The Effects of Nonionic Hyperosmolarity and of High Temperature on Cell-Associated Low Molecular Weight Saccharides from Two Peanut Rhizobia Strains

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Abstract

The adaptation to nonionic hyperosmolarity and to high temperature was examined in two rhizobia strains which nodulate *Arachis hypogaea* (peanut). When grown under hyperosmolarity, the pattern of periplasmic low molecular weight saccharides was noticeably changed. The cellular content of this saccharide species was also modified by the tested conditions of stress: it was decreased by the presence of a nonpermeating osmoticum (PEG) in the growth medium, but it was augmented either under hyperosmolarity imposed by a readily permeating solute (glycerol) or by high temperature. Furthermore, in the strain ATCC 10317 grown at 37°C, trehalose was the major component of the increased low molecular weight saccharides.

Keywords: Peanut rhizobia, nonionic hyperosmolarity, high temperature, low molecular weight saccharides

1. Introduction

Soil microorganisms must adapt to whatever conditions they encounter in the environment. Osmolarity and temperature are important factors in the survival

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and function of rhizobia in soil, because they can influence both nodulation and nitrogen fixation (Streeter, 1994). Furthermore, hyper-osmolarity and high temperatures can affect rhizobial persistence in inoculants during shipment or in storage (Graham, 1992).

In bacteria, one common mechanism of adaptation to osmotic stress is the accumulation of inorganic and/or organic solutes in the cytoplasm to restore cell turgor. Long-term osmoadaptation of bacteria was achieved by the accumulation of compatible solutes that are not deleterious for the cell's physiology, such as the nonreducing disaccharide trehalose (Hoelzle and Streeter, 1989; Madkour et al., 1990; Lucht and Bremer, 1994). Trehalose was well recognized in bacteria for its role in protection against dehydration and heat-shock (Dinnbier et al., 1988). The level of trehalose increased at the stationary growth phase of some salt-stressed rhizobia (Hoelzle and Streeter, 1989; Breedveld et al., 1990 a; Talibart et al., 1994; Ghittoni and Bueno, 1995, 1996) and of hyperosmotic-shocked *Bradyrhizobium japonicum* (Pfeffer et al., 1994). In addition, the amount of periplasmic glucans synthesized by *B. japonicum* decreased when cells were grown in high osmolarity media (Tully et al., 1990; Miller and Gore, 1992).

On the other hand, by growing *Rhizobium trifolii* at greater than optimal temperatures, a considerably increased production of periplasmic glucans was obtained and a drastic change in the glucan production pattern was observed (Breedveld et al., 1990b). Although the biochemistry and the genetics of trehalose accumulation in heat-stressed *Saccharomyces cerevisiae* have been extensively investigated, no such analysis has been conducted in rhizobia under hypercaloric conditions.

Arachis hypogaea (peanut) is a commercially important crop in Córdoba State (Argentina). Because peanut rhizobia are poorly studied, we are particularly interested in rhizobia which are able to nodulate peanut cultivars from the region. We had identified trehalose as the counteracting osmolyte which is accumulated to significant levels in salt-stressed peanut rhizobia (Ghittoni and Bueno, 1995; 1996). In order to study the adaptation of these rhizobia to other environmental stresses, in this paper we report how nonionic hyperosmolarity and high temperature of the medium affect low molecular weight saccharides from rhizobia which nodulate peanut.

2. Materials and Methods

Bacteria, media, and culture conditions

Bacterial strains used in this study were recommended as peanut inoculants by international culture collections: *Rhizobium* sp. ATCC 10317 was kindly

provided by the American Type Culture Collection, Rockville, MD, USA, and *Rhizobium* sp. TAL 1000 was received by courtesy of the NifTAL Microbiological Resource Center, Paia, HI, USA. The strains were selected for our study on the basis of their abilities to nodulate Blanco Manfredi 68 INTA peanut cultivar, currently used in Córdoba State (Argentina). Nodulation tests were done as described by Wilson et al (1989) and the two strains formed abundant large pink nodules. The strains were also chosen because their cells accumulated trehalose when grown under hypersalinity (Ghittoni and Bueno, 1996).

The strains were maintained as previously described (Ghittoni and Bueno, 1995) and were routinely grown in Vincent's (1970) YEM basal medium to serve as an inoculum for all experiments. Osmotic strength of the medium was increased by adding nonionic solutes, either sucrose, glycerol, or polyethyleneglycol 8000 (PEG). Osmolality of media was measured with a vapor pressure osmometer (Model 8500 C, Wescor Logan, UT, USA). The conversion of osmolality values to -MPa, according to Lee-Stadelmann and Stadelmann (1989) is presented in Table 1. For all experiments, bacteria were grown on 10 ml of medium in sidearm 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). For the hypercaloric shock, temperature of incubation was increased to 37°C. Growth was followed turbidimetrically at 620 nm with a metrolab VD40 spectrophotometer. At the stationary phase of growth, small samples were taken for determination of dry weight after lyophylization (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). The number of viable cells was measured as CFU by the plating technique described by Somasegaran and Hoben (1994).

Table 1. Osmotic potential of growth media for *Rhizobium* spp. before and after addition of different osmotica

Osmoticum	Concentration	Osmotic potential (MPa)
None (basal medium)		−0.22
Sucrose	350 mM	−0.98
Sucrose	650 mM	−2.03
Glycerol	350 mM	−0.92
PEG-8000	16% W/V	−1.87
PEG-8000	24% W/V	−2.30

Extraction and identification of periplasmic oligosaccharides

Starting from a 2% inoculum, bacteria were grown in Erlenmeyer flasks containing 500 ml of growth medium. At the stationary phase of growth, cells were harvested by centrifugation, washed, and pellets were treated with chloroform as described by Ames et al. (1984). Proteins were removed with 1% trichloroacetic acid (TCA) as described by Miller et al. (1986). TCA-extracts were neutralized, concentrated, and fractionated on a BioGel P4 column, with a fractionation range between molecular weights of 800 and 4000; the column was previously calibrated with carbohydrate molecular weight markers. All chromatografic and chemical procedures have been described elsewhere (Ghittoni and Bueno, 1995; 1996). Briefly, eluted fractions of 3 ml were collected, and the hexose content of the fractions was measured with the anthrone-sulfuric acid reagent (Trevelyan and Harrison, 1952) using glucose as the standard. Anthrone-positive fractions recovered from the BioGel column were pooled, concentrated, and loaded on a Dowex 50W-X8 column. The hexose containing peaks eluted from the Dowex column were concentrated under vacuum. The concentrated fractions were passed through a DEAE-Sephadex A-25 column which was subsequently eluted with a NaCl linear gradient. The monosaccharide composition of samples was determined by total acid hydrolysis, followed by two different TLC-systems.

Identification of trehalose and sucrose

Fractions (0.1 mg) which were eluted from the Dowex column, and concentrated as described above, were derivatized, and gas chromatography was performed on a Hewlett-Packard 5890 Series II instrument, equipped with a 25 m HP1 capillary column, a flame ionization detector, and a HP-3396 (II) integrator; nitrogen was employed as the carrier gas. Trehalose and sucrose were used as standards, retention times were 19.2 ± 0.2 min (n = 9) and 16.8 ± 0.1 (n = 4), respectively. ^{13}C nuclear magnetic resonance spectra were recorded on a Bruker Ac 200 spectrometer operating at 50.32 Mhz; the samples were dissolved in D_2O at a concentration of approximately 20 mg/ml; 1,4-dioxane was used as an internal reference; the 90°C pulse width was 5.4 μs.

3. Results and discussion

Cells from two *Rhizobium* strains recommended as peanut inoculants by ATCC and by NifTAL, were grown in high osmotic media. Table 2 shows the results obtained when high osmotic potentials were imposed either by permeating solutes (glycerol, sucrose) or by the nonpermeating solute PEG

which was utilized by several authors (Zaharan and Sprent, 1986; Busse and Bottomley, 1989; Mohammad et al., 1991) to simulate drought stress. In the strain TAL 1000, the production of biomass was strongly decreased in all imposed hyperosmotic conditions, but the protein content of cells was only diminished by sucrose. In contrast, in the strain ATCC 10317, 350 mM sucrose increased biomass production, however the protein content of cells was not augmented. In spite of cell alterations, almost all media allowed the production of 10^9 viable cells per ml of medium; the presence of 650 mM sucrose in the medium was needed to produce a 10-fold decrease in cell viability.

Table 2. Growth responses of two peanut *Rhizobium* strains to hyperosmotic stress imposed by nonionic osmolytes

Strain / Osmolyte (conc.)	Biomass mg/ml medium	Proteins mg/mg biomass	Viability CFU/ml medium
TAL 1000			
None	1.45±0.21	0.24±0.03	5.4×10^9
Sucrose (350 mM)	0.91±0.28 (a)	0.08±0.02 (c)	2.6×10^9
Sucrose (650 mM)	0.90±0.20 (a)	0.06±0.01 (c)	0.4×10^9
Glycerol (350 mM)	0.17±0.02 (c)	0.19±0.04	5.5×10^9
PEG (16% P/V)	0.52±0.28 (b)	0.19±0.03	1.1×10^9
PEG (24% P/V)	0.39±0.13 (c)	0.26±0.02	1.0×10^9
ATCC 10317			
None	0.64±0.07	0.26±0.11	7.1×10^9
Sucrose (350 mM)	1.25±0.04 (c)	0.12±0.04	2.9×10^9
Sucrose (650 mM)	0.64±0.04	0.12±0.02	0.3×10^9
Glycerol (350 mM)	0.54±0.07	0.18±0.03	1.7×10^9
PEG (16% P/V)	0.79±0.20	0.16±0.03	3.4×10^9
PEG (24 % P/V)	0.53±0.19	0.14±0.02	1.1×10^9

Data are the means of three to four independent experiments. Biomass and proteins are expressed +/− standard deviations. Significant differences: (a) $p<0.05$; (b) $p<0.01$; (c) $p<0.001$. The standard deviation for CFU was ±10%.

Low molecular weight saccharides (LMWS), most of them cyclic glucans, are present in the periplasmic space of rhizobia, where they play a role in osmotic adaptation (Miller et al., 1986; Dylan et al., 1990). Fig. 1 shows patterns of LMWS obtained from cells grown in basal media and eluted from BioGel P4

column. In the strain TAL 1000 (Fig. 1A) a single anthrone-positive peak eluted from the column was identified as trehalose by gas chromatography and by ^{13}C-NMR spectroscopy. On the other hand, the major LMWS from the strain ATCC 10317 (Fig. 1B) eluted from the BioGel column as a peak with a Kav = 0.093 that corresponded to an apparent molecular weight of 3750. Material from the peak did not adsorb onto DEAE-Sephadex A-25 and, after complete acid hydrolysis followed by TLC, revealed only one spot that comigrated with glucose standard. Results suggest that the major LMWS from the strain ATCC 10317 is a neutral glucan with a degree of polymerization of about 22 glucose residues. Amemura et al. (1985) determined similar degree of polymerization of glucans isolated from some tropical strains of rhizobia.
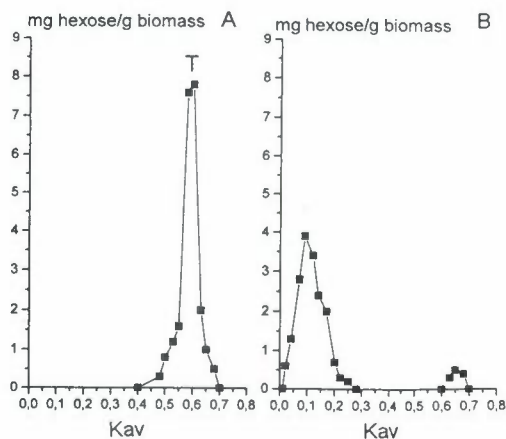

Figure 1. Chromatography on BioGel P4 of periplasmic low molecular weight saccharides (LMWS) from stationary phase *Rhizobium* spp. TAL 1000 (A) and ATCC 10317 (B) growing on basal conditions (Vincent, 1970). LMWS are expressed as miligrams of equivalent hexose per gram of biomass (mg hexose/g biomass). Kav = (Ve-Vo)/(Vt-Vo) in which Ve is the elution volume, Vo is the void volume, and Vt is the total volume. Data are representative of at least three experiments. T: this peak was shown by GC and by NMR to contain trehalose.

When LMWS from cells which maintained their viability in hyper-osmolarity were investigated, BioGel chromatography (Fig. 2) revealed that bacteria replaced their LMWS with other saccharide species: trehalose could not be found in strain TAL 1000 (Fig. 2A, B, C) and the neutral glucan with Kav = 0.093 was not detected in strain ATCC 10317 (Fig. 2D, E, F). When hyperosmolarity was imposed by 350 mM sucrose, excess of osmoticum was accumulated in the periplasmic space (Fig. 2A and D). The periplasmic

concentration of LMWS from both strains was increased by 350 mM glycerol (Fig. 2B and E) and it was decreased by 16% W/V PEG (Fig. 2C and F). Therefore, changes either in LMWS metabolism or in their cell localization, or the sum of both, might allow the adaptation and the survival of peanut rhizobia in environments of high osmotic potential.

By raising the growth temperature of the two tested strains from 28 to 37°C, the biomass production was slightly reduced and the cell content of LMWS was highly increased (Table 3), the ratio between LMWS and proteins was 2–3 fold augmented. BioGel P4 chromatography of increased LMWS from the strain TAL 1000 yielded two major peaks (Fig. 3A), one of them eluted close the void volume of the column, and the other contained neither trehalose nor sucrose. Although trehalose was synthesized by the strain TAL 1000 under basal conditions of growth, the disaccharide was detected neither in osmotic-stressed nor in heat-stressed cells. On the other hand, in the strain ATCC 10317, the neutral glucan synthesized at 28°C was fully suppressed at 37°C (Fig. 3B), instead, significant amounts of anthrone-positive material was eluted as three sharp peaks of lower molecular weight than the glucan,; material from the largest peak was identified as trehalose by gas chromatography. When cells from the strain ATCC 10317 had been grown under hypersalinity, trehalose was also detected (Ghittoni and Bueno, 1996).

As far as we know, no reports in the literature are available on the adjustment of peanut rhizobia to environmental stresses. Our results demonstrate some similarities in the responses of the two tested peanut

Table 3. Biomass, proteins and cellular low molecular weight saccharides (LMWS) from two peanut *Rhizobium* strains growing at 28 and 37°C.

Strain	Temp. °C	Biomass mg/ml medium	Proteins mg/g biomass	LMWS	
				mg/g biomass	mg/g protein
TAL 1000	28	1.45	240	22	92
	37	0.94	310	65	210
ATCC 10317	28	0.64	250	18	72
	37	0.41	340	78	229

Data are the means of two to four independent experiments, standard deviations never exceeded 5%. LMWS were extracted, identified and determined as described in Materials and Methods; they are expressed as miligrams of equivalent hexose.
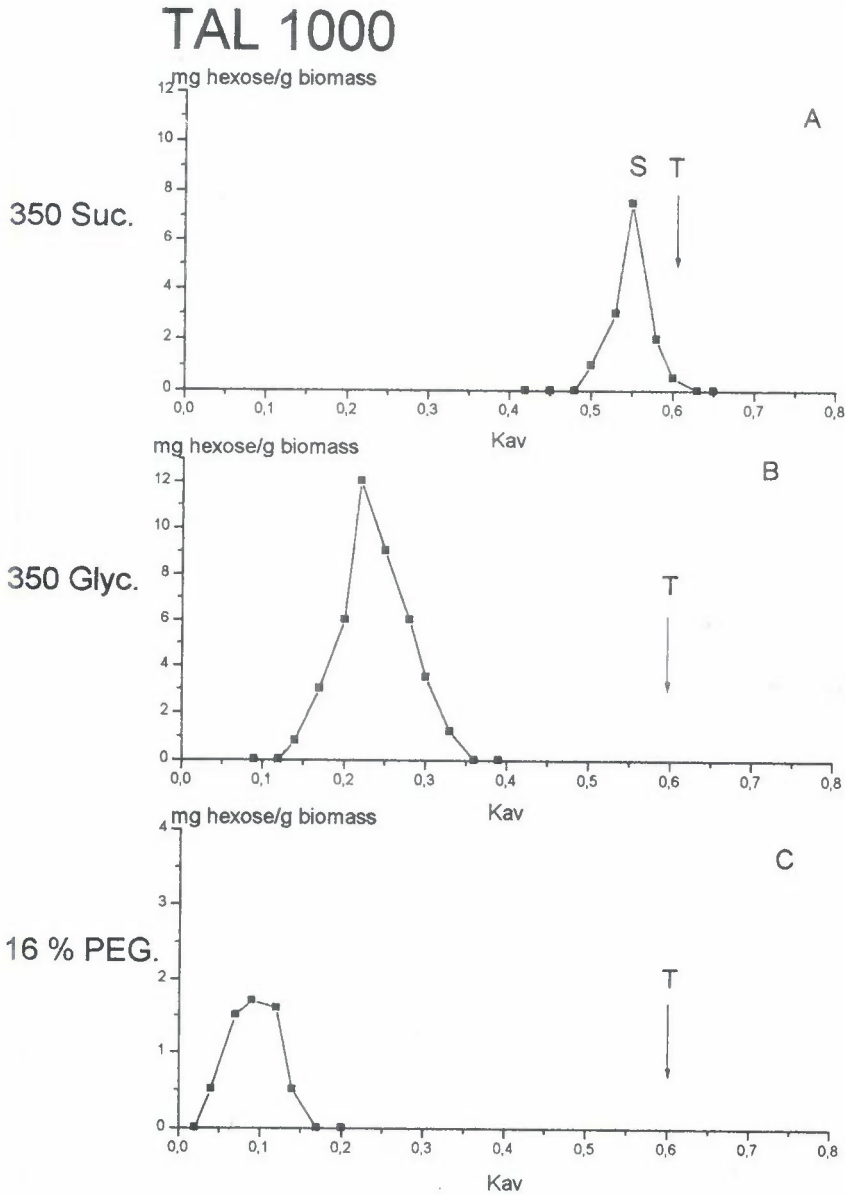
Figure 2. Effect of nonionic hyperosmolarity on periplasmic LMWS from stationary phase peanut *Rhizobium* spp. TAL 1000 (A, B, C) and ATCC 10317 (D, E, F). Bacteria were grown in Vincent's medium (1970) and the following osmolytes were included: 350 mM sucrose (A, D), 350 mM glycerol (B, E), and 16% W/V PEG (C, F). Data are expressed as in Fig. 1, and they are representative for at least two independent experiments done at each hyperosmotic condition. S: peak identified as sucrose by gas chromatography. The arrows indicate the positions of trehalose (T) from control TAL 1000, and of glucan (G) from control ATCC 10317.
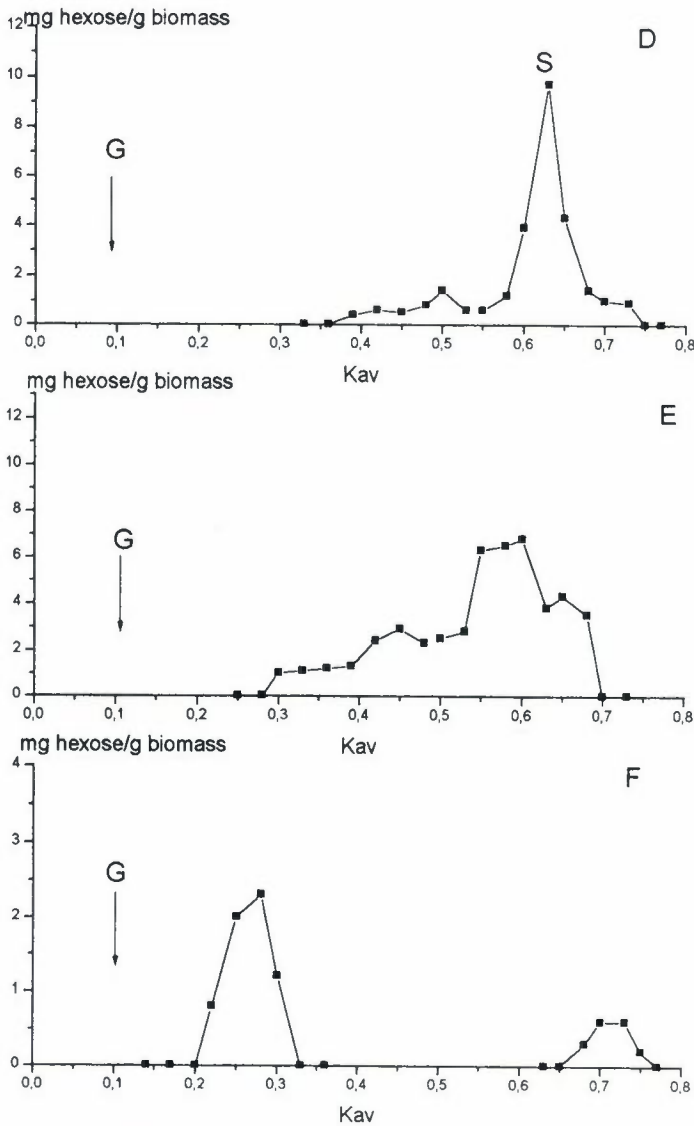
ATCC 10317

Figure 2. Continuation.

rhizobia strains: the content of periplasmic LMWS was augmented by greater than optimal temperatures and by a readily permeating solute (glycerol), but it was decreased by a nonpermeating osmoticum (PEG). In addition to the observed quantitative changes, component analysis of LMWS also revealed
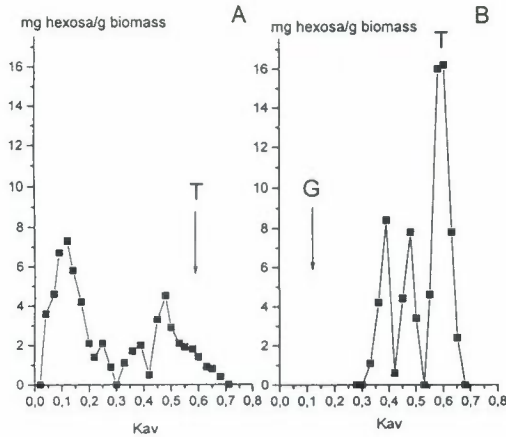
Figure 3.   Periplasmic LMWS from stationary phase peanut *Rhizobium* spp. TAL 1000 (A)
and ATCC 10317 (B) growing at 37°C on Vincent's medium (1970). Data are
expressed as in Fig. 1, and they are representative for at least two independent
experiments. T: peak identified as trehalose by gas chromatography. The arrows
indicate the positions of trehalose (T) from control TAL 1000 and of glucan (G)
from control ATCC 10317.

qualitative alterations: it is particularly intriguing that trehalose
disappeared in stressed cells from the strain TAL 1000, while the disaccharide
appeared in salt- and in heat-stressed cells from the strain ATCC 10317. As
pointed out by Smith et al. (1988) rhizobia would be expected to have a
sophisticated   array of regulatory mechanisms of adjustment to harsh
environmental conditions. Based on our observations, it is tempting to speculate
that trehalose may be involved in some of those regulatory mechanisms.
Therefore, in our laboratory experiments are in progress to study the effect of
stressful conditions on enzymes of trehalose metabolism in peanut rhizobia.

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