Sinorhizobium meliloti Regulator MucR Couples Exopolysaccharide Synthesis and Motility

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In order to enter symbiosis with its legume partner, *Sinorhizobium meliloti* requires regulatory systems for the appropriate responses to its environment. For example, motility is required for the chemotactic movement of bacteria toward the compounds released by its host, and exopolysaccharides (EPS) are required for bacterial attachment to the root or for invasion of the infection thread. Previous research has shown that ExoR/ExoS/ChvI as well as the ExpR/Sin quorum-sensing system inversely regulate both motility and EPS production, although the regulation mechanisms were unknown. We were able to attribute the ExpR-mediated regulation of motility to the ability of ExpR to bind a DNA sequence upstream of *visN* when activated by *N*-acyl-homoserine lactone. Furthermore, MucR, previously characterized as a regulator of EPS production, also affected motility. MucR inhibited expression of *rem* encoding an activator of motility gene expression and, consequently, the expression of Rem-regulated genes such as *flaF* and *flgG*. Binding of MucR to the *rem* promoter region was demonstrated and a sequence motif similar to the previously identified MucR binding consensus was identified within this region. The swarming ability of *S. meliloti* Rm2011 was shown to depend on a functional ExpR/Sin quorum-sensing system and the production of both flagella and EPS. Finally, we propose a model for the coordination of motility and EPS synthesis in *S. meliloti*.

Rhizobia are of considerable relevance for agriculture because they are capable of assimilating atmospheric nitrogen through symbiosis with leguminous plants. Survival of these bacteria during starvation and various other stress situations encountered in the soil depends upon properties such as biofilm formation and motility. Bacterial motility is probably also a critical factor for the establishment of symbiosis under natural soil conditions. Despite the lack of an absolute requirement of motility for the establishment of symbiosis under standard laboratory conditions that include addition of the bacteria directly to the roots (Hoang et al. 2008; Truchet et al. 1985), it has been demonstrated that motile bacteria are more competitive over nonmotile bacteria for sites of nodule initiation on the host root (Ames and Bergman 1981; Caetano-Anollés et al. 1992).

To colonize a surface or to move toward a better environment, bacteria use different types of motility (Harshey 2003). The

most frequently observed are swimming and swarming (Fraser and Hughes 1999), both of which require flagella. Other modes of motility include twitching (requiring type IV pili) (Mattick 2002), gliding (Burchard 1981; McBride 2001), and sliding (Murray and Kazmierczak 2008). Extensively investigated in *Escherichia coli*, swimming is described as a movement of a single cell in low-viscosity liquid medium (e.g., $\leq 0.3\%$ agar), where several flagella, typically four to eight, emerge from the cell surface and are each driven by their own motor located at the base of the flagellum (Harshey 2003). In contrast, swarming is described as the movement of a cell population across a semisolid surface (e.g., $\geq 0.4\%$ agar), requiring both a functional flagellum and the production of surface wetting agents (Sharma and Anand 2002).

Sinorhizobium meliloti, a model organism for the study of rhizobia–legume interactions, possesses four to eight peritrichous flagella that propel the cell exclusively by clockwise rotation. A change in direction of bacterial migration is mediated by differential rotary speeds (Attmannspacher et al. 2005). *S. meliloti* is positively chemotactic toward several compounds found in the root exudates, such as nutrients, sugar, and dicarboxylic and hydroxyaromatic acids, and toward the nodulation gene-inducing flavonoids secreted by its host, *Medicago sativa* (Cooper 2004).

Until recently, all known *S. meliloti* genes related to motility and its regulation were found within a 56-kb contiguous chromosomal region named the flagellar regulon (Galibert et al. 2001). These genes are expressed in a temporal hierarchy. At the top of the hierarchy, class Ia is composed of an operon consisting of two genes, *visN* and *visR*, that encode the LuxR-type subunits of the global transcriptional activator of the motility and chemotaxis genes. Class Ib consists of *rem*, which is regulated by the class Ia genes (Rotter et al. 2006). A disruption of *visN*, *visR*, or *rem* leads to the loss of flagella and, consequently, the complete loss of swimming and swarming motility. Rem positively regulates the expression of the motility and chemotaxis genes by binding to the promoters of at least *flgB*, *fljF*, and *orf 38*, in addition to autoregulation of the *rem* gene. Class II comprises flagellar assembly (class IIa) and motor (class IIb) genes, and class III comprises flagellin and chemotaxis genes that require functional class I and class IIa genes for expression (Rotter et al. 2006; Sourjik et al. 2000).

New regulators of motility have been found recently outside of this regulon, increasing our understanding of the complex motility regulation network. For example, a disruption in *cbrA*, initially described as coding for a regulator of symbiosis (Gibson et al. 2006), leads to a decrease in expression of several motility genes, including *visN-visR* and *rem*. A *cbrA* mutant is also severely affected in its swimming ability (Gibson et al. 2007). More recently, Hoang and associates (2008) demon-

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strated that the ExpR/Sin quorum-sensing system downregulates levels of mRNA from the genes controlling swimming motility in *S. meliloti* via the *visN-visR* operon, although they point out that the mechanism by which this regulation occurs is unknown.

The motility of *S. meliloti* is also reduced in strains carrying either a mutation in *exoR* (*exoR95*) or *exoS* (*exoS96*) (Wells et al. 2007; Yao et al. 2004). The *exoR* gene encodes a protein of 268 amino acids that is transported to the periplasm (Reed et al. 1991; Wells et al. 2007) whereas the *exoS* gene encodes the membrane-bound sensor of the ExoS/ChvI two-component regulatory system. In addition to reduced motility, the gain-of-function mutation *exoS96*, resulting in a constitutively active sensor, and the loss-of-function mutation *exoR95* cause overproduction of succinoglycan (Yao et al. 2004). This implies that regulation of exopolysaccharide (EPS) production and cellular motility are coupled.

Recently, the regulation of production of the second EPS galactoglucan has also been demonstrated to be coordinated with regulation of motility through the ExpR/Sin quorum-sensing system (Hoang et al. 2008). In this study, both the ExoS-dependent two-component system and the Sin quorum-sensing system were shown to suppress motility gene expression through VisN and Rem in the presence of a functional ExpR whereas, in another study, the ExoS-dependent two-component system did not regulate *visN/visR* in a strain devoid of ExpR (Yao et al. 2004).

In this study, we add MucR to the regulatory network controlling motility in *S. meliloti*. MucR is a zinc finger protein

that shares 80% identity with the transcriptional regulator Ros from *Agrobacterium tumefaciens*. Ros controls the expression of different virulence genes by binding to a consensus sequence called ROS box (Chou et al. 1998) via its zinc finger (Archdeacon et al. 2000). MucR is known to influence the composition of the EPS matrix surrounding the bacterium by activating succinoglycan and inhibiting galactoglucan synthesis through binding to promoter regions in both EPS biosynthesis gene clusters (Bahlawane et al. 2008; Bertram-Drogatz et al. 1998). Here, we report that MucR negatively regulates *rem*, the regulator of motility, by binding upstream of the *rem* gene, and that the previously reported negative regulation of the *visN-visR* operon by the Sin quorum-sensing system (Hoang et al. 2008) is mediated by binding of *N*-acyl-homoserine lactone (AHL)-activated ExpR upstream of *visN*.

RESULTS

In order to identify additional MucR target genes, global expression profiles of *mucR* mutant Rm101 and the wild-type strain *S. meliloti* Rm2011 were compared using microarray analyses. This general screening revealed 282 upregulated and 16 downregulated genes in the early stationary growth phase of the *mucR* mutant cultured in tryptone yeast (TY) medium (Supplementary Table 1). Several genes related to motility appeared among the differentially expressed genes in the *mucR* mutant compared with the wild-type Rm2011 (Table 1). Therefore, we searched for MucR binding sequences within the flagellar regulon.

MucR binds specifically to a DNA region upstream of *rem*.

Similar to the ROS box, a consensus sequence has been identified for MucR (Bertram-Drogatz et al. 1998). Searching the genome by a Hidden Markov Model generated for the MucR consensus binding site led to the identification of a putative MucR binding site upstream of *rem* (Fig. 1). Rem has recently been identified as regulator of motility, hierarchically situated between the VisN/VisR master regulator and the class II genes in the *S. meliloti* wild-type strain RU11/001 (Rotter et al. 2006) and strain Rm8530, a derivative of wild-type strain Rm1021 carrying a functional copy of *expR* (Hoang et al. 2008). 5' Rapid amplification of cDNA ends (RACE) and computational analysis identified two transcription start sites

Table 1. Genes related to motility that displayed differential expression in the *mucR* mutant compared with the wild-type Rm2011 cultured in tryptone yeast medium to the early stationary phase^a

Gene	<i>M</i>	<i>P</i>
<i>flaB</i>	1.20	0.019
<i>flgL</i>	1.68	0.028
<i>flgG</i>	1.81	0.019
<i>flaF</i>	1.68	0.000
<i>fliK</i>	1.87	0.012
<i>rem</i>	1.00	0.029

^a *M* is the normalized logarithmic ratio of signal intensities and *P* is the probability that the data are random.

Fig. 1. A, Schematic view of the SMc03045-*rem* intergenic region; +1 and arrows mark the transcription start sites. The Rem binding site, confirmed in strain RU11/001, is marked by a gray box and the MucR consensus sequence by a dark bar. **B,** Alignment of experimentally verified MucR binding sites with the *rem* promoter sequence. The bases are colored to indicate similarity and the dark boxes (below) represent the highest homology between the different sequences.

(TSS) for the proximal and distal promoters upstream of *rem* in strain RU11/001 (Rotter et al. 2006). We experimentally confirmed the presence of the proximal TSS by 5' RACE in *S. meliloti* strain Rm2011 (data not shown) but could not confirm the presence of the distal TSS.

In this study, purified N-terminally flag-tagged MucR protein (Bahlawane et al. 2008) was used to assess whether MucR binds to the putative binding site in front of *rem*. Two DNA fragments covering the entire intergenic region between *rem* and the upstream gene *SMc03045* were selected for electrophoresis mobility shift assays (EMSA) (Fig. 2). The Flag-MucR protein caused a shift in the migration of the DNA fragment corresponding to the distal promoter (dP) (Fig. 2A) but not of the proximal fragment (pP) (Fig. 2B). Reduced mobility was not observed for several heterologous DNA fragments and control fragments from *S. meliloti* (data not shown).

Zinc finger motifs are rare among prokaryotes; thus, it was of interest to determine if MucR, like Ros, binds DNA through its C2H2 motif. To assess this question, we substituted histidine 92, shown to be essential for the protein–DNA interactions in case of Ros (Archdeacon et al. 2000), with alanine by site-directed mutagenesis. The modified protein was also purified as a Flag fusion protein but did not exhibit any DNA-binding properties (Fig. 2). Moreover, the addition of the divalent ion chelator EDTA at concentrations higher than 25 mM prevented the mobility shift. This demonstrates that the purified Flag-MucR protein binds specifically to a DNA fragment containing the sequence similar to the MucR binding-site consensus. Binding seems to occur via the zinc finger motif of MucR, because it was disturbed by either the depletion of zinc through the addition of EDTA or by a single amino-acid exchange within the C2H2 motif.

MucR inhibits *rem* transcription in all growth phases.

In order to define the role of MucR in the control of *rem* transcription, the *rem* promoter activity was monitored in several strains through the use of the promoter probe vector pSRPP18, which fuses the promoterless *lacZ* reporter gene to the promoter of interest and is integrated as a single copy into the genome (Bahlawane et al. 2008). In this study, the *SMc03045*–*rem* intergenic region was inserted into this vector

Fig. 2. Electrophoretic mobility shift assay using the Cy3-labeled DNA corresponding to the region between the end of the *SMc03045* gene and **A**, the first transcription start site (TSS) (distal promoter [dP]) and **B**, the DNA region from the distal TSS to the beginning of the *rem* translational start (proximal fragment [pP]). Purified Flag-MucR or Flag- H92A (0.2 µg) was mixed with 5 ng of Cy3-labeled DNA. A black bar marks the previously indicated DNA sequence (Fig. 1) exhibiting homology to the MucR binding consensus sequence. The gray box indicates the DNA region protected by Rem as determined by footprinting analysis in strain RU11/001 (Rotter et al. 2006).

and β-galactosidase activity conferred by this construct was measured in *S. meliloti* strain Rm2011 as well as in *mucR* (Rm101), *rem* (Sm2B5001), *mucR/rem* (Sm2B5002), *sinI* (Sm2B6016), and *visN* (Sm2B6015) mutants (Fig. 3). For these experiments, cells were grown in rhizobium basal (RB) medium as previously described (Rotter et al. 2006; Sourjik et al. 2000). The β-galactosidase activity found in the *mucR* mutant was significantly higher (up to three times) than in the wild type, especially during the stationary phase. This inhibitory effect of MucR on *rem* transcription was more obvious when we compared the β-galactosidase activities in the *rem* and *mucR/rem* mutants where, apparently, the inhibitory effect of MucR on *rem* transcription was greater in the absence of Rem. The inhibitory effect of MucR on *rem* expression could be complemented by the pPHU231 plasmid carrying either native *mucR* or Flag-*mucR* but not Flag-H92A-*mucR*. These results suggest that MucR inhibits *rem* expression by directly binding to the DNA site through its zinc finger motif.

The β-galactosidase activity measured from the *rem* promoter in the *rem* mutant was maintained between 0.8 and 1.8 times the activity measured in the wild type. This result appears somewhat analogous to the previously reported regulation of *rem* transcription in the *S. meliloti* strain RU11/001 (Rotter et al. 2006), where the expression activity of the *rem* promoter in the *rem* mutant compared with the wild type was initially higher but then declined more dramatically to a lower expression activity during progression of growth, consistent with a scenario where transcription of *rem* is subject to auto-regulation.

Fig. 3. Representation of the *rem* gene expression during growth in Rm2011 and several mutants. The growth curve is represented by an unbroken line and *rem* promoter activity (in Miller units) as columns, as monitored through the use of the promoter probe vector pSRPP18 in Rm2011 (gray columns) and derivative strains carrying disruption mutations in *mucR* (black column), *rem* (gray dashed column), *mucR/rem* (black dashed column), *sinI* (filled gray columns), and *visN* (black dotted column). Columns represent the average of six replicates.

A reduction of β -galactosidase activity to background levels in the *visN* mutant confirmed the position of Rem under the control of the *visN-visR* locus, as previously described (Rotter et al. 2006). Furthermore, compared with the wild-type Rm2011, the AHL-deficient mutant Rm2011 *sinI* did not exhibit significant changes in *rem* expression. This implies that synthesis of AHL by SinI is not required for VisN/VisR-mediated activation of *rem* expression.

MucR inhibition is relayed to *rem*-controlled motility genes.

In order to confirm the dependency of expression of several motility genes on the regulation of *rem* by MucR, we measured their mRNA level by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) in *S. meliloti* Rm2011, and its *rem* (Rm2B5001) and *mucR* (Rm101) mutant derivatives. In addition to measuring the mRNA of *visN* and *rem*, *flaF*, *flgG*, and *fliK* were also included (Fig. 4). *flaF* is one of the class IIa genes, which codes for a flagellar biosynthesis regulatory protein (Capela et al. 2001), while *flgG* encodes a component of the basal-body rod and *fliK* a regulator of flagellar hook length (Eggenhofer et al. 2006; Platzer et al. 1997). *visN* expression appeared constitutive throughout the growth curve and unaffected by a disruption in either *rem* or *mucR*. *flaF* and *flgG* showed a downregulation in the *rem* mutant, especially strong at low optical density when *S. meliloti* is expected to be more motile. In contrast, *fliK* was weakly or not influenced by a mutation in *rem* until 68 h after inoculation but showed repression at high optical density.

rem, *flaF*, and *flgG* were upregulated in a *mucR* mutant 34 h after inoculation. This confirms the conclusion derived from the promoter probe vector or β -galactosidase assays that MucR inhibits *rem* and indicates that this effect is relayed to the *rem*-controlled genes such as *flaF* and *flgG*. At low cell density (10 h after inoculation), no significant difference in expression of the motility genes could be observed between Rm2011 and its *mucR* mutant. This was also observed in the β -galactosidase activity assays where, at low cell density, only a weak repression of *rem* expression was conferred by MucR in the presence of Rem.

In the *mucR* mutant, *fliK* showed a reduced level of expression at 69 h of cultivation. Based on these results, we suggest that MucR inhibits the expression of *rem* through binding to the promoter region of *rem* and thereby also affects the expression of at least some of the motility genes, such as *flaF* and *flgG*.

ExpR is required for swarming but not for swimming.

Swimming and swarming tests using a series of *S. meliloti* mutants were performed to learn more about the effect of MucR on cell motility. Characteristic images are shown of the various strains grown on swimming (Fig. 5A) and swarming (Fig. 5B and 5C) plates. Strains capable of motility showed an increased diameter in colony size after 3 days, compared with the strains incapable of swimming (Fig. 5A). After 3 days, the colony diameter of the motile strains did not increase significantly further. Mutations in either *visN*, *rem*, or the *expR*-deficient strain Rm2011, and a *visN* mutation in Sm2B3001 containing a functional copy of *expR*, resulted in loss of the ability to swim or to swarm on medium supplemented with 0.3 or 0.6% agar, respectively. This observation is consistent with previously published results from strains RU11/001 (Rotter et al. 2006) and Rm8530 (Hoang et al. 2008). Other strains incapable of swimming are Sm2B5005 (Fig. 5A), Sm2B5006 (not shown), and Sm2B5007 (not shown), because each of these strains carried a mini-Tn5 insertion in one of the following genes: *flgE* (flagellar hook protein), *flgK* (putative flagellar hook-associated protein), and *fliF* (flagellar M-ring transmem-

brane protein), respectively. All other strains tested were capable of swimming. Therefore, the presence of MucR, ExpR, and Sin AHL all affect the expression of motility genes but are not required for swimming, whereas both VisN and Rem are.

Interestingly, only strains carrying a functional *expR* locus (Rm8530 [Rm1021 *expR*⁺], Rm2B3001 [Rm2011 *expR*⁺], Rm2011 pJN*expR*⁺, and Sm2B5004 [Rm2011 *mucR*⁻, *expR*⁺]) demonstrated the ability to swarm on 0.6% agar. In contrast, strains with an interrupted *expR* locus (Rm2011, Sm2B5001 [Rm2011 Δ *rem*] and Rm101 [Rm2011 *mucR*⁻]) could not swarm, regardless of the presence or absence of a functional copy of *rem* or *mucR* (Fig. 5B).

We note that, although MucR reduces expression of *rem* and, consequently, the downstream motility genes, *mucR* was required for neither swimming nor swarming. Induction of galactoglucan biosynthesis genes resulted in a mucoid phenotype of the *mucR* mutant similar to that of strains carrying a functional *expR* locus. Nevertheless, the *mucR* mutant was not able to swarm in the absence of a functional copy of *expR*.

The composition and amount of EPS of *expR*⁺ strains differ from that of a *mucR* mutant. Galactoglucan produced by *expR*⁺ strains contains a low molecular weight fraction while this is not the case for a *mucR* mutant (González et al. 1996), and an *expR*⁺ strain synthesizes more succinoglycan than a *mucR*

Fig. 4. Motility genes expression monitored by quantitative reverse-transcriptase polymerase chain reaction. Columns indicate expression of *flaF* (black), *flgG* (gray), *fliK* (white), *rem* (dashed gray), and *visN* (dashed black) genes at 10, 34, and 69 h after inoculation. **A**, Sm2B5001 (*rem*⁻) versus Rm2011 and **B**, Rm101 (*mucR*⁻) versus Rm2011. The relative expression is calculated as the fold change ($2^{-\Delta C(T)}$) between the corresponding mutant and Rm2011. Each figure is the result of measurements based on two biological and two technical replicates.

Fig. 5. A, Swimming and **B** and **C**, swarming tests on several *Sinorhizobium meliloti* strains. Plates containing **A**, 0.3% or **B** and **C**, 0.6% agar were spotted with 3 μ l of a stationary culture of Rm2011, Sm2B3001 (*expR*⁺), Sm8530 (*expR*⁺), Rm2011/pJN*expR*, Sm2B5001 (*expR*⁻, Δ *rem*), Sm2B5005 (*expR*⁺, *flgE*⁻), Sm2B6005 (*expR*⁺, *visN*⁻), Sm8530 *wgeB*::*lacZ*, Rm101 *mucR*⁻, Sm2B5004 (*expR*⁺, *mucR*⁻), and Sm2B6006 (*expR*⁺, *sinI*⁻). **C**, For *N*-acyl-homoserine lactone complementation, oxo-C14-HL and oxo-C16-HL were added during agar pouring to a final concentration of 10 μ M. For the swimming tests, colony diameter in millimeters was measured after 3 days of incubation and the mean was calculated from 24 measurements and included under each image. To the right of each diameter value is the *P* value, where a value <0.05 indicates a significant difference to the reference strain Rm2011.

mutant (Bertram-Drogatz et al. 1998). In addition, it was shown that ExpR increases depolymerization of high molecular weight succinoglycan to low molecular weight oligosaccharides by induction of endoglycanase-encoding genes (Glenn et al. 2007; Hoang et al. 2008). Therefore, we checked if the motility of *expR*⁺ strains on swarming agar was due to active swarming or the result of EPS production. *expR*⁺ strains unable to produce galactoglucan (Rm8530 [Rm1021 *expR*⁺, *wgeB*⁻]) or Sin AHL (Sm2B6006 [Rm2011 *expR*⁺, *sinI*⁻]) or that were defective in flagellum production—Sm2B5005 (Rm2011 *expR*⁺, *flgE*⁻) (Fig. 5B), Sm2B5006 (Rm2011 *expR*⁺, *flgK*⁻) (not shown), Sm2B5007 (Rm2011 *expR*⁺, *fliF*⁻) (not shown), and Sm2B6005 (Rm2011 *expR*⁺, *visN*⁻) (Fig. 5B)—were tested for their swarming ability. In each case, these mutants were nonmotile on 0.6% agar. Hence, swarming appears to be dependent on active production of galactoglucan, Sin AHL, and functional flagellar in addition to a functional ExpR.

Swarming ability was recovered in Sm2B6006 (Rm2011 *expR*⁺, *sinI*⁻) after the addition of 10 μ M of either synthetic oxo-C14-HL or oxo-C16-HL, previously found to be important for motility (Gao et al. 2005), to the swarming medium (Fig. 5C). Addition of these AHL did not recover swarming of Rm2011 (*expR*⁺) or Rm101 (Rm2011 *mucR*⁻) (Fig. 5C).

ExpR inhibits both *visN* and *rem* expression by specific binding to the upstream region of *visN*.

The influence of ExpR on *rem* and *visN* expression in RB medium was monitored in Rm2011 *expR*⁺ and *expR*⁻ strains as well as in quorum sensing-deficient and -proficient strains by using the promoter probe vector pSRPP18, which fuses the whole intergenic region preceding *rem* and a 274-bp upstream region of *visN*, respectively, to the promoterless *lacZ* reporter gene. At 69 h after inoculation, expression of *visN* in either Rm2011 (*expR*⁺) or Sm2B6006 (Rm2011 *expR*⁺, *sinI*⁻) was three times higher than in the AHL-producing *expR*⁺ strain

Fig. 6. *visN* expression (in Miller units) as monitored through the use of the promoter probe vector pSRPP18 in Rm2011, Sm2B3001 (*expR*⁺), and their respective *sinI* mutants (Sm2B6016 [*expR*⁺, *sinI*⁻] and Sm2B6006 [*expR*⁺, *sinI*⁻]) in cultures in rhizobium basal medium. *N*-acyl-homoserine lactones (oxo-C14-HLs and oxo-C16-HLs) were included at the time of inoculation at a final concentration of 10 μ M.

Sm2B3001 (Fig. 6). Addition of either oxo-C14-HL or oxo-C16-HL to the medium restored the inhibition of *visN* in the *sinI* mutant Sm2B6006. Likewise, we found that *rem* expression was reduced by approximately 50% in the presence of ExpR (data not shown). Thus, it appears that ExpR inhibits the expression of both *visN* and *rem* in an AHL-dependent manner, as has been recently published (Hoang et al. 2008).

ExpR has been recently shown to activate the expression of the genes *wgaA* and *wgeA* (involved in galactoglucan production), *exsH* and *exoI* (involved in succinoglycan production), and *sinI* (encoding the AHL synthase) in the presence of oxo-C14-HL, through binding to the promoters of these genes (Bartels et al. 2007; McIntosh et al. 2008). We postulated that ExpR-dependent inhibition, evident in the case of *visN* and *rem*, may also be mediated through binding of ExpR to the *visN* promoter in the presence of oxo-C14-HL and, consequently, resulting in an indirect inhibition of *rem*.

An EMSA including (his)₆ExpR, a Cy3-labeled DNA fragment representing a 274-bp upstream region of *visN* and oxo-C14-HL, demonstrated that the AHL-activated ExpR binds to the *visN* promoter (Fig. 7A). EMSA involving various shorter fragments from the *visN* upstream region (data not shown) identified a 35-bp sequence (Fig. 7B) that contains an ExpR binding site. The 35-bp fragment shares some homology to other known ExpR binding sites (Fig. 7C).

DISCUSSION

Motility and chemotaxis offer microorganisms a distinct advantage when faced with starvation, toxic substances, or the requirement to approach an appropriate host. However, the motility machinery places high energy demands on the cell. Therefore, bacteria strictly regulate expression of motility-related genes to avoid inappropriate energy expenditure. Bacteria also invoke alternative strategies for the regulation of motility when faced with starvation or reduced nutrient conditions. In some bacteria (e.g., *Serratia liquefaciens* and *E. coli*), this strict regulation results in an increase in the ability to migrate when faced with reduced nutrient conditions (Sharma and Anand 2002). Apparently, the strategy is to engage the motility machinery only in response to stress or starvation conditions by moving toward a better environment. In other bacteria (e.g., *Sinorhizobium meliloti*), the strict regulation results in migration toward chemo-attractants under nutrient-sufficient or -excess conditions and the development of the vegetative state and biofilm upon encountering stress or starvation conditions (Wei and Bauer 1998). Thus, *S. meliloti* was found to be motile throughout active growth, when nutrients are readily available, and to become nonmotile upon transition to the stationary phase (Rotter et al. 2006).

Regulation of bacterial motility appears to be a complex system involving multiple processes. For example, results from a recent study with *Rhizobium etli* indicate that genes responsible for quorum sensing, production of EPS, amino acid and polyamine metabolism, and production of flagella are required for swarming motility, because transposon insertions into these genes resulted in a nonswarming phenotype (Braeken et al. 2008). Furthermore, it is likely that additional processes are involved in motility, because various strains or isolates of *S. meliloti* display different motility behaviors, possibly due to unidentified environmental factors (Wei and Bauer 1999).

In *S. meliloti*, the processes of motility and EPS biosynthesis appear to be contrarily regulated (Hoang et al. 2008; Reed et al. 1991; Wells et al. 2007; Yao et al. 2004). The repression of genes related to motility occurred in concert with upregulation of EPS biosynthesis genes. Transposon mutations leading to a

faster swarming phenotype have been associated with a reduction in EPS production (Wei and Bauer 1999). In this study, we investigated the molecular basis for this coordinated regulation.

Rem was identified as activator of motility and chemotaxis genes that relays the regulatory effect of the LuxR-type regulators VisN and VisR to these genes (Rotter et al. 2006). It was previously shown that the Sin/ExpR quorum-sensing system (Marketon and González 2002; Marketon et al. 2002) down-regulates motility and chemotaxis genes and that the *visN-visR* operon is a target for the LuxR-type regulator ExpR (Hoang et al. 2004, 2008). We confirmed the inhibition of *visN* expression by AHL-activated ExpR and demonstrated binding of ExpR to the promoter region of *visN* in the presence of AHL implying that ExpR directly regulates transcription of *visN-visR*. A consensus sequence for AHL-activated ExpR has been described for the activation of genes controlling succinoglycan and galactoglucan production (McIntosh et al. 2008). In each of these cases, the gene or genes directly downstream of the binding sites are activated in the presence of ExpR and Sin AHL. In the case of *visN*, the DNA fragment containing the ExpR binding site contains some homology to the previously identified sites.

ExpR is involved in the regulation of a multitude of *S. meliloti* genes (Gao et al. 2005; Glenn et al. 2007; Hoang et al. 2004, 2008; Pellock et al. 2002; Yao et al. 2004), including genes related to motility and chemotaxis as well as to the biosynthesis of the EPS succinoglycan and galactoglucan, thereby coupling regulation of motility and EPS. It functions as an activator of the expression of galactoglucan genes in the presence of Sin AHL and positively affects expression of *exoK*- and *exoH*-encoding endoglycanases that specifically depolymerize succinoglycan (Glenn et al. 2007; Hoang et al. 2004; McIntosh et al. 2008).

MucR also affects the biosynthetic pathways of galactoglucan and succinoglycan. It represses galactoglucan production and stimulates succinoglycan synthesis (Bahlawane et al. 2008; Bertram-Drogatz et al. 1998; Rüberg et al. 2003). In this study, we demonstrated that MucR also affects motility by regulation of *rem* expression. Binding of MucR to the promoter

region upstream of *rem* was shown and a sequence sharing similarities with the MucR binding site consensus (Bertram-Drogatz et al. 1998) was identified in this region. This sequence motif overlaps with the previously reported Rem binding site found in strain RU11/001 (Rotter et al. 2006), possibly causing competition between MucR and Rem for the target sequence. Considering that MucR inhibits while Rem activates *rem* expression at low cell density, we speculate that the ratio of Rem/MucR levels in the cell may contribute to the regulation of motility in *S. meliloti*. Consistent with this postulation, we observed that, at low cell densities, the expression of the *rem* promoter was inhibited in the presence of an intact *mucR* locus and a disrupted *rem* locus but not in the presence of a functional *rem* locus. In such a scenario, a ratio that favored Rem (e.g., low cell density) would mask the inhibiting effect of MucR, leading to the upregulation of *rem*, whereas a ratio favoring MucR (e.g., high cell density) would result in the inhibition of *rem*.

Although *rem* expression levels are regulated by several regulators, including ExpR, VisN, and VisR, as demonstrated in this study and elsewhere (Hoang et al. 2008; Rotter et al. 2006), it is unknown what factors affect expression of the autoregulated *mucR* gene (Müller et al. 1993). However, MucR appears to function as a repressor in both the presence and absence of a functional Sin quorum-sensing system (Bahlawane et al. 2008; McIntosh et al. 2008), suggesting that the regulation of *mucR* expression is not dependent on quorum sensing, although this remains to be confirmed.

This study highlights another interesting aspect of motility in *S. meliloti*; namely, that swarming but not swimming depends on the presence of a functional quorum-sensing system and galactoglucan production. Only those strains with an intact ExpR/Sin quorum-sensing system and capable of galactoglucan and flagella production showed the ability to swarm under our conditions. Swimming required flagella production but not the ExpR/Sin system and galactoglucan biosynthesis. Whereas swimming is a type of individual behavior, swarming is the coordinated movement of a population of bacteria in high viscosity media or over a surface and has been shown to be wide-

Fig. 7. *N*-acyl-homoserine lactone-enhanced DNA binding of purified (his)₆ExpR to the *visN* promoter region. **A**, Above, the Cy3-labeled DNA fragment (274 bp) containing the promoter region of *visN* was used in an electrophoretic mobility shift assay (EMSA) with purified (his)₆ExpR and oxo-C14-HL. Below, a 35-bp oligonucleotide covering the ExpR binding site within the *visN* promoter was cloned into vector pCR to form pCRvisN-oligo. A DNA fragment (250 bp) from pCRvisN-oligo was amplified and Cy3-labeled and included in the EMSA. **B**, For each DNA fragment, lane 1 is Cy3-labeled DNA only; lane 2 is Cy3-labeled DNA mixed with (his)₆ExpR; and lane 3 is a mixture of Cy3-labeled DNA, (his)₆ExpR, and oxo-C14-HL. **C**, ExpR binding site consensus. The identified ExpR binding sites from the promoter regions of *sinI*, *wgaA*, and *wgeA* aligned in the 5'-3' orientation to show homology (McIntosh et al. 2008). Underneath is the 35-bp oligonucleotide from the *visN* promoter that was cloned into pCRvisN-oligo and binds to oxo-C14-HL activated (his)₆ExpR. The oligonucleotide contains sequence homology to the previously identified ExpR binding sites.

spread among flagellated bacteria, particularly in association with quorum sensing (Harshey 2003; Sharma and Anand 2002). Hence, the involvement of quorum sensing in swarming but not in swimming may be an important feature in coordination of motility.

Coupling of EPS production and cell motility has been also been found in *Ralstonia solanacearum* (Brumbley and Denny 1990), *Vibrio cholerae* (Watnick and Kolter 1999; Ali et al. 2000), and *Salmonella enterica* (Cano et al. 2002), and presumably increases the ability of the cells to interact with their prospective host cells. Coordination of these processes in *Sinorhizobium meliloti* may also contribute to the establishment of symbiosis. The regulatory network is summarized in Figure 8; ExpR and MucR play central roles as key regulators that modulate both EPS production and motility. Figure 8 also emphasizes how the presence of Sin AHL influences the level of motility gene expression that is controlled by the LuxR-type regulators ExpR and VisN/VisR. Whereas ExpR required Sin AHL to reduce expression of *visN-visR*, VisN/VisR did not require these AHL for induction of the expression of *rem* that encodes the class Ib activator of motility genes. Under quorum conditions, production of succinoglycan (Glenn et al. 2007) and galactoglucan oligosaccharides (Pellock et al. 2002; Marketon et al. 2003) that promote invasion of the root nodules appears to be enhanced. It remains to be investigated how ExoR and the two-component regulatory system ExoS/ChvI integrate into this regulatory network.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Strains and plasmids used in this study are listed in Table 2.

Media and growth conditions.

E. coli strains were grown in Luria-Bertani (LB) medium (Sambrook 1989) at 37°C; *S. meliloti* strains were typically grown in TY (Beringer 1974) at 30°C. Measurements of transcription of motility-related genes via β -galactosidase assays were made after growth in *S. meliloti*-motility medium (RB; 0.2% mannitol and 2% tryptone-yeast extract) (Rotter et al. 2006) at 30°C. Swimming plates (0.3% agar) and swarming plates (0.6% agar) were prepared using Bromfield medium (0.04% tryptone, 0.01% yeast extract, and 0.0067% CaCl_2). For measurement of motility, an overnight TY culture (3 μl) was inoculated on the surface of the agar and incubated at 30°C for 3 days. When necessary, nalidixic acid (10 $\mu\text{g/ml}$), neomycin (120 $\mu\text{g/ml}$), or spectinomycin (200 $\mu\text{g/ml}$) was added.

Gene expression profiling by microarray hybridizations.

cDNA synthesis, hybridization of *S. meliloti* microarrays, microarray image processing, and data analysis were carried out as described previously (Krol and Becker 2004). Mean signal and mean background intensities were obtained for each spot of the microarray images using the ImaGene Software 6.0 software (Biodiscovery Inc., Los Angeles). Spots were flagged

Fig. 8. Model of the regulatory network correlating exopolysaccharide production and motility in *Sinorhizobium meliloti* through MucR and ExpR. To increase comprehension, all regulatory levels are represented where rectangles indicate the gene, parallelograms mark the corresponding transcripts, and rectangles with round edges indicate the encoded proteins. Arrows and bar-ending arrows indicate activation and repression, respectively. The formation of protein complexes is marked by dark circles at the junction of the proteins forming the complex. AHL = acyl homoserine lactones, SAM = S-adenosylmethionine, ACP = acyl carrier protein, and LMW = low molecular weight.

as “empty” if $R \leq 0.5$ in both channels, where $R = (\text{signal mean} - \text{background mean})/\text{background standard deviation}$. The remaining spots were considered for further analysis. After subtractions of the local background intensities from the signal intensities and introduction of a floor value of 20, the $\log_2$ value of the ratio of intensities was calculated for each spot: $M_i = \log_2(R_i/G_i)$, where $R_i = I_{\text{ch1}(i)} - B_{\text{gch1}(i)}$, $G_i = I_{\text{ch2}(i)} - B_{\text{gch2}(i)}$, $I_{\text{ch1}(i)}$ or $I_{\text{ch2}(i)}$ is the intensity of a spot in channel 1 or channel 2, respectively, and $B_{\text{gch1}(i)}$ or $B_{\text{gch2}(i)}$ is the background intensity of a spot in channel 1 or channel 2, respectively. The mean intensity was calculated for each spot as $A_i = \log_2(R_i G_i)^{0.5}$. A normalization method based on local regression was applied: $M_i = \log_2(R_i/G_i) \rightarrow \log_2(R_i/G_i) - c(A) = \log_2(R_i/[k_j(A)G_i])$, where $c(A)$ is the locally weighted scatter-plot smoothing (LOWESS) fit to the MA plot. Significantly up- or downregulated genes were identified by t statistics. Genes were accounted as differentially expressed if $P \leq 0.05$ and $M \geq 1.00$ or ≤ -1.00 . Normalization and t statistics were carried out using the EMMA 2.2 microarray data analysis software developed at Bielefeld University, Bioinformatics Resource Facility, Center for Biotechnology website (Dondrup et al. 2003).

Construction of *S. meliloti* strains.

GeneSOEing was used to construct the *expR*⁺ strain Sm2B3001 and its derivatives *visN* (Sm2B6005) and *sinI* (Sm2B6006), as well as the Rm2011 (*expR*[−]) *rem* (Sm2B5001), *visN* (Sm2B6015), and *sinI* (Sm2B6016) mutants. Primers used for the construction of mutants are listed Table 3.

To construct the Rm2011 Δrem mutant, two products, upstream (SMc03046A-SMc03046B) and downstream (SMc03046C-SMc03046D) of *rem*, were PCR amplified from Rm2011 genomic DNA. The outside primers included an *XbaI* restriction site for cloning into the pK18*mobsacB* vector while the inside primers included complementary regions. A third PCR using both products as templates and the outside primers yielded a 1,500-bp construct with downstream and upstream *rem* DNA sequences. Within this construct, a portion of the *rem* gene was deleted from 17 bp after the translation start to 75 bp before the translation stop. This product was cloned into the pK18*mobsacB* vector and sequenced. After transfer to the Rm2011 chromosome via conjugation with S17-1, single crossover mutants were selected using neomycin. Several clones of the double crossover mutant, named Sm2B5001 (*rem*[−], *expR*[−]), were selected after growth on 10% sucrose-LB plates. The deletion was then checked by PCR and by swimming tests.

In Rm2011, the *expR* locus, located on the 3.4-Mb main chromosome, is nonfunctional and contains an insertion sequence (IS_{Rm2011-1}) within the coding region of the gene, as is the case with strain Rm1021 (Pellock et al. 2002). For the construction of an *expR*⁺ derivative of Rm2011 (Sm2B3001), the functional *expR* locus in *S. meliloti* strain Rm8530 (Pellock et al. 2002), with the exception of the ATG translation start, was PCR amplified using primers with the restriction sites *EcoRI* and *HindIII* (Table 3) and cloned into pK18*mobsacB*. The resulting construct (pK18*mobsacB*::

Table 2. Strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>Sinorhizobium meliloti</i>		
Rm2011	Wild type, Nx ^r , Sm ^r	Casse et al. 1979
Rm101	Rm2011 <i>mucR</i> ::Spec ^r	Becker et al. 1997
2011mTn5STM.1.03.E07	Rm2011 <i>flgE</i> ::mini-Tn5	Pobigaylo et al. 2006
2011mTn5STM.1.08.D04	Rm2011 <i>flgK</i> ::mini-Tn5	Pobigaylo et al. 2006
2011mTn5STM.1.09.G02	Rm2011 <i>fliF</i> ::mini-Tn5	Pobigaylo et al. 2006
Sm2B5001	Rm2011 Δrem	This work
Sm2B5002	Rm101, <i>expR</i> [−] , <i>mucR</i> ::Spec ^r , Δrem	This work
Sm2B6016	Rm2011, <i>expR</i> [−] , <i>sinI</i> ::Spec ^r	This work
Sm2B6015	Rm2011, <i>expR</i> [−] , <i>visN</i>	This work
Rm8530	Rm1021 <i>expR</i> ⁺ , (formerly <i>expR101</i>)	Pellock et al. 2002
Rm8530 <i>wgeB</i> ::lacZ	Rm8530 <i>wgeB</i> ::lacZ::Gm ^r	Pellock et al. 2002
Sm2B3001	Rm2011 with a restored <i>expR</i> gene	This work
Sm2B5004	Sm2B3001 <i>mucR</i> ::Spec ^r	This work
Sm2B5005	Sm2B3001 <i>flgE</i> ::mini-Tn5	This work
Sm2B5006	Sm2B3001 <i>flgK</i> ::mini-Tn5	This work
Sm2B5007	Sm2B3001 <i>fliF</i> ::mini-Tn5	This work
Sm2B6006	Sm2B3001, <i>expR</i> ⁺ , <i>sinI</i> ::Spec ^r	This work
Sm2B6005	Sm2B3001, <i>visN</i> ::Spec ^r	This work
<i>Escherichia coli</i>		
DH5 α	For plasmid construction	Grant et al. 1990
S17-1	Used for conjugation with <i>S. meliloti</i>	Simon et al. 1983
Plasmids		
pFLAGMucR	pT7/T3 α 19 plasmid for over-expression of FLAG-MucR fusion protein	Bahlawane et al. 2008
pWH844(his) ₆ <i>expR</i>	pWH844 plasmid for overexpression of (his) ₆ -tagged ExpR	Bartels et al. 2007
pSRPP18	Promoter probe vector, pUC derivative, promoterless <i>lacZ</i> gene, integrates between the <i>exoD</i> terminator and the <i>thiD</i> gene	Bahlawane et al. 2008
pJN <i>expR</i>	pJN105 containing <i>expR</i>	Bartels et al. 2007
pHP45 Ω	Vector carrying a 2.0-kb spectinomycin/streptomycin resistance cassette flanked by transcription terminators	Prentki and Krisch 1984
pK18 <i>mobsacB</i>	Km ^r ; <i>lacZ</i> , <i>mob</i> , <i>sacB</i>	Schäfer et al. 1994
pK18SMc03046	pK18 <i>mobsacB</i> with upstream and downstream SMc03046 DNA region for geneSOEing	This work
pK18 <i>mobsacBexpR</i> ^{ATG}	pK18 <i>mobsacB</i> carrying <i>expR</i> from strain Rm8530, minus the ATG start codon	This work
pK18 <i>mobsacBsinI</i> Ω	pK18 <i>mobsacB</i> carrying the spectinomycin/streptomycin cassette from pHP45 Ω inserted into the <i>sinR/sinI</i> region	This work
pK18 <i>mobsacBvisN</i> Ω	pK18 <i>mobsacB</i> carrying a spectinomycin/streptomycin cassette from pHP45 Ω inserted into <i>visN</i>	This work
pCR <i>visN</i> -oligo	ExpR binding site upstream of <i>visN</i> cloned into the vector pCR (Invitrogen) using the TOPO cloning technique	This work

^a Nx^r, Sm^r, Spec^r, Gm^r, and Km^r = resistant to nalidixic acid, streptomycin, spectinomycin, gentamycin, and kanamycin, respectively.

expR^{ATG-}) was inserted into the Rm2011 chromosome via conjugation with S17-1 and single crossover mutants were selected using neomycin. Double-crossover mutants were selected on LB agar containing 10% sucrose. Clones in which the double crossover resulted in the replacement of the non-functional *expR* locus containing the IS element with the *expR* locus from pK18*mobsacB*::*expR*^{ATG-} were visibly mucoid colonies after growth on agar, indicative of a functional *expR*.

For the *visN* mutants, Sm2B6005 (*expR*⁺, *visN*⁻) and Sm2B6015 (*expR*⁻, *visN*⁻), two *visN*-derived fragments of 548 bp (consisting of 289 bp upstream of *visN* and 260 bp of the 5' end of *visN*) and 563 bp (consisting of 355 bp of the 3' end of *visN*, the 12-bp intergenic region between *visN* and *visR*, and 196 bp of the 5' end of *visR*) were cloned into pK18*mobsacB* via the indicated restriction digest sites (Table 3). Between these fragments was inserted a 2.0-kb cassette carrying streptomycin and spectinomycin (Sm^r/Sp^r) resistance from plasmid pHP45Ω (Prentki and Krisch 1984) via the *Hind*III restriction sites to yield the vector construct pK18*mobsacB*Δ*visN*Ω.

Similarly, for the *sinI* mutants, Sm2B6006 (*expR*⁺, *sinI*⁻) and Sm2B6016 (*expR*⁻, *sinI*⁻), two fragments of 637 bp (consisting of 583 bp of the 3' end of *sinR* and 54 bp of the intergenic region between *sinR* and *sinI*) and 603 bp (consisting of 578 bp of the 3' end of *sinI* and 25 bp of the DNA sequence downstream of *sinI*) were cloned into pK18*mobsacB* via the indicated restriction digest sites (Table 3). Between these fragments was inserted the 2.0-kb Sm^r/Sp^r cassette from plasmid pHP45Ω (Prentki and Krisch 1984) via the *Hind*III restriction sites to yield the vector construct pK18*mobsacB*Δ*sinI*Ω.

The *mucR* disruption locus was transferred from strain Rm101 *mucR* to strains Rm2B3001 (*expR*⁺) and Sm2B5001 (*expR*⁻, *rem*⁻) via phage (ΦM12) transduction to produce strains Rm2B5004 (*mucR*⁻, *expR*⁺) and Sm2B5002 (*rem*⁻, *mucR*⁻, *expR*⁻), respectively. Likewise, the mini-Tn5-disrupted loci of *flgE*, *flgF*, and *fliF* were transferred from strains

2011mTn5STM.1.03.E07, 2011mTn5STM.1.08.D04, and 2011mTn5STM.1.09.G02, respectively (Pobigaylo et al. 2006) to strain Rm2B3001 via phage transduction to produce strains Rm2B5005, Rm2B5006, and Rm2B5007. The mutants were checked by PCR.

Mutation of Flag-MucR gene by site-directed mutagenesis.

The exchange between the histidine 92 of MucR and alanine was performed using the Chameleon double-stranded site-directed mutagenesis kit from Stratagene, after the manufacturer's instructions. Primer sequences are listed in Table 3.

Construction of the promoter probe vectors.

The *rem* and *visN* promoters were PCR amplified using the primers listed in Table 3 and cloned into the pSRPP18 promoter probe vector using the *Kpn*I and *Bam*HI restriction sites, as has been described (Bahlawane et al. 2008). After sequencing, the plasmids were transferred to S17-1 for conjugation with *S. meliloti*. Several clones were selected and the correct integration of the plasmid construct into the *exoP* gene on the *S. meliloti* chromosome was verified by PCR.

Construction of the vector containing the ExpR binding site upstream of *visN*.

For cloning of the ExpR binding site upstream of *visN*, complementary oligonucleotides (Table 3) were annealed and cloned into vector pCR, using the TOPO cloning kit (Invitrogen GmbH, Karlsruhe, Germany) according to manufacturer's instructions. The DNA fragment from the pCR*visN*-oligo construct was amplified and Cy3-labeled using the pUC18 universal sequencing primers (Table 3).

Protein purification.

Flag-MucR and H92AFlag-MucR proteins were purified using an ANTI-FLAG M2 affinity gel, as described previously (Bahlawane et al. 2008). Expression and purification of (his)₆ExpR was performed as described previously (Bartels et al. 2007).

Table 3. Primers used for DNA manipulation

Primer name	Sequence	Used for ^a
SMc03046A	TCTAGAGCGATCTAGAGCATGCGATCAACTACC	<i>rem</i> deletion
SMc03046B	ACCGAGCTTCTTGCAGCTTCAACCACCACGATCATTC	<i>rem</i> deletion
SMc03046C	GAATGATCGTGGTGGTTGAACTGCGCAAGAAGCTCGGT	<i>rem</i> deletion
SMc03036D	TCTAGAGATATCTAGAAGCAATGAGGCCGTCCGAAG	<i>rem</i> deletion
ScaI_mut	CTGTGACTGGTGAGACCTCAACCAAGTCATTC	H92A exchange
MucR_H92_A92	GGTTGTGGTGCATCATGAGCCCGCTCAACGACTTGAAGG	H92A exchange
rem up <i>Kpn</i> I	CCCGGTACCCGACAATGCCGACAGATT	pSRPP18/ <i>rem</i>
rem low <i>Bam</i> HI	CCCGGATCCTAGCCGTCTTCAACAAGA	pSRPP18/ <i>rem</i>
visN-up <i>Kpn</i> I	TAGCGAATTGGTCGGAAAAG	pSRPP18/ <i>visN</i>
visN-low <i>Bam</i> HI	GTGCGTCCGACCGGACATTT	pSRPP18/ <i>visN</i>
expR-up <i>Hind</i> III	ACACAAGCTTCTTCTGAACGGCGTATTACACA	<i>expR</i> restoration
expR-low <i>Eco</i> RI	TGATGAATTCCATTCCGTCGGCAGATAGT	<i>expR</i> restoration
sinR-E-fwdII	GCGAATTCCTGCCGCTGCATCACACTT	<i>sinI</i> disruption
sinI-B-revII	CGCGGATCCTAGATTTGTGGGGGAATCTG	<i>sinI</i> disruption
sinI-B-fwdII	ATAGGATCCACTGCGTAAGCGCGTGTTC	<i>sinI</i> disruption
sinI-H-rev	CGCCAAGCTGTGTAATGAGGAGCGCGCCACC	<i>sinI</i> disruption
visN-E-fwd	CGGAATTCATCGCCGGATGCCGTTAATA	<i>visN</i> disruption
visN-B-rev	AATAGGATCCACTGCAGAGGCGCGAACGATGTCGAA	<i>visN</i> disruption
visN-B-fwd	CTGCAGTGGATCCTATTGCGCCGTGACCTTCAG	<i>visN</i> disruption
visN- <i>Hind</i> III-rev	CGCCAAGCTTATTGTGTGAGGACGCGAGGTGAG	<i>visN</i> disruption
visN-ExpR- binding oligo	AATCGCGCAACGGGACGCCCTGTCTTTTGGGGG	pCR <i>visN</i> -oligo
Cy3-pUC18 -48	[Cy3]AGCGGATAACAATTTACACAGGA	EMSA <i>visN</i> -oligo
pUC18 -40	GTTTCCCAGTCACGAC	EMSA <i>visN</i> -oligo
rem-up121	TGGCGACAATGCCGACAGAT	EMSA dP <i>rem</i>
rem-low121_Cy3	[Cy3]TCGGTGGCGATTGTGGCTAT	EMSA dP <i>rem</i>
rem_proximal	GGC CAA ATC GGC ACG CAA TTC	EMSA pP <i>rem</i>
rem_full- length_CY3	[Cy3]TAGCCGTCTTCAACAAGAGCT	EMSA pP <i>rem</i>

^a EMSA = electrophoresis mobility shift assay, dP = distal promoter, and pP = proximal fragment.

EMSA.

EMSA were performed similarly as previously reported (Baumgarth et al. 2005), but with some minor modifications. Cy3-labeled DNA fragments representing the *rem* promoter were PCR amplified from the Rm2011 genome using the primers *rem-up121/rem-low121_Cy3* for the distal fragment (dP), and the primers *rem_proximal/rem_full-length_Cy3* for the proximal one (pP). For EMSA with MucR, 0.2 µg of Flag-MucR or H92AFlag-MucR was mixed with 5 ng of Cy3-labeled DNA in binding buffer (50 mM Tris HCl, pH 8.0; 100 mM NaCl, 0.1 mM MgSO₄, 4.5% glycerol, sonicated herring testes DNA at 0.05 mg ml⁻¹, and bovine serum albumin at 0.5 mg ml⁻¹) in a final volume of 20 µl and incubated at 20°C for 15 min. The mixture was then loaded on a 2% agarose gel and subjected to electrophoresis (4.5 V cm⁻²) for 3 h at 4°C. For EMSA with (his)₆ExpR, the *visN* promoter region was amplified and Cy3 labeled using the primer *visN-up KpnI* with a Cy3 label and *visN-low BamHI* (Table 3). The DNA fragment from the pCRvisN-oligo construct was amplified and Cy3 labeled using the pUC18 universal sequencing primers (Table 3). The Cy3-labeled DNA fragments were mixed with purified (his)₆ExpR in a reaction buffer containing sonicated herring testes DNA at approximately 0.1 mg ml⁻¹ and bovine serum albumin at 1.0 mg ml⁻¹ in a final volume of 20 µl of DNA binding buffer (20 mM Tris-HCL, pH 8.0; 5 mM KCl). Cy3-labeled DNA was included at 0.05 pmol, (his)₆ExpR at 3.0 pmol, and oxo-C14-HL at 20 pmol. The reactions were incubated at 22°C for 20 min. Loading buffer (5 µl, 78% glycerol) was added, and the reaction mixtures were loaded onto a 2% agarose gel. Following electrophoresis (5 V cm⁻²) for 2 h at 22°C, gel images were acquired using a Typhoon 8600 variable mode imager (Amersham Bioscience, Freiburg, Germany).

β-Galactosidase assays of *S. meliloti* strains carrying the integrated promoter probe vector pSRPP18 with *rem* or *visN* fragment inserts.

S. meliloti strains were grown in TY to stationary phase and diluted to an optical density of 0.03 (600 nm) in RB medium, supplemented with 2% TY and 0.2% mannitol. The assays for β-galactosidase activity were performed as previously described (Bahlawane et al. 2008; Miller 1972).

Real-time PCR analysis.

For RT-PCR analysis, cells were grown for 2 days in TY, diluted with RB to an optical density of 0.03 (600 nm) in 15 ml, loaded onto Bromfield Agar plates, and incubated on a slowly rotating platform at 30°C as described (Rotter et al. 2006). Cells were harvested with a centrifuge step and snap-frozen in liquid nitrogen, and the RNA was isolated using the RNeasy kit from Qiagen (Hilden, Germany). RT-PCR assays were performed using the Sensimix one-step RT-PCR kit from Quantace (Berlin, Germany). The gene *SMc00128* was used as a loading control. The primer sequences corresponding to the various monitored genes are listed in Table 3. For each reaction (25 µl), 100 ng of RNA was included. The experiments were performed with the Opticon real time-PCR detection system (Bio-rad, Munich, Germany) and included the following steps: i) a reverse transcription of RNA into cDNA at 50°C for 30 min; ii) a 95°C incubation of cDNA for 15 min for “hot start” polymerase activation; iii) a series of incubations at 95°C for 20 s, 58°C for 30 s, and 72°C for 40 s, repeated 40 times with a measurement of product levels at the end of the elongation step; and iv) the inclusion of a melting gradient from 60 to 90°C. The change in gene expression was calculated as 2^{-ΔCT}, where C_T indicates the level of gene expression in the specified strain relative to the expression of the *SMc00128* control gene.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Bioinformatics Resource Facility EMMA software:
www.cebitec.uni-bielefeld.de/groups/brf/software/emma_info