Structural and Biochemical Analysis of the Essential Diadenylate Cyclase CdaA from *Listeria monocytogenes*

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Jonathan Rosenberg 1, Achim Dickmanns 1, Piotr Neumann 5, Katrin Gunka 6, Johannes Arens 6, Volkhard Kaever 6, Jörg Stülke 1, Ralf Ficner 1, and Fabian M. Commichau 1,2

From the Departments of 6 General Microbiology and 3 Molecular Structural Biology, Institute for Microbiology and Genetics, Georg-August University Göttingen, D-37077 Göttingen, Germany and the 5 Research Core Unit Metabolomics, Hannover Medical School, D-30625 Hannover, Germany

Background: *Listeria monocytogenes* CdaA is an essential diadenylate cyclase.

Results: CdaA activity depends on manganese and cobalt ions.

Conclusion: CdaA has an unusual requirement for metal cofactors.

Significance: Characterization of essential enzymes is important for developing novel antibiotics.

The recently identified second messenger cyclic di-AMP (c-di-AMP) is involved in several important cellular processes, such as cell wall metabolism, maintenance of DNA integrity, ion transport, transcription regulation, and allosteric regulation of enzyme function. Interestingly, c-di-AMP is essential for growth of the Gram-positive model bacterium *Bacillus subtilis*. Although the genome of *B. subtilis* encodes three c-di-AMP-producing diadenylate cyclases that can functionally replace each other, the phylogenetically related human pathogens like *Listeria monocytogenes* and *Staphylococcus aureus* possess only one enzyme, the diadenylate cyclase CdaA. Because CdaA is also essential for growth of these bacteria, the enzyme is a promising target for the development of novel antibiotics. Here we present the first crystal structure of the *L. monocytogenes* CdaA diadenylate cyclase domain that is conserved in many human pathogens. Moreover, biochemical characterization of the cyclase revealed an unusual metal cofactor requirement.

Bacteria use second messenger molecules like the cyclic and linear nucleotides cAMP and (p)ppGpp, respectively, as well as the cyclic dinucleotides c-di-GMP and c-di-AMP to regulate and coordinate cellular processes in response to extra- and intracellular stimuli (1, 2). The functions of cAMP, (p)ppGpp, and c-di-GMP have been studied extensively in several bacteria (3–5). However, fascinating novel discoveries (e.g. the control of *Streptomyces* development by c-di-GMP (6)) reveal that the cellular functions of second messengers, even of well studied signaling molecules like c-di-GMP, are not yet fully explored. Moreover, some bacteria, such as the cyanobacterium *Synechocystis* PCC6803, are capable of producing the cyclic nucleotide cGMP (7). However, although the cyclase that synthesizes cGMP has been biochemically and structurally characterized, the physiological role of the nucleotide remains to be elucidated (8).

The second messenger c-di-AMP was coincidently identified in 2008 (9) in the course of the structural analysis of the DNA integrity scanning protein A (DisA), an enzyme involved in proper genome replication and maintenance of genome integrity in the Gram-positive model organism *Bacillus subtilis* (10, 11). A detailed biochemical characterization of DisA revealed that the nucleotide-binding domain of the protein is a diadenylate cyclase (DAC) that converts two molecules of ATP to c-di-AMP (9). It was also observed that the DAC activity of DisA is strongly inhibited by the presence of non-standard DNA secondary structures like Holliday junctions (9). A recent study emphasized a function of DisA in controlling genome replication during outgrowth of *B. subtilis* spores to prevent propagation of damaged DNA to the germinating cells (12). Meanwhile, homologs of DisA have been identified in several other bacteria like *Mycobacterium tuberculosis* (13–15). It is interesting to note that some bacteria possess more than one DAC-containing protein (14, 16). In addition to the disA gene, the genome of *B. subtilis* harbors the cdaA and cdaS genes that code for the DACs CdaA and CdaS, respectively (17). Whereas the disA and cdaA genes are both constitutively expressed during vegetative growth, the cdaS gene encodes the sporulation-specific DAC CdaS that was shown to be required for efficient germination of *B. subtilis* spores (18).

Although c-di-AMP was discovered only recently, several targets have been identified that bind to the signaling nucleotide. The first target that was shown to bind c-di-AMP is the DNA-binding transcription factor DarR from *Mycobacterium smegmatis* (19). Cyclic di-AMP stimulates the DNA-binding activity of DarR, which in its nucleotide-bound form acts as a repressor and prevents transcription of three target genes. In three recent systematic approaches, several additional protein targets of c-di-AMP that are widely distributed among other bacteria were identified in *Staphylococcus aureus*, *Listeria monocytogenes*, and *B. subtilis* (20–22). As yet, most of the mechanistic details of how c-di-AMP affects the activity of its
targets are unknown. However, it was shown that c-di-AMP controls potassium uptake in *S. aureus* and *Streptococcus pneumoniae* (20, 23) and allosterically regulates the activity of the *L. monocytogenes* pyruvate carboxylase (21). Moreover, c-di-AMP is capable of binding to a conserved riboswitch that controls the expression of the ydaO and ktrAB genes (24, 25), of which the latter encodes a potassium transporter in *B. subtilis* (26). As stated above, c-di-AMP also directly controls potassium uptake (20, 23). Thus, c-di-AMP is the first signaling molecule that regulates the expression of a gene and controls the activity of the encoded protein.

The growing resistance of pathogenic bacteria to the majority of antibiotics that are used in both community and health care settings is an enormous threat to infected patients (27, 28). Moreover, some isolates of *S. aureus* are even resistant to multiple antibiotics (29). Unfortunately, over the past 25 years, only a handful of new antibiotics have been launched (30, 31). Therefore, there is an urgent demand for the identification of essential targets in bacterial pathogens that are suitable for the development of novel antibiotic substances to fight multiresistant isolates (32). As described above, c-di-AMP plays a central role in bacterial physiology. In several bacteria, c-di-AMP homeostasis seems to be also crucial for cell wall metabolism, cell division, and cell size control (17, 33–35). Moreover, some bacteria like the prominent human pathogens *S. aureus*, *S. pneumoniae*, and *L. monocytogenes* possess only the DAC CdaA, which is essential for their growth (36–38). The observation that CdaA is essential and that c-di-AMP does not exist in humans makes the DAC an excellent target for novel antibiotics.

So far, only the structures of the DACs DisA and CdaS from *Thermotoga maritima* and *Bacillus cereus*, respectively, are available (9) (PDB code 2FB5 for the *B. cereus* enzyme) (39). By contrast, no structure of a DAC is available that belongs to the many structurally available restriction enzymes, T4 DNA ligase, and DNA polymerases were used as recommended by the manufacturers. DNA fragments were purified using the PCR purification kit (Qiagen, Hilden, Germany). DNA sequences were determined by the dideoxy chain termination method (SeqLab, Göttingen, Germany). Chromosomal DNA of *L. monocytogenes* was isolated as described previously (41).

**Plasmid Construction**—To assess the biochemical activity of the DAC CdaA from *L. monocytogenes*, the enzyme was produced in *E. coli* BL21(DE3). For this purpose, the truncated cdaA (*lmo2120*) alleles ∆240cdaA and ∆300cdaA, lacking the nucleotides 1–240 and 1–300, respectively, were amplified using the primer pairs JR07 (5′-AAAGAGCTCTGTTTCCAAACCCGAATTTAGCCCGG)/JR08 (5′-TTTGGATCCCTATTGCGTCTTTGGCCTTTTTCGCTTTTTC) and iGEM9 (5′-AAAGGCGCCATGCGACAAAGACATTAAAA)/iGEM10 (5′-AAAGGATCCTTACC-GCGAGTCCTTGCTTTTCAT), respectively. Chromosomal DNA of the *L. monocytogenes* wild-type strain EGD-e served as the template. The PCR products were cloned between the SacI and BamHI sites of the expression vector pGP172 (42). The resulting plasmids pBP119 and pBP33 encode the N-terminally truncated DACs ∆80CdaA and ∆100CdaA, respectively, lacking the first 80 and 100 amino acids, and carry an N-terminal Strep-tag for affinity purification.

**Site-directed Mutagenesis of the cdaA Allele**—To identify the amino acid residues involved in the reaction catalyzed by the truncated DAC, we generated the cdaA mutant alleles G511A, G515C, and C604A/G605T using the combined chain reaction (43). For this purpose, a truncated cdaA gene lacking nucleotides 1–240 was amplified from chromosomal DNA of *L. monocytogenes* EGD-e using the primer pair JR07/JR08 and the mutagenic primer JR18 (5′-P-GAAATACACCGCTTCATAATGGACGGTTATTATTAA), JR19 (5′-P-CGGCTTACGATGCGAGCTTTTTATTAA). The combined chain reaction products were cloned between the SacI and BamHI sites of plasmid pGP172 (42), giving plasmids pBP124 (∆240-cdaA (G511A)), pBP125 (∆240cdaA(G515C)), and pBP126 (∆240cdaA(G604/G605T)). The plasmids pBP124, pBP125, and pBP126 encode the N-terminally truncated mutant variants ∆80CdaA (D171N), ∆80CdaA (G172A), and ∆80CdaA (T202N), respectively, that harbor N-terminal Strep-tags for affinity purification. The numbers of residues indicated in the truncated DAC variants correspond to the full-length CdaA protein (UniProt code Q8Y5E4).

**Analysis and Quantification of Cyclic Dinucleotide Pools**—Intracellular c-di-AMP pools were determined by the liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) method (18, 44). Quantification of c-di-AMP was performed as described previously (18).

**Protein Purification**—*E. coli* BL21(DE3) was used as the host for the production of the truncated protein that was purified using the Streptactin:Strep-tag purification system. The cultures were grown in 1 liter of LB medium at 37 °C. Gene expression was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside (final concentration 1 mM) to logarithmically growing cultures (A600 of 0.5). Cells were collected 3 h after induction, and the cell pellets were resuspended in 8 ml of disruption buffer (10 mM Tris/His, 200 mM NaCl, pH 7.5). The
cells were disrupted by using a French press (18,000 p.s.i., 138,000 kilopascals; Spectronic Instruments, G. Heinemann, Schwäbisch Gmünd, Germany), and the crude extracts were passed over a 1-ml Streptactin column (IBA, Göttingen, Germany). The column was washed five times with 2.5 ml of disruption buffer, and the proteins were eluted from the column with 2.5 ml of elution buffer containing 2.5 mM desthiobiotin (Sigma-Aldrich). The Bio-Rad dye-binding assay was used to determine the protein concentrations. Bovine serum albumin was used as the standard.

Assay for Monitoring Diadenylate Cyclase Activity in Vitro—The in vitro DAC activity assays were carried out in buffer containing 10 mM Tris/Cl, pH 7.5, and 0.1% bovine serum albumin. Depending on the assay conditions, different amounts of ATP/MgCl₂ (equimolar), divalent metal ions, and purified DAC were mixed to a final volume of 400 μl in 1.5-ml reaction tubes. The reaction mixtures were incubated for 4 h at 37 °C with agitation. Reactions were stopped by heating the samples for 5 min at 95 °C. Next, the samples were incubated for 5 min on ice and centrifuged for 10 min at 20,800 × g and 4 °C. 350 μl of the supernatant were added to 1.4 ml of extraction solution (acetonitrile/methanol mixture (1:1, v/v)). The extraction mixture was stored at −20 °C overnight. Samples were then centrifuged at 20,800 × g and 4 °C, and then the supernatants were transferred to fresh vials and dried in a SpeedVac. The dried supernatants were solved with 100 or 200 μl of H₂O (depending on the expected amount of c-di-AMP). After repeated centrifugation and the addition of the internal standard [¹³C,¹⁵N-c-di-AMP], part of the supernatants was analyzed by LC-MS/MS. Subsequently, the samples were heated for 10 min at 95 °C in Laemmli buffer. Finally, the proteins were separated by 12% SDS-PAGE and visualized by silver staining as described previously (45).

Size Exclusion Chromatography and Multilangle Light Scattering (SEC-MALS) Analysis of the Diadenylate Cyclase—The oligomerization status of the DACs Δ80CdaA and Δ100CdaA was determined by SEC-MALS using a setup of an S75 Superdex 10/300GL on an Äkta purifier (both from GE Healthcare) subsequent to a degasser (model 2003 from Biotech AB, Onsala, Sweden) for the buffer (50 mM Tris/Cl, pH 7.5, 150 mM sodium chloride) in line with a miniDawn Treos multilangle light scattering system followed by an Optilab T-rEX RI detector (both from Wyatt Technology Europe). Data analysis was performed using the ASTRA version 6.1 software (Wyatt Technology).

Crystallization and Data Collection—The CdaA DAC domain was subjected to initial crystallization trials at a concentration of 9.4 mg/ml with a 5-fold molar excess of ATP. Best diffracting crystals grew in 6–16% 1,6-hexanediol, 2 mM spermine, 20 mM MgCl₂, 0.1 mM Na-PIPES, pH 7.5. The oscillation images were collected at PETRA III (Beamline P13, EMBL, Hamburg, Germany) and processed with XDS (46). The scaling process revealed a tetragonal lattice and unit cell parameters of a = b = 130.7 Å, c = 178.2 Å, α = β = γ = 90.00°. Systematic absences indicated the space group to be either P4₁2₁2 or P4₁2₁₂. The Matthews coefficient (Vₘ = 4.5 Å³/Da) suggested four molecules occupying the asymmetric unit corresponding to a solvent content of 72%. The data collection statistics are summarized below (see Table 1).

**Structure Determination and Refinement—**Initial phases were obtained by molecular replacement method with Phaser using the structure of the DAC CdaS from B. cereus (PDB code 2BFS) as the search model. Template candidate has been identified based on an HHpred (47, 48) search and trimmed to the last common Cα atoms using phenix.sculptor prior to the MR search. The model was manually rebuilt using Coot (49) and refined with Phenix (50) using standard parameters. A random set of 5% of reflections generated in thin shells was excluded from refinement to monitor Rfree (51, 52). The final model has been refined at a resolution of 2.8 Å to R and Rfree factors of 24.0 and 25.6%, respectively. The model consists of four protein molecules per asymmetric unit encompassing residues 0–4 of the N-terminal Strep-tag and residues 1–157 (155 for molecule D) of the DAC domain, three water molecules, one ATP molecule, and one Mg ion per protein molecule and one 1,6-hexanediol. The refinement statistics are summarized below (see Table 1). Surface complementarity coefficients and solvent-accessible surface areas were calculated with the SC program using a 1.7-Å radius probe (53). Possible hydrogen bonds, salt bridges, and van der Waals contacts were detected with HBPLUS and CONTACTSYM (54) using default parameters. Surface potentials were calculated with PyMOL using the APBS plugin (55). The quality of the model was assessed using MOLROBITY (56) as implemented in Phenix. Secondary structure predictions were performed using DSSP (57, 58). Coordinate were superimposed with LSQKAB (59) from the CCP4 program suite (53) or as implemented in PyMOL. Structure-based sequence alignment was performed using Espross (60) and optimized for presentation using ESPRIPT (61). Structure coordinates and structure factors of the CdaA-DAC domain from L. monocytogenes have been deposited as PDB entry 4RV7.

**RESULTS**

**Purification and Structure Determination of Δ100CdaA—**It was suggested previously that the DACs of the CdaA family are membrane-bound enzymes containing three N-terminally located α-helices that form a transmembrane (TM) domain (Fig. 1) (14). The TM domain could hamper the purification of the enzyme. Indeed, previous attempts to express and purify the full-length B. subtilis and L. monocytogenes CdaA proteins failed. Therefore, the truncated L. monocytogenes cdaA allele was cloned for heterologous production in E. coli and for in vitro characterization of the DAC. For this purpose, the Δ300cdaA allele encoding the N-terminally truncated Δ100CdaA protein was introduced into the E. coli expression vector pGP172 (see “Experimental Procedures”). The truncated DAC carries an N-terminal Strep-tag for affinity purification

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and lacks the predicted TM domain and the coiled-coil motif that separates the TM domain from the cyclase domain (Fig. 1). The truncated Δ100CdaA protein was purified from E. coli, and the evaluation of the elution fractions by SDS-PAGE revealed that the protein can be purified in high amounts and with high purity (data not shown). Next, the protein was subjected to crystallization in the presence of ATP. The crystals obtained diffracted up to 2.8 Å resolution (Table 1, top). The structure was solved by means of molecular replacement using the model of the B. cereus DAC CdaS that was identified by HHPred server search (PDB code 2FB5). The CdaA-DAC domain structure was refined at 2.8 Å and yielded a model of good quality with reasonable data statistics (see Table 1, bottom). Δ100CdaA exhibits an overall globular fold with the long N-terminally located helix (α1) flanking the core (Fig. 2, A and B). A slightly twisted central β-sheet, made up of seven mixed-parallel and antiparallel β-strands (β1–β7), forms the core of the globular part. Both sides of the β-sheets are flanked by a total of five α-helices (α1–α5), resulting in the observed globular shape. In addition to the complete sequence of Δ100CdaA, the structure contains 3–4 residues of the N-terminal Strep-tag, which are well defined in the electron density map and precede the N-terminally located α-helix.

Comparison of the DAC Domains of CdaA, DisA, and CdaS—Because c-di-AMP synthesis is thought to require the close proximity of two ATP molecules, the active sites of two DAC domains have to be face-to-face orientated. In order to make an educated guess about the structural arrangement of the dimer assembly, we compared the structure of Δ100CdaA with the most prominent hits obtained by HHpred (Fig. 3, A–C). The first hit that was already used for molecular replacement is the sporulation-specific B. cereus DAC CdaS (PDB code 2FB5), which exists as a trimer in the asymmetric unit. Unfortunately, the analysis of the crystal packing and contacts to symmetry-related neighboring molecules revealed that there is also no proper arrangement in a face-to-face manner allowing for c-di-AMP formation (PDB code 2FB5). A recently suggested model positions six molecules in a circular arrangement with dimers interacting in a face-to-face manner, connected via their hydrophobic tails (18). The second hit was the DAC DisA from T. maritima (PDB code 3C23, ATP analog-bound; 3C1Z, apo-form, 3C1Y c-di-AMP-bound). Interestingly, DisA forms a homo-octamer, thereby arranging pairs of molecules in such a way that two active sites face each other. This arrangement is compatible with binding of two ATP molecules and subsequent c-di-AMP formation (PDB codes 3C21 and 3C23).

Interestingly, there are four Δ100CdaA molecules present in the asymmetric unit but in an arrangement incompatible with the formation of c-di-AMP (PDB code 4RV7), which would require a face-to-face orientation of two active sites to be able to form the cyclic sugar phosphate backbone ring (9). The observed assembly within an asymmetric unit is probably due to the crystallization conditions and probably does not reflect the situation in aqueous solution (see below). However, superposition of either two Δ100CdaA or CdaS DAC molecules with the DAC domains of two corresponding DisA molecules resulted in a dimer model of CdaA with a high degree of overlap (Fig. 3D).

The fully refined overall structure of the Δ100CdaA DAC domain exhibited a prominent unoccupied electron density (Fig. 2C), which, based on its shape, could be interpreted as a molecule of ATP that was present in the crystallization condition. This ATP molecule is bound in a well defined cavity made up by helix α4 and strands β1 and β5 as well as the loops connecting α1 and β1, α3 and β3, β4 and α4, and β5 and β6 (Fig. 2B). The bottom of this pocket is formed by the hydrophobic patch of residues G(A/G)L131I of strand β1 conserved in all three known structures of CdaA, CdaS (PDB code 2FB5), and DisA (PDB code 3C21) (see Fig. 2D). The structure-based sequence alignment with the other two structures found by structure similarity also reveals the conservation of residues in two more distinct patches (Fig. 2D). One patch is made up by the loop connecting α3 and β3, which harbors the active site, a highly conserved D171GA motif followed by three hydrophobic residues (Fig. 2, C and D). The aspartic acid Asp-171 localizes in

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**Figure 1.** Schematic illustration of the domain architecture of *L. monocytogenes* DAC CdaA. The protein contains a TM domain, two CC motifs, and a cyclase domain. The Δ80CdaA protein lacks the TM domain. The Δ100CdaA lacks the TM domain and the CC motif. Both truncated DACs harbor at their N terminus a Strep-tag for affinity purification. The numbers relate to the full-length CdaA protein (UniProt code Q8Y5E4).

### Table 1

| Table 1: Crystallographic data collection and refinement statistics |
|---|
| **Δ100CdaA-ATP** |
| **Crystallographic data** |
| Beamline | Petra III-P13, EMBL, Hamburg |
| Wavelength (Å) | 0.9100 |
| Resolution (Å)* | 50.280 (2.89-2.94) |
| Unique reflections | 38,360 (5,240) |
| Redundancy | 6.5 (6.5) |
| Completeness (%) | 99.0 (99.7) |
| Space group | P4 3 2 |
| a (Å) | 130.67 |
| b (Å) | 130.67 |
| c (Å) | 178.15 |
| Rmerge (%) | 5.1 (68.1) |
| I/σ (I) | 24.42 (2.74) |
| CC1/2 (%) | 100 (83.1) |

| **Refinement statistics** |
| Resolution range (Å) | 48.86–2.8 (2.87–2.80) |
| Completeness (%) | 99.16 (99.7) |
| Rmerge/Rfree (%) | 22.97/25.39 (30.57/33.51) |
| No. of atoms | 4,996 |
| Average B factor (Å²) | 87.1 |

| **Ramachandran plot** |
| Favorable (%) | 96.64 |
| Allowed (%) | 2.72 |
| Outliers (%) | 0.64 |

*Values for the data in the highest resolution shell are shown in parentheses.

* The CC1/2 is the correlation coefficient between two randomly selected half-data sets as described (71).

* Rmerge = \( \frac{\sum|I(I) - \langle I(I) \rangle|}{\sum I(I)} \) where “Test” is a test set of about 5% of the data in the highest resolution shell.

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close proximity to the ribose and the α-phosphate of the neighboring subunit in the CdaA dimer model (Fig. 3D), well suited for catalysis (Fig. 4A). This region positions the adenosine moiety in a conformation highly similar to the adenosine moieties of c-di-AMP in DisA (PDB code 3C21; Fig. 4B). In the dimer model of CdaA, the two ATPs would be oriented similarly to the ATP analogs in the DisA dimer (PDB code 3C23; Fig. 4A). The loop subsequent to 5 contains a short patch of 2–3 residues subsequent to the highly conserved Ser-222 (see Fig. 2C). This patch interacts with the β- and γ-phosphate and is involved in coordination of the magnesium ion. The other conserved patch, a GXRHRRA motif, resides in helix α4 and harbors an arginine, Arg-203, which is involved in coordination of the ribose and the phosphates (see Fig. 2D). Within the long loop connecting the preceding strand β4 with helix α4, a leucine is located (Leu-188) whose carbonyl group interacts with the exocyclic N6 of the adenosine moiety (see Fig. 2, C and D). Taken together, the identical arrangement of the residues within the structures used in the structure-based sequence alignment seems to be required for binding of ATP and catalysis, which explains the high conservation of the residues and their function.

In Vivo Activities of Truncated Diadenylate Cyclases—As described above, four molecules are present in the asymmetric unit in an arrangement incompatible with c-di-AMP formation. As shown in Fig. 1, the Δ100CdaA protein lacks the TM domain and the predicted coiled-coil (CC) motif that separates the TM and DAC domains from each other. The lack of either of the domains or both together might be the reason for the molecular arrangement of the Δ100CdaA molecules. To evaluate whether the TM domain and the CC motif are important for enzyme catalysis, we assessed the in vivo activities of the truncated DACs Δ80CdaA and Δ100CdaA. Whereas the latter var-
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FIGURE 3. The cyclase domain of the truncated Δ100CdaA protein is similar to that of DisA and CdaS. The two structures with the highest similarity to Δ100CdaA (pink), DisA (PDB code 3C21) from T. maritima (pale cyan) and the sporulation-specific DAC CdaS (PDB code 2FB5) from B. cereus (gray) are shown. A, superposition of all three structures indicating a high similarity in the central part of the DAC domain. Shown is pairwise superposition of Δ100CdaA with DisA (B) and Δ100CdaA with CdaS (C). D, generation of a dimer model of CdaA by superposition to a DisA dimer. The second molecules of the dimer are shown in pink and pale cyan for CdaA and DisA, respectively. The two ATPs were colored according to Fig. 2.

FIGURE 4. The Δ100CdaA cyclase domain binds ATP similar to DisA and dimer modeling and superposition reveals highly conserved patches, suggesting an identical reaction mechanism. A, magnification and overlay of Δ100CdaA active site model formed by the two subunits (dark and light pink) focusing on divergent residues after superposition of the two molecules in the proper arrangement with respect to DisA (PDB code 3C23) from T. maritima (dark and light gray). Selected important active site residue side chains for both molecules are labeled and shown in stick mode on opposing molecules and in line mode on the other molecule. One molecule may be converted into the other by a 180° turn in the plane of the paper. The numbers of residues indicated in the DACs correspond to the full-length CdaA and DisA proteins (CdaA, UniProt code Q8Y5E4, black; DisA, UniProt code Q9WY43, gray). Mg2+ ions are depicted in green. B, a model c-di-AMP in the active site of Δ100CdaA after arrangement of two Δ100CdaA molecules with respect to DisA (PDB code 3C23).

A variant that was used for crystallization lacks the TM domain and the CC motif, the newly constructed Δ80CdaA variant only lacks the TM domain (Fig. 1). Because E. coli does not produce c-di-AMP, this organism is well suited to analyze the production of the nucleotide in vivo (14, 17, 35). Therefore, both enzymes were expressed in E. coli (see “Experimental Procedures”). The amounts of c-di-AMP in in the bacteria harboring the plasmids pBP119 and pBP33 encoding the truncated DACs Δ80CdaA and Δ100CdaA, respectively, were compared with those produced by the same strain carrying the empty plasmid pGP172. As expected, no c-di-AMP was detectable in cells of the control strain that carries the empty vector (Fig. 5A). In contrast, 31,019 ng and 4,772 ng of c-di-AMP/mg of protein were detected in cells carrying the plasmids pBP33 (Δ100CdaA) and pBP119 (Δ80CdaA), respectively (Fig. 5A). Δ100CdaA and Δ80CdaA were expressed in similar amounts, as shown by Coomassie-stained SDS-polyacrylamide gels of 100/600 μl from the cultures (Fig. 5B). Although the shorter DAC variant was 6.5-fold more active than the longer variant, the results clearly indicate that both truncated DACs form c-di-AMP in vivo. This suggests that the DACs must be transiently face-to-face oriented in vivo to allow the formation of c-di-AMP.

Oligomerization of the Truncated Diadenylate Cyclases—Although the precise mechanism of the cyclase reaction remains to be elucidated, structural and biochemical analyses of the DAC DisA from T. maritima revealed that two cyclase domains must come into close proximity with each other to allow the conversion of two molecules of ATP to c-di-AMP (9). To determine the oligomerization of the truncated L. monocytogenes DACs, we performed an in vitro cross-linking experiment. For this purpose, the purified proteins Δ80CdaA and Δ100CdaA were incubated with different amounts of the cross-linker glutardialdehyde, and the oligomerization was analyzed by SDS-PAGE. As shown in Fig. 6A, the untreated proteins exclusively exist as monomers. By contrast, Δ80CdaA as well as Δ100CdaA formed dimers when the proteins were treated with 0.1 or 0.2% glutardialdehyde. Thus, both enzymes are capable of forming dimers in the presence of a cross-linker that helps to stabilize oligomeric states of proteins under denaturing conditions (Fig. 6A).

We also performed SEC-MALS with Δ80CdaA and Δ100CdaA (Fig. 6B). The determined masses were 38.3 and 46.9 kDa for Δ100CdaA and Δ80CdaA, respectively. This almost perfectly matches the doubled mass of 42.5 and 47.2 kDa calculated for Δ100CdaA and Δ80CdaA, respectively, supporting the idea of a more or less stable dimer formation of both variants. To conclude, despite the lack of the TM domain in the DAC Δ80CdaA or the TM domain and the CC motif in the Δ100CdaA protein...
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![Graph]

**FIGURE 5.** *In vivo* activity assay with the truncated DACs Δ80CdaA and Δ100CdaA, and the Δ80CdaA mutants D171N, G172A, and T202N. A, detection of intracellular c-di-AMP in *E. coli* cells harboring the empty plasmid pGP172 (control) and the plasmids pBP33 and pBP119 that encode the truncated DACs Δ80CdaA and Δ100CdaA, respectively. The mutant variants Δ80CdaA(D171N), Δ80CdaA(G172A), and Δ80CdaA(T202N) are encoded on plasmids pBP124, pBP125, and pBP126, respectively. The cells were grown in LB medium, and cell extracts for the determination of c-di-AMP by HPLC-MS/MS were prepared as described under “Experimental Procedures.” S.D. values (error bars) based on three biological replicates are indicated. B, Coomassie-stained SDS gels of 100/ΔCdaA μl from the cultures used for the determination of the *in vivo* activity of the CdaA variants in A. The variants are expressed in similar amounts, suggesting that the amount of c-di-AMP in the cultures corresponds with the activity of the enzymes.

![Graph]

**FIGURE 6.** Oligomerization analyses of the truncated DACs by cross-linking and SEC-MALS. A, *in vitro* cross-linking experiment with the purified Δ80CdaA and Δ100CdaA proteins. 50 pmol of the purified proteins Δ100CdaA (21.26 kDa) and Δ80CdaA (23.6 kDa) were incubated with increasing amounts of the cross-linking agent glutaraldehyde (X-link). To analyze oligomerization of the DACs, samples were analyzed by 12% SDS-PAGE, and the proteins were visualized by silver staining. B, SEC-MALS analysis of the purified Δ80CdaA and Δ100CdaA proteins performed as described under “Experimental Procedures.” The elution volume and the molecular mass calculated from the scattered signal indicate dimer formation. The light scattered (LS) by the Δ80CdaA and Δ100CdaA proteins, which allows measurement of the molecular mass is shown in dark blue and red, respectively. The UV light detector signals for the Δ80CdaA and Δ100CdaA proteins are shown in light blue and orange, respectively. The short blue and red lines within the peaks indicate the estimated molecular masses for the Δ80CdaA and Δ100CdaA proteins, respectively.

(see Fig. 1), both enzymes are capable of forming dimers, which is the prerequisite for c-di-AMP formation. The fact that the buffer that was used for size exclusion chromatography was not supplemented with divalent ions suggests that the metal ion is required for catalysis and not for dimerization of the enzyme. Moreover, the observation shows that the assembly observed within an asymmetric unit is due to the crystallization conditions and does not exist in aqueous solution.

*In Vitro Activity of the L. monocytogenes Diadenylate Cyclases—* Next, we performed *in vitro* enzyme assays with the DAC Δ80CdaA protein that was purified from *E. coli* (see “Experimental Procedures”). The truncated DAC showed no activity in a buffer containing 10 mM MgCl₂ (see Fig. 7A). The shorter Δ100CdaA variant also did not produce c-di-AMP under these conditions (data not shown). It has been shown that the regulatory protein CdaR stimulates the CdaA homolog from *B. subtilis* (17). However, the fact that the DACs Δ80CdaA and Δ100CdaA produced significant amounts of c-di-AMP in *E. coli* (see Fig. 5A), although the organism does not contain a CdaR-like protein, suggests that the *L. monocytogenes* DAC does not require a protein as a cofactor for being active *in vitro*.

To identify the cofactor that enables the DAC Δ80CdaA to produce c-di-AMP *in vitro*, we performed a metal ion screening with the divalent chloride salts of magnesium, cobalt, copper, nickel, zinc, manganese, and calcium. As shown in Fig. 7A, the Δ80CdaA enzyme only produced c-di-AMP when either Mn²⁺ or Co²⁺ was present in the reaction mixture. The shorter DAC variant Δ100CdaA required the same cofactors (data not shown). Unlike other DACs, such as DisA from *M. tuberculosis* and *T. maritima*, that synthesize c-di-AMP in the presence of Mg ions (9, 13), the activity of the *L. monocytogenes* DAC unequivocally depends on Mn²⁺ or Co²⁺.

*c-di-AMP Production by Δ80CdaA in the Presence of Co²⁺ and Mn²⁺—*To identify the best cofactor concentration for the truncated DAC Δ80CdaA, we performed *in vitro* activity assays with different concentrations of the divalent chloride salts of...
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In conclusion, the truncated DAC even more c-di-AMP than with MnCl₂ (Fig. 7). Below 0.75 mM and above 5 mM MnCl₂, the activity of the enzyme decreased, and no c-di-AMP was produced below 0.5 mM and above 100 mM MnCl₂. The c-di-AMP levels were determined by HPLC-MS/MS as described under “Experimental Procedures.” S.D. values (error bars) based on three technical replicates are indicated.

cobalt and manganese. As shown in Fig. 7B, when Mn²⁺ ions were present in the reaction mixture, the Δ80CdaA protein showed the highest DAC activity in the range between 0.75 and 20 mM. Below 0.75 mM and above 5 mM MnCl₂, the activity of the enzyme decreased, and no c-di-AMP was produced below 0.5 mM and above 100 mM MnCl₂. The in vitro assay that was performed with various amounts of CoCl₂ revealed that the Δ80CdaA protein was active with low amounts of Co²⁺, and in the presence of this ion, the enzyme produced even more c-di-AMP than with MnCl₂ (Fig. 7B). With 20 mM CoCl₂, the activity of the enzyme was strongly reduced, and no c-di-AMP was produced below 0.5 mM and above 20 mM CoCl₂. In conclusion, the truncated DAC Δ80CdaA from L. monocytogenes preferentially uses Co²⁺ to produce c-di-AMP from ATP.

Importance of Conserved Residues for the Cyclase Reaction—

It has been suggested previously that the conserved basic and acidic side chains in the RHR and DGA motifs, respectively, are involved in the cyclase reaction (see Fig. 2D) (9). In order to test the importance of these residues for ATP binding and c-di-AMP formation, the residues Asp-171, Gly-172, and Thr-202 directly preceding the RHR motif were selected and replaced by Asn, Ala, and Asn, respectively, in the truncated DAC Δ80CdaA. In the dimeric model, the active site asparagine, Asp-171, is involved in coordination of one ribose and phosphates of the neighboring ATP mediated by the metal ion. The additional methyl group of alanine replacing glycine at position 172 is expected to sterically interfere with binding of the adenosine and the sugar moiety of the ATP molecule (Figs. 2C and 4A). Moreover, the T202N mutation should disrupt the stabilization of the sugar α-phosphate through interaction between the asparagine and the α-phosphate. The three respective Δ240cdaA mutant alleles were expressed in E. coli and tested for in vivo activity (see “Experimental Procedures”). All mutants expressed like the non-mutated DAC Δ80CdaA (data not shown). In contrast to the non-mutated variant, the mutated enzymes were unable to form c-di-AMP in vivo (Fig. 5A). These results unequivocally indicate that the conserved residues are important for the cyclase reaction.

DISCUSSION

In the present study, we have structurally and biochemically analyzed truncated variants of the essential DAC of the human pathogen L. monocytogenes. Interestingly, the biochemical characterization of the DAC revealed that this enzyme requires either a divalent manganese or cobalt ion for in vitro activity. By contrast, other DACs like the DisA orthologs from T. maritima, B. subtilis, Bacillus thuringiensis, and M. tuberculosis were all shown to be active in vitro with magnesium ions (9, 62, 63). DisA from M. tuberculosis also produced c-di-AMP when manganese was provided as a cofactor (62, 63). However, in none of the characterized DACs can magnesium be fully replaced by cobalt. Because L. monocytogenes is an invasive human pathogen that is able to penetrate and to survive in different cell types like macrophages (64), it has been suggested that competition between the pathogen and the host cell for divalent metal ions is important for survival (65). Therefore, mechanisms must have evolved in pathogenic bacteria, allowing them to survive in very specialized ecological niches like macrophages that are believed to be nutrient-restricted environments. For instance, one could imagine that an alteration of the cofactor requirement of the essential DAC CdaA by adaptive evolution might improve survival of L. monocytogenes inside the host cells. However, it remains to be elucidated whether the physiological role of cobalt is indeed important for pathogenesis of L. monocytogenes.

The structural analysis of the truncated L. monocytogenes D. CdaA, lacking the first 100 amino acids, revealed a structural arrangement in the crystals that is incompatible with a functional dimer of CdaA (PDB code 4RV7). Recently, a similar observation was made when the sporulation-specific DAC CdaS from B. cereus was structurally analyzed (PDB code 2FB5). In the case of CdaS, the arrangement of the molecules in the crystals is also incompatible with a functional DAC. However, the data obtained by the enzyme assays, in vitro cross-linking experiments, and SEC-MALS analysis show that CdaA forms dimers and suggest that DAC is functional as a dimer in solution (Figs. 6 and 7). Therefore, the observed structural arrangements of the B. cereus DAC CdaS and the truncated L. monocytogenes DAC CdaA is most likely a consequence of
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the crystallization conditions used. However, the structures of the CdaA DAC domain as well as of the deposited structure of CdaS are highly similar to that of the DAC domain of DisA from *T. maritima* (9). The superposition of the DAC domains demonstrates the proper arrangement of the important residues within the three identified highly conserved patches. These patches that are required for ATP coordination and synthesis of c-di-AMP are present in all three structures compared (Figs. 2 (C and D) and 4 (A and B)). Taken together, our observations strongly argue for a similar arrangement of the dimers in the truncated DAC CdaA in a face-to-face fashion and an identical mechanism in c-di-AMP formation.

As illustrated in Fig. 1, the full-length DAC CdaA contains an N-terminal TM domain. It has been proposed that this domain acts as a sensor for the perception of stimuli from the cell membrane or cell wall and that the altered c-di-AMP production in turn affects essential cellular processes that remain to be uncovered (14). For CdaA from *B. subtilis*, it has been shown that the DAC activity is stimulated through a direct protein-protein interaction with CdaR (17), a protein that is also present in *L. monocytogenes*. Thus, alternatively to the model proposed above, CdaR might serve as the sensor that perceives an environmental stimulus and transduces the information to CdaA, which changes its activity accordingly. However, the precise physiological role of the regulatory interaction between CdaA and CdaR is rather unclear.

In contrast to other bacteria like *B. subtilis* that possesses three DACs (17, 18), *L. monocytogenes* synthesizes only the membrane-bound DAC CdaA. It is not yet clear why CdaA in *L. monocytogenes* and other bacteria is localized at the membrane. Recently, it has been shown that growth of an *L. monocytogenes* conditional cdaA mutant that was depleted for CdaA could be rescued by expressing the DAC domain of DisA from *B. subtilis* (35). Similarly, a *B. subtilis* δdisA ΔcdaA mutant strain was able to grow when cdaS, encoding the sporulation-specific DAC CdaS, was overexpressed (17). These observations suggest that c-di-AMP production by a cytosolic DAC is *per se* sufficient to ensure survival of the bacteria. However, the situation might be completely different if the bacteria grow in their natural habitats. Under these conditions, in particular for bacteria having multiple DACs, the enzymes might perceive different signals and generate local c-di-AMP pools that trigger different downstream targets. Recently, it has indeed been suggested that signaling specificity of second messengers can be achieved by generating local pools through the localization of enzymes that are involved in synthesis and in degradation of the signaling molecule (16, 66).

As described above, the emergence of multiresistant human pathogens is a serious threat for humankind because some isolates are untreatable with the antibiotics that are currently used. Among the DACs, CdaA is the most abundant enzyme that is conserved and essential in several human pathogenic bacteria like *L. monocytogenes* and multiresistant *S. aureus* isolates (14, 36 – 38). Moreover, c-di-AMP proves to be a second messenger that has multiple cellular targets like riboswitches, signal transduction proteins, and metabolic enzymes (21, 22, 24). In addition to this, perturbation of c-di-AMP homeostasis was shown to severely affect cell wall metabolism in Gram-positive bacteria (17, 33) and to influence the susceptibility of bacteria to cell wall-targeting antibiotics (34, 67). Therefore, the DAC CdaA might be a promising target for the development of novel antimicrobial substances to fight multiresistant pathogenic bacteria. Since the discovery of c-di-AMP, several methods for monitoring its production in small scale have been developed (68, 69). These methods seem to be suitable for high throughput screenings to identify substances that inhibit bacterial DACs (70).

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REFERENCES

1. Gomelsky, M. (2011) cAMP, c-di-GMP, c-di-AMP and now cGMP: bacteria use them all! *Mol. Microbiol.* 79, 562–565

2. Kalia, D., Merey, G., Nakayama, S., Zheng, Y., Zhou, J., Luo, Y., Guo, M., Roembke, B. T., and Sintim, H. O. (2013) Nucleotide, c-di-GMP, c-di-AMP, cGMP, cAMP, ppGpp signaling in bacteria and implications in pathogenesis. *Chem. Soc. Rev.* 42, 305–341

3. Magnusson, L. U., Farewell, A., and Nyström, T. (2005) ppGpp: a global regulator in *Escherichia coli*. *Trends Microbiol.* 13, 236–242

4. Hengge, R. (2009) Principles of c-di-GMP signalling in bacteria. *Nat. Rev. Microbiol.* 7, 263–273

5. Gancedo, J. M. (2013) Biological roles of CAMP: variations on a theme in the kingdoms of life. * Biol. Rev. Camb. Philos. Soc.* 88, 645–668

6. Tschowri, N., Schumacher, M. A., Schlimpert, S., Chinnam, N. B., Findlay, K. C., Brennen, R. G., and Buttner, M. J. (2014) Tetrameric c-di-GMP mediates effective transcription factor dimerization to control *Streptomyces* development. *Cell 158*, 1136–1147

7. Ochoa de Alda, J. A. G., Ajiani, G., and Houmard, J. (2000) *Synecocystis* strain PCC6803 *cyoA2*, a prokaryotic gene encodes a guanylyl cyclase. *J. Bacteriol.* 182, 3839–3842

8. Rauch, A., Leipelt, M., Russwurm, M., and Steegborn, C. (2008) Crystal structure of the guanylyl cyclase *Cya2*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 15720–15725

9. Witte, G., Hartung, S., Büttner, K., and Hopfner, K. P. (2008) Structural biochemistry of a bacterial checkpoint protein reveals diadenylate cyclase activity regulated by DNA recombination intermediates. *Mol. Cell* 30, 167–178

10. Bejerano-Sagie, M., Oppenheimer-Shaanan, Y., Berlatzky, I., Rouvinski, A., Meyerovich, M., and Ben-Yehuda, S. (2006) A checkpoint protein that scans the chromosome for damage at the start of sporulation of *Bacillus subtilis*. *Cell 125*, 679–690

11. Oppenheimer-Shaanan, Y., Wexselblatt, E., Katzhendler, J., Yavin, E., and Ben-Yehuda, S. (2011) c-di-AMP reports DNA integrity during sporulation in *Bacillus subtilis*. *EMBO Rep.* 12, 594–601

12. Campos, S. S., Ibarra-Rodriguez, J. R., Barajas-Ornelas, R. C., Ramirez-Guardiana, F. H., Obregón-Herrera, A., Selтов, P., and Pedraza-Reyes, M. (2014) Interaction of apurinic/apyrimidinic endonucleases Nfo and ExoA with the DNA integrity scanning protein DisA in the processing of oxidative DNA damage during *Bacillus subtilis* spore outgrowth. *J. Bacteriol.* 196, 568–578

13. Bai, Y., Yang, J., Zhou, X., Ding, X., Eisele, L. E., and Bai, G. (2012) Mycobacterium tuberculosis *Rv3586* (DacA) is a diadenylate cyclase that con-
Characterization of an Essential Diadenylate Cyclase

Luo, Y., and Helmann, J. D. (2012) Analysis of the role of Bacillus subtilis σ^54 in β-lactam resistance reveals an essential role for c-di-AMP in penicillin-resistant homeostasis. Mol. Microbiol. 83, 623–639

Witte, C. E., Whiteley, A. T., Burke, T. P., Sauer, J. D., Portnoy, D. A., and Woodard, J. J. (2013) Cyclic di-AMP is critical for Listeria monocytogenes growth, cell wall homeostasis, and establishment of infection. MBio 4, e00282–13

Song, J. H., Ko, K. S., Lee, J. Y., Baek, J. Y., and Oh, W. S. (2005) Identification of essential genes in Streptococcus pneumoniae by allelic replacement mutagenesis. Mol. Cells 19, 365–374

Chaudhuri, R. R., Allen, A. G., Owen, P. J., Shalom, G., Stone, K., Harrison, M., Burgis, T. A., Lockyer, M., Garcia-Lara, J., Foster, S. J., Pleasance, S. J., Peters, S. E., Maskell, D. J., and Charles, I. G. (2009) Comprehensive identification of essential Staphylococcus aureus genes using transposon-mediated differential hybridization (TMDH). BMC Genomics 10, 291

Woodward, J. J., Iavarone, A. T., and Portnoy, D. A. (2010) c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response. Science 328, 1703–1705

Kiefer, F., Arnold, K., Künzl, M., Bordoli, L., and Schwede, T. (2009) The SWISS-MODEL repository and associated resources. Nucleic Acids Res. 37, D387–D392

Sambrook, J., and Russell, D. (2001) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Monk, I. R., Gahan, C. G., and Hill, C. (2008) Tools for functional post-genomic analysis of Listeria monocytogenes. Appl. Environ. Microbiol. 74, 3921–3934

Merzbacher, M., Detsch, C., Hillen, W., and Stülke, J. (2004) Mycoplasma pneumoniae HPr kinase/phosphorylase. Eur. J. Biochem. 271, 367–374

Bi, W., and Stambrook, P. J. (1997) CCR: a rapid and simple approach for mutation detection. Nucleic Acids Res. 25, 2949–2951

Spangler, C., Böhm, A., Jenal, U., Seifert, R., and Kaever, V. (2010) A liquid chromatography-coupled tandem mass spectrometry method for quantification of cyclic di-guanosine monophosphate. J. Microbiol. Methods 81, 226–231

Blum, H., Beier, H., and Gross, H. J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8, 93–99

Kabsch, W. (2010) XDS. Acta Crystallogr. D Biol. Crystallogr. 66, 125–133

Söding, J. (2005) Protein homology detection by HMM-HMM comparison. Bioinformatics 21, 951–960

Söding, J., Biegert, A., and Lupas, A. N. (2005) The HHpred interactive server for protein homology detection and structure prediction. Nucleic Acids Res. 33, W244–W248

Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501

Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., Moriarty, N. W., Mountain, M., Oxford, J. A., Raunak, S. J., Richardson, J. C., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX: a comprehensive Phenix-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221

Brünger, A. T. (1993) Assessment of phase accuracy by cross validation: the free R value: methods and applications. Acta Crystallogr. D Biol. Crystallogr. 49, 24–36

Kleywegt, G. J., and Brünger, A. T. (1996) Checking your imagination: applications of the free R value. Structure 4, 897–904

Wain, M. D., Ballard, C. C., Cowtan, K. D., Dodson, E. J., Emsley, P., Evans, P. R., Keegan, R. K., Krissinel, E. B., Leslie, A. G., McCoy, A., McNicholas, S. J., Murshudov, G. N., Pannu, N. S., Potterson, E. A., Powell, H. R., Read, R. J., Vagin, A., Wilson, K. S. (2011) Overview of the CCP4 suite and current developments. Acta Crystallogr. D Biol. Crystallogr. 67, 235–242

Sherriff, S., Hendrickson, W. A., and Smith, J. L. (1987) Structure of myohemerythrin in the azidomet state at 1.7/1.3 Å resolution. J. Mol. Biol. 197, 273–296

Baker, N. A., Sept, D., Joseph, S., Holst, M. J., and McCammon, J. A. (2001) Electrostatics of nanosystems: application to microtubules and the ribosome. Proc. Natl. Acad. Sci. U.S.A. 98, 10037–10041
56. Chen, V. B., Arendall W. B., 3rd, Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S., and Richardson, D. C. (2010) MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 12–21

57. Kabsch, W., and Sander, C. (1983) Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* **22**, 2577–2637

58. Joosten, R. P., te Beek, T. A., Krieger, E., Hekkelman, M. L., Hooft, R. W., Schneider, R., Sander, C., and Vriend, G. (2011) A series of PDB related databases for everyday needs. *Nucleic Acids Res.* **39**, D411–D419

59. Kabsch, W., Kabsch, H., and Eisenberg, D. (1976) Packing in a new crystalline form of glutamine synthetase from *Escherichia coli*. *J. Mol. Biol.* **100**, 283–291

60. Armougom, F., Moretti, S., Poirot, O., Audic, S., Dumas, P., Schaeili, B., Kedusas, V., Notredame, C. (2006) Expresso: automatic incorporation of structural information in multiple sequence alignments using 3D-coffee. *Nucleic Acids Res.* **34**, W604–W608

61. Robert, X., and Gouet, P. (2014) Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Res.* **42**, W320–W324

62. Bai, Y., Yang, J., Zhou, X., Ding, X., Eisele, L. E., and Bai, G. (2012) *Mycobacterium tuberculosis* Rv3586 (DacA) is adiadenylate cyclase that converts ATP to ADP into c-di-AMP. *PLoS One* **7**, e35206

63. Zheng, C., Wang, J., Luo, Y., Fu, Y., Su, J., and He, J. (2013) Highly efficient enzymatic preparation of c-di-AMP using the diadenylate cyclase DisA from *Bacillus thuringiensis*. *Enzyme Microb. Technol.* **52**, 319–324

64. Vázquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., and Kreft, J. (2001) *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* **14**, 584–640

65. Agranoff, D. D., and Krishna, S. (1998) Metal ion homeostasis and intracellular parasitism. *Mol. Microbiol.* **28**, 403–412

66. Abel, S., Chien, P., Wassmann, P., Schirmer, T., Kaever, V., Laub, M. T., Baker, T. A., and Jenal, U. (2011) Regulatory cohesion of cell cycle and cell differentiation through interlinked phosphorylation and second messenger networks. *Mol. Cell* **43**, 550–560

67. Dengler, V., McCallum, N., Kiefer, P., Christen, P., Patrignani, A., Vorholt, J. A., Berger-Bächi, B., and Senn, M. M. (2013) Mutation in the c-di-AMP cyclase dacA affects fitness and resistance of methicillin resistant. *Staphylococcus aureus*. *PLoS One* **8**, e73512

68. Underwood, A. J., Zhang, Y., Metzger, D. W., and Bai, G. (2014) Detection of cyclic di-AMP using a competitive ELISA with a unique pneumococcal cyclic di-AMP binding protein. *J. Microbiol. Methods* **107**, 58–62

69. Zhou, J., Sayre, D. A., Zheng, Y., Szmacinski, H., and Sintim, H. O. (2014) Unexpected complex formation between coraline and cyclic diadenosine monophosphate providing a simple fluorescent turn-on assay to detect this bacterial second messenger. *Anal. Chem.* **86**, 2412–2420

70. Zheng, Y., Zhou, J., Sayre, D. A., and Sintim, H. O. (2014) Identification of bromphenol thiodydantoin as an inhibitor of DisA, a c-di-AMP synthase, from a 1000 compound library, using the coraline assay. *Chem. Commun. (Camb.)* **26**, 11234–11237

71. Karplus, P. A., and Diederichs, K. (2012) Linking crystallographic model and data quality. *Science* **336**, 1030–1033