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Research Letter

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Expression of the *Sinorhizobium meliloti* small RNA gene *mmgR* is controlled by the nitrogen source

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One sentence summary: Nitrogen controls expression of *mmgR* sRNA in *Sinorhizobium meliloti*.
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ABSTRACT

Small non-coding regulatory RNAs (sRNAs) are key players in post-transcriptional regulation of gene expression. Hundreds of sRNAs have been identified in *Sinorhizobium meliloti*, but their biological function remains unknown for most of them. In this study, we characterized the expression pattern of the gene encoding the 77-nt sRNA MmgR in *S. meliloti* strain 2011. A chromosomal transcriptional reporter fusion (P*mmgR-gfp*) showed that the *mmgR* promoter is active along different stages of the interaction with alfalfa roots. In pure cultures, P*mmgR-gfp* activity paralleled the sRNA abundance indicating that *mmgR* expression is primarily controlled at the level of transcriptional initiation. P*mmgR-gfp* activity was higher during growth in rhizobial defined medium (RDM) than in TY medium. Furthermore, P*mmgR-gfp* was induced at 60 min after shifting growing cells from TY to RDM medium, i.e. shorter than the cell doubling time. In defined RDM medium containing NO_3^-, both P*mmgR-gfp* and MmgR level were repressed by the addition of tryptone or single amino acids, suggesting that *mmgR* expression depends on the cellular nitrogen (N) status. *In silico* analysis failed to detect conserved motifs upstream the promoter RNA polymerase binding site, but revealed a strongly conserved motif centered at −28 that may be linked to the observed regulatory pattern by the N source.

Keywords: Riboregulation; sRNA; *Sinorhizobium meliloti*; *mmgR*; promoter regulation; nitrogen

INTRODUCTION

Small non-coding regulatory RNAs of the *trans*-encoded class (sRNAs) are ubiquitous in prokaryotes as mediators of signal transduction cascades and they usually control gene expression at the level of mRNA stability and/or translation efficiency (Wagner and Romby 2015). Most sRNAs act by base-pairing the 5′-UTR of target mRNAs with the assistance of the RNA chaperone Hfq, thus directly competing with ribosome access (Vogel and Luisi 2011; Wagner and Romby 2015). sRNA activity depends on its cellular abundance, which in turn is determined by the balance between their transcription and degradation rates (Levine *et al.* 2007). Of these two processes, usually—but not exclusively—the sRNA transcription rate is modulated by environmental stimuli by means of classical transcriptional regulators such as members of two-component systems (TCS) or alternative sigma factors (Udekwu and Wagner 2007; Valverde and Haas 2008).

Sinorhizobium meliloti is a soil dwelling α-proteobacterium that induces formation of root nodules in legumes of the genus *Medicago*, *Trigonella* and *Melilotus*, wherein they differentiate into nitrogen-fixing bacteroids (Jones *et al.* 2007). The composite genome of *S. meliloti* (the 3.65-Mb chromosome, and the 1.35-Mb

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pSymA and 1.68-Mb pSymB megaplasmids) encodes hundreds of sRNAs (Schluter *et al.* 2013), being a subset of them expressed in the symbiotic stage (Roux *et al.* 2014). With only a few exceptions, the biological role (i.e. direct regulatory targets and mechanism of action) of this repertoire of sRNAs is unknown (Torres-Quesada *et al.* 2013; Baumgardt *et al.* 2015; Robledo *et al.* 2015). One approach to understand their biological function is to identify factors controlling their expression. It is well known that sRNAs are key mediators of signal transducing cascades mastered by TCS that regulate their expression (Gopel and Gorke 2012). Nevertheless, the knowledge about the control of sRNA expression is incipient in α-proteobacteria. For instance, in *Rhodobacter sphaeroides*, the expression of PcrZ sRNA is controlled by oxygen tension through the redox state-sensing TCS PrrB/PrrA; PcrZ in turn, negatively controls formation of photosynthetic complexes (Sallet, Gouzy and Schiex 2014). In *Caulobacter crescentus*, the expression of the CrfA sRNA, which is involved in control of a variety of cell membrane transporters, is induced by carbon starvation, although no mechanism has been identified for such induction (Landt *et al.* 2010). In *S. meliloti*, two sRNAs encoded in tandem (AbcR1 and AbcR2) have different expression pattern, being AbcR1 preferentially expressed in actively dividing bacteria, whereas AbcR2 accumulates in stationary phase cells or under abiotic stress (Torres-Quesada *et al.* 2013). Another *S. meliloti* sRNA, the cell cycle regulator EcpR1, is expressed upon entry into stationary phase and under stress conditions (Robledo *et al.* 2015). Finally, the cellular level of the sRNA RcsR1 linking stress responses to quorum sensing, is downregulated under salt stress but upregulated at low temperature (Baumgardt *et al.* 2015). However, the mechanisms underlying their regulation of transcription are unknown.

The *S. meliloti* MmgR sRNA is member of the αr8 RNA family, widely distributed in α-proteobacteria (Lagares Jr, Roux and Valverde 2016). MmgR is required to limit intracellular accumulation of poly-hydroxybutyrate granules under conditions of carbon surplus (Lagares Jr 2015). MmgR is transcribed from the chromosomal coordinate 3046287 (Schluter *et al.* 2013) as a discrete 77-nt RNA as revealed by Northern blot and RNAseq (Valverde *et al.* 2008; Schluter *et al.* 2010, 2013; Lagares Jr, Roux and Valverde 2016). MmgR is bound to and stabilized by the RNA binding protein Hfq (Sobrero and Valverde 2011; Torres-Quesada *et al.* 2014). The abundance of MmgR sRNA is higher in stationary phase than in exponential phase; in addition, cells growing in rhizobial defined medium (RDM) achieve higher MmgR levels than cells growing in complex tryptone-yeast extract medium (Valverde *et al.* 2008; Sobrero and Valverde 2011). Thus, MmgR level seems to be regulated, yet by unknown signals and mechanism. Here, we have characterized the expression pattern of the *S. meliloti* 2011 *mmgR* gene, and provide evidences of the influence of the growth medium nitrogen (N) source in the control of *mmgR* promoter activity.

MATERIALS AND METHODS

Bacterial strains, plasmids and oligonucleotides

Strains, plasmids and oligonucleotides used are listed in Table S1 (Supporting Information). *Escherichia coli* was grown aerobically at 37°C and 200 rpm in nutrient yeast broth (NYB; in g l-1: nutrient broth, 20; yeast extract, 5) and *S. meliloti* was cultured aerobically at 28°C and 200 rpm in tryptone-yeast extract (TY; in g l-1: tryptone, 5; yeast extract, 3, $CaCl_2$, 0.7), or RDM (a potassium phosphate-buffered medium containing sucrose and nitrate as C and N sources, respectively, Mg and Ca divalent ions and vitamins; (Vincent 1970)). When required, media were supplemented with (in μg ml-1): for *E. coli*, ampicillin 100, kanamycin 25, chloramphenicol 20, gentamicin 10, tetracycline 25; for *S. meliloti*, streptomycin 400, neomycin 100, gentamicin 40 and tetracycline 5.

DNA manipulations

DNA preparations, electrophoretic analyses in agarose gels and cloning steps were done according to standard protocols (Sambrook, Fritsch and Maniatis 1989). Small-scale plasmid preparations were done with the one-tube CTAB method (Del Sal, Manfioletti and Schneider 1988) and high quality plasmid preparations with the JetQuick miniprep spin kit (Genomed GmbH, Löhne, Germany). PCR reactions were carried out as reported previously (Valverde 2009). DNA fragments were purified from agarose gels with QiaexII (Qiagen Inc., Hilden, Germany). All cloned PCR products were verified by sequencing from both ends by Macrogen Inc. (Korea).

Construction of transcriptional reporter fusions and genomic integration in *S. meliloti* strains

The promoter region of *mmgR* (606 bp), *sm12* (415 bp), *sm26* (592 bp) and *sm145* (471 bp) genes, encoding putative small RNA transcripts (Valverde *et al.* 2008), were PCR amplified using the oligonucleotide pairs sm8Kf5/sm8Kr, sm12f/sm12r, sm26f/sm26r, sm145f/sm145r, respectively (Table S1, Supporting Information). The PCR products were cloned into the pCR4-TOPO® vector and subsequently subcloned into the transcriptional reporter vectors pTH1705 or pTH1945 using restriction sites *Xho*I (for *mmgR*, *sm12* and *sm26* promoters) or *Kpn*I (for *mmgR* and *sm145* promoters). The promoter reporter constructs were transferred by triparental mating from *E. coli* DH5α into *S. meliloti* strains using the mobilization helper *E. coli* MT616. Single recombinants were selected in TY plates containing appropriate antibiotics. The correct genomic integration of the reporter constructs was verified by PCR.

Reporter strains carrying ectopic chromosomal reporter P*mmgR-gfp* fusions were generated using forward oligonucleotides sm8Kf1 or sm8Kf5, and reverse primer sm8Kr, to generate amplicons having 600 or 100 bp upstream the *mmgR* transcription start site (TSS). PCR fragments were cloned into pSRmig vector using its *Kpn*I restriction site (Fig. S1c, Supporting Information). The constructs were mobilized from *E. coli* S17-1λpir into *S. meliloti* 2011 by mating and single recombination events were selected by plating onto TY supplemented with Sm and Nm. The correct chromosomal integration was confirmed by PCR with oligonucleotides exoP and gfp-R-pSRmig (Table S1, Supporting Information).

RNA manipulations

RNA preparations and Northern blotting were done as described previously (Valverde *et al.* 2008). Densitometric analysis of the RNA bands was done with the software ImageJ v1.38 (Abramoff, Magelhaes and Ram 2004). For qRT-PCR analysis, 100 pg-1 ng of total DNAse-treated RNA were used as template for each 10-μl reaction using 20 pmol of 8f and 8r primers (Table S1, Supporting Information), and following the protocol supplied by the manufacturer for the KAPA SYBR FAST One-step qRT-PCR (KAPA Biosystems, Wilmington, MA, USA). As reference, the abundance of the 5S rRNA was assessed by amplification of each cDNA with primers 5Sf and 5Sr (Table S1, Supporting Information).

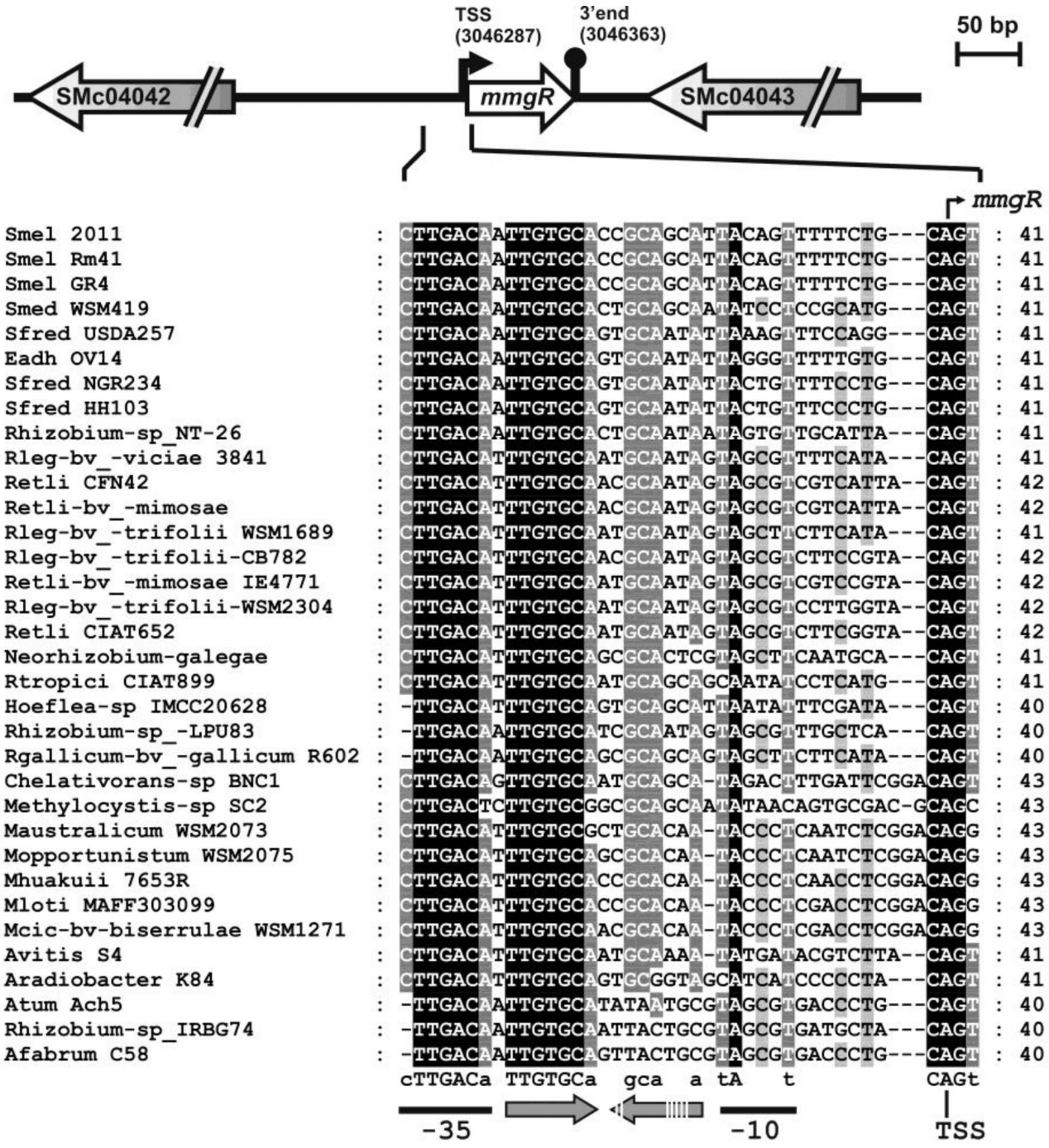

Figure 1. Genomic context of the *mmgR* gene in *S. meliloti* strain 2011 and multiple sequence alignment of the *mmgR* promoter region of members of the Rhizobiales. The absolute chromosomal coordinates of the *mmgR* 5′ and 3′end are indicated (Schluter *et al.* 2013). The ORFs flanking *mmgR* are designated with their synonym gene names from strain 1021 (not drawn to scale). Clustal Omega multiple sequence alignment (Goujon *et al.* 2010) was performed with *mmgR* promoter sequences using default parameters, and GeneDoc (Nicholas, Nicholas and Deerfield 1997) was used to visualize and format the alignment. Fully conserved nucleotides are shaded in black, whereas partially conserved nucleotides are shaded in gray. The consensus sequence derived from the alignment is shown at the base; nucleotides in upper case denote fully conserved bases, whereas nucleotides in lower case represent partially conserved bases. The −35 and −10 RNA polymerase recognition sequences are underlined. Inverted arrows denote a sequence region with partial dyadic symmetry, with a fully conserved 5′ heptamer (solid arrow) and a partially conserved 3′ heptamer (discontinuous arrow). The list of species' sequences used for the alignment is detailed in Table S2 (Supporting Information).

Flow cytometry analysis of promoter reporter fusions

Cells were collected by centrifugation, washed once with saline solution (NaCl 0.9% w/v), and appropriately diluted in IsoFlow (BD Biosciences, San José, CA, USA). Single-cell fluorescence was directly measured on a BD FACSCalibur (BD Biosciences) programmed to determine 100.000 events per run. Single cells were identified on the basis of forward and side scatter, while GFP fluorescence was analyzed using 488 nm for excitation and 530 nm for detection. Bacteria were gated in FSC-H and analyzed for the expression of GFP in FL1-H. Fluorescence cut-off was determined

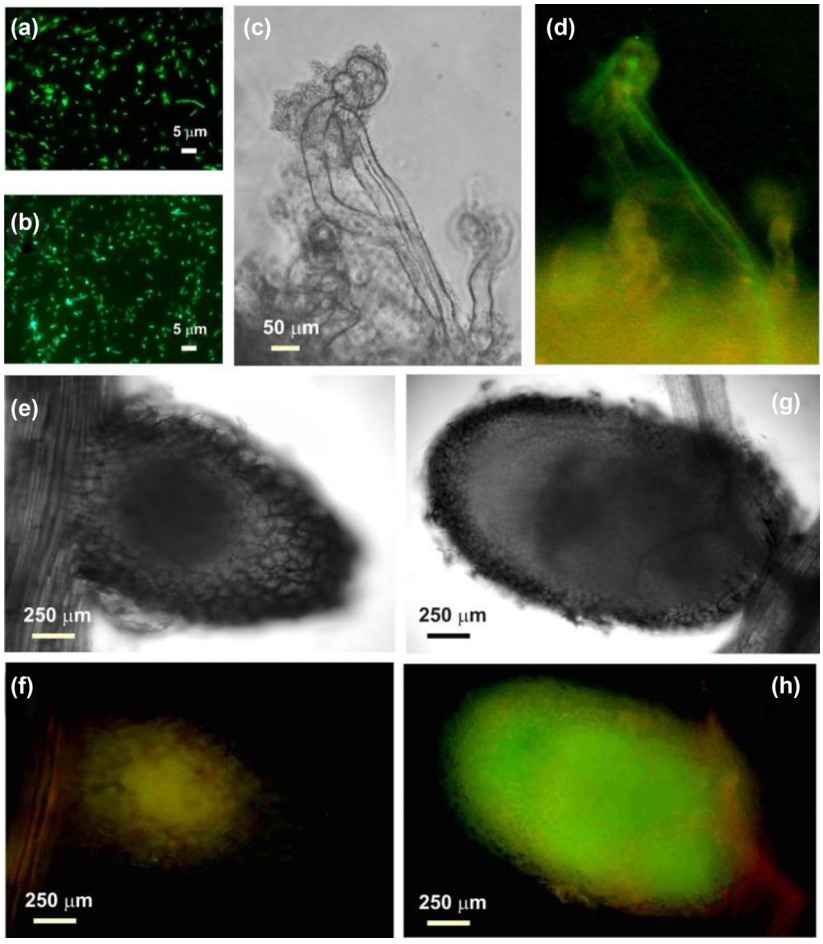

Figure 2. The *S. meliloti mmgR* promoter is active during the symbiotic interaction with *Medicago sativa* roots. (**a**) Expression of plasmid-borne P*mmgR-gfp* fusion in *E. coli* cells. (**b**) Expression of chromosomal P*mmgR-gfp* fusion in *S. meliloti* 2011 cells. (**c**) Root hair with curled tip and infection thread visualized under white light. (**d**) The same field as in (c) under fluorescent light, revealing the infection focus in the root tip and the infection thread filled with rhizobial cells expressing P*mmgR-gfp*. (**e**, **f**) A twenty-one-day-old nodule induced by wild-type *S. meliloti* 2011 cells, visualized under white (e) or fluorescent (f) light. (**g**, **h**) A twenty-one-day-old nodule induced by *S. meliloti* 2011 cells bearing a chromosomal P*mmgR-gfp* fusion, visualized under white (g) or fluorescent (h) light. The green color in (a), (b), (d) and (h) corresponds to the expression of the P*mmgR-gfp* reporter fusion; the yellow and red zones in (d), (f) and (h) correspond to background autofluorescence from plant root tissues.

upon passage of the non-fluorescent parental strain. Data were captured using Cell Quest Pro (BD Biosciences) and further analyzed using FlowJo data analysis software (TreeStar, USA). All data profiles gave a normal distribution. The fluorescence geometric mean of flow histograms (fluorescence versus number of events) was used to compare the promoter activity between treatments.

Promoter expression analysis in microplate multimode reader

Pre-cultures of the reporter strains were grown in TY or RDM; cells were collected by centrifugation, washed twice with saline solution and finally resuspended into the appropriate test growth medium at a normalized OD_{600} of 0.05. Triplicate 450 μl-aliquots of each strain's normalized suspension were transferred into 48-well flat-bottom plates (GBO, Kremsmünster, Austria), covered with a clear lid, sealed with Parafilm M® and incubated in a multimode microplate reader-incubator-shaker (POLARstar Omega microplate reader; BMG Labtech, Ortenberg, Germany). Cultures were grown for 30–60 h with orbital shaking at 700 rpm double orbital movement. Repeated measurements of the OD_{600} and fluorescence were performed every 30 min. The fluorescence base line was set up with wild-type strain *S. meliloti* 2011. Fluorescence reads (registered as fluorescence units, FU) were done with excitation at 485 nm and emission at 520 nm; the gain was set at 800. All experiments were performed in triplicate and repeated at least three times. Differences in growth and expression profiles of randomly chosen strains between shake flask batch cultures and microplate reader plates were found to be negligible. Promoter expression values were pondered by the OD_{600} of the reporter strain culture and are presented as relative fluorescence units ($RFU = FU/OD_{600}$) as a function of culture OD_{600}.

Nodulation and fluorescence microscopy

Medicago sativa cv. Key1 seeds were surface sterilized, germinated and grown in pouches as described previously (Sobrero and Valverde 2011). Three days after transfer to pouches, seedlings were inoculated with 100 μl of a bacterial suspension containing ~10^7 exponential-phase cells/ml. Plants were grown in a greenhouse with a light: dark cycle of 16:8 h and temperatures ranging 20°C–28°C. Nodule appearance was monitored between 5 and 21 days after inoculation. Microscopy was performed on control (non-inoculated) and inoculated roots and

mature nodules at 21 days after inoculation, in an inverted microscope Leica DMI6000B (Leica Microsystems, GmbH, Wetzlar, Germany). Images were captured with a digital camera (Leica DFC345 FX, Leica Microsystems, GmbH) using either bright field or a GFP filter (excitation at 470/440 nm; emission at 525/550 nm).

RESULTS AND DISCUSSION

Construction of a P*mmgR-gfp* reporter fusion and its activity in planta

The *mmgR* gene is encoded within the *SMc04042–SMc04043* inter-ORF region (Fig. 1), a genomic location that is highly syntenic among members of the Rhizobiales (Lagares Jr, Roux and Valverde 2016). However, in spite of the strong linkage with the *SMc04043* gene, there is no evident sequence conservation in the promoter region upstream the TSS (chromosomal nucleotide 3046287; (Schluter *et al.* 2013)), except for a short stretch spanning the −35 element of the promoter and a fully conserved A within the −10 box (Fig. 1).

In order to study the expression pattern of *mmgR*, the promoter-less *gfp* was coupled to a 600-bp fragment of DNA upstream the *mmgR* TSS. The P*mmgR-gfp* reporter construct was functional in *E. coli* cells (Fig. 2a). The reporter fusion was mobilized and integrated by site-specific single homologous recombination into the chromosomal *mmgR* locus of *S. meliloti* strain 2011, thus ensuring *gfp* expression from the original context without affecting transcription of the native sRNA. The P*mmgR-gfp* fusion was active in free-living as well as in symbiotic cells (Fig. 2), both in early (root hair infection and progression) and late stages (N-fixing nodules) of *S. meliloti* interaction with alfalfa roots (Fig. 2c–h). These observations match the detection of the MmgR transcript by RNAseq of nodule symbiotic zones (Roux *et al.* 2014).

Comparative analysis of promoter activity of *mmgR* and other *S. meliloti* sRNA genes

The promoter sequence of three other putative sRNA genes (*sm12*, *sm26* and *sm145*) (Valverde *et al.* 2008) resembles that of *mmgR*, and matches Motif1, a recently reported consensus promoter (Schluter *et al.* 2013) (Fig. 3b). However, the absolute expression level of their chromosomal transcriptional fusions was significantly different among the three tested promoters and that of *mmgR* under the same growth conditions and equivalent reporter constructs (Fig. 3a). In addition, the expression pattern of *sm12* and *sm145* was similar to that of *mmgR*, whereas expression of *sm26* slightly decreased along growth (Fig. 3a). Data suggest that *mmgR*, *sm12* and *sm145* are transcriptionally activated as cells progress in the growth curve, although the fold-activation is stronger for *mmgR* (Fig. 3a). This is consistent with reported changes in the abundance of the corresponding transcripts between growth phases (Valverde *et al.* 2008; Sobrero and Valverde 2011). These results indicate that the four promoters seem to be regulated differentially under the tested condition, and that the expression level and pattern observed for the *mmgR* promoter is not a signature of the Motif1-type promoters (Fig. 3b).

Effect of growth medium on *S. meliloti mmgR* expression

In agreement with our previous reports (Valverde *et al.* 2008; Sobrero and Valverde 2011), qRT-PCR analysis showed that the MmgR transcript level was higher in stationary phase than

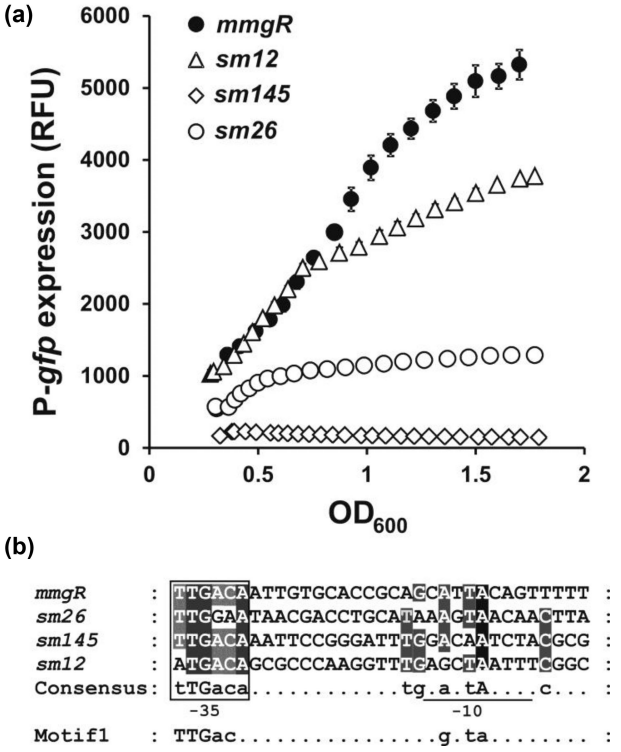

Figure 3. Comparison of the expression pattern and promoter sequence features of *mmgR* and other *S. meliloti* sRNA genes. (**a**) sRNA promoter expression along the growth of strain 2011 in Rhizobial defined medium (RDM) as reported by chromosomal *gfp* fusions. Error bars denote ±SE from triplicate cultures (some error bars may not be visible because they are smaller than the symbols). (**b**) Comparison of the promoter elements of sRNA genes and the recently identified promoter type Motif 1 (Schluter *et al.* 2013). The alignment was done as described for Fig. 1. In the consensus sequence, nucleotides in upper case denote fully conserved bases among the four promoters (dark grey shading), whereas nucleotides in lower case represent partially conserved bases (light gray shading). The −35 and −10 RNA polymerase recognition sites are indicated. The *mmgR* sequence shown corresponds to positions −37 to −5 relative to the *mmgR* TSS (Fig. 1).

in exponential phase cells, for both defined (RDM) and complex (TY) media, and that growth in RDM medium resulted in higher MmgR cellular levels (Fig. 4b). MmgR transcript abundance mostly paralleled P*mmgR-gfp* expression pattern (Fig. 4b), suggesting that the MmgR level primarily increases upon activation of its promoter. However, it cannot be excluded that down regulation of MmgR RNA degradation rate also contributes to the accumulation of MmgR in stationary phase. Given that quorum-sensing mechanisms are often responsible for coordinated activation of gene expression upon reaching high cell densities (Gray *et al.* 1996), we studied the expression pattern of P*mmgR-gfp* in a quorum-sensing mutant unable to produce AHL signals (McIntosh, Meyer and Becker 2009), but we found that the activation of the *mmgR* promoter did not require AHL signaling (data not shown).

The regulation of *mmgR* expression pattern operates within the 100 bp upstream the transcriptional start site

The cloned *mmgR* promoter region (600 bp) could be progressively trimmed down to 100 bp upstream the *mmgR* TSS without losing induction in stationary growth phase nor repression

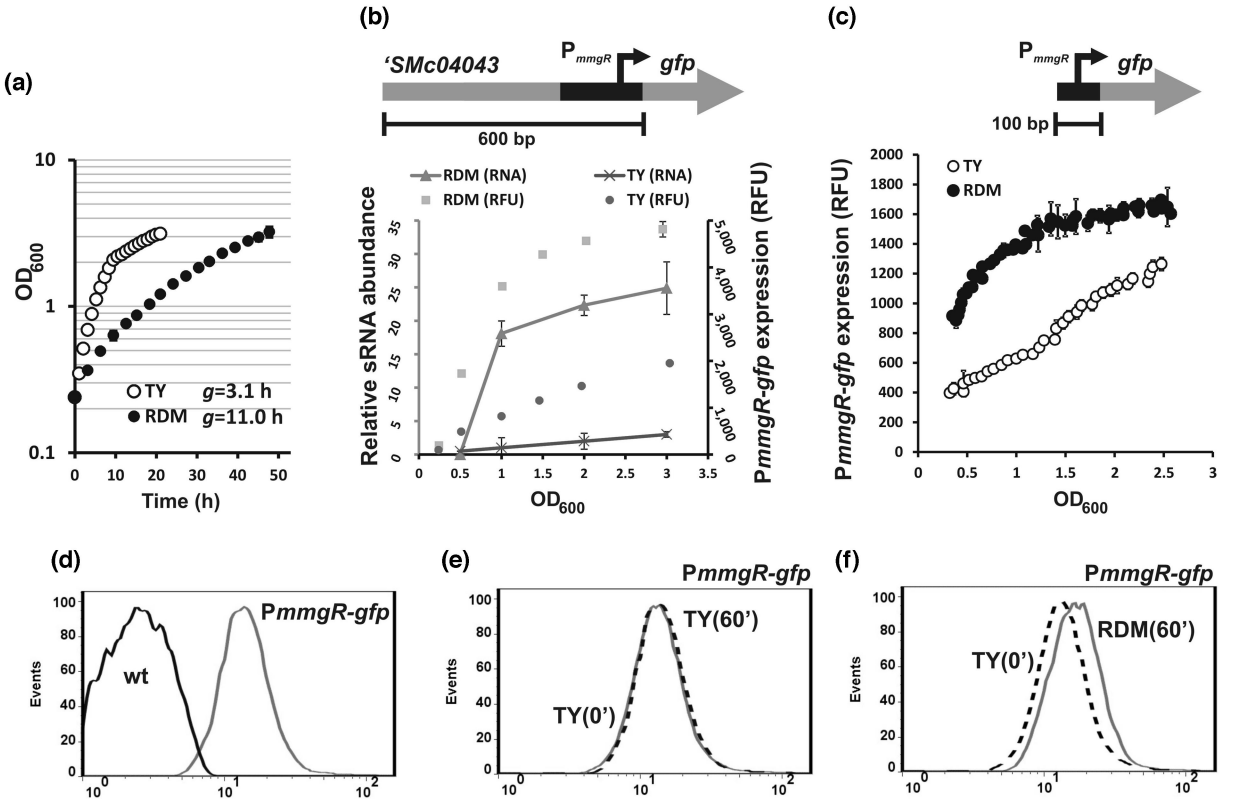

Figure 4. Effect of growth medium on *S. meliloti mmgR* expression. (**a**) Growth curves of the reporter strain 2011 bearing a chromosomal P*mmgR-gfp* fusion in TY and RDM media. Error bars denote ±SE from triplicate cultures (some error bars may not be visible because they are smaller than the symbols). The generation time for each medium is indicated. (**b**) Relative abundance of MmgR transcript along the growth of wild-type *S. meliloti* strain 2011 (left axis) and expression pattern of a chromosomal P*mmgR-gfp* in strain 2011 (right axis) growing in complex TY medium and rhizobial defined medium (RDM). Error bars denote ±SE from triplicate cultures (some error bars may not be visible because they are smaller than the symbols). The architecture of the reporter P*mmgR-gfp* fusion is shown above the plot. (**c**) Same as in (b), but reporter cells contained a shorter version of the P*mmgR-gfp* fusion (as shown schematically). Error bars denote ±SE from triplicate cultures. (**d**) Flow cytometry analysis of *S. meliloti* 2011 cells bearing the 600 bp-P*mmgR-gfp* fusion. wt, histogram of the background fluorescence distribution from the wild-type *S. meliloti* cells. (**e**) Comparison of P*mmgR-gfp* expression histograms after 60 min of shifting exponential phase cells growing in TY medium (dotted line) to fresh TY medium (solid line). (**f**) Comparison of P*mmgR-gfp* expression histograms after 60 min of shifting exponential phase cells growing in TY medium (dotted line) to fresh RDM medium (solid line).

in TY medium, also when this shortened P*mmgR-gfp* fusion was inserted ectopically in the chromosome (Fig. 4c; Fig. S1c, Supporting Information). The lower level of promoter activity measured for these reporter constructs, integrated into the *exoP-thiD* chromosomal locus (Fig. 4c; Fig. S1c, Supporting Information), may be due to the influence of the genomic context on transcription (Bryant *et al.* 2014). Nevertheless, the data showed that the mechanism controlling *mmgR* expression operates within the 100 bp upstream the *mmgR* TSS, under the conditions tested in this work. As a matter of fact, rhizobial *mmgR* promoter regions did not contain any conserved nucleotide stretch that may serve as a binding site for a typical transcriptional activator upstream the −35 box (Fig. 1); instead, there is a strongly conserved heptamer (TTGTGCA) located immediately downstream the −35 box, which is also followed by a less conserved inverted repeat motif (nGCAnnA) that may be targeted by a DNA-binding protein (Fig. 1).

mmgR expression is activated upon shifting from complex TY medium to defined RDM medium

Flow cytometry analysis of single cells revealed that P*mmgR-gfp* expression followed a unimodal distribution at the population level, irrespective of the growth condition (Fig. 4d, and data not shown). When exponential phase cells from TY medium were resuspended into fresh TY medium, the mean fluorescence intensity of the population was only marginally shifted towards higher values after 60 min (+4%; Fig. 4e). In contrast, when cells were resuspended into fresh RDM medium, we detected a significantly higher shift in *mmgR* expression (+20%, $P = 0.05$ following Mann–Whitney's test; Fig. 4f). This enhancement of P*mmgR-gfp* expression took place after 60 min, i.e. at one-tenth of a generation time in RDM (11 h; Fig. 4a). This short-term activation of the *mmgR* promoter—in comparison to cell doubling time—may be the consequence of a signal transduction cascade that perceives altered nutrient availability upon the TY to RDM shift, thus resulting in higher *mmgR* expression.

Amino acids repress *S. meliloti mmgR* expression

The results described above pointed to a possible relationship between the extent of *mmgR* expression and the N source present in the growth medium (tryptone in TY and NO_3^- in RDM). Accordingly, supplementing RDM with tryptone (TTN) repressed P*mmgR-gfp* (Fig. 5a). Moreover, individual amino acids at equivalent millimolar N concentrations mimicked the repressive effect of TTN (Fig. 5b). The repressive effect of individual amino acids was also reflected at the MmgR transcript level

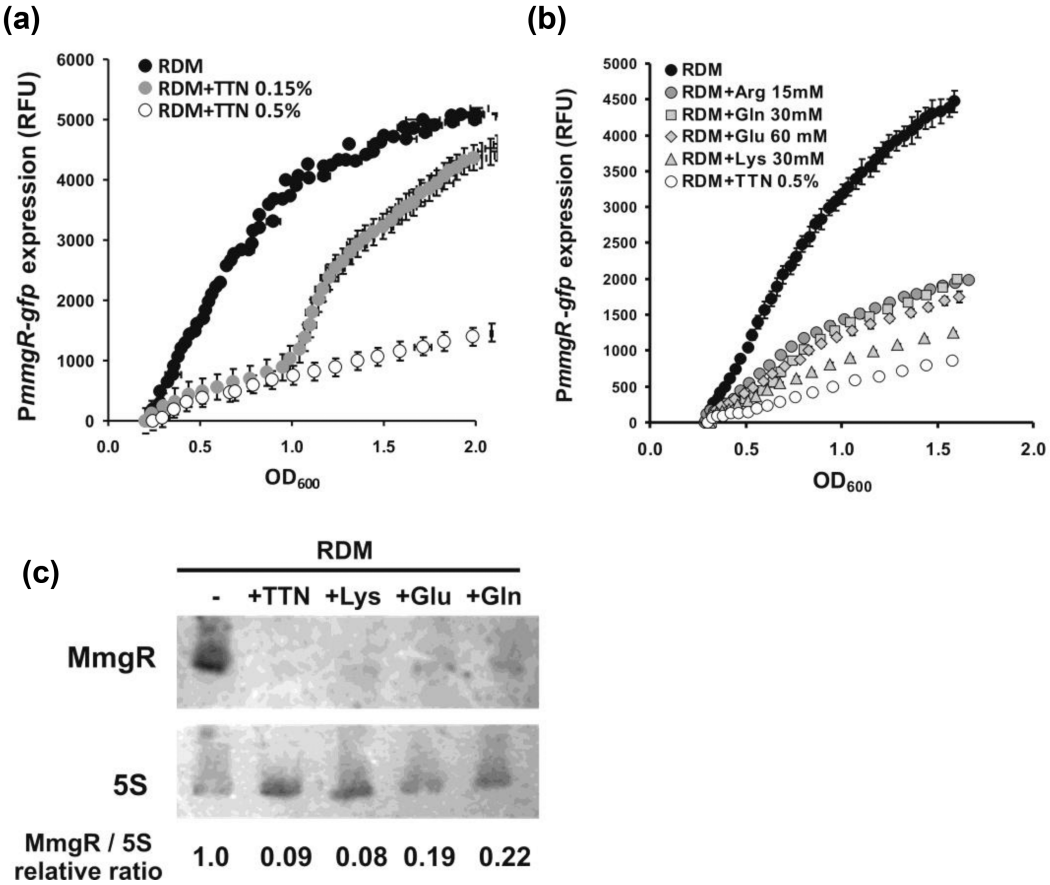

Figure 5. Amino acids repress *S. meliloti mmgR* expression.(**a**) Effect of addition of tryptone (TTN) to RDM medium on the expression pattern of a chromosomal P*mmgR-gfp* fusion in strain 2011. (**b**) Effect of individual amino acids at equimolar N concentrations on P*mmgR-gfp* expression. For (a) and (b), error bars denote ±SE from triplicate cultures (some error bars may not be visible because they are smaller than the symbols). (**c**) Northern blot showing the effect of TTN or individual amino acids at equimolar N concentrations on MmgR abundance in *S. meliloti* 2011 grown in RDM medium. See Materials and Methods for experimental details. Arg, arginine; Glu, glutamate; Gln, glutamine; Lys, lysine. The relative abundance of MmgR sRNA is indicated below the blot. The MmgR/5S ratio of RDM was set as the reference (=1.0).

(Fig. 5c). These were not osmotic or saline effects, as addition of up to 50 mM NaCl to RDM medium did not reduce *mmgR* expression (Fig. S1a, Supporting Information); neither was it related to the addition of carbon, as an equivalent millimolar increment of medium C source (sucrose) did not repress *mmgR* promoter activity (Fig. S1b, Supporting Information). These results indicate that the *mmgR* promoter responds to the availability of N in the culture medium, being activated when N is limiting (RDM) and down regulated when N availability increases by addition of the tested amino acids or TTN (Figs 4 and 5). This is consistent with the fact that both *mmgR* expression and MmgR abundance are higher in stationary than in exponential phase in RDM (5 mM NO_3^-) (Fig. 4); such effect is markedly less pronounced in TY having a higher content of N (*ca.* 45 mM at inoculation). Provided that MmgR limits formation of poly-hydroxybutyrate granules under C/N surplus (as found in RDM medium) (Lagares Jr 2015), MmgR may be a post-transcriptional arm of a global regulatory system for control of C and N fluxes (Schumacher *et al.* 2013; Goodwin and Gage 2014).

A working model for control of *mmgR* expression by the nitrogen source

The N control over *mmgR* transcription takes place within the 100-bp lying upstream the *mmgR* TSS (Fig. 4c). In *S. meliloti*, many N-regulated promoters are recognized by the sigma factor RpoN (σ^{54}) (Dombrecht *et al.* 2002). However, the *mmgR* promoter consensus did not contain RpoN binding sites (Schluter *et al.* 2013) (Fig. 6), thus excluding an RpoN-dependent control over *mmgR* expression. Instead, the consensus *mmgR* promoter displays a −35 signature typical of RpoD (σ^{70})-dependent promoters and of their recently identified variant Motif 1 (Schluter *et al.* 2013) (Fig. 6). Still, the *mmgR* promoter bears two unique features that distinguish it from the RpoD and Motif 1 consensus: (i) the −10 region has different conserved nucleotides at equivalent positions (Fig. 6); (ii) there is a conserved motif lying just in between the −35 and −10 elements, with a fully conserved 5′ heptamer and a moderately conserved 3′ inverted repeat (Fig. 6). We hypothesize that this dyadic motif is a binding site for a regulatory protein that controls *mmgR* transcription and whose DNA binding properties responds to the medium N source. One candidate regulator is NtrC (the DNA-binding partner of the NtrB/C sensory-transducing system (Dombrecht *et al.* 2002; Davalos *et al.* 2004)), which has been shown to bind to the heptamer TTTTGCA at the −15 region of the *S. meliloti nifH* promoter (Ow *et al.* 1983); this is fairly similar to the fully conserved heptamer TTGTGCA within the *mmgR* promoter (Fig. 6). It follows that both a site-directed mutagenesis of this heptamer, as well as a random mutagenesis approach over

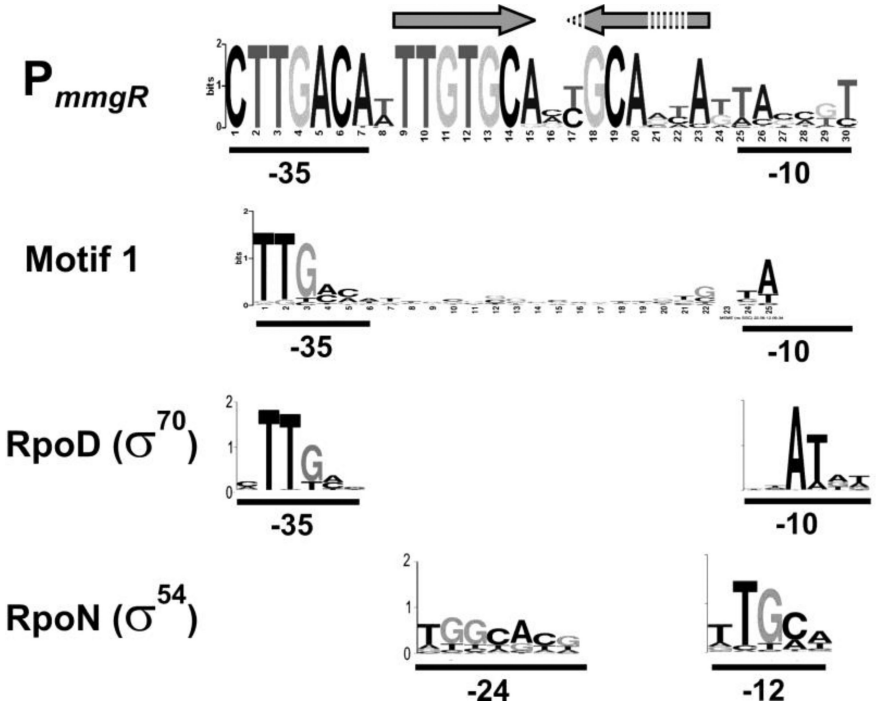

Figure 6. Sequence features of the *mmgR* promoter region. The sequence logo of the *mmgR* promoter was generated with the MEME motif discovery tool (http://meme-suite.org/tools/meme) using the *mmgR* promoter sequences shown in Fig. 1. The sequence logos of the RpoD (σ^{70})- and RpoN (σ^{54})-dependent promoters and of the Motif 1-type promoters, were adapted from those reported in (Schluter *et al.* 2013). Inverted arrows denote a sequence region with partial dyadic symmetry, with a fully conserved 5′ heptamer (solid arrow) and a partially conserved 3′ heptamer (discontinuous arrow).

the *S. meliloti* genome to identify the putative transcriptional regulator of *mmgR*, would shed light on the molecular mechanism involved in control of *mmgR* expression by the N source of the growth medium.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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