Expression of the small regulatory RNA gene \textit{mmgR} is regulated negatively by AniA and positively by NtrC in \textit{Sinorhizobium meliloti} 2011

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Abstract

In the \textit{N}_2-fixing symbiont of alfalfa root nodules, \textit{Sinorhizobium meliloti} 2011, the \textit{mmgR} gene encodes a 77 nt small untranslated RNA (sRNA) that negatively regulates the accumulation of polyhydroxybutyrate (PHB) when the bacterium is grown under conditions of surplus carbon (C) in relation to nitrogen (N). We previously showed that the expression of \textit{mmgR} is primarily controlled at the transcriptional level and that it depends on the cellular N status, although the regulatory mechanism and the factors involved were unknown. In this study, we provide experimental data supporting that: (a) \textit{mmgR} is induced upon N limitation with the maximum expression found at the highest tested C/N molar ratio in the growth medium; (b) a conserved heptamer TTGTGCA located between the −35 and −10 \textit{mmgR} promoter elements is necessary and sufficient for induction by N limitation; (c) induction of \textit{mmgR} requires the N-status regulator NtrC; (d) under C limitation, \textit{mmgR} transcription is repressed by AniA, a global regulator of C flow; (e) the \textit{mmgR} promoter contains a conserved dyadic motif (TGC[N]_2GCA) partially overlapping the heptamer TTGTGCA, which was also found in the promoters of the PHB-related genes \textit{phaP1}, \textit{phaP2}, \textit{phaZ} and \textit{phaR} (aniA) of \textit{S. meliloti} and other alpha-proteobacteria. Taken together, these results suggest that the \textit{mmgR} promoter would integrate signals from the metabolism of C and N through – at least – the global regulators NtrC and AniA, to provide an optimal level of the MmgR sRNA to fine-tune gene expression post-transcriptionally according to varying C and N availability.

INTRODUCTION

Prokaryotic genomes encode hundreds of RNA molecules that are neither translated into polypeptides nor engaged in translation in the ribosomes [1]. A major class of such non-coding RNA species includes the so-called sRNAs, which are small transcripts that regulate gene expression at the post-transcriptional level, typically by means of controlling the translational activity or the stability of target mRNAs [1]. One broadly occurring mechanism of sRNA action involves the formation of imperfect antisense base-pairings between the target mRNA and the sRNA, an interaction that may require the assistance of chaperoning proteins like Hfq or FinO/ProQ [2–5]. For instance, a sRNA molecule that binds to the 5’-UTR of a target mRNA at the RBS will impede its translation, but this will only happen when the intracellular sRNA concentration surpasses a threshold level [6]. In turn, the intracellular level of a given sRNA, and therefore its activity, will depend on the balance between its synthesis (transcription rate) and its stability (degradation rate) [7]. In most documented cases, the cellular concentration of a sRNA is controlled at the level of transcription initiation, and is determined by canonical mechanisms involving DNA-binding regulatory proteins and/or specific sigma factors that are part of signal transduction cascades associated with the perception of a variety of physicochemical stimuli [8–11]. Thus, sRNA genes serve to adjust gene expression at the post-transcriptional level in response to fluctuations in environmental conditions [11]. As expected, there is a functional link between the physicochemical signal that controls transcription of a given sRNA and the biological pathways or processes commanded by its mRNA targets [11]; e.g. the cellular Fe^{2+} level modulates the synthesis of the enterobacterial RyhB sRNA, which in turn fine-tunes the expression of a number of mRNAs, encoding proteins that store or use iron as a cofactor [12].

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Abbreviations: PHB, polyhydroxybutyrate; RDM, rhizobial-defined medium; RFU, relative fluorescence units; sRNA, small regulatory RNA; TY, tryptone-yeast extract.

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One supplementary table and five supplementary figures are available with the online version of this article.
In the alpha-proteobacterium Sinorhizobium meliloti, the N2-fixing root nodule symbiont of alfalfa (Medicago sativa), recent RNA-seq approaches applied to free-living cells as well as to nodule symbiotic cells, led to an inventory of over 500 sRNAs that are encoded on all three replicons of the multipartite genome [13, 14]. The biological functions of this wealth of sRNAs are largely unknown, except for the cell cycle regulator EcR1 [15], the nodule formation efficiency RNA NfeR1 [16], the quorum-sensing regulator RcsR1 [17], the tandemly encoded orthologues AbcR1 and AbcR2 [18], and MmgR, which is the subject of this study. The S. meliloti sRNA MmgR is a 77 nt transcript that binds to and is stabilized by Hfq [19, 20]. Orthologues of the mmgR gene are widely distributed within the alpha-proteobacteria in which they are usually flanked by the same neighbouring genes [21], suggesting the existence of an ancient and shared conserved function for this gene. However, its biological relevance has only been explored in S. meliloti, in which MmgR limits the accumulation of the carbon- and reducing power-storage polymer polyhydroxybutyrate (PHB) under conditions of N starvation and C surplus [22]. In the absence of a functional MmgR sRNA and when growing in a medium with a C/N molar ratio of 30 (i.e. threefold over the balanced C/N ratio), S. meliloti cells accumulate 20% more PHB and four times more phasin than the wild-type strain [22]. With regards to the expression of the mmgR gene, we have recently found that in free-living S. meliloti cells the activity of a P_mmgR::gfp reporter fusion paralleled the abundance of MmgR along the growth curve, pointing to a primary control of mmgR expression at the level of transcriptional initiation [23]. Both the cellular level of the MmgR transcript and its promoter expression are higher in stationary phase than in exponential phase [20, 23]. In addition, MmgR expression is modulated in response to the quality and amount of the available N source, reaching the highest intracellular level with an inorganic N source or upon starvation of the organic N sources [20, 23]. Thus, it seems that the availability and/or quality of the N source in the growth medium are relevant for controlling the promoter activity of mmgR. Interestingly, we could not find conserved motifs usually serving as DNA recognition sites for positive regulatory proteins, upstream of the RNA polymerase-binding region of the mmgR promoter. We found, however, a conserved motif of dyadic symmetry lying just between the −35 and −10 elements, with a fully conserved 5′-heptamer and a moderately conserved 3′-inverted repeat. We hypothesize that this motif is instrumental for the expression pattern of mmgR [23]. Despite these recent findings, further experimental testing is required to deepen insights into the mechanisms that control expression of the S. meliloti mmgR sRNA gene. To this end, we here report the impact that nt replacements within the mmgR promoter and that knocking out well-characterized transcriptional regulators of nitrogen metabolism and PHB synthesis have on mmgR expression at different C/N conditions in the growth medium.

METHODS

Bacterial strains, plasmids and oligonucleotides

The strains and plasmids are listed in Table 1. Oligonucleotides are listed in Table S1 (available in the online version of this article). E. coli was grown aerobically at 37°C and 200 r.p.m. in nutrient yeast broth (NYB; in g l−1: yeast broth, 25; yeast extract, 5). Pre-cultures of S. meliloti were cultured aerobically at 28°C and 200 r.p.m. in tryptone-yeast extract (TY; in g l−1: tryptone, 5; yeast extract, 3; CaCl2, 0.7). For GFP-expression assays, S. meliloti strains were grown in rhizobial-defined medium (RDM; [24]), with the following modifications: nitrate was replaced by ammonium, MOPS was incorporated into the buffer at pH 7.2, and micronutrients were added. The composition of the modified RDM was: KH2PO4, 1 g l−1, K2HPO4, 1 g l−1, CaCl2·2H2O, 0.1 g l−1, MgSO4·7H2O, 0.25 g l−1, MOPS, 10 g l−1 (pH 7.2), FeCl3, 37 µM; biotin, 1 µM; thiamine, 33 µM; H2BO3, 50 µM; MnSO4·H2O, 10 µM; ZnSO4·7H2O, 1 µM; NaMoO4·2H2O, 0.5 µM; CoCl2·6H2O, 0.5 µM. The following modifications did not alter the expression pattern of the wild-type strain S. meliloti 2011 carrying a chromosomal P_mmgR::gfp reporter fusion, despite the higher growth rate in the modified RDM (Fig. S1). The amount of C source (as sucrose) and N source (as NH4Cl) in the medium was set according to Table 2. When required, media were supplemented with (in µg ml−1): for E. coli, ampicillin 100, kanamycin 25, chloramphenicol 20 and gentamicin 10; for S. meliloti, streptomycin 400, neomycin 100 and gentamicin 40.

DNA manipulations

DNA preparations, electrophoretic analyses in agarose gels and cloning steps were done according to standard protocols [25]. Small-scale plasmid preparations were performed with the one-tube cetyltrimethylammonium bromide method [26] and high-quality plasmid preparations with the JetQuick miniprep spin kit (Genomed GmbH, Löhne, Germany). DNA fragments were purified from agarose gels by QiaexII (Qiagen, Hilden, Germany). DNA manipulations, electrophoretic analyses in agarose gels and cloning steps were done according to standard protocols [25]. Small-scale plasmid preparations were performed with the one-tube cetyltrimethylammonium bromide method [26] and high-quality plasmid preparations with the JetQuick miniprep spin kit (Genomed GmbH, Löhne, Germany). DNA preparations, electrophoretic analyses in agarose gels and cloning steps were done according to standard protocols [25]. Small-scale plasmid preparations were performed with the one-tube cetyltrimethylammonium bromide method [26] and high-quality plasmid preparations with the JetQuick miniprep spin kit (Genomed GmbH, Löhne, Germany). DNA fragments were purified from agarose gels by QiaexII (Qiagen, Hilden, Germany). All cloned PCR products were verified by sequencing from both ends by Macrogen (Korea).

Inactivation of ntrC and aniA alleles

The ntrC (smc01043) and aniA (smc03880) mutants of S. meliloti 2011 were generated by site-directed vector integration mutagenesis. The donor strain E. coli S17-1 (S17-1. PLG1PHEL05D12) carries the plasmid pK19mob8HMB [27] (kanamycin-resistant) with an internal DNA region of the ntrC-coding sequence (chromosomal positions 1569958 to 1570264), whereas the donor strain E. coli S17-1 (S17-1. PLG1PHEL1E11) carries the plasmid pK19mob9HMB with an internal DNA region of the aniA-coding sequence (chromosomal positions 3551280 to 3551590). The recombinant plasmids were transferred by biparental conjugation from strain S17-1 to S. meliloti 2011 [28]. ntrC and aniA mutants that had been generated after site-specific integration of the plasmid (single crossover) were selected by their expected...
streptomycin and neomycin resistance. The correct insertion of the integrative plasmid was verified by PCR (see Fig. S2).

**Construction of transcriptional reporter fusions with site-directed mutations and genomic integration in S. meliloti strains**

Site-directed nt replacements were introduced in the promoter sequence of mmgR and sm145 gfp-reporter vectors by PCR amplification using a combination of one wild-type forward primer and a reverse primer with the desired base exchanges annealing at the RNA polymerase-binding site (see Table S1). The PCR products were cloned into the pCR4-TOPO vector and subsequently subcloned into the pTH1705 reporter vector. The wild-type and mutated variants of 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.

**Analysis of promoter expression**

Pre-cultures of the reporter strains were grown in TY; 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 (Greiner), covered with a clear lid, sealed with Parafilm M and incubated in a multimode microplate reader–incubator–shaker (POLARstar Omega; BMG Labtech). Cultures were grown for 15–25 h with shaking at 700 r.p.m. of double orbital movement. Repeated measurements of the OD_{600} and fluorescence were performed every 30 min. The fluorescence baseline 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.

**Table 1.** Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype and features</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
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<tr>
<td>DH5α</td>
<td>F endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1Δ(lacZΔM15lacZYA-argF)U169 deoR (Φ80lacZΔM15)</td>
<td>[25]</td>
</tr>
<tr>
<td>MT616</td>
<td>MT607 (pro-82 thi-1 hsdR17 supE44)·pRK600, Cm³</td>
<td>[45]</td>
</tr>
<tr>
<td>S17-1</td>
<td>F pro thi hsdR recA; chromosome: RP4-2 Tc: Mu Km: Tn7 Tp³, Sp³</td>
<td>[46]</td>
</tr>
<tr>
<td><strong>S. meliloti</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>Wild-type, Sm³</td>
<td>[47]</td>
</tr>
<tr>
<td>2011-Psm8G</td>
<td>2011 with a chromosomal PmmgR-gfp transcriptional fusion in psm8G, Sm³, Gm³</td>
<td>[23]</td>
</tr>
<tr>
<td>2011-Psm8mut</td>
<td>2011 with a chromosomal mutant PmmgR-gfp transcriptional fusion in psm8Gmut, Sm³, Gm³</td>
<td>This work</td>
</tr>
<tr>
<td>2011-Psm8(100)</td>
<td>2011 with a chromosomal PmmgR-gfp transcriptional fusion in psm8100, Sm³, Nm³</td>
<td>[23]</td>
</tr>
<tr>
<td>2011-Psm145</td>
<td>2011 with a chromosomal Psm145-gfp transcriptional fusion in psm145, Sm³, Gm³</td>
<td>[23]</td>
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<tr>
<td>2011-Psm145mut</td>
<td>2011 with a chromosomal mutant Psm145-gfp transcriptional fusion in psm145mut, Sm³, Gm³</td>
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<td>2011-aniA</td>
<td>2011 with an insertion of plasmid pK19mobHMB within the aniA allele, Sm³, Nm³</td>
<td>This work</td>
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<td>2011aniA with a chromosomal PmmgR-gfp transcriptional fusion in psm8G, Sm³, Gm³, Nm³</td>
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<td>This work</td>
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<td>2011-ntrC-Psm8G</td>
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<td>This work</td>
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<td><strong>Plasmids</strong></td>
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<td></td>
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<tr>
<td>pCR 4-TOPO</td>
<td>Cloning vector, pUC ori, Ap³, Km³</td>
<td>Invitrogen</td>
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<tr>
<td>psm8G</td>
<td>pTH1705 carrying the mmgR promoter region, Gm³</td>
<td>[23]</td>
</tr>
<tr>
<td>psm8Gmut</td>
<td>pTH1705 carrying the mmgR promoter region with a replacement of the heptamer TTGTGCA by AGAATAC, Gm³</td>
<td>This work</td>
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<td>[23]</td>
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<td>psm145</td>
<td>pTH1705 carrying the sm145 promoter region, Gm³</td>
<td>[23]</td>
</tr>
<tr>
<td>psm145mut</td>
<td>pTH1705 carrying the sm145 promoter region with a replacement of the heptamer ATTCGCG by TTGTGCA, Gm³</td>
<td>This work</td>
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</tbody>
</table>

**Table 2.** Nitrogen and carbon content, and C/N ratio of the growth media tested in this work

<table>
<thead>
<tr>
<th>mM NH₄⁺ (N)</th>
<th>mM sucrose</th>
<th>mM C</th>
<th>C/N molar ratio</th>
<th>Limited in</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.50</td>
<td>2.63</td>
<td>31.56</td>
<td><strong>12.6</strong></td>
<td>N (high C surplus)</td>
</tr>
<tr>
<td>2.50</td>
<td>2.05</td>
<td>24.56</td>
<td><strong>9.8</strong></td>
<td>N (low C surplus)</td>
</tr>
<tr>
<td>5.00</td>
<td>2.05</td>
<td>24.56</td>
<td><strong>4.9</strong></td>
<td>C</td>
</tr>
</tbody>
</table>
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 relativized to the OD\textsubscript{600} of the reporter strain culture and are presented as relative fluorescence units (RFU=FU/OD\textsubscript{600}) as a function of culture OD\textsubscript{600}.

**RNA extraction and purification**

Total RNA from the bacterial cells was extracted with acid phenol/guanidinium isothiocyanate (Quick-zol, Kalium Technologies) and chloroform, following manufacturer instructions. The RNA was then purified by precipitation with isopropanol. Before reverse transcription, the RNA was treated with DNase I for 1 h at 37 °C (Thermo Scientific, 1 U DNase I per µg RNA). DNase I was then inactivated by incubation at 65 °C after the addition of 0.1 volumes of 50 mM EDTA. The purified RNA was then quantified by UV absorbance (Nanodrop, Thermo Scientific, USA) and the quality of the preparation further assessed by denaturing agarose gel electrophoresis [25].

**Northern blot analysis**

Northern blot analyses were performed as reported elsewhere [22, 29]. Overall, 2 µg of total RNA from each sample were initially electrophoresed for 45 min at constant current (15 mA) in polyacrylamide gels [8.3 M urea, 8 % (w/v) acrylamide, 0.2 % (w/v) bisacrylamide in 1× TBE buffer]. With the Low-Range-RNA Ladder (Thermo Scientific, USA) serving as a molecular weight marker, the corresponding lane was cut, stained separately with ethidium bromide, and the image registered with a UV transilluminator. The remaining gel was electrobotted at 150 mA for 30 min onto a Hybond-N membrane in 1× TBE buffer. After a twofold washing of the membrane with SSC 2×solution (30 mM Na-citrate, 0.3 M NaCl), the RNA was cross-linked with the hybridization buffer containing the specific DIG-labeled dsDNA probe, previously generated by PCR amplification of the \textit{mmgR} genomic region with primers subBF (TGTCGCTCTCTGCGAGGG) and subBr (TTTCGG CGGCTATCTGCCC). The hybridized membranes were washed under standard stringent conditions, incubated with an alkaline phosphatase-coupled anti-DIG antibody solution, washed with the same buffer, and covered with the LUMIPHOS chemiluminescent reagent (Lumigen, USA) in the dark at room temperature for 5 min. The membranes were exposed for 5–180 min to photographic films and then further developed. The membranes were stripped by a twofold incubation with a boiling 0.1 % (w/v) SDS solution for 30 min. The prehybridization, hybridization and developing steps were repeated by using an anti-5S rRNA probe, in order to provide an indication of total RNA load [22].

Densitometric analysis of the RNA bands was done with the software ImageJ v1.38 [30].

**RESULTS**

The \textit{mmgR} promoter is induced upon exiting exponential growth and the final activity is determined by the C/N ratio in the growth medium

We assayed different C and N concentrations to ensure that cultures growing exponentially become limited by deprivation of either macronutrient at OD\textsubscript{600}<1.0 for appropriate data collection in the microplate reader (Table 2). The only condition for which the cultures kept increasing the OD\textsubscript{600} upon exiting the exponential phase was at a C/N molar ratio=12.6 (Fig. 1a). A likely explanation for this observation would be that the increase in cell density is due to the accumulation of PHB granules, a process that is triggered by the relative excess of C over N (i.e. C/N>10) [22]. When comparing the expression pattern of \textit{mmgR} under the medium conditions shown in Table 2, we found that the promoter was activated for a C/N=9.8 or higher, whereas it remained almost silent for C/N<5.0 (Fig. 1a). Induction of the \textit{mmgR} reporter fusion, both at C/N=9.8 or 12.6, took place when the cultures exited exponential growth, thus suggesting that the promoter was activated by the onset of N limitation in the growth medium (Fig. 1a, Table 2). The final promoter activity was inversely correlated with the C/N ratio in the growth medium, being maximal for a C/N=12.6 (Fig. 1a). This pattern was unaltered upon trimming 500 pb from the 5′-end of the promoter fusion, indicating that any regulatory mechanism determining induction of \textit{mmgR} expression and its modulation by the medium C/N ratio operates within the 100 bp lying upstream the \textit{mmgR} transcription start site (Fig. 1b). Finally, the observed regulatory pattern (Fig. 1a) was specific for the \textit{mmgR} promoter, because the activity of a chromosomal P\textsubscript{sm145}gfp fusion reporting the expression of an unrelated sRNA [23, 29] remained indistinguishable along most of the growth curve under the three different tested conditions (Fig. 1c).

A highly conserved heptameric motif located at -30/-24 relative to the \textit{mmgR} transcription start site is required for induction of \textit{mmgR} expression

Although \textit{mmgR} orthologues are widespread among the alpha-proteobacteria [21], the \textit{mmgR} promoter lacks conserved sequence motifs upstream of the RNA polymerase-binding site [23]. Nevertheless, there is a strongly conserved heptameric sequence (TTGTGCA) just downstream of the −35 promoter element, which also seems to be part of a dyadic sequence motif with partial inverted symmetry (Fig. 2a; [23]). We explored the requirement of this conserved heptamer for \textit{mmgR} expression by replacement of the TTGTGCA motif by AGAATAC. As shown in Fig. 2a, the replacement had a strong impact on the P\textit{mmgR}gfp expression profile in the wild-type strain 2011, with a drastic reduction in the final activity of the promoter under conditions of C surplus (C/N=12.6). Based on the observation that the sm145 promoter was relatively insensitive to the C/N ratio of the
growth medium (Fig. 1c), we asked whether transplantation of the conserved heptamer from the mmgR promoter to the equivalent position within the sm145 promoter (Fig. 2b) would make the latter inducible under high C/N conditions. Indeed, the sole grafting of the TTGTGCA heptamer resulted in a strong activation of P_{sm145}-gfp fusion under N-limiting and C excess conditions (C/N=12.6) (Fig. 2b). Together, the results shown in Fig. 2 suggest that the conserved TTGTGCA heptamer located at -30/-24 relative to the mmgR transcription start site is necessary and sufficient to confer induction by N limitation in the growth medium.

Full activation of the mmgR promoter requires NtrC under N-limiting conditions

Two observations drew our attention to the transcriptional regulator NtrC as a possible factor involved in the control of mmgR expression in S. mellioti strain 2011 under N limitation. First, the phosphorylated form of NtrC is the transcriptional activator of genes involved in N catabolism and assimilation of ammonia when organic N is limiting. The phosphorylation state of NtrC depends on the activity of the sensor protein NtrB, which in turn responds to the uridylation level of PII proteins that master the N stress response [31]. Second, the S. mellioti nifH promoter and the promoters of several enterobacterial genes like glmA, dhuA, argT and nifL, all of them being involved in N fixation or assimilation and regulated at the transcriptional level by the corresponding orthologue proteins NtrC and GlnG, all contain a conserved heptamer sequence TTGTGCA (with at most one mismatch) [32]. This heptameric string is strikingly similar to the conserved heptamer TTGTGCA within the mmgR promoter that is required for activation of mmgR under N-limiting conditions (Fig. 2a) and that is sufficient to confer inducibility by N limitation to the heterologous sm145 promoter (Fig. 2b). Thus, we hypothesized that NtrC may be required for mmgR expression under N-limiting conditions. We constructed an ntrC insertional mutant which, as expected [33], had severe difficulties growing in RDM containing nitrate as the sole N source (Figs S2 and S3), but it could grow when supplied with ammonium (Figs 3a, b and S3). When the P_{mmgR}-gfp reporter fusion was mobilized into the ntrC mutant strain, we observed that the activity of the mmgR promoter was strongly depressed under N-limiting conditions, quite in contrast to what is observed in the wild-type background of strain 2011 (Fig. 3a). At N surplus conditions, the expression pattern of the P_{mmgR}-gfp reporter fusion in the ntrC mutant strain was undistinguishable from that of the wild-type strain, and showed the lowest activity (Fig. 3b). Spontaneous revertants that lost the ntrC interrupting suicide plasmid recovered their ability to grow in RDM with nitrate as the sole N source, and exhibited strong gfp expression from the P_{mmgR} promoter fusion under N-limiting conditions (Fig. S3) consistent with a restored MmgR sRNA abundance to wild-type levels (as determined by qRT-PCR; Fig. S4). These observations rule out unexpected secondary mutations accompanying ntrC inactivation.

AniA (directly or indirectly) represses mmgR expression under C-limiting conditions

We have recently reported that the MmgR sRNA is a negative regulator of PHB storage in S. mellioti 2011 [22]. As PHB levels in S. mellioti as well as in Rhizobium etli are regulated by the product of the smc03880 gene (aniA), encoding a global
carbon flux regulator required for symbiotic nitrogen fixation and having a DNA-binding domain [34, 35], we explored the requirement of the S. meliloti aniA gene for the control of mmgR expression (see Fig. S2 for verification of the aniA insertional inactivation). Interestingly, we found that under C-limiting growth conditions, the PmmgR-gfp reporter was activated in the aniA mutant strain, in sharp contrast to the wild-type strain in which the reporter fusion remained repressed (Fig. 3d). Under N-limiting conditions, the behaviour of the PmmgR-gfp fusion was similar for both wild-type and aniA mutant strains, with the sole difference that in stationary phase, the aniA mutant showed an additional activation phase of the mmgR promoter (Fig. 3c) that coincides with the physiological stage of the culture in which storage of PHB has already achieved a plateau [22].

Impact of ntrC and aniA knock-outs in the cellular level of MmgR sRNA

We have previously shown that the cellular level of MmgR matches the activity of a chromosomal PmmgR-gfp reporter fusion, indicating that the MmgR level is primarily dependent on the expression of its promoter [23]. On the basis of this observation and in light of the results for the PmmgR-gfp activity along the growth curve under N- or C-limiting conditions (Fig. 3), it is expected that upon N limitation the abundance of the MmgR transcript in an ntrC mutant should be lower than in the wild-type strain (Fig. 3a), but higher in an aniA mutant than in the wild-type upon C limitation (Fig. 3d). We then compared the abundance of the MmgR sRNA in stationary phase cultures of wild-type and of ntrC and aniA mutant strains by Northern blot. As shown in Fig. 4, the insertional inactivation of ntrC results in a 70 % reduction in the cellular level of MmgR RNA under conditions of N limitation, which essentially reproduced the lower expression of the PmmgR-gfp fusion achieved in stationary phase (Fig. 3a). By contrast, inactivation of aniA allowed accumulation of MmgR sRNA at growing conditions of C limitation (and relative N excess), a condition that otherwise avoided detection of MmgR in the wild-type strain (Fig. 4). The impact of ntrC or aniA insertional inactivation on mmgR expression was
fully validated by qRT-PCR analysis of independent N-limited or C-limited cultures, with an average of a 2.6× reduction in the MmgR transcript level in the ntrC mutant and a 30× increase in the absence of AniA (Fig. S4). These and the previous results (Fig. 3) strongly suggest that both transcriptional regulators, NtrC and AniA, control expression of the mmgR sRNA gene.

The mmgR promoter contains a conserved putative-binding site for AniA

In an attempt to identify additional S. meliloti genes sharing their regulatory pattern with mmgR, we carried out a genomic search with the DNA pattern tool of the RSAT server [36] across all intergenic regions of the S. meliloti 2011 replicons, using the sequence string TTGTGCANNGCA NNA as a query; this sequence corresponds to the strongly conserved and partially dyadic DNA string present between the −35 and −10 elements of the mmgR promoter (Fig. 5a). To our surprise, we found that among the 53 hits with scores>0.92 (corresponding to no or 1 mismatches with respect to the query string), six hits were located within the promoter regions of genes directly involved in storage and degradation of PHB: e.g. three hits within the promoter of the phasin gene phaP1, one hit within the promoter of the phasin gene phaP2, and one hit within the promoter of each of the two PHB depolymerase genes phaZ and sma1961 (a phaZ-like ORF). The sequence alignment of the promoter regions of the phaP1, phaP2 and phaZ of S. meliloti 2011
and of related alpha-proteobacteria confirmed the presence of the conserved sequence stretches containing the identified motif matching the query string (Fig. S5), and also allowed detection of a second conserved motif within the phaP2 promoter which escaped our DNA pattern search because it contains two mismatches (Fig. S5). It has been previously reported in two other alpha-proteobacterial species, *Rhodobacter sphaeroides* and *Paracoccus denitrificans*, that the promoter regions of phasin genes *phaP* and of the PHB-degrading gene *phaZ* contain binding sites for the transcriptional regulatory protein PhaR (an orthologue of the *S. meliloti* AniA protein), and that PhaR also binds to its own promoter [37, 38]. Based on these reports we inspected an alignment of the promoter regions of *aniA* (*phaR*) homologues and also detected a conserved sequence motif highly similar to the one present in the promoter regions of *phaP1*, *phaP2* and *phaZ* (Fig. S5). In all these cases, the location of the identified conserved motifs is consistent with a negative effect on the transcription upon binding of the presumed AniA (PhaR) protein. The findings are summarized in Fig. 5, which illustrates not only the high conservation of the short palindromic motif TGCnnnGCA in all four promoters of the genes directly involved in storage and regulation of PHB granules, but also within the promoter of the *mmgR* sRNA. This observation is in agreement with the results shown in Figs 3(d) and 4, and thus strongly suggests that the *S. meliloti* *mmgR* sRNA gene is repressed directly by the AniA (PhaR) protein. In line with this hypothesis, we observed that the replacement of the TTGTGCA heptamer located downstream of the −35 motif of the *mmgR* promoter (a mutation that simultaneously removes the 5′ arm of the putative AniA (PhaR) recognition site, TGC), resulted in a higher promoter activity under growth conditions that would promote binding of AniA to the *mmgR* promoter (C/N=4.9). The wild-type reporter fusion reached ca. 2500 RFU at the end of the experiment (Fig. 1a; C/N=4.9), whereas the promoter fusion lacking the TTGTGCA

![Figure 4](image)

**Fig. 4.** Transcriptional regulators NtrC and AniA control expression of the *mmgR* sRNA gene. Northern blot analysis of *MmgR* and 5S rRNA transcript level in *S. meliloti* wild-type and ntrC or *aniA* insertional mutant strains in stationary phase under N-limiting (C/N=12.6) or C-limiting (C/N=4.9) conditions. See Methods for the experimental details.

![Figure 5](image)

**Fig. 5.** The *mmgR* promoter contains a conserved putative-binding site for AniA, which is also present in the promoter regions of PHB-related genes. Multiple sequence alignment of promoter regions of *mmgR* and PHB-related genes containing the identified motif TGC[n]GCA. The alignments were done with Clustal Omega [48] and edited manually. Partially conserved positions are shaded in grey whereas fully conserved positions are shaded in black. *phaP1*/*phaP2*, genes encoding the PHB granule coat proteins (phasins); *phaZ* encodes a PHB depolymerase; *aniA* (*phaR*) encodes the transcriptional repressor of *phaP1*, *phaP2*, *phaZ* and *phaR* (*aniA*) genes [37, 38]. Sm, *S. meliloti*; Pd, *Paracoccus denitrificans*. I, II and III correspond to the different number of motifs identified within a single gene promoter (see Fig. S5).
heptamer reached ca. 6000 RFU (Fig. 2a, C/N=4.9). Thus, the effect on mmgR expression of altering the putative AniA (PhaR)-binding site within the mmgR promoter is comparable to that of inactivating the aniA gene (Fig. 4). These findings strongly suggest that the mmgR promoter is recognized by the AniA (PhaR) regulatory protein.

**DISCUSSION**

In previous studies with *S. meliloti* strain 2011, it was demonstrated that the expression of the sRNA gene *mmgR* (based on the activity of a P_<sub>mmgR</sub>*gfp* reporter fusion and on the cellular MmgR steady-state level) was higher in stationary phase than in exponential phase [20, 23]. With respect to the composition of the growth medium, the abundance of MmgR sRNA was much higher in the defined RDM containing 5 mM nitrate as the sole N source than in the complex TY medium containing 45 mM of organic N in the form of amino acids [20, 23]. Moreover, addition of tryptone or single amino acids to RDM resulted in repression of mmmR expression [23]. These observations suggested a physiological link between the induction of mmmR expression and the quality and/or amount of N in the growth medium, as well as with the transition from exponential to stationary phase of growth. On the other hand, a mutation that drastically reduces the cellular level of the MmgR sRNA results in a higher content of PHB distributed in a higher number of irregularly shaped granules when cells grow in a medium with a C surplus over the balanced C/N ratio, thus pointing to the role of MmgR in setting up a limit for storage of the major C reserve polymer in *S. meliloti* [22]. These findings suggest that the MmgR sRNA somehow connects N availability with C storage as part of a regulatory network in which MmgR acts at the post-transcriptional level, and this complements recent evidences of a transcriptional layer of regulation linking PHB storage with N utilization in *S. meliloti* [39]. Here, we studied the expression of mmmR in growth media with different concentrations of N and C (Table 2). The results presented in this work allow us to delineate the working model shown in Fig. 6. We propose that expression of the sRNA gene mmmR of *S. meliloti* 2011 is controlled at the level of its promoter by (at least) two regulatory proteins, NtrC and AniA (PhaR), specifically operating at conserved sequence motifs present in the −30 to −16 region of the promoter, and both having opposing roles on the transcription of mmmR.

NtrC is required for full expression of mmmR under conditions of N limitation (Figs 3 and 4). In our experimental setup, either the insertional inactivation of *ntrC* or the replacement of the conserved heptamer TTGTTGCA in the mmmR promoter resulted in a similar expression pattern (Figs 2 and 3). Thus, we hypothesize that limiting N supply results in activation of the N stress response ending in phosphorylation of the NtrC response regulator, which in turn activates mmmR transcription, either directly or indirectly through a yet unidentified regulatory factor that recognizes the conserved heptameric motif located at −30/−24 (Fig. 6). As the location of the sequence required for NtrC activation of transcription is rather atypical for a positive regulator and as it lies between the RNA polymerase recognition motifs centred at positions −10 and −35, it seems plausible that the mechanism of transcriptional activation would be similar to that of the members of the MerR family of regulators [40]. As a consequence, the MmgR sRNA level would rise inside *S. meliloti* cells leading to post-transcriptional regulation of yet uncovered target mRNAs possibly involved in the response to N starvation (Fig. 6). The inverse relationship between mmmR expression level and N availability observed in free living *S. meliloti* cells has an interesting correlation with the abundance pattern of MmgR sRNA in mature nitrogen-fixing nodules; recent dual RNAseq of *Medicago truncata* nodules [14] revealed that the number of MmgR transcript reads is very low (<50 copies) in the nodule zone I, low in nodule zones IIp and IIId (70–100 copies) and is much more abundant in nodule zones IIId and IIIp (>300 copies), in which bacteroids are experiencing N starvation and depend on plant supply of specific amino acids [41]. Thus, it appears that the cellular level of MmgR sRNA is dependent on the N status both in free-living and in symbiotic *S. meliloti*.

The regulatory protein AniA (PhaR) would act as a repressor of mmmR expression operating at the conserved dyadic motif TGCnnnGCA (Figs 4, 5 and 6). The fact that this motif perfectly matches the binding sites for AniA (PhaR) in other alpha-proteobacteria [37, 38] strongly suggests that AniA (PhaR) may directly bind the mmmR promoter to out-compete the RNA polymerase. Under conditions of C surplus and proper reducing power availability, *S. meliloti* would synthesize PHB that requires an adequate supply of phasin proteins for stable granule formation (Fig. 6). It is expected that the amount of phasin proteins is adequately balanced according to the amount of PHB inside the cell. The MmgR sRNA contributes to fine-tuning of the amount of PHB and phasin proteins that are stored within *S. meliloti* cells under conditions of C surplus, and this control is executed by setting a limit to the amount of phasin proteins at a post-transcriptional level (Fig. 6) [22]. It follows that AniA and MmgR represent a case of a regulatory coherent feed-forward negative loop that contains both transcriptional regulation by a protein and post-transcriptional regulation by a sRNA [42]. Such modules were shown to be superior to direct regulation alone in both regulation efficiency and tolerance to noise [43]. In this way, upon the onset of PHB production, AniA sequestration into the growing PHB granule would relieve both its own repression and the transcriptional repression of phasin genes and of mmmR, leading to accumulation of phasins; upon exhaustion of the carbon excess and/or reducing power, PHB synthesis would decelerate and AniA would be in excess to restore the transcriptional repression over phasin production and mmmR expression (Fig. 6). During the growing stage of PHB granules, the feed-forward negative loop involving AniA and MmgR may serve to fine-tune the supply of phasin proteins to the size and number of PHB granules, as demonstrated by the uncontrolled accumulation of...
PHB granules that has been observed in S. meliloti cells having reduced MmgR levels [22].

We conclude that expression of the S. meliloti gene mmgR is subject to a dual control by transcriptional regulators whose output determines the level of MmgR sRNA as a function of two major inputs: the cell N status (with NtrC activating mmgR expression) and the availability of carbon and reducing power for storage as PHB (with AniA acting as a repressor). The involvement of NtrC in the regulation of PHB synthesis in response to varying C/N ratios has only been suggested in Azospirillum brasilense Sp7, although the mechanistic basis was not explored further [44]. Additional experimental work is required to demonstrate the direct interaction of AniA and NtrC – or of a subordinated regulator – with the presumed target sequences identified in this work within the mmgR promoter.

Fig. 6. Proposed model for the regulation and function of the sRNA gene mmgR in S. meliloti. The transcriptional regulator NtrC activates the transcription of mmgR under N-limiting conditions and this induction requires a conserved heptameric motif present in the –30 to –24 region of the promoter. Activation by NtrC may be direct or indirect through an unknown intermediate regulatory factor (X). In parallel, the mmgR promoter is subject to negative regulation by the transcriptional regulator of C flux AniA (PhaR), most likely by acting on the conserved dyadic motif TGC[N3]GCA located at –27 to –19 within the mmgR promoter. Given that MmgR is a negative regulator of phaP1 and phaP2 expression [22], AniA and MmgR constitute a coherent feed-forward negative loop over expression of phasin and phaZ genes. See Discussion for further details.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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