ANALYSIS OF PHOSPHOLIPIDS FROM COXIELLA BURNETII BY FAST ATOM BOMBARDMENT MASS SPECTROMETRY. A RAPID METHOD FOR DIFFERENTIATION OF VIRULENT PHASE I AND LOW VIRULENT PHASE II CELLS

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Summary. – Phospholipids extracted from the *Coxiella burnetii* strain Nine Mile virulent phase I and low-virulent phase II cells were directly analyzed by fast atom bombardment mass spectrometry (FAB-MS). Constant neutral loss (CNL) scanning mass spectra (MS) were acquired to identify various phospholipids within phospholipid classes. Phospholipids from the phase I *C. burnetii* cells were much more complex than those from the phase II cells. Moreover, in the latter, the absence of phospholipids of the phosphatidylinositol class could be noticed. The results indicate that CNL scanning of phospholipid samples provides a rapid and simple method for identification of the phase state of the bacterium.

Key words: Coxiella burnetii; phospholipids; FAB-MS; phase state

Introduction

Rapid and reliable detection and identification of microorganisms is of great importance in numerous fields including clinical medicine, public health, food production and processing, biotechnology, biological warfare, etc. Microbiological techniques currently used need often hours and even days to yield a positive identification, which is frequently too late for certain applications. Therefore, a variety of physicochemical techniques has been developed for a fast identification of microorganisms. They rely on the analysis of typical biomarkers, which are indicative of the genus, species, or even strain of the microorganism involved. Among such biomarkers, membrane phospholipids offer several potential advantages. They are present at relatively high concentrations in all living cells, can easily be extracted, and their analysis can be very specific (Shaw, 1974).

The development of FAB and electrospray ionizations has permitted a direct analysis of underivatized glycerophospholipids (Heller *et al.*, 1987; Cole and Enke, 1991; Bryant *et al.*, 1991, Goodacre *et al.*, 1999; Fang *et al.*, 2000). CNL scanning for the polar head functional groups has been shown to be very useful for both the detection and differentiation of other phospholipid classes in complex matrices and mixtures (Cole and Enke, 1991; Heller *et al.*, 1988). In this mode of operation, the only ions that are detected are those that undergo loss of a specific neutral fragment. In the positive ion mode, one of the main fragmentation pathways is cleavage of the phosphate-glycerol bond resulting in the elimination of the polar head group as a neutral species. By a sequential selection of the mass of each class characteristic neutral fragment, a series of mass spectra are obtained for each particular phospholipid species in a complex lipid extract from a microorganism (Heller *et al.*, 1988).

C. burnetii, the etiological agent of Q fever, is found world-wide. The bacterium is unique among *Rickettsiae* in

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Abbreviations: CNL = constant neutral loss; FAB = fast atom bombardment; MS = mass spectrometry

Table 1. Phospholipids detected in the *C. burnetii* strain Nine Mile phase I and II cells using the CNL scan MS method

Phospholipid class	Phase I cells (m/z)	Phase II cells (m/z)
Phosphatidic acid	575.3(27:3), 577.3(27:1), 601.3(29:5), 603.3(29:3), 604.3(29:2), 675.4(34:1)	575.3(27:3), 603.3(29:3)
Phosphatidylethanolamine	744(36:2), 848(44:12)	752(38:5;1-O-Alkyl-2-acyl), 850(44:10)
Phosphatidylserine	784.5(36:4)	784.5(36:4)
Phosphatidylinositol	833.6(34:4), 835(34:2), 837.6(34:0), 849.5(35:4), 851.6(35:2), 859.6(36:5), 861.6(36:3), 873.6(37:8), 875.6(37:6), 877.6(37:4)	
Phosphatidylcholine	732.7(32:1), 734.7(32:0), 748.6(34:0; 1-O-Alkyl-2-acyl), 758.6(34:2), 760.6(34:1), 762.6(34:0), 774.6(36:1; 1-O-Alkyl-2-acyl), 776.6(36:0; 1-O-Alkyl-2-acyl), 786.6(36:2), 788.6(36:0), 802.6(38:1; 1-O-Alkyl-2-acyl)	760.6(34:1), 788.6(36:0)
Phosphatidylglycerol	747.5(34:2), 749.5(34:1)	747.5(34:2), 749.5(34.1), 775.5(36:2), 777.5(36:1)

m/z = a mass to charge ratio.
The values in parentheses represent the xx:y ratios, in which xx = total number of carbon atoms in both radyl groups, and y = total degree of unsaturation.

that it undergoes a host-dependent phase variation (Williams and Waag, 1991; Ftáček *et al.*, 2000). The cells in virulent phase I are isolated from nature whereas the cells in low-virulent phase II are selected by serial laboratory passaging in immunologically incompetent hosts such as eggs or tissue culture cells (Baca and Paretsky, 1983). There are only a few preliminary data on the composition of *C. burnetii* phospholipids (Schramek, 1985). Two glycerophospholipid classes, namely phosphatidylethanolamine and phosphatidylglycerol were detected. Therefore, in our continuing efforts to establish the composition and structure of outer membrane components of *C. burnetii*, we focused our attention on a more detailed characterization of phospholipids present in this bacterium. Moreover, it was of interest to see whether the phase variation of the microorganism affects the composition of its phospholipids.

Materials and Methods

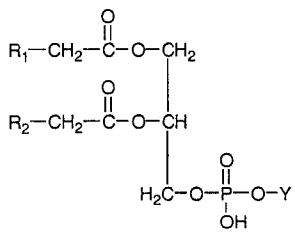
Cultivation and purification of C. burnetii. C. burnetii strain Nine Mile I, serologically in the virulent phase I (yolk sac passage 3 in this laboratory), and the strain Nine Mile II, serologically in the low-virulent phase II (yolk sac passage 162 in this laboratory), were obtained originally from the WHO Collaborating Centre for Rickettsial Reference and Research at the Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic. Both strains were propagated in embryonated hen eggs as described earlier (Toman and Škultéty, 1996; Škultéty *et al.*, 1998). After cultivation, the phase I and phase II cells were purified by a Renografin density gradient centrifugation (Williams *et al.*, 1981).

Isolation of phospholipids was performed as described by Smith *et al.* (1995). Briefly, 500 µl of chloroform-methanol (2:1, v/v) was added to the bacteria (1 mg), the mixture was vortexed for 1 min at room temperature, and 100 µl of water was added. The lower layer was separated and subjected to the analysis by FAB-MS.

FAB-MS. Approximately 10 µg of phospholipids dissolved in chloroform was mixed with a drop of NBA matrix on the FAB probe tip. FAB- and tandem-MS spectra were obtained with a Micromass AutoSpecQ (UK). The instrument with EBEqQ geometry was equipped with a cesium ion gun. The accelerating voltage of 8 kV and the cesium beam intensity of 3 µA at 20 kV were used. In this work, the instrument was employed as an E-B-E instrument. Tandem mass spectra were acquired by selecting the desired ion with the EB section of the mass spectrometer and by colliding the selected ion at 8 kV in the collision cell with a sufficient amount of argon gas to reduce the selected ion beam intensity by approximately 50%. The resulting daughter ions were determined by a scan of the second electric sector. CNL scans were obtained by maintaining a constant $B^2(1-E)/E^2$. The parent spectra were obtained by a linked scan of B^2/E. The data acquisition was carried out with a Micromass OPUS V 3.4X data system interfaced to the mass spectrometer by a VG SIOS unit.

Results and Discussion

As mentioned above, each phospholipid class has a characteristic neutral loss in the FAB mass spectra. Thus, performing the CNL linked scan it is possible to assign the major mass spectra peaks to various glycerophospholipid classes. Table 1 shows the results of those scans performed with phospholipids isolated from the *C. burnetii* phase I and phase II cells. Structures of glycerophospholipid classes are depicted in Fig. 1. From Table 1 it is evident that phospholipids detected in the virulent phase I *C. burnetii* cells are much more complex than those present in the low-virulent phase II cells. In addition, our measurements revealed absence of phospholipids of the phosphatidylinositol class in phase II *C. burnetti*. These are important



Phospholipid Class	Head Group (Y)
Phosphatidic acid	H
Phosphatidylethanolamine	NH_2
Phosphatidylcholine	$N(CH_3)_3$
Phosphatidylglycerol	OH
Phosphatidylserine	OH, NH_2
Phosphatidylinositol	OH

Fig. 1

Structures of glycerophospholipid classes

R_1, R_2, = higher alkyls.

findings since it has been believed (Williams and Waag, 1991) so far that a lipopolysaccharide is the only outer membrane component that undergoes modifications during the phase variation of the microorganism. Nevertheless, it has already been assumed that the phospholipid composition in microorganisms can be influenced by environmental factors (Smith *et al.*, 1995). Modifications of the outer membrane macromolecules enable the bacterium to be a more versatile, more heterogeneous microorganism that can cope better with a variety of different environments (Appelmelk *et al.*, 1998). Environmental cues are probably important parts of this regulatory process. This switching ability should be advantageous within different physiological environments of the host body (Kwan and Isaacson, 1998).

Using relatively simple extraction procedure, phospholipids were isolated from the *C. burnetii* virulent phase I and low-virulent phase II cells and subjected to FAB-MS measurements. Different MS data were obtained for these two cell species, suggesting that the technique used provides a rapid and simple detection method of the phase state of the bacterium under investigation.

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