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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

Photometer, Thermo Scientific, United States). Arsenic concentrations and incubation times for each microorganism are described above.

1.4. Isolation and quantification of EPS

EPS were extracted by the modified procedure of Mueller and González (2011). Bacterial cultures at stationary phase were centrifuged at 10,000 r/min 15 min at 4°C, the supernatant containing EPS was reserved and the bacterial pellet was dried in an oven (60°C) until constant weight. Then, 10 mL of supernatant was taken and was resuspended in three volumes of 96% cold ethanol. The mixture was incubated overnight at 4°C and centrifuged at 10,000 r/min 4°C during 20 min. The pellet was washed 3 times with ethanol, dried and then resuspended in 6 mL of distilled water to be dialyzed for 24 h. Finally, the samples were dried, resuspended in distilled water and then used for EPS content determination using the anthrone method (Dische, 1962). The amount of EPS was expressed as mg EPS/mg of bacterial biomass.

1.5. Effect of As on *B. japonicum* E109 and *A. brasilense* Az39 motility: swarming and swimming

Swarming and swimming of both strains were evaluated in the presence of As. Motility of *B. japonicum* E109 and *A. brasilense* Az39 was analyzed in diluted 1/10 TY and LB, respectively, solid medium (0.3% agar for swimming and 0.5% for swarming) supplemented with 10, 25, 50 and 100 μmol/L As(V) and 10 or 25 μmol/L As(III) for E109 and 25 μmol/L, 500 μmol/L and 5 mmol/L of both As salts for Az39. Once inoculated, the plates were inverted and incubated at 28 ± 2°C and the diameter of the ring was measured.

1.6. Analysis of As(V) and As(III) metabolism and As accumulation in bacterial biomass

For As(V) and As(III) metabolism experiments, bacterial strains were grown at 28 ± 2°C, with continuous shaking at 200 rpm, in 50 mL YEM or LB medium with or without 25 μmol/L of both As salts. Cultures were incubated until stationary phase, then they were centrifuged (10,000 rpm for 10 min) and the supernatants were used for As(V) and As(III) removal estimations. Then, the bacterial pellets were washed once with 1 mL of distilled water to eliminate possible As adsorbed to the cells. After that, the pellets were dried until constant weight to measure total biomass and total As accumulation. A non-inoculated control medium was included to evaluate possible metalloid loss by flask adsorption or evaporation as well as other unspecific transformations (abiotic control). Total residual As, species (As(V) and As(III)) concentration in the culture medium as well as As accumulation in bacterial pellet were measured by a graphite furnace atomic absorption spectrophotometric (GF-AAS) technique after a wet acid digestion with nitric acid.

1.7. Statistical analysis

All experiments were performed three times in independent assays. Results were analyzed with InfoStat (version 2012e) software. To determine the statistical difference between at least one pair of means, analysis of variance test (ANOVA) was used. If the assumptions of homogeneity of variance (*Levene* test) and normality (*Shapiro–Wilk* test) were not possibly checked, corresponding transformations were performed using the appropriate functions. To determine significant differences between treatments, the Tukey test was applied, with a significance level of 0.05 ($p < 0.05$).

2. Results and discussion

2.1. Effect of As on bacterial growth and cell viability

In order to analyze to what extent As affects *B. japonicum* E109 and *A. brasilense* Az39 growth, OD_{620nm} values and the number of viable cells of both strains were measured. As it is shown in Fig. 1, different As concentrations were used for each bacterial strain based on previous studies of As tolerance carried out in plates containing the proper solid culture medium supplemented with different As(V) and As(III) concentrations. In those conditions at which bacterial growth was observed, including the highest As concentration at which bacteria could grow (maximum tolerated concentration, MTC) were selected for the evaluation of growth in liquid medium (data not shown).

Fig. 1A shows the absorbance values of *B. japonicum* E109 grown under different As concentration treatments. Bacterial growth was not significantly affected by 25 or 100 μmol/L As(V). However, it was considerably reduced by As(III) reaching low OD values at 96 hr (0.55 for 10 μmol/L As(III)) compared with control. This effect was more evident with increasing concentrations of As(III), and the growth was completely inhibited at 100 μmol/L As(III). On the contrary, *A. brasilense* Az39 was able to grow in all tested conditions even at high As(III) concentration (500 μmol/L), with the exception of 5 mmol/L As(III) where growth was completely inhibited (Fig. 1B). The above results were confirmed by determining viable cells as it is shown in Table 1. As it could be observed, *A. brasilense* Az39 showed higher As tolerance than *B. japonicum* E109 probably reflecting different As metabolic capacities.

Bacterial tolerance to As is mostly mediated by genes belonging to *ars* operon (Rosen, 2002). Despite the conformation of *ars* operon changes between species, there are some genes that are always present and they confer a basal As resistance to microorganisms, such as those found in *ars*RBC operon and pI258 plasmid from *Escherichia coli* and *Staphylococcus aureus*, respectively (Carlin et al., 1995; Silver et al., 1981). Those basal operons can be present in a strain and they can also be complemented by other *ars* genes related to the resistance, such as *ars*H (Muller et al., 2007) and *ars*N (Chauhan et al., 2009). In *ars*RDABC operon, five genes give resistance to higher As concentrations. In this sense, considering that *A. brasilense* Az39 was able to tolerate and to grow under higher As(III) concentrations than *B. japonicum* E109, a difference in operon composition would be suggested as the one responsible of the high As tolerance that was observed.

Since genomes of the strains used in the present work are completely sequenced, we could verify that both of them have *ars*C and *ars*B genes which codify for an As(V) reductase and

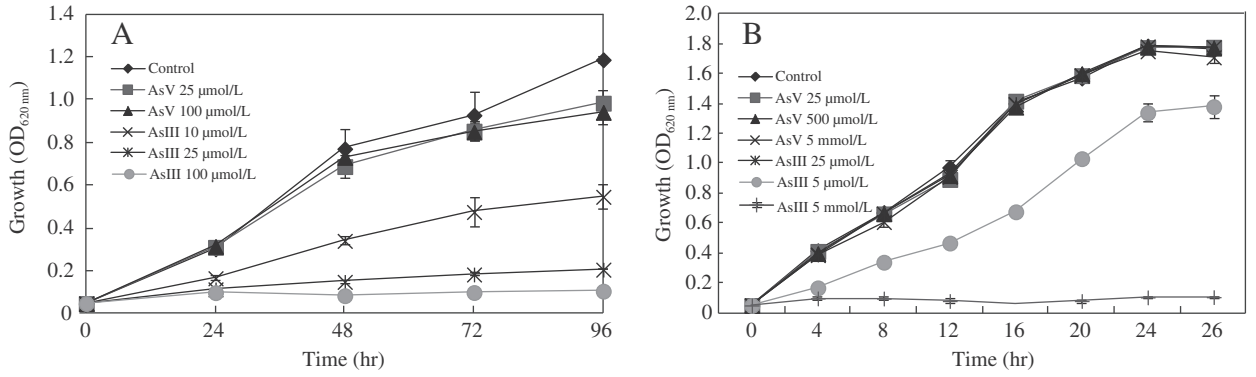

Fig. 1 – Growth of *B. japonicum* E109 in TY liquid medium containing As(III) (A) and *A. brasilense* Az39 in LB liquid medium containing both As salts (B).

As(III) efflux pump, respectively. However, *A. brasilense* Az39 has two additional genes, *ars*H and *Acr*3. The role of the protein codified by *ars*H gene is not well known, although it has been described as a NADPH:FMN oxide-reductase that forms H_2O_2 in *Sinorhizobium meliloti* (Ye et al., 2007) and *Shigella flexneri* (Vorontsov et al., 2007). Moreover, a reduction in As(III) resistance has been seen in *E. coli* cells in which *ars*H gene was removed (Branco et al., 2008). Acr3 is a homologue of arsB, which is an As(III) efflux protein (Achour et al., 2007). Thus, the presence of *ars*H and *Acr*3 genes in *A. brasilense* Az39 would be partly responsible for the higher tolerance compared with *B. japonicum* E109, along with other characteristics related to protective mechanisms that are described below.

At this point it is important to remark that the following characteristics (biofilm and EPS production, motility, and As metabolism) were studied including those concentrations that produced reduction or inhibitory effect on growth, in order to link or elucidate if the behavior of bacteria is a response to As toxicity and hence a tolerance mechanism.

2.2. Effect of As on biofilm formation

A biofilm consists of a multicellular structure attached to a surface where bacteria are surrounded by extracellular polymers (Stoodley et al., 2002). Since the capacity to form biofilm has shown changes in microorganisms exposed to heavy metal treatments, the effect of As on biofilm formation of *B. japonicum* E109 and *A. brasilense* Az39 was tested.

Biofilm formation significantly increased as the treatment was more severe for both strains. As it is shown in Fig. 2, biofilm production significantly increased when *B. japonicum* E109 was exposed to 25 μmol/L As(III) while for *A. brasilense* Az39 strain it significantly increased in all tested conditions compared with controls, with the exception of 25 μmol/L As(V).

It is important to note that in both microorganisms, biofilm production was promoted by As(III) concentrations that inhibited bacterial growth. This result supports the idea that biofilm can be associated with a protection mechanism, allowing bacteria to survive and thrive in environments containing high concentrations of heavy metals or metalloids (Guibaud et al., 2006; Muller et al., 2007). In this sense, biofilm production induction has been described for *Herminiimonas arsenicoxydans* (Marchal et al., 2010) and *Thiomonas arsenitoxydans* (Marchal et al., 2011) at high As(III) concentration (0.67 and 2.67 mmol/L, respectively), which were isolated from an activated sludge and a mine contaminated with As, respectively. Conversely, Andres et al. (2013) detected a reduction in biofilm production in the presence of 8 mmol/L As(III) in *Rhizobium* sp.

Table 1 – Cell viability (log_{10} CFU/mL) of *B. japonicum* E109 and *A. brasilense* Az39 at different As concentrations.

Strain		24 hr	48 hr	72 hr
E109	Control	8.7 ± 0.05	9.5 ± 0.01	9.7 ± 0.07
	As(V) 25 μmol/L	8.6 ± 0.04	9.3 ± 0.02	9.3 ± 0.06
	As(III) 10 μmol/L	8.1 ± 0.01	7.9 ± 0.35	7.2 ± 0.01
	As(III) 25 μmol/L	7.8 ± 0.04	7.8 ± 0.07	6.3 ± 0.02
		6 hr	10 hr	24 hr
Az39	Control	8.5 ± 0.31	8.9 ± 0.29	9.9 ± 0.08
	As(V) 25 μmol/L	8.5 ± 0.30	8.9 ± 0.26	9.8 ± 0.07
	As(V) 500 μmol/L	7.8 ± 0.19	8.9 ± 0.21	9.7 ± 0.13
	As(III) 25 μmol/L	8.3 ± 0.21	8.9 ± 0.36	10 ± 0.09
	As(III) 500 μmol/L	7.8 ± 0.01	8.3 ± 0.08	8.7 ± 0.06

Data are presented as means ± standard error.

Table 2 – Diameter of swimming and swarming rings of *B. japonicum* E109 and *A. brasilense* Az39 grown under different As(V) and As(III) concentrations.

	Treatment	Swarming (cm)	Swimming (cm)
E109	Control	0.50 ± 0.04[a]	2.3 ± 0.0[a]
	As(V) 10 μmol/L	0.50 ± 0.00[a]	2.0 ± 0.05[a]
	As(V) 25 μmol/L	0.40 ± 0.02[a]	2.1 ± 0.11[a]
	As(V) 50 μmol/L	0.40 ± 0.02[a]	1.9 ± 0.02[bc]
	As(V) 100 μmol/L	0.40 ± 0.02[a]	1.5 ± 0.06[c]
	As(III) 10 μmol/L	0.40 ± 0.02[a]	1.8 ± 0.07[bc]
	As(III) 25 μmol/L	0.03 ± 0.00[b]	ND[d]
Az39	Control	0.46 ± 0.02[a]	1.5 ± 0.16[a]
	As(V) 25 μmol/L	0.30 ± 0.05[a]	1.3 ± 0.03[a]
	As(V) 500 μmol/L	0.51 ± 0.02[a]	2.1 ± 0.09[a]
	As(III) 25 μmol/L	0.41 ± 0.00[a]	1.8 ± 0.06[a]
	As(III) 500 μmol/L	0.20 ± 0.00[b]	0.6 ± 0.08[b]

ND: not detectable.
Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$).

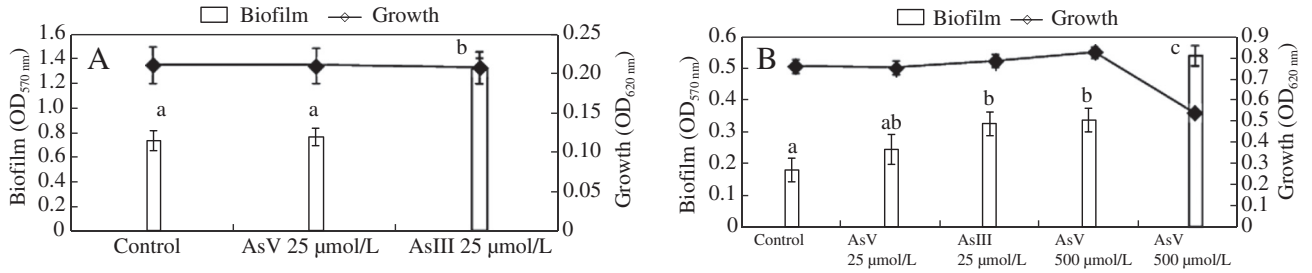

Fig. 2 – Biofilm formation of *B. japonicum* E109 in TY liquid medium containing As(V) or As(III) (A) and *A. brasilense* Az39 in LB liquid medium containing both As salts (B). Data are presented as means ± standard error. Different letters are indicative of significant differences (Tukey's test, $p < 0.05$).

NT-26 strain, which indicates its preferential development as motile planktonic cells rather than sessile under this condition. The different behavior on biofilm formation shown for NT-26 strain compared with that of *B. japonicum* E109 and *A. brasilense* Az39 could be related to the precedence of *Rhizobium* sp. NT-26 since it was isolated from an As containing goldmine. This fact could have exerted a selective pressure upon this strain that should give an inherent way of dealing with As. In addition, *Rhizobium* sp. NT-26 has lost the major colonizing capabilities needed for symbiosis with legumes (Andres et al., 2013). These results illustrate the impact that environmental pressure can have on the evolution of bacterial genomes, improving bacterial performance by the acquisition of novel functions even in detriment of others, such as colonization.

2.3. Production of EPS by E109 and Az39 under As treatment

EPS bind to cells forming a vast net-like structure that protects cells from toxic substances (Sutherland, 2001). Since EPS are bounded mainly through ionic interactions with multivalent metals, metal concentration in the external media may influence EPS content. In order to analyze the effect of As on EPS production, EPS were extracted from stationary phase cultures of *B. japonicum* E109 and *A. brasilense* Az39 strains and quantified by the anthrone method. As shown in Fig. 3A, EPS production in *B. japonicum* E109 increased significantly under 25 μmol/L As(III) treatment. Similarly, *A. brasilense* Az39 showed a considerable increase on EPS after treatment with 500 μmol/L As(III) (Fig. 3B). An increased production of EPS was also reported in *Rhodopseudomonas acidophila* as the severity of the treatment with other toxic metal (CrVI) was intensified (Sheng et al., 2005).

As it was expected, EPS production was induced by the same conditions that promoted biofilm formation, since biofilm extracellular matrix is composed mostly of EPS, which play a key role in biofilm endurance (Stoodley et al., 2002).

2.4. Arsenic effect on *B. japonicum* E109 and *A. brasilense* Az39 motility: swarming and swimming

Swarming is a rapid multicellular bacterial surface movement powered by rotating flagella. Swimming is a movement in liquid media also mediated by rotating flagella but, unlike swarming, involves individual cells (Kearns, 2010). It has been suggested that motility plays a substantial role in some survival processes and functions of microorganisms under adverse conditions, for instance in biofilm development (Drenkard, 2003). Little is known about As effect on bacterial motility, thus, different concentrations of sodium arsenate and sodium arsenite were assayed to study swarming and swimming of *B. japonicum* E109 and *A. brasilense* Az39 (Table 2).

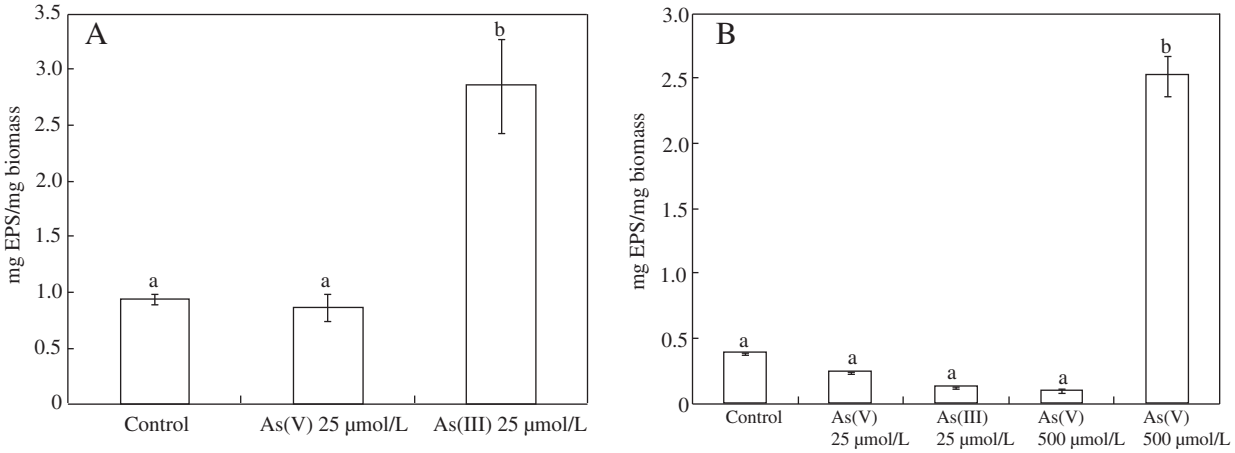

Fig. 3 – EPS production of *B. japonicum* E109 in TY liquid medium containing As(V) or As(III) (A) and *A. brasilense* Az39 in LB liquid medium containing both As salts(B). Data are presented as means ± standard error. Different letters are indicative of significant differences (Tukey's test, $p < 0.05$).

In control conditions (without As), *B. japonicum* E109 exhibited swarming behavior, showing the typical colonies with irregular edges and filamentous extrusions. However, when As(V) and As(III) were added, the normal consolidation was not observed. Treatments with 10 to 100 μmol/L As(V) or 10 μmol/L As(III) did not affect swarming compared with control conditions, while 25 μmol/L As(III) produced a significant reduction. Swimming was significantly affected with the highest tested As(V) concentrations (50 and 100 μmol/L) and 10 μmol/L As(III), while the diameter of the swimming ring was completely reduced under 25 μmol/L As(III) treatment. Hence, at this concentration, the strain was unable to swim.

For *A. brasilense* Az39, swarming and swimming were not significantly different at all tested As(V) concentrations, whereas both types of motilities were significantly reduced with 500 μmol/L As(III).

As it could be perceived, bacterial motility diminished in those As(III) concentrations that also reduced growth in liquid medium, such as 25 μmol/L and 500 μmol/L As(III) for *B. japonicum* E109 and *A. brasilense* Az39, respectively. Nonetheless, despite the fact that multicellular motility was reduced, this was not an impediment to form biofilm, which reinforces the idea that biofilm formation constitutes an important protection mechanism.

In a previous work, reduced motility and growth of *B. japonicum* E109 under 25 μmol/L As(III) treatment was demonstrated and associated to a minor soybean nodulation capacity under this condition (Talano et al., 2013). This was related with the fact that an effective bacterial colonization of root surface depends, at least partially, on bacterium motility, even for free-living bacteria such as *Azospirillum* strains (Kanbe et al., 2007; Burdman et al., 2000). Moreover, in the rizosphere as well as in other niches, the establishment of those strains with adaptive advantages will be favored and the success often depends on the competition with native strains in which the motility is one of the involved factors (Hibbing et al., 2010). Considering that *A. brasilense* Az39 growth and motility were not reduced under 25 μmol/L As(III) treatment, a better root colonization performance would be expected, compared with *B. japonicum* E109 under As stress.

2.5. As(V) and As(III) metabolism and accumulation in *B. japonicum* E109 and *A. brasilense* Az39

Microorganisms have specific enzymes or multienzymatic complexes that mediate As redox transformations, where As compounds can be used as electron donors or acceptors. This has been correlated with the presence of As resistance genes (*ars*), As respiratory reduction genes (*arr*), or AsII oxidation genes (*aox*/*aro*/*aso*) (Kashyap et al., 2006; Chang et al., 2010). In order to examine redox capabilities of *B. japonicum* E109 and *A. brasilense* Az39, As(V) and As(III) concentrations in the culture medium were monitored and total As was determined in cell biomass to evaluate metalloid accumulation. To our knowledge, it has not been found any description related to the As metabolism of these bacteria in the literature available.

When *B. japonicum* E109 grew in the presence of 25 μmol/L As(V), it was able to reduce around 34% of initial As(V), similar to the reduction percentage reached by *A. brasilense* Az39 (36%). However, under this same condition, the strains differed in As accumulation capability, since *B. japonicum* E109 accumulated 11.3% of As in biomass while *A. brasilense* Az39 was not able to accumulate the metalloid (Fig. 4A). As shown in Fig. 4B, both microorganisms were able to oxidize As(III) to As(V) when they grew in the presence of 25 μmol/L As(III). Nonetheless, *A. brasilense* Az39 showed a higher efficiency for As(III) oxidation (around 53%) compared with *B. japonicum* E109 ability (17%). Regarding accumulation under As(III) treatment, only *A. brasilense* Az39 was able to accumulate an 8.5% of the total As in cell biomass.

In general it can be stated that, some bacteria can reduce As(V) during anaerobic respiration or as means of As detoxification, while others oxidize As(III) during their chemolithoautotrophic/heterotrophic metabolism (Oremland et al., 2005). Our results showed that *B. japonicum* E109 and *A. brasilense* Az39 strains were

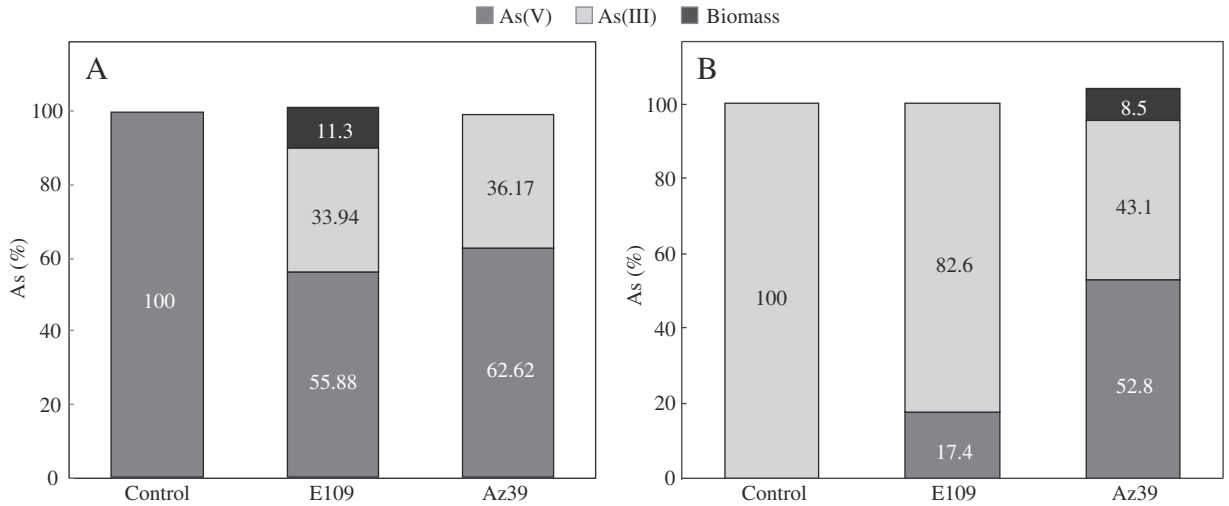

Fig. 4 – Percentage of As(V) or As(III) present in the culture medium of *B. japonicum* E109 or *A. brasilense* Az39 and accumulation of As in cell biomass of bacteria exposed to (A) 25 μmol/L As(V) or (B) 25 μmol/L As(III) after 96 and 24 hr, respectively.

able to carry out As(V) reduction as well as As(III) oxidation. Although this simultaneous ability for both metabolic activities is unusual, it has been reported in some bacteria (Gihring and Banfield, 2001; Muller et al., 2007).

Arsenic produces negative effects on the growth of several plant species and, in many cases, it is highly accumulated in fronds and/or grains (Das et al., 2004; Srivastava and Sharma, 2013) which constitute a risk for consumers. As(III) is more toxic than As(V) for plants. Thus the ability of *A. brasilense* Az39 for accumulating As from solutions that initially contained As(III), is interesting for phytotoxicity mitigation. In this context, the high tolerance of *A. brasilense* Az39 to As, because of their own genetic background that allowed the development of protective mechanisms, encourages us to consider this bacterium as an interesting alternative for plant inoculation. Lyubun et al. (2006) showed that the inoculation of wheat plants with an *Azospirillum* strain produced a minor As accumulation in plant tissues compared with those non-inoculated plants. These authors suggested that this bacterium could be attractive for agricultural practices due to its plant growth promoting properties and As removal performance. Furthermore, since this bacterium gives greater productivity to corn, wheat and sorghum in field, it would be expected to improve the growth of these crops exposed to As.

In this sense, *A. brasilense* Az39 could be considered not only for inoculation of different plants for improving As tolerance but also for co-inoculation with *B. japonicum* E109, particularly in soybean crop. Soybean inoculation with *B. japonicum* E109 is a well established and adopted practice. In spite of this, in a previous work, we showed that soybean inoculation with this bacterium could not attenuate As toxicity (Talano et al., 2013). This was attributed, in part, to a reduction in the number of effective nodules under this condition. Accordingly, water and/or soil containing As would impact negatively on soybean production or even in plants inoculated with *B. japonicum* E109. Thus, it is interesting to consider the combination of bacteria with different and complementary plant growth promoting properties as well as different tolerance performances for inoculation strategies in order to improve yield crop under unfavorable conditions. These assays are currently being carried out in our laboratory in order to corroborate if plant inoculation with the most tolerant microorganism, as it was shown by *in vitro* assays, contributes *in vivo* with plant tolerance to As.

3. Conclusions

One of the major contributions of the present work is shedding light on the different strategies evolved by *B. japonicum* E109 and *A. brasilense* Az39 to deal with As stress. In this sense, our results suggest that root colonization potential of this bacteria can be negatively affected by As. Growth and motility of *B. japonicum* E109 was severely affected by 25 μmol/L As(III), while *A. brasilense* Az39 motility and biofilm/EPS production remained unchanged, which correlates with the observed higher tolerance of this strain to As(V) and to the more toxic form As(III). Therefore, the higher As(III) tolerance, the ability for As(III) oxidation and As accumulation of *A. brasilense* Az39 compared to *B. japonicum* E109 indicates that the former has adaptive advantages for surviving in an As contaminated environment. This suggests that *A. brasilense* Az39 would be more efficient in the establishment of plant colonization under this condition. Thus, inoculation strategies using *A. brasilense* Az39 combined with other plant promoting bacteria would improve the development of plants under As stress.

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