Structural analysis of the lipid A isolated from *Hafnia alvei* 32 and PCM 1192 lipopolysaccharides[S]

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Abstract ***Hafnia alvei*, a Gram-negative bacterium, is an opportunistic pathogen associated with mixed hospital infections, bacteremia, septicemia, and respiratory diseases. The majority of clinical symptoms of diseases caused by this bacterium have a lipopolysaccharide (LPS, endotoxin)-related origin. The lipid A structure affects the biological activity of endotoxins predominantly. Thus, the structure of *H. alvei* lipid A was analyzed for the first time. The major form, asymmetrically hexa-acylated lipid A built of β-D-Glc*p*N4P-(1→6)-α-D-Glc*p*N1P substituted with (*R*)-14:0(3-OH) at N-2 and O-3, 14:0(3-(*R*)-O-12:0) at N-2', and 14:0(3-(*R*)-O-14:0) at O-3′, was identified by ESI-MS^n and MALDI-time-of-flight (TOF) MS. Comparative analysis performed by MS suggested that LPSs of *H. alvei* 32, PCM 1192, PCM 1206, and PCM 1207 share the identified structure of lipid A. LPSs of *H. alvei* are yet another example of enterobacterial endotoxins having the *Escherichia coli*-type structure of lipid A. The presence of hepta-acylated forms of *H. alvei* lipid A resulted from the addition of palmitate (16:0) substituting 14:0(3-OH) at N-2 of the α-Glc*p*N residue. All the studied strains of *H. alvei* have an ability to modify their lipid A structure by palmitoylation.**—Lukasiewicz, J., W. Jachymek, T. Niedziela, L. Kenne, and C. Lugowski. **Structural analysis of the lipid A isolated from *Hafnia alvei* 32 and PCM 1192 lipopolysaccharides. *J. Lipid Res.* 2010.** 51: **564–574.**

Supplementary key words Polish Collection of Microorganisms • endotoxin • mass spectrometry • palmitoylation

Lipopolysaccharide (LPS, endotoxin) is the main surface antigen (O-antigen) and important virulence factor of most of the Gram-negative bacteria pathogenic for humans and animals (1). LPS contributes greatly to the structural integrity of the bacterial cell wall and constitutes a "pathogen-associated molecular pattern" for host infection (1).

Most of the known structures of LPS are smooth-type molecules built of O-specific polysaccharide, core oligosaccharide, and lipid A. Among all these defined regions, the glycolipid part of LPS called lipid A constitutes a center of biological activity of the endotoxin that stimulates different cells of the immune system (2). The most common type of lipid A that is also one of the highly endotoxic forms consists of the bisphosphorylated carbohydrate backbone disaccharide β-D-Glc*p*N4P-(1→6)-α-D-Glc*p*N1P, substituted by six asymmetrically distributed fatty acids linked via ester and amide linkages: 14:0[3-(*R*)-OH] at O-2 and O-3 of α-D-Glc*p*N and 14:0[3-(*R*)-*O*-12:0] or 14:0[3-(*R*)-*O*-14:0] at O-2′ and O-3′ of β-D-Glc*p*N (2, 3). Such hexa-acylated lipid A displays the highest immunostimulatory or endotoxic activity in the mammalian host (1, 2) and it was identified for the first time in *Escherichia coli* LPS (2, 3). Interaction of such asymmetric, hexa-acylated lipid A region of LPS with mCD14/TLR4/MD-2 receptor complex on the surface of monocytes/macrophages constitutes a major mechanism responsible for innate immune response to Gram-negative infection (1, 2). High levels of inflammatory mediators [tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8, INFγ, INFα, nitric oxide, platelet-activating factor, and endorphins], as a response of the immune system to large amount of LPS released into a bloodstream, have profound effects on the cardiovascular system, kidneys, lungs, liver, and central nervous system and trigger the coagulation cascade. Excessive in-

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Abbreviations: LPS, lipopolysaccharide; P group, phosphate group; PCM, Polish Collection of Microorganisms; TOF, time-of-flight.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of three figures.

flammatory response of the innate immune system finally leads to sepsis and septic shock (1).

Hafnia alvei lipopolysaccharides have been studied for many years because of frequent reports about opportunistic infections caused by this bacterium but also several interesting structural features of their core oligosaccharides and O-specific polysaccharides. As an opportunistic pathogen, *H. alvei* has been associated with respiratory diseases and mixed hospital infections in humans (4). Most cases of *H. alvei*-related bacteremia usually originate from gastrointestinal and respiratory infections. The bacteremia (mostly monomicrobic infection) and sepsis seem to be the most common syndromes reported for this bacterium (4). These Gram-negative bacteria were isolated from blood, hepatic abscesses, pancreatic pseudocyst fluid, sputum, feces, and central venous catheter. *H. alvei* has also been isolated from a variety of foods, e.g., cow milk, honey, corned beef, hard goat cheese, and fish (5). *H. alvei* infections can cause serious diseases and also substantial loss in farm animal production. Hemorrhagic septicemia in rainbow trout and in laying hens, pneumonic diseases in goats, and mastitis in cows were reported as a consequence of such infections (4). The clinical symptoms of *H. alvei* septicaemia of pullets are similar to those caused by *Salmonella* spp. in different host species (6).

Lipopolysaccharides of *H. alvei* are unusual examples of enterobacterial endotoxins, among the few elucidated so far, having various Kdo-containing motifs in the outer core regions of the core oligosaccharides (7). Thus, this feature distinguishes them from classical enterobacterial endotoxins. Even though many O-specific polysaccharide and core oligosaccharide structures have been elucidated for *H. alvei* LPSs (8–17), the structure of the *H. alvei* lipid A, the center of toxicity of the endotoxin, has not been analyzed so far. Because the majority of clinical symptoms of diseases caused by this bacterium have LPS-related origin and the lipid A structure predominantly affects the biological activity of endotoxins, it seems to be a gap in our knowledge of *H. alvei* LPSs. Moreover, recent studies on the invasion and intracellular survival of different *H. alvei* strains in a HeLa cell line revealed that these bacteria were able to enter and persist in human epithelial cells (18). For Gram-negative bacteria that employ intracellular survival mechanism during invasion, e.g., *Salmonella typhimurium* (19, 20), various lipid A modifications are needed. To survive within mammalian cells, an addition of 4-amino-4-deoxy-L-arabinose (Ara4N) and phosphoethanolamine (PEtn) and palmitate residues is often required. Thus, it was interesting to investigate *H. alvei* lipids A, the last uncharacterized region of their LPS.

The studies presented herein describe, for the first time, lipid A of *H. alvei* LPS. Recently we have determined the structure of the carbohydrate backbone of *H. alvei* 32 lipid A as a part of N,O-deacylated LPS, which is built of the bisphosphorylated disaccharide, Glc*p*N4P-(1→6)-Glc*p*N1P (7). We now report on the structural studies of *H. alvei* 32 and 1192 lipid A regions, with emphasis on fatty acid analysis and their distribution performed by ESI-MS^n and MALDI-time-of-flight (TOF) MS. The presented results and recently published data (7) connect the lipid A and core oligosaccharide of *H. alvei* 32 LPS. Elucidation of the *H. alvei* lipid A structure is a precondition for future examination of a possible relationship between atypical core oligosaccharide (outer core Kdo-containing motifs), lipid A structure, and biological activity of *H. alvei* LPSs.

MATERIALS AND METHODS

Bacteria

H. alvei strains 32, PCM 1192, PCM 1206, and PCM 1207 were obtained from the Polish Collection of Microorganisms (PCM) at the Institute of Immunology and Experimental Therapy (Wroclaw, Poland). The bacteria were grown in Davis medium, killed with 0.5% phenol, centrifuged using a CEPA flow laboratory centrifuge, suspended in water, and freeze-dried (16).

LPS and lipid A isolation and purification

LPS was extracted from bacterial cells by the hot phenol/water method (21). Briefly, freeze-dried bacteria were suspended in 45% phenol solution (2g/50 ml) and incubated at 65° C with intermittent stirring for 15 min. The suspension was cooled down to the temperature below 10° C and centrifuged (3,000 *g*, 30 min). Water phase was collected. Water was added to compensate for the collected volume of water phase and the cycle was repeated. The water phases were combined and dialyzed against deionized water to remove residual phenol, filtered, and freeze-dried. The obtained crude LPS was dissolved in water and purified by ultracentrifugation (105,000 *g*, 6 h) as previously reported (16). Lipid A was isolated from the LPS (200 mg) as a water-insoluble fraction by treatment with 1.5% acetic acid (45 min at 100°C) followed by centrifugation (40,000 *g*, 20 min). The sediment (lipid A) was resuspended in water and freeze-dried.

Partial O-deacylation of lipid A

The partial liberation of the ester-linked fatty acids from lipid A was done by mild alkali treatment (22). Lipid A (0.5 mg) was suspended in 25% ammonium hydroxide (1 mg/ml) and incubated with stirring at 50° C for 5 h followed by drying with a stream of nitrogen. The partially O-deacylated lipid A samples were further analyzed by ESI-MS.

Analytical procedures

Prior to analyses, LPS and lipid A were additionally purified by extraction with 2:1:3 chloroform/methanol/water mixture (v/v/v) to remove membrane phospholipids. The absolute configuration of monosaccharides was determined on dephosphorylated and O,N-deacylated lipid A as described by Gerwig et al. (23, 24), using (*R*)-2-butanol for the formation of 2-butyl glycosides. N,O-deacylation and dephosphorylation of lipid A were performed by hydrolysis with 4 M hydrochloric acid followed by 48% HF treatment as previously described (25). The trimethylsilylated butyl glycosides were then identified by comparison with samples produced from carbohydrate standards (Sigma, St. Louis, MO) and (*R/S*)-2-butanol (Fluka, Buchs, Switzerland) on GC-MS. GC-MS analysis was carried out with a Hewlett-Packard 5971A system using an HP-1 fused-silica capillary column (0.2 mm × 12 m) and a temperature program from 100 to 270°C at 8°C/min. Qualitative analysis of lipid A was done separately for amide- and ester-bound fatty acids using chemical analysis followed by GC-MS (26). Ester-bound fatty acids were released from intact LPS by treatment with sodium CH_3ONa in methanol and the amide-linked fatty acids were released from deO-acylated LPS

by aqueous 4 M KOH at 120°C for 16 h. The absolute configuration of 3-hydroxy fatty acids was determined by GC-MS (HP-5 column, 0.25 mm × 30 m; temperature program from 150 to 270°C at 8°C/min) using 3-methoxy-(*S*)-phenylethylamide derivatives (27).

Mass spectrometry

MALDI-TOF MS was carried out on a Kratos Kompact-SEQ instrument as described previously (25). 9H-Pyrido[3,4-b]indole [10 mg/ml in a 1:1 acetonitrile/water mixture (v/v)] and 2,4,6-trihydroxyacetophenone [25 mg/ml in a 1:1 acetonitrile/water mixture (v/v)] were used as matrices for the analysis of lipid A samples in negative and positive ion modes, respectively. Negative-ion electrospray mass spectra of lipid A were recorded using ESQUIRE-LC ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) for ESI-MS^n analyses of native lipid A and micrOTOF-Q spectrometer (Bruker Daltonics, Bremen, Germany) for analysis of the partially deO-acylated lipid A. The lipid A samples were desalted and dissolved in a methanol/chloroform mixture (1:1, v/v, 1 mg/ml). In ESI-IT MS^n analyses, the samples were continuously infused through the capillary head at 4 kV into the ion source using a linear syringe pump at a rate of 2 μl/min. Spectra were scanned in the *m/z* 200–2200 range. The mass isolation window for the precursor ion selection was set to 4 Da in all the MS^n analyses. In analyses performed on micrOTOF-Q spectrometer, the samples were dissolved in a 1:1 methanol/chloroform mixture (v/v, 1 mg/ml) and analyzed by direct infusion at a rate of 3 μl/min with an electrospray capillary high voltage of 4,5 kV. Spectra were scanned in the ranges of *m/z* 100–2250 (MS) and *m/z* 250–2000 (MS^2 of an ion at *m/z* 1291.9). The ion source temperature was 200°C, the N_2 flow rate was set at 4 l/min, and the pressure of nitrogen was 0.4 bar. External calibration in the negative-ion mode was applied using the Tunemix™(neg) mixture (Bruker Daltonics, Germany) in quadratic regression mode and *m/z* range of 113–2234 Da. The isolation width for MS^2 experiments was $\Delta m/z = 10$, and the collision energy of the quadrupole was −45 eV.

RESULTS

Isolation of lipopolysaccharide and lipid A

LPS of *H. alvei* 32, PCM 1192, PCM 1206, and PCM 1207 were extracted from bacterial cells by the hot phenol/water method (21) and purified as previously described (16). The yields of LPS preparations were 2%–3.5%. Lipid A was liberated by acidic hydrolysis of LPS (1.5% acetic acid, 45 min, at 100°C). The reaction mixture was cooled and centrifuged. The sediment was resuspended in water and freeze-dried. The yield of lipid A preparations were between 11% and 16% of LPS.

Compositional analysis

Chemical analyses of lipid A from *H. alvei* 32 and PCM 1192 indicated the presence of GlcN, phosphate group (P group), dodecanoic acid (12:0), tetradecanoic acid (14:0), 3-hydroxy-tetradecanoic acid [14:0(3-OH)] and hexadecanoic acid (16:0) in the relative proportions 2.0:1.6:0.9:1.0:3.7:0.1. GC-MS analysis of the trimethylsilylated (*R*)-2-butyl glycosides of sugar constituents of lipid A showed the presence of the D-GlcN isomer. Qualitative analysis of fatty acids was also done separately for amide- and ester-linked fatty acids. GC-MS data revealed the presence of (*R*)-14:0(3-OH), 14:0, 12:0, and 16:0 as ester-bound fatty acids and (*R*)-14:0(3-OH) as the only amide-linked fatty acid. Identification of the methoxy derivative, the methyl ester of 3-methoxytetradecanoic acid, among fatty acid methyl esters obtained by transestrification of acyloxyacyl residues in lipid A with sodium methanolate, indicated that some of 14:0(3-OH) fatty acids were substituted with the "secondary" fatty acid in the native lipid A.

ESI-MS/MS^n analyses of lipid A

ESI mass spectra obtained for lipid A isolated from *H. alvei* 32, 1192, 1206, and 1207 showed their heterogeneity and identical pattern of ions (**Fig. 1**), thus suggesting a common structure of the lipid A. On the basis of compositional analyses and taking into account previously published structure of the N,O-deacylated *H. alvei* 32 LPS (7), the most abundant ions at *m/z* 1796.4 and *m/z* 1716.5 could be attributed to bis- and mono-phosphorylated hexa-acylated forms of lipid A carrying four (*R*)-14:0(3-OH), one 14:0 and one 12:0 fatty acids (Fig. 1A). Additional forms of lipid A were also identified but observed ions were of much lower intensity. Ions at *m/z* 1954.6 and 2034.6 could be attributed to mono- and bis-phosphorylated hepta-acylated forms of lipid A, respectively (Fig. 1, inset structure). The mass difference 238 Da between *m/z* 1954.6 and *m/z* 1716.5 indicates the presence of hexadecanoic acid (16:0) as the seventh acyl residue. The lipid A variant represented by ions at *m/z* 1768.3 and 1688.4 could be explained by bis- and mono-phosphorylated hexa-acylated molecules that are substituted by one shorter fatty acid in comparison with the major form described above (mass difference of 28 Da observed between ions at *m/z* 1796.4 and 1768.3 and at *m/z* 1716.5 and 1688.4). The peaks at lower *m/z*-values and intensities could represent different bisphosphorylated lipid A species devoid of 14:0 (*m/z* 1586.1), 14:0 and 14:0(3-OH) (*m/z* 1360.1) or monophosphorylated species devoid of 14:0 (*m/z* 1506.3), 14:0(3-OH) (*m/z* 1490.2), and 14:0 and 14:0(3-OH) (*m/z* 1279.9) (Fig. 1). The presence of ions with lower *m/z* values and those related to monophosphorylated forms can be explained by partial degradation of lipid A during work-up and lability of some acyl side chains and the P group at O-1 during the acid hydrolysis (28).

The distribution of fatty acids was determined for the lipid A isolated from *H. alvei* 32 and PCM 1192 by multiple-stage ESI-MS^n (n = 2 and 3). Detected ions were interpreted according to the rules described previously in ESI-MS^n studies of lipid A (25, 29). ESI-MS^2 was performed on the selected ions at *m/z* 1716.5 (**Fig. 2**), *m/z* 1506.3 (**Fig. 3A**) and *m/z* 1279.9 (supplementary Fig. I). Because the obtained data suggested the *E. coli*-type structure of studied lipid A, ESI-MS^n spectra were compared with data published previously for lipid A of *E. coli* (29–31).

To determine the structure of the most abundant component corresponding to the most abundant ion (*m/z* 1716.5) in lipid A from both *H. alvei* 32 and PCM 1192, this ion was isolated in MS^1 and fragmented in MS^2 (Fig. 2, outlined inset structure). The spectra of lipid A from both strains showed almost identical pattern of daughter ions.

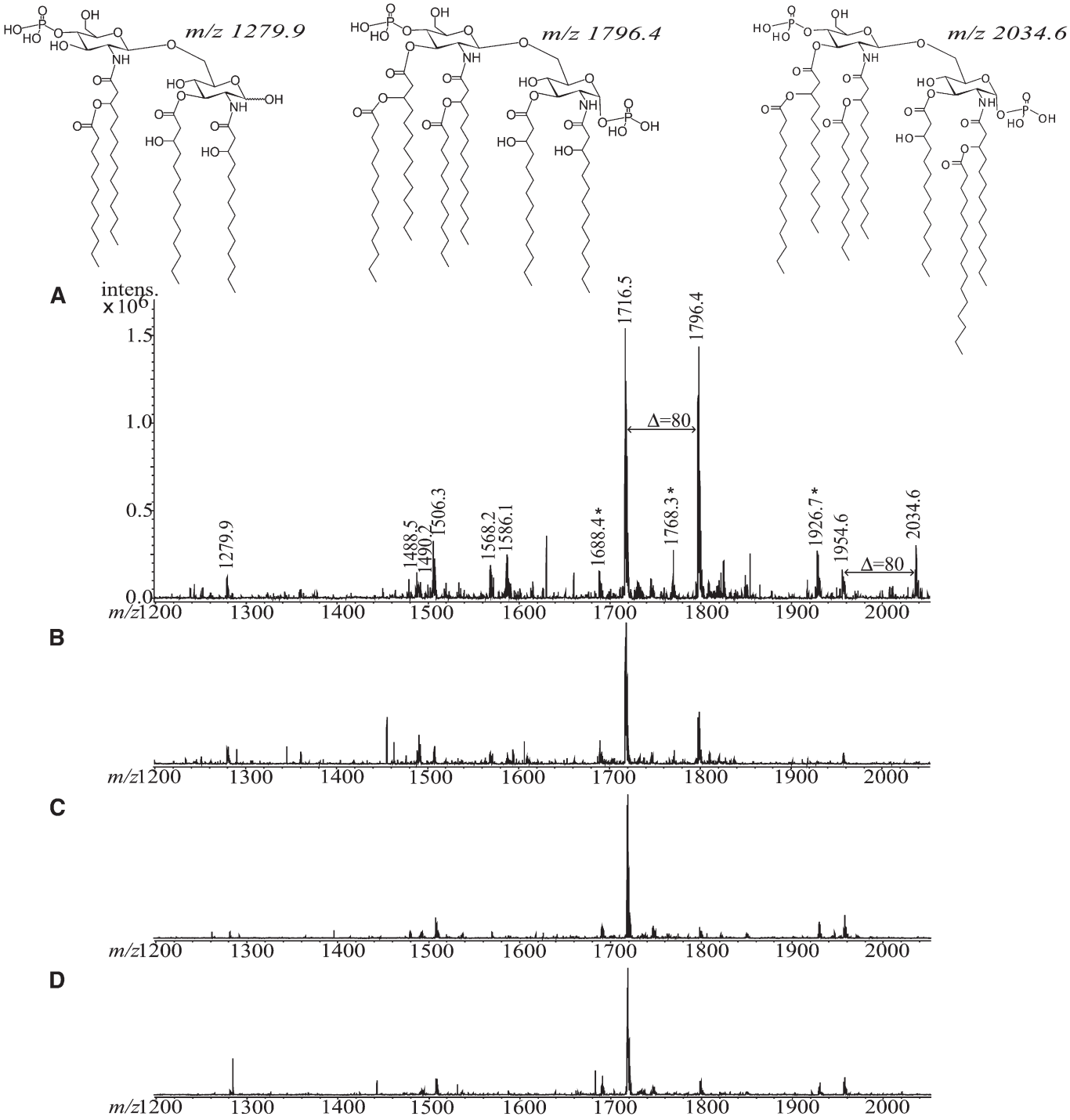

Fig. 1. Negative ion mode ESI mass spectra obtained for lipid A (inset structures) isolated from LPSs of *H. alvei* 1192 (A), 32 (B), 1206 (C), and 1207 (D). The ions denoted with an asterisk correspond to a mass difference of 28 due to the decrease in carbon chain length of fatty acids in comparison with the main forms (inset structures). Mass difference of 80 Da corresponds to the difference between mono- and bisphosphorylated molecules.

Chemical analysis of lipid A constituents suggested that this ion could be attributed to the monophosphorylated, hexa-acylated lipid A form containing two GlcN, one P group, four 14:0(3-OH), one 14:0, and one 12:0 (Fig. 2, outlined inset structure). Ions at *m/z* 1516.1, *m/z* 1488.0, and *m/z* 1472.0, correspond to elimination of 12:0, 14:0 and 14:0(3-OH), respectively, from the parent ion. Product ions from elimination of two acyl groups were also detected: *m/z* 1287.8 (elimination of 12:0 and 14:0), *m/z* 1279.9 [elimination of 14:0(3-*O*-14:0) as a ketene derivative], *m/z* 1261.8 [elimination of 14:0(3-*O*-14:0) as fatty acid]. The most abundant daughter ion at *m/z* 1243.8, corresponded to the elimination of two ester-linked fatty acids, 14:0(3-OH) at O-3 and secondary-bound 14:0 at O-3′ (Fig. 2, outlined inset structure). Two double bonds were formed as a result of the eliminations. The mass of the ion

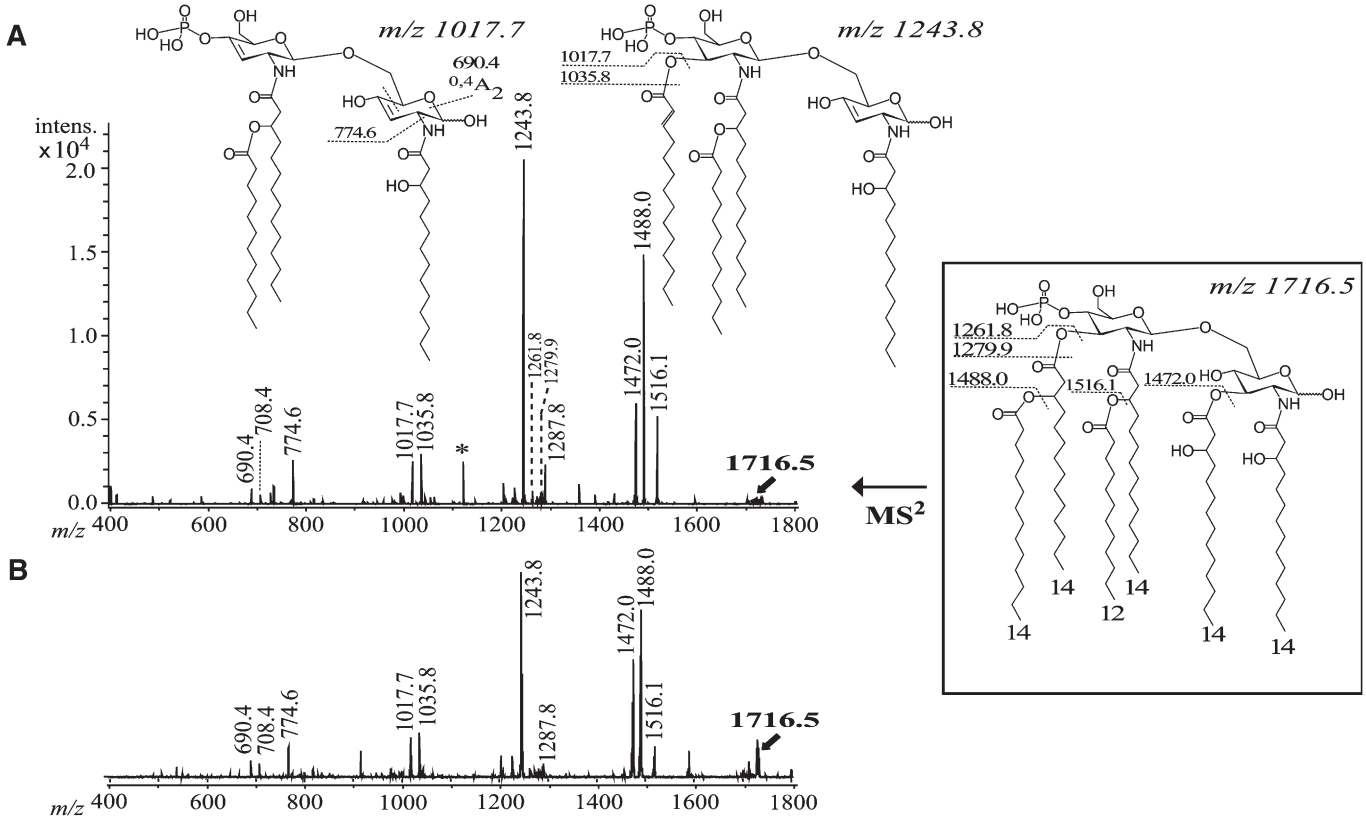

Fig. 2. Negative ion mode ESI-MS^2 of lipid A from *H. alvei* 32 (A) and 1192 (B) represented by the ion at *m/z* 1716.5 (outlined inset structure). Interpretation of the observed fragment ions is presented in the inset structures. The representation $^{0,4}A_2$ is according to the nomenclature of Domon and Costello (32). Numerals 12 or 14 indicate number of carbons in the fatty acids. The asterisk denotes ions that were not interpreted.

at *m/z* 1243.8 was in agreement with the calculated mass of two GlcN (one unsaturated), one P group, two 14:0(3-OH) fatty acids, one 12:0 fatty acid, and one 14:1 Δ^2 fatty acid derivative. Subsequent loss of a 14:1 Δ^2 from O-3′ as a ketene derivative and free fatty acid yielded a characteristic pair of ions at *m/z* 1035.8 and *m/z* 1017.7, respectively (Fig. 2). The observed elimination of 14:1 Δ^2 by both charge-driven (loss of a ketene derivative) and charge-remote processes (loss of a free fatty acid) suggested its location at O-3′ and also its previous substitution by 14:0 (ion at *m/z* 1243.8). The ions at *m/z* 708.4 and 690.4 represented $^{0,4}A_2$ fragments [according to the nomenclature of Domon and Costello (32)] and could be formed from the ions at *m/z* 1035.8 and 1017.7, respectively. These ions corresponded to the in-source intra-ring fragmentation and confirmed the distribution of the identified fatty acids. Interpretation of these types of ions makes it possible to define fatty acid distribution on each GlcN residue. In the case of MS^2 of the ion at *m/z* 1716.5, such fragments were identified only for daughter ions and not for the isolated parent ion. Thus, an interpretation of intra-ring fragmentation ions was described below for MS^n analyses of ions with lower *m/z* ratio (MS^2 of the ions at *m/z* 1506.3 and 1279.9, and MS^3 of the ion at *m/z* 1261.8).

Further investigation of fatty acid distribution was performed by MS^2 and MS^3 of the ion at *m/z* 1506.3 observed in MS analysis of lipid A preparations from both *H. alvei* 32 and PCM 1192 (Fig. 3A). On the basis of chemical analysis and types of the observed fragment ions (described below), the ion at *m/z* 1506.3 could represent pentaacylated lipid A that is monophosphorylated at O-4', consisting of two GlcN, one P group, four 14:0(3-OH) fatty acids, and one 12:0 fatty acid (Fig. 3A, outlined inset structure). Almost identical spectra were obtained for the lipid A of *H. alvei* 1192 (data not shown). Peaks with lower intensity at *m/z* 1488.3 and *m/z* 1305.9 correspond to elimination of water and 12:0 fatty acid, respectively. The most abundant ion at *m/z* 1261.8 could be the result of elimination of 14:0(3-OH) at O-3 or O-3′ as a free fatty acid. Because elimination of the acyloxyacyl group at O-3′ of the distal GlcN could occur by both charge-driven (elimination as a ketene derivative) and charge-remote processes (elimination as free fatty acid), the pair of ions at *m/z* 1279.8 and *m/z* 1261.8 corresponds to such elimination of 14:0(3-OH) at O-3′. Elimination of both 14:0(3-OH) from O-3 as a free fatty acid and from O-3′ as a free fatty acid and a ketene derivative corresponds to the characteristic pair of ions at *m/z* 1035.8 and 1017.7. The presence of $^{0,2}A_2$ (*m/z* 1221.0) and $^{2,5}A_2$ (*m/z* 1202.6) fragments of the parent ion confirmed the substitution of GlcN by 14:0(3-OH) fatty acid at N-2 (Fig. 3A, inset structures).

The most abundant ion at *m/z* 1261.8 derived from the MS^2 fragmentation was selected for MS^3 in order to determine the distribution of the remaining fatty acids (Fig.

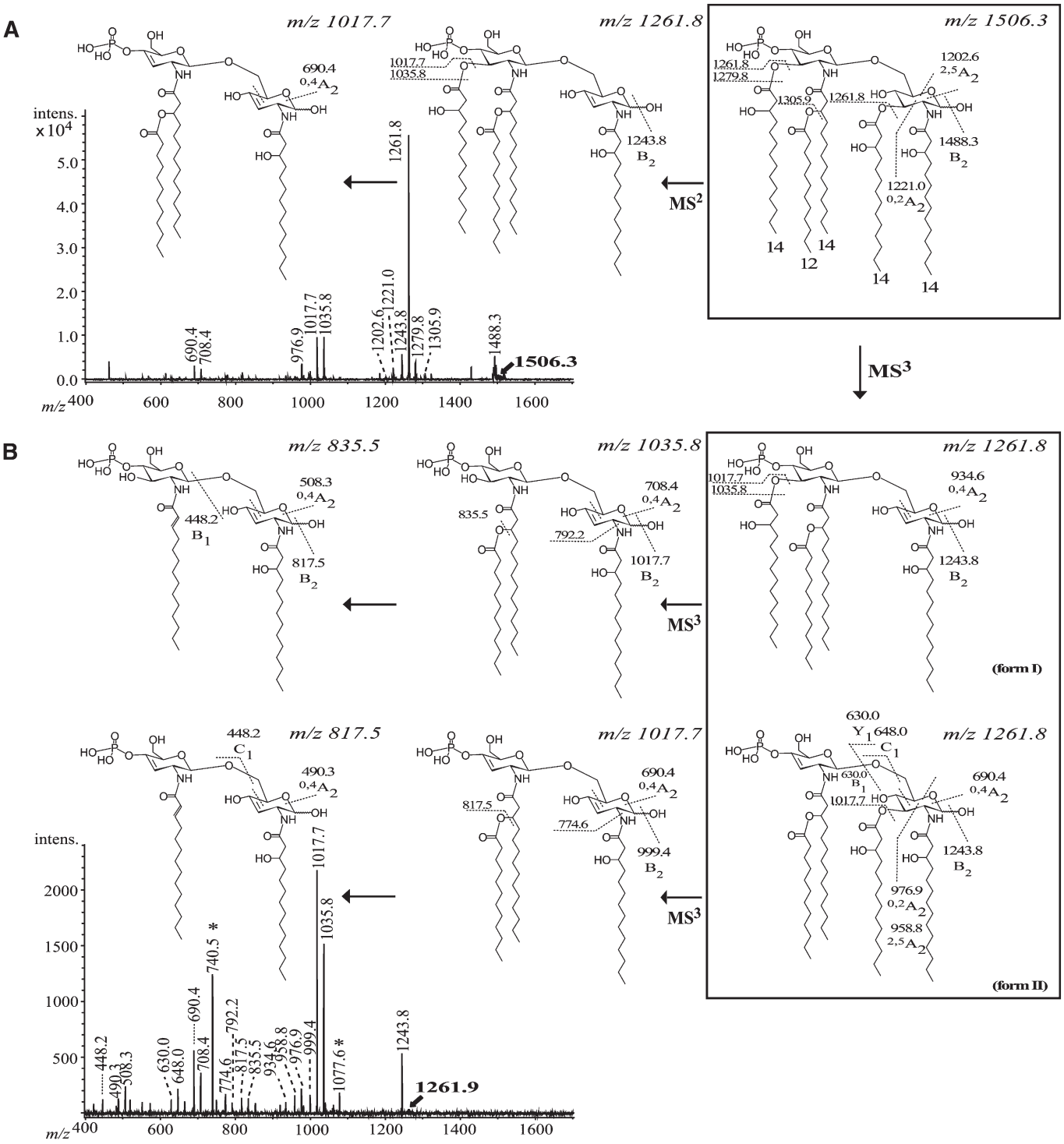

Fig. 3. Negative ion mode ESI-MS^n of lipid A from *H. alvei* 32 represented by an the ion at *m/z* 1506.3 (outlined inset structure). A: MS^2 of the ion at *m/z* 1506.3. B: MS^3 of the ion at *m/z* 1261.8 (form I and II). Interpretation of observed fragment ions is presented using inset structures and described as explained in the legend to Fig. 2.

3B). The *m/z* value of this isolated ion was in agreement with the calculated mass of two GlcN, one P group, three 14:0(3-OH), and one 12:0 fatty acid. Considering the high stability of acyl amides, the ion at *m/z* 1261.8 was attributed to two forms of the following structure: *I*) a monophosphorylated lipid A, substituted with 14:0(3-OH) at N-2 and O-3′ and 14:0(3-*O*-12:0) at N-2' and *II*) a monophosphorylated lipid A, substituted with 14:0(3-OH) at N-2 and O-3 and 14:0(3-*O*-12:0) at N-2' (Fig. 3B, outlined inset structures). These two possible variants differ by the presence or lack of 14:0(3-OH) at O-3 or O-3′. Obtained MS^3 spectrum contained ions originating from both forms. The peak at *m/z* 1243.8 is consistent with elimination of water (−18 Da). Subsequent elimination of 14:0(3-OH) at O-3′

(form *I*) yielded a characteristic pair of ions at *m/z* 1035.8 and 1017.7, corresponding to the loss of a 14:0(3-OH) as a ketene derivative (−180 Da) or the loss of a free fatty acid (−198 Da), respectively. Alternatively, the ion at *m/z* 1017.7 could arise from elimination of 14:0(3-OH) as free fatty acid at O-3 (form *II*), which explains its higher abundance in comparison with the ion at *m/z* 1035.8. Ions of lower intensity correspond to molecules that have lost 14:0(3-OH), have a double bond at O-3, and a hydroxyl group or double bond at O-3′. Several types of diagnostic fragment ions, e.g. $^{0,2}A_2$ (*m/z* 976.9), $^{0,4}A_2$ (*m/z* 690.4), $^{2,5}A_2$ (*m/z* 958.8), C_1 (*m/z* 648.0), and Y_1 (*m/z* 630.0), were detected from the parent ion (Fig. 3B, outlined inset structures). These ions confirmed the location of the 14:0(3-OH) at N-2, O-3 or O-3′ and 14:0(3-O-12:0) at N-2'. Ions corresponding to the elimination of 12:0 from amide-bound 14:0(3-O-12:0) were also detected (*m/z* 835.5 from the ion at *m/z* 1035.8 and *m/z* 817.5 from the ion at *m/z* 1017.7), but due to the higher stability of acyl amides, the peaks from these ions exhibited very low intensities. Two structural forms for the parent ion (*m/z* 1261.8) and interpretation of ions corresponding to the in-source intra- and inter-ring fragmentation are presented in Fig. 3B. The observed pattern of ions confirmed that lipid A, represented by the fragment ion at *m/z* 1506.3, is a monophosphorylated GlcN disaccharide, substituted with 14:0(3-OH) at N-2, O-3 and O-3′ and 14:0(3-*O*-12:0) at N-2'.

ESI-MS^n analysis (supplemental Fig. I) of the fatty acid distribution in lipid A from both strains was also performed for the most abundant ion, *m/z* 1279.9, in the low mass region of the ESI mass spectrum (Fig. 1). Observed fragment ions indicate that this ion could represent a tetraacylated lipid A. ESI-MS^2 analysis indicated that this lipid A form of *H. alvei,* represented by the ion at *m/z* 1279.9, has the glucosamine backbone structure, phosphorylated at O-4' and substituted with 14:0(3-OH) at N-2 and O-3, and 14:0(3-*O*-12:0) at N-2' (supplementary Fig. I, outlined inset structure).

To confirm that the identified fatty acid distribution in monophosphorylated form of *H. alvei* lipid A follows the same pattern as bisphosphorylated and hexa-acylated molecule, the ion at *m/z* 1797.3 was isolated and ESI-MS^2 analysis was carried out for *H. alvei* 32 (**Fig. 4B**) and *H. alvei* PCM 1192 (supplementary Fig. II). Thus, the results confirmed identical distribution of fatty acids in the bisphosphorylated forms of lipid A isolated from both strains (supplementary Fig. II). Product ions were formed mainly by the loss of phosphoric acid at O-1 (*m/z* 1698.3) with subsequent elimination of ester-bound acyl and acyloxyacyl groups [14:0 (*m/z* 1470.1), 14:0(3-OH) (*m/z* 1454.1), 14:0(3-O-14:0) (*m/z* 1243.9)] (Fig. 4B). The frequency of the loss of phosphoric acid from the position O-1 was significantly higher than from O-4' during ESI-MS^2 analysis due to the lability of glycosidically linked P group (28, 31). Interpretation of the observed fragment ions is presented in Fig. 4 (inset structures). Ion at *m/z* 1498.1 attributed to the elimination of secondary-bound 12:0 fatty acid at N-2' exhibited low intensity. Elimination of acyl residues with preservation of the P group at O-1 was also observed (*m/z* 1568.0, 1552.1, 1323.8).

The results of compositional analysis of *H. alvei* 32 lipid A and the mass difference (238 Da) between the ion at *m/z* 1797.3 (bisphosphorylated and hexa-acylated lipid A) and an ion at *m/z* 2034.6 (bisphosphorylated and hepta-acylated lipid A) suggested the presence of a 16:0 fatty acid in hepta-acylated lipid A. To confirm that 16:0 is a secondary-linked fatty acid substituting amide-linked 14:0(3-OH) at N-2, MS^2 analysis of the ion at *m/z* 2034.6 was performed (Fig. 4A). Observed fragmentation was similar to that observed for the ion at *m/z* 1797.3 (Fig. 4B). Fragment ions were formed mainly by the loss of the glycosidically linked P group and elimination of primary and secondary, ester-bound acyl and acyloxyacyl groups at O-3 and O-3′ [14:0 (*m/z* 1708.5), 14:0(3-OH) (*m/z* 1692.4), 14:0(3-O-14:0) (*m/z* 1500.1/1482.2)]. Elimination of acyl residues with the preservation of P group at O-1 was also observed (*m/z* 1806.5, 1790.4, 1580.3, 1562.2, 1336.2). Several ions resulting from elimination of 16:0 fatty acid were identified (e.g., *m/z* 1778.4, 1680.4, 1436.2, 1208.0), further supported the presence of 16:0 in the analyzed structure. The mass difference (200 Da) between pairs of related ions *m/z* 999.7/981.7 and *m/z* 799.6/781.7 indicated that 12:0 is a substituent of amide-linked 14:0(3-OH) at N-2' of the bisphosphorylated, hepta-acylated lipid A.

ESI-MS analysis of partially deO-acylated lipid A

ESI-MS and ESI-MS^2 analyses of partially deO-acylated lipid A samples allowed the determination of type and location of the seventh fatty acid in the hepta-acylated forms of *H. alvei* lipid A. The NH_4OH treatment leads to partial liberation of ester-linked acyl and acyloxyacyl residues (22, 33). Negative-ion mass spectra of the partially deO-acylated lipid A (**Fig. 5A**) followed by MS^2 analysis (Fig. 5B) gave further information concerning hepta-acylated forms of lipid A from *H. alvei* 32 and 1192. Several molecular ion peaks were observed at *m/z* 1371.9, *m/z* 1291.9, *m/z* 1133.7, *m/z* 1053.7, and *m/z* 951.5 together with their doubly charged variants (Fig. 5A, inset structures). Two of them could be explained by the bisphosphorylated (*m/z* 1371.9) and monophosphorylated (*m/z* 1291.9) tetra-acylated lipid A that were formed by hydrolysis of 14:0(3-OH) and 14:0(3-O-14:0) at O-3 and O-3′, respectively, from the hepta-acylated lipid A. The ion at *m/z* 1263.9, contributing to an additional microheterogeneity of the lipid A (mass difference of 28 Da). The MS analysis also revealed the presence of ion at *m/z* 453.4 that corresponded to the deprotonated 14:0(3-O-14:0) released upon NH_4OH hydrolysis.

To confirm the presence of 16:0 as a secondary fatty acid at N-2 of the proximal GlcN, MS^2 analysis of an ion at *m/z* 1291.9 was performed with the use of an ESI-(Q)-TOF mass spectrometer (Fig. 5B). The fragmentation pattern observed for the singly charged ion at *m/z* 1291.9 revealed the structure showed in Fig. 5B (inset structure), corresponding to the GlcN disaccharide monophosphorylated at O-4' and substituted with 14:0(3-O-16:0) and 14:0(3-O-12:0) at N-2 and N-2', respectively. Two ions at *m/z* 1035.7 and *m/z* 1091.7 could correspond to elimination of 16:0 and 12:0, respectively. Subsequent loss of both fatty acids yielded the

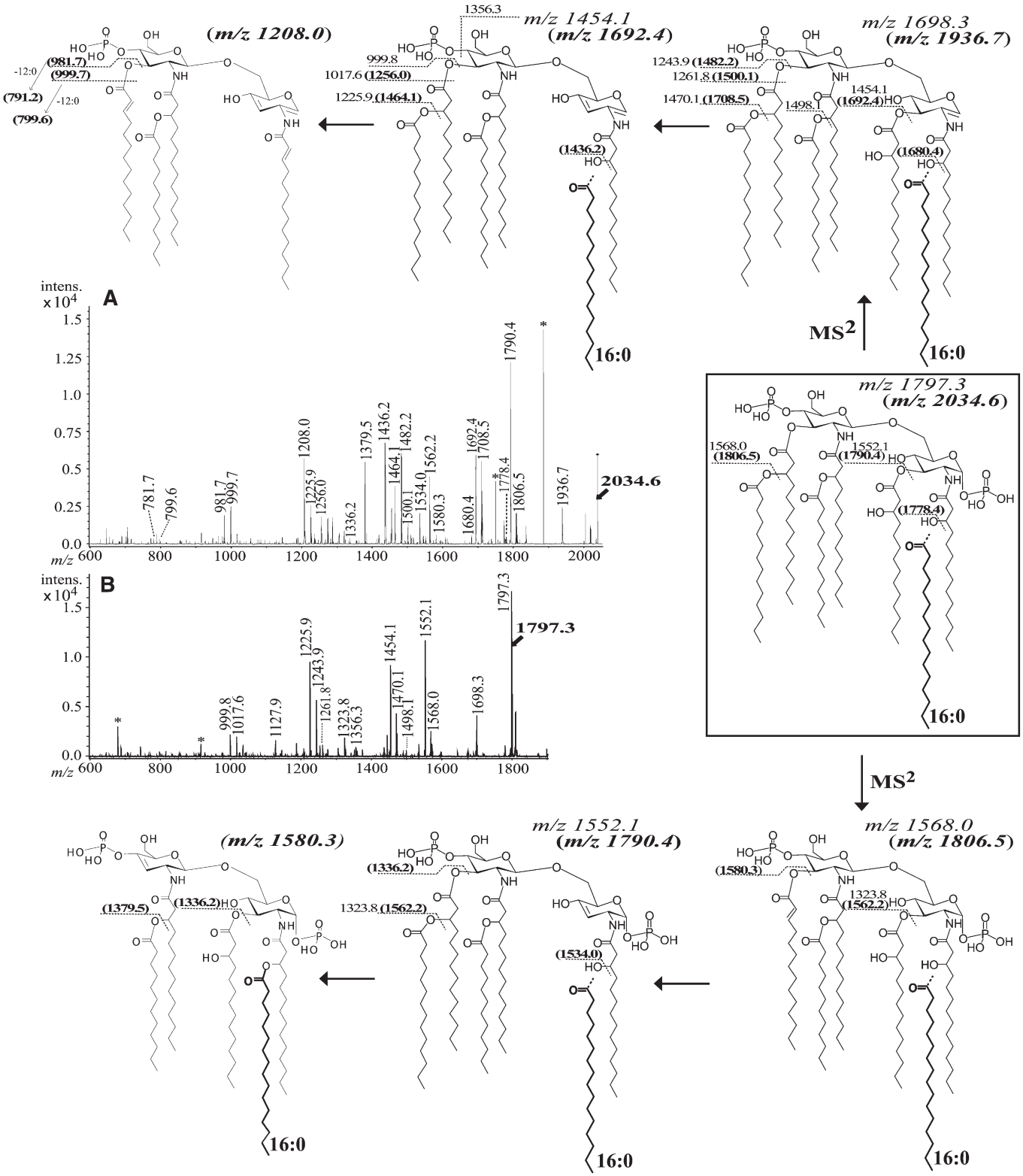

Fig. 4. Negative ion mode ESI-MS^2 of lipid A isolated from *H. alvei* 32 LPS. A: The MS^2 of the ion at *m/z* 2034.6 (bisphosphorylated and hepta-acylated lipid A, outlined inset structure). B: The MS^2 spectrum of the ion at *m/z* 1797.3 (bisphosphorylated and hexa-acylated lipid A, outlined inset structure). Interpretation of the observed fragment ions is presented in the inset structures for both parent ions. Regular font denotes ions for the hexa-acylated lipid A. *m/z* Values in brackets, written in bold, are related to MS^2 of ion at *m/z* 2034.6 contributing to the bisphosphorylated and hepta-acylated lipid A. Abbreviations 12:0 and 16:0 stand for dodecanoic and hexadecanoic acid, respectively. The asterisk denotes ions that were not interpreted.

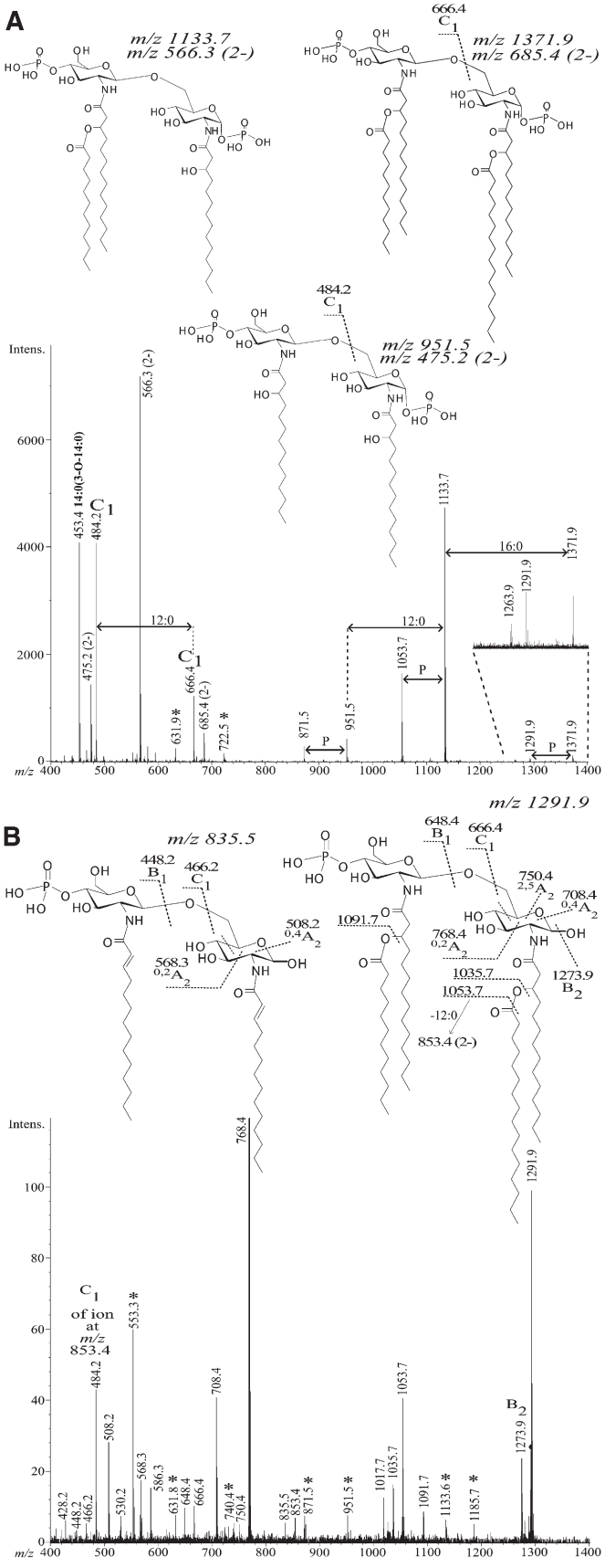

Fig. 5. A: Negative ion mode ESI-MS mass spectrum obtained for partially deO-acylated lipid A (inset structures) isolated from LPS of *H. alvei* 32. The difference of 80 Da corresponds to the difference in mass between mono- and bis-phosphorylated molecules. Arrows assigned with abbreviations 12:0 or 16:0 correspond to the difference in mass attributed to dodecanoic and hexadecanoic acids. Most of ions are singly charged. Those indicated with (2-) are doubly charged ions. B: Negative ion mode ESI-MS^2 spectra of partially deO-acylated lipid A from *H. alvei* 32 (inset structure) represented by the singly charged ion at *m/z* 1291.9. Interpretation of the observed fragment ions is presented using inset structures and described as explained in the legend to Fig. 2. The asterisk denotes ions that were not interpreted.

ion at *m/z* 835.5 (Fig. 5B, inset structure). The diagnostic fragment ions, e.g., $^{0,2}A_2$ (*m/z* 768.4), $^{0,4}A_2$ (*m/z* 708.4), $^{2,5}A_2$ (*m/z* 750.4), C_1 (*m/z* 666.4), and B_1 (*m/z* 648.4) supported the presence of amide-bound 14:0(3-O-12:0) at N-2' and the free hydroxyl group at position O-3. Most of these ions had analogs resulting from elimination of 12:0 (*m/z* 568.3, 508.2, 466.2, 448.2). Several ions corresponded to molecules devoid of one or two secondary 12:0 and/or 16:0 fatty acids (*m/z* 1053.7, 871.5, 853.4) and this could be a result of dissociation of solvated deprotonated species at the skimmer of electrospray source (31). The presence of described ions combined with the ESI-MS^n analysis of native lipid A allowed the determination of the seventh fatty acid as a constituent of the acyl substituent 14:0(3-O-16:0) at N-2.

MALDI-TOF analysis

The structural information provided by the negative ESI-MS analysis was compared with MALDI-TOF MS data to confirm the proposed structure of lipid A and above all, the substitution positions of 14:0(3-OH) at O-3 and 14:0 at O-3′ as the "secondary" ester-linked fatty acids. These fatty acids were eliminated first during the ESI-MS^2 experiments. MALDI-TOF mass spectra of *H. alvei* 32 lipid A obtained at high laser power in the positive-ion mode showed the presence of the oxonium ion B_1^+, which identified the fatty acids that substitute the β-D-GlcN4P residue (supplementary Fig. III). The most abundant ions represented the sodium $[M+Na]^+$ (*m/z* 1741) and potassium $[M+K]^+$ (*m/z* 1757) adducts of the monophosphorylated form of hexa-acylated lipid A and the B_1^+ ions (*m/z* 1087 and 887), arising from the cleavage of the glycosidic linkage between the two GlcN residues at high laser power settings. The ion at *m/z* 1087 corresponds to the average mass of the phosphorylated tetraacylated GlcN, substituted with 14:0(3-O-12:0) at N-2' and 14:0(3-O-14:0) at O-3′. The B_1^+ ion devoid of the secondary-bound 12:0 fatty acid at N-2' was also detected (*m/z* 887). Elimination of the secondary fatty acid at N-2' is preferred in positive-ion mode MS in comparison to negative-ion mode experiments (34). The presence of such ions provided additional evidence that the N-2' amide-bound 14:0(3-OH) fatty acid is substituted by a 12:0 and not by a 14:0 fatty acid.

DISCUSSION

The lipopolysaccharide constitutes the "pathogen-associated molecular pattern" for host infection by Gram-negative bacteria and is among the most powerful natural activators of the innate immune system (1). Lipid A, the

center of biological activity of the LPS, has been the last uncharacterized region of endotoxins isolated from *H. alvei*.

To complete the recently published structure of the N,O-deacylated LPS of *H. alvei* 32 (7), the lipid A isolated from LPS 32 was investigated by qualitative analysis of fatty acids and their location in the lipid A disaccharide backbone was established. Comparative analysis performed by MS suggested that LPS of *H. alvei* 32, PCM 1192, PCM 1206, and PCM 1207 have the same lipid A structure (Fig. 1, inset structure).

We have now demonstrated that lipid A of *H. alvei* LPS consists of hexa- and hepta-acylated molecules. Detailed mass spectrometric analysis carried out on lipid A isolated from LPS of *H. alvei* 32 and PCM 1192 showed that their major form was built of β-Glc*p*N4P-(1→6)-α-Glc*p*N1P substituted with saturated "primary" (*R*)-3-hydroxylated and "secondary" nonhydroxylated acyl residues of 12 and mostly 14 carbon atoms (Fig. 1), conforming to a classical form of enterobacterial-type lipid A. The analyzed lipids A of *H. alvei* are identical with those of *E. coli* (2, 3), *S. typhimurium* (35, 36), *Serratia marcescens* (37), *Providencia rettgeri* (37), *Klebsiella oxytoca* (33), and *Shigella flexneri* Sc576 (*msbB* mutant) (29). The carbohydrate backbone and primary-linked fatty acids of *H. alvei* lipid A are also similar to those of *Klebsiella pneumoniae, Proteus mirabilis*, *Aeromonas sp.*, *Haemophilus influenzae*, *Campylobacter jejuni*, *Shigella sonnei*, *Yersinia pestis*, *Enterobacter agglomerans*, *Ervinia corotovora*, and *Rhizobium etli* (36).

The described form of lipid A warrants maximal immunostimulatory activities of LPSs (2). Because most of *H. alvei* LPSs studied to date are smooth-type molecules and have *E. coli*-type lipid A structure, they would most likely induce high inflammatory response of the innate immunity system during sepsis as well.

The presence of hepta-acylated forms of *H. alvei* lipid A resulted from the addition of a palmitate (16:0) to 14:0(3-OH) at N-2 of Glc*p*N. This type of a single modification of lipid A, called palmitoylation, was previously observed only in a few enterobacterial lipids A of *S. enterica* serovar *Typhimurium* (38), *E. coli* (39), and *K. pneumoniae* (40). Similar modification of an acyl moiety of lipid A was also observed in *Legionella pneumophila*, *Pseudomonas aeruginosa, Bordetella bronchiseptica*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis* LPS, but in most of the cases the palmitoylation position was different (41). It is known that palmitoylated lipid A usually coexists as a substoichiometric component with other lipid A variants. A palmitate chain originating from a phospholipid is incorporated into lipid A by an outer membrane enzyme (PagP). The PagP enzyme transfers palimitate chain from the *sn*-1 position of a phospholipid to the hydroxyl group of the 14:0(3-OH) at N-2 of proximal Glc*p*N of lipid A (41). The gene *pagP* and its functional homologs were identified among several bacteria, for example *Salmonella*, *E. coli*, *L. pneumophila*, *B. bronchiseptica*, *Y. enterocolitica*, and *Y. pseudotuberculosis* (41). The palmitoylation, together with the regulated addition of Ara4N and PEtn, is dependent on the environmental conditions and can directly protect the bacterium from certain host immune defenses (41). Such a modification attenuates the ability of lipid A to activate defense mechanisms through the TLR4 signal transduction pathway (41) and provides resistance to certain cationic antimicrobial peptides (38). The addition of palmitate presumably increases the hydrophobic and van der Waals interactions in the outer membrane of the cell wall, thus, preventing translocation of the cationic antimicrobial peptides across the bilayer. The palmitoylation helps bacteria to maintain and monitor the outer membrane permeability and lipid asymmetry as well (41). *Salmonella*, as an enteropathogen, utilizes lipid A modifications to survive within macrophages (41). Studies of the immune signaling in human cell lines showed that chemically synthesized hepta-acylated lipid A has 10- to 100-fold lower activity in comparison with its hexa-acylated analogs (42).

Some LPSs of *H. alvei* and *K. pneumoniae* share their general structure, having structural Kdo-containing motifs in the outer core region (7) and a basic lipid A structure. LPS isolated from the polymyxin-resistant *K. pneumoniae* O3 mutant contained approximately five times more of the 4-amino-4-deoxy-L-arabinopyranose and an increased ratio of hepta-acylated lipid A (the addition of 16:0 fatty acid) compared with that of a polymyxin-sensitive parent strain (40).

Inasmuch as many of the already known cases of Gram-negative bacteria employing the palmitoylation of lipid A regulate this process through the PhoP/PhoQ-activated *pagP* gene or its homologs (41), it is possible that *H. alvei* could regulate this process in a similar way. Recent studies showed that some *H. alvei* strains were able to enter and persist in human epithelial cells, but little is known about factors contributing to this invasion strategy (18). Thus, *H. alvei* lipid A structure elucidation and the identification of hepta-acylated variant among other forms of *H. alvei* lipid A are important for the understanding of the pathogenicity of this bacterium.

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