Correction

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Correction for “c-di-AMP hydrolysis by the phosphodiesterase AtaC promotes differentiation of multicellular bacteria,” by Andreas Latoscha, David Jan Drexler, Mahmoud M. Al-Bassam, Adrian M. Bandera, Volkhard Kaever, Kim C. Findlay, Gregor Witte, and Natalia Tschowri, which was first published March 18, 2020; 10.1073/pnas.1917080117 (Proc. Natl. Acad. Sci. U.S.A. 117, 7392–7400).

The authors note that the Streptococcus pneumoniae gene (sequence ID CVN04004.1) used in the study likely comes from Micrococcus luteus. The error was caused by a misannotation in the misassigned contig embl:FCSG01000028.1. The eight AtaC hits to Mycobacterium tuberculosis presented in the study were found in various contigs but not in complete genomic sequences of M. tuberculosis H37Rv. AtaC homologs are present in M. avium 104, M. smegmatis MC2 155, and other mycobacteria. The authors thank Michael Galperin, NIH/National Library of Medicine/National Center for Biotechnology Information, for bringing this matter to their attention. This misassignment does not change the conclusions of the article describing AtaC as the c-di-AMP degrading phosphodiesterase in Streptomyces and many other actinobacteria.

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

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Antibiotic-producing Streptomyces use the diadenylate cyclase DisA to synthesize the nucleotide second messenger c-di-AMP, but the mechanism for terminating c-di-AMP signaling and the proteins that bind the molecule to effect signal transduction are unknown. Here, we identify the AtaC protein as a c-di-AMP-specific phosphodiesterase that is also conserved in pathogens such as Streptococcus pneumoniae and Mycobacterium tuberculosis. AtaC is monomeric in solution and binds Mn²⁺ to specifically hydrolyze c-di-AMP to AMP via the intermediate S₂′-O-pApA. As an effector of c-di-AMP signaling, we characterize the RCK C domain protein CpeA. c-di-AMP promotes interaction between CpeA and the predicted cation/proton antiporter, CpeB, linking c-di-AMP signaling to ion homeostasis in Actinobacteria. Hydrolysis of c-di-AMP is critical for normal growth and differentiation in Streptomyces, connecting ionic stress to development. Thus, we present the discovery of two components of c-di-AMP signaling in bacteria and show that precise control of this second messenger is essential for ion balance and coordinated development in Streptomyces.

c-di-AMP | Streptomyces | phosphodiesterase | development | osmstress

Bacteria use mono-, di-, and trinucleotides as second messengers to control fundamental physiological functions in response to signal sensing (1). Among these molecules, cyclic di-3′,5′-adenosine monophosphate (c-di-AMP) is the only nucleotide messenger that must be precisely balanced since both its depletion and overproduction can be toxic (2). Its core function is to control cellular integrity by setting homeostasis of osmoles that in many bacteria are used for osmoregulation (3, 4). Changes in external osmolarity trigger water fluxes across the membrane, which can lead to cell dehydration or swelling and, finally, collapse or burst when osmobalance mechanisms fail to respond properly (5). As a key component of these mechanisms, c-di-AMP directly targets transport systems for osmotic and osmoprotective substances such as potassium ions and low-molecular-weight compatible solutes in many bacteria (6–10).

c-di-AMP also plays a central role in host–pathogen interactions and bacterial virulence (11). Secreted c-di-AMP is recognized by host innate immunity receptors STING, DDX41, ERAdP, and RECON to regulate type I interferon expression and NF-κB pathways, respectively (12–16). Modulation of intracellular c-di-AMP has been reported to affect virulence of Streptococcus pyogenes (17), Listeria monocytogenes (18), Streptococcus pneumoniae (19), and Mycobacterium tuberculosis. Thus, the molecule is considered an attractive antimicrobial target (20).

c-di-AMP synthesis out of two ATP molecules is catalyzed by the diadenylate cyclase (DACA) activity of the DisA_N domain (Pfam PF02457), which was identified in the structural and biochemical analysis of the DNA integrity scanning protein A (DisA) of Thermotoga maritima (21). DisA is mainly present in sporulating Firmicutes and Actinobacteria (22) and has a conserved domain organization consisting of an N-terminal DAC domain and a C-terminal DNA-binding helix–helixpin–helix domain separated by a linker region (21). c-di-AMP hydrolysis is mediated by the DHH-DHHA1 domain containing the Asp–His–His motif. The multidomain membrane-associated GdPD protein in Bacillus subtilis was the first characterized DHH-DHHA1-type phosphodiesterase (PDE) (23). In addition, HD domains, which contain a catalytic His–Asp motif and were first identified in the PgPHP protein in L. monocytogenes, also degrade c-di-AMP (18).

Most Actinobacteria contain DisA for c-di-AMP synthesis; however, the majority of them do not encode DHH-DHHA1 domain–containing or HD-type c-di-AMP PDEs. Hence, we wondered how actinomycetes balance intracellular c-di-AMP levels. Within Actinobacteria, Streptomyces are the most extensively studied mycelial organisms and the richest natural source of antibiotics (24). For growth and reproduction, Streptomyces undergo a complex developmental life cycle, which involves the conversion between three morphologically and physiologically distinct forms of cell existence. During exponential growth, they proliferate by extension and branching of vegetative hyphae. The switch to the stationary phase and onset of the reproductive phase are marked by the erection of aerial hyphae. These filaments

**Significance**

Bacteria use the nucleotide cyclic di-3′,5′-adenosine monophosphate (c-di-AMP) for adaptation to changing environments and host–pathogen interactions. Enzymes for nucleotide synthesis and degradation and proteins for binding of the second messenger are key components of signal transduction pathways. It was long unknown how the majority of Actinobacteria, one of the largest bacterial phyla, stop c-di-AMP signals and which proteins bind the molecule to elicit cellular responses. Here, we identify a c-di-AMP phosphodiesterase that bacteria evolved to terminate c-di-AMP signaling and a protein that forms a complex with c-di-AMP in Streptomyces. We also demonstrate that balance of c-di-AMP is critical for developmental transitions from filaments to spores in multicellular bacteria.

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The authors declare no competing interest.

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Data deposition: Small-angle scattering data have been deposited in the Small Angle Scattering Biological Data Bank (SASDB) with accession numbers SASD2H25 (AtaC) and SASD2H35 (CpeA).

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elongate and divide into unigenomic spore compartments that ultimately mature into chains of spores. Completion of the developmental program is easily visible by eye since mature *Streptomyces* spores accumulate a spore pigment. For example, our model species, the chloromphenicol producer *S. venezuelae*, is characterized by a green spore pigment such that colonies turn green at the end of the life cycle (25, 26). Importantly, antibiotic production and morphological differentiation are coregulated in *Streptomyces*. Hence, studying their developmental biology also provides a better understanding of the control of their secondary metabolism.

In this work, we identified and characterized the PDE superfamily protein AtaC as a c-di-AMP–specific hydrolase unrelated to canonical c-di-AMP PDEs. AtaC is broadly distributed in bacteria and the only known c-di-AMP PDE in most actinomycetes. Among others, pathogens such as the causative agent of pneumonia, *S. pneumoniae*, contain an AtaC homolog that we characterize here to be a functional c-di-AMP hydrolase. Our biochemical and structural analyses show that AtaC is a monomeric Mn²⁺-dependent PDE with high affinity for c-di-AMP. Moreover, we provide direct biochemical evidence that *Streptomyces* DisA is an active DAC and that c-di-AMP produced by DisA is crucial for survival under ionic stress conditions. Further, we show that accumulation of c-di-AMP in the *S. venezuelae* ataC mutant results in profound developmental and growth defects and report the identification of the RCK C domain (RCK stands for regulator of conductance of K⁺) containing protein CpeA as a c-di-AMP binding protein in *Streptomyces*. Overall, in this study we identified and functionally characterized core components of c-di-AMP signaling in *Streptomyces* and linked c-di-AMP regulation to ion homeostasis to control differentiation in multicellular bacteria.

**Results**

**DisA Synthesizes c-di-AMP in *S. venezuelae***. DisA is the sole DAC protein encoded in the *S. venezuelae* genome and is conserved in all sequenced *Streptomyces* strains. To demonstrate DisA DAC activity, we purified N-terminally His-tagged DisA and an inactive DisAΔ966A, that carries an alanine instead of aspartate in the active site (21). We included His-tagged *B. subtilis* DisA (DisA650) as a positive control for enzymatic activity (21). [³²P]-labeled ATP was added as a substrate for in vitro DAC assays, and the reactions were separated by thin-layer chromatography (TLC). DisA synthesized c-di-AMP, whereas the mutated DisAΔ966A failed, demonstrating that *S. venezuelae* DisA is a functional DAC, which requires the conserved catalytic aspartate D₈₀ for activity (Fig. L4).

In vivo, DisA is the major source for c-di-AMP during onset of sporulation (14 to 16 h) and the sporulation phase (18 to 20 h) (Fig. 1B) (27). However, we reproducibly detected low c-di-AMP levels in ΔdisA during vegetative growth (10 and 12 h), suggesting that *S. venezuelae* might contain a non-DAC domain enzyme capable of c-di-AMP production (Fig. 1B). The presence of c-di-AMP throughout the wild-type *S. venezuelae* life cycle suggested that disA expression is constitutive. To confirm this, we complemented the disA mutant by chromosomal insertion of a *C. terminally FLAG*-tagged disA under control of its native promoter. Using a monoclonal anti-FLAG antibody, we detected constant DisA-FLAG expression in all developmental stages, which correlated with c-di-AMP production in the wild type under the conditions tested (Fig. 1C and SI Appendix, Fig. S1A).

Altogether, our data show that DisA is a functional DAC in vitro and in vivo and the major enzyme for c-di-AMP production during *S. venezuelae* sporulation.

The Phosphodiesterase Superfamily Protein AtaC (*Vnz_27310*) Degradates c-di-AMP. Streptomyces do not possess PDEs with a DHH-DHHAI domain or a PglH-type HD domain, known to degrade c-di-AMP in other bacteria (18, 23), raising the question of how *S. venezuelae* removes c-di-AMP from the cytoplasm. To find a potentially novel c-di-AMP PDE, we used interproscan (28) to search for Pfam PF01663, which is associated with putative type I phosphodiesterases/nucleotide pyrophosphatases. Among others, we found two proteins (*Vnz_27310* and *Vnz_31010*) belonging to the phosphodiesterase and metallophosphatase superfamilies, respectively, that we selected for in vitro PDE activity tests.

Purified N-terminally His-tagged *Vnz_27310* and *Vnz_31010* were assayed in vitro using [³²P]-labeled c-di-AMP as a substrate. While we could not detect [³²P]-c-di-AMP cleavage activity for *Vnz_31010*, *Vnz_27310* clearly degraded c-di-AMP to 5′-pApA and finally to AMP (Fig. 2A), so that we named *Vnz_27310* AtaC for actinobacterial PDE targeting c-di-AMP. The addition of unlabeled c-di-AMP but not of c-di-GMP or cAMP competed with [³²P]-c-di-AMP and led to reduced cleavage of the radiolabeled substrate, showing specificity for c-di-AMP (Fig. 2A).

We analyzed the kinetics of c-di-AMP hydrolysis activity of AtaC using anion exchange chromatography assays and determined a catalytic rate constant (kₐ) of 0.2 s⁻¹ (SI Appendix, Fig. S2 A and B), while only a negligible c-di-GMP hydrolysis activity was detected (SI Appendix, Fig. S2C). We also compared AtaC-dependent hydrolysis of the linear dinucleotides 5′-pApG and 5′-pApA to the hydrolysis of 5′-pApA and observed a high hydrolysis activity for 5′-pApA (kₐ = 2.1 s⁻¹), whereas other substrates tested were only degraded to a small extent (Fig. 2B and SI Appendix, Fig. S2 D–F).

Using the PATRIC database (https://www.patricbrc.org), we examined the distribution of the here discovered c-di-AMP PDE (PF0_00172869) and found at least 5,374 prokaryotic species containing homologs to AtaC (Dataset S1), including pathogens such as *S. pneumoniae* and *M. tuberculosis*. AtaC from *S. pneumoniae* (AtaCSpn; sequence ID: CVN04004.1) and from *M. tuberculosis* (AtaC_Mtu; sequence ID: CNE38097.1) share 41 and 47%, respectively, identical residues with AtaC from *S. venezuelae*. In agreement with the high degree of protein identity, enzyme assays data shown in Fig. 2C demonstrate that AtaCSpn is a PDE that hydrolyzes c-di-AMP and AtaC_Mtu likely has the same function.

In summary, we identified and functionally characterized a c-di-AMP hydrolase in *Streptomyces* and a c-di-AMP signaling component in pathogens and showed that AtaC is a conserved...
AtaC Is a Monomeric Mn$^{2+}$-Dependent Phosphodiesterase. To further characterize the c-di-AMP hydrolysis mechanism of AtaC and to gain some structural insights into this PDE, we used ITC and confirmed by nanoDSF experiments that showed a shift in the melting curve with increasing ligand concentration (Fig. 3D). Using isothermal titration calorimetry (ITC) analysis, we determined the dissociation constant (K_D) of AtaC_D269N for c-di-AMP to be 949 ± 360 nM, whereas binding of c-di-GMP could not be detected (Fig. 3G and H and SI Appendix, Fig. S4B).

Altogether, our combined structural analysis and biochemical data strongly suggest that AtaC uses a similar metal ion-dependent mechanism as its structural homolog PhnA for substrate cleavage.

AtaC Hydrolyzes c-di-AMP In Vivo. We quantified c-di-AMP in cell extracts isolated from wild-type S. venezuelae and the ataC null mutant using liquid chromatography tandem mass spectrometry (LC-MS/MS). Our data show that c-di-AMP levels are elevated in the ataC mutant during all developmental stages when compared to the wild type, demonstrating that AtaC degrades c-di-AMP in vivo and thus is an important component of c-di-AMP metabolism in S. venezuelae (Fig. 4A). Western blot analysis showed that AtaC levels slightly increase during the life cycle (Fig. 4B and SI Appendix, Fig. S1B).

Inactivation of AtaC Delays S. venezuelae Development. To investigate the physiological functions of disA and ataC and thus of c-di-AMP in S. venezuelae, we first analyzed the developmental phenotypes of mutant strains. Colonies of S. venezuelae ΔdisA and ΔataC
Over, many of the aerial hyphae of the mutant had largely sporulated, with sporadic nondifferentiated development. After extended incubation (7 d), the aerial hyphae of the mutant showed mainly undifferentiated aerial hyphae, in contrast to the wild type (Fig. 5B). The mutants did not turn green like the wild type (Fig. 5B).

Thus, neither the DisA protein nor the c-di-AMP produced by AtaCD269N mutant with morphology identical to those of the wild type (Fig. 5B). A heat resistance assay, we found that neither spores formed by the ataC mutant nor those produced by ΔdisA and ΔdisA ΔataC strains were defective in spore viability (SI Appendix, Fig. S5A).

We could fully complement the defects of ΔataC in development and growth by introduction of the ataC wild-type allele under the control of its native promoter from the pL10770 vector (32) that integrates into the chromosomal attB site (Fig. 5A and C and SI Appendix, Figs. S4 and S5B). In contrast, expression of ataC ΔataC integrate vector, did not restore the developmental defects caused by ataC deletion (Fig. 5A), showing that the cleavage of c-di-AMP by AtaC is crucial for normal development of Streptomyces.

became green (Fig. 5A), and scanning electron microscopy (SEM) confirmed that the disA mutant produced spore chains with morphology identical to those of the wild type (Fig. 5B). Thus, neither the DisA protein nor the c-di-AMP produced by DisA is required for differentiation.

In contrast, the ataC mutant showed a severe delay in development. After 4 d, the ΔataC strain developed aerial hyphae but did not turn green like the wild type (Fig. 5A), and SEM imaging showed mainly undifferentiated aerial hyphae, in contrast to the fully sporulated hyphae seen in the wild type (Fig. 5B). Moreover, many of the aerial hyphae of the ataC mutant had lysed. After extended incubation (7 d), the aerial hyphae of the ataC mutant had largely sporulated, with sporadic nondifferentiated and lysed filaments still detected (Fig. 5B).

The lysed hyphae seen in the SEMs led us to analyze the growth of the ΔataC strain in liquid maltose–yeast extract–malt extract (MYM) medium. As shown in Fig. 5C, the ataC mutant grew slower than the wild type in the exponential phase but reached a similar final optical density at 578 nm after 20 h. Notably, deletion of disA had no effect on growth (Fig. 5C). Using a heat resistance assay, we found that neither spores formed by the ataC mutant nor those produced by ΔdisA and ΔdisA ΔataC strains were defective in spore viability (SI Appendix, Fig. S5A).

We could fully complement the defects of ΔataC in development and growth by introduction of the ataC wild-type allele under the control of its native promoter from the pL10770 vector (32) that integrates into the chromosomal attB site (Fig. 5A and C and SI Appendix, Figs. S4 and S5B). In contrast, expression of ataC ΔataC integrate vector, did not restore the developmental defects caused by ataC deletion (Fig. 5A), showing that the cleavage of c-di-AMP by AtaC is crucial for normal development of Streptomyces.
Altogether, these results demonstrate that elevated levels of c-di-AMP impair growth and development, whereas reduced levels of c-di-AMP do not affect differentiation under standard growth conditions.

The disA Mutant Is More Susceptible to Ionic Stress. Since regulation of osmotic balance is a major function of c-di-AMP in many bacteria (3), we next investigated the osmotic stress resistance of strains with altered c-di-AMP levels due to mutations in either ataC or disA. We spotted serially diluted spores on nutrient agar (NA) medium plates supplemented with 0.5 M NaCl and a control plate without extra added NaCl. On both plates, growth of the ΔataC strain was slightly impaired, resulting in smaller colony size compared to the wild type (Fig. 5D). This phenotype reflects the growth defect of the mutant (Fig. 5C) and could be complemented by expression of the wild-type allele from pIJ10770 (Fig. 5D).

In contrast, when grown on NA plates containing 0.5 M NaCl, ΔdisA and disAΔ86A showed pronounced reduction in growth. Expression of wild-type disA from pIJ10770 fully complemented the growth defect of ΔdisA (Fig. 5D). The identical ΔdisA and disAΔ86A phenotypes demonstrate that c-di-AMP produced by DisA is crucial for osmotic stress resistance in S. venezuelae (Fig. 5D).

In summary, our data revealed that accumulation of c-di-AMP due to ataC inactivation delays development and slows down Streptomyces growth in the exponential phase. On the other hand, depletion of c-di-AMP due to disA inactivation renders S. venezuelae highly susceptible to ionic stress.

The RCK_C Domain Protein CpeA (Vnz_28055) Binds c-di-AMP. RCK_C domains are established direct targets of c-di-AMP that have the (I/L)(I/L)X2DXRX2N(I/L)(I/L) signature for ligand binding (Fig. 6A) (33). We found the RCK_C domain protein CpeA (Vnz_28055) with a putative c-di-AMP binding motif (Fig. 6A) in 93 Streptomyces species for which complete genome sequences are available (34). We purified N-terminally His-tagged CpeA and applied differential radial capillary action of ligand assays (DRAcALA) to probe interaction between CpeA and c-di-AMP. DRAcALA allows visualization of protein-bound radiolabeled ligand as a concentrated spot or ring after the application of the protein-ligand mixture onto nitrocellulose (35). With this assay, we confirmed that CpeA binds [32P]-labeled c-di-AMP (Fig. 6B).

In competition experiments, we found that unlabeled c-di-AMP interfered with binding of [32P]-c-di-AMP to CpeA, while excess of c-di-GMP, cAMP, 5′-pApA, or ATP still allowed [32P]-c-di-AMP-CpeA complex formation (Fig. 6B). NanoDSF analysis revealed that addition of c-di-AMP in the micromolar range significantly increased the melting point of CpeA. In contrast, high concentrations of other nucleotides such as 5′-pApA, ATP, AMP, or cAMP were needed for CpeA stabilization to the same extent, and c-di-GMP even destabilized the protein (Fig. 6C and SI Appendix, Fig. S6). Taken together, these data indicate that CpeA specifically binds c-di-AMP. To determine the Ki, we analyzed c-di-AMP binding through surface plasmon resonance (SPR) assays. Since we faced protein instability problems during CpeA coupling to the chip and using ITC, we applied biotinylated c-di-AMP on chip and CpeA as a ligand in SPR analysis. We determined a Ki of 37 μM, probably reflecting an upper limit for the Ki as the boitin at the 2′-OH of the ribose moiety likely interferes with binding of c-di-AMP to CpeA (SI Appendix, Fig. S7A). SAXS and static light scattering experiments showed that CpeA is a stable dimer in solution and does not dissociate at concentrations down to 260 nM, as observed in analytical size exclusion chromatography (SI Appendix, Fig. S7 B–E), independent of the presence of c-di-AMP. In summary, we could identify CpeA as a c-di-AMP binding protein in the genus Streptomyces.

cpeA forms a conserved operon with cpeB (vnz_28050). Some Streptomyces species, such as S. venezuelae, contain the small open reading frame cpeC (vnz_28045) in the same operon (Fig. 6D). CpeB is a structural homolog of the sodium/proton antiporter NapA (PDB code 5BZ3_A) from Thermus thermophilus (36), as predicted with 100% probability using HHpred (29). To test whether CpeA and CpeB form a functional interacting unit, we used a bacterial two-hybrid system in which an interaction between bait and target proteins reconstitutes a functional adenylate cyclase (Cya) that allows the Escherichia coli cya mutant to utilize maltose as a carbon source (37). We found that CpeA and CpeB form a complex (Fig. 6E). The interaction between these two proteins was strongly enhanced when enzymatically active DisA-FLAG was coexpressed, while coexpression of the inactive DisA ΔP66A-FLAG variant had no effect (Fig. 6E), demonstrating that c-di-AMP produced by DisA stimulates CpeA-CpeB interaction. These data are consistent with our model that c-di-AMP controls ion transport activity of CpeB by stimulating complex formation with the regulatory unit, CpeA. Thus, we renamed Vnz_28055-28045 to CpeABC for cation proton exchange family protein that is monomeric in solution (SI Appendix, Fig. 2).

Discussion

In this work, using the chloramphenicol producer S. venezuelae as a model and a combination of bioinformatic, biochemical, structural, and genetic analyses, we identified AtaC as a c-di-AMP-specific PDE. AtaC is widely distributed in bacteria and represents the only c-di-AMP PDE in the majority of Actinobacteria and a c-di-AMP signaling component in pathogens, such as S. pneumoniae (Fig. 2 and Dataset S1).

AtaC is a soluble, single-domain phosphodiesterase superfamily protein that is monomeric in solution (SI Appendix, Fig.
S. venezuelae wild type and ΔdisA formed spores, but ΔataC consisted predominantly of nonsporulating aerial hyphae (white arrows) and formed flat, likely lysed hyphae (red arrows). After 7 d of growth, ΔataC produced wild-type-like spore chains, but occasional nondifferentiated and lysed hyphae were still detectable. (C) Deletion of ataC leads to a growth defect in S. venezuelae. c-di-AMP mutants were grown in a liquid sporulation medium (MYM) at 30 °C, and optical density was measured at 578 nm. ΔataC growth is delayed by 3 h and can be restored by expression of the wild-type allele under the control of its native promoter from the attBATT site. (D) Osmotic stress resistance of c-di-AMP mutants. Serial dilutions of spores were spotted on NA without additional salt or supplemented with 0.5 M NaCl and grown at 30 °C for ~2 d. ΔdisA and disAΔTolA, (expressing inactive DisA) are hypersensitive to salt stress.

In solution, AtaC is structurally similar to the alkaline phosphatase superfamily domain of the C-P bond-loving enzyme PnhA from S. melliloti 1021 (Fig. 3A) (30). As described for DHH-DHHA1 domain-containing proteins GdpP and DhlpP and the HD domain PDE PppH, AtaC binds Mn2+ to hydrolyze c-di-AMP, and we showed that residue D269 participates in metal ion coordination and is crucial for hydrolysis activity (Fig. 3) (18, 23, 38). AtaC has a kcat of 0.2 s-1, which is comparable to the reported kcat of GdpP (0.55 s−1). Hydrolytically inactive AtaCD269N has a dissociation constant of 0.9 μM, which is highly similar to the Kd of wild-type PppH (0.3 to 0.4 μM) (Fig. 3 G and H) (18, 23). Since we determined the AtaC dissociation constant using a protein carrying the D269N mutation lacking Mn2+ coordination, the Kd value represents an upper limit as the metal ions bound by the wild-type protein likely contribute to protein stability and c-di-AMP binding. However, while PpgH- and GdpP-type PDEs hydrolyze c-di-AMP exclusively to the linear S−pApA, AtaC cleaves c-di-AMP and the intermediate product S−pApA to AMP, which has also been shown for some DhlpP-type PDEs (Fig. 2A and B and SI Appendix, Fig. S2 A, B, and D) (18, 23, 38). The substrate specificity of AtaC is strictly dependent on two adenosine bases as it shows only weak hydrolysis activity for 5′-pApG and 5′-pGpG, in contrast to the DhlpP-type PDE TmpPDE, which does not distinguish between different nucleobases (Fig. 2B and SI Appendix, Fig. S2 E and F) (31).

In Streptomyces, AtaC and the DAC DisA are important regulators of c-di-AMP (Figs. 1B and 4A). On standard growth medium, elevation of intracellular c-di-AMP in ΔataC interferes with growth and ordered hyphae-to-spores transition, while reduction of the second messenger in ΔdisA does not have any noticeable consequence on these cell functions. On the other hand, when incubated at high external NaCl concentrations, ΔdisA is severely inhibited in growth, whereas ΔataC grows similarly to the wild type (Fig. 5), indicating that c-di-AMP stimulates an osmoprotective function. We found that the RCK_C domain protein CpeA binds c-di-AMP and that ligand binding induces interaction between CpeA and CpeB, a structural homolog of the Na+/H+ antiporter NapA from T. thermophilus and a member of the large monovalent cation/proton antiporter (CPA) superfamily (36) (Fig. 6). Na+/H+ antiporters exist in all living cells, where they regulate intracellular pH, sodium levels, and cell volume (39). In some bacteria, Na+/H+ antiporters use the proton motive force to extrude sodium out of the cell and are activated at alkaline pH (40). However, in Staphylococcus aureus, the CPA family transporter CpaA has a cytosolic RCK_C domain that binds c-di-AMP to regulate transport activity (6, 41). Similarly, the regulatory RCK_C domain proteins KtrA and KtrC bind c-di-AMP to control the activity of the corresponding potassium transport units KtrB and KtrD, respectively (33). Thus, in agreement with this general concept and our data, we propose that c-di-AMP sensed by the regulatory RCK_C domain protein CpeA induces CpeA-CpeB complex formation to activate sodium export via CpeB in Streptomyces. At low c-di-AMP, CpeB is presumably inactive, allowing accumulation of toxic Na+ ions in the cell and leading to growth defects of ΔdisA on NaCl-containing medium. However, on the other hand, likely constant activity of CpeB at high c-di-AMP in ΔataC may result in continuous proton influx affecting...
Protein Overexpression and Purification. Cultures were grown in the presence of required antibiotics at 37 °C and induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cells were harvested and lysed using a French press, and the proteins were purified from the cell lysate using Ni-NTA chromatography as described in ref. 31. Reaction solutions contained 50 mM Tris (pH 7.5), 20 mM NaCl, 100 μM MnCl₂, 62.5 to 2,000 μM of 6xHis-AtaC and were incubated at 37 °C for 1 h. The reaction was stopped by separating the reaction products from the protein by ultrafiltration (Centricon, 30 kDa cut-off). The filtrate was diluted to 500 μL with running buffer A (50 mM Tris, pH 9) and loaded on a 1 mL Resource Q anion exchange column (GE Healthcare Life Sciences). A linear gradient to 40% running buffer B (50 mM Tris, 1 M NaCl, pH = 9) over 20 column volumes was used to separate the nucleotides. The product peaks were identified by comparison to nucleotide standards, c-di-NMP, pNpN, N = A or G, obtained from Biolog.

Biochemical Characterization of DisA and AtaC Variants. Biochemical assays using radioactive-labeled substrates were conducted as described in ref. 31. For DAC assays, 5 μM 6xHis-tagged DisA (DisA₅₆₃), DisA_G205D, or DisA_TRACE were incubated with 83 nM [32P]-ATP (Hartmann Analytic) in DisA cyclase buffer. For PDE assays, 100 nM 6xHis-AtaC or 8 μM 6xHis-Vnz_31010 were mixed with 2 nM [32P]-c-di-AMP (Hartmann Analytic, synthesized using purified 6xHis-DisA_TRACE) in PDE buffer. For characterization of biophysical properties of 6xHis-AtaC, proteins were stored at −20 °C. For characterization of biophysical properties of 6xHis-AtaC and 6xHis-AtaC_TRACE, the protein elution was concentrated prior to size exclusion chromatography, flash frozen in liquid nitrogen, and stored at −80 °C.

Differential Radial Capillary Action of Ligand Assay. DRaCALAs were performed using 5 μg of purified 6xHis-CpeA (Vnz_28055) as described in Roelofs et al. (35) with minor modifications. For competition, reactions were incubated with 42 nM [32P]-c-di-AMP for 5 min at room temperature prior to addition of unlabeled c-di-AMP, c-di-GMP, 5'-pApA, or ATP was added to the binding reaction containing 10 nM c-di-AMP and 6xHis-CpeA. (C) Inhibition points from nanoDSF thermal shift assays of 20 μM CpeA with different concentrations of c-di-AMP, ATP, AMP, cAMP, 5'-pApA, and c-di-GMP (0 to 10 mM) at a heating rate of 1.5 K/min. Shown are mean values of $n = 3$ independent experiments with SD. (D) CpeA (Vnz_28055) and cpeB (Vnz_28050) and cpeC (Vnz_28045) form an operon in S. venezuelae. CpeA has an N-terminal domain (NTD) of unknown function and a C-terminal RCK_C domain. CpeB is a predicted structural homolog of the Na⁺/H⁺ antiporter NapA (36). It consists of 13 transmembrane (TM) domains and a cytosolic fraction at the C terminus (CTD). CpeC is a predicted membrane protein with 3 TM domains. (E) Adenylyl cyclase-based two-hybrid assays revealing that CpeA and CpeB interact in vivo and that c-di-AMP production by coexpressed DisA-FLAG stimulates protein–protein interaction. Using pKT25 and pUT18, the T25 and T18 fragments of adenylyl cyclase were attached to the C termini of CpeB and CpeA, respectively. disA-FLAG and disA_TRACE-FLAG were expressed from pUT18-cpeA. The leucine zipper part of the yeast GCN4 protein was used as a positive control. Spotted cotransformants were grown for 24 h at 30 °C.

In summary, in this study we identified AtaC as a component of c-di-AMP metabolism in bacteria and uncovered CpeA as a potential link between c-di-AMP and ion balance in Streptomyces. In nature, these bacteria primarily inhabit the upper layer of the soil, where they often face fluctuating osmotic conditions through desiccation and rainfall. The c-di-AMP pathway described here is likely crucial for adaptation in such a challenging ecosystem.

Materials and Methods

For a full explanation of the experimental protocols, see Extended Experimental Procedures in SI Appendix.

Bacterial Strains and Plasmids. All strains, plasmids, and oligonucleotides used in this study are listed in SI Appendix, Table S1. Plasmids and strains were constructed as described in SI Appendix.

Protein Overexpression and Purification. E. coli BL21 (DE3) pLysS and Rosetta (DE3) were used for protein overexpression. Cultures were grown in the presence of required antibiotics at 37 °C and induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG) in the logarithmic phase and transferred for growth at 16 °C overnight. Strains expressing 6xHis-AtaC, 6xHis-AtaC_TRACE, 6xHis-Vnz_31010, and 6xHis-AtaC_TRACE were supplemented with MnCl₂ (18). Cultures were harvested and lysed using a French press, and the proteins were purified via nickel-nitrilotriacetic acid (Ni-NTA) chromatography. 6xHis-DisA variants and 6xHis-CpeA were dialyzed twice in DisA cyclase buffer (ref. 42 and SI Appendix), and tested PDEs were dialyzed twice in PDE buffer (SI Appendix) with 5 to 10% glycerol (18) at 4 °C. Dialedized proteins were stored at −20 °C. For characterization of biophysical properties of 6xHis-AtaC and 6xHis-AtaC_TRACE, the protein elution was concentrated prior to size exclusion chromatography, flash frozen in liquid nitrogen, and stored at −80 °C.

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of 100 μM of nonlabeled nucleotides. Samples were spotted on nitrocellulose after 10 min reaction at room temperature.

**Western Blotting.** For detection of FLAG-tagged DisA, Western blot analysis was performed as described in ref. 34 using 5 μg total protein of *S. venezuelae* ΔDisA expressing the FLAG-tagged disA allele from the pBAD integration site under the control of the native promoter. Anti-FLAG primary antibody (Sigma) and the anti-mouse IgG-HRP (Thermo Fisher Scientific) were used for detection. AtaC was detected in the wild-type strain (10 μg total protein) using polyclonal rabbit anti-AtaC antisera as the primary antibody (purchased by Pineda GmbH using purified ΔHis-AtaC and donkey anti-rabbit-HRP as the secondary antibody (GE Healthcare). Enhanced chemiluminescent detection reagent (Perkin-Elmer) was used for visualization.

c-di-AMP Extraction and Quantification. The nucleotide extraction protocol from ref. 2 was adapted to Streptomyces. Wild-type, ΔDisA, and ΔAtaC strains were grown in MYM. Samples for c-di-AMP extraction and for determination of the protein concentration were taken every 2 h after initial growth for 10 h. c-di-AMP was extracted using acetonitrile/methanol from cells disrupted with the BeadBlaster (Biozym). Samples were analyzed using LC-MS/MS as described in ref. 2.

**Bacterial Adenylate Cyclase Two-Hybrid (BACTH) Assays.** The BACTH system was used to assay c-di-AMP–dependent protein–protein interaction between CpeA and CpeB to T18 and T25 fragments of *L. monocytogenes* genomes with interproscan (version 5.27-66.0, ref. 28) and searching for proteins harboring type I phosphodiesterase/nucleotide pyrophosphatase domain (Pfam PF01663).

**Scanning Electron Microscopy.** SEM was performed as previously described (48).

**Data Availability Statement.** Small-angle scattering data have been deposited in the Small Angle Scattering Biological Data Bank (SASDBB) with accession numbers SASDH25 (AtaC, https://www.sasdb.org/data/SASDH25) and SASDH35 (CpeA, https://www.sasdb.org/data/SASDH35). All data discussed in the paper will be made available to readers.

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