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Mutation in the *ntrR* Gene, a Member of the *vap* Gene Family, Increases the Symbiotic Efficiency of *Sinorhizobium meliloti*

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In specific plant organs, namely the root nodules of alfalfa, fixed nitrogen (ammonia) produced by the symbiotic partner *Sinorhizobium meliloti* supports the growth of the host plant in nitrogen-depleted environment. Here, we report that a derivative of *S. meliloti* carrying a mutation in the chromosomal *ntrR* gene induced nodules with enhanced nitrogen fixation capacity, resulting in an increased dry weight and nitrogen content of alfalfa. The efficient nitrogen fixation is a result of the higher expression level of the *nifH* gene, encoding one of the subunits of the nitrogenase enzyme, and *nifA*, the transcriptional regulator of the *nif* operon. The *ntrR* gene, controlled negatively by its own product and positively by the symbiotic regulator *syrM*, is expressed in the same zone of nodules as the *nif* genes. As a result of the nitrogen-tolerant phenotype of the strain, the beneficial effect of the mutation on efficiency is not abolished in the presence of the exogenous nitrogen source. The *ntrR* mutant is highly competitive in nodule occupancy compared with the wild-type strain. Sequence analysis of the mutant region revealed a new cluster of genes, termed the "*ntrPR* operon," which is highly homologous to a group of *vap*-related genes of various pathogenic bacteria that are presumably implicated in bacterium–host interactions. On the basis of its favorable properties, the strain is a good candidate for future agricultural utilization.

One of the major growth-limiting factors for plants is the amount of available nitrogen. Plants utilize fixed nitrogen obtained from various sources such as photorespiration, mineral nitrogen fertilizers, metabolic pathways (e.g., decomposition of amino acids and phenylpropanoids), or from the soil in the form of nitrate. Leguminous plants have a unique additional ability to develop symbiotic interactions with soil bacteria (*Rhizobium*, *Sinorhizobium*, *Azorhizobium*, and *Bradyrhizobium* species), which are capable of reducing atmospheric nitrogen to ammonia in the root nodules. Nitrogen-fixing bacteroids in nodules provide reduced nitrogen for the host plant that, in turn, supplies bacteroids with carbon sources and energy.

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Biological nitrogen fixation is secondary only to photosynthesis as the most important biochemical process. Because of its significance, efforts to optimize and increase the nitrogen fixation potential of symbiotic systems were reported (Maier and Triplett 1996). To construct better bacterial inoculants, either the nodulation potential and the ability to compete successfully with indigenous strains or the nitrogen fixation capacity was improved. Promising derivatives, however, often failed to increase plant yield when exogenous nitrogen was not limiting or if only one of the required abilities was optimized. Here, we show that in a derivative of *Sinorhizobium meliloti*, the mutation of the *ntrR* gene results in a pleiotropic effect on the symbiotic properties with alfalfa.

Previously, we have shown that exogenous nitrogen (ammonium) repressed the expression of nodulation (*nod)* genes involved in the Nod factor production of *S. meliloti*, resulting in decreased root nodule formation (Dusha et al. 1989). As a result of a mutation in the chromosomal *ntrR* gene, the nitrogen repression of *nod* genes was released. The *ntrR* mutant strain *S. meliloti* 399 formed nodules on alfalfa roots earlier and in higher numbers than in the wild-type strain, even in the presence of fixed nitrogen (Dusha et al. 1989). These data demonstrate that the bacterium contributes to controlling nodule initiation by adjusting the optimum number of nodules, depending upon the level of fixed nitrogen.

Preliminary data indicated that alfalfa plants inoculated with *S. meliloti* 399 developed not only a higher number of nodules, but the plants also had higher dry weight values when compared with those of the plants inoculated with wild-type bacteria. Increased symbiotic efficiency may imply an effect of *ntrR* mutation on nitrogen fixation by the bacteroids. Our aim was to elucidate the molecular basis of this phenomenon; therefore, the possible influence of *ntrR* mutation was examined at two levels: the expression of bacterial *nif* genes encoding for i) one of the subunits of the nitrogenase enzyme (*nifH*) and ii) the positive regulator protein (*nifA*), which was studied in bacteroids formed by the mutant strain.

The reduction of molecular nitrogen to ammonia catalyzed by the nitrogenase enzyme is very tightly controlled at the transcriptional level in *S. meliloti*. The regulatory protein

NifA, induced at low free oxygen concentrations, activates the transcription of *nif* genes. The signal of microaerobiosis is conveyed to *nifA* by the two-component sensor–regulator system FixLJ (David et al. 1988). FixJ acts as a transcriptional activator when phosphorylated by FixL (Agron et al. 1993; Reyrat et al. 1993), which senses the environmental oxygen level as a signal. Microaerobiosis seemed sufficient to induce *nifA* expression and occurred also in the presence of fixed nitrogen (Ditta et al. 1987). Therefore, oxygen concentration was considered to be the primary regulatory factor of *nif* expression in symbiotic nodules. Contradictory results were obtained when ammonium was applied as a nitrogen source, i.e., the repression of *nifA* was observed under microaerobic conditions (Noonan et al. 1992). As a result of conflicting data, we investigated whether the mutation in the *ntrR* gene affected *nif* gene expression and whether the regulation was altered at various levels of exogenous nitrogen. Furthermore, we characterized the *ntrR* gene, its regulation in free-living and symbiotic conditions, and its homology with the *vap* gene family.

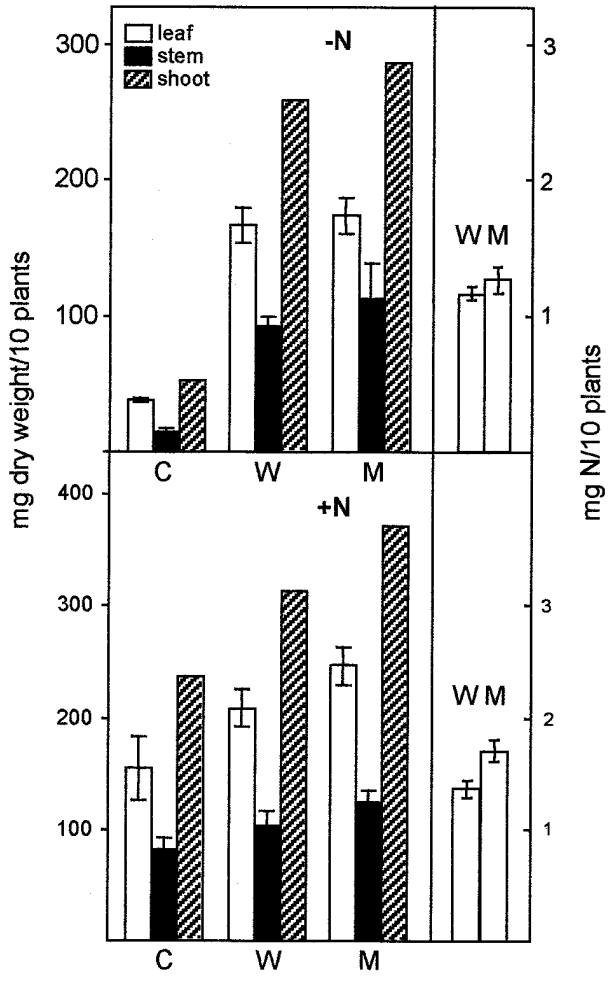

Fig. 1. Dry matter yield and nitrogen content of alfalfa plants inoculated with wild-type or *ntrR* mutant *Sinorhizobium meliloti*. Plants grown under nitrogen-free conditions (–N: upper panel) or with 2 mM ammonium sulfate (+N: lower panel) were either uninoculated (C) or infected with wild-type (W) or *ntrR* mutant (M) strains. Left: Dry weights of leaves (empty bar) and stems (filled bar) and the sum of the two values (hatched bar). Right: Nitrogen content. Standard deviation was calculated from the data of 500 individual plants. Shoot dry weight and nitrogen content of plants inoculated by the *ntrR* mutant were significantly higher when grown with nitrogen compared with the wild-type values ($P = 5$ and 1%, respectively).

RESULTS

Mutant strain *S. meliloti* 399 exhibits improved symbiotic efficiency.

Strain *S. meliloti* 399 induced nodules on *Medicago sativa* earlier and at higher number than did the wild-type strain *S. meliloti* 1021 (Dusha et al. 1989). The mutant strain also retained its increased nodulation ability in the presence of fixed nitrogen. To verify that inoculation by the mutant strain results in a more effective symbiosis, alfalfa plants were infected with either the wild-type strain or the mutant *S. meliloti* 399, and the plants were analyzed for dry matter yield and nitrogen content. To decrease the effect of biological variation, a high number of individual plants (500 seedlings for each treatment) was inoculated.

Under nitrogen-free conditions, the leaf and stem dry weight of the mutant-inoculated plants increased by 4 and 21%, respectively, compared with wild-type-inoculated samples (Fig. 1). The total shoot weight was 10% higher, although the number of leaves was nearly identical (98 ± 5.9 and 96 ± 3.6; data not shown in Fig. 1). In medium containing 2 mM ammonium sulfate, a more expressed difference was observed in favor of the mutant strain. Plants inoculated by *S. meliloti* 399 developed approximately 10% more leaves (115 ± 3.5 compared with 105 ± 3.4; data not shown in Fig. 1) and exhibited an 18 and 20% increase in leaf and stem dry weight, respectively. The nitrogen content of the plants also increased by 9 and 24% in nitrogen-free and nitrogen-supplemented medium, respectively, compared with the wild-type control (Fig. 1). Similar results were obtained with the use of different varieties of alfalfa.

Competition of the wild-type strain and the *ntrR* mutant for nodule occupancy was tested by inoculating alfalfa seedlings with either the wild type or the *ntrR* mutant bacteria or with a 1:1 mixture of the two strains. A *S. meliloti* derivative carrying a Tn*5* insertion in a symbiotically indifferent position (provided by P. Putnoky) was used as a control. Bacteria (400 colonies from each inoculation) were recovered from nodules and tested for kanamycin resistance, the marker of the mutant strain. When the *ntrR* mutant or the control strain with a Tn*5* insertion was used, 100% of the reisolated colonies were kanamycin resistant. The nodules infected by the wild-type strain contained only 0.5 to 4% Km^R bacteria. When the mixture of the wild-type and the mutant strains was used for inoculation, all 400 colonies were Km^R, indicating that probably as a result of its ability to nodulate earlier, the *ntrR* mutant had an advantage to occupy nodules, even in the presence of wild-type bacteria.

Cloning and sequencing of the *ntrR* region.

An approximately 13-kb *Eco*RI fragment of *S. meliloti* 399 carrying the Tn*5* insertion responsible for the *ntrR* phenotype was identified and cloned in vector pACYC184, resulting in plasmid pID120. This fragment was used to isolate the corresponding wild-type region from a clone bank of *S. meliloti* 1021. A 4.4-kb *Eco*RI–*Sal*I fragment was obtained from a

hybridizing cosmid clone, inserted in pBluescript vector (resulting in plasmid pZV522.4), and used subsequently for DNA sequencing. On a 2,300-bp *Sma*I fragment, three forward-directed open reading frames (ORF) were identified (ORF1 to ORF3; Fig. 2). A fourth ORF, ORF4, was located on the other strand and disrupted by the *Sma*I site. The stop codon of ORF1 overlapped with the start codon of ORF2. On the basis of the Tn*5* insertion site, which was determined with the use of a Tn*5*-specific primer, ORF2 was designated *ntrR*. The putative translation start point of *ntrR* is the rare TTG codon. ORF1, designated *ntrP*, has a coding capacity for a 10.3-kDa protein consisting of 90 amino acids. The putative protein encoded by *ntrR* (ORF2) consists of 132 amino acids and represents a 14.2-kDa molecule.

The deduced amino acid sequences of NtrP and NtrR showed high similarity to a group of hypothetical proteins, all homologous to the *vapBC* region of *Dichelobacter nodosus* (Katz et al. 1992), a strict anaerobe animal pathogen (Fig. 2). Additionally, *vapBC* homologs were identified in *Synechocystis* sp. strain PCC6803 (Kaneko et al. 1995) and *Haemophilus influenzae* (Fleischmann et al. 1995) by whole-genome sequencing programs as well as in *Shigella flexneri* (Radnedge et al. 1997). In addition to their homologous protein sequences, the organization of these ORFs is highly conserved. The *vapB* and *vapC* and their homologs form one operon, and the start codon of the second gene overlaps the stop codon of the first by one or four bases (Radnedge et al. 1997). In *S. meliloti*, the overlap extends to four bases.

The comparison of NtrR with annotated protein motifs revealed the presence of a highly conserved PIN domain. The function of the PIN domain in signaling has been proposed on the basis of its presence in StbB and DIS3 proteins (Makarova et al. 1999).

Regulation of *ntrR* expression.

In order to examine the regulation of the *ntrR* gene, the *lacZ* reporter gene was fused to *ntrR* at the *Hin*dIII site. The resulting construct, pID115, was introduced into the wild-type strain and the *ntrR* mutant *S. meliloti* 399. Previously, the involvement of *ntrR*, the nitrogen regulatory gene *ntrC*, and the symbiotic regulator *syrM* was demonstrated in the nitrogen control of *nod* genes (Dusha and Kondorosi 1993; Dusha et al. 1989). Therefore, a possible regulatory link between them also was tested by determining the expression

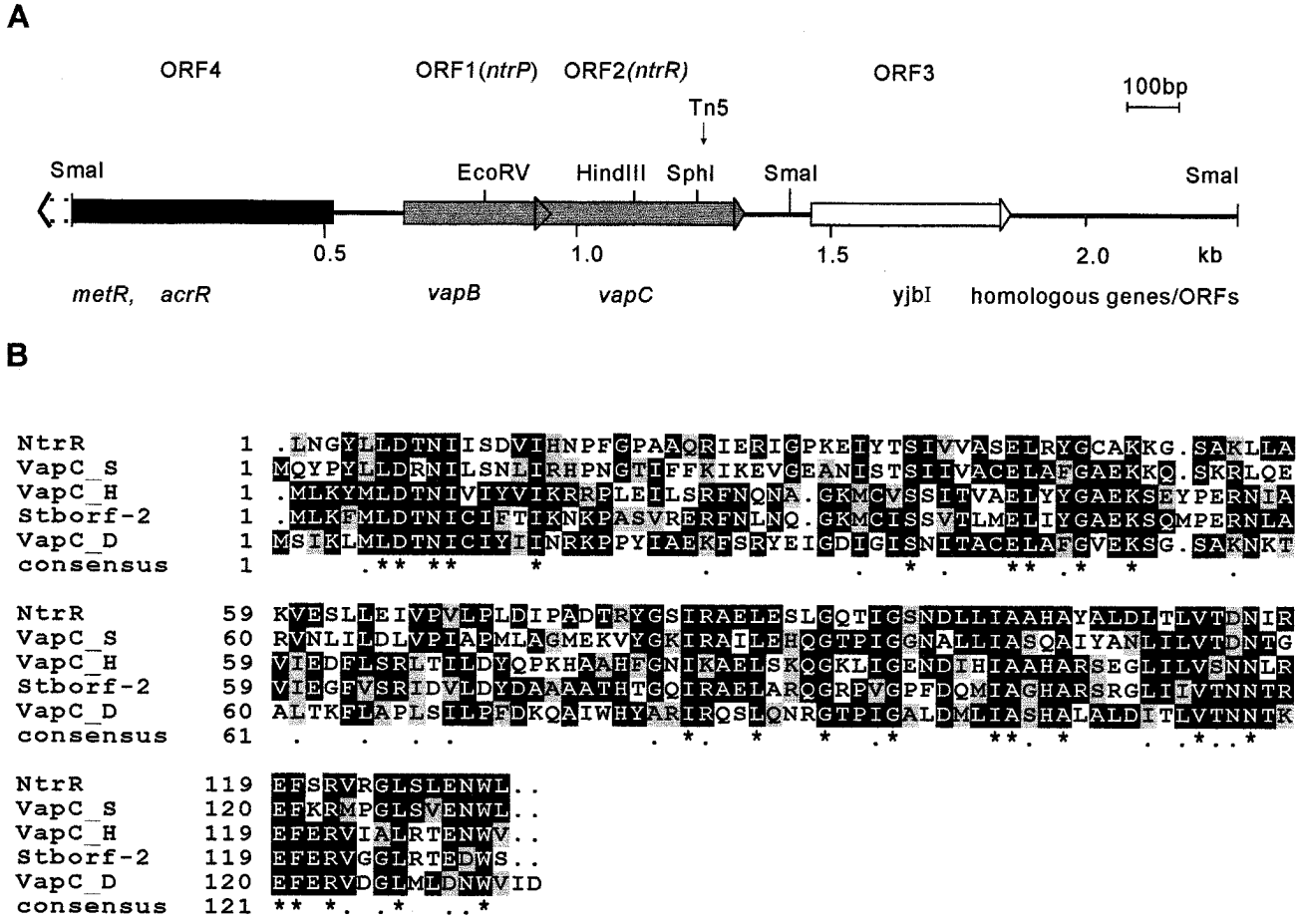

Fig. 2. Map of the *ntrPR* region and comparison of the deduced NtrR sequence to VapC-related sequences. **A,** Thick, horizontal arrows represent the localization of open reading frames (ORFs) 1–4. Restriction sites used are shown above the line. Vertical arrow indicates the Tn*5* insertion site in *Sinorhizobium meliloti* 399. Designations of homologous genes or ORFs are below the arrows: *acrR* of *Escherichia coli* (NCBI accession no. P34000), *vapBC* of *Dichelobacter nodosus* (GenBank accession no. L22308), and *yjbI* of *Bacillus subtilis* (NCBI accession no. Z99110). **B,** Homology of VapC-related proteins. Origin of sequences: VapC_S, *Synechocystis sp.*; VapC_H, *Haemophilus influenzae*; Stborf_2, *Shigella flexneri*; and VapC_D, *D. nodosus*. Black and gray boxes show identical and similar amino acids, respectively. *ntrPR* sequence: EMBL accession no. AJ277847.

of *ntrR* in the *ntrC* mutant *S. meliloti* 5002 (Szeto et al. 1987), in the *syrM* mutant *S. meliloti* EK217 (Kondorosi et al. 1991), and in the *syrM–ntrR* double mutant *S. meliloti* 871. In the *ntrR* mutant, the enzyme activity of the *ntrR–lacZ* fusion was considerably higher than in the wild-type background, indicating that *ntrR* may strongly repress its own expression (Fig. 3). The expression of *ntrR* is not controlled by nitrogen because the enzyme activities obtained at low or excess nitrogen concentration (0.01 and 0.2%, respectively) were comparable. In the *ntrC* mutant, the level of *ntrR* expression was as low as in the wild-type cells. The lack of putative NtrC binding sites in the upstream region of the *ntrPR* operon and the nitrogen-independent expression of *ntrR* supported the conclusion that *ntrR* is not controlled by NtrC. In the *syrM* mutant, the *ntrR–lacZ* fusion also was derepressed, although to a lower level than in the *ntrR* mutant. In the *ntrR–syrM* double mutant, derepression at an intermediate level was observed. If the effects of the two mutations were independent, enzyme activities as high as in the *ntrR* mutant could be expected. A possible explanation for the lower derepression level may be the positive regulatory effect by *syrM* on *ntrR* expression.

Symbiotic phenotype of the ntrP mutant.

The DNA sequence of the *ntrP–ntrR* region suggests that it represents a single transcription unit controlled by the putative promoter upstream of *ntrP*. On the basis of high sequence conservation and the identical arrangement of the two coding regions in various genetically distant species, we supposed that both genes may participate in the same function. Therefore, a *ntrP* mutant was constructed by inserting a Km^R cassette into the *ntrP* gene and tested in symbiosis with alfalfa. The effect of *ntrP* mutation on the symbiotic phenotype was comparable to that of *ntrR*: earlier nodule formation and higher plant dry matter yield (approximately 19 to 20% increase compared with wild type-inoculated plants) was obtained (data not shown) both in nitrogen-free and nitrogen-containing medium, suggesting either a polar effect of mutation and/or the participation of the two genes in a common process.

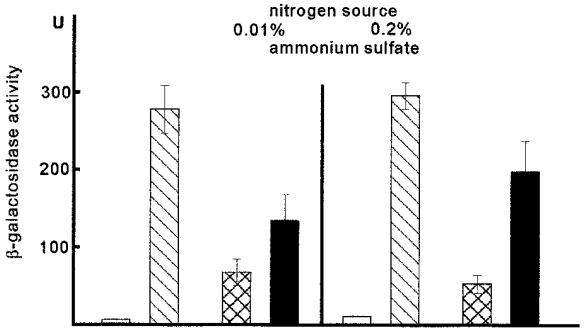

Fig. 3. Regulation of *ntrR* expression. β-Galactosidase activities expressed from the *ntrR–lacZ* fusion (pID115) were determined in the wild-type strain *Sinorhizobium meliloti* 1021 (empty bar), *ntrR* mutant *S. meliloti* 399 (hatched bar), *syrM* mutant EK217 (cross-hatched bar) and *syrM–ntrR* double mutant *S. meliloti* 871 (black bar). Enzyme activities were calculated as Miller units from parallel samples of 4–12 independent measurements.

Effect of *ntrR* mutation on *nifA* and *nifH* expression in nodules.

To clarify whether the elevated symbiotic efficiency in nodules formed by the *ntrR* mutant is the result of increased nitrogen-fixing ability of the mutant bacteroids, the expression level of the transcriptionally regulated *nifH* gene was examined in wild-type and mutant bacteroids. The expression of *nifH–lacZ* fusion was higher in the mutant than in the wild-type nodules both in the presence and absence of combined nitrogen (Fig. 4). In nitrogen-free conditions, the transcription of *nifH* was high (Fig. 4A) and showed significant enhancement in both types of bacteroids during the examination period (Fig. 4B). In the presence of exogenous nitrogen, a significantly lower level of transcription was observed (Fig. 4C). Gene expression permanently increased in the mutant, however, whereas the wild-type strain exhibited constant values, resulting in an increasing difference of *nifH* expression between the mutant and the wild-type bacteria (Fig. 4C and D).

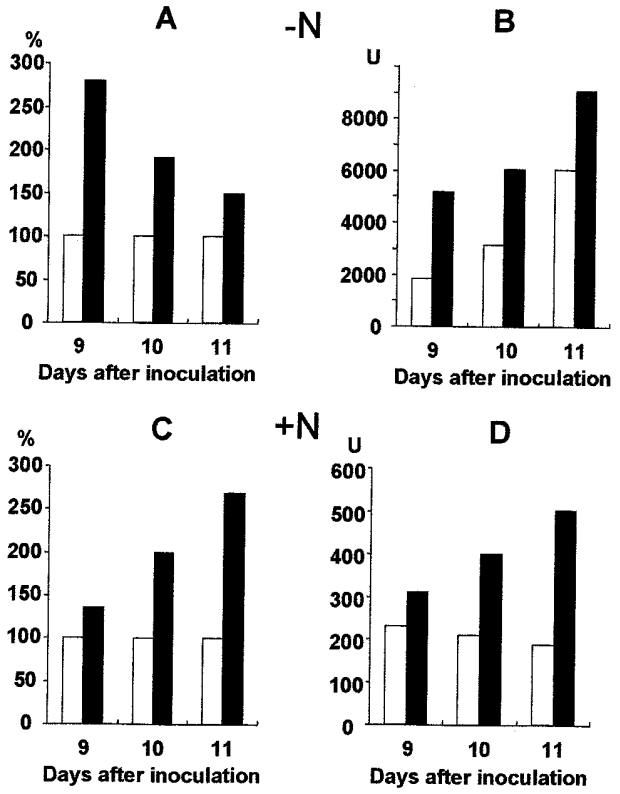

Fig. 4. Expression of *nifH–lacZ* in nodules elicited by wild-type and *ntrR* mutant strains carrying plasmid pRKP9. Plants were grown in **A and B,** nitrogen-free medium or **C and D,** the presence of 2 mM ammonium sulfate. β-Galactosidase activities of the mutant nodules (black bars) are presented as a percentage of wild-type activity (empty bars) determined on the same day (**A and C**) or as a comparison of the calculated enzyme activities (**B and D**). Standard deviation of activity measurements was 6–10%, within one experiment. Plant tests producing variable levels of β-galactosidase activities were repeated four times. The differences between the activities of the wild-type and mutant nodules were well reproducible. In nitrogen-free medium, activity in the mutant nodules was 3.4 times higher (± 0.6) on day 9, which decreased to 1.6 (± 0.05) by day 11. In contrast, in the presence of nitrogen, activity only was 1.3 (± 0.15) times higher in the mutant on day 9, which increased to 2.5 (± 0.15) by day 11. The data of one representative experiment are shown.

The enhanced expression of *nifH* observed in the *ntrR* mutant bacteroids, even in the presence of exogenous nitrogen source, indicates elevated nitrogen fixation ability and provides a likely explanation for the increased dry matter yield and specific nitrogen content of the plants.

To examine the regulatory level at which the *ntrR* may act, the expression of the positive regulator *nifA* was measured under the same conditions as *nifH*. The β-galactosidase activities coincided with the results obtained for *nifH–lacZ*, in that the expression of *nifA–lacZ* was higher in the mutant background in both growth conditions (Fig. 5). The presence of nitrogen resulted in an approximately 50% decrease of activity (Fig. 5C and D). These data suggest that *ntrR* may affect nitrogen fixation via control of the NifA regulator.

The expression pattern of *ntrR* was compared with that of the *nifH* gene in nodules induced by the wild-type or *ntrR* mutant strains carrying *ntrR–lacZ* or *nifH–lacZ* fusions (Fig. 6). In nodule sections stained histochemically for β-galactosidase activity, the expression of *ntrR* was localized in the same zone (nitrogen fixing zone III), as determined earlier for *nifH* (Trepp et al. 1999). A similar expression pattern was obtained for both genes when alfalfa plants were grown in the presence of exogenous nitrogen (data not shown). The level of *nifH* expression considerably exceeded that of *ntrR* both in the wild-type and mutant background (compare Fig. 6A and B to C and D), in accordance with the low expression level of *ntrR* in free-living, wild-type bacteria. The histochemical staining obtained with the *ntrR–* and *nifH–lacZ* fusions was stronger in the *ntrR* mutant, supporting data from the β-galactosidase measurements (Figs. 3 and 4).

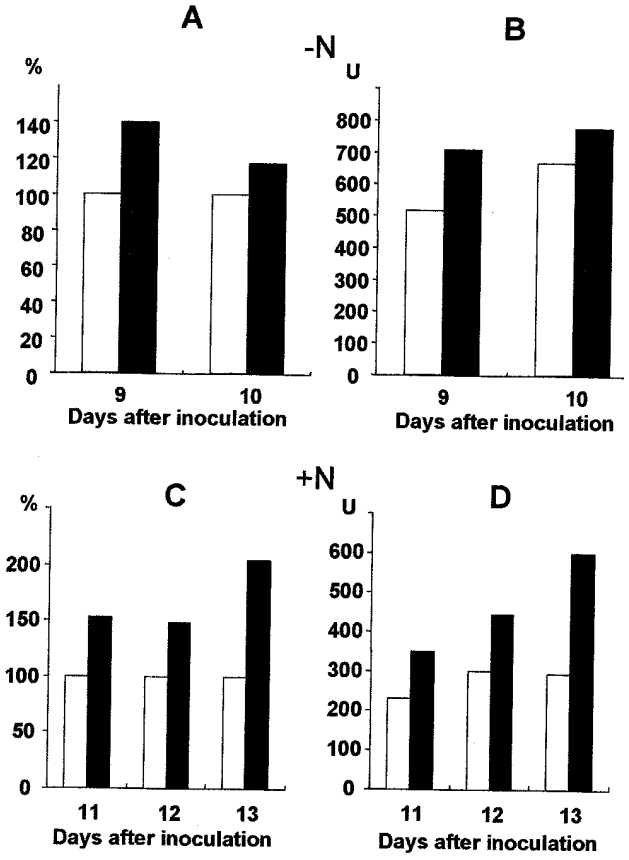

Fig. 5. β-Galactosidase activities expressed from *nifA–lacZ* fusion (pCHK57) in nodules induced by wild-type and *ntrR* mutant strains. Conditions of plant growth and calculation of activities are as described in Figure 4. The standard deviation of activity measurements was 6–10% within one experiment. β-Galactosidase activities were determined in four independent plant tests. Although the activity values varied in the same manner as those of the *nifH–lacZ* fusions (Fig. 4), the differences were reproducible. Under nitrogen-free conditions, activity in the mutant nodules was 1.6 times higher (± 0.2), decreasing to 1.3 (± 0.1) by day 10. In the presence of nitrogen, the ratio remained constant (1.4–1.5 ± 0.05). Data from one representative experiment are shown.

DISCUSSION

In this paper, we report the characterization of the *ntrPR* operon of *S. meliloti* and demonstrate that the mutations created in this operon improve the nitrogen fixation efficiency in alfalfa nodules. We show that the mutation in the *ntrR* gene results in an altered expression of *nifH* and *nifA* and, as a re-

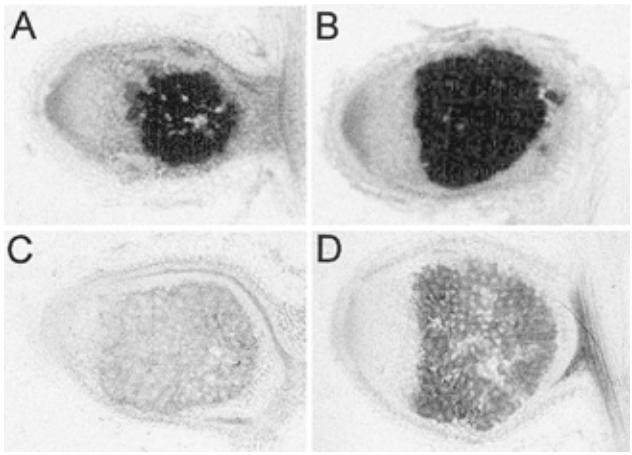

Fig. 6. Histochemical localization of the expression of *nifH* and *ntrR* genes in nodules. Sections of the **A and C,** wild-type and **B and D,** *ntrR* mutant nodules carrying a *nifH–lacZ* (**A and B**) or *ntrR–lacZ* (**C and D**) fusion were prepared and stained for β-galactosidase activity.

Table 1. Homology of the predicted *ntrP*- and *ntrR*-encoded proteins of *S. meliloti* to hypothetical proteins of various origin

Homologous sequence		Origin	Amino acid (%)		Citation
			Identity	Similarity	
NtrP	VapB	*Dichelobacter nodosus*	32	57	Katz et al. 1992
	Hi0948	*Haemophilus influenzae*	17	27	Fleischmann et al. 1995
	sII0690	*Synechocystis* sp.	19	45	Kaneko et al. 1995
	StbORF1	*Shigella flexneri*	17	36	Radnedge et al. 1997
NtrR	VapC	*D. nodosus*	41	60	Katz et al. 1992
	Hi0947	*H. influenzae*	39	56	Fleischmann et al. 1995
	sII0691	*Synechocystis* sp.	47	66	Kaneko et al. 1995
	StbORF2	*S. flexneri*	37	56	Radnedge et al. 1997

sult of the nitrogen-tolerant phenotype of the mutant, the beneficial effect on symbiosis is not abolished in the presence of external nitrogen.

Earlier, we described that in *S. meliloti* 399, the nitrogen repression of *nod* genes was released and more nodules were formed by the mutant than by the wild-type bacteria. Here, we demonstrate that the strain also has a more efficient symbiotic capacity, which is reflected by the higher dry matter yield and nitrogen content of alfalfa (Fig. 1). Moreover, the effect is more pronounced when exogenous nitrogen is available for the host plant.

The *ntrPR* operon showed a high homology to the *vapBC* genes of *D. nodosus* (Katz et al. 1992) and to further genes of various origins (Fleischmann et al. 1995; Kaneko et al. 1995; Radnedge et al. 1997). The homology between deduced VapB- and VapC-related proteins is remarkable (Fig. 2 and Table 1), and the organization of the operons also is conserved. The high sequence conservation may reflect important functions of the Vap proteins.

The *vap* region was present in all virulent isolates of *D. nodosus* but was absent in 67% of the benign isolates (Cheetham et al. 1995). Hence, these sequences were referred to as required potentially for virulence, although no direct proof is available for their role. The proposed implication in virulence and the presence in various pathogenic bacteria suggest that *vap* genes may be involved in bacterium–host interactions. Our results, described here, provide evidence for the important function of the *ntrPR* operon. As a result of a single mutation in the *ntrR* gene, identified originally by selecting for the loss of nitrogen repression of *nod* genes (Dusha et al. 1989), the efficiency of nitrogen fixation also was altered. Our data indicate that this is the result of an increased *nifH* transcription in mutant bacteroids, which was observed both in the presence and absence of combined nitrogen. A similar conclusion was drawn from data on *nifA* expression, indicating that *ntrR* may regulate *nif* genes via controlling the NifA regulator.

In symbiotic nodules, oxygen concentration was suggested to be the main regulatory factor for *nif* expression (Ditta et al. 1987). Our measurements of *nifH–* and *nifA–lacZ* fusions in bacteroids, however, indicate that exogenous nitrogen also may control *nif* expression (Fig. 4) because the transcription of *nif* genes was reduced to a significantly lower level when the host plant was supplied by nitrogen. Contrary to the generally accepted primary role of oxygen concentration, the repression of *nifA* gene was demonstrated also in free-living *S. meliloti* under microaerobic conditions when ammonium was added to the medium (Noonan et al. 1992). The FixLJ regulatory system appeared to mediate the ammonium effect, and the possible involvement of another intracellular factor acting as a repressor of *nifA* also was proposed (Noonan et al. 1992). Our observation that *nifH* and *ntrR* genes are expressed in the same zone of nitrogen fixing nodules (Fig. 5) supports the hypothesis that NtrR may be responsible for this repressor function.

Over the last 50 years, agriculture has become highly dependent on chemical nitrogen fertilizers, whereas the input of nitrogen derived from biological nitrogen fixation decreased. As the environmental risks of nitrogen fertilization (e.g., pollution of soil and water and the greenhouse effect) and the increasing costs of energy for chemical synthesis became evident, a renewed attention was paid to symbiotic nitrogen fixation. This process has the advantage of utilizing photosynthetically produced carbon and energy and locally provides nitrogen to the host plant.

In the last 10 to 15 years, various approaches have been worked out to develop more efficient bacterium–host interactions and to increase nitrogen fixation potential by means of genetic manipulation (Maier and Triplett 1996). To improve nodulation ability, a flavonoid-independent hybrid nodulation gene was constructed (Spaink et al. 1989). In other experiments, strains with altered expression of cytochromes (Miranda et al. 1996; Soberon et al. 1989) or with a mutation in a transcriptional activator (Sharypova et al. 1999) that resulted in higher symbiotic performance, were selected. Recombinant strains were constructed, with increased expression of the positive regulatory *nifA* gene and/or the C4-dicarboxylate transport gene *dctA* (Bosworth et al. 1994; Cannon et al. 1988; Rastogi et al. 1992). Although in these systems nitrogenase activity increased in the bacteroids, it either did not result in enhanced plant biomass production (Rastogi et al. 1992) or the beneficial effect (a 22% increase in the nitrogen content of the plant) was not examined further (Soberon et al. 1989). Field experiments carried out by Bosworth et al. (1994) demonstrated a 12.9% higher alfalfa biomass production. This yield, however, was obtained only in soil with low nitrogen content and a low endogenous *Rhizobium* spp. population, although the seeds were coated with a 10^2- to 10^3-fold higher number of rhizobia than the commercial average.

Our derivative *S. meliloti* 399 combines the advantageous nodulation and nitrogen fixation properties that result from the pleiotropic effect of *ntrR* mutation on the symbiosis with alfalfa. The mutant strain induces nitrogen-fixing nodules earlier and in higher number, thus outcompeting the wild-type strain during nodule occupation. Preliminary data of field experiments indicate that the mutant also is highly competitive with endogenous rhizobia. In addition, bacteroids have an improved nitrogen fixation capacity (higher dry matter yield. nitrogen content, and leaf number) as a result of the increased expression level of nitrogenase. Because the mutant strain has a nitrogen-tolerant phenotype, it also retains these properties in a nitrogen-containing environment. On the basis of these beneficial effects, *S. meliloti* 399 is a good candidate for agricultural utilization as an inoculant to improve nitrogen fixation in symbiosis with alfalfa.

MATERIALS AND METHODS

Microbiological techniques.

Bacterial strains and plasmids are described in Table 2. Growth conditions for *S. meliloti* strains and conjugation of recombinant pRK290 derivatives to *S. meliloti* were performed as described previously (Dusha et al. 1989).

DNA manipulation and sequence analysis.

DNA manipulations were performed by standard methods (Maniatis et al. 1982). The DNA sequence was determined by the dideoxy-chain termination method, according to Maniatis et al. (1982). The position of the Tn*5* insertion was determined with the use of a Tn*5*-specific oligonucleotide primer (5′-GCAAAACGGGAAAGGTTCCG-3′). DNA sequence analysis was performed with the PC/Gene software package (Intel-

liGenetics, Mountain View, CA, U.S.A.). Amino acid homology searches were performed with the BLASTX program. Sequence alignments were performed with the University of Washington GCG program package (Genetics Computer Group, Madison, WI, U.S.A.).

Construction of a *ntrR–lacZ* fusion and mutant strains with *ntrP* of *ntrR–syrM* double mutations.

A 2.6-kb *Hin*dIII fragment containing *ntrP* with the promoter region and 182 bp of *ntrR* was isolated from pZV522.4 and ligated into the *Hin*dIII site of plasmid pNM482 carrying the *lacZ* gene (Minton 1984). By this procedure, a region coding for 61 amino acids of *ntrR* was fused to the 8 codon of *lacZ* (pID8). The fusion construct was recloned into the mobilizable vector pRK290, resulting in plasmid pID115.

The 1.15-kb *Sma*I–*Hin*dIII fragment carrying *ntrP*, a fragmant of *ntrR*, and the upstream region in pUC19 was cut at the single *Eco*RV site present in the *ntrP* coding region and a Km^R cassette was inserted (pID167). This construct was cloned in pRK290 (pID170) and conjugated into wild-type *S. meliloti*. Double recombinants carrying the Km^R cassette in *ntrP* were selected and verified by Southern hybridization. One derivative, designated *S. meliloti* 866, was selected for further experiments.

To construct strain *S. meliloti* 871, which is deficient in producing SyrM and NtrR, the *ntrR* mutation was transferred from *S. meliloti* 399 to strain JM142, carrying a *uidA*::Spc resistance cassette in the *syrM* gene, with the help of phage M12.

Determination of symbiotic efficiency and competitiveness.

Plant tests were performed in a greenhouse at 20 to 22ºC with 16 h day–8 h night period. Surface-sterilized seeds (*M. sativa* spp. *sativa* cv. Sitel, Nagyszenasi, or Moapa 69) were planted into pots containing perlite washed previously with distilled water and nitrogen-free or nitrogen-supplemented (2 mM ammonium sulfate) Gibson medium (Dusha et al. 1989). Five-hundred seedlings were planted in each treatment for reliable statistical evaluation. *S. meliloti* 1021 (wild-type control) and *S. meliloti* 399 were used for inoculation (0.1 ml per plant). Five-hundred seedlings in nitrogen-free and nitrogen-containing perlite also were grown without inoculation as controls. After 2 months, leaves and stems were collected separately, and the number of leaves per plants was counted. The material collected from 10 plants was combined, dried for 48 h at 68ºC, and the dry weight was measured. Total nitrogen content was determined from dried leaves and stems of 500 plants (combined in five groups) with the use of an automatic Kjeldahl analyzer (Kjeltec 2300-unit; Foss Tecator, Padova, Italy).

To test nodule occupancy, bacteria were reisolated from surface-sterilized crushed nodules, and 400 colonies from each treatment were checked for kanamycin resistance.

β-Galactosidase measurements from plant nodules and free-living bacterial cultures.

Alfalfa seedlings were inoculated by either the wild type or the *ntrR* mutant strain carrying plasmids pRKP9 or pCHK57 with *nifH–lacZ* or *nifA–lacZ* fusions, respectively. Nodules of 20 to 30 mg were collected daily between days 9 and 11 postinoculation, crushed in 1 ml of ice-cold buffer (250 mM mannitol; 50 mM Tris, pH 7.0), kept on ice for 30 min, then centrifuged. β-Galactosidase activity and protein concentration (Bio-Rad protein microassay, Richmond, CA, U.S.A.) was determined from the supernatant. Enzyme activity from four independent plant tests was calculated as $1{,}000 \times OD_{420}/\text{min} \times \text{mg} \times \text{ml}$.

Free-living bacteria carrying plasmid pID115 were grown in complete medium, then washed with GTS-N medium (Dusha et al. 1999) and diluted to $OD_{600} \times 0.05$–0.1. Ammonium sulfate was added as a nitrogen source at two concentrations (0.01 or 0.2%). Cells were grown to OD_{600} 0.6–0.7, then β-galactosidase activities were determined from parallel samples of four to 12 independent clones. Enzyme activities were calculated as $1{,}000 \times OD_{420}/OD_{600} \times \text{min} \times \text{ml}$.

Histochemical detection of gene expression in nodules.

Alfalfa plants were inoculated with *S. meliloti* strains 1021 and 399 carrying *the ntrR–lacZ* and *nifH–lacZ* constructs. Nine-day-old nodules were collected and 80-µm longitudinal

Table 2. Bacterial strains and plasmids

Strain–plasmid	Relevant genotype	References
Strains		
Sinorhizobium meliloti		
1021	Wild type, Sm^R derivative of SU47	F. M. Ausubel
399	1021, *ntrR*::Tn*5*	Dusha et al. 1989
866	1021, *ntrP*::Km^R	This work
EK217	1021, *syrM*::Tn*5*	Kondorosi et al. 1991
JM 142	1021, *syrM–gusA* fusion	Swanson et al. 1993
5002	1021, *ntrC*::Tn*5*	Szeto et al. 1987
Plasmids		
pID120	13-kb *Eco*RI fragment in pACYC184	This work
pZV522.4	4.4-kb *Eco*RI–*Sal*I fragment in Bluescript carrying the *ntrP–ntrR* region	This work
pID8	2.6-kb *Hin*dIII fragment from pZV522.4 cloned into the *Hin*dIII site of pNM482, resulting in a *ntrR–lacZ* fusion	This work
pID115	*Eco*RI–*Dra*I fragment of pID8 in pRK290	This work
pID167	1.15-kb *Sma*I–*Hin*dIII fragment in pUC19 carrying the Km^R cassette in the *Eco*RV site of *ntrP*	This work
pID170	pID167 linearized by *Eco*RI ligated into the *Eco*RI site of pRK290	This work
pNM482	Cloning vector, Ap^R	Minton, 1984
pRK290	Cloning vector, Tc^R, IncP	Ditta et al. 1980
pRKP9	*nifH–lacZ* fusion in pRK290	Szeto et al. 1987
pCHK57	*nifA–lacZ* fusion in pRK290	Ditta et al. 1987

slices of nodules were prepared and stained histochemically, as described (Boivin et al. 1990).

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