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Rhizobium rubi^T: A Gram-Negative Phytopathogenic Bacterium Expressing the Lewis B Epitope on the Outer Core of its Lipooligosaccharide Fraction

Valentina Gargiulo,[a] Domenico Garozzo,[b] Rosa Lanzetta,[a] Antonio Molinaro,[a] Luisa Sturiale,[b] Cristina De Castro,*[a] and Michelangelo Parrilli[a]

The structure of the core oligosaccharide from the phytopathogenic bacterium Rhizobium rubi *was deduced by combining information from complementary chemical approaches (alkaline and acid hydrolysis), similar to the "overlap peptide" strategy. This structure is new and it contains two main oligosaccharide backbones that differ in the substitution degree of the external Kdo unit. The relevant feature shared by both oligosaccharides is the presence of a tetrasaccharide motif that is similar to the blood group Lewis B antigen (Le^B). This epitope differs from Le^B in the glycosidic configuration of the glucosamine unit (α and not β) and in the occurrence of acetyls substituents at O3 and/or O4 of the galactose moiety. Other notable structural features are the location of the Dha residue, the presence of a α-glucose unit that is linked to the inner Kdo unit, the high number of acid sugars and the highly branched core structure.*

Introduction

Rhizobium rubi is a Gram-negative, capsulated, soil-borne bacterium that was previously named *Agrobacterium rubi*, but recently reclassified.[1]

It displays phytopathogenic activity and causes tumours on the fruiting canes of *Rubus* plants like raspberries and blackberries; the galls develop as whitish eruptions that later turn brown and black and begin to disintegrate, and their presence causes the production of dry, seedy berries and prevents the formation of new canes.

Its pathogenesis mechanism is similar to that described for *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*),[1] which is responsible for the crown gall disease. In fact, *R. rubi* has the same characteristic tumour-inducing plasmid *Ti* that harbours the genes required for tumourogenesis. The tumour formation is due to the ability of the pathogen to transfer the T-DNA, a part of the plasmid *Ti*, into the nuclear genome of the host cells, an event that is conditioned from the recognition and absorption of the bacterium on the host. This last event is crucial because it conditions the successive steps, and it is mediated by the polysaccharide components at the membrane surface,[2] which makes their characterisation of importance to the understanding of this interaction.

Previous studies have determined the structure of one of the membrane components produced from *R. rubi*, a capsular polysaccharide that is composed entirely from 6-deoxy-talose, with an O-acetylation pattern that is modulated during the growth stadium of the organism.[3]

In the present paper, the chemical characterisation of the other glycidic membrane constituent, the lipooligosaccharide (LOS), is achieved by the combined used of chemical and spectroscopic techniques.

Results and Discussion

LOS isolation and chemical composition data

Freeze-dried bacterial cells were extracted with a hot phenol/water mixture,[4] LOS was found principally in the water phase and it was composed of L-fucose (Fuc), D-galacturonic acid (GalA), D-glucuronic acid (GlcA), D-galactose (Gal), D-glucose (Glc), 2-amino-2-deoxy-D-glucosamine (GlcN), 3-deoxy-*manno*-oct-2-ulosonic acid (Kdo) and 3-deoxy-*lyxo*-2-heptulosaric acid (Dha).

Methylation analysis led to the identification of terminal fucose, terminal Dha, terminal and 4- and 3-substituted GalA residues, 4-substituted GlcA, 4,6-substituted and 3-linked Glc units, 2-substituted Gal, 3,4-substituted GlcN, 4,5-linked and 7-substituted Kdo and a small amount of terminal Kdo.

The analysis of the lipid fraction showed the presence of C14:0 3-OH, C16:0 3-OH, C18:1 3-OH, and C28:0 27-OH, in agreement with that reported for other phytopathogenic *Rhizobiaceae*.[5]

NMR spectroscopic analysis of core oligosaccharide

The primary structure of the entire core oligosaccharide, **1** (Figure 1), was deduced by combining the NMR data of the

[a] *Dr. V. Gargiulo, Prof. R. Lanzetta, Prof. A. Molinaro, Dr. C. De Castro, Prof. M. Parrilli*
Department of Organic Chemistry and Biochemistry
University of Napoli "Federico II"
Via Cintia 4, 80126 Napoli (Italy)
Fax: (+39) 81674393
E-mail: decastro@unina.it

[b] *Dr. D. Garozzo, Dr. L. Sturiale*
Istituto di Chimica e Tecnologia dei polimeri, CNR
Viale R. Margherita 6, 95123 Catania (Italy)

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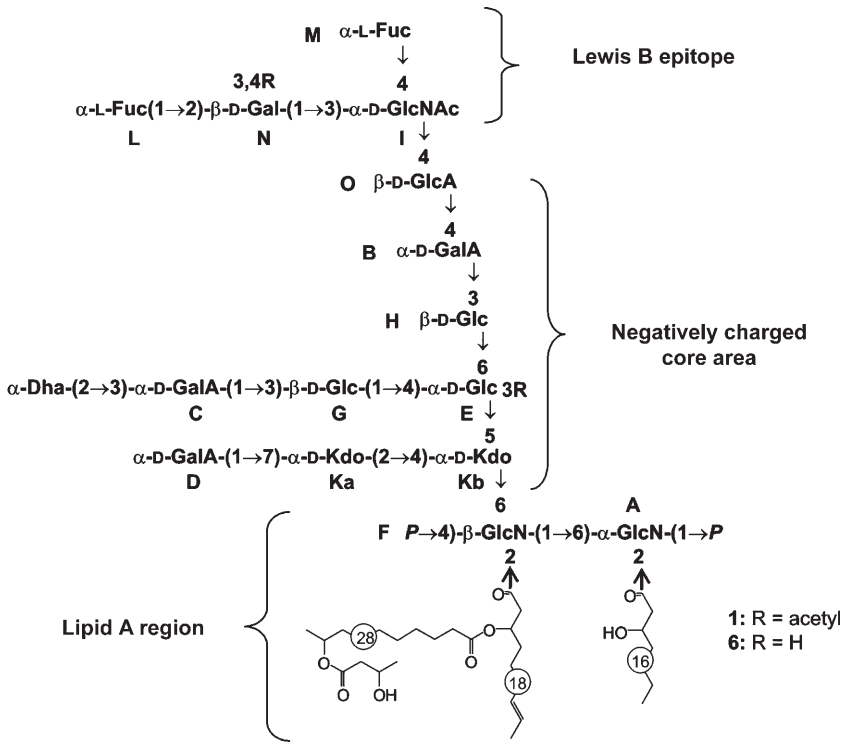

Figure 1. 1: *R. rubi*[T] complete LOS structure (primary fatty acids of the lipid A moiety are not included); **6**: LOS structure after aqueous ammonia treatment.

products that were isolated by alkaline degradation and acid hydrolysis. The first approach provided an incomplete core oligosaccharide, and information regarding the sequence of the fragment that was lost during the alkaline treatment was recovered by analysing the product from the mild acid hydrolysis.

Core oligosaccharides from strong alkaline hydrolysis

Purification of the products from the strong alkaline degradation provided two truncated core oligosaccharides (**2** and **3**, Figure 2) that included the lipid A sugar backbone and were capped with an uronic acid derivative that bore an α,β-unsaturated carboxyl group.

The α,β-unsaturated residue resulted from a β-elimination process that is effective only for those uronic acids that are substituted at O4. Products **2** and **3** differed only in the sugar unit, which was visible in the low-field region of the ^{1}H NMR spectrum; in fact product **2** (Figures 3A and B) contained eight anomeric signals whereas oligosaccharide **3** (Figure 3C) displayed only seven anomeric protons. In the high-field region of the spectrum of both products, three sets of diastereotopic methylene signals were present due to two Kdo and one Dha residue (Figure not shown).

The resonances of the eleven residues of **2** were assigned by comparing the product at both neutral and alkaline pH (Figures 3A and B, Tables S1 and S2). By exploiting the proton chemical shift sensitivity to the solution pH, analysis of the spectra that were acquired under both conditions avoided any ambiguous assignment. The spectra that were recorded at neutral pH were studied first and the anomeric protons were sequentially labelled with a capital letter (**A**–**H**, Figure 3A) in order of decreasing chemical shift; the three sets of methylene signals are indicated as **Ka**, **Kb** and **Dha**.

Residue **A** was the GlcN-I of the lipid A backbone, in fact its H2 (3.44 ppm) correlated with a nitrogen-bearing carbon (55.9 ppm), the anomeric position was phosphorylated ($^3J_{H1,P}$ = 8.1 Hz), and the anomeric carbon configuration was α on the basis of its chemical shift (91.9 ppm) and of its $^3J_{H1,H2}$ of 3.3 Hz. In addition, the low-field displacement of the C6 resonance indicated that this unit was glycosylated at O6.[6]

Similarly, residue **F** was GlcN-II of lipid A (C2 at 56.8 ppm and phosphorylated at O4), the $^3J_{H1,H2}$ value of 8.6 Hz proved the β configuration, and the glycosylation shift of 3.5 ppm at its C6 was diagnostic of a ketose unit at that position, namely the internal Kdo (**Kb**).

The spin system of **B** contained only four protons; the last in the sequence occurred at 5.80 ppm and belonged to the β carbon (108.5 ppm) of an α,β-unsaturated carboxyl system. **B** was the Hex-4-enuronic residue that resulted from the β-elimination process promoted from the lipid-removal procedure.

The residues **C** and **D** were identified as galacturonic residues because of their COSY H3/H4 and H4/

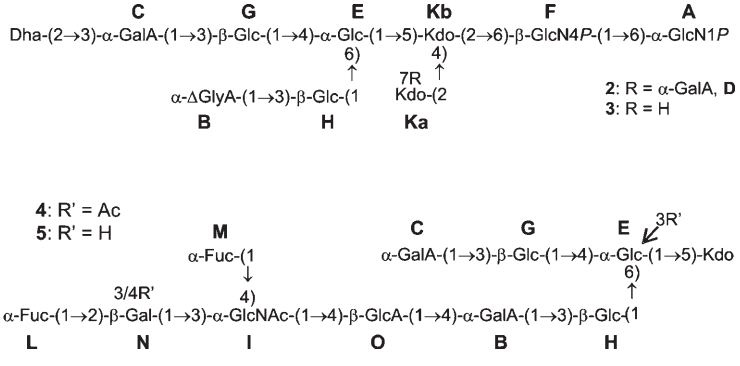

Figure 2. Structure of oligosaccharides that are derived from delipidation of *Rhizobium rubi*[T] LOS: **2** and **3**, oligosaccharides from strong alkaline de-*O*-acylation; **4** oligosaccharide from acid hydrolysis, **5** was obtained from **4** after mild de-*O*-acylation. The Kdo and Dha residues are α configured. ΔGlyA is a hex-4-enuronic residue that resulted from the β-elimination process of a galacturonic acid and was produced during the lipid-removal procedure.

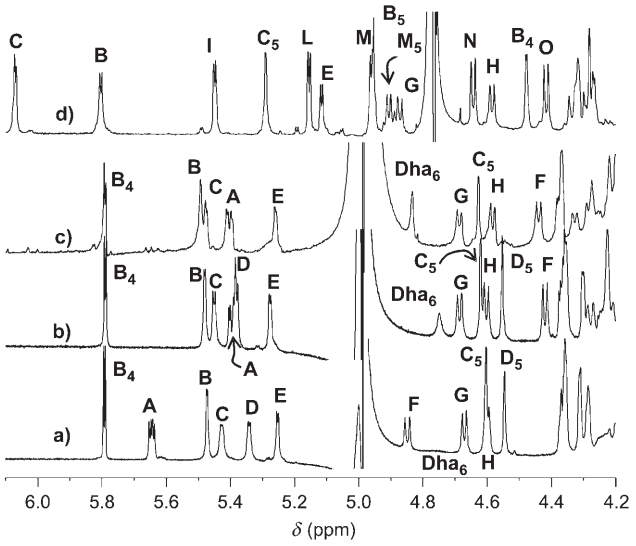

Figure 3. Anomeric area expansion of ^{1}H NMR spectra at 600 MHz of oligosaccharides that are derived from *R. rubi*^{T} LOS, signals are labelled according to the notation used in Figure 1: a) product **2** from strong alkaline de-*O*-acylation (283 K, neutral pH, D_2O), b) product **2** (283 K, 10 mM NaOD in D_2O), c) product **3** from strong alkaline de-*O*-acylation (283 K, 10 mM NaOD in D_2O), it differs from **2** for the lack of **D** residue; d) oligosaccharide **5** from LOS acid hydrolysis (303 K, neutral pH, D_2O).

H5 correlation magnitude and because of their long-range correlation that related H5 to a carbonyl signal at 177.3 ppm. The ^{13}C chemical shift of C1 (100.5 ppm for **C** and 101.4 ppm for **D**) and the coupling constant $^{3}J_{H1,H2}$ values for both **C** and **D** (3.2 Hz) indicated their α configuration, and analysis of their carbon chemical shifts classified **D** as a terminal unit and **C** as a 3-linked GalA unit. In this last case, the small glycosylation shift that was found for the C3 of **C** (74.2 ppm) suggested that this residue was glycosylated at its O3 by a ketose sugar.

Residue **E** was an α-configured glucose unit (C1 at 100.5 ppm and $^{3}J_{H1,H2}=3.2$ Hz), signal attribution was hindered by the overlapping of H3 and H4 resonances and by the absence of the scalar coupling between H5 and both H6a and H6b. These last two protons were identified through the NOE contact with H5. The exact position of H4 was assigned by its COSY correlation with H5, and H3 was assigned by its COSY correlation with H2. Finally, the C4 and C6 chemical shifts were shifted with respect to the standard values[6] so that **E** resulted in a 4,6-linked Glc unit.

G and **H**, were both classified as β-configured glucose units (anomeric carbons at 103.5 ppm and 103.4 ppm, respectively, $^{3}J_{H1,H2}=7.9$ Hz in both cases), their C3 was displaced at low field due to glycosylation.

The assignment of **Ka**, **Kb** and **Dha** was made by starting from the diastereotopic methylene protons, whose chemical shift was also diagnostic of their α-anomeric configuration.[7]

For both **Ka** and **Kb**, protons from H3 to H5 were easily identified by using scalar connectivities, whereas H6 was deduced by exploiting the NOE with both H4 and H5. The assignment of H7 and both H8a and H8b was made by analysing the COSY spectrum by starting from the H6 signal. **Ka** was identified as an O7-substituted unit and its location at O4 of **Kb** was inferred from the NOE between its H6 with $H3_{eq}$ of **Kb**.[8]

In the case of **Dha**, proton H6 was poorly identifiable in the spectrum that was recorded at neutral pH because its signal was very broad; its position was deduced from the TOCSY and ROESY (Figure 4) spectra, and no correlation in the HSQC spectrum was present. The **Dha** analysis problem was solved by analysing the spectrum at alkaline pH (Table S2, Figure 3B), in this case, the H6 signal appeared as a broad singlet and it correlated to C6 and exhibited the expected long-range correlation with a carbonyl at 177.0 ppm. The carbon chemical shift values were diagnostic of its terminal location.[7]

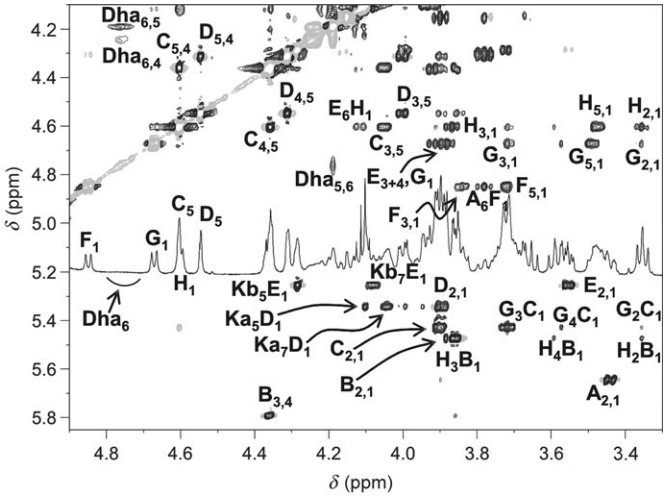

Figure 4. Expansion of ROESY spectrum of **2**: relevant NOEs are indicated together with the position of the H6 proton of the Dha residue (600 MHz, 283 K, neutral pH, D_2O).

The monosaccharide sequence was deduced from the following NOE contacts (Figure 4): H1 of **B** with H3 of **H**, H1 of **H** with H6 of **E**, H1 of **E** with both H5 and H7 of **Kb**, which in turn was linked to O6 of residue **F** of the lipid A, H1 of **C** with H3 of **G**, H1 of **G** with both H3 and H4 of **E**, and H1 of **D** with both H7 and H5 of **Ka**. The **Dha** position was deduced from the NOE between H6 and H3 of **C**; this was supported by the small glycosylation effect at C3 of this last residue.[6]

Oligosaccharide **3** differed from **2** in the absence of the α-GalA unit **D** (Figure 2C, Table S3) on the external Kdo residue (**Ka**), which is terminal.

Core oligosaccharide from mild acid hydrolysis

The product from the mild acid hydrolysis, **4** (Figure 2) lacked the lipid A moiety, the Dha and the external Kdo residues, but conserved the oligosaccharide fragment at the uronic acid **B** together with the acetyl substituents. The structure of the de-*O*-acetylated derivative **5** (Figure 2) was elucidated first.

The proton spectrum of **5** (Figure 3d, Table S4) presented ten anomeric signals. Residues **B**, **C**, **E**, **G**, **H** and the **Kdo** at the reducing end were expected on the basis of the product **2** structure, whereas the others (**I**–**O**) belonged to the previously

lost fragment; importantly, **B** was present in its native form, namely as an α-galacturonic residue.

The new residues were assigned by using the same strategy as was reported for oligosaccharide **2. I** was a α-GlcN unit that was substituted at both O3 and O4, **L** and **M** were two terminal α-fucose units, **N** was an O2-linked β-Gal unit, and **O** was a β-GlcA unit linked at O4.

The following NOE effects elucidated the sequence: H1 of **L** with H2 of **N**, H1 of **N** with H3 of **I**, H1 of **M** with H4 of **I**, H1 of **I** with H4 of **O** and H1 of **O** with H4 of **B**. Finally, the nonstoichiometric O-acetyl substituents were clarified by analysing the 2D spectra (data not reported) of product **4**. They were located on **N** and **E**; the β-Gal unit **N** was either acetylated at O3 or at O4, likewise residue **E** was incompletely acetylated at position O3. The possibility that an irregular distribution of the acetyl groups could be induced from the acid hydrolysis was discarded. Actually this protocol is routinely applied for the LOS (or lipopolysaccharide), and the different products that are obtained always display the intact original pattern of acetyl groups.[9,10]

MALDI analysis of ammonia-treated LOS

MALDI analysis of the intact LOS gave inconclusive results, but the data that were collected from the ammonia-treated LOS (sample **6**) were consistent with the NMR spectroscopic information for compounds **2**, **3**, **5** and with the ammonia-treated lipid A structure from the related *R. radiobacter* C58.[5]

The negative-ion MALDI mass spectrum in Figure 5 shows a main series of peaks between 3300 and 4300 Th, which result from the molecular ions of the partially O-deacylated LOS that still retains the amide and the acyloxyacylamide moieties. In addition, in the mass range below 2600 Th, further peaks originated from the *in source* cleavage of the glycosydic linkage between the internal Kdo (**Kb** unit) and the lipid A distal GlcN residue.

This well-known fragmentation pattern[11] allowed us to assign the molecular masses of the components that belong to the O-deacylated LOS species. The peak at *m/z* 1542.6 corresponded to the Y-type ion (Domon and Costello nomenclature)[12] of a bis-phosphorylated, triacylated lipid A moiety that carries a 16:0 (3-OH) and a 18:1 (3-OH) in an amide linkage; the 18:1(3-OH) is, in turn, esterified with a 28:0 (27-OH) and one 3-hydroxy-butyroyl group. The second peak, which differed by 26 Th (*m/z* 1516.9) was consistent with the same species, but carrying a 16:0 (3-OH) instead of a 18:1 (3-OH). A further intense ion fragment at *m/z* 2491.9 (B-type ion) matched the *core* oligosaccharide that was delineated through NMR spectroscopic and compositional analyses.

Minor peaks were assigned to species with a different sugar composition, this last depended on the nonstoichiometric presence of a terminal hexuronic acid ($\Delta m/z \sim 176$ Th), or to the *in source* cleavage of the Dha residue ($\Delta m/z \sim 204$ Th). The above-described structural variety of LOS substructures was reflected even more strongly in the high mass spectrum region, where a plethora of pseudomolecular ions $[M-H]^-$, which were due to either the number and/or composition of lipid A fatty acids and to the *core* heterogeneity were found. The base peak at *m/z* 4035.4 was consistent with the intact molecular ion $\mathbf{M_1}$ which components were the highly branched *core* oligosaccharide at *m/z* 2941.9 and the triacyl lipid A at *m/z* 1542.6. The more significant molecular ions that were attributed to the LOSs mixture are listed in Table S5 together with the corresponding composition.

Conclusions

Rhizobium rubi^T produces a lipooligosaccharide with a rather complex structure, the predominant species, **1** (Figure 1), is composed by sixteen residues and can be divided into two parts, an inner and an outer one. The inner part is proximal to and comprehensive of the lipid A moiety, it possesses a strong ionic character due to the presence of two phosphates and seven acidic monosaccharides: two Kdo units, four uronic acids and the bis-carboxylic Dha residue. This anionic character is consistent with the functional role that is generally attributed to this molecule; according to this model, doubly charged cations such as Ca^{2+} or Mg^{2+} bridge different LOS units and lead to the mechanical stabilisation of the bacterial outer membrane and to the creation of an effective permeability barrier towards external stress factors.[13]

In contrast with the functional role of the internal part, the outer core usually is considered to be of importance for the interaction of the bacterium with the plant cell wall.

The structure of this moiety is rather lipophilic (nonstoichiometric O-acetyl groups and two fucose residues) and contains

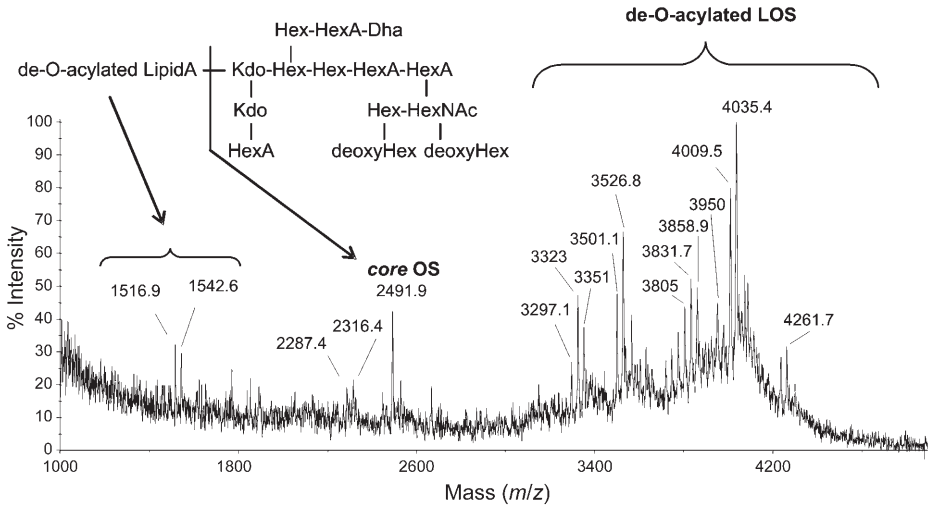

Figure 5. MALDI spectrum of *R. rubi*^{T} LOS **6** (Figure 1) de-*O*-acylated with aqueous ammonia solution.

structural motifs of biological relevance. Actually, the disaccharide Fuc–Gal (**L** and **N** residues) is widespread in plants as well, where it constitutes the terminal part of the xyloglucan oligosaccharides that reside in the ventral and lateral area of the guard cell wall.[14]

This disaccharide is considered to be a signalling molecule that is able to initiate physiological functions such as laminarase activity in *Rubus* cells,[15] and the existence of a high-affinity receptor for this moiety has been demonstrated.[16]

In this context, it seems that *R. rubi* presents an epitope for which the appropriate plant receptors exist and might promote the adhesion of the bacterium on the plant cell wall.

Further speculation is possible by considering the last four sugar residues of the outer core; actually, they define a structure that is similar to the blood group epitope Lewis B. This motif is reported for the first time in a plant pathogenic bacterium, and it differs in the anomeric configuration (α and not β) of the glucosamine unit **I** and in the presence of nonstoichiometric acetyl decorations at the galactose moiety **N**.

The occurrence of blood group antigens in human pathogenic bacteria is known (*Neisseria*,[17] *Helicobacter*,[18] *Campylobacter*[18] and *Haemophilus*[17]), and the advantages gained by the bacteria are clear: they become invisible to the host immune system and can better colonise the host tissue by using the dedicated receptors.[19]

In contrast to human pathogenic bacteria, the importance of the Lewis B epitope for *R. rubi* is less clear, but it suggests the presence of a complementary receptor on the plant cell wall. This hypothesis is probable because the existence of a lectin that is specific for Lewis B determinant has already been reported from *Griffonia simplicifolia* seeds.[20]

A more tempting speculation, which is raised from a parallel with the human pathogenic bacteria, suggests that *R. rubi* might mime those epitopes that are already present in the plant cell wall. This possibility is acceptable in the case of the Fuc-Gal disaccharide, but it is not supported in the case of the Le^B antigen, although it must be underscored that plants already display another similar human epitope, Lewis A, which differs from B in that it lacks the fucose unit **L**.[21]

In addition to the above considerations, a fruitful implication of this discovery is the use of this bacterium for biotechnological purposes: it is a "safe-to-handle" organism (biosafety level 1) that is attractive for its enzymatic machinery that is dedicated to the synthesis of the Lewis B epitope.

Experimental Section

***Rhizobium rubi*^{T}, bacterial cultivation and LOS isolation**: *R. rubi* strain DMS 6772 (type strain) was grown at 28 °C in liquid shake culture (200 rpm) in Nutrient Broth (Fluka) for 48 h. Cells were collected by centrifugation at 7000 rpm for 15 min at 4 °C, washed with distilled water and freeze-dried (yield 180 $mg_{cells}\,L_{culture}^{-1}$).

Dried cells were extracted according to the phenol–water method.[4] Both phases were separately dialyzed against distilled water, freeze-dried and screened by discontinuous SDS-PAGE[20] by using a 12% gel on a miniprotean gel system from Bio-Rad (Hercules, CA, USA); the samples were run at constant voltage (150 V) and stained according to the procedure of Kittelberger and Hilbink.[23]

LOS was present principally in the water phase; it was present only in traces in the phenol phase.

General and analytical methods: Monosaccharides were analysed as acetylated O-methyl glycoside derivates and lipids as methyl esters, as reported elsewhere.[24] The absolute configuration was determined by analysis of the chiral 2-octyl[25] or 2-butyl[26] derivatives.

Methylation analysis was modified for the detection of the acidic residues, namely, LOS was first carboxy-methylated with methanolic 0.1 M HCl (5 min), and then with diazomethane. The product was methylated with CH_3I and powdered NaOH in DMSO,[27] then hydrolysed with 2 M trifluoroacetic acid (TFA; 100 °C, 1 h). The carbonyl was then reduced with $NaBD_4$ (4 °C, 18 h) and the carboxyl group was methylated as before (with methanolic 0.1 M HCl and then diazomethane), reduced with $NaBD_4$ (4 °C, 18 h), acetylated and analysed by GC–MS. This approach led to the assignment of the terminal Kdo and the Dha, the rest of the residues of the LOS were detected by repeating the whole procedure but by adopting stronger hydrolysis conditions (4 M TFA, 100 °C, 4 h).

GC–MS analysis conditions for the acetylated *O*-methyl glycoside derivates, lipids and the chiral alcohol derivates were the same and were performed with on a Agilent 5973 instrument by using a SPB-5 capillary column (Supelco, Bellefonte, PA, USA; 30 m × 0.25 i.d. flow rate 0.8 mL min^{-1}, He as carrier gas, temperature program: 150 °C for 3 min, 150 → 300 °C at 10 °C min^{-1}, 300° 12 min, ionisation energy of 70 eV and an ionising current of 0.2 mA).

The analysis of the partially methylated alditol acetates was performed on the same GC–MS instrument by using a different temperature program: 150 °C for 3 min, 150 → 240 °C at 2 °C min^{-1}, 240 → 300 °C at 10 °C min^{-1}, 300° 5 min.

Isolation of oligosaccharides 2–5: LOS (150 mg) was treated with 1 M hydrazine in THF (3 mL, 30 min at 37 °C) to yield to the de-*O*-acylated LOS. The solution was cooled, poured into ice-cold acetone (50 mL), and the de-*O*-acylated LOS (LOS_{OH}) was collected by centrifugation, washed twice with ice-cold acetone, dried, dissolved in distilled water and precipitated by the addition of ice-cold acetone.

The LOS_{OH} (43 mg) was treated with 4 M KOH (2 mL) for 16 h at 120 °C to de-*N*-acylate the substrate. After neutralisation, the sample was desalted by SEC chromatography (Sephadex G-10, Pharmacia, 1.5 × 120 cm, eluent H_2O, flow rate 16 mL min^{-1}). The resulting oligosaccharide mixture (12 mg) was fractionated by analytical HPAEC on a Carbopac PA-100 column (4.6 × 250 mm) by using a flow rate of 1 mL min^{-1} and a gradient of 1 M NaOAc (40 → 65% over 90 min and a final regeneration 65 → 90% in 5 min) in 0.1 M NaOH. Analytical runs were monitored by using a pulse amperometric detector (Dionex, Sunnyvale, CA, USA). Two principal species were isolated: oligosaccharide **2** (RT: 56 min, 2 mg) and **3** (RT: 52 min, 0.5 mg).

Another portion of the water phase (10 mg) was hydrolysed in aq. 1% AcOH (100 °C, 1 h), the precipitate (lipid A) was removed by centrifugation and the supernatant was lyophilised and fractionated by GPC on a TSK HW-40 column. The oligosaccharide **5** was obtained by the de-*O*-acetylation (14% aq. ammonia, 37 °C, 1 h) of the first fraction of TSK-40 (product **4**, 3 mg).

NMR spectroscopy: ^1H and ^1H,^{13}C NMR experiments on all the products were carried out on a Bruker DRX-600 spectrometer that

was equipped with a cryogenic probe. Spectra were calibrated with respect to internal acetone ($\delta_h = 2.225$ ppm; $\delta_C = 31.45$ ppm). ^{31}P NMR and with $^{1}H,^{31}P$ HSQC sequence were measured for oligosaccharide **2** at 162 MHz on a Bruker DRX 400 MHz spectrometer that was equipped with a reverse broadband probe with z-gradients; 85% H_3PO_4 was used as external standard for calibration.

For all the homonuclear spectra, experiments were measured with data sets of 2048×512 points, and 32 scans were acquired, a mixing time of 200 and 120 ms was employed for ROESY and TOCSY, respectively. Each data matrix was zero-filled in both dimensions to give a matrix of 4096×2048 points and the resolution was enhanced in both dimensions by a shifted sine-bell function before Fourier transformation.

The HSQC experiment was measured by using a data set of 2048×512 points whereas for the HMBC experiment it was 2048×256; 64 scans were acquired for each t_1 value, and the HMBC sequence was optimised for a 6 Hz long-range coupling constant. In all the heteronuclear experiments the data matrix was extended to 2048×1024 points by forward linear prediction extrapolation.[28] All NMR spectra were acquired and transformed with Topspin 1.3 program, and studied with Pronto software.[29]

For products **2** and **3**, spectra were recorded at alkaline pH, which was made by adding 4 M NaOD (5 μL) to the sample solution (500 μL), to give a final concentration of 10 mM, no pH measurement was performed.

MALDI mass spectrometry: The negative-ion MALDI mass spectrum of intact *O*-deacylated LOS was acquired in linear mode on a Voyager STR instrument (Applied Biosystems, Framingham, MA, USA). Ions that were extracted by the pulsed laser beam (nitrogen $\lambda = 337$) were accelerated through 24 kV. Each spectrum is the result of 256 laser shots. Sample preparation was performed according with the *thin-layer* procedure[30] by using a homogeneous film of 2,4,6- trihydroxyacetophenone (THAP, 200 mg mL^{-1} in methanol) and nitrocellulose (Trans-blot membrane, BioRad, 15 mg mL^{-1} in acetone/propan-2-ol, 1:1 *v/v*) mixed in a 4:1 (*v/v*) ratio as a matrix. The sample was first suspended in a mixture of methanol/water (1:1) that contained 5 mM ethylenediaminetetraacetic acid (EDTA), then converted into the ammonium form with cation-exchange beads (Dowex 50WX8–200, Sigma–Aldrich) and finally deposited (~0.3 μL), together with the same volume of 20 mM dibasic ammonium citrate, on the matrix film that was already spotted on the MALDI plate.

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Keywords: Dha · Lewis B · lipooligosaccharide · *Rhizobium rubi*

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