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Lipid A structural determination from a single colony

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Abstract

We describe an innovative use for the recently reported Fast Lipid Analysis Technique (FLAT) that allows for the generation of MALDI tandem mass spectrometry data suitable for lipid A structure analysis directly from a single Gram-negative bacterial colony. We refer to this tandem MS version of FLAT as FLAT^n. Neither technique requires sophisticated sample preparation beyond selection of a single bacterial colony, which significantly reduces overall analysis time (~ one hour), as compared to conventional methods. Moreover, the tandem mass spectra generated by FLAT^n provides comprehensive information of fragments of lipid A, e.g., ester bonded acyl chain dissociations, cross-ring cleavages, and glycosidic bond dissociations all of which allows facile determination of novel lipid A structures or confirmation of expected structures. In addition to generating tandem mass spectra directly from single colonies, we also show that FLAT^n can be used to analyze lipid A structures taken directly from a complex biological clinical sample without the need for *ex vivo* growth. From a urine sample from a patient with an *E.coli* infection, FLAT^n identified the organism and demonstrated that this clinical isolate carried the mobile colistin resistance-1 gene (*mcr-1*) that results in the addition of a phosphoethanolamine moiety and subsequently resistance to the antimicrobial, colistin (polymyxin E). Moreover, FLAT^n allowed

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for determination of the existence of a structural isomer in *E.coli* lipid A that had either a 1 or 4'-phosphate group modification by phosphoethanolamine generated by a change of bacterial culture conditions.

Keywords

Lipid A; Gram-negative bacteria; Tandem Mass Spectrometry; FLATn; Structural Analysis

Introduction

Gram-negative bacteria cause a variety of human infectious diseases, including urinary tract infections, pneumonia, and bloodstream infections (sepsis).[1] Thus, understanding the pathogenic microbial molecules responsible for such maladies is important. One well-known molecule present in the outer membrane of Gram-negative bacteria is lipopolysaccharide (LPS).[2] LPS consists of three structural elements: Oantigen, the most exterior region and composed of repeating saccharide units, followed by a carbohydrate core region, and anchored in the membrane by lipid A.[3] Most Gram-negative bacteria share a canonical lipid A base structure consisting of two glucosamine backbones connected by a β(1',6)-glycosidic bond with each sugar having a terminal phosphate group at the 1 and 4' carbon position of the diglucosamine backbone, respectively.[4] Fatty acid moieties are linked to the sugar backbone at the O-3', N-2', O-3, or N-2 positions by ester or amide bonds and can vary in length and number of acyl chains, both of which can be influenced by environment conditions during growth.[5]

Structural modification of lipid A plays a critical role in resistance to antibiotics, such as polymyxins including colistin (polymyxin E).[6] Modification of lipid A by attachment of positively-charged groups, such as phosphoethanolamine (PEtN) and 4-amino-4-deoxy-l-arabinopyranose (Ara4N) decrease the overall negative charge of the bacterial membrane, thereby resulting in resistance to cationic antimicrobial peptides, including colistin.[7,8] Thus, structural determination is a critical component to provide a better understanding of the lipid A structure activity relationship (SAR) with the host innate immune system.

Historically, lipid A chemical structure was solved by tandem mass spectrometry (MS/MS)-based analysis (alone or together with nuclear magnetic resonance), collision induced dissociation (CID)[9–13] using either standard MS2 approaches in quadrupole based mass analyzers, or MS^n [14–16] approaches in ion trapping devices. Due to the presence of salts, which can hamper ionization and interpretation in crude preparations of lipid A, highly purified lipid A, free of nucleic acid and protein contamination has been essential to generate high quality MS^n data. However, current LPS/lipid A extraction protocols are labor-intensive and require the use of harmful organic chemicals, including phenol and chloroform to produce milligram quantities of lipid A for tandem MS analysis.[17] Routine lipid A structural analysis has typically required a minimum 10 milligrams to fully solve a structure of a single lipid A molecule.[18] Therefore, enabling rapid analysis without labor- and time-intense purification methods would significantly decrease the bottleneck of lipid A structure analysis.

For rapid lipid-based identification of bacterial species, our group previously developed a method for on-surface and/or on-tissue release of lipid A from LPS allowing detection of lipid A by matrix-assisted laser desorption ionization (MALDI) MS in the negative ion mode.[19,20] This new method, termed Fast Lipid Analysis Technique (FLAT) selectively cleaves the glycosidic linkage between the first core sugar, 3-deoxy-D-manno-octulsonic acid (Kdo), and the non-reducing glucosamine (6' position) of lipid A and core. Since FLAT requires only a single colony and a single step to generate lipid A from intact bacteria, the entire process takes approximately one hour.[19] In this study, we demonstrate that a MALDI trapped ion-mobility time-of-flight (timsTOF) mass spectrometer can produce detailed structural information, including cross-ring fragmentation patterns that allowed for the rapid definition of the chemical structure of lipid A. We refer to this tandem MS adoption of FLAT as FLAT^n and demonstrate that FLAT^n can produce all the key fragment ions needed to interpret lipid A structure from a single colony using various Gram-negative bacterial backgrounds. With the ability to expedite the structural analysis of lipid A, we can accelerate the rate at which we associate a given structure with an observed activity,[21–24] which is key to development of objective SAR predictions for lipid A's interaction with the MD2/TLR4 complex that can be either antagonistic or agonistic for future studies.

Experimental Section

A single colony (<2 mm) of each bacterial strain (*E. coli*, *P. aeruginosa*, *mcr-1 A. baumannii*, *mcr-1 E. coli, mcr-1 K. pneumoniae*, and *mcr-1 P. aeruginosa)* was picked from lysogeny broth (LB, Becton-Dickenson, Hunt Valley, MD, USA). agar plate using a 0.2 – 10 μL capacity pipette tip after 18 hours of growth at 37°C. The single colony was directly smeared on the ITO-glass slide. To confirm reproducibility, three individual, well separated colonies were chosen and used for FLAT^n from each bacterial plate. In order to determine compatibility of FLAT^n for liquid cultured samples, *E. coli* was used for control experiment. In detail, the single colony of *E. coli* was picked from the LB agar plate using a disposable inoculation loop. The colony was resuspended in deionized water (200 μL). Then, the colony was spun down (8000 × g, 5 min), supernatant was discarded by a pipette, and the remaining 10 μL of slurry was used for MS analysis. For each FLATn, only one microliter of slurry was used. Next, FLAT was conducted according to the previous literature.[19,20] Briefly, 1 μL of the prepared citrate buffer solution (0.2 M citric acid, 0.1 M trisodium citrate, pH 3.5) was deposited onto the sample spot on the ITO slide. The plate was incubated in a humidified, closed glass chamber for 30 min at 110°C. After heating, the ITO slide was removed from the chamber, cooled, and the plate was thoroughly washed with water using a pipettor several times and left to dry on the laboratory bench.

For liquid culture, single colonies were picked from agar plates of *E. coli, P. aeruginosa, mcr-1 A. baumannii*, *mcr-1 E. coli, mcr-1 K. pneumoniae, and mcr-1 P. aeruginosa* and inoculated into 5 mL of LB broth (Becton-Dickenson, Hunt Valley, MD, USA). Cultures were incubated at 37°C with 200 rpm shaking to mid-log phase (approximately 18 hours). For isolation of bacteria from media, 1.0 mL of liquid culture of *E. coli*, *P. aeruginosa*, *mcr-1 A. baumannii*, *mcr-1 E. coli, mcr-1 K. pneumoniae, and mcr-1 P. aeruginosa* were harvested into a microfuge tube, pelleted (8000 × g, 5 min), and 900 μL of supernatant discarded. To remove remaining growth media, 900 μL of deionized water was added into the tube and

the cell harvesting procedure was conducted one more time and as described above. The remaining 100 μL of bacteria solution were used to resuspend the bacterial pellet and 1 μL of this suspension was used for analysis. The sample was deposited on the ITO glass slide and allowed to dry. FLAT was conducted according to the aforementioned method.

A urine specimen was obtained from the University of Maryland Medical Center clinical microbiology laboratory. The sample was confirmed to be positive by the clinical laboratory for a urinary tract infection (UTI) after quantification via culture contained greater than 100,000 cfu/mL (Institutional Review Board (IRB) HP-00064919). Lipid analysis via MALDI-TOF MS identified the presence of *E. coli* with an addition of a phosphoethanolamine indicative of the presence of an *mcr-1* gene.[25] Pathogen identification of the urine specimen was confirmed by the clinical microbiology laboratory via standard culture methods. The presence of the *mcr-1* gene was confirmed via polymerase chain reaction (PCR).25 For urine analysis, the sample was concentrated before use as follows: 1.0 mL of human urine were harvested into a microfuge tube, pelleted (8000 × g, 5 min), and 990 μL of supernatant discarded. The remaining 10 μL of bacteria solution was used to re-suspend the bacterial pellet and 1 μL of this suspension was used for analysis. The sample was deposited on the ITO glass slide and allowed to dry. FLAT was conducted according to the aforementioned method.

Expanded materials, bacteria strains, bacterial growth conditions, instrumentation information, and data processing are available in the Supporting Information.

Results

Determination of structure of canonical lipid A from a single bacterial colony

FLAT is compatible with various MALDI MS instruments with different configurations for vacuum pressure (high-vacuum or intermediate-pressure) and different laser wavelengths (337 nm from N2 versus 355 nm from Nd:YAG).[20] Previously, we used the Gram-negative organisms, *Escherichia coli* (*E. coli)* and *Pseudomonas aeruginosa* (*P. aeruginosa)* to examine the efficiency of FLAT to produce sufficient lipid A signal from a single colony of bacteria. However, the extent to which lipid A fragment ions are important for structure determination could reproducibly be generated from a single colony was not determined. Using FLAT^n, we found that there was sufficient biomass in a single colony to produce lipid A signature ions historically used for identification[26] and tandem MS to determine the structure of these two lipid A molecules from their respective signature ions. To our knowledge, this is the first successful demonstration of structural analysis of lipid A from a single bacterial colony.

Figure 1 illustrates FLAT^n results from a single colony of *P. aeruginosa* used for lipid A structure derivation. Figure 1A (arrow) shows the exact colony chosen for analysis (>2 mm). Figure 1B shows a mass spectrum obtained from the colony after FLAT processing (MS^1). Using the precursor ion at *m/z* 1445.8673 (Figure 1C), we carried out tandem MS (MS^2) and used that resultant data to confirm the structure of this lipid A molecule. Figure 1D indicates key bond cleavages by CID from FLAT^n. As expected, many neutral losses are observed, such as acyl chains from ester bonds, phosphoric acid (H3PO4), and cross-ring cleavages.

The list of key fragment ions generated by FLAT^n is shown in Figure 1D. For instance, the loss of H_3PO_4 from *P. aeruginosa* lipid A corresponds to an ion at *m/z* 1347.8804, which is labeled as B_2 according to accepted nomenclature.[13,27] Multiple losses of acyl chains from ester bonds, such as *m/z* 1159.7391 (B_2 with 3'α), 943.5666 (B_2 with 3'α and 3ε), and 743.3889 (B_2 with 3'α, 3ε, and 2' ε) are shown in the Figure 1E. The cross-ring cleavage produces ions at *m/z* 722.3886 and 662.3675 corresponded to $^{0,2}A_2$ and $^{0,4}A_2$, respectively. These selective dissociations of acyl chains from ester bonds and crossring cleavage of the backbone were used to deduce the location of additional acylations and modifications of acyl chains.

Since the *m/z* error in these experiments resulting from the difference between theoretical and experimental value is approximately 20 ppm, fragment ions are readily identified using the high mass accuracy data. We note that FLAT^n of *E. coli* lipid A was conducted with similar results (see Figures S2 and S3). In order to test reproducibility of FLAT^n, three different individual colonies were selected and subjected to FLAT^n. These results suggest that FLAT^n provides highly reproducible data (see Figure S4). Interestingly, the acyl chain in the amide bond was not dissociated and the cross-ring cleavage was only observed in the reducing-end of the glucosamine backbone; however sufficient fragment ion detail was present to solve the structure represented by the ion at *m/z* 1347.8804 (B_2), 1159.7391 (B_2 + 3'α), 943.5666 (B_2 + 3'α + 3ε), 743.3889 (B_2 + 3'α + 3ε + 2'ε), 722.3886 ($^{0,2}A_2$ + 3'α), and 662.3675 ($^{0,4}A_2$ + 3'α).These type fragmentation phenomena of lipid A have been previously observed.[28,29]

Determination of lipid A structures that correlate with antibiotic resistance from a single bacterial colony

Electrostatic interaction between positively charged antibiotics, such as colistin and negatively charged LPS is a critical mechanism underlying the mechanism through which antibiotics target bacterial membranes.[30] As colistin is often used as the 'last resort' antibiotic to treat multi-drug resistant Gram-negative bacterial infections, the emergence of colistin resistance is a high risk factor for patients.[7] Colistin resistance in Gram-negative bacteria can be encoded chromosomally[31–32] or be plasmid-based[33,34]. For this work, we focused on the recently identified Mobilized Colistin Resistance (*mcr*) genes that mediate bacterial colistin resistance via a bacterial plasmid.[35] Previously, our group and others have shown *mcr-1* gene products function as a lipid A phosphoethanolamine transferase resulting in the modification of the terminal phosphate groups of lipid A by addition of phosphoethanolamine (PEtN).[33,34] However, it is not possible to assess which phosphate group position is modified without MS2 data. Here, we demonstrate that FLAT^n can be used to determine the exact location of the phosphate group modified by PEtN addition. All FLAT^n tandem mass spectra for each *mcr-1* expressing bacterial (*mcr-1 A. baumannii*, *mcr-1 K. pneumoniae*, and *mcr-1 P. aeruginosa*) and their assigned structures are shown in Figures S5 to S8. Moreover, using the FLAT^n data, we were also able to identify acyl chain modifications from colistin-resistant bacteria, *Acinetobacter baumannii* (*mcr-1 A. baumannii*) and *Klebsiella pneumoniae* (*mcr-1 K. pneumoniae*). These data are shown in Figures S9 to S12. As shown for *E. coli* and *P. aeruginosa* above, FLAT^n tandem mass spectra used for structure elucidation could be obtained from analysis of a single colony.

Data in all Supplemental Figures were produced from a single colony for each bacterial background. We were able to structurally analyze multiple lipid A structures from a single colony using FLAT^n (Figure S7 – 10).

Furthermore, we demonstrate the dependence on growth conditions of the location of PEtN modification on phosphate in *E. coli* lipid A. Figure 2A shows FLAT^n tandem mass spectra from solid media (Top) and *mcr-1 E. coli* obtained from a human urine sample (Bottom). Although the two mass spectra share the same precursor ion at *m/z* 1919.2206, each has a different tandem MS fragmentation pattern. This result indicates that the PEtN modification site is variable depending on growth conditions. The characteristic ion at *m/z* 1267.8095, representing the 4' phosphate group modified with PEtN was also observed from single colony experiments (Figure 2A, Top) or when grown in the presence of gentamicin (liquid culture) as shown in Figure 2B (Top). Conversely, the characteristic ion at *m/z* 833.4335 representing the 1-phosphate group modification with PEtN, generated from Y1, was the predominant ion in the human urine sample (Figure 2A, Bottom) and after liquid culture without gentamicin (Figure 2B, Bottom). The structure of *mcr-1 E. coli* lipid A modification with PEtN on the 1 or 4'-phosphate group are shown in Figure 2C and D, respectively. These figures show the base peak and characteristic ions of each *mcr-1 E. coli* lipid A at *m/z* 1919.2206. When we expand any of these mass spectra, each one contains both the characteristic ion at *m/z* 1267.8095 and 833.4335. A bar graph in Figure 2E shows the ratio of intensities of two ions (*m/z* 1267.8095 and 833.4335), which represents the 4'-and 1-phosphate group modification, respectively. Individual data sets, except for the human urine sample, which was a single collection, were obtained from three independent biological replicates. The error bars in Figure 2E indicate the standard deviation that arise from biological replicates. This result indicates FLAT^n unambiguously distinguished structure isomers of lipid A from various growth conditions. Interestingly, other *mcr-1 A. baumannii, mcr-1 K. pneumoniae*, and *mcr-1 P. aeruginosa* did not show any alteration of the site of PEtN modification when growth conditions were changed. All data used to determine PEtN modification are provided in Table S1.

Conclusion

We developed FLAT^n, a method that provides highly reliable and robust tandem MS data for lipid A structural analysis without prior extraction. FLAT^n provided sufficient fragment ions from a single colony to confirm the structures of several Gram-negative bacteria and to discern structural changes induced by differences in growth conditions. Like FLAT, the FLAT^n method is conducted entirely after on-surface extraction of lipid A in a time that is significantly shorter compared to conventional methods (e.g., 48 hours vs 1 hr). Given the rapid rate that lipid A tandem MS data may now be generated we expect FLAT^n to play an important role in our quest to develop a better understanding of the lipid A SAR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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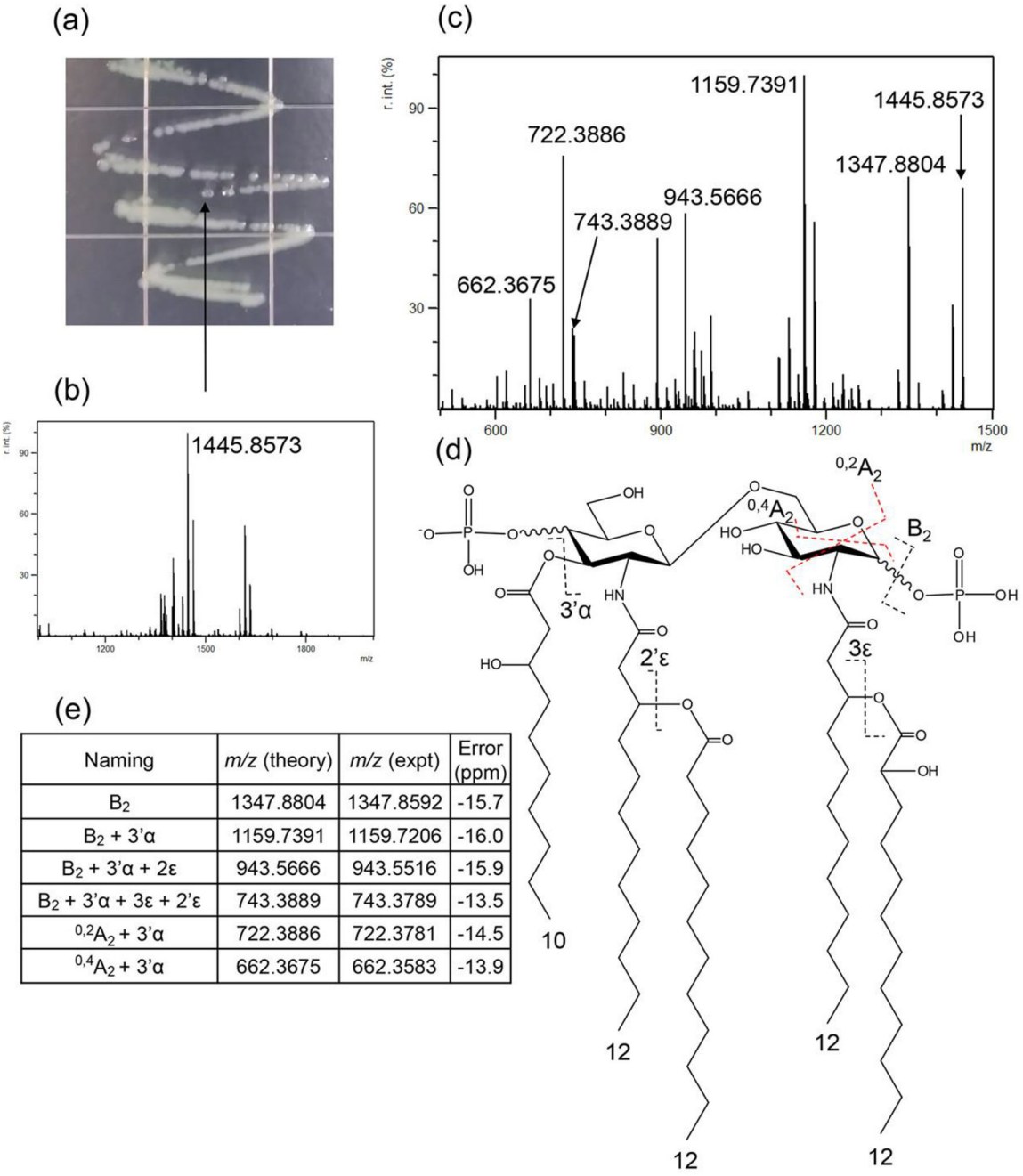
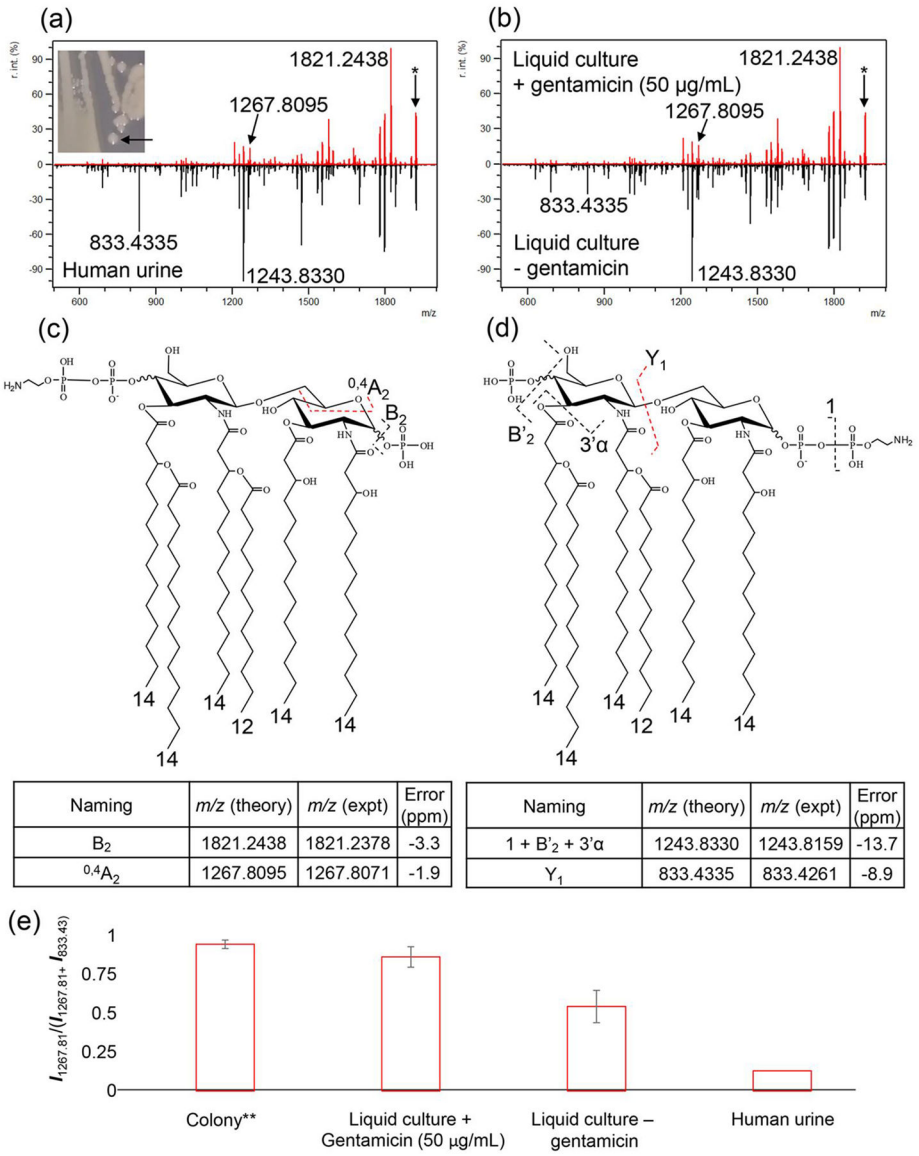


(e)

Naming	*m/z* (theory)	*m/z* (expt)	Error (ppm)
B_2	1347.8804	1347.8592	-15.7
B_2 + 3'α	1159.7391	1159.7206	-16.0
B_2 + 3'α + 2ε	943.5666	943.5516	-15.9
B_2 + 3'α + 3ε + 2'ε	743.3889	743.3789	-13.5
$^{0,2}A_2$ + 3'α	722.3886	722.3781	-14.5
$^{0,4}A_2$ + 3'α	662.3675	662.3583	-13.9

Figure 1.

(a) An image of *Pa* single colonies on agar plate. (b) FLAT mass spectrum obtained from the colony (c) FLAT^n mass spectrum from precursor ion at *m/z* 1445.8573 (d) Structure of lipid A from FLAT^n, and (e) list of assigned ions.



Naming	*m/z* (theory)	*m/z* (expt)	Error (ppm)
B_2	1821.2438	1821.2378	-3.3
$^{0,4}A_2$	1267.8095	1267.8071	-1.9

Naming	*m/z* (theory)	*m/z* (expt)	Error (ppm)
1 + B'_2 + 3'α	1243.8330	1243.8159	-13.7
Y_1	833.4335	833.4261	-8.9

Figure 2.

FLAT^n mass spectra of *m/z* 1919.2206, where the samples were obtained from (a) Single colony (Top) and human urine sample (Bottom), (b) liquid culture with gentamicin (50 μg/mL, Top) and without gentamicin (Bottom). Asterisk mark (*) in the mass spectra indicates precursor ion at m/z 1919.2206. The structure of 4' (c) and 1-phosphate group (d) modification with PEtN (e) a bar graph of ratio of intensities of two ions between 1267.8095 and 833.4335, which represents 4'- and 1-phosphate group modification by PEtN addition, respectively. **Agar plate with gentamicin (50 μg/mL)