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Biological Activity of (Lipo)Polysaccharides of the Exopolysaccharide-Deficient Mutant Rt120 Derived from *Rhizobium leguminosarum* bv. trifolii Strain TA1*

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Abstract—Lipopolysaccharides (LPS) from *Rhizobium leguminosarum* biovar trifolii TA1 (RtTA1) and its mutant Rt120 in the *pssB–pssA* intergenic region as well as degraded polysaccharides (DPS) derived from the LPS were elucidated in terms of their chemical composition and biological activities. The polysaccharide portions were examined by methylation analysis, MALDI-TOF mass spectrometry, and ^{1}H NMR spectroscopy. A high molecular mass carbohydrate fraction obtained from Rt120 DPS by Sephadex G-50 gel chromatography was composed mainly of L-rhamnose, 6-deoxy-L-talose, D-galactose, and D-galacturonic acid, whereas that from RtTA1 DPS contained L-fucose, 2-acetamido-2,6-dideoxy-D-glucose, D-galacturonic acid, 3-deoxy-3-methylaminofucose, D-glucose, D-glucuronic acid, and heptose. Relative intensities of the major ^{1}H NMR signals for O-acetyl and N-acetyl groups were 1 : 0.8 and 1 : 1.24 in DPS of Rt120 and RtTA1, respectively. The intact mutant LPS exhibited a twice higher lethal toxicity than the wild type LPS. A higher *in vivo* production of TNFα and IL-6 after induction of mice with Rt120 LPS correlated with the toxicity, although the mutant LPS induced the secretion of IL-1β and IFNγ more weakly than RtTA1 LPS. A polysaccharide obtained by gel chromatography on Bio-Gel P-4 of the high molecular mass material from Rt120 had a toxic effect on tumor HeLa cells but was inactive against the normal human skin fibroblast cell line. The polysaccharide from RtTA1 was inactive against either cell line. The potent inhibitory effect of the mutant DPS on tumor HeLa cells seems to be related with the differences in sugar composition.

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Key words: lipopolysaccharide, *Rhizobium leguminosarum*, 3-deoxy-3-methylaminofucose, lethal toxicity, TNFα

Lipopolysaccharides (LPS, endotoxins) are a family of glycolipids found in the outer membrane of Gram-negative bacteria, generally possessing potent immunomodulating and immunostimulating properties. LPS of various bacteria are composed of three distinct regions, including the outermost saccharide moiety divided into an O-specific polysaccharide (O-chain, O-antigen) and a core oligosaccharide covalently linked to the lipid A moiety, which anchors the molecule in the outer membrane. Each of the domains has different structural and biological properties [1, 2].

Abbreviations: 6dHex, 6-deoxyhexose; 2,3-di-Me-Glc, 2,3-di-O-methylglucose; DPS, degraded polysaccharide(s); 6dTal, 6-deoxy-L-talose; GalA, galacturonic acid; GC, gas-liquid chromatography; GlcA, glucuronic acid; Hep, heptose; Hex, hexose; HexA, hexuronic acid; HSF, human skin fibroblast; IC_{50}, 50% inhibitory concentration; IFNγ, interferon γ; IL-1β, interleukin-1β; IL-6, interleukin-6; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LD_{50}, 50% lethal dose; LPS, lipopolysaccharide(s); MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; 3-Me-6dTal, 6-deoxy-3-O-methyl-L-talose; 2-Me-Fuc, 2-O-methylfucose; 3-NMe-Fuc, 3-deoxy-3-methylaminofucose; PMAA, partially methylated alditol acetates; QuiNAc, 2-acetamido-2,6-dideoxy-D-glucose (N-acetylquinovosamine); Rha, rhamnose; TLR4, toll-like receptor 4; TNFα, tumor necrosis factor α.

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LPS can induce activation of macrophages, which results in the production of bioactive lipids, reactive oxygen species, and peptide mediators such as tumor necrosis factor α (TNFα), and interleukins: IL-1, IL-6, IL-8, and IL-10. Lipid A is the immunomodulatory center of the endotoxin, which is recognized during animal and human infection by different classes of receptors, including toll-like receptors. The endotoxicity of LPS is modulated by the core and O-chain [3]. Mild acid hydrolysis of LPS released polysaccharides, which contain the O-chain together with the core constituents, including 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), and posses an intrinsic immunopotentiation activity [2, 4].

Most information about the biological role of LPS comes from studies of enteric bacteria. Studies of endotoxins from bacteria of a different phylogenetic origin, e.g. *Rhizobium*, could explain the role of LPS in nature. The structures of LPS from *R. leguminosarum* bv. trifolii [5, 6], *R. leguminosarum* bv. viciae [7], and a closely related species *R. etli* have been defined [8]. Both lipid A and core regions of these bacteria are structurally different from those of enteric bacteria. The most variable LPS domain is the O-specific polysaccharide [9]. In *R. leguminosarum* and *R. etli*, a Kdo residue occurs at the reducing end of the O-chain [10].

LPS from *R. etli*, *R. leguminosarum*, and *Rhizobium* sp. Sin-1 as well *Salmonella enterica* serovar Typhimurium stimulated the production of an inducible LPS receptor, CD14, in bone marrow cells of normal mice. *Salmonella enterica* sv. Typhimurium LPS failed to induce CD14 production in bone marrow cells from mutant mouse cell lines that were defective in the toll-like receptor 4 gene (*tlr4*). By contrast, the rhizobial LPS still stimulated CD14 production in the mutant cells, suggesting that in mice the rhizobial LPS may be acting via an alternative mechanism that does not involve TLR4 [11].

Several studies have indicated that composition or size of the O-antigen might be a reliable indicator of virulence potential. In general, O-antigen modifications seem to play an important role at several stages of the infection process, including the colonization (adherence) step and the ability to bypass or overcome host defense mechanisms. In addition to the observed variability of LPS in bacterial pathogens, the structure of LPS is also altered in bacterial symbionts in response to signals from the plant during symbiosis. This appears to be part of molecular communication between the bacterium and the host plant [12]. The unusual array of carbohydrate structures of rhizobial LPS confer resistance to plant defense mechanisms and may serve as signals that trigger the plant to allow the infection to proceed [7].

Studies on mutants lacking the O-antigen or producing a truncated O-chain suggest that an intact O-antigen is not essential for bacterial penetration of the cell walls of the root hair but is required to sustain the growth of the infection thread and effective colonization of nodule cells. It seems that poor immunogenicity of LPS is a prerequisite for effective nodulation of *R. leguminosarum* species [5]. A prevalence of 6-deoxy sugars not only in *Rhizobium* but also in phytopathogenic species of *Agrobacterium* does not appear to correlate with virulence. Interestingly, although a high content of rhamnose in LPS is shared by *Agrobacterium* and other phytopathogenic bacteria, a high content of fucose and 6-deoxytalose is much less common [13].

Many fungal and bacterial polysaccharides are useful antitumor agents, the activity of which is a consequence of stimulation of cell-mediated immune response [14]. Among them neutral glucans, such as lentinan from *Lentinus edodes* with predominantly β-(1,3) and β-(1,6) linkages, demonstrated anticarcinogenic effects in both animals and humans [15].

Our previous studies demonstrated that mutant Rt120 of *R. leguminosarum* bv. trifolii TA1 (RtTA1) with Tn5 insertion in the *pssB–pssA* intergenic region was completely deficient in exopolysaccharide biosynthesis and had an altered sugar composition of the O-antigen [16]. In the present study, chemical composition of degraded polysaccharides (DPS) released from LPS of mutant Rt120 and wild type RtTA1 by mild acid hydrolysis was analyzed. The lethal toxicity and cytokine production ability of LPS from Rt120 and RtTA1 were compared. The cytotoxic activity of O-chain fractions on human normal (HSF, human skin fibroblast) and tumor HeLa cell lines was determined. The relationship between the biological activity and chemical composition of LPS is discussed.

MATERIALS AND METHODS

Bacterial strains and cultivation. *Rhizobium leguminosarum* bv. trifolii wild type strain RtTA1 and its mutant derivative Rt120 with a Tn*5* insertion in the *pssB–pssA* intergenic region have been described earlier [16]. The strains were cultured in liquid medium (yeast extract–mannitol) on a gyratory shaker (160 rpm) at 28°C. The culture of Rt120 was supplemented with kanamycin (40 μg/ml).

Isolation of LPS and Tricine SDS-PAGE. LPS were isolated by the hot phenol–water procedure and purified by affinity chromatography on Polymyxin-agarose (Sigma, USA) as described [17]. The purified LPS preparations were analyzed by Tricine SDS-PAGE and visualized by silver staining as described [18].

Degradation of LPS and fractionation of DPS. LPS from each strain was hydrolyzed with aqueous 1% AcOH (10 mg/ml) at 100°C for 2.5 h. The resulting lipid A precipitate was removed by centrifugation (15,000 rpm, 20 min), washed three times with hot distilled water, and lyophilized. The supernatant and the first washings were combined and evaporated to dryness to yield a degraded polysaccharide (DPS).

DPS from each strain was fractionated by size-exclusion chromatography on a column (1.0 × 50 cm) of Sephadex G-50 fine (Pharmacia, Sweden) in 1% AcOH at a flow rate of 20 ml/h; fractions of 1 ml were collected. High molecular mass fractions (>2 kDa) from Sephadex G-50 were applied to a column (2.0 × 60 cm) of Bio-Gel P-4 (BioRad Laboratories, USA) and eluted with 1% AcOH at a flow rate of 15 ml/h. Monitoring for total carbohydrates was performed using the anthrone sulfuric acid assay. Appropriate oligosaccharide fractions were combined.

Sugar and linkage analyses. For neutral and amino sugar analysis, the polysaccharides were carbonyl-reduced, hydrolyzed with 2 M CF_3CO_2H (120°C, 2 h), N-acetylated, reduced with $NaBD_4$, and acetylated with a 1 : 1 (v/v) pyridine–acetic anhydride mixture. To release acidic monosaccharides, poly- and oligosaccharides were subjected to methanolysis (1 M HCl in MeOH, 85°C, 16 h), carboxyl-reduced with $NaBD_4$ in aqueous 50% methanol, hydrolyzed with 2 M CF_3CO_2H (120°C, 2 h), reduced with $NaBD_4$, and acetylated [19].

For analysis of esterification of uronic acids, DPS were carboxyl-reduced with $NaBD_4$ in 50% aqueous methanol, hydrolyzed, reduced with $NaBD_4$, and acetylated. The appearance of an ion at *m/z* 219 and the ratio of the ions at *m/z* 219 and 217 in the peak of a hexitol in the mass spectra of the acetylated alditols would indicate the presence and the content of an uronic acid ester.

The absolute configurations of the monosaccharides were established by GC of the acetylated *R*-(–)-2-butyl glycosides according to Gerwig and coauthors [20].

Glycosyl linkage analysis was performed by permethylation with CH_3I in dimethylsulfoxide in the presence of sodium methylsulfinylmethanide [21]. The products were purified on a Sep-Pak C18 cartridge (Waters Associates, USA), hydrolyzed with 2 M CF_3CO_2H (100°C, 4 h), and converted to partially methylated alditol acetates (PMAA).

Disaccharide alditols were obtained by partial acid hydrolysis of LPS (0.5 M CF_3CO_2H, 120°C, 0.5 h) followed by N-acetylation, reduction with $NaBD_4$, methanolysis (0.5 M HCl in MeOH, 80°C, 2 h), carboxyl reduction with $NaBD_4$, and conversion to PMAA.

The alditol acetates and PMAA were analyzed by GC-mass spectrometry using a Hewlett-Packard 5890 gas chromatograph/5973 mass selective detector equipped with an HP-5 capillary column (30 m × 0.25 mm; Supelco, USA). The analyses were performed routinely in the electron impact mode (ionization energy 70 eV, source temperature 150°C). The temperature program was 150°C for 3 min, then raised to 250°C at 3°C/min, then to 320°C at 25°C/min, and 10 min at the final temperature.

MALDI-TOF MS and NMR spectroscopy. MALDI-TOF MS was performed using a Voyager-Elite 106 instrument (PE Biosystems, USA). The mass spectra were recorded in the positive and negative ion mode. The matrix solution prepared by dissolving 2,5-dihydroxybenzoic acid in acetonitrile was placed on a MALDI-TOF plate together with samples dissolved in water and allowed to dry under a stream of warm air.

^{1}H NMR spectra were recorded on a Bruker Avance DRX-300 NMR spectrometer (Germany) in D_2O at 40°C using acetone as internal reference, δ_H 2.225.

LPS-induced cytokine production *in vivo*. Production of interleukins IL-1β and IL-6, interferon γ (IFNγ), and TNFα were tested as described [22].

Cell cultures and spectrophotometric assay. Sensitivity of human cervical adenocarcinoma HeLa cells (ATCC No. CCL-2) and the normal HSF cell line (obtained from, and deposited at, the Department of Virology and Immunology, Maria Curie-Sklodowska University, Lublin, Poland) to polysaccharides was determined by the standard spectrophotometric assay [23]. Cells grown in 96-well multiplates were incubated for 24-96 h with polysaccharides in Eagle's medium supplemented with 10% fetal calf serum, and then for 3 h with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (Sigma) (5 mg/ml). The yellow tetrazolium salt was metabolized by viable cells to purple crystals of formazan. The crystals were solubilized overnight in 10% SDS in 0.01 M HCl. The product of incubation was quantified spectrophotometrically by measurement of absorbance at 570 nm using an E-max Microplate Reader (Molecular Devices Corporation, USA) [24].

RESULTS

Isolation of LPS and SDS-PAGE. Most LPS isolated by the hot phenol–water method from cultured *R. leguminosarum* bv. trifolii RtTA1 and Rt120 cells was recovered from the water layer in yields of 1.5 and 2.7% of dry cell mass, respectively. The higher yield of Rt120 LPS was due to exopolysaccharide deficiency of the mutant. The LPS were purified by affinity chromatography on Polymyxin-agarose using a method described by Kannenberg and Carlson [17] and examined by Tricine SDS-PAGE [19] (Fig. 1, inset).

The LPS of strain RtTA1 exhibited two equally abundant bands corresponding to LPS I and LPS II, which matched a description of the LPS from *R. etli* CE3 [25]. The slow-migrating LPS I corresponded to smooth-type LPS containing the O-polysaccharide, and the fast-migrating LPS II represented the core–lipid A species. LPS of mutant Rt120 showed two equally intense bands in the region corresponding to LPS II. A set of several discrete bands without regular spacing and having masses slightly higher than LPS II suggested either an altered susceptibility of LPS I to silver staining or the presence of LPS with truncated O-chains.

Fractionation and composition analysis of DPS. Fractionation on Sephadex G-50 of the polymeric carbo-

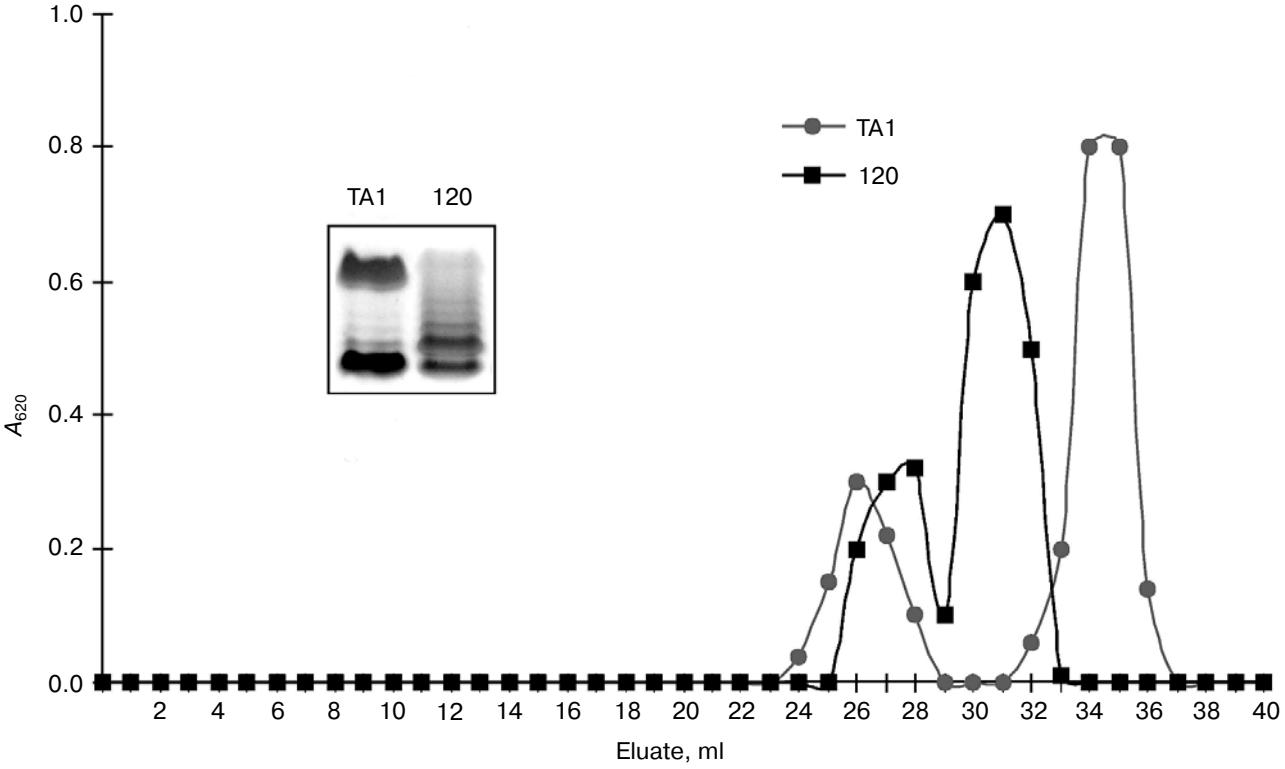

Fig. 1. Fractionation of DPS obtained by mild acid hydrolysis from LPS of *R. leguminosarum* bv. trifolii RtTA1 and Rt120 on a Sephadex G-50 column (0.5 × 50 cm) eluted with 1% acetic acid. Fractions (1 ml) were screened for carbohydrate content by the anthrone assay. The inset shows silver-stained Tricine SDS-PAGE profiles of LPS from the wild type (TA1) and the Rt120 mutant (120).

hydrate portion (degraded polysaccharide, DPS) released by mild acid hydrolysis of Rt120 LPS afforded two distinct peaks: an abundant peak of <2000 Da average molecular mass and a twice less intense peak with a molecular mass centered at around 3000 Da. The molecular mass of the obtained fractions corresponded to the approximate size of 10-12 and 16-18 sugar units, respectively. Sugar analysis of Rt120 DPS by GC of alditol acetates showed the presence of seven neutral sugars (Rha, 6dTal, 2,3-di-Me-Glc, Man, Glc, Gal, and Hep), one amino sugar (QuiNAc), and two acidic sugars (GalA and Kdo) at a molar ratio of 1 : 1 : 1 : 1 : 0.5 : 1.7 : 1 : 1 : 6 : 1, respectively (Table 1). The L-configuration of all 6-deoxyhexoses and the D-configuration of hexoses and uronic acids were determined by GC of the trimethylsilylated (–)-2-butyl glycosides.

The DPS from RtTA1 was separated into two well-resolved peaks: a major peak of 1500 Da and a three times less intense peak of 3300 Da (Fig. 1). The DPS from RtTA1 contained the same sugars as the LPS of this strain [26], except for N-acetyl-D-glucosamine, which was a lipid A component. Monosaccharide analysis indicated that RtTA1 DPS was composed of six neutral sugars (2-Me-Fuc, L-Fuc, D-Man, D-Glc, D-Gal, and Hep), two amino sugars (QuiNAc and 3-NMe-Fuc), and three acidic sugars (D-GlcA, D-GalA, and Kdo) at a ratio of 1 : 1 : 1 : 3 : 1.5 : 1 : 1 : 1 : 1.7 : 3 : 0.5, respectively (the mass spectrum and the fragmentation pathway of 3-NMe-Fuc are shown in Fig. 2).

The high molecular mass fractions of Rt120 and RtTA1 DPS eluted from Sephadex G-50 were further submitted to size-exclusion chromatography on Bio-Gel P-4. As a result, two poorly separated equally intense peaks were observed, and the corresponding fractions of 3000 and 2500 Da were recovered from the Rt120 material and found to differ in chemical composition. The fraction of 3000 Da was composed mainly of 6dTal, Rha, and GalA at a ratio of 1 : 1 : 1, whereas the composition of the 2500-Da fraction, besides increased galactose content, was similar to that of the non-fractionated DPS. In contrast, the RtTA1 DPS eluted from Bio-Gel P-4 as a single broad peak within the molecular mass range of 3100-3300 Da. All sugar components of the RtTA1 DPS were found also in this fraction, but the content of Glc, GlcA, and 3-NMe-Fuc was higher (Table 1).

Analysis of the low molecular mass fraction from Sephadex G-50, corresponding by size to the core oligosaccharide, revealed significant differences in composition between the mutant and the wild type strain. The composition of this fraction from RtTA1 DPS (Man, Glc, QuiNAc, Fuc, Gal, GalA, and Kdo at a ratio of 2 : 1 : 0.5 : 0.5 : 1 : 2 : 4), except for the presence of glucose,

Table 1. Compositional analysis of DPS obtained by mild acid hydrolysis of LPS from wild type *R. leguminosarum* RtTA1, Rt120 mutant, and their 3-kDa fractions from Bio-Gel P-4

Monosaccharide	DPS/3-kDa fraction (mol%)	
	RtTA1	Rt120
2-Me-Fuc	6.2/2.5	–
L-Fuc	7.8/6.0	–
L-Rha	–	7.5/29.7
L-6dTal	–	6.6/27.3
QuiNAc	4.7/6.0	4.9/–
3-NMe-Fuc	6.4/11.2	–
2.3-di-Me-Glc	–	5.2/3.0
D-Man	6.1/5.7	5.0/3.5
D-Glc	20.6/37.7	3.5/1.0
D-GlcA	10.4/16.2	–
D-Gal	9.0/0.6	19.9/7.7
D-GalA	20.0/0.4	36.8/30.7
Hep	6.3/12.1	4.8/–
Kdo	2.5/1.1	5.4/–

was similar to that described for core oligosaccharides in LPS from *R. etli* and *R. leguminosarum* [7, 27]. In addition to the sugars occurring in *R. leguminosarum* and *R. etli* core oligosaccharides, such as Man, QuiNAc, Gal, GalA, and Kdo, the low molecular mass fraction from Rt120 DPS contained Glc, 6dTal, and Rha at a ratio of 1.5 : 1 : 1 : 3 : 1 : 0.2 : 1 : 2.5, respectively.

Methyl esterification of glucuronic acid has been reported in the O-chain of *R. etli* LPS [8, 28]. When the alditol acetates were prepared from the DPS that were carboxyl-reduced with $NaBD_4$ without methanolysis, no products derived from (methyl) esters of uronic acids were detected.

Linkage analyses. Linkage analysis of neutral sugars was performed by methylation (Hakomori method), conversion to PMAA, and GC-mass spectrometry analysis. Disaccharide alditols terminated with uronosyl residues were determined after partial acid hydrolysis of DPS (0.5 M TFA), reduction, methanolysis, carboxyl reduction, and acetylation. When applied to RtTA1 DPS, the above procedure resulted in the isolation of the following disaccharide alditols: GlcA-1,3-2-Me-fucitol, GlcA-1,3-fucitol, and GlcA(GalA)-1,4-hexitol. In case of Rt120 DPS, only GalA-1,4-hexitol was found.

Relative PMAA recoveries from the methylated polysaccharides are listed in Table 2. The results showed that the linkage and substitution patterns were highly heterogeneous. The terminal residues identified in the oligosaccharides released by mild acid hydrolysis of the mutant LPS were 6dTal, Rha, Hep, Glc, and GalA at a ratio of 2 : 2 : 1 : 0.5 : 1. For comparison, in the mixture of products released from the wild type LPS, the major terminal residues identified were 2-Me-Fuc and/or Fuc, Hep, Glc, GlcA, and GalA at a ratio of 1 : 2 : 1 : 1 : 1.

Table 2. Linkage methylation analysis of DPS from LPS of *R. leguminosarum* bv. trifolii RtTA1 and Rt120

Inferred linkage of sugar component*	Mol%	
	RtTA1	Rt120
L-Fucose (2-O-methylfucose)/terminal**	5.5	–
6-Deoxy-L-talose/terminal	–	9.9
6-Deoxy-L-rhamnose/terminal	–	12.5
6-Deoxy-L-talose/2-substituted	–	6.3
6-Deoxy-L-rhamnose/2-substituted	–	7.9
6-Deoxy-L-fucose/2-substituted	6.5	–
L-Fucose/3-substituted	5.0	–
L-Rhamnose/3-substituted	–	9.8
L-Fucose/4-substituted	5.0	–
L-Fucose/3,4-disubstituted	4.0	–
6-Deoxy-L-talose/3,4-disubstituted	–	9.5
3-NMe-Fuc/terminal	1.3	–
3-NMe-Fuc/4-substituted	4.7	–
Heptose/terminal	11.0	6.7
Glucose/terminal**	7.5	2.3
Mannose/3-substituted	3.3	2.0
Mannose/4-substituted	1.0	3.6
Glucose/2-substituted	6.0	1.8
QuiNAc/3-substituted	3.9	1.8
Galactose/6-substituted	2.6	6.3
Glucose/2,3-disubstituted	3.9	–
Glucose/2,4-disubstituted	0.9	–
Mannose/4,6-disubstituted	3.4	4.1
Glucose/2,3,6-trisubstituted	2.3	–
D-GlcA/terminal	5.8	–
D-GlcA/4-substituted	1.5	–
D-GalA/terminal	5.1	6.6
Kdo/4-substituted	5.5	4.6
Kdo/5-substituted	2.6	1.8
Kdo/4,5-disubstituted	2.2	2.5

* Terminal L-fucose was inferred from 1,5-di-O-acetyl-2,3,4-tri-O-methylfucitol, 2-linked 6-deoxy-L-talose from 1,5-di-O-acetyl-3,4-di-O-methyl-6-deoxytalitol, etc.

** Part of the terminal fucose and glucose detected could derive from their methylated derivatives in the intact LPS and could be distinguished by permethylation with C^2H_3I.

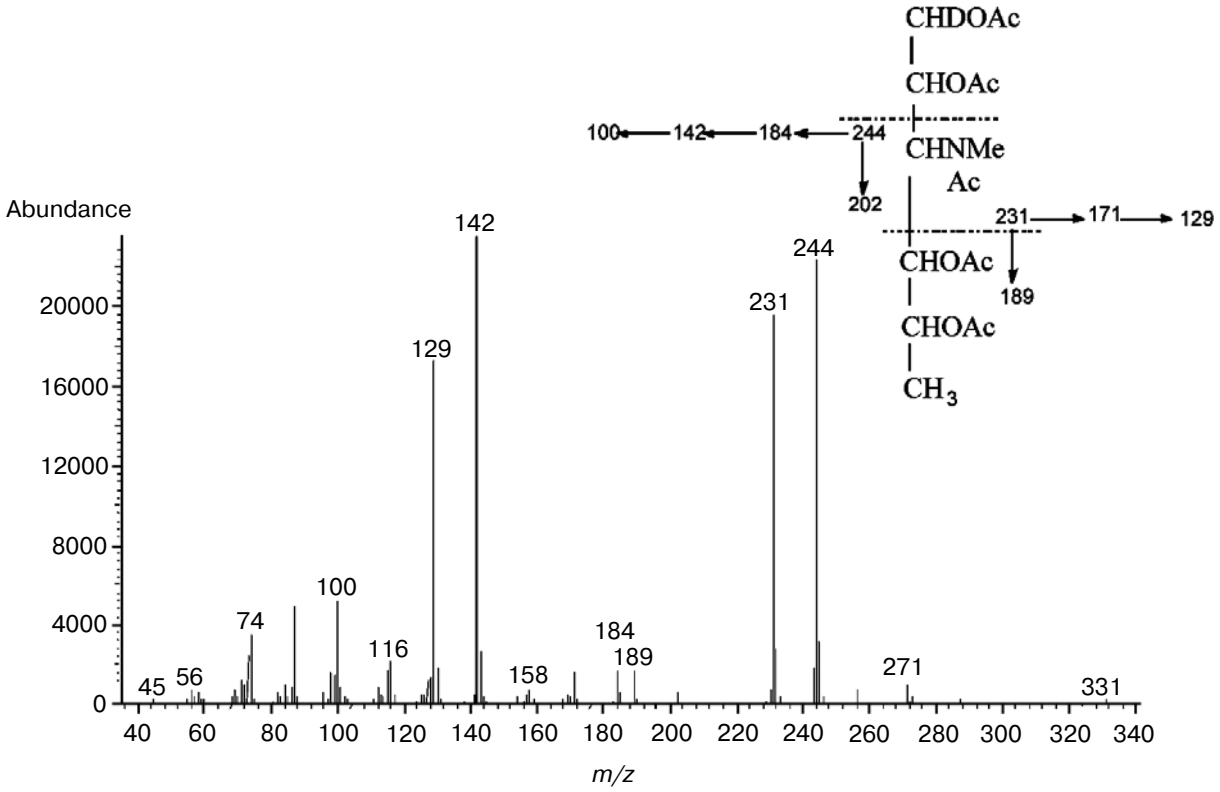

Fig. 2. Mass spectrum and fragmentation pathway of 1,2,4,5-tetra-O-acetyl-3,6-dideoxy-3-N-methylacetamidohexitol(galactitol) derived from DPS of *R. leguminosarum* bv. trifolii RtTA1.

Many of the glycosyl residues had multiple linkage patterns, although heptose occurred only as a terminal sugar in both DPS. In Rt120 DPS, the residues of 6dTal were mainly 2-substituted and those of Rha were 2- and 3-substituted. Additionally, both 6-deoxy sugars occurred at branching points. The linkage pattern of fucose in RtTA1 DPS was similar to that of rhamnose in mutant DPS. The results indicated that mild acid hydrolysis of Rt120 LPS released products with terminal 6dTal, Rha, Hep, and 3,4-branched 6dTal residues. Among the products of mild acid hydrolysis of RtTA1 DPS, terminal Fuc, Hep, and 3,4-branched Fuc residues were found. The significant proportions of terminal and 2-linked Glc residues in the wild type DPS may suggest that at least part of Glc derived from a 1,2-linked glucan contamination.

The results of PMAA identification were confirmed by analysis of methylation products prior to hydrolysis, which allowed identification of the following oligosaccharides in the DPS from Rt120: 6-Man-1,5-Kdo; Hex-1,2-Hex-1,3-QuiNAc; GalA-1,4-Hex, and 6dHex-1,2-6dHex. For comparison, the DPS of the wild type RtTA1 contained 3-NMe-Fuc-1,5-[HexA-1,4]-Kdo; 6dHex-1,3-6dHex, Hep-1,3-Hex; Hex-1,3-Hex-1,4-Hex; GalA(GlcA)-1,4-Hex, and GlcA-1,3-Fuc(2-OMe-Fuc).

The high molecular mass fractions eluted from Sephadex G-50 were further analyzed by MALDI-TOF MS (Table 3). The mass spectra of DPS obtained in the negative ion mode showed multiple peaks distributed in different mass ranges at *m/z* 300-747.7 and 300-1112.6 for the fractions from Rt120 and RtTA1, respectively. Two ions were present only in Rt120: a singly charged ion at *m/z* 747.7, expected for the sequence of HexA–6dHex–QuiNAc–Kdo, and an ion at *m/z* 538.9 corresponding to a Na-adduct of a trisaccharide composed of Hex, 2,3-di-Me-Hex, and 6dHex. Distinctive of RtTA1 were negative ions at *m/z* 823.8, corresponding to a pentasaccharide composed of 3-NMe-Fuc, 6dHex, Hex_2, and HexA, and *m/z* 369.0, for which a disaccharide of 3-NMe-Fuc and Hep was proposed. The remaining ions were identified as common to both LPS: an ion at *m/z* 686.3 corresponding to the sequence HexA-Hex_2-6dHex (Na-adduct), an ion at *m/z* 577.3 (not assigned), and ions at *m/z* 395.5 and 332.9 corresponding to Hep-Hex and 6dHex-6dHex disaccharides, respectively. The presence of the short disaccharides composed of 6-deoxyhexoses could indicate that at least a part of the O-chains of both LPS was released as small-size fragments during mild acid hydrolysis.

The heterogeneity of the material from both RtTA1 and Rt120 was apparent also from the ^{1}H NMR spectrum (Fig. 3). An additional source of anomeric region complexity was non-stoichiometric O-acetylation. The relative intensities of the signals for the major O-acetyl groups

Table 3. MALDI-TOF MS data and proposed composition of the oligosaccharides identified in DPS from LPS of *R. leguminosarum* bv. trifolii RtTA1 and Rt120

Experimental *m/z* value	Calculated mass for ion	Proposed composition	RtTA1	Rt120
823.3	823.8 $[M - H]^-$	3-NMe-6dHex, 6dHex, Hex_2, HexA	+	–
747.7	746.7 $[M - H]^-$	6dHex, HexA, QuiNAc, Kdo	–	+
686.3	687.6 $[M + Na]^+$	HexA, Hex_2, 6dHex	+	+
538.9	539.4 $[M + Na]^+$	Hex, 2,3-di-Me-Hex, 6dHex	–	+
395.5	395.4 $[M + Na]^+$	Hep, Hex	+	+
369.0	368.3 $[M - H]^-$	Hep, 3-NMe-6dHex	+	–
332.9	333.3 $[M + Na]^+$	6dHex, 6dHex	+	+

(2.25 and 2.26 ppm) and N-acetyl groups (2.10-2.12 ppm) were 1 : 0.8 and 1 : 1.24 in DPS from RtTA1 and Rt120, respectively. Apart from 14 minor signals, the anomeric region (between 4.50 and 5.55 ppm) of the spectrum of DPS from the wild type strain (Fig. 3a) showed four abundant anomeric proton signals at 4.95, 4.98, 5.31, and 5.33 ppm. Besides that, the spectrum showed signals for sugar ring protons at 3.5-4.5 ppm and four methyl groups at 1.28, 1.49, 1.56, and 3.05 ppm belonging to H6 of Fuc, 3-NMe-Fuc, QuiNAc, and N-methyl group of 3-NMe-Fuc, respectively. A signal for O-methyl group of 2-Me-Fuc was observed at δ 3.51, i.e. within the ring proton region. In the spectrum of Rt120 DPS (Fig. 3b), four methyl signals occurred at δ 1.32, 1.34, 1.40, and 1.42 ppm, which were attributable to H6 of 6dTal and Rha. In the anomeric region of Rt120 DPS, there were four signals (all doublets) of similar integral intensities at 5.08, 5.25, 5.31 and 5.51 ppm, the last one being assigned to the anomeric proton of GalA.

Biological properties of LPS and DPS. Sugar and fatty acid composition of lipid A from Rt120 was identical to that described for the wild type lipid A [22]. The lethal toxicity of LPS was analyzed in galactosamine-treated NIH male mice. LD_{50} for Rt120 LPS was 10 μg per mouse, and it was 1.8 times more toxic than RtTA1 LPS showing LD_{50} 17.8 μg per mouse (Table 4). To estimate the ability of the mutant LPS to induce cytokine production (TNFα, IL-1β, IL-6, and IFNγ), NIH mice were injected with an 80 μg/ml dose of LPS, and cytokine concentrations in serum were determined using ELISA as described [22]. The concentrations of TNFα and IL-6 detected 1.5 h after injection of Rt120 LPS were slightly higher (1.3 and 1.2, respectively), but the amount of IL-1β after 3 h was 2.2 times lower in comparison with the wild type LPS. Both LPS induced similar levels of IFNγ (Table 4).

The 3-kDa fractions eluted from Bio-Gel P-4 (Table 1) were used to study the influence of oligosaccharides, containing mainly the O-chain fragments of RtTA1 and Rt120 LPS, on the viability of human normal and tumor cell lines. The viability of HSF cells was over 80% during 24, 48, 72, and 96 h of incubation with increasing concentrations (1-10 μg/ml) of the 3-kDa fraction of Rt120 DPS (Fig. 4). In the case of HeLa cells, a growing cytotoxic effect, expressed as IC_{50}, was observed: 9.75, 10.53, 8.05, and 8.36 μg/ml after 24, 48, 72, and 96 h of incubation. The same concentrations of 3-kDa fraction from RtTA1 DPS did not affect the viability of either cell culture.

DISCUSSION

The polysaccharide portions released from the LPS of the wild type strain of *R. leguminosarum* and the strain harboring mutation in the intergenic *pssB–pssA* region differed in their molecular size distribution and sugar composition. DPS from the wild type strain consisted of

Table 4. Lethal toxicity and *in vivo* production of TNFα, IL-6, IL-1β, and IFNγ (pg/ml) after induction of mice (six mice per group) with LPS (80 μg per mouse) from *R. leguminosarum* bv. trifolii RtTA1 and Rt120

LPS	LD_{50}, μg per mouse	TNFα after 1.5 h	IL-6 after 1.5 h	IL-1β after 3 h	IFNγ after 3 h
RtTA1 [22]	17.8	5516	14 240	136	33
Rt120	10	7186	16 630	62	37

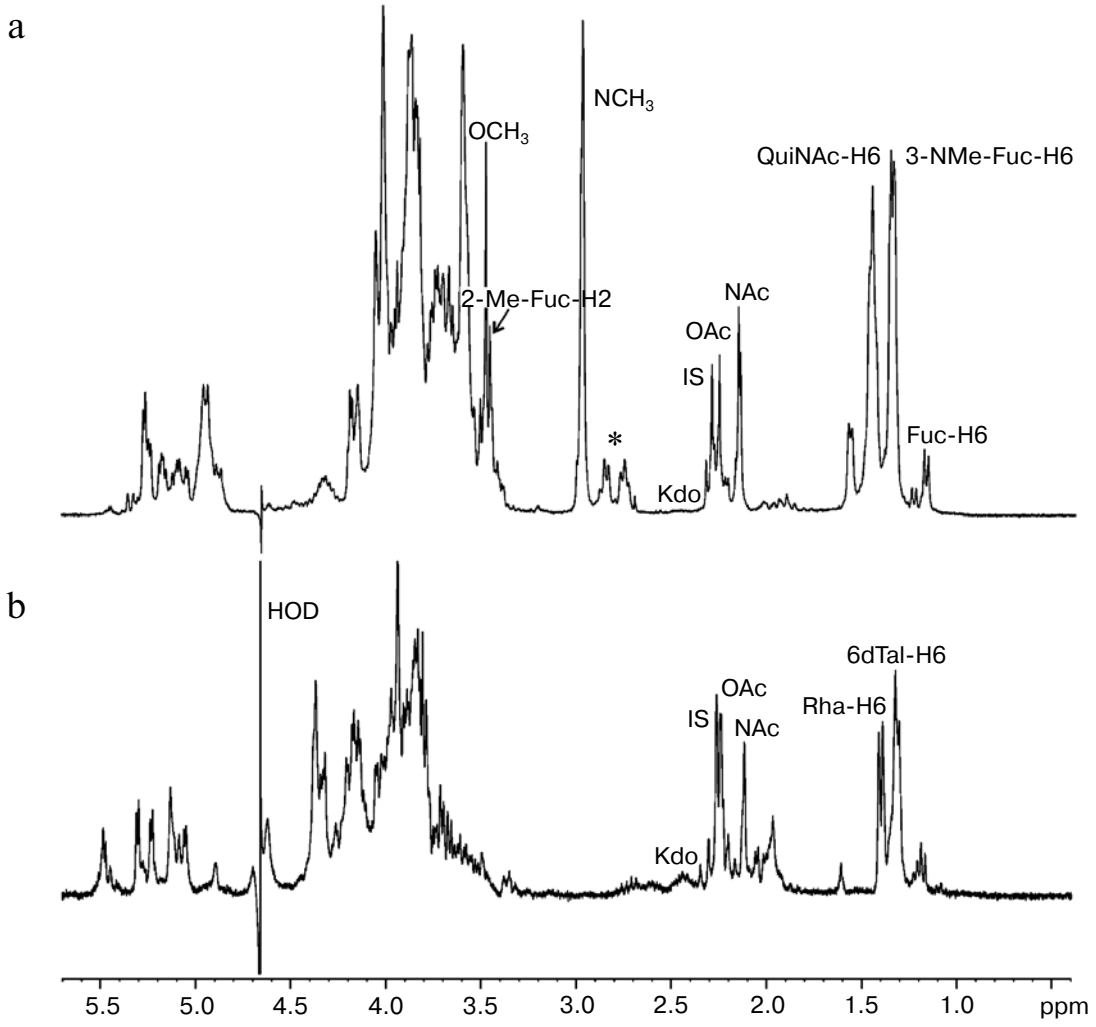

Fig. 3. ^{1}H NMR (300 MHz) spectra of DPS containing the O-antigen and core oligosaccharides of *R. leguminosarum* bv. trifolii (a) RtTA1 and (b) Rt120. The presence in the spectrum of RtTA1 DPS of signals at 2.47 and 2.57 ppm marked with an asterisk suggests that 3-NMe-Fuc may be substituted with an aspartyl residue. Similar signals at δ 2.62 (H3) and 2.71 (H4) have been observed in the ^{1}H NMR spectrum of the O-polysaccharide of *Proteus mirabilis* O38 [29]. The region of methylene protons of anhydro-Kdo is designated as Kdo.

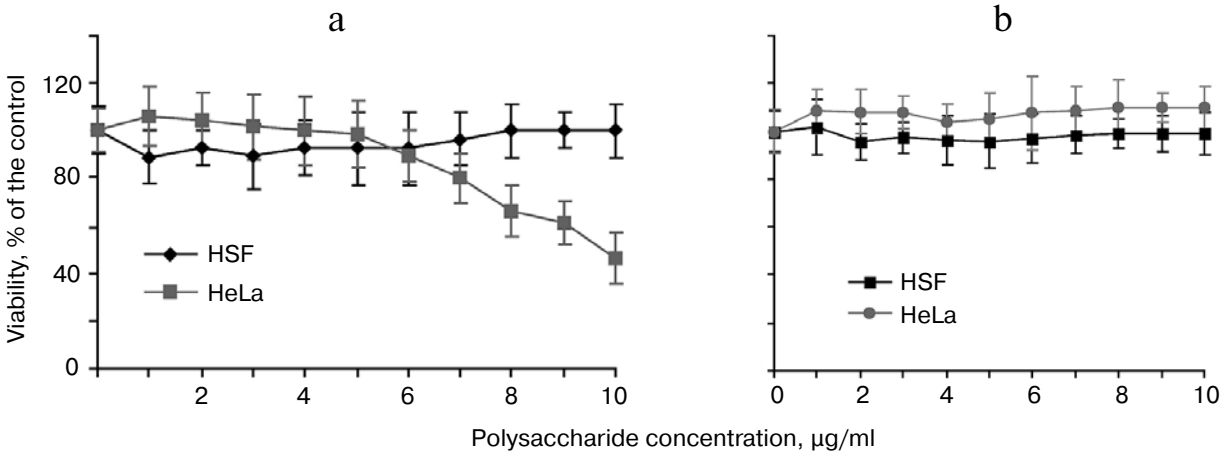

Fig. 4. The cytotoxicity effect (% of the control ± SD) of 3-kDa fractions from DPS of RtTA1 (a) and Rt120 (b) on normal HSF and HeLa cell lines.

two fractions with a molecular mass difference ~1800 Da, which is larger than that of 1000 Da for the fractions derived from the mutant (Fig. 1). For comparison, the molecular masses of the major core oligosaccharide species and the O-polysaccharide of *R. etli* LPS were 1531 and ~3100 Da, respectively [30]. In the present study, mild acid hydrolysis of the mutant LPS released the O-chain and the core portion having similar molecular masses. The electrophoretic pattern indicated a more homogeneous size distribution of the LPS species from Rt120 in comparison with the wild type LPS and similar lengths of the O-chain and core oligosaccharide in the intact LPS (Fig. 1).

The similarity in size between the O-polysaccharide portion and the core part was confirmed by differences in carbohydrate composition between the low and high molecular mass fractions of DPS, which were less distinct for Rt120 than for RtTA1. Sugar composition of the carbohydrate part of RtTA1 LPS was similar to that of *R. leguminosarum* bv. trifolii wild type strain 0403, both containing 2-Me-Fuc and 3-NMe-Fuc. Moreover, the ratio of the four 6-deoxyhexoses, namely 2-Me-Fuc, Fuc, 3-NMe-Fuc, and QuiNAc was 1 : 1 : 1 : 0.5 in the O-polysaccharide from strain 0403 [5], and in RtTA1 the content of these monosaccharides was approximately equimolar.

Rt120 LPS lacked 2-Me-Fuc, Fuc, 3-NMe-Fuc, and GlcA, i.e. the sugars that are characteristic of the wild type RtTA1 O-chain. Instead, two other 6-deoxyhexoses (6dTal and Rha) along with 2,3-di-Me-Glc were found. In the RtTA1 O-chain, Fuc and 2-Me-Fuc are substituted by GlcA at position 3. For comparison, the repeating unit of the *R. etli* CE3 O-polysaccharide consists of branching Fuc residues substituted by GlcA at O4 (the majority of GlcA residues are methyl esterified) and by 3-Me-6dTal at O3 [8, 28]. The 6-deoxy sugars, i.e. L-Fuc in RtTA1 and L-6dTal and L-Rha in Rt120, occur both as terminal and interior sugar residues. A high content of deoxy sugars is characteristic of O-antigens from most *Rhizobium* species and related genera. Fuc and 6dTal seem to represent the characteristic features of the rhizobial LPS. Contrary to *Mesorhizobium*, in *Rhizobium* fucose appears mostly as a terminal side chain sugar [13], although in *R. leguminosarum* bv. viciae 128C53 [31] and *R. etli* CE3 [28] fucose occurs in the main chain. The O-polysaccharides from two biovars of *R. leguminosarum* consist of L-Rha, D-GlcNAc, and D-ManNAc (bv. trifolii strain 4S) [6] or 3-Me-6dTal and L-QuiNAc in addition to L-Fuc (bv. viciae strain 3841) [7]. In addition, a new monosaccharide, 3-acetimidoylamino-3-deoxy-D-glucuronic acid (rhizoaminouronic acid) has been identified in an *ex planta* synthesized O-chain of *R. leguminosarum* strain bv. viciae 3841 [7]. The O-chain of *R. etli* CE3 LPS is composed of methyl ester of GlcA, L-Fuc, and 3-Me-6dTal [32].

One of the effects of the mutation in the *pssB–pssA* region was the inability of Rt120 to form effective nitrogen fixing nodules on clover [16]. Mutants that lack the O-polysaccharide or have a reduced amount of the O-chain, fail to infect host cells, resulting in root nodules that are devoid of bacteria and are unable to fix nitrogen [32]. Although numerous studies have demonstrated that the presence of O-antigen is important in the symbiosis, the specific structural features that are required have not been established yet.

Dazzo et al. [5] demonstrated that biologically active LPS from *R. leguminosarum* bv. trifolii 0403 produced in the early stationary phase had less Fuc but more 2-Me-Fuc, 3-NMe-Fuc, QuiNAc, and non-carbohydrate substituents (O-methyl, N-methyl, and acetyl groups) than had inactive LPS in the mid-exponential phase. The activity manifested itself as infiltration of LPS across the root hair wall and an increase in infections of root hairs with the bacteria [5].

The pleiotropic effects of mutation in the *pssB* encoding inositol monophosphatase [26] as well as the various effects caused by complementation of Exo$^-$ mutants by the *pssA* gene coding for glucosyl-isoprenylphosphate-transferase [33] suggest that within the long regulatory region of the genes related to exopolysaccharide biosynthesis in *R. leguminosarum* bv. trifolii, a putative open reading frame or regulatory RNA involved in LPS synthesis could be encoded. Mutations in genes involved in exopolysaccharide synthesis (*pssA*, *pssD*, or *pssE*) of *R. leguminosarum* bv. viciae and *pssA* of *R. leguminosarum* bv. trifolii ANU794 also had pleiotropic effects; a minimum of 22 protein differences were observed for two *pssA* mutant strains [34]. Mutation in a single gene coding for UDP-Glc dehydrogenase in *R. leguminosarum* bv. viciae RBL5808 resulted in multiple phenotypic changes: a loss of exopolysaccharide, the absence of GalA in LPS, and a reduced level of the O-polysaccharide expression [27].

A possibility has been suggested that the product of the *pssB* gene influences the biosynthetic pathway of 6-deoxyhexoses or enables the expression of an alternative form of the O-antigen in *R. leguminosarum* TA1. In *Actinobacillus actinomycetemcomitans* NCTC 9710 (serotype c), two dTDP-6-deoxy-L-*lyxo*-hexos-4-ulose reductases of weak sequence homology, which are involved in synthesis of dTDP-6-deoxy-L-talose and dTDP-L-rhamnose from dTTP and D-glucose 1-phosphate, have been reported [35].

The compositional differences between the studied LPS were partially ascribed to structural features. The structures with terminal 6dTal, Rha, Hep, and 3,4-branched 6dTal residues were predominant in Rt120 LPS, whereas mainly terminal Fuc, Hep, and 3,4-branched Fuc residues were found in RtTA1 LPS. Furthermore, GlcA residues substituting the position 3 of 2-Me-Fuc and Fuc occurred in RtTA1 DPS but not in the DPS of Rt120.

A higher degree of O-acetylation of sugars was observed in RtTA1 DPS. As the polysaccharide portions

studied clearly differed in the content of hydrophobic groups (O-methyl, O-acetyl, N-methyl), it is likely that these groups are of significance for the biological activity of the polysaccharides. The rare carbohydrate component 3-NMe-Fuc was found only in the LPS of *R. leguminosarum* bv. trifolii 0403, *R. leguminosarum* 128C63, *Rhizobium phaseoli* 127K17 (later renamed to *R. etli*), and *R. leguminosarum* bv. trifolii ANU 843. Moreover, in the LPS of strain ANU 843, this sugar derivative was found in PS1 only and not in PS2. The PS1 fraction resulting from mild acid hydrolysis of LPS was immunodominant and contained 2-Me-6dHex, Fuc, QuiN, Hep, GlcA, and Kdo, whereas PS2 represented a core oligosaccharide and included Man, Gal, GlcA, and Kdo [5].

The remarkably high content of glucose in the RtTA1 polysaccharide portion is unexpected; it could be related to contamination with glucan produced by RtTA1 [36], or the sugar might be a constituent of LPS. Recently, the presence of glucose as an O-chain component was demonstrated in the LPS of an *R. leguminosarum* mutant defective in UDP-Glc dehydrogenase, which produced an O-polysaccharide having an O-acetylated 4-α-D-Glc*p*-1,3-α-D-Qui*p*NAc-1 repeating unit, identical to that of the parent strain but at a reduced level [27]. Neither the signals at 4.70-4.72 ppm characteristic of β-configured glucose nor those at 3.38-3.44 ppm that correspond to H2 protons involved in β-1,2-linkage reported by Roset et al. [37] were observed in the ^{1}H NMR spectrum of the RtTA1 DPS (Fig. 3).

Certain biological activities of both LPS and their polysaccharide portions of *R. leguminosarum* bv. trifolii were compared. The LPS of Rt120 was 1.8 times more toxic than the LPS of RtTA1 in galactosamine-sensitized mice. It was demonstrated that the sugar and fatty acid composition of lipid A from wild type RtTA1 and mutant strains of *R. leguminosarum* were identical [22]. Both LPS could be defined as toxic also using criteria of other authors [38, 39]. TNFα and IL-6 secretion was 1.3 and 1.2 higher, when induced *in vivo* by the LPS from Rt120 in comparison to the wild type LPS. In contrast, LPS-induced production of IL-1β was twice lower for Rt120 than for RtTA1. IFNγ production was the same for both LPS.

Studies on non-enterobacterial species, e.g. *Rhodobacter capsulatus* or *Bradyrhizobium* sp. (Lupinus), have demonstrated that *in vivo* lethality correlates with induction of TNFα, IL-1, and IL-6 in mouse macrophages [38]. In bone marrow cells, LPS of *Rhizobium* species Sin-1 and three strains of *Legionella pneumophila*, which do not belong to the Enterobacteriaceae family too, required TLR2 rather than TLR4 to elicit the expression of CD14. The data have shown selective action of different LPS via different TLRs [40].

Biological activities of LPS from *Sinorhizobium meliloti*, *Mesorhizobium loti*, and *Escherichia coli*, including lethality, production of TNFα and nitric oxide, adjuvant action, and Limulus activity, have shown that LPS from *S. meliloti* exhibited stronger actions than LPS from *M. loti* but weaker than LPS from *E. coli* [39]. On the other hand, *M. loti* LPS showed a higher ability to activate human complement than *S. meliloti* LPS. These characteristic biological actions were related to a difference in the lipid A structure between *S. meliloti* and *M. loti*. The sugar and fatty acid composition of lipid A from the wild type strain RtTA1 and the mutant Rt120 were identical. A possible explanation for the discrepancy in the lethal toxic effect could be the different shape of the LPS molecules and different affinity of their lipid A to bactericidal/permeability increasing protein, which, in a complex with LPS, is known to inhibit synthesis of cytokines after initial stimulation of macrophages by LPS [2, 38].

Various naturally occurring carbohydrate-containing compounds are receiving continuous attention as immunomodulators in cancer therapy. The results of our studies indicated that treatment of normal human cells with growing concentrations of the fractions having the same molecular size but different sugar composition (i.e. characterized by prevailing contents of Fuc, 3-NMe-Fuc, Glc, GlcA, and Hep in RtTA1 or Rha, 6dTal, and GalA in Rt120) elicited different reactions, but only in the tumor HeLa cells. The viability of these cells decreased with increasing concentrations of the preparation, reaching 40% of the initial value at 10 μg/ml of the oligosaccharides derived from Rt120 LPS. The treatment of the normal and cancer cells with the same concentrations of the corresponding fractions of RtTA1 LPS did not influence their viability. The mechanism of the cytotoxic activity of Rt120 LPS requires further studies.

Recently, a growing number of studies are undertaken on glycomimetics, i.e. synthetic or natural molecules based on the structures of ligands that are capable of interrupting some routes in cancer malignancy or allergic responses [41]. The specificity of carbohydrate interactions in an orderly cell–cell or cell–substratum interplay has been proven by inhibition of the interaction of mouse lymphoma clones *in vivo* and *in vitro* by NeuAcα-2,3-Galβ-1,4-Glc but not NeuAcα-2,6-Galβ-1,4-Glc [42]. The mechanism of the antitumor effect of polysaccharides, such as β-glucans from the mushroom *Agaricus* or lentinan, a β-1,3 pentasaccharide glucan with two β-1,6-linked branches, extracted from *Lentinus edodes* (Shiitake mushroom), has not yet been determined, but the action may be host-mediated and not directly cytocidal [15, 43].

REFERENCES

1. Raetz, C. R., and Whitfield, C. (2002) *Annu. Rev. Biochem.*, **71**, 635-700.
2. Trent, M. S., Stead, C. M., Tran, A. X., and Hankins, J. V. (2006) *J. Endotoxin Res.*, **12**, 205-223.

3. Alexander, C., and Zahringer, U. (2002) *Trends Glycosci. Glycotechnol.*, **14**, 69-86.
4. Rietschel, E. T., Kirikae, T., Schade, U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, H. J., Zahringer, U., Seydel, U., Di Padova, F., Schreier, M., and Brade, H. (1994) *FASEB J.*, **8**, 217-225.
5. Dazzo, F. B., Truchet, G. L., Hollingsworth, R. I., Hrabak, E. M., Pankratz, H. S., Philip-Hollingsworth, S., Salzwedel, J. L., Chapman, K., Appenzeller, L., Squartini, A., Gerhold, D., and Orgambide, G. (1991) *J. Bacteriol.*, **173**, 5371-5384.
6. Wang, Y., and Hollingsworth, R. I. (1994) *Carbohydr. Res.*, **260**, 305-317.
7. Forsberg, L. S., and Carlson, R. W. (2008) *J. Biol. Chem.*, **283**, 16037-16050.
8. Noel, K. D., Box, J. M., and Bonne, V. J. (2004) *Appl. Environ. Microbiol.*, **70**, 1537-1544.
9. Lerouge, I., and Vanderleyden, J. (2001) *FEMS Microbiol. Rev.*, **26**, 17-47.
10. Carlson, R. W., Shatters, R., Duh, J., Turnbull, E., Hanley, B., Rolfe, B. G., and Djordjevic, M. A. (1987) *Plant Physiol.*, **84**, 421-427.
11. Jeyaretnam, B., Glushka, J., Kolli, V. S. K., and Carlson, R. W. (2002) *J. Biol. Chem.*, **277**, 41802-41810.
12. Caroff, M., and Karibian, D. (2003) *Carbohydr. Res.*, **338**, 2431-2447.
13. De Castro, C., Molinaro, A., Lanzetta, R., Silipo, A., and Parrilli, M. (2008) *Carbohydr. Res.*, **343**, 1924-1933.
14. Kogan, G., Sandula, J., Korolenko, T. A., Falameeva, O. V., Poteryaeva, O. N., Zhanaeva, S. Ya., Levina, O. A., Filatova, T. G., and Kaledin, V. I. (2002) *Int. Immunopharmacol.*, **2**, 775-781.
15. Laroche, C., and Michaud, P. (2007) *Recent Patents Biotechnol.*, **1**, 59-73.
16. Janczarek, M., Krol, J., Kutkowska, J., Mazur, A., Wielbo, J., Borucki, W., Kopcinska, J., Lotocka, B., Urbanik-Sypniewska, T., and Skorupska, A. (2001) *J. Plant Physiol.*, **158**, 1565-1574.
17. Kannenberg, E. L., and Carlson, R. W. (2001) *Mol. Microbiol.*, **39**, 379-391.
18. Lesse, A. J., Campagnari, A. A., Bittner, W. E., and Apicella, M. A. (1990) *J. Immunol. Methods*, **126**, 109-117.
19. Russa, R., Urbanik-Sypniewska, T., Lindstrom, K., and Mayer, H. (1995) *Arch. Microbiol.*, **163**, 345-351.
20. Gerwig, G. J., Kamerling, J. P., and Vliegenthart, J. F. G. (1978) *Carbohydr. Res.*, **62**, 349.
21. Hakomori, S.-I. (1964) *J. Biochem.*, **55**, 205-208.
22. Urbanik-Sypniewska, T., Choma, A., Kutkowska, J., Kaminska, T., Kandefer-Szerszen, M., Russa, R., and Dolecka, J. (2000) *Immunobiology*, **202**, 408-420.
23. Mosman, T. (1983) *J. Immunol. Methods*, **65**, 55-63.
24. Matysik, G., Wojciak-Kosior, M., and Paduch, R. (2005) *J. Pharm. Biomed. Anal.*, **38**, 285-292.
25. Noel, K. D., Forsberg, L. S., and Carlson, R. W. (2000) *J. Bacteriol.*, **182**, 5317-5324.
26. Kutkowska, J., Janczarek, M., Kopcinska, J., Urbanik-Sypniewska, T., and Skorupska, A. (2007) *Acta Biol. Cracov. Bot.*, **49**, 81-89.
27. Muszynski, A., Laus, M., Kijne, J. W., and Carlson, R. W. (2011) *Glycobiology*, **21**, 55-68.
28. D'Haeze, W., Leoff, C., Freshour, G., Noel, K. D., and Carlson, R. W. (2007) *J. Biol. Chem.*, **282**, 17101-17113.
29. Kondakova, A. N., Senchenkova, S. N., Gremyakov, A. I., Shashkov, A. S., Knirel, Y. A., Fudala, R., and Kaca, W. (2003) *Carbohydr. Res.*, **338**, 2387-2392.
30. Forsberg, L. S., Bhat, U. R., and Carlson, R. W. (2000) *J. Biol. Chem.*, **275**, 18851-18863.
31. Kannenberg, M. J., Reuhs, B. L., Forsberg, L. S. S., and Carlson, R. W. (1998) in *The Rhizobiaceae* (Spaink, H. P., Kondorosi, A., and Hooykaas, P. J. J., eds.) Kluwer Academic, Dordrecht, pp. 119-154.
32. Forsberg, L. S., Noel, K. D., Box, J. M., and Carlson, R. W. (2003) *J. Biol. Chem.*, **278**, 3957-3968.
33. Janczarek, M., and Skorupska, A. (2003) *Res. Microbiol.*, **154**, 433-442.
34. Guerreiro, N., Ksenzenko, V. N., Djordjevic, M. A., Ivashina, T. V., and Rolfe, B. G. (2000) *J. Bacteriol.*, **182**, 4521-4532.
35. Nakano, Y., Suzuki, N., Yoshida, Takashi Nezu, Y., Yoshihisa Yamashita, Y., and Koga, T. (2000) *J. Biol. Chem.*, **275**, 6806-6812.
36. Breedveld, M. W., Zevenhuizen, L. P. T. M., and Zehnder, A. J. B. (1992) *J. Bacteriol.*, **174**, 6336-6342.
37. Roset, M. S., Ciocchini, A. E., Rodolfo, A., Ugalde, R. A., and de Iannino, N. I. (2006) *J. Bacteriol.*, **188**, 5003-5013.
38. Loppnow, H., Libby, P., Freudenberg, M., Krauss, J. H., Weckesser, J., and Mayer, H. (1990) *Infect. Immun.*, **58**, 3743-3750.
39. Tsukushi, Y., Kido, N., Saeki, K., Sugiyama, T., Koide, N., Mori, I., Yoshida, T., and Yokochi, T. (2004) *J. Endotoxin Res.*, **10**, 25-31.
40. Girard, R., Pedron, T., Uematsu, S., Balloy, V., Chignard, M., Akira, S., and Chaby, R. (2003) *J. Cell Sci.*, **116**, 293-302.
41. Hakomori, S. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 10231-10233.
42. Hakomori, S. (1991) *Pure Appl. Chem.*, **63**, 473-482.
43. Liu, C., Li, X., Li, Y., Feng, Y., Zhou, S., and Wang, F. (2008) *Food Chem.*, **110**, 807-813.