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Carbohydrate Research 336 (2001) 213–223

CARBOHYDRATE RESEARCH

Structural analyses of the lipopolysaccharides from *Chlamydophila psittaci* strain 6BC and *Chlamydophila pneumoniae* strain Kajaani 6

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Received 1 June 2001; accepted 2 September 2001

Abstract

Lipopolysaccharides (LPSs) of *Chlamydophila psittaci* 6BC and *Chlamydophila pneumoniae* Kajaani 6 contain 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), GlcN, organic bound phosphate, and fatty acids in the molar ratios of ~3:2:2.2:4.8 and ~2.9:2:2.1:4.9, respectively. The LPSs were immunoreactive with a monoclonal antibody against a family-specific epitope of chlamydial LPS. This finding, together with methylation analyses of both LPSs and MALDI-TOF MS experiments on de-*O*-, and de-*O*,*N*-acylated LPSs, indicate the presence of a Kdo trisaccharide proximal to lipid A having a structure α-Kdo-(2→8)-α-Kdo-(2→4)-α-Kdo, which appears to be the main component of the core region in the native chlamydial LPSs. In the de-*O*-acylated LPSs from *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6, two major molecular species are present that differ in distribution of amide-bound hydroxy fatty acids over both GlcN. It appears that either two (*R*)-3-hydroxy-18-methylicosanoic acids or one (*R*)-3-hydroxy-18-methylicosanoic acid and one (*R*)-3-hydroxyicosanoic acid are attached to the GlcN residues. In contrast, the de-*O*-acylated LPS of *Chl. psittaci* PK 5082 contains one major molecular species that has two (*R*)-3-hydroxy-icosanoic acid residues attached to two GlcN residues.

Keywords: *Chlamydophila psittaci*; *Chlamydophila pneumoniae*; Lipopolysaccharide; Chemical composition; Structure

1. Introduction

Chlamydia are pathogenic, obligatory intracellular Gram-negative bacteria, which cause a variety of acute and chronic diseases in humans and animals.[1] The natural reservoir of *Chlamydophila psittaci* are animals in which the bacterium is responsible for organ-specific infections such as abortion, pneumonia, arthritis, encephalitis and enteritis. Human infections are known by avian strains, particularly those from parakeets and related birds, causing severe, atypical pneumonia (ornithosis, psittacosis).[2] *Chlamydophila pneumoniae* is a common cause of respiratory infections worldwide.[3,4] Further studies have suggested possible association of the microorganism with several clinical conditions such as sarcoidosis,[5] adult onset asthma,[6] arteriosclerosis[7,8] in general, and myocardial infarction[9,10] in particular.

A lipopolysaccharide (LPS) is the major surface antigen of chlamydia, against which antibodies are raised in all types of chlamydial infections. It contains[11] a linear trisaccharide

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0008-6215/01/$ - see front matter
PII: S0008-6215(01)00263-4

of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) having the sequence α-Kdo-(2→8)-α-Kdo-(2→4)-α-Kdo (**1**) that has not been found in any other LPS thus far and represents a family-specific epitope of diagnostic importance. Although all chlamydial species studied to date contain this epitope, it was indicated[12] that the LPS of *Chl. psittaci* from an ewe abortion strain PK 5082[13] differs considerably in its composition from those of *C. trachomatis* and *Chl. pneumoniae*.[14,15] It has been suggested[12] that the *Chl. psittaci* PK 5082 LPS contains, in addition to the LPS population with **1** in the core, other LPS populations having α-Kdo-(2→4)-α-Kdo-(2→4)-α-Kdo (**2**) and α-Kdo-(2→4)-[α-Kdo-(2→8)]-α-Kdo-(2→4)-α-Kdo (**3**) in their core regions. These assumptions were based on the studies[16] of an LPS isolated from a recombinant *Escherichia coli* F515-140 strain. We showed[17] earlier that the *Chl. psittaci* PK 5082 LPS isolated from the same source as that given in Ref. 13 contained only GlcN from amino sugars. This was in contrast with the previous study[13] where GlcN and GalN were found in a molar ratio of ~2:1. Our recent reinvestigation[18] of this LPS showed Kdo, GlcN, phosphate, and fatty acids in the molar ratio of ~2.6:2.0:2.4:4.4. Again, GalN was not detected. Structural studies established **1** in the core region. No evidence was found for the presence of an appreciable amount of **2** and/or **3**. We came to a similar conclusion when the LPS from *Chl. psittaci* strain 6BC was analyzed.[19]. Again, the trisaccharide (**1**) was found to be the main component of the core region. The LPSs from both strains PK 5082 and 6BC differed[19] in fatty acid composition. Most recently, however, it has been reported[20] that the LPS of *Chl. psittaci* 6BC contains **1** and **3** as the major components of the core region. The presence of **3** in the LPS would be of a diagnostic value in human and veterinary medicine, since application of specific monoclonal antibody directed against this unique branched region (epitope) could identify *Chl. psittaci* and differentiate it from other chlamydial strains. For these reasons, we decided to reinvestigate our previous findings[19] on the LPS isolated from *Chl. psittaci* 6BC.

The *waaA* gene of *Chl. pneumoniae* TW-183, encoding the Kdo transferase, was expressed[21] in *E. coli* K-12 strain. Analyses of a recombinant LPS revealed the major LPS population with **1** in the core. However, this work resulted in structural and immunochemical investigations of only the core region, and not in that of the lipid A moiety. In addition, it is still not clear whether the Kdo structures formed in the *E. coli* background are the same as those synthesized in *Chl. pneumoniae* which posses a different lipid A moiety. Our early investigations[15] on an LPS isolated from *Chl. pneumoniae* strain Kajaani 6 indicated the presence of three Kdo units having the structure identical with **1**. Due to a limited amount of LPS available, detailed analyses of fatty acid composition and other possible constituents could not be undertaken at that time. With improvements in both methodology and instrumentation, the amount of LPS needed for structural analysis could be considerably reduced, and here, we also report on a more detailed chemical characterization of a deep-rough (*R*) LPS obtained from *Chl. pneumoniae* Kajaani 6.

2. Experimental

Cultivation and purification of chlamydial elementary bodies (EBs).—*Chl. psittaci* strain 6BC and *Chl. pneumoniae* strain Kajaani 6 (a gift from Dr P. Saikku, National Public Health Institute, Helsinki, Finland), were propagated in embryonated hen eggs. After cultivation, EBs were purified as follows. A 20% suspension of yolk sacs with the chlamydial EBs in 2 M NaCl containing 0.2% formaldehyde was allowed to stand at 5 °C for 5 days. It was then centrifuged at 20,000*g* at 20 °C for 40 min. The pellet was suspended in phosphate-buffered saline and treated with 0.2% trypsin (EC 3.4.21.4, 1:250, from bovine pancreas, Serva, Germany) at 39 °C for 90 min. After addition of saline, the suspension was extracted four times with Et_2O. Purified EBs were centrifuged from aqueous phase and stored at 5 °C in 0.15 M NaCl containing 0.2% formaldehyde.

Isolation of the LPSs.—EBs (350 mg each) from *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6 were extracted by a phenol–water method.[22] The water layers were extensively dialyzed against distilled water for 4 days at 18 °C and freeze-dried to give the crude LPSs in the yields of 14.7 mg (*Chl. psittaci*) and 13.3 mg (*Chl. pneumoniae*). The crude LPS extracts were purified further by electrophoresis on a Model 491 Prep Cell (Bio-Rad, USA) as described.[23] Buffer salts and deoxycholate were removed by dialysis and the LPS solutions were lyophilized. The final yields were 9.8 and 7.7 mg of the LPSs of *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6, respectively.

Gel electrophoresis.—Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% gels with or without 4 M urea. The gels were stained with $AgNO_3$[24] and Coomasie Blue[25] for control. The *Salmonella minnesota* Re 595 LPS was purchased from Sigma, USA.

Western blot analysis.—In the immunoblotting analysis, membranes with the transferred LPS bands were blocked overnight with 3% skimmed milk in Tris-buffered saline (TBS, pH 8.0) and then incubated for 3 h with murine monoclonal antibody (MAb) against a family-specific epitope of chlamydial LPS (Pathfinder, Kallestadt, Chaska, USA) diluted 1:100 in TBS, and with goat anti-mouse IgG peroxidase conjugate (Bio-Rad Laboratories, USA) diluted 1:2500 in TBS. Subsequently, membranes were rinsed in TBS and stained with the 4-chloro-1-naphthol peroxidase substrate system.

General methods.—Kdo, protein, phosphate, and hexosamine contents in the LPSs were determined colorimetrically as given[26] elsewhere. In gas chromatography (GC) and GC-mass spectrometry (GC-MS), amino sugars were analyzed as alditol acetates after hydrolysis of the LPS with 3 M HCl for 8 h. Kdo was determined as trimethylsilyl (Me_3Si) methyl glycosides after methanolysis (2 M HCl) of the LPS (100 μg) for 2 and 6 h at 60 °C and Me_3Si derivatization. To accomplish the latter procedure, a Tri–Sil reagent (100 μL, Pierce, USA) was added to the dry and HCl-free residue and the mixture was heated in a sealed tube at 80 °C for 20 min. In order to estimate the molar response of Kdo, a monomeric Kdo (Sigma, USA) and the *S. minnesota* Re 595 LPS were methanolyzed and trimethylsilylated simultaneously with the LPS samples under identical conditions. Total fatty acids were determined by GC and GC-MS after their release with 4 M HCl in dry MeOH at 100 °C for 16 h and trimethylsilylation. Ester-linked fatty acids were determined after their release with 2 M HCl in MeOH at 60 °C for 2 h. The stereochemistry of 3-hydroxy fatty acids present in the LPS was established as described.[27]

Methylation analysis.—The LPS (1.5 mg) was solubilized in Me_2SO (0.3 mL) and methylated[28] with MeI (0.1 mL) in the presence of NaOH (20 mg) for 30 min at 20 °C. After dialysis, the methylated LPS was carboxyl-reduced with lithium triethylborodeuteride (150 μL, Super-Deuteride, Aldrich, Germany) for 60 min at 20 °C. After neutralization with glacial AcOH and dialysis, the recovered material (0.9 mg) was hydrolyzed with 0.1 M trifluoroacetic acid (0.3 mL) at 100 °C for 30 min and then carbonyl-reduced with NaB^2H_4 (3 mg) in 1 M NH_4OH (0.4 mL) for 16 h at 20 °C. The dry residue was then acetylated and analyzed by GC and GC-MS.

O,N-*Deacylation of the LPS.*—Deacylation of the LPS (2 mg) was accomplished as given[20] elsewhere. After extraction of the released fatty acids with $CHCl_3$, the water phase was dialyzed in tubings (Spectra/Por, Spectrum Medical Industries, Inc., USA, molecular weight cut off, 500) at 20 °C for 4 days. The lyophilized material (0.8 mg) was dissolved in ultra-pure water and separated by HPLC. One major oligosaccharide (0.6 mg) was isolated at an elution volume corresponding approximately to that of the standard pentasaccharide.

GC and GC-MS.—GC was performed with a Shimadzu model 17A chromatograph equipped with flame-ionization detector using helium as the carrier gas. Alditol acetates of amino sugars were analyzed on an HP-5 column (25 m × 0.32 mm, Hewlett–Packard, USA) at 160 (3 min) increasing to 245 °C at 2 °C/min. Me_3Si methyl glycosides and fatty acid methyl esters were separated on a DB-1

column (60 m × 0.25 mm, Fison, UK) at 180 (2 min) increasing to 280 °C at 3 °C/min with a final 30 min hold for the latter compounds. Acetylated and methylated glycose derivatives were analyzed on a DB-5 column (30 m × 0.25 mm) at 80 (2 min) increasing first to 160 °C at 8 °C/min and then to 300 °C at 4 °C/min. GC-MS was performed on a Finnigan MAT SSQ 710 mass spectrometer with helium as the carrier gas. Electron impact (EI) mass spectra were recorded at 70 eV and an ion-source temperature of 150 °C. Pyridine was used[29] as the reaction gas for chemical ionization (CI). GC-MS was run with the columns and temperature programs already described.

HPLC.—Semi-preparative HPLC was carried out on a Biospher SI120C18 column (5 μm/8 × 250 mm, Labio, Prague, Czech Republic) using ultra-pure water as the eluent at 20 °C and a flow rate of 0.6 mL/min. The HPLC system used involved an LKB (Bromms, Sweden) 2152 LC controller, an LKB 2150 HPLC programmable pump, a Rheodyne (Lotati, USA) 7125 injector valve equipped with 100 μL loop, an RIDK 102 (Laboratorní přístroje, Prague, Czech Republic) differential refractometer, and a DataApex (Prague, Czech Republic) CSW v. 1.6 integrator. D-Glucose and its α-(1→4)-linked oligomers (from dimer to heptamer) were used for the column calibration.

Matrix assisted laser desorption/ionization-time-of-flight mass spectrometry.—MALDI-TOF MS of LPSs, de-*O*-, and de-*O*,*N*-acylated LPSs was performed with a KOMPACT MALDI II (Shimadzu–Kratos Analytical, Japan) in the linear time-of-flight configuration at an acceleration voltage of 20 kV. LPSs, de-*O*-, and de-*O*,*N*-acylated LPSs (< 10 nmol) were dispersed in aq 0.2 M Et_3N (15 μL). The solutions were mixed with an equal volume of matrix (satd solution of 2,4,6-trihydroxyacetophenone in MeOH; Aldrich, Germany) and aliquots of 0.2 μL were deposited on a metallic sample holder and analyzed after drying. The samples were analyzed in a negative ion mode. For analyses in a positive ion mode, the de-*O*,*N*-acylated LPSs were dissolved in distilled water at a concentration of 20 μg/L. Aliquots (5 μL) of the sample solutions were mixed with an equal volume of saturated solution of 2,5-dihydroxybenzoic acid (Aldrich, Germany) in MeOH, and aliquots of 0.5 μL were deposited on a metallic sample holder and analyzed after drying. The mass spectra shown are the sum of 250 laser shots. Mass scale calibrations were performed externally with similar compounds of known chemical structure.

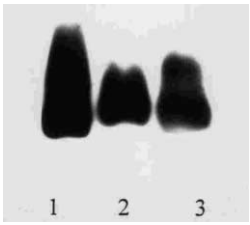

Fig. 1. SDS-PAGE silver stain of the LPSs from *S. minnesota* Re 595, *Chl. psittaci* 6BC, and *Chl. pneumoniae* Kajaani 6 (lanes 1, 2, and 3).

3. Results and discussion

Both *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6 LPSs were isolated from the purified EBs by a conventional hot phenol–water extraction. The LPSs were found in the aqueous layer. After dialysis and lyophilization, the preparations contained ~14% of protein. The presence of nucleic acid could also be detected by UV spectrometry. Further purification of the LPSs by the preparative gel electrophoresis gave pure LPSs in the final yields of 2.8 (*Chl. psittaci* 6BC) and 2.2% (*Chl. pneumoniae* Kajaani 6). In SDS-PAGE followed by staining with alkaline $AgNO_3$, the LPSs of *S. minnesota* Re 595 and *Chl. psittaci* 6BC exhibited single bands (Fig. 1). An indication for the presence of two bands was seen with the *Chl. pneumoniae* Kajaani 6 LPS. A better resolution of chlamydial LPSs was achieved by the addition of 4 M urea to the gel. The *Chl. pneumoniae* LPS showed clearly two bands indicating the presence of two distinct LPS species whereas two fused bands were obtained with the *Chl. psittaci* LPS (Fig. 2). Two LPS species in both LPSs were immunoreactive with MAb against a family-specific epitope of chlamydial LPS (Fig. 3). The MAb also reacted with the LPS of *Chlamydia trachomatis* but it did not react with the LPS of *S. minnesota* Re 595 and the LPS II of

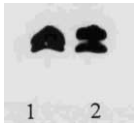

Fig. 2. SDS-PAGE silver stain of the LPSs from *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6 (lanes 1 and 2). 4 M urea added to the gel.

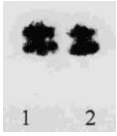

Fig. 3. Western blot of the LPSs from *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6 (lanes 1 and 2). Immunoidentification of the LPS bands transferred from the SDS-PAGE gel (Fig. 2) with MAb against the family-specific epitope of chlamydial LPS.

Table 1
Chemical compositions (nmol/mg) of the *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6 LPSs

Compound	*Chl. psittaci* 6BC		*Chl. pneumoniae* Kajaani 6	
	Colorimetry	GC	Colorimetry	GC
Kdo	786 (2.9) [a]	806 (3.1)	792 (2.8)	819 (3.0)
GlcN	542 (2.0)	520 (2.0)	566 (2.0)	539 (2.0)
Phosphate	596 (2.2)		589 (2.1)	
Fatty acids		1251 (4.8)		1327 (4.9)

[a] Molar ratio in parentheses.

Coxiella burnetii[26] indicating that it is chlamydia-specific (data not shown). From SDS-PAGE, a relative size homogeneity of the investigated LPSs can be anticipated which, however, does not exclude major microheterogeneity of the lipid A moiety with regard to the substitution by different fatty acids, and which has been shown[30] in detail recently for lipid A from *C. trachomatis*.

In the *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6 LPSs, the colorimetric assays gave the molar ratios of Kdo, hexosamine, and phosphate of $\sim$2.9:2.0:2.2 and $\sim$2.8:2.0:2.1, respectively (Table 1). For amino sugars, only GlcN was found in both LPSs.

The quantitative determination of Kdo by the thiobarbituric acid assay yielded 786 and 792 nmol/mg of the LPSs of *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6, respectively. These amounts were considerably lower than those already found[20,15] for both *Chl. psittaci* 6BC (1047 nmol/mg) and *Chl. pneumoniae* Kajaani 6 (940 nmol/mg) LPSs. Therefore, an additional assay was used to determine the Kdo content in the investigated LPSs. The LPSs were methanolyzed under the conditions which were shown[18] to release quantitatively Kdo from the LPSs with minimal degradation. Subsequent trimethylsilylation of the mixtures revealed on GC and GC-MS the corresponding Kdo derivatives which amounted to 806 and 819 nmol/mg of the LPSs of *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6, respectively.

In our further studies, the methylation linkage-analysis of the Kdo region of the investigated LPSs was of primary interest. Thus, the LPSs were permethylated, carboxyl-reduced with lithium triethylborodeuteride in tetrahydrofuran, and hydrolyzed under mild conditions. The hydrolyzates were carbonyl-reduced with NaB^2H_4, acetylated, and directly analyzed by GC-MS. Isomeric 1,2,6-tri-*O*-acetyl-3-deoxy-4,5,7,8-tetra-*O*-methyl(1,1,2-3^{2}H)-octitols, 1,2,4,6-tetra-*O*-acetyl-3-deoxy-5,7,8-tri-*O*-methyl(1,1,2-3^{2}H)octitols, 1,2,6,8-tetra-*O*-acetyl-3-deoxy-4,5,7-tri-*O*-methyl(1,1,2-3^{2}H) octitols, and 1,2,4,6,8-penta-*O*-acetyl-3-deoxy-5,7-di-*O*-methyl(1,1,2-3^{2}H)octitols giving characteristic fragment-ions[31,32] in the EI mode were found in the analyzed samples. CI with protonated pyridine as the reagent[29] produced very stable quasimolecular $[M+PyrH]^+$ adduct ions with molecular masses of 491, 519, 519, and 547. Their peak area ratio was $\sim$1:1.2:0.9:0.2 and $\sim$1:1.1:0.8:0.1 for the *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6 LPSs, respectively. From these results, the presence of terminal, 4-, 8-, and a small amount of 4,8-linked Kdo residues could be established in both LPSs.

Fatty acids were analyzed by GC and GC-MS after mild and strong methanolyses of the LPSs followed by trimethylsilylation. In both LPSs, the analyses revealed the presence of eight different straight or branched (iso and anteiso) nonhydroxy fatty acids and of six (*R*)-configured 3-hydroxy fatty acids (Table 2). Fatty acids amounting less than 1 mol% were not considered. The major nonhydroxy fatty acids were octadecanoic and icosanoic

acids for the *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6 LPSs, respectively. In the *Chl. pneumoniae* Kajaani 6 LPS, the most prominent 3-hydroxy fatty acid was (*R*)-3-hydroxy-18-methylicosanoic acid followed by (*R*)-3-hydroxyicosanoic acid. In contrast, these two fatty acids, present in an equal amount, followed by (*R*)-3-hydroxy-16-methyloctadecanoic acid represented the major hydroxylated fatty acids in the *Chl. psittaci* 6BC LPS. It should be noted that nonhydroxy fatty acids were ester-linked and hydroxylated fatty acids were amide-linked.

Table 2
Fatty acid composition of the *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6 LPSs

Fatty acid	Amount in mol%	
	Chl. psittaci	*Chl. pneumoniae*
n14:0	4.9	4.6
i15:0	4.6	3.5
a15:0	7.8	7.7
n16:0	7.3	5.6
n18:0	16.3	3.4
a19:0	4.5	1.7
i20:0	2.3	1.4
n20:0	9.7	10.2
(*R*)-3-OH-i19:0	2.6	4.2
(*R*)-3-OH-a19:0	9.6	7.7
(*R*)-3-OH-i20:0	3.5	3.9
(*R*)-3-OH-n20:0	12.5	17.2
(*R*)-3-OH-i21:0	1.9	3.9
(*R*)-3-OH-a21:0	12.5	25.0

n, normal fatty acids; i, a, iso, anteiso-branched fatty acids.

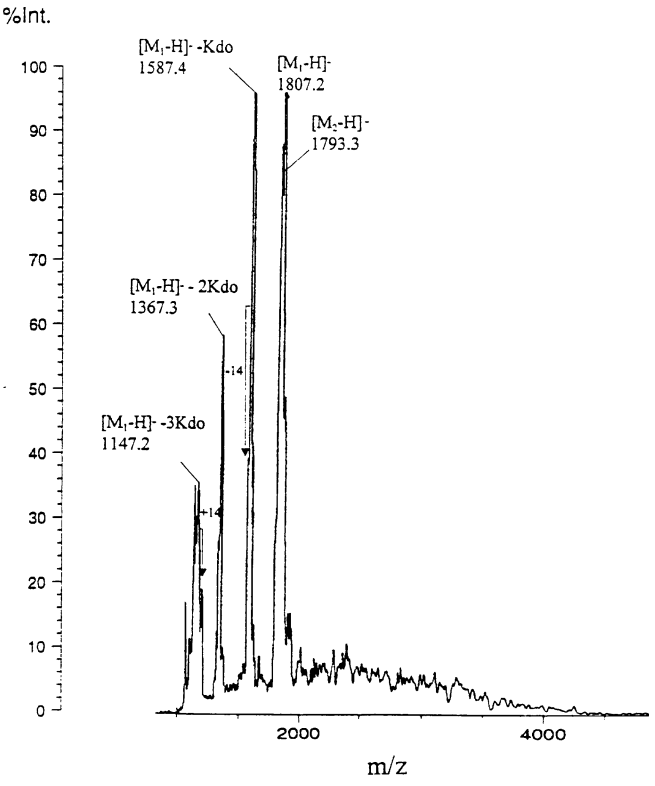

Fig. 4. Negative-ion MALDI-TOF MS spectrum of de-*O*-acylated LPS from *Chl. psittaci* 6BC.

MALDI-TOF MS analyses of the investigated LPSs gave ions consisting of lipid A (two GlcN and two phosphate residues, and 4–5 fatty acid residues) alone or substituted by one, two, or three Kdo units (data not shown). Higher masses were not detected. Because the mass resolution was low due to a considerable microheterogeneity in fatty acid substitution, de-*O*-acylated LPSs were prepared and analyzed. The negative-ion mass spectrum of de-*O*-acylated LPS of *Chl. psittaci* 6BC (Fig. 4) exhibited two major molecular ions at m/z 1807.2 and 1793.3 corresponding to the $[M_1 - H]^-$ and $[M_2 - H]^-$ ions consisting of three Kdo, two GlcN, two phosphates and two 3-hydroxy-18-methylicosanoic acids for the former ion, and of 3-hydroxyicosanoic and 3-hydroxy-18-methylicosanoic acid residues for the latter ion, respectively. Under the conditions of analysis, strong laser-induced in source fragment ions were observed that represented molecular species representing de-*O*-acylated lipid A alone or substituted by one or two Kdo units. In addition, signals with mass differences of ± 14 (CH_2) were identified indicating also heterogeneity of amide-linked fatty acids. These types of ions were also seen in the mass spectra of other chlamydial LPSs. For comparison, the mass spectrum of de-*O*-acylated LPS from *Chl. psittaci* PK 5082 is depicted (Fig. 5). In this case, the spectrum exhibited one major ion at m/z 1779.4 corresponding to an $[M - H]^-$ ion consisting of three Kdo, two GlcN, two phosphates, and two amide-bound 3-hydroxyicosanoic acid residues. Thus, in both *Chl. psittaci* LPSs, higher masses which could indicate the presence of four or five Kdo units, were not detected. With the de-*O*-acylated *Chl. pneumoniae* Kajaani 6 LPS, the negative ion mass spectrum (Fig. 6) revealed two major molecular ions $[M_1 - H]^-$ and $[M_2 - H]^-$ at

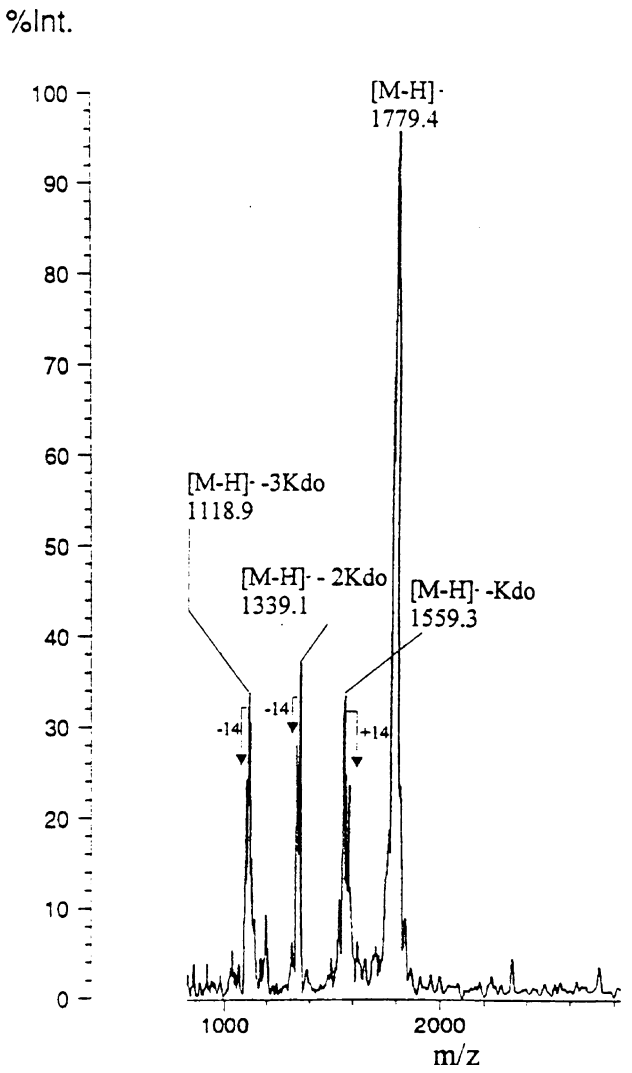

Fig. 5. Negative-ion MALDI-TOF MS spectrum of de-*O*-acylated LPS from *Chl. psittaci* PK 5082.

m/*z* 1807.8 and 1793.4 indicating the presence of similar molecular species as those already found in the LPS of *Chl. psittaci* 6BC. The intense fragment ions showing a consecutive loss of Kdo residues were present, too.

To analyze the carbohydrate backbones of the *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6 LPSs, we applied a deacylation procedure that involved hydrazinolysis and successive hot KOH treatment. After usual work up, the sugar residues were analyzed by HPLC using a C_{18} column that was calibrated internally with a standard mixture of mono- to hepta-saccharides. Only one major peak could be seen in both cases at an elution volume corresponding approximately to a pentasaccharide. The oligosaccharide samples collected by HPLC were then analyzed by MALDI-TOF MS. The positive-ion mass spectrum of the oligosaccharide obtained on deacylation of the *Chl. psittaci* 6BC LPS exhibited (Fig. 7) an $[M + K]^+$ ion at *m*/*z* 1199.2 consisting of three Kdo, two GlcN, and two phosphates. A peak at *m*/*z* 1119.4 indicates a loss of phosphate from the former ion. $[M + Na]^+$ ions at *m*/*z* 1023.3, 803.8, and 583.4 indicate the losses of two phosphates and one or two Kdo residues as the result of prompt fragmentation occurring in the source. The negative-ion mass spectrum of the oligosaccharide (Fig. 8) confirmed the results from the positive-ion mode. It gave an $[M - H]^-$ ion at *m*/*z* 1159.3 and an intense fragment ion at *m*/*z* 859.9 showing a loss of phosphate and one Kdo. A similar mass spectrum gave the oligosaccharide obtained on deacylation of the *Chl. pneumoniae* Kajaani 6 LPS (not shown). Thus, another evidence has been obtained for the presence of a pentasaccharide backbone as the main carbohydrate component in both *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6 LPSs.

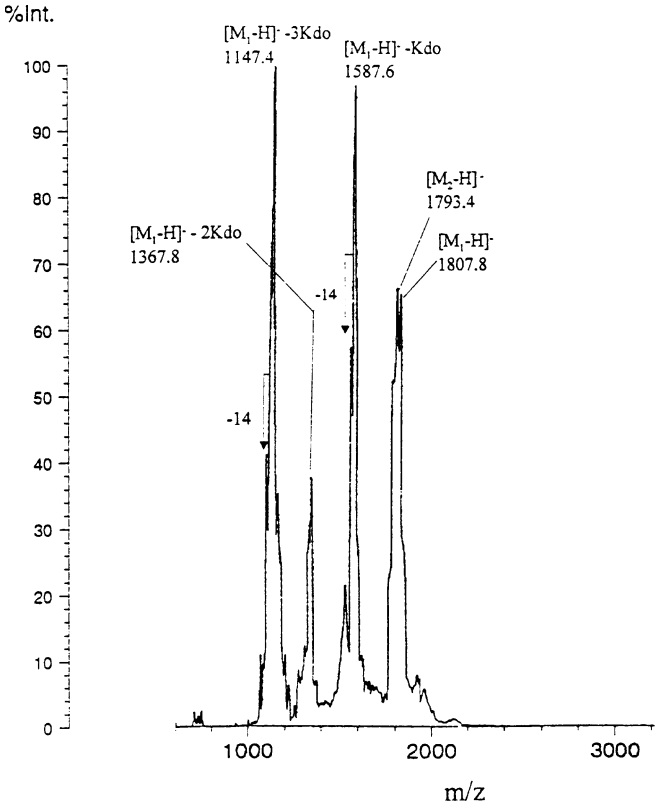

Fig. 6. Negative-ion MALDI-TOF MS spectrum of de-*O*-acylated LPS from *Chl. pneumoniae* Kajaani *6*.

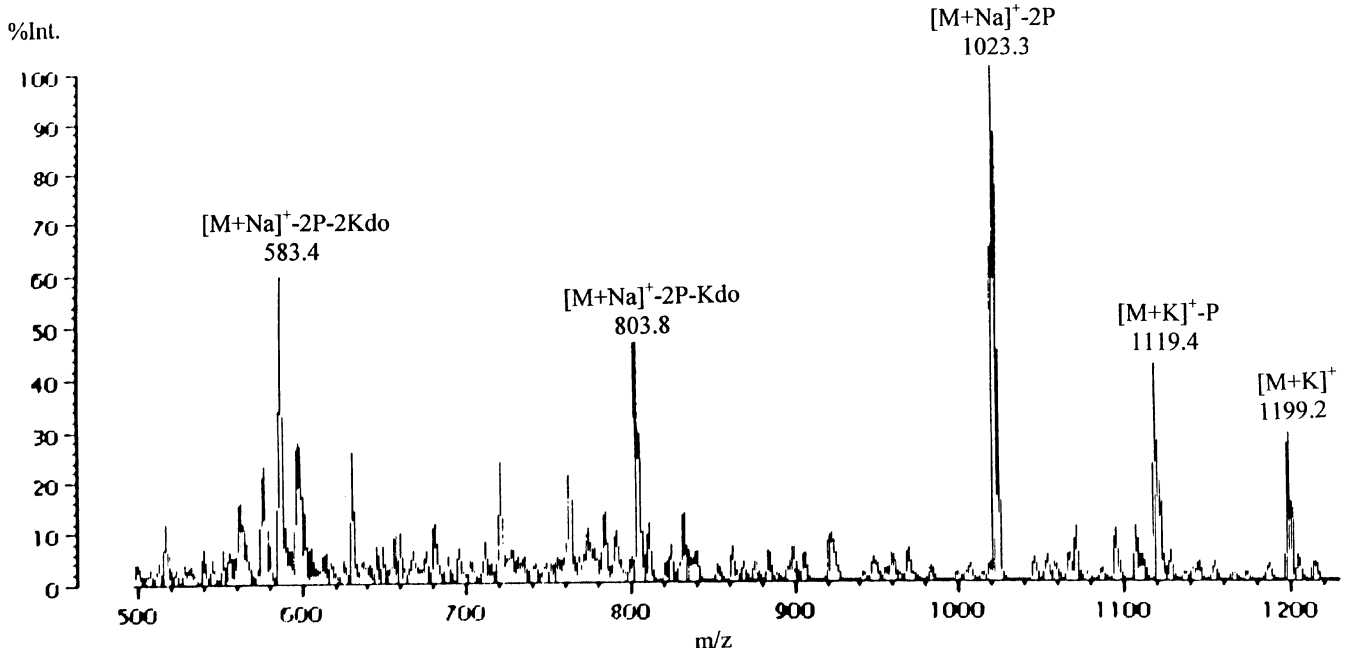

Fig. 7. Positive-ion MALDI-TOF MS spectrum of de-*O*,*N*-acylated LPS from *Chl. psittaci* 6BC.

From the new data reported herein and from those published[15,18,19] earlier, the chemical structures of the major molecular species of de-*O*-acylated LPSs from *Chl. psittaci* 6BC and PK 5082, and from *Chl. pneumoniae* Kajaani 6 can be depicted as shown in Fig. 9.

Structural analyses of a recombinant LPS from *E. coli* F515-140 carrying the *Chl. psittaci waaA* gene on a plasmid revealed[16] the presence of several LPS populations having **1**, **2**, **3**, and α-Kdo-(2→4)-α-Kdo (**4**) in their core regions whereby an LPS with **4** was the prevailing one.[20] The existence of Kdo oligomers has been ascribed to the action of a multifunctional *Chl. psittaci* Kdo transferase, which is supposed[12,33] to have different properties as compared to the Kdo transferase from *C. trachomatis*. In the light of our results it appears, however, that the cloned chlamydial LPS is a hybrid molecule composed of both *Chlamydia* and *E. coli* components. Since this recombinant strain still expressed its own Kdo transferase, an interference with the activity of the corresponding host enzyme can be anticipated. When the host Kdo transferase was inactivated in the *waa* CF negative strain of *E. coli* K-12 and the chlamydial *waaA* genes were transferred, the resulting transformants synthesized[21] less complex LPSs. Mainly the Kdo trimer (**1**) was found in the

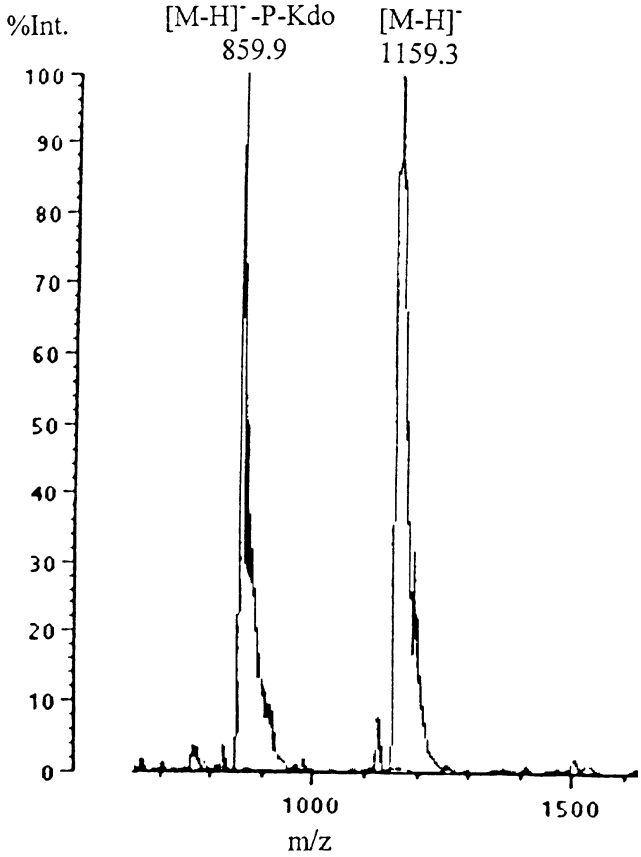

Fig. 8. Negative-ion MALDI-TOF MS spectrum of de-*O*,*N*-acylated LPS from *Chl. psittaci* 6BC.

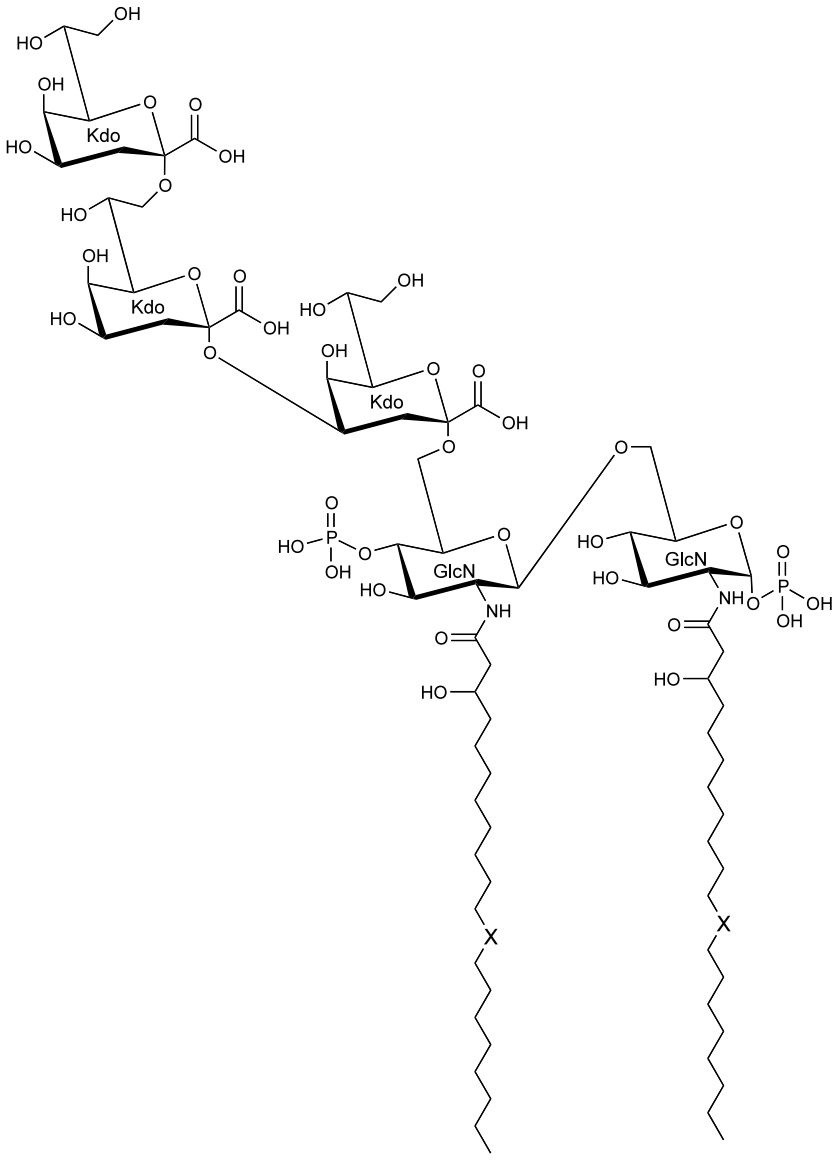

Fig. 9. Basic chemical structure of de-*O*-acylated chlamydial LPSs. Two major molecular species with two (*R*)-3-hydroxy-18-methylicosanoic acids (X = 21) and one (*R*)-3-hydroxy-18-methylicosanoic acid, and one (*R*)-3-hydroxyicosanoic acid (X = 20) were identified in de-*O*-acylated LPSs from *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6. One major species with two (*R*)-3-hydroxyicosanoic acids was identified in de-*O*-acylated LPS from *Chl. psittaci* PK 5082.

deacylated LPS from *E. coli* with *waaA* of *Chl. pneumoniae* and the tetrasaccharide (**3**) in the deacylated LPS from *E. coli* with *waaA* of *Chl. psittaci* 6BC. The former finding is in agreement with our results presented here on the native *Chl. pneumoniae* Kajaani 6 LPS but the latter is not consistent with the data we have obtained[18] recently on the *Chl. psittaci* PK 5082 LPS and with those of the *Chl. psittaci* 6BC LPS reported in this paper. It appears that the product specificity of *waaA* of *Chl. psittaci* 6BC differs significantly from those of *C. trachomatis* and *Chl. pneumoniae*. Evidence for this assumption can also be found in two publications[20,21] already cited in this paper. In the former publication, a high-performance anion-exchange chromatogram of the deacylated LPS of *Chl. psittaci* 6BC showed the presence of oligosaccharides with **1** and **3** in the peak area ratio of $\sim$1.5:1, whereas in the latter, with the deacylated LPS from recombinant *E. coli*, the ratio was $\sim$1:6.3. It has to be found out which additional genetic or biochemical factors are involved in this phenomenon. Our results of methylation analyses of the investigated LPSs

might indicate an occurrence of a limited number of LPS populations with **3** in the core. However, other analytical approaches used in this study have not brought any evidence in this respect.

For chlamydial LPSs, a presence of long chain fatty acids is typical. This fact, together with the lower number of acyl chains contributes to much lower endotoxic potency of the chlamydial lipid A as compared to enterobacterial lipid A. We have found only quantitative differences in the fatty acid composition of the LPSs isolated from *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6. Nevertheless, both qualitative and quantitative differences are apparent from the fatty acid composition of the LPSs isolated from the *Chl. psittaci* strains 6BC and PK 5082.[18] Moreover, our data differ from those published[20,13] by others. This finding is not quite unexpected since it is well known[34] that the fatty acid composition in an LPS may vary with the cultivation conditions of a microorganism.

In summary, our data indicate that **1** is the main component of the core regions in the LPSs we have isolated from *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6. In the former LPS, no evidence was found for the presence of two major LPS populations having **1** and **3** in their cores as it has been published[20] recently. It should be clarified in future whether this fact is due to different cultivation and isolation conditions. Compositional analyses of the LPS from *Chl. psittaci* 6BC indicated that per two GlcN units in lipid A, 2.8 nonhydroxy fatty acid residues are present in an ester linkage and 2.1 hydroxy fatty acid residues in amide linkages. In addition, 2.2 phosphate groups were determined. In contrast, the LPS from *Chl. pneumoniae* Kajaani 6 contains per two GlcN residues in lipid A, 1.9 nonhydroxy and 3.0 hydroxy fatty acid residues, and 2.1 phosphate groups. These data indicate the presence of a bisphosphorylated glucosamine backbone in both LPSs, in analogy to many lipid A structures, but without any ester-linked hydroxy fatty acids. MALDI-TOF MS data of the de-*O*-acylated LPSs from *Chl. psittaci* 6BC and *Chl. pneumoniae* Kajaani 6 have shown that two major molecular species are present that differ in distribution of amide-bound hydroxy fatty acids over both GlcN. It appears that either two (*R*)-3-hydroxy-18-methylicosanoic acids or one (*R*)-3-hydroxy-18-methylicosanoic acid and one (*R*)-3-hydroxyicosanoic acid are attached to the GlcN residues. In contrast, the de-*O*-acylated LPS of *Chl. psittaci* PK 5082 contains one major molecular species that has two (*R*)-3-hydroxyicosanoic acid residues attached to two GlcN. In conclusion, our data indicate that the LPSs from various chlamydial species may differ in fatty acid composition and distribution.

Acknowledgements

This research was supported in part by grant No. 2/7032/20 of the Scientific Grant Agency of Ministry of Education of the Slovak Republic and the Slovak Academy of Sciences.

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