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

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Abstract

In the N_2-fixing symbiont of alfalfa root nodules, *Sinorhizobium meliloti* 2011, the *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 *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) *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 *mmgR* promoter elements is necessary and sufficient for induction by N limitation; (c) induction of *mmgR* requires the N-status regulator NtrC; (d) under C limitation, *mmgR* transcription is repressed by AniA, a global regulator of C flow; (e) the *mmgR* promoter contains a conserved dyadic motif (TGC[N_3]GCA) partially overlapping the heptamer TTGTGCA, which was also found in the promoters of the PHB-related genes *phaP1*, *phaP2*, *phaZ* and *phaR* (*aniA*) of *S. meliloti* and other alpha-proteobacteria. Taken together, these results suggest that the *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 N_2-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 EcpR1 [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 proteins, which results in larger cells containing much more PHB granules 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}: nutrient broth, 20; 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; $CaCl_2$, 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: KH_2PO_4, 1 g l^{-1}, K_2HPO_4, 1 g l^{-1}, $CaCl_2.2H_2O$, 0.1 g l^{-1}, $MgSO_4.7H_2O$, 0.25 g l^{-1}, MOPS, 10 g l^{-1} (pH 7.2), $FeCl_3$, 37 µM; biotin, 1 µM; thiamine, 33 µM; H_3BO_3, 50 µM; $MnSO_4.H_2O$, 10 µM; $ZnSO_4.7H_2O$, 1 µM; $NaMoO_4.2H_2O$, 0.5 µM; $CoCl_2.6H_2O$, 0.5 µM. These 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 NH_4Cl) 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, gentamicin 10 and 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 [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 with 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.PI. G1PELR05D12) carries the plasmid pK19mob*6*HMB [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.PI. G1PELR1E11) carries the plasmid pK19mob*6*HMB 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

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and features	Reference or source
E. coli		
DH5α	F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96* *relA1* Δ(*lacZYA-argF*)U169 *deoR* (Φ80d*lacZ*ΔM15)	[25]
MT616	MT607 (*pro-82 thi-I hsdR17 supE44*) :: pRK600, Cm^R	[45]
S17-1 λ*pir*	F$^-$ *pro thi hsdR recA*; chromosome :: RP4-2 Tc :: Mu Km :: Tn*7* Tp^R, Sp^R λ*pir*	[46]
S. meliloti		
2011	Wild-type, Sm^R	[47]
2011-Psm8G	2011 with a chromosomal P*mmgR-gfp* transcriptional fusion in psm8G, Sm^R, Gm^R	[23]
2011-Psm8mut	2011 with a chromosomal mutant P*mmgR-gfp* transcriptional fusion in psm8Gmut, Sm^R, Gm^R	This work
2011-Psm8(100)	2011 with a chromosomal P*mmgR-gfp* transcriptional fusion in psm8_100, Sm^R, Nm^R	[23]
2011-Psm145	2011 with a chromosomal P*sm145-gfp* transcriptional fusion in psm145, Sm^R, Gm^R	[23]
2011-Psm145mut	2011 with a chromosomal mutant P*sm145-gfp* transcriptional fusion in psm145mut, Sm^R, Gm^R	This work
2011*aniA*	2011 with an insertion of plasmid pK19mob*6*HMB within the *aniA* allele, Sm^R, Nm^R	This work
2011*aniA*-Psm8G	2011*aniA* with a chromosomal P*mmgR-gfp* transcriptional fusion in psm8G, Sm^R, Gm^R, Nm^R	This work
2011*ntrC*	2011 with an insertion of plasmid pK19mob*6*HMB within the *ntrC* allele, Sm^R, Nm^R	This work
2011*ntrC*-Psm8G	2011*ntrC* with a chromosomal P*mmgR-gfp* transcriptional fusion in psm8G, Sm^R, Gm^R, Nm^R	This work
Plasmids		
pCR 4-TOPO	Cloning vector, pUC *ori*, Ap^R, Km^R	Invitrogen
psm8G	pTH1705 carrying the *mmgR* promoter region, Gm^R	[23]
psm8Gmut	pTH1705 carrying the *mmgR* promoter region with a replacement of the heptamer TTGTGCA by AGAATAC, Gm^R	This work
psm8_100	pSRmig carrying a shortened *mmgR* promoter region (100 bp), Km^R	[23]
psm145	pTH1705 carrying the *sm145* promoter region, Gm^R	[23]
psm145mut	pTH1705 carrying the *sm145* promoter region with a replacement of the heptamer ATTCCGG by TTGTGCA, Gm^R	This work

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.

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

mM NH_4^+ (N)	mM sucrose	mM C	C/N molar ratio	Limited in
2.50	2.63	31.56	**12.6**	N (high C surplus)
2.50	2.05	24.56	**9.8**	N (low C surplus)
5.00	2.05	24.56	**4.9**	C

Analysis of promoter expression

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 (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. 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_{600} of the reporter strain culture and are presented as relative fluorescence units ($RFU=FU/OD_{600}$) as a function of culture OD_{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 electroblotted 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 to the membrane by exposure to UV light for 5 min. The membranes were then blocked with prehybridization buffer [50 % (w/v) formamide, 5×SSC, 50 mM phosphate buffer pH 7.0, blocking reagent 2 % (w/v), N-laurylsarcosine 0.1 % (w/v)] for 1 h at 43 °C in a hybridization oven and then incubated overnight at 50 °C with the hybridization buffer containing the specific DIG-labelled dsDNA probe, previously generated by PCR amplification of the *mmgR* genomic region with primers suhBf (TGTCGCTCTCTGCGAGGG) and suhBr (TTTCGGCGCCTATCTGCC). 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 *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 or both macronutrients at $OD_{600}<1.0$ for appropriate data collection in the microplate reader (Table 2). The only condition for which the cultures kept increasing the OD_{600} upon exiting the exponential phase was 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 *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 *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 *mmgR* expression and its modulation by the medium C/N ratio operates within the 100 bp lying upstream the *mmgR* transcription start site (Fig. 1b). Finally, the observed regulatory pattern (Fig. 1a) was specific for the *mmgR* promoter, because the activity of a chromosomal P_{sm145}-*gfp* fusion reporting the expression of an unrelated sRNA [23, 29] remained undistinguishable 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 *mmgR* transcription start site is required for induction of *mmgR* expression

Although *mmgR* orthologues are widespread among the alpha-proteobacteria [21], the *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 *mmgR* expression by replacement of the TTGTGCA motif by AGAATAC. As shown in Fig. 2a, the replacement had a strong impact on the P_{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)

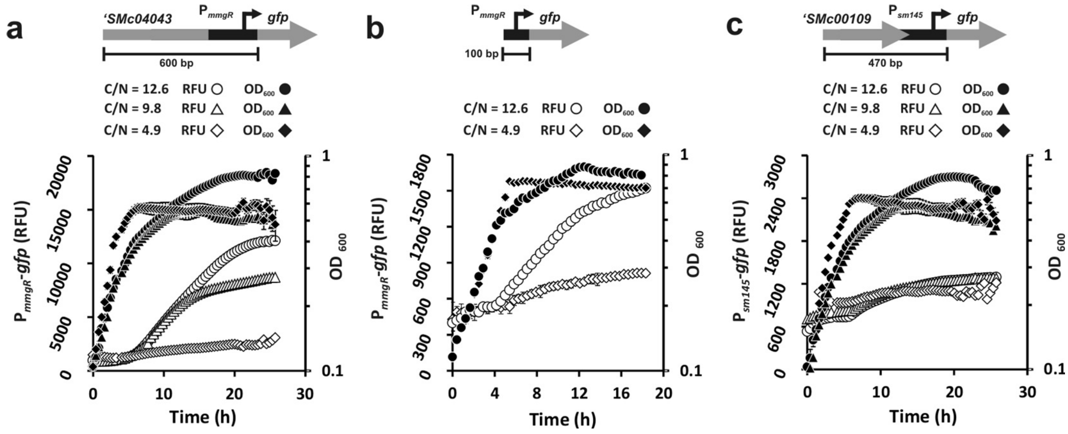

Fig. 1. Effect of the medium C/N ratio on *S. meliloti mmgR* expression. (a) Comparative expression pattern of a chromosomal P_{mmgR}-*gfp* fusion in strain 2011 (shown schematically above the expression curves) growing in modified RDM containing different C/N ratios (see Methods and Table 2). Curves with empty symbols correspond to P_{mmgR}-*gfp* activity whereas curves with filled symbols correspond to growth estimated as culture OD at 600 nm. (○, •) C/*N*=12.6 (limited in N, with a high C surplus); (△, ▲) C/*N*=9.8 (limited in N, with a low C surplus); (◊, ♦) C/*N*=4.9 (limited in C). (b) Same as (a), but cells contained a shorter version of the P_{mmgR}-*gfp* fusion (as shown schematically). (○, •) C/*N*=12.6 (limited in N, with a high C surplus); (◊, ♦) C/*N*=4.9 (limited in C). (c) Same as (a), but cells contained a chromosomal P_{sm145}-*gfp* fusion, involving the promoter of the unrelated sRNA gene *sm145* (as shown schematically). (○, •) C/*N*=12.6 (limited in N, with a high C surplus); (△, ▲) C/*N*=9.8 (limited in N, with a low C surplus); (◊, ♦) C/*N*=4.9 (limited in C). In all cases, error bars denote ±SE from triplicate cultures.

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. meliloti* 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. meliloti nifH* promoter and the promoters of several enterobacterial genes like *glnA*, *dhuA*, *argTr* 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 TTTTGCA (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 as the cause of *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. meliloti* 2011 [22]. As PHB levels in *S. meliloti* 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

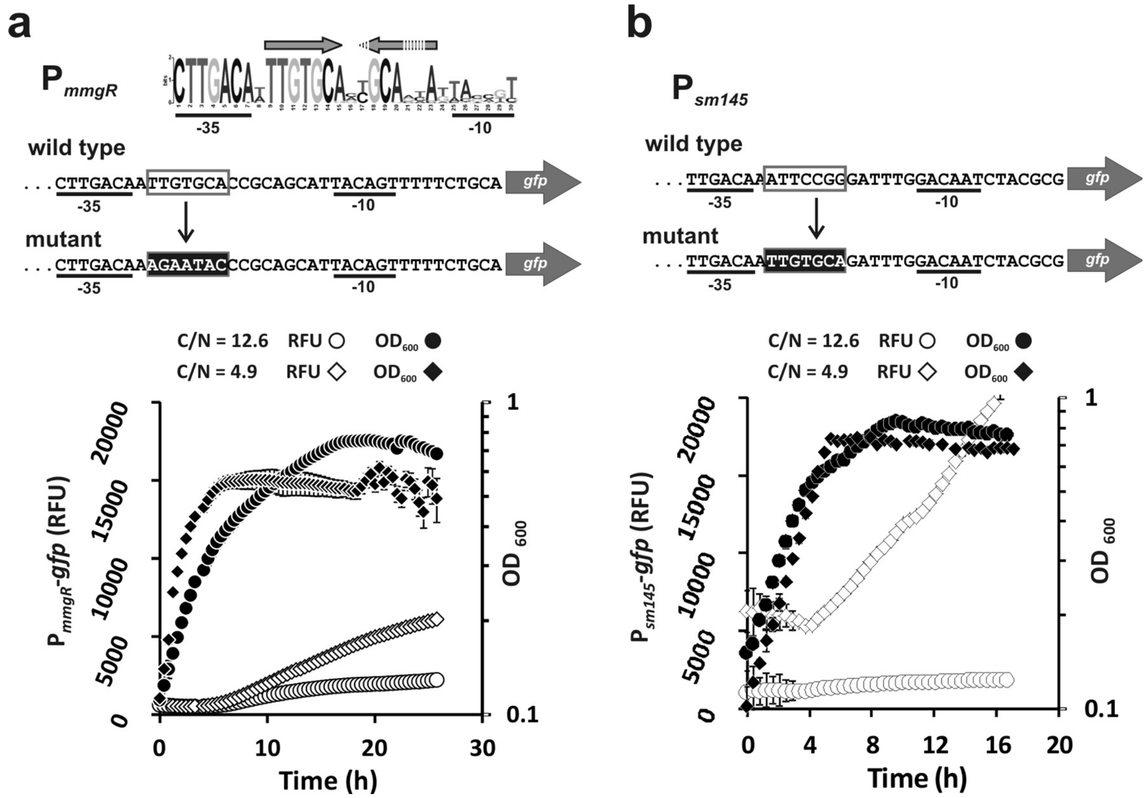

Fig. 2. Conserved heptamer TTGTGCA of the *mmgR* promoter is necessary and sufficient for the induction of expression by N limitation. (a) Top of the panel: sequence logo of the *mmgR* promoter [23] and detail of the sequence replacement to remove the conserved heptamer TTGTGCA from the wild-type P_{mmgR}-*gfp* fusion. The graph below the sequences shows the comparative expression pattern of the mutant P_{mmgR}-*gfp* fusion (TTGTGCA→AGAATAC) in strain 2011 in modified RDM containing different C/N ratios. Curves with empty symbols correspond to P_{mmgR}-*gfp* activity whereas curves with filled symbols correspond to growth estimated as culture OD at 600 nm. (○, •) C/*N*=12.6 (limited in N, with a high C surplus); (◊, ♦) C/*N*=4.9 (limited in C). (b) Detail of the sequence replacement made to the promoter of the *sm145* gene to insert the conserved heptamer TTGTGCA from the wild-type *mmgR* promoter. The graph below the sequences shows the comparative expression pattern of the mutant P_{sm145}-*gfp* fusion in strain 2011 in modified RDM containing different C/N ratios. (○, •) C/*N*=12.6 (limited in N, with a high C surplus); (◊, ♦) C/*N*=4.9 (limited in C). In all cases, error bars denote ±SE from triplicate cultures.

mmgR expression (see Fig. S2 for verification of *aniA* insertional inactivation). Interestingly, we found that under C-limiting growth conditions, the P_{mmgR}-*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 P_{mmgR}-*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 P_{mmgR}-*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 P_{mmgR}-*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 than in the wild-type for an *aniA* mutant 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 P_{mmgR}-*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

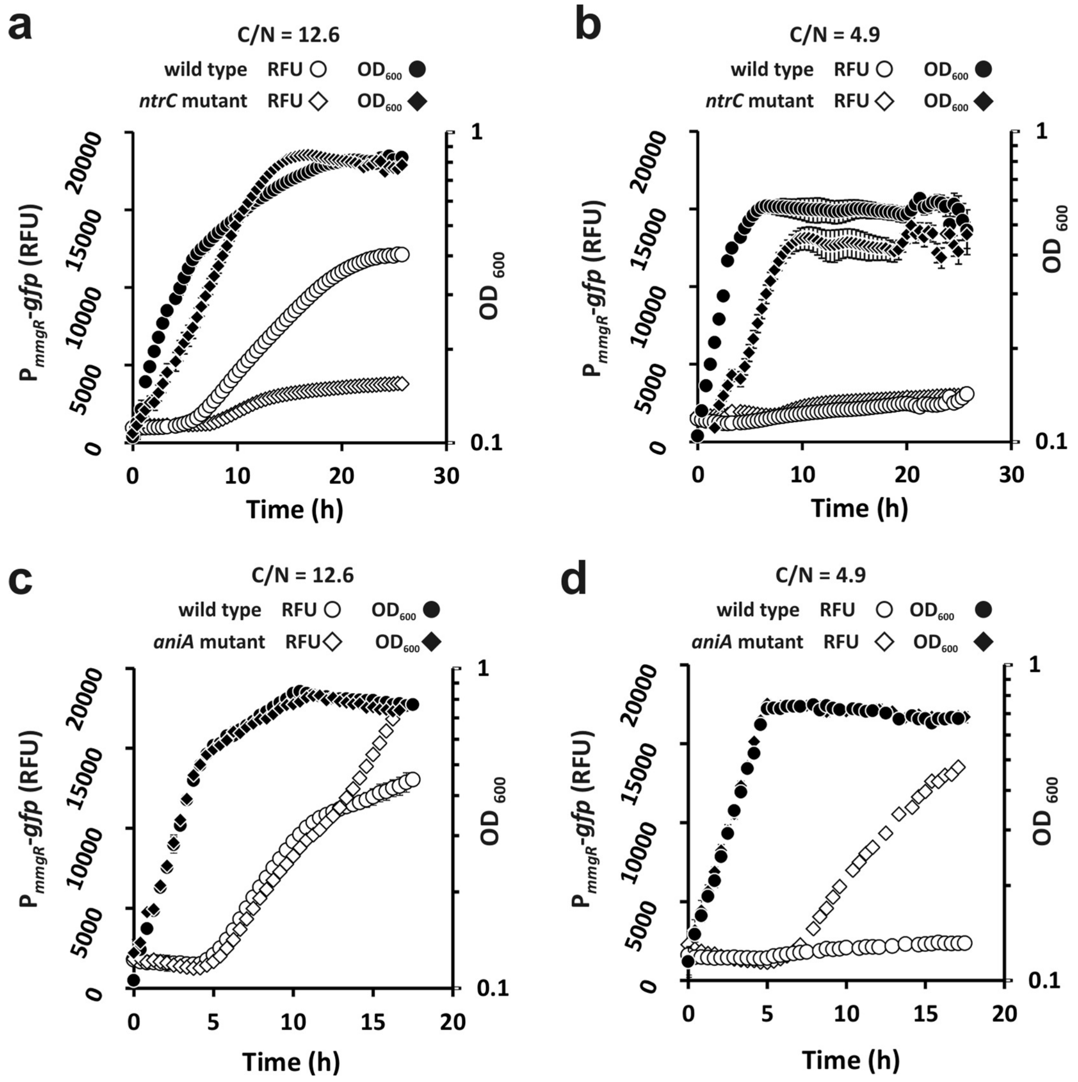

Fig. 3. Impact of insertional inactivation of *ntrC* and *aniA* on *S. meliloti mmgR* expression. Curves with empty symbols correspond to P_{mmgR}-*gfp* activity whereas curves with filled symbols correspond to growth estimated as culture OD at 600 nm. (a) wild-type (○, ●) and *ntrC* mutant (◇, ◆) at C/*N*=12.6 (limited in N, with a high C surplus). (b) wild-type (○, ●) and *ntrC* mutant (◇, ◆) at C/*N*=4.9 (limited in C). (c) wild-type (○, ●) and *aniA* mutant (◇, ◆) at C/*N*=12.6 (limited in N, with a high C surplus). (d) wild-type (○, ●) and *aniA* mutant (◇, ◆) at C/*N*=4.9 (limited in C). In all cases, error bars denote ±SE from triplicate cultures.

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

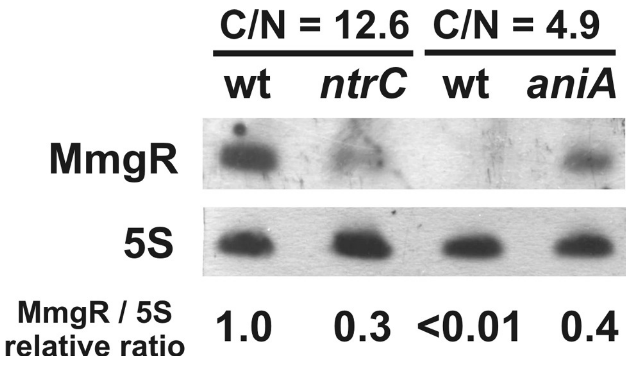

Fig. 4. Transcriptional regulators NtrC and AniA control expression of the *mmgR* sRNA gene. Northern blot analysis of MmgR and 5S rRNA transcript level in *S. meliloti* wild-type and *ntrC* or *aniA* insertional mutant strains in stationary phase under N-limiting (C/*N*=12.6) or C-limiting (C/*N*=4.9)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

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 heptamer reached

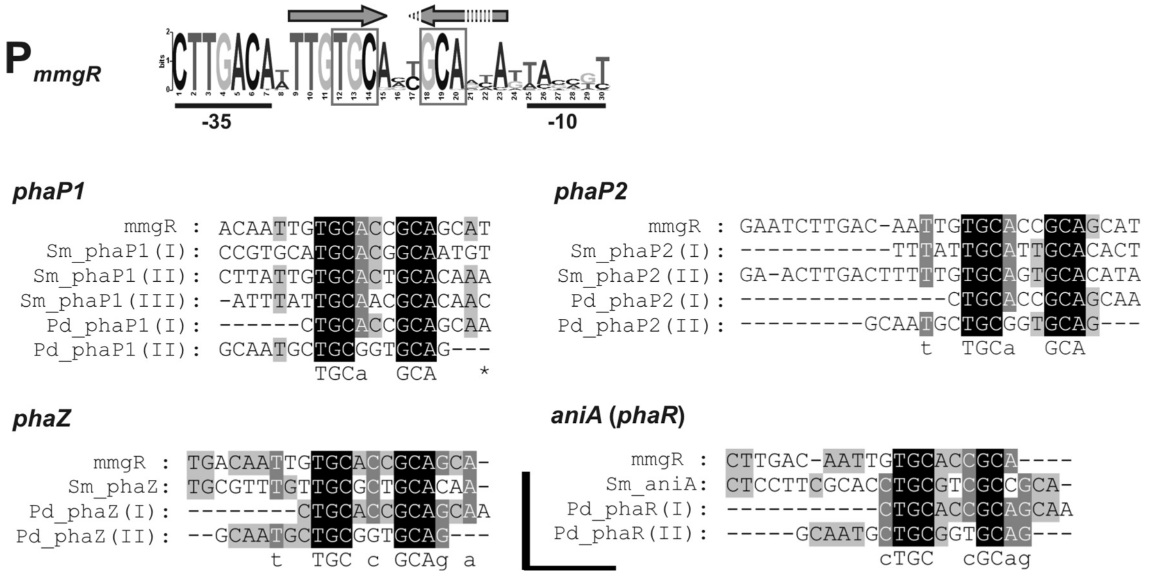

Fig. 5. The *mmgR* promoter contains a conserved putative-binding site for AniA, which is also present in the promoter regions of PHB-related genes. Multiple sequence alignment of promoter regions of *mmgR* and PHB-related genes containing the identified motif TGC[N$_3$]GCA. The alignments were done 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* encode a PHB depolymerase; *phaR* (*aniA*) encode 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).

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_{mmgR}-*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 *mmgR* expression [23]. These observations suggested a physiological link between the induction of *mmgR* 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 *mmgR* 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 *mmgR* 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 *mmgR*.

NtrC is required for full expression of *mmgR* 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 TTGTGCA in the *mmgR* 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 *mmgR* 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 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 *mmgR* 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 truncatula* 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 IId (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 *mmgR* 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 *mmgR* promoter to outcompete 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, as well as the post-transcriptional repression through MmgR, 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 dual repression over phasin production (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].

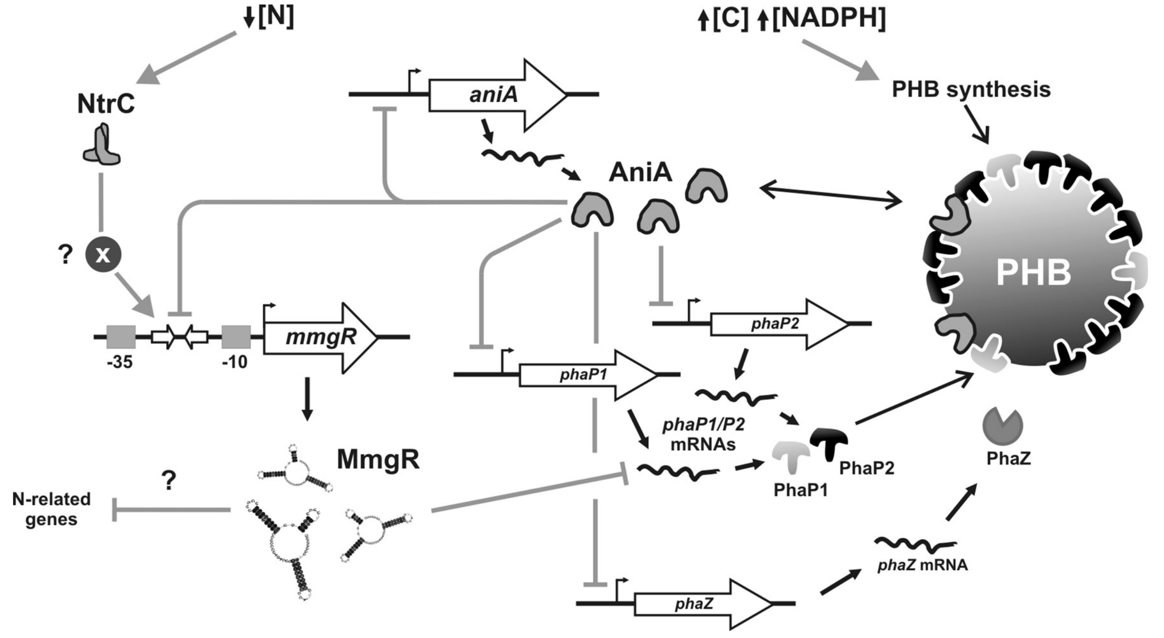

Fig. 6. Proposed model for the regulation and function of the sRNA gene *mmgR* in *S. meliloti* 2011. The transcriptional regulator NtrC activates the transcription of *mmgR* under N-limiting conditions and this induction requires a conserved heptameric motif present in the −30 to −16 region of the promoter. 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.

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.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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