Changes in the lipid composition of *Bradyrhizobium* cell envelope reveal a rapid response to water deficit involving lysophosphatidylethanolamine synthesis from phosphatidylethanolamine in outer membrane

Adriana B. Cesari, Natalia S. Paulucci, María A. Biasutti, Gustavo M. Morales, Marta S. Dardanelli

PII: S0923-2508(18)30082-2

DOI: 10.1016/j.resmic.2018.05.008

Reference: RESMIC 3670

To appear in: *Research in Microbiology*

Received Date: 20 September 2017

Revised Date: 30 April 2018

Accepted Date: 25 May 2018

Please cite this article as: A.B. Cesari, N.S. Paulucci, M.A. Biasutti, G.M. Morales, M.S. Dardanelli, Changes in the lipid composition of *Bradyrhizobium* cell envelope reveal a rapid response to water deficit involving lysophosphatidylethanolamine synthesis from phosphatidylethanolamine in outer membrane, *Research in Microbiologoy* (2018), doi: 10.1016/j.resmic.2018.05.008.

Changes in the lipid composition of *Bradyrhizobium* cell envelope reveal a rapid response to water deficit involving lysophosphatidylethanolamine synthesis from phosphatidylethanolamine in outer membrane

Adriana B. Cesari[a], Natalia S. Paulucci[a]*, María A. Biasutti[b], Gustavo M. Morales[b], Marta S. Dardanelli[a]

a- Departamento de Biología Molecular, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina

b- Departamento de Química, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina

acesari@exa.unrc.edu.ar

npaulucci@exa.unrc.edu.ar "*Correspondence and reprints"

abiasutti@exa.unrc.edu.ar

gmorales@exa.unrc.edu.ar

mdardanelli@exa.unrc.edu.ar

Abstract

We evaluate the behavior of the membrane of *Bradyrhizobium* sp. SEMIA6144 during adaptation to polyethylene glycol (PEG). A dehydrating effect on the morphology of the cell surface, as well as a fluidizing effect on the membrane was observed 10 min after PEG shock; however, the bacteria were able to restore optimal membrane fluidity.

Shock for 1 h caused an increase of lysophosphatidylethanolamine in the outer membrane at the expense of phosphatidylcholine and phosphatidylethanolamine, through an increase in phospholipase activity. The amount of lysophosphatidylethanolamine did not remain constant during PEG shock, but after 24 h the outer membrane was composed of large amounts of phosphatidylcholine and less amount of lysophosphatidylethanolamine similar to the control. The inner membrane composition was also modified after 1 h of shock, observing an increase of phosphatidylcholine at the expense of phosphatidylethanolamine, the proportions of these phospholipids were then modified to reach 24 h of shock values similar to the control.

Vesicles prepared with the lipids of cells exposed to 1 h shock presented higher rigidity compared to the control, indicating that changes in the composition of phospholipids after 1 h of shock restoring fluidity after the PEG effect and would allow cells to maintain surface morphology.

Keywords: *Bradyrhizobium* peanut-nodulating, water deficit, envelope cell topography, membrane fluidity, phospholipids remodeling.

1. Introduction

Bradyrhizobium sp. SEMIA6144 is a Gram-negative bacterium that is able to fix atmospheric nitrogen (N) in symbiotic association with peanut plants to enhance plant N-content [1,2]. However, physiological stresses such as water deficit, salinity and extreme temperature negatively affect this symbiotic interaction. Water deficit is the most important limiting factor for crop production in many parts of the world, and fluctuating water availability is one of the primary environmental factors that affects the activity and viability of soil microorganisms [3,4]. High-molecular-weight (i.e., a molecular weight of 6000 Da) polyethylene glycol (PEG) has been utilized extensively in microbial and plant studies examining responses to reduced water content and is believed to simulate water deficit [5].

The lipid matrix of bacterial membranes comprises structured phospholipid (PL) assemblies in a bimolecular layer and integrated proteins [6]. There is general agreement that phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG) and cardiolipin (CL) are the major PL in rhizobia [6]. However, PC is restricted to a limited number of genera, being a clear example of this is the genus *Bradyrhizobium* [2]. Microbial fatty acids (FA) are typically 14-20 carbons in length [7, 8, 9]. Acyl chains of this length balance the stability and fluidity needs of the membrane [6]. Paulucci [10] reported that the major fatty acid (FA) in SEMIA6144 are the unsaturated FA (UFA) 18:1Δ11 and the saturated FA (SFA) 16:0 and 18:0.

Environmental factors can alter the packing order of the bacterial cell membrane and change the bilayer structure [6]. The cell envelope is in constant contact with the environment, and thus flexibility of the membranes and its components to facilitate

adaptation is a primary determinant of cell survival. There are several techniques for evaluating membrane integrity within which atomic force microscopy (AFM) is used to monitor changes in the cell surface produced by different environmental factors [11,12]. Lipid membranes can undergo changes upon exposure to adverse environmental factors through alterations in the polar head groups or acyl chains of PL [2, 13, 14]. These changes may be accomplished by varying the amount of total PL and/or their class distribution, changing the type of FA incorporated into PL during synthesis or turnover via phospholipase activity, or regulating the degree of unsaturation of FA moieties [15, 13, 14]. As intermediates in PL metabolism and turnover, lysophospholipids, as lysophosphatidylethanolamine (LPE), are produced via phospholipase A (PLA) mediated hydrolysis of the acyl group of a PL to lysophospholipids and FA [16].

Water deficits affect membranes integrity. Scherber [17] reported an increase in the membrane phase temperature transition of *Escherichia coli* after extensive cell water deficit (1 week at 20 % - 40 % relative humidity). These authors also found that *E. coli* membranes exposed to water deficit exhibit high levels of SFA compared to undried cells. In addition, Cesari [14] showed that *Azospirillum brasilense* can perceive osmotic changes in medium by sensing modifications in membrane fluidity. Under these conditions, the bacterium responds by increasing PC and SFA levels to maintain membrane integrity.

Several studies in bacteria have elucidated the roles of membrane components in water deficit tolerance [17, 18, 13, 14]; however, there have been few studies exploring how within the cell envelope, the membranes and its components change during a stress situation to achieve optimal fluidity and ensure survival. In addition, many questions remain regarding the physiological and biochemical responses of rhizobia to water deficit;

prior work in this area has focused on improving the long-term tolerance of inoculant-based rhizobia rather than investigating bacterial physiology under water deficit conditions.

Escherichia coli has been widely used as a model for predicting behaviors of both outer and inner membrane of other bacteria against different environmental conditions. However, in the case of rhizobia it is very difficult to make such comparisons due to the fact that they contain PC in their membranes, which is absent in *E. coli*.

The objective of this work was to elucidate the dynamic changes that occur in the lipid components of the cellular envelope of a strain of the genus *Bradyrhizobium* peanut nodulant, exposed to water deficit.

We have placed special emphasis on elucidating the commitment of each of the membranes (outer and inner) in the response to water deficit. Moreover, in this paper, we observed a dehydrating effect of PEG on the cell surface and explained how *B.* sp. SEMIA6144 responded rapidly to the presence of PEG and counteracted that effect on the membrane by modifying the lipid composition. We also demonstrate that this first response is modified during adaptation to achieve optimal membrane fluidity.

The identification of physical and physiological factors that determine the survival of *B.* sp. SEMIA6144 under water deficit conditions may aid in the strategic development of rhizobial strains with improved ability to survive water deficit.

2. Materials and Methods

2.1. Bacteria and growth conditions

Bradyrhizobium sp. SEMIA6144, provided by MIRCEN/FEPAGRO, Brazil (SEMIA6144 in the rest of text), was maintained in yeast extract mannitol (YEM) medium [18] and

grown on B-modified medium [2]. Bacterial media were supplemented with the nonpermeating solute polyethylene glycol (PEG, average MW 5489 Da, Sigma Chemical Co., St. Louis, MO, USA) to simulate moderate water deficit [14].

All cultures were incubated at 28 °C with shaking at 150 rpm (Allied Fisher Scientific). The water potential of 15 mmol l^{-1} (90 g l^{-1}) PEG medium at 25 °C was measured using an osmometer (Semi Micro K-7400, Knauer) and was found to be -0.8 MPa.

Viable SEMIA6144 cells were counted as colony forming units per ml of culture (CFU ml^{-1}) using the droplet technique [20].

2.2. Effects of PEG Shock on SEMIA6144 Viability

SEMIA6144 cells were grown until the exponential growth phase was reached (48 h); then to induce osmotic shock, 15 mmol l^{-1} PEG was added to the cultures. The cultures were then incubated for 1 h, 5 h and 24 h, and viability was determined using the droplet technique [20].

2.3. Atomic Force Microscopy Imaging of SEMIA6144 Cells

We used AFM to investigate if PEG caused changes in cell morphology. Coverslips coated with polyethylenimine (0.1 %) were used to firmly immobilize bacterial cells so they could withstand the lateral forces inflicted by the cantilever during AFM imaging. Briefly, coverslips were cleaned in 70 % ethanol for 10 min, rinsed with MilliQ water, and air-dried in upright position. Then polyethylenimine (0.1 %) was added on each coverslip, air-dried 20 min at room temperature. Finally, excessive polyethylenimine were removed by submerging coverslips in MilliQ water. The coverslips were air-dried, and stored for a maximum of 2 weeks at 4 °C.

144 We immobilized cells by applying 20 µL cell suspension to the coverslips covered with
145 polyethylenimine, and let the cells settle for 15 min at room temperature before removing
146 non-adhered cells by rinsing with MilliQ water.
147 The images were obtained through the use of an atomic force microscope (AFM, Agilent,
148 Technologies, SPM model 5500) working in acoustic mode. AFM probes (Micromasch,
149 HQ:XSC11/A1 BS) with a cantilever resonance frequency and force constant of 155 kHz
150 and 7 N/m were used in all the measurements.
151 Images were processed by using the Gwyddion v2.39 free software for scanning probe
152 microscopy data visualization and analysis (http://gwyddion.net/).
153

154 **2.4. Incorporation of Labeled Acetate**

155 A total of 0.5 µCi of [1-^{14}C] sterilized acetate sodium salt (43 mCi mmol^{-1}, New England
156 Nuclear) was added to 25 ml bacterial cultures at the time of inoculation. Cells grown under
157 the above conditions were harvested by centrifugation at 8,000 rpm for 10 min in a
158 Beckman Allegra 64R refrigerated centrifuge. Pellets were washed twice with 0.9 % NaCl
159 and used for subsequent studies.
160

161 **2.5. Preparation of Outer and Cytoplasmic Membranes**

162 Fractions of internal and external membrane were obtained according to the technique of
163 Mizuno et al., (1978) [21]. Cells control and PEG shocked were harvested by centrifugation
164 at 8,000 rpm for 10 min at room temperature and washed with saline buffer pH 7. Cells (ca.
165 1.5 g wet weight) were suspended in 18ml of ice-cold 20 % (w/v) sucrose and ice-cold
166 reagents were slowly added to the suspension in an ice-bath in the following order: 9 ml of
167 2 M sucrose, 10 ml of 0.1 M Tris-HCl (pH 7.8), 0.8 ml of 1 % Na-EDTA (pH 7.0), and 1.8

ml of 0.5 % lysozyme. Then the mixture was warmed to 30 ºC within a few minutes and kept at that temperature for 60 min. During the incubation, the mixture became viscous due to breakage of a part of the cells. Then the suspension was centrifuged to remove the spheroplasts at 13,000 rpm for 15 min at 30 ºC. Crude outer membranes were recovered from the supernatant by centrifugation at 30,000 rpm for 60 min. The spheroplasts were burst in 40 ml of 5mM $MgCl_2$ and the spheroplast membranes were recovered by centrifugation at 15,000 rpm for 20 min.

Two membrane markers, the KDO (2-ketodeoxyoctonate) content for the outer membrane and the NADH oxidase activity for the inner membrane [22] were used. The KDO content was 4.16 µg/100 µg protein and 0.63 µg/100 µg protein for the outer and inner membrane respectively. NADH oxidase activity was 760 U/mg protein/min and 17.6 U/mg protein/min for the inner and outer membrane respectively. The content of these markers demonstrates efficient separation and purity of both membrane fractions.

2.6. Lipid extraction

Lipids were extracted from bacterial cells and outer and inner membrane and washed with chloroform/methanol/water [23]. The lower phase containing lipids was dried under N_2 and dissolved in an appropriate volume of 2:1 chloroform/methanol (v/v).

2.7. Separation and Quantification of [1-14C]-labelled Phospholipids

Thin layer chromatography (TLC) plates (silica gel HLF, 250 ml) were purchased from Analtech. Aliquots of total lipid extracts were analyzed by TLC using a solution of 40:15:14:12:7 chloroform/acetone/methanol/acetic acid/water (v/v/v/v/v) as the solvent. All solvents were of analytical or high-performance liquid chromatography grade. Lipids were

192 detected with iodine vapors, and the separated lipids were identified by comparing with
193 authentic purified standards purchased from Sigma. The TLC plates were scraped, and
194 fractions were quantified by performing radioactivity measurements using a liquid
195 scintillation counter (Beckman LS 60001 C).
196
197 **2.8. Analysis of Fatty Acids by GC-MS**
198 FA methyl esters (FAME) from SEMIA6144 cells were analyzed using a gas
199 chromatograph (GC) Agilent 7890B coupled to a Mass Spectrometer Agilent 5977A (MS)
200 equipped with a ZB-WAX (30 mm x 0.25 mm ID) Zebron column. The following GC-MS
201 conditions were used: injector temperature, 240 °C; column temperature, 180 °C,
202 maintained for 30 min; increase of 5 °C/min to 240 °C, maintained for 10 min. Run time:
203 46 min. MS: full SCAN, 40-500. Injection volume: 1 µl. Split: 1:10.
204 The peaks for each FA were identified using a mixture of commercial standards obtained
205 from Sigma-Aldrich Chemical Co. and Supelco [24]. FAME were identified by comparing
206 retention times to commercial standards (Sigma Chemical Co., St. Louis, MO, USA).
207 To determine the FA composition of every specific PL class, PL were separated as
208 described above, and each spot was scraped from plates and methyl-esterified. The
209 resulting FAME were analyzed by GC-MS as described above.
210
211 **2.9. Preparation of Phospholipid Vesicles**
212 Multilamellar vesicles (MLVs) were prepared according to Yamazaki [25] by adding 1 ml
213 of distilled water to dry SEMIA6144 lipids (total, outer or inner membrane) and then
214 heating the mixture to approximately 10 °C above the phase transition temperature for 20
215 min. The suspension was vortexed several times for approximately 5 min each.

2.10. Determination of Membrane Fluidity

Fluidity of cells and MLVs was determined by measuring the fluorescence polarization of the 1,6-diphenyl-1,3,5-hexatriene (DPH) probe (Invitrogen) inserted into the membrane. Fluorescence polarization quantifies the degree of depolarization of light emitted by the embedded fluorescence probe and is a measure of membrane state [26]. The probe polarization ratio and membrane fluidity are inversely correlated. As the fluidity of the bacterial membrane decreases, the polarization ratio increases and vice versa [27]. Following procedures described by Trevors [28], SEMIA6144 cells were grown in B-modified medium to exponential phase, harvested, washed in sterile 15 mmol l^{-1} Tris-HCl buffer (pH 7.0) and resuspended in the same buffer up to 0.2 OD at 600 nm. Then, 1 µl of the fluorescent probe (stock solution diluted to 12 mmol l^{-1} in tetrahydrofuran) was added to 3 ml aliquots of resuspended cells to obtain a final probe concentration of 4 µmol l^{-1}. Aliquots were incubated with a magnetic stirrer at 200 rpm for 10 min in the dark at room temperature. The culture aliquots were then divided; one part was maintained as the control and the other part was subjected to 15 mmol l^{-1} PEG treatment for different periods of time (10, 20, 30 and 60 min). Aliquots were collected, and the degree of polarization was determined.

To measure the fluidity of the vesicles directly 1 µl of DPH was added to 3 ml of the suspension of MLVs and the polarization was measured.

Fluorescence polarization measurements were performed using a FluoroMax®-4 Horiba spectrofluorometer with a polarizer. The excitation wavelength for the DPH probe was 358 nm, and the emission wavelength was 428 nm. The slit widths for the excitation and emission beams were 12 and 10 nm, respectively. The degree of polarization was calculated from the polarization ratio (P) using the equation $P = (IVV – IVHG)/(IVV + IVHG)$, where

IVV and IVH are the intensities of vertically and horizontally polarized light components emitted after excitation by vertically polarized light, respectively, and G is the sensitivity factor of the detection system [29].

2.11. Phospholipase Assay

Total protein extracts were obtained from SEMIA6144 cultures (200 ml) grown to exponential phase under control conditions or PEG shock for 1 h, 5 h and 24 h. Outer membrane was extracted as explained above and used as enzyme source. Protein concentrations were determined using the Bradford method [30]. To determine the PLA activity in the extracts, PLA assays were performed at 30 °C. The standard reaction mixture contained 50 mM Tris-HCl (pH 9), 10 mM $CaCl_2$, 450 µM Triton X-100, and [1-^{14}C]-labeled PE of *E. coli* DH5α as the substrate [16, 31]. Assays were initiated by adding 100 µg of protein (enzyme extract) to the reaction mixture and incubating for 60 min. The reaction was stopped with the addition of 150 µl of methanol and 125 µl of chloroform. Following centrifugation, the bottom layer was transferred to a glass tube and dried under an N_2 stream. The residue was dissolved in 20 µl of chloroform/methanol 2:1 (v/v) and subjected to TLC detection. PE and LPE fractions were scraped and quantified by performing radioactivity measurements using a liquid scintillation counter. Finally, the radioactivity present in the LPE fraction was used to calculate the enzymatic activity of phospholipase A, which was expressed as percent of substrate hydrolyzed.

2.12. Statistical Analyses

All experiments were carried out in triplicate. One-way ANOVA was used to analyze the results. When ANOVA indicated a significant treatment effect, the least significant differences test (Tukey, $p<0.05$) was applied to compare the mean values.

3. Results

3.1. PEG Shock Negatively Affects the Viability of SEMIA6144 Cells

The viability (measured as CFU ml^{-1}) of SEMIA6144 cells under water deficit conditions was determined (Fig. 1). PEG shock from 48 h growth caused a decrease in bacterial growth compared to control conditions, with bacterial counts reaching $7.3.10^{8}$ CFU ml^{-1} and $2.2.10^{12}$ CFU ml^{-1}, respectively, after 110 h of growth.

3.2. PEG Shock Modifies the Surface Morphology of the Cells of SEMIA6144

AFM were used to determine the effect of PEG on cell surface morphology (Fig. 2). The control cells were rod-shaped with a relatively rough surface (Fig. 2A). Perturbations on the surface and membrane dehydration were observed 10 min after the addition of PEG (Fig. 2B). However, after 1 h of shock (Fig. 2C, D), we found that many of the cells had a similar surface morphology to control cells, without the marked effects observed at 10 min. For this reason, we decided to study the physical state of the membrane after PEG shock.

3.3. PEG shock fluidizes the SEMIA6144 membrane, but the cells are able to restore fluidity to optimal values

The effects of PEG addition on the physical state of the membrane in SEMIA6144 cells were evaluated by measuring the fluorescence polarization (*P*) of the probe DPH.

285 We found that the *P* value decreased from 0.20 ± 0.01 to 0.15 ± 0.02 immediately (10 min)
286 after the addition of PEG (Fig. 3). However, after 30 min of PEG exposure, the *P* value
287 returned to a value similar to that obtained in the absence of PEG. These results suggest
288 that PEG exerts a fluidizing effect on the SEMIA6144 membrane, and this effect reverts
289 after 30 min of PEG exposure, after reaching 1 h of shock values similar to the control.
290

291 **3.4. SEMIA6144 Cells Change the Composition of Totals Phospholipid and Fatty**
292 **acids**

293 Based on the results described above, the following question can be posed: how does the
294 SEMIA6144 membrane return to optimal fluidity values after undergoing the fluidizing
295 effects of PEG? To investigate this question, we studied the total PL and FA composition in
296 PEG shocked cells for different periods of time (1 h, 5 h and 24 h).
297 During exponential growth, the predominant labeled PL (Fig. 4) were PC (34.25 %), PE
298 (30.6 %) and PG (21.3 %), followed in descending order by CL (7.15 %) and LPE (6.7 %).
299 This implies that the PL forming the bilayer (PC and PG) comprised 55 % of the total PL,
300 while non-bilayer-forming PL (PE and LPE) comprised 37.3 % of the total. In addition, the
301 FA detected were 18:1Δ11 (78.1 %), 18:0 (5.2 %), 16:1 (0.5 %) and 16:0 (15.9 %) (Table
302 1).
303 The response to PEG shock for 1 h revealed an increase in PC (22 %) and a marked
304 increase in LPE (205 %), while PE decreased (76.6 %) with respect to control conditions
305 (Fig. 4). Unlike SEMIA6144 control cells, shocked cells exhibited membranes containing
306 63.8 % bilayer-forming PL and 29.3 % non-bilayer-forming PL. When SEMIA6144 was
307 exposed to PEG for 1 h, the percentage of FA 18:1Δ11 declined from 78.1 to 72.9 %, while
308 the percentage of FA 16:0 increased from 15.9 to 18.5 %. The UFA/SFA ratio decreased

309 from 3.7 to 2.9 with respect to control conditions due to a decrease in UFA (Table 1). These
310 changes in PL and FA composition may be responsible for the restoration of membrane
311 fluidity of SEMIA6144 after undergoing the fluidizing effects of PEG (Fig. 3).
312 To determine whether the response discussed above is constant or dynamic in time, we
313 evaluated the effects of prolonged exposure to PEG (5 h and 24 h) on total PL and FA
314 composition.
315 After 5 h of exposure to PEG, rearrangements in the levels of different PL were observed
316 with respect to the composition after 1 h of exposure. The amount of LPE decreased from
317 20.47 to 14.18 %, but the amount of PE was constant, unlike PC, whose percentage
318 increased from 41.8 to 48.8 % (Fig. 4). Interestingly, after 24 h of PEG shock, a novel
319 rearrangement in PL was observed compared to 5 h of shock. Under these conditions, the
320 amount of PE significantly increased from 8.5 to 23.7 %, the amount of PC decreased from
321 48.8 to 41 %, and the amount of LPE remained constant (Fig. 4). The total membrane
322 composition comprised 55 % bilayer-forming PL and 38 % non-bilayer-forming PL.
323 When the FA composition of SEMIA6144 cells exposed to PEG for 5 h and 24 h was
324 evaluated and compared to exposure for 1 h, FA 18:1Δ11 increased from 72.9 to 82 % and
325 from 72.9 to 84.6 %, respectively (Table 1). These results suggest that SEMIA6144 rapidly
326 responds to the presence of PEG, and this response is modified as the exposure time
327 increases.
328 Because SEMIA6144 is a Gram-negative bacterium and has a complex cell envelope with
329 two membranes (outer and inner), and due to the effect, that PEG exerts on the cell surface,
330 we decided to study the role of each of them in the response to PEG.
331
332

3.5. Both Outer and Inner Membranes Modify Their Phospholipid Composition in Response to PEG Shock

The PL composition of both membranes (outer and inner) of SEMIA6144 cells was evaluated (Fig. 5). Both membranes presented PC, PE, PG, CL and LPE as main PL, however the outer membrane was more enriched in LPE than the inner membrane.

PEG shock for 1 h, caused an increase in LPE in both membranes, being more evident in the outer membrane (165 %) compared to the control (Fig. 5A). In addition, the percentage of PE decreased in both membranes (49 % in the outer membrane and 26.5 % in the inner membrane). Regarding the behavior of PC was different in both membranes, increased in inner membrane (9.3 %) and decreased in outer membrane (24 %) with respect to the control. Compared to 1 h of shock, a decrease in LPE in both membranes (51 % in the outer membrane and 34 % in the inner membrane) was observed at 5 h of shock, which resulted in an increase of PE in both membranes (33 % in outer membrane and 13 % in inner membrane), in this condition the percentage of PC remained constant in both membranes. 24 h of shock caused again changes in both membranes with respect to the previous condition, obtaining a composition of PL similar to the control without PEG. For this PC, increased external membrane (34 %) and decreased inner membrane (9 %) in which PE increased (8 %). An increase in the anionic PL, PG and CL, was observed in outer membrane, after 1 h (21 % and 8 % respectively) and 5 h (37 % and 31 % respectively) shock. At 24 h the percentages of PG and CL decreased in outer membrane, reaching values similar to control. In inner membrane, the percentages of PG and CL were not modified during the shock.

Due to the complexity of the cellular envelopes of this type of bacteria, the use of model systems is very useful to be able to study the behavior of the lipid components without the interference of other components. For this reason, we studied the fluorescence polarization of DPH in MLVs synthesized with lipids of different cell fractions.

3.6. The MLVs synthesized with lipids from shocked cells 1 h have greater rigidity

MLVs From Total Lipids

When the fluidity of the vesicles prepared with the total lipids of the SEMIA6144 cells was analyzed (Fig. 6), greater *P* (less fluidity) was observed in the MLVs derived from lipids of cells exposed to 1 h with PEG (0.20) as compared to control MLVs (0.14). MLVs prepared with total lipids of cells exposed to 5 h and 24 h at PEG, showed lower *P* (0.17 and 0.12 respectively) than the MLVs prepared with total lipids from cells exposed 1 h to PEG. In addition, MLVs prepared with total lipids of cells exposed to 24 h to PEG presented practically the same polarization as the control MLVs.

MLVs From Outer and Inner Membrane Lipids

When the fluidity of the MLVs prepared with the outer membrane lipids of the SEMIA6144 cells was analyzed (Fig. 6), higher *P* value (greater rigidity) was observed in the MLVs derived from the lipids of cells exposed by 1 h to PEG (0.14) compared to MLVs control (0.12). These results suggest that the PL composition of these vesicles would contribute a greater rigidity to the outer membrane after 1 h of shock compared to the control. However, the MLVs prepared with the outer membrane lipids of cells shocked for 5 h and 24 h showed fluidity similar to the control. The fluorescence polarization of DPH in

MLVs prepared with the internal membrane lipids of the SEMIA6144 showed a similar behavior to that observed in MLVs from outer membrane lipids (Fig. 6), observing an increase in rigidity in those vesicles prepared with the lipids of bacteria shocked for 1 h.

3.7. LPE increase after 1 h of PEG shock involved an increase in PLA activity in the outer membrane

Our results suggested that the turnover between LPE and PE during PEG shock may involve PLA activity and may be affected by PEG shock.

When outer membranes of SEMIA6144 grown under control conditions were used as enzyme extract, PLA activity was observed, indicating that under optimum conditions of growth SEMIA6144 produces lysophospholipids, as was demonstrated when the PL composition of outer membrane was evaluated. Interestingly an increase in PLA activity (70 %) was observed in outer membranes of SEMIA6144 shock for 1 h with PEG compared to the control. Outer membranes from 24 h shock-cell presented a decrease in enzyme activity (55 %) respect to 1 h of shock (Table 2).

4. Discussion

Lipid bilayers are essential for the survival of microorganisms that are not isolated from extrinsic physical and chemical factors [6, 32, 33, 34]. PEG dehydrates the lipid bilayer, leading to formation of nonlamellar structures [35], raising the gel-to-fluid phase transition temperature [36] and enhancing membrane permeability [37]. In addition, PEG creates osmotic imbalance that leads to mechanical stress in membranes and may induce local membrane curvature [38]. The visualization of SEMIA6144 cells through AFM showed alteration of the surface morphology with areas of evident membrane dehydration by effect to PEG shock. A fluidizing was observed 10 min after the addition of PEG, this effect was maximal after 20 min of exposure and was then reversed until optimum fluidity was achieved. The membrane fluidity of SEMIA6144 cells exposed to PEG for 1 h was equivalent, indicating that the cells were able to alter this parameter to adjusting after the immediate effects induced by PEG. In this condition, many of the cells presented a superficial morphology similar to the control cells, indicating that they have strategies to mitigate the effects of PEG.

The response of the SEMIA6144 membrane to quickly revert to optimal fluidity after encountering the fluidizing effects of PEG involved changes in both PL and FA composition.

When evaluating the total PL composition of the SEMIA6144 cells after 1 h of PEG shock, we observed an increase in the amount of bilayer-forming PL (PC) and a reduction in non-bilayer-forming PL (PE and LPE) compared to control. Among the non-bilayer-forming PL, a surprising increase in LPE was observed as well as a decrease in its precursor (PE).

The LPE content in bacterial membranes usually does not exceed 2-5 %; however, various stressors generate a dramatic increase in LPE content [39]. In *Yersinia pseudotuberculosis* exposed to phenol, increased LPE caused an increase in the phase transition temperature of the membrane, resulting in greater membrane rigidity [39]. In SEMIA6144 shocked cells for 1 h, LPE increased at the expense of PE, which appeared to be a rapid response since we find that the total lipid MVLs of exhibited higher rigidity compared to control, indicating that the turnover between LPE and PE contributes to rigidify the membranes. Besides an increase in PC after shock PEG for 1 h may also help counteract the fluidizing effects, stabilizing the membrane. Furthermore, this rapid response of SEMIA6144 involved a change in the composition of FA with a decrease in the FA unsaturation index, which may promote increased membrane rigidity in response to the fluidizing effects of PEG.

When SEMIA6144 was shocked with PEG for longer periods of time (5 h and 24 h), LPE was not maintained at a high percentage in the membrane. LPE is an intermediate in the metabolism of PL [40] derived from the PLA activity on PE [41] and is quickly recycled and likely metabolized due to its ability to create membrane instability [42, 43, 39].

Although the amount of LPE decreases, the rearrangements that occur in the membrane after SEMIA6144 is exposed to PEG for longer periods of shock allow optimal fluidity to be maintained (*P*: 0.23), as well as the balance between bilayer-forming and non-bilayer-forming PL.

The envelope of Gram-negative bacteria is composed of two distinct lipid membranes: an inner membrane and outer membrane. Both, the outer and the inner membrane of SEMIA6144 presented PC and PE as main PL. The most important difference was the high

content of LPE in the outer membrane, in addition to a greater proportion of anionic PL, PG and CL.

The detailed analysis of the PL composition of each SEMIA6144 membrane under PEG shock, indicated that 1 h of shock caused a large increase of LPE in the outer membrane and concomitant decrease of PC and PE. This increase in LPE was related to an increase in the outer membrane PLA activity on PE. Nevertheless, after 24 h of PEG shock, the outer membrane was composed of large amounts of PC and PE and less amount of LPE, due to a decrease in PLA activity. The inner membrane composition was also modified after 1 h of shock, observing an increase of PC at the expense of PE. The proportions of these PL were then modified to reach 24 h of shock values similar to the control.

The role of lysophospholipids in bacteria remains poorly studied, it has been proposed that the geometric form of inverted cone that these shows could alleviate the curvature stress caused on the membrane in certain situations [44]. LPE was also found to become dominant (12 %) in the outer membrane of *Y. pseudotuberculosis* after shifting of the growth temperature from 8 ºC to 37 ºC or after exposure of the cells to heat shock [45], it is unclear why heat-stressed cells are able to retain LPE at such a high level. In this paper, we demonstrate that in SEMIA6144 cells after PEG shock, the amount of LPE does not remain high for a long time, but other phospholipids are synthesized (PC and PE).

All the MLVs synthesized with lipids of cells exposed 1 h to shock presented a greater polarization, which could indicate that the increase of LPE and PC at the expense of PE could be the response that allows the cells to recover from the fluidizing effect of PEG. Although the use of model systems such as MLVs are very important to predict the behavior of the lipid component of cell membranes, we must not forget that they are much

475 more complex and other components such as proteins and lipopolysaccharides could also
476 be involved in the bacterial response to water deficit.
477 Finally, we conclude that SEMIA6144 is able to tolerate moderate water deficiency by a
478 rapid response in the outer membrane, product of an increase in PLA activity, which
479 generates LPE from PE. This response is not maintained over time, but the cells modify
480 again the composition of both membranes to maintain homeostasis and ensure the integrity
481 of the cellular envelope under this condition. The findings reported in this study are
482 significant because they describe for the first time the importance of phospholipases as key
483 enzymes in PL turnover for rapid adaptation to stress in this bacterial genus.
484

485 **Conflict of interest**

486 The authors do not have any possible conflicts of interest.

487 **Acknowledgment**

488 This study was financially supported by PIP CONICET (Grant No. 112-201501-00232),
489 UNRC (Grant No. 161/126), PICT (Grant No. 2278/13) and PICT (Grant No. 1065/15).
490 A.B.C. is a fellow of CONICET-Argentina. N.S.P, M.A.B, G.M.M and M.S.D. are
491 members of the Research Career of CONICET-Argentina. The authors are grateful to
492 Mónica E. Hourcade (Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad
493 Nacional de Rosario) for technical assistance with the analysis of FAME.
494

495 **References**

496 [1] Angelini J, Taurian T, Morgante C, Ibáñez F, Castro S, Fabra, A. Peanut nodulation
497 kinetics in response to low pH. *Plant Physiol Bioch* 2005;43:754-759.

[2] Medeot D, Sohlenkamp C, Dardanelli MS, Geiger O, García M, López-Lara I. Phosphatidylcholine levels of peanut-nodulating *Bradyrhizobium sp.* SEMIA6144 affect cell size and motility. *FEMS Microbiol Lett* 2010; 303:123-131.

[3] Watt M, Silk WK, Passioura J. Rates of root and organism growth, soil conditions and temporal and spatial development of the rhizosphere. *Ann Bot* 2006;97:839-855.

[4] Niles MT, Lubella M, Brownc M. How limiting factors drive agricultural adaptation to climate change. *Agr Ecosyst Environ* 2015;200:178-185.

[5] Johnson DR, Coronado E, Moreno-Forero S, Heipieper J, van der Meer R. Transcriptome and membrane fatty acid analyses reveal different strategies for responding to permeating and non-permeating solutes in the bacterium *Sphingomonas wittichii*. *BMC Microbiol* 2011;11:250-262.

[6] Wilkinson SG. Gram-negative bacteria. In: Ratledge C, Wilkinson SG (eds) Microbial lipids, Vol. 1. Academic Press, New York, 1998, pp 299-488.

[7] Finean JB, Michell RH. Isolation, composition and general structure of membranes. In: Finean JB, Michell RH (eds) Membrane structure. Elsevier, New York, 1981, pp 19-25.

[8] Russell NJ, Fukunaga N. A comparison of thermal adaptation of membrane lipids in psychrophilic and thermophilic bacteria. *FEMS Microbiol* 1990;75:171-182.

[9] Russell NJ. Psychrotrophy and adaptation to low temperatures: microbial membrane lipids. Proceedings of the 19th International Congress on Refrigeration. Workshop Refrigeration and Microbiology: Health, Food, Drinks and Flowers 1995;1:359-365.

[10] Paulucci NS, Medeot DB, Dardanelli MS, de Lema MG. Growth temperature and salinity impact fatty acid composition and degree of unsaturation in peanut nodulating rhizobia. *Lipids* 2011;46: 435-444.

[11] Wu L, Zaborina O, Zaborin A, Chang E, Musch M, Holbrook C et al. High-Molecular-Weight Polyethylene Glycol Prevents Lethal Sepsis Due to Intestinal *Pseudomonas aeruginosa*. *Gastroent* 2004;126:488-498.

[12] Priyanka K, Ashutosh V, Kothari D, Varsha K. Morphostructural changes induced in *E.coli* exposed to copper ions in water at increasing concentrations. *J Pharma Research* 2015;4: 837-852.

[13] Paulucci NS, Gallarato LA, Reguera YB, Vicario JC, Cesari AB et al. *Arachis hypogaea* PGPR isolated from Argentine soil modifies its lipids components in response to temperature and salinity. *Microbiol Res* 2015;173: 1-9.

[14] Cesari AB, Paulucci NS, Biasutti MA, Reguera YB, Gallarato LA, Kilmurray C, Dardanelli MS. Reorganization of *Azospirillum brasilense* cell membrane is mediated by lipid composition adjustment to maintain optimal fluidity during water deficit. *J Appl Microbiol* 2016;120: 185-194.

[15] Pereyra, MA, Zalazar SA, Barassi CA. Root phospholipids in *Azospirillum* inoculated wheat seedlings exposed to water stress. *Plant Physiol Bioch* 2006;44: 87-879.

[16] Song JK, Rhee JS. Enhancement of stability and activity of phospholipase A in organic solvents by directed evolution. *BBA-Protein Struct M* 2001;1547:370-378.

[17] Scherber CM, Schottel JL, Aksan A. Membrane phase behavior of *Escherichia coli* during water deficit, rehydration, and growth recovery. *BBA-Biomembrane* 2009;1788:2427-2435.

[18] Mhamdi R, Nouairi I, Hammouda T, Mhamdi RB, Mhadhbi H. Growth capacity and biochemical mechanisms involved in rhizobia tolerance to salinity and water deficit. *J Basic Microbiol* 2002;54: 1-11.

544 [19] Somasegaran P, Hoben H. Handbook for Rhizobia. Methods in legume-*Rhizobium*
545 technology, 1st edn. Spring-Verlag, New York, 1994, pp. 332-341.
546 [20] da Silva R. (1996) Tecnica de Microgota para contagem de células bacterianas viaveis
547 em uma suspensao. Universidade Federal de Vi1osa, Vi1osa, Minas Gerais, Brazil.
548 [21] Mizuno T, Kageyama M. Separation and characterization of the outer membrane of
549 *Pseudomonas aeruginosa*. *J Biochem* 1978;84:179-191.
550 [22] de Maagd R, Lugtenberg B. Fractionation of *Rhizobium leguminosarum* cells into
551 outer membrane, cytoplasmic membrane, periplasmic, and cytoplasmic components. *J of*
552 *bacteriol* 1986;167:1083-1085.
553 [23] Bligh E, Dyer W. A rapid method of total lipid extraction and purification. *Can J*
554 *Biochem Phys* 1959;37: 911-918.
555 [24] Kates M. Techniques of lipidology. Work T, Work E (eds). North Holland Publishing
556 Company,1973, pp 465-469.
557 [25] Yamazaki M, Ohnishi S, Ito T. Osmoelastic coupling in biological structures: decrease
558 in membrane fluidity and osmophobic association of phospholipid vesicles in response to
559 osmotic stress. *Biochemistry* 1989;28: 3710-3715.
560 [26] Mykytczuk NC, Trevors JT, Leduca LG, Ferroni GD. Fluorescence polarization in
561 studies of bacterial cytoplasmic membrane fluidity under environmental stress. *Prog*
562 *Biophys Mol Biol* 2007;95:60-82.
563 [27] Litman BJ, Barenholz Y. Fluorescent probe: diphenylhexatriene. *Method Enzymol*
564 1982;81:678-685.
565 [28] Trevors, JT. Fluorescent probes for bacterial cytoplasmic membrane research. *J*
566 *Biochem Bioph Meth* 2003;57:87-103.

[29] Lakowicz JR. Principles of Fluorescence Spectroscopy. 2nd edn. Kluwer Academic, New York 1999.

[30] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 1976;72: 248-254.

[31] Zavaleta-Pastor M, Sohlenkamp C, Gao JL, Guan Z, Zaheer R, Finan TM et al. *Sinorhizobium meliloti* phospholipase C required for lipid remodeling during phosphorus limitation. *Proc Natl Acad Sci* 2010;107:302-307.

[32] Bloom M, Evans E, Mouritsen OG. Physical properties of the fluid lipid-bilayer component of cell membrane; a perspective. *Quarterly Reviews of Biophysics* 1999;24: 293-397.

[33] Konings WN, Albers MV, Koning S, Driessen JM. The cell membrane plays a crucial role in the survival of bacteria and archaea in extreme environments. *Antoine van Leeuwenhoek* 2002;81:61-72.

[34] Bohuszewicz O, Liu J, Low H. Membrane remodelling in bacteria. *J Struct Biol* 2016;196: 3-14.

[35] Boni LT, Stewart TP, Alderfer JL, Hui SW. Lipidpolyethylene glycol interactions: II. Formation of defects in bilayers. *J Membr Biol* 1981;62:71-77.

[36] Tilcock CP, Fisher D. Interaction of phospholipid membranes with polyethylene glycols. *Biochim. Biophys. Acta* 1979;557:53-61.

[37] Aldwinckle TJ, Ahkong QF, Bangham AD, Fisher D, Lucy, J.A. Effects of polyethylene glycol on liposomes and erythrocytes. Permeability changes and membrane fusion. *Biochim Biophys Acta* 1982;689:548-560.

590 [38] Malinin VS, Federik P, Barry R. Lentz Osmotic and Curvature Stress Affect PEG-
591 Induced Fusion of Lipid Vesicles but Not Mixing of Their Lipids. *Biophysc J*
592 2002;82:2090-2100.
593 [30] Nina S, Ludmilaa D, Svetlanab B, Olgab N, Olgaa P, et al. Effect of phenol-induced
594 changes in lipid composition on conformation of OmpF-like porin of *Yersinia*
595 *pseudotuberculosis. FEBS Lett* 2013;587:2260-2265.
596 [40] Fuller N, Rand RP. The Influence of lysolipids on the spontaneous curvature and
597 bending elasticity of phospholipid membranes. *Biophys J* 2001;81: 243-254.
598
599 [41] Beney L, Simonin H, Mille Y. Membrane physical state as key parameter for the
600 resistance of the gram-negative *Bradyrhizobium japonicum* to hyperosmotic treatments.
601 *Arch Microbiol* 2007;187: 387-396.
602 [42] Eibl H, Hill EE, Lands WE. The subcellular distribution of acyltranferases which
603 catalyze the synthesis of phosphoglycerides. *European J Biochem* 1969;9:250-258.
604 [43] Van Golde LM, Fleischer B, Fleischer S. Some studies on the metabolism of
605 phospholipids in Golgi-complex from bovine and rat liver in comparison to other
606 subcellular fraction. *Biochim Biophys Acta* 1979;249: 318-330.
607 [44] Zheng L, Lina Y, Lua S, Zhanga J, Bogdanov J. Biogenesis, transport and remodeling
608 of lysophospholipids in Gram-negative bacteria. *Biochim Biophy Acta* 2016*;*
609 *doi.org/10.1016/j.bbalip.2016.11.015*
610 [45] Davydova L, Bakholdina S, Barkina M, Velansky P, Bogdanov P, Nina S. Effects of
611 elevated growth temperature and heat shock on the lipid composition of the inner and outer
612 membranes of *Yersinia pseudotuberculosis. Biochimie* 2016;123:103-109.
613

614

615

616

617

618

619 **Figure legends**

620

621 **Fig. 1.** Effects of PEG shock on the viability of *B.* sp. SEMIA6144.

622 Viability is expressed as LOG CFU.ml^{-1}. Values represent the mean ± SEM of three
623 independent experiments. (■) Control without PEG; (●) control cells grown for 48 h and
624 then shocked with PEG.

625

626 **Fig.2.** Topography AFM images of *B.* sp. SEMIA6144. Three-dimensional images of
627 control (A) and exposed to PEG by 10 min (B) and 1 h (C). Two-dimensional image of the
628 population of *B.* sp. SEMIA6144 cells exposed for 1 h to PEG (D).

629

630 **Fig. 3.** Fluorescence polarization of DPH in *B.* sp. SEMIA6144 cells after PEG shock for
631 different amounts of time.
632 Cells cultivated in B-modified medium were harvested at the exponential phase (48 h),
633 washed and resuspended in Tris-HCl buffer to an OD 660 nm of 0.2. The fluorescent
634 membrane probe was added to the resuspended culture and incubated to facilitate
635 incorporation of the probe. Fluorescence polarization was measured in one aliquot as the
636 control sample (absence of PEG). The remainder of the culture was treated with PEG.
637 Aliquots were collected at different times (10, 20, 30 and 60 min) to determine the degree
638 of polarization. Values represent the mean ± SEM of three independent experiments.

639

640

641

642

643 **Fig. 4.** Effects of PEG shock on the composition of total phospholipids of *B.* sp.
644 SEMIA6144.
645 Cells grown at exponential phase with [1-^{14}C] acetate sodium salt (37KBq ml^{-1}) followed
646 by exposure to PEG for 1 h, 5 h and 24 h were harvested and the total phospholipids
647 analyzed by TLC.
648 Lysophosphatidylethanolamine (LPE), phosphatidylcholine (PC), phosphatidylglycerol
649 (PG), phosphatidylethanolamine (PE), cardiolipin (CL). Values represent the mean ± SEM
650 of three independent experiments. * indicates a statistically significant difference ($p < 0.05$)
651 for each condition studied with respect to control conditions.
652
653 **Fig. 5.** Effects of PEG shock on the composition of phospholipids of outer (a) and inner (b)
654 *B.* sp. SEMIA6144 membrane.
655 Cells grown at exponential phase with [1-14C] acetate sodium salt (37KBq ml-1) followed
656 by exposure to PEG for 1 h, 5 h and 24 h were harvested and the outer and inner membrane
657 fraction were obtained. The phospholipids were analyzed by TLC.
658 Lysophosphatidylethanolamine (LPE), phosphatidylcholine (PC), phosphatidylglycerol
659 (PG), phosphatidylethanolamine (PE), cardiolipin (CL). Values represent the mean ± SEM
660 of three independent experiments. * indicates a statistically significant difference ($p < 0.05$)
661 for each condition studied with respect to control conditions.
662
663 **Fig. 6.** Fluorescence polarization of DPH in MLVs prepared with lipids of *B.* sp.
664 SEMIA6144.
665 Cells grown at exponential followed by exposure to PEG for 1 h, 5 h and 24 h were
666 harvested and the outer and inner membrane fraction were obtained. Phospholipids of each

667 fraction were used to prepare MLVs as indicated in the materials and methods section. The
668 fluorescent membrane probe (DPH) was added to the suspension of vesicles and incubated
669 to facilitate incorporation of the probe. Fluorescence polarization was measured. Values
670 represent the mean ± SEM of three independent experiments.
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690

691

692 **Table 1.** Effects of PEG shock on the fatty acid composition of *B.* sp. SEMIA6144.

Fatty acid type (%)	Exponential Phase (48 h)			
	Control	PEG shock		
		1 h	5 h	24 h
Saturated				
16:0	15.9 ± 1.3	18.2 ±1.1*	14.7 ± 1.1	13.7 ± 1*
18:0	5.22 ± 1.7	7.2 ± 1.4*	2.4 ± 0.9*	1.2 ± 0.8*
Unsaturated				
16:1Δ9	0.53 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.8 ± 0.1*
18:1Δ11	78.1 ± 3	72.9 ± 3*	82 ± 3.2	84.6 ±3.1*
UFA/SFA†	**3.7**	**2.9**	**4.8**	**5.7**

693 To study the effects of growth under PEG on FA composition, *B.* sp. SEMIA6144 cells
694 were grown until the exponential phase was reached and then were exposed to PEG for 1 h,
695 5 h and 24 h. Cells were harvested, and FAME were obtained from total lipids and analyzed
696 by GC-MS. The percentage of each FA is relative to the total FA defined as 100 %.
697 † Ratio between the sums of unsaturated and saturated FA (UFA/SFA). Values represent
698 the mean ± SEM of three independent experiments. * indicates a statistically significant
699 difference ($P < 0.05$).

700

701

702

703

704

705

706 **Table 2.** Phospholipase A activity of *B.* sp. SEMIA6144 outer membrane of cells exposed
707 to PEG.

Growth conditions	PLA activity (% of substrate hydrolyzed)
Control	7.2 ± 0.7
1 h PEG	12.5 ± 0.5
5 h PEG	11.9 ± 0.9
24 h PEG	5.7 ± 0.5

708 Cells were grown until the exponential phase was reached and then were exposed to PEG
709 for 1 h, 5 h and 24 h; cells were then harvested, and protein fraction of the outer membrane
710 was used as enzymatic extracts. [1-^{14}C]-labeled PE of *E. coli* DH5α was used as substrate
711 and after expiry of the reaction time, the fraction of PE was scraped and the fraction
712 quantified by radioactivity measurements, activity is expressed as percent of substrate
713 hydrolyzed. Values represent mean ± SEM of two independent experiments.