Modifications in the glycerophospholipid composition between the *Coxiella burnetii* phase I and phase II cells suggest an association with phase variation of the bacterium

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Summary. – Glycerophospholipids (GP) extracted from the *Coxiella burnetii* strain Nine Mile in virulent phase I (NM I) and low virulent phase II (NM II) were analyzed by Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry (MS) that gave a superior mass resolution and mass accuracy allowing unambiguous peak recognition and precise assignment of ions. We showed that GP present in the pathogen's outer membrane underwent considerable modifications during the phase variation that might be related to impact of various environmental factors. It was found that GP from phase I cells were much more complex than those from phase II cells. While glycerophosphoethanolamines (PE), glycerophosphocholines (PC) and glycerophosphoglycerols (PG) were present in both phases of *C. burnetii*, major differences were observed in the presence of glycerophosphates (PA) and glycerophosphoserines (PS). Thus, PA but no PS were detected in NM I variant in contrast with NM II cells where PS but no PA were identified. It is suggested that enzymes for PA head group modifications to form PS, PE, and PG become active during the phase variation of the bacterium.

Introduction

Coxiella burnetii is the etiological agent of Q fever, a zoonosis which is capable of being transmitted from animals to humans and which is endemic throughout the world (Marrie and Raoult, 1997; Arricau-Bouvery and Rodolakis, 2005). The most common acute form of the disease is manifested in humans as a self-limited febrile illness or pneumonia, or less frequently as hepatitis. Persistent infection can lead to a chronic form of Q fever, which may be associated with a fatal endocarditis (Marrie and Raoult, 1997). In animals, Q fever affects livestock and is associated with pneumonia and reproductive disorders like abortion, stillbirth, placentitis, endometritis and infertility (Arricau-Bouvery and Rodolakis, 2005).

C. burnetii, an obligate intracellular parasite of eukaryotic cells, is a pleomorphic coccobacillus with a gram-negative cell wall (Baca and Paretsky, 1983). The envelope surrounding *C. burnetii* is comprised of two membranes (McCaul and Williams, 1981). Both leaflets of the inner membrane are composed of phospholipids (PL), while the outer leaflet of the outer membrane is dominated in naturally occurring virulent phase I cells by a lipopolysaccharide (LPS) that is considered to be one of the major factors of virulence expression and infection of the bacterium (Toman *et al.,* 2009). The primary function of PL is the formation of a semipermeable

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Abbreviations: CL = cardiolipin; FT-ICR = Fourier transform ion cyclotron resonance; GP = glycerophospholipids; MS = mass spectrometry; *m/z* = mass-to-charge ratio; NM I = strain Nine Mile in virulent phase I; NM II = strain Nine Mile in low virulent phase II; PA = glycerophosphates; PC = glycerophosphocholines; PE = glycerophosphoethanolamines; PG = glycerophosphoglycerols; PL = phospholipids; PS = glycerophosphoserines

diffusion barrier which allows the intracellular accumulation of metabolites in physiologically relevant concentrations, selective uptake of nutrients, defined release of secretory molecules, and prevention of entry of harmful substances (Cronan, 2003). The PL composition of the membrane is crucial for bacteria to cope with environmental hazards such as extreme pH, high osmolarity, or exposure to surfactant molecules (Kuhn *et al.,* 2015). Moreover, PL are involved in bacterial infection as they represent both, target and barrier for antibiotics and host defense mechanisms such as cationic antimicrobial peptides and enzymes produced by phagocytes or epithelial cells (Kraus and Peschel, 2008).

Despite an important role PL play in the live cycle/survival of *C. burnetii* in host cells, data on their composition are scarce. It is well known that the bacterium occurring in nature in virulent phase I changes to low virulent phase II upon serial passages in a culture medium. This phase variation is connected with modifications in both composition and structure of the *C. burnetii* LPS, proteins and PL (Narasaki and Toman, 2012; Skultety *et al.,* 2011; Domingues *et al.,* 2002). The phenomenon has a noticeable impact on biological properties of the bacterium but is still poorly understood. In the past, we published the first report on the composition of glycerophospholipids (GP) isolated from virulent and low virulent cells of *C. burnetii* (Domingues *et al.,* 2002). Using fast atom bombardment (FAB) mass spectrometry (MS) we detected six GP classes in virulent and five classes in low virulent cells. In addition, we showed that composition of GP within the individual classes was much more complex in virulent than in low virulent cells. It appeared that a more detailed identification and characterization of these biopolymers would enable to better understand their structural and functional peculiarities and their role in pathogenesis and immunity of Q fever. Fourier transform ion-cyclotron resonance (FT-ICR) MS is a perfect tool in this respect. It can provide superior mass resolution and mass accuracy, which allows unambiguous peak recognition and precise assignment of exact masses. Thus, it allows identification of molecular formulae with increased confidence. We have used this technique for further, more detailed analysis of GP from *C. burnetii.* The results are reported herein.

Materials and Methods

Cultivation and purification of C. burnetii strains. C. burnetii strains Nine Mile in virulent phase I (NM I) and low virulent phase II (NM II) were obtained from the strain collection of the Laboratory for Diagnosis and Prevention of Rickettsial and Chlamydial Infections, Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic. Both strains were propagated in embryonated, antibiotic-free, and pathogen-free hen eggs as described earlier (Toman and Škultéty, 1996; Škultéty *et al.,* 1998).

All laboratory procedures involving the handling of live bacteria were carried out at Biosafety level 3. The propagated bacteria were inactivated with 0.5% phenol and purified by rate-zonal sedimentation in gradients of Renografin as described previously (Fodorova *et al.,* 2011).

Isolation of PL. PL were extracted from the NM I and NM II cells with chloroform-methanol (2:1, v/v) at 20°C overnight (Folch *et al.,* 1957). The cells were separated by low-speed centrifugation (9,300 x g for 10 min) and extracted again with the chloroform-methanol mixture at 20°C for 2 hr. After centrifugation, the PL extracts were combined, evaporated to dryness and stored at +5°C.

Direct infusion FT-ICR MS. Following the Folch extraction in triplicate preparations, 10 mg of each dry sample were dissolved in 100 μl of a mixture of methanol-chloroform (50:50 v/v). To reduce ionization suppression or enhancement effects, each lipid extract (20 μl) was then diluted with 180 μl of 90% acetonitrile containing 0.2% formic acid. The solutions were infused through a syringe pump (KDS Scientific, USA) at a flow rate of 2.0 μl/min into a 12T-solariX XR FT-ICR mass spectrometer (Bruker Daltonics, USA). Data were acquired in a positive-ion mode using broadband detection with a data acquisition size of 1.024 kb/sec and within *m/z* 600 to 900. Typical electrospray ionization (ESI) mass spectrometer parameters were: a capillary electrospray voltage of -4500 V, source ion accumulation time of 0.01 sec, and collision cell ion accumulation time of 0.2 sec. Survey scan mass spectra were averaged from the accumulation of 8 scans per spectrum. The mass spectrometer was externally calibrated using the NaTFA (sodium trifluoroacetate) clusters.

Data processing. Raw MS data were processed using the mMass software package that was freely available online (Strohalm *et al.,* 2010). Monoisotopic peaks corresponding to the isotopic pattern distributions were automatically determined, and those with a signal-to-noise ratio of at least 3 were picked. The peak intensities of all the monoisotopic masses were subsequently normalized to the total ion intensity calculated from a mass spectrum. PL identification was done by searches against Lipid Maps database (website: http://www.lipidmaps.org/) within 10 ppm tolerance across all data. Identities observed at least in two biological samples of at least one of the sample groups (phase I or phase II) were aligned and combined in a table.

Results and Discussion

Two sets of PL were isolated from highly purified *C. burnetii* phase I and phase II cells by chloroform-methanol extraction. The extracts were subjected to exact mass measurements in the selected mass range of *m/z* 600–900 by FT-ICR MS (Figs. 1 and 2). To evaluate mass spectra, the open source MS tool mMass was used to search against the Lipid Maps database. The analyses were focused on the identification of major GP classes present in the isolated materials. At first glance, it was evident that GP detected in

Table 1. Glycerophospholipids found in virulent phase I and low virulent phase II cells of *C. burnetii* strain Nine Mile

Class	Subclass	Theoretical *m/z*	Observed mass difference ppm	Phase I cells[a]	Phase II cells[a]	Common name[b]
PA	DiAcPA	657.4490	-2.0	LMGP10010145 [c]		PA(15:0/18:3(9Z,12Z,15Z))
		679.4309	-2.8	LMGP10010145 Na[d]		
		685.4779	1.5	LMGP10010910 Na		PA(16:0/17:0)
		701.4518	-7.2	LMGP10010910 K[e]		
		671.4646	-1.8	LMGP10010975		PA(16:0/18:3(9Z,12Z,15Z))
		693.4466	-2.5	LMGP10010975 Na		
		713.5116	-1.8	LMGP10010353		PA(18:2(9Z,12Z)/19:1(9Z))
		735.4935	-2.0	LMGP10010353 Na		
		685.4803	-2.0	LMGP10010372		PA(18:3(6Z,9Z,12Z)/17:0)
		707.4622	-2.3	LMGP10010372 Na		
	1-(1Z-alkenyl),2-AcPA	725.5456	4.0	LMGP10030044 Na		PA(P-18:0/19:0)
		741.5195	6.3	LMGP10030044 K		
PE	DiAcPE	686.4755	-2.0	LMGP02010374	LMGP02010374	PE(12:0/20:3(8Z,11Z,14Z))
		708.4575	-2.8	LMGP02010374 Na	LMGP02010374 Na	
		670.4442	8.5	LMGP02010394		PE(13:0/18:4(6Z,9Z,12Z,15Z))
		692.4262	9.3	LMGP02010394 Na		
		700.4912	0.0	LMGP02010459		PE(15:0/18:3(6Z,9Z,12Z))
		722.4731	-1.5	LMGP02010459 Na		
		672.4599	9.8	LMGP02010594		PE(17:2(9Z,12Z)/14:1(9Z))
		694.4418	8.4	LMGP02010594 Na		
		704.5225	1.1	LMGP02010638		PE(18:1(9Z)/15:0)
		726.5044	1.9	LMGP02010638 Na		
		740.5225	-4.2		LMGP02011197	PE(18:1(9Z)/18:3(9Z,12Z,15Z))
		762.5044	-1.5		LMGP02011197 Na	
		714.5068	-1.5	LMGP02010659		PE(18:2(9Z,12Z)/16:1(9Z))
		736.4888	-1.9	LMGP02010659 Na		
PC	DiAcPC	692.5225	1.0	LMGP01011246		PC(8:0/21:0)
		714.5044	1.8	LMGP01011246 Na		
		730.4784	1.3	LMGP01011246 K		
		678.5068	1.1	LMGP01010414	LMGP01010414	PC(11:0/17:0)
		700.4888	1.7	LMGP01010414 Na	LMGP01010414 Na	
		716.4627	-0.3	LMGP01010414 K	LMGP01010414 K	
		734.5694	1.5	LMGP01010418		PC(11:0/21:0)
		756.5514	-0.4	LMGP01010418 Na		
		664.4912	1.2	LMGP01011317		PC(12:0/15:0)
		686.4731	1.5	LMGP01011317 Na		
		726.5068	-1.4	LMGP01011332		PC(12:0/20:4(5Z,8Z,11Z,14Z))
		748.4888	-3.3	LMGP01011332 Na		
		760.5851	2.2	LMGP01011372		PC(14:0/20:1(11Z))
		782.5670	1.9	LMGP01011372 Na		
		758.5694	2.2	LMGP01011373	LMGP01011373	PC(14:0/20:2(11Z,14Z))
		780.5514	1.2	LMGP01011373 Na	LMGP01011373 Na	
		718.5381	0.6	LMGP01010535		PC(15:0/16:1(9Z))
		740.5201	-1.0	LMGP01010535 Na		
		712.4912	-1.8		LMGP01011701	PC(18:4(6Z,9Z,12Z,15Z)/13:0)
		734.4731	-2.1		LMGP01011701 Na	
	DialkylPC	774.5796	7.5		LMGP01040090	
		796.5615	7.1		LMGP01040090 Na	
		812.5355	8.3		LMGP01040090 K	

Class	Subclass	Theoretical m/z	Observed mass difference ppm	Phase I cells[a]	Phase II cells[a]	Common name[b]
PS	DiAcPS	790.5593	2.8		LMGP03010134	PS(14:1(9Z)/22:0)
		812.5412	1.2		LMGP03010134 Na	
	1-alkyl,2-AcPS	776.5800	-0.9		LMGP03020012	PS(O-16:0/20:1(11Z))
		798.5619	-1.1		LMGP03020012 Na	
		774.5643	1.9		LMGP03020013	PS(O-16:0/20:2(11Z,14Z))
		796.5463	-1.1		LMGP03020013 Na	
		804.6113	2.6		LMGP03020015	PS(O-16:0/22:1(11Z))
		826.5932	-1.2		LMGP03020015 Na	
		802.5956	1.9		LMGP03020016	PS(O-16:0/22:2(13Z,16Z))
		824.5776	-1.3		LMGP03020016 Na	
PG	DiAcPG	717.4701	-2.6	LMGP04010399		PG(18:3(9Z,12Z,15Z)/14:0)
		739.4521	-1.4	LMGP04010399 Na		
		745.5014	-1.8	LMGP04010955		PG(18:3(9Z,12Z,15Z)/16:0)
		767.4834	-2.1	LMGP04010955 Na		
		759.5171	-0.1	LMGP04010404	LMGP04010404	PG(18:3(9Z,12Z,15Z)/17:0)
		781.4990	2.4	LMGP04010404 Na	LMGP04010404 Na	
		757.5014	-1.8		LMGP04010405	PG(18:3(9Z,12Z,15Z)/17:1(9Z))
		779.4834	-2.5		LMGP04010405 Na	
		755.4834	2.5	LMGP04010483 Na		PG(19:1(9Z)/14:1(9Z))
		771.4573	6.3	LMGP04010483 K		

[a]Lipid Maps Glycerophospholipid (LMGP) Identification Number, [a,b]Lipid Maps Structure Database (LMSD), http://www.lipidmaps.org/data/databases. html. The ions are in [c] [M + H]+, [d] [M + Na]+, and [e] [M + K]+ forms. For abbreviations, see text.
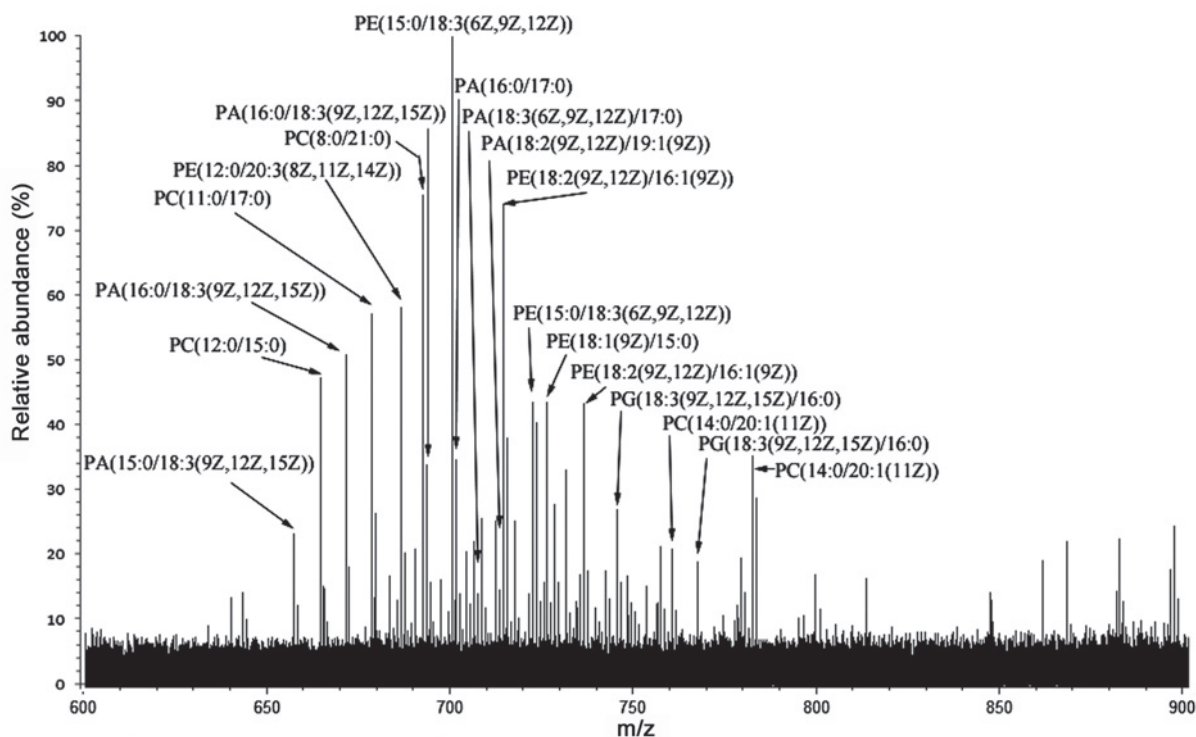

Fig. 1

FT-ICR mass spectrum in the region of *m/z* 600–900 obtained for chloroform-methanol extract of *C. burnetii* in virulent phase I
The selected ion peaks are labeled with the Common name as given for GP at http://www.lipidmaps.org/data/databases.html. The peaks represent [M + H]+, [M + Na]+, and [M + K]+ ions.

virulent *C. burnetii* phase I cells were more complex than those present in low virulent phase II cells. The analyses revealed an absence of the glycerophosphoserines (PS) class in phase I and of glycerophosphates (PA) class in phase II cells (Table 1, Fig. 3). Additionally, in contrast, with the previous work (Domingues *et al.*, 2002), glycerophosphoinositols (PI) were not found in either of both investigated cell forms. This finding is, however, in agreement with an earlier report showing that the genes predicted to be involved in the synthesis of PI are absent from the organism's genome (Sheshadri *et al.,* 2003).

The results show a high variability in the ratio of individual GP classes present in the investigated cells. In phase I cells, 33% of glycerophosphocholines (PC), 25% of glycerophosphoethanolamines (PE), 25% of PA, and 17% of glycerophosphoglycerols (PG) were detected (Fig. 3). Glycerophosphocholines have a role in the maintenance of cell-membrane integrity and are vital to all of the basic biological processes, especially with formation of cellular energy and intracellular communication or signal transduction (Cronan, 2003). One of the primary roles for PE in bacterial membranes is to spread out the negative charge

caused by anionic membrane PL. The PE also enable bacterial multidrug transporters to function properly. They allow the formation of intermediates that are needed for the transporters to properly open and close (Cronan, 2003).

Like previous two PL, PA and PG are important constituents of cell membranes as well. Glycerophosphates play several roles in the functioning of cells being utilized as precursors in the biosynthesis of other lipids, facilitating vesicle fission/fusion *via* its biophysical properties and acting as a signaling lipids (Athenstaedt and Daum, 1999). Glycerophosphoglycerols also serve as structural components of cell membranes. Furthermore, they are utilized as a precursor for cardiolipin (CL) synthesis (Cronan, 2003; Kuhn *et al.,* 2015).

As shown in Fig. 3, 39% of PS, 31% of PC, 15% of PE, and 15% of PG were detected in phase II cells. Glycerophosphoserines not found in phase I cells are actively held facing the cytosolic side of the cell membrane by the enzyme flippase (Pomorski and Menon, 2006). This is in contrast to the normal behavior of PL in cell membrane which can freely flip their heads between the two faces of the membrane they comprise. However, when a cell undergoes apoptosis PS are
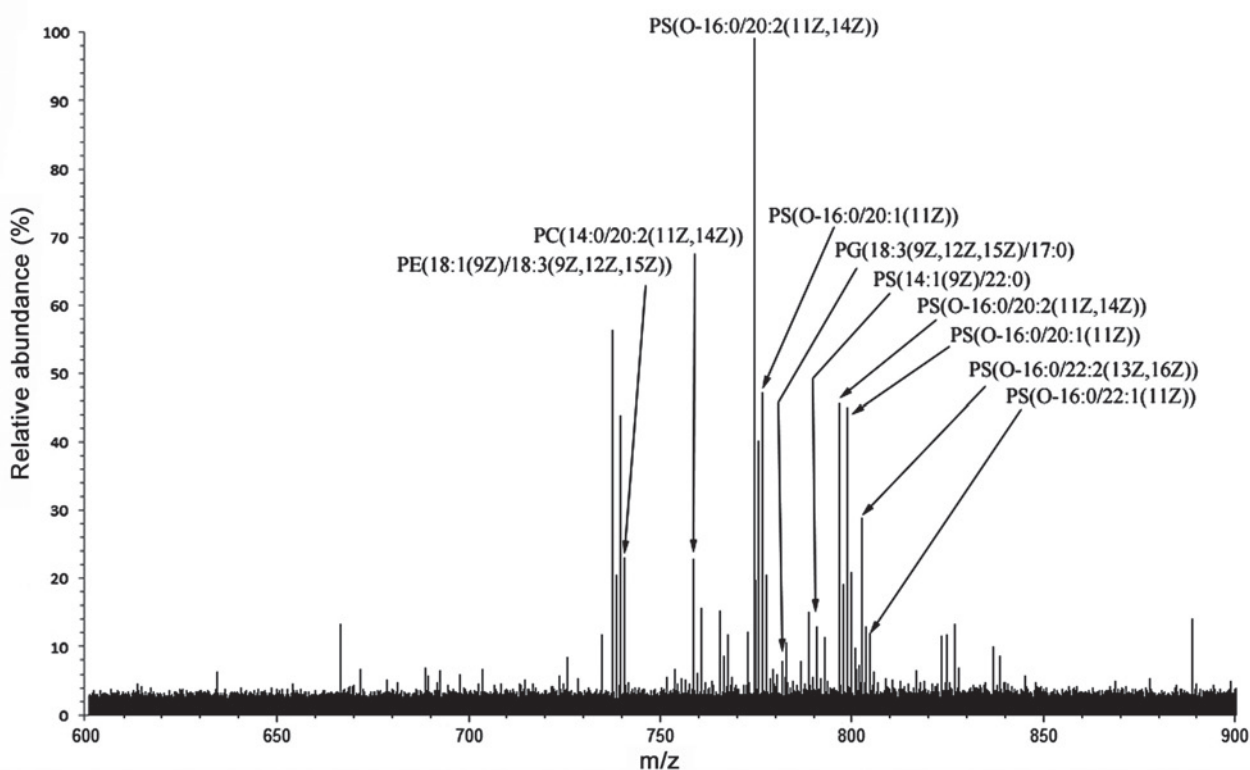

Fig. 2

FT-ICR mass spectrum in the region of *m/z* 600–900 obtained for chloroform-methanol extract of *C. burnetii* in low virulent phase II
The selected ion peaks are labeled with the Common name as given for GP at http://www.lipidmaps.org/data/databases.html. The peaks represent [M + H]⁺, [M + Na]⁺, and [M + K]⁺ ions.

Fig. 3
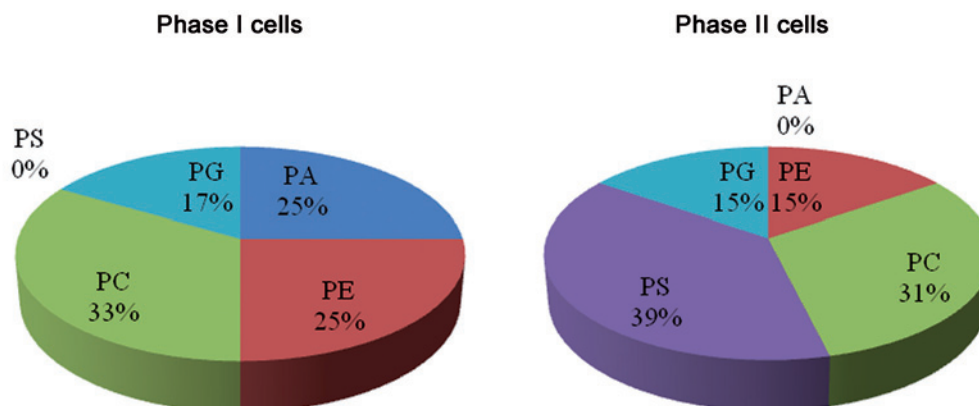**Relative distribution (in %) of GP classes in *C. burnetii* phase I and II cells**
For abbreviations, see text.

no longer restricted to the cytosolic domain by flippase. When PS naturally flip to the extracellular surface of the cell, they act as a signal for macrophages to engulf the cells (Verhoven *et al.,* 1995).

In this work, we have shown that GP present in the *C. burnetii* outer membrane underwent considerable modifications during the phase variation. This ability is advantageous for the pathogen because a more versatile microorganism can cope better with a variety of different environments. *C. burnetii* utilizes host cell lipids for membrane biogenesis and possibly energy. In addition, bacterial manipulation of host cell lipid signaling pathways is thought to support the establishment of the intracellular niche (Gilk, 2012). It has been suggested that effectors secreted by the *C. burnetii* type IV secretion system (Segal *et al.,* 2005) may either directly or indirectly modify host cell lipids (Gilk, 2012). Because PL determine membrane-protein topology, the composition of zwitterionic (*e.g.,* PE) and anionic (*e.g.,* PG and CL) PL in membranes is tightly controlled. Regulation of the bacterial envelope PL composition is suggested to play a critical role in the environmental stability of small cell variant (SCV) *versus* large cell variant (LCV) forms of *C. burnetii*, or its ability to grow in the acidic environment of the parasitophorous vacuole (Gilk, 2012). For example, it has been shown that *Bacillus stearothermophilus* increases the PG and CL content in order to adapt to an acidic environment (Card and Trautman, 1990). *C. burnetii* contains enzymes for PA head group modifications to form PS, PE, and PG (Gilk, 2012). Our results strongly indicate that these enzymes might become active during the phase variation of the pathogen because PA was found only in phase I and PS in phase II cells (Table 1, Fig. 3).

In conclusion, we have demonstrated an enormous potential of FT-ICR MS combined with Lipid Maps database for rapid and reliable detection of GP in highly complex extraction mixtures of virulent and low virulent cells of *C. burnetii*. For the first time, a more detailed composition and characterization of the individual GP was performed and the role of phase variation on their modifications and compositional changes was demonstrated. Further investigations in this direction, particularly of GP from the SCV and LCV as well as of those isolated from *C. burnetii* grown under different growth conditions or media might provide an important insight into the basic processes governing the bacterium's development and survival in host cells.

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