

Cultivable endophytic bacteria from heavy metal(loid)-tolerant plants

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Abstract To evaluate the interactions among endophytes, plants and heavy metal/arsenic contamination, root endophytic bacteria of *Prosopis laevigata* (Humb and Bonpl. ex Willd) and *Sphaeralcea angustifolia* grown in a heavy metal(loid)-contaminated zone in San Luis Potosi, Mexico, were isolated and characterized. Greater abundance and species richness were found in *Prosopis* than in *Sphaeralcea* and in the nutrient Pb–Zn-rich hill than in the poor nutrient and As–Cu-rich mine tailing. The 25 species identified among the 60 isolates formed three groups in the correspondence analysis, relating to *Prosopis*/hill (11 species), *Prosopis*/mine tailing (4 species) and *Sphaeralcea*/hill (4 species), with six species ungrouped. Most of the isolates showed high or extremely high resistance to arsenic, such as ≥ 100 mM for As(V) and ≥ 20 mM for As(III), in mineral medium. These results demonstrated that the abundance and community composition of root endophytic bacteria were strongly affected by the concentration and type

of the heavy metals and metalloids (arsenic), as well as the plant species.

Keywords Endophyte · Resistance · Heavy metal(loid) · Bacteria · Phylogeny · Association

Introduction

Heavy metal(loid)s (HMs) contamination is a serious problem for deterioration of soil, which cause decrease in microbial abundance and diversity in soil and contamination of plants, and in turn formed a threaten for animals and human health. The heavy metals and metalloids in soils may come from the weathering of mother rocks, but the great accumulation of these toxic elements in many of the contaminated zones is caused by the human activities, such as the mining activities. As by-products of mining, a large amount of residues (mine tailings) with high levels of heavy metals (Ni, Cd, Pb, Mn, Cu and Zn) and metalloids (As and Sb) are normally deposited in open areas, which cause heavy metal and metalloid pollution of soil, surface water and groundwater (Navarro-Noya et al. 2010). Mexico is one of the most important mining countries, as the most important producer of silver (Ag) and the fifth producer of lead (Pb) in the world (The Economist 2003). Therefore, huge mine spoils have been accumulated throughout the country, especially in the most important mining regions at North and Central Mexico (Franco-Hernández et al. 2010).

As the mining district, Santa María de la Paz is located between the municipalities of Villa de la Paz and Matehuala in the state of San Luis Potosí, Mexico. A skarn deposit of Pb–Zn–Ag (Cu–Au) [metamorphic rocks made of silicates of calcium (Ca), iron (Fe) and magnesium (Mg) derived from a protolith limestone and dolomite in which

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great quantities of aluminum (Al), Fe and Mg have been introduced] is found in this district. In Villa de la Paz, mining has been taking place for the past 200 years, and it has generated a great amount of waste, which lies in open air and is exposed to environmental weathering, presenting a risk of environmental pollution (Espinosa-Reyes et al. 2014). Several studies have been done in that mining region to determine whether risk to human health and diverse biological components exists. The previous studies have revealed the environmental characteristics (Chiprés et al. 2007), evaluated the risk for the health of local people by exposing to heavy metals and As (Gamiño-Gutiérrez et al. 2013) and estimated the effects of heavy metal(loid) pollution on diversity of flora and fauna (Jasso-Pineda et al. 2007; Machado-Estrada et al. 2013).

Generally, mine tailings have geochemical properties that inhibit plant establishment. However, some plant species tolerating high concentrations of phytotoxic heavy metal(loid)s have been found on the mine tailings. The microbial communities associated with these plants may have capacity to detoxify metals by transforming them into insoluble salts or relatively non-toxic oxidized states (Kuiper et al. 2004; Mastretta et al. 2006). Previously, some studies on the endophytic microorganisms associated with the heavy metal-resistant plants have been performed focused upon characterization of the bacteria (Barzanti et al. 2007; Bhojiya and Joshi 2012; Idris et al. 2004; Sun et al. 2010; Wei et al. 2009; Zhu et al. 2014), their effects on growth of the host plants and/or on their potential in bioremediation of the contaminated soils (Guo et al. 2010; Luo et al. 2011; Mastretta et al. 2006; Sheng et al. 2008; Shin et al. 2012; Zhang et al. 2011). These studies have revealed that genera *Sphingomonas*, *Bacillus*, *Microbacterium*, *Arthrobacter*, *Pseudomonas* and *Serratia* are the common rhizospheric and endophytic bacteria associated with hyperaccumulating plants from mine tailings and heavy metal-contaminated soils (Barzanti et al. 2007; Guo et al. 2010; Idris et al. 2004; Shin et al. 2012; Zhang et al. 2011). However, effects of the heavy metals and arsenic contaminants and the host plants on the abundance and the community composition of endophytes have not been well revealed. Previously, it has been described that the plant-associated microorganisms, like the endophytic bacteria, were selected by both of the environmental factors and the plant species (Deng et al. 2011; Zhang et al. 2011); therefore, we hypothesized that the heavy metals and arsenic contaminants in soils have selected the plants and the plant-associated microbial communities. Considering the potential use in bioremediation of the heavy metal-resistant plants and microorganisms associated with them, investigation of both the plants and their microbial partners is an important field of microbial ecology,

for understanding the interactions among the microbes, plants and environmental factors.

Based upon the information described above and the absence of study about the endophytic bacteria from endemic plants growing on tailings in mine district of Villa de la Paz, San Luis Potosí, Mexico, we performed this study to evaluate the interactions among the endophytic bacteria, the host plants and the environmental factors. In this study, the endophytic bacteria were isolated from two plant species grown in a mine tailing and a natural hill at Villa de la Paz, while the correlations among the bacterial species, the soil characters and the host plants species were estimated.

Materials and methods

The sampling sites

The sampling sites are located in Villa de la Paz in the state of San Luis Potosí (23.7 N, 178.7 W), a zone with mean annual temperature of 18 °C and average annual precipitation of 486 mm. The two sampling sites are a mine tailing with altitude of 1557 m and a natural hill with altitude 1830 m, with a distance about 5 km between them. In a previous study, arsenic (As) above 8420 mg kg⁻¹, lead (Pb) above 754 mg kg⁻¹, copper (Cu) greater than 1154 mg kg⁻¹ and zinc (Zn) above 1386 mg kg⁻¹ have been detected in the mine tailing (Franco-Hernández et al. 2010). However, the levels of these metals in the hill were not reported yet.

Sampling of plants and soils

For plant sampling, two endemic and common plant species in both sites were sampled, which were identified as *Prosopis laevigata* (Humb and Bonpl. ex Willd) (Smooth Mesquite) and *Sphaeralcea angustifolia* (commonly named as copper globemallow, an herbaceous perennial plant) by botanist in our school. For each species, three individuals were randomly sampled by digging out the whole roots (for *S. angustifolia*) or lateral roots (for *P. laevigata*) in each site. About one kg of soil surrounding the roots was also sampled. Once collected, the root and soil samples were stored in polyethylene bags at 4 °C for several days until further analysis.

Soil characterization

Three soil samples of the same plant at the same site were air-dried, sieved with a 2-mm sieve and mixed to form a compile sample. The physicochemical features of the compile soil sample were analyzed with the techniques used by Vásquez-Murrieta et al. (2006) and Franco-Hernández

et al. (2010). For soil pH determination, soil–H₂O suspension (1:2.5, w/w) was prepared and measured by using a 716 DMS Titrimo pH meter (Metrohm Ltd. CH-901 Herisau, Switzerland). For organic matter determination, soil was oxidized with potassium dichromate. For total N determination, the Kjeldahl method was used by digesting the sample with concentrated H₂SO₄, K₂SO₄ and HgO. The soil texture was estimated with the hydrometer method. The total phosphorous content was measured with the Olsen method. The concentrations of metal elements in soil were determined with an inductively coupled plasma-optical emission spectrometer (4600DV-PerkinElmer, USA) as described by Franco-Hernández et al. (2010).

Isolation and preservation of the endophytic bacteria

For isolation of endophytes, 1 g of thin roots (<0.5 mm in diameter) was washed with sterile distilled water, rinsed in 70 % (v/v) ethanol for 30 s, immersed in sodium hypochlorite solution (2.5 % available Cl⁻) for 5 min and finally washed five times with sterile distilled water (Marquez-Santacruz et al. 2010). Subsequently, the samples were ground in 9 mL of 0.85 % NaCl under aseptic condition (Barzanti et al. 2007). Aliquots (100 µL) of tissue extracts (10⁻¹–10⁻³) were plated in triplicate on tryptic soy agar (TSA, Difco), which is a widely used medium for isolating endophytic bacteria (Arvind et al. 2009), Luria–Bertani (LB) medium (Praveena and Bhore 2013) and peptone-yeast extract (PY) medium (Wang et al. 2006) in plates. The three media were used to ensure the isolation of diverse bacteria. To confirm that the disinfection process was successful, the aliquots of the sterile distilled water used in the final rinse were set on TSA, LB and PY plates and incubated at 28 °C for one to 14 days (Sun et al. 2010).

The plates inoculated with root extracts were incubated at 28 °C for one to 14 days (Marquez-Santacruz et al. 2010). Colonies formed in the plates were counted to estimate the abundance of endophytes bacteria (CFU g⁻¹ of fresh tissue). Single colonies with different morphologies were selected and purified by repeated cross-streaking, until very similar colonies in the same isolate were obtained. The conservation of the isolates was performed by growing the bacteria on TSA plates and then in 5 mL of nutrient broth at 28 °C for 24–48 h. The bacterial cells were preserved in 50 % glycerol (w/v) and stored at –70 °C.

Genomic DNA extraction, PCR amplification and sequencing of bacterial 16S rRNA

Genomic DNA of endophytic bacteria was extracted from each isolate (in 5 mL of TSA medium at 28 °C with agitation of 150 rpm, 18 h) using the protocols described previously by Román-Ponce et al. (2015) and was used as

template to amplify 16S rRNA genes. The 16S rRNA gene was amplified by PCR with a thermocycler (Maxygene Thermal Cycler Therm 1061 Axygen Scientific) using an initial denaturing step of 5 min at 94 °C followed by 30 cycles of 45 s at 94 °C, 1 min of annealing at 57 °C, 90 s extension at 72 °C and a final polymerization step for 8 min at 72 °C. The PCR mixture (25 µL) contains 10–100 ng of DNA template, 1.5 mM MgCl₂, 2.5 U *Taq* DNA polymerase (Invitrogen, USA), 1× PCR buffer, 100 pmol of primers fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rD1 (5'-AAG GAG GTG ATC CAG CC-3') and 200 µM of each dNTP. Amplification products were visualized after electrophoresis in agarose gel (1 %, w/v) in the buffer of 1× TAE, by staining with an aqueous solution of ethidium bromide (0.5 µg mL⁻¹); then, they were purified with a commercial kit PureLink (Invitrogen 310002) and sequenced under Big Dye™ terminator cycling conditions with the same primers using Automatic Sequencer 3730XL in MacroGen (Korea).

Phylogenetic analysis

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 were aligned using CLUSTAL X (2.0) software (Larkin et al. 2007), and the presence of chimerical sequences was checked with the RDP Chimera Check program. The problem sequences were manually edited with SEAVIEW software (Galtier et al. 1996). Phylogenetic relationships were constructed by maximum likelihood using PhyML [<http://www.atgc-montpellier.fr/phyml>] (Guideon and Gascuel 2003). jModelTest 3.06 software (Darriba et al. 2012) was used to select appropriate models of sequence evolution by the AIC (Akaike information criterion). The GTR + I + G model ($\alpha = 0.4450$) for the gamma distribution and $p\text{-inv} = 0.1720$ were used. The confidence at each node was assessed by 100 bootstrap replicates, and *Flavobacterium phragmitis* was used as out-group. Similarities among sequences were calculated using the MatGAT v.2.01 software (Campanella et al. 2003). Taxonomic assignment was obtained by using the Roselló-Mora prokaryotes criteria (Roselló-Mora and Amman 2001).

Species richness and community composition analysis

The distance matrixes (generates in Phylip 3.695 software) were used to obtain the operational taxonomic units (OTUs) for each site of sampling. A 3 % distance level between sequences was considered as the cutoff among different OTUs. Rarefaction curve, richness estimator (bias-corrected Chao1) and Simpson's diversity index (D) were

evaluated using DOTUR software version 1.51 (Schloss and Handelsman 2005) for each site.

Physiological characterization of the isolates

Range and optimum pH and salinity for growth were estimated on TSA plates. The pH values of TSA medium were adjusted to 4.5 through 13 with interval of 1 by adding 1 N HCl or KOH after autoclaved at 121 °C for 15 min. For salinity analysis, concentrations of NaCl in TSA (pH 7.3) were adjusted to 1 through 20 % (w/v) with interval of 2 %. Inoculation of microorganisms was performed by pitting with sterile toothpicks. The plates were incubated at 28 °C for 24–48 h, and then, the diameter of the colonies was measured and compared with those grown in TSA plates at pH 7.3 without addition of salt.

Enzymatic activity

The productions of following enzymes were analyzed for each isolate: amylase, cellulase, xylanase and pectinase. The enzymatic activities were performed by initially growing the isolates in TSA for 24 h at 28 °C. For detection of amylase and pectinase, the culture medium was prepared according to Mendoza-Gamboa (2007) consisted of (g L⁻¹): KH₂PO₄, 0.375; NaCl, 0.25; MgSO₄, 0.275; Na₂CO₃, 0.375, yeast extract, 1; casamino acids, 1; and agar 15. The pH was adjusted to 6.2; the soluble starch and pectin were added at the final concentration of 1 % according to Tomova et al. (2013) and Tenorio-Sánchez et al. (2010). In the case of the cellulose and xylanase, the isolates were tested on mineral medium as previously described by Trujillo-Cabrera et al. (2012), separately supplied with carboxy methyl cellulose (CMC, amorphous cellulose), and xylan (hemicellulose) at final concentration of 2 % (w/v) and Congo red (CR) was used as indicator (Ghush et al. 2007). Each bacterium was inoculated by pricking in twenty squares of 1 × 1 cm in a plate, using sterile toothpicks. The cultures were incubated at 28 °C during 96 h. The enzymatic index (EI) was determined within 24, 48, 72 and 96 h of incubation, according to specific methodologies for each investigated enzyme. The EI was expressed by the relationship between the average diameter of the transparent (degradation) ring and the average diameter of the colony (Ramos-Garza et al. 2016).

Plant growth-promoting characters

Siderophore production was performed following the protocol described by Perez-Miranda et al. (2007) and Achari and Ramesh (2014). Mineral phosphate solubilization activity was assayed according to Kuklinsky-Sobral et al. (2004). Indoleacetic acid (IAA) production was analyzed

using a modified qualitative method (Ma et al. 2013), and the ability of nitrogen fixing was estimated according to Mohan and Rajendran (2014). After being treated with Salkowski reagent for 30 min, appearance of a pale pink ring on the filter paper from LB medium was positive for the IAA assay. An orange annulus appeared around the colony was defined as positive for siderophore production. Growth in the nitrogen-free medium was taken as indicator of nitrogen fixation. The presence of a clear ring around the colony in the medium supplemented with inorganic phosphate was considered as positive for solubilization of phosphate.

Heavy metal(loid)s resistance

The minimum inhibitory concentration (MIC) of heavy metals and arsenic was carried out by inoculating triplicate in plates of LB medium supplemented with various concentrations of Cu(II) (2, 4, 12, 20, 40 mM CuSO₄), Zn(II) (2, 4, 12, 22 mM ZnSO₄), and metalloids As(V) (3, 6, 18, 30, 72, 144, 203, 305, 360, 480 mM NaH₂AsO₄) and As(III) (5.8, 14.42, 28.8 mM NaAsO₂). For Pb(II), minimal medium was used (Pastor et al. 2012), and the Pb(II) concentrations were 3.12, 12.5, 20 mM [Pb(NO₃)₂]. MIC was considered as the lowest concentration of heavy metals and metalloids that completely inhibited the bacterial growth (Luo et al. 2011). Resistance of endophytic bacteria to the HMs was also determined using the MES-buffered minimal medium (MBMM) (Rathnayake et al. 2013) supplemented with different amounts (0.05, 0.1, 0.5, 1, 1.5 and 5 mM) of Cu(II) (CuSO₄), Zn(II) (ZnSO₄) and Pb(II) [Pb(NO₃)₂], and of metalloids As(V) (0.25, 0.5, 1, 5, 20, 50, 100 mM NaH₂AsO₄) and As(III) (0.0125, 0.025, 0.05, 0.5, 5, 10, 20 mM NaAsO₂).

Statistical analysis

This analysis was performed to evaluate the difference on the heavy metals and metalloids minimum inhibitory concentration (MIC) for the isolates between the sites and plants. The data from three replications (n = 3) were subjected to a one-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/>).

For estimation of correlation between the strains in their enzymatic activities and plant growth-promoting characters, the positive properties were marked as “1” and negatives as “0.” NTSYSpc (Numerical Taxonomy and Multivariate Analysis System) version 2.1 software (Rohlf 2000) was used to perform the similarity matrix and cluster analysis. Phenotypic association among the strains was measured by the SM similarity coefficient (Shaffer and Rogers 1993)

Table 1 Physicochemical characters of the sampled soils from the root zones of two plants grown in Villa de la Paz, SLP

Soil characters	Mine tailing			Natural hill		
	<i>Sphaeralcea</i>	<i>Prosopis</i>	Average \pm SD	<i>Sphaeralcea</i>	<i>Prosopis</i>	Average \pm SD
Texture	Sandy loam			Loam		
pH	6.90	6.96	6.93 \pm 0.03	7.06	6.91	6.98 \pm 0.08
CEC ^a (cmolc kg ⁻¹)	37.50	40.20	38.85 \pm 1.35	80.00	75.83	77.92 \pm 2.08
EC ^a (dS m ⁻¹)	2.37	1.65	2.01 \pm 0.29	0.74	0.38	0.56 \pm 0.18
OM ^a (%)	3.12	0.70	1.91 \pm 1.21	5.73	9.69	7.71 \pm 1.98
Total N (mg kg ⁻¹ soil)	0.11	0.07	0.09 \pm 0.02	0.40	0.25	0.32 \pm 0.08
Total PO ₄ ³⁺ (mg kg ⁻¹ soil)	40.36	26.60	33.48 \pm 6.88	62.20	76.03	69.11 \pm 6.92
Metal (mg kg ⁻¹)						
As ^b	2816.30	4332.70	3574.50 \pm 758.20	841.70	1301.30	1071.50 \pm 229.80
Cd	100.06	158.11	129.08 \pm 29.02	39.47	85.25	62.36 \pm 22.89
Co	2.84	2.87	2.86 \pm 0.02	0.24	0.00	0.12 \pm 0.12
Cr	17.39	11.25	14.32 \pm 3.07	13.89	5.71	9.80 \pm 4.09
Cu	1605.00	2599.30	2012.15 \pm 497.15	744.70	753.90	749.30 \pm 4.60
Fe	31083.00	39438.30	34760.65 \pm 3677.65	29083.00	42266.00	35674.50 \pm 6591.50
Li	6.21	4.83	5.52 \pm 0.69	5.62	4.89	5.26 \pm 0.36
Mg	2975.70	2350.30	2663.00 \pm 312.70	2560.60	2167.70	2364.15 \pm 196.45
Mn	984.83	1780.50	1382.66 \pm 397.84	1532.50	4576.40	3054.45 \pm 1521.95
Mo	7.78	10.31	9.04 \pm 1.26	5.53	8.21	6.87 \pm 1.34
Ni	6.58	3.78	5.18 \pm 1.40	8.88	5.49	7.18 \pm 1.70
Pb	506.87	604.03	555.45 \pm 48.56	3083.90	7893.30	5488.60 \pm 2404.70
Sb	28.85	36.33	32.59 \pm 3.74	50.29	98.39	74.34 \pm 24.05
Ti	410.05	300.92	355.48 \pm 54.56	325.23	202.34	263.78 \pm 61.44
V	60.83	66.99	63.91 \pm 3.08	45.95	34.22	40.08 \pm 5.86
Zn	1058.28	1618.10	1337.19 \pm 280.91	1152.50	2193.20	1672.85 \pm 520.35

^a CEC cation exchange capacity, EC electrolytic conductivity, OM organic matter

^b Elements in boldface are those with higher concentration than the normal ranges in the soil. The natural range in agricultural soils (mg kg⁻¹): As, 0.1–40; Cd, 0.01–2.0; Co, 1–40; Cr, 5–1000; Cu, 2–100; Fe, 1–5 %; Mn, 850; Mo, 2; Ni, 5–500; Pb, 2–300; Zn, 20

with the SIMQUAL (Similarity for qualitative data program in NTSYS) module of NTSYS-pc software. The similarity matrix was subjected to cluster analysis of UPGMA (unweighted pair group method with arithmetic mean), and a dendrogram was generated by using the SAHN (sequential, agglomerative hierarchical and nested clustering) module of NTSYS-pc. The trees were statistically assessed by means of the cophenetic correlation coefficient (CCCr) using Mantel's test.

Simple correspondence analysis between the sampling sites, host species and the genomic species of endophytic bacteria defined in this study was performed by using the Correspondence 1.0 program in the SPSS 12.0 package. The levels of the variables were two sampling sites, two hosts and 21 genomic species. The Pearson coefficient was used to calculate the correlation, and the results were presented in a two-dimensional figure. The multiple relationships between soil factors (nutrients, physicochemical and heavy metal and metalloid contents) and genospecies of

the endophytic bacteria isolated from the two sites and two host plants were estimated by the canonical correspondence analysis (CCA) using CANOCO software (Microcomputer Power, Ithaca, NY) (Šilaver and Lepš 2014).

Results

Soil physicochemical characters

The physicochemical characters summarized in Table 1 demonstrated that the soils were sandy loam in the mine tailing and loam in the natural hill, with neutral pH 6.90–7.06. Both sites had soils with concentrations of As, Cd, Cu, Mn, Mo, Pb and Zn much higher than the normal ranges; therefore, both sites were contaminated with heavy metals, and both *S. angustifolia* and *P. laevigata* were multiple heavy metal and arsenic-resistant plants. The concentrations of As, Cd, Cr, Cu and Mo were greater in mine

tailing than those in the hill, while the concentrations of Mn, Pb and Zn were reverse. Furthermore, the concentrations of As, Cd, Cu, Mn, Mo, Pb, Sb and Zn in the root zones of *S. angustifolia* were always lower than those in the root zones of *P. laevigata*, demonstrating that the former might be a potential accumulator for these heavy metals, while *P. laevigata* might be more efficient for removing Cr, Ni and Ti (Table 1).

Isolation of endophytic bacteria

The surface sterilization protocol used in this study was efficient since no bacterial colony was observed in the control plates. A total of sixty aerobic heterotrophic endophytic isolates were obtained, including 40 from the hill site (8 from *Sphaeralcea* and 32 from *Prosopis*) and 20 from the mine tailing (7 from *Sphaeralcea* and 13 from *Prosopis*) (Table 2, detailed information available as Supplementary Table S1). The abundance of endophytic bacteria (CUF g⁻¹ fresh weight) ranged from 4.41×10^4 in *Prosopis* to 2.91×10^4 in *Sphaeralcea* in the hill site and from 1.35×10^3 for *Prosopis* to 2.3×10^2 for *Sphaeralcea* in mine tailing. The ratio of bacterial abundances was 21.5 and 191.7 between the natural site and mine tailing for *S. angustifolia* and *P. laevigata*, and was 1.5 and 5.9 between the plants *P. laevigata* and *S. angustifolia* in the natural site and in the mine tailing, respectively.

Identification of the endophytic bacteria

The sequences of 16S rRNA genes obtained from the 60 bacterial isolates have been deposited in GenBank under the accession numbers KM874394 through KM874453, each presented about 1400 bp. The phylogenetic analyses of the sequences identified them within ten genera: *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Kocuria*, *Leucobacter*, *Microbacterium*, *Micrococcus*, *Nocardiopsis*, *Pseudomonas* and *Staphylococcus* (Fig. 1). Fifty-five isolates showing sequence similarities $\geq 97\%$ with those of the defined species were designed as 20 described species (Table 2). While the five isolates NE1E1p, NE1E3, NM2E3, NM3E10 and CM1E1 showing similarities between 95.0 and 96.1 % with the defined species were designed as putative novel species in the genera *Staphylococcus*, *Microbacterium*, *Brevibacterium*, *Bacillus* and *Kocuria*, respectively (Supplementary Table S1 for detail). The ratio of species numbers between the natural site and mine tailing was 7:4 and 15:8 for *S. angustifolia* and *P. laevigata*, respectively (Table 2). *Bacillus endophyticus* was detected from *P. laevigata* in both sites, but not from *S. angustifolia*. “*Bacillus aryabhatai*”, *Microbacterium schleiferi* and *Micrococcus luteus* were found in both plants in the hill, while “*Staphylococcus warneri*” was found in both plants in the mine tailing.

Arthrobacter scleromae, “*Bacillus axarquiensis*”, *Microbacterium arborescens* and *Pseudomonas stutzeri* were found in both sites, but associated with different plants. The remaining isolates were originated from one of the two host plants grown in one of the two sites (Table 2).

Community richness and diversity analysis

Rarefaction analysis showed different degrees of diversity in the two sites (available as Supplementary Fig. S2). A decline in the rate of OTUs indicated that only the most dominant bacteria were detected in this study. The coverages percent was 72.5 and 44 % for the hill and mine tailing sites, respectively. Analysis with the DOTUR software also showed that the hill site had higher richness (bias-corrected Chao1 = 29.25) and diversity ($D = 0.0679$) than the mine tailing site (bias-corrected Chao1 = 18; $D = 0.17$).

Physiological characterization

The physiological characterization (as summarized in supplementary Table S2) demonstrated that the optimum pH for growth of all the isolates was 8.0. More than 85 % of the endophytic bacteria could tolerate a wide range of pH from 6.0 to 10.0. About 63 % of the isolates grow well at pH 11.0, while 22 % of the isolates could grow at pH 12.0. The endophytic bacteria *Micrococcus luteus* NM2E1, *Pseudomonas stutzeri* NM2E2, NM2E4, *Arthrobacter scleromae* NM3E2 and *Staphylococcus saprophyticus* NM3E5 could grow at pH 13. All the isolates grew well in medium with 1 % (w/v) of NaCl; about 50 % of the endophytic bacteria could grow in medium with 13 % (w/v) of NaCl; 18 % of the isolates could tolerate 15 % (w/v) of NaCl and two isolates (*Staphylococcus* spp. CE3E4 and CE3E5) could grow in medium supplied with 20 % (w/v) of NaCl.

Analysis of enzymatic activities and plant growth-promoting characteristics

The results revealed that 59.3 % (35/59, positive isolates/grown isolates in the medium), 54.9 % (28/51), 47.8 % (22/46), and 72.5 % (29/40) of the isolates showed activities of amylases, xylanases, cellulases and pectinases, respectively (Table 2 and Supplementary Fig. S1). Among the endophytes of *Sphaeralcea*, more bacteria with activities of amylase and pectinase and fewer bacteria with activities of xylanase and cellulase were found in the mine tailing than in the natural hill (Table 2, also Supplementary Fig. S1). For the endophytes of *Prosopis*, fewer bacteria with activities of amylase and cellulase and more bacteria with activities of xylanase and pectinase were found in the mine tailing than in the natural hill. Nine isolates, *Bacillus* spp. NM1E3, NM1E5, NM2E8, NM2E15, NM2E18 and CM1E6,

Table 2 Identification, origin of host, sampling site and abundance of endophytic bacteria associated with roots of heavy metal-resistant plants *Sphaeralcea angustifolia* and *Prosopis laevis* in a mine zone of San Luis Potosí, Mexico

No.	Species	Natural hill		Mine tailing		Total
		<i>Sphaeralcea</i>	<i>Prosopis</i>	<i>Sphaeralcea</i>	<i>Prosopis</i>	
1	<i>Arthrobacter scleromae</i>		1	1		2
2	“ <i>Bacillus aryabhatai</i> ” ^a	1	4			5
3	<i>Bacillus atrophaeus</i>				1	1
4	“ <i>Bacillus axarquiensis</i> ” ^b		10	2		12
5	<i>Bacillus cereus</i>		2			2
6	<i>Bacillus endophyticus</i>		2		3	3
7	<i>Bacillus niacini</i>				1	1
8	<i>Bacillus simplex</i>		1			1
9	<i>Bacillus</i> sp.		1			1
10	<i>Bacillus vallismortis</i>				3	3
11	<i>Brevibacterium</i> sp.		1			1
12	<i>Kocuria rhizophila</i>		1			1
13	<i>Kocuria</i> sp.				1	1
14	<i>Leucobacter aridicollis</i>		2			2
15	<i>Microbacterium arborescens</i>	1			1	2
16	<i>Microbacterium</i> sp.	1				1
17	<i>Microbacterium oxydans</i>	1				1
18	<i>Microbacterium schleiferi</i>	1	1			2
19	<i>Micrococcus luteus</i>	1	1			2
20	<i>Nocardiopsis dassonvillei</i>		1			1
21	<i>Pseudomonas stutzeri</i>		3	1		4
22	<i>Staphylococcus epidermidis</i>	1				1
23	<i>Staphylococcus saprophyticus</i>		1			1
24	<i>Staphylococcus</i> sp.	1				1
25	“ <i>Staphylococcus warneri</i> ” ^c			3	3	6
Total number of strains/species/genera		8/8/4	32/15/10	7/4/4	13/7/4	60/25/10
Abundance (CFU g ⁻¹ of fresh root)		2.9 × 10 ⁴	4.4 × 10 ⁴	1.4 × 10 ³	2.3 × 10 ²	–
Coveratures		72.5 %		44 %		
Bias-corrected Chao1		29.25		18.00		
<i>D</i>		0.0679		0.1700		
Siderophore producers	%	0	21.4	57.1	7.7	19.3
	Positive/total strains ^d	0/8	6/28	4/7	1/13	11/57
P-solubilizers	%	0	28.0	20.0	0	18.2
	Positive/total strains ^d	0/2	7/25	1/5	0/13	8/44
IAA producers	%	25.0	19.2	0	0	9.2
	Positive/total strains ^d	2/8	5/26	0/7	0/12	5/54
Potential N-fixers	%	12.5	14.3	40.0	0	15.4
	Positive/total strains ^d	1/8	5/29	2/5	0/10	8/52

^a These strains showed 99.6 % sequence similarity of 16S rRNA gene with both *Bacillus megaterium* and *B. aryabhatai*

^b These strains showed 97.0 % sequence similarity of 16S rRNA gene with *Bacillus axarquiensis* and *B. malacitensis* or *B. subtilis*

^c These strains showed 98.0–99.9 % sequence similarity of 16S rRNA gene with *Staphylococcus warneri* and *S. pasteri*

^d The strains that could not grow were not considered for calculating the percentage

Nocardiopsis dassonvillei NM1E1, *Microbacterium schleiferi* NM1E4 and *Leucobacter aridicollis* NM2E13 showed activities for all the four enzymes (Supplementary Fig. S1).

The highest amylase activity was found in NM1E1 (EI = 7), and all the other isolates presented EI ≤ 3.5. The isolate *Microbacterium oxydans* NE2E3 presented the highest

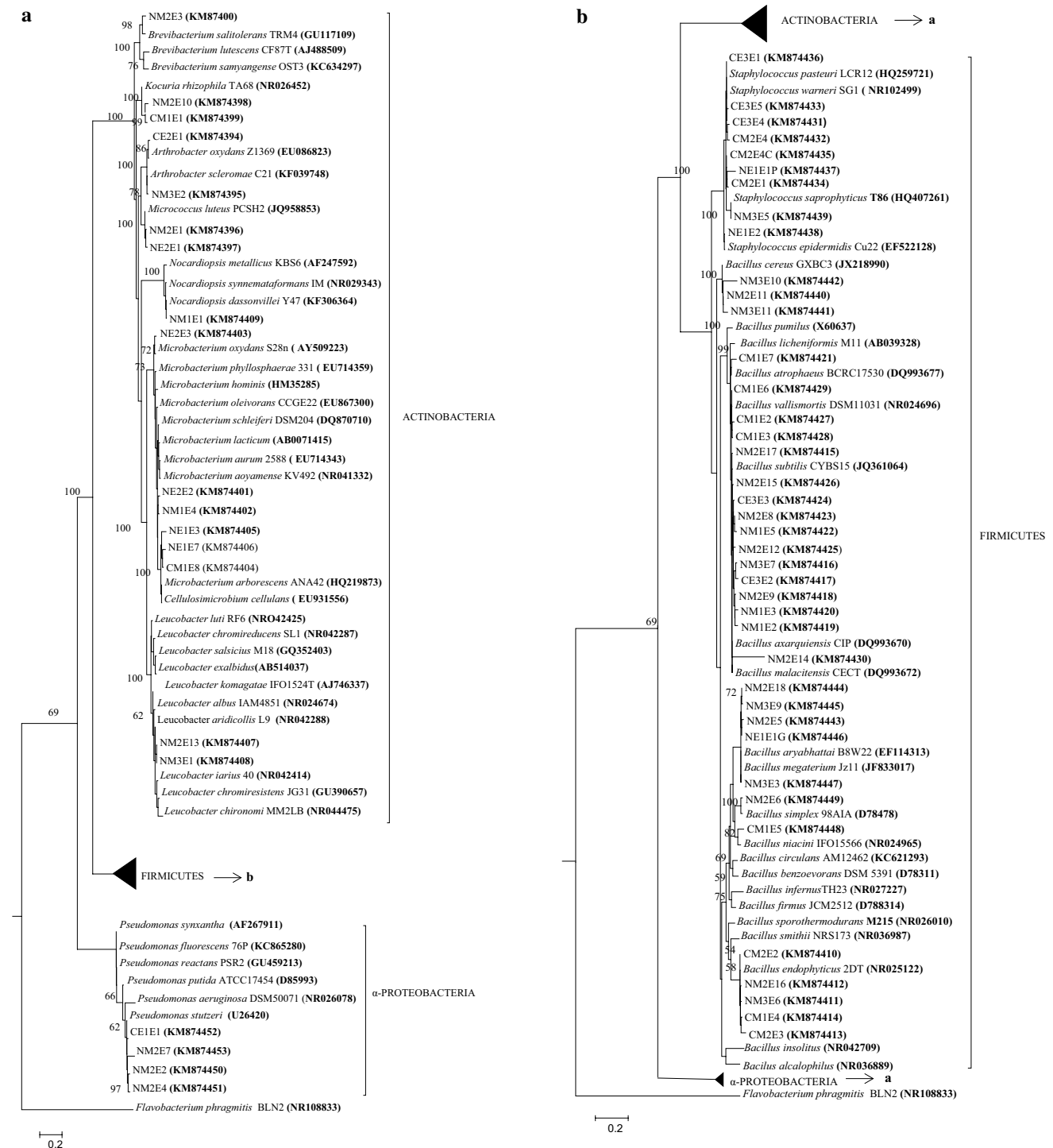


Fig. 1 Maximum likelihood tree based on 16S rRNA genes 60 sequences of endophytic bacteria isolated from two heavy metal-resistant plants (–InL = 19470.8225). Number above branches

indicate bootstrap support (>50 %). *Flavobacterium phragmitis* was included as out-group in the analysis. The scale bar presented 0.2 substitution of the nucleotide

xylanase activity (EI = 2), while the other strains showed $EI \leq 1.83$. The maximum EI for cellulase was 4.0 for *Microbacterium oxydans* NE2E3, and the other strains presented $EI \leq 3.5$. For pectinase, the highest EI was 1.80 for two

Bacillus strains (NM3E10, CM1E6), followed by several *Bacillus* strains and a *Kocuria* strain with EI between 1.60 and 1.75; the other strains presented $EI \leq 1.55$ (see Supplementary Table S1 for detail).

Among the endophytes of *Sphaeralcea*, more bacteria able to produce siderophore, to solubilize phosphate and to fix nitrogen and less IAA producers were found in the mine tailing than in the natural hill. While the bacteria presented each of the four activities were less in the endophytes of *Prosopis* in the mine tailing than in the natural hill (Table 2 and see Supplementary Fig. S1). The strains *M. arborescens* NE1E7, *Bacillus* sp NM1E3, NM2E5, NM3E9, CE3E3, *Brevibacterium salitolerans* NM2E3 and *Bacillus aryabhatai* NM3E3 showed positive for multiple of the evaluated traits.

Minimum inhibitory concentration (MIC)

The isolated endophytic bacteria showed a high degree of resistance to heavy metals and metalloids, especially to Cu, As(III) and As(V). The order of the toxicity of the metals to the isolates was Pb > Zn > Cu > As(III) > As(V) in the mineral medium, with mean MIC of 1.1, 3.1, 4.3, 11.0 and 94.3 mM, respectively (Table 3). And the toxic order changed to Pb > Zn > As(III) > Cu > As(V) in the LB medium with mean MIC of 6.6, 8.3, 13.0, 16.7 and 77.1, respectively (Table 3). The Tukey test showed significant differences between sampling sites, plant species and isolates (data not shown). The resistance to Cu, Pb and Zn for most of the isolates in minimal medium MES (Table 3) was 4 times lower than that observed in LB agar plates, while higher MIC values for As(III) and As(V) were observed in the MES medium. Several exceptions were found; *Bacillus axarquiensis* CE3E2 and *B. vallismortis* CM1E3 showed lower Zn resistance in LB (2 mM) than in MES (>5 mM), while “*S. warneri*” CE3E4 and CE3E5 presented greater As(III) resistance in LB (5.8 mM) than in MES (0.0125 and 0.5 mM) (Table 3).

Correspondence analyses

According to the simple correspondence analysis (available as Supplementary Fig. S3), it was clear that (1) the endophytes associated with the two plants were clearly different in the two sites: eighteen species in the natural hill and 7 in the mine tailing; (2) the *Prosopis* plants showed closer correlations with some bacteria, mainly the *Bacillus* species, in the two sites, while the *Sphaeralcea* plants closely associated with four species of *Microbacterium* and *Staphylococcus* in the natural hill, but did not show special association with any bacterial species in the mine tailing. Several bacterial species, such as *M. arborescens*, *M. luteus* and *S. warneri*, showed distribution across the sites or hosts.

The CCA results (Fig. 2) confirmed the correlation in correspondence analysis and revealed the determinants for bacterial distribution: Group 1 (*Sphaeralcea*-associated bacteria in natural hill) was positively related to the soil pH

and contents of Li and Ni; Group 2 (*Prosopis*-associated bacteria in natural hill) was positively related to the contents of organic matter, Zn, Pb, Sb and Mn; Group 3 (*Prosopis*-associated bacteria in mine tailing) was positively related to Co, Cu, As and Cd. In addition, *M. luteus* and *M. schleiferi* mainly selected by high contents of TN, TP, OC and CEC; *Bacillus endophyticus* mainly selected by high contents of Mo, Cd and Fe; *Microbacterium arborescens* mainly correlated with high Mg, Cr, Ti, V and BC.

Discussion

In this study, the soils in both of the sampling sites were seriously contaminated by HMs such as As, Cd, Mn, Mo, Pb and Zn (Table 1). The high concentrations of the heavy metals and arsenic in the natural hill might come directly from the weathering of mother rock. The higher values of CEC, OC, TN, TP, Mn, Sb, Pb and Zn, but lower As, Cd, Co, Cr, Cu and Mo in the natural hill in comparing with the mine tailing make them very different habitats for plants and microorganisms, allowing us to estimate the effects of environmental factors on the endophytes of the studied plants. Although endophytic bacteria isolated from plants in heavy metal-contaminated soils have been characterized in several reports (Barzanti et al. 2007; Guo et al. 2010; Sheng et al. 2008; Shin et al. 2012; Zhang et al. 2011), most of the previous studies focused upon the plants and/or the plant-associated bacteria with resistance to single heavy metals, such as nickel (Barzanti et al. 2007), cadmium (Luo et al. 2011) or lead (Sheng et al. 2008; Shin et al. 2012). Therefore, the high concentrations of multiple heavy metals and arsenic in the soils formed a special selective stress for both the plants and the associated endophytic bacteria, different from that involved in the previous studies.

The less abundance of endophytic bacteria (10^2 – 10^3 g⁻¹ of root tissue) in the mine tailing compared with that in the hill (10^4 g⁻¹ of root tissue) (Table 2) might be related to the low concentration of nutrients and the great concentration of the high toxic heavy metals and metalloids (As, Cd, Cr and Cu) in that environment (Table 1). In addition, the abundance of endophytes detected in this study was rather low comparing with the values in previous reports (10^3 – 10^6 g⁻¹) (Wang et al. 2006), which might be a result of effects by the multiple heavy metals and metalloids contamination and the dry season (February). These results demonstrated that the endophytes were very sensitive to the soil conditions (high heavy metals and arsenic contamination and low nutrients) (Table 2), while those in *P. laevigata* were more sensitive than those in *Sphaeralcea*. In addition, the *Prosopis* plant harbored more endophytic bacteria than the *Sphaeralcea* plants in both the sampling sites, reflecting the effects of host species on the endophytic communities.

Table 3 Minimum inhibitory concentration in LB medium and MES-buffered minimum medium of the endophytic bacteria isolated from contaminated area

Strain (species)	MIC (mM) in LB medium					MIC (mM) in MBMM				
	Cu(II)	Zn(II)	Pb(II)	As(III)	As(V)	Cu(II)	Zn(II)	Pb(II)	As(III)	As(V)
Natural hill- <i>Sphaeralcea</i>										
NE1E1p (<i>Staphylococcus</i> sp.)	12	12	3.12	14.4	72	NG	NG	NG	NG	NG
NE1E1g (<i>Bacillus</i> sp.)	12	4	3.12	14.4	72	5	5	1.5	10	100
NE1E2 (<i>Staphylococcus epidermidis</i>)	12	2	3.12	5.8	72	NG	NG	NG	NG	NG
NE1E3 (<i>Microbacterium arborescens</i>)	20	2	3.12	14.4	72	5	5	0.5	>20	100
NE1E7 (<i>Microbacterium arborescens</i>)	12	2	3.12	14.4	72	1.5	0.5	0.5	5	100
NE2E1 (<i>Micrococcus luteus</i>)	12	12	>12.5	14.4	72	5	5	>1.5	10	100
NE2E2 (<i>Microbacterium schleiferi</i>)	12	12	>12.5	14.4	480	>5	1.5	>1.5	>20	>100
NM1E1 (<i>Nocardiopsis dassonvillei</i>)	4	12	3.12	5.8	72	NG	NG	NG	NG	NG
NM1E2 (<i>Bacillus</i> sp.)	12	2	3.12	5.8	72	1.5	1.5	0.5	0.5	50
NM1E3 (<i>Bacillus</i> sp.)	12	12	3.12	14.4	72	5	>5	>1.5	10	50
NM1E4 (<i>Microbacterium schleiferi</i>)	40	12	>12.5	14.4	72	1.5	1.5	0.5	0.5	20
NM1E5 (<i>Bacillus</i> sp.)	20	4	3.12	>28.8	305	5	5	>1.5	10	50
NM2E1 (<i>Micrococcus luteus</i>)	20	2	3.12	>28.8	6	5	1.5	0.5	10	50
NM2E2 (<i>Pseudomonas stutzeri</i>)	40	22	3.12	5.8	72	5	0.5	0.5	5	20
NM2E3 (<i>Brevibacterium</i> sp.)	40	2	>12.5	5.8	72	NG	NG	NG	NG	NG
NM2E4 (<i>Pseudomonas stutzeri</i>)	40	12	>12.5	14.4	72	5	5	0.5	5	50
NM2E5 (<i>Bacillus</i> sp.)	12	4	3.12	28.8	360	5	1.5	1.5	10	50
NM2E6 (<i>Bacillus simplex</i>)	2	2	3.12	14.4	3	5	5	>1.5	10	50
NM2E7 (<i>Pseudomonas stutzeri</i>)	12	2	>12.5	14.4	30	>5	5	>1.5	10	50
NM2E8 (<i>Bacillus</i> sp.)	12	12	>12.5	5.8	30	5	5	1.5	10	20
NM2E9 (<i>Bacillus</i> sp.)	40	12	3.12	5.8	72	5	5	1.5	10	50
NM2E10 (<i>Kocuria rhizophila</i>)	2	2	>12.5	>28.8	3	5	0.5	1.5	10	50
NM2E11 (<i>Bacillus cereus</i>)	20	22	>12.5	14.4	72	5	0.5	1.5	10	20
NM2E12 (<i>Bacillus</i> sp.)	12	4	3.12	5.8	72	>5	1.5	1.5	5	50
NM2E13 (<i>Leucobacter aridicollis</i>)	12	4	3.12	5.8	72	5	1.5	1.5	>20	50
NM2E14 (<i>Bacillus</i> sp.)	12	22	3.12	5.8	72	5	0.5	1.5	5	50
NM2E15 (<i>Bacillus</i> sp.)	12	12	3.12	14.4	72	5	5	1.5	0.5	50
NM2E16 (<i>Bacillus endophyticus</i>)	40	4	>12.5	28.8	144	5	5	1.5	>20	50
NM2E17 (<i>Bacillus</i> sp.)	40	4	3.12	5.8	72	5	1.5	1.5	>20	50
NM2E18 (<i>Bacillus</i> sp.)	40	4	>12.5	28.8	144	5	1.5	1.5	20	>100
NM3E1 (<i>Leucobacter aridicollis</i>)	2	2	>12.5	28.8	3	5	0.5	0.5	5	100
NM3E3 (<i>Bacillus aryabhatai</i>)	20	12	>12.5	28.8	144	5	0.5	1.5	5	>100
NM3E5 (<i>Staphylococcus saprophyticus</i>)	4	4	3.12	5.8	72	NG	NG	NG	NG	NG
NM3E6 (<i>Bacillus endophyticus</i>)	12	2	3.12	5.8	72	NG	NG	NG	NG	NG
NM3E7 (<i>Bacillus</i> sp.)	20	22	3.12	5.8	3	5	5	1.5	>20	50
NM3E9 (<i>Bacillus</i> sp.)	12	22	3.12	>28.8	72	5	1.5	0.5	0.5	50
NM3E10 (<i>Bacillus</i> sp.)	40	22	3.12	14.4	72	5	0.5	1.5	5	50
NM3E11 (<i>Bacillus cereus</i>)	4	2	>12.5	5.8	72	5	0.5	0.05	10	100
Natural hill- <i>Prosopis</i>										
NM3E2 (<i>Arthrobacter scleromae</i>)	2	2	>12.5	28.8	3	5	0.1	0.5	>20	5
NE2E3 (<i>Microbacterium oxydans</i>)	20	22	>12.5	14.4	72	>5	1.5	>1.5	>20	>100
Number of high resistant strains	33/37	18	40	25	35	31	13	0	8	33
% of high resistant strains ^a	82.5	45.0	100	62.5	87.5	91.2	36.1	0	23.5	97.0
Average MIC	17.6	8.7	6.9	13.7	266.4					

Table 3 continued

Strain (species)	MIC (mM) in LB medium					MIC (mM) in MBMM				
	Cu(II)	Zn(II)	Pb(II)	As(III)	As(V)	Cu(II)	Zn(II)	Pb(II)	As(III)	As(V)
Mine tailing										
CE1E1 (<i>Pseudomonas stutzeri</i>)	40	4	>12.5	5.8	72	5	1.5	0.1	10	100
CE2E1 (<i>Arthrobacter scleromae</i>)	12	22	3.12	5.8	72	>5	>5	1.5	>20	>100
CE3E1 (<i>Staphylococcus</i> sp.)	12	12	3.12	5.8	72	5	>5	1.5	>20	>100
CE3E2 (<i>Bacillus</i> sp.)	12	2	3.12	5.8	72	>5	>5	1.5	>20	>100
CE3E3 (<i>Bacillus</i> sp.)	12	4	3.12	5.8	72	>5	>5	>1.5	>20	>100
CE3E4 (<i>Staphylococcus</i> sp.)	12	12	3.12	5.8	72	5	>5	0.1	0.0125	50
CE3E5 (<i>Staphylococcus</i> sp.)	12	12	3.12	5.8	72	1.5	>5	0.1	0.5	5
CM1E1 (<i>Kocuria</i> sp.)	40	12	>12.5	28.8	72	1.5	>5	0.5	20	20
CM1E2 (<i>Bacillus vallismortis</i>)	12	4	3.12	5.8	20	>5	>5	0.5	10	50
CM1E3 (<i>Bacillus vallismortis</i>)	12	2	3.12	5.8	72	1.5	>5	1.5	20	>100
CM1E4 (<i>Bacillus endophyticus</i>)	2	2	3.12	28.8	72	5	1.5	1.5	>20	>100
CM1E5 (<i>Bacillus niacini</i>)	40	12	3.12	5.8	72	1.5	0.5	1.5	10	50
CM1E6 (<i>Bacillus vallismortis</i>)	40	22	>12.5	5.8	72	NG	NG	NG	NG	NG
CM1E7 (<i>Bacillus atrophaeus</i>)	20	4	12.5	5.8	72	1.5	5	1.5	10	20
CM1E8 (<i>Microbacterium arborescens</i>)	4	2	3.12	5.8	3	1.5	0.5	0.5	10	50
CM2E1 (<i>Staphylococcus</i> sp.)	40	12	3.12	5.8	72	0.5	1.5	0.5	0.5	50
CM2E2 (<i>Bacillus endophyticus</i>)	12	2	>12.5	5.8	3	>5	1.5	1.5	>20	20
CM2E3 (<i>Bacillus endophyticus</i>)	20	2	12.5	28.8	20	5	5	1.5	>20	50
CM2E4 (<i>Staphylococcus</i> sp.)	12	4	3.12	14.4	30	5	0.5	0.05	0.5	50
CM2E4c (<i>Staphylococcus</i> sp.)	12	4	3.12	14.4	72	5	0.5	0.05	0.5	5
Number of high resistant strains	19/20	8	20	5	18	11	11	0	9	17
% of high resistant strains ^a	95.0	40.0	100	25.0	90.0	63.2	57.9	0	47.4	89.5
Average MIC	18.9	7.6	5.9	10.1	57.8	3.6	3.3			
Average MIC for total strains	16.7	8.3	6.6	13.0	77.1	4.3	3.1	1.1	11.0	94.3

NG no growth

^a The high MICs for the metals were identified according to Zhu et al. (2014) for As(V) and As(III); Chen and Wang (2007), Hasnain et al. (1993) and Wei et al. (2009) for Pb(II); Wei et al. (2009), Bhojiya and Joshi (2012) and Barzanti et al. (2007) for Zn(II); Wei et al. (2009), Altimira et al. (2012) and Luo et al. (2010) for Cu(II)

Based upon the 16S rRNA gene sequence analysis, 25 genomic species within 10 genera were identified (Fig. 1a, b; Table 2) among the 60 isolates. The identification of 12 isolates in *Bacillus* and *Staphylococcus* was uncertain because the isolates in these groups showed similar high similarities with two or three defined species. These further evidenced the inability of 16S rRNA gene for distinguishing the closely related species, as reported in other studies (Menna et al. 2006). The exact identification of these isolates needs further analysis, such as the multilocus sequence analysis (MLSA) (Soufiane et al. 2013; Vinuesa et al. 2005).

The rarefaction analysis (available as Supplementary Fig. S2) suggested relatively low coverages (72.5 % in hill and 44.0 % in mine tailing), demonstrating that more isolates were necessary to accurately reveal the diversity of endophytes in this study; however, these low values may also be related to the low abundance of endophytes in these sampling sites and the growing season (Fig. 2).

The extremely predominance of Gram-positive bacteria (56/60) in our study (Table 2) was consistent with previous reports (Barzanti et al. 2007; Guo et al. 2010; Sun et al. 2010), indicating that the G⁺ bacteria might be more resistant to the heavy metals and arsenic than Gram-negative bacteria. Most of the genera detected in the present study have been reported as endophytic bacteria associated with heavy metal-resistant plants, like *Bacillus*, *Staphylococcus* (Barzanti et al. 2007; Guo et al. 2010; Shin et al. 2012; Sun et al. 2010; Zhang et al. 2011), *Microbacterium*, *Micrococcus*, *Leucobacter*, *Arthrobacter*, *Brevibacterium*, *Nocardiosis* (Barzanti et al. 2007; Sun et al. 2010) and *Pseudomonas* (Barzanti et al. 2007; Zhang et al. 2011). However, differences could be found between our study and the other studies in the community composition, such as the genus *Curtobacterium* reported by Barzanti et al. (2007) was not found and *Kocuria* was only recorded in our study. The identification of *Bacillus* sp. NM3E10, *Brevibacterium*

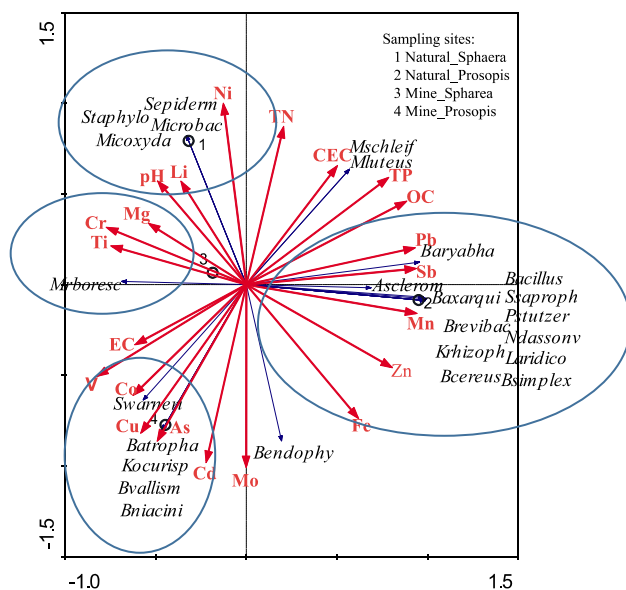


Fig. 2 Triplot from a canonical correspondence analysis (CCA) of the endophytic bacteria species, host plants/sampling sites and the soil characters. Environmental variables are represented by red arrows, sampling sites by small open circles and numbers, and species by their names. See Table 1 for the soil characters. Abbreviations for the bacteria: *Ascleromae*, *Arthrobacter scleromae*; *Baryabhatai*, *Bacillus aryabhatai*; *Batrophaeus*, *Bacillus atrophaeus*; *Baxarquensis*, *Bacillus axarquensis*; *Bcereus*, *Bacillus cereus*; *Bendophyticus*, *Bacillus endophyticus*; *Bniacini*, *Bacillus niacin*; *Bsimplex*, *Bacillus simplex*; *Bacillusp*, *Bacillus* sp.; *Bvallismortis*, *Bacillus vallismortis*; *Brevibacteriumsp*, *Brevibacterium* sp.; *Krhizophila*, *Kocuria rhizophila*; *Kocurisp*, *Kocuria* sp.; *Laridicollis*, *Leucobacter aridicollis*; *Mrborescens*, *Microbacterium arborescens*; *Microbacteriumsp*, *Microbacterium* sp.; *Micoxydans*, *Microbacterium oxydans*; *Mschleiferi*, *Microbacterium schleiferi*; *Mluteus*, *Micrococcus luteus*; *Ndassonvillei*, *Nocardiopsis dassonvillei*; *Pstutzeri*, *Pseudomonas stutzeri*; *Sepidermidis*, *Staphylococcus epidermidis*; *Ssaprophyticus*, *Staphylococcus saprophyticus*; *Staphylococcussp*, *Staphylococcus* sp.; *Swarmeri*, “*Staphylococcus warneri*” (color figure online)

sp. NM2E3, *Kocuria* sp. CM1E1, *Microbacterium* sp. NE1E3 and *Staphylococcus* sp. NE1E1p evidenced that the two involved host plants harbored some novel bacteria (Table 2). Furthermore, *B. endophyticus* was originally isolated from cotton plants (*Gossypium* sp.) (Reva et al. 2002); *Kocuria rhizophila* was isolated from the rhizoplane of narrow-leaved cattail (*Typha angustifolia*) (Kovács et al. 1999); and *Nocardiopsis dassonvillei* was recovered as an agent to cause cutaneous and pulmonary infections (Brocq-Rousseau 1904); therefore, our study also improved the knowledge about the distribution of these bacteria. The host plant species has been reported as a major determinant for the endophytic bacterial communities (Ding et al. 2013), as well as for the relative abundances of specific endophytic bacteria in the microbial community (Ding and Melcher 2016). In addition, the community structure and function of endophytic microbes are also related to

environmental factors (Hartman et al. 2008, Bannert et al. 2011, Peralta et al. 2013), including the soil metal contamination (Azarbad et al. 2013). Our results evidenced that the endophytic communities varied according to the host plant and the environments. The simple correspondence analysis and CCA results (Fig. 2) revealed that the type and concentration of nutrients and HMs in soils were determinants for the endophyte compositions and that *P. laevigata* could interacted with more diverse endophytic bacteria than *S. angustifolia* (Fig. 2; Table 2).

The plant-polymer-degrading enzymes such as cellulases, amylases, xylanases and pectinases can facilitate the process of colonization of host plants by the endophytic bacteria. However, little information was available about the activities of these enzymes in endophytic bacteria associated with heavy metal-tolerant plants. It was interesting that the activities of the four enzymes were found in about half or more of the isolates, with pectinase activity most universal (72.5 %, Table 2). Our data imply that these enzymes might play some role in the invasion and movement inside the plant roots.

The endophytic bacteria may promote plant growth by production of plant hormones, siderophores synthesis, nitrogen fixation, solubilization of phosphorous, and suppression of ethylene synthesis by 1-aminocyclopropane-1-carboxylate (ACC) deaminase, etc. (Ali et al. 2014). The detection of some of these characters among the isolates in the present study (Table 2) indicates that the endophytic bacteria may help their host plants to colonize the heavy metal-contaminated and arsenic-contaminated sites. The proportion of bacteria with siderophore production, phosphate solubilization, IAA production and nitrogen fixation varied according to the host plant and the sampling sites (Table 2). The absence of siderophore production, phosphate solubilization and nitrogen fixation in the endophytic bacteria of *S. angustifolia* grown in the hill site, and the presence of these activities in 57.1, 20.0 and 40 % of the isolates from the same plant in the mine tailing (Table 2) demonstrated that the bacteria with these activities were necessary for *S. angustifolia* to colonize the mine tailing, similar to previous reports about the plants faced to other stresses (Barzanti et al. 2007; Sheng et al. 2008; Zhang et al. 2011). For *P. laevigata*, the decrease or disappearance of bacteria with siderophore production, phosphate solubilization and nitrogen fixation in mine tailing might indicate that bacteria with these functions were not selected by this plant in that environment. For both the host species, the disappearance of IAA-producing bacteria in plants grown on mine tailing demonstrates that this function is not necessary for the plants in this environment with poor nutrient and rich in HMs, although IAA-producing endophytic bacteria associated with plants grown on mine tailings have been reported (Sheng et al. 2008; Zhang et al. 2011).

In the present study, all the 60 endophytic isolates exhibited multiple resistances to the HMs, but the resistance patterns and MIC values varied (Table 3). Previously, bacteria and yeasts presenting the MICs of 10 mM for As(V) or As(III) (Zhu et al. 2014), 1–5 mM for Pb(II) (Chen and Wang 2007; Wei et al. 2009), 3 mM (Wei et al. 2009) to 10 mM (Bhojiya and Joshi 2012) or 15 (Bazanti et al. 2007) for Zn(II), 0.2 mM (Wei et al. 2009) to 4.7 mM (very high resistance) (Altimira et al. 2012; Luo et al. 2011) for Cu(II) have been reported. Comparing with these data, great proportion of the isolates originated from the hill (82.5, 45.0, 100, 62.5 and 87.5 %) and from the mine tailing (95.0, 40.0, 100, 25.0 and 90.0 %) showed high resistance to Cu, Zn, Pb, As(III) and As(V) in LB medium, respectively (Table 3). About 100, 57.9, 52.6, 73.7 and 89.5 % of the 20 test isolates were resistant in the MES medium to Cu, Zn, Pb, As(III) and As(V), respectively, in which 63.2, 57.9, 0, 47.4 and 89.5 % were highly resistant to the corresponding metals (Table 3). The high proportion of the great resistance might be a selection results by the high concentration of heavy metals and arsenic in the sampling region, and the endophytic bacteria might have been developed resistant mechanisms to these heavy metals. Previously, metal exclusion by permeability barriers, active transport of the metal away from the cell, intracellular sequestration of the metal by protein binding, extracellular sequestration, enzymatic detoxification of the metal to a less toxic form and reduction in the sensitivity of cellular targets to metal ions have been evidenced as mechanisms in bacteria for resistance of HMs (Agrawal et al. 2011; Das et al. 2016; Williams et al. 2012).

Previously, effects of medium composition on the HM resistance of microbes have been reported (Rathnayake et al. 2013). Indeed, the MICs varied apparently in the two media for the same strain, as well as for different heavy metals and arsenic. The lower MIC values in minimum medium MES for Cu, Pb and Zn than those in LB medium (Table 3) might be referred to the fact that the high level of organic constituents affects the bioavailability of the metal in the culture medium (Rathnayake et al. 2013) and/or helped the bacteria to develop HM-/arsenic-resistant mechanisms.

The extremely high resistance to arsenic of the isolates, like *Microbacterium schleiferi* NE2E2 and *Microbacterium oxydans* NE2E3 (MIC >480 mM arsenic in LB and MIC >100 mM of arsenate in MES-buffered medium), might be referred to their adaptation to the extremely high As concentration in the sampling sites. It was interesting that the MICs for As(III) and As(V) were greater in mineral (MES) medium than those in LB for most isolates, and several isolates even presented MICs more than 100 mM. To explain this situation, it could be estimated that the endophytes may have developed special mechanism to adapt the

local environment, such as metabolize arsenic as substrate for respiration (Stolz et al. 2006), arsenite oxidation, arsenate reduction, methylation and demethylation of arsenic, organic acids production, ligand production (siderophores), membrane transporters, biosorption, adsorption and compartmentalization (Nair et al. 2007, Mailloux et al. 2009, Ahsan et al. 2011, Kruger et al. 2013, Prasad et al. 2013, Yang and Rosen 2016). Further study is worthy for clarifying the mechanisms in our isolates.

Conclusions

P. laevigata and *S. angustifolia* plants grown in the soils seriously contaminated by multiple heavy metals and metalloids, especially As and Pb, associated with diverse endophytic bacteria, with Firmicutes and Actinobacteria as predominant groups and Proteobacteria as minor group. Some of them represented novel species or novel endophytic distribution. The abundance and community composition of the endophytes were strongly affected by the soil conditions and also by the plant species. Many of the isolated endophytic bacteria were resistant to very high concentrations of As and multiple heavy metals, as well as presented some plant growth-promoting characteristics. Future study to investigate the mechanisms of resistance to HMs is undergoing.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests and they are notified about the content of the manuscript.

Human and animal rights This article does not contain any studies with human or animal subjects.

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