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Adaptational Changes in Lipids of *Bradyrhizobium* SEMIA 6144 Nodulating Peanut as a Response to Growth Temperature and Salinity

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Abstract. Phospholipids provide the membrane with its barrier function and play a role in a variety of processes in the bacterial cell, as responding to environmental changes. The aim of the present study was to characterize the physiological and metabolic response of *Bradyrhizobium* SEMIA 6144 to saline and temperature stress. This study provides metabolic and compositional evidence that nodulating peanut *Bradyrhizobium* SEMIA 6144 is able to synthesize fatty acids, to incorporate them into its phospholipids (PL), and then modify them in response to stress conditions such as temperature and salinity. The fatty acids were formed from [1-^{14}C]acetate and mostly incorporated in PL (95%). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) were found to be the major phospholipids in the bacteria analyzed. The amount and the labeling of each individual PL was increased by NaCl, while they were decreased by temperature stress. The amount of PC, PE, and PG under the combined stresses decreased, as in the temperature effect. The results indicate that synthesized PL of *Bradyrhizobium* SEMIA 6144 are modified under the tested conditions. Because in all conditions tested the PC amount was always modified and PC was the major PL, we suggest that this PL may be involved in the bacteria response to environmental conditions.

Rhizobia are soil bacteria that can elicit the formation of nitrogen-fixing nodules on the legumes. Within the nodule, differentiated intracellular forms of rhizobia called bacteroids reduce molecular dinitrogen to ammonia. Rhizobia are thus economically important not only for the increase of agricultural legume yields, but also for the enrichment of the soil with nitrogen for other crops [4]. Environmental conditions usually affect symbiosis between rhizobia and its host.

Cellular envelopes are the first barriers that protect the bacterium against different environmental stresses; changes in their composition may represent adaptive mechanisms against the stress exerted [7].

The lipid bilayer forms the framework of the cytoplasmic membranes. The primary lipid components of the bilayer are phospholipids (PL). They provide the membrane with its barrier function and play a role in a variety of processes in the bacterial cell, such as energy transduction, signal transduction, solute transport, and cell–cell recognition [8]. Phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) are characteristic phospholipids of most Gram-negative bacteria. Phosphatidylcholine (PC) has also been found in bacteria, and it is more widespread than originally thought, although its role is unclear [17]. The PL pattern of rhizobia still is not entirely clear, but there is general agreement that PE and PC are present in appreciable amounts and that PG is also consistently present. In addition, monomethylphosphatidylethanolamine (MMPE) and dimethylphosphatidylethanolamine (DMPE) may also be formed [29]. Among the PL mentioned, PC may play a particularly important role, because it has been found that *Bradyrhizobium japonicum* PC participates in successful interaction with the eukaryotic host [18]. PL changes have been reported in response to environmental stress such as that produced by temperature [20] and by

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salt [25]. In addition, low-oxygen conditions have been shown to increase PE and PG amounts and also to decrease PC amount [27].

Little is yet known about the biochemical and physiological basis of saline and temperature tolerance by rhizobia nodulating *Arachis hypogaea* (peanut) roots. Previous work from our laboratory suggests that the trehalose metabolism of peanut rhizobia changes in response to the salinity of the medium [11]. To the best of our knowledge, the incorporation of [1-^{14}C]acetate into PL of *Bradyrhizobium* strain nodulating peanut have not been reported. The aim of the present study was to characterize the physiological and metabolic response of *Bradyrhizobium* SEMIA 6144 to saline and temperature stress. By using [1-^{14}C]acetate, we followed the labeling of the fatty acids (FA) acyl moieties of PL of strain under control and stressed conditions.

Materials and Methods

Bacterial Strain and Culture Conditions. The strain used in this study, *Bradyrhizobium* SEMIA 6144 [12], was provided by MIRCEN/FEPAGRO. The bacterial strain was maintained in yeast extract mannitol (YEM) plates [22] at 28°C. The pH of the medium was adjusted to 7 before autoclaving. For the determination of bacterial growth, viability, and biochemical parameters, the strain was grown in B medium [24] for 120 h in a shaking water bath at 28°C and 37°C for temperature stress treatment. The medium was supplemented with 50 mM NaCl for saline stress treatment. Bacterial growth was followed by measuring the optical density at 620 nm (OD_{620}), with three replicates. Viable cells were counted by removing samples at various times. Serial dilutions of each sample were spotted onto YEM plates in triplicate and incubated at 28°C. Viable cells were counted after 4–5 days.

Incorporation of Labeled Acetate. Previously sterilized [1-^{14}C]acetate, sodium salt, (2.26 GBq mmol^{-1}), New England Nuclear, was added to the media (37 KBq mL^{-1}). Cells were harvested at late exponential phase by centrifugation at 6,000 *g* for 10 min, in a Beckman Allegra 64R refrigerated centrifuge. Pellets were washed two times with 0.9% NaCl and used for further studies. The same procedures and conditions were used for unlabeled samples.

Lipid Extractions. The lipids were extracted from the washed bacteria with chloroform/methanol/water extraction [3]; 0.1 M KCl in 50% methanol was added to obtain a lower chloroform phase and an upper phase. The lower phase, containing the lipids, was washed once with the KCl solution, dried under N_2 and dissolved in a suitable volume of chloroform/methanol 2:1 (vol/vol).

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

Processing of Nonradioactive Samples. Aliquots of the extracts were taken for the determination of total lipid phosphorus. PL were resolved from neutral lipids using the TLC procedure just described for labeled samples. PL classes were scraped and the fractions were quantified by phosphorus determination [19].

Statistical Analyses. All these statistical analyses were performed using a one-way analysis of variance test.

Results and Discussion

Bacterial Strain and Culture Conditions. Soils during the summer season are subjected to high temperature stress and as a consequence the saline concentration increases, which may have detrimental effects on the introduced rhizobia. Temperature can affect rhizobial persistence in inoculants during shipment or in storage. In addition, salt and temperature stress can influence survival in soil and can limit both nodulation and nitrogen fixation. An understanding of the growth of *Rhizobium* is likely when the physiology of these organisms has been carefully studied under these suboptimal conditions [1, 15].

Figure 1a shows the growth of *Bradyrhizobium* SEMIA 6144 in control and stressed conditions. The strain was able to grow in the presence of 50 mM NaCl and at 37°C, although growth rates decreased especially with the combination of both stresses.

When the viability of *Bradyrhizobium* SEMIA 6144 was tested, it was observed that it was negatively affected by the combination of both stresses, although they were able to survive with salt and temperature stress (Fig. 1b). These results are in agreement with those obtained by Kulkarni and Nautiyal [15]. Changes in growth rates of rhizobia have also been reported to be related to adaptation to different environmental situations such as pH, salt, and temperature [7, 9, 21, 23].

Incorporation of Labeled Acetate. Regarding lipid metabolism, [1-^{14}C]acetate was incorporated into total lipids of *Bradyrhizobium* SEMIA 6144. The precursor was incorporated mostly (94–95%) into PL, the rest being recovered in the neutral lipid fractions. Most of the radioactivity incorporated was in FA esterified to lipids. The incorporation of [1-^{14}C]acetate into the total lipids of *Bradyrhizobium* SEMIA 6144, which was 10,126 Bq.g wet wt^{-1} when the strain was grown at 28°C, was affected by salt (15,815 Bq.g wet wt^{-1}) and by temperature stress (5,943 Bq.g wet wt^{-1}). NaCl increased the incorporation of radioactive substrate into total PL (from 9,650 to 15,097 Bq.g wet wt^{-1}), while the contrary effect was observed with temperature stress (from 9,650 to 5,590 Bq.g wet wt^{-1}). Consistent with the results from [1-^{14}C]acetate labeling, the most significant change associated with the stresses was a sustained

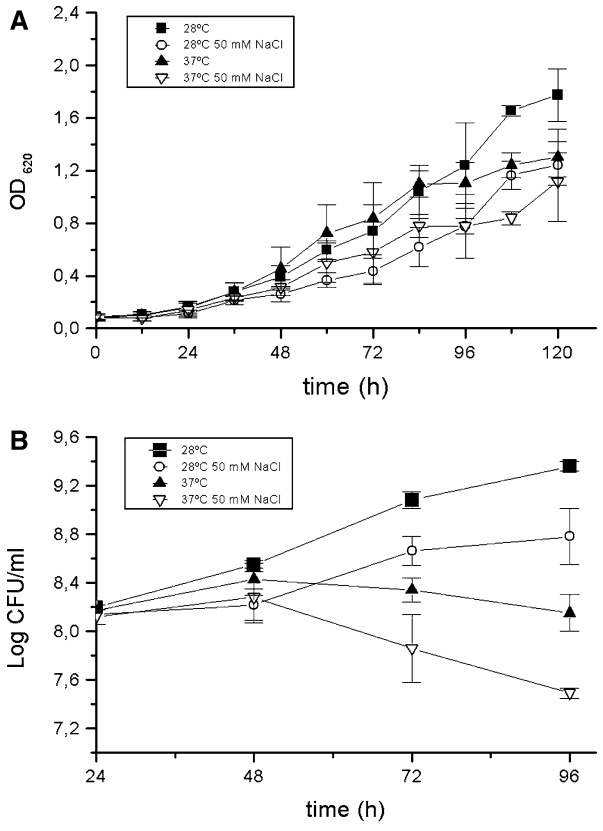

Fig. 1. Effect of NaCl and temperature on growth **(a)** and survival **(b)** of *Bradyrhizobium* SEMIA 6144. Growth was measured by the increase in absorbance at 620 nm. Viability results are expressed as CFU mL^{-1}. Values represent means ± S.E.M. of three independent experiments. (a) $P < 0.05$, 28°C, 50 mM NaCl 48 h and 37°C; $P < 0.01$, 28°C, 50 mM NaCl 72 h, 96 h, 108 h, 37°C, 108 h and 37°C 50 mM NaCl 96 h; $P < 0.001$, 28°C, 50 mM NaCl 60 h and 37°C 50 mM NaCl 108 h, 120 h, related to 28°C. (b) $P < 0.05$, 28°C, 50 mM NaCl 48 h; $P < 0.01$, 28°C 50 mM NaCl 72 h, 96 h; $P < 0.001$ 37°C 72 h, 96 h, and 37°C 50 mM NaCl 72 h, 96 h, related to 28°C.

increase in the total amount of PL (measured by phosphorus concentration) with NaCl (from 4.5 to 6.2 mg PL.g wet wt^{-1}) and a decrease with temperature stress (from 4.5 to 2.7 mg PL.g wet wt^{-1}). These findings agree with previous results of our laboratory using *Bradyrhizobium* sp strain TAL 169 [5]. Surprisingly, when both stresses were applied together, only the amount of PL was affected (from 4.5 to 2.9 mg PL.g wet wt^{-1}), because the labeling from acetate was not modified. The fact that PL amount and the cell number (Fig. 1b) were decreased while Bq.g wet $wt.^{-1}$, was not modified would indicate that *Bradyrhizobium* SEMIA 6144 cells are active metabolically.

Separation and Analysis of PL. Table 1 shows that PC was the most actively labeled PL, followed by PE, PG, CL, DMPE, and phosphatidic acid (PA). The PL composition of *Bradyrhizobium* SEMIA 6144 resembles that described for other rhizobia such as *Mesorhizobium loti* [7], *Bradyrhizobium japonicum* [14], *Mesorhizobium ciceri* [6], and *Rhizobium meliloti* [13]. The PL patterns were found to be qualitatively the same in all conditions. The PL labeling of each individual PL was significantly increased by NaCl, whereas a significantly negative effect was revealed by temperature stress. Other changes were also observed when the bacteria were grown with NaCl; thus, the ratio zwitterionic (PE, DMPE, PC) to anionic (PG, CL, PA) PL was decreased (15%). When the bacteria were grown at 37°C this ratio also was decreased (40%). Both modifications may be a consequence of the increased amount of the anionic PL, suggesting an increase of negative charges on the membrane provoked by temperature stress [10]. In relation to that, Bakholdina et al. [2] observed changes in the relative percentages of each PL and in the ratio between zwitterionic to anionic PL with growth temperature. Also, Lewis et al. [16] noted that the accumulation of the acidic PL might be considered as a factor of stabilization for membrane lipid bilayer.

The combination of both stresses provoked differential effects on the labeling of PL. Thus, CL labeling tended to increase (16%), whereas DMPE, PG, and PA labeling tended to decrease (25%, 24%, and 39%, respectively). The most relevant changes observed under both stresses were a significant increase of PC (30%) and a significant reduction of PE (44%). The decrease in the labeling of several PL might be offset by the increase in the labeling of PC, the major PL, maintaining the total labeling and the ratio zwitterionic to anionic PL similar to control (28°C). The increase of PC and the concomitant decrease of PE would indicate that *Bradyrhizobium* SEMIA 6144 is able to synthesize PC from PE by methylation pathway. However, when the amounts of PC, PE, and PG were measured under combined stresses, it was observed that they significantly decreased, similarly as under the temperature effect, even though the distribution of labeling among PL showed a different behavior, as was described above. A similar conduct was described for other organisms, where it was observed that the total amount of label in lipids increased even though the distribution of label among lipids did not change significantly [28]. Because in all conditions tested the PC amount was always modified and PC was the major PL, we suggest that this PL may be involved in the bacterial response to environmental conditions. The importance of PC in other responses like the successful interaction with the eukaryotic host has been noted by Minder et al. [18]. The other PL, i.e., PE, which would be the substrate for PC synthesis in this organism,

Table 1. Effect of NaCl and temperature on phospholipids composition of *Bradyrhizobium* SEMIA 6144[a]

	28°C						37°C					
	B medium			B medium + NaCl			B medium			B medium + NaCl		
PL	mg PL.g wet wt^{-1} × 10	Bq.g wet wt^{-1}	DL %	mg PL.g wet wt^{-1} × 10	Bq.g wet wt^{-1}	DL %	mg PL.g wet wt^{-1} × 10	Bq.g wet wt^{-1}	DL %	mg PL.g wet wt^{-1} × 10	Bq.g wet wt^{-1}	DL %
PC	12.0 ± 1.7	4993 ± 548	43.7	19.9 ± 3.5*	7973 ± 595**	50.2	7.7 ± 2.6*	3208 ± 483***	54.6	7.6 ± 1.4*	5170 ± 653*	57.1
DMPE	1.3 ± 0.5	270 ± 120	2.9	1.8 ± 0.9	320 ± 180	1.9	ND	127 ± 52	2.3	1.8 ± 0.9	204 ± 22	2.0
PG	4.8 ± 1.1	945 ± 112	10.1	8.9 ± 1.3*	1343 ± 33***	8.9	1.6 ± 0.2*	453 ± 138*	6.4	1.9 ± 0.3***	723 ± 207	6.8
PE	9.7 ± 2.3	2205 ± 37	24.4	13.0 ± 1.9*	2890 ± 20***	21.4	4.3 ± 0.4*	797 ± 175**	19.0	3 ± 0.5***	1232 ± 167***	15.5
CL	2.3 ± 0.2	702 ± 175	6.6	3.8 ± 1.1*	1090 ± 320	6.1	4.7 ± 0.8*	183 ± 60**	3.1	2.6 ± 1.0	813 ± 177	7.0
PA	0.6 ± 0.2	138 ± 23	1.2	0.9 ± 0.1	160 ± 32	0.9	0.5 ± 0.2	72 ± 15	1.8	0.3 ± 0.1	85 ± 23	1.0
NI	11.7 ± 4.1	994 ± 322	11.1	13.3 ± 3.2	1316 ± 366	10.6	9.4 ± 1.4	743 ± 169	12.7	10.1 ± 1.3	1024 ± 177	10.5

[a] *Bradyrhizobium* SEMIA 6144 was grown in B medium and harvested at late exponential phase. [1-^{14}C]acetate, sodium salt (37 KBq mL^{-1}) was added when Bq/g wet wt was measured. Values represent means ± S.E.M. of three independent experiments.
DL distribution of label. ND no detected, NI no identified, PL phospholipids, PC Phosphatidycholine, DMPE dimethylphosphatidylethanolamine, PG phosphatidylglycevol, PE phosphatidylethanolamine, CL cardiolipin, PA phosphatidic acid.
* $P < 0.05$, *** $P < 0.01$, ** $P < 0.001$ related to 28°C in B medium.

was modified in every condition. The PE variation in both the labeling and the amount would implicate the PE participation in the response to stress, as has been noted for other factors [7, 10]. On the other hand, the effect of oxygen tension on membrane chemistry is especially interesting because, similarly to our present results, the changes involving PC and PE from *Bradyrhizobium japonicum* were also found under low-oxygen conditions. Thus, besides salt and temperature stresses, in future studies it should be considered the oxygen tension, because it is critical to the physiology of the bacterium once this is inside the plant [27]. These results taken together indicate that saline and temperature stress affect both labeling of fatty acids and polar head of PL. These changes maintain the optimal physical state of membrane lipid matrix that is necessary for the normal functioning of the bacterial cell [26].

In this study, we showed that *Bradyrhizobium* SEMIA 6144 growth is affected by salt and temperature stress. These conditions induce modifications in the composition of PL, suggesting that these changes may be adaptive responses to salt and temperature stress. Thus, *Bradyrhizobium* SEMIA 6144 is able to respond and adapt their cell envelopes to changing environments in order to survive.

Experiments are in progress in our laboratory in order to determine FA composition of *Bradyrhizobium* SEMIA 6144 to then consider both PL and FA composition under nodulation effect.

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