Cellular envelope phospholipids from *Legionella lytica*

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**Keywords**

*Legionella lytica*; phospholipid; branched-chain fatty acid.

**Abstract**

The composition of phospholipids from the cellular envelope of *Legionella lytica* grown on artificial medium was determined by two-dimensional thin-layer chromatography. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidyl-N-monomethyllethanolamine were the predominant phospholipids, while diphosphatidylglycerol, phosphatidylglycerol, and phosphatidyl-N,N-dimethylethanolamine were present at low concentrations. A trace amount of lipids carrying glycosyl residues was also observed. The fatty acids and their distribution in individual phospholipids were characterized using liquid chromatography/mass spectrometry (LC/MS), matrix-assisted laser desorption ionization-time of flight, and gas chromatography/MS methods. The characteristic feature of *L. lytica* phospholipids was the presence of an unbranched chain (which differentiates this bacterium from *Legionella pneumophila*) and branched *iso* and *anteiso* fatty acids as well as cis-9,10-methylenehexadecanoic acid. According to spectroscopic LC/MS data, the localization of saturated and unsaturated fatty acid residues on phosphatidylglycerol was determined. Some aspects of the significance of phosphatidylcholine, one of the main phospholipids in *L. lytica*, are addressed and taxonomic implications of the data are discussed.

**Introduction**

*Legionella lytica* is a Gram-negative aerobic, rod-shaped bacterium that is motile by means of three to five polar flagella (Drożanski, 1991). The bacterium appears to survive as an intracellular parasite of free-living amebae (Drożanski, 1963) and also multiplies intracellularly in human monocyte line U937 (B.S. Fields, pers. commun.). The pathogenicity of *L. lytica* for humans was supported by the presence of antibodies from patients with community-acquired pneumonia (Benson et al., 1995; McNally et al., 2000).

The nutrients required by *L. lytica* indicate the need for an intracellular environment. Under laboratory conditions, the bacterium exhibits limited growth on buffered charcoal yeast extract agar supplemented with ferric pyrophosphate, potassium α-ketoglutarate, l-cysteine, and glycine (Giles et al., 1995). These levels of nutrients are rarely found in the environment and, if present, they only serve to amplify faster growing bacteria that compete with *L. lytica*. However, ‘necrophitic’ growth of *Legionella pneumophila* on heat-killed bacterial cells or in a biofilm has been described recently (Temmerman et al., 2006).

*Legionella lytica* belongs to the γ subclass of the *Proteobacteria* and is closely related to other *Legionellae* species (Springer et al., 1992; Adeleke et al., 1996; Birtles et al., 1996; Hookey et al., 1996). All legionellae share phenotypic characteristics that include absence of growth on blood agar, nitrate not being reduced, negative reaction for urease, and requirement for l-cysteine and iron salts. They are chemoorganotrophic, using amino acids as carbon and energy sources, and do not ferment or oxidize carbohydrates. Branched-chain fatty acids predominate in their cell envelope (Brenner, 1986; Benson & Fields, 1998).

*Legionella lytica* and other legionellae have an intracellular lifestyle within macrophages and protozoa (Abu Kwaik et al., 1998). The pathogenicity of intracellular parasites, such as legionellae, largely depends on the components of the cell envelope: proteins, peptidoglycan, and lipids (Dowling et al., 1992). The inner leaflet of the bacterial outer membrane consists of a phospholipid bilayer. The major structural and functional components of their inner membrane are also phospholipids.

Both *Escherichia coli*, the Gram-negative model bacterium, and Gram-positive *Bacillus subtilis*, contain...
phosphatidylethanolamine, phosphatidylglycerol, and di-
phosphatidylglycerol as major membrane phospholipids. Therefore, it was thought that this holds true for most bacteria. However, a recently reported estimation suggests that probably more than 10% of all bacteria, principally those that associate with eukaryotic cells, contain phospha-
tidylylcholine as a major membrane phospholipid (Sohlen-
kamp et al., 2003).

Phosphatidylylcholine is the major phospholipid in eukar-
yotic cells. It is a structural constituent of membranes and plays an important role in signal transduction as a major source of lipid second messengers (Exton, 1994). In eukar-
yotic organisms, phosphatidylylcholine can be synthesized by two alternative biosynthetic pathways: the cytidine-5'-di-
phosphate choline (CDP-choline) pathway or the methyla-
tion pathway (Kent, 1995).

The components of the cellular envelope of Legionellaceae – lipopolysaccharides, proteins, and peptidoglycan – have been more or less thoroughly described (Hindahl & Iglewski, 1984; Zähringer et al., 1995; Steinert et al., 2002). The cellular membrane structure and its phospholipid composition, as well as the function of phospholipids for L. lytica and for all of the Legionellaceae are still obscure. The total percentages of particular phospholipid classes and the predominant fatty acids in each class described for L. pneumophila (Finnerty et al., 1979) are the only literature information available on the phospholipid composition of this bacterial family.

The aim of this study was to investigate the chemical structure of phospholipid species from L. lytica, especially the composition of fatty acids bound to the glycerol backbone of these molecules, and to determine whether the fatty acids characteristic of L. lytica phospholipids can differenti-
ate this bacterium from other legionellae.

Materials and methods

Organism and growth conditions

Legionella lytica, strain L2T (PCM 2298), formerly named Sarcobium lyticum, was isolated from soil. The bacterium was grown on buffered charcoal yeast extract agar medium at 32 °C for 3 days (Edelstein, 1981). The bacterial cells were harvested in the stationary phase of growth. The final cell liofilizate mass was c.1.6 g.

Lipid analysis

The cellular envelope lipids and phospholipids were ex-
tracted (Bligh & Dyer, 1959), yielding 124 mg total lipids (7.75% of cell material), and 20 mg portions of these were separated into fractions according to Kunsman (1970). The neutral lipid fraction (8.1 mg; 45.5% of total lipids) was discarded. The polar lipid fraction (9.7 mg; 54.5% of total lipids) was used for thin-layer chromatography (TLC) and MS analysis.

The two-dimensional TLC analysis was conducted on silica gel 60 F254 plates (Merck, Darmstadt, Germany). A chloroform/methanol/water (14:6:1, v/v/v) mixture was used for the first dimension and chloroform/methanol/ glacial acetic acid (13:5:2, v/v/v) was used for the second dimension. Lipids were visualized with H2SO4 or with iodine vapor, phosphorus using the Bochner method (Bochner et al., 1981), carbohydrates with α-naphthol, amino groups with ninhydrin, and quaternary nitrogen groups with the Dragendorff reagent (Kates, 1986). The unseparated spot of phosphatidylethanolamine and phosphatidyl-N-mono-
ethanolamine was scraped off from iodine-stained material and reseparated on silica gel with n-propanol/ propionic acid/chloroform/water (3:2:2:1, v/v/v/v) (Higgins, 1987). The proportions of individual lipid classes were determined using standard calibration curves (OD of spot vs. standard amount). Synthetic phosphatidylylcholine, phosphatidylethanolamine, phosphatidyl-N,N-dimethylethanolamine, phosphatidylglycerol, and diphosphatidylgly-
erol from bovine heart (Sigma–Aldrich Chemical Co., St. Louis, MO) were used as standards.

Fatty acid analysis

The fatty acids were released from dried lipid extract and converted into methyl esters (Wollenweber & Rietschel, 1990), analyzed on a Hewlett-Packard gas chromatograph (model HP 5890A) equipped with a capillary column (HP-5MS, 30 m x 0.25 mm) and mass selective detector (HP5971). Helium (0.7 mL min−1) was used as carrier gas. The temperature program was as follows: 150 °C for 5 min, then raised to 310 °C at a ramp rate of 5 °C min−1, and held for a further 10 min. The structure of fatty acids was elucidated from the m/z values of their [M]+ and [M-32]+ ions and by comparison of retention parameters with the data obtained for standard bacterial acid methyl ester mixture (C12–C18) (Supelco, Bellefonte, PA). The positions of the branching methyl group, the double bonds, and cyclopropane ring were determined by analysis of fatty acid pyrrollides (Andersson & Holman, 1975).

MS

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS was performed on a Voyager-Elite instrument (PE Biosystems, Foster City, CA) using delayed extraction. The dry lipid extract was dissolved in a CHCl3/ CH3OH mixture (2:1, v/v). The sample constituents, mixed with a 50% solution of 2,5-dihydrobenzoic acid in acetonitrile as a matrix, were desorbed and ionized with a nitrogen laser at an extraction voltage of 20 kV. Each spectrum was the average of about 256 laser shots.
The LC/MS was performed on a Finnigan LCQ Advantage Max ion-trap mass spectrometric system (ThermoElectron Corporation, San Jose, CA), using a 5 μm, 4.6 mm × 250 mm cyan column and acetonitrile–formic acid–ammonia gradient elution. The column effluent was ionized by electrospray (ESI). The ESI needle potential was 4.5 kV in the positive and 4 kV in the negative ionization mode. The instrument was programmed to acquire data-dependent scans up to the fourth stage of fragmentation. More detailed information about LC/MS conditions is contained in supplementary material Appendix S1.

Results

Identification of the main lipid classes by two-dimensional TLC

The chromatographic profiles of polar lipids on a two-dimensional TLC plate are shown in Fig. 1. The number of spots indicated the presence of six distinct lipid classes. Five of them were phospholipids, identified by chromatographic behavior, comigration with authentic standards (marked with a dotted line in Fig. 1), and specific staining properties. The sixth one, reactive with α-naphthol, was found to be a phosphorus-free compound and further analyses of this lipid are in progress. The major phospholipids were phosphatidylcholine and phosphatidylethanolamine, followed by phosphatidyl-N,N-monomethylethanolamine; diphosphatidylglycerol, phosphatidylglycerol, and phosphatidyl-N,N-dimethylethanolamine were relatively minor components (see Table 1).

GC/MS analysis of fatty acid composition

A summary of the isomeric structures of fatty acids contained in the total phospholipid extract and their relative contents is presented in Table 2. The fatty acid composition showed the presence of up to 19 different acids including both unbranched saturated and unsaturated fatty acids as well as branched ones (iso and anteiso). The palmitic and stearic acids were the major normal chain hydrophobic components of the examined lipids. The major branched acids were 12-methyltetradecanoic (a15:0) and 12-methyltridecanoic acid (i14:0). A cyclopropane, cis-9-10-methylenehexadecanoic acid (C17:0cyc), was also found.

Phospholipid identification by MALDI-TOF and LC/MS

The positive ionization MALDI-TOF spectrum, indicative for molecular masses of the individual phospholipids, is presented in Figs 2 and S1. Two clusters were clearly seen in the spectrum. One represents mainly phosphatidylcholines (m/z 660.5–774.7, Fig. 2) and the second one (m/z 1173.7–1255.7, supplementary Fig. S1) may represent diphosphatidylglycerols.

The systematic identification of the class and structure of individual phospholipids was possible by analyzing the parent spectra and fragmentation data obtained by LC/MS/MS in both positive and negative ionization modes. The procedure applied for identification of individual phospholipids for particular phospholipid classes is described in supplementary material Appendix S1. The most abundant sample constituents were phosphatidylcholines: mainly 16:0/15:0, 16:0/14:0, 18:0/16:1, and 17:1/15:0 or 17:0cyc/15:0. The major identified phosphatidylethanolamines were 15:0/15:0, 16:0/15:0, and 15:0/14:0, the main phosphatidyl-N,N-dimethylethanolamines 14:0/15:0, 16:0/17:1 or 16:0/17:0cyc, 16:1/15:0, and the major phosphatidyl-N,N-dimethylethanolamine 17:1/17:0 or 17:0cyc/17:0. For ethanolamine phospholipids, the highest overall peak intensities were for phosphatidylethanolamines, whereas the lowest were for phosphatidyl-N,N-dimethylethanolamines. Phosphatidylglycerol 14:1/14:0 was also detected. The higher peaks in the second ion cluster on the MALDI spectrum (supplementary Fig. S1) were provisionally identified as sodium adducts of diphosphatidylglycerols 50:3, 51:3, 52:4, 52:3, 53:4, 53:3, 54:4, 54:3, 55:5, 55:4, and 56:4.
Table 1. Class identification and relative amounts of phospholipids extracted from Legionella lytica

<table>
<thead>
<tr>
<th>Spot number (on TLC plate)</th>
<th>Reagent*</th>
<th>Identification</th>
<th>Relative amount (weight %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>P</td>
<td>D</td>
</tr>
<tr>
<td>1</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>3A</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>3B</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Reagents: N, ninhydrin; P, molybdenum blue (reagent for phosphorus); D, Dragendorff reagent; C, α-naphthol (reagent for sugars); (+), positive reaction; (−), lack of reaction.

†Mean value ± SD, n = 3. The weight of each phospholipid class was determined from standard calibration curves and expressed as percent of total weight.

Table 2. Fatty acid composition of total phospholipids from Legionella lytica determined by GC/MS analysis of fatty acid methyl esters

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Nature of fatty acids*</th>
<th>Relative content (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.81</td>
<td>a13:0</td>
<td>0.4</td>
</tr>
<tr>
<td>10.91</td>
<td>i14:0</td>
<td>6.2</td>
</tr>
<tr>
<td>11.75</td>
<td>n14:0</td>
<td>4.1</td>
</tr>
<tr>
<td>13.19</td>
<td>i15:0</td>
<td>0.2</td>
</tr>
<tr>
<td>13.44</td>
<td>a15:0</td>
<td>26.2</td>
</tr>
<tr>
<td>14.03</td>
<td>n15:0</td>
<td>1.5</td>
</tr>
<tr>
<td>15.43</td>
<td>i16:0</td>
<td>5.3</td>
</tr>
<tr>
<td>16.27</td>
<td>n16:0</td>
<td>18.9</td>
</tr>
<tr>
<td>17.54</td>
<td>i17:0</td>
<td>0.2</td>
</tr>
<tr>
<td>17.74</td>
<td>a17:0</td>
<td>4.8</td>
</tr>
<tr>
<td>18.31</td>
<td>n17:0</td>
<td>1.9</td>
</tr>
<tr>
<td>19.58</td>
<td>i18:0</td>
<td>2.3</td>
</tr>
<tr>
<td>20.32</td>
<td>n18:0</td>
<td>6.3</td>
</tr>
<tr>
<td>21.69</td>
<td>a19:0</td>
<td>1.4</td>
</tr>
<tr>
<td>Unsaturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.48</td>
<td>14:1†</td>
<td>1.8</td>
</tr>
<tr>
<td>13.67</td>
<td>15:1o6c</td>
<td>0.7</td>
</tr>
<tr>
<td>15.8</td>
<td>16:1o7c</td>
<td>9.6</td>
</tr>
<tr>
<td>17.94</td>
<td>17:1†</td>
<td>0.9</td>
</tr>
<tr>
<td>Cyclopropane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.03</td>
<td>17:0cyc</td>
<td>7.3</td>
</tr>
<tr>
<td>Total branched</td>
<td></td>
<td>54.3</td>
</tr>
<tr>
<td>Total unbranched</td>
<td></td>
<td>45.7</td>
</tr>
</tbody>
</table>

*Numbers before colons refer to the number of carbon atoms; number after colons refer to the number of double bonds; a, methyl branch at the anteiso carbon atom; i, methyl branch at the iso carbon atom; n, unbranched acid; o, double bond position from the hydrocarbon end of the carbon chain; cyc, cyclopropane ring structure.

†Uncorrected relative area (%).

Discussion

The characteristic chemotaxonomic feature of Legionellaceae (Finney et al., 1979) is the significant content of branched fatty acids in their cellular envelope. The results obtained confirmed the considerable amount (54%) of these fatty acids in the phospholipids of L. lytica. Such a quantity of branched acids indicates an interesting metabolic relationship with amino acid metabolism, especially in the case of some of these peaks might represent protonated pseudomolecular ions of diphosphatidylglycerols 53:0, 54:0, 55:0, 56:1, 56:0, and 57:0. The lack of negative and doubly charged positive ions in respective parts of MALDI and LC/MS spectra and too small signal intensities to register fragmentation information made the definite identification impossible.

The full structural data for individual compounds, constituting the phospholipid profile of L. lytica, are summarized along with the LC/MS data in supplementary Table S1. A kind of tentative illustration of the quantitative relationship between individual L. lytica phospholipids is provided by Figs 3 and S2. These figures show the pseudomolecular ions that fulfill the primary criterion of detection for each of the more abundant phospholipid classes identified in the samples. For phosphatidylcholines, the spectra correspond to neutral loss of m/z 60 from the [M+HCOO]− ion (Fig. 3). The analogous spectra for ethanolamine phospholipids are presented in supplementary Fig. S2.

Two solutions of dry lipid sample were analyzed by LC/MS in order to obtain structural information about more phospholipids. For this reason, Figs 3 and S2 have two parts for each phospholipid class presenting the ions registered for the solutions of the sample in acetonitrile (part a) and the chloroform–methanol mixture (part b). Only a part of the lipid sample was soluble in acetonitrile. Thus, the spectra from part (a) contain the dependent scan signals from some phospholipids that were masked up by some others (better soluble in chloroform) during the analysis of chloroform–methanol solution.

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valine, leucine, and isoleucine, which are the donors of branched α-keto acids for the synthesis of branched-chain fatty acids. While isoleucine is the precursor of anteiso-branched-chain fatty acids, leucine and valine give rise to the primers of iso-branched-chain fatty acids (Kaneda, 1991). According to the GC/MS data (Table 2), the phospholipids from *L. lytica* contain not only branched fatty acids but also 46% of normal chain acids. It is worth mentioning at this point that the isolates from ten other Legionnaires disease bacteria only have branched fatty acids (Finnerty et al., 1979). Thus, the presence of unbranched fatty acids in *L. lytica* phospholipids may be a diagnostic feature of this species. The identified cis-9,10-methylenehexadecanoic acid also occurs in the cells of other legionellae (Diogo et al., 1999).

The two-dimensional TLC results presented in Fig. 1 and Table 1 demonstrate that phosphatidylcholine and phosphatidylethanolamine are the major phospholipid classes of *L. lytica*, whereas phosphatidyl-N-monomethylethanolamine, diposphatidylglycerol, phosphatidylglycerol, and phosphatidyl-N,N-dimethylethanolamine are present as minor components. As indicated by the MALDI and LC/MS data, the contents of individual diposphatidylglycerols in the investigated sample are very low. The low stability of phosphatidylglycerol fragment ions in the ion trap spectrometer can explain the under-representation of this phospholipid class in LC/MS experiments.

The general conclusion arising from the present investigation is the predominance of phosphatidylcholine and phosphatidylethanolamine species among all of the identified phospholipids. This result confirms the presence of the same phospholipid classes as described for *L. pneumophila* (Finnerty et al., 1979).

The simultaneous presence of phosphatidylcholine and phosphatidylethanolamine as the main phospholipids is a striking peculiarity of the cellular envelope of *L. lytica* that clearly distinguishes it from other Gram-negative bacteria. While phosphatidylethanolamine is a common phospholipid in the majority of Gram-negative bacteria, phosphatidylcholine is the dominant phospholipid in eukaryotic membranes and only a few bacteria, principally intracellular replicating, possess this phospholipid.

The presence of both phosphatidylethanolamine derivatives, phosphatidyl-N-monomethylethanolamine and phosphatidyl-N,N-dimethylethanolamine, indicates the existence of a typical bacterial pathway for phosphatidylcholine biosynthesis, in which this phospholipid is formed by three successive N-methylations of phosphatidylethanolamine (Vance & Ridgway, 1988). However, this observation does not exclude the possibility of the existence of additional ways for phosphatidylcholine synthesis, similar to *L. pneumophila*. *Legionella pneumophila* possesses an alternative...
pathway for phosphatidylcholine synthesis that is based on a one-step reaction of free choline condensation directly with CDP-diacylglyceride. This reaction is catalyzed by phosphatidylcholine synthase (Martinez-Morales et al., 2003). The novel enzyme activity enables bacteria (Pseudomonas aeruginosa, L. pneumophila, Borrelia burgdorferi) to produce phosphatidylcholine from exogenous choline, provided by eukaryotic organisms with which they associate (Lopez-Lara & Geiger, 2001). In this way, choline can be used by pathogenic bacteria as a nutrient, an osmoprotectant, or as a building block for membrane lipid biosynthesis causing persistent bacterial infections in their host.

Moreover, as phosphatidylcholine synthase homologs are only found in prokaryotes, for example in the genomes of L. pneumophila (28% amino acid identity), P. aeruginosa (36% amino acid identity), and B. burgdorferi (27% amino acid identity), which are pathogenic to humans, phosphatidylcholine synthase might be an attractive target for drug development. Drugs directed against this enzyme might selectively inhibit bacteria utilizing phosphatidylcholine synthase and might therefore show an antibiotic effect (Sohlenkamp et al., 2003).

The results obtained provide comprehensive information on the phospholipid profile and the nature of fatty acids in the particular phospholipids of L. lytica. This information can be useful in assessing the molecular architecture of the outer membrane and understanding the susceptibility of the organism to various antibiotics.

In conclusion, it is worth stressing that the data obtained constitute the first structural description of phospholipids from bacteria belonging to the Legionellaceae family. The fatty acids pattern and the localization of fatty acids residues, in particular phospholipids, might be used as a taxonomic signature for L. lytica, helpful in identification of this species of bacteria.

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**Supplementary material**

The following supplementary material is available for this article:

Fig. S1. Positive ion mode MALDI-TOF spectrum of the lipid mixture from *L. lytica* (m/z range from 1140 to 1330).
Fig. S2. Pseudomolecular ions undergoing neutral losses in the first fragmentation step under positive ionization.

Table S1. Phospholipids identified by LC/MS in the extract from cells of *L. lytica*.

Appendix S1. Additional information about mass spectrometry conditions and structural identification procedure.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2008.01177.x (This link will take you to the article abstract).

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