Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Reorganization of *Azospirillum brasilense* cell membrane is mediated by lipid composition adjustment to maintain optimal fluidity during water deficit

A.B. Cesari[1], N.S. Paulucci[1], M.A. Biasutti[2], Y.B. Reguera[1], L.A. Gallarato[1], C. Kilmurray[1] and M.S. Dardanelli[1]

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

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

Keywords

Azospirillum brasilense, fatty acids, membrane fluidity, phospholipids, response, water deficit.

Correspondence

Marta Susana Dardanelli, Departamento de Biología Molecular, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta Nacional N° 36, Km 601. CPX5804BYA Río Cuarto, Córdoba, Argentina.

E-mail: mdardanelli@exa.unrc.edu.ar

2015/1849: received 8 July 2015, revised 23 October 2015 and accepted 26 October 2015

doi:10.1111/jam.12994

Abstract

Aims: We study the *Azospirillum brasilense* tolerance to water deficit and the dynamics of adaptive process at the level of the membrane.

Methods and Results: *Azospirillum brasilense* was exposed to polyethylene glycol (PEG) growth and PEG shock. Tolerance, phospholipids and fatty acid (FA) composition and membrane fluidity were determined. *Azospirillum brasilense* was able to grow in the presence of PEG; however, its viability was reduced. Cells grown with PEG showed membrane fluidity similar to those grown without, the lipid composition was modified, increasing phosphatidylcholine and decreasing phosphatidylethanolamine amounts. The unsaturation FAs degree was reduced. The dynamics of the adaptive response revealed a decrease in fluidity 20 min after the addition of PEG, indicating that the PEG has a fluidizing effect on the hydrophobic region of the cell membrane. Fluidity returned to initial values after 60 min of PEG exposure.

Conclusion: *Azospirillum brasilense* is able to perceive osmotic changes by changing the membrane fluidity. This effect is offset by changes in the composition of membrane phospholipid and FA, contributing to the homeostasis of membrane fluidity under water deficit.

Significance and Impact of the Study: This knowledge can be used to develop new *Azospirillum brasilense* formulations showing an adapted membrane to water deficit.

Introduction

Drought stress is common in many parts of the world, and more than 50% of the globe is arid, semiarid or subjected to some type of drought stress (Arzanesh *et al.* 2011). In Argentina, 75% of the total area of the country consists of arid, semi-arid, or dry regions, accounting for 60 million hectares (http://inta.gob.ar/noticias/desertificacion-en-argentina-el-problema-de-las-60-millones-de-hectareas). Water deficiency is the major problem in agriculture, and is of immense economic importance identify strategies to mitigate the negative effects.

In soil, the drought resulting in plant growth reduction and similar to plants, bacterial species is also subjected to water deficiency, and some of their physiological functions are affected by stress. The application of biofertilizers, such as plant growth-promoting rhizobacteria (PGPR), e.g. *Azospirillum* sp., enhanced growth of plants under stress (Arzanesh *et al.* 2011). *Azospirillum brasilense* has been successfully inoculated under abiotic stress in peanut, tomato, maize, bean, and rice (German *et al.* 2000; Casanovas *et al.* 2002; Rodríguez-Salazar *et al.* 2009; Cohen *et al.* 2009; Ruíz Sánchez *et al.* 2011; Volfson *et al.* 2013). Specifically it has been reported that

A. brasilense ameliorates the response of *Arabidopsis thaliana* to drought mainly via enhancement of ABA levels (Cohen *et al.* 2015).

Bacteria of the genus *Azospirillum* (α-subclass of proteobacteria) are Gram-negative free-living nitrogen-fixing rhizosphere bacteria. Under certain environmental and soil conditions, *Azospirillum* can positively influence plant growth, crop yields and N-content of the plant. This plant stimulatory effect exerted by *Azospirillum* has been attributed to several mechanisms, including biological nitrogen fixation and auxin production (Steenhoudt *et al.* 2011).

Studies of bacterial water stress physiology have generally examined the genetic and physiological mechanisms of adaptation to osmotic stress caused by permeating solutes as NaCl, used to lower the water potential of the growth medium (Csonka 1989). In many nonsaline soils, however, lowering of the water potential is due primarily to a reduction in the water content and not to an increase in the concentration of permeating solutes. High molecular-weight (i.e. a molecular weight higher than 3000) polyethylene glycol (PEG) has been used extensively in plant and microbial studies focused on responses to reduced water content. The importance of PEG use is that this compound is too large to penetrate cell walls and lower the water potential of a medium like a dry soil (Steuter *et al.* 1981).

Survival of bacteria in stressful conditions is often determined by their capacity to adapt. The cytoplasmic membranes are important sites of injury in bacteria exposed to water deficiency. The barrier function of the cytoplasmic membrane is known to be critically dependent on the physical state of lipid bilayers, making it susceptible to environmental changes (Cronan *et al.* 1987). In fact, normal cellular function requires membrane lipid that are largely fluid and the bilayers of most organisms are entirely or mostly fluid at physiological temperatures (Paulucci *et al.* 2013). The fluidity of biological membranes may be modulated by metabolic regulation of lipid composition (Thompson 1980). The primary lipid components of the bilayer are phospholipids (PL), with fatty acids (FAs) being the major component (Paulucci *et al.* 2013). The major membrane-forming PL in bacteria are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) (Denich *et al.* 2003). Microbial FA are typically 14–20 carbons long, saturated or unsaturated (Russell 1995). There is a complex relationship between membrane damage, susceptibility to stress, and lipid composition (Hardwood 1994). Increasing the osmotic pressure of the medium may induce structural modifications, such as lipids phase transitions that could damage the barrier properties of the membranes (Crowe and Crowe 1992), leading to cell death. However, many micro-organisms have been shown to modify lipid composition in order to maintain membrane fluidity by a process known as homeoviscous adaptation (Sinensky 1974). These modifications may be achieved by varying the amount of total PL and/or their class distribution, by changing the type of FA incorporated into them during synthesis or turnover, or by regulating the degree of unsaturation of the FA moieties. Not all bacterial species exhibit fully efficacious homeoviscous adaptation mechanisms (McElhaney 1982). In this regard, Cossins and Synensky (1984) introduced the concept of a variable homeoviscous efficacy that allows a quantitative estimation of the tendency of organisms to show a more or less ideal membrane homeostatic response.

Many previous studies on *Azospirillum* lipid composition have revealed the presence of saturated FA (SFA), such as lauric (12:0), myristic (14:0), palmitic (16:0), stearic (18:0) and the unsaturated FA (UFA) *cis*-vaccenic (18:1) (Mehnaz *et al.* 2007). UFA has an important effect on the fluidity and function of biological membranes (Aguilar and de Mendoza 2006). Numerous studies have demonstrated that FA plays a role in the adaptive response in many micro-organisms and is therefore an important factor in maintenance of biological activity (Drouin *et al.* 2000; Paulucci *et al.* 2011, 2013). However, little is known about the function of cytoplasmic membrane and its components in response to different environmental conditions in *A. brasilense*.

Our research group has extensive experience in the study of the *Arachis hypogaea.* L - rhizobacteria interaction (Dardanelli *et al.* 2003; Fabra *et al.* 2010; Paulucci *et al.* 2015). This legume is sensitive to water stress (Fernández *et al.* 2006; Dardanelli *et al.* 2009), affecting their ability to interact with rhizobia. Recently *A. hypogaea.* L has begun inoculating with *A. brasilense* for improved productivity in seeding areas under abiotic stresses (http://inta.gob.ar/documentos/nuevas-estrategias-para-mejorar-la-respuesta-a-la-inoculacion-de-mani-coin-oculacion-con-azospirillum-1). The study of *A. brasilense* tolerance to different environmental conditions and the mechanisms involved in the adaptation is necessary to generate strategies for a successful interaction between bacteria and plant even under unfavourable conditions.

The aim of this study was to evaluate the tolerance of *A. brasilense* to water stress value supported by the peanut plant at seedling emergence and to elucidate the mechanisms underlying the response at membrane lipids level. We evaluated the immediate effects of water deficit on the physical state of the bilayer and how membrane components are modified to compensate for this change. In addition we also evaluated the dynamics of the processes by which *A. brasilense* adapted to water stress.

Materials and methods

Bacteria and growth conditions

Azospirillum brasilense Az39 (Rodríguez Cáceres 1982) cells were maintained in plates with NFb medium containing 0·1% NH_4Cl (Dobereiner and Day 1976) at 28°C. The strain was stored at −80°C in 50% glycerol. To determine bacterial growth, viability and others parameters, the strain was grown in 25 ml of a high carbon–nitrogen (C–N) modified liquid medium D&D (Burdman *et al.* 1999) for 24 h in a shaking water bath (Allied Fisher Scientific) at 28°C. For treatment of water deficit, nonpermeating solute polyethylene glycol (average MW 5489; Sigma Chemical Co., St. Louis, MO) was added to the medium. In this paper a concentration of 15 mmol l^{-1} (90 g l^{-1}) PEG was used to generate a moderate stress. This concentration was selected by comparison of colony forming units per millilitre (CFU ml^{-1}) reached by *A. brasilense* Az39 after 24 h of growth without and with 15 mmol l^{-1} (90 g l^{-1}), 20 mmol l^{-1} (116 g l^{-1}), 30 mmol l^{-1} (180 g l^{-1}) and 40 mmol l^{-1} (232 g l^{-1}) PEG ($9{\cdot}7 \times 10^{10}$ CFU ml^{-1}, $1{\cdot}2 \times 10^{10}$ CFU ml^{-1}, 1×10^{10} CFU ml^{-1}, 5×10^{5} CFU ml^{-1}, no CFU ml^{-1} detected respectively). Addition of 15, 20, 30 and 40 mmol l^{-1} PEG gave water potential of −0·8, −1·07, −1·35 and −1·9 MPa respectively.

Based on this previous characterization, 15 $mmol^{-1}$ PEG concentration was selected for this study. Bacterial growth was followed by measuring optical density (OD) at a wavelength 600 nm. The viable cell count was performed in triplicate using the technique of the droplet of Miles and Misra (Somasegaran and Hoben 1994), where each dilution aliquot was plated on the respective solid media. The count values are expressed as colony forming units per ml of culture (CFU ml^{-1}). The growth rate and the generation time were calculated from growth curve at the exponential growth phase. Small samples were taken to determine the biomass and the protein content of cells by the method of Bradford (1976) after solubilization in 0·2 m l^{-1} NaOH in a boiling water bath for 10 min (bovine serum albumin was used as the standard).

Bacterial cell size determination

Bacterial cells from the control and stress treatment groups were stained by Gram staining and observed under a Zeiss microscope (Axiophot Carl Zeiss, Weimar, Germany) equipped with a Canon PC1089 Powershot G6 7.1-megapixel digital camera (Canon Inc., Japan). Photographs were processed and bacterial size was determined using the AXIOVISION 4.1 software (Carl Zeiss) (Medeot *et al.* 2010).

Determination of membrane fluidity

Membrane fluidity was determined by measuring fluorescence polarization of the 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) probe (Invitrogen) inserted into the cytoplasmic membranes. Fluorescence polarization quantifies the degree of depolarization of light emitted by the embedded fluorescence probe and is a measure of membrane state (Mykytczuk *et al.* 2007). The probe polarization ratio and cytoplasmic membrane fluidity are inversely correlated. As the bacterial cytoplasmic membrane fluidity decreases, the polarization ratio increases and vice versa (Litman and Barenholz 1982). Following procedures described by Trevors (2003), *A. brasilense* Az39 cells were grown in D&D media (Dobereiner and Day 1976) to OD 600 nm 0·8–1·00, 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 600 nm. Then, 1 μl of the fluorescent probe (stock solution diluted to 12 mmol l^{-1} in tetrahydrofuran) was added to each 3 ml aliquot of resuspended culture to obtain a final probe concentration of 4 μmol l^{-1}. Cultures were incubated with on a magnetic stirrer at 200 rev min^{-1} for 10 min in the dark at room temperature to allow DPH incorporation into cytoplasmic membranes. The culture was then divided; one part was maintained as the control and the other part was subject to 15 mmol l^{-1} PEG treatment at different times (10, 20, 30 and 60 min). Aliquots were collected and their degree of polarization was determined. *Azospirillum brasilense* Az39 grown in D&D medium with or without 15 mmol l^{-1} PEG were treated similarly until the beginning of the stationary phase (OD 600 nm 0·6) and then incubated with DPH and subject to similar measurements.

Fluorescence polarization measurements were performed in a Hitachi 2500 spectrofluorometer (Chiyoda, Tokyo, Japan) with a Glam-Thomson 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 expression $P = (IVV - IVHG)/((IVV + IVHG)$, where IVV and IVH are the intensities of vertically and horizontally polarized components of light emitted after excitation by vertically polarized light, and G is the sensitivity factor of the detection system (Lakowicz 1999).

Study of the lipid response of *Azospirillum brasilense* Az39 in response to water deficit

Azospirillum brasilense Az39 cells grown under the same conditions as used for membrane fluidity measurements were used to evaluate the role of membrane lipids in response to water deficit. For PL quantification, 0·5 μCi of sodium acetate was added to 25 ml of culture at the time of inoculation.

Total lipid extraction

Lipids were extracted from bacterial cells washed with chloroform/methanol/water (Bligh and Dyer 1959). The lower phase containing lipids was dried under N_2 and dissolved in an appropriate volume of chloroform/methanol (2 : 1 by vol).

Separation and analysis of PL

Thin layer chromatography (TLC) plates (silica gel HLF, 250 ml) were purchased from Analtech. Aliquots of the total lipid extracts were analysed by TLC using a solution of chloroform/acetone/methanol/acetic acid/water 40 : 15 : 14 : 12 : 7 (by vol) as the solvent. All solvents were of analytical or high-performance liquid chromatography grade. Lipids were detected with iodine vapours and the separated lipids were identified by comparison with authentic purified standards purchased from Sigma. The TLC plates were scraped and the fractions quantified by radioactivity measurements using a liquid scintillation counter (Beckman LS 60001 C, Brea, CA).

Analysis of fatty acids by gas chromatography

FA methyl esters from *A. brasilense* Az39 cells were analysed using a Hewlett Packard 5890 II gas chromatograph equipped with a highly polar column (HP 88) cyanopropyl (length 60 m, inner diameter 0·25 mm, film thickness 0·2 micron) and a flame ionization detector. The following gas chromatography conditions were used: injector temperature 250°C, detector temperature 300°C, and nitrogen as carrier gas. The temperature was programmed at 120°C for 1 min and then increased to 10°C min^{-1} to 175°C for 10 min, 5°C min^{-1} to 210°C for 5 min and 5°C min^{-1} to 230°C for 5 min. The peaks for each FA were identified using a mixture of commercial standards obtained from Sigma - Aldrich Chemical Co. and Supelco (Kates 1972). FAs were identified by comparing retention times to commercial standards (Sigma Chemical Co.).

Statistical analyses

All experiments were carried out in triplicate. One-way ANOVA was used to analyse the results of the inoculum size assay using InfoStat. When the analysis of variance showed significant treatment effect, the least significant differences test (Tukey, $P < 0{\cdot}05$) was applied to compare the mean values.

Results

Azospirillum brasilense Az39 growth under water deficit

Azospirillum brasilense Az39 (taken from the collection of the National Institute of Agricultural Technology) was grown with the nonpermeating solute PEG, which is used to simulate water deficit. 15 mmol l^{-1} PEG (−0·8 MPa) was selected as this is the concentration that produces a moderate water deficit as that found in peanut crop soils (Fernández *et al.* 2006).

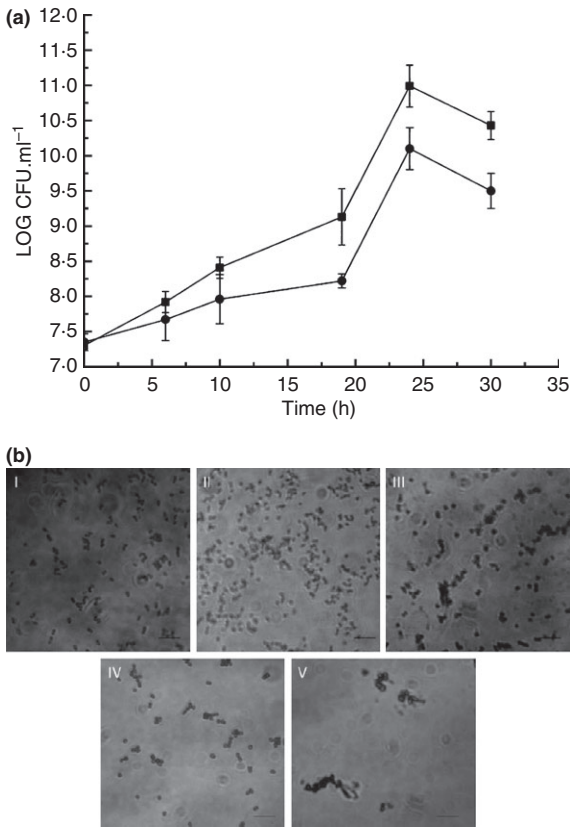

Figure 1 Effect of polyethylene glycol (PEG) growth on viability (a) and cells size (b) of *Azospirillum brasilense* Az39. Viability is expressed as LOG CFU ml^{-1}. Values represent mean ± SEM of three independent experiments. (■) Control; (●) 15 mmol l^{-1} PEG. Micrograph of cells grown without PEG (I) and with PEG 15 mmol l^{-1} PEG; (II); 20 mmol l^{-1} PEG (III); 30 mmol l^{-1} PEG (IV). Bar length 10 μm.

Lower CFU values were obtained after 24 h of growth in the presence of PEG compared to control ($1{\cdot}2 \times 10^{10}$ CFU ml^{-1} to $9{\cdot}7 \times 10^{10}$ CFU ml^{-1} respectively) (Fig. 1a). This effect was accompanied by a decrease in other parameters such as diminution of dry biomass (37·91%), growth rate (20%), increased generation time (from 2 to 2·5 h) and total protein content (13·82%) (data not shown).

Azospirillum brasilense Az39 cell size examined by optical microscopy showed a statistically significant increase from 1·6 to 2·15 μm when grown in the presence of PEG (Fig. 1b, II).

To determine if the cell size depends on the dose of PEG used, we evaluate the size of cells exposed to 20, 30 and 40 mmol l^{-1} PEG. The increase in the length of the cells resulted dose dependent (2·7, 3·2 and 3·5 μm respectively) (Fig. 1b).

Physical state and phospholipids and fatty acid composition of *Azospirillum brasilense* Az39 cell membrane when grown with PEG

The physical state of cell membranes of *A. brasilense* Az39 cells grown for 24 h with or without the addition of PEG was evaluated by fluorescence polarisation of the probe DPH (*P*). We found similar *P* values (0·21 ± 0·03 and 0·20 ± 0·01 respectively), which would indicate that *A. brasilense* Az39 cells grown with the addition of PEG have membranes with similar fluidity to those of cells grown without the addition of PEG.

To determine how cells grown for 24 h with PEG manage to maintain membrane fluidity, we investigated the PL and FA composition in membranes of *A. brasilense* Az39 grown for 24 h with or without PEG. In *A. brasilense* Az39, the predominant labelled PL was PE, followed by PC, PG, CL and lysophosphatidylethanolamine (LPE) in descending order. The growth of *A. brasilense* Az39 in the presence of PEG mainly affected PC and PE biosynthesis. There was a significant increase in PC (51%) and decrease in PE (67%) compared to the control (Table 1).

The FA composition of *A. brasilense* Az39 growth with or without PEG is shown in Table 2. The major FA are *cis*-vaccenic acid (18:1Δ11) followed by palmitoleic acid (16:1Δ9), palmitic acid (16:0), stearic acid (18:0), lauric acid (12:0) and myristic acid (14:0). Growth with PEG showed an increase in 18:0 (60·5%) and 16:0 (19·7%) but a decrease in 18:1Δ11 (6·9%). These changes mainly affected the degree of unsaturation, modifying the UFA/SFA (sum of UFA to sum of SFA) ratio from 7 to 4·75.

The observed changes in FA composition of *A. brasilense* Az39 when it was exposed to 15 mmol l^{-1} PEG were more pronounced when the bacteria grew with 30 mmol l^{-1} PEG (Fig. 2). An increase in saturated 16:0 FA (from 8·5 to 27·4) and 18:0 (from 2·2 to 34·9) accompanied by a decrease in 18:1Δ11 (from 66·6 to 30) was observed. In addition UFA/SFA ratio always showed decrease in treated cells in response to PEG (4·75, 3·06 and 0·6 for 15, 20 and 30 mmol l^{-1} PEG respectively). These findings suggest that the adaptive response to PEG growth is dose dependent. Like when *A. brasilense* Az39 grew with 15 mmol l^{-1} PEG, we observed that cells growing at different PEG

Table 1 Effect of polyethylene glycol (PEG) growth on the incorporation of [^{14}C] acetate into phospholipids (PL) of *Azospirillum brasilense* Az39

	Culture conditions	
PL	Control	15 mmol l^{-1} PEG
LPE	2·86 ± 1·3	1·81 ± 0·6
PC	28·5 ± 3·6*	55·7 ± 5·6*
PG	16·4 ± 4·5*	18·8 ± 4·1*
PE	35·0 ± 1·5*	11·6 ± 2·1*
CL	10·6 ± 2·8*	3·30 ± 0·2*
NL	2·72 ± 0·4	2·70 ± 0·8

Cells grown in the appropriate media for both test conditions with [1-^{14}C] sodium acetate (37 KBq ml^{-1}) were harvested in the stationary phase, and PL were analysed by thin layer chromatography. Phospholipids measured: lysophosphatidylethanolamine (LPE), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanoamine (PE), cardiolipin (CL), neutral lipids (NL). Values represent mean ± SEM of three independent experiments. The * indicates statistically significant differences ($P < 0{\cdot}05$).

Table 2 Effect of polyethylene glycol (PEG) growth on fatty acids composition of *Azospirillum brasilense* Az39

	Culture conditions	
FA (%)	Control	15 mmol l^{-1} PEG
Saturated		
12:0	1·54 ± 0·4	1·31 ± 0·4
14:0	0·44 ± 0·2	0·50 ± 0·01
16:0	8·19 ± 0·4*	10·2 ± 1·9*
18:0	2·14 ± 0·2*	5·42 ± 0·4*
Unsaturated		
16:1Δ^{9}	22·5 ± 2·5	23·8 ± 2·9
18:1Δ^{11}	63·6 ± 2·2*	59·2 ± 2·4*
NI	1·2 ± 0·4*	2·06 ± 0·8*
UFA/SFA†	7	4·75

A. brasilense Az39 cells were cultured for 24 h at 28°C with or without PEG. Fatty acid methyl esters were obtained from total lipids and analysed by gas chromatography. The percentage of each FA is relative to total FA defined as 100%. NI: not identified.
†Ratio between the sum of unsaturated and the sum of saturated FA. Values represent mean ± SEM of three independent experiments. The * indicates statistically significant differences ($P < 0{\cdot}05$).

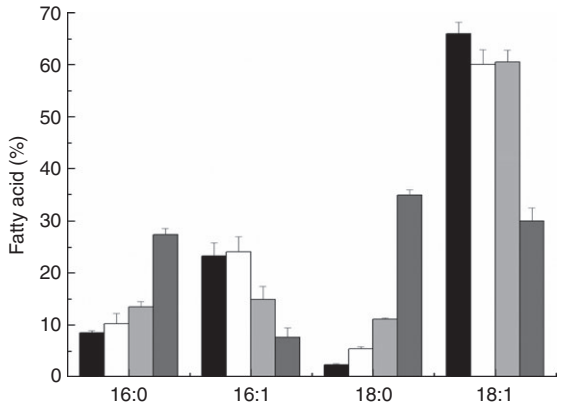

Figure 2 Effect of polyethylene glycol (PEG) concentration on the fatty acid composition of *Azospirillum brasilense* Az39. Cells were cultured for 24 h at 28°C exposed or not to 15, 20 and 30 mmol l^{-1} PEG. Fatty acid methyl esters were obtained from total lipids and analysed by gas chromatography. The percentage of each FA is relative to total FA defined as 100%. Values represent mean ± SEM of three independent experiments. (■) Control; (□) 15 mmol l^{-1} PEG; (▒) 20 mmol l^{-1} PEG; (▓) 30 mmol l^{-1} PEG.

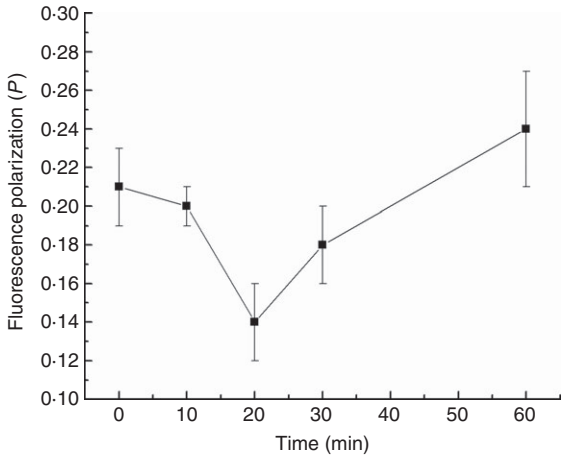

Figure 3 Fluorescence polarization of DPH in *Azospirillum brasilense* Az39 cells after polyethylene glycol (PEG) shock at different times. Cells cultivated in D&D medium were harvested at the late exponential phase, washed and resuspended in Tris-HCl buffer to an OD660 nm of 0·2. The fluorescent membrane probe was added to the resuspended culture and incubated to allow probe incorporation into the cytoplasmic membrane. Fluorescence polarization was measured in one aliquot as the control sample (PEG absence). The rest of the culture was treated with 15 mmol l^{-1} PEG. Aliquots were collected at different times (10, 20, 30 and 60 min) to determine the degree of polarization. Values represent mean ± SEM of three independent experiments.

concentrations (20, 30, 40 mmol l^{-1}), had *P* values similar to control. These results suggest that when grown in the presence of PEG, *A. brasilense* Az39 can maintain membrane fluidity by modifying its PL and FA composition. To determine the dynamics of the adaptive response, we evaluated the effect of PEG on the physical state and the composition of the membrane after varying lengths of PEG exposure.

Behaviour of *Azospirillum brasilense* Az39 membrane during adaptation to water deficit

When we evaluated membrane fluidity, we found that *P* decreased from 0·21 ± 0·02 immediately after the addition of PEG, reaching a *P* value of 0·14 ± 0·02 after 20 min of PEG exposure (Fig. 3). However, as shown in Fig. 3, after 60 min of PEG exposure, *P* returned to a similar value to the one obtained in the absence of PEG treatment ($P = 0{\cdot}24 \pm 0{\cdot}03$), indicating that the response of the cell membrane occurs after 60 min of PEG exposure. Our results suggest that the fluidizing effect of PEG (Fig. 3) was counteracted in the cell membranes. To determine the potential mechanisms involved in maintenance of membrane fluidity, we studied bacterial response to the presence of PEG in terms of the composition of membrane PL and FAs. When the bacteria were exposed to PEG for 60 min, a decrease in 18:1Δ11 FA (from 70·5 to 63·6) and an increase in 16:0 (from 6·7 to 9·2) were observed compared to absence PEG treatment (0 min) (Fig. 4a). Among membrane PL, a marked increase in PC (from 22·3 to 40·2) and LPE (from 5·7 to 12·4) and a decrease in PE (from 34·8 to 5·7) was observed (Fig. 4b).

Discussion

Water deficit is one of the main environmental conditions affecting performance, survival and the quality of crops and is part of the most significant risks associated with agricultural production in Argentina (Straschnoy *et al.* 2006). One strategy to increase plant growth and yield is the use of bacterial PGPR inoculants. An association between *Azospirillum* and the plant root can be successful only if the bacterium is able to survive in the soil and tolerate stress conditions. The ability of any bacterium to grow in a medium with a water potential of −0·73 MPa was considered as drought tolerant (Chakraborty *et al.* 2013). Our results show that although its viability decreased significantly, *A. brasilense* Az39 was able to adapt and grow in the presence of a moderate water deficit (−0·81 MPa). Growth parameters were negatively modified in the presence of PEG. Consistent with our results, Mhamdi *et al.* (2014) reported a progressive decrease in the number of viable cells of rhizobia strains belonging to *Mesorhizobium*, *Sinorhizobium* and *Rhizobium* genera with an increase in drought stress (15% to 30% of PEG).

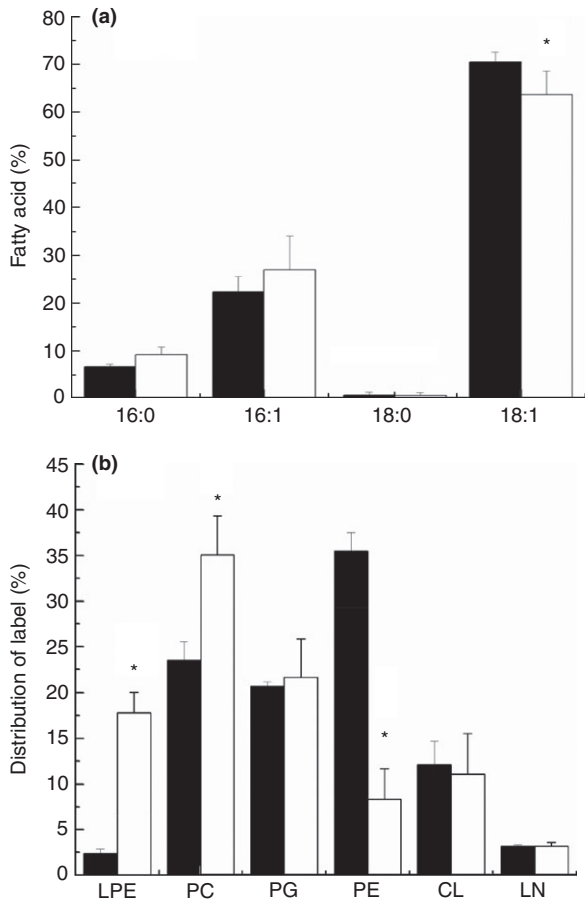

Figure 4 Effect of 60 min exposure of polyethylene glycol (PEG) on the composition of fatty acids (a) and phospholipids (b) of *Azospirillum brasilense* Az39. Values represent mean ± SEM of three independent experiments. The * indicates statistically significant differences ($P < 0{\cdot}05$). (■) Control; (□) 15 mmol l^{-1} PEG (60 min).

Examination of *A. brasilense* Az39 by optic microscopy revealed a difference in the morphology of the bacteria. Compared to the control sample, the size of *A. brasilense* Az39 varied when grown in the presence of PEG. The length of cells grown under water deficit increased significantly from 1·6 to 2·15 μm. An increase in cell size has also been observed for *Rhizobium* sp. NBRI330 when grown in the presence of high salt for 24 h (Kulkarni and Nautiyal 2000). Neumann *et al.* (2005) observed that *Pseudomonas putida* changed its cell shape when grown in the presence of aromatic compounds such as phenol and 4-chlorophenol. The cells showed an increase in the diameter depending on the toxic effects of the applied concentrations of both solvents. The same effect was measured for *Enterobacter* sp., in the presence of n-butanol. Bigger cells are better protected against toxic organic compounds than smaller cells of the same species.

Biological membranes are cellular structures that can sense and respond to environmental changes. Membrane PL have considerable freedom of movement, and the term membrane fluidity represents the dynamic properties of lipids within the bilayer. This structure, called the fluid-crystalline lamellar phase, is dominant in functional membranes (Gervais and Beney 2001). Here, we investigated how the presence of PEG alters membrane fluidity. These results indicate that the *A. brasilense* Az39 membrane adapts to osmotic environmental changes. The perturbation of membrane fluidity by extrinsic factors initiates an active response that tends to counteract these effects by bringing about intrinsic chemical changes. In *E. coli*, two cultures grown at 27 and 37°C have similar membrane fluidity. The dynamic characteristics of lipids in growing bacteria are the same regardless of the temperature of the culture (Gervais and Beney 2001). This characteristic is described as the homeoviscous adaptation (Sinensky 1974).

The mechanisms developed by the cell to stabilize the cytoplasmic membrane against environmental changes include modifications in PL and FA compositions, among others. In this study, *A. brasilense* Az39 grown in the presence of PEG was found to modify the PL composition by increasing PC and decreasing PE synthesis. PC is a phospholipid that tends to form in the membrane bilayer structure so that membranes with a greater amount of PC (bilayer forming) tend to be packaged more than those containing greater amounts of PE (nonbilayer forming). Balancing bilayer and nonbilayer forming lipids within the membrane is crucial in maintaining structural and functional integrity (Denich *et al.* 2003). In strain *A. brasilense* Az39, the increase in PC with water deficiency could be indicative of an important adaptive mechanism for maintenance of the structure and function of the membrane under stress conditions. Numerous studies have reported the involvement of PC in abiotic stress responses. Paulucci *et al.* (2015) observed that *Ochrobactrum* strain L115 modifies the PL composition when grown at high temperatures (37°C) and under high salinity conditions (300 mmol l^{-1} NaCl), increasing PC and decreasing PE synthesis. Rhizobial strains show an increase in PC when exposed to 37°C, NaCl and combined conditions (Paulucci *et al.* 2011).

In addition to the modification in the PL composition, the modification of the FA unsaturation degree was reported to be the most important mechanism for maintaining the physical properties of the rhizobia membrane cells (Paulucci *et al.* 2011). Our results indicate that after 24 h of growth under control conditions, the main FA found in *A. brasilense* Az39 were the UFA 18:1Δ11, followed by 16:1Δ9. The majority of SFA detected were 16:0 and 18:0. It has been shown that bacteria with high UFA

content have high PC levels to compensate for the increased volume generated by cis UFA and maintain the stability of the lipid bilayer (Geiger *et al.* 2013). Changes in membrane FA composition were observed when *A. brasilense* Az39 was grown in the presence of PEG. The main change was an increase in the percentage of SFA (16:0 and 18:0) and decrease in UFA (16:1 and 18:1 Δ11). This effect becomes more pronounced depending on the PEG dose applied. Our findings indicate that *A. brasilense* Az39 is capable to sense different PEG concentrations and to respond accordingly. Thereby the bacteria maintain optimal membrane fluidity by adjusting lipid composition. Similar to us, Boumahdi *et al.* (2001) showed that small reductions in water activities of the growth media for *Sinorhizobium melilotti* and for *Bradyrhizobia* resulted in a gradual decrease in the degree of unsaturation.

Although FA profiles for different strains of *Azospirillum* have been reported (Schenk and Werner 1988), our work is the first to demonstrate that FA plays a role in the response to water deficit for this genus of bacteria and specifically for the *A. brasilense* Az39 strain. Mhamdi *et al.* (2014) showed that for *Sinorhizobium* strains, the main modification in response to osmotic stress (PEG) is preferential accumulation of the 19:0 cyclo and 18:0 SFA. When *A. brasilense* Az39 cells were exposed to PEG, the amount of SFA was approx. 30% higher than that of control samples and resulted in a decrease in the UFA/SFA ratio. Increases in the degree of saturation of the membrane FA reduce the fluidity of the cell membrane and slows the rate of water loss in low water membrane potential environments (Kates 1986). Although, Johnson *et al.* (2011) had an opposite effect on the cytoplasmic membrane FA of *Sphingomonas wittichii* when expose that to short and long term of PEG 8000. These results provide physiological evidence that PEG has different effect on the cytoplasmic membrane depending on the micro-organism and the FA membrane composition.

The dynamic and structural characteristics of membranes are very sensitive to environmental conditions, mainly because of their liquid-crystalline nature, and are used by cells in the perception of stresses (Gervais and Beney 2001). To determine how the *A. brasilense* Az39 cells perceive water deficiency at the membrane l evel and gradually adapt to this condition, PEG exposure studies were performed at different times evaluating changes in the fluorescence depolarization probe DPH and in PL and FA composition. *Azospirillum brasilense* Az39 starts sensing PEG from 10 min after exposure by increased membrane fluidity. This early effect in membrane fluidity is then reversed from 20 min of PEG exposure, reaching fluidity values similar to the initial fluidity of cells without PEG treatment or with up to 60 min of PEG exposure. These results indicate a high efficiency homeoviscous of *A. brasilense* Az39 membrane in response to water deficit. When we evaluated the composition of PL and FA after 60 min of PEG exposure, the response observed was similar to that observed after growing *A. brasilense* Az39 for 24 h in the presence of PEG (increased PC and SFA and decreased PE levels). Moreover, the early response also involved an increased LPE. The PE headgroup in LPE should increase the van der Waals interactions and the lipid packing (Kalani *et al.* 2006). Our results demonstrate that simultaneous changes to the head group and chain acyl can cancel out the effects of PEG on lipid fluidity. It is plausible that alterations in composition could be used to change membrane fluidity and activate specific stress response pathways.

Beney *et al.* (2007) observed that treatment with glycerol solution for 30 min had a fluidizing effect on *B. japonicum* membrane lipids.

Our results indicate that *A. brasilense* Az39 cells are able to quickly perceive changes in the osmotic pressure of the medium by changing their membrane fluidity. This effect is then offset by changes in the composition of FA and PL, being PC, the PL more involved in this particular stress response.

Based on these results, we are currently investigating whether the bacterium is able to maintain its PGPR properties and whether is capable of alleviating the adverse effects of water stress on peanuts plants during its early development. Considering the importance of the broad application of PGPR inoculants in Argentina, this knowledge can be used to develop new *A. brasilense* Az39 formulations showing an adapted membrane to water deficit.

Acknowledgements

Financial assistance was provided by PIP CONICET 112-201101-00309. A.B.C, Y.B.R, L.A.G and C.K are fellows of CONICET-Argentina. N.S.P, M.A.B and M.S.D. are members of the Research Career of CONICET-Argentina.

Conflict of Interest

No conflict of interest declared.

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