THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1995 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 270, No. 11, Issue of March 17, pp. 6050–6055, 1995
Printed in U.S.A.

Wild Type *Rhizobium etli*, a Bean Symbiont, Produces Acetylfucosylated, *N*-Methylated, and Carbamoylated Nodulation Factors*

(Received for publication, December 8, 1994)

Rémy Poupot‡, Espéranza Martinez-Romero§, Nicolas Gautier‡, and Jean-Claude Promé‡¶

From the ‡Laboratoire de Pharmacologie et Toxicologie Fondamentales, Centre National de la Recherche Scientifique, 31077 Toulouse Cedex, France and the §Departamento de Genética Molecular, Centro de Investigacion sobre Fijacion de Nitrogeno, Universidad Nacional Autonoma de Mexico, Ap. 565-A Cuernavaca, Morelos, Mexico

***Phaseolus vulgaris* (common bean) can be nodulated by different *Rhizobium* species. A new species has been recently proposed: *Rhizobium etli*. Following transcriptional activation of the bacterial nodulation genes using naringenin or bean seed exudate, we have isolated, purified, and characterized *R. etli* extracellular nodulation factors. They are chitopentameric compounds that are *N*-methyl-*N*-vaccenoylated at their non-reducing end. At position 6 of the reducing *N*-acetyl-D-glucosamine, they are 4-*O*-acetyl-L-fucosylated. Minor compounds bear a carbamate group on the terminal non-reducing saccharidic residue.**

Azorhizobium, *Bradyrhizobium*, and *Rhizobium*, collectively referred to as Rhizobia, specifically trigger stem or root nodule organogenesis on leguminous plants. In these symbiotically elicited organs, the bacteria reduce atmospheric nitrogen to ammonia (1).

During the infection process, signal exchange between the two symbionts controls the specificity of this interaction (2). Legume roots secrete flavonoids that regulate the expression of the nodulation genes of the bacteria (the *nod* and *nol* genes) (3). In turn, these genes are involved in the synthesis and excretion of the so-called nodulation factors (Nod factors)[1] (2, 4, 5).

Nod factors are lipooligosaccharides that share the same common backbone: an oligochitin core bearing a fatty acid that *N*-acylates the non-reducing end. These factors may also carry a variety of substitutions that complete the backbone and are involved in the specificity of the interaction between the plant and the bacteria (6). At picomolar to nanomolar concentrations, these molecules provoke the root hair deformation effect in nodulation-competent root hairs (2, 7, 8). They also induce rapid membrane depolarization (9), expression of early nodulins (10), and mitosis in the root cortex (11, 12), which, in some cases, leads to nodule organogenesis (13–16). Thus they appear to play a key role in the establishment of the nodule symbiosis.

Recognition of Nod factors by host plants is relatively specific, and the nature and position of the Nod factor substitutions are of crucial importance. It has been proposed that a way for a bacterium to exhibit a wide host range is to synthesize a large variety of Nod factors, suggesting that each variety of plant is specifically triggered by Nod factors possessing a similar set of substitutions. For example, all Nod factors from bacteria that nodulate soybeans possess a 2-*O*-methylfucosyl substitution (8, 12, 16, 17). A 6-*O*-sulfate group together with an *N*-(2*E*)(9*Z*)-hexadienoyl substitution seems specific for nodulation of alfalfa (13, 18). *Rhizobium* NGR234, which nodulates more than 60 different legumes, synthesizes a very complex mixture of Nod factors bearing an *N*-methyl group and zero, one, or two *O*-carbamoyl groups at the non-reducing end together with a 2-*O*-methylfucose moiety, which may or may not be substituted by acetate or sulfate (19).

To examine whether a strict relationship between the structure of Nod factors secreted by bacteria and their host plant range exists for all symbiotic associations, we examined the Nod factors from two different bacterial species that nodulate the common bean (*Phaseolus vulgaris*). One species is *Rhizobium tropici*, from which the Nod factor structure has been described earlier (20). The species that will be dealt with in the present paper is *Rhizobium etli*.

R. etli (21), previously named *Rhizobium leguminosarum* bv. *phaseoli* type I, nodulates the common bean, as does *R. tropici*. However, the geographical origin and breadth of the host range of these two species are different. The former, isolated from Mexican soils, seems to have a narrow host range (21). The latter, which originated in South America, has a broad specificity as it nodulates several tropical legumes and some trees (*e.g. Leucaena*) (22). In this paper we show that Nod factors from *R. etli* do not share identical structures with those described for *R. tropici* and represent a new type of Nod factor.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Cell Cultures—*R. etli* strains CFN 42 (wild type strain) and CFN 2001 (wild type strain cured of its symbiotic plasmid) were grown in a minimal medium in the same conditions used for *R. tropici* (20). Induction of the bacterial nodulation genes was achieved by using 1.5 μM naringenin (4′,5,7-trihydroxyflavanone, Sigma) or by adding bean seed exudates that were prepared as follows.

Preparation and Testing of Bean Seed Exudates—After surface sterilization (23, 24), 20 g of bean seeds were germinated (in darkness at 28 °C) in 50 ml of sterile water in a 2-liter Erlenmeyer flask stirred at 180 rpm. The aqueous solution was collected after 8 h and replaced by 50 ml of sterile water. This operation was repeated 5 times. The pooled aqueous exudates were evaporated to dryness, and the residue was dissolved in 20 ml of ethanol. 50 μl, 200 μl, or 1 ml of this solution were tested for its ability to induce Nod factors synthesis in 50 ml of *R. etli* radiolabeled cultures.

Thin-layer Chromatography and Radioactivity Assays—The experimental protocols for labeling Nod factors using [^{35}S]sulfate or [^{14}C]acetate were identical to those already described for *R. tropici* (20).

Purification of NodRe Factors—4 liters of culture medium were extracted twice, first with 1 liter and then with 500 ml of butanol. The

* This work was financially supported by a VLIR/ABOS grant from Belgium, CNRS, Université Paul Sabatier, Région Midi-Pyrénées, and European Economic Community (Biotech and Science programs). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Laboratoire de Pharmacologie et Toxicologie Fondamentales, Centre National de la Recherche Scientifique, 205 route de Narbonne, 31077 Toulouse Cedex, France. Tel.: 33-61-17-59-35; Fax: 33-61-17-59-94.

[1] The abbreviations used are: Nod factor, nodulation factor; GC, gas chromatography; GC-MS, gas chromatography coupled to mass spectrometry; HPLC, high-pressure liquid chromatography; NodRe factor, nodulation factor of *R. etli*; NodRt, nodulation factor of *R. tropici*; NodRm, nodulation factor of *R. meliloti*.

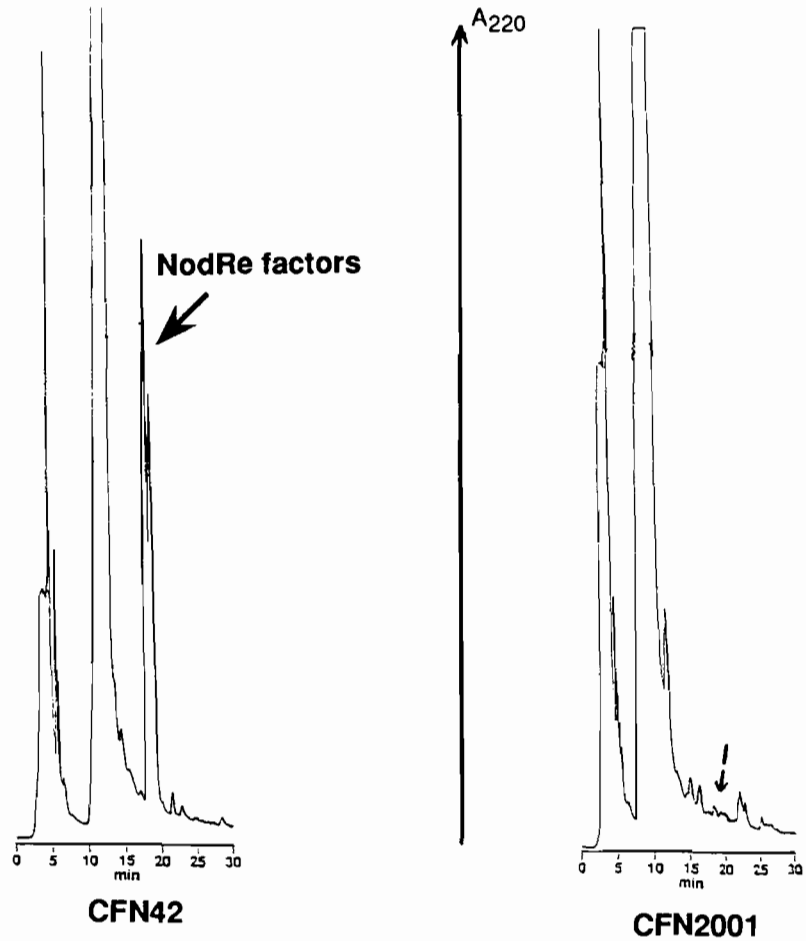

FIG. 1. **Comparative analytical C_{18} HPLC chromatograms of the butanol extracts of the culture media of the wild type *R. etli* strain CFN 42 and the pSym$^-$ strain CFN 2001.**

butanol extract was vacuum evaporated to dryness. The residue was dissolved in water and purified by HPLC on a semi-preparative C_{18} reversed-phase column (250 × 7.5 mm, Spherisorb ODS2, 5 μm, ColoChrom), using a water/acetonitrile gradient. Detection was done by UV absorbance at 220 nm. The Nod factor-containing fraction was collected and purified again on an analytical C_{18} reversed-phase column (250 × 4.6 mm, Spherisorb ODS1, 5 μm, ColoChrom) using a linear gradient from 80:20, v/v to 40:60, v/v water/acetonitrile in 10 min at a 1 ml/min flow rate.

Analytical Methods—Gas chromatography analyses were done on a Girdel 30 gas chromatograph equipped with an OV1 bound capillary column (0.32 mm × 30 m, Spiral France). The temperature gradient was 2 °C/min from 100 to 280 °C.

Mass spectra were recorded on an AutoSpec instrument (VG Analytical, Manchester, U.K.) fitted with a cesium bombardment ion source. The matrix was a mixture of *m*-nitrobenzyl alcohol/glycerol (1:1, v/v) spiked either with 1% trichloroacetic acid in water or with a solution of sodium iodide (1 mg/ml). The location of double bond of fatty acid was determined by remote charge fragmentation of the carboxylate anions as described (25) using a capillary gas chromatograph coupling to fractionate the fatty acids as pentafluorobenzyl esters, followed by negative ion ionization and collision-activated dissociation mass-analyzed ion kinetic energy spectrometry.

GC-MS experiments were performed on a Hewlett-Packard 5989A mass spectrometer in electronic impact ionization mode. One- and two-dimensional correlation spectroscopy ^{1}H NMR spectra were measured on a Brücker AC-300 spectrometer (Karlsruhe, Germany) using 2 mg of sample dissolved in 0.5 ml of perdeuterated dimethyl sulfoxide (Sigma).

Carbohydrate Determination and Methylation Studies—Carbohydrate determination was carried out after complete hydrolysis using 3 N HCl (3 h at 80 °C) followed by extraction with diethyl ether to remove the fatty acids. Sugars from the aqueous phase were derivatized as alditol acetates and analyzed by capillary GC-MS. Alternatively, 2-butyl glycosides of individual sugars were prepared using 3 N HCl in (±)-2-butanol or (−)-2-butanol (3 h at 60 °C). These glycosides were further acetylated (acetic anhydride/pyridine mixture (1:1, v/v), 3 h at 40 °C) and analyzed by capillary GC. Permethylation analysis was done according to the method of Ciucanu and Kerek (26) as described for NodRt factors (20).

Deacetylation of 1 mg of NodRe factors was achieved by 1 ml of 0.5 N sodium methanolate in methanol at 30 °C overnight. After acidification with acetic acid, the evaporated residue was dissolved in 1 ml of water and applied to a Sep-Pak C_{18} cartridge. After washing with water, deacetylated NodRe factors were eluted with methanol.

Fatty Acid Analysis—Fatty acids extracted from the acid hydrolysis were either methylated by diazomethane and analyzed by GC or derivatized as pentafluorobenzyl esters for negative ion GC coupled to tandem mass spectrometry. Fatty acids (100 μg) were dissolved into 60 μl of a mixture of dry methanol and acetonitrile (1:5, v/v) followed by the addition of 2 μl of pentafluorobenzyl bromide and 2 μl of diisopropylethylamine. After 1 h at room temperature, reagents were removed by evaporation. The pentafluorobenzyl esters were dissolved in cyclohexane for GC-MS analysis (see "Analytical Methods") (25).

RESULTS

Detection of NodRe Factors after nod Gene Induction—R. etli (CFN 42) and the same strain cured of the symbiotic plasmid (CFN 2001) were grown in the presence or absence of naringenin with the addition of sodium [^{14}C]acetate or sodium [^{35}S]sulfate to the medium. Lipophilic compounds were extracted on C_{18} reversed-phase material, eluted with methanol, and analyzed by reversed-phase thin-layer chromatography. Autoradiography showed that a spot at R_F = 0.53 was detected only in the lane corresponding to an induced culture of CFN 42 after sodium [^{14}C]acetate labeling. When treated with chitinase, this compound disappeared and a new spot was observed at R_F = 0.42, thus suggesting that the compound has a chitooligomeric backbone. Thus, the compound with a R_F of 0.53 possesses the characteristics of Nod factors: a chitooligomeric structure whose biosynthesis is dependent on the presence of *nod* genes and flavonoid induction.

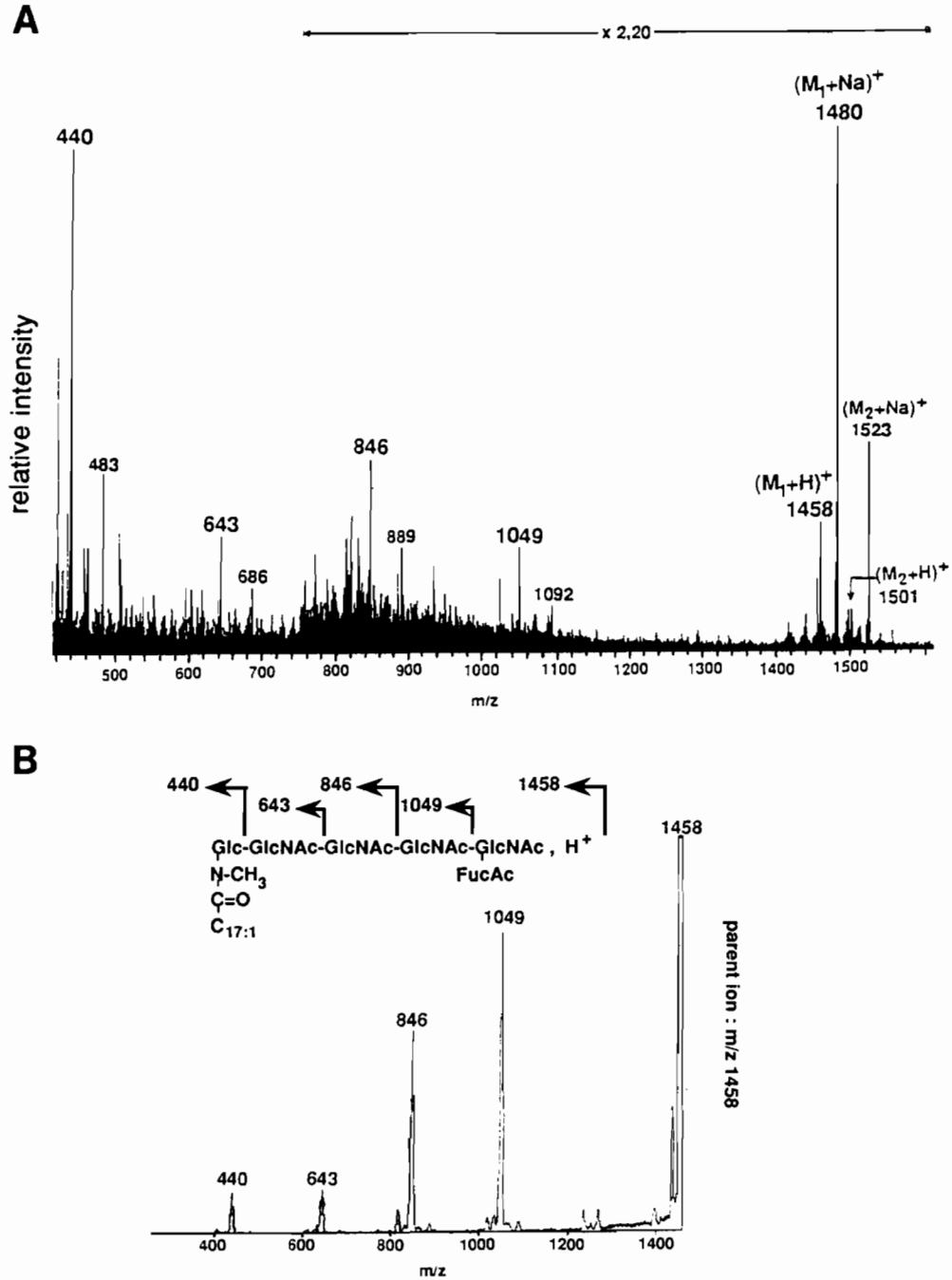

FIG. 2. *A*, positive ion fast atom bombardment mass spectrum of the NodRe factors. Molecular ions are cationizated with the sodium ion (NaI doped matrix). *B*, positive ion fast atom bombardment mass-analyzed ion kinetic energy spectrum of the protonated ion at *m/z* 1458.

Purification of NodRe Factors—4 liters of sterile medium from naringenin-induced culture were extracted with butanol. The butanol extract was fractionated by HPLC using two consecutive purifications, first on a C_{18} reversed-phase HPLC semi-preparative column and then on an analytical column; Nod factors gave a double HPLC peak (Fig. 1). About 1.0 mg of NodRe factors was purified from a 4-liter culture.

Induction of R. etli Nodulation Genes by Bean Seed Exudates—Exudates of bean seeds in ethanol were used to induce a 50-ml culture of *R. etli* after ^{14}C and ^{35}S radiolabeling. Reversed-phase thin-layer chromatography analysis showed the same result as with induction with naringenin: a compound with a R_f of 0.53, labeled only by growing the bacteria in the presence of sodium [^{14}C]acetate. Using a 4-liter culture induced with bean seed exudates, the same molecules in the same proportions were isolated as for the naringenin-induced culture.

Constituent Analysis of NodRe Factors—After complete acid hydrolysis with 3 N HCl for 3 h at 80 °C, *N*-acetylglucosamine, *N*-methylglucosamine, and fucose were detected as water-soluble compounds. GC analysis of the peracetylated (−)-2-butyl glycosides (27) assigned *N*-acetylglucosamine and *N*-methylglucosamine to the D series, whereas fucose was assigned to the L series. A single fatty acid was liberated by acid hydrolysis that was identified as *cis*-vaccenic acid both by studying the decomposition of its carboxylate anion by tandem mass spectrometry (25) and by comparison of the GC retention time of its methyl ester with an authentic standard.

Mass Spectrometry Analysis—In the positive ion mode, the Nod factor-containing fraction exhibited two $(M+H)^+$ ions at *m/z* 1458 and 1501. Cationization with the sodium ion resulted

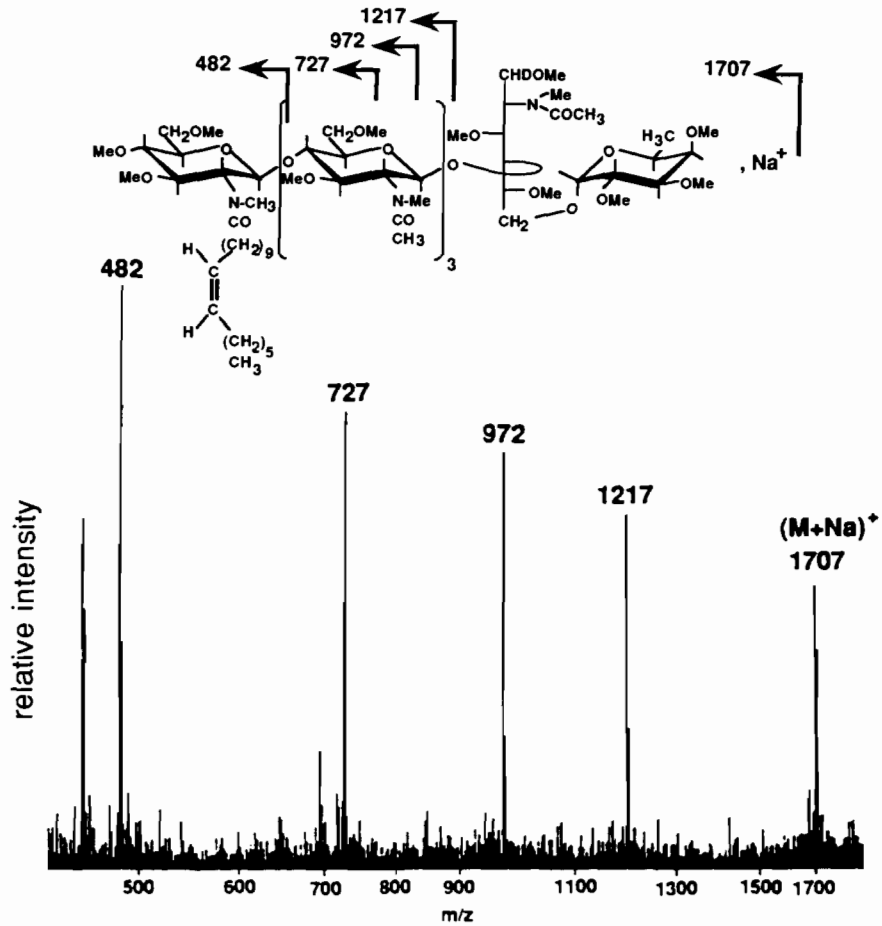

FIG. 3. **Positive ion fast atom bombardment mass spectrum of the $NaBD_4$-reduced and permethylated NodRe factors.** The molecular ion is cationizated with the sodium ion (NaI doped matrix).

in two $(M+Na)^+$ ions at *m/z* 1480 and 1523, respectively (Fig. 2*A*). Metastable ion spectrum from the $(M+H)^+$ ion at *m/z* 1458 (Fig. 2*B*) showed the clear fragmentation of the *N*-acetylglucosamine backbone giving a series of peaks separated by 203 mass units. The highest peak of this series at *m/z* 1049 was 409 mass units below the $(M+H)^+$ ion. The decomposition of *m/z* 1501 showed a corresponding series of fragment ions shifted up 43 mass units.

When NodRe factors were treated with 0.5 N sodium methanolate prior to analysis, the fast atom bombardment-mass spectrum in the positive ion mode showed $(M+H)^+$ ions at *m/z* 1416 and 1459. However, all fragmentations remained unchanged. Thus an acetate group was present near the reducing end. The mass difference between the first fragment ions (*m/z* 1049 or 1092) and the $(M+H)^+$ ions corresponded to the loss of both L-fucose and *N*-acetyl-D-glucosamine.

The fragmentation series ended at *m/z* 440 (or *m/z* 483), which was attributed to oxenium ions from the first sugar residue at the non-reducing end. *m/z* 440 was attributed to a *N*-methyl-*N*-vaccenoyl-D-glucosamine (20). *m/z* 483, which was 43 mass units higher, indicated the presence of an additional carbamoyl substitution. Thus, NodRe factors are a mixture of carbamoylated and non-carbamoylated molecules, the former representing one-quarter to one-third of the total molecules. All NodRe factors bore a L-fucose residue linked to the terminal reducing *N*-acetyl-D-glucosamine together with an additional *O*-acetyl group.

Permethylation Studies—Permethylation studies of NodRe factors were performed in order to confirm the oligochitin core and to precisely locate the L-fucose residue.

$NaBD_4$ reduction followed by permethylation produced a single compound with a molecular mass of 1684 Da as determined by fast atom bombardment-mass spectrometry (Fig. 3). This result indicated that the acetyl and carbamoyl (28) groups had been lost during the chemical process and that all the hydroxyl and amide groups had been methylated.

The partially methylated monosaccharides were reduced with $NaBH_4$ and peracetylated. The partially methylated alditol acetates were then analyzed by GC-MS. Four compounds were identified: 1,5-di-*O*-acetyl-3,4,6-tri-*O*-methyl-*N*-acetyl-*N*-methylglucosaminitol derived from the glucosamine residue at the non-reducing end of the molecules; 1,4,5-tri-*O*-acetyl-3,6-di-*O*-methyl-*N*-acetyl-*N*-methylglucosaminitol arising from the three internal residues; 4,6-di-*O*-acetyl-1,3,5-tri-*O*-methyl-*N*-acetyl-*N*-methylglucosaminitol produced from the reducing glucosaminyl residue; and 1,5-di-*O*-acetyl 2,3,4-tri-*O*-methylfucositol provided by the L-fucose residue. Therefore, this study confirmed the (1→4) glycosidic linkages between the five *N*-acetyl-D-glucosamine residues of the NodRe factors and indicated a (1→6) glycosidic linkage between the L-fucose residue and the reducing *N*-acetyl-D-glucosamine.

^1H NMR Analysis—The two-dimensional proton homonuclear spectrum of NodRe factors was used both to locate the acetyl group and to indicate the anomeric configuration of the sugar linkages. The anomeric proton of the L-fucose residue showed a doublet at 5.05 ppm with a coupling constant of 3.7 Hz with the H-2 proton of the cycle. These data were consistent with an α-linked L-fucose (12, 29). From this anomeric proton, a complete assignment of the L-fucosyl protons was obtained on the two-dimensional spectrum (Fig. 4). The cross-peak observed from H-1 enabled the location of the H-2 signal at 3.80 ppm. This proton showed a scalar connection with H-3 at 3.58 ppm ($J_{H\text{-}2\text{–}H\text{-}3}$ = 9 Hz). Connectivity was also observed through H-3 and H-4 protons ($J_{H\text{-}3\text{–}H\text{-}4}$ = 3.9 Hz), thereby locating the

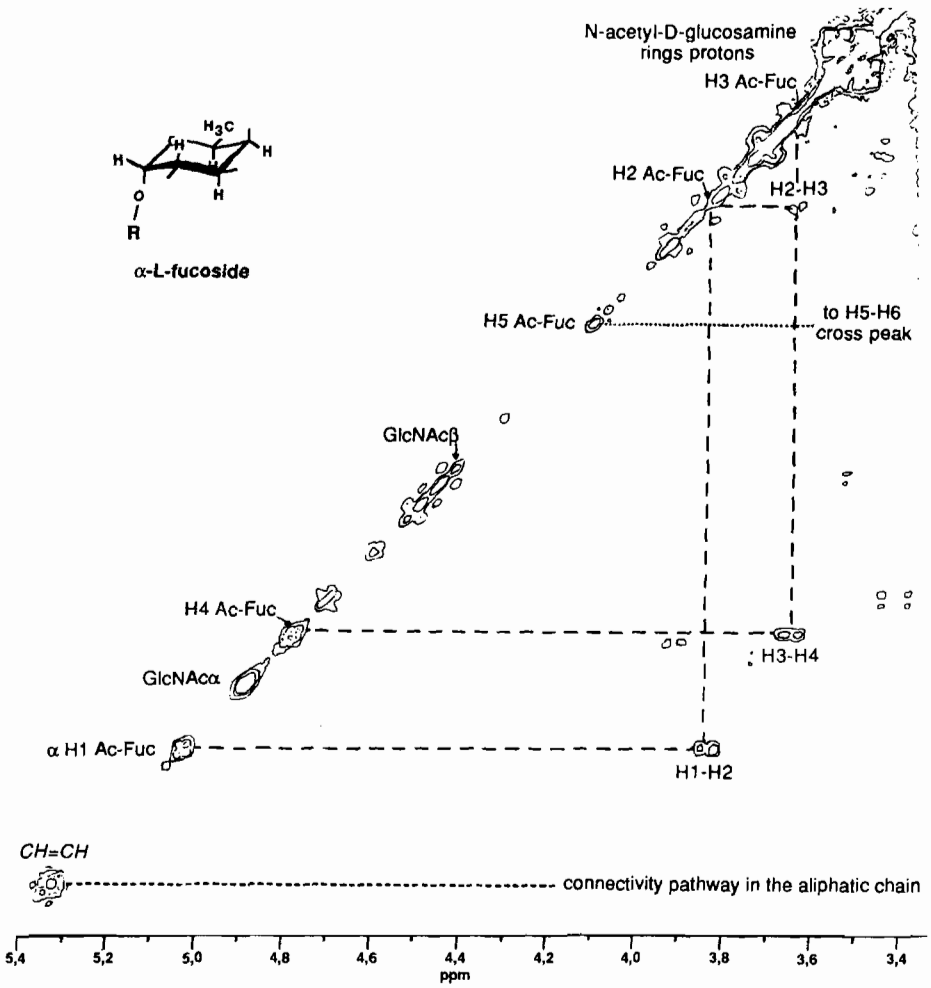

FIG. 4. **Two-dimensional homonuclear (^{1}H-^{1}H) NMR spectrum of the NodRe factors.**

resonance of H-4 at 4.75 ppm. This downfield signal (more than 1 ppm over the expected value for a non-substituted fucose residue) (12) enabled the acetate group to be located on the O-4 of the α-L-fucose residue. Because of the low coupling constants $J_{H\text{-}4\text{-}H\text{-}5}$ in the L-fucose residue (0.8–0.9 Hz) (29), the resulting low amplitude connectivity peak could not be seen on the spectrum (12, 30). The three 6-deoxymethyl protons ($\delta_{H\text{-}6}$ = 0.95 ppm) correlated with the H-5 at 4.05 ppm.

For the D-glucosamine residues, α ($J_{H\text{-}1\text{-}H\text{-}2}$ = 3.5 Hz at the reducing end) and β ($J_{H\text{-}1\text{-}H\text{-}2}$ = 9 Hz) anomeric configurations were characterized. Other signals, classically described for the NMR spectra of nodulation factors, were also identified (upfield signals not shown).

DISCUSSION

The wild type strain (CFN 42) of *R. etli* produced enough Nod factors to enable structural studies without the need of amplifying the Nod factors production by genetic engineering. The structural characteristics of the *R. etli* nodulation factors are as follows: (i) they have a chitopentameric core; (ii) a 4-*O*-acetyl-α-L-fucose residue is linked to carbon 6 of the reducing *N*-acetyl-D-glucosaminyl unit; (iii) the glucosaminyl residue at the non-reducing end of the molecules is *N*-methyl-*N*-vaccenoyl substituted; and (iv) one-quarter to one-third of these symbiotic signals is *O*-carbamoylated at their non-reducing end. According to the nomenclature proposed by Roche and co-workers (31), we name these factors NodRe-V (Me, Ac-Fuc) and NodRe-V (Carb, Me, Ac-Fuc), respectively (Fig. 5*A*).

The structure of NodRe factors represents a new variation on the Nod factors structure. L-Fucose, as a substituent of chito-

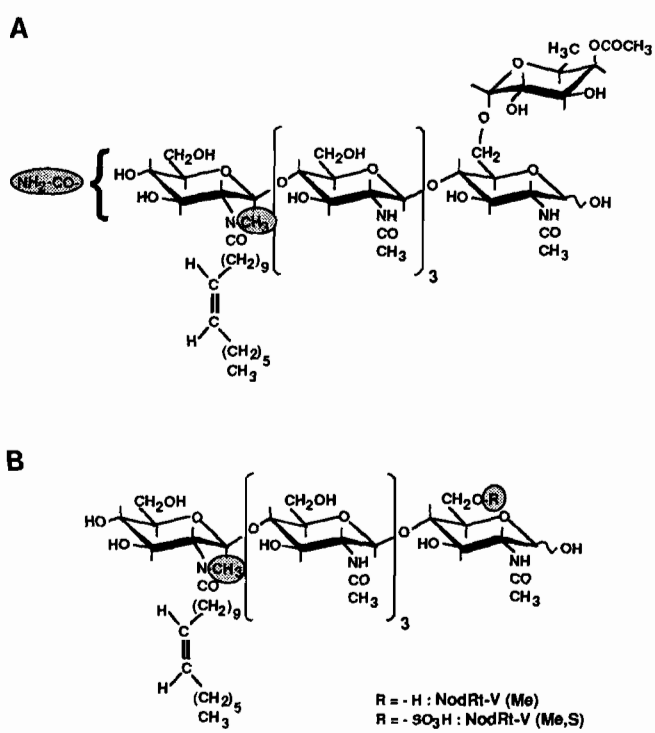

FIG. 5. *A*, structural features of the NodRe-V (Me, Ac-Fuc) and NodRe-V (Carb, Me, Ac-Fuc) factors. *B*, structure of the NodRt-V (Me, S) and NodRt-V (Me) factors.

oligomers, has been previously found as a minor component of Nod factors from *Rhizobium fredii* (8) and *Bradyrhizobium elkanii* (16). However, none of these structures bears an additional acetyl group on L-fucose. An L-fucose ring with an acetyl group on O-4 has been found in *Rhizobium* NGR234, but an *O*-methyl group was also present on fucosyl O-2 (19). Carbamoyl and *N*-methyl groups on the non-reducing end are more common because they have been found in *Rhizobium* NGR234 (19), *Azorhizobium caulinodans* (15), *Bradyrhizobium japonicum* (17), and *B. elkanii* (16). The precise location of the carbamoyl group needs a sufficient amount of material to perform heteronuclear ^{1}H-^{13}C bidimensional NMR. When we tried to cultivate *R. etli* on a larger scale (20 liters) to purify enough NodRe factors for this analysis, we found that the proportion of carbamoylated molecules was lower than in the first experiments, and thus the position of the carbamoyl group could not be reliably assigned.

Common bean is also nodulated by *R. tropici*, a bacterium with a broader host range than *R. etli*. When comparing Nod factors from both bacteria, no common substituents can be found as NodRt factors are non-fucosylated, non-carbamoylated molecules (20). Instead, part of them possesses a sulfate group on O-6 of the reducing *N*-acetyl-D-glucosamine. It has been found that these sulfated molecules are able to elicit root nodule organogenesis on bean at 10^{-5} to 10^{-8} M (14).

Purified Nod factors from *R. etli* were also found to elicit root nodules on bean in the same concentration range as sulfate-containing NodRt factors.[2] When cultivating *R. etli* using bean extracts as *nod* gene inducers, no change in the NodRe factors pattern was found. In particular, no NodRe factors with a sulfate group could be detected by sodium [^{35}S]sulfate labeling.

It thus appears that bean is able to be nodulated by two rhizobial species that seem to produce Nod factors with structurally different substitutions.

However, purified fractions of Nod factors from either *R. tropici* or *R. etli* seemed to be active on bean at concentrations higher than those used to induce nodules on alfalfa by NodRm factors, for example. It remains possible that some sulfated components, being under the detection level, still contaminate the fucosylated fractions from NodRe factors, that fucosylated molecules are present in *R. tropici*, or that very low proportions of another common component are present in all tested fractions.

With reversed-phase HPLC, sulfated factors of *R. tropici* and acetylfucosylated factors of *R. etli* have very different retention volumes. Thus cross-contaminations with an identical component seemed unlikely. However, because reversed-phase HPLC separations are very sensitive to the hydrophilic/hydrophobic balance of the analytes, it cannot be excluded that a minor structural variation having a weak effect on the activity (such as the acyl chain length) may induce a dramatic effect on the HPLC retention volumes and thus permit the elution of analogs of cross-contaminants in the different fractions.

We are currently reinvestigating both strains to search for impurities that could be a common signal for bean nodulation.

[2] E. Martinez-Romero, personal communication.

Acknowledgments—We thank M. A. Rogel for technical assistance and M. Bon and M. Vedrenne for recording NMR spectra. We are grateful to J. Cullimore for helpful reviewing of the manuscript.

REFERENCES

1. Long, S. R. (1989) *Cell* **56,** 203–214
2. Dénarié, J., and Cullimore, J. (1993) *Cell* **74,** 951–954
3. Peters, N. K., Frost, J. W., and Long, S. R. (1986) *Science* **233,** 977–980
4. Fisher, R. F., and Long, S. R. (1992) *Nature* **357,** 655–660
5. Göttfert, M. (1993) *FEMS Microbiol. Rev.* **104,** 39–64
6. Lerouge, P. (1994) *Glycobiology* **4,** 127–134
7. Relic, B., Talmont, F., Kopcinska, J., Golinowski, W., Promé, J.-C., and Broughton, W. J. (1994) *Mol. Plant-Microbe Interact.* **6,** 764–774
8. Bec-Ferté, M.-P., Krishnan, H. B., Promé, D., Savagnac, A., Pueppke, S. G., and Promé, J.-C. (1994) *Biochemistry* **33,** 11782–11788
9. Ehrhardt, D. W., Atkinson, E. M., and Long, S. R. (1992) *Science* **256,** 998-1000
10. Nap, J.-P., and Bisseling, T. (1990) *Science* **250,** 948–954
11. Spaink, H. P., Sheeley, D. M., van Brussel, A. A. N., Glushka, J., York, W. S., Tak, T., Geiger, O., Kennedy, E. P., Reinhold, V. N., and Lugtenberg, B. J. J. (1991) *Nature* **354,** 125–130
12. Carlson, R. W., Sanjuan, J., Bhat, U. R., Glushka, J., Spaink, H. P., Wijfjes, A. H. M., van Brussel, A. A. N., Stokkermans, T. J. W., Peters, N. K., and Stacey, G. (1993) *J. Biol. Chem.* **268,** 18372–18381
13. Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Promé, J.-C., and Dénarié, J. (1991) *Nature* **351,** 670–673
14. Martinez, E., Poupot, R., Promé, J.-C., Pardo, M. A., Segovia, L., Truchet, G., and Dénarié, J. (1993) in *New Horizons in Nitrogen Fixation* (Palacios, R., Mora, J., and Newton, W. E., eds) pp. 171–175, Kluwer Academic Publishers, Dordrecht, The Netherlands
15. Mergaert, P., Van Montagu, M., Promé, J.-C., and Holsters, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 1551–1555
16. Stokkermans, T. J. W., and Peters, N. K. (1994) *Planta (Heidelb.)* **193,** 413–420
17. Sanjuan, J., Carlson, R. W., Spaink, H. P., Bhat, U. R., Barbour, W. M., Glushka, J., and Stacey, G. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 8789–8793
18. Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J.-C., and Dénarié, J. (1990) *Nature* **344,** 781–784
19. Price, N. P. J., Relic, B., Talmont, F., Lewin, A., Promé, D., Pueppke, S. G., Maillet, F., Dénarié, J., Promé, J.-C., and Broughton, W. J. (1992) *Mol. Microbiol.* **6,** 3575–3584
20. Poupot, R., Martinez-Romero, E., and Promé, J.-C. (1993) *Biochemistry* **32,** 10430–10435
21. Segovia, L., Young, J. P. W., and Martinez-Romero, E. (1993) *Int. J. Syst. Bacteriol.* **43,** 374–377
22. Martinez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A., Graham, P., and Pardo, M. A. (1991) *Int. J. Syst. Bacteriol.* **41,** 417–426
23. Hungria, M., Joseph, C. M., and Phillips, D. A. (1991) *Plant Physiol.* **97,** 751–758
24. Hungria, M., Joseph, C. M., and Phillips, D. A. (1991) *Plant Physiol.* **97,** 759–764
25. Promé, J.-C., Aurelle, H., Couderc, F., and Savagnac, A. (1987) *Rapid Commun. Mass Spectrom.* **1,** 50–52
26. Ciucanu, I., and Kerek, F. (1984) *Carbohydr. Res.* **131,** 209–217
27. Gerwig, G. J., Kamerling, J. P., and Vliegenthart, J. F. G. (1979) *Carbohydr. Res.* **77,** 1–7
28. Isono, K., and Suzuki, S. (1968) *Tetrahedron Lett.* **9,** 1133–1137
29. Altona, C., and Haasnoot, C. A. G. (1980) *Org. Magn. Reson.* **13,** 417–429
30. Yu, R. K., Koerner, T. A. W., Scarsdale, J. N., and Prestegard, J. H. (1986) *Chem. Phys. Lipids* **42,** 27–48
31. Roche, P., Debellé, F., Maillet, F., Lerouge, P., Faucher, C., Truchet, G., Dénarié, J., and Promé, J.-C. (1991) *Cell* **67,** 1131–1143