Identification of phosphatidylserylglutamate: a novel minor lipid in *Escherichia coli*

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Abstract Advances in mass spectrometry have facilitated the identification of novel lipid structures. In this work, we fractionated the lipids of *Escherichia coli* B and analyzed the fractions using negative-ion electrospray ionization mass spectrometry to reveal unknown lipid structures. Analysis of a fraction eluting with high salt from DEAE cellulose revealed a series of ions not corresponding to any of the known lipids of *E. coli*. The ions, with m/z 861.5, 875.5, 887.5, 889.5, and 915.5, were analyzed using collision-induced dissociation mass spectrometry (MS/MS) and yielded related fragmentation patterns consistent with a novel diacylated glycerophospholipid. Product ions arising by neutral loss of 216 mass units were observed with all of the unknowns. A corresponding negative product ion was also observed at m/z 215.0. Additional ions at m/z 197.0, 171.0, 146.0, and 128.0 were used to propose the novel structure phosphatidylserylglutamate (PSE). The hypothesized structure was confirmed by comparison with the MS/MS spectrum of a synthetic standard. Normal phase liquid chromatography-mass spectrometry analysis further showed that the endogenous PSE and synthetic PSE eluted with the same retention times. PSE was also observed in the equivalent anion exchange fraction of total lipids extracted from the wild-type *E. coli* K-12 strain MG1655.

Supplementary key words mass spectrometry • lipid structure determination • electrospray ionization

Phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) are the major membrane glycerophospholipids of *Escherichia coli*, constituting about 10% of the dry weight of the cell (1, 2). Important minor lipids include the precursors phosphatidic acid, CDP-diacylglycerol, phosphatidylserine, and phosphatidylglycerol phosphate (3). For each of these lipids, there can exist several molecular species arising from the different lengths, unsaturation, and/or cyclopropane analogs of the acyl chains (4). The complexity of the *E. coli* lipidome, however, is even greater than can be explained by acyl chain heterogeneity. Numerous additional minor lipids are present in wild-type cells, as judged by isotopic labeling experiments and two-dimensional TLC (3). Many of these species cannot be identified by their migration with standards of known lipids or biosynthetic precursors.

Electrospray ionization-mass spectrometry (ESI-MS) is well suited to the analysis of intact lipids (5). Fragmentation during ionization is minimized and the sensitivity is high (6, 7). In addition, collision-induced dissociation mass spectrometry (MS/MS) allows for the structural analysis of the lipid ion of interest and, when combined with the high mass accuracy of time-of-flight mass spectrometers, can be used to propose a molecular formula for a particular ion.

Current applications of mass spectrometry to lipid analysis have focused mainly on the quantification of known lipid species, often coupling liquid chromatography directly to the mass spectrometer (8–10). These analyses compare levels of known lipid species present under various growth conditions or disease states. However, important changes in levels of unknown or minor lipids are difficult to analyze without knowledge of their structures and the availability of appropriate standards. Furthermore, some important, minor *E. coli* lipids, such as CDP-diacylglycerol-phosphate...
lycerol or the peptidoglycan precursor lipid II, are not detectable unless the total lipids are first fractionated to remove the major species (11, 12).

In this work, we prefractionated 0.5 g of E. coli B lipids using anion exchange chromatography. A fraction that eluted just after cardiolipin with high salt from a DEAE cellulose column was analyzed by negative-ion ESI-MS. Here, we report the identification of the novel glycerophospholipid phosphatidylserglycylglutamate (PSE), in which the seryl-glutamate dipeptide is linked to the phosphate moiety via the serine hydroxyl group.

MATERIALS AND METHODS

Materials

Tryptone and yeast extract were from FisherBiotech (Fairlawn, NJ). Glass-backed Silica Gel 60 TLC plates (0.25 mm) and high-performance TLC (HPTLC) plates were from E. Merck; solvents were reagent grade from Mallinckrodt. Other chemicals were purchased from VWR (West Chester, PA). Total E. coli B lipids and 1, 2-dioleoyl-snglycero-3-phosphoserylglutamate were from Avanti Polar Lipids (Alabastar, AL).

Ion exchange chromatography

The total lipid of E. coli B (0.5 g) was dissolved in 100 ml of CHCl₃/CH₃OH/H₂O (2:3:1, v/v/v) and loaded onto a 15 ml DEAE-cellulose column (Whatman DE52) pre-equilibrated with ammonium acetate as the counter-ion in the same solvent (13). The column was washed with 500 ml of CHCl₃/CH₃OH/H₂O (2:3:1, v/v/v) and then with 75 ml each of CHCl₃/CH₃OH/NaH₂Ac (2:3:1, v/v/v) containing sequentially 15, 30, 60, 90, 120, 240, and 480 mM NaH₂Ac in the aqueous component. Fractions (15 ml) were collected, and a portion of each was spotted onto an HPTLC plate. The plate was developed in CHCl₃/CH₃OH/H₂O/(25:15:4:2, v/v/v/v) and charred with 10% sulfuric acid in ethanol. This TLC analysis was used to direct the pooling of fractions as shown in Table 1. The pooled fractions were converted to a two-phase Bligh-Dyer system (14) through the addition of water and chloroform (final ratios, CHCl₃:CH₃OH:aqueous, 2:2:1.8, v/v/v). Upper and lower phases were resolved by centrifugation for 15 min at 2,600 × g and washed with 1 liter of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄).

Normal phase liquid chromatography-mass spectrometry

Normal phase liquid chromatography-mass spectrometry (LC-MS) was adapted from Becart, Chevalier, and Biesse (16). An Agilent 1100 system with quaternary pumps was used with three mobile phase solvents and coupled to the QSTAR XL quadrupole-time-of-flight mass spectrometer. The LC was operated with an Ascentis silica column (5 µm, 25 cm × 2.1 mm) at a flow rate of 300 µl/min with a three solvent gradient program. The 62 min program begins with 100% mobile phase A [CHCl₃:CH₃OH:NH₄OH (800:195:5, v/v/v)] and is decreased linearly from 100% to 0% mobile phase B as mobile phase B is decreased from 0% to 100% over 14 min. Mobile phase B is held for 11 min at 100% and then decreased linearly to 0% as mobile phase C [CHCl₃:CH₃OH:H₂O:NH₄OH (450:450:95:5, v/v/v/v)] is increased from 0% to 100% over the next 13 min. Mobile phase C is held at 100% C for 2 min. The column was regenerated by decreasing mobile phase C from 100% to 0% as mobile phase B is increased from 0% to 100% over 5 min and then held at 100% mobile phase B for 2 min. Finally, mobile phase B was decreased from 100% to 0% as mobile phase A was increased from 0% to 100% over 5 min and then held at 100% mobile phase A for 18 min.

In a typical run, the dried lipid film from a pooled fraction (see Table 1) was redissolved in 2.0 ml of CHCl₃:CH₃OH (2:1, v/v), and 0.5 ml was transferred to a separate tube, dried under nitrogen gas, and then redissolved in 0.3 ml of CHCl₃:CH₃OH:H₂O (73:25:3, v/v/v). Twelve microliters was injected onto the column at 300 µl/min with mobile phase A. The column flow was split 1 to 10 prior to introduction into the mass spectrometer. Mass spectra were obtained scanning from 200 to 2000 Da in negative-ion mode with the electrospray ionization source operating at the following settings: nebulizer gas, 21 kPa, curtain gas, 27 kPa, ion-spray voltage, −4,500 V, declustering potential, −55 V, focusing potential, −265 V, declustering potential 2, −15 V, ionization temperature, ambient room temperature. In some LC-MS runs, collision-induced dissociation was performed using information-dependent acquisition mode (IDA), a collision energy of −52 V (laboratory frame of reference) and N₂ as the collision gas. Data acquisition, analysis, and elemental composition calculations were performed using the Analyst QS 1.1 software. Exact masses of lipid species and product ions were obtained using CS Chem Draw Pro, version 8.0. The PSE synthetic standard was diluted to 0.1 µg/ml using CHCl₃:CH₃OH (2:1, v/v) and analyzed by direct infusion mass spectrometry and MS/MS, as described above.

Mass spectrometry

The dried lipid film remaining in the tube following solvent removal was redissolved in 2.0 ml of CHCl₃:CH₃OH (2:1, v/v). This solution was directly infused into the Turbo electrospray ionization source of a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (ABI/MDS-Sciex, Toronto, Canada) at 6 µl/min (15). The instrument was calibrated using polypropylene glycol (PPG) (Applied Biosystems, Foster City, CA). The resolution of the instrument under normal operating conditions was 10,000 to 15,000. The mass accuracy of the instrument was between 5 and 20 ppm, and as such measured masses are given to three decimal places. MS/MS was performed with a collision energy of −50.0 V (laboratory frame of reference) and N₂ as the collision gas. Data acquisition, analysis, and elemental composition calculations were performed using the Analyst QS 1.1 software. Exact masses of lipid species and product ions were obtained using CS Chem Draw Pro, version 8.0. The PSE synthetic standard was diluted to 0.1 µg/ml using CHCl₃:CH₃OH (2:1, v/v) and analyzed by direct infusion mass spectrometry and MS/MS, as described above.

Growth of E. coli

E. coli K-12 strain MG1655 was cultured at 37°C in Luria broth (LB) consisting of 10 g of NaCl, 5 g of yeast extract, and 10 g of tryptone per liter (17). The cells were grown overnight in LB medium at 37°C and then diluted into 2 liters of LB medium to an A₆₀₀ of 0.01. The culture was grown at 37°C, shaking at 225 rpm until the A₆₀₀ was about 3.0. Cells were harvested by centrifugation for 45 min at 2,600 g and washed with 1 liter of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄).
**Extract**ion of *E. coli* MG1655 total lipids

The final cell pellet was resuspended in 50 ml of PBS and transferred to a Teflon-lined centrifuge bottle. The cellular lipids were extracted using the method of Bligh and Dyer (14). Briefly, 62.5 ml of chloroform and 125 ml of methanol were added to the cell suspension to generate a single phase extraction mixture of CHCl₃:CH₃OH:H₂O (1:2:0.8, v/v/v). After incubation at room temperature for 20 min, the mixture was centrifuged at 2,600 g for 15 min. The supernatant was transferred to a clean bottle and converted to a two-phase Bligh-Dyer extraction mixture (CHCl₃:CH₃OH:PBS, 2:2:1.8, v/v/v) by the addition of 62.5 ml of chloroform and 62.5 ml of PBS. The extraction mixture was centrifuged as above to resolve the phases. The lower phase was washed with 237 ml of preequilibrated neutral upper phase, and the final lower phase dried using rotary evaporation. The MG1655 total lipid extract was redissolved in 150 ml of CHCl₃:CH₃OH:H₂O (2:3:1, v/v/v) and fractionated on a 15 ml DEAE cellulose column as described above. The fractions that eluted with CHCl₃:CH₃OH:240 mM NH₄Ac (2:3:1, v/v/v) and CHCl₃:CH₃OH:480 mM NH₄Ac (2:3:1, v/v/v) were pooled and converted to a two-phase Bligh-Dyer extraction mixture. The lower phase was washed down and the lipids redissolved in 100 µl of CHCl₃:CH₃OH:H₂O (73:23:3, v/v/v). Seventy microliters of the sample was analyzed by normal phase LC-MS as described above.

**RESULTS**

**Ion exchange chromatography of *E. coli* lipids**

*E. coli* B lipids were separated on a DEAE cellulose column based on their charge. The fractions were pooled as shown in **Table 1**. Care was taken to minimize the overlap among the major lipid species, PE, PG, phosphatidic acid (PA), and CL and further analyzed by TLC (**Fig. 1**). As expected, PE did not adhere to the column and was found predominantly in the run through (**Fig. 1, fraction 1**). Fraction 2, the first part of the CHCl₃:CH₃OH:15 mM NH₄Ac (2:3:1, v/v/v) elution, contained residual traces of PE and some PG. Fractions 3–5 contained the majority of the PG. PA and some CL eluted at higher NH₄Ac concentrations in fractions 6 and 7. Most of the CL eluted in fractions 8 and 9. Fraction 10 contained the least lipid (**Fig. 1**). Small amounts of CL are present as well as at least two more slowly migrating, unknown species. Initial mass spectrometry analysis was conducted on this fraction because it contained the least amount of a known, major glycerophospholipid and would allow for more efficient ionization of a novel minor lipid species.

**Mass spectrometry of Fraction 10**

**Figure 2** shows the negative ion ESI mass spectrum of the m/z 500–950 region, obtained by direct infusion of fraction 10. The predominant ions between m/z 670 and 740 represent doubly charged species [M-2H]²⁺ of CL, as determined by exact mass and MS/MS analysis (18–20). The singly charged ion at m/z 813.447 represents phosphatidylglycerol phosphate, the biosynthetic precursor to PG (21–23), again determined by exact mass and MS/MS analysis.

A series of peaks at m/z 861.506, 875.521, 887.525, 889.532, and 915.548, interpreted as [M-H]⁻ ions based on the one mass unit spacing of their ¹³C-isotope ions, did not correspond to any of the known lipids of *E. coli* (**Fig. 2, inset**). However, this pattern of ions is consistent with a series of related *E. coli* glycerophospholipids; the difference of 28 mass units corresponds to two methylenes (CH₂); the difference of 26 mass units corresponds to two methylenes and an unsaturation; and the difference of 14 amu reflects the presence of cyclopropane fatty acids in *E. coli* (24).

**Elucidation of the structure of the unknown ions by ESI-MS/MS**

To determine the structures of the compounds responsible for these ions, MS/MS was performed on each of the ions, revealing similar fragmentation patterns. **Figure 3** shows the results for the ion at m/z 889.5. The product ion at m/z 78.959 corresponds to PO₂⁻, and the product ion at m/z 152.985 corresponds to C₆H₇O₄P⁻ (5, 19, 25), consistently seen with all glycerophospholipids. The product ion at m/z 845.572 corresponds to the loss of 44 mass units,
tenth formula, C₅H₈NO₄, had the same elemental composition as the [M-H]/H⁺ ion of the amino acid glutamate. The product ion at m/z 128 is consistent with water loss from one of the carboxyl groups of the glutamate ion. From this data, we hypothesized that the novel head group consisted of a serine residue with a glutamate moiety attached via an amide linkage to the carboxyl of serine. The proposed structure of the 889.5 ion is shown in the inset of Fig. 3, a new glycerophospholipid, which we designate as PSE.

Figure 3B shows the proposed structures of the product ions derived from the head group of PSE. These product ions are all consistent with glutamate and serine linked via an amide bond between the carboxyl group of the serine and the amine group of the glutamate. The alternative linkage, between the amine of the serine and either carboxyl of the glutamate, would not yield the glutamate product ion at m/z 146.0459 (Fig. 3B).

The other major peaks in the product ion spectra are attributable to losses of acyl chains and/or the head group from the precursor ion. The ions at m/z 673.481, 685.521, and 693.316 correspond to the neutral loss of CO₂ from the head group of a novel diacylated glycerophospholipid. The negative-ion of this head group is seen as a prominent peak at m/z 215.068. The product ions at m/z 197.057, 171.078, 146.047, and 128.036 also appear to be derived from the head group because all of the unknown [M-H]⁻ ions yielded these species upon collision-induced dissociation (Fig. 3B). The ion at m/z 197.057 corresponds to water loss from the 215.068 ion. The ion at m/z 171.078 is interpreted as loss of CO₂ from the ion at m/z 215.068. The ion at m/z 128.036 corresponds to water loss from the ion at m/z 146.047.

We used an elemental composition calculator to obtain a list of possible molecular formulas for the product ion at m/z 146.047. With a mass error tolerance of 30 ppm, 13 possible molecular formulas containing the elements C, H, O, N, S, and P (maximum 20 of each) were predicted (Table 2). Several of the formulas were not consistent with naturally occurring molecules. However, it was observed that the consistent with loss of CO₂ from the precursor ion. Acyl chains corresponding to palmitate (16:0, m/z 255.233) and octadecenoate (18:1, m/z 281.249) are also seen. As this is an E. coli derived lipid, the octadecenoate observed above is likely cis-vaccenate (26). However, this analysis cannot definitively identify the location of the unsaturation.

A prominent neutral loss of 216 amu leads to the formation of the product ion at m/z 673.481, which is interpreted as a 16:0, 18:1 phosphatidic acid anion (C₃₇H₇₀O₈P⁻, exact mass 673.4814) (27) and is consistent with a diacylated glycerophospholipid. The 216 mass units loss, consistently seen in the MS/MS spectra of all of the unknown [M-H]⁻ ions at m/z 861.506, 875.521, 887.525, and 915.548 (data not shown), could correspond to the loss of a part of the head group of a novel diacylated glycerophospholipid. The negative-ion of this head group is seen as a prominent peak at m/z 215.068. The product ions at m/z 197.057, 171.078, 146.047, and 128.036 also appear to be derived from the head group because all of the unknown [M-H]⁻ ions yielded these species upon collision-induced dissociation (Fig. 3B). The ion at m/z 197.057 corresponds to water loss from the 215.068 ion. The ion at m/z 171.078 is interpreted as loss of CO₂ from the ion at m/z 215.068. The ion at m/z 128.036 corresponds to water loss from the ion at m/z 146.047.

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Fig. 3. MS/MS of the negative ion of the unknown lipid at m/z 889.5. A: The MS/MS spectrum of m/z 889.5 is shown. The inset shows the proposed structure of PSE and the proposed major product ion fragments. B: The proposed structures of the product ions derived from the PSE head group.

| Exact mass of product ion | Molecular formula | Proposed structure of product ion |
|--------------------------|-------------------|----------------------------------|
| 215.0673                 | C₈H₁₁N₂O₅⁻        | ![Proposed structure]            |
| 197.0568                 | C₈H₉N₂O₄⁻         | ![Proposed structure]            |
| 171.0775                 | C₇H₁₁N₂O₃⁻         | ![Proposed structure]            |
| 146.0459                 | C₅H₆NO₄⁻          | ![Proposed structure]            |
| 128.0353                 | C₅H₆NO₃⁻          | ![Proposed structure]            |
neutral loss of 216 mass units, the ion at m/z 215, and the other head group derived ions were all observed (data not shown). The differences in the product ion masses are attributable to the differences in the acyl chain composition among the different ions. Table 3 summarizes the variety of PSE molecular species detected and their acyl chain compositions, as inferred from the exact mass and MS/MS analysis. MS/MS can suggest the location of the acyl chain on the glycerol backbone with two oleate residues (18:1<sup>20</sup>) and has a predicted [M-H]<sup>-</sup> of m/z 915.524 amu, isobaric with an endogenous PSE esterified with two 18:1 fatty acids, presumed to be cis-vaccenates residues (18:1<sup>±11</sup>) based on the fatty acid composition of E. coli (32, 33). Figure 5 compares the MS/MS of the endogenous ion at m/z 915.5 (panels A and B) with the synthetic PSE (panels C and D). The synthetic PSE has a fragmentation pattern very similar to the endogenous lipid with m/z 915.5. Both displayed the characteristic 216 mass unit neutral loss and the presence of head group derived ions at m/z 215, 197, 171, 146, and 128 (panels B and D). Because the endogenous PSE at m/z 915.5 can arise from acylation with acyl chains other than 18:1, additional fragment ions are present in the product ion spectra (panel B). For example, acylation with cis-11, 12-methylene-palmi tolate residue (17cp, m/z 267.214) and cis-13,14-methylene-vaccenate residue (19cp, m/z 295.249), instead of two 18:1 residues, also gives rise to m/z 915.5.

The presence of additional fragment ions, such as m/z 267.215 and m/z 295.249, in the endogenous PSE spectrum strongly suggest the presence of such isobaric PSE species in fraction 10. As stated above, we cannot definitively assign these product ions as cyclopropane fatty acids using this mass spectrometry analysis.

To confirm the proposed structure of the unknown lipids, normal phase LC-MS was performed to demonstrate that the endogenous PSE species have the same retention time as the synthetic PSE. Fraction 10 and the synthetic PSE were independently analyzed by normal phase LC-MS. The extracted ion current of the synthetic PSE ion at m/z 915.5 and its mass spectrum are shown in Fig. 6A and B. It elutes between minutes 21 and 22. The endogenous PSE ion at m/z 889.5 from fraction 10 also eluted between 21 and 22 min (Fig. 6C). The mass spectrum of the fraction 10 lipids eluting between minutes 21 and 22 of is shown in Fig. 6D. Several ions corresponding to PSE with 32:1, 33cp, 34:2, 34:1, 35:1cp, and 36:2 acyl chains (the number of carbons in the acyl chains: number of unsaturations or cyclopropane units in the acyl chains; see Table 3) are detected (Fig. 6D). In addition, when synthetic PSE was added to a portion of fraction 10 and analyzed by LC-MS, the standard and the endogenous PSE coeluted, as seen by an increase in the ion intensity of the ion at m/z 915.5 (Fig. 6E, F).

**Detection of PSE in MG1655 wild-type E. coli K-12**

PSE was first identified in lipids of E. coli B (ATCC 11303). To demonstrate that PSE is not unique to E. coli B strains, we looked for PSE in wild-type E. coli K-12 MG1655. Following anion exchange fractionation, PSE was again detected in fraction 10 using normal phase LC-MS as the final step in the identification. As with the synthetic PSE (Fig. 6A) and the endogenous PSE from E. coli B (Fig. 6C), ions corresponding to PSE were detected in MG1655 fraction 10 between minutes 21 and 22 (Fig. 7A, peak marked with an asterisk). The mass spectra of the lipids eluting between minutes 21 and 22 are shown in Fig. 7B. The ions at m/z 861.531, 863.534, 873.542, 875.540, 887.546, and

### Table 2. Possible elemental compositions of the ion at m/z 146.047

| Molecular Formula [M-H] | Exact Mass [146.047] | Difference (Observed Mass – Exact Mass) | Difference (ppm) |
|-------------------------|----------------------|----------------------------------------|-----------------|
| C<sub>13</sub>H<sub>8</sub>N<sub>2</sub>O<sub>13</sub>P | 146.0469 | 0.0001 | 0.1 |
| C<sub>14</sub>H<sub>10</sub>N<sub>2</sub>O<sub>13</sub>S <sub>2</sub> | 146.0468 | 0.0002 | 0.7 |
| C<sub>15</sub>H<sub>10</sub>N<sub>2</sub>O<sub>13</sub> | 146.0466 | 0.0004 | 2.2 |
| C<sub>16</sub>H<sub>12</sub>N<sub>2</sub>O<sub>13</sub> | 146.0473 | −0.0005 | −3.9 |
| C<sub>17</sub>H<sub>12</sub>N<sub>2</sub>O<sub>13</sub>P | 146.0480 | −0.0010 | −6.9 |
| C<sub>18</sub>H<sub>12</sub>N<sub>2</sub>O<sub>13</sub>P<sub>2</sub> | 146.0481 | −0.0011 | −7.9 |
| C<sub>19</sub>H<sub>12</sub>N<sub>2</sub>O<sub>13</sub> | 146.0483 | −0.0013 | −9.0 |
| C<sub>20</sub>H<sub>12</sub>N<sub>2</sub>O<sub>13</sub>P<sub>2</sub> | 146.0456 | 0.0014 | 9.3 |
| C<sub>21</sub>H<sub>12</sub>N<sub>2</sub>O<sub>13</sub>P | 146.0486 | −0.0016 | −11.1 |
| C<sub>22</sub>H<sub>12</sub>N<sub>2</sub>O<sub>13</sub>P<sub>2</sub> | 146.0453 | 0.0017 | 11.4 |
| C<sub>23</sub>H<sub>12</sub>N<sub>2</sub>O<sub>13</sub> | 146.0487 | −0.0017 | −11.4 |
| C<sub>24</sub>H<sub>12</sub>N<sub>2</sub>O<sub>13</sub>P | 146.0447 | 0.0023 | 15.1 |
| C<sub>25</sub>H<sub>12</sub>N<sub>2</sub>O<sub>13</sub>P<sub>2</sub> | 146.0496 | −0.0026 | −18.2 |

### Table 3. PSE species detected in E. coli B lipids

| Acyl Chain Composition | Total No. of Carbons | No. of Unsaturations | Acyl Chain Combinations | Observed<sup>a</sup> |
|------------------------|----------------------|----------------------|-------------------------|----------------------|
| [M-H] = m/z 861.5      | 32:1                 |                      | 16:0, 16:1              | 14:0, 18:1           |
| 863.5                  | 32:0                 |                      | 16:0, 16:0              | 16:0, 16:0           |
| 873.5                  | 33cp<sup>b</sup> 1    |                      | 16:1, 17cp              | 16:1, 17cp           |
| 875.5                  | 33cp                 |                      | 16:0, 17cp              | 17cp, 17cp           |
| 887.5                  | 34:2                 |                      | 16:1, 18:1              | 17cp, 17cp           |
| 889.5                  | 34:1                 |                      | 16:0, 18:1              | 16:0, 18:1           |
| 901.5                  | 35:1cp               |                      | 16:1, 19cp              | 16:1, 19cp           |
| 903.5                  | 35:1                 |                      | 16:0, 19cp              | 17cp, 18:1           |
| 915.6                  | 36:2                 |                      | 18:1, 18:1              | 17cp, 19cp           |

<sup>a</sup> The position of the acyl chain cannot be determined from this data.

<sup>b</sup> Cyclopropane.
889.542 correspond to PSE species with 32:1, 32:0, 33:1cp, 33cp, 34:2, and 34:1 acyl chains. PSE species containing cyclopropane fatty acids were the most abundant species in the MG1655 lipid extract in contrast to the E. coli B PSE species. This is consistent with the different growth conditions that were employed. MG1655 cells were grown to stationary phase ($A_{600} \sim 3.0$) compared with the E. coli B, which were grown to log phase (Avanti Polar Lipids). Growth to stationary phase increases the proportion of cyclopropane fatty acids in the glycerophospholipid pool.

Fig. 4. Overlay of the predicted isotope distribution on the mass spectrum of endogenous PSE. Analyst QS1.1 software was used to calculate the predicted isotope distribution (dashed lines) of the molecular formula $C_{45}H_{82}N_2O_{13}P$, which is overlaid on the observed mass spectrum of the PSE species with an exact mass near $m/z$ 889.5.

Fig. 5. MS/MS of the endogenous PSE compared with synthetic PSE. The MS/MS spectrum of endogenous PSE (A) and synthetic PSE (C). B and D show the expansion of the low mass region of A and C, respectively, to show that the head group-specific product ions are the same between the two spectra.
Fig. 6. Extracted ion current and mass spectra of endogenous PSE and synthetic PSE. A: Extracted ion current during normal phase LC-MS of the synthetic PSE ion at m/z 915.5. B: Mass spectra of the lipids eluting between minutes 21 and 22. C: Extracted ion current during normal phase LC/MS of the PSE ion from fraction 10 with m/z 889.5. D: Mass spectra of the lipids eluting between minutes 21 and 22. E: Extracted ion current of m/z 915.5 following LC-MS analysis of fraction 10 spiked with synthetic PSE. F: Mass spectra of the lipids eluting between minutes 21 and 22.
Targeted MS/MS analysis of the ion at \( m/z \) 875.5 revealed the same neutral loss of 216 mass units and the presence of the head group ion at \( m/z \) 215 (data not shown). The combination of similar elution time during normal phase LC, the exact mass, and the MS/MS analysis show that PSE is present in common \( E. coli \) K-12 laboratory strains.

**DISCUSSION**

The advent of ESI-MS has confirmed the complexity of the lipidome of \( E. coli \), which was already suspected based on early radiochemical studies (3). The major ions observed in the direct negative ion ESI-MS analysis of an \( E. coli \) total lipid extract, PG, PE, PA, and CL, often obscure the additional minor lipid ions. Prefractionation of the lipids prior to analysis by mass spectrometry uncovers ions arising from some of these minor lipids. For example, in this work, we were able to identify a series of ions corresponding to molecules of phosphatidylglycerol phosphate, the immediate precursor of PG (21, 36, 37) only after prefractionation of the total lipids using anion exchange chromatography (Fig. 2). Likewise, we have now identified a new family of molecules, the PSEs, as minor lipids from two strains of \( E. coli \).

The structure of PSE was confirmed by accurate mass measurements, MS/MS, and comparison to a synthetic standard. In addition to the detection of this novel lipid in \( E. coli \) B lipid extracts, five different PSE species were observed in fractionated lipid extracts of MG1655.

The biosynthetic pathway responsible for the formation of PSE remains to be determined. Several possibilities can be envisioned. Phosphatidylserine synthase may be able to use a Ser-Glu dipeptide, perhaps the by-product of protein degradation, instead of serine. While it has been shown that the phosphatidylserine synthase of \( E. coli \) is very specific for serine, it does slowly transfer the CDP-DAG phosphatidyl moiety to glycerol, glycerol 3-P, and phosphatidylglycerol (38, 39). Accordingly, purified phosphatidylserine synthase needs to be tested to determine if PSE can be formed in vitro using CDP-DAG and serylglutamate as substrates (39). If so, PSE should be missing in the available \( pss \) deletion mutants (40).

Alternatively, Shibuya, Yamagoe, and Miyazaki (41) have described the formation of novel \( E. coli \) glycerophospholipids in which a phosphatidyl group is transferred to the sugar alcohol, mannitol, to form phosphatidylmannitol. Cardiolipin synthase is implicated as the phosphatidyl donor in the formation of this and other sugar alcohol analogs. A similar activity could be at work in the formation of PSE. The free hydroxyl on the serine of a Ser-Glu dipeptide could be used in an alcoholysis reaction catalyzed by cardiolipin synthase (41). If that is the case, cardiolipin synthase deletion mutants should lack PSE.

PSE also may be formed by the addition of a glutamate directly to PS. However, PS levels in wild-type \( E. coli \) are very low (42). Only when the phosphatidylserine decarboxylase is inhibited by mutation do phosphatidylserine levels become readily detectable (42). In that scenario, PSE might accumulate in strains harboring \( psd \) mutations. In addition to the favorable equilibrium for the decarboxylation of PS to PE (43), PS levels may be low because of other enzymatic reactions, such as formation of PSE.

A final hypothetical possibility is that PSE may arise from the processing of a protein, modified with a phosphatidyl moiety on a Ser-Glu dipeptide within its primary sequence. Such a lipid modification is not without precedent. The lipidation of the yeast autophagy factor Apg8 involves the linkage of a C-terminal glycine to the free amine of phosphatidylethanolamine (44). To our knowledge, protein lipidation of this sort has not been observed in \( E. coli \). However, the known lipoproteins of \( E. coli \) are conjugated with a diacylglycerol moiety linked via a thioether to an N-terminal cysteine residue (45). A third acyl chain is attached to the N-terminal amine of the same cysteine (45). The identification and structure of PSE suggests that a new
type of protein lipidation might exist in *E. coli*. The phosphatidyl group may be transferred to a seryl-glutamate dipeptide within a protein from CDP-diacylglycerol.

Interestingly, no ions corresponding to phosphatidylserine linked to any of the other common amino acids were detected. Phosphatidylseralpstatate, in particular, which should be enriched in the same anion exchange fraction (10) as PSE, was not present (data not shown). This finding suggests that PSE is not an artifact of the extraction and therefore may have a defined role in the cell. In future investigations, the biosynthesis and degradation of PSE will be examined to determine its role in *E. coli*.

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