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Effect of arsenic on tolerance mechanisms of two plant growth-promoting bacteria used as biological inoculants

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ABSTRACT

Bacterial ability to colonize the rhizosphere of plants in arsenic (As) contaminated soils is highly important for symbiotic and free-living plant growth-promoting rhizobacteria (PGPR) used as inoculants, since they can contribute to enhance plant As tolerance and limit metalloid uptake by plants. The aim of this work was to study the effect of As on growth, exopolysaccharide (EPS) production, biofilm formation and motility of two strains used as soybean inoculants, *Bradyrhizobium japonicum* E109 and *Azospirillum brasilense* Az39. The metabolism of arsenate (As(V)) and arsenite (As(III)) and their removal and/or possible accumulation were also evaluated. The behavior of both bacteria under As treatment was compared and discussed in relation to their potential for colonizing plant rhizosphere with high content of the metalloid. *B. japonicum* E109 growth was reduced with As(III) concentration from 10 μM while *A. brasilense* Az39 showed a reduction of growth with As(III) from 500 μM. EPS and biofilm production increased significantly under 25 μM As(III) for both strains. Moreover, this was more notorious for *Azospirillum* under 500 μM As(III), where motility was seriously affected. Both bacterial strains showed a similar ability to reduce As(V). However, *Azospirillum* was able to oxidize more As(III) (around 53%) than *Bradyrhizobium* (17%). In addition, both strains accumulated As in cell biomass. The behavior of *Azospirillum* under As treatments suggests that this strain would be able to colonize efficiently As contaminated soils. In this way, inoculation with *A. brasilense* Az39 would positively contribute to promoting growth of different plant species under As treatment.

Introduction

Arsenic (As) is frequently found at high concentrations in Argentinian agriculture soils and groundwater (Smedley and Kinniburgh, 2002; Farías et al., 2003). Groundwater is increasingly being used for irrigation schemes due to the expansion of crops to desert regions, thus raising the risk of As incorporation into the food chain through its accumulation in different crops.

In As contaminated soils, rhizospheric microorganisms play a crucial role since their metabolic abilities (reduction, oxidation and methylation) can affect As speciation and bioavailability, and consequently As phytotoxicity (Oremland and Stolz, 2003; Islam et al., 2004). As it is well known, arsenate (As(V)) is less toxic than arsenite (As(III)) but, surprisingly, resistance to As(V) requires its reduction to As(III), which can then be stored in vacuoles or extruded outside. Furthermore, As(III) oxidation, which constitutes an

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electron source for some microorganism metabolism, transforms As(III) to As(V) (Silver and Phung, 2005). In the last years, the selection of symbiotic or free-living plant growth promoting rhizobacteria (PGPR) strains with remediation capabilities has been emphasized, since they could contribute to plant development in contaminated soils or they could even limit the incorporation of contaminants into plant tissues.

In particular, inoculants based in nitrogen fixing bacteria which are being used as an alternative to avoid the indiscriminate use of synthetic fertilizers are of special interest. Several strains of *Bradyrhizobium* are currently used for soybean inoculation. Specially, *Bradyrhizobium japonicum* E109 was selected as the most suitable for soybean inoculant formulation in Argentina, after an intensive selection program initiated in 1980 (Cassán et al., 2009). This PGPR has shown to increase significantly soybean productivity (Hume and Blair, 1992; Ressia et al., 2003). In spite of this, this strain could be applied for growth promotion of both legume and non-legume plant species due to the fact that phytohormone production was identified as the most important bacterial mechanism for plant growth promoting besides symbiotic nitrogen fixation (Cassán et al., 2009). Regarding *Azospirillum brasilense* Az39, it was also selected in the 1980s by an intensive agricultural program of Agriculture Zoology and Microbiology Institute (IMYZA) and National Institute of Agricultural Technology (INTA), Castelar, Argentina and it showed ability to improve productivity of wheat, corn and sorghum crops, as it was demonstrated by numerous field experiments (Díaz-Zorita et al., 2004; Díaz-Zorita and Grove, 2006). Furthermore, this free-living bacterial strain alone or co-inoculated with *B. japonicum* E109 promoted seed germination and early seedling growth of corn and soybean (Cassán et al., 2009). Moreover, co-inoculation of soybean plants with *B. japonicum* E109 and *A. brasilense* Az39 produced a larger amount of nodules and higher percentage of nodulated plants than inoculation with the symbiont alone (Cassán et al., 2009).

Successful rhizosphere colonization is a fundamental step for optimum inoculation results. In relation to this, the interaction between soybean and *B. japonicum* for nodule development requires a cell-to-cell communication. The properties of the bacterial cell surface may play an important role in this symbiotic process (Park and So, 2000). Moreover, colonization potential greatly depends on bacterial adhesion and growth capabilities on different surfaces. In this sense, the extracellular polymeric substances produced by microorganisms, such as exopolysaccharides (EPS) that lead to the formation of aggregated microbial communities called biofilms are of great importance (Costerton et al., 1999; Borucki et al., 2003). In fact, biofilm formation is the most common strategy for bacterial life and survival in terrestrial habitats (Fujishige et al., 2006), and EPS matrix plays a role as an impermeable barrier to heavy metals and/or other toxic compounds, improving bacterial tolerance (Teitzel and Parsek, 2003; Harrison et al., 2007). However, little is known about the effect of As on tolerance mechanisms of *B. japonicum* E109 and *A. brasilense* Az39 and how these mechanisms can affect its colonization ability of root plants in the presence of As. Thus, the aim of this study was to evaluate and compare the effect of As(V) and As(III) on growth, EPS production, biofilm formation and motility of *B. japonicum* E109 and *A. brasilense* Az39. The ability of these two PGPR strains for As removal and accumulation was also studied.

1. Materials and methods

1.1. Bacterial strains

B. japonicum E109 and *A. brasilense* Az39 were used in this work. They are collection strains from the and they were gently provided by our colleague Dr. Fabricio Cassán.

1.2. Growth and cell viability of *B. japonicum* E109 and *A. brasilense* Az39 under As treatments

Growth of *B. japonicum* E109 and *A. brasilense* Az39 in liquid culture medium supplemented with As was evaluated. For *B. japonicum* E109, a proper volume of sterile stock solutions of sodium arsenate ($AsHNa_2O_4$ $7H_2O$) (SIGMA) and sodium arsenite ($NaAsO_2$) (SIGMA) was added to culture flasks containing 20 mL of TY medium ((g/L): 5 tryptone; 3 yeast extract; 0.52 $CaCl_2$) in order to reach final concentrations of 25 or 100 μmol/L As(V) and 10 or 25 μmol/L As(III). For *A. brasilense* Az39 culture flasks with 20 mL of LB medium ((g/L): 10 tryptone; 5 yeast extract; 5 NaCl) were supplemented with 25 μmol/L, 500 μmol/L and 5 mmol/L of both As salts. For both strains the specific medium without metalloid was considered as control. The culture flasks were inoculated with an appropriate volume of *B. japonicum* E109 or *A. brasilense* Az39 cultures previously grown in TY or LB medium without As, respectively, to achieve an initial value of 0.05 at OD_{620nm}. Both microorganisms were incubated in orbital shaker at 200 rpm and 28 ± 2°C. The OD_{620nm} was monitored for each strain at different times until stationary phase. Cell viability was determined by the microdroplet method according to Somasegaran and Hoben (1994).

1.3. Biofilm formation assay

The biofilm-forming ability of *B. japonicum* E109 and *A. brasilense* Az39 was quantitatively assayed by measuring the amount of cells attached to Khan's tube, using a well-established crystal violet staining method (O'Toole and Kolter, 1998) with slight modifications. For this, bacteria grown until stationary phase were centrifuged at 10,000 rpm for 15 min at 4°C and then re-suspended in an appropriate volume of saline solution (0.9% NaCl) to achieve initial OD_{620nm} of 1. Thereafter, 400 μL of that suspension was inoculated into Khan's tube containing 400 μL of TY or LB culture medium for *B. japonicum* E109 or *A. brasilense* Az39, respectively, with or without the addition of As salts. Uninoculated tubes with culture medium were considered as controls. After incubation, an aliquot (200 μL) was taken and OD_{620nm} was measured in order to estimate free cell quantity. The culture remaining was removed from the tubes and they were carefully washed with saline solution. Then, biofilm staining was performed with 1 mL of 0.1% crystal violet for 15 min at room temperature. Dye solution was gently removed and successive washes were performed with saline solution. Later, the stained biofilm ring was homogenized with 1 mL 96% ethanol in vortex and finally OD_{570nm} was measured using an ELISA reader (Multiskan™ FC Microplate