Conservation of *nolR* in the *Sinorhizobium* and *Rhizobium* Genera of the *Rhizobiaceae* Family

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In *Sinorhizobium meliloti* the NodR repressor displays differential negative regulation of nodulation genes and is required for optimal nodulation. Here, we demonstrate that the NodR function is not unique to *S. meliloti* but is also present in other species of the *Rhizobiaceae* family. DNA hybridization indicates the presence of *nolR* homologous sequences in species belonging to the *Rhizobium* and *Sinorhizobium* genera while no hybridization signal was detected in species from the *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, and *Agrobacterium* genera. We isolated the *nolR* gene from the *Rhizobium leguminosarum* bv. *viciae* strain TOM and showed that the TOM *nolR* gene acts similarly to *S. meliloti* *nolR* by repressing the expression of both the *nodABCII* and the *nodD* genes, resulting in decreased Nod factor production. The presence of a functional *nolR* gene in *R. leguminosarum* is correlated with an increased rate and extent of nodulation of pea. The conserved primary structure, the location of the DNA-binding domain, and the similar size of NodR proteins, compared with a family of small bacterial regulatory proteins including HlyU, SmtB, and the ArsR-type regulators, revealed that NodR belongs to this family.

Symbiotic interactions between rhizobia and leguminous plants result in the development of nitrogen-fixing root nodules. Nodule organogenesis is encoded by the plant and triggered by the bacterial Nod signal molecules. The core structure of these Nod factors is conserved in all rhizobia and consists of tetra- or pentamers of β -1,4-linked *N*-acetylglucosamines that are *N*-acylated on the nonreducing terminus. In different strains, these molecules can be modified by various substitutions on the reducing and nonreducing termini. The synthesis of the core structure is catalyzed by the *nodABC* gene products, which are present in all rhizobia whereas deco-

ration of the reducing and nonreducing termini involves various other nodulation genes. After synthesis is completed, the Nod factors are secreted by a protein complex involving the *nodII* gene products that are members of the ABC-transporter family (reviewed in Downie 1998; Schultze et al. 1994; Dénaire et al. 1996).

Expression of nodulation (*nod*, *nol*, *noe*) genes is induced by flavonoids excreted by the host plant root, in conjunction with the bacterial NodD activator proteins. NodD (belonging to the bacterial LysR regulatory family) binds to the conserved *nod* box sequence in the promoter of *nod* operons and, in the presence of inducing flavonoids, activates the transcription of nodulation genes. Expression of nodulation genes and the consequent production of Nod factors are finely controlled in most rhizobia via either multiple NodD proteins or additional regulatory elements (reviewed in Schlaman et al. 1998). The only well-characterized *nod* gene repressor is NodR from *Sinorhizobium meliloti* (Kondorosi et al. 1991). The *nolR* gene has been localized on the *S. meliloti* chromosome and encodes a 13-kDa protein. In dimeric form, NodR binds to the (A/T)TTAG-N(9)-A(T/A) target sequences present in its own promoter as well as in the *n1*, *n4*, and *n6* *nod* promoters (Cren et al. 1995). The NodR-binding motif is absent from the *n2*, *n3*, and *n5* promoters controlling the expression of the host-specific *nod* genes *nodFEG*, *nodH*, and *nodL*, respectively. Thus, in *S. meliloti*, NodR differentially downregulates the expression of genes involved in core Nod factor synthesis and elicits preferential synthesis of fully decorated Nod factors that, in low amounts, appear to be optimal for nodulation.

Negative regulation of *nod* genes has been suggested in other rhizobia (Bañfalvi et al. 1988; Schlaman et al. 1990; Bellato et al. 1996). Here, we tested the conservation of *nolR* in the *Rhizobiaceae* family. Recent development in taxonomy of *Rhizobiaceae* resulted in the separation of the family into seven genera. Molecular evolutionary systematics on legume symbionts (Martinez-Romero and Caballero-Mellado 1996; van Berkum and Eardly 1998; and Figure 1B), proposed the separation of the *Rhizobium* genus into the genera *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium*, leading to the classification of legume-nodulating bacteria in five genera, *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, and *Azorhizobium*. Based on this proposi-

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tion, *S. meliloti*, *Sinorhizobium fredii*, and *Sinorhizobium* sp. NGR234, previously members of the *Rhizobium* genus, are now classified as members of the *Sinorhizobium* genus, while *Mesorhizobium loti* belongs to the *Mesorhizobium* genus, in contrast to *Rhizobium leguminosarum*, *Rhizobium tropici*, and *Rhizobium etli*, which remained in the *Rhizobium* genus. *Azorhizobium caulinodans* falls into a separate genus and the phylogenetic position of *Rhizobium galageae* is still unclear, being as distant from *Agrobacterium* as it is from *Rhizobium*. In this paper, representative species from each group were tested for the presence of *nolR*. We show the conservation of *nolR* in a subset of rhizobia and the identification of the *nolR* gene from *R. leguminosarum* bv. *viciae* strain TOM (*nolR*_{TOM}) is also reported.

RESULTS

Conservation of *nolR* in rhizobia.

Conservation of *nolR*_{Sm} was tested by Southern blot analysis in different genera of legume symbionts as well as in *Agrobacterium* spp. (Fig. 1A). The *nolR* probe, a 285-bp fragment representing 80% of the *nolR*_{Sm} coding region, was hybridized to *Eco*RI-digested genomic DNA from *S. meliloti* AK631 (Fig. 1A, lane 1), *Sinorhizobium* sp. NGR234 (Fig. 1A, lane 2), *S. fredii* (Fig. 1A, lane 3), the *R. leguminosarum* bv. *viciae* strains 248 (Fig. 1A, lane 4) and TOM (Fig. 1A, lane 5), *R. leguminosarum* 8401 (Fig. 1A, lane 7), *R. leguminosarum* bv. *trifolii* (Fig. 1A, lane 8), *R. etli* (Fig. 1A, lane 9), *R. leguminosarum* bv. *phaseoli* 8002 (Fig. 1A, lane 10), *R. galegae* (Fig. 1A, lane 11), *M. loti* (Fig. 1A, lane 12), *Bradyrhizobium japonicum* (Fig. 1A, lane 13), *A. caulinodans* (Fig. 1A, lane 14), *Agrobacterium tumefaciens* (Fig. 1A, lane 15), and *Agrobacterium rhizogenes* (Fig. 1A, lane 16). The hybridization signals detected in Figure 1 lanes 1 to 10 indicated that the *nolR* homologous sequences were conserved only in the species belonging to the genera *Rhizobium* and *Sinorhizobium* while they were absent from other genera of legume symbionts as well as from *Agrobacterium* spp. (Fig. 1B). Based on the strength of hybridization, conservation of the *nolR*-related sequences was higher in the *Sinorhizobium* genus than in the *Rhizobium* genus (in Fig. 1A, lanes 4 and 6, the amount of loaded DNA was higher than in the other lanes).

Identification and cloning of *nolR*_{Sm} homologous region from the *R. leguminosarum* bv. *viciae* strain TOM.

To demonstrate that the *nolR* homologous sequences encode functional NodR repressors, we cloned the *nolR* hybridizing region from *R. leguminosarum* bv. *viciae* TOM, where repressor function was expected (Firmin et al. 1993). This strain differs from other strains of *R. leguminosarum* bv. *viciae* in two key aspects: (i) it expresses the *nod* genes at significantly lower levels and subsequently synthesizes the Nod factors in about 20-fold lower amounts (Firmin et al. 1993); and (ii) it harbors the *nodX* gene, and therefore it can O-acetylate the reducing terminus of the Nod factors, thereby extending the host range of strain TOM to the ancient pea cultivar Afghanistan (Götz et al. 1985; Firmin et al. 1993). To localize the *nolR* homologous regions in the genome of strain TOM, the pRL5JI symbiotic plasmid from TOM was transferred into the *R. leguminosarum* strain 8401. The absence of the characteristic hybridization signal with DNA of the transconjugant (data not shown) indicated that the *nolR*_{Sm}-hybridizing region

was not encoded by the pSym, and therefore may be located on the chromosome of strain TOM, as was observed for the *nolR* gene in *S. meliloti*.

The hybridization signal detected on the 3.8-kb *Eco*RI fragment was further delimited to a 2.8-kb fragment by *Eco*RI-*Bam*HI double digestion (Fig. 1A, lanes 5 and 6). In order to clone *nolR*, a partial genomic library was constructed from strain TOM by cloning *Bam*HI-*Eco*RI-digested DNA fragments in the size range of 3 to 2.5 kb into the broad host range vector pBBR1MCS-2 (Kovach et al. 1995). *Escherichia coli* transformants were screened by colony hybridization with the 285-bp *nolR*_{Sm} probe. Out of

Fig. 1. Occurrence of *nolR* homologous sequences in different species belonging to the *Rhizobiaceae*. **A**, Southern analysis of genomic DNA of the following species, digested with *Eco*RI: Lane 1, *Sinorhizobium meliloti* AK631; lane 2, *Sinorhizobium* sp. NGR234; lane 3, *S. fredii* USDA 203; lane 4 *Rhizobium leguminosarum* bv. *viciae* 248; lane 5, *R. leguminosarum* bv. *viciae* TOM; lane 6, *R. leguminosarum* bv. *viciae* TOM (*Bam*HI-*Eco*RI digestion); lane 7, *R. leguminosarum* strain 8401; lane 8, *R. leguminosarum* bv. *trifolii* ANU843; lane 9, *R. etli* CE3; lane 10, *R. leguminosarum* bv. *phaseoli* 8002; lane 11, *R. galegae*; lane 12, *M. loti*; lane 13, *Bradyrhizobium japonicum* USDA 110; lane 14, *Azorhizobium caulinodans*; lane 15, *Agrobacterium tumefaciens* C58; lane 16, *Agrobacterium rhizogenes* 1724. **B**, Phylogenetic tree, based on 16S rDNA sequences, of *Rhizobiaceae* (Martinez-Romero and Caballero-Mellado 1996). Numbers refer to rhizobia used in **A**. The *Rhizobium*-*Sinorhizobium* branch, consisting of the strains containing *nolR* homologous sequences, is shaded.

1,500 transformants 14 hybridizing clones were found. The physical map of the positive clones revealed that they were identical and contained the same 2.8-kb DNA fragment. *NcoI*, which cuts once in the *S. meliloti nolR* gene, also splits the 2.8-kb *EcoRI-BamHI* fragment, resulting in a 1.5-kb *NcoI-BamHI* and a 1.3-kb *EcoRI-NcoI* fragment, both of which hybridized to the *nolR* probe (data not shown). These DNA fragments were then subcloned in the pUK21 vector and used for nucleotide sequencing.

The nucleotide sequence of a 708-bp DNA region contained a 315-bp open reading frame (ORF) between positions 300 and 614. The putative translation product of the ORF is a protein of 105 amino acid residues with a calculated molecular mass of 13.4 kDa that corresponds to the size of NolR from *S. meliloti*. The amino acid sequence shares 75% identity with the NolR_{Sm} sequence. Analysis of annotated domains by BEAUTY (BLAST Enhanced Alignment Utility; Worley et al. 1995) revealed a helix-turn-helix (HTH) motif from amino acid residues 39 to 58, in a similar position to the HTH DNA-binding motif of NolR_{Sm} (Kondorosi et al. 1991; Cren et al. 1995). Computer-aided structural analysis (Eisenberg et al. 1984) indicated that NolR is a cytoplasmic protein.

In the 5' untranslated region a potential ribosome binding sequence was detected 6 bp upstream of the translational initiation codon. Moreover, the ATTAGCCGTGATGCAT sequence, 110 bp upstream of the translational start codon, corresponding to the consensus NolR target sequence (A/T)TTAG-N(9)-A(T/A), suggests that the *nolR* gene might be autoregulated in strain TOM as it is in *S. meliloti* (Cren et al. 1995).

NolR belongs to a family of small bacterial regulatory proteins.

While previous data base searches showed homology between NolR and the LysR regulatory proteins around their DNA-binding domain (Kondorosi et al. 1991), a recent search revealed that NolR is most similar to a group of small bacterial regulatory proteins (Fig. 2). The highest similarity (67%) was between NolR and HlyU, a transcriptional activator of the hemolysin *hlyA* gene from *Vibrio cholerae* (Williams et al. 1993). NolR was also homologous to SmtB (61%), the repressor of the *smtA* metallothionein gene of a *Synechococcus* sp. (Huckle et al. 1993). SmtB, like NolR_{Sm}, acts as a dimeric repressor and its three-dimensional structure indicates that it is a "winged helix" DNA-binding protein (Cook et al. 1998). NolR exhibits 53% similarity with MerR (Sedlmeier and Altenbuchner 1992),

47% with ArsR (Rosenstein et al. 1992), and 49% with CadC (Ivey et al. 1992). The homology among the proteins is confined to the central region while the N- and C-termini are variable (Fig. 2). All these small (12 to 13.8 kDa) proteins contain HTH DNA-binding motifs in similar positions and are, with the exception of HlyU, repressors of heavy metal resistance operons. This suggests that NolR is member of the ArsR family of metalloregulatory proteins. However, such proteins contain several conserved cysteine and histidine residues involved in metal binding (Cook et al. 1998; Shi et al. 1994; Turner et al. 1996) that are absent both in NolR and HlyU. Thus, the functions of NolR and HlyU are most likely to be independent of heavy metals and evolved for the regulation of *nod* gene and virulence gene expression, respectively.

In addition to the above-mentioned proteins with known functions, a BLASTP search of data bases revealed similarity to many putative proteins predicted from bacterial genome sequencing projects. In the list of 50 best hits the following were found: 11 *Mycobacterium tuberculosis* sequences; five *Bacillus subtilis* sequences; four *Archaeoglobus fulgidus* sequences; and four *Methanobacterium thermoautotrophicum* sequences. The former two are gram-positive species and the latter two belong to the *Archaea*. In the *E. coli* genome, only one similar sequence was found, an ORF that was designated as a homolog of the *V. cholerae hlyU* gene product.

Repression of *nod* gene expression by *nolR*_{TOM}.

In *S. meliloti*, downregulation of the *nodABC* operon and the *nodD1* gene by *nolR*_{Sm} was demonstrated by measuring the β -galactosidase activity of translational fusions of *nod* genes to *lacZ* (Kondorosi et al. 1989b). Based on the strong similarity of the putative NolR_{TOM} protein to NolR_{Sm} we presumed that NolR_{TOM} might substitute for the NolR_{Sm} function. To test this, the *nolR*_{TOM} gene was introduced into the *S. meliloti* strain JM57 (Mulligan and Long 1985), which lacks the active NolR_{Sm} repressor and carries a *nodC-lacZ* fusion on its symbiotic megaplasmid. As shown in Table 1, expression of the *nodABC* operon in JM57 was induced threefold by the *nod* gene inducer luteolin, whereas the presence of either *nolR*_{Sm} or *nolR*_{TOM} in JM57 repressed this induction. An inactivated derivative of *nolR*_{TOM}, constructed via insertion of a Km-resistance cassette in the *NcoI* site (plasmid pAT465), failed to repress the luteolin-induced expression of the *nodABC* genes. These data confirmed that NolR_{TOM} acts as a highly conserved counterpart of NolR_{Sm}, and encodes a functional repressor.

Repressor activity of NolR_{TOM} was also investigated in *R. leguminosarum* by introducing the *nodC-lacZ* or the *nodD-lacZ* fusions from *R. leguminosarum* bv. *viciae* (Table 2). In strain TOM (harboring the intact, endogenous *nolR* gene), β -galactosidase activity of the *nodC-lacZ* fusion was induced 14-fold by hesperetin (strain AT483) whereas inactivation of the *nolR* gene (strain AT484) increased the induction to 26-fold. In a 8401pRL1JI background (*R. leguminosarum* 8401 carrying the *R. leguminosarum* bv. *viciae* symbiotic plasmid pRL1JI), the *nodC-lacZ* fusion was induced 60-fold (strain AT485). This induction was reduced to 20-fold by introduction of *nolR*_{TOM} on a multicopy plasmid (pKE66) into 8401pRL1JI (resulting in strain AT486).

Table 1. Repression of the common nodulation genes in *Sinorhizobium meliloti* by *nolR*_{TOM}

<i>S. meliloti</i> strain	Regulator gene on plasmid	β -Galactosidase activity $\pm$ standard deviation of <i>nodC-lacZ</i> fusion (Miller units)	
		-L	+L ^a
JM57	—	5.3 $\pm$ 0.1	16 $\pm$ 0.5
JM57(pEK722)	<i>nolR</i> _{Sm}	5 $\pm$ 0.5	7.7 $\pm$ 0.2
JM57(pKE66)	<i>nolR</i> _{TOM}	7 $\pm$ 0.5	8.6 $\pm$ 0.5
JM57(pAT465)	<i>nolR</i> _{TOM} ::Km ^r	6.8 $\pm$ 0.8	20.6 $\pm$ 0.7

^a Luteolin (5 μ M).

TOM strain or by its *nolR* mutant (data not shown). Therefore, the plasmid pKE66 carrying the *nolR*_{TOM} was introduced in the *R. leguminosarum* bv. *viciae* strain 8401pRL1JI. Upon addition of naringenin, strain 8401pRL1JI produced a high amount of Nod factors while the derivative

carrying *nolR*_{TOM} (AT482; Table 3) produced much less (Fig. 3). Quantification of these products showed that both the total amount of the radioactive Nod metabolites and the radioactivity of each separate spot were about 10-fold reduced by *nolR*_{TOM}.

Table 2. Repression of the *Rhizobium leguminosarum* bv. *viciae* *nodC* and *nodD* genes by *nolR*_{TOM}

<i>R. leguminosarum</i> bv. <i>viciae</i> strain	Genetic background	<i>nolR</i> _{TOM} ^a	<i>nod-lacZ</i> fusion	β-Galactosidase activity ± standard deviation (Miller units)	
				–H	+H ^b
AT483	TOM	+	<i>nodC</i>	45 ± 3	624 ± 31
AT484	TOM	–	<i>nodC</i>	63 ± 4	1645 ± 122
AT485	8401pRL1JI	–	<i>nodC</i>	113 ± 3	6909 ± 387
AT486	8401pRL1JI	+++	<i>nodC</i>	129 ± 10	2786 ± 102
AT487	TOM	+	<i>nodD</i>	98 ± 6	80 ± 5
AT488	TOM	–	<i>nodD</i>	385 ± 15	360 ± 12
AT489	8401pRL1JI	–	<i>nodD</i>	314 ± 10	123 ± 5
AT490	8401pRL1JI	+++	<i>nodD</i>	112 ± 6	51 ± 3

^a – = *nolR*_{TOM} is absent or mutated by Tn5; + = *nolR*_{TOM} is present as a single genomic copy; +++ = multiple *nolR*_{TOM} copies present on a multicopy plasmid (pKE66).

^b Hesperetin (5 μM).

Table 3. Bacterial strains and plasmids

Strain/plasmid	Relevant genotype	Reference
Strains		
<i>Sinorhizobium meliloti</i>		
AK631	<i>Rm41 exoB nolR</i> ⁺	Kondorosi et al. 1989a
JM57	<i>Rm1021 nolR pSym nodC::lacZ</i>	Mulligan and Long 1985
KE73	JM57(pEK722)	This work
KE74	JM57 (pKE66)	This work
AT469	JM57 (pAT465)	This work
<i>Rhizobium leguminosarum</i>		
TOM	<i>R. leguminosarum</i> bv. <i>viciae</i> wild-type strain, nodulating also pea cv. Afghanistan	Lie 1978
AT475	TOM <i>nolR::Km</i> ^r	This work
AT483	TOM (pIJ1477)	This work
AT484	AT475 (pIJ1477)	This work
AT487	TOM (pIJ1478)	This work
AT488	AT475 (pIJ1478)	This work
8002	<i>R. leguminosarum</i> bv. <i>phaseoli</i> carrying the pSym plasmid pRP2JI	Johnston et al. 1982
8401	pSym-cured derivative of strain 8002	Downie et al. 1983
8401pRL1JI	8401 carrying the <i>R. leguminosarum</i> bv. <i>viciae</i> symbiotic plasmid pRL1JI	Downie et al. 1983
8401pRL5JI	8401 carrying the TOM symbiotic plasmid pRL5JI	This work
AT482	8401pRL1JI (pKE66)	This work
AT485	8401pRL1JI (pIJ1477)	This work
AT486	AT482 (pIJ1477)	This work
AT489	8401pRL1JI (pIJ1478)	This work
AT490	AT482 (pIJ1478)	This work
248	<i>R. leguminosarum</i> bv. <i>viciae</i> wild-type strain	Josey et al. 1979
ANU843	<i>R. leguminosarum</i> bv. <i>trifolii</i>	Rolfe and Shine 1980
Other rhizobia		
CE3	<i>R. etli</i>	Noel et al. 1984
USDA 205	<i>S. fredii</i>	Keyser et al. 1982
NGR234	<i>Sinorhizobium</i> sp.	Trinick 1980
USDA110	<i>Bradyrhizobium japonicum</i>	USDA, Beltsville, MD
	<i>R. galegae</i>	Lindstrom 1989
NZP2037	<i>R. loti</i>	DSIR Culture Collection
ORS571	<i>Azorhizobium caulinodans</i>	Dreyfus et al. 1988
C58	<i>Agrobacterium tumefaciens</i>	Hamilton and Fall 1972
1724	<i>Agrobacterium rhizogenes</i>	Isogai et al. 1988
Plasmids		
pUK21	Cloning vector, <i>Km</i> ^r	Vieira and Messing 1991
pIJ1477	<i>R. leguminosarum</i> bv. <i>viciae</i> <i>nodC-lacZ</i>	Rossen et al. 1985
pIJ1478	<i>R. leguminosarum</i> bv. <i>viciae</i> <i>nodD-lacZ</i>	Rossen et al. 1985
pBBR1MCS-2	Broad host range plasmid vector, <i>Km</i> ^r	Kovach et al. 1995
pKE66	2.2-kb <i>nolR</i> region from strain TOM in pBBR1MCS-2	This work
pEK722	<i>S. meliloti</i> <i>nolR</i> gene in pPR33	Kondorosi et al. 1991
pAT465	pRK290:: <i>nolR</i> _{TOM} :: <i>Km</i> ^r	This work

Effect of NolR on nodulation.

Previously, NolR-producing *S. meliloti* strains were shown to nodulate alfalfa more effectively than their NolR⁻ isogenic derivatives, suggesting that NolR is necessary for optimal nodulation (Kondorosi et al. 1989b). In order to investigate the effect of NolR_{TOM} on nodulation, *Pisum sativum* (cv. Wisconsin Perfection) plants were inoculated with *R. leguminosarum* strains harboring or lacking the TOM *nolR* gene (Fig. 4). The *nolR* mutant derivative of TOM (AT475) had a significantly reduced rate and extent of nodulation, compared with the TOM control (Fig. 4A). Moreover, introduction of the cloned *nolR*_{TOM} gene (on pKE66) into *R. leguminosarum* bv. *viciae* 8401pRL1JI (resulting in strain AT482) stimulated nodulation, compared with the control strain 8401pRL1JI (Fig. 4B).

Fig. 3. Thin-layer chromatography (TLC) analysis of in vivo radioactively labeled Nod factors. Nod factors were extracted from strains 8401(pRL1JI) (lacking *nolR*_{TOM}: *nolR*⁻) and 8401pRL1JI (pKE66) (carrying *nolR*_{TOM}: *nolR*⁺) that were grown in the presence of [2-¹⁴C]-acetate and in the presence (+) or absence (-) of the *nod* gene inducer naringenin. Nf indicates region where Nod factors migrate. O is origin of TLC where samples were applied. F is the solvent front.

DISCUSSION

The *nolR* gene in *S. meliloti* contributes to the optimal nodulation of *Medicago sativa* via negative regulation of *nod* gene expression, resulting in the synthesis of modified Nod factors in lower amounts (Cren et al. 1995). Thus, overproduction of Nod factors does not necessarily promote nodulation efficiency. In *S. meliloti*, elevated expression of *nod* genes in *nolR* mutants led to delayed nodulation. Moreover, application of Nod factors at high (micromolar) concentration elicited defenselike responses in *M. sativa* roots (Savouré et al. 1997). Similarly, overexpression of *nod* genes in *R. leguminosarum* had detrimental effects on nodulation (Knight et al. 1986). Therefore, for optimal nodulation, negative control of Nod factor production may be required also in other *Rhizobium*-plant interactions.

Here we report on the conservation of *nolR* in a subset of rhizobia. The presence of *nolR* homologous sequences was tested by Southern hybridization in selected species from different genera of the *Rhizobiaceae* family. Conservation of *nolR* was found in the *Rhizobium* and *Sinorhizobium* genera but not in other branches of the *Rhizobiaceae* family. Negative regulation of nodulation genes was further supported by sequence analysis of the nodulation gene promoters that revealed the coexistence of the *nolR* homologous sequences and NolR-binding sites found in the *Rhizobium* and *Sinorhizobium* genera (Fig. 5). These data indicate common evolution of bacteria belonging to the *Rhizobium* and *Sinorhizobium* genera that might have adopted the *nolR* function from other soil bacteria. The existence of *nolR* homologs in *M. tuberculosis*, *B. subtilis*, *A. fulgidus*, and *M. thermoautotrophicum* in multiple copies indicates that the *nolR*-type gene regulation might be widely used in bacteria.

In order to confirm that the *nolR* hybridization signal encodes a functional NolR repressor, we identified the *nolR* gene from the *R. leguminosarum* bv. *viciae* strain TOM and demonstrated its repressor function. In the five *nod* operons of *R. leguminosarum* bv. *viciae*, only the overlapping promoter region of *nodD* and *nodABCIJ* contains a sequence characteristic of an NolR-binding site (Fig. 5). Expression of both the *nodD* and *nodABCIJ* genes was downregulated by *nolR* in *R. leguminosarum* bv. *viciae*. Moreover, the 75% homology of the TOM and the *S. meliloti* NolR proteins was sufficient for repression of the *S. meliloti* *nodD1* and *nodABC* genes by the NolR_{TOM}. Computer-based analysis revealed the lack of an NolR target site in the promoter sequences of the *nodFEL* and the *nodO* genes (Fig. 5), indicating that, as in *S. meliloti*, host-specific functions are not controlled by NolR. However, regulation of the *nodM* and *nodN* genes in the two species appears to differ. The NolR-binding site is only partially conserved in the *R. leguminosarum* *nodM* promoter and therefore *nodM* is probably not controlled by NolR, whereas in *S. meliloti* NolR affects negatively the expression of the *nodM-nolFGHI-nodN* transcription unit (Cren et al. 1995). The conservation of the NolR-binding sites in the *nolR* promoter suggests autoregulation of *nolR* in strain TOM, as found in *S. meliloti* (Cren et al. 1995). In addition to the apparent downregulation of the *nodD* and *nodABC* genes by the NolR_{TOM}, other regulatory factors might contribute to the low expression of the *nod* genes in strain TOM, compared with *R. leguminosarum* bv. *viciae* (Table 2). We found that *R. legumi-*

nosarum strains expressing the *nod* genes at higher level than strain TOM also had a *nolR* hybridization signal. In these strains the putative *nolR* gene might be inactive, as is the case in the *S. meliloti* strain 1021, where a point mutation in the coding region abolished DNA-binding ability of the NolR protein (Cren et al. 1994).

NolR shares homology to and corresponds in both its size and structure to a group of small bacterial regulatory proteins. Most of the known members of this family are involved in the regulation of heavy metal resistance operons. SmtB, the cyanobacterial metallothionein repressor, represents a separate branch of the ArsR, MerR, and CadC proteins and it is as distant from these as from the NolR-HlyU branch. The repressors of the heavy metal resistance operons have metal-binding sites that regulate the repressor activity in the presence of heavy metals (Cook et al. 1998; Shi et al. 1994; Turner et al. 1996). Since NolR and HlyU lack the conserved metal-binding sites, the regulatory function of the latter two is probably independent of heavy metals.

Fig. 4. Influence of *nolR* on nodulation of pea (*Pisum sativum* cv. Wisconsin Perfection) by strains of *Rhizobium leguminosarum* bv. *viciae*. **A**, Comparison of nodulation of TOM and the *nolR* mutant (AT475). **B**, Comparison of nodulation of 8401pRL1JI and its derivative carrying cloned *nolR*_{TOM} gene (AT482). Bars show standard errors. **A**, A significant difference (95% confidence level) is from day 11. **B**, Difference is significant from day 15.

Moreover, it is not excluded that not only the *nod* genes are regulated by NolR. Since *nolR* is expressed at high levels in bacteroids it might have a function also in the late stage of symbiotic nodule development (Cren et al. 1995).

Nod factor production correlated with the level of expression of *nod* genes and was decreased by NolR. The *nolR*-harboring *R. leguminosarum* bv. *viciae* strains nodulated pea 1 or 2 days earlier than those that lacked the NolR repressor. Similar observations on the effect of *nolR* on nodulation and Nod factor production were made in the *S. meliloti*-*M. sativa* system. This slight acceleration of nodulation by *nolR* should not be underestimated since it might reflect higher competitiveness of the *nolR*-harboring strains. However, *nolR* is not the only factor regulating the amounts of Nod factors. Strains lacking the *nolR* function, like *R. leguminosarum* 8401pRL1JI, nodulate the host plant efficiently as well, albeit with slight delay. In the absence of *nolR*, the Nod factor concentration might be adjusted by the host plants producing Nod factor-degrading enzymes, as was demonstrated in the case of *M. sativa* (Staehelin et al. 1995). This concerted action of the symbiotic partners is likely responsible for the optimal adjustment of Nod factor concentration for nodulation.

MATERIALS AND METHODS

Microbiological techniques.

Bacterial strains and plasmids used are described in Table 3. Media, antibiotic concentrations, and general growth condi-

Rl bv viciae		
<i>nodA</i>	TATAGAAAACCCGGAA	(+)
<i>nodF</i>	CATAGCAGGGCAGCCG	(-)
<i>nodM</i>	ATTAGCAGCGCTGGA	(-)
<i>nodO</i>	GATAAGGGGCACAGGC	(-)
<i>nolR</i> (strain TOM)	ATTAGGAAGATAACAT	(+)
Rl bv trifolii		
<i>nodA</i>	ATTAGAAAGGCCGGAA	(+)
<i>nodF</i>	CATAGCACAAAACAG	(-)
S. meliloti		
<i>nodA</i> - <i>nodD1</i> *	ATTAGAGAACCCTGAA	+
<i>nodM</i>	TTTAGGAGACCCTGAA	+
<i>nodD2</i> *	ATTAGAGAACCCTGAA	+
<i>nolR</i>	ATTAGCCGTGATGCAT	+
<i>nodF</i>	GATATGTAGCACAAAGC	-
<i>nodH</i>	ATTAAAACGCTAAGCA	-
<i>nodL</i>	GATAGGAGACAAGGCG	-
<i>syrM</i>	ATTACAAGAACGTTAG	(+)
<i>nodD3</i> *	ATTACACCGGTAGGAA	(+)
S. fredii		
<i>nodA</i>	ATTAGAAGATGCTCAC	(+)
NGR234		
<i>nodA</i>	ATTAGAAGATGCTCAC	(+)
<i>nodZ</i>	ATTAGGAAGCTCTGAA	(+)
<i>noeK</i>	ATTAGTGGATGCGAAT	(+)
R. etli		
<i>nodA</i>	ATTAGAAGCGCCCCAA	(+)
<i>nodB</i>	ATTAGACGAGCATCTT	(-)
<i>nolE</i>	AATAGATTTCACCAAT	(+)

consensus sequence: (A/T) TTAG - N(9) - A(T/A)

*reverse complementary sequence

+: demonstrated NolR control

-: experimental evidence for NolR-independent regulation

(+): putative NolR-binding site

(-): probably NolR-independent control

Fig. 5. Potential NolR-binding sites in the *nod* promoters of different rhizobia.

tions were as described previously (Beringer 1974; Kondorosi et al. 1984).

DNA manipulations.

DNA isolation, restriction enzyme digestions, agarose gel electrophoresis, and purification and ligation of DNA fragments were done by standard methods (Maniatis et al. 1982). The enzymes applied in recombinant DNA experiments were used as recommended by the suppliers. The 2.8-kb *nodR*_{TOM} region was subcloned into pUK21 (Vieira and Messing 1991) and the DNA sequence was determined by the dideoxy-chain termination method (Sanger et al. 1977) with [³⁵S]dATP and the Sequenase kit as described in the USB Corporation Sequence Protocols (USB, Cleveland, OH).

Computer-assisted sequence analysis.

DNA sequence analysis was done with computer programs from PC/Gene software package (designed by Amos Bairoch, Intelligenetics, Mountain View, CA). Amino acid homology searches were performed against the "nonredundant" data base of the NCBI BLAST E-mail server with the BLASTX program (Altschul et al. 1990). Sequence alignments were done with the UWGCG program package for the VAX (Devereux et al. 1984).

Southern hybridization.

Filters for Southern hybridization were made by the downward alkaline capillary transfer method (Chomczynski 1992). Eighty percent of the *nodR*_{Sm} gene was amplified from *S. meliloti* genomic DNA by the polymerase chain reaction (PCR) with the primers 5'-CAGCCTCTTCCGCCTGA-3' and 5'-AATGTCGGATAGTGCGC-3' (3 min at 94°C; 30 s at 94°C, 1 min at 50°C, 1 min at 72°C, for 30 cycles; 2 min at 72°C) in the presence of [α -³²P]dCTP. The labeled PCR fragment was used as a hybridization probe for Southern blot analysis of genomic DNA from different *Rhizobium* and related species. Hybridizations were done in G+C buffer (Maniatis et al. 1982) at 65°C for 16 to 20 h. The filters were washed in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), three times for 30 min at 55°C. After autoradiography the filters were washed again with the same solution but at 60°C.

Construction of partial library from TOM.

Genomic DNA (15 µg) of TOM was digested with *Eco*RI and *Bam*HI for 3 h and separated on a 0.8% agarose gel. A region containing the size range of 2.5 to 3 kb was cut out. Pools of the reisolated fragments were ligated into pBRR1MCS-2 and transformed into JM109 competent cells. Clones harboring the *nodR*_{TOM} region were selected by colony hybridization with the *nodR*_{Sm} PCR fragment as a probe.

Measurement of β -galactosidase activity.

S. meliloti and *R. leguminosarum* strains carrying translational *lacZ* fusions to *nodD* or *nodC* genes were grown in TA (10 g of trypton per liter, 1 g of yeast extract per liter, 5 g of NaCl per liter, 1 mM MgCl₂, 1 mM CaCl₂) and TY (5 g of trypton per liter, 3 g of yeast extract per liter, 13.4 mM CaCl₂) media, respectively, in the presence of the appropri-

ate antibiotics for plasmid selection. Bacteria from overnight cultures were diluted to OD₆₀₀ = 0.05 and the *nod* genes were induced with luteolin or hesperetin at a final concentration of 5 µM. Bacteria were grown approximately to OD₆₀₀ = 0.5 and β -galactosidase activity was determined from four independent experiments according to Miller (1972).

Detection of Nod factors by TLC.

Cultures of strains 8401pRL1JI and AT482 (Table 3) were grown overnight at 30°C in TY medium (Beringer 1974). The cultures were diluted 10-fold in fresh TY medium to a final volume of 1 ml, grown for 1 h at 30°C, and then induced with 5 µM naringenin. Two hours after induction, 25 mCi [2-¹⁴C]-acetate (Amersham, Aylesbury, U.K.) was added and the cultures were further incubated for 16 h at 30°C.

Nod factors were extracted from the cultures twice with 500 ml of water-saturated *n*-butanol. The combined butanol phases were washed with water and then dried in a vacuum dryer. The residue was resuspended in 500 ml of water and the water phase was washed three times with 500 ml of ethyl acetate. The water phase was then dried and the residue was dissolved in 10 ml of 70% methanol.

These radioactive Nod factor preparations were analyzed on octadecyl reverse phase TLC with acetonitrile/water (1:1) as the mobile phase. Radioactive compounds on the TLC plate were visualized and quantified with a PhosphorImager with the Image Quant software (both from Molecular Dynamics, Sunnyvale, CA).

Nodulation assay.

Pisum sativum cv. Wisconsin Perfection seedlings (a minimum of 20 plants per test) were grown in flasks for 4 days prior to inoculation with *R. leguminosarum* strains as described previously (Knight et al. 1986). The data presented are from a single experiment; essentially the same differences were observed in a separate independent test.

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