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# *Rhizobium nodM* and *nodN* Genes Are Common *nod* Genes: *nodM* Encodes Functions for Efficiency of Nod Signal Production and Bacteroid Maturation

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**Earlier, we showed that *Rhizobium meliloti nodM* codes for glucosamine synthase and that *nodM* and *nodN* mutants produce strongly reduced root hair deformation activity and display delayed nodulation of *Medicago sativa* (Baev et al., Mol. Gen. Genet. 228:113–124, 1991). Here, we demonstrate that *nodM* and *nodN* genes from *Rhizobium leguminosarum* biovar viciae restore the root hair deformation activity of exudates of the corresponding *R. meliloti* mutant strains. Partial restoration of the nodulation phenotypes of these two strains was also observed. In nodulation assays, galactosamine and *N*-acetylglucosamine could substitute for glucosamine in the suppression of the *R. meliloti nodM* mutation, although *N*-acetylglucosamine was less efficient. We observed that in nodules induced by *nodM* mutants, the bacteroids did not show complete development or were deteriorated, resulting in decreased nitrogen fixation and, consequently, lower dry weights of the plants. This mutant phenotype could also be suppressed by exogenously supplied glucosamine, *N*-acetylglucosamine, and galactosamine and to a lesser extent by glucosamine-6-phosphate, indicating that the *nodM* mutant bacteroids are limited for glucosamine. In addition, by using derivatives of the wild type and a *nodM* mutant in which the *nod* genes are expressed at a high constitutive level, it was shown that the *nodM* mutant produces significantly fewer Nod factors than the wild-type strain but that their chemical structures are unchanged. However, the relative amounts of analogs of the cognate Nod signals were elevated, and this may explain the observed host range effects of the *nodM* mutation. Our data indicate that both the *nodM* and *nodN* genes of the two species have common functions and confirm that NodM is a glucosamine synthase with the biochemical role of providing sufficient amounts of the sugar moiety for the synthesis of the glucosamine oligosaccharide signal molecules.**

Rhizobia are capable of establishing symbiotic associations with their legume hosts. During this process, the bacteria undergo rapid developmental changes in order to make the transition from a soil environment through the root surface environment to a new ecological niche inside the plant host cells. The partnership between a given plant host and its microsymbiont is highly specific; most leguminous plants can be nodulated by a more or less limited number of *Rhizobium* species. For example, *Rhizobium meliloti* nodulates species of the genera *Medicago*, *Melilotus*, and *Trigonella* (11).

Numerous bacterial and plant genes are expressed specifically during nodule development (19). The two partners interact by one partner transmitting signal molecules to activate sets of symbiotic genes in the other partner. The first signals are specific flavonoid compounds exuded by the plant roots which can activate expression of the nodulation (*nod* and *nol*) genes. The nodulation gene products are involved in producing signal molecules, the Nod factors, which elicit curling and deformation of the root hairs and meristematic cell division, leading to nodule formation (12). Using *R. meliloti* strains overproducing the Nod factors, Lerouge and coworkers (17) identified a major alfalfa-specific signal molecule as an acylated and sulfated glucosamine tetrasaccharide. Using a similar approach, we showed that *R. meliloti* produces a family of biologically active Nod signal molecules (26). Host-specific signal molecules secreted by *Rhizobium leguminosarum* have also been identified (30).

In different rhizobia, common and host-specific *nod* genes have been identified (12). The biochemical pathway involved in generating the Nod factors is still far from being understood, although the common *nodABC* as well as the host-specific *nodE*, *nodF*, *nodH*, *nodP*, *nodQ*, and *nodL* genes were shown to be involved in production of the Nod factors (2, 7, 17, 23, 27, 29, 30).

Recently, we described a new *nod* gene cluster (*nodM*, *nolFGHI*, and *nodN*) in *R. meliloti* which is required for optimal nodulation of alfalfa (1). The first gene of this operon, *nodM*, was shown on the basis of the following findings to code for glucosamine synthase: (i) its strong homology with the *Escherichia coli* glucosamine synthase gene (*glmS*), (ii) the complementation of an *E. coli glmS* mutant by *nodM*, and (iii) suppression by exogenously supplied glucosamine of delayed nodulation of alfalfa by the *nodM* mutant.

Mutations in *nodM* or *nodN* of *R. meliloti* or *R. leguminosarum* bv. viciae had no significant effect on nodulation of

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certain plant hosts (1, 32), while in other hosts, *nodM* was required for efficient nodulation (1). Moreover, in *R. leguminosarum* bv. viciae, *nodM* appeared to reduce nodulation when the gene was present on a multicopy vector (4). In *R. leguminosarum* bv. trifolii, the *nodM* gene was also reported to act negatively on certain cultivars of *Trifolium subterraneum* (18). It was proposed that *nodM* controls cultivar specificity, possibly by modifying the host specificity of the signal molecules. This suggestion seemed to contradict reports that *nodM* is a glucosamine synthase gene under plant signal control (1, 21). Therefore, we set out to identify various possible functions of *nodM* ex planta or in planta and to investigate whether these functions are involved in determining host specificity.

Here, we report that in *nodM* mutants of *R. meliloti*, production of the members of the Nod factor family is severely reduced, but no difference in the chemical structures of factors produced by wild-type or *nodM* mutant bacteria could be detected. We found that in contrast to *E. coli glmS*, for which the requirement for glucosamine was reported to be satisfied also by *N*-acetylglucosamine but much less efficiently by galactosamine (34), not only *N*-acetylglucosamine but also galactosamine could complement *nodM*-deficient *R. meliloti* strains. We observed that the *nodM* mutants induced nodules which were not only delayed but were also less efficient in symbiotic nitrogen fixation (1). In the present study, the ultrastructures of host cells induced by *nodM* mutants of *R. meliloti* indicated that the development of bacteroids was impaired. Phenotypic restoration of nodulation and bacteroid development by glucosamine and two related amino sugars was observed. In addition, we showed that the *nodM* and *nodN* genes of *R. leguminosarum* bv. viciae complemented the *R. meliloti nodM* and *nodN* mutations, respectively, indicating that the *nodM* and *nodN* genes of the two species have essentially the same functions, respectively, in the two species.

## MATERIALS AND METHODS

**Bacterial strains, bacteriophages, and plasmids.** AK631, a compact colony morphology variant of *R. meliloti* 41, and *R. meliloti* 1021 were used as wild-type strains (15). *R. meliloti* DN4-15 and DN4-18 strains are AK631 derivatives mutated in the *nodM* and *nodN* genes, respectively (1). For analyzing the effect of *nodM* on Nod factor production, a *nodM* mutation was generated in *R. meliloti* 1021 lacking the repressor NolR (15, 16) by introduction and homogenotization of pDN4-3 carrying a Tn*5* insertion in the *nodM* gene on the cosmid clone pPP346 (1). The *nodC* and *nodH* mutations in strain 1021 were generated in the same way but using pAK1644 for the *nodC* mutation (14) and pAK1693 for the *nodH* mutation (10). Overexpression of the *nod* genes in wild-type strain 1021 and in the *nod* mutant derivatives was achieved by introduction of pNID6 carrying the positive regulatory genes *nodD3* and *syrM* (15). The *glmS* mutant *E. coli* strain was E110 (34).

For complementation of *R. meliloti nodM* and *nodN* mutant strains, the *nodMN* operon from *R. leguminosarum* bv. viciae was recloned from pIM169 (kindly provided by A. Downie [32]) in two steps. First, the 2.5-kb *Sal*I fragment was cloned in pACYC184 (5), which was then linearized with *Bam*HI and cloned into the *Bgl*II site of pRK290 (6). Then, the resulting plasmid, pDX1, was mobilized into *R. meliloti nodM* and *nodN* mutant strains DN4-15 and DN4-18, respectively. DNA manipulations were performed by standard procedures (20).

**Microbiological techniques.** Media and growth conditions for *R. meliloti* and *E. coli* were described earlier (3). Bacterial conjugation was carried out as described previously (1). Antibiotic concentrations for *E. coli* and *R. meliloti* were (in micrograms per milliliter) 10 and 10 for tetracycline, 30 and 30 for chloramphenicol, 20 and 200 for kanamycin, 20 and 200 for gentamicin, and 100 for ampicillin for *E. coli*. *E. coli* E110 was propagated in Luria-Bertani medium supplemented with 0.2 mg of D-glucosamine (GlcN) per ml. The effect of replacing GlcN with other amino sugars (D-glucosamine-6-phosphate [GlcN-6-P], *N*-acetyl-D-glucosamine [GlcNAc], and D-galactosamine [GalN], all purchased from Sigma) on the growth of *E. coli* E110 was tested as follows: overnight cultures of *E. coli* E110 were diluted to an optical density at 600 nm ($OD_{600}$) of 0.05 in Luria-Bertani medium without supplements and grown for 2.5 h. The bacteria were washed once and resuspended in Luria-Bertani medium containing 0.2 mg of amino sugar per ml. The $OD_{600}$ was measured during a growth period of 7 hours.

**Nod metabolite purification.** For the purification of Nod metabolites, *R. meliloti* 1021 *nodM*::Tn*5*(pNID6) was grown in 10 liters of Vincent minimal medium (26) at 30°C to and $OD_{600}$ of 1.1. The bacteria were pelleted by centrifugation for 15 min at 18,000 × *g*. The supernatant was extracted with 3 liters of 1-butanol by vigorous shaking for 1 h. The organic phase was cleared from residual aqueous phase by centrifugation and evaporated under reduced pressure at 50°C. The residue was suspended in 10 ml of 50% acetonitrile–$H_2O$, centrifuged, and filtered through a Gelman Acro LC25 filter (0.45-μm pore size). Aliquots (1 ml each) were fractionated on a C18 reverse-phase high-pressure liquid chromatography (HPLC) column (ODS Ultrasphere, 5 μm; 10 by 250 mm; Beckman). Elution was at 5 ml/min with 30% acetonitrile–$H_2O$–0.1 M ammonium acetate isocratic for 10 min followed by a 30-min gradient to 60% acetonitrile–$H_2O$–0.1 M ammonium acetate. Fractions were collected around the time of elution of the major peaks absorbing at 220 nm. After evaporation of the solvent, the different fractions were repurified on the same column by elution with 20% acetonitrile–$H_2O$ isocratic for 5 min followed by a 20-min gradient to 60% acetonitrile–$H_2O$ without the addition of ammonium acetate. Fractions absorbing at 205 nm were lyophilized, taken up in 60% methanol–$H_2O$, and analyzed by fast atom bombardment mass spectrometry (FAB-MS) as described elsewhere (26).

For analytical HPLC, aliquots of a butanol extract from 200-ml cultures of *Rhizobium* spp. were analyzed on an analytical reverse-phase HPLC column (3.9 by 150 mm) (Waters $C_{18}$ Novapak). Elution was with 20% acetonitrile–0.2 M ammonium acetate (pH 6.8) isocratic for 5 min followed by a gradient to 70% acetonitrile–0.2 M ammonium acetate within 60 min. Chromatograms were recorded at multiple wavelengths with a Waters 990 system connected to a photodiode array detector.

**TLC analysis.** In vivo labeling was carried out as described elsewhere (29) by using 1-ml cultures in minimal medium (26) containing 25 μCi of [1-$^{14}$C]acetate (Amersham; specific activity, 55 mCi/mmol). The supernatants of cultures with $OD_{600}$ of 0.2 were extracted with 0.5 ml of 1-butanol. The residue after lyophilization was taken up in 20 μl of butanol, and 2 μl was applied to a thin-layer chromatography (TLC) plate (5 by 10 cm) (HPTLC, RP-18; Merck). The plate was developed with 45% acetonitrile–$H_2O$ and exposed for 2 days to X-Omat AR film (Kodak).

To visualize purified Nod factors and nonradioactive butanol extracts on TLC plates, the plates were soaked

briefly in a solution of 2% (wt/vol) diphenylamine and 2% (vol/vol) aniline in 5 volumes of acetone and 1 volume of *ortho*-phosphoric acid and then heated at 250°C. Purified standards for the TLC analysis comprised NodRm-IV($C_{16:2}$,S), NodRm-IV($C_{16:0}$,S) (26), and desulfated NodRm-IV($C_{16:2}$), which was obtained by mild acid hydrolysis of NodRm-IV($C_{16:2}$,S) in methanol-HCl as described previously (33) followed by purification on a reverse-phase HPLC column as detailed above.

**Plant tests.** Plant cultivation and nodulation assays were carried out with alfalfa (*Medicago sativa* L. Nagyszenasi) seedlings according to the methods described in reference 13. Each assay was repeated three or four times, and in each assay, 20 or 25 seedlings were inoculated with the different *R. meliloti* strains.

The amino sugars were mixed into the media at 25-μg/ml final concentrations before seedlings were placed into the test tubes. The number of nodulated plants was recorded every day for 3 weeks. Then, nodules were collected for microscopic studies.

**Root hair deformation (Had) assays.** Preparation of bacterial exudates and Had assays were done by the methods of Banfalvi and Kondorosi (2). Bacteria in logarithmic phase ($3 \times 10^8$ bacteria per ml) were harvested by centrifugation, and their filter-sterilized exudates were mixed into the plant medium at concentrations of 25, 10, 5, and 1% (vol/vol). Assays were repeated at least three times.

**Microscopic studies.** For each strain and condition, at least 30 nodules from 20 different plants were collected 3 weeks after inoculation. Five to ten nodules were analyzed by light microscopy and electron microscopy. Nodule samples were fixed overnight at 0°C in 4% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 6.8. After two rinses with the same buffer, the samples were postfixed in 2% (wt/vol) aqueous $OsO_4$ at 0°C for 6 h (9). Then, the samples were washed three times in cold distilled water and dehydrated in a graded acetone series at 0°C. Finally, they were embedded in Spurr standard low-viscosity medium (31). For light microscopy, thick sections (0.5 μm) were cut and stained with 0.1% (wt/vol) methylene blue. For electron microscopy, ultrathin sections were collected on 300-mesh copper grids and stained with uranyl acetate-lead citrate (22). Grids were examined in a JEM 100 B electron microscope operated at 80 kV.

## RESULTS

**Production of the Nod factor family is severely reduced in *nodM* mutant derivatives of *R. meliloti* without observable changes in chemical structures.** Previously, we demonstrated that culture exudates of *nodM* and *nodN* mutant derivatives of *R. meliloti* AK631 did not elicit root hair deformation on *M. sativa* (1). Despite this, the mutant bacteria were able to induce root hair curling and nodulation, although the latter was delayed and less efficient. We wanted to test whether *nodM* mutants produced modified Nod metabolites which were no longer active in root hair deformation or whether the amount of unmodified Nod factors was reduced to levels too low to be detected by the Had assay.

In order to obtain sufficient amounts of Nod factors for analysis, it was necessary to construct mutant derivatives of *R. meliloti* in which the expression of the *nod* genes was upregulated. This was achieved by transferring the *nodM*::Tn*5* allele from the AK631 background into strain 1021 lacking the *nod* gene repressor NolR (15, 16) and replacing the wild-type gene with the mutated one by homogenotization (see Materials and Methods). In addition, extra copies of the positive regulatory genes *syrM* and *nodD3* were introduced on plasmid pNID6 (15). The Nod metabolites excreted by this overproducing *nodM* mutant strain were compared with those excreted by strain Rm1021 carrying pNID6 and by its *nodC* and *nodH* mutant derivatives.

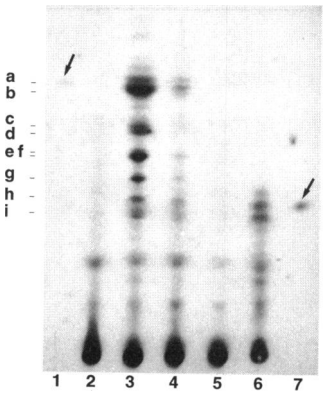


FIG. 1. TLC (RP-18) of in vivo-labeled Nod metabolites with 45% acetonitrile–$H_2O$ as mobile phase. Lanes 1 and 7, purified Nod factors stained with diphenylamine-aniline and indicated by arrows; lane 1, 50 ng of NodRm-IV($C_{16:2}$,S); lane 7, 100 ng of NodRm-IV($C_{16:2}$); lanes 2 through 6, butanol extracts of $^{14}C$-labeled Nod metabolites. The amount of material loaded in each lane was equivalent to 0.1 ml of culture supernatant of the following strains: lane 2, Rm1021; lane 3, Rm1021(pNID6); lane 4, Rm1021 *nodM*::Tn*5*(pNID6); lane 5, Rm1021 *nodC*::Tn*5*(pNID6); lane 6, Rm1021 *nodH*::Tn*5*(pNID6). Major Nod metabolites for strain Rm1021 (pNID6) and its *nodM* mutant derivative are indicated (a through i).

Nod factors labeled in vivo by [1-$^{14}C$]acetate were extracted from culture supernatants by 1-butanol, and aliquots were fractionated by reverse-phase TLC (Fig. 1). As reported previously for a similar overproducing derivative of Rm1021 containing in addition to *syrM* and *nodD3* extra copies of the *nodE*, *nodF*, *nodG*, *nodH*, *nodP*, and *nodQ* genes (26), a family of Nod factors was produced in high amounts by Rm1021(pNID6), whereas the *nodC* mutant was unable to produce butanol-extractable Nod metabolites (17) (Fig. 1, lanes 3 and 5). Extracts from Rm1021 lacking plasmid pNID6 did not show any spot in addition to the background visible with the *nodC* mutant extract (lanes 2 and 5). Purified nonlabeled Nod factors were separated on the same plate as standards. NodRm-IV($C_{16:2}$,S) cofractionated with the major labeled Nod metabolites produced by Rm1021(pNID6) (lanes 1 and 3). The nonsulfated NodRm-IV($C_{16:2}$) showed a retention similar to that of one of the major labeled compounds excreted by the *nodH* mutant which is unable to produce sulfated Nod factors (23) (lanes 6 and 7).

For the *nodM* mutant, we could not observe a difference in the numbers and mobilities of labeled Nod metabolites in comparison with those of Rm1021(pNID6) (Fig. 1, lanes 3 and 4, spots a through i). However, the intensities of all spots were significantly reduced, suggesting that *nodM* affects the efficiency of Nod factor production but not the chemical structures of the factors.

Interestingly, the Nod factor-overproducing strain Rm1021(pNID6) excretes not only sulfated compounds but also apparently significant quantities (about 10%) of nonsulfated compounds. These molecules were not detected in

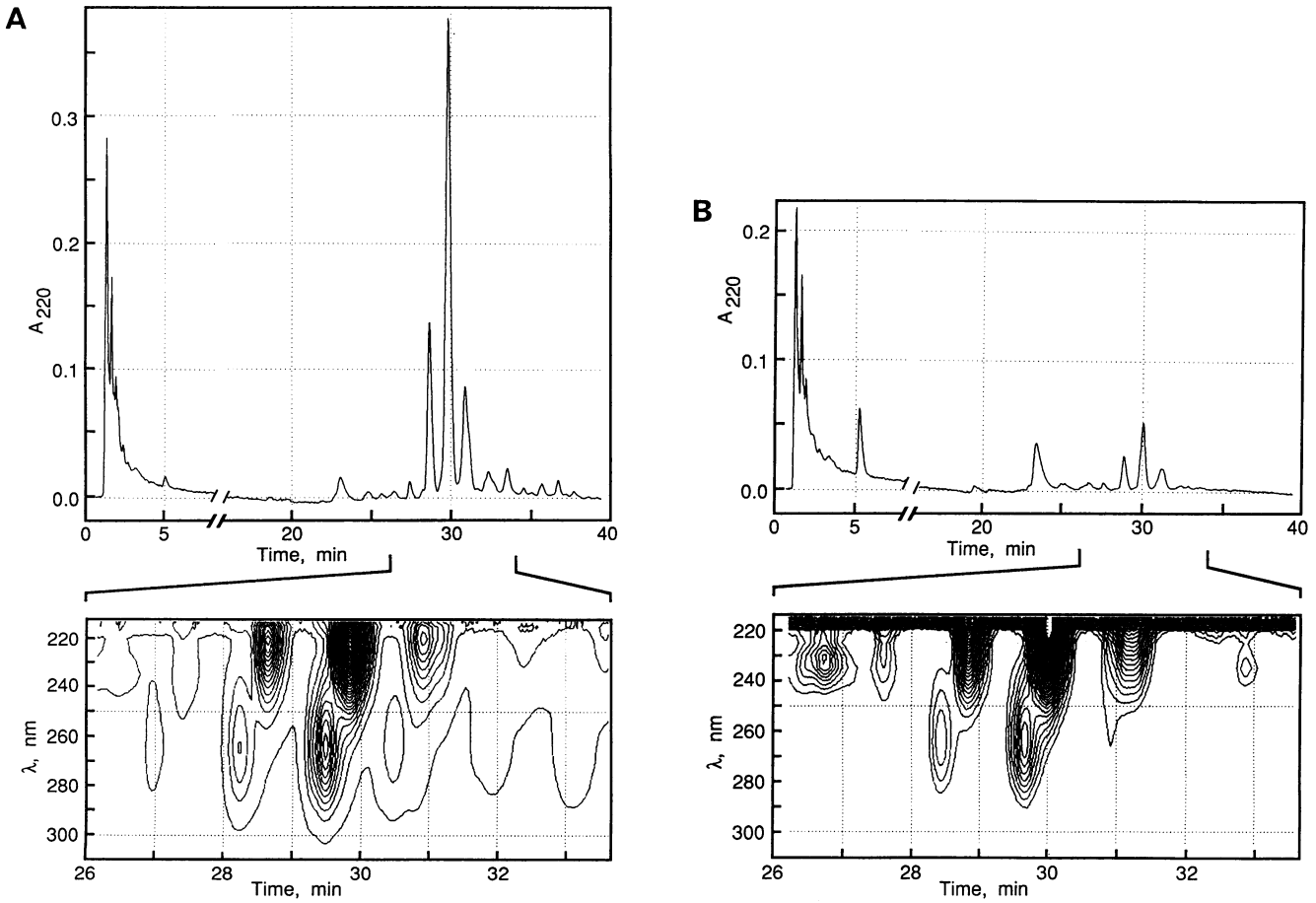


FIG. 2. Analytical reverse-phase HPLC of butanol extracts from culture supernatants of Rm1021(pNID6) (A) and Rm1021 *nodM*::Tn*5*(pNID6) (B). Upper panels, chromatograms recorded at 220 nm; lower panels, photodiode array detections, demonstrating the presence of additional peaks with an absorption maximum at 265 nm. The chromatograms at 220 nm are presented on the same scale, whereas the scales for the diode array detections are different in order to demonstrate the qualitative similarity.

strain Rm1021(pEK327) containing in addition to *syrM* and *nodD3* the host-specific *nodG*, *nodE*, *nodF*, *nodH*, *nodP*, and *nodQ* genes on a plasmid (26) (data not shown), a fact probably reflecting the higher dosage of genes (*nodHPQ*) involved in Nod factor sulfation (23). The reduction in Nod metabolite production by the *nodM* mutant in comparison with that by Rm1021(pNID6) was less for the nonsulfated compounds (Fig. 1, lanes 3 and 4, spots h and i). The *nodM* mutant therefore showed an increased ratio of nonsulfated to sulfated Nod factors.

Butanol extracts of culture supernatants were analyzed more precisely on reverse-phase HPLC connected to a photodiode array detector. Extracts from Rm1021(pNID6) and the *nodM* mutant showed similar patterns of molecules displaying absorption maxima at 220 and 265 nm with similar retention times (Fig. 2). Quantitation of the Nod metabolites revealed a reduction of the sulfated forms in the *nodM* mutant to 5 to 15% of the levels in Rm1021(pNID6) (Fig. 1 and 2), varying somewhat in different experiments. In agreement with these data, root hair deformation assays with *M. sativa* showed a difference of 1 order of magnitude between supernatants of Rm1021(pNID6) and the *nodM* mutant (data not shown).

Slight modifications in the structures of the Nod metabolites of the *nodM* mutant in comparison with those of Rm1021(pNID6) could not be excluded by TLC and HPLC analysis. In order to test whether the *nodM* mutant was still able to produce the cognate NodRm-IV($C_{16:2}$,S) and its related sulfated and nonsulfated derivatives, a number of Nod metabolites were purified from the supernatant of a large-scale culture of Rm1021 *nodM*::Tn*5*(pNID6). The butanol extract was first fractionated on reverse-phase HPLC by an acetonitrile-water gradient in the presence of ammonium acetate. Sulfated and nonsulfated metabolites which coeluted under these conditions were separated in a second HPLC fractionation in the absence of ammonium acetate. They were subsequently analyzed by FAB-MS.

Table 1 shows the molecular weights of the major Nod metabolites purified from the *nodM* mutant and of some of the minor compounds, not all of which were analyzed. The molecular weights are compatible with the indicated types of Nod factors. The purified molecules were fractionated together with a butanol extract of Rm1021(pNID6) on RP-18 TLC (Fig. 3). Staining with diphenylamine-aniline revealed a similar pattern of spots (a through i) for the butanol extract, as did [$^{14}$C]acetate labeling (compare Fig. 1 and 3). Thus, the major spots of the *nodM* mutant (a, b, h, and i) can be assigned to sulfated penta- and tetrasaccharides and nonsulfated tetra- and trisaccharides containing unsaturated acyl chains. Spot c comigrates with purified NodRm-IV($C_{16:0}$,S)

TABLE 1. Nod metabolites purified from Rm1021 *nodM*::Tn*5*(pNID6)

| Compound group | Mol wt[a] | Nod metabolite[b] |
|---|---|---|
| Major | 1,305 | NodRm-V($C_{16:2}$,S) |
| | 1,307 | NodRm-V($C_{16:1}$,S) |
| | 1,102 | NodRm-IV($C_{16:2}$,S) |
| | 1,104 | NodRm-IV($C_{16:1}$,S) |
| | 1,064 | NodRm-IV(Ac,$C_{16:2}$) |
| | 861 | NodRm-III(Ac,$C_{16:2}$) |
| Minor | 1,106 | NodRm-IV($C_{16:0}$,S) |
| | 1,100 | NodRm-IV($C_{16:3}$,S) |
| | 660 | NodRm-II(Ac,$C_{16:1}$) |

[a] Predicted nominal molecular weight as determined by negative FAB-MS.
[b] Nomenclature is according to references 23 and 26.

obtained by catalytic hydrogenation (26). Spots d, e, f, and g could not be recovered during the purification scheme.

**Complementation of *R. meliloti nodM* and *E. coli glmS* mutants with different amino sugars.** In our previous work, we showed that exogenously supplied GlcN suppressed delayed nodulation by *nodM* mutants of *R. meliloti* on *M. sativa* (1). An *E. coli glmS* mutant could be complemented not only by GlcN but also by GlcNAc and, less efficiently, GalN (34). Therefore, we tested whether these last two amino sugars also suppress the delayed-nodulation phenotype of a *R. meliloti nodM* mutant. In nodulation assays on *M. sativa* with the *R. meliloti nodM* mutant strain DN4-15, GlcNAc, GalN, and, as a further sugar, GlcN-6-P were supplied exogenously. As shown in Fig. 4, GalN was almost as effective as GlcN (1-day delay compared with GlcN). Complementation was observed also with GlcNAc, although in the first 2 weeks, not all the test plants were nodulated. When GlcN-6-P was used, nodulation remained delayed, which could be explained by a lack of efficient uptake of this compound. In control experiments, the nodulation delay caused by *nodN* or *nolG* mutations was not suppressed by any of the four amino sugars added exogenously (data not shown). The nodulation kinetics of the wild-type strain supplemented with either of these amino sugars did not differ significantly from that of the same strain without added amino sugars.

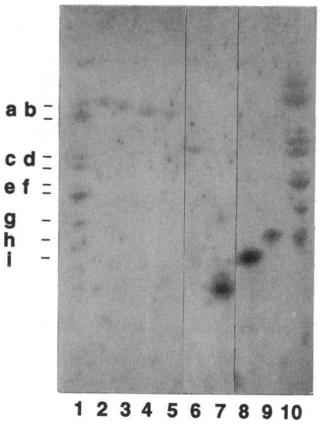


FIG. 3. TLC of Nod factors purified from Rm1021 *nodM*::Tn*5*(pNID6). The metabolites were stained with diphenylamine-aniline. Lanes 1 and 10, 1 and 2 μg of butanol extract obtained from supernatant of strain Rm1021(pNID6); major Nod metabolites are indicated (a through i); lane 6, 20 ng of purified NodRm-IV($C_{16:0}$,S); lanes 2 through 5 and 7 through 9, aliquots of purified factors having molecular weights as follows: lane 2, 1,305; lane 3, 1,307; lane 4, 1,102; lane 5, 1,104; lane 7, 660; lane 8, 861; lane 9, 1,064.

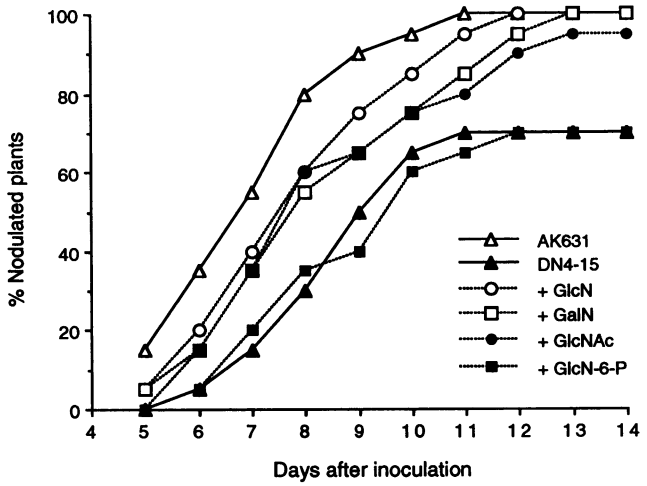


FIG. 4. Nodulation kinetics of *R. meliloti nodM* mutant (DN4-15) complemented with GlcN, GalN, GlcNAc, and GlcN-6-P. AK631, wild-type bacterium. Maximum standard deviation ($n$ = 4) was ±6.6%.

We tested *E. coli glmS* mutant strain E110 for growth in the presence of GlcN, GlcN-6-P, GlcNAc, or GalN. As shown in Fig. 5, the first three amino sugars satisfied the growth requirement with about the same efficiency, whereas GalN did not complement the mutant under our experimental conditions.

**Microscopic analysis of *M. sativa* nodule cells invaded by *R. meliloti nodM* mutant strain without and with exogenously**

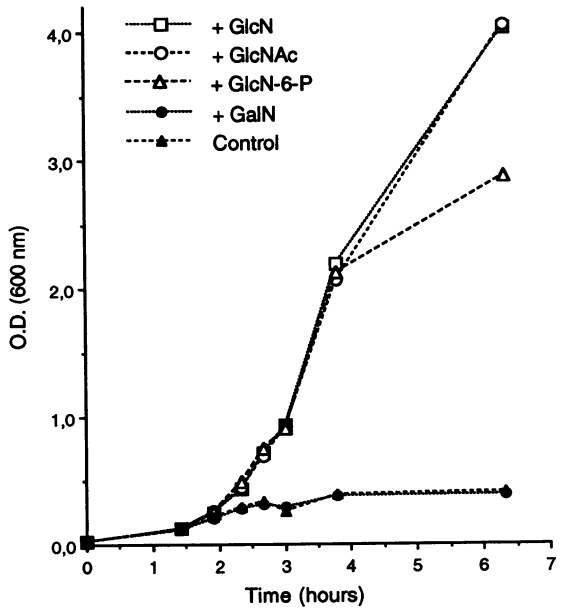


FIG. 5. Growth of *glmS* mutant *E. coli* E110 on Luria-Bertani medium supplemented with different amino sugars, each at a concentration of 200 μg/ml. Maximum standard error of the mean ($n$ = 3) was ±11% for each time point.

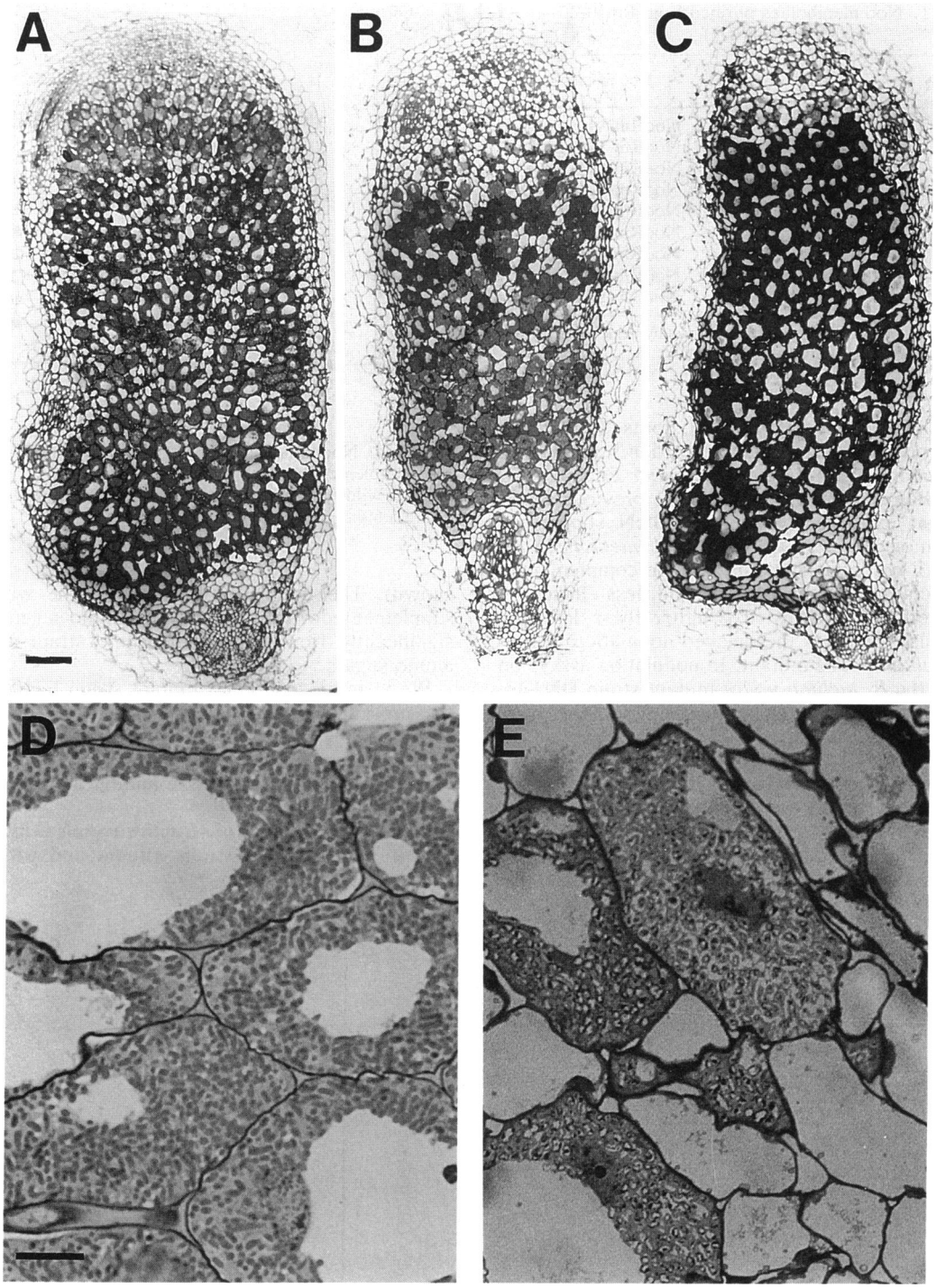


FIG. 6. *M. sativa* nodules induced by wild-type and *nodM* mutant *R. meliloti* strains. (A through C) Longitudinal sections of nodules induced by wild type (A), *nodM* (B), and *nodM* (C) in the presence of GlcN. Bar = 100 μm. (D and E) Infected cells and bacteroids in nodules induced by wild-type (D) and *nodM* (E) strains. Bar = 10 μm.

**supplied amino sugars.** We observed that the dry weights of plants inoculated with *nodM* mutants remained below those of plants infected with the wild-type strain, suggesting that the mutations might affect not only nodule initiation but also the development of nitrogen-fixing symbiosis (1). To elucidate the basis of this effect, the structures of the *M. sativa* nodules induced by the wild type or the *nodM* mutants were compared by microscopy. It was observed by light microscopy (Fig. 6) that invasion of plant cells by the bacteria was less pronounced in nodules induced by the *nodM* mutant strain DN4-15 (Fig. 6B) than in nodules induced by wild-type bacteria (Fig. 6A). In addition, the morphologies of the wild

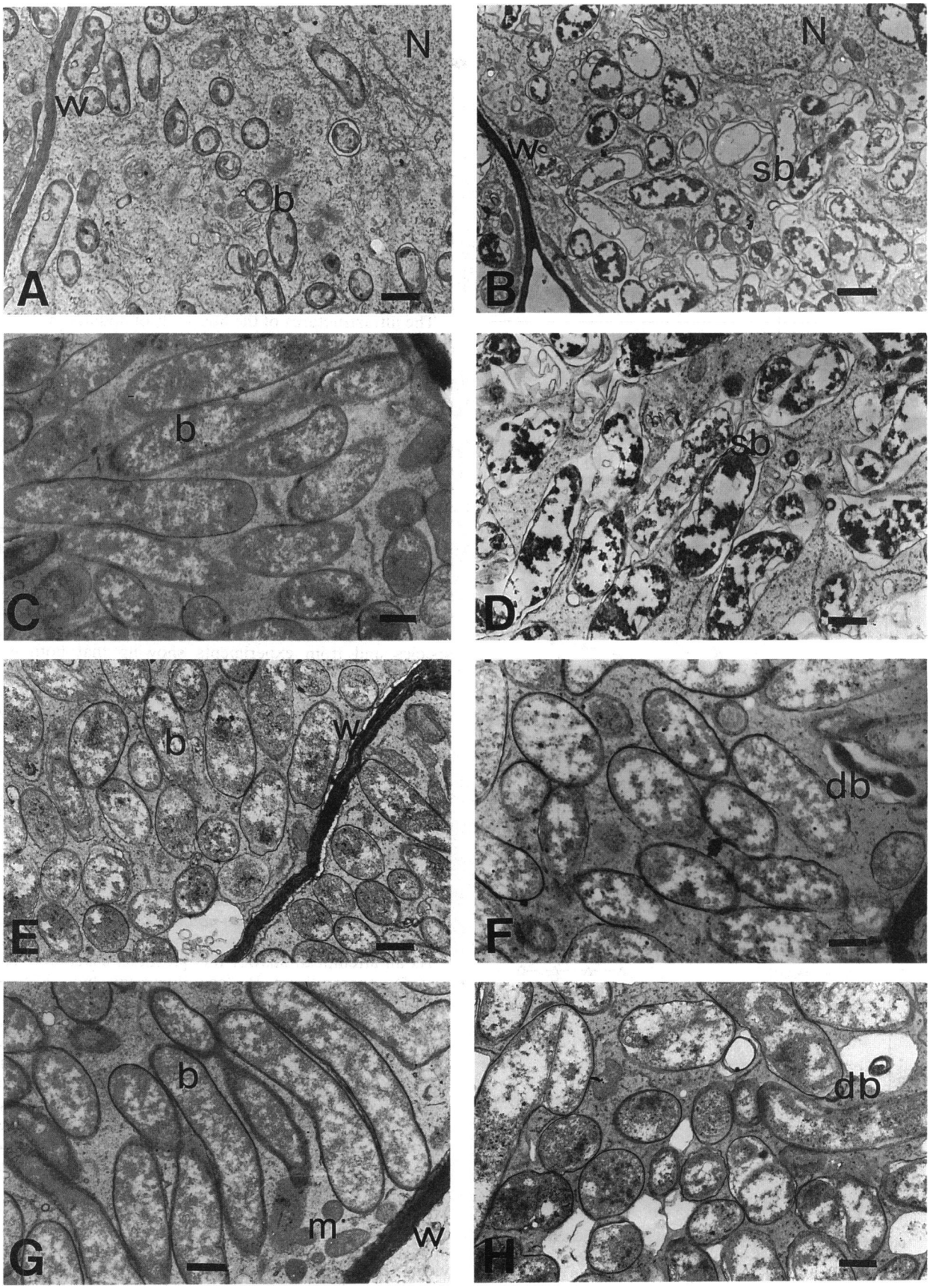


FIG. 7. Ultrastructures of nodule cells induced by wild type and *nodM* mutant of *R. meliloti* in the absence and presence of exogenously added amino sugars. (A and C) Bacteroids of wild-type *R. meliloti* AK631; (B and D) mutant strain DN4-15; (E, F, G, and H) DN4-15 complemented with GlcN, GlcNAc, GalN, and GlcN-6-P, respectively. Panels A and B show host cells of 3-week-old nodules in the early symbiotic zone. All other panels represent host cells of 3-week-old nodules in the late symbiotic zone. Abbreviations: b, bacteroid; db, deteriorated bacteroid; m, mitochondrion; N, nucleus; sb, senescent bacteroid; w, cell wall. Bars = 1 μm.

TABLE 2. Restoration of Had phenotypes of *R. meliloti* *nodM* and *nodN* mutants with the *nodMN* (pDX1) operon from *R. leguminosarum* bv. viciae

| Strain | Mutation | Had phenotype at exudate concn (vol/vol) of[a]: | | | |
|---|---|---|---|---|---|
| | | 25% | 10% | 5% | 1% |
| AK631 | Wild type | + | + | + | + |
| DN4-15 | *nodM* | − | − | − | − |
| DN4-15 + pDX1 | *nodM* | + | + | + | + |
| DN4-18 | *nodN* | − | − | − | − |
| DN4-18 + pDX1 | *nodN* | + | + | + | + |

[a] +, heavily deformed root hairs; −, no plant response.

type (Fig. 6D) and the *nodM* bacteroids (Fig. 6E) were different. In contrast, nodules induced in the presence of GlcN by the *nodM* mutant showed a morphology similar to that of wild-type-induced nodules (Fig. 6C). Electron microscopic analysis of the nodule cells (Fig. 7) confirmed that the morphologies of the invaded cells and bacteroids were altered in nodules induced by *nodM* in comparison with those induced by the wild type. As shown in Fig. 7B (early symbiotic zone of the nodule) and Fig. 7D (late symbiotic zone), the host cell cytoplasm occupied by strain DN4-15 was heterogenous compared with those of cells invaded by the wild-type bacteroids (Fig. 7A, early symbiotic zone, and 7C, late symbiotic zone). The mutant bacteroids seemed to be blocked in their development, often having abnormal shapes that suggested early deterioration. The peribacteroid membranes around the mutant bacteroids were not distinguishable from those found in wild-type nodules.

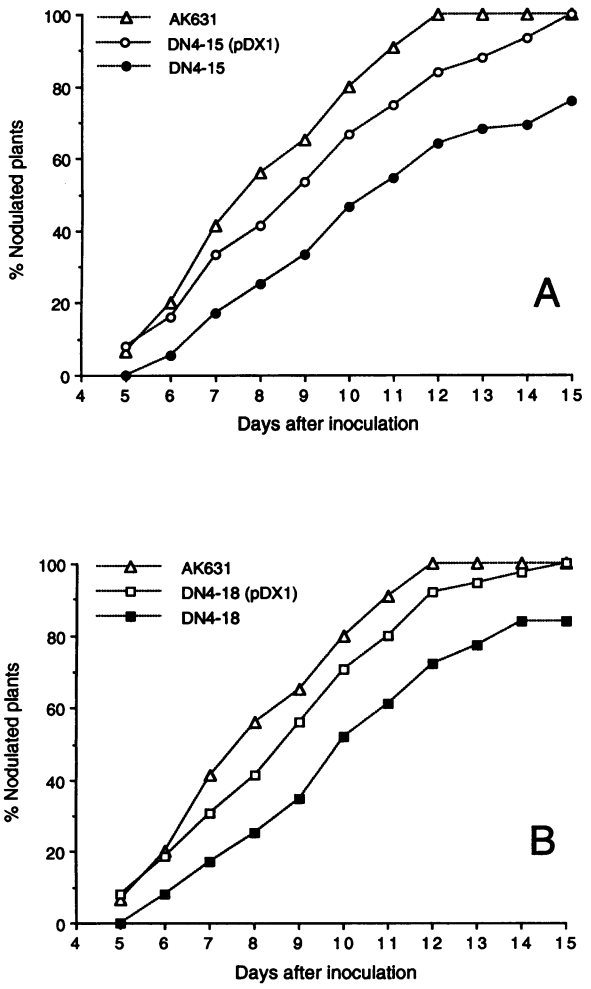


FIG. 8. Nodulation kinetics of wild-type strain AK631, the *nodM* mutant DN4-15 (A), and the *nodN* mutant DN4-18 (B) of *R. meliloti* and of the two mutants carrying the *nodMN*(pDX1) operon from *R. leguminosarum* bv. viciae. Maximum standard deviations ($n$ = 3) were ±6.11% (A) and ±4.62% (B).

The ultrastructures of the host cells of nodules induced by the *nodM* mutant were also examined when amino sugars (GlcN, GlcNAc, GalN, and GlcN-6-P) were added to the plant medium prior to the assay. As seen in Fig. 7E, F, and G, GlcN, GlcNAc, and GalN restored the wild-type appearance of the bacteroids and the host cell cytoplasm. In the cytoplasms of host cells to which GlcNAc was added, a few deteriorated bacteroids were found (Fig. 7F). Addition of GlcN-6-P, however, restored the ultrastructures of the cells to a lesser extent (Fig. 7H).

**Complementation of *R. meliloti nodM* and *nodN* mutants with corresponding genes from *R. leguminosarum* bv. viciae.** We reported that *nodM* from *R. meliloti* codes for glucosamine synthase (1). The same was recently concluded for *nodM* from *R. leguminosarum* bv. viciae (21). From the high score of sequence homology of *nodM* genes of the two species and from experiments showing that both genes coded for the same enzyme arose the inevitable question: can the two genes substitute for each other on different plant hosts, and if they can, can we consider *nodM* a common *nod* gene, at least for these two *Rhizobium* species, even though the *nodM* genes from both species were found to affect nodulation differently depending on the host plant species or cultivars (1, 4, 18)? The *nodN* genes from both species were also found to influence nodulation in a host-dependent manner (1). Unfortunately, no data about the possible biochemical function of the translational products of *nodN* from both species are available. Even so, the high sequence homology of *nodN* genes from the two species as well as the mutant phenotype similar to *nodM* mutants (1) suggest that *nodN* probably has a common function in the two species.

As an attempt to answer the questions asked above, the *nodMN* operon from *R. leguminosarum* bv. viciae was recloned in the broad-host-range vector pRK290, and the resultant construct (pDX1) was mobilized into strains DN4-15 and DN4-18 (*nodM* and *nodN* mutant derivatives, respectively, of *R. meliloti* AK631). As was presented earlier (1), mutations in *nodM* or *nodN* did not abolish root hair curling but affected root hair deformation (Had phenotype) of the respective mutant strains. As shown in Table 2, introduction of the *nodM* and *nodN* genes from *R. leguminosarum* bv. viciae into the *R. meliloti nodM* and *nodN* mutants did restore the Had$^+$ phenotype.

On the basis of the data given above, the nodulation abilities of the same transconjugants were tested. As shown in Fig. 8, the *nodMN* operon from *R. leguminosarum* bv. viciae partially restored the nodulation phenotypes of DN4-15 and DN4-18. The test plants were nodulated within 15 days after inoculation, which is 3 days of delay compared with nodulation time of the wild type. The mutant strains themselves nodulated within 20 (DN4-15) and 19 (DN4-18) days after inoculation (data not shown).

## DISCUSSION

We show here that the *nodM* gene coding for glucosamine synthase is required for efficient Nod signal production and that this function of *nodM* is common in the two species, *R. meliloti* and *R. leguminosarum*, in which the *nodM* gene was detected. In a *R. leguminosarum* bv. viciae *nodM* mutant, a slight decrease in Nod factor production was also observed (29). Recent work from several laboratories indicates that the overwhelming majority of *nod* genes are involved in the production of Nod signals (1, 2, 17, 21, 23, 26, 27, 30). The common *nodABC* genes are essential for the synthesis of any Nod metabolite (2, 7, 17, 29, 30), while several other genes (*nodE*, *nodF*, *nodH*, *nodP*, *nodQ*, and *nodL*) were shown to be involved in the chemical modification of a basic lipooligosaccharide structure (23, 29, 30, 33), and in the case of *nodE*, *nodF*, *nodH*, *nodP*, and *nodQ*, the decoration of the oligosaccharide with a specific fatty acid or a sulfate group altered the host specificity of the molecule (23, 30). Moreover, *nodL* was shown to play a role in the O-acetylation which is required for increasing the efficiency of the factor in evoking cortical cell division (30, 33). In the *nodN*, *nolF*, *nolG*, *nolH*, and *nolI* mutants, a decrease in Nod factor production was suggested on the basis of a significant decrease in root hair deformation activity by exudates of the mutants, but quantitative and structural analyses of the Nod factors have not been performed (1).

We have shown recently that *R. meliloti* produces a family of Nod factors (26). The six identified compounds were sulfated penta-, tetra-, and trisaccharides containing either a $C_{16:2}$ or a $C_{16:3}$ acyl chain. A chemical analysis of Nod factors produced by a *R. meliloti nodM* mutant indicated a 7- to 20-fold decrease in the amounts of the sulfated Nod factors. In addition to these Nod metabolites, the overproducing *nodM* mutant apparently still produced significant quantities of the minor Nod factors (penta- and tetrasaccharides containing $C_{16:1}$ acyl chains) and nonsulfated but acetylated tetrasaccharide NodRm-IV(Ac,$C_{16:2}$) and trisaccharide NodRm-III(Ac,$C_{16:2}$). The ratio of nonsulfated to sulfated compounds was increased in the *nodM* mutant in comparison with that in Rm1021(pNID6). A number of other Nod metabolites were detected in both overproducing wild-type and *nodM* strains. These seem to represent variations in the length and saturation of the acyl chain as well as in the length of the oligosaccharide chain similar to what was reported previously (17, 23, 26). Whereas a variety of different Nod metabolites are produced by a single wild-type *Rhizobium* strain (26, 30, 33), our studies suggest that the relative amount of minor Nod factors is elevated in the *nodM* mutant. To what extent these analogs of the cognate Nod signals themselves display a biological activity, as was reported for the pentasaccharides (26), or to what extent they may interfere with the action of the specific Nod signals remains to be investigated. A specifically modified Nod factor which could be assigned to the NodM function was not detected.

The preferential decrease of sulfated versus nonsulfated compounds in the *nodM* mutant is surprising. One possible explanation might be that the housekeeping enzyme (GlmS) partially provides GlcN as substrate in a Nod factor biosynthetic pathway for which the sulfate transfer is inefficient. It is possible that the $K_m$s of the housekeeping GlmS and NodM are different, which may explain the specific involvement of NodM in Nod factor production. Alternatively, an effective Nod signal synthesis might require some kind of "compartmentalization" via multienzyme complexes that involves a number of Nod proteins or a specific subcellular localization (e.g., periplasmic space). At the same time, this would explain why housekeeping functions are found among several Nod proteins (e.g., NodM, NodP, and NodQ). A specific biosynthetic pathway for the cognate Nod signals (already postulated in reference 23) might also explain why in the *nodM* mutant, the production of analogs is relatively less decreased and why, for example, the major nonsulfated metabolites are acetylated but the sulfated ones are not.

By altering the ratio of sulfated versus nonsulfated Nod factors, one may predict, in fact, a widening of the host range for the overproducing *nodM* mutant so that the host range is similar to that of the *nodPQ* mutants (23). Interestingly, in contrast to Rm1021 *nodM*::Tn*5*, the overproducing strain Rm1021 *nodM*::Tn*5*(pNID6) is as efficient in nodulating *M. sativa* as the wild-type strain Rm1021 (3a). Therefore, the phenotype of the *nodM* mutation on *M. sativa* is suppressed by the overproduction of Nod factors, and it remains to be shown whether the host range could be extended to *Vicia sativa*, which requires the presence of nonsulfated Nod factors.

It has been reported previously that *R. meliloti nodH* mutants lose the ability to nodulate *M. sativa* but are still able to form nodules on *Melilotus albus*, suggesting that the nonsulfated Nod factors are active on *M. albus* (10). Similarly, when mutations significantly reduce the production of sulfated but not nonsulfated Nod factors, *nodM* mutants show a delayed nodulation on *M. sativa* but nodulate efficiently on *M. albus* (1). It is intriguing to speculate that this host range effect of the *nodM* as well as the *nodH* mutation is mediated in both cases by the amount of nonsulfated factors or by the altered ratio of sulfated to nonsulfated Nod factors.

In *R. leguminosarum* bv. viciae, mutations in *nodM* were reported to have no or only slight effects on nodulation, again depending on the plant host (32). For *R. leguminosarum* bv. trifolii, a cultivar-specific interaction with *T. subterraneum* was detected and shown to be negatively controlled by *nodM* (18). In both species, *nodM* was shown to be a *glmS* gene coding for the same enzyme, glucosamine synthase (1, 21). Our work indicates that mutation in *nodM* affects only the amounts but not the structures of the Nod metabolites and the ratio of modified and nonmodified forms of Nod factors. In this way, it may alter the ratio of Nod factors active on different plants and thereby lead to changes in host range.

In previous studies, Tn*5*-induced mutations did not show a polar effect on genes located downstream of the insertion site because of constitutive expression of these genes via promoter elements located within the transposon, which were shown to be active in *R. meliloti* (24). In the present analysis of the *nodM*::Tn*5* insertion mutants in an overproducing background, we could not exclude the possibility that the levels of constitutive expression of the downstream *nolF*, *nolG*, *nolH*, *nolI*, and *nodN* genes from the Tn*5* promoter were lower than those of the other upregulated *nod* genes. Thus, a polar effect contributing to the inefficiency of Nod factor production in these strains could not be ruled out completely. It is clear, however, that the *nodM* mutation, irrespective of its possible polar effect, did not lead to visible changes in the structures of the Nod metabolites.

As reported earlier (1), *nodM* is not the only gene that codes for glucosamine synthase in *R. meliloti*. In *R. leguminosarum*, the chromosomal *glmS* gene has been identified, and a *glmS nodM* double mutant, in contrast to the *nodM* mutants, required GlcN for growth (21). Our studies do not

indicate that the production of GlcN from *nodM* or from the chromosomal *glmS* has any distinct features, such as the site of production or the availability of GlcN for different biosynthetic pathways. The *nodM* gene has been identified only in *R. meliloti* and *R. leguminosarum*. It seems that other *Rhizobium* strains may not need this extra pool of GlcN or that in certain *Rhizobium*-plant interactions, such a pool may even be deleterious for efficient nodule induction (18).

We found that in the restoration of the nodulation phenotype of the *R. meliloti nodM* mutant, GalN and GlcNAc could substitute for GlcN, although the second amino sugar was less efficient. Wu and Wu (34) found that an *E. coli glmS* mutant could be complemented equally well by GlcN and GlcNAc. They also reported that GalN, although less efficient than GlcNAc, could complement the mutant, whereas it was ineffective in our study. While we cannot explain this discrepancy, these results suggest that there is a different degree of interconversion of the epimeric isomers in *R. meliloti* and *E. coli*. Wu and Wu (34) also observed that the growth of the *glmS* mutant on GalN did not depend on the presence of functional uridine diphosphate-D-galactose-4-epimerase (UDP-D-Gal-4-Ep), which catalyzes the interconversion of uridine diphosphate-D-glucose and uridine diphosphate-D-galactose. This enzyme is essential in the utilization of galactose by many microorganisms and by mammals as well as being required for the synthesis of uridine diphosphate-D-galactose, the precursor of galactose residues in a wide variety of polysaccharides, glycoproteins, and glycolipids. On the other hand, GlcN and GalN are not substrates for UDP-D-Gal-4-Ep (8). We may speculate that in the suppression of the observed phenotype of the *R. meliloti nodM* mutant with GalN, another epimerase is probably involved. Alternatively, in order to achieve such effective complementation, *R. meliloti* might make use of a yet unknown pathway for the utilization of GalN.

The possibility that the requirement for GlcN and its fate are not the same in *E. coli* and *R. meliloti* cells is not excluded. Both bacteria need GlcN as a precursor for cell wall synthesis. The observation that GalN acted as a suppressor of the *nodM* mutation nearly as efficiently as GlcN but more effectively than GlcNAc supports once again the conclusion that at least at the initial stage of establishment of symbiosis, the GlcN produced by NodM is not primarily utilized as a precursor of cell wall synthesis. Rather, it could be used as a substrate of an enzyme(s) encoded by the *nod* genes and serving as precursor for synthesis of the Nod factors.

Earlier, we reported that mutations in *nodM* decreased the symbiotic nitrogen fixation efficiency of *R. meliloti* (1). Mutations in none of the other *R. meliloti nod* genes, in which the mutations cause delay of nodulation, were reported to affect symbiotic nitrogen fixation. Therefore, we investigated at what symbiotic developmental stage the *nodM* gene might be required for nitrogen-fixing symbiosis. We found that the ultrastructures of the nodule cells, particularly the appearances of the bacteroids, were affected by the *nodM* mutation. Three weeks after inoculation, the mutant bacteroids looked somewhat different from those found in the nodules induced by the wild-type bacteria, and some of them were deteriorated. It seems that the *nodM* gene is involved in the development and maintenance of the bacteroids. An explanation for this abnormal outlook of the bacteroids might be that the outer surfaces (the cell wall, lipopolysaccharide, etc.) of the developing bacteroids require high amounts of GlcN, which is partly provided by NodM. The peribacteroid membranes (which are of plant origin) of the mutant bacteroids looked normal. In light of recent reports that *nod* genes are switched off after bacteroid development (25, 28) it is unlikely that *nodM* would affect symbiotic nitrogen fixation in the fully developed nitrogen-fixing nodule tissue. Our finding that the delayed-nodulation phenotype and the changes in nodule ultrastructure observed with *nodM* mutants on *M. sativa* could be suppressed by GlcN or related amino sugars again did not reveal differences between the functions of *glmS* and *nodM*.

The *nodM* and *nodN* genes of *R. leguminosarum* completely restored the $Had^+$ phenotype and partially restored the nodulation phenotype of the corresponding *R. meliloti* mutants. The less-efficient complementation might be due to the lower expression level of the *R. leguminosarum* genes with the *R. meliloti*-specific *nod* gene activators, *syrM* and *nodD3*, which consequently results in less Nod factor production. This possibility is supported by the finding that nodule induction by purified Nod factors required at least 100-fold-higher concentrations than the Had assay (26, 33). The complementation data provide further support for the common function of the *nodM* and *nodN* genes in the two species. It seems, therefore, that the gene products catalyze the same biochemical reaction in both organisms. Perhaps not only NodM but also NodN provides a substrate(s) for the anabolic pathway leading to the synthesis of sufficient amounts of Nod factors. Alternatively, NodN might be involved in the excretion of Nod factors.

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