Leguminosae native nodulating bacteria from a gold mine As-contaminated soil: Multi-resistance to trace elements, and possible role in plant growth and mineral nutrition

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\textbf{ABSTRACT}

Efficient N\textsubscript{2}-fixing Leguminosae nodulating bacteria resistant to As may facilitate plant growth on As-contaminated sites. In order to identify bacteria holding these features, 24 strains were isolated from nodules of the trap species \textit{Crotalaria spectabilis} (12) and \textit{Stizolobium aterrimum} (12) growing on an As-contaminated gold mine site. 16S rRNA gene sequencing revealed that most of the strains belonged to the group of \textalpha-Proteobacteria, being representatives of the genera \textit{Bradyrhizobium}, \textit{Rhizobium}, \textit{Inquilinus}, \textit{Labrys}, \textit{Bosea}, \textit{Starkeya}, and \textit{Methyllobacterium}. Strains of the first four genera showed symbiotic efficiency with their original host, and demonstrated \textit{in vitro} specific plant-growth-promoting (PGP) traits (production of organic acids, indole-3-acetic-acid and siderophores, 1-aminocyclopropane-1-carboxylate deaminase activity, and Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} solubilization), and increased resistance to As, Zn, and Cd. In addition, these strains and some type and reference rhizobia strains exhibited a wide resistance spectrum to \beta-lactam antibiotics. Both intrinsic PGP abilities and multi-element resistance of rhizobia are promising for exploiting the symbiosis with different legume plants on trace-element-contaminated soils.

\textbf{KEYWORDS}

plant growth-promoting; biological N\textsubscript{2} fixation; trace elements multi-resistance; \beta-lactam antibiotics resistance

\textbf{Introduction}

Soil microorganisms delivering ecosystem services (e.g. biological N\textsubscript{2} fixation (BNF)) have been recognized as important allies for phytotechnologies. Their ability to improve the nutritional status of the plant positively influences the tolerance of plants to excess of trace elements.

Symbiotic relationships between legume plants and rhizobia possess a great potential for phytostabilization of hostile environments (Franco et al. 1995; Dary et al. 2010; Hao et al. 2014). They are, for instance, (i) protecting soils, (ii) enriching ecosystems with N, (iii) supplying land cover, (iv) restoring soil functions, and (v) increasing diversity of flora and fauna (Dary et al. 2010; Siqueira et al. 2008). Moreover, the symbiosis rhizobia-legume can replace ammonium-based fertilizers, and thus reduce risks on soil acidification, which might increase availability of trace elements. Further, the presence of a vegetation cover prevents the dispersal of contaminated dusts through wind and water erosion from formerly bare or sparsely vegetated sites, and markedly decreases leaching of contaminants to groundwater. In fact, contamination is “inactivated” in place, preventing further spreading and transfer into food chains (Dary et al. 2010; Vangronsveld et al. 1995a; b, 1996, 2009). All these contribute to an attenuation of the impacts of the contaminants on site and on adjacent ecosystems (Dary et al. 2010; Vangronsveld et al. 2009).

Trace elements resistance has been demonstrated for different rhizobia genera (Trannin et al. 2001; Matsuda et al. 2002a; Carrasco et al. 2005; Rangel et al. 2016). Moreover, the intrinsic plant-growth-promoting features of rhizobia enlarge the horizons for exploiting the symbiosis with different legume plants on both single and multi-trace-element-contaminated soils (Dary et al. 2010; Hao et al. 2014; Rangel et al. 2016; Ma et al. 2011).

Inoculating legume plants with rhizobia or stimulating native rhizobia strains efficient… in N\textsubscript{2} fixation and equipped with other plant-growth-promoting traits, and well adapted to trace-element-induced stress, features high relevance from both ecological and economic points of view (Franco et al. 2012; Dary et al. 2010; Rangel et al. 2016). Thus, investigating native rhizobia populations from soils with high trace elements contents (like mining areas) can provide essential information concerning genetic or phenotypic resources that are better adapted to trace elements stress, in function of phytoremediation approaches (Trannin et al. 2001; Rangel et al. 2016; Weyens et al. 2009a).

Therefore, this study aimed to (a) isolate and characterize native N\textsubscript{2}-fixing bacteria from nodules of \textit{Crotalaria spectabilis} and \textit{Stizolobium aterrimum} growing on an arsenic-contaminated mining site; (b) identify them by the partial sequencing of their 16S rRNA genes; (c) evaluate their multi-resistance to As, Cd, and Zn, and their resistance to \beta-lactam antibiotics; (d) investigate...
their in vitro plant-growth-promoting (PGP) traits, and (e) evaluate their symbiotic efficiency with their original host plant.

Materials and methods

Isolation and strain characterization

Bacteria were isolated from *S. aterrimum* (17° 10'59.88"S 46° 52'24.11"W) and *C. spectabilis* (17° 8'10.99"S 46° 51'31.75"W) nodules collected in a gold mine area contaminated with arsenic, in the northwest region of Minas Gerais, Brazil. Soil chemical and physical parameters (0–20 cm) from the As-contaminated gold mine area are presented in Table 1. Phosphorus and potassium were determined by Mehlich-1 extraction (HCl 0.05 mol L⁻¹); calcium, magnesium, and aluminum were determined after KCl extraction (1 mol L⁻¹). The potential acidity (H + Al) was estimated by SMP extraction, and organic matter was determined by oxidation using Na₂Cr₂O₇ + H₂SO₄ (10N) (EMBRAPA 2011). According to the 5th Approach (Guidelines for use of lime and fertilizers in Minas Gerais) (Ribeiro et al. 1999), the soil active acidity was chemically classified as medium acidity; the phosphorus availability considering the clay content and P-rem value was classified as good; the soil fertility (based on organic matter and cation exchange capacity) was classified as very good for P, low for potassium, calcium, and magnesium, very low for aluminum, hydrogen + aluminum, and organic matter content. The soil texture was determined by the pipette method as described by ref. (Day 1965), and according to the classification of the normative guideline number 2 from the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA) October 9th 2008, the soil texture was identified as silt loam.

Nodules were surface-sterilized according to ref. (Vincent 1970), and the nodule inhabiting bacteria were isolated on 79 solid culture medium (Vincent 1970; Fred and Waskman 1928) with bromothymol blue (pH 6.9, 28 °C). After purification of the single colonies, the following characteristics of the colonies were evaluated: pH change of the culture medium, morphological features (diameter, form, edge, lifting, surface, light transmission, color, and bromothymol blue absorption) and exopolysaccharide (EPS) production (Moreira et al. 1993). The range of EPS production was classified as scarce, low, moderate, and abundant.

Strains were clustered based on their characteristics including type or reference strains of the genera Azorhizobium (*A. caulinodans*—ORS571T; *A. doebereinerae*—BR 5401T), Mesorhizobium (*M. plurifarium*—BR 3804), Rhizobium (*R. tropici*—CIAT 899T), Burkholderia (*B. cepacia*—LMG 1222T), and strains of the genus Bradyrhizobium (*B. bradyrhizobium* sp.—BR 2001 e BR 2811). The strain BR 2811 is the inoculum for *C. spectabilis* and *S. aterrimum* plant species, approved by the Brazilian Ministry of Agriculture, Livestock and Food Supply. All strains were clustered considering 11 characteristics; a similarity dendrogram was composed using the Ward’s minimum variance method using the package Cluster in R 2.12.2 (Figure S1).

Genotypic characterization

Genomic DNA was extracted from the bacterial cultures according to the extraction kit protocol ZR Fungal/Bacterial DNA (Zymo Research Corp). Strains were identified by sequencing the 16S rRNA genes.

PCR was performed using 50 ng of the extracted DNA, 45 μL PCR reaction mixture containing 0.2 mM dNTP, 2.5 mM MgCl₂, 0.2 μM 27F primer (5’-AGAGTTT-GATCCTGCGTCAAG-3’), 0.2 μM 1492R primer (5’-GTTACCTTGTAGGACCT-3’) (Lane 1991), 1 U Taq DNA polymerase (Fermentas), 10X KCl buffer, and ultrapure sterile water. The amplification was done using an Eppendorf Mastercycler® under the following conditions: initial denaturation step at 94°C for 5 minutes; 40 cycles of denaturation at 94°C for 40 seconds; annealing step at 55°C for 40 seconds; extension step at 72°C for 1.5 minutes; final extension at 72°C for 7 minutes. The obtained PCR products were purified and sequenced by Macrogen (South Korea).

The quality of the sequences was verified using the BioNumerics 6.5 program (Applied Maths, Sint-Martens-Latem, Belgium), and they were blasted against the GenBank sequences (NCBI—National Center for Biotechnology Information).

Nucleotide sequence accession numbers—The sequences determined in this work were deposited in GenBank under accession numbers KT694150 to KT694173.

| Chemical parameters¹ | pH | H₂O | P-rem | K² | Ca³⁺ | Mg²⁺ | Al³⁺ | H+Al⁴⁺ | OM⁵⁺ | As⁶⁺ |
|----------------------|----|-----|-------|----|------|------|------|--------|-------|-------|
| mg L⁻¹               | _mg dm⁻³ | _cmol, dm⁻³ | _dag kg⁻¹ | _mg kg⁻¹ |
| 5.5                  | 61.36 | 40.1 | 25.4    | 0.68 | 0.22 | 0.1   | 0.9   | 0.5    | 13.2  | 395.9 |

Table 1. Soil chemical and physical parameters (0–20 cm) from the As-contaminated mining area in Minas Gerais.

| Soil physical size group and texture |
|-------------------------------------|
| Sand                                |
| 160                                  |
| Clay                                |
| 760                                  |
| Silt loam                           |
| 80                                   |

¹Chemical parameters: pH – H₂O pH (ratio 1:2.5); P-rem (remaining phosphorus); K (potassium) – Mehlich-1 extractor (HCl 0.05 mol L⁻¹ + H₂SO₄ 0.0125 mol L⁻¹); Ca (calcium); Mg (magnesium); Al (aluminum) – KCl extractor 1 mol L⁻¹; H + Al (hydrogen + aluminum) – SMP extractor; OM (Organic matter) – oxidation using Na₂Cr₂O₇ + H₂SO₄ 10N (15); Arsenic available and semitotal content.
**Strain authentication and symbiotic efficiency**

The nodulation capacity (authentication), i.e. the ability to establish symbiosis with its original host, and the symbiotic efficiency of the 24 nitrogen-fixing bacterial strains isolated from nodules of the trap species *Crotalaria spectabilis* and *Stizolobium atterrimum* were examined in a greenhouse experiment for each trap species under axenic conditions.

The seeds were scarified using H_{2}SO_{4} p.a. (*C. spectabilis* for 5 minutes and *S. atterrimum* for 45 minutes) and placed on sterile Petri dishes containing moistened cotton incubated at 28°C until radicle emergence. The strains were grown in 79 liquid medium shaking (125 rpm, 28°C) for 120 hours. At the moment of sowing, each seed was inoculated with 1 mL of the bacterial inoculum containing about 10^8 cells. After inoculating the seeds, a thin layer of the sterile mixture of sand-benzene-paraffin was disposed on the top to avoid contamination. Two plants were grown in sterile Leonard jars for 45 days. Sand and vermiculite (1:1 ratio) were used as substrate in the topmost portion of the jars, and in the lower portion, a fourfold diluted modified Hoagland nutrient solution (Hoagland and Arnon 1950) was added. The inoculated plants and the non-inoculated control plants were supplied with a low nitrogen concentration (5.25 mg L^{-1}) in the nutrient solution, which is considered a starting dose for, and not an inhibitor of, the biological nitrogen fixation process. The following quantities of the stock solutions were added to 4 L of water: 0.4 mL of 236.16 g L^{-1} Ca(N_{2}O_{4})_{2}·4H_{2}O; 0.1 mL of 115.03 g L^{-1} NH_{4}H_{2}PO_{4}; 0.6 mL of 10.11 g L^{-1} KNO_{3}; 2.0 mL of 246.9 g L^{-1} MgSO_{4}·7H_{2}O; 3.0 mL of 87.13 g L^{-1} K_{2}SO_{4}; 10 mL of 12.6 g L^{-1} CaH_{2}PO_{4}·2H_{2}O; 200 mL of 1.72 g L^{-1} CaSO_{4}·2H_{2}O; 1 mL of 10 g L^{-1} FeCl_{3}, and 1 mL of micro-nutrients (2.86 mg L^{-1} H_{3}BO_{3}; 2.03 mg L^{-1} MnSO_{4}·4H_{2}O; 0.22 mg L^{-1} ZnSO_{4}·7H_{2}O; 0.08 mg L^{-1} CuSO_{4}·5H_{2}O, and 0.09 mg L^{-1} Na_{2}MoO_{4}·2H_{2}O). In addition, a control treatment supplemented with a high inorganic nitrogen concentration (52.5 mg L^{-1}) was also provided, composed by the following quantities of the stock solutions added to 4 L of water: 4.0 mL of 236.16 g L^{-1} Ca(N_{2}O_{4})_{2}·4H_{2}O; 1.0 mL of 115.03 g L^{-1} NH_{4}H_{2}PO_{4}; 6.0 mL of 10.11 g L^{-1} KNO_{3}; 2.0 mL of 246.9 g L^{-1} MgSO_{4}·7H_{2}O; 1.0 mL of 10 g L^{-1} FeCl_{3}, and 1.0 mL of micro-nutrients solution (composition as described above). Besides the negative control treatments (low and high N content), a positive control treatment was inoculated with *Bradyrhizobium* sp. Strain BR 2811, which has been approved as inoculant for both plant species by the Brazilian Ministry of Agriculture, was also included. The assays were fully randomized, and four replicates were implemented for each treatment. Harvesting was performed after 45 days including the following measurements: nodule number (NN) and nodule dry weight (NDW), shoot dry weight (SDW), and relative efficiency (RE%). The RE% of each inoculated treatment was calculated in relation to the shoot dry matter production by the control treatment supplied with high inorganic nitrogen content, using the formula RE = [(inoculated SDW/high N SDW) × 100] where RE means relative efficiency, inoculated SDW means shoot dry weight of the inoculated treatment, and high N SDW means shoot dry weight of the control treatment supplied with high inorganic nitrogen content.

The data were analyzed by one-way analysis of variance using the statistical program SISVAR (Ferreira 2011). The NN and NDW were transformed using the formula (x + 0.5)^0.5. The average of the treatments was grouped by the Scott-Knott test at 5% significance.

**Phenotypic characterization**

**Arsenic minimum inhibitory concentration (As MIC)**

Bacterial strains representative for the different “cultural” groups, which were formed after colony characterization, were selected for the As MIC assay. Seven type or reference strains belonging to the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Burkholderia* were also included in this assay.

Strains were grown in 30 mL of 79 liquid medium at pH 6.9, using an orbital shaker (125 rpm) at 28°C. Subsequently, 1 mL of each strain containing about 10^8 cells was transferred to sterile microtubes (1.5 mL), which were centrifuged at 8,000 g at 25°C for 4 min. The supernatant was discarded, the cells were resuspended in 1 mL sterile NaCl (8.5 g L^{-1}), and centrifuged again. This washing procedure was repeated three times. After that, 20 μL aliquots of the cell suspension were inoculated on 79 solid media containing different As concentrations. Arsenic (Na_{2}HAsO_{4}·7H_{2}O) was used at concentrations of 50, 100, 150, and 200 mmol L^{-1}, in addition to a control treatment without As. After adding As to the medium, pH was adjusted to 6.9 using HCl (0.5 M). The susceptibility of the strains to As was examined by determining the MIC, which was defined as the lowest concentration at which there are no colony-forming units (CFU) on the medium after 9 days of incubation at 28°C. Each treatment (strains and controls) was evaluated in three replicates.

After composing the similarity dendrogram using the colony characteristics of the strains, the frequencies of resistant individuals at different As concentrations within the groups formed by the characterization of the colonies were analyzed using the chi-square test at 5% significance.

**Pattern of β-lactam antibiotics resistance**

Bacteria were grown on 79 liquid medium for 72 hours at 28°C, after which 0.1 mL aliquots of the bacterial inoculum were spread on 79 solid medium using a Drigalski spatula. Susceptibility was determined using the disk diffusion method (Cecon- Sensobiodisc) for amoxicillin (AMO) (10 μg), ampicillin (AMP) (10 μg), ceftriaxone (CEF) (30 μg), cefoxitin (CFOX) (30 μg), oxacillin (OXA) (1 μg), and vancomycin (VAN) (30 μg) (Bauer et al. 1996). The strains were defined as "sensitive" in case a radius zone was observed or "resistant" in case no radius zone was formed after 48 hours at 28°C. Strains were grown in triplicate.

**Plant growth-promoting traits**

Besides the native bacterial strains isolated from nodules of both *Crotalaria spectabilis* and *Stizolobium atterrimum*, growing on a gold mine area contaminated with arsenic, five type or reference strains, *Azorhizobium cauliformans* ORS751^T, *A. doberi-enerae* BR 5401^T, *Mesorhizobium plurifarium* BR 3804, *Rhizobium tropici* CIAT 899^T, *Burkholderia* sp. BR 11340, were also investigated in vitro for their PGP traits. Bacterial organic
acid (OA) production was assessed based on a colorimetric method (Cunningham and Kuiack 1992), indole-3-acetic acid (IAA) production capacity was tested using the Salkowski assay (adapted from ref. (Patten and Glick 2002)), 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity was evaluated using a slightly modified protocol according to ref. (Belimov et al. 2005), siderophore production was qualitatively evaluated by a widely used colorimetrical method (Schwyn and Neilands 1987), and Ca$_3$(PO$_4$)$_2$ phosphate solubilization ability was evaluated in solid medium (Nautiyal 1999) (Table 2). Their multi-

**Table 2.** Original host species, most similar sequence 16s rRNA gene available in NCBI, and qualitative plant growth-promoting traits of the strains isolated from As-contaminated mining soil.

| Host species | Strains   | bp* of 16s rRNA | Identity                        | Phylum/class                     | OA** | IAA** | ACC** | SID** | Ca$_3$(PO$_4$)$_2$ Sol*** |
|--------------|-----------|----------------|---------------------------------|----------------------------------|------|-------|-------|-------|--------------------------|
| Crotalaria spectabilis | UFLA 05-01 | 688 | 100% | Bradyrhizobium sp. (FR872439.1) | a-Proteobacteria | –   | –     | –     | +++++ | I**** |
| Crotalaria spectabilis | UFLA 05-02 | 557 | 100% | Bradyrhizobium sp. (FR872439.1) | a-Proteobacteria | –   | –     | –     | +++++ | GNFH |
| Crotalaria spectabilis | UFLA 05-03 | 505 | 100% | Bradyrhizobium sp. (FR872439.1) | a-Proteobacteria | –   | –     | –     | +++++ | I |
| Crotalaria spectabilis | UFLA 05-04 | 516 | 100% | Bradyrhizobium sp. (FR872439.1) | a-Proteobacteria | –   | –     | –     | +++++ | I |
| Crotalaria spectabilis | UFLA 05-06 | 953 | 99% | Bradyrhizobium sp. (FR872439.1) | a-Proteobacteria | –   | –     | –     | +++++ | L**** |
| Crotalaria spectabilis | UFLA 05-07 | 773 | 100% | Inquilinus sp. MG-2011-30-B (FR872493.1) | a-Proteobacteria | –   | –     | –     | +++++ | L |
| Crotalaria spectabilis | UFLA 05-08 | 992 | 99% | Bradyrhizobium sp. (FR872439.1) | α-Proteobacteria | –   | –     | –     | +++++ | L |
| Crotalaria spectabilis | UFLA 05-09 | 653 | 100% | Bradyrhizobium sp. (FR872439.1) | α-Proteobacteria | –   | –     | –     | +++++ | I |
| Crotalaria spectabilis | UFLA 05-10 | 888 | 100% | Bradyrhizobium sp. (FR872439.1) | α-Proteobacteria | –   | –     | –     | +++++ | L |
| Stizolobium atenarum | UFLA 05-11 | 1096 | 100% | Bradyrhizobium elkanii (HQ231447.1) | α-Proteobacteria | –   | +     | +     | +++++ | GNFH |
| Stizolobium atenarum | UFLA 05-12 | 512 | 100% | Bradyrhizobium sp. UFLA 03-143 (JX284230.1) | α-Proteobacteria | ++  | +++   | +     | –   | L |
| Stizolobium atenarum | UFLA 05-13 | 835 | 99% | Bradyrhizobium sp. UFLA 03-174 (JX284219.1) | α-Proteobacteria | –   | –     | +     | –   | L |
| Crotalaria spectabilis | UFLA 05-14 | 414 | 98% | Bradyrhizobium sp. (DQ202330.1) | α-Proteobacteria | –   | –     | –     | +++++ | GNFH |
| Stizolobium atenarum | UFLA 05-15 | 553 | 99% | Bacillus sp. DB170 (HM566884.1) | Firmicutes | –   | –     | –     | +++++ | I |
| Crotalaria spectabilis | UFLA 05-16 | 952 | 100% | Rhizobium tropici CIAT 899 (NR 102511) | α-Proteobacteria | –   | +     | +     | +++++ | I |
| Stizolobium atenarum | UFLA 05-17 | 378 | 100% | Bradyrhizobium sp. UFLA 03-182 (JX284238.1) | α-Proteobacteria | –   | +     | +     | +++++ | I |
| Stizolobium atenarum | UFLA 05-18 | 642 | 100% | Bradyrhizobium sp. UFLA 03-140 (JX284229.1) | α-Proteobacteria | –   | –     | +     | –   | L |
| Stizolobium atenarum | UFLA 05-19 | 295 | 100% | Bradyrhizobium elkanii IAR12 (JQ809927.1) | α-Proteobacteria | ++  | +++   | +     | +++++ | L |
| Stizolobium atenarum | UFLA 05-20 | 1097 | 99% | Bradyrhizobium sp. CCBAU 23005 (GU334461.1) | α-Proteobacteria | ++  | +++   | +     | +++++ | I |
| Stizolobium atenarum | UFLA 05-21 | 1068 | 99% | Methylobacterium sp. AMS19(R) (AB60008.1) | α-Proteobacteria | –   | –     | +     | +++++ | I |
| Stizolobium atenarum | UFLA 05-22 | 794 | 100% | Bosea sp. S41RM2 (GU731243.1) | α-Proteobacteria | –   | –     | +     | +++++ | GNFH |
| Stizolobium atenarum | UFLA 05-23 | 578 | 98% | Starkeya novelloe DMS 506 (CP002026.1) | α-Proteobacteria | –   | +     | –     | –   | GNFH |
| Stizolobium atenarum | UFLA 05-24 | 307 | 100% | Bradyrhizobium sp. LmjM3 (JX514883.2) | α-Proteobacteria | ++  | +++   | +     | +++++ | I |

*bp = base pairs of 16s rRNA gene sequence.
*Classification conferred according to the color intensity. OA: organic acid production; IAA: indole-3-acetic acid production; ACC: 1-aminocyclopropane-1-carboxylate deaminase activity; SID: siderophores production.
**Based on the Ca$_3$(PO$_4$)$_2$ solubilization index, the strains were classified as Low (L) with solubilization index < 2.00, Intermediate (I) 2.00 ≤ SI < 4.00 or High (H) SI ≥ 4.00.
1Grown but did not form a halo (GNFH) by the 15th day.
element resistance was also studied including Cd and Zn (Table 4) (Weyens et al. 2013a and Croes et al. 2013). Resistance was appraised visually examining growth and polysaccharide (mucus) production on the plate. The range of exopolysaccharide production that was used was the same one used for strain characterization… (i.e. scarce, low, moderate, and abundant).

**Results**

**Isolation, strain characterization, and identification by 16S rRNA gene partial sequencing**

All strains isolated from nodules of *C. spectabilis* and *S. aterrimum* were characterized and clustered together with reference strains for the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Burkholderia*. In general, strains are clustered in two main groups (Figure S1; Table S1), using the Ward’s hierarchical clustering method.

Group A was formed by strains that alkalinized the 79 solid medium, and showed low or scarce exopolysaccharide (EPS) production. Six strains isolated from nodules of *C. spectabilis*, identified as *Bradyrhizobium* sp., and three strains isolated from nodules of *S. aterrimum*, identified as *Bosea* sp., *Starkeya novella* and *Methylobacterium* sp., clustered into this group (Table 2), as well as the two reference strains for the genus *Azorhizobium*, BR 5401T and ORS571T.

Within group A, two small subgroups were formed. One subgroup was represented by strains belonging to the *Bradyrhizobiaceae* family (*Bradyrhizobium* sp. and *Bosea* sp. strains). The second subgroup was composed of strains belonging to the *Bradyrhizobiaceae* family (*Bradyrhizobium* sp.), in addition to strains from the *Methylobacteriaceae* (Methyllobacterium sp.), the *Xanthobacteraceae* (Starkeya novella), and the reference strains *A. cauliformans*-ORS571T and *A. deleymera*-BR 5401T. The strains belonging to the *Bradyrhizobiaceae* family in group A exhibited low EPS production (Table S1).

The nucleotide sequence of the 16S rRNA gene of the *Bosea* sp. strain isolated in our study showed 100% similarity with the sequence of *Bosea* sp. S41RM2, deposited in GenBank with the accession number GU731243.1. The origin of that strain is also an As-contaminated soil (Sultana et al. 2012).

**Table 3.** Authentication and symbiotic efficiency of native bacteria isolated from nodules of legume species growing on As-contaminated soil.  

| Bacteria and control treatments | NN | g pot⁻¹ | SDW | NAS mg pot⁻¹ | RE % |
|--------------------------------|----|---------|-----|--------------|------|
|                                |    |         |     |              |      |
| *Crotalaria spectabilis*        |    |         |     |              |      |
| UFLA 05-01 *Bradyrhizobium* sp.| 304±28b | 0.19±0.03a | 3.9±0.6c | 97.5±0.04c | 65.6±8.5b |
| UFLA 05-02 *Bradyrhizobium* sp.| 312±42b | 0.17±0.01a | 5.2±0.4b | 148.7±4.5b | 80.2±1.1b |
| UFLA 05-03 *Bradyrhizobium* sp.| 309±49b | 0.24±0.04a | 8.8±0.03a | 242.5±12.1a | 123.6±3.3a |
| UFLA 05-04 *Bradyrhizobium* sp.| 262±30b | 0.16±0.03a | 6.9±1.0b | 150.5±12.37b | 79.2±7.9b |
| UFLA 05-05 *Burkholderia* sp.  | 422±87a | 0.19±0.04a | 7.8±1.0a | 180.0±10.9b | 94.6±7.4b |
| UFLA 05-06 *Burkholderia* sp.  | 215±13b | 0.05±0.01b | 5.4±1.2b | 132.6±24.7b | 70.1±14.2b |
| UFLA 05-07 *Inquilinus* sp.     | 308±59b | 0.14±0.003a | 6.9±0.7b | 174.9±10.0b | 106.2±9.9a |
| UFLA 05-08 *Labrys monachus*    | 330±58b | 0.07±0.03b | 6.2±0.1b | 163.5±7.4b | 85.6±1.9b |
| UFLA 05-09 *Bradyrhizobium* sp.| 418±17a | 0.21±0.003a | 9.1±0.6a | 226.6±5.7a | 138.8±17.5a |
| UFLA 05-10 *Bradyrhizobium* sp.| 481±26a | 0.17±0.08a | 6.1±0.3b | 151.0±10.5b | 92.7±13.5b |
| UFLA 05-14 *Bradyrhizobium* sp.| 303±39b | 0.22±0.05a | 8.5±0.5a | 189.3±7.4b | 116.6±17.5a |
| UFLA 05-16 *Rhizobium tropici*  | 362±31a | 0.23±0.01a | 6.7±1.7b | 143.0±26.3b | 93.0±31.8b |
| BR 2811 *Bradyrhizobium* sp.   | 299±36b | 0.03±0.01b | 1.3±0.01d | 13.6±2.5d | 8.0±0.7c |
| 5.25 mg N L⁻¹                  | 0c   | 0c       | 5.3±0.2b | 169.0±22.5b | 100.0b |
| 52.5 mg N L⁻¹                  | 0c   | 0c       | 5.3±0.2b | 169.0±22.5b | 100.0b |

NN, number of nodules; NDW, nodule dry weight; SDW, shoot dry weight; NAS, nitrogen accumulation in shoot; RE, relative efficiency. Values followed by the same letter on the column comparing strains do not differ by Scott-Knott test, p < 0.05.
5.25 mg L⁻¹. In the greenhouse experiment, no nodulation was observed for Strain authentication and symbiotic efficiency of all strains except BR 2811 (Table 3). Without inoculation with the BR 2811 strain, the efficiency of all strains was higher even in comparison with strain BR 2811 that was approved by MAPA as an inoculant for C. spectabilis. The strains UFLA 05-03 (Bradyrhizobium sp.) and UFLA 05-09 (Bradyrhizobium sp.) were able to provide more N to the plants, even more than in case of application of the high concentration of inorganic N (52.5 mg L⁻¹). Moreover, the two above-mentioned strains and UFLA 05-14 (Bradyrhizobium sp.) could establish symbiosis with the plants, even more than in case of application of the high concentration of inorganic N (52.5 mg L⁻¹). Furthermore, the two above-mentioned strains and UFLA 05-14 (Bradyrhizobium sp.) also demonstrated higher RE% than the control treatment supplied with the high concentration of inorganic N (52.5 mg L⁻¹) (Table 3). Interestingly, this latter strain and also UFLA 05-08 (Labrys monachus) are atypical genera in nodulating legume plants. Although these strains were able to efficiently nodulate and fix N₂ in symbiosis with C. spectabilis, a further study examining for specific genes for those processes needs to be performed.

Eight strains out of the 12 isolated from nodules of S. aterrimatum were able to establish symbiosis and induced nodule formation on the roots of this plant species (Table 3). The strains UFLA 05-11 (B. elkanii), UFLA 05-12 (Bradyrhizobium sp.), UFLA 05-13 (Bradyrhizobium sp.), UFLA 05-17 (Bradyrhizobium sp.), UFLA 05-18 (Bradyrhizobium sp.), UFLA 05-19 (B. elkanii), UFLA 05-20 (Bradyrhizobium sp.), and UFLA 05-24 (Bradyrhizobium sp.) induced nodule formation in S. aterrimatum plants, in addition to the control strain BR 2811. The strains UFLA 05-11 (B. elkanii) and UFLA 05-12 (Bradyrhizobium sp.) showed higher NN, even in comparison to the control strain BR 2811, but this feature did not increase their RE%.

Table 4. Cadmium and zinc resistance (in 284 medium) of strains isolated from nodules of legume species growing in As-contaminated mining soil.

| Strains     | Closest related strain by NCBI | Cadmium 0.4 mM | Cadmium 0.8 mM | Zinc 0.6 mM | Zinc 1.0 mM |
|-------------|--------------------------------|----------------|----------------|-------------|-------------|
| UFLA 05-01  | Bradyrhizobium sp.             | +              | +              | +           | +           |
| UFLA 05-02  | Bradyrhizobium sp.             | +              | +              | +           | +           |
| UFLA 05-03  | Bradyrhizobium sp.             | +              | +              | +           | +           |
| UFLA 05-04  | Bradyrhizobium sp.             | +              | +              | +           | +           |
| UFLA 05-06  | Burkholderia sp. JPY321        | +              | +              | +           | +           |
| UFLA 05-07  | Inquilinus sp. MG-2011-30-BD    | +              | +              | +           | +           |
| UFLA 05-08  | Labrys monachus                | ++             | ++             | ++          | ++          |
| UFLA 05-09  | Bradyrhizobium sp.             | +              | +              | +           | +           |
| UFLA 05-10  | Bradyrhizobium sp.             | +              | +              | +           | +           |
| UFLA 05-11  | Bradyrhizobium elkanii         | +              | +              | +           | +           |
| UFLA 05-12  | Bradyrhizobium sp. UFLA 03-143 | +              | +              | +           | +           |
| UFLA 05-13  | Bradyrhizobium sp. UFLA 03-174 | +              | +              | +           | +           |
| UFLA 05-14  | Bradyrhizobium sp.             | +              | +              | +           | +           |
| UFLA 05-15  | Bacillus sp. DB170             | +              | +              | +           | +           |
| UFLA 05-16  | Rhizobium tropici CIAT 899     | ++             | ++             | ++          | ++          |
| UFLA 05-17  | Bradyrhizobium sp. UFLA 03-182 | +              | +              | +           | +           |
| UFLA 05-18  | Bradyrhizobium sp. UFLA 03-140 | +              | +              | +           | +           |
| UFLA 05-19  | Bradyrhizobium elkanii IAR12   | +              | +              | +           | +           |
| UFLA 05-20  | Bradyrhizobium sp. CCBAU 23005 | +              | +              | +           | +           |
| UFLA 05-21  | Methyllobacterium sp. AMS19    | +              | +              | +           | +           |
| UFLA 05-22  | Bosea sp. S41RM2               | +              | +              | +           | +           |
| UFLA 05-23  | Starkeya novella DMS 506      | +              | +              | +           | +           |
| UFLA 05-24  | Bradyrhizobium sp. LmjM3      | +              | +              | +           | +           |

Type or Reference rhizobia strains:

- CIAT 899: Rhizobium tropici
- BR 3804: Mesorhizobium plurifarium
- LMG 571: Azorhizobium caulodons
- BR 5401: Azorhizobium doebereinerae
- BR 11340: Burkholderia cepacia

- +: Scarce, ++: Low, +++: Moderate and +++++: Abundant - Growth plus polysaccharide production under in vitro contamination.

phyllum Firmicutes belonging to the Bacillaceae family was isolated (Figure S1; Table 2). The following type or reference strains also clustered in group B: Bradyrhizobium sp. (BR 2001 and BR 2811), Mesorhizobium plurifarium (BR 3804), Rhizobium tropici (CIAT 899), and Burkholderia cepacia (LMG 1222). The 16S rRNA gene sequencing revealed representatives of two phyla, Firmicutes and Proteobacteria (Table 2). Most of the strains belonged to the β-Proteobacteria such as Bradyrhizobium, Rhizobium, Bosea, Inquilinus, Labrys, Starkeya, and Methyllobacterium. A few strains belonged to the α-Proteobacteria.

**Strain authentication and symbiotic efficiency**

In the greenhouse experiment, no nodulation was observed for the control treatments (without inoculation and supplied with 5.25 mg L⁻¹ or 52.5 mg L⁻¹ of inorganic N) of both C. spectabilis and S. aterrimatum plants. Taking this into account, it was possible to verify the authentication of the symbiosis and the symbiotic efficiency of the isolated strains. C. spectabilis plants established symbiosis with all strains tested, including BR 2811 (Table 3). Without exception, all strains exhibited N₂ fixation in symbiosis with C. spectabilis, showing NAS and RE% higher or similar to the control treatment supplied with the high concentration of inorganic N (52.5 mg L⁻¹). Only the UFLA 05-01 (Bradyrhizobium sp.) strain displayed a lower SDW production in comparison with the high inorganic N treatment, but it was still higher when compared with the low concentration of inorganic N and inoculation with the BR 2811 strain. The efficiency of all strains was higher even in comparison with strain BR 2811 that was approved by MAPA as an inoculant for C. spectabilis. The strains UFLA 05-03 (Bradyrhizobium sp.) and UFLA 05-09 (Bradyrhizobium sp.) were able to provide more N to the plants, even more than in case of application of the high concentration of inorganic N (52.5 mg L⁻¹). Moreover, the two above-mentioned strains and UFLA 05-14 (Bradyrhizobium sp.) and UFLA 05-07 (Inquilinus sp.) also demonstrated higher RE% than the control treatment supplied with the high concentration of inorganic N (52.5 mg L⁻¹) (Table 3). Interestingly, this latter strain and also UFLA 05-08 (Labrys monachus) are atypical genera in nodulating legume plants. Although these strains were able to efficiently nodulate and fix N₂ in symbiosis with C. spectabilis, a further study examining for specific genes for those processes needs to be performed.
Unlike the other strains, which showed low NN but high NDW, strains UFLA 05-13 (Bradyrhizobium sp.), UFLA 05-17 (Bradyrhizobium sp.), UFLA 05-18 (Bradyrhizobium sp.), UFLA 05-19 (B. elkanii), UFLA 05-20 (Bradyrhizobium sp.), and UFLA 05-24 (Bradyrhizobium sp.), were more efficient in N₂ fixation and showed higher RE%. Interestingly, these strains demonstrated higher efficiencies than the control strain BR 2811, which is approved as inoculant for C. spectabilis. Among the six strains that showed higher RE% than the high inorganic N treatment, four—UFLA 05-18 (Bradyrhizobium sp.), UFLA 05-19 (B. elkanii), and UFLA 05-24 (Bradyrhizobium sp.)—showed SDW productions statistically similar to the control treatment supplied with the high concentration of inorganic N (52.5 mg L⁻¹). Although the SDW productions by the other 2 strains, UFLA 05-13 (Bradyrhizobium sp.) and UFLA 05-17 (Bradyrhizobium sp.), were lower than the control treatment with high inorganic N, these strains displayed higher RE% than the control treatment with high inorganic N (52.5 mg L⁻¹). The atypical genera, Bosea sp., Methylobacterium sp., and Starkeya novella, did not induce nodule formation on S. aterrimum roots in the authentication test (Table 3).

Phenotypic characterization

Arsenic minimal inhibitory concentration (MIC)

The MIC screening revealed 8 As-resistant strains (Figure 1). The strains UFLA 05-21 (Methylobacterium sp.) and UFLA 05-23 (Starkeya novella), isolated from nodules of S. aterrimum, tolerated up to 150 and 200 mmol As L⁻¹, respectively. Among the strains isolated from nodules of C. spectabilis, only UFLA 05-16 (Rhizobium tropici) tolerated the highest As concentration tested. Other strains isolated from S. aterrimum and C. spectabilis did not survive any of the As concentrations applied and were therefore considered to be sensitive to As. Among the type and reference strains, A. caulodans ORS571T, Mesorhizobium plurifarium BR 3804, Rhizobium tropici CIAT 899T, and Burkholderia cepacia LMG 1222T were resistant to As, since they could grow until the highest As concentration tested. The strain A. doebereinereae BR 5401T was also As resistant, but it survived only until 100 mmol As L⁻¹. On the other hand, Bradyrhizobium sp. BR 2001 and BR 2811 strains were considered to be As sensitive, since they did not tolerate any of the As concentrations applied (Figure 1).

Pattern of β-lactams antibiotics resistance

The patterns of β-lactams antibiotics resistance indicated that most of the As-resistant strains had a similar resistance spectrum to AMO/AMP/CEFT/OXA and the As-sensitive strains had a similar resistance spectrum to VAN/OXA/AMO/AMP (Figure 2). The As-resistant strains UFLA 05-21 (Methylobacterium sp.), BR 5401T (A. doebereinereae), and ORS571T (A. caulodans) showed the same pattern of β-lactams antibiotics resistance to AMO, AMP, CEFT, OXA, and VAN, and sensitivity to CFD. The strains UFLA 05-16 (R. tropici) and CIAT 899T (R. tropici) showed the same pattern of β-lactams antibiotic resistance to AMO, AMP, CFD, CEFT, and OXA, and sensitivity to VAN. Strain BR 3804 (M. plurifarium) demonstrated a similar pattern, being resistant to the same β-lactams: AMO, AMP, CFD, CEFT, and OXA, but it was sensitive to CFD, and VAN as well. Strain LMG 1222T (B. cepacia) was resistant to all β-lactams studied: AMO, AMP, CFD, CEFT, OXA, and VAN. On the other hand, the strain UFLA 05-23 (S. novella) was only resistant to CFD, and sensitive to all the other β-lactams under investigation.

Plant growth-promoting traits

Alongside the native bacterial strains isolated from nodules of both Crotalaria spectabilis and Stizolobium aterrimum, five type or reference strains, Azorhizobium caulodans ORS571T, A. doebereinereae BR 5401T, Mesorhizobium plurifarium BR 3804, Rhizobium tropici CIAT 899T, and Burkholderia cepacia BR 3804, were also screened for their in vitro PGP traits (Table 2), and their multi-resistance to Zn and Cd (Table 4). In general, the number of strains demonstrating potential PGP traits in in vitro tests, such as production of OA, IAA, ACC deaminase, siderophores, and solubilization of P, is remarkably high.

Figure 1. Dendrogram based on colony characteristics and As resistance of the strains isolated from nodules of S. aterrimum and C. spectabilis species, growing on As-contaminated soil. UFLA 05-01, UFLA 05-02, UFLA 05-12, UFLA 05-13, UFLA 05-14, UFLA 05-18 e UFLA 05-20 (Bradyrhizobium sp.), UFLA 05-11 (Bradyrhizobium elkanii), UFLA 05-16 (Rhizobium tropici), UFLA 05-22 (Bosea sp.), UFLA 05-21 (Methylobacterium sp.), UFLA 05-23 (Starkeya novella), and UFLA 05-15 (Bacillus sp.). Reference and type strains Azorhizobium (BR 5401T and ORS 571T), Bradyrhizobium (BR 2001 and BR 2811), Mesorhizobium (BR 3804), Rhizobium (CIAT 899T), and Burkholderia (LMG 1222T).

Figure 2. The patterns of β-lactams antibiotics resistance indicated that most of the As-resistant strains had a similar resistance spectrum to AMO/AMP/CEFT/OXA and the As-sensitive strains had a similar resistance spectrum to VAN/OXA/AMO/AMP. The As-resistant strains UFLA 05-21 (Methylobacterium sp.), BR 5401T (A. doebereinereae), and ORS571T (A. caulodans) showed the same pattern of β-lactams antibiotics resistance to AMO, AMP, CEFT, OXA, and VAN, and sensitivity to CFD. The strains UFLA 05-16 (R. tropici) and CIAT 899T (R. tropici) showed the same pattern of β-lactams antibiotic resistance to AMO, AMP, CFD, CEFT, and OXA, and sensitivity to VAN. Strain BR 3804 (M. plurifarium) demonstrated a similar pattern, being resistant to the same β-lactams: AMO, AMP, CFD, CEFT, and OXA, but it was sensitive to CFD, and VAN as well. Strain LMG 1222T (B. cepacia) was resistant to all β-lactams studied: AMO, AMP, CFD, CEFT, OXA, and VAN. On the other hand, the strain UFLA 05-23 (S. novella) was only resistant to CFD, and sensitive to all the other β-lactams under investigation.
Phytostabilization of trace-element-contaminated soils has been proposed as a sustainable and low-cost technology (Vangronsveld and Cunningham 1998). Symbiotic interactions between legume plants and rhizobia possess a great potential to improve the efficiency and sustainability of phytostabilization (Franco et al. 1995; Dary et al. 2010; Hao et al. 2014).

The interactions between plants with their associated microorganisms indeed are highly important to enhance the success of phytostabilization. Plant-associated microorganisms can perform several essential biological processes, such as biological N₂ fixation and improving and promoting plant growth (Weyens et al. 2013a). Both the selection of the appropriate plant species and the most beneficial associated microorganisms are crucial steps in phytoremediation projects (Matsuda et al. 2002a; Carrasco et al. 2005; Weyens et al. 2013a; Matsuda et al. 2002b; Pajuelo et al. 2008; Weyens et al. 2009b).

In this study, bacterial strains were isolated from nodules of C. spectabilis and S. aterrimum plants growing on As-contaminated soils of a gold mine area. The isolated strains were characterized phenotypically (Figure S1; Table S1) and genotypically (Table 2). In previous experiments, using the same soil, both plant species, C. spectabilis (Lopes et al. 2015) and S. aterrimum (Rangel et al. 2014), showed potential for phytostabilization of As-contaminated soils.

N₂-fixing bacteria have been isolated from several soils contaminated with different trace elements (Carrasco et al. 2005; Weyens et al. 2013a; Croes et al. 2013; Drewniak et al. 2008; Oliveira et al. 2009; Becerra-Castro et al. 2011). Two main mechanisms for adaptation of bacteria to increase As concentrations have been proposed. The first mechanism comprises the reduction of arsenate to arsenite, using the As resistance system (ars)… and the second mechanism would be activated at the same time, to oxidize arsenite on the respiratory system, producing energy required for growth.

In this study, 13 strains representative for the different “cultural” groups were investigated in vitro for their resistance to As (Figure 1). The strains UFLA 05-16 (Rhizobium tropici) and UFLA 05-23 (Starkeya novella), and reference or type strains ORS571T (A. caulinodans), BR 3804 (Mesorhizobium plurifarium), CIAT 899T (Rhizobium tropici), and LMG 1222T (Burkholderia cepacia) tolerated up to 200 mmol As L⁻¹, the strain UFLA 05-21 (Methyllobacterium sp.) up to 150 mmol As L⁻¹, and the type strain BR 5401T (A. doebereinereae) tolerated up to 100 mmol As L⁻¹. These results emphasize the As resistance of these strains. All strains that could cope with the highest As concentration (200 mmol L⁻¹) that was used were members of α-Proteobacteria.

Our results confirm that the α-Proteobacteria contain a high diversity of As-resistant bacteria, including several rhizobia genera like Azorhizobium, Mesorhizobium, Rhizobium, Burkholderia, and Starkeya (Table 2). This fact makes the use of biological nitrogen fixation (BNF) for phytostabilization purposes more promising, since it increases the number of host legume species for these genera of rhizobia that can be tested in the field.

Already in 1982, Mobley and Rosen (Mobley and Rosen 1982) demonstrated that As resistance is genetically conferred, and the genes are located on a plasmid. The resistance mechanism is an energy-dependent efflux system, linked to the cellular membrane (Messens and Silver 2006). Plasmids that contain resistance to trace elements may also confer resistance to β-lactams antibiotics (Baker-Austin et al. 2006). Different resistance mechanisms to β-lactams antibiotics have been well

**Figure 2.** Pattern of β-lactam antibiotics resistance of arsenic-resistant strains by disk diffusion method. AMO: Amoxicillin, AMP: Ampicillin, CFD: Cefadroxil, CEFT: Ceftriaxon, OXA: Oxacillin, and VAN: Vancomycin.
characterized, such as the reduction of membrane permeability to trace elements and antibiotics, drug and trace elements inactivation and modification, and rapid efflux of the trace elements and antibiotics (Silver 1996; Mukhopadhyay and Rosen 2002; Wright 2005). In this study, most of the As-resistant strains also showed a pattern of multiple resistance to β-lactam antibiotics (Figure 2). The production of β-lactamase, an enzyme capable of modifying and inactivating the β-lactams antibiotics, is part of the resistance mechanism to β-lactams antibiotics (Williams 1999). In Gram-negative bacteria, β-lactamases are produced constitutively, even when the antibiotic is not present (Marchou et al. 1987). In contrast to Gram-positive bacteria, Gram-negative bacteria retain this enzyme within the periplasmic space, which results in a more efficient resistance mechanism. This fact might explain why the strains sensitive to As (Figure 1) also showed a pattern of multiple resistance to β-lactams antibiotics (Figure 2), since most of them are Gram negative. Most of the As-sensitive bacteria (Figure 1; Table 2) belonged to the genus Bradyrhizobium, which is known for producing and using outer membrane proteins as an uptake system for siderophore-metal complexes (Plessner et al. 1993). Overall, these observations might suggest that the β-lactams antibiotics resistance and As resistance are related only by the permeability of the membrane for trace elements and antibiotics. Both trace elements and antibiotics may be taken up through the same outer membrane proteins (porins), which are responsible for increasing or decreasing membrane permeability (Silver 1996; Wright 2005). However, the resistance to As depends on the presence of arsenate reductases and related enzymes (Mukhopadhyay and Rosen 2002).

Plant-growth-promoting rhizobacteria (PGPR) can have different direct beneficial effects on the growth of their host plant. PGPR may synthesize and provide their host plant with fixed nitrogen or phytohormones such as IAA; they may facilitate uptake of nutrients such as P and Fe, or may synthesize enzymes such as ACC deaminase, which lowers plant ethylene levels and in this way may affect plant growth (Glick et al. 2007).

The total percentage of P solubilizing strains was 73%, which is high (Table 2). The Nautiyal protocol (Nautiyal 1999) is based on extracellular oxidation of glucose via quinoprotein glucose dehydrogenase, which produces gluconic acid and mobilizes insoluble phosphates very efficiently. Moreover, apart from the production and excretion of gluconic acid, other mechanisms such as the production of chelating substances, the release of protons originating from NH\textsuperscript{4+} assimilation, and the production of inorganic acids, have also been proposed to be responsible for phosphate solubilization by bacteria (Illmer and Schinner 1995).

Plants growing on trace-element-contaminated soils might become severely deficient in the amount of available iron (Glick 2003). Fortunately, plants can produce siderophores that bind iron, which allows them to take up more iron. Moreover, plants can also assimilate complexes of iron with bacterial siderophores. Even though plants can produce siderophores themselves, the affinity of plant siderophores for iron is considerably lower than that of bacterial siderophores (Glick 2003). Therefore, plants are considered to depend to a great extent on bacterial siderophore production. In trace-element-contaminated soils, plants are often unable to accumulate sufficient amounts of iron unless bacterial siderophores are present (Glick 2003). A high number of our bacterial strains (69%) produced siderophores (Table 2). This high amount of siderophores-producing strains was not surprising since they were isolated from legume nodules, and most of the isolated strains were rhizobia (Table 2) that perform BNF. The latter process is highly Fe demanding (Tang et al. 1990; Brear et al. 2013) since not only the nitrogenase enzyme complex but also cytochromes, ferredoxins, and hydrogenases contain iron. Moreover, iron is highly important for nodule formation. Nodules of iron-adequate plants contained more than the double of leghemoglobin in comparison to iron-deficient plants (Tang et al. 1990). These authors demonstrated that the leghemoglobin production by Lupinus angustifolius inoculated with Bradyrhizobium lupini WU425 was depressed under iron deficiency. Even if BNF and symbiosis between leguminous plants and rhizobia is highly iron demanding, there are some Bradyrhizobium strains which do not produce siderophores: UFLA 05-10, UFLA 05-11, UFLA 05-12, UFLA 05-13, and UFLA 05-18 (Table 2). This can partly be explained by the fact that rhizobia strains that do not produce siderophores do synthesize outer membrane receptors instead. These outer membrane specific receptors are able to bind siderophore-metal complexes, allowing the uptake of iron chelates (Small et al. 2009). In rhizobia, Fe\textsuperscript{3+}-siderophore complexes are recognized by different outer membrane receptors depending on the siderophores. Such siderophores are referred to as xenosiderophores, since they are used by one organism but are synthesized and secreted by other organisms. An example was reported by Plessner et al. (1993) who have shown that Bradyrhizobium japonicum USDA 110, reclassified as B. diazoefficiens (Delamuta et al. 2013), and 61A152 strains are able to use ferrichrome and rhodotorulic acid, siderophores of fungal origin, as iron source.

OA production by bacteria is another way to promote plant growth, especially in trace-element-contaminated soils, where plant growth is often inhibited. By producing OA, bacteria can improve plant uptake of essential mineral nutrients (Weyens et al. 2013a; Braud et al. 2009), which are usually limiting in mining soils (Rangel et al. 2016; Lopes et al. 2015; Rangel et al. 2014). However, by enhancing the availability of essential mineral nutrients, OA production may also increase the availability of potentially toxic trace elements for the plants.

The low number (26%) of OA-producing bacterial strains isolated from the As-contaminated soil is remarkable (Table 2). Interestingly, most of the strains which did not produce OA were able to solubilize Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{2}. Therefore, the Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} solubilization by these strains is most likely not due to release of OA. We hypothesize that these strains do not produce OA in As-contaminated soil in order to avoid As solubilization since OA release may cause arsenopyrite (FeAsS) dissolution, even if little efficient (Drewniak et al. 2014).

Plants growing on contaminated mining soils often show growth inhibition as a consequence of nutrient deficiency as well as exposure to toxic amounts of potentially toxic trace elements. Since these conditions threaten the survival of the plants, the plant growth promotion potential of bacterial strains might be of high importance. The auxin IAA is involved in enhancing root growth and root length, as well as in the
proliferation and elongation of root hairs (Taghavi et al. 2009). A more extended root system due to bacterial IAA production might reduce nutrient deficiency as a result of the bigger soil volume that can be explored by the roots (Weyens et al. 2011). Several studies have shown that rhizosphere bacteria, phyllosphere bacteria, and endophytes can improve phytoremediation efficiency by different mechanisms, including IAA production (Weyens et al. 2009; Croes et al. 2013; Weyens et al. 2009b; Becerra-Castro et al. 2011; Taghavi et al. 2009; Weyens et al. 2011; Mastretta et al. 2009; Weyens et al. 2009c, 2013b). Fifty-two percent of the isolated bacterial strains are able to produce IAA in vitro (Table 2). Given the high number of native IAA-producing strains isolated from mining soils, we hypothesize that this trait might be of high importance for plant growth and development on these contaminated soils.

Besides directly affecting plant growth, IAA can also induce the transcription of ACC synthase, which catalyzes the formation of ACC (Tang et al. 1990). ACC is the immediate precursor of ethylene. At high levels, ethylene inhibits plant growth and induces early senescence. Therefore, plant-associated bacteria that are able to cleave ACC by ACC deaminase act as a sink for plant ACC, reducing the amount of ethylene released in the plant tissues, and thus also the consequences of high ethylene levels for plant growth and development (Glick et al. 2007; Glick and Stearns 2011). The high percentage (73%) of ACC deaminase-producing strains isolated from As-contaminated soils is noteworthy. Also Croes et al. (2013) and Tuyens et al. (2014) reported that trace element contamination pushes the plant-associated bacterial community to trace-element-resistant, phosphorus-solubilizing, nitrogen-fixing, and ACC deaminase and IAA-producing phenotypes.

Along the remarkable potential of the native rhizobia isolated from As-contaminated soils, also the results for the type or reference rhizobia strains that were included in this study are noteworthy (Table 2). Several strains belonging to different rhizobia genera exhibited positive results for most of the PGP traits we tested for in vitro. Altogether, our promising results are encouraging to continue rhizobium research in the framework of phytostabilization. These intrinsic PGP abilities of rhizobia increase the number of legume plant species that can be considered for phytoremediation purposes in different soil contamination conditions (e.g., mining, smelting etc.).

In addition to their capacity to promote plant growth on trace-element-contaminated soils, it is interesting that these bacteria possess multi-element resistance since almost all mining soils contain a multi-element pollution. The bacteria we have investigated in this work originated from mining soils. This implies that they were exposed for many generations to a toxic and hostile environment and thus were forced to adapt in order to survive under this selective pressure. Without exception, all bacterial strains isolated from the As-contaminated soil were resistant to both low and high Zn and Cd concentrations (Table 4). Among those strains, we highlight the Zn- and Cd-resistant Rhizobium tropici UFLA 05-16 strain, which also showed high As resistance (Figure 1). Labrys monachus strain UFLA 05-08 and Inquilinus sp. UFLA 05-07 also showed high Zn and Cd resistance.

Conclusions
The group of α-Proteobacteria harbors a high diversity of strains resistant to As, Cd, and Zn;

The symbiosis between UFLA 05-16 (R. tropici) and C. spectabilis plants has potential to be used on As-contaminated soils for phytostabilization purposes;

The potential PGP abilities together with the multi-element resistance of rhizobia are promising for exploiting the symbiosis with different legume species on trace-element-contaminated soils.

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