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Short Review

Recent advances and perspectives in nucleotide second messenger signaling in bacteria

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Abstract

Nucleotide second messengers act as intracellular 'secondary' signals that represent environmental or cellular cues, i.e. the 'primary' signals. As such, they are linking sensory input with regulatory output in all living cells. The amazing physiological versatility, the mechanistic diversity of second messenger synthesis, degradation, and action as well as the high level of integration of second messenger pathways and networks in prokaryotes has only recently become apparent. In these networks, specific second messengers play conserved general roles. Thus, (p)ppGpp coordinates growth and survival in response to nutrient availability and various stresses, while c-di-GMP is *the* nucleotide signaling molecule to orchestrate bacterial adhesion and multicellularity. c-di-AMP links osmotic balance and metabolism and that it does so even in Archaea may suggest a very early evolutionary origin of second messenger signaling. Many of the enzymes that make or break second messengers show complex sensory domain architectures, which allow multisignal integration. The multiplicity of c-di-GMP-related enzymes in many species has led to the discovery that bacterial cells are even able to use the same freely diffusible second messenger in local signaling pathways that can act in parallel without cross-talking. On the other hand, signaling pathways operating with different nucleotides can intersect in elaborate signaling networks. Apart from the small number of common signaling nucleotides that bacteria use for controlling their cellular "business," diverse nucleotides were recently found to play very specific roles in phage defense. Furthermore, these systems represent the phylogenetic ancestors of cyclic nucleotide-activated immune signaling in eukaryotes.

Keywords: biofilm, c-di-AMP, c-di-GMP, cGAMP, ppGpp, Ap_4A, CBASS

Introduction

By acting as ubiquitous intracellular representatives of environmental or cellular cues, nucleotide second messengers connect sensory input to regulatory output in all living cells. Early research with bacteria focused on cyclic (3′,5′)-adenosine phosphate (cAMP) and guanosine-(penta)tetra-phosphate ((p)ppGpp) and their roles in gene expression mostly in the model bacterium *Escherichia coli* (Busby and Ebright 1999, Hauryliuk et al. 2015). In more recent years, however, and in fact following the discovery of bis-(3′,5′)-cyclic di-guanosine-mono-phosphate (c-di-GMP), a striking diversity, mechanistic complexity, unprecedented specificity and manifold physiological roles of bacterial second messengers have been revealed (Bush et al. 2015, Jenal et al. 2017, Römling and Galperin 2017, Commichau et al. 2019, Irving et al. 2020). New signaling nucleotides such as bis-(3′,5′)-cyclic di-adenosine-mono-phosphate (c-di-AMP) and its roles in cell wall homeostasis, osmoregulation, and development in Gram-positive bacteria (Commichau et al. 2019, Latoscha et al. 2019, Stülke and Krüger 2020, Yin et al. 2020) or mixed purine di-nucleotides such as (3′,3′)-cyclic-AMP–GMP (c-AMP–GMP or cGAMP) and their links to virulence and the mammalian innate immune response were found (Yoon and Waters 2021). In parallel, classics such as (p)ppGpp are experiencing a revival as numerous new targets are being discovered in all kinds of bacteria (Irving et al. 2020, Anderson et al. 2021, Bange et al. 2021). Nucleotide second messengers have now been shown to centrally control the most fundamental physiological processes in bacteria including growth rate, metabolic homeostasis, general stress responses, the transition to multicellularity and developmental differentiation, and most recently, mechanisms of phage resistance (Hallez et al. 2017, Jenal et al. 2017, Galperin 2018, Martinez-Gil and Ramos 2018, Latoscha et al. 2019, Hengge 2020, Stülke and Krüger 2020, Athukoralage and White 2022).

In recognition of the dynamics of this field of research, the priority programme *Nucleotide Second Messenger Signaling in Bacteria* was established in 2016 in Germany (SPP1879, funded by the Deutsche Forschungsgemeinschaft; www.spp1879.de). Besides funding research projects (until 2023), this programme has allowed the organization of dedicated international conferences to monitor the newest findings and developments in the field. This review serves both as an introduction to a collection of topical minireviews written by members of this SPP1879 consortium

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as well as a report on the final *DFG-SPP1879 International Symposium on Nucleotide Second Messenger Signaling in Bacteria* organized by Regine Hengge and Mihaela Pruteanu (Humboldt-Universität zu Berlin) in Berlin (Germany) from 22nd to 25th May 2022. Notably, for most participants this was the very first in-person scientific conference after more than 2 years of online conferences due to the Covid-19 pandemic, which gave this meeting a very special flavor. The meeting built upon two previous international symposia of similar format that also took place in Berlin in 2015 and 2019 (Hengge et al. 2016, 2019). This conference series covered the entire range from signaling input via sensory domains as well as the structure and function of enzymes that make and break nucleotide second messengers to the molecular and physiological functions controlled by these signaling molecules in diverse bacteria, both in the environment and in their interactions with hosts.[1]

(p)ppGpp—orchestrating general stress responses at the ribosome and beyond

For more than 50 years, the (p)ppGpp alarmone has been known to play an important general role in slowing down translation, especially when bacterial cells have to orchestrate and adjust their growth to allow an ordered transition into stationary phase. Synthesis and breakdown of this versatile second messenger can be controlled by different signal input, e.g. various nutrient and other stress sensing pathways. This allows to integrate and transmit environmental signals feeding into the control of not only translation, but also of transcriptional regulation of different pathways involved in various metabolic activities, such as amino acid or nucleotide synthesis.

The classical stringent response is induced upon amino acid starvation, i.e. sensed as an increased concentration of uncharged tRNAs that form a complex with the bifunctional Rel protein, which contains (p)ppGpp synthetase, hydrolase Rel, and regulatory domains (RSH). This complex is recruited to stalled ribosomes, where the Rel hydrolase activity is shut off, while its synthetase activity is strongly upregulated. In Gram-negative bacteria, a key target for (p)ppGpp—triggering the stringent response as well as the general stress response—is RNA polymerase, but recent work has been revealing a striking number of additional effectors and target processes in diverse bacteria (Liu et al. 2015, Pausch et al. 2020, Anderson et al. 2021, Bange et al. 2021, Roghanian et al. 2021).

Jade Wang (University of Wisconsin-Madison) demonstrated that (p)ppGpp can directly control the activity of a transcription factor (PurR), which was not observed before in *Bacillus subtilis*. By directly competing with the inducer phosphoribose-pyrophosphate (PRPP) for binding to PurR, the anti-inducer (p)ppGpp allows PurR to repress various genes for purine synthesis upon amino acid starvation, thereby downregulating ATP and GTP synthesis (Fig. 1) (Anderson et al. 2022).

Gert Bange (Philipps-Universität Marburg) reported that (p)ppGpp competes with GTP binding both to the signal recognition particle (SRP) and its receptor FtsY, which prevents the interaction of the two factors. As a result, co- as well as post-translational preprotein membrane targeting and secretion are inhibited (Fig. 2) (Czech et al. 2022). Interestingly, another highly conserved second messenger, diadenosine tetraphosphate (Ap_4A), is synthesized by tRNA synthetases as a side reaction, when the respective amino acids are in short supply. When investigating the cellular role of this alarmone, Gert Bange, Jade Wang, and colleagues observed that in *B. subtilis* Ap_4A interacts with inosine-5′-monophosphate dehydrogenase (IMPDH), a key branching point enzyme for the biosynthesis of adenosine or guanosine nucleotides. By affecting the oligomeric state, and thereby the activity of this enzyme, Ap_4A can control GTP synthesis (Giammarinaro et al. 2022).

In *Caulobacter crescentus* the phosphorylated $EIIA^{Ntr}$ enzyme of the nitrogen-sensing PTS system can interact with Rel (RSH) protein, and thereby inhibit its hydrolase activity upon nitrogen starvation (Ronneau et al. 2016, 2019). Regis Hallez (University of Namur) reported that also under normal growth conditions $EIIA^{Ntr}$ is involved in regulating the basal levels of (p)ppGpp, and that these basal (p)ppGpp levels oscillate during the *C. crescentus* cell cycle. Overall, the control of cellular (p)ppGpp levels by the interaction of regulatory proteins with Rel (RSH) seems a recurring theme, as later in the conference also Jörg Stülke (Georg-August Universität Göttingen) presented the regulatory interaction of the c-di-AMP-binding DarB protein with Rel (RSH) in *B. subtilis* (see below in the c-di-AMP section) (Krüger et al. 2021a, Heidemann et al. 2022).

Gemma Atkinson (Lund University) reported the discovery of widespread new toxin/antitoxin (TA) elements, where the toxin proteins are related to the Rel (RSH) protein family (Jimmy et al. 2020) and the cognate antitoxins contain a Panacea domain (Kurata et al. 2022). These Rel-like toxin elements either act as hyperactive synthetases for (p)ppApp, which leads to ATP depletion (Ahmad et al. 2019, Jimmy et al. 2020), or pyrophosphorylate the CCA end of tRNAs (Kurata et al. 2021), which are both toxic processes for bacterial cells. As further detailed below, Michael Laub (MIT) described how *E. coli* uses such a tRNA-pyrophosphorylating toxin in a phage protection system. In this case, the CapRel antiphage protein is an autoinhibited, fused toxin/antitoxin protein, whose inhibited toxin activity is relieved by the direct and specific interaction of the CapRel C-terminal domain with capsid proteins of certain phages that are synthesized during the infection cycle (Zhang et al. 2022a).

C-di-GMP—from unprecedented intracellular specificity to bacterial multicellularity

Diguanylate cyclases (DGC) are characterized by the GGDEF domain, which synthesize c-di-GMP from two GTP molecules (the GGDEF amino acid motif corresponds to the conserved active site or A-site). Most, but not all DGCs also bind c-di-GMP at a secondary and inhibitory binding site (I-site), thereby controling c-di-GMP levels by direct homeostatic feedback (Schirmer 2016). Degradation of c-di-GMP is mediated by specific phosphodiesterases (PDE), which can feature either EAL or HD-GYP domains, whose names have also been derived from highly conserved and functionally crucial amino acid motifs (Schirmer and Jenal 2009, Galperin and Chou 2022). Often GGDEF and EAL domains occur together in single ‘composite’ proteins, with mostly one of the two domains providing enzymatic activity and the other, usually degenerate domain having a regulatory function. Most bacteria possess multiple DGCs and PDEs, which often play surprisingly specific roles (Jenal et al. 2017, Hengge 2021). Since in recent years knowledge about these molecular processes as well as the diverse physiology of c-di-GMP signaling has exploded, about half of the talks at the conference were dedicated to this ubiquitous signaling nucleotide.

[1]The following report focuses on the 28 oral presentations given during the 2022 conference, with only a few of the 59 poster presentations mentioned. We try to provide relevant literature wherever possible (published until December 2022), but we also apologize to all colleagues whose work with relevance to a specific question or the field in general could not be cited here due to space constraints. Unreferenced statements refer to unpublished results presented at the conference, with their inclusion here being authorized by the respective presenters.

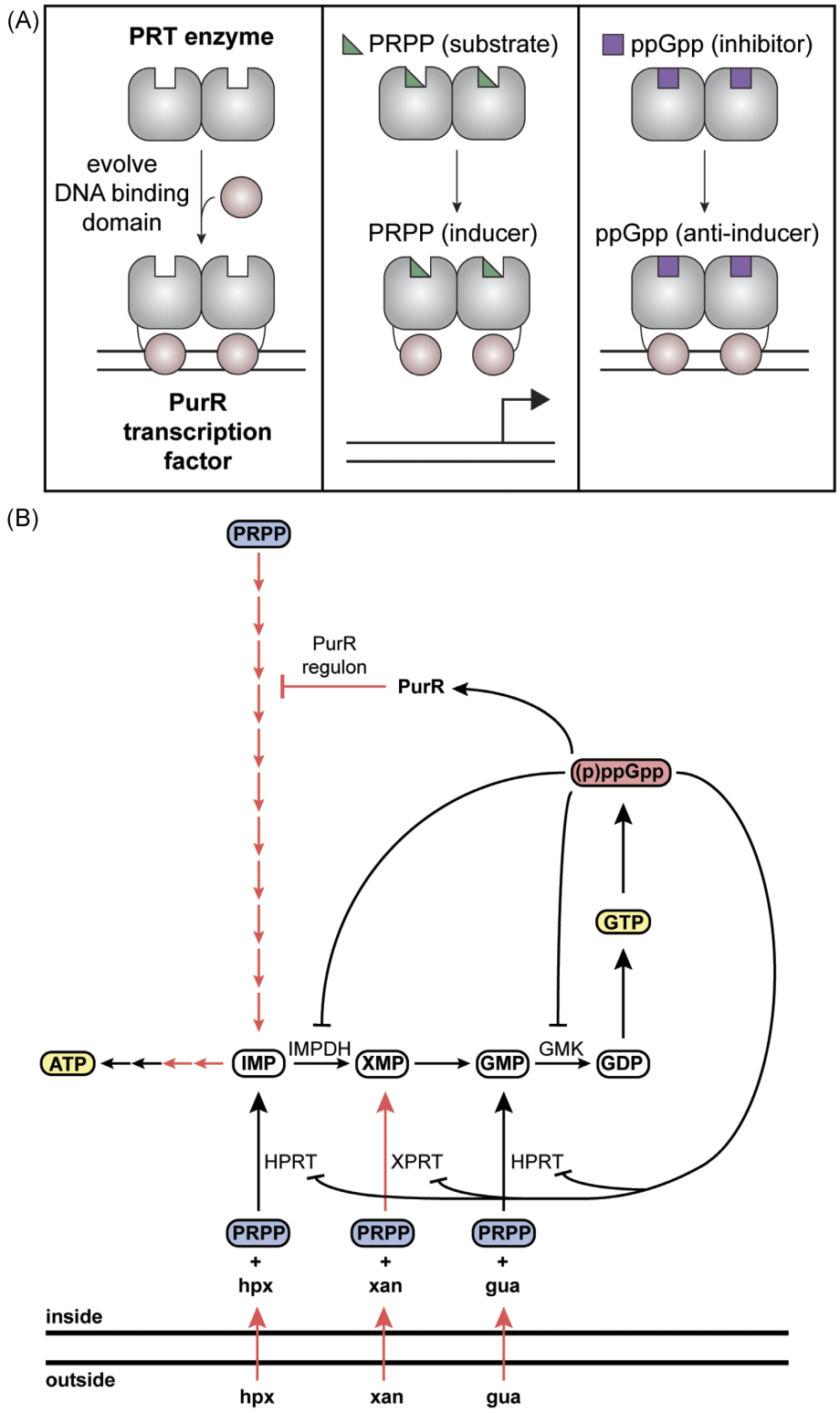

Figure 1. Model of (p)ppGpp regulation of PurR and global (p)ppGpp regulation of purine nucleotide synthesis in *B. subtilis*. (A) Model of evolution of PurR from PRT enzymes, with the active site of the latter becoming an effector binding pocket. (B) GTP and ATP synthesis in *B. subtilis* is regulated at multiple points by (p)ppGpp. This figure was previously published (Anderson et al. 2022) under the CC Attribution License.

Sensory input into c-di-GMP signaling

Most DGCs and PDEs, many of which are membrane-associated, contain diverse N-terminal sensory input domains (Galperin 2004, Hengge et al. 2015) that control their enzymatic activities in response to intra- or extracellular cues such as the binding of small ligands, proteins, or oxygen as well as redox changes or light (Ishitsuka et al. 2008, Barends et al. 2009, Tschowri et al. 2009, Tuckerman et al. 2009, Zähringer et al. 2013, Hufnagel et al. 2014, Wilde and Mullineaux 2017, Herbst et al. 2018, Palardini et al. 2018, Randall et al. 2022). Since a DGC monomer binds only one GTP

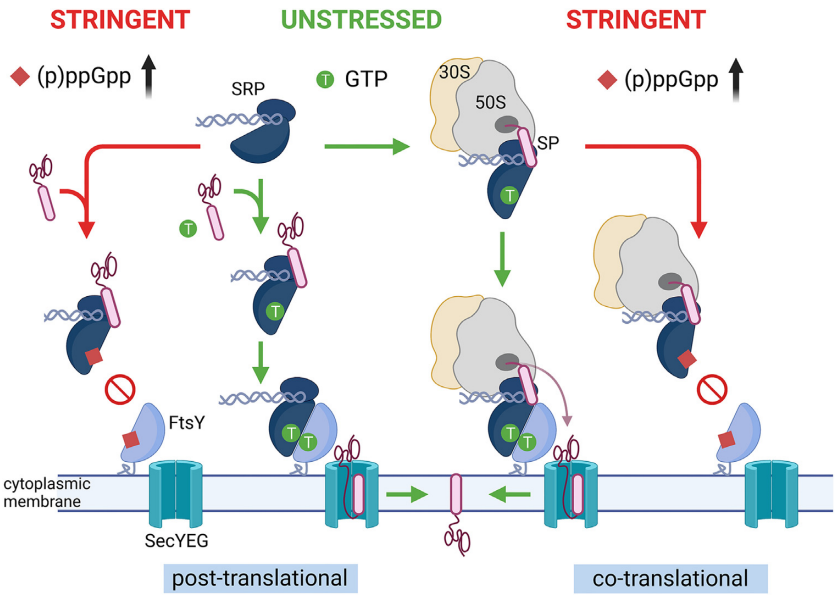

Figure 2. (p)ppGpp interferes with the post- and cotranslational SRP-dependent membrane targeting pathway. In unstressed cells, SRP (Ffh protein in blue and the RNA in gray) can recognize a signal peptide (SP, pink) cotranslationally or post-translationally. Binding of GTP (green) then allows the formation of the SRP–FtsY targeting complex, which leads to GTP hydrolysis and transfer of the protein to the SecYEG translocon (light green). Under stringent conditions, (p)ppGpp (red) binds to SRP and prevents the formation of the SRP–FtsY targeting complex. This figure was previously published (Czech et al. 2022) under the CC Attribution License.

molecule, these enzymes are active in the dimeric or oligomeric state, and thus signal input via their N-terminal domains usually controls quaternary structure (Schirmer 2016).

An intriguing example of complex signal input into a bifunctional DGC/PDE enzyme, DcpG from *Paenibacillus dentritiformis*, was presented by Emily Weinert (Penn State University). Upstream of its GGDEF and EAL domains, this enzyme carries a globin-coupled sensor (GCS) domain, which can bind different ligands at its heme/Fe group. In contrast to several other DGCs that are activated by O_2, the Fe(II)-O_2 state of DcpG shows high PDE activity, whereas NO or CO binding promotes DGC activity, which stimulates biofilm formation (Patterson et al. 2021a). In mechanistic terms, very minor differences in heme conformation with either O_2 or NO bound are translated into rather large conformational changes of DcpG, which result in dimerization of either the EAL domain or of the GGDEF domain, respectively. Based on structural and genetic evidence, single amino acids in close vicinity to the heme could be identified that play crucial roles in this inter-domain signal transduction (Patterson et al. 2021b).

The RmcA protein of *P. aeruginosa* (PA0575) is a multisignal-integrating c-di-GMP-degrading PDE that consists of an N-terminal transmembrane venus-fly-trap (VFT) domain followed by four PAS domains, $GGDEF^{deg}$ and EAL domains (Okegbe et al. 2017). High activity of RmcA was previously shown to result in reduced extracellular matrix production and therefore thicker and nonwrinkled colonies on agar plates. A low NADH/NAD+ ratio—as induced by the presence of secreted and diffusible phenazines, which can deliver electrons to oxygen close to the colony surface—activates RmcA's PDE activity via the PAS domains (Sporer et al. 2017). As reported by Serena Rinaldo (Sapienza University of Rome), this redox signal input is integrated with other RmcA-stimulating signals such as GTP binding to the $GGDEF^{deg}$ domain and arginine sensing via the VFT domain (Palardini et al. 2018). Arginine is a key metabolite at the crossroads of carbon and nitrogen metabolism. It is a precursor in polyamine synthesis, plays an important role in acid tolerance and is also involved in NO production by the host immune system (Brameyer et al. 2022, Scribani Rossi et al. 2022). The rationale behind arginine being an important signal is not yet fully apparent, its effects seem to depend on the species and specific circumstances. Thus, *P. aeruginosa* biofilm formation is inhibited by activation of the PDE RmcA (Palardini et al. 2018). By contrast, as reported by Manuel Espinosa-Urgel (CSIC, Granada), in *Pseudomonas putida* high arginine levels—e.g. in the presence of exogenous arginine or in an *argR* mutant, where arginine synthesis is turned on—stimulate the RpoS-dependent expression of the DGC CfcR and thereby c-di-GMP accumulation and biofilm formation. Moreover, this pathway seems homeostatically feedback-controlled by the c-di-GMP-binding transcription factor FleQ that regulates ArgR expression (Scribani Rossi et al. 2022). Also in *Salmonella* arginine seems to stimulate c-di-GMP production and thus biofilm formation (Mills et al. 2015).

C-di-GMP-binding effectors and their targets

A variety of c-di-GMP-sensing effector components, which can be proteins or riboswitches, that interact with various downstream targets to mount appropriate molecular responses, have been reported (Chou and Galperin 2016). Among these the widespread PilZ protein domain has been an early paradigm. The PilZ domain can occur as a stand-alone protein or as a part of otherwise diverse larger proteins and binds c-di-GMP with K_d's in the (sub)micromolar range (Galperin and Chou 2020). In addition, various types of transcription factors use c-di-GMP as an allosteric ligand (Hickman and Harwood 2008, Krasteva et al. 2010, Baraquet et al. 2012, Tschowri et al. 2014, Chakrabortti et al. 2022). Also, some enzymatically inactive or 'degenerate' GGDEF domains (with still intact I-sites) or EAL domains have evolved into c-di-GMP-sensing effectors (Collins et al. 2020). More recently, the c-di-GMP-binding MshEN domain was identified as a regulatory domain of an ATPase involved in type IV pilus formation (Wang et al. 2016).

The inner membrane-integral cellulose synthase subunit BcsA with its C-terminal PilZ domain, which serves both as glycosyltransferase (GT) and a central part of the cellulose secretion pore, has been the classical example of a c-di-GMP-activated system producing an exopolysaccharide as a component of the extracellular biofilm matrix (Morgan et al. 2014, Serra and Hengge 2019b). More recently, additional components of the *E. coli* cellulose synthase complex have been identified, including BcsE—a second c-di-GMP-binding protein with a REC^{deg}-$GGDEF^{deg}$ domain (previously called a 'GIL' domain)—that has been implicated in the stable assembly of the Bcs complex and in the control of phosphoethanolamine (pEtN) modification of cellulose (Fang et al. 2014, Thongsomboon et al. 2018, Zouhir et al. 2020). Cryo-EM data presented by Petya Krasteva (Université de Bordeaux) showed cellulose synthase as a rather huge oligomeric and asymmetric multicomponent complex of unexpected stoichiometry ($BcsAB_{5-6}R_2Q_2E_2F_2$) that features multisite c-di-GMP binding (at BcsA and BcsE) and even ATP-dependent regulation via the $BcsR_2Q_2$ complex located at the cytoplasmic base of the large Bcs complex (Krasteva et al. 2017, Abidi et al. 2021, 2022).

Yet another type of c-di-GMP-binding effector is RsiG, an anti-sigma factor in *Streptomyces*, which requires c-di-GMP binding in order to form a complex with, and thus sequester the late sporulation sigma factor σ^{WhiG}. Mark Buttner (John Innes Centre, Norwich) showed that two c-di-GMP molecules bind to an antiparallel coiled–coiled structure of RsiG and become partially intercalated into the RsiG-σ^{WhiG} interface (Gallagher et al. 2020). The two helices involved (α1 and α5 in RsiG) both carry the same c-di-GMP-binding motif and also the domain structure suggests that modern RsiG in *Streptomyces* arose by duplication of a single helix in an ancestral protein. This is further supported by the existence of smaller homologs of RsiG in nonsporulating actinobacteria, e.g. *Rubrobacter radiotolerans*, that have only one interaction helix but homodimerize to form a structure, which is similar to RsiG-$(c\text{-}di\text{-}GMP)_2$ of *Streptomyces*. Genetic evidence indicates that the target regulated in *R. radiotolerans* may be type IV pilus expression (Schumacher et al. 2021).

c-di-GMP can also control type III secretion systems (T3SS) directly as exemplified by its binding to the export ATPase of the flagellum of *Pseudomonas fluorescens* (Trampari et al. 2015). Similar c-di-GMP binding to the T3SS ATPase (HrcN) in the plant pathogen *Pseudomonas syringae*, which controls HrcN dodecamerization, has now been reported by Danny Ward (from the group of Jacob Malone, John Innes Centre, Norwich). Strains with point mutations in HrcN that affect c-di-GMP binding are not impaired for growth in plant tissues, but were shown to be avirulent due to compromised effector translocation.

The MshEN domain was identified as an N-terminal c-di-GMP-binding regulatory domain of an ATPase that controls the assembly of type IV MSHA pili in *Vibrio cholerae* (Jones et al. 2015, Floyd et al. 2020). *Escherichia coli* possesses a single MshEN domain protein, NfrB, which—together with the outer membrane protein NfrA—was found to serve as a receptor system for bacteriophage N4 (Kiino et al. 1993). As now reported by Eike Junkermeier (from the group of Regine Hengge, Humboldt Universität zu Berlin), the inner membrane-located NfrB does not only bind c-di-GMP at its C-terminal MshEN domain, but contains a GT domain, which is distantly related to that of glycogen synthase and synthesizes a novel exopolysaccharide, most likely a polymer of N-acetyl-mannosamine. As c-di-GMP binding and GT activity are both required for N4 infection, the primary phage receptor seems this exopolysaccharide rather than the NfrA protein itself. Moreover, specifically DgcJ, which is one of 12 DGCs in *E. coli* K-12, is

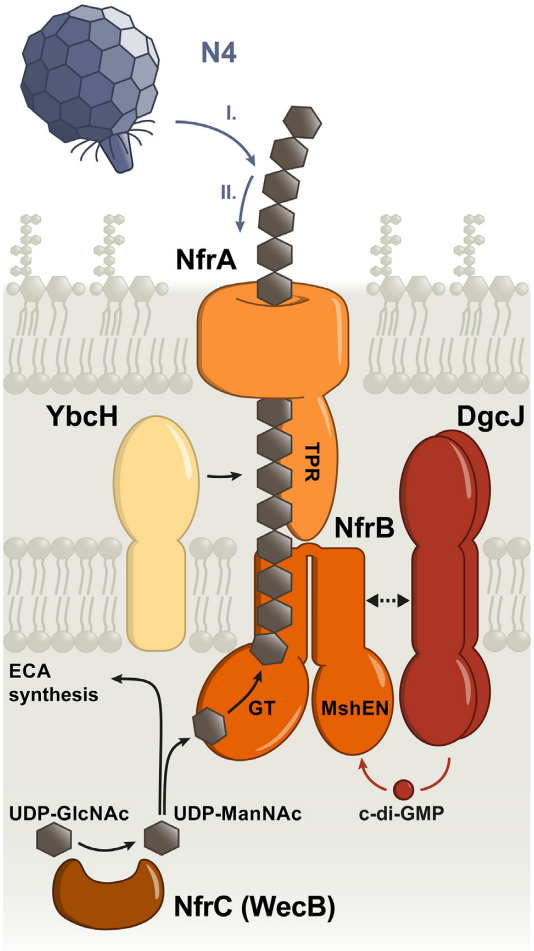

Figure 3. Model of the Nfr/DgcJ system and its role in locally c-di-GMP-activated exopolysaccharide production and bacteriophage N4 adsorption. DgcJ and NfrB colocalize via a direct protein–protein interaction. The C-terminal MshEN domain of NfrB binds c-di-GMP specifically produced by DgcJ, leading to an allosteric activation of the N-terminal GT domain of NfrB. WecB converts UDP-GlcNAc into UDP-ManNAc, which is used for the biosynthesis of the enterobaterial common antigen (ECA). In addition, the GT domain of NfrB uses UDP-ManNAc as a substrate to produce a putative ManNAc-polymer, which is secreted via the outer membrane protein NfrA. YbcH is a periplasmatic protein, which may play an auxiliary role, but is not essential for polysaccharide secretion. Phage N4 binds the exopolysaccharide secreted by the Nfr system as an initial receptor (I.) before interacting with NfrA (II.), which leads to the irriversible adsorption of the phage. This figure was previously published (Junkermeier and Hengge 2021) under the CC Attribution License.

required for phage N4 infection and was shown to directly interact with NfrB (Fig. 3) (Junkermeier and Hengge 2021, Sellner et al. 2021). Just as cellulose synthase (BcsA) is directly activated by the colocalized DgcC (Richter et al. 2020), NfrB activation by direct interaction with DgcJ is thus a novel case of 'local' c-di-GMP signaling, which allows bacterial cells to use the diffusible second messenger c-di-GMP to control several pathways in parallel with high signal input and target output specificity (Fig. 4) (Hengge 2021).

In a search for novel c-di-GMP-sensing effectors in *Myxococcus xanthus*, the small ribbon-helix–helix DNA-binding protein CdbA was found to bind c-di-GMP and DNA in a mutually exclusive manner. CdbA is essential and, as a nucleoid-associated protein, it seems to play a role in chromosome organization and segregation (Skotnicka et al. 2020). Michael Seidel (from Lotte

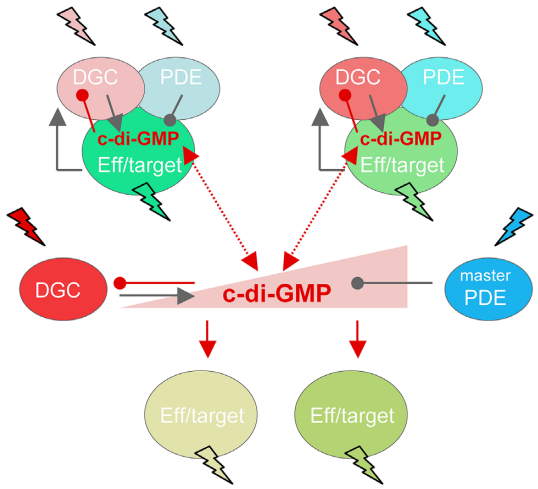

Figure 4. Locally acting c-di-GMP control modules and global control of the cellular c-di-GMP level. If a strongly expressed master PDE keeps the cellular c-di-GMP pool low, local c-di-GMP production can become mandatory to activate specific effector/target systems. However, locally produced c-di-GMP would not significantly contribute to the global pool due to constant drainage of the latter by the master PDE. On the other hand, a strong DGC, which is expressed or activated under some particular conditions, could drive up cellular c-di-GMP to concentrations that can activate some effectors directly (depending on their K_D), thus making local c-di-GMP production at these systems dispensable. Production of c-di-GMP is symbolized by gray arrows, degradation of c-di-GMP by gray lollypop symbols. Red arrows and lollypop symbols stand for activation and inhibition, respectively, by c-di-GMP. Bolts indicate signal input or regulatory output. Eff/target, effector/target component(s). This figure was previously published by R.H. (Hengge 2021) and is used here with permission.

Søgaard-Andersen's group, Max Planck Institute for Terrestric Microbiology, Marburg) reported that knocking out CdbZ, a PilZ domain protein that also binds c-di-GMP, suppresses the lethality of the *cdbA* mutation. CdbZ is induced in the *cdbA* mutant and its artificial overexpression phenocopies the *cdbA* mutation, which in turn could be suppressed by knocking out two other PilZ domain-containing and DnaK-like proteins. This suggests a link between c-di-GMP, chaperone-involving stress responses and chromosome segregation that warrants further study.

Finally, cyclic dinucleotide binding to riboswitches present in the 5′-untranslated regions of certain mRNAs can control transcriptional elongation or translation of the mRNA and thereby control the expression of downstream-encoded genes (Sudarsan et al. 2008, Smith et al. 2009, Nelson et al. 2013, Kellenberger et al. 2015). In *Geobacter* species, the Hypr subclass of GGDEF enzymes are promiscuous for both ATP and GTP as substrates and are capable of producing three cyclic dinucleotides, i.e. c-di-GMP and c-di-AMP as well as the hybrid second messenger c-AMP-GMP (3',3'-cGAMP) (Hallberg et al. 2016). The corresponding riboswitches regulate genes associated with extracellular electron transfer to insoluble metal oxides during bacterial colonization of surfaces (Kellenberger et al. 2015, Nelson et al. 2015). Based on rapid preparation of several transcriptional intermediates studied by ITC analysis, NMR analysis and computational modeling, Harald Schwalbe (Goethe Universität, Frankfurt am Main) reported on the dynamics of c-GAMP control of the *pilM* riboswitch of Geobacter metallireducens, which functions as an ON-switch to control transcriptional elongation of downstream genes, i.e. binding of c-GAMP prevents the formation of a terminator conformation in the *pilM* riboswitch. The presented results highlighted key transcriptional intermediates within a short kinetic reaction window of 25 nucleotides associated with changes in binding affinity, binding capability, and the ability to terminate transcription. To gain insights into the transcriptional folding pathways of riboswitches, the experimental results were incorporated into a Markov simulation quantifying regulation as a function of cotranscriptional folding, ligand affinity taking into account *in vivo* concentrations, kinetics of ligand binding and the switching efficiency depending on transcription and refolding speed. Their overall model, which also included similar findings for the homologous c-di-GMP-responsive OFF-switch Cd1 from *Clostridium difficile*, shows the ability to predict riboswitch-based gene regulation and its dependence on transcription speed, pausing and nucleotide ligand concentration (Landgraf et al. 2022).

C-di-GMP heterogeneity and phenotypic diversification in bacterial populations

Recent advances in single-cell analyses using c-di-GMP-specific biosensors have revealed that heterogeneity of c-di-GMP levels within a population is common in bacteria even if exposed to an isotropic environment (Weiss et al. 2019, Kaczmarczyk et al. 2023). This variation drives phenotypic diversification, which can be beneficial at the population levels by enabling a single genotype to survive drastic changes in the environment (bet hedging) or by allowing the division of labor. However, the mechanisms and cues that lead to c-di-GMP heterogeneity can be diverse even in single bacterial species. In *P. aeruginosa*, surface sensing by the Wsp system during an early stage of biofilm formation leads to high levels of c-di-GMP due to activation of the DGC WspR in a fraction of cells, which become the founders of a biofilm. On the other hand, a subpopulation with lower c-di-GMP displays surface motility to explore the surface (Armbruster et al. 2019). In planktonic cells, *P. aeruginosa* achieves c-di-GMP heterogeneity through asymmetrical partitioning of c-di-GMP-metabolizing enzymes and effectors. Thus, the PDE Pch becomes recruited to and activated at the flagellated cell pole via phosphorylation by the histidine kinase component of the chemotaxis machinery, CheA, which ensures manifestation of a motile and chemotactic subpopulation (Kulasekara et al. 2013). Surface sensing by planktonic *P. aeruginosa* can also trigger asymmetric division generating a surface-attached subpopulation, which is characterized by stimulated pili assembly through the c-di-GMP receptor FimW that acts at the cell poles (Laventie et al. 2019).

Vanessa Kreiling (from the group of Kai Thormann, Justus-Liebig-Universität Gießen) reported that *Shewanella putrefaciens* makes use of a similar mechanism of monopolar localization of a c-di-GMP signaling component to achieve c-di-GMP heterogeneity (Fig. 5). The lowly expressed composite $GGDEF^{deg}$-EAL domain protein PdeB is recruited to the flagellated cell pole by the polar landmark protein HubP. Full activation of its PDE activity depends on interaction of the enzymatically inactive, but still GTP-binding $GGDEF^{deg}$ domain of PdeB with the FimV domain of HubP. Upon cell division, this asymmetric localization of active PdeB results in only the flagellated daughter cell having a low c-di-GMP level and being motile, whereas the other daughter cell remains surface-attached due to its high c-di-GMP inducing the Bpf surface adhesion system and MshA pili formation (Rick et al. 2022).

In the opportunistic nosocomial pathogen *C. difficile*, phase variation drives c-di-GMP and phenotypic heterogeneity, as presented by Rita Tamayo (University of North Carolina). Here, the '*pdcB* switch' sequence in the promoter region of the *pdcB* gene, encoding an active PDE, undergoes inversion through site-specific

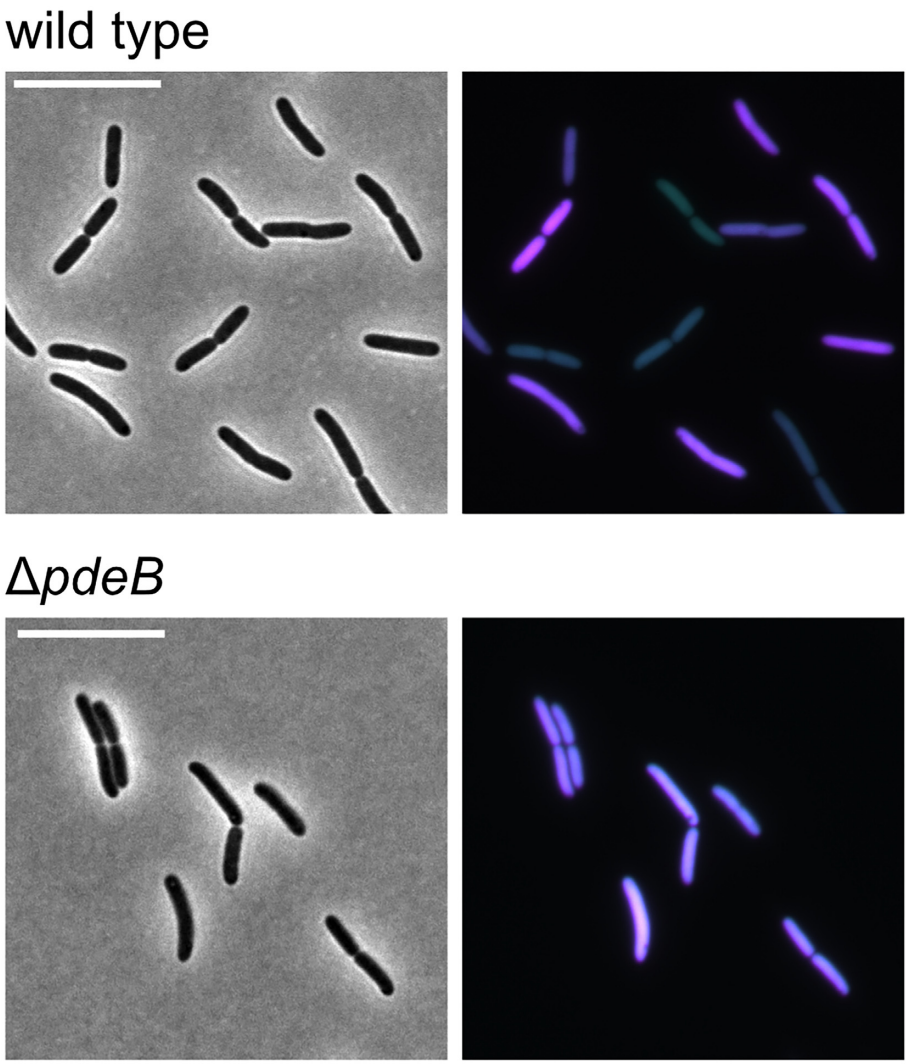

Figure 5. The PDE PdeB is crucial to generate heterogeneity in *Shewanella* populations. Variation in protein copy number, late appearance at the cell pole and the following activation by interaction with HubP lead to high degree of variability in c-di-GMP levels. A plasmid-based reporter system (Zhou et al. 2016) was introduced in *Shewanella* for visualization of c-di-GMP content *in vivo*. The wild type (upper panel) shows much greater heterogeneity in c-di-GMP levels than a mutant lacking PdeB (lower panel). The scale bar equals 10 μm. This figure was kindly provided by Kai Thormann.

recombination. The orientation of the switch determines expression of *pdcB* and is highly heterogeneous within populations. The analysis of mutants with locked orientation of *pdcB* showed that strains in the ON state had reduced c-di-GMP levels when compared to those in the OFF state, in line with increased and decreased PdcB expression and activity, respectively. As a consequence, this phase variation-driven heterogeneity of c-di-GMP within a population leads to phenotypic diversification with respect to biofilm formation, surface motility, type 4 pili and adhesin expression (at high c-di-GMP levels) or swimming, toxin production, and sporulation (at low c-di-GMP levels) (Reyes Ruiz et al. 2022).

C-di-GMP control of bacterial multicellularity: biofilm formation, cell differentiation, and sporulation

Bacterial biofilms are multicellular aggregates held together and protected by a self-produced elastic extracellular matrix (ECM). This matrix is composed of distinct exopolysaccharides, diverse proteins some of which may form very stable amyloid fibers and/or exo-DNA. These aggregates often form on external surfaces, but they can also occur as free-floating flocs of aggregated cells in various environments. Although flagella often play a not well-characterized role in very early biofilm formation, a decisive first step to a multicellular community is the adhesion of 'founder' cells to a surface. Very often this adhesion and swimming motility are inversely coordinated by c-di-GMP, which therefore, has been seen as key regulator of motile-to-sessile 'lifestyle' transitions (Römling et al. 2013). Notably, using the undefined term of bacterial 'life-style' has contributed to uncritically equating 'motility vs. sessility' (as properties of single cells) with 'planktonic vs. biofilm' (although the term 'biofilm' implies multicellularity), which has caused quite some conceptual confusion (Hengge 2020).

While adhered cells multiply to form microcolonies, they may in some cases give rise to a subpopulation of motile daughter cells taking off again (see above), but daughter cells that remain in the aggregate will eventually start to produce extracellular matrix components. Just as initial adhesion, this matrix synthesis is usually under elaborate control of c-di-GMP signaling pathways. At this stage, these pathways respond to spatiotemporal cues that

reflect the structured anisotropic extracellular environment that builds up inside a growing biofilm (Klauck et al. 2018). In particular, these cues relate to metabolic gradients of primary nutrients, secreted metabolic products (which can become secondary nutrients), oxygen, or extracellular signaling molecules (e.g. quorum sensing signals) (Stewart and Franklin 2008, Serra et al. 2015, Sporer et al. 2017). A consequence of the resulting spatially different microconditions is a high physiological heterogeneity and division of labor among cells inside a biofilm, which may be further enhanced by endogenous causes such as bistable switches in regulatory pathways (Serra and Hengge 2019b). One of the most readily detectable results of this spatiotemporal control are specific large-scale matrix architectures within mature biofilms, which arise from the spatial distribution of matrix-producing and nonmatrix-producing cells. These matrix architectures are functionally important for allowing the emergence of macroscopic form based on tissue-like folding and wrinkling without breakage (Serra and Hengge 2021). In sporulating bacteria, spatially controlled physiological heterogeneity in biofilms can culminate in spore formation in specific macroscopic structures emerging from these multicellular aggregates such as fruiting bodies or aerial hyphae (Aguilar et al. 2007, Bush et al. 2015, Bretl and Kirby 2016, Skotnicka et al. 2016).

The early stages of cellular adherence, microcolony formation, beginning matrix production, and the crucial regulatory role of c-di-GMP in these processes have been quite well-studied in *V. cholerae* (Conner et al. 2017, Teschler et al. 2022). In her talk, Fitnat Yildiz (University of California, Santa Cruz) focused on the very early motility vs. adhesion decision in this bacterium. Here, 'roaming' and 'orbiting' cells can be observed to move on a surface, with the latter being committed to adhesion and thus seeding microcolonies. This surface behavior depends on the c-di-GMP-controlled assembly of type IV MshA pili (Jones et al. 2015). Moreover, the lack of the flagellar filament also generates a signal, which is transduced by functional flagellar stators leading to elevated c-di-GMP levels. When the flagellum fails to assemble, three specific diaguanylate cyclases are responsible for an increase of the second messenger to activate biofilm gene expression (Wu et al. 2020), which can occur via c-di-GMP binding to the biofilm-controlling VpsR–VpsT system (Krasteva et al. 2010, Zamorano Sánchez et al. 2015, Chakrabortti et al. 2022, Hsieh et al. 2022) and the c-di-GMP-mediated inhibition of the master regulator of flagella production, FlrA (Srivastava et al. 2013). In addition, the role of *V. cholerae* PilZ domain proteins in c-di-GMP signaling has been dissected by the Yildiz group. Both PlzC and PlzD were shown to bind c-di-GMP and to control motility. Altogether, the link between proper assembly of the flagellum and limitation of c-di-GMP accumulation seems to give motility-associated functions priority over surface commitment and biofilm formation.

In her talk, Regine Hengge (Humboldt Universität zu Berlin) reported on the formation of the structurally complex extracellular matrix architecture in mature macrocolony biofilms of *E. coli* (Hengge 2020). Remarkably, this 3D matrix architecture, which consists of amyloid curli fibers and/or pEtN-cellulose in locally different ratios and patterns, is two orders of magnitude larger than the millions of cells that reproducibly generate it when they enter into stationary phase in these very crowded and therefore nutrient-limited multicellular aggregates (Serra and Hengge 2014, Klauck et al. 2018, Thongsomboon et al. 2018). Functionally, this matrix architecture is not only protective, but also a prerequisite for the emergence of stable macroscopic form, i.e. the tissue-like behavior of folding and wrinkling of macrocolony biofilms (Serra et al. 2015, Serra and Hengge 2019). Underlying its formation is a highly integrated regulatory network that relies on several sigma factors of RNA polymerase and a small number of transcriptional hubs as central players (Fig. 6). By conveying information about the actual microenvironments, nucleotide second messengers—cAMP, (p)ppGpp and in particular c-di-GMP—are the key triggers and drivers that promote either growth or stress resistance and structured multicellularity within a self-organized environment of long-range metabolic gradients (Hengge 2020, Serra and Hengge 2021). While explaining the formation of the large-scale spatial matrix architecture, this regulatory network also features paradigmatic examples of a novel signal-input pathway that integrates GTP, (p)ppGpp and c-di-GMP (Pfiffer et al. 2019), bistable switches (Yousef et al. 2015, Serra and Hengge 2019a), a c-di-GMP-degrading PDE, which acts as a c-di-GMP-sensing trigger enzyme (Hengge 2016) and local c-di-GMP signaling within multiprotein complexes (Sarenko et al. 2017, Richter et al. 2020, Hengge 2021).

In addition to its central functions as a regulator of the initial 'swim-or-stick switch' and subsequent extracellular matrix synthesis to organize the 3D biofilm space, c-di-GMP plays a fundamental role in orchestrating bacterial differentiation and spore development. In *M. xanthus*, type IV pili-dependent motility and the starvation-induced developmental program, which results in the formation of spore-filled fruiting bodies, are regulated by c-di-GMP. The DGC DmxB and the HD-GYP-type PDE PmxA are important for development and represent direct targets of the CRP-like transcription factor MrpC (Kuzmich et al. 2022). During development, DmxB is responsible for an increase in c-di-GMP that binds to the transcriptional regulator EpsI/Nla24 to activate EPS synthesis, which is essential for fruiting body formation and sporulation (Skotnicka et al. 2016). In addition, c-di-GMP binding by the two PilZ domains in PixB were shown to be involved in the formation of fruiting bodies and spores (Kuzmich et al. 2021).

The mechanisms behind the role of c-di-GMP in controlling cell differentiation are particularly well-understood in the sporulating Gram-positive model species *Streptomyces*. These are nonmotile, filamentous antibiotic producers that are characterized by a sophisticated life cycle involving two central modes of cell differentiation: the transition from vegetative to aerial hyphae and the switch from aerial hyphae to spores (Latoscha et al. 2019). High c-di-GMP levels block differentiation at both transition stages, yet by completely different mechanisms that were revealed through structural and genetic analyses. To maintain vegetative growth, a c-di-GMP tetramer binds to and activates the developmental regulator BldD by inducing protein dimerization, which is essential for BldD activity as a repressor of developmental genes (Tschowri et al. 2014). To avoid precocious transition from aerial hyphae to spores, c-di-GMP stabilizes the complex between the developmental sigma factor σ^{WhiG} and the antisigma RsiG, thus making σ^{WhiG} inaccessible to RNA polymerase for the transcription of sporulation genes (as reported by Mark Buttner and already described above) (Gallagher et al. 2020). Furthermore, two PDEs (RmdA and RmdB) and two DGCs (CdgB and CdgC) are key c-di-GMP enzymes in *Streptomyces venezuelae* developmental control by affecting FtsZ-driven cell division and the production of the hydrophobic cellular layer, i.e. needed for the formation of aerial hyphae and spores (Haist et al. 2020).

In addition, c-di-GMP influences *Streptomyces* cell differentiation also via an indirect route by affecting energy storage metabolism. Natalia Tschowri (Leibniz Universität Hannover) presented the discovery of her team that the activity of the glycogen-debranching enzyme, GlgX, is activated by c-di-GMP in *S. venezuelae*. Structural analysis of the GlgX–c-di-GMP complex in the

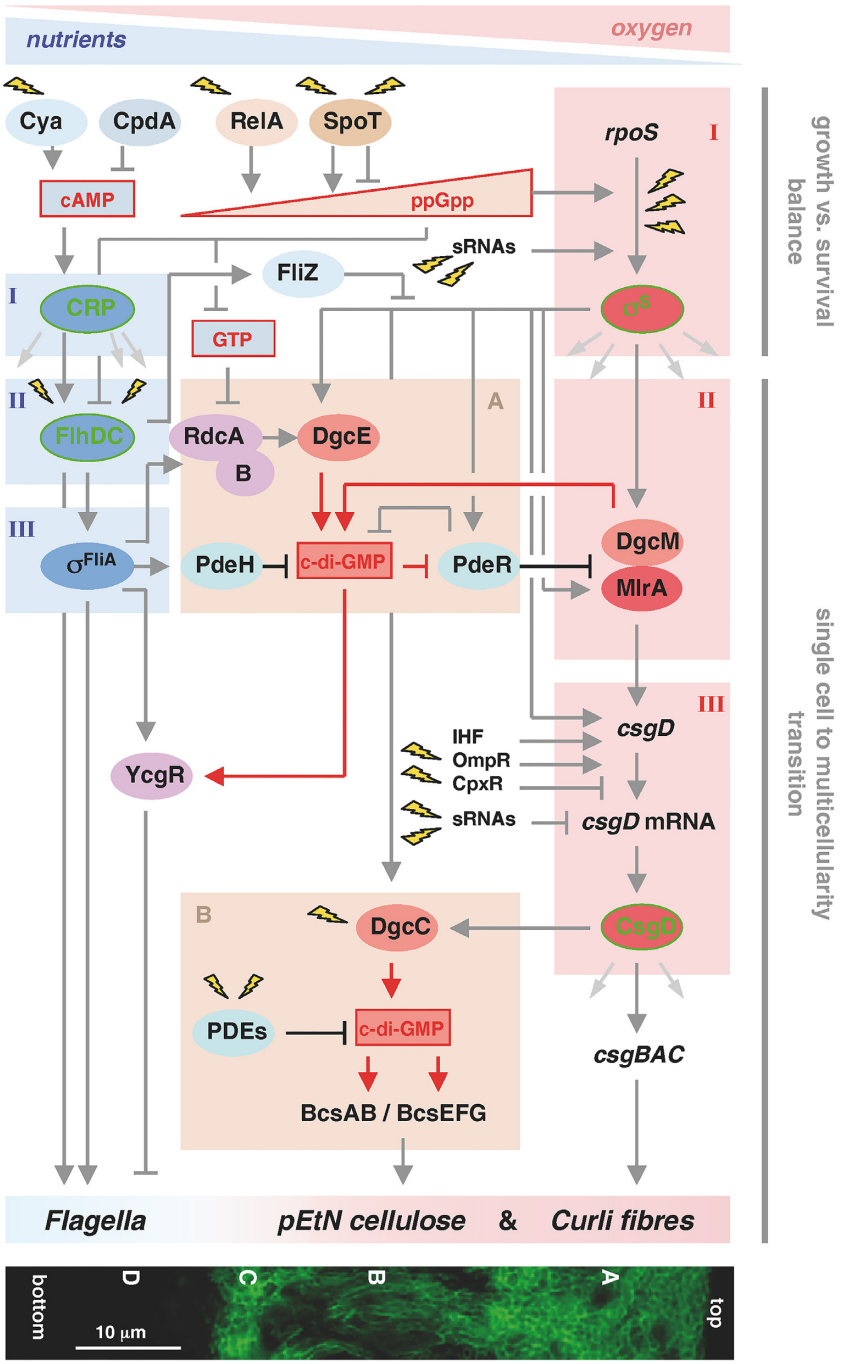

Figure 6. Regulatory network controlling the growth-vs.-survival balance and the integrated transition to adhesive multicellularity of *E. coli*. Signaling and regulatory proteins are shown by ovoid symbols (blue color indicates components that support growth and flagella-based activities, red color represents factors that drive maintenance, resilience, and multicellularity, and purple color designates components deployed by the flagellar control cascade, which, however, counteract flagellar activity and promote multicellularity). Transcriptional hubs are highlighted in light green. At the bottom, a vertical cryosection through a macrocolony biofilm of *E. coli* is shown that was rotated clockwise by 90° to align it roughly with the spatial distribution of the activities of the relevant network components. Extracellular matrix components, i.e. pEtN-cellulose and amyloid curli fibers, which are major targets of the control network, were stained with the fluorescent dye Thioflavin-S in order to visualize the morphologically different zones of the matrix architecture. At the top, the direction of nutrient and oxygen gradients in the biofilm is indicated. This figure was previously published by R.H. (Hengge 2020) and is used here with permission.

group of Maria Schumacher (Duke University School of Medicine) revealed that binding of the ligand to GlgX stabilizes the protein dimer and induces long-range conformational changes leading to a rearrangement of the active site and activation of the enzyme. α-Glucan granules can be observed in prespore chains, but seem to have disappeared in mature single spores (Fig. 7). A drop of glycogen levels due to overexpression of GlgX compromises sporulation (Schumacher et al. 2022).

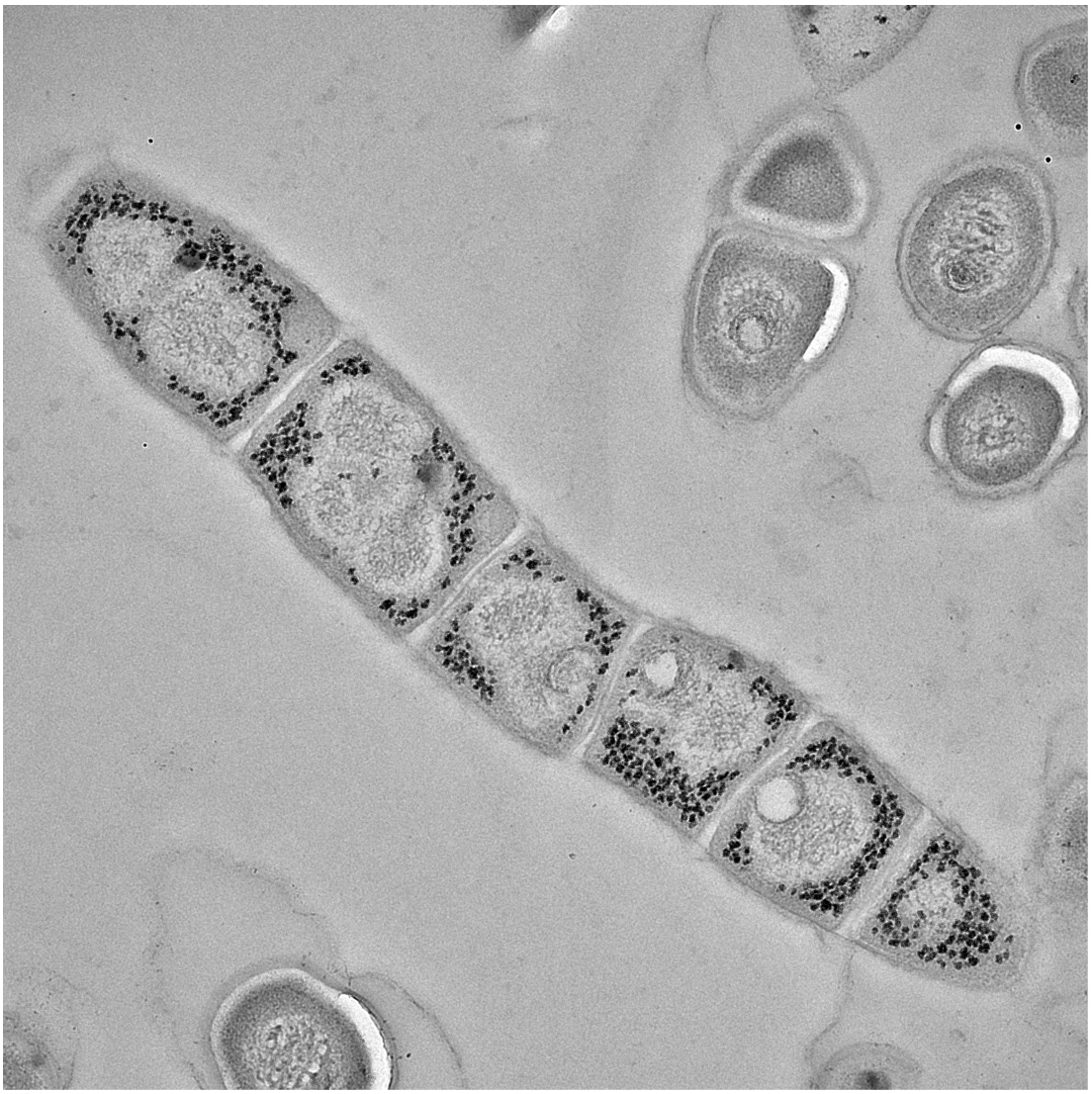

Figure 7. Glycogen granules in prespore chains of *S. venezuelae*. Transmission electron micrographs of wild type *S. venezuelae* stained for α-glucans. Colonies were grown for 5 days on maltose/yeast-extract/malt-extract (MYM) agar at 30°C. This figure was kindly provided by Natalia Tschowri.

Getting rid of the signal: enzymes involved in c-di-GMP degradation

c-di-GMP-degrading PDEs, which are key for the termination of c-di-GMP-mediated signaling cascades in bacteria, exist as two structurally unrelated enzyme families characterized by either EAL or HD-GYP domains. EAL-type PDEs hydrolyze c-di-GMP in the presence of Mg^{2+} or Mn^{2+} to the linear 5′-phosphoguanylyl-(3′-5′)-guanosine (pGpG) dinucleotide (Schirmer 2016, Jenal et al. 2017). HD-GYP domain enzymes are members of the HD superfamily with the ability to hydrolyze c-di-GMP not only to pGpG, but also further to two molecules of GMP and typically require two ferrous ions or manganese (Bellini et al. 2014). HD-GYP domains are almost as widespread as EAL domains but remain far less characterized (Al-Bassam et al. 2018, Sun and Pandelia 2020, Galperin and Chou 2022). As discussed by Michael Galperin (NCBI—NIH, Bethesda), a main reason for this was the absence of a well-defined sequence model of the HD-GYP domain in the majority of public domain databases such as Pfam or SMART, which has now been addressed by the work of Galperin and Chou (2022). Just as EAL domains, also HD-GYP domains occur in association with a variety of signaling or sensory domains (see above) or are occasionally linked to a GGDEF domain, but most commonly are stand-alone HD-GYP domain proteins.

Since the discovery of the 3′-to-5′exonuclease oligoribonuclease Orn as an important enzyme for the hydrolysis of pGpG in *P. aeruginosa*, much progress has been made in our understanding of the components needed for the removal of this linear intermediate in c-di-GMP degradation (Lee et al. 2022). Hydrolysis of pGpG is crucial to maintain c-di-GMP homeostasis since its accumulation inhibits EAL domain-mediated c-di-GMP linearization by a product feedback mechanism (Cohen et al. 2015, Orr et al. 2015). Homologs of *orn* are found in beta-proteobacteria, gamma-proteobacteria, delta-proteobacteria, and actinobacteria, while firmicutes and alpha-proteobacteria possess exoribonucleases NrnA, NrnB, and NrnC, which are functionally analogous to Orn (Orr et al. 2018). Holger Sondermann (Centre for Structural Systems Biology, Deutsches Elektronen-Synchrotron DESY) presented a structural view on the dinucleases Orn and NrnC (Fig. 8), which share a strong preference for dinucleotides, which is determined by unifying structural properties like the $DEDD^{h/y}$ active site, the P-cap, and the L-wedge. Both types of enzymes link RNA degradation and clearance of cyclic di-nucleotide

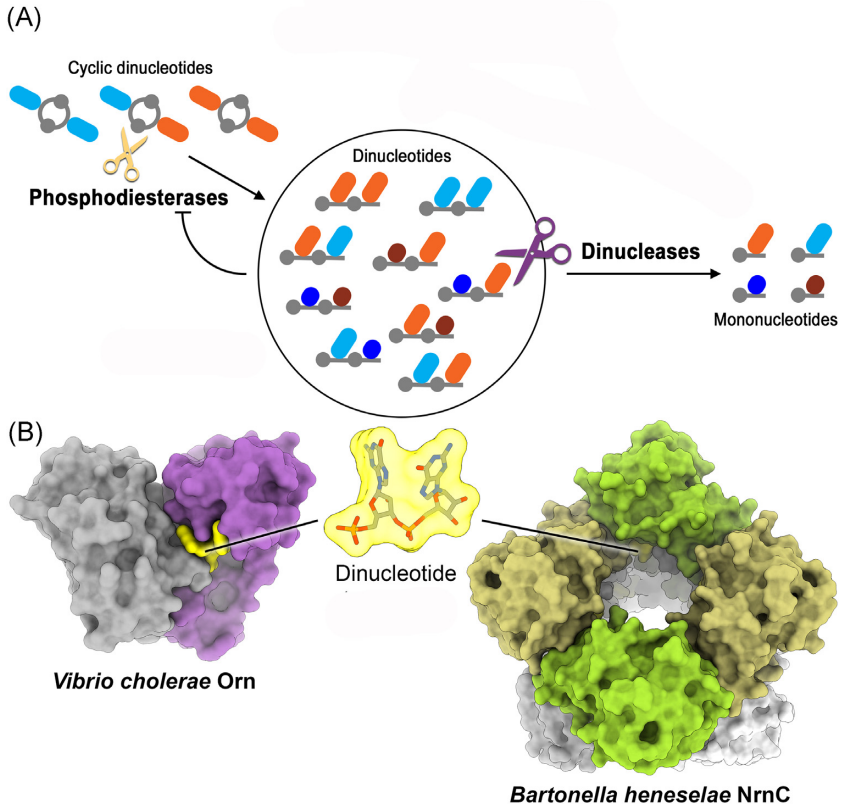

Figure 8. Structure and function of dinucleases. (A) PDE with EAL or HD-GYP domains hydrolyze cyclic dinucleotides like c-di-GMP to linear molecules. Dinucleases of the Orn- and NrnC-type degrade these linear dinucleotides to mononucleotides. (B) Structure of substrate-bound Orn from *V. cholera* (left) and nano-RNAse C (NrnC) of *Bartonella henselae* (right). This figure was kindly provided by Holger Sondermann.

intermediates as they evolved independently from two different classes of RNases, i.e. RNaseD and RNaseT, respectively, and are essential in many species (Kim et al. 2019, Lormand et al. 2021).

Cyclic di-AMP: from osmotic regulation to global control of cellular activities

In the course of the structural analysis of the DNA integrity-scanning protein DisA from *B. subtilis*, a novel second messenger, c-di-AMP, was discovered (Witte et al. 2008). Subsequently, this nucleotide was implicated in resistance against cell wall-targeting antibiotics as well as in the human immune response to pathogens, and has thus attracted much attention. Given the fact that numerous studies have revealed c-di-AMP as a key signaling molecule linking osmotic balance with metabolism, it is still not clear, how this connects to its apparent role in DNA repair, where it was first discovered. c-di-AMP is also outstanding among signaling nucleotides for being essential for many of the bacteria that produce it. On the other hand, also excessive accumulation of c-di-AMP inhibits growth. Just like c-di-GMP, c-di-AMP regulates cellular processes by binding to very different target molecules, i.e. to various types of proteins and in some cases both to a mRNA as well as to the encoded target protein (Corrigan and Gründling 2013, He et al. 2020, Stülke and Krüger 2020, Yin et al. 2020).

With the notable exception of gamma-proteobacteria, c-di-AMP is produced in many bacteria, including firmicutes, actinobacteria, cyanobacteria, delta-proteobacteria, *Chlamydia*, and spirochaetes, as well as in many euryarchaeota, i.e. a large archaeal subgroup. c-di-AMP homeostasis is controlled by diadenylate cyclases (DAC) that are characterized by the presence of the DAC domain. This domain can be fused to a multitude of other domains, among them DNA-binding or a membrane-spanning domains. While most species have one type of DAC, the spore-forming species of *Clostridium* and *Bacillus* possess two or even three distinct DACs. c-di-AMP is degraded by specific PDE that contain either DHH-DHHA1 or HD domains (Commichau et al. 2019). Moreover, c-di-AMP can be secreted from the producing bacterial cell (Woodward et al. 2010, McFarland et al. 2018). In many bacteria, the intracellular levels of c-di-AMP respond to the availability of potassium, which is the by far most abundant cation in any living cell, and to osmotic stress (Gundlach et al. 2017a,b, Gibhardt et al. 2019). Unfortunately, the molecular mechanisms that control the activities of the enzymes that make and break c-di-AMP are only poorly understood.

A variety of c-di-AMP targets have been identified in different bacteria. Importantly, many of these targets are related to potassium and osmotic homeostasis. c-di-AMP binds and inhibits several types of potassium uptake systems, and it activates potassium export by binding to exporters. Moreover, c-di-AMP down-regulates the expression of high-affinity potassium transporters by binding to their regulatory elements, e.g. to a sensor kinase or a riboswitch (Corrigan et al. 2013, Nelson et al. 2013, Gundlach et al. 2019). Other important targets are transporters for osmotically compatible solutes and the regulators of their expression (Schuster et al. 2016, Devaux et al. 2018, Bandera et al. 2021). Moreover, in *Streptomyces* a c-di-AMP-binding riboswitch controls the expression of a cell wall-degrading enzyme (St-Onge et al. 2015). Finally, c-di-AMP binds to signal transduction proteins that then act as a "third messenger." Functions of these stand-alone c-di-AMP-binding effector proteins have been identified only recently and were an important topic at the conference.

Jörg Stülke (Georg-August-Universität Göttingen) showed how c-di-AMP controls cellular processes even beyond potassium and osmotic regulation in the model organism *B. subtilis*. There are two c-di-AMP-binding signal transduction proteins in *B. subtilis* and other firmicutes, i.e. DarA and DarB. For DarB, two activities have been identified. In both cases apo-DarB, which is present at low potassium concentrations when the intracellular c-di-AMP levels are low, binds other proteins to modulate their activity. Potassium starvation has profound consequences for many cellular processes such as translation or buffering of the negative DNA charge. This is where DarB comes into play. The protein can bind to the bifunctional GTP pyrophosphokinase Rel, which synthesizes and degrades the alarmone (p)ppGpp. The interaction stimulates (p)ppGpp production and inhibits the degrading activity of Rel. This activation of the stringent response under conditions of potassium starvation allows the cell to immediately switch off many of its growth-related activities until conditions get better. This control of (p)ppGpp homeostasis by DarB has been described for *Listeria monocytogenes* and *B. subtilis* (Peterson et al. 2020, Krüger et al. 2021b). The structure of the *B. subtilis* DarB-c-di-AMP complex suggests that the nucleotide protrudes from the protein and prevents the interaction with Rel in a sting-like manner (Heidemann et al. 2022). In addition, *B. subtilis* apo-DarB binds to and stimulates the activity of pyruvate carboxylase (PycA), an enzyme that replenishes the citric acid cycle by producing oxaloacetate from pyruvate. This is important since amino acids derived from glutamate play a major role in buffering the negative charge of DNA if potassium is limiting (Krüger et al. 2022). Potassium and glutamate are the most abundant cation and anion, respectively, in any cell, and their concentrations are tightly coupled to each other (Gundlach et al. 2018). Accordingly, a *B. subtilis* strain lacking c-di-AMP is not only sensitive to potassium but also to glutamate and other amino acids. This phenotype allowed the identification of the major glutamate transporters in *B. subtilis* as well as an exporter for histidine (Krüger et al. 2021a, Meißner et al. 2022). Interestingly, c-di-AMP controls PycA in both *B. subtilis* and *L. monocytogenes*; however, while this control is mediated by the stringent response and the DarB–PycA interaction in *B. subtilis* (see above), it is achieved by direct binding of c-di-AMP to the enzyme in *L. monocytogenes* (Sureka et al. 2014). Independent of the specific mechanism, the final outcome is high PycA activity under conditions of potassium starvation, and low activity at potassium abundance in both organisms (Fig. 9).

PII-like proteins bind c-di-AMP in firmicutes and cyanobacteria. While the role of the DarA protein in *B. subtilis* and related bacteria still awaits elucidation, more is known about the cyanobacterial SbtB protein as presented by Michael Haffner (from the group of Karl Forchhammer, Eberhard-Karls-Universität Tübingen). Both the single DAC DacA and the c-di-AMP target SbtB are required for growth of *Synechocystis sp.* in a day–night cycle. SbtB in complex with c-di-AMP interacts with an enzyme of glycogen synthesis, and loss of the interaction reduces the production of this important carbon storage molecule, and thus strongly impairs nighttime survival (Selim et al. 2021).

c-di-AMP also binds to BusR, a transcriptional regulator of genes involved in the uptake of compatible solutes in lactic acid bacteria. Gregor Witte (Ludwig-Maximilians-Universität München) presented the structure of the BusR–c-di-AMP complex. BusR is unique in that it binds a bipartite operator site in front of the target genes. Moreover, BusR has a C-terminal RCK_C domain. Such domains are normally found in potassium transporters and are known to bind c-di-AMP. Binding of c-di-AMP to the RCK_C domain of BusR results in a repositioning of the N-terminal DNA-binding domains with respect to the major groove of DNA and allows efficient repression of osmolyte uptake (Bandera et al. 2021).

New results on the role of c-di-AMP in the human pathogen *L. monocytogenes* were presented by Inge Schwedt (from the group of Fabian Commichau, Universität Hohenheim). They studied mutants that either lack or hyperaccumulate c-di-AMP. In both cases, the mutants were sensitive to osmotic stress indicating that c-di-AMP plays a central role in osmotic homeostasis also in this organism. Moreover, suppressor mutants of the strain lacking the single DAC CdaA—and therefore, c-di-AMP entirely—were affected in the Opp oligopeptide uptake system indicating that the uncontrolled influx of oligopeptides in this mutant is toxic for the cells. c-di-AMP indirectly controls the expression of the *opp* operon in *L. monocytogenes*, and thus expression of a reporter construct can be used to monitor the intracellular c-di-AMP concentration (Wang et al. 2022). Similarly, Igor Kwiatkowski (from the group of Angelika Gründling, Imperial College London) presented a c-di-AMP-specific biosensor for *Staphylococcus aureus*, i.e. based on the c-di-AMP-controlled riboswitch of the *B. subtilis kimA* gene and its use for the identification of factors that modulate intracellular levels of c-di-AMP.

Finally, c-di-AMP signaling is not limited to bacteria but occurs also in many euryarchaeota. Sonja-Verena Albers (Albert-Ludwigs-Universität Freiburg) presented results on c-di-AMP signaling in the model archaeon *Haloferax volcanii*. As in many bacteria, c-di-AMP is essential for this organism, and the control of potassium homeostasis is a key function of this second messenger in *H. volcanii* (Braun et al. 2021).

The role of signaling nucleotides in recently discovered phage defense systems

Enzymes related to RelA-SpoT homologs (RSH), which synthesize the alarmone (p)ppGpp, have also been implicated in bacterial phage defense. RSHs include long RSHs (that combine RelA and SpoT domains as in *E. coli*) as well as small alarmone synthetases (SASs) and small alarmone hydrolases (SAHs). SAS members contain the (p)ppGpp synthesis domain, but lack the hydrolysis domain and regulatory C-terminal domains of the long RSHs (Irving et al. 2020). Various SASs have been found to act as toxins (toxSAS) in toxin–antitoxin (TA) systems by synthesizing ppGpp and its unusual analogue ppApp. Also some RSH enzymes can synthesize (p)ppApp or pyrophosphorylate tRNAs (Ahmad et al. 2019, Jimmy et al. 2020, Kurata et al. 2021). Tas1, which was the first (p)ppGpp synthase-like enzyme identified that produces (p)ppApp, is actually an interbacterial toxin delivered by a type VI secretion system in *P. aeruginosa*. The rapid accumulation of (p)ppApp due to very high enzyme activity depletes existing pools of ATP, leading to dysregulation of essential metabolic pathways and ultimately to cell death (Ahmad et al. 2019).

In his talk, Michael Laub addressed the physiological functions of toxic RSHs that pyrophosphorylate the 3′-CCA end of tRNAs, which his group has studied in a collaboration with the Atkinson, Hauryliuk and Garcia-Pino groups (Zhang et al. 2022a). In contrast to canonical bicistronic TA systems, several representatives of the CapRel subfamily of these pyrophosphorylating enzymes combine a toxSAS-like N-terminal domain with a C-terminal domain that acts as a corresponding antitoxin. The molecular basis of phage-induced activation of bacterial immunity was studied with a CapRel enzyme encoded by the *Salmonella* phage SJ46, which is also encoded (with 100% amino acid sequence identity) in prophages of several *E. coli* strains. The C-terminal region of CapRel^{SJ46} was shown to directly counteract the toxic N-terminal

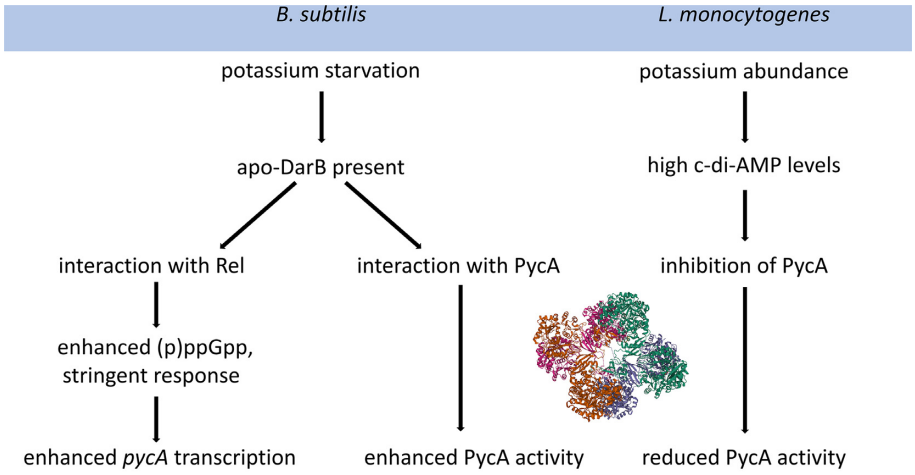

Figure 9. Different mechanisms to achieve potassium-dependent regulation of pyruvate carboxylation in *B. subtilis* and *L. monocytogenes*. Regulation is achieved indirectly via DarB-mediated control of the stringent response (expression of the *pycA* gene) and the DarB–PycA protein–protein interaction in *B. subtilis*. In contrast, c-di-AMP directly binds PycA in *L. monocytogenes*. Nevertheless, the regulatory logic and outcome is the same in both organisms. This figure was kindly provided by Jörg Stülke.

region as well as to serve as a sensor for phage infection, with the pseudo-zinc finger domain (pseudo-ZFD) of CapRel confering phage specificity. The structural analysis of CapRel^{SJ46} revealed an autoinhibited and an active state. Activation occurs by direct binding of the major capsid protein Gp57 of SECΦ27 (or homologous capsids of other phages) to the pseudo-ZFD in the C-terminal region of CapRel^{SJ46}. This interaction relieves autoinhibition and enables the N-terminal toxin domain to pyrophosphorylate tRNA, which disrupts translation. This happens long before enough capsid protein for phage assembly has been synthesized and, therefore, results in abortive infection. Thus, fused CapRel homologs are single component TA systems that robustly protect bacteria against various phages.

As presented in an overview talk by Rotem Sorek (Weizmann Institute of Science, Rehovot), novel cyclic nucleotide signaling molecules play crucial roles in a variety of prokaryotic antiphage defense systems including type III CRISPR (a cyclic oligoadenylate (cOA) signaling pathway), cyclic oligonucleotide-based antiphage signaling system (CBASS), Pycsar (pyrimidine cyclase system for antiphage resistance), and Thoeris (Lopatina et al. 2020, Athukoralage and White 2022). In vertebrates, the cyclic GMP–AMP synthase (cGAS)-STING signaling pathway is an important cytosolic DNA-sensing pathway enabling cells to produce cyclic GMP–AMP (2′3′-cGAMP) that binds to the STING protein and initiates interferon-mediated gene expression in response to foreign DNA from viruses, bacteria, or tumors (Chen et al. 2016). *Vibrio cholerae* produces 3′3′-cGAMP to activate a phospholipase that degrades the inner bacterial membrane (Severin et al. 2018). Subsequently, it was found that this cGAMP signaling is part of an antiphage defense system, i.e. common in bacteria, with hundreds of homologs of the *dncV* gene, which encodes the *V. cholerae* cGAS, in thousands of genomes and usually located in the genomic vicinity of known defense systems (Cohen et al. 2019). Mutational analyses in the *E. coli*-derived system showed that the defense against a broad range of phages depends on cGAMP production and phospholipase activity (encoded by the *capV–dncV* gene pair). Therefore, this system was termed the CBASS. Structural and functional data, obtained in collaboration with the Kranzusch lab, indicate that CBASS is the evolutionary ancestor of the animal cGAS-STING antiviral pathway (Morehouse et al. 2020). CBASS operons occur widely and generally encode a cGAS/DncV-like nucleotidyltransferase (CD-NTase) enzyme that synthesizes a nucleotide second messenger in response to viral infection, and a CD-NTase-associated protein (Cap) effector that functions as an effector that specifically binds the CD-NTase-produced nucleotide second messenger signal and induces cell death to prevent phage propagation. CBASS cyclases synthesize a wide range of cyclic nucleotides (cyclic dipurines, dipyrimidines, mixed purine/pyrimidine dinucleotides, and trinucleotides) associated with diverse effector proteins (Duncan-Lowey and Kranzusch 2022).

In addition to cyclic purine nucleotides that are found across all domains of life, also cCMP and cUMP can mediate bacterial immunity against phages (Tal et al. 2021). Based on the observation that genes encoding uncharacterized adenylate cyclase-like proteins often colocalize with phage defense-associated genomic islands, such an enzyme from *E. coli* E831 was shown to be a cytidylate cyclase and this type of defense systems was, therefore, called Pycsar (pyrimidine cyclase system for antiphage resistance). This novel family of bacterial pyrimidine cyclases, which is widespread in bacteria and archaea, synthesizes cCMP or cUMP following phage infection, and these signaling molecules activate effector proteins that mediate abortive infection through membrane impairment or depletion of cellular NAD^+. Their sensor domains are fused to transmembrane–helix domains predicted to disrupt cell membranes or TIR domains that degrade NAD^+.

Although the Thoeris antiphage defense system also utilizes a cyclic nucleotide signaling molecule and a catalytic TIR domain, it differs from CBASS and Pycsar systems in many aspects. The Thoeris system consists of TshA, which has NAD^+ cleavage activity, and TshB, which resembles TIR domain proteins (Ka et al. 2020). The Sorek lab has shown that upon viral infection, the TIR domain of TshB converts NAD^+ into a variant cyclic ADP ribose (cADPR) molecule. This cADPR isomer binds to the C-terminal SLOG domain of ThsA and activates—through a change in the ThsA oligomerization state—its N-terminal SIR2 NADase domain, which depletes the cell of NAD^+, leading to growth arrest and abortive infection. The bacterial Thoeris system is clearly related to TIR domain-containing plant defense systems, and thus may have been the ancestor of plant TIR-containing innate immunity

mechanisms (Ofir et al. 2021). In yet another turn of the arms race between phages and bacteria, an Thoeris antidefense system was recently found in phages that uses Tad1 proteins, which act as 'sponges' that sequester the cADPR signaling molecule produced by TshB protein and thereby prevent the activation of the Thoeris phage defense system (Leavitt et al. 2022).

Conclusions and perspectives

The presentations at the International Symposium on 'Nucleotide Second Messenger Signaling in Bacteria' as well as the collection of minireviews put together here clearly demonstrate that this complex topic has moved forward from an 'emerging'—as it was considered 6 years ago when the SPP1879 consortium was established—to a fully flourishing field.

We now have a relatively clear overview of the widely conserved global physiological "division of labor" between the major nucleotide signals in bacteria, whose pathways nevertheless intersect in complex networks as can be seen in certain model bacteria (Hengge 2020). Thus, (p)ppGpp coordinates growth and survival in response to nutrient availability and various stresses, which includes far-reaching stationary phase responses (Anderson et al. 2021). C-di-GMP generally orchestrates bacterial adhesion and multicellularity, but also plays major roles in coordinating the cell cycle and the interplay between bacterial biofilm formation and development or virulence functions in pathogens (Jenal et al. 2017, Valentini and Filloux 2019). C-di-AMP links osmoregulation with central metabolism, but also controls developmental functions (Latoscha et al. 2019, Stülke and Krüger 2020). Furthermore, we can now appreciate the mechanistic diversity of signal input into the enzyme activities, which control dynamic nucleotide levels and effector/target mechanisms that sense and react to signaling nucleotides—even though many more will certainly be discovered in the future. Second messengers are certainly always good for surprises, as illustrated by the recent finding reported by Lars Dietrich (Columbia University) that R-bodies (or 'javelins'), which are very long, extendable protein polymers with potential roles in virulence that form in the cytoplasm of some bacteria, are under the control of a transcription factor that resembles cAMP-binding proteins, but that seems to sense another not yet identified cNMP (Wang et al. 2021).

The multiplicity of second messenger-related enzymes (above all c-di-GMP) is no longer a mystery, but we see that—despite their use of a diffusible second messenger—these enzymes can operate specifically in parallel in global and local signaling pathways. The prerequisites, theoretical possibility, molecular mechanisms, and experimental detection have been studied in detail (Richter et al. 2020, Hengge 2021). The analysis of local signaling has so far focused on local sources of nucleotide production in synthase–effector protein complexes on a background of a low global nucleotide pool drained by master nucleotide PDEs, but the opposite—local nucleotide sinks generated by specific signaling PDEs on a background of high global nucleotide level maintenance—is equally conceivable.

A field where we expect rapid future progress is the regulatory integration in complex nucleotide signaling networks (Hengge 2020). This involves both the integration of different signals into single nucleotide synthetic or degrading enzymes with multiple sensory domains as well as the regulatory intersection of different nucleotide signaling pathways (e.g. ppGpp/c-di-GMP/GTP or Ap4A/c-di-AMP). To name just a few mechanistic possibilities, different signaling nucleotides can control synthesis or degradation of others or they can compete for the same effector binding sites with different regulatory consequences.

Moreover, the elucidation of second messenger functions and networks beyond the most studied model bacteria is highly desirable. A fruitful starting point are genomic analyses that identify novel types of genes that are colocalized or coregulated with genes for second messenger-related enzymes or for functions known to be generally controlled by nucleotides. This approach has been strikingly successful in the discovery of novel nucleotide signaling-based phage-defense systems that drive abortive infection and include phage-based antidefense systems (Athukoralage and White 2022, Duncan-Lowey and Kranzusch 2022). To set them apart from the common and (nearly) ubiquitous nucleotides that bacteria use for controlling their cellular "business," the nucleotides used in phage defense are diverse and highly specific. Furthermore, these systems represent the phylogenetic ancestors of cyclic nucleotide-activated immune signaling in plants and animals (Wein and Sorek 2022).

Overall, joint research in the SPP1879 consortium as well as the highly inspiring SPP1879 International Symposium in May 2022 in Berlin has made it amply clear that nucleotide second messenger research will continue to provide us with exciting discoveries and to have both fundamental and applied implications in molecular and environmental microbiology as well as in biotechnology, infection biology, and even immunology.

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