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ORIGINAL PAPER

Mechanism of arsenic resistance in endophytic bacteria isolated from endemic plant of mine tailings and their arsenophore production

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Abstract

Arsenic contamination is an important environmental problem around the world since its high toxicity, and bacteria resist to this element serve as valuable resource for its bioremediation. Aiming at searching the arsenic-resistant bacteria and determining their resistant mechanism, a total of 27 strains isolated from roots of *Prosopis laevigata* and *Sphaerealcea angustifolia* grown in a heavy metal-contaminated region in Mexico were investigated. The minimum inhibitory concentration (MIC) and transformation abilities of arsenate (As^{5+}) and arsenite (As^{3+}), arsenophore synthesis, arsenate uptake, and cytoplasmatic arsenate reductase (*arsC*), and arsenite transporter (*arsB*) genes were studied for these strains. Based on these results and the 16S rDNA sequence analysis, these isolates were identified as arsenic-resistant endophytic bacteria (AREB) belonging to the genera *Arthrobacter, Bacillus, Brevibacterium, Kocuria, Microbacterium, Micrococcus, Pseudomonas*, and *Staphylococcus*. They could tolerate high concentrations of arsenic with MIC from 20 to > 100 mM for As^{5+} and 10–20 mM for As^{3+}. Eleven isolates presented dual abilities of As^{5+} reduction and As^{3+} oxidation. As the most effective strains, *Micrococcus luteus* NE2E1 reduced 94% of the As^{5+} and *Pseudomonas zhaodongensis* NM2E7 oxidized 46% of As^{3+} under aerobic condition. About 70 and 44% of the test strains produced arsenophores to chelate As^{5+} and As^{3+}, respectively. The AREB may absorb arsenate via the same receptor of phosphate uptake or via other way in some case. The cytoplasmic arsenate reductase and alternative arsenate reduction pathways exist in these AREB. Therefore, these AREB could be candidates for the bioremediation process.

Keywords Arsenic resistance · Mine tailings · Endophytic bacteria · Resistance mechanism · As transformation · Arsenophore

Introduction

Arsenic is one of the 20 most abundant elements in the Earth's crust. Arsenic catastrophes are occurring worldwide and resulting in serious health problems in many countries such as Bangladesh, India, Chile, Argentina, Sweden, Mexico, the USA, and China (Singh et al. 2015; Smedley and Kinniburgh 2002). Arsenic concentration in natural soils is

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normally less than 24 mg kg^{-1} (Singh et al. 2015), but it may reached up to 600 mg kg^{-1} in the highly polluted soils in different parts of the world (Arco-Lázaro et al. 2016; Franco-Hernández et al. 2010; Gunduz et al. 2010; Karczewska et al. 2007; Nriagu et al. 2007; Román-Ponce et al. 2016; Vásquez-Murrieta et al. 2006). The arsenic concentration can be increased considerably as a result of anthropogenic activities, including the uses of herbicides, insecticides, fungicides, and phosphate fertilizers, cattle and sheep baths, dyes, food additives, mining and smelting, industrial processes, coal combustion, and timber preservatives (Bundschuh et al. 2011; Mondal et al. 2008). Problems related to arsenic contamination have drawn attention worldwide and several physical and chemical remediation processes have been developed (Singh et al. 2015). Unfortunately, the available technologies (physicochemical processes) for remediation of arsenic-contaminated areas are expensive, time-consuming, with risks for workers and large amounts of secondary wastes (Lombi et al. 2000). Considering the limitations of conventional remediation techniques, biological strategies could be explored as alternative mitigation options (Chandraprabha and Natarajan 2011; Singh and Minsker 2008). As the environmental-friendly and low-cost technology, bioremediation refers to the methods relying on the use of plants, microorganisms, and their enzymes individually or in combination to reclaim contaminated natural environments by different agents, including arsenic (Pearce et al. 2003).

Using plants and plant-associated microbes, phytoremediation has been applied for the remediation of arsenic-contaminated sites to clean up contaminated air, soil, and water (Behera 2014; Cherian and Oliveira 2005; Dickinson et al. 2009; Lasat 2002). Endophytic bacteria colonize the internal plant tissues without causing adverse effects on their host (Khan and Doty 2009; Long et al. 2011). Some of their beneficial effects on plant growth have been attributed to their ability in indole acetic acid (IAA) synthesis, siderophore production, phosphate solubilization (Rajkumar et al. 2009) and improving mineral nutrient uptake (Luo et al. 2011). Indeed, the endophytes have the potential for phytoremediation and some of them could alleviate plants from heavy metals (HM) toxicity and enhance the phytoremediation.

In the environment, the arsenic biogeochemical cycle was strongly influenced by microbial transformation. The microorganisms are able to transform the arsenic in different states of oxidation, affecting the arsenic mobility, solubility, and toxicity (Gadd 2010; Paez-Espino et al. 2009). Some mechanisms in the arsenic-resistant microorganisms have been described, such as using arsenic in their respiration (Silver and Phung 2005; Stolz et al. 2006), oxidizing (Chang et al. 2010) or reducing (Govarthanan et al. 2015a) the arsenic salts, methylating inorganic As species (Xue et al. 2017) or demethylating organic As counterparts (Silver and Phung 2005), having specific phosphate transporters (Pit) and non-specific phosphate transporters (Pst) (Rosen and Liu 2009), having arsenite transporters (GLpF) (Meng et al. 2004; Paez-Espino et al. 2009), solubilizing arsenic by organic acids production (Mailloux et al. 2009), producing organic ligands (Drewniak et al. 2010; Nair et al. 2007), compartamentalization (Joshi et al. 2009), biosorption (Prasad et al. 2013), and adsorption (Ahsan et al. 2011). Recently, a few studies on endophytes associated with arsenic hyperaccumulator plant *Pteris vittata* (Chinese brake fern) and their arsenic transformation capacity have been reported (Han et al. 2016, 2017; Selvankumar et al. 2017; Tiwari et al. 2016; Xu et al. 2016; Zhu et al. 2014). Likewise, some arsenic-resistant endophytic bacteria (AREB) showed plant growth promoting features and increased the arsenic uptake by plant (Das et al. 2016; Govarthanan et al. 2016; Han et al. 2016; Mallick et al. 2018; Qmar et al. 2017; Tiwari et al. 2016; Xu et al. 2016; Zhu et al. 2014). Until now, there is limited information about the arsenic tolerance, transformation and other mechanisms of arsenic resistance by endophytes associated with endemic plants in mine tailings.

According to the information mentioned above, we performed the present study to characterize AREB associated with *Prosopis laevigata* and *Sharealcea angustifolia*, two endemic plant species in the arsenic-contaminated soils at Villa de la Paz, San Luis Potosí, Mexico. The aims of this study were (a) evaluating the arsenic accumulation or translocation by *P. laevigata* and *S. angustifolia* and (b) evaluating the arsenic-resistant mechanism developed for these endophytes. Our findings are important for better understanding arsenic tolerance and transformation mechanisms in arsenic-resistant endophytes and substantiate the potential application of native bacterial species in the detoxification of arsenic in contaminated soil environments and could guide us to develop eco-friendly and cost-effective remediation techniques.

Materials and methods

Site description and sampling of plants

P. laevigata and *S. angustifolia* plants together with roots and soils in the root zone were sampled in February 2012 as endemic plants from Villa de la Paz (23.7 N, 178.7 W) located in the mining district of Santa María at the State of San Luis Potosi, Mexico. All the samples were immediately transported in polyethylene bags and stored at 4 °C for 1–3 days until further analysis. The climate of the sampling area is dry-temperate with a mean annual temperature of 18 °C and an average annual precipitation of 486 mm. The two sampling sites were a mine tailing with altitude of 1557 m and a natural hill with altitude of 1830 m, with a

distance of about 5 km between them. This area was highly contaminated by multiple heavy metals and arsenic (Ramos-Garza et al. 2016; Román-Ponce et al. 2016). Total arsenic concentrations were 2816 and 4332 mg kg^{-1} in the rhizosphere soils of *S. angustifolia* and *P. laevigata*, respectively, in the mine tailing; and were 841 and 1301 mg kg^{-1}, respectively, at the hill site (Román-Ponce et al. 2016).

Analysis of arsenic in plant tissues

Arsenic content in *P. laevigata* and *S. angustifolia* tissues was determined using the methodologies proposed by Franco-Hernández et al. (2010) and Vásquez-Murieta et al. (2006). Briefly, aerial plant material and roots were oven-dried for 48 h at 80 °C and hammer-milled. One gram of hammer-milled dry aerial parts or roots was mineralized with 2 ml HCl, 6 ml HNO_3, and 2 ml H_2O_2. The solution was analyzed for arsenic with an inductively coupled plasma-optical emission spectrometer (ICP-OES) (4600DV-Perkin Elmer, USA) (Franco-Hernández et al. 2010). The arsenic removing ability of the plants, biological accumulation coefficient (BAC), and biological translocation coefficient (BTC) in both plants were calculated as described by Li et al. (2007).

Screening of AREB

The endophytic bacterial strains isolated from root samples in our previous study (Román-Ponce et al. 2016) were used for screening arsenic resistance using 96-well microliter plates. Each well was filled with 190-µl sterile MES buffered minimal medium (Rathnayake et al. 2013) and supplemented with arsenite (As^{3+}) from 1 to 20 mM, and arsenate (As^{5+}) from 5 to 100 mM. Strains were grown in 5 mL TSI medium without As for 24 h at 28 ± 2 °C on a rotary shaker (150 rpm). Aliquot of 10 mL of bacterial inoculum (1.0 OD at 600 nm) was placed in each well. Medium without arsenic but with the bacterial inoculum (bacterial growth control), and medium with arsenic but without bacteria (abiotic control) was included. Plates were incubated at 28 ± 2 °C. Bacterial growth was measured after 4 days of incubation using an EZ Read 400 Microplate Reader (Biochrom) at 620 nm.

Identification of selected AREB based on 16S rRNA

Genomic DNA was extracted from each of the AREB using the protocols described previously (Román-Ponce et al. 2015) and was used as template to amplify the 16S rRNA genes. The PCR was performed in a thermal cycle with the reaction conditions as described by Román-Ponce et al. (2016). Partial nucleotide sequence was determined using an Automatic Sequencer 3730XL in Macrogen (Korea). The acquired sequences were compared with those in the GenBank database using the program BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All the sequences acquired in this work were aligned together with the reference sequences using CLUSTAL X (2.0) software (Larkin et al. 2007). The problem sequences were manually edited with SEAVIEW software (Galtier et al. 1996). Similarities among sequences were calculated using the MatGAT v.2.01 software (Campanella et al. 2003). Taxonomic assignment was obtained using the Roselló-Mora prokaryotes criteria (Roselló-Mora and Amman 2001).

Screening for arsenic-transforming ability

The silver nitrate screening was performed based on the interaction of $AgNO_3$ with AsO_3^{3-} ion that generates bright yellow precipitate of Ag_3AsO_3; and on the interaction with AsO_4^{3-} ion that generates brownish precipitate of Ag_3AsO_4 (Simeonova et al. 2004). Bacterial strains able to grow at 10–20 mM of As^{3+} and 20–100 mM of As^{5+}, respectively, were used for testing arsenic transformation ability. Modified chemically defined medium (CDM) (Weeger et al. 1999) supplemented with 1 mL of vitamin solution and 0.5 ml trace element solution, the medium pH was adjusted to 7.2 (Wu et al. 2013). Each isolate was incubated in 5 mL of CDM amended with 2 mM of $NaAsO_2$ or $NaH_2AsO_4 \cdot 7H_2O$ in the dark at 28 °C for 4–14 days. Then, the culture was centrifuged at 47,500×*g* for 10 min. Each well of the 96-well microliter plate was filled with 100 µL of the culture supernatant and 100 µL of the 0.1 M $AgNO_3$ (Krumanova et al. 2008). The arsenite-oxidizing reaction was recognized by a change of the medium from bright yellow to brownish color, while the change from brownish to bright yellow color indicated an arsenate-reducing reaction (Lett et al. 2001). *Pseudomonas aeruginosa* ATCC 25,619 and *Escherichia coli* DH5α were used as positive reference for As^{3+} oxidation and As^{5+} reduction, respectively (Chang et al. 2008; Parvatiyar et al. 2005). Abiotic control were used in each assay without bacteria added.

Arsenic transformation

For determination, the ability of As^{3+} oxidation and As^{5+} reduction by the AREB, aliquot (0.2 mL) of an 18–20 h culture was inoculated in 20 mL of CDM broth supplied with 0.15 mM arsenate (reduction test) or arsenite (oxidation test) and incubated a 28 °C, with agitation of 150 rpm for 48 h. Due to the limit of spectrophotometric analysis for arsenic speciation, the concentration of 0.15 mM was used in this assay. After incubation, the culture was centrifuged at 5700×*g* at 4 °C for 10 min. The speciation and amounts of arsenate and arsenite were measured with the protocol of Hu et al. (2012). Briefly, 3 mL of a cell-free extract were neutralized with 1% HCl (until pH 7) and 10 µM K_2HPO_4.

The supernatant was divided into three parallel 1 mL subsamples and treated separately with 100 µL of an oxidizing agent ($KMnO_4$), a reducing agent ($Na_2S_2O_3$), or deionized water (untreated sample). Then, each treatment was incubated 30 min at room temperature, except for the reduction treatment that was incubated 1 h. After the incubation, 100 µL of coloring reagent [a mixture of 10.8% $C_6H_8O_6$; 3% $(NH_4)_6MO_7O_{24}{\cdot}4H_2O$; 0.56% $C_8H_4K_2O_{12}Sb_2{\cdot}3H_2O$ and 13.98% H_2SO_4 in a 2:2:1:5 volume ratio] were added and the absorbance at 880 nm was measured after 5 min. The concentrations of As^{5+} and As^{3+} were calculated using the following equations: $[As^{3+}]$ = oxidized–untreated; $[As^{5+}]$ = untreated–reduced. The percentage of reduction of As^{5+} and oxidation of As^{3+} was calculated using the equation: $(\%R) = (Co - Ce) \times 100/Co$, where Co and Ce are the initial concentration and equilibrium concentration, respectively, of As^{5+} or As^{3+} (mg L^{-1}) in the solution (Mallick et al. 2014).

Organic ligand production (arsenophores) by AREB

The arsenophore synthesis was tested for the AERB in Luria–Bertani (LB) medium supplied with Chromo-*S*-Azurol (CAS) (Schwyn and Neilands 1987), hexadecyltrimethylammonium bromide (CTAB) and the metaloid salt $NaH_2AsO_4{\cdot}7H_2O$ or $NaAsO_2$ at the concentration of 56 ppm that was calculated from Fe^{3+} concentration in CAS assay as described previously (Nair et al. 2007; Schwyn and Neilands 1987). The plates were incubated at 28 °C for 24–48 h to observe the color change.

Competent phosphate and arsenate uptake by AREB

Five AREB strains NM2E5, NE2E2, NE2E3, CM2E4, and CM2E3 were selected based on their feature of no arsenophore production and As^{5+} resistance > 50 mM. The phosphate uptake and As^{5+} reduction were measured in 25 mL of MMBMES medium (Rathnayake et al. 2013) containing 0, 0.1, or 1 mM As^{5+} and 0.1 or 1 mM PO_4^{3-}. The cultures were incubated at room temperature for 48 h. The bacterial growth (OD_{600}) was determined spectrophotometrically. Phosphate concentration was measured by molybdenum blue method (Tsang et al. 2007), while arsenic speciation was evaluated as described by Hu et al. (2012).

Arsenic-resistant gene detection in the AREB

Genes *arsC* and *arsB* related to As^{5+} reduction and As^{3+}transportation, respectively, in prokaryotes were targeted in this assay. Genomic DNA was isolated from each strain with the protocol of Román-Ponce et al. (2015) and was used as template to amplify the *arsC* and *arsB* genes by PCR with the protocols and primers specific to these genes (Achour et al. 2007; Sun et al. 2004). *E. coli* DH5α and *Cupriavidus metallidurans* were used as the positive control for *arsC* and *arsB*, respectively (Chang et al. 2008; Sun et al. 2004). The PCR products were visualized and sequenced similar to the 16S rRNA genes. The acquired gene sequences were used for Blast searching in GenBank database to verify the gene identity.

Statistical analysis

All experiments were performed in triplicate and mean values were calculated. The data from three replications ($n = 3$) were subjected to a two-way analysis of variance (ANOVA) and Tukey's post hoc test (Tukey's honest significant difference) in the R package (R Development Core Team 2012, http://cran.r-project.org/).

Results

Arsenic contents in plant tissues

The arsenic concentration in roots and aerial parts varied according to the plant species and sampling sites (Supplementary Table S1). *S. angustifolia* showed arsenic concentrations (mg kg^{-1}) of 33.73 and 256.21 in root and aerial parts grown in mine tailing; and the corresponding values were 11.33 and 12.82, respectively, in the plants grown in the natural hill. The accumulation coefficient (BAC) in this plant in both sampling sites was less than 1. According to the values of translocation coefficient (BTC) determined for *S. angustifolia* (Supplementary Table S1) in the mine tailing 7.59 and natural hill 1.13, suggested that these endemic plant was arsenic excluder (BTC > 1).

P. laevigata tissues showed different arsenic concentration (mg kg^{-1}) in both sampling sites (Supplementary Table S1), while the arsenic concentration in the aerial parts from the mine tailing (55.20) was 8.9 times higher than that from the natural hill (6.16). The BAC coefficients in the plants in both sampling sites were less than 1, suggesting that *P. laevigata* was not an arsenic accumulator. The BTC value was 4.51 in the mine tailing showed that *P. laevigata* was arsenic excluders only in the mine tailing. The results obtained for both plants species suggested these plants unable accumulating arsenic.

Screening and identification of AREB

In the present report, a total of 27 AREB were obtained by the screening procedure, including 16 from the natural hill and 11 from the mine tailing (Table 1), while most of them (93%) were Gram-positive. All of them presented high arsenic resistance, e.g., to 20–100 mM for As^{5+} and/

Table 1 Endophytic bacteria used in this study and their resistance to arsenic

Strain	Related species and accession number	% Identity	Arsenic resistance (mM)	
			As^{5+}	As^{3+}
Natural hill-*Spharealcea*				
NE1E7 (*Microbacterium arborescens*)	*Microbacterium arborescens* ANA42 (HQ219873)	97.2	100	> 20
NE2E1 *(Micrococcus luteus*)	*Micrococcus luteus* PCSH2 (JN378531)	97.9	100	10
NE2E2 (*Microbacterium schleiferi*)	*Microbacterium schleiferi* DSM 2014 (NR044936)	97.7	> 100	> 20
NE2E3 (*Microbacterium* sp.)	*Microbacterium oxydans* S28n (AY509223) *Microbacterium phyllosphaerae* 331 (EU714359)	98.0 97.1	> 100	> 20
Natural hill-*Prosopis*				
NM2E3* (*Brevibacterium metallicus*)	*Brevibacterium metallicus* NM2E3 (KM87400)	100.0	72	5.8
NM2E5 (*Bacillus* sp.)	*Bacillus megaterium* Jz11 (KJ843149) *Bacillus aryabhattai* B8W22 (JF792521)	98.3 98.3	50	10
NM2E6 (*Bacillus simplex*)	*Bacillus simplex* 98AIA (NR024695)	97.4	50	10
NM2E7(*Pseudomonas zhaodongensis*)	*Pseudomonas zhaodongensis* NEAUST521 (NR134795)	97.2	50	10
NM2E10 (*Kocuria* sp.)	*Kocuria rhizophila* TA68 (NR026452 *Kocuria arsenatis* CM1E1 (KM874399)	97.3 97.6	50	10
NM2E14 (*Bacillus* sp.)	*Bacillus axarquiensis* (GU568196)	90.0	50	5
NM2E15 (*Bacillus* sp.)	*Bacillus axarquiensis* (GU568196) *Bacillus malacitensis* CECT (NR115930) *Bacillus subtilis* CYBS15 (JQ361064) *Bacillus vallismortis* DSM 11,031 (NR024696)	98.8 98.8 98.8 98.4	50	0.5
NM2E16 (*Bacillus endophyticus*)	*Bacillus endophyticus* 70BC7 (KF254667)	99.7	50	> 20
NM2E18 (*Bacillus* sp.)	*Bacillus aryabhattai* B8W22 (JF792521) *Bacillus megaterium* Jz11 (KJ843149)	98.9 98.9	> 100	20
NM3E2 (*Arthrobacter scleromae*)	*Arthrobacter scleromae* C21 (KF039748)	98.4	5	> 20
NM3E3 (*Bacillus* sp.)	*Bacillus aryarbhattai* B8W22 (JF792521) *Bacillus megaterium* Jz11 (KJ843149)	99.2 99.2	> 100	5
NM3E7 (*Bacillus* sp.)	*Bacillus subtilis* CYBS15 (JQ361064) *Bacillus malacitensis* CECT (NR115930) *Bacillus vallismortis* DSM 11,031 (NR024696)	98.8 98.8 98.4	50	> 20
Mine tailing-*Spharealcea*				
CE1E1 (*Pseudomonas stutzeri*)	*Pseudomonas stutzeri* (U26420)	97.5	100	10
CE2E1 (*Arthrobacter scleoromae*)	*Arthrobacter scleoromae* C21 (KF039748)	98.5	> 100	> 20
CE3E1 (*Sthaplylococcus* sp.)	*Staphylococcus pasteuri* LCR12 (HQ259721) *Staphylococcus warneri* SG1 (NR102499)	99.2 99.2	> 100	> 20
CE3E2 (*Bacillus* sp.)	*Bacillus axarquiensis* (GU568196) *Bacillus malacitensis* CECT (NR115930) *Bacillus subtilis* CYBS15 (JQ361064) *Bacillus vallismortis* DSM11031 (NR024696)	98.5 98.5 98.4 98.2	> 100	> 20
CE3E3 (*Bacillus* sp.)	*Bacillus axarquiensis* (GU568196) *Bacillus malacitensis* CECT (NR115930) *Bacillus subtilis* CYBS15 (JQ361064) *Bacillus vallismortis* DSM 11,031 (NR024696)	98.5 98.5 98.4 98.1	> 100	> 20
Mine tailing-*Prosopis*				
CM1E1 (*Kocuria arsenatis*)	*Kocuria arsenatis* CM1E1 (KM874399)	100.0	20	20
CM1E4 (*Bacillus endophyticus*)	*Bacillus endophyticus* 70BC7 (KF254667)	99.4	> 100	> 20
CM1E5 (*Bacillus niacini*)	*Bacillus niacini* IFO15566 (NR024695)	97.4	50	10
CM2E2 (*Bacillus endophyticus*)	*Bacillus endophyticus* 70BC7 (KF254667)	98.9	20	> 20
CM2E3 (*Bacillus endophyticus*)	*Bacillus endophyticus* 70BC7 (KF254667)	98.3	50	> 20
CM2E4 (*Staphylococcus* sp.)	*Staphylococcus warneri S*G1 (NR102499) *Staphylococcus pasteuri* LCR12 (HQ259721)	98.3 98.3	50	0.5

or 10–20 mM for As^{3+} in MMBM medium (Table 1). The 16S rRNA gene phylogeny (Fig. 1) affiliated them to the phyla Firmicutes (59%), Actinobacteria (33%) and α-Proteobacteria (8%), and identified them within eight genera: *Arthrobacter, Bacillus, Brevibacterium, Kocuria, Microbacterium, Micrococcus, Pseudomonas*, and *Staphylococccus* (Fig. 1; Table 1). *Bacillus* (51%) was the most common AREB associated with *P. laevigata* and *S. angustifolia* in the studied zone.

Arsenic transformation by the AREB

With the silver nitrate assay (Table 2, also Supplementary Fig. S1), 16 As^{5+} reducers and 15 As^{3+} oxidizers were detected, in which 11 strains presented both the transformation capacities. Seven strains (*Bacillus endophyticus* CM2E2, NM2E16, CM2E3, CM1E4, *Bacillus niacini* CM1E5, *Pseudomonas stutzeri* CE1E1, and *Staphylococcus* sp. CE3E1) did not show transformation of arsenic under the experiment conditions (Table 2).

Quantitative arsenic transformation by the AREB

This assay was performed only for the 20 AREB strains that showed the As oxidizing and/or reducing activities. With the molybdenum blue method, only three As^{5+} reducers were confirmed. *Micrococcus luteus* NE2E1 showed the highest (94%) As^{5+} reduction effectiveness, while *Bacillus* sp. NM2E15 and CE3E2 reduced As^{5+} at the rate of 69 and 25%, respectively (Table 2). Only the strain *Pseudomonas zhaodongensis* NM2E7 presented oxidation (46%) of As^{3+} under the experiment conditions (Table 2).

Production of arsenophores to chelate As^{5+} and/or As^{3+}

Results indicated that 12 strains, including *Staphylococcus* (2), *Bacillus* sp. (4), *Microbacterium schleiferi* NE2E2, *Microbacterium* sp. NE2E3, *Pseudomonas stutzeri* CE1E1, *Bacillus endophyticus* NM2E16, *Pseudomonas zhaodongensis* NM2E7, and *Kocuria arsenatis* CM1E1 produced arsenophores against both As^{5+} and As^{3+}. Seven strains covering *Bacillus endophyticus* (2), *Bacillus* sp. (3), *Microbacterium arborescens* NE1E7, and *Brevibacterium metallicus* NM2E3 only produced arsenophores for As^{5+} (Table 2 and Supplementary Fig S2). None strain produced arsenophore only for As^{3+}.

Competent uptake of phosphate and arsenate by AREB

In this work, we evaluated the impacts of PO_4^{3-} and As^{5+} on the uptake of each other and of PO_4^{3-} on the arsenic reduction using the five representative strains (Table 3). The data in Table 3 showed that the PO_4^{3-} uptake of four strains decreased with the increase of As^{5+} concentration in the medium; except strain *Microbacterium* sp. NE2E3 that showed increased P uptake with the increase of As^{5+} concentration. In addition, 100% of As^{5+} was removed for all the five strains at presence of 0.1 mM PO_4^{3-}, and it was maintained at the high removing rate (95.9–100%) for four strains, except strains NE2E3 again that was decreased to 88.9%. The reduction efficiency of As^{5+} varied dramatically among the five strains under both the PO_4^{3-} concentrations: 13.5–100% at 0.1 mM PO_4^{3-} and 3.5–88.1% at 1 mM of PO_4^{3-}. In addition, interesting situation was observed again for *Microbacterium* sp. NE2E3: its reduction of As^{5+} was increased (from 50.0 to 88.1%), but it was decreased in the other four strains, as the PO_4^{3-} concentration increased. Furthermore, the growth of the four strains, except NE2E3, was better in medium at 0.1 mM of As^{5+} than that at 1 mM of As^{5+} (data not shown). According to the statistical analysis, the effects of 1 mM of arsenate on uptake of phosphate and 1 mM of PO_4^{3-} on reduction of arsenate by the test strains were significant ($P < 0.05$) compared with the effects of low concentration of them.

Amplification of genes involved in arsenic resistance (Ars operon)

The cytoplasmatic arsenate reductase (*ars*C) gene with a length of 304–376 bp (Supplementary Fig. S3) was amplified in 16 strains (Table 2). Sequences of the amplified fragments showed a similarity from 94 to 99% with the homologous genes in *E. coli* K12, *Agromyces* sp. H90, *Ochrobacterium* sp. kAs5-1, *Vibrio* sp. Ma14, *Pseudomonas* sp. pHAs-1, *Pseudoxanthomonas* sp. kAs5-1, and *Rhizobiaceae* sp. kAs5-1 published in GenBank database (data not showed). Despite efforts to optimize the PCR conditions, the *ars*B gene was not amplified from the AREB strains, while it was amplified from the positive control *Cupriavidus metallidurans* with approximate length of 750 bp (Supplementary Fig S4).

Discussion

A prerequisite for plants to accumulate and detoxify arsenic is that they must tolerate arsenic in the surrounding environment, such as soil, and/or in the plant tissue. In the present study, both *P. laevigata* and *S. angustifolia* were endemic in the area with arsenic and multiple HM contaminated soils; therefore, they could be arsenic/HM-resistant plants with potential in phytoremediation, just like the *Brassica juncea* and *Andropogon scoparius* reported in the previous studies (Pickering et al. 2000; Rocovich and West 1975).

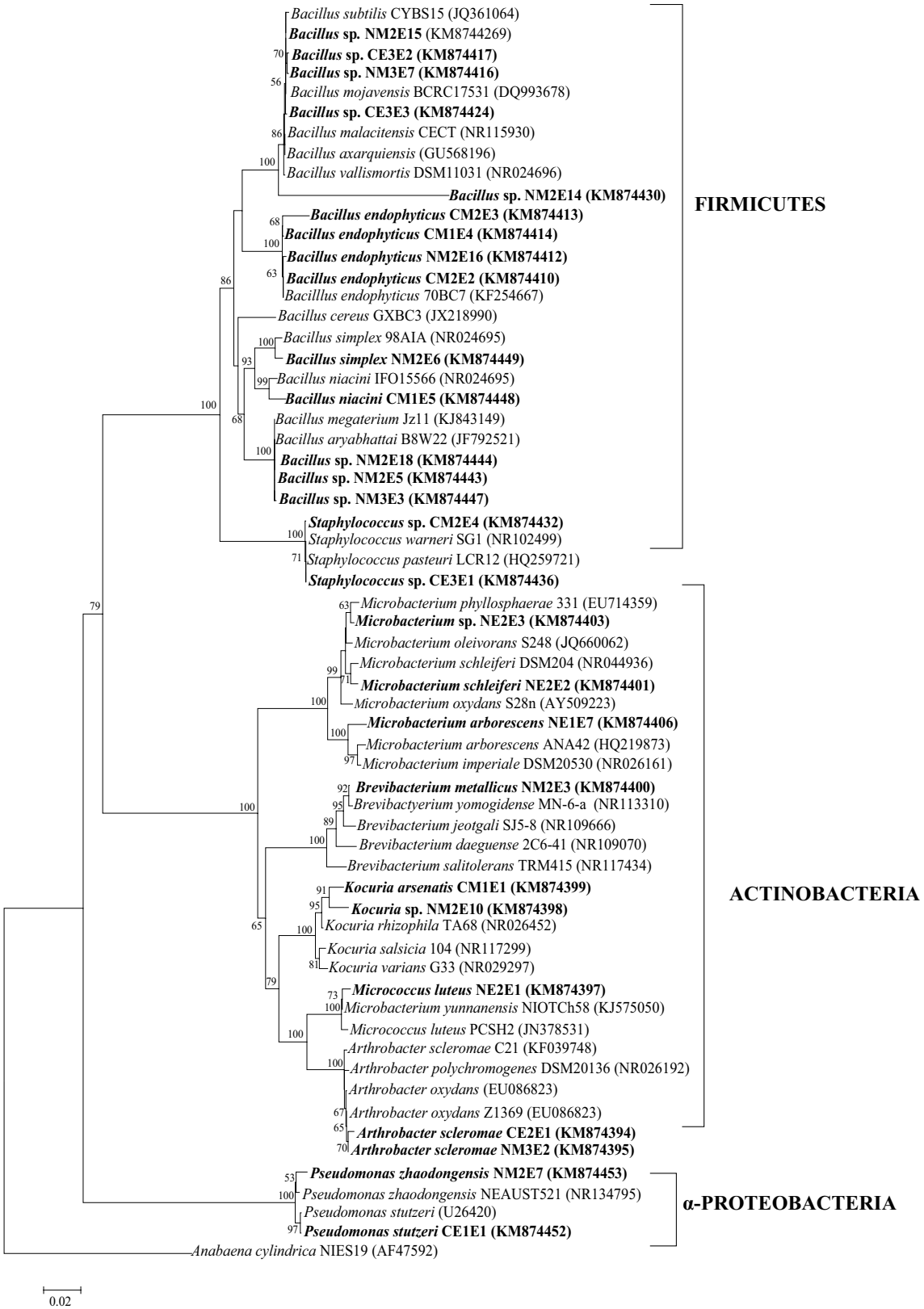

Fig. 1 Neighbor-joining (NJ) tree based on 16S rRNA genes sequences showing the genus affiliation of the 27 AREB isolated from two arsenic excluder plants (*P. laevigata* and *S. angustifolia*). Number above branches indicate bootstrap support (> 50%). *Anabaena cylindrica* NIES19 was included as outgroup. The scale bar presented 0.2 substitution of the nucleotide

Table 2 Arsenic transformation, arsenophores production and genes involved in the arsenic-resistance mechanism of AREB associated with *P. laevigata* and *S. angustifolia* plant

Strain	Arsenic transformation[a]		% of Arsenic transformation[b]		Arsenophore production		*Ars* Operon	
	AsO_4^-	AsO_3^-	AsO_4^-	AsO_3^-	AsO_4^-	AsO_3^-	*ars*C	*ars*B
Natural hill-*Spharealcea*								
NE1E7 (*Microbacterium arborescens*)	+	−	−	−	+	NG	−	−
NE2E1 (*Micrococcus luteus*)	+	+	94 ± 0.36	−	NG	NG	+	−
NE2E2 (*Microbacterium schleiferi*)	+	+	−	−	+	+	+	−
NE2E3 (*Microbacterium* sp.)	+	−	−	−	+	+	+	−
Natural hill-*Prosopis*								
NM2E3(*Brevibacterium metallicus*)	+	−	−	−	+	NP	−	−
NM2E5 (*Bacillus* sp.)	−	+	−	−	+	NG	+	−
NM2E6 (*Bacillus simplex*)	−	+	−	−	NG	NG	−	−
NM2E7 (*Pseudomonas zhaodongensis*)	+	+	−	46 ± 2.92	+	+	+	−
NM2E10 *Kocuria rhizophila*	+	+	−	−	NG	NG	+	−
NM2E14 (*Bacillus* sp.)	+	+	−	−	+	+	−	−
NM2E15 (*Bacillus* sp.)	+	+	69 ± 1.14	−	+	+	+	−
NM2E16 (*Bacillus endophyticus*)	−	−	−	−	+	+	−	−
NM2E18 (*Bacillus* sp.)	+	−	−	−	NG	NG	−	−
NM3E2 (*Arthrobacter scleorame*)	−	+	−	−	NG	NG	+	−
NM3E3 (*Bacillus* sp.)	+	−	−	−	+	NP	−	−
NM3E7 (*Bacillus* sp.)	+	+	−	−	+	+	+	−
Mine tailing-*Spharealcea*								
CE1E1 (*Pseudomonas stutzeri*)	−	−	−	−	+	+	+	−
CE2E1 (*Arthrobacter scleorame*)	+	+	−	−	NG	NG	+	−
CE3E1 (*Sthaplylococcus* sp.)	−	−	−	−	+	+	−	−
CE3E2 (*Bacillus* sp.)	+	+	25 ± 4.69	−	+	+	+	−
CE3E3 (*Bacillus* sp.)	+	+	−	−	+	NG	+	−
Mine tailing-*Prosopis*								
CM1E1 *Kocuria arsenatis*	−	+	−	−	+	+	+	−
CM1E4 (*Bacillus endophyticus*)	−	−	−	−	NG	NG	−	−
CM1E5 (*Bacillus niacini*)	−	−	−	−	NG	NG	−	−
CM2E2 (*Bacillus endophyticus*)	−	−	−	−	+	NP	+	−
CM2E3 (*Bacillus endophyticus*)	−	−	−	−	+	NP	−	−
CM2E4 (*Staphylococcus* sp.)	+	+	−	−	+	+	+	−

+ Positive or present; − negative or absent; *NP* not production

NG not growth

[a]By $AgNO_3$ test for qualitative detection the transformation ability

[b]By molybdenum blue method for quantitatively determination of the transformation ability

Our results (Supplementary Table S1) demonstrated that both the plant species presented arsenic contents in roots and shoots (6.16–256.21 mg kg^{-1}) significantly lower than those in the soils (841–4332 mg kg^{-1}). As suggested previously, the hyperaccumulator plant for arsenic should accumulate > 1000 mg kg^{-1} (Ma et al. 2001) or ten times greater than the background in plants: 0.01–1 mg kg^{-1} (Chaney 1989). Applying these criteria to the sampled plants in the present work, none of the plants involved in this study was hyperaccumulator for arsenic. However, the arsenic content in the two plants (Supplementary Table S1) was much greater than their normal content in plants (0.009–1.7 mg kg^{-1}) (Pais and Jones 2000). Therefore, these two plants could have contributed to the removing of arsenic from soil.

Baker (1981) divided the HM-resistant plants into three groups: accumulators that concentrate the element in the aerial parts, indicators in which the element content reflects the external concentrations and excluders that prevents element uptake until the soil concentration gets too high. According to the values of BAC and BTC (Supplementary

Table 3 Competent uptake of phosphate vs arsenate reduction and arsenate uptake of AREB

Strain	PO_4^{3-} (1 mM) uptake (%) at presence of			AsO_3^- removing (%) at presence of		AsO_3^- reduction (%) at presence of	
	0 AsO_3^-	0.1 AsO_3^-	1 AsO_3^-	0.1 PO_4^{3-}	1 PO_4^{3-}	0.1 PO_4^{3-}	1 PO_4^{3-}
NE2E2	57.9 ± 0.4aA	47.3 ± 0.5aA	7.8 ± 1.3cB	100 ± 0aA	99.6 ± 0.7aA	100 ± 0aA	12.1 ± 5.2aB
NE2E3	10.6 ± 18.3bA	23.1 ± 3.8bA	67.4 ± 28.3bB	100 ± 0aA	88.9 ± 2.1bB	50.0 ± 23.3bA	88.1 ± 20.1bB
NM2E5	60.9 ± 12.9aA	52.3 ± 2.2aA	16.0 ± 8.2aB	100 ± 0aA	95.9 ± 1.8cB	75.4 ± 5.7aA	25.5 ± 11.4cB
CM2E3	58.7 ± 1.9aA	52.3 ± 2.3aA	33.0 ± 6.1aB	100 ± 0aA	100 ± 0aA	13.5 ± 0.9cA	5.2 ± 0.7dB
CM2E4	56.6 ± 3.7aA	54.4 ± 6.9aA	50.8 ± 2.7aB	100 ± 0aA	100 ± 0aA	38.3 ± 5.5bA	3.5 ± 0.7dB

The assay was carried out after 48 h incubation in minimal buffer medium MES (MMBMES) added with 0.1 or 1 mM PO_4^{-3} and 0.1 or 1 mM As^{5+}. The values shown are the mean ± standard deviation of three replicates ($n = 3$). Values with the same letter in lower are not significantly different between strains (within the column), while values with the same capital letters are not significantly different between treatments (within the line) ($P < 0.05$ level with Tukey Post Hoc Test)

Table S1), *S. angustifolia* are excluder (BAC < 1, BTC > 1), while the BAC coefficients in the plants of *P. laevigata* in both sampling sites were much less than 1, suggesting that *P. laevigata* was not an arsenic accumulator. Nevertheless, the BTC value was 4.51 and 0.17 for *P. laevigata* in the mine tailing and in the hill, respectively, evidencing this plant an arsenic excluder only in the mine tailing. Our results of arsenic content in the plant tissues (Supplementary Table S1) also demonstrated that the arsenic contents in aerial parts and in roots varied according to the plant species and the sampling sites.

Based upon the plant analyses, we can conclude that both *S. angustifolia* and *P. laevigata* are highly arsenic-resistant plants than can accumulate arsenic in their tissues at a concentration much higher than the normal levels in other plants, although they are not hyperaccumulator for arsenic. Both the plant species have the character of excluder, but *P. laevigata* did not show this phenomenon in the hill, where the arsenic concentration was 70% (1301 mg kg^{-1}) lower than that (4332 mg kg^{-1}) at the mine tailing. Therefore, there should be other factors or mechanisms affected its resistance to the high concentration of arsenic, which may be related to the microbiome associated with the plant. Indeed, diverse AREB were isolated from these two plant species.

In the present study, eight genera were detected as AREB associated with *P. laevigata* and *S. angustifolia* (Table 1), in which *Bacillus, Pseudomonas, Microbacterium, Micrococcus*, and *Staphylococcus* have been previously reported as arsenate reducers (Guo et al. 2015; Jareonmit et al. 2012; Zhu et al. 2014). The dominance of *Bacillus*, which represented 74% of the arsenic AREB, was also similar to the previous report for *Pteris* plants (Zhu et al. 2014). These data demonstrated that these bacteria are the commons arsenic-resistant microbes in the contaminated environments. The detection of AREB belonging to the genera *Arthrobacter, Brevibacterium* and *Kocuria*, especially *Kocuria* as arsenate reducers was novel record in the present study. Although arsenic-resistant bacteria with MICs > 100 mM for As^{5+} have been isolated in the previous studies (Andrades-Moreno et al. 2014; Lampis et al. 2015), the AREB with high resistance to both As^{5+} (≥ 100 mM) and As^{3+} (> 20 mM) in the genera *Arthrobacter, Bacillus, Microbacterium* and *Staphylococcus* (Table 2) were not common. To estimate the mechanisms of high arsenic resistance in these bacteria, several representatives were further characterized.

First, the arsenic transformation by the AREB was investigated and many strains were identified as arsenic transformers with both the As^{3+} oxidation and As^{5+} reduction (Table 2). Very high As^{5+} reduction rate (94%) was detected in *Micrococcus luteus* NE2E1, while these values were 25 and 69% for *Bacillus* sp. CE3E2 and *Bacillus* sp. NM2E15, respectively, that were greater or in the range reported for endophytic bacteria of *P. vittate* and *P. multifida* (3–55%) (Zhu et al. 2014). The detection of As^{3+} oxidation in the genera *Pseudomonas, Bacillus, Micrococcus, Staphylococcus, Microbacterium, Arthrobacter*, and *Kocuria* demonstrated that this ability was more common in the AREB of *P. laevigata* and *S. angustifolia* grown in the studied area and *Pseudomonas* and *Bacillus* might be widely distributed arsenite oxidants as reported previously (Majumder et al. 2013). It has been known that As^{5+} is less toxic than As^{3+} (Fitz and Wenzel 2002); therefore, the oxidation by the AREB might help the host plant in resistance to arsenic. However, the real role of the AREB with high efficiency of arsenic transformation in the resistance of their host plant needs further study.

Until the date, the information of ligands production for chelate salts of As^{5+} and As^{3+} was limited, and only Nair et al. (2007) reported the arsenic ligands production by *Pseudomonas azotoformans*. In this study, we assayed the ligands production of AREB and we propose the term "arsenophore" for ligand able to chelate arsenic salts. (arseno from (arsenic) + phore (carry), referring to the ability of chelate arsenate and arsenite). The production of arsenophores by AREB was verified in diverse bacteria, including those in the genera

Microbacterium, Bacillus, Staphylococcus, Brevibacterium, Micrococcus, Pseudomonas, and *Kocuria* (Table 2). About 70% of the test strains produced arsenophores to chelate As^{5+}, whereas 44% synthesized arsenophores for As^{3+}. The chelation of arsenic irons might reduce its toxicity to the plants, since it has been reported that organic arsenicals are less toxic than the inorganic arsenicals; for example, arsenobetaine $[(CH_3)_3As + CH_2COOH]^-$ and arsenocholine $[(CH_3)_3As + CH_2CH_2OH]^-$ are not toxic (Fitz and Wenzel 2002).

Since the similar physicochemical features between phosphate and arsenate, the main pathway of entry for arsenate into the plant and bacterial cells is through the phosphate transport system (Mukhopadhyay et al. 2002; Oremland and Stolz 2003). Therefore, uptake competence between PO_4^{3-} and AsO_4^{3-} would be expected in numerous biological species (Willsky and Malamy 1980). In addition, Slaughter et al. (2012) reported that at high PO_4^{3-} concentrations, the reduction of AsO_4^{3-} was inhibited. These two estimations were consistent with our results for four of the five representative AREB, but opposite results were obtained in *Microbacterium* sp. NE2E3 (Table 3), that increased the phosphate uptake when the AsO_4^{3-} concentration increased and increased the AsO_4^{3-} reduction when PO_4^{3-} concentration increased. This increased P uptake by NE2E3 was coincided with the report of Ghosh et al. (2015) and it is possible that *Microbacterium* sp. NE2E3 has to increase the uptake of PO_4^{3-} to overcome the negative effect of AsO_4^{3-} inside its cells. Another interesting finding was that decreased removal of AsO_4^{3-} (from 100 to 88.9%) and increased AsO_4^{3-} reduction (50–88.1%) by strain NE2E3 at the presence of 1 mM PO_4^{3-} compared with those at 0.1 mM PO_4^{3-}. These data mean that half (0.5 mM) of AsO_4^{3-} was absorbed and another half was reduced by the bacterium at the presence of 0.1 mM PO_4^{3-}, while the presence of 1 mM PO_4^{3-} almost completely inhibited the AsO_4^{3-} uptake and the removal of AsO_4^{3-} was by the reduction.

For the other four strains, the decreased phosphate uptake in the cultures at the higher AsO_4^{3-} concentration (Table 3) evidenced the competition between the uptakes of AsO_4^{3-} and PO_4^{3-}. However, the increased PO_4^{3-} concentration has no or only slightly affected the removal of AsO_4^{3-} by the bacteria, since 100–95.9% AsO_4^{3-} was removed from the medium at the presence of 1 mM PO_4^{3-}. Meanwhile, the reduction of AsO_4^{3-} was significantly inhibited at 1 mM PO_4^{3-} compared with that at 0.1 mM PO_4^{3-}, which might be related the AsO_4^{3-} reduction to the tyrosine phosphatase, since it showed arsenate-reducing function and its gene (Wzb) expression was inhibited by high AsO_4^{3-} concentration (100 mg L^{-1}) in *Herbaspirillum* sp. GW103 (Govarthanan et al. 2015b). Therefore, the removal of AsO_4^{3-} by these four strains was mainly via the cell uptake. In another word, for these four strains, AsO_4^{3-} was more competent than PO_4^{3-}. These contrary phenomena between strain *Microbacterium* sp. NE2E3 and implied existence of different arsenic-resistant mechanisms in the tested AREB.

The *Ars* operon conferring arsenic resistance/tolerance in bacteria has been extensively studied (Carlin et al. 1995; Oremland and Stolz 2003). The essential genes of the system include transcriptional repressor (*Ars*R), efflux pump (*Ars*B), and arsenate reductase (*Ars*C) (Xu et al. 1998). The *ars*C gene has been located at chromosome or at plasmids in a large number of Gram negative bacteria belonging to Alpha- and Gamma proteobacteria, as well as Gram-positive bacteria such as Firmicutes (Páez-Espino et al. 2015). At the present study, *ars*C gene was amplified from 16 strains belonging to *Pseudomonas, Bacillus, Staphylococcus, Microbacterium, Kocuria*, and *Arthrobacter*, among them *ars*C has been previously reported in *Bacillus* and *Staphylococcus* (Anderson and Cook 2004). The amplification of *ars*C was failure in about 40% of AREB, suggesting that alternative pathway might be involved in As^{5+} reduction, or divergent *ars*C gene existed in the AREB strains. The amplification of *ars*B was failure in all the tested strains, implying the possibility that these AERB presented other As efflux pump, such as the ATP-dependent efflux pump (*Ars*B proteins) or the arsenite-carrier families (ACR3) (Páez-Espino et al. 2009). The *ars*B genes are more frequent in Firmicutes and Gamma Proteobacteria, whereas ACR3 carriers are more common in Actinobacteria and Alpha-proteobacteria (Achour et al. 2007).

Conclusions

Our research evidenced the arsenic-resistant plants *P. laevigata* and *S. angustifolia* as arsenic excluders. Diverse AREB associated with these two plants grown in the area with high arsenic contamination, and *Bacillus* was the most dominant group. Some of the AREB presented high resistance to both As^{5+} and As^{3+}, and different mechanisms of arsenic resistance, including arsenic oxidation/reduction and arsenophores production, have been developed in these bacteria. The arsenate may be absorbed by the AREB via the same receptor of phosphate uptake or via an alternative way in other case. Some endophyte bacteria may have arsenate reduction pathway different from the cytoplasmic arsenate reductase; therefore, these AREB could be candidate for improving the bioremediation process.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with humans or animals performed by any of the authors.

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