Lipids (2011) 46:435–441
DOI 10.1007/s11745-011-3545-1

ORIGINAL ARTICLE

Growth Temperature and Salinity Impact Fatty Acid Composition and Degree of Unsaturation in Peanut-Nodulating Rhizobia

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Received: 6 July 2010 / Accepted: 21 February 2011 / Published online: 10 March 2011

Abstract Growth and survival of bacteria depend on homeostasis of membrane lipids, and the capacity to adjust lipid composition to adapt to various environmental stresses. Membrane fluidity is regulated in part by the ratio of unsaturated to saturated fatty acids present in membrane lipids. Here, we studied the effects of high growth temperature and salinity (NaCl) stress, separately or in combination, on fatty acids composition and de novo synthesis in two peanut-nodulating *Bradyrhizobium* strains (fast-growing TAL1000 and slow-growing SEMIA6144). Both strains contained the fatty acids palmitic, stearic, and *cis*-vaccenic + oleic. TAL1000 also contained eicosatrienoic acid and cyclopropane fatty acid. The most striking change, in both strains, was a decreased percentage of *cis*-vaccenic + oleic (≥80% for TAL1000), and an associated increase in saturated fatty acids, under high growth temperature or combined conditions. Cyclopropane fatty acid was significantly increased in TAL1000 under the above conditions. De novo synthesis of fatty acids was shifted to the synthesis of a higher proportion of saturated fatty acids under all tested conditions, but to a lesser degree for SEMIA6144 compared to TAL1000. The major adaptive response of these rhizobial strains to increased temperature and salinity was an altered degree of fatty acid unsaturation, to maintain the normal physical state of membrane lipids.

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Keywords Fatty acid composition · Fatty acid synthesis · Adaptive response · Peanut-nodulating rhizobia

Abbreviations

CFU	Colony forming unit
FAME	Fatty acid methyl esters
FA	Fatty acids
GC	Gas chromatography
HPLC	High performance liquid chromatography
PL	Phospholipids
Ptd_2Gro	Cardiolipin
DMPtdEtn	Dimethyl phosphatidylethanolamine
LPtdEtn	Lysophosphatidylethanolamine
PtdCho	Phosphatidylcholine
PtdEtn	Phosphatidylethanolamine
PtdGro	Phosphatidylglycerol
SEM	Standard error of the mean
TLC	Thin layer chromatography
UFA	Unsaturated fatty acids
U/S	Ratio between sum of unsaturated to sum of saturated fatty acids
Z/A	Ratio of zwitterionic to anionic phospholipids

Introduction

Increased agricultural production plays a key role in enhancing food supply, economic growth, and the overall standard of living in developing countries. Bacteria termed "rhizobia", which have the ability to fix atmospheric nitrogen in symbiosis with roots of legumes, are economically important for increasing the yield of legume crops. Peanut is an important legume crop used for direct human

consumption and a variety of food products. It is a major agricultural crop in many countries such as China, United States and Argentina. Peanut is nodulated by the slow growing strain *Bradyrhizobium* sp. SEMIA6144 [1]. In addition, peanut is also nodulated by *Bradyrhizobium* sp. TAL1000 [2]. In peanut, the rhizobial infection mechanism differs from other legumes since rhizobia penetration into the root occurs without intracellular infection thread formation and involves intercellular penetration (crack entry) [3].

Environmental conditions, e.g., temperature and salinity, affect the symbiosis between rhizobia and host plant. Suboptimal temperatures reduce the competitiveness of rhizobia for nodulation [4], delay root infection and inhibit nodule development and nitrogenase activity [5]. Application of 100 mM NaCl to peanut plants completely inhibited nodule formation by *Bradyrhizobium* strains ATCC10317, TAL1000, and SEMIA6144 [6].

Survival of bacteria in stressful conditions is often determined by their capacity to adapt by altering the composition of the lipid bilayer of the cell surface membranes, which regulate or integrate many vital processes [7, 8]. The primary lipid components of the bilayer are phospholipids (PL). In bacterial membranes PL play key roles in energy transduction, signal transduction, solute transport, and cell–cell recognition [9]. Many microorganisms have been shown to modify lipid composition in order to maintain membrane fluidity within an optimal range [10]. In general, perturbation of membrane fluidity by extrinsic chemical agents or other factors initiates an active response based on intrinsic chemical changes such as the modification of existing lipids and the de novo synthesis that tend to counteract the perturbation [11, 12].

Previous studies of our laboratory demonstrated that, both PL composition and synthesis were modified by salt and temperature stresses in the peanut-nodulating *Bradyrhizobium* sp. SEMIA6144. The amount and the labeling of each individual PL was increased by NaCl, while they were decreased by temperature stress. The amount of PtdCho, PtdEtn, and PtdGro under the combined stresses decreased, as in the temperature effect [13]. Similar PL changes in response to salt and temperature stress have been observed in other microorganisms [8, 14]. Additional mechanisms to stabilize membrane fluidity in bacteria involve changes in fatty acids (FA), the major component of PL. Such mechanisms, which may occur in combination, involve changing the ratio of saturation to unsaturation; *cis* to *trans* unsaturation; branched to unbranched structures, type of branching; acyl chain length and formation of cyclopropane FA [15, 16].

Increased degree of unsaturation in response to reduced temperature has been described for many microorganisms [17, 18], and can be regarded as a universally conserved adaptation response [19]. In *Aeromonas*, alteration of growth temperature induced changes in unsaturation, branching, and chain length of the FA. At temperatures below 15 °C or above 25 °C, three species of *Aeromonas*, *A. caviae*, *A. hydrophila* and *A. sobria*, showed significant decrease of *cis*-vaccenic acid (*cis*-11-C18:1) content. In cells exposed to high NaCl concentration, maintenance of growth ability was related to a reduced ratio of unsaturated to saturated FA, reflecting membrane rigidification [20].

How the FA composition of membrane lipids is altered in response to change of growth temperature depends on the mechanism of unsaturated FA (UFA) synthesis [21]. In bacteria, UFA synthesis involves both anaerobic and aerobic mechanisms. UFA synthesis in response to low temperature was characterized in vivo for the gram-positive bacteria *Bacillus subtilis*, which desaturates palmitate to delta 5-hexadecenoate [22]. The molecular mechanism of UFA synthesis in response to temperature change has been well studied in the gram-negative bacteria *Escherichia coli*. Since membrane of *E. coli* lacks of PtdCho, its composition is quite different from that of the rhizobia [23]. However, little is known regarding the control of FA synthesis in legume-nodulating rhizobia under abiotic stress.

The FA composition profiles of *Bradyrhizobium* and *Rhizobium* are quite different, and have been used for chemotaxonomic purposes [24]. Effects of growth phase [25, 26] and low temperature [4, 27] on FA synthesis and composition in these genera have been studied, but not the effects of high temperature or high salinity.

While we have previously determined the composition of PL in SEMIA6144 [13], in this study we describe for the first time the composition of FA and the effect of high growth temperature and salinity on the FA composition and FA synthesis in this strain and in TAL1000, peanut-nodulating rhizobia. We also describe the composition of PL in TAL1000 and the effect of high growth temperature and salinity on this composition and on the survival of this strain. Our purpose was to clarify the role of cell membrane modifications in resistance and adaptation of these rhizobia to environmental stresses. The results may identify new strategies for increasing symbiotic efficiency between rhizobia and peanut.

Materials and Methods

Bacterial Strains and Culture Conditions

The fast-growing strain TAL1000 was kindly provided by NifTAL Microbiological Resource Center, Paia, HI (USA), and the slow-growing strain SEMIA6144 was provided by MIRCEN/FEPAGRO (Brazil). The strains were kept on yeast extract mannitol plates [2] at 28 °C, with the pH of

the medium adjusted to 7 before autoclaving. For the determination of bacterial growth, viability and biochemical parameters, the strains were grown in B^- medium [28] for 24 h (TAL1000) or 120 h (SEMIA6144) with an initial optical density of 0.1 (620 nm), in a shaking water bath at 28 °C or 37 °C for high growth temperature. Based on differential NaCl tolerance of each strain (data not shown), the medium was supplemented with 300 mM NaCl (TAL1000) or 50 mM NaCl (SEMIA6144) for saline stress experiments. Viable TAL1000 cells were counted (CFU) by removing samples at intervals, as described by da Silva [29].

Incorporation of Labelled Acetate

[1-^{14}C]acetate, sodium salt (43 mCi mmol^{-1}, New England Nuclear), sterilized, was added to the medium (1 μCi ml^{-1}) at the time of inoculation. Cells were harvested at the late exponential phase (24 h for TAL1000 and 96 h for SEMIA6144) by centrifugation at 6000×*g* for 10 min, in a Beckman Allegra 64R refrigerated centrifuge. Pellets were washed twice with 0.9% NaCl and used for further studies. The same procedures and conditions were used for unlabelled samples.

Lipid Extraction

Lipids were extracted from washed bacteria with chloroform/methanol/water [30]. The lower phase, containing lipids, was dried under N_2, and dissolved in appropriate volume of chloroform/methanol 2:1 (v/v).

Separation and Quantification of ^{14}C-labelled Phospholipids

Aliquots of total lipid extracts were analyzed by Analtech thin layer chromatography (TLC) plates (silica gel HLF, 250 μm) using chloroform/acetone/methanol/acetic acid/water (40:15:14:12:7, by vol) as solvent. All solvents were of analytical or HPLC grade. Lipids were detected by iodine vapors and separated lipids were identified by comparison with purified standards (Sigma Chemical Co., St. Louis, MO, USA). TLC bands were scraped, 3 mL Optiphase Hisafe 2 (PerkinElmer, USA) was added to each vial, and radioactivity was measured by liquid scintillation counter (Beckman LS 60001 C, USA) [31].

Separation and Quantification of ^{14}C-labelled Fatty Acids Based on the Degree of Unsaturation

Fatty acid methyl esters (FAME) were prepared from total lipid extracts with 10% BF_3 in methanol [32], and resolved according to number of double bonds on TLC plates impregnated with $AgNO_3$ (10%, w/v), using hexane/ethyl ether/acetic acid (94:4:2, by vol) as solvent. FAME bands were detected under UV light after spraying the plates with dichlorofluorescein, elution [33], and drying in counting vials as described above.

Analysis of Fatty Acids by GC-FID

FAME prepared as above were analyzed using a Hewlett Packard 5890 II gas chromatograph (GC) equipped with a methyl silicone column (length 50 m; inner diameter 0.2 mm; film thickness 0.33 μm) and a flame ionization detector (FID).

GC conditions: injector temperature 250 °C; detector temperature 300 °C; carrier gas nitrogen. Temperature program: 180 °C, 25 min isothermal; 3 °C min^{-1} to 250 °C. Peak areas of carboxylic acids in total ion were used to determine relative amounts.

Fatty acids were identified by comparison of retention times with commercial standards. (Sigma Chemical Co., St. Louis, MO, USA).

Statistical Analyses

Data were compared by one-way analysis of variance (ANOVA) test.

Results

Effects of High Growth Temperature (37 °C) and Salinity on TAL1000 Survival

Viability of TAL1000 (measured as CFU ml^{-1}) was reduced by NaCl stress alone, and by a combination of NaCl stress and high growth temperature (Fig. 1), although TAL1000 was able to survive in these conditions. Lowest CFU values were obtained with 300 mM NaCl. Viability at high growth temperature, 37 °C, was not significantly affected.

Effect of High Growth Temperature and Salinity on TAL1000 Phospholipid Metabolism

[1-^{14}C]acetate sodium salt was incorporated mostly (90–92%) into PL, and the rest into neutral lipid fraction. The predominant labeled PL was phosphatidylcholine (PtdCho), followed in descending order by phosphatidylglycerol (PtdGro), phosphatidylethanolamine (PtdEtn), dimethyl phosphatidylethanolamine (DMPtdEtn), cardiolipin (Ptd_2Gro), and lysophosphatidylethanolamine (LPtdEtn) (Table 1).

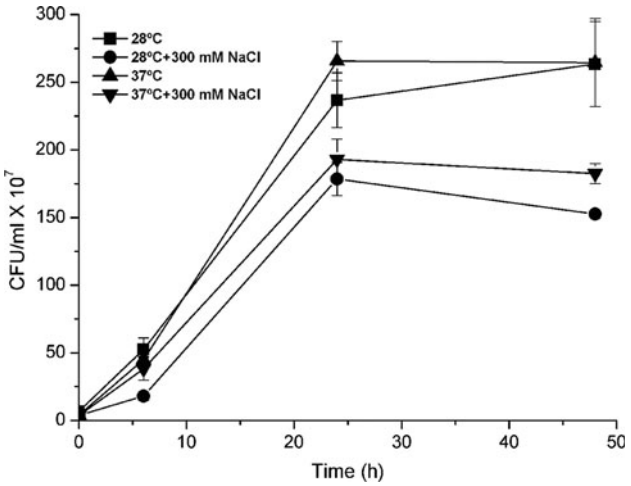

Fig. 1 Effect of NaCl and temperature on viability of fast-growing *Bradyrhizobium* strain TAL1000. Viability is expressed as CFU ml^{-1}. Values represent means ± SEM from three independent experiments

Table 1 Effect of temperature and salinity stress on the incorporation of [^{14}C]acetate into phospholipids of *Bradyrhizobium* TAL1000

PL (%)	Growth condition			
	28 °C	28 °C + NaCl	37 °C	37 °C + NaCl
PtdCho	44.5 ± 2.9	45.7 ± 2.2	49.4 ± 2.6*	54.0 ± 4.0*
DMPtdEtn	9.90 ± 1.5	9.70 ± 0.9	10.9 ± 1.4	10.8 ± 1.7
LPtdEtn	0.88 ± 0.3	2.50 ± 1.3	3.60 ± 0.9*	2.50 ± 0.9*
PtdEtn	13.7 ± 1.7	13.5 ± 2.0	7.60 ± 2.4*	8.10 ± 2.7*
Ptd_2Gro	4.20 ± 0.3	3.90 ± 0.1	3.00 ± 0.8*	4.10 ± 0.3
PtdGro	17.5 ± 1.1	15.4 ± 1.9	18.2 ± 1.5	12.4 ± 0.5*
NL	9.30 ± 3.5	9.60 ± 3.1	7.80 ± 3.0	9.00 ± 1.8

Values represent means ± SEM of three independent experiments

PL phospholipids, *PtdCho* phosphatidylcholine, *DMPtdEtn* Dimethyl phosphatidylethanolamine, *LPtdEtn* lysophosphatidylethanolamine, *PtdEtn* Phosphatidylethanolamine, *Ptd_2Gro* cardiolipin, *PtdGro* phosphatidylglycerol, *NL* neutral lipids

* Difference from control (28 °C) value statistically significant at $P < 0.05$ level

PL patterns for TAL1000 were qualitatively similar for all experimental conditions, but quantitative changes were observed for individual PL. High growth temperature caused an increase in PtdCho from 44 to 49.4% and combined conditions increased PtdCho from 44 to 54%. Compared to the control condition (28 °C), the amount of radiolabel in LPtdEtn (identity confirmed by ninhydrin spray reagent) increased about twofold in response to salt stress and increased about threefold in response to high growth temperature.

Decreased labeling was observed for PtdEtn under high growth temperature from 13.7 to 7.6% and combined conditions from 13.75 to 8.1%, for PtdGro under combined conditions from 17.5 to 12.4% and for Ptd_2Gro by high growth temperature from 4.2 to 3%. Under combined conditions, the ratio of zwitterionic to anionic PL (Z/A) increased (data not shown).

Fatty Acid Composition of TAL1000 and SEMIA6144

Major FA detected in TAL1000 and SEMIA6144 were *cis*-vaccenic (18:1n-7) + oleic (18:1n-9), stearic acid (18:0) and palmitic acid (16:0). Eicosatrienoic acid (20:3n-6) and cyclopropane fatty acid ($19:0_{cyclo}$) were only detected in TAL1000, while palmitoleic acid (16:1n-7) was only detected in SEMIA6144 (Table 2).

Effect of Growth Conditions on Fatty Acid Composition of TAL1000 and SEMIA6144

FA composition of the two strains under experimental conditions tested is shown in Table 2. In TAL1000 the FA showing greatest change in response to tested conditions was 18:1n-7 + 18:1n-9, whose percentage declined from 63.3 to 8.2% under high growth temperature, and to 4.3% under temperature plus NaCl stress. Conversely, 16:0 increased from 8.4 to 20% at 37 °C, and to 16.1% under the combined conditions. The other saturated FA, 18:0, increased from 12.6 to 24% at 37 °C, and to 29% under combined conditions. $19:0_{cyclo}$ increased from 3.4 to 10% at 37 °C and to 14.5% under combined conditions. The changes in FA percentages led to alteration of the ratio between unsaturated to saturated FA (U/S in Table 2), which decreased in all experimental conditions.

Of all the tested conditions, the growth temperature increase was the one causing the most significant changes in the level of FA in TAL1000.

Notably, in SEMIA6144, 18:1n-7 + 18:1n-9 decreased from 84 to 73.5% under temperature stress, and to 68.5% under combined stresses, while 16:0 increased from 11 to 18.6% under temperature stress, and to 19.7% under combined stresses. FA values under NaCl stress alone were not significantly different from control values. The U/S ratio for SEMIA6144 decreased under all experimental conditions, but not as markedly as in TAL1000.

Effect of Growth Conditions on Fatty Acid Metabolism of TAL1000 and SEMIA6144

[1-^{14}C]acetate sodium salt was used as precursor for study of FA metabolism. Radioactivity distribution of various FA in TAL1000, separated by TLC according to degree of unsaturation, is shown in Fig. 2. Under control conditions (28 °C), labeling in TAL1000 was found predominantly in monounsaturated FA, followed by triunsaturated, saturated, and diunsaturated fractions. Consistent with findings for

Table 2 Effects of temperature and salinity stress on fatty acid composition of two peanut-nodulating rhizobia

Fatty acid type (%)	Strain	Growth condition			
		28 °C	28 °C + NaCl	37 °C	37 °C + NaCl
Saturated					
Stearic acid (18:0)	TAL1000	12.6 ± 1.5	14.6 ± 1.6*	24.0 ± 2.7*	29.0 ± 0.9*
	SEMIA6144	1.40 ± 0.3	1.66 ± 0.3	2.00 ± 0.6	2.16 ± 0.9
Palmitic acid (16:0)	TAL1000	8.40 ± 1.7	9.30 ± 1.5	20.0 ± 2.3*	16.1 ± 1.8*
	SEMIA6144	11.0 ± 1.2	12.6 ± 2.7	18.6 ± 3.9*	19.7 ± 3.5*
Unsaturated					
Palmitoleic acid (16:1n-7)	TAL1000	ND	ND	ND	ND
	SEMIA6144	0.42 ± 0.0	0.90 ± 0.4	0.65 ± 0.2	0.56 ± 0.2
cis-vaccenic acid + oleic acid (18:1)	TAL1000	63.3 ± 5.4	55.8 ± 2.7	8.20 ± 0.4*	4.30 ± 0.0*
	SEMIA6144	84.0 ± 2.2	82.4 ± 2.9	73.5 ± 3.4*	68.5 ± 6.8*
Eicosatrienoic acid (20:3)	TAL1000	6.30 ± 1.9	9.20 ± 1.2	15.8 ± 1.6*	19.0 ± 4.7*
	SEMIA6144	ND	ND	ND	ND
Cyclopropane					
$19:0_{cyclo}$	TAL1000	3.40 ± 0.7	3.85 ± 0.3	10.0 ± 1.5*	14.5 ± 1.7*
	SEMIA6144	ND	ND	ND	ND
Others	TAL1000[a]	6.70 ± 1.6	7.10 ± 0.8	23.2 ± 2.6*	17.1 ± 1.9*
	SEMIA6144	1.10 ± 0.0	2.55 ± 0.7	5.00 ± 0.2	5.30 ± 1.3
U/S[b]	TAL1000	3.3	2.7	0.5	0.5
	SEMIA6144	7.0	6.1	3.6	3.1

Lipids were extracted, and fatty acids of total lipid were converted to methyl esters and analyzed by GC, as described in the text

Percentage of each fatty acid is relative to total fatty acids defined as 100%. Values represent means ± SEM of three independent experiments

ND not detected

[a] Correspond to two peaks of retention times of 36 min and 37.4 min. Such peaks could correspond to FA of more than 18 carbon atoms

[b] Ratio between sum of unsaturated and sum of saturated fatty acids

* Difference from control (28 °C) value statistically significant at $P < 0.05$ level

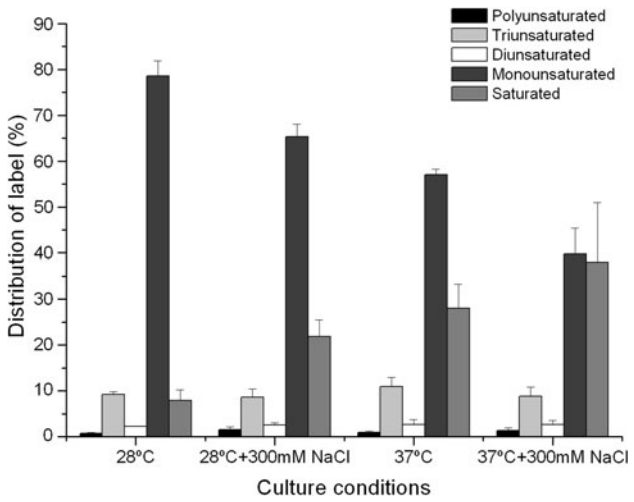

Fig. 2 Effect of NaCl and temperature on incorporation of [1-^{14}C]acetate in fatty acids of *Bradyrhizobium* TAL1000. FAMEs were prepared from total lipids, and separated according to unsaturation degree using TLC plates impregnated with 10% $AgNO_3$. Results are expressed as the percentage of total radioactivity incorporated in each FA fraction. Values represent means ± SEM from three independent experiments

FA composition (Table 2), high growth temperature and combined conditions decreased the [1-^{14}C]acetate incorporation in monounsaturated FA, 27.4 and 49.4% respectively. [1-^{14}C]acetate incorporation in saturated FA increased 3.5-fold by high growth temperature and 4.7-fold by combined conditions. [1-^{14}C]acetate incorporation in monounsaturated FA decreased from 78 to 65% and that of saturated FA increased from 8 to 22% by NaCl stress. Based on modified incorporation of labelled acetate, the U/S ratio decreased from 11.5 to 3.5 for NaCl stress, from 11.5 to 2.5 for high growth temperature, and from 11.5 to 1.6 for combined conditions.

Incorporation of [1-^{14}C]acetate in FA of SEMIA6144 was also tested. Labeling was observed primarily in monounsaturated FA, followed by saturated, diunsaturated, and triunsaturated FA. [1-^{14}C]acetate incorporation in saturated FA increased 28% under NaCl stress, 39% at 37 °C, and 45% under combined conditions. In contrast, radioactive incorporation in diunsaturated FA decreased 73% under NaCl stress, 66% at 37 °C, and 82% under combined conditions. As a consequence of these changes, the U/S

ratio decreased from 14.7 to 11.5 for NaCl stress, from 14.7 to 10.7 for high growth temperature, and from 14.7 to 10 for combined conditions.

Discussion

Our previous biochemical studies showed that *Bradyrhizobium* SEMIA6144, exhibited reduced viability and increased levels of PtdCho, when exposed to 37 °C, NaCl 50 mM and combined conditions [13]. In the present study we found that, viability of TAL1000 was reduced by NaCl 300 mM and by combined conditions (NaCl 300 mM plus 37 °C). Viability of salt-tolerant TAL1000 was unaffected by high temperature, consistent with studies that indicate a relationship between salt tolerance and temperature tolerance in rhizobial strains [34].

Adaptive mechanisms induced in cells in response to changes in environmental conditions, to maintain membrane fluidity involve modification of PL at the level of FA components and PL head groups [15, 35]. Behavior of SEMIA6144 PL labeling in response to stress [13] differed from those of TAL1000, resulting in different degree of modification in the Z/A ratio. We suggest that the two strains, although possessing similar PL composition, have different mechanisms for stabilizing membrane fluidity. The other adaptive mechanism developed by bacteria is alteration of membrane FA [36]. The FA composition of total lipids in the two strains was different, since TAL1000 contained 20:3n-6 and 19:0_{cyclo} FA, which were not present in SEMIA6144. Since the FA profile of TAL1000 is similar to that reported for *Rhizobium* [24], and both growth velocity and FA profile of TAL1000 differ from those of *Bradyrhizobium*, the taxonomic classification of TAL1000 may need to be reconsidered. High growth temperature and combined conditions caused significant reduction of 18:1 and increase of 18:0 and 16:0 (in TAL1000), or 16:0 (in SEMIA6144). These changes that were more pronounced in TAL1000, provoked modifications at the level of FA unsaturation degree coincident with results obtained for other rhizobia in which different environmental changes caused modifications in the U/S ratio [4, 26].

TAL1000 at 37 °C showed a decreased in the U/S ratio and enhanced formation of 19:0_{cyclo} (Table 2). Cyclic FA in the membranes of rhizobia could represent a mechanism to reduce membrane fluidity, similar to lactobacillus [37]. SEMIA6144, showed change in degree of FA unsaturation and increased 16:0/18:0 ratio under temperature stress, which may reflect decreased chain length and it may alter transition temperature for change from gel to liquid crystalline phase [19]. Results using [1-^{14}C]acetate labeling are consistent with FA composition studies and similar with studies in other gram-negative bacteria [38, 39] since showed synthesis of both saturated and monounsaturated FA. Tested experimental conditions altered incorporation of labeled acetate in FA of both rhizobial strains. We found no studies on effects of abiotic stress on FA synthesis in rhizobia, but a study showing increase of saturated FA synthesis at high temperature in *Bacillus subtilis* was consistent with our results [22].

We conclude that peanut-nodulating rhizobia strains are able to adapt to tested environmental conditions through FA modification, and that fast-growing TAL1000 is more efficient in this respect than slow-growing SEMIA6144. The most important mechanism for maintaining physical properties of the membrane is modification of the FA unsaturation degree. The ability of TAL1000 to alter its content of FA 19:0_{cyclo} may account for its tolerance of high temperature, while adaptation to environmental stresses in SEMIA6144 may involve shortening of the FA chain length [20].

The FA composition of strains of rhizobia used in commercial formulations should be considered as an indicator of whether or not an organism can adapt to changing environmental conditions, since differences in the capacity of rhizobia to adapt to environmental conditions may be related to differences in the FA composition.

Acknowledgments Financial assistance was provided by SECyT-UNRC/Argentina. N.S.P. is a fellow of CONICET-Argentina. D.B.M. was a fellow of CONICET-Argentina. M.S.D. is a member of the Research Career of CONICET-Argentina. The authors thank Dr. Laura Villasuso for her valuable assistance in the preparation of the figures and Dr. Ricardo Lema for his help with the language.

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