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Biochemistry

A 2-*O*-methylfucose moiety is present in the lipo-oligosaccharide nodulation signal of *Bradyrhizobium japonicum*

(*nod* genes/symbiosis/soybean)

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ABSTRACT ***Bradyrhizobium japonicum* is a soil bacterium that forms nitrogen-fixing nodules on the roots of the agronomically important legume soybean. Microscopic observation of plant roots showed that butanol extract of *B. japonicum* strain USDA110 cultures induced for *nod* gene expression elicited root hair deformation, an early event in the nodulation process. The metabolite produced by *B. japonicum* responsible for root hair deformation activity was purified. Chemical analysis of the compound revealed it to be a pentasaccharide of *N*-acetylglucosamine modified by a $C_{18:1}$ fatty acyl chain at the nonreducing end. In these respects, the *B. japonicum* metabolite is similar to the lipo-oligosaccharide signals described from *Rhizobium* species. However, the *B. japonicum* compound is unique in that an additional sugar, 2-*O*-methylfucose, is linked to the reducing end. Comparative analysis of the *B. japonicum* Nod metabolite and those characterized from *Rhizobium* species suggests that the presence of the fucosyl residue plays an important role in the specificity of the *B. japonicum*–soybean symbiosis. The availability of the purified *B. japonicum* nodulation signal should greatly facilitate further studies of soybean nodulation.**

Bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* have the ability to establish a symbiotic relationship with leguminous plants. In this association, the bacteria induce the plant to develop a new organ, the root nodule, in which the symbiont resides and reduces atmospheric nitrogen to ammonium. Root hair deformation and curling are the first microscopically visible responses of the legume host to its compatible rhizobial counterpart. Three different classes of bacterial genes have been shown to be required for eliciting these early responses (1). The common nodulation genes (i.e., *nodABC*) are found in all rhizobial species and are functionally interchangeable among different species. Mutations in these genes completely abolish the ability to induce root hair deformations. The host-specific *nod* genes determine the ability to nodulate particular host plant species. Hence, these genes are not interchangeable between different species. The *nodD* gene, which can be present in up to three copies, encodes a regulatory protein that activates transcription of the other *nod* genes, but only in the presence of specific plant-produced flavonoids (1, 2). In the case of *Bradyrhizobium japonicum* these *nod* gene-inducing flavonoids are isoflavones (e.g., genistein or daidzein) (3).

The nodulation genes are required for the biosynthesis of N-fatty acylated chitin oligomer signals that elicit early nodulation events on the host plant. These signal molecules have been purified and chemically characterized from *Rhizobium meliloti* and *Rhizobium leguminosarum* biovar *viciae* (4–7). In this report, we show that butanol extracts of *B. japonicum* cultures induced for *nod* gene expression elicit root hair deformations on soybean (*Glycine max* and *Glycine soja*) and siratro (*Macroptilium atropurpureum*) and report on the purification and chemical characterization of the bacterial metabolite responsible for this activity.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *B. japonicum* strains were maintained on RDY agar as described (8). For Nod factor detection and purification, bacteria were grown on a minimal medium (MM) (9) with glycerol as a carbon source and sodium glutamate as the nitrogen source. When required for *nod* gene induction, genistein (2 μM final concentration) or soybean (*G. max* cv. Essex) seed extract, prepared as described (10), was added. Strains used were the wild-type *B. japonicum* USDA110 and transposon Tn*5* mutants of USDA110 in the *nodB* gene (strain AN279) or the *nodC* gene (strain AN122) (8).

Detection of Nod Metabolites by Thin-Layer Chromatography (TLC). Cells were grown in liquid RDY medium at 30°C until the cultures reached an OD_{600} of 0.5–0.6. Bacteria were pelleted in a microcentrifuge, washed once with liquid MM, and diluted in this medium to an OD_{600} of 0.1. Cells were then induced by the addition of 2 μM genistein or soybean seed extract. At the time of inducer addition, 50 μCi of [1-^{14}C]acetate (56 mCi/mmol, ICN; 1 Ci = 37 GBq) was added and the cultures were incubated overnight. The induction of the nodulation genes was indirectly monitored by the induction of β-galactosidase in a strain containing a *nodY–lacZ* fusion (i.e., ZB977) (3). Supernatants of labeled cultures were extracted with 1-butanol and applied to octadecyl silica TLC plates (Sigma) as described (11). Plates were dried and exposed to x-ray film (Kodak X-Omat AR) for 2–4 days at room temperature.

Purification of the Nod Metabolite. In order to purify the Nod metabolite, typically the [^{14}C]acetate butanol extract from a 100-ml culture of strain USDA110, labeled as above, was mixed with nonradioactive butanol extract (⅓ volume butanol) of a 5-liter culture grown on MM in the presence of soybean seed extract. The butanol was removed by rotary evaporation and the residue was resuspended in acetonitrile/water (50:50, vol/vol). This mixture was then chromatographed on a silica gel 60 column (Pharmacia LKB, 1.6 × 100 cm). Fractions were eluted with a 60:40 acetonitrile/water gradient and analyzed for root hair curling activity on *G. soja* seedlings (see below). In addition, samples spiked with ^{14}C-labeled material were monitored by TLC and subsequent autoradiography. Biologically active fractions were further purified by reverse-phase HPLC (Waters 501 with a Waters 484 detector at a wavelength of 206 nm), with a Pharmacia

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LKB SuperPac Pep-S column (5-μm, 4 × 250 mm) using 20:80 acetonitrile/water and 40:60 acetonitrile/water gradients. At this point, the purity of the sample was confirmed by mass spectrometry (MS) and NMR analysis.

Chemical Analysis of the Nod Factor. Glycosyl composition analysis was performed by GLC–MS analysis of alditol acetates prepared as described by York *et al.* (12). Glycosyl linkages were determined by GLC–MS analysis of partially methylated alditol acetates prepared by the Hakomori procedure as described previously (12). Either a 30-m DB1 column (J&W Scientific, Rancho Cordova, CA) or a 30-m SP2330 capillary column (Supelco) was used in these analyses. Fatty acids were analyzed by GLC–MS analysis of their methyl esters prepared by acid-catalyzed methanolysis (13). Prior to methanolysis, fatty acids could also be isolated by acid hydrolysis, or by alkaline hydrolysis followed by acidification, and extracted into chloroform. The DB1 column was used for the analysis of fatty acid methyl esters. The fatty acyl residue that was attached to glucosamine was determined by mild methanolysis (4, 14) followed by trimethylsilylation and analysis by GLC–MS. Again, a DB1 column was used for these analyses. The location of the double bond in the fatty acyl residue was determined by oxidation of the double bond to a diol, followed by acid-catalyzed methanolysis and trimethylsilylation (15). The resulting product was identified by GLC–MS analysis using the DB1 column. Fast-atom bombardment MS was carried out on a VG-ZAB SE instrument at an accelerating voltage of 8 kV in the positive mode, with thioglycerol as the matrix. NMR spectra were collected on a Bruker AM500 spectrometer with deuterated dimethyl sulfoxide as the solvent at 23°C. Prior to analysis, the samples were resuspended three times in ^2H_2O and lyophilized.

Root Hair Deformation Assays. Seeds of *G. max* cv. Essex, *G. soja* PI468397, *Macroptilium atropurpureum*, and *Medicago sativa* cv. Buffalo were surface sterilized and germinated as described (8, 16). Seedlings were transferred to sterile tubes containing 1 ml of plant nutrient solution (8, 16), a filter paper strip, and serial dilutions of the Nod factor or crude butanol extract. Roots were examined microscopically for root hair deformations 1, 2, 3, and 4 days after inoculation. At least six plants were examined for each dilution of the Nod metabolite. Activity of a given dilution was scored as positive when at least 50% of the seedlings showed strong root hair deformation.

RESULTS

Identification and Purification of a Nod Factor from *B. japonicum* USDA110. Using the TLC system previously described (11), we found that *B. japonicum* USDA110 produces one major, unique metabolite when grown in the presence of genistein or soybean seed extract in order to induce *nod* gene expression (Fig. 1*A*). This compound was absent from uninduced cultures and was not produced by mutant strains AN279 (NodB$^-$) and AN122 (NodC$^-$), regardless of the presence of the *nod* gene inducers. In addition, the compound was found to be sensitive to chitinase, suggestive of an *N*-acetylglucosamine polymer (data not shown). The material corresponding to the radioactive spot shown in Fig. 1*A* was subsequently purified as described in *Materials and Methods*. At each step in the purification, the biological activity was determined by bioassay for root hair deformation activity on *G. soja* seedlings. In addition, samples spiked with ^{14}C material were monitored by TLC and subsequent autoradiography. The final HPLC purification step yielded a single peak (Fig. 1*B*) corresponding to the radioactive spot produced by induced cultures of USDA110 as detected in the TLC system.

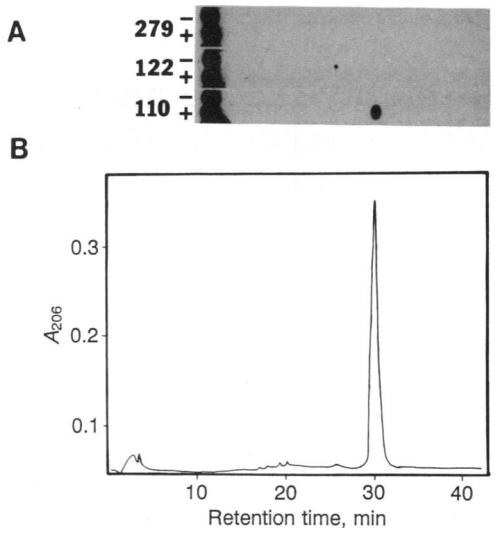

FIG. 1. Detection and purification of the Nod factor from *B. japonicum* USDA110. (*A*) Separation and detection of the radiolabeled Nod metabolite by TLC (11). The major spot produced by strain USDA110 in the presence of the isoflavone genistein (+) is not present in the absence of the inducer (−). Likewise, cultures of strain AN279 (*nodB*::Tn*5*) and AN122 (*nodC*::Tn*5*) did not produce this metabolite regardless of the presence of genistein. (*B*) Analytical HPLC of the purified *B. japonicum* USDA110 Nod factor. The single peak shown in the chromatogram was identified as the radioactive spot produced by strain USDA110, as detected in the above TLC system (see text for details).

Structural Analysis of the Nod Factor. The structure of the Nod metabolite was determined by chemical composition analysis, NMR spectroscopy and MS. Glycosyl composition analysis (12) revealed that the Nod metabolite contained 2-*O*-methyl-6-deoxyhexose and glucosamine in a 1:5 ratio. The 2-*O*-methyl-6-deoxyhexose was identified by its mass spectrum, which showed characteristic ions of m/z 118 and 275, and as a fucosyl residue by comparing its retention time with that of an authentic standard. Analysis of partially methylated alditol acetates (12) by GLC–MS showed the presence of terminal fucose along with terminal, 4-linked and 4,6-linked glucosamine. The respective glucosamine residues were present in a 1:3:1 ratio. Fig. 2 *Lower* shows a typical fast-atom bombardment mass spectrum of the USDA110 Nod factor. The difference in mass, 203, between the fragment ions is characteristic of an oligosaccharide composed of *N*-acetylglucosamine residues. The difference of 381 between the molecular ion, 1416, and the first fragment ion, 1035, shows that the reducing end of this Nod factor consists of a branching *N*-acetylglucosamine residue with the branching glycosyl residue having a mass of 160, exactly what would be expected for 2-*O*-methylfucose. The results of the methylation analysis, showing only one branched *N*-acetylglucosamine residue, 4,6-linked glucosamine, together with the fast-atom bombardment MS results, indicate that the 2-*O*-methylfucose residue is linked to C-6 of the reducing 4-linked *N*-acetylglucosamine. Acidic or alkaline hydrolysis of the compound with subsequent chloroform extraction and methanolysis in methanolic 1 M HCl released $C_{18:1}$ along with $C_{16:0}$ and $C_{18:0}$ fatty acid methyl esters. The presence of $C_{16:0}$ and $C_{18:0}$ fatty acid methyl esters may be due to a slight contamination by other lipids. Mild methanolysis (4, 14) followed by trimethylsilylation released trimethylsilylated methylglycosides of 2-*O*-methylfucose, *N*-acetylglucosamine, and *N*-acylglucosamine. GLC–MS analysis (using both chemical ionization and electron impact MS) showed that the trimethylsilylated methylglycoside of *N*-acylglucosamine had a pseudomolecular ion of 674 $(M+H)^+$ and characteristic

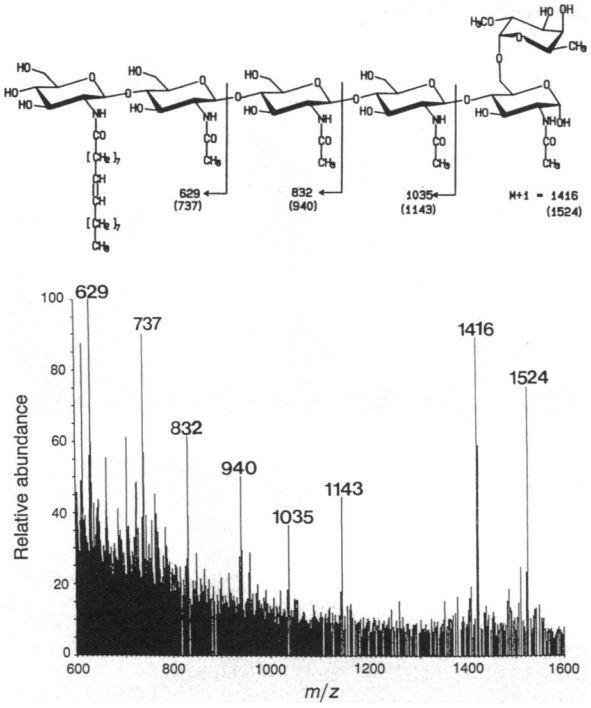

FIG. 2. (*Upper*) Chemical structure of the *B. japonicum* Nod factor. Following the mentioned convention (see text) for naming of such structures (6, 7, 17), this compound is NodBj-V ($C_{18:1}$, Me-Fuc). (*Lower*) Fast-atom bombardment mass spectrum of the Nod factor. When thioglycerol is used as the matrix, thioglycerol adducts are observed (+108, ions in parentheses) for the pseudomolecular ion and all fragment ions. The difference in mass between the fragment ions, 203, is characteristic of an oligosaccharide composed of *N*-acetylglucosamine.

fragment ions of m/z 204 (the fragment containing C-3 and C-4 of glucosamine) and m/z 395 (the fragment containing C-2 and C-3 of glucosamine and the $C_{18:1}$ fatty acyl substituent). The location of the double bond in the $C_{18:1}$ fatty acyl group was determined by oxidation to a diol, preparation of the fatty acid methyl ester, and trimethylsilylation (15). Analysis by electron impact MS resulted in ions from cleavage between the carbons containing the trimethylsiloxy groups, m/z 259 and 214, and showed that the double bond was between C-9 and C-10 of the $C_{18:1}$ fatty acyl residue. The proposed structure for this metabolite is shown in Fig. 2 *Upper*. The *B. japonicum* Nod factor is a β-1,4-linked pentasaccharide of one *N*-acyl-D-glucosamine and four *N*-acetyl-D-glucosamines that bears a 2-*O*-methylfucose substituent on C-6 of the reducing *N*-acetylglucosamine. The aliphatic chain, carried by the nonreducing terminal glucosamine, is a 9-octadecenoyl (oleoyl) N-acyl group. The structure proposed for this metabolite is further supported by the NMR spectrum of this compound (Fig. 3). The resonance at δ 4.85 is consistent with an anomeric proton of the reducing α-*N*-acetylglucosamine residue as reported for the *R. meliloti* Nod factors (4, 14). The resonance at δ 4.95 ($J_{1,2}$ = 3.7 Hz) is consistent with that of an α-linked fucosyl residue and has been shown by two-dimensional NMR (homonuclear Hartmann–Hann) to be part of the glycosyl ring system that contains the methyl protons which resonate at $\delta \approx 1.15$ (data not shown). Thus, this resonance can be assigned to H on C-1 of the α-linked 2-*O*-methylfucosyl residue. The methoxy substituent of this residue resonates as a sharp singlet at $\delta \approx 3.5$. Following the accepted nomenclature for Nod metabolites from *Rhizobium* (6, 7, 17), we will subsequently refer to the *B. japonicum* Nod signal as NodBj-V ($C_{18:1}$, Me-Fuc).

Biological Activity of the Nod Factor. The activity of NodBj-V ($C_{18:1}$, Me-Fuc) was tested in the root deformation assay. The addition of as little as 10 pM purified Nod factor to roots of *G. soja* or siratro elicited marked root hair deformation (Fig. 4). No activity was detected on alfalfa, a heterologous host plant, up to concentrations 10,000-fold higher than those found active on soybean. Thus, NodBj-V ($C_{18:1}$, Me-Fuc) is a host-specific symbiotic signal produced by *B. japonicum*. However, under the conditions tested, the addition of this compound did not induce cortical cell division on soybean.

DISCUSSION

We have found that *B. japonicum* USDA110, when grown in the presence of isoflavones in order to induce the *nod* genes, produces a metabolite that is able to induce early nodulation responses on normal host legumes. The functional presence and induction of the *nod* genes are required for the production of this signal molecule, since strains carrying a Tn*5* insertion

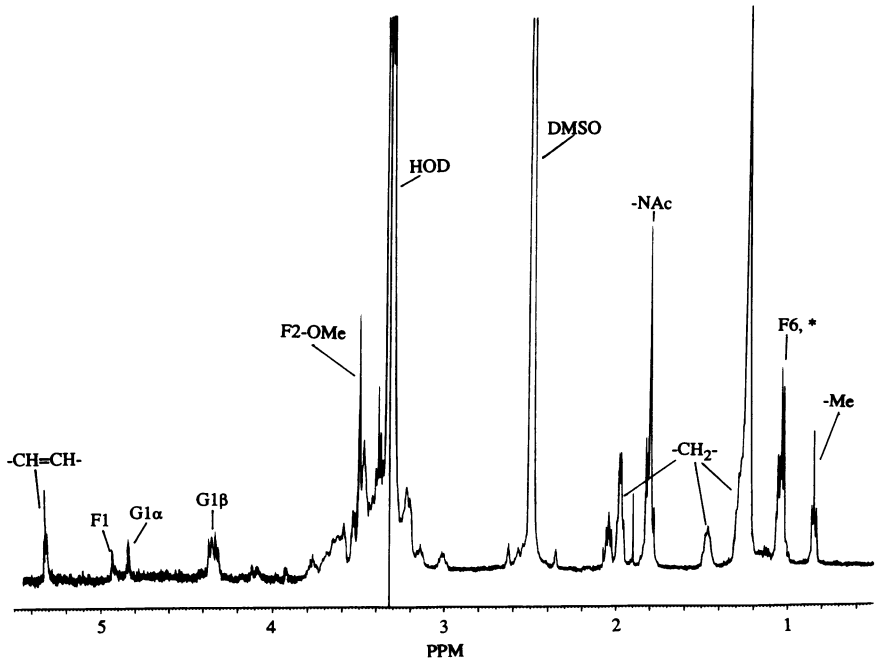

FIG. 3. NMR spectrum of the *B. japonicum* Nod metabolite from strain USDA110. Some assignments of the 2-*O*-methylfucose (F) and glucosamine residues (G) are indicated, as well as signals due to the $C_{18:1}$ acyl protons. Chemical shifts were measured relative to that of dimethyl sulfoxide (DMSO) (δ 2.49). A peak due to a contaminant is marked by an asterisk. This contaminant can be found in varying amounts in different Nod factor preparations and has been shown to come from the butanol used in the extraction procedure. HOD, 'HO^{2}H.

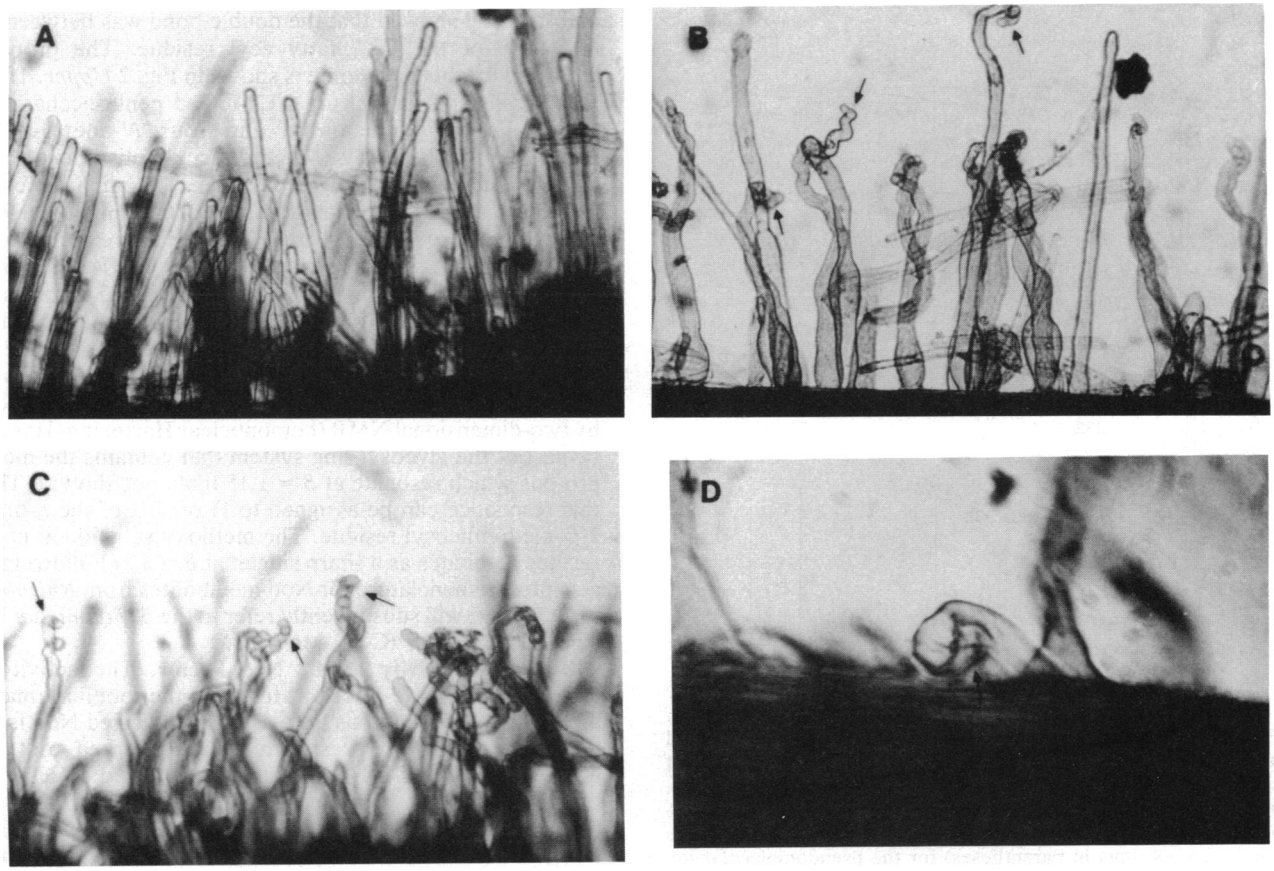

FIG. 4. Root hair deformation activity of the NodBj-V ($C_{18:1}$, Me-Fuc) nodulation factor. (*A*) Root hairs of control siratro seedlings. (*B*) Root hairs of siratro seedlings inoculated with 10 pM Nod factor. (*C* and *D*) Root hair deformation and curling in *G. soja* seedlings in the presence of 100 pM NodBj-V ($C_{18:1}$, Me-Fuc). No activity was observed on alfalfa with Nod factor up to concentrations estimated to be 10,000-fold higher than those found active on soybean and siratro. (*A–C*, ×250; *D*, ×800.)

in the *nodB* or *nodC* gene were unable to produce it. Chemical and structural analysis of this metabolite revealed it to be a β-1,4-linked pentasaccharide consisting of one *N*-acyl-D-glucosamine and four *N*-acetyl-D-glucosamines, bearing a 2-*O*-methylfucose substituent on C-6 of the reducing *N*-acetylglucosamine. The aliphatic chain, carried by the nonreducing terminal glucosamine, is a 9-octadecenoyl N-acyl group. This metabolite, named NodBj-V ($C_{18:1}$, Me-Fuc), was able to induce early nodulation responses—i.e., root hair curling—on soybean and siratro at concentrations as low as 10 pM. Two features of this compound, chemical structure and strong biological activity on specific legume roots, resemble the properties of the nodulation signals produced by *Rhizobium* species (4–7). However, the *B. japonicum* Nod signal is unique in that it contains a sugar other than *N*-acetylglucosamine, 2-*O*-methylfucose. Comparative analysis of the Nod metabolites isolated from *R. meliloti*, *R. leguminosarum* bv. *viciae*, and *B. japonicum* reveals similarities in that all three have a chitin oligomer backbone (4–7). The specificity of these compounds is apparently due to the specific modification of a basic structure. In the case of *R. meliloti*, the critical modification for specificity appears to be sulfation of the reducing-end sugar (4, 5). The products of the host-specific *nodH*, *nodP*, and *nodQ* genes are required for this reaction (17, 18). The host-specific character of the *R. leguminosarum* bv. *viciae* Nod metabolite appears to be determined by the specific acetylation of the nonreducing terminal sugar and the addition of a polyunsaturated fatty acyl group to this same residue (7). Data indicate that the product of the *nodL* gene is a transacetylase, whereas the *nodE* and *nodF* gene products are apparently involved in the synthesis of the fatty acyl group (7). In the case of the NodBj-V ($C_{18:1}$, Me-Fuc) factor, removal of the 2-*O*-methylfucose would yield a compound structurally similar to one of the nonspecific metabolites purified from *R. leguminosarum* bv. *viciae* (7). Based on these differences and similarities of the various Nod metabolites characterized so far, we postulate that the presence of the 2-*O*-methylfucose residue on the reducing end of the *B. japonicum* Nod factor gives the molecule its host-specific character.

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