The Nodulation Protein NodG Shows the Enzymatic Activity of an 3-Oxoacyl-Acyl Carrier Protein Reductase

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The acyl carrier protein NodF is required for the synthesis of unusual polyunsaturated fatty acids that confer specificity to lipochitin oligosaccharide nodulation (Nod) factors of *Rhizobium leguminosarum*. In this study, homogeneous NodF protein was used as a ligand to identify proteins of *R. leguminosarum* that specifically interact with NodF and presumably are involved in the biosynthesis or transfer of the unusual fatty acids. The N-terminal amino acid sequence of a 29-kDa protein that interacts strongly with NodF revealed high similarity to NodG of *Rhizobium* sp. N33 and to NodG of *Sinorhizobium meliloti*. We cloned and sequenced the gene coding for the NodG-like protein of *R. leguminosarum* and found it to be the product of the constitutively expressed gene *fabG*. FabG is the 3-oxoacyl-acyl carrier protein reductase that catalyzes the first reduction step in each cycle of fatty acid elongation. FabG of *R. leguminosarum* and NodG of *Rhizobium* sp. N33 were expressed in *Escherichia coli*. In both cases, the purified protein showed 3-oxoacyl-acyl carrier protein reductase activity in vitro. Therefore, NodG has the same biochemical function as FabG, and the high degree of similarity at the protein and DNA level suggest that *nodG* is a duplication of the housekeeping gene *fabG*.

Additional keywords: fatty acid biosynthesis, gene duplication.

Rhizobial bacteria have the unique ability to induce nitrogen-fixing nodules on the roots or stems of leguminous plants. The development of legume nodules is largely controlled by the reciprocal signal exchange between these symbiotic partners. Legume roots secrete specific flavonoids or isoflavonoids that induce the transcription of many bacterial genes (*nod*, *nol*, and *noe* genes). Most of these genes are involved in the synthesis and secretion of signal molecules that are essential to trigger the nodule formation and are known as Nod

factors. In all cases, their basic structure consists of a chitin oligosaccharide backbone that is *N*-acylated on the nonreducing terminal residue and are referred to as lipochitin oligosaccharides (LCOs). The nature of the fatty acids and diverse chemical substitutions provide host specificity to the LCOs produced by a given rhizobial strain (Dénarié et al. 1996; Downie 1998; Perret et al. 2000).

Although there is a large variability of LCO structures, two major structural groups can be distinguished in accordance with the type of *N*-acyl substitution (Mergaert et al. 1997; Spaink 1996; Yang et al. 1999). The first group is formed by rhizobial species infecting plants of the *Trifolieae*, *Vicieae*, and *Galegeae* tribes that produce LCOs carrying specific polyunsaturated fatty acids. The second group is synthesized by rhizobia associated with other plants that produce LCOs with common fatty acids and, in most cases, the N atom carrying the fatty acid also is substituted with a methyl group.

The biochemical functions for most of the nodulation genes involved in the biosynthesis of LCOs have been shown directly or indirectly. The *nodABC* genes are a necessity for the synthesis of LCOs: NodC produces chitooligosaccharides, NodB removes an *N*-acetyl group from the sugar of its nonreducing end, and NodA is involved in the addition of the acyl chain. Modifications of the LCO core are dependent on *nod* genes that are strain specific. Some nodulation proteins have been purified to homogeneity and in vitro analysis of their substrate specificities has provided information about their position in the LCO biosynthesis pathway (Dénarié et al. 1996; Downie 1998; Kamst et al. 1998).

Substitution of LCOs with a highly unsaturated C18:4 fatty acid is essential for the ability of the purified LCOs to induce nodule primordia and preinfection thread structures on *Vicia sativa* (Spaink et al. 1991; van Brussel et al. 1992). In *Rhizobium leguminosarum*, the nodulation genes *nodFE* are the only specialized genes required for the production of these unusual polyunsaturated fatty acids (Geiger et al. 1994). The gene products of *nodF* and *nodE* have recognizable homologies to acyl carrier protein (ACP) and to 3-oxoacyl-ACP synthase, respectively (Bibb et al. 1989; Shearman et al. 1986). An ACP (NodF) and a condensing enzyme (NodE), however, are not sufficient to allow for the synthesis of such fatty acids (Geiger et al. 1998).

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ACPs play a key role during fatty acid biosynthesis and transfer and interact with the enzymes that catalyze a certain step of fatty acid synthesis or transfer. In several instances it was possible to purify proteins that interact with ACPs during their catalytic cycle by means of an AcpP affinity column (Bayan and Therisod 1989; Cooper et al. 1989; Lambalot and Walsh 1995).

In this study, homogeneous NodF protein was used as a ligand to identify proteins of *R. leguminosarum* that specifically interact with NodF and, therefore, are presumably involved in the biosynthesis or transfer of the unusual fatty acid. We identified a 29-kDa protein in cell-free extracts of *R. leguminosarum* that elutes from the NodF affinity column with 2 M NaCl. The determined N-terminal amino acid sequence showed high similarity to NodG of *Rhizobium* sp. N33 and NodG of *Sinorhizobium meliloti*. We cloned the region carrying the gene coding for the NodG-like protein of *R. leguminosarum* and found that it codes for the constitutive enzyme 3-oxoacyl-ACP reductase (FabG). FabG and NodG were over-produced as histidine (His)-tagged proteins, and the purified proteins show the same activity.

RESULTS

Identification of a soluble protein of *R. leguminosarum* that shows strong interaction with NodF.

Previously it was shown that expression of the *nodFE* and *nodD* genes was sufficient to allow for biosynthesis of the C18:4 highly unsaturated fatty acid in a strain (*R. leguminosarum* LPR5045) lacking the symbiotic plasmid (Geiger et al. 1994). Initially we used soluble proteins prepared from *R.*

leguminosarum LPR5045 (pMP280) (pMP1255) that had been induced with naringenin to investigate the binding of proteins to a NodF protein-affinity column. We prepared a control column to test for proteins that were retained by the column matrix or by contaminants copurified with NodF. Portions of 1 ml from a cell extract preparation were applied to the NodF and control columns and eluted with increasing concentrations of NaCl, followed by an elution with 6 M guanidine-HCl. The protein patterns of equivalent fractions eluting from the NodF or control affinity column were compared using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by silver staining, in order to identify proteins interacting specifically with NodF (Fig. 1 and data not shown). There were at least six proteins eluting at 150 mM NaCl that interact specifically with NodF. Their subunit molecular weights are 51, 43, 32, 27, 23, and 20 kDa (Fig. 1A). There was a major and almost unique protein of 29 kDa eluting from the NodF column with 2 M NaCl, whereas no protein was eluting from the control column at this step (Fig. 1B). There were no detectable differences in the patterns of proteins eluting from the NodF and control column with 500 mM NaCl or at 6 M guanidine-HCl (data not shown).

To obtain sufficient protein for N-terminal sequencing, we prepared a large-scale NodF-affinity column of 6.5 ml. On this column, 6.5 ml of soluble proteins of a cell-free extract from *R. leguminosarum* LPR5045 (pMP280) (pMP1255) that had been induced with naringenin were chromatographed. The proteins eluting at 2 M NaCl were concentrated and, after separation on SDS-PAGE and blotting to a polyvinylidene difluoride (PVDF) membrane, the 29-kDa protein was subjected to N-terminal sequencing. A sequence of 50 amino acids was obtained, and a BLAST-P search with this fragment revealed homology to NodG of *Rhizobium* sp. N33 and to NodG of *S. meliloti* (82 and 72% identity, respectively) (Fig. 2A).

In order to establish whether the presence of the 29-kDa NodF binding protein in the crude extract was dependent on the addition of naringenin and the regulatory gene *nodD* (meaning that it might be the product of a possible nodulation gene located on the chromosome), we obtained proteins from soluble cell extracts prepared from *R. leguminosarum* LPR5045 that had not been induced with naringenin. Chromatography of this extract at the 6.5-ml NodF-affinity column again yielded a protein of 29 kDa eluting at 2 M NaCl. This protein was prepared, as described above, and subjected to N-terminal sequencing. The first 20 amino acids were determined and were identical to those of the 29-kDa protein obtained from extract induced with naringenin. Therefore, the 29-kDa NodF binding protein was the product of a constitutively expressed gene rather than the product of a nodulation gene.

Isolation and characterization of the gene coding for the NodG-like protein.

We designed a pair of degenerate oligonucleotides on the basis of the amino acid sequence of the N terminus of the 29-kDa protein in order to clone a fragment of the encoding gene (see below and Fig. 2A). From genomic DNA of *R. leguminosarum* LPR5045, a fragment of the expected size was amplified and subsequently cloned in pUC19. The internal core sequence of 67 nucleotides (nt) of the insert fragment in

Fig. 1. NodF-Affi-Gel 15 affinity chromatography. **A**, Elution at 150 mM NaCl. **B**, Elution at 2 M NaCl. **A and B**, Fraction eluting from the control column (lanes 1) is compared with an equivalent fraction eluting from the NodF column (lanes 2). Lanes 3, Molecular weight protein markers. Protein subunit molecular weights are indicated in kilodaltons; 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis with silver staining.

pTB5057 had a deduced amino acid sequence corresponding to amino acids 15 to 35 of the N-terminal sequence (Fig. 2A). The pTB5057 (109 bp) insert was used as a probe against genomic DNA of *R. leguminosarum* LPR5045 that was completely digested with different restriction enzymes. A unique fragment of approximately 5.1 kb was observed in the *Eco*RI digestion (data not shown). A size-selected library of *Eco*RI-digested fragments of approximately 5 kb was constructed and, with the use of the same DNA probe, several hybridizing clones were identified. Sequencing from one of the borders of the selected plasmid pTB5101 identified a partial open reading frame (ORF) that corresponded to the recently identified *acpP* of *R. leguminosarum* (López-Lara and Geiger 2000). Upstream of the *acpP* gene, we identified an ORF of 735 nt coding for a polypeptide of 245 amino acids from which the N-terminus matched that of the 29-kDa NodF binding protein (Fig. 2B). The predicted protein shows highest similarity to *Rhizobium* sp. N33 NodG (87% identity) and to the NodG of *S. meliloti* 1021 (80% identity). Significant homologies also are found with FabG proteins from different organisms. FabG or 3-oxoacyl-ACP reductase is responsible for the first

NADP-dependent reduction step during elongation cycles in fatty acid biosynthesis. Upstream of the gene coding for the 29-kDa NodF binding protein, an ORF that was probably coding for FabD was located. Previously we had shown that the *fabF* gene is located downstream of *acpP* (López-Lara and Geiger 2000). This same organization, *fabD*–*fabG*–*acpP*–*fabF* (Fig. 2B), is found in other bacteria such as *Escherichia coli* (Rawlings and Cronan 1992) and represents a cluster of genes involved in fatty acid biosynthesis. Several lines of evidence support that the 29-kDa NodF binding protein identified in this study corresponds to the constitutive 3-oxoacyl-ACP reductase of *R. leguminosarum* because it is expressed constitutively, shows homology to FabG, and the encoding gene is located within a cluster of genes involved in fatty acid biosynthesis.

We isolated two overlapping cosmids of *S. meliloti* 1021 carrying the *fab* cluster and upstream of *acpP*, also in *S. meliloti*, the *fabG* gene was found. *S. meliloti* FabG shows 89% identity along the whole protein to *R. leguminosarum* FabG. The NodG of *Rhizobium* sp. N33 is slightly more similar to rhizobial FabG proteins than it is to the NodG from

A

B

Fig. 2. Strategy for cloning of the gene encoding the 29-kDa NodF-binding protein. **A**, Alignment of the N terminus obtained for the 29-kDa NodF-binding protein of *Rhizobium leguminosarum* LPR5045 with NodG of *Rhizobium* sp. N33 and NodG of *Sinorhizobium meliloti* 1021. Identical residues are shaded in black. Degenerate oligonucleotides (oTBC23 and oTBC24) used for amplification are depicted above the corresponding amino acid sequence. The polymerase chain reaction (PCR) product obtained with oTBC23 and oTBC24 was cloned to produce plasmid pTB5057. **B**, Fatty acid biosynthesis (*fab*) cluster in *R. leguminosarum* LPR5045. Region carrying the gene encoding the 29-kDa protein was identified on a 5.1-kb *Eco*RI fragment that had been cloned in pUC18 (pTB5101) with the insert in pTB5057 as a probe. pTB5101 carries sequences located upstream of the *Eco*RI restriction site (R) located in the *acpP* gene. Location of an open reading frame corresponding to *fabF* downstream of *acpP* was shown previously (López-Lara and Geiger 2000). The same genetic organization was found in *S. meliloti*.

S. meliloti 1021 (Table 1). All four proteins, however, are very similar, with identities above 79.6% (Table 1).

***nodG* is a reiteration of *fabG*.**

Genomic DNAs from *R. leguminosarum* LPR5045, *S. meliloti* 1021, *Rhizobium* sp. N33, and plasmid pGMI515 that carries *nodG* of *S. meliloti* (Faucher et al. 1988) were analyzed in Southern blots with a digoxigenin-11-ddUTP (DIG)-labeled probe carrying *fabG* of *R. leguminosarum*. DNAs were digested with *Eco*RI or *Bam*HI (enzymes that do not cut in either of the *nodG* genes). Strong hybridizing signals were found for plasmid pGMI515 at the expected size of fragments carrying *nodG* (15.25 and 3.9 kb for the *Eco*RI and *Bam*HI digestion, respectively). Genomic DNA digests of *S. meliloti* showed two hybridizing bands, each of different intensity. The weak bands correspond to fragments carrying *nodG*, whereas the strongest bands correspond to fragments carrying *fabG* (7.2 and 6.1 kb for the *Eco*RI and *Bam*HI fragments, respectively). In genomic DNA of *Rhizobium* sp. N33, two bands of equal intensity were detected for each digestion. *Eco*RI and *Bam*HI fragments carrying *nodG* were detected at the expected sizes of 4.15 and 7.1 kb, respectively. Therefore, the 5.05-kb *Eco*RI fragment and the *Bam*HI fragment larger than 20 kb are the ones carrying *fabG* of *Rhizobium* sp. N33. Only a single band hybridizing to *fabG* is detected in genomic DNAs of *R. leguminosarum* LPR5045 and of the strain carrying the symbiotic plasmid pRL1J1 (in both cases, at 5.1 and over 20 kb for the *Eco*RI and *Bam*HI digestion, respectively), indicating that there are no homologs to *fabG* or *nodG* in pRL1J1. A high similarity between FabG and NodG can be detected at the DNA level, and it is likely that *nodG* originated from a duplication of the chromosomal counterpart *fabG*.

NodG functions as 3-oxoacyl-ACP reductase.

The high similarity between the NodG and FabG proteins of rhizobia suggest that both have the same enzymatic activity. The genes coding for *Rhizobium* sp. N33 NodG and for *R. leguminosarum* FabG were cloned in the expression vector pET16b that allowed for overproduction of the respective His-tagged proteins (see below). Both gene products were highly overproduced as soluble proteins in the host strain *E. coli* BL21(DE3) pLysS (Fig. 3; compare lanes 3 and 4 to 2). The presence of the His-tag on the overproduced proteins was confirmed with an anti-His antibody (data not shown). Although an overexpressed protein was not observed in cell extracts of uninduced BL21(DE3) pLysS carrying pTB5111 or pTB5116 after SDS-PAGE and subsequent staining with

Coomassie blue, weak signals were observed with the anti-His antibody, indicating the expression of a minor amount of protein, even in the absence of isopropyl- β -D-thiogalactopyranoside (IPTG) induction (data not shown). Therefore, the BL21(DE3) pLysS strain was used as a negative control.

Soluble cell extracts of BL21(DE3) pLysS (negative control) and of the strain carrying pTB5011 (overproduction of His₁₀-FabG) or pTB5116 (overproduction of His₁₀-NodG) were chromatographed on a Ni²⁺ column. The fractions eluting with 500 mM imidazole and containing possible contaminants from the mock purification (Fig. 3, lane 5), the purified His-FabG (Fig. 3, lane 6), or the purified His-NodG (Fig. 3, lane 7) were tested for 3-oxoacyl-ACP activity with the substrate analog acetoacetyl-coenzyme A (CoA). The specific activities for FabG and NodG with acetoacetyl-CoA as substrate were 41 and 46 μ mol of NADPH, respectively, oxidized per minute per milligram of protein. The FabG and NodG activities were completely dependent on NADPH because NADH produced no activity. Additionally, no activity was detected in the fraction obtained from the mock purification with either NADPH or NADH. Therefore, NodG shows 3-oxoacyl-ACP reductase activity and, as is the case for FabG, uses NADPH as cosubstrate.

DISCUSSION

We used affinity chromatography and found a strong interaction between the NodF protein and the constitutive enzyme 3-oxoacyl-ACP reductase of *R. leguminosarum*. 3-Oxoacyl-ACP reductase, also known as FabG because it is the product

Fig. 3. Expression and purification of histidine (His)-FabG and His-NodG. Lanes 2 to 4, Soluble cell extracts obtained from isopropyl- β -D-thiogalactopyranoside-induced cultures from strains BL21(DE3) pLysS (lane 2), BL21(DE3) pLysS $\times$ pTB5111 (lane 3), and BL21(DE3) pLysS $\times$ pTB5116 (lane 4). Lanes 5 to 7, Fractions obtained after chromatography on Ni²⁺ columns used for the 3-oxoacyl-ACP-reductase activity. Lane 5, Negative control; lane 6, His₁₀-FabG; lane 7, His₁₀-NodG. Lane 1, Broad-range protein marker (New England BioLabs, Beverly, MA, U.S.A.). Molecular weights are indicated in kilodaltons; 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis stained with Coomassie blue.

Table 1. Percentage of identity (similarity) among FabG and NodG proteins^a

	FabG1021	FabG5045	NodGN33	NodG1021
FabG1021		89.4 (94)	87.8 (93)	79.6 (89)
FabG5045			87.8 (94)	80.0 (89)
NodGN33				86.9 (91)
FabGE. coli	52.8 (73)	52.2 (70)	52.4 (72)	48.8 (70)

^a FabG1021: FabG from *Sinorhizobium meliloti* 1021. FabG5045: FabG from *Rhizobium leguminosarum* LPR5045. NodGN33: NodG from *Rhizobium* sp. N33. NodG1021: NodG from *S. meliloti* 1021. FabGE. coli: FabG from *Escherichia coli*. Accession numbers of proteins used for comparison are NodG1021, CAA68649; NodGN33, P72332; FabGE. coli, P25716.

of the *fabG* gene, catalyzes the first reduction step in each cycle of fatty acid elongation. We found that FabGs of rhizobia are very similar to the nodulation protein NodG. Therefore, *nodG* probably originated from a recent duplication of *fabG* and its product still has the same enzymatic function as FabG.

A *nodG* gene has been reported for three rhizobia. In each case (*S. meliloti*, *Rhizobium* sp. N33, and *Mesorhizobium* sp. 7653R), *nodG* is located in the same operon and downstream immediately of *nodFE* (Cloutier et al. 1997; Debellé and Sharma 1986; Horvath et al. 1986; Zhang et al. 2000). Strong sequence similarity of NodG to 3-oxoacyl-ACP reductases was reported earlier (Rawlings and Cronan 1992; Slabas et al. 1992), and the fact that mutations in the *nodG* of *S. meliloti* do not result in any detectable change in the Nod factor fatty acid structure (Demont et al. 1993) suggests the presence of a chromosomal counterpart that could substitute for NodG. Nevertheless, the study of the symbiotic phenotypes of rhizobia carrying a mutation in *nodG* has shown that the function of NodG, in some instances, cannot be complemented (fully) by the chromosomal counterpart. *S. meliloti nodG* mutants inoculated on alfalfa either do not show any defect in nodulation (Swanson et al. 1987) or cause only a slight delay in nodulation (Horvath et al. 1986). A *nodG* mutant of *Rhizobium* sp. N33 has a slight defect in nodulation and forms a reduced number of nodules in the host *Astragalus cicer*. Moreover, the nodules formed on *Onobrychis viciifolia* are smaller (Cloutier et al. 1997). Recently it was shown that mutants of *S. meliloti* RCR2011 that are deficient in *nodG* or *nodE* formed smaller nodules on *Sesbania macrocarpa* than the *S. meliloti* wild type (Krishnan and Pueppke 1998).

NodG is not the first example of a nodulation protein representing a duplication of a constitutive (housekeeping) enzyme. A well-studied example is NodM, which is a second glutamine synthetase with the same function as the chromosomal GlnS (Baev et al. 1991; Baev et al. 1992; Marie et al. 1992). In contrast to mutations in *S. meliloti nodG* that are reported to have no effect on the structure or amount of Nod factors produced (Demont et al. 1993), *nodM* mutant strains produced up to 20-fold lower amounts of Nod signals in *S. meliloti* (Baev et al. 1992) and threefold lower amounts in *R. leguminosarum* (Spaink et al. 1992). It is therefore thought that NodM provides glucosamine in sufficient amounts for the synthesis of Nod factors (Baev et al. 1991).

S. meliloti also contains multiple copies of the sulfate activating genes *nodP₁nodQ₁*, *nodP₂nodQ₂*, as well as a third locus, *saa*, which is required for the synthesis of sulfur-containing amino acids (Schwedock and Long 1992). A similar redundancy of genes for sulfate activation has been reported for strain *Rhizobium* sp. BR816 (Laeremans et al. 1997). The *saa* locus of *Rhizobium* sp. BR816 can provide activated sulfate for LCO sulfation (Laeremans et al. 1997), whereas the *S. meliloti saa* locus cannot (Roche et al. 1991).

The presence in *R. leguminosarum* bv. *viciae* of a chromosomal ORF homologous to *nodT* may explain the lack of a clear nodulation-deficient phenotype for *nodT* mutants of *R. leguminosarum* bv. *viciae* (Rivilla and Downie 1994). The strain *R. leguminosarum* bv. *trifolii* TA1, which naturally lacks *nodT*, however, is defective in symbiosis with the *Trifolium subterraneum* cv. Woogenellup. The nodulation defect can be complemented with extra copies of *nodT* (Lewis-Henderson and Djordjevic 1991).

It is not clear at this point why in some instances symbiosis-specific copies of housekeeping-gene functions are required. Compartmentalization for the biosynthesis of LCOs has been proposed as one explanation (Laeremans et al. 1997; Schultze et al. 1994), but providing extra enzyme activity for the elimination of biosynthetic "bottlenecks" that normally limit the availability of building blocks for Nod factor synthesis is expected to lead to a much higher synthesis of Nod factors that require those building blocks.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

The bacterial strains and plasmids used in this study are listed in Table 2. *E. coli* DH5 α was used for cloning purposes. *E. coli* BL21(DE3) (Studier et al. 1990), which expresses the T7 polymerase under the control of the *lac* promoter, was used as a host for the overproduction of NodF, FabG, and NodG. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium, except for a large-scale overproduction of NodF grown in M9 minimal medium (Miller 1972). Cultures of *R. leguminosarum* were grown on medium B⁻ (Spaink et al. 1989) at 30°C on a gyratory shaker. For induction, naringenin (1.5 μ M final concentration) was added at a cell density of 5×10^7 per ml. Cells usually were harvested after three generations of further growth. Antibiotics were added when required to the following final concentration per ml: 100 μ g of carbenicillin, 50 μ g of kanamycin, 20 μ g of chloramphenicol for *E. coli* and, for rhizobia, 2 μ g of tetracycline and 500 μ g of streptomycin.

Preparation of affinity columns.

NodF protein was obtained from *E. coli* BL21(DE3) (pMP2301) to be used as a ligand for affinity chromatography (Ritsemá et al. 1994). NodF was purified to homogeneity in two steps, as previously described (Ghose et al. 1996). As a control, a fraction that had been purified in an identical way from a BL21(DE3) strain containing a pET9a vector without the *nodF* gene was used for coupling to control columns. Either 20 mg of purified NodF or an equivalent fraction from the mocked purification was coupled to Affi-Gel 15 (Bio-Rad Laboratories, Hercules, CA, U.S.A), following the manufacturer's instructions, and columns containing 1 ml of gel were prepared. For a large-scale Affi-Gel 15-NodF affinity column, 75 mg of NodF was used for coupling for a 6.5-ml column (1 cm in diameter $\times$ 8.3 cm in height). Prior to chromatography, columns were equilibrated with buffer A (50 mM Tris-HCl, pH 8).

Preparation of extracts for affinity chromatography.

The strains *R. leguminosarum* LPR5045 (for uninduced cultures) and *R. leguminosarum* LPR5045 (pMP280) (pMP1255) (for induced cultures) were grown in medium B⁻ to an optical density (OD) of 620 nm at approximately 0.8. All procedures for extract preparation were performed at 4°C, unless otherwise indicated. The cells were collected by centrifugation at $9,000 \times g$ for 40 min. Cell pellets from each 1-liter culture were washed with 150 ml of 50 mM Tris-HCl, pH 6.8, containing 0.1 M KCl, in order to eliminate most of the exopolysaccharide. The pellets were then resuspended in a volume equal to the wet cell mass of the same buffer and kept frozen. After thawing, the cells were disrupted by three pas-

sages through a French pressure cell at 20,000 lb per in². The lysate was centrifuged at 5,000 × g for 10 min to remove debris and unbroken cells. The 5,000 × g supernatant was ultracentrifuged at 150,000 × g for 90 min. The resulting supernatant carrying the soluble proteins of the extract was either frozen or treated further, as described below.

Proteins of the supernatant from the ultracentrifugation were precipitated by adding ammonium sulfate to 95% saturation. After stirring the suspension gently for 1 h and keeping it for an additional hour at 4°C, the precipitated proteins were sedimented at 10,000 × g for 30 min. The pellet was redissolved in buffer A and dialyzed twice against the starting buffer A for affinity chromatography. Before applying samples to affinity columns, dialysates were centrifuged at 100,000 × g for 1 h.

Chromatography at affinity columns and analysis of eluted fractions.

In each chromatography, 1 ml of a solution containing the soluble proteins from approximately 1 g of wet cells of *R. leguminosarum* was applied to a 1-ml column. After the column was washed with 10 ml of starting buffer A, the column was eluted with 5-ml portions of buffer containing 150 mM, 500 mM, and 2 M NaCl. Finally, the column was eluted with 6 ml of 6 M guanidine-HCl in buffer A. After this step, extensive washing of the column with 80 to 100 ml of buffer A allowed for the refolding of NodF protein and the columns were reused.

Elution was performed at a flow rate of 7 ml per h and 0.5-ml fractions were collected. For analysis of the eluted proteins, 0.25 ml of each fraction were precipitated by adding

trichloroacetic acid (TCA) to a final concentration of 10%. Proteins eluting at 6 M guanidine-HCl were dialyzed before TCA precipitation. The precipitated proteins were resuspended in 7 µl of 2× treatment buffer (0.125 M Tris-HCl, pH 6.8; 4% SDS; 25% glycerol; and 10% β-mercaptoethanol) plus 2 µl of 1 M Tris in order to neutralize the sample. Fractions eluting from a NodF column were compared by SDS-PAGE to those from the control column (Laemmli 1970), followed by silver staining (Nesterenko et al. 1994).

For the 6.5-ml NodF affinity column, the amount of sample was scaled up 6.5 times and the volumes of buffers used for washing and elution were scaled up five times. The fraction size was 2.5 ml, and 0.25 ml of each fraction was used for analysis, as described above.

Electroblotting of proteins and N-terminal sequencing.

For N-terminal sequencing, the fractions containing the protein of interest from one elution over the 6.5-ml Affi-Gel 15 NodF affinity column were pooled, concentrated by TCA precipitation (10% final concentration) and redissolved in a small volume of 2× treatment buffer. A few microliters of 1 M Tris were added to neutralize the sample. The proteins were separated by SDS-PAGE and subsequently transferred to PVDF membranes, according to the method described (Matsudaira 1987). N-terminal sequencing was performed, and amino acids sequences were analyzed with the BLAST-P search program (Altschul et al. 1997).

DNA techniques and DNA sequence analysis.

DNA manipulations were performed by standard procedures (Sambrook et al. 1989). Labeling of DNA probes was per-

Table 2. Bacterial strains and plasmids^a

Strain, plasmid	Relevant characteristics	Source, reference
<i>Escherichia coli</i>		
DH5α	Host for cloning	Bethesda Research Laboratory, Gaithersburg, MD, U.S.A.
BL21(DE3)	Host for expression	Studier et al. 1990
Rhizobia		
<i>Sinorhizobium meliloti</i> 1021	Sm ^R of wild type SU47	Meade et al. 1982
<i>Rhizobium leguminosarum</i> LPR5045	Rif ^R , symbiosis plasmid-cured strain	Hooykaas et al. 1981
<i>Rhizobium</i> sp. N33	Wild type	Prévost et al. 1987
Plasmids		
pUC18, pUC19	Cloning vectors, Cb ^R	Yanisch-Perron et al. 1985
pGMI515	RP4 prime, 26-kb insert from pSymA carrying <i>S. meliloti</i> <i>hsn</i> region, Tc ^R	Faucher et al. 1988; Truchet et al. 1985
pMP280	IncP carrying <i>nodD</i> from <i>R. leguminosarum</i> bv. <i>viciae</i> , Tc ^R	Spaink et al. 1987
pMP1255	IncQ carrying <i>nodFE</i> , Sm ^R	Geiger et al. 1991
pMP2301	overexpression NodF, Kn ^R	Ritsema et al. 1994
pET16b	Vector for histidine (His) ₁₀ -overexpression, Cb ^R	Novagen, Heidelberg, Germany
pLysS	Produces lysozyme, Cm ^R	Studier et al. 1990
pTB5057	109-bp insert in pUC19 coding for N terminus of FabG	This study
pFAB1 and pFAB2	Overlapping cosmids carrying <i>S. meliloti</i> 1021 <i>fab</i> cluster, Tc ^R	This study
pTB5086	6-kb <i>Bam</i> HI fragment carrying <i>S. meliloti</i> 1021 <i>fab</i> cluster in pUC18	This study
pTB5101	5.1 <i>Eco</i> RI fragment carrying <i>R. leguminosarum</i> LPR5045 <i>fabG</i> in pUC18	This study
pTB5111	<i>Pfu</i> -amplified <i>fabG</i> from LPR5045 in pET16b, overproduction His ₁₀ -FabG	This study
pTB5116	<i>Pfu</i> -amplified <i>nodG</i> from N33 in pET16b, overproduction His ₁₀ -NodG	This study

^a Sm^R, streptomycin; Rif^R, rifampicin; Cb^R, carbenicillin; Tc^R, tetracycline; Cm^R, chloramphenicol; and Kn^R, kanamycin resistance.

formed with the DIG-DNA labeling kit (Boehringer Mannheim, Mannheim, Germany). Chemiluminescent detection of the DIG label was performed with CSPD substrate (Boehringer Mannheim). Hybridizations were performed at 62°C. DNA sequencing was carried out following the Sequenase 2.0 protocol with alkaline denaturation for double-stranded DNA sequencing or handled commercially by Meixner AG (Berlin, Germany). Plasmids for DNA sequencing were isolated with a QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany). When required, polymerase chain reaction (PCR) products were purified with the QIAquick PCR purification kit (Qiagen). DNA and derived protein sequences were analyzed with the BLAST search program (Altschul et al. 1997). Alignment of amino acids pairs (or DNA) sequences was performed with the LALIGN, which is part of the IGH Genestream network server (Institut de Génétique Humaine, Montpellier, France), and BLAST-2 (version 2.0) programs.

Isolation of the gene coding for the 29-kDa NodF-binding protein.

Two degenerate primers were created for PCR amplification of a fragment of DNA coding for the N terminus of the 29-kDa NodF binding protein (Fig. 2A). The forward primer oTBC23, based on the amino acid sequence KALVTGA, adds a *Bam*HI site (5'-AAAGGATCC AA(A/G) GCI (C/T)TI ACI GGI G C-3'). The backward primer oTBC24, based on sequences complementary to the coding for the amino acid sequence GTRVEKLE, also adds a *Bam*HI restriction site (5'-AAAGGATCC TC IA(A/G) (T/C)TT (T/C)TC IAC IC(G/T) IGT ICC-3'). With the use of genomic DNA of *R. leguminosarum* LPR5045 as a template, PCR was carried out with degenerate primers (oTBC23 and oTBC24), *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania), and a PCR program designed especially for degenerate oligonucleotides. After a heating step of 95°C for 5 min, the PCR amplification started with three cycles consisting of 45 s at 94°C, 45 s at 42°C, and 3 min at 72°C, followed by 30 cycles with identical parameters except that the annealing temperature was 60 rather than 42°C. The major product was a 125-bp fragment, which was expected, as well as some unspecified bands of 0.5 to 2 kb. The 125-bp fragment was purified over a 2% agarose gel, digested with *Bam*HI, and ligated in pUC19 that had been digested with *Bam*HI. Fragments from three independent clones were sequenced and all showed an identical 67-bp core region (coding for amino acids 15 to 35 of the N-terminal amino acid sequence). The insert in pTB5057 was used as a probe, and the gene for the 29-kDa NodF-binding protein was localized in a 5.1-kb *Eco*RI fragment of *R. leguminosarum* LPR5045.

In order to get the complete sequence of the 29-kDa NodF-binding protein, a size-selected partial library of *Eco*RI fragments of approximately 5 kb was constructed. Clones carrying the gene of interest were selected by colony hybridization with the fragment cloned in pTB5057 as probe. Several positive clones were obtained, and the presence of the gene for the 29-kDa NodF binding protein was confirmed by PCR with the degenerate primers oTBC23 and oTBC24. Plasmid pTB5101 was chosen to sequence the gene.

A *Kpn*I-*Sal*I fragment of 850 bp, obtained by inverse PCR from genomic DNA of *S. meliloti* 1021 corresponding to sequences upstream of *acpP* (López-Lara and Geiger 2000), was

used as a probe against a pLAFR3-based gene bank of *S. meliloti* 1021 (Sohlenkamp et al. 2000). Two cosmids sharing an overlapping region of 11 kb were isolated and named pFAB1 and pFAB2. An internal 6-kb *Bam*HI fragment of pFAB1 carrying the *S. meliloti* 1021 *fab* cluster region was cloned in pUC18, and the resulting plasmid was named pTB5086. The DNA sequence of *S. meliloti fabG* was obtained from this plasmid and its subclones.

Construction of expression vectors.

The genes for *fabG* of *R. leguminosarum* LPR5045 and *nodG* of *Rhizobium* sp. N33 were amplified from the respective genomic DNAs. Primers created novel restriction sites for *Nde*I at the N-terminal methionine and *Bam*HI downstream of the stop codon. PCR was performed with *Pfu* DNA polymerase and, after purification, the fragments were digested with *Nde*I and *Bam*HI and ligated into *Nde*I- and *Bam*HI-digested pET16b. Sequencing analysis confirmed that plasmid pTB5111 carried an insert with an identical DNA sequence to *fabG* of *R. leguminosarum* LPR5045, whereas the insert on pTB5116 matched the sequence of N33 *nodG* (accession no. U53327).

Expression and purification of His₁₀-tagged proteins.

For overproduction of the His₁₀-tagged recombinant proteins, strain BL21(DE3) pLysS was used as a host. Cultures of 30 ml in LB medium containing the required antibiotics were grown at 37°C to OD₆₀₀ at approximately 0.4, IPTG was added to a final concentration of 0.1 mM, and, 3 h later, cells were harvested by centrifugation. Cell pellets were resuspended by adding 1.5 ml of ice-cold 20 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl and 10 mM imidazole and kept frozen. The presence of His-tagged proteins in cell extracts was confirmed by Western blot with an anti-His antibody (Amersham Pharmacia, Uppsala, Sweden), according to the manufacturer's instructions.

For purification of recombinant proteins, 1 ml of frozen cell pellets was thawed and frozen three times to allow the residual lysozyme (from pLysS) to lyse the cells. All of the following steps were carried out at room temperature. Cell extracts were treated for 20 min with DNase I (15 units per ml) and centrifuged for 20 min at 20,000 × *g* (24°C). The 20,000-*g* supernatants were applied to a 1-ml Ni²⁺ agarose column (HiTrap, Pharmacia Biotech, Uppsala, Sweden) and washed with 40 mM imidazole-containing metal chelation affinity chromatography buffer (20 mM sodium phosphate, pH 7.4; and 0.5 M NaCl), followed by washing with 200 mM imidazole in the same buffer. His₁₀-tagged proteins were eluted with 500 mM imidazole in the same buffer and assayed for 3-oxoacyl-ACP reductase activity shortly after elution from the Ni²⁺-column. Each extract was purified on an independent column in order to avoid cross contamination. Proteins were analyzed by SDS-PAGE, followed by Coomassie blue staining.

3-Oxoacyl-ACP reductase assay.

The 3-oxoacyl-ACP reductase activity was determined spectrophotometrically by measuring the decrease of E₃₄₀ that occurred as a result of the consumption of NADPH during the reduction of acetoacetyl-CoA, as described previously (Sheldon et al. 1990). The standard incubation mixture in a

total volume of 200 µl contained 100 mM potassium phosphate (pH 7), 100 µM NADPH or NADH, and 1 to 6 µl of purified protein fraction. The reaction was started by the addition of acetoacetyl-CoA to a final concentration of 2.5 mM.

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