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Qualitative Analysis of Lipid A from Intestinal Bacteria by Quadrupole Time-of-Flight Mass Spectrometry

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■ Abstract

This report describes an example of using a high-resolution quadrupole time-of-flight mass spectrometer to estimate the structure of natural compounds produced by bacteria. Lipid A is a bioactive molecule on the cell surface of gram-negative bacteria that induces an immune response in hosts such as humans. It is known that human intestinal bacteria, *Bacteroides fragilis*, have lipid A, but its structure has not been investigated in detail. In this study, the structures of lipid A from *B. fragilis* were elucidated by taking advantage of the accuracy and reliability of mass information obtained using an LCMS™-9030 spectrometer. It is expected that high-resolution mass spectrometers will contribute to discovering useful natural compounds and expediting analysis of their structure.

1. Introduction

Gram-negative bacteria have characteristic macromolecules called lipopolysaccharides in their cell membrane (Fig. 1). Lipid A, which plays a role as an anchor that binds polysaccharides to the cell surface, acts on the host's pattern recognition receptor (toll-like receptor 4) to trigger an innate immune response. The structure of lipid A differs depending on the types of bacteria and the environment, which alters the reactivity with host receptors.[1)] Therefore, it is important to clarify the structure of lipid A in each bacterium in detail in order to understand the interaction between a host and bacteria. One of the predominant intestinal bacteria, *Bacteroidetes fragilis* have the effect of attenuating the signal of toll-like receptors 4 acted on by *Escherichia coli* lipid A. It has also been suggested that the effect is due to structural differences in lipid A.[2)] However, the detailed structure has not yet been determined based on the analysis of product ion spectra. Therefore, this report describes a case study where we inferred the structure of lipid A of *B. fragilis* using a liquid chromatograph-quadrupole time-of-flight mass spectrometer (LC-Q-TOF-MS).[3)]

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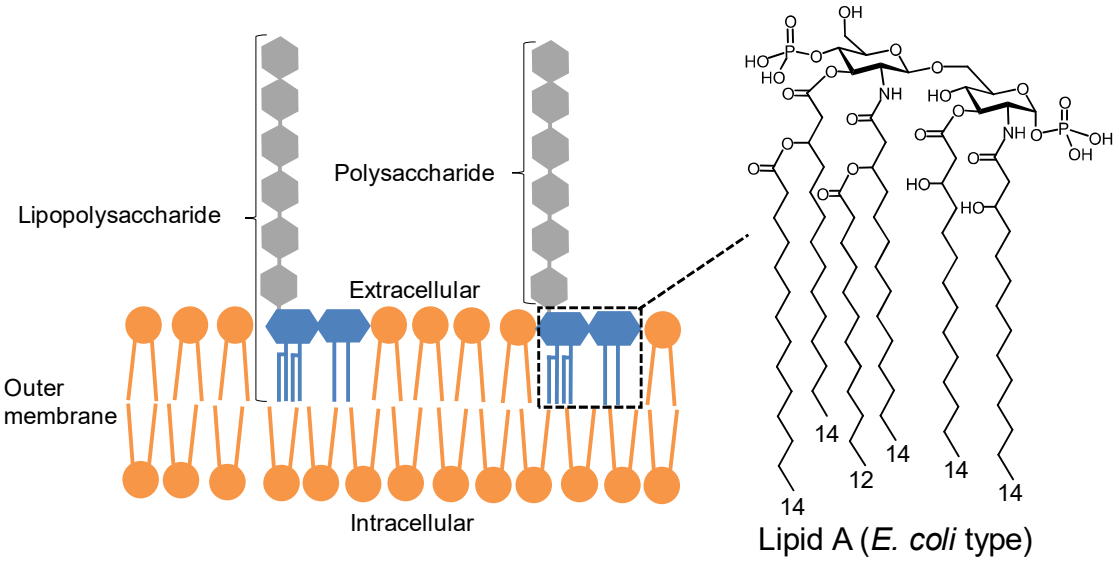

Fig. 1 Structure of Lipopolysaccharide and Lipid A in Membranes of Gram-Negative Bacteria

2. Purification of Lipid A from Bacteria

In this study, the structure of lipid A in *B. fragilis*, a type of human intestinal bacteria, was analyzed. Crude lipid A was prepared according to the report by Yi et al.[4] First, *B. fragilis* was cultured overnight in 10 mL of modified Gifu anaerobic medium in an anaerobic environment with 10 % H_2, 10 % CO_2, 80 % N_2, and 5 ppm or less O_2 concentration. The bacteria cells were collected by centrifugation. Subsequently, lipopolysaccharides were extracted from the bacteria cells using commercially available TRI-Reagent (Table 1, steps 1 to 15). Then the lipopolysaccharides were heat-treated in a weakly acidic condition to remove their glycan and finally obtain crude products of lipid A (Table 1, steps 16 to 26).

3. Analysis of Lipid A by LC-Q-TOF-MS

Crude lipid A isolated from *B. fragilis* was analyzed by LC-Q-TOF-MS in the DDA mode (Table 2). That resulted in observing a series of peaks with *m/z* differences of 14.015 in the chromatogram (Fig. 2). This suggested that the lipid A in the *B. fragilis* was a mixture of molecules with fatty acids of different carbon chain lengths. The molecular formula for *m/z* 1688.2519, which had the highest intensity, was presumed to be $[C_{93}H_{176}N_2O_{21}P]^-$ (with an *m/z* measurement error of 0.6 ppm). It was assumed to be lipid A that had a diglucosamine backbone with one phosphate group, and acyl chains with a total carbon chain length of 81, a total unsaturation number of 0, and a total of 4 hydroxyl groups.

Next, a product ion scan was performed for the molecule as a precursor ion to infer the detailed structure (Fig. 3). The product ion spectra obtained presumably corresponded to fatty acids and glucosamine phosphates, with less than 2 ppm error (Δ) from each theoretical *m/z* value (upper Fig. 3). That suggested the molecule of interest was lipid A. Neutral losses of acyl chains, such as C15:0 and C16:0+O, were assumed to be either the primary acyl chain ester-linked to the 3 or 3′-position on its diglucosamine backbone, or the secondary acyl chain further ester-linked to the hydroxy fatty acid (arrows (a), (b) and (c) in the middle of Fig. 3). This is consistent with the previous report that the ester bond of lipid A is generally predominantly cleaved.[5] The ion at *m/z* 490.2576 (with a 0.2 ppm error (Δ) from the theoretical value), generated by cleavage of the 1′-6 glycosidic bond of the diglucosamine backbone (Fig. 3(d)), suggests that the hydroxy fatty acid amide-bonded at the 2-position is C17:0. The ion at *m/z* 633.3156, produced by the cleavage shown in Fig. 3(e) (Δ = 0.3 ppm), indicates that the fatty acid amide-bonded at the 2′-position is C17:1 and this unsaturated bond comes from the secondary acyl being ester-bonded. The cleavage shown in Fig. 3(f) suggests that the hydroxy group is in the β position, with such fragmentation known to be observed specifically when the phosphate group is in the 1-position.[3] Although no fragment ion indicating the position of the ester-linked fatty acid was observed, based on a previous report[6] that the main chain of lipid A consists of hydroxy fatty acids, it was assumed that the 3 and 3'-positions are C17:0+O, and the 2'-position is C15:0. Based on the above information, it was concluded that the *B. fragilis* lipid A at *m/z* 1688.2519 has a diglucosamine backbone with one phosphate group at the 1-position, and a structure with four hydroxy fatty acids bound to the primary acyl chain and a fatty acid bound to the secondary acyl chain in the 2'-position, as shown in the lower part of Fig. 3, suggesting that the structure is different from that of lipid A in *Escherichia coli* (Fig. 1).

Similarly, the structure of other lipids A observed in the chromatogram in Fig. 2 (Fig. 4) were also estimated. As a result, the carbon chain length of the hydroxy fatty acids amide-linked in the 2 or 2′-position and ester-linked in the 3 or 3′-position of the diglucosamine backbone are presumably 16 or 17 and 15 or 16, respectively, whereas all secondary fatty acids in the 2′-position are C15:0. Similar analyses of other bacteria belonging to the phylum Bacteroidetes found that the abundance ratio of lipid A with different structures correlated with the taxonomic proximity in bacteria.[3]

Table 1 Experimental Procedure for Lipid A Extraction

1	Culture the bacteria cells (cell density (OD_{600}) × the volume of culture broth (mL) needs about 10).
2	Centrifuge the culture broth to form cell pellets and remove the supernatant.
3	Wash the cell pellets with phosphate-buffered saline and repeat step 2.
4	Add 200 µL TRI Reagent (Molecular Research Center, Inc.) to the pellets to form a suspension.
5	Incubate the solution for 15 min at 37 °C.
6	Add 40 µL chloroform to the solution.
7	Shake vigorously and incubate for 10 min at room temperature.
8	Centrifuge the solution at 12,000 *g* for 10 min.
9	Collect 100 µL of the supernatant in a new tube.
10	Add 100 µL ultrapure water to the remaining solution.
11	Shake vigorously and incubate for 10 min at room temperature.
12	Centrifuge the solution at 12,000 *g* for 10 min.
13	Collect 100 µL of the supernatant in the same tube used in step 9.
14	Repeat steps 10 to 13 twice.
15	Dry the recovered supernatant at 60 °C under reduced pressure to obtain crude lipopolysaccharides.
16	Add 500 µL of water adjusted to pH 4.5 with 6*N* hydrochloric acid to the crude lipopolysaccharide extract containing 1 % (w/v) sodium dodecyl sulfate and 10 mM sodium acetate.
17	Shake and dissolve well.
18	Incubate the solution for 1 hour at 100 °C.
19	Dry under reduced pressure.
20	Add 100 µL ultrapure water and 500 µL of 95 % (v/v) ethanol containing 20 mM hydrochloric acid.
21	Centrifuge the solution at 2,000 *g* for 10 min.
22	Remove the supernatant.
23	Add 500 µL of 95 % (v/v) ethanol and to create a suspension.
24	Centrifuge the solution at 2,000 *g* for 10 min.
25	Remove the supernatant.
26	Dry under reduced pressure to obtain a powder of isolated lipid A.

Table 2 Analytical Conditions

LC	
Instrument:	Nexera™ X2
Column:	YMC triart C18 (2.0 mmI.D. × 50 mm, 1.9 µm, YMC)
Flowrate:	0.3 mL/min
Mobile Phase A:	200 mM ammonia* - 8:2 (v/v) MeOH/H_2O
Mobile Phase B:	200 mM ammonia*- 2-propanol
Time Program (B Conc.):	0 % (0-2 min) – 95 % (17 min) – 0 % (17.1 min) – 0 % (20 min)
Column Temp.:	45 °C
Injection Volume:	1 µL
MS	
Instrument:	LCMS-9030
Ionization:	ESI
Nebulizing Gas Flow:	2.0 L/min
Drying Gas Flow:	10 L/min
Heating Gas Flow:	10 L/min
DL Temp.:	300 °C
BH Temp.:	400 °C
Interface Temp.:	300 °C
Mode:	Data-dependent acquisition - MS scan (250 msec), MS/MS scan (ten 100 msec events)
MS Scan Range:	*m/z* 650 – 2000
MS/MS Scan Range:	*m/z* 70 – 2000
Q1 Resolution:	Low
Collision Energy:	85 V
Collision Energy Spread (±):	20 V

*Prepared by adding 1.5 mL of 25 % aqueous ammonia (Merck) per 100 mL

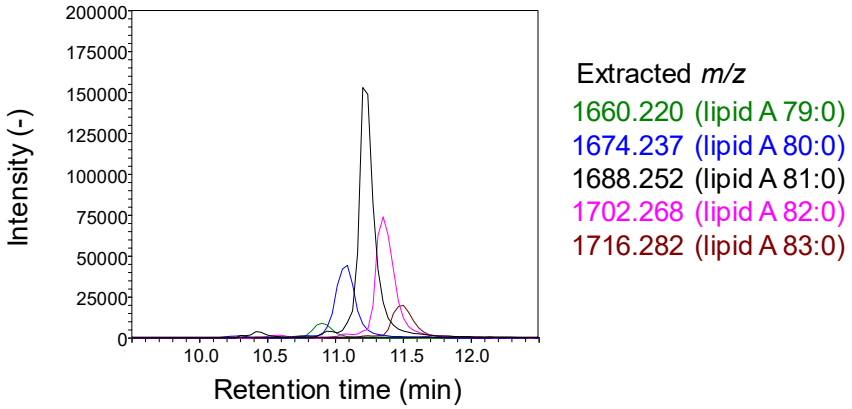

Fig. 2 Extracted-Ion Chromatograms of Lipid A Obtained from *B. fragilis*

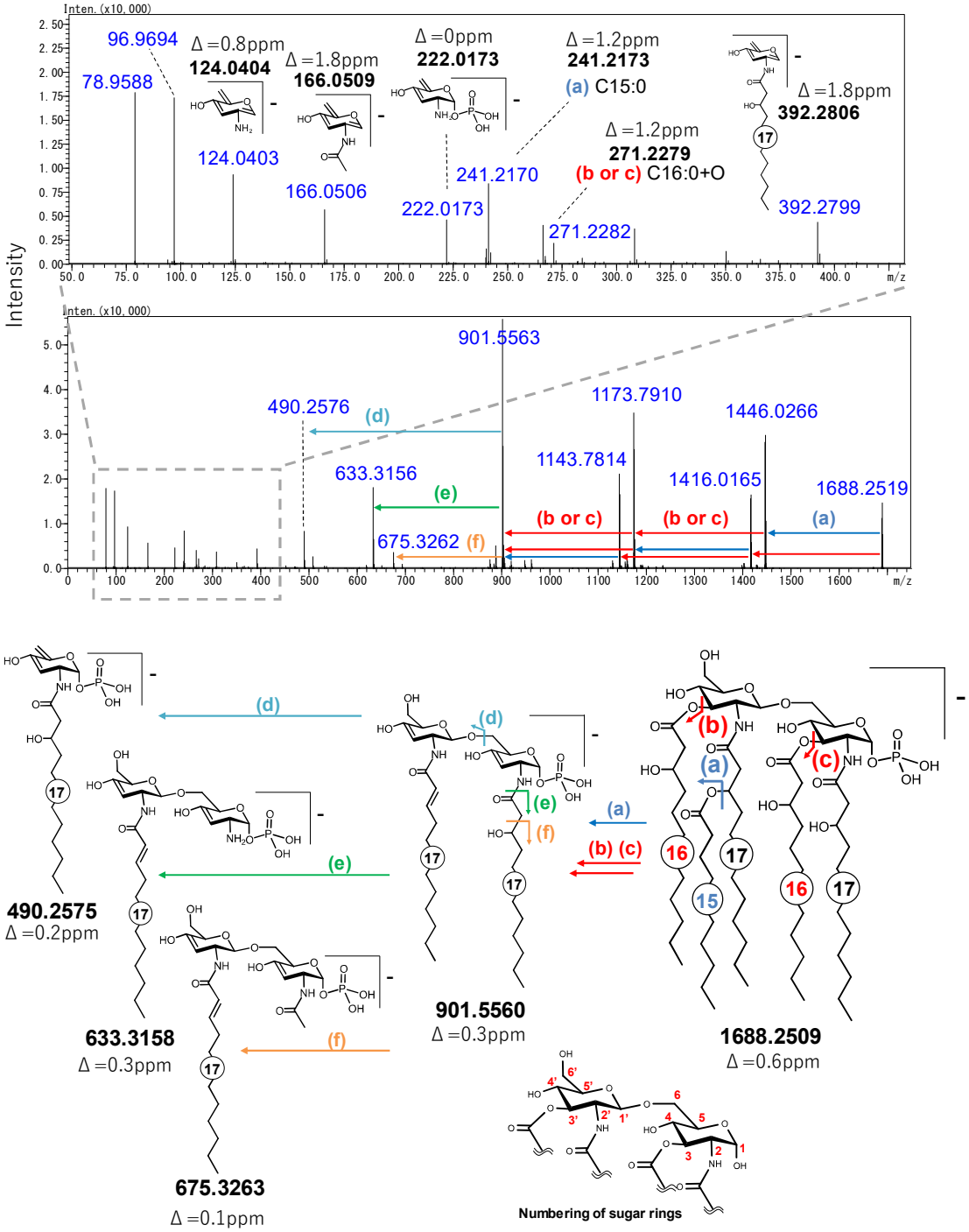

Fig. 3 Estimated Structure of *B. fragilis* Lipid A
Upper: MS/MS spectra of the precursor ion (*m/z* 1688.251). Arrows indicate the neutral losses. Δ indicates the error between the theoretical and measured *m/z* values.
Bottom: Proposed fragmentation scheme of lipid A. Circled numbers on acyl chains indicate chain length.

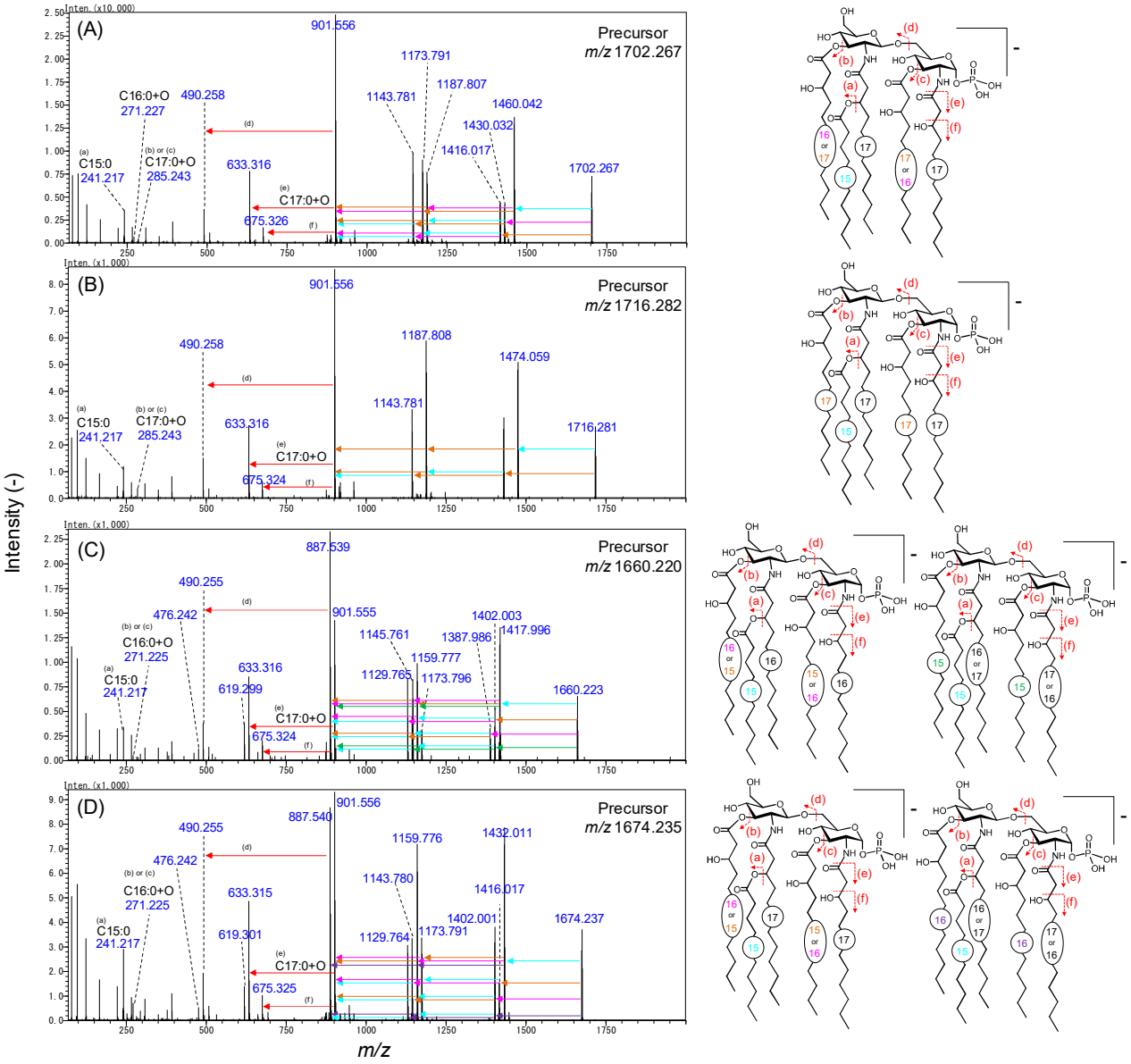

Fig. 4 MS/MS Spectra and Proposed Structure of Lipid A in *B. fragilis*
Colored arrows indicate the neutral loss of acyl chains with of the corresponding color. Circled numbers on acyl chains indicate the chain length.

4. Conclusion

We analyzed lipid A in *B. fragilis*, a kind of intestinal bacteria, using an LC-Q-TOF-MS spectrometer and successfully revealed the structure. Accurate mass information obtained using an LCMS-9030 system was very useful for inferring the structure of lipid A. In this analysis, it was possible to efficiently narrow down candidates for estimating structures using highly accurate mass information. The differences between theoretical and measured *m/z* values were less than 1 ppm in the mid-to-high *m/z* region, and less than 2 ppm even in the low *m/z* region. We anticipate that such a mass spectrometer with high accuracy and mass reliability will contribute to increasing throughput for discovering and identifying novel compounds and bioactive substances.

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