European Journal of Soil Biology 76 (2016) 46–52

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi

Original article

Effect of osmotic stress on *Astragalus cicer* microsymbiont growth and survival

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ARTICLE INFO

Article history:
Received 16 May 2016
Received in revised form
6 July 2016
Accepted 26 July 2016
Available online 3 August 2016

Handling Editor: C.C. Tebbe

Keywords:
Osmotic stress
Rhizobia
Glycine betaine
Biofilm

ABSTRACT

In this study, the effect of various concentrations of stress inductors such as KCl, sucrose, and polyethylene glycol (PEG) on the growth and survival of *Astragalus cicer* microsymbionts in the presence and absence of an osmoprotectant, glycine betaine (GB), was determined. It was found that the higher concentrations of the stress factors lengthened the generation time and, additionally, in the case of sucrose and PEG, caused entry of the cells into the viable but nonculturable state (VBNC). The presence of the osmoprotectant, glycine betaine (GB), significantly decreased the effects of stress induced by KCl, sucrose, and PEG, as indicated by reduction of the generation time of *A. cicer* isolates, an increase in the proportion of living to dead cells in comparison to bacteria under stress conditions but in the absence of GB. We also investigated the ability of *A. cicer* symbionts to form biofilm in the different stress conditions. It was demonstrated that PEG enhanced biofilm formation, whereas KCl and sucrose at higher concentrations negatively affected this process.

1. Introduction

Drought, soil salinity and other osmotic stresses are important environmental factors affecting the productivity and sustainability of agriculture and whole natural environment. Osmotic stress in the soil influences the growth and survival of bacteria and root colonisation by rhizobia, inhibits infection of fabaceans, nodule development, and symbiotic nitrogen fixation, and decreases relative water content (RWC) and plant growth [1,2].

Microorganisms in the natural environment are constantly exposed to stress factors such as osmotic stress, extreme temperature, pH changes, heavy metals, biocides, and parasites. Some microorganisms possess an ability to survive and function in severe environments. In response to stress, bacteria can modulate the composition of the cell envelope or accumulate compatible solutes such as amino acids (glutamate, glutamine, proline), amino acid derivatives (betaines, peptides, N-acetylated amino acids), polyols, and sugars (trehalose, sucrose) [3,4]. Under osmotic stress, the accumulation of compatible solutes may occur in two ways. Firstly, by altering their metabolism (increased biosynthesis or/and decreased degradation of osmoprotectants) and, secondly, by transport mediated by specific transporters (increased uptake or/and decreased export of osmoprotectants). Under osmotic stress, compatible solutes are accumulated at a high concentration in the cell cytoplasm. It has been demonstrated that they take part in the maintenance of cell turgor and protect cellular structures against damage caused by ions or dehydratation [4–6].

Glycine betaine (*N,N,N*-trimethyl glycine; GB) is a major osmoprotectant in bacteria, algae, plants, and animals. It has been established that many microorganisms cannot synthesise glycine betaine and they take up GB from the medium during osmotic stress [4,5,7].

Rhizobia are generally more tolerant to salt and other osmotic stresses than their host plants and the use of osmotic tolerant soil bacteria as inoculants can greatly improve biological nitrogen fixation under stress conditions.

The aim of this study was to determine the survival of milkvetch symbionts in stress conditions induced by KCl, PEG, and sucrose and to determine the osmoprotective effects of glycine betaine on the growth of these bacteria under stress conditions.

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http://dx.doi.org/10.1016/j.ejsobi.2016.07.005
1164-5563/

2. Materials and method

2.1. Bacterial strain and media

Mesorhizobium sp. (*Astragalus cicer*) strain USDA3350 from the culture collection of the United States Department of Agriculture (USDA) and strain ACM18 from our collection [8] were used in this study. Bacteria were maintained at 4 °C on the Yeast Extract Mannitol (YEM) medium [9]. Their osmotolerance phenotype and glycine betaine osmoprotective properties were determined in a modified YEM medium, in which mannitol was substituted by 1% lactate (v/v) and yeast extract by 1% NH_4NO_3 (w/v). The YEM medium was prepared with different final concentrations of potassium chloride (0.5%; 1%; 1.5%; 2%; w/v), sucrose (5%; 10%; 15%; 30% w/v), and polyethylene glycol-PEG (9%; 17%; 24%; 30% w/v). Glycine betaine used in these studies was prepared as a 0.5 M solution and sterilised by filtration before addition into the medium. The final concentration of GB in the medium for osmoprotection assays was 1 mM. The control bacteria were cultivated in the modified YEM medium (as described above).

2.2. Culture conditions for KCl, sucrose, PEG tolerance studies and analysis of glycine betaine osmoprotective properties

5-ml volumes of the modified YEM medium supplemented with different KCl (0.5%; 1%; 1.5%; 2%), sucrose (5%; 10%; 15%; 30%), and PEG (9%; 17%; 24%; 30%) concentrations (w/v) and with/without 1 mM glycine betaine were prepared and inoculated with 0.5 ml of an exponential phase culture of the bacteria studied. Cells were grown at 28 °C in an orbital shaker (160 rev min^{-1}). Growth was monitored spectrophotometrically for 72 h by optical density (OD) at 600 nm and by counts of the cell number 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.

2.3. DEAD/LIVE cell enumeration

The analysis of the relative number of dead and living bacterial cells growing in the modified YEM medium supplemented with different KCl (0.5%; 1%; 1.5%; 2%), sucrose (5%; 10%; 15%; 30%), and PEG (9%; 17%; 24%; 30%) concentrations (w/v), in the presence and absence of 1 mM glycine betaine, was carried out using the LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies) and a confocal laser scanning microscope (Zeiss KLM150) according to manufacturer's instruction as described earlier [10].

2.4. HPLC analysis of the amino acid pool

Cultures of the studied bacteria were grown to the logarithmic phase in the modified YEM medium in the presence of different KCl (0.5%, 1%, 1.5%, 2%), sucrose (5%, 10%, 15%) and PEG (9%, 17%) concentrations (w/v) with and without 1 mM (w/v) of the osmoprotectant, glycine betaine. Next, the bacterial cells were harvested by centrifugation at 15 000*g* for 15 min and pellets of freshly harvested cells were suspended in 80% (v/v) ethanol and stirred for 30 min at 85 °C. Next, the bacteria were centrifuged and the supernatants (ethanol-soluble fractions) were evaporated to dryness at 40 °C and dissolved in distilled water. The samples were filtered using a sterile syringe filter with a pore size of 0.2 μm (Millipore) and stored at −20 °C until needed for further analysis.

Qualitative and quantitative analysis of the amino acid pool were performed using LC/MS system from Finnigan (LCQ Advantage Max) equipped with the ion-trap mass spectrometric system (ThermoElectron Corporation, San Jose, CA) - LC/ESI/IT/MS. 100 mm × 4.6 mm i.d., 3 μm, Gemini C18 column from Phenomenex (Torrance, CA, USA) was applied.

Chromatographic separation was performed using mobile phase composed of solvent A (95%) and solvent B (5%). Solvent A was 25 mM formic acid in water and solvent B was 25 mM formic acid in acetonitrile. The mobile phase flow rate was 0.5 mL/min. The column effluent was ionised by electrospray (ESI). The ESI needle potential was 4.5 kV in the positive ionisation mode.

For qualitative and quantitative analysis, the following target ions for amino acid analysis by Single Ion Monitoring (SIM) were selected: 76 *m/z* – glycine, 90 *m/z* – alanine, 116 *m/z* - proline, 147 *m/z* – lysine and glutamine, 148 *m/z* – glutamic acid and 175 *m/z* – arginine.

The amounts of amino acids in the examined samples were calculated using the calibration curve of their standards.

2.5. Biofilm formation

To analyse the biofilm formation by the studied bacteria under various osmotic stress conditions, the method described by Rinaudi and Gonzales [11] was used. *Astragalus cicer* symbionts were grown in RDM medium [9] supplemented with different concentrations of KCl (0.5%, 1%, 2%), sucrose (5%, 10%, 15%, 30%), and PEG (9%, 17%, 24%, 30%) at 28 °C for 48 h. Next, they were resuspended in the same media to OD_{600} = 0.2 and 100 μl of bacterial cultures were added into polystyrene microplate wells. 100 μl RDM medium was the control. The microplate was incubated with agitation (100 rpm) at 28 °C for 48 h. After this time, the OD_{600} was read in a BioRad Microtiter Plate reader and the medium was removed from the wells. The wells were washed three times with 150 μl of 0.85%NaCl. Next, the biofilms were stained with 0.01% crystal violet for 20 min. After this time, the wells were washed three times with sterile water. Crystal violet that stained the biofilm was solubilised with 95% ethanol and the amount of the dye was quantified by measuring the absorbance at 560 nm.

2.6. Statistical analysis

Each experiment was performed in duplicate. The results were statistically analysed using Statistica (StatSoft, Poland). Data were analysed with two-way factorial ANOVA tests. The statistical significance was set at the $p < 0.05$ confidence level.

3. Results

3.1. Amino acid pool

In this study, the size of the amino acid pool in *A. cicer* symbionts growing in the presence of stress factors with and without the osmoprotectant, glycine betaine (1 mM) was determined by HPLC analysis. Osmotic stress significantly increased the total amino acid pool in the milkvetch nodule isolates.

The results showed that glutamate, alanine, and glycine were present in significant quantities in bacterial cells stressed by sucrose and PEG. Relative to control bacteria grown in the absence of factors causing osmotic stress, the level of glutamate in the bacterial cells grown at 15% sucrose and 17% PEG increased approx. 33-fold and 58.7-fold, respectively. Addition of glycine betaine to the medium with 15% sucrose and 17% PEG caused reduction of the glutamate level compared to bacteria grown in the media without the osmoprotectant. In the medium with GB, a 26-fold and 48-fold increase in glutamate was observed, compared to the control medium. With the increase in the sucrose concentration in medium, the level of alanine in bacterial cells increased and in the presence of 15% sucrose it reached a value ca. 47-fold greater in the medium without GB and 26.9-fold greater in the medium with the