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Application of electrospray ionization mass spectrometry to characterize glycerophospholipids in *Francisella tularensis* subsp. *novicida*

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ABSTRACT

Francisella tularensis is a highly infectious human pathogen that can cause tularemia, but little is known about the lipid composition in membranes of this bacterium. Our recent study has shown that lipid A in membranes of *F. tularensis* subsp. *novicida* is related to its infection ability [X. Wang, A.A. Ribeiro, Z. Guan, S.N. Abraham, C.R. Raetz, Attenuated virulence of a *Francisella* mutant lacking the lipid A 4′-phosphatase, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 4136–4141.]. Here we purified the glycerophospholipids of *F. novicida*, and analyzed by using thin-layer chromatography, electrospray ionization mass spectrometry and gas chromatography/mass spectrometry. Phosphatidylglycerol, phosphatidylethanolamine, cardiolipin, phosphatidylcholine and lyso-phosphatidylethanolamine were found in *F. novicida*. These glycerophospholipids contain fatty acids with broad range of chain lengths, and acyl chains have unusually different length in a single glycerophospholipid molecule. The special fatty acyl composition of glycerophospholipids in *F. tularensis* could potentially be used to detect the bacteria.

1. Introduction

Cell membranes are very important for the survival of cells. They are composed of lipids and proteins; the former makes membranes impermeable to most water-soluble solutes, and the latter serves as transporters and signaling devices. The characteristics of the lipid matrix depend upon the physical properties of the individual lipid components in the membrane. Membrane phospholipids composition is closely related to the structure and function of membrane proteins and the function of the cell [1,2]. Therefore, it is important to identify the lipid composition in different cell membranes.

Francisella tularensis, a Gram-negative bacterium, is a highly infectious human pathogen that causes tularemia, but the lipids in their membranes have not been well characterized. Previously, we have demonstrated that lipid A in membranes of *F. tularensis* subsp. *novicida* is related to the bacterial infection ability [3–5]. In this study, we have extracted glycerophospholipids from *F. novicida*, purified to homogeneity, and analyzed using thin-layer chromatography (TLC), electrospray ionization mass spectrometry/tandem mass spectrometry (ESI/MS/MS), and gas chromatography/mass spectrometry (GC/MS). Phosphatidylglycerol, phosphatidylethanolamine, cardiolipin, phosphatidylcholine and lyso-phosphatidylethanolamine were found in *F. novicida*. These lipids contain fatty acids with a broad range of chain lengths. Interestingly, the two acyl chains on some of the glycerophospholipids are very different in chain length. The special structure and composition of glycerophospholipids in *F. tularensis* may potentially be utilized to detect the bacteria.

2. Experimental procedures

2.1. Materials

Glass backed 0.25 mm Silica Gel 60 thin-layer chromatography plates were from E. Merck, Darmstadt, Germany. Chloroform, ammonium acetate, and sodium acetate were obtained from EMD Science, Gibbstown, NJ. Pyridine, methanol, and formic acid were from Mallinckrodt, Phillipsburg, NJ. Trypticase soy broth, yeast extract and tryptone were purchased from Difco, Detroit, MI. DEAE cellulose was purchased from Whatman, Florham Park, NJ. $^{32}P_i$ was purchased from NEN Life Science Products.

2.2. Isolation of lipids from ^{32}P-labeled cells

The ^{32}P-labeled lipids were isolated from *E. coli* and *F. novicida* as previously published [3]. Typically, 20 ml cells, inoculated from

Abbreviations: PE, phosphatidylethanolamine; Lyso-PE, lyso-phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PC, phosphatidylcholine; ESI/MS/MS, electrospray ionization mass spectrometry/tandem mass spectrometry; GC/MS, gas chromatography/mass spectrometry; TLC, thin-layer chromatography.

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an overnight culture to A_{600} = 0.02, were grown in the presence of 5 Ci/ml ^{32}Pi to A_{600} of 1.2. The cells were collected by centrifugation and washed with phosphate-buffered saline. The cell pellets were resuspended in 3 ml of a single-phase Bligh–Dyer mixture [7] consisting of chloroform, methanol and water (1:2:0.8, v/v/v), incubated at room temperature for 60 min, and centrifuged to remove insoluble debris. The supernatant was removed and converted to a two-phase Bligh–Dyer system by adding chloroform and water to generate a mixture consisting of chloroform, methanol and water (2:2:1.8, v/v/v). The upper phases were washed once with a fresh pre-equilibrated Bligh–Dyer lower phases. The lower phases were pooled and dried under a stream of nitrogen. The ^{32}P-labeled lipids were re-dissolved in chloroform and methanol (4:1, v/v) and spotted onto a Silica Gel 60 TLC plate (10,000 cpm per lane), which was developed in a solvent mixture consisting chloroform, pyridine, 88% formic acid and water (50:50:16:5, v/v/v/v). After drying, the plates were exposed to a PhosphorImager Screen overnight, and the ^{32}P-labeled lipids were detected with a Molecular Dynamics Storm PhosphorImager.

2.3. Extraction and purification of phospholipids from F. novicida U112

One liter of *F. novicida* U112 cells were grown at 37 °C in TSB-C medium (3% trypticase soy broth and 0.1% cysteine [6]), harvested by centrifugation, and washed with phosphate-buffered saline. About 5 g wet cells were obtained. The cells were extracted for 1 h at room temperature with 1 l of a single-phase Bligh–Dyer mixture [7] and centrifuged to remove insoluble debris. The supernatant was converted to a two-phase Bligh–Dyer system, and the lower phases were dried by rotary evaporation. About 130 mg lipids were obtained from the initial supernatant.

The dried lipids were dissolved in chloroform, methanol and water (2:3:1, v/v/v), and applied to a 2 ml DEAE-cellulose column in the acetate form equilibrated with the same solvent. The run-through was saved. The column was washed with 10 column volumes of chloroform, methanol and water (2:3:1, v/v/v). The various lipid components were then eluted stepwise with 5 column volumes of chloroform, methanol and ammonium acetate (2:3:1, v/v/v) with ammonium acetate concentrations of 60 mM, 120 mM, 240 mM, 360 mM and 480 mM, respectively. Fractions equal to one column volume were collected, and 20 μl of each fraction was spotted onto a TLC plate to monitor the lipid elution profile. The plates were developed in the solvent of chloroform, methanol, acetic acid and water (25:15:4:4, v/v/v/v), and the lipids were visualized by spraying the plates with 10% sulfuric acid in ethanol, followed by charring. The fractions were pooled according to their lipid contents and converted to two-phase Bligh–Dyer mixtures. The lower phases were recovered and dried by rotary evaporation.

To further purify the lipid species, preparative thin-layer chromatography was employed. Lipids obtained from the DEAE column were dissolved in chloroform, methanol (4:1, v/v) and applied to a TLC plate, which was developed with chloroform, methanol, pyridine, acetic acid, water (25:10:5:4:3, v/v/v/v/v). While the plates were drying, the lipid bands could be seen transiently as white zones. These bands were marked with a pencil and scraped off after the plates were dry. The silica chips were extracted with a single-phase Bligh–Dyer mixture for 1 h at room temperature. The suspension was centrifuged. The supernatant was passed through a small column fitted with a small glass wool plug and converted into a two-phase Bligh–Dyer system. The two phases were separated by centrifugation, and the lower phase was dried. The total lipids and the purified lipids were analyzed by ESI/MS/MS.

2.4. ESI/MS/MS analysis

All MS/MS spectra were acquired on a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems Inc., Foster City, CA, USA) equipped with an ESI source. Lipid samples were dissolved in chloroform and methanol (2:1, v/v) at 25 μg/ml and subjected to ESI/MS in the negative ion mode. Nitrogen was used as collision gas for MS/MS experiments. Data acquisition and analysis were performed using the instrument's Analyst QS software.

2.5. The extraction of fatty acids from the total lipids

About 10 mg total lipids extracted from *F. novicida* were hydrolyzed by adding 3.8 ml single-phase Bligh–Dyer mixture consisting of chloroform, methanol, and 6 M NaOH (1:2:0.8, v/v/v) and incubating at room temperature for 1 h. The reaction mixture was then neutralized by adding 3.8 ml mixture consisting of chloroform, methanol, and 6 M HCl (1:2:0.8, v/v/v). After adding 2 ml distilled water and 2 ml chloroform, the system was converted to a two-phase Bligh–Dyer mixture. The lower phase, containing the released fatty acids, was dried under a stream of nitrogen.

2.6. GC/MS analysis

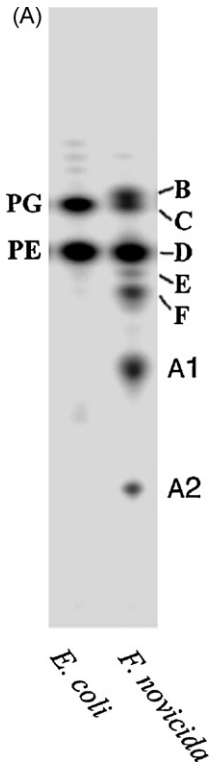
The dried fatty acids were mixed with 50 μl bistrimethylsilyl trifluoroacetamide. The mixture was incubated at 70 °C for 30 min, then dried under a stream of nitrogen. The samples were dissolved in 100 μl hexane for the GC/MS analysis. The fatty acid analysis was performed on a gas chromatography/mass spectrometer (1200L-GC/MS, Varian, USA). A silica capillary column (Varian VF-5MS) was used for the separation of fatty acids. After applying onto the column, the samples were held at 120 °C for 0.5 min, then the temperature was increased to 220 °C at 10 °C/min, to 240 °C at 5 °C/min, to 280 °C at 12 °C/min and held at 280 °C for 7 min. Helium was used as the carrier gas at a constant flow rate of 0.8 ml/min. The mass spectrometer was operated in the electron impact mode at 70 eV with the scan range of 50–500 *m*/*z*. The data acquisition and analysis were performed using the Varian workstation software. The fatty acids were identified by database searching.

3. Results

3.1. TLC and MS analysis of total lipids of F. novicida

E. coli and *F. novicida* were grown to late-log phase with or without $^{32}P_i$. The ^{32}P-labeled lipids extracted from both bacteria were separated on TLC (Fig. 1A). Two major lipid species were detected from the *E. coli* sample. In the *F. novicida* sample, however, seven different lipid species were detected; they are labeled as A1, A2, B, C, D, E and F, respectively (Fig. 1A). Based on the intensity of radioactivity, the percentages of A1, A2, B, C, D, E and F in the total lipids of *F. novicida* were estimated and shown in Fig. 1B. The most abundant lipid species (49%) is D; while the least abundant (1%) is E. A1 and A2, making up 17% of the total lipids, have been previously confirmed as lipid A species [3]. Similar separation pattern on TLC was observed for the total lipids extracted from unlabeled cells of *E. coli* and *F. novicida* (data not shown), suggesting that these lipids are the major lipids in membranes of *F. novicida*.

The total lipids extracted from unlabeled cells of *E. coli* and *F. novicida* were analyzed by ESI/MS in the negative ion mode (Fig. 2). Based on MS/MS data analysis, the major ions in the spectrum of *E. coli* sample were derived from phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), consistent with the observation of two lipid species separated on TLC (Fig. 1A). Since PE is known as the most dominant phospholipid in the membrane composition of



(B)

Labels	ID	Percentage (%)
B	CL	5
C	PG	14
D	PE	49
E	Lyso-PE	1
F	PC	14
A1	Lipid A	15
A2	Lipid A	2

Fig. 1. Analysis of lipids extracted from *E. coli* and *F. novicida*. (A) TLC analysis of lipids extracted from uniformly ^{32}P-labeled *E. coli* or *F. novicida* by the method of Bligh and Dyer [7]. Lipids were separated by using solvent of chloroform, pyridine, 88% formic acid and water (50:50:16:5, v/v/v/v) and visualized with a PhosphorImager. About 10,000–15,000 cpm of ^{32}P-labeled lipids was loaded on each lane. (B) The percentage of the seven lipid species identified in *F. novicida*. Previously, A1 and A2 have been characterized as free lipid A molecules [3]. Glycerophospholipids CL, PG, PE and PC were identified in this work by ESI/MS/MS analysis. The percentage of lipids was estimated according to their radioactivity on TLC.

E. coli [8], the major lipid species (the lower band) on the TLC of *E. coli* sample should be PE, and the upper lipid band PG (Fig. 1A). Because they migrate at the similar rate to *E. coli* PG and PE on TLC, lipid species C and D in *F. novicida* could also be PG and PE, respectively (Fig. 1A).

More lipid species exist in *F. novicida* as judged by MS/MS analysis, consistent with the multiple lipid species detected on TLC (Fig. 1A). For example, in the range of *m*/*z* 600–900, more than 30 different peaks were observed in the ESI/MS spectrum of *F. novicida* lipid sample (Fig. 2B). Based on the MS/MS analysis, ions at *m*/*z* 536.43 and 562.45 were derived from lyso-phosphatidylethanolamine (lyso-PE) molecules which contain only one single fatty acyl chain; ions at *m*/*z* 688.47, 690.46, 702.49, 716.47, 718.47, 732.51, 734.51, 744.54 and 746.54 were derived from PE; ions at *m*/*z* 693.48, 721.47, 749.51, 773.53, 775.53, 777.53, 801.56, 803.56, 829.58, 831.59, 857.63, 859.63 and 887.66 were derived from PG; minor doubly-charged ions at *m*/*z* 647.45 and 675.46 were derived from cardiolipin (CL).

Compared with the mass spectrum of *E. coli* lipids, *F. novicida* lipids yield more ions in the *m*/*z* ranges of 500–600 and 800–900 (Fig. 2). Based on the MS/MS analysis, the ions in the *m*/*z* 500–600 are mainly derived from lyso-PE and ions in the *m*/*z* 800–900 are mainly derived from PG. All these molecules contain longer fatty acyl chains. To further confirm this, the fatty acids were extracted from *F. novicida* lipids, and analyzed by GC/MS (Fig. 3). About 13 fatty acid species were detected. Among them, eight are saturated, three mono-unsaturated, and two are hydroxyl fatty acids. The hydroxyl fatty acids are likely derived from the free lipid A molecules in *F. novicida* [3]. The most dominated fatty acid (18%) is hexadecanoic acid (C16:0). The long chain fatty acids (C20:0, C22:0, C24:0 and C24:1), however, make up 47% of the total fatty acids, consistent with the MS/MS analysis.

3.2. Separation of *F. novicida* lipids by DEAE column chromatography

To identify the phospholipids composition in *F. novicida*, a large scale extraction of lipids from *F. novicida* was performed. The total lipids extracted were fractionated by DEAE column. The lipids in

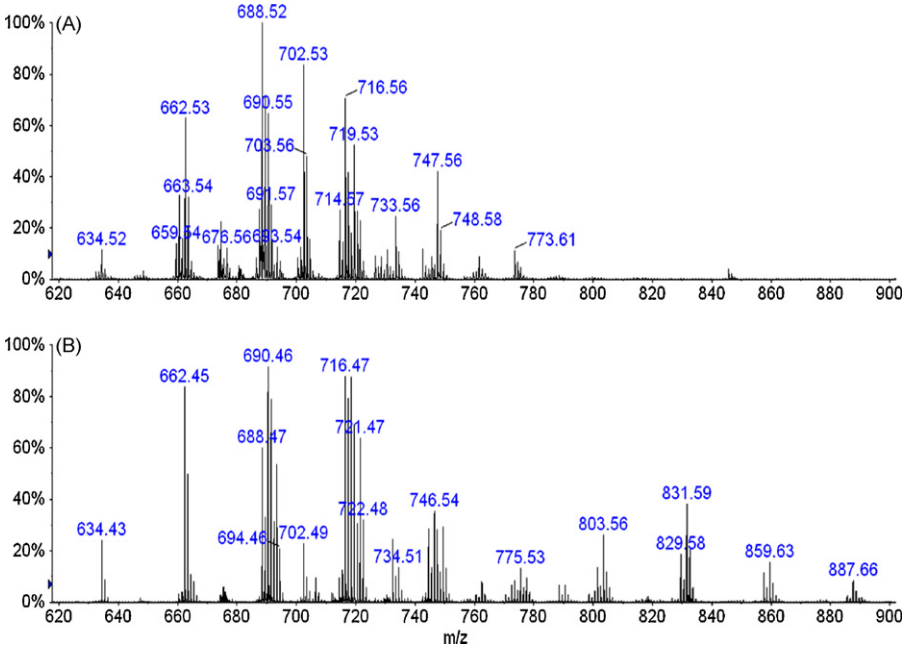


Fig. 2. Negative ion ESI/MS spectra of the total lipids extracted from *E. coli* (A) and *F. novicida* (B). The lipids were extracted from the bacteria by the method of Bligh and Dyer [7]. Only partial spectra in the range of *m*/*z* 500–900 were shown. All the major ions shown in the spectra were identified by MS/MS.

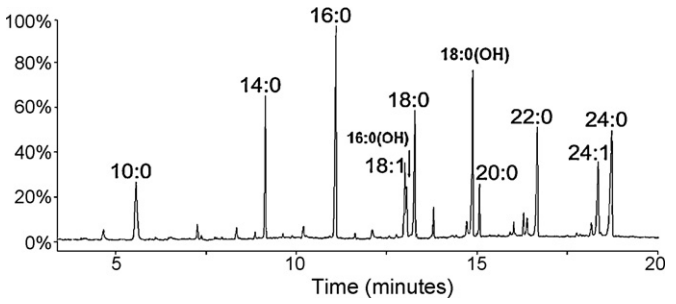


Fig. 3. The GC/MS analysis of the total fatty acids extracted from *F. novicida*. The total lipids extracted from *F. novicida* were hydrolyzed, and the released free fatty acids were analyzed by GC/MS.

different fractions were analyzed by TLC. Lipid species A1, A2, D, E and F were observed in the run-through fraction, lipid species C were eluted in the fraction containing 60 mM ammonium acetate, and lipid species B mainly in the fraction containing 120 mM ammonium acetate. These three lipid fractions were analyzed by using ESI/MS (Fig. 4); the major ions were subjected to MS/MS analysis. Lipid species A1, A2, D, E and F did not retain on the DEAE column, suggesting that they are either neutral or positively changed. Based on the MS/MS data, the major ions in the run-through fraction at *m*/*z* 634.43, 662.46, 688.48, 690.47, 702.49, 716.49, 718.49, 732.50, 734.50, 744.55 and 746.55 (Fig. 4A) were derived from PE (including lyso-PE) species. The ions corresponding to A1 and A2 [3] were also observed in the run-through fraction at *m*/*z* 1665.4 and 1827.5, respectively (data not shown).

Although lipid species B and C cannot be well separated on the TLC (Fig. 1A), they can be separated on the DEAE column. Lipid species C were observed in the fraction of 60 mM ammonium acetate, while lipid species B were observed in the fraction of 120 mM ammonium acetate. In the ESI/MS spectrum of the 60 mM fraction (Fig. 4B), based on MS/MS analysis, the major ions at *m*/*z* 665.42, 693.43, 719.45, 721.46, 747.48, 749.48, 773.52, 775.52, 777.52, 801.54, 803.54, 829.56, 831.56, 857.61, 859.61 and 887.65 were identified as PG species; in the ESI/MS spectrum of the 120 mM fraction (Fig. 4C), the major doubly-charged ions at *m*/*z* 647.45, 661.45, 675.46, 689.47, 703.47 and 730.51 were identified as CL species, and the singly-charged ions at *m*/*z* 693.46, 721.47, 749.51 and 803.57 were derived from the residual PG species. These data suggest that the lipid species B and C of *F. novicida* observed on the TLC (Fig. 1) are CL and PG, respectively.

3.3. Identification and characterization of phosphatidylethanolamine in F. novicida

For lipid species A1, A2, D, E and F existed in the run-through fraction of the DEAE column, we know that A1 and A2 are lipid A species, and PE and lyso-PE species also exist, but the identification of D, E and F are not clear. To this end, D, E and F lipid species were purified by prep-TLC to homogeneity, and analyzed by ESI/MS/MS. The spectrum of the purified lipid species D showed the similar patterns to that of flow-through fraction (Fig. 4A), containing the major ions at *m*/*z* 634.43, 662.46, 688.48, 690.47, 702.49, 716.49, 718.49, 732.50, 734.50, 744.55 and 746.55, suggesting that lipid species D is PE. Further MS/MS analysis showed that *F. novicida* PE molecules contain much longer fatty acyl chains than those of *E. coli*, and prefer unsymmetrical fatty acid chains distribution on a single glycerol backbone. For example, the ion at *m*/*z* 690.47 contains fatty acids C10:0/C22:0 or C14:0/C18:0, the ion at *m*/*z* 716.49 contains fatty acids of C10:0/C24:1, and the ion at *m*/*z* 718.49 contains fatty acids of C10:0/C24:0.

In the ESI/MS spectrum of the purified lipid species E, major peaks at *m*/*z* 536.37 and 562.36 were derived from lyso-PE (Fig. 5A). This was confirmed by high resolution ESI/MS/MS analysis of fragment ions at *m*/*z* 536.37 (Fig. 5B) and 562.36 (Fig. 5C). In both spectra, the peak at *m*/*z* 78.97 confirms the presence of a phosphate group $[PO_3]^-$, while peaks at *m*/*z* 140.02 and 196.05 were derived from ions $[HPO_4CH_2CH_2NH_2]^-$ and $[CH_2C(OH)CH_2PO_4CH_2CH_2NH_2]^-$, respectively, confirming the presence of a PE head group. The only prominent peaks at *m*/*z* 339.34 and 365.37 in both spectra are the carboxylic anions of the C22:0 and C24:1 fatty acids, respectively.

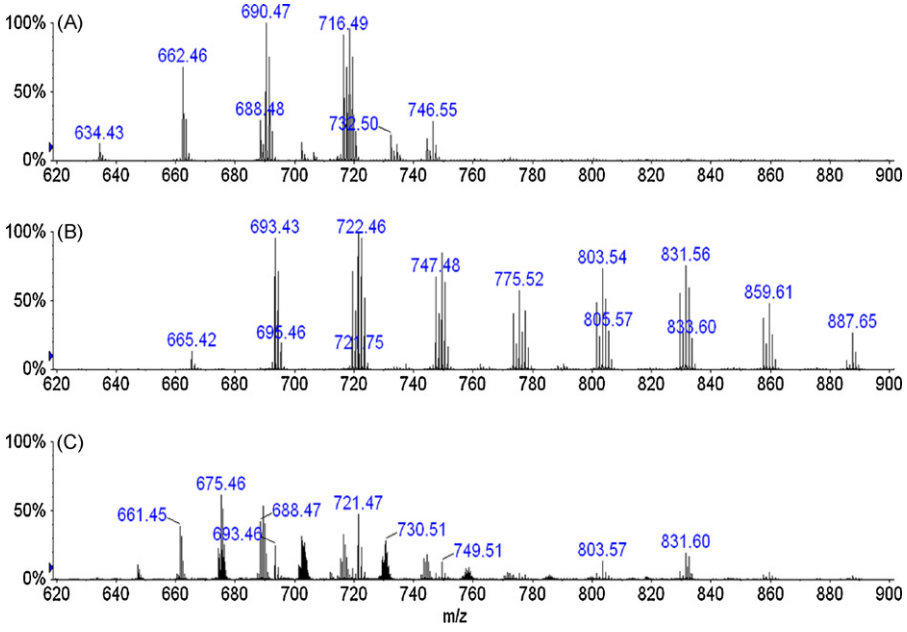


Fig. 4. Negative ion ESI/MS spectra of *F. novicida* lipids in different fractions of DEAE column. Only partial spectra in the range of *m*/*z* 500–900 were shown. (Panel A) The spectrum of lipids in the run-through fraction. (Panel B) The spectrum of lipids in the 60 mM fraction. (Panel C) The spectrum of lipids in the 120 mM fraction.

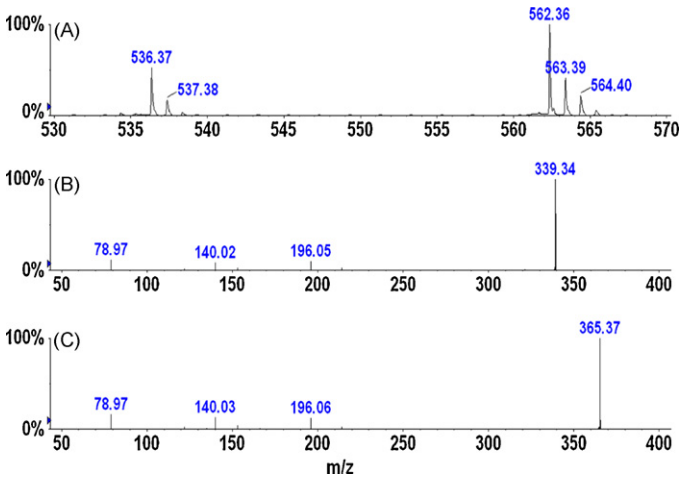


Fig. 5. Structure identification of lyso-PE extracted from *F. novicida*. (Panel A) The ESI/MS spectrum of the purified lyso-PE from *F. novicida*. (Panel B) The ESI/MS/MS analysis of the ion at *m*/*z* 536.4 in Panel A. (Panel C) The ESI/MS/MS analysis of the ion at *m*/*z* 562.4 in Panel A.

3.4. Identification and characterization of phosphatidylcholine in *F. novicida*

The negative ion ESI/MS spectrum of lipid species F was not informative, as a result ESI/MS was carried out in the positive ion mode (Fig. 6A). The difference between the *m*/*z* values of major ions in the spectrum is 28 or 26, suggesting these ions may contain fatty acids with 2 carbon difference. The major molecular ion at *m*/*z* 784.55 was further analyzed by high resolution ESI/MS/MS (Fig. 6B). The fragment ion at *m*/*z* 146.98 is a characteristic ion for the phosphocholine moiety (sodiated cyclophosphane). The ion at *m*/*z* 784.55 is the sodiated molecular ion $[M+Na]^+$. The ion at *m*/*z* 725.48 is a fragment ion, $[M+Na-59]^+$, by losing the trimethylamine from the molecular ion. Other major fragment ions at *m*/*z* 601.49 and 579.51 result from loss of non-sodiated and sodiated choline phosphate, $[M+Na-183]^+$ and $[M+Na-205]^+$, respectively. Similar ESI/MS/MS patterns of the other major ions at *m*/*z* 700.43, 728.46, 756.51, 784.55, 812.58, 838.60 and 866.63 were observed (data not shown), suggesting these ions were all derived from sodiated phosphatidylcholine (PC). Because PC contains the positively charged quaternary nitrogen in the head group, it could not be detected by ESI/MS in negative ion mode. The identification of lipid species F as PC is consistent with its absence in *E. coli* sample (Fig. 1A), and its flow through the DEAE column.

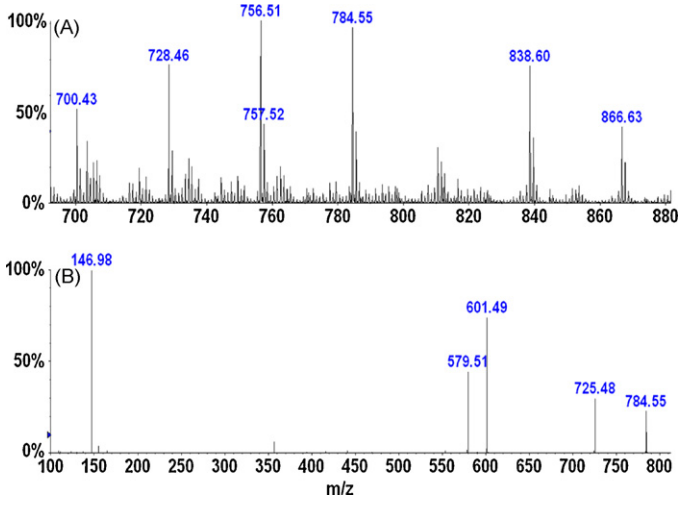


Fig. 6. Structure identification of PC extracted from *F. novicida*. (Panel A) The positive ion ESI/MS spectrum of the purified PC from *F. novicida*. (Panel B) The positive ESI/MS/MS spectrum of the ion at *m*/*z* 784 in Panel A.

4. Discussion

Membrane lipids in Gram-negative bacteria mainly include glycerophospholipids and lipid A. Glycerophospholipids make up the inner membrane and the inner layer of outer membrane, while lipid A makes up the outer layer of the outer membrane. Different types of bacteria make different types of membrane lipids, suggesting that these lipids have functions other than as the building blocks of the cell barrier. In this study, we analyzed the glycerophospholipids in *F. novicida*, using TLC purification followed by ESI/MS analysis. This method takes longer time but gives more accurate results than HPLC/MS. The later method is fast and simple, therefore can be developed to detect bacteria. Except for PE and PG which are made by most Gram-negative bacteria such as *E. coli*, *F. novicida* also makes PC, CL and lyso-PE.

PC which is usually not synthesized in most Gram-negative bacteria such as *E. coli* was detected in *F. novicida* in this study. PC is the major structural constituents of the eukaryotic membranes and the major source of the lipid second messenger involved in signal transduction pathways. PC has been found in a number of prokaryotes that establish close interactions with eukaryotes, such as symbionts, pathogens, and photosynthetic bacteria [9]. PC is required for the survival of the respiratory pathogen *Legionella pneumophila* within host cells [10]. The PC deletion mutant of *Brucella abortus* displays a virulence defect in mice, indicating that PC is necessary to sustain a chronic infection [11]. Amounts of CL in bacteria usually depend on their physiologic state. Only a trace amount of CL is produced during exponential growth, but it may become the most dominant phospholipid under certain conditions [12]. For instance, the levels of CL have been shown to increase in the stationary phase [13], in response to energy deprivation [14], and in response to osmotic stress [15,16]. Accordingly, the expression level of cardiolipin synthase and its catalytic activity increase when *E. coli* cultures reach the stationary phase [17,18]. The observation of CL in *F. novicida* lipid sample but not in *E. coli* lipid sample (Fig. 1A) may be due to their physiologic state and growth conditions. Lysophospholipids are usually bioactive. For example, lysophosphatidic acid and cyclic-phosphatidic acid stimulate a variety of responses that include cell survival, proliferation, migration, invasion, wound healing, and angiogenesis.

Only PE (76%) and PG (24%) were reported as lipids of *F. tularensis* LVS grown in a chemically defined medium [19]. It is necessary to analyze the lipid profiles in other subspecies of *Francisella* to see if they have the same pattern as that in *F. novicida*. Growth media and phase conditions might be also be considered. Nevertheless in this study we showed that *F. novicida* clearly makes specific phospholipids which are not found in the model Gram-negative bacterium *E. coli*. To elucidate the important role of these specific lipids in *Francisella*, more studies on all subspecies of *Francisella* are need to be done.

5. Conclusions

In this study, we have used ESI/MS to analyze the glycerophospholipids in membranes of *F. novicida*. Phosphatidylglycerol, phosphatidylethanolamine, cardiolipin, phosphatidylcholine and lyso-phosphatidylethanolamine were found. These glycerophospholipids contain fatty acids with broad range of chain lengths, and the chain lengths of the two fatty acids on a single glycerophospholipid molecule are unusually different. The special fatty acyl composition of glycerophospholipids in *F. tularensis* could poten-

tially be used to detect the *F. tularensis*, a highly infectious human pathogen.

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