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Structural analysis of the lipoteichoic acid anchor glycolipid: Comparison of methods for degradation of the glycerophosphate backbone polymer

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ABSTRACT

Acetic acid treatment [98% (v/v), 100 °C, 3 h] was proposed as a new method for degrading the glycerophosphate polymer moiety of Gram-positive bacterial lipoteichoic acid. We demonstrated that this method resulted in partial *O*-acetylation on the carbohydrate residues of the anchor glycolipid. Hence, the acetic acid treatment is not suitable for the chemical structural analysis of lipoteichoic acid.

Lipoteichoic acid (LTA) is an amphiphilic polymer and one of the major cell surface components of Gram-positive bacteria. Typical LTA is comprised of a poly-glycerophosphate (GroP polymer) backbone and an anchor glycolipid (Shiraishi et al., 2016). The GroP polymer needs to be removed for determination of chemical structure of the LTA anchor glycolipid. Conventionally, the removal is carried out by hydrofluoric acid treatment. Jang et al. (2011) reported the use of 98% (v/v) acetic acid for the preparation of anchor glycolipid. In the current study, we examined anchor glycolipid fractions prepared from *Lactobacillus gasseri* JCM 1131^T LTA by two methods: 98% (v/v) acetic acid treatment and 48% (w/v) hydrofluoric acid treatment. The two methods yielded products with different spectra of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The contradictory data gave the different interpretations of fatty acid compositions of the anchor glycolipids between the methods, thus either assignment should be considered to be wrong. We verified chemical structures of the glycolipid products prepared using these two methods.

L. gasseri JCM 1131^T was obtained from the Japan Collection of Microorganisms, RIKEN BioResource Center (Tsukuba, Japan). The cells were grown anaerobically to a logarithmic phase (optical density value at 660 nm of approximately 0.60) in half-strength (1/2) Difco Lactobacilli MRS broth (Becton, Dickinson and Co., Franklin Lakes, NJ) at 37 °C using AnaeroPack-Anaero (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan). The bacterial cells were disrupted by French pressure cell (Ohtake Works, Tokyo, Japan). Disruption was confirmed by the microscopic observation. LTA was purified from disrupted cells by 1-butanol extraction, followed by hydrophobic interaction chromatography using an Octyl-Sepharose 4 Fast Flow column (GE Healthcare UK Ltd., Little Chalfont, UK), as described previously (Shiraishi et al., 2013).

LTA was treated with hydrofluoric acid or acetic acid as described below. After the removal of acid by flash evaporation, the product was partitioned into chloroform/methanol/water (1:1:0.9, v/v/v). The organic layer was used as the anchor glycolipid fraction. It was analyzed by MALDI-TOF MS as described previously (Shiraishi et al., 2013). The samples were dissolved in chloroform/methanol (2:1, v/v) at a concentration of 1 μg/μL, and mixed with an equal amount of matrix [10 mg/mL 2,5-dihydroxybenzoic acid in water/methanol (7:3, v/v) containing 0.1% (w/v) trifluoroacetic acid] on a target plate. After co-crystallization, MALDI-TOF MS spectra were acquired by integration of over 2000 random shots in the positive ion and reflectron modes, and the molecular masses were determined using the Autoflex III smartbeam with the FlexControl software (Bruker Daltonics Inc., Billerica, MA). The experiments were performed at least three times.

First, LTA was treated with 48% (w/v) hydrofluoric acid at 4 °C for 3 h (Seo et al., 2008). The anchor glycolipid gave two series of mass peaks (Fig. 1A). The peaks at *m/z* 1266 and 1292 (Group 1) were attributed to the $(M + Na)^+$ molecular ions of tetrahexosyl diacylglycerol (Hex_4DAG) containing $C_{16:0}/C_{18:1}$ and $C_{18:1}/C_{18:1}$, respectively. The peaks at *m/z* 1530 and 1556 (Group 2) were attributed to those of acyltetrahexosyl diacylglycerol ($acylHex_4DAG$) containing $C_{16:0}/C_{18:1}/C_{18:1}$ and $C_{18:1}/C_{18:1}/C_{18:1}$, respectively.

Abbreviations: $acylHex_4DAG$, acyltetrahexosyl diacylglycerol; GroP polymer, poly-glycerophosphate; Hex_4DAG, tetrahexosyl diacylglycerol; LTA, lipoteichoic acid; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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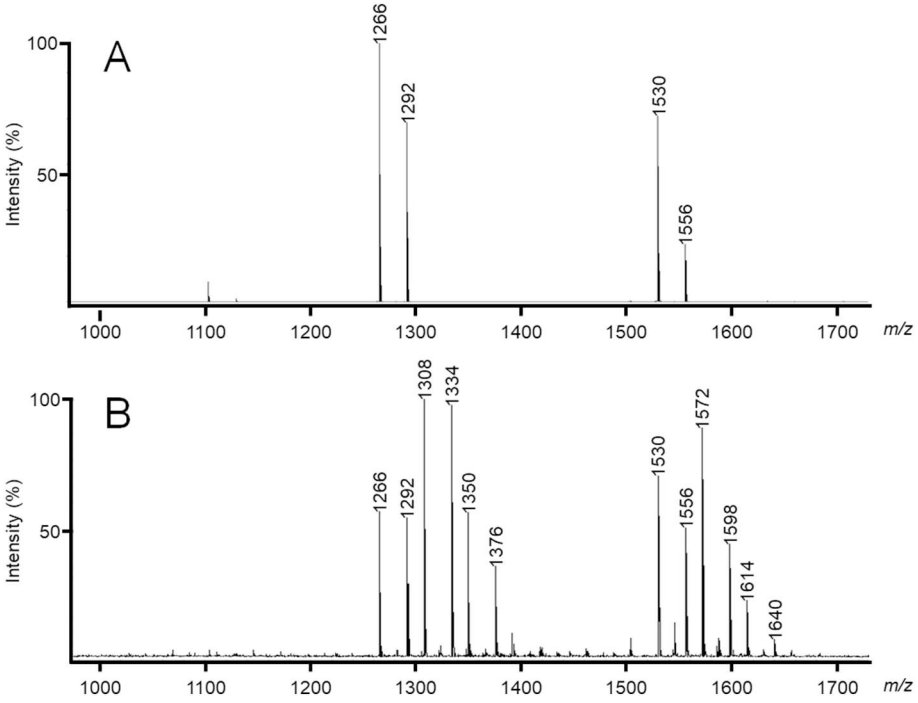

Fig. 1. MALDI-TOF MS spectra of the anchor glycolipid fractions obtained by the treatments with hydrofluoric acid (A) and acetic acid (B).

Second, the anchor glycolipid obtained by treatment with 98% (v/v) acetic acid for 3 h at 100 °C (Jang et al., 2011) was also analyzed. Multiple peaks were observed, which contrasted with the spectrum of that obtained by the hydrofluoric acid treatment. Two series of peaks were observed: Group 1 peaks (*m/z* 1266, 1292, 1308, 1334, 1350, and 1376) and Group 2 peaks (*m/z* 1530, 1556, 1572, 1598, 1614, and 1640) (Fig. 1B). Group 1 and Group 2 peaks were attributed to the $(M + Na)^+$ molecular ions of Hex_4DAG and $acylHex_4DAG$, respectively. The peak pattern was similar to that of a glycolipid fraction of *Lactobacillus plantarum* KCTC 10887BP LTA (Jang et al., 2011), except that the peaks were smaller by *m/z* 162 than those of *L. gasseri* JCM 1131^T. Because the glycolipid anchor of *L. plantarum* is trihexosyl glycolipid. As stated by Jang et al. (2011), the multiple peaks could reflect the heterogeneity of fatty acid composition. For example, the peaks of Group 1 were attributed to the $(M + Na)^+$ molecular ions containing $C_{16:1}/C_{18:0}$ (*m/z* 1266), $C_{18:0}/C_{18:2}$ (*m/z* 1292), $C_{18:0}/C_{19:1}$ (*m/z* 1308), $C_{18:0}/C_{21:2}$ (*m/z* 1334), $C_{19:0}/C_{21:1}$ (*m/z* 1350), and $C_{21:0}/C_{21:2}$ (*m/z* 1376). However, many of these fatty acids are rare or undiscovered in the living world (LIPID MAPS® Lipidomics Gateway, 2003-2019). According to previous reports, $C_{18:1}$ and $C_{16:0}$ are the major fatty acids in lactobacilli LTA (Shiraishi et al., 2013) and cell membranes (O'leary and Wilkinson, 1988). The membrane lipid composition of *L. plantarum* accounts for 80% at $C_{16:0}$, $C_{18:1}$, and $C_{19:cy}$ (O'leary and Wilkinson, 1988). Lines of evidence strongly suggest that the large number of peaks cannot be explained by the heterogeneity of fatty acids.

To resolve this contradiction, we carefully examined the MALDI-TOF MS spectra of anchor glycolipid fraction obtained by the acetic acid treatment. The peaks at *m/z* 1308 and 1350 were *m/z* 42 and 84 different from that at *m/z* 1266 (Hex_4DAG with $C_{16:0}/C_{18:1}$), respectively. The peaks at *m/z* 1334 and 1376 were *m/z* 42 and 84 different from that at *m/z* 1292 (Hex_4DAG with $C_{18:1}/C_{18:1}$), respectively. The peaks at *m/z* 1572 and 1614 were *m/z* 42 and 84 different from that at *m/z* 1530 ($acylHex_4DAG$ with $C_{16:0}/C_{18:1}/C_{18:1}$), respectively. The peaks at *m/z* 1598 and 1640 were *m/z* 42 and 84 different from that at *m/z* 1556 ($acylHex_4DAG$ with $C_{18:1}/C_{18:1}/C_{18:1}$), respectively. This suggested that the acetic acid treatment introduced one or two substitutions with a molecular mass of 42 into the anchor glycolipid.

We hypothesized that the functional group with *m/z* 42 is an acetyl group. To test that, we treated methyl-α-D-glucopyranoside with 98% (v/v) acetic acid for 3 h at 100 °C, and analyzed the products by MALDI-TOF MS. Whereas the peak for methyl-α-D-glucopyranoside (*m/z* 217) was only detected before the treatment, the peaks of *m/z* 259 and 301, which are *m/z* 42 and 84 larger than that of *m/z* 217, respectively, appeared after the treatment (data not shown). Furthermore, proton signals (δ 2.15 ppm; peak intensity was 1.47H compared to the anomeric proton) attributed to methyl groups of *O*-acetyl groups appeared in ^{1}H NMR spectrum after the acetic acid treatment (data not shown). These suggested that the acetic acid treatment introduced one or two acetyl groups at the hydroxyl groups of methyl-α-D-glucopyranoside.

We next examined that the anchor glycolipid fractions obtained by the acetic acid and hydrofluoric acid treatments were per-*O*-acetylated by treatment with acetic anhydride/pyridine (1:1, v/v) at 80 °C for 2 h. Acetic anhydride and pyridine were then removed by flash evaporation. The two per-*O*-acetylated anchor glycolipid fractions yielded a similar peak pattern (Fig. 2A, B). The peaks at *m/z* 1812 and 1838 were *m/z* 546, which corresponds to 13 acetyl residues, larger than the peaks at *m/z* 1266 and 1292 (Hex_4DAG) in the spectra of the anchor glycolipid fractions (Fig. 1A, B), respectively. Hence, these were completely *O*-acetylated Hex_4DAG containing $C_{16:0}/C_{18:1}$ and $C_{18:1}/C_{18:1}$, respectively. The peaks at *m/z* 2034 and 2060 were *m/z* 504, which corresponds to 12 acetyl residues, larger than the peaks at *m/z* 1530 and 1556 ($acylHex_4DAG$) observed in the spectra of glycolipid fractions (Fig. 1A, B), respectively. This agreed with one of the three acyl groups in $acylHex_4DAG$ being linked to one hydroxyl group of the saccharide portion of the molecule.

Structural analysis of the anchor glycolipid moiety of LTA requires the removal of GroP polymer moiety. Hydrofluoric acid is conventionally used for cleavage of phosphodiester bonds in the GroP polymer (Seo et al., 2008). However, hydrofluoric acid is highly hazardous to humans and highly caustic. Since the handling is problematic, the acetic acid treatment appeared to be a promising alternative (Jang et al., 2011; Shiraishi et al., 2013; Jeong et al., 2015). However, we demonstrated here that partial *O*-acetylation of the carbohydrate residues occurs via acetolysis during the 98% (v/v) acetic acid treatment. The partial *O*-acetylation would mislead the determination of

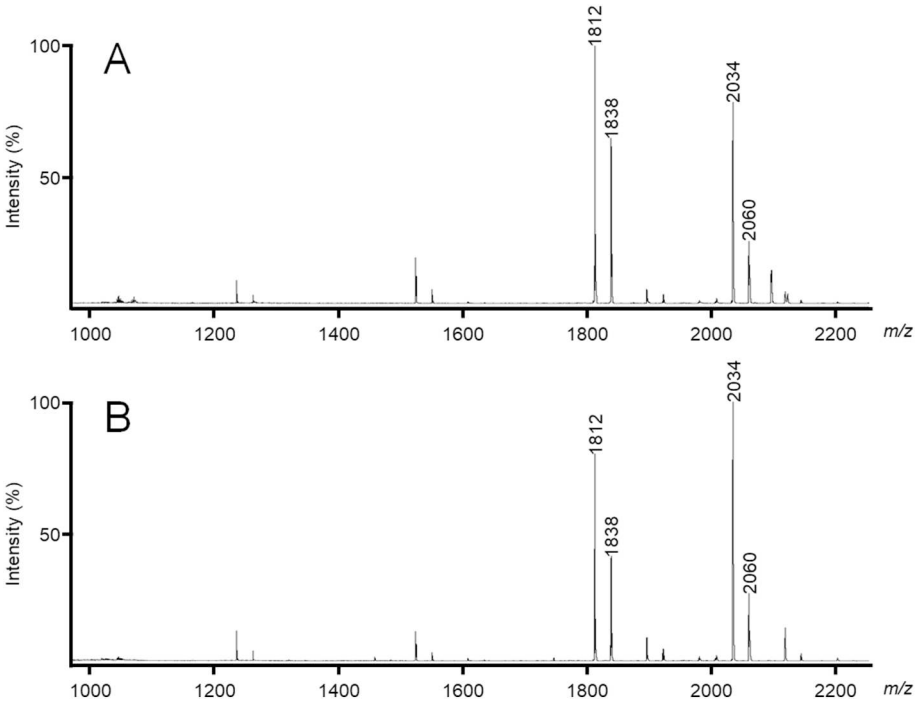

Fig. 2. MALDI-TOF MS spectra of the per-*O*-acetylated anchor glycolipid fractions obtained by the treatments with hydrofluoric acid (A) and acetic acid (B).

chemical structure of LTA anchor glycolipid. In conclusion, the 98% (v/v) acetic acid treatment is not suitable for the chemical structural analysis of LTA.

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Declaration of Competing Interest

None.

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