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# Structural characterization of the lipid A region of *Aeromonas salmonicida* subsp. *salmonicida* lipopolysaccharide

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**Abstract**—The lipid A components of *Aeromonas salmonicida* subsp. *salmonicida* from strains A449, 80204-1 and an in vivo rough isolate were isolated by mild acid hydrolysis of the lipopolysaccharide. Structural studies carried out by a combination of fatty acid, electrospray ionization-mass spectrometry and nuclear magnetic resonance analyses confirmed that the structure of lipid A was conserved among different isolates of *A. salmonicida* subsp. *salmonicida*. All analyzed strains contained three major lipid A molecules differing in acylation patterns corresponding to tetra-, penta- and hexaacylated lipid A species and comprising 4′-monophosphorylated β-2-amino-2-deoxy-D-glucopyranose-(1→6)-2-amino-2-deoxy-D-glucopyranose disaccharide, where the reducing end 2-amino-2-deoxy-D-glucose was present primarily in the α-pyranose form. Electrospray ionization-tandem mass spectrometry fragment pattern analysis, including investigation of the inner-ring fragmentation, allowed the localization of fatty acyl residues on the disaccharide backbone of lipid A. The tetraacylated lipid A structure containing 3-(dodecanoyloxy)tetradecanoic acid at *N*-2′,3-hydroxytetradecanoic acid at N-2 and 3-hydroxytetradecanoic acid at O-3, respectively, was found. The pentaacyl lipid A molecule had a similar fatty acid distribution pattern and, additionally, carried 3-hydroxytetradecanoic acid at O-3′. In the hexaacylated lipid A structure, 3-hydroxytetradecanoic acid at O-3′ was esterified with a secondary 9-hexadecenoic acid. Interestingly, lipid A of the in vivo rough isolate contained predominantly tetra- and pentaacylated lipid A species suggesting that the presence of the hexaacyl lipid A was associated with the smooth-form lipopolysaccharide.


*Keywords:* *Aeromonas salmonicida*; Lipopolysaccharide; Lipid A; NMR; ESIMS

## 1. Introduction

*Aeromonas salmonicida* subsp. *salmonicida* is one of the lethal aquatic pathogens and is the cause of furunculosis in salmonid fish, resulting in high mortalities in aquaculture.[1] *A. salmonicida* subsp. *salmonicida* strains possess an outer-membrane LPS, which is partially exposed on the cell surface and plays an important role in the maintenance of cell surface protein array, host-cell invasion and bacterial survival.[2,3] Similar to other gram-negative bacteria, *A. salmonicida* subsp. *salmonicida* LPS is composed of three distinct structural regions: O-chain polysaccharide, core oligosaccharide and lipid A moiety. The lipid A portion of LPS is linked to the core oligosaccharide *via* 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and serves as the hydrophobic anchor of LPS in the outer membrane. Previous reports have suggested that lipid A, as the principal endotoxic component of LPS, plays a major role in the pathogenesis of bacterial infections[4–6] and is an important contributor to massive inflammation, sepsis and septic shock leading to fatalities in gram-negative bacterial infections. It also promotes the activation of the innate immune system *via* the induction of inflammatory cytokines released

*Abbreviations*: CF, cystic fibrosis; COSY, correlated spectroscopy; 2D, two-dimensional; ESIMS, electrospray ionization-mass spectrometry; GLC, gas liquid chromatography; HSQC, heteronuclear single quantum correlation; IL-8, interleukin-8; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo); LPS, lipopolysaccharide; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; RNA, ribonucleic acid; TOCSY, total correlated spectroscopy; TSB, Tryptic Soy Broth

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by human cells. The chemical structure of lipid A from a number of different bacterial species has been investigated.[7–9] It usually contains a diglucosamine backbone substituted with both ester- and amide-linked fatty acyl side-chains and may carry phosphate groups at O-1 and O-4′. In addition, other sugar constituents, such as 4-amino-4-deoxy-L-arabinose, have been found.[10] Recently we established structures of the O-chain polysaccharide and core oligosaccharide region of *A. salmonicida* subsp. *salmonicida*.[11–13] In the present study, we describe the structural determination of the lipid A region of *A. salmonicida* subsp. *salmonicida* LPS from strains A449 and 80204-1 and an in vivo rough isolate.

## 2. Experimental

### 2.1. Bacterial culture and growth conditions

*A. salmonicida* subsp. *salmonicida* strains A449 and 80204-1 and an in vivo-derived rough isolate were obtained from the Institute for Marine Biosciences, National Research Council of Canada (Halifax, Nova Scotia). The bacteria were cultured in Tryptic Soy Broth (TSB) at 18 °C for 48–72 h. The cells were killed with 1% (w/v) phenol soln (22 °C, 4 h), washed with 10 mM phosphate buffered saline (PBS, pH 7.4) and harvested by low-speed centrifugation (3000*g*, 25 min).

### 2.2. LPS isolation

Bacterial cells were washed successively with 2.5% saline (w/v). LPS from *A. salmonicida* subsp. *salmonicida* strains A449 and 80204-1 was extracted by the method of Westphal and Jann,[14] while LPS from the in vivo rough isolate was extracted from dried cells with 5:5:8 phenol–$CHCl_3$–light petroleum at 60–95 °C and precipitated as described previously.[15,16] Crude LPS was purified by ultracentrifugation (105,000*g*, 4 °C, 16 h).

### 2.3. Mild acid hydrolysis of LPS and purification of lipid A

LPS was hydrolyzed by treatment with 2% AcOH at 100 °C for 2 h. The soln was cooled down on ice, and the precipitated lipid A was collected by low-speed centrifugation (3000*g*, 20 min). The lipid A pellet was dissolved in 1:10 MeOH–$CHCl_3$ soln, subjected to silica gel chromatography and sequentially eluted with 1:10, 1:5, 1:2 and 2:1 MeOH–$CHCl_3$ soln. Each fraction was collected separately and analyzed by NMR and ESIMS.

### 2.4. O-Deacylation of lipid A

The lipid A isolated by mild acid hydrolysis (2–3 mg) was treated with 0.25 N sodium methoxide in absolute MeOH[17] (0.5 mL). The solution was kept at rt for 24 h and acidified with 1 M HCl to pH 5–6. Free fatty acid methyl esters were extracted with hexane and analyzed by GLC–MS as described below. The hexane-insoluble material was subjected to ESIMS analysis and also analyzed by GLC–MS following the treatment with 3% (w/v) methanolic hydrogen chloride (100 °C, 16 h) and neutralization with silver carbonate (Aldrich).

### 2.5. NMR spectroscopy

NMR spectra were performed on Varian INOVA 400 MHz spectrometer using a standard software. All NMR experiments were performed at 25 °C using a 5 mm indirect detection probe with the $^1$H coil nearest to the sample in $CDCl_3$–$CD_3OD$ (2:1, v/v). Chloroform ($^1$H, $\delta$ 7.26 ppm) and MeOH ($^{13}$C, $\delta$ 49.15 ppm) were used as internal references. Standard homo- and heteronuclear correlated 2D techniques were used for general assignments: COSY, TOCSY and HSQC.[18]

### 2.6. General methods

Fatty acids were determined by GLC–MS analysis of their methyl esters derived by the sealed-tube hydrolysis of LPS with 3% (w/v) methanolic hydrogen chloride at 100 °C for 16 h and neutralized with silver carbonate. For GLC analysis, a 30 m DB-5 capillary column [160 °C (2 min) to 260 °C at 1 °C/min] was used and the identity of each fatty acid was established by comparison of its MS profile with that of the reference compound.

### 2.7. Electrospray ionization-mass spectrometry (ESIMS)

All experiments were performed with a capillary electrophoresis interface as described in detail previously.[19,20] Briefly, a Crystal Model 310 CE instrument (ATI Unicam, Boston, MA, USA) was coupled to a mass spectrometer, API 3000 or 4000 Q-Trap (Applied Biosystems/MDS Sciex, Concord, Canada) *via* a microionspray interface. The lipid A samples were dissolved in 2:1 MeOH–$CHCl_3$ soln. The sheath soln (2:1 isopropanol–MeOH) was delivered at a flow rate of 1 μL/min. An electrospray stainless steel needle (27-gauge) was butted against the low dead vol tee, which enabled the delivery of the sheath soln to the end of the capillary column.

## 3. Results

LPSs were extracted from the enzyme-digested cells of *A. salmonicida* subsp. *salmonicida* strains A449 and 80204-1 using hot aqueous phenol method and from the dried cells of the in vivo rough isolate by a modified phenol–chloro-

form–light petroleum (PCP) method and purified by ultracentrifugation. Purified LPS was free of RNA as inferred by the absence of ribose in the sugar analysis and its protein content was ca. 3% (data not shown). SDS-PAGE analysis of LPSs indicated that *A. salmonicida* subsp. *salmonicida* strains A449 and 80204-1 contained high molecular mass smooth-form LPS, while an in vivo isolate produced a low molecular mass rough-form LPS, as described in previous reports[12,13] (not shown). Lipid A was released from LPS by mild acid hydrolysis. Fatty acid analysis of the crude lipid A revealed the presence of 3-hydroxytetradecanoic acid [C14:0(3-OH)], dodecanoic acid (C12:0) and 9-hexadecenoic acid (C16:1n9). Following alkaline O-deacylation, the reaction mixture was extracted with hexane to remove released fatty acids. Both the residual lipid A and the hexane extract were analyzed by GLC as methyl ester derivatives, which confirmed that the residual lipid A contained C14:0(3-OH) exclusively and that the hexane extract possessed a mixture of the above mentioned fatty acids suggesting that C12:0 and C16:1n9 were ester-linked and C14:0(3-OH) was both ester and amide bound.

The ESIMS analysis confirmed that lipid A isolated by mild acid hydrolysis from *A. salmonicida* subsp. *salmonicida* strains A449, 80204-1 and an in vivo isolate exhibited similar MS profiles (Fig. 1). Three major lipid A species corresponding to tetra-, penta- and hexaacylated lipid A components were observed at *m*/*z* 1280.3, *m*/*z* 1506.3 and *m*/*z* 1743.8, suggesting that the lipid A structure was conserved among *A. salmonicida* subsp. *salmonicida* isolates. Significantly less hexaacylated lipid A form was found in lipid A from the in vivo-derived rough isolate as compared with lipid A from *A. salmonicida* subsp. *salmonicida* strains A449 and 80204-1. Following O-deacylation with MeONa, the residual lipid A was subjected to ESIMS analysis, which revealed the presence of the fragment ion at *m*/*z* 871.6 corresponding to a molecular species containing 2 glucosamine (GlcN) residues, 1 phosphate (P) and 2 C14:0(3-OH), thus suggesting that the diglucosamine backbone in lipid A species of *A. salmonicida* subsp. *salmonicida* LPS was substituted with C14:0(3-OH) at both N-2 and N-2′.

The crude lipid A from *A. salmonicida* subsp. *salmonicida* strain A449 was subjected to purification by silica gel chromatography and sequential fractionation with 1:10, 1:5, 1:2 and 2:1 (v/v) MeOH in $CHCl_3$ soln. MS analysis of the fraction 1:2 MeOH–$CHCl_3$ revealed the presence of the molecular ions $[M-H_2O-H]^-$ at *m*/*z* 1489.5 and *m*/*z* 1726.5 suggesting that it contained both penta- and hexaacylated lipid A species. This was followed by NMR analysis, which confirmed the presence of the classical diglucosamine backbone in *A. salmonicida* subsp. *salmonicida* lipid A structure. The $^1H$ and $^{13}C$ NMR resonances corresponding to the disaccharide backbone were completely assigned using 2D COSY (Fig. 2), TOCSY and HSQC experiments (Table 1). Two anomeric resonances were observed at 4.77 ppm/91.6 ppm for the reducing end GlcN **A** and 4.39 ppm/102.1 ppm for the non-reducing end GlcN **A**′, respectively. The presence of aminosugars was confirmed by correlation of their H-2 protons at the nitrogen-bearing carbon to the corresponding C-2 carbons at $\delta$ 3.77 ppm/51.5 ppm for GlcN **A** and $\delta$ 3.54 ppm/54.2 ppm for GlcN **A**′. In our previous report,[12] the structure of 1,4′-bisphosphorylated β-D-GlcN-(1→6)-α-D-GlcN (**A**′→**A**) disaccharide backbone of lipid A was identified in the deacylated LPS of *A. salmonicida* subsp. *salmonicida* LPS (LPS-OH). In the current investigation, significant upfield chemical shifts were observed for H-1, H-5 and H-6 of GlcN **A** (Table 1) as compared to their previous positions in the structure of 1,4′-bisphosphorylated β-D-GlcN-(1→6)-α-D-GlcN (**A**′ → **A**),[12] indicating the absence of a phosphate group at O-1 of GlcN **A**. Similar effects were observed in structural investigations of lipid A backbone of *Escherichia coli*[21] and *Salmonella enterica* sv. Minnesota Re[22] LPS. Based on NMR analysis, the reducing end GlcN **A** residue was present primarily in the α-pyranose form. MS analysis of the crude lipid A isolated after mild acid hydrolysis (Fig. 3) confirmed the presence of the monophosphoryl lipid A species (see below). The lability of the phosphate group at O-1 during hydrolysis was previously observed by other research groups.[23,24] In addition, significant downfield chemical shifts observed for H-3 of GlcN **A** (4.86 ppm) and H-3′ of GlcN **A**′ (4.92 ppm), as compared with the previously reported data,[12] indicated that positions O-3 and O-3′ of the lipid A were acylated. The complete structural elucidation of the lipid A from *A. salmonicida* subsp. *salmonicida*, including the distribution of acyl residues, was based on the fragmentation analysis using tandem mass spectrometry (MS/MS) in the negative-ion mode as discussed below.

### 3.1. MS/MS analysis of the hexaacylated lipid A species at *m*/*z* 1743.8

The pseudomolecular ion at *m*/*z* 1743.8 corresponded to the monophosphorylated hexaacylated lipid A species containing four C14:0(3-OH), one C12:0 and one C16:1n9 (Fig. 1). Tandem mass spectrometry strategy was used to confirm the fatty acid substitution pattern in the hexaacylated lipid A species at *m*/*z* 1743.8, which provided a series of fragment ions corresponding to the acyl residue elimination (Fig. 3a and Table 2). The fragmentation pattern analysis and corresponding lipid A structures with expected *m*/*z* values are shown in Scheme 1. As discussed above, NMR analysis suggested that the phosphate group was located at O-4′, which was also supported by MS/MS analysis (see below).

Kussak and Weuntraub[25] have recently proposed that phosphorylation of lipid A backbone at O-4′ results in elimination of the acyl group at O-3′ by two competing

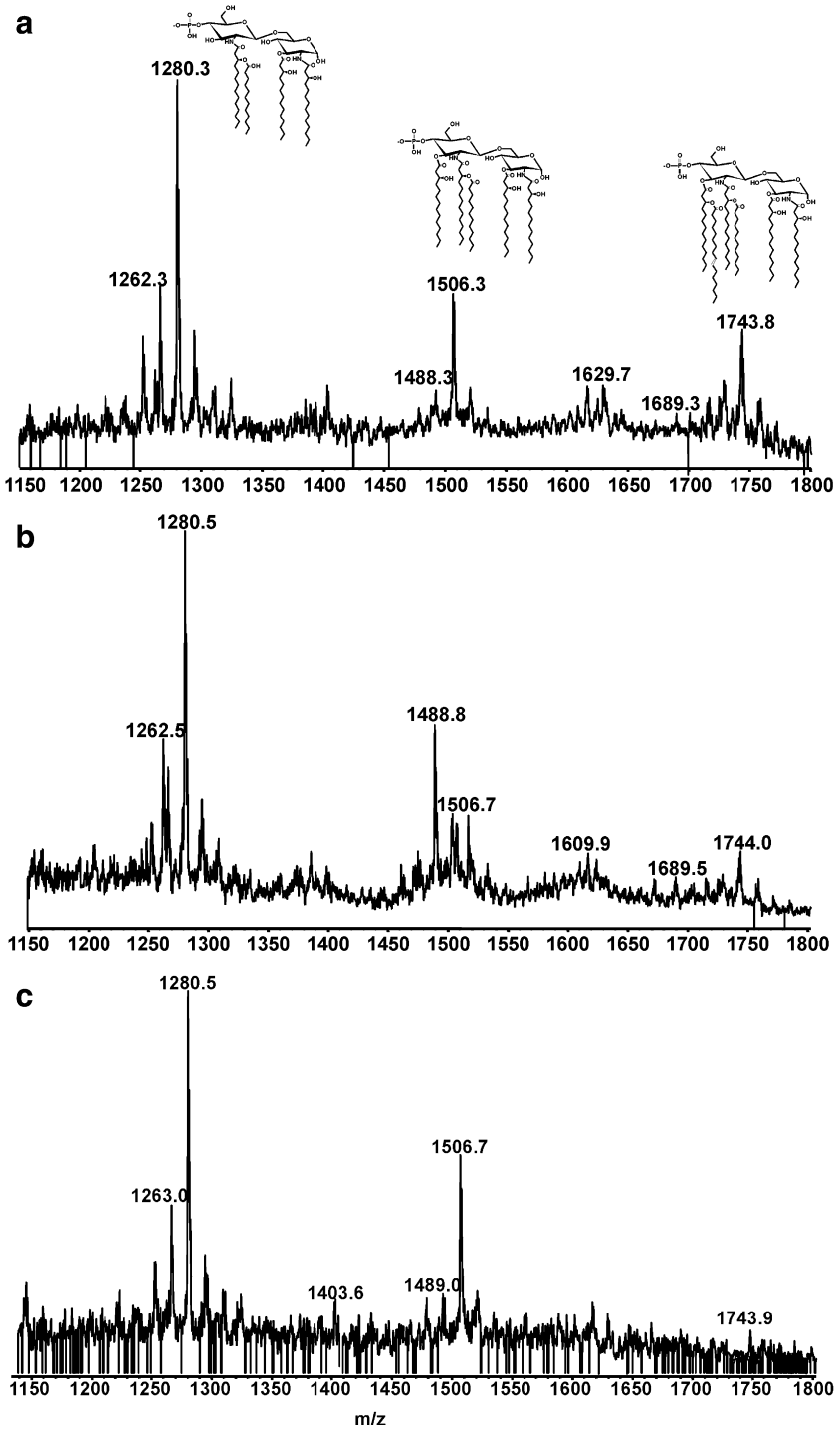



**Figure 1.** ESIMS spectra (-ion mode) of lipid A isolated by mild acid hydrolysis of *A. salmonicida* subsp. *salmonicida* LPS; major molecular structures are indicated: (a) strain A449; (b) strain 80204-1; (c) in vivo-derived rough isolate.

fragmentation processes, either in a form of a ketene derivative (charge-driven process) or as a free fatty acid (charge-remote process). Other acyl groups lacking an adjacent phosphate group, such as fatty acid at O-3 or at the secondary substitution site, are usually eliminated as free fatty acids by a charge-remote process. MS/MS of the pseudomolecular ion at *m*/*z* 1743.8 resulted in the fragment ion at *m*/*z* 1281.1 corresponding to the loss of an acyl–oxyacyl group from the secondary ester-bound C16:1n9 of C14:0(3-OH) {C14:0[3-O(C16:1n9)]} as a ketene derivative (charge-driven process) (Scheme 1). The fragment ion at *m*/*z* 1263.0 indicated that the acyl–oxyacyl group at O-3′ could also be eliminated as a free acid by a charge-remote process (Scheme 1).

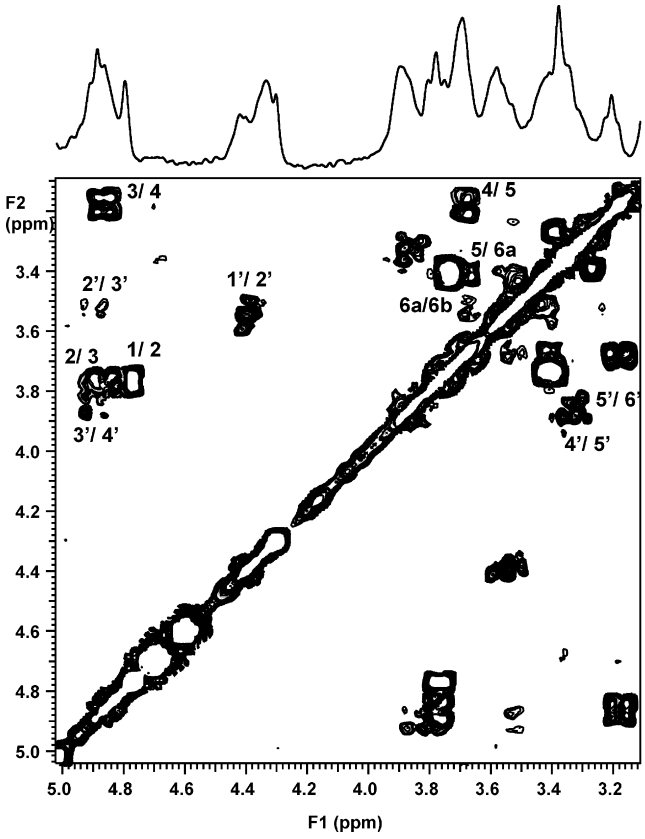



**Figure 2.** A portion of $^{1}H$–$^{1}H$ COSY spectrum of lipid A fraction 1:2 MeOH–$CHCl_3$ from *A. salmonicida* subsp. *salmonicida* strain A449 LPS with assignments.

**Table 1.** Partial NMR data of lipid A from *A. salmonicida* subsp. *salmonicida* strain A449 recorded in 2:1 $CD_3Cl$–$CD_3OD$ at 25 °C

| Residue | Atom no. | Chemical shift (ppm) | | | |
|---|---|---|---|---|---|
| | | $^{1}H$ | | $^{13}C$ | |
| | | Lipid A[a] | Lipid A backbone[b] | Lipid A | Lipid A backbone |
| GlcN (**A**) | 1 | 4.77 | 5.64 | 91.6 | 90.9 |
| | 2 | 3.77 | 3.37 | 51.5 | 54.8 |
| | 3 | 4.86 | 3.90 | 73.7 | 70.1 |
| | 4 | 3.19 | 3.48 | 68.0 | 70.1 |
| | 5 | 3.68 | 4.17 | 70.2 | 72.4 |
| | 6 | 3.74, 3.43 | 4.34, 3.89 | 69.3 | 69.8 |
| GlcN (**A′**) | 1 | 4.39 | 4.88 | 102.5 | 99.5 |
| | 2 | 3.54 | 3.08 | 54.2 | 55.8 |
| | 3 | 4.92 | 3.85 | 72.7 | 73.0 |
| | 4 | 3.86 | 3.82 | 67.6 | 73.5 |
| | 5 | 3.34 | 3.71 | 75.6 | 74.4 |
| | 6 | 3.87 | 3.56, 3.70 | 62.3 | 62.7 |

[a] Lipid A fraction 1:2 MeOH–$CHCl_3$ containing penta- and hexa-acylated lipid A species.

[b] NMR data for 1,4′-bisphosphorylated β-D-GlcN-(1→6)-α-D-GlcN (**A′** → **A**) disaccharide backbone are from Ref. 12.

Observation of major fragment ions at *m*/*z* 1035.6 and *m*/*z* 1017.6 in MS/MS spectrum of the pseudomolecular ion at *m*/*z* 1743.8 was consistent with the cleavage of

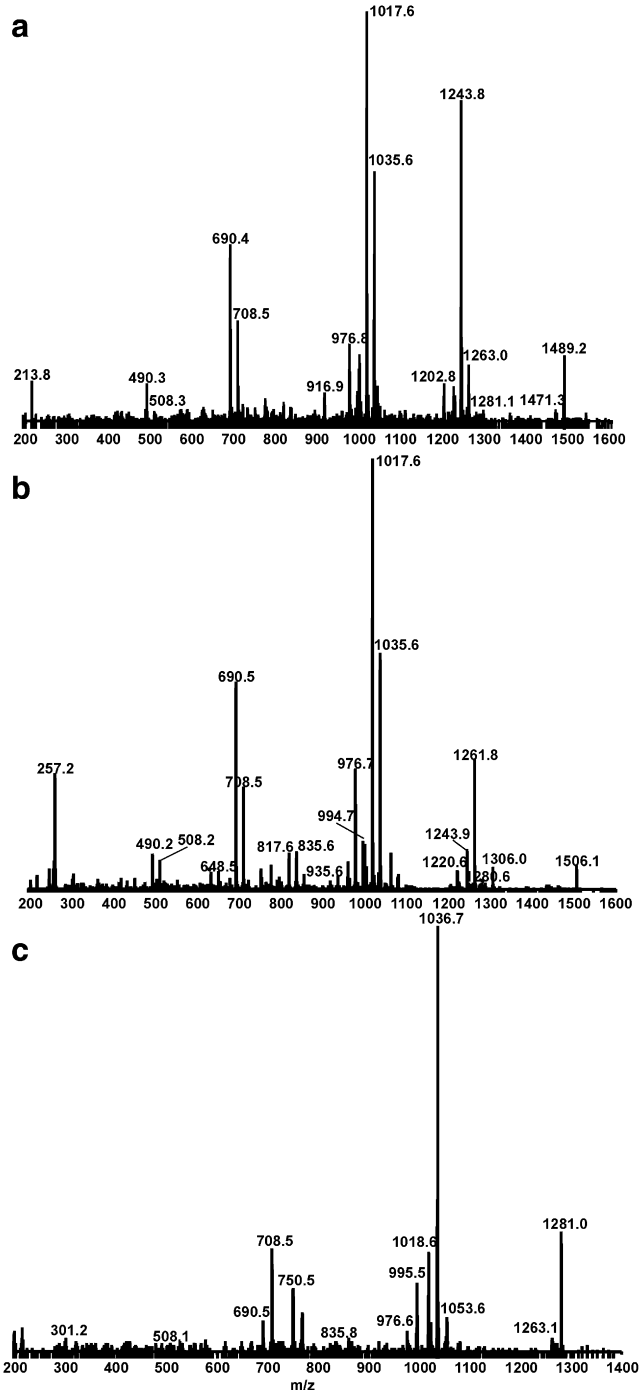



**Figure 3.** ESIMS/MS spectra (-ion mode) of lipid A isolated by mild acid hydrolysis of *A. salmonicida* subsp. *salmonicida* strain A449: (a) MS/MS of *m*/*z* 1743.8; (b) MS/MS of *m*/*z* 1506.3; (c) MS/MS of *m*/*z* 1280.3.

C14:0[3-O(C16:1n9)] by both charge-driven and charge-remote processes, respectively, and with a simultaneous elimination of C14:0(3-OH) as compared with fragment ions at *m*/*z* 1281.1 and *m*/*z* 1263.0 (Scheme 1). Elimination of C14:0[3-O(C16:1n9)], either as a ketene or fatty acid, confirmed that it was located at O-3′. The fragment ion at *m*/*z* 1489.2 corresponded

**Table 2.** Assignment of ions observed in ESIMS and MS/MS spectra of lipid A species of *A. salmonicida* subsp. *salmonicida* strain A449

| Molecular mass (Da) | | Proposed interpretation[a] |
|---|---|---|
| Observed | Calculated | |
| MS | | |
| 1743.8 | 1743.4 | $[M_1-H]^-$ |
| 1506.3 | 1507.0 | $[M_2-H]^-$ |
| 1488.3 | 1488.9 | $[M_2-H_2O-H]^-$ |
| 1280.3 | 1280.6 | $[M_3-H]^-$ |
| 1262.3 | 1262.7 | $[M_3-H_2O-H]^-$ |
| MS/MS of *m/z* 1743.8 | | |
| 1489.2 | 1489.0 | $[M_1-H]^-$−C16:1n9 |
| 1471.3 | 1471.0 | $[M_1-H]^-$−C16:1n9−$H_2O$ |
| 1281.1 | 1280.6 | $[M_1-H]^-$−C14:0[3-O(C16:1n9)][c] |
| 1263.0 | 1262.6 | $[M_1-H]^-$−C14:0[3-O(C16:1n9)] |
| 1243.8 | 1244.6 | $[M_1-H]^-$−C16:1n9−C14:0(3-OH) |
| 1202.8 | 1203.5 | $^{0,2}$A[b]−C16:1n9 |
| 1035.6 | 1036.2 | $[M_1-H]^-$−C14:0(3-OH)–C14:0[3-O(C16:1n9)][c] |
| 1017.6 | 1018.2 | $[M_1-H]^-$−C14:0(3-OH)–C14:0[3-O(C16:1n9)] |
| 976.8 | 977.2 | $^{0,2}$A−C14:0[3-O(C16:1n9)] |
| 916.9 | 917.2 | $^{0,4}$A−C16:1n9 |
| 708.5 | 708.8 | $^{0,4}$A−C14:0[3-O(C16:1n9)][c] |
| 690.4 | 690.8 | $^{0,4}$A−C14:0[3-O(C16:1n9)] |
| 508.3 | 508.5 | $^{0,4}$A−C14:0[3-O(C16:1n9)][c]−C12:0 |
| 490.3 | 490.5 | $^{0,4}$A−C14:0[3-O(C16:1n9)]−C12:0 |
| MS/MS of *m/z* 1506.3 | | |
| 1261.8 | 1262.6 | $[M_2-H]^-$−C14:0(3-OH) |
| 1243.9 | 1244.6 | $[M_2-H]^-$−C14:0(3-OH)–$H_2O$ |
| 1220.6 | 1221.4 | $^{0,2}$A |
| 1035.6 | 1036.2 | $[M_2-H]^-$−C14:0(3-OH)−C14:0(3-OH)[c] |
| 1017.6 | 1018.2 | $[M_2-H]^-$− 2 × C14:0(3-OH) |
| 994.7 | 995.2 | $^{0,2}$A−C14:0(3-OH)[c] |
| 976.7 | 977.2 | $^{0,2}$A−C14:0(3-OH) |
| 935.6 | 935.2 | $^{0,4}$A |
| 835.6 | 835.9 | $[M_2-H]^-$−C14:0(3-OH)–C14:0(3-OH)[c]-C12:0 |
| 817.6 | 817.9 | $[M_2-H]^-$− 2 × C14:0(3-OH)−C12:0 |
| 708.5 | 708.8 | $^{0,4}$A−C14:0(3-OH)[c] |
| 690.4 | 690.8 | $^{0,4}$A−C14:0(3-OH) |
| 508.2 | 508.5 | $^{0,4}$A−C14:0(3-OH)[c]−C12:0 |
| 490.2 | 490.5 | $^{0,4}$A−C14:0(3-OH)−C12:0 |
| MS/MS of *m/z* 1280.3 | | |
| 1263.1 | 1262.6 | $[M_3-H]^-$−$H_2O$ |
| 1036.7 | 1036.2 | $[M_3-H]^-$−C14:0(3-OH) |
| 1018.6 | 1018.2 | $[M_3-H]^-$−C14:0(3-OH)−$H_2O$ |
| 995.5 | 995.2 | $^{0,2}$A |
| 976.6 | 977.2 | $^{0,2}$A−$H_2O$ |
| 835.8 | 835.9 | $[M_3-H]^-$−C14:0(3-OH)−C12:0 |
| 750.5 | 750.8 | $^{0,2}$A−C14:0(3-OH) |
| 708.5 | 708.8 | $^{0,4}$A |
| 690.5 | 690.8 | $^{0,4}$A−$H_2O$ |
| 508.1 | 508.5 | $^{0,4}$A−C12:0 |

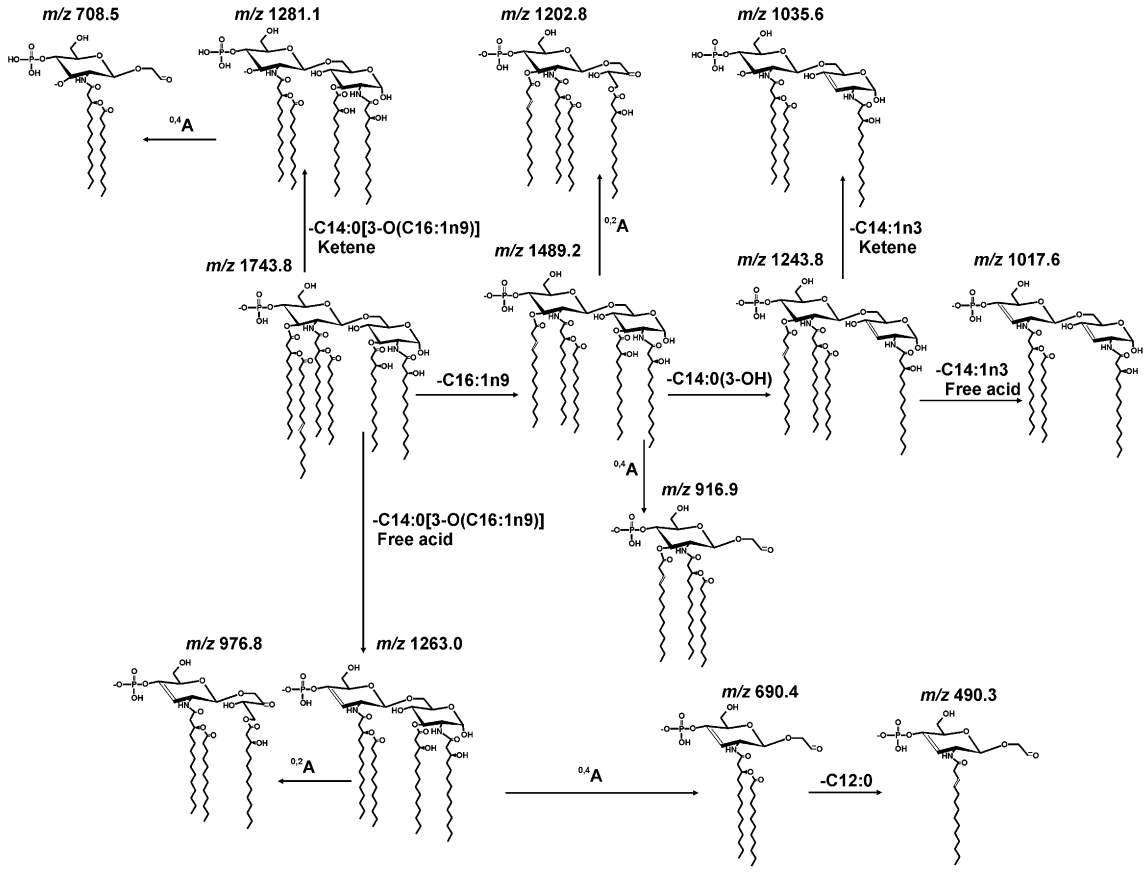
[a] $M_1$, $M_2$ and $M_3$ correspond to molecular mass of hexaacyl, pentaacyl and tetraacyl lipid A species, respectively.
[b] $^{0,2}$A and $^{0,4}$A correspond to the cross-ring fragments with cleavage in the sugar ring of GlcN **A**.
[c] Fatty acid eliminated as ketene.

to the elimination of C16:1n9. The observed fragment ion at *m/z* 1243.8 was obtained due to the loss of two acyl groups, C16:1n9 and C14:0(3-OH). Both fatty acids were eliminated in the charge-remote process. As already mentioned, C14:0(3-OH) at O-3′ was substituted with the secondary ester-bound C16:1n9. Taking into account a greater stability of acyl amides at N-2 and N-2′ it was assumed that the second C14:0(3-OH) was located at O-3 of GlcN **A**. This conclusion was reinforced by NMR analysis and further corroborated by the inter-ring fragmentation pattern discussed below.

Cross-ring fragmentation of the reducing end GlcN **A** has been widely used in structural studies of lipid A to reveal the distribution of acyl residues in lipid A. The cross-ring fragmentation mechanisms have been discussed in depth by Costello and Vath.[26,27] The MS/MS analysis of the pseudomolecular ion at *m/z* 1743.8 indicated that a series of $^{0,4}$A fragment ions (following the nomenclature of Costello and Vath[26]) detected at *m/z* 916.9, *m/z* 708.5, *m/z* 690.4 and *m/z* 490.3 were produced from the ring opening of GlcN **A** as shown in Figure 3. The fragment ions at *m/z* 916.9, *m/z* 708.5 and *m/z* 690.4 differing only in the acyl group substitution at O-3′ corresponded to the fragmentation of GlcN **A**. The ion at *m/z* 916.9 corresponded to $^{0,4}$A fragment ion, lacking the secondary C16:1n9 at O-3′ and suggesting that C14:0(3-OH) of N-2′ was substituted by the secondary C12:0 {C14:0[3-O(C12:0)]} as shown in Figure 3a and Scheme 1. The C14:0[3-O(C16:1n9)] at O-3′ has undergone elimination either as a free acid or ketene resulting in the formation of $^{0,4}$A fragment ions at *m/z* 690.4 and *m/z* 708.5. The fragment ion at *m/z* 490.3 was produced similarly to the ion at *m/z* 690.4, but with the simultaneous elimination of the secondary C12:0 of C14:0(3-OH) at O-2′. Furthermore, $^{0,2}$A fragment ions from the cross-ring fragmentation of GlcN **A** were observed at *m/z* 1202.8 and *m/z* 976.8. The former corresponded to $^{0,2}$A fragment where the secondary C16:1n9 at O-3′ was eliminated and the latter was assigned to $^{0,2}$A fragment where the acyl–oxyacyl group C14:0[3-O(C16:1n9)] at O-3′ was cleaved. Taken together, the above data confirmed that O-3 of GlcN **A** was substituted by C14:0(3-OH) since it was present in all $^{0,2}$A fragment ions. Fatty acid composition of the pseudomolecular ion at *m/z* 1743.8 containing four C14:0(3-OH), one C12:0 and one C16:1n9, suggested that O-2 of the reducing end GlcN **A** should be substituted by C14:0(3-OH). This conclusion was consistent with the results of alkaline O-deacylation discussed above. The combined evidence allowed for the complete assignment of acyl residues in the hexaacylated lipid A species at *m/z* 1743.8.

### 3.2. MS/MS analysis of the pentaacylated lipid A species at *m/z* 1506.3

The mass difference between ions at *m/z* 1743.8 and *m/z* 1506.3 (Δ 237.5 Da) suggested that pseudomolecular ion

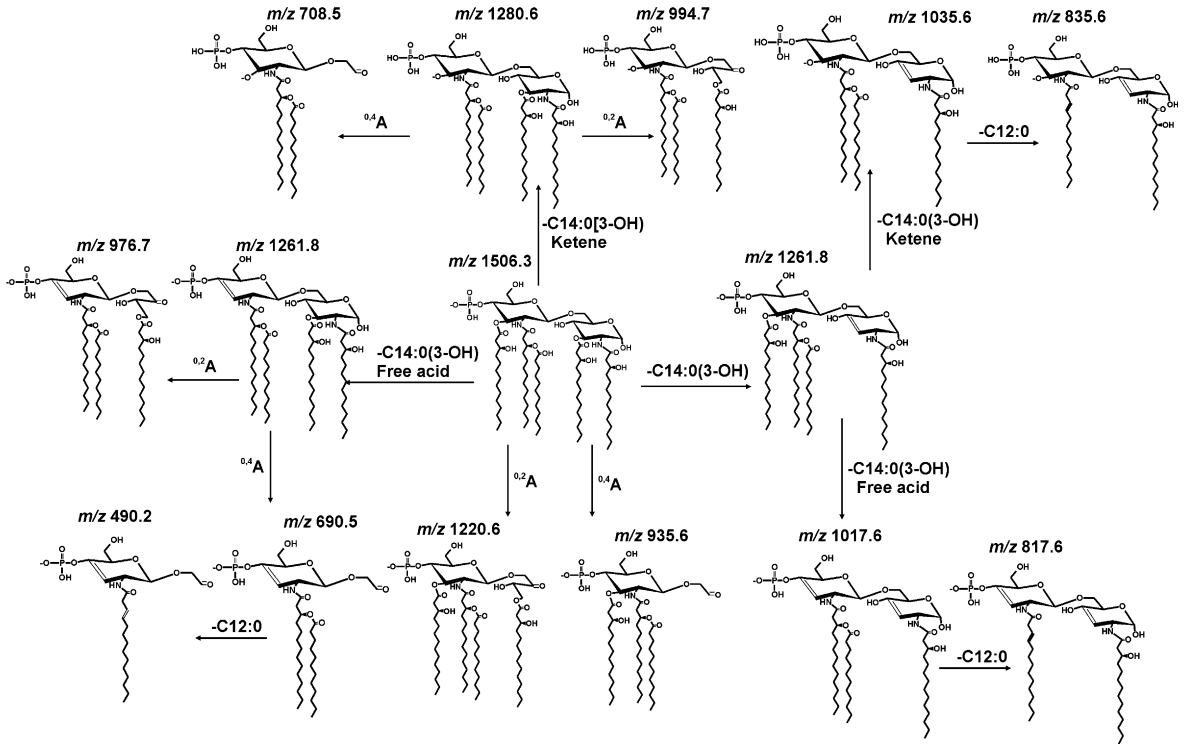



**Scheme 1.** Fragmentation pathway of the lipid A precursor ion at *m/z* 1743.8 with proposed structures.

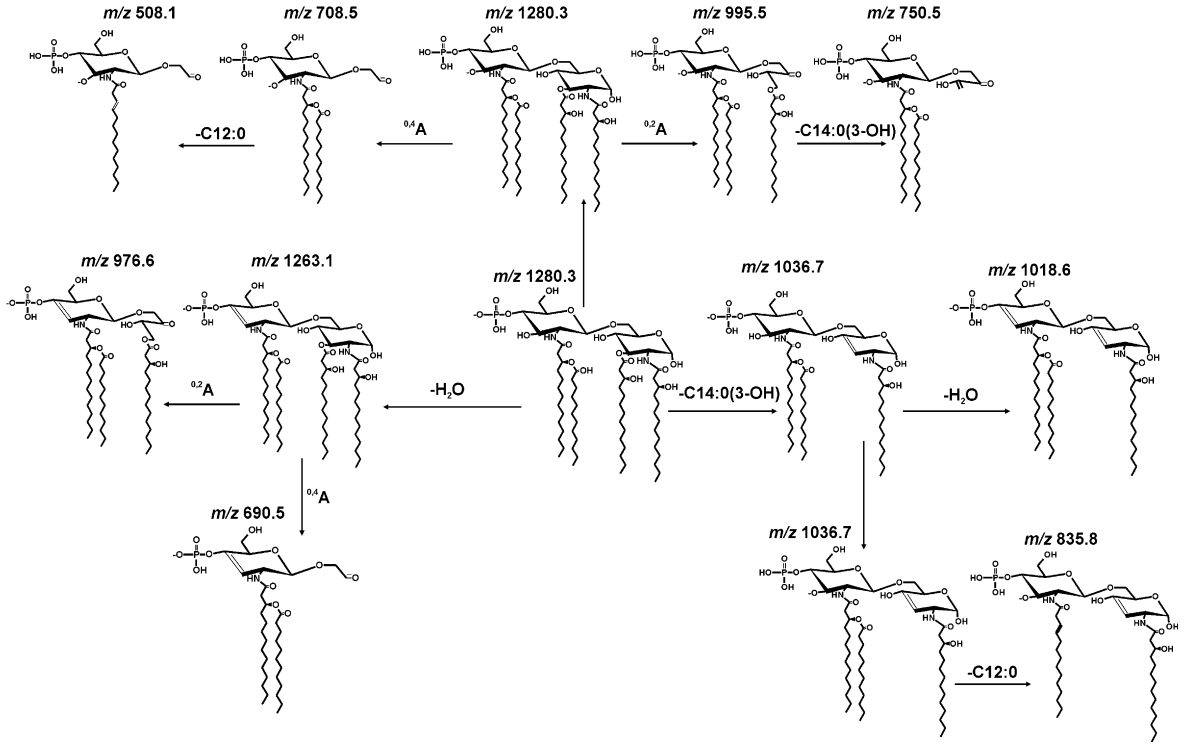
at *m/z* 1506.3 corresponding to a monophosphorylated pentaacylated lipid A species was lacking C16:1n9 as compared with the fragment ion at *m/z* 1743.8. The MS/MS spectrum of the molecular ion at *m/z* 1506.3 (Fig. 3b) contained a number of ions related to the loss of acyl residues (Scheme 2 and Table 2). The fragment ion at *m/z* 1261.8 was consistent with the loss of one C14:0(3-OH). The presence of major fragment ions at *m/z* 1017.6 and *m/z* 1035.6 was consistent with the loss of the second C14:0(3-OH) eliminated either as a ketene derivative or a free fatty acid and suggesting that a pentaacylated lipid A species was substituted by the primary C14:0(3-OH) at O-3′. Due to a greater stability of amide-bound fatty acids the first C14:0(3-OH) was located at O-3, either as the primary or the secondary fatty acid, and not at N-2 or N′-2. The ions at *m/z* 708.5, *m/z* 690.4 and *m/z* 490.2 were assigned to $^{0,4}$A fragments. Their corresponding structures were identical to the ones deduced for the molecular ion at *m/z* 1743.8 suggesting that N-2′ in the non-reducing end GlcN **A**′ of this lipid A species was also substituted by an acyl–oxyacyl group C14:0[3-O(C12:0)]. In addition, $^{0,2}$A fragment ions at *m/z* 1220.6 and *m/z* 976.7 were consistent with the presence of C14:0(3-OH) at O-3. Similarly to the molecular ion at *m/z* 1220.6, the fragment ion at *m/z* 976.7 was attributed to the cross-ring fragmentation of the reducing end GlcN **A** with an additional elimination of C14:0(3-OH) at O-3′. As a result, the structure of the pentaacylated lipid A at *m/z* 1506.3 was found to be similar to that of the hexaacylated lipid A species but was devoid of the secondary C16:1n9 at O-3′ of GlcN **A**′.

### 3.3. MS/MS analysis of the tetraacylated lipid A species at *m/z* 1280.3

The molecular ion at *m/z* 1280.3 represented a monophosphorylated tetraacylated lipid A species containing three C14:0(3-OH) and one C12:0 (Table 2 and Scheme 3). MS analysis of the O-deacylated lipid A mixture indicated the presence of two C14:0(3-OH) at positions O-2 and O-2′, respectively. The MS/MS spectrum of the ion at *m/z* 1280.3 provided the evidence for the substitution pattern of the remaining C14:0(3-OH) and C12:0, as shown in Figure 3c. The ion at *m/z* 1036.7 was generated due to cleavage of C14:0(3-OH), and a simultaneous elimination of C12:0 resulting in the fragment ion at *m/z* 835.8. The $^{0,4}$A fragments were observed at *m/z* 708.5 and *m/z* 508.1. The ion at *m/z* 708.5 contained C12:0, which was further eliminated resulting in the ion at *m/z* 508.1. The mass difference of 200.4 Da between




**Scheme 2.** Fragmentation pathway of the lipid A precursor ion at *m*/*z* 1506.3 with proposed structures.




**Scheme 3.** Fragmentation pathway of the lipid A precursor ion at *m*/*z* 1280.3 with proposed structures.

the ions at *m*/*z* 708.5 and *m*/*z* 508.1 indicated that C12:0 was eliminated as a free fatty acid by the charge-remote process and suggested that C14:0(3-OH) at O-2′ of GlcN **A**′ was further substituted by the secondary C12:0, while position O-3′ remained unsubstituted. The ion at *m*/*z* 995.5 corresponded to the inner-ring $^{0,2}$A cleavage,

establishing that the remaining C14:0(3-OH) was present at O-3 in the tetraacylated lipid A species.

## 4. Conclusion

The lipid A of gram-negative bacteria contains a complex mixture of structurally similar components varying in number, position and length of fatty acids, phosphate substitution and presence or absence of glycosyl residues other than glucosamine. While these structural variations are mainly attributed to the incomplete biosynthesis of lipid A, mild acid hydrolysis could also contribute to the degradation of fatty acid residues.[23,24] Heterogeneity of lipid A has limited the application of classical chemical degradation methods and NMR analysis techniques to the elucidation of its structure. Together with soft ionization techniques, such as fast atom bombardment[28] laser desorption[29] and plasma desorption,[30] mass spectrometry has been widely applied to the structural characterization of lipid A. In particular, recently developed very soft ionization methods, such as matrix-assisted laser desorption/ionization time-flight[31] and electrospray ionization, in combination with tandem mass spectrometry techniques[31–33] have facilitated the localization of fatty acid residues in lipid A region leading to rapid identification of its complete structure.

In the present report, we have established structures of the major lipid A components of *A. salmonicida* subsp. *salmonicida* from strains A449, 80204-1 and an in vivo isolate by a combination of chemical methods and NMR and ESIMS/MS analyses. ESIMS/MS analysis of lipid A indicated the presence of various fragment ions, especially cross-ring fragments, permitting the assignment of acyl residues in the lipid A backbone. Additionally, tandem MS spectra of each lipid A species afforded fragment ions corresponding to cleavage of ester-bound fatty acids and confirming a greater stability of acyl amide linkages,[33] thus allowing the identification of O-substituted acyl residues.

El-Aneed and Banoub reported the lipid A structure of *A. salmonicida* from strains SJ-15 and SJ-83, which was determined by electrospray ionization quadrupole time-of-flight tandem mass spectrometry techniques and showed that it contained a main constituent comprising β-D-GlcN4P-(1→6)-α-D-GlcN1P disaccharide substituted with phosphate groups at positions 4′ and 1, where positions 2/2′ and 3/3′ were substituted by amide-linked and ester-linked C14:0(3-OH), respectively, and primary acyl chains on positions 2′ and 3′ were further esterified by C12:0. No C16:1n9 was detected.[34] This lipid A component was also detected as a minor species in lipid A of *A. salmonicida* subsp. *salmonicida* from strains A449 and 80204-1, and corresponding molecular ions at *m*/*z* 1689.3 and *m*/*z* 1689.5, respectively, were observed (Fig. 1a and b).

The significance of the acylation pattern variation in lipid A of *A. salmonicida* subsp. *salmonicida* is still unclear, but it may play a role in biological activities. It was evidenced that the structural heterogeneity of lipid A could affect the toxicity and virulence of other gram-negative bacteria, such as *E. coli*[35] and *Salmonella typhimurium*.[36] Enteric bacteria can synthesize different forms of lipid A in response to the host environment. Earlier work by Ernst et al.[31] indicated that *Pseudomonas aeruginosa* isolates from the airway of cystic fibrosis (CF) patients synthesized lipid A species with an increased content of C16:0 leading to increased stimulation of IL-8 expression and bacterial resistance to cationic antimicrobial peptides when compared with the clinical non-CF lung infection.

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## References

1. Bullock, G. L.; Griffin, B. R.; Stuckey, H. M. *Can. J. Fish Aquat. Sci.* **1980**, *37*, 719–721.
2. Dooley, J. S.; Lallier, R.; Shaw, D. H.; Trust, T. J. *J. Bacteriol.* **1985**, *164*, 263–269.
3. Bricknell, I. R.; Bowden, T. J.; Lomax, J.; Ellis, A. E. *Fish Shell Immunol.* **1997**, *7*, 1–16.
4. Khan, S. A.; Everest, P.; Servos, S.; Foxwell, N.; Zähringer, U.; Brade, H.; Rietschel, E. T.; Dougan, G.; Charles, I. G.; Maskell, D. J. *Mol. Microbiol.* **1998**, *29*, 571–579.
5. Schromm, A. B.; Brandenburg, K.; Loppnow, H.; Moran, A. P.; Koch, M. H. J.; Rietschel, E. T.; Seydel, U. *Eur. J. Biochem.* **2000**, *267*, 2008–2013.
6. Rose, J. R.; Christ, W. J.; Bristol, J. R.; Kawata, T.; Rossignol, D. P. *Infect. Immun.* **1995**, *63*, 833–839.
7. Suda, Y.; Ogawa, T.; Kashihara, W.; Oikawa, M.; Shimoyama, T.; Hayashi, T.; Tamura, T.; Kusumoto, S. *J. Biochem.* **1997**, *121*, 1129–1133.
8. Aussel, L.; Thérisod, H.; Karibian, D.; Perry, M. B.; Bruneteau, M.; Caroff, M. *FEBS Lett.* **2000**, *465*, 87–92.
9. Silipo, A.; Molinaro, A.; Cescutti, P.; Bedini, E.; Rizzo, R.; Parrilli, M.; Lanzetta, R. *Glycobiology* **2005**, *15*, 561–570.
10. Bhat, R.; Marx, A.; Galanos, C.; Conrad, R. S. *J. Bacteriol.* **1990**, *172*, 6631–6636.
11. Wang, Z.; Larocque, S.; Vinogradov, E.; Brisson, J.-R.; Dacanay, A.; Greenwell, M.; Brown, L. L.; Li, J.; Altman, E. *Eur. J. Biochem.* **2004**, *271*, 4507–4516.
12. Wang, Z.; Li, J.; Vinogradov, E.; Altman, E. *Carbohydr. Res.* **2006**, *341*, 109–117.
13. Wang, Z.; Vinogradov, E.; Larocque, S.; Harrison, B. A.; Li, J.; Altman, E. *Carbohydr. Res.* **2005**, *340*, 693–700.

14. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.
15. Helander, I. M.; Hurme, R.; Haikara, A.; Moran, A. P. *J. Bacteriol.* **1992**, *174*, 3348–3354.
16. Brade, H.; Galanos, C. *Eur. J. Biochem.* **1982**, *122*, 233–237.
17. Vinogradov, E.; Perry, M. B.; Conlan, J. W. *Eur. J. Biochem.* **2002**, *269*, 6112–6118.
18. Brisson, J.-R.; Sue, S. C.; Wu, W. G.; McManus, G.; Nghia, P. T.; Uhrin, D. In *NMR Spectroscopy of Glycoconjugates*; Jimenez-Barbero, J., Peters, T., Eds.; Wiley-VCH: Weinheim, 2002; pp 59–93.
19. Li, J.; Wang, Z.; Altman, E. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 1305–1314.
20. Li, J.; Thibault, P.; Martin, A.; Richards, J. C.; Wakarchuk, W. W.; van der Wilp, W. *J. Chromatogr. A* **1998**, *817*, 325–336.
21. Müller-Loennies, S.; Holst, O.; Lindner, B.; Brade, H. *Eur. J. Biochem.* **1999**, *260*, 235–249.
22. Janusch, H.; Brecker, L.; Lindner, B.; Alexander, C.; Gronow, S.; Heine, H.; Ulmer, A. J.; Rietschel, E. T.; Zähringer, U. *J. Endotoxin Res.* **2002**, *8*, 343–356.
23. Kelly, J.; Masoud, H.; Perry, M. B.; Richards, J. C. *Anal. Biochem.* **1996**, *233*, 15–30.
24. Melaugh, W.; Phillips, N. J.; Campagnari, A. A.; Karalus, R.; Gibson, B. W. *J. Biol. Chem.* **1992**, *267*, 13434–13439.
25. Kussak, A.; Weintraub, A. *Anal. Biochem.* **2002**, *307*, 131–137.
26. Costello, C. E.; Vath, J. E. *Methods Enzymol.* **1990**, *193*, 738–768.
27. Corsaro, M. M.; Piaz, F. D.; Lanzetta, R.; Parrilli, M. *J. Mass Spectrom.* **2002**, *37*, 481–488.
28. Karunaratne, D. N.; Richards, J. C.; Hancock, R. E. W. *Arch. Biochem. Biophys.* **1992**, *299*, 368–376.
29. Kulshin, V. A.; Zähringer, U.; Lindner, B.; Jäger, K. E.; Dmitriev, B. A.; Rietschel, E. T. *Eur. J. Biochem.* **1991**, *198*, 697–704.
30. Cole, R. B.; Domelsmith, L. N.; David, C. M.; Laine, R. A.; Delucca, A. J. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 616–622.
31. Ernst, R. K.; Yi, E. C.; Guo, L.; Lim, K. B.; Burns, J. L.; Hackett, M.; Miller, S. I. *Science* **1999**, *286*, 1561–1565.
32. Harrata, A. K.; Domelsmith, L. N.; Cole, R. B. *Biol. Mass Spectrom.* **1993**, *22*, 59–67.
33. Chan, S.; Reinhold, V. N. *Anal. Biochem.* **1994**, *218*, 63–73.
34. El-Aneed, A.; Banoub, J. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 1683–1695.
35. Guo, L.; Lim, K. B.; Poduje, C. M.; Daniel, M.; Gunn, J. S.; Hackett, M.; Miller, S. I. *Cell* **1998**, *95*, 189–198.
36. Guo, L.; Lim, K. B.; Gunn, J. S.; Bainbridge, B.; Darveau, R. P.; Hackett, M.; Miller, S. I. *Science* **1997**, *276*, 250–253.