Structure and inflammatory activity of the LPS isolated from Acetobacter pasteurianus CIP103108

To cite this version:

HAL Id: hal-01956104
https://hal.archives-ouvertes.fr/hal-01956104
Submitted on 14 Dec 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives| 4.0 International License
Structure and inflammatory activity of the LPS isolated from Acetobacter pasteurianus CIP103108

Mateusz Pallach a, Flaviana Di Lorenzo a, Fabio Alessandro Facchini b, Djamel Gully d, Eric Giraud d, Francesco Peri b, Katarzyna A. Duda c,e, Antonio Molinaro a, Alba Silipo a

a Department of Chemical Sciences, University of Napoli Federico II, Complesso Universitario Monte Sant’Angelo, Via Cintia 4, I-80126 Napoli, Italy
b Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milano, Italy
c Junior Group of Allergobiochemistry, Research Center Borstel, Leibniz Lung Center, 23845 Borstel, Germany
d IRD, Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), UMR IRD/SupAgro/INRA/CIRAD, TA-A82/J – Campus de Baillarguet, 34398 Montpellier Cedex 5, France
e Airway Research Center North (ARCN), German Center for Lung Research, Germany

A R T I C L E   I N F O
Article history:
Received 16 May 2018
Received in revised form 6 August 2018
Accepted 7 August 2018
Available online 8 August 2018

Keywords:
Lipopolysaccharides (LPS)
Gram-negative bacteria
Innate immunity
MD2-TLR4
Acetobacter pasteurianus

A B S T R A C T
Acetobacter pasteurianus is an acetic acid-producing Gram-negative bacterium commonly found associated with plants and plant products and widely used in the production of fermented foods, such as kefir and vinegar. Due to the acid conditions of the bacteria living habitat, uncommon structural features composing its cell envelope are expected. In the present work we have investigated the A. pasteurianus CIP103108 lipopolysaccharide (LPS) structure and immunoactivity. The structure of the lipid A and of two different O-polysaccharides was assessed. Furthermore, immunological studies with human cells showed a low immunostimulant activity of the isolated LPS, in addition to a slight capability to lower the NF-κB activation upon stimulation by toxic LPS.

© 2018 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Gram-negative bacteria can be found in different habitats, many used within human activities as production and processing of traditional foods and beverages, in biotechnology and genetic engineering, in the production of pharmaceuticals [1]. A fascinating example is given by Acetobacter pasteurianus, an acetic acid-producing bacterium [2,3] commonly found associated with plants and plant products and present on sugar-rich substrates such as fruits, flowers and vegetables. A. pasteurianus is used in the production of fermented foods, such as kefir and vinegar [4] and, together with other microorganisms such as Aspergillus oryzae, Saccharomyces cerevisiae and lactic acid bacteria, is used in production process of Japanese black rice vinegar, called kurozu [5], whose consumption is believed to carry significant benefits to human health in cancer prevention and relief of hypertension [6].

A. pasteurianus, as most Gram-negative bacteria, possesses an outer membrane which predominantly contains lipopolysaccharides (LPS) on the outer leaflet [7]. The LPS is an amphiphilic molecule typically constituted by three different domains in the so called “smooth” form (S-LPS): the glycolipid anchoring the LPS to the outer membrane is called lipid A and is commonly made up of a variously acylated bis-phosphorylated glucosamine disaccharide backbone [8,9]. A core oligosaccharide (core OS) is attached to the lipid A through the 3-deoxy-o-manno-oct-2-ulosonic acid (Kdo), a sugar marker of Gram-negative bacteria. To the core OS is, in turn, connected a polysaccharide termed O-specific polysaccharide or O-chain [10]. Being the most exposed moiety of the LPS, the O-chain is in direct contact with the surrounding environment and is involved in interaction with host cells in case of pathogen and symbiotic bacteria [11,12].

The LPS is classified as a microbe associated molecular pattern (MAMP) and it can activate the innate immune response following its recognition by immune receptors referred to as pattern recognition receptors (PRR) [8]. In mammals, LPS is recognized by the Toll-like Receptor 4/Myeloid differentiation factor-2 (TLR4/MD-2) receptor complex [13,14]. The center of LPS immunoactivity lies in the lipid A portion, specifically bound by the TLR4/MD-2 complex and differently activating the production of pro-inflammatory cytokines depending on its fine structure [8]. This event is beneficial to the host, enhancing resistance to infecting microbes, nonetheless excessive activation of TLR4/MD-2 signalling by agonistic LPS leads to massive and uncontrolled cytokines release finally leading to septic shock and multi-organ failure. Intriguingly, some lipid A showed a weak or no immunopotency and, in
some cases, an inhibition of the toxic effects of pathogenic LPS on TLR4/ MD-2 expressing cells [15–21]. Given this premise, investigation on novel lipid A structures, which might possess inhibitory activity towards TLR4/MD-2 mediated release of pro-inflammatory cytokines by toxic lipid A, are considered of high relevance.

Here, we report the structural characterization of LPS O-antigen and lipid A from A. pasteurianus strain CIP 103108, performed by the combined use of chemical, NMR spectroscopy and mass spectrometry (MS) approaches. Furthermore, we also evaluated the immunostimulant activity of the isolated LPS on human cell lines showing interesting inhibitory effects against potent toxic LPS.

2. Experimental

2.1. Extraction and purification of A. pasteurianus CIP103108 LPS

Dried A. pasteurianus CIP103108 cells were washed with ethanol, water and acetone. Afterwards, the hot phenol/water procedure was employed to extract the LPS material [22]. After dialysis against distilled water, each phase, namely the water phase (WP) and phenol phase (PP) underwent enzymatic digestion using DNAse, RNAse and proteinase K. WP and PP were analyzed by 14% sodium deoxycholate-polyacrylamide gel electrophoresis (DOC-PAGE) analysis followed by silver nitrate gel staining. A size exclusion chromatography on a Sephacryl S200 column in denaturing conditions (0.25% DOC at pH 8) was performed to further purify the LPS material. Fractions were lyophilized and DOC was removed by ethanol washing, followed by extensive dialysis against distilled water. Each fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.2. Monosaccharide compositional analysis of A. pasteurianus CIP103108 LPS

The neutral sugar constitution was established using acetylated alditols method [23]. An aliquot of sample was hydrolyzed using 2 M TFA solution at 120 °C for 120 min. The so obtained monosaccharides were reduced to alditols using NaBH₄ and then acetylated twice with equal amounts of acetic anhydride in pyridine at 85 °C for 10 min. The acetylated alditols were analyzed by gas-liquid chromatography/mass spectrometry (GLC-MS). For quantification 5 μg of xylose as internal standard was added to the sample. Amino sugars were characterized using 16 h hydrolysis in 4 M HCl at 100 °C, followed by reduction and acetylation as described above. The uronic acids composition was characterized using the same method as neutral sugars but preceded by additional reduction of the carboxyl group with NaBD₄. Uronic acids were identified using acetylated methyl glycosides method [23]. The sample underwent 16 h methanolysis with 1.25 M HCl/MeOH at 85 °C, therefore was acetylated as described above. Linkage analysis was performed using Cucanu-Kerek's method [24]. An aliquot of LPS was suspended in DMSO and methylated with iodomethane in presence of NaOH. Therefore, the product was hydrolyzed with 2 M TFA for 2 h at 120 °C, reduced with NaBD₄ and acetylated with acetic anhydride in pyridine. Partially methylated acetylated alditols were analyzed by GC–MS.

2.3. Fatty acid compositional analysis of A. pasteurianus CIP103108 LPS

Total fatty acid content was characterized as fatty acid methyl esters. An aliquot of LPS was hydrolyzed using 4 M HCl at 100 °C for 4 h. After the sample was neutralized with 5 M NaOH for 30 min at 100 °C, liberated fatty acids were extracted with chloroform and methylated using diazomethane at room temperature. In order to elucidate the position of hydroxy group of hydroxy fatty acids 2 h derivatization with N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) at 65 °C was performed. Ester linked fatty acid content was characterized using 30 min hydrolysis with 0.5 M NaOH in water:methanol solution (1:1) at 100 °C for an hour. The ester linked fatty acids were extracted and methylated as described above. Fatty acid methyl esters were detected by GLC [HP 6890 N gas chromatograph with FID and a column (Agilent Technologies, 30 m × 0.25 mm, film thickness 0.25 μm) of Phenyl MethylSiloxane HP-5MS] with temperature program as follows: 120 °C for 3 min, then 5 °C min⁻¹ to 320 °C.

2.4. Isolation of lipid A and O-poly saccharide moieties

To separate the lipid A and the polysaccharide portion constituting the A. pasteurianus CIP103108 LPS, a mild acid hydrolysis with acetate buffer (pH 4.4) for 5 h at 100 °C was performed. The glycolipid part was separated from the hydrophilic carbohydrate part using Bligh-Dyer’s method [25]. Briefly, a mixture of methanol and chloroform was added to the sample to reach a ratio of chloroform/methanol/hydrolysat 2:1:8 (v/v/v). The mixture was shaken and centrifuged. The water-soluble phase was then applied on a GE Healthcare Life Sciences Superdex 75 column eluted with 50 mM ammonium bicarbonate. The chromatography fractions were then monitored by NMR spectroscopy. The lipid A part was also collected, lyophilized and analyzed by MALDI MS.

2.5. MALDI-TOF MS and MS/MS

Reflectron MALDI-TOF MS and MS/MS analysis were performed on an ABSCIEX TOF/TOF™ 5800 Applied Biosystems mass spectrometer equipped with an Nd:YLF laser with a λ of 345 nm, a ~500-ps pulse length and a repetition rate of up to 1000 Hz. The lipid A fraction was dissolved in CHCl₃/MeOH (1:1, v/v) as previously described [26,27]. The matrix was the trihydroxyacetophenone (THAP) dissolved in CH₃OH:0.1% TFA/CH₃CN (7:2.1, v/v/v) at a concentration of 75 mg ml⁻¹. Lipid A solution and the matrix solution, both 0.5 μl, were deposited on the MALDI plate and dried at room temperature. All of spectra were a result of the accumulation of 1500 laser shots, whereas 6000–7000 shots were summed for the MS/MS data acquisitions [28].

2.6. NMR spectroscopy

For structural assignments of isolated polysaccharides ¹H NMR and 2D NMR spectra were recorded in D₂O at 278 K at pD 7 with a cryoprobe-equipped Bruker 600 DRX spectrometer. Total correlation spectroscopy (TOCSY) experiments were performed with spinlock times of 100 ms using data sets (t1 × t2) of 4096 × 512 points. Rotating-frame Overhauser enhancement spectroscopy (ROESY) and nuclear Overhauser enhancement spectroscopy (NOESY) experiments were performed using data sets (t1 × t2) of 4096 × 512 points with mixing times between 100 and 400 ms. Double-quantum-filtered phase-sensitive correlation spectroscopy (DQF-COSY) experiments were performed using data sets of 4096 × 912 points. The data matrix in all homonuclear experiments was zero-filled in both dimensions to give a matrix of 4 K × 2 K points and was resolution-enhanced in both dimensions using a cosinebell function before Fourier transformation. Coupling constants were determined by 2D phase-sensitive DQF-COSY measurements [29,30]. Heteronuclear single–quantum coherence (HSQC) and heteronuclear multiple–bond correlation (HMBC) experiments were performed in the ¹H-detection mode by single–quantum coherence with proton decoupling in the ¹³C domain using data sets of 2048 × 400 points. HSQC was performed using sensitivity improvement and in the phase-sensitive mode using echo/antecho gradient selection, with multiplicity editing during the selection step [31]. HMBC was optimized on long-range coupling constants, with a low-pass filter to suppress one-bond correlations, using gradient pulses for selection. Moreover, a 80 ms delay was used for the evolution of long-range correlations. HMBC spectra were optimized for 6–15 Hz coupling constants. The data matrix in all the heteronuclear experiments was extended to
2048 × 1024 points by using a forward linear prediction extrapolation [32].

2.7. TLR4 activation assay with HEK-Blue hTLR4 cells

HEK-Blue hTLR4 cells were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, antibiotics and 1× HEK-Blue™ Selection (Invivogen). Cells were detached using a cell scraper, counted and seeded in a 96-well multiwell plate at a density of 4 × 10^4 cells per well. After overnight incubation (37 °C, 5% CO₂, 95% humidity), supernatants were replaced with only DMEM w/o Phenol Red. The activation assay was conducted stimulating cells with different concentrations (0.1, 1, 10, 100 ng/ml) of A. pasteurianus CIP103108 LPS or E. coli O55:B5 LPS (Sigma-Aldrich) for 16 h. While, the competition assay was performed pre-incubating cells with A. pasteurianus CIP103108 LPS (0.1, 1, 10, 100 ng/ml) for 30 min and then adding cells 1 ng/ml of E. coli O55:B5 LPS. After 16 h of incubation SEAP-containing supernatants were collected and incubated with paranitrophenylphosphate (pNPP) for 2–4 h in the dark at room temperature. The wells optical density (OD) was determined using a microplate reader set to 405 nm. Regarding activation assay, SEAP levels (OD about 75%.

3. Results

3.1. LPS isolation, purification and compositional analysis

The LPS of A. pasteurianus CIP103108 was isolated using the hot phenol-water procedure [22]; DOC-PAGE analysis revealed the presence of a mixture of R-type LPS (the O-polysaccharide lacking form) and S-type LPS in the extract (Fig. S1). Purification steps, including enzymatic digestion followed by size exclusion chromatography in denaturing conditions, allowed to isolate S-type LPS and R-type LPS (Fig. S1).

In the following conditions, allowed to isolate S-type LPS and R-type LPS (Fig. S1). Purification steps, including enzymatic digestion followed by size exclusion chromatography in denaturing conditions, allowed to isolate S-type LPS and R-type LPS (Fig. S1).

3.2. Isolation, purification of lipid A and O-polysaccharide fractions

An aliquot of pure S-LPS underwent a mild acid hydrolysis in order to separate the lipid A moiety from the sugar part, therefore the O-chain containing fraction was recovered and further purified using gel filtration chromatography. NMR spectra and compositional analysis revealed the presence of two polysaccharide containing fractions, PS1 and PS2 (Fig. 1). PS2 was eluted first and thus possessed higher molecular mass compared to PS1. The two fractions were characterized as follows.

3.3. Structural characterization of PS1 and PS2

The spin systems and monosaccharide sequence were characterized using a combination of 1D and 2D NMR spectra. The anomeric configuration of the monosaccharides were assigned on the basis of the ^3J_H1,H2 and ^3J_C1,H1 coupling constants and confirmed by the intra-residual NOE contacts; the vicinal ^3J_H1,H2 coupling constants and intra residual NOE contacts revealed the relative configuration of the sugar residues.

The anomeric region of PS1 revealed the presence of four different anomeric signals (Fig. 1a, Table 1). Spin systems A, B and D at 5.21, 5.07 and 4.97 ppm were identified as β-D-galactopyranosyl units. The downfield shift of all carbon signals, the strong downfield shift of carbon C-4 and the intra-residual long-range correlation between positions 1 and 4 found in the HMBC spectrum (Fig. 2) were diagnostic of a furanose ring [33]. The β-anomeric configuration was assessed on the basis of 1^3C chemical shift [34]. Spin system C was identified as an α-d-glucopyranose (Glc); its anomeric configuration was assigned on the basis of ^3J_C1,H1 (Table 1) and the ^3J_H1,H2 (~4 Hz) whereas high ^3J_H1,H2 (9.1 Hz) and intra-residual NOE connectivity of H-3 and H-2 confirmed the gluco configuration.

The downfield shift of carbon resonances suggested glycosylated positions at O-2 and O-6 of A, O-6 of B and O-3 of D. Spin system C was identified as a terminal Glc residue. The inter-residual connectivity (Fig. S2) along with long-range correlations found on the HMBC spectrum (Fig. 2) of A/D, B/D, D/A and C/A allowed to define the saccharide sequence. Residue C was found to be not stoichiometrically linked at position O-2 of the galactofuranose residue A, substituted for about 75%.

<table>
<thead>
<tr>
<th>A/B</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>-6</td>
<td>-(d-Glc-p(α1-2))−Gal/(β1-3)-D-Gal/(β1-C*)</td>
</tr>
</tbody>
</table>

*non-stoichiometric substitution

The NMR spectrum of PS2 unveiled the presence of five anomeric signals (Figs. 1b and 3, Table 2). Spin systems A and B, H-1 at 5.10 and 5.03 ppm respectively (Table 2), were characterized as α-galactopyranosyl units. In both cases, correlations of the ring protons with methyl signals were visible from the TOCSY spectrum. The manno configuration of both spin systems was assigned on the basis of C-6a and C-6b coupling constants, the α-configuration from intra-residual NOE contact of H-1 with H-2 and the chemical shift value of C-5. Analogously, spin systems C and D were assigned as α-glucopyranose units while spin system E was identified as α-mannopyranose. The downfield shift of carbon resonances identified the glycosylated positions: O-2 and O-4 of A, O-2 of B, O-6 of D and O-2 of E. Unit C was identified as a terminal residue. The inter-residual NOE contacts observed from the ROESY spectrum (Fig. S3) together with the long-range correlations, derived from the HMBC spectrum (Fig. 3), between A1 with E2; E1 with D6, D1 with B2; B1 with A2 so as C1 with A4 confirmed the following repeating unit for the polysaccharide PS2:

C

-6|-(d-Glc-p(α1-2)|-Rhap-(α1-2)−[GlcA-(α1-4)]-|−Rhap-(α1-2)-D-Manp-(α1-2) |

3.4. MALDI MS on A. pasteurianus CIP103108 lipid A

A. pasteurianus CIP103108 lipid A structure was investigated by MALDI-MS and MS/MS. The negative-ion MALDI MS spectrum (Fig. 4) revealed the presence of several cluster of peaks in the mass range m/z 1543.9–2434.7, indicative of lipid A species with a different acylation degree. Each cluster was characterized by the presence of mass differences of 14 amu (−CH₂− unit), attributable to lipid A species differing by the length of their acyl chains (Fig. 4). In detail and based on the compositional analysis, cluster of peaks at around m/z 2406.7 matched with hexa-acylated lipid A species with a pentasaccharide sugar backbone containing one hexose (identified as Man), one DAG, one GlcN, one acid (identified as GlcA) and one Ko
unit and acylated by two 14:0 (3-OH), two 18:0 (3-OH) and two 16:0, in accordance with previously reported structure on A. pasteurianus NBRC 3283 lipid A [35]. The cluster at m/z 2168.4 was assigned to penta-acylated lipid A species made up of two 14:0 (3-OH), two 18:0 (3-OH) and one 16:0, thereafter, tetra-acylated lipid A species at m/z 1942.2, devoid of one 14:0 (3-OH) with respect to the species at m/z 2168.4, was also identified. Furthermore, tetra-acylated species lacking one hexose and/or K0 units and minor tri-acylated species were also present in the MALDI spectrum (Fig. 4).

MS/MS experiments were performed in order to characterize the location of the lipid A acyl chains with respect to the saccharide backbone [26,36]. The negative-ion MS/MS spectrum of precursor ion m/z 1942.2 (Fig. S4 and S5), consistent with a tetra-acylated pentasaccharidic species, showed an intense peak at m/z 1706.1 attributed to an ion originated from the loss of K0 residue. Peaks at m/z 1766.1 and 1780.1 were assigned to fragments lacking the GlcA and the hexose unit respectively. A lipid A fragment devoid of the 16:0 moiety was attributed to the peak at m/z 1686.1. More importantly, peaks originating from the sugar ring fragmentations 1,3A3 (m/z 1582.5), 1,4A3 (m/z 1552.5), 0,3A3 (m/z 1393.3) and 0,4A3 (m/z 1362.4) were very informative as they clearly demonstrated that the proximal GlcN was decorated by a GlcA and an amide-bound 18:0 (3-OH) moiety whereas the distal DAG was substituted by K0, hexasac and acylated by one 18:0 (3-OH), one 14:0 (3-OH) and one secondary 16:0 fatty acid. Finally, the peak at m/z 1450.0 was ascribed to a fragment ion where both K0 and 16:0 were absent.

MS/MS experiments conducted on precursor ion at m/z 2168.4 (Fig. S6) showed an ion peak at m/z 1932.3 originating from the loss of K0 unit, as well as from the loss of the hexose at m/z 2006.3. Lipid A fragments lacking the K0 and one 14:0 (3-OH) (m/z 1688.2) and one 16:0 (m/z 1432.3) were also identified. Moreover, ions originated from the loss of the hexose unit and 16:0 (m/z 1750.3) and from the loss of the hexose and one 14:0 (3-OH) (m/z 1762.3) were also detected. The absence of an ion originating from the loss of a whole hydroxylated 18:0 or 14:0 bearing a 16:0 suggested that the secondary fatty acid was linked to the acyloxyacyl amides, thus excluding its presence on the proximal GlcN as a substituent of the primary ester-bound 14:0 (3-OH). Furthermore, in support of this hypothesis, a peak was found at (Fig. S4 and S5), consistent with a tetra-acylated pentasaccharidic species, showed an intense peak at m/z 1706.1 attributed to an ion originated from the loss of K0 residue. Peaks at m/z 1766.1 and 1780.1 were assigned to fragments lacking the GlcA and the hexose unit respectively. A lipid A fragment devoid of the 16:0 moiety was attributed to the peak at m/z 1686.1. More importantly, peaks originating from the sugar ring fragmentations 1,3A3 (m/z 1582.5), 1,4A3 (m/z 1552.5), 0,3A3 (m/z 1393.3) and 0,4A3 (m/z 1362.4) were very informative as they clearly demonstrated that the proximal GlcN was decorated by a GlcA and an amide-bound 18:0 (3-OH) moiety whereas the distal DAG was substituted by K0, hexasac and acylated by one 18:0 (3-OH), one 14:0 (3-OH) and one secondary 16:0 fatty acid. Finally, the peak at m/z 1450.0 was ascribed to a fragment ion where both K0 and 16:0 were absent.

MS/MS experiments conducted on precursor ion at m/z 2168.4 (Fig. S6) showed an ion peak at m/z 1932.3 originating from the loss of K0 unit, as well as from the loss of the hexose at m/z 2006.3. Lipid A fragments lacking the K0 and one 14:0 (3-OH) (m/z 1688.2) and one 16:0 (m/z 1432.3) were also identified. Moreover, ions originated from the loss of the hexose unit and 16:0 (m/z 1750.3) and from the loss of the hexose and one 14:0 (3-OH) (m/z 1762.3) were also detected. The absence of an ion originating from the loss of a whole hydroxylated 18:0 or 14:0 bearing a 16:0 suggested that the secondary fatty acid was linked to the acyloxyacyl amides, thus excluding its presence on the proximal GlcN as a substituent of the primary ester-bound 14:0 (3-OH). Furthermore, in support of this hypothesis, a peak was found at

### Table 1

<table>
<thead>
<tr>
<th>Residue</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>1J_C1,H1</th>
<th>2J_H1,H2</th>
<th>3J_H2,H3</th>
<th>4J_H3,H4</th>
<th>5J_H4,H5</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5.21</td>
<td>4.08</td>
<td>4.15</td>
<td>3.86</td>
<td>3.91</td>
<td>3.53/3.78</td>
<td>172.3 Hz</td>
<td>3.4 Hz</td>
<td>9.5 Hz</td>
<td>9.4 Hz</td>
<td></td>
</tr>
<tr>
<td>2,6-β-D-Gal</td>
<td>152.2</td>
<td>86.6</td>
<td>75.0</td>
<td>82.3</td>
<td>69.0</td>
<td>69.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>5.07</td>
<td>4.02</td>
<td>3.97</td>
<td>3.84</td>
<td>3.86</td>
<td>3.77/3.50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>4.98</td>
<td>3.47</td>
<td>3.61</td>
<td>3.34</td>
<td>3.67</td>
<td>3.77/3.67</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>4.97</td>
<td>4.21</td>
<td>4.09</td>
<td>4.00</td>
<td>3.80</td>
<td>3.62/3.57</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. 1H NMR spectra of (a) PS1 and (b) PS2.
m/z 1984.4 resulting from the loss of 184 amu arising from the primary O-linked 14:0 (3-OH) and promoted by its free 3-OH group. Unfortunately, no ions originating from sugar ring fragmentations were detected. In addition, MS/MS spectrum of precursor ion at m/z 2406.7 (Fig. S7), relative to a hexa-acylated lipid A species, presented the ion peak derived from the sugar ring fragmentation at m/z 1791.5 that was fundamental to further corroborate the location of the 16:0 residues as secondary acyl substituents of the DAG unit. Therefore, the lipid A and O-chain structure from A. pasterianus CIP103108 were as reported in Fig. 5.

3.5. Immunological studies on A. pasterianus CIP103108 LPS

Studies on immunological properties of A. pasterianus CIP103108 LPS were performed using HEK-Blue hTLR4 cells. These cells are designed for studying the activation of human TLR4 by monitoring the activation of NF-κB and AP-1 transcription factors. HEK-Blue hTLR4 cells are HEK cells transfected in order to stably express hTLR4, hMD-2 and hCD14 receptor genes and a secreted embryonic alkaline phosphatase (SEAP) reporter gene placed under the control of NF-κB and AP-1. Stimulation with a TLR4 ligand activates NF-κB and AP-1 inducing the production and secretion of SEAP in cells culture medium. Levels of SEAP can be easily determined incubating the enzyme with para-nitrophenylphosphate (pNPP). In order to evaluate the capacity of A. pasterianus CIP103108 LPS to trigger TLR4-mediated signalling (activation assay) cells were exposed to different concentrations (0.1, 1, 10, 100 ng/ml) for 16 h. As a positive control E. coli O55:B5 LPS was used. As expected, E. coli O55:B5 LPS strongly activated TLR4-mediated pathway (Fig. S8 A). Furthermore, the capability of A. pasterianus CIP103108 LPS to interfere with E. coli LPS-triggered TLR4-mediated signalling was also evaluated (competition assay). To investigate this aspect, cells were pre-incubated with different concentration of A. pasteurianus CIP103108 LPS (0.1, 1, 10, 100 ng/ml) for 30 min and then exposed to E. coli O55:B5 LPS (1 ng/ml) for 16 h. Results showed that the pre-incubation with A. pasteurianus CIP103108 LPS slightly reduced the capacity of cells to respond to E. coli O55:B5 LPS, as demonstrated by the minor activation of the TLR4-mediated pathway (Fig. S8 B).

4. Discussion

In this work we have focused our attention on the structural characterization of the LPS isolated from A. pasteurianus CIP103108. Two novel LPS O-chains were found, PS1 and PS2, both possessing structural analogies with those isolated from other Acetobacter strains. The presence of two different O-chains is not considered as a common feature of LPSs, nevertheless this phenomenon was reported among Burkholderia species [37]. PS1 possessed a Galp disaccharide repeating unit carrying a non-stoichiometric Glc substitution; it showed subtle compositional similarities with the O-chains isolated from two Acetobacter methanolicus strains, MB58/4 [38] and MB70 [39]. Moreover, we have also found that a similar exopolysaccharide was produced by another acidophilic bacterium, Zymomonas mobilis, which was proven to have relevance with the outer milieu of bacterium (manuscript in preparation). The second polysaccharide PS2 contained a pentasaccharide repeating unit constituted by a tetrasaccharide skeleton containing glucose, mannose and two rhamnose residues, one of which carrying a terminal glucose as an appendage. A similar O-antigen was reported for Acetobacter tropicalis SKU1100 [40], whose backbone also contained two rhamnose residues, although in an α-(1→3) linkage, instead of the α-(1→2) here reported, with one rhamnose further substituted by a glucose residue. Furthermore, other O-
polysaccharides possessing structural similarities were also found in *Acetobacter diazotrophicus* PAL5 [41]. None of the above structures possessed mannose residues, though for *A. diazotrophicus* PAL5 contained a manno-configured sugar.

Recently, Hashimoto et al. [35] reported about the capability of *A. pasteurianus* NBRC 3283 LPS to remain stable in acidic conditions for long periods and correlated this capability to its unique lipid A structure. Indeed, the authors demonstrated that the LPS was characterized by the occurrence of a d-glycer-o-talo-oct-2-ulosonic acid (Ko), in place of Kdo, as the first sugar of the core OS, directly linked to the lipid A domain. This was explained with the acid-lability of Kdo that would be degraded in the acid environmental conditions of bacterium life. Thus, the substitution of Kdo with the acid-stable Ko is considered as an adaptation phenomenon necessary for bacterial survival. The presence of Ko has been so far described in the core oligosaccharides from *Burkholderia*, *Acinetobacter*, *Yersinia*, and *Serratia* species [42], although a direct linkage of Ko to the lipid A has so far reported only for *Acinetobacter haemolyticus* [43], in which the Kdo is almost completely replaced by Ko residues. Furthermore, *A. pasteurianus* LPS is devoid of phosphate groups and the lipid A is characterized by tetrasaccharide backbone composed of Man-DAG-GlcN-GlcA. Interestingly, lipid A devoid of phosphate groups and with a similar sugar skeleton were already reported for other plant associated bacteria belonging to the *Alphaproteobacteria* family, as *Rhizobiales* and *Bradyrhizobiales*. The lipid A isolated from *Rhodopseudomonas palustris* [19] possessed nearly the same tetrasaccharide skeleton but with a complete DAG backbone. *Bradyrhizobium* strains possess instead an α-(1 → 6)-mannose disaccharide linked to the non-reducing DAG [44,45]. Moreover, a DAG-Nac disaccharide skeleton was previously reported for *Campylobacter jejuni* [46] while a DAG backbone has been found in the lipid A of other strains like *Azorhizobium caulinodans* [47], *Phyllobacterium trifoli* [48], *Leptospira interrogans* [49], *Acidithiobacillus ferroxoxidans* [50], *Thiobacillus* [51], *Bartonella* [21] and *Brucella* [52]. The occurrence of this phenomenon is explained by the presence of two enzymes, GnaA and GnaB in the lipid A biosynthesis process which are pivotal for synthesis of UDP-D-GlcNP3N from UDP-D-GlcNAc [53].

---

**Fig. 3.** Overlapped sections of $^1$H $^13$C HMBC (blue) and $^1$H $^13$C HSQC (red and green) NMR of PS2. The anomeric signals and key inter-residual long range correlations involving sugar residues of PS2 are indicated. Letters found in Table 2.

**Table 2**

Values of $^1$H and $^13$C (italic) chemical shifts of *A. pasteurianus* CIP103108 PS2.

<table>
<thead>
<tr>
<th>Residue</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5.10</td>
<td>4.11</td>
<td>3.97</td>
<td>3.49</td>
<td>3.82</td>
<td>1.32</td>
</tr>
<tr>
<td>2-α-L-Rhap</td>
<td>173.4 Hz</td>
<td>3J_{1,H1}</td>
<td>173.4 Hz</td>
<td>3J_{1,H1}</td>
<td>173.4 Hz</td>
<td>3J_{1,H1}</td>
</tr>
<tr>
<td>2-α-L-Rhap</td>
<td>99.9</td>
<td>71.5</td>
<td>65.6</td>
<td>65.6</td>
<td>69.5</td>
<td>69.5</td>
</tr>
<tr>
<td>2-α-L-Rhap</td>
<td>174.2 Hz</td>
<td>3J_{1,H1}</td>
<td>174.2 Hz</td>
<td>3J_{1,H1}</td>
<td>174.2 Hz</td>
<td>3J_{1,H1}</td>
</tr>
<tr>
<td>C</td>
<td>8.0</td>
<td>7.15</td>
<td>6.28</td>
<td>6.28</td>
<td>6.28</td>
<td>6.28</td>
</tr>
<tr>
<td>2-α-L-Glc</td>
<td>174.2 Hz</td>
<td>3J_{1,H1}</td>
<td>174.2 Hz</td>
<td>3J_{1,H1}</td>
<td>174.2 Hz</td>
<td>3J_{1,H1}</td>
</tr>
<tr>
<td>D</td>
<td>4.95</td>
<td>3.50</td>
<td>3.69</td>
<td>3.43</td>
<td>3.43</td>
<td>3.43</td>
</tr>
<tr>
<td>6-α-L-Glc</td>
<td>14.1 Hz</td>
<td>3J_{1,H1}</td>
<td>14.1 Hz</td>
<td>3J_{1,H1}</td>
<td>14.1 Hz</td>
<td>3J_{1,H1}</td>
</tr>
<tr>
<td>E</td>
<td>4.80</td>
<td>3.95</td>
<td>3.84</td>
<td>3.38</td>
<td>3.63</td>
<td>3.76</td>
</tr>
<tr>
<td>2-α-Mann</td>
<td>173.2 Hz</td>
<td>3J_{1,H1}</td>
<td>173.2 Hz</td>
<td>3J_{1,H1}</td>
<td>173.2 Hz</td>
<td>3J_{1,H1}</td>
</tr>
</tbody>
</table>
The lipid A is considered as the center of toxicity of the LPS. It is well known that this part of the molecule is recognized by the TLR4/MD-2 receptor complex resulting in immunostimulation activity. A widely known fact is that hexa-acylated bis-phosphorylated lipid A produced by *E. coli* possesses high affinity to TLR4/MD-2 receptor complex, triggering powerful innate immune response. Overstimulation by agonistic LPS can deregulate innate immune system signalling, finally effecting in uncontrolled, massive proinflammatory cytokine release. Nevertheless, it was shown that modifications of the lipid A structure can tune and modulate the innate immune response. Within this frame, research and investigation of natural LPS able to inhibit the TLR4/MD-2 dependent signalling is an important and interesting topic.

The results obtained on TLR4/MD-2 mediated NF-kB activation on *A. pasteurianus* CIP103108 LPS have shown significantly lower response than *E. coli* O55:B5 LPS. A weak effect of inhibition of the TLR4/MD-2 mediated activation of NF-kB induced by *E. coli* O55:B5 LPS was also observed. The very weak agonist activity may be explained by lipid A structural features as i) the absence of phosphate groups [54], ii) the presence of penta-, tetra- and tri-acylated species, besides the hexa-acylated form, iii) the occurrence of a DAG unit.

![MALDI mass spectrum](image)

**Fig. 4.** Reflectron MALDI mass spectrum, recorded in negative polarity, of the *A. pasteurianus* CIP103108 lipid A. Lipid A species are outlined. In the inset, the proposed structural composition of the peak at *m/z* 2406.7 is listed.

![Structure](image)

**Fig. 5.** Structure of the a) lipid A and two O-polysaccharides, b) PS1 c) PS2 isolated from *Acetobacter pasteurianus* CIP103108.
Acknowledgements

AS, AM, FP and MP acknowledge the European Commission (H2020-MSCA-ETN-642157 TOLLerant) project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2018.08.035.

References


[24] I. Ciucanu, F. Kerek, A simple and rapid method for the permethylation of carbohy-


