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Salt Tolerance in *Astragalus cicer* Microsymbionts: The Role of Glycine Betaine in Osmoprotection

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Abstract In this study, we have investigated intrinsic salt tolerance of *Astragalus cicer* microsymbionts (USDA3350, ACMP18) and the role of exogenous glycine betaine in osmoprotection in these bacteria. Salt stress was imposed by NaCl concentrations ranging from 0.5 to 2 %. *A. cicer* mesorhizobia were capable of tolerating up to 2 % sodium chloride with a population count that was inversely proportional to the salt content. When the extracellular concentration of NaCl was raised to 2 %, the generation time of the UDSA3350 strain in the mid-exponential phase of growth was 3.9-times greater than that in the no-salt control medium, whereas the ACMP18 strain survived under the same conditions but did not multiply. Application of 1 mM glycine betaine into the salt-stressed rhizobium cultures increased the number of culturable bacteria, pointing out that this molecule was involved in restoration of osmotic balance. The decline in *A. cicer* symbiont viability in the medium with sodium chloride and the osmoprotective role of glycine betaine for these bacteria was confirmed in the experiment using the live/dead Bac Light Bacterial Vibility Kit. Data presented in this study showed the presence of *proU*-like genes in the genomes of *A. cicer* rhizobia with high sequence similarity to the genes of the ProU-like system in *Sinorhizobium meliloti* and the *proU* operon of *Escherichia coli.*

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Introduction

Soil bacteria called rhizobia are frequently exposed to osmotic stress that adversely affects their metabolic activity and, in consequence, the growth of bacteria is generally strongly reduced. Rhizobia exhibit significant differences in salt tolerance [14–16, 47]. While strains of the genera *Rhizobium* and *Bradyrhizobium* are inhibited by 100–300 mM NaCl, *Sinorhizobium meliloti* strains grow at salt concentrations higher than 300 mM. The studies of salt tolerance in bacteria of the *Mesorhizobium* genus showed that most of these strains were more sensitive to salt stress than *Rhizobium* and *Sinorhizobium* strains but more resistant than bacteria of the *Bradyrhizobium* genus. The growth of most mesorhizobial strains was inhibited by 100 mM NaCl with some exceptions, e.g., *Mesorhizobium thiogangenicum* and *Mesorhizobium ciceri* (they tolerated up to 200 mM NaCl) [8, 23]. To maintain the osmotic equilibrium between the cytoplasm and the saline habitat, rhizobia accumulate high levels of small, highly water-soluble organic compounds termed compatible solutes, i.e., proline, glutamate, glycine betaine, and trehalose [7, 9 , 11, 18, 29, 38, 46]. Such solutes, which do not have a detrimental effect on vital processes in stressed cells and function as osmoprotectants, stabilize enzymes, DNA, membranes, and whole cells against different stresses [10]. Compatible solutes are released into the ecosystem by primary microbial producers upon dilution stress, decaying plant and animal cells, and as excretion fluids of mammals (urine) [34]. A very important compatible solute for most rhizobia is glycine betaine [3]. In the best osmotically characterized rhizobia, *S. meliloti*, glycine betaine can be taken up from the environment or synthesized from choline or choline-*O*-sulfate [27, 35]. Glycine betaine transport systems in *S. meliloti* are poorly understood; however, it was found that their activity is strongly stimulated when bacteria are grown at elevated

osmolarity [4, 6]. One of these transporters is a constitutively expressed BetS system, rapidly posttranslationally activated by high medium osmolarity, which transports glycine betaine and proline betaine into stressed cells [1, 6]. In *S. meliloti,* ProU-like systems Cho and Hut were identified. It is interesting that HutXVW proteins showed higher similarity to ProU transporter proteins than to the *Escherichia coli* histidine transport protein, although the Hut system is involved in histidine but not glycine betaine uptake. The Cho system showed homology to the *proU* glycine betaine transport system but it is engaged in choline rather than glycine betaine uptake [1, 5, 13, 17]. In *E. coli,* two transporter systems, ProP and ProU, are primarily responsible for the import of glycine betaine [10, 22, 32, 26, 45]. ProP is a single-component transport system, regulated mainly at the activity level, which functions as an H^+ symporter and imports a wide variety of compatible solutes related to proline and glycine betaine as well as ectoine. ProU is a high-affinity ATP-binding transport system regulated at the level of both transcription and activity. A sudden osmotic upshock results in a rapid increase in *proU* operon transcription which is proportional to the osmolarity level of the growth medium, and expression of *proU* operon genes is kept at an elevated level as long as the osmotic stimulus exists. The ProU transporter, a member of the ABC superfamily, consists of three proteins: ProV, ProW, and ProX. ProV is a protein associated with the cytoplasmic side of the membrane and shares considerable sequence identity with the ATP-binding protein. ProW is a transmembrane component of the transport system, whereas ProX is the periplasmic protein binding glycine betaine.

Bacterial cells subjected to a number adverse environmental stresses (high salt concentration, O_2 limitation, temperature and pH variations, nutrient deficiencies) can die, exist in a metabolically active state, and proliferate or survive as "dormant" cells called a viable but nonculturable (VBNC) with reduced metabolism and suppressed reproduction [2, 28, 39, 42]. Viable but nonculturable bacteria cannot be cultured on conventional laboratory growth media but it is possible to demonstrate whether they still alive using the live/dead BacLight bacterial viability kit (Life Technologies).

In the present study, we examined the tolerance of *Astragalus cicer* symbionts to salt stress and the possible role of glycine betaine in NaCl tolerance. We also analyzed whether hyperosmolarity induces the VBNC state in *A. cicer*-specific rhizobia.

Materials and Methods

Bacterial Strains and Media

Mesorhizobium sp. (*A. cicer*) strain USDA3350 from the culture collection of the United States Department of Agriculture (USDA) and strain ACMP18 from our collection [44] were used throughout this study. The rhizobia were isolated from *A. cicer* root nodules which were surface sterilized in 70 % ethanol (2 min) and next in 0.1 % $HgCl_2$ solution (2 min), and were rinsed in sterile water several times. The nodules were individually crushed, and the nodule suspension was streaked onto yeast mannitol agar (YEM) plates. The plates were incubated at 28 °C for 3–5 days. The purity of the bacteria was checked by repeated streaking on the YEM medium. The phenotypic characterization of ACMP18 and USDA3350 strains was presented in the paper by Wdowiak et al. [44]. The bacteria were maintained at 4 °C on the YEM rich medium [41]. Their osmotolerance phenotype and glycine betaine osmoprotective properties were determined in a modified YEM medium, in which mannitol was substituted by 1 % lactate and the yeast extract by 1 % NH_4NO_3. The osmotic strength of the medium was increased by NaCl addition to achieve final concentrations of 0.5, 1, 1.5, and 2 %. Glycine betaine used in these studies was prepared as a 0.5 M solution and sterilized by filtration before incorporation into the medium. Its final concentration in the medium was 1 mM for osmoprotection assays and 10 mM when used as sole carbon and nitrogen sources.

Culture Conditions for NaCl Tolerance Studies and Analysis of glycine Betaine Osmoprotective Properties

5 ml volumes of a modified YEM medium supplemented with different NaCl concentrations and with NaCl + 1 mM glycine betaine were prepared and inoculated with 0.5 ml of a late exponential phase culture of the bacteria studied. Cells were grown at 28 °C in an orbital shaker (160 rev min^{-1}). Growth was monitored for 72 h spectrophotometrically by optical density at 600 nm and by plate counts of cell numbers as colony-forming units (CFU) on the YEM medium at 24 h intervals. The generation time from the log phase of the bacterial growth was also determined. Additionally, the relative number of dead and living bacterial cells growing under the above-mentioned conditions was estimated using the staining live/dead BacLight Bacterial Viability Kit (Life Technologies). This kit consists of two nucleic acid stains: SYTO 9 which penetrates most membranes freely and propidium iodide which is highly charged and permeates only damaged membranes. Simultaneous application of both these dyes results in green fluorescence of viable cells with intact membranes, whereas dead cells with an injured membrane show intense red fluorescence. The rapid visualization and evaluation of the viability of cells were performed by the live/dead BacLight bacterial viability kit and a confocal laser

scanning microscope (Zeiss KLM150) in accordance with the manufacturer's instruction.

The growth of Rhizobia with Glycine Betaine as the Sole Carbon and Nitrogen Source

To determine the growth of rhizobia with glycine betaine as the sole carbon or nitrogen source, precultures from overnight bacterial cultures on the modified YEM medium were pelleted, resuspended in the YEM medium without carbon or nitrogen source, and used to inoculate (3 %) the same medium containing glycine betaine as a sole carbon or nitrogen source. The rhizobial growth was determined by monitoring the optical density at 600 nm for 72 h at 24 h intervals.

DNA Isolation

Genomic DNAs from 30 ml of 72 h bacterial liquid cultures in the YEM medium were prepared according to the method described by Pitcher et al. [31]. The purities and concentrations of DNAs were determined with the spectrophotometer (Bio-Rad, SmartSpec^{TM}3000).

PCR Amplification and Sequencing of Amplicons

PCRs were performed with the ReadyMixTM Taq kit and bacterial genomic DNA according to the manufacturer's instructions (Sigma-Aldrich). The 640-bp fragment of *proV* gene was amplified and sequenced with primer proVf (5'-TCATCATCATGGGCCTGTCGG-3') and reverse primer proVr (5'-CGGTCGACGTTGCGGGTGAAC-3'). To amplify and sequence the 450-bp fragment of the *proW* gene, primers proWf (5'-GCGCACAAGCCGAAGGTC-TAC -3' and proWr (5'-ACGATGGCCAGCACGACGA T-3') were used. The 718-bp fragment of the *proX* gene was amplified and sequenced using primers proXf (5'-GCGGCTCTGTTGTCCACGAG-3') and proXr (5'-TTG AACTTCAGGTTGGCGATG-3'). The PCR protocols for the *proV, proW*, and *proX* genes consisted of an initial denaturation step at 95 °C for 2 min followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 52 °C for 1 min, extension at 72 °C for 2 min, and final extension at 72 °C for 5 min. The amplification products were purified with the PCR purification Clean up kit (A&A Biotechnology) as recommended by the manufacturer and next they were sequenced using the ABI Prism BigDye Terminator Cycle sequence kit (Applied Biosystems model 310 DNA sequencer).

The nucleotide sequences determined in this study have been deposited in the GenBank database under the following accession numbers: KC155290 and KC155291 for the *proV* genes of USDA3350 and ACMP18, respectively; KC155293 and KC155292 for the *proW* genes of USDA3350 and ACMP18, respectively; and KC155294 and KC155295 for the *proX* genes of USDA3350 and ACMP18, respectively.

Results

Growth Characteristics Under Saline Conditions and Osmoprotection By Glycine Betaine

The intrinstic osmotolerance in two *Mesorhizobium* sp. (*A. cicer*) strains USDA3350 and ACMP18 was evaluated by their growth in the medium with five different NaCl concentrations (0, 0.5, 1, 1.5, and 2 %). This was done by measuring absorbance at 600 nm and the cell titer for 72 h at 12 and 24 h intervals. In the absence of NaCl (control medium), the strains studied showed different growth characteristics, i.e., the generation time of the ACMP18 strain (in the middle of the exponential growth phase) was about half an hour longer (5.20 h) than that of the USDA3350 strain (4.70 h), and the final bacterial yield was higher in the case of USDA3350 rhizobia (Table 1). However, both strains reached the stationary phase at the same time i.e., after 48 h. The *A. cicer* microsymbionts survived the entire period of cultivation in the medium with all four sodium chloride concentrations. It was found that the recovered viable count of the USDA3350 bacteria growing for 72 h in the presence of 0.5, 1, 1.5, and 2 % increased about 52-, 22-, 11-, and 5-fold, respectively, compared to the ~70-fold increase in the bacterial count in the no-salt control YEM medium. In the case of the ACMP18 strain growing over 72 h period in the presence of 0.5, 1, and 1.5 % NaCl, the culturable cell number increased ~37-, ~15-, and ~7-fold, respectively. In turn, the number of CFU ml^{-1} recovered after 72 h of bacterium incubation in the medium with 2 % salt remained at the same level as at the beginning of the experiment. It is noteworthy that the increase in the number of CFU ml^{-1} of the ACMP18 strain growing in the no-salt control medium from time 0 to 72 h was only 58-fold. Furthermore, the doubling times for both studied strains growing in the presence of sodium chloride was significantly longer compared to that in the no-salt control medium. In the case of the USDA3350 strain, the generation time of bacteria cultivated in the medium with 1, 1.5, and 2 % NaCl was 1.8-, 2.7-, and 3.9-times greater, respectively, than in the control medium. The reduction in the growth rate under conditions of salt stress was much more pronounced for the ACMP18 strain. Its generation time in the YEM medium without salt was 5.20 h and increased to 10.20 h at 1 % NaCl and 22.9 h in the medium with 2 % sodium chloride (Table 1). In the presence of 2 % NaCl, the bacteria

survived but did not multiply. To determine the effect of glycine betaine in relieving salt stress in *Mesorhizobium* sp. (*A. cicer*) strains USDA3350 and ACMP18, the bacteria were cultivated in the modified YEM medium containing 0, 0.5, 1, 1.5, and 2 % NaCl as well as under the same conditions plus the osmoprotectant supplied at a final concentration of 1 mM (Table 1). Addition of exogenous GB to the salt-containing medium had a beneficial effect on the growth of both strains and led to an increase in the growth rate and culturable cell number (data not shown) in comparison to the medium without the osmoprotectant; however, their growth was only partially recovered. For example, the addition of 1 mM of GB to the USDA3350 culture growing in the presence of 1.5 and 2 % NaCl reduced the doubling time of bacteria from 12.6 to 10.9 h and from 18.5 to 15.6 h, respectively. Incorporation of 1 mM GB to the culture medium with 1.5 % NaCl reduced the generation time of the ACMP18 strain from 16.0 to 13.3 h. The addition of 1 mM GB to the medium with 2 % NaCl decreased the doubling time of this strain from 22.9 to 18.5 h. The beneficial effect of GB on the growth rate of USDA3350 and ACMP18 under elevated salinity was less pronounced in the presence of 0.5 and 1 % NaCl.

Live/Dead Analysis

The live/dead Bac Light Bacterial Viability Kit was used to determine the relative proportions of viable and dead USDA3350 and ACMP18 bacterial cells under salt stress conditions (0.5, 1, 1.5, and 2 % NaCl) and in the salt solution plus glycine betaine as an osmoprotectant. In this assay containing two fluorescent nucleic acid stains, bacteria with intact membranes stain fluorescent green, whereas cells with damaged membranes (dead bacteria) stain fluorescent red. The percentage of live USDA3350 cells recovered after 72 h incubation at 0.5, 1, and 1.5 % NaCl in the medium with and without 1 mM glycine betaine remained at similar levels. However, the number of CFU/mL in the medium with NaCl but without GB in relation to the control clearly decreased and significantly increased after GB addition (data not shown). At the strong growth-inhibiting 2 % NaCl concentration, the percentage of dead cells clearly increased. The presence of GB in the medium with 2 % sodium chloride significantly elevated the percentage of viable cells in comparison to the percentage of live cells in the medium with 2 % NaCl but without glycine betaine (Fig. 1). The fluorescent dye treatment of the ACMP18 strain growing for 72 h in the presence of 1, 1.5, and 2 % NaCl showed that the percentage of viable cells declined gradually with the increasing concentrations of salt. Incorporation of GB to these growth media clearly alleviated viability inhibition by NaCl and clear improvement of the viable cell fraction in the presence of this osmoprotectant (5–11 %) was found. In the presence of 0.5 % NaCl and the non-salt control, the percentage of live ACMP18 cells (fluorescent green) was similar to that of the USDA3350 strain (Fig. 1).

Glycine Betaine as Sole Carbon and/or Nitrogen Sources

To test whether the *Mesorhizobium* sp. (*A. cicer*) strains USDA3350 and ACMP18 can catabolize GB and use it as potential carbon and nitrogen sources at low and elevated

Table 1 Effects of salt stress on the growth of *A. cicer* microsymbionts in the YEM modified medium with different NaCl concentrations with and without glycine betaine GB (1 mM)

Strain	Salt (%)	Generation time (h)		Yield[a]					
				24 h		48 h		72 h	
		−GB[b]	+GB	−GB	+GB	−GB	+GB	−GB	+GB
ACMP18	0	5.25	5.6	100	100	100	100	100	100
	0.5	6.9	6.7	66.2	92.2	51.18	75.0	50.0	72.9
	1	10.2	9.1	52.6	63.4	25.6	38.9	23.4	37.2
	1.5	16.0	13.3	39.9	52.8	13.4	22.9	12.0	20.1
	2	22.9	18.5	22.6	28.9	5.3	8.4	4.9	7.8
USDA3350	0	4.7	4.6	100	100	100	100	100	100
	0.5	5.8	5.3	75.9	80.5	65.6	91.3	62.0	98.4
	1	8.4	7.1	47.7	74.9	26.0	52.2	24.5	49.5
	1.5	12.6	10.9	35.8	43.8	14.3	23.8	13.4	22.4
	2	18.5	15.6	24.0	27.4	7.4	10.6	6.58	9.6

[a] The yield is the cell density (optical density at 600 nm) reached at the stationary phase. A value of 100 % corresponds to the optical density obtained with cells grown in the absence of salt

[b] Effect of 1 mM glycine betaine (GB) on the growth of rhizobial species in the YEM modified medium without or with NaCl

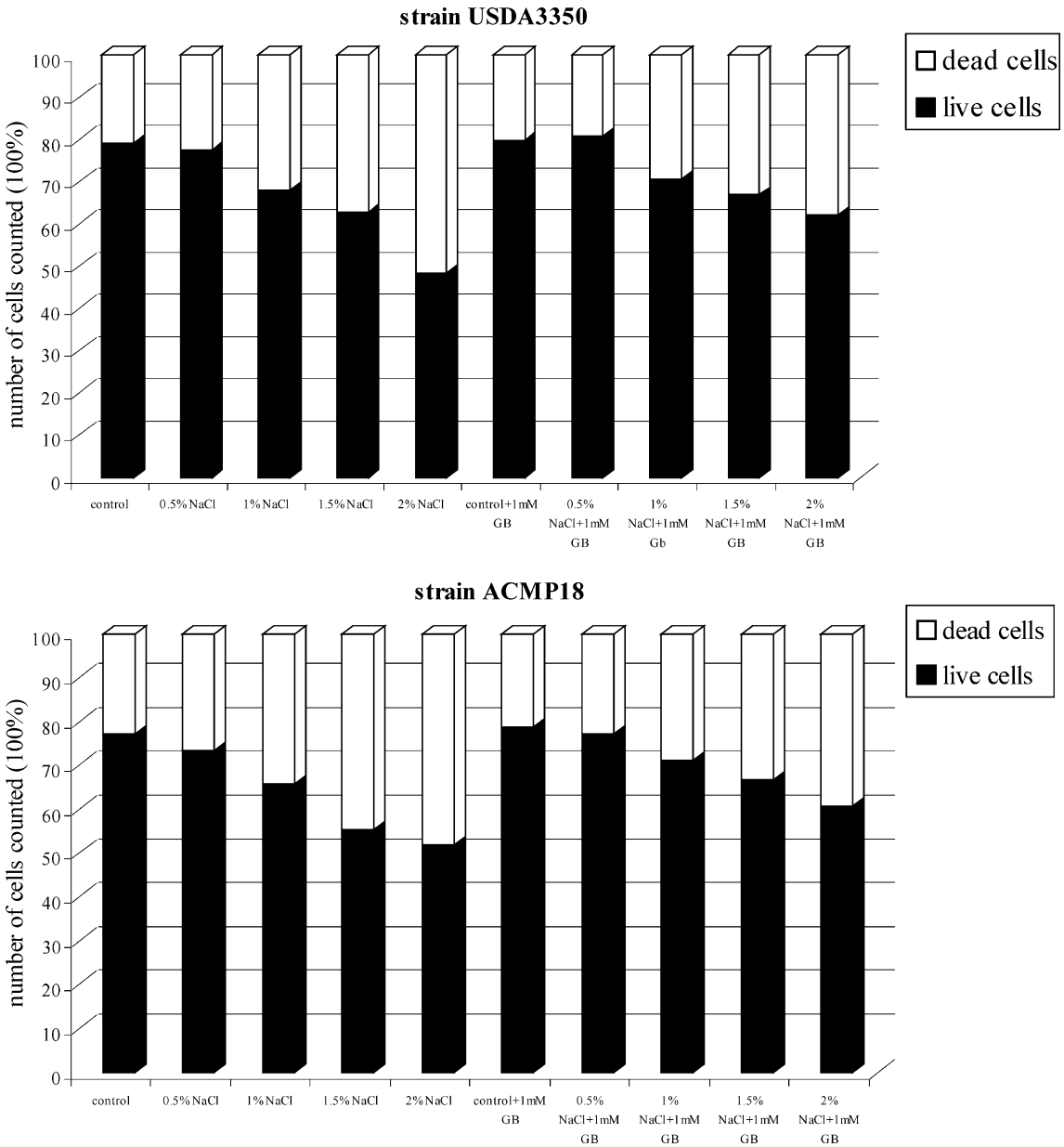

Fig. 1 Percentage of live/dead cells of *Mesorhizobium* sp. (*A. cicer*) ACMP18 and USDA3350 strains during growth on the modified YEM medium without and with NaCl and 1 mM glycine betaine (GB) determined by microscopic enumeration with live/dead BacLight Bacterial Viability Kit. Standard deviations never exceeded 10 %

osmolarities, the growth of bacteria was monitored in the presence of 10 mM GB added to the carbon- and nitrogen-free YEM medium with and without NaCl. As shown in Fig. 2, both strains were able to assimilate GB at low osmolarity and grow in such a medium at a rate comparable to that in the YEM medium. In osmotically stressed *A. cicer* mesorhizobia, glycine betaine was not used as a carbon and nitrogen nutrient and the growth of the USDA3350 as well as ACMP18 strains was not supported by this compatible solute (data not shown).

Identification of *proU* Operon Genes

The PCR strategy was used to identify *proU* operon genes encoding a high-affinity glycine betaine transporter of *E. coli* in the *Mesorhizobium* sp. (*A. cicer*) strains USD A3350 and ACMP18. PCR amplification and sequencing of DNA with primers specific for the *proX* gene provided, in the case of two *A. cicer* microsymbionts, reliable 718-bp gene sequences sharing 41–42 % identical sequences with the *E. coli proX* gene and 92–91 % identical

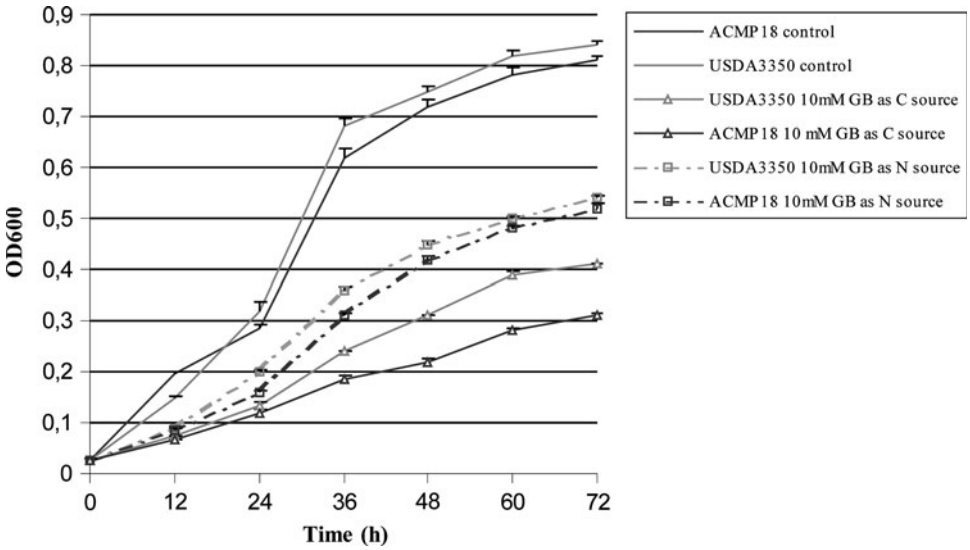

Fig. 2 Growth curves of *A. cicer* ACMP18 and USDA3350 microsymbionts in the medium with 10 mM glycine betaine (GB) as a sole carbon or nitrogen source. Growth was measured by absorbance readings at 600 nm. The standard error of the means is less than 5 %

nucleotides with the *proX*-like gene of *Mesorhizobium loti* MAFF303099 (Table 2). The *proX*-like gene *hutX* of *S. meliloti* 1021 showed 68–69 % sequences identical to the *proX* sequences of *A. cicer* nodulators. PCR amplification and sequencing of DNA with specific primers was also used to identify *proV*- and *proW*-like genes of *E. coli* in *A. cicer* symbionts. 643-bp *proV* gene amplicons of the USDA3350 and ACMP18 strains, with 95 % sequence identity to each other, shared 58–60 % identical nucleotides with the *E. coli proV* gene, 90–92 % sequence identity with the *proV*-putative gene of *M. loti* MAFF303099, and 68 % sequence similarity with the *proV*-like *hutV* gene of *S. meliloti* 1021 (Table 2). It was also found that the 450-bp fragments of *proW* genes of the *A. cicer* rhizobia, with 98 % sequence identity to each other, displayed 54–55 % sequence similarity to the *proW* gene of *E. coli*, 73 % identical nucleotides with the *proW*-like *hutW* gene of *S. meliloti* 1021, and many more, i.e., 89–90 %, identical nucleotides with the *proW*-like gene of *M. loti* MAFF303099 (Table 2).

Table 2 Similarity values for the *proV*, *proX*, and *proW* gene sequences of *Mesorhizobium* sp. (*Astragalus cicer*) rhizobia and reference strains

		(%) similarity to:	
Gene	Strain	ACMP 18	USDA3350
proV	ACMP18 (KC155291)[a]	100 %	95 %
	USDA3350 (KC155290)	95 %	100 %
	M. loti MAFF303099 (BA000012)	92 %	90 %
	S. meliloti 1021 (AL591688)	68 %	68 %
	E. coli O103:H2 (AP010958)	60 %	58 %
	Rhizobium leguminosarum bv. *viciae* 3841 (NC_008381)	64 %	65 %
proX	ACMP18 (KC155295)	100 %	97 %
	USDA3350 (KC155294)	97 %	100 %
	M. loti MAFF303099 (BA000012)	92 %	91 %
	S. meliloti 1021 (AL591688)	69 %	68 %
	E. coli O103:H2 (AP010958)	42 %	41 %
	Rhizobium leguminosarum bv. *viciae* 3841 (NC_008381)	48 %	48 %
proW	ACMP18 (KC155292)	100 %	98 %
	USDA3350 (KC155293)	98 %	100 %
	M. loti MAFF303099 (BA000012)	90 %	89 %
	S. meliloti 1021 (AL591688)	73 %	73 %
	E. coli O103:H2 (AP010958)	54 %	55 %
	Rhizobium leguminosarum bv. *viciae* 3841 (NC_008381)	65 %	64 %

[a] GenBank accession numbers are shown in parentheses

Discussion

As soil bacteria, Rhizobia frequently cope with high osmolarity of the environment. As the osmolarity of the surrounding environment increases, the turgor pressure drops because of cytoplasm dehydration, metabolic processes are inhibited, and bacterial growth slows down or is halted [28, 40, 43, 48]. Rhizobia differ in their tolerance to salinity. Generally, fast-growing rhizobia are more salt-tolerant than slow-growing *Bradyrhizobium* strains [15, 24, 33, 36, 49]. To present day, *A. cicer* microsymbionts have not been studied in terms of NaCl stress, although it is known that in nature elevated osmolarity affects both plant and bacterium growth as well as legume-rhizobium symbiosis. To characterize the intrinsic NaCl tolerance of *A. cicer* symbionts, two strains, USDA3350 from Canada and ACMP18 from Poland, were chosen based on our previous work, and studied in detail for their growth rate in the YEM medium with NaCl concentrations ranging from 0.5 to 2 %. Both strains were able to withstand even 2 %

sodium chloride over 72 h. The ACMP18 strain was more sensitive to salt than USDA3350 (Table 1). It only survived in the YEM medium with 2 % NaCl and the titer of these bacteria was maintained under these conditions at a similar level within 72 h. The USDA3350 strain withstands the presence of NaCl in the culture medium better and its growth parameters (the generation time, growth rate) were less affected by sodium chloride. It was found that multiplication of USDA3350 bacteria occurred even in the presence of 2 % NaCl. Evaluation of salinity resistance of the *Mesorhizobium* USDA3350 and ACMP18 strains forming symbiosis with *A. cicer* allowed us to classify them as moderately NaCl-tolerant bacteria.

Rhizobia exposed to increased salinity defend themselves against the outflow of water from the cells by accumulation of organic osmolytes called compatible compounds [7, 18, 19, 24]. These highly water-soluble osmoprotectants can be accumulated to very high levels in stressed cells without disturbing vital cellular functions [3, 4, 25, 34]. It has been shown that glycine betaine is a very potent osmoprotectant in many bacteria including rhizobia [4, 29]. Its accumulation not only allows the cells to withstand a given osmolarity but also permits the cell to proliferate under unfavourable conditions. The growth of *A. cicer* mesorhizobia inhibited in the presence of 0.5–2 % NaCl was clearly stimulated by the addition of 1 mM glycine betaine to the culture medium (Table 1). The beneficial effect of glycine betaine on the growth of *A. cicer* rhizobia in the elevated osmolarity medium was particularly pronounced in the 48–72 h stationary phase culture.

The rapid decline in the culturability (CFU) of the ACPM18 and USDA3350 strains after 72 h incubation in the salted medium was also supported by the live/dead *Bac* Light Bacterial Viability Kit (Fig. 1). This assay with two fluorescent nucleic acid dyes differentiated fluorescent green viable cells from dead bacteria which stain with red fluorescent dye. The decrease in the culturability of the *A. cicer* symbionts that occurred in response to the addition of NaCl to the medium can be due to cell death or entry of the bacteria into the viable but nonculturable state (VBNC). In the medium with sodium chloride and glycine betaine, the number of metabolically active ACMP18 cells able to produce colonies and the proportion of viable to dead cells estimated as a percentage of green versus red fluorescent cells increased in comparison to these parameters in the salt-supplemented medium without the osmoprotectant. We conclude from these data that the compatible solute glycine betaine really protects ACMP18 strain cells from the detrimental effect of salt stress. It is interesting that the live/dead viability staining of USDA3350 cells growing at 0.5, 1, and 1.5 % NaCl with and without glycine betaine showed a comparable ratio of live to dead cells under both conditions. However, the number of colony-forming cells in the salt medium with the osmoprotectant was larger than that in the medium without glycine betaine (data not shown). We suppose that in the salt medium part of live USDA3350 bacterial cells are culturable and form colonies on a stable medium but part of this population enter the viable but nonculturable state. In the presence of the osmoprotectant glycine betaine, metabolically active but nonculturable cells of the USDA3350 strain affected by salt stress resuscitate to bacterial colony formation. Since elevated NaCl concentrations in the culture medium can induce the viable but nonculturable state in rhizobia, we can suppose that such bacteria exist under soil adverse conditions. The capability of rhizobia to enter the VBNC state can be an advantage for their survival in soil under stress conditions and further studies are needed to elucidate the effect of this metabolic shift on rhizobium-legume symbiosis.

Depending on their growth medium osmolarity, *A. cicer* rhizobia, like *S. meliloti*, use glycine betaine as an osmolite or carbon and nitrogen source [3, 20, 29, 30, 35, 37]. In unstressed cells of the ACMP18 and USDA3350 strains, glycine betaine was catabolized and used as a nitrogen or carbon nutrient (Fig. 2). Under osmotic stress, glycine betaine was an effective osmoprotectant but not a nitrogen and carbon source (data not shown). The osmotic strength of the environment can modulate the pathway of glycine betaine metabolism [35]. It was found that in low-salt grown *S. meliloti* cells, glycine betaine stimulated the enzymes involved in the catabolism of glycine betaine into serine and then to pyruvate. Under high-osmolarity growth conditions, the activities of these enzymes are inhibited and glycine betaine is accumulated in the cytoplasm as an osmoprotectant. The ability to accumulate and catabolize glycine betaine, liberated into the environment from plant and animal residues, may be beneficial to bacteria and allows them to survive as well as thrive in natural habitats.

The mechanism of the glycine betaine transport into the *A. cicer* symbiont cells is not known and no genes have been attributed to this function. As shown from the fully annotated nucleotide sequences of the *M. loti* MAFF30 3099 genome, the genes encoding transporter-related proteins constitute 10.5 % of the total DNA. The data presented in this paper show that the *Mesorhizobium* sp. (*A.cicer*) USDA3350 and ACMP18 strains have *proX, proW,* and *proV*-like genes which encode a high-affinity glycine betaine ProU uptake system of *E. coli* [2, 12, 21, 22, 26]. The ProU transporter consists of a periplasmic substrate binding protein ProX, a transmembrane component ProW, and an inner membrane-associated ATPase protein ProV. Comparison of *proX, proW,* and *proV* gene sequences of *A. cicer* symbionts with those in the data libraries revealed their highest sequence similarity with

genes encoding components of the ProU-like glycine betaine system of *M. loti* (89–92 %). Lower identity scores were noted between the nucleotide sequences of *proX, proW,* and *proV* genes of the USDA3350 and ACMP18 strains and those of *S. meliloti* (68–73 %) as well as *E. coli proU* operon sequences (41–60 %). Based on the annotated sequences in the *A. cicer* symbionts studied, the presence of a potential glycine betaine ProU-like transporter that allows bacteria to cope with salt stress was revealed.

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