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Mercury-resistant rhizobial bacteria isolated from nodules of leguminous plants growing in high Hg-contaminated soils

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Abstract A survey of symbiotic bacteria from legumes grown in high mercury-contaminated soils (Almadén, Spain) was performed to produce a collection of rhizobia which could be well adapted to the environmental conditions of this region and be used for restoration practices. Nineteen Hg-tolerant rhizobia were isolated from nodules of 11 legume species (of the genera *Medicago*, *Trifolium*, *Vicia*, *Lupinus*, *Phaseolus*, and *Retama*) and characterized. Based on their growth on Hg-supplemented media, the isolates were classified into three susceptibility groups. The minimum inhibitory concentrations (MICs) and the effective concentrations that produce 50% mortality identified the patterns of mercury tolerance and showed that 15 isolates were tolerant. The dynamics of cell growth during incubation with mercury showed that five isolates were unaffected by exposure to Hg concentrations under the MICs. Genetic analyses of the 16S rRNA gene assigned ten strains to *Rhizobium leguminosarum*, six to *Ensifer medicae*, two to *Bradyrhizobium canariense*, and one to *Rhizobium radiobacter*. Inoculation of host plants and analysis of the *nodC* genes revealed that most of them were symbiotically effective. Finally, three isolates were selected for bioremediation processes with restoration purposes on the basis of their levels of Hg tolerance, their response to high concentrations of this heavy metal, and their genetic affiliation and nodulation capacity.

Keywords Mercury-tolerant rhizobia · Hg-contaminated land · Rhizobia–legume symbiosis · Bioremediation

Introduction

The effects of soil contamination vary widely and directly affect water quality and the natural environment and therefore constitute a serious risk to human health and the environment. One of the biggest problems affecting terrestrial ecosystems is contamination with heavy metals, including mercury (Boening 2000). This is the case in the Almadén area (Spain), where the presence of the largest cinnabar (HgS, the main ore of the element) deposit in the world and its exploitation for centuries for the production of mercury have caused the dispersion of the metal, and is recognized as one of the world's most mercury-polluted areas (Gray et al. 2004; Higueras et al. 2006; Molina et al. 2006; among others). Mercury is a highly polluting element, although the toxicity of its chemical species varies from relatively inert to highly toxic (Barkay et al. 2003). The toxicity and distribution of mercury in soil and its content in plants of Almadén were previously investigated (Millán et al. 2006; Molina et al. 2006; Moreno-Jiménez et al. 2006). However, up to date, it is not known how mercury contamination affects the diversity of soil microorganisms, which are essential for carrying out the biogeochemical cycles of the most important elements. Among soil microorganisms, the nitrogen-fixing bacteria are especially relevant in the nitrogen cycle. They

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associate with the roots of leguminous plants to form symbiotic structures called nodules in which atmospheric nitrogen is fixed. In this symbiosis, the plant receives nitrogen, while the bacterium receives carbohydrates and a favorable environment for nitrogen fixation. So far, 76 species in 13 genera of bacteria that can establish symbiosis with legumes have been identified (Weir 2010). Most of them are grouped in the rhizobial lineages, within the alpha-proteobacteria, comprised mainly by genera *Rhizobium*, *Bradyrhizobium*, *Ensifer*, *Azorhizobium*, and *Mesorhizobium* (generally denominated as "rhizobia"), although other alpha- and beta-proteobacteria capable of forming symbiosis with legumes have been reported (Weir 2010).

Legumes have proven their effectiveness in soil restoration and its preparation for colonization by other species, and because of this natural fertilizing function, they are regarded as pioneers (de Andrés et al. 2007; Villar-Salvador et al. 2008). The optimal establishment of the legume requires the presence in the soil of symbiotic bacteria necessary for the fixation of atmospheric nitrogen. Therefore, soil restoration with legumes under limiting environmental conditions requires not only the selection of compatible rhizobia but also that these rhizobia are able to resist the stressing agent, in this case, the high mercury concentration in the soil.

By having resistance mechanisms which detoxify chemical forms of mercury, resistant bacteria may play an important role in its biogeochemistry in mercury-contaminated environments (Chadhain Ní et al. 2006). Thus, exploitation of bacterial mercury tolerance may potentially be used as a method of detoxifying mercury-contaminated sites (Barkay et al. 2003). Considering this fact and the important role of rhizobial–legume symbiosis for the soils, knowledge of the abundance, diversity, and activity of the mercury-tolerant members of the rhizobial communities become paramount. On the other hand, it is well known that heavy metals, and especially mercury, are toxic to bacteria even at low concentrations (Boening 2000), and to date, no mercury-tolerant rhizobial strains have been identified. However, rhizobial strains tolerant to other heavy metals have been isolated from a variety of herbaceous (Carrasco et al. 2005; Pajuelo et al. 2008) and shrubby legumes (Ruiz-Díez et al. 2009). Different ecotypes of rhizobia can exhibit varying degrees of tolerance to stress by heavy metal contamination, so prior to the use of biotechnologies for inoculation with these microorganisms for the production of plants, it is necessary to establish a recruitment program of bacterial strains.

The aim of this work was to isolate and genotypically characterized the rhizobia associated with legumes from mercury-contaminated areas of South Central Spain to be used for restoration purposes. In order to select the highest tolerant isolates as specific inoculants for autochthonous legumes, their susceptibility to mercury has been analyzed.

Materials and methods

Bacterial isolates

Rhizobial isolates were obtained from root nodules of legumes grown in five soils from three areas of Almadén, Spain (Table 1). Physicochemical characteristics of the soils included a neutral pH (from 6.17 to 7.44) and three levels of mercury: San Quintín (212 mg Hg kg^{-1}), Las Cuevas (970 mg Hg kg^{-1}), and Almadenejos (3,870 mg Hg kg^{-1}). The whole Almadén area comprised 300 km^2. Isolation was performed from surface-sterilized nodules recovered directly from the legumes found in the sampling areas. The nodules of *Lupinus albus* and *Phaseolus vulgaris* were obtained by sowing their seeds (cultivars G1 and Planchada, respectively) in pots containing rhizospheric soils collected from the same natural sites. Briefly, nodules were surface-sterilized with ethanol 95% (v/v) for 30 s and $HgCl_2$ 0.1% (v/v) for 45 s and washed with sterilized, distilled water. Nodules were then cut, and a loopful of tissue containing bacteria was transferred to solid yeast extract mannitol (YM) medium (Vincent 1970). Single colonies were picked up and checked for purity by repeated streaking and by microscopic examination of cellular morphology. Isolates were routinely cultivated at 28 °C, maintained in YM agar (YMA) slants at 4 °C and in YM broth containing 20% (v/v) glycerol at -80 °C. Randomly chosen nodules from plants of each area were examined for the existence of different bacteria. Previously characterized rhizobia isolated from the same legume species grown in non-contaminated areas of Central Spain were used as references (Table 1).

Isolation of genomic DNA

A loopful of each isolate was used to inoculate 20 ml of YM broth. After incubation to saturation, the cells were harvested and washed twice with PBS, and DNA was isolated using the UltraClean TM Microbial DNA Isolation Kit (MOBIO). The concentration and integrity of DNA was analyzed with a spectrophotometer (Thermo Scientific) and by electrophoresis with 0.8% agarose gels and comparison with known amounts of phage lambda DNA (Sambrook et al. 1989). The DNA was further employed in all genetic tests.

PCR amplifications and restriction analyses

The primers fD1 and rP2 (Weisburg et al. 1991) were employed for 16S rDNA and NodCF and NodCI for *nodC* gene (Laguerre et al. 2001). All were synthesized by Genotek (Spain). The DNA which codified for 16S rRNA was amplified as previously described (Laguerre et al. 1994), with modifications according to Ruiz-Díez et al. (2009). The *nodC* PCR mixture was the same as described for PCR of 16S rRNA

Table 1 Strains isolated in the area of Almadén and rhizobia used as controls in these studies. Their provenance, original host, and genetic characterization based on chromosomal gene 16S rRNA

	Geographical origin/coordinates[a]	Original host	Genetic characterization[b]	
			ARDRA	16S rRNA
Isolate/species				
R-7Q	San Quintín/38° 49.15′N 4° 16.31′W	*Retama sphaerocarpa*	1	*Rhizobium radiobacter*
L-7AH	Almadenejos Hornos/38.1° 44.3′N 4.1° 42.5′W	*Lupinus albus*	2	*Bradyrhizobium canariense*
L-7Q	San Quintín	*L. albus*	2	*B. canariense*
M-7AH	Almadenejos Hornos	*Medicago sativa*	3	*Ensifer medicae*
M-7Q	San Quintín	*Medicago polimorfa*	3	*E. medicae*
M-7C	Las Cuevas/38° 49.21′N 4° 45.35′W	*M. polimorfa*	3	*E. medicae*
J-7AH	Almadenejos Hornos	*Phaseolus vulgaris*	3	*E. medicae*
J-7QC	San Quintín Norte/38.1° 49.2′N 4° 16.4′W	*P. vulgaris*	3	*E. medicae*
J-7C	Las Cuevas	*P. vulgaris*	3	*E. medicae*
V-7A	Almadenejos/38° 44.27′N 4° 42.44′W	*Vicia sativa* L.	4	*Rhizobium leguminosarum*
V-7Q	San Quintín	*V. sativa* L.	4	*R. leguminosarum*
V-7C	Las Cuevas	*V. sativa* L.	4	*R. leguminosarum*
TT-7A	Almadenejos	*Trifolium tomentosum*	4	*R. leguminosarum*
TT-7AH	Almadenejos Hornos	*T. tomentosum*	4	*R. leguminosarum*
TE-7AH	Almadenejos Hornos	*Trifolium scabrum*	4	*R. leguminosarum*
T-7Q	San Quintín	*Trifolium glomeratum*	4	*R. leguminosarum*
TX-7C	Las Cuevas	*T. glomeratum*	4	*R. leguminosarum*
TT-7C	Las Cuevas	*T. tomentosum*	4	*R. leguminosarum*
TS-7C	Las Cuevas	*Trifolium repens*	4	*R. leguminosarum*
Reference strains				
Bradyrhizobium sp. (*Lupinus*) CIFA ISLU-16	Sevilla	*Lupinus* sp.	2	*B. canariense*
B. canariense CCMA L-3	La Higueruela/40°4′N 4°26′W	*L. albus* L.	2	*B. canariense*
Ensifer meliloti CCMA ALF-3	La Higueruela	*M. sativa*	3	*E. meliloti*
Rhizobium leguminosarum bv. *trifolii* CCMA T-3	La Higueruela	*Trifolium subterraneum*	4	*R. leguminosarum*
Rhizobium gallicum CCMA J-2	La Higueruela	*P. vulgaris*	5	*R. gallicum*

CIFA Centro de Investigación y Formación Agraria-Las Torres-Tomejil, Sevilla, *CCMA* Centro de Ciencias Mediambientales, CSIC

[a] Geographical locations from different areas of Almadén (Spain) and two agricultural lands of the La Higueruela Experimental Station (CCMA, CSIC), Santa Olalla, Toledo, Spain

[b] ARDRA type obtained by analyses of 16S rRNA PCR amplified region digested with four endonucleases *Msp*I, *Hinf*I, *Dde*I, and *Hha*I. Different numbers were assigned to represent each ARDRA group. 16S rRNA determined by comparison of the full nucleotide sequence with the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/index.html)

gene, with the exception of the primers and $MgCl_2$ whose final concentrations were 0.8 μM and 2.5 mM, respectively. The amplifications were performed in a thermal cycler (Veriti 96 Well, Applied Biosystems) with the same temperature program as used by Ruiz-Díez et al. (2011). The PCR products were analyzed by electrophoresis on 0.8% agarose gels (40 V).

The restriction endonucleases *Msp*I, *Hinf*I, *Dde*I, and *Hha*I (New England Biolabs) were used in separate digestion reactions with PCR-amplified 16S rRNA gene from all isolates listed in Table 1. A 15-μl portion of amplification reactions was digested according to the manufacturer's recommendations, and restriction analyses of amplified 16S rDNA (ARDRA) were performed as in Ruiz-Díez et al. (2009).

Determination of DNA sequences

Nearly 1,500 bp of 16S rRNA gene from the selected isolates (Fig. 1) were purified with the EZNA CYCLE-PURE kit (Omega), and approximately 930 bp of *nodC* from selected isolates (Fig. 2) were run on 0.8% agarose gels

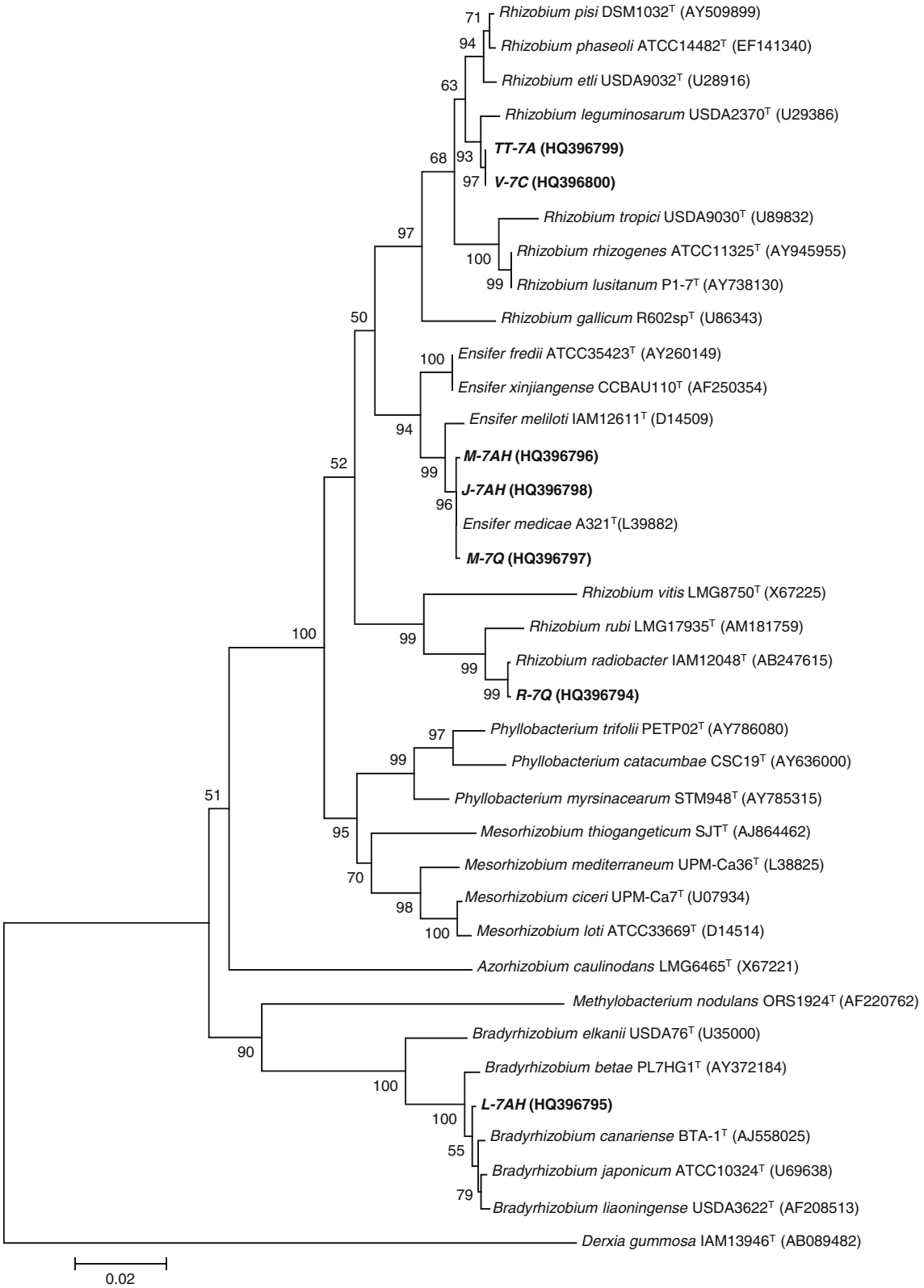

Fig. 1 Phylogenetic tree derived from neighbor-joining analysis of 1,420-bp-long 16S rRNA gene sequences from legume-nodulating isolates and representative type strains from the GenBank. Numbers above the branches are bootstrap values based on 1,000 replicates (only values of 50% or above are shown). *Derxia gummosa* was used as outgroup to root the tree (the *bold letters* indicate the sequences obtained in this study). *Scale bar*, 2 nt substitution per 100 nt

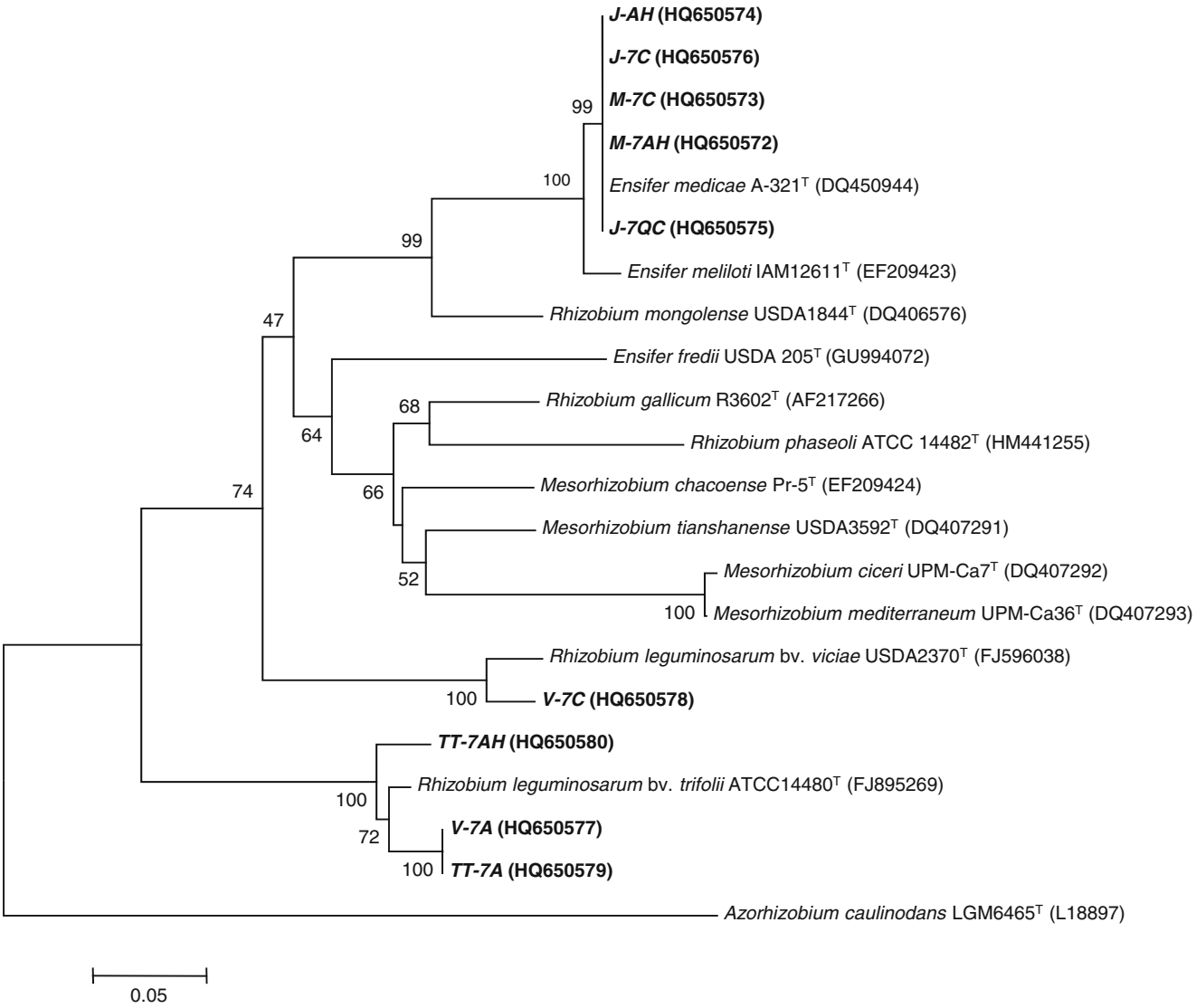

Fig. 2 Phylogenetic tree derived from neighbor-joining analysis of 933-bp-long *nodC* gene sequences from legume-nodulating isolates and representative related type strains from the GenBank. Numbers above the branches are bootstrap values (only values of 50% or above are shown). *Azorhizobium caulinodans* was used as outgroup to root the tree (*bold letters* indicate the sequences obtained in this study). *Scale bar*, 5 nt substitution per 100 nt

and purified with illustraTM GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). Sequences of PCR products were obtained with an ABI PRISM 3700 (Applied Biosystems) sequencer using the Taq Dyedeoxi Terminator cycle systems (Automatic Sequencing Service SAAD, CIB, CSIC, Madrid). The 16S rRNA gene was sequenced using each of the primers 1050R, 800R, 800 F, and IRF1 (Lane 1991), which allowed for determination of almost the full 16S rRNA gene sequence. The *nodC* PCR products were sequenced using the primers NodCF and NodCI. The sequences were determined by pairwise alignments employing the Clustal W program, and similarities were identified and analyzed by comparison with databases using the BLASTN program on the network service of GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The percentage values of homology with respect to the 16S rRNA gene were calculated with Clustal W multiple alignments (http://www.npsa-pbil.ibcp.fr/). Phylogenetic analyses were performed using the MEGA program version 4 (Tamura et al. 2007). The phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei 1987) based on the two parameter distance model of Kimura. To assess the relative support for each clade, bootstrap values were calculated from 1,000 replicated analysis.

Nodulation tests

The nodulation ability of all isolates was checked by inoculating the host plants of origin as previously described (Vincent 1970). The seeds were surface sterilized by

immersion in $HgCl_2$ 1% (v/v) for 5 min and thoroughly washed with sterilized, distilled water. *Retama sphaerocarpa* seeds were first mechanically scarified. Subsequently, seeds were transferred to pots filled with sterile vermiculite and inoculated with 2 ml of the bacterial inoculum (heavy suspension of the log-phase culture). Four replicates were established for each plant and treatment, incubated in a growth chamber (15-h photoperiod, 250 μmol photon m^{-2} s^{-1}, day/night temperature of 25/20 °C) and watering with sterile nitrogen-free nutrient solution. Plants were harvested after 1 month of growth and checked for root nodules. Nitrogen fixation activity of plant–rhizobia symbioses was measured by the acetylene-reduction assay as previously described (Fernández-Pascual et al. 1988).

Screening for mercury tolerance

Mercury tolerance of the isolates and reference strains was determined in YM broth according to Ruiz-Díez et al. (2009). Mercury was assayed as mercury chloride ($HgCl_2$ analysis grade ACS, Merck), prepared in distilled water (MILLI-Ro Systems, Millipore), sterilized by membrane filtration (Millipore 0.22 μm), and added to the sterilized YM medium. The $HgCl_2$ concentrations tested were 0, 3, 6, 12.5, 25, 30, 60, 125, and 250 μM. Briefly, 5 ml of the medium was inoculated with 50 μl of bacterial inoculum (suspension of the log-phase culture in 5 ml of YM broth), reaching a final density of 10^6 cells/ml and incubated at 28 °C in a rotary shaker (100 rpm). The log-phase was previously determined by means of growth curves for each strain, and the log-phase was defined by a constant doubling time. Growth measurements were performed in the mid-log-phase by optical density determinations (absorbance at 680 nm). The minimum inhibitory concentration (MIC) was defined as the lowest heavy-metal concentration that completely prevents visible growth. The experiments were repeated at least twice, with three replicates each time, and paired samples always gave the same end-point.

Determination of exact range of toxicity

Once the MIC was established, the rhizobial strains were cultured with serially increasing concentrations of $HgCl_2$ up to the MIC in the log-phase as described previously. By plotting the relative growth rates (relative to the controls without mercury) against the concentrations of mercury, a direct correlation between the concentration of metal and its growth-inhibiting effect was observed, as previously described for other bacteria (OECD 2003), and a polynomial regression was performed (grade 2, $R^2 \approx 1$). From these regressions, the effective concentrations of mercury leading to 50% growth inhibition (EC50) were calculated by extrapolation and listed in Table 2.

Table 2 Patterns of resistance/tolerance to mercury, given as MIC, and toxicity, given as EC50, of the rhizobial strains isolated from nodules of leguminous plants in different soils of the area of Almadén and rhizobia used as controls (values obtained from three to six different replicates)

	Species[a]	MIC (μM)[b]	EC50 (μM)[c]
Strain			
R-7Q	*Rhizobium radiobacter*	30	16.69
L-7AH	*Bradyrhizobium canariense*	12.5	4.39
L-7Q	*B. canariense*	< 3	*
M-7AH	*Ensifer medicae*	6	2.82
M-7Q	*E. medicae*	6	3.94
M-7C	*E. medicae*	25	9.96
J-7AH	*E. medicae*	12.5	5.83
J-7QC	*E. medicae*	< 3	*
J-7C	*E. medicae*	25	9.36
V-7A	*Rhizobium leguminosarum* bv. *trifolii*	6	3.77
V-7Q	*R. leguminosarum*	3	1.91
V-7C	*R. leguminosarum* bv. *viceae*	6	4.54
TT-7A	*R. leguminosarum* bv. *trifolii*	12.5	5.54
TT-7AH	*R. leguminosarum* bv. *trifolii*	12.5	6.82
TE-7AH	*R. leguminosarum*	12.5	6.21
T-7Q	*R. leguminosarum*	< 3	1.92
TX-7C	*R. leguminosarum*	12.5	6.85
TT-7C	*R. leguminosarum*	12.5	7.32
TS-7C	*R. leguminosarum*	12.5	5.11
Reference strains			
ISLU-16	*Bradyrhizobium* sp. (*Lupinus*)	< 3	*
L-3	*B. canariense*	< 3	*
ALF-3	*Ensifer meliloti*	< 3	*
T-3	*R. leguminosarum* bv. *trifolii*	< 3	*
J-2	*Rhizobium gallicum*	< 3	*

Asterisks indicate that strains showed no growth at any of the concentrations tested

[a] Biovarieties determined by means of *nodC* phylogeny

[b] MIC is the lowest concentration of $HgCl_2$ that completely prevents visible growth in comparison with the control without mercury

[c] EC50 is the concentration of mercury that inhibits the exponential growth of bacteria by 50% compared to the control

Effect of mercury on the growth of rhizobial strains

Liquid cultures of 25 ml of YM medium supplemented with $HgCl_2$ at final concentrations of 0, 3, 6, 12.5, and 25 μM (prepared as described previously) were inoculated with 250 μl of a log-phase culture and grown at 28 °C for 7 days in a rotary shaker (100 rpm). No volatilization of mercury or any measurable sorptions of metal on the glassware surface were detected under these conditions. Growth was monitored at 24-h intervals as the absorbance at 680 nm with a

spectrophotometer (Spectronic 2000, Bausch α Lomb). Each strain was tested in triplicate at each point in the time series. A blank with the medium culture alone, with no added bacteria, was also analyzed.

Nucleotide sequence accession numbers

The sequences of the 16S rRNA genes were deposited in the GenBank database under accession numbers HQ396794–396800 (provided in Fig. 1). The sequences of the *nodC* gene were also deposited in the GenBank (accession no. HQ 650572–650580 displayed in Fig. 2). The most promising rhizobial isolates obtained in this study (L-7AH, M-7C, and TT-7C) were deposited with the Colección Española de Cultivos Tipo (CECT, Valencia, Spain), accession numbers CECT 8017, 8018, and 8019, respectively.

Results

Isolation of rhizobia

Nineteen different rhizobial strains were isolated from nodules of 11 legumes grown in five soils with three different levels of mercury in the area of Almadén (Table 1). Isolates obtained from different nodules of the same host plant always rendered the same 16S genotype; consequently, one strain was chosen from each legume species. The examination of single colonies revealed that they could be identified as rhizobia. Most isolates were classified as fast growers, according to the classification of Odee et al. (1997), since their colonies were >2 mm in diameter after 5 days in YM medium at 28 °C. Only strains L-7AH and L-7Q were classified as slow growers because their colonies were ≤1 mm in diameter after 7 days in YM medium at 28 °C.

Genetic identification

In order to obtain an initial genetic classification, the 16S rRNA gene of all isolates (including references) was amplified, and the PCR products were digested with four endonucleases. Restriction analyses showed five genotypes (1–5), representing different combinations of patterns which were retrieved from all strains (Table 1). The 19 root-nodule isolates were grouped in four different rRNA genotypes (1–4), with group 4 including the majority of fast-growing isolates (10) and exhibiting the same genotype pattern as *Rhizobium leguminosarum* CCMA T-3. The 16S rRNA gene is not able to discriminate the biovars. Slow-growing isolates belonged to genotype 2 and were grouped with the *Bradyrhizobium canariense* CCMA L-3 reference strain. Genotype 3 included isolates from *Medicago* sp. and *Phaseolus vulgaris* as well as the reference strain for *Ensifer meliloti* CCMA ALF-3. The isolate R-7Q (genotype 1) was separate from all strains including references. These preliminary ARDRAs allowed us to choose seven isolates which represented each genotype (and were also from different soils and plant hosts) for the determination of 16S rRNA gene sequences to achieve the species affiliation, i.e., their genetic identity. The sequenced isolates from the same ARDRA genotype grouping showed identical or almost identical nucleotide composition. Sequence similarity searches (BLASTN) confirmed that all strains belonged to the Rhizobiales lineages within the α-proteobacteria (Table 1). Consequently, a phylogenetic analysis was performed to assess the evolutionary relationships with related-type strains within the major Rhizobiales lineages (Fig. 1). The phylogenetic tree was divided into two major branches: One included the major genera *Rhizobium*, *Ensifer*, *Phyllobacterium*, and *Mesorhizobium*, whereas the second clade was mainly comprised of genera from the family Bradyrhizobiaceae. The two representative sequences of genotype 4 (TT-7A and V-7C) were grouped into a branch with the type strain of *Rhizobium leguminosarum* and presented 100% homology with the closest relative *Rhizobium leguminosarum* strain ALM-2 (GenBank accession number DQ660316) and 99.5% with *Rhizobium leguminosarum* type strain USDA2370. The strains M-7AH, J-7AH, and M-7Q (genotype 3) were included in the clade of genus *Ensifer* and closely related with *Ensifer medicae* (99.8% homology with the closest *E. medicae* type strain A321). The isolate R-7Q was very close to *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*), with 99.9% homology with respect to the closest relative *Rhizobium radiobacter* type strain IAM12048. Finally, isolate L-7AH stayed apart in the second main branch within the family Bradyrhizobiaceae, where it grouped with *B. canariense* (Fig. 1). This strain exhibited 100% homology with the closest *B. canariense* strain MCLA23 (GenBank no. EF694743) and 99.8% with *B. canariense* type strain BTA-1.

Analyses of nodulation capacity

The ability of the new rhizobial isolates to form symbioses with their own host plant was tested. The control uninoculated plants did not nodulate. All isolates nodulated their hostplant when they were reinoculated and had fixing nodules with the exception of R-7Q (identified as *Rhizobium radiobacter*).

The symbiosis-specific *nodC* gene, a common *nod* gene essential for nodulation in the majority of rhizobial species, was analyzed in selected strains of different rhizobial species (Fig. 2). This demonstrated their infective capacity for each soil type and plant host. R-7Q was also included in order to verify the results of the infectivity tests. The strains ascribed to *B. canariense* were excluded as a result of the

difficulty in amplifying the *nodC* gene in *Bradyrhizobium*, probably due to low annealing of the primers. *Bradyrhizobium nod* genes are complex and have different regulation (Stacey 1995). A partial fragment of approximately 950 bp of *nodC* gene was obtained for all strains tested. However, the isolate R-7Q did not amplify for the *nodC* gene. The *nodC* phylogenetic tree (Fig. 2) grouped the *E. medicae* strains in the *Ensifer* clade with the *E. medicae* reference strain, forming a robust phylogenetic group (bootstrap value of 99%) which demonstrate a diverse range of host specificity for this rhizobial species. The strain V-7C was closely related to *Rhizobium leguminosarum* bv. *viceae* (100% bootstrap), confirming the specificity of *Vicia sativa* for this biovar. Whereas the strains from *Trifolium* (TT-7AH and TT-7A) clustered with *Rhizobium leguminosarum* bv. *trifolii* in a well-defined branch (100% bootstrap), the strain V-7A from *V. sativa* was surprisingly fitted in this clade.

Evaluation of the susceptibility to mercury

The discrimination and subsequent selection between mercury-tolerant and mercury-sensitive rhizobia require an indispensable and accurate cultivation-based approach before performing any other molecular approach.

The effect of Hg on growth of the isolates (Fig. 3), reported as a percentage growth relative to the controls, allowed us to identify the bacteria in relation to mercury tolerance and classify them into three susceptibility groups. The sensitive group (growths of 0–2% related to the control) included all reference strains employed in these assays (Table 2) and isolates L-7Q, J-7QC, V-7Q, and T-7Q. The MICs of this group were ≤3 μM (MICs summarized in Table 2). The intermediate group integrated strains M-7AH, M-7Q, V-7A, and V-7C, which showed similar moderate tolerance to metal concentrations (MICs of 6 μM). The remaining 11 isolates (the most numerous group) were classified as tolerant, with MICs of ≥12.5 μM. Although L-7AH and TS-7C were included in this group, they displayed a significant reduction of growth at 6 μM (Fig. 3). In contrast, the strains R-7Q, M-7C, and J-7C were all extremely tolerant, and R-7Q had the highest tolerance. The determination of MICs allowed us to calculate the exact range of toxicity of each strain measured by EC50 (Table 2). The reference isolates did not grow at any of the concentrations assayed. As already observed for the MICs, each isolate showed a different degree of tolerance to mercury, and the EC50 values were of a similar order in each of the previously described tolerance groups. Again, M-7C, J-7C, and especially R-7Q presented the highest levels of tolerance compared to other strains.

Dynamics of cell growth during incubation with mercury

The dynamics of rhizobial growth at different incubation times were examined in all strains, and Fig. 4a, b shows the growth curves of six selected strains with a similar logarithmic-phase, representing different susceptibility groups. M-7C and J-7C represented the most tolerant, TE-7AH and J-7AH represented tolerant, and M7-AH and J-7QC represented the intermediate and sensitive isolates, respectively. The curves demonstrate that the most tolerant strains (M-7C and J-7C) grew at 12.5-μM $HgCl_2$, the tolerant (TE-7AH and J-7AH) grew at 6 μM, the intermediate M-7AH displayed reduced growth at 3 μM, and the sensitive (J-7QC) displayed no growth at any concentration. In addition, the growth curves presented two different profiles: The majority of the isolates displayed less absorbance than the control at any of the concentrations tested (J-7C, TE-7AH, M-7AH, and J-7QC shown in Fig. 4a, b), whereas isolates M-7C and J-7AH (shown in Fig. 4a, b) and R-7Q, L-7AH, and TT-7C (shown in Electronic Supplementary Material) exhibited similar growth rates as the control at

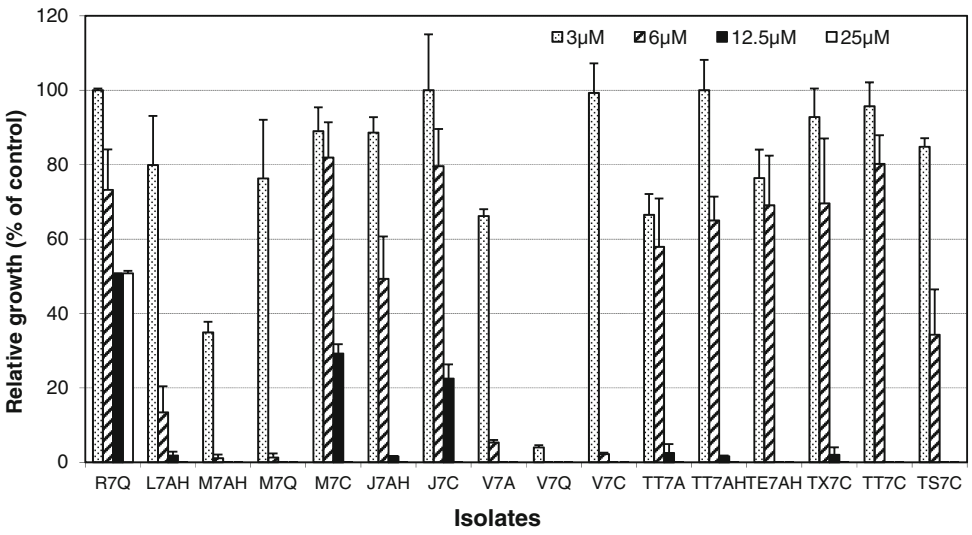

Fig. 3 Effect of Hg concentrations (3, 6, 12.5, and 25 μM) on the growth of the bacterial isolates. The isolates which did not grow at any of the concentrations tested (including control strains) are not shown in the bar diagram. Results are expressed as percentages relative to controls cells in media not supplemented with $HgCl_2$. Data are means + SE from three to six replicate experiments

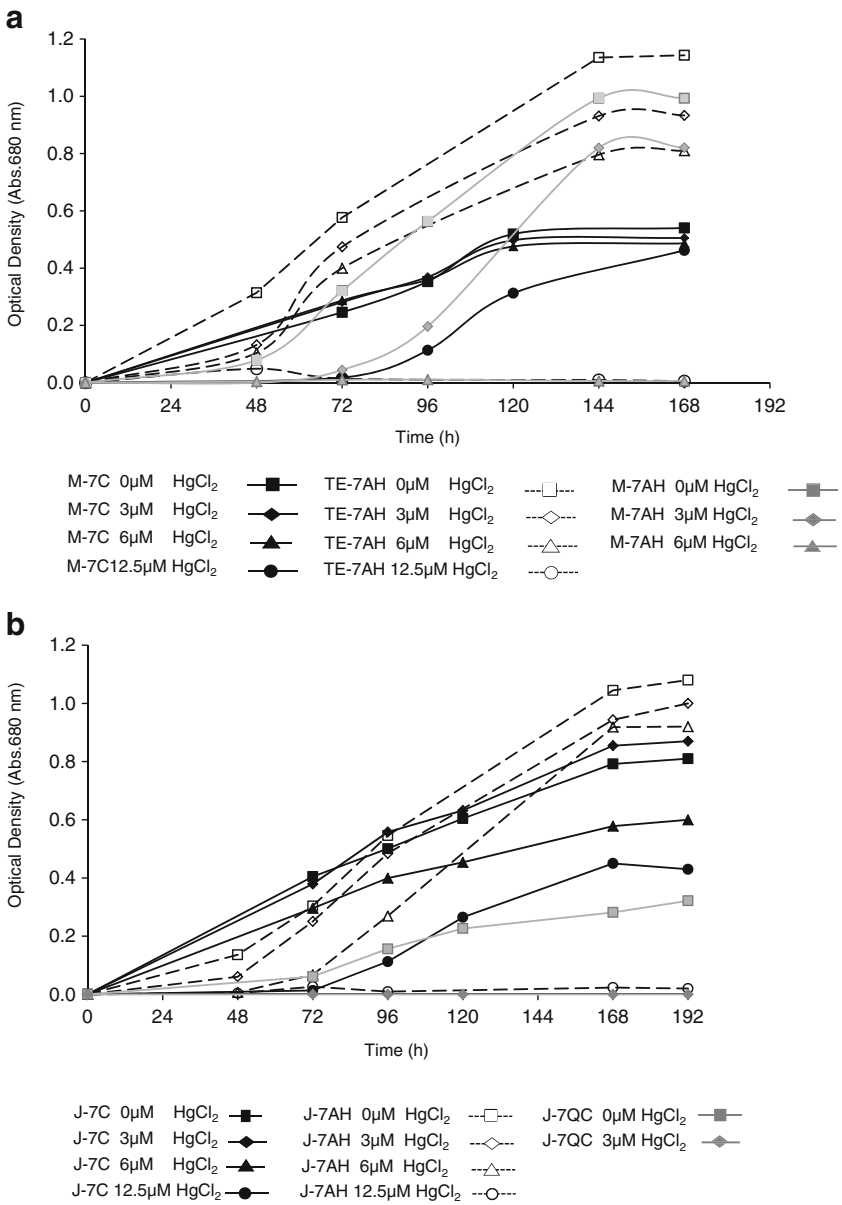

Fig. 4 Growth of rhizobia dependent on increasing mercury concentrations (0, 3, 6, 12.5, and 25-μM $HgCl_2$). **a** Strains M-7C, TE-7AH, and M-7AH. **b** J-7C, J-7AH, and J-7QC. In each treatment, three flasks were used and sampled in the time series. Data shown are the means of three replicates. Growth curves for all 19 rhizobial strains isolated from the Almadén area, plus reference strains, are included in the Electronic Supplementary Material, with means ± SE for each one

the Hg concentrations at which they grew and reached the same absorbance as the control in the stationary phase. Hence, five strains demonstrated the best response to the different concentrations of mercury and also displayed the highest EC50 values (Table 2) within each of the susceptibility groups which they represented. These strains were selected for the amplification of known genes of mercury resistance (Barkay et al. 2003), either *merA* (Liebert et al. 1997) or *merR*, *merT*, and the 5′ end of *merP* (Abou-Shanab et al. 2007); however, our attempts repeatedly failed.

Discussion

Legume–rhizobia symbiosis biodiversity in areas contaminated with heavy metals has received little attention (Carrasco et al. 2005), and to our knowledge, this is the first study in mercury-contaminated land. Our surveys resulted in only a few species of leguminous plants being found in the five areas of Almadén, mostly belonging to *Trifolium* and *Medicago*. However, their relative abundance probably contributes to the distribution and diversity of rhizobial bacteria. Furthermore, we did not find differences in terms of the legume species growing in any of the areas. The ability of different herbaceous legumes to act as trap plants was tested with soils from the same areas, but only nodules from *Phaseolus vulgaris* and *Lupinus albus* were obtained.

The 16S RNA gene has demonstrated the existence of at least four different bacterial species within the 19 strains isolated, with most of them belonging to *Rhizobium leguminosarum* (10 isolates), but also to *E. medicae* (six isolates), *B. canariense* (two isolates), and *Rhizobium radiobacter* (one

isolate). The large group of *Rhizobium leguminosarum* strains confirm our previous investigations with rhizobia nodulating agricultural legumes (Ruiz-Díez et al. 2011), which demonstrated the high success rate of this rhizobial species in Central Spain. The nodulation of *P. vulgaris* by *E. medicae* is atypical because this species usually nodulates legumes from the alfalfa cross-inoculation group, but reports of different species of *Ensifer* in association with *P. vulgaris* are increasing (e.g., Zurdo-Piñeiro et al. 2009). With respect to other species, as far as we know, this is the first time that *Rhizobium radiobacter* has been described in association with nodules of the leguminous shrub *Retama sphaerocarpa*. To summarize, the limited number of rhizobial species associated with these host plants was, in part, due to the small sampling area and its particular soil conditions in comparison with other studies. In general, the genetic diversity of mercury-acclimated bacterial communities is relatively low (Rasmussen and Sorensen 2001).

Analyses of nodulation ability by the rhizobial strains are necessary because increasing concentrations of heavy metals in the soil tend to reduce the nodulation of legumes (Zornoza et al. 2002; Pastor et al. 2003). In our experiments, all inoculated legumes had fixing nodules and amplified for the *nodC* gene, with the exception of those inoculated with R-7Q (identified as *Rhizobium radiobacter*, formerly *Agrobacterium tumefaciens*). These nodulation results could suggest that *Rhizobium radiobacter* colonized the *Retama sphaerocarpa* nodule just after its formation, forming part of the consortium of endophytic bacteria living in nodules (Muresu et al. 2008). This would agree with the fact that the former genus *Agrobacterium* generally does not form symbiotic root nodules unless they harbor a Sym plasmid or other transposable element with nodulation genes (Young et al. 2001). *Rhizobium radiobacter* isolates have also been found colonizing active nodules of *P. vulgaris* and modulating, either positively or negatively, the nodulation by native rhizobia (Mhamdi et al. 2005).

The phylogeny of *nodC* grouped the strains by their host plants. Only V-7A (from *V. sativa*) was clustered with *Trifolium* strains (TT-7AH and TT-7A). The three strains came from the same area with the highest mercury contamination (Almadenejos). Although *nodC* is employed as a molecular marker to identify the specific biovar (Moschetti et al. 2005), this symbiotic gene is carried on plasmids which can be interchanged between closely related rhizobial species (Franche et al. 2009). This lateral transfer of symbiotic genes between closely related rhizobial species isolated from very small areas with special characteristics has previously been documented (e.g., Hou et al. 2009). However, in general, the *nodC* analysis established that the capacity of soil rhizobia to form nodules is also determined by their host plants.

The effect of Hg on the growth of isolates allowed us to classify them into three susceptibility groups. The rhizobial MIC values for Hg were lower than the ones obtained for other bacteria (Abou-Shanab et al. 2007). In contrast to our isolates, their mercury-tolerant phenotype was mainly demonstrated with solid media, which, due to colonial growth, may not give an accurate estimate of resistance level. Besides, most of our isolates could be considered as tolerant to higher or lower degree since their MICs were significantly higher than the values for the reference strains growing in a nearby non-Hg-contaminated area. This phenotypic trait represents a very important finding because, to date, there are no previous reports of rhizobial strains tolerant to mercury isolated from Hg-contaminated soils. Isolate R-7Q from *Retama sphaerocarpa* was extremely tolerant to mercury, confirming our previous observations on the exceptional tolerance to natural stresses of rhizobia from shrubby legumes (Ruiz-Díez et al. 2009). The EC50 studies demonstrated the high toxicity of mercury: Half of the bacterial cells died at very low concentrations. In fact, this toxicity parameter is used as an indirect index of a pollutant's bioavailability for specific organisms and is commonly applied in ecotoxicity studies (OECD 2003). Furthermore, EC50 will allow us to define the concentrations of mercury to use in trials with these bacteria.

All strains classified as sensitive were isolated from legumes grown in the soil with low mercury concentrations (San Quintín). However, mercury MICs and EC50s of the other two susceptibility groups seemed to be independent of the soils in which the legumes grew and mostly came from either medium or high Hg levels, with the exceptions of R-7Q and M-7Q (also from San Quintín). The continuous exposure to mercury in the soils of origin, even at low levels, probably led to selection of tolerance among members of rhizobial group as reported for other gram-positive and gram-negative bacteria (Abou-Shanab et al. 2007; Rasmussen and Sorensen 2001). Until now, it has been impossible to establish a direct relationship between ecotoxicity parameters, such as EC50, and soil characteristics (Maliszewska-Kordybach et al. 2007). In addition, no correlations were found between any of the Hg-susceptibility groups and the rhizobial species or host plant, so the tolerance to Hg seems strain dependent, same with other heavy metals in different rhizobial symbioses (e.g., Hungria et al. 2001; Almeida Pereira et al. 2006; Ruiz-Díez et al. 2009). Our results also showed that the two tolerance indices considered (EC50 and MIC) can be successfully used for measuring Hg tolerance of rhizobia, as reported for other heavy metals and/or bacteria (e.g., Hungria et al. 2001; Pepi et al. 2009; Ruiz-Díez et al. 2009).

Measurement of the rate of processes at a large number of toxic concentrations is a good way to study the effects of a contaminant on a specific microorganism (Van Beelen and Doelman 1997). Our response curves showed that mercury inhibited growth to different degrees in all isolates and reflected two different responses: inhibition at any mercury concentration or only at the MICs. This second response could be explained from a toxicity point of view as a

hormetic effect (Calabrese 2005), that is, a generally favorable biological response to low exposures of pollutant and inhibition at large doses. Strains that can survive better in soils containing high mercury levels can readily start their multiplication if mercury decreases, for example, after a volatilization or filtration event. Thus, the percentage of viable cells and the evolution of mercury tolerance are important parameters to be evaluated as they depend essentially on the concentration of mercury in the soil and the intrinsic capacity for mercury bioremediation and/or alleviation of each strain. However, our attempts to amplify the mercury resistance genes (Barkay et al. 2003) in these tolerant strains failed, possibly due to the utilization of other mechanisms to deal with Hg toxicity (Boening 2000). Consequently, this phenomenon, i.e., "phenotypic tolerance," could be accounted for by the mercury-mediated enrichment of subpopulations of rhizobial strains which are physiologically tolerant but genetically susceptible to mercury.

Considering all the susceptibility tests performed, the selection of strains with phenotypic traits of mercury tolerance comprises the species *Rhizobium radiobacter* (R-7Q), *B. canariense* (L-7AH), *E. medicae* (M-7C), and *Rhizobium leguminosarum* bv. *trifolii* (TT-7C). Although the most tolerant R-7Q did not nodulate, corroborating reports that resistant microorganisms often fail to perform specific ecological functions in polluted ecosystems (Van Beelen and Doelman 1997), this strain will be employed as a control of tolerance in future susceptibility studies. The isolates with high Hg tolerance and nodulation capacity will be utilized for analyzing the response of the symbioses to Hg, first in a growth chamber and thereafter in Almadén soils.

This report reveals the existence of rhizobial bacteria able to survive in areas contaminated with mercury. Genetic analyses infer that the genetic diversity of rhizobia in this small area is a result of interactions among the specific environment/soil conditions, the host legumes, and bacterial genomic backgrounds. The phenotypic analyses indicate that most strains have unique properties of mercury tolerance, differing from strains isolated from near uncontaminated lands. This feature is important to allow these rhizobia to cope with the high mercury concentrations in Almadén soils. At present, we have selected the most promising strains (L-7AH, M-7C, and TT-7C) to be used with their host legume species for bioremediation processes on the basis of their levels of Hg tolerance and their response to its high concentrations, together with their genetic identification and nodulation capacity.

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