Review

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Physiology of guanosine-based second messenger signaling in *Bacillus subtilis*

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Abstract: The guanosine-based second messengers (p)ppGpp and c-di-GMP are key players of the physiological regulation of the Gram-positive model organism *Bacillus subtilis*. Their regulatory spectrum ranges from key metabolic processes over motility to biofilm formation. Here we review our mechanistic knowledge on their synthesis and degradation in response to environmental and stress signals as well as what is known on their cellular effectors and targets. Moreover, we discuss open questions and our gaps in knowledge on these two important second messengers.

Keywords: alarmone; *Bacillus subtilis*; c-di-GMP; (p)ppGpp; signaling.

Introduction: nucleotide-based second messengers

Living cells must sense, respond and adapt to an immense variety of rapidly changing conditions in their environment. In bacteria, this can be achieved by sophisticated regulation of cellular processes such as through two- and one-component regulatory systems, alternative sigma factors or second messenger signaling. Second messengers, which can be nucleotide-derivatives, lipids and other small molecules are essential and universally found elements in signal transduction cascades (Galperin 2018; Parkinson 1993). They mediate the perception, transduction and processing of intra- and extra-cellular stimuli and ensure an appropriate and specific response inside the

cell (Figure 1A). Second messengers integrate, amplify and convert the signals (the first messengers) received through extra- and/or intra-cellular receptors into a cellular response. This signal transduction occurs through the interaction of a wide but defined set of cellular targets including proteins and RNA molecules.

Second messenger signaling was conceptualized in the 1950s, when Sutherland and colleagues discovered that the hormones epinephrine and glucagon (first messengers) instead of entering the cell, stimulated liver glycogenolysis via the production of the adenosine-based second messenger cyclic adenosine 3',5'-monophosphate (cAMP) (Sutherland and Rall 1958). Since this hallmark discovery, a plethora of second messenger molecules including a variety of adenosine and guanosine nucleotides-based second messengers have been identified in all organisms studied.

This review will summarize our understanding of second messenger signaling in the Gram-positive model organism *Bacillus subtilis* with a focus on the guanosine-derived second-messengers (p)ppGpp and c-di-GMP (Figure 1B). The metabolism of these guanosine nucleotide-based second messengers is intimately linked to the cellular nucleotide pools because their synthesis requires nucleotides as substrates as well as their degradation yields nucleotide products (Figure 1A). In response to specific signals, dedicated synthetases and hydrolytic enzymes tightly control the corresponding cellular levels. Essentially bacteria often contain a plethora of these enzymes for a specific nucleotide derivative resulting in the constitution of highly complex sensory and responsive circuits (Dahlstrom and O'Toole 2017; Steinchen and Bange 2016).

The ecophysiology of *Bacillus subtilis*

The rod-shaped *B. subtilis* (also hay or grass bacillus, originally named *Vibrio subtilis*) is a non-pathogenic, Gram-positive bacterium belonging to the phylum of the

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Figure 1: Nucleotide-based second messengers. (A) Scheme of a signaling circuit. The second messengers (p)ppGpp and c-di-GMP are colored according to the contribution of their basic nucleotides GDP (red), GTP (blue) and ATP (green). (B) Chemical structures of the second messengers (p)ppGpp and c-di-GMP. The color code is as defined in panel (A).

Firmicutes. Already in 1877, Ferdinand Cohn described the characteristic features of this bacterium including its ability to develop spores (summarized in: Soule 1932). Since the dawn of microbiology it has then paved its way into one of the most investigated model organisms. While most commonly found in soil, *B. subtilis* is meanwhile also considered as a commensal of the human gut, which is exemplified best by the finding of up 10^4 spores per gram of human faeces (Hong et al. 2009). As a beneficial plant-associated bacterium, *B. subtilis* colonizes plant roots, thereby stimulating their growth and protecting them from pathogens in the rhizosphere. Therefore, *B. subtilis* and other *Bacillus* species are becoming increasingly important in agricultural settings serving as natural alternatives to chemical pesticides (Molina-Santiago and Matilla 2019; Vlamakis et al. 2013). In return, *B. subtilis* profits from plant exudations, which comprise a huge diversity of organic compounds such as sugars and amino acids enabling microbial proliferation and the formation of multicellular communities, commonly known as biofilms (e.g. (Beauregard et al. 2013; Dennis et al. 2010; Lucy et al. 2004)). Especially in such a heterogeneous and nutrient-poor habitat like soil, association to plant-roots conveys a huge advantage in this ecological niche for microorganisms.

Undomesticated strains of *B. subtilis* such as NCIB3610, are able to form floating biofilms at air-liquid

interfaces (pellicles), structured colony biofilms on semi-solid agar surfaces and are furthermore capable of colonizing plant roots (Branda et al. 2001; Cairns et al. 2014) (Figure 2). Laboratory strains such as 168 and PY79 have largely lost the ability to spread on semi solid agar and to form biofilms as a consequence of domestication (McLoon et al. 2011; Zeigler et al. 2008). The formation of different biofilm forms in natural settings, however, requires the ability of *B. subtilis* to be mobile and to differentiate into different cell types. The undomesticated *B. subtilis* strain NCIB3610 exhibits a peritrichous flagellation pattern with approximately 25 flagella visible at its lateral sides allowing the bacterium to swim and swarm (Guttenplan et al. 2013; Schuhmacher et al. 2015). Moreover, *B. subtilis* was amongst the first bacterial species for which a cellular heterogeneity within a genetically identical population was noted (Kearns and Losick 2005). In a hallmark study, Daniel Kearns showed that during mid-exponential growth this bacterium exhibits two cell forms; one being highly flagellated and motile, while the other appears as long chains of sessile cells (Figure 2). Cell population heterogeneity is thought to enable this microbe to exploit its present location as well as to conquer new resources and habitats through the motile cell type. Under conditions of nutrient starvation and harmful stress such as temperature, drought and salinity, *B. subtilis* forms endospores

Figure 2: Lifestyles of *Bacillus subtilis*. *B. subtilis* can exist as sessile chain, swimmer cell or spore. Moreover, the bacterium can form biofilms stabilized by extracellular matrix (ECM) consisting of proteins, DNA and exopolysaccharide (EPS). In the biofilm, different cell types exist. Further description can be found in the text.

(McKenney et al. 2013) (Figure 2), which are resistant to highly extreme conditions such as space flights (Bucker 1974; Bucker et al. 1974).

Yet, differentiation into endospores and motile cells are not the only cellular differentiation pathways for this bacterium. During biofilm development (pellicle and colony biofilms; Figure 2), strikingly different genetic programs are activated leading to differentiation of an isogenic progenitor population into subpopulations of functionally distinct, coexisting cell types (among others: motile cells, competent cells, matrix producers, spores) and the production of extracellular matrix (ECM) components, which are shared with the entire community (Cairns et al. 2014). ECM components are indispensable for biofilm formation and conservation as their nature determines the physicochemical and biological qualities of a biofilm and ensures the structural and functional integrity of the community. The major ECM component secreted by undomesticated *B. subtilis* NCIB3610 is a chemically poorly characterized exopolysaccharide (EPS), which is synthesized by the products of the 15 gene long *epsA-O* operon whose expression is ensured by a mechanism termed processive riboantitermination (Artsimovitch 2010, Irnov and Winkler 2010). Within the *epsA-O* cluster, the *epsHIJK* locus has been proposed to encode proteins able to synthesize the conserved bacterial exopolysaccharide poly-N-acetylglucosamine that may function as a scaffold and anchoring substrate for polysaccharides produced by other

epsA-O encoded proteins (Roux et al. 2015). In addition to the EPS produced by the gene products of *epsA-O*, various *B. subtilis* strains are able to synthesize Levan, a prominent structural extracellular polymeric substance consisting of β -(2–6) linked D-fructose molecules with branching β -(2–1) units (Benigar et al. 2014; Marvasi et al. 2010). Besides the two structural ECM components described above, some wild type isolates including *B. subtilis* B-1 and *B. subtilis* (natto) also synthesize the extracellular, anionic polymer poly- γ -glutamic acid (PGA), eventually being required for the sorption of ions and/or charged molecules (summarized in: [Candela and Fouet 2006]). In addition to EPSs, biofilms of *B. subtilis* contain various extracellular protein components. The synthesis of amyloid-like fiber structures accomplished by proteins encoded within the *tapA-sipW-tasA* operon is essential for *B. subtilis* biofilm formation in some, but not in all experimental set ups (Branda et al. 2006; Earl et al. 2020; Hamon et al. 2004; Romero et al. 2010). The correct development of biofilms furthermore depends on the secretion of the amphiphilic protein BslA forming an organized protein layer at the biofilm surface allowing the assembly of amyloid-like fibers and EPS (Hobley et al. 2013; Kobayashi and Iwano 2012; Ostrowski et al. 2011). Taken together, *B. subtilis* biofilms exhibit a population of genetically identical but phenotypically distinct cells all of which presumably benefit from an efficient division of labor in order to optimize survival and fitness of the population (Dragos et al. 2018a, b; Kearns, 2008).

In order to enable all these lifestyle features, *B. subtilis* has to continuously adapt its metabolism and physiology. The guanosine nucleotide-based second messenger (p) ppGpp and c-di-GMP constitute the regulatory heart, which translates environmental and intracellular signals into a response enabling the continuous adaptation of this bacterium to ever-changing conditions. In the next chapters, we will summarize our current knowledge on nucleotide-based second messenger signaling on this model organism and point to open questions and current twists.

The magic nucleotides (p)ppGpp in *B. subtilis*

Synthesis and degradation of (p)ppGpp

The nucleotides ppGpp and pppGpp (collectively: (p) ppGpp or the ‘alarmones’) are central for the bacterial adaptation to nutritional limitations in the framework of the stringent response and to other environmental factors

such as heat stress (as detailed below). They have been initially discovered as the so-called “magic spots”, appearing on in thin-layer chromatography experiments from ^{32}P -radio-labeled cellular extracts of the Gram-negative bacterium *Escherichia coli* stressed by amino acid limitation (Cashel and Gallant 1969). Shortly after, (p)ppGpp could also be identified as critical element of the stringent response in Gram-positive *Bacilli* including the well-studied model organism *B. subtilis* (Ogilvie et al. 1975; Rhaese et al. 1975; Setlow 1974). To date; it is clear that the alarmones are being synthesized by synthetases, which catalyze the transfer of the β and γ -pyrophosphate moiety of ATP to the 3' hydroxyl end of GDP and guanosine-triphosphate (GTP) in order to produce ppGpp and pppGpp, respectively (Figure 1) (Steinchen and Bange 2016; Steinchen et al. 2015). Conversely, (p)ppGpp hydrolases catalyze the removal of the same pyrophosphate moiety from the two alarmones (Figure 1A).

Both synthetases and hydrolases belong to the RelA/SpoT homology (RSH)-family with distinct sequence features and catalytic motives that allow their straight forward identification by *in silico* methods (Atkinson et al. 2011). RSH-family proteins are divided into long and small forms, with the long ones being multi-domain proteins containing a structurally and functionally coupled hydrolase and a synthetase (also: NTD) besides other regulatory domains (Figure 3A, B). It is important to note that bacterial species shows substantial differences in their enzymatic repertoire of (p)ppGpp metabolism. This is best exemplified when comparing the RSH-enzymes found in *B. subtilis* to those in the Gram-negative model organism *E. coli* (Figure 3A, B). While *B. subtilis* is equipped with the long RSH enzyme, named Rel, and two small alarmone synthetases named RelQ and RelP (Figure 3A); *E. coli* contains the long RSH enzymes, RelA and SpoT, besides the pppGpp to ppGpp-converting enzyme GppA absent in *B. subtilis* (Figure 3B).

The long RSH-enzyme Rel

This enzyme is a multi domain protein, which shares high sequence and domain identity to the RelA protein of *E. coli* (Figure 3). As its *E. coli* counterpart, Rel can bind to uncharged tRNAs in the context of the ribosomal A-site and synthesize (p)ppGpp in order to signal the lack of amino acids (Arenz et al. 2016; Loveland et al. 2016; Wendrich et al. 2002) (Figure 3C). However and in stark contrast to *E. coli* RelA, *B. subtilis* Rel is also able to degrade (p)ppGpp through its N-terminal hydrolase domain (Figure 3C). The (p)ppGpp synthetase and hydrolase activities of Rel proteins are regulated in an antagonistic manner and are

mutually exclusive (Hogg et al. 2004, Steinchen and Bange 2016). Besides the long RSH-type enzyme Rel, *B. subtilis* as most other species of the genus *Firmicutes* is equipped with two small alarmone synthetases (SASs), which complement the enzymatic repertoire of (p)ppGpp metabolism.

The small alarmone synthetases RelP and RelQ

B. subtilis as well as *Staphylococcus aureus*, *Enterococcus faecalis* and *Listeria monocytogenes* contains two SAS enzymes, named RelP (YwaC, SAS2) and RelQ (YjbM, SAS1) (Geiger et al. 2014; Lemos et al. 2007; Nanamiya et al. 2008). Both enzymes consist of a single (p)ppGpp synthetase domain and form highly symmetric homotetramers with a prominent cleft in their center (Figure 3C) (Steinchen et al. 2015). For RelQ, it was shown that this cleft can bind two pppGpp – however not ppGpp – molecules, which stimulate the (p)ppGpp synthetase activity of the enzyme by approximately 10-fold (Steinchen et al. 2015). *In vitro*, the catalytic activity of RelQ from *E. faecalis* can be negatively influenced by single stranded RNA, suggesting that RelQ proteins might bind to one or more RNAs (Beljantseva et al. 2017) (Figure 3C). However, the true nature of the cognate RelQ-binding RNA(s) remains subject to identification. The functional role of the cleft in the RelP protein is unknown. However, it is tempting to speculate that might also represent a regulatory site (Manav et al. 2018; Steinchen et al. 2015; Steinchen et al. 2018) (Figure 3C).

While both enzymes are well characterized at the structural and mechanistic level, their cellular function is still rather enigmatic. The transcriptional analysis shows that RelP and RelQ are expressed during different phases of cell growth. While *relQ* is transcribed as part of an operon during exponential growth of *B. subtilis*, a single mRNA encoding YwaC is detected from the early stage of vegetative growth through the transition phase of this bacterium (Nanamiya et al. 2008; Tagami et al. 2012). Moreover, alkaline stress can serve as inducer for the transcription of *relP* and the enzymatic activity of the corresponding enzyme (Nanamiya et al. 2008). However, the precise functional roles of the small alarmone synthetases RelQ and RelP are far from being understood, despite the huge amount of biochemical and structural information being available.

Targets of (p)ppGpp and the physiology of *B. subtilis*

The alarmones (p)ppGpp are involved in the regulation of many – if not all aspects – of metabolism and physiology of

Figure 3: The alarmones (p)ppGpp. (A, B) Composition and architectures of RelA/SpoT homology (RSH)-enzymes found in *B. subtilis* and *Escherichia coli* differ from each other. Hydrolase and synthesase domains are colored in orange and magenta, respectively. RelA contains an inactive hydrolase at its N-terminus colored in yellow. (C) Synthesis and degradation of ppGpp (yellow) and pppGpp (red) by SAS1 (RelQ), SAS2 (RelP) and Rel in *B. subtilis*. The so-produced alarmones modulate a wide range of cellular processes (further detailed in the text).

B. subtilis. In the following, we will frankly summarize which targets of (p)ppGpp have been identified in *B. subtilis* (Figure 3C).

GTP biosynthesis and purine-salvage

In *B. subtilis*, the alarmones (p)ppGpp are involved in inhibiting a variety of enzymes required for the biosynthesis of guanosine-triphosphate (GTP). The GTP biosynthetic pathway formally begins with inositol-monophosphate (IMP), which represents at the branching point of ATP- and GTP biosynthesis. Thus, conversion of IMP into xanthosine-monophosphate (XMP) through the Inositol-monophosphate-dehydrogenase (IMPDH, GuaB in *B. subtilis*) represents the first step of the GTP biosynthesis route. While early studies in *E. coli* have suggested IMPDH as target of (p)ppGpp inhibition (Gallant et al. 1971; Pao and Dyess 1981), *B. subtilis* IMPDH does not seem to be strongly affected by the alarmones ((Kriel et al. 2012) (Bange and Steinchen, unpublished observations)). Structural and biochemical studies showed that *B. subtilis* Guanylate kinase (GMK), which phosphorylates GMP into GDP, is a bona-fide target of (p)ppGpp with inhibitory constants in the micromolar range (Liu et al. 2015b). Moreover, (p)ppGpp inhibits the purine salvage pathway enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT), converting hypoxanthine and guanine bases to IMP and GMP, respectively (Anderson et al., 2019, 2020). Thus, (p)ppGpp plays important roles in modulating

guanosine (and adenosine) nucleotide metabolism through the GTP biosynthetic route and associated salvage pathways.

Biosynthesis of amino acids

The (p)ppGpp-dependent stringent response has been initially identified as response to amino acid starvation. Thus, it is not surprising that (p)ppGpp also affects amino acid metabolism in *B. subtilis*. One main route is proposed via the transcriptional regulator CodY, which regulates over 200 promoters, including those involved in the biosynthesis of branched amino acids (Belitsky and Sonenshein 2013). Because activation of CodY depends on GTP (Handke et al. 2008), (p)ppGpp can alter the activity of this transcriptional regulator indirectly through its action on the GTP biosynthesis (see previous chapter). A *B. subtilis* strain unable to produce (p)ppGpp (the “(p)ppGpp0 mutant”) is auxotrophic for the amino acids leucine, isoleucine, valine, methionine, and threonine due to failure of their gene regulation (Kriel et al. 2014). This auxotrophy can be completely rescued by spontaneous suppressors that reduce GTP levels (Bittner et al. 2014). Comparative transcriptome analysis in wild type, (p)ppGpp0 and suppressors showed that reduction of GTP levels activates the transcription of genes responsible for the five amino acids by inactivating the transcription factor CodY, which represses the *ybgE*, *ilvD*, *ilvBHC-leuABCD*, *ilvA*, *ywaA*, and *hom-thrCB* operons, and by a

CodY-independent activation of transcription of the *ilvA*, *ywaA*, *hom-thrCB*, and *metE* operons. Finally, the CodY-independent activation is due to a mechanism involving regulating the initiating nucleotide (mostly an A instead of G) for the amino acid synthesis operons (Krasny et al. 2008). Besides that, no direct interaction of (p)ppGpp with enzymes involved in the amino acid metabolism of *B. subtilis* have been shown so far. However, it is tempting to speculate that such direct regulation exists and future experiments might address this point in more detail.

Replication

In *B. subtilis* the alarmones (p)ppGpp were shown to inhibit DNA replication in a dose-dependent manner (Denapoli et al. 2013; Wang et al. 2007). Biochemical and structural studies identified the DNA primase DnaG, which synthesizes short RNA primers during replication of DNA, as the target of (p)ppGpp. Structural analysis of the RNA polymerase domain of DnaG (from *Staphylococcus aureus*) showed that (p)ppGpp binds into the active site of the enzyme to interfere with primer synthesis (Rymer et al. 2012).

Regulation of translation

The process of mRNA translation is conceptually divided into four stages: initiation, elongation, termination, and ribosome recycling (for a review of translation in prokaryotes see (Rodnina 2018)). A number of GTP-binding proteins facilitate and catalyze specific functional steps of the ribosome during these subsequent stages of translation. These GTPases include translation initiation factor 2 (IF2), elongation factor Tu (EF-Tu), elongation factor G (EF-G), release factor 3 (RF3), and HflX. Already in the past century, numerous studies indicated that (p)ppGpp affect all stages of translation by interacting with these GTPases (Cashel 1996; Faxén and Isaksson 1994; Liu et al. 2015a; Milon et al. 2006; Rojas et al. 1984; Zhang et al. 2018). Given the highly conserved nature of these translational GTPases and translation-associated factors, it is tempting to propose that (p)ppGpp also regulates them in *B. subtilis*. However, future studies need to address the correctness of this assumption.

Hibernation of ribosomes

Nutrient deprivation, stress conditions or stationary phase conditions is often accompanied by the formation of 100S ribosomal particles, which are dimers of 70S ribosomes (Gohara and Yap 2018; Matzov et al. 2019; Prossliner et al. 2018). Formation of *B. subtilis* 100S ribosome dimers is

mediated by the hibernation-promoting factor (HPF) protein YvyD, which is upregulated under stress conditions in a (p)ppGpp-dependent manner (Eymann et al. 2002; Schafer et al. 2020). Structural analysis of 100S ribosomes from *B. subtilis* visualized how YvyD enables dimerization of two 70S ribosomes: YvyD consists of an N-terminal and a C-terminal domain (NTD and CTD, respectively), which are connected by a disordered linker (Beckert et al. 2017). The CTD enables homodimerization of YvyD and interacts exclusively with ribosomal proteins S2 and S18. Each CTD contacts the r-protein S2 from the 70S to which the corresponding NTD is bound, whereas the interaction with the N-terminal extension of S18 is from the second 70S ribosome.

Assembly of ribosomal subunits

The ribosome is a mega-Dalton ribonucleoprotein complex, which consists of a large and a small subunit (50S and 30S respectively). Biogenesis of both ribosomal subunits is highly cost-intensive and requires up to 40% of the cellular resources during proliferation. It requires the transcription of the ribosomal RNAs (rRNAs), the production of 54 ribosomal proteins (r-proteins) and approximately 100 additional biogenesis factors enabling the maturation of the subunits and their intermediates (Lecompte et al. 2002; Nierhaus and Lafontaine 2004). In *B. subtilis*, rRNA transcription is not directly affected by (p)ppGpp through the RNA polymerase (RNAP) (Krasny and Gourse 2004). Instead, synthesis of rRNAs seems to be influenced by its initiating nucleotide, which is typically GTP (Krasny and Gourse 2004). Thus, it seems that increasing (p)ppGpp pools might lead to a reduction of available GTP (see above), which in turn affects the activity of rRNA promoters indirectly. Ribosome-assembly GTPases (RA-GTPases) guide the process of small and large subunit maturation and serve as checkpoints at multiple assembly intermediates (Britton 2009; Corrigan et al. 2016). Accumulation of (p)ppGpp during the stringent response leads to the direct inhibition of multiple RA-GTPases – with Era, RsgA, ObgE, RbgA and HflX being affected in both Gram-negative and -positive bacteria – and thus a down-regulation of newly formed ribosomes (for a review on (p)ppGpp-mediated inhibition of ribosome assembly see (Bennison et al. 2019)). Structural analysis of the (p)ppGpp-bound RA-GTPases Obg and RbgA from *B. subtilis* show that the alarmones bind in the same way with similar affinities to the active site of the GTPases as their counterparts GDP and GTP (Buglino et al. 2002; Pausch et al. 2018). Thus, (p)ppGpp act as competitive inhibitors of the guanosine nucleotides for RA-GTPases, and might therefore

control ribosome biogenesis in a concentration-dependent manner.

Regulation of motility, cellular heterogeneity and spore formation

As described above, *B. subtilis* exhibits cellular heterogeneity in that it can form sessile and non-flagellated cell chains, besides a flagellated motile cell type (Figure 2A). Deletion of the gene encoding for the long RSH-type enzyme Rel (also referred to as *relA*) leads to a significant change in the ratio of these cell types when compared to the wildtype situation (Ababneh and Herman 2015b). The *relA* deletion strain grew exclusively as unchained, motile cells, which shows how (p)ppGpp levels can affect decision making during cellular heterogeneity programs. Albeit the precise molecular mechanisms await clarification, the transcription factor SigD and transcriptional regulator CodY will play key roles in the underlying mechanistic framework (Ababneh and Herman 2015a,b). It is important to mention that seminal work by Freeze and coworkers showed that (p)ppGpp induces sporulation through a decrease of GTP or GDP (Ochi et al., 1981, 1982) (see also above).

Heat-shock and thermo-tolerance

The alarmones (p)ppGpp have recently been shown to be important players during the heat shock response and critical for the successful adaptation of *B. subtilis* to heat (thermo tolerance) (Schafer et al. 2020). Heat-induced (p)ppGpp is produced by the long RSH-type enzyme Rel, while the small alarmone synthetases RelP and RelQ do not contribute. The (p)ppGpp generated under heat conditions primarily affects translation and associated processes (such as *yvyD*; see above). The study also indicated an interplay of (p)ppGpp with the stress-associated transcription factor Spx during heat stress, albeit the underlying molecular mechanism remains unclear. Taken together, it seems that at elevated temperatures (p)ppGpp regulates the capacity of the translation machinery of *B. subtilis* in order to optimize the cellular response to proteotoxic stress.

Cyclic di-guanosine-monophosphate (c-di-GMP) in *B. subtilis*

The second messenger c-di-GMP has been extensively studied in many Gram-negative and -positive bacteria and

is prominently known to mediate the motile to sessile lifestyle transition and *vice versa*. Commonly, high intracellular c-di-GMP concentrations negatively interfere with motility-based processes, while positively affecting extracellular matrix (ECM) production, which eventually favors bacterial biofilm formation. Conversely, low levels promote biofilm dispersal and the planktonic mode of bacterial life (summarized in Hengge 2009; Jenal et al. 2017; Romling et al. 2013). Studies investigating the intracellular concentrations of c-di-GMP in *B. subtilis* suggested growth phase-dependent differences. While c-di-GMP could not be detected during vegetative growth in standard rich medium (Gao et al. 2013), relative high concentrations were detected for cells at the onset of sporulation and for cells grown in biofilm-promoting medium (Diethmaier et al. 2014; Orr et al. 2018). Therefore, it seems that equivalent to the c-di-GMP signaling in Gram-negative bacteria such as *E. coli*, the concentration of this second messenger rises at the onset of stationary phase (Sommerfeldt et al. 2009). These findings are further supported by the investigation of c-di-GMP abundance at the single-cell level showing that intracellular c-di-GMP levels can vary significantly among different cell types within a population (Weiss et al. 2019). While motile and competent cells exhibited low c-di-GMP levels, matrix-production and endospore formation correlated with high c-di-GMP levels (Figure 4).

Synthesis of c-di-GMP in *B. subtilis*

Generally, biosynthesis of c-di-GMP relies on the condensation of two GTP molecules accompanied by the release of pyrophosphate. This reaction is catalyzed by enzymes of the diguanylate cyclases (DGCs) family which is characterized by the presence of a so-called GGDEF domain, whose designation derives from the existence of the signature motif sequence GG(D/E)EF (active site, A-site) that has been demonstrated to be conserved and essential for substrate binding, divalent cation coordination and the catalytic action (summarized in: (Schirmer 2016)).

A prerequisite for the formation of the two intermolecular phosphodiester linkages within the c-di-GMP molecule is the assembly of a two-fold symmetric GGDEF domain dimer. Within these assemblies both domains are arranged in an antiparallel manner where each monomer is thought to bind one GTP substrate molecule to form an active site at the dimer interface (De et al., 2008, 2009; Paul et al. 2007; Wassmann et al. 2007). In addition to the aforementioned catalytic residues, roughly half of all annotated GGDEF domains possess a regulatory feature that allows allosteric, noncompetitive product inhibition

Figure 4: Cyclic-di-GMP in *B. subtilis*. Three diguanylate cyclases (DGCs) (DgcK, DgcP, DgcW) synthesize c-di-GMP from GTP. The sole phosphodiesterases (PDE) PdeH degrades c-di-GMP into pGpG which can be further degraded into GMP by the oligoribonucleases NrnA and NrnB. MotI, YdaK and Ykul serve as c-di-GMP receptors. Color code for schematic representation of protein domains: putative soluble signaling domains and TM domains (grey); active GGDEF domains (magenta); active EAL domains (orange); in case of DgcW, the EAL domain activity has not been tested so far *in vitro*; c-di-GMP receptor proteins (blue). (A) C-di-GMP represses flagellar motility via MotI. MotI accepts c-di-GMP from DgcK and DgcP. Upon c-di-GMP-binding MotI inhibits flagellar motility by interacting with the flagellar stator element MotA, to disengage and sequester it from the flagellar rotor FliG. (B) C-di-GMP-dependent activation of EPS synthesis via YdaK. Upon perception of an unknown extracellular signal, c-di-GMP is synthesized in close proximity to its integral membrane receptor YdaK through the activity of the physically associated DGC DgcK. C-di-GMP binding to the I-site motif YdaK enables synthesis and export of the unknown EPS. Figure 4A and B were inspired by a book chapter by Weiss and Winkler (2020). (C) Function of DgcW and of the c-di-GMP receptor Ykul are not known. (D) Experimentally confirmed transcriptional regulators of *pdeH*, *ydaJ-N* and *dgcW*. (E) c-di-GMP levels vary between different subtypes of cells.

through a four-residue motif called autoinhibitory I-site (Seshasayee et al. 2010). I-sites are formed by the arginine and aspartic acids of the conserved RxxD motif

positioned upstream of the A-site motif. Binding of c-di-GMP to the distal I-site prevents overconsumption of the substrate, non-physiological accumulation of this second

messenger and potentially maintains c-di-GMP in defined concentration ranges (Christen et al. 2006). However, several recent studies have uncovered additional biological functions for this motif. I-sites do not only contribute to the maintenance of c-di-GMP homeostasis as negative regulatory features, but can furthermore positively participate as important elements in protein-protein interactions between DGCs and c-di-GMP receptors (Dahlstrom et al. 2016; Giacalone et al. 2018). Additionally, conserved I-sites within non-canonical GGDEF domains (enzymatically inactive DGCs) can serve as c-di-GMP receptor sites to drive EPS production for example (Chen et al. 2014; Gao et al. 2013; Kunz et al. 2020). *In toto*, I-site motives of active and inactive DGCs enable the modulation of signal transduction pathways at diverse levels. However, outside of the GGDEF domain, the domain organization diverges between different DGCs. The majority of predicted GGDEF domains is associated with a variety of different preceding sensory and/or regulatory domains, including most commonly PAS (PER/ARNT/SIM), GAF (cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA) and REC (CheY-homologous receiver domain) domains. These accessory domains, including also integral membrane sensor domains, are qualified to sense different signals (first messengers) thereby affecting the activation/inactivation of the downstream domain and thus connecting extra- and intracellular signals to c-di-GMP synthesis or degradation (Romling et al. 2013). The genome of *B. subtilis* NCIB3610 conceals three active DGC-domain containing proteins, which will be discussed in the following:

DgcK

DgcK (YhcK) is transmembrane protein with five predicted transmembrane helices and a C-terminal GGDEF domain residing in the cytoplasm (Figure 4A, B). Together with the phosphorelay sensor kinase LytS, DgcK was selected as a representative putative sensor protein of the 5TMR-LYT family (PF07694) (Anantharaman and Aravind 2003). In *B. subtilis*, DgcK and DgcP regulate repression of motility and activate synthesis of an uncharacterized EPS in the biofilm context (Bedrunka and Graumann 2017a, Kunz et al. 2020) (see below).

DgcP

DgcP (YtrP) is the only soluble DGC in *B. subtilis*, and has been reported to be an active diguanylate cyclase *in vivo* and *in vitro* (Gao et al. 2013). The protein contains two

predicted GAF sensory domains N-terminally to the GGDEF domain shown to stimulate diguanylate cyclase activity *in vitro* (Figure 4A, B). GAF domains are prominent regulatory input domains of DGCs such as REC and PAS domains and are able to bind small molecules such as cAMP and cGMP (Biswas et al. 2015; Charbonneau et al. 1990). However, it is unknown which molecules bind to the GAF domains present in DgcP.

DgcW

As DgcK, DgcW (also known as YkoW) is also a transmembrane protein with seven predicted transmembrane helices, which share homology to the so-called MHYT domain proposed to sense diatomic gases (O₂, CO or NO) through conserved residues that may coordinate copper ions (Li et al. 2013). DgcW contains a large cytoplasmic fraction at its C-terminus that can be further subdivided into the PAS sensory domain, GGDEF and EAL domains (Figure 4). *In vitro* analysis of the GGDEF domain revealed elevated c-di-GMP levels when the adjacent PAS domain was included implying also a potential stimulatory activity of this sensory domain *in vivo* (Gao et al. 2013). Overproduction of full-length DgcW in *B. subtilis* resulted in increased cellular level of c-di-GMP. Therefore, it was concluded that DgcW primarily possesses DGC activity. In contrast to DgcK and DgcP, which are both involved in motility repression and EPS production (see above), the function of DgcW is still unclear. Interestingly, *dgcW* belongs to the SigD regulon and is co-transcribed with various chemotaxis genes including *yoaH*, *mcpB* and *mcpA* (Arrieta-Ortiz et al. 2015; Nicolas et al. 2012), suggesting an involvement in motility control. This idea may seem counterintuitive at first glance, since DgcW was reported to act mainly as a DGC (Gao et al. 2013). However, DgcW harbors both, a GGDEF and an EAL domain, and it is unknown under which conditions or at all the EAL domain might be active. Importantly, DgcW displays a similar domain arrangement as MucR and NdbA from *Pseudomonas aeruginosa* (Hay et al. 2009; Li et al. 2013). These proteins are linked to nitric oxide (NO) sensing and biofilm dispersal. In particular, sensing of NO, most likely mediated by the MHYT domains of these proteins, leads to the activation of the downstream PDE domains and consequently to a drop in c-di-GMP levels. Low c-di-GMP levels have been shown to trigger biofilm dispersal in several other species (Lahiri et al. 2014). Therefore, it is tempting to speculate that DgcW, in particular its EAL domain, which needs further biochemical investigations, regulates motility upon biofilm dispersing conditions.

Degradation of c-di-GMP in *B. subtilis*

Two types of c-di-GMP specific phosphodiesterases (PDEs) degrade the second messenger c-di-GMP. EAL-type PDEs operate as magnesium- or manganese-dependent dimers and catalyze the opening of the cyclic nucleotide through hydrolysis of one phosphodiester bond giving rise to the linear dinucleotide 5'-phosphoguanylyl- (3',5')-guanosine (pGpG). In contrast to EAL-domain PDEs, the unrelated HD-GYP PDEs contain a HHExxDGxxGYP motif, are less abundant and are capable of complete c-di-GMP hydrolysis into GMP in a one-step reaction (Bellini et al. 2014). Similar to GGDEF-domain containing proteins, EAL and HD-GYP domains are frequently found in combination with sensory domains allowing for signal-dependent and regulated degradation of c-di-GMP. In *B. subtilis*, the EAL domain-containing protein PdeH is the only functional PDE capable of linearizing c-di-GMP into pGpG (Figure 4A). Of note; PdeH does not contain any additional sensing domains, which would allow a signal-dependent regulation of its activity. Hence, it was postulated that PdeH is mainly regulated at the level of gene expression (Chen et al. 2012), indicating a rather global role of this enzyme. Studies on the transcriptional regulation of *pdeH* further support this idea. The -35 and -10 promoter regions of *pdeH* harbor the promoter of the primary housekeeping σ -factor σ^A of *B. subtilis*, indicating that *pdeH* is constitutively transcribed during vegetative growth (Johnston et al. 2009; Weinrauch et al. 1989). Thus, c-di-GMP levels are presumably kept low during vegetative growth due to the c-di-GMP degrading activity of PdeH (Gao et al. 2013). Most notably, *pdeH* expression declines in the course of sporulation (Nicolas et al. 2012), and is under the negative control of the master regulator Spo0A~P (Molle et al. 2003; Weiss et al. 2019), further linking c-di-GMP signaling towards stationary phase and biofilm formation and/or sporulation (see also above). Noteworthy, the EAL domain of PdeH is followed by a HD-domain, predicted to have a metal-dependent phosphohydrolase activity (Aravind and Koonin 1998) (Figure 4). However, it remains to be clarified whether the EAL domain is solely responsible for c-di-GMP degradation or whether both domains are required for the breakdown.

Physiology and targets of c-di-GMP in *B. subtilis*

Inhibition of motility via MotI

Deletion of the only PDE enzyme PdeH results in elevated c-di-GMP levels and transient swimming and swarming

defects (Chen et al. 2012; Gao et al. 2013; Subramanian et al. 2017). Inhibition of motility could be rescued through deletion in the *motI* gene, encoding for a paralog of the *E. coli* YcgR. The MotI protein contains a conserved PilZ- and YcgR-domain and was shown to bind c-di-GMP *in vitro* with high affinity (Gao et al. 2013, Subramanian et al. 2017). When *motI* was constitutively expressed upon *pdeH* deletion, swarming motility was not only transiently blocked but almost completely suppressed suggesting different levels of *motI* expression under swarming conditions (Gao et al. 2013). In addition to its negative effect on swarming behavior, overproduced MotI also inhibited swimming behavior of *B. subtilis* as suggested by light microscopic analysis (Chen et al. 2012). Likewise, deletion of *motI* resulted in slightly altered biofilm morphologies reflected by accelerated wrinkle development and delayed disassembly of pellicles. C-di-GMP-bound MotI directly inhibits similarly to other YcgR-type proteins motility through its interaction with the flagellar motor component MotA to disturb motor-rotor interactions of the flagellar apparatus (Chen et al. 2012; Subramanian et al. 2017) (Figure 4A). However, it remains enigmatic to which extent DgcK, DgcP and DgcW contribute to regulation of motility. Importantly, MotI showed direct interactions with the GGDEF domain of DgcK and with full-length DgcP *in vitro* (Figure 4A) (Kunz et al. 2020). Additionally, fluorescent DgcK-mVenus and DgcP-mVenus fusions were drastically affected in their mobility when *motI* was absent as revealed by single molecule microscopy in living cells. Under these conditions (Δ *motI*), DgcK-mVenus and DgcP-mVenus arrested less frequently at the cell membrane. Thus, MotI most likely accepts c-di-GMP directly from the two DGCs DgcK and DgcP to regulate motility upon sensing of yet to be identified signals. It not clear whether the GGDEF-EAL domain protein DgcW provides further input into this modulation.

Activation of EPS synthesis via YdaK

Besides MotI, two other targets of c-di-GMP have been proposed in *B. subtilis*. These include the transmembrane protein YdaK, which harbors a degenerated GGDEF domain with a conserved I-site motif at its C-terminus (Chen et al. 2012; Gao et al. 2013) (Figure 4B). Deletion and overexpression of *ydaK* does not alter colony biofilm formation under standard laboratory conditions. Tiling microarrays suggested that *ydaK* is part of a general stress sigma factor σ^B -dependent operon (Figure 4D) together with the *ydaJKLMN* genes (Nicolas et al. 2012; Petersohn et al. 1999). *In silico* analysis suggested that this operon encodes for a polysaccharide-synthesizing machinery,

potentially controlled by c-di-GMP (Bedrunka and Graumann 2017a). Overexpression of *ydaJKLMN* led to cell clumping during exponential growth in liquid culture, increased Congo Red staining of colony biofilms and strongly altered biofilm architectures, implying a change in ECM composition. Furthermore, these phenotypes were depended on the presence of YdaK's I-site, its transmembrane domain and *dgc* genes (Bedrunka and Graumann, 2017a,b). Further biochemical and *in vivo* studies on YdaK showed that c-di-GMP interacts with the I-site motif present within its the GGDEF domain (Kunz et al. 2020). Biochemical studies showed that the c-di-GMP/I-site interaction of YdaK leads to major conformational changes within its entire GGDEF domain to propagate signal transduction. However, the way in which YdaK mediates c-di-GMP signal transfer to activate synthesis of the unknown EPS is unclear to date. Considering recent studies on c-di-GMP receptors, two scenarios are conceivable. Binding of c-di-GMP to YdaK's I-site may either link the protein to an unknown binding partner, presumably the proposed glycosyltransferase YdaM, or may instead favor an interruption of protein-protein interactions. Further biochemical studies are required to distinguish between these possibilities and to delineate the molecular mechanism.

As already stated, overexpression of *ydaJKLMN* does only result in detectable phenotypes when the *dgc* genes are present. However, under standard laboratory conditions, single deletion of *dgcK* is sufficient to inhibit YdaJKLMN-associated EPS synthesis (Bedrunka and Graumann 2017a). These and recently published experiments suggest that YdaK receives the second messenger c-di-GMP directly from DgcK to regulate EPS synthesis (Kunz et al. 2020) (Figure 4B). The idea that YdaK and DgcK form a local signaling pair is further supported by their colocalization *in vivo* as inferred from fluorescence microscopic experiments (Bedrunka and Graumann, 2017a,b). Moreover, the soluble GGDEF domains of YdaK and of DgcK are able to interact with each other *in vitro*. This interaction depends on a functional I-site in YdaK (Kunz et al. 2020). Whether DgcK's I-site does also participate in this interaction remains to be shown. Also an involvement of DgcP is conceivable. In summary, YdaK regulates the production of a yet to be characterized polysaccharide within the ECM of *B. subtilis* through c-di-GMP. Especially in the context of such localized structural machinery, it is reasonable to assume that the maintenance of a local c-di-GMP pool or gradient would allow the achievement of a rapid and efficient physiological response (EPS synthesis) with a minimal absolute change in the intracellular c-di-GMP concentration.

Putative functions of the newly identified EPS

Bacterial EPSs have been demonstrated to serve a plethora of functions. For example, they can have a role in water retention and serve as nutrient sources and energy sinks respectively, and furthermore offer protection against extracellular stress factors by providing a physical barrier (Nwodo et al. 2012). The physiological role of the EPS synthesized by YdaJ-N could not be determined so far. As mentioned earlier, the *ydaJ-N* operon is assigned to the σ_B -regulon, which is reflected in the upregulated transcription of corresponding elements upon stress treatment including ethanol stress (Boylan et al. 1993) (Figure 4D). This suggests that the unknown EPS might convey some stress resistance. Besides conveying stress resistance, the unknown EPS could be potentially also involved in biofilm formation, more precisely in the initial steps involving reversible attachment to surfaces. In addition to altered colony morphologies, induction of YdaJ-N also leads to the formation of cell aggregates in liquid media during exponential phase, indicating that the unknown EPS mediates cell-to-cell adhesion of proliferating cells. Distinct localization patterns of the YdaJ-N components (cell poles and mid cell) further support the view of locally administrated EPS synthesis. The observed cell aggregates could be easily resolved through vortexing, suggesting that only weak physical interactions are established. Such weak interactions represent an appropriate prerequisite for an EPS that might mediate reversible attachment of cells to a surface and/or reversible cell aggregation in the early stages of biofilm formation on a surface. Contrarily, a constitutive derepression of the major EPS synthesis operon *epsA-O* results in hyper-aggregated cells (Winkelman et al. 2009). Notably an induction of YdaJ-N cannot compensate for the loss of the biofilm essential EPS. The corresponding EPS, produced by the *epsA-O* products, does apparently convey stronger interactions in comparison to YdaJ-N's EPS, which are eventually not compatible with a reversible settlement of cells. Thus, it is possible that *B. subtilis* synthesizes distinct EPSs during different stages of biofilm formation.

The unknown signaling component YkuI

Despite MotI and YdaK, the protein YkuI has been proposed to serve as a c-di-GMP effector protein. Although YkuI has been structurally characterized in its apo- and c-di-GMP-bound states more than a decade ago as one of

the first EAL-domain structures (Minasov et al. 2009), its concrete functional role in *B. subtilis* is still mysterious. YkuI is a canonical EAL-domain protein that despite its capability to bind the second messenger did not exhibit PDE activity towards c-di-GMP *in vitro*. Furthermore, deletion of the *ykuI* gene did not alter motility as demonstrated for *pdeH* deletion (Chen et al. 2012). Consequently, it is now widely considered as a c-di-GMP output effector. Structural analysis of YkuI documented a TIM- (tri-osephosphate isomerase) barrel fold as the basic constituent of c-di-GMP-specific PDEs. Furthermore, Minasov and coworkers argued that YkuI contains, in contrast to active EAL domain proteins, a degenerated $\beta 5$ - $\alpha 5$ loop, which results in a nonproductive arrangement of active site residues and thus in an inability to degrade c-di-GMP (Minasov et al. 2009). Noteworthy, YkuI harbors besides the N-terminal located EAL domain an adjacent C-terminal domain that resembles a PAS-domain fold. Both domains are connected through a long linker helix and participate in the formation of a tight homodimer. The pocket-like C-terminal domain has the potential to accommodate a ligand and may therefore also function as a signaling domain affecting the quaternary structure and potentially also the catalytic activity towards c-di-GMP (Minasov et al. 2009). Hence, it still remains to be determined whether the putative sensory domain functions as a signal input domain to affect c-di-GMP binding and potentially hydrolysis by the EAL domain or as an output domain upon c-di-GMP binding to the EAL-domain altering its conformation (Wolfe and Visick 2008). Very recently, YkuI has been implicated to control zinc homeostasis in *B. subtilis* (Chandrangsu and Helmann 2016). Inactivation of *ykuI* and of the *fla-che* operon conferred resistance to high Zn (II) concentrations and it was hypothesized that these disruptions restrict the access of zinc presumably by increasing ECM production. While not reported for *B. subtilis* YkuI, the ortholog protein CdgJ (55% amino acid identity, 99% sequence coverage) harboring a degenerated EAL domain motif (EVV), influences biofilm formation and sporulation in *B. cereus* (Fagerlund et al. 2016).

Concluding remarks

Our review on the metabolism and physiology of the guanosine-based second messengers (p)ppGpp and c-di-GMP in the Gram-positive model organism *B. subtilis* impressively shows what has been learnt until now on the diversity of processes both molecule classes are involved in. However, the following major questions remain: What are the sensory inputs of the small alarmone synthetases

RelP and RelQ and what targets are addressed by their (p)ppGpp synthesized? Is our understanding of the diversity of (p)ppGpp targets complete or are there other yet to be discovered processes regulated by the alarmones? Equivalently, which mechanisms regulate the activities of the c-di-GMP synthetases DgcK, DgcP and DgcW? Are there further processes besides EPS production and motility regulated by c-di-GMP? Last but not least, a cross-regulation involving both, (p)ppGpp and c-di-GMP, in *B. subtilis* awaits further investigation. Taken together, our review summarizes the enormous regulatory potential of two ancient molecules from the former RNA world (Nelson and Breaker 2017).

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References

- Ababneh, Q.O., and Herman, J.K. (2015a). CodY regulates SigD levels and activity by binding to three sites in the *fla-che* operon. *J. Bacteriol.* 197: 2999–3006.
- Ababneh, Q.O., and Herman, J.K. (2015b). RelA inhibits *Bacillus subtilis* motility and chaining. *J. Bacteriol.* 197: 128–137.
- Anantharaman, V., and Aravind, L. (2003). Application of comparative genomics in the identification and analysis of novel families of membrane-associated receptors in bacteria. *BMC Genom.* 4: 34.
- Anderson, B.W., Hao, A., Satyshur, K.A., Keck, J.L., and Wang, J.D. (2020). Molecular mechanism of regulation of the purine salvage enzyme XPRT by the alarmones pppGpp, ppGpp, and pGpp. *J. Mol. Biol.* 432: 4108–4126.
- Anderson, B.W., Liu, K., Wolak, C., Dubiel, K., She, F., Satyshur, K. A., Keck, J.L., and Wang, J.D. (2019). Evolution of (p)ppGpp-HPRT regulation through diversification of an allosteric oligomeric interaction. *eLife* 8, <https://doi.org/10.7554/elife.47534>.
- Aravind, L., and Koonin, E.V. (1998). The HD domain defines a new superfamily of metal-dependent phosphohydrolases. *Trends Biochem. Sci.* 23: 469–472.
- Arenz, S., Abdelshahid, M., Sohmen, D., Payoe, R., Starosta, A.L., Berninghausen, O., Haurlyuk, V., Beckmann, R., and Wilson, D.N. (2016). The stringent factor RelA adopts an open conformation on the ribosome to stimulate ppGpp synthesis. *Nucleic Acids Res.* 44: 6471–6481.
- Arrieta-Ortiz, M.L., Hafemeister, C., Bate, A.R., Chu, T., Greenfield, A., Shuster, B., Barry, S.N., Gallitto, M., Liu, B., Kacmarczyk, T., et al.

- (2015). An experimentally supported model of the *Bacillus subtilis* global transcriptional regulatory network. *Mol. Syst. Biol.* 11: 839.
- Artsimovitch, I. (2010). A processive riboantiterminator seeks a switch to make biofilms. *Mol. Microbiol.* 76: 535–539.
- Atkinson, G.C., Tenson, T., and Haurlyuk, V. (2011). The RelA/SpoT homolog (RSH) superfamily: distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life. *PLoS One* 6: e23479.
- Beauregard, P.B., Chai, Y., Vlamakis, H., Losick, R., and Kolter, R. (2013). *Bacillus subtilis* biofilm induction by plant polysaccharides. *Proc. Natl. Acad. Sci. U. S. A.* 110: E1621–E1630.
- Beckert, B., Abdelshahid, M., Schafer, H., Steinchen, W., Arenz, S., Berninghausen, O., Beckmann, R., Bange, G., Turgay, K., and Wilson, D.N. (2017). Structure of the *Bacillus subtilis* hibernating 100S ribosome reveals the basis for 70S dimerization. *EMBO J.* 36: 2061–2072.
- Bedrunka, P., and Graumann, P.L. (2017a). New functions and subcellular localization patterns of c-di-GMP components (GGDEF Domain Proteins) in *B. subtilis*. *Front. Microbiol.* 8: 794.
- Bedrunka, P., and Graumann, P.L. (2017b). Subcellular clustering of a putative c-di-GMP-dependent exopolysaccharide machinery affecting macro colony architecture in *Bacillus subtilis*. *Environ. Microbiol. Rep.* 9: 211–222.
- Belitsky, B.R., and Sonenshein, A.L. (2013). Genome-wide identification of *Bacillus subtilis* CodY-binding sites at single-nucleotide resolution. *Proc. Natl. Acad. Sci. U. S. A.* 110: 7026–7031.
- Beljantseva, J., Kudrin, P., Andresen, L., Shingler, V., Atkinson, G. C., Tenson, T., and Haurlyuk, V. (2017). Negative allosteric regulation of *Enterococcus faecalis* small alarmone synthetase RelQ by single-stranded RNA. *Proc. Natl. Acad. Sci. U. S. A.* 114: 3726–3731.
- Bellini, D., Caly, D.L., McCarthy, Y., Bumann, M., An, S.Q., Dow, J.M., Ryan, R.P., and Walsh, M.A. (2014). Crystal structure of an HD-GYP domain cyclic-di-GMP phosphodiesterase reveals an enzyme with a novel trinuclear catalytic iron centre. *Mol. Microbiol.* 91: 26–38.
- Benigar, E., Dogsa, I., Stopar, D., Jamnik, A., Kralj Cigic, I., and Tomsic, M. (2014). Structure and dynamics of a polysaccharide matrix: aqueous solutions of bacterial levan. *Langmuir* 30: 4172–4182.
- Bennison, D.J., Irving, S.E., and Corrigan, R.M. (2019). The impact of the stringent response on TRAFAC GTPases and prokaryotic ribosome assembly. *Cells* 8: 1313.
- Biswas, K.H., Badireddy, S., Rajendran, A., Anand, G.S., and Visweswariah, S.S. (2015). Cyclic nucleotide binding and structural changes in the isolated GAF domain of *Anabaena* adenyl cyclase, CyaB2. *PeerJ* 3: e882.
- Bittner, A.N., Kriel, A., and Wang, J.D. (2014). Lowering GTP level increases survival of amino acid starvation but slows growth rate for *Bacillus subtilis* cells lacking (p)ppGpp. *J. Bacteriol.* 196: 2067–2076.
- Boylan, S.A., Redfield, A.R., Brody, M.S., and Price, C.W. (1993). Stress-induced activation of the sigma B transcription factor of *Bacillus subtilis*. *J. Bacteriol.* 175: 7931–7937.
- Branda, S.S., Chu, F., Kearns, D.B., Losick, R., and Kolter, R. (2006). A major protein component of the *Bacillus subtilis* biofilm matrix. *Mol. Microbiol.* 59: 1229–1238.
- Branda, S.S., Gonzalez-Pastor, J.E., Ben-Yehuda, S., Losick, R., and Kolter, R. (2001). Fruiting body formation by *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U. S. A.* 98: 11621–11626.
- Britton, R.A. (2009). Role of GTPases in bacterial ribosome assembly. *Annu. Rev. Microbiol.* 63: 155–176.
- Bucker, H. (1974). The biostack experiments I and II aboard Apollo 16 and 17. *Life Sci. Space Res.* 12: 43–50.
- Bucker, H., Horneck, G., Wollenhaupt, H., Schwager, M., and Taylor, G.R. (1974). Viability of *Bacillus subtilis* spores exposed to space environment in the M-191 experiment system aboard Apollo 16. *Life Sci. Space Res.* 12: 209–213.
- Buglino, J., Shen, V., Hakimian, P., and Lima, C.D. (2002). Structural and biochemical analysis of the obg GTP binding protein. *Structure* 10: 1581–1592.
- Cairns, L.S., Hobley, L., and Stanley-Wall, N.R. (2014). Biofilm formation by *Bacillus subtilis*: new insights into regulatory strategies and assembly mechanisms. *Mol. Microbiol.* 93: 587–598.
- Candela, T., and Fouet, A. (2006). Poly-gamma-glutamate in bacteria. *Mol. Microbiol.* 60: 1091–1098.
- Cashel, M. (1996). The stringent response. *Escherichia coli* and *Salmonella typhimurium*: Cell. Mol. Biol. 2: 1458–1496.
- Cashel, M., and Gallant, J. (1969). Two compounds implicated in the function of the RC gene of *Escherichia coli*. *Nature* 221: 838–841.
- Chandrangsu, P., and Helmann, J.D. (2016). Intracellular Zn(II) intoxication leads to dysregulation of the PerR regulon resulting in heme toxicity in *Bacillus subtilis*. *PLoS Genet.* 12: e1006515.
- Charbonneau, H., Prusti, R.K., Letrong, H., Sonnenburg, W.K., Mullaney, P.J., Walsh, K.A., and Beavo, J.A. (1990). Identification of a noncatalytic cGMP-binding domain conserved in both the cGMP-stimulated and photoreceptor cyclic nucleotide phosphodiesterases. *Proc. Natl. Acad. Sci. U. S. A.* 87: 288–292.
- Chen, Y., Chai, Y., Guo, J.H., and Losick, R. (2012). Evidence for cyclic Di-GMP-mediated signaling *Bacillus subtilis*. *J. Bacteriol.* 194: 5080–5090.
- Chen, L.H., Koseoglu, V.K., Guvener, Z.T., Myers-Morales, T., Reed, J. M., D'orazio, S.E., Miller, K.W., and Gomelsky, M. (2014). Cyclic di-GMP-dependent signaling pathways in the pathogenic Firmicute *Listeria monocytogenes*. *PLoS Pathog.* 10, e1004301.
- Christen, B., Christen, M., Paul, R., Schmid, F., Folcher, M., Jenoe, P., Meuwly, M., and Jenal, U. (2006). Allosteric control of cyclic di-GMP signaling. *J. Biol. Chem.* 281: 32015–32024.
- Corrigan, R.M., Bellows, L.E., Wood, A., and Grundling, A. (2016). ppGpp negatively impacts ribosome assembly affecting growth and antimicrobial tolerance in Gram-positive bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 113: E1710–E1719.
- Dahlstrom, K.M., Giglio, K.M., Sondermann, H., and O'toole, G.A. (2016). The inhibitory site of a diguanylate cyclase is a necessary element for interaction and signaling with an effector protein. *J. Bacteriol.* 198: 1595–1603.
- Dahlstrom, K.M., and O'Toole, G.A. (2017). A symphony of cyclases: specificity in diguanylate cyclase signaling. *Annu. Rev. Microbiol.* 71: 179–195.
- De, N., Navarro, M.V., Raghavan, R.V., and Sondermann, H. (2009). Determinants for the activation and autoinhibition of the diguanylate cyclase response regulator WspR. *J. Mol. Biol.* 393: 619–633.

- De, N., Pirruccello, M., Krasteva, P.V., Bae, N., Raghavan, R.V., and Sondermann, H. (2008). Phosphorylation-independent regulation of the diguanylate cyclase WspR. *PLoS Biol.* 6: e67.
- Denapoli, J., Tehranchi, A.K., and Wang, J.D. (2013). Dose-dependent reduction of replication elongation rate by (p)ppGpp in *Escherichia coli* and *Bacillus subtilis*. *Mol. Microbiol.* 88: 93–104.
- Dennis, P.G., Miller, A.J., and Hirsch, P.R. (2010). Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? *FEMS Microbiol. Ecol.* 72: 313–327.
- Diethmaier, C., Newman, J.A., Kovacs, A.T., Kaever, V., Herzberg, C., Rodrigues, C., Boonstra, M., Kuipers, O.P., Lewis, R.J., and Stulke, J. (2014). The YmdB phosphodiesterase is a global regulator of late adaptive responses in *Bacillus subtilis*. *J. Bacteriol.* 196: 265–275.
- Dragos, A., Kiesewalter, H., Martin, M., Hsu, C.Y., Hartmann, R., Wechsler, T., Eriksen, C., Brix, S., Drescher, K., Stanley-Wall, N., et al. (2018a). Division of labor during biofilm matrix production. *Curr. Biol.* 28: 1903–1913 e5.
- Dragos, A., Martin, M., Falcon Garcia, C., Kricks, L., Pausch, P., Heimerl, T., Balint, B., Maroti, G., Bange, G., Lopez, D., et al. (2018b). Collapse of genetic division of labour and evolution of autonomy in pellicle biofilms. *Nat. Microbiol.* 3: 1451–1460.
- Earl, C., Arnaouteli, S., Bamford, N.C., Porter, M., Sukhodub, T., Macphée, C.E., and Stanley-Wall, N.R. (2020). The majority of the matrix protein TapA is dispensable for *Bacillus subtilis* colony biofilm architecture. *Mol. Microbiol.*, 1–14. <https://doi.org/10.1111/mmi.14559>.
- Eymann, C., Homuth, G., Scharf, C., and Hecker, M. (2002). *Bacillus subtilis* functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. *J. Bacteriol.* 184: 2500–2520.
- Fagerlund, A., Smith, V., Rohr, A.K., Lindback, T., Parmer, M.P., Andersson, K.K., Reubsaet, L., and Okstad, O.A. (2016). Cyclic diguanylate regulation of *Bacillus cereus* group biofilm formation. *Mol. Microbiol.* 101: 471–494.
- Faxén, M., and Isaksson, L.A. (1994). Functional interactions between translation, transcription and ppGpp in growing *Escherichia coli*. *Biochim. Biophys. Acta Gene Struct. Expr.* 1219: 425–434.
- Gallant, J., Irr, J., and Cashel, M. (1971). The mechanism of amino acid control of guanylate and adenylate biosynthesis. *J. Biol. Chem.* 246: 5812–5816.
- Galperin, M.Y. (2018). What bacteria want. *Environ. Microbiol.* 20: 4221–4229.
- Gao, X., Mukherjee, S., Matthews, P.M., Hammad, L.A., Kearns, D.B., and Dann, C.E. (2013). Functional characterization of core components of the *Bacillus subtilis* cyclic-di-GMP signaling pathway. *J. Bacteriol.* 195: 4782–4792.
- Geiger, T., Kastle, B., Gratani, F.L., Goerke, C., and Wolz, C. (2014). Two small (p)ppGpp synthases in *Staphylococcus aureus* mediate tolerance against cell envelope stress conditions. *J. Bacteriol.* 196: 894–902.
- Giacalone, D., Smith, T.J., Collins, A.J., Sondermann, H., Koziol, L.J., and O’toole, G.A. (2018). Ligand-mediated biofilm formation via enhanced physical interaction between a diguanylate cyclase and its receptor. *mBio* 9: e01254-18, <https://doi.org/10.1128/mbio.01254-18>.
- Gohara, D.W., and Yap, M.-N.F. (2018). Survival of the drowsiest: the hibernating 100S ribosome in bacterial stress management. *Curr. Genet.* 64: 753–760.
- Guttenplan, S.B., Shaw, S., and Kearns, D.B. (2013). The cell biology of peritrichous flagella in *Bacillus subtilis*. *Mol. Microbiol.* 87: 211–229.
- Hamon, M.A., Stanley, N.R., Britton, R.A., Grossman, A.D., and Lazazzera, B.A. (2004). Identification of AbrB-regulated genes involved in biofilm formation by *Bacillus subtilis*. *Mol. Microbiol.* 52: 847–860.
- Handke, L.D., Shivers, R.P., and Sonenshein, A.L. (2008). Interaction of *Bacillus subtilis* CodY with GTP. *J. Bacteriol.* 190: 798–806.
- Hay, I.D., Remminghorst, U., and Rehm, B.H. (2009). MucR, a novel membrane-associated regulator of alginate biosynthesis in *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 75: 1110–1120.
- Hengge, R. (2009). Principles of c-di-GMP signalling in bacteria. *Nat. Rev. Microbiol.* 7: 263–273.
- Hobley, L., Ostrowski, A., Rao, F.V., Bromley, K.M., Porter, M., Prescott, A.R., Macphée, C.E., Van Aalten, D.M., and Stanley-Wall, N.R. (2013). BslA is a self-assembling bacterial hydrophobin that coats the *Bacillus subtilis* biofilm. *Proc. Natl. Acad. Sci. U. S. A.* 110: 13600–13605.
- Hogg, T., Mechold, U., Malke, H., Cashel, M., and Hilgenfeld, R. (2004). Conformational antagonism between opposing active sites in a bifunctional RelA/SpoT homolog modulates (p)ppGpp metabolism during the stringent response. *Cell* 117: 57–68.
- Hong, H.A., Khaneja, R., Tam, N.M., Cazzato, A., Tan, S., Urdaci, M., Brisson, A., Gasbarrini, A., Barnes, I., and Cutting, S.M. (2009). *Bacillus subtilis* isolated from the human gastrointestinal tract. *Res. Microbiol.* 160: 134–143.
- Irnov, I., and Winkler, W.C. (2010). A regulatory RNA required for antitermination of biofilm and capsular polysaccharide operons in Bacillales. *Mol. Microbiol.* 76: 559–575.
- Jenal, U., Reinders, A., and Lori, C. (2017). Cyclic di-GMP: second messenger extraordinaire. *Nat. Rev. Microbiol.* 15: 271–284.
- Johnston, E.B., Lewis, P.J., and Griffith, R. (2009). The interaction of *Bacillus subtilis* sigmaA with RNA polymerase. *Protein Sci.* 18: 2287–2297.
- Kearns, D.B. (2008). Division of labour during *Bacillus subtilis* biofilm formation. *Mol. Microbiol.* 67: 229–231.
- Kearns, D.B., and Losick, R. (2005). Cell population heterogeneity during growth of *Bacillus subtilis*. *Genes Dev.* 19: 3083–3094.
- Kobayashi, K., and Iwano, M. (2012). BslA(YuaB) forms a hydrophobic layer on the surface of *Bacillus subtilis* biofilms. *Mol. Microbiol.* 85: 51–66.
- Krasny, L., and Gourse, R.L. (2004). An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation. *EMBO J.* 23: 4473–4483.
- Krasny, L., Tisero, H., Jonak, J., Rejman, D., and Sanderova, H. (2008). The identity of the transcription +1 position is crucial for changes in gene expression in response to amino acid starvation in *Bacillus subtilis*. *Mol. Microbiol.* 69: 42–54.
- Kriel, A., Bittner, A.N., Kim, S.H., Liu, K., Tehranchi, A.K., Zou, W.Y., Rendon, S., Chen, R., Tu, B.P., and Wang, J.D. (2012). Direct regulation of GTP homeostasis by (p)ppGpp: a critical component of viability and stress resistance. *Mol. Cell* 48: 231–241.
- Kriel, A., Brinsmade, S.R., Tse, J.L., Tehranchi, A.K., Bittner, A.N., Sonenshein, A.L., and Wang, J.D. (2014). GTP dysregulation in *Bacillus subtilis* cells lacking (p)ppGpp results in phenotypic amino acid auxotrophy and failure to adapt to nutrient downshift and regulate biosynthesis genes. *J. Bacteriol.* 196: 189–201.
- Kunz, S., Tribensky, A., Steinchen, W., Oviedo-Bocanegra, L., Bedrunka, P., and Graumann, P.L. (2020). Cyclic di-GMP

- signaling in *Bacillus subtilis* is governed by direct interactions of diguanylate cyclases and cognate receptors. *mBio* 11: e03122-19, <https://doi.org/10.1128/mbio.03122-19>.
- Lahiri, T., Luan, B., Raleigh, D.P., and Boon, E.M. (2014). A structural basis for the regulation of an H-NOX-associated cyclic-di-GMP synthase/phosphodiesterase enzyme by nitric oxide-bound H-NOX. *Biochemistry* 53: 2126–2135.
- Lecompte, O., Ripp, R., Thierry, J. C., Moras, D., and Poch, O. (2002). Comparative analysis of ribosomal proteins in complete genomes: an example of reductive evolution at the domain scale. *Nucleic Acids Res.* 30: 5382–5390.
- Lemos, J.A., Lin, V.K., Nascimento, M.M., Abranches, J., and Burne, R. A. (2007). Three gene products govern (p)ppGpp production by *Streptococcus mutans*. *Mol. Microbiol.* 65: 1568–1581.
- Li, Y., Heine, S., Entian, M., Sauer, K., and Frankenberg-Dinkel, N. (2013). NO-induced biofilm dispersion in *Pseudomonas aeruginosa* is mediated by an MHYT domain-coupled phosphodiesterase. *J. Bacteriol.* 195: 3531–3542.
- Liu, K., Bittner, A.N., and Wang, J.D. (2015a). Diversity in (p) ppGpp metabolism and effectors. *Curr. Opin. Microbiol.* 24: 72–79.
- Liu, K., Myers, A.R., Pisithkul, T., Claas, K.R., Satyshur, K.A., Amador-Noguez, D., Keck, J.L., and Wang, J.D. (2015b). Molecular mechanism and evolution of guanylate kinase regulation by (p) ppGpp. *Mol. Cell* 57: 735–749.
- Loveland, A.B., Bah, E., Madireddy, R., Zhang, Y., Brilot, A.F., Grigorieff, N., and Korostelev, A.A. (2016). Ribosome*RelA structures reveal the mechanism of stringent response activation. *eLife* 5: e17029, <https://doi.org/10.7554/elife.17029>.
- Lucy, M., Reed, E., and Glick, B.R. (2004). Applications of free living plant growth-promoting rhizobacteria. *Antonie Leeuwenhoek* 86: 1–25, <https://doi.org/10.1023/b:anto.0000024903.10757.6e>.
- Manav, M.C., Beljantseva, J., Bojer, M.S., Tenson, T., Ingmer, H., Haurlyuk, V., and Brodersen, D.E. (2018). Structural basis for (p) ppGpp synthesis by the *Staphylococcus aureus* small alarmone synthetase RelP. *J. Biol. Chem.* 293: 3254–3264.
- Marvasi, M., Visscher, P.T., and Casillas Martinez, L. (2010). Exopolymeric substances (EPS) from *Bacillus subtilis*: polymers and genes encoding their synthesis. *FEMS Microbiol. Lett.* 313: 1–9.
- Matzov, D., Bashan, A., Yap, M.N.F., and Yonath, A. (2019). Stress response as implemented by hibernating ribosomes: a structural overview. *FEBS J.* 286: 3558–3565.
- McKenney, P.T., Driks, A., and Eichenberger, P. (2013). The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. *Nat. Rev. Microbiol.* 11: 33–44.
- McLoon, A.L., Guttenplan, S.B., Kearns, D.B., Kolter, R., and Losick, R. (2011). Tracing the domestication of a biofilm-forming bacterium. *J. Bacteriol.* 193: 2027–2034.
- Milon, P., Tischenko, E., Tomšić, J., Caserta, E., Folkers, G., La Teana, A., Rodnina, M.V., Pon, C.L., Boelens, R., and Gualerzi, C.O. (2006). The nucleotide-binding site of bacterial translation initiation factor 2 (IF2) as a metabolic sensor. *Proc. Natl. Acad. Sci. U. S. A.* 103: 13962–13967.
- Minasov, G., Padavattan, S., Shuvalova, L., Brunzelle, J.S., Miller, D.J., Basle, A., Massa, C., Collart, F.R., Schirmer, T., and Anderson, W.F. (2009). Crystal structures of YkuL and its complex with second messenger cyclic Di-GMP suggest catalytic mechanism of phosphodiester bond cleavage by EAL domains. *J. Biol. Chem.* 284: 13174–13184.
- Molina-Santiago, C., and Matilla, M.A. (2019). Chemical fertilization: a short-term solution for plant productivity?. *Microb. Biotechnol.* 13: 1311–1313.
- Molle, V., Fujita, M., Jensen, S.T., Eichenberger, P., Gonzalez-Pastor, J. E., Liu, J.S., and Losick, R. (2003). The Spo0A regulon of *Bacillus subtilis*. *Mol. Microbiol.* 50: 1683–1701.
- Nanamiya, H., Kasai, K., Nozawa, A., Yun, C.S., Narisawa, T., Murakami, K., Natori, Y., Kawamura, F., and Tozawa, Y. (2008). Identification and functional analysis of novel (p)ppGpp synthetase genes in *Bacillus subtilis*. *Mol. Microbiol.* 67: 291–304.
- Nelson, J.W., and Breaker, R.R. (2017). The lost language of the RNA World. *Sci. Signal.* 10, <https://doi.org/10.1126/scisignal.aam8812>.
- Nicolas, P., Mader, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., Bidnenko, E., Marchadier, E., Hoebeke, M., Aymerich, S., et al. (2012). Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. *Science* 335: 1103–1106.
- Nierhaus, K.H., and Lafontaine, D.L. (2004). Ribosome assembly. *Protein Synth. Ribosome Struct.:* 85–143.
- Nwodo, U.U., Green, E., and Okoh, A.I. (2012). Bacterial exopolysaccharides: functionality and prospects. *Int. J. Mol. Sci.* 13: 14002–14015.
- Ochi, K., Kandala, J.C., and Freese, E. (1981). Initiation of *Bacillus subtilis* sporulation by the stringent response to partial amino acid deprivation. *J. Biol. Chem.* 256: 6866–6875.
- Ochi, K., Kandala, J., and Freese, E. (1982). Evidence that *Bacillus subtilis* sporulation induced by the stringent response is caused by the decrease in GTP or GDP. *J. Bacteriol.* 151: 1062–1065.
- Ogilvie, A., Wiebauer, K., and Kersten, W. (1975). Stringent control of ribonucleic acid synthesis in *Bacillus subtilis* treated with granaticin. *Biochem. J.* 152: 517–522.
- Orr, M.W., Weiss, C.A., Severin, G.B., Turdiev, H., Kim, S.K., Turdiev, A., Liu, K., Tu, B.P., Waters, C.M., Winkler, W.C., and Lee, V.T. (2018). A subset of exoribonucleases serve as degradative enzymes for pGpG in c-di-GMP signaling. *J. Bacteriol.* 200: e00300-18, <https://doi.org/10.1128/jb.00300-18>.
- Ostrowski, A., Mehert, A., Prescott, A., Kiley, T.B., and Stanley-Wall, N.R. (2011). YuaB functions synergistically with the exopolysaccharide and TasA amyloid fibers to allow biofilm formation by *Bacillus subtilis*. *J. Bacteriol.* 193: 4821–4831.
- Pao, C.C., and Dyess, B.T. (1981). Effect of unusual guanosine nucleotides on the activities of some *Escherichia coli* cellular enzymes. *Biochim. Biophys. Acta* 677: 358–362.
- Parkinson, J.S. (1993). Signal transduction schemes of bacteria. *Cell* 73: 857–871.
- Paul, R., Abel, S., Wassmann, P., Beck, A., Heerklotz, H., and Jenal, U. (2007). Activation of the diguanylate cyclase PleD by phosphorylation-mediated dimerization. *J. Biol. Chem.* 282: 29170–29177.
- Pausch, P., Steinchen, W., Wieland, M., Klaus, T., Freibert, S.A., Altegoer, F., Wilson, D.N., and Bange, G. (2018). Structural basis for (p)ppGpp-mediated inhibition of the GTPase RbgA. *J. Biol. Chem.* 293: 19699–19709.
- Petersohn, A., Antelmann, H., Gerth, U., and Hecker, M. (1999). Identification and transcriptional analysis of new members of the sigmaB regulon in *Bacillus subtilis*. *Microbiology* 145: 869–880.
- Prossliner, T., Skovbo Winther, K., Sørensen, M.A., and Gerdes, K. (2018). Ribosome hibernation. *Annu. Rev. Genet.* 52: 321–348.

- Rhaese, H.J., Dichtelmuller, H., and Grade, R. (1975). Studies on the control of development. Accumulation of guanosine tetraphosphate and pentaphosphate in response to inhibition of protein synthesis in *Bacillus subtilis*. *Eur. J. Biochem.* 56: 385–392.
- Rodnina, M.V. (2018). Translation in prokaryotes. *Cold Spring Harb. Perspect. Biol.* 10: a032664.
- Rojas, A.-M., Ehrenberg, M., Andersson, S.G., and Kurland, C. (1984). ppGpp inhibition of elongation factors Tu, G and Ts during polypeptide synthesis. *Mol. Gen. Genet.* 197: 36–45.
- Romero, D., Aguilar, C., Losick, R., and Kolter, R. (2010). Amyloid fibers provide structural integrity to *Bacillus subtilis* biofilms. *Proc. Natl. Acad. Sci. U. S. A.* 107: 2230–2234.
- Romling, U., Galperin, M.Y., and Gomelsky, M. (2013). Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol. Mol. Biol. Rev.* 77: 1–52.
- Roux, D., Cywes-Bentley, C., Zhang, Y.F., Pons, S., Konkol, M., Kearns, D.B., Little, D.J., Howell, P.L., Skurnik, D., and Pier, G.B. (2015). Identification of poly-N-acetylglucosamine as a major polysaccharide component of the *Bacillus subtilis* biofilm matrix. *J. Biol. Chem.* 290: 19261–19272.
- Rymer, R.U., Solorio, F.A., Tehranchi, A.K., Chu, C., Corn, J.E., Keck, J. L., Wang, J.D., and Berger, J.M. (2012). Binding mechanism of metalNTP substrates and stringent-response alarmones to bacterial DnaG-type primases. *Structure* 20: 1478–1489.
- Schafer, H., Beckert, B., Frese, C.K., Steinchen, W., Nuss, A.M., Beckstette, M., Hantke, I., Driller, K., Sudzinova, P., Krasny, L., et al. (2020). The alarmones (p)ppGpp are part of the heat shock response of *Bacillus subtilis*. *PLoS Genet.* 16: e1008275.
- Schirmer, T. (2016). C-di-GMP synthesis: structural aspects of evolution, catalysis and regulation. *J. Mol. Biol.* 428: 3683–3701.
- Schuhmacher, J.S., Thormann, K.M., and Bange, G. (2015). How bacteria maintain location and number of flagella?. *FEMS Microbiol. Rev.* 39: 812–822.
- Seshasayee, A.S., Fraser, G.M., and Luscombe, N.M. (2010). Comparative genomics of cyclic-di-GMP signalling in bacteria: post-translational regulation and catalytic activity. *Nucleic Acids Res.* 38: 5970–5981.
- Setlow, P. (1974). Percent charging of transfer ribonucleic acid and levels of ppGpp and pppGpp in dormant and germinated spores of *Bacillus megaterium*. *J. Bacteriol.* 118: 1067–1074.
- Sommerfeldt, N., Possling, A., Becker, G., Pesavento, C., Tschowri, N., and Hengge, R. (2009). Gene expression patterns and differential input into curli fimbriae regulation of all GGDEF/EAL domain proteins in *Escherichia coli*. *Microbiology* 155: 1318–1331.
- Soule, H.M. (1932). Identity of *Bacillus subtilis*, Cohn 1872. *J. Infect. Dis.* 51: 191–215.
- Steinchen, W., and Bange, G. (2016). The magic dance of the alarmones (p)ppGpp. *Mol. Microbiol.* 101: 531–544.
- Steinchen, W., Schuhmacher, J.S., Altegoer, F., Fage, C.D., Srinivasan, V., Linne, U., Marahiel, M.A., and Bange, G. (2015). Catalytic mechanism and allosteric regulation of an oligomeric (p)ppGpp synthetase by an alarmone. *Proc. Natl. Acad. Sci. U. S. A.* 112: 13348–13353.
- Steinchen, W., Vogt, M.S., Altegoer, F., Giammarinaro, P.I., Horvatek, P., Wolz, C., and Bange, G. (2018). Structural and mechanistic divergence of the small (p)ppGpp synthetases RelP and RelQ. *Sci. Rep.* 8: 2195.
- Subramanian, S., Gao, X., Dann, C.E. 3RD, and Kearns, D.B. (2017). MotI (DgrA) acts as a molecular clutch on the flagellar stator protein MotA in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U. S. A.* 114: 13537–13542.
- Sutherland, E.W., and Rall, T.W. (1958). Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. *J. Biol. Chem.* 232: 1077–1091.
- Tagami, K., Nanamiya, H., Kazo, Y., Maehashi, M., Suzuki, S., Namba, E., Hoshiya, M., Hanai, R., Tozawa, Y., Morimoto, T., et al. (2012). Expression of a small (p)ppGpp synthetase, YwaC, in the (p) ppGpp(0) mutant of *Bacillus subtilis* triggers YvyD-dependent dimerization of ribosome. *Microbiologyopen* 1: 115–134.
- Vlamakis, H., Chai, Y., Beauregard, P., Losick, R., and Kolter, R. (2013). Sticking together: building a biofilm the *Bacillus subtilis* way. *Nat. Rev. Microbiol.* 11: 157–168.
- Wang, J.D., Sanders, G.M., and Grossman, A.D. (2007). Nutritional control of elongation of DNA replication by (p)ppGpp. *Cell* 128: 865–875.
- Wassmann, P., Chan, C., Paul, R., Beck, A., Heerklotz, H., Jenal, U., and Schirmer, T. (2007). Structure of BeF₃⁻-modified response regulator PleD: implications for diguanylate cyclase activation, catalysis, and feedback inhibition. *Structure* 15: 915–927.
- Weinrauch, Y., Guillen, N., and Dubnau, D.A. (1989). Sequence and transcription mapping of *Bacillus subtilis* competence genes comB and comA, one of which is related to a family of bacterial regulatory determinants. *J. Bacteriol.* 171: 5362–5375.
- Weiss, C.A., Hoberg, J.A., Liu, K., Tu, B.P., and Winkler, W.C. (2019). Single-cell microscopy reveals that levels of cyclic di-GMP vary among *Bacillus subtilis* subpopulations. *J. Bacteriol.* 201: e00247-19, <https://doi.org/10.1128/jb.00247-19>.
- Weiss, C.A., and Winkler, W.C. (2020). Cyclic di-GMP signaling in *Bacillus subtilis*. In: Chou, S.H., Guilian, N., Lee, V.T., and Römling, U. (Eds.). *Microbial cyclic di-nucleotide signaling*. Springer, Cham, pp. 241–260, <https://doi.org/10.1007/978-3-030-33308-9>.
- Wendrich, T.M., Blaha, G., Wilson, D.N., Marahiel, M.A., and Nierhaus, K.H. (2002). Dissection of the mechanism for the stringent factor RelA. *Mol. Cell* 10: 779–788.
- Winkelman, J.T., Blair, K.M., and Kearns, D.B. (2009). RemA (YlzA) and RemB (YaaB) regulate extracellular matrix operon expression and biofilm formation in *Bacillus subtilis*. *J. Bacteriol.* 191: 3981–3991.
- Wolfe, A.J., and Visick, K.L. (2008). Get the message out: cyclic-Di-GMP regulates multiple levels of flagellum-based motility. *J. Bacteriol.* 190: 463–475.
- Zeigler, D.R., Pragai, Z., Rodriguez, S., Chevreux, B., Muffler, A., Albert, T., Bai, R., Wyss, M., and Perkins, J.B. (2008). The origins of 168, W23, and other *Bacillus subtilis* legacy strains. *J. Bacteriol.* 190: 6983–6995.
- Zhang, Y., Zborníková, E., Rejman, D., and Gerdes, K. (2018). Novel (p) ppGpp binding and metabolizing proteins of *Escherichia coli*. *mBio* 9, e02188-17, <https://doi.org/10.1128/mbio.02188-17>.