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An Essential Poison: Synthesis and Degradation of Cyclic Di-AMP in *Bacillus subtilis*

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ABSTRACT

Gram-positive bacteria synthesize the second messenger cyclic di-AMP (c-di-AMP) to control cell wall and potassium homeostasis and to secure the integrity of their DNA. In the firmicutes, c-di-AMP is essential for growth. The model organism *Bacillus subtilis* encodes three diadenylate cyclases and two potential phosphodiesterases to produce and degrade c-di-AMP, respectively. Among the three cyclases, CdaA is conserved in nearly all firmicutes, and this enzyme seems to be responsible for the c-di-AMP that is required for cell wall homeostasis. Here, we demonstrate that CdaA localizes to the membrane and forms a complex with the regulatory protein CdaR and the glucosamine-6-phosphate mutase GlmM. Interestingly, *cdaA*, *cdaR*, and *glmM* form a gene cluster that is conserved throughout the firmicutes. This conserved arrangement and the observed interaction between the three proteins suggest a functional relationship. Our data suggest that GlmM and GlmS are involved in the control of c-di-AMP synthesis. These enzymes convert glutamine and fructose-6-phosphate to glutamate and glucosamine-1-phosphate. c-di-AMP synthesis is enhanced if the cells are grown in the presence of glutamate compared to that in glutamine-grown cells. Thus, the quality of the nitrogen source is an important signal for c-di-AMP production. In the analysis of c-di-AMP-degrading phosphodiesterases, we observed that both phosphodiesterases, GdpP and PgpH (previously known as YqfF), contribute to the degradation of the second messenger. Accumulation of c-di-AMP in a *gdpP pgpH* double mutant is toxic for the cells, and the cells respond to this accumulation by inactivation of the diadenylate cyclase CdaA.

IMPORTANCE

Bacteria use second messengers for signal transduction. Cyclic di-AMP (c-di-AMP) is the only second messenger known so far that is essential for a large group of bacteria. We have studied the regulation of c-di-AMP synthesis and the role of the phosphodiesterases that degrade this second messenger. c-di-AMP synthesis strongly depends on the nitrogen source: glutamate-grown cells produce more c-di-AMP than glutamine-grown cells. The accumulation of c-di-AMP in a strain lacking both phosphodiesterases is toxic and results in inactivation of the diadenylate cyclase CdaA. Our results suggest that CdaA is the critical diadenylate cyclase that produces the c-di-AMP that is both essential and toxic upon accumulation.

In order to process environmental information in the cell, many organisms are capable of synthesizing so-called second messengers. These small molecules are formed in response to primary signals and perceived by cellular targets. Bacteria often use specific nucleotides as second messengers. These nucleotides include cyclic mononucleotides, such as cyclic AMP (cAMP) and cyclic GMP (cGMP), as well as cyclic dinucleotides, such as cyclic di-AMP (c-di-AMP), cyclic di-GMP (c-di-GMP), and (p)ppGpp (1–3).

The investigation of c-di-AMP-mediated signaling has recently attracted much attention (2). This molecule is formed by many bacteria and archaea, including deltaproteobacteria, spirochaetes, actinobacteria, firmicutes, and cyanobacteria (see references 4 and 5 for recent reviews). Interestingly, c-di-AMP is essential in all firmicutes that have been studied in this respect, among them the model organism *Bacillus subtilis* and the pathogens *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Listeria monocytogenes* (6-10). This makes c-di-AMP unique among all second messengers, since it is the only essential signaling nucleotide (5).

Cyclic di-AMP is synthesized and degraded by diadenylate cyclases and phosphodiesterases, respectively. All diadenylate cyclases are characterized by a conserved catalytically active domain, the so-called DAC (diadenylate cyclase) domain (11, 12). *B. subtilis* encodes three diadenylate cyclases: DisA, CdaA, and CdaS (7) (Fig. 1A). DisA is involved in the control of DNA integrity, and the activity of this enzyme is controlled by a protein-protein interaction with the DNA methyltransferase RadA, as first observed for *Mycobacterium smegmatis* (13–15). CdaA is the most widespread diadenylate cyclase, and it is the only such enzyme in pathogenic firmicutes. CdaA has been implicated in the control of cell wall and potassium homeostasis (6, 16–19). The *cdaA* gene is the first gene of a widely conserved gene cluster that also encodes a regulatory protein, CdaR, and enzymes that generate glucosamine-1-

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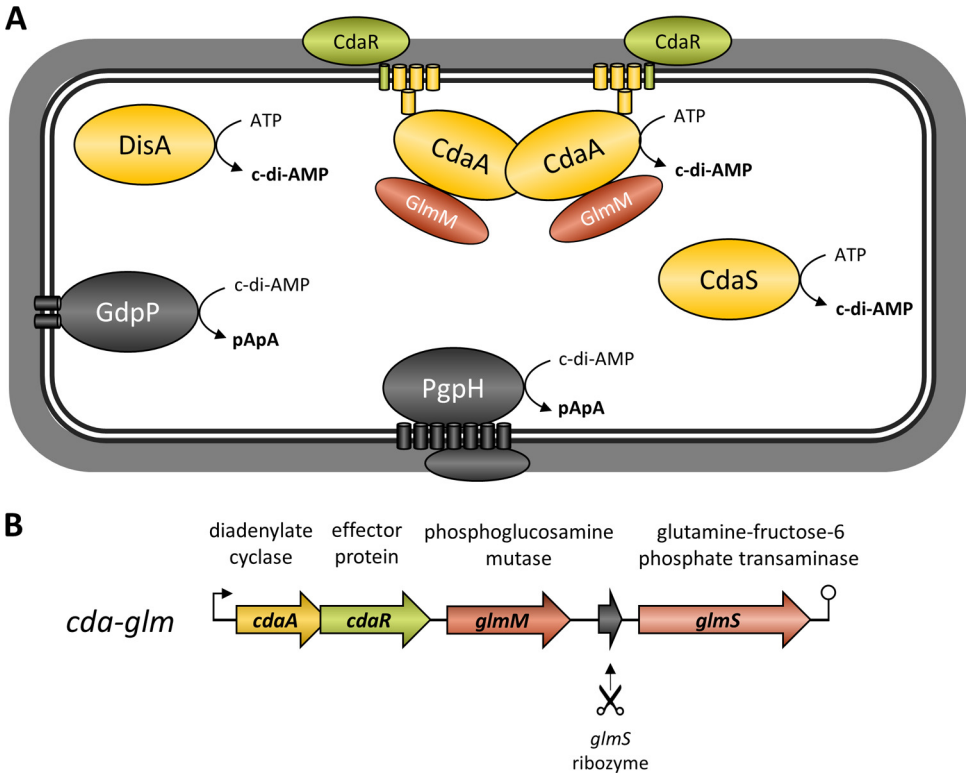

FIG 1 Major components of c-di-AMP signaling in *B. subtilis* studied in this work. (A) Cyclic di-AMP signaling in *B. subtilis*. Transmembrane regions are depicted as barrels, with one barrel per transmembrane domain. The three diadenylate cyclases, DisA, CdaA, and CdaS, are shown in yellow. CdaR and GlmM are shown in green and red, respectively. The phosphodiesterases GdpP and PgpH are shown in black. Please note that the illustration is not shown to scale. (B) Genetic organization of the highly conserved *cda-glm* operon in firmicutes. The operon is expressed under the control of a constitutive promoter; in addition, *glmS* is controlled by the glucosamine 6-phosphate-responsive *glmS* ribozyme.

phosphate, the precursor for the sugar backbone of peptidoglycan, from fructose-6-phosphate and glutamine (GlmM and GlmS). In *B. subtilis*, the *cdaAR-glmMS* operon is constitutively expressed, and the distal *glmS* gene is under the control of a glucosamine-6-phosphate-responsive ribozyme (7) (Fig. 1B). Interestingly, *cdaA*, *cdaR*, and *glmM* form a conserved gene cluster in most firmicutes, suggesting a functional relationship between the encoded proteins. The third diadenylate cyclase of *B. subtilis*, CdaS, is expressed exclusively during sporulation and is required for spore germination. The activity of CdaS is controlled by its N-terminal autoinhibitory domain (20).

Two distinct classes of phosphodiesterases are implicated in the degradation of c-di-AMP. The first class is characterized by a catalytically active Asp-His-His (DHH) motif, and *B. subtilis* GdpP is the representative of this family. The activity of GdpP is inhibited upon binding of another second messenger, ppGpp, which is synthesized in response to amino acid starvation (21). The second class of phosphodiesterases contains a His-Asp motif in the active center (HD domain). The first, and so far only, representative of this family, PgpH, was only recently discovered and characterized in *L. monocytogenes* (22). PgpH has a homolog in *B. subtilis* (YqfF; here renamed PgpH) but is absent from *S. aureus*.

Recent work on c-di-AMP signaling identified several target molecules, including both proteins and riboswitches. In several Gram-positive bacteria, c-di-AMP binds and inhibits KtrA, the cytoplasmic gating component of the KtrAB potassium transporter (17, 19). Moreover, c-di-AMP also binds the *ydaO* riboswitch, which is located upstream of the *ktrAB* operon and controls its expression (23). In all firmicutes that have been studied so far, c-di-AMP binds to DarA, a small PII-like regulatory protein. However, the function of this protein has so far remained elusive (17, 18, 24). Finally, c-di-AMP controls the activity of the pyruvate carboxylase in *L. monocytogenes* (18). However, all these identified targets cannot answer the question of why c-di-AMP is essential in the firmicutes. Recently, a study using *L. monocytogenes* identified conditions that allow growth of this bacterium in the absence of c-di-AMP: c-di-AMP production was dispensable if the bacterium grew in minimal medium or if the stringent response was inactivated due to a loss of ppGpp synthesis. This suggests that c-di-AMP is required for *L. monocytogenes* to cope with the potential toxicity of ppGpp (25).

Two observations suggest a link between c-di-AMP signaling and nitrogen sensing. First, the conserved c-di-AMP binding protein DarA represents a protein family that is most closely related to nitrogen-sensing PII proteins. Moreover, the *cdaA* gene is clustered with *glmM* and *glmS*. As mentioned above, the encoded enzymes are required for the synthesis of glucosamine-1-phosphate, the universal precursor for peptidoglycan biosynthesis, from fructose-6-phosphate and glutamine (26; http://subtiwiki.uni-goettingen.de/subtipathways/index.php?pathway=LlfNo). Glutamine is the preferred nitrogen source for *B. subtilis*, but this bacterium can use several other nitrogen sources, such as glutamate, ammonium, or amino acids (27). However, a possible relationship between c-di-AMP and nitrogen metabolism has so far not been explored.

TABLE 1 *Bacillus subtilis* strains used in this study

Strain	Genotype[a]	Source[b] or reference
168	*trpC2*	Laboratory collection
BKE25330	Δ*pgpH*::*ermC*	Bacillus Genetic Stock Center (BGSC)
GP588	*trpC2* Δ*glmM*::*spc*; complemented with pGP400	33
GP983	*trpC2* Δ*cdaS*::*ermC*	7
GP985	*trpC2* Δ*cdaAR*::*cat*	7
GP987	*trpC2* Δ*disA*::*tet*	7
GP991	*trpC2* Δ*cdaS*::*ermC* Δ*disA*::*tet*	7
GP997	*trpC2* Δ*cdaA*::*cat*	7
GP998	*trpC2* Δ*gdpP*::*spc*	7
GP999	*trpC2* Δ*cdaR*::*cat*	7
GP1320	*trpC2 ganA*::(p_{xylA} $glmM_{Bsu}$ *aphA3*)	pGP2572 → 168
GP1321	*trpC2 ganA*::(p_{xylA} $glmM_{Bsu}$ *aphA3*) Δ*disA*::*tet* Δ*cdaS*::*ermC*	GP1320 → GP991
GP1327	*trpC2 ganA*::(p_{xylA} *cdaS aphA3*) Δ*cdaS*::*ermC* Δ*disA*::*tet* Δ*cdaR*::*cat*	7
GP1331	*trpC2 ganA*::(*cdaR*-Strep tag *aphA3*) *cdaA*-3×FLAG *ermC*	7
GP1365	*trpC2 ganA*::(p_{xylA} $glmM_{Eco}$ *aphA3*)	pGP2566 → 168
GP1367	*trpC2 ganA*::(p_{xylA} $glmM_{Eco}$ *aphA3*) Δ*glmM*::*spc*	GP588 → GP1365
GP1374	*trpC2 ganA*::(p_{xylA} $glmM_{Eco}$ *aphA3*) Δ*glmM*::*spc* Δ*cdaS*::*ermC* Δ*disA*::*tet*	GP983 + GP987 → GP1367
GP1376	*trpC2* Δ*gdpP*::*spc* Δ*cdaA*::*cat*	GP997 → GP998
GP1381	*trpC2 cdaA*-3×FLAG *ermC*	pGP1966 → 168
GP1383	*trpC2 ganA*::(p_{xylA} $glmM_{Bsu}$ *aphA3*) Δ*glmM*::*spc* Δ*cdaS*::*ermC* Δ*disA*::*tet*	GP588 → GP1321
GP2032	*trpC2* Δ*cdaS*::*ermC* Δ*cdaA*::*cat*	GP997 → GP983
GP2034	*trpC2* Δ*pgpH*::*ermC*	BKE25330 → 168
GP2035	*trpC2* Δ*gdpP*::*spc* Δ*pgpH*::*ermC* Δ*disA*::*tet*	GP987 → GP2040
GP2040	*trpC2* Δ*gdpP*::*spc* Δ*pgpH*::*ermC*	BKE25330 → GP998
GP2041	*trpC2* Δ*gdpP*::*spc* Δ*cdaA*::*cat* Δ*pgpH*::*ermC*	BKE25330 → GP1376
GP2042	*trpC2* Δ*gdpP*::*spc* Δ*pgpH*::*ermC* $cdaA_{1–795}$ Δ*cdaR*::*cat*	GP999 → GP2040 (suppressor)
GP2051	*trpC2* Δ*gdpP*::*spc* Δ*pgpH*::*ermC* Δ*cdaAR*::*cat*	GP985 → GP2040
GP2064	*trpC2* Δ*gdpP*::*spc* Δ*pgpH*::*ermC cdaA*-ins295	Suppressor of GP2040
GP2065	*trpC2* Δ*gdpP*::*spc* Δ*pgpH*::*ermC cdaA*-del381	Suppressor of GP2040
GP2134	*trpC2* Δ*pgpH*::*ermC* Δ*disA*::*tet* Δ*gdpP*::*spc* $cdaA_{SD\text{-}mut}$	Suppressor of GP2035

[a] $glmM_{Bsu}$, *B. subtilis glmM* gene; $glmM_{Eco}$, *E. coli glmM* gene; $cdaA_{1-795}$, truncated *cdaA* gene; *cdaA*-ins295, *cdaA* with insertion after base 285; *cdaA*-del381, *cdaA* with deletion of base 382; $cdaA_{SD\text{-}mut}$, *cdaA* with mutated Shine-Dalgarno sequence.
[b] Arrows indicate construction by transformation.

We are interested in c-di-AMP-mediated signal transduction in the Gram-positive model organism *B. subtilis*. In this bacterium, the nucleotide is essential, but its excessive accumulation leads to a defect in cell wall biosynthesis resulting in impaired growth (7). Moreover, accumulation of c-di-AMP due to a loss of the phosphodiesterase GdpP results in increased resistance to β-lactam antibiotics that target cell wall synthesis (6). In this study, we address the control of c-di-AMP levels by different nitrogen sources and the differential role of the c-di-AMP-degrading phosphodiesterases. We demonstrate that the nitrogen source in the medium has a profound effect on the intracellular c-di-AMP pools. Moreover, we identify PgpH as the major c-di-AMP-hydrolyzing phosphodiesterase. Our data indicate that c-di-AMP not only is essential but also is toxic for cells at high concentrations and that bacteria respond to such poisoning by accumulating suppressor mutations in the diadenylate cyclase CdaA.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. subtilis* strains were derived from the laboratory strain 168 (*trpC2*) and are listed in Table 1. *Escherichia coli* DH5α (28) was used for cloning experiments. *B. subtilis* and *E. coli* were grown in LB medium. LB and SP plates were prepared by addition of 17 g Bacto agar/liter (Difco) to LB and SP media, respectively (28, 29). To study *in vivo* protein-protein interactions and for the quantification of c-di-AMP, we used CSE minimal medium (30) and Spizizen minimal medium (SM medium) (31), respectively. If required, ammonium sulfate was used to supplement the cultures with ammonium.

DNA manipulation. Transformation of *E. coli* and plasmid DNA extraction were performed using standard procedures (28). All commercially available plasmids, restriction enzymes, T4 DNA ligase, and DNA polymerases were used as recommended by the manufacturers. DNA fragments were purified using a peqGOLD Cycle-Pure kit (Peqlab, Erlangen, Germany). DNA sequences were determined using the dideoxy chain termination method (28). Chromosomal DNA of *B. subtilis* was isolated as described previously (29).

Transformation and phenotypic analysis. Standard procedures were used to transform *E. coli* (28), and transformants were selected on LB plates containing ampicillin (100 μg/ml). *B. subtilis* was transformed with plasmid and genomic DNAs according to the two-step protocol (29). Transformants were selected on SP plates containing erythromycin (2 μg/ml) plus lincomycin (25 μg/ml), chloramphenicol (5 μg/ml), kanamycin (10 μg/ml), tetracycline (12.5 μg/ml), or spectinomycin (150 μg/ml).

Construction of a strain that allows controlled expression of glucosamine-6-phosphate mutases. First, we constructed plasmid pGP2566, which allows the expression of *E. coli glmM* under the control of a xylose-inducible promoter. Briefly, *E. coli glmM* was amplified using the primer pair FX32 (5′-AAATCTAGAGATGAGTAATCGTAAATATTTCGGTAC CGATGG)/FX33 (5′-TTTGGATCCCTATTAAACGGCTTTTACTGCA TCGGCGATG), digested with XbaI and KpnI, and inserted into the expression vector pGP888 (32) linearized with the same enzymes. Plasmid pGP2566 allows the integration of the expression cassette into the *ganA*

locus in the *B. subtilis* chromosome. The corresponding strain was GP1365. In the next step, the *B. subtilis glmM* gene was deleted by transforming GP1365 with chromosomal DNA of *B. subtilis* GP588. In the latter strain, the chromosomal copy of *glmM* is deleted, and *glmM* is provided ectopically from a plasmid (33). The loss of the *B. subtilis glmM* gene was verified by PCR. Finally, the diadenylate cyclase genes *disA* and *cdaS* were deleted, giving rise to strain GP1374, which encodes CdaA as the only diadenylate cyclase. In parallel, we constructed a strain expressing *B. subtilis glmM* at the ectopic *ganA* locus. For this purpose, *B. subtilis glmM* was amplified using the primer pair FX133 (5′-AAATCTAGAGAT GGGCAAGTATTTTGGAACAGACGG)/FX135 (5′-TTTGGTACCCTA TTACTCTAATCCCATTTCTGACCGGAC), and all further steps were performed as described above. The resulting plasmid was pGP2572. See Table 1 for the details of strain construction.

***In vivo* detection of protein-protein interactions.** To detect interaction partners of the CdaR protein, we made use of SPINE technology (34). Briefly, 1-liter cultures of *B. subtilis* GP1331 expressing Strep-tagged CdaR were grown in CSE medium supplemented with 0.5% glucose at 37°C to an optical density at 600 nm (OD_{600}) of 1.0. The culture was split in two 500-ml aliquots. One aliquot was harvested immediately and stored at −20°C, whereas the second aliquot was treated with paraformaldehyde (0.6% [wt/vol], 20 min, 37°C) to cross-link interacting proteins. These cells were then harvested and stored at −20°C. The Strep-tagged proteins in both cultures and their potential interaction partners were then purified from crude extracts by use of a Streptactin column (IBA, Göttingen, Germany), with 2.5 mM D-desthiobiotin as the eluent. The eluted proteins were separated in an SDS gel, and potential interacting partners were analyzed by silver staining and identified by mass spectrometry (MS) analysis.

Protein identification by mass spectrometry. Excised polyacrylamide gel pieces of stained protein bands were digested with trypsin as described previously (35). Tryptic peptides were separated in a water-acetonitrile gradient on an Acclaim PepMap RSLC 75-mm by 15-cm column within an Ultimate3000 RSLC-nano system (Thermo Scientific). Peptides were ionized online by using a Nanospray Flex ion source in front of an Orbitrap Velos Pro mass spectrometer (Thermo Scientific). MS spectra and MS2 spectra (data dependent, top 10, dynamic exclusion) were taken with an FT-Orbitrap mass analyzer and a Velos Pro ion trap mass analyzer, respectively. MS data analysis and protein identification were done with the SequestHT and Mascot search engines and the results bundled within the Proteome Discoverer 1.4 platform (Thermo Scientific), using a *B. subtilis*-specific protein database. At least two unique unambiguous high-scoring peptides with a false discovery rate of ≤0.01 for both search engines were expected for positive protein identification.

B2H assay. Primary protein-protein interactions were identified by bacterial two-hybrid (B2H) analysis (36). 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-catabolite gene activator protein (CAP)-dependent promoter of the *E. coli lac* operon. Plasmids pUT18C and p25N allow the expression of proteins fused to the T18 and T25 fragments of CyaA, respectively. For these experiments, we used the plasmids pGP1989 and pGP1990, which encode T18-CdaR and CdaA-T25, respectively (7). To test the interaction capability of GlmM, we constructed the plasmids pGP2576 (GlmM-T25) and pGP2688 (T18-GlmM). Briefly, the *glmM* gene was amplified using the primer pair FX133 (see above)/FX134 (5′-TTTGGTACCCGC TCTAATCCCATTTCTGACCGGACG) and cloned between the XbaI and KpnI sites of p25N and pUT18C. These plasmids 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, 40 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The plates were incubated for a maximum of 36 h at 30°C. For quantitative assays, the transformants were grown overnight in LB containing 0.5 mM IPTG and the appropriate antibiotics and subsequently inoculated into 10 ml fresh medium to an OD_{600} of 0.1. The cultures were grown at 30°C to the exponential growth phase, and the β-galactosidase activities were determined as described previously (37).

Localization of CdaA. To determine the localization of CdaA, we constructed strain GP1381, which expresses CdaA carrying a C-terminal FLAG tag to facilitate protein detection. For this purpose, *B. subtilis* 168 was transformed with plasmid pGP1966 (7). Cultures of *B. subtilis* GP1381 were cultivated in LB medium and harvested by centrifugation (4,400 × *g*, 10 min, 4°C). The following steps were done as described previously (31). Briefly, the cells were lysed by sonication, the cellular debris was removed, and the fractions of the cell extract were separated by ultracentrifugation. The membrane pellet was washed three times and finally resuspended in phosphate buffer (50 mM Na_2HPO_4, 50 mM NaH_2PO_4, pH 6.8). To assess the quality of the preparations, the fractions were analyzed for the presence of CggR and RNase Y by using polyclonal rabbit antibodies raised against these proteins (38, 39).

Western blotting. For Western blotting, *B. subtilis* cell extracts were separated in 12.5% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) by electroblotting. Proteins were detected using specific antibodies or antibodies recognizing the FLAG tag (Sigma). The primary antibodies were visualized by using anti-rabbit IgG–alkaline phosphatase (AP) secondary antibodies (Promega) and the CDP* detection system (Roche Diagnostics) (40).

Analysis of cyclic dinucleotide pools. The concentration of c-di-AMP in *B. subtilis* cells was determined by a liquid chromatography-tandem MS (LC-MS/MS) method, essentially as described previously. Briefly, *B. subtilis* cells (20 ml) were grown in SM medium supplemented with the desired nitrogen source at a concentration of 68 mM at 37°C to an OD_{600} of 1.0. The samples (10 ml) were centrifuged (0°C, 20,800 × *g*), shock frozen in liquid nitrogen, and stored at −80°C. These samples were used for c-di-AMP quantification. Two additional aliquots (1 ml each) of each sample were harvested for total protein determination (see below). Pellets were stored at −20°C. The samples for c-di-AMP quantification were resuspended in 150 μl lysozyme solution (2 mg/ml in Tris-EDTA [TE] buffer) and incubated for 30 min at 25°C with agitation (750 rpm). The resulting cell lysates were again shock frozen in liquid nitrogen and subsequently boiled for 10 min. Eight hundred microliters of extraction mixture I (acetonitrile-methanol [1:1]) was added and vortexed for 45 s, followed by 15 min of incubation at 4°C. The supernatant was collected by centrifugation (10 min, 4°C, 20,800 × *g*) and transferred to a new tube. The remaining pellet was resuspended with 200 μl of extraction mixture II (acetonitrile-methanol-water [2:2:1]) and vortexed for 45 s, followed by 15 min of incubation at 4°C. The supernatant was collected as described above and pooled with the previous supernatant. Again, the pellet was resuspended as described above, and the supernatant was again collected and pooled. The total supernatant was incubated overnight at −20°C and then centrifuged (20 min, 4°C, 20,800 × *g*). The resulting supernatant was air dried at 40°C and dissolved with 200 μl of H_2O. After repeated centrifugation and addition of the internal standard ([^{13}C,^{15}N]c-di-AMP), part of the extract was analyzed by LC-MS/MS.

Quantification of total protein amount. The cell pellets for protein quantification were dissolved in 800 μl 0.1 M NaOH and boiled for 10 min. After a short centrifugation (5 min, 20,800 × *g*), the supernatant was transferred to a new tube. The pellet was processed a second time as described above. The resulting supernatant was pooled with the previous one. This sample was used for Bradford quantification.

Quantification of cyclic dinucleotides by MS/MS. Chromatographic separation was performed on a series 200 high-pressure liquid chromatography (HPLC) system (PerkinElmer Life Sciences) as described previously (7). The analyte detection was performed on an API 3000 triple-quadrupole mass spectrometer equipped with an electrospray ionization

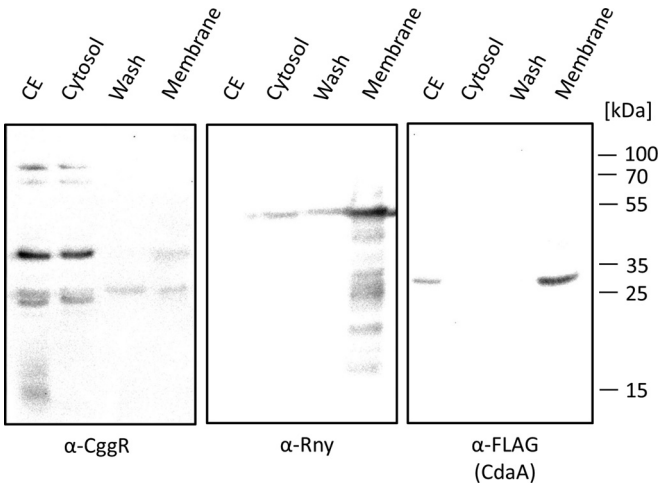

FIG 2 The diadenylate cyclase CdaA is a membrane-associated enzyme. A crude extract of *B. subtilis* GP1381 (*cdaA*-FLAG) was separated by ultracentrifugation to obtain cytosolic and membrane fractions. To check for successful separation, the cytosolic and membrane fractions were tested with specific antibodies recognizing CggR and Rny, respectively. CdaA-FLAG was detected using commercial antibodies. CE, cell extract.

source (AB Sciex), using selected reaction monitoring (SRM) analysis in positive ionization mode. The SRM transitions labeled "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 relative concentration of c-di-AMP was determined as follows: relative amount of c-di-AMP = raw data for HPLC-MS analyses (nanomolar)/protein concentration (micrograms per milliliter of cells) × value obtained for the control sample (*B. subtilis* 168 with glutamine).

RESULTS

Localization of diadenylate cyclase CdaA. Of the three diadenylate cyclases in *B. subtilis*, only CdaA is predicted to contain transmembrane helices. However, the intracellular localization has so far not been studied. To investigate the localization of CdaA, we constructed a strain (*B. subtilis* GP1381) in which a C-terminal FLAG tag is attached to the CdaA protein. In this strain, the *cdaR* and *glmMS* genes downstream of *cdaA* are expressed from an IPTG-inducible promoter. GP1381 was cultivated in LB medium containing IPTG, and subcellular fractions were separated by analytical ultracentrifugation. The cytosolic and membrane fractions were then analyzed for the presence of the tagged CdaA protein. To ensure the specificity of fractionation, we used the cytosolic CggR repressor protein and the membrane-bound RNase Y as controls. As shown in Fig. 2, CggR and RNase Y were detected in the cytosol and membrane fractions, respectively. This indicates that the fractionation of the cellular compartments was successful. For CdaA, we observed a signal exclusively in the membrane fraction, demonstrating that this diadenylate cyclase is indeed a membrane protein.

Identification of interaction partners of CdaR. The diadenylate cyclase CdaA is encoded in an operon with the regulatory protein CdaR, the essential glucosamine-6-phosphate mutase GlmM, and the essential glutamine-fructose-6-phosphate transaminase GlmS (7). The *cdaAR glmM* operon is widely conserved among Gram-positive bacteria, suggesting a functional link between the encoded proteins. Indeed, we have provided evidence for a direct protein-protein interaction between CdaA and CdaR (7). To gain novel insights into the functions of these proteins, we performed an unbiased screen for proteins that interact with CdaR. For this purpose, we made use of strain GP1331, which expresses CdaR carrying a Strep tag at its C terminus to facilitate purification. This strain was cultivated in minimal CSE medium containing glucose. A part of the culture was treated with formaldehyde to cross-link interacting proteins. CdaR was then purified by affinity chromatography. Two proteins were detectable irrespective of the presence of Strep-tagged CdaR (data not shown). These proteins were previously identified as AccB and PycA, the only two biotin-containing proteins of *B. subtilis*, which bind intrinsically to Streptactin columns (41). These proteins were also found as contaminants of CdaR purification. Even though PycA has been identified as a c-di-AMP binding protein in *L. monocytogenes* (18), we did not study this protein further due to the lack of interaction specificity in our assay. As shown in Fig. 3A, one band corresponding to a protein of about 35 kDa was specifically copurified with CdaR in the absence of the cross-linker, whereas two potentially interacting proteins (about 50 and 80 kDa) were observed upon cross-linking. These proteins were identified as CdaA, GlmM, and MetE, respectively. The interaction between CdaA and CdaR has been observed previously (7). MetE is found in many screens for interaction partners in *B. subtilis*, suggesting that this protein is somewhat sticky and not a physiologically relevant interaction partner (41, 42). In contrast, the interaction between CdaR and GlmM has not previously been identified.

To confirm the interaction between CdaR, CdaA, and GlmM and to distinguish primary from indirect interactions, we studied the binary interactions by using a bacterial two-hybrid screen. In this system, interacting proteins reconstitute the *B. pertussis* adenylate cyclase, resulting in cAMP synthesis and subsequent activation of β-galactosidase synthesis. As shown in Fig. 3B and C, CdaR and CdaA showed a strong direct interaction. This observation is in excellent agreement with previous results (7). Moreover, GlmM exhibited a strong self-interaction, which corresponds to its previously reported formation of homodimers (43). Moreover, we detected a weak but significant interaction between CdaA and GlmM. It is possible that this interaction is stimulated by cofactors that are absent or not present in sufficient amounts in *E. coli*. Finally, no binary interaction was detected between GlmM and CdaR. Taken together, our data suggest that the three proteins CdaR, CdaA, and GlmM might form a complex in which CdaA, as the central component, interacts directly with both CdaR and GlmM.

GlmM and GlmS are not the essential targets of c-di-AMP. Three lines of evidence suggest a functional relationship between c-di-AMP formation by CdaA and the GlmM and GlmS proteins that synthesize precursors for cell wall biosynthesis. (i) CdaA is encoded in a conserved operon with GlmM, and sometimes also with GlmS (7), and (ii) the CdaA protein interacts with CdaR and GlmM. (iii) Moreover, both GlmM and GlmS are essential proteins, and c-di-AMP is an essential second messenger. Since the reason for the essentiality of c-di-AMP is unknown, GlmM and GlmS are attractive candidates as c-di-AMP binding proteins that are controlled by the dinucleotide. Therefore, we designed experiments to study the roles of GlmM and GlmS in the essentiality of c-di-AMP.

In order to investigate the c-di-AMP dependence of GlmM, we took advantage of the fact that there is no c-di-AMP present in *E. coli* (7). In consequence, the *E. coli* GlmM protein is active whether

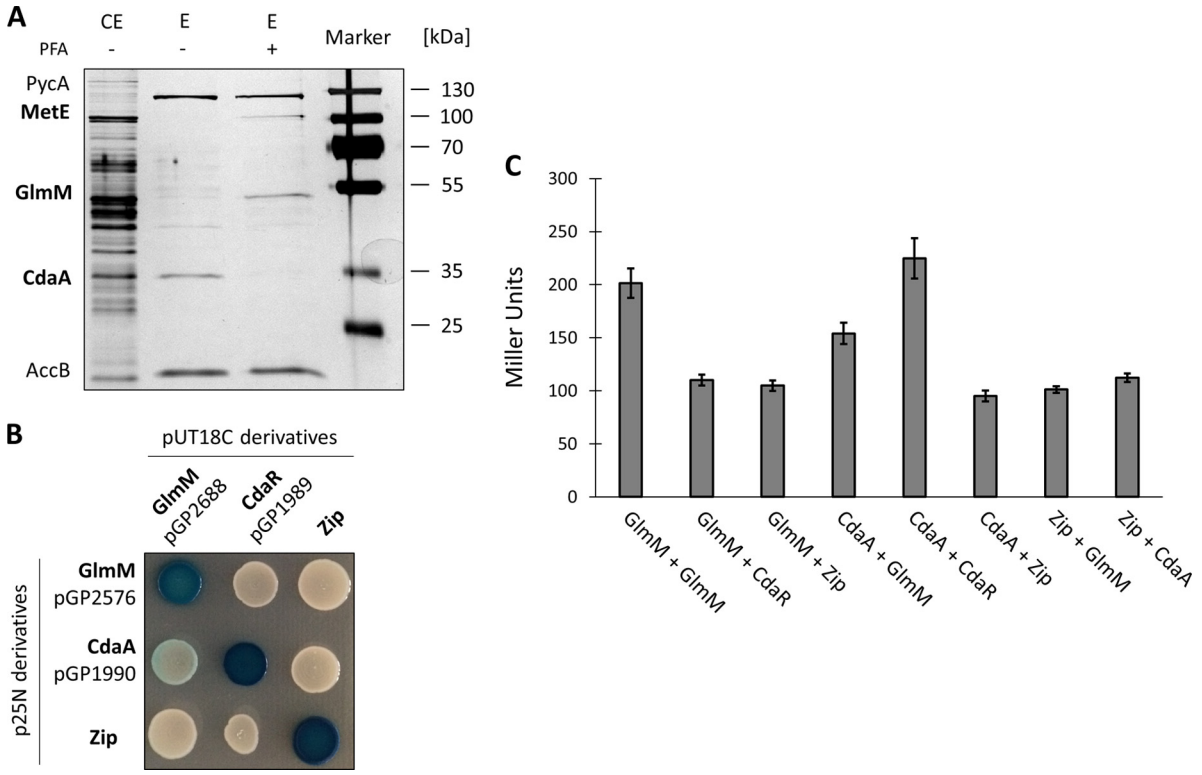

FIG 3 Interactions between CdaA, CdaR, and GlmM. (A) Detection of *in vivo* interaction partners of CdaR. *B. subtilis* cells (GP1331) were grown in CSE minimal medium supplemented with glucose in the presence or absence of the cross-linker paraformaldehyde (PFA). The copurified proteins were analyzed in an SDS-polyacrylamide gel and visualized by silver staining. Proteins were identified by mass spectrometry. CE, cell extract; E −, elution fraction without cross-linking; E +, elution fraction after cross-linking with paraformaldehyde. (B) B2H screen. (C) β-Galactosidase activities in B2H screen.

c-di-AMP is present or not. Therefore, we ectopically expressed the *E. coli* GlmM protein in *B. subtilis*, and we deleted the cognate *glmM* gene. With such a strain at hand, we could test whether c-di-AMP would still be essential for the bacterium. This strain was viable and did not exhibit any growth defect compared to the wild type. This result indicates that *E. coli* GlmM is active in *B. subtilis* and that it can meet the metabolic demand for glucosamine-1-phosphate for cell wall biosynthesis. In the next step, we attempted to delete the *cdaA* gene from *B. subtilis* GP1374. In this strain, CdaA is the only diadenylate cyclase, and *E. coli* GlmM produces glucosamine-1-phosphate. If GlmM were the essential target of c-di-AMP in *B. subtilis*, it should have been possible to delete the last diadenylate cyclase gene in this strain that expresses the c-di-AMP-independent *E. coli* GlmM protein. However, all our attempts to delete the *cdaA* gene in *B. subtilis* GP1374 failed, demonstrating that c-di-AMP is still essential in this strain. This result indicates that either GlmM is not an essential target of c-di-AMP or there is an another essential target in addition to GlmM.

To address the involvement of GlmS in the essentiality of c-di-AMP, we made use of the fact that this enzyme is dispensable if cells are provided with external glucosamine. Glucosamine is then transported and phosphorylated by a dedicated phosphotransferase system (GamP) to generate glucosamine-6-phosphate, the substrate for GlmM (44, 45). Therefore, if GlmS were the essential target of c-di-AMP, a strain lacking all three diadenylate cyclases would be able to grow in the presence of glucosamine to bypass the need for GlmS. This hypothesis was tested using strain GP1327, in which all diadenylate cyclase genes have been deleted and which is able to grow in the presence of xylose due to the presence of an ectopic copy of the *cdaS* gene expressed under the control of a xylose-inducible promoter (7). We cultivated this strain in the presence of glucosamine and recorded its growth after the removal of xylose from the medium. While the bacteria grew well in the presence of both glucosamine and xylose, no growth was possible in the absence of the inducer. Thus, neither GlmM nor GlmS is the single reason for the essentiality of c-di-AMP. Our results indicate the existence of either an additional or alternative essential target.

Control of the c-di-AMP pool by the nitrogen source. Based on the interaction between GlmM and CdaA observed in both the SPINE and two-hybrid analyses (see above), we hypothesized that GlmM might provide a signal input for CdaA. If this was the case, it seemed likely that the production of c-di-AMP might be responsive to the availability of nutrients that feed into the reactions catalyzed by GlmM and GlmS. These are fructose-6-phosphate, a glycolytic intermediate, glutamine, and glucosamine-6-phosphate. Since fructose-6-phosphate is an abundant metabolite and glucosamine-6-phosphate is essential for growth (see above), we focused on glutamine as the nitrogen source for the biosynthesis of cell wall precursors. Thus, we cultivated *B. subtilis* 168 in SM minimal medium with glutamine, ammonium, glutamate, and their combinations as the nitrogen source, extracted the metabolites, and determined the levels of the c-di-AMP pools. As shown in Fig. 4, small amounts of c-di-AMP were detected in the presence of glutamine, whereas the pools were increased up to 2-fold in the presence of glutamate as the only source of nitrogen. With

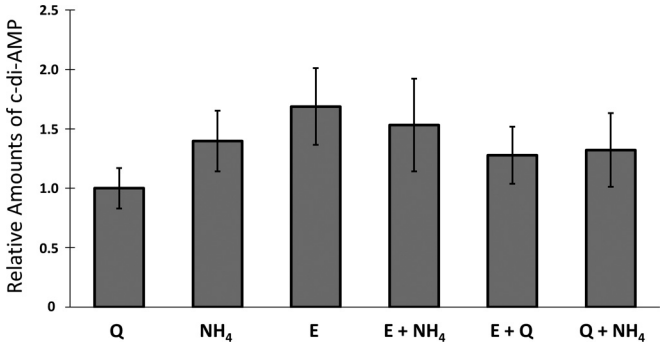

FIG 4 The intracellular c-di-AMP concentration depends on the nitrogen source. Cells were grown in SM minimal medium supplemented with the indicated nitrogen sources (68 mM [each]). Data for three biological replicates are shown; error bars show standard deviations. E, glutamate; Q glutamine.

ammonium, we observed an intermediate level of c-di-AMP. Moreover, the additional presence of glutamine resulted in slightly reduced c-di-AMP levels. These differences in c-di-AMP levels during growth with different nitrogen sources might simply result from differences in the growth rates that can be reached in the presence of these nitrogen sources. Alternatively, GlmS/GlmM might directly sense the quality of the nitrogen source and relay this information to CdaA. To distinguish between these possibilities, we determined the generation times in the different media. With glutamate, ammonium, and glutamine as the only nitrogen sources, the generation times were 85 ± 4, 66 ± 6, and 42 ± 2 min, respectively. The combinations of the different nitrogen sources all allowed generation times of 40 to 42 min (for glutamate plus glutamine, 42 ± 2 min; for glutamate plus ammonium, 41 ± 1 min; and for glutamine plus ammonium, 40 ± 0 min). Thus, the c-di-AMP levels with the nitrogen source combinations did not correspond to those observed in glutamine-grown cells. Similarly, all cultures containing ammonium exhibited similar relative c-di-AMP levels, even though their generation times differed substantially (for ammonium, 66 min; and for ammonium plus glutamate, 42 min). This suggests that the intracellular c-di-AMP levels depend on the nature of the nitrogen source rather than on the growth rate.

Impact of phosphodiesterases on intracellular c-di-AMP levels. Recently, PgpH was identified as a second c-di-AMP-specific phosphodiesterase in *L. monocytogenes* (22). This prompted us to test whether the corresponding protein of *B. subtilis* (YqfF; here renamed PgpH) might also have this function and how the two potential phosphodiesterases, GdpP and PgpH, act together to control the intracellular c-di-AMP concentration. To address this question, we determined the c-di-AMP levels in strains with deletion of one or both enzymes. As shown in Fig. 5, deletion of the *gdpP* gene resulted in only a weak increase of the c-di-AMP pool. In contrast, loss of PgpH resulted in a 2-fold higher c-di-AMP level. This finding suggests that PgpH is the major c-di-AMP-degrading phosphodiesterase in *B. subtilis*. In the *gdpP pgpH* double mutant GP2040, the intracellular amount of c-di-AMP was even somewhat higher than that in any of the two single mutants, indicating that both enzymes act in concert in the degradation of the nucleotide. It is interesting that in both phosphodiesterase mutants, the levels of c-di-AMP were significantly higher when the cells were grown with glutamate than with glutamine. This was even the case in the absence of both phosphodiesterases, suggesting that the regulation is exerted at the level of c-di-AMP synthesis.

Loss of phosphodiesterases results in the inactivation of CdaA. We noticed that the *gdpP pgpH* double mutant was lysed when the cells were incubated for two or more days on plates. This was not the case for the two single mutants, suggesting that lysis results from the increased levels of intracellular c-di-AMP in the double mutant that lacks both phosphodiesterases. However, we observed the appearance of suppressor mutants that were able to grow in the absence of any c-di-AMP-specific phosphodiesterase. We suspected that the synthesis of c-di-AMP might be reduced in these mutants, and we sequenced the *cdaA* and *disA* alleles of two mutants. Interestingly, in both mutants, we found frameshift mutations in *cdaA* that result in the synthesis of truncated, and most likely inactive, proteins. Strains GP2064 and GP2065 harbor an insertion of an extra T after base 285 in *cdaA* and a deletion of base 382, respectively. Both resulting truncated proteins lack the DGA (amino acids [aa] 171 to 173) and RHR (aa 203 to 205) motifs, which are essential for the enzymatic activity of CdaA (12) (see Fig. 6 for the localization of the essential motifs and the mutations). When we deleted *disA* and *cdaA* individually in the *gdpP pgpH* double mutant, we observed immediate lysis of the *gdpP pgpH disA* mutant GP2035, whereas the *gdpP pgpH cdaA* mutant

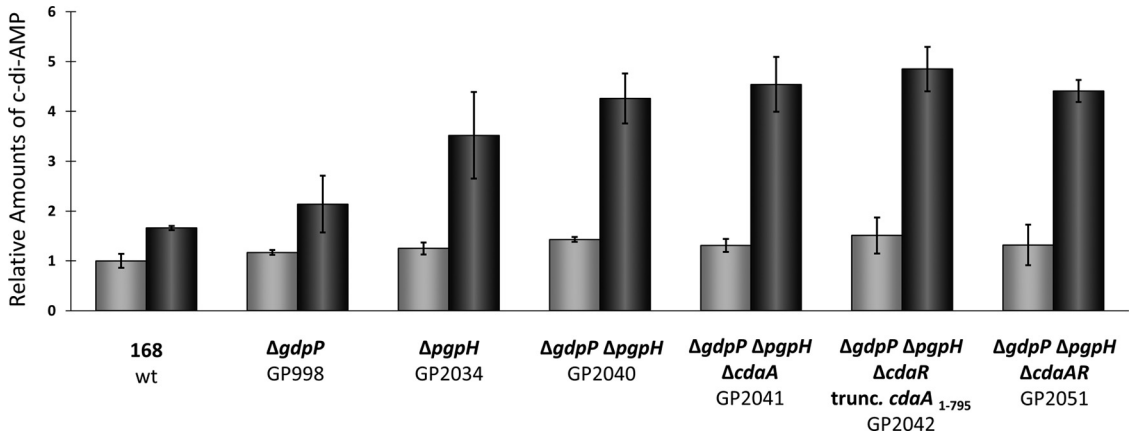

FIG 5 Identification of PgpH as the major c-di-AMP-hydrolyzing enzyme. Cells were grown in SM minimal medium supplemented with glutamine (gray) or glutamate (black). Data for three biological replicates are shown; error bars show standard deviations. wt, wild type.

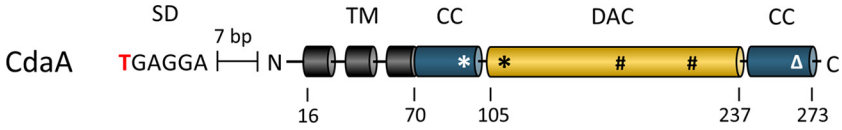

FIG 6 Mutations affecting the diadenylate cyclase CdaA. TM, transmembrane region; CC, coiled-coil domain; DAC, diadenylate cyclase domain; #, DGA and RHR active site motifs; SD, Shine-Dalgarno sequence (the first G of the sequence is replaced by a T in the suppressor mutant GP2134); *, frameshift mutations (both mutations result in the production of inactive truncated proteins); △, C-terminal deletion.

GP2041 was more stable. We obtained one suppressor of the highly instable strain GP2035 and designated this strain GP2134. Analysis of the *cdaA* allele of the suppressor mutant GP2134 indicated a mutation in the Shine-Dalgarno sequence of *cdaA* (Fig. 6), suggesting reduced expression of CdaA. The high instability of the strain that lacks both phosphodiesterases but expresses CdaA, together with the detection of three independent suppressor mutants of *cdaA*, indicates that the accumulating pool of c-di-AMP generated by CdaA is toxic for the survival of bacteria.

We also attempted to construct a strain devoid of the phosphodiesterases GdpP and PgpH and the regulatory protein CdaR. Initial attempts failed; however, simultaneous deletion of the *cdaAR* segment was possible (GP2051). After prolonged incubation of primary transformation plates to generate the *gdpP pgpH cdaR* mutant, we found one single colony. Since the expression of *cdaA* is obviously toxic in the mutant lacking both phosphodiesterases, we suspected that this colony (strain GP2042) might contain a mutation in *cdaA*. This was indeed the case; we found a deletion of a region from the 3′ end of *cdaA* to the 5′end of *cdaR* (note that the first base pairs of *cdaR* are not affected by the *cdaR* gene deletion in GP2042). Interestingly, this deletion occurred within a 9-bp repeat. As a result, the CdaA protein is truncated in GP2042 (Fig. 6), suggesting that the protein is inactive. This finding reinforces the hypothesis that c-di-AMP generated by CdaA is toxic for the strain lacking the phosphodiesterases.

To address the idea that the truncated CdaA variant is inactive, we measured the intracellular c-di-AMP pools of *B. subtilis* GP2042. As controls, we used the otherwise isogenic *cdaA* and *cdaAR* deletion mutants GP2041 and GP2051, respectively. As shown in Fig. 5, none of the mutations affecting *cdaA* had a significant impact on the intracellular c-di-AMP concentration. This suggests that CdaA might build up a local pool of c-di-AMP that is essential but is toxic if this local pool is too high (see Discussion).

DISCUSSION

Cyclic di-AMP is unique among all known second messengers: *B. subtilis* and other firmicutes are not able to live without this signaling molecule (5). In this work, we discovered a second peculiarity of c-di-AMP: not only is this nucleotide essential, but at high levels it becomes toxic to cells, resulting in lysis or in the accumulation of mutations that inactivate the diadenylate cyclase CdaA or prevent its expression. This finding substantially extends the previous observation that large amounts of c-di-AMP are detrimental to the growth of *B. subtilis*, and it is in agreement with the growth defect of a *gdpP pgpH* double mutant of *L. monocytogenes* (7, 22).

The simultaneous essentiality and toxicity of c-di-AMP suggest that it is implicated in the control of a biological process that depends on homeostasis. Both insufficient and excessive activities of this essential process may impair growth of bacteria. Several lines of evidence suggest that it is the c-di-AMP produced by CdaA that is required for the control of this essential process, and moreover, the evidence indicates that this essential process is the control of cell wall homeostasis. (i) Of the three diadenylate cyclases present in *B. subtilis*, only CdaA is conserved among the firmicutes, and in all bacteria that contain CdaA as the only diadenylate cyclase, this protein is essential (5). (ii) The common property of all firmicutes is the formation of a thick cell wall composed of many layers of peptidoglycan. It is well established that cell wall homeostasis is subject to intricate regulation (46, 47), and c-di-AMP might add another level of complexity to this control. (iii) Indeed, weak accumulation of c-di-AMP can cause resistance to β-lactam antibiotics that target cell wall synthesis in *B. subtilis* and can bypass the requirement for lipoteichoic acid in *S. aureus* (6, 16). (iv) Moreover, the defects observed upon manipulation of the c-di-AMP pool in *B. subtilis* could at least partially be rescued by the presence of elevated magnesium concentrations, which is commonly observed for defects in cell wall homeostasis (6, 7). (v) Both the genomic context and the subcellular localization of CdaA provide further support for an implication of this diadenylate cyclase in cell wall homeostasis. The *cdaA* gene is part of a gene cluster that also comprises the *cdaR* and *glmM* genes (Fig. 1B). This gene cluster is conserved in the firmicutes. Such a conserved clustering of genes is often related to functional and physical interactions of the encoded proteins (48). Indeed, CdaA and CdaR are both membrane proteins, and they interact physically (7; M. Gomelsky, personal communication; the present study). Based on the observation that CdaR is inserted into the membrane, with the major part of the protein being exposed to the extracellular space, we suggest that CdaR and CdaA interact via their transmembrane helices, whereas GlmM may directly interact with the cytosolic part of CdaA (Fig. 1A). (vi) Finally, the results of our suppressor analysis strongly point toward CdaA as the enzyme producing the toxic molecule c-di-AMP, as expression or activity of CdaA was affected in all four independent mutants that were isolated in this study. Taken together, the data indicate that there is an intricate link between CdaA, the essentiality and toxicity of c-di-AMP, and cell wall homeostasis.

The presence of three diadenylate cyclases in *B. subtilis* raises the questions of how these proteins contribute to the cellular pool of c-di-AMP and how they are involved in specific signal transduction processes. The presence of a variety of synthesizing and degrading enzymes is also characteristic of signaling by c-di-GMP. It has been proposed that the different enzymes form temporally and spatially distinct pools of c-di-GMP which can then participate in specific signal transduction processes (2, 49, 50). Since CdaS is expressed exclusively under conditions of sporulation, and since the c-di-AMP formed by this enzyme is implicated in spore germination (20), we can assume that CdaS produces a specific pool of c-di-AMP that is relevant only under very specific conditions. For CdaA and DisA, the data suggest a functional spe-

cialization: while CdaA seems to be involved in the control of cell wall homeostasis, there is increasing support for the idea that DisA contributes to the control of DNA integrity and to DNA repair (5, 13, 15, 51). The localizations of the two enzymes, in the membrane and attached to the DNA (51; the present study), are in excellent agreement with the differential functional assignments. However, expression of any of the three diadenylate cyclases of *B. subtilis* is sufficient to satisfy the requirement for c-di-AMP. As explained above, this can be explained most straightforwardly if we assume that CdaA synthesizes the essential pool of c-di-AMP at the cell membrane and that this particular pool is required for cell wall homeostasis. Moreover, the cell responds to the simultaneous inactivation of both phosphodiesterases that degrade c-di-AMP by the inactivation of CdaA. In contrast, a strain lacking both phosphodiesterases and DisA is poorly viable and acquires mutations that reduce the expression of CdaA. These findings support the idea that the c-di-AMP pool generated by CdaA is critical for the cell.

It is interesting that the inactivation of *cdaA* in strain GP2042 results from a precise deletion of one part of a 9-bp direct repeat. Such deletions are not unprecedented in *B. subtilis*. In the laboratory strain 168, the *gudB* gene, encoding a catabolic glutamate dehydrogenase, is inactive due to a duplication of 9 bp which results in a duplication of three amino acids in the active center (52). However, upon deletion of the second glutamate dehydrogenase-encoding gene, *rocG*, *gudB* becomes rapidly decryptified by the accurate deletion of one part of the direct repeat (30, 53).

So far, the primary signals which result in the accumulation of c-di-AMP have largely remained enigmatic. Here, we show that the nature of the nitrogen source affects the cellular pool of this second messenger. The intracellular concentration of c-di-AMP is low if cells are grown in the presence of glutamine, which is a direct substrate of the first enzyme in the production of cell wall precursors, i.e., GlmS. In contrast, the concentration is significantly increased if the cells are grown with glutamate as the only source of nitrogen. In this context, it is worth noting that CdaA is encoded in an operon with GlmS and GlmM. These two enzymes catalyze the formation of glucosamine-1-phosphate from fructose-6-phosphate and glutamine. This coincidence as well as the interaction of GlmM with CdaA and CdaR suggests a functional link between these proteins. In this study, we excluded the possibility that GlmS and GlmM are the sought-after essential targets of c-di-AMP. Therefore, it seems more likely that they are implicated in the signal transduction to the DAC domain that results in nitrogen source-sensitive control of c-di-AMP synthesis.

The results obtained in this study, as well as the recent identification of the anaplerotic enzyme pyruvate carboxylase (PycA) as a c-di-AMP target in *L. monocytogenes* (18), suggest that c-di-AMP links the control of metabolism to central cellular processes, such as cell wall homeostasis. This conclusion is underlined by the isolation of suppressor mutants of *L. monocytogenes* that are able to grow in the absence of c-di-AMP. The common theme of those mutants is a global regulatory defect in the CodY regulon that encompasses genes subject to nutritional signaling (25).

The work presented in this study shows for the first time that c-di-AMP synthesis is controlled by the quality of the nitrogen source and that this second messenger is not only essential for the growth of *B. subtilis* but also toxic if it accumulates. Future work will focus on the identification of the molecular target(s) that causes both the essentiality and toxicity of this unique second messenger.

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