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Subcellular Localization of the *Rhizobium leguminosarum nodI* Gene Product

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By the use of antibodies raised against a fusion protein of *lacZ'-nodI* (produced in *Escherichia coli*) which specifically react with NodI protein, it was shown that in wild-type *Rhizobium leguminosarum* biovar viciae NodI protein (i) is recovered with the cytoplasmic membrane fraction and (ii) is translated as part of the *nodABCIJ* operon. In addition, it was found that the bacterial chromosomal background strongly influences the expression of several *nod* genes.

Bacteria of the genus *Rhizobium* can form nitrogen-fixing root nodules on leguminous plants in a host-specific way. Many of the bacterial genes that are involved in the nodulation process are localized on a symbiosis (Sym) plasmid and have been designated *nod* (for nodulation) genes. The product of the constitutively expressed *nodD* gene acts as a positive regulator; upon activation with an inducer of a flavonoid nature (23, 25, 32), it induces transcription of the other, inducible *nod* genes. Except for some suggested functions resulting from homology studies at the predicted amino acid level, the biochemical functions of these inducible *nod* gene products are unknown. One way to contribute to the elucidation of the function of gene products is to establish their subcellular location. Knowledge of the location excludes at least some of their possible functions.

The *nodI* and *nodJ* genes are inducible *nod* genes identified in both *Rhizobium leguminosarum* bv. viciae and bv. trifolii (7, 11, 28). The genes are very homologous in both biovars, and the open reading frames of the two genes are separated by only three nucleotides in *R. leguminosarum* bv. viciae (11) and have overlapping stop and start codons in bv. trifolii (28), suggesting a transcriptional coupling. While a Tn*5* insertion in *nodI* or *nodJ* of *R. leguminosarum* bv. trifolii results in poor nodulation and no nodulation within 4 weeks on the host plants *Trifolium repens* and *Trifolium pratense*, respectively (4, 8), such mutations in *R. leguminosarum* bv. viciae cause far less severe effects on nodulation (4, 9). The NodI and NodJ proteins are therefore involved in the efficiency of nodulation and probably play a role in the normal development of infection threads (8, 17). On the basis of homology with histidine and maltose transport systems of *Salmonella typhimurium* and *Escherichia coli*, respectively, it is assumed that NodI and NodJ proteins are involved in active transport of a low-molecular-weight product (11). These data suggest that NodI and NodJ proteins act together in or near the cytoplasmic membrane. To test this hypothesis, we have determined the subcellular location of NodI protein in wild-type *R. leguminosarum* bv. viciae cells.

Production of specific antibodies. In order to obtain antibodies against NodI protein, plasmid pMP2004 was constructed. This plasmid contains a translational fusion between the 5'-terminal 30 base pairs of *lacZ* and the *nodI* gene of *R. leguminosarum* bv. viciae under the control of the *lac* promoter (Fig. 1A). In this construct, the entire *nodI* gene was fused to *lacZ* by using the *Pst*I site 124 base pairs upstream of the presumed translational start of *nodI* but in the same open reading frame (Fig. 1B). The putative product encoded by pMP2004 is designated LacZ'-NodI hybrid protein, in which the N terminus is formed by *lacZ* sequences and the C terminus consists of NodI protein. To identify the products encoded by pMP2004, total cell proteins of *E. coli* JM101 (Table 1) harboring pMP2004 were analyzed by using sodium dodecyl sulfate–11% polyacrylamide gels (21). A plasmid-dependent 38-kilodalton (kDa) protein was observed when the growth medium was supplemented with isopropyl-β-D-galactopyranoside (20 $\mu g \cdot ml^{-1}$). This molecular mass is in good agreement with the predicted mass of the LacZ'-NodI hybrid protein (39.9 kDa). To isolate hybrid protein for antibody production, cell envelopes and soluble proteins were first separated. It was found that the LacZ'-NodI hybrid protein was present only in the cell envelope fraction (data not shown). This membrane fraction was subjected to preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the hybrid protein was isolated by electroelution (14) and used for immunization of a rabbit, and antiserum was obtained as described previously (26).

Plasmid pMP2004 also contains the entire *nodJ* gene (Fig. 1A). However, despite several attempts, we could not detect a protein corresponding to the *nodJ* product, the predicted size of which is 27.7 kDa (11), in *E. coli*. A possible reason may be the use of a heterologous system for expression which can result in (i) RNA instability, (ii) an inefficient translation start (for a review, see reference 5), (iii) inefficient translation due to other codon usage, or (iv) proteolytic degradation of the gene product (19).

The specificity of the antiserum raised against the LacZ'-NodI hybrid protein was determined by using Western blots (immunoblots) containing total cell proteins of wild-type *Rhizobium* strain 248 and of the *nodI*::Tn*5* mutant strain RBL1417 (Table 1). Only a protein with an apparent molecular mass of 36.5 kDa showed specific immunoreaction with the antiserum. This reaction was observed only when the bacteria were grown in the presence of the inducer naringenin (1.0 μM) (Fig. 2A, lanes 1 and 2). Except for some background reaction, no signal was found with a total protein preparation derived from the *nodI* mutant strain RBL1417 (Fig. 2A, lanes 3 and 4). As another approach to determine whether the antiserum is specific for NodI protein, the copy number of the *nodI* gene in *R. leguminosarum* was increased. The *nodI* gene was cloned downstream of the

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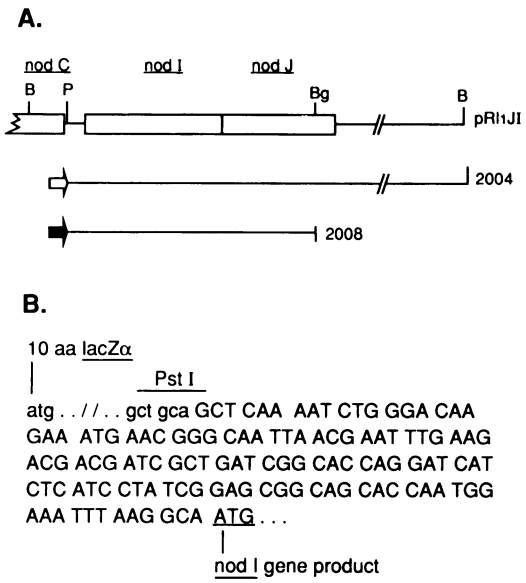

FIG. 1. *nod* sequences present in plasmids used in this study. (A) Part of pRL1JI is shown on the top line; open reading frames of the *nod* genes are represented as open boxes. Plasmid pMP2004 contains all of the *nodIJ* genes, and pMP2008 contains the entire *nodI* gene and *nodJ* sequences up to the *Bgl*II site. The *lac* promoter and the *nodA* promoter are indicated by open and black arrows, respectively; directions of transcription are indicated. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I. (B) Site of fusion between *lacZ* and pRL1JI sequences in pMP2004. Lowercase letters and capital letters represent pIC19H and pRL1JI sequences, respectively. Nucleotides are grouped in coding triplets.

nodA promoter in an IncP plasmid, resulting in pMP2008 (Fig. 1A). Analysis of the proteins from cells of induced *Rhizobium* strain 248(pMP2008) on Western blots showed a signal of the putative NodI protein eightfold higher than the signal in corresponding material of induced cells of wild-type *Rhizobium* strain 248 (data not shown). Since the observed positive-reacting protein has a mass which corresponds to the predicted size of the NodI protein (34.5 kDa) (11) and was also present in total cell proteins of induced cells of *nodJ* mutant strain RBL1418 (data not shown), it was concluded that the antiserum against LacZ'-NodI hybrid protein is specific for NodI protein.

Subcellular localization of NodI protein. To determine the subcellular location of NodI protein in *R. leguminosarum* bv. viciae, wild-type cells were disrupted by using a French press and various cell fractions were isolated by methods used routinely in our laboratory (6). Total membranes were collected by centrifugation of cell fractions for 16 h at 120,000 × *g*. The proteins in preparations of these membranes, as well as proteins in the soluble (combined periplasmic and cytoplasmic) fraction, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently analyzed on Western blots. NodI protein was detected in the total membrane fraction, but not in the soluble protein fraction, of the two tested wild-type *Rhizobium* strains, RBL5560 (data not shown) and 248 (Fig. 2B, lanes 1 to 3). Detection of the NodI protein in this location was not affected by the presence of either 0.4 M KCl or 1 M NaCl during harvesting of the membranes (data not shown). After separation of cytoplasmic and outer membranes of *Rhizobium* strain 248 on a sucrose gradient, it appeared that NodI protein was present only in the cytoplasmic membrane fraction (Fig. 2B, lanes 4 and 5). In some experiments, we detected NodI protein in preparations of an at least 10-fold-concentrated soluble protein fraction. Although we expect this to be due to contamination with some membranes, we cannot exclude the fact that, of the total NodI protein, less than 10% is present in the cytoplasm or periplasmic space. Computer analysis (10) of the amino acid sequence derived from the nucleotide sequence of *nodI* (11) shows that a potential membrane-integrated region may be present near the N terminus of the protein (Fig. 3).

From these data, it can be concluded that 90% or more of the NodI protein of wild-type *R. leguminosarum* bv. viciae cells is associated with the inner membrane. The exact nature of the association between NodI protein and the cytoplasmic membrane has yet to be elucidated. The profile obtained by using the algorithm of Engelman et al. (10) (Fig. 3) makes it unlikely that NodI protein is a membrane protein with strong overall interactions with the phospholipid bilayer. However, the fact that the membrane association is resistant to high salt concentrations excludes the possibility that the association is based on electrostatic interactions. The combined data, presented here and by others (11), suggest a hydrophobic interaction of NodI protein with either the phospholipid bilayer or an integral membrane protein. NodJ protein has been proposed as a candidate for the latter role (11, 28). However, it is not likely to serve this role since NodI protein is also recovered with the membrane fraction in the *nodJ* mutant strain RBL1418 (data not shown). NodI protein shares homology with a class of ATP-binding proteins involved in transport (1, 11, 16). To this superfamily belong bacterial proteins involved in periplasmic binding protein-dependent transport of low-molecular-weight products, e.g., maltose or histidine (15) (for a review, see reference 2), as well as proteins which function

TABLE 1. Relevant characteristics of bacterial strains and plasmids

Bacterial strains and plasmids	Characteristics	Reference
Rhizobium strains		
248	Wild-type *R. leguminosarum* bv. viciae containing Sym plasmid pRL1JI	18
RBL1417	248(pRL1JI), *nodI*82::Tn*5*	9
RBL1418	248(pRL1JI), *nodJ29*::Tn*5*	9
RBL5505	*R. leguminosarum* bv. trifolii cured of its Sym plasmid pRtr5a	24
RBL5560	Wild-type *R. leguminosarum* containing pRL1JI	32
RBL5729	RBL5505(pRL1JI), *nodI82*::Tn*5*	9
RBL5633	RBL5505(pRL1JI), *nodA10*::Tn*5*	30
RBL5634	RBL5505(pRL1JI), *nodB11*::*Tn5*	30
RBL5632	RBL5505(pRL1JI), *nodC9*::Tn*5*	30
RBL5705	RBL5505(pRL1JI::Tn*5*), *nodC7*, a 300-base-pair deletion in *nodC*	30
RBL5736	RBL5505(pRL1JI), *nodJ29*::Tn*5*	9
E. coli JM101	*supE thi* Δ(*lac-proAB*)(F' *traD36 proAB lacI*^{q}ZΔM15)	31
Plasmids		
pIC19H	IncColMP1, cloning vector	22
pMP2004	IncColMP1, production of LacZ'-NodI hybrid protein	This study
pMP2008	IncP, with *nodI* gene downstream of *NodA* promoter	This study

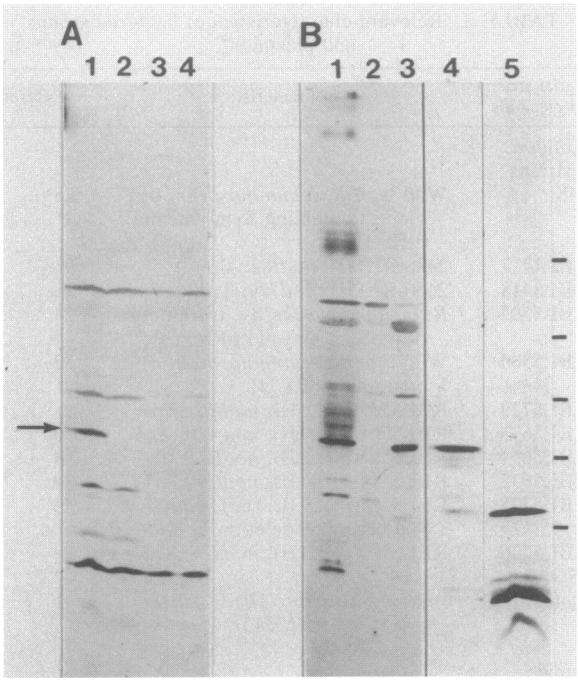

FIG. 2. (A) Western blot showing the specificity of antibodies raised against the LacZ'-NodI hybrid protein. Lanes contain proteins from whole cells of *R. leguminosarum* bv. viciae 248 (lanes 1 and 2) and from *nodI* mutant strain RBL1417 (lanes 3 and 4). The bacteria were grown either in the presence (odd-numbered lanes) or in the absence (even-numbered lanes) of 1.0 μM naringenin. The position of NodI protein is indicated by an arrow. (B) Determination of the subcellular location of NodI protein in induced cells of *R. leguminosarum* bv. viciae 248 by using Western blot analysis. Material is derived from total cells (lane 1), combined cytoplasmic and periplasmic fractions (lane 2), unseparated membranes (lane 3), cytoplasmic membrane (lane 4), and outer membrane (lane 5). Lanes 1 to 3 contain protein samples derived from the same number of cells. Horizontal bars at right indicate the positions of molecular size markers (from top to bottom: 66, 55, 45, 36, and 29 kDa).

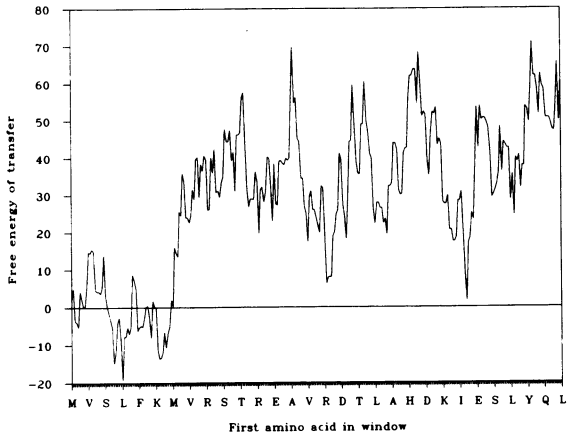

FIG. 3. Computer-generated analysis of the predicted amino acid sequence of *nodI* of pRL1JI (11) using the algorithm of Engelman et al. (10), performed with a window of 20 amino acids. The profile shows the free energy of transfer from water to oil (in kilocalories per mole [1 cal = 4.184 J]). A value of free energy equal to or less than -20 kcal · mol^{-1} indicates a potential membrane-spanning region (hydrophobic and α-helical in structure).

without a periplasmic binding protein, such as the product of the mammalian multidrug resistance (*mdr*) gene (13) and the VirB4, VirB11, and ChvA proteins from *Agrobacterium tumefaciens* (3, 29). All examples of these proteins examined so far are peripherally associated with the cytoplasmic membrane, possibly via another protein (see reference 16 and references therein). Most of the proteins of this class do not show typical membrane protein characteristics, and some of them are even very hydrophilic (such as the oligopeptide permease subunit OppF [12]).

Molecular proof of the presence of *nodI* in the *nodABC* operon. The nucleotide sequences of *nodI* and *nodJ* of *R. leguminosarum* bv. viciae have been determined (11), and it is assumed that these *nod* genes are in the same operon as *nodABC* because no promoterlike structure has been identified in the 140-base-pair region which separates *nodC* and *nodI* (27). Additional complementation assays confirm this assumption (4). With the NodI protein-specific antiserum, we were able to test this operon model further. In protein samples obtained from total cell lysates from strains RBL5633, RBL5634, and RBL5632, carrying Tn*5* insertions in *nodA*, *nodB*, and *nodC*, respectively, we could not detect any NodI protein, while we obtained a positive signal on Western blots with material derived from strain RBL5705, a deletion mutant in *nodC*, and from strain RBL5636, a Tn*5* insertion mutant in *nodJ* (data not shown). Thus, we demonstrated at the molecular level that *nodI* is part of the same operon as *nodABC*.

Influence of the chromosomal background on expression of *nod* genes. When equal amounts of total cell protein of various wild-type *R. leguminosarum* bv. viciae strains were compared, it was remarkable to observe that at least 10-fold less NodI protein was present in strain RBL5560 than was present in strain 248 (data not shown). Such a difference between the strains was also found for NodE and NodD proteins (data not shown). These data indicate a strong influence of the chromosomal background on the expression of various *nod* genes and are supported by the observation that in several, but not all, *Rhizobium meliloti* strains a repressor of *nod* gene expression acts which is encoded by a chromosomal locus (20).

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