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Serine Residue 45 of Nodulation Protein NodF from *Rhizobium leguminosarum* bv. viciae Is Essential for Its Biological Function

TITA RITSEMA,* OTTO GEIGER,† PIETER VAN DILLEWIJN, BEN J. J. LUGTENBERG, AND HERMAN P. SPAINK

Clusius Laboratory, Institute of Molecular Plant Sciences, Leiden University, 2333 AL Leiden, The Netherlands

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A system for testing the role of the *Rhizobium nodF* gene in the production of host-specific lipochitin oligosaccharides and in nodulation was developed. We show that a mutant *nodF* gene, in which the codon for serine residue 45 was changed to that for threonine, still expresses NodF, which, however, is no longer functional.

In *Rhizobium leguminosarum* bv. viciae, host specificity of the lipochitin oligosaccharide molecules (LCOs) is determined by a highly unsaturated fatty acyl chain, for which the *nodE* gene is essential (12, 19). *nodF* precedes *nodE* in one transcriptional unit. NodF is homologous to acyl carrier protein (ACP) (16) and carries a 4′-phosphopantetheine group (5). Therefore, NodF has, like NodE, been presumed to be involved in the synthesis of the fatty acid moiety. Induction of *nodFE* is sufficient for the synthesis of multiunsaturated fatty acids (6). Here, we demonstrate that the serine 45 of NodF is essential for the synthesis of the host-specific LCOs and for nodulation on *Vicia sativa* in the absence of *nodO*.

System for analysis of the function of NodF. The study of the function of NodF has been hampered because (i) Tn*5* insertions in the *nodF* gene are polar and therefore affect *nodE* gene transcription and (ii) mutations in *nodF* and *nodE* have only a slight effect on nodulation (26). Downie and Surin (4) have explained the latter phenomenon by demonstrating that a defect in the *nodFE* genes can be complemented by the *nodO* gene. To analyze the biological function of the *nodF* gene, we have developed a test system. The *R. leguminosarum* bv. viciae Sym plasmid pRL1JI carrying deletion A69 (4) was introduced into the Sym plasmid-cured strain LPR5045 (8), resulting in strain RBL5900. The *nod* genes present in this strain are *nodABCIJ* and *nodD*. As a source for the *nodL*, *nodE*, and *nodF* genes of *R. leguminosarum* bv. viciae, the plasmids pMP2109, pMP258, and pMP2368, respectively, were used. The *nodL* gene under control of the *nodA* promoter was present on pMP2109, which was constructed by cloning the *Hin*dIII fragment of pMP2107 (1) into the IncW vector pRI40 (9) which encodes spectinomycin (100 μg/ml) resistance. The *nodE* gene under control of the *nodA* promoter is present on the IncP vector pMP258 (20) which encodes tetracycline (2 μg/ml) resistance. The *nodF* gene under control of the T7 promoter is present on pMP2368. This plasmid was constructed by cloning pMP2301, the pET9a-derived expression vector which encodes kanamycin (50 μg/ml) resistance and that is used in *Escherichia coli*, into the IncQ vector pMP190 (18) which encodes chloramphenicol (10 μg/ml) resistance and streptomycin (500 μg/ml) resistance (Fig. 1). The T7 promoter constitutively expresses *nodF* in *Rhizobium* strains (data not shown). The plasmid pMP2387 is a control plasmid which is similar to pMP2368 but lacks the *nodF* gene. The IncW, IncP, and IncQ plasmids can be maintained in each other's presence when the appropriate antibiotics are present (21).

Functional analysis of NodF. Using the plasmids listed above, we constructed a set of derivatives of *Rhizobium* strain RBL5900 which contains all combinations of the *nodF*, *nodE*, and *nodL* genes. This set of strains was tested in nodulation assays on *V. sativa* subsp. *nigra* (25). The results (Table 1) show that nodulation occurred only when, in addition to *nodABCIJ* and *nodD*, *nodF*, *nodE*, and *nodL* were also present. In that case, nodulation was as good as with the wild-type strain RBL5560. These results indicate that both the *nodE* and the *nodF* genes are essential for nodulation of *Vicia* plants in the absence of the *nodO* gene.

The strains were also analyzed for the production of LCOs by thin-layer chromatography (17) of radiolabeled compounds and high-pressure liquid chromatography (HPLC) separation of LCOs linked to diode array spectroscopic detection (19). Thin-layer chromatography analysis showed that strains which do not contain *nodL* produce only very small amounts of LCOs (data not shown), confirming previous results with a *nodL* mutant (17). HPLC analysis of *nodL*-containing strains (Fig. 2 and data not shown) showed that only in the presence of both the *nodF* and *nodE* genes, LCOs with an absorption maximum of 303 nm were observed. This UV absorption is characteristic of the C18:4 fatty acid moiety of the LCOs of *R. leguminosarum* bv. viciae. We therefore conclude that both *nodF* and *nodE* are required for the production of the host-specific LCOs. This is in agreement with the observation that in our system both *nodF* and *nodE* are necessary for nodulation of *Vicia* plants (Table 1). With *Rhizobium meliloti*, it has been shown that *nodF* is essential for the synthesis of the C16:2 acyl moiety of the LCOs (3). However, the biological importance of NodF was not shown.

Construction and functional analysis of mutant NodF S45T. In the ACP of *E. coli*, the 4′-phosphopantetheine prosthetic group, attached to serine 36, is essential for the function of ACP in fatty acid biosynthesis and transfer. The active-site

* Corresponding author. Mailing address: Institute of Molecular Plant Sciences, Leiden University, Clusius Laboratory, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands. Phone: 31-71-275072. Fax: 31-71-275088.

† Present address: TU Berlin, Institut für Biotechnologie, FG Technische Biochemie, 13353 Berlin, Germany.

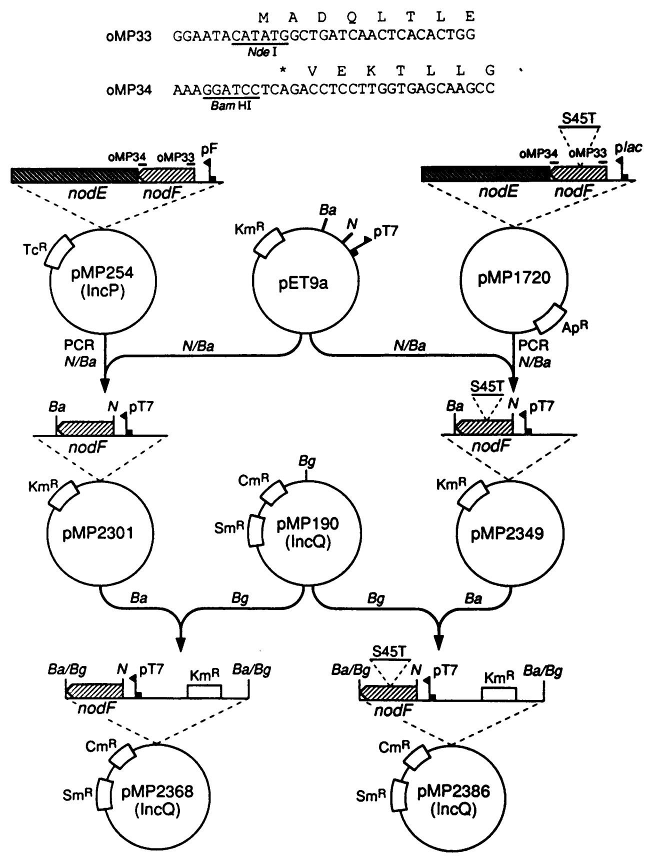

FIG. 1. Construction of plasmids. pMP254 contains a *Bgl*II-*Eco*RI fragment of pRL1JI, the symbiotic plasmid of *R. leguminosarum* bv. viciae. This fragment contains part of *nodD* and the complete *nodF* and *nodE* genes. pMP1720 contains the same genes, with a point mutation in the *nodF* gene which changes the codon for serine 45 to a codon for threonine. pMP2301 and pMP2349 are expression plasmids for the overproduction of NodF protein and NodF S45T protein, respectively, in *E. coli*. Both plasmids contain the *nodF* gene behind the T7 promoter. Suitable restriction sites for cloning of the *nodF* genes in expression vector pET9a (22) were introduced by PCR with primers oMP33 and oMP34 (whose nucleotide [bottom line] sequences and the corresponding NodF amino acids [top lines] are shown above the diagram). pMP2301 and pMP2349 were cloned into pMP190 (18), resulting in pMP2368 and pMP2386, respectively. These last two plasmids were used for expression of the NodF and NodF S45T proteins in *Rhizobium* strains. *nod* gene sequences (hatched boxes), antibiotic resistances (open boxes), and promoters (solid boxes with flags) are represented. Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Tc, tetracycline. The restriction sites indicated are *Bam*HI (Ba), *Bgl*II (Bg), and *Nde*I (N).

serine residue and the neighboring amino acids, aspartic acid and leucine, are highly conserved within presumed ACPs of various organisms (5). In NodF of *Rhizobium* species, this conserved serine is present at position 45. The ACP of *E. coli* is also involved in a *trans*-glycosylation reaction during the synthesis of membrane-derived oligosaccharides (24). For this function the 4′-phosphopantetheine group is not needed (23).

To investigate whether the prosthetic group is important for the functioning of NodF in the production of the host-specific LCOs and in nodulation, we have constructed a *nodF* mutant in which the codon for serine 45 is changed to that for threonine (S45T). We expect this substitution to have little influence on the secondary and tertiary structures of the protein.

For the construction of the mutant, in which nucleic acid 133 is changed from thymine to adenine, we made use of the methods of Kramer et al. (11) and Carter et al. (2). The PCR technique was used to obtain the plasmids pMP2349 and pMP2386 which differ from the wild-type *nodF*-containing plasmids pMP2301 and pMP2368 only by the mentioned point mutation (Fig. 1). Nucleotide sequence analysis (14) of the cloned PCR product showed that besides the desired mutation no other alteration of the original sequence was present (data not shown). In *E. coli* JM101, the mutant NodF S45T is expressed at approximately the same level as the wild-type NodF (data not shown). To test whether NodF S45T contains a 4′-phosphopantetheine prosthetic group, we have performed radiolabeling studies using [^{3}H]β-alanine, a biosynthetic precursor of the prosthetic group, as described previously (5). In the control strain, which contains the wild-type *nodF* gene, the radiolabel was incorporated into NodF (Fig. 3A). However, we could not detect a radiolabeled mutant NodF (Fig. 3A). We therefore conclude that mutant NodF S45T does not contain a 4′-phosphopantetheine group.

Plasmid pMP2386, the IncQ derivative of pMP2349, was introduced into strain RBL5900.pMP2109.pMP258. The resulting strain, containing the *nodF* mutant, was tested for the production of LCOs and nodulation on *V. sativa*. The results show that this strain does not produce 303-nm-absorbing LCOs (Fig. 2C) and is completely unable to produce nodules on *V. sativa* (Table 1).

To investigate whether the mutant *nodF*-containing *Rhizobium* strain produces NodF as efficiently as the wild-type *nodF*-containing strain, we had to raise antibodies against NodF. For this purpose, NodF was purified from RBL5560. pMP1255 by the procedure of Geiger et al. (5). Homogeneous NodF (150 μg) was injected into a rabbit by standard methods (7), and serum was collected. Using this serum, we could detect NodF in immunoblots with a titer in serum of 1:10,000. Immunoanalysis of lysates of induced strains RBL5900.

TABLE 1. Nodulation characteristics of different *R. leguminosarum* bv. viciae strains on *V. sativa*

Strain	*nod* genes present	No. of nodules per plant[a]	Nodulated plants (%)
RBL5560	All	4.2 ± 1.3	100
RBL5900.pMP258.pMP2368	*nodDABCIJEF*	0[b]	0
RBL5900.pMP2109.pMP258	*nodDABCIJLE*	0[b]	0
RBL5900.pMP2109.pMP2368	*nodDABCIJLF*	0[b]	0
RBL5900.pMP2109.pMP258.pMP2368	*nodDABCIJLEF*	5.6 ± 1.3	100
RBL5900.pMP2109.pMP258.pMP2386	*nodDABCIJLEF**[c]	0[b]	0
RBL5900.pMP2109.pMP258.pMP2387	*nodDABCIJLE*	0[b]	0

[a] The number of nodules was scored after 14 days. For each strain, 12 plants were tested. Means ± standard deviations are indicated.
[b] Also, no nodulation was observed after 21 days.
[c] *nodF** is the mutant *nodF* S45T gene.

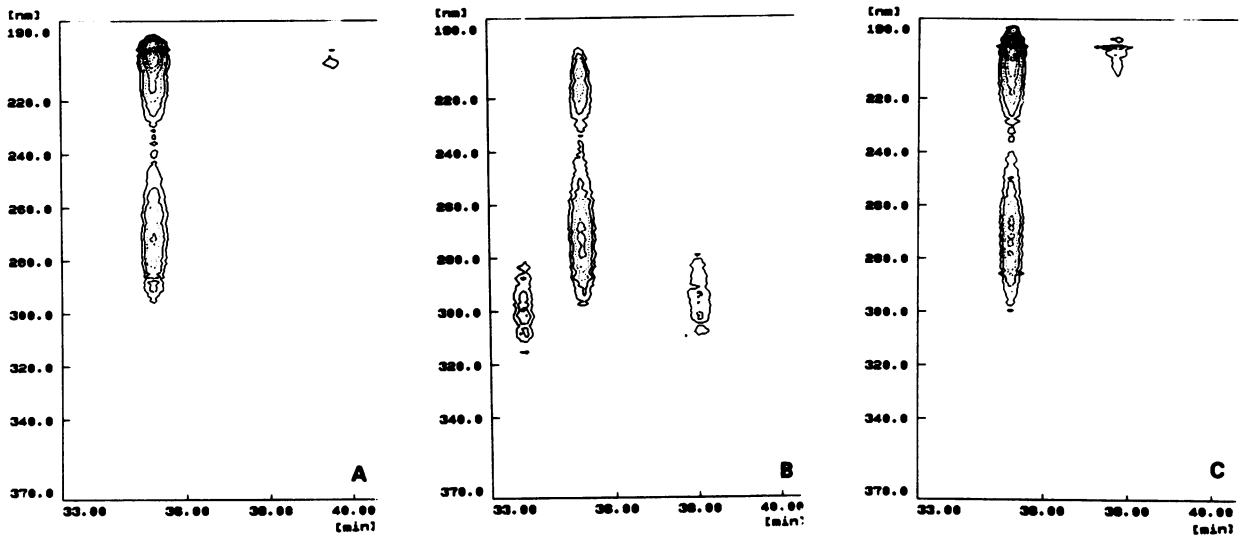

FIG. 2. HPLC analysis of lipochitin oligosaccharides. *n*-Butanol extracts of overnight-induced cultures were prepurified by using a Bakerbond C18 column (J. T. Baker, Deventer, The Netherlands) and were separated on a pepS HPLC column (Pharmacia LKB, Uppsala, Sweden), and the absorption of the eluent was analyzed with a photodiode array detector (Pharmacia LKB). The strains analyzed were RBL5900.pMP2109.pMP2368 (A), RBL5900.pMP2109.pMP258.pMP2368 (B), and RBL5900.pMP2109.pMP258.pMP2386 (C).

pMP2109.pMP258.pMP2368 and RBL5900.pMP2109.pMP 258.pMP2386 showed that approximately equal amounts of NodF S45T and wild-type NodF were produced (Fig. 3B). NodF S45T and wild-type NodF migrate at the same position in a native polyacrylamide gel (Fig. 3B), suggesting that they display the same hydrodynamic parameters and therefore should possess nearly identical three-dimensional structures.

In conclusion, in NodF the mutation S45T results in a protein which is no longer functional, indicating the crucial importance of the 4′-phosphopantetheine. These results give further support for the presumption that the function of NodF is that of an ACP. ACPs are relatively small proteins which presumably have to be recognized by various enzymes. Hardly anything is known about the structure-function relationship of ACPs. NodF has an advantage over conventional ACPs in that it is not involved in essential cellular functions and that it is not toxic when overexpressed (13). Our results show that the system we have developed for analysis of NodF can be a valuable tool for studies on the structure-function relationship of NodF as a model for ACPs in general.

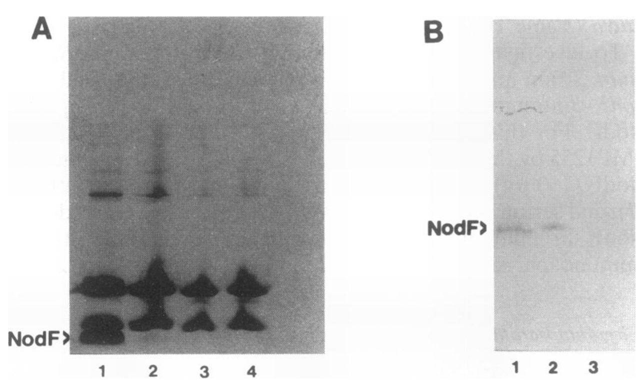

FIG. 3. (A) [^{3}H]β-alanine labeling studies of NodF in *E. coli*. Soluble proteins were analyzed on Tricine-sodium dodecyl sulfate-polyacrylamide gels (15), blotted onto nitrocellulose, sprayed with an enhancer, and autoradiographed. Lanes: 1, JM101.pMP2301 (induced); 2, JM101.pMP2301 (uninduced); 3, JM101.pMP2349 (induced); 4, JM101.pMP2349 (uninduced). (B) Western blot detection of NodF and NodF S45T in *Rhizobium* strains. Soluble proteins were analyzed on native polyacrylamide gels (10), blotted onto nitrocellulose, and immunodetected with antibodies against NodF protein diluted 1/5000. Lanes: 1, RBL5900.pMP2109.pMP258.pMP2368; 2, RBL5900.pMP2109.pMP258.pMP2386; 3, RBL5900.pMP2109.pMP 258.pMP2387.

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