The *ntrPR* Operon of *Sinorhizobium meliloti* Is Organized and Functions as a Toxin-Antitoxin Module

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The chromosomal *ntrPR* operon of *Sinorhizobium meliloti* encodes a protein pair that forms a toxin-antitoxin (TA) module, the first characterized functional TA system in *Rhizobiaceae*. Similarly to other bacterial TA systems, the toxin gene *ntrR* is preceded by and partially overlaps with the antitoxin gene *ntrP*. Based on protein homologies, the *ntrPR* operon belongs to the *vapBC* family of TA systems. The operon is negatively autoregulated by the NtrPNtrR complex. Promoter binding by NtrP is weak; stable complex formation also requires the presence of NtrR. The N-terminal part of NtrP is responsible for the interaction with promoter DNA, whereas the C-terminal part is required for protein-protein interactions. In the promoter region, a direct repeat sequence was identified as the binding site of the NtrPNtrR complex. NtrR expression resulted in the inhibition of cell growth and colony formation; this effect was counteracted by the presence of the antitoxin NtrP. These results and our earlier observations demonstrating a less effective downregulation of a wide range of symbiotic and metabolic functions in the *ntrR* mutant under microoxic conditions and an increased symbiotic efficiency with the host plant alfalfa suggest that the *ntrPR* module contributes to adjusting metabolic levels under symbiosis and other stressful conditions.

Additional keywords: autoregulation, direct repeat sequences.

Under conditions of nitrogen deprivation, soil bacteria belonging to the genera *Rhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium*, and *Bradyrhizobium* develop symbiotic interactions with their leguminous host plants. This highly complex multistep process, governed by signal exchanges between the partners, results in the formation of root nodules in which bacteroids are capable of reducing atmospheric nitrogen to ammonia. The fixed nitrogen is utilized by the host plant, which in turn, provides carbon sources and energy for the bacteroids.

The symbiotic efficiency of the bacterial partner is controlled at several levels of plant-bacterium interaction by the available exogenous nitrogen sources. Nodulation (*nod*) genes involved in the production of the first bacterial signal molecules, the Nod factors, are repressed in the presence of ammonium (Dusha et al. 1989). At a later stage of nodule develop-

ment, nitrogen fixation efficiency is also reduced, due to the decreased expression of *nif* genes encoding the nitrogenase enzyme (Oláh et al. 2001).

Previously, we showed that a Tn5 insertion into the chromosomal gene *ntrR* of *Sinorhizobium meliloti* resulted in increased transcription of *nod* and *nif* genes as compared with that of the wild-type strain, and this effect was more pronounced in the presence of an external ammonium source (Dusha et al. 1989; Oláh et al. 2001). As a result of this pleiotropic effect, alfalfa plants inoculated by the mutant strain had increased nitrogen content and biomass production (Oláh et al. 2001). Based on these observations, *ntrR* and the upstream *ntrP* gene were first proposed to function as nitrogen regulatory genes in *S. meliloti*. However, when the gene expression patterns of the entire genomes of wild type and *ntrR* mutant strains were compared under oxic and microoxic conditions, an unexpectedly large number of genes exhibited altered expression in the mutant strain. Since a relatively wide range of unrelated functions was modulated, we suggested that *ntrR* is not a nitrogen regulatory gene and a more general function for NtrR was proposed (Puskás et al. 2004).

Earlier the comparison of DNA sequence (and the deduced amino-acid sequence) of the *ntrPR* operon with databases revealed homology to the *vapBC* genes of *Dichelobacter nodosus* (Katz et al. 1992) and to related genes of various microorganisms (Puskás et al. 2004). Since the functions of the homologous systems were unknown or apparently unrelated, the molecular mechanism of NtrPR remained a question. As a further attempt, NtrP and NtrR were searched for conserved domains to elucidate their function. The NtrP protein was shown to carry a SpoVT/AbrB-like domain between amino acids 15 and 65 (Puskás et al. 2004). The AbrB domain, first described in a protein of *Bacillus subtilis* (Strauch et al. 1989), regulates a set of unrelated genes that contribute to the adaptive capacity for survival under suboptimal conditions and represents a novel class of DNA recognition domain (Vaughn et al. 2000). The NtrR protein had high homology to the PIN domain, which was described as the N-terminal domain of the PilT protein (Wall and Kaiser 1999; Wolfgang et al. 2000), and later was identified in many bacteria, *Archaea*, and eukaryota (Makarova et al. 1999). Apart from suggesting a possible role for the PIN domain in signaling processes (Noguchi et al. 1996), no information is available about its functional role in different proteins.

By using sequence analysis, motif-based studies, and secondary structure predictions, Clissold and Ponting (2000) identified PIN domains in proteins participating in nonsense-mediated mRNA decay and RNAi in eukaryotic cells. They found significant similarities between the 5' nuclease domain families and the PIN domain, which suggested a phosphodiesterase,

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more specifically RNase activity for PIN domain proteins. Based on characteristic amino-acid conservations, a metal-binding ability was also predicted.

By using bioinformatic approaches, Anantharaman and Aravind (2003) examined the evolutionary relationship between the eukaryotic nonsense-mediated RNA degradation system and bacterial toxin-antitoxin (TA) modules. Superfamilies of small uncharacterized proteins of bacteria and *Archaea* were described, in which characteristic organization of the coding regions was revealed by gene-neighborhood analysis. Two small genes formed an operon in which the first gene encoded an unstable antitoxin and the second gene determined a stable toxin protein. The toxins and antitoxins of different TA pairs may belong to unrelated superfamilies. In one of these families, the operon organization and domain architecture resembled that of the NtrPR proteins; the antitoxin was an AbrB/MazE homolog and the toxin belonged to the PIN domain family, which is also present in the proteins of the eukaryotic nonsense-mediated RNA decay system. Therefore, the PIN domain in toxin proteins was suggested to have an RNA-binding ability and to function similarly to known toxin proteins in cleaving ribosome-associated transcripts.

The first TA modules were identified on plasmids acting as post-segregational killing systems (Engelberg-Kulka and Glaser 1999; Gerdes 2000; Gerdes et al. 2005). Their function was to prevent the proliferation of plasmid-free progeny. In further experiments, TA loci were also identified on chromosomes and were considered to be associated with modulation of the global level of translation under conditions of nutrient limitation (Christensen et al. 2001) or under various stress conditions (Hazan et al. 2004; Sat et al. 2001, 2003). Chromosomal TA modules are characterized by a well-conserved structural organization, transcriptional autoregulation, and a similar function, although their targets in cells are different. The two adjacent genes of the operon partly overlap at the stop/start codons. The two proteins form a complex, thus the antitoxin prevents the lethal or bacteriostatic effect of the toxin. By binding to its own promoter, the complex negatively autoregulates the transcription of the module. Under nutritional stress (e.g., amino-acid starvation), transcription of TA modules decreases. Specific proteases, such as Lon or ClpA, activated under these conditions degrade antitoxin molecules faster than the stable toxins. The free toxin then cleaves mRNA positioned at the ribosomal A site between the second and third nucleotides. The site specificities of the cleavage described for RelE toxin (Pedersen et al. 2003) and MazF toxin (Muñoz-Gómez et al. 2004; Zhang et al. 2003a) are different. This RNase function reduces the global level of translation, resulting in a bacteriostatic state (Pedersen et al. 2002) or cell death (Amitai et al. 2004; Engelberg-Kulka and Glaser 1999).

The organization of the *ntrPR* operon identified in *S. meliloti* exhibits remarkable similarities to bacterial TA systems. The two partly overlapping genes encode two small proteins (90 and 134 amino acids in NtrP and NtrR, respectively) (Puskás et al. 2004). Preliminary data also indicated a negative autoregulatory function for NtrR (Oláh et al. 2001). The domain organization resembled one family of TA systems, the presence of the AbrB domain in the first and a PIN domain in the second protein (Anantharaman and Aravind 2003). Furthermore, based on the homology to the VapBC proteins described earlier (Oláh et al. 2001), the NtrPR protein pair may belong to the most highly abundant class of TA systems, the *vapBC* family (Gerdes et al. 2005).

Our objective was to examine whether or not, on the basis of their autoregulatory properties, complex formation, and function, the proteins NtrP and NtrR represent an active TA system in *Sinorhizobium meliloti*.

RESULTS

Autoregulation of the *ntrPR* operon.

Mapping of the transcription start site. By comparing the β -galactosidase activities expressed from an *ntrR-lacZ* fusion in wild-type strain *S. meliloti* 1021 and its *ntrR* mutant derivative (*S. meliloti* 399), we have shown previously that the NtrR protein may repress its own transcription (Oláh et al. 2001). If the *ntrPR* operon functions as a TA module, this observation may be due to the negative autoregulatory properties of NtrP and NtrR acting on their promoter as a protein complex. To further investigate this regulation, we performed electrophoretic mobility shift assays (EMSA).

We first determined the transcriptional starting point of the *ntrPR* operon by primer extension analysis. A 397-bp fragment of the *ntrPR* promoter region (obtained by polymerase chain reaction [PCR] amplification using the primers PPF and PPR; Table 1) was cloned in the pSEM211 vector and the resulting plasmid (pBM174) was mobilized into the *S. meliloti* 1021 wild-type strain by triparental conjugation. Total RNA was isolated from the resulting strain BM175, which was subjected to primer extension using primer 7 (Table 1). The signal obtained indicates that the transcriptional start point is the A nucleotide located 22 bp upstream of the translational start codon of the *ntrP* gene (Fig. 1).

Binding of NtrP and NtrR to the *ntrPR* promoter. We examined the binding abilities of NtrP and NtrR proteins to the 397-bp segment of the *ntrPR* promoter region in EMSA experiments. The fragment cloned in plasmid pBM24 was recovered by *XhoI*-*BamHI* digestion, was labeled by Klenow polymerase using α [³²P]-dCTP, and then, was exposed to crude extracts containing NtrP, NtrR, or both. Since, in *S. meliloti* 1021, the expression of the *ntrPR* operon was low (Oláh et al. 2001), we constructed recombinant plasmids carrying the *ntrP* gene (pBM136), or the *ntrR* gene (pBM135), or the whole *ntrPR* operon (pBM138) under the control of the arabinose-inducible promoter of the vector pBAD24 (Guzman et al. 1995). These constructs were introduced into *Escherichia coli* DH5 α , and the resulting strains (BM136, BM135, and BM138, respectively) were used to obtain crude cell extracts containing the corresponding proteins. To confirm that other proteins present in *E. coli* cells are not able to recognize the *ntrPR* promoter, a control binding reaction with crude extracts of *E. coli* containing the empty pBAD24 vector was performed (Fig. 2A, lane 2). As expected, a retarded band was obtained only when the promoter DNA was incubated with the extract containing both the NtrP and NtrR proteins (Fig. 2A, lane 5). When only the NtrP protein was present in the crude extract, a protein-DNA complex was also obtained, but the stability of the complex was very weak (Fig. 2A, lane 3). Although the concentration of the extract was 10-fold higher than that seen in lane 5, the protein-DNA complex remained unstable. Finally, when the crude extract containing the NtrR protein was incubated with the promoter fragment, no retarded complex appeared in the gel (Fig. 2A, lane 4).

To prove that the NtrPR complex binds specifically to the 397-bp promoter fragment, various negative control reactions were carried out such as the complex formation in the absence and presence (of various amounts) of competitor DNA, the addition of unlabeled promoter DNA to compete with the labeled fragment, and also the testing of two randomly selected DNA fragments (*groESL3* promoter and an internal fragment of the *nifA* gene) as target DNA (data not shown). The results confirmed that under the conditions shown in Figure 2, only the NtrPR complex was able to interact with the promoter region.

Data obtained on various TA systems proved that the N- and C-terminal parts of antitoxin and toxin proteins play different

roles in DNA-protein and protein-protein complex formation and in toxicity (Bernard and Couturier 1991; Ruiz-Echevarria et al. 1995; Zhang et al. 2003b). The presence of the AbrB-like domain in the N-terminal region of NtrP suggested that this part of the protein might be responsible for binding to the promoter DNA, whereas the second half of the molecule might be involved in the interactions with the NtrR protein.

In order to examine this, truncated versions of the *ntrP* and *ntrR* genes encoding the N- or C-terminal regions of the corresponding proteins were constructed (Fig. 2C). The truncated proteins, expressed together with the other member of the protein pair were placed under the control of the arabinose-inducible promoter in vector pBAD24. The constructs were introduced in *E. coli* DH5 α , and crude cell extracts were prepared for EMSA experiments. The 397-bp fragment carrying the *ntrPR* promoter region, prepared and labeled as in the experiment in Figure 2A, was used for binding assays. When the N-terminal part of NtrP (amplified region between primers 105 and 112, representing residues 1 to 56) was expressed together with the full-length NtrR (amplified region between primers 107 and 109) from plasmid pBM139, a complex was formed with the promoter fragment (Fig. 2B, lane 1). The migration distance differed from that of the complex obtained with full-length NtrP and NtrR (Fig. 2A, lane 5), which can be explained by the reduced size of the NtrP protein. Another construct on plasmid pBM140 carried the C-terminal part of NtrP (residues 46 to 90, plus the full-length NtrR amplified region between primers 110 and 109). The lack of a retarded band when using the cell extract of *E. coli* with pBM140 also supports the major role of the N-terminal part of the NtrP protein in the interaction with promoter DNA (Fig. 2B, lane 2). Two further constructs contained the full-length NtrP and the C- or N-terminal part of NtrR in plasmids pBM137 and pBM141, respectively. pBM137 was obtained by cloning NtrP (amplified region between primers 105 and 106) and a 79-amino acid long C-terminal region of NtrR (amplified region between primers 111 and 109). pBM141 was constructed by using the primers 105 and 113, therefore the N-terminal 64 residues of NtrR were present on the plasmid. When *E. coli* cell extracts carrying proteins expressed from either of

these plasmids were incubated with the promoter DNA fragment, the resulting complexes were less stable than those obtained with the full-length NtrR protein (Fig. 2B, lanes 3 and 4 compared with 2A, lane 5). This suggests an important role of

Fig. 1. Determination of the transcriptional start point of the *ntrPR* operon. **A**, Lane 1, Primer extension reaction using the total RNA of the strain BM175; lanes 2 to 5, sequencing reactions. The position of the band corresponding to the 5' end of *ntrP* transcript is indicated by an arrow and an asterisk in the sequence (left side). **B**, Nucleotide sequence of the *ntrPR* promoter region. The arrows show the transcriptional starting site (P) and the start codon of *ntrP*.

Table 1. Oligonucleotide sequences

Primer	Sequence 5' to 3'	Orientation and localization ^a
100 ^b	GATATCGGCATTCGTCTCCTTTGTAT	-(+3 to +22)
105 ^c	CCATGGTGGCCGTCCTTCGCGTCA	+(+24 to +44)
106 ^d	AGATCTAAGATAGCCGTTCAAAAG	-(+289 to +306)
107 ^b	GATATCTTTTGAACGGCTATC	+(+287 to +304)
108 ^c	CCATGGACGGCTATCTTCTCGATACGAA	+(+296 to +317)
109	AAAACGTGTGCGCTATCGCTC	-(+688 to +708)
110 ^c	CCATGGGACTCATTATCGAACC	+(+156 to +171)
111 ^d	AGATCTCTGGAAATCGTCCCGGTGCTG	+(+481 to +501)
112	GCTCCACGATATCGGCTG	-(+183 to +200)
113 ^e	CAGCACCGGGACGATTTACAG	-(+481 to +501)
PPF	AGGTCATCGAAGAGGGCGT	+(+317 to +299)
PPR	GGTTATTGCGGAACAGCTTT	-(+61 to +80)
116	TGTTTGTGCGCGCCGAGGCAGATCA	+(+61 to +38)
117	TTGCTTCTTCTGGAGGGGGCGCT	+(+144 to +121)
118	ATACGGCGTCGAGGGTCA	+(+232 to +215)
7	GGCGCGCCCCGGGTACC	
123 ^f	TCTAGAATGGTTCGGCATATACATTTAGGCATATACAAAGGAGACGAAGTCGAC	+(+24 to +18)
124 ^g	GTCGACTTCGTCTCCTTTGTATATGCCTAAATGTATATGCCGAACCATTTCTAGA	-(+24 to +18)
125	TTTAGGCATATACAAAGGAGACGAATGCCA	+(+7 to +23)
126	TGGCATTCGTCTCCTTTGTATATGCCTAAA	-(+7 to +23)

^a Positions are shown relative to the transcription start site. + and - before the parentheses indicate lower or upper DNA strand, respectively.

^b The primer contains an *EcoRV* site at the 5' end.

^c The primer contains an *NcoI* site at the 5' end.

^d The primer contains a *BglII* site at the 5' end.

^e The fourth nucleotide from the 3' end of the primer was changed from C to A to obtain a stop codon.

^f The oligo has an *XbaI* site at the 5' end and a *SalI* site at the 3' end.

^g The oligo has a *SalI* site at the 5' end and a *XbaI* site at the 3' end.

both parts of NtrR in stabilizing the DNA-NtrP/NtrR complex or in maintaining the correct structure of the protein required for a probable oligomerization.

Localization of the binding site for the NtrPR complex. To localize the binding sites for the autoregulatory proteins in the *ntrPR* promoter region, we first aimed to determine a shorter region necessary for the binding of the complex. In addition to the 397-bp fragment (cloned in plasmid pBM24), the following regions were amplified and cloned: a 312-bp fragment obtained with primers 118 and PPR (plasmid pBM170), a 224-bp fragment between primers 117 and PPR (plasmid pBM171), and a 141-bp fragment between primers 116 and PPR (plasmid pBM172). The localization of primers 118, 117, and 116 relative to the transcription start site is shown in Table 1. The cloned fragments were recovered from these plasmids by *XhoI*-*Bam*HI digestion, were labeled by Klenow polymerase with α [³²P]-dCTP, and were used in EMSA experiments. All of these DNA regions were fully active in complex formation with the *E. coli* extract containing the NtrP and NtrR proteins.

To define the exact binding site, DNase I footprint experiments were carried out using the 141- and 224-bp DNA fragments for the lower and upper strand reactions, respectively. When, prior to DNase I digestion, increasing amounts of *E. coli*(pBM138) crude extracts (carrying both NtrP and NtrR

under the control of the arabinose-inducible promoter) were added to the DNA fragments, a 31-bp sequence of the upper strand and a 34-bp sequence of the lower strand were protected by the protein complex (Fig. 3). This segment of DNA covers the transcription start site and is located close to the putative ribosome binding site GGAGA. The nucleotide sequence of this region revealed a direct repeat sequence 5'-GGCATATACA-(N₄)-GGCATATACA-3'. The data presented in Figure 3 indicate that these exact direct repeats are involved in binding the repressor complex. Interestingly, a palindrome sequence located 9 bp further upstream of the direct repeats is not protected by NtrP and NtrR under the conditions tested.

In order to investigate whether both direct repeats are required for protein binding, two oligonucleotide pairs were synthesized. One pair, 123 and 124 (35 bp) covered the complete repeated sequence (plus 7 nt upstream and 4 nt downstream; Table 1). The other oligonucleotide pair, 125 and 126 (30 bp) carried only the second half of the direct repeat (plus 4 nt upstream and 16 bp downstream). The oligonucleotide fragments were labeled by T₄ polynucleotide kinase using γ [³²P]-ATP and were tested in EMSA experiments. After incubating the labeled oligonucleotide fragments with extracts of *E. coli*(pBM138), complex formation was observed in both cases (Fig. 4), indicating that one of the direct repeated sequences (10 bp) is sufficient to accommodate the repressor complex.

Fig. 2. The binding ability of NtrP and NtrR (full-length and truncated proteins) to the *ntrPR* promoter region. The *ntrPR* promoter fragment (397 bp), amplified by PPF and PPR primers was exposed to crude cell extracts from *Escherichia coli* DH5 α carrying the constructs as indicated in part C. **A**, Lane 1, Free DNA fragment; lane 2, 1- μ g extract of *E. coli* DH5 α (pBAD24); lane 3, 0.5- μ g extract of *E. coli* DH5 α (pBM136); lane 4, 0.5- μ g extract of *E. coli* DH5 α (pBM135); and lane 5, 0.05- μ g extract of *E. coli* DH5 α (pBM138). **B**, The 397-bp fragment was incubated with the following extracts. Lane 1, 0.5- μ g extracts of *E. coli* DH5 α (pBM139); lane 2, 0.5- μ g extract of *E. coli* DH5 α (pBM140); lane 3, 0.5- μ g extract of *E. coli* DH5 α (pBM141); and lane 4, 0.5- μ g extract of *E. coli* DH5 α (pBM137). **C**, Schematic representation of the *ntrPR* operon. Gray and black bars represent the *ntrP* and *ntrR* genes, respectively. Arrows with numbers demonstrate the site and orientation of primers used for amplification. The lines between the arrows show the corresponding parts of *ntrP* or *ntrR* genes; above the lines the length of the proteins is indicated (AA = amino acid). The resulting recombinant plasmid constructs are listed on the right.

The function of the *ntrP* module of *S. meliloti*.

Toxicity of the NtrR protein. In order to examine the function of NtrP and NtrR, we tested the growth and viability of *E. coli* derivatives carrying plasmids with *ntrR*, *ntrP*, or both genes (plasmids pBM135, pBM136, and pBM138, respectively) controlled by the arabinose-inducible promoter (Fig. 5A). The expression of NtrP or NtrP and NtrR together did not affect the growth of *E. coli* cells, as was demonstrated by the identical optical densities of these cultures and the control cells carrying the pBAD24 vector. About 2 h after induction of the expression of the NtrR protein, the optical density of the culture was lower than those of the other cultures. By the end of the experiment (after 23 h), an optical density more than 2.5-fold lower than the controls was detected for the sample expressing NtrR. To determine the number of viable cells, samples from the cultures were plated and colonies were counted. The difference in living cell numbers observed already after 2 h resulted in a final decrease of about four orders of magnitude (Fig. 5B). These results demonstrate that the NtrR protein profoundly affects cell viability and this toxic effect can be prevented by the presence of the NtrP protein.

Response to amino-acid deprivation. During stringent response elicited by amino-acid starvation, guanosine 3',5'-bisphosphate (ppGpp) is synthesized by the RelA protein. Under such conditions, the expression of *mazEF* in *E. coli* was shown to be negatively regulated by ppGpp (Aizenman et al. 1996). In contrast, the transcription of another TA system, *relBE*, was

strongly induced during nutritional stress, and this activation was independent of ppGpp or *relA* (Christensen et al. 2001).

The accumulation of ppGpp following amino-acid starvation was also documented in the strain *S. meliloti* 1021 (Howorth and England 1999). We examined whether the expression from the *ntrPR* promoter is dependent on the level of ppGpp. Plasmid pBM90 carrying the *lacZ* gene under the control of the *ntrPR* promoter was introduced into the wild-type strain *S. meliloti* 1021, the Tn5 insertion mutant *S. meliloti* 399, and the *ntrPR* deletion derivative BM111. As expected, β -galactosidase activity was low in the wild-type strain and showed a similar low level also in the amino acid-starved samples (Fig. 6A, samples 1 and 4, respectively). Due to the lack of negative autoregulation, the activity in the mutant strains increased but the activities in samples incubated in the presence of serine hydroxamate (SH) (Fig. 6A, samples 5 and 6) were only slightly higher than the values in the control cultures (Fig. 6A, samples 2 and 3). The viability of strains *S. meliloti* 1021 and BM111 also showed a similar pattern when starved for amino acids for 22 h (Fig. 6B). Therefore, the expression of the *ntrPR* genes in free-living bacteria seems to be independent of starvation-induced ppGpp levels.

Effect of antibiotics on the viability of *AntrPR* mutant strain. Previously, it was described that antibiotics known to inhibit transcription and translation can trigger *mazEF*-mediated cell death by reducing the MazE antitoxin level (Sat et al. 2001). Three of these antibiotics, rifampicin, chloramphenicol, and

Fig. 3. DNase I footprint analysis of the *ntrPR* promoter. **A** and **B**, Protection of the lower and the upper DNA strand, respectively, by increasing amounts of crude extract of strain *Escherichia coli* DH5 α (pBM138). Lanes labeled with A, G, T, or C show sequencing reactions. Lane 1, No extract was added prior to DNase I digestion; lanes 2 to 6, 0.1, 0.2, 0.5, 0.7, and 1.2 μ g of protein extracts were added to protect DNA. A negative control reaction performed by adding 1 μ g of protein extract of the strain *E. coli* DH5 α (pBAD24) prior to DNase I digestion, resulted in the same pattern as shown in lane 1 of A and B (data not shown). **C**, The nucleotide sequence of the *ntrPR* promoter. Arrows indicate the transcription start site (+1) and the start codon of *ntrP*. Regions protected by protein binding are marked with bold letters. The two direct repeats are underlined. The palindrome sequence upstream of the protected region is shown by italic letters.

spectinomycin, were tested on the viability of wild-type *S. meliloti* 1021 and the BM111 strain carrying a deletion in the *ntrPR* operon (Fig. 7). The effect of these antibiotics on the survival of wild-type cells was less dramatic than that observed with *E. coli* cells (Sat et al. 2001); however, after a 10-min incubation, CFU per milliliter values were 15 to 30% higher with the BM111 strain than with the wild-type *S. meliloti* 1021. As a control, a similar experiment was carried out with claforan, an antibiotic inhibiting cell-wall synthesis, and the number of CFU decreased equally in the cultures of wild type and *AntrPR* mutant strains (Fig. 7).

DISCUSSION

In this paper, we demonstrated that the *ntrPR* operon of *S. meliloti*, which is structurally similar to bacterial TA modules, also exhibits the characteristic autoregulatory circuit of such modules. In addition, our results suggest that the function of the *ntrPR* operon is also related to that of chromosomal TA systems such as *mazEF* and *relBE* (Engelberg-Kulka and Glaser 1999; Gerdes 2000; Pedersen et al. 2003; Zhang 2003a). The present characterization of *ntrPR* is the first description of a functional TA system in members of family *Rhizobiaceae*.

TA modules identified up to the present were classified by Gerdes and associates (2005). Seven typical TA gene families were described; one of them is the most abundant *vapBC* family occurring in gram-positive and gram-negative bacteria and in *Archaea*. Based on amino acid sequence homology, secondary structure predictions, and domain organization, the *ntrPR* operon can be classified as a member of the *vapBC* family (Anantharaman and Aravind 2003; Gerdes et al. 2005). We have shown that NtrR, when expressed in the absence of the

NtrP protein, inhibits cell growth and viability (Fig. 5). The increased *nod* and *nif* gene expression in the *ntrR* mutant strain *S. meliloti* 399 (Dusha et al. 1989; Oláh et al. 2001) and the increased survival of *AntrPR* cells in the presence of antibiotics (controlling translation) might indicate that the cellular target of (at least this member of) the *vapC* toxin family could also be translation.

The autoregulatory functions of NtrPR have been investigated by electrophoretic mobility shift assays and DNase I footprinting. The antitoxin NtrP was able to recognize a DNA segment in the promoter region of the *ntrPR* operon, but the binding was weak, resulting in an unstable DNA-protein complex (Fig. 2A). The toxin component alone was not able to bind to the same DNA region, but cellular extracts containing both NtrP and NtrR shifted the promoter fragment (Fig. 2A), indicating that both proteins participate in the complex formation.

The observed mechanism of autoregulation seems to be general for TA modules, located either chromosomally or on plasmids. The antitoxin protein carries a DNA-binding motif and interacts with the promoter region with variable affinities (Engelberg-Kulka and Glaser 1999; Gerdes et al. 2005). The toxin, unable to bind DNA independently, enhances the stability of the antidote-DNA complex. Therefore, for the full transcriptional repression of the operon, both proteins are required. As an exception, ParD of the RK2 plasmid was found to be sufficient for full transcriptional regulation of the *parDE* operon (Eberl et al. 1992; Roberts et al. 1993).

Functional analysis of TA systems allows different roles to be attributed to the N- and C-terminal parts of toxins and antitoxins in protein-protein and protein-DNA interactions. In EMSA experiments, we examined various truncated proteins containing the N- or C-terminal half of the T or A proteins expressed together with the other full-length component of the module (Fig. 2B). Our results are in agreement with data obtained on other TA systems. The DNA-binding domain resides

Fig. 4. The role of direct repeats in the binding of NtrPR complex. **A**, Oligonucleotides 123 and 124 covering both direct repeats were used as DNA target incubated with extracts of *Escherichia coli* DH5 α (pBM138). Lane 1, No extract added; lane 2, 3.0- μ g extract of *E. coli* DH5 α (pBAD24); lanes 3 to 5, 1.0-, 3.0-, and 5.0- μ g extracts of *E. coli* DH5 α (pBM138) were used in binding reactions. **B**, Oligonucleotides 125 and 126 representing only one of the repeated sequences were incubated with cell extracts. Lane 1, Free DNA; lane 2, 3.0- μ g extract of *E. coli* DH5 α (pBAD24); lanes 3 to 5, 1.0-, 3.0-, and 5.0- μ g extracts of *E. coli* DH5 α (pBM138) were applied. **C**, Bold letters in the nucleotide sequences represent the oligonucleotides of the upper strand used in the experiments in panels A and B. Underlined letters show the direct repeats involved in DNA-protein interaction.

Fig. 5. Graphic representation of **A**, cell growth and **B**, viability of *Escherichia coli* derivatives with plasmids encoding NtrP (pBM136), NtrR (pBM135), and either both NtrP and NtrR (pBM138) proteins or none (pBAD24). The expression of these proteins was induced by adding 1% L-arabinose to the cultures. In panel B, the time scale is interrupted after 420 min, since the last samples were tested after 23 h. The experiments were repeated twice.

on the N-terminal region of antitoxin. In MazE and in seven homologous proteins, a conserved region designated as the N-box was identified, in which three conserved amino acids (Fig. 8, marked with asterisks) were important in DNA-binding functions (Zhang et al. 2003b). In the C-terminal part, a conserved Hp box with conserved hydrophobic residues was identified. These regions show homology to the corresponding parts of NtrP (Fig. 8), although the global homology among antitoxins, particularly at the C terminus, is rather low. The region from residue 38 to 75 of MazE, also including the Hp box, was shown to be responsible for the interaction with MazF toxin; residues L55 and L58 were essential (Zhang et al. 2003b; Fig. 8, marked by asterisks). We have no direct evidence for the role of the C-terminal region of NtrP in the interaction with NtrR, but the presence of a region homologous to the Hp box suggests a similar biological function.

The binding site for the NtrP protein in the promoter region was delimited. Even the shortest fragment of 141 bp exhibited the same protein-binding ability as was determined for the 397-bp fragment (data not shown). The 141-bp fragment comprises a 61-bp region upstream of the transcription start site, a 22-bp sequence between the transcription start and the translation start codon, and an additional 58-bp region downstream of ATG. Within this region a palindrome sequence, 5'-CAGATCA-TATC-TGATCTG-3', and a direct repeat, 5'-GGCATATACA-TTTA-GGCATATACA-3', were identified as potential binding sites (Fig. 3). DNase I footprint experiments revealed that the direct-repeat sequences are involved in the interaction with the autoregulatory NtrPR complex (Fig. 3), and these results were confirmed in DNA-binding assays, using oligonucleotide fragments as DNA targets (Fig. 4). Since the second repeat overlaps

Fig. 6. A, Expression of the *ntrPR* operon in amino acid-starved *Sinorhizobium meliloti* strains. β-Galactosidase activity expressed under the control of the *ntrP* promoter was determined in wild-type *S. meliloti* 1021 (black column), in the Tn5 insertion mutant *S. meliloti* 399 (gray column), and in the deletion derivative BM111 (empty column). Control samples (1 to 3) were grown without serine hydroxamate (SH), samples 4 to 6 were cultured with 1 mg of SH per milliliter. **B**, Viability of *S. meliloti* strains under amino-acid starvation. CFU per milliliter were calculated as a percent of viable cell numbers determined prior to SH addition. Triangles and filled squares represent the values of wild-type *S. meliloti* 1021 and the mutant strain BM111, respectively.

the transcription start site, the repressor activity of the complex is very probably due to interference with RNA polymerase binding.

The role of two palindrome sequences in the binding of anti-toxin-toxin complex has also been demonstrated for other TA systems, such as PemIK (Masuda et al. 1993; Tsuchimoto and Ohtsubo 1993) and PhdDoc (Magnuson and Yarmolinsky 1998). In the chromosomal *mazEF* promoter region, a unique structure with several palindromes located between -34 to +6 was identified. These sequences may exist in two possible configurations, and the MazEF complex can bind to either of these structures. Interestingly, a nearby perfect palindrome was not involved in protein binding (Marianovsky et al. 2001). In the promoter region of *ntrPR*, the role of the direct repeat sequences in complex formation was demonstrated, but the perfect palindrome sequence was not protected by the NtrPR complex in the DNase I footprint experiment (Fig. 3). Since, in the binding experiments, *E. coli* extracts containing NtrR and NtrP were used, we cannot exclude that this palindrome sequence may be involved in binding a protein present in *S. meliloti* cells. Interestingly, the binding of the factor for inversion stimulation (FIS) was also observed in the promoter region of *mazEF*, upstream of the alternating palindrome sequences, and the binding of FIS activated the *mazEF* promoter (Marianovsky et al. 2001).

Fig. 7. The effect of antibiotics on cell viability. Wild-type *Sinorhizobium meliloti* 1021 (empty bars) and the *ΔntrPR* mutant strain BM111 (filled bars) were treated for 10 min with rifampicin (10 μg/ml, Rif10), chloramphenicol (50 μg/ml, Cm50), spectinomycin (100 μg/ml, Sp100), and claforan (100 μg/ml, Cl100). Viable cell numbers (CFU/ml) are shown as percent of the corresponding control samples incubated without antibiotics.

Fig. 8. Comparison of the N box and Hp box identified in MazE (Zhang et al. 2003b) with the corresponding amino acid sequence of NtrP. In the N box, three amino acids essential for DNA-binding ability are marked by asterisks. In the Hp box, residues participating in the interaction with MazF toxin are labeled by asterisks. Homologous regions in the NtrP sequence are underlined.

Expression of the NtrR protein resulted in the inhibition of cell growth and colony formation. The effect of NtrR was less drastic than that of RelE or MazF (Aizenman et al. 1996; Gotfredsen and Gerdes 1998; Pedersen et al. 2002; Zhang et al. 2003a) when expressed in *E. coli* cells. While the number of colony-forming cells decreased by three to four orders of magnitude within 30 to 60 min when RelE or MazF was induced (Aizenman et al. 1996; Christensen et al. 2001; Pedersen et al. 2002), a similar decrease was detected only after 23 h in the presence of NtrR. The weaker inhibition could be due to a difference between the action mechanisms of NtrR and *E. coli* toxins. *S. meliloti*, as a soil bacterium, is adapted to survive in oligotrophic environments (Roszak and Colwell 1987), in which low oxygen tension and nutrient deprivation may limit growth in the free-living state. Soil bacteria may therefore tolerate longer periods of starvation by developing distinct mechanisms for their persistence under unfavorable conditions (Davey and de Bruijn, 2000). This assumption seems to be supported by the less powerful impact of the $\Delta ntrPR$ mutation on the survival of strains in the presence of antibiotics influencing either transcription, translation, or both.

The possible effect of altered ppGpp levels following amino-acid deprivation was also examined by measuring *lacZ* expression under the control of the *ntrPR* promoter in mutant *S. meliloti* derivatives defective in NtrPR production. This approach allowed testing of the regulatory effects on the promoter without the interference of autoregulation exerted by the variable levels of NtrP and NtrR or the possible influence of proteases on NtrP protein. The similar levels of β -galactosidase activities in starved and control cultures indicated that, in contrast to *mazEF* in *E. coli* (Aizenman et al. 1996), ppGpp has no direct regulatory role in controlling *ntrPR* expression. Our results show a ppGpp-independent expression of the *ntrPR* module in *S. meliloti* similar to that found for the *relBE* system in *E. coli* (Christensen et al. 2001).

Previously higher expression levels of plant factor-induced *nod* genes under free-living conditions and increased expression of *nif* genes in symbiosis with the host plant were demonstrated in strain *S. meliloti* 399 mutated in the *ntrR* gene as compared with wild-type *S. meliloti* 1021 (Dusha et al. 1989; Oláh et al. 2001). Investigations using the methods of functional genomics detected considerable changes in the general metabolic pattern of bacteria under such conditions (Ampe et al. 2003; Becker et al. 2003). Under microaerobic conditions, the expression of a large set of transcription and translation-related genes decreased to a low level (Puskás et al. 2004). However, downregulation was less effective in the *ntrR* mutant, resulting in a higher relative level of expression for these genes. Bacteria entering the plant root via the infection thread also encounter stressful conditions, which elicit physiological changes required for the transition from the free-living to the symbiotic state (Calderon-Flores et al. 2005; Moris et al. 2005; Wells and Long 2002). These results, together with our earlier observation on the wide-range transcriptional modulating effect of *ntrR* (Puskás et al. 2004), strongly suggest that the *ntrPR* module contributes to the adjustment of metabolic processes under such conditions. Experiments to test this assumption and to determine the cellular target of *ntrR* are in progress.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains and plasmids used in this work are described in Table 1. *E. coli* strains were grown at 37°C in LB medium (Maniatis et al. 1982) supplemented with the appropriate antibiotics (ampicillin, 100 µg/ml; kanamycin (Km), 50 µg/ml). L-arabinose was used at a final concentration of 1%. *S. meliloti*

strains were grown at 31°C in TA-enriched medium (Kondorosi et al. 1984) supplemented with the appropriate antibiotics (Km, 200 µg/ml, streptomycin, 200 µg/ml), unless otherwise stated.

Viability tests.

E. coli DH5 α cells with plasmids pBM120, pBM121, pBM124 or the vector pBAD24 were grown overnight in LB medium supplemented with ampicillin. The cultures were diluted in the same medium to obtain equal optical densities ($OD_{600} = 0.1$), and were induced with 1% L-arabinose. Cultures were grown at 37°C, and samples were removed to determine optical density and viable cell number.

The effect of antibiotics on viability.

S. meliloti 1021 wild type and BM111 deletion-derivative ($\Delta ntrPR$) strains were grown in complete TA medium to $OD_{600} = 0.4$, were centrifuged and washed with M9 minimal medium (Dusha et al. 1989), and then, were suspended in M9 containing antibiotics as indicated (rifampicin, 10 to 25 µg/ml; chloramphenicol, 50 to 75 µg/ml; spectinomycin, 100 to 200 µg/ml; claforan, 100 to 200 µg/ml). Samples were incubated for 10 min at 31°C and then were diluted and plated to determine the number of CFU per milliliter.

The expression of the *ntrPR* operon and viability of amino acid-starved *S. meliloti* strains.

Wild-type (*S. meliloti* 1021) and mutant (*S. meliloti* 399, BM111) strains with the plasmid pBM90 carrying the *ntrPR* promoter-*lacZ* fusion were grown in complete TA medium with appropriate antibiotics to obtain $OD_{600} = 0.4$. The cultures were centrifuged and resuspended in chemically defined RMM medium (Hooykaas et al. 1977) supplemented with tetracycline (Tc; 15 µg/ml). The samples were treated with SH (1 mg/ml final concentration) and were incubated for 17 h. The control samples were further cultured without SH to obtain OD_{600} values similar to those in the amino acid-starved samples after 17 h of incubation. β -galactosidase activities were determined in three independent experiments as described earlier (Dusha et al. 1989).

For viability tests, the wild type (*S. meliloti* 1021) and the deletion mutant (BM111) strains were grown in complete TA medium to $OD_{600} = 0.3$. After centrifugation, the cells were washed and resuspended in RMM medium supplemented with SH (1 mg/ml final concentration) and were further incubated for 22 h. Control samples were cultivated without SH addition. At different timepoints, viable cell numbers were determined. The experiments were repeated four times.

DNA manipulations.

Plasmids were transferred into *S. meliloti* strains by conjugation using the triparental mating system (Ditta et al. 1980). Preparation of plasmid DNA, digestion with restriction enzymes, agarose gel electrophoresis, fragment isolation, cloning procedures, and transformation of *E. coli* cells were performed according to Maniatis and associates (1982). Enzymes used for restriction and modification of DNA samples were obtained from Fermentas (Vilnius, Lithuania) or Promega (Madison, WI, U.S.A.).

Databases and software.

The complete genomic sequence of *Sinorhizobium meliloti* was available online. All other sequence analyses were done using the Bioedit program.

Strain and plasmid constructions.

Construction of an ntrPR deletion derivative of S. meliloti. A 4.4-kb *EcoRI*-*Sall* restriction fragment of the plasmid

pZV522.4 (Oláh et al. 2001) carrying the *ntrPR* operon was recloned in pBluescriptIISK. By *MunI*-*SphI* digestion, a fragment was removed that covered 184 bp upstream of the translation start of *ntrP*, the complete *ntrP* gene, and a large part of *ntrR* gene up to the *SphI* restriction site (located 89 bp upstream of the stop codon of *ntrR*). In this way, a 771-bp deletion was created. The *MunI* and *SphI* sites were filled by Klenow polymerase and T₄ DNA polymerase, respectively, and the Km^r gene was inserted between them. The deletion derivative of the *EcoRI*-*SalI* fragment with the Km^r marker was cut and recloned in the vector pRK290 at the *EcoRI* site (also filled by Klenow polymerase). This recombinant plasmid was introduced into wild-type *S. meliloti* 1021 by triparental mating, and the deletion was introduced into the chromosome by homologous recombination at the flanking regions. The resulting Km^r and Tc^s colonies were checked by Southern hybridization, and the strain BM111 was selected for further manipulations.

Plasmids containing the promoter region of *ntrPR* operon. To measure the expression of the *ntrPR* operon, a 339-bp fragment carrying the promoter region was amplified by PCR with primers PPF and 100 (Table 1), was filled by Klenow polymerase, and was inserted in pEP82 plasmid (Elö et al. 1998) upstream of the promoterless *lacZ* gene. The resulting plasmid pBM90 was introduced into wild type and mutant *S. meliloti* strains by conjugation. For transcriptional start determination, a DNA fragment was amplified by PCR with primers PPF and PPR (Table 1), the fragment was filled by Klenow polymerase and was cloned in the *EcoRI* site (also filled) of pSEM211 (Ferenczi et al. 2004) transcriptional fusion vector, resulting in the plasmid pBM174. The 397-bp fragment covered a 339-bp region upstream of the translational start point of *ntrP* and a 58-bp 5' fragment of the *ntrP* gene (Fig. 3B).

To localize the binding site of the autoregulatory NtrPR complex, plasmids carrying the promoter region on fragments of various lengths were constructed (Fig. 3B). DNA regions on 397-, 312-, 224-, and 141-bp fragments were amplified using the primers shown in Figure 1, were filled by Klenow polymerase, and were cloned in the *EcoRV* site of BluescriptIISK vector resulting in the plasmids pBM24, pBM170, pBM171, and pBM172, respectively. The *XhoI*-*BamHI* restriction fragments of these pBluescriptIISK derivatives were used for electrophoretic mobility shift assays.

Plasmids carrying different regions of the *ntr* operon under the arabinose-inducible promoter of pBAD24. Plasmids carrying intact or truncated genes of the *ntrPR* operon were constructed by cloning the appropriate PCR fragments first in pBM88, and then, the inserts were recloned in the vector pBAD24 (Fig. 2C). pBM88 is a derivative of pBluescriptII lacking the *EcoRI*-*SalI* region from the multicloning site. The following plasmids were obtained: pBM121 with the 105 to 106 PCR fragment, pBM120 with the 108 to 109 PCR fragment, pBM124 with the 105 to 109 PCR fragment, pBM125 with the 105 to 112 PCR fragment, pBM126 with the 107 to 109 PCR fragment, pBM128 with the 110 to 109 PCR fragment, pBM122 with the 111 to 109 PCR fragment, and pBM130 with the 105 to 113 PCR fragment. Two additional plasmids were created in consecutive steps, pBM123 carrying 105 to 106 and 111 to 109 fragments fused in-frame at a *BglII* restriction site and pBM127 containing 105 to 112 and 107 to 109 fragments fused in-frame at an *EcoRV* restriction site. For the creation of plasmid BM123, we used plasmids pBM121 and pBM122 carrying the PCR fragments mentioned above. pBM121 was digested by *BglII* (restriction site present on the 5' end of primer 106) and *BamHI* (present on the polylinker) and subsequently used as a vector for cloning the *BglII*-*BamHI* fragment isolated from pBM122. In this way, the *ntrP* gene was fused in-frame to the second part of the *ntrR* gene at a

BglII site. In the case of plasmid pBM127, plasmids pBM125 and pBM126 were used. pBM125 was digested with *EcoRV* and *BamHI* and was used as a vector for cloning the *EcoRV*-*BamHI* fragment isolated from pBM126, obtaining in this way an in-frame fusion of the first part of the *ntrP* gene with the *ntrR* gene.

The oligonucleotide primers used for the various constructions are listed in Table 1. The pBM88 derivatives carrying the cloned fragments were digested with *NcoI* (present on the fragment) and *BamHI* (present on the multicloning site of the vector), and the inserts were recloned in the *NcoI*-*BamHI*-digested pBAD24 vector, resulting in the plasmids listed in Figure 2C. In the case of pBM141, the cloned fragment was cut with *NcoI*-*KpnI* enzymes, and after filling the *KpnI* site with Klenow polymerase, the fragment was cloned in the *NcoI*-*BamHI*-digested pBAD24 vector (also filled at the *BamHI* site). Thus, the *NcoI* restriction site of pBAD24 and the translational start codon ATG were restored in plasmids pBM135, pBM136, pBM137, pBM138, pBM139, pBM140, and pBM141, due to the presence of an *NcoI* site at the 5' end of the corresponding primers.

Crude extracts of cellular proteins.

Cellular extracts were made from *E. coli* DH5 α (pBAD24) as control and *E. coli* DH5 α derivatives carrying pBM135, pBM136, pBM137, pBM138, pBM139, pBM140, and pBM141 plasmids described in Table 2. The strains were grown at 37°C in LB medium supplemented with ampicillin to OD₆₀₀ = 0.8. The arabinose promoter of pBAD24 vector was next induced by adding 1% L-arabinose to the cultures, and the strains were grown for additional 30 min. Cells were harvested, sonicated, and centrifuged to remove cell debris. After the addition of 10% glycerol, supernatants were stored at -80°C. Protein concentrations of the samples were measured by the Bradford assay (Bio-Rad, Hercules, CA, U.S.A.).

Gel mobility-shift assays.

Crude extracts were diluted to obtain various protein concentrations (discussed above) by using the binding buffer (0.1 M Tris HCl, pH 7.4, 2 mM EDTA, 1 mM dithiothreitol, 5 mM MgCl₂, 5% glycerol). To inactivate nucleases, the samples were heated at 65°C for 3 min. Mixtures were cooled to room temperature, and then, 2 μ g of herring sperm DNA and 3 μ l of labeled DNA fragment were added to 10 μ l of crude extract (Marianovsky et al. 2000). DNA fragments for gel mobility-shift assays were obtained from plasmids pBM24, pBM170, pBM171, and pBM172 by *XhoI*-*BamHI* digestions, and the fragments were labeled with α [³²P]-dCTP using Klenow polymerase. When oligonucleotides (123 to 126) were applied as target DNA, the samples were end-labeled with γ [³²P]-ATP using T₄ polynucleotide kinase. Binding reactions were carried out at room temperature for 10 min and were then loaded onto 4 or 8% native polyacrylamide gels and run in tris-borate-EDTA buffer (pH 8) at 150 V at 4°C.

Transcription start determination.

Total RNA was extracted from *S. meliloti* BM175 using the RNeasy mini kit (Qiagen, Basel, Switzerland). Primer extension experiments were carried out with Tth DNA polymerase (Promega). Primer 7, which corresponds to the sequence located within the 5' end of the *lacZ* gene on the vector plasmid pSEM211, was end-labeled with γ [³²P]-ATP by using T₄ polynucleotide kinase. The reaction mixture (50 μ l) contained 2 pmoles of end-labeled primer, 46 μ g of total RNA from *S. meliloti* BM175, 0.2 mM each dNTP, 2 M betaine, 1 mM MnCl₂, and 15 units of Tth DNA polymerase. The reaction was performed at 75°C for 30 min and was terminated by adding EGTA

(final concentration of 20 mM). The extended products were resolved on a denaturing 6% polyacrylamide gel. Sequencing reactions as controls were performed on plasmid pBM174 with primer 7 and were run on the gel parallel to the primer extension reaction. The gel was analyzed using Storm 840 Phosphorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

DNase I footprinting.

Binding reactions were carried out as described for gel mobility-shift assays. Various amounts of cellular extracts, calculated according to the binding efficiencies determined previ-

ously, were added to the reaction mixtures. After incubation for 10 min, reaction mixtures were treated with 0.2 U of DNase I at room temperature for 3 min and reactions were inactivated at -80°C . For the lower strand, a 224-bp DNA fragment (obtained by PCR amplification with primer 117 and PPR) and, for the upper strand, a 141-bp DNA fragment (obtained by PCR amplification with primers 116 and PPR) were used and labeled with $\gamma[^{32}\text{P}]\text{-ATP}$ by using T_4 polynucleotide kinase. Markers were generated by carrying out a Sanger AG-only sequencing reaction. The products were separated by electrophoresis on a 6% sequencing gel and were visualized by autoradiography.

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	References
Strains		
<i>E. coli</i>		
DH5 α	<i>supE44 D lacU169 hsdR17 recA1 endA1gyrA96 thi-1 relA1</i>	Maniatis et al. 1982
<i>Sinorhizobium meliloti</i>		
1021	Wild type, Sm ^r derivative of SU47	F. M. Ausubel
<i>S. meliloti</i> 399	<i>S. meliloti</i> 1021 with a Tn5 insertion in the <i>ntrR</i> gene	Dusha et al. 1989
BM175	<i>S. meliloti</i> 1021 carrying the pBM174, Sm ^r , Km ^r	This work
BM111	<i>S. meliloti</i> 1021 carrying a deletion in the <i>ntrPR</i> operon	This work
Plasmids		
pBluescriptII (SK+/-)	Amp ^r , f1 (+) <i>ori</i>	Alting-Mees and Short 1989
pSEM211	Km ^r , pCU1 replicon, RP4 <i>mob</i> , promoterless <i>lacZ</i>	Ferenczi et al. 2004
pEP82	Tet ^r , derivative of pRK290 carrying a promoterless <i>lacZ</i> gene	Élö et al. 1998
pBM174	Km ^r , derivative of pSEM211 carrying the PPF-PPR polymerase chain reaction (PCR) product cloned in the <i>EcoRI</i> site	This work
pBM88	Amp ^r , derivative of pBluescriptII(SK+/-) lacking the <i>EcoRI-SalI</i> region	This work
pBM90	Tet ^r , derivative of pEP82 carrying the 339 bp <i>ntrPR</i> promoter fragment upstream of the <i>lacZ</i> gene	This work
pBAD24	Amp ^r , ColE1 replication origin, pBAD promoter	Guzman et al. 1995
pBM120	Amp ^r , derivative of pBM88 carrying the 108 to 109 PCR fragment cloned in the filled <i>XhoI</i> site (<i>ntrR</i> gene)	This work
pBM121	Amp ^r , derivative of pBM88 carrying the 105 to 106 PCR fragment cloned in the filled <i>XhoI</i> site (<i>ntrP</i> gene)	This work
pBM122	Amp ^r , derivative of pBM88 carrying the 111 to 109 PCR fragment cloned in the filled <i>XhoI</i> site (second part of the <i>ntrR</i> gene)	This work
pBM123	Amp ^r , derivative of pBM121 and pBM122 carrying the 105 to 106 and 111 to 109 PCR fragments (<i>ntrP</i> and the second part of <i>ntrR</i> fused in-frame through a <i>BglII</i> site)	This work
pBM124	Amp ^r , derivative of pBM88 carrying the 105 to 109 PCR fragment cloned in the filled <i>XhoI</i> site (<i>ntrPR</i> gene)	This work
pBM125	Amp ^r , derivative of pBM88 carrying the 105 to 112 PCR fragment cloned in the filled <i>XhoI</i> site (first part of <i>ntrP</i> gene)	This work
pBM126	Amp ^r , derivative of pBM88 carrying the 107 to 109 PCR fragment cloned in the filled <i>XhoI</i> site (<i>ntrR</i> gene)	This work
pBM127	Amp ^r , derivative of pBM88 carrying the 105 to 112 and 107-109 PCR fragments (first part of <i>ntrP</i> and the <i>ntrR</i> gene fused in frame through an <i>EcoRV</i> site)	This work
pBM128	Amp ^r , derivative of pBM88 carrying the 110 to 109 PCR fragment cloned in the filled <i>XhoI</i> site (second part of <i>ntrP</i> and the <i>ntrR</i> gene)	This work
pBM130	Amp ^r , derivative of pBM88 carrying the 105 to 113 PCR fragment cloned in the filled <i>XhoI</i> site (<i>ntrP</i> and the first part of the <i>ntrR</i> gene)	This work
pBM135	Amp ^r , derivative of pBAD24 carrying the <i>NcoI-PstI</i> fragment of pBM120 (<i>ntrR</i> gene)	This work
pBM136	Amp ^r , derivative of pBAD24 carrying the <i>NcoI-PstI</i> fragment of pBM121 (<i>ntrP</i> gene)	This work
pBM137	Amp ^r , derivative of pBAD24 carrying the <i>NcoI-PstI</i> fragment of pBM123 (<i>ntrP</i> gene and the second part of <i>ntrR</i>)	This work
pBM138	Amp ^r , derivative of pBAD24 carrying the <i>NcoI-PstI</i> fragment of pBM124 (<i>ntrPR</i> gene)	This work
pBM139	Amp ^r , derivative of pBAD24 carrying the <i>NcoI-PstI</i> fragment of pBM127 (first part of <i>ntrP</i> and the <i>ntrR</i> gene)	This work
pBM140	Amp ^r , derivative of pBAD24 carrying the <i>NcoI-PstI</i> fragment of pBM128 (second part of <i>ntrP</i> and the <i>ntrR</i> gene)	This work
pBM141	Amp ^r , derivative of pBAD24 carrying the <i>NcoI-KpnI</i> (<i>KpnI</i> filled in) fragment of pBM130 (<i>ntrP</i> and the first part of the <i>ntrR</i> gene)	This work
pBM24	Amp ^r , derivative of pBluescriptII(SK+/-) carrying the PPF-PPR PCR fragment in the <i>EcoRV</i> site (397 bp <i>ntrP</i> promoter region)	This work
pBM170	Amp ^r , derivative of pBluescriptII(SK+/-) carrying the 116-PPR PCR fragment in the <i>EcoRV</i> site (312-bp <i>ntrP</i> promoter region)	This work
pBM171	Amp ^r , derivative of pBluescriptII(SK+/-) carrying the 117-PPR PCR fragment in the <i>EcoRV</i> site (224-bp <i>ntrP</i> promoter region)	This work
pBM172	Amp ^r , derivative of pBluescriptII(SK+/-) carrying the 118-PPR PCR fragment in the <i>EcoRV</i> site (141-bp <i>ntrP</i> promoter region)	This work
pRK2013	Km ^r , helper plasmid for mobilization of pRK290 derivatives, tra (RK2)	Ditta et al. 1980

^a Sm^r, Km^r, Amp^r, and Tet^r = streptomycin, kanamycin, ampicillin, and tetracycline resistant.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

RhizoBase, the genome database for rhizobia:
www.kazusa.or.jp/rhizobase/