Chapter 94

Potential Plant-Growth-Promoting and Nitrogen-Fixing Bacteria Associated with Pioneer Plants Growing on Mine Tailings

Yendi E. Navarro-Noya
Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, IPN, Mexico

Esperanza Martínez-Romero
Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Mexico

César Hernández-Rodríguez
Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, IPN, Mexico

94.1 INTRODUCTION

Mine tailings are solid residues produced by the mining industry with high concentrations of heavy metals (HMs) and often deposited under open sky (Dudka and Adriano, 1997). Mining tailings are known to have high environmental effects, exacerbated by the incidence of erosion and lixiviation, contaminating surrounding areas and ground water with HM (Wong et al., 1998). Mine tailings have low concentration of plant nutrients, such as nitrogen, organic matter, and phosphorus. Also, these residues retain high HM concentrations after extraction procedures, such as arsenic, copper, iron, manganese, nickel, lead, and cadmium (Krzaklewski and Pietrzykowski, 2002; Mendez et al., 2008). These areas are difficult to remediate because HM cannot be degraded to harmless products. Besides, HMs have a profound impact on the structure and function of soil microbial communities (Gleeson et al., 2006; Bamborough and Cummings, 2009). However, some pioneer plant species can grow on mine tailings and have developed mechanisms that allow them to resist phytotoxic HM concentrations (Lei and Duan, 2008). These plants are promising for use in phytoremediation processes for the management of mining residues or HM-polluted soils (Salt et al., 1995; Escarré et al., 2011).

Endemic metal-tolerant plants have been used to vegetate and control soil erosion on metal mine tailings and thereby reduce the spread of these metals in the environment. Plants may harbor microbial rhizospheric communities that are known to play a key role in modifying the availability and toxicity of these elements to the plants (Wu et al., 2006; Li et al., 2011). Among them, plant-growth-promoting bacteria (PGPB) are potentially capable of fixing atmospheric nitrogen, of solubilizing phosphorus and iron, and producing phytohormones.

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PGPB have found wide use in agriculture and are now being explored for environmental applications (Bashan and de-Bashan, 2002; Khan et al., 2009; see Chapter 53).

The bacterial diversity of the metallophyte plant rhizosphere under HM stress has been estimated by culture-based methods, ribosomal libraries, denaturing gradient gel electrophoresis (DGGE), and PhyloChips (see Chapters 5, 6, 9, 10). Despite adverse conditions, the mine tailing environments displayed a considerable potential metabolic diversity as deduced from the heterotrophic and chemolithotrophic indigenous microbial populations detected. Specifically, chemolithotrophic ammonia oxidizers, iron-reducers and iron-oxidizers, methanogens, and sulfate-reducers were shown to be present in tailings contaminated with HM (Zhang et al., 2007a, b; Sun et al., 2009; Wang et al., 2009; Navarro-Noya et al., 2010; Rastogi et al., 2011).

Several benefits of PGPBs for the establishment of vegetation on desertified or contaminated sites, including mine tailings located in arid regions, have been recognized (Bashan and de-Bashan, 2002; Wu et al., 2006; Grandlic et al., 2008). Particularly, biological nitrogen fixation is an important biological process to provide this macroelement to mine tailings (Vitousek and Howarth, 1991; Arp, 2000; see Chapter 44). Thus, it could be expected that such a nitrogen input would stimulate the nitrogen cycle by improving the fertility of mine spoils. Mine tailings provide an opportunity to study the ecological effects of HM on the growth of plants and the activity of soil microbial communities in the rhizosphere of plants, which are important for the survival and succession of plant species on mine tailings (Li et al., 2011).

Little attention has been paid to the occurrence of nitrogen-fixing bacteria in the rhizosphere of plants growing on HM and nitrogen-deficient substrates, such as HM-contaminated soils or mine tailings. However, some papers have evaluated the nitrogen-fixing potential of plant-diazotrophic bacteria in these environments. *Azospirillum* spp.-tolerant strains were isolated from dumping sites near a zinc-processing industry in Brazil. These diazotrophic bacteria were able to grow and fix nitrogen *in vitro* with assimilable nitrogen or in nitrogen-free conditions and moderate concentrations of Zn^{2+} and Cd^{2+} (Moreira et al., 2008).

The rhizosphere of *Gymnostoma webbianum* and *Serianthes calycina*, pioneer plants from serpentine soils, exhibit *in situ* nitrogen-fixing activity and reveal the presence of *nif*H genes associated with the *Bradyrhizobium–Beijerinckia nif*H gene cluster (Héry et al., 2005). In addition, from the rhizosphere of pioneer plants growing on copper mine tailings from East China *nifH* gene sequences have been detected, suggesting the presence of a moderate diversity of nitrogen-fixing Alpha- and Betaproteobacteria and Cyanobacteria (Zhan and Sun, 2012; see Chapter 44). Recently, it was demonstrated that the legume *Anthyllis vulneraria* subsp. *carpatica* is able to establish a symbiosis with *Mesorhizobium metallidurans*, both species originating from highly polluted mine tailings from Saint-Laurent-le-Minier, France. Plant and bacteria were able to form nodules, survive, and grow in soil contaminated with zinc, lead, and cadmium, fixing nitrogen and increasing nitrogen concentration in soils, a set of tasks potentially useful for phytostabilization (Mahieu et al., 2011). On the other hand, *Frankia* and/or the mycorrhizal fungus *Paxillus involutus* did not improve *Alnus incana* plant survival and growth on HM mine tailings (Markham, 2005).

Consequently, more studies are required to understand the biological nitrogen input to metallophyte plant rhizospheres. Previously, we described that the bacterial community associated with the rhizosphere of HM-tolerant and pioneer plants in Zacatecas, Mexico, included nine major phylogenetic groups, but only *Azoarcus evansii* was recognized as a potential diazotroph in mine tailing samples in this region (Navarro-Noya et al., 2010). In this chapter, we present the characterization of culturable bacteria from the rhizosphere of HM-tolerant, endemic, and pioneer plants, which were previously studied.

94.2 METHODS

94.2.1 Rhizosphere Soil Sampling and Characterization

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 at 2558 m altitude) and Noria de los Angeles (22° 28.009′N, 101° 54.305′W, and at 2208 m altitude) in Zacatecas, Mexico. The plants were identified as *Viguiera linearis*, *Polygonum aviculare*, and *Aster gymnocephalus* from Sombrerete, and *Bahia xylopoda*, *Haplopappus venetus*, and *Chloris virgata* from Noria de los Angeles. The physicochemical characterization, HM and organic matter content, particle distribution, and cation exchange capacity (CEC) of these soils were published previously (González and González-Chávez, 2006). Also, samples of nonrhizospheric or bulk soil were obtained.

94.2.2 Isolation of Nitrogen-Fixing Heterotrophic Bacteria

Serial decimal dilutions of soil in sterile water were spread on nitrogen-free Bridges and WAT4C agar media and incubated at 28 °C for several days for selective isolation of nitrogen-fixing bacteria (Bridges, 1981; Berge et al., 1991). In order to screen the ability to fix

atmospheric nitrogen in liquid medium, each isolate was grown serially thrice in nitrogen-free media.

94.2.3 Identification of the Strains by the Analysis of the 16S rRNA Gene Fragments

Isolated microorganisms were identified by phylogenetic analyses of the 16S rRNA gene partial sequence.

Total DNA from bacterial DNA from isolated strains was extracted using a previously described protocol (Cullen and Hirsch, 1998). The amplification of 16S rRNA genes was performed by PCR using universal bacterial primers (Relman, 1993). PCR products were 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).

94.2.4 Phylogenetic Analysis of the Bacteria

Details of the phylogenetic analysis were previously given (Navarro-Noya et al., 2010). The species identification was achieved considering genus and species limits of 95% and 97.5%, respectively, (Rossello-Mora and Amann, 2001). In addition, the phylogenetic approach considered the similarity percentage and clustering in trees.

94.2.5 Phenotypic and Genotypic Features of the Strains

94.2.5.1 Amplification of acdS Gene Encoding ACC (1-Aminocyclopropane-1-Carboxylate) Deaminase. Deamination of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), by ACC enzyme is a key plant-beneficial trait found in PGPB (Glick, 2003; see Chapter 53). The *acd*S gene, encoding ACC, was detected among the nitrogen-fixing isolates through a PCR procedure designed in this work. In brief, primers were chosen from conserved regions detected in multiple sequence alignments of a broad collection of *acd*S genes. Forward and reverse primer correspond to *Pseudomonas fluorescens acd*S gene (EF635249) N148-N170 (AAYAARMCGCGSAAGCTCGAATA) and N828-N847 (CGCACAGDCGRATWGCYTCC), respectively. 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 between 60 °C 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.

94.2.6 Acetylene Reduction Assay

Acetylene reduction assays (ARA) were carried out with isolated bacteria grown in WAT4C semisolid media (Berge et al., 1991) for 72 h at 28 °C, using acetylene-enriched atmospheres to a final concentration of 1% and 10%. The acetylene reduction activity was measured with a Varian 3300 (Walnut Creek, CA) gas chromatograph with a flame ionization detector. *Klebsiella variicola* ATCC BAA-830T and *Escherichia coli* DH10b were included as the positive and negative controls, respectively.

94.2.7 Determination of the Minimal Inhibitory Concentrations of Heavy Metals

The minimal inhibitory concentrations (MIC) of HM were determined by broth microdilution according to the CLSI document 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. The isolates growing in 1 mM of Cu^{2+}, Ni^{2+}, and Zn^{2+}, and 0.5 mM of Co^{2+} and Cr^{3+} were considered resistant according to previous criteria (Brim et al., 1999; Nieto et al., 1987). Similarly, MIC assays in nitrogen-free WAT4C medium were performed.

94.2.8 Cellulose-Degrading Activity and Mineral Phosphate Solubilization

The cellulolytic activity of isolates was detected by a clear halo around the colonies in cellulose-Congo red agar plates (Hendricks et al., 1995). Phosphate-solubilizing bacteria colonies were recognized by the formation of clear halos using phosphate growth media (Wu et al., 2006). Activity was indexed as the diameter of the halo divided by the diameter of the colony (Teather and Wood, 1982).

94.2.9 Indole Acetic Acid Production

Indole acetic acid (IAA) production was quantified spectrophotometrically in bacterial overnight culture supernatants using Salkowski R2 reagent ($FeCl_3$, 4.5 g/l in 10.8 M H_2SO_4) (Glickmann and Dessaux, 1995).

94.3 RESULTS

94.3.1 Enrichments and Isolation of Bacteria Capable of Growing in Nitrogen-Free Media

A total of 49, 58, and 45 isolates with different colonial morphologies were obtained using nitrogen-free Winogradsky, Bridges, and WAT4C agar culture media, respectively. Only 36 bacterial isolates of a total of 61 strains tested could grow after three serial transfers in nitrogen-free media. Similarly, only 3 isolates of a total of 11 strains tested from two samples of nonrhizospheric soil were able to grow in nitrogen-free liquid media. No isolates were shared between rhizospheric and nonrhizospheric or bulk soils.

94.3.2 Molecular Identification of the Bacterial Isolates

The 16S rRNA neighbor-joining phylogenetic tree of 28 bacteria isolated from rhizospheric and nonrhizospheric HM-contaminated soils and GenBank sequences is shown in Figure 94.1. Isolates were grouped in four different phyla: Bacteroidetes, Firmicutes, Actinobacteria, and Alpha- and Deltaproteobacteria. The majority of the isolates were members of the Alphaproteobacteria class (20 out of 38) and identified as *Azospirillum lipoferum*, *Brevundimonas diminuta*, *Sphingomonas xenophaga*, *Rhizobium etli*, *Rhizobium huautlense*, *Rhizobium* sp., *Rhizobium cellulosilyticum*, *Agrobacterium tumefaciens*, *Agrobacterium* sp., and *B. japonicum*. Isolates grouped with phylum Firmicutes were identified as different *Paenibacillus* species, including *Paenibacillus azotofixans*, *P. caespitis*, *P. graminis*, *P. odorifer*, *P. illinoisensis*, *P. pabuli*, *P. borealis*, and *P. xylanexedens*. A pair of members of phyla Actinobacteria and Bacteroidetes was identified as *Agromyces mediolanus* and *Olivibacter soli*, respectively.

94.3.3 Characterization of the Isolates

The phenotypic and genotypic characteristics of 34 selected bacterial isolates are summarized in Table 94.1. The nitrogen-fixing phenotype of only 12 strains was confirmed by ARA using WAT4C semisolid media. The majority of the strains with nitrogen-fixing capacity belonged to the *Paenibacillus* genus but *A. lipoferum* and *B. japonicum* were also included. Some strains, such as *P. azotofixans* (BR_30) and *P. graminis* (BR_35) showed high levels of activity (169.45 and 148.08 nmolC_2H_4/h/culture, respectively). The nitrogen-fixing ability of *P. graminis*, *P. odorifer*, and *P. caespitis*, isolates that exhibited the highest level of HM resistance and enzymatic activity of nitrogenase, were also determined. Cr^{6+} and Co^{2+} were the HM that displayed the most significant inhibitory effect on the acetylene reduction in all the strains tested, while moderate concentrations of Ni^{2+} and Zn^{2+} did not significantly reduce the nitrogen-fixing activities (data not shown).

Table 94.1 also shows the resistance to Cu, Ni, Zn, Co, and Cr. All isolates were Cu^{2+} resistant in culture medium containing nitrogen, but sensitive in nitrogen-free culture medium. All bacterial strains tested had a particular range of HM resistances. In general, most of them were resistant to Ni^{2+} and Zn^{2+} in culture media. *A. mediolanus* KYR_C8 was resistant to all the HM tested.

94.3.4 Plant-Growth-Promoting Activities

Table 94.1 shows the characteristics of potential PGPB isolates. At least one strain of each species exhibited phosphate solubilization (see Chapter 58), but this capacity was broadly distributed among *Paenibacillus* spp. and *Olivibacter* spp. In addition, variable cellulolytic activity and auxin production were detected. Particularly, *Agrobacterium*, *Azospirillum*, and *Rhizobium* exhibited a significant IAA production. The *acd*S gene was only detected in *P. azotofixans* BR_30, *Agrobacterium* sp. WR_5.6-10, and *Rhizobium* sp. WR_5.6-2.

94.4 DISCUSSION

Nitrogen-fixing bacteria were isolated from the rhizospheres of six HM-tolerant and pioneer plants growing on mine tailings, and several relevant phenotypic traits were assayed *in vitro*.

In general, *Paenibacillus* species reached the highest nitrogen-fixing activities *in vitro*, although *B. japonicum* and *A. lipoferum* exhibited significant acetylene reduction activities as well. The highest nitrogen-reduction activity was observed in *P. azotofixans*, a rhizospheric or endophytic bacterium frequently isolated from different grasses and maize (Seldin et al., 1984, 1998). Paradoxically, this strain isolated from a highly HM-contaminated environment was unable to grow and fix nitrogen in the presence of low HM concentrations.

In contrast, other *Paenibacillus* species (*P. graminis*, *P. odorifer*, and *P. caespitis*) fixed nitrogen in moderate HM concentrations. These apparent inconsistent data could be explained if a mosaic of microenvironments with different HM bioavailabilities exist in the rhizosphere of plants (Ranjard et al., 2000). In addition, the plant-growth promoting activities of *P. azotofixans* strains isolated in this work were more significant than

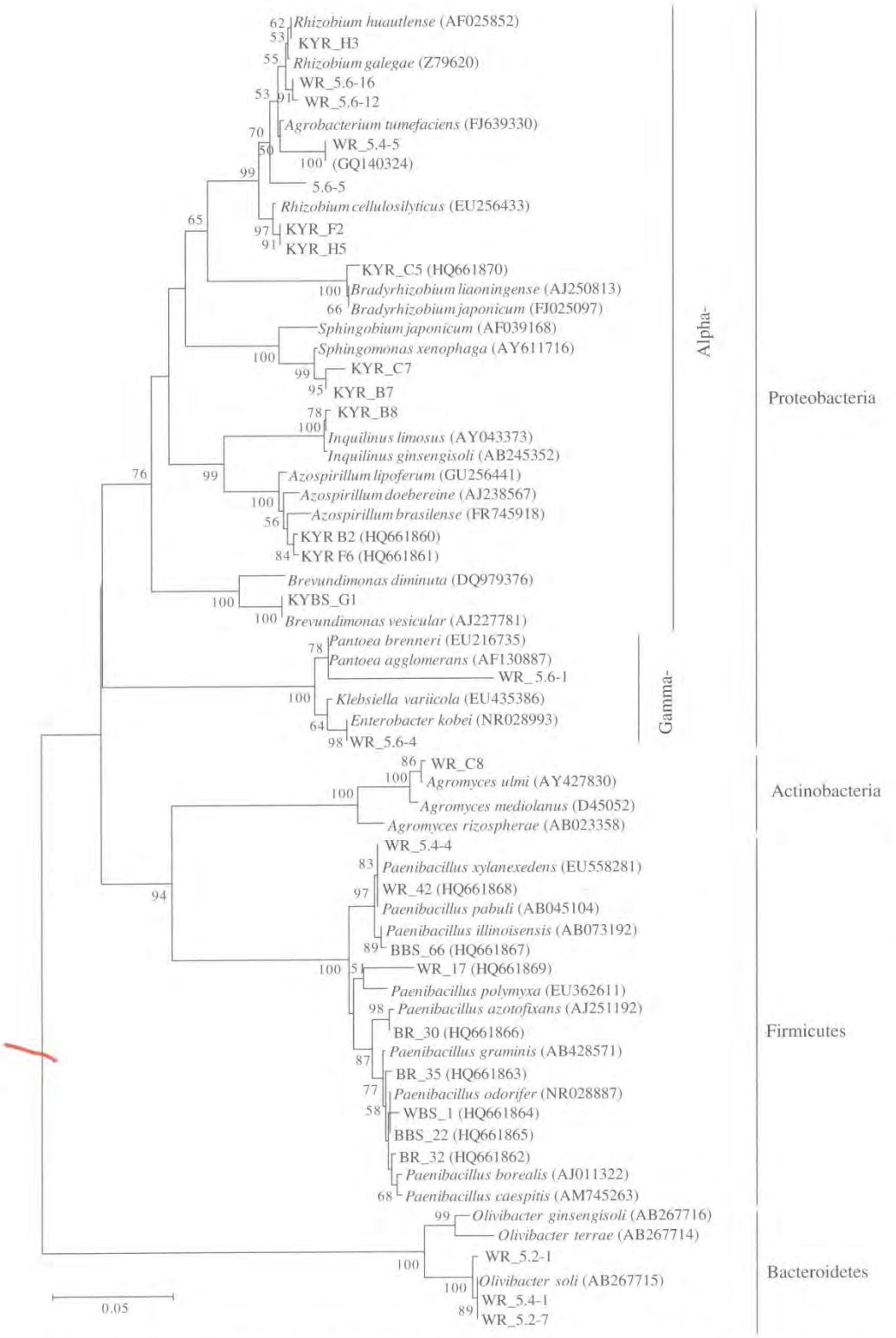

Figure 94.1 Neighbor-joining phylogenetic tree of 16S rRNA gene sequences of bacteria isolated from rhizospheric and nonrhizospheric HM-contaminated soils. Distances were calculated with the Kimura 2-parameter substitution model. The scale bars indicate the nucleotide substitutions per site. Numbers at the branches indicate the bootstrap values of 1000 samplings. The Bacteroidetes phylum served as an outgroup.

Table 94.1 Plant-growth-promoting activities in bacterial strains isolated from the rhizosphere of pioneer plants growing on heavy-metal-contaminated soils

Isolate	Potential Plant-Growth-Promoting Activities					
	P-SI[a]	CA	*acdS*[b]	AP (μg AIA/ml)	AR (mmol/h/culture)	HM-R
Paenibacillus azotofixans BR_30	2 ± 0	+	+	8.52 ± 3.3	169.45 ± 18.01	Cu
Paenibacillus caespitis BR_32	4 ± 0	–	–	10.5 ± 4.5	106.05 ± 12.75	Ni, Cu
Paenibacillus graminis BR_35	3 ± 0	–	–	0	148.08 ± 8.83	Ni, Zn, Cu
Paenibacillus sp. WR_17	0	+	–	11.83 ± 4.6	0.54 ± 0.02	Co, Ni, Zn, Cr, Cu
Paenibacillus pabuli WR_42	0	–	–	0	0.27 ± 0.006	Ni, Cu
Paenibacillus odorifer BBS_22	2 ± 0	–	–	7.12 ± 5.3	13.59 ± 2.07	Ni, Cu
Paenibacillus illinoisensis BBS_66	4 ± 0	–	–	0	0.14 ± 0.04	Ni, Zn, Cu
Paenibacillus borealis WBS_1	3 ± 0	–	–	5.52 ± 2.8	18.98 ± 3.67	Ni, Cr, Co
Paenibacillus xylanexedens WR_5.4-4	4.0 ± 0	–	+	10 ± 5.7	–	Cu
Azospirillum lipoferum KYR_B2	0	+	–	20.64 ± 2.47	33.84 ± 5.12	Ni, Zn, Cu
Azospirillum lipoferum KYR_F6	5 ± 0	+	–	21.37 ± 1.51	29.91 ± 9.28	Ni, Zn, Cr, Cu
Bradyrhizobium japonicum KYR_C5	0	+	–	12.45 ± 3.67	0.89 ± 0.0.078	Ni, Zn, Cu
Agrobacterium tumefaciens WR_5.2-2	4.0 ± 1.1	–	–	0	–	Zn, Cu
Agrobacterium tumefaciens WR_5.6-16	–	+	–	10 ± 5.7	–	Ni, Zn, Cu
Agrobacterium sp. WR_5.2-6	–	–	–	10 ± 5.3	–	Ni, Zn, Cu
Agrobacterium sp. WR_5.6-10	–	–	+	0	–	Cu
Agrobacterium sp. WR_5.6-12	–	–	–	10.0	–	Cu
Agrobacterium sp. WR_5.6-14	–	+	–	10.0 ± 5.7	–	Ni, Zn, Cu
Agrobacterium sp. WR_5.6-15	–	–	–	0	–	Ni, Zn, Cu
Rhizobium huahutlense HYR_H3	–	+	–	23 ± 3.3	–	Ni, Cu
Rhizobium etli KYR_H5	2.0 ± 0.3	–	–	13 ± 3.3	–	Co, Cu
Rhizobium etli WR_5.4-5	–	–	–	0	–	Zn, Cu
Rhizobium cellulosilyticum WR_5.6-5	–	–	–	10.0 ± 2.1	–	Ni, Zn, Cu
Rhizobium sp. WR_5.6-2	–	+	+	0	–	Cu
Rhizobium sp. KYR_E6	–	–	–	20 ± 5.7	–	Co, Ni, Zn, Cr, Cu
Rhizobium sp. KYR_F2	3.0 ± 0	+	–	13 ± 8.8	–	Ni, Cu
Olivibacter soli WR_5.2-1	–	+	–	10 ± 5.7	–	Ni, Zn, Cu
Olivibacter soli WR_5.4-1	1.5 ± 0	–	–	0	–	Zn, Cu
Brevundimonas vesicular KYR_G1	–	+	–	40 ± 15.2	–	Cu
Enterobacter kobei WR_5.6-4	–	+	–	0	–	Cu
Pantoea sp. WR_5.6-1	–	+	–	6.6 ± 3.3	–	Ni, Cu
Sphingomonas xenophaga KYR_C7	–	+	–	20.1 ± 5.7	–	Ni, Cu
Inquilinus ginsengisoli KYR_B8	–	+	–	20 ± 1.6	–	Co, Ni, Zn, Cu
Agromyces mediolanus KYR_C8	–	+	–	16.6 ± 6.6	–	Ni, Zn, Cu
Klebsiella variicola [c] ATTC BAA-830T	3.33 ± 0	–	–	4.52 ± 1.7	178.23 ± 22.57	Zn, Cu
Azospirillum sp.[d]	–	–	–	26.6 ± 8.8	+	–
Escherichia coli [e]	–	–	–	0	–	–

Abbreviations: P-SI, phosphate-solubilizing index; CA, cellulolytic activity; AP, auxin production; AR, acetylene reduction; HM-R, heavy metal resistance

[a] P-SI was determined as the diameter of the colony plus the clear zone divided by the diameter of the colony.

[b] *acdS*, *acdS* gene detection.

[c] Used as the positive control for acetylene reduction.

[d] Used as the positive control for indole acetic acid production.

[e] Used as the negative control.

all other strains, confirming previous studies performed with nitrogen-fixing *Paenibacillus* (Rosado et al., 1998; Seldin et al., 1998; Beneduzi et al., 2008).

Paenibacillaceae have been previously detected in HM-contaminated soils (Zhang et al., 2007a, 2007b) and recognized as endophytic bacteria in hyperaccumulator plants (Barzanti et al., 2007). Moreover, the nitrogen-fixing capacity of several *Paenibacillus* spp. has been studied *in vitro*, but to our knowledge no studies evaluating the effect of HM on nitrogen fixation by *Paenibacillus* spp. have been performed.

In this work, *P. caespitis* 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 nitrogen-fixing activities were detected in relatively high concentrations of Co (1 mM), Ni (2.5 mM), and Zn (2.5 mM). However, these HM concentrations are fourfold lower than those observed in the rhizosphere of the plants growing on this tailing environment.

The presence in mine tailings of a community of taxonomically related, but phenotypically and physiologically different, HM-tolerant and nitrogen-fixing *Paenibacillus* suggests the possible existence of functional redundancy, a scenario in which the competitive exclusion principle must be functioning (Hardin, 1960). Rhizospheric soils could consist of different microenvironments with different HM concentrations and availabilities. Consequently, several niches in the mine tailings are open to coexistence of the nitrogen-fixing bacterial guild, in this case several members of the *Paenibacillus* genus. The mine tailings are an extreme environment with severe nitrogen deficiency, low organic matter content, and strong selection pressure derived from high HM content. Fluctuating metal concentrations may allow nitrogen fixation to occur when levels are lower, similar to what takes place under other changing environmental conditions (Kennedy and Smith, 1995).

Nitrogen-fixing activity of all the nitrogen-fixing species included in this work was decreased by the presence of HM (data not shown), an effect that has been broadly 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 the nitrogen fixation is the unique input mechanism of this element to the environment.

The PGPB capacities of *Azospirillum* spp. are frequently recognized (Costacurta and Vanderleyden, 1995; Steenhoudt and Vanderleyden, 2000; Bashan and De-Bashan, 2002), but the nitrogen-fixing capacities of *Azospirillum* spp. isolated from HM-contaminated environments has been scarcely studied. In particular, some strains of *Azospirillum amazonense* and *Azospirillum brasilense* maintained its *in vitro* nitrogen-fixation activity in the presence of moderate concentrations of Zn^{2+} and Cd^{2+} (Moreira et al., 2008). Evidently, more research must be performed to reveal the protection mechanisms that make nitrogen fixation possible in these nitrogen-deficient mine tailings and HM-contaminated soils.

Interestingly, a diazotrophic *B. japonicum* strain was isolated. Normally, *Bradyrhizobium* isolates are recovered from legume nodules and we do not know if the isolate reported here is symbiotic or nonsymbiotic. *Bradyrhizobium* spp. are also known as producers of siderophores, IAA, and phosphate solubilizers (Abd-Alla, 1994; Antoun et al., 2004; Wittenberg et al., 1996), and are frequently used as PGPB in phytoremediation processes (Khan, 2005). Particularly, *B. japonicum* KYR_C5 exhibited IAA production and growth tolerance to Ni^{2+}, Zn^{2+}, and Cu^{2+}, although no phosphate solubilization and nitrogen-fixing activity in the presence of HM was obtained. However, this set of phenotypic features contributes to plant pioneering and survival.

Remarkably, *A. mediolanus* KYR_C8 isolated from a rhizospheric sample soil was resistant to all the HM tested, a capacity frequently detected in many Actinomycetes (Golab et al., 1990; Richards et al., 2002; Asatiani et al., 2004).

Several strains were also isolated in nitrogen-free media and grew after three successive transfers, but no acetylene reduction activity could be demonstrated. Probably, they are "nitrogen scavengers" that are able to grow with traces of combined nitrogen (NH_3 and N_2O) from the atmosphere (Hurek et al., 1988). However, it is possible that those strains require particular conditions for nitrogen fixation not yet tested. *Rhizobium* spp. are known to fix nitrogen only in nodules and attempts to obtain nitrogen-fixing bacteria in free-living conditions have failed. In pioneer plant rhizospheres, consortia or communities with the nitrogen-fixing bacteria described here and seemingly others that were not recovered with a culture-dependent approach may contribute to the plant adaptation to toxic stress and high nutrient deficiency in environments such as mine tailings.

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