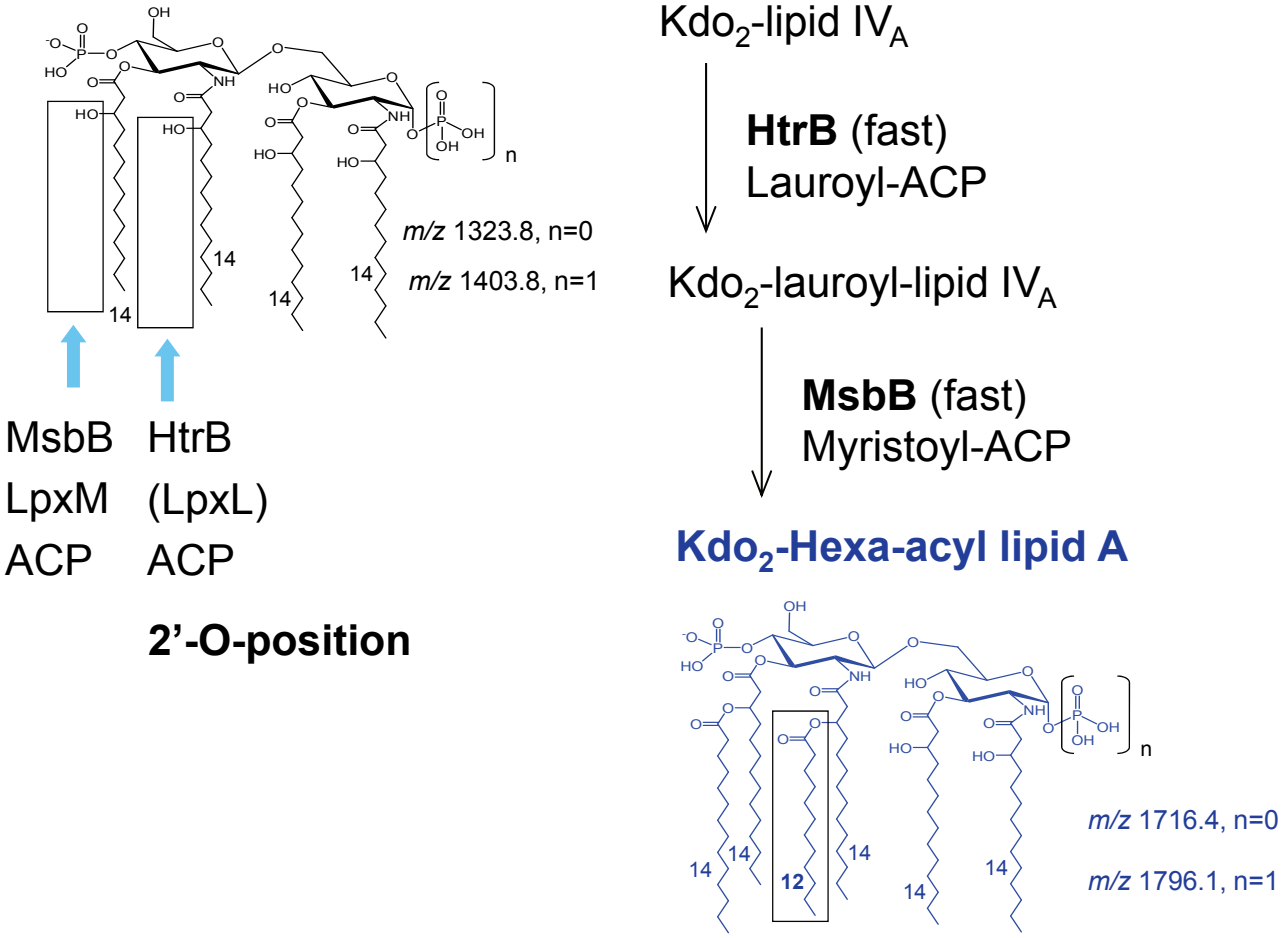
# Supplementary Figure S1

## *E. coli* "late" Acylations of lipid A precursor ($Kdo_2$-lipid $IV_A$)



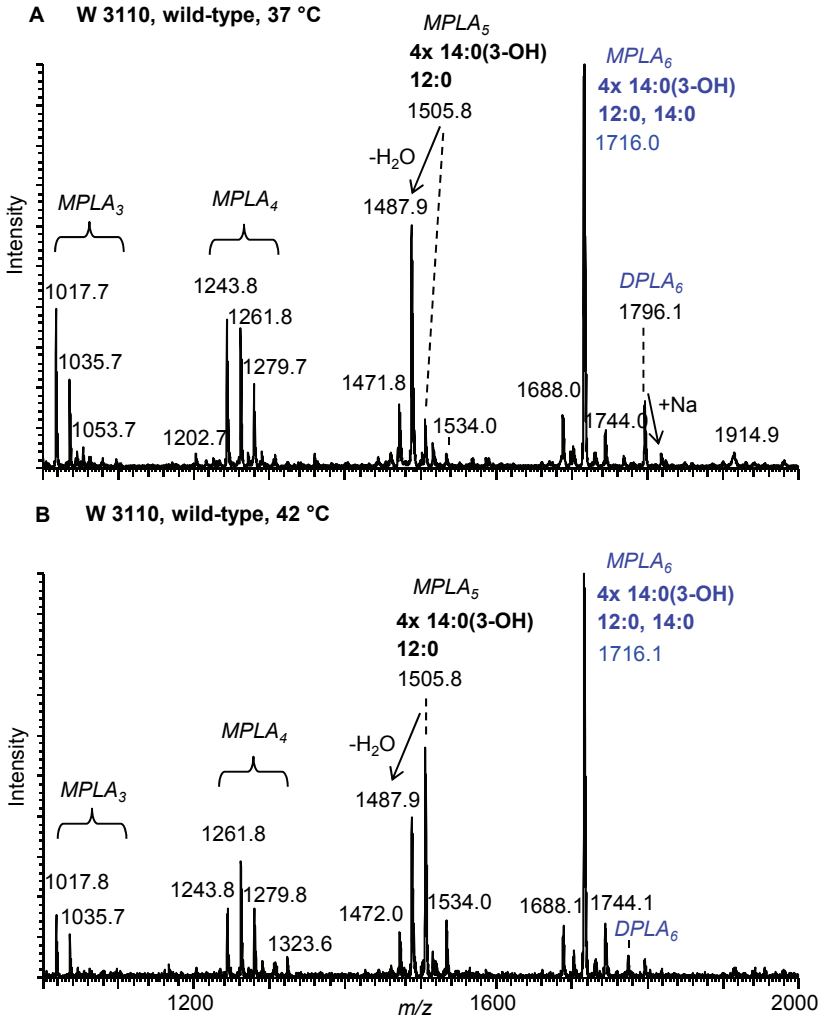

**Supplementary Figure S1:** *E. coli* "late" Acylations of lipid A precursor ($Kdo_2$-lipid $IV_A$). Biosynthetic pathway from tetra acyl lipid A to hexa acyl lipid A as suggested by various studies, i.e., by Clementz et al., **1996**. *J. Biol. Chem.* 271:12095–12102. Briefly, in *E. coli*, late acyltransferase enzymes LpxL and LpxM sequentially add the fifth and sixth fatty acids, respectively, to precursor lipid $IV_A$ (tetra-acyl lipid A) after glycosylation with two 3-deoxy-D-manno-octulosonic acid (Kdo) sugars. In *E. coli*, laurate transferase, encoded by *lpxL* (formerly *htrB*), acylates $(Kdo)_2$-lipid $IV_A$, adding a lauric acid (12:0) to 3-hydroxymyristic acid [14:0(3-OH)] at the 2' position of the distal glucosamine. *E. coli* myristate transferase, encoded by *lpxM* (formerly *msbB*), uses the penta-acylated lipid A structure as a substrate, adding a myristic acid (14:0) onto 3-hydroxymyristic acid [14:0(3-OH)], which is located at the 3' position of the distal glucosamine.

# Supplementary Table S1: Primer Table

| Target Gene | Primer/Probe | Sequence | Length (bp) |
|---|---|---|---|
| *rrsA* | Forward | CGTGGCTTCCGGAGCTAAC | 60 |
| | Reverse | TTTAACCTTGCGGCCGTACT | |
| | Probe | CGTTAAGTCGACCGCCT | |
| *lpxL* * | Forward | TGCGCGGCAGTTTGG | 53 |
| | Reverse | TCGTTCGGGCGATAAACG | |
| | Probe | TGCAGGAACCGGGTAT | |

* *lpxL* formerly known as *htrB*

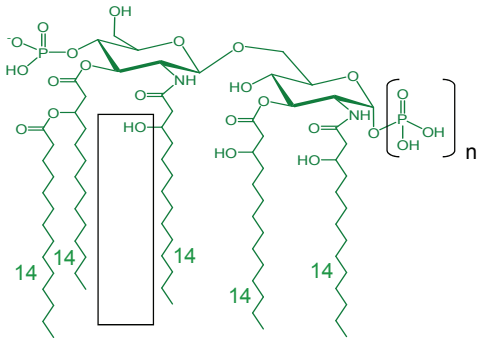
# Supplementary Figure S2




**Supplementary Figure S2:** Negative-ion MALDI-MS spectra from *E. coli* wild-type strain W3110. MALDI-MS of lipid A isolated from *E. coli* wild-type strain W3110 grown A) at 37°C and B) at 42°C. Lipid A mass peaks are annotated with the assigned composition of fatty acids. As expected, the major lipid A species was observed at *m/z* 1716.0 and *m/z* 1716.1 at 37 and 42°C that correspond to the typical wild-type mono-phoshoryl hexa-acyl lipid A ($MPLA_6$) containing four primary 3-hydroxymyristic acids, one secondary myristic acid, and one secondary lauric acid, [4x 14:0(3-OH), 14:0, 12:0]. Other components of the heterogeneous lipid A mixture were observed at *m/z* 1505.8, corresponding to penta-acyl $MPLA_5$, as well as tetra-acyl $MPLA_4$ at *m/z* 1279.7, consisting of one lauric and three or four 3-hydroxymyristic acids, respectively.

# Supplementary Figure S3 *E. coli – lpxL-* mutant lipid A structures

**Pentaacyl lipid A**

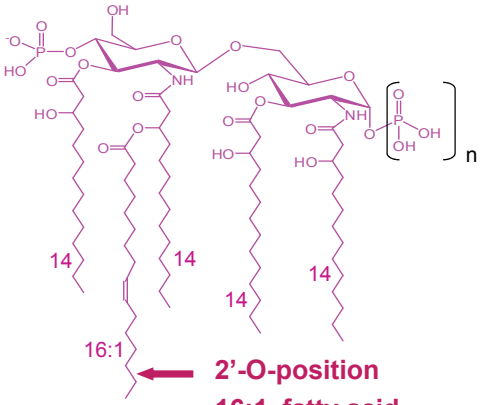


***MPLA$_5$*** *m/z* 1533.8, n=0

***DPLA$_5$*** *m/z* 1614.0, n=1

**expected pentaacyl lipid A species (observed at 30, 37, and 42 ºC)**

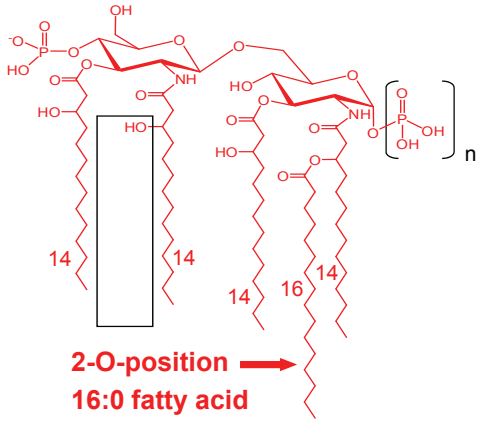
**Pentaacyl lipid A**




***MPLA$_5$*** *m/z* 1559.8, n=0

**predominantly observed at 37 ºC**

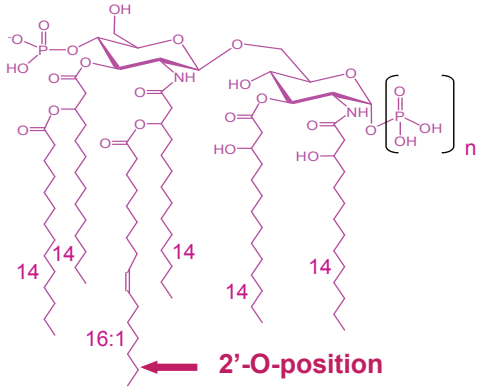
**Pentaacyl lipid A**




***MPLA$_5$*** *m/z* 1561.8, n=0

**predominantly observed at 42 ºC**

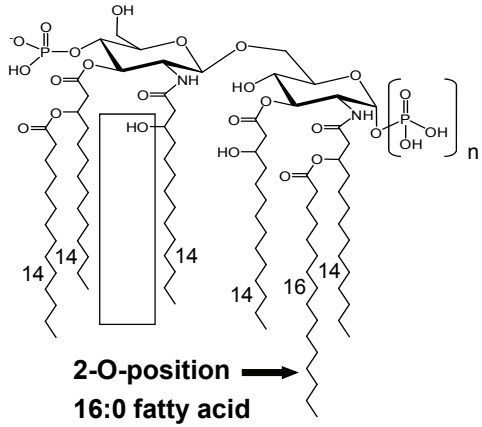
**Hexaacyl lipid A**




***MPLA$_6$*** *m/z* 1769.8, n=0

**predominantly observed at 37 ºC**

**Hexaacyl lipid A**

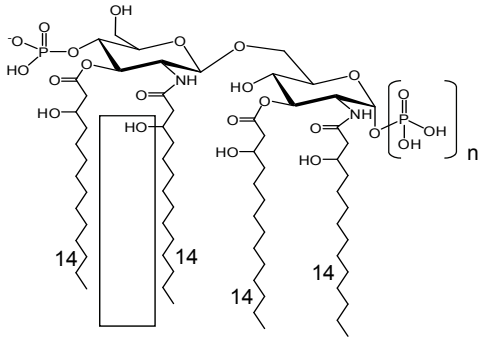



***MPLA$_6$*** *m/z* 1772.1, n=0

**predominantly observed at 42 ºC**

# Supplementary Figure S3 - continued

**Tetraacyl lipid A**




***MPLA$_4$*** *m/z* 1323.8, n=0

***DPLA$_4$*** *m/z* 1403.8, n=1

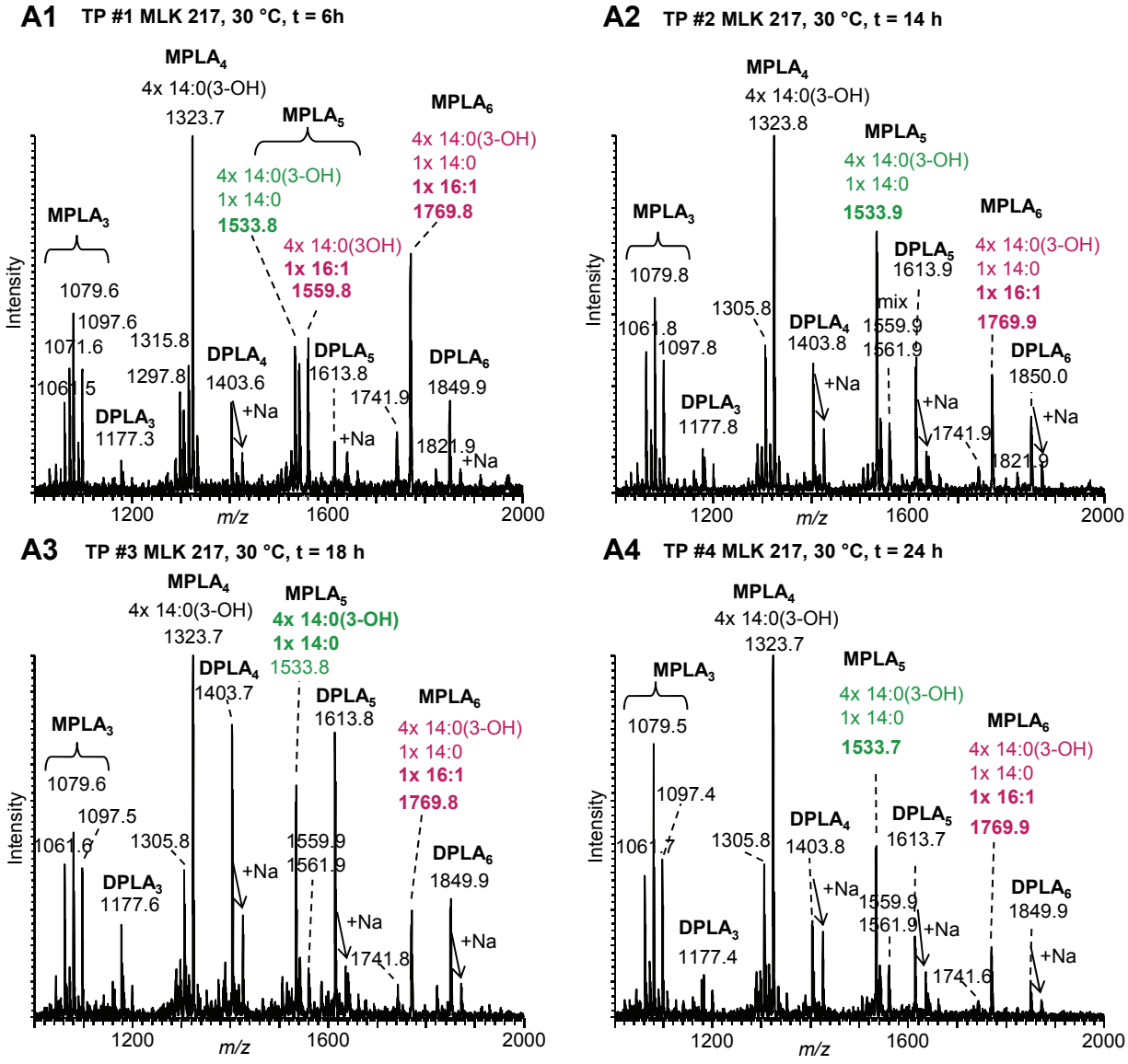
**Tetraacyl lipid A species (observed at 30, 37, and 42 ºC)**

**Supplementary Figure S3:**
Overview of observed lipid A species from *E. coli lpxL* mutant strain grown at different temperatures (at 30°C, at 37°C, and at 42°C). *E. coli lpxL* mutant strain MLK217 is lacking a lauric acid in its structure (12:0 fatty acid, ΔM= -182 Da) and accordingly *lpxL* mutant strain showed the expected pentaacyl lipid A species (marked in green) consisting of four 3-hydroxymyristic acids [4x 14:0(3OH)] and one myristic acids [14:0] in secondary 3'-O position (distal glucosamine) under all temperature growth conditions.

At 37°C the predominant pentaacyl species consisted of four 3-hydroxymyristic acids [4x 14:0(3OH)] and one palmitoleic acid [16:1] in secondary 2'-O position (distal glucosamine), marked in pink. In contrast, at 42°C, the lipid A profile changed to a pentaacyl species consisting of four 3-hydroxymyristic acids [4x 14:0(3OH)] and one palmitic acid [16:0] in secondary 2-O position (proximal glucosamine). Similarly, corresponding hexaacyl species were observed at 37°C with the following fatty acid composition, specifically [4x 14:0(3OH), 14:0, 16:1] with the palmitoleic acid [16:1] in secondary 2'-O position (distal glucosamine).

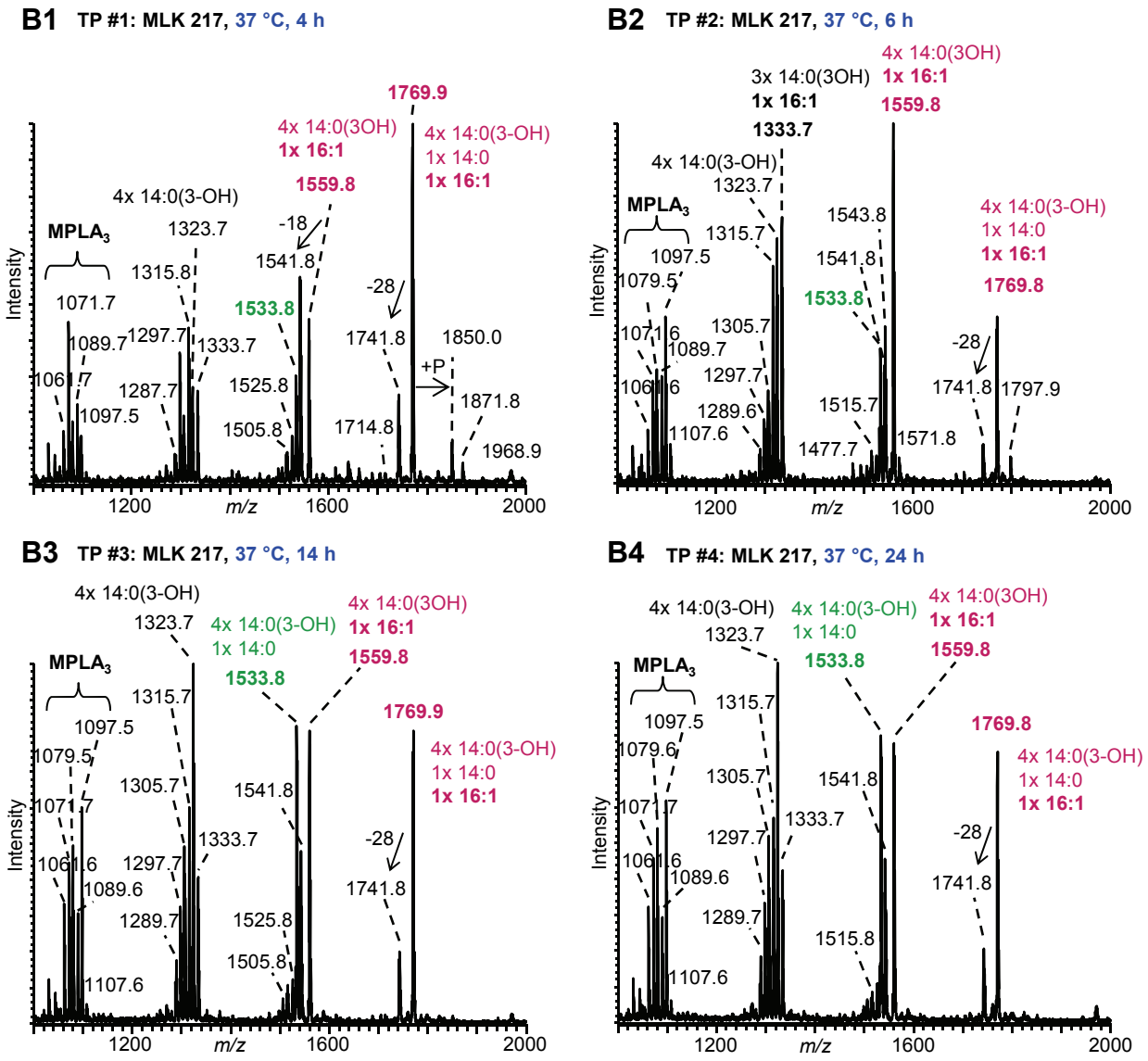
At 42°C a hexaacyl lipid A species was observed with a fatty acid composition of [4x 14:0(3OH), 14:0, 16:0] with the palmitic acid [16:0] in secondary 2-O position (proximal glucosamine). The tetraacyl lipid A species consisting of four 3-hydroxymyristic acids [4x 14:0(3OH)] was observed under all temperature conditions.

# Supplementary Figure S4-A



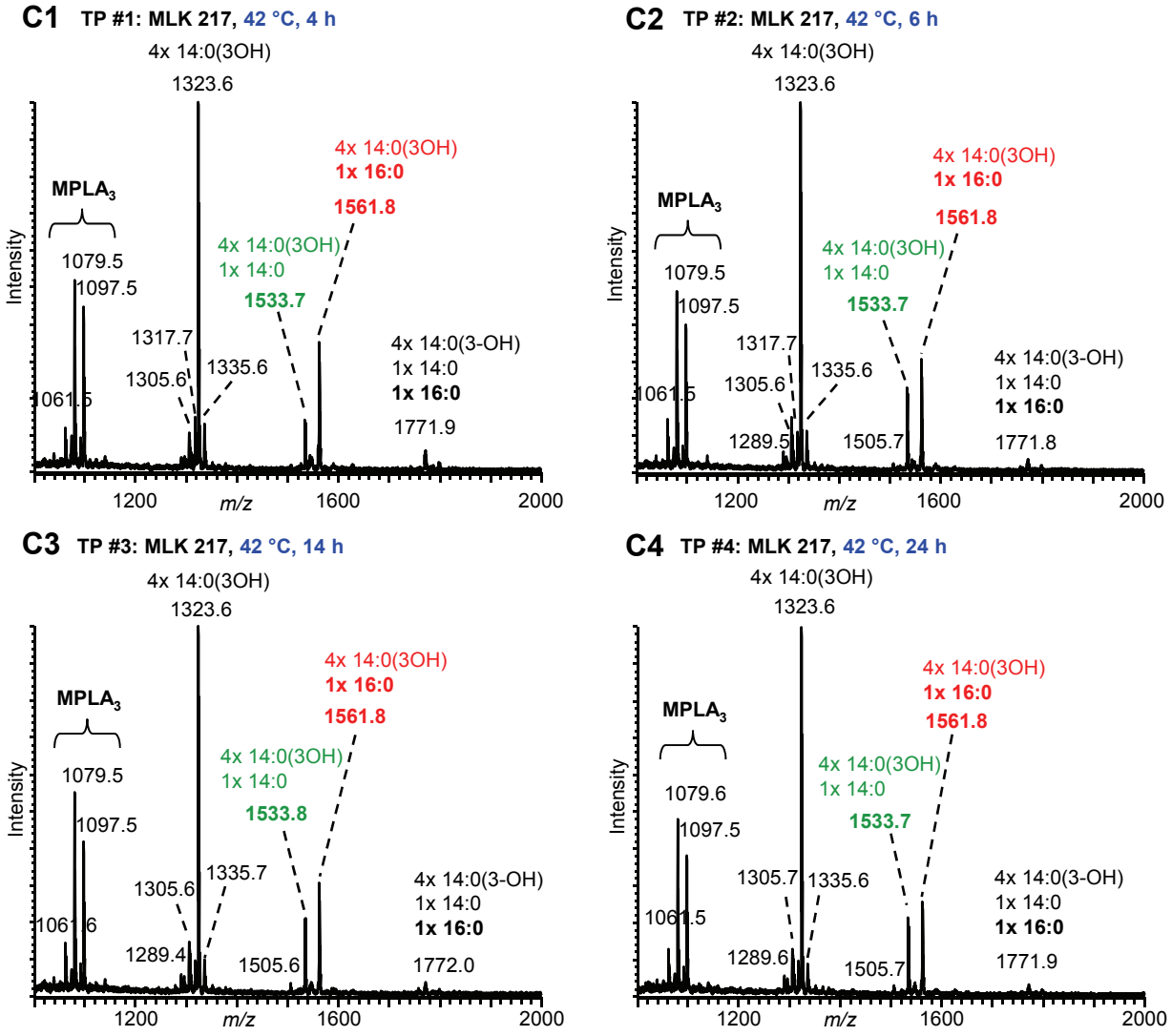

**Supplementary Figure S4-A:** Negative-ion MALDI-MS profiles of lipid As following a time-course study. MALDI-LIT MS of lipid A isolated from *E. coli lpxL* mutant strain MLK217 grown at 30°C for A1) 6 hours, A2) 14 hours, A3) 18 hours, and A4) 24 hours. *E. coli lpxL* mutant strain MLK217 is lacking a lauric acid in its structure (12:0 fatty acid, ΔM= -182 Da). Lipid A mass peaks are annotated with the assigned composition of fatty acids. Major lipid A species observed were the tetraacyl species consisting of four 3-hydroxymyristic acids [4x 14:0(3OH)] (at *m/z* 1323.8), and the expected pentaacyl species consisting of four 3-hydroxymyristic acids [4x 14:0(3OH)] and one myristic acids [14:0] in secondary 3'-O position (at *m/z* 1533.9). A less abundant hexaacyl species was observed at *m/z* 1769.9 corresponding to a fatty acid composition of [4x 14:0(3OH), 14:0, 16:1] with the palmitoleic acid [16:1] in secondary 2'-O position. During the time-course lipid A acylation patterns and acyl group compositions do vary slightly but not significantly.

# Supplementary Figure S4-B



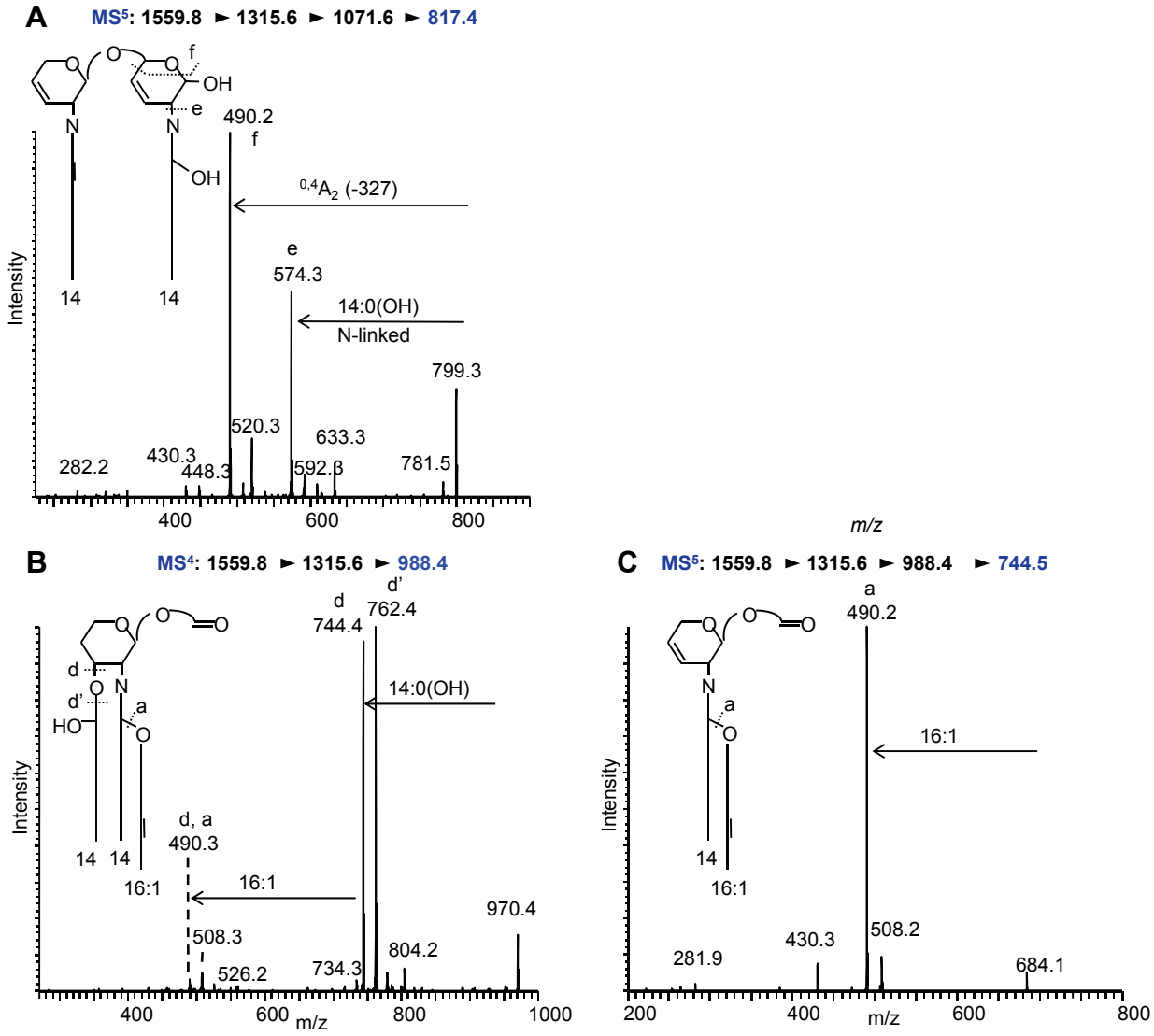

**Supplementary Figure S4-B:** Negative-ion MALDI-MS of lipid A following a time-course study. MALDI- MS of lipid A isolated from *E. coli lpxL* mutant strain MLK217 grown at 37°C for B1) 4 hours, B2) 6 hours, B3) 14 hours, and B4) 24 hours. *E. coli lpxL* mutant strain MLK217 is lacking a lauric acid in its structure (12:0 fatty acid, ΔM= -182 Da). Lipid A mass peaks are annotated with the assigned composition of fatty acids. Besides the tetraacyl species at *m/z* 1323.8 [4x 14:0(3OH)], and the expected pentaacyl species at *m/z* 1533.9 [4x 14:0(3OH), 14:0] with 14:0 in secondary 3'-O position, major lipid A species were observed at *m/z* 1559.8 [4x 14:0(3OH), 16:1] and at *m/z* 1769.9 [4x 14:0(3OH), 14:0, 16:1], both of the latter structures featuring the palmitoleic acid [16:1] in secondary 2'-O position (distal glucosamine). Lipid A acylation patterns and acyl group compositions do not significantly vary during the time-course.

# Supplementary Figure S4-C



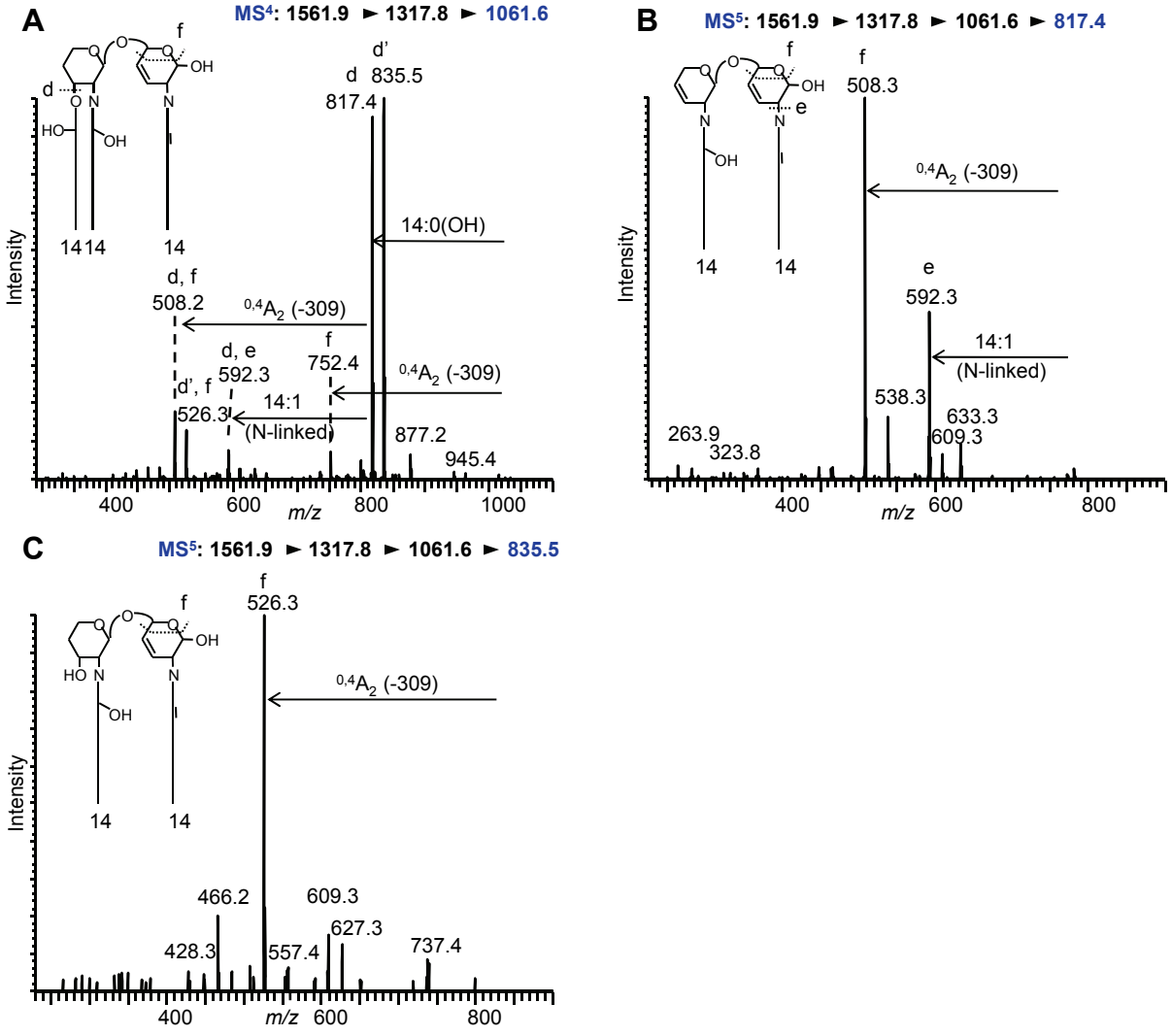

**Supplementary Figure S4-C:** Negative-ion MALDI-MS of lipid A following a time-course study. MALDI-MS of lipid A isolated from *E. coli lpxL* mutant strain MLK217 grown at 42°C for C1) 4 hours, C2) 6 hours, C3) 14 hours, and C4) 24 hours. *E. coli lpxL* mutant strain MLK217 is lacking a lauric acid in its structure (12:0 fatty acid, ΔM= -182 Da). Lipid A mass peaks are annotated with the assigned composition of fatty acids. Besides the tetraacyl species at *m/z* 1323.8 [4x 14:0(3OH)], and the expected pentaacyl species at *m/z* 1533.9 [4x 14:0(3OH), 14:0] with 14:0 in secondary 3'-O position, major lipid A species were observed at *m/z* 1561.8 [4x 14:0(3OH), 16:0] and at *m/z* 1772.0 [4x 14:0(3OH), 14:0, 16:0], both of the latter structures featuring the palmitic acid [16:0] in secondary 2-O position (proximal glucosamine). Lipid A acylation patterns and acyl group compositions do not significantly vary during the time-course.

# Supplementary Figure S5




**Supplementary Figure S5:** Additional negative-ion MALDI-MS$^n$ spectra of pentaacyl lipid A from *E. coli* mutant strain MLK217 (*lpxL*) grown at 37°C. MS$^n$ spectra of penta-acyl lipid A species at *m/z* 1559.8 [4x 14:0(3-OH), 16:1] with the palmitoleic acid [16:1] in 2'-O-position (on the distal glucosamine); also see Fig. 5 (main manuscript). (A) MS$^5$ of *m/z* 817.4 (selected from MS$^4$ 1071.6, selected from MS$^3$ 1315.6, selected from MS$^2$ 1559.8); (B) MS$^4$ of *m/z* 988.4 (selected from MS$^3$ 1315.6, selected from MS$^2$ 1559.8); and (C) MS$^5$ of *m/z* 744.5 (selected from MS$^4$ 988.4, selected from MS$^3$ 1315.6, selected from MS$^2$ 1559.8).

# Supplementary Figure S6

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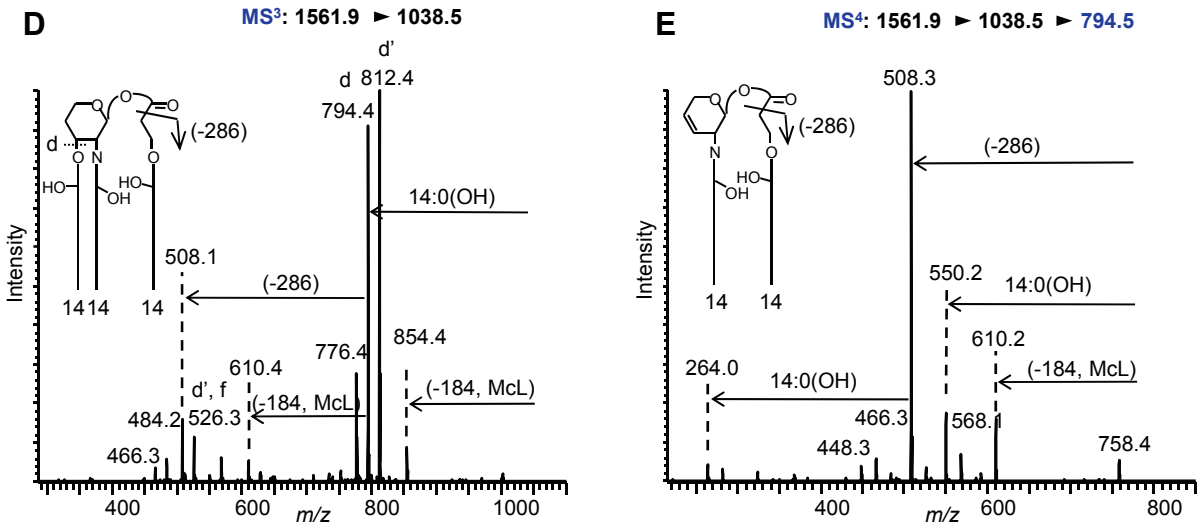
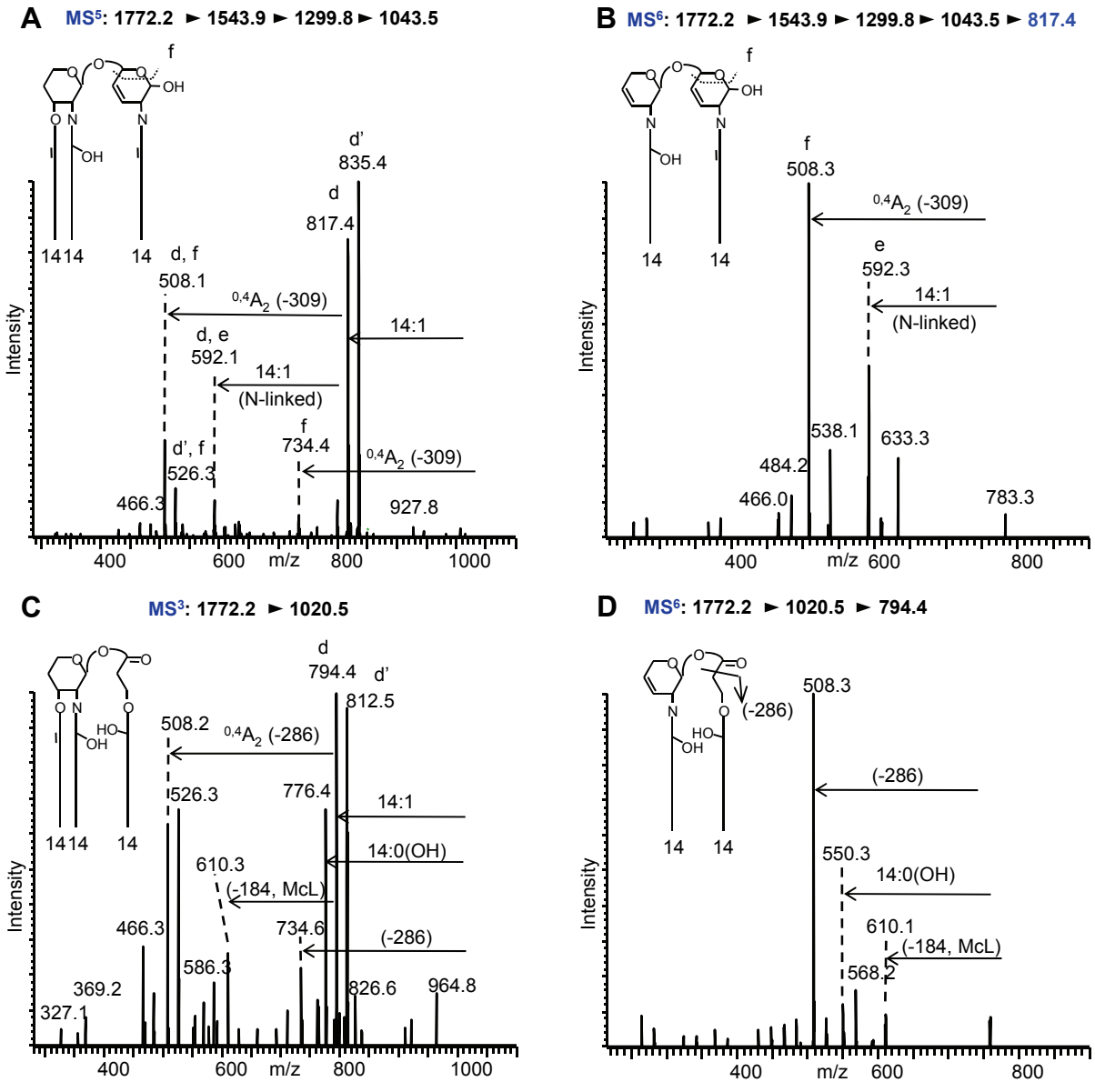
# Supplementary Figure S6 - continued




**Supplementary Figure S6:**
Additional negative-ion MALDI-MS$^n$ spectra of pentaacyl lipid A from *E. coli* mutant strain MLK217 (*lpxL*) grown at 42°C. MS$^n$ spectra of pentaacyl lipid A species at *m/z* 1561.9 [4x 14:0(3-OH), 16:0] with the palmitic acid [16:0] in 2-O-position (on the proximal glucosamine); also see Fig. 6 (main manuscript). (A) MS$^4$ of *m/z* 1061.6 (selected from MS$^3$ 1317.8, selected from MS$^2$ 1561.9); (B) MS$^5$ of *m/z* 817.4 (selected from MS$^4$ 1061.6, selected from MS$^3$ 1317.8, selected from MS$^2$ 1561.9); (C) MS$^5$ of *m/z* 835.5 (selected from MS$^4$ 1061.6, selected from MS$^3$ 1317.8, selected from MS$^2$ 1561.9); (D) MS$^3$ of *m/z* 1038.5 (selected from MS$^2$ 1561.9); (E) MS$^4$ of *m/z* 794.5 (selected from MS$^3$ 1038.5, selected from MS$^2$ 1561.9). The abbreviation 'McL' indicates a McLafferty rearrangement.

# Supplementary Figure S7

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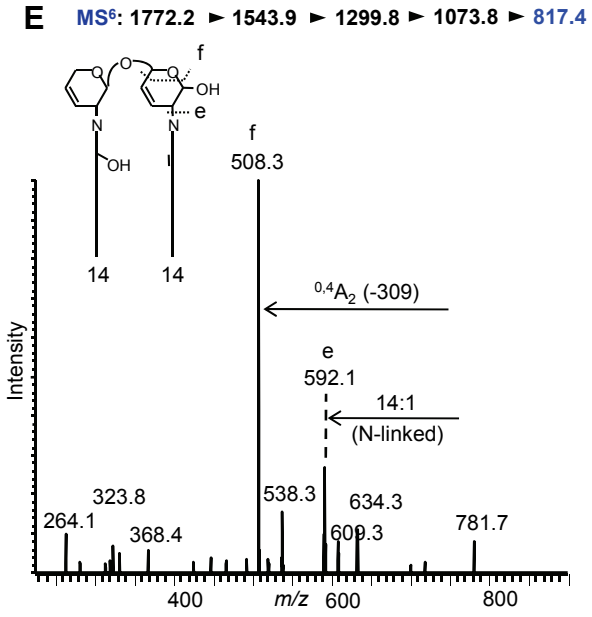
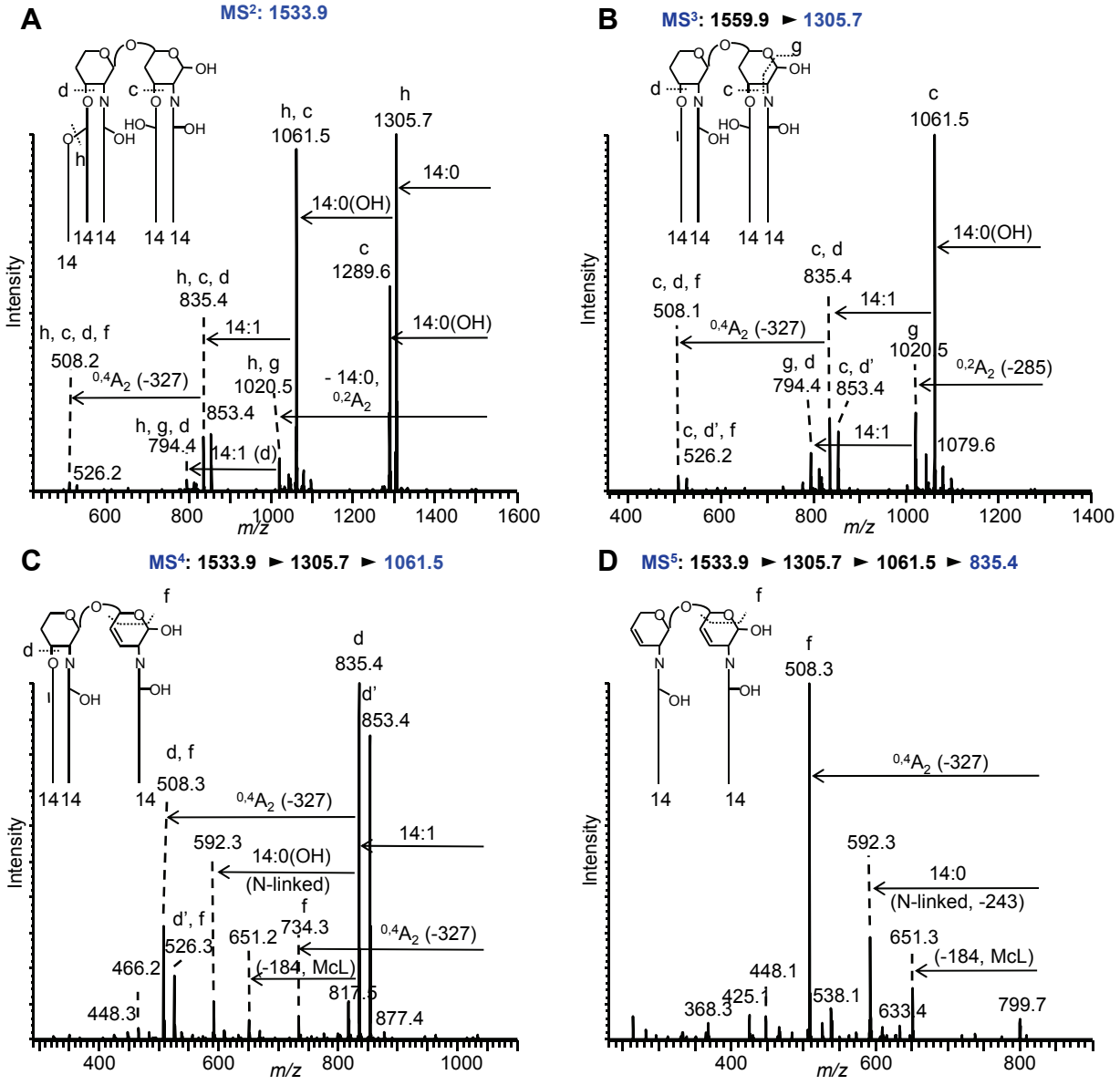
# Supplementary Figure S7 - continued




**Supplementary Figure S7:**
Negative-ion MALDI- $MS^n$ spectrum of hexaacyl lipid A at *m/z* 1772.2 from *E. coli* mutant strain MLK217 (*lpxL*) grown at 42°C. $MS^n$ spectra of hexaacyl lipid A species at *m/z* 1772.2 [4x 14:0(3-OH), 14:0, 16:0] with the palmitic acid [16:0] in 2-O-position (on the proximal glucosamine) are shown in Figure 7. Additional fragmentation details are shown here with (A) $MS^5$ of *m/z* 1043.5 (selected from $MS^4$ of *m/z* 1299.8, selected from $MS^3$ 1543.9, selected from $MS^2$ 1772.2); (B) $MS^6$ of *m/z* 817.4 (selected from $MS^5$ of *m/z* 1043.5, selected from $MS^4$ of *m/z* 1299.8, selected from $MS^3$ 1543.9, selected from $MS^2$ 1772.2); (C) $MS^3$ of *m/z* 1020.5 (selected from $MS^2$ 1772.2); (D $MS^4$ of *m/z* 794.4 (selected from $MS^3$ *m/z* 1020.5, selected from $MS^2$ 1772.2); and (E) $MS^6$ of *m/z* 817.4 (selected from $MS^5$ of *m/z* 1073.8, selected from $MS^4$ of *m/z* 1299.8, selected from $MS^3$ 1543.9, selected from $MS^2$ 1772.2). The abbreviation 'McL' indicates a McLafferty rearrangement.

# Supplementary Figure S8




**Supplementary Figure S8:**

Negative-ion MALDI-MS$^n$ spectra of pentaacyl lipid A at *m/z* 1533.9 from *E. coli* mutant strain MLK217 (*lpxL*) grown at 42°C lacking a lauric acid in its structure (12:0 fatty acid, ΔM= -182 Da) [corresponding fragmentation patterns are obtained from *E. coli* mutant strain MLK217 (*lpxL*) grown at lower temperature (i.e., 30° and 37°C)]. MS$^n$ spectra of penta-acyl lipid A species at *m/z* 1533.9 [4x 14:0(3-OH), 14:0] with the myristic acid [14:0] in 2'-O-position (on the distal glucosamine). (A) MS$^2$ of *m/z* 1533.9; (B) MS$^3$ of *m/z* 1305.7 (selected from MS$^2$ 1533.9); (C) MS$^4$ of *m/z* 1061.5 (selected from MS$^3$ 1305.7, selected from MS$^2$ 1533.9); (D) MS$^5$ of *m/z* 835.4 (selected from MS$^4$ 1061.5, selected from MS$^3$ 1305.7, selected from MS$^2$ 1533.9); (E) MS$^3$ of *m/z* 1289.6 (selected from MS$^2$ 1533.9); (F) MS$^4$ of *m/z* 1061.5 (selected from MS$^3$ 1289.6, selected from MS$^2$ 1533.9); (G) MS$^5$ of *m/z* 835.5 (selected from MS$^4$ 1061.5, selected from MS$^3$ 1289.6, selected from MS$^2$ 1533.9). The abbreviation 'McL' indicates a McLafferty rearrangement.

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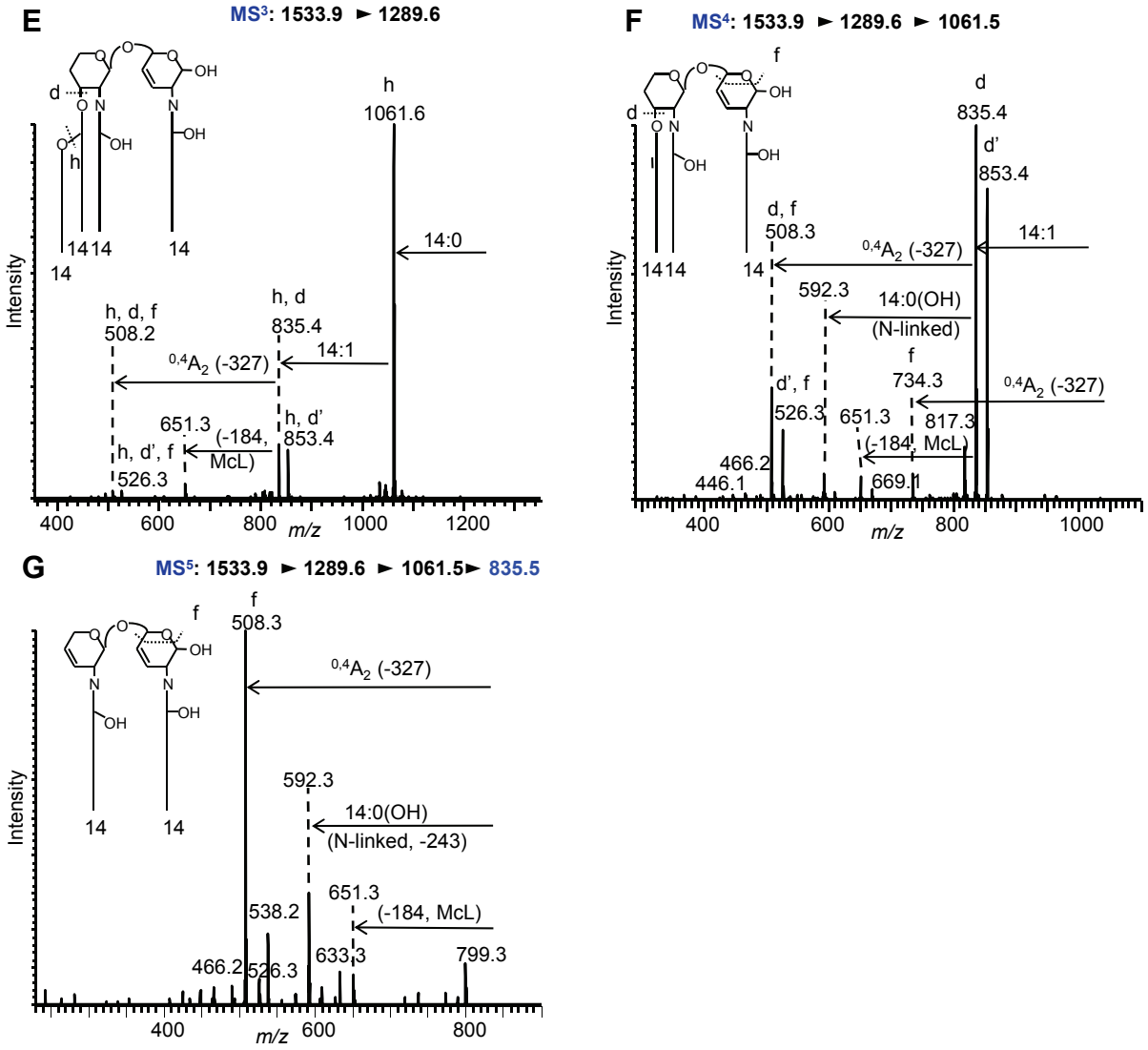
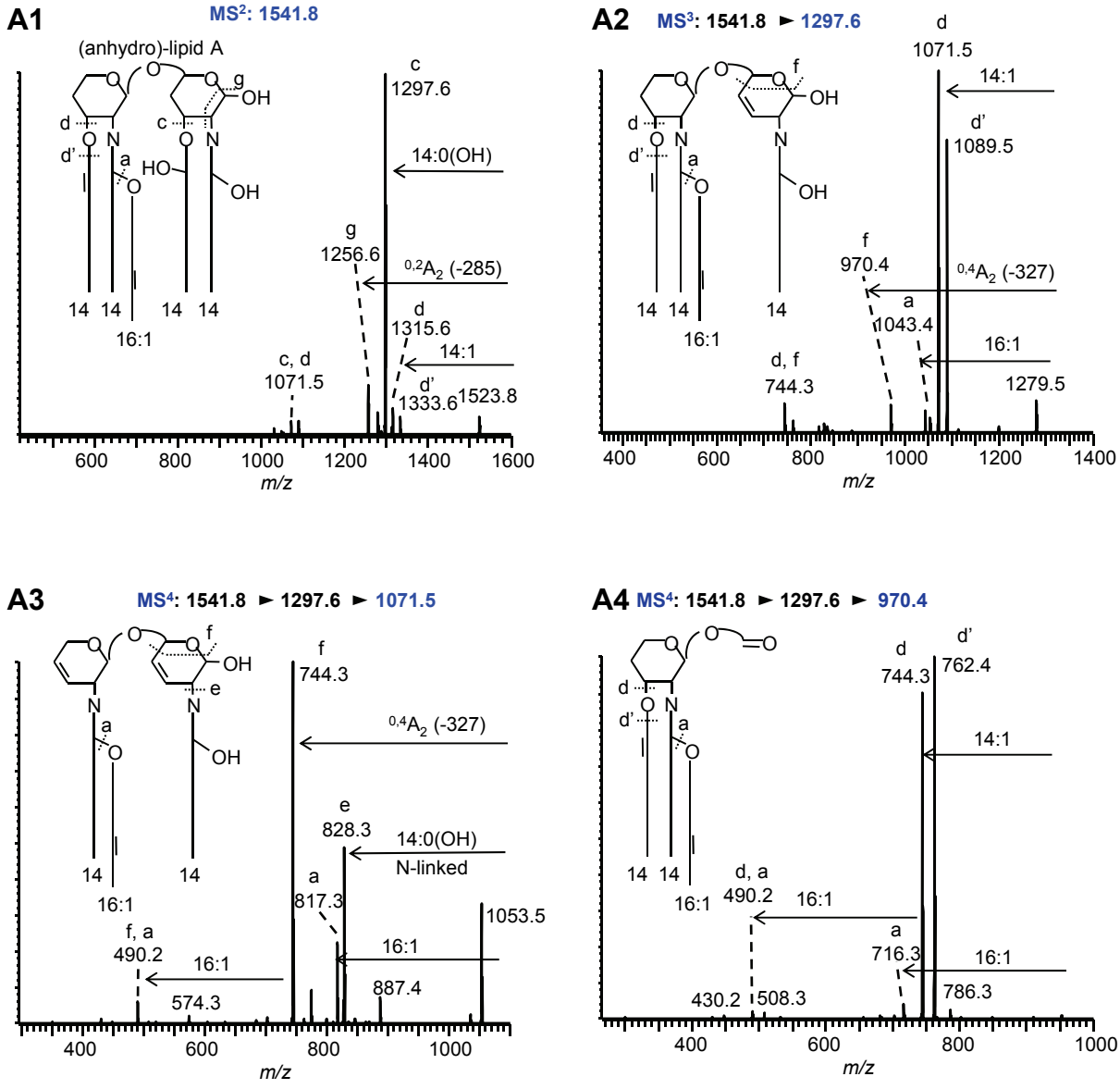
# Supplementary Figure S8 - continued



# Supplementary Figure S9-A



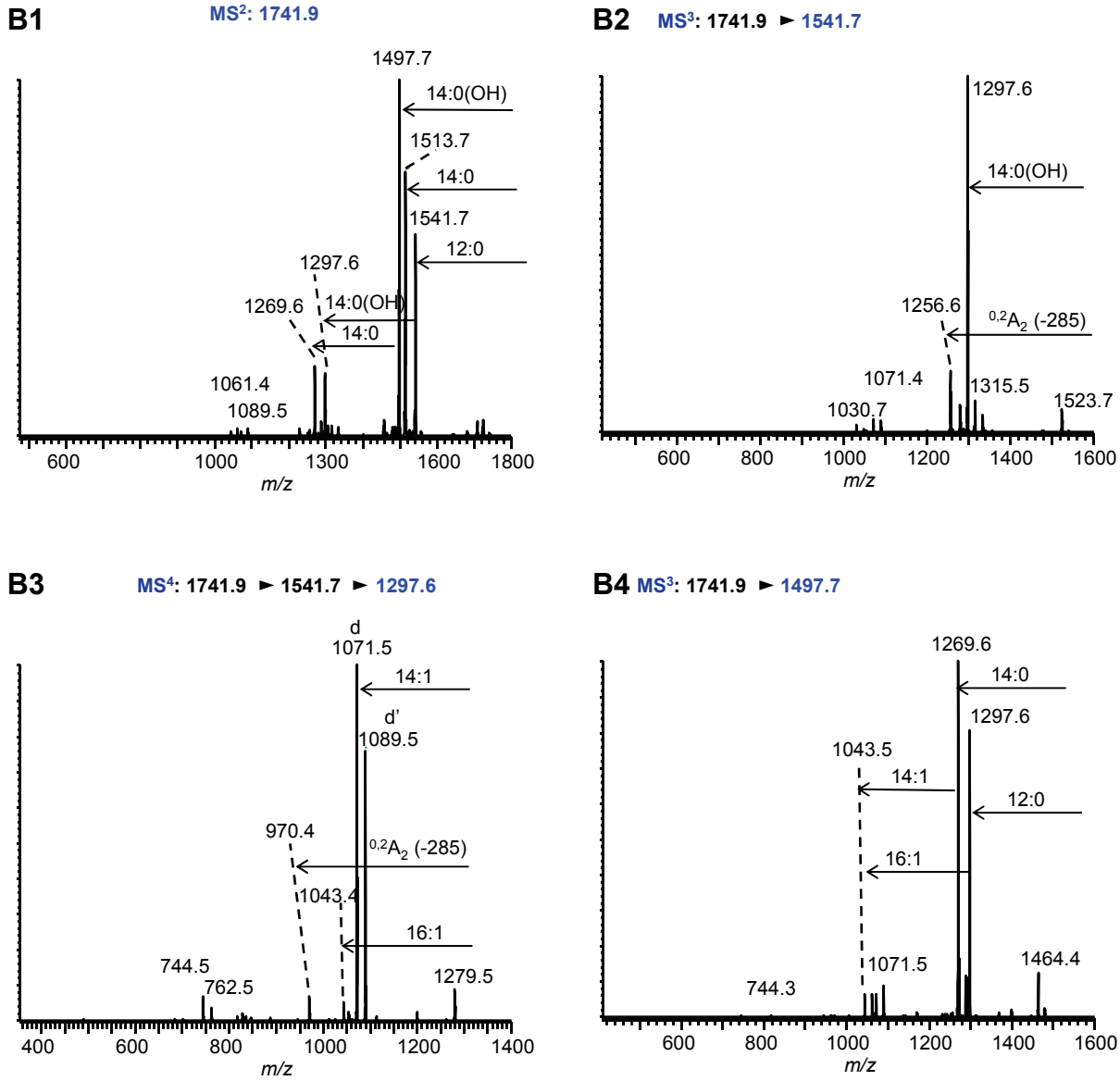

**Supplementary Figure S9-A:** Negative-ion MALDI-MS$^n$ spectra of pentaacyl lipid A from *E. coli* mutant strain MLK217 (*lpxL*) grown at 37°C. MS$^n$ spectra of pentaacyl lipid A species at *m/z* 1541.8 anhydro-[4x 14:0(3-OH), 16:1] with the palmitoleic acid [16:1] in 2'-O-position (on the distal glucosamine); lipid A species with *m/z* [1559.8 – $H_2O$]. (A1) MS$^2$ of *m/z* 1541.8; (A2) MS$^3$ of *m/z* 1297.6; (A3) MS$^4$ of *m/z* 1071.5; (A4) MS$^4$ of *m/z* 970.4.

# Supplementary Figure S9-B

Low abundant isoform mixture of lipid A species with fatty acid compositions such as

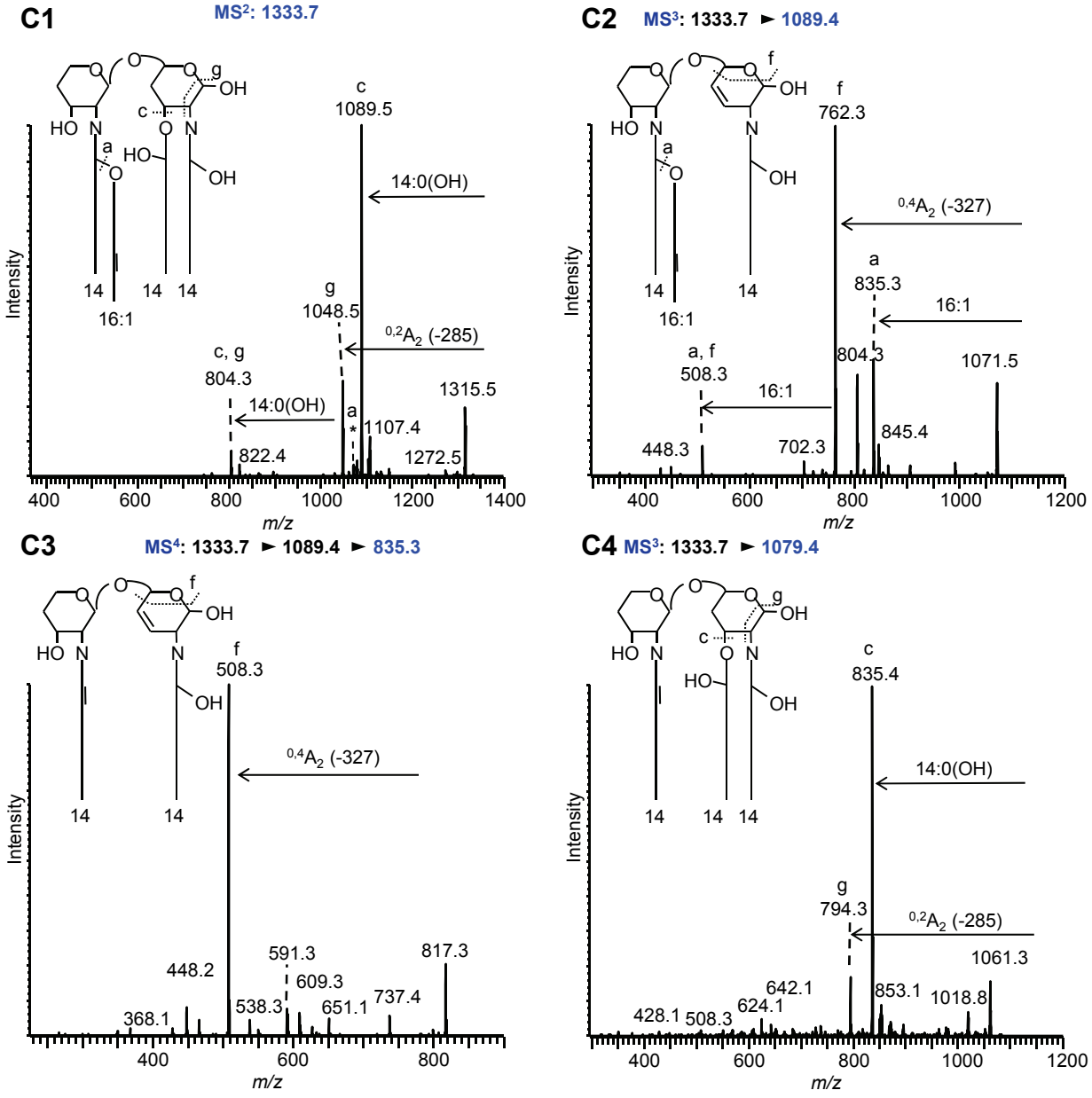
[4x 14:0(3-OH), 14:0, 14:1]

[4x 14:0(3-OH), 12:0, 16:1]



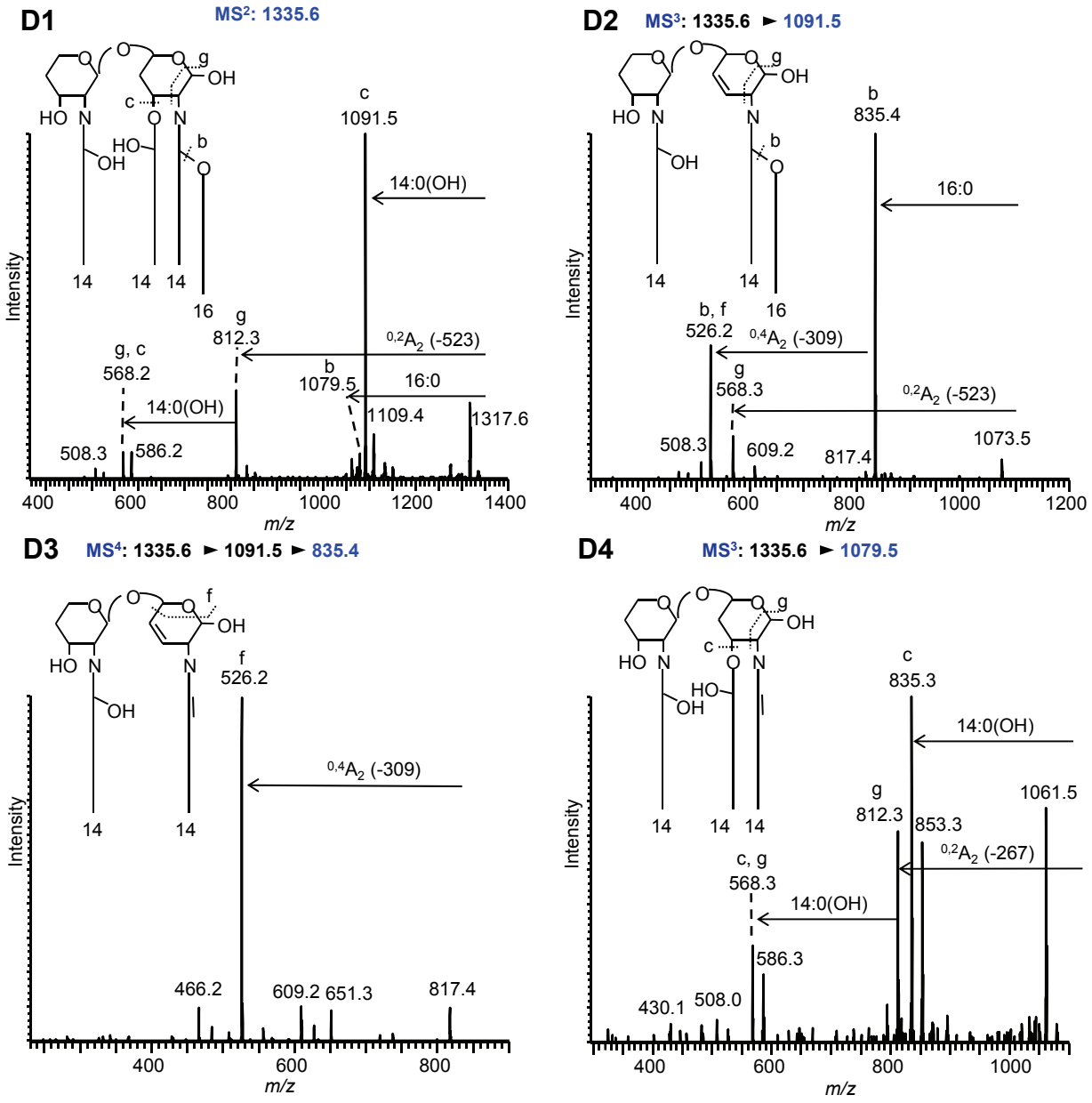

**Supplementary Figure S9-B:** Negative-ion MALDI-MS$^n$ spectra of hexaacyl lipid A from *E. coli* mutant strain MLK217 (*lpxL*) grown at 37°C. MS$^n$ spectra of hexaacyl lipid A species at *m/z* 1741.8 that appears to be a mixture of different lipid A species, i.e., [4x 14:0(3-OH), 14:0, 14:1], and [4x 14:0(3-OH), 12:0, 16:1] with the unsaturated acyl group [16:1 and 14:1, respectively] in 2'-O-position (on the distal glucosamine). These lipid A species were detected at low levels (Fig. 2B). (B1) MS$^2$ of *m/z* 1741.9; (B2) MS$^3$ of *m/z* 1541.7; (B3) MS$^4$ of *m/z* 1297.6; (B4) MS$^3$ of *m/z* 1497.7.

# Supplementary Figure S9-C




**Supplementary Figure S9-C:** Negative-ion MALDI-MS$^n$ spectra of tetraacyl lipid A from *E. coli* mutant strain MLK217 (*lpxL*) grown at 37°C. MS$^n$ spectra of tetraacyl lipid A species at *m/z* 1333.7 [3x 14:0(3-OH), 16:1] with the palmitoleic acid [16:1] in 2'-O-position (on the distal glucosamine). (C1) MS$^2$ of *m/z* 1333.7; the asterisk * indicates the minor fragment ion *m/z* 1079.4 which in panel (C4) is selected for further MS$^3$ fragmentation. (C2) MS$^3$ of *m/z* 1089.4; (C3) MS$^4$ of *m/z* 835.3; (C4) MS$^3$ of *m/z* 1079.4.

# Supplementary Figure S9-D




**Supplementary Figure S9-D:** Negative-ion MALDI-MS$^n$ spectra of tetraacyl lipid A from *E. coli* mutant strain MLK217 (*lpxL*) grown at 42°C. MS$^n$ spectra of tetraacyl lipid A species at *m/z* 1335.7 [3x 14:0(3-OH), 16:0] with the palmitic acid [16:0] in 2-O-position (on the proximal glucosamine). (D1) MS$^2$ of *m/z* 1335.6; (D2) MS$^3$ of *m/z* 1091.5; (D3) MS$^4$ of *m/z* 835.4; (D4) MS$^3$ of *m/z* 1079.4.

**Supplementary Table S2: Fragmentation Pathways of *lpxL*- lipid A with different acyl substitution patterns**

| lipid A at *m/z* | Substitutions | $^{0,4}A_2$ fragment loss * | $MS^n$ fragment ion, *m/z* | $^{0,2}A_2$ fragment loss |
|---|---|---|---|---|
| 1559.9 (lpxL-, 37 °C ) | 16:1 in 2'-O ; 2-O-H (free) | - 327 Da | 490 [Z] | - 285 Da |
| 1561.9 (lpxL-, 42 °C ) | 2'-O-H (free) ; 2-O- 16:0 | - 309 Da [#] | 508 [Z] | - 523 Da [W] |
| 1769.9 (lpxL-, 37 °C ) | 16:1 in 2'-O ; 2-O-H (free) | - 327 Da | 490 | - 285 Da |
| 1771.9 (lpxL-, 42 °C ) | 2'-O-H (free) ; 2-O- 16:0 | - 309 Da [#] | 508 | - 523 Da [W] |
| 1553.9 (lpxL-, 30 °C ) | 2'-O-H (free) ; 2-O-H (free) | - 327 Da | 508 | - 285 Da |

[*] $^{0,4}A_2$ fragmentation is observed after initial loss of fatty acid in proximal 3- position of the glucosamine

[#] fatty acid in secondary 2-O position was eliminated first and subsequently the acyl group in primary 2- position carries a double bond, $\Delta^2$-14:1 (after eliminating 16:0 off proximal 2-O position)

[Z] fragment ion as shown in Figure 8A and 8B

[W] $^{0,2}A_2$ fragmentation includes combined loss of secondary 16:0 (in 2-O-position ) bound to hydroxy group of hydroxymyristic acid in 2-position, also see Figure 6.