Against the rules: A marine bacterium, *Loktanella rosea*, possesses a unique lipopolysaccharide

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Bacteria are an inimitable source of new glyco-structures potentially useful in medicinal and environmental chemistry. Lipopolysaccharides (LPS; endotoxins) are the major components of the outer membrane of Gram-negative bacteria; being exposed toward the external environment they can undergo structural changes and thus, they often possess peculiar chemical features that allow them to thrive in harsh chemical and physical environments. Marine bacteria have evolved and adapted over millions of years in order to succeed in different environments, finding a niche for their survival characterized by severe physical or chemical parameters. The present work focuses on the structural investigation of the LPS from *Loktanella rosea*, a marine Gram-negative bacterium. Through chemical analysis, 2D nuclear magnetic resonance and matrix-assisted laser desorption ionization mass spectrometry investigations, a unique LPS carbohydrate backbone has been defined. The lipid A skeleton consists of a trisaccharide backbone lacking the typical phosphate groups and is characterized by two β-glucosamines and an α-galacturonic acid. The core region is built up of three ulosonic acids, with two 3-deoxy-D-manno-oct-2-ulopyranosonic acid residues, one of which is carrying a neuraminic acid. This carbohydrate structure is an exceptional variation from the typical architectural skeleton of endotoxins which consequently implies a very different biosynthesis.

**Keywords:** lipid A/lipopolysaccharide/*Loktanella rosea*/NMR spectroscopy

Introduction

Gram-negative bacteria possess an asymmetric outer membrane which surrounds a thin layer of peptidoglycan. In these microorganisms, the external membrane is the first and immediate line of defense against harsh environment and antimicrobial molecules and makes the cytoplasmic membrane more efficiently protected. Lipopolysaccharides (LPS), the major components of the outer membrane, are pivot molecules for bacterial life and possess within their chemical structure some invariable moieties that are strictly savied by any biosynthesis such as the lipid A carbohydrate backbone which is built up of two amino-sugars, one β− and another α-anomeric configured. Actually, in the most cases, it possesses a β−GlcN-(1→6)-α-GlcN disaccharide backbone phosphorylated at positions 1 and 4′ and acylated with primary 3-hydroxy fatty acids at positions 2 and 3 of both glucosamine (GlcN) residues; the hydroxyl groups of the primary fatty acids can be further acylated by secondary acyl moieties (Holst and Molinaro 2009). LPS lacking the O-chain portion are termed rough LPS (R-LPS) or lipo-oligosaccharides (LOS). In the core oligosaccharide region, the inner and outer core oligosaccharide regions are present: the inner core, proximal to the lipid A, consists of archetypal residues among which the 3-deoxy-D-manno-oct-2-ulopyranosonic acid (Kdo), a chemical hallmark for Gram-negative bacteria; moreover, the inner core is often rich in negatively charged residues as phosphate groups. The outer core region is more variable and is usually composed by hexoses.

The present work focuses on the structural investigation of the LPS from *Loktanella rosea*, a mesophilic and chemo-organotroph marine Gram-negative bacterium (Ivanova et al. 2005; Van Trappen et al. 2004) isolated from sediments of Chazma Bay, Sea of Japan. The genus *Loktanella* was created in 2004 in order to classify some new heterotrophic *Alphaproteobacteria* collected from Antarctic lakes.

The LPS from *L. rosea* has been defined through sugar analysis, 2D nuclear magnetic resonance (NMR) and matrix-assisted laser desorption ionization (MALDI) mass spectrometry investigation. A unique highly negatively charged carbohydrate backbone has been identified. The lipid A skeleton lacks the typical phosphate groups and is characterized by two β−GlcNs and an α-galacturonic acid (GalA). This is the first example of a lipid A saccharide backbone in which the α-GlcN-phosphate residue is replaced by a β−GlcN-(1→1)-α-GalA in a mixed trehalose-like linkage. The core region is built up of three ulosonic acids, with two 3-deoxy-D-manno-oct-2-ulosonic acid residues, one of which is carrying a neuraminic acid (Neu). The overall carbohydrate structure is an exceptional variation from the typical
architectural skeleton of endotoxins and also implies a very different biosynthesis.

**Results**

**Extraction of LOS and compositional analysis**

Dried cells were extracted and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) of *L. rosea* LPS fraction revealed that it was an R-LPS, i.e., an LOS. Structural investigations on extracted LOS revealed the following monosaccharide composition: 2-amino-2-deoxy-D-glucose (D-GlcN), D-GalA, 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and 5-amino-3,5-dideoxy-D-glycero-D-galacto-non-2-ulosonic acid (Neu). Methylation analysis showed the presence of 6-substituted GlcN, terminal GalA, 4,8-di-substituted Kdo and terminal Neu, all in pyranose form. Fatty acids analysis revealed the presence of (R)-3-hydroxydecanoic (C10:0 (3-OH)) and dodecanoic acid (C12:0), confirming chemotaxonomic data (Ivanova et al. 2005). Phosphate assays gave negative results.

**Isolation and structural elucidation of fully deacylated oligosaccharide (OS) from *L. rosea***

The LOS was completely deacylated with a strong alkaline treatment as reported (Holst and Molinaro 2009) and then purified by gel-permeation chromatography that yielded the oligosaccharide fraction (OS). The monosaccharide analysis of OS gave the same results of intact LOS that means that no sugar was lost or altered following chemical treatment. The oligosaccharide product was characterized through a full 2D NMR analysis and supported by mass spectrometry (MS) data. In the anomeric region of OS $^1$H NMR spectrum (Figure 1), three anomic signals were identified (A–C) (Table I). Furthermore, the signals at 1.62/2.67, 1.65/2.05 and 1.73/2.01 ppm were identified as three pairs of H-3 methylene protons belonging to the non-substituted Neu (D) and Kdo (E) residues and to the 4,8-di-substituted-D-Kdo residue (F). A complete assignment of the spin systems was carried out assigning the proton resonances in double quantum filtered correlated spectroscopy (DQF-COSY) and total correlation spectroscopy (TOCSY) spectra and the carbon resonances in the heteronuclear single quantum coherence (HSQC) spectrum.
The anomic configuration of each aldose monosaccharide unit was assigned on the basis of the $^{3}J_{H_{1},H_{2}}$ coupling constants obtained by the DQF-COSY, by the $^{1}J_{C_{1},H_{1}}$ derived by F2-coupled HSQC (Figure 3, main text and Table I) and confirmed by the intra-residual nuclear Overhauser effect (NOE) contacts observed in the rotating frame nuclear Overhauser effect spectroscopy (ROESY) and nuclear Overhauser enhancement spectroscopy (NOESY) spectra (Figure 2, main text and Table I), whereas the values of vicinal $^{3}J_{H_{1},H_{2}}$ ring coupling constants allowed the identification of the relative configuration of hydroxyl groups within each sugar residue.

Spin system A (H-1 at 5.23ppm) was identified as the $\alpha$-GalA. Its $^{3}J_{H_{13},H_{14}}$ and $^{3}J_{H_{14},H_{15}}$ values (3 and 1Hz, respectively) were diagnostic of a galacto configuration; the chemical shifts of H-1 and C-1 (5.23 and 100.6ppm), the $^{1}J_{C_{1},H_{1}}$ and $^{3}J_{H_{1},H_{2}}$ values (172.5 and 3.6Hz, respectively) and the intra-residual NOE contact of H-1 with H-2 were all in agreement with an $\alpha$-anomeric configuration. Furthermore, in the $^{1}H,^{13}C$ heteronuclear multiple bond correlation (HMBC) spectrum (Figure 3), it was a visible correlation of H-5 (4.33ppm) with a carboxyl group (175.6ppm), thus defining residue A as a uronic acid residue.

Spin systems B and C (H-1 at 4.86 and 4.48ppm, respectively) were both identified as GlcN residues as indicated by their large $^{3}J_{H_{1},H_{2}}$ coupling constants of ring protons (around 10Hz). Furthermore, the $^{1}H,^{13}C$ HSQC spectrum showed the correlation of both H-2 B (3.09ppm) and H-2 C (2.92ppm) with nitrogen bearing carbon signals at 55.4 and at 55.7ppm, respectively. The chemical shifts of H-1 and C-1 of B (4.86 and 99.6ppm) and C (4.48 and 100.8ppm), the $^{1}J_{C_{1},H_{1}}$ (162.0Hz for B and 163.1Hz for C, Figure 1) and the intra-residual NOE contacts (Figure 2, main text) of H-1 with H-3 and H-5 univocally and surprisingly demonstrated $\beta$-anomeric configuration for both residues.

Spin systems E and F were identified as Kdo residues and were assigned starting from the diastereotopic H-3 methylene protons, resonating in a shielded region at 1.65/2.05 and 1.73/2.01ppm (H-3ax and H-3eq, respectively, for E and F). The $\alpha$-anomeric configuration at C-2 for both residues was attributed by the chemical shift values of H-3 and by $^{3}J_{H_{7},H_{8a}}$ and $^{3}J_{H_{7},H_{8b}}$.
The primary structure of the oligosaccharide fraction OS derived by NMR investigations is reported in Figure 4 and sketched below:

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<tr>
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<tr>
<td>α-Neu-(2→8)[α-D-Kdo-(2→4)]β-1-D-GlcN-(1→6)-β-D-GalA</td>
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The OS oligosaccharide structure was also confirmed through MALDI mass spectrometry. The mass spectrum acquired in negative polarity gave a peak at m/z 1186.3, matching with the ion [OS–H₂O]⁻ (Figure 5), composed by a GalA, an Neu, two Kdo and two GlcN residues.

**Discussion**

The LOS turned out to be characterized by a novel and unique hexasaccharide skeleton comprising (i) a very small core region exclusively composed of ulosonic sugars and containing an Neu attached to a Kdo unit: α-Neu-(2→8)[α-D-Kdo-(2→4)]-α-D-Kdo-(2→) and (ii) an exceptional lipid A backbone: β-D-GlcN-(1→6)-β-D-GlcN-(1→1)-α-D-GalA in which both GlcN residues were present with a β-anomeric configuration. Moreover, it lacked the classical phosphate residues at O-4’ and O-1, this latter was replaced by an α-GalA linked in a mixed trehalose-like linkage. At the best of our knowledge, this kind of glycosydic linkage was never found in biomolecules; its presence obviously implies profound biosynthetic differences from the canonical LPS lipid A pathway (Raetz et al. 2007). In fact, current studies showed that, in Gram-negative bacteria, the initial steps of lipid A biosynthesis are common and catalyzed by a set of intracellular enzymes constitutively expressed and generally not subjected to regulation (Raetz et al. 2007). These earlier stages lead to (Kdo)₂LipidIVₐ intermediate constituted by a β-(1→6)-GlcN disaccharide backbone phosphorylated at positions 1 of the reducing α-configured unit (GlcN I) and at 4’ of the non-reducing β-configured unit (GlcN II), acylated at positions 2 and 3 by 3-hydroxy fatty
acids and glycosylated at O-6 of GlcN II by an α-(2→4)-Kdo disaccharide. Instead, lipid A modifications, which characterize the maturation process, are catalyzed by extracytoplasmic enzymes and are different from bacteria to bacteria since they are generally induced and strongly influenced by growth conditions. For instance, it has been proven the existence of two inner membrane phosphatases, LpxE and LpxF, as described for the highly infectious human pathogen *Francisella tularensis* lipid A, that act on the periplasmic surface of the inner membrane and promote the cleavage of the phosphate groups on the lipid A moiety (Raetz et al. 2007). Likewise, in *L. rosea* the lipid A precursor could be processed by two homologues of 1- and 4′-phosphatase, thus explaining the lack of both phosphate groups. For what concerns the presence of the two GlcNs exclusively in the β-anomeric orientation, it can be speculated that the GalA tranferase only recognizes the equatorial GlcN I free anomer obtained by phosphatase cleavage or, conversely, it catalyzes the transfer of GalA on a different substrate, the canonical phosphorylated α-GlcN, thus achieving an inversion of configuration at the anomeric position.

The replacement of phosphates with GalA residues has been already detected in lipid A deriving from other bacterial lipid A (De Castro et al. 2008), for example, a lipid A with a 2,3-diamino-2,3-dideoxyglucose (DAG) disaccharide backbone substituted at both 1 and 4′ positions by α-D-GalA moieties has been identified for the first time in the extremely thermophilic bacterium *Aquifex pyrophilus* (Plötz et al. 2000); further, the lipid A from the symbiotic bacterium *Mesorhizobium huakuii* (Choma and Sowinski 2004) consists of a DAG backbone.

![Fig. 4. The structure of the oligosaccharide isolated by the LOS from *L. rosea*. Monosaccharides are labeled as in Table I.](image)

![Fig. 5. Negative-ion MALDI mass spectrum of the OS obtained after O/N deacylation of *L. rosea* R-LPS. The mass spectrum acquired in negative polarity shows a peak at *m/z* 1186.3, matching with the ion [OS–H₂O]⁻. The ion [OS–H₂O + Na]⁻ is also visible.](image)
bearing an α-GalA only at O-1 of distal DAG residue. In other lipid A belonging to Rhizobiaceae, as for Rhizobium etli and Rhizobium leguminosarum (De Castro et al., 2008; Que et al. 2000; Jeyaretnam et al. 2002), LPS lacking phosphate groups are constituted by a mixture of species bearing α-GalA moiety at 4′ and carrying the GlcN I oxidized to aminoglucosinate; for what concerns GalA units, in a later step of the biosynthesis (Kdo)2-lipid IVa precursor is processed by 1-phosphatase and then by a GalA tranferase (Raetz et al. 2007). The uronic acids are known to be more resistant to the hydrolysis than the phosphate groups thus reinforcing the lipid A and, consequently, membrane stability. The absence of the phosphate group at position 4′ has been reported so far in few lipid A species as the case of Azospirillum lipoferum (Choma and Komaniecka 2008), a plant growth-promoting Rhizobacterium, and of the genus Francisella as mentioned above (Raetz et al. 2007; Vinogradov et al. 2002; Wang et al. 2006), whose absence strongly contributes to the LPS low endotoxicity (Loppnow et al. 1989). The reason of the absence in L. rosea of the 4′ phosphate group is unknown.

The “wrong” anomeric configuration on the GlcN I might be crucial in the supramolecular assembly of the LOS and so in determining the rheological and biological properties of outer membrane. In fact, the intrinsic conformation of the lipid A strongly influences its supramolecular properties, and the inclination angle of the glycoside linkage allows the reducing GlcN to be exposed toward the external environment (Brandenburg and Wiese 2004).

As for the core region of L. rosea LPS, even its carbohydrate sequence is very peculiar. Actually, the lipid A moiety is glycosylated by a Kdo unit which is further substituted at O-4 and O-8 positions by two 2-ulosonic residues: a further Kdo, sitting at O-4 and an Neu at position O-8. Neu5Ac residues have been found in the outer core region, as the case of Campylobacter jejuni, Helicobacter pylori (Holst 2002) that can express either Lewis antigens, or resemble structural similarities with glycosphingolipids of the ganglioside group, all attempts to evade host immune response although has never been found in an environmental or marine bacterial LPS. Chlamydia trachomatis produces a deep-R-LPS characterized by a linear sequence of three Kdo residues: α-Kdo-(2→8)-α-Kdo-(2→4)-α-Kdo-(2→6)-lipid A, whereas in Chlamydia psittaci a non-stoichiometric fourth Kdo is located at O-4 of the second one (Holst 2002). However, in few cases, the first Kdo has been found carrying two ulosonic acid residues, as the case of the LPS from Acinetobacter lwofii F7816 (Hanuszkiwicz et al. 2008) but never an Neu residue has been found directly linked to the Kdo residue.

The deep-R-LPS from L. rosea has a short oligosaccharide moiety that at physiological pH is negatively charged: overall, of six residues, four bear negatively charged carboxyl groups. This peculiar sugar backbone conforms to the whole LOS the ability to strongly and extensively interact with divalent cations, especially Ca2+ and Mg2+, on the surface of the outer membrane. The establishment of such wide network of ionic bridges can contribute to the network of interactions that shield the outer membrane and give rigidity and resistance to bacterial cells. This could give a key explanation on why this class of microorganisms is allowed to survive in conditions of unusual pressure and salinity.

Materials and methods

Bacterial growth and LOS extraction

The type strain of L. rosea KMM 6003T was cultivated on a liquid medium containing (g/L) pepton (5), yeast extract (1), citric acid (0.1), FeSO4 (0.07) and sea water (1L), pH 7.6. Cells were collected by centrifugation, washed with water and next dried with acetone (three times) to yield around 10g of dried cells from 20L of the cultural fluid.

Dried cells (6g) were treated with Westphal method as reported (Westphal and Jann 1965). In details, they were suspended in hot 90% phenol and extracted three times with hot water. Then the two phases obtained were dialyzed and purified through enzymatic treatment, the LOS was present only in the water phase (3% of bacterial dried mass). After this, an SDS–PAGE (13.5%) was performed in order to detect LPS and LOS after staining with silver nitrate.

Isolation of oligosaccharide OS

An aliquot of LOS (10mg) was dissolved in 1mL of anhydrous hydrazine, stirred at 37°C for 90min, cooled, poured into ice-cold acetone and allowed to precipitate. The precipitate was collected by centrifugation (4°C, 7000rpm, 30min), washed twice with ice-cold acetone and dried. The sample was further N-deacylated adding 4M KOH (120°C, 16h). After desalting on a Sephadex G-10 column, the resulting oligosaccharide was purified on a Biogel P-2 column.

General and analytical methods

Determination of sugar residues and of their absolute configuration, gas–liquid chromatography and gas–liquid chromatography-mass spectrometry (GLC-MS) were all carried out as described (Leontin and Lönngren 1978). Monosaccharides were identified as acetylated O-methyl glycosides derivatives. After methanalysis (2M HCl/MeOH, 85°C, 2h) and acetylation with acetic anhydride in pyridine (85°C, 30 min), the sample was analyzed by GLC-MS. Linkage analysis was carried out by methylation of the complete core region as described (Hakomori 1964). The sample was hydrolyzed with 2M trifluoroacetic acid (100°C, 2h), carbonyl-reduced, carboxy-methylated, carboxy-reduced with NaBD4 (4°C, 20h), acetylated and analyzed by GLC-MS.

Total fatty acid content was obtained by acid hydrolysis. LOS was first treated with 4M HCl (4h, 100°C) and then neutralized with 5M NaOH (30min, 100°C). Fatty acids were then extracted in CHCl3, methylated with diazomethane and analyzed by GLC-MS. The absolute configuration of fatty acids was determined as described (Rietschel 1976).

NMR spectroscopy

For structural assignments, 1D and 2D 1H NMR spectra were recorded on a solution of 0.5mg in 0.5mL of D2O (5), yeast extract (1), citric acid (0.1), FeSO4 (0.07) and sea water (1L), pH 7.6, at pD 7 (uncorrected value) on Bruker 600 DRX equipped with a cryoprobe. Spectra were calibrated with internal acetone [δH 2.225, δC 31.45]. 2D DQF-COSY spectra were acquired with 4096 × 512 data points in both F2 and F1 dimensions. Quadrature indirect dimensions were achieved through States-time proportional phase incrementation method; spectra were processed applying a Qsine function to both dimensions, and data matrix was zero-filled by factor of 2 before Fourier trans-
formation. Coupling constants were determined on a first order basis from 2D phase-sensitive DQF-COSY (Piantini et al. 1982; Rance et al. 1983). NOESY and ROESY spectra were measured using data sets \((t_1 \times t_2)\) of 4096 × 256 points, mixing times of 200–400 ms were used. TOCSY experiments were performed with a spinlock time of 100 ms, using data sets \((t_1 \times t_2)\) of 4096 × 256 points. In these homonuclear experiments, the data matrix was zero-filled in the \(F_1\) dimension to give a matrix of 4096 × 2048 points and was resolution enhanced in both dimensions by a 90° shifted Qsine function before Fourier transformation. HSQC, HSQC-TOCSY and HMBC experiments were measured in the \(^1\)H-detected mode via single quantum coherence with proton decoupling in the \(^1\)H domain and in negative polarity on a Perseptive (Framingham, MA) Voyager STR instrument equipped with delayed extraction technology. Such acidic OS was first converted in the ammonium form by a home-made miniaturized column of cation-exchange resin (Dowex 50WX8-200, Sigma Aldrich) previously equilibrated with a 5% NH\(_4\)OH solution. The obtained sample was analyzed in a matrix solution of dihydroxybenzoic acid (50 mg/ml) in trifluoroacetic acid 0.1%-acetonitrile 80/20, utilizing the classic dried drop method: 1 \(\mu\)L of a sample/matrix solution mixture (1:1, v/v) was deposited onto the MALDI sample plate utilizing data sets of 2048 × 256 points. Experiments were carried out in the phase-sensitive mode according to the method of States et al. (1982). \(^1\)H, \(^13\)C HMBC spectra were optimized for 4, 6 and 10 Hz coupling constants, and \(^1\)H, \(^31\)P HSQC for 8 Hz coupling constant. In all heteronuclear experiments, the data matrix was extended to 2048 × 1024 points using forward linear prediction extrapolation. F2-coupled HSQC was recorded with 4096 × 128 data points, the free induction decay was apodized in both dimensions with a 90° shifted cosine function and zero-filled to give, after Fourier transformation, a 2D spectrum of 8192 × 512.

Matrix-assisted laser desorption/ionization time-of-flight analysis

MS analysis of the core oligosaccharide was performed in reflector mode and in negative polarity on a Perseptive (Framingham, MA) Voyager STR instrument equipped with delayed extraction technology. Such acidic OS was first converted in the ammonium form by a home-made miniaturized column of cation-exchange resin (Dowex 50WX8-200, Sigma Aldrich) previously equilibrated with a 5% NH\(_4\)OH solution. The obtained sample was analyzed in a matrix solution of dihydroxybenzoic acid (50 mg/ml) in trifluoroacetic acid 0.1%-acetonitrile 80/20, utilizing the classic dried drop method: 1 \(\mu\)L of a sample/matrix solution mixture (1:1, v/v) was deposited onto the MALDI sample plate and left to dry at room temperature.

Conflict of interest statement

None declared.

Abbreviations

DAG, 2,3-diamino-2,3-dideoxyglucose; DQF-COSY, double quantum filtered correlated spectroscopy; GalA, galacturonic acid; GlCN, glucosamine; GLC-MS, gas–liquid chromatography-mass spectrometry; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; LOS, lipooligosaccharides; LPS, lipopolysaccharides; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; Neu, neuraminic acid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; ROESY, rotating frame nuclear Overhauser effect spectroscopy; R-LPS, rough LPS; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TOCSY, total correlation spectroscopy.

References


