PERIODICUM BIOLOGORUM
VOL. 112, No 4, 459–467, 2010

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Original scientific paper

Genetic analysis of a *nodA-nodD* region of autochthonous strains of *Rhizobium leguminosarum* biovar viciae that showed effective nodulation of host plants

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Key words: *nodA* and *nodD* genes, *nodA* promoter, nitrogen fixation, nodulation

Received October 9, 2009.

Abstract

Two autochthonous strains of R. leguminosarum bv. viciae, designated 201ZG and 301ZG, were isolated from large and numerous root nodules of peas and vetches where these symbiotic bacteria fix nitrogen into ammonia. In preliminary biological experiments, both local isolates showed a better nodulation efficacy than control referent strain R. leguminosarum bv. viciae 248.

The most important nodulation genes nodA and nodD, and the bidirectional promoter region between them, were cloned, sequenced and compared with the referent strain.

Many changes in the primary structure of nod genes and the major promoter region were detected in both strains. Amino acid sequences of NodA and NodD proteins have changed up to 6.6% and 3.6%, respectively. The changes were conservative, however, and located at positions where variations had already been detected in both proteins. The nod boxes, which are essential to the function of the promoter region, were well conserved in both strains.

The results of this study provide an insight into the primary structure of nodA-nodD region in both autochthonous strains and in the possible association with the better nodulation and nitrogen fixation which showed biological experiment in greenhouse.

INTRODUCTION

In nitrate-poor soils, strains of *Bradyrhizobium*, *Azorhizobium*, *Mesorhizobium* and *Rhizobium* (jointly known as rhizobia) are able to establish a symbiosis with leguminous plants in a host-specific way by forming root nodules in which, after differentiation of the bacteria to bacteroids, they fix atmospheric nitrogen into ammonia (28,1). Differentiation of *Rhizobium* species and biovars is based on their ability to successfully nodulate a particular group of host plants. Flavonoids released by legume roots are one of the first signals that the two symbionts take turns in the molecular dialog between them. Bacterial genes, which are located on large Sym (symbiosis) plasmid, are involved in important stages of nodule formation. Some of these *nod* genes are functionally interchangeable between different *Rhizobium* species, and

they have been designated as common *nod* genes, while other genes determine the host specificity of nodulation (*hsn* genes). These bacterial genes most likely encode enzymes which synthesize a family of signal molecules that trigger a set of morphological changes in the plant root. The process culminates in the development of a mature nitrogen-fixing root nodule. Thus, plant flavonoids are recognized by regulatory NodD protein of the rhizobia. This recognition activates the transcription of nodulation genes (*nod*, *noe* and *nol*), which are involved in the biosynthesis of nodulation signals – the Nod factors, required for bacterial entry into root hairs. Inducible *nod* genes are arranged in operons which are preceded by remarkably conserved DNA sequences that have been termed »*nod* boxes« (25), and serve as binding sites for NodD. Thus, NodD has a crucial role during the nodulation process between rhizobia and host plants.

NodD belongs to the family of LysR-type transcriptional regulators (LTTRs) (26). Different studies on LTTRs have identified three basic functional domains: an N-terminal DNA-binding domain, a coinducer recognition / response domain and a conserved C-terminal domain (26, 19).

Some rhizobia species, like *Sinorhizobium meliloti*, *Rhizobium tropici*, *Rhizobium* sp. NGR234 and *Bradyrhizobium japonicum* harbour two to five copies of *nodD* genes, whereas *Rhizobium leguminosarum* bv. *viciae* and *Rhizobium leguminosarum* bv. *trifolii* have only one *nodD* gene (33). In *R. leguminosarum* bv. viciae, *nod* genes are arranged in the symbiotic plasmid pRL1JI. The *nodD* gene is transcribed divergently from the *nodABCIJ* operon (24). Beside this operon, *nodD* activates the transcription of all three other nodulation operons (*nodFEL*, *nodMNT* and *nodO*) (29, 31, 20). NodD auto regulates its own transcription (13). DNase I footprints show that NodD specifically protects the *nod* box region (11,8). Furthermore, it is predicted that NodD binds to target DNA as a dimer or tetramer (9,8). NodD binds to two separate sites in the *nod* box and these sites lie on the same side of the DNA helix (8), inducing a bend in the DNA because of its binding at the *nod* box.

The most important nodulation genes *nodA* and *nodD*, as well as the main promoter region between them, were analyzed in details. In our previous work (36) many differences in restriction maps between strains were detected. Here we describe the primary structure of *nodA*, *nodD* genes and the promoter region with the aim to associate these findings with the biological characteristics of autochthonous strains.

MATERIALS AND METHODS

Bacterial strains and plasmids

Autochthonous strains of *R. leguminosarum* bv. viciae, named 201ZG and 301ZG, were isolated from peas and vetches root nodules by the standard procedure (34). *R. leguminosarum* bv. viciae 248 (14) was used as a reference strain. Yeast-mannitol agar (34) was used for selection and maintenance of the strains of *R. leguminosarum*. For the isolation of bacterial DNA, cells were grown in Yeast--mannitol broth (YMB) at 28 °C for 48 hours with extensive aeration.

We used plasmid pCR2.1-TOPO (*Invitrogen*) for cloning of *nodA-nodD* region (from plasmid pRL1JI).

DNA preparation

High molecular weight DNA from *R. leguminosarum* was isolated as described by Hopwood et al. (12). Isolation of large plasmids was done by method described by Kado & Liu (16).

Cloning and DNA sequencing

The primers used for the polymerase chain reaction (PCR) to amplify »*nodA-nodD*« region were: JZ3 (5' – AGGACGTCTTCAGTCCAAC – 3') and JZ5 (5' – AGAGCCGGCCATTGGACAG – 3'). The amplicons were cloned in pCR2.1-TOPO vector using »TOPO TA Cloning Kit« (*Invitrogen*).

The primary structure of cloned *nodA-nodD* DNA fragments was determined on »ALFexpress™ DNA Sequencer« using kits »ALFexpress AutoRead Sequencing Kit« and »Thermo Sequenase Cy 5 Dye Terminator Kit« (Amersham Pharmacia Biotech). Sequencing was performed in several steps using different primers (universal and reverse from the kit, and specially designed primers SJZ2 (5'-TCATTAGAGCGTCGAGCGC-3') and AJZ (5'-TCGTAAGTGCCGGACCAAGG-3').

DNA and protein sequences were analyzed and stored using Lasergene sequence analysis software (DNAStar, MADISON, WI). Homology searches and sequences retrieval were done via Internet server BLAST (NCBI, NIH, Bethesda, MD, USA: http://www.ncbi.nlm.nih.gov). Multiple sequences alignments (MSA) and constructions of the phylogenetic tree from the MSA were performed with CLUSTAL X program (32). The degree of support for internal branches was further assessed by bootstrap analysis. Programs GeneDoc (22) and TreeView, version 1.6.6. (23) were used for graphic presentation of the results.

RESULTS

The nucleotide sequences of *nodA-nodD* region of autochthonous strains of *R. leguminosarum* bv. viciae, 201ZG and 301ZG, are deposited under acc. nos. DQ286900 and DQ286867, respectively.

The sequence includes complete ORF of 588 bp of *nodA* gene (196 amino acids long NodA protein), bidirectional promoter region (254 and 253 bp in autoch-

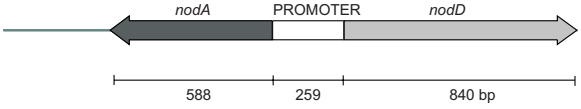

Figure 1. *Scheme of* nod *gene cluster on plasmid pRL1JI from* R. leguminosarum *bv. viciae.*

thonous strains 201ZG and 301ZG, respectively), and the partial ORF of 840 bp of *nodD* gene (280 amino acids of NodD protein; about 30 amino acids are missing from the C-terminus of NodD), (Fig. 1).

Promoter region

The sequence similarity in bidirectional promoter region is very high (91%). The length of this region was 254 bp for the *R. leguminosarum* bv. viciae 201ZG, and 253 bp for the strain *R. leguminosarum* bv. viciae 301ZG. The sequences were than compared with the promoter sequence of the control strain *R. leguminosarum* bv. viciae 248 (Fig. 2). Similarities with the control strain were lower: 84 % with 201ZG strain, and 82 % with 301ZG strain. Mutations have not changed the basic structure of certain important regulatory sequences of the promoter. The most important regulatory sequence, »*nod* box«, is completely conserved in 301ZG strain. In 201ZG strain, the presence of two substitutions at positions 39 and 40, were found (CA → TT; see Fig. 2). We have confirmed the presence of restriction site *Eco*RI in 301ZG strain, missing in 201ZG and wild type 248, which was detected in our previous work (36). Unchanged are also transcription start sites for *nodA* and *nodD*. However, we have detected several mutations, (including deletions and insertions of 1-2 bp) which resulted in shorter regions in both autochthonous strains (5 bp shorter in 201ZG strain, and 6 in 301ZG strain). There are two important regions where NodD binds, A2 and A3 (21). Region A2 is responsible for activation of *nodD* transcription, and A3 for the NodD dependent repression of *nodD* transcription. Region A2 is completely conserved in both autochthonous strains. Nevertheless, it is important to notice that dyad symmetry of A2 region, due to mutation G → A in both strains, is two nucleotides longer on both sides, »CT → A2 → AG«. In A3 region, C → T change is present at position 172 in both strains, and A → T at position 171 only in 301ZG.

nod genes

Primary structures of *nod* genes were also analyzed. Sequences of two autochthonous strains and the control 248 strain were aligned and compared (data not shown). Similarity of *nodA* was 96 % in the case of 201ZG:248, 93 % for the ratio 201ZG:301ZG and 92 % for 301ZG:248. The most related were autochthonous strain 201ZG and the control strain 248. Comparison of *nodD* genes were: 96 % for 201ZG:301ZG, and 93 % for 201ZG:248 and 301ZG:248. The most related were two autochthonous strains, which were than equally close to the control strain 248.

The number of nucleotide changes in two genes were assessed (Table 1). For 201ZG *nodA,* the number of changes was 21 (3.57 %): only 4 out of of 21 changes affected 4 amino acids (19.04 %), and 17 were silent (80.95 %). Interestingly, in 301ZG *nodA*, the percentage of difference was 7.99 %; 17 out of 47 changes affected 13 amino acids (36.17 %), and 30 were silent (63.83 %).

In 201ZG *nodD* gene, the percentage of difference was 6.43 % in: 11 out of 54 changes affected 9 amino acids (20.37 %), and 43 were silent (79.63 %). In 301ZG *nodD*, the number of nt changes was 57 (6.78 %): 14 out of 57 changes affected 12 amino acids (24.56 %), and 43 were silent (73.43 %).

Nucleotide sequencing confirmed some changes in restriction maps of autochthonous strains that have been determined in our previous work (36): the absence of

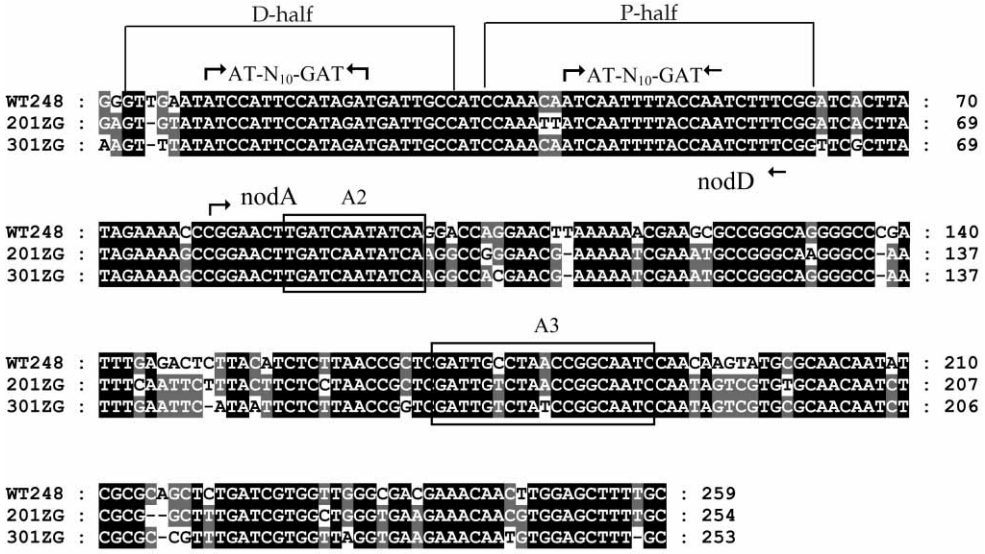

Figure 2. *The alignment of the promoter regions of all strains of* R. l. *bv. viciae, produced by Clustal X. Amino acids (aa) conserved in all three proteins are shown in white on black and those conserved in two out of three proteins in white on gray. WT248 is wild type strain; 201ZG and 301ZG are autochthonous strains. The consensus sequence of »*nod *box« region with AT-N_{10}-GAT motif is boxed. The two protected half-sites of the* nod *box are named D-half and P-half, respectively. The other* nod*D binding sites (A2 and A3) are boxed too. Transcriptional starting sites for* nod*A and* nod*D are marked with arrows.*

TABLE 1

The number of changes in two autochthonous strains in comparison with the control strain 248.

Nod proteins / strains	Substitutional mutations	Silent mutations	Total number of point mutations
NodA / 201ZG strain	4 (4 aa)	17	21
NodA / 301ZG strain	17 (13 aa)	30	47
NodD / 201ZG strain	11 (9 aa)	43	54
NodD / 301ZG strain	14 (12 aa)	43	57

*Bgl*II site in *nodD* of 201ZG strain, and the absence of *Hind*III site in *nodA* of 301ZG strain.

We analyzed and compared amino acid sequences of autochthonous strains of *R. leguminosarum* bv. viciae 201ZG and 301ZG with the control strain *R. leguminosarum* bv. viciae 248 and detected changes in amino acids (Fig. 3 for NodA and Fig. 4 for NodD).

In 201ZG NodA protein, there were detected 4 changes in amino acids (in total of 196 amino acids it is 2.0 %), and in 301ZG NodA protein13 amino acids have changed (6.6 %). The most of the mutations occurred in the less conserved regions.

In 201ZG NodD protein, there were 9 changed amino acids (3.2 %) in total of 280 amino acids that we analyzed, and in 301ZG NodD protein, 12 changed amino acids were detected or 3.6 % of the 280 amino acids that we had analyzed.

We also compared these strains with few other related rhizobia (Fig. 3 for NodA and Fig. 4 for NodD). We chose 4 NodA proteins and 6 NodD from SwissProt data bank. The analysis of homology revealed different levels of relationships between different rhizobia.

On the basis of NodA protein, the most closely related were autochthonous strain *R. leguminosarum* bv. viciae 201ZG and the control strain *R. leguminosarum* bv. viciae 248; statistically 97 %. Less related was autochthonous strain *R. leguminosarum* bv. viciae 301ZG (93 %). Than followed *R. leguminosarum* bv. trifolii, *R. meliloti* and *R. galegae*. More distant were *R. tropici* and *A. caulinodans*, and quite distant was the group created from *B. elkanii*, *R. etli* and *Rhizobium* sp. NGR234.

The NodD protein analysis revealed the following relationships: the most closely related were two autochthonous strains *R. leguminosarum* bv. viciae 201ZG and 301ZG (97 %). The wild type *R. leguminosarum* bv. viciae 248 was next (83 % similar to 201ZG and 82 % similar to 301ZG). More distant were *R. meliloti* (NodD2), *R. galegae* and *R. leguminosarum* bv. trifolii. As regards *R. leguminosarum* bv. phaseoli (NodD1 and NodD3), *Rhizobium* sp. NGR234 (NodD1), *B. japonicum* (NodD1 and NodD2), *B. elkanii* (NodD1) and *A. caulinodans*, these species made up quite a separate group.

The unrooted trees of all analysed NodA and NodD proteins are shown in Fig. 5a and 5b, respectively. We chose 8 NodA proteins and 12 NodD from SwissProt data bank. NodA as well as NodD proteins from *Rhizo-*

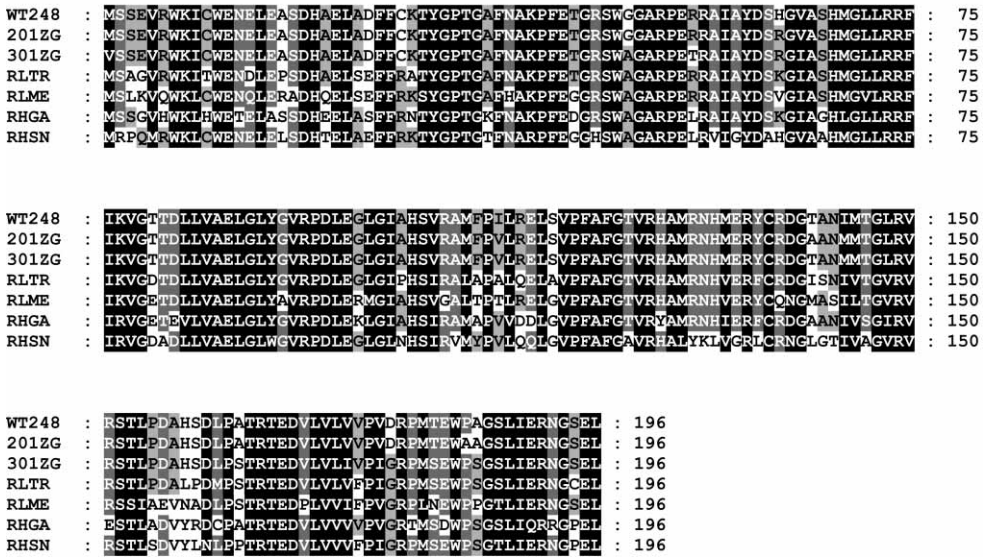

Figure 3. *Multiple alignment of seven NodA proteins from different rhizobia. 100% conserved aa (identical + similar) are shown in white on black and 75% conserved aa in white on gray. WT248 is wild type strain; 201ZG and 301ZG are autochthonous strains. RLTR (*R. leguminosarum *bv. trifolii; acc. no. P04673); RLME (*R. meliloti*; acc. no. P02962); RHGA (*R. galegae*; acc. no. P50348); RHSN (*Rhizobium sp. *NGR234; acc. no. P50349).*

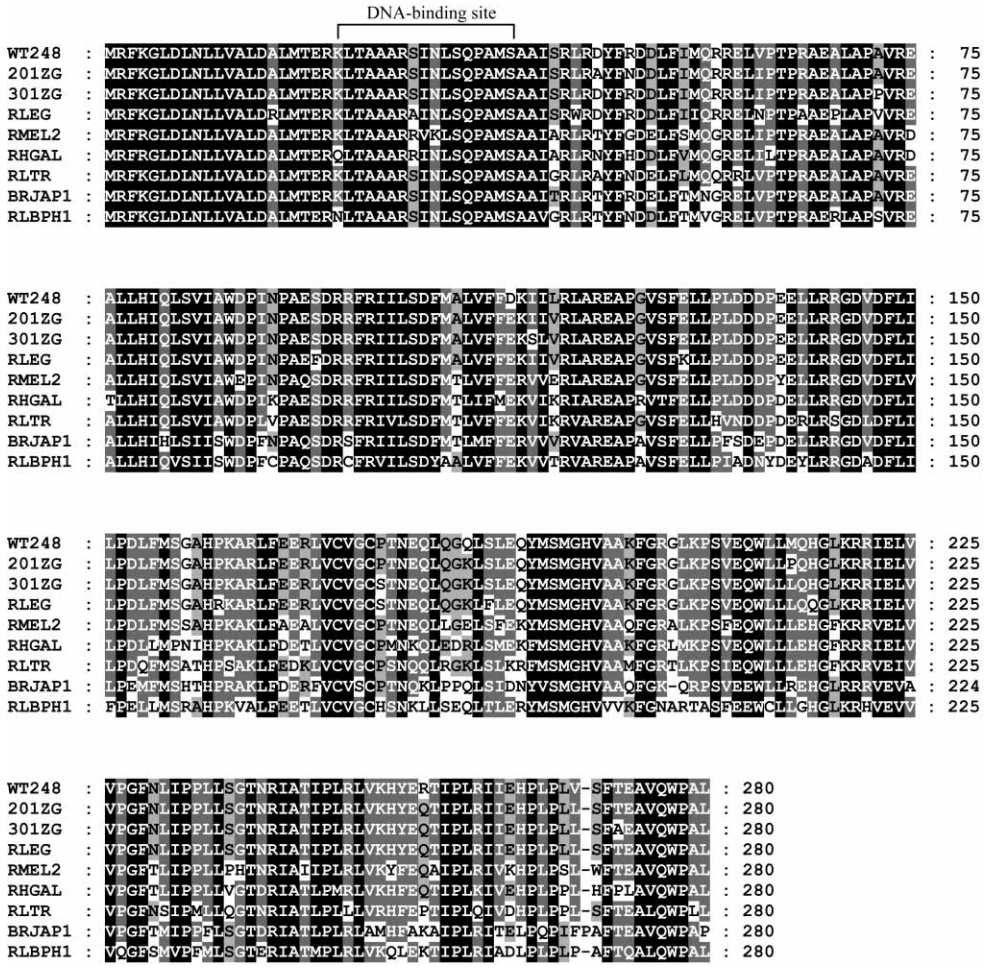

Figure 4. *Multiple alignment of seven NodD proteins from different rhizobia. 100% conserved aa (identical + similar) are shown in white on black and 75% conserved aa in white on gray. DNA-binding site is boxed. WT248 is wild type strain; 201ZG and 301ZG are autochthonous strains. RLEG (R. leguminosarum strain 1001; acc. no. P16556); RMEL2 (*R. meliloti *(nodD2); acc. no. P08719); RHGAL (*R. galegae*; acc. no. P50348); RLTR (*R. leguminosarum *bv. trifolii; acc. no.P04680); BRJAP1 (*Bradyrhizobium japonicum *(nodD1); acc. no. P12232); RLBPH1 (*R. leguminosarum *bv. phaseoli (nodD1); acc. no. P23718).*

bium leguminosarum bv. viciae strains cluster together at very high bootstrap values. Phylogenetic analysis revealed the closest relation between autochthonous strain 301ZG and *R. leguminosarum* strain 1001 (isolated from Italian soil). NodA and NodD proteins from other rhizobia were much more divergent.

Biological experiment in greenhouse

Total nitrogen content in the stem and leaves of the plants

Results obtained from measuring total nitrogen in the stem and leaves of the plants are in accordance with nodulation efficacy indicators. The values are shown in Tables 2a (pea) and 2b (vetch). They represent nitrogen content expressed in percentage as average values obtained from five repetitions for each variant in the experiment. The data were statistically interpretated using the analysis of variance method (ANOVA), which determines whether there are differences among different specimens, and using the LSD test, which points to the statistical justifiability thereof.

Amide nitrogen content in xylem

Amide nitrogen represents a transitive form of nitrogen from ammonium to nitrate. In this form, nitrogen is transported from the root to the leaves of peas and vetches. Amide nitrogen (concentration is measured only in the stem) is a good activity indicator, when speaking of nitrogen fixation. The values are listed in Tables 3a (pea) and 3b (vetch).

DISCUSSION

The number and the organization of rRNA operons in two new strains of *R. leguminosarum* bv. viciae named 201ZG and 301ZG were analyzed in previous work (36). Three rRNA operons that were found in both autoch-

TABLE 2a

Total nitrogen content in the stem and leaves of the plants (PEA).

EXPERIMENT VARIANTS	Nitrogen in leaf (%)	Nitrogen in stem (%)	Total nitrogen (leaf+stem %)
BACTERIZED *Rhizobium leguminosarum* bv. viciae 201ZG	2.790	1.374	4.164
BACTERIZED *Rhizobium leguminosarum* bv. viciae 248	2.836	1.222	4.058
NO BACTERIZATION AND NO MINERAL FERTILIZATION – N	1.900	1.068	2.968
NO BACTERIZATION + MINERAL FERTILIZATION – N	2.722	1.280	4.002

LSD P=5% =0.436, LSD P=1% =0.61 for leaf;
LSD P=5% =0.163, LSD P=1% =0.23 for stem/stalk

TABLE 2b

Total nitrogen content in the stem and leaves of the plants (VETCH).

EXPERIMENT VARIANTS	Nitrogen in leaf (%)	Nitrogen in stem/stalk (%)	Total nitrogen (leaf+stem %)
BACTERIZED *Rhizobium leguminosarum* bv. viciae 301ZG	2.274	1.394	3.668
BACTERIZED *Rhizobium leguminosarum* bv. viciae 248	1.136	0.878	2.014
NO BACTERIZATION AND NO MINERAL FERTILIZATION – N	1.198	0.730	1.928
NO BACTERIZATION + MINERAL FERTILIZATION – N	2.578	1.446	4.024

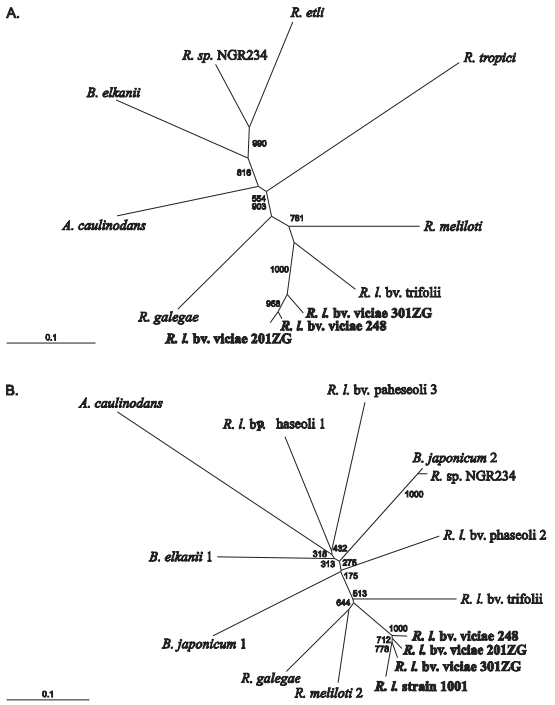

Figure 5. *Unrooted phylogenetic tree of NodA (A) proteins and NodD (B) proteins.* R. l. *bv. viciae 248, 201ZG, 301ZG strains were compared with several other rhizobia. Our strains cluster together with the high level of confidence, as determined by bootstrap analysis (see numbers at the nodes). The bar corresponds to 0.1 (10%) substitutions per site. Figure 5A.* A. caulinodans*, acc. no. Q07739; B. elkanii, acc. no. P50326;* R. etli*, acc. no. P24154; R. tropicii, acc. no. Q53252. Figure 5B.* R. l. *strain 1001, acc. no. P16556;* Bradyrhizobium japonicum (nodD2)*, acc. no. P12233;* B. elkanii*, acc. no. P50323;* A. caulinodans*, acc. no. P20669; R. l. bv. phaseoli 2, acc. no. P23719;* R. l. *bv. phaseoli 3, acc. no. P23720;* R. *sp. NGR234, acc. no. P55359. See figure 3 and 4 for other accession numbers.*

thonous strains and Southern hybridization profiles were almost identical to the profiles of control strain 248. We also sequenced the variable region between 200 and 300 nucleotides of 16S rRNA genes, finding the primary structure to be identical in all strains (data not shown).

nod genes

From the greenhouse experiment we know that both autochthonous strains are very efficient in nodulation. Thus, the mutations that we detected do not have any negative influence on the biological function of NodA and NodD proteins.

Mutations in *nodA* are more likely to be the sign of diversity in these proteins among different rhizobia. Sequences are more different on nucleotide level than on amino acid level. Fig. 3 shows that most mutations occurred in the less conserved regions of the NodA protein that is 196 amino acids long. There are several changes that are worth pointing out: Met → Val at starting position in 301ZG strain, which is one of the alternative starting codons in bacteria; Ile → Met (position 144) in both strains (mutation that was not found in any of the analyzed rhizobia); and His → Arg (position 63) in both strains, also not found in other rhizobia. Since other changes that are present in other rhizobia are changes between similar amino acids, they could not dramatically change the structure of Nod proteins. This diversity is easy to understand because of the NodA's role in nodulation process. One part of the host specificity is regulated on the level at which NodA recognizes acyl chains and transfers them to Nod factor, as a final structure that is specific for the host plant.

The primary structure of NodD differs between the strains, and a significant number of amino acids is chan-

TABLE 3a

Amide nitrogen content in the xylem (PEA).

EXPERIMENT VARIANTS	Amide nitrogen (μmol/g)
BACTERIZED *Rhizobium leguminosarum* bv. viciae 201ZG	7.976
BACTERIZED *Rhizobium leguminosarum* bv. viciae 248	7.428
NO BACTERIZATION AND NO MINERAL FERTILIZATION – N	4.112
NO BACTERIZATION + MINERAL FERTILIZATION – N	7.720

LSD P=5% =1.375, LSD P=1% =1.924

TABLE 3b

Amide nitrogen content in the xylem (VETCH).

EXPERIMENT VARIANTS	Amide nitrogen (μmol/g)
BACTERIZED *Rhizobium leguminosarum* bv. viciae 301ZG	7.776
BACTERIZED *Rhizobium leguminosarum* bv. viciae 248	2.420
NO BACTERIZATION AND NO MINERAL FERTILIZATION – N	2.442
NO BACTERIZATION + MINERAL FERTILIZATION – N	3.790

LSD P=5% =1.542, LSD P=1% =2.158

ged in autochthonous strains (Fig. 4). NodD proteins vary in length between different rhizobia. In *R. leguminosarum* bv. viciae 248 it is 303 amino acids long (in our analyses last 23 amino acids are missing in autochthonous strains 201ZG and 301ZG, because it was not possible to design primers for PCR amplification of the variable 3'-end of *nodD* gene). There were also more differences in nucleotide sequences than on the amino acid level. Nevertheless, more changes were detected when compared with NodA protein. Towards the C-terminus of NodD protein, we detected more mutations, as expected, because this part is highly variable among different rhizobia due to its role in the interaction with the host plant. N-terminus of NodD is more conserved than C-terminus because it has important regulatory function located in the highly conserved sequence, »helix-turn--helix« motif (10). This motif binds DNA to a highly conserved region in the promoter region called »*nod* box«. Some of the changes in *nodD* genes were the same in both autochthonous strains (Figs. 3 and 4; see results). Most mutations that were detected are changes between similar amino acids and occur in the less conserved regions. Interesting changes when compared to the control strain 248 are: Met → Pro (position 214), which was found only in the autochthonous strain 201ZG; Ala → Pro (position 72), unique to the 301ZG strain among all the analyzed rhizobia. This autochthonous strain also had another two changes not found in other rhizobia: Ile → Ser (position 115) and Thr → Ala (position 272).

We assume that neither mutation causes the insufficient regulatory function of NodD, nor the overexpression of *nodD*, because it could have detrimental effect on nodulation and nitrogen fixation (2, 17). Two autochthonous strains are the most related to the control strain 248; they cluster together with high bootstrap value (Fig. 5). NodA and NodD proteins from other rhizobia included in our phylogenetic analysis are much more divergent.

nod box

There is genetic evidence that interaction between NodD and flavonoid inducer activates transcription (30, 3) of *nod* genes, but direct proof is still missing (27). In some instances, the addition of appropriate flavonoids increased NodD – *nod* box binding, but in most cases, the affinity and/or binding of NodD for *nod* boxes seem not to be affected (7). Tetrameric NodD binds to promoter through anchoring the two half-sites of *nod* box. Feng et al. (6) suggested that the most important nucleotides for the specific NodD binding are located in a 2-fold inverted repeat AT-N10-GAT-N16-ATC-N10-AT, which is in agreement with some other proposed models (35). This target site is usually divided into two binding sub--sites or half-sites (Fig. 2, see results). The D-half-site consists of an imperfect inverted repeat (9) and the P--half-site, in which inverted repeat is usually not obvious, likely due to possible dual roles of this region as target sites for both NodD and RNA polymerase (5,15,18,4). Besides the conserved nucleotides in the proposed motif, other nucleotides of the *nod* box region also appear to be required for the natural optimal NodD binding (6). In our autochthonous strains of *R. leguminosarum* bv. viciae 201ZG and 301ZG the basic structure of the *nod* box region was unchanged. Also important to note is the fact that no insertions or deletions were detected in either of the two autochthonous strains, something that could possibly lead to insufficient binding of NodD to »*nod* box« region. It is known from the literature what type of mutation could lead to inefficient recognition between NodD and »*nod* box« (21,6). In our study, no such muta-

tion was detected in the critical regions. Classical consensus (25), as well as the other proposed models of *nod* box (35, 9, 6) are fully recognizable.

There are two more regions that are important for NodD binding: A2 and A3 (21). A2 is responsible for the activation of *nodD* transcription, and A3 for the NodD dependent repression of *nodD* transcription. In part A3, some substitutions occurred (C → T at position 175 in both strains, and A → T at position 179 in 301ZG strain). The first substitution weakened the symmetry of the region, which could have caused a weaker affinity of NodD to this region. Region A2, responsible for the NodD dependent activation of *nodD,* is completely conserved in both autochthonous strains, as well as the transcription starts of *nodA* and *nodD*. However, it is important to notice that dyad symmetry of A2 region, due to mutation G → A in both strains, is extended for two nucleotides on each side, »CT → A2 → AG«, which makes it more stabile and could lead to stronger binding of NodD to this region. This could lead to weaker repression, as well as stronger transcription of NodD dependent transcription of *nodD* gene.

All the results and analysis support the conclusion that the highest mutation rates were detected in the promoter regions of our two isolates; in comparison, *nod* genes were much more conserved, especially if we look at the protein level. None of the mutations disrupted the basic structure of the promoter regions important in the regulation of *nodA* and *nodD* transcriptions, or the »*nod* box« region crucial for the contact with NodD protein.

As the normal ratio between silent and substitutional mutations in the wild is 30:70 %, one may assume that many mutations were eliminated from *nod* genes because of the interference with their conserved biological role which is essential for the nodulation process.

Biological experiment in greenhouse

Total nitrogen content in the stem and leaves of the plants

The analysis of nitrogen level found in the pea leaf (Table 2a; see results) showed that there are no statistically significant differences between samples P201ZG, P248 and PN. However, the value of the negative P- control is substantially lower than any of the three samples (LSD P=1%). Having interpreted the results for nitrogen content in the pea stem (Table 2a; see results), there are no statistically significant differences among samples P201ZG, P248 and PN. However, the lowest value was produced by sample P248, which makes it not significantly different from the negative P- control. Contrary to this, a significant difference was noted between samples P201ZG (highest value) and P- (LSD P=1%). Furthermore, a difference was noted between PN and P- values. This difference, however, proved not to be as significant (LSD P=5%).

The highest values of total nitrogen were noted in samples where the bacteria had been inoculated with autochthonous strains, whereas the wild type strain produced lower values. Negative controls proved to yield the lowest values, as expected. However, the samples with added mineral nitrogen gave equally high values as the samples with autochthonous strains.

The data regarding the vetch were statistically interpreted (Table 2b; see results). The values for total nitrogen content in the leaf show that there is no statistically relevant difference between samples V301ZG and VN. There is no justified difference between samples V248 and V- either. Quite to the contrary – highly significant differences were found between the former and the latter pair, i.e. between the values of samples V301ZG and VN, on the one side, and V248 and V-, on the other side (LSD P=1%). An identical ratio was noted in the values of total nitrogen content in the stem.

Amide nitrogen content in xylem

The data regarding amide nitrogen content in the xylem of a pea were statistically interpreted (Table 3a; see results). The highest value was obtained in bacterization with an autochthonous strain of *R. leguminosarum* bv. viciae 201ZG. No statistical differences were found among samples P201ZG, P248 and PN. However, a highly significant difference was found between the same samples and the negative P- control (LSD P=1%).

After the interpretation of the results of measuring amide nitrogen in the xylem of a vetch (Table 3b; see results), it can be noted that, in relation to all other samples (LSD P=1%), the significantly highest value occurred in the case of bacterization by autochthonous strain of *R. leguminosarum* 301ZG. At the same time, there is no statistically significant difference among samples VN, V248 and V-.

Our genetic analysis indicate that differences in *nod* region could be related to the highly efficient nodulation and to nitrogen fixation in the two autochthonous strains of *R. leguminosarum* bv. viciae 201ZG and 301ZG, observed in biological experiment in greenhouse.

Acknowledgements: The molecular genetic part of this work was supported by grant to V. Gamulin from the Croatian Ministry of Science, Technology and Education (MZOS-P0098072). We are grateful to Prof. J. H. Okker from Leiden University for providing wild type *R. leguminosarum* bv. viciae 248. This work was supported by the Ministry of Science and Technology of Republic Croatia (projects: 4-01-047, 0119-113, 119-126).

REFERENCES

1. BROUGHTON W J, JABBOURI S, PERRET X 2000 Keys to symbiotic harmony. *J Bacteriol 182*:5641–5652
2. BURN J, ROSSEN L, JOHNSTON A W B 1987 Four classes of mutations in the *nodD* gene of *Rhizobium leguminosarum* biovar. viciae that affect its ability to autoregulate and/or activate other *nod* genes in the presence of flavonoid inducers. *Genes Dev 1*:456–464
3. BURN J E, WOOTTON H J C, JOHNSTON A W B 1989 Single and multiple mutations affecting properties of the regulatory gene *nodD* of *Rhizobium*. *Mol Microbiol 3*:1567–1577
4. CHANG M, CRAWFORD I 1990 The roles of indoleglycerol phosphate and the TrpI protein in the expression of *trpBA* from *Pseudomonas aeruginosa*. *Nucleic Acids Res 18*:979–988

5. COCO W M, PARSEK M R, CHAKRABARTY A M 1994 Purification of the LysR family regulator, ClcR and its interaction with the *Pseudomonas putida clcABD* chlorocatechol operon promoter. *J Bacteriol 176*:5530–5533
6. FENG J, QIANG L, HAI-LIANG H, XIAO-CHUN C, GUO--FAN H 2003 Inactivation of the *nod* box distal half-site allows tetrameric NodD to activate *nodA* transcription in an inducer-independent manner. *Nucleic Acids Res 31*:3143–3156
7. FISHER R F, LONG S R 1989 DNA footprinting analysis of the transcriptional activator proteins NodD1 and NodD3 on inducible *nod* gene promoters. *J Bacteriol 171*:5492–5502
8. FISHER R F, LONG S R 1993 Interaction of NodD at the *nod* box: NodD binds to two distinct sites on the same face of the helix and induces a bend in the DNA. *J Mol Biol 233*:336–348
9. GOETHALS K, VAN MONTAGU M, HOLSTERS M 1992 Conserved motifs in a divergent *nod* box of *Azorhizobium caulinodans* ORS571 reveal a common structure in promoters regulated by the LysR-type protein. *Proc Natl Acad Sci USA 89*:1646–1650
10. HENIKOFF S, HAUGHN G W, CALVO J M, WALLACE J C 1988 A large family of bacterial activators. *Proc Natl Acad Sci USA 85*:6602–6606
11. HONG G F, BURN J E, JOHNSTON A W B 1987 Evidence that DNA involved in the expression of nodulation (*nod*) genes in *Rhizobium leguminosarum* binds to the product of the regulatory gene *nodD*. *Nucleic Acids Res 15*:9677–9690
12. HOPWOOD D A, BIBB M J, CHATER K F, KIESER T, BRUTON C J, KIESER H M, LYDIATE D J, SMITH C P, WARD J M, SCHREMPF H 1985 Genetic manipulation of Streptomyces: A laboratory manual. The John Innes Foundation, Norwich, UK
13. HU H L, LIU S T, YANG Y, CHANG W Z, HONG G F 2000 In *Rhizobium leguminosarum*, NodD represses its own transcription by competing with RNA polymerase for binding sites. *Nucleic Acids Res 28*:2784–2793
14. JOSEY D P, BEYNON J L, JOHNSTON A W B, BERINGER J B 1979 Strain identification in *Rhizobium* using intrinsic antibiotic resistance. *J App Microbiol 46*:343–350
15. JOURLIN-CASTELLI C, MANI N, NAKANO M M, SONENSHEIN A L 2000 CcpC, a novel regulator of the LysR family required for glucose repression of the citB gene in *Bacillus subtilis*. *J Mol Biol 295*:865–878
16. KADO C I, LIU S T 1981 Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol 145*:1365–1373
17. KNIGHT C D, ROSSEN L, ROBERTSON J G, WELLS B, DOWNIE J A 1986 Nodulation inhibition by *Rhizobium leguminosarum* multicopy *nodABC* genes and analysis of early stages of plant infection. *J Bacteriol 166*:552–558
18. LAMBLIN A J, FUCHS J A 1994 Functional analysis of the *Escherichia coli* K-12 *cyn* operon transcriptional regulation. *J Bacteriol 176*:6613–6622
19. LOCHOWSKA A, HRYNIEWICZ M M 2001 Functional dissection of the LysR – type CysB transcriptional regulator. *J Biol Chem 276*:2098–2107
20. MAAGD R A D, WIJFJIES A H M, LUGTENBERG B J J 1989 *nodO*, a new *nod* gene of the *Rhizobium leguminosarum Biovar viciae* Sym plasmid pRL1JI, encodes a secreted protein. *J Bacteriol 171*: 6764–6770
21. MAO C, DOWNIE J A, HONG G 1994 Two inverted repeats in the *nodD* promoter region are involved in *nodD* regulation in *Rhizobium leguminosarum*. *Gene 144*:87–90
22. NICHOLAS K B, NICHOLAS H B, DEERFIELD D W 1997 GeneDoc: Analysis and Visualization of Genetic Variation. *EMBNEW NEWS 4*:14
23. PAGE R D M 1996 TREEVIEW: An application to display phylogenetic trees on personal computers. *Comput Appl Biosci 12*:357–358
24. ROSSEN L, SHEARMAN A W B, DOWNIE J A 1985 The *nodD* gene of *Rhizobium leguminosarum* is autoregulatory and in the presence of plant exudate induces the *nodA,B,C* genes. *EMBO J 4*: 3369–3373
25. ROSTAS K, KONDOROSI E, HORVATH B, SIMONCSITS A, KONDOROSI A 1986 Conservation of extended promoter regions of nodulation genes in *Rhizobium*. *Proc Natl Acad Sci USA 83*: 1757–1761
26. SCHELL M A 1993 Molecular biology of the LysR family of transcriptional regulators. *Annu Rev Microbiol 47*:597–626
27. SCHLAMAN H R M, PHILIPS D A, KONDOROSI E 1998 Genetic organization and transcriptional regulation of rhizobial nodulation genes. *In*: SPAINK H P, KONDOROSI A, HOOYKAAS P J J (*eds*) *The Rhizobiaceae*, Kluwer Academic Publishers, Dordrecht, Boston, London, pp 361–386
28. SCHULTZE M, KONDOROSI A 1998 Regulation of symbiotic root nodule development. *Annu Rev Genet 32*:33–57
29. SHEARMAN C A, ROSSEN L, JOHNSTON A W B, DOWNIE J A 1986 The *Rhizobium leguminosarum* nodulation gene NodF encodes a polypeptide similar to acyl-carrier protein and is regulated by NodD plus a factor in pea root exudates. *EMBO J 5*:647–652
30. SPAINK H P, WIJFFELMAN C A, PEES E, OKKER R J H, LUGTENBERG B J J 1987 *Rhizobium* nodulation gene *nodD* as a determinant of host specificity. *Nature 328*:337–339
31. SURIN B P, DOWNIE J A 1988 Characterization of the *Rhizobium leguminosarum* genes *nodLMN* involved in efficient host – specific nodulation. *Mol Microbiol 2*:173–183
32. THOMPSON J D, GIBSON T J, PLEWNIAK F, JEANMOUGIN F, HIGGINS D G 1997 The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res 24*:4876–4882
33. VAN RHIJN P J, FEYS B, VERRETH C, VANDERLEYDEN J 1993 Multiple copies of *nodD* in *Rhizobium tropici* CIAT899 and BR816. *J Bacteriol 175*:438–447
34. VINCENT J M 1970 A Manual for the practical study of root-nodule bacteria. Burgess and son (Abingdon) LTD, Berkshire, UK
35. WANG S P, STACEY G 1991 Studies of the *Bradyrhizobium japonicum nodD1* promoter: a repeat structure for the *nod* box. *J Bacteriol 173*:3356–3365
36. ZAFRAN J, REDZEPOVIC S, GAMULIN V 1996 Characterization of two new strains of *Rhizobium leguminosarum* biovar viciae efficient in the nodulation of host plants. *Poljop Znan Smotra 61*: 157–166