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Localization and Symbiotic Function of a Region on the *Rhizobium leguminosarum* Sym Plasmid pRL1JI Responsible for a Secreted, Flavonoid-Inducible 50-Kilodalton Protein

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A previously described (R. A. de Maagd, C. A. Wijffelman, E. Pees, and B. J. J. Lugtenberg, J. Bacteriol. 170:4424–4427, 1988) Sym plasmid-dependent, naringenin-inducible 50-kilodalton protein of *Rhizobium leguminosarum* biovar *viciae* is further characterized in this paper. The protein was overproduced by constructing a strain containing multiple copies of the *R. meliloti nodD* gene, which facilitated its purification. An antiserum was used to screen Tn*5* insertion mutants located in the pRL1JI region found to be responsible for the production of the 50-kilodalton protein. These inserts define a new *nod* locus left of the *nod* genes identified previously. Mutations in this region affect the nodulation ability in a way which is dependent on the bacterial background as well as on the host plant. The mutants nodulate normally in a strain RBL1532 (*R. leguminosarum* biovar *viciae* strain 248, cured of its Sym plasmid) background on all three tested host plant species. In contrast, in a strain RBL5045 (*R. leguminosarum* biovar *trifolii* strain RCR5, cured of its Sym plasmid) background, nodulation on *Vicia sativa* is severely impaired, whereas nodulation on *Vicia hirsuta* and *Trifolium subterraneum* is apparently unaltered.

Rhizobia are gram-negative soil bacteria that infect the roots of plants of the family Leguminosae. The infection process leads to the formation of root nodules, in which bacteria, in the form of bacteroids, fix atmospheric nitrogen (1).

Most of the identified genes that are involved in nodulation (*nod* genes) and nitrogen fixation (*nif* and *fix* genes) are located on large, so-called Sym plasmids (3, 16). Expression of all but one (*nodD*) of these *nod* genes is induced by certain flavonoids present in root exudates of host plants (8, 13, 22, 24, 35, 36) and requires a functional *nodD* gene (21, 26). It has been shown in our laboratory that the presence of several commercially available flavonoids, such as naringenin, causes efficient induction of the *nod* gene promoters (29, 35, 36) and also causes the nodulation-related Tsr (thick short roots) phenomenon and root hair responses (curling and deformation) (37). We have tried to identify *nod* gene products in fractionated *Rhizobium* cells grown in the presence of naringenin. The overall detectability of *nod* gene products was rather low in that only one naringenin-inducible, Sym plasmid-dependent, 50-kilodalton (kDa) protein was detected. This protein was detected in the spent culture medium. Mutations in the *nod* genes *nodA*, *nodB*, *nodC*, *nodE*, *nodF*, *nodI*, and *nodJ* did not affect the production of this protein, whereas production did not occur in a *nodD* mutant (5).

In this study we have used an antiserum raised against the partially purified secreted 50-kDa protein to isolate Tn*5*-induced, nonproducing mutants. The mutations were localized in a Sym plasmid region near, but not within, the known *nod* region. The mutations in this region appeared to affect nodulation in a certain bacterial (chromosomal) background and on a certain host plant, whereas in other combinations of bacterial (chromosomal) background and host plant, no effects on nodulation were observed.

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MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are listed in Table 1.

Detection and partial purification of the 50-kDa protein. For visualization of the 50-kDa protein on gels, bacteria were grown overnight in tryptone-yeast extract (TY) medium (2) supplemented with 20% B^- medium (32), the appropriate antibiotics, and, if appropriate, inducer (luteolin or naringenin; final concentration, 4 μM). Bacteria were pelleted, and macromolecules were precipitated from the supernatant fluid by incubation for 1 h at 0°C with a final concentration of 5% (wt/vol) trichloroacetic acid with lysozyme (0.1 mg/ml) as a carrier. The precipitate was collected by centrifugation, dissolved in a small volume of water, and used for gel electrophoresis. For the production of an antiserum against the 50-kDa protein, strain LPR5045 containing pRL1JI and the IncQ plasmid pMP165 was grown overnight in 2 liters of TY–20% B^- medium supplemented with 2 μg of tetracycline per ml, 1 mg of streptomycin per ml, and 4 μM naringenin. Cells were pelleted by centrifugation at 8,000 × *g* for 15 min, and the supernatant fluid was removed and centrifuged again. Subsequently, the resulting supernatant was centrifuged for 1 h at 160,000 × *g* to pellet fragments of membranes and flagella. The resulting supernatant fluid was made 40% saturated with ammonium sulfate and kept at 4°C overnight. The resulting fine precipitate was pelleted by centrifugation at 160,000 × *g* for 30 min. Since this precipitate still contained some contaminating lipopolysaccharide and flagellar material, it was redissolved in water and centrifuged again at 160,000 × *g* for 1 h. The ammonium sulfate precipitation procedure was repeated on the resulting supernatant fluid. The final precipitate, which contained approxi-

TABLE 1. Relevant characteristics of strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
R. leguminosarum		
248	Wild type bv. *viciae*, containing pRL1JI	17
LPR5045	bv. *trifolii* RCR5, Sym plasmid cured, Rif^r	14
RBL5560	LPR5045 carrying pJB5JI(=pRL1*mep*::Tn*5*)	14, 34
248Δ5580	pRL1::Tn*1831*, 50 kb deleted from within *nodE* to the left	29
248 *nod-31*	pRL1::Tn*1831*, 12 kb deleted from within *nodB* to the right	34
248 nodD2	pRL1*nodD2*::Tn*5*	34
RBL1387	248 cured of pRL1JI	23
RBL1532	RBL1387 Rif^r Spc^r	This study
RBL1531	RBL1532 containing pIJ1089	This study
RBL1	Wild type bv. *viciae*	33
PRE	Wild type bv. *viciae*	18
TOM	Wild type bv. *viciae*	18
RCC1012	Wild type bv. *viciae*	RCC[a]
RCC1044	Wild type bv. *viciae*	RCC[a]
128C53k	Wild type bv. *viciae*	NC[a]
T1a,T2c	Wild type, isolated from broad bean	This study
K1b,K1d,K2c	Wild type, isolated from marrow fat pea	This study
RBL1520 to RBL1524	RBL1531(pIJ1089::Tn*5*)	This study
E. coli		
KMBL1164	Δ(*lac-proAB*) *thi* F$^-$	P. van de Putte
HB101(pRK2013)		7
1280	(X9368) *hflA*::Tn*5*	M. Howe
Plasmids		
pMP225	IncP carrying the pRL1 *nod* region	29
pMP165	IncQ carrying *nodD1AB* of *R. meliloti*	This study
pIJ1089	IncP carrying a 30-kb pRL1 fragment	9

[a] RCC, Rothamsted Culture Collection, Harpenden, United Kingdom; NC, Nitragin Co., Milwaukee, Wis.

mately 90% pure 50-kDa protein and some residual flagellar protein and lipopolysaccharide as contaminants, was dissolved in a small volume of PBS (0.9% [wt/vol] sodium chloride in 10 mM sodium hydrogen-dihydrogen phosphate [pH 7.4]) and used for immunization.

Production of antiserum. A 100-μg sample of protein dissolved in 0.5 ml of PBS was homogenized with an equal volume of Freund complete adjuvant and injected subcutaneously into a New Zealand White rabbit. At 1 and 2 months after the primary injection, booster injections of 100 μg of protein in PBS without adjuvant were given. The rabbit was bled 1 week after the second booster.

Isolation of non-50-kDa protein-producing mutants. Plasmid pIJ1089 was transformed into *Escherichia coli* 1280, which contains Tn*5* in its genome. From this strain, pIJ1089 was transferred to RBL1532 in the presence of the helper strain HB101(pRK2013). To select for transfer of Tn*5*-containing pIJ1089 to strain RBL1532, we used the conjugation mixture plated on YMB agar (32) supplemented with rifampin, tetracycline, and kanamycin. Non-50-kDa protein-producing mutants were identified by applying streaks of colonies on nitrocellulose filters located on top of TY agar supplemented with rifampin, tetracycline, kanamycin, and naringenin. After growth overnight, the filters were removed from the agar and excess bacteria were washed off with running tap water. Production of the secreted 50-kDa protein was detected by using 500-fold-diluted antiserum and, in a second incubation step, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin serum (Sigma Chemical Co., St. Louis, Mo.) as described below for Western immunoblotting.

Plasmid isolation and hybridization. Plasmid pIJ1089 and its Tn*5*-containing derivatives were isolated by the method of Birnboim and Doly (4) and purified by isopycnic gradient centrifugation in cesium chloride. Extraction of DNA and digestions with restriction endonucleases were carried out as described by Maniatis et al. (20). DNA fragments were transferred from agarose gels to nitrocellulose filters by the method of Southern (20). Conditions for hybridization with ^{32}P-labeled DNA probe, prepared by nick translation, were as described by Maniatis et al. (20). The transposon insertions were localized by analyzing *Bam*HI, *Hin*dIII, and *Eco*RI restriction enzyme digests of mutant DNA.

Analysis of polypeptide patterns. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described previously. Samples were prepared by mixing protein solutions or growth medium with concentrated sample buffer (19). Proteins were separated on 11% polyacrylamide gels and stained with Fast Green FCF (Sigma). All samples were routinely heated for 10 min at 95°C prior to electrophoresis.

Western blotting and immunodetection. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose sheets (BA85; Schleicher & Schuell, Inc., Dassel, Federal Republic of Germany) by electroblotting (31). The nitrocellulose was blocked with 2% (wt/vol) dried, defatted milkpowder in Tween buffer (0.1% Tween 20 in PBS) for 1 h. Subsequently, the nitrocellulose sheets were incubated with appropriate dilutions of antiserum in Tween buffer for 1 h. After being washed in Tween buffer for 30 min, blots were incubated for 1 h with 2,000-fold-diluted alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin serum in Tween buffer. After being washed in Tween buffer for 30 min, the blots were developed with

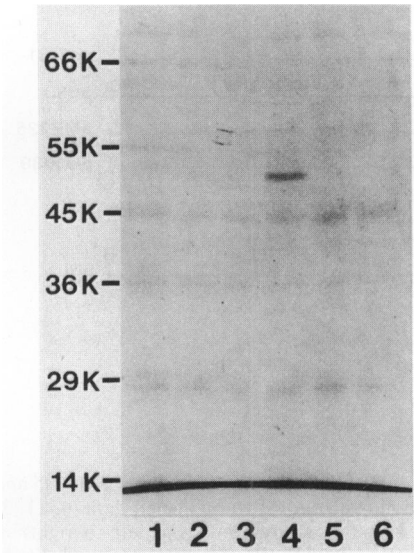

FIG. 1. Fast-Green-stained polypeptide profiles of trichloroacetic acid-precipitated culture supernatant fluids of strains RBL5560 (lanes 1 and 2), RBL5560(pMP165) (lanes 3 and 4), and LPR5045 (pMP165) (lanes 5 and 6), grown in the absence (lanes 1, 3, and 5) and in the presence (lanes 2, 4, and 6) of naringenin. Positions of molecular weight markers (in thousands) are indicated at the left.

Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates (12).

Nodulation tests. Nodulation tests on *Vicia sativa*, *V. hirsuta*, and *Trifolium subterraneum* were performed as described earlier (33). The ability of bacteria to induce root hair curling (Hac) was assessed 7 days after infection.

RESULTS

Production and isolation of the 50-kDa protein. The amount of 50-kDa protein produced in cultures of the naringenin-induced wild-type strains 248 and RBL5560 is so small (in the order of 10 ng/ml) that concentration of the culture supernatant by precipitation with trichloroacetic acid and a sensitive silver-staining procedure are required for its detection (5). However, during our studies we observed that substantially larger amounts (±50 times more), which were detectable by Fast Green staining, were produced when multiple copies of a *nodD* gene were present. By testing various *nodD* genes (of *R. leguminosarum* biovars *viciae* and *trifolii* and of *R. meliloti*), we found that this overproducing effect was largest when pMP165, containing the *R. meliloti nodD1*, *nodA*, and *nodB* genes on an IncQ plasmid, was present in RBL5560. This plasmid was obtained by cloning a *Hin*dII fragment of pRmeSL26, which contains the *nodD1*, *nodA*, *nodB*, and part of the *nodC* gene of *R. meliloti*, into the vector pMP190 (29).

Figure 1 shows the trichloroacetic acid-precipitated, Fast-Green-stained culture supernatant components after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The 50-kDa protein could not be detected in the culture supernatant of strain RBL5560 grown in the absence (lane 1) or in the presence (lane 2) of naringenin. However, the same strain containing pMP165 did produce detectable amounts of the 50-kDa protein, provided that it had been grown in the presence of luteolin, an inducer of the *R. meliloti nod* genes (22) (compare lanes 3 and 4). Detection of the 50-kDa protein requires the presence of the Sym plasmid pRL1JI of strain RBL5560, since its cured derivative, LPR5045, does not produce detectable amounts of the 50-kDa protein after introduction of pMP165 (see lanes 5 and 6).

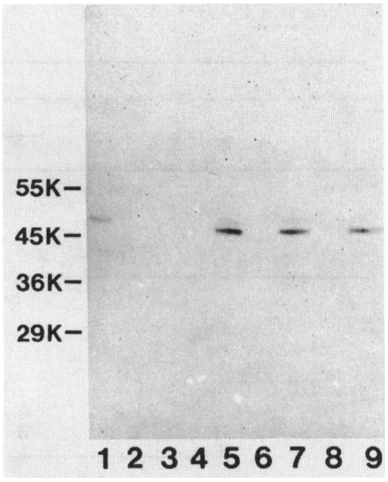

FIG. 2. Western blot of culture supernatant fluids (lanes 1 to 3 and 5 to 9) and of the cellular fraction (lane 4) of strains 248 (lane 1), RBL1387 (lane 2), 248 *nodD2*::Tn*5* (lane 3), 248 (lanes 4 and 5), RBL1387(pMP225) (lane 6), 248 *nod-31* (lane 7), 248Δ5580 (lane 8), and RBL1531 (lane 9), all grown in the presence of naringenin and incubated with antiserum against the 50-kDa protein. Positions of molecular weight markers (in thousands) are indicated at the left.

Immunological detection of the 50-kDa protein. The overproduction of the 50-kDa protein in strains with multiple copies of the *R. meliloti nodD* gene allowed its isolation in sufficient amounts and at sufficient purity to produce an antiserum. The protein was isolated from the culture supernatant by precipitation with 40% ammonium sulfate as described in Materials and Methods. The higher sensitivity of Western blotting with a specific antiserum compared with electrophoresis and protein staining allowed a much more sensitive detection of the 50-kDa protein, which did not require concentration by trichloroacetic acid precipitation. Figure 2 shows a Western blot of components of the medium separated by sodium dodecyl sulfate-gel electrophoresis without prior concentration. Production of the 50-kDa protein was observed in cultures of the pRL1JI-containing wild-type strain 248, grown in the presence of naringenin (Fig. 2, lane 1), whereas RBL1387, which is strain 248 cured of pRL1JI, and 248*nodD2*::Tn*5*, which has no functional *nodD* gene, show no production of this protein when grown in the presence of naringenin (Fig. 2, lanes 2 and 3, respectively). When cell extracts and culture supernatants of the naringenin-induced wild-type strain 248 were applied to blots in amounts derived from the same number of cells (Fig. 2, lanes 4 and 5 respectively), Western blotting showed that the protein was present exclusively in the supernatant fluid. When naringenin was omitted, the 50-kDa protein was not detected in any of the cultures.

Localization of the pRL1JI region responsible for production and secretion of the 50-kDa protein. The sensitive immunological detection method allowed us to screen a number of *nod* mutants and clones of the pRL1JI *nod* region for the production of the 50-kDa protein. Strain RBL1387 containing pMP225, an IncP plasmid carrying all known *nod* genes (Fig. 3), does not produce the 50-kDa protein (Fig. 2, lane 6), showing that a locus involved in production or secretion is located outside the established *nod* region.

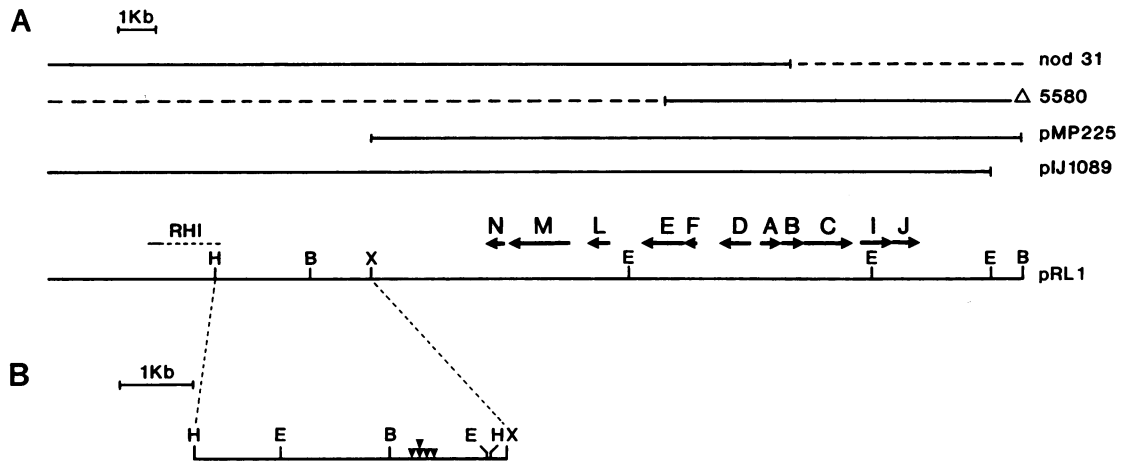

FIG. 3. (A) Genetic organization of the *R. leguminosarum* biovar *viciae* Sym plasmid pRL1JI. —, Cloned regions of pMP225 and pIJ1089; -----, deletions *nod-31* and Δ5580. Localization of the known *nod* genes and the *rhi* region is according to published data (6, 11, 25, 28–30). (B) Detail of the region of plasmid pIJ1089, containing the Tn*5* insertions described in this study (▼) (from left to right): RBL1523, RBL1522-RBL1524, RBL1520, and RBL1521. Restriction sites indicated are *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), and *Xho*I (X). Kb, Kilobases.

Strain 248 *nod-31*, carrying pRL1JI with a 12-kilobase deletion from within *nodB* to the right (Fig. 3), does secrete the 50-kDa protein, whereas strain 248(pRL1JIΔ5580), carrying pRL1JI with a 50-kilobase deletion from within *nodE* to the left, does not (Fig. 2, lanes 7 to 8, respectively). Since this result shows that a responsible region is located somewhere to the left of the known *nod* region, a large cosmid clone of pRL1JI, pIJ1089 (9), containing a large region left of the known *nod* region, was tested and found to cause production of the 50-kDa protein (Fig. 2, lane 9). Mutants containing plasmids with random Tn*5* insertions isolated as described previously were screened for production of the 50-kDa protein in an immunoblot assay. Bacteria were grown on nitrocellulose placed on TY agar containing naringenin. The 50-kDa protein absorbed to the nitrocellulose was detected with the antiserum in a color-producing reaction, resulting in the isolation of 11 mutants from a total of 220 tested colonies. Since the 50-kDa protein could not be detected in the cellular fractions of cultures of these mutants, these strains are likely to be impaired in the production rather than the secretion of the protein. To discriminate between mutants directly involved in the production of the 50-kDa protein and *nodD* mutants, tested the strains for their ability to induce root hair curling upon inoculation of *V. sativa* roots. Only 1 of the 11 mutants was unable to induce root hair curling. Since this strain was expected to carry a *nodD* mutation, it was not studied further. Plasmid DNA of 5 (RBL1520 to RBL1524) of the remaining 10 mutants was isolated, digested with restriction enzymes, and used for Southern blotting with a labeled Tn*5* probe to establish the localization of the Tn*5* insertions in these mutants. All five Tn*5* insertions appeared to be located in the same 1.6-kilobase *Bam*HI-*Xho*I fragment of the pRL1JI fragment of pIJ1089, 4 kilobases to the left of the end of the *nodN* gene (Fig. 3). The exact positions of the Tn*5* insertions are given in Fig. 3B.

Nodulation phenotype of 50-kDa protein-negative Tn*5* mutants. The nodulation ability of Tn*5* mutants with mutations in pIJ1089 of the *R. leguminosarum* 248 choromosomal background (RBL1532) was tested on three different host plants and compared with results obtained with the RBL1531(pIJ1089) parental strain. No changes in nodulation ability on *V. sativa*, *V. hirsuta*, and *T. subterraneum* were found in the strain RBL1532 background (data not shown). Results were clearly different in the strain LPR5045 background. The nodulation ability on *V. hirsuta* and *T. subterraneum* was unaffected, but nodulation on *V. sativa* was severely impaired. Figure 4 shows the nodulation kinetics of LPR5045(pIJ1089), RBL1520 and RBL1521 [LPR5045 (pIJ1089::Tn*5*)], and LPR5045(pMP225) on *V. sativa*. The onset of nodule formation was shifted from 8 days for pIJ1089 to 12 days in Tn*5* mutants, and eventually only 40% of the *V. sativa* plants were nodulated by the Tn*5* mutants, as opposed to 95% nodulated by the parental strain. Root hair curling (Hac) and infection thread formation (Inf) were unaltered in the Tn*5* mutants. This is comparable to, although slightly better than, the result with the strain containing pMP225. These results show that the pRL1JI region defined by these Tn*5* insertions is involved in the efficient nodulation of one of the host plant species, *V. sativa*, in one bacterial background, strain LPR5045, whereas it is not required in the background of strain RBL1532 or for nodulation of two other host plant species.

Occurrence in other *Rhizobium* strains of proteins immunologically related to the 50-kDa protein. To test whether proteins homologous to the 50-kDa protein are common throughout the genus *Rhizobium*, we screened for immunologically related proteins in a number of other *Rhizobium* strains by using Western blotting with the specific antiserum. Typical results of such an experiment are shown in Fig. 5. Flavonoid-inducible proteins cross-reacting with the 50-kDa protein antiserum were found in 20 of the 29 tested *R. leguminosarum* bv. *viciae* strains (indicated by the solid arrow in Fig. 5; lanes 2 and 3 are examples of negative results). In some positive strains, cross-reacting, naringenin-inducible proteins with clearly different molecular masses (ranging from 45 to 55 kDa) were detected (Fig. 5). These proteins were visible only after induction. Some reaction of the antiserum with noninducible proteins with molecular masses of 30 to 40 kDa occurs (indicated by the open arrow in Fig. 5). These proteins were visible after growth both in the presence and in the absence of *nod* gene inducers. No flavonoid-inducible proteins, immunologically related to the 50-kDa protein, were detected in culture supernatant fluids

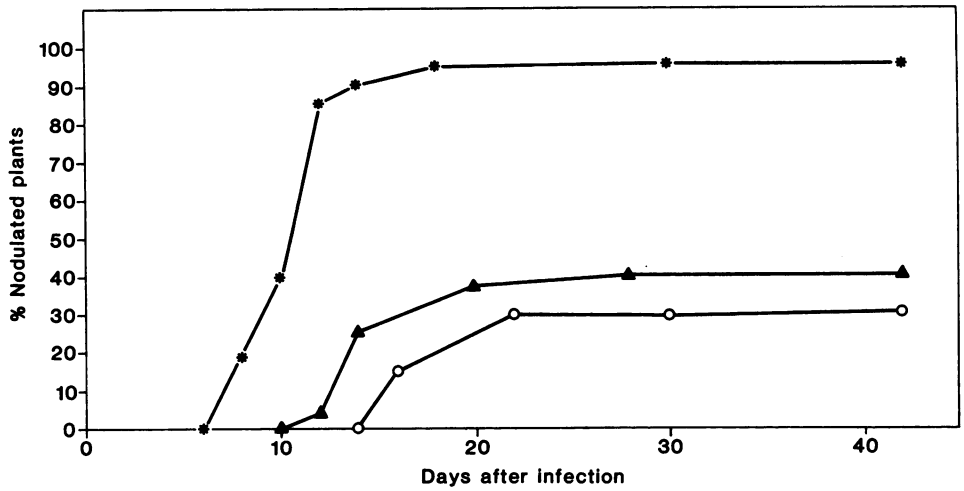

FIG. 4. Kinetics of nodule formation on *Vicia sativa* by LPR5045(pIJ1089) (*), LPR5045(pMP225) (○), and LPR5045(pIJ1089::Tn*5*) (RBL1520 and RBL1521) (▲). The results are means of three experiments, each involving at least 10 plants per strain.

of *R. leguminosarum* biovar *trifolii* (17 strains tested), *R. leguminosarum* biovar *phaseoli* (9 strains tested), *R. meliloti* (6 strains tested), or *Agrobacterium tumefaciens* (4 strains tested).

DISCUSSION

In this study we have shown that the *R. leguminosarum* biovar *viciae* Sym plasmid pRL1JI contains a previously unknown locus involved in nodulation and in production of a 50-kDa protein. This protein, whose production was previously shown to be naringenin and *nodD* dependent (5), is secreted in small amounts by wild-type strain 248. We have shown in the present paper that it is produced in much larger amounts when the *nodDAB* genes are present in multiple copies. A higher expression of *nod* genes in the presence of multiple copies of *nodD* has also been reported by others (21) and is probably also the cause of the increased production of the 50-kDa protein. An important factor in differences in the behavior of various *nodD* genes may be that, in contrast to the *R. leguminosarum nodD* gene, the *R. meliloti nodD1* gene is not autoregulated (21).

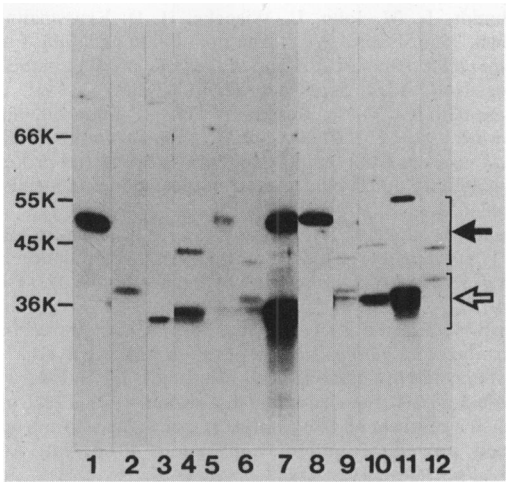

FIG. 5. Western blot of culture supernatant fluids of *R. leguminosarum* biovar *viciae*, strains 248 (lane 1), PRE (lane 2), TOM (lane 3), RCC1012 (lane 4), RCC1044 (lane 5), 128C53k (lane 6), RBL1 (lane 7), T1a (lane 8), T2c (lane 9), K1b (lane 10), K1d (lane 11), and K2c (lane 12), all grown in the presence of naringenin, incubated with antiserum against the 50-kDa protein. Symbols: ◊, reactions with noninducible proteins; ◆, reactions with the 50-kDa protein or cross-reacting, naringenin-inducible proteins. Positions of molecular weight markers (in thousands) are indicated at the left.

The overproduction of the 50-kDa protein under the conditions described above facilitated isolation of the protein and production of an antiserum, which in turn facilitated detection of the protein and allowed the use of a simple screening procedure for mutants. The five Tn*5* insertions in pIJ1089, which result in loss of production, are located close to each other in a 1.6-kilobase *Bam*HI-*Xho*I fragment, well outside the *nod* region as defined up to now, including the recently identified *nodL*, *nodM*, and *nodN* genes (30) (Fig. 3). Testing of the nodulation phenotype of these mutants showed that this region has no apparent function for nodulation in the strain 248 chromosomal background but is necessary for efficient nodulation of *V. sativa*, but not of *V. hirsuta* and *T. subterraneum*, in the strain LPR5045 background. This phenomenon may explain earlier results of Spaink et al. (29), who observed that pMP225, a large clone containing all known *nod* genes (including *nodL*, *nodM*, and *nodN*), did not contain sufficient information for efficient nodulation of *V. sativa* when used in a strain LPR5045 background. However, the same clone is sufficient in a strain 248 background (E. Pees, unpublished results). It now appears that the presence of one or more genes in the region defined by the Tn*5* insertions made in this study, which are lacking in pMP225, can be responsible for these results. Surin and Downie (30) found a similar dependence of host plant species for the nodulation phenotypes of mutations in the *nodL* and, to a lesser extent, *nodM* and *nodN* genes. For mutations in the locus responsible for production of the 50-kDa protein, we have demonstrated an additional dependence of the bacterial chromosomal background.

The observation that no immunologically related proteins were detected in other *R. leguminosarum* biovars or in *R. meliloti* and that the 50-kDa protein may not even be common among *R. leguminosarum* biovar *viciae* strains allows some speculation about its function, for which further investigation is required. Since the new *nod* region seems not to be essential under all circumstances, one might

speculate that the distribution of this region among wild-type strains may modulate the ability of these strains to nodulate some host plants. The presence of this locus may even stimulate competition for nodulation on plants on which it is not strictly essential. Possible functions of the protein are currently being studied in our laboratory. One might speculate that the protein has an enzymatic function in modifying the cell surface of either the bacterium or the host plant, allowing infection to proceed more efficiently.

Although *nodD* and flavonoid inducers are needed for expression of the 50-kDa protein, no *nod* box has been demonstrated so far outside the known *nod* region, which suggests that either a so far undetected new *nod* box is present in this region or a new mechanism of regulation is used. The 50-kDa protein is, besides the *nodA* and *nodC* products (10, 15, 27), one of the few nodulation-related proteins that can be demonstrated in cultures of wild-type cells, and it is the first rhizobial protein that has been shown to be secreted.

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