Mgl2 Is a Hypothetical Methyltransferase Involved in Exopolysaccharide Production, Biofilm Formation, and Motility in *Rhizobium leguminosarum* bv. *trifolii*

Małgorzata Marczak,^{1,†} Kamil Żebracki,¹ Piotr Koper,¹ Anna Turska-Szewczuk,¹ Andrzej Mazur,¹ Jerzy Wydrych,² Magdalena Wójcik,¹ and Anna Skorupska¹

¹ Department of Genetics and Microbiology, Institute of Microbiology and Biotechnology, Maria Curie-Skłodowska University, Akademicka 19 St, 20-033 Lublin, Poland

² Department of Comparative Anatomy and Anthropology, Institute of Biology, Faculty of Biology and Biotechnology, Maria Curie-Skłodowska University

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In this study, functional characterization of the *mgl2* gene located near the Pss-I exopolysaccharide biosynthesis region in *Rhizobium leguminosarum* bv. *trifolii* TA1 is described. The hypothetical protein encoded by the *mgl2* gene was found to be similar to methyltransferases (MTases). Protein homology and template-based modeling facilitated prediction of the Mgl2 structure, which greatly resembled class I MTases with a S-adenosyl-L-methionine-binding cleft. The Mgl2 protein was engaged in exopolysaccharide, but not lipopolysaccharide, synthesis. The *mgl2* deletion mutant produced exopolysaccharide comprised of only low molecular weight fractions, while overexpression of *mgl2* caused overproduction of exopolysaccharide with a normal low to high molecular weight ratio. The deletion of the *mgl2* gene resulted in disturbances in biofilm formation and a slight increase in motility in minimal medium. Red clover (*Trifolium pratense*) inoculated with the *mgl2* mutant formed effective nodules, and the appearance of the plants indicated active nitrogen fixation. The *mgl2* gene was preceded by an active and strong promoter. Mgl2 was defined as an integral membrane protein and formed homodimers in vivo; however, it did not interact with Pss proteins encoded within the Pss-I region. The results are discussed in the context of the possible involvement of the newly described potential MTase in various metabolic traits, such as the exopolysaccharide synthesis and motility that are important for rhizobial saprophytic and symbiotic relationships.

Keywords: biofilm, exopolysaccharide, methyltransferase, motility, *Rhizobium*, symbiosis

Rhizobia dwell and multiply in the soil and can enter into a symbiotic relationship with plants and fix atmospheric nitrogen inside de novo-created plant organs called nodules (Jones et al. 2007). Rhizobia produce extracellular polysaccharides (EPS), which play several physiological roles, provide protection from desiccation, form biofilm matrices, and are essential for a specific symbiotic interaction with galegoid-type host plants (Kawaharada et al. 2015; Marczak et al. 2017; Skorupska et al. 2006). *Rhizobium leguminosarum* bv. *trifolii* produces EPS in two forms that differ in the degree of polymerization (i.e., high and low molecular weight [HMW and LMW, respectively]) (Mazur et al. 2002, 2003). It has long been postulated that the LMW fraction is important for efficient infection of nodules (Battisti et al. 1992; Pellock et al. 2000). However, recent data show unequivocally that *Sinorhizobium meliloti* mutants that do not produce any LMW succinoglycan can establish productive symbiosis, albeit with lower efficiency than the wild-type (WT) strain (Mendis et al. 2016). HMW fractions of EPS have been recognized as beneficial at the infection stage (Marczak et al. 2013; Mazur et al. 2003) and as preserving bacteria from desiccation (Gharzouli et al. 2013).

Exopolysaccharide is synthesized in a process termed a Wzx/Wzy-dependent pathway, because of the involvement of the key Wzx flippase and the Wzy polysaccharide polymerase proteins. Synthesis is initiated by glycosyltransferases, which catalyze the assembly of the repeating unit on the lipid carrier undecaprenyl pyrophosphate. Subunits are then translocated to the periplasmic face of the inner membrane and polymerized. Translocation through the periplasm and the outer membrane is thought to be coordinated with polymerization (Islam and Lam 2014; Whitfield 2006), and several studies report the existence of a multiprotein transenvelope channel composed of polymerase, copolymerase, and a tunnel outer membrane protein (Collins et al. 2007; Islam et al. 2013; King et al. 2014; Marczak et al. 2013, 2014; Papadopoulos et al. 2016; Taylor et al. 2013).

Most of the known genes participating directly in EPS synthesis in *R. leguminosarum* bv. *trifolii* (i.e., assembly of the subunit, its modification with noncarbohydrate substituents, polymerization, and transport of the nascent chain outside the cell) are located on the chromosome in the Pss-I cluster (Król et al. 2007). Genes encoding proteins with established or predicted functions of glycosyltransferases (Janczarek and Skorupska 2003; Janczarek et al. 2015; Król et al. 1998), flippase, polymerase, copolymerase, and an outer membrane channel

[†]Corresponding author: M. Marczak;
malgorzata.marczak@poczta.umcs.lublin.pl

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protein (Marczak et al. 2006; Mazur et al. 2002, 2003, 2005) were identified in this region. The PssL protein is similar to Wzx flippase (Mazur et al. 2005), the PssT protein has characteristics of exopolysaccharide polymerases (Marczak et al. 2013; Mazur et al. 2003), and PssP plays a role as a polysaccharide copolymerase, which is not engaged in polymerization itself, but serves an essential role in determining the length of the growing polymer (Mazur et al. 2002). The results of studies on the architecture and mode of action of the EPS transport system in *R. leguminosarum* bv. *trifolii* indicate that Pss proteins form a complex network of interactions, wherein the various components form both homo- and heterooligomers (Marczak et al. 2006, 2013, 2014). Several other genes outside the Pss-I region were identified as being directly or indirectly involved in the EPS biosynthesis (e.g., the *pssA* gene encoding a priming glycosyltransferase [Janczarek and Skorupska 2003] and the *pssP2* gene encoding yet another copolymerase modulating the HMW/LMW proportion of EPS in *R. leguminosarum* bv. *trifolii* [Marczak et al. 2014, 2017]).

Biosynthesis of EPS is regulated at transcriptional and posttranscriptional levels. Factors influencing EPS synthesis include medium osmolarity, availability of ammonium and phosphate, flavonoids, and carbon source (Janczarek 2011). Considering the importance of exopolysaccharides, it is obvious that their biosynthesis must be strictly regulated and coordinated with other cellular processes. Several two-component systems were shown to be necessary for regulation of exopolysaccharide synthesis, motility, stress adaptation, and biofilm formation. The ExoS/ChvI two-component system together with ExoR protein influence EPS synthesis, biofilm formation, motility, nutrient utilization, and free-living viability (Chen et al. 2008; Cheng and Walker 1998; Yao et al. 2004). *S. meliloti* *emmABC* genes are associated with EPS and motility and encode a histidine kinase and a response regulator (Morris and González 2009). The ExrR/Sin system is involved in LMW succinoglycan (EPS I) production and motility in *S. meliloti* (Hoang et al. 2008).

Lipopolysaccharide (LPS) synthesis involves regulatory mechanisms that modulate its production in a dynamic fashion in response to environmental factors through two-component systems, similar to EPS synthesis. There are several two-component regulatory systems for LPS modification in *Salmonella enterica* serovar Typhimurium, each activated by different stimuli and independently promoting transcription of the *wzz* genes (Delgado et al. 2006), which encode chain length-determining proteins similar to PssP in *R. leguminosarum* bv. *trifolii*. Dam methylation plays a role in the regulation of LPS synthesis through its control of *wzz* expression (Sarnacki et al. 2009). Dam can also regulate other genes attributed to virulence (i.e., those involved in motility, adherence, and host colonization) (Heusipp et al. 2007).

In searching for additional factors that determine EPS production and the extent of polymerization, we focused on the *mgl2* gene located near the Pss-I gene cluster, encoding glycosyltransferases and other genes required for the assembly of the EPS repeating unit and its modification and polymerization in *R. leguminosarum* bv. *trifolii* TA1 (RtTA1). The *mgl2* encodes a hypothetical S-adenosyl-L-methionine-dependent methyltransferase (SAM-MTase). Protein homology and template-based modeling helped to ascribe a potential function of the Mgl2 protein. The *mgl2* deletion strain phenotype revealed the involvement of the gene in EPS synthesis and indirectly in the biofilm formation and motility of the bacteria. A possible direct or indirect role of Mgl2 in EPS synthesis and its connection with other RtTA1 cellular processes was proposed and discussed in the context of *mgl2* conservation in a narrow group of related bacteria.

RESULTS

mgl2 gene is conserved in a narrow group of related rhizobia.

The *mgl2* gene encoding a hypothetical MTase is located close to the Pss-I region (GeneBank DQ384110), the largest cluster of EPS synthesis genes (Król et al. 2007). *mgl2* is adjacent to the divergently transcribed *pssV* gene encoding a hypothetical protein with an amino acid kinase (AAK) motif and the *regA* gene encoding a hypothetical DNA-binding protein with a helix-turn-helix (HTH) domain (Fig. 1A). The genetic context of *mgl2* suggests that putative Mgl2 can be classified as an orphan MTase with a signature of SAM-dependent MTases (also found in other hypothetical MTases), among which only ExpE3 was shown to be linked with EPS synthesis in *S. meliloti* (Becker et al. 1997) (Supplementary Table S1).

Sequences highly similar to *mgl2* were identified only in *R. leguminosarum* bv. *trifolii*, *R. etli*, and *Rhizobium* sp. genomes. An ortholog of *mgl2* was found in the chromosome of *R. leguminosarum* bv. *viciae* 3841, not linked with the Pss-I region, but with *celABCD*, which is involved in cellulose synthesis. A putative paralog of the *mgl2* gene can be predicted in a distant locus of the RtTA1 chromosome (*mgl22* gene approximately 2.5 Mb from Pss-I) and 7 kb apart from *celABCD* genes. In the genome of *R. etli* CFN42, two copies of *mgl2* were identified: one close to the Pss-I region, like in RtTA1, and the other close to cellulose synthesis genes.

mgl2 is preceded by strong promoter.

mgl2 in RtTA1 is preceded by a large (>700 bp) intergenic region shared with the divergently transcribed *pssV* gene (Fig. 1A and B), in which promoter motifs, potentially recognized by σ^{70} (RpoD) and σ^{54} (RpoN), can be predicted.

Based on the promoter prediction, a series of reporter plasmids were constructed. Fragments of the *mgl2-pssV* intergenic region differing in length at the 5' or 3' ends, as well as one fragment covering the 5' end of *mgl2*, were cloned into the β -galactosidase reporter plasmid (Fig. 1B). The activity of β -galactosidase in RtTA1 cells carrying transcriptional fusion plasmids (Fig. 1C) revealed that *mgl2* was preceded by a promoter whose strength was comparable to that of promoters preceding the *pssO* and *pssA* genes (Janczarek and Urbanik-Sypniewska 2013; Wielbo et al. 2004). It is noteworthy that the activity of pMPmgl2/4 reporter fusion was significantly higher than fusions 1-3 and 5-6, and the relation was generally independent of the medium used (Fig. 1C). High transcriptional activity in fusion pMPmgl2/4, significant activity in fusion pMPmgl2/5, and lack of activity in the pMPmgl2/6 fusion indicate that the promoter of the *mgl2* gene is composed of sequences within fragments P4 and P5, with the strong candidate being the (−35)TTGACG(21N)(−10)TATTCT motif inside fragment 4. The activity of the pMPmgl2/1 reporter fusion did not change in the background of the *mgl2* deletion mutant cells (the construction of the mutant is described later), indicating no autoregulation of *mgl2* expression.

The activity of Pmgl2 was modulated by the composition of the media used. It was significantly higher ($P < 0.01$) in 79CA than in M1 and M1 without NH_4Cl (Fig. 1C). Moreover, Pmgl2 activity was comparable in M1 and in M1 without NH_4Cl . If the predicted binding site for σ^{54} was functional, the activity of the promoter should be upregulated in the absence of NH_4Cl in the medium, at least for fusions 1 and 2 (Fig. 1C).

Mgl2 as a putative SAM-dependent MTase.

The predicted secondary structure of Mgl2 resembles Rossmann-like fold (class I) MTases. A clear majority of MTases belonging

to this group have a catalytic domain consisting of a seven-stranded β -sheet adjoined by α -helices (Kozbial and Mushegian 2005). In the central part of Mgl2, a domain shared with SAM-MTases (InterPro motif number IPR029063; Pfam motif number PF13489) was predicted; in the N-terminal part of Mgl2, a conserved N₍₄₆₎GCGTG₍₅₀₎-C motif constituting part of the putative binding site for the adenosyl part of SAM was found.

The structural model generated for Mgl2 (99% of residues were modeled at >90% confidence) possesses a central β -sheet

that is flanked by α -helices on both sides. In the model, the GCG portion of the N₍₄₆₎GCGTG₍₅₀₎-C motif is predicted to form a part of the SAM and S-adenosyl homocysteine ligand-binding site (Supplementary Fig. S1).

To check on the possibility that the Mgl2 protein could be a DNA MTase, restriction analyses of the genomic DNA were performed. No methylation of adenines in GATC sequences occurred in the WT R_tTA1, and the methylation profile did not change in the *mgl2* mutant. Moreover, the *Hinf*I restriction showed that Mgl2 had no adenine MTase activity specific for

Fig. 1. Overview of the promoter mapping experiment. **A**, Physical and genetic map of the Pss-I region. **B**, Schematic representation of the *mgl2*-*pssV* intergenic region. The -10 and -35 motifs for the σ^{70} predicted with the BDGP Neural Network Promoter Prediction tool (solid lines with probability of functioning as the promoter over the set threshold of 0.85) are depicted. The σ^{54} hypothetical motif was found based on the consensus sequence available in the literature (GGCAC-N₅-TTGC around -25/-12 relative to the transcription start site). The motif found in this region is GGCAC-N₄-TTGC (sigma 54 line). Fragments Pmgl2-1 to Pmgl2-6 of the intergenic region cloned into pMP220 are shown as solid lines. **C**, Transcriptional activity of the predicted *mgl2* promoter, as determined by measuring β -galactosidase in the wild-type strain carrying promoter probe plasmids pMPmgl2/1 to 6. The strains were cultured in 79CA, M1, or M1 without NH₄Cl media. Values represent mean activity relative to the strain bearing the pMP220 vector control (approximately 100 Miller units) and not the arbitrary values. The bars labeled with different letters represent mean relative β -galactosidase activity values that are significantly different at $P < 0.01$ for 79CA, $P < 0.02$ for M1, and $P < 0.001$ for M1 without NH₄Cl.

GATC sequences. The genomic DNA of the WT strain was not digested by *Hin*FI, the activity of which is blocked by GATC methylation. However, the genomic DNA of the *mg*l2 mutant also was resistant to digestion, proving that the GATC methylation status of the genome was not altered (decreased) in the mutant, indicating that Mgl2 is not a DNA methylase (Supplementary Fig. S2).

Mgl2 is a membrane protein.

Subcellular and signal peptide predictions for Mgl2 produced conflicting results; however, Mgl2 was shown to reside presumably in the membrane. Mgl2 subcellular localization was examined in the WT/pQB*mg*l2 strain overproducing Mgl2 as an N-terminally His₆-tagged protein. The cells were subjected to a fractionation procedure previously used to analyze the cellular localization of several Pss proteins (Marczak et al. 2006, 2008). Considering that the expression of His₆-Mgl2 took place due to the activity of a promoter other than *Pmg*l2, the fractionation procedure included a clarification step—the aim of which was to get rid of potential inclusion bodies (Fig. 2). Western blotting with anti-His antibodies not only revealed that such a tagged protein was produced in the cells and revealed that it did not have a cleaved signal sequence at the N terminus, it also confirmed that His₆-Mgl2 was localized to a membrane fraction and was not extracted from it with 1 M NaCl, indicating it is not membrane-associated but is an integral membrane protein (Fig. 2).

Mgl2 protein oligomerization status, as well as its propensity to interact with Pss proteins and proteins encoded within the nearest neighborhood, was examined further using a bacterial two-hybrid (BTH) system. A set of the following genes was chosen: direct genomic neighbors of *mg*l2 (i.e., *pss*V, and *reg*A) and previously characterized *pss* genes (i.e., *pss*T, *pss*N, *pss*O, *pss*P, *pss*L, *pss*P2, *pss*A, and *pss*C). The Mgl2 protein interacted with itself in vivo. Concomitantly, no interaction with any of the tested Pss proteins was observed. Hypothetical kinase PssV also appeared to have the ability to form homo-oligomeric forms in vivo (Supplementary Fig. S3).

Phenotypic consequences of *mg*l2 deletion and overexpression.

The *mg*l2 genomic context and the encoded protein functional predictions suggested that the Mgl2 protein might be engaged in EPS synthesis and/or amino acid metabolism/chemotaxis. To determine the Mgl2 function, an in-frame deletion of the *mg*l2 gene, Δ *mg*l2 mutant, was constructed (Supplementary Fig. S4).

Fig. 2. Subcellular localization of Mgl2. Identification of the His₆-Mgl2 protein in subcellular fractions of the WT/pQB*mg*l2 strain: P, periplasmic fraction; U, unbroken cells and spheroplasts and inclusion bodies (if produced) after centrifugation at 10,000 × g; L, cleared lysate after centrifugation at 10,000 × g; SOL, soluble proteins recovered from cleared lysate; TM, membranes recovered from cleared lysate; SOL_{NaCl}, proteins recovered from TM via washing with 1 M NaCl; U_{NaCl}, integral membrane proteins unrecoverable from membranes with 1 M NaCl. The blot was developed twice: 1) with anti-His antibodies; 2) with anti-PssN antibodies. PssN protein was previously shown to fractionate to the periplasm and outer membrane, thus it served as a fractionation quality control.

In 79CA medium, the Δ *mg*l2 mutant overproduced EPS (Fig. 3A), which compared with the WT strain was almost exclusively composed of LMW fractions with molecular masses ranging from 7.4 to 125 kDa (Fig. 3B).

To complement Δ *mg*l2 mutation a genomic fragment covering the *mg*l2 gene, with an upstream promoter and downstream sequences important for efficient transcription termination, was cloned into a broad host-range vector under *Plac*, and the resulting plasmid pBK*mg*l2-12 was introduced to the Δ *mg*l2 cells.

Gel permeation chromatography of EPS produced by the Δ *mg*l2 mutant-complemented derivative Δ *mg*l2/pBK*mg*l2-12 revealed that it had a HMW/LMW composition similar to the WT strain, ranging from 6.8 to 359.4 kDa. Minor fractions of 683.3 kDa for the WT and 527.5 kDa for the complemented strain also were present (Fig. 3C). The WT/pQB*mg*l2 strain also was included in the EPS production analysis. Similar to the Δ *mg*l2 mutant, WT/pQB*mg*l2 overproduced EPS (Fig. 3A). However, even though overexpression of the *mg*l2 gene resulted in significant EPS overproduction, the composition of HMW and LMW EPS was not different from that of the WT strain (Fig. 3D).

It was shown that mutations in EPS synthesis genes, in addition to affecting EPS production, may be accompanied by changes in the LPS (Janczarek et al. 2001; Król et al. 1998). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) LPS profiles of the Δ *mg*l2 mutant and WT/pQB*mg*l2 strains appeared to not be different from those of the WT strain (Supplementary Fig. S5), indicating the *mg*l2 locus plays a direct or indirect role in the biosynthesis of EPS, but not LPS.

Quantitative and qualitative changes in EPS production in the *mg*l2 mutant strain corresponded with changed biofilm formation properties. Taking into account the composition, characteristics, and influence of different media on EPS production, a comparative study was performed in 79CA and tryptone-yeast (TY) high-energy media and M1 minimal medium. The 79CA medium promotes EPS production, but in the TY medium EPS production is significantly decreased. Crystal violet staining revealed that in the TY medium the Δ *mg*l2 mutant produced amounts of biofilm similar to the WT strain, whereas in the 79CA medium the amount of biofilm formed by the mutant decreased significantly ($P < 0.01$) and in the M1 minimal medium biofilm formation increased significantly ($P < 0.001$). The WT/pQB*mg*l2 strain exhibited a reduced amount of biofilm in 79CA medium (Fig. 4A), even though the quality of EPS produced by the strain was not changed. Microscopic examination of the structure and depth of the biofilm confirmed that *mg*l2 deletion significantly ($P < 0.05$) increased the volume of produced biofilm, but only in the minimal medium (Fig. 4B and C). No significant structural differences were observed for the strains grown in TY (residual fragmented biofilm) and 79CA (similar firm structure and depth) (Fig. 4B and C).

Symbiotic performance of Δ *mg*l2 and WT/pQB*mg*l2 strains.

The symbiotic properties of the Δ *mg*l2 and *mg*l2-gene overexpressing strains were assessed in plant tests. Nodules induced on the roots of clover infected with the Δ *mg*l2 mutant appeared with no delay (Fig. 5A), and the overall number of nodules that formed on the roots was not significantly different from the WT strain. The appearance of clover plants infected with the Δ *mg*l2 mutant corresponded with nodule morphology, with no signs of nitrogen starvation, which was reflected in a wet green mass of clover shoots harvested 35 days after inoculation (Fig. 5B). It is worth emphasizing that the decrease in the molecular weight of EPS produced by Δ *mg*l2 did not affect

its symbiotic capabilities. The symbiotic performance of the WT/pQBmgl2 strain was not significantly changed compared with the WT in terms of the kinetics of nodulation, the overall number of nodules, and the wet mass of clover shoots and roots 35 days after inoculation (Fig. 5A and B). This is in accordance with the quality of EPS produced by the strain.

mgl2 deletion affects motility and stress tolerance.

Considering the changed EPS phenotype, we sought to examine other phenotype characteristics, such as motility, which is related to EPS production. Both $\Delta mgl2$ and WT/pQBmgl2 were tested for their motility properties in a semisolid (0.3% agar) medium plate assay.

In complete 79CA with mannitol as a carbon source, the motility of the $\Delta mgl2$ mutant was comparable to the WT and WT/pQBmgl2 strains (Fig. 6A). In complete TY medium, both the $\Delta mgl2$ mutant and its complemented derivative were the least motile (Fig. 6A). Interestingly, even though the motility of rhizobia in M1 minimal medium is generally substantially reduced compared with the complete media, a significant increase in motility of the $\Delta mgl2$ and

$\Delta pssO$ mutants was repeatedly observed in this medium (Fig. 6A).

Disturbances in exopolysaccharide production are often correlated with changes in sensitivity to stressors such as detergents or increased osmolarity. In keeping with this, we tested the $\Delta mgl2$ mutant and WT/pQBmgl2 strains for their sensitivity to ethanol, sodium deoxycholate (DOC), SDS, and NaCl. The $\Delta mgl2$ mutant appeared to be more resistant, while the WT/pQBmgl2 strain was slightly more sensitive to the membrane stressor DOC (Fig. 6B). No significant differences in sensitivity to other stressors were observed (Fig. 6B).

DISCUSSION

The *mgl2* gene was identified near the Pss-I region, comprising most of the genes involved in EPS synthesis in *R. leguminosarum* bv. *trifolii*. In this work, we have shown that the product of *mgl2* plays a direct or indirect role in exopolysaccharide production, stress survival, and motility.

Database similarity searches unequivocally indicated that *mgl2* encodes an MTase. The genomic context of the gene in

Fig. 3. Exopolysaccharide production by the *Rhizobium leguminosarum* bv. *trifolii* TA1 (RfTA1) wild type and $\Delta mgl2$ mutant. **A**, Quantitation of the amount of extracellular polysaccharides (EPS) produced in the 79CA with mannitol medium. The bars labeled with asterisks represent EPS values that are significantly different at $P < 0.01$, and the extended bars indicate the standard deviation; the amount of EPS is expressed as glucose equivalents. Gel permeation chromatography of EPS produced by **B**, the $\Delta mgl2$ mutant; **C**, its complemented derivative; and **D**, the *mgl2*-overexpressing strain in the 79CA with glycerol medium was performed. The retention times of dextran blue (2 MDa) and dextran T10 (10 kDa) molecular mass markers are indicated by arrows above the graphs.

RtTA1 and genomes of other rhizobia are interesting. The genomic location of *mgl2* and *mgl22* suggest involvement in polysaccharide synthesis: exopolysaccharide and cellulose. In fact, deletion of the *mgl2* gene resulted in an increase in the level of EPS production, accompanied by a decrease in the molecular mass of polymers. Furthermore, overexpression of *mgl2* caused substantial overproduction of EPS. On the other hand, the LPS profiles of the *mgl2* mutant and overexpression strains were not changed, thus the gene is not linked to LPS synthesis.

Fig. 4. Biofilm formation by the *Rhizobium leguminosarum* bv. *trifolii* TA1 (RtTA1) wild type and *Δmgl2* mutant. **A**, Biofilm formation capabilities were examined by staining with crystal violet and expressed as the ratio of the amount of crystal violet solubilized by ethanol (OD_{560}) to the bacterial growth (OD_{600}). The experiment was performed in triplicate and averaged. The bars labeled with different letters represent biofilm amounts that are significantly different at $P < 0.001$ for M1 and $P < 0.01$ for 79CA media, and the extended bars indicate the standard deviation. **B**, Structures of biofilms analyzed by confocal laser scanning microscopy. For each medium, the left panel presents images of biofilms that are 5 days old, with live (SYTO-9 fluorescence) and dead (propidium iodide fluorescence) cells, while the right panel presents cross-sections of the formed biofilms. **C**, Table summarizing the depth of the biofilms formed in different growth media by the individual strains. Numbers followed by different letters are significantly different at $P < 0.05$.

Homology modeling of Mgl2 was possible due to the huge representation of MTases in the Protein Data Bank (PDB) archive. The comparable protein folding and the existence of equivalent amino acids in similar secondary and tertiary positions indicate that many SAM-MTases have a common catalytic domain structure. This facilitates tertiary structure prediction based on their amino acid sequences, even though the overall similarity at the amino acid sequence level is low (Petrosian and Clarke 2009). Mgl2 resembled class I MTases, with a SAM-binding site composed of a β -sheet and surrounded by α -helices. However, restriction analyses of the RtTA1 and *mgl2* mutant genomic DNA strongly suggested that Mgl2 is not a DNA methylase. It is noteworthy that several classes of proteins representing Rossmann-fold SAM-binding proteins do not have MTase activity (Kozbial and Mushegian 2005).

Deletion of the *mgl2* locus did not affect the symbiotic performance of the RtTA1. The RtTA1 mutants producing residual LMW EPS previously were shown to infect plants, and even though they induced fewer nodules on clover, the green mass of shoots was almost identical to that of the plants infected with the WT strain (Mazur et al. 2002). In the case of *mgl2* deletion such an uncompromised symbiotic phenotype may also result from increased biofilm formation. The latter result is even more interesting, considering the fact that the mutant produces EPS characterized by the higher LMW fraction component. Both crystal violet staining and confocal laser scanning microscopy (CLSM) revealed that *mgl2* deletion increased the ability of the strain to form biofilm in the M1 minimal medium. In the case of 79CA medium, significantly less biofilm was detected by

Fig. 5. Symbiotic capability of the *Δmgl2* mutant and *mgl2*-overexpressing derivative of *Rhizobium leguminosarum* bv. *trifolii* TA1 (RtTA1) wild type. The mean number of nodules induced on **A**, 30 clover plants roots and **B**, the fresh masses of shoots and roots of clover plants assessed 35 days after inoculation. Bars labeled with different letters represent clover shoot and root masses that are significantly different at $P < 0.0001$ (shoot) and $P < 0.03$ (root). Control plants were not inoculated with rhizobia.

crystal violet staining then by microscopic examination of $\Delta mgl2$. However, such a result is not without precedence. van Duuren et al. (2017) compared biofilm produced by polysaccharide-compromised mutants of *Pseudomonas aeruginosa* with crystal violet staining and CLSM. A strain producing more EPS than the WT in a crystal violet test produced biovolume significantly lower when measured through CLSM. One of the reasons is the different localization of biofilm structures detected by both techniques in a well of a microtiter plate (van Duuren et al. 2017). Several studies have shown a connection between the production of bacterial polysaccharides and motility (Bahlawane et al. 2008; Hoang et al. 2008; Rachwał et al. 2015). Deletion of the *mgl2* gene caused a decrease in motility in the TY complete semisolid medium, but its swimming motility in the minimal medium was significantly higher than that of the WT strain. In rhizobia, regulation of EPS production and motility are intertwined in an inverse manner: an increase in EPS production is accompanied by a decrease in motility. A decrease in viscosity caused by a decrease in EPS production is accompanied by an increase in motility (Hoang et al. 2008). In the case of the $\Delta mgl2$ mutant, this inverse relation between EPS synthesis and motility is far more complex. Changes in the amount and quality of EPS produced by the mutant correlate with decreased motility in TY medium and increased motility in M1 minimal medium. Taking EPS, motility, and biofilm phenotypes together, involvement of Mgl2 in an unknown regulatory mechanism can be postulated.

The deletion of the *mgl2* gene caused an increase in tolerance to the membrane stressor DOC. Susceptibility to membrane

stress is often associated with defects in LPS production (Campbell et al. 2003; Nikaido 2003). In this work, no defect in LPS and increased resistance to membrane stress was revealed, suggesting stress-protective mechanisms that have not yet been determined. Moreover, the sensitivity or resistance of the *mgl2*-overexpressing strain was barely distinguishable from that of the WT. Exopolysaccharides have been associated with protection against H_2O_2 , as well as low pH (Hawkins et al. 2017; Lehman and Long 2013). Even though the $\Delta mgl2$ mutant produced EPS containing more LMW exopolysaccharide, no differences in sensitivity to H_2O_2 and low pH were detected compared with the WT strain and the *mgl2*-overexpressing strain overproducing EPS (Supplementary Fig. S6).

Biosynthesis of EPS is achieved through a complex network of protein–protein interactions. The process of EPS synthesis is regulated at transcriptional and posttranscriptional levels. Several two-component systems are engaged in regulation of exopolysaccharide synthesis and motility: the ExoS/ChvI and ExoR protein (Chen et al. 2008; Cheng and Walker 1998; Yao et al. 2004), the EmmABC proteins (Morris and González 2009), and the ExpR/Sin system (Hoang et al. 2008).

Two chromosomal regions identified in RfTA1, containing the *mgl2* gene and its paralogue *mgl22*, also carry genes for hypothetical transcriptional regulators and kinases, suggesting roles as components of the two-component regulatory systems (TCS) or chemotaxis-like clusters. A TCS is usually composed of one histidine kinase and one response regulator (Gao and Stock 2009). The genes are involved in different physiological processes, including nitrogen metabolism, polysaccharide

Fig. 6. Effects of deletion of the *mgl2* gene on motility and sensitivity to stressors. **A**, Swim plate assay performed on 79CA with mannitol, tryptone-yeast (TY), and M1 plates solidified with 0.3% agar. The plates were inoculated by puncturing with the suspension of washed bacteria with equal optical density. The diameters of the motility zones were measured 5, 9, and 13 days after inoculation. The experiment was repeated four times, with two technical repeats in each. The bars labeled with different letters represent mean zone diameters (at 13 days) that are significantly different at $P < 0.01$ in TY, $P < 0.05$ in 79CA, and $P < 0.002$ in M1 media. **B**, The sensitivity of the strains to ethanol (%), sodium dodecyl sulfate (SDS) (%), NaCl (mM), and sodium deoxycholate (DOC) (%) was determined by plating serial fivefold dilutions of bacteria on 79CA supplemented with appropriate concentrations of stressors. The experiment was repeated four times. Presented photographs were taken of the same replica.

production, and flagellum formation (Hoch 2000). The *mgl2* gene is preceded by an intergenic region shared with the *pssV* gene encoding a hypothetical kinase protein. In silico analyses revealed the presence of motifs, which could hypothetically serve as promoters recognized by σ^{70} and σ^{54} . However, our analyses revealed that nitrogen availability did not influence *mgl2* gene expression. No other specific binding sites for regulators were found in the *mgl2-pssV* intergenic region, thus it could be postulated that the increase in activity in the pMPmgl2/4 fragment may have resulted from the lack of upstream sequences that would compete for binding of proteins responsible for transcription from *mgl2* and *pssV* promoters. It was shown that the *PpssV* promoter is active and that its activity is comparable to that of *PpssP* and two times lower than that of *Pmgl2* (Janczarek et al. 2015). It was also previously shown that *mgl2* activity is not modulated by RosR, the general regulatory protein in *R. leguminosarum* bv. *trifolii* (Janczarek et al. 2015).

Homotypic interactions between the two fusion proteins carrying Mgl2 at the C terminus were observed. Mgl2 was shown to be a membrane protein, thus dimerization may occur

through transmembrane segments. From the potential Mgl2 interactors, no heterotypic interactions with previously characterized Pss proteins, PssV, and RegA were revealed. In this work a hypothetical MTase Mgl2 was proved to affect various cellular processes that are critical for both saprophytic and symbiotic rhizobial relationships, like EPS production, biofilm formation, and motility. It cannot be excluded that Mgl2 belongs to the broader family of Rossmann-fold SAM-binding proteins that do not exhibit MTase activity (Kozbial and Mushegian 2005). It will be interesting to determine whether *regA*, *mgl2*, and *pssV* are indeed part of a two- or three-component system. Mutagenesis of the remaining genes could reveal any potential relationship.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

Strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown in lysogeny broth (LB) medium at 37°C (Sambrook and Russell 2001), and *R. leguminosarum* bv. *trifolii* strains were grown in TY (Beringer 1974), M1

Table 1. Strains and plasmids used in this work

Strain/plasmid	Relevant description	Reference
<i>Escherichia coli</i>		
JM101	Δlac <i>proAB thi supE F'</i> <i>traD36 proAB lacI</i> ^q Δ M15	Yanisch-Perron et al. 1985
S17-1	294 derivative RP4-2-Tc::Mu-Km::Tn7 chromosomally integrated	Simon et al. 1983
DH5 α	<i>supE44</i> $\Delta lacU169$ ($\Delta 80 lacZ\Delta$ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Sambrook and Russell 2001
DHM1	Reporter strain for bacterial two-hybrid (BTH) system; F- <i>glnV44(AS) recA1 endA1 gyrA96 thi-1 hsdR17 spoT1 rfbD1 cya-854</i>	Karimova et al. 1998
<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i>		
RtTA1	Wild-type strain, Sm ^r , Rf ^r	Chakravorty et al. 1982
RtO112	RtTA1, $\Delta pssO$, Gm ^r , Km ^r	Marczak et al. 2008
TA1 Δ mgl2(Gm)	$\Delta mgl2::Gm^r$ <i>R. leguminosarum</i> bv. <i>trifolii</i> TA1	This work
TA1 Δ mgl2[pCM157]	$\Delta mgl2$ <i>R. leguminosarum</i> bv. <i>trifolii</i> TA1, carrying pCM157 <i>cre</i> expressing vector	This work
TA1 Δ mgl2	$\Delta mgl2$ <i>R. leguminosarum</i> bv. <i>trifolii</i> TA1	This work
WT/pQBmgl2	RtTA1, carrying pQBmgl2, Km ^r	This work
WT/pQB	RtTA1, carrying pQB, Km ^r	This work
Plasmids		
pQE30	Expression vector, Ap ^r	Qiagen
pQE30mgl2	pQE30 with <i>mgl2</i> gene cloned into <i>Bam</i> HI/ <i>Hind</i> III	This work
pBBR1MCS-2	<i>mob</i> , Km ^r	Kovach et al. 1995
pBBR1MCS-5	<i>mob</i> , Gm ^r	Kovach et al. 1995
pQBmgl2	pBBR1MCS-2 with expression cassette from pQE30mgl2 subcloned into <i>Xho</i> I- <i>Hind</i> III	This work
pQB	pBBR1MCS-2 with expression cassette from pQE30 subcloned into <i>Xho</i> I- <i>Hind</i> III	This work
pBKmgl2-12	pBBR1MCS-2 with 1,530-bp fragment carrying <i>Pmgl2</i> , <i>mgl2</i> gene, and downstream sequences, cloned into <i>Sac</i> I in the direction corresponding to <i>Plac</i> ; tetracycline cassette in <i>Sma</i> I	This work
pCM351	<i>ori</i> ColE1, <i>oriT</i> , Ap ^r , Gm ^r , Tc ^r , allelic exchange vector	Marx and Lidstrom 2002
pCM157	<i>ori</i> IncP, <i>oriT</i> , Tc ^r , <i>cre</i> expression vector	Marx and Lidstrom 2002
pCGmgl2U	pCM351 with <i>Kpn</i> I- <i>Not</i> I fragment comprising 603 bp upstream of <i>mgl2</i>	This work
pCGmgl2UD	pCGmgl2U with 600-bp <i>Apa</i> I- <i>Sac</i> I fragment comprising 175 bp downstream of <i>mgl2</i> and 425 bp of <i>regA</i>	This work
pMP220	IncP, <i>mob</i> , promoterless <i>lacZ</i> , Tc ^r	Spaink et al. 1987
pMPO1	pMP220 carrying <i>pssO</i> promoter	Wielbo et al. 2004
pMP-P2	pMP220 carrying <i>pssP2</i> promoter	Marczak et al. 2014

(Continued on next page)

(Brown and Dilworth 1975), or 79CA with 1% mannitol or glycerol medium at 28°C (Kowalczyk and Lorkiewicz 1979). BTH reporter strain *E. coli* DHM1 was grown at 30°C. Antibiotics were used at the following final concentrations: ampicillin at 100 µg/ml, kanamycin at 40 µg/ml, gentamicin at 5 µg/ml (*E. coli*) or 10 µg/ml (*R. leguminosarum* bv. *trifolii*), tetracycline at 10 µg/ml, and rifampin at 40 µg/ml.

DNA manipulations.

Standard protocols for DNA isolation, PCR, molecular cloning, transformation, and DNA analysis were used (Sambrook and Russell 2001). Plasmids and primers used in this work are listed in Supplementary Table S2. Oligonucleotide primers were supplied by Genomed. Plasmid DNA purification was performed using MiniPrep Express Matrix (MP Biomedicals) and GeneElute plasmid miniprep kit (Sigma-Aldrich). PCR was performed with high-fidelity SuperFi DNA polymerase (Thermo Fisher Scientific). DNA sequencing of plasmid constructs prepared in this work was performed by Genomed. Sequence data were analyzed with DNASTAR Lasergene analysis software.

Bioinformatic analyses.

Putative homologs of Mgl2 were identified via BLASTp (Altschul et al. 1990). The HHpred tool was used to search against existing structures of proteins similar to Mgl2 in the PDB archive. Protein homology modeling and modeling of the SAM-binding site were performed using Phyre2 (Kelley et al. 2015) and 3DLigandSite (Wass et al. 2010), respectively. Secondary structure prediction was performed using PredictProtein (Yachdav et al. 2014). For identification of functional motifs Pfam (Finn et al. 2016) and InterPro (Finn et al. 2017) were used. Promoter prediction in the sequence upstream of the *mgl2* gene was performed using the BDGP Neural Network Promoter Prediction (Reese 2001) and BPROM (Solovyev and Salamov 2011) tools, and Rho-independent terminators were searched for using the ARNold tool (Lesnik et al. 2001).

Construction of the $\Delta mgl2$ mutant and the strain overexpressing *mgl2* gene.

The RtTA1 mutant deleted for *mgl2* was generated using the pCM351 allelic exchange vector (Marx and Lidstrom 2002).

Table 1. (Continued from previous page)

Strain/plasmid	Relevant description	Reference
pMP-Y	pMP220 carrying <i>pssY</i> promoter	Marczak et al. 2014
pMP-P	pMP220 with 133-bp fragment carrying the <i>pssP</i> promoter cloned into <i>XbaI-PstI</i>	This work
pMP-V	pMP220 with 635-bp fragment carrying the <i>pssV</i> promoter cloned into <i>KpnI</i>	This work
pMP-A	pMP220 with 766-bp fragment carrying the <i>pssA</i> promoter cloned into <i>PstI-BglII</i>	This work
pMP- <i>mgl2</i> /1	pMP220 with 737-bp fragment carrying the <i>mgl2</i> promoter fragment 1 cloned into <i>BglII-KpnI</i>	This work
pMP- <i>mgl2</i> /2	pMP220 with 513-bp fragment carrying the <i>mgl2</i> promoter fragment 2 cloned into <i>BglII-KpnI</i>	This work
pMP- <i>mgl2</i> /3	pMP220 with 306-bp fragment carrying the <i>mgl2</i> promoter fragment 3 cloned into <i>BglII-KpnI</i>	This work
pMP- <i>mgl2</i> /4	pMP220 with 86-bp fragment carrying the <i>mgl2</i> promoter fragment 4 cloned into <i>BglII-KpnI</i>	This work
pMP- <i>mgl2</i> /5	pMP220 with 102-bp fragment carrying 30 bp upstream of <i>mgl2</i> and 72 bp of <i>mgl2</i> gene cloned into <i>BglII-KpnI</i>	This work
pMP- <i>mgl2</i> /6	pMP220 with 431-bp fragment carrying the <i>mgl2</i> promoter fragment 6 cloned into <i>BglII-KpnI</i>	This work
pUT18	Two-hybrid plasmid for <i>cyaAT18</i> fusion construction, Ap ^r	Karimova et al. 1998
pUT18C	Two-hybrid plasmid for <i>cyaAT18</i> fusion construction, Ap ^r	Karimova et al. 1998
pKTN25	Two-hybrid plasmid for <i>cyaAT25</i> fusion construction, Km ^r	Karimova et al. 1998
pUT18C-zip	Two-hybrid control plasmid, Ap ^r	Karimova et al. 1998
pKT25-zip	Two-hybrid control plasmid, Km ^r	Karimova et al. 1998
<i>pssP/pssT/pssL/pssO/pssP2/pssA/pssC</i> pUT18	Two-hybrid plasmid containing <i>pssP/pssT/pssL/pssO/pssP2/pssA/pssC-cyaAT18</i> fusion, respectively	Marczak et al. 2013, 2014
pUT18C <i>pssP/pssT/pssL/pssO/pssP2/pssA/pssC</i>	Two-hybrid plasmid containing <i>cyaAT18-pssP/pssT/pssL/pssO/pssP2/pssA/pssC</i> fusion, respectively	Marczak et al. 2013, 2014
pKT25 <i>pssO/pssL/pssA/pssC</i>	Two-hybrid plasmid containing <i>cyaAT25-pssO/pssL/pssA/pssC</i> fusion, respectively	Marczak et al. 2013, 2014
<i>mgl2/regA/pssV</i> pUT18	Two-hybrid plasmid containing <i>mgl2, regA, pssV-cyaAT18</i> fusion, respectively	This work
pUT18C <i>mgl2/regA/pssV</i>	Two-hybrid plasmid containing <i>cyaAT18-mgl2, regA, pssV</i> fusion, respectively	This work
<i>mgl2/regA/pssV</i> pKTN25	Two-hybrid plasmid containing <i>mgl2, regA, pssV-cyaAT25</i> fusion, respectively	This work

The regions immediately flanking *mgl2* were amplified by PCR using RtTA1 genomic DNA as a template. The purified 603-bp PCR product for the *mgl2* upstream region was cloned between the *KpnI* and *NotI* sites of pCM351 to produce pCGmgl2U. Subsequently, the purified 0.6-kb PCR product for the *mgl2* downstream region was introduced between the *ApaI* and *SacI* sites of pCGmgl2U, resulting in pCGmgl2UD. The $\Delta mgl2::Gm^R$ RtTA1 mutant was generated by introducing pCGmgl2UD by conjugation from *E. coli* S17-1 (Simon et al. 1983). Bacterial mating experiments were performed as described by Reeve et al. (2016). Gentamicin-resistant transconjugants obtained on TY medium containing rifampicin and gentamicin were subsequently screened for tetracycline sensitivity to identify potential null mutants. The frequency of double-crossover events was 13.3%. One such $\Delta mgl2::Gm^R$ mutant, called TA1 $\Delta mgl2$ (Gm), was chosen for further study. Analytical PCRs were performed with WT RtTA1 and TA1 $\Delta mgl2$ (Gm) genomic DNAs to confirm successful allelic exchange. This result was also confirmed with restriction analysis of PCR-amplified products. To remove the gentamicin resistance cassette, the plasmid pCM157 was introduced into TA1 $\Delta mgl2$ (Gm) by electrotransformation, as described by Garg et al. (1999). Tetracycline-resistant transformants were streaked for purity using two passages to obtain a strain, called TA1 $\Delta mgl2$ [pCM157], that produced only gentamicin-sensitive colonies. Next, pCM157 was cured from TA1 $\Delta mgl2$ [pCM157] using six consecutive transfers on medium lacking tetracycline to obtain the $\Delta mgl2$ mutant strain TA1 $\Delta mgl2$. Analytical PCRs were performed with WT RtTA1 and TA1 $\Delta mgl2$ genomic DNAs to confirm successful deletion of the gentamicin resistance cassette. The sequencing of PCR-amplified product indicated expected recombination between *loxP* sites. pQBmgl2 encoding a His-tagged version of Mgl2 was constructed using the pQE30 expression vector (QIAGEN) and pBBR1MCS-2. For that purpose, *mgl2* was amplified with Pfu polymerase (Thermo Fisher Scientific) and cloned into *BamHI* and *HindIII* restriction sites in the pQE30 expression vector and used to transform *E. coli* JM101. The entire expression cassette that comprised the *mgl2* ORF with the His₆-tag in the corresponding reading frame, as well as the promoter and the operator sequences, was subcloned into *XhoI*-*HindIII* of the broad host range vector pBBR1MCS-2. The resultant plasmid was then transferred to RtTA1 WT through electrotransformation (Garg et al. 1999). The presence of His-tagged protein in the obtained WT/pQBmgl2 strain with a predicted molecular mass was evidenced through Western blotting with anti-His antibodies (Roche). For construction of pBKmgl2-12 plasmid used in complementation analyses, a 1,530-bp PCR fragment comprising *mgl2* with upstream promoter and predicted downstream terminator sequences was cloned into the pGEM-T Easy vector. The fragment was then subcloned to pBBR1MCS-2 with *SacI* under the *Plac* in the vector. Next, a tetracycline cassette amplified with Pfu polymerase was cloned into *SmaI*, resulting in pBKmgl2-12. Plasmids were transferred to the $\Delta mgl2$ mutant through conjugation from *E. coli* S17.1 and electrotransformation.

DNA methylation analysis.

Genomic DNA was isolated from the WT RtTA1, $\Delta mgl2$ mutant, and WT/pQBmgl2 strains using a Genomic Mini kit (A&A Biotechnology). DNA was incubated with *MboI*, *DpnI*, and *HinfI* restriction enzymes for 2 h at 37°C in mixtures with the buffers recommended by the manufacturer (Thermo Fisher Scientific). DNA was analyzed in agarose gels along with DNA subjected to the same conditions in the absence of enzymes.

Promoter activity assays.

Promoter probe constructs bearing transcriptional *mgl2/pssV/pssA/pssP-lacZ* fusions resulted from cloning of PCR products covering putative promoters into the respective restriction sites of the pMP220 vector. Obtained plasmids, along with the previously constructed pMPO1, pMP-Y, and pMP-P2, were introduced into the RtTA1, $\Delta mgl2$ mutant, and WT/pQBmgl2 cells by electroporation. Activities of promoters were measured in cultures of respective strains as the activity of the β -galactosidase reporter enzyme. Strains were grown in 79CA with 1% mannitol for 48 h, then the cells were washed twice with sterile water and resuspended in respective medium: 79CA with 1% mannitol, M1, or M1 without NH₄Cl (nitrogen source) in 1 ml of final culture volume, at 28°C for 16 h. M1 minimal medium was additionally supplemented with a vitamin solution (Brown and Dilworth 1975). The assay was performed in a plate format. Briefly, to 1 ml of culture 25 μ l of 0.1% SDS solution and 25 μ l of chloroform were added, and cultures were shaken for 30 min at 28°C. Permeabilized cells (20 to 80 μ l) were added to the well of a 96-well polystyrene microtiter plate and diluted with buffer Z (40 mM Na₂HPO₄ $\times$ 7H₂O, 60 mM NaH₂PO₄ $\times$ H₂O, 10 mM KCl, 1 mM MgSO₄ $\times$ 7H₂O, and 50 mM 2-mercaptoethanol) to the final volume of 160 μ l. The mixture was incubated at 28°C for 5 min, and then 40 μ l of the substrate *o*-nitrophenyl β -D-galactopyranoside solution (MP Biomedicals) in buffer Z was added. The reaction was developed for 5 to 15 min at 28°C and stopped by the addition of 80 μ l of 1 M NaCO₃ solution. A *R. leguminosarum* strain bearing the pMP220 vector served as a negative control. The reported values are the means of activity calculated in relation to pMP220 and represent averages from at least three independent experiments.

Plant tests.

Seeds of red clover (*T. pratense* L. cv. Nike) were surface-sterilized and germinated on nitrogen-free medium. Two-day-old clover seedlings were planted in sterile nitrogen-free slants and allowed to grow for 4 to 5 days before being inoculated with 0.1 ml of bacterial suspension at an approximate density of 1.0×10^9 cells/ml. The plants were grown in a greenhouse under natural light supplemented with an artificial light regime (14/10 h of light/dark). After 35 days, the plants were harvested, and the fresh mass of shoots and roots was examined. Nodule formation was examined every week throughout the duration of the experiment. Twenty clover plants were used for each strain.

Stress tolerance tests.

The sensitivity of the $\Delta mgl2$ mutant and WT/pQBmgl2 strains to SDS, DOC, NaCl, and ethanol was studied. Bacteria were cultured in 79CA medium with 1% mannitol as a carbon source for 48 h. Bacteria were pelleted, washed, and resuspended in sterile water to an OD₆₀₀ of 0.2. Aliquots (5 μ L) of fivefold serial dilutions of these suspensions were spotted on the surface of 79CA agar with defined concentrations of SDS (0.01 to 0.05%, w/v), DOC (0.025 to 0.1%, w/v), NaCl (25 to 100 mM), and ethanol (1 to 5%, v/v). The growth of the strains was determined after 4 days of incubation at 28°C. The experiment was repeated three times.

Cell-surface polysaccharide analysis.

Extracellular polysaccharides were precipitated with 3 vol of ethanol from the supernatants of bacterial cultures grown with shaking for 3 days in 79CA medium with 1% glycerol. The strains tested were previously grown on 79CA agar with 1% glycerol instead of mannitol. The molecular mass range of the EPS was determined by gel permeation chromatography on a

column (1.0 cm × 90 cm) of Sepharose CL-6B (Sigma-Aldrich) using 1 M NaOH as eluent and a gravity flow at 0.2 ml/min. Fractions of 1 ml were collected; the total sugar content was determined according to DuBois et al. (1956) and calculated in glucose equivalents. Blue dextran (2 MDa) and dextran T10 (10 kDa) were used as molecular weight standards. Glycosyl composition analysis of EPS was performed as described previously (Marczak et al. 2014). LPS was isolated by whole-cell microextraction using proteinase K digestion as described by Apicella (2008), analyzed by electrophoresis on a 12.5% SDS-PAGE polyacrylamide gel using a tricine buffer system, and visualized by silver staining after oxidation with periodate according to the published method (Tsai and Frasch 1982). The experiment was repeated twice.

Biofilm formation assays.

Biofilm formation was assessed according to a method described by Stasiak et al. (2014) and in CLSM. For crystal violet staining, rhizobial strains were grown in TY, M1, and 79CA media at 28°C for 48 h. The cultures were diluted to an OD₆₀₀ of 0.1 in the same medium, inoculated into 96-well microtiter plates in 100-μl aliquots, and incubated with agitation (100 rpm) at 28°C for 24 h. Next, bacterial growth was assessed by measuring the OD₆₀₀. The planktonic cells were gently removed, stained for 15 min with 180 μl of 0.1% crystal violet, and then rinsed three times with water. Biofilm formation was quantified by the addition of 150 μl of 95% ethanol and measurement of absorbance at 560 nm. The experiment was performed in triplicate. For the three-dimensional biofilm structure characterization, bacterial cultures prepared as above were inoculated into 48-well microtiter plates in 250-μl aliquots and incubated under static conditions at 28°C for 5 days. Afterward, the planktonic cells were removed, and each well was washed three times with 0.85% NaCl. Bacteria present in the biofilm were stained with the dye mixture containing 5 μM Syto-9 and 30 μM propidium iodide (LIVE/DEAD BacLight bacterial viability kit, Molecular Probes, Inc.). After the dyes were removed, the wells were again washed with 0.85% NaCl and observed using a laser scanning confocal microscope (LSC 5 PASCAL, Carl Zeiss).

General analyses of proteins.

Proteins were routinely analyzed by SDS-PAGE and either visualized using PageBlue staining solution (Thermo Fisher Scientific) or electroblotted onto polyvinylidene difluoride PVDF membrane (Millipore). Immunoblots were probed with the primary: anti-PssN (Marczak et al. 2006), anti-CyaA (Santa Cruz Biotechnology) and anti-His antibodies (Roche), and secondary antirabbit and antimouse IgG antibodies conjugated with alkaline phosphatase (Sigma-Aldrich). French press disruption of cells and protein fractionation were performed as described previously (Marczak et al. 2006, 2008).

BTH system complementation assays.

The vectors pKTN25, pUT18C, and pUT18 and the control plasmids pKT25-zip and pUT18C-zip were used in Mgl2, PssV, and RegA interaction analyses. To construct plasmids encoding fusion proteins with CyaA, *mgl2*, *pssV*, and *regA* genes were PCR-amplified using appropriate primers and subcloned into the pKTN25, pUT18C, and pUT18 vectors. Plasmids were then cotransformed into the *E. coli* DHM1 reporter strain, and screening for the interaction was performed on LB agar plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (40 μg/ml), isopropyl-β-D-galactopyranoside (0.5 mM), and ampicillin (100 μg/ml) and kanamycin (40 μg/ml). For a quantitative measurement of interaction, β-galactosidase activity was measured as described in the previous section

Promoter Activity Assays. Construction of BTH plasmids with other *pss* genes was described previously (Marczak et al. 2013, 2014). The presence of fusion proteins in either case was evidenced through Western blotting with anti-CyaA antibodies.

Motility assays.

To compare the motility behavior of RfTA1 WT and its derivatives, bacteria were grown in 79CA medium for 24 h, washed (centrifugation at 6,000 × g), and resuspended in sterile water at an OD₆₀₀ of 0.4. Agar swim plates (0.3%) were inoculated using an inoculation needle that was first submerged in a bacterial suspension and then stabbed into the agar. The diameter of migration zones was assessed after 5, 9, and 13 days of upright incubation of the plates at 28°C. The experiment was repeated three times.

Statistical analyses.

The results of promoter activity assays, plant tests, BTH quantitative assays, biofilm formation, and EPS production were submitted for statistical analyses, which were performed with STATISTICA software (TIBCO Software), using one-way analysis of variance and the posthoc Tukey's test.

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