Review

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Cyclic di-GMP signaling controlling the free-living lifestyle of alpha-proteobacterial rhizobia

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Abstract: Cyclic-di-GMP (c-di-GMP) is a ubiquitous bacterial second messenger which has been associated with a motile to sessile lifestyle switch in many bacteria. Here, we review recent insights into c-di-GMP regulated processes related to environmental adaptations in alphaproteobacterial rhizobia, which are diazotrophic bacteria capable of fixing nitrogen in symbiosis with their leguminous host plants. The review centers on *Sinorhizobium meliloti*, which in the recent years was intensively studied for its c-di-GMP regulatory network.

Keywords: cyclic dinucleotide second messengers; extracellular polysaccharides; motility; Rhizobiaceae; *Sinorhizobium meliloti*; sessile-motile switch.

Introduction

In their natural environment, bacteria constantly face changes of conditions and need to adapt their behavior in order to maximize propagation and minimize the risk of damage. Signal perception and transmission are key processes for generating physiological, genetic and cellular adaptive responses. In bacteria, various signaling systems are based on direct sensing of the cue by regulatory proteins, for example transcription factors and sensors belonging to two-component regulatory systems (Stock et al. 2000). Other signaling pathways involve second messenger molecules. Nucleotide second messengers include the stringent response alarmones guanosine tetra- and pentaphosphate, and versatile function cyclic mono-, di and trinucleotides (Gomelsky 2011; Gründling and Lee 2016; Krasteva and Sondermann 2017; Severin and Waters 2019).

Bis-(3′,5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) has emerged as one of the most ubiquitous and versatile bacterial second messengers. Activities of c-di-GMP metabolizing enzymes are subject to regulation by internal or environmental factors, whereas binding of the second messenger to the cognate receptors exerts action on the regulatory targets and triggers the physiological response. Computational prediction of c-di-GMP metabolizing proteins as well as experimental evidence revealed their broad distribution among the major phylogenetic branches of bacteria, such as Proteobacteria, Spirochetes, Cyanobacteria, Actinobacteria and Firmicutes (Galperin et al. 2001). Genes encoding these proteins are present in multiple copies per genome and their number can vary significantly even between species from the same genus (Bobrov et al. 2011). Comparative genomic analyses indicated that free-living bacteria with complex environmental lifestyles carry far more c-di-GMP metabolizing enzymes than obligate parasites (Galperin 2005), consistent with an important role of this second messenger in environmental adaptation. Regulation based on c-di-GMP signaling takes place at transcriptional, post-transcriptional and post-translational levels. It has key roles in physiological regulation determining bacterial lifestyle, including regulation of cellular processes such as biofilm formation and dispersal, motility, virulence, cell cycle and differentiation (Jenal et al. 2017). The number of identified targets and mechanisms of c-di-GMP-mediated regulation is constantly increasing.

Biological nitrogen fixation, performed exclusively by bacteria, is a primary source of combined nitrogen in any given ecosystem. It is catalyzed by the nitrogenase, an

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enzyme that requires microoxic conditions and has a concomitant high energy demand for the N_2-fixing reaction. Along with diverse free-living water- and plant-associated soil bacteria, plant endosymbionts are capable of nitrogen fixation. Root nodule symbiosis between soil bacteria from the alpha-proteobacterial Rhizobiales order, collectively called alpha-rhizobia, and leguminous plants accomplishes a direct supply of bioavailable nitrogen to the host. The process begins with a signal exchange between the partners, leading to bacterial chemotaxis towards the plant roots, attachment to the root hair and their entrapment inside the curling root hair tip. Subsequently, an infection thread is formed inside the root hair, in which bacteria proliferate and progress towards the root cortex. In the root cortex, bacteria invade the plant cells and differentiate into nitrogen-fixing forms, called bacteroids. Symbiosis culminates in formation of a novel plant organ, a nitrogen fixing root nodule in which bacteria provide combined nitrogen to the plant (Roy et al. 2020).

During the free-living stage, rhizobia live as soil saprophytes and become exposed to adverse environmental conditions such as temperature, pH and osmotic pressure challenges, nutrient limitation and desiccation. Therefore, alternating phases of a motile lifestyle, characterized by proliferation and spreading in favorable conditions, and a sessile lifestyle, supporting bacterial survival under unfavorable conditions, are likely prevalent in bacterial soil communities. Processes typically controlled by c-di-GMP signaling, such as motility, exopolysaccharide production and surface attachment have been reported to be important for survival of rhizobia in the soil and for competitive establishment of symbiosis (Figure 1) (Caetano-Annolés et al. 1988; Cheng and Walker 1998; Downie 2010; Skorupska et al. 2006). In this review, we summarize the current knowledge on alpha-rhizobial c-di-GMP regulated processes related to environmental adaptation. We focus the review on *Sinorhizobium meliloti* as it provides the best studied c-di-GMP regulatory network in alpha-rhizobia to date. *S. meliloti* emerged as a model alpha-rhizobium for studying not only its symbiotic interaction with its host plants from the genus *Medicago*, but also properties associated with sessile or motile lifestyle (Jones et al. 2007; Janczarek 2011).

c-di-GMP metabolic enzymes

In bacteria, c-di-GMP is synthesized from two GTP molecules by diguanylate cyclases (DGCs). Their active site contains a GGD(E)EF signature motif, therefore the corresponding catalytic domains were named GGDEF domains

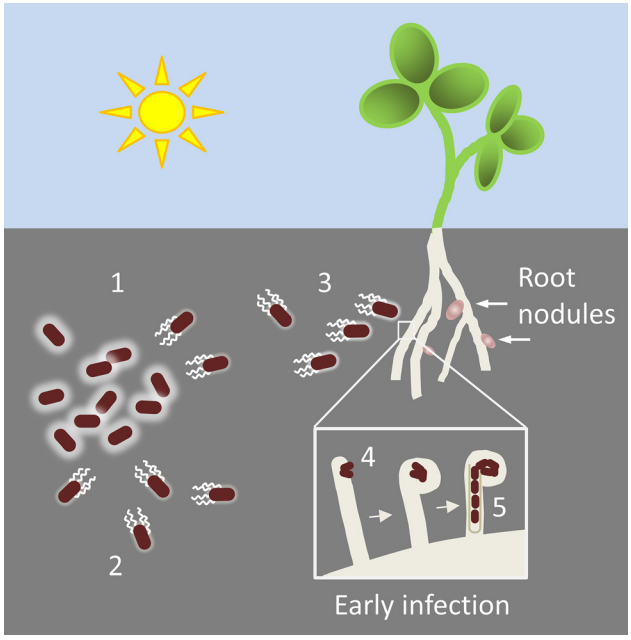

Figure 1: Physiological processes, influenced by c-di-GMP-mediated regulation in alpha-rhizobia.
1: Sedentary lifestyle under unfavorable conditions. 2: Motile lifestyle, spreading. 3: Motile lifestyle, plant chemotaxis. 4: Symbiosis, potential role in attachment to the root hair. 5: Symbiosis, potential role in infection thread progression (EPS biosynthesis).

(Figure 2). For c-di-GMP synthesis, a GGDEF domain homodimer is required (Chan et al. 2004; Paul et al. 2007). The glycine residues in the active site are involved in GTP binding, the aspartate/glutamate at the third position is required for the formation of a phosphodiester bond, and the glutamate in the fourth position coordinates an Mg^{2+} or Mn^{2+} ion, involved in GTP binding (Chan et al. 2004; Wassmann et al. 2007). In about half of all annotated GGDEF domains, an inhibitory c-di-GMP binding site with an RxxD signature motif is present (Seshasayee et al. 2010). Binding of c-di-GMP at this site prevents homodimerization of GGDEF domains, resulting in feedback inhibition (Chan et al. 2004; Christen et al. 2006).

Hydrolysis of c-di-GMP is catalyzed by EAL and HD-GYP domains, named after their signature active site motifs (Galperin et al. 2001) (Figure 2). Phosphodiesterase (PDE) cleavage of c-di-GMP by EAL domains results in the formation of the linear nucleotide pGpG. The glutamate residue of the active site is directly involved in coordination of a Mg^{2+} or Mn^{2+} metal ion required for c-di-GMP binding (Schmidt et al. 2005; Tchigvintsev et al. 2010). The vast majority of EAL domains form homodimers or higher-order oligomers *in vitro*, and a dimer is the most probable functional unit of the EAL domain *in vivo* (Barends et al. 2009; Bellini et al. 2017; Sundriyal et al. 2014). Unlike EAL

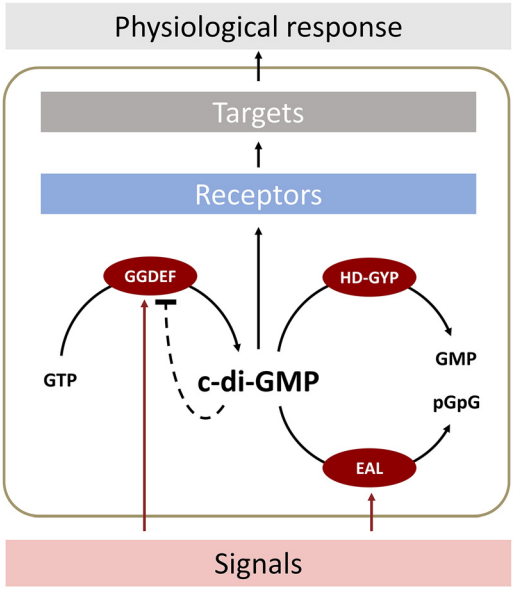

Figure 2: C-di-GMP mediated regulation in bacteria. Abundance of the second messenger is defined by enzymatic activities of GGDEF, EAL and HD-GYP domains. These are subject to regulation by abiotic factors, small molecules and protein-protein interactions (signals). C-di-GMP binds to receptor molecules, which mediate regulation of the targets, leading to a physiological response. Dashed line represents negative feedback regulation of DGC activity by binding of c-di-GMP at the inhibitory site.

domains, HD-GYP domains degrade c-di-GMP in a one-step reaction that yields two molecules of GMP (Bellini et al. 2014) (Figure 2).

Within a given protein, GGDEF and EAL domains are often arranged in tandem (Römling et al. 2013). This apparent contradiction is resolved by their differential regulation or sequence aberrations in the active site, resulting in conditionally or permanently determined single enzymatic activity. For instance, the enzymatic activity of *Agrobacterium tumefaciens* GGDEF-EAL domain protein DcpA is shifted from a DGC to a PDE in presence of pteridine reductase PruA (Feirer et al. 2015). In *Vibrio parahaemolyticus*, dual-function protein ScrC switches from DGC to PDE activity in response to autoinducer molecules at high cell densities (Trimble and McCarter 2011).

The alpha-rhizobial *S. meliloti* type strain Rm2011 carries 18 intact genes encoding c-di-GMP metabolizing proteins, with six containing only a GGDEF domain, one containing only an EAL domain and eleven tandem-type proteins containing both domains. 10 out of 17 GGDEF domains contain a canonical GG(D/E)EF catalytic site motif and 10 out of 11 EAL domains carry an intact active site (Schäper et al. 2016). This array of c-di-GMP metabolizing proteins is medium-size relative to other nitrogen-fixing alpha-rhizobial species, which contain up to 51 c-di-GMP metabolizing enzymes (Gao et al. 2014) or the enterobacterium *Escherichia coli* with 29 enzymes (Hengge et al. 2015). No proteins with HD-GYP domains were identified in *S. meliloti*. Like many c-di-GMP metabolizing proteins in other bacterial species, most of the *S. meliloti* GGDEF and EAL proteins contain additional cytoplasmic or periplasmic sensory input domains.

An effort towards understanding the roles of different *S. meliloti* enzymes was made employing gene overexpression coupled with c-di-GMP quantification (Schäper et al. 2016). This analysis identified five DGCs as enzymatically active. These included the cytoplasmic proteins PleD (SMc01370) and BgrR (SMb20447), and inner membrane proteins SMb20523, SMc01464, and SMc03178. Overproduction of active DGCs resulted in elevated cellular c-di-GMP levels, reduced swimming motility, alterations in exopolysaccharide production and increased biofilm formation (Schäper et al. 2016).

REC domains, which are known as receiver part of two-component systems using phosphorylation for signal transmission (Bourret 2010), are frequently encountered in c-di-GMP metabolizing proteins (Römling et al. 2013). Phosphorylation of the REC domain as a cue for DGC activation was demonstrated for PleD from alpha-proteobacterium *Caulobacter crescentus* and WspR from gamma-proteobacterium *Pseudomonas aeruginosa* (Hickman et al. 2005; Paul et al. 2007; Wassmann et al. 2007). *C. crescentus* PleD was activated by beryllium fluoride mimicking phosphorylation, resulting in dimerization and DGC activity (Paul et al. 2007). Similarly to its *C. crescentus* homolog, *S. meliloti* PleD contains two REC domains. *C. crescentus* cell division produces a surface-attached stalked mother cell and a flagellated swarmer daughter cell. Enzymatic activity of *C. crescentus* PleD is linked to its localization at the old cell pole of the sessile mother cell, which results in higher c-di-GMP content in this cell compared to the motile daughter cell (Christen et al. 2010; Paul et al. 2004). In *S. meliloti*, PleD transiently localized to the old pole of the daughter cell, equivalent to the *C. crescentus* motile swarmer cell (Schäper et al. 2016). It remains to be determined if the c-di-GMP content of the two *S. meliloti* progeny cells differ.

Sinorhizobium meliloti is a rod-shaped bacterium that proliferates by asymmetric cell division and exhibits unipolar cell wall growth at the new cell pole (Brown et al. 2012; Frage et al. 2016; Schäper et al. 2018). RgsP (SMc00074) is an active PDE responsible for about half of the net c-di-GMP degradation (Schäper et al. 2018). RgsP additionally contains PAS (regulatory Per-Arndt-Sim domain) and 7TMR-DISM (seven-transmembrane

receptors with diverse intracellular signal modules) domains, and a non-consensus GGDEF domain. Moreover, RgsP is an essential protein, localized to sites of cell wall growth at one cell pole and the septal site. RgsP is involved in interactions with an array of other essential transmembrane or periplasmic proteins and the Tol-Pal system, localized at the new cell pole in both progeny cells and the septal site in a dividing cell (Krol et al. 2020; Schäper et al. 2018) (Figure 3). Although the enzymatic portion of RgsP is not essential for its cell growth-related function, the question if localized PDE activity of RgsP could generate a c-di-GMP pole-to-pole gradient in *S. meliloti* cells is the subject of future studies.

The GGDEF-EAL tandem protein SMc03178 is potentially the most active *S. meliloti* DGC, since its overexpression resulted in the highest c-di-GMP accumulation (Schäper et al. 2016). In addition to a GGDEF domain, it contains an EAL domain, and extracellular sensory CHASE (Cyclases/Histidine kinases Associated Sensory Extracellular) and regulatory PAS domains, implying direct regulation by a yet-unknown external factor and potential dual c-di-GMP synthesis and degradation functionality.

Although GG(D/E)EF is the canonical motif of the catalytic DGC domain, an active DGC with AGDEF motif was described in *Vibrio cholerae* suggesting that the first position of the motif is less conserved (Hunter et al. 2014). *S. meliloti* tandem protein BgrR represents another example of an active DGC with AGDEF motif (Schäper et al. 2016). The gene encoding BgrR is the first of the *bgrRSTUWV* operon (Baena et al. 2019). BgrUVW constitute a putative partner-switching system, composed of transmembrane phosphatase BgrU and protein kinase BgrW, both acting upon BgrV. If dephosphorylated by BgrU, BgrV inhibits DGC activity of BgrR (Figure 3) (Baena et al. 2019). A yet-unknown environmental signal is suggested to modulate the BgrU-mediated BgrV dephosphorylation, leading to changes in BgrR DGC activity. Additionally, BgrS and BgrT might modulate BgrU phosphatase activity via methylation (Baena et al. 2019).

Gene deletion analysis revealed that all *S. meliloti* GGDEF domain proteins except for RgsP, which has no DGC activity, can be eliminated from the cells (Schäper et al. 2016). The resulting c-di-GMP^0 strain lacking 16 GGDEF domain proteins displayed attenuated growth in acidic conditions, however no further free-living or symbiotic defects were found (Schäper et al. 2016). This constitutes an important difference to other model bacteria such as *Salmonella* Enteritidis and *C. crescentus*, which showed

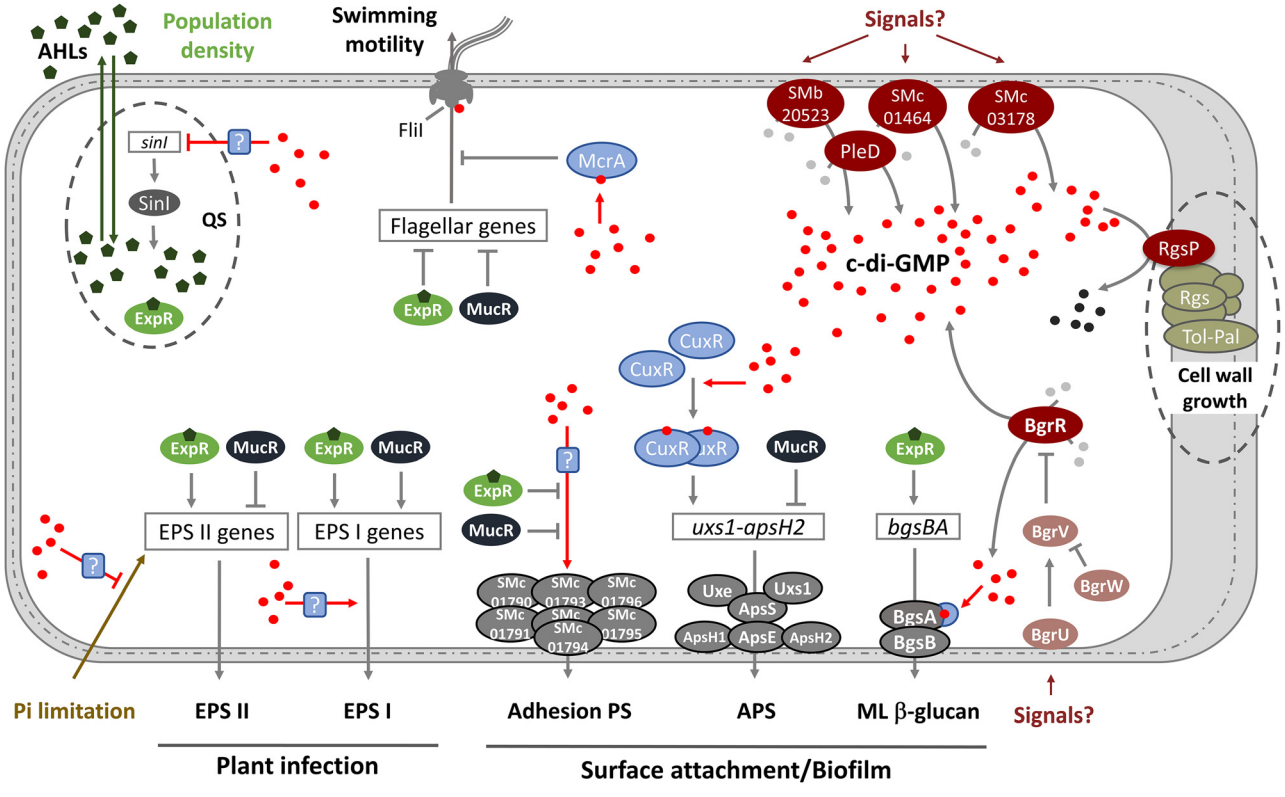

Figure 3: Regulatory network of c-di-GMP control on exopolysaccharide production and swimming motility in *S. meliloti*.
Red circles: c-di-GMP, gray circles: GTP, black circles: pGpG. Blue boxes with question marks represent yet-unknown c-di-GMP receptors.

defects in swimming motility and cell cycle progression in cells lacking DGC enzymes (Abel et al. 2011; Solano et al. 2009).

c-di-GMP receptors

c-di-GMP binding proteins are key components in the delivery of second messenger signals to the cognate targets at transcriptional, post-transcriptional and post-translational levels. Some of these proteins display distinct structural properties, like a PilZ domain (see below) and enzymatically inactive GGDEF and EAL domains, and possess defined c-di-GMP binding sites (Benach et al. 2007; Duerig et al. 2009; Petters et al. 2012; Ramelot et al. 2007). In addition, proteins with diverse non-conserved c-di-GMP binding sites were shown to receive and transmit the second messenger signal (Fang et al. 2014; Gallagher et al. 2020; Wang et al. 2016; Sprecher et al. 2017). Moreover, c-di-GMP can serve as a cofactor, which promotes enzymatic activity of the protein upon binding, in the absence of a specific c-di-GMP binding domain (Steiner et al. 2013).

The PilZ domain, named after the *P. aeruginosa* type IV pilus control protein, is the first discovered and most commonly known type of c-di-GMP receptors. PilZ domains are composed of approximately 100 amino acids and contain the c-di-GMP binding motifs RXXXR and (D/N)X(S/A)XXG (Benach et al. 2007; Ramelot et al. 2007). The c-di-GMP-regulated function discovered first, cellulose biosynthesis in *Acetobacter xylinum*, relies on binding of the second messenger to the PilZ domain of cellulose synthase CeSA (Fujiwara et al. 2013; Weinhouse et al. 1997). In addition to being part of multidomain proteins, PilZ domains can exist as stand-alone proteins, tandem dimers or imperfect tandems, consisting of one functional and one non-consensus PilZ domain (Galperin and Chou 2020). An example of an imperfect PilZ domain tandem is *E. coli* YcgR. Upon c-di-GMP binding, it undergoes structural rearrangement, which promotes its interaction with MotA and FliGMN, resulting in a negative effect on swimming motility (Boehm et al. 2010; Fang and Gomelsky 2010; Hou et al. 2020; Paul et al. 2010).

In the *S. meliloti* genome, two stand-alone PilZ domain proteins are encoded, SMc00999 and McrA (SMc00507). At elevated c-di-GMP levels, McrA was shown to mediate repression of swimming motility resulting from *pleD* overexpression (Figure 3), whereas SMc00999 did not mediate any phenotypic change in the conditions tested (Schäper et al. 2016). McrA bound c-di-GMP *in vitro* and underwent a conformational change upon c-di-GMP binding (Schäper et al. 2016). Factors mediating the repression of swimming motility by McrA remain unknown. Similarly to McrA, stand-alone PilZ domain proteins DgrA and DgrB regulate c-di-GMP-dependent motility in *C. crescentus* through a yet unknown mechanism (Christen et al. 2007).

An example of a c-di-GMP receptor protein with a non-conserved c-di-GMP binding site is *S. meliloti* (1 → 3)(1 → 4)-β-D-glucan (mixed linkage β-glucan) biosynthesis glycosyltransferase BgsA displaying similarities to cellulose synthases (Perez-Mendoza et al. 2015). Such cellulose synthases perceive the c-di-GMP signal via PilZ domains (Morgan et al. 2014). In contrast, allosteric activation of BgsA proceeds via c-di-GMP binding to its C-terminal portion, which is non-homologous to PilZ domains (Perez-Mendoza et al. 2017). A 139 amino acid long C-terminal portion of BgsA contains residues R599 and H615 crucial for both c-di-GMP binding and mixed linkage β-glucan biosynthesis in cells with elevated c-di-GMP (Perez-Mendoza et al., 2015, 2017).

c-di-GMP-binding transcription factors are c-di-GMP receptors that mediate second messenger-dependent regulation at the level of gene expression. Unlike PilZ or non-consensus c-di-GMP-metabolizing domains, they do not have a common conserved c-di-GMP binding motif at the amino acid sequence level. Transcriptional activators and repressors of various types like CRP/FNR, TetR-like, XRE, NtrC or FixJ-LuxR-CsgD were reported to regulate gene expression dependent on c-di-GMP (Chin et al. 2010; Hsieh et al. 2018; Li and He 2012; Tschowri et al. 2014).

In *S. meliloti*, the AraC-type transcription activator CuxR was identified as c-di-GMP receptor (Figure 3) (Schäper et al. 2017). The mode of c-di-GMP-CuxR interaction, involving an RxxxR motif and an additional distal binding site, is reminiscent of that of PilZ domains (Schäper et al. 2017). Binding of a c-di-GMP dimer to CuxR is proposed to promote structural rearrangements in CuxR favoring its dimerization and hence its DNA-binding ability, required for promoter activation (Schäper et al. 2017).

Regulatory network controlling processes, related to environmental adaptation in *S. meliloti*

Alpha-rhizobia are able to produce a complex array of glucidic molecules, such as exopolysaccharides (EPSs), lipopolysaccharide (LPS), capsular polysaccharide, K-antigen polysaccharide, cyclic glucans, glucomannan

and gel-forming polysaccharide (Fraysse et al. 2003; Laus et al. 2006). Rhizobial surface polysaccharides play a role in the infection process and in the free-living state, contribute to nutrient gathering, surface attachment, biofilm formation, and protection against environmental stresses and antimicrobial compounds (Downie 2010; Skorupska et al. 2006). Noteworthy, alpha-rhizobia from the genus *Rhizobium* are able to produce cellulose, which plays an important role in surface attachment and biofilm formation (Perez-Mendoza et al. 2014; Robledo et al. 2012; Laus et al. 2005). Swimming or swarming motility is required for both spreading during the free-living state and efficient establishment of the contact with the plant host at the early stages of symbiotic interaction (Caetano-Anollés et al. 1988). Rhizobia are equipped with quorum sensing (QS) systems that use *N*-acyl-homoserine lactones (AHLs) as messenger molecules (Marketon et al. 2002). QS regulates polysaccharide production and motility and influences symbiotic interaction (Edwards et al. 2009; Hoang et al. 2004; Sanchez-Contreras et al. 2007).

Sinorhizobium meliloti forms peritrichous flagella enabling swimming motility and chemotactic response during exponential growth as well as chemotaxis towards the plant (Rotter et al. 2006; Schmitt 2002; Sourjik et al. 2000; Webb et al. 2014). Two major EPSs are produced by *S. meliloti*. EPS I, succinoglycan, is an acidic heteropolymer consisting of repeating octasaccharide subunits (Reinhold et al. 1994). EPS II, galactoglucan, consists of disaccharide repeating units (Her et al. 1990). The ability to produce at least one of the major EPS is crucial for infection thread progression (Battisti et al. 1992; Cheng and Walker 1998; González et al. 1996; Urzainqui and Walker 1992). Furthermore, recent research provided evidence that at elevated c-di-GMP levels, *S. meliloti* with intact quorum sensing system genes *expR*, *sinI* and *sinR* is able to produce mixed-linkage β-glucan (Pérez-Mendoza et al. 2015).

Moreover, c-di-GMP stimulates production of arabinose-containing polysaccharide (APS) and an adhesion polysaccharide of unknown composition, likely similar to *A. tumefaciens* unipolar adhesion polysaccharide UPP. These polysaccharides facilitate cell aggregation, surface attachment and biofilm formation, however they are not required for effective symbiotic interaction (Pérez-Mendoza et al. 2015; Schäper et al. 2016; Schäper et al. 2019; Xu et al. 2012).

C-di-GMP-mediated control of *S. meliloti* EPS biosynthesis and swimming motility is implicated into a complex regulatory network governing these processes (Janczarek 2011; Rotter et al. 2006; Scharf and Schmitt 2002; Sourjik et al. 2000). Of notice, opposing control of EPS I production and swimming motility is exerted by a regulatory system composed of ExoR (periplasmic repressor), ExoS (sensory histidine kinase) and ChvI (transcription regulator) (Wang et al. 2010; Yao et al. 2004). In general, stress factors, such as starvation, generally negatively affect expression of genes controlling swimming motility and simultaneously activate expression of EPS biosynthesis genes (Chao et al. 2005; Domínguez-Ferreras et al. 2006; Hoang et al. 2008; Krol and Becker, 2004, 2011).

The mechanisms of stress response regulation of EPS biosynthesis and swimming motility are only partially known. For example, expression of EPS II biosynthesis genes under phosphate limitation is stimulated by PhoB, the response regulator of the PhoR-PhoB two-component system (Bahlawane et al. 2008a; Krol and Becker 2004). Furthermore, the global regulators MucR and ExpR modulate *S. meliloti* exopolysaccharide production and swimming motility (Figure 3). MucR is a zinc-finger transcriptional regulator. It is transcriptionally autoregulated and couples both EPS biosynthetic pathways by positive regulation of EPS I production and negative regulation of EPS II production (Bertram-Drogatz et al. 1998; Rüberg et al. 1999). MucR also negatively regulates swimming motility by repressing the transcription of flagellar gene regulator *rem* (Bahlawane et al. 2008b; Rotter et al. 2006). ExpR is a LuxR-type regulator of the Sin/ExpR QS system. *S. meliloti* QS AHL molecules are produced by the synthase SinI (Gao et al. 2005; McIntosh et al. 2009). ExpR-AHL regulates various target processes at the transcription level, exerting a positive effect on polysaccharide biosynthesis and a negative effect on flagellar motility (Figure 3) (Charoenpanich et al. 2013; Mueller and González 2011; Zatakia et al. 2014).

c-di-GMP-mediated regulation of motility

Bacterial swimming motility is crucial at the early steps of plant-microbial interaction, which culminate in a physical contact between the partners. Bacteria sense the plant-derived compounds and respond with chemotaxis. Motility and chemotaxis quantitatively affect important traits that facilitate the initial contact and adsorption of symbiotic rhizobia to the host root surface (Caetano-Anollés et al. 1988).

Regulation of bacterial motility by c-di-GMP is complex. Low levels proved to be beneficial for this trait, whereas either artificial accumulation or removal of c-di-GMP resulted in motility inhibition (Abel et al. 2011; Bhasme et al. 2020; Pallegar et al. 2020; Yang et al. 2016).

The mechanisms of c-di-GMP-mediated control of bacterial motility range from regulation of flagellar gene expression to direct binding to proteins interacting with flagellar components and regulation of chemotaxis (Boehm et al. 2010; Fang and Gomelsky 2010; Hou et al. 2020; Paul et al. 2010; Sun et al. 2019). Noteworthy, not only does c-di-GMP affect swimming motility, but also control of c-di-GMP levels is interwoven with regulation of flagellar motility. In *A. tumefaciens*, the master activator VisN of flagellar motility genes represses the DGC genes *dgcB* and *dgcC*, providing a pathway to inversely correlate flagellar gene expression with c-di-GMP levels (Xu et al. 2013). Upon c-di-GMP-binding, *P. aeruginosa* PilZ-domain protein MapZ affects chemoreceptor methylation via interaction with chemotaxis methyltransferase CheR1 (Xu et al. 2016). In turn, asymmetrical inheritance of the chemotaxis apparatus after cell division results in differential activation of PDE Pch via the phosphorylation status of CheA (Kulasekara et al. 2013).

C-di-GMP-mediated regulation of motility in *S. meliloti* shows both similarities and differences to the general paradigm. In contrast to c-di-GMP^0 strains of *Salmonella* Enteritidis and *C. crescentus*, which show a flagellar motility defect, the *S. meliloti* c-di-GMP^0 strain performs normally in swimming motility assays (Schäper et al. 2016). Unlike *E. coli* and *P. aeruginosa*, whose flagellar gene expression is repressed in c-di-GMP-dependent manner (Hickman and Harwood 2008; Nieto et al. 2019), no effect on flagellar gene transcript abundances was observed in *S. meliloti* overproducing DGC PleD (Schäper et al. 2017). Artificial increase of c-di-GMP levels upon DGC overproduction in *S. meliloti* and the two related alpha-rhizobia *Rhizobium etli* and *Rhizobium leguminosarum* resulted in inhibition of swimming motility, consistent with the general paradigm (Schäper et al. 2016; Perez-Mendoza et al. 2014). One possible pathway of c-di-GMP control of *S. meliloti* swimming motility is its binding to flagellar export ATPase FliI (Trampari et al. 2015). Moreover, PilZ domain protein McrA mediates inhibition of swimming motility upon overexpression of *pleD* (Figure 3).

Regulatory connections to QS

Quorum sensing (QS) is a powerful mechanism of global gene regulation in response to changes in population density which relies on secretion and uptake of small signal molecules. Functional QS is advantageous for bacterial performance in both pathogenic and symbiotic microbe-plant interactions (Calatrava-Morales et al. 2018; Lowe-Power et al. 2018; Sanchez-Contreras et al. 2007). Although *S. meliloti* QS is not essential for a successful symbiotic interaction, strains lacking AHL synthase SinI showed attenuated symbiotic performance (Gurich and Gonzalez 2009; Marketon et al. 2002).

Reciprocal interaction between QS and c-di-GMP regulatory circuits are well documented, with a general trend of dominant negative control of QS by c-di-GMP (Hochstrasser et al. 2019; Lin Chua et al. 2017; Waldron et al. 2019; Yang et al. 2017). In *S. meliloti*, elevated levels of c-di-GMP repressed transcription of AHL synthase enzyme *sinI* and lowered abundance of QS signal AHL molecules in the culture medium (Schäper et al. 2016) (Figure 3). However, absence of QS regulator ExpR had no effect on *S. meliloti* intracellular c-di-GMP content (Schäper et al. 2016). Inhibition of the QS system by c-di-GMP provides an additional level of control on swimming motility and polysaccharide production by this second messenger.

Regulation of surface attachment and biofilm formation by c-di-GMP

A positive regulatory effect of c-di-GMP on bacterial surface attachment and formation of higher-order three-dimensional structures designated biofilms is the most prominent and best understood function of this second messenger in promoting a sedentary lifestyle (for recent reviews, see Hengge 2020; Maunders and Welch 2017; Purcell and Tamayo 2016). c-di-GMP-dependent regulation of biofilm formation takes place in a non-uniform distribution across the biofilm and follows a defined spatiotemporal pattern during colony maturation (Nair et al. 2017). Biofilm formation is associated with survival in free-living conditions (Rinaudi and Giordano 2010) and diverse pathogenic interactions (Kumar et al. 2017).

Biofilms are composed of bacterial cells encased in extracellular matrix, which can contain cellulose and other exopolysaccharides, adhesive pili, non-fimbrial adhesins and extracellular DNA. Its composition defines multicellular morphotypes and cooperative bacterial movements (Gao et al. 2012; Serra et al. 2013; Whitchurch et al. 2002; Zogaj et al. 2001). c-di-GMP dependent regulation of polysaccharide biosynthesis is important for extracellular matrix formation (Liang 2015; McDougald et al. 2012).

In *S. meliloti* and the related plant pathogen *A. tumefaciens*, biofilm formation is enhanced at high c-di-GMP levels. One of the underlying molecular mechanisms comprises activation of *A. tumefaciens* adhesion polysaccharide UPP production in a yet-unknown manner (Xu et al. 2012). In *A. tumefaciens*, UPP biosynthesis is

controlled by an *uppABCDEF* gene cluster encoding two glycosyltransferases, two polysaccharide transport proteins and two hypothetical proteins (Xu et al. 2012). The *S. meliloti* gene cluster *SMc01796-SMc01790* is highly similar to *uppABCDEF*, however it contains an additional glycosyltransferase gene (Schäper et al. 2016). In both *A. tumefaciens* and *S. meliloti*, removal of EPS I due to knockout of *exoY* increased the biofilm forming capacity of cultures of strains with elevated c-di-GMP levels, indicating that EPS I diminishes cell aggregation and surface attachment (Schäper et al. 2016; Xu et al. 2013). Consistent with this finding, the global regulators ExpR and MucR, stimulating EPS I production, negatively affected biofilm formation by *S. meliloti* (Schäper et al. 2016). The molecular mechanism of c-di-GMP action on UPP biosynthesis and the UPP sugar composition are subjects of future studies.

Biosynthesis of mixed-linkage β-glucan in strains with elevated levels of c-di-GMP requires c-di-GMP binding glycosyltransferase BgsA and putative export protein BgsB (Figure 3). It contributes to cell aggregation and biofilm formation and is required for efficient attachment to plant roots (Perez-Mendoza et al., 2015, 2017).

Furthermore, at high c-di-GMP levels, *S. meliloti* produced arabinose-containing polysaccharide (APS) (Figure 3). Its biosynthesis is controlled by the *uxs1-uxe-apsS-apsH1-apsE-apsH2* operon (Schäper et al. 2019). Expression of the *uxs1-aspH2* operon is repressed by MucR and is activated by the cognate transcription regulator CuxR, encoded upstream of the *uxs1-apsH2* operon (Schäper et al. 2019). CuxR requires c-di-GMP for DNA binding and therefore transcription of the operon is activated at high c-di-GMP levels (Schäper et al. 2017). The gene products of the first two genes in the operon, Uxs1 and Uxe, act as UDP-xylose synthase and UDP-xylose 4-epimerase, respectively (Gu et al. 2011). ApsS is a putative glycosyl transferase, ApsE is a putative endoglycanase and the two remaining proteins are of unknown function. APS production significantly increased biofilm formation in strains that lacked the ability to produce the putative polar adhesion polysaccharide, whose biosynthesis is controlled by the *SMc01796-SMc01790* gene cluster (Schäper et al. 2019).

c-di-GMP-mediated regulation of symbiosis-relevant polysaccharide production

Artificial increase in c-di-GMP levels upon DGC overproduction in *S. meliloti*, *R. etli* and *R. leguminosarum* affected biosynthesis of EPSs. Cellulose production was enhanced in *R. etli* and *R. leguminosarum* (Perez-Mendoza et al. 2014). In *S. meliloti* Rm2011, not containing a functional *expR* gene, EPS I production was increased at high c-di-GMP levels (Schäper et al. 2016). This was not observed in *S. meliloti* Rm8530 carrying functional *expR* (Perez-Mendoza et al. 2015). These EPSs are required at the early stages of plant infection (Muszyński et al. 2016; Nieahus and Becker 1998; Staehelin et al. 2006).

At high c-di-GMP levels, transcription of *S. meliloti exoY*, encoding the first, production rate-determining enzyme of the EPS I biosynthesis pathway, was not altered, which implies a posttranscriptional control by c-di-GMP (Schäper et al. 2016). In contrast, production of EPS II, stimulated by phosphate starvation, was negatively affected by elevated c-di-GMP levels. This correlated with repression of *wgeA*, a gene from the EPS II biosynthesis cluster, indicating that c-di-GMP repressed EPS II biosynthesis at transcription level (Schäper et al. 2016). Moreover, negative regulation of AHL production by c-di-GMP constitutes an additional pathway of EPS II biosynthesis regulation via QS (Figure 3).

Interestingly, at elevated c-di-GMP levels, activation of *uxs1-uxe-apsS-apsH1-apsE-apsH2* operon transcription can have an indirect positive effect on EPS I and EPS II biosynthesis. The UDP-sugar epimerase Uxe, initially reported to perform conversion of UDP-xylose and UDP-arabinose, appeared to be also able to convert UDP-glucose and UDP-galactose. UDP-galactose, a component of LPS and both symbiotically relevant EPS, is normally synthesized by *S. meliloti* ExoB, encoded within the EPS I biosynthesis gene cluster, and previously the only known UDP-glucose 4-epimerase in this *S. meliloti* strain. Activating *uxs1-apsH2* operon expression complemented the symbiotic defect conferred by an *exoB* mutation (Schäper et al. 2019). Thus, c-di-GMP-mediated regulation can promote the symbiotically important EPS production by increasing nucleotide sugar precursor abundance.

Effect of c-di-GMP on the symbiotic interaction

In plant pathogenic bacteria, c-di-GMP regulates virulence traits in a positive as well as a negative fashion. In the soft rot pathogen *Dickeya dadantii*, production of plant cell wall lytic enzymes is repressed by c-di-GMP (Yuan et al. 2020). In the phytopathogen *Xanthomonas campestris*, DGCs were reported to inhibit virulence, whereas PDEs increased it, suggesting that low c-di-GMP levels are

beneficial for plant infection (Su et al. 2016; Xue et al. 2018; Yang et al. 2016). In *Erwinia amylovora*, c-di-GMP promotes virulence determinants such as amylovoran production and biofilm formation. However, motility and the type III secretion system are expressed and functional when cyclic di-GMP is absent (Kharadi et al. 2018). Moreover, c-di-GMP-mediated modulation of protein secretion systems of virtually any known type was reported in plant pathogenic or beneficial bacteria (Lopez-Baena et al. 2019).

In alpha-rhizobia, only adverse effects of elevated c-di-GMP levels on symbiotic performance were described. Removal of c-di-GMP did not affect the symbiotic efficiency of *S. meliloti* (Schäper et al. 2016). In contrast, strongly increased c-di-GMP levels in the related rhizobia *R. etli* and *R. leguminosarum* enhanced bacterial attachment to the plant roots, at the same time reducing the ability of the bacteria to promote growth of the respective host plants, despite normal number, size and visual appearance of the nodules (Perez-Mendoza et al. 2014). The adverse effect of increased c-di-GMP levels during symbiotic interaction was corroborated by observation of a massive loss of the plasmid conferring high DGC activity during the symbiotic interaction (Perez-Mendoza et al. 2014). *S. meliloti* ML β-glucan, which is produced at high c-di-GMP levels, was required for increased bacterial attachment to the host plant roots (Perez-Mendoza et al. 2015). However, this did not provide an advantage for the symbiotic performance in general (Perez-Mendoza et al. 2015).

Concluding remarks

Since its discovery, the picture of bacterial c-di-GMP mediated regulation has become more and more fine-grained and widely conserved roles for this almost ubiquitous second messenger in controlling the switch between sessile and motile lifestyles have been recognized in diverse bacteria. In the recent years, several studies provided insights into the c-di-GMP regulatory network in alpha-rhizobial root nodule symbionts and pointed to a role in the free-living rather than in the symbiotic state. So far, mechanistic studies of c-di-GMP mediated regulation mostly focused on *S. meliloti*. Studies in *S. meliloti* contributed novel types of c-di-GMP binding proteins and thus broadened the repertoire of known receptors of this second messenger in bacteria. Yet, there is certainly much to discover in this and other alpha-rhizobia. The most important gap in our knowledge of c-di-GMP signaling in these bacteria comprise the environmental signals controlling c-di-GMP mediated regulation as well as perception and transduction of these signals.

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