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Isolation and characterization of nitrogen fixing heterotrophic bacteria from the rhizosphere of pioneer plants growing on mine tailings

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ABSTRACT

Mine tailings are extreme environments containing high concentrations of heavy metals and deficient in nitrogen and organic matter. However, some plant species tolerant to heavy metals are capable of growing on mine tailings. In this environment, it is expected that biological nitrogen fixation is the major source of nitrogen available for plants. In this work, three different nitrogen-free culture media were used to isolate nitrogen-fixing bacteria associated with nine plant species growing on mine tailings in Zacatecas, Mexico. Acetylene reduction assays (ARA) with and without heavy metals were carried out, as were the determination of minimum inhibitory concentration (MIC) of Ni, Cu, Co, Cr and Zn, and plant growth promotion (PGP) activities of each bacterial isolate. The major nitrogen-fixing bacteria distributed among rhizospheres of *Juniperus* sp., *Aster gymnocephalus*, *Gringelia* sp., *Lygodesmia* sp., and *Haplopappus* sp. were members of the genus *Paenibacillus*. However, *Azospirillum lipoferum* and *Bradyrhizobium japonicum* were also isolated from the rhizosphere of *Haplopappus* sp., and *Viguiera linearis*. Functionally, *Paenibacillus durus* BR_30 had the highest ARA and PGP activities, but was highly sensitive to heavy metals. However, *Paenibacillus graminis* BR_35 and *Paenibacillus borealis* BR_32 maintained a significant ARA activity in the presence of relatively high concentrations of Ni and Zn. Also, *B. japonicum* KYR_C5 and *A. lipoferum* showed moderate ARA and PGP activities. The majority of the isolates grew in moderate concentrations of heavy metals but ARA activities were inhibited at low Co and Cr concentrations. The diversity of nitrogen-fixing bacteria in the rhizosphere of pioneer plants may contribute to their adaptation to toxic stress and nutrient-deficiency in environments such as mine tailings.

1. Introduction

Mining activities often generate high amounts of solid wastes. Among them, mine tailings are known to have a significant environmental impact (Dudka and Adriano, 1997). They are stored outside, retain high concentrations of heavy metals (HM), maintain low pH, and can be dispersed by wind or leached by water, thus contaminating surrounding areas and ground water (Wong et al., 1998). Furthermore, mine tailings often have geochemical properties that inhibit the establishment of plants. However, there are some plant species (*e.g. Polygonum aviculare* and *Jatropha dioica*) that can grow on mine tailings and have developed mechanisms that allow them to resist metal concentrations, which are toxic to most plants (González and González-Chávez, 2006; Reeves and Baker, 2000).

Additionally, plants may harbor microbial communities that play a role in modifying the availability and toxicity of these elements to the plants (Wong et al., 1998; Wu et al., 2006). However, in spite of the increasing knowledge of metal–microorganism interactions, few studies have attempted to characterize the rhizosphere soil bacterial communities of metallophyte plants under HM stress (Navarro-Noya et al., 2010; Sun et al., 2009; Wang et al., 2009; Zhang et al., 2007a,b). The complexity of mine tailings mainly depends on the microbial diversity, redox state and mobility of metals, humidity, temperature, pH and root exudates released by metallophyte plants. There is little information concerning biogeochemical cycles in the rhizospheres of plants growing on mine tailings, a substrate rich in HM and strongly deficient in essential elements such as carbon and nitrogen (Mendez et al., 2007).

Biological nitrogen fixation, which catalyzes the reduction of atmospheric N_2 gas to biologically available ammonium, is ecologically important as an input of fixed nitrogen in many terrestrial and aquatic habitats limited in combined nitrogen sources (Arp, 2000; Vitousek and Howarth, 1991). Such nitrogen input would stimulate the nitrogen cycle by improving the fertility of mine spoils. More

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studies are required to understand the biological nitrogen input to metallophyte plant rhizospheres.

Most studies on associative nitrogen fixation have focused on crops of agronomic interest such as rice or sugar cane (Engelhard et al., 2000; Steenhoudt and Vanderleyden, 2000; Ueda et al., 1995) that have high fertilizer requirements. However, little attention has been paid to the occurrence of nitrogen-fixing heterotrophic bacteria in the rhizospheres of plants growing on nitrogen-deficient substrates, such as heavy metal contaminated soils. The rhizospheres of *Gymnostoma webbianum* and *Serianthes calycina*, pioneer plants from serpentine soils, exhibited *in situ* nitrogen fixing activity and revealed the presence of *nif*H genes associated with the *Bradyrhizobium-Beijerinckia nif*H gene cluster (Héry et al., 2005).

In this work, we attempt to characterize the cultivable nitrogen-fixing bacteria associated with nine plant species growing on mine tailings in Zacatecas, Mexico. Given the physicochemical properties of the tailing environment, the lack of organic matter, and that nitrogen-fixing activity is sensitive to many variables; we expected to isolate bacteria with traits to make them competitive in this environment. Thus, nitrogenase activity with and without heavy metals were carried out, as were the determination of heavy metal resistance to Ni, Cu, Co, Cr and Zn, and plant growth promotion (PGP) activities of each bacterial isolate.

2. Material and methods

2.1. Rhizosphere soil sampling and characterization

The HM-contaminated soils were collected from the rhizosphere of plants growing in mine tailings in Sombrerete (23°40.087′N, 103°44.868′W and 2558 m of altitude) and Noria de los Angeles (22°28.009′N, 101°54.305′W and 2208 m of altitude), Zacatecas, Mexico. Sombrerete and Noria de los Angeles mines are known because of its huge mineral deposits of gold, silver, lead, tin, cadmium, zinc and mercury. Calcopyrite, sphalerite, bornite, tetrahedrite, native silver, arsenopyrite, pyrrotite, stibnite and galena minerals are extracted by froth flotation, and milling and cyanidation circuit processes. The soil firmly adhering to the roots, designated as rhizosphere soil, was collected by brushing. Bulk soil was collected from the areas surrounding the tailings heaps. Plants and bulk soils were sampled in triplicate, and 33 samples were obtained. All samples were transferred to polyethylene bags and transported to the laboratory at 4 °C. The plants were identified as *Viguiera linearis, Asphodelus* sp., *Grindelia* sp., *Lygodesmia* sp., *Juniperus* sp. and *Aster gymnocephalus* from Sombrerete, and *Bahia* sp., *Haplopappus* sp., and *Chloris virgata* from Noria de los Angeles. The physicochemical characterization of the sample soil of each location, HM and organic and inorganic carbon and total nitrogen were determined as described by Conde et al. (2005) and González and González-Chávez (2006).

2.2. Isolation of nitrogen-fixing heterotrophic bacteria

To obtain nitrogen-fixing bacteria, 1 g of soil was suspended in 100 mL of water. After shaking for 1 h at 200 rpm, a 1.5 mL sample of the suspension was inoculated into N-free Winogradsky culture medium (Tchan, 1984) and incubated at 28 °C until turbidity of the medium, measured at 600 nm, was evident. Three successive subcultures into fresh N-free media were performed to enrich the nitrogen-fixing bacterial yield. Finally, bacteria were isolated on solid N-free Winogradsky culture medium.

Additionally, serial decimal dilutions of soil in sterile water were performed and 10^{-3} to 10^{-7} dilutions were spread on N-free Bridges and N-free WAT4C media and incubated at 28 °C for several days (Berge et al., 1991; Bridges, 1981).

Five representative clones of each morphotype observed were conserved in 25% (v/v) glycerol at −70 °C.

2.3. DNA extraction

Total DNA from enriched subcultures and from isolated strains was extracted using the method of Cullen and Hirsch (1998). DNA quality was estimated by electrophoresis in 1% agarose gels in 1× TAE buffer (40 mM Tris, pH 8.3; 20 mM acetic acid; 1 mM EDTA) and stained with 0.5 μg mL^{-1} ethidium bromide solution (ETB). DNA concentrations were determined spectrophotometrically and a A260/A280 ratio of 1.8 was considered to be acceptable.

2.4. PCR-DGGE of V3 region of bacterial 16S rRNA

To monitor the stabilization of consortia in the Winogradsky culture medium, we carried out PCR-DGGE using a DCode mutation detection system (Bio-Rad Laboratories, Hercules, CA). The DNAs extracted from successive subcultures in each culture medium were used as templates to amplify the V3 hypervariable region of the bacterial 16S rRNA gene by PCR with primers and conditions described by Muyzer et al. (1993). The PCR products were applied to 8% (w/v) polyacrylamide gels prepared in 1× TAE buffer. The denaturing gradients contained 40–60% denaturant [100% denaturant corresponds to 7 M urea and 40% (w/v) formamide]. Electrophoresis was performed at 85 V and 60 °C for 16 h. DGGE gels were fixed and silver stained by a procedure reported by Sanguinetti et al. (1994).

2.5. Differentiation of bacteria by random amplification polymorphic DNA (RAPD) fingerprints

RAPD fingerprints were obtained to distinguish the morphologically similar strains. RAPD fingerprints were generated using the primer OPB01 and a procedure reported by Williams et al. (1990). The RAPD products were electrophoresed in 1.5% agarose gels in 1× TAE and stained with ETB, as mentioned above. At least two isolates from each RAPD group were selected for 16S rRNA sequencing.

2.6. Amplification and sequencing of the 16S rRNA gene fragment of the strains

Isolated microorganisms were identified by the similarity and phylogenetic analysis of the 16S rRNA gene partial sequence. The amplification of 16S rRNA genes was performed with a PCR System 9700 (Applied Biosystems, Foster City, CA). Reaction mixtures contained 5 ng of template DNA, 1× reaction buffer, 25 mM $MgCl_2$, 0.25 mM of each dNTP, 10 pM of each primer, and 1 U of *Taq* Polymerase, adjusted to 25 μL. Ribosomal 16S rRNA genes were amplified using the universal bacterial primer 8 (5′-GCG GAT CCG CGG CCG CTG CAG AGT TTG ATC CTG GCT CAG-3′) forward and 1492 (5′-GGC TCG AGC GGC CGC CCG GGT TAC CTT GTT ACG ACT T-3′) reverse (Relman, 1993). The following PCR conditions were used: an initial denaturation step at 94 °C for 5 min; 35 cycles of 45 s at 94 °C, 45 s at 55 °C, and 90 s at 72 °C; and a final extension step at 72 °C for 10 min. The PCR products were electrophoresed and stained as mentioned above. PCR products corresponding to the 16S rRNA genes were purified using the QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen Inc., Valencia, CA) and sequenced using the Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Piscataway, NJ) and an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Applied Biosystem, Inc., Boston, MA).

Table 1
Oligonucleotides used to amplify *nif*H, *nif*D, *anf*H and *acd*S genes. All the oligonucleotides exhibited in this table were designed in this work.

Gene	Oligonucleotide	Sequence (5′ → 3′)	Fragment amplified[a]	Alignment temperature (°C)	Related Microorganism (GenBank access)	Expected size (nt)
*nif*H	nifHEnter-F	ACACCATTATGGAGATGG	N167–N184	63	*Klebsiellapneumonie*	655
	nifHEnter-R	GATGCCGAACTCCATCAG	N805–N822		(X13303)	
	nifHAzo-F	GACTCCACCCGCCTGATCCT	N130–N152	60	*Azotobactervinelandii*	567
	nifHAzo-R	GATCGTATTCGATCACGGTCAT	N676–N697		(M11579)	
	nifHClo-F	GCTATTTAYGGAAARGTTG	N13–N26	62	*Clostridium pasteurianum*	457
	nifHClo-R	GCATAYADTGCCATCAT	N454–N470		(AY603957)	
	nifHRFZ-F	TYGGCAAGTCCACCACC	N41–N57	55	*Azospirillumbrasilense*	431
	nifHRFZ-R	GCGCCATCATCTCRCCGGA	N454–N472		(M64344)	
	nHPb-F	GAACCIGGBGTIGGYTGTGC	N286–N305	60	*Paenibacillusdurus*	150
	nHPb-F	ATGCCGATYCGBGARAABAA	N418–N437		(31620968)	
*nif*D	nifD-F	CGCGGCTGCGCCTAYGCMGG	N181–N200	64	*Klebsiellapneumoniae*	1147
	nifD-R	GARTGCATCTGRCGGAAMGG	N1309–N1328		(X13303)	
*anf*H	anfHPb-F	ATGATTCTCGGCGGCAAACC	N187–N207	63	*Paenibacillusdurus*	245
	anfHPb-R	AATCGGCATTGCGAAACCGCC	N410–N432		(AJ515294)	
*acd*S	acdS-F	AAYAARMCGCGSAAGCTCGAATA	N148–N170	57	*Pseudomonas fluorescens*	699
	acdS-R	CGCACAGDCGRATWGCYTCC	N828–N847		(EF635249)	

[a] DNA template used to design the pairs of oligonucleotides.

2.7. Phylogenetic analysis of the bacteria

16S rRNA gene sequences were subjected to BLAST (Altschul et al., 1990) and Analysis Tools of Ribosomal Database Project-II Release 10 (http://rdp.cme.msu.edu) were used. A collection of taxonomically related sequences obtained from the National Center for Biotechnology Information (NCBI) Taxonomy Homepage (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/) and Ribosomal Database Project-II Release 10 (http://rdp.cme.msu.edu) were included in the multiple alignment analyses with CLUSTAL X (Thompson et al., 1997) that were manually edited using SEAVIEW software (Galtier et al., 1996). Only common 16S rRNA gene regions were considered in the phylogenetic and similarity analyses. All alignment gaps were treated as missing data. Maximum likelihood analyses were done using MEGA version 5 (Tamura et al., 2007). The "Find best model" tool was used to evaluate the substitution models. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (*lnL*), and the number of parameters (including branch lengths) were calculated (Nei and Kumar, 2000). The confidence at each node was assessed by 1000 bootstrap replicates (Hillis and Bull, 1993). The similarity percentages among sequences were calculated using MatGAT v. 2.01 software (Campanella et al., 2003). The species identification was achieved considering genus and species limits of 95 and 97.5%, respectively (Rosselló-Mora and Amann, 2001) and the phylogenetic clustering in the trees. The sequence data reported in this paper have been deposited in the GenBank database, under accession numbers HQ661860 to HQ661871.

2.8. PCR amplification of genes encoding nitrogenase (nifH, nifD and anfH)

*nif*H, *nif*D and *anf*H gene fragments were amplified from samples using primers designed in this work. These primers were chosen from conserved regions detected in multiple sequence alignments of a broad collection of four *nif*H gene groups: Enterobacteria, *Azotobacter*, Clostridia and *Azospirillum-Rhizobium-Frankia*. The PCR reactions were carried out in accordance with an initial denaturation step at 94 °C, for 10 min; 35 cycles of 94 °C for 60 s, alignment temperature shown in Table 1 for 60 s, and 72 °C for 60 s; and a final extension of 72 °C for 10 min. PCRs contained 5 ng of template DNA, 1× reaction buffer, 50 mM $MgCl_2$, 0.25 mM of each dNTP, 10 pM of each primer, and 1 U of *Taq* Polymerase, adjusted to 25 μL.

2.9. Amplification of acdS gene encoding ACC (1-aminocyclopropane-1-carboxylate) deaminase

Deamination of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) is a key plant-beneficial trait found in plant growth-promoting rhizobacteria (PGPR) (Glick, 2003) and the presence of the corresponding gene (*acd*S) was detected among the nitrogen fixing isolates through a PCR procedure designed in this work (Table 1).

2.10. Phenotypic features of the nitrogen fixing strains

2.10.1. Acetylene reduction assay

Acetylene reduction assay (ARA) was performed with a single colony of bacteria grown in N-free WAT4C semisolid medium. The cultures were incubated for 72 h at 28 °C, and then acetylene-enriched atmospheres to a final concentration of 1% and 10% were injected. Acetylene was produced by dissolving calcium carbide in tap water and injected to a final concentration of 1% (v/v) by replacement of an identical volume of air. The acetylene reduction activity was measured with a Varian 3300 (Walnut Creek, CA, USA) gas chromatograph with a flame ionization detector. *Klebsiella varicola* ATCC BAA-830T and *Escherichia coli* DH10b were included as positive and negative controls, respectively.

2.10.2. Determination of the minimal inhibitory concentration of heavy metals

The minimal inhibitory concentration (MIC) of HM was determined by broth microdilution according to the CLSI Approved Standard M27-A2 (National Committee for Clinical Laboratory Standards, 2002). The metals were used as $NiCl_2{\cdot}6H_2O$, $CuSO_4{\cdot}5H_2O$, $ZnCl_2$, $CoSO_4{\cdot}7H_2O$ and $K_2Cr_2O_4$ salts. An inoculum suspension was adjusted to 0.5 McFarland (OD_{600} of 0.125) and diluted 1:50 then 1:20 in PY medium (Yao et al., 2002). The MIC microplates were incubated at 28 °C.

Isolates growing at 1 mM of Cu^{2+}, Ni^{2+} and Zn^{2+}; and 0.5 mM of Co^{2+} and Cr^{3+} were considered resistant (Brim et al., 1999; Nieto et al., 1987). MIC assays in nitrogen-free medium were performed as described above in WAT4C semisolid medium supplemented with the HM.

2.10.3. Cellulose degrading activity

Bacterial isolates were spread on plates of Congo red agar containing the following ingredients per liter: K_2HPO_4, 0.5 g; $MgSO_4$, 0.25 g; powdered cellulose, 1.88 g; Congo red 0.2 g, gelatin 2 g, soil

Table 2
Phylogenetic affiliation of bacterial strains isolated from the rhizosphere of pioneer plants growing on heavy-metal contaminated soils.

Medium	RAPD profile[a]	Phylogenetic relationship			Source of isolation/locality
		Representative isolate	Closest species in GenBank (Accession number)[b]/similarity (%)[c]	Microbial group affiliation[d]	
Direct culture in Bridges	B-A	BR_30	*Paenibacillus durus* (NR_028887)/99.3	*Paenibacillus durus*	*Asphodelus* sp./Sombrerete
	B-B	BR_32	*Paenibacillus borealis* (AJ011322)/98.6	*Paenibacillus borealis*	*Juniperus* sp./Sombrerete
	B-C	BR_35	*Paenibacillus graminis* (AB428571)/97.9	*Paenibacillus graminis*	*Aster gymnocephalus*/Sombretete
	B-D	BR_36	*Paenibacillus graminis* (AB428571)/97.6	*Paenibacillus graminis*	*Grindelia* sp./Sombrerete
	B-E	BBS_22	*Paenibacillus odorifer* (NR_028887)/99.5	*Paenibacillus odorifer*	Bulk soil/Sombrerete
	B-F	BBS_66	*Paenibacillus illinoisensis* (DQ870759)/98.2	*Paenibacillus illinoisensis*	Bulk soil/Noria de los Ángeles
Direct culture in WAT4C	W-B	WR_17	*Paenibacillus illinoisensis* (DQ870759)/97.2	*Paenibacillus* sp.	*Asphodelus* sp./Sombrerete
	W-H	WR_42	*Paenibacillus pabuli* (X60630)/99.2	*Paenibacillus pabuli*	*Lygodesmia* sp./Sombrerete
	W-A	WBS_1	*Paenibacillus odorifer* (NR_028887)/98.3	*Paenibacillus odorifer*	Bulk soil/Sombrerete
Enrichment in Winogradky	KY-G	KYR_B2	*Azospirillum lipoferum* (DQ787328)/99.2	*Azospirillum lipoferum*	*Haplopappus* sp./Noria de los Ángeles
	KY-H	KYR_F6	*Azospirillum lipoferum* (DQ787328)/98.8	*Azospirillum lipoferum*	*Haplopappus* sp./Noria de los Ángeles
	KY-I	KYR_C5	*Bradyrhizobium japonicum* (FJ025097)/99.4	*Bradyrhizobium japonicum*	*Viguiera linearis*/Sombrerete

[a] Representative isolate selected by RAPD profile.
[b] The best match was selected using the closest sequence from the phylogenetic tree.
[c] Similarity percentage was estimated by considering the number of nucleotide-substitutions between a pair of sequences divided by the total number of compared bases × 100%.
[d] The nucleotide similarity percentages between the sequence and its closest match sequence to define genus and species were 95% and 97.5%, respectively (Rosselló-Mora and Amann, 2001).

extract 100 mL, agar 15 g. Plates were incubated at 30 °C for 3–5 days. The cellulolytic activity of microorganisms was detected by a clear zone around the colonies.

2.10.4. Mineral phosphate solubilization

Bacterial isolates were tested by plate assay using phosphate growth media containing per liter: glucose, 10 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.3 g; KCl, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.03 g; $MnSO_4 \cdot 4H_2O$, 0.03 g; $Ca_3(PO_4)_2$, 10.0 g; agar, 20.0 g at pH 7 (Wu et al., 2006). Phosphate solubilizing bacteria colonies were recognized by clear halos after 5 days of incubation at 30 °C and the activity was indexed as the diameter of the halo divided by the diameter of the colony (Teather and Wood, 1982).

2.10.5. Indoleacetic acid production

An overnight culture of each isolate was used to inoculate flasks containing 50 mL of King B broth supplemented with 2.5 mM L-tryptophan, then incubated for 6 days at 28 °C. After incubation, 2 mL of cell suspension was centrifuged at 13,000 × *g* for 5 min, the supernatants were transferred into a tube and mixed vigorously with 2 mL of Salkowski R2 reagent ($FeCl_3$, 4.5 g L^{-1} in 10.8 M H_2SO_4) (Glickmann and Dessaux, 1995) and incubated at 26 °C for 30 min until pink color development. The indoleacetic acid (IAA) was quantified spectrophotometrically at 540 nm by interpolation on an IAA calibration curve.

2.10.6. Germination assay on filter paper culture

The germination promotion activity of the isolated bacteria was determined using the modified assay of Belimov et al. (2001). Bacteria were grown in PY medium for 48 h at 28 °C, then centrifuged and suspended in sterile saline solution to reach McFarland 0.5 standard. Six mL of bacterial suspensions or sterile water (negative control) were added to Petri dishes with sterile filter paper. Wheat seeds were surface-disinfected with 10% hypochlorite solution for 20 min, washed several times with sterile water and placed on wetted filter paper. Germination of the seedlings was measured after incubation during 6 days at 28 °C. The assay was repeated two times with three dishes per assay (containing 10 seeds per dish).

3. Results

3.1. Enrichments and isolation of bacteria on N-free media

Eight different HM-contaminated soil samples with no detectable total nitrogen (data not shown) and organic carbon content in the range of 1–3.5 g kg^{-1} were used for isolation of nitrogen-fixing bacteria. After five continuous subcultures in nitrogen free media, the stabilization of bacterial consortia was confirmed by similar DGGE profiles. A total of 49, 58 and 45 isolates with different colony morphologies were obtained using N-free Winogradsky, Bridges and WAT4C agar culture media, respectively, but only 30, 12 and 19 RAPD profiles were recognized. Most of the bacterial colonies growing on Winogradsky agar plates were flat, smooth, and brilliant, with regular margins and corresponded to

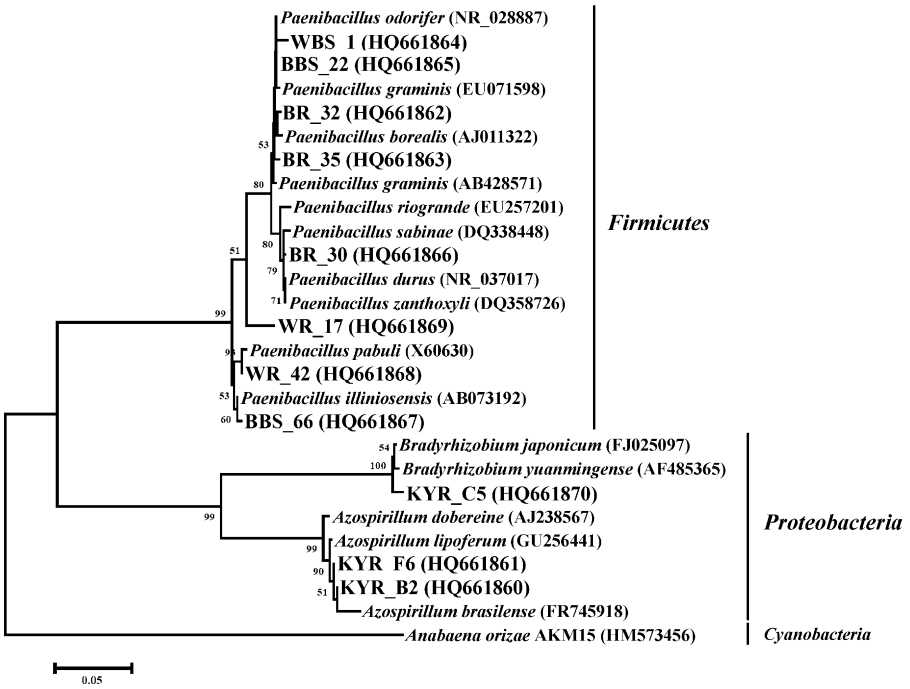

Fig. 1. Maximum likelihood phylogenetic tree of isolates based on 16S rRNA gene partial sequences. The scale bars indicate the nucleotide substitutions per site. Numbers at the branches indicate the bootstrap values of 1000 resamplings. Only values above 50% are shown. *Anabaena oryzae* served as outgroup.

Gram negative bacilli, and to a lesser extent to Gram positive bacilli, while bacterial colonies growing on Bridges and WAT4C agar plates were white, mucoid, smooth, and brilliant, with irregular margins and corresponded to Gram positive bacilli.

To screen for the ability to fix atmospheric nitrogen in liquid medium, a representative isolate of each RAPD profile was grown serially three times in each nitrogen-free medium. Only 36 bacterial isolates of a total of 61 strains could grow after three serial transfers.

Two samples of non-rhizospheric soil were included in the study. A total of 12 isolates and 9 RADP profiles were obtained. Only three RADP profiles were shared between rhizospheres and the bulk soil and three non-rhizospheric isolates were able to grow in nitrogen-free liquid media.

3.2. Molecular identification of the bacteria isolates

Only 11 strains with nitrogen-fixing phenotypes were identified. All sequences were identified to species level, except the isolate WR_17 identified only to genus level (Table 2). Three isolates were members of the *alpha-Proteobacteria* and identified as *Azospirillum lipoferum* and *Bradyrhizobium japonicum;* the others members of the phylum *Firmicutes* corresponded to *Paenibacillus* spp. (Fig. 1).

3.3. Nitrogen fixing activity

In order to corroborate the ability to fix atmospheric nitrogen by the isolates, the enzymatic activity of nitrogenase was quantified by ARA. Some strains, such as *Paenibacillus durus* (BR_30) and *Paenibacillus graminis* (BR_35) reached high activity levels (169.45 and 148.08 nmol C_2H_4/h/culture, respectively). Other isolates were able to reduce acetylene; however, the levels reached were not as high. We also evaluated the nitrogen fixing ability of the isolates that exhibited the highest level of HM resistance, thus, *P. graminis*, *P. odorifer* and *P. borealis* were analyzed. Low concentrations of Cr^{6+} and Co^{2+} inhibited the acetylene reduction in all the tested strains; while Ni^{2+} and Zn^{2+} were mild inhibitors (see Fig. 2).

*nif*D and *nif*H genes were detected in the majority of the strains that grew in N-free media except in *Paenibacillus pabuli* WR_42 and *P. odorifer* WBS_1. Only in *P. durus* BR_30 was the *anf*H gene, encoding an alternative nitrogenase, detected (Table 3).

3.4. Determination of minimum inhibitory concentration of heavy metals

HM resistance was tested in culture media with and without N (Table 4). All isolates were Cu^{2+}-resistant in culture media containing nitrogen, but sensitive in nitrogen-free media. In general, the MICs were influenced by N and most isolates were resistant to Ni^{2+} and Zn^{2+} in both culture media conditions.

3.5. Plant growth-promoting ability

Phosphate solubilization, cellulolytic activity, indole acetic acid production and germination promotion were evaluated. Most of the isolates exhibited clear zones of solubilization around their colonies. The phosphate solubilization index ranged from two to five (Table 5). This preliminary observation suggested the existence of bacterial isolates exhibiting different degrees of mineral phosphate solubilizing efficiencies in the soil samples collected. Furthermore, some of them exhibited cellulolytic activity and a different range of IAA production. The *acd*S gene was only detected in *P. durus* BR_30. All the bacterial strains isolated in this work had a range of heavy metal resistance and they were resistant to two or three heavy metals (Table 5).

4. Discussion

Some plants have phytostabilization and phytoextraction capacities applicable to man-made mine tailings highly contaminated with HMs (Vassilev et al., 2004). However, there is scarce evidence of the contribution of bacteria to plant adaptation to this environment. Bacterial traits, such as tolerance to HM, production of phytohormones and siderophores, as well as nitrogen-fixation

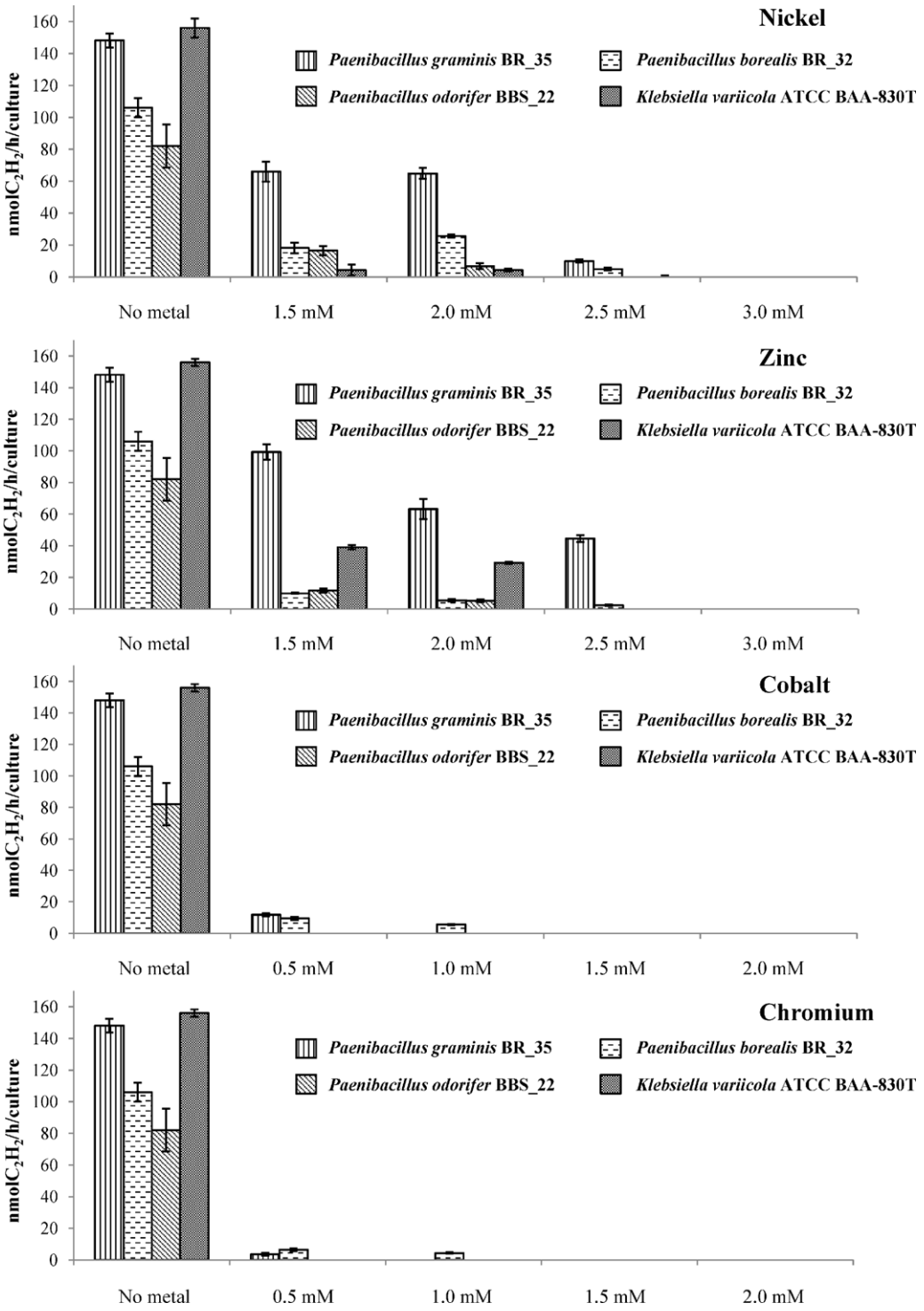

Fig. 2. Acetylene reduction assays in presence of heavy-metals of diazotrophic isolates. Determinations were performed using WAT4C semisolid media with an acetylene-enriched atmosphere to a final concentration of 10%. Media were supplemented with a heavy-metal stock solution 125 mM to reach the final concentrations. Error bars indicate standard deviation from triplicate measurements.

and phosphate solubilizing capacities represent a potential contribution to plant adaptation (Glick, 2003). The nitrogen-fixing bacteria were isolated from the rhizospheres of nine HM tolerant and pioneer plants in mine tailings, and some relevant phenotypic traits were assayed *in vitro*. Importantly, we found that the bacterial community associated with the rhizosphere of heavy metal-tolerant and pioneer plants in Zacatecas, Mexico, included nine major phylogenetic groups (Navarro-Noya et al., 2010).

A total of three media were used for enriching nitrogen-fixing bacteria. Each medium containing a different carbon source allowed the isolation of *Paenibacillus*, *Azospirillum* and *Bradyrhizobium* species. In general, *Paenibacillus* species reached the highest nitrogen-fixing activities *in vitro*, although *B. japonicum* and *A. lipoferum* exhibited significant acetylene reductions. Nitrogen-reduction activity was observed in *P. durus* BR_30, a rhizospheric or endophytic bacterium frequently isolated from different grasses

Table 3
Nitrogen-fixing genes and acetylene reduction activities in nitrogen fixing bacterial strains isolated from the rhizosphere of pioneer plants growing on heavy-metal contaminated soils.

Bacterial strain	PCR gene detection			Acetylene reduction (nmol C_2H_4/h/culture)
	*nif*H	*nif*D	*anf*H	
Nitrogen fixing bacteria				
Paenibacillus durus BR_30	+	+	+	169.4 ± 18.0
Paenibacillus borealis BR_32	+	+	–	106.0 ± 12.7
Paenibacillus graminis BR_35	+	+	–	148.0 ± 8.8
Paenibacillus sp. WR_17	+	+	–	0.6 ± 0.02
Paenibacillus pabuli WR_42	–	–	–	0.3 ± 0.006
Azospirillum lipoferum KYR_B2	+	+	–	33.8 ± 5.1
Azospirillum lipoferum KYR_F6	+	+	–	29.9 ± 9.2
Bradyrhizobium japonicum KYR_C5	+	+	–	0.9 ± 0.08
Paenibacillus odorifer BBS_22	–	–	–	13.6 ± 2.1
Paenibacillus illinoisensis BBS_66	+	+	–	0.1 ± 0.004
Paenibacillus odorifer WBS_1	+	+	–	19.0 ± 3.7
Positive and negative controls				
Klebsiella variicola F2R9	+	+	–	178.2 ± 22.6
Escherichia coli DH10b	–	–	–	–
Negative control[a]	–	–	–	–

[a] Sterile medium amended with acetylene.

Table 4
Minimum inhibitory concentrations (MIC) of nitrogen-fixing bacterial strains isolated from the rhizosphere of pioneer plants growing on heavy-metal contaminated soils.

Bacterial strain	Minimum inhibitory concentration (mM)									
	PY medium					Nitrogen fixing conditions[a]				
	Co	Ni	Zn	Cr	Cu	Co	Ni	Zn	Cr	Cu
Paenibacillus durus BR_30	0.5	0.5	0.25	0.25	1.0	0.12	0.25	2.5	0.5	0.06
Paenibacillus borealis BR_32	0.5	1.0	0.5	0.12	2.0	0.5	2.5	2.5	0.25	0.12
Paenibacillus graminis BR_35	0.25	2.0	2.0	0.25	4.0	0.5	2.5	2.5	0.25	0.12
Paenibacillus sp. WR_17	1.0	1.0	2.0	0.5	2.0	0.12	0.25	2.5	0.5	0.12
Paenibacillus pabuli WR_42	0.5	2.0	1.0	0.25	2.0	0.12	0.25	2.0	0.06	2.0
Azospirillum lipoferum KYR_B2	0.5	1.0	1.0	0.25	2.0	0.25	2.5	2.0	0.06	0.25
Azospirillum lipoferum KYR_F6	0.5	2.0	1.0	0.5	2.0	0.25	1.5	2.5	0.06	0.25
Bradyrhizobium japonicum KYR_C5	0.25	2.0	4.0	0.12	2.0	0.25	1.5	2.0	0.06	0.25
Paenibacillus odorifer BBS_22	0.25	1.0	0.5	0.25	2.0	0.25	0.25	2.0	0.5	0.12
Paenibacillus illinoisensis BBS_66	0.25	2.0	4.0	0.25	4.0	0.12	0.25	2.0	0.12	0.25
Paenibacillus odorifer WBS_1	0.25	1.0	0.5	0.5	2.0	0.25	0.25	2.0	0.06	0.12
Klebsiella variicola F2R9	0.25	0.5	1.0	0.12	2.0	0.12	0.25	1.0	0.5	0.12

[a] MIC values determined on WAT4C semisolid medium amended with the metals.
Isolates growing at 1 mM of Cu^{2+}, Ni^{2+} and Zn^{2+}; and 0.5 mM of Co^{2+} and Cr^{3+} were considered resistant (Brim et al., 1999; Nieto et al., 1987).

Table 5
Plant growth promotion activities in nitrogen-fixing bacterial strains isolated from the rhizosphere of pioneer plants growing on heavy-metal contaminated soils.

Bacterial strain	P solubilization index[a]	Cellulolytic activity	*acd*S detection	IAA production (μg AIA/mL)	Spermosphere (% germination)	Heavy metal resistance
Paenibacillus durus BR_30	2 ± 0	+	+	8.52 ± 3.3	46.6 ± 8.8	Cu
Paenibacillus borealis BR_32	4 ± 0	–	–	10.5 ± 4.5	16.6 ± 6.6	Ni, Cu
Paenibacillus graminis BR_35	3 ± 0	–	–	0	13.3 ± 3.3	Ni, Zn, Cu
Paenibacillus sp. WR_17	0	+	–	11.83 ± 4.6	10 ± 0	Co, Ni, Zn, Cr, Cu
Paenibacillus pabuli WR_42	0	–	–	0	6.6 ± 3.3	Ni, Cu
Azospirillum lipoferum KYR_B2	0	+	–	20.64 ± 2.47	10.0 ± 0	Ni, Zn, Cu
Azospirillum lipoferum KYR_F6	5 ± 0	+	–	21.37 ± 1.51	20.0 ± 0	Ni, Zn, Cr, Cu
Bradyrhizobium japonicum KYR_C5	0	+	–	12.45 ± 3.67	6.6 ± 3.3	Ni, Zn, Cu
Paenibacillus odorifer BBS_22	2 ± 0	–	–	7.12 ± 5.3	26.6 ± 8.8	Ni, Cu
Paenibacillus illinoisensis BBS_66	4 ± 0	–	–	0	6.6 ± 3.3	Ni, Zn, Cu
Paenibacillus odorifer WBS_1	3 ± 0	–	–	5.52 ± 2.8	0	Ni, Cr, Co
Klebsiella variicola F2R9	3.3 ± 0	–	–	4.52 ± 1.7	0	Zn, Cu
Escherichia coli DH10b	0	–	–	3.25 ± 1.2	0	
Azospirillum sp.	0	–	–	8.52 ± 3.9	48.15 ± 6.8	
Negative control	–	–	–	–	6.6 ± 3.3	

[a] Index of solubilization was determined as the diameter of the colony plus the clear zone divided by the diameter of the colony.
Isolates growing at 1 mM of Cu^{2+}, Ni^{2+} and Zn^{2+}; and 0.5 mM of Co^{2+} and Cr^{3+} were considered resistant (Brim et al., 1999; Nieto et al., 1987).

and maize (Seldin et al., 1998, 1984). Paradoxically, this strain isolated from an environment highly contaminated with heavy metals was unable to grow and fix nitrogen in the presence of heavy metals. In contrast, the other *Paenibacillus* species (*P. graminis* BR_35, *P. odorifer* BBS_22 and *P. borealis* BR_32) fixed nitrogen in moderate HM concentrations. Although no specific viable counts of *P. durus* could be determined, the total populations of culturable *Paenibacillus* species were approximately 10^3–10^4 CFU g^{-1} in the rhizospheric tailings (data not shown). Remarkably, only in *P. durus* BR_30 was an alternative nitrogenase gene detected, the product of which apparently confers a selective advantage to strains in nutrient deficient environments (Raymond et al., 2004). PGP activities of *P. durus* strains isolated in this work were more significant than all other strains, confirming previous studies (Beneduzi et al., 2008; Rosado et al., 1998; Seldin et al., 1998).

Paenibacillus genus has been frequently detected or isolated as free-living bacteria in HM contaminated soils (Zhang et al., 2007a,b), and as endophytic bacteria in hyperaccumulator plants (Barzanti et al., 2007). Moreover, the nitrogen-fixing capacity of several *Paenibacillus* species has been studied *in vitro*. Unfortunately, the data and activity units cannot be compared with ARA data due to the different techniques performed to estimate the nitrogen-fixing capacity of the strains (Beneduzi et al., 2008; Ding et al., 2005; Rosado et al., 1998). To the best of our knowledge, no studies evaluating the effect of heavy metals on nitrogen-fixation by *Paenibacillus* spp. have been conducted. In this work *P. borealis* BR_32, *P. graminis* BR_35 isolated from the rhizosphere, and *P. odorifer* BBS_22 isolated from the bulk soil were efficient as nitrogen-fixing bacteria without HMs, but moderate activities were detected in relatively high concentrations of Co (1 mM), Ni (2.5 mM), and Zn (2.5 mM). Also, *P. illinoisensis* strains were commonly isolated in rhizospheres and in bulk soil, and were resistant to HM. However, they exhibited low levels of nitrogenase activity.

The presence of a community of taxonomically related bacteria with different HM sensibility and nitrogen-fixing capacities suggests the possible existence of functional redundancy. However, if the competitive exclusion principle is assumed, the niche redundancy is not allowed (Hardin, 1960). Thus phenotypically different *Paenibacillus* spp. isolated from the same habitat could be occupying different ecological niches. This work suggests that whereas some strains hardly fixed nitrogen and were HM resistant, others moderately fixed nitrogen and were HM resistant, and still others exhibited relevant nitrogen fixing capacities but were sensitive to HM. Another possibility is a dynamic scenario for the ecological niche, where the redundancy in functions helps to maintain processes in changing environmental conditions (Kennedy and Smith, 1995). Evidently, more experiments are necessary to define the theoretical ecological niches in the rhizosphere of HM tolerant plants.

Nitrogen-fixing activity of all the strains included in this work decreased with increasing HM concentration, an effect that has been widely reported (Chen et al., 2003; Obbard and Jones, 1993; Obbard et al., 1993). Many papers speculate on the importance of nitrogen fixation in phytoremediation processes of mine tailings, human-made residues where nitrogen fixation is the unique input mechanism of this element to the environment (Glick, 2003; Mendez et al., 2007; Wu et al., 2006). Apart from evident capacities of *Azospirillum* spp. in PGP (Bashan and De-Bashan, 2002; Costacurta and Vanderleyden, 1995), only one study describing the nitrogen-fixing capacities of *Azospirillum* spp. isolated from HM contaminated environments has been performed (Moreira et al., 2008). Although there are important differences between the ARA performed in our work and that of Moreira et al. (2008), *A. lipoferum* isolated from mine tailings in Brazil maintained the nitrogen fixing activity up to 1.9 mM Zn^{2+} and 0.04 mM Cd^{2+}, while *A. lipoferum* KYR_B2 isolated in our work exhibited nitrogen fixing activity with 1.0 mM, 2.5 mM, and 2.5 mM of Co, Ni and Zn, respectively. In another work, physiological responses of *Azospirillum brasiliensis* to low concentrations (up to 0.2 mM) of Co^{2+}, Cu^{2+} and Zn^{2+} (Kamnev et al., 2005) were determined, however, nitrogen fixation was not tested. In any case, these assays were carried out *in vitro* reducing the complexity of the tailing or soil ecosystem. Evidently, in this scenario more research must be performed to reveal the protection mechanisms that make nitrogen fixation possible in this nitrogen deficient environment.

Finally, the diazotrophic *B. japonicum* KYR_C5 strain was isolated in one sample. *Bradyrhizobium* spp. are also known as producers of siderophores, IAA, and P solubilizers (Abd-Alla, 1994; Antoun et al., 2004; Wittenberg et al., 1996), and are frequently used as PGPR in phytoremediation processes (Khan, 2005). Particularly, *B. japonicum* KYR_C5 produced IAA and was resistant to Ni^{2+}, Zn^{2+} and Cu^{2+}, although no P-solubilization and nitrogen-fixing activity in the presence of HM was obtained.

5. Conclusion

We isolated and characterized rhizospheric nitrogen-fixing bacteria associated with HM tolerant plants. Most of the isolates were identified as different species of the *Paenibacillus* genus, being *P. durus* the isolate with the highest nitrogenase activity, but was sensitive to heavy metals. Some strains maintained nitrogenase activity in high concentrations of Ni and Zn. Apparent redundancy in microbial functions in the rhizosphere probably helps to maintain processes in a range of environmental conditions.

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