Cyclic di-AMP homeostasis in *Bacillus subtilis*: both lack and high-level accumulation of the nucleotide are detrimental for cell growth

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Running title: c-di-AMP homeostasis in *Bacillus subtilis*

Key words: c-di-AMP, signalling, cell wall metabolism, protein-protein interaction, DAC domain, diadenylate cyclase

Background: *Bacillus subtilis* encodes three diadenylate cyclases.

Results: Cyclic di-AMP is essential for the viability of *B. subtilis*; however, excess c-di-AMP does also harm the cells. The activity of the cyclases is subject to regulation.

Conclusion: The control of c-di-AMP homeostasis is crucial for *B. subtilis*.

Significance: c-di-AMP is the first essential signalling nucleotide in bacteria.

SUMMARY

The genome of the Gram-positive soil bacterium *Bacillus subtilis* encodes three potential diadenylate cyclases that may synthesize the signalling nucleotide cyclic di-AMP (c-di-AMP). These enzymes are expressed under different conditions, in different cell compartments, and they localize to distinct positions in the cell. Here we demonstrate the diadenylate cyclase activity of the so far uncharacterized enzymes CdaA (previously known as YbbP) and CdaS (YojJ). Our work confirms that c-di-AMP is essential for the growth of *B. subtilis*, and shows that an excess of the molecule is also harmful for the bacteria. Several lines of evidence suggest that the diadenylate cyclase CdaA is part of the conserved essential cda-glm module involved in cell wall metabolism. In contrast, the CdaS enzyme seems to provide c-di-AMP for spores. Accumulation of large amounts of c-di-AMP impairs the growth of *B. subtilis* and results in the formation of aberrant curly cells. This phenotype can be partially suppressed by elevated concentrations of magnesium. These observations suggest that c-di-AMP interferes with the peptidoglycan synthesis machinery. The activity of the diadenylate cyclases is controlled by distinct molecular mechanisms: CdaA is stimulated by a regulatory interaction with the CdaR (YbbR) protein. In contrast, the activity of CdaS seems to be intrinsically restricted, and a single amino acid substitution is sufficient to get a drastic increase of the enzyme’s activity. Taken together, our results support the idea of an important role for c-di-AMP in *B. subtilis* and suggest that the levels of the nucleotide have to be tightly controlled.

Introduction

In order to respond appropriately to changing environments, bacteria have evolved a variety of signaling strategies. Many of these strategies involve changes of gene expression programs. However, quick responses may often be important for the bacterial cell. For this purpose, protein activities have to be modulated. This can occur by either covalent modification of the proteins, e. g. by phosphorylation or acetylation, by degradation of the proteins, or by non-covalent interaction with other proteins or low molecular weight effectors. The low molecular weight effectors may either be metabolites that are part of the normal metabolism, or they may be dedicated
signaling molecules that are produced by the cell for the purpose of signal transduction (1).

Among the best-studied signaling molecules produced by bacteria are the so-called auto-inducers, either acylated homoserine lactones or peptides in Gram-negative and Gram-positive bacteria, respectively (2). In addition, specific signaling nucleotides have been discovered in all bacteria where they have been searched for (3). While the autoinducers serve mainly for purposes of cell-cell communication (quorum sensing), the signaling nucleotides are used for intracellular signaling. In addition to cyclic AMP and (p)ppGpp that are involved in carbon catabolite repression and the stringent response, respectively, many bacteria do also synthesize cyclic dinucleotides such as cyclic di-AMP (c-di-AMP) and cyclic di-GMP (c-di-GMP). These nucleotides are often involved in the control of motility and biofilm formation, i.e., in the switch between motile and sessile lifestyles (4). Their synthesis is catalyzed by dedicated diadenylate or diguanylate cyclases. These proteins do always contain conserved catalytic domains that may be combined with additional domains for signal input and output. In addition, bacteria that produce cyclic dinucleotides do also contain nucleotide-specific phosphodiesterases for the degradation of the molecules (5, 6).

The synthesis, mode of action, and degradation of c-di-GMP have been studied in detail in many bacteria (see ref. 4 for review). In contrast, much less is known about the metabolism and physiological function of c-di-AMP. Diadenylate cyclase activity was first described for the DisA protein of *B. subtilis* (7). This octameric protein has two interdependent activities: (i) it binds DNA via its RuvA-like C-terminal DNA-binding domain and scans its integrity and (ii) it synthesizes c-di-AMP. If the protein arrives at branched DNA molecules as they are present in Holliday junctions, the catalytic activity is inhibited, and this reduction in c-di-AMP concentration results in the delay of sporulation (7, 8). DisA contains a catalytic domain, called diadenylate cyclase (DAC) domain (previously referred to as domain of unknown function DUF147) (7). The discovery of this c-di-AMP producing enzyme revealed that proteins with similar DAC domains are present in many bacteria, both Gram-positive and Gram-negative, and archaea (5). This observation suggests that c-di-AMP might be a wide-spread signaling nucleotide. Moreover, DAC domains are not only coupled to RuvA-like DNA-binding domains, but also to a wide variety of different domains of unknown function. These domains may control the signal in- and/or output of the proteins (5). The presence of DAC domains in so many different organisms and in varying domain arrangements indicates that c-di-AMP levels may respond to a number of distinct stimuli and that c-di-AMP may play an important role in the control of different cellular activities.

We are interested in the molecular biology of the Gram-positive model bacterium *Bacillus subtilis*. Signaling in this bacterium involves a variety of transcription factors, alternative sigma factors, RNA-mediated regulation via RNA-binding proteins or riboswitches, and protein phosphorylation. Moreover, signal transduction via small molecules plays an important role in *B. subtilis*. In this bacterium, small peptides control the initiation of sporulation and the induction of genetic competence (9). Moreover, ppGpp mediates the stringent response by inhibiting GTP synthesis and thus by differential control of transcription of mRNAs that use an A or a G as the first nucleotide (10 - 12). The genome of *B. subtilis* encodes several potential diguanylate cyclases, and potential c-di-GMP specific phosphodiesterases (13, 14). Very recently, c-di-GMP was found to control motility by binding the YpfA protein which in turn interacts with and inhibits the motor protein MotA in *B. subtilis* (14). In addition to these well-established signaling nucleotides, c-di-AMP synthesis by the DisA protein was recently discovered for the first time (7). In contrast to most other bacteria that contain only one diadenylate cyclase, *B. subtilis* encodes two additional proteins with DAC domains that may possibly be involved in c-di-AMP synthesis. Finally, the c-di-AMP specific phosphodiesterase, GdpP (previously referred to as YybT, 15) degrades cyclic di-AMP (16). Recently, c-di-AMP was implicated in the control of cell wall homeostasis in *B. subtilis* and it was demonstrated that this signaling nucleotide is essential for the growth of the bacterium (15).

In this work, we have analyzed the activity of the so far unknown diadenylate cyclases of *B. subtilis*, YbbP and YojJ. Our results indicate that both proteins have enzymatic activity. Moreover, we demonstrate...
that the activity of YbbP is modulated by a protein-protein interaction with the modulator protein YbbR. The activity of the sporulation-specific diadenylate cyclase YojJ is self-restricted. Full YojJ activity and accumulation of c-di-AMP result in impaired growth. Thus, the cells need a certain level of c-di-AMP and strongly reduced or increased amounts of the nucleotide seem to be deleterious for the cell. Based on our results, YbbP, YojJ, and YbbR were re-named CdaA (cyclic di-AMP synthase A, YbbP) and CdaS (cyclic di-AMP synthase S, sporulation specific, YojJ), and CdaR (cyclic di-AMP synthase A regulator, YbbR). These designations will be used from now on.

**Experimental procedures**

**Bacterial strains, oligonucleotides and growth conditions**

The *B. subtilis* strains were derived from the laboratory strain 168 (*trpC2*) and they are listed in Table 1. *E. coli* XL1-Blue (Stratagene), BL21 (DE3) and BTH101 (17, 18) were used for cloning experiments, protein over-production, and bacterial two-hybrid assays, respectively.

*E. coli* was grown in LB medium. *B. subtilis* was grown in LB medium or in C minimal medium (19) supplemented with carbon sources and auxotrophic requirements (at 50 mg l\(^{-1}\)) as indicated. CSE medium is C medium supplemented with potassium succinate and potassium glutamate (6 and 8 g/l, respectively). LB and SP plates were prepared by addition of 17 g Bacto agar/l (Difco) to LB and SP, respectively (17, 20).

When required, media were supplemented with antibiotics at the following concentrations: ampicillin (100 μg ml\(^{-1}\)), kanamycin (50 μg ml\(^{-1}\)), (for *E. coli*), spectinomycin (150 μg ml\(^{-1}\)), kanamycin (10 μg ml\(^{-1}\)), tetracycline (12.5 μg ml\(^{-1}\)), chloramphenicol (5 μg ml\(^{-1}\)), and erythromycin (2 μg ml\(^{-1}\)) plus lincomycin (25 μg ml\(^{-1}\)) (for *B. subtilis*).

The oligonucleotides and plasmids used in this study are listed in the supplementary Tables S1 and S2, respectively.

**DNA manipulation**

Transformation of *E. coli* and plasmid DNA extraction were performed using standard procedures (17). All commercially available plasmids, restriction enzymes, T4 DNA ligase and DNA polymerases were used as recommended by the manufacturers. DNA fragments were purified from agarose gels using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). DNA sequences were determined using the dideoxy chain termination method (17). Chromosomal DNA of *B. subtilis* was isolated as described (20).

**Transformation and phenotypic analysis**

Standard procedures were used to transform *E. coli* (17) and transformants were selected on LB plates containing ampicillin (100 μg/ml). *B. subtilis* was transformed with plasmid DNA according to the two-step protocol (20). Transformants were selected on SP plates containing the appropriate antibiotics.

In *B. subtilis*, amylase activity was detected after growth on plates containing nutrient broth (7.5 g/l), 17 g Bacto agar/l (Difco) and 5 g hydrolyzed starch/l (Connaught). Starch degradation was detected by sublimating iodine onto the plates.

Quantitative studies of *lacZ* expression in *B. subtilis* were performed as follows: cells were grown in C minimal medium containing succinate and glutamate and sugars as indicated. Cells were harvested in the logarithmic growth phase at an OD\(_{600}\) of 0.6 to 0.8. β-Galactosidase specific activities were determined with cell extracts obtained by lysozyme treatment as described previously (20). One unit of β-galactosidase is defined as the amount of enzyme which produces 1 nmol of o-nitrophenol per min at 28° C.

**Construction of deletion and complementation strains**

Deletion of the *cdaA*, *cdaR* and *cdaS* genes was achieved by transformation with PCR products constructed using oligonucleotides (see Table S1) to amplify DNA fragments flanking the target genes and intervening antibiotic resistance cassettes (21) as described previously (22).

In order to obtain regulated ectopic expression of *cdaS*, we used plasmid pGP1959. This plasmid was obtained as follows: The *cdaS* gene was amplified using the primer pair FX46/ FX47 and digested with XbaI and KpnI. The fragment was cloned into the vector pGP888 (23) linearized with the same enzymes. Plasmid pGP1959 was then linearized with Scal and used to transform the relevant *B. subtilis* mutants. In the resulting
strains, the cdaS gene under the control of the xylose-regulated promoter is ectopically integrated into the lacA locus.

Construction of integrative vectors that allow the ectopic expression of proteins carrying a Strep-tag in B. subtilis
To facilitate the purification of proteins that are fused to a Strep-tag directly from B. subtilis, we constructed two plasmids that allow the integration of the expression cassette into the lacA gene. For the fusion of the Strep-tag to the N-terminus of the target protein, the integration vector pGP882 (23) was linearized with BamHI and Smal and ligated in a three-arm-ligation to a kanamycin resistance cassette and to a PCR product corresponding to the constitutive promoter of the degQHy gene, the DNA coding for the Strep-tag and a multiple cloning site. The resistance gene was amplified from plasmid pDG780 (21) using the primers KG46 and KG47 and digested with SmaI and EcoRI. The promoter Strep-tag fragment was obtained by PCR using plasmid pGP380 (24) and the primer pair M13-fwd/HE307 and subsequent digestion with EcoRI and BglII. The resulting plasmid was pGP1459. For the fusion of the Strep-tag to the C-terminus of the target protein we constructed plasmid pGP1460 essentially in the same way. However, the fragment covering the promoter of the degQHy gene, the multiple cloning site and DNA coding for the Strep-tag was obtained with pGP382 (24) and the primer pair M13-fwd/HE308.

Construction of strains containing tagged proteins
To facilitate the analysis of protein-protein interactions involving CdaA and CdaR, we constructed strains expressing CdaA and CdaR carrying a triple FLAG-tag and a Strep-tag, respectively, at their C-termini. To express CdaA fused to a triple FLAG-tag at its native locus, we used plasmid pGP1966. This plasmid was obtained by amplification of about 300 bp of the 3’ end of the cdaA gene using the primer pair FX62/ FX63 and insertion of the PCR product into the vector pGP1087 (23) linearized with KpnI and HindIII. For the ectopic expression of CdaR carrying a C-terminal Strep-tag we used plasmid pGP1969. This plasmid was constructed by cloning the cdaR gene (amplified using the primer pair FX56/ FX57) into the integration vector pGP1460 linearized with XbaI and PstI. Prior to transformation of the relevant B. subtilis strains, plasmid pGP1969 was digested with NotI.

Detection of protein-protein interactions
The isolation of protein complexes from B. subtilis cells was performed by the SPINE technology (24). Briefly, growing cultures of B. subtilis were treated with formaldehyde (0.6% w/v, 20 min) to facilitate cross-linking of interacting proteins (24). The Strep-tagged proteins and their potential interaction partners were then purified from crude extracts using a Streptactin column (IBA, Göttingen, Germany) and desthiobiotin as the eluent. Interacting proteins were identified by Western blot analysis.

Primary protein-protein interactions were identified by bacterial two-hybrid (B2H) analysis (18). The B2H system is based on the interaction-mediated reconstruction of Bordetella pertussis adenylate cyclase (CyaA) activity in E. coli. Functional complementation between two fragments (T18 and T25) of CyaA as a consequence of the interaction between bait and prey molecules results in the synthesis of cAMP, which is monitored by measuring the β-galactosidase activity of the cAMP-CAP-dependent promoter of the E. coli lac operon. Plasmid p25-N allows the expression of proteins fused to the N-terminus of the T25 fragment of CyaA, whereas pUT18C allows the expression of proteins fused to the C-terminus of the T18 fragment (18). The plasmids constructed for the B2H assay (see Table S2) were used for cotransformation of E. coli BTH101 and the protein-protein interactions were then analyzed by plating the cells on LB plates containing 100 µg/ml ampicillin, 50 µg/ml kanamycin, 80 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside), respectively. The plates were incubated for a maximum of 72 h at 30°C.

Western blotting
For Western blot analysis, proteins were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad) by electroblotting. Rabbit anti-FLAG polyclonal antibodies (Sigma-Aldrich; 1:10 000) served as primary antibodies. The antibodies were visualized by using anti-rabbit immunoglobulin alkaline phosphatase secondary antibodies (Promega)
and the CDP-Star detection system (Roche Diagnostics), as described previously (19).

**Construction of strains carrying lacZ fusions**

Plasmid pAC6 (25) was used to construct transcriptional fusions of the cdaA or glmM control regions with the lacZ gene. The promoter regions were amplified using the primer pairs FX85/ FX87 and FX86/ FX88, respectively. The PCR product for cdaA and glmM were digested with EcoRI/ BamHI and MfeI/ BamHI, respectively, and cloned into pAC6 linearized with EcoRI and BamHI. The resulting plasmids pGP1980 and pGP1981, respectively, (see Table 1, Table S2 for details) were linearized with Scal and used to transform *B. subtilis* 168.

**Northern blot analysis**

Preparation of total RNA and Northern blot analysis were carried out as described previously (26). Digoxigenin (DIG) RNA probes were obtained by *in vitro* transcription with T7 RNA polymerase (Roche Diagnostics) using PCR-generated DNA fragments as templates. The primer pairs used to amplify DNA fragments specific for cdaA, cdaR, glmM, and glmS are listed in Table S1. The reverse primers contained a T7 RNA polymerase recognition sequence. *In vitro* RNA labelling, hybridization and signal detection were carried out according to the instructions of the manufacturer (DIG RNA labelling kit and detection chemicals; Roche Diagnostics).

**Reverse transcription-PCR (RT-PCR)**

*B. subtilis* 168 total RNA was converted to first strand cDNA using Maxima reverse transcriptase (Fermentas) following the manufacturers protocol (+RT). To get rid of genomic DNA contaminations the cDNA was digested with DNaseI (Fermentas) followed by purification with the RNeasy Plus Mini Kit (Qiagen) which includes a gDNA eliminator column. As a negative control, an additional RNA sample (-RT) was treated equally column. An additional sample was taken for the determination of total protein amount for normalization purposes. The cell culture (10 ml) was harvested by quick centrifugation at 4°C. Samples were then treated by a heating step for 10 min at 95°C followed by centrifugation for 10 min at 20,800 x g at 4°C. The extraction of the resulting pellet was repeated twice with 200 µl extraction mixture at 4°C omitting the heating step. Supernatants were pooled and stored at -20°C over night. After centrifugation...
for 10 min at 20,800 x g at 4°C the supernatant was removed and dried in a speed vac.

For the determination of c-di-AMP in cells of *B. subtilis*, the cultures (45 ml) were grown in SP medium supplemented with xylose (1%, to induce the ectopic cdaS<sub>L44F</sub> allele) at 37°C to an OD<sub>600</sub> of 1.0. The cells were harvested by quick centrifugation at 4°C. An additional sample was taken for the determination of total protein amount for normalization purposes. The cell pellet was resuspended in 200 µl H<sub>2</sub>O and lysed in a Micro Dismembrator S (Sartorius) for 3 min at 1,800 rpm. The resulting cell powder was resuspended in 800 µl extraction buffer and incubated on ice for 15 min. Samples were snap frozen in liquid nitrogen and subsequently treated by a heating step for 10 min at 95°C. After centrifugation for 10 min at 20,800 x g the supernatant was collected and stored at 4°C. The remaining sample mixture was resuspended in 200 µl extraction buffer, vortexed for 45 sec, incubated on ice for 15 min and centrifuged again. The supernatant was collected and pooled with the previous one. Once again, samples were resuspended in 200 µl extraction buffer, vortexed for 45 sec, incubated on ice for 15 min and centrifuged. All supernatants were pooled and stored at -20°C over night. After centrifugation for 10 min at 20,800 x g at 4°C the supernatant was dried in a speed vac.

The dried supernatants were solved with 200 µl H<sub>2</sub>O. After repeated centrifugation and addition of the internal standard <sup>13</sup>C<sup>15</sup>N-c-di-AMP part of the supernatants was analyzed by LC-MS/MS.

Quantification of c-di-AMP by MS/MS

The chromatographic separation was performed on a Series 200 HPLC system (Perkin Elmer Instruments) equipped with a binary pump system and a 200 µl sample loop. A combination of column saver (2.0 µm filter, Supelec Analytical), security guard cartridge (C18, 4 x 2 mm) in an analytical guard holder (Phenomenex) and an analytical NUCLEODUR C18 Pyramid RP column (50 x 3 mm, 3 µm particle size, Macherey-Nagel) temperature controlled (Series 200 Peltier column oven, Perkin Elmer Instruments) at 30°C was used. Eluent A consisted of 10 mM ammonium acetate and 0.1% (v/v) acetic acid in water and eluent B was methanol. The injection volume was 50 µl and the flow rate was 0.4 ml/min throughout the chromatographic run. 100% A was used from 0 to 5 minutes followed by a linear gradient from 100% A to 70% A until 9 minutes. Re-equilibration of the column was achieved by constantly running 100% A from 9 to 13 minutes. The internal standard <sup>13</sup>C<sup>15</sup>N-c-di-AMP and c-di-AMP were eluted with identical retention times of 9.1 minutes. The analyte detection was performed on an API 3000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (AB SCIEX) using selected reaction monitoring (SRM) analysis in positive ionization mode. The following SRM transitions using a dwell time of 40 ms were detected: <sup>13</sup>C<sup>15</sup>N-c-di-AMP: +689/146 (quantifier), +689/345 (identifier) and c-di-AMP: +659/136 (quantifier), +659/330 (identifier) and +659/524 (identifier). The SRM transitions labeled as “quantifier” were used to quantify the compound of interest whereas “identifier” SRM transitions were monitored as confirmatory signals. The quantifier SRM transitions were most intense and were therefore used for quantification. The mass spectrometer parameters were as follows: IS voltage: 5500 V, temperature: 350 °C, nebulizer gas: 6 psi, curtain gas: 15 psi. MS/MS was performed using nitrogen as collision gas. The following collision energies were applied: 61 eV (+689/146), 29 eV (+689/345), 63 eV (+659/136), 29 eV (+659/330), 33 eV (+659/524).

Results

Diadenylate cyclases in *Bacillus subtilis*

Diadenylate cyclases are characterized by the presence of a specific domain, the DAC domain (formerly known as DUF147) (5). The genome of *B. subtilis* encodes three proteins with a DAC domain that may be potential diadenylate cyclases, DisA, CdaA, and CdaS (5, 13). The diadenylate cyclase activity of DisA has been demonstrated experimentally (7, 8). *disA* is the promoter-distal gene of the hexacistronic *ctsR-mcsA-mcsB-clpC-radA-disA* operon, moreover a monocistronic *disA* mRNA is expressed by RNA polymerase containing the alternative sigma factor, σ<sup>M</sup> (29, 30). Recent transcription studies show that *cdaS* is specifically expressed in sporulating cells suggesting a role for c-di-AMP in spore formation (31). Finally, *cdaA* seems to be the first gene of a potential operon that also
contains the downstream gene *cdaR* and the essential *glmM* gene (31).

Transcriptional organization of the *cda-glm* gene cluster

To get more insights into the expression of the potential diadenylate cyclase CdaA, we performed Northern blot analyses. Only very faint bands were detectable using a probe against cdaA in RNA isolated from a culture grown in minimal medium containing glucose and glutamate (see Fig. 1A, CSE glc). Since the accumulation of the cdaA and glutamate (see Fig. 1A, CSE glc). Since the accumulation of the cdaA mRNA is negatively affected by the essential RNase Y (26), we compared the expression of the gene in a strain that allows xylose-controlled expression of RNase Y, and thus depletion of the essential RNase in the absence of xylose. As shown in Fig. 1A, a major transcript of 4 kb was detectable upon RNase Y depletion. Since there is essentially no read-through from the upstream rsiW gene into cdaA (31), this transcript is likely to cover the cdaA, cdaR, and glmM genes. Indeed, the same band corresponding to a 4 kb transcript was detected using riboprobes specific for cdaR and glmM (see Fig. 1B, C). Moreover, a faint 5.7 kb signal was detected with the glmM probe. This signal might correspond to a tetracistronic cdaAR-glmMS mRNA (see Fig. 1F). In addition, signals corresponding to mRNAs of 1.3 and 3.5 kb were observed with the glmM riboprobe. These signals correspond probably to monocistronic glmM and bicistronic glmMS transcripts, respectively. Using a riboprobe specific for glmS, a single 2.0 kb transcript corresponding to a monocistronic glmS mRNA was detected (Fig. 1D). This transcript was very abundant, thus preventing the concomitant detection of the bicistronic glmMS mRNA. The glmS mRNA was less abundant when the bacteria were grown in the presence of glucosamine, the precursor of glucosamine 6-phosphate. This latter metabolite acts as the trigger of the glmS ribozyme that initiates degradation of the transcript (32, 33). In order to detect the transcript of the tetracistronic cdaAR-glmMS operon in a wild type strain, we used RNA from *B. subtilis* 168 for reverse transcription and subsequent PCR analysis. As shown in Fig. 1E, PCR products were obtained with primer pairs that allow the amplification of regions between the coding genes. This result supports the observations of the Northern blot analysis.

Diadenylate cyclase activity of CdaA and CdaS

Both CdaA and CdaS possess the conserved DAC domain that is thought to be required for c-di-AMP formation. In order to test whether these proteins do indeed exhibit diadenylate cyclase activity, we took advantage of the inability of *Escherichia coli* to produce c-di-AMP (27). The cdaA and cdaS genes were cloned into the expression vector pET28a, and the amounts of c-di-AMP in *E. coli* BL21(DE3) carrying the corresponding plasmids pGP1973 and pGP1974, respectively, were compared to those of the same strain carrying the empty vector pET28a. As shown in Fig. 2, no c-di-AMP was present in the strain with the empty vector. In contrast, the expression of CdaA or CdaS resulted in the detection of 150 and 670 ng c-di-AMP/ mg of protein, respectively. This result provides unequivocal evidence for the diadenylate cyclase activity of both proteins.
c-di-AMP formation during exponential growth is essential in B. subtilis
In order to identify roles for the diadenylate cyclases, we deleted the three genes individually from the chromosome of B. subtilis. Moreover, we combined the mutations to construct double and triple mutants. The three single mutant strains GP983 (∆cdaS), GP987 (∆disA), and GP997 (∆cdaA) were viable and did not exhibit an obvious phenotype during log phase growth. Similarly, the disA cdaS and cdaA cdaS double mutants, GP991 and GP989, respectively, grew like the wild type. In contrast, we were unable to construct a cdaA disA double mutant strain or a triple mutant devoid of all three diadenylate cyclases. This observation is in good agreement with the recent report that c-diadenylate cyclases. This observation is in good agreement with the recent report that c-diadenylate cyclases are essential for the viability of B. subtilis. In order to test this hypothesis, we attempted to purify CdaR with its attached potential interaction partners. For this purpose, we constructed strain GP1331 that ectopically encodes a variant of CdaR carrying a C-terminal Strep-tag for affinity purification. Moreover, this strain codes for a CdaA variant labeled with a FLAG-tag.
Parallel cultures of strain GP1331 were grown in C minimal medium with glucose as the carbon source, and the cross-linker formaldehyde was added to one of the cultures to fix the interaction between CdaR and its possible partners. CdaR-Strep was then purified from protein extracts of these cultures, and the elution fractions were analyzed by SDS-PAGE and subsequent Western blot analysis with antibodies recognizing the FLAG-tag to detect the CdaA protein. As shown in Fig. 3A, CdaA-FLAG was present in the crude extract of GP1331. Similarly, CdaA-FLAG was detected in the elution fractions of Strep-tagged CdaA, even when CdaA had been purified from the culture that had not been treated with the cross-linker. When CdaR was purified from the cross-linked cultures, a second larger protein was recognized by the antibodies. This band corresponds to CdaA dimers that can be resolved by prolonged heating of the samples. To exclude non-specific detection of FLAG-tagged CdaA, two different controls were performed:

1. First, we used strain GP1332 that expresses CdaR-Strep and a FLAG-tagged version of the DEAD-box RNA helicase CshA. Again, CshA-FLAG was present in the crude extract. However, no CdaA-FLAG was detectable in the elution fraction (see Fig. 3B). Finally, strain GP1333 encoding Strep-tagged CshA and FLAG-tagged CdaA served as a control to exclude non-specific binding of CdaA-FLAG to the StrepTactin affinity matrix. When CshA-Strep was purified, no CdaA-FLAG was present in the elution fraction (see Fig. 3C). This result demonstrates that the observed co-purification of CdaR and CdaA is specific, and that it is indeed the result of a physical interaction between the two proteins. The co-purification of CdaA with CdaR even in the

Physical interaction between CdaR and the diadenylate cyclase CdaA
The cdaA gene is located upstream of the cdaR gene, and the two genes are part of the cda-glm operon identified in this work (see above). The function of the cdaR gene or its protein product has so far not been identified. The CdaR protein contains a repeat of four similar domains (called YbbR-like domains, 35). Interestingly, in some δ-proteobacteria and Streptococci DAC domains are organized in one protein with YbbR-like domains (5). This suggests that the YbbR domains might somehow control the activity of the DAC domain. Specifically, the genetic arrangement and co-expression of the cdaA and cdaR genes implies that B. subtilis CdaR might control the diadenylate cyclase activity of CdaA.
If CdaR would regulate the activity of CdaA, one would expect that the two proteins interact physically in the cell. To test this hypothesis, we attempted to purify CdaR with its attached potential interaction partners. For this purpose, we constructed strain GP1331 that ectopically encodes a variant of CdaR carrying a C-terminal Strep-tag for affinity purification. Moreover, this strain codes for a CdaA variant labeled with a FLAG-tag.
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1. First, we used strain GP1332 that expresses CdaR-Strep and a FLAG-tagged version of the DEAD-box RNA helicase CshA. Again, CshA-FLAG was present in the crude extract. However, no CdaA-FLAG was detectable in the elution fraction (see Fig. 3B). Finally, strain GP1333 encoding Strep-tagged CshA and FLAG-tagged CdaA served as a control to exclude non-specific binding of CdaA-FLAG to the StrepTactin affinity matrix. When CshA-Strep was purified, no CdaA-FLAG was present in the elution fraction (see Fig. 3C). This result demonstrates that the observed co-purification of CdaR and CdaA is specific, and that it is indeed the result of a physical interaction between the two proteins. The co-purification of CdaA with CdaR even in the
absence of the cross-linker suggests that the interaction is rather strong.

The co-purification of two proteins indicates that they are part of common protein complexes, but does not exclude the possibility that the interaction is indirect. To test whether CdaA and CdaR are capable of interacting directly, we used the bacterial adenylate cyclase two-hybrid system (18). As shown in Fig. 3D, a primary interaction between the two proteins was observed thus confirming our in vivo results.

**CdaR stimulates the diadenylate cyclase activity of CdaA**

The observed physical interaction between CdaA and CdaR supports the idea that CdaR might control the activity of the diadenylate cyclase CdaA. To get a first impression of the role of CdaR, we used a genetic approach: For this purpose, we constructed three isogenic strains with deletions of disA and cdaS and the ectopic inducible allele of cdaS. In these strains, we deleted either cdaA, cdaR, or both genes. As described above, strain GP1327 with the deletion of all three diadenylate cyclase-encoding genes was unable to grow in the absence of the inducer xylose. This result allows drawing two important conclusions: First, the construct used here does not interfere with the expression of the essential downstream glmM gene thus excluding the possibility that cdaA was required due to possible polar effects. Second, CdaA had retained its diadenylate cyclase activity even in the absence of CdaR. For strain GP1329 with a deletion of both cdaA and cdaR genes, xylose was again required to allow growth of the bacteria. Taken together, these observations suggest that CdaR might act as a negative effector of CdaA. Alternatively, CdaA might a priori have diadenylate cyclase activity that can be enhanced upon interaction with CdaR.

To test the role of CdaR for the activity of CdaA more directly, we co-expressed the two proteins in E. coli, and determined the concentration of c-di-AMP of this strain and of strains expressing either CdaA or CdaR alone (see Table 3). In this experiment, we detected 237 ng c-di-AMP/ mg of protein in the strain carrying the cdaA gene. This is in good agreement with earlier observations (see above, Fig. 2). No c-di-AMP was detectable in the strain expressing the cdaR gene. In the strain expressing both cdaA and cdaR a more than 20-fold increase in the c-di-AMP concentration (5,256 ng c-di-AMP/ mg of protein) was observed. Since all three diadenylate cyclases of B. subtilis share the conserved DAC domain, we could not exclude the possibility that CdaR might also stimulate the activities of CdaS and DisA. To address this question, we determined the formation of c-di-AMP by CdaS and DisA in the presence or absence of CdaR. As shown in Table 3, CdaR did not affect the activities of either CdaS or DisA. Taken together, these results demonstrate that CdaR specifically stimulates the enzymatic activity of CdaA.

**Isolation and characterization of a hyperactive variant of CdaS**

As mentioned above, B. subtilis GP1327 was unable to grow in the absence of the inducer xylose, i. e. if no active diadenylate cyclase was present. However, after prolonged incubation this strain gave rise to small colonies that grew even in the absence of xylose. We hypothesized that this suppression might result (i) from an inactivation of the xylR gene that would lead to constitutive cdaS expression, (ii) from a mutation in the promoter region that would lead to constitutive promoter activity, or (iii) from a mutation within the cdaS gene that allows the production of sufficient c-di-AMP even at very low cdaS expression levels. Of these possibilities, the inactivation of xylR seemed rather unlikely since the strain GP1327 carries two copies of xylR, one at the native locus and a second copy at the ectopic lacA site. Thus, we sequenced the p_{xylA}cdaS copies present in the lacA locus of two suppressor mutants in order to distinguish between the second and third possibility. In one mutant, GP1334, we found a single substitution of C130 by T in the coding sequence of the cdaS gene. This mutation results in a replacement of Leu-44 of CdaS by Phe (L44F). In the second suppressor mutant, GP1348, two mutations that both affect the binding site for XylR were detected: An extra A is inserted at the end of the spacer between the two parts of the palindrome that is recognized by XylR, and the A at position 4 of the second unit of the palindrome is replaced by a T. Together, these mutations affect the length of the spacer and disturb the palindrome. Both spacer length and
c-di-AMP in the wild type strain GP1173 and determined the intracellular concentrations of included degraded by the latter enzyme. Therefore we confirmed that the suppressor phenotype did indeed result from the mutation of cdaS and not from secondary mutations. For this purpose, chromosomal DNA of the suppressor mutant GP1334 was used to transform strain GP991 that is deleted for disA and cdaS. Selection for resistance against both kanamycin, tetracyclin and chloramphenicol allowed the isolation of clones that had the three chromosomal genes encoding the diadenylate cyclases deleted, and the mutant cdaS copy at the lacA locus. These clones were tested for growth in the absence of xylose to verify that the mutation in cdaS was causative for the suppressor phenotype. Indeed, all clones were able to grow in the absence of xylose.

The findings reported above suggest that the mutant CdaS<sub>L44F</sub> protein is capable of producing sufficient c-di-AMP even at very low expression. To test this hypothesis, the mutant allele was cloned into the expression vector pET28a, and the formation of c-di-AMP was assayed in the heterologous host E. coli. While the expression of wild type CdaS resulted in the detection of 670 ng c-di-AMP/ mg of protein, 56,400 ng c-di-AMP/ mg of protein were detected in the E. coli strain expressing CdaS<sub>L44F</sub>. Thus, the mutation resulted in a nearly 100-fold increase in c-diAMP production. This result is in excellent support of the idea that the mutant enzyme exhibits increased activity.

**Accumulation of c-di-AMP inhibits growth and leads to aberrant cell morphology**

As shown above, c-di-AMP is required for the growth of B. subtilis. The availability of the cdaS<sub>L44F</sub> allele allowed us to investigate the consequences of increased c-di-AMP accumulation. Since B. subtilis does also possess the c-di-AMP specific phosphodiesterase GdpP, it was possible that the increased amount of c-di-AMP would be degraded by the latter enzyme. Therefore we included gdpP mutants in our study. First, we determined the intracellular concentrations of c-di-AMP in the wild type strain GP1173 and the isogenic cdaS<sub>L44F</sub> gdpP mutant GP1344. In the wild type, we detected 5.6 (± 2.82) ng of c-di-AMP per mg of protein. In contrast, a concentration of 17.9 (± 2.49) ng of c-di-AMP per mg of protein was determined for GP1344. Thus, the intracellular concentration of c-di-AMP was indeed increased upon expression of the hyperactive cdaS allele and deletion of the phosphodiesterase-encoding gene gdpP.

To study the consequences of c-di-AMP accumulation for bacterial growth and morphology, we used a set of four strains that were wild type for the diadenylate cyclases and that contained additionally either the cdaS wild type or the cdaS<sub>L44F</sub> mutant allele under the control of the xylose promoter. Moreover, each cdaS allele was also combined with a deletion of the gdpP gene to prevent degradation of c-di-AMP. When the cells were cultivated in the absence of xylose (i. e. when the ectopic cdaS alleles were not expressed), the growth was similar for the four tested strains (see Fig. 4). However, strain GP1344 encoding the hyperactive CdaS diadenylate cyclase variant in the absence of the phosphodiesterase GdpP exhibited a somewhat slower growth (generation time of 53.6 min vs. 48.5 min for the isogenic strain with wild type CdaS, GP1343). The differences became more drastic when the ectopic cdaS alleles had been induced with xylose: While the growth of the strain expressing wild type cdaS in the presence of the phosphodiesterase (GP1341) was not affected, the isogenic strain GP1343 from which the gdpP gene was deleted grew somewhat slower (see Fig. 4) (generation times: 49.7 min for GP1341 vs. 60.0 min for GP1343). Similarly, expression of the mutant allele cdaS<sub>L44F</sub> in the presence of a functional phosphodiesterase (GP1342) had only a slight effect (76.4 min for GP1342 vs. 49.7 for GP1341). However, when the hyperactive CdaS variant was expressed in the absence of the functional phosphodiesterase GdpP (GP1344), growth was severely impaired (see Fig. 4, generation time 89.1 min). These data suggest that the accumulation of excess c-di-AMP is detrimental to the normal growth of B. subtilis.

In order to get a first glimpse how c-di-AMP might interfere with the growth of B. subtilis, we observed the morphology of the strains GP1173 (no accumulation of additional c-di-AMP) and GP1344 (isogenic construct with strong accumulation of c-di-AMP due to the expression of the hyperactive diadenylate cyclase CdaS<sub>L44F</sub> and the absence of the
phosphodiesterase GdpP). During the logarithmic growth phase, we observed the formation of curled non-separated cell filaments in the strain accumulating c-di-AMP (GP1344 in the presence of xylose) (see Fig. 5). Interestingly, the curled morphology disappeared when the cells had entered sporulation (Fig. 5). In this phase, the cells were short with a significant portion already containing spores, a phenotype indistinguishable from that of the control strain GP1173. This wild type-like morphology of the sporulating cells may result from either the acquisition of a suppressor mutation that prevents the accumulation of c-di-AMP or from the insensitivity of sporulating cells to increased amounts of c-di-AMP. To distinguish between these two possibilities, we tested the phenotype of cells that had undergone sporulation after a new growth cycle. Again, exponentially growing cells exhibited the curled filament morphology whereas the sporulating cells were short and contained spores (data not shown). Thus, the consequences of c-di-AMP accumulation are different during the different phases of growth: while a process related to cell division seems to be impaired during logarithmic growth, this is obviously not the case during sporulation.

Aberrant cell morphologies are often observed with mutants impaired in cell wall metabolism. Recently, a depletion of c-di-AMP was shown to cause a defect in peptidoglycan biosynthesis that could be suppressed by the addition of magnesium (15). To test whether an excess of c-di-AMP does also cause a defect in peptidoglycan metabolism, we tested the effect of an addition of magnesium on the morphology of the bacteria that accumulate c-di-AMP. As shown in Fig. 6, the addition of magnesium did not affect the (normal) morphology of strain GP1173 that can degrade c-di-AMP. In contrast, the curly appearance of strain GP1344 that accumulates an excess of c-di-AMP was abolished at a high magnesium concentration. However, many cells did not divide properly as seen by the formation of long cell chains suggesting that magnesium cannot completely counteract the effect of a massive c-di-AMP accumulation. Thus, our results suggest that increased levels of c-di-AMP lead to a defect in the peptidoglycan synthesizing machinery.

Discussion

In this work, we have demonstrated that the intracellular concentration of the signaling nucleotide c-di-AMP has to be adjusted to a certain level; too low or too high amounts of the nucleotide are disadvantageous for the cell.

The genetic evidence presented here and in a previous study demonstrates that the three diadenylate cyclases of B. subtilis can replace each other in the generation of a c-di-AMP pool sufficient for growth. However, the three enzymes seem to have distinct functions: DisA scans the DNA and stops c-di-AMP production if it encounters problems with DNA integrity such as Holliday junctions. The reduced DisA-mediated c-di-AMP synthesis results in a delay of sporulation (8, 38). This specific function is rather unlikely to be involved in the essential role of c-di-AMP. The diadenylate cyclase CdaS is specifically expressed during sporulation, most likely by RNA polymerase containing the late forespore-specific sigma factor σG (31). In agreement with this observation, CdaS was unable to provide the cell with sufficient c-di-AMP in the absence of DisA and CdaA unless it was expressed from a regulated promoter that is also active during exponential growth. Thus, the function of CdaS seems to be limited to the spore.

Several lines of evidence suggest that CdaA is implicated in the control of cell wall biosynthesis. First, CdaA is encoded in an operon with proteins involved in cell wall biosynthesis. Interestingly, the genetic clustering of CdaA-related diadenylate cyclase genes with the essential glm genes involved in the generation of glucosamine-1-phosphate, a key precursor for cell wall biosynthesis is conserved in most δ-proteobacteria and firmicutes (with the notable exception of cell wall-less mollicutes). Second, a link of at least one of the diadenylate cyclases to cell wall metabolism is also supported by the recent study of Luo et al. (15). These authors report that accumulation of c-di-AMP due to the inactivation of the gdpP gene encoding the specific phosphodiesterase results in increased resistance to cell wall antibiotics such as β-lactams and conclude that c-di-AMP plays an essential role in peptidoglycan homeostasis. Third, this conclusion is supported by our observation that severe overproduction of c-di-AMP interferes with cell morphology in an Mg2+-dependent manner (see Fig. 6). This is
reminiscent of the phenotypes of other mutations that affect cell wall synthesis (39 - 42).

The obvious functional specialization of the three diadenylate cyclases suggests that the c-di-AMP synthesized by the individual proteins is active in a time- and compartment-specific manner. This is rather obvious for CdaS which is expressed only in the forespore, suggesting that c-di-AMP produced by CdaS has a spore-specific function. In the vegetative cell, DisA is associated to the DNA whereas CdaA contains three trans-membrane domains and is associated to the membrane (43). Thus, the enzymes seem to form distinct c-di-AMP pools in a temporally and spatially ordered manner. In this way the c-di-AMP may also be close to its potential target proteins. A similar hypothesis has been proposed for c-di-GMP which can be produced by more than a dozen different proteins in a single bacterial cell (44).

The rather specialized functions of DisA and CdaS suggest that CdaA is the major player in c-di-AMP in B. subtilis that is also key for the essential function of the nucleotide. In this context, the conserved genetic linkage with the essential glmM and glmS genes is noteworthy. The idea of a crucial function for CdaA is further supported by the observation that the single diadenylate cyclases of the Gram-positive pathogens Listeria monocytogenes, Staphylococcus aureus, and Streptococcus pneumoniae are highly similar to CdaA (including the domain organization), and that the encoding genes are essential (45 - 47). Moreover, the genes homologous to cdaA and cdaR are also linked to glmM and glmS in those organisms. The intimate genetic, transcriptional and functional linkage of the cdaAR and glmMS genes suggests that they form a conserved module, the cda-glm module.

As mentioned above, the c-di-AMP concentration has to be tightly controlled to ensure that it does not fall below nor exceed a certain physiological concentration. In the case of CdaA, the enzyme has a basal activity; and this activity can be increased by a specific regulatory interaction with the CdaR protein. CdaR proteins are widespread in bacteria; however, this is the first report of their function. These proteins consist of a repeated conserved domain (the YbbR domain); B. subtilis CdaR is made up of four such domains. The structural analysis of YbbR domains from Desulfitobacterium hafniense (35) revealed a striking similarity to the ribosomal protein L25. Since L25 proteins bind the 5S ribosomal RNA (48, 49), it is tempting to speculate that CdaR might also bind RNA and that this binding might in turn control the interaction with and activation of CdaA.

The activity of CdaS seems to be limited in order to keep the c-di-AMP levels at a physiologically acceptable level. Interestingly, a single amino acid substitution is sufficient to increase the activity of the protein by a factor of 100. The structure of CdaS can be inferred by modelling based on the known structure of the homologous protein from Bacillus cereus (see Fig. 7). The protein is made up of three identical subunits, and each monomer contains two long α-helices at the N-terminus that are followed by the rather globular DAC domain. Interestingly, the mutation resulting in the hyperactive CdaS protein is located in the loop that connects the two N-terminal helices. This might result in a repositioning of the two helices with respect to each other and the catalytic DAC domain. Based on this finding it is tempting to speculate that the N-terminal helices are involved in the control of the activity of the DAC domain, and thus in the spore-specific synthesis of c-di-AMP. Similarly, inhibitory protein domains have been found in the B. subtilis transcription factor RocR and the alternative sigma factor σ54 (51, 52).

Future studies will focus on the distinct molecular mechanisms controlling the c-di-AMP levels in B. subtilis. Moreover, we will search for the receptors of c-di-AMP and thus for the precise cellular functions of this fascinating signalling molecule.

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References

1. Petsko, G. A., and Ringe, D. (2004) Primers in Biology: Protein structure and function. New Science Press, London.
2. Thoendel, M., and Horswill, A. R. (2010) Biosynthesis of peptide signals in gram-positive bacteria. *Adv. Appl. Microbiol.* 71, 91-112.
3. Gomelsky, M. (2011) cAMP, c-di-GMP, c-di-AMP and now cGMP: bacteria use them all! *Mol. Microbiol.* 79, 562-565.
4. Hengge, R. (2009) Principles of c-di-GMP signaling in bacteria. *Nat. Rev. Microbiol.* 7, 263-273.
5. Röm ling, U. (2008) Great times for small molecules: c-di-AMP, a second messenger candidate in bacteria and archaea. *Sci. Signal.* 1, pe39.
6. Schirmer, T. and Jenal, U. (2009) Structural and mechanistic determinants of c-di-GMP signalling. *Nat. Rev. Microbiol.* 7, 724-735.
7. 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.
8. Oppenheimer-Shaanan, Y., Wexselblatt, E., Katzenhendler, J., Yavin, E., and Ben-Yehuda, S. (2011) c-di-AMP reports DNA integrity during sporulation in *Bacillus subtilis*. *EMBO Rep.* 12, 594-601.
9. Pottathil, M., and Lazazzera, B. A. (2003) The extracellular Phr peptide-Rap phosphatase signaling circuit of *Bacillus subtilis*. *Front. Biosci.* 8, d32-45.
10. Kriel, A., Bittner, A. N., Kim, S. H., Liu, K., Tehranchi, A. K., Zou, W. Y., Rendon, S., Chen, R., Tu, B. P., and Wang, J. D. (2012) Direct regulation of GTP homeostasis by (p)pGpp: A critical component of viability and stress resistance. *Mol. Cell* 48, 231-241.
11. Krásný, L., Tiserová, H., Jonák, J., Rejman, D., and Sanderová, H. (2008) The identity of the transcription +1 position is crucial for changes in gene expression in response to amino acid starvation in *Bacillus subtilis*. *Mol. Microbiol.* 69, 42-54.
12. Tojo, S., Kumamoto, K., Hirooka, K., and Fujita, Y. (2010) Heavy involvement of stringent transcription control depending on the adenine or guanine species of the transcription initiation site in glucose and pyruvate metabolism in *Bacillus subtilis*. *J. Bacteriol.* 192, 1573-1585.
13. Mäder, U., Schmeisky, A. G., Flórez, L. A., and Stülke, J. (2012) *SubtiWiki* – a comprehensive community resource for the model organism *Bacillus subtilis*. *Nucleic Acids Res.* 40, D1278-D1287.
14. Chen, Y., Chai, Y., Guo, J. H., and Losick. (2012) Evidence for cyclic di-GMP-mediated signaling in *Bacillus subtilis*. *J. Bacteriol.* 194, 5080-5090.
15. Luo, Y., and Helmann, J. D. (2012) Analysis of the role of *Bacillus subtilis σM* in β-lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. *Mol. Microbiol.* 83, 623-639.
16. Rao, F., See, R. Y., Zhang, D., Toh, D. C., Ji, Q., and Liang, Z. X. (2010) YybT is a signaling protein that contains a cyclic dinucleotide phosphodiesterase domain and a GGDEF domain with ATPase activity. *J. Biol. Chem.* 285, 473-482.
17. Sambrook, J., and Russell, D. (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor.
18. Karimova, G., Pidoux, J., Ullmann, A., and Ladant, D. (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc. Natl. Acad. Sci. USA* 95, 5752-5756.
19. Commichau, F. M., Herzberg, C., Tripal, P., Valerius, O., and Stülke, J. (2007) A regulatory protein-protein interaction governs glutamate biosynthesis in *Bacillus subtilis*: The glutamate dehydrogenase RocG moonlights in controlling the transcription factor GltC. *Mol. Microbiol.* 65, 642-654.
20. Kunst, F., and Rapoport, G. (1995) Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. *J. Bacteriol.* 177, 2403-2407.
21. Guérout-Fleury, A. M., Shazand, K., Frandsen, N., and Stragier, P. (1995) Antibiotic resistance cassettes for *Bacillus subtilis*. *Gene* 167, 335-336.
22. Wach, A. (1996) PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. *Yeast* **12**, 259-265.

23. Diethmaier, C., Pietack, N., Gunka, K., Wrede, C., Lehnik-Habrink, M., Herzberg, C., Hübner, S., and Stülke, J. (2011) A novel factor controlling bistability in *Bacillus subtilis*: the YmdB protein affects flagellin expression and biofilm formation. *J. Bacteriol.* **193**, 5997-6007.

24. Herzberg, C., Flórez-Weidinger, L. A., Dörrecker, B., Hübner, S., Stülke, J., and Commichau, F. M. (2007) SPINE: a method for the rapid detection and analysis of protein-protein interactions in *vivo*. *Proteomics* **7**, 4032-4035.

25. Stülke, J., Martin-Verstraete, I., Zagorec, M., Rose, M., Klier, A., and Rapoport, G. (1997) Induction of the *Bacillus subtilis* ptsGHI operon by glucose is controlled by a novel antiterminator, GlcT. *Mol. Microbiol.* **81**, 1459-1473.

26. Lehnik-Habrink, M., Schaffer, M., Mäder, U., Diethmaier, C., Herzberg, C., and Stülke, J. (2011) RNA processing in *Bacillus subtilis*: Identification of targets of the essential RNase Y. *Mol. Microbiol.* **81**, 4032-4035.

27. Corrigan, R. M., Abbott, J. C., Burhenne, H., Kaever, V., and Gründling, A. (2011) c-di-AMP is a new second messenger in *Staphylococcus aureus* with a role in controlling cell size and envelope stress. *PLoS Pathog.* **7**, e1002217.

28. Spangler, C., Böh m, 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. Meth.* **81**, 226-231.

29. Krüger, E., Msadek, T., and Hecker, M. (1996) Alternate promoters direct stress-induced transcription of the *Bacillus subtilis* clpC operon. *Mol. Microbiol.* **20**, 713-723.

30. Jervis, A. J., Thackray, P. D., Houston, C. W., Horburgh, M. J., and Moir, A. (2007) SigM-responsive genes of *Bacillus subtilis* and their promoters. *J. Bacteriol.* **189**, 4534-4538.

31. Nicolas, P., Mäder, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., Bidnenko, E., Marchadier, E., Hoebeke, M., Aymeric, S., Becher, Bisicchia, P., Botella, E., Delumeau, O., Doherty, G., Denham, E. L., Devine, K. M., Fogg, M., Fromion, V., Goelzer, A., Hansen, A., Härtig, E., Harwood, C. R., Homuth, G., Junes, M., Klipp, E., Le Chat, L., Leconte, F., Lewis, P., Liebermeister, W., March, A., Mars, R. A. T., Nannapaneni, P., Noone, D., Pohl, S., Rinn, B., Rügheimer, F., Sappa, P. K., Samson, F., Schaffer, M., Schwikowski, B., Steil, L., Stülke, J., Wiegert, T., Wilkinson, A. J., van Dijl, J. M., Hecker, M., Volker, U., Bessières, P., and Noirot, P. (2012) The condition-dependent whole-transcriptome reveals high-level regulatory architecture in bacteria. *Science* **335**, 1103-1106.

32. Klein, D. J., and Ferré-D’Amaré, A. R. (2006) Structural basis of glmS ribozyme activation by glucosamine-6-phosphate. *Science* **313**, 1752-1756.

33. Collins, J. A., Irnov, I., Baker, S., and Winkler, W. C. (2007) Mechanism of mRNA destabilization by the glmS ribozyme. *Genes Dev.* **21**, 3356-3368.

34. Schilling, O., Frick, O., Herzberg, C., Ehrenreich, A., Heinzle, E., Wittmann, C., and Stülke, J. (2007) Transcriptional and metabolic responses of *Bacillus subtilis* to the availability of organic acids: Transcription regulation is important but not sufficient to account for metabolic adaptation. *Appl. Env. Microbiol.* **73**, 499-507.

35. Barb, A. W., Cort, J. R., Seetharaman, J., Lew, S., Lee, H. W., Acton, T., Xiao, R., Kennedy, M. A., Tong, L., Montelione, G. T., and Prestegard, J. H. (2011) Structures of domains I and IV from YbbR are representative of a widely distributed protein family. *Protein Sci.* **20**, 396-405.

36. Gärtn er, D., Degenkolb, J., Ripperger, J. A., Allmannsberger, R., and Hillen, W. (1992) Regulation of the *Bacillus subtilis* W23 xylose utilization operon: interaction of the Xyl repressor with the xyl operator and the inducer xylose. *Mol. Gen. Genet.* **232**, 415-422.

37. Dahl, M. K., Degenkolb, J., and Hillen W. (1994) Transcription of the xyl operon is controlled in *Bacillus subtilis* by tandem overlapping operators spaced by four base-pairs. *J. Mol. Biol.* **243**, 413-424.

38. Bejerano-Sagie, M., Oppenheimer-Shaanan, Y., Berlatzky, I., Rouvinski, A., Meyervich, M., and Ben-Yehuda, S. (2006) A checkpoint protein that scans the chromosome for damage at the start of sporulation in *Bacillus subtilis*. *Cell* **125**, 679-690.
39. Murray, T., Popham, D. L., and Setlow, P. (1998) *Bacillus subtilis* cells lacking penicillin-binding protein 1 require increased level of divalent cations for growth. *J. Bacteriol.* **180**, 4555-4563.

40. Görke, B., Foulquier, E., and Galinier, A. (2005) YvcK of *Bacillus subtilis* is required for a normal cell shape and for growth on Krebs cycle intermediates and substrates of the pentose phosphate pathway. *Microbiology* **151**, 3777-3791.

41. Formstone, A., and Errington, J. (2005) A magnesium-dependent mreB null mutant: implications for the role of MreB in *Bacillus subtilis*. *Mol. Microbiol.* **55**, 1646-1657.

42. Kawai, Y., Daniel, R. A., and Errington, J. (2009) Regulation of cell wall morphogenesis in *Bacillus subtilis* by recruitment of PBP1 to the MreB helix. *Mol. Microbiol.* **71**, 1131-1144.

43. Hahne, H., Wolff, S., Hecker, M., and Becher, D. (2008) From complementarity to comprehensiveness – targeting the membrane proteome of growing *Bacillus subtilis* by divergent approaches. *Proteomics* **8**, 4123-4136.

44. Seshasayee, A. S., Fraser, G. M., and Luscombe, N. M. (2010) Comparative genomics of cyclic-di-GMP signaling in bacteria: post-translational regulation and catalytic activity. *Nucleic Acids Res.* **38**, 5970-5981.

45. Chaudhuri, R. R., Allen, A. G., Oween, P. J., Shalom, G., Stone, K., Harrison, M., Burgis, T. A., Lockyer, M., Garcia-Lana, 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.

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

47. 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.

48. Gongadze, G. M., Meshcheryakov, V. A., Serganov, A. A., Fomenkova, N. P., Mudrik, E. S., Jonsson, B. H., Liljas, A., Nikonov, S. V., and Garber, M. B. (1999) N-terminal domain, residues 1-91, of ribosomal protein TL5 from *Thermus thermophilus* binds specifically and strongly to the rRNA containing loop E. *FEBS Lett.* **451**, 51-55.

49. Schmalisch, M., Langbein, I., and Stülke, J. (2002) The general stress protein Ctc of *Bacillus subtilis* is a ribosomal protein. *J. Mol. Microbiol. Biotechnol.* **4**, 495-501.

50. Commichau, F. M., Rothe, F. M., Herzberg, C., Wagner, E., Hellwig, D., Lehnik-Habrink, M., Hammer, E., Völker, U., and Stülke, J. (2009) Novel activities of glycolytic enzymes in *Bacillus subtilis*: Interactions with essential proteins involved in mRNA processing. *Mol. Cell. Proteomics* **8**, 1350-1360.

51. Gardan, R., Rapoport, G., and Débarbouillé, M. (1997) Role of the transcriptional activator RocR in the arginine-degradation pathway of *Bacillus subtilis*. *Mol. Microbiol.* **24**, 825-837.

52. Cannon, W., Gallegos, M. T., Casaz, P., and Buck, M. (1999) Amino-terminal sequences of sigmaN (sigma54) inhibit RNA polymerase isomerisation. *Genes Dev.* **13**, 357-370.

53. Lehnik-Habrink, M., Pfrörter, H., Rempe ters, L., Pietack, N., Herzberg, C., and Stülke, J. (2010) The RNA degradosome in *Bacillus subtilis*: identification of CshA as the major RNA helicase in the multi-protein complex. *Mol. Microbiol.* **77**, 958-971.

54. Kiefer F., Arnold, K., Künzli, M., Bor doli, L., and Schwede, T. (2009) The SWISS-MODEL repository and associated resources. *Nucleic Acids Res.* **37**, D387-D392.
| Strain   | Genotype                                      | Source         |
|----------|-----------------------------------------------|----------------|
| 168      | *trpC2*                                       | laboratory collection |
| GP193    | *trpC2 Ωrny::pGP774 (p~gfp-rny cat)*          | 50             |
| GP983    | *trpC2 ΔcdaS::ermC*                           | LFH-PCR → 168  |
| GP985    | *trpC2 ΔcdaAR::cat*                           | LFH-PCR → 168  |
| GP987    | *trpC2 ΔdisA::tet*                            | YA5 → 168      |
| GP989    | *trpC2 ΔcdaS::ermC ΔcdaAR::cat*               | GP983 → GP985  |
| GP991    | *trpC2 ΔcdaS::ermC ΔdisA::tet*                | GP983 → GP987  |
| GP997    | *trpC2 ΔcdaA::cat*                            | LFH-PCR → 168  |
| GP999    | *trpC2 ΔcdaR::cat*                            | YA188 → 168    |
| GP1026   | *trpC2 cshA-Strep aphA3*                      | LFH-PCR → 168  |
| GP1173   | *trpC2 lacA::(p~gfp-yfp aphA3)*               | 23             |
| GP1319   | *trpC2 amyE::lacZ cat*                        | pAC6 → 168     |
| GP1323   | *trpC2 lacA::(p~gfp,ΔcdaS aphA3) ΔdisA::tet ΔcdaS::ermC ΔcdaA::cat* | pGP1959 → GP991 |
| GP1327   | *trpC2 lacA::(p~gfp,ΔcdaS aphA3) ΔdisA::tet ΔcdaS::ermC ΔcdaA::cat* | GP997 → GP1323 |
| GP1328   | *trpC2 lacA::(p~gfp,ΔcdaS aphA3) ΔdisA::tet ΔcdaS::ermC ΔcdaR::cat* | GP999 → GP1323 |
| GP1329   | *trpC2 lacA::(p~gfp,ΔcdaS aphA3) ΔdisA::tet ΔcdaS::ermC ΔcdaAR::cat* | GP985 → GP1323 |
| GP1330   | *trpC2 lacA::(cdaR-Strep aphA3)*              | pGP1969 → 168  |
| GP1331   | *trpC2 lacA::(cdaR-Strep aphA3) cdaA-3xFLAG ermC* | pGP1966 → GP1330 |
| GP1332   | *trpC2 lacA::(cdaR-Strep aphA3) cshA-3xFLAG spc* | pGP1333 → GP1330 |
| GP1333   | *trpC2 cshA-Strep aphA3 cdaA-3xFLAG ermC*     | pGP1966 → GP1026 |
| GP1334   | *trpC2 lacA::(p~glucose,ΔcdaS L44F aphA3) ΔdisA::tet ΔcdaS::ermC ΔcdaA::cat* | GP1327 on a plate without xylose |
| GP1339   | *trpC2 amyE::(cdaA-lacZ cat)*                 | pGP1980 → 168  |
| GP1340   | *trpC2 amyE::(glmM-lacZ cat)*                 | pGP1981 → 168  |
| GP1341   | *trpC2 lacA::(p~gfp,ΔcdaS aphA3)*             | pGP1959 → 168  |
| GP1342   | *trpC2 lacA::(p~gfp,ΔcdaS L44F aphA3)*        | GP1334 → 168   |
| GP1343   | *trpC2 lacA::(p~gfp,ΔcdaS aphA3) ΔgdpP::spc*  | pGP1959 → GP998 |
| GP1344   | *trpC2 lacA::(p~gfp,ΔcdaS L44F aphA3) ΔgdpP::spc* | GP1334 → GP998 |
| GP1346   | *trpC2 ΔcdaS::ermC ΔdisA::tet ΔcdaR::cat*     | GP999 → GP991  |
| GP1348   | *trpC2 lacA::(p~glucose,ΔcdaS aphA3) ΔdisA::tet ΔcdaS::ermC ΔcdaA::cat* | GP1327 on a plate without xylose |
| YA5      | ΔdisA::tet*                                   | 38             |
| YA188    | ΔgdpP::spc*                                   | 8              |

*a.* Arrows indicate construction by transformation.
Table 2

Determination of promoter activities upstream of the *cdaA* and *glmM* genes.

| Carbon source\(^a\) | Enzyme activity in units/mg of protein\(^b\) |
|---------------------|------------------------------------------|
|                     | GP1319 \(_{-lacZ}\) | GP1339 \(_{p_{cdaA}-lacZ}\) | GP1340 \(_{p_{glmM}-lacZ}\) |
| - (CSE)             | 2 (0.2)                  | 46 (0.2)                 | 2 (0.3)                   |
| Glucose             | 0.6 (0.1)                | 34 (4.6)                 | 1 (0.2)                   |
| Glucosamine         | 0.6 (0.1)                | 42 (3.2)                 | 0.8 (0.3)                 |

\(^a\) added to CSE medium

\(^b\) All measurements were performed at least in triplicate. Values in parentheses indicate the standard deviation.

Table 3

Control of diadenylate cyclase activities by the regulatory protein CdaR

| Diadenylate cyclase | plasmid       | -CdaR | +CdaR (pGP1984) |
|---------------------|---------------|-------|-----------------|
| none                | empty vector  | 0 (0) | 0 (0)           |
| CdaA                | pGP1970       | 237 (72) | 5,256 (1,038) |
| CdaS                | pGP1972       | 562 (140) | 342 (22)     |
| DisA                | pGP2563       | 79,187 (25,875) | 70,511 (2,313) |

\(^a\) *E. coli* cultures harboring the relevant plasmids were grown in LB medium and cell extracts were prepared as described in Experimental procedures. c-di-AMP levels were determined by HPLC-MS/MS analysis.

\(^b\) Mean values from three individual experiments were calculated. Standard deviations are shown in parentheses.
Figure legends

Fig. 1. Genetic organization of the cda-glm operon. (A) to (D) Northern blot analysis of the cda-glm gene cluster using riboprobes specific for cdaA (A), cdaR (B), glmM (C), glmS (D). RNA was isolated from B. subtilis 168 grown in CSE minimal medium supplemented with glucose (0.5%) (glc) or glucosamine (0.5%) (glcNH₂). For the depletion of the essential RNase Y, B. subtilis GP193 was grown in CSE supplemented with glucose and with (+) or without (-) xylose (2.0%). 5 µg of total RNA were separated by electrophoresis in 1.0% agarose gels and, after blotting, nylon membranes were hybridized to gene-specific riboprobes as indicated. Note that the probes cross-hybridized with the 16S and 23S rRNAs. The sizes of 16S rRNA and 23S rRNA are indicated by arrows. Moreover, the exposure time for glmS (D) was reduced compared to the other blots and thus, the 3.5 kb transcript was not detected. (E) Reverse transcription-PCR (RT-PCR) analysis. Total RNA was used as a template in the reverse transcription reaction with a random nonamer primer. Regular PCRs were carried out subsequently for the amplification of different parts of the mRNA transcript using primer pairs indicated in (F). As a negative control, RNA template without addition of reverse transcriptase was used (-RT). Experiments were carried out with two biological replicate samples at least three times each. (F) Summary of the genetic and transcriptional organization.

Fig. 2. Detection of intracellular c-di-AMP in E. coli cultures harboring the empty vector pET28a, the expression vector pGP1973 (CdaA), pGP1974 (CdaS) or pGP1975 (CdaS Δ44F). Cells were grown in LB medium and cell extracts were prepared as described in Materials and Methods. c-di-AMP levels were determined by HPLC-MS/MS analysis. Standard deviations based on three biological replicates are indicated. Note the logarithmic scale of the y-axis.

Fig. 3. Detection of in vivo interactions between CdaA and CdaR by Western blot (A - C) and bacterial two-hybrid (D) analyses. Cells were grown in CSE minimal medium supplemented with glucose in the absence and presence of the cross-linker formaldehyde (FA). The protein complexes were isolated from B. subtilis GP1331 (A) and GP1332 (B) with an ectopically encoded CdaR protein carrying a C-terminal Strep-tag or GP1333 (C) with CshA encoded in the native locus carrying a C-terminal Strep-tag. A C-terminal FLAG-tag was attached to the putative interaction partner CdaA. 28 µl of the elution fractions from each purification without (-FA) or with (+FA) cross-linking by formaldehyde and 15 µg of untreated crude extracts were analyzed by 12% SDS-PAGE. After electrophoresis and blotting onto a PVDF membrane, interaction partners were detected by an α-FLAG antibody. As a control, CshA and CdaA (B and C, respectively) were detected. For the bacterial two-hybrid analysis (D), the cdaR and cdaA, genes were cloned into p25-N. In addition, cdaR was cloned into the plasmid pUT18C. Plasmid p25-N allows the expression of the selected enzymes fused to the N-terminus of the T25 domain of the adenylate cyclase. Plasmids pUT18C allows the expression of the selected proteins fused to the C-terminus of the T18 domain of the B. pertussis adenylate cyclase. The E. coli transformants were incubated for 72 h at 30°C. The degradation of XGal (blue colour) indicates the presence of a functional adenylate cyclase owing to the interaction of the two proteins of interest.

Fig. 4. Impact of intracellular c-di-AMP levels on growth of B. subtilis. The strains GP1341 (diamonds, pXylA cdaS), GP1342 (squares, pXylA cdaS Δ44F), GP1343 (circles, pXylA cdaS ΔgdpP) and GP1344 (triangles, pXylA cdaS Δ44F ΔgdpP) were grown in CSE minimal medium supplemented with glucose in the absence or presence of the inducer xylose. Cell density was measured at 600 nm (OD₆₀₀). Three experiments were performed individually and showed similar growth patterns. One representative growth curve from each experiment is shown.

Fig. 5. Morphology of B. subtilis strains with different c-di-AMP levels. Strains GP1173 and GP1344 were grown in SP medium in the absence or presence of the inducer xylose and visualized by microscopy after 6 and 23 hours. The scale bar corresponds to 10 µm.

Fig. 6. Impact of magnesium ions on the morphology of c-di-AMP overproducing bacteria. Strains GP1173 (control) and GP1344 were grown in SP medium in the presence of the inducer xylose and visualized by microscopy after 7 hours. MgSO₄ was added to the cultures as indicated; the
concentration in the upper panel (1 mM) corresponds to the magnesium concentration in the standard medium. The scale bar corresponds to 10 µm.

Fig. 7. A model of the three-dimensional structure of CdaS based on the known structure of the corresponding YojJ protein of *Bacillus cereus* (PDB 2FB5) using the SWISS-MODEL homology-modeling server (54). The DAC domain and the N-terminal helices are shown in light blue and dark blue, respectively. The position of the amino acid exchange (L44F) resulting in the hyperactive CdaS protein is highlighted in red.
Mehne et al. Fig. 2

The graph shows the expression levels of different constructs: pET28a, CdaA, CdaS, and CdaS_{L44F} in ng c-di-AMP/mg protein. The y-axis represents the concentration in ng c-di-AMP/mg protein, ranging from 1 ng to 100,000 ng. The x-axis lists the constructs tested, with pET28a showing the lowest expression, followed by CdaA, CdaS, and CdaS_{L44F} showing the highest expression.
### Table A

| CE | CdaR-Strep | CdaA-FLAG |
|----|------------|-----------|
| -  | 10         | 10        |
| +  | 10         | 5         |
| +  | 10         | 10        |
| +  | 20         | 20        |
| +  | 30         | 30        |

- **kDa**
  - 80
  - 58
  - 46
  - 30

### Diagram B

- **CdaR-Strep CshA-FLAG**
  - CE: 10, 10, 20
  - FA: 98 °C [min]
  - CshA

### Diagram C

- **CshA-Strep CdaA-FLAG**
  - CE: 30, 30, 30
  - FA: 98 °C [min]
  - CdaA

### Diagram D

**pUT18C derivatives**
- CdaR-T18
- Zip-T18
- T25-CdaA
- T25-CdaR
- T25-Zip

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*Mehne et al. Fig. 3*
- xylose

+ xylose

![Graphs showing growth kinetics](image)

- OD₆₀₀ vs. time (t [h])
- Different strains and conditions:
  - GP1341: lacA::p₅₅lacCdaS
  - GP1342: lacA::p₅₅lacCdaS₄₄₅
  - GP1343: lacA::p₅₅lacCdaS ΔgdpP
  - GP1344: lacA::p₅₅lacCdaS₄₄₅ ΔgdpP

Mehne et al. Fig. 4
Mehne et al. Fig. 5

GP1173
wild type

GP1344
lacA::p_{xyf}cdaS_{L44F} \Delta gdpP

- xylose

6 h 23 h

+ xylose

6 h 23 h
Mehne et al. Fig. 6

GP1173
wild type

GP1344
lacA::pxylΔcdaS_{L44F} ΔgdpP

1 mM Mg$^{2+}$

25 mM Mg$^{2+}$

7 h
