The *Sinorhizobium meliloti* EmrR Regulator Is Required for Efficient Colonization of *Medicago sativa* Root Nodules

Mário R. Santos,¹ Andreia T. Marques,¹ Jörg D. Becker,² and Leonilde M. Moreira^{1,3}

¹IBB-Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico (IST), Lisbon, Portugal; ²Instituto Gulbenkian de Ciência, Rua da Quinta Grande No. 6, 2780-156 Oeiras, Portugal; ³Department of Bioengineering, IST, University of Lisbon, Av. Rovisco Pais, 1049-001 Lisbon, Portugal

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The nitrogen-fixing bacterium *Sinorhizobium meliloti* must adapt to diverse conditions encountered during its symbiosis with leguminous plants. We characterized a new symbiotically relevant gene, *emrR* (*SMc03169*), whose product belongs to the TetR family of repressors and is divergently transcribed from *emrAB* genes encoding a putative major facilitator superfamily-type efflux pump. An *emrR* deletion mutant produced more succinoglycan, displayed increased cell-wall permeability, and exhibited higher tolerance to heat shock. It also showed lower tolerance to acidic conditions, a reduced production of siderophores, and lower motility and biofilm formation. The simultaneous deletion of *emrA* and *emrR* genes restored the mentioned traits to the wild-type phenotype, except for survival under heat shock, which was lower than that displayed by the wild-type strain. Furthermore, the Δ *emrR* mutant as well as the double Δ *emrAR* mutant was impaired in symbiosis with *Medicago sativa*; it formed fewer nodules and competed poorly with the wild-type strain for nodule colonization. Expression profiling of the Δ *emrR* mutant showed decreased expression of genes involved in Nod-factor and rhizobactin biosynthesis and in stress responses. Expression of genes directing the biosynthesis of succinoglycan and other polysaccharides were increased. EmrR may therefore be involved in a regulatory network targeting membrane and cell wall modifications in preparation for colonization of root hairs during symbiosis.

Sinorhizobium meliloti 1021 interacts with legume roots inducing the formation of nodules, within which this bacterium fixes nitrogen to ammonia that becomes available to the plant. The *S. meliloti* TolC protein is required to establish a successful nitrogen-fixing symbiosis with *Medicago* plants and is necessary for protein and exopolysaccharide secretion and protection against osmotic and oxidative stresses. TolC interacts with membrane transporters to control cellular import and export of various compounds (Cosme et al. 2008). Comparison

of the transcriptomes of *S. meliloti* 1021 and the *tolC* mutant confirmed that the absence of functional TolC protein compromises cell homeostasis as reflected by the simultaneous increase in expression of many genes involved in cytoplasmic and extra-cytoplasmic stress responses (Santos et al. 2010). In the same transcriptomic study, *SMc03167*, *SMc03168*, and *SMc03169* genes were found to be significantly upregulated in the *tolC* mutant. The two first encode a putative major facilitator superfamily (MFS)-multidrug resistance (MDR) efflux pump, and gene *SMc03169* encodes a putative transcriptional repressor of the TetR family. Other expression profiling analyses also showed that genes *SMc03167*, *SMc03168*, and *SMc03169* were inducible by the presence of the flavonoid luteolin (Capela et al. 2005) under acidic pH (Hellweg et al. 2009) and under heat shock (Barnett et al. 2012), suggesting their involvement in response to stress.

Despite the importance of MDR efflux pumps in the active extrusion of toxic compounds from cells, their role is relatively unknown in plant symbiotic bacteria such as rhizobia. The flavonoid-inducible MFS-type multidrug efflux pump RmrAB from *Rhizobium etli* was shown to be required for nodulation of *Phaseolus vulgaris* and resistance toward phytoalexins, flavonoids, and salicylic acid (González-Pasayo and Martínez-Romero 2000). In *Bradyrhizobium japonicum*, loss of the resistance-nodulation-cell division (RND)-type RagCD pump had no effect on the sensitivity to antimicrobial compounds or nodulation of soybean roots (Krummenacher and Narberhaus 2000), but another RND-type efflux pump, BdeAB, although not required for nodule formation, was essential for effective symbiotic nitrogen fixation in soybean nodules (Lindemann et al. 2010). In *S. meliloti*, a study involving the systematic deletion of genes encoding MFS-type and RND-type proteins resulted in the identification of the SmeAB RND-type pump as being important in nodulation competitiveness and in mediating resistance towards several antimicrobials, including plant-derived compounds (Eda et al. 2011).

Deletion of the putative MFS-type transporter encoded by *S. meliloti* 1021 *SMc03167* and *SMc03168* genes did not affect either the sensitivity to several toxic compounds or the symbiotic properties of the bacteria (Capela et al. 2005; Eda et al. 2011). Nevertheless, the comparison of the transcriptome of *S. meliloti* 1021 in the presence or absence of the flavonoid luteolin, showed that the expression of genes *SMc03167* and *SMc03168* as well as the divergently transcribed gene *SMc03169*, encoding a putative regulator of the TetR family, was highly induced (Capela et al. 2005). Similar induction of gene expression by flavonoids was observed for other MFS-type encoding genes relevant for the interaction of bacteria such as *Erwinia amylovora* (Burse et al. 2004) and *Agrobacte-*

Microarray data were deposited in the Gene Expression Omnibus (GEO) repository at National Center for Biotechnology Information under accession number GSE44607.

Corresponding author: L. M. Moreira; Telephone: (+351)218419031; Fax: (+351)218419199; E-mail: lmoreira@ist.utl.pt

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rium tumefaciens (Palumbo et al. 1998) with host cells. Recently, the expression of genes *freC*, *freA*, and *freB* encoding a putative RND-type efflux pump of *B. japonicum* was shown to be dependent on the flavonoid responsive transcriptional repressor FrrA of the TetR-family (Wenzel et al. 2012). Nevertheless, the deletion of *frrA* or *freC* genes had no effect on nodule number and plant appearance. As TetR-type regulators are encoded next to the genes encoding the efflux pump and some are flavonoid-responsive (Burse et al. 2004; González-Pasayo and Martínez-Romero 2000; Palumbo et al. 1998; Wenzel et al. 2012), we have investigated the role of the TetR regulator-encoding gene *SMc03169* (*emrR*) of *S. meliloti* in symbiosis and other phenotypic traits including adaptation to stress conditions, exopolysaccharide and siderophore production, biofilm formation, and motility.

RESULTS

Genomic location of *SMc03167* to *SMc03169* genes from *S. meliloti*.

In *S. meliloti* 1021, genes with the locus tag *SMc03167* and *SMc03168* encode a putative efflux system of the MFS, with *SMc03168* having the characteristics of a membrane fusion protein and *SMc03167* the MFS-type transporter protein. Due to their similarity to the EmrA and EmrB efflux pump of *Escherichia coli*, this designation was kept (Fig. 1A), with *SMc03168* and *SMc03167* being designated *emrA* and *emrB*, respectively. Located about 300 bp downstream of the *SMc03168* coding region and in opposite orientation is gene *SMc03169* (Fig. 1A). *SMc03169* encodes a putative protein with 236 amino acids that shares an N-terminal sequence and structural similarities to members of the TetR family of transcriptional repressors. In conformity with the *E. coli* repressor controlling the expression of *emrA* and *emrB* genes (Lomovskaya et al. 1995), this gene was named *emrR*. The search for conserved regions showed the DNA-binding helix-turn-helix motif in the N-terminal (Fig. 1B). The predicted ligand-binding domain is located within the C-terminal region, although, due to the diverse nature of this region, no conservation at the amino acid level was observed. As previous expression profiling showed that genes *SMc03167*, *SMc03168*, and *SMc03169* were inducible by luteolin and under

several stress conditions, our assumption was that these gene products might be important for *S. meliloti* adaptation to changing environments with possible influence on symbiosis.

Phenotypes of $\Delta emrB$, $\Delta emrR$, and $\Delta emrAR$ deletion mutant strains.

S. meliloti 1021 deletion of *emrB*, *emrR* or *emrAR* genes was performed as described below. The deletion mutant of the *emrB* gene (Sm1021-7) showed similar growth behavior as wild-type Sm1021 in rich tryptone yeast (TY) and minimal glutamate-mannitol salts (GMS) media (data not shown). Antimicrobial susceptibility tests against an antibiotic (novobiocin), a dye (ethidium bromide), detergents (sodium dodecyl sulfate [SDS], deoxycholate [DOC]), oxidative stress agents (H_2O_2 , cumene hydroperoxide), and plant-derived compounds (berberine, *p*-coumaric acid, genistein) showed no difference in sensitivity between the $\Delta emrB$ mutant and the wild-type Sm1021 (data not shown). Similarly, *Medicago sativa* seedlings inoculated with the wild-type Sm1021 and $\Delta emrB$ mutant strains gave rise to a slightly lower nodule number in the presence of the deletion mutant, but plant dry weights were similar. Our results are in line with those obtained for the simultaneous deletion of *SMc03167* and *SMc03168* genes (Eda et al. 2011) or the *SMc03167* gene only (Capela et al. 2005), which could not identify one or more substrates of this putative efflux pump or attribute a role in symbiosis.

The deletion mutant for the *emrR* gene (Sm1021-9) showed a specific growth rate similar to the one of the wild-type Sm1021 in complex TY medium (data not shown) and in minimal GMS (pH 7.0) medium (Fig. 2A). Nevertheless, when growing in this last medium, it showed higher growth deceleration after 24 h than the wild-type Sm1021, as indicated by optical density measurement (Fig. 2A). Determination of colony forming units (CFU) under those conditions confirmed the decreased growth of the $\Delta emrR$ mutant when entering the stationary phase by comparison with the wild-type strain (data not shown). As previous expression profiling data in *S. meliloti* showed that gene *emrR* (*SMc03169*) was responsive to an acidic pH shift (Hellweg et al. 2009), we measured the external pH during growth in GMS medium. It was observed that, between 24 and 48 h of growth, the $\Delta emrR$ mutant acidified

Fig. 1. A, Physical organization of *Sinorhizobium meliloti* 1021 *SMc03167* (*emrB*) and *SMc03168* (*emrA*) genes encoding a putative major facilitator superfamily-type transporter and *SMc03169* (*emrR*) encoding a putative TetR transcriptional regulator. The flanking genes are *SMc03170*, encoding a putative transcriptional regulator of the AraC family, and *SMc03166*, encoding a putative metallophosphoesterase. N = nucleotides. **B**, Partial multiple sequence alignment of helix-turn-helix (HTH) DNA-binding motif present in TetR-type regulators. The accession numbers of the proteins are *S. meliloti* 1021 (NP_387006.1), *Bradyrhizobium japonicum* USDA 110 (NP_770962.1), *Methylosinus trichosporium* OB3b (EFH05139.1), *Azospirillum* sp. strain B510 (YP_003450612), *Xanthobacte autotrophicus* Py2 (YP_001416416), *Anaeromyxobacter* sp. strain FW109-5 (YP_001377768), *Mesorhizobium loti* MAFF303099 (3BHQ), *Pseudomonas syringae* pv. *tomato* Dc3000 (3CDL). Asterisks indicate the amino acid residues that are identical in all the proteins; one or two dots indicate semiconserved or conserved substitutions, respectively.

the medium by 1 pH unit, while only a slight decrease was observed for the wild-type strain (Fig. 2A). To evaluate whether buffering the medium would prevent the acidification and the growth defect of the $\Delta emrR$ mutant, strains were grown in morpholinepropanesulfonic acid (MOPS) buffered medium (pH 7.0). Results illustrated in Figure 2B showed the same growth behavior as well as the same increase in the medium pH for both strains.

For complementation analysis, plasmid pMAS17 harboring the complete *emrR* gene under the control of its own promoter region in the broad-host range vector pPHU231 was constructed. In trans complementation of the $\Delta emrR$ mutant with pMAS17 restored growth in GMS medium as well as the pH profile of the culture medium to the wild-type level (Fig. 2A).

To determine if the $\Delta emrR$ mutant was more sensitive to acidic pH, cells were exposed to an acid shift and growth behavior was monitored. At pH 5.0 and below, none of the strains were growing. At pH 5.5, the $\Delta emrR$ mutant grew more poorly than Sm1021, although it still exhibited considerable growth and this phenotypic defect could be complemented by expressing the *emrR* gene from pMAS17 (Fig. 2C). pH measurement of the culture supernatant during a time period of 4 days showed a slight increase for the $\Delta emrR$ mutant, while the Sm1021 wild-type and the complemented mutant were able to increase the pH value by approximately 1 unit (Fig. 2C). When the medium was buffered with morpholineethanesulfonic acid (pH 5.5), there was no growth difference between the $\Delta emrR$

mutant and the wild-type strain, with both strains growing poorly (data not shown).

The previous GMS culture medium acidification by the $\Delta emrR$ mutant suggests that this strain secretes one or more products of an acidic nature. To test whether this phenotype was dependent on the presence of the EmrAB MFS transporter, a double mutant $\Delta emrAR$ was tested for growth in the different media and for external pH variation. The double mutant behaved as the wild-type strain under all tested growth conditions (data not shown), suggesting that the negative effect on growth and medium acidification caused by the absence of EmrR was suppressed by additional inactivation of the MFS-transporter encoding gene. It is important to say that the $\Delta emrAR$ double mutant is effectively a triple mutant, since the *emrB* gene cannot be expressed due to the absence of the *emrAB* promoter region. Nevertheless, it will be referred to in this text as a double mutant, since only the region spanning the beginning of *emrA* until the end of *emrR* was deleted.

Loss of *emrR* function strongly decreases competitive nodulation ability of *S. meliloti*.

To examine the symbiotic phenotype of the $\Delta emrR$ deletion mutant, plant inoculation experiments were performed in agar plates on the host plant *Medicago sativa*. After 4 weeks, plants inoculated with the wild-type Sm1021 were healthy, with an average number of approximately four nodules per plant, whereas the plants inoculated with the $\Delta emrR$ mutant were

Fig. 2. Growth curves of *Sinorhizobium meliloti* strains as estimated by optical density at 600 nm during a 96-h period at 30°C (open symbols) and fluctuation of the pH culture supernatant (filled symbols) of cells growing in **A**, glutamate-mannitol salts (GMS) medium at pH 7, **B**, morpholinepropanesulfonic acid medium at pH 7, and **C**, GMS medium at pH 5.5. *S. meliloti* 1021/pPHU231 (■, □); $\Delta emrR$ /pPHU231 (▲, △); and $\Delta emrR$ /pMAS17 (●, ○). Standard deviation of the growth curves is below 5%. pH values are the mean $\pm$ standard deviation of at least three independent experiments.

Fig. 3. **A**, Rate of root nodules induced by strains Sm1021/pPHU231 (■), $\Delta emrR$ /pPHU231 (▲), $\Delta emrAR$ /pPHU231 (◇), and $\Delta emrR$ complemented in trans with plasmid pMAS17 expressing the *emrR* gene (●). **B**, Plant dry weight determined 4 weeks postinoculation with the indicated bacteria. **C**, Competition for nodule occupancy between the wild-type strain Sm1021 (white bars) and the $\Delta emrR$ mutant or the $\Delta emrAR$ double mutant (gray bars). Values are the mean $\pm$ standard deviation of at least three independent experiments. A *P* value < 0.05 was considered significant compared with Sm1021/pPHU231 (*).

smaller with an average number of two nodules per plant (Fig. 3A). Plant dry-weight determination confirmed a reduction of growth by approximately 25% when inoculated with the $\Delta emrR$ mutant (Fig. 3B). Nevertheless, this value is 30% higher than the uninoculated plants, implying that the $\Delta emrR$ mutant is Fix⁺. The symbiotic defective phenotype of $\Delta emrR$ mutant was complemented by the introduction of pMAS17. Pink nodule crush from plants inoculated with the $\Delta emrR$ mutant followed by bacterial plating in TY media confirmed that these nodules were colonized by mutant cells in numbers that did not differ significantly from wild-type levels (data not shown). The symbiotic phenotype of the $\Delta emrAR$ mutant was similar to the one of the $\Delta emrR$ mutant, both for induced nodule number and plant size (Figs. 3A and B).

Due to the lower amount of nodules induced by the $\Delta emrR$ and the $\Delta emrAR$ mutant strains, we performed competitive nodulation assays. For that, the wild-type strain and the mutant $\Delta emrR$ or $\Delta emrAR$ were combined at a 1:9, 1:1, and 9:1 ratio and were then applied onto *M. sativa* roots. Four weeks after inoculation, bacteria were re-isolated from nodules, and more

than 85% of the analyzed colonies corresponded to the wild-type, independently of the proportion tested (Fig. 3C). These results suggest that the absence of the *emrR* gene in both mutants interferes with their ability to establish a full symbiosis.

Deletion of *emrR* has consequences in motility, biofilm formation, and adhesion to roots.

To evaluate if the lower competitive ability of the $\Delta emrR$ and $\Delta emrAR$ mutants is caused by some delay in the early steps of symbiotic establishment, we quantified motility, biofilm formation, and root-hair attachment. To mimic symbiotic conditions, motility and biofilm formation were tested in the presence of luteolin, known to induce the expression of the *emrR* gene (Capela et al. 2005). As regards swimming motility, the $\Delta emrR$ mutant showed a slightly lower motility than the wild-type strain Sm1021, restored by the introduction of plasmid pMAS7 (Fig. 4A). The double mutant $\Delta emrAR$ was as motile as the wild-type strain. Due to the shape of colony ramifications, it was not possible to quantify swarming motility. Nevertheless, a different colony shape of $\Delta emrR$ mutant was

Fig. 4. Motility and adhesion properties of *Sinorhizobium meliloti* 1021 $\Delta emrR$ mutant. **A**, Swimming motility measured by the colony diameter obtained after 3 days of incubation in 0.3% purified agar glutamate-mannitol salts (GMS) medium supplemented with luteolin at 30°C; **B**, Swarming motility obtained after 48 h of incubation in 0.6% purified agar GMS medium supplemented with luteolin at 30°C; **C**, Biofilm formation measured by crystal violet staining assay using static broth cultures. Bacteria were cultured in test tubes with Vincent medium supplemented with luteolin for 72 h at 30°C. An image of the biofilm formed at the liquid and air interface before crystal violet solubilization is also shown. **D**, Root-hair attachment by bacteria as determined by CFU count after 8 h of incubation. The percentage of attachment for the $\Delta emrR$ mutant and complemented mutant are relative to the wild-type strain, considered 100%. Values are the mean $\pm$ standard deviation of at least three independent experiments. A *P* value < 0.05 was considered significant compared with Sm1021/pPHU231 (*).

observed in comparison with the wild-type Sm1021, the complemented mutant, and the $\Delta emrR$ deletion mutant (Fig. 4B). We then measured biofilm formation in glass test tubes containing 3 ml of Vincent medium supplemented with 10 μ M luteolin and incubated at 30°C for 3 days. After removal of the liquid culture and the washing steps, the biofilm formed at the liquid and air interface (Fig. 4C) was quantified. Data obtained indicated that the final biomass was reduced in the $\Delta emrR$ mutant in comparison with the wild-type strain and the $\Delta emrR$ -complemented mutant (Fig. 4C). This difference in biofilm formation of the $\Delta emrR$ mutant may be indicative of different attachment to surfaces. Therefore, we analyzed the attachment of $\Delta emrR$ mutant to roots using seedlings of the host plant *M. sativa* by co-incubating bacteria and roots in nitrogen-free medium as previously described (Rolfe et al. 1980) for 8 h. After this time, seedlings were washed to remove unbound bacteria and were vortexed to release attached bacteria into solution, followed by plating into TY solid medium. A CFU count indicated a reduction of about 50% in the number of attached bacteria of the $\Delta emrR$ mutant when compared with the wild-type strain (Fig. 4D). Complementation of the mutant with the *emrR* gene from pMAS17 restored adhesion to levels close to the wild-type Sm1021 strain. To exclude a negative effect of the nitrogen-free medium in cell viability and root attachment, CFU of the $\Delta emrR$ mutant and wild-type strain at the beginning and after 8 h of incubation without a root were determined. During the tested period no significant differences were obtained in colony number of each strain (data not shown), suggesting that the reduced adhesion of $\Delta emrR$ mutant to roots is not caused by loss of cell viability.

Identification of altered pathways in the $\Delta emrR$ mutant.

Due to the symbiotic impairment of the $\Delta emrR$, we performed a transcriptional analysis of three biological replicates of wild-type *S. meliloti* 1021 and the $\Delta emrR$ deletion mutant grown in GMS medium supplemented with 10 μ M luteolin for 4 h. Comparison of the transcriptome of the $\Delta emrR$ deletion mutant with the one of the wild-type strain using a lower confidence bound fold change cutoff of 1.2 resulted in 285 genes differentially expressed, with a false discovery rate (FDR) of $\leq 0.9\%$, in which 172 genes showed increased levels of expression and 113 genes showed decreased levels of expression (Supplementary Table S1). Replicon distribution of the 285 *emrR*-dependent differentially expressed genes indicated that 59% were chromosomal, 22% from pSymB, and 19% from pSymA, which corresponds to approximately 5% of the genes

present in each replicon. The distribution of genes by functional groups showed a significant decrease in the expression of genes related to symbiotic signaling, stress response, and inorganic ion transport and metabolism. In contrast, the expression level of genes encoding transcriptional regulators, energy conversion, and metabolism as well as cell envelope biogenesis was increased in the $\Delta emrR$ mutant transcriptome. As a validation of the microarray data, the expression of several genes was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Table 1). The expression values are in good agreement with the microarray data.

Genes with increased expression in the $\Delta emrR$ deletion mutant.

Genes with the highest value of fold change are located in the vicinity of gene *emrR*. These include downstream the putative AraC family transcription regulator *SMc03170* (3.4-fold) and upstream the genes *emrA* and *emrB* (3.8- and 3.6-fold, respectively), and the putative metallophosphoesterase encoding gene *SMc03166*, with an increased expression of 7.3-fold in the $\Delta emrR$ mutant when compared with the wild-type Sm1021 strain. Most likely the expression of those genes is repressed when protein EmrR is present and, therefore, the putative promoter regions of *SMc03170*, *emrA*, and *SMc03166* genes are good targets for the direct binding of EmrR protein.

Another gene with increased expression in the $\Delta emrR$ mutant encodes the transcriptional regulator ChvI (1.6-fold). This regulator is known to be involved in polysaccharide biosynthesis, motility, and metabolism (Bélanger et al. 2009; Chen et al. 2009; Wang et al. 2010), and many of the genes identified as ChvI targets (Chen et al. 2009) showed increased expression in our microarray data set. These include all the *exo* genes involved in succinoglycan biosynthesis (Reuber and Walker 1993; Reuber et al. 1991), the *msbA2* operon (*SMb21188* to *SMb21191*) also involved in polysaccharide biosynthesis and symbiosis (Beck et al. 2008; Bélanger and Charles 2013), the *sinI* gene encoding the *N*-acyl-L-homoserine lactone synthetase, the *ropB1* gene encoding an outer membrane protein that influences phage sensitivity of *S. meliloti* (Campbell et al. 2003), and several other genes of unknown function, such as *SMc01580* and *SMb21440*, whose intergenic upstream regions were demonstrated as direct binding sites of the ChvI regulator (Chen et al. 2009). To determine whether the increased expression of the *exo* genes had influence on the amount of succinoglycan produced, the wild-type Sm1021, the $\Delta emrR$ and $\Delta emrAR$ deletion mutants, and the complemented $\Delta emrR$ mutant strain were grown for 10 days in GMS medium supplemented with luteolin, followed by cell-free supernatant precipitation. The results shown in Figure 5 confirmed that, in the $\Delta emrR$ mutant, the amount of total carbohydrates present in the cetyl pyridinium chloride (CPC)-precipitated supernatant was much higher than the one for the wild-type and complemented mutant. The double mutant $\Delta emrAR$ produced similar amount of carbohydrates as the wild-type strain (Fig. 5). To confirm the nature of this polysaccharide as being succinoglycan, an *exoY* mutation was transduced to Sm1021, $\Delta emrR$ mutant, and $\Delta emrAR$ double mutant. These Sm1021 *exoY-lacZ/aacC1*, $\Delta emrR$ *exoY-lacZ/aacC1*, and $\Delta emrAR$ *exoY-lacZ/aacC1* CPC-precipitated culture supernatants showed a very low amount of total carbohydrates (less than 2 mg of glucose equivalents per liter after 10 days of growth), confirming the absence of succinoglycan (Fig. 5).

Genes with decreased expression in the $\Delta emrR$ deletion mutant.

Among the genes displaying decreased expression in the $\Delta emrR$ deletion mutant when compared with the Sm1021

Table 1. Quantitative real-time reverse transcription-polymerase chain reaction analysis performed in *Sinorhizobium meliloti* 1021 and $\Delta emrR$ mutant cells

Gene identifier (gene name)	LB-FC ^a	Real-time fold change ^b
SMa0869 (<i>nodA</i>)	-1.6	-1.7 $\pm$ 0.5
SMa1442	1.7	7.5 $\pm$ 1.6
SMa2402 (<i>rhhB</i>)	-1.3	-1.5 $\pm$ 0.2
SMb20198 (<i>rbcL</i>)	1.4	4.1 $\pm$ 1.2
SMb20961 (<i>exoP</i>)	1.9	2.7 $\pm$ 1.3
SMb21174 (<i>phoT</i>)	-2.6	-4.6 $\pm$ 2.4
SMb21191 (<i>msbA2</i>)	1.6	2.9 $\pm$ 1.3
SMc00168 (<i>sinI</i>)	1.2	2.5 $\pm$ 1.0
SMc00646 (<i>rpoH1</i>)	-1.3	-1.5 $\pm$ 0.3
SMc00658	1.6	4.1 $\pm$ 0.8
SMc00913 (<i>groEL1</i>)	-1.4	-1.5 $\pm$ 0.3
SMc01944 (<i>cpo</i>)	-1.3	-1.6 $\pm$ 0.3
SMc02560 (<i>chvI</i>)	1.6	4.5 $\pm$ 2.3
SMc03170	3.4	5.4 $\pm$ 2.1
SMc04267 (<i>lpsS</i>)	1.6	4.1 $\pm$ 1.7

^a Microarray lower bound of fold change.

^b Fold change $\pm$ standard deviation.

wild-type strain are sixteen *nod* and *noe* genes involved in Nod factor biosynthesis. None of these genes encodes a transcriptional regulator of the NodD family whose activity positively controls *nod*, *nol*, and *noe* gene expression, and the process by which protein EmrR affects *nod* and *noe* gene expression is unknown. Nevertheless, the comparison of the differentially expressed genes between the data sets $\Delta emrR$ mutant vs. wild-type and *nodD1* mutant vs. wild-type (Capela et al. 2005) obtained in the presence of luteolin showed 31 genes in common. The common genes with decreased expression were the *nol*, *nod*, *groEL5*, and *ibpA* genes and the ones with increased expression were *Sma1809* and *Sma1821*, encoding a nonheme haloperoxidase and a hydrolase, respectively, *SMc02446*, encoding a putative pilus assembly protein, and *edd*, encoding a phopophogluconate dehydratase.

Another set of genes with differential expression are the ones involved in iron and phosphate acquisition. Under iron limitation, *S. meliloti* produces the siderophore rhizobactin (Lynch et al. 2001). Here, we observed that the genes *rhbB*, *rhbC*, *rhbD*, *rhbE*, *rhbF*, *rhtA*, and *Sma2339* had a decreased expression in the $\Delta emrR$ mutant strain. To assess whether the decreased expression of those genes had a negative effect on the $\Delta emrR$ mutant growth under iron limitation, two different assays were performed. In one of them, Sm1021 wild-type, the $\Delta emrR$ mutant, and the complemented mutant, grown overnight in GMS medium, were spot-inoculated onto GMS-chrome azurol S (CAS) medium and were incubated for 3 days. Under these conditions, the wild-type strain and complemented mutant formed a colony with an orange halo due to siderophore diffusion and iron chelation, while the $\Delta emrR$ mutant strain was unable to grow (Fig. 6A). The $\Delta emrAR$ mutant showed a siderophore production identical to the wild-type strain (data not shown). In the second approach, cells were grown in GMS supplemented with 175 μ M of the iron chelating agent dipyriddy. As seen in Figure 6B, there was a negative effect on the growth of the $\Delta emrR$ deletion mutant, as this strain most likely produced a lower amount of siderophores, while Sm1021 and the complemented mutant presented higher growth rate.

Genes showing the greatest decrease in expression encode the high-affinity phosphate uptake ABC transporter PhoCDET (2.3 to 3.0-fold). To determine whether the $\Delta emrR$ deletion

Fig. 5. Exopolysaccharide production assessed by measuring the total carbohydrates content of the cetyl pyridinium chloride-precipitate obtained from growing *Sinorhizobium meliloti* 1021/pPHU231 (□), the $\Delta emrR$ /pPHU231 mutant (△), the complemented mutant $\Delta emrR$ /pMAS17 (○), the $\Delta emrAR$ double mutant (◇), and the $\Delta emrR$ *exoY*/pPHU231 mutant (*) in GMS medium containing 10 μ M luteolin at 30°C for 10 days. Values are the mean $\pm$ standard deviation of at least three independent experiments. A *P* value < 0.05 was considered significant compared with Sm1021/pPHU231 (*).

mutant had a growth defect under phosphate limitation, it was grown in GMS medium without addition of phosphate or supplemented with 0.05 and 1.0 mM. Results showed no difference between the $\Delta emrR$ mutant and the wild-type strain for each of the tested phosphate concentrations (data not shown).

Another gene with a decreased level of expression was *rpoH1*, encoding the alternative sigma factor RpoH1. The comparison between our expression data and those obtained for the *S. meliloti* 1021 *rpoH1* deletion mutant under acidic pH stress (de Lucena et al. 2010) and heat-shock (Barnett et al. 2012) showed seven genes in common. Genes with increased expression encode a D-3-phosphoglycerate dehydrogenase, SerA, a putative membrane-bound lytic transglycosylase, SMc01855, and the beta subunit of glutamate synthase GltD. Genes with decreased expression were *SMb21295* and *SMb21566*, encoding a Hsp20 family heat-shock protein and a GroEL5 chaperonin, respectively, *SMc00591*, encoding a hypothetical protein, and *ibpA*, encoding another heat-shock protein. Despite this limited overlap between the three datasets, we have shown the increased sensitivity of the $\Delta emrR$ mutant under acidic condition (Fig. 2). To expand this analysis, we also tested heat and osmotic shocks and oxidative stress. Heat-shock response was tested by incubating exponential growth-phase cultures (grown for 4 h in GMS medium supplemented with 10 μ M luteolin) for 15 and 30 min at 42 and 30°C, followed by CFU determination. Viability at 42°C was compared with 30°C, a non-heat shock temperature used as control. Results showed an increased survival at 42°C of the $\Delta emrR$ mutant at both time points when compared with the wild-type strain (Fig. 7A). In contrast, the double $\Delta emrAR$ mutant showed the lowest

Fig. 6. A, Siderophore production by *Sinorhizobium meliloti* strains assayed in glutamate-mannitol salts (GMS)-chrome azurol S agar plates spotted with 10 μ l of bacteria and incubated at 30°C for 3 days. B, Growth curve of Sm1021/pPHU231 (■), $\Delta emrR$ /pPHU231 (▲), and $\Delta emrR$ mutant complemented in trans with plasmid pMAS17 expressing the *emrR* gene (●) in GMS medium supplemented with 175 μ M of dipyriddy as estimated by optical density measurement at 30°C. Values are the mean $\pm$ standard deviation of at least three independent experiments.

survival ability under heat shock, followed by the complemented $\Delta emrR$ mutant (Fig. 7A).

Osmotic shock was tested by adding 0.3 M of NaCl to the GMS medium used for growing strains, but no difference in growth behavior was observed (data not shown). Resistance against oxidative stress was tested in the presence of 1 M hydrogen peroxide. Results indicated that the $\Delta emrR$ mutant was slightly more sensitive to this compound than the other tested strains (Fig. 7B).

In addition, we also evaluated the sensitivity of the $\Delta emrR$ deletion mutant to malachite green and crystal violet, two lipophilic inhibitors of cell growth that have been used to screen for cell-wall perturbations, and the anionic detergent SDS. As seen in Figure 7B, cells deleted for the *emrR* gene showed higher sensitivity than the wild-type Sm1021 and the double $\Delta emrAR$ mutant to all the tested compounds, implying differences in membrane integrity of this mutant. The introduction of pMAS17 into the $\Delta emrR$ deletion mutant rescued the phenotype to wild-type levels. These results suggest that EmrR protein may play a role in maintaining membrane stability during stress conditions.

DISCUSSION

In the current study, we characterized the *emrR* gene encoding a putative transcriptional repressor of the TetR family and the neighbor genes *emrA* and *emrB*, encoding a putative MFS-type transporter. Deletion of the *emrB* gene had no significant impact in *S. meliloti* physiology, but the absence of the EmrR regulator caused alteration of cellular properties, such as motility, membrane permeability, siderophore production, adhesion to biotic and abiotic surfaces, polysaccharide production, acidic and heat shock responses, and competitive nodulation efficiency. Interestingly, the simultaneous deletion of *emrA* and *emrR* genes restored all the phenotypes to wild-type levels, except for the symbiotic properties and survival under heat-shock conditions. These observations suggest that most of the free-living $\Delta emrR$ phenotypes and microarray expression data are a pleiotropic consequence of aberrant expression of genes *emrA*, *emrB*, and *SMc03166*. The most frequent function performed by TetR family proteins is the regulation of efflux pumps and transporters involved in antibiotic resistance and tolerance to toxic chemicals, but some others participate in regulatory networks involved in metabolism homeostasis, osmotic stress, quorum sensing, biosynthesis of antibiotics, and pathogenicity

(Cuthbertson and Nodwell 2013; Ramos et al. 2005). One of the most studied MFS-type transporters is the *E. coli* AcrAB-TolC efflux transporter leading to a multiple antibiotic resistance phenotype (Okusu et al. 1996). The *acrA* and *acrB* genes form an operon whose transcription is regulated by the product of the divergently transcribed *acrR* gene encoding a transcriptional repressor of the TetR family (Ma et al. 1996). Similarly, *S. meliloti* *emrR* gene is transcribed in the opposite direction of genes *emrA* and *emrB* and these genes are targets for EmrR repression. In fact, the expression data confirmed that in the absence of EmrR, the genes *emrA* and *emrB* had increased expression, suggesting a possible repression in the presence of EmrR. Also, gene *SMc03166*, encoding a putative metallophosphoesterase, and *SMc03170*, encoding a putative regulator of the AraC family, showed increased expression. Thus, it is very likely that these genes act together. Perhaps, in the presence of the EmrR protein, the expression of *SMc03166*, *emrAB*, and *SMc03170* genes is repressed, but in its absence, they could be expressed directly or indirectly through activation by the *SMc03170* transcriptional regulator. This needs to be experimentally determined.

The expression of *S. meliloti* *emrAB* genes has been shown to increase under heat shock (Barnett et al. 2012), acidic pH shift (de Lucena et al. 2010), in the absence of a functional TolC protein (Santos et al. 2010), in the presence of luteolin (Barnett et al. 2004; Capela et al. 2005), and in mature nodules (Barnett et al. 2004), and it has been shown to decrease in the *exoR* mutant (Wells et al. 2007). As the above stresses have strong effect on the cell envelope, it is possible that *emrAB* is important in response to stresses acting on the cell envelope. In that sense, the increased expression of *emrAB* in the $\Delta emrR$ mutant should have a positive effect on the response to stresses such as low pH, osmotic stress, and heat shock. At least for heat-shock conditions, that positive effect on survival of the $\Delta emrR$ deletion mutant was observed. Additionally, the simultaneous deletion of *emrR* and *emrA* genes had a negative effect on survival, suggesting that the MFS transporter EmrAB is required under heat shock. This observation is further supported by the low survival rate of the complemented $\Delta emrR$ mutant. The overexpression of the EmrR repressor in this strain must decrease the expression levels of *emrAB* genes and, consequently, cells have increased deficiency in mounting a heat-shock response. The comparison between our expression data and those obtained for acidic pH (de Lucena et al. 2010) and heat shock (Barnett et al. 2012) have in common the increased expression of the *chvI* gene encoding

Fig. 7. Survival of *Sinorhizobium meliloti* strains under stress conditions. **A**, Cell viability at 42°C when compared to 30°C after 15 min (gray bars) and 30 min (white bars) of exposure as determined by CFU count. **B**, Sensitivity to stress-inducing agents (1% crystal violet [CV], 5% malachite green [MG], 10% sodium dodecyl sulfate [SDS], and 1 M H₂O₂) determined by measuring growth-inhibition halos after incubation of plates at 30°C for 3 days. Values are the mean $\pm$ standard deviation of at least three independent experiments. A *P* value < 0.05 was considered significant compared with Sm1021/pPHU231 (*).

the response regulator of *S. meliloti* ChvG(ExoS)/ChvI two-component regulatory system (Bélanger et al. 2009; Wang et al. 2010), the *exo* genes directing the biosynthesis of succinoglycan (Leigh and Lee 1988), *lpsS* gene encoding a sulfotransferase involved in LPS modification (Cronan and Keating 2004), and other genes with unknown function, such as *Smb21440* or *SMc02278*. Genes with decreased expression in the $\Delta emrR$ mutant, whose products are involved in rhizobactin biosynthesis (Lynch et al. 2001), sulfur assimilation (Abola et al. 1999), and synthesis of phosphorus-free membrane lipids (López-Lara et al. 2005), were common to the osmotic and heat-shock gene expression datasets (Barnett et al. 2012; de Lucena et al. 2010). From this analysis is evident that the $\Delta emrR$ mutant is under stress and the displayed phenotypes are most likely due to overexpression of the adjacent genes. It remains to be determined whether that stress is caused by extra copies of EmrAB proteins at the membrane or is caused by excessive hydrolysis of the SMc03166 substrate and secretion, possibly mediated by EmrAB transporter.

One of the most evident phenotypes displayed by the $\Delta emrR$ deletion mutant is overproduction of succinoglycan. Although speculative, that increase in succinoglycan could explain the decrease in biomass observed in GMS medium when cells enter stationary phase and perhaps the acidification of the growth medium. Overproduction of succinoglycan may imply an increase in nutrient uptake and in the precursor's formation, namely the activated nucleotide sugars and the acetyl, pyruvyl, and succinyl modifications. These needs are reflected in the expression data by the increased expression of the *exo* genes, the *dctA* gene encoding the C4-dicarboxylate transporter, and several genes involved in the biosynthesis of tricarboxylic acid cycle enzymes, namely *sucA*, *sucB*, *sucC*, *sucD*, and *mdh*. Besides the $\Delta emrR$ mutant, other mutants are known to overproduce succinoglycan, namely the mutants with a Tn5 insertion in *exoS* and *exoR* genes (Cheng and Walker 1998), the *podJ* mutant (Fields et al. 2012), the *cbrA* mutant (Gibson et al. 2006), and the *emmABC* mutant (Morris and González 2009). As these mutants also shared other phenotypes, such as decreased motility, have cell-envelope defects, and are impaired in symbiosis, the increase in

succinoglycan production can be a response to cell-envelope deficiency, or EPS production and cell-envelope integrity share common regulatory pathways.

Previous transcriptomic analysis of the wild-type *S. meliloti* in the presence of luteolin showed increased expression of *emrAB* and *emrR* genes when compared with the noninducing condition (Barnett et al. 2004; Capela et al. 2005). In addition, *emrA* expression was induced in bacteroids (Barnett et al. 2004). Our work shows the importance of EmrR in symbiosis, as both the $\Delta emrR$ and the $\Delta emrAR$ mutants, despite being Fix⁺, showed reduced nodule formation and reduced competitiveness against the wild-type strain in coinoculation experiments. Considering that overexpression of *emrA* (through the deletion of *emrR* gene) and the deletion of *emrA* (as part of the *emrAR* deletion) have the same symbiotic phenotype, it excludes the idea of the EmrAB MFS transporter having an important role in symbiosis. Therefore, it could be that the main function of EmrR is to mount a response to stress caused by the presence of luteolin or a derived compound. In *E. coli*, luteolin seems to have a negative effect in protein and peptide synthesis and in alteration of inner and outer membrane permeability (Eumkeb et al. 2012). Perhaps in the presence of this compound, *S. meliloti* cells need to restructure the cell envelope, possibly mediated by the putative metallophosphoesterase SMc03166 protein. Whether the symbiotic defect of the $\Delta emrR$ and of the $\Delta emrAR$ mutants can be due to overexpression of the *SMc03166* gene product still needs to be determined, but that could explain why the *emrR* symbiotic phenotype is not relieved with the additional mutation of the *emrA* gene.

In conclusion, this work suggests that when *S. meliloti* is in the presence of flavonoids, a gene expression program is put into action to produce the signaling molecule Nod factors but, also, to adjust metabolic functions and cell-envelope composition. One possible mechanism is through the release of EmrR repressor from certain promoters and directly or indirectly activating the expression of genes involved in either stress response or cell-wall composition or both while repressing polysaccharide biosynthesis, perhaps exposing cellular structures important for adhesion and invasion of plant root hairs. Because

Table 2. Strains, plasmids, and phage used in this study

Strain or plasmid	Relevant characteristics ^a	Source/reference
Bacterial strains		
<i>Sinorhizobium meliloti</i>		
Sm1021	Wild-type, Su47, Sm ^r	Meade et al. 1982
Sm1021-9	Sm1021 $\Delta emrR$, Sm ^r	This work
Sm1021-7	Sm1021 $\Delta emrB$, Sm ^r	This work
Sm1021-8/9	Sm1021 $\Delta emrA \Delta emrR$, Sm ^r	This work
RmAR9007	Sm2011 <i>exoY-lacZ/aacC1</i>	Keller et al. 1995
Sm1021Y	Sm1021 <i>exoY-lacZ/aacC1</i> , Gm ^r , Sm ^r	This work
Sm1021-9Y	Sm1021 $\Delta emrR$ <i>exoY-lacZ/aacC1</i> , Gm ^r , Sm ^r	This work
Sm1021-8/9Y	Sm1021 $\Delta emrA \Delta emrR$ <i>exoY-lacZ/aacC1</i> , Gm ^r , Sm ^r	This work
<i>Escherichia coli</i>		
DH5 α	<i>recA1</i> $\Delta lacU169$, $\phi 80$ <i>lacZ</i> $\Delta M15$	Hanahan 1983
S17-1	<i>E. coli</i> 294, <i>thi</i> RP4-2-Tc::Mu-Km::Tn7 chromosomally integrated	Simon et al. 1983
Plasmids		
pPHU231	Broad host-range low-copy vector, Tc ^r	Reyes et al. 1996
pK19mobG	<i>oriColE1</i> <i>Mob</i> ⁺ <i>lacZ'</i> , Km ^r in <i>E. coli</i> , Neo ^r in <i>S. meliloti</i>	Katzen et al. 1999
pAFM01-2	pK19mobG containing the 2040 and the 2020 bps fragments upstream and downstream, respectively, of <i>emrB</i> gene, Km ^r	This work
pAFM09-2	pK19mobG containing the 2,223- and the 1,959-bp fragments upstream and downstream, respectively, of <i>emrR</i> gene, Km ^r	This work
pLM135-1	pK19mobG containing the 2,067- and the 1,959-bp fragments upstream of <i>emrA</i> and downstream of <i>emrR</i> genes, respectively, Km ^r	This work
pMAS17	pPHU231 containing a 1,207-bp fragment with the <i>emrR</i> gene under the control of its own promoter, Tc ^r	This work
Phage		
Φ M12	<i>S. meliloti</i> transducing phage	Finan et al. 1984

^a Sm^r, Tc^r, Neo^r, Km^r, and Gm^r = streptomycin, tetracycline, neomycin, kanamycin, and gentamicin resistance, respectively.

the early steps of symbiosis are relatively unknown and EmrR is believed to play a role in this step, further studies on the function of this TetR family regulator are justified.

MATERIALS AND METHODS

Bacterial strains and general growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 2. *E. coli* strains were grown in Lennox Broth (Lennox 1955) at 37°C. *S. meliloti* strains were grown in TY medium (Beringer 1974), MOPS buffered medium supplemented with 0.1 mM phosphate (Neidhardt et al. 1974), or GMS medium (York and Walker 1997) at 30°C. Starter cultures for RNA extraction were grown in TY medium and subcultured in GMS medium supplemented with 10 µM luteolin. Antibiotics were supplemented as required at the following concentrations (µg/ml): for *S. meliloti*, streptomycin, 600; gentamicin, 40; tetracycline, 10; and neomycin, 120; for *E. coli*, tetracycline, 10; and kanamycin, 50. Luteolin was dissolved in 95% dimethyl sulfoxide (DMSO) at a 4 mg/ml stock solution, and 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide CHA salt was dissolved in *N,N*-dimethylformamide at an 80 mg/ml stock solution. An aqueous solution of 2-2'-dipyridyl was used to supplement GMS medium at a final concentration of 175 µM. Media were solidified by the addition of 2.0% (wt/vol) agar. Liquid cultures were shaken at 180 rpm.

Computational analysis of protein sequences.

The BLAST algorithm (Altschul et al. 1997) was used to compare the deduced amino acid sequence of EmrR protein to database sequences available at the National Center for Biotechnology Information. Alignments were performed using the program CLUSTAL W (Thompson et al. 1994).

DNA manipulations.

Preparation of plasmid DNA, DNA restriction, DNA amplification, agarose gel electrophoresis, cloning, and transformation of *E. coli* cells were carried out according to established protocols (Sambrook and Russell 2001). Genomic DNA extraction from *S. meliloti* 1021 was carried out using glass beads to lyse cells, followed by purification by phenol/chloroform and precipitation with isopropanol (Sambrook and Russell 2001). Mutations were transferred between *S. meliloti* strains by phage ΦM12-mediated general transduction as described previously (Finan et al. 1984).

Construction of *emr* gene deletion mutants.

The flanking regions of *emrB* gene were amplified by PCR using *S. meliloti* 1021 genomic DNA as template and the oligonucleotide primers SMc03167_L (fw/rev) and SMc03167_R (fw/rev) shown in Supplementary Table S2 and were cloned into the multiple cloning site of suicide vector pK19mobG (Katzen et al. 1999). The same procedure was followed for the deletion of the *emrR* (SMc03169) gene, but the primers used were SMc03169_L (fw/rev) and SMc03169_R (fw/rev). Deletion of *emrA* and *emrR* simultaneously was accomplished by amplifying the *emrA* upstream region (primers SMc03168_L (fw/rev)) and *emrR* downstream region SMc03169_R (fw/rev). This mutation affects the promoter region of genes *emrA* and *emrB* and, consequently, the *emrB* gene is not expressed. The plasmids obtained were designated pAFM01-2, pAFM09-2, and pLM135-1, characterized by deletions of *emrB*, *emrR*, and *emrAR* genes, respectively. These plasmids were mobilized from strain *E. coli* S17-1 to *S. meliloti* 1021. Homogenization of these mutations was carried out as previously described (Moreira et al. 2000). The mutants obtained were named Sm1021-7 (*ΔemrB*), Sm1021-9 (*ΔemrR*), and Sm2012-8/9

(*ΔemrAR*) (Table 2). The candidate deletion mutants were further confirmed by PCR amplification and DNA sequencing.

In order to complement the *S. meliloti* *ΔemrR* deletion mutant, a 1,207-bp fragment containing the *emrR* gene under the control of its own promoter was amplified by PCR, using primers P03169-fw and P03169-rev and Sm1021 genomic DNA as template. The amplified fragment was inserted into the *EcoRI* site of broad host-range vector pPHU231, and the resulting plasmid was named pMAS17 (Table 2).

Nodulation assays.

Medicago sativa genotype Europa seeds were surface-sterilized by concentrated sulfuric acid for 10 min and were washed several times with large volumes of sterile water. Seeds were kept at 4°C over a two-day period and were then allowed to germinate on 1.5% water agar plates at room temperature in the dark for 24 h. Seedlings were then grown on nitrogen-free medium (Rolfe et al. 1980) and were inoculated with overnight cultures of *S. meliloti* strains that were washed in saline buffer. Each plate, containing three *M. sativa* seedlings, was inoculated with 200 µl of cell suspension (10^8 to 10^9 cells). Plants were grown under a 16-h light, 8-h dark cycle for 4 weeks. At the end of the experiment, each plant was dried at 65°C, until no further reduction in mass was obtained, and weighted.

In coinoculation assays, the seedlings were inoculated with a cell suspension containing different proportions of Sm1021 wild-type strain and *ΔemrR* or *ΔemrAR* mutants, i.e., 10:90, 50:50, and 90:10, respectively. After four weeks, nodules were harvested and crushed in TY medium as previously described (Cosme et al. 2008) and bacterial suspensions were plated on TY plates. Random colonies were tested for the presence or absence of the *emrR* gene by colony PCR using primers SMc03169C-(fw/rev). Briefly a colony was resuspended in 50 µl of H₂O and was boiled at 100°C for 5 min, followed by centrifugation. The supernatant was then used as DNA template in the PCR reaction.

Motility assays.

Motility was carried out as previously described (Soto et al. 2002). Swimming plates with 0.3% bacto agar (Difco, Detroit) and swarming plates with 0.6% Noble agar (Difco) were prepared using GMS medium supplemented with 10 µM luteolin. For estimation of motility, overnight GMS cultures (5 µl) were inoculated into the agar and were incubated at 30°C for 1 and 3 days to measure swarming and swimming motility, respectively. Three separate experiments, each containing five technical replicates, were performed.

Biofilm formation.

Bacteria were grown in Vincent minimal medium (Vincent 1970) supplemented with 10 µM luteolin at 30°C until mid-exponential phase, diluted to an optical density at 600 nm (OD_{600nm}) of 0.1, and 3 ml of these cell suspensions were inoculated into glass test tubes. Cell suspensions were incubated at 30°C without agitation for 72 h. After removal of planktonic cells and three washing steps with saline buffer, the biofilm was stained with a crystal violet solution, followed by dye solubilization with ethanol and measurement of the solution absorbance at 590 nm as previously described (Ferreira et al. 2007).

Root attachment assays.

Strains were grown overnight in TY medium, and 10^8 cells were resuspended in nitrogen-free medium (Rolfe et al. 1980). A volume of 1 ml of each bacterial suspension was then placed into a microcentrifuge tube containing a sterile germi-

nated alfalfa seedling with a root of about 1.5 cm and was incubated for 8 h at room temperature. Following that period, the seedling was washed with water three times, and then, attached bacteria were released by vortex in the presence of silica beads. Bacterial suspensions were plated onto TY agar plates, and CFU were counted. Three separate experiments, each containing three technical replicates, were performed for each strain.

Isolation and processing of RNA samples.

Cells from three biological replicates of the wild-type Sm1021 or the *ΔemrR* mutant grown in GMS medium supplemented with luteolin for 4 h were harvested, resuspended in RNeasy lysis reagent (Qiagen, Hilden, Germany), and total RNA extraction was carried out using the RNeasy mini kit (Qiagen) with DNase treatment, following manufacturer's recommendations. Once absence of residual DNA was confirmed, concentration and purity were determined using a Nanodrop ND-1000 UV-visible spectrophotometer. RNA integrity was checked with an Agilent 2100 Bioanalyser using an RNA Nano assay (Agilent Technologies, Santa Clara, CA, U.S.A.). RNA was processed for use on Affymetrix (Santa Clara, CA, U.S.A.) GeneChip Medicago/Sinorhizobium Genome Arrays as previously described (Santos et al. 2010).

Microarray analysis.

Scanned arrays were first analyzed using Affymetrix Expression Console software to assure that all quality parameters were in the recommended range. Subsequent analysis was carried out with DNA-Chip Analyzer 2008. First a digital mask was applied, leaving for analysis only the 8,305 probe sets on the array representing *S. meliloti* transcripts. Then the six arrays were normalized to a baseline array with median CEL intensity by applying an invariant set-normalization method (Li and Wong 2001b). Normalized CEL intensities of the arrays were used to obtain model-based gene expression indices based on a perfect match-only model (Li and Wong 2001a). Replicate data (triplicates) for each of the *S. meliloti* wild-type and *ΔemrR* deletion mutant strains were weighted gene-wise by using inverse squared standard error as weights. Genes compared were considered to be differentially expressed if the 90% lower confidence bound of the fold change between experiment and baseline was above 1.2, resulting in 285 differentially expressed transcripts with a median FDR of 0.9%. The lower confidence bound criterion meant that we can be 90% confident that the fold change was a value between the lower confidence bound and a variable upper confidence bound. Li and Wong (2001b) have shown that the lower confidence bound is a conservative estimate of the fold change and, therefore, more reliable as a ranking statistic for changes in gene expression.

Real-time qRT-PCR.

DNA microarray data were validated by real-time qRT-PCR. For reverse transcription, 1 µg of total RNA from *S. meliloti* 1021 and the *ΔemrR* deletion mutant strains, derived from three independent samples, was used. cDNA was synthesized using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer's instructions. Primers used to amplify selected *S. meliloti* genes were designed using Primer Express 3.0 software (Applied Biosystems). RT-PCR amplification mixtures used 400 ng of template cDNA, 2× SYBR Green PCR Master Mix, and 0.4 mM of reverse and forward primers for each gene in a total volume of 25 µl. Reactions containing nuclease-free water instead of the reverse transcriptase were included as negative control. Reactions were performed using a model 7500 Ther-

mocycler (Applied Biosystems). The expression ratio of the target genes was determined relative to reference gene *hema*, which showed no variation in the transcript abundance under the experimental conditions used. Relative quantification of gene expression by real-time RT-PCR was determined by applying the $\Delta\Delta C_t$ method (Pfaffl 2001).

Production and recovery of extracellular polysaccharides from bacterial culture supernatants.

To assess succinoglycan production, the different *S. meliloti* strains were cultivated in 50 ml of GMS medium supplemented with 10 µM luteolin for 10 days at 30°C, with orbital agitation. Bacterial cells were removed by centrifugation (20,000 × g, 15 min), and the polysaccharide present in culture supernatants was precipitated with 3% (wt/vol) CPC, was air dried, and was resuspended in the same volume of 3% (wt/vol) NaCl. Total sugars were quantified by the HCl-L-Cysteine method (Chaplin and Kennedy 1986).

Siderophore production.

The presence of siderophores was measured by CAS assay (Schwyn and Neilands 1987) with modifications. Briefly, overnight cultures of *S. meliloti* 1021, the *ΔemrR* deletion mutant, and complemented mutant grown in TY medium were diluted to an initial OD_{600nm} = 0.1 in GMS medium supplemented with luteolin (10 µM). Strains in these conditions were cultured overnight at 30°C, were concentrated tenfold, were inoculated (10 µl) on the surface of the CAS-GMS agar plates, and were incubated at 30°C for 3 days. Modified CAS-GMS agar plates were prepared as follows: 60.5 mg CAS was dissolved in 50 ml of deionized water and was mixed with 10 ml of iron (III) solution (1 mM FeCl₃·6H₂O, 10 mM HCl). With stirring, this solution was slowly mixed with 72.9 mg of hexadecyltrimethylammonium dissolved in 40 ml of water. The resultant dark-blue solution was sterilized and mixed with 900 ml of GMS agar before plating.

Heat and osmotic stresses.

For heat shock, cells were grown in GMS supplemented with 10 µM luteolin for 4 h and were then submitted to a static incubation at 30 and at 42°C in a water bath for 30 min. At minutes 15 and 30, an aliquot of 100 µl was serially diluted and plated onto TY agar. After 72 h of incubation at 30°C, CFU were counted. For osmotic stress, cells were grown in GMS supplemented with 10 µM luteolin for 18 h, followed by the addition of 0.3 M NaCl. OD_{600nm} was measured until cultures reached 65 h of growth.

Zone inhibition assays.

Bacterial cultures were grown overnight and approximately 10⁶ cells were plated in TY agar. Sterile paper disks 6 mm in diameter were placed on the surface of the agar. A total of 10 µl of *p*-coumaric acid (1.5 mg/ml dissolved in 95% ethanol), berberine (3 mg/ml), genistein (3 mg/ml dissolved in DMSO), SDS (10% [wt/vol]), 1 M H₂O₂, cumene hydroperoxide (5% [wt/vol]), DOC (10% [wt/vol]), novobiocin (20 mg/ml), malachite green (5% [wt/vol]), or crystal violet (1% [wt/vol]) was pipetted onto separate disks. The plates were incubated for 72 h, and the diameters of the zones of inhibition were measured (in millimeters).

Statistical analysis.

All quantitative data was obtained from at least three independent assays. Standard deviation was used to calculate error bars. The *t*-tests to determine *P* values were performed using GraphPad Prism 5.0 software. Differences were considered to be statistically significant if the *P* value was lower than 0.05.

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