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Physiological Response of Two Peanut Rhizobia Strains to Acid pH

TANIA TAURIAN, STELLA CASTRO, and ADRIANA FABRA*
Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Fco-Qcas y Naturales, Universidad Nacional de Río Cuarto, 5800 Río Cuarto (Córdoba), Argentina, Tel. +54-58-676114, Fax. +54-58-676230, E-mail. afabra@exa.unrc.edu.ar

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Abstract

Soil acidity is a stress factor that negatively affects the persistence of *Rhizobium* strains and the nodulation and nitrogen fixation in legumes. To identify peanut symbiont acid-tolerant strains we tested the ability of *Bradyrhizobium* sp. USDA 3187 and *Bradyrhizobium* sp. TT 001 (isolated from nodules of peanut) to grow at pH 5 in unbuffered broth medium. Only *Bradyrhizobium* sp. TT 001 grew at this pH and tolerance to this condition was not related with the synthesis of polyamines, lipopolysaccharide or with the production of extracellular polysaccharides. Cells of TT 001 were markedly resistant to the effects of crystal violet and was not affected by pH 5. These results suggest that the outer membrane structure could be involved in pH tolerance.

Keywords: *Bradyrhizobium* sp, peanut , acid-tolerance, soil acidity

1. Introduction

Soil acidity may significantly reduce the symbiotic nitrogen fixation in tropical and temperate soils, limiting rhizobia survival and persistence and reducing nodulation (Graham et al., 1982, 1994; Brockwell et al., 1991). Presently, soil acidity is frequently a major constraint on the cultivation of

*The author to whom correspondence should be sent.

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leguminous crops, not only due to the hydrogen toxicity, but also to other nutritional imbalances that are often associated with acid soils (i.e. calcium and molybdenum deficiencies and aluminium and manganese toxicities (Graham, 1992). Many studies have shown that in acid upland soils the rhizobial population is small and with a preponderance of ineffective strains (Masterson, 1961; Jones, 1963). In addition, acidity can also drastically change the host plant's "preference" for particular strains of nodule bacteria (Thurman et al., 1985). Approximately a quarter of the world's agricultural soils are acidic and there is increasing concern about soil acidification (Munns, 1986). Since in some cases the root nodule bacteria represent the acid-sensitive component of symbiosies (Munns, 1986), the development of inoculum strains of rhizobia with enhanced ability to survive and to nodulate in acidic conditions may provide a means of maintaining high levels of symbiotic nitrogen by legumes.

The physiological basis for pH tolerance among strains of *Rhizobium* and *Bradyrhizobium* is still not clear, although some reports have shown that the cytoplasmic pH is less affected by external acidity in acid-tolerant strains. In fact, most bacteria that have been studied (Caldwell, 1956; Waddel and Bates, 1969; Thomas et al., 1976; Booth, 1985) maintain a relatively constant internal pH over a wide range of external pH. This control of pH may involve different mechanisms for regulating the influx and efflux of protons across the cytoplasmic membrane (Glenn and Dilworth, 1994), but little is know about such mechanisms in *Rhizobium* and *Bradyrhizobium*. Aarons and Graham (1991) reported high cytoplasmic potassium levels in acid-stressed *R. leguminosarum* bv. *phaseoli*. Chen et al (1993) observed differences in lipopolysaccharides (LPS) composition in *R. leguminosarum* bv. *trifolii* and Fujihara and Yoneyama (1993) reported the accumulation of cellular polyamines in *R. fredii* and in *B. japonicum* associated with their growth at acid pH.

With reference to the genetic basis for acid tolerance, Tiwari et al. (1996) have proposed that an acid-sensitive mutant strain *R. meliloti* TG5-46 is defective in a regulator gene (*act*A) and that a sensor-regulator pair is essential for acid tolerance in wild-type *R. meliloti* WSM 419.

Although there are several reports about soil pH effects on different *Rhizobium* strains, relatively few studies have examined this effect on the peanut (*Arachis hypogaea* L.) symbiont. In this study, in order to know the mechanisms involved in acid tolerance in this microsymbiont, we compared the physiological response of the strain *Bradyrhizobium* sp. TT 001 growing at pH 5 or 7 with the acid sensitive *Bradyrhizobium* sp. USDA 3187 growing under the same conditions.

2. Materials and Methods

Bacterial strains

Bradyrhizobium sp. USDA 3187 was gently provided by Dr. Nora Ghittoni, Universidad Nacional de Río Cuarto and *Bradyrhizobium* sp. TT 001 was isolated from field-grown peanut nodules. Nodules were surface sterilized, crushed and the nodule extracts were spread on YEMA medium (Vincent, 1970). Plates were incubated for 48 h at 28°C. Colonies with the typical colonial morphology of rhizobia were tested for nodulation on peanut (Vincent,1970). For the experiments, bacteria were grown at 28°C in YEM medium. The pH was adjusted after or before autoclaving to values from pH 5 to 7. Growth was followed by absorbance (620 nm) determinations.

Intracellular potassium content

Cultures were assayed for potassium concentration using the procedure of Bernard et al. (1993). Cells, in logarithmic growth phase, were centrifuged, washed twice in saline buffer solution (PBS) (K_2HPO_4 0.05 M, KH_2PO_4 0.05 M, $MgSO_4$ 0.002 M and NaCl 0.140 M, pH 7) and pellets suspended in 0.1 M perchloric acid for 24 h. K+ levels were determined using a Tecnolab-T410 model ion analyzer.

Polyamine determination

The technique employed for polyamine determination was based on the method used by Smith and Best (1977). Cells were collected from a logarithmic growth phase culture by centrifugation at 15,000 × g for 10 min. Lysis of bacteria was carried out by grinding the cellular pellets with alumina (2 g/g of wet cells) in a mortar for 5 min. The mixture was resuspended in 2 ml of PBS and centrifuged at 3,500 × g for 15 min. Proteins in the resulting supernatants were precipitated with 50% trichloroacetic acid and centrifuged at 420 × g. Fifty mg sodium bicarbonate and 0.2 ml dansyl chloride solution (30 mg/ml acetone) were added and tubes were kept in darkness for 16 h. A 0.1 ml aliquot of proline solution (1.5 mg/ml) was added, and each sample was incubated at room temperature for 30 min and subsequently dried with a current of warm air. The dry residue was dissolved in water: toluene (1:2 (V/V)) and, after mixing for a few minutes, the sample was centrifuged. The toluene layer was separated and 20 μl was placed on a TLC plate (Silica gel G 60, Sigma). The chromatography was run for 2 h using chloroform:triethylamine (5:1). Polyamine locations were detected by means of UV light, extracted with acetone and the concentration

determined with a spectrofluorometer (excitation 359 nm and emission 500 nm). The results were compared with dansylated polyamine standards.

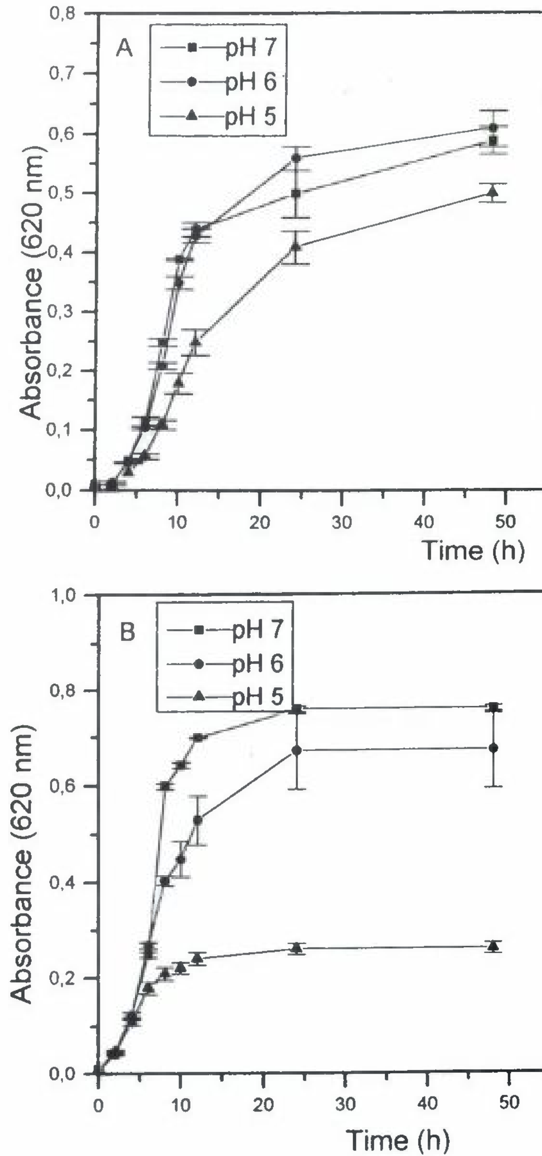

Figure 1. Effect of pH on growth. A) *Bradyrhizobium* sp. TT 001. B) *Bradyrhizobium* sp. USDA 3187. Data are means ± S.E. of 3 determinations.

Lipopolysaccharide and exopolysaccharide production

Lipopolysaccharide was measured using the method described by Westphal-Jahn (1965) with the following modifications. Cultures in logarithmic phase of growth were centrifuged at 15,000 × g for 10 min and pellets were washed with buffer KH_2PO_4 10 mM (pH 7). Cells were resuspended in twice destillated water. Phenol 45% (W/V) (5 ml of phenol/g cells) was added and the mixture was heated for 15 min at 70°C in a water bath. Tubes were cooled quickly in ice-water, centrifuged at 15,000 × g for 10 min, and the top phases were dialyzed against water for 3 days. Lipopolysacharides were quantified using the anthrone method (Trevelyan and Harrison, 1952).

Exopolysaccharide production was qualitatively determined on the basis of colonies fluorescence under UV light on YEMA medium containing Calcofluor (0.05%) and by examining cells under fluorescence microscopy for calcofluor-binding material on bacteria.

Crystal violet sensitivity

Crystal Violet was added to cultures in logarithmic phase of growth (25 mg/l culture medium) and cultures were incubated on a shaker at 28°C for 60 min. Before and after the incubation period, the culture pH was measured (in order to determine alterations produced by the crystal violet addition) and the number of viable cells was determined by plating a culture sample onto YEMA and counting the cells after 2 days of incubation at 28°C.

Statistical analysis

Experimental differences were compared by the Student t-test at the 0.05 significance level.

Chemicals

All the chemicals were obtained from Sigma Chemical (St. Louis, MO) or Merck Quìmica Argentina. Homospermidine was kindly provided by Dr. S. Fujihara.

3. Results and Discussion

Effect of low pH on growth

Growth of *Bradyrhizobium* sp. USDA 3187 and TT 001 was examined over a range of pH values in liquid culture medium. Strain TT 001 was able to grow at

pH 5, whereas with strain USDA 3187 growth was significantly decreased at this pH (Fig. 1). In both cases, the pH of the medium set initially at 5, decreased by up to 0.5 pH units after 36 h of incubation and no differences in the growth were observed when pH was adjusted after or before medium autoclaving. At the time the cells were collected for the experiments (12 h of incubation), no changes in the pH value compared with the beginning of the incubation were determined. Howieson (1985) used MES to buffer media used in the identification of acid-tolerant strains of *R. meliloti*. Unfortunately, MES is only an effective buffer from pH 5.5 to pH 6.7, while several alternative buffers (i.e., citrate or glutamate) may be metabolized by rhizobia (Graham et al., 1994). Because our studies included pH values outside the effective buffering range for MES, they were undertaken without buffers.

Potassium content

It is possible that metabolic changes could contribute to the ability of TT 001 to tolerate acid pH. Aarons and Graham (1991) reported higher potassium levels in cells of *R. tropici* following exposure to acid pH, but, as it is shown in Table 1, in our study we did not find elevated potassium levels either in USDA 3187 or in TT 001.

Polyamine production

Recent studies with *Escherichia coli* have emphasized the role of lysine decarboxylase (enzyme involved in the polyamine biosynthesis) in the acidification response (Watson et al., 1992). Fujihara and Yoneyama (1993) reported an enhanced polyamine synthesis by *Rhizobium* cells subjected to acid and osmotic stress. Because of these results, we undertook studies to determine polyamine production in *Bradyrhizobium* sp. USDA 3187 and TT 001 as a function of culture pH. Results shown in Table 2 demonstrated that both strains contained spermidine, homospermidine, cadaverine and putrescine, but surprisingly it was the acid-sensitive strain (USDA 3187) that showed a greater production of polyamines (specially spermidine) at acid pH. In *Bradyrhizobium* sp. TT 001 growing at pH 5, there was a diminution in this amine. Thus, it appears from our results that polyamine production in USDA 3187 is a consequence of pH stress, rather than a factor in recovery. Similar results were obtained by Graham et al (1994) in *R. tropici*.

Cellular envelope and permeability

Lipopolysaccharides (LPS) are a major component of the outer membrane of

gram-negative bacteria, including the members of the family Rhizobiaceae. Changes in LPS structure or production have been related to adaptation to different environmental conditions such as pH, oxygen concentration and osmotic pressure (Bhat and Carlson, 1992; Kannenberg and Brewin, 1989; Tao et al., 1992; Zahran et al., 1994). On the other hand, extracellular polysaccharides (EPS) production has been previously correlated with acid pH tolerance in bean rhizobia (Cunningham and Munns, 1984).

In order to investigate the possible role of *Bradyrhizobium* sp. cellular envelope in acid pH tolerance, bacteria that had been growing in liquid medium at pH 5 or 7 were transferred to solid medium at pH 5 or 7, respectively. No changes in colony morphology were noticed on TT 001 cells grown at pH 5 when compared with cells grown at pH 7 (data not shown). However, when the same studies were done with the acid-sensitive strain USDA 3187, morphology of the colonies was different from that of the acid-tolerant strain, resembling the semirough colonies produced by LPS mutants. These results suggest that in *Bradyrhizobium* sp. TT 001 both exopolysaccharides and lipopolysaccharides were not altered by acid pH. No decrease in fluorescence on YEMA-Calcofluor plates was observed when acid-tolerant bacteria were grown at pH 5, suggesting that there was no affect on exopolysaccharide production. The same results were observed when bacteria were examined by fluorescence microscopy (data not shown). Furthermore, when the lipopolysaccharide content was measured in this acid-tolerant strain, it was not affected by pH 5. However, there was a significant decrease in the lipopolysaccharide content in the acid-sensitive bacteria when growing at pH 5 (Table 3).

Crystal violet is a compound whose uptake is affected by the permeability of the outer membrane. To test whether these alterations were affecting bacteria permeability, the resistance to this compound was determined. The addition of crystal violet to culture medium did not alter its pH neither before nor after the incubation period. Table 4 shows that there was a decrease of 66.7% in the survival of the acid-sensitive strain, *Bradyrhizobium* sp. USDA 3187, after exposure to crystal violet at pH 5, compared with the same strain growing at pH 7. In contrast, the percent survival of TT 001 following exposure to crystal violet in a culture medium at pH 5 did not change significantly when it was compared with data obtained at pH 7. Therefore, it is possible to assume that the outer membrane of this organism may play a part in pH tolerance, as reported for *R. tropici* by Graham et al. (1994). Lloret et al. (1995) also reported modification of the bacterial cell wall LPS as an adaptative mechanism of *R. meliloti* for ionic and osmotic stress.

The identification in this study of *Bradyrhizobium* sp. TT 001 as acid-tolerant strain opens the way for more detailed genetic and physiological studies of acid pH tolerance in *Bradyrhizobium* strains. The results presented

Table 1. Potassium content

Strain	pH	K^+ (mmoles/l)
TT 001	7	1.1 ± 0.088
	5	1.1 ± 0.14
USDA 3187	7	0.8 ± 0.16
	5	0.1 ± 0.08 *

Data are means ± S.E. of 10 determinations. *$p<0.0005$.

Table 2. Polyamine content

		Polyamine (μg/g pellet)			
Strain	pH	Homospermidine	Spermidine	Cadaverine	Putrescine
TT 001	7	2.55±0.98	7.71±2.29	1.28±0.51	12.77±4.04
	5	3.06±0.73	2.32±0.74*	1.47±0.41	11.78±3.02
USDA3187	7	0.72±0.22	0.16±0.03	1.13±0.37	3.53±1.50
	5	0.75±0.03	0.31±0.10**	3.02±0.97	5.75±2.80

Data are means ± S.E. of 3–14 determinations. * $p<0.0005$, ** $p< 0.05$.

Table 3. Lipopolysaccharide content

Strain	pH	LPS (μg/g pellet)
TT 001	7	0.7 ± 0.032
	5	0.9 ± 0.23
USDA 3187	7	0.7 ± 0.28
	5	0.1 ± 0.062 *

Data are means ± S.E. of 5–6 determinations. *$p<0.005$.

Table 4. Survival after exposure to crystal violet

Strain	pH	Survival (%)
TT 001	7	47
	5	45
USDA 3187	7	0.18
	5	0.06

Data are means ± S.E. of 5–6 determinations.

here emphasize the possible implications, in the response of *Bradyrhizobium* peanut symbiont to this stress, of the cellular permeability and its relationship with the outer membrane structure.

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