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RESEARCH ARTICLE

# Characterization of N-Succinylation of L-Lysylphosphatidylglycerol in *Bacillus subtilis* Using Tandem Mass Spectrometry

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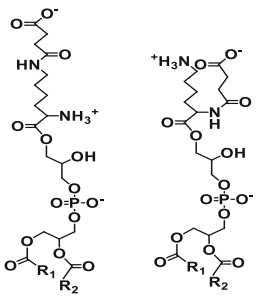

**Abstract.** Phospholipids generally dominate in bacterial lipids. The negatively charged nature of phospholipids renders bacteria susceptible to cationic antibiotic peptides. In comparison with Gram-negative bacteria, Gram-positive bacteria in general have much less zwitterionic phosphatidylethanolamine. However, they are known for producing aminoacylated phosphatidylglycerol (PG), especially positively charged L-lysyl-PG, which is catalyzed by lysyl-PG synthase MprF, which appears to have a broad range of specificity for L-aminoacyl transfer RNAs. In addition, many Gram-positive bacteria also have a *dlt*-gene-coded D-alanylation pathway for lipoteichoic acids and wall teichoic acids covalently attached to a glycolipid or peptidoglycan. D-Alanylation also masks the dominant negative charge of the phosphate-rich polymers of teichoic acids. Using mass spectrometry, we have recently observed that precursor scans in negative mode for deprotonated amino acid fragments were most sensitive for ester-linked amino acids. Such a scan for precursors generating an *m/z* 145 lysyl anion revealed lysyl-PG as well as an additional species 100 *m/z* units greater than lysyl-PG. This unexpected species corresponded precisely to the expected mass of N-succinylated lysyl-PG. Tandem mass spectrometry revealed a precise match to the fragmentation pattern of this putative new species. PG, lysyl-PG, and *N*-succinyl-lysyl-PG may form a complete loop of charge reversal from -1 to +1 and then back to -1. Analogous charge reversal by N-succinylation of lysine residues in the bacterial as well as eukaryotic proteomes has been recently discovered as a major posttranslational modification. Such modification in bacterial lipids is possibly catalyzed by an enzyme homologous to the enzymes that modify lysine residues in proteins.

**Keywords:** Phospholipid, Phosphatidylglycerol, lysyl-phosphatidylglycerol, Mass Spectrometry, succinyl-lysyl-phosphatidylglycerol, Lysine succinylation, Charge modulation, Lipid, MS2, MS3

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## Introduction

Dominant cell membrane components of phospholipids in most bacteria [1] render the bacterial cell surface negatively charged and susceptible to host immune molecules such as cationic antibiotic peptides [2–4]. In response to changes in the environment, bacteria constantly modulate membrane components to adjust fluidity and electrostatic charge [1, 5]. Three known pathways contribute to surface charge modulation in Gram-positive bacteria: biosynthesis of zwitterionic phosphatidylethanolamine, biosynthesis of positively charged L-lysylphosphatidylglycerol (lysyl-PG), and D-alanylation of lipoteichoic acids and wall teichoic acids. The latter two are typically lacking in Gram-negative bacteria, which makes Gram-positive bacteria fascinating targets for studying surface charge modulation.

Aminoacylated PGs, especially lysyl-PG, are a major group of polar lipids in *Bacillus subtilis* as well as many other Gram-positive bacteria [6]. Such lipids are produced by MprF which is also known as "lysylphosphatidylglycerol synthase" [7, 8].

Metin Atila and George Katselis contributed equally to this work.

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They are important for resistance to host immune peptides such as defensins [9]. The crystal structures of two such enzymes have been determined recently, and may serve as targets for novel antibiotics [10]. Covalent modification of phospha tidylglycerol (PG)-bound lysine or other amino acids has never been reported. On the other hand, N-succinylation of lysine residues in proteins has been recently discovered in *Escherichia coli* by mass spectrometry (MS) [11]. This post-translational modification of lysine, as well as acetylation and acylation by other dicarboxylic acids, was soon found to be widespread among prokaryotic and eukaryotic proteomes [12, 13].

We have recently developed a sensitive MS profiling method by searching for precursors of deprotonated amino acid ions in bacterial lipids [14]. The search for the *m*/*z* 145 lysyl anion revealed a cluster of lysyl-PG as expected, with the dominant species being 32:0 lysyl-PG at *m*/*z* 849. Surprisingly, a group of another lipid species with *m*/*z* values 100 larger than *m*/*z* of lysyl-PG were also found. Precise match of this molecular anion as well as its major fragments supports the assignment of this novel species to either α-*N*-succinyl-lysyl-PG or ε-*N*-succinyl-lysyl-PG.

## Methods

### *Bacterial Strain and Cell Culture*

Competent cells (50 µL) of *B. subtilis* strain 168 (Bacillus Genetic Stock Center) were mixed on ice with a linear DNA with an *ampR* gene inserted in the middle of the coding sequence of DltB protein, and electroporated at 2.1 kV/mm for 5 ms. The cells were recovered in 1 mL of lysogeny broth (LB). After incubation at 37 °C for 1–2 h, 200 µL of the broth was plated on LB agar supplemented with ampicillin at 25 µg/mL. An ampicillin-resistant strain was persistently observed and was cultured in LB at 37 °C and at 220 rpm in an environmental shaking incubator. Bacterial cells (100 mL) were harvested by centrifugation when the cell culture reached an optical density of 2.0 at 600 nm. Sodium acetate (1.0 M, 2.0 mL) buffer at pH 4.6 was added to the cell culture before centrifugation at 5500 rpm with a Beckman JLA-8.1 rotor for 16 min at 4 °C. The wet cell pellet was subjected to lipid extraction.

### *Lipid Extraction*

High-performance liquid chromatography grade organic solvents (Fisher Scientific, Ottawa, ON, Canada) and distilled deionized water were used throughout the experiment. Bacterial lipids were extracted following the Bligh and Dyer method [15] with ice-chilled solvents as previously described [16]. The chloroform-rich phase was mixed and vortexed for 3 s with 0.5 mL 0.5 M sodium chloride and centrifuged at 1,300 rpm for 5 min to assist phase separation. The heavier chloroform-rich phase was collected for storage at -80 °C.

### *Lipid Profiling by MS*

The lipid sample was mixed with two volumes of ice-chilled methanol before direct infusion at a rate of 0.6 mL/h into a QTRAP 4000 liquid chromatography–MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo V Ion Spray electrospray ionization source. Optimal electrospray ionization was achieved at 400 °C. The collision energy in the ion trap was optimized as -95 eV for the most efficient detection of precursors that produce the *m*/*z* 145 lysyl anion. SCIEX Analyst 1.6 was used to acquire and export averaged mass spectra. Mass spectra were also analyzed by Mass++ 2.7.4 [17] and figures were generated by Microsoft Excel or Mass++ 2.7.4.

### *Tandem MS*

The targeted MS/MS spectra were first acquired with the QTRAP 4000 mass spectrometer by use of five collision energy settings ranging from 50 to 90 eV in increments of 10 eV. The MS/MS/MS spectra were also acquired with the QTRAP system with specified collision energy and AF2 settings. High-accuracy MS/MS spectra were acquired with a 6550 iFunnel quadrupole time-of-flight mass spectrometer (Agilent Technologies, Mississauga, ON, Canada) equipped with a dual Agilent Jet Stream electrospray ionization source. The collision energies ranged from 30 to 80 eV in increments of 5 eV. Direct infusion at a faster flow rate of 2.0 mL/h was used for the 6550 iFunnel quadrupole time-of-flight mass spectrometer. The Agilent MassHunter BioConfirm (B.06.00) was used to process mass spectra obtained with the 6550 iFunnel quadrupole time-of-flight mass spectrometer.

## Results and Discussion

### *Profiling and MS/MS of Polar Lipids with an Ester-Linked Lysine Residue*

The initial transformation of *B. subtilis* was intended to generate a *dltB*-knockout mutant. Verification by polymerase chain reaction, however, indicated that the recovered ampicillin-resistant mutant strain of *B. subtilis* contained the intact *dltB* locus as well as the *ampR* gene. We first extracted polar lipids from this ampicillin-resistant *B. subtilis* using the Bligh-Dyer method [15] and performed lipid profiling for aminoacylated-PGs in negative and positive modes. Unlike lipids extracted from the wild-type strain, which had two major fatty acyl compositions of 30:0 and 32:0 for PG, alanyl-PG, and lysyl-PG, lipids from the mutant strain had only one major composition of 32:0. The most sensitive methods for profiling aminoacyl-PGs were the precursor scans for deprotonated amino acid fragments [14]. Lipids from *B. subtilis* were extremely abundant in lysyl-PG and somewhat rich in alanyl-PG [14]. The precursor scan for the *m*/*z* 145 [lysine − H]$^-$ ion (Figure 1a) revealed lysyl-PGs with a major peak at *m*/*z* 849 corresponding to 32:0 lysyl-PG surrounded by minor peaks 14 *m*/*z* units (–$CH_2$–) apart. MS/MS spectra of the *m*/*z* 849 ion (data not

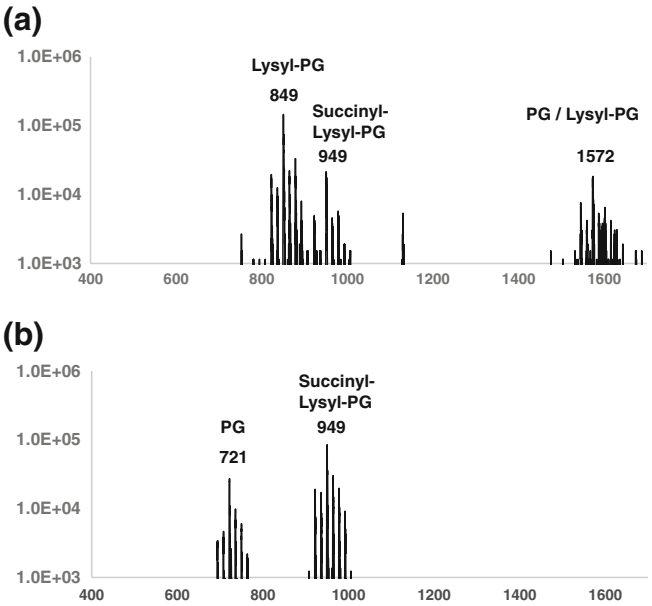


**Figure 1.** Precursor scans for anionic fragments of *m/z* 145 and *m/z* 245. The electrospray ionization mass spectrometry (MS) spectra were acquired in negative ion mode by direct infusion of lipid extracts from cultured mutant cells of *Bacillus subtilis* strain 168. **a** The precursor scan for the *m/z* 145 lysine anion was acquired at a collision energy of -95 eV. **b** The precursor scan for the *m/z* 245 *N*-succinyl-lysine anion was acquired at a collision energy of -65 eV. Groups of lipids were assigned on the basis of the MS/MS spectra. *PG* phosphatidylglycerol

shown), which revealed prominent fatty acyl and lysyl anions as expected, further confirmed this assignment. Another cluster of anions centered at *m/z* 1572 were identified as PG/lysyl-PG adducts by MS/MS (data not shown). The *m/z* 1572 anion dissociated into a cluster of $[PG - H]^-$ ions, with the major peak at *m/z* 721 corresponding to the 32:0 PG anion, as well as a second cluster of $[lysyl\text{-}PG - H]^-$, with the major peak at *m/z* 849. Surprisingly, the scan also revealed a cluster with the major peak at *m/z* 949, 100 *m/z* units greater than the 32:0 lysyl-PG anion at *m/z* 849. This unexpected cluster of anions shared a fragment ion of *m/z* 245, as revealed by a precursor scan (Figure 1b). This second precursor scan also revealed a second cluster, which was assigned as PG anions on the basis of MS/MS spectra that contained the most prominent anions of fatty acids (FAs) and cyclic glycerolphosphate at *m/z* 153 [18]. The result of the precursor scan for the *m/z* 245 fragment can be explained by the fact that PG anions can undergo two successive neutral losses of fatty acyl ketenes to produce the *m/z* 245 anion of diglycerol phosphate. This unexpected cluster centered around *m/z* 949 was observed in all lipids extracted from this mutant strain grown in unbuffered medium or medium buffered with 10 mM potassium phosphate at pH 6, 7, and 8, as well as from cells harvested when the optical density at 600 nm reached the range between 0.8 and 2.0. Lipid extraction with or without 0.1 M sodium acetate at pH 4.6 did not affect this outcome either. In retrospect, we detected small peaks in this cluster when the more sensitive multiple channel acquisition (MCA) option was used for profiling lipids extracted from the wild-type bacteria (see the electronic supplementary material).

We then acquired MS/MS spectra of the *m/z* 949 anion (Figure 2) as well as the related *m/z* 921 anion (see the electronic supplementary material). The two MS/MS spectra share peaks corresponding to head group fragments with *m/z* values of 98, 145, 153, 165, 183, 227, 245, 381, and 399. Their differences can be fully explained by the fatty acyl composition, which was highlighted by the spectrum of the fatty acyl anions, which are known to be the most prominent for phospholipid anions [18]. A higher collision energy of -90 eV provided the most information on fragments less than 350 *m/z* units (Figure 2b), whereas a lower collision energy of 60 eV provided the most information on fragments greater than 350 *m/z* units (Figure 2c). The $[M - H]^-$ ions matched that of succinylated lysyl-PG (observed 949.614 vs calculated 949.613). It produced a major $[lysyl\text{-}PG - H]^-$ peak (849.598 vs 849.597) by a neutral loss matching that of succinic

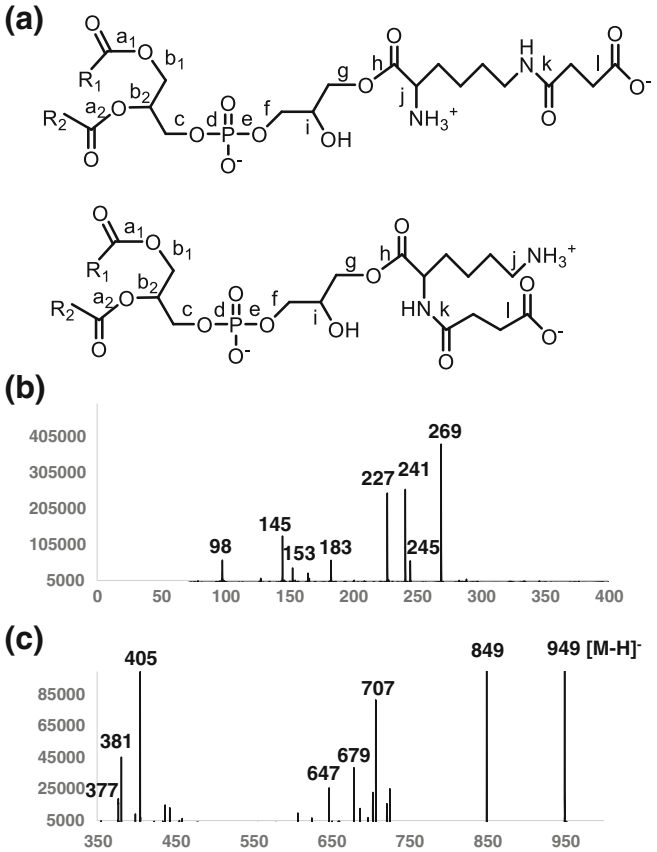


**Figure 2.** MS/MS spectra of the putative α-*N*-succinyl-lysyl-PG or ε-*N*-succinyl-lysyl-PG (*m/z* 949) anion. **a** The alternative molecular structures were drawn with ChemDraw with scissile bonds labeled alphabetically. The *horizontal axis* denotes *m/z* values and the *vertical axis* denotes ion counts. **b** MS/MS spectrum between *m/z* values of 50 and 400 acquired at a collision energy of -90 eV. **c** MS/MS spectrum between *m/z* values of 350 and 1000 acquired at a collision energy of -60 eV

anhydride (100.016 vs 100.016), implying a terminal location of this putative moiety. A second reaction of the lysyl-PG fragment likely produced an intense peak corresponding to the *m/z* value of [lysine − H]$^-$ (145.098 vs 145.098). The *m/z* 949 species also produced succinyl-lysine anion (245.114 vs 245.114), cyclic/dehydrated succinyl-lysine anion (227.102 vs 227.103), and the latter's decarboxylated residue (183.112 vs 183.113). These observations imply the formation of an amide bond between succinic acid and lysine as well as the presence of a free terminal carboxylate. The anion of the entire dehydrated head group of succinyl-lysyl-glycerol phosphate (381.105 vs 381.106) was also observed. In addition, two dominant peaks corresponded to FA anions of 15:0 [FA − H]$^-$ (241.217 vs 241.217) and 17:0 [FA − H]$^-$ (269.249 vs 269.248) respectively. Weaker peaks matched the sizes of lysosuccinyl-lysyl-PG and phosphatidic acid (Table 1). Using the ChemCalc server [19], we found no chemical formula other than $C_4H_4O_3$ that matched the 100.016-amu neutral loss within an error range of 13 ppm. The $C_4H_4O_3$ formula corresponds to either methylmalonic anhydride with a four-membered ring or succinic anhydride with a five-membered ring. The high intensity of the lysyl-PG anion appears to suggest that the lost neutral fragment of 100.016 amu may correspond to the stabler succinic anhydride. This assignment is further supported by the fact that succinic acid is a major component of the citric acid cycle in all aerobic organisms. Therefore, our data support the production of either α-N-succinylated lysyl-PG or ε-N-succinylated lysyl-PG in this ampicillin-resistant mutant of *B. subtilis*.

## *MS/MS/MS Spectra and Putative Dissociation Reactions*

Fragmentation patterns of phosphoglycerides such as PG have been well studied [20, 21] and summarized by Murphy and Axelsen [18]. The fragmentation pattern of the putative N-succinylated lysyl-PG resembles that of PG [18] and aminoacylated PG [14] and can be fully explained by dissociations at multiple ester bonds and the amide bond between lysine and succinic acid residues (see the electronic supplementary material). For instance, phosphoglyceride anions all undergo collision-induced dissociation to produce prominent fragments of fatty acyl anions, aminoacyl anions, and cyclized and intact head group anions.

**Table 1.** Fragments of the 32:0 succinyl-lysylphosphatidylglycerol anion

| Observed *m/z* | Calculated *m/z* | Cleavage | Description |
|---|---|---|---|
| 98.0240 | 98.0242 | Figure 7 | Succinimide − H |
| 145.0984 | 145.0978 | g and k | Lys − H |
| 152.9956 | 152.9953 | c and g | Gro-P − $H_3O$ |
| 183.1119 | 183.1134 | h and l | Suc-Lys − $CO_2$ − $H_3O$ |
| 227.1035 | 227.1032 | h | Suc-Lys − $H_3O$ |
| 245.1143 | 245.1138 | g | Suc-Lys − H |
| 241.2171 | 241.2169 | $b_1$ or $b_2$ | 15:0 FA − H |
| 269.2487 | 269.2482 | $b_1$ or $b_2$ | 17:0 FA − H |
| 377.2087 | 377.2094 | $b_1$ and f | 15:0 PA − $H_3O$ |
| 405.2391 | 405.2408 | $b_2$ and f | 17:0 PA − $H_3O$ |
| 381.1051 | 381.1064 | d | Suc-Lys-Gro-P − $H_3O$ |
| 647.4723 (<20 counts) | 647.4655 | f | 32:0 PA − H |
| 679.3634 (<60 counts) | 679.3573 | $b_1$ or $b_2$ | 15:0 Suc-Lys-PG − $H_3O$ |
| 707.3920 | 707.3886 | $b_1$ or $b_2$ | 17:0 Suc-Lys-PG − $H_3O$ |
| 849.5978 | 849.5973 | k | 32:0 Lys-PG − H |
| 949.6140 | 949.6133 | [M − H]$^-$ | 32:0 Suc-Lys-PG − H |

The alphabetically labeled scissile bonds are shown in Figure 2a
*DAG* diacylglycerol, *FA* fatty acid, *Gro* glycerol, *Lys* lysine, *P* phosphate, *PA* phosphatidic acid or lysophosphatidic acid, *PG* phosphatidylglycerol or lysophosphatidylglycerol, *Suc* succinic acid

Unlike the related lysyl-PG anion, the *m/z* 949 ion has an acidic proton in the ammonium group that can catalyze dissociation reactions. This feature is consistent with the observation that the MS/MS spectrum of the *m/z* 949 anion revealed more intense fragments than that of the related lysyl-PG anion. Despite the fact that one free amino group is protonated, it can become deprotonated by the terminal carboxylate, and along with the hydroxyl, carboxylate, and phosphate groups may serve as the nucleophile in nucleophilic substitution that would fragment the molecular anion at the scissile bonds shown in Figure 2a. For instance, the *m/z* 227 ion may form because of a nucleophilic substitution with an amino group as the nucleophile that renders the fragment ion cyclic (Figure 3, reaction 1). On the other hand, with either the phosphate group or the hydroxyl group in the PG backbone as the nucleophile, the noncyclic *m/z* 245 ion can be produced (Figure 3, reaction 4). This *m/z* 245 anion is equivalent to prominent fatty acyl fragments observed for phosphoglyceride anions [18] or aminoacyl anion dissociated from aminoacylated PG [14].

To support the assignment of the modified lysyl group, we also acquired MS/MS/MS spectra of the anionic fragments at *m/z* values of 245, 227, and 183 (Figure 3, bottom). Major reactions of the three head group fragments are proposed (Figure 3, top) for α-*N*-succinyl-lysyl-PG. The mechanism for ε-*N*-succinyl-lysyl-PG would be essentially identical except for the relative location of the functional groups and ring sizes. The MS/MS/MS spectrum of the *m/z* 245 species suggests that the *m/z* 227 ion is likely the result of the formation of lactam (Figure 3, reactions 5 and 8) and that the *m/z* 145 ion is likely the product resulting from the neutral loss of succinic anhydride (Figure 3, reaction 7). The MS/MS/MS spectrum of the *m/z* 227 ion suggests that formation of the *m/z* 183 ion is likely due to decarboxylation. We speculate that the presence of a carbonyl or amide proton in the *m/z* 227 anion is crucial for decarboxylation as the carbonyl may accept a pair of electrons to form oxyanion as shown in reactions 2 and 6 in Figure 3. Besides, the *m/z* 183 ion likely dehydrates to form the *m/z* 165 ion (Figure 3, reactions 3), as observed in the MS/MS/MS spectrum of the *m/z* 183 ion. The *m/z* 245 anion also produced an *m/z* 241 fragment, which may be due to two successive dehydrogenation reactions in the succinyl group (Figure 4). Because of the existence of two carbonyl groups, all hydrogen atoms in the succinyl residue are weakly acidic. One of the protons can be approached by the terminal carboxylate by forming a five-membered ring and be abstracted (reaction 9). In the resulting structure with a carbanion, torsional rotation of the carboxylate would cause the formation of a second five-membered ring where two hydrogen atoms are juxtaposed to

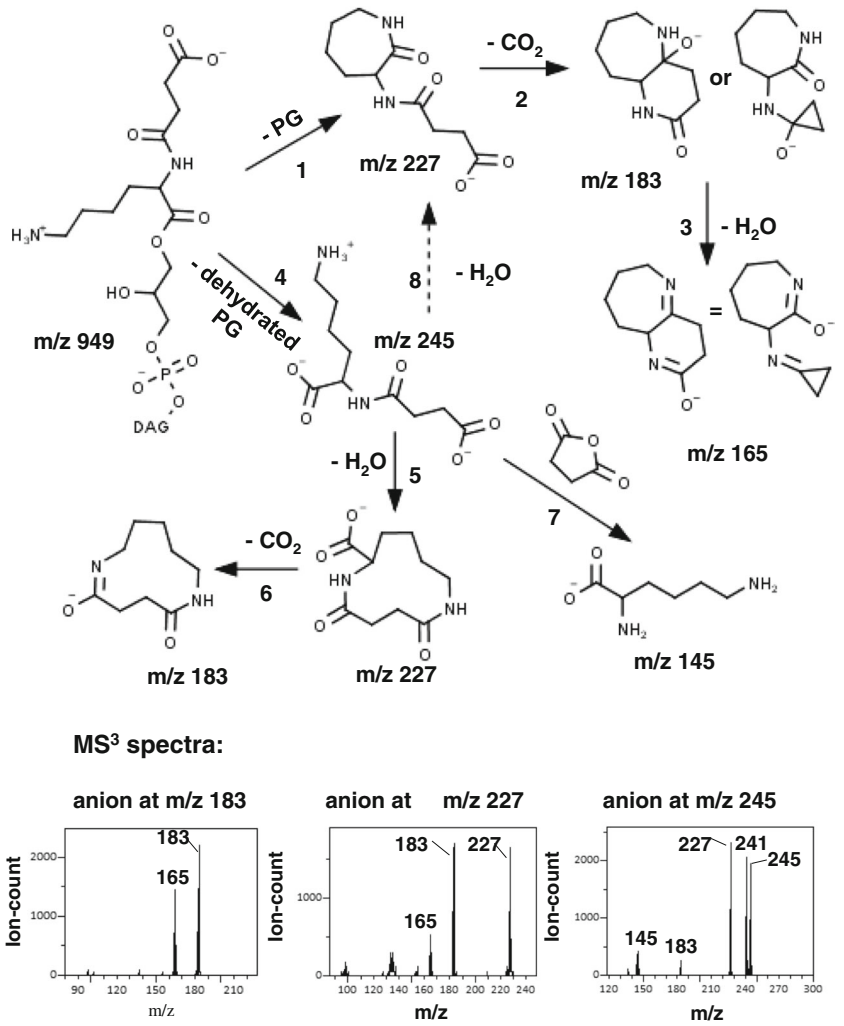


Figure 3. MS/MS/MS spectra of head group fragments (*m/z* 183, 227, and 245 anions) and putative dissociation mechanisms. Putative reactions were drawn with MarvinSketch. Each reaction is numbered. *DAG* diacylglycerol

allow double bond formation and loss of hydrogen gas (reaction 10). This dehydrogenation process can repeat itself once more (reactions 11 and 12) and cause a successive neutral loss of 2 amu, producing the *m/z* 241 anion.

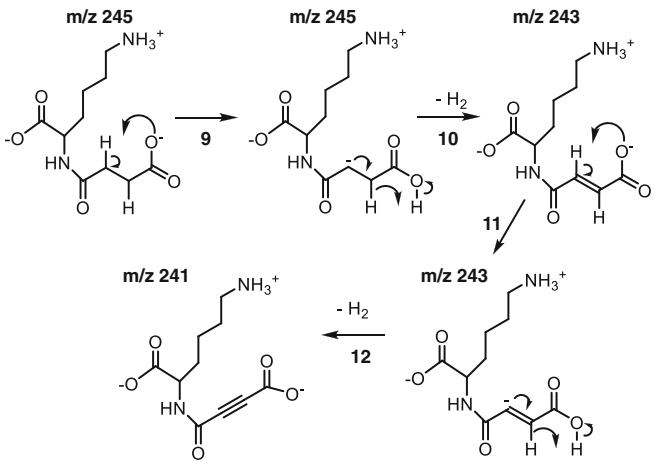


Figure 4. Putative dissociation mechanism for double losses of $H_2$. Putative reactions were drawn with ChemDraw 14.0.0. Each reaction is numbered

PG anions dissociate to produce an intense peak at *m/z* 153 that corresponds to the cyclic glycerol phosphate anion [18]. The equivalent fragment anion of *N*-succinyl-lysyl-PG has an *m/z* value of 381 (Figure 5, top). As for the *m/z* 245 anion, the MS/MS/MS spectrum (Figure 5, bottom) indicated that the *m/z* 381 anion underwent the dissociation reaction that results in a neutral loss of 4 amu (reaction 13). The resultant *m/z* 377 anion can undergo a nucleophilic substitution (reaction 14) that produces a tricyclic glycerol phosphate, a 136-amu neutral species as summarized by Murphy and Axelsen [18], and an *m/z* 241 anion. The *m/z* 381 and *m/z* 377 anions can both undergo a nucleophilic substitution (reaction 15) to form the common *m/z* 153 cyclic glycerol phosphate anion as well as a cyclic 246- or 242-amu neutral fragment.

PG anions also dissociate to produce a noticeable peak at *m/z* 171, which corresponds to the intact head group glycerol phosphate anion [18]. The *m/z* 171 peak is much weaker than the cyclic head group anion of PG at *m/z* 153. The equivalent intact head group anion of *N*-succinyl-lysyl-

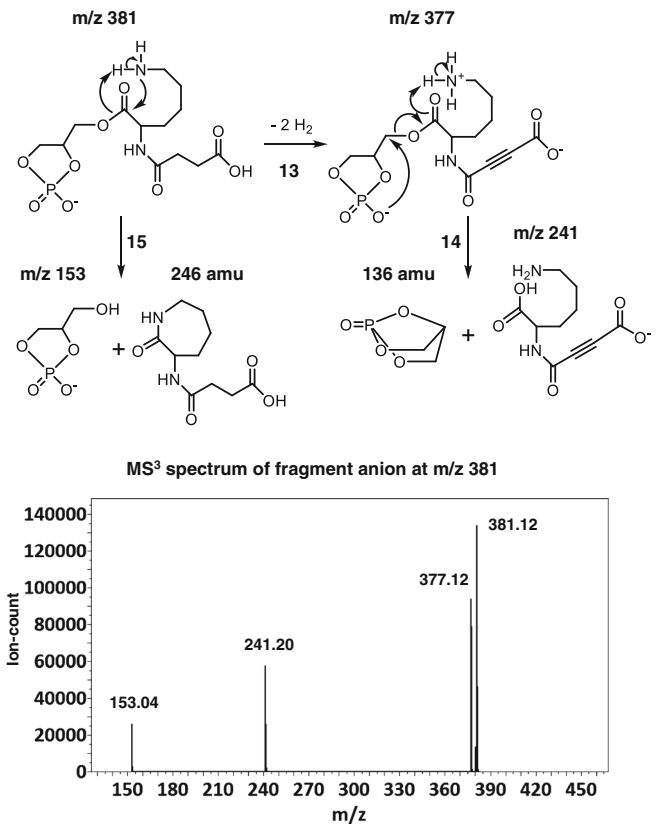


Figure 5. MS/MS/MS spectrum of the cyclized head group fragment at *m/z* 381 at a collision energy of 60 eV and an AF2 setting of 90 V and putative dissociation mechanisms. Putative reactions were drawn with ChemDraw 14.0.0. Each reaction is numbered

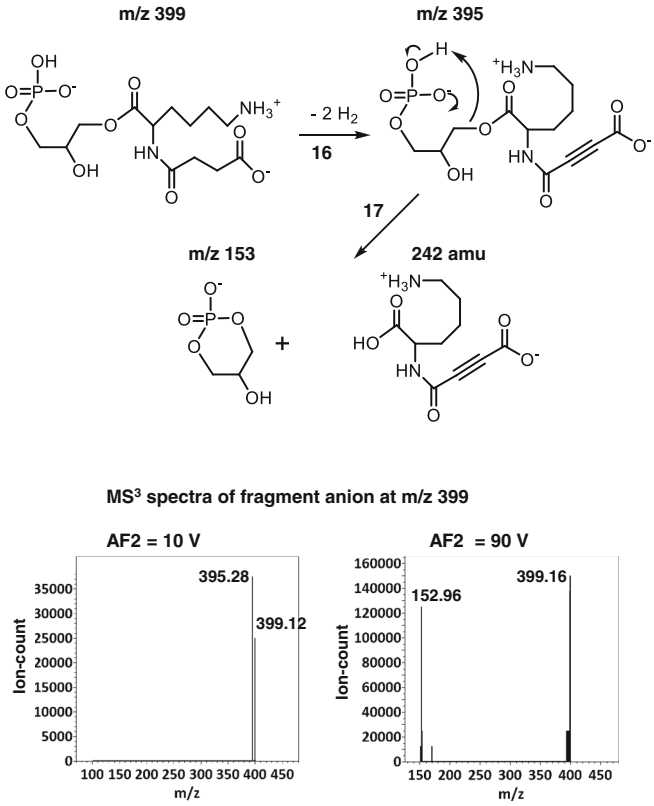


Figure 6. MS/MS/MS spectra of the head group fragment at *m/z* 399 at a collision energy of 60 eV and AF2 settings of 10 V and 90 V and putative dissociation mechanisms. Putative reactions were drawn with ChemDraw 14.0.0. Each reaction is numbered

PG has an *m/z* value of 399 (Figure 6, top). As for PG, this anion was much less intense than the cyclized head group anion at *m/z* 381. As for the *m/z* 245 anion, the MS/MS/MS spectrum (Figure 6, bottom) indicated that the *m/z* 399 anion readily underwent the dissociation reaction that results in a neutral loss of 4 amu (reaction 16) even at a low AF2 setting of 10 V (Figure 6, bottom right). At a high AF2 setting of 90 V, the *m/z* 395 anion further dissociated to form the common *m/z* 153 species of the cyclic glycerol phosphate anion. This reaction is likely a nucleophilic substitution with the phosphate as the most potent nucleophile because of the formation of a six-membered ring and the terminal carbon of the glycerol residue as the electron-deficient center (reaction 17). Before the reaction, the phosphate group likely has a p$K_a$ value similar to that of p$K_{a2}$ of phosphoric acid (approximately 7). As the nascent bond forms, the negative charge on this phosphate group is transferred to the carboxylate leaving group, and the phosphate group likely becomes strongly acidic with a p$K_a$ value closer to the p$K_{a1}$ value of phosphoric acid (approximately 2). As such, the leaving carboxylate (p$K_a$ 4–5 in aqueous solution) would strip the proton from the strongly acidic phosphate to produce the *m/z* 153 fragment anion (reaction 17). On the other hand, the phosphate group is unlikely to stay bound to this proton to form the neutral 154-amu cyclic glycerol phosphate and the *m/z* 241 anion observed as a prominent fragment of the cyclized head group anion at *m/z* 381 (Figure 5).

## *Putative Dissociation Mechanism for the Formation of the m/z 98 Anion*

MS/MS spectra of the *m/z* 921 and *m/z* 949 anions revealed an *m/z* 98 anion at relatively high collision energy settings. This ion showed very low counts in the MS/MS spectra acquired with the quadrupole time-of-flight system. Nevertheless, an accurate *m/z* value of 98.024 was observed, which corresponds precisely to a formula of $C_4H_4NO_2$. No other formula matched within 10 ppm of the observed *m/z* value. This formula suggested succinimide as well as many other compounds that are

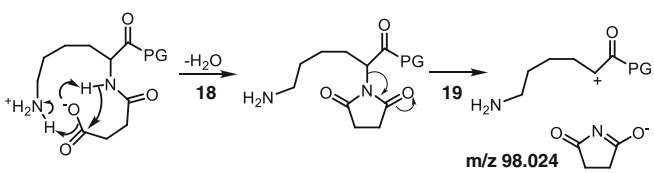


Figure 7. Putative dissociation mechanism for formation of succinimide anion. Putative reactions were drawn with ChemDraw 14.0.0. Each reaction is numbered

not known as metabolites. In the literature, proteins and peptides containing aspartate or asparagine residues spontaneously form succinimide, which leads to protein degradation [22]. On the basis of this mechanism, a mechanism for the production of succinimide anion is proposed in Figure 7. Reaction 18 in Figure 7 is derived from the reaction for the formation of succinimide from aspartate- or asparagine-containing proteins because the two carbonyl groups are separated by two carbon atoms in both amino acids and in the succinamate moiety of the lipid. After succinimide formation, which can be catalyzed by a proton donor in the lysyl residue, succinimide anion can form at high collision energy via reaction 19. The two carbonyl groups are separated by one carbon atom in methylmalonate. As such, the *m/z* 98 anion would not form if N-methylmalonylation instead of N-succinylation were the modification.

## Conclusion

Aminoacylated PGs may play a role in surface charge modulation of Gram-positive bacteria [5]. The D-alanylation pathway of lipoteichoic acids also produces a pool of D-alanyl-PG [14]. Except for a few instances of horizontal gene transfer, the production of aminoacylated lipids is a unique trait in Gram-positive bacteria and apparently plays important roles in antibiotic resistance. The recently determined crystal structures of aminoacyl-PG synthases [10] could serve as the target for rational design of novel antibiotic compounds. In addition, fine-tuning of surface charge appears to be an important survival strategy in response to environmental challenges. The Gram-negative bacterium *Pseudonomas aeruginosa* apparently acquired an MprF homolog which has more than 30% sequence identity with MprF in *Staphylococcus aureus* [23]. This homolog corresponds to the biosynthesis of alanyl-PG in response to acidification [23]. In addition, *P. aeruginosa* also has a pH-dependent promoter-controlled gene which codes for alanyl-PG hydrolase [24]. This pair of enzymes in *P. aerugi nosa* forms a closed circuit of forming and breaking alanylated PG. Our finding resembles this circuit of charge reversal. However, the biological relevance of N-succinylation of lysy l-PG remains to be elucidated.

Succinylation of lysine residues in proteins has recently been discovered in *E. coli* [11]. Subsequent proteomic studies have found that this posttranslational modification, as well as modification by other dicarboxylic acids, is widespread in prokaryotes as well as eukaryotes [12, 13]. Such modifications on histone apparently imply a regulatory role in gene expression [25]. Our finding implies that succinylation of lysine may extend from the proteome to the lipidome, although it remains a challenge to find the enzyme responsible for its synthesis. It is also possible that succinylation of amino groups in the proteome and lipidome may be the result of nonenzymatic reactions due to the fact that cells maintain a pool of succinyl-coenzyme A that can spontaneously cause succinylation of amino groups and other nucleophilic groups such as a thiol group. If this is true, cells must be equipped with hydrolases such as sirtuins to reverse such nonenzymatic modification [26]. Except for *N*-succinyl-lysyl-PG, we did not observe any anionic species that would match the expected fragmentation pattern of analogous *N*-acetyl-lysyl-PG, *N*-malonyl-lysyl-PG, or *N*-glutaryl-lysyl-PG. Even though alanyl-PG anions were detected as the second most intense aminoacyl-PG anions in the bacterial lipids extracted from the mutant strain, no analogous *N*-acyl-alanyl-PG anions were detected by MS/MS and precursor scans for *N*-acylalanine anion. It appeared that N-succinylation of lipids in this strain is specific to lysyl-PG but not alanyl-PG.

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