A partner-switching system controls activation of mixed-linkage β-glucan synthesis by c-di-GMP in *Sinorhizobium meliloti.*

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Originality-Significance Statement.

The symbiotic soil bacterium *Sinorhizobium meliloti* can synthesize at least three different exopolysaccharides: succinoglycan (EPS I), galactoglucan (EPS II) and the recently described mixed-linkage β-glucan (MLG). Although not essential for symbiosis, the MLG seems to be important for biofilm formation and is required for efficient attachment to the host plant root. The synthesis of this polysaccharide is activated by c-di-GMP binding to the MLG synthase. In this report, we identify a partner-switching system that regulates MLG synthesis by modulating the activity of a genetically linked diguanylate cyclase. This work highlights the complex regulatory networks that control the synthesis of the ubiquitous bacterial second messenger c-di-GMP.

Summary

Sinorhizobium meliloti synthesizes a linear mixed-linkage (1→3)(1→4)-β-D-glucan (ML β-glucan, MLG) in response to high levels of cyclic diguanylate (c-di-GMP). Two proteins BgsA and BgsB are required for MLG synthesis, BgsA being the glucan synthase which is activated upon c-di-GMP binding to its C-terminal domain. Here we report that the product of *bgrR* (SMb20447), is a diguanylate cyclase (DGC) that provides c-di-GMP for the synthesis of MLG by BgsA. *bgrR* is the first gene of a hexacistronic *bgrRSTUWV* operon, likely encoding a partner-switching regulatory network where BgrR is the final target. Using different approaches, we have determined that the products of genes *bgrU* (containing a putative PP2C serine phosphatase domain) and *bgrW* (with predicted kinase effector domain), modulate the phosphorylation status and the activity of the STAS domain protein BgrV. We propose that unphosphorylated BgrV inhibits BgrR DGC activity, perhaps through direct protein-protein interactions as established for other partner switchers. A *bgrRSTUWV* operon coexists with MLG structural *bgsBA* genes in many rhizobial genomes but is also present in some MLG non-producers, suggesting a role of this partner-switching system in other processes besides MLG biosynthesis.

Introduction

Bacterial partner-switching systems (PSS) constitute regulatory modules that work through reversible protein-protein interactions and phosphorylation events. They are characterized by having a central partner able to switch binding to one or another component of the system, depending on the activation status of the signaling pathway (Francez-Charlot *et al.*, 2015). So far, the best characterized PSS are those regulating the spore development and the general stress response in *Bacillus subtilis*. In these systems, the targets are sigma factors which are required for the transcription of their corresponding regulons (Alper *et al.*, 1994; Dufour and Haldenwang, 1994). For instance, in the general stress response σ^B is retained by its cognate anti-sigma factor RsbW, preventing its transcriptional activity in non-stress situations. RsbW also displays a serine kinase activity and phosphorylates its antagonist RsbV in unstressed conditions. Upon stress sensing, RsbV is dephosphorylated by the serine phosphatases RsbU or RsbP. Unphosphorylated RsbV then binds to RsbW and releases the sigma factor from the σ^B-RsbW complex, thereby allowing the transcription of σ^B target genes (Hecker *et al.*, 2007). Furthermore, RsbU serine phosphatase activity is regulated by its interaction with a second PSS formed by the switching kinase RsbT and the stressosome (Chen *et al.*, 2003).

PSS that regulate other traits have been described in diverse bacteria, such as biofilm formation in *Vibrio fischeri* (Morris and Visick, 2010), type III secretion system in *Bordetella* (Mattoo *et al.*, 2004), biofilm formation and motility in *Pseudomonas aeruginosa* (Bordi *et al.*, 2010; Bhuwan *et al.*, 2012), or hormogonium development in *Nostoc punctiforme* (Riley *et al.*, 2018). Proteins containing specific domains are shared by all these systems: a C-terminal phosphatase 2C (PP2C phosphatase) like in RsbU, an ATPase/kinase domain (GHKL domain) like in RsbW and a sulfate transporter and anti-

sigma factor antagonist (STAS) domain protein like in RsbV (Sharma *et al.*, 2011; Bouillet *et al.*, 2018). In some cases like in *V. fischeri* and *Bordetella* the target proteins of the PSS are not yet identified (Kozak *et al.*, 2005; Thompson and Visick, 2015). Additionally in the Gram-positive *Moorella thermoacetica*, the stressosome complex regulates the activity of a diguanylate cyclase (DGC) encoded within the stressosome gene cluster (Quin *et al.*, 2012). In this case, the DGC possesses a N-terminal RsbU like domain, which is necessary for the interaction with the RsbT-like partner switching kinase. Thus, PSS might represent versatile mechanisms to regulate multiple processes in response to different environmental cues.

The α-proteobacterium *Sinorhizobium meliloti* can establish symbiotic relationships with several leguminous plants, including alfalfa. In this symbiosis, the bacterium induces the formation of nitrogen-fixing nodules in the plant root by setting up a molecular dialogue with the host. Bacterial exopolysaccharides (EPS) are known to participate in the complex mechanisms necessary for root nodule development (Jones *et al.*, 2007; Gibson *et al.*, 2008). *S. meliloti* is able to produce two symbiotically active EPS, succinoglycan (EPS I) and galactoglucan (EPS II). The structural genes involved in their synthesis are clustered in separate regions of pSymB, the largest megaplasmid in *S. meliloti*. Up to 12% of the genes in the pSymB might be involved in the synthesis of cell surface carbohydrates (Finan *et al.*, 2001; Galibert *et al.*, 2001).

We have described that *S. meliloti* produces another EPS, a linear mixed-linkage (1→3)(1→4)-β-D-glucan (ML β-glucan; MLG) that is not essential for symbiosis establishment but is important for bacterial adhesion to biotic and abiotic surfaces, (Pérez-Mendoza *et al.*, 2015). The two genes involved in its synthesis, *bgsBA*, are located in a single pSymB operon transcriptionally regulated by the ExpR/Sin quorum sensing system. The ExpR/Sin system is a master regulator of several important processes,

including the synthesis of EPS II and also EPS I production (Pellock *et al.*, 2002; Marketon *et al.*, 2003; Glenn *et al.*, 2007). BgsB contains a HlyD-like domain and might function in the export of the polysaccharide, whereas BgsA is the glycosyltransferase that synthesizes the MLG. The production of the MLG is boosted when the levels of cyclic diguanylate (c-di-GMP) are increased, through a mechanism involving c-di-GMP binding to the C-terminus of BgsA (Pérez-Mendoza *et al.*, 2015, 2017). Cyclic-di-GMP is a ubiquitous bacterial second messenger that has been involved in diverse physiological processes, such as biofilm formation, exopolysaccharide production, motility, cell cycle and virulence. Cyclic-di-GMP promotes a wide range of responses, from transcriptional regulation of target genes to enzyme activity modulation (Romling *et al.*, 2013). Diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) are responsible for the synthesis and degradation of the c-di-GMP, respectively (Tal *et al.*, 1998). DGCs contain a characteristic common domain GGDEF while PDEs may present either EAL or HD-GYP domains. Numerous species, particularly those with complex life cycles, encode dozens of c-di-GMP metabolic enzymes in their genomes, whose activities can be modulated by environmental and physiological cues. Furthermore, some proteins bear both types of domains, what hinders predicting their function as DGC, PDE or both. All these features suggest functional redundancies, which often is a handicap to identify separate c-di-GMP signaling pathways (Romling *et al.*, 2013). For instance, the *S. meliloti* genome contains at least 20 putative DGC and/or PDE-encoding genes.

In this report we identify a DGC (SMb20447) involved in the synthesis of MLG in *S. meliloti*. *SMb20447* is the first gene of large operon (*SMb20447-SMb20452*) likely encoding a partner-switching system where SMb20447 is the final target. Thus, by regulating the DGC activity of SMb20447, this PSS controls the activation of the ML β-glucan synthase by c-di-GMP and the production of the polysaccharide MLG.

Results

SMb20450 is a negative regulator of ML β-glucan production in *S. meliloti*.

In order to identify genes involved in the regulation of the synthesis of the MLG in *S. meliloti*, we performed a random general mutagenesis of strain Rm8530 (Rm1021 *expR*$^+$) with the transposon Tn*5* (Simon *et al.*, 1983). Approximately 10^5 Km^r transposants were pooled and around 8,000 were screened in minimal medium supplemented with Congo red (CR), which is known to stain *S. meliloti* colonies overproducing MLG (Pérez-Mendoza *et al.*, 2015). Amongst a great majority of Km^r white transposants (CR$^-$), several red ones (CR$^+$) were found and ten were selected for further analysis. Southern blots indicated that all insertions mapped to a 12 kb StuI DNA fragment. Three of them were sequenced and located to either *SMb20449* or to the downstream gene *SMb20450* (data not shown; Fig 1A).

Since Tn*5* transposon insertions usually cause strong polar effects on downstream genes, we constructed a strain with a non-polar, unmarked in-frame deletion of *SMb20450* to verify the implication of this gene in the CR$^+$ phenotype. This mutant strain IBR503 (Δ*SMb20450*) also formed CR$^+$ colonies (Fig. S1A). This CR$^+$ phenotype was reverted to CR$^-$ when a wild type copy of *SMb20450* was provided in trans (pJLR1110; Fig. S1A). Furthermore, IBR503 also showed a calcofluor-positive (CF$^+$) phenotype, similar to the MLG overproducing strain Rm8530 (pJBpleD*) (Fig. 1B). The CR$^+$ and CF$^+$ phenotypes were unrelated to EPS I and EPS II, as a strain carrying additional mutations in genes essential for the synthesis of these EPSs, *exoY* and *wgaB*, exhibited the same CF$^+$ and CR$^+$ phenotypes (strain IBR508, Fig. 1B and Fig. S1A). Additionally, we compared the substance produced by IBR503 with the known MLG produced by Rm8530 (pJBpleD*). The polysaccharides isolated from both strains were analyzed by FTIR and both showed

virtually identical spectra (Fig. S1B**)**. These results indicated that mutations in *SMb20450* lead to activating MLG production. To confirm this point, we combined the Δ*SMb20450* deletion with a *lacZ*::Gn insertion in the gene coding for the ML β-glucan synthase BgsA, to produce strain IBR515 (Fig. 1B and S1A). This double mutant displayed CF^{-} and CR^{-} phenotypes, which were reverted to CF^{+} and CR^{+} by a plasmid carrying the *bgsA* wild-type gene (data not shown). We also analyzed the transcriptional level of *bgsA* in Rm8530 (IBR513) and IBR515 and a slight increase in the β-galactosidase activity was observed in the strain IBR515 (259.0 ± 24.43) compared to IBR513 (214.7 ± 8.68). However, previous results suggested that similar small variations in the transcription levels of *bgsA* were not related to the strong effect in MLG production observed (Pérez-Mendoza *et al.*, 2015). All these data suggested that *SMb20450* negatively regulates MLG production.

Identification of an operon involved in the regulation of ML β-glucan synthesis.

We analyzed in depth the gene cluster containing *SMb20450* (Fig. 1A). First, we determined what other genes were transcriptionally coupled with *SMb20450*. RT-PCR analysis showed that six genes, from *SMb20447* to *SMb20452*, were organized in a single operon (Fig. 1C). This was in concordance with RNAseq and transcription start sites data published previously (Sallet *et al.*, 2013; Schlüter *et al.*, 2013). Since MLG synthesis is transcriptionally dependent on the Sin/ExpR regulatory system (Pérez-Mendoza *et al.*, 2015), we explored if the *bgr* operon could also be subjected to the same transcriptional control. Transcriptional *SMb20447-lacZ* and *SMb20450-lacZ* fusions were integrated in the strains Rm8530 (*expR*$^{+}$) and Rm1021 (*expR*$^{-}$). Both fusions showed similar expression levels in both genetic backgrounds (data not shown), indicating that the *bgr* operon is not regulated by ExpR/Sin.

To determine the importance of each individual gene in the synthesis of MLG, we obtained strains with non-polar unmarked in-frame deletions of each gene of the operon. The production of the polysaccharide was visualized on TY plates supplemented with calcofluor and quantified by measuring the CF-derived fluorescence of the strains grown in liquid minimal medium supplemented with CF. The results showed that mutants in *SMb20449*, *SMb20450* or *SMb20452* overproduced the MLG, whereas strains lacking *SMb20447*, *SMb20448* or *SMb20451* behaved like the wild type (Fig 2A and Table 1). Opposite results were obtained when each gene was individually expressed from a multicopy plasmid in the wild type background. Overexpression of *SMb20447*, *SMb20448* or *SMb20451* led to enhanced production of the MLG, whereas overexpression of *SMb20449*, *SMb20450* or *SMb20452* did not (Fig 2B and Table 1). Thus, individual genes within the operon seemed to exert contrasting effects on the production of the MLG. The absence of some of them produced the same phenotype as the overexpression of some others.

SMb20447 codes for a protein with GGDEF and EAL domains (Fig. S2) and has been reported as a likely DGC (Schäper *et al.*, 2016). SMb20448 carries no described domains but is conserved in other rhizobial genomes, whereas SMb20449 contains a methyltransferase domain. On the other hand, the products of *SMb20450*, *SMb20451* and *SMb20452* resemble components of partner-switching systems (PSS). SMb20450 possesses central CACHE and HAMP domains, and a C-terminal PP2C serine phosphatase-like domain, the latter similar to RsbU from *B. subtilis* (Fig. 3A). SMb20451 exhibits a GHKL domain with a characteristic Bergerat fold (Dutta and Inouye, 2000). This fold is also present in *B. subtilis* RsbW, which has serine-kinase activity (Fig. 3B). SMb20452 possesses a STAS domain like *B. subtilis* RsbV (Fig. 3C).

Given the similarities with several proteins of the *B. subtilis* partner-switching mechanism

194 that controls σ^B activity and the role played by the *S. meliloti* genes in the regulation of
195 the MLG production (see below), we propose the operon to be renamed as *bgrRSTUWV*,
196 "bgr" standing for "*b*eta-*g*lucan *r*egulation" and the letters conserving those assigned to
197 equivalent genes of the *B. subtilis* σ^B regulatory network (Fig. 1A).

198

199 The *bgrRSTUWV* operon regulates the synthesis of ML β-glucan through BgrR.

200 MLG production is known to boost upon increasing the intracellular c-di-GMP levels
201 (Perez-Mendoza *et al*., 2015). SMb20447 (BgrR) is a likely DGC since its overexpression
202 increases intracellular c-di-GMP contents and promotes EPS production in the related
203 strain *S. meliloti* Rm2011 (Schäper *et al.*, 2016). We also observed that a BgrR plasmid
204 increased 9-fold the intracellular c-di-GMP contents (not shown) and produced a strong
205 CF^+ phenotype in the wild-type strain (Fig. 2B and Table 1). These phenotypes were not
206 observed in a BgrR derivative ($BgrR^{E215A}$) in which the degenerate AGDEF motif was
207 altered to AGDAF (Table 1). These results agreed with BgrR being a DGC providing c-
208 di-GMP for MLG biosynthesis. Therefore, we investigated the interactions between *bgrR*
209 and the remaining *bgr* genes for MLG production. For this, we obtained double mutants
210 in the *bgr* operon and determined their phenotypes in relation to MLG production. The
211 results are summarized in Table 1. Strikingly, the CF^+ phenotype displayed by mutants
212 lacking *bgrT*, *bgrU* or *bgrV* turned to CF^- upon removal of *bgrR* (see double mutants
213 IBR518, IBR519 and IBR520; Table 1). On the other hand, the CF^+ phenotype shown by
214 the wild type strain carrying the *bgrW* plasmid was also dependent on a functional *bgrR*
215 gene (Table 1). Thus, the positive or negative effects of individual *bgr* genes on MLG
216 production seemed all dependent on the presence of a wild type *bgrR* gene.
217 We also measured the c-di-GMP contents in some strains displaying enhanced MLG
218 production. Strains harboring a deletion in either *bgrU* (IBR503) or *bgrV* (IBR505)

showed very significant increments of their c-di-GMP contents (Fig. 4). These enhanced c-di-GMP levels were significantly reduced in the double mutants *bgrU bgrR* (strain IBR519) and *bgrV bgrR* (strain IBR520) (Fig. 4). Thus, there was a correlation between the CF^+ phenotype and high c-di-GMP contents. These results further supported BgrR as a DGC and indicated that *bgrRSTUWV* operon likely regulates MLG production through modulating BgrR-dependent c-di-GMP levels.

A likely partner-switching system regulates diguanylate cyclase activity of BgrR.

The domain conservation among BgrU, BgrW, BgrV with some components of PSS, and their influence in the BgrR-dependent intracellular c-di-GMP contents were suggestive of a partner-switching mechanism regulating the DGC activity of BgrR. Here, BgrU and BgrW would be the phosphatase and kinase, respectively, which would determine the unphosphorylated or phosphorylated state of BgrV, respectively. To test this hypothesis, we first obtained a double mutant *bgrU bgrV* (IBR521) and a triple mutant *bgrU bgrW bgrV* (IBR522). Both strains displayed a CF^+ phenotype (Table 1), however they responded differently to the introduction of a plasmid expressing *bgrV*. While the CF^+ phenotype of IBR521 remained unchanged, the phenotype of IBR522 was reverted to CF^- by the *bgrV* plasmid (Table 1). Thus, in the absence of both the putative kinase (BgrW) and phosphatase (BgrU), the STAS domain protein BgrV inhibited MLG production. Since *bgrU* and *bgrV* genes do not cross-complement (Table 1), we hypothesized that either the absence of BgrV or its putative permanent phosphorylation in the absence of BgrU would be sufficient to boost the synthesis of the MLG.

To confirm that the phosphorylated/dephosphorylated status of BgrV was at the crossroads of MLG regulation, we constructed a BgrV derivative in which a conserved putative phosphoryl-accepting serine (Ser61) was replaced with an alanine (Fig 3C). In

other STAS-containing proteins equivalent mutations lead to a permanent non-phosphorylated state of the protein (Diederich *et al.*, 1994; Kozak *et al.*, 2005; Hua *et al.*, 2006). The introduction of a plasmid expressing $BgrV^{S61A}$ into the Δ*bgrU* strain IBR503 as well as in the double mutant *bgrU bgrV* IBR521 reverted the CF^+ phenotypes to CF^-, in contrast to the wild type form of BgrV (Table 1).

We determined if these different phenotypes were actually due to differences in the phosphorylated states of BgrV and $BgrV^{S61A}$. For this, N-terminal c-Myc tagged derivatives of BgrV and $BgrV^{S61A}$ were obtained. The plasmids bearing these constructions were introduced into the wild type Rm8530, as well as in Δ*bgrU* and Δ*bgrW* strains, and the level of phosphorylated BgrV (P-BgrV) was assessed. Total protein extracts were separated by normal or Phos-tag SDS-PAGE (Barbieri and Stock, 2008), transferred to a membrane and proteins detected with anti c-Myc HRP conjugated antibody. A unique protein isoform was observed in all strains after normal SDS-PAGE (Fig. 5A). In contrast, Phos-tag gels revealed that two isoforms of BgrV were produced in the wild type background (Fig. 5B, lane 1). The P-BgrV disappeared after phosphatase treatment of the protein extracts (Fig. 5B, lane 2). In the absence of BgrU only P-BgrV was detected (Fig. 5B, lane 4), which again became unphosphorylated after CIP treatment (Fig. 5B, lane 5). On the contrary, all BgrV was in the non-phosphorylated state in the absence of the kinase-like BgrW (Fig. 5B, lane 7). As expected, wild type and IBR503 only produced a non-phosphorylated form of the tagged $BgrV^{S61A}$ (Fig. 5B, lanes 3 and 6), supporting the important role of Ser61 in phosphorylation of BgrV. The data indicated a dynamic system involving BgrU and BgrW that determines the phosphorylation status of BgrV. Interestingly, the amount of BgrV protein detected was lower in the strains producing P-BgrV, perhaps due to a reduced stability of the phosphorylated form of BgrV.

The *bgr* operon is conserved among Rhizobiales.

It was previously shown that the *bgsBA* operon responsible for the synthesis of the MLG is present in the genomes of *Sinorhizobium*, *Rhizobium*, *Agrobacterium* and *Methylobacterium* genera within the Rhizobiales (Pérez-Mendoza *et al.*, 2015). A *bgr* operon seems also widespread in the Rhizobiaceae but absent from Methylobacteriaceae family. Sinorhizobial and most *Rhizobium* genomes contain the full 6-gene *bgrRSTUWV* operon (Fig. 6). *Agrobacterium radiobacter* presents a degenerated *bgrW*-like kinase gene, where some of the critical residues for ATP binding are not conserved. A *bgr*-like operon is also recognizable in the genomes of *Mesorhizobium loti* MAFF303099 and the non-symbiotic *Ensifer adhaerens* OV14 and *Aminobacter aminovorans* KCTC 2477, despite neither of these bacteria carry *bgsBA*-like genes needed for the synthesis of the MLG. The lack of coexistence of *bgs* and *bgr* genes in some genomes suggests that *bgr* may regulate other activities besides MLG synthesis.

Discussion

Biosynthesis of the recently described MLG in *S. meliloti* is triggered upon an artificial increment of intracellular levels of cyclic diguanylate. Resembling bacterial cellulose synthases, c-di-GMP binding to the ML β-glucan synthase is required for enzyme activity and polysaccharide synthesis (Pérez-Mendoza *et al.*, 2015, 2017). We have identified an operon of 6 genes, *bgrRSTUWV*, that regulates the activation of MLG biosynthesis. One of the encoded proteins is BgrR, a DGC that provides the c-di-GMP needed for MLG synthesis and that seems to be the target of a PSS likely composed by proteins BgrU,

BgrV and BgrW. Figure 7 shows a proposed model of how this partner-switching complex regulates the synthesis of the MLG exopolysaccharide through BgrR.

BgrR (SMb20447) has DGC activity in spite of a non-canonical AGDEF motif, nevertheless similar to an active DGC from *V. cholerae* (Hunter *et al.*, 2014) or the SGDEF motif present in the DGC ECA3270 from *Pectobacterium atrosepticum* (Pérez-Mendoza *et al.*, 2011). Previously, Schäper *et al.* (2016) have shown that BgrR overproduction determines significant increments of cellular c-di-GMP contents and associated phenotypes in the MLG non-producer *S. meliloti* strain Rm2011. Likewise, we also found a correlation of BgrR overexpression with increased c-di-GMP contents and enhanced MLG synthesis in strain Rm8530. These two effects were lost after changing the BgrR AGDEF motif to AGDAF ($BgrR^{E215A}$), further supporting the AGDEF as a bona fide DGC active site. BgrR also possesses a well conserved EAL domain and therefore putative PDE activity, although there are no clues so far about possible PDE activity of this protein.

Proteins BgrU, BgrV and BgrW do likely form a partner-switching mechanism that regulates the activity of BgrR through the phosphorylation status of BgrV. Strains lacking BgrU and/or BgrV display enhanced c-di-GMP levels and production of MLG, both phenotypes dependent on a functional *bgrR* gene. This suggests that in the wild type situation, where little or no MLG is produced, the main roles of BgrU and BgrV are the inhibition of the DGC activity of BgrR. We have shown here that the STAS protein BgrV can be phosphorylated in a conserved serine residue (Ser61). Indeed, BgrV was found in both phosphorylated and unphosphorylated forms in the wild type strain Rm8530. *bgrU* mutants contain only the phosphorylated form of BgrV, what determines the same phenotypes as the complete lack of BgrV: enhanced c-di-GMP levels and MLG production. This indicates the phosphatase activity of BgrU acts on phosphorylated BgrV

and that dephosphorylated BgrV is required to inhibit BgrR. We have also shown that phosphorylation of BgrV requires the putative kinase BgrW, as *bgrW* mutants only produce the unphosphorylated form of BgrV. Similar to other PSS, we hypothesize that BgrV directly interacts with BgrR and currently investigate which domains of BgrR are important for this interaction. However, this system might be subjected to additional regulation through protein degradation, as we consistently found weaker signals in protein samples where P-BgrV was present.

Among the remaining proteins encoded in the *bgrRSTUWV* operon, BgrS and BgrT might have secondary roles in the PSS controlling MLG production, although they are present in other *bgr*-like operons detected in diverse rhizobial genomes. The homology of BgrT with methyltransferases suggests its possible role in the signal transduction cascade through BgrU. BgrU carries a HAMP domain which is found in histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins and some phosphatases (Hazelbauer and Lai, 2010). This domain may respond to environmental stimuli and be subjected to conformational changes mediated by methylation of specific residues within the protein (Zhou *et al.*, 2009). BgrU also contains a putative extracellular ligand-binding CACHE domain (Hazelbauer and Lai, 2010), which could also be involved in regulating its phosphatase activity in response to yet unknown signal compounds. BgrS shows no known functional domains but the CF^{+} phenotype of strains overexpressing *bgrS* suggest a positive role in MLG activation.

PSS are well known in *B. subtilis* and other Gram-positive bacteria as regulators of sporulation and stress responses, through the interaction with RNA polymerase sigma factors (Yudkin and Clarkson, 2005; Hecker *et al.*, 2007). However, similar PSS have been described for different processes in unrelated bacteria such as *Chlamydia* (Hua *et al.*, 2006) or the proteobacteria *Bordetella* and *V. fischerii* (Mattoo *et al.*, 2004; Visick,

2009). In *P. aeruginosa* the HptB-HsbR-HsbA PSS is known to regulate flagellar gene expression through the interaction of the dephosphorylated STAS domain protein HsbA with the anti-sigma factor FlgM (Bhuwan *et al.*, 2012). It also regulates biofilm formation by the interaction of the DGC HsbD and phosphorylated HsbA (Valentini *et al.*, 2016). In *M. thermoacetica* the switching partners formed by the stressosome and the kinase MtT modulate the activity of the DGC MtG. Besides a canonical GGDEF domain, MtG displays also N-terminal RsbU and GAF domains. The regulation of DGC activity by MtT is mediated by this RsbU N-terminal domain, resembling the protein-protein interaction mechanism that regulates RsbU phosphatase activity by the stressosome in *B. subtilis* (Quin *et al.*, 2012). However, the physiological response regulated by c-di-GMP levels through the stressosome in *M. thermoacetica* remains unknown. These connections between c-di-GMP levels and partner-switching systems point out that it might be a widespread mechanism to regulate the concentration of this second messenger within the cell. Nonetheless, different systems would have specific characteristics that determine the interactions between the proteins involved.

MLG synthesis is dependent on quorum sensing as the transcription of the biosynthetic operon *bgsBA* is dependent on the Sin/ExpR regulatory system (Pérez-Mendoza *et al.*, 2015). This seems not the case for the *bgr* operon, which has similar levels of expression in $ExpR^+$ and $ExpR^-$ strains. Probably the *bgr* operon is constitutively expressed, regulating c-di-GMP associated phenotypes by modulating the activity of BgrR. To understand the functioning of the system, it will be necessary to unravel the mechanism by which dephosphorylated BgrV is capable of turning down the synthesis of c-di-GMP by BgrR. It is also to be determined the signal(s) that turn on/off the system, as well as how the BgrR perceives it, since the BgrR protein carries no clear protein-protein

366 interaction domains. Nevertheless, we anticipate that BgrR likely interacts with either
367 phosphorylated or, more plausible, unphosphorylated BgrV.

368 The co-occurrence of the *bgr* operon in rhizobia that also bear the *bgsBA* operon suggests
369 a widespread similar control of the MLG synthesis. On the other hand, the effect that this
370 operon has on the homeostasis of a crucial second messenger like c-di-GMP, may have
371 important consequences on several other traits which have not been considered here.
372 Schäper and coworkers (2016) have reported that overexpression of SMb20447 (BgrR)
373 has effects on motility, EPS I production and cell morphology of the MLG non-producer
374 strain Rm2011. Thus, further studies will be necessary to determine the importance of the
375 *bgr* operon in other traits of *S. meliloti*. It will also be interesting to analyze the role(s) of
376 *bgr*-like operons in other bacteria that do not harbor *bgsBA* genes, which might widen our
377 understanding of the relationships between partner-switching systems and c-di-GMP
378 regulatory networks.

379

380 **Experimental procedures**

381 Strains and growth conditions.

382 The strains and most relevant plasmids used in this study are listed in Table S1.
383 *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37℃.
384 *Sinorhizobium meliloti* strains were grown in TY medium (Beringer, 1974) YMB
385 (Vincent, 1970) or MM (Robertsen *et al.*, 1981) at 28℃. When required, antibiotics and
386 other compounds were added at the following final concentrations: streptomycin (Sm),
387 300 µg ml^{-1}; gentamicin (Gm), 10 µg ml^{-1} for *E. coli* and 25-50 µg ml^{-1} for *S. meliloti*;
388 neomycin (Nm), 100 µg ml^{-1}; tetracycline (Tc), 10 µg ml^{-1}; ampicillin (Amp), 100 µg ml^{-}

389 [1]; chloramphenicol (Cm), 30 µg ml^{-1}; trimethoprim (Tmp), 400 µg ml^{-1}; calcofluor (CF),
390 100 µg ml^{-1} for liquid medium and 200 µg ml^{-1} for plates; Congo red (CR), 50 µg ml^{-1}.

391 Construction of *S. meliloti* strains and plasmids.

392 Transductions with ΦM12 were performed as described previously (Finan *et al.*, 1984).
393 Random general mutagenesis and tri-parental matings were done as previously described
394 (Glazebrook and Walker, 1991). PCR fragments containing partial or whole gene
395 amplifications were routinely cloned into pGEM®-T Easy cloning vector (Promega) to
396 be sequenced and to obtain additional flanking restriction sites. All deletion mutants were
397 obtained by double homologous recombination events using derivatives from plasmid
398 pK19*mobsacB* (Schäfer *et al.*, 1994). Briefly, for each gene around 1kb up- and
399 downstream DNA fragments were amplified with Hot Start High Fidelity Polymerase
400 (Roche Diagnostics). The fragments were excised from pGEM®-T Easy with the
401 corresponding restriction enzymes and ligated into plasmid pK19*mobsacB* to generate in-
402 frame deletion derivatives of each gene. Plasmids for overexpression of genes were
403 constructed by amplifying the DNA fragments containing at least the complete ORF and
404 cloned into pGEM®-T Easy. Then, the fragments were subcloned into the broad host
405 range plasmids pBBR1MCS-5 under the control of the internal *lac* promoter, except for
406 *bgrR* which was cloned under the control of the *bgr* operon promoter. *bgrU* was
407 subcloned into pFAJ1708 due to putative gene-dose toxic effects in the higher copy
408 number plasmid pBBR1MCS-5, as it is predicted to code for a membrane protein. When
409 only one restriction enzyme was used, the direction of the insert was confirmed by PCR.
410 Insertion mutants were obtained by inserting a *lacZ*-Gn^r cassette in the gene of interest
411 and cloned into pK19*mobsacB* to look for double homologous recombination. Mutant
412 strains were checked by PCR, Southern blot using probes from the corresponding gene

413 and complementation analysis. All primers used for the constructions and the analysis are
414 listed in table S2.

415 For the construction of the c-Myc tagged version of BgrV (pJLR1130), a forward primer
416 containing a KpnI site followed by an ATG start codon and the c-Myc tag sequence was
417 used in combination with a reverse primer containing HindIII site (Table S2). PCR
418 products were cloned into pGEM®-T Easy and the sequences of several clones were
419 verified. The selected clone and the expression vector pMLBAD were digested with the
420 KpnI isoschizomer Acc65I and the cohesive ends were refilled using the Klenow
421 fragment from the *E. coli* DNA polymerase I (Thermo Scientific Inc). Finally, samples
422 were digested with HindIII to generate blunt-HindIII fragment and ligated. The purpose
423 of this strategy was to obtain in-frame translational fusions with the Ribosome Binding
424 Site located in the pMLBAD vector. The sequences of the resulting pMLBAD derivatives
425 were verified.

426 RNA extraction and RT-PCR.

427 *S. meliloti* Rm8530 RNA was extracted from 50 ml of late exponential TY cultures with
428 Trizol® according to the manufacturer's specifications (Invitrogen). DNA was eliminated
429 with RQ1 RNase-Free DNase treatment (Promega) for 30 minutes at 37°C and the RNA
430 was purified again with Trizol®. RNA concentration was determined with a Nanodrop®
431 spectrophotometer and the integrity was verified in agarose gels. All RNA samples were
432 stored at -80°C.

433 First-strand cDNA was prepared with the iScript™ cDNA Synthesis Kit from Bio-Rad
434 by using 1 µg of total RNA in the reaction. For reverse-transcription PCR, 1 µl (50 ng)
435 of cDNA was used in each mixture. Reactions with the same amount of RNA or genomic
436 DNA templates instead of cDNA were used as negative and positive controls

437 respectively. The primers pairs used to amplify the intergenic regions are listed in table
438 S2.

439 Site-directed mutagenesis.

440 The construction of BgrR^{E215A} with an alanine replacing the glutamic acid in the AGDEF
441 active site was done by using two rounds of PCR. In the first round forward and reverse
442 primers with the desired nucleotide substitution, OB129 and OB130 (Table S2), were
443 used in conjunction with upstream or downstream primers, OB73 and OB74. The PCR
444 products were subjected to a second round of PCR with OB73 and OB74 to obtain a full
445 length *bgrR* gene with the substitution. The PCR product was cloned into pGEM®-T Easy
446 and sequenced. A correct point-mutated *bgrR* was cloned into pBBR1MCS-5 for further
447 analysis. Substitution of the serine-61 of BgrV with an alanine was performed according
448 to the instructions from the Q5® Site-Directed Mutagenesis Kit (New England Biolabs
449 Inc.), using primers with the desired nucleotide changes (OB149 and OB150) and plasmid
450 pJLR1046 as the template. Several clones were sequenced and a correct substituted one
451 was selected for subcloning into pBBR1MCS-5. The BgrV c-Myc tagged version
452 harbouring S61A substitution was obtained by digesting plasmid pJLR1130 with PstI and
453 HindIII, which creates a 170 bp deletion. The deleted fragment was substituted by a
454 corresponding fragment amplified from pJLR1137 with OB162 and OB163 and digested
455 with the same enzymes, and the substitution was confirmed by sequencing.

456 Calcofluor binding assays.

457 The CF assay used was based on the method described elsewhere (Pérez-Mendoza *et al.*,
458 2015). Overnight TY cultures of the desired strains where centrifuged and the pellets were
459 washed three times with minimal medium (MM). Cultures were diluted 1:100 in flasks
460 containing 10 ml of MM supplemented with CF (100 µM final concentration) and
461 incubated at 30ºC and 180 rpm for 24-28 hours. The cultures were centrifuged for one

hour at 4000 × *g*. Supernatants containing unbound CF were removed and pellets resuspended in 2 ml of distilled water and placed in 24-well plates. Similar growth of the strains was confirmed by measuring the OD_{600}. Fluorescence was measured with a FluoDiaT70 Microplate Reader (Photon Technology International) by exciting the samples at 365nm and recording the emission at 450 nm. Three biological replicates per strain were analyzed. The results were expressed as arbitrary units ± SD and subjected to the ANOVA test using $P<0.05$ as a threshold for significant differences.

β-galactosidase assays.

Overnight cultures of the desired strains were used to start 5 ml TY cultures incubated until they reached an OD_{600} close to 0.4. Transcriptional activity was measured as described elsewhere (Miller, 1972). At least two biological samples and three technical replicates were used for each strain. The results were expressed as arbitrary units ± SD and subjected to the ANOVA test using $P<0.05$ as a threshold for significant differences.

Bioinformatic analysis

Amino acid sequence comparison, alignments and homology detection of the different proteins were performed with BLAST, T-Coffee and MPI Bioinformatics Toolkit software (Altschul *et al.*, 1990; Notredame *et al.*, 2000; Zimmermann *et al.*, 2018). The homology of the *bgrRSTUWV* operon in other bacteria species was analyzed by using the KEGG data base (Kanehisa *et al.*, 2016).

ML β-glucan isolation and FTIR analysis.

To purify the exopolysaccharide, the protocol described previously (Pérez-Mendoza *et al.*, 2015) was followed, with some modifications. Briefly, a starting culture of the desire strain was diluted 1:100 in YMB for three days with slow shaking (80 rpm). The flocks

were collected using a sieve with a 90-µm cutoff and subjected four consecutive rounds of a process consisting of washing with boiling Milli-Q water, cooling down to room temperature and centrifugation for 20 min at 3,220 × g. The exopolysaccharide was frozen and lyophilized. Infrared spectra were obtained on a FTIR Bruker IFS66v, using potassium bromide (KBr) discs. The transmittance was measure from 550 to 4000 cm^{-1} at resolution of 4 cm^{-1}.

Quantification of intracellular c-di-GMP.

Extraction and quantification of c-di-GMP for IBR500/Rm8530 comparison was performed using the method previously described in Pérez-Mendoza *et al.*, 2014. Samples shown in Fig. 4 were obtained by the method described in Burhenne and Kaever, 2013. Briefly, 5 ml TY cultures of the desired strains grown to an approximately OD600 of 0.8 were centrifuged, washed twice with TY and the nucleotides were extracted from the pellets with cold extraction solvent (acetonitrile/methanol/water; 2/2/1; v/v/v). Samples were dried, resuspended in water and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) at Research Service Centre Metabolomics, Hannover Medical School (Germany). The c-di-GMP content was normalized to total protein, determined by the BCA method (Smith *et al.*, 1985). Three biological replicates of each strain were measured and values were expressed as pmol c-di-GMP /mg protein ± standard error.

In-vivo phosphorylation assays.

Overnight cultures of the desired *S. meliloti* stains harbouring pMLBAD, pJLR1130 or pJLR1149 were used to start new 50 ml TY cultures, which were induced with 2% arabinose at an initial OD=0.4 until they reach an OD=0.8. Cells were harvested by centrifugation (10 min/6000 x g/10ºC). Pellets were resuspended in 1ml H_2O, centrifuged again at 13,000 x g for 5 min, frozen with liquid nitrogen and stored at -20ºC. Total protein

511 extraction was performed by resuspending the aliquots in the BugBuster® Plus
512 Lysonase™ Kit (40 µl for 1 OD_{600}; Novagen) supplemented with protease inhibitors
513 (Complete, Mini, EDTA-free, Roche) and incubating at room temperature for 1 hour.
514 Lysates were centrifuged (20 min/20,000 x g/4ºC). 2 OD_{600} units of the pellets were
515 loaded into a 12% SDS-polyacrylamide gels (Gallagher, 2012) non-supplemented or
516 supplemented with 25 µM Phos-tag™ (NARD Chemicals, Hiroshima) and 50 µM $MnCl_2$.
517 For samples containing phosphorylated form of BgrV, 2 OD_{600} units of the pellets were
518 treated with 25 units of calf intestinal phosphatase at 37°C for 90 min. prior loading into
519 the gels. Proteins were transferred to a Protran BA85 nitrocellulose membrane (GE
520 Healthcare Life Sciences) and stained with Red Ponceau to verify the transferences and
521 similar protein loads. Membranes were subjected to a Western blot analysis (Gallagher *et*
522 *al.*, 2008) using a c-Myc HRP antibody (Santa Cruz Biotechnology Inc) according to the
523 manufacturer's instructions.
524

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705 **Table 1.** Calcofluor-fluorescent phenotype of the strains used in this study.

706

Strain	Mutated genes	Gene *in trans*	CF Phenotype
Rm8530	None	None	-
		bgrR	+
		$bgrR^{E215A}$	-
		bgrS	+
		bgrT	-
		bgrU	-
		bgrW	+
		bgrV	-
IBR500	Δ*bgrR*	None	-
		bgrW	-
IBR501	Δ*bgrS*	None	-
		bgrS	+
IBR502	Δ*bgrT*	None	+
		bgrT	-
IBR503	Δ*bgrU*	None	+
		bgrU	-
		bgrV	+
		$bgrV^{S61A}$	-
IBR504	Δ*bgrW*	None	-
		bgrR	+
IBR505	Δ*bgrV*	None	+
		bgrU	+
		bgrV	-
		$bgrV^{S61A}$	-
IBR518	Δ*bgrR* Δ*bgrT*	None	-
		bgrR	+
IBR519	Δ*bgrR* Δ*bgrU*	None	-
		bgrR	+
IBR520	Δ*bgrR* Δ*bgrV*	None	-
		bgrR	+
IBR521	Δ*bgrU* Δ*bgrV*	None	+
		bgrV	+
		$bgrV^{S61A}$	-
IBR522	Δ*bgrU* Δ*bgrW* Δ*bgrV*	None	+
		bgrV	-

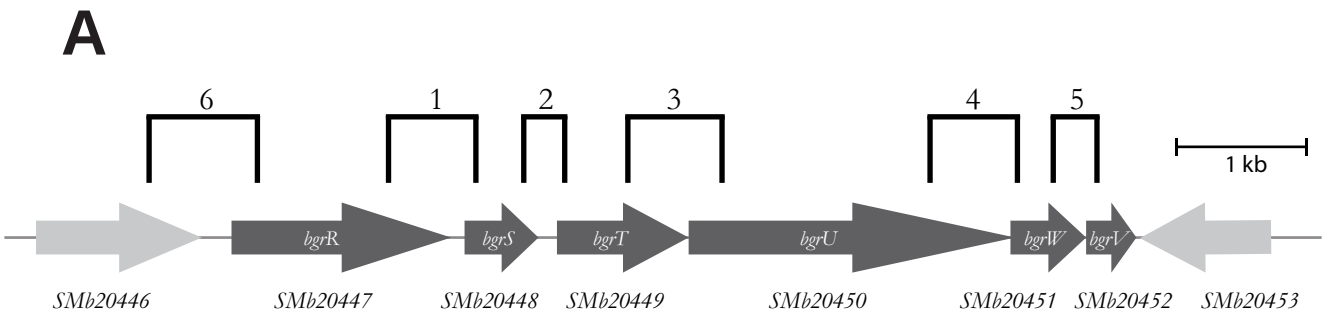
Fig. 1. *SMb20450* regulates the synthesis of ML β-glucan and forms part of a hexacistronic operon. (**A)** Genetic map of the pSymB region regulating the synthesis of ML β-glucan. The new proposed names are shown inside the ORFs, whereas the genome annotated numbers are displayed below. **(B)** Calcofluor fluorescence phenotype on TY plates of (a) Rm8530, (b) IBR503 (Δ*SMb20450*), (c) Rm8530 (pJBpleD*), (d) IBR503 (pJLR1110), (e) IBR508 (Δ*SMb20450 exoY210*::Tn*5-233, wgaB*::Tn*5*), (f) IBR515 (Δ*SMb20450 SMb20391*::*lacZ-Gn*). (**C**) RT-PCR analysis *bgrRSTUWV* transcription. Shown are the amplicons of the corresponding intergenic regions marked in A.

Fig. 2. Contrasting effects of *bgr* genes on the synthesis of ML β-glucan. Quantification of CF-derived fluorescence of cells grown in minimal medium supplemented with CF. Results are expressed in relative fluorescence compared to the wild type strain (WT, black bars). The numbers correspond to the genes either deleted **(A)** or overexpressed **(B)**. *SMb20047*/*bgrR* (1), *SMb20448*/*bgrS* (2), *SMb20449*/*bgrT* (3), *SMb20450*/*bgrU* (4), *SMb20451*/*bgrW* (5), *SMb20452*/*bgrV* (6). Different letters above the bars indicate statistically significant differences ($p<0.05$)

Fig. 3. Predicted protein structures of *S. meliloti* PSS-like proteins regulating ML β-glucan and comparison with other well characterized PSS. Conserved residues, motifs and domains important for protein function are shown. *Sm, Sinorhizobium meliloti*; *Bb, Bordetella bronchiseptica*; *Ct, Chlamydia trachomatis*; *Bs, Bacillus subtilis*. **(A)** BgrU contains two putative transmembrane (TM), a putative Cache (C), a HAMP (H) and PP2C serine-phosphatase domains. **(B)** BgrW contains the four motifs present in the

731 Bergerat ATP-binding fold (N, G1, G2 and G3), found in different ATPases/kinases.
732 **(C)** BgrV contains a STAS domain found in anti-sigma factor antagonists, including a
733 conserved phosphorylatable serine residue (S61 in BgrV).
734
735 **Fig. 4.** Quantification of c-di-GMP in Rm8530 and *bgr* derivatives. IBR503 (Δ*bgrU*),
736 IBR519 (Δ*bgrR* Δ*bgrU*), IBR505 (Δ*bgrV*), IBR520 (Δ*bgrR* Δ*bgrV*).
737
738 **Fig. 5**. BgrV phosphorylation status is regulated by BgrU and BgrW. *In vivo*
739 phosphorylation analysis of c-Myc tagged BgrV and $BgrV^{S61A}$ overexpressed in
740 different genetic backgrounds: Rm8530 (lanes 1-3), IBR503 (lanes 4-6), and IBR504
741 (lane 7). Total protein extracts were subjected to PAGE without **(A)** and with Phos-tag
742 reactive added **(B)**. Tagged proteins were revealed with anti-c-Myc antibody. Samples
743 showing a retarded mobility protein form were also subjected to a calf intestinal
744 phosphatase treatment (CIP). A control lane with extracts from Rm8530 harbouring an
745 empty plasmid and CIP treatment was included (lane 8).
746
747 **Fig. 6.** Conservation of the *bgr* operon in Rhizobiales. The characteristic domains of the
748 encoded proteins are indicated (see also Legend to Fig. 3).
749
750 **Fig. 7.** Proposed model for control of the synthesis of the ML β-glucan through regulation
751 of the DGC activity of BgrR. Both BgrW (kinase) and BgrU (phosphatase) act on a
752 specific serine in BgrV, but under MLG non-producing conditions (right) the phosphatase
753 BgrU dominates and maintains most BgrV dephosphorylated, which leads to the

754 inhibition of BgrR DGC activity; therefore no c-di-GMP is available to activate the
755 glycosyl transferase BgsA, and no or very little MLG is produced. Upon unknown signals
756 for MLG production (left), BgrU PP2C activity is inhibited and most BgrV stays in the
757 phosphorylated state; in this situation the DGC of BgrR becomes uninhibited and
758 provides sufficient c-di-GMP for MLG synthesis. Putative roles of BgrS and BgrT in the
759 modulation of BgrU phosphatase activity are presumed.


A
6
1
2
3
4
5
1 kb
bgrR
bgrS
bgrT
bgrU
bgrW
bgrV
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SMb20447
SMb20448
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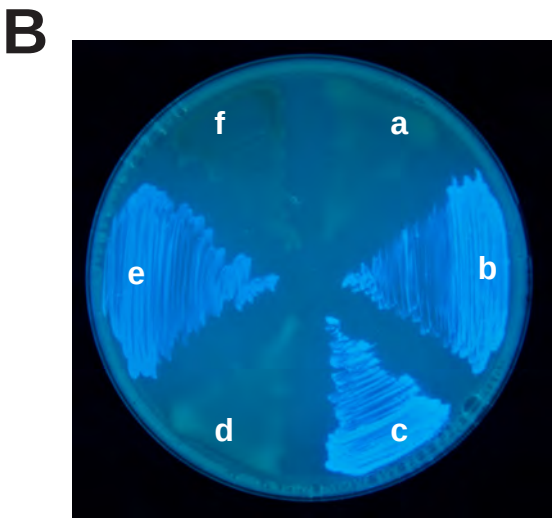
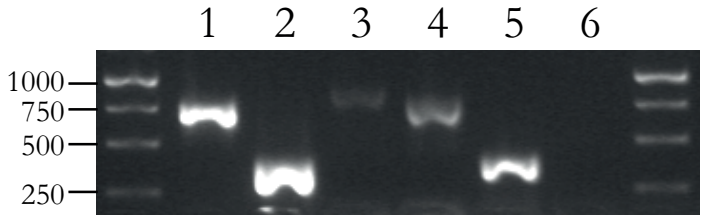

B
f
a
e
b
d
c


C
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2
3
4
5
6
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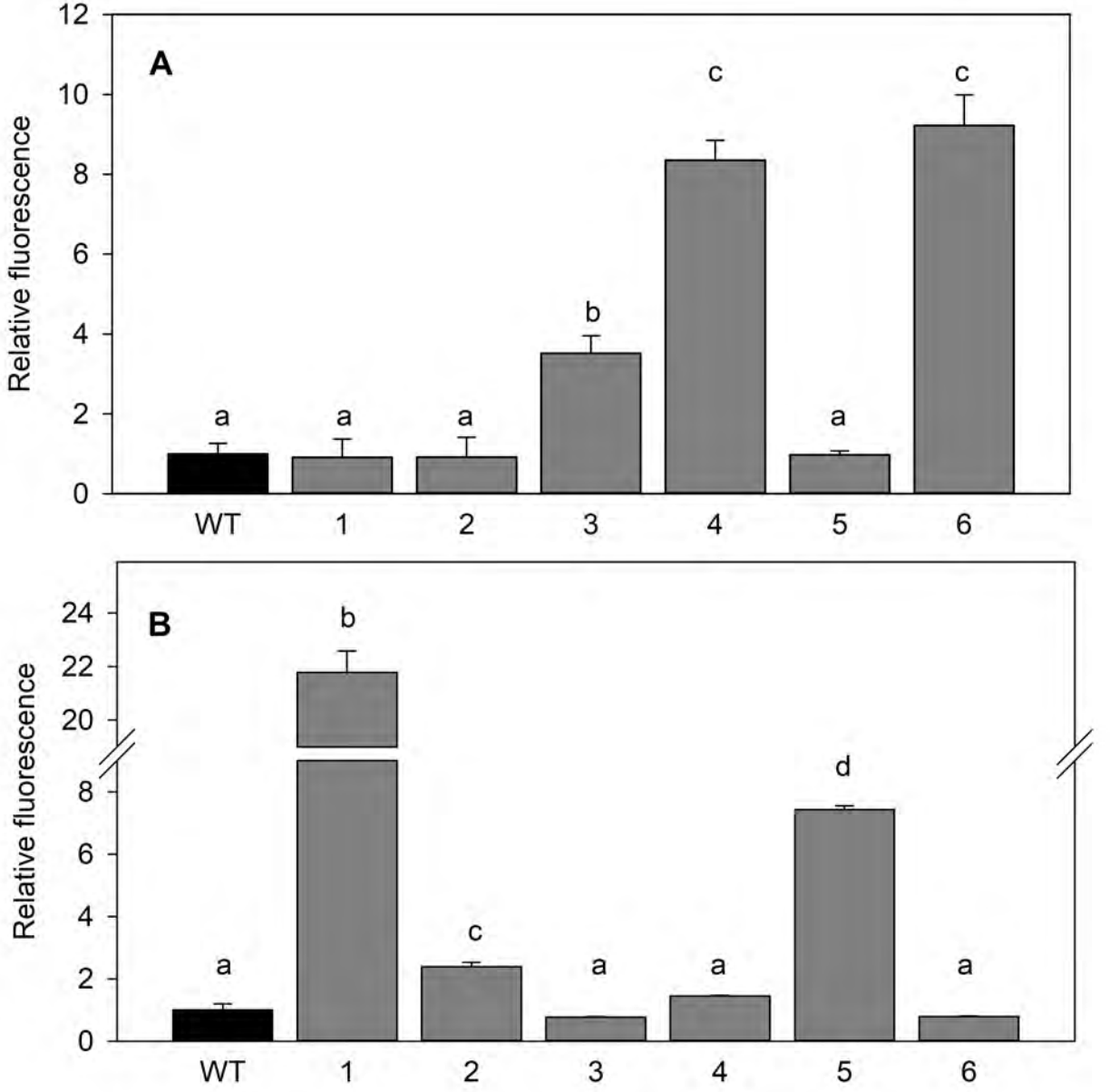
750
500
250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Relative fluorescence
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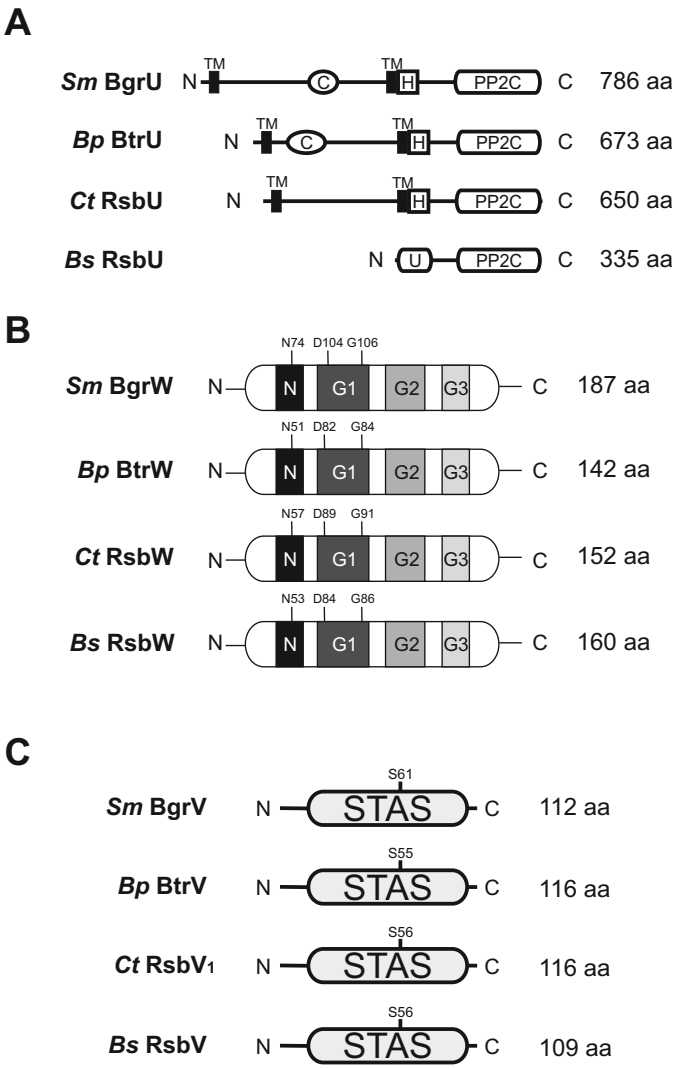
10
8
6
4
2
0
a
a
a
b
c
a
c
WT
1
2
3
4
5
6
B
Relative fluorescence
24
22
20
8
6
4
2
0
a
b
c
a
a
d
a
WT
1
2
3
4
5
6


A
Sm BgrU
TM
TM
N
C
H
PP2C
C
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Bp BtrU
673 aa
Ct RsbU
650 aa
Bs RsbU
U
335 aa
B
N74 D104 G106
Sm BgrW
N
G1
G2
G3
187 aa
N51 D82 G84
Bp BtrW
142 aa
N57 D89 G91
Ct RsbW
152 aa
N53 D84 G86
Bs RsbW
160 aa
C
S61
Sm BgrV
STAS
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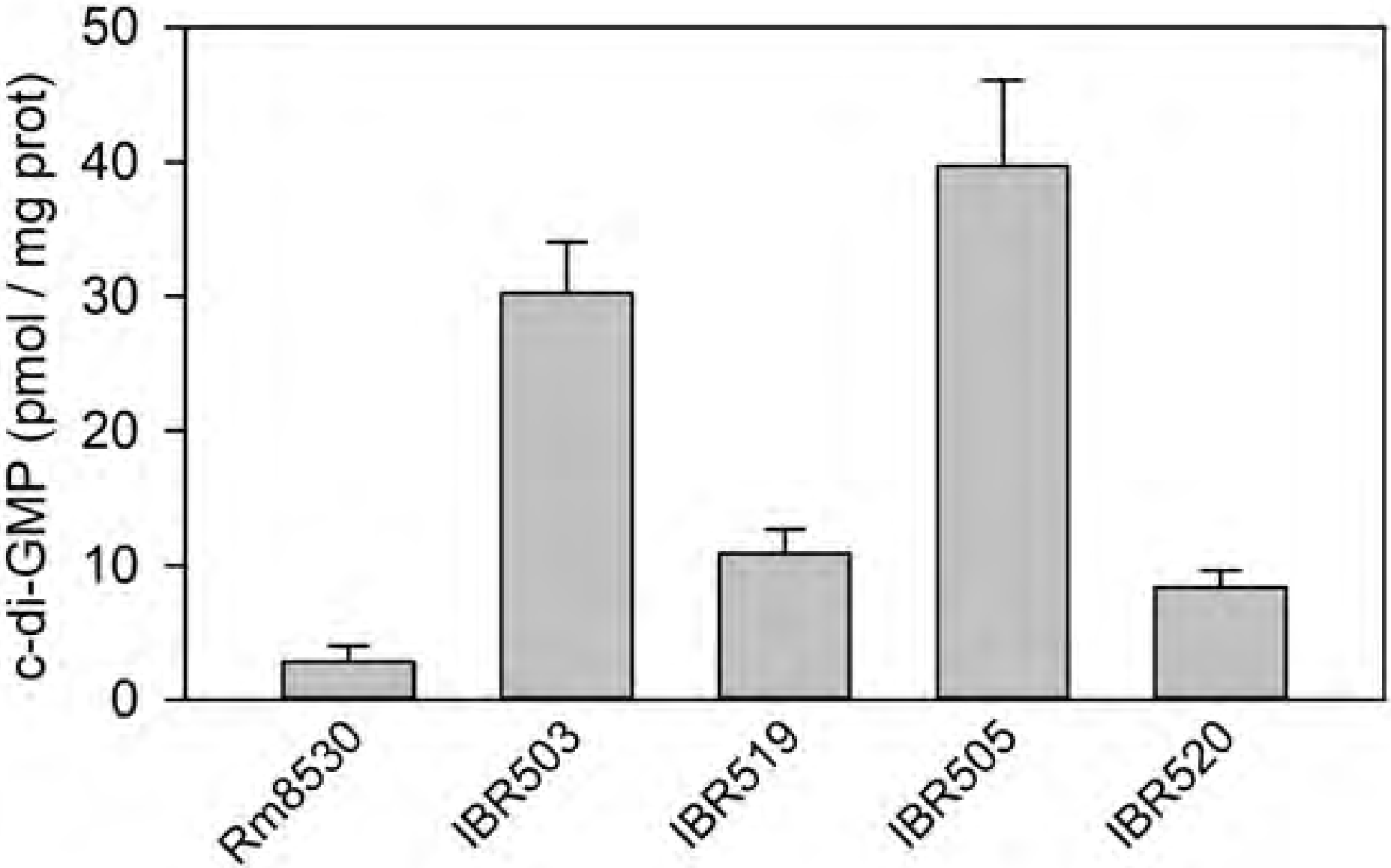
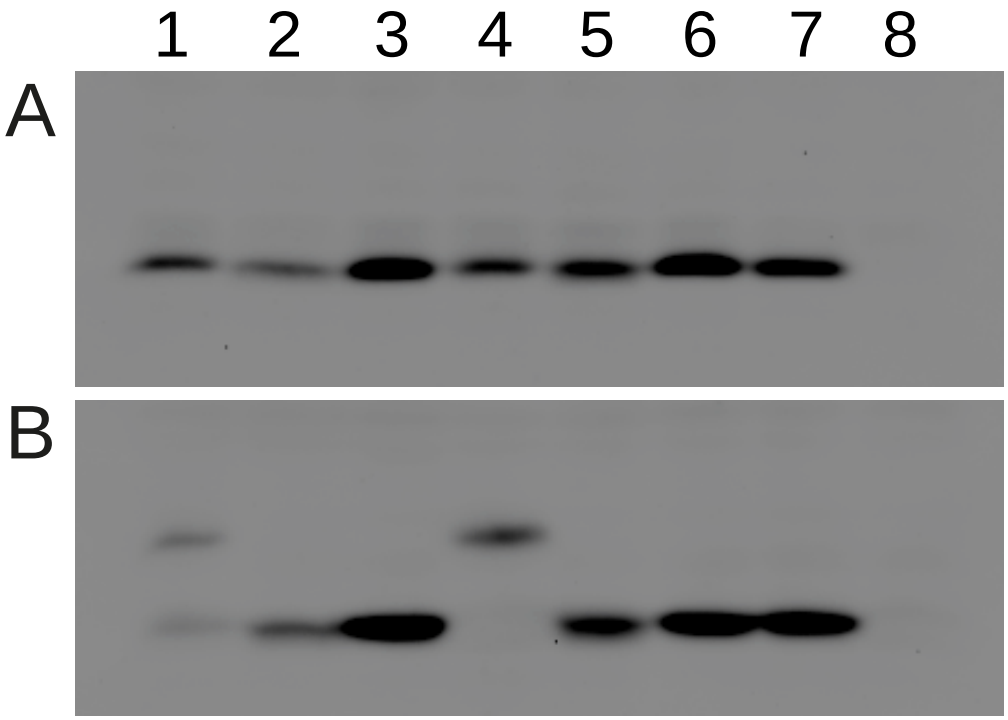
116 aa
S56
Ct RsbV1
116 aa
Bs RsbV
109 aa


c-di-GMP (pmol / mg prot)
50
40
30
20
10
0
Rm8530
IBR503
IBR519
IBR505
IBR520



BgrV	+	+	-	+	+	-	+	-
$BgrV^{S61A}$	-	-	+	-	-	+	-	-
pMLBAD	-	-	-	-	-	-	-	+
CIP	-	+	-	-	+	-	-	+

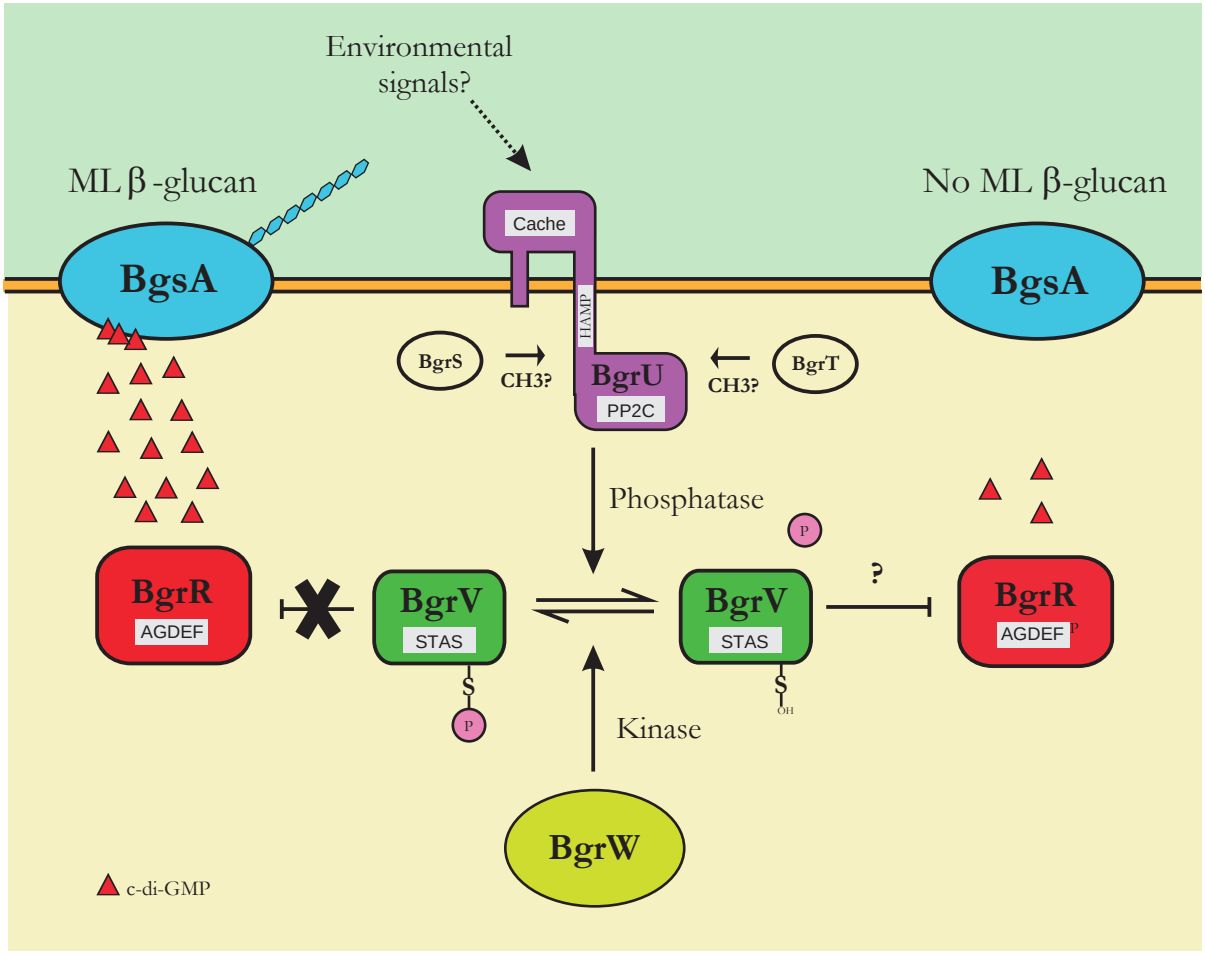
Sinorhizobium/Ensifer group

Rhizobium sp.

Agrobacterium radiobacter

Aminobacter aminovorans

Mesorhizobium loti MAFF303099


Environmental signals?
ML β-glucan
No ML β-glucan
BgsA
BgsA
Cache
HAMP
BgrS
CH3?
BgrU
PP2C
CH3?
BgrT
Phosphatase
BgrR
AGDEF
BgrV
STAS
P
BgrV
STAS
OH
?
BgrR
AGDEF
P
Kinase
BgrW
c-di-GMP

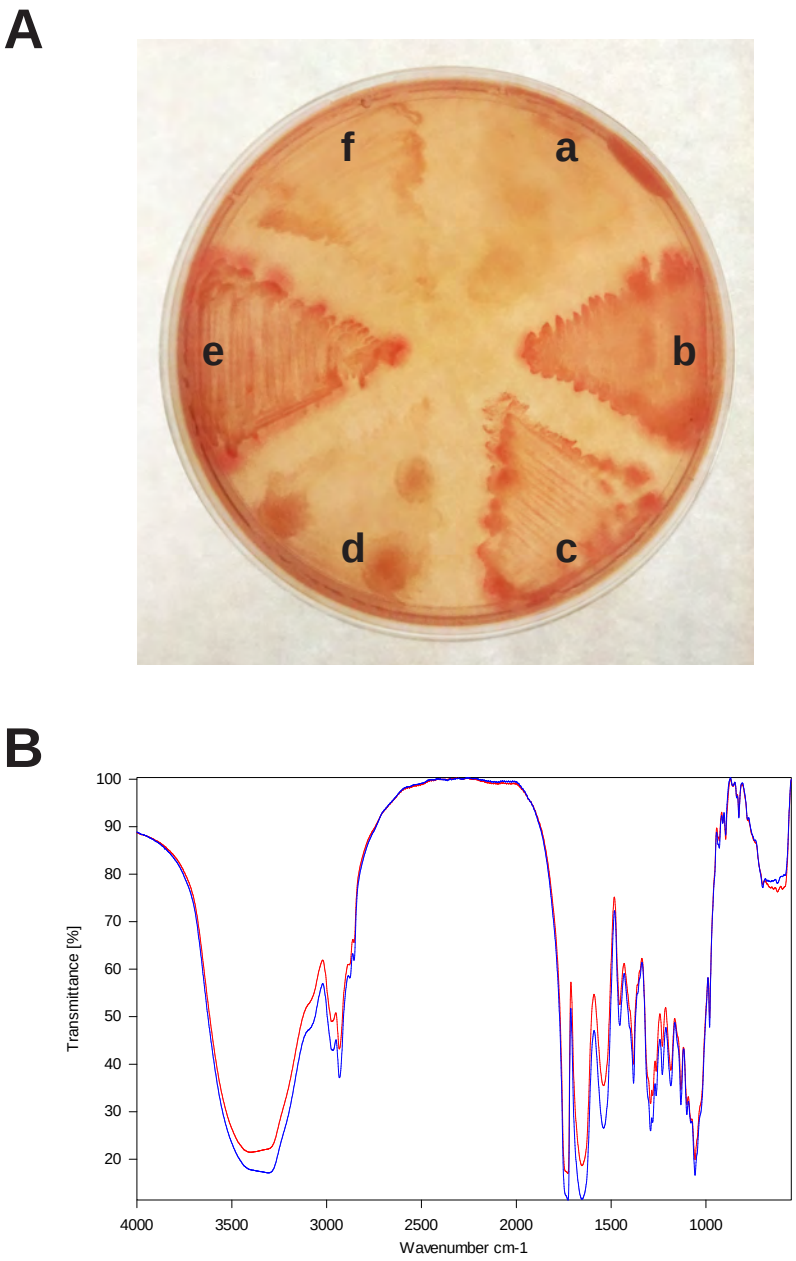


Figure S1. A deletion in *SMb20450* promotes the synthesis of ML β-glucan. **(A)** Congo red phenotype on TY plates of (a) Rm8530, (b) IBR503 (Δ*SMb20450*), (c) Rm8530 (pJBpleD*), (d) IBR503 (pJLR1110), (e) IBR508 (Δ*SMb20450 exoY210::*Tn*5-233 wgaB::*Tn*5*), (f) IBR515 (Δ*SMb20450 SMb20391::lacZ-Gn*). **(B)** FTIR transmission spectra from the ML β-glucan (blue line) and the polymer produced by IBR503 (red line).

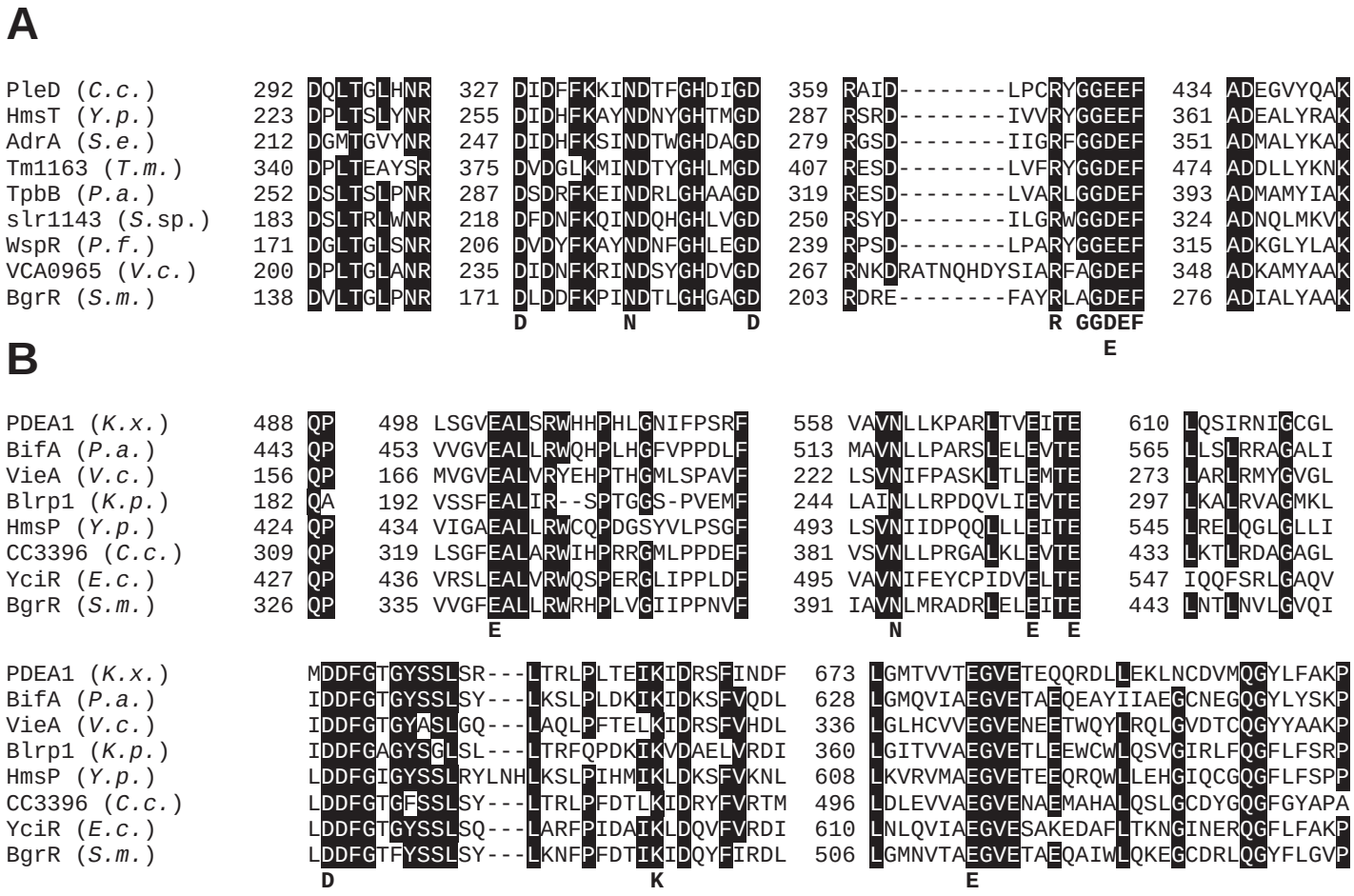


Figure S2. Multiple alignment of characterized DGC (A) and PDE (B) with BgrR. Conserved residues are shade in black and residues described as critical for domain activity are shown in bold letters at the botton. Bacterial species are indicated between brackets: *Caulobacter crescentus* (*C.c.*), *Yersinia pestis* (*Y.p.*) *Salmonella enterica* (*S.e.*), *Thermotoga maritima* (*T.m.*), *Pseudomonas aeruginosa* (*P.a.*), *Synechocystis* sp. (*S*.sp.), *Pseudomonas fluorescens* (*P.f.*), *Vibrio Cholerae* (*V.c.*), *Komagataeibacter xylinus* (*K.x.*), *Klebsiella pneumoniae* (*K.p.*), *Escherichia coli* (*E.c.*), *Sinorhizobium meliloti* (*S.m.*).

Supplemental Table 1. Strains and plasmids used in this work.

Strains	Genotype	Reference
S. meliloti strains		
Rm1021	SU47, Sm^r	(Galibert et al. 2001)
Rm8530	Rm1021 *expR*$^+$*;* Sm^r	(Glazebrook and Walker 1989)
IBR500	Rm8530 Δ*bgrR;* Sm^r (Δ*SMb20447*)	This study
IBR501	Rm8530 Δ*bgrS;* Sm^r (Δ*SMb20448*)	This study
IBR502	Rm8530 Δ*bgrT;* Sm^r (Δ*SMb20449*)	This study
IBR503	Rm8530 Δ*bgrU;* Sm^r (Δ*SMb20450*)	This study
IBR504	Rm8530 Δ*bgrW;* Sm^r (Δ*SMb20451*)	This study
IBR505	Rm8530 Δ*bgrV;* Sm^r (Δ*SMb20452*)	This study
IBR508	IBR503 *exoY210*::Tn*5-233*, *wgaB*::Tn5*;* Sm^r, Gn^r, Nm^r	This study
IBR509	Rm8530 *bgrU*::*lacZ-Gn;* Sm^r, Gn^r	This study
IBR510	Rm1021 *bgrU*::*lacZ-Gn;* Sm^r, Gn^r	This study
IBR511	Rm8530 *bgrR*::*lacZ-Gn;* Sm^r, Gn^r	This study
IBR512	Rm1021 *bgrR*::*lacZ-Gn;* Sm^r, Gn^r	This study
IBR513	Rm8530 *bgsA*::*lacZ-Gn;* Sm^r, Gn^r	This study
IBR515	IBR503 *bgsA*::*lacZ-Gn;* Sm^r, Gn^r	This study
IBR518	Rm8530 Δ*bgrR* Δ*bgrT;* Sm^r	This study
IBR519	Rm8530 Δ*bgrR* Δ*bgrU*; Sm^r	This study
IBR520	Rm8530 Δ*bgrR* Δ*bgrV;* Sm^r	This study
IBR521	Rm8530 Δ*bgrU* Δ*bgrV;* Sm^r	This study
IBR522	Rm8530 Δ*bgrUWV;* Sm^r	This study
E. coli strains		
DH5α	*endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1 Δ(lacZYA-argF)U169 Φ80d lacZΔM15*	Gibco-BRL
Plasmids		
pRK600	Helper plasmid for triparental mating; Cm^r	(Finan et al. 1986)
pGEM® T-Easy	Cloning vector for PCR; Amp^r	Promega Corp.
pK19*mob*	Suicide vector in *S. meliloti*; *oriV*, Kn^r	(Schäfer et al. 1994)
pK19*mobsacB*	Suicide vector in *S. meliloti*; *oriV, sacB*, Kn^r	(Schäfer et al. 1994)
pBBR1MCS-5	Broad-host-range plasmid; Gn^r	(Kovach et al. 1995)

pFAJ1708	Broad-host-range plasmid with *nptII* promoter; Tc^r	(Dombrecht et al. 2001)
pSUP2021	pSUP202 derivative containing Tn*5*; Ap^r, Cm^r, Km^r	(Simon et al. 1983)
pAB2001	pUC6S derivative containing a *lacZ*-Gn^r cassette; Ap^r	(Becker et al. 1995)
pJBPleD*	pJB3Tc19 derivate bearing a 1423 bp XbaI/EcoRI fragment containing *pleD**; Ap^r, Tc^r	(Pérez-Mendoza et al. 2014)
pMLBAD	Expression vector inducible with arabinose; Tmp^r	(Lefebre and Valvano 2002)
pJLR1046	pGEM® T-Easy with complete amplified *bgrV* cloned; Ap^r	This study
pJLR1086	pBBR1MCS-5 with *bgrR* cloned; Gn^r	This study
pJLR1090	pK19*mobsacB* with *bgrR*::*lacZ*-Gn^r	This study
pJLR1095	pK19*mobsacB* with *bgrU*::*lacZ*-Gn^r	This study
pJLR1102	pK19*mobsacB* with *bgsA*::*lacZ*-Gn^r	
pJLR1105	pFAJ1708 with *bgsA* cloned; Tc^r	This study
pJLR1110	pFAJ1708 with *bgrU* cloned; Tc^r	This study
pJLR1119	pBBR1MCS-5 with *bgrV* cloned; Gn^r	This study
pJLR1120	pBBR1MCS-5 with *bgrS* cloned; Gn^r	This study
pJLR1121	pBBR1MCS-5 with *bgrT* cloned; Gn^r	This study
pJLR1122	pBBR1MCS-5 with *bgrW* cloned; Gn^r	This study
pJLR1124	pJLR1086 derivative with a point mutation at the AGDEF site of BgrR (E215A); Gn^r	This study
pJLR1130	pMLBAD with a c-Myc tagged version of BgrV; Tmp^r	This study
pJLR1137	pBBR1MCS-5 with *bgrV* harbouring a Ser61 to alanine substitution (S61A); Gn^r	This study
pJLR1149	pJLR1130 derivative with a bgrV harbouring Ser61 to alanine substitution (S61A); Tmp^r	This study

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Supplemental Table 2. Primers used in this study. Specific restriction sites are indicated with bold letters.

Primer pair	PCR product	Sequence
Construction of mutants		
OB118 OB119	1048 bp *bgrR* up-stream fragment	5'TCGTGAAATGACGGAGGAGC 5'**AGATCT**CGATGGAAGATGAA
OB120 OB121	1204 bp *bgrR* down-stream fragment	5'**AGATCT**ATGTTACCGCCGAAGGAGTG 5'GCGCCAGGTCATTTTCGTTT
OB122 OB123	1129 bp *bgrS* up-stream fragment	5'ACGAGTTCGCGGTCATTCAGTGG 5'**GCGGCCGC**CTCCCTTACGCAAAAAC
OB116 OB70	1242 bp *bgrS* down-stream fragment (including a NotI internal site)	5'GAGTCGCGAGGCTGTTCTTCTG 5'GATGGAGGGCGGCAAAAATGTGGT
OB120 OB117	1056 bp *bgrT* up-stream fragment	5'**AGATCT**CGATGGAAGATGAA 5'**GGATCC**ACGACGCTCGAGGTCAC
OB91 OB86	800 bp *bgrT* down-stream fragment	5'**GGATCC**GGTCGCGAGGCTGTTTAG 5'**GCATGC**GGAAGCGGAAGGTGGTTC
OB85 OB86	885 bp *bgrU* up-stream fragment	5'**GGATCC**GCGGGCGGGTCTATA 5'**GCATGC**GGAAGCGGAAGGTGGTTC
OB87 OB88	1769 bp *bgrU* down-stream fragment	5'**GCATGC**GGCGATCTACAAGAACC 5'**AAGCTT**AAGCAGGTGGCGGAACTT
OB93 OB126	1008 bp *bgrW* up-stream fragment	5'CAAGCGCATCGAGGCGAAGGACT 5'**CCCGGG**TCCCGACGGCAAGATC
OB161 OB125	1617 bp *bgrW* down-stream fragment	5' **CCCGGG**TAACGATAGGAGAT 5' ACTCGAACTATAAGTGAACC
OB87 OB127	1048 bp *bgrV* up-stream fragment	5'**GCATGC**GGCGATCTACAAGAACC 5'**AAGCTT**GATTTCCATGTTTTCG
OB128 OB125	1082 bp *bgrV* down-stream fragment	5'**AAGCTT**CACCCTGGCGTCGATC 5'ACTCGAACTATAAGTGAACC
OB100 OB101	Complete *bgsA* gene for the insertion of the *lacZ*-Gn^r cassette	5' **GGATCC**GGCGCAGGTTCGTTTCTC 5' CGCTCCCGCACGCTCTAT
RT-PCR		
OB98 OB74	783 bp fragment between *bgrR* and *bgrS*	5' GCTTCTGGAGCGTACGATAAACAA 5' **GGTACC**GGGGGACACGGGAAAGT
OB116 OB117	404 bp fragment between *bgrS* and *bgrT*	5' GAGTCGCGAGGCTGTTCTTCTG 5' **GGATCC**ACGACGCTCGAGGTCAC
OB85 OB86	885 bp fragment between *bgrT* and *bgrU*	5'**GGATCC**GCGGGCGGGTCTATA 5'**GCATGC**GGAAGCGGAAGGTGGTTC
OB87 OB88	806 bp fragment between *bgrU* and *bgrW*	5'**GCATGC**GGCGATCTACAAGAACC 5'**AAGCTT**AAGCAGGTGGCGGAACTT
OB111 OB110	414 bp fragment between *bgrW* and *bgrV*	5' CCTGCTTTCTGAAATAACGATAGG 5' GAACTGCAGCCCCGTGAGAT

Complementation/ Overexpression		
OB73 OB74	Complete *bgrR* gene	5' **GGATCC**CACGCGACCTATGGAC 5' **GGTACC**GGGGGACACGGGAAAGT
OB120 OB117	Complete *bgrS* gene	5'**AGATCT**ATGTTACCGCCGAAGGAGTG 5'**GGATCC**ACGACGCTCGAGGTCAC
OB116 OB86	Complete *bgrT* gene	5'GAGTCGCGAGGCTGTTCTTCTG 5'**GCATGC**GGAAGCGGAAGGTGGTTC
OB91 OB92	Complete *bgrU* gene	5' **GGATCC**GGTCGCGAGGCTGTTTAG 5' **GGATCC**GGGCCGTCGAAGAGAC
OB87 OB110	Complete *bgrW* gene	5' **GCATGC**GGCGATCTACAAGAACC 5' GAACTGCAGCCCCGTGAGAT
OB124 OB84	Complete *bgrV* gene	5' **CCCGGG**ACCTCTCGAATTGACG 5' CGCCGGACGAGGCGCGACTTTA
OB100 OB101	Complete *bgsA* gene	5' **GGATCC**GGCGCAGGTTCGTTTCTC 5' CGCTCCCGCACGCTCTAT
OB134 OB135	N-terminal c-Myc tagged *bgrV*.	5'**GGTACC**ATGGAGCAGAAACTCATCTC AGAAGAGGATCTGATGGAAATCAAGGA AGAC 5'**AAGCTT**TCAGCTTAGCCTCAGAT
Point mutations		
OB129 OB130	Substitutes the glutamic acid residue of the AGDEF domain with an alanine (E215A) in BgrR	5'GTCTAGCCGGGGACG**C**GTTCGCGGTCATTC AGT 5'CTGAATGACCGCGAAC**G**CGTCCCCGGCTAG ACG
OB149 OB150	Substitutes the serine at position 61 with an alanine in BgrV (S61A)	5' GTT CCT CAA T**G**C CTC CGG AAT CAA C 5' TGC AGC CCC GTG AGA TCC
OB162 OB163	Amplifies a 282 bp fragment of *bgrV* that includes the internal PstI site.	5' ACGGGACGATGCGCTTGCCG 5'-GGGAG**AAGCTT**TCAGCTTAGCCTCAGAT