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Use of Endophytic and Rhizosphere Bacteria To Improve Phytoremediation of Arsenic-Contaminated Industrial Soils by Autochthonous *Betula celtiberica*

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ABSTRACT The aim of this study was to investigate the potential of indigenous arsenic-tolerant bacteria to enhance arsenic phytoremediation by the autochthonous pseudometallophyte *Betula celtiberica*. The first goal was to perform an initial analysis of the entire rhizosphere and endophytic bacterial communities of the above-named accumulator plant, including the cultivable bacterial species. *B. celtiberica*'s microbiome was dominated by taxa related to *Flavobacteriales*, *Burkholderiales*, and *Pseudomonadales*, especially the *Pseudomonas* and *Flavobacterium* genera. A total of 54 cultivable rhizobacteria and 41 root endophytes, mainly affiliated with the phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*, were isolated and characterized with respect to several potentially useful features for metal plant accumulation, such as the ability to promote plant growth, metal chelation, and/or mitigation of heavy-metal stress. Seven bacterial isolates were further selected and tested for *in vitro* accumulation of arsenic in plants; four of them were finally assayed in field-scale bioaugmentation experiments. The exposure to arsenic *in vitro* caused an increase in the total nonprotein thiol compound content in roots, suggesting a detoxification mechanism through phytochelatin complexation. In the contaminated field, the siderophore and indole-3-acetic acid producers of the endophytic bacterial consortium enhanced arsenic accumulation in the leaves and roots of *Betula celtiberica*, whereas the rhizosphere isolate *Ensifer adhaerens* strain 91R mainly promoted plant growth. Field experimentation showed that additional factors, such as soil arsenic content and pH, influenced arsenic uptake in the plant, attesting to the relevance of field conditions in the success of phytoextraction strategies.

IMPORTANCE Microorganisms and plants have developed several ways of dealing with arsenic, allowing them to resist and metabolize this metalloid. These properties form the basis of phytoremediation treatments and the understanding that the interactions of plants with soil bacteria are crucial for the optimization of arsenic uptake. To address this in our work, we initially performed a microbiome analysis of the autochthonous *Betula celtiberica* plants growing in arsenic-contaminated soils, including endosphere and rhizosphere bacterial communities. We then proceeded to isolate and characterize the cultivable bacteria that were potentially better suited to enhance phytoextraction efficiency. Eventually, we went to the field application stage. Our results corroborated the idea that recovery of pseudometallophyte-associated bacteria adapted to a large historically contaminated site and their use in

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bioaugmentation technologies are affordable experimental approaches and potentially very useful for implementing effective phytoremediation strategies with plants and their indigenous bacteria.

KEYWORDS contaminated soil, arsenic, root endophytes, rhizobacteria, *Betula*, field-scale study, phytoextraction, bioaugmentation

Arsenic (As) is a natural component of the earth's crust and is widely distributed throughout the environment (air, water, and land). Other sources of environmental arsenic are anthropogenic, e.g., insecticides, mining, industrial processes, coal combustion, timber preservatives, etc. (1). High concentrations of As lead to environmental damage and health problems (2, 3). Arsenic exists in four oxidation states, predominantly arsenate [As(V)] and arsenite [As(III)] and to a lesser extent arsenic [As(0)] and arsine As(−III) (4, 5). As(V) is a phosphate analogue and interferes with essential cellular processes, such as oxidative phosphorylation and ATP synthesis, whereas the toxicity of As(III) is due to its tendency to bind to sulfhydryl groups, affecting general protein functioning (6).

A sustainable technology for cleaning As-contaminated soils is phytoremediation, which is defined as the use of plants to remove or reduce toxic concentrations of hazardous substances in the environment (7). As part of As detoxification, plants produce metabolites, like nonprotein thiols (NPTs), cysteine, glutathione (GSH), or phytochelatins (PCs) synthesized from GSH, which are involved in the defense pathways of plants against As-induced oxidative stress (8). PCs have been identified in plants and some microorganisms (9). Phytoremediation of As-, Cd-, and Pb-contaminated soils is more cost-effective, more efficient, and less time-consuming than most other remediation technologies (10). Phytoremediation can reduce the available concentrations of inorganic compounds through different processes, such as phytoextraction, rhizofiltration, phytostabilization, or phytovolatilization (11, 12). Phytoextraction seeks to remove inorganic contaminants, especially heavy metals, metalloids, and radionuclides, from contaminated soils through uptake by plants and accumulation in harvestable plant biomass (13).

Microbial processes play a major role in As cycling in the plant-soil-microbe system, and effective phytoremediation of contaminated soils involves interactions with plant-associated microbes (14). Some bacterial mechanisms that enhance phytoremediation consist of plant growth by bacterial metabolites, such as indole-3-acetic acid (IAA), metal chelation by siderophores, and organic acid production, soil acidification, solubilization of metal phosphates, methylation, and moderation of heavy-metal stress by bacterial 1-amino-cyclopropane-1-carboxylic acid deaminase (ACCD) (15, 16). Rhizobacteria that colonize the vicinity of roots accelerate metal mobility and plant availability by various processes, including redox transformations and the release of protons and organic acids, whereas endophytic bacteria colonize the internal tissues of plants and promote plant growth through mechanisms such as phosphate solubilization, IAA and siderophore production, and/or supplying essential vitamins to plants (17, 18). In addition to their tolerance for heavy-metal stress, plant-associated bacteria can act as biocontrol agents against certain pathogenic organisms and ensure nitrogen fixation and the production of growth regulators (19).

In Asturias (northeastern Spain), several areas are contaminated with arsenic as a result of recent industrial (chemical, metallurgical, and siderurgical) dismantling. A representative example of a large-scale contaminated site is Nitrastur, a highly contaminated abandoned fertilizer industrial site located in the municipality of Langreo (20). The main waste found at this site that affects soil quality is pyrite ashes, composed largely of iron oxides and hydroxides, and other metal(loid)s that were produced as by-products of roasting sulfur ores. *Betula celtiberica* (Rothm. & Vasc.) is a deciduous tree, pseudometallophyte (21), and fast-growing high-biomass plant with well-developed root systems colonizing the study area. Birches are widely used in repopulations, and the genus *Betula* includes several pioneer species found in soils contaminated with heavy metals displaying high tolerance to Zn, Pb, and Cd (22,

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23). Its distribution is restricted; *Betula* was only found in mountain ranges in the Sistema Central (one of the largest mountain ranges on the Iberian Peninsula) (24). Thus, the autoecology of this species suggests that it might be a suitable candidate to bioremediate contaminated soils in Asturias.

Consequently, a question that arises when considering the role of As in the plant-bacterium relationship is how a natural mechanism, such as phytoremediation, can be enhanced in the autochthonous pseudometallophyte *Betula celtiberica*. Additionally, it is worth asking what the importance of the associated bacteria is in this process. Thus, the objectives of this study were to assess how the abundance, richness, community composition, and activities of *B. celtiberica*'s microbiome respond to long-term As contamination in the abandoned fertilizer industrial site, and subsequently, to evaluate the effect of bioaugmentation with indigenous endophytic and rhizospheric bacteria on As accumulation in *Betula*; leaves and roots were also studied. To achieve these goals, we analyzed the *Betula*-associated bacterial community through DNA-dependent approaches (pyrosequencing of 16S rRNA genes), we isolated and characterized cultivable root endophytic and rhizosphere strains capable of augmenting As phytoextraction, and finally, we tested their behavior *in vitro* and in field bioaugmentation experiments.

RESULTS

Rhizosphere soil characterization. The total As content of the rhizosphere soils varied from 865 to 3,349 mg · kg^{-1}, with a mean value of 1,900 mg · kg^{-1}, which was 9.5-fold higher than the Spanish regulatory limits for total As content in industrial soils (25). Furthermore, the samples also had Pb concentrations exceeding Spanish regulatory limits for industrial soils. Organic matter content was 9 to 16.36%, whereas the texture was found to be similar in all samples. In contrast, the pH of 6.01 to 6.97, in the vicinity of the roots, was relatively consistent among plants (Table S1).

Bacterial diversity of rhizosphere and endosphere communities associated with *Betula celtiberica* growing in Nitrastur. High-throughput 16S rRNA gene sequencing of the total community DNA was performed on the endophytic and rhizospheric fractions and was subsequently used to analyze cultivable bacteria. The raw sequence data from the samples consisted of 20,098 and 72,730 sequences from endosphere and rhizosphere, respectively. The number of operational taxonomic units (OTUs) based on a 97% similarity threshold was smaller in the endosphere than in the rhizosphere for all trees sampled (119 to 595 compared to 798 to 1,463, respectively; Table S2).

Among trees, microbial communities exhibited greater diversity in the rhizosphere (average number of observed OTUs, Chao1, Shannon index, and Simpson index were 1,068, 1,122, 7.5, and 0.96, respectively) than in the endosphere (average number of observed OTUs, Chao1, Shannon index, and Simpson index were 296, 322, 5.49, and 0.87, respectively). Bacterial diversity was higher in the rhizosphere than in the endosphere for the different alpha diversity indices (Fig. 1A). Rarefaction curves revealed that endophytic bacterial communities were less diverse than rhizosphere communities. In the current analysis, the asymptotic shape of the curves indicates that sequencing depth was sufficient to capture the entire bacterial diversity (Fig. 1B). In addition, microbial community diversity from the endosphere and rhizosphere, estimated with the Chao1 index, was negatively affected by the soil As content but correlated positively with soil pH (Fig. 1C).

From the classifiable sequences, the bacterial community composition was analyzed at three different taxon levels (phylum, order, and genus), considering abundant OTUs to be sequences present in more than one sample and representing more than 1% of the total sequence. Some sequences could not be assigned to any taxon at the phylum level (0.9 to 21.3% in the endosphere and 7.3 to 15.5% in the rhizosphere). At the phylum level, six bacterial phyla were overrepresented for both communities. *Proteobacteria* was the most abundant phylum (accounting for 64% of the total reads), followed by *Bacteroidetes* (17%), *Actinobacteria* (7.9%), *Firmicutes* (1.8%), and *Chlamydiae* (1.3%). *Proteobacteria* were represented by *Alphaproteobacteria*, *Betaproteobacteria*,

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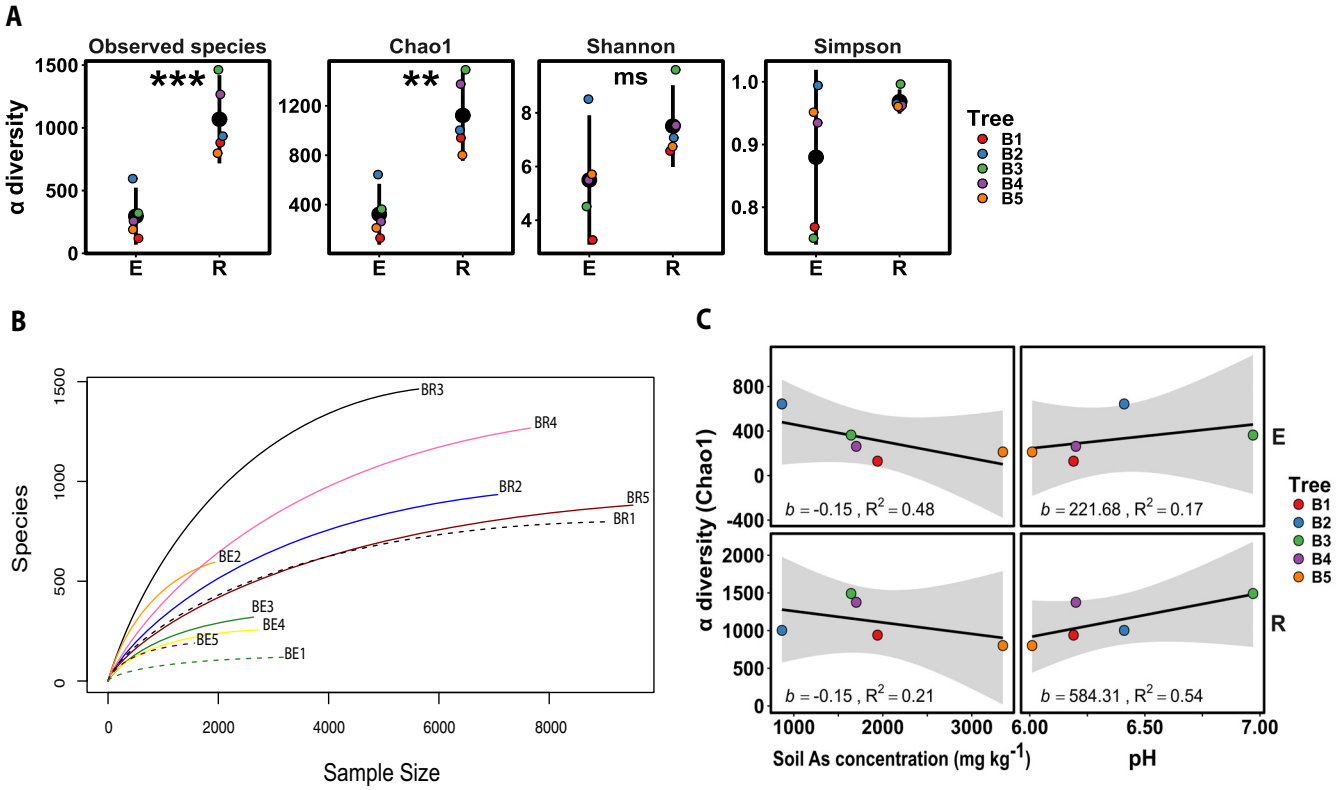

FIG 1 (A) Values of several alpha diversity metrics for the endosphere (E) and rhizosphere (R) microbial populations of five *B. celtiberica* trees (colorful dots) and their corresponding E and R means and 95% confidence intervals (solid black dots and vertical error bars, respectively). The significance levels of the difference between E and R means for each alpha metric resulting from ANOVA tests are also indicated (P = 0.050 to 0.100, 0.001 to 0.010, or 0.000 to 0.001 corresponding, respectively, to ms, which denotes marginally significant, **, or ***). (B) Rarefaction curves at the 3% distance cutoff microbial communities. (C) Relationship between microbial community diversity of E and R and As concentration or pH of the rhizosphere soils. The shaded areas represent the 95% confidence intervals of each linear relationship (black solid lines). The slope (b) and the R^2 (proportion of variance explained) of each relationship are also shown.

and *Gammaproteobacteria* (Fig. 2A). On closer inspection, some OTUs belonging to *Sphingobacteriales*, *Rhizobiales*, and *Xanthomonadales* orders and *Aquicella*, *Flavobacterium*, *Burkholderia*, and *Pseudomonas* genera were shared by both endosphere and rhizosphere communities. In the rhizosphere, *Pseudomonas* (37.07%), *Burkholderiales* (*Oxalobacteraceae* and *Xanthomonadaceae*, 9.62%), and *Flavobacterium* (7.64%) were the dominant OTUs. In the endosphere, *Flavobacterium* (14.99%), *Pseudomonas* (13.83%), and *Burkholderiales* (*Comamonadaceae* and *Oxalobacteraceae*, 11.39%) were the most frequently observed OTUs (Fig. 2B). Consistent with these observations, the nonparametric analyses of variance on 16S data confirmed the similarity of the rhizo- and endophytic communities (analysis of similarity [ANOSIM], $P = 0.20$, $R^2 = 15\%$; adonis, $P = 0.11$, $R^2 = 13\%$).

Isolation and identification of autochthonous cultivable bacteria associated with *Betula celtiberica*. To explore the diversity of cultivable root endophytes (E) and rhizobacteria (R) associated with *B. celtiberica*, isolation was performed on nonselective media. The number of CFU on tryptic soy agar (TSA) and 869 media varied in samples taken from different trees. The total numbers of cultivable endophytes and rhizosphere strains were $11.7 \times 10^4 \pm 3.16 \times 10^4$ CFU · g^{-1} of plant material (fresh weight) to $15.8 \times 10^7 \pm 6.2 \times 10^7$ CFU · g^{-1} of rhizosphere soil (fresh weight), respectively. Ninety-five bacterial strains were isolated as axenic cultures, with 41 endophytic strains and 54 rhizospheric strains. The phylogeny of the *B. celtiberica*-associated cultivable bacteria (based on the 16S rRNA genes) separated clearly into four strongly supported phyla: *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. Each of these phyla accounted for 7.3%, 7.3%, 36.6%, and 48.8% of endophytic strains and 38.9%, 13%, 20.3%, and 27.8% of rhizospheric strains, respectively (Fig. 3). Based on 16S rRNA gene

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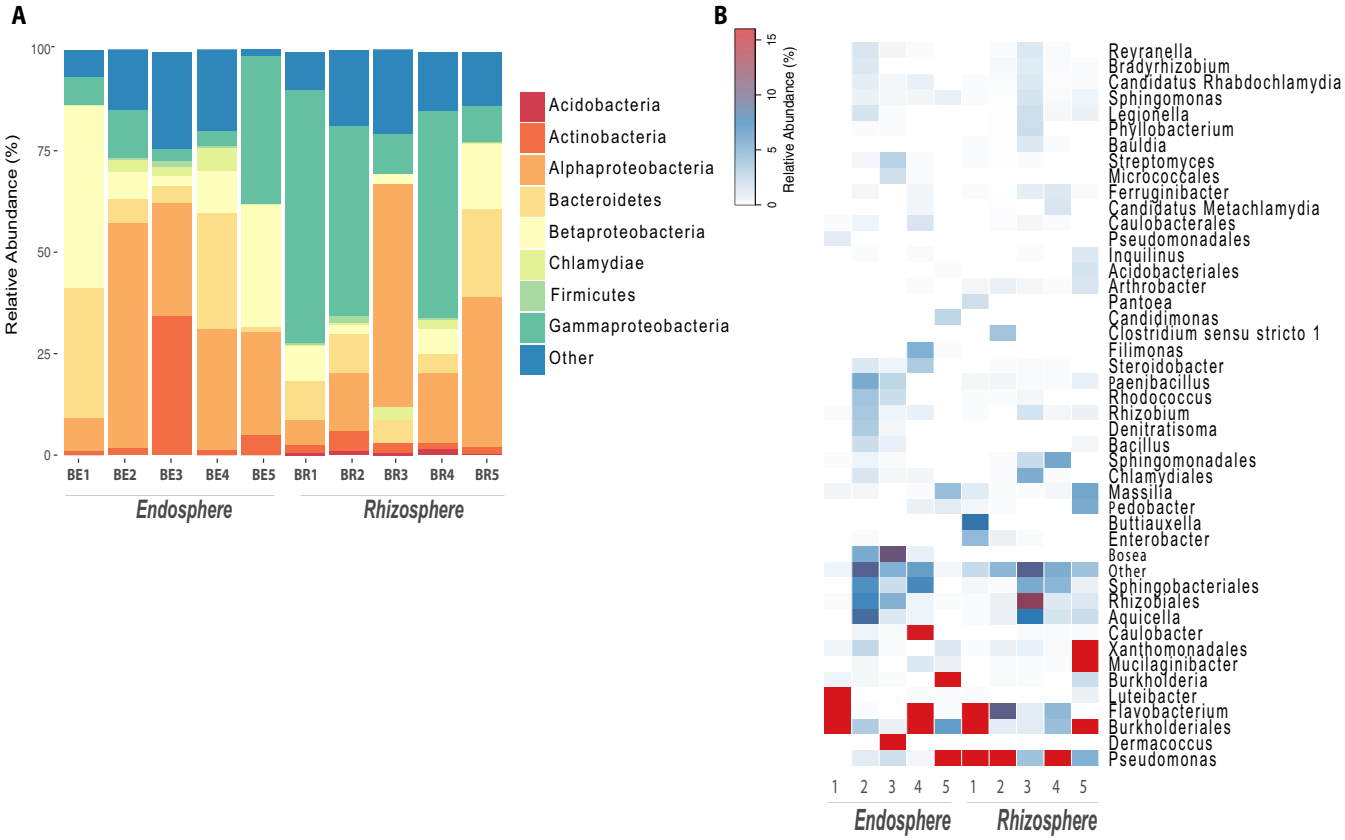

FIG 2 Microbial community analysis plots based on 16S rRNA gene pyrosequencing in endosphere and rhizosphere. (A) Bar chart showing the relative abundance of major bacterial phyla (over 1%). (B) Heatmap showing the distribution of bacterial order and genus level (over 1%).

sequences, redundant strains were eliminated, ending up with 39 endophytes and 46 rhizobacteria (Table S3). Strains from the endosphere belonged to 19 genera; among the most predominant were *Bacillus* (24%), *Rhizobium* (11%), and *Flavobacterium* and *Pseudomonas* (8% each), while rhizobacteria were principally *Streptomyces* (23%), *Bacillus* (17%), *Flavobacterium* (9%), and *Pseudomonas* (10%).

Characterization and selection of endosphere and rhizosphere isolates based on their IAA, ACCD, and siderophore production and As resistance. The isolated strains were evaluated for their plant growth-promoting traits and resistance to As. Isolates produced various amounts of IAA. Of the 39 endophytic isolates, 68% were able to produce more than 10 $\mu g \cdot ml^{-1}$ IAA, and 46 rhizobacteria (53%) were able to produce more than 10 $\mu g \cdot ml^{-1}$ IAA. High IAA production was exhibited by rhizospheric strain 91R (closely related to *Ensifer adhaerens* Sx1) (30.01 $\mu g \cdot ml^{-1}$) and root endophyte 89R (closely related to *Aminobacter aminovorans* LZ1304-3-1) (23.92 $\mu g \cdot ml^{-1}$). Another important plant growth-promoting (PGP) trait, ACCD production, was detected in 30% of rhizospheric and 5% of endophytic bacteria. Siderophore production was found in 32% of the endophytes and 36% of the rhizobacteria. The MIC of As(V) for 30.7% of the endophytic strains and 34% of the rhizobacteria was 100 mM As(V), while 10% of the endophytes and 8.7% of the rhizobacteria grew up to 50 mM As(V). More than one in five (20.5%) of the endophytes were sensitive to the lowest As(V) concentration, while 6.5% of the rhizobacteria were sensitive to As. The As(III) MIC was 20 mM for 12.8% of the endophytes and 6.5% of the rhizobacteria, but 15% of the endophytes and 13% of the rhizobacteria were able to grow between 10 and 15 mM As(III). Table S3 illustrates that 30.7% and 41% of the endophytes and rhizobacteria were sensitive to the lowest As(III) concentration, respectively.

Seven isolates revealing resistance to high concentrations of As and/or potential promotion of growth by at least one PGP trait were selected (Table 1). Five bacteria

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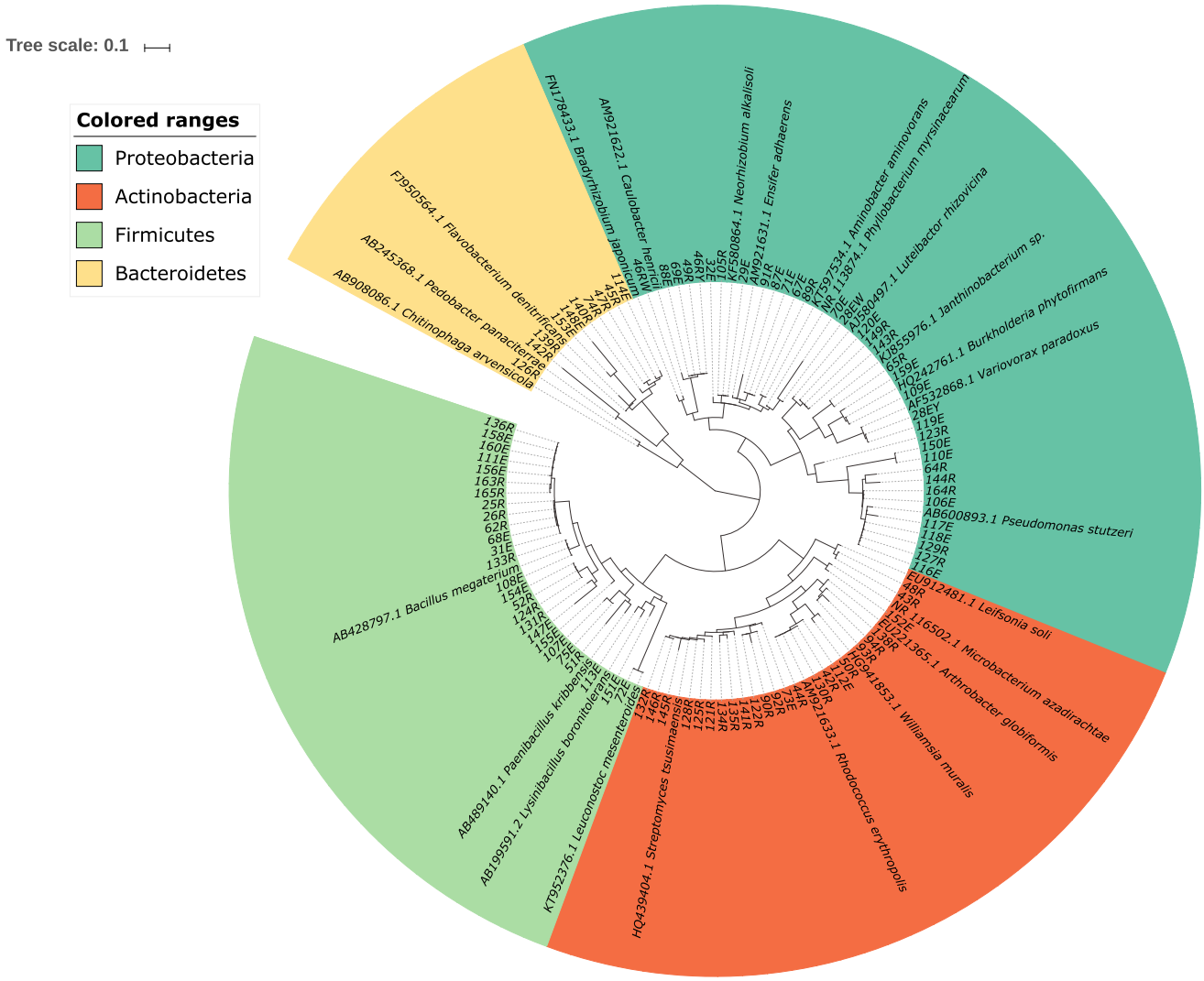

FIG 3 Phylogenetic correlations of endophytic and rhizospheric strains isolated from *B. celtiberica* trees (endosphere, $n = 41$; rhizosphere, $n = 54$). Neighbor-joining tree inferred using MEGA version 6.0. Species associated with rhizosphere and roots were used as reference strains; the numbers to the left of the organism names are their accession numbers. The scale bars indicate the number of substitutions per nucleotide, 323 substitutions per site.

were tested separately, and two endophytic bacteria were tested in a consortium (28EY and 28EW) after evaluating their compatibility by cultivating the strains together on asparagine-yeast extract (GAE) medium and plating serial dilutions of the culture. All selected bacteria were resistant to high concentrations of As(V) (100 mM), whereas only

TABLE 1 Bacterial strains selected for inoculation in *in vitro* culture[a]

Strain[b]	Most closely related species	MIC (mM) for: As(V)	MIC (mM) for: As(III)	IAA (± SD) ($\mu g \cdot ml^{-1}$)	SID[c]	ACCD[d]
29E	*Neorhizobium alkalisoli* ZY-4s	>100	<2	13.26 (± 0.38)	++	−
32E	*Rhizobium herbae* CCBAU 83011	>100	<2	13.06 (± 0.43)	++	−
28EY	*Variovorax paradoxus* S110	>100	<2	10.29 (± 0.51)	+	−
28EW	*Phyllobacterium myrsinacearum* NBRC 100019	>100	2	13.77 (± 0.22)	+	−
44R	*Rhodococcus erythropolis* TS-TYKAKK-12	>100	20	12.39 (± 0.36)	++	++
89R	*Aminobacter aminovorans* LZ1304-3-1	>100	<2	23.92 (± 0.91)	++	++
91R	*Ensifer adhaerens* Sx1	>100	5	30.01 (± 0.82)	++	+

[a]Phylogenetic affiliations are based on sequence analysis of about 800 bp of 16S rRNA genes.
[b]The letters of the strains indicate origin: E, endosphere; R, rhizosphere.
[c]Siderophore production (determined qualitatively as color intensity after the reaction [see the supplemental material]).
[d]1-Amino-cyclopropane-1-carboxylic acid deaminase (ACCD) activity (++, strains with the maximum rate of reaction; +, strains with less than the maximum rate of reaction).

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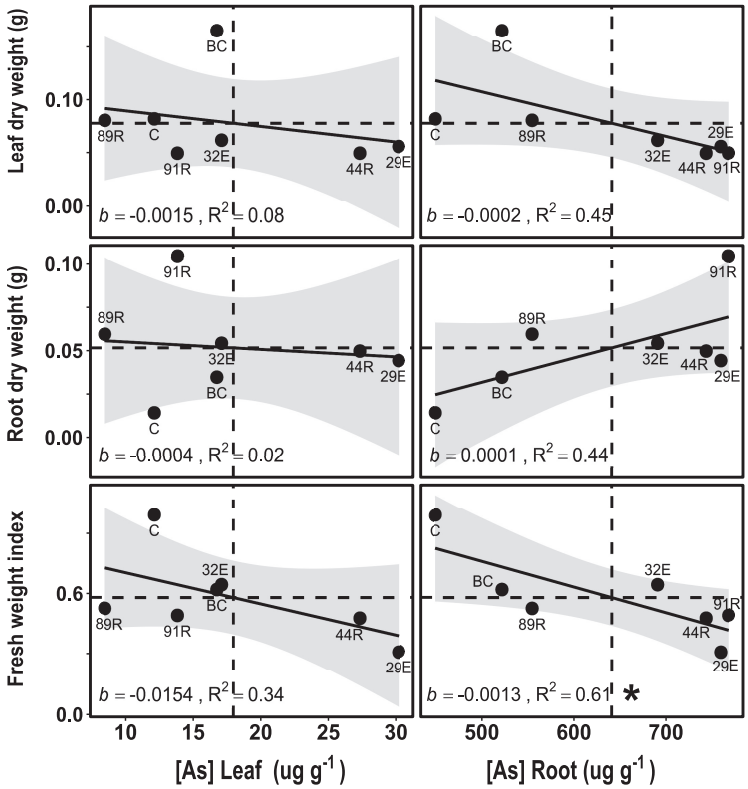

FIG 4 *In vitro* evaluation under arsenic (150 μM) exposure. Relationship between mean leaf and root biomass (grams [dry weight]) ($n = 3$) or mean plant fresh weight index (FWI) of *B. celtiberica* plants ($n = 6$) and their mean leaf or root arsenic concentration (micrograms per gram [dry weight], $n = 3$). Dots indicate whether mean values correspond to noninoculated (control [C]) or inoculated (*Neorhizobium* sp. strain 29E, *Rhizobium herbae* strain 32E, *Rhodococcus erythropolis* strain 44R, *Aminobacter aminovorans* strain 89R, *Ensifer adhaerens* strain 91R, or bacterial consortium [BC], which includes *Variovorax paradoxus* strain 28EY and *Phyllobacterium myrsinacearum* strain 28EW) *B. celtiberica* plants. The shaded areas represent the 95% confidence intervals of the linear relationships (black solid lines). The dashed lines mark the mean for each variable. The slope (b) and the R^2 (proportion of variance explained) of the linear correlations are also shown. Significant relationships ($P = 0.01$ to 0.05) are denoted with *.

one, strain 44R (closely related to *Rhodococcus erythropolis*), was resistant to a high concentration of As(III) (20 mM). In addition, endophytic strains 29E, 32E, 28EY, and 28EW (closely related to *Neorhizobium alkalisoli*, *Rhizobium herbae*, *Variovorax paradoxus*, and *Phyllobacterium myrsinacearum*, respectively) produced siderophore and IAA. Rhizosphere strains 44R and 89R (closely related to *Rhodococcus erythropolis* and *Aminobacter aminovorans*, respectively) and 91R (closely related to *Ensifer adhaerens*) produced siderophore, IAA, and ACC deaminase.

***In vitro* evaluation of arsenic uptake by noninoculated and inoculated *Betula celtiberica* plants.** The seven selected strains mentioned above were used to inoculate *B. celtiberica* plants. Plant performance was evaluated under *in vitro* cultivation ($n = 6$ per treatment). After 30 days, no visible symptoms of As toxicity were observed in any *B. celtiberica* plants; however, As affected plant biomass ($P < 0.05$) (Table S6). Regarding As accumulation in plant tissues, root As concentration was higher and had a greater effect on leaf and root biomass than leaf As concentration, which showed almost no effect (Fig. 4). Moreover, the effect of root As concentration was negative on leaf biomass but positive on root biomass. Leaf and root As accumulation negatively impacted the fresh weight index (FWI), although root As concentration exerted a more pronounced effect. Figure 4 also illustrates that only *B. celtiberica* clones inoculated with *Ensifer adhaerens* strain 91R and *Rhizobium herbae* strain 32E showed both root As concentration and root biomass above the mean. Furthermore, plants inoculated with the *Rhizobium herbae* strain 32E were the only ones with root As concentration and FWI above the mean.

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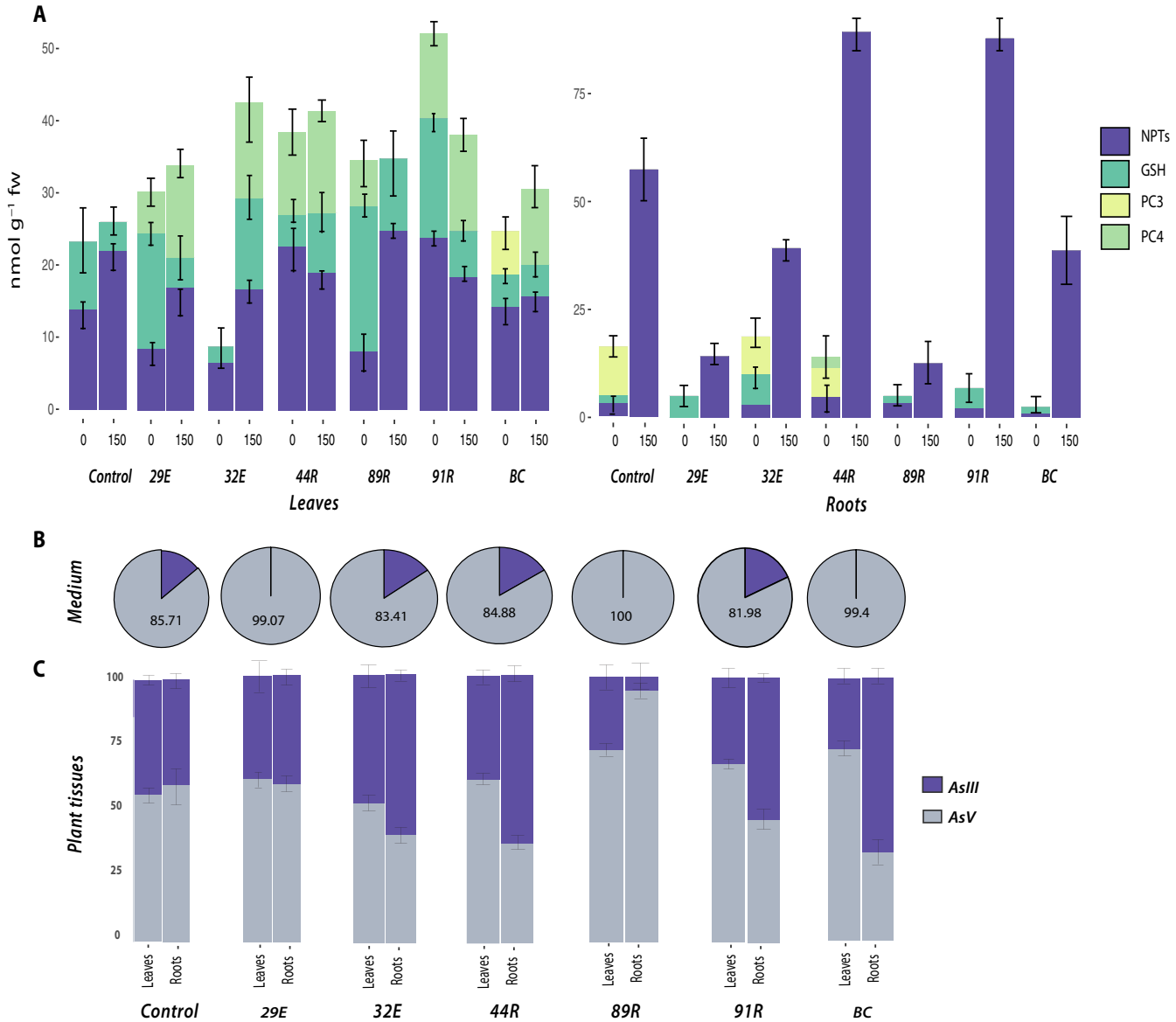

FIG 5 (A to C) Effect of As(V) (150 μM) on NPTs, GSH, and PCs (A) and percentages of As(V) and As(III) found in medium (B) and different parts of the plant leaves and roots (C) by ICP-MS. Treatments: noninoculated (control) or inoculated *B. celtiberica* plants (*Neorhizobium alkalisoli* strain 29E, *Rhizobium herbae* strain 32E, *Rhodococcus erythropolis* strain 44R, *Aminobacter aminovorans* strain 89R, *Ensifer adhaerens* strain 91R, or bacterial consortium [BC] with *Variovorax paradoxus* strain 28EY and *Phyllobacterium myrsinacearum* strain 28EW). Values in bar graphs are reported as the means ± SD.

An estimation of total NPT content would aid in analyzing its hypothetical role in As toxicity mitigation (Fig. 5). Different NPT analyses in the absence of arsenic quantified higher concentrations of NPTs in leaves than in roots in the different treatments (represented as ratio of leaf to root NPTs [L/R]) (Table S4). The exception to this was plants inoculated with *Rhizobium herbae* strain 32E, the only treatment yielding an L/R ratio of <1, with the particularity of higher GSH content in roots than in leaves. Exposed to arsenic, NPT concentrations were higher in roots than in leaves; hence, the ratio was reversed (L/R $<$ 1). *Neorhizobium* sp. strain 29E and *Aminobacter aminovorans* strain 89R treatments are the exceptions to this trend and displayed higher contents in leaves than roots.

Overall, in the presence of arsenic, both leaves and roots had increased total content of NPTs, except in the leaves of *Rhodococcus erythropolis* strain 44R- and *Ensifer adhaerens* strain 91R-treated plants (Fig. 5A). With these treatments, leaf NPT contents

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decreased slightly compared to that in noninoculated plants (C), even though they had higher levels of phytochelatin 4 (PC_4). *De novo* PC synthesis was found in roots treated with *Rhizobium herbae* strain 32E (8.62 nmol · g^{-1} [fresh weight {fw}]) and *Rhodococcus erythropolis* strain 44R (9.06 nmol · g^{-1} [fw]) under As exposure. In leaves, PCs were also detected after inoculation with the bacterial consortium (BC; *Variovorax paradoxus* strain 28EY and *Phyllobacterium myrsinacearum* strain 28EW) (10.54 nmol · g^{-1} [fw]), *Rhizobium herbae* strain 32E (13.21 nmol · g^{-1} [fw]), *Neorhizobium* sp. strain 29E (12.82 nmol · g^{-1} [fw]), *Rhodococcus erythropolis* strain 44R (14.13 nmol · g^{-1} [fw]), and *Ensifer adhaerens* strain 91R (13.30 nmol · g^{-1} [fw]).

The As speciation analysis in the culture medium (Fig. 5B) enables us to conclude that *B. celtiberica* metabolism was responsible for some 20% of the reduction of As(V) to As(III) present in the medium. Bacterial inocula with *Neorhizobium alkalisoli* strain 29E, *Aminobacter aminovorans* strain 89R, or *Ensifer adhaerens* strain 91R would thereby oxidize this 20% of As(III) generated by plants, resulting in about 100% As(V) in the medium. No further reduction was seen with any of the strains under these culture conditions. In plant tissues (Fig. 5C), inoculation with *Aminobacter aminovorans* strain 89R resulted in As(III) oxidation of 16% in the leaves and 35% in the roots versus controls. Furthermore, treatment with *Ensifer adhaerens* strain 91R led to 11% of the As(III) oxidation in leaves and 13% of the As(V) reduction in roots. The BC treatment oxidized 17% of the As(III) in leaves and reduced 26% of the As(V) in roots. Finally, As(III) levels in roots were higher after inoculation with *Ensifer adhaerens* strain 91R, *Rhodococcus erythropolis* 44R, *Rhizobium herbae* strain 32E, and with BC; these treatments were also characterized by high NPT content.

Field-scale evaluation of arsenic uptake by noninoculated and inoculated *Betula celtiberica* plants. Bacterial strains were chosen for field testing based on *in vitro* As uptake and physiological traits. Another important feature was their ability to grow in the laboratory media, since high biomass is a factor of great practical relevance for field-scale bioaugmentation experiments. Four strains were selected: the siderophore and IAA- and ACC deaminase-producing *Ensifer adhaerens* strain 91R (As uptake in roots, >700 $\mu g \cdot g^{-1}$ [dry weight {dw}]; As uptake in leaves, >10 $\mu g \cdot g^{-1}$ [dw]) (treatment R), the siderophore and IAA-producing *Rhizobium herbae* strain 32E (As uptake in roots, >600 $\mu g \cdot g^{-1}$ [dw]; As uptake in leaves, >15 $\mu g \cdot g^{-1}$ [dw]) (treatment E), and the endophytic BC that included the siderophore and IAA-producing strains *Variovorax paradoxus* strain 28EY and *Phyllobacterium myrsinacearum* strain 28EW (As uptake in roots, >500 $\mu g \cdot g^{-1}$ [dw]; As uptake in leaves, >15 $\mu g \cdot g^{-1}$ [dw]). Soil fertilization was also evaluated in the field for the BC inoculation (BC+F) and for the control (FC). Soil sample analyses performed prior to the experiment on the different phytoextraction field subplots detected a wide range of pH values (2.56 to 6.04), As levels (1,219 to 3,034 mg · kg^{-1}), and Zn and Pb levels (Table S5). Inoculations affected *B. celtiberica* plant heights and shoot biomass ($P < 0.05$) (Table S6) in the field after 90 days of cultivation. In general, soil conditions, such as As concentration and pH, in the different plots might account for a large proportion of the variance in As concentrations in roots and leaves, shoot biomass, and plant height index (PHI) (Fig. 6). Correlations between variables can be positive or negative, according to independent variables (soil As concentration or pH) and the type of dependent variable (As concentration in leaves and roots, shoot biomass, or PHI). However, the effect of soil As and pH varies from one variable to the next; while root and leaf As contents were positively and negatively correlated with soil As concentration or pH, respectively, the opposite occurred for shoot biomass and PHI.

A model selection showed that both soil As content and pH must be considered when accounting for the effect of soil variability among plots on leaf and root As concentration, shoot biomass, and PHI. In fact, a model using soil As and pH as independent variables resulted in the lowest Akaike information criterion (AIC), or was at least fairly similar to that in the other candidate models (i.e., ΔAIC, <2, Table S7; more details about the statistical analysis in Materials and Methods). The residuals from this model for leaf As concentration were uncorrelated with those

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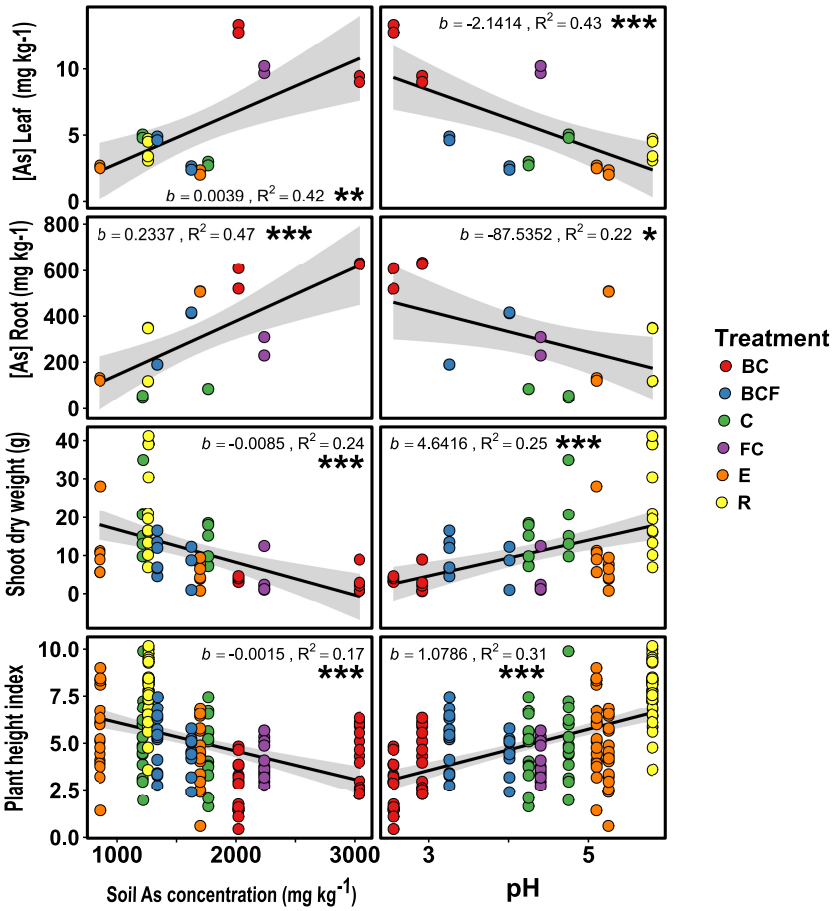

FIG 6 Field evaluation. Correlation between leaf and root As concentration (milligrams per kilogram [dry weight]), shoot biomass (grams [dry weight]), or PHI of *B. celtiberica* plants and their soil As concentration (milligrams per kilogram) or pH of their corresponding plot. Dots indicate whether plants were inoculated (E, with *Rhizobium herbae* strain 32E; R, with *Ensifer adhaerens* strain 91R; BC, with the bacterial consortium of *Variovorax paradoxus* strain 28EY and *Phyllobacterium myrsinacearum* strain 28EW; BCF, with BC plus fertilizer) or not (C and FC for control and fertilized control, respectively). The shaded areas represent 95% confidence intervals of the linear correlations (black solid lines). The slope (*b*) and the R^2 (proportion of variance explained) of each regression are also shown. The significance level of the correlations is also indicated (*P* value = 0.010 to 0.050, 0.001 to 0.010, or 0.000 to 0.001 correspond to *, **, or ***, respectively).

from shoot biomass and PHI (Fig. S2). However, the shoot biomass and PHI residuals correlated somewhat negatively with the root As concentration residuals. This coincides with those from the *in vitro* experiments, where root As concentration also impacted plant growth more than leaf As concentration. We also observed that only those *B. celtiberica* clones inoculated with *Ensifer adhaerens* strain 91R (R) and BC showed positive residual means for PHI, shoot biomass, and root As concentration (Fig. S2A and B).

The indices involved in plant–trace-metal interactions (bioconcentration factor [BCF], mobility ratio [MR], and translocation factor [TF]; Table S8) and determination of indices involved in As phytoextraction efficiency in the supplemental material were all lower than 1. Arsenic BCF was increased in plants inoculated with BC compared to noninoculated plants. Likewise, the mobility ratio (MR) was also increased in plants inoculated with the bacterial consortium (BC), with an average value of 0.009. In general, a mobility ratio of <1 indicates that the plants exclude As or have a low uptake rate of this metalloid. BCF and MR rose 10% and 33%, respectively, in plants inoculated with BC with respect to noninoculated plants. TF exhibited low translocation rates of As from roots to leaves.

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DISCUSSION

Microorganisms and plants have developed several constitutive or adaptive mechanisms to cope with As enabling them to resist and metabolize it. Phytoextraction has emerged as an alternative remediation approach to restore contaminated sites (10). Moreover, the use of common native plants appears to be a good approach to guarantee phytoextraction success, since it limits plant competition and ensures autoecological plant requirements (26). In this context, the recovery of pseudometallophyte-associated bacteria adapted to a large historically contaminated site may improve phytoremediation through bioaugmentation technologies (27). In Asturias, the location of this study, the endemic *B. celtiberica* prospers in numerous places and has naturally colonized the Nitrastur industrial area. On this site, *B. celtiberica* trees grow in soils containing various amounts of As (up to 3,349 mg · kg^{-1}). This, together with birch autoecology, makes this species an ideal candidate to investigate its phytoextraction capabilities. Our approaches include analyzing the *B. celtiberica* endosphere and rhizosphere microbiome via culture-independent and culture-dependent techniques as a first step to later proceed to field application using these bacteria in bioaugmentation-assisted phytoextraction of *B. celtiberica* clones.

Culture-independent techniques indicated that *B. celtiberica*'s microbiome was dominated by *Bacteroidetes*, *Betaproteobacteria*, and *Gammaproteobacteria*, represented by the taxa related to *Flavobacterium*, *Burkholderiales*, and *Pseudomonas*. At the genus level, *Pseudomonas* displays the most promising levels of colonization and ability to persist in the endophytic niche, probably due to its wide spectrum of fatty acid and carbon source utilization (18). Predominant bacterial strains in the rhizosphere are Gram negative, and *Pseudomonas* and *Flavobacterium* are among the most represented genera. This may be attributed to the efficiency of Gram-negative bacteria to utilize the root exudates and organic compounds released by living plant roots into their surrounding environment (28, 29). The number of OTUs in the endophytic bacterial communities (296 ± 183 OTUs) was much lower than in the rhizosphere (1,068 ± 283 OTUs); similar results have been described for mature poplar trees growing in natural ecosystems (30). Interestingly, root endophytic and rhizospheric communities differ in richness estimators and diversity indices, while they do not differ in community composition. The two communities share many bacterial species, and the nonparametric analyses of variance on 16S data bore out these similarities. Regarding the impact of microbial diversity in As-contaminated soils, our results show that the diversity of the rhizosphere and endosphere microbial communities negatively correlated with As soil contents. Hence, arsenic affected microbial diversity, not only of the rhizospheric fraction, but also of the endophytic fraction of roots, as previously noted by Hu et al. (31). Likewise, soil pH appeared also to be an important factor impacting the structure of the bacterial community in Nitrastur soils. At lower pH, lower microbial diversity was reported in different environmental contexts (32, 33).

Cultured bacteria were predominantly represented by *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Alphaproteobacteria*, and *Gammaproteobacteria*, among which the main genera were *Streptomyces*, *Flavobacterium*, *Bacillus*, *Rhizobium*, and *Pseudomonas*. Many endophytes are members of common soil bacterial genera, such as *Pseudomonas* and *Bacillus*. *Rhizobium*, *Pseudomonas*, and *Bacillus* have been reported to be typical rhizobacteria (18, 28). The composition of the cultivable bacterial community of *B. celtiberica* at the phylum level appears to be similar to the root microbiome of other plants (17, 34, 35). Although more strains were present in the total communities than in the cultivable bacterial community, the cultivated bacterial strains gave a good overview of the most dominant genera present in *B. celtiberica* plants. However, *Actinobacteria* and *Firmicutes* were more represented in our culture-dependent isolation method than in the pyrosequencing surveys. As expected, the population density of cultivable bacteria was considerably higher in the rhizosphere than in the endosphere recovered from root tissues (36). Rhizosphere soils are indeed rich environments, due to nutrients exuded from the roots of most plants which are accustomed to supporting bacterial growth

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and metabolism. In addition, rhizosphere soils are described as mesotrophic, favoring suitable conditions for microbial growth (12, 37). As in previous reports (17), we found the ability to produce IAA mainly among endophytes, whereas siderophore and ACCD production was more common among rhizobacteria, which were also the most resistant to high concentrations of arsenate and arsenite.

The selection criteria for the most promising strains to improve phytoextraction efficiency were As resistance, suppression of stress ethylene production (due to ACC deaminase activity), stimulation in plant growth and biomass, phytohormone (such as IAA) production, or improvement in plant nutrition, due to the presence of siderophore producers (38). Considering all the properties studied, the strains selected were those closely related to *Neorhizobium alkalisoli* ZY-4s, *Rhizobium herbae* CCBAU 83011, *Rhodococcus erythropolis* TS-TYKAKK-12, *Aminobacter aminovorans* LZ1304-3-1, and *Ensifer adhaerens* Sx1. Moreover, strains that were highly similar to *Variovorax paradoxus* S110 and *Phyllobacterium myrsinacearum* NBRC 100019 were also tested in a consortium. In an initial phase, bioaugmentation was performed under sterile conditions (*in vitro* experiment) to exclusively determine the effect of the inoculated strain. The *in vitro* inoculations of *B. celtiberica* clones showed a positive effect of the siderophore-, IAA-, and ACCD-producing *Ensifer adhaerens* strain 91R and the siderophore- and IAA-producing *Rhizobium herbae* strain 32E on growth and root As concentration.

To minimize the toxic effects of metals accumulated inside the plant, internal tolerance mechanisms include (i) sequestration of the trace elements, i.e., transport to cell components not involved in physiological processes (vacuole and cell wall), and (ii) complexation with metal-binding peptides, i.e., metallothioneins and PCs (38). PCs are cysteine-rich peptides synthesized enzymatically from glutathione (GSH) by PC synthase under metal exposure (9). From the analysis of components involved in thiol metabolism (total NPTs), the *in vitro* evaluation showed that the concentration of GSH in roots with respect to leaves was reduced to undetectable levels, probably due to their involvement in the synthesis of long-chain compounds responsible for As detoxification (39). Under exposure to arsenic, the content of NPTs in leaves of plants inoculated with *Rhodococcus erythropolis* strain 44R or *Ensifer adhaerens* strain 91R decreased in comparison to noninoculated plants (C) but showed increased levels of phytochelatin 4 (PC_4). This might point to a defense mechanism for As chelation by PCs. Additionally, we found that As exposure caused increased NPT content in roots, suggesting that As was complexed in the roots through enhanced synthesis of NPTs (39). Among the conditions with high As accumulation in roots are inoculations with *Rhodococcus erythropolis* 44R (750 mg · kg^{-1}) and with *Ensifer adhaerens* 91R (up to 790 mg · kg^{-1}). Likewise, these conditions showed the highest NPT values in roots, at 89 and 87 nmol · g^{-1} (fw) for the two inoculations, respectively.

Arsenic accumulation in roots correlated positively with NPT content, indicating that As, in the form of As(V) introduced in the culture media, was complexed in roots through enhanced synthesis of NPTs. Indeed, we found a positive relation between NPT and As(III) concentration in roots of the treatments inoculated with *Ensifer adhaerens* strain 91R, *Rhodococcus erythropolis* 44R, *Rhizobium herbae* strain 32E, and BC. It has been demonstrated that the presence of As(III) as a dominant As species indicates the prevalence of PC-based As detoxification mechanism in plants (40).

In a next step toward a field trial, species of *Ensifer*, *Rhizobium*, *Variovorax*, and *Phyllobacterium* taxa were selected, based on *in vitro* evaluation results. Interestingly, previous studies indicated that these strains exhibit traits of interest for phytoextraction. Thus, species of the *Ensifer* genus were found in association with *Medicago* plants, where inoculated plants exhibited a reduction in the stress response, thereby indicating the protective effect of As-tolerant rhizobia (41). It has been demonstrated that *Rhizobium* species promoted plant growth in *Lolium multiflorum* (Lam.) and Cd uptake in *Glycine max* (L.) Merr. (42). Furthermore, a *Variovorax strain* with siderophore- and IAA-producing traits was found in the rhizosphere of *Pteris vittata*, where it contributed to increased arsenic accumulation in fronds upon inoculation (14). Additionally, *Phyl-*

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lobacterium has been described as a plant growth-promoting bacterium (PGPB) that stimulates *Brassica napus* root morphogenesis (43).

In the field trials conducted at the Nitrastur site, the plants inoculated with the endophytic BC (composed of the siderophore- and IAA-producing strains *Variovorax paradoxus* strain 28EY and *Phyllobacterium myrsinacearum* strain 28EW) achieved high As uptake (mean ± standard deviation [SD], 11 ± 2 mg · kg^{-1} in leaves and 595 ± 52 mg · kg^{-1} in roots). The effect of the fertilizer was reflected by an increase in shoot biomass, and the treatment supplemented with fertilizer (BC+F) increased shoot biomass (mean ± SD, 9 ± 4 g [dw]) compared to nonfertilized plants inoculated with BC (mean ± SD, 3.45 ± 2 g [dw]).

Usually, the highest BCF levels are observed in soils with decreased contamination; however, BCFs also vary depending on the metal and plant species, irrespective of bioaugmentation (15). For As, the translocation from roots to shoots in most plant species is generally not very effective, supporting the high As concentrations found in roots and low As translocation by *B. celtiberica* (40). We can hypothesize that arsenate in the roots is rapidly reduced to arsenite, complexed to PCs, and subsequently sequestered in root vacuoles, thus limiting its translocation to the leaves (44).

Furthermore, the amounts of metals extracted by plants from the soil, as well as their extraction performance, are directly related to bioavailability, which in turn was influenced by soil characteristics, such as pH (15). In our study, soil conditions for BC treatment were pH 2.74 and 2,527 mg · kg^{-1} As (mean ± SD As uptake in leaves of 11.11 ± 2.21 g [dw] and in roots of 595.15 ± 52 g [dw]), while soil characteristics for the BC+F treatment were pH 3.64 and 1,484 mg · kg^{-1} As (mean ± SD As uptake in leaves of 3.65 ± 1.29 g [dw] and in roots of 302.18 ± 78 g [dw]) (Fig. 6). Thus, As uptake might be influenced by soil conditions. The effect on As accumulation in the BC inoculation would be expected, given the lower pH (38). However, the statistical model without the effect of the pH variable (Fig. S2) suggests that pH was not the sole factor that determines the accumulation, and we propose that the bacterial inoculation had a decisive influence. Consequently, our data highlight the difficulty in conducting bioaugmentation experiments under field conditions and underline the necessity to take into account all the environmental variables and their subsequent consideration in the interpretation of the final results (45). This also applies to the effects on plant growth of rhizophytic *Ensifer* spp. upon metal exposure. We observed an increase of some 1.5-fold in plant height in the presence of *Ensifer adhaerens* strain 91R compared to noninoculated plants; nonetheless, the control pH and assay plots with the bacterium were more similar (Fig. 6). These findings are a potent indicator of the indigenous rhizospheric bacterium's ability to promote plant growth, which is consistent with its capacity to produce high levels of IAA, the highest production among all isolates.

In this study, bacterial responses to long-term As contamination were investigated in an abandoned industrial area. The arsenic content and pH of soils affected the diversity of the entire bacterial community. The effect of plant inoculation with *Rhodococcus erythropolis* and *Ensifer adhaerens* tested *in vitro* caused an increase in total NPT content in roots, suggesting a detoxification mechanism through phytochelatin complexation. Likewise, bacterial inoculation affected the As speciation in plants. The field trials suggested that inoculation with *Ensifer adhaerens* can increase plant growth and that inoculation with the consortium consisting of *Variovorax paradoxus* and *Phyllobacterium myrsinacearum* enhances As accumulation in roots. Extraction performance is directly related to and influenced by soil characteristics, such as pH. From these results, we conclude that the inoculation of plants with indigenous bacteria exhibiting As resistance, producing growth-promoting factors, and having the capacity to reduce As(V) to As(III), thereby facilitating As detoxification, can improve As phytoextraction efficiency by pseudometallophyte species, such as those of *Betula*. Additionally, the use of autochthonous plants and indigenous bacteria alleviated the autoecological requirement of both partners, ensuring successful plant establishment and site remediation. This approach appears to be particularly useful for metal(loid)-contaminated sites produced as a by-product of roasting sulfur ores at the Nitrastur industrial site researched in this investigation.

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MATERIALS AND METHODS

Plant material, rhizosphere soil sampling, and chemical analysis. Details of the contaminated site are given in the supplemental material. Five *B. celtiberica* trees growing on contaminated sites in NC5 and NC6 plots of the Nitrastur industrial area (Fig. S1) were sampled in March 2013. Root samples were randomly collected from each tree. and adherent rhizosphere soils were pooled from around each tree. Rhizosphere-containing soil, defined as soil in the immediate vicinity of the roots, was obtained by shaking the roots, thereby collecting the soil that had been attached to the roots. Soils were air-dried and sieved to a grain size of up to 2 mm. A 250-mg representative of each sample was digested in a microwave (Multiwave 3000; Anton Paar) with *aqua regia* at 800 W for 15 min. The solutions were diluted to 50 ml with ultrapure water and filtered through a 0.45-μm-pore-size polytetrafluoroethylene (PTFE) filter prior to analysis. As, Cd, Zn, Cr, Ni, Cu, Pb, and Hg were quantified using an inductively coupled plasma mass spectrometer (ICP-MS; Agilent Technologies 7700 ICP-MS) using isotopic dilution analysis (IDA), as previously described (46).

Collection of rhizospheric and endophytic fractions and isolation of cultivable *B. celtiberica*-associated bacteria. To obtain rhizospheric fractions, 5 g of rhizosphere soils was shaken in 10 ml of 0.1% sodium pyrophosphate (Sigma-Aldrich, USA). Soil particles were allowed to settle for 1 h. The supernatants were diluted to 10^{-8} and plated on two different media, 1:10-diluted tryptic soy agar (TSA) (47) and 1:10-diluted 869 solid medium (48). The plates were incubated for 7 days at 30°C. The remaining supernatants were stored at −80°C until DNA extraction. To obtain the endophytic fraction, roots were rinsed under running tap water. Root samples were surface sterilized for 10 min in 2% active chloride solution supplemented with one droplet of Tween 80 (Merck, Germany) per 100 ml of solution and subsequently rinsed three times for 1 min in sterile distilled water. After surface sterilization, root samples were macerated in 10 ml of 10 mM $MgSO_4$ with a mortar. To isolate endophytes, the extracts obtained from the macerated roots were serially diluted to 10^{-5}, plated on the same medium, and incubated. Colonies with different morphotypes were selected and repeatedly streaked until axenic cultures were obtained (49). The identification and phylogenetic affiliation of cultivable *B. celtiberica*-associated bacteria are detailed in the supplemental material (section "Identification and phylogenetic affiliation of cultivable *B. celtiberica*-associated bacteria"). Methods of characterization of endosphere and rhizosphere isolates based on the production of indole-3-acetic acid (IAA), 1-amino-cyclopropane-1-carboxylic acid deaminase (ACCD) activity, siderophores, and As resistance are included in "Characterization of cultivable isolates" in the supplemental material.

DNA extraction and 454 pyrosequencing. DNA samples were obtained from the 10 rhizospheric and endophytic fractions using the PowerSoil DNA isolation kit (Mo Bio, USA). The V5 to V7 region of the 16S rRNA gene was amplified using the primer set 799F (5′-AACMGGATTAGATACCCKG-3′) (50) and 1391R (5′-GACGGGCGGTGWGTRCA-3′) (51). Each 25-μl PCR mixture contained ~10 ng of DNA and PCR was performed using the FastStart high-fidelity PCR system (Roche Applied Science, Mannheim, Germany), according to the instructions provided by the manufacturer. Cycling conditions included initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min; a final extension phase was conducted at 72°C for 10 min. PCR amplicon pools were cleared from residual primers and primer dimers by separating the PCR products on a 1.5% agarose gel. Bacterial amplicons were excised from the gels using the QIAquick gel extraction kit (Qiagen Benelux N.V., The Netherlands). The amplicon length of sequences produced by primer set 799F-1391R was reduced by amplifying the samples with primer set 967F (5′-CAACGCGAAGAA CCTTACC-3′) (52) and 1391R in a second round. The forward primer was fused to the Roche 454 pyrosequencing adaptor A and a sample-specific 10-bp barcode (multiplex identifiers [MIDs]), and the reverse primer was fused to adaptor B (Roche Applied Science, Germany). The PCR cycling conditions were identical to the ones previously described, with the exception of the number of PCR cycles, which was lowered to 25. Sequencing was carried out on one-eighth of a picotiter plate on a Roche Genome Sequencer FLX+ using Titanium chemistry (Roche Applied Science, Germany) by LGC Genomics (Germany).

Sequencing data analysis. Sequences were analyzed using the Quantitative Insights into Microbial Ecology (QIIME) package (53). First, reads were assigned to samples based on their nucleotide barcode. This step also performed quality filtering (minimum quality score of 30, eliminating reads with lengths >200 bp and 0 ambiguous bases and mismatches in primer sequences). Chimeric sequences were checked and removed using USEARCH (54), and the resulting sequences obtained were clustered into operational taxonomic units (OTUs) using a minimum identity of 97% based on their similarity according to UCLUST (55). Representative sequences from each OTU were aligned with the SILVA databases version 119 (56), using the PyNAST algorithm (57), and singletons were excluded from the analysis. Sequences classified as chloroplasts (0.3%) or mitochondria (0.5%) were removed from the alignment. Alpha diversity was calculated through observed species (observed OTUs), richness estimator (Chao1), and diversity indices (Shannon and Simpson) to compare the diversity of the bacterial community within samples. Richness and diversity were estimated using the phyloseq R package (version 1.7.12) (58). Bacterial diversity, measured as OTU richness, was estimated by rarefaction analysis, and rarefaction curves were generated based on OTUs found in each sample using vegan R package (version 2.4-0) (59).

Propagation of plant clones and bacterial inoculation under *in vitro* arsenic exposure experiments. The micropropagated birch clone BC-K (*B. celtiberica*) was used. This clone has been selected because of its great biomass, high tolerance to Cd accumulation, and well-developed root system (60). Birches were micropropagated *in vitro* from apical shoot segments of ~10 to 15 mm in a slightly modified Murashige and Skoog medium (MS) (61) in which macronutrients were diluted by half and 30 g · liter^{-1} saccharose and 7 g · liter^{-1} agar were added. The medium pH was adjusted to 5.7. Plants were grown for 2 months in a growth chamber with a 16-h photoperiod at 25°C. Bacteria were grown

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separately in 500-ml Erlenmeyer flasks containing 100 ml of glucose-asparagine-yeast extract (GAE) medium (62) with continuous shaking at 30°C to reach 10^8 to 10^9 cells per ml (24 to 48 h). Cells were recovered by centrifugation (8,000 × *g*, 10 min, 4°C), and cell pellets were resuspended in MS liquid medium to avoid changes in the state of the plants. For the bacterial inoculation, plants were placed in Falcon tubes containing 10-ml bacterial suspensions (see below) for 20 min, ensuring proper immersion of the roots.

Seven treatments were tested with six biologically independent replicates per treatment: (i) noninoculated plants used as controls (C), (ii) plants inoculated with endophytic strain 29E, (iii) plants inoculated with endophytic strain 32E, (iv) plants inoculated with an endophytic bacterial consortium (BC) composed of strains 28EY and 28EW (inoculum containing equal numbers of each strain), (v) plants inoculated with rhizospheric strain 44R, (vi) plants inoculated with rhizospheric strain 89R, and (vii) plants inoculated with rhizospheric strain 91R. Treated and control plants were transferred to polycarbonate magenta vessels (Magenta Corp., USA) containing 80 ml of MS liquid medium supplemented with 150 μM As(V) (arsenic is provided as Na_2HAsO_4), and 1 ml of each bacterial suspension was added to the medium of each plant (except for the control). Plants were randomly placed in the growth chamber and after 30 days of As(V) exposure, the leaves and roots of each plant were separated and rinsed with doubly deionized water (Milli-Q 185 Plus system). Increases in the fresh weight of plants were determined (FWI, calculated as [final plant fresh weight − initial plant fresh weight]/[initial plant fresh weight]). Fresh plant material from three individual plants was reserved to estimate the total nonprotein thiol compound content. The other three plants were individually dried at 40°C for 72 h, and dry weights of leaves and roots were determined. Dried plant material was used for further chemical analyses, arsenic content, and speciation in plant tissues.

Determination of total NPT compounds by HPLC, arsenic content, and speciation in plant tissues. NPT content was analyzed in the leaves and roots of each treatment (63), with slight modifications (64). Chemical As was determined in leaves and roots by ICP-MS. As speciation was quantified in leaves, roots, and the culture medium in which the plants grew (65). Details of these methods are provided in the corresponding section of the supplemental methods.

Field experiments. Field trials were performed on a 600-m^2 experimental plot located within the Nitrastur industrial zone (Fig. S1) from July to October 2014 with 216 *B. celtiberica* plants. This experimental plot was divided into 2 by 2-m subplots with at least 1 m between plots to minimize near-neighbor effects. Plants were transferred to the field when they were 9 cm tall and cultivated in 12 different plots (6 treatments × 2 replicate plots for each treatment × 18 plants in each plot). Based on results obtained in the previous study from *in vitro B. celtiberica* inoculation, four bacterial strains were selected for inoculation in the field. The experimental design included six treatments: (i) noninoculated plants used as a control (C), (ii) noninoculated plants supplemented with NPK fertilizer (N:P:K, 6:8:15; Phenix; Italpollina) used as a fertilized control (FC), (iii) plants inoculated with endophytic strain 32E (E), (iv) plants inoculated with rhizospheric strain 91R (R), (v) plants inoculated with the endophytic BC, and (vi) plants inoculated with endophytic BC and supplied with the NPK fertilizer (BC+F). At the end of the experiment, plant heights were measured. Six plants per treatment were harvested, and the roots and shoots were sampled separately. They were washed with tap water, rinsed with deionized distilled water, and oven-dried at 40°C for 72 h. The dried plant material was ground in a cutting mill (Pulverisette; Fritsch, Germany) to a size of up to 1 mm. As content was determined in dried plant material (leaves and roots). The effects of inoculations were evaluated by the increase in plant height (plant height index [PHI], calculated by [final height − initial height]/[initial height]) and the estimation of biomass through shoot dry weight. Root dry weights were not determined given the difficulty of uprooting entire roots. As concentration and pH measurements in the different plots and the successive stages of *in vitro* propagation and acclimatization of the plants in greenhouse before their final planting in the field are detailed in "Field experiments" in the supplemental methods. Determination of As phytoextraction efficiency indices is also described in the corresponding section of the supplemental methods.

Statistical analysis. The analysis of variance (ANOVA) and Tukey's mean grouping were applied in *in vitro* experiments to determine the significance of the interactions between treatment means. Analyses were performed with SPSS software package version 22.0 (SPSS, Inc., Chicago, IL). We also used linear regression techniques to investigate the correlation between fresh weight index (FWI), defined as (final plant fresh weight − initial plant fresh weight)/initial plant fresh weight or leaf and root dry weights, and leaf and root As concentrations in the *B. celtiberica* clones harvested under *in vitro* conditions and inoculated with different bacterial strains.

For the field experiment, data were processed by ANOVA, and differences between specific pairs of mean values were evaluated using Tukey's test (SPSS, Inc., Chicago, IL). In contrast to *in vitro* experiments, the soil conditions among the plots where the *B. celtiberica* clones were exposed to different treatments could have varied in terms of soil pH and As concentrations. This might affect As concentrations in leaves and roots, leaf dry weight, and PHI. Thus, we had to account for this medium condition variability before exploring the effect of leaf and root As content on the leaf dry weight and PHI or find a possible treatment effect on these variables. To do so, we first conducted a model selection using the Akaike information criterion (AIC) for each variable comparing three different models: (i) $y = a + \text{pH} = \varepsilon$, (ii) $y = a + \text{As soil} + \varepsilon$, and (iii) $y = a + \text{As soil} + \text{pH} + \varepsilon$, in which y could be the leaf or root As concentration, leaf dry weight, or PHI, a represents the intercept, and ε was the error term or residual component. From the selected best model, we extracted the residual part for each variable and studied the relationship between the residuals for leaf or root As concentration and those for leaf dry weight or PHI. For each variable, we also inspected the existence of significant differences in these residuals between treatments using Tukey's honestly significant difference (HSD) test.

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In the bacterial diversity analysis, an ANOVA was applied to estimate potential differences in microbial community diversity between the rhizosphere and endosphere of the five *B. celtiberica* trees. Simple linear regression analyses were then used to study the effect of rhizosphere pH and As concentration on this microbial diversity (in particular on the Chao1 diversity index). Nonparametric analyses of variance on 16S data were performed to evaluate differences between bacterial communities with permutation-based hypothesis tests, namely, analysis of similarity (ANOSIM) and adonis (permutational multivariate analysis of variance), with 99 and 999 permutations, respectively (66). All the above-mentioned statistical analyses were conducted with R (version 3.2.4) (67). Plots were made using the ggplot2 package for R (version 2.1.0) (68).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.03411-16.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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We declare no conflicts of interest.

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