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Rhizosphere Microbial Community Composition Affects Cadmium and Zinc Uptake by the Metal-Hyperaccumulating Plant *Arabidopsis halleri*

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The remediation of metal-contaminated soils by phytoextraction depends on plant growth and plant metal accessibility. Soil microorganisms can affect the accumulation of metals by plants either by directly or indirectly stimulating plant growth and activity or by (im)mobilizing and/or complexing metals. Understanding the intricate interplay of metal-accumulating plants with their rhizosphere microbiome is an important step toward the application and optimization of phytoremediation. We compared the effects of a "native" and a strongly disturbed (gamma-irradiated) soil microbial communities on cadmium and zinc accumulation by the plant *Arabidopsis halleri* in soil microcosm experiments. *A. halleri* accumulated 100% more cadmium and 15% more zinc when grown on the untreated than on the gamma-irradiated soil. Gamma irradiation affected neither plant growth nor the 1 M HCl-extractable metal content of the soil. However, it strongly altered the soil microbial community composition and overall cell numbers. Pyrosequencing of 16S rRNA gene amplicons of DNA extracted from rhizosphere samples of *A. halleri* identified microbial taxa (*Lysobacter*, *Streptomyces*, *Agromyces*, *Nitrospira*, "*Candidatus* Chloracidobacterium") of higher relative sequence abundance in the rhizospheres of *A. halleri* plants grown on untreated than on gamma-irradiated soil, leading to hypotheses on their potential effect on plant metal uptake. However, further experimental evidence is required, and wherefore we discuss different mechanisms of interaction of *A. halleri* with its rhizosphere microbiome that might have directly or indirectly affected plant metal accumulation. Deciphering the complex interactions between *A. halleri* and individual microbial taxa will help to further develop soil metal phytoextraction as an efficient and sustainable remediation strategy.

Elevated metal concentrations in agricultural soils decrease the yield and quality of crops. One of these metals is cadmium (Cd), which is present in the waste materials and waters of many industries (1) and in phosphate fertilizers used in agriculture (2, 3). Besides causing a decrease in crop yield (4), Cd is also a risk to human health if larger quantities (>2.5 μg kg^{-1} body weight $week^{-1}$, according to the EFSA [5]) are consumed (6–8). To preserve crop yield and quality, sustainable biotechnologies are urgently needed to relieve agricultural soils of metal contaminants. One promising soil remediation technology is phytoremediation—the use of plants for removing inorganic and organic contaminants from soils (9–11). Phytoremediation is attractive, as it is sustainable and relatively inexpensive compared to other soil cleanup strategies. So far, it has rarely been employed on the field scale (12) due to its low efficiency, slow progress, and the need to develop individual implementation strategies depending on the field site conditions and plant type.

Arabidopsis halleri has been studied as a model plant for Cd as well as zinc (Zn) hyperaccumulation in order to apply the plant for phytoremediation of Cd- and Zn-contaminated soils (10, 13). Wild *A. halleri* plants have been shown to accumulate up to 354 μg Cd (14) and 21.5 mg Zn (15) g^{-1} dry above-ground biomass. *A. halleri*'s efficiency to take up Cd and Zn from soil is limited by (i) the plant's overall low biomass and slow growth, (ii) a restricted radius and expansion of the plant's root system (10), and (iii) the general bioavailability of metals in soil for plant root uptake (11, 16). Recent research aiming at optimizing plant metal accumulation focused on plant growth-promoting substances and/or processes that provide metals to the plant, e.g., metal mobilization from minerals or enhanced metal mobility by metal complexation with organic ligands and chelators (11, 12). As soil microorganisms are known to affect plant growth (17) and the mobility of nutrients, metals, and contaminants in soils (18), phytoremediation research increasingly focuses on the complex interplay of the plant with the soil microbial community (17, 19). Several studies have previously shown that soil microorganisms can promote the accumulation of various metals in different plant species (20–29). Most of these studies focused on specific groups of microorganisms or were performed with soil extracts in artificial cultivation systems under laboratory conditions (hydroponics cultures). Currently, not much is known about the impact of the soil microbial community at heavy metal-contaminated sites on Cd and Zn accumulation by *A. halleri*. We therefore ask the following ques-

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tions. Will Cd and Zn accumulation by *A. halleri* be affected when the plant is grown on soil from a heavy metal-contaminated field site that either contains the "native" soil microbial community or a microbial community that has been strongly altered in species richness and evenness? Can the comparison of the two soil microbial communities help to identify microbial taxa living in the rhizosphere of *A. halleri* that might promote metal accumulation by *A. halleri*?

We set up plant microcosms (see Fig. S1A in the supplemental material) using Cd/Zn-contaminated soil that either was not treated or was gamma irradiated. Plant Cd and Zn accumulation, soil geochemical parameters, and bacterial cell numbers were quantified. Pyrosequencing of 16S rRNA gene amplicons was used to analyze differences in microbial diversity and the relative abundance of individual taxa in the rhizospheres of *A. halleri* plants grown in the untreated and gamma-irradiated soil microcosms.

MATERIALS AND METHODS

Plant and soil characterization. *Arabidopsis halleri* subsp. *halleri* (L.) O'Kane & Al-Shehbaz (accession Langelsheim Lan 3.1, Germany) plants were maintained in the greenhouse of the Department of Plant Physiology, Ruhr University Bochum, Bochum, Germany. For experiments, offshoot clones of similar sizes and with comparable numbers of leaves were separated from well-grown mother plants just before the experiment. Cadmium-contaminated soil was collected at Langelsheim in the Harz Mountains, Germany (lat 51.9429, long 10.3489) in October 2009 and July 2010. The soil was sieved (2 mm) and stored in the dark at 4°C.

Experimental setup. For microcosm setup, regular quartz sand (Hornbach gardening center, Germany) was cleaned with 6 M HCl for 48 h, neutralized to pH 5.5 by washing with water, dried at 25°C, and baked at 180°C for 4.25 h. The sand was mixed with soil at a ratio of 1:3 (wt/wt), in order to allow the plant to grow more easily on the soil (see Fig. S1B in the supplemental material). Half of the soil-sand mix was sterilized by gamma radiation at 35 kGy at the Beta-Gamma-Service (BGS), Wiehl, Germany. For initial wetting of the dry soil to a water content of approximately 35% (wt/wt), a soil extract instead of pure water was used in order to avoid shifts in ionic strength and mobility of mineral-bound ions. The soil extract was prepared by mixing the soil with sterile water (ratio of 1:1.4) (see Fig. S1C in the supplemental material). After incubation for 6 h, the soil extract was obtained by collecting the supernatant after sedimentation for 5 min. The soil extract was gamma radiation sterilized before use. Nine hundred grams of sterile and nonsterile soil-sand mixes per plant microcosm was amended with 33 ml of sterile soil extract and homogenized using a sterile spatula. The bottom of each plant microcosm was covered with the soil-sand mix. Three root bags (polyester mesh, 50.0-μm pore size, 4-cm diameter, 10-cm length) were filled with the prepared soil and evenly distributed in each microcosm. The remaining soil was filled in between the root bags (see Fig. S1D in the supplemental material). *A. halleri* offshoot clones were washed three times in sterile water and placed into the root bags (see Fig. S1E in the supplemental material). Ten milliliters of sterile water was added to each plant directly and an additional 50 ml to the whole pot. For each soil, three microcosms with three plants each were set up. Microcosms with the same soil were placed onto the same trays and covered with clear plastic hoods to prevent cross-contamination (see Fig. S1A in the supplemental material). Microcosms were irrigated with approximately 15 ml of sterile water once a week. Microcosms were moved randomly within their trays. Trays were also moved once per week in order to ensure unbiased light conditions in the greenhouse. Plants were grown for 4 weeks at 20 to 26°C with a 16-h/8-h day/night cycle. The experiment was repeated three times.

Sampling. Samples for geochemical and microbiological characterization of the soil and for plant metal uptake were taken in the beginning of the experiment and after plant microcosm incubation. Plant clones were weighed at the start of the experiment in order to use plants of similar weight in the experiment. This also allowed for calculation of the increase in plant biomass after incubation. The total element (Cd, Zn) content of plant clones was determined at the start of the experiment using inductively coupled plasma optical emission spectrometry (ICP-OES) (see the supplemental material) by washing plants in water and drying at 60°C. As no additional rooting agent was added to the soil, some plants did not grow well within the 4 weeks of incubation, independent of the treatment. Only plants that developed a root system were considered for analysis. The above-ground material was sampled, washed in water, and dried with tissue paper. The green plant tissue was weighed and ground in liquid nitrogen. The homogenized material was dried at 60°C for total elemental (Cd, Zn) analysis with ICP-OES. Thin roots could not be harvested for element analysis because it was not possible to completely separate them from the soil.

For water content and pH measurements, soil samples were taken at the start and end of incubation of the plant microcosms and stored at 4°C until analysis (see the supplemental material). Total and 0.1 M HCl-extractable Cd and Zn were quantified in bulk soil sampled at the start and in bulk soil and rhizosphere soil at the end of incubation (see the supplemental material). Soil samples for molecular analysis were stored at −20°C to determine total bacterial 16S rRNA gene copy numbers and the analysis of the microbial community composition by pyrosequencing. Start bulk soil samples were taken from the initially wetted sand-soil mix. End bulk soil samples were taken from a 4-cm depth between two plants. End rhizosphere samples were taken from root washes by first shaking soil off roots (this soil was used for metal extraction) and then by incubating roots in 20 ml of sterile water for 30 min. Roots were removed from the suspension, which was centrifuged for 10 min at 7,500 × *g*. The supernatant was filtered through 0.22-μm filters (Durapore membrane filters, GVWP02500; Millipore). Soil suspension pellet and filter were combined and frozen at −20°C (see the supplemental material).

Geochemical analyses and molecular biology techniques. All geochemical and molecular analyses are described in detail in the supplemental material.

Nucleotide sequence accession number. Sequencing reads are available at the European Nucleotide Archive (ENA) under accession no. PRJEB8016.

RESULTS

Geochemical parameters in the soil microcosms. The pH of the Langelsheim soil/sand mixture was 5.9 (see Table S1 in the supplemental material) and did not change during microcosm incubation. Langelsheim soil contained 12.2 ± 1.9 μg Cd and 3.3 ± 0.5 mg Zn g^{-1} dry soil (see Table S1). Further information on soil metal and carbon content is available in Table S1 in the supplemental material.

Cd and Zn in the soil. Cd and Zn concentrations extractable by 0.1 M HCl were not affected by gamma irradiation (Fig. 1A). The initial 0.1 M HCl-extractable Cd content was 6.7 ± 0.4 μg Cd g^{-1} dry soil. After 1 month of incubation, the 0.1 M HCl-extractable Cd content decreased to 5.7 ± 0.5 and 5.5 ± 0.3 μg Cd g^{-1} dry soil in the untreated bulk and rhizosphere soils, respectively. In the gamma-irradiated bulk and rhizosphere soils, the 0.1 M HCl-extractable Cd content decreased to 6.0 ± 0.5 and 5.9 ± 0.5 μg Cd g^{-1} dry soil, respectively. The initial 0.1 M HCl-extractable Zn content was 2,047.9 ± 123.8 μg Zn g^{-1} dry soil. After 1 month of incubation, the 0.1 M HCl-extractable Zn content decreased to 1,677.9 ± 152.1 and 1,637.6 ± 158.7 μg Zn g^{-1} dry soil in the untreated bulk and rhizosphere soils, respectively. In the gamma-irradiated bulk and rhizosphere soils, Zn contents decreased to 1,761.5 ± 166.0 and 1,738.5 ± 230.4 μg Zn g^{-1} dry soil, respectively. Aqua regia extractions did not reveal significant changes in

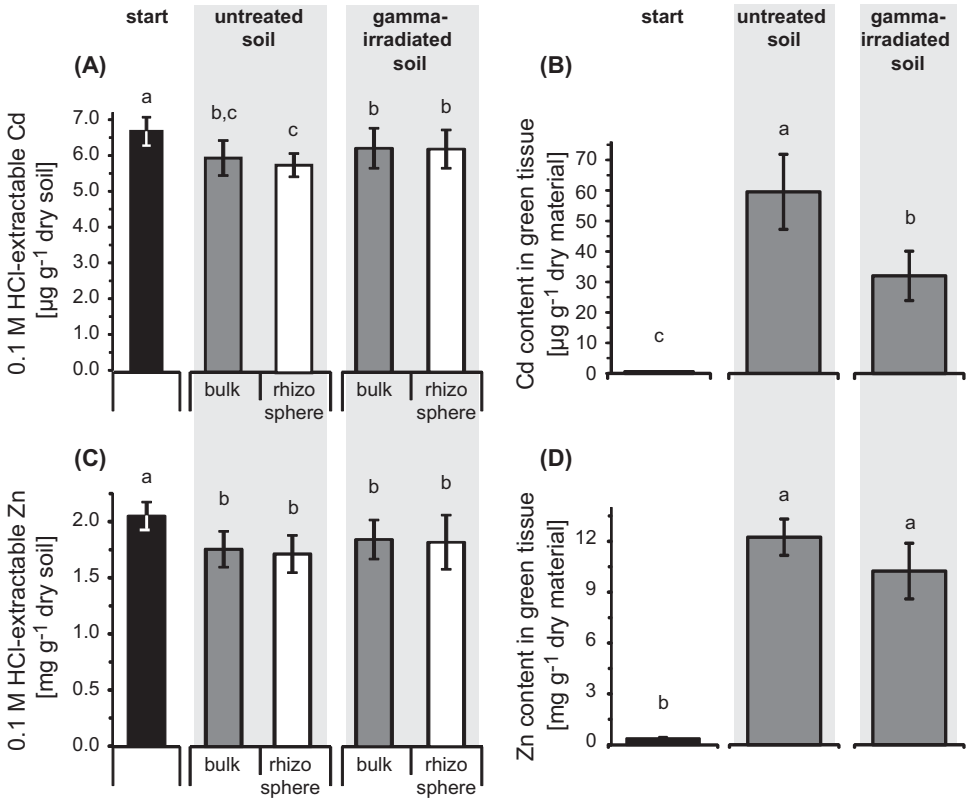

FIG 1 Cd and Zn concentrations in the soil and in above-ground plant biomass initially and at the end of the microcosm experiment. *A. halleri* was grown in microcosms with untreated soil containing a native microbial community or with gamma-irradiated soil containing an altered microbial community. HCl (0.1 M)-extractable Cd (A) and Zn (C) were quantified before microcosm incubation (black bars) and at the end of the experiment in the bulk soil (gray bars) and rhizosphere (white bars) of *A. halleri* plants (mean ± standard deviation, $n = 6$ to 12). The Cd (B) and Zn (D) content in *A. halleri* biomass was quantified in the above-ground green tissue of plants at the beginning and at the end of the microcosm experiment for both soil treatments (mean ± standard deviation, $n \geq 7$). Please note the differences in scales and units when comparing Cd and Zn concentrations. Mean values were compared to each other using the unpaired *t* test at a 95% confidence interval. Identical lowercase letters indicate that the mean values were not significantly different from each other ($P > 0.05$), while different lowercase letters indicate that the mean values were significantly different from each other ($P < 0.05$).

total Cd and Zn contents in the soil before and after incubation (see Fig. S2 in the supplemental material).

Cd and Zn accumulation by *A. halleri*. The initial average above-ground weight of *A. halleri* offshoot clones was 0.47 ± 0.23 g, which increased by 60% during 4 weeks of incubation (Fig. S1E and F in the supplemental material show the different sizes of plants before and after incubation). Plant growth was unaffected by soil gamma irradiation, as a similar plant biomass increase was observed for plants grown on untreated and on gamma-irradiated soils (data not shown). Initially, *A. halleri* clones did not contain more than 3 μg of Cd g^{-1} dry above-ground biomass (Fig. 1B). After cultivation, *A. halleri* plants accumulated 61.7 ± 12.8 μg and 33.2 ± 8.4 μg Cd g^{-1} dry plant material on untreated or gamma-irradiated soil, respectively, which are significantly different at a 95% confidence interval. *A. halleri* clones contained initially approximately 0.4 mg of Zn g^{-1} dry above-ground biomass (Fig. 1D). After 4 weeks of cultivation, *A. halleri* accumulated 12.2 ± 1.1 and 10.7 ± 1.7 mg of Zn g^{-1} dry above-ground biomass on untreated and gamma-irradiated soils, respectively, which are not significantly different at a 95% confidence interval. However, at a confidence interval of 94% the difference in Zn concentrations in the green biomass of plants grown on untreated and on gamma-irradiated soils becomes significant.

Microbial community composition: bacterial 16S rRNA gene copy numbers. Quantitative PCR analysis targeting the 16S rRNA gene was used to approximate total bacterial cell numbers in the soil (see Fig. S3 in the supplemental material). Initial bacterial 16S rRNA gene copy numbers in the native, untreated soil were $1.2 \times 10^8 \pm 2.0 \times 10^7$ 16S rRNA gene copies g^{-1} dry soil, which remained unchanged in *A. halleri* rhizospheres after 1 month of incubation. Total bacterial 16S rRNA gene copy numbers in the gamma-irradiated soil were initially significantly lower than those in the untreated soil ($2.1 \times 10^7 \pm 5.3 \times 10^5$ 16S rRNA gene copies g^{-1} dry soil). Bacterial 16S rRNA gene copy numbers in the rhizosphere of *A. halleri* increased during incubation on gamma-irradiated soil to $1.3 \times 10^8 \pm 3.0 \times 10^7$ 16S rRNA gene copies g^{-1} dry soil.

Microbial community richness and diversity. Pyrosequencing of bacterial 16S rRNA gene amplicons was used to compare the composition of the microbial communities present in the initial native bulk soil and in the rhizosphere of *A. halleri* after 1 month of plant growth in the untreated and gamma-irradiated soil microcosms (Fig. 2; see also Fig. S4 and Tables S2 and S3 in the supplemental material). For the initial bulk soil, 7,461 sequence reads were obtained after quality processing of the raw data. For the *A. halleri* rhizosphere samples collected after incubation a total

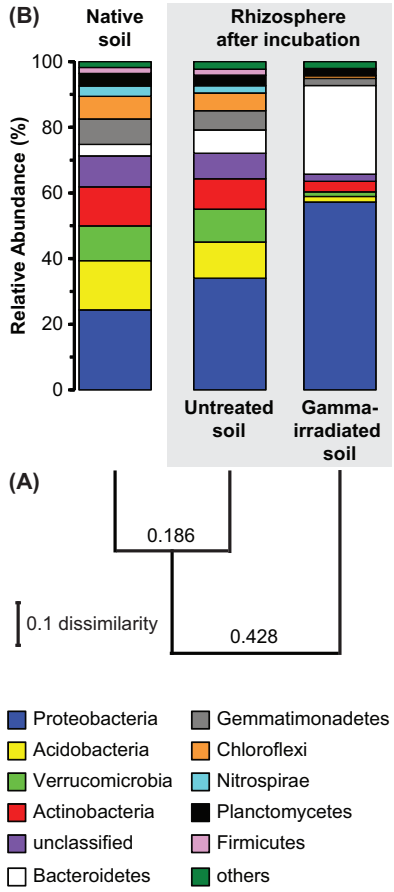

FIG 2 Cluster diagram and microbial community composition of the initial native soil and of the rhizosphere microbial community of *A. halleri* grown on untreated and gamma-irradiated soils after 1 month of soil microcosm incubation. (A) The cluster diagram was calculated using the Bray-Curtis dissimilarity based on 97% operational taxonomic unit (OTU) clusters. (B) The relative sequence abundances at the phylum level are shown as stacked bar graphs above the cluster diagram.

of 10,124 and 11,305 sequence reads were obtained after quality processing for the untreated and gamma-irradiated microcosms, respectively. Archaeal 16S rRNA gene PCR products could be generated only for the untreated soil samples. After gamma irradiation, no archaeal 16S rRNA genes could be amplified (data not shown). Unfortunately, this precluded further comparative analysis of the archaeal communities from untreated and gamma-irradiated microcosms.

Calculation of the Chao1 and abundance-based coverage estimator (ACE) richness estimators for 97% operational taxonomic unit (OTU) clusters after random subsampling revealed that our sequencing approach covered 36 to 52% and 38 to 53% of the bacterial OTU richness for the untreated soil samples before and after incubation, respectively. For the gamma-irradiated soil, richness coverage for 97% OTU clusters as estimated by Chao1 and ACE ranged from 45 to 55% (see Table S2 in the supplemental material). Rarefaction curves calculated for 97% OTU clusters did not reach saturation for any of the three sequenced soil samples (see Fig. S4A in the supplemental material). Both bacterial richness estimators (Chao1 and ACE) and diversity indexes (Shannon and Simpson) calculated for 97% OTU clusters indicated a higher richness and diversity in the initial bulk soil and in rhizospheres from plants grown on untreated soil than in rhizospheres from plants grown on gamma-irradiated soils (see Table S2 in the supplemental material).

Microbial community shifts with time. Cluster analysis based on Bray-Curtis dissimilarity showed that after 1 month of incubation the rhizosphere microbial community of the untreated soil was more similar to the initial, native bulk soil microbial community than the rhizosphere microbial community of the gamma-irradiated microcosms (Fig. 2A). After plant cultivation, the relative abundance of sequences affiliated with the phyla *Proteobacteria* and *Bacteroidetes* increased under both microcosm conditions, although a much stronger increase of the relative abundance of both taxa was observed in the rhizosphere of *A. halleri* grown on gamma-irradiated soil (Fig. 2B). Lists of the most abundant taxa (at the family level) found in the native bulk soil and the rhizosphere of plants grown on untreated or gamma-irradiated soil are given in Table S3 in the supplemental material.

Microbial community shifts in the rhizosphere. The microbial community compositions of rhizospheres of *A. halleri* grown on untreated, native soil and on gamma-irradiated soil were compared. 16S rRNA gene sequence reads obtained from the rhizosphere of plants grown on untreated soil could be clustered into 1,449 OTUs using a 97% sequence similarity cutoff (see Fig. S4B in the supplemental material). The five most abundant OTUs with relative sequence abundances ranging between 0.9 and 3% in the untreated soil were classified as "*Candidatus* Chloracidobacterium" (3.0%), *Nitrospira* (2.2%), *Acidovorax* (1.3%), *Gemmatimonas* (1.0%), and "*Candidatus* Solibacter" (0.9%) (Table 1). Of 1,449 OTUs, 1,191 were found only in the untreated soil samples but not in the gamma-irradiated soil. Among these OTUs specific to untreated soil, the five most abundant OTUs were classified as *Lysobacter* (0.7%), *Streptomyces* (0.5%), *Nocardioides* (0.3%), *Kineosporia* (0.3%), and *Steroidobacter* (0.3%) (Table 2). After gamma irradiation, the microbial community richness in the

TABLE 1 Most abundant genera found in the rhizosphere of *A. halleri* grown on untreated and gamma-irradiated soils[a]

Untreated soil			Gamma-irradiated soil		
Genus	No. of sequences	Relative abundance (%)	Genus	No. of sequences	Relative abundance (%)
"*Candidatus* Chloracidobacterium"	307	3.0	*Acidovorax*	901	8.0
Nitrospira	226	2.2	*Sediminibacterium*	508	4.5
Acidovorax	129	1.3	*Sphingopyxis*	332	2.9
Gemmatimonas	99	1.0	*Sphingobium*	305	2.7
"*Candidatus* Solibacter"	94	0.9	*Phenylobacterium*	295	2.6

[a] A complete list of all genera and their relative abundances is provided in Table S4 in the supplementary material.

TABLE 2 Most abundant genera present only in the rhizosphere of *A. halleri* under either one of the two soil treatments[a]

Untreated soil			Gamma-irradiated soil		
Genus	No. of sequences	Relative abundance (%)	Genus	No. of sequences	Relative abundance (%)
Lysobacter	66	0.7	*Sphingopyxis*	332	2.9
Streptomyces	47	0.5	*Rhizobium*	99	0.9
Nocardioides	32	0.3	*Microbacterium*	52	0.5
Kineosporia	30	0.3	*Bosea*	33	0.3
Steroidobacter	28	0.3	*Gordonia*	6	0.1

[a] A complete list of all genera and their relative abundances is provided in Table S4 in the supplementary material.

rhizosphere of *A. halleri* was strongly diminished. The total sequence reads obtained for the gamma-irradiated soil could be clustered into 406 OTUs. The five most abundant OTUs belonged to the genera *Acidovorax* (8.0%), *Sediminibacterium* (4.5%), *Sphingopyxis* (2.9%), *Sphingobium* (2.7%), and *Phenylobacterium* (2.6%) (Table 1). Also, the gamma-irradiated rhizosphere of *A. halleri* contained specific OTUs (148 OTUs) not found in the untreated soil. The five most abundant OTUs found only in the rhizosphere of plants grown on gamma-irradiated soil were classified as *Sphingopyxis* (2.9%), *Rhizobium* (0.9%), *Microbacterium* (0.5%), *Bosea* (0.3%), and *Gordonia* (0.1%) (Table 2). When comparing the sequence reads obtained from the rhizosphere of untreated and gamma-irradiated soils, we found that 258 OTUs were present under both conditions, although with various sequence frequencies (see Fig. S4B in the supplemental material). The genera "*Candidatus* Chloracidobacterium," *Nitrospira*, *Agromyces*, *Aquicella*, and *Haliangium* were present in untreated and gamma-irradiated soils, but their relative sequence abundance was 61- to 11-fold higher in the rhizosphere of plants grown on untreated soil (Table 3). In contrast, the genera *Polaromonas*, *Azospirillum*, *Bradyrhizobium*, *Larkinella*, and *Novosphingobium* had a much higher relative sequence abundance (76- to 34-fold) in the rhizosphere of plants grown on gamma-irradiated soil (Table 3).

DISCUSSION

The plant *A. halleri* is able to take up large amounts of Cd and Zn from soil and can accumulate these metals in its above-ground tissue (30, 31). This was also confirmed by our study, in which *A. halleri* accumulated 33 to 66 μg Cd and 10.7 to 12.2 mg Zn g^{-1} dry above-ground plant biomass during incubation. Plants capable of Cd hyperaccumulation can store more than 100 μg Cd g^{-1} dry above-ground biomass (13). The lower Cd values observed in our study are most likely due to the short incubation time of only 1 month. Furthermore, Cd hyperaccumulation has been described to not be constitutive among *A. halleri* species (13), causing differences in absolute Cd uptake levels among individual plants. For Zn hyperaccumulation, values exceeding 3,000 μg Zn g^{-1} dry above-ground biomass have been reported (13), and such values were reached in the experiments described here. In contrast to Cd, Zn is an essential element and is enriched to higher levels in the above-ground plant biomass (200-fold more than Cd) (10).

Soils containing high concentrations of heavy metals, such as the soil used in the experiments here, affect not only plant diversity and growth but also the soil microbial community composition. Using 16S rRNA gene-targeted quantitative PCR (qPCR), 1×10^8 bacterial 16S rRNA gene copies g^{-1} dry soil were quantified. Total cell numbers in the metal-contaminated soil of Langelsheim were about 1 order of magnitude lower than the average bacterial cell numbers reported for noncontaminated soils (1×10^9 bacteria g^{-1} [32–34]). Nonetheless, the microbial community composition of the Langelsheim soil was typical for soils in general with slight shifts toward an elevated abundance of the phyla *Actinobacteria* and *Proteobacteria*, which are known to comprise many taxa that contain metal resistance and tolerance mechanisms (33, 35–37).

TABLE 3 Genera found in the rhizosphere of *A. halleri* grown on untreated and gamma-irradiated soil (shared) at different sequence abundances[a]

Genus	No. of sequences in:		Ratio of untreated to gamma-irradiated	Ratio of gamma-irradiated to untreated
	Untreated soil	Gamma-irradiated soil		
"*Candidatus* Chloracidobacterium"	**307**	5	**61.40**	0.02
Nitrospira	**226**	11	**20.55**	0.05
Agromyces	**41**	2	**20.50**	0.05
Aquicella	**29**	2	**14.50**	0.07
Haliangium	**22**	2	**11.00**	0.09
Polaromonas	1	**76**	0.01	**76.00**
Azospirillum	3	**198**	0.02	**66.00**
Bradyrhizobium	4	**230**	0.02	**57.50**
Larkinella	1	**37**	0.03	**37.00**
Novosphingobium	8	**272**	0.03	**34.00**

[a] The values for the 5 most abundant genera identified under each soil condition are indicated in boldface. A complete list of all genera and their relative abundances is provided in Table S4 in the supplementary material.

Cd and Zn accumulation in *A. halleri*. *A. halleri* was grown on metal-contaminated soil that was either not treated or gamma irradiated. The main finding from the comparison of these two soil microcosms was that *A. halleri* accumulated approximately 100% more Cd and 15% more Zn when grown on untreated soil containing the native microbial community than on gamma-irradiated soil (Fig. 1B and D). The removal of metals from contaminated soils by plants is determined by the soil geochemistry, the activity of the plant itself, and the structure and function of the soil microbial community (reviewed by Garbeva et al. [38]). Gamma irradiation did not alter the soil geochemistry with respect to the 0.1 M HCl-extractable Cd and Zn contents (Fig. 1A and C). Gamma irradiation also did not affect overall plant growth, as no change in above-ground green biomass of plants grown on untreated and gamma-irradiated soil was observed. However, gamma irradiation drastically affected overall bacterial cell numbers (initially 10-fold-lower 16S rRNA gene copy numbers in gamma-irradiated soil) and microbial community composition, as soil microorganisms possess different resistance and tolerance mechanisms to gamma irradiation (39–43) or were physically

protected by soil particles during radiation. Nonetheless, during the 1 month of soil microcosm incubation, 16S rRNA gene copy numbers reached almost initial values, indicating regrowth of the microbial community that survived the gamma irradiation treatment. The newly established microbial community had a lower diversity and a lower overall species richness (see Table S2 in the supplemental material) and revealed considerable differences in relative sequence abundance of individual taxa (e.g., an increased relative sequence abundance of *Proteobacteria* and *Bacteroidetes*) compared to the untreated soil after incubation (Fig. 2). This suggests that the irradiation-induced change in microbial community evenness and richness might have impacted the ability of *A. halleri* to accumulate Cd and Zn.

Microbially enhanced uptake of metals by plants has been observed for various plants, microorganisms, and metals (20–29). Farinati et al. (28, 29) grew *A. halleri* in hydroponic cultures, which they inoculated with an "extracted" rhizosphere microbial community. *A. halleri* accumulated more Cd and Zn in the presence than in the absence of the extracted rhizosphere microbial community. Other studies also used hydroponics instead of soil microcosms for experiments addressing the effect of microorganisms for plant metal uptake (21, 22, 26). However, experiments performed in hydroponic cultures using defined mineral media do not reproduce the complexity of soils, and Cd concentrations in solution usually exceed Cd bioavailability in soil. Plant metal accumulation is directly correlated to metal (bio)availability, mobility, and transport in soil (in relation to total metal content). These parameters can directly and indirectly be affected by plant-microbe interactions. The ability of microorganisms to mobilize metals from immobile metal pools in soils is not considered in hydroponic culture experiments. Abou-Shanab et al. (20), Jia et al. (24), Li et al. (25), and Whiting et al. (27) performed metal uptake experiments with plants in soil microcosms but focused on specific rhizosphere bacteria or microbial consortia. Recently, Gomez-Balderas et al. used restriction fragment length polymorphism analysis and conventional clone libraries to describe the total bacterial community associated with the hyperaccumulator *A. halleri* grown on a Zn- and Cd-polluted soil (44). In the study here, a high-throughput sequencing approach was used to gain insights into the complex interplay of soil-plant-microbe interactions that could impact plant metal accumulation and might help to explain the observed differences in Cd and Zn accumulation by *A. halleri* grown on untreated and gamma-irradiated soils.

Plant growth and metal accumulation. Soil microorganisms are known to stimulate plant growth and performance (45). Vice versa, plants are known to stimulate or inhibit microorganisms in proximity to their roots in order to select for a rhizosphere microbial community that improves overall plant health (45–47). Plant growth promotion by so-called plant growth-promoting bacteria includes the solubilization of essential nutrients (e.g., phosphate, sulfate, iron, and potassium), nitrogen fixation, senescence retardation, stress limitation, and the production of precursors of the plant growth hormone auxin (17, 42, 48). Bacterial genera known to promote plant growth were present in the rhizosphere of *A. halleri* grown on untreated and gamma-irradiated soils, e.g., *Acidovorax facilis* (49), *Bradyrhizobium* spp. (50), *Azospirillum* spp. (51), *Pseudomonas saccharophila* (52, 53), *Chryseobacterium* sp. (54), *Lysobacter* sp. (55), *Agromyces* sp. (56), and *Massilia* spp. (57).

In our experiment, the genus *Sediminibacterium* had a high relative sequence abundance in the rhizosphere of *A. halleri* on both soil types, but potential plant growth-promoting interactions between *Sediminibacterium* and *A. halleri* have so far not been described.

Some visible effects of microbial plant growth promotion are increased root growth and above-ground biomass proliferation, which would result in a larger rhizosphere radius for metal uptake and increased shoot biomass for metal storage (48). In the study presented here, *A. halleri* plants grown on untreated and gamma-irradiated soils did not show any differences in above-ground biomass over the duration of the experiment. Although gamma irradiation altered the soil microbial community composition, no differential effect on plant growth (quantified by green biomass increase) was observable between soil treatments, indicating that potential changes in the composition or relative abundance of plant growth-affecting microbial taxa had no effect on plant biomass increase in our microcosm experiments. Furthermore, the observed differences in Cd and Zn accumulation by *A. halleri* grown on untreated and gamma-irradiated soils are not a result of differences in plant biomass increase during microcosm incubation. However, it should be kept in mind that plant performance and health are not indicated by just an increase in plant biomass. Farinati et al. (28, 29) also did not observe an increase in *A. halleri* biomass in the presence of microorganisms in the hydroponic nutrient solution. In contrast, they found that the presence of microorganisms in the hydroponic nutrient solution increased the plant's production of chlorophyll and photosynthesis-related proteins, which might have enhanced photosynthetic energy production, providing more energy for metal accumulation, while plant biomass increase remained unchanged.

Metal hyperaccumulation gives plants a selective advantage over other plant species to grow on soils with a high metal content (13). Metal-hyperaccumulating plants have to allocate their photosynthesis-derived energy, among other things, for processes associated with plant growth, metal acquisition, and microbial community control (pathogens and commensals). Pathogenic microorganisms might be able to thrive more competitively in gamma-irradiated soils, since other microbial taxa controlling their proliferation were either completely erased or strongly decreased in abundance during gamma irradiation of the soil. We found species of known plant pathogens, such as *Pseudomonas syringae* (58) and *Pseudoxanthomonas* spp. (59, 60), in the rhizosphere of *A. halleri* grown on gamma-irradiated soil that showed a considerable lower relative sequence abundance in the untreated microcosms. However, further studies are necessary to identify the physiological response mechanisms induced by *A. halleri* grown on gamma-irradiated soil and to verify if pathogen control mechanisms provide a possible explanation for the lower Cd concentrations in *A. halleri*'s green biomass after growth on gamma-irradiated soil.

Plant- and microbe-mediated metal-mobilizing processes in the rhizosphere of *A. halleri*. Although greater amounts of Cd and Zn were taken up by *A. halleri* grown on the untreated soil than on the gamma-irradiated soil, this did not result in a quantifiable decrease in 0.1 M HCl-extractable Cd and Zn concentrations in the rhizosphere (Fig. 1A and C). However, Menzies et al. (61) found that soil trace metal concentrations determined by acid extractants such as 0.1 M HCl are only poorly correlated to plant phytoavailability. Both plants and microorganisms can mediate or directly catalyze processes that can mobilize metals in the rhizo-

sphere, thereby improving metal transport kinetics and root-metal accessibility (16, 17).

For example, plants can lower the pH in proximity to their roots to dissolve poorly crystalline and easily soluble minerals (11, 16, 62–64). Plants are also able to directly reduce Fe(III) in Fe minerals, which dissolves the mineral (64, 65). Metals present in small amounts in many minerals (66) can be mobilized via the acid- or reduction-mediated dissolution of minerals (18). Furthermore, plants mobilize metals by excreting low-molecular-weight chelators (11, 16, 62, 63). Such organic compounds capture metals and thereby increase transport and bioavailability of the chelated metals.

However, plants are able to mobilize only metals in direct proximity to their roots and metals that are not strongly bound to soil mineral phases (17). Bacteria are able to mobilize metals beyond the rhizosphere and from more crystalline mineral phases. Abou-Shanab et al. (20) showed that rhizosphere bacteria enhance the availability of Ni in soil, which stimulated the uptake of Ni into the plant *Alyssum murale*. Also, Li et al. (25) showed that *Pseudomonas* spp. are able to increase the amount of plant-available Cu, which enhanced Cu uptake of maize plants. Whiting et al. (27) have shown that soil microorganisms increased the water-soluble fraction of Zn in soils, leading to an enhanced accumulation of Zn in the hyperaccumulator plant *Thlaspi caerulescens*. Fe(III)-reducing bacteria were shown to mobilize mineral-associated Cd from Fe(III) minerals during Fe(III) mineral reduction, resulting in mineral dissolution (67, 68). The stimulation of soil microbial Fe(III) reduction for the (phyto-)bioremediation of Cd-contaminated soils has previously been discussed (68). In general, metals can be mobilized by the activity of mineral-dissolving bacteria, which besides Fe(III)-reducing bacteria also comprise sulfur-oxidizing, phosphate-solubilizing, fermenting, and organic acid- and proton-releasing functional groups of microorganisms (17, 18). In this study, we found elevated sequence abundances of *Lysobacter*, *Chryseobacterium*, and *Pseudomonas* in the rhizosphere of *A. halleri* (see Table S4 in the supplemental material). *Lysobacter* spp. were especially interesting as they were present only in the rhizosphere of *A. halleri* grown on untreated soil (Table 1). *Lysobacter* spp. have previously been shown to increase in abundance in the presence of clays and iron mineral oxides (69), but so far it is not known if they are also involved in the release of metals from these minerals. *Pseudomonas* spp. can oxidize sulfur compounds (70), and *Pseudomonas monteilii* can mobilize Zn (27), while *Chryseobacterium* spp. have been shown to solubilize soil phosphates (54). Sulfur and phosphorus are important nutrients for plant growth. However, phosphate- and sulfur-associated Cd could also be mobilized during the transformation of sulfur- and phosphate-containing minerals. Thus, microbial phosphate and sulfur mineral dissolution could also increase metal bioavailability.

Microbially produced extracellular organic chelators, siderophores, and ligands can complex mobilized metals and could thereby facilitate their transport and make them more plant accessible (17). We found bacterial genera in the rhizosphere of *A. halleri* grown on untreated and gamma-irradiated soils that have previously been reported to stimulate metal solubility by secretion of siderophores and other organic compounds, e.g., *Agromyces* sp. strain AR33 (56), *Streptomyces* spp. (71), and *Pseudomonas* (72). Different *Pseudomonas* sp. strains have been shown to exert diverse catalytic activities in soil and engage in complex interactions with plants. For example, Cd-resistant *Pseudomonas* sp. strain RJ10 leached Cd from $CdCO_3$ by producing organic acids (73), while highly Cd-resistant *Pseudomonas aeruginosa* and *Pseudomonas putida* 06909 were reported to release Cd-binding siderophores and peptides (74, 75).

Many of the above-mentioned processes are often interlinked, and members of a specific microbial taxon might simultaneously support and/or suppress metal uptake by *A. halleri* by various mechanisms. In this discussion, we focused mainly on microbial taxa for which specific interactions with metals and/or plants have been reported. However, for many taxa identified in the rhizosphere of *A. halleri* in our soil microcosms, no specific information on metal or plant interactions have previously been described. If and how these taxa affect Cd and Zn uptake by *A. halleri* and thereby the plant's applicability to remediate metal-contaminated soils are currently unknown. "*Candidatus* Chloracidobacterium," for example, is the most abundant genus in the rhizosphere of *A. halleri* grown on untreated soil. However, "*Candidatus* Chloracidobacterium thermophilum" has been described to inhabit only microbial mats associated with geothermal hot springs (76). Genus *Gemmatimonas* spp. have been found in metal-contaminated soils (77), but no specific mineral-metal-plant interactions have so far been reported. Further specific association experiments are necessary to elucidate how individual strains of the many genera identified in this study and their specific metal-mineral-plant interactions can promote Cd and Zn hyperaccumulation by *A. halleri*.

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