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# Effects of salinity on protein and lipopolysaccharide pattern in a salt-tolerant strain of *Mesorhizobium ciceri*

**M. Soussi[1], M. Santamaría[2], A. Ocaña[1] and C. Lluch[1]**

*[1]Departamento de Biología Vegetal, Facultad de Ciencias, Universidad de Granada, and [2]Departamento de Bioquímica y Biología Molecular, Universidad de La Laguna, Tenerife, Spain*

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**Aims:** To characterize the physiological and metabolic responses of *Mesorhizobium ciceri* strain ch-191 to salt stress, investigating the changes induced by salinity in protein and lipopolysaccharide profiles, as well as determining the accumulation of amino acids, glutamate and proline.

**Methods and Results:** Strain ch-191 of *M. ciceri* was grown with different NaCl concentrations. Protein and lipopolysaccharide patterns were determined by electrophoresis. The strain ch-191 tolerated up to 200 mmol $l^{-1}$ NaCl, although higher salt dosages limited its growth and induced changes in the protein profile. The most noteworthy change in the LPS-I pattern was the decrease in the slowest band and the appearance of an intermediate mobility band. The accumulation of proline in response to salt stress surpassed that of glutamate.

**Conclusions:** The protein profile showed major alterations at salinity levels which inhibited growth. However, the alterations in the LPS profile and accumulation of compatible solutes were evident from the lowest levels, suggesting that these changes may constitute adaptative responses to salt, allowing normal growth.

**Significance and Impact of the Study:** The selection and characterization of salt-tolerant strains, which also show efficient symbiotic performance under salinity, may constitute a strategy for improving *Cicer arietinum–Mesorhizobium ciceri* symbiosis in adverse environments.

## INTRODUCTION

Salinity considerably restrains symbiotic nitrogen fixation (Elsheikh and Wood 1995) and therefore, the cultivation of tolerant nitrogen-fixing leguminous crops can help improve the fertility of saline soils (Zahran 1997). For example, chick-pea in symbiosis with an efficient strain of *Meshorizobium ciceri* constitutes an important component of crop systems, being capable of supplying between 80 and 120 kg N $ha^{-1}$ to the soil (Herridge *et al*. 1995).

Salt tolerance of symbiotic nitrogen fixation reportedly depends both on the plant and on *Rhizobium* genotypes (Pessarakli and Zhou 1990; Cordovilla *et al*. 1995). Studies on cultivar–strain interactions indicate that the plant is a determining factor for symbiosis tolerance (Craig *et al*. 1991; Soussi *et al*. 1999), although the salt-tolerant *Bradyrhizobium* strain fixes more nitrogen than the sensitive one (Elsheikh and Wood 1990), and there is a marked difference in nodulation and nitrogen fixation parameters between salt-tolerant and salt-sensitive strains (Kumar *et al*. 1999). Furthermore, a salt-sensitive *Rhizobium* has been correlated with ineffective nodulation in soybean (Ohwada *et al*. 1998).

One approach to understanding the ability of *Rhizobium* to tolerate salt stress has been to identify stress-induced changes of individual proteins (Natarajan *et al*. 1996) under the assumption that stress adaptation results from alterations in gene expression. Salt-stress responses also lead to changes in lipopolysaccharides (LPS) (Lloret *et al*. 1995), which, as important components of the external cell wall of Gram-negative bacteria, may play a major role in the adaptation of bacteria to the environment (Tao *et al*. 1992). Also, these changes in LPS structure may affect the capacity of *Rhizobium* to infect the roots and form effective nodules.

Other responses of *Rhizobium* to salt stress include transient adjustments in ionic balance and pronounced changes in the metabolism of cytoplasmic low-molecular-weight

*Correspondence to: Dr Carmen Lluch, Departamento de Biología Vegetal, Facultad de Ciencias, Universidad de Granada, E-18071 Granada, Spain (e-mail: clluch@goliat.ugr.es)*

compounds (Yap and Lim 1983). The accumulation of these compounds, referred to as osmotically-active solutes, is compatible with metabolic functions, and help in osmoregulation and prevention of cytoplasmic dehydration in saline environments (Fujihara and Yoneyama 1994). Examples of these compatible solutes have been proposed for many compounds, such as glutamate, proline, glycine-betaine and polyoles (Botsford and Lewis 1990; Fougère *et al*. 1991; Miller and Wood 1996). Glutamate functions as a compatible solute in various micro-organisms (Botsford and Lewis 1990), and its accumulation is central to osmoregulation in *Sinorhizobium fredii* under salt-stress conditions (Fujihara and Yoneyama 1994). Under such conditions, the intracellular glutamate pool might contribute 90% or more of the total free amino acid in the cell (Tempest *et al*. 1970).

The selection and characterization of salt-tolerant strains which also show efficient symbiotic performance under salinity may provide a strategy to improve *Cicer arietinum–Mesorhizobium ciceri* symbiosis in adverse environments. The aim of the present study was to characterize the physiological and metabolic responses of *Mesorhizobium ciceri* strain ch-191 to salt stress. The changes induced by salinity in protein and lipopolysaccharide profiles were investigated, and the accumulation of amino acids, glutamate and proline was determined.

## MATERIALS AND METHODS

### *Rhizobium* strain and growth conditions

Strain ch-191 of *Mesorhizobium ciceri*, obtained from the International Center for Agricultural Research in Dry Areas (ICARDA, Syria), was used throughout this study. For growth curves, 100 ml volumes of YEM medium (Vincent 1970) supplemented with different NaCl concentrations were prepared in 250 ml Erlenmeyer flasks and inoculated with 1 ml of a late exponential phase culture of ch-191. Cells were grown at 28°C in an orbital shaker (170 rev $min^{-1}$). Growth was monitored for 72 h by optical density (O.D.) at 600 nm in a Spectronic 20D spectrophotometer (Spectromic Instruments Inc., NY, USA) at intervals of 6 and 12 h. Generation time and maximum number of viable cells $ml^{-1}$ were also determined.

### Protein patterns

Cells grown in liquid YEM medium to the late logarithmic phase were harvested by centrifugation at 15 000 *g* for 5 min at 4°C, washed in 50 mmol $l^{-1}$ Tris-HCl (pH 7·5) and resuspended in 200 $\mu$l of the same buffer. The cells were sonicated for 5 min at 15 s intervals in a refrigerated circuit at 4°C using a sonicator Vibra-Cell 100 Watt model (Sonic & Materials Inc., Danbury, CT, USA). Lysates were centrifuged at 30 000 *g* for 20 min to remove cell debris. The total protein content of the extracts was determined by the Lowry method modified by Markwell *et al*. (1978). Proteins were subjected to SDS-PAGE according to Laemmli (1970), using 30 $\mu$g of protein per lane. Protein electrophoresis was carried out in 15% SDS-polyacrylamide gels. Gels were fixed and stained with silver nitrate as described by Blum *et al*. (1987).

### Lipopolysaccharide analysis

Bacterial samples from the late logarithmic phase were prepared for electrophoresis as described by Hitchcok and Brown (1983). Lyophilized cells were suspended in distilled water and 1 ml of this suspension was centrifuged at 10 000 *g* for 10 min. Pellets were mixed with 0·5 ml lysis buffer containing 2% SDS, 5% dithiothreitol, 10% glycerol and 0·02% bromophenol blue, and boiled for 10 min. Aliquots of each extract were treated with proteinase K (Boehringer) at 2·5 mg $ml^{-1}$. Electrophoresis was carried out by the method of Laemmli (1970), at room temperature, in 0·75 mm thick 17·5% polyacrylamide slab gels. The acrylamide concentration ranged from 15 to 20%, but the best resolution of the significant bands was achieved with 17·5% acrylamide. A 5 $\mu$l sample was applied to each lane and lipopolysaccharides were detected after silver staining (Tsai and Frasch 1982). The polyacrylamide gels were analysed with a Pharmacia-LKB Ultroscan XL densitometer (Pharmacia Biotech, Barcelona, Spain). Due to the width of the bands, each lane was scanned three times and the resulting profiles were analysed separately. Pharmacia-LKB analysis software, GelScan XL, was used for analysis of the densitometric traces. The steps involved were: (i) normalization of the profiles to the same length; (ii) background subtraction; (iii) integration of the peaks. Two bands in different profiles were considered to be the same when the difference in $R_F$ values (measured as the gel length travelled by the main absorbance of the band divided by the front distance) was less than 5%.

### Amino acid, proline and glutamate determination

Cultures of strain Ch-191 were grown in YEM medium in the presence of various NaCl concentrations (0, 100, 200, 300 and 400 mmol $l^{-1}$) to the late logarithmic phase. The cultures were harvested by centrifugation at 15 000 *g* for 10 min. The pellets were washed with 0·85% saline solution and re-centrifuged twice. The procedure of Prusiner *et al*. (1972) was followed to extract the hydrosoluble materials. The pellets were extracted in ethanol (70% v/v), centrifuged, and the supernatant fluid resuspended in water and stored at −20°C prior to analysis.

The intracellular amino acid pools of cells were determined by the reaction with the ninhydrin reagent (Sigma).

Proline was determined according to Irigoyen *et al.* (1992), and a calibration curve was made using L-proline (Sigma). Glutamate content was assayed enzymatically according to the procedure of Bernt and Bergmeyer (1974) after protein precipitation with 5% trichloroacetic acid (TCA). Bacterial cytosolic glutamate content was assayed at 30°C by monitoring the change in absorbance at 340 nm due to the reduction of NAD for 45 min. The reaction mixture included 1·6 mmol $l^{-1}$ $NAD^+$, 4·5 U $ml^{-1}$ GDH and 1 mmol $l^{-1}$ ADP in 300/250 mmol $l^{-1}$ glycine-hydrazine buffer (pH 9·0).

## RESULTS

Growth responses of *M. ciceri* strain ch-191 varied with different NaCl dosage (Fig. 1). Growth gradually decreased as the salt concentration increased. This strain could tolerate up to 200 mmol $l^{-1}$ in the YEM medium, whereas 300 mmol $l^{-1}$ reduced the total amount of growth (Fig. 1). The mean generation time, approximately 5 h under these conditions, proved twice as long as in control bacteria (Table 1), reflecting impaired bacterial growth. At 400 mmol $l^{-1}$, maximum growth diminished more than 10-fold.

The electrophoretic protein pattern of *M. ciceri* strain ch-191, under control conditions and with salt treatments, are shown in Fig. 2. The SDS-PAGE revealed, in the bacteria grown in salt-free medium, at least 30 polypeptides which ranged from 29 to more than 116 kDa. Comparative analysis of the lanes showed that up to 200 mmol $l^{-1}$ NaCl, the protein pattern did not change appreciably (Fig. 2). The dosage of 300 mmol $l^{-1}$ NaCl affected several polypeptides;

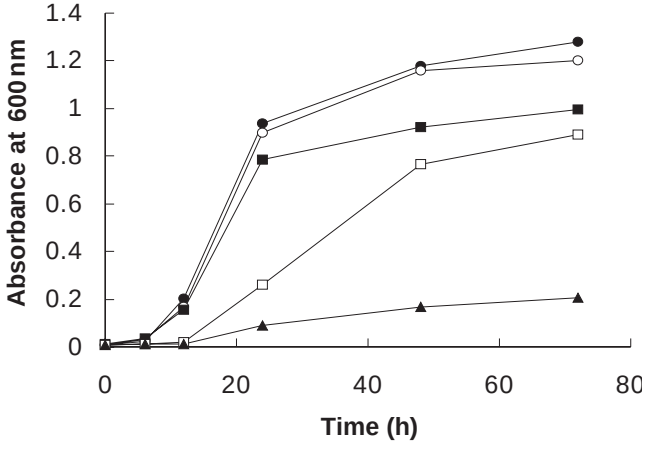


**Fig. 1** Effect of NaCl on growth of *Mesorhizobium ciceri* strain ch-191 grown in YEM medium and incubated at 28°C. Growth was measured by the increase in absorbance at 600 nm. Control (●), 100 mmol $l^{-1}$ (○), 200 mmol $l^{-1}$ (■), 300 mmol $l^{-1}$ (□) and 400 mmol $l^{-1}$ NaCl (▲). Standard error of the means are less than 5%

**Table 1** Growth conditions, generation time and maximum number of viable cells $ml^{-1}$ in strain ch-191 of *Mesorhizobium ciceri* grown in YEM medium and incubated at 28 °C with different NaCl concentrations

| NaCl concentration (mmol $l^{-1}$) | Generation time (h) | Maximum growth ($10^9$ cells $ml^{-1}$) |
|---|---|---|
| 0 | 2·83 | 3·36 |
| 100 | 2·96 | 2·52 |
| 200 | 3·01 | 1·91 |
| 300 | 4·96 | 0·93 |
| 400 | 5·89 | 0·29 |

the protein bands of 36·4 and 34·9 kDa observed in the control lane disappeared, whereas new polypeptides of molecular weights 41·5, 38·6 and 34 kDa appeared. Furthermore, protein bands of molecular weight 112·7, and one band of a molecular weight more than 116 kda, were intensified at this salt concentration. This trend was more pronounced when bacteria were grown with 400 mmol $l^{-1}$ NaCl.

Cell extracts of *M. ciceri* ch-191 growing on YEM medium with additional sodium chloride were subjected to SDS-PAGE and silver staining for LPS detection. The zone of interest corresponded to the LPSI (Fig. 3), changes detected in the zone of the LPSII proving inconsistent (data not shown). Figure 3 shows a silver-stained polyacrylamide gel in which the changes in the LPSI pattern induced by NaCl are visible. The NaCl effect is more clearly shown after densitometric analysis of the lanes, as described in the Material and

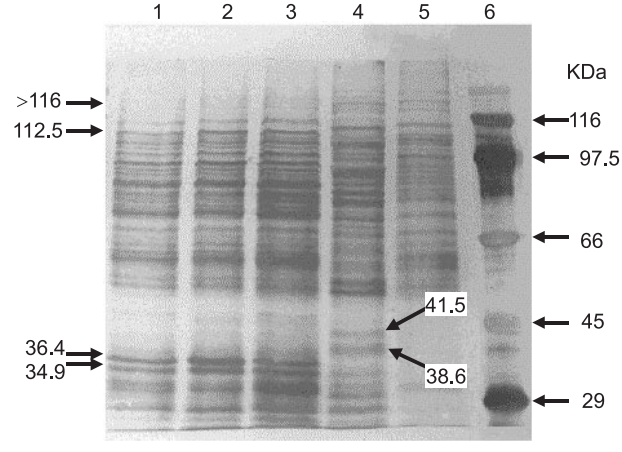


**Fig. 2** Stained SDS-PAGE of total soluble protein of *Mesorhizobium ciceri* strain ch-191 grown in YEM medium and incubated at 28 °C, under control conditions (lane 1) and with 100 mmol $l^{-1}$ (lane 2), 200 mmol $l^{-1}$ (lane 3), 300 mmol $l^{-1}$ (lane 4) and 400 mmol $l^{-1}$ NaCl (lane 5). Lane 6, molecular mass standards (Kit MW-GF-1000, Sigma). The arrows on the left and on lane 4 indicate the changes observed that are explained in the text

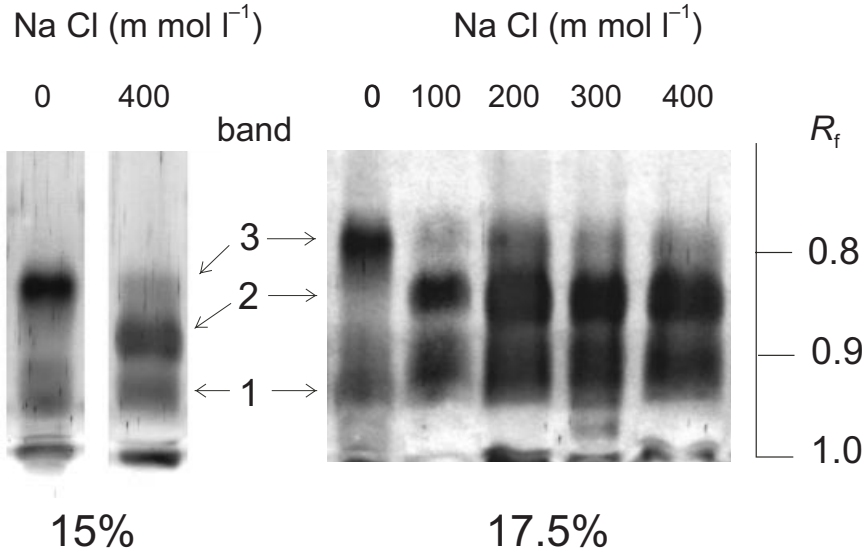


**Fig. 3** Gel electrophoresis at 15% and 17·5% acrylamide of LPS from strain 191 grown under different NaCl concentrations. Note the decrease in band 3 and the appearance of band 2 when NaCl was added to the culture medium

Methods section. Quantification of the changes in the percentage of each band in total LPSI is shown in Fig. 4.

The most noteworthy change is the decrease in the slowest band 3, and the appearance of an intermediate mobility band (band 2), which depends on the presence of NaCl in the medium. Furthermore, band 1 showed a different profile in the absence and presence of NaCl; without salt, band 3 was composed of two poorly-resolved bands, whereas with increasing salt concentration, band 1 was clearly homogeneous (see Fig. 3, 15% acrylamide gel).

The total amino acid content increased significantly with salinity (Fig. 5), and the highest concentration was reached in cells grown with 200 mmol l$^{-1}$ NaCl. A high proline content was found in this rhizobial strain, representing up to 45% of total amino acid content under control conditions and 28% in cells grown with 200 mmol l$^{-1}$ NaCl. It is noteworthy that salt at 200 mmol l$^{-1}$ boosted proline accumulation up to twofold compared with control cells. However, at levels higher than 300 mmol l$^{-1}$, proline accumulation sharply declined (Fig. 5). Glutamate levels

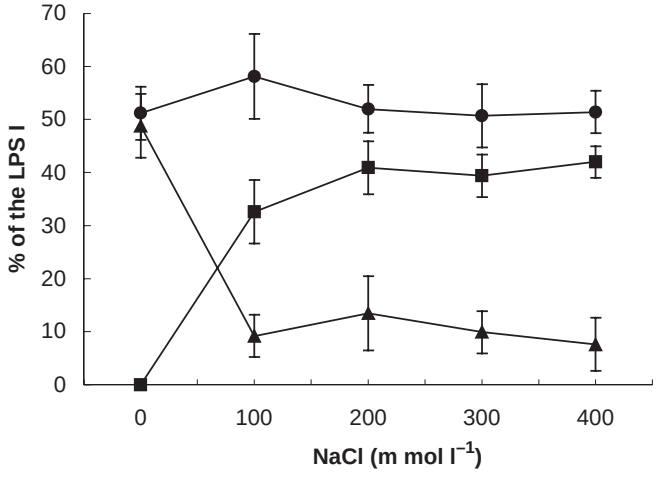


**Fig. 4** Percentage of the band in total LPSI in function of the NaCl added to the growth medium. (●), band 1; (■), band 2; (▲), band 3. The numbering of the bands is the same as that in the previous figure. Each point is the mean ± S.E. of three electrophoreses

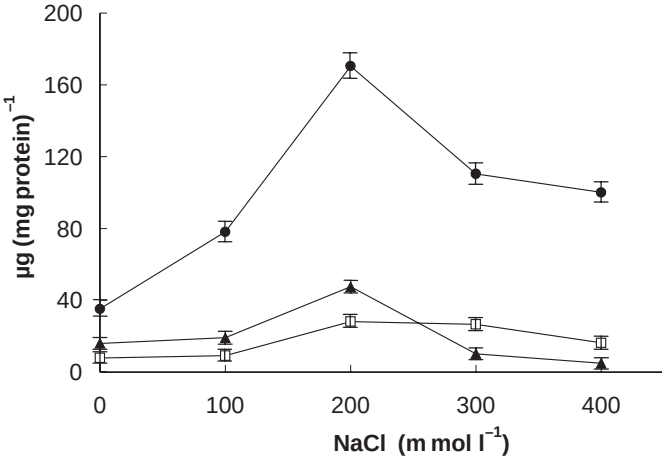


**Fig. 5** Total soluble amino acids (●), proline (▲) and glutamate (□) contents of *Mesorhizobium ciceri* strain ch-191 grown in YEM medium and incubated at 28 °C with different NaCl concentrations. Each point is the mean ± S.E. of four analyses

also rose with salinity, especially at 200 mmol $l^{-1}$ NaCl (Fig. 5), but fell at 300 mmol $l^{-1}$, as in the case of proline. In addition, at low and medium NaCl concentrations, proline levels were consistently higher than glutamate levels.

## DISCUSSION

*Mesorhizobium ciceri* strain ch-191 tolerates up to 200 mmol $l^{-1}$ NaCl (Fig. 1), although higher salt dosages limited growth. The lag phase of cell multiplication was greater with increasing salt, as indicated for other *Rhizobium*-nodulating *Cicer arietinum* (Zurayk *et al*. 1998). Nevertheless, ch-191 cells survived even in the presence of 300 and 400 mmol $l^{-1}$ NaCl. Based on earlier findings reported for chick-pea *Rhizobium* strains (Elsheikh and Wood 1989), ch-191 could be considered to be moderately salt tolerant, showing stable symbiotic properties under saline conditions (Soussi *et al*. 1998).

In media with 100 or 200 mmol $l^{-1}$ NaCl, the protein patterns in ch-191 did not change compared with control conditions (Fig. 2). However, at 300 mmol $l^{-1}$ NaCl, prominent qualitative and quantitative differences were detected, mainly the lack of some low-molecular-weight polypeptides and an increase in the relative abundance of several high-molecular-weight polypeptides. These findings agree with results reported for a *Sesbania*-nodulating *Rhizobium* strain (Natarajan *et al*. 1996). The shift towards high-molecular-weight polypeptides in protein patterns may be due to general protease inhibition (Saxena *et al*. 1996).

Rhizobial LPS are important for the interaction between bacteria and the legume host plant (Stacey *et al*. 1991). Successful infection and nodulation was dependent upon LPS integrity (Tao *et al*. 1992). However, little information is available on rhizobial LPS under salinity. In *M. ciceri* ch-191, densitometric traces of LPSI show that salt induces the disappearance of the slow migration bands and a reorganization of the faster bands. These changes in the LPS profile, being detected at lower NaCl dosages, could signify specific adaptive responses to salinity. However, the physiological meaning of these alterations is unclear. Zahran *et al*. (1994) found changes in LPSI only in sensitive strains or in moderately tolerant ones, whereas such changes were not evident in tolerant strains. By contrast, no alteration in LPS electrophoretic profile was detected in a salt-sensitive strain of *Rhizobium meliloti* (Lloret *et al*. 1995). Therefore, no direct relationship has been established between salt tolerance and alteration of the LPS.

Salt significantly increased the total amino acid pool in *M. ciceri* ch-191 (Fig. 5), as has been reported in other *Rhizobium* species (Yap and Lim 1983). It has been suggested that increases in total amino acids may be a consequence of protein degradation (Botsford and Lewis 1990), although the accumulation of high-molecular-weight polypeptides in *M. ciceri* ch-191 may suggest the opposite. The accumulation of glutamate in response to osmotic stress has been widely studied in *Rhizobium* (Yap and Lim 1983; Botsford and Lewis 1990; Miller and Wood 1996). This glutamate accumulation was related to stimulation of glutamate dehydrogenase activity by $K^+$ influx (Yap and Lim 1983), thereby neutralizing $Na^+$ accumulation promoted by salt stress. In the present study, the salt induced a marked accumulation of glutamate, and the content of this solute was highest at 200 mmol $l^{-1}$ (Fig. 5). Such increases in the intracellular glutamate pool of *M. ciceri* are not great in comparison with results for *Rhizobium meliloti* (Botsford and Lewis 1990; Fujihara and Yoneyama 1994). In addition, salt induced proline accumulation in *M. ciceri* strain ch-191, and as far as is known this is the first report of significant proline accumulation in a *Rhizobium* strain. However, the highest dosages of salt diminished proline levels, indicating that these compounds may contribute to osmoregulation within salinity ranges tolerated by *M. ciceri* strain ch-191.

In summary, the results presented here confirm that ch-191 is a moderately salt-tolerant strain. The protein profile showed major alterations at salinity levels which inhibited growth. On the other hand, the alterations in the LPS profile and accumulation of compatible solutes were evident from the lowest levels, suggesting that these changes may constitute adaptive responses to salt, allowing normal growth. In *M. ciceri*, in response to salt stress, the accumulation of proline surpasses that of glutamate.

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