Symbiosis, 20 (1996) 117–127
Balaban, Philadelphia/Rehovot

Changes in the Cellular Content of Trehalose in Four Peanut Rhizobia Strains Cultured under Hypersalinity

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Received July 16, 1995; September 22, 1995

Abstract

The adaptation to saline stress was examined in four *Rhizobium* strains which nodulate *Arachis hypogaea* (peanut). When cultured on basal yeast extract-mannitol liquid medium (1.7 mM NaCl), the strains ATCC 10317, ATCC 51466, and TAL 1000 behaved as fast-growers, while the strain USDA 3187 behaved as slow-grower. After a prolonged lag time, the four strains were able to grow under hypersalinity, albeit at a reduced rate. The slow-grower was less salt-tolerant than the fast-growers. On the other hand, the disaccharide trehalose was identified in the four strains by gas chromatography and by ^{13}C-NMR spectroscopy. Strains ATCC 10314 and ATCC 51466 contained negligible amounts of trehalose, while strains TAL 1000 and USDA 3187 accumulated significant quantities of the disaccharide. Under hypersalinity, fast-growers increased the cellular content of trehalose, regardless of the carbon source mannitol, sucrose, or lactose; when cultured with mannitol as the carbon source, the slow-grower also increased the accumulation of cellular trehalose. When cultured with trehalose as the carbon source, the increase of NaCl in the growth medium did not change the cellular trehalose content in the strain ATCC 10317, but the accumulation of the disaccharide decreased in the strains TAL 1000 and USDA 3187. Results were correlated with changes in the metabolism of trehalose in peanut rhizobia exposed to hypersalinity.

Keywords: trehalose, peanut *Rhizobium*, hypersalinity

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0334-5114/96/$05.50

1. Introduction

Rhizobia must be able to proliferate in the soil before the legume host can be infected and a productive symbiotic association established. One parameter that changes frequently is the osmotic pressure of the surrounding medium. The sensitivity of symbiotic rhizobia to environmental osmotic conditions could affect the productivity of useful legume crops. Hypersalinity is a form of osmotic stress responsible for major crop losses. The response of legumes to salt stress is not a property of the host plant alone, but demands consideration of the inoculant rhizobia (Craig et al., 1991; Graham, 1992). Detrimental effects of salt to the survival and growth of rhizobia have been reported, and the tolerance to NaCl of rhizobia from different species was found to vary widely from 100 to 600 mM (Elsheikh and Wood, 1989; 1990).

A common mechanism of osmotic stress adaptation in bacteria is the rapid intracellular accumulation of osmolytes, which results in the restoration of turgor (Csonka, 1989). In some rhizobia grown under conditions of high osmotic pressure, several authors identified glutamate, proline, betaines, and trehalose, as the prominent species of accumulated osmolytes (Botsford, 1984; Le Rudulier and Bernard, 1986; Botsford and Lewis, 1990; Madkour et al., 1990; Pfeffer et al., 1994). When subjected to hypersaline stress, only some strains of *Rhizobium leguminosarum, R. loti,* and *R. meliloti* increased the net synthesis of trehalose (Hoelzle and Streeter, 1989; Breedveld et al., 1990; 1991).

Arachis hypogaea (peanut) is a commercially important crop in Córdoba State (Argentina), and we are particularly interested in rhizobia which nodulate peanut. *Arachis hypogaea* nodulates with an array of bacteria isolated from a wide range of tropical and subtropical legumes (Singleton et al., 1992). Because the infection process in peanut takes place through the junction where secondary roots emerge (crack entry), and that peanut nodules have unique ultrastructural characteristics (Bal et al., 1989), it may not be expected that peanut rhizobia exhibit the same mechanism of osmotic stress adaptation as other rhizobia. As far as we know, no reports in the literature are available on the osmotic adaptation of rhizobia which nodulate peanut.

In this study, we identified trehalose as the osmolyte which is accumulated to significant levels in hypersaline-stressed cells of four peanut rhizobia.

2. Materials and Methods

Bacteria, media and culture conditions

The following peanut *Rhizobium* strains were used: ATCC 10317 was kindly provided by the American Type Culture Collection, Rockville, MD, USA; TAL

1000 was received by courtesy of the NifTAL Microbiological Resource Center, Paia, HI, USA; USDA 3187 was obtained from the IPAGRO *Rhizobium* Culture Collection, Porto Alegre, RS, Brazil; and ATCC 51466 was isolated from nodules of *Arachis hypogaea* growing at Rio Cuarto, Córdoba, Argentina. These bacteria were maintained on agar slants of YEM medium, which contains yeast extract, mannitol, and mineral salts (Vincent, 1970).

The four strains were examined for their abilities to nodulate Blanco Manfredi 68 INTA (Argentina) peanut cultivars. Nodulation tests were done as described by Wilson et al. (1989). All of the tested bacteria formed abundant and large pink nodules.

Vincent's (1970) yeast extract-mannitol-mineral salts medium (YEM) was used as a basal growth medium. For all experiments, bacteria were first grown at 28°C in the basal medium, and cells from the late logarithmic phase (absorbance at 620 nm: 0.8–0.9) were used as an inoculum. Carbon sources for growth media were: 55 mM mannitol (basal); 2.9 mM sucrose; 27.5 mM sucrose; 27.5 mM lactose; and 27.5 mM trehalose. For the hypersaline media, NaCl was added before autoclaving to give final concentrations of 100 or 400 mM. For all experiments, small samples (1% v/v) of the inoculum were used for 10 ml of medium in side-arm conical flasks, which were never filled to more than 20% capacity. The flasks were incubated at 28°C and 80 rpm on a Fisher Versa-Bath (Model 224). Growth was followed turbidimetrically at 620 nm with a Metrolab VD40 spectrophotometer. Maximum doubling times were calculated from the exponential phase of the growth curves. Small samples were taken for determination of dry weight after lyophilization (biomass) and of protein content of cells by the method of Bradford (1976) after solubilization in 0.2 N NaOH in a boiling water bath for 10 min. Bovine serum albumin was used as the standard.

Extraction and identification of trehalose

Starting from 2% inoculum, bacteria were grown in Erlenmeyer flasks containing 500 ml of growth medium. Cells were harvested at early stationary growth (absorbance at 620 nm: 0.9–1.2) by centrifugation at 10,000 × g for 10 min and washed once with 30 mM Tris-HCl buffer at pH 8.2. After centrifugation at 10,000 × g for 10 min, the cellular pellet was treated with chloroform as described by Ames et al. (1984) and centrifuged. Proteins were removed from the supernatant by addition of 1% trichloroacetic acid (TCA) as described by Miller et al. (1986). The TCA extracts were neutralized to pH 7.0 with ammonium hydroxide, concentrated by lyophilization, and subjected to gel filtration on Bio-Gel P4 (BioRad Laboratories, Richmond, CA, USA). The column (dimensions 70 × 1.8 cm, bed volume 178 ml) was equilibrated and eluted

with 50 mM sodium acetate, pH 5.2 (York et al., 1980), at room temperature at the rate of 0.12 ml/min. Fractions of 3 ml were collected, and the hexose content of the fractions was measured with anthrone-sulfuric acid reagent (Trevelyan and Harrison, 1952) using glucose as standard. Kav was calculated as (Ve-Vo)/(Vt-Vo), in which Ve is the elution volume, Vo is the void volume, and Vt is the total volume.

Anthrone-positive fractions eluting from the Bio-Gel column at Kav between 0.50 and 0.75 were pooled, concentrated under vacuum, and loaded on a Dowex 50W-X8 column (10 × 1 cm); the elution buffer was Tris-HCl 30 mM, pH 8.2. The hexose-containing peak recovered from the Dowex column was concentrated under vacuum, 0.1 mg of sample was dissolved in 70 µl bis(trimethyl) formamide, derivatization was completed by adding 70 µl bis(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchloro-silane (Alltech Assoc. Inc., Deerfield, IL, USA) by shaking and heating at 70°C for 10 min. Gas chromatographic analysis was performed on a Hewlett-Packard 5890-Series II instrument equipped with a 25 m HP1 (0.33 µm film thickness) capillary column, a flame ionization detector, and a HP-3396(II) integrator. Nitrogen was used as the carrier gas. The injector and detector temperatures were 250 and 300°C, respectively. A temperature program starting at 190°C with 2 min hold and 30°C/min ramp to 250°C was employed, after which the temperature was kept at 250°C for 4 min, followed by temperature increase of 30°C/min to 280°C, and subsequently the temperature was kept at 280°C for 14 min. Glucose, sucrose, and trehalose were used as standards.

^{13}C nuclear magnetic resonance spectra were also recorded on a Bruker Ac 200 spectrometer operating at 50.32 MHz. The samples were dissolved in D_2O at a concentration of approximately 200 mg/ml. 1,4-Dioxane was used as an internal reference (67.6 ppm relative to tetramethylsilane). The 90°C pulse width was 5.4 µs.

Chemicals

Unless otherwise stated, all reagents and standards were obtained from Sigma Chemical Co., St. Louis, MO, USA.

3. Results and Discussion

We examined the growth behavior of four peanut rhizobia strains under stress due to salinity (Table 1). Although the four strains were able to multiply under conditions of hypersalinity, the cells underwent an increase in lag time, from 130 to 400% of lag time of control values. The extended lag phase was

Table 1. Effect of NaCl on the growth and the protein content of cultures of four peanut rhizobia grown with different carbon sources.

Carbon source (mM)	NaCl (mM)	Lag time (h)	Doubling time (h)	Biomass (g/l medium)	Protein (mg/g biomass)
***Rhizobium* ATCC 51466**					
Mannitol (55)	1.7	7.3	2.1	0.9	0.33
	400	20.5	3.8	0.89	0.26
Sucrose (2.9)	1.7	8.0	5.1	1.23	0.22
	400	19.0	5.9	1.55	0.18
Lactose (27.5)	1.7	8.0	6.3	0.62	0.38
	400	13.0	6.5	0.55	0.31
Trehalose (27.5)	1.7	6.0	2.9	1.03	0.18
	400	15.0	4.9	0.59	0.25
***Rhizobium* ATCC 10317**					
Mannitol (55)	1.7	9.0	4.1	0.63	0.15
	400	33.5	4.7	0.60	0.20
Sucrose (2.9)	1.7	12.0	11.7	0.57	0.34
	400	48.0	15.7	0.37	0.33
Lactose (27.5)	1.7	14.0	6.8	0.74	0.32
	400	43.0	7.5	0.41	0.36
Trehalose (27.5)	1.7	5.0	4.0	0.61	0.20
	400	9.0	4.2	0.44	0.22
***Rhizobium* TAL 1000**					
Mannitol (55)	1.7	4.5	2.2	0.32	0.15
	400	6.5	2.6	0.27	0.12
Sucrose (27.5)	1.7	4.5	5.0	0.34	0.17
	400	10.0	5.8	0.58	0.14
Lactose (27.5)	1.7	10.0	2.6	0.39	0.19
	400	14.5	10.7	0.58	0.16
Trehalose (27.5)	1.7	7.0	4.3	0.40	0.26
	400	9.0	7.3	0.36	0.30
***Rhizobium* USDA 3187**					
Mannitol (55)	1.7	20.0	11.2	0.57	0.12
	100	38.0	27.2	0.53	0.11
Sucrose (27.5)	1.7	10.0	5.8	0.86	0.52
	400	20.0	10.4	0.82	0.38
Lactose (27.5)	1.7	10.0	6.0	0.82	0.34
	100	18.0	10.0	0.29	0.28
Trehalose (27.5)	1.7	15.0	6.3	0.52	0.34
	400	25.0	10.6	0.92	0.39

The data shown here are representative of two (TAL 1000, lactose) to ten (ATCC 51466, mannitol) independent experiments, among which values varied not more than 5%.

Table 2. Effect of NaCl on the oligosaccharides from four peanut rhizobia grown with different carbon sources.

Carbon source (mM)	NaCl (mM)	Oligosaccharides (mg hexose equiv./ g cellular biomass)	Trehalose (μmoles/g cellular biomass)
***Rhizobium* ATCC 51466**			
Mannitol (55)	1.7	29	undetectable*
	400	28	41
Sucrose (2.9)	1.7	11	undetectable*
	400	29	35
Lactose (27.5)	1.7	22	undetectable*
	400	36	45
Trehalose (27.5)	1.7	20	4
	400	43	56
***Rhizobium* ATCC 10317**			
Mannitol (55)	1.7	21	traces**
	400	25	35
Sucrose (2.9)	1.7	24	3
	400	33	44
Lactose (27.5)	1.7	16	6
	400	28	32
Trehalose (27.5)	1.7	58	38
	400	34	41
***Rhizobium* TAL 1000**			
Mannitol (55)	1.7	34	47
	400	63	88
Sucrose (27.5)	1.7	58	82
	400	99	143
Lactose (27.5)	1.7	48	56
	400	61	88
Trehalose (27.5)	1.7	36	50
	400	57	18
***Rhizobium* USDA 3187**			
Mannitol (55)	1.7	11	9
	100	15	15
Sucrose (27.5)	1.7	12	15
	400	13	16
Lactose (27.5)	1.7	30	38
	100	21	23
Trehalose (27.5)	1.7	35	38
	400	24	26

*Undetectable by the anthrone reagent; **values were lower than the limit of detection by the anthrone reagent. Experiments were performed from one to ten times, the values obtained differed maximally by 5%.

possibly due to the initial inhibitory effect of the high NaCl concentration in the growth medium. After the increased lag, cells grew, albeit at a noticeably reduced rate. With mannitol and lactose as carbon sources, the slow-growing strain USDA 3187 grew poorly at NaCl above 100 mM; with sucrose and trehalose as carbon sources, bacterial increased the salt-tolerance to 400 mM NaCl. TAL 1000 behaved as the most salt-tolerant strain; it grew in media with 800 mM NaCl, but copious amounts of exopolysaccharides increased the viscosity of the cultures and interfered with spectrophotometric measurements.

When biomass and protein content of cells were determined (Table 1), different responses of the four strains to hypersalinity were observed; bacteria responded also differently with different carbon sources.

Examination of TCA-extracts of the stationary cultures by chromatography on a Bio-Gel P4 column revealed the presence of oligosaccharides in the four strains (Table 2). In basal media (1.7 mM NaCl) the oligosaccharides accounted for about 4±2% of the cellular biomass. Table 2 also shows that, on increasing the NaCl concentration in the culture media, the amount of cellular oligosaccharides varied from 59 to 436% of the control levels, and they accounted for about 7±4% of the cellular biomass.

The hexose-containing material eluting from the Bio-Gel column at Kav between 0.50 and 0.75 was analyzed as described in Materials and Methods. When subjected to gas chromatography, the relative retention times of trimethylsilyl-derivatives were 19.09±0.16 min for the unknown material and 19.14±0.16 min for the authentic trehalose standard. The material was also subjected to ^{13}C-NMR spectroscopy with authentic trehalose as a reference (Fig. 1). The spectra from the unknown material showed strong signals at 61.6, 70.7, 72.0, 73.2, and 73.5 ppm; these signals corresponded to those given by the standard of trehalose. The peak at 67.6 ppm arose from the dioxane internal standard. Signals at 64.2, 70.3, and 71.7 ppm were also observed in the unknown material, possibly due to contaminants.

Variable contents of trehalose were detected in the four rhizobia strains. When cultured in basal media (1.7 mM NaCl) (Table 2): ATCC 51466 and ATCC 10314 contained negligible amounts of the disaccharide, while strains USDA 3187 and TAL 1000 accumulated significant quantities of trehalose. When cultured in hypersaline media, cells from strains ATCC 51466, ATCC 10317, and TAL 1000 increased the cellular content of trehalose, independently of the carbon source mannitol, sucrose, or lactose. Cells from the strain USDA 3187 grown at hypersalinity with mannitol as the carbon source also increased the accumulation of trehalose. In all these salt-stressed cells, the accumulation of trehalose could be due to increased synthesis of the disaccharide as an osmoprotectant; a similar effect was demonstrated in *Escherichia coli* grown at high osmolarity (Boos et al., 1990). However, the possibilities of decreased

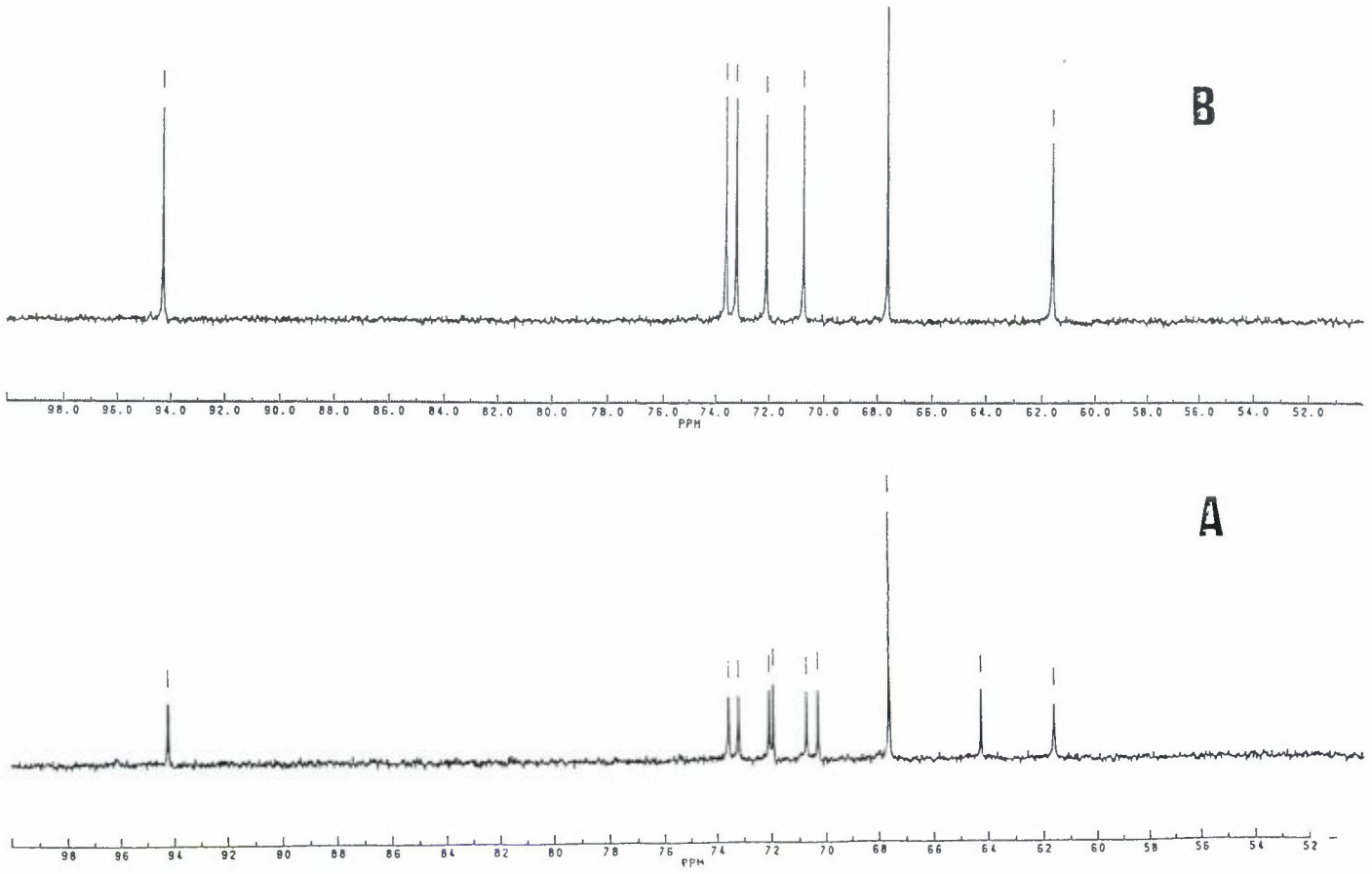

Figure 1. ^{13}C-NMR spectroscopic analyses of (A) the unknown material (obtained from stationary cells grown on hypersaline media) compared with that of authentic trehalose (B). Spectra were recorded as described in Material and Methods.

breakdown of trehalose, decreased efflux of the disaccharide to the medium, or the sum of both, are also supported by our results.

Trehalose plays an important role in many organisms as a stress-protectant (Crowe et al., 1984; Wiemken, 1990). Demonstration that some rhizobia accumulated trehalose in response to salt stress is not new (Hoelzle and Streeter, 1989; Breedveld et al., 1991; Talibart et al., 1994), however, the effect was not reported for rhizobia which nodulate peanut. Moreover, accumulation of trehalose in a *Rhizobium* strain which did not produce detectable amounts of the disaccharide under basal conditions, was only observed when *R. meliloti* SU-47 was exposed to hypersalinity (Breedveld et al., 1990). We found the same effect on strains ATCC 51466 and ATCC 10317.

On the other hand, our four strains grew with trehalose as the carbon source at both low and high osmolarity (Table 1). When medium was supplemented with NaCl, the amount of cellular trehalose did not change in the strain ATCC 10317, but the accumulation of trehalose decreased in the strains USDA 3187 and TAL 1000 (Table 2); the decrease could be explained as a consequence of changes in trehalose metabolism, such as the increased hydrolysis of the disaccharide. *E. coli* can also grow on trehalose as the carbon source. This bacterium has developed two different systems of trehalose metabolism, one for low osmolarity and one for high osmolarity, but external trehalose does not contribute to osmoprotection (Boos et al., 1990; Rimmele and Boos, 1994). As far as we know, trehalose metabolism has not been studied in bacterial from the family Rhizobiaceae. The results presented here suggest that in peanut rhizobia the metabolism of trehalose is regulated by the salinity of the medium. Our future studies will have to concentrate on the role of trehalose as well as on the metabolism of the disaccharide in peanut rhizobia.

Acknowledgements

The authors are gratefully indebted to Marta S. Dardanelli, Paola S. González, and Mariela E. Woelke, for their assistance in some experiments. Funding support from Secretaria de Ciencia Y Técnica de la Universidad Nacional de Rio Cuarto to N. Ghittoni is acknowledged.

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