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# Functional *nodFE* Genes Are Present in *Sinorhizobium* sp. Strain MUS10, a Symbiont of the Tropical Legume *Sesbania rostrata*▽

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**We have cloned the *nodFE* operon from *Sinorhizobium* sp. strain MUS10. MUS10 NodF shows sequence homology to acyl carrier protein and enables an *S. meliloti nodF* mutant to effectively nodulate alfalfa. Our results demonstrate the occurrence of *nodFE* in a symbiont that nodulates a legume host not belonging to the galegoid group.**

Nod factors (NFs) are lipochitoologosaccharides consisting of a backbone of four or five glucosamine residues. Most rhizobium species produce NFs that carry a stearic (C18:0), palmitic (C16:0), or vaccenic (C18:1) acid at the nonreducing end. The reducing end is decorated with various substituents such as fucose, arabinose, or sulfate, which are important determinants of the host range (6, 11, 18). For example, it has been shown that arabinosyl substitution at the reducing end of NFs produced by *Azorhizobium caulinodans*, *Sinorhizobium saheli* bv. *sesbaniae*, and *S. terangae* bv. *sesbaniae* strains is required for nodulation of *Sesbania* species (7, 13). The structure of NFs has also been used as a molecular marker to study the phylogentic relationship between rhizobia and legumes (3).

*Sinorhizobium meliloti*, *Rhizobium leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii*, *R. galegae*, *Mesorhizobium huakuii*, and *Mesorhizobium* sp. strain N33 (*Oxytropis arctobia*) produce NFs that contain α,β-unsaturated fatty acids (3). Interestingly, the legume hosts of these diverse rhizobia all belong to the galegoid group, which includes the phylogentically related tribes Trifolieae, Vicieae, and Galegeae. This observation implied that members of galegoid group in the course of evolution of rhizobium-legume symbiosis have developed the unique ability to recognize NFs with α,β-unsaturated fatty acids (3).

*Sinorhizobium* sp. strain MUS10 (hereafter called MUS10) isolated from South India is able to form both stem and root nodules on *S. rostrata* (9). The NF structures of MUS10 have been elucidated (16) and were found to be identical to those produced by the *Azorhizobium caulinodans*, *S. saheli* bv. *sesbaniae*, and *S. terangae* bv. *sesbaniae* strains originating in Africa. However, MUS10 also produced unique NFs that were not reported from the studies of the African strains. MUS10 produced NFs with N-linked fatty acids with a Δ-hydroxy group or with one carbonyl-conjugated double bond (Fig. 1). Nod factors with carbonyl-conjugated double bonds are exclusively found in rhizobia that nodulate legumes belonging to Galegeae tribe (19). However, *Sesbania* does not belong to Galegeae tribe and yet its symbiont MUS10 is able to produce NFs with carbonyl-conjugated double bonds. The biosynthesis of fatty acids carrying *trans* double bonds conjugated to the carbonyl group requires functional *nodFE* genes (4, 17). This observation indicated that *nodFE* genes may also be present in MUS10. To verify this possibility we performed Southern blot analysis (Fig. 2). Genomic DNA isolated from *Azorhizobium caulinodans*, *S. saheli* bv. *sesbaniae*, *S. terangae* bv. *sesbaniae*, and *Sinorhizobium* sp. strain MUS10 was hybridized with $^{32}$P-labeled *nodF* of *R. leguminosarum* bv. *viciae*. The coding region of *nodF* of *R. leguminosarum* bv. *viciae* was isolated from the plasmid pMP2301 (17) by digestion with BamHI and NdeI. Strong hybridization with 10- and 8-kb DNA fragments was observed in the results obtained with *S. saheli* bv. *sesbaniae* and *Sinorhizobium* sp. strain MUS10, respectively. A weak hybridizing signal was also detected with *S. terangae* bv. *sesbaniae* genomic DNA (Fig. 2). However, no hybridization was observed with *A. caulinodans*, indicating the absence of *nodF* homologous sequences in this strain.

To isolate *nodF* homologous sequences we screened a genomic cosmid library of MUS10 utilizing *nodF* of *R. leguminosarum* bv. *viciae* as a hybridization probe. Four positive cosmids were identified by colony hybridization. Subsequently, we were able to locate a *nodF* homologous sequence within a 6-kb BamHI fragment. To define precisely the location of *nodF*, the DNA sequence of a 2.8-kb region was determined. MUS10 NodF showed significant homology to NodF from different rhizobia and to the acyl carrier protein from *Escherichia coli* (Fig. 3).

Previously it was shown that *nodF* mutants of *S. meliloti* exhibited reduced root hair curling and initiated very low numbers of infection threads on their host plants. However, once the infection threads were formed, they grew and ramified in the root cortex and resulted in the formation of nitrogen-fixing nodules (1). Since the *nodF* mutant had reduced capacity to elicit infection threads, they produced lower numbers of nodules than the wild type when examined at 15 days after inoculation (1). To examine whether the *nodF* of MUS10 could complement this defect, we mobilized pMUS1519 (a cosmid clone which carries the *nodFE* genes of MUS10) into GMI5877, a *nodF* mutant of *S. meliloti* (14) and examined the symbiotic phenotype. Nodulation experiments were repeated twice, with 12 plants used for each treatment. Sterile alfalfa seedlings inoculated with wild-type *S. meliloti*, GMI5877, and

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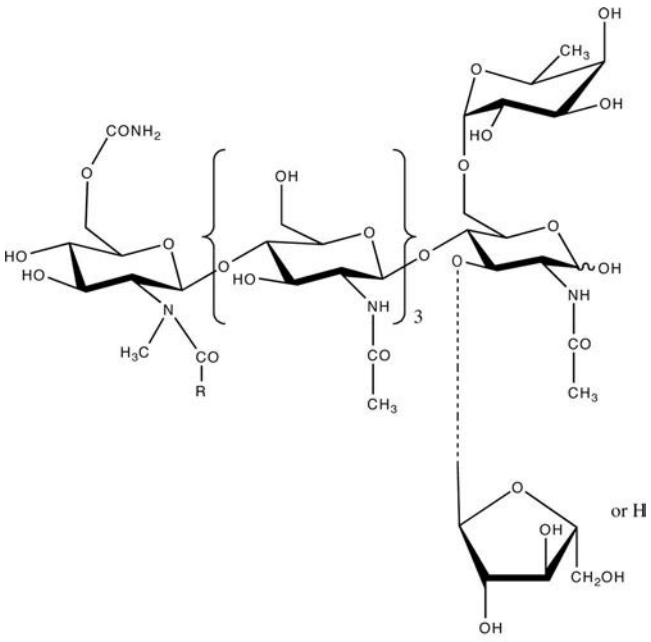


| Rhizobia | Fatty acids |
|---|---|
| *Azorhizobium caulinodans* | C18:0<br>C18:1 Δ11 |
| *Sinorhizobium saheli* | C16:0<br>C18:1 Δ11 |
| *Sinorhizobium teranga* bv *sesbaniae* | C16:0<br>C18:1 Δ11 |
| *Sinorhizobium* sp. strain MUS10 | C16:0<br>C18:1 Δ9<br>C18:1 Δ11<br>C18:1 Δ2<br>C18:0 (Δ-OH) |

FIG. 1. Structure of Nod factors produced by *Sesbania* spp. nodulating rhizobia. Note that MUS10 produces Nod factors with carbonyl-conjugated double bonds, which are exclusively produced by rhizobia that nodulate plants belonging to the Galegeae tribe. The second column of the table describes the nature of the N-linked fatty acids, with the carbon chain length and the number of double bonds indicated. The Nod factor structure was adapted from Promé et al. (16).

GMI5877 (pMUS1519) strains were examined 15 days after inoculation. The *nodF* mutant had a mean of 6 ± 2.0 nodules, while the wild-type and *nodF*-complemented strain had 14 ± 4.8 and 12 ± 3.0 nodules per plant, respectively. This observation suggests that the MUS10 *nodF* can complement the symbiotic defect of *S. meliloti nodF* mutant, presumably by restoring the production Nod factors with unsaturated C16 fatty acids at the terminal nonreducing end. Confirmation of this possibility awaits the elucidation of the structure of Nod factors produced by the complemented strain.

*Azorhizobium caulinodans*, *S. saheli* bv. *sesbaniae*, *S. terangae* bv. *sesbaniae*, and *Sinorhizobium* sp. strain MUS10, rhizobia

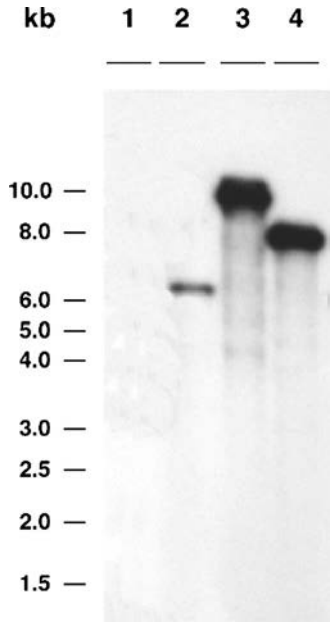


FIG. 2. Southern blot analysis of *nodF* in *Sesbania*-nodulating rhizobia. Genomic DNA from *Azorhizobium caulinodans* (lane 1), *S. terangae* bv. *sesbaniae* (lane 2), *S. saheli* bv. *sesbaniae* (lane 3), and *Sinorhizobium* sp. strain MUS10 (lane 4) was restricted with EcoRI and electrophoresed in 0.8% agarose. The gel was blotted onto nitrocellulose and probed with $^{32}$P-labeled *R. leguminosarum* bv. *viciae nodF* gene. Molecular mass markers in kilobases are shown on the left side of the figure.

belonging to taxonomically different groups, all have the ability to effectively form stem nodules on the tropical legume *Sesbania rostrata*, a legume belonging to Robinieae tribe. All these *Sesbania*-nodulating strains produce Nod factors with a terminal reducing glucosamine bearing arabinosyl and fucosyl substitutions (13, 15, 16). The arabinosyl group is a structural determinant for *Sesbania* nodulation (7). Unlike the African strain, MUS10 elaborates NFs containing α,β-unsaturated acyl substituents (16), which possibly enables this strain to nodulate legumes that are not nodulated by the African strains. Thus, a comparative investigation of the host range of African and Indian *Sesbania*-nodulating strains will shed light on the role of structural variability of NFs in host range extension. It will be interesting to examine whether MUS10 can form nodules on the legume hosts belonging to the Galegeae tribe. *Sesbania*-nodulating MUS10 has a geographically distinct origin from the African strains and presumably evolved under very different environmental conditions. The acquisition of *nodFE* by MUS10 could have resulted by horizontal gene transfer.

At least four acyl carrier proteins (AcpP, NodF, RkpF, and AcpXL) have been identified in rhizobia (12). These proteins are involved in the biosynthesis and transfer of fatty acids. The amino acid homologies among these four proteins are limited, ranging from 26 to 32% (2). The results of Southern blot analysis under stringent hybridization conditions indicate the presence of *nodF* homologous sequences in *S. saheli* bv. *sesbaniae* and *S. terangae* bv. *sesbaniae*. Yet these *Sesbania*-nodulating strains do not produce NFs with carbonyl-conjugated double bonds. Previous studies have shown that *nodFE* genes are sufficient for the synthesis of unsaturated fatty acids (5, 8).


FIG. 3. Multiple sequence alignment of NodF from different rhizobia. The sequences from *E. coli* (Swiss-Prot accession no. P0A6A8), *Sinorhizobium meliloti* (UniProt-EMBL accession no. Y00604), *Rhizobium leguminosarum* bv. *viciae* (UniProt-EMBL accession no. AM236084), *Rhizobium leguminosarum* bv. *trifolii* (Swiss-Prot accession no. P04686), *Mesorhizobium* sp. strain N33 (Swiss-Prot accession no. P72330), *Sinorhizobium* sp. strain BR816 (UniProt-EMBL accession no. AJ518946), *Sinorhizobium medicae* (RefSeq accession no. NZ_AATG01000001), and *Mesorhizobium loti* (RefSeq accession no. NC_002678) are shown aligned with MUS10 NodF sequences. Positions of amino acid identity are shown with white characters of a black background, and residues exhibiting similarity are shown with black characters on a gray background.

One possible explanation for the apparent absence of NFs with α,β-unsaturated fatty acids is that *nodFE* genes in these strains are defective. Since rhizobia undergo frequent genetic rearrangements, including deletions, mutations, and duplications, the possibility of acquiring defective *nod* genes cannot be ignored. Such an instance has been reported from a study of *S. fredii* USDA257, where *nodSU* was shown to be defective due to a deletion in the promoter sequences (10). The other possibility is that both *S. saheli* bv. *sesbaniae* and *S. terangae* bv. *sesbaniae* may indeed produce NFs with α,β-unsaturated fatty acids but in such minute amounts as to have precluded their identification. A reexamination of the NF structures of these strains utilizing highly sensitive analytical techniques may verify this possibility.

**Nucleotide sequence accession number.** The DNA sequence of the 2.8-kb region determined in this work was submitted to the GenBank database (accession number EF621916).

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The mention of product names is necessary to report factually on available data; however, the University of Missouri and the USDA do not guarantee or warrant the standard of the products mentioned herein, and the use of product names implies no approval by the University of Missouri or the USDA of the product to the exclusion of others that may be suitable.

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