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Detection and Subcellular Localization of Two Sym Plasmid-Dependent Proteins of *Rhizobium leguminosarum* Biovar *viciae*

RUUD A. DE MAAGD,* CAREL A. WIJFFELMAN, ELLY PEES, AND BEN J. J. LUGTENBERG

Department of Plant Molecular Biology, Botanical Laboratory, Leiden University, Nonnensteeg 3, 2311 VJ Leiden, The Netherlands

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The previously described Sym plasmid-dependent 24-kilodalton *rhi* protein of *Rhizobium leguminosarum* biovar *viciae* was localized in the cytosol fraction. Another Sym plasmid-dependent protein of 50 kilodaltons is secreted into the growth medium, and its expression is dependent on both the *nodD* gene and a *nod* gene inducer.

Expression of Sym plasmid-localized *nod* genes of *Rhizobium* spp. is induced by root exudates of host plants (9) and requires the presence of the *nodD* gene product (14, 18). The inducing factors of fast-growing rhizobia in root exudates have been identified as flavones and flavanones (15, 17, 25, 26). Several commercially available flavonoids, such as naringenin, can replace the inducer(s) present in root exudates (25, 26). At the molecular level, nothing is known about the functions of the approximately 10 inducible *nod* gene products. Homology with genes with known functions suggests that *nod* gene products may play a role in transport processes and in polysaccharide synthesis (8, 20). Subcellular localization of the *nod* gene products, which has recently started (10, 19), is likely to contribute to elucidating their functions. In an attempt to detect and localize cell constituents that play a role in symbiosis, and particularly in nodulation, we have compared protein and lipopolysaccharide (LPS) profiles of subcellular fractions (4) of *Rhizobium leguminosarum* biovar *viciae* strain 248 and its Sym plasmid-cured derivative grown in the absence and in the presence of the *nod* gene inducer naringenin.

Wild-type *R. leguminosarum* biovar *viciae* strain 248, which harbors Sym plasmid pRL1JI (11), and its derivative RBL1387, which is cured of pRL1JI (16), were pregrown in tryptone-yeast extract (TY) medium (1), diluted 1:28, and grown for 16 h in TY medium on a rotary shaker at 28°C to an A_{620} of 0.2 to 0.3. For induction of *nod* genes, the growth medium was supplemented with 1 μg of naringenin (Sigma Chemical Co., St. Louis, Mo.) per ml. These conditions cause good *nod* gene induction in this background, as judged from β-galactosidase activities determined (13) for a derivative of strain 248. This derivative contains a transcriptional fusion of the *nodA* promoter of pRL1JI and the *lacZ* structural gene on pMP240, a plasmid of the IncP incompatibility group.

To detect Sym plasmid-dependent or naringenin-inducible proteins, the spent growth medium, total membranes, and total soluble cell fractions were isolated. Briefly, membrane fractions were isolated by sonication and differential centrifugation as described elsewhere (5). Macromolecules of the soluble cell fractions and of the spent TY medium, the latter after the addition of 0.1 mg of lysozyme per ml, were precipitated separately with 5% (wt/vol) trichloroacetic acid for 1 h at 0°C. Precipitated material was collected by centrifugation, resolubilized, and used for electrophoresis. Polypeptides of the various fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 11 or 13% acrylamide gels (12) and visualized by fast green FCF (12) or by silver staining (2). For the analysis of LPS, samples of cell envelopes were heated for 10 min at 95°C, incubated for 60 min at 60°C with 0.2 mg of proteinase K per ml, diluted 15-fold with sample buffer without β-mercaptoethanol, and separated on 15% polyacrylamide gels. After electrophoresis, LPSs were visualized by silver staining (23). For immunodetection, protein and LPS profiles were blotted to nitrocellulose paper (22) and allowed to react with a polyclonal rabbit antiserum raised against *R. leguminosarum* biovar *viciae* strain 248 cells grown in the presence of naringenin. The antiserum was prepared by injecting a New Zealand White rabbit subcutaneously with 2×10^8 cells in phosphate-buffered saline and Freund complete adjuvant (1:1). Injection without adjuvant was repeated 1 and 2 months later, and immune serum was collected 1 week after the last injection. To detect Sym plasmid- or naringenin-dependent antigens, the antiserum was preadsorbed with a sonicated suspension of RBL1387 cells grown without naringenin. Immunodetection was performed with alkaline phosphatase-conjugated goat anti-rabbit serum as described elsewhere (R. A. de Maagd, R. de Rijk, I. Mulders, and B. J. J. Lugtenberg, submitted for publication). The results of comparison of LPSs and proteins by electrophoresis and of antigens by Western blotting (immunoblotting) are shown (see Fig. 1, Fig. 2, and Fig. 3, respectively).

LPS profiles. Figure 1 shows the silver-stained LPS profiles of cell envelopes of strain 248 grown with and without naringenin (lanes 1 and 2, respectively) and of strain RBL1387 grown with naringenin (lane 3). These profiles contain only two major LPS species, which is in contrast with the heterogeneity of LPS length observed in other bacterial species. However, this apparent simplicity of LPS profiles occurs in several other *Rhizobium* strains as well. In these cases, the lower band may correspond to an unsubstituted core LPS, while the upper band corresponds to the O antigen containing LPS (3, 5). No changes in LPS profiles caused by the presence of the Sym plasmid or of naringenin were detected. The antiserum described above reacts with the slowest-moving species of the LPS (see Fig. 3, right

* Corresponding author.

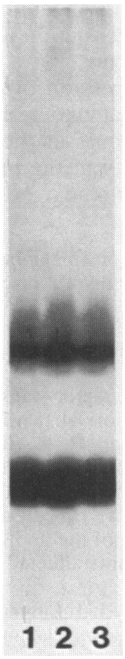

FIG. 1. Silver-stained profiles of proteinase K-treated membrane fractions of strain 248 grown with (lane 1) and without (lane 2) naringenin and of RBL1387 grown with naringenin (lane 3).

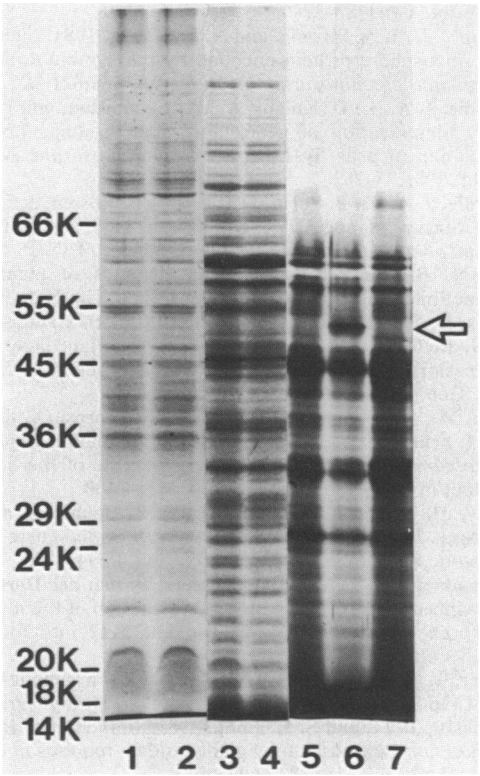

FIG. 2. Protein profiles of membrane fractions (lanes 1 and 2), soluble cell fractions (lanes 3 and 4), and medium fractions (lanes 5, 6, and 7) of cultures of strain 248 grown without (lanes 1, 3, and 5) and with (lanes 2, 4, and 6) naringenin and of RBL1387 grown with naringenin (lane 7). Lanes 1 to 4 were stained with fast green FCF. Lanes 5 to 7 were silver stained. The arrow indicates the 50-kDa naringenin-induced protein (see text). Positions of molecular weight (in thousands) marker proteins are indicated on the left.

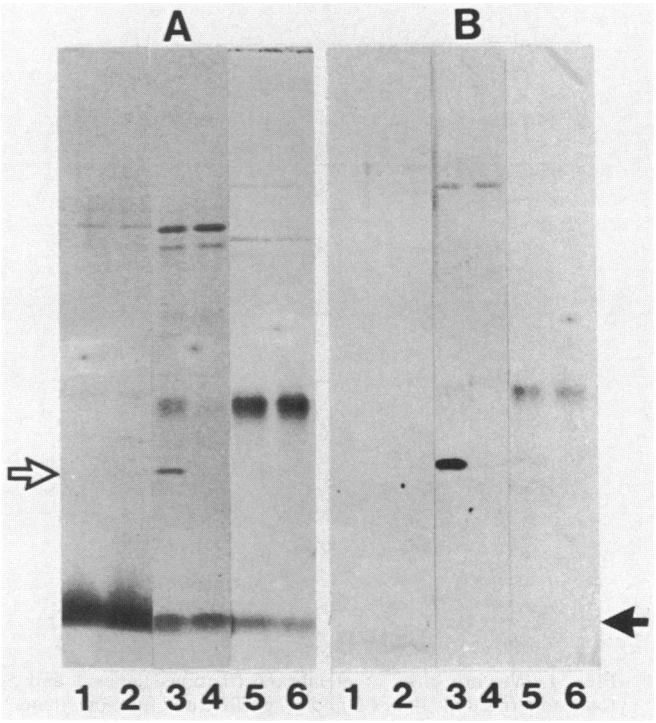

FIG. 3. (A) Western blots of membrane fractions (lanes 1 and 2), soluble cell fractions (lanes 3 and 4), and medium fractions (lanes 5 and 6) of strain 248 grown in the presence of naringenin (lanes 1 , 3, and 5) and of strain RBL1387 grown without naringenin (lanes 2, 4, and 6), incubated with rabbit antiserum to cells of strain 248 grown in the presence of naringenin. (B) Western blot of the same fractions incubated with antiserum that was preadsorbed with sonicated cells of RBL1387. The arrow on the right indicates the reaction of the antiserum with the electrophoretically slowest-moving species of LPS (Fig. 1 and text). The arrow on the left indicates the reaction with the 24-kDa (*rhi*) protein.

arrow). No antigenic changes were observed either before (see Fig. 3A) or after (see Fig. 3B) preabsorption of the antiserum. The LPS profiles of Sym plasmid pRL1JI-localized *nod* mutants were analyzed in the background of strain 248. No significant changes were detected as a result of the presence of the mutations *nodA10*, *nodB11*, *nodC9*, *nodD2*, *nodE1*, *nodF4* (24), *nodI82*, or *nodJ29* (7).

A 50-kDa protein, dependent on *nodD* and naringenin, is secreted into the medium. Figure 2 shows the protein profiles of the membrane fractions, soluble cell fractions, and medium fractions for cultures of strain 248 grown in the absence and in the presence of naringenin. No significant differences caused by the presence of the inducer were observed in the membrane (lanes 1 and 2) or in the soluble cell fractions (lanes 3 and 4). However, in the medium fraction (lanes 5 and 6) a naringenin-dependent protein with an apparent molecular mass of 50 kilodaltons (kDa) was detected. Comparison with the medium fraction of the cured derivative RBL1387 grown with naringenin (Fig. 2, lane 7), showed that the occurrence of this protein is dependent on the presence of the Sym plasmid. The use of antisera did not reveal additional naringenin-dependent molecules.

The *nod* mutants described previously were tested for the production of the 50-kDa protein. Mutants with Tn*5* insertions in *nodA*, *nodB*, *nodC*, *nodE*, *nodF*, *nodI*, and *nodJ*, respectively, all produced the protein. The *nodD* mutant, which lacks the positive regulatory gene required for the

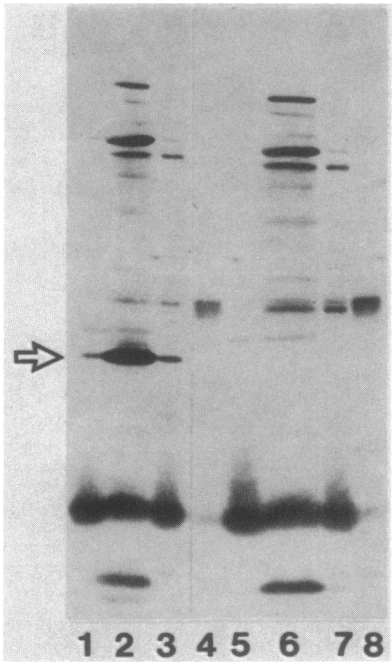

FIG. 4. Western blots of membrane fractions (lanes 1 and 5), cytoplasmic fractions (lanes 2 and 6), periplasmic fractions (lanes 3 and 7), and medium fractions (lanes 4 and 8) of strains 248 (lanes 1 to 4) and RBL1387 (lanes 5 to 8) grown without naringenin, incubated with antiserum against cells of strain 248. All fractions were applied in amounts that represented the same culture volume. The arrow indicates the reaction with the 24-kDa (*rhi*) protein.

activation of all known inducible *nod* genes, did not produce this protein. The predicted molecular mass of the *nodD* gene product and the constitutive and autoregulated transcription of *nodD* (7, 21) exclude the possibility that the 50-kDa protein is a product of the *nodD* gene itself. The result indicates that the 50-kDa protein is either the product of one of the newly identified genes *nodL* or *nodM* (8), for which no mutations have been described yet, or the product of a so far unidentified *nod* or nodulation-related gene. The localization of the structural gene for the naringenin-dependent 50-kDa protein, observed in this study, and its possible function in symbiosis are currently being studied in our laboratory.

Sym plasmid-dependent 24-kDa protein is located in the cytosol. Although fast green staining did not reveal a Sym plasmid-dependent protein in the absence of naringenin, immunoblotting did reveal such a protein with an apparent molecular mass of 24 kDa in the soluble cell fraction (Fig. 3A, lane 3). Since the protein was not detected in strain RBL1387(pIJ1211), which contains pRL1JI with a Tn*5* mutation in the *rhi* gene (6), it must be identical to the 24-kDa protein described by Dibb et al. (6). The use of antiserum preabsorbed with RBL1387 (Fig. 3B) did not reveal additional Sym plasmid- or naringenin-dependent antigens. This antiserum has strongly decreased reactions with all antigens except the 24-kDa protein (Fig. 3B). We have used this reaction of the antiserum in combination with methods described earlier for the isolation of subcellular fractions (4) to establish the localization of the protein in either the periplasmic or the cytoplasmic fraction (Fig. 4). Whereas Dibb et al. (6) made no distinction between cytoplasmic and periplasmic fractions, our results clearly show that the *rhi* protein is localized in the cytoplasmic fraction (Fig. 4, lane 3). Comparison of fractions of cells from strain 248 grown either with or without naringenin showed that naringenin influences neither the production nor the localization of the protein (results not shown).

The induction of expression of *lacZ* regulated by a *nod* promoter has shown that under the conditions used here, *nod* gene expression occurs at the transcription level (21). Surprisingly, detectability at the protein level was severely low and requires further study.

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