, T. I., Hintze, B., Hung, L. W., Jain, S., McCoy, A. J., Moriarty, N. W., Oeffner, R. D., Poon, B. K., Prisant, M. G., Read, R. J., Richardson, J. S., Richardson, D. C., Sammito, M. D., Sobolev, O. V., Stockwell, D. H., Terwilliger, T. C., Urzhumtsev, A. G., Videau, L. L., Williams, C. J., and Adams, P. D. (2019) Macromolecular structure determination using X-rays, neutrons and electrons: recent developments in Phenix. *Acta Crystallogr D Struct Biol* **75**, 861-877
47. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* **66**, 486-501
48. Headd, J. J., Echols, N., Afonine, P. V., Grosse-Kunstleve, R. W., Chen, V. B., Moriarty, N. W., Richardson, D. C., Richardson, J. S., and Adams, P. D. (2012) Use of knowledge-based restraints in phenix.refine to improve macromolecular refinement at low resolution. *Acta Crystallogr D Biol Crystallogr* **68**, 381-390
49. Ashkenazy, H., Abadi, S., Martz, E., Chay, O., Mayrose, I., Pupko, T., and Ben-Tal, N. (2016) ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules. *Nucleic Acids Research* **44**, W344-W350
50. Förster, S., Apostol, L., and Bras, W. (2010) Scatter: software for the analysis of nano- and mesoscale small-angle scattering. *Journal of Applied Crystallography* **43**, 639-646
51. Franke, D., and Svergun, D. I. (2009) DAMMIF, a program for rapid ab-initio shape determination in small-angle scattering. *J Appl Crystallogr* **42**, 342-346
52. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* **25**, 1605-1612
53. Burkholder, P. R., and Giles, N. H., Jr. (1947) Induced biochemical mutations in *Bacillus subtilis*. *Am J Bot* **34**, 345-348
54. Laskowski, R. A., and Swindells, M. B. (2011) LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. *J Chem Inf Model* **51**, 2778-2786

749 **Tables**

750 **Table 1:** Crystallographic data and refinement statistics

	$CdaA_{CD}$	GlmM (PO_4 bound)	GlmM (metal bound)	$CdaA_{CD}$ and $GlmM_{F369}$ Complex 1	$CdaA_{CD}$ and $GlmM_{F369}$ Complex 2
Data collection					
Space group	P 4_3 2_1 2	P 3_2 2 1	P 3_2 2 1	P 1 2_1 1	P 1 2_1 1
Cell dimensions					
a,b,c (Å)	62.88, 62.88, 187.32	134.87, 134.87, 69.18	134.41, 134.41, 69.01	62.20, 227.56, 151.60	62.80, 228.53, 153.19
α,β,γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 120.00	90.00, 90.00, 120.00	90.00, 99.66, 90.00	90.00, 99.86, 90.00
Resolution (Å)	93.66 (2.8)*	48.290 (3.0)*	48.15 (2.9)*	61.32 (3.65)*	76.18 (4.2)*
Unique reflections	9974 (1396)*	14081 (2497)*	15955 (2503)*	45925 (4441)*	31005 (4490)*
*R*pim	0.05 (0.52)*	0.13 (2.58)*	0.09 (1.46)*	0.019 (0.15)*	0.06 (0.22)*
CC(1/2)	0.99 (0.97)*	0.54 (0.54)*	0.99 (0.39)*	0.99 (0.53)*	0.78 (0.39)*
I / σ(I)	18.2 (5.9)*	6.9 (2.4)*	12.6 (4.4)*	5.5 (1.2)*	2.1 (0.9)*
Completeness (%)	100.0 (100.0)*	99.9 (99.5)*	98.8 (97.3)*	99.6 (99.2)*	99.9 (99.9)*
Redundancy	23.2 (24.1)*	19.6 (17.3)*	20.2 (17.5)*	6.9 (7.1)	3.8 (3.8)*
Refinement					
Resolution (Å)	2.8	3.0	2.9	3.6	4.2
*R*work / *R*free(%)[a]	0.2545/ 0.2966	0.1996/ 0.2589	0.1868/ 0.2348	0.2418/0.2615	0.2473/ 0.2976
Rms deviations					
Bond lengths (Å)	0.008	0.016	0.0015	0.003	0.003
Bond angles (°)	1.192	1.533	1.396	0.658	0.703
Ramachandran plot (%) Most favoured/ outliers	89.82/ 2.11	95.71/ 0.68	87.36/ 1.81	92.49/1.44	92.94/ 0.88
PDB code	6HUW	7OJR	7OML	7OLH	7OJS

751 * refers to the highest resolution shell

752 [a]Rfree was computed same as Rwork, using a test set (~5%) of randomly selected reflections that
753 were omitted from the refinement

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Figures and Figure Legends

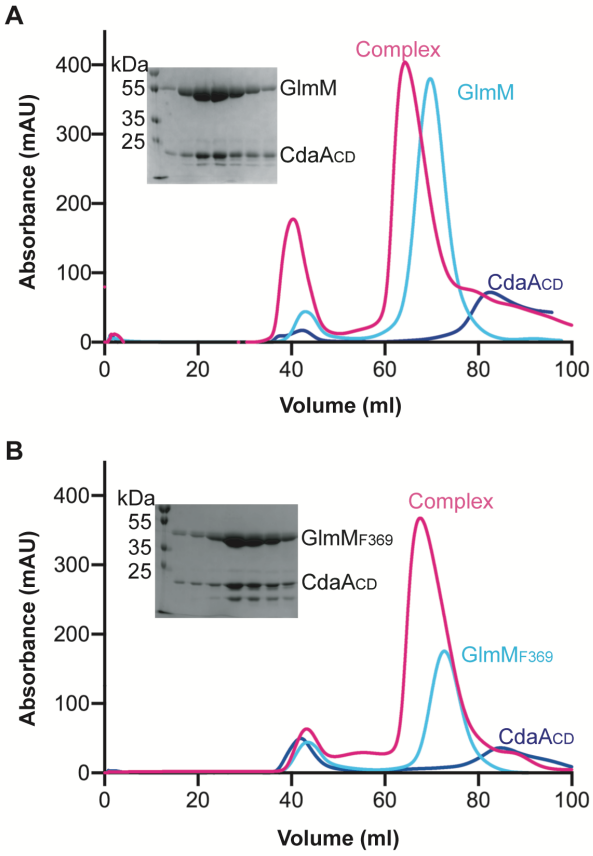

Figure 1: **The *B. subtilis* GlmM and CdaA$_{CD}$ proteins form a complex *in vitro***. *A,* FPLC chromatograms and SDS-PAGE gel analysis of the *B. subtilis* CdaA$_{CD}$ and GlmM complex. The *B. subtilis* proteins CdaA$_{CD}$, GlmM and the CdaA$_{CD}$/GlmM complex were purified by Ni-affinity chromatography and size-exclusion chromatography. The FPLC elution profiles, recorded at a wavelength of 280 nm, are shown for CdaA$_{CD}$ (blue), GlmM (cyan) and the CdaA$_{CD}$/GlmM complex (pink). Proteins from the peak fractions of the CdaA$_{CD}$-GlmM complex were separated on a 12% SDS PAGE gel and proteins visualized by Coomassie staining (shown in the insert). *B,* FPLC chromatograms and SDS-PAGE gel analysis of the *B. subtilis* CdaA$_{CD}$ and GlmM$_{F369}$ complex. Same as in (A) but using the C-terminally truncated GlmM$_{F369}$ variant in place of the full-length GlmM protein. The experiments were performed in triplicates and representative chromatograms and gel images are shown.

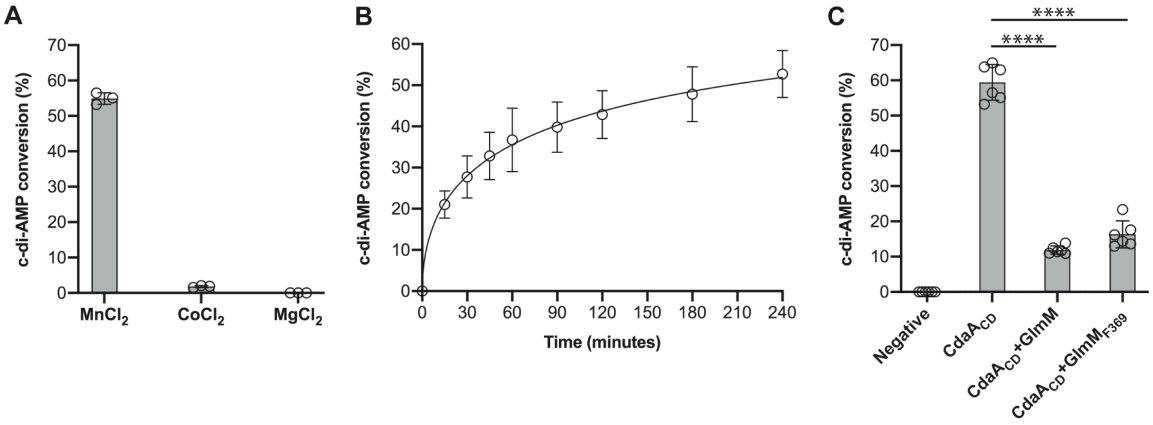

Figure 2: Enzymatic activity of the *B. subtilis* $CdaA_{CD}$ enzyme is inhibited by GlmM or $GlmM_{F369}$. *A,* Metal-dependency of the *B. subtilis* $CdaA_{CD}$ enzyme. The metal-dependency of the *B. subtilis* $CdaA_{CD}$ was assessed by performing enzyme reactions using 5 µM of purified $CdaA_{CD}$ in buffer containing 1 mM Mn^{+2}, Mg^{+2} or Co^{+2} ions. After 4 h of incubation, the reactions were stopped, separated by TLC and the percentage conversion of radiolabelled ATP to c-di-AMP determined. The average values and standard deviations (SDs) from three experiments were plotted. *B,* $CdaA_{CD}$ activity time-course experiment. Enzyme reactions were set up with the *B. subtilis* $CdaA_{CD}$ enzyme in buffer containing 1 mM Mn^{+2}, aliquots removed, and reactions stopped at the indicated time points and separated by TLC. The percentage conversion of radiolabelled ATP to c-di-AMP was determined and the average values and SDs from three independent experiments plotted. *C,* Enzyme activity of the *B. subtilis* $CdaA_{CD}$ enzyme in the presence of *B. subtilis* GlmM. The enzyme activity of $CdaA_{CD}$ was measured in the absence or presence of GlmM or $GlmM_{F369}$ at a molar ratio of 2:1 GlmM or $GlmM_{F369}$ to $CdaA_{CD}$. After 4 h of incubation, the reactions were stopped, separated by TLC and the percentage conversion of radiolabelled ATP to c-di-AMP was determined. The average values and SDs from six independent experiments plotted. One-way ANOVA tests followed by Dunnett's multiple comparison were performed to identify statistically significant differences in cyclase activity in the absence or presence of GlmM or $GlmM_{F369}$. *** indicates $p<0.0001$.

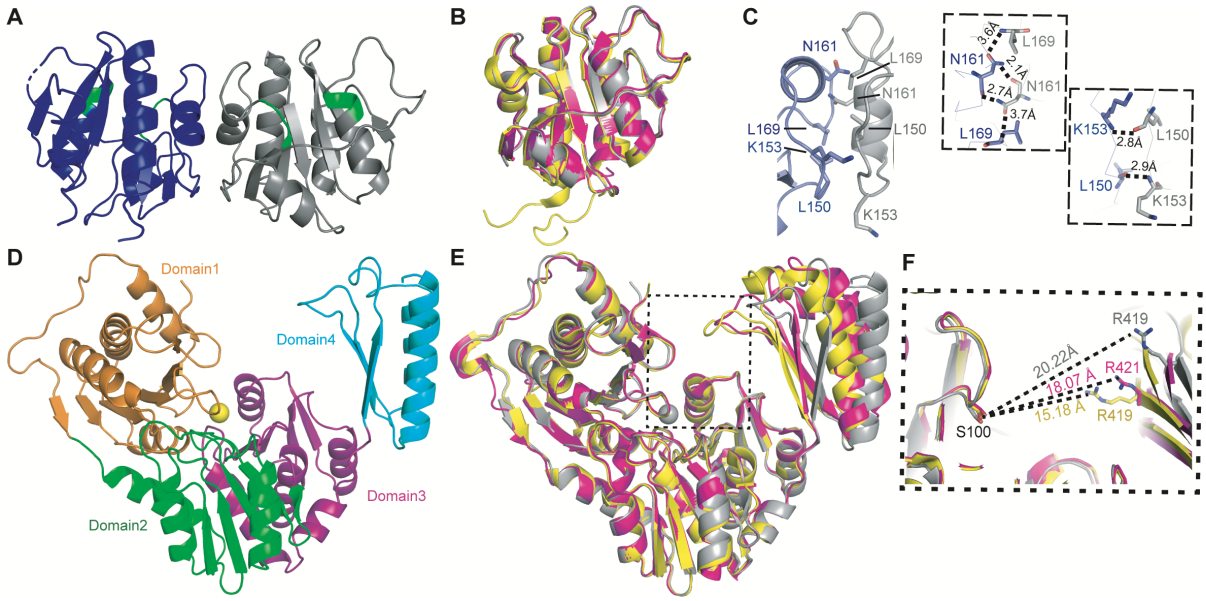

Figure 3: Crystal structures of the *B. subtilis* CdaA$_{CD}$ and GlmM enzymes. *A, B. subtilis* CdaA$_{CD}$ structure in cartoon representation. The CdaA$_{CD}$ protein crystallized as dimer and individual CdaA$_{CD}$ monomers are shown in blue and grey, with active site DGA and RHR motifs highlighted in green. *B,* Superimposition of the *B. subtilis, L. monocytogenes* and *S. aureus* CdaA$_{CD}$ (DacA$_{CD}$) structures. Monomers of the *B. subtilis* CdaA$_{CD}$ (grey), *L. monocytogenes* CdaA$_{CD}$ (yellow; PDB ID 4RV7) and *S. aureus* DacA$_{CD}$ (pink; PDB ID 6GYW) protein structures were superimposed using COOT (47). *C,* CdaA$_{CD}$ dimer interface and zoomed in view showing residues Leu150, Lys153, Asn161 and Leu169, which form hydrogen-bonds, in stick representation. *D*, *B. subtilis* GlmM structure in cartoon representation. The GlmM protein crystalized as a dimer (Fig. S2) with one monomer in the asymmetric unit. The GlmM monomer displayed the typical 4 domain architecture, and the individual domains are represented in different colours. A metal-ion was bound to the protein and is shown as yellow sphere. *E,* Superposition of the *B. subtilis, S. aureus* and *B. anthracis* GlmM structures. *B. subtilis* GlmM (grey), *B. anthracis* GlmM (yellow; PDB 3PDK) and *S. aureus* GlmM (pink; PDB 6GYX) monomer structures were superimposed using COOT (47). *F,* Zoomed in view of the superimposed *B. subtilis* (grey; metal-ion bound structure)*, S. aureus* (pink) and *B. anthracis* (yellow) GlmM structures showing the distances between the indicated Arg residue within the phosphate binding site and the active site Ser residue. Images were generated using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

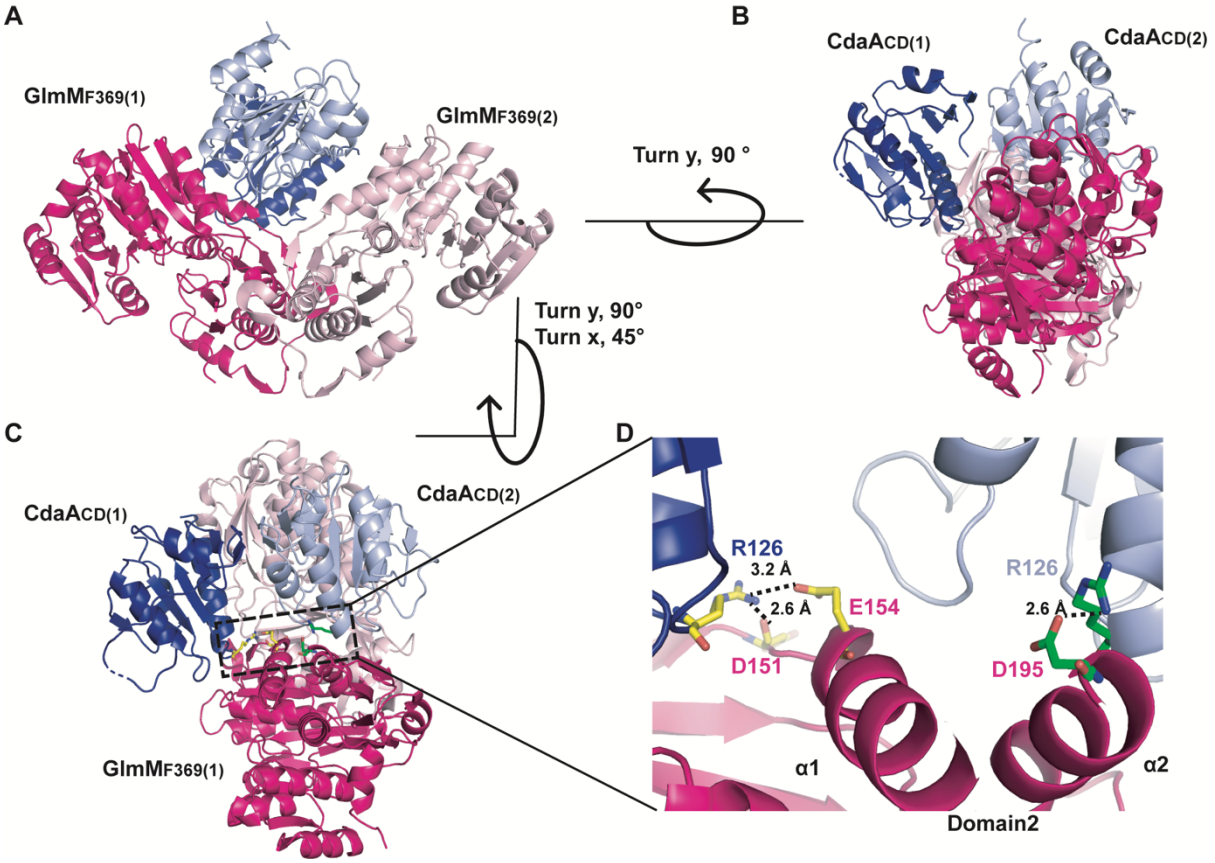

Figure 4: Crystal structure of the *B. subtilis* $CdaA_{CD}$:$GlmM_{F369}$ complex 1. (*A-D*) Structure of the *B. subtilis* $CdaA_{CD}$:$GlmM_{F369}$ complex 1 shown in cartoon representation. The *B. subtilis* $CdaA_{CD}$:$GlmM_{F369}$ complex crystallized as a dimer of dimers with individual $GlmM_{F369}$ monomers shown in dark pink [$GlmM_{F369}$(1)] and light pink [$GlmM_{F369}$(2)] and individual $CdaA_{CD}$ monomers shown in dark blue [$CdaA_{CD}$(1)] and light blue [$CdaA_{CD}$(2)], respectively. The complex is show in *A,* in front view, *B,* in side view (rotated 90º along the y-axis) and *C*, in top-side view rotated at the angle as indicated with respect to (A). *D,* a zoom in view of the $CdaA_{CD}$/$GlmM_{F369}$ interface is shown. Residue Arg126 from $CdaA_{CD}$(1) forms H-bond and ionic interactions with Asp 151 and Glu154 of $GlmM_{F369}$(1) (residues shown in yellow), and residue Arg126 from $CdaA_{CD}$(2) forms ionic interactions with Asp195 in $GlmM_{F369}$(1) (residues shown in green). The images were prepared in PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

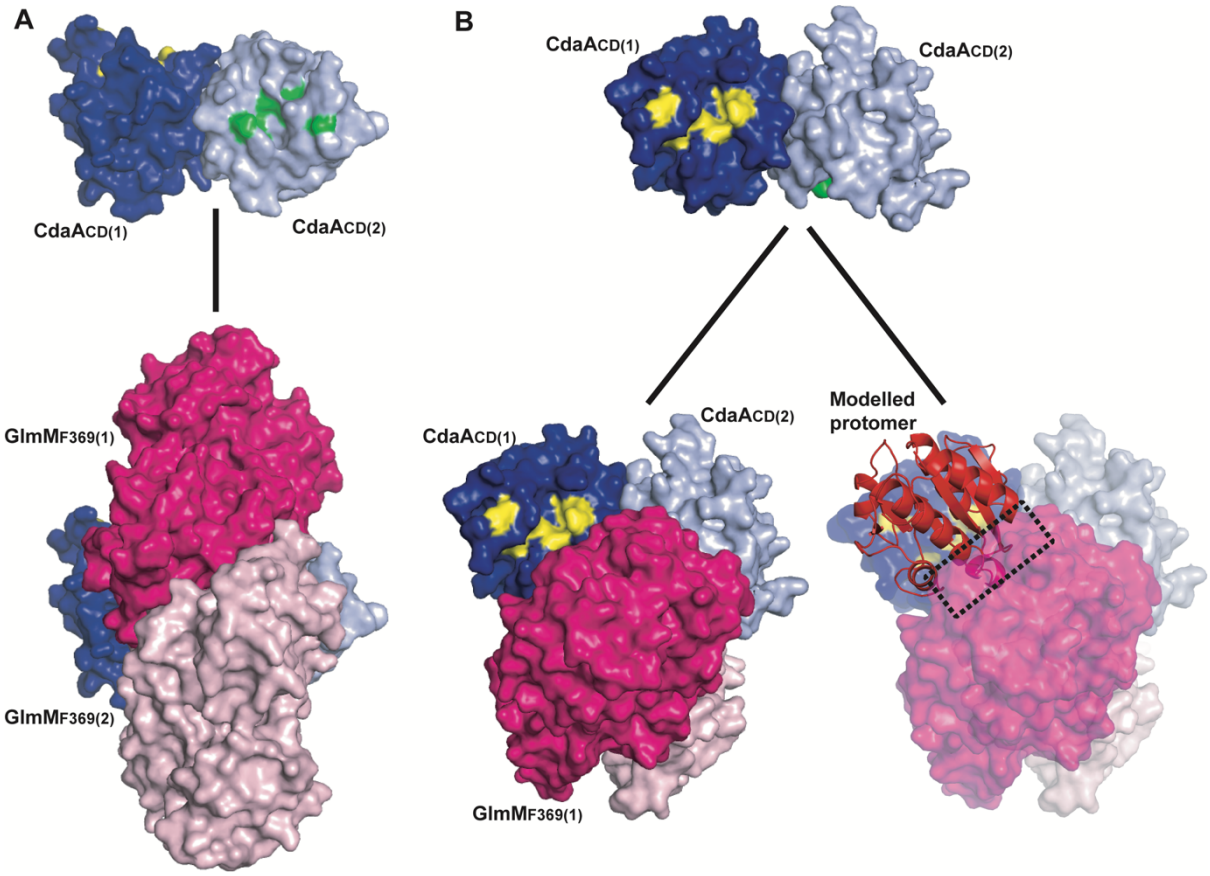

Figure 5: **Formation of an active $CdaA_{CD}$ dimer is blocked in the complex.** (*A-B*) Structure of the *B. subtilis* $CdaA_{CD}$ dimer from complex 1 (top panels) and the complete $CdaA_{CD}$:$GlmM_{F369}$ complex 1 (bottom panels) shown as space filling models. The active site DGA and RHR motifs in $CdaA_{CD}(1)$ (dark blue protomer) are shown in yellow and in $CdaA_{CD}(2)$ (light blue protomer) in green. *A*, Bottom panel, complex model showing that the active site (green residues) in $CdaA_{CD}(2)$ (light blue protomer) are completely occluded in the complex and *B,* the active site (yellow residues) of $CdaA_{CD}(1)$ (dark blue protomer) remains partially exposed. However, the modelling of another $CdaA_{CD}$ protomer (in red) that forms an active dimer with $CdaA_{CD}(1)$ (dark blue protomer) results in protein-protein clashes with the $GlmM_{F369}(1)$ monomer in the complex. The active $CdaA_{CD}$ dimer was modelled based on the *L. monocytogenes* Δ100CdaA (PDB 6HVL) structure (30).

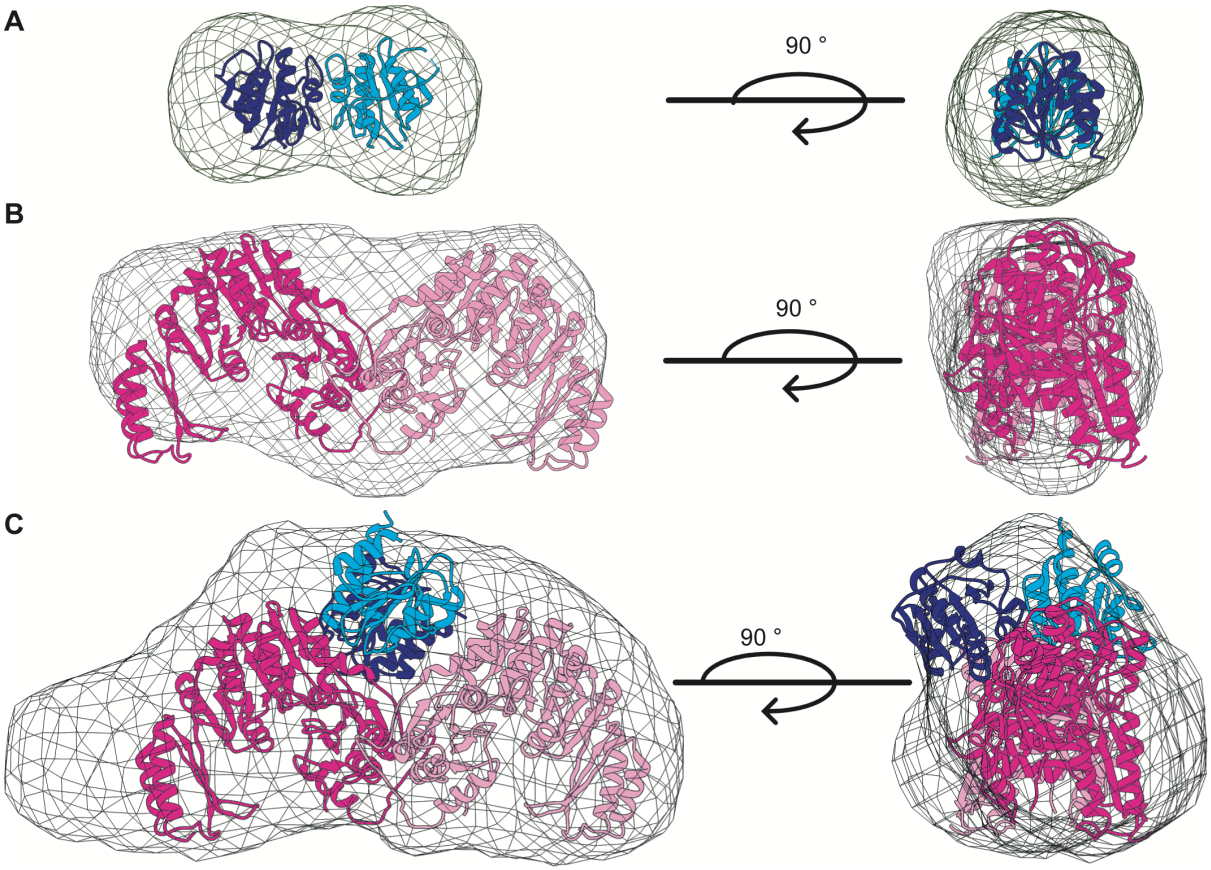

Figure 6: Small Angle X-ray Scattering (SAXS) data of the *B. subtilis* $CdaA_{CD}$, GlmM proteins and the *B. subtilis* and *S. aureus* $CdaA_{CD}$:GlmM complex. *A-C,* SAXS envelopes with fitted protein structures. For the SAXS experiment, 50 µl of $CdaA_{CD}$ (5 mg/ml), GlmM (24 mg/ml) and the complex $CdaA_{CD}$:GlmM (24 mg/ml) were injected onto a high pressure Shodex column coupled to the B21 Small-Angle X-Ray beamline at the Diamond Light Source (Didcot, UK). The data analysis and envelope reconstruction were performed using ScÅtter (50). The program Chimera (52) was used to visualise the reconstructed envelopes as maps into which the protein structures were fitted. ***A,*** SAXS envelope of the *B. subtilis* $CdaA_{CD}$ protein with a $CdaA_{CD}$ dimer protein structure fitted into the envelop. ***B,*** SAXS envelope of the *B. subtilis* GlmM protein with the GlmM protein dimer structure fitted into the envelop. ***C,*** SAXS envelope of the *B. subtilis* $CdaA_{CD}$:GlmM complex with a $CdaA_{CD}$:GlmM dimer model structure fitted into the envelope.

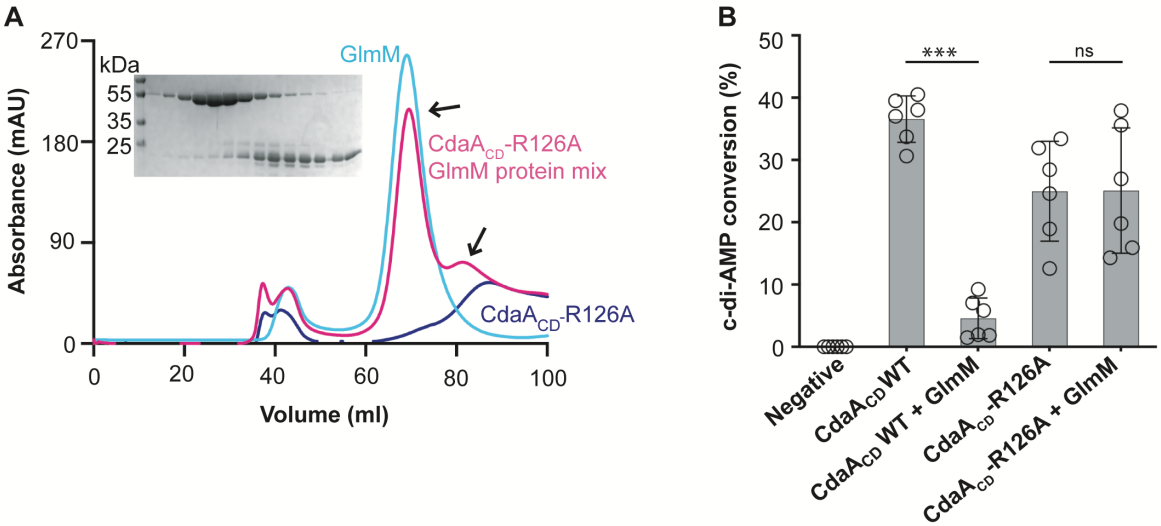

Figure 7: **Residue Arg126 in *B. subtilis* CdaA$_{CD}$-R126A is important for complex formation with GlmM**. *A,* FPLC chromatograms and SDS-PAGE gel analysis of the *B. subtilis* CdaA$_{CD}$-R126A, GlmM and a mix of both proteins. The *B. subtilis* CdaA$_{CD}$-R126A, GlmM and the CdaA$_{CD}$-R126A/GlmM protein mixture were purified by Ni-affinity chromatography and size-exclusion chromatography. The FPLC elution profiles, recorded at a wavelength of 280 nm, are shown for CdaA$_{CD}$-R126A (blue), GlmM (cyan) and the CdaA$_{CD}$-R126A/GlmM mixture (pink). Proteins from the peak fractions of the CdaA$_{CD}$-R126A/GlmM sample were separated on a 12% SDS PAGE gel and proteins visualized by coomassie staining (shown in the insert). The experiments were performed in triplicates and representative chromatograms and gel images are shown. *B,* Enzymatic activity of wild type CdaA$_{CD}$ and the CdaA$_{CD}$-R126A protein variant in the absence or presence of GlmM. Enzyme activity assays were performed with 5 µM purified CdaA$_{CD}$ or CdaA$_{CD}$-R126 in absence or presence of 10 µM GlmM protein. After 4 h of incubation, the reactions were stopped, separated by TLC and the percentage conversion of radiolabelled ATP to c-di-AMP determined. The average values and SDs from six experiments were plotted. T-tests were performed to determine statistically significant differences in enzyme activity of CdaA$_{CD}$ or CdaA$_{CD}$-R126A in the absence or presence of GlmM. ns indicates not statistically significant; *** indicates $p<0.001$.