Research Note

***Rhizobium* sp. BR816 Produces a Complex Mixture of Known and Novel Lipochitooligosaccharide Molecules**

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Rhizobial lipochitooligosaccharide (LCO) signal molecules induce various plant responses, leading to nodule development. We report here the LCO structures of the broad-host range strain *Rhizobium* sp. BR816. The LCOs produced are all pentamers, carrying common C_{18:1} or C_{18:0} fatty acyl chains, *N*-methylated and C-6 carbamoylated on the nonreducing terminal *N*-acetylglucosamine and sulfated on the reducing/terminal residue. A second acetyl group can be present on the penultimate *N*-acetylglucosamine from the nonreducing terminus. Two novel characteristics were observed: the reducing/terminal residue can be a glucosaminitol (open structure) and the degree of acetylation of this glucosaminitol or of the reducing residue can vary.

Additional keywords: host specificity, Nod factors, nodulation.

The establishment of a nitrogen-fixing symbiosis between rhizobia and legumes involves specific signals to be exchanged by both partners. Particular combinations of symbionts result in the formation of bacteroid-containing nitrogen fixing nodules (Mylona et al. 1995). For successful recognition, rhizobia need to synthesize the appropriate lipochitooligosaccharides (LCOs) in response to specific plant-secreted flavonoids. The LCOs, also known as Nod factors (NFs), are β -1,4-linked trimers, tetramers, or pentamers of *N*-acetyl-D-glucosamine (GlcNAc) that are *N*-acylated on the nonreducing terminal residue and carry various substitutions on reducing and nonreducing terminal glucosamine residues (Mergaert et al. 1997). In recent years, several variations on this theme have been reported. Two novel LCO structures were discovered in *Mesorhizobium loti*; more specifically, a dimer of GlcNAc units and a NF structure bearing a fucose residue α -1,3 linked to the *N*-acetylglucosamine residue proximal to the nonreducing terminus (Olsthoorn et al. 1998). Also, Yang et al. (1999) described that the LCOs produced by two strains of *Rhizobium galegae* can be 3-acetylated on the glucosamine residue next to the nonreducing terminus. The specific substituents of the NF backbone have been shown to be important

determinants of host specificity (Long 1996). Transconjugant *Rhizobium* spp. strains producing a changed set of NFs can extend their host range to certain plants (Krishnan et al. 1992). Moreover, substituted NFs can be protected against degradation by plant chitinases (Stachelin et al. 1994). It seems likely that the NF side chains modulate nodulation efficiency in a host-specific and cultivar-dependent manner (Fernández-López et al. 1998; Laeremans et al. 1999).

Rhizobium sp. BR816, an isolate from *Leucaena leucocephala*, is able to nodulate a wide range of tropical leguminous plants, including *Phaseolus vulgaris* (Hernández-Lucas et al. 1995). The elucidation of different host-specific genetic determinants of *Rhizobium* sp. BR816 (Laeremans et al. 1998; Van Rhijn et al. 1993; Van Rhijn et al. 1994; Vlassak et al. 1998) prompted us to determine the chemical structures of its NFs. In addition to the common LCO structures described to date (Perret et al. 2000), we detected a number of novel LCO molecules that can possess a glucosaminitol instead of a glucosamine as the reducing terminal residue. In this study, we report on the analysis of structurally different LCO molecules of *Rhizobium* sp. BR816.

LCOs were purified from 6-liter cultures of *Rhizobium* sp. BR816. Cells were grown overnight in 60 ml of minimal medium (10 g of sodium glutamate, 0.22 g of K₂HPO₄, 0.1 g of MgSO₄, 0.1 g of NaCl, 20 mg of FeCl₃, and 0.5 mg of biotin per liter of water) at 30°C and added to 6 liters of fresh medium. After induction with 500 nM apigenin, cells were grown for 16 h. Filtered culture supernatants were mixed with Amberlite XAD-4 (Sigma Chemical, St. Louis, MO, U.S.A.). Adsorbed components were eluted from the resin with 100% methanol and then with 80% aqueous methanol. These fractions were pooled, dried, dissolved in water, and extracted with 1-butanol. The resulting organic fraction was dried, redissolved in water, and washed with ethyl acetate. Finally, the aqueous fraction first was purified through an open C₁₈ reversed-phase column (1.5 × 5 cm, Lichroprep, RP-18; Merck, Darmstadt, Germany). Elution was performed consecutively with water, 50% aqueous acetonitrile, and pure acetonitrile. The water–acetonitrile fraction (1:1 vol/vol) was concentrated and subjected to high-performance liquid chromatography (HPLC) on a semipreparative C-18 reversed-phase HPLC column (7.5 × 250 mm, Waters Spherisorb, ODS2, 5 μ m; Supelco, Lisle d'Abeau, France) with a linear gradient of water–

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acetonitrile (80:20 vol/vol) to pure acetonitrile for 50 min at a flow rate of 2 ml per min. The HPLC profile of the NF extract showed two major peaks eluting with 52 (fraction 1) and 56% acetonitrile in water (fraction 2), respectively, followed by an unresolved bulk eluting 62 to 80% acetonitrile (Fig. 1). By comparison with the HPLC profiles of the purified extracts from the noninduced versus the induced wild-type strain BR816 and the induced *nodA* mutant strain, which no longer produce LCOs (K. Vlassak and E. Luyten, *personal communication*; data not shown), we analyzed fractions 1 and 2. It cannot be excluded, however, that LCOs also are present in the bulk. Fractions 1 and 2 were analyzed by liquid secondary ion mass spectrometry (LSIMS) in the positive ion mode. The fractions are mixtures because only a crude fractionation of LCOs was obtained with HPLC. Both fractions exhibited complex patterns in the molecular ion region (1,300 to 1,550 mass units). In order to suppress the signals, which were a result of salt adducts (Na^+ and K^+), both fractions were submitted to negative ion mode analysis (Fig. 2). The spectra showed four groups of NF signals. The four $[\text{M}-\text{H}]^-$ distributions are separated by 42 mass units and centered around ions at m/z 1,393, 1,435, 1,477, and 1,519. Each group is composed of ions for three different compounds that are separated from each other by two mass units. The 42 mass-unit increments might indicate increasing numbers of acetyl substituents, whereas the two mass-unit increments might be a result of LCOs bearing diunsaturated, monounsaturated, or saturated fatty acids on the nonreducing residue or the reduction of the carbohydrate. Therefore, we proceeded with the reduction of the possible fatty acid double bonds by catalytic hydrogenation. It was demonstrated by subsequent LSIMS analysis (negative ion mode; data not shown) that, on the basis of the comparison of the intensities of the different MH^+ ions, only half of the MH^+ ions of each group exhibited a two mass-unit increment, which means that approximately 50% of the detected LCOs bear an unsaturated $\text{C}_{18:1}$ instead of a $\text{C}_{18:0}$ fatty acyl chain. From this comparative analysis, it was postulated that the remaining two mass-unit increment was a result of the reduction of the carbohydrate. The positive ion mode mass spectrum clearly showed B1 ions at m/z 483 and 485 (Fig. 2). These odd values indicate the presence of one supplementary nitrogen on the glucosamine residue, which is consistent with a carbamoyl substitution. Furthermore, the B1 values indicate the presence of a C_{19} *N*-acyl substituent (low probability) or an *N*-methyl with a C_{18} *N*-acyl group.

To confirm the latter possibility, we performed mass spectrometry-mass spectrometry (MS-MS) studies of the B1 ion at m/z 485 (Fig. 3). First, we demonstrated the presence of an *N*-methyl group by analyzing the helium CID spectrum of the m/z 485 ion because the ratio (m/z 84:98) is less than one (Treilhou et al. 2000), by which the presence of a C_{18} -acyl substitution and a methyl group on the same nitrogen was attested. To demonstrate the presence and localization of the carbamoyl group, we recorded the metastable MS-MS spectrum of the same B1 ion. The abundant loss of water compared with the small amount of the combined loss of water and carbamic acid clearly indicated that the carbamoyl substituent is located at position 6 of the nonreducing end (Fig. 3) (Treilhou et al. 2000). Therefore, the B1 ion at m/z 485 is assigned as corresponding to a carbamoylated, *N*-methylated glucosamine carrying a $\text{C}_{18:0}$ saturated fatty acyl chain. Con-

sistent with the data described above (two mass-unit increments), the B1 m/z 483 ion corresponds to the analogous compound bearing the $\text{C}_{18:1}$ acyl chain. The FAB^+ mass spectrum also contains ions arising from the systematic loss of 80 mass units from the corresponding MH^+ ions, which is characteristic of the presence of a sulfate group.

Subsequently, we further investigated the varying degrees of acetylation and the remaining increment of two mass units, thus recording the metastable B/E spectrum of the different $[\text{M}+\text{H}]^+$ ions. As presented in Figure 4 for MH^+ m/z 1,439

Fig. 1. High-performance liquid chromatography profile of lipochitooligosaccharide extracts from apigenin-induced cultures of *Rhizobium* sp. BR816. A semipreparative Spherisorb RP18 column (Supelco, Lisle d'Abeau, France) was used with a 20 to 100% aqueous acetonitrile gradient (flow rate: 2 ml per min). Absorbance of the eluate was monitored at 206 nm.

(monoacetylated NF), the MS–MS spectrum exhibits two series of fragmentations. As shown in the first pattern, after the loss of 80 mass units resulting from the loss of the sulfate group (m/z 1,359), a difference of 223 ($221 + 2$) mass units compared with the following B4 ion (m/z 1,136) was detected. This mass difference corresponds to a reducing-terminal *N*-acetylglucosaminitol rather than *N*-acetylglucosamine. The following two B-type ions are separated by 203 mass units corresponding to *N*-acetylglucosamine subunits, and a 245 ($203 + 42$) mass-unit difference between B1 and B2 indicates that the *N*-acetylglucosamine residue sugar next to the nonreducing end bears an acetyl residue. The second and more abundant pattern starts (after loss of sulfate) with a deficit of 265 mass units ($221 + 2 + 42$), resulting in the B4 ion at m/z 1,094 and demonstrating acetylation of the *N*-acetylglucosaminitol that is located at the reducing terminus instead of on the penultimate sugar. All of the following B-type ions are separated by 203 mass units (unsubstituted *N*-acetyl glucosamine). Both series demonstrate that these NFs are pentameric LCOs. Similar analysis of the MH^+ ion at m/z 1,481 shows a mass difference of 265 or 307 units ($265 + 42$) between $[MH-SO_3]^+$ and the B4 ion (data not shown). In this case, a second supplementary acetate is located on the reducing terminal *N*-acetylglucosaminitol. Unfortunately, the compound with MH^+ at m/z 1,523 is present in too small of an amount to obtain a useful MS–MS spectrum. Therefore, we were unable to de-

termine the precise position of the third acetate. According to the preceding observations, two of the acetates are probably located on the reducing terminal *N*-acetylglucosaminitol, whereas the third could be located on the penultimate monosaccharide residue from the nonreducing terminus. Another possibility is the presence of the three acetyl groups on the reducing terminal residue. In addition, it cannot be excluded from the obtained mass spectra (low detection level) that one acetyl group is located on the reducing terminus, one on the penultimate *N*-acetylglucosamine proximal to the nonreducing terminus, and one on the nonreducing terminus, respectively.

To ensure that both B4 ions (m/z 1,136 and 1,094) observed in the MS–MS spectrum (Fig. 4) were derived from the same MH^+ ion at m/z 1,439, we performed a parent ion scan in the B^2/E mode (data not shown). In this way, it could be confirmed that one of the parent ions of the B4 ion at m/z 1,094 is indeed the MH^+ at m/z 1,439 (with $[MH-SO_3]^+$ at 1,359), whereas the other is the ion with MH^+ at m/z 1,395 (with $[MH-SO_3]^+$ at m/z 1,315) (Fig. 5). Simultaneously it was shown that the B4 ion at m/z 1,136 has the same parent, MH^+ at m/z 1,439 (with $[MH-SO_3]^+$ at m/z 1,359), in addition to the parent MH^+ at m/z 1,481 (with $[MH-SO_3]^+$ at m/z 1,401). The other molecular species were examined in a similar way (data not shown). The structures of the detected LCOs produced by *Rhizobium* sp. BR816 are summarized in Table 1.

Fig. 2. Liquid secondary ion mass spectrometry (LSIMS) spectrum of high-performance liquid chromatography fraction 1 in the FAB^+ mode. Insert shows the simplified molecular domain $(M-H)^-$ in the FAB^- mode. All spectra were recorded on an Autospec 6F Instrument (Micromass, Manchester, U.K.) fitted with an LSIMS source and a cesium ion gun. Acceleration voltage was set to 8 kV; cesium gun to 28 kV. Samples (1 μ l) dissolved in methanol–water (1:1 vol/vol) were deposited on the LSIMS target with the appropriate matrix (glycerol–metanitrobenzyl alcohol, 1:1 vol/vol). For the positive mode, the target was acidified with 0.5 μ l of 10% trichloroacetic acid. For the negative mode, alkalization was performed with 0.5 μ l of diisopropylmethyl amine. Mass spectrometry–mass spectrometry spectra were recorded in the B/E constant scanning mode.

To determine the presence of the *N*-acetylglucosaminitol (reduced *N*-acetylglucosamine), we hydrolyzed fraction 1 with trifluoroacetic acid. Ten micrograms of fraction 1 was placed in a screw-cap Teflon microvial, followed by the addition of 20 μ l of 2M trifluoroacetic acid (TFA). After heating for 2 h at 120°C, the reagent was evaporated under a gentle nitrogen flux. To remove residual TFA, drops of isopropylalcohol were added and evaporated. In the next step, 20 μ l of pyridine and 20 μ l of acetic anhydride were added to the dried vial and heated for 20 min at 70°C. The solvents were again evaporated under a gentle nitrogen flux. Residual pyridine was totally removed by adding drops of toluol and subsequent evaporation. The peracetylated sample was dissolved in 0.1 ml of ethyl acetate, and 1 μ l of the solution was injected onto the gas chromatograph. We performed a gas chromatography (GC)–MS analysis in the electron-impact and chemical-ionization mode of the peracetylated released sugars (with acetic anhydride in pyridine). GC–MS was carried out on the Autospec 6F Instrument, coupled to a Hewlett Packard HP 5890 II gas chromatograph with an optima-5-ms column (25 μ m, 30 m $\times$ 0.25 mm; Macherey-Nagel, Düren, Germany), with helium as the carrier gas, set to 50 kPa. The temperature program used was 80°C (2 min), 80 to 180°C (10°C per min), 180 to 280°C (3°C per min), and 280°C (5 min). The *N*-acetylglucosaminitol present in this fraction can maximally represent only one-fifth of the released monosaccharides, which is a minority in comparison to the *N*-acetylglucosamines in the total ion chromatogram (TIC). Nevertheless,

we obtained the characteristic fragmentation pattern and retention times on chromatograms with the use of both ionization modes compared with the reference glucosaminitol compound (data not shown).

The broad-host range strain *Rhizobium* sp. BR816 is a tropical bacterium, which is closely related genetically to *Sinorhizobium meliloti*, *Sinorhizobium fredii*, and *Rhizobium* sp.

Table 1. Summary of the lipochitooligosaccharide structures produced by *Rhizobium* sp. BR816^a

MH ⁺	Nomenclature
1393	V(C18:1, NMe, Carb, S)
1395	V(C18:0, NMe, Carb, S); V-GlcOHNAc(C18:1, NMe, Carb, S)
1397	V-GlcOHNAc(C18:0, NMe, Carb, S)
1435	V(C18:1, NMe, Carb, Ac, S)
1437	V(C18:0, NMe, Carb, Ac, S); V-GlcOHNAc(C18:1, NMe, Carb, Ac, S)
1439	V-GlcOHNAc(C18:0, NMe, Carb, Ac, S)
1477	V(C18:1, NMe, Carb, Ac ₂ , S)
1479	V(C18:0, NMe, Carb, Ac ₂ , S); V-GlcOHNAc(C18:1, NMe, Carb, Ac ₂ , S)
1481	V-GlcOHNAc(C18:0, NMe, Carb, Ac ₂ , S)
1519	V(C18:1, NMe, Carb, Ac ₃ , S)
1521	V(C18:0, NMe, Carb, Ac ₃ , S); V-GlcOHNAc(C18:1, NMe, Carb, Ac ₃ , S)
1523	V-GlcOHNAc(C18:0, NMe, Carb, Ac ₃ , S)

^a Nod-factor nomenclature is as previously described (Roche et al. 1991). Ac, acetate; Carb, carbamoyl; NMe, *N*-methyl; S, sulfate.

Fig. 3. Liquid secondary ion mass spectrometry B/E mass spectrometry–mass spectrometry spectrum of B1 ion $m/z = 485$. Left panel : He CID spectrum showing m/z 97 > 84. Right panel: metastable spectrum showing water and water + carbamic acid losses, allowing the location of the carbamoyl group on position 6.

NGR234 (Hernández-Lucas et al. 1995). In this paper, the structures of LCOs produced by BR816 were determined. All LCOs are pentamers, which are *N*-acylated with vaccenic ($C_{18:1}$) or stearic acid ($C_{18:0}$) and *N*-methylated and C-6 carbamoylated on the nonreducing terminal *N*-acetylglucosamine. All LCO molecules are sulfated on the reducing terminal residue. Interestingly, some LCO molecules produced by BR816 have an *N*-acetylglucosaminitol as the reducing terminal residue. Moreover, the degree of acetylation of the NFs can vary. An additional acetylation can be present on the penultimate residue from the nonreducing terminus, and one to three supplementary acetyl decorations were on the reducing residue. The exact position of the acetyl group on the penultimate *N*-acetylglucosamine could not be determined. Recently, 3-*O* fucosylation or 3-*O* acetylation of the same glucosamine residue in *M. loti* and *R. galegae* LCOs, respectively, were reported (Olsthoff et al. 1998; Yang et al. 1999). Classification of rhizobia on the basis of the correlation between NF structural diversity and the type of host plant (determinate versus indeterminate nodules) revealed that NFs from rhizobia isolated from determinate nodules (especially tropical legumes) are mostly pentamers with household fatty acids and *N*-methyl and carbamoyl substitutions on the nonreducing residue, bearing glycosyl groups. In contrast, NFs produced by rhizobia isolated from indeterminate nodules (mostly temperate legumes) are generally *N*-acetyl glucosamine tetramers and contain specific polyunsaturated fatty acyl chains. Acetyl

and sulfate substitutions were present in both groups (Mergaert et al. 1997; Spaink 1996). The BR816 NF family fits in the former group. Phylogenetically, members of both groups are positioned distantly (Martínez-Romero et al. 2000). Lortet et al. (1996) classified a large number of rhizobial strains isolated from *Acacia* and *Sesbania* spp. nodules on the basis of NF thin-layer chromatography (TLC) profiles. Whatever their taxonomic position, isolated strains from the same plant genus exhibit similar TLC profiles, although clearly different for

Fig. 5. Relationship between fragment ions and their respective precursor ions.

Fig. 4. Liquid secondary ion mass spectrometry metastable B/E mass spectrometry-mass spectrometry spectrum of m/z 1,439 showing presence of *N*-acetylglucosaminitol, major acetylation on the *N*-acetylglucosaminitol, and minor acetylation on the sugar next to the nonreducing residue.

Acacia and *Sesbania* spp. isolates. LCOs from BR816 are very similar to those produced by the *Acacia* spp. nodulating strains *Sinorhizobium teranga* bv. *acacia* and *Rhizobium loti* cluster U, isolated from *Acacia* spp. nodules in Senegal (Lorquin et al. 1997). The LCOs produced by the latter strains are pentamers with household fatty acids, *N*-methylated and carbamoylated on the nonreducing residue and sulfated on the reducing terminal residue. Moreover, the TLC pattern of *Rhizobium* sp. BR816 LCOs (Laeremans et al. 1998) shows a remarkable similarity with those from certain strains belonging to cluster U (Lortet et al. 1996). As far as we know, these African *Acacia* spp. isolates successfully interact with *Prosopis*, *Leucaena*, and *Acacia* spp. (Lortet et al. 1996). This could imply that *Rhizobium* sp. BR816, which has a broader host range, possibly possesses genetic determinants typical for host plants other than *Prosopis*, *Leucaena*, and *Acacia* spp.

Different regulatory and structural nodulation genes of *Rhizobium* sp. BR816 already were identified and subsequently characterized. BR816 contains four *nodD* genes, which differ in their interaction with flavonoids (Van Rhijn et al. 1994). In addition to the regulatory *nodD* genes, research has been carried out on the elucidation of the function of some structural *nod* genes. BR816 possesses one copy of *nodSU* and *nodH* and two copies of *nodPQ* encoding proteins involved in methylation, carbamoylation, and sulfation of the Nod factor backbone, respectively (Laeremans et al. 1998; Van Rhijn 1994; C. Snoeck, unpublished results). Although the exact location of the sulfate was not determined, the presence of a functional NodH sulfotransferase suggest 6-O sulfation of the reducing terminal residue (Roche et al. 1991; C. Snoeck, unpublished results). In BR816, one *nodFE* copy was characterized (Van Rhijn 1994). The *nodFE* genes probably determine the nature of the acyl chain linked to the core molecule (Spaink et al. 1991), although no polyunsaturated fatty acids were detected in the LCOs of BR816. It already has been shown that *nodE* mutations of *Rhizobium leguminosarum* bv. *trifolii* and *S. meliloti* could not be complemented by the *nodE* gene of BR816 (Van Rhijn 1994). Genes encoding putative acetyltransferases involved in the numerous acetylations of the LCOs have not yet been identified but are currently under investigation. It is possible that two different enzymes are involved in the acetylation of the penultimate monosaccharide from the nonreducing residue and the reducing terminal residue because enzymes involved in acetylation of the nonreducing terminus (NodL), the reducing-terminal residue (NodX), or the glycosyl group on the reducing residue (NolL) all belong to different families (Corvera et al. 1999). Acetylation of the *N*-acetylglucosamine next to the nonreducing residue was first reported recently on the Nod factors of two *R. galegae* strains of different geographical origins. It was hypothesized that it is involved in determining the particular host range of this species (Yang et al. 1999). Otherwise, it could be suggested that these numerous acetylations contribute not only to host-range broadening but might provide protection against degradation (as is the case for sulfation) by a particular host plant enzyme (e.g., chitinase), as shown previously (Schultze et al. 1998; Staehelin et al. 1994).

The biological activity of the different LCOs produced by BR816 has not yet been examined. Referring to the novel compound with an *N*-acetylglucosaminitol as the reducing terminal residue, it will be of particular interest to investigate

whether this type of molecule has the capacity to extend the host range. Preliminary studies revealed a similarity between a particular sequence in the nodulation region of BR816 and an *Escherichia coli* aldo-ketoreductase (GenBank accession no. U29581). This might indicate a possible gene encoding an enzyme for the reduction of the reducing *N*-acetylglucosamine, resulting in the formation of an *N*-acetylglucosaminitol. The elucidation of the chemical structures of the LCOs of BR816 will allow us to further analyze the function of known nodulation genes in BR816 and to search for new genes in *Rhizobium* spp. signaling.

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