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RESEARCH ARTICLE

Profiling and tandem mass spectrometry analysis of aminoacylated phospholipids in *Bacillus subtilis* [version 1; peer review: 1 approved, 2 approved with reservations]

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Abstract

Cationic modulation of the dominantly negative electrostatic structure of phospholipids plays an important role in bacterial response to changes in the environment. In addition to zwitterionic phosphatidylethanolamine, Gram-positive bacteria are also abundant in positively charged lysyl-phosphatidylglycerol. Increased amounts of both types of lipids render Gram-positive bacterial cells more resistant to cationic antibiotic peptides such as defensins. Lysyl and alanyl-phosphatidylglycerol as well as alanyl-cardiolipin have also been studied by mass spectroscopy. Phospholipids modified by other amino acids have been discovered by chemical analysis of the lipid lysate but have yet to be studied by mass spectroscopy. We exploited the high sensitivity of modern mass spectroscopy in searching for substructures in complex mixtures to establish a sensitive and thorough screen for aminoacylated phospholipids. The search for deprotonated aminoacyl anions in lipid extracted from *Bacillus subtilis* strain 168 yielded strong evidence as well as relative abundance of aminoacyl-phosphatidylglycerols, which serves as a crude measure of the specificity of aminoacyl-phosphatidylglycerol synthase MprF. No aminoacyl-cardiolipin was found. More importantly, the second most abundant species in this category is D-alanyl-phosphatidylglycerol, suggesting a possible role in the D-alanylation pathway of wall- and lipo-teichoic acids.

Keywords

Gram-positive , Antibiotic resistance , Membrane , Charge , Phospholipid , Teichoic acid , alanyl-phosphatidylglycerol , lysyl-phosphatidylglycerol , aminoacylated phospholipids, D-alanylation

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Introduction

In most bacteria, phospholipids are the dominant cell membrane component¹. The phosphate moieties in these lipid molecules dictate the overall negative nature of bacterial membranes. This electrostatic feature makes them susceptible to cationic antibiotic peptides such as defensins^{2–4}. In response to environmental challenges, bacteria constantly change their membrane composition^{1,5}. Incorporation of less saturated and shorter fatty acyl chains makes the membrane more fluidic¹. Gram-negative bacteria generally have a high concentration of zwitterionic phosphatidylethanolamine (PE) which masks this anionic surface feature^{1,6}. In comparison, Gram-positive bacteria in general have much less PE¹. However, they are abundant in aminoacylated phosphatidylglycerol (aminoacyl-PG), especially L-lysyl-PG^{3,7}. The pivotal protein for aminoacyl-PG biosynthesis from L-aminoacyl-tRNA and PG is the lysyl-PG synthase MprF (multiple peptide resistance factor)⁸ which appears to have a broad range of specificity for L-aminoacyl-tRNAs^{9,10}. The crystal structures of the cytoplasmic catalytic domains of two MprFs, with one specific for lysyl- the other for alanyl-PG biosynthesis, have recently been elucidated¹¹. The catalytic domains of the two MprF enzymes have a long tunnel for accommodating PG with the catalytic site located at the narrowest part of the tunnel¹¹. The primary and tertiary structures of MprF resemble that of FemX which catalyzes L-alanyl transfer from tRNA to a peptidoglycan precursor¹². Both proteins are potential targets for novel antibiotics. MprF of *Bacillus subtilis* over-expressed in *Escherichia coli* has been observed to synthesize both L-lysyl-PG and L-alanyl-PG in the presence of aminoacyl-tRNA¹⁰. We expect to find both lysyl- and alanyl-PGs in *B. subtilis* lipids.

In comparison to Gram-negative bacteria, Gram-positive bacteria have profoundly different cell-envelope structures; they lack the outer membrane, and the cell wall is usually much thicker, with multiple peptidoglycan layers. In addition to PE and aminoacyl-PG biosynthesis, which modulate bacterial surface charge, one constant signature of Gram-positive cell envelopes, however, is the presence of additional glycopolymers including peptidoglycan-attached wall-teichoic acids and lipid-anchored lipoteichoic acids. This type of cell surface polymer was discovered in the late 1950s¹³. It carries multiple negative charges due to its phosphodiester bonds between repetitive glycerol or ribitol residues. Its association with the cell envelope is anchored by covalent attachment to either membrane glycolipids or peptidoglycan^{14–16}. The most common modification of this biopolymer is D-alanine esterification^{13,15,17}, which is carried out by four proteins (DltA, DltB, DltC and DltD) coded by the *dlt* operon¹⁸. This surface charge modulation by D-alanylation appears to have profound effects on the antigenicity of the bacteria and immune response of host cells⁷. The D-alanyl carrier protein ligase DltA (~500 amino acid residues)¹⁸ is an enzyme resembling the adenylation domains (also called AMP-forming domains) found in modular nonribosomal peptide synthetases¹⁹. Its remote homologues include the acyl-coenzyme A synthetases and firefly luciferases²⁰. DltA catalyzes the ATP-driven adenylation of the carboxyl group of D-alanine and the transfer of the activated D-alanyl to the thiol group of 4'-phosphopantetheine which is covalently attached to a serine side chain of D-alanyl carrier protein DltC (~80 amino acid residues)^{18,21,22}. The functional role has not been firmly established for DltB (~400 amino acid residues), an integral membrane protein.

DltD (~400 amino acid residues), a membrane-bound protein via a putative N-terminal transmembrane helix, appears to bind DltC and possibly catalyzes the final D-alanyl transfer from DltC to teichoic acid²³. We suspect that a D-alanylated lipid species may serve as the intermediate between cytosolic D-alanyl-DltC and lipo- and wall-teichoic acids on the outside of cell membrane.

Lipid profiling of aminoacyl-PG using mass spectroscopy has been reported recently for *E. coli* and *B. subtilis*²⁴. L-alanyl-PG has been found to be abundant in Gram-negative *Pseudomonas aeruginosa*²⁵, which has a MprF homolog specific for L-alanyl-tRNA substrate¹⁰. D-alanyl- and L-lysyl-cardiolipin (CL) have also been separated from *Vagococcus fluvialis*^{26,27}. However, only lysyl-PG has been identified in *B. subtilis* lipid by mass spectrometry²⁴. Serine, glycine and ornithine-containing lipids are also known to exist in bacteria²⁸. Here we report a more thorough profiling of aminoacyl-PGs. We also established sensitive scans for lysyl-PE as well as alanyl-PE. Importantly, the second most abundant aminoacyl-PG, alanyl-PG, appeared to be D-alanyl-PG, implying a role in the D-alanylation pathway of wall- and lipo-teichoic acids.

Materials and methods

Bacterial strain and cell culture. The BL21 (DE3) strain of *E. coli* was acquired from Novagen. Strain 168 of *B. subtilis* was acquired from Bacillus Genetic Stock Center (BGSC). Both types of cells were first plated on LB-agar media. A single colony was inoculated into 10 ml of LB media. After over-night incubation at 37°C and 220 rpm in an environmental shaker, it was transferred to 1 liter of LB media. When the cell culture just reached an optical density of ~2.0 at 600 nm, the cell pellet was collected by centrifugation at 5,500 rpm for 16 min in a Beckman JLA-8.1 rotor at 4°C.

Lipid extraction. HPLC-grade organic solvents (Fisher Scientific) are used throughout the experiment. The lipid extraction procedure was adapted to get maximal yield of aminoacylated lipids based on the protocol developed by Folch²⁹. The wet cell pellet was re-suspended in equal weight of distilled and deionized water. The lipid extraction was carried out at a room temperature of 21°C except that the cells were kept on ice. 1.8 ml of the cell suspension was transferred to a glass centrifuge tube. Addition of 4 ml chloroform and 2 ml of methanol was followed by vortexing for 1 minute. 2 ml of methanol was added followed by 1 minute of vortexing. 2 ml of buffer solution (0.1 M NaAc at pH 4.5) was added followed by 1 minute of vortexing. Then the tube was placed on a rocking incubator for 3 hours. After that, the phase separation was assisted by centrifugation at 1,300 rpm for 5 minutes with a Beckman Allegro X-22R centrifuge. The heavier chloroform-rich phase was transferred by a glass syringe to a second glass centrifuge tube. The water-rich phase in the first tube was further extracted three times. Each time, 2 ml of chloroform was added, the mixture vortexed, the phase separation assisted by centrifugation, and the chloroform-rich phase transferred to the second glass tube. The combined chloroform-rich phase (~10 ml) in the second tube was first washed by adding 1 ml DI water followed by vortexing for 5 seconds. The lighter water-rich phase was removed after centrifugation. Another wash and dehydration cycle with 1.0 ml 0.5 M NaCl followed. After vortexing and subsequent centrifugation at 1,300 rpm, the chloroform-rich phase was collected into a third tube. This final

sample was placed in a heater at 30°C and dried in an argon stream for approximately 2–3 hours. The empty tube was weighted, and again after drying. Typically, approximately 5 mg of total lipids were obtained and dissolved in chloroform to a concentration of 4 mg/ml.

Chemical syntheses of aminoacylated derivatives of PE – Lipids of PE with fatty acyl chains 16:0–18:1 were acquired from Avanti Polar Lipids. Fluorenylmethyloxycarbonyl chloride (Fmoc)-protected L-alanine as well as Fmoc and t-Butyloxycarbonyl (Boc)-protected L-Lysine were purchased from Sigma-Aldrich. 10 ml of dichloromethane (DCM) was added into a round bottom flask on ice with continuous stirring. 0.014 mmol Fmoc-Ala or Fmoc-Lys-Boc (2.0 × equivalents) was dissolved in the solvent followed by the addition of 0.016 mmol (2.2 × equivalents) NN-Dicyclohexylcarbodiimide (DCC). The PE chloroform solution was washed with saturated sodium bicarbonate. Then 0.007 mmol (1 × equivalent) PE was added dropwise in 1 minute. After 5 minutes of incubation on ice, the reaction mixture was placed in a water bath at the room temperature of 21°C for 1–2 hours. The reaction mixture was filtered through a 100 ml glass filter with fritted disc and then washed first with 1.0 ml of saturated sodium bicarbonate and then 1.0 ml of 0.5% HCl. The organic phase was dried by rotary evaporation, and was redissolved in 50% piperidine in dimethylformamide for the deprotection of Fmoc. Deprotection of Fmoc was carried out at room temperature for 4 hours, followed by the double wash and drying procedure described above. The lysyl-PE product was dissolved in DCM containing 10–20% trifluoroacetic acid (TFA) and incubated at room temperature for 30 minutes to remove Boc protection. The final product was double washed, dried, and redissolved in chloroform for storage at -80°C.

Lipid analysis by thin-layer chromatography. A total volume of 15 µl of lipid samples (4 mg/ml) were spotted 1.5 cm above the bottom edge on 0.25 mm thick silica gel on plastic sheet (Millipore) cut to a size of 10 cm × 20 cm. Alternatively, 100 µl of lipid samples were spotted on 1.0 mm thick silica gel on a glass plate (Fluka). After drying over a heater set at 50°C for ~10 minutes, the TLC sheet/plate was placed into a TLC chamber pre-equilibrated with a mixed solvent of chloroform : methanol : water (65:25:4). After ~30 minutes, the TLC sheet/plate was removed from the TLC chamber and dried for 5 minutes at 50°C. The TLC sheet/plate was first stained by spraying 0.01% primuline (Sigma-Aldrich) solution in acetone : water (80:20), dried in air or with mild heating for ~5 minutes. The fluorescent image was recorded with a Syngene G:BOX system. The fluorescent bands on the thicker gel were lifted and extracted by 100 µl of chloroform in a glass tube. The gel debris was discarded after centrifugation at 1,300 rpm for 1 minute. The TLC sheet was stained again by 0.1% ninhydrin (Sigma-Aldrich) in acetone : acetic acid (100:1), dried in air for ~5 minutes and heated at 100°C until purple spots appeared in a few minutes. The visible light image was recorded with the Syngene system.

Lipid profiling by mass spectroscopy. The lipid samples were diluted by adding 9-fold volume of methanol to a concentration of 0.4 mg/ml (or 400 ppm) for direct infusion at a rate of 0.6 ml/hour to a SCIEX 4000 QTRAP mass spectrometer. Electrospray ionization was achieved at a temperature of 500°C and a pressure of 20 psi for curtain gas as well as ion source gas 1 and 2. The collision

energy in the ion trap was set at +45 or -65 electronvolts in positive and negative mode, respectively. A total of 30 MCA cycles of ion counts were accumulated as the mass spectra of precursor scans and neutral loss scans. The SCIEX Analyst software (version 1.6) was used to acquire and export averaged mass spectra. MS spectra in the figures were generated by Mass++ software (version 2.7.3)³⁰ or Microsoft Excel.

Tandem mass spectroscopy. The targeted MS/MS spectra were first acquired using the SCIEX 4000 QTRAP system. The parameters were the same as those for the profiling. High-accuracy MS/MS spectra were later acquired using an Agilent Q-TOF 6550 system. Direct infusion was set at a slower rate of 0.1 ml/hour for the Q-TOF 6550 system.

Alkaline hydrolysis of lipids – All lipids from one extraction procedure from 0.9 g of wet cells, dissolved in ~0.6 ml chloroform, was partially hydrolyzed by adding 0.25 ml of methanol and 0.04 ml of 1.0 M NH₄OH and incubating at 37°C for 90 minutes without stirring. A volume of 0.05 ml 1.0 M formic acid was added to the solution followed by 0.5 ml of water. The mixture was vortexed for a few seconds and gently shaken in hand to partially remove bubbles. Then the mixture was centrifuged at 1,300 rpm for 5 minutes. The top water-rich layer was collected into a glass beaker and thoroughly dried at 90°C. The residue was dissolved in 0.1 ml water.

Alkaline hydrolysis of bacterial cells – 1.5 ml of cells at early stationary phase with an OD₆₀₀ value (optical density at 600 nm) of ~2.0, were centrifuged at 13,000 rpm for 5 minutes. The cell pellet was subjected to 3 rounds of washing with 1.0 ml of water and centrifugation to discard the wash. The cells were deactivated by heating in boiling water bath for 10 minutes. Then the cells were suspended in 0.1 ml of 1.0 M NH₄OH. The sample was incubated at 37°C for 90 minutes without stirring. The supernatant after centrifugation at 13,000 rpm for 5 minutes was transferred to a glass beaker and thoroughly dried (1 minute). The residue was dissolved in 0.1 ml of water.

Conjugation with Marfey's reagent – 0.1 ml of 2 mM L- or D-alanine, or the samples from alkaline hydrolysis, was transferred to a glass vial with cap. Then 0.2 ml acetone, 0.05 ml 1% (~30 mM) Marfey's reagent³¹ in acetone, and 0.04 ml 1.0 M NaHCO₃ were added and mixed by gentle shaking. The reaction solution was kept at 37°C for 90 minutes without stirring. As the reaction progressed, the bright yellow color of the solution turned into a darker color resembling maple syrup. The reaction was stopped by adding 0.05 ml 1.0 M formic acid.

LC/MS analysis of conjugated alanine – The reaction solution with Marfey's reagent was diluted 10-fold into acetone. 2 µl of the diluted sample was injected into the LC system for inline LC/MS analysis using the SCIEX 4000 QTRAP system. An Agilent ZORBAX Eclipse Plus C18 (2.1 mm × 100 mm) reverse-phase column was used. The aqueous solution A contained 10 mM NH₄Ac at pH 4.6. The organic solution B is HPLC-grade acetone. The flow rate was set at 0.2 ml/minute. The gradient from 20% B to 80% B was developed in 10 minutes, followed by 2 extra minutes of column regeneration at 80% B and 8 minutes of equilibration at 20% B.

The molecular anion of 340 amu corresponding to the alanyl-derivative of Marfey's reagent was monitored by the mass spectrometer. The mixed alanine standard was a 1:1 mix of the diluted reaction solutions of D- and L-alanine.

Results

Dataset 1. MS scans in search for aminoacylated phospholipids and tandem mass spectra of aminoacylated phosphatidylglycerol and aminoacylated phosphatidylethanolamine

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The data sets are named as Figure 3B and such according to their appearance in the figures of the article.

Figure 2A: MS/MS spectrum of (30:0) lysyl-phosphatidylglycerol (lysyl-PG) anion (821 amu)

Figure 2B: MS/MS spectrum of (32:0) alanyl-PG anion (792 amu)

Figure 3A: Precursor scan for 145 amu lysyl anion

Figure 3B: Precursor scan for 88 amu alanyl anion

Figure 3C: Precursor scan for 130 amu leucyl/isoleucyl anion

Figure 3D: Precursor scan for 132 amu aspartyl anion

Figure 4A: Precursor scan for predominant 523 amu [DAG-OH]⁺ ion

Figure 4B: Scan for neutral loss of 269 amu head group of lysyl-phosphatidylethanolamine (lysyl-PE)

Figure 4C: Precursor scan for sodiated lysyl-PE head group of 292 amu

Figure 4D: Precursor scan for sodiated alanyl-PE head group of 235 amu

Figure 5: MS/MS spectra of chemically synthesized (16:0–18:1) lysyl- and alanyl-PE

Figure 5A: MS/MS spectrum protonated (16:0–18:1) lysyl-PE (846 amu)

Figure 5B: MS/MS spectrum of sodiated (16:0–18:1) lysyl-PE (868 amu)

Figure 5C: MS/MS spectrum of protonated (16:0–18:1) alanyl-PE (789 amu)

Figure 5D: MS/MS spectrum of sodiated (16:0–18:1) alanyl-PE (811 amu)

Figure 6: High-accuracy MS/MS spectrum of (32:0) lysyl-PG (873 amu)

Figure 7: High-accuracy MS/MS spectrum of (32:0) alanyl-PG (816 amu)

Profiling of major bacterial lipids - We first modified the lipid extraction protocol using chloroform and methanol based on Folch method²⁹. Polar lipids from *E. coli* strain BL21(DE3) and *B. subtilis* strain 168 were extracted. Thin-layer chromatography was carried out to analyze the major components. Every primuline-stained major band on the TLC plate was collected using a razor and redissolved in 100 μ l chloroform. We then acquired MS spectra of the total lipids as well as the TLC-separated lipids and tandem MS/MS spectra of dominant molecular ions. The major component of each primuline-stained band on the TLC plate was assigned (Figure 1) based on the MS and MS/MS spectra. As expected, lipids extracted from *E. coli* strain BL21(DE3) were mainly composed of PE and PG (Figure 1)⁶. As expected, lipids from *B. subtilis* strain 168 were also rich in lysyl-PG and cardiolipin (CL) (Figure 1). The relative abundance of PE was much lower in *B. subtilis* than *E. coli*.

Figure 1. Thin-layer chromatogram of total lipids extracted from *E. coli* strain BL21(DE3) and *B. subtilis* strain 168. Major components of the bands are shown. **Bright panels:** Primuline-stained TLC sheet. **Dark panels:** Ninhydrin-stained TLC sheet. The fluorescent primuline (bright spots) is absorbed to hydrophobic molecules. Ninhydrin reacts with amino groups to produce purple products (dark spots). Lysyl-phospholipids form a distinct band with slow mobility. Alanyl-PG migrates slightly faster than PG, but significantly slower than PE. The Gram-negative *E. coli* has little CL.

The most abundant ninhydrin-stained amino group-containing band was that of lysyl-PG. A third ninhydrin-stained band, which was visible in only a small fraction of lipid preparations, overlapped with the PG-rich band. The amino group in this band likely comes from alanyl-PG as indicated by mass spectroscopy. The ninhydrin-stained spot at the origin likely corresponds to free amino acids.

Tandem MS/MS spectra of lysyl- and alanyl-PGs - The most abundant fragments of negative (deprotonated) molecular ions of phospholipids were fatty acyl anions ([FA-H]⁻) of various sizes. Deprotonated molecular ions of PG and CL also dissociated to form a cyclo-glycerol-phosphate anion (153 amu), which is widely used to search for precursors that have a phosphoglycerol backbone³². Lipids from the *E. coli* strain were richest in saturated pentadecanoic acid (15:0) and hexadecanoic acid (16:0). The *B. subtilis* lipids, on the other hand, were most abundant in pentadecanoic acid (15:0) and heptadecanoic acid (17:0). The dominance of odd numbers of carbon atoms in the fatty acyl chain indicates that both bacteria likely utilize leucine or isoleucine primers for branch-chain fatty acid biosynthesis³³. The loss of a neutral head group from positive (protonated or sodiated) molecular ions of phospholipids produced dehydroxyl-diacylglycerol cations ([DAG-OH]⁺) as the

most abundant fragments. This neutral loss feature is commonly used to search for phospholipids with certain head groups³⁴. For instance, PE can be identified by a scan for the neutral loss of phosphoethanolamine (141 amu). We acquired tandem MS/MS spectra of lysyl- and alanyl-PGs in both positive and negative mode. The spectra in negative mode with a collision energy of -65 electronvolts were dominated by [FA-H]⁻ and deprotonated aminoacyl ions: [Ala-H]⁻ (88 amu) and [Lys-H]⁻ (145 amu) (Figure 2).

Precursor scans for aminoacyl-PGs – Besides lysyl- and alanyl-PGs, there were no molecular ions in the MS spectra which matched expected m/z values for other types of aminoacyl-PG ions. The abundance of the deprotonated aminoacyl ions prompted us to utilize this structural feature to search with high sensitivity for lipid precursors which produce such fragment ions. We first tested this protocol on lysyl- and alanyl-PGs and similarly esterized CL. The dominant molecular anions from the precursor scans at a collision energy of -65 electronvolts matched the expected m/z values of lysyl-PG and alanyl-PG species with two (15:0) or (17:0) fatty acyl chains (Figure 3A & 3B). The two types of lipid species with less abundant fatty-acyl compositions were also identified as peaks separated by 14 amu that corresponds to a methylene group. No aminoacyl-CL was identified in higher mass

range around 1500 amu. We then applied such precursor scans to search for other aminoacylated PGs or CLs. With the exception of cysteine, the scans identified correct sized candidates of molecular anions of 17 aminoacyl-PGs with two clearest examples of leucyl-PG and aspartyl-PG shown in Figure 3C & 3D. The scans were not able to differentiate between glutamine and lysine, or between leucine and isoleucine, which share similar or identical molecular mass.

Neutral loss and precursor scans for aminoacyl-PEs – There were no major peaks which corresponded to molecular ions of aminoacyl-PEs. We first employed a scan at an optimized collision energy of +45 electronvolts that searches for precursors of the most abundant [(30:0) DAG-OH]⁺ fragment ion (523 amu). The major hits corresponded to protonated as well as sodiated PE, PG, lysyl-PG, alanyl-PG as well as several other less abundant species including one at 792 amu which corresponded to the expected size of a protonated lysyl-PE species (Figure 4A). No other ions matched expected m/z values of aminoacyl-PEs. We then employed scans for molecular cations, also at +45 electronvolts, which produced fragments that resulted from the neutral loss of head groups (269 amu for lysyl-phosphoethanolamine, 212 amu for alanyl-phosphoethanolamine). The scan for the neutral loss of 269 amu found strong candidates

Figure 2. Tandem mass spectra of deprotonated lysyl-PG and alanyl-PG. Major peaks in the MS/MS spectra are labeled. FA – fatty acid, LPG – (30:0) lysyl-PG, APG – (32:0) alanyl-PG, cGP – cyclo-glycerol-phosphate. **A.** MS/MS spectrum of lysyl-PG. **B.** MS/MS spectrum of alanyl-PG.

Figure 3. Precursor scans for aminoacyl-PGs. Major peaks in the spectra are labeled with fatty acid composition (number of carbon atoms: number of desaturation). **A.** Scan for precursors of a 145 amu anion. **B.** Scan for precursors of a 88 amu anion. **C.** Scan for precursors of a 130 amu anion. **D.** Scan for precursors of a 132 amu anion.

Figure 4. Neutral loss and precursor scans for aminoacyl-PEs. Major peaks in the MS/MS spectra are labeled. LPG – lysyl-PG, APG – alanyl-PG, LPE – lysyl-PE, APE – alanyl-PE. **A.** Scan for precursors of a 523.3 amu DAG fragment. **B.** Scan for a neutral loss of a 269.1 amu fragment. **C.** Scan for precursors of a 292.1 amu fragment. **D.** Scan for precursors of a 235.1 amu fragment.

for lysyl-PEs (Figure 4B). It is worth noting that their putative fatty acyl compositions (30:0, 31:0, 32:0) matched those of the dominant molecular ions of PE, PG, lysyl-PG and alanyl-PG. We then synthesized (16:0–18:1) lysyl-PE and alanyl-PE. The tandem MS/MS spectra of chemically synthesized lysyl-PE and alanyl-PE were also acquired (Figure 5). In addition to the major fragment [DAG-OH]⁺ ion, the sodiated molecular cations also dissociate to

produce intense fragment peaks which corresponded to the sizes of the sodiated head groups (292 amu and 235 amu, respectively). Additional scans at a collision energy of +45 electronvolts for precursors which generate such sodiated head group ions revealed hits which were in agreement with the neutral loss scan for lysyl-PE (Figure 4B & 4C), and implied the existence of alanyl-PE (757, 771 and 785 amu ions in Figure 4D).

Figure 5. MS/MS spectra of chemically synthesized lysyl-PE and alanyl-PE. Protonated and sodiated ions are shown. LPE – (16:0–18:1) lysyl-PE, APE – (16:0–18:1) alanyl-PE, PA – phosphatidyl acid, DAG – diacylglycerol, Head – head group, cAE – cyclo-alanyl-ethanolamine, cK – cyclo-lysine, cKE – cyclo-lysyl-ethanolamine. **A & B.** Lysyl-PE. **C & D.** Alanyl-PE.

Irreproducibility of aminoacyl-PEs – Although the presence of aminoacyl-PEs was interesting at first, we no longer found their presence when several new batches of the bacterial polar lipids were extracted without the final drying step at 30°C. It appears that the drying process may have caused existing species PE and aminoacyl-PGs in the lipids to chemically react to produce aminoacyl-PEs. This should be a caution which needs attention while sensitive profiling by mass spectrometry is employed.

Tandem mass spectra of L-lysyl-PG – The 4000 QTRAP system for liquid profiling has a practical resolution of 0.7 amu and accuracy of 0.2 amu. The Q-TOF 6550 system, optimized for proteomic research in positive mode, was tuned to reach a much higher accuracy of ~2 ppm or within 0.001 amu. We acquired high-accuracy MS/MS spectra of putative molecular ions of lysyl- and alanyl-PGs to further verify our assignments and to obtain clues for devising sensitive scans for lipid profiling. There were only two aminoacyl-PGs, lysyl-PG and alanyl-PG, that produced abundant enough (over 1000 counts) molecular ions for tandem mass spectrometry study. In fact, lysyl-PG was expectedly one of the most abundant phospholipids in the bacterium. It is certainly L-lysyl-PG as its lysyl group is known to have originated from L-lysyl-tRNA. In negative mode with collision energy set at -50 electronvolts, lysyl-PG ions of 821 and 849 amu produced an abundance of deprotonated lysyl ions besides two major – (15:0) and (17:0) - fatty acyl anions at 241 and 269 amu. The observed mass of [Lys-H]⁻ was 145.0972, matching the calculated monoisotopic mass of 145.0978. A deprotonated glutamine ion would have a distinctively different mass of

145.0613. We also acquired MS/MS spectrum of sodiated (32:0) lysyl-PG cation (873 amu) at a collision energy of +40 electronvolts (Figure 6). Although protonated lysyl-PG ions were also abundant (823 amu and 851 amu), they produced less prominent fragments than the sodiated ions. Since a sodiated lysyl-PG but not a protonated lysyl-PG has a potent neutral amino group for intramolecular nucleophilic substitution, it is not surprising that we observed plenty of prominent structural features (Table 1) from the sodiated lysyl-PG ion (873 amu). For instance, it produced sodiated cyclo-lysine (151.0841 amu vs a calculated mass of 151.0848 amu) to verify the presence of lysyl residue along with the deprotonated lysyl ion in negative mode. It produced cyclo-lysyl-glycerol in both protonated (185.1275 vs 185.1291) and sodiated form (225.1205 vs 225.1216) to further indicate that the lysyl residue is attached to the glycerol head group. The fragments of sodiated cyclo-lysyl-glycerolphosphate (305.0868 vs 305.0879) and lysyl-glycerolphosphate (323.0972 vs 323.0985) finally completed the head group assignment. The outstanding abundance of the 323 amu fragment made a precursor scan for this fragment in positive mode the second best behind the precursor scan for deprotonated lysyl ion (145 amu) in negative mode. Both protonated and sodiated dehydrated DAG fragments were abundant (551 and 573 amu, respectively). So was sodiated DAG (591 amu). The neutral loss of the cyclo-lysyl-glycerol head group (202 amu) produced sodiated phosphatidic acid (671 amu) and that of cyclo-lysine (128 amu) produced sodiated PG (745 amu). It is worth noting that neutral losses of fatty acid (RCOOH) or ketene (R-C=O) produced minor peaks less than 100 counts, which are not shown in Figure 6.

Figure 6. Tandem MS spectrum of (32:0) lysyl-PG. MS/MS spectrum of sodiated lysyl-PG (873 amu) ions is shown. The molecular structure is shown on the top with scissile bonds labeled alphabetically. The horizontal axis represents m/z values. The vertical axis represents ion counts.

Table 1. Accurate masses of fragments from lysyl-PG.

Observed mass	Calculated Msss	Cleavage	Description
120.9655	120.9667	c & f	Pho + Na ⁺
151.0841	151.0848	h	cyclo-Lys + Na ⁺
176.9913	176.9929	c & g	cyclo-Gro-Pho + Na ⁺
185.1275	185.1291	f & i	cyclol-Lys-Gro - OH
195.0022	195.0035	c & h	Gro-Pho + Na ⁺
225.1205	225.1216	f	cyclo-Lys-Gro + Na ⁺
305.0868	305.0879	d	cyclo-Lys-Gro-Pho + Na ⁺
323.0972	323.0985	c	Lys-Gro-Pho + Na ⁺
551.5018	551.5040	c	DAG - OH
573.4839	573.4862	c	DAG -H ₂ O + Na ⁺
591.4942	591.4968	d	DAG + Na ⁺
671.4604	671.4631	f	PA + Na ⁺
745.4967	745.4999	h	PG + Na ⁺

Note: The alphabetically labeled scissile bonds are shown in Figure 6 and Figure 7. PA – phosphatidic acid; PG – phosphatidylglycerol; DAG – diacylglycerol; Pho – phosphate; Lys – lysine; Gro – glycerol. A cyclic compound in mass is equivalent to a dehydrated compound.

Tandem mass spectra of alanyl-PG – Since alanyl-PG appeared to be much less abundant than lysyl-PG, we first chose the lipid preparation with the highest abundance of alanyl-PG as revealed by TLC analysis (Figure 1) for tandem mass spectra acquisition. The most abundant putative alanyl-PG anions were observed at 764 and 792 amu, corresponding to (30:0) and (32:0) alanyl-PG, respectively. Besides the fatty acyl anions, they produced an abundance of alanyl anion at 88.0396 amu, closely matching expected value of 88.0399. Fragmentation at putative protonated alanyl-PG ions (766 and 794 amu) did not produce meaningful results. We conclude that they are not abundant enough for tandem MS analysis. The sodiated alanyl-PG cation (816 amu), corresponding to (32:0) alanyl-PG, was abundant enough to produce a simpler MS/MS spectrum (Figure 7 and Table 2) than that of its lysyl-PG counter-part (Figure 6 and Table 1). The presence of the 168 amu ion was critical for the assignment of the alanyl-glycerol attachment, as it corresponds to sodiated cyclo-alanyl-glycerol (168.0623 vs 168.0640). The presence of the whole alanyl-glycerolphosphate head group was verified by the presence of sodiated cyclo-alanyl-glycerolphosphate (248.0287 vs 248.0300) and sodiated alanyl-glycerolphosphate (266.0395 vs 266.0410). In fact, the outstanding abundance of the 266 amu cation makes a precursor scan for this fragment the second most sensitive lipid profiling scan for alanyl-PG behind the scan for deprotonated alanine (88 amu). As for lysyl-PG, the DAG residues were also abundant (551 and 573 amu).

Figure 7. Tandem MS spectrum of (32:0) alanyl-PG. MS/MS spectrum of sodiated alanyl-PG (816 amu) ions is shown. The molecular structure is shown on the top with scissile bonds labeled alphabetically. The horizontal axis represents m/z values. The vertical axis represents ion counts.

Table 2. Accurate masses of fragments from alanyl-PG.

Observed mass	Calculated mass	Cleavage	Description
168.0623	168.0640	f	cyclo-Ala-Gro + Na ⁺
248.0287	248.0300	d	cyclo-Ala-Gro-Pho+ Na ⁺
266.0395	266.0410	c	Ala-Gro-Pho + Na ⁺
551.5018	551.5040	c	DAG - OH
573.4839	573.4860	c	DAG - H ₂ O + Na ⁺

Note: The alphabetically labeled scissile bonds are shown in [Figure 6](#) and [Figure 7](#). The abbreviations are the same as in [Table 1](#). Ala - alanine.

LC/MS analysis of D- and L-alanine in lipid and whole cell lysates – Alanyl groups in the bacterial lipids as well as on bacterial cell surface are known to be labile under mild alkaline conditions^{35,36}. We established a set of alkaline hydrolysis and alanine extraction protocols that were practically complete at both hydrolysis and extraction stages. Since ammonia and formic acid residues were removed by evaporation, these reagents did not pose any interference with later experimental steps of TLC, conjugation with Marfey's reagent, and mass spectrometry. By Marfey's design, the derivatives of D-amino acids tend to have longer retention times on a reverse-phase column than their respective L-amino acid derivatives. The D-alanyl-derivative of Marfey's reagent eluted significantly later and as a higher and sharper peak at 9.16 minutes than that of the L-alanyl-derivative which eluted at 7.55 minutes ([Figure 8](#)).

A. L- & D-alanine standards

B. Cell lysate

C. Lipid lysate

Figure 8. LC/MS chromatograms of alanine in cell and lipid lysates. The 340 amu molecular anion was monitored. The horizontal axis corresponds to retention time (minute). Peak retention times are marked. The peaks at 8.05 minutes, which correspond to a background 339 amu anion, is marked with "339".

The alanine released by alkaline hydrolysis of bacterial cells was predominantly D-alanine (~90%), while that of lipids was exclusively D-alanine. By comparing the ion counts with the standard D- and L-alanine solution (equivalent to 1 mM each), the 0.1 ml lysate from 1.5 ml of bacterial cells contained ~0.05 mM D-alanine, while the 0.1 ml lipid lysate contained ~0.05 mM D-alanine. Considering that the lipid lysate was derived from ~100-fold more bacterial cells than the whole cell sample, there appeared to be 1000-fold more D-alanine on the cell surface than that in the membrane. We typically obtained 5–10 mg dried lipids from 0.9 g wet cell pellet, which corresponds to a weight ratio of approximately 100. By taking into account this weight ratio, we estimate that whole cell still has ~10-fold denser D-alanine than membrane. Importantly, the alanylated phosphatidylglycerol is D-alanyl-PG, it is therefore not synthesized from tRNA-carried L-alanyl by a reaction catalyzed by MprF. Instead, another well-known source of activated alanine carried by D-alanine carrier protein DltC in the form of thioester^{22,37} may be the most likely origin.

Discussion

Aminoacylated lipids play an important role in regulating the surface charge of Gram-positive bacteria⁵. It appears that mass spectrometry can be exploited to successfully search for trace amounts of aminoacylated phospholipids. Mass spectrometry also makes identification of known as well as unknown lipids possible even without separation or chemical synthesis. The positive results on a broad range of aminoacylated-PGs are consistent with previous work on lipid hydrolysate⁷. The intensities of various aminoacyl anions dissociated from the bacterial lipids span at least 3 orders of magnitude with lysyl anion being the strongest (6×10^6) followed by alanyl (9×10^5), leucyl/isoleucyl (4×10^4) and aspartyl (7×10^3) (Figure 3). It is worth noting that the latter two molecular ions did not show appreciable peaks in the MS spectrum. The aminoacyl-PG synthase MprF is known to have a broad range of aminoacyl-tRNA specificity¹⁰. Our results may have provided a semi-quantitative measure of the specificity of *B. subtilis* MprF.

Since PE is a major component of bacterial lipids, we also searched for aminoacylated derivatives of PE. The amide-linked PE derivative cannot be identified by their PG counterparts' dissociation into deprotonated aminoacyl ions. We therefore employed the neutral loss (NL) scanning methodology based on those commonly used for identifying phosphatidylethanolamine (NL of 141 amu head group), phosphatidylserine (NL of 185 amu head group), phosphatidylacid (NL of 115 amu ammoniated head group) and phosphatidylinositol (NL of 277 amu ammoniated head group)³⁴. We also searched for precursors of the most abundant dehydroxyl-diacylglycerol cation (523 amu) which has the dominant fatty acyl composition of (30:0). The resulting spectrum provided a representative survey of all major species of phospholipids (Figure 4A). MS/MS spectra of chemically synthesized lysyl-PE and alanyl-PE revealed intense peaks corresponding to sodiated head groups, which led to high-sensitivity precursor scans. The sodium ion appeared to have played an important role in generating intense peaks of head group fragments as well as contributing to high yield in lipid extraction due to its inert chemical property in comparison to commonly used ammonium salt. Other metal ions such as cesium which, like sodium, has only one stable isotope, can be further exploited for enhanced sensitivity in lipid

profiling³⁸. Although the presence of lysyl- and alanyl-PEs appeared to be accidentally introduced in the lipid drying process, we did have established a sensitive enough lipid profiling method to rule out their biological relevance in *B. subtilis*.

Importantly, the identification of D-alanyl-PG rather than L-alanyl-PG apparently rules out the relevance of the aminoacyl-tRNA-dependent MprF in its biosynthesis. Instead, the *dlt* operon, which codes four proteins named sequentially as DltA-D, comes into focus. The cytosolic DltC protein serves as the alanyl carrier protein with a serine-attached 4'-phosphopantetheine as the site for alanyl-thioester formation in the presence of ATP and catalyzed by DltA. Biological functions of the two membrane-bound proteins DltB and DltD have yet to be fully characterized. Mysteriously, the targets of DltC-carried alanyl group are lipoteichoic acid located at the outer leaflet of cytoplasmic membrane, and wall-teichoic acid covalently attached to peptidoglycan. We have long suspected the presence of a D-alanylated lipid as an intermediate for the eventual transfer of D-alanine from the cytosol to lipoteichoic acid. D-alanyl-PG may just be this putative intermediate D-alanyl carrier. Figure 9 illustrates a list of possible pathways for the transfer of D-alanine to lipo- and wall-teichoic acids. First, the D-alanylated lipid may be produced by DltD, and transported to the outer leaflet by a flippase such as the integral membrane protein DltB or the pore domain of MprF which is known to transport L-lysyl-PG and other L-aminoacyl-PGs⁹. Second, D-alanyl-PG can be transferred to lipo- and wall-teichoic acids by a transferase such as DltB, a putative membrane-bound O-acyltransferase³⁹, or incorporated as D-alanyl-glycerolphosphate units from D-alanyl-PG into the growing ends of teichoic acids by their respective polymerases LtaS^{40,41} and TagF^{42,43}. It is worth noting that D-alanyl-CL has been reported before²⁷. As alanyl-PG and alanyl-CL share an ester bond with the glycerol head group, both are candidates for the lipid intermediate for D-alanylation of teichoic acids. Our hypothesis is also based on the best biochemical evidence, or the lack thereof, on DltB and DltD. DltD was previously observed to bind specifically to DltC and possess thioesterase activity on D-alanyl-acyl carrier protein²³. If we substitute the water nucleophile in the thioesterase-catalyzed reaction for hydroxyl in the head group of PG, DltD would become a D-alanyl transferase. In addition, our bioinformatics analysis of crystal structure of *Streptococcus pneumoniae* DltD (PDB entry 3BMA, deposited by New York SGX Research Center for Structural Genomics) using ProFunc⁴⁴ revealed a Ser-His-Asp triad embed in a structure (Figure 10) with overall similarity to platelet-activating factor, which belongs to the phospholipase A2 category. Apparently, the putative catalytic triad is conserved in all known DltD orthologs in Gram-positive bacteria. Since many phospholipid synthases belong to a superfamily of phospholipase D¹, a synthase in the superfamily of phospholipase A2 would not be surprising. We therefore hypothesize that DltD may serve as the synthase of D-alanyl-PG, which may serve as the key lipid D-alanyl carrier for the D-alanylation pathway of teichoic acids.

Data availability

F1000Research: Dataset 1. MS scans in search for aminoacylated phospholipids and tandem mass spectra of aminoacylated phosphatidylglycerol and aminoacylated phosphatidylethanolamine, [10.5256/f1000research.7842.d112021](https://doi.org/10.5256/f1000research.7842.d112021)⁴⁵

Figure 9. Possible D-alanylation pathways of lipo- and wall-teichoic acids. Arrows depict either transport or transfer processes. The dihexosyl parts of teichoic acids are shown as twin hexagons. The head group of phosphatidylglycerol and repeating glycerolphosphate units in teichoic acids are shown in grey circle and ellipse, respectively.

Figure 10. Structure of DltD from *Streptococcus pneumoniae* in stereo. A, Ribbons diagram of DltD. The side chains in the Ser-His-Asp triad are shown in ball-and-stick model. B, Space-filling model of DltD. The most conserved residues are shown in red, and the least conserved in green. The putative catalytic triad is shown in blue.

Author contributions

YL conceived the study, carried out the bioinformatics analysis of DltD, optimized alkaline hydrolysis of lipids and bacterial cell, characterized the D-enantiomer of alanine by LC/MS, interpreted the QTOF MS/MS spectra, and wrote the manuscript. MA carried out cell culture, chemical synthesis, lipid extraction and lipid profiling, and drew Figure 9.

Competing interests

No competing interests were disclosed.

Grant information

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I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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References

- Sohlenkamp C, Geiger O: **Bacterial membrane lipids: diversity in structures and pathways.** *FEMS Microbiol Rev.* 2016; **40**(1): 133–59.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Weidenmaier C, Kristian SA, Peschel A: **Bacterial resistance to antimicrobial host defenses—an emerging target for novel anti-infective strategies?** *Curr Drug Targets.* 2003; **4**(8): 643–9.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Peschel A, Otto M, Jack RW, *et al.*: **Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides.** *J Biol Chem.* 1999; **274**(13): 8405–10.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Kristian SA, Lauth X, Nizet V, *et al.*: **Alanylation of teichoic acids protects *Staphylococcus aureus* against Toll-like receptor 2-dependent host defense in a mouse tissue cage infection model.** *J Infect Dis.* 2003; **188**(3): 414–23.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Roy H: **Tuning the properties of the bacterial membrane with aminoacylated phosphatidylglycerol.** *JUBMB Life.* 2009; **61**(10): 940–53.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Epand RF, Savage PB, Epand RM: **Bacterial lipid composition and the antimicrobial efficacy of cationic steroid compounds (Ceragenins).** *Biochim Biophys Acta.* 2007; **1768**(10): 2500–9.
[PubMed Abstract](#) | [Publisher Full Text](#)
- den Kamp JA, Redai I, van Deenen LL: **Phospholipid composition of *Bacillus subtilis*.** *J Bacteriol.* 1969; **99**(1): 298–303.
[PubMed Abstract](#) | [Free Full Text](#)
- Peschel A, Jack RW, Otto M, *et al.*: ***Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine.** *J Exp Med.* 2001; **193**(9): 1067–76.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Slavetinsky CJ, Peschel A, Ernst CM: **Alanyl-phosphatidylglycerol and lysyl-phosphatidylglycerol are translocated by the same MprF flippases and have similar capacities to protect against the antibiotic daptomycin in *Staphylococcus aureus*.** *Antimicrob Agents Chemother.* 2012; **56**(7): 3492–7.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Roy H, Ibba M: **Broad range amino acid specificity of RNA-dependent lipid remodeling by multiple peptide resistance factors.** *J Biol Chem.* 2009; **284**(43): 29677–83.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Hebecker S, Krausz J, Hasenkampf T, *et al.*: **Structures of two bacterial resistance factors mediating tRNA-dependent aminoacylation of phosphatidylglycerol with lysine or alanine.** *Proc Natl Acad Sci U S A.* 2015; **112**(34): 10691–6.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Biarrotte-Sorin S, Maillard AP, Delettré J, *et al.*: **Crystal structures of *Weissella viridescens* FemX and its complex with UDP-MurNAc-pentapeptide: insights into FemABX family substrates recognition.** *Structure.* 2004; **12**(2): 257–67.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Armstrong JJ, Baddiley J, Buchanan JG, *et al.*: **Composition of teichoic acids from a number of bacterial walls.** *Nature.* 1959; **184**: 247–8.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Neuhaus FC, Baddiley J: **A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria.** *Microbiol Mol Biol Rev.* 2003; **67**(4): 686–723.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Fischer W: **Physiology of lipoteichoic acids in bacteria.** *Adv Microb Physiol.* 1988; **29**: 233–302.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Percy MG, Gründling A: **Lipoteichoic acid synthesis and function in gram-positive bacteria.** *Annu Rev Microbiol.* 2014; **68**: 81–100.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Hyrylainen HL, Vitikainen M, Thwaite J, *et al.*: **D-Alanine substitution of teichoic acids as a modulator of protein folding and stability at the cytoplasmic membrane/cell wall interface of *Bacillus subtilis*.** *J Biol Chem.* 2000; **275**(35): 26696–703.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Perego M, Glaser P, Minutello A, *et al.*: **Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis*. Identification of genes and regulation.** *J Biol Chem.* 1995; **270**(26): 15598–606.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Stachelhaus T, Mootz HD, Marahiel MA: **The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases.** *Chem Biol.* 1999; **6**(8): 493–505.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Conti E, Franks NP, Brick P: **Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes.** *Structure.* 1996; **4**(3): 287–98.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Kiriukhin MY, Neuhaus FC: **D-alanylation of lipoteichoic acid: role of the D-alanyl carrier protein in acylation.** *J Bacteriol.* 2001; **183**(6): 2051–8.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Zimmermann S, Pfennig S, Neumann P, *et al.*: **High-resolution structures of the D-alanyl carrier protein (Dcp) DltC from *Bacillus subtilis* reveal equivalent conformations of apo- and holo-forms.** *FEBS Lett.* 2015; **589**(18): 2283–9.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Debabov DV, Kiriukhin MY, Neuhaus FC: **Biosynthesis of lipoteichoic acid in *Lactobacillus rhamnosus*: role of DltD in D-alanylation.** *J Bacteriol.* 2000; **182**(10): 2855–64.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Gidden J, Denson J, Liyanage R, *et al.*: **Lipid Compositions in *Escherichia coli* and *Bacillus subtilis* During Growth as Determined by MALDI-TOF and TOF/TOF Mass Spectrometry.** *Int J Mass Spectrom.* 2009; **283**(1–3): 178–184.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Klein S, Lorenzo C, Hoffmann S, *et al.*: **Adaptation of *Pseudomonas aeruginosa* to various conditions includes tRNA-dependent formation of alanyl-phosphatidylglycerol.** *Mol Microbiol.* 2009; **71**(3): 551–65.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Peter-Katalinic J, Fischer W: **alpha-d-glucopyranosyl-, d-alanyl- and L-lysylcardiolipin from gram-positive bacteria: analysis by fast atom bombardment mass spectrometry.** *J Lipid Res.* 1998; **39**(11): 2286–92.
[PubMed Abstract](#)
- Fischer W, Arneth-Seifert D: **D-Alanylcardiolipin, a major component of the unique lipid pattern of *Vagococcus fluvialis*.** *J Bacteriol.* 1998; **180**(11): 2950–7.
[PubMed Abstract](#) | [Free Full Text](#)

28. Geiger O, González-Silva N, López-Lara IM, *et al.*: **Amino acid-containing membrane lipids in bacteria.** *Prog Lipid Res.* 2010; **49**(1): 46–60.
[PubMed Abstract](#) | [Publisher Full Text](#)
29. Folch J, Lees M, Sloane Stanley GH: **A simple method for the isolation and purification of total lipides from animal tissues.** *J Biol Chem.* 1957; **226**(1): 497–509.
[PubMed Abstract](#)
30. Tanaka S, Fujita Y, Parry HE, *et al.*: **Mass++: A Visualization and Analysis Tool for Mass Spectrometry.** *J Proteome Res.* 2014; **13**(8): 3846–3853.
[PubMed Abstract](#) | [Publisher Full Text](#)
31. Marfey P: **Determination of D-Amino Acids. 2. Use of a Bifunctional Reagent, 1,5-Difluoro-2,4-Dinitrobenzene.** *Carlsberg Res Commun.* 1984; **49**(6): 591–596.
[Publisher Full Text](#)
32. Welti R, Wang X, Williams TD: **Electrospray ionization tandem mass spectrometry scan modes for plant chloroplast lipids.** *Anal Biochem.* 2003; **314**(1): 149–52.
[PubMed Abstract](#) | [Publisher Full Text](#)
33. Kaneda T: **Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance.** *Microbiol Rev.* 1991; **55**(2): 288–302.
[PubMed Abstract](#) | [Free Full Text](#)
34. Welti R, Li W, Li M, *et al.*: **Profiling membrane lipids in plant stress responses. Role of phospholipase D alpha in freezing-induced lipid changes in Arabidopsis.** *J Biol Chem.* 2002; **277**(35): 31994–2002.
[PubMed Abstract](#) | [Publisher Full Text](#)
35. Archibald AR, Baddiley J, Heptinstall S: **The alanine ester content and magnesium binding capacity of walls of Staphylococcus aureus H grown at different pH values.** *Biochim Biophys Acta.* 1973; **291**(3): 629–34.
[PubMed Abstract](#) | [Publisher Full Text](#)
36. Childs WC 3rd, Neuhaus FC: **Biosynthesis of D-alanyl-lipoteichoic acid: characterization of ester-linked D-alanine in the in vitro-synthesized product.** *J Bacteriol.* 1980; **143**(1): 293–301.
[PubMed Abstract](#) | [Free Full Text](#)
37. Debabov DV, Heaton MP, Zhang Q, *et al.*: **The D-Alanyl carrier protein in Lactobacillus casei: cloning, sequencing, and expression of dltC.** *J Bacteriol.* 1996; **178**(13): 3869–76.
[PubMed Abstract](#) | [Free Full Text](#)
38. Griffiths RL, Bunch J: **A survey of useful salt additives in matrix-assisted laser desorption/ionization mass spectrometry and tandem mass spectrometry of lipids: introducing nitrates for improved analysis.** *Rapid Commun Mass Spectrom.* 2012; **26**(13): 1557–66.
[PubMed Abstract](#) | [Publisher Full Text](#)
39. Hofmann K: **A superfamily of membrane-bound O-acyltransferases with implications for wnt signaling.** *Trends Biochem Sci.* 2000; **25**(3): 111–2.
[PubMed Abstract](#) | [Publisher Full Text](#)
40. Grundling A, Schneewind O: **Synthesis of glycerol phosphate lipoteichoic acid in Staphylococcus aureus.** *Proc Natl Acad Sci U S A.* 2007; **104**(20): 8478–83.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
41. Lu D, Wörmann ME, Zhang X, *et al.*: **Structure-based mechanism of lipoteichoic acid synthesis by Staphylococcus aureus LtaS.** *Proc Natl Acad Sci U S A.* 2009; **106**(5): 1584–9.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
42. Fitzgerald SN, Foster TJ: **Molecular analysis of the tagF gene, encoding CDP-Glycerol:Poly(glycerophosphate) glycerophosphotransferase of Staphylococcus epidermidis ATCC 14990.** *J Bacteriol.* 2000; **182**(4): 1046–52.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
43. Lovering AL, Lin LY, Sewell EW, *et al.*: **Structure of the bacterial teichoic acid polymerase TagF provides insights into membrane association and catalysis.** *Nat Struct Mol Biol.* 2010; **17**(5): 582–9.
[PubMed Abstract](#) | [Publisher Full Text](#)
44. Laskowski RA, Watson JD, Thornton JM: **ProFunc: a server for predicting protein function from 3D structure.** *Nucleic Acids Res.* 2005; **33**(Web Server issue): W89–93.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
45. Luo Y, Atila M: **Dataset 1 in: Profiling and tandem mass spectrometry analysis of aminoacylated phospholipids in Bacillus subtilis.** *F1000Research.* 2016.
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The manuscript by Atila and Luo presents a helpful, descriptive mass spectrometric analysis of the aminoacylated phospholipids in *Bacillus subtilis*. The authors acquired product ion scans of lysyl and alanyl phosphatidylglycerol, as well as precursor ion scans and neutral loss scans for different aminoacyl phosphatidylglycerols and aminoacyl phosphatidylethanolamine. They found 17 different aminoacyl phosphatidylglycerols but no aminoacyl phosphatidylethanolamine in the lipid extracts. Furthermore, D- and L- alanine were quantified after alkaline hydrolysis of the lipid extracts, and only the D enantiomer was found. The authors conclude that D-alanyl phosphatidylglycerol is present, which is a D-alanine carrier from the cytosol to lipoteichoic acid.

As already mentioned by the referee 1, the most interesting finding of this study is that alanyl phosphatidylglycerol is composed solely of the D enantiomer. However additional control measurements which support this finding are highly recommended. I support the suggestion of referee 1 to test if alanyl-PG is formed in MprF deficient mutants, other controls with chemical standards are possible as well.

In Figure 8, the ion count of D-alanine in the cell lysate seems much larger as in the lipid lysate; it is difficult to understand how this leads to the same concentration estimate (0.05 mM). In general the assumption that “the cell has ~10 fold denser d-alanine than membrane” appears speculative and is based on several assumptions (linear response of the instrument, quantitative hydrolysis and extraction). I suggest to remove this part or to make a more elaborate analysis. Have the authors analysed alanyl PGs in the chloroform rich phase after hydrolysis to check the efficiency of the hydrolysis? Furthermore the authors could measure the concentrations of free alanine by applying the same protocols without adding NH₄OH to check the efficiency of the hydrolysis step.

The authors reported an irreproducible measurement of aminoacyl PE, potentially caused by the drying process. How long did this 30C drying procedure take? The formation of aminoacyl PEs due

to chemical reactions of PE and aminoacyl PGs would indicate an instability of the aminoacyl PGs. Therefore, how reproducible are the measurements of the aminoacyl PGs (variation in ion count)? Are these also influenced by the drying process?

The authors used collision energies of +45 and -65eV with the QTRAP MS and -40 and +50 eV with the Q-ToF system. Potential caveats resulting from the differently chosen fragmentation settings should be discussed.

What is the m/z of the precursor ion for the tandem MS measurements of deprotonated lysyl and alanyl PG (Figure 2)? m/z 821.5 and 792.5?

We suggest to remove figure 10, as it provides no relevant information in the context of the findings reported in this study.

Competing Interests: No competing interests were disclosed.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response () 14 Mar 2016

Yu Luo, University of Saskatchewan, Saskatoon, Canada

YL: Thank you Dr. Rasler for your comments. We will incorporate part of our response to your comments in the revised version. For your comments on the methodologies, our response is listed below.

The manuscript by Atila and Luo presents a helpful, descriptive mass spectrometric analysis of the aminoacylated phospholipids in *Bacillus subtilis*. The authors acquired product ion scans of lysyl and alanyl phosphatidylglycerol, as well as precursor ion scans and neutral loss scans for different aminoacyl phosphatidylglycerols and aminoacyl phosphatidylethanolamine. They found 17 different aminoacyl phosphatidylglycerols but no aminoacyl phosphatidylethanolamine in the lipid extracts. Furthermore, D- and L- alanine were quantified after alkaline hydrolysis of the lipid extracts, and only the D enantiomer was found. The authors conclude that D-alanyl phosphatidylglycerol is present, which is a D-alanine carrier from the cytosol to lipoteichoic acid.

YL: We hypothesize that D-alanyl-PG may serve as the intermediate for D-alanylation of wall- and lipo-teichoic acids. We haven't proved this.

As already mentioned by referee 1, the most interesting finding of this study is that alanyl phosphatidylglycerol is composed solely of the D enantiomer. However additional control measurements which support this finding are highly recommended. I support the suggestion of referee 1 to test if alanyl-PG is formed in MprF deficient mutants, other controls with chemical standards are possible as well.

YL: We have acquired and studied the lipid composition of the mprF mutant strain. This mprF

mutant strain indeed produced alanyl-PG, which is consistent to our hypothesis.

In Figure 8, the ion count of D-alanine in the cell lysate seems much larger as in the lipid lysate; it is difficult to understand how this leads to the same concentration estimate (0.05 mM). In general the assumption that "the cell has ~10 fold denser d-alanine than membrane" appears speculative and is based on several assumptions (linear response of the instrument, quantitative hydrolysis and extraction). I suggest to remove this part or to make a more elaborate analysis.

YL: It was an error. Thanks for pointing this out. The correct value of the alanine concentration in the 0.1 ml lipid lysate should be ~0.005 mM. The peak width stayed the same, and the peak high was linear to the concentration of D-alanine derivative. The linear intrapolation was valid.

Have the authors analysed alanyl PGs in the chloroform rich phase after hydrolysis to check the efficiency of the hydrolysis? Furthermore the authors could measure the concentrations of free alanine by applying the same protocols without adding NH_4OH to check the efficiency of the hydrolysis step.

YL: We found that HCl and NaOH at less than 50 mM was compatible with TLC analysis without distorting the lanes. So was 1.0 M of volatile NH_4OH , formic acid and acetic acid. Completion of hydrolysis and partitioning of alanine in the aqueous phase were both traced by TLC with ninhydrin staining. The three acidic conditions were inefficient in releasing alanine. NaOH and NH_4OH were found to be efficient in releasing alanine. We further chose to use the low concentration of NaOH in lipid hydrolysis for its relative ease to acidify by formic acid, which facilitates free fatty acid partitioning in the chloroform-rich phase.

The authors reported an irreproducible measurement of aminoacyl PE, potentially caused by the drying process. How long did this 30C drying procedure take? The formation of aminoacyl PEs due to chemical reactions of PE and aminoacyl PGs would indicate an instability of the aminoacyl PGs. Therefore, how reproducible are the measurements of the aminoacyl PGs (variation in ion count)? Are these also influenced by the drying process?

YL: The drying process typically took a few hours. Unlike aminoacylated PEs, aminoacylated PGs were always detected in ~10 samples with a range of ion counts within 10 fold prior to manuscript preparation. The fluctuation could be partially addressed to constant optimization process which changes experimental parameters such as pH and buffer concentration. Now we have standardized cell culture protocol with 10 mM pH 7 sodium phosphate buffer in the LB media and standardized lipid extraction protocol, the most recent triplicates showed ion counts within 2 fold.

The authors used collision energies of +45 and -65eV with the QTRAP MS and -40 and +50 eV with the Q-ToF system. Potential caveats resulting from the differently chosen fragmentation settings should be discussed.

YL: The design of the two system are too different to address the implication of different energies on the fragmentation pattern. As far as we know, the tandem MS spectra were consistent within a 20 eV range with changes in ion counts but not in the overall fragmentation pattern. Tandem MS

spectra were acquired at multiple collision energies. Generally, higher collision energy enriches smaller fragments, while lower energy enriches larger fragments. Only the spectra with the most fragments detected with adequate abundance were analysed.

What is the m/z of the precursor ion for the tandem MS measurements of deprotonated lysyl and alanyl PG (Figure 2)? m/z 821.5 and 792.5?

YL: The [M-H]⁻ precursor ion of the alanyl-PG had an observed m/z value of 792.5405 amu. Its calculated mass is 792.5394 amu. The precursor lysyl-PG anion had an observed m/z value of 821.5672 amu versus the calculated value of 821.5660 amu. However, figure 2 shows the data acquired with the Sciex 400 QTRAP system which has a lower resolution than the Agilent Q-TOF 6550 system.

We suggest to remove figure 10, as it provides no relevant information in the context of the findings reported in this study.

YL: We felt it is somewhat useful to describe the bioinformatics analysis which indicated that DltD is likely a lipase-like enzyme.

Competing Interests: No competing interests were disclosed.

Reviewer Report 09 March 2016

<https://doi.org/10.5256/f1000research.8441.r12662>

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Zeeshan Ahmed

The Jackson Laboratory, Farmington, CT, USA

The manuscript "Profiling and tandem mass spectrometry analysis of aminoacylated phospholipids in *Bacillus subtilis*" reports the contribution of authors in profiling and tandem mass spectrometry analysis of aminoacylated phospholipids in *Bacillus subtilis*. In general manuscript is

- very well written,
- language is good,
- citations are up to date,
- writing is to the point,
- in scope of the journal,
- justified **Introduction**,
- very well described **Materials and methods**,
- very well presented and discussed **Results**,

- Quality of figure is good and legends are well described,
- Good points raised in **Discussion**,
- Data is provided.

I am personally satisfied with the paper and I would like to congratulate authors of good work. I wanted to mention some points but most of those have been already addressed by the other reviewer (Otto Geiger). I agree with Dr Geiger's publicly available comments, especially:

- Addressing "why *Escherichia coli* is used at all in this study as a reference strain?".
- Minor comments.

I would like to request authors to please address these before final submission.

Thanks.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response () 14 Mar 2016

Yu Luo, University of Saskatchewan, Saskatoon, Canada

YL: Thank you Dr. Ahmed for your comments.

I am personally satisfied with the paper and I would like to congratulate authors of good work. I wanted to mention some points but most of those have been already addressed by the other reviewer (Otto Geiger). I agree with Dr Geiger's publicly available comments, especially:

- Addressing "why *Escherichia coli* is used at all in this study as a reference strain?".

YL: This strain was used as the negative reference for its absence of aminoacylated lipids. Indeed its lipid composition was essentially identical to that reported for the K12 strain in reference 24. We will describe this in the revised version.

YL: As suggested by Dr. Geiger, we have now acquired and studied the mprF mutant strain of B. subtilis 168. It indeed produced alanyl-PG but not lysyl-PG. We will mention this result in the revised discussion section. Since we are in the process of acquiring and studying several other lipid synthase mutant strains, we will report this piece of experimental data in a future publication.

Competing Interests: No competing interests were disclosed.

Reviewer Report 23 February 2016

<https://doi.org/10.5256/f1000research.8441.r12605>

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Otto Geiger

Centre for Genomic Sciences, National Autonomous University of Mexico, Cuernavaca, Mexico

This manuscript by Atila and Luo reports on the profiling and tandem mass spectrometry analysis of aminoacylated phospholipids in *Bacillus subtilis*. Lipids from *Escherichia coli* and *B. subtilis* are analyzed by thin-layer chromatography and mass spectrometry, but no mass spectral data are shown for *E. coli* lipids or anything that supports the claim that *E. coli* lipids were rich in C15:0 fatty acid. Other mass spectral data presented suggest that phosphatidylglycerol (PG) can be substituted with most proteinogenic amino acids, however, lysyl- and alanyl-PG are certainly the most abundant. The authors report extensively (Figs. 4, 5) on lysyl- and alanyl-phosphatidylethanolamine (PE) but come to the conclusion that these structures were artifacts generated during the isolation procedure. The potentially most interesting finding is that alanyl-PG is almost exclusively substituted with the D-alanyl isomer. In general, the manuscript seems scientifically sound. The mainly mass spectral analysis data make the manuscript somehow descriptive and leave much room for speculation. Instead, one would like to see some complementary data that solidify some of the ideas presented here.

Major Comments:

It is not exactly clear why *Escherichia coli* is used at all in this study. – As a reference strain? If so, why then a strain that is used for expression of genes? What really surprises me is that the authors find that “Lipids from the *E. coli* strain were richest in saturated pentadecanoic acid (C15:0) and ...”. – Whatever previous references you consult, C15:0 is never mentioned as a major fatty acyl residue in *E. coli*. For example, see Mejía *et al.* (1999). If it is true that C15:0 is a major fatty acid in *E. coli*, this finding would certainly put in doubt some dogmas.

Membrane lipid biosynthesis and composition of *Bacillus subtilis* strain 168 has been studied by numerous groups to a considerable extent. One of the major results of the present paper is that alanyl-PG carries exclusively the D-alanyl form and the authors discussed extensively that D-alanyl-PG should be formed by a mprF-independent pathway. There are MprF-deficient mutants available from John Helman’s group (Salzberg and Helman, 2008) and if the authors are correct, one would expect that these mutants still can make D-alanyl-PG.

Fig. Legend 8: Mention here or somewhere that the “340 amu molecular anion” corresponds to alanine + Marley’s ?

I don’t understand the last part of the Discussion “with overall similarity to platelet-activating factor”. Platelet-activating factor is a relatively hydrophilic lipid; so which similarity can it have to a protein?

Minor Comments:

1. Page 2, second paragraph of Introduction: change “peptitoglycan” to “peptidoglycan”

2. Page 3, left column, third paragraph, and elsewhere: The symbol for “micro” used here is an “u” not the Greek symbol as it should be.
3. Table 1: change “Calculated Msss” to “Calculated mass”.

References

1. Mejía R, Gómez-Eichelmann MC, Fernández MS: Fatty acid profile of *Escherichia coli* during the heat-shock response. *Biochem Mol Biol Int.* 1999; **47** (5): 835-44 [PubMed Abstract](#)
2. Salzberg LI, Helmann JD: Phenotypic and transcriptomic characterization of *Bacillus subtilis* mutants with grossly altered membrane composition. *J Bacteriol.* 2008; **190** (23): 7797-807 [PubMed Abstract](#) | [Publisher Full Text](#)

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response () 02 Mar 2016

Yu Luo, University of Saskatchewan, Saskatoon, Canada

This manuscript by Atila and Luo reports on the profiling and tandem mass spectrometry analysis of aminoacylated phospholipids in *Bacillus subtilis*. Lipids from *Escherichia coli* and *B. subtilis* are analyzed by thin-layer chromatography and mass spectrometry, but no mass spectral data are shown for *E. coli* lipids or anything that supports the claim that *E. coli* lipids were rich in C15:0 fatty acid. Other mass spectral data presented suggest that phosphatidylglycerol (PG) can be substituted with most proteinogenic amino acids, however, lysyl- and alanyl-PG are certainly the most abundant. The authors report extensively (Figs. 4, 5) on lysyl- and alanyl-phosphatidylethanolamine (PE) but come to the conclusion that that these structures were artifacts generated during the isolation procedure. The potentially most interesting finding is that alanyl-PG is almost exclusively substituted with the D-alanyl isomer. In general, the manuscript seems scientifically sound. The mainly mass spectral analysis data make the manuscript somehow descriptive and leave much room for speculation. Instead, one would like to see some complementary data that solidify some of the ideas presented here.

YL: We appreciate Dr. Otto Geiger's comments and suggestions. Our response as listed below and clarification will be incorporated in the revised version. It was a fact, perhaps an unfortunate distraction, that we had spent a lot of time on aminoacylated PE.

Major Comments:

It is not exactly clear why *Escherichia coli* is used at all in this study. – As a reference strain? If so, why then a strain that is used for expression of genes?

YL: This strain is first used as a reference strain which is not expected to produce aminoacylated PG. It is also planned to be used in the near future as the host for expressing DltABCD proteins.

What really surprises me is that the authors find that “Lipids from the *E. coli* strain were richest in saturated pentadecanoic acid (C15:0) and ...”. - Whatever previous references you consult, C15:0 is never mentioned as a major fatty acyl residue in *E. coli*. For example, see Mejía *et al.* (1999). If it is true that C15:0 is a major fatty acid in *E. coli*, this finding would certainly put in doubt some dogmas.

YL: The mass spectra of the *E. coli* strain were similar to that reported in reference 24 with (33:1) PG and PE being the most abundant. It was richest in (16:0) and (14:0) saturated fatty acids along with (17:1) and (18:1) monounsaturated or cyclopropane fatty acids. There was a less abundant 241 amu fragment ion matching deprotonated (15:0) saturated fatty acid or (14:0) epoxy fatty acid. However, it was not the aim of this study to characterize the fatty acid composition.

Membrane lipid biosynthesis and composition of *Bacillus subtilis* strain 168 has been studied by numerous groups to a considerable extent. One of the major results of the present paper is that alanyl-PG carries exclusively the D-alanyl form and the authors discussed extensively that D-alanyl-PG should be formed by a mprF-independent pathway. There are MprF-deficient mutants available from John Helman's group (Salzberg and Helman, 2008) and if the authors are correct, one would expect that these mutants still can make D-alanyl-PG.

YL: We have a plan to gather mutants of *Bacillus subtilis* strain 168, which are relevant to lipid biosynthesis. Since MprF's substrate is L-aminoacylated tRNA, we reasoned that the overwhelming abundance of D- over L-alanine in the lipid lysate implied that an mprF-dependent pathway likely produced D-alanyl-PG. During the past week, the MprF-deficient strain has been acquired as suggested and its lipid extract studied. As expected, the lipid did not contain lysyl-PG. Importantly, it did contain alanyl-PG, which is consistent to our hypothesis.

Fig. Legend 8: Mention here or somewhere that the “340 amu molecular anion” corresponds to alanine + Marfey's ?

YL: We will add a statement that the 340 amu molecular anion corresponds to alanine derivatized by Marfey's reagent.

I don't understand the last part of the Discussion “with overall similarity to platelet-activating factor”. Platelet-activating factor is a relatively hydrophilic lipid; so which similarity can it have to a protein?

YL: We are sorry to have missed the word “acetylhydrolase”. The remote homolog is platelet-activating factor acetylhydrolase. ProFunc server found a possible match with an E-score of 0.078 to a platelet-activating factor acetylhydrolase (PDB entry 1BWR). A

hydrogen-bonded triad of Ser-47 / Asp376 / His 379 corresponding to the active site of this acetylhydrolase was found conserved in the DltD structure.

Minor Comments:

1. Page 2, second paragraph of Introduction: change "peptitoglycan" to "peptidoglycan"
2. Page 3, left column, third paragraph, and elsewhere: The symbol for "micro" used here is an "u" not the Greek symbol as it should be.
3. Table 1: change "Calculated Msss" to "Calculated mass".

YL: We will correct these errors in the revised version.

References

1. Mejía R, Gómez-Eichelmann MC, Fernández MS: Fatty acid profile of Escherichia coli during the heat-shock response. *Biochem Mol Biol Int.* 1999; **47** (5): 835-44 [PubMed Abstract](#)

YL: We appreciate this reference.

2. Salzberg LI, Helmann JD: Phenotypic and transcriptomic characterization of Bacillus subtilis mutants with grossly altered membrane composition. *J Bacteriol.* 2008; **190** (23): 7797-807 [PubMed Abstract](#) | [Publisher Full Text](#)

YL: We plan to request all the mutant strains produced by the authors of this article. We have since acquired and studied the pssA and mprF mutants.

Competing Interests: As authors, we do not have competing interests.

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