Evolutionarily Conserved Core Rhizosphere Microbiota Promotes Host Performance and Fitness in Heavy Metal Accumulating Plants

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Research

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Abstract

Background: Persistent microbial symbioses offer the potential to confer greater fitness to the host under unfavorable conditions, but manipulation of such beneficial interactions requires a mechanistic understanding of the consistently important microbiome members for the plant. Here, use five phylogenetically divergent heavy metal (HM) accumulating plants as a model, we examined the composition, assembly and relationships of the core and active rhizosphere microbiota across diverse soils with varying concentrations of HMs and further explored their roles in host performance.

Results: Our results showed that the rhizosphere bacterial communities were primarily determined by soil type, with plant species having a stronger influence on the microbial diversity and composition than rhizocompartment and soil pollution level. We found that different HM accumulating plants harbored a unique set of core taxa in the rhizosphere with *Sphingomonas* and *Burkholderiaceae* shared among them. Use of RNA-SIP further revealed that the core rhizosphere taxa phylogenetically overlapped with the active rhizobacteria feeding on carbon-rich rhizodeposits, suggesting that the specific root exudate components driving the core microbiomes may be common across different plant species. Several keystone taxa were part of the core microbiota and facilitated plant metal tolerance and accumulation when inoculated with SynCom comprising the core cohorts.

Conclusions: Our results suggest that a conserved core root microbiota has evolved with HM accumulating plants via root metabolic cues and exhibited potential to increase plant fitness and phytoextraction of HM. This study has important implications for harnessing the persistent microbiome members to improve host performance and accelerate the plant-assisted restoration of contaminated soil ecosystems.

Background

Land plants provide a multitude of niches for the growth and proliferation of a diverse community of microorganisms that thrive on and inside host tissues. These microorganisms form symbioses with their host plants and exert influences on plant phenotypes, and ultimately fitness, by modulating the plant growth, nutrient acquisition and adaptation to biotic and abiotic stresses [1–3]. A ‘cry for help’ strategy is often employed by plants to attract beneficial microbes to overcome environmental stresses [4]. Therefore, the plant microbiome can be seen as the complementary functional repertoire that expands the capacity of the plants to mitigate the negative effects of abiotic stresses on plant growth and productivity, and manipulation of these microbiomes may offer a promising strategy to combat stresses in field settings [5, 6]. However, deploying microbiomes to increase plant resistance to environmental stresses and host performance is largely challenged by ascertaining which members are consistently important for host plants [7, 8].

Plant roots exude a substantial amount (11-40%) of photosynthesis-derived carbon-rich compounds [9], which play an active role in the enrichment and maintenance of the taxonomically structured rhizosphere

microorganisms [10]. Despite the variation in local soil microbial reservoir across locations and environmental conditions, a subset of microbial members are persistently associated with a given host regardless of changes in space, time and plant genotypes [11–13]. The core microbiota may encompass key microbial taxa carrying genes with essential functions for 'holobiont' (plant and its co-evolved microbiome) fitness [14] and can result in mutualistic interactions [13], a trait that could be exploited to improve the host performance [7]. Of note, the functioning of the core microbiota might be more prominent if they comprise keystone taxa [15] that are highly connected in the community and can act as mediators between the plant and its associated microbiome [16]. Identifying the core microorganisms that consist of keystone taxa would be a plausible step for steering plant-microbiome interactions towards desired beneficial outcomes, such as increased plant growth and fitness [7, 13]. Thus far, however, most existing core microbiome studies have focused on the taxonomic characterization, and very few have attempted to experimentally test their potential to enhance plant performance [12, 17–19].

Heavy-metal (HM) accumulating plants can serve as a model to study the importance of core microbiota and keystone taxa for plants to combat against environmental stresses as they thrive in HM contaminated soils. These plants have a superior ability to extract HMs from soil and concentrate them in the aboveground biomass [20, 21]. Their bioconcentration factor (the shoot-to-soil ratio of element concentration) and the shoot-to-root ratio of HM concentration are usually greater than one [22], indicative of an efficient root-to-shoot HM transport. These plants have garnered considerable attention in recent years as they offer a cost-effective and sustainable solution to reclaiming soil ecosystems polluted with anthropogenic contaminants [22, 23]. Although plant HM accumulation is often considered to be driven by genetic and physiological bases [21, 24], certain microorganisms associated with these plants have been shown to play a part in this process by promoting plant growth, regulating metal bioavailability and consequently uptake of HM [25–27]. Hence, HM-accumulating plants present a useful system to elucidate the relationships between plants and core microorganisms and their causal roles in plant performance.

Here, we aimed to uncover the core rhizosphere microbiota of five phylogenetically divergent cadmium (Cd)-accumulating plants, Indian mustard (*Brassica juncea* L., Brassicaceae), *Sedum alfredii* (Crassulaceae), *Solanum nigrum* L. (Solanaceae), and Ganges and Prayon ecotypes of *Noccaea caerulescens* (Brassicaceae), and harness them to improve the phytoextraction efficiency and productivity of their host plants. Our experimental design allowed us to investigate the extent to which microbiomes living in association with phylogenetically diverse, but functionally similar (Cd accumulation) hosts overlap with each other. We hypothesized that despite the phylogenetic variability, different Cd-accumulating plants share a subset of the core rhizosphere microbiota that may be linked to their performance and fitness. To achieve this, we first examined the structural diversification and assembly of the rhizosphere and soil microbiota associated with the Cd-accumulating plants grown in the three types of paddy soils with varying concentrations of Cd/Zn [highly polluted (HP) vs. slightly polluted (SP)] and different geographical origins [HP and SP soils vs. non-polluted paddy soil (NP)]. We then characterized the active rhizosphere bacterial communities using RNA-based stable isotope probing (RNA-SIP). Lastly, we investigated the functions of the core microbiota using synthetic communities

(SynComs) isolated from Cd-accumulating plant and linked them to plant growth and Cd accumulation using hydroponic cultivation.

Materials And Methods

Source soils

Three soils of different origin or distinct Cd/Zn pollution levels were used: highly polluted (HP) soil, slightly polluted (SP) soil, and non-polluted (NP) paddy soils. The HP and SP soils, collected from the rice paddy fields near Hangzhou, Zhejiang Province, China (30°04'38.3"N; 119°24'39.7"E), have the same origin. This site has been primarily contaminated with Cd and Zn derived from local metal smelters that have been active for more than thirty years. The long history of smelter activity resulted in depositional gradients of HMs across the site, and the HP and SP soils that were collected depended on distance apart from the region of various smelters. The non-polluted paddy (NP) soil was collected from a paddy field in Jian City, Jiangxi Province, China (27°21'24"N; 114°22'46"E). The HP, SP and NP soils were subjected to similar water and fertilization regimes. Total Cd concentration in SP soil was below the detection limit and in HP and SP soils was 12.54 and 0.98 mg kg^{-1}, which was higher than the environmental quality standard (0.4 mg kg^{-1}; GB15618-2018). Physicochemical properties of the three soils were determined by standard procedures and are shown in Supplementary Table S5.

Bacterial strains used in hydroponic experiment

Five single bacterial strains, i.e. *Sphingomonas* sp. SaMR12, *Burkholderia* sp. SaZR4, *Burkholderia* sp. SaMR10, *Burkholderia* sp. SaZRH13, *Burkholderia* sp. SaCRH14, used in this study were selected from our Culture Collection of *Sedum alfredii*-associated bacterial isolates [54]. *Sphingomonas* spp. SaHR01 was isolated from the highly-polluted (HP) rhizosphere soil in the present study, according to our previously described protocols [55]. These six bacterial isolates share a similar phylogenetic relatedness with the core rhizosphere taxa common to the five Cd-accumulating plants (i.e. *Sphingomonas*, *Burkholderia*), which exhibit high resistance to Cd and Zn stress, and carry at least one potential plant-growth promoting trait, such as ACC deaminase activity, IAA production, siderophore production, and phosphate solubilization [54]. Thus, these bacterial isolates might be the ideal object to test the role of core microbiota in host fitness and metal accumulation.

Experimental design, plant growth and sample collection

We have characterized the overall and core root bacterial microbiomes of the five Cd-accumulating plants, Indian mustard (*Brassica juncea* L.) [56], *Sedum alfredii* [55], *Solanum nigrum* L. [57], and the Ganges and Prayon ecotype of *Noccaea caerulescens* [58], grown in three different soils using two sets of experiments; these plants exhibit distinct ability to accumulate Cd in their root and shoot tissues (Fig. S6). First, for the “overall bacterial community” experiment, seeds of the Cd-accumulating plants were surface sterilized, germinated and pre-conditioned in sterile potting media for three weeks. After preconditioning four uniform seedlings of each Cd-accumulating plant were transplanted to the rhizobox containing 5 kg

of air-dried soil, which is designed to obtain separate and intact rhizosphere soil as previously described [59]. Control rhizoboxes without transplantation were maintained in parallel (i.e. unplanted soil). For each plant grown in a given soil, five biological replicates were established. Rhizoboxes were randomly distributed in trays and placed in a greenhouse under natural light, with an average day/night temperature of 26/20℃ and relative humidity of 70/85%. Plants and soils were watered periodically every two days to maintain the soil moisture at 55-65% of water-holding capacity. After four months of growth and soil microcosm incubation, plant shoot samples, root samples, rhizosphere and unplanted soils were collected from five replicated blocks as previously described [59]. Rhizosphere soil was collected as the wash containing the root-adhering soil layer after removing the excess soil attaching the roots according to our previous study [60].

Second, for the “active rhizosphere bacterial community” experiment, healthy *S. alfredii* seedlings with the uniform size were transferred to the rhizobox containing 3 kg of HP and SP soils, respectively. We chose *S. alfredii* because it has the strongest ability to extract Cd from the soils among the five Cd-accumulating plants and thus can represent the assembly pattern of the active rhizobiomes in other Cd-accumulators. The continuous ^{13}C labelling initiated 45 days later when *S. alfredii* were in an active photosynthesis status, and the rhizosphere microbiota reaches a stable state. *S. alfredii* were labelled with $^{13}CO_2$ (98 atom % ^{13}C, Cambridge Isotope Laboratories, Inc., Andover, USA) between 9 am and 5 pm (8 h) for 10 consecutive days [61]. During the labelling period, the total concentration of CO_2 inside the growth chamber was maintained at 350-400 μl l^{-1} by mixing with additional $^{13}CO_2$. Simultaneously, parallel microcosms as controls were also established with $^{12}CO_2$ labelling. At the end of labelling, the rhizosphere and unplanted soils were sampled as previously described.

Lastly, we conducted a hydroponic cultivation to examine the effect of core synthetic community (SynCom) on plant growth and Cd accumulation in Indian mustard, *Sedum alfredii* and *Solanum nigrum*. Four uniform and healthy Cd-accumulating plant seedlings were transferred into axenic nutrient solution either with or without 25 μM $Cd(NO_3)_2$. The nutrient solutions were inoculated with the SynCom consisting of six potential core rhizospheric bacterial isolates (based on the results of the first experiment) affiliated with *Burkholderia* sp. and *Sphingomonas* sp.. Treatments included Control (axenic nutrient solution), 25 μM Cd^{2+} treatment (Cd) (equivalent to the minimum value of available Cd concentration in highly polluted field soil), and Cd treatment plus the SynCom (Cd + SynCom). Preparation and inoculation of SynCom in hydroponic experiment was similar to our previously described methods [55]. SynComs were inoculated to a final OD_{600} of 0.01–0.05 (approximately 10^7 CFU ml^{-1} water solution). Each treatment had five independent replicates, and plants were harvested after 45 days of cultivation under a 16 h light cycle, 26/22℃ average day/night temperature and relative humidity of 70%. At harvest, the root and shoot tissues were sampled and were rinsed with tap water and thoroughly washed with deionized water. Afterwards, the roots were immersed in 20 mM Na_2-EDTA for 15 min to remove the metal ions attached to the root surface. The root and shoot tissues were oven-dried at 105℃ for 1 h, then at 70℃ to a constant weight and their dry weights were recorded.

Determination of Cd in root and shoot tissues, H_2O_2 and superoxide anion

Dry root and shoot samples (~0.1 g) were digested with HNO_3–$HClO_4$ (5:1, v/v) in a microwave dissolver, and the digestion solution was filtered (0.45-μm pore size) and Cd concentration were determined using Agilent 5100 ICP-OES (Agilent Technologies, CA, USA). In hydroponic experiment, the concentration of H_2O_2 in roots was determined by H_2O_2–titanium (Ti) complex reaction [62] and concentration of $O_2^{\bullet -}$ was measured by methods with 2–naphthylamine–4–aminobenzenesulfonic acid [63].

DNA extraction and 16S rRNA gene amplicon sequencing

Microbial genomic DNA was extracted from the rhizosphere and unplanted soil samples using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. Extracted DNA was subjected to quality-check using NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA) and gel electrophoresis (1.5% agarose). Amplicon libraries were prepared from two independent PCR reactions. During the first PCR run, the bacterial 16S rRNA gene of V3-V4 region (~480 bp) was amplified using the Illumina iTags primer pairs of 338F (5'-ACTCCTACGGGAGGCAGCA-3') /806R (5'-GGACTACHVGGGTWTCTAAT-3') [64]. The PCR conditions were as follow: 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 min. PCR reactions were carried out in triplicate. In the second PCR run, each sample was tagged with a unique dual-index barcode. Amplicons were purified (Beckman, USA), pooled in equimolar ratios and were combined into one pooled sample and subjected to Illumina Hiseq sequencing (San Diego, CA, USA).

RNA-SIP gradient fractionation

Soil total RNA was extracted from ~2 g of soil sample using the PowerSoil® Total RNA Isolation Kit (MoBio Laboratories, Carlsbad, USA). The extracted rRNA (~500 ng) was mixed well with cesium trifluoroacetate (CsTFA) to achieve an initial buoyant density (BD) of 1.790 g ml^{-1} before ultracentrifugation at 130, 000×*g* for 65 h at 20℃. Fifteen RNA fractions (~380 μl for each) were fractionated according to a previous report [65], and the CsTFA BD of each fraction was measured by determining the refractive index. Then, RNA in each fraction was precipitated with isopropanol as described previously [65]. Precipitation from gradient fractions were washed once with 70% ethanol and re-eluted in 30 μl Tris-NaCl for subsequent determination of total RNA. The rRNA from each fraction of each sample was quantified in triplicate by real-time reverse transcription PCR in an Applied Biosystems7900HT real-time system (Carlsbad, CA USA) with primer Ba519f/Bac907r [65]. Selected density fractions of rRNA were reversely transcribed into complementary DNA using the PrimeScriptTM RT reagent kit (Takara, Dalian, China), and subjected to 16S rRNA gene sequencing similar to the aforementioned protocols.

16S rRNA sequence processing

A total of 4216175 high-quality sequence was obtained with a median read count per sample of 46846 (range: 24369-60990). Raw sequence data was processed using QIIME2 (v.2020.6) pipeline. The DADA2 method [66] was used to trim the primer pairs, perform quality control (QC) of sequences, and assemble quality-filtered reads into error-corrected amplicon sequence variants (ASVs), which represent unique bacterial taxa and reveal cryptic diversity. A minimum of 25 bp of overlap was required for the paired-end reads merging step. The script '*data2 denoise-paired*' in QIIME2 was used to perform DADA2, which is a pipeline for detecting and correcting (where possible) Illumina amplicon sequence data. The QC process filtered any phiX reads and chimeric sequences. Taxonomic classification of the assembled ASVs was conducted by training the naïve Bayes classifier against the Silva 138 99% OTUs (Operational Taxonomic Units) full-length reference sequences. Next, using the R package 'phyloseq' [67], we removed any ASVs without a bacterial phylum assignment, or assigned to archaea, or mitochondria. To facilitate downstream composition and differential abundance analyses, we applied a prevalence and abundance threshold for filtered ASVs, where taxa were retained only if they were found in 2% of samples (three samples) and at a frequency of 20 sequence reads per sample. The filtered ASVs comprised 85% of the total number of sequences. Subsequently, we normalized the counts of individual ASV in a sample by dividing the total counts of the filtered ASVs within that sample resulting in relative abundance. Without application of this threshold, we rarefied community profiles to 10000 sequences to eliminate the bias from sequencing depth. Alpha diversity index of inverse Simpson's diversity was calculated using the rarefied ASV table in 'phyloseq' R package, and phylogenetic diversity (Faith's pd) was calculated using 'picante' R package.

Defining the core rhizosphere microbiota

To detect the ecologically robust core microbial members, two methods were applied to identify the core rhizosphere microbiota for each Cd-accumulating plant [17]. We first performed differential abundance analyses with the DESeq2 package to find the ASVs that significantly enriched in the rhizosphere compared with the unplanted soil [68]. We then defined the core root microbiota for each Cd-accumulating plant as the rhizosphere-enriched bacterial ASVs found in more than 75% [11, 19] of a particular sample type (rhizosphere) across the three soils using the rarefaction-normalized abundance table. In parallel, the core microbiota members were also identified using the indicator species analysis with the 'multipat' function of the R 'indicspecies' package, with *indval* value > 0.6 and $P < 0.05$ considering as the strong indicator for an ASV. This method is used to find indicator species and species assemblages characterizing groups of niches [17, 69], e.g. distinct plants or rhizocompartments[70], and combines a species relative abundance with its relative frequency of occurrence in the various groups of niches [69]. Next, we compared the core taxa identified by the two analyses, and those that were completely overlapped phylogenetically were referred to as the commonly occurring core rhizosphere microbiota and were retained for subsequent analyses.

Statistical analyses

Differences in alpha-diversity indices between rhizosphere and unplanted soil were determined by Wilcoxon rank tests. Testing for statistical differences in plant parameters, metal concentration, alpha-diversity indices and community similarities among plants or soil types was performed using analysis of variance (ANOVA) with the Tukey's HSD *post hoc* test (n = 5 for each group). Hierarchical clustering analysis based on Bray-Curtis dissimilarities was used to visualize the differences in active rhizobiomes, and principal coordinate analysis (PCoA) of the weighted UniFrac distance to characterize the rhizobiomes of different Cd-accumulating plants in the package 'ape' in R [71]. Permutational multivariate analysis of variance (PERMANOVA) was performed to test the significance of the biotic (plant species and rhizocompartment) and abiotic (soil type and soil pollution) factors in bacterial community divergences using 'adonis' test in 'vegan' package [72]. Differences in bacterial community composition between ^{13}C-labelled and ^{12}C-labelled rhizosphere samples and between HP and SP soils in RNA-SIP experiment were tested by analysis of similarities (ANOSIM). The effects of plant species, soil type and rhizocompartment on the differential abundance of bacterial taxa were analyzed using DESeq2 [68], which fits negative binomial generalized linear models to count data (ASVs) and estimates their log2-fold change in abundance across one or more experimental factors.

To reduce rare ASVs in the data set, we only retained ASVs with relative abundance more than 0.01% of the total read count and were found in 20% of the compartment samples of each Cd-accumulating plant. Spearman correlation matrixes were calculated with the 'WGCNA' package [73], and all *P*-values were adjusted for multiple testing using Benjamini and Hochberg false discovery rate (FDR) controlling procedure. Only robust (Spearman's $\rho > 0.6$ or $\rho < -0.6$) and statistically significant ($p < 0.05$) correlations were kept. The co-occurrence network complexity was evaluated by the topological parameters of average degree, betweenness centrality and assortativity according to a previous study [74], with higher average degree, and smaller betweenness and assortativity representing greater network complexity. The nodes with high average degree (> 50), high closeness centrality (> 0.2) and low betweenness centrality (< 5000) were defined as keystone taxa in co-occurrence networks that were visualized in Gephi [16, 75].

The main microbial predictors for root and shoot biomasses, and root and shoot Cd uptake associated with Cd-accumulating plants were identified by a classification Random Forest (RF) regression analysis using the 'randomForest' package [76]. In these RF models, ASVs from the filtered dataset were served as predictors for plant biomass and Cd uptake indexes. The importance of each predictor variable is determined by evaluating the decrease in prediction accuracy (that is, increase in the MSE (mean squared error) of variables: high MSE% values indicate more important variables) when the data for that predictor is randomly permuted. The significance of the importance of each predictor on plant biomass and Cd uptake, and the total variance explained by the predictors was assessed using the 'rfPermute' package [77]. Significance of the models was assessed with 5000 permutations of the response variable, by using the "A3" package. Afterwards, a linear regression model was performed to assess the RF analysis outcome, and to validate the relationships between the core bacterial taxa, plant biomass and Cd uptake.

Results

Microbial composition, diversity and differentially abundant taxa

Principal coordinate analysis (PCoA) and PERMANOVA of the weighted UniFrac distance demonstrated that the primary source of bacterial community β-diversity was soil type (R^2 = 41%, p < 0.001) and plant species (R^2 = 12%, p < 0.001), followed by rhizosphere (R^2 = 6.5%, p < 0.001) (Fig. 1a, b; Table S1). The notable influence of plant interspecies variation on β-diversity was also discernible across the three tested soils, with the largest influence being observed in HP soil (R^2 = 59%, p < 0.001; Fig. 1). Similarly, soil pollution was a strong driver of rhizosphere bacterial communities, solely explaining 31% of the observed community compositional variance (p < 0.001; Table S1). Moreover, α-diversity of the soil bacterial communities significantly varied between plant species (ANOVA, F value = 4.16, p < 0.005 and F value = 5.58, p < 0.001 for Simpson's D^{-1} and Faith's PD, respectively; Fig. 1c, Fig. S2a). Phylogenetic diversity (Faith's pd) also differed among soils (ANOVA, F value = 5.58, p < 0.01) and between pollution levels (Wilcoxon test, p < 0.005) (Fig. 1d, Fig. S2b). Among the 10 most abundant phyla, *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobia* exhibited higher relative abundance while *Firmicutes* and *Planctomycetes* were less abundant in the rhizosphere compared to the unplanted soil [generalized linear model (GLM): p < 0.05 after false discovery rate (FDR) correction] (Fig. S3, S4). Six of the abundant phyla were strongly affected by plant interspecies variation whereas eight of the ten abundant phyla were impacted by the soil (Fig. 1e). Plant biomass, the concentration of Cd in HM-accumulating plants, and differential ASV abundance analysis are detailed in the Supplementary Results and Supplementary Fig. S5 and S6.

Core rhizosphere microbiome

We found a large number of rhizosphere ASVs that were shared among the three tested soils (544 taxa, mean 22.5%, range 20.5–25%; Fig. S7a). There was a small but notable overlap of ASVs among the five Cd-accumulating plants (302 taxa, mean 15.6%, range 14.1–16.5%) (Fig. S7b). Next, we identified the core microbiota for each Cd-accumulating plant based on the following criteria: a) the ASVs that were significantly enriched in the rhizosphere compared to the unplanted soil, b) found in at least 75% of the rhizosphere samples across the soils [11, 19] and c) had an overlapped phylogenetic relatedness with the core genera identified by the indicator species analysis as well [17]. Across the plants, 26 ASVs from 63 bacterial genera and 10 families were identified as the core rhizosphere taxa (Fig. 2a, b), including the abundant *Sphingomonadaceae* (24 bacterial taxa) with a dominant taxon classified as *Sphingomonas* (mean relative abundance of 0.7%) and *Burkholderiaceae* (15 bacterial taxa, mean relative abundance of 0.5%) (Fig. 2; Table S2). When grown in the controlled condition, different plants also harbored unique but overlapping core rhizosphere microbiota across diverse soils (Fig. 3). A large fraction of core ASVs (65%) could be classified at the genus level, with these rhizosphere cores comprising several *Alphaproteobacteria* (*Sphingomonas* and *Rhizobium*) and *Gammaproteobacteria* (*Burkholderiaceae* and *Ralstonia*), followed by *Bacteroidia* (*Flavisolibacter* and *Mucilaginibacter*), Bacilli (*Sporosarcina*), *Actinobacteria* (*Micrococcaceae*), Verrucomicrobiae (*Udaeobacter*) and *Acidobacteriales* (Fig. 3a-e). Interestingly, five core ASVs, affiliated with *Sphingomonas* and *Burkholderiaceae*, were shared among the five Cd-accumulating plants (Fig. 3f).

To unravel the role of root exudation in the assembly of core rhizosphere microbiome, we characterized the soil bacterial community actively utilizing the *S. alfredii* root exudates using RNA-SIP (Fig. 4). Bacterial communities differed between ^{13}C-labelled and ^{12}C-labelled rhizosphere samples (ANOSIM, R = 0.99, P = 0.003), but the structure of ^{13}C-labelled rhizobiomes in HP and SP soils was similar (ANOSIM, R = -0.04, P = 0.56) (Fig. 4c), suggesting a possible host-driven selection for active bacterial rhizosphere microbiota. ^{13}C-labelled rhizosphere samples were significantly enriched for *Proteobacteria*, *Firmicutes* and *Bacteroidetes* compared to the ^{12}C-labelled rhizospheres (GLM: $p < 0.05$ after FDR correction) (Fig. 4c; Table S4). Notably, the most abundant members of the core rhizosphere assemblage, belonging to *Mucilaginibacter*, *Sphingomonas* and *Burkholderiaceae*, were significantly ($p < 0.05$) more abundant in ^{13}C-labelled than ^{12}C-labelled rhizosphere or bulk samples while *Flavisolibacter* and *Micrococcaceae* were present in low abundance in ^{13}C-labelled rhizosphere samples (Fig. 4d, 4e).

Keystone taxa in the core rhizosphere microbiota

Network complexity of the rhizosphere microbiota, as evaluated by average degree, betweenness centrality and assortativity, differed between soils and among the HM-accumulating plants (Fig. S8, S9; Table S3), with the rhizosphere having a lower network complexity than the unplanted soil (Fig. S9). The number of negative associations greatly increased from unplanted soil to the rhizosphere, suggesting a stronger competition for resources (Table S3). Cd-accumulating plant rhizosphere consistently harbored keystone taxa belonging to *Sphingomonas*, *Gemmatimonas*, *Bryobacter*, *Bradyrhizobium* and *Rhizobium* (Fig. S10), with *Gemmatimonas* common across five Cd-accumulating plants and *Sphingomonas* and *Bryobacter* common across four. Some of the rhizospheric keystone taxa, such as *Sphingomonas*, *Rhizobium*, *Mucilaginibacter*, *Udaeobacter* and *Burkholderia*, were also part of the core microbiota (Fig. S10a-e), suggesting their consistent occurrence in the community.

Core rhizosphere microbiota and plant performance

Random Forest (RF) models indicated that a larger number of core rhizosphere ASVs were identified as important predictors for plant biomass compared to plant Cd accumulation (Fig. S11-14). Seven core ASVs belonging to *Burkholderiaceae*, *Ralstonia* and *Mucilaginibacter* were notable predictors of root biomass, and six of them (besides *Ralstonia*) were positively ($p < 0.05$) related to root biomass (Fig. S11a, b). Twelve core ASVs were strong predictors of shoot biomass, with six of these affiliated with *Burkholderiaceae*, *Sphingomonas*, *Mucilaginibacter*, *Rhizobium* and *Rhizobiaceae* showing positive relationships with shoot biomass ($p < 0.05$; Fig. S12a, b). Moreover, seven and nine of the core taxa were found as significant predictors of root and shoot Cd accumulation, respectively (Fig. S13, S14). Two core ASVs identified as *Sphingomonas* and *Micrococcaceae* were strongly associated with Cd accumulation in roots (Fig. S13b), and only one core belonging to *Micrococcaceae* with Cd accumulation in shoots (Fig. S14b). These results suggest the functional importance of core rhizosphere microbiota for plant growth and Cd accumulation.

We further assessed the role of the core microbiota in plant performance using synthetic communities (SynComs) consisting of core isolates (see methods). Our results showed that core SynComs could alleviate root Cd toxicity and increase Cd concentration in the hyperaccumulator plants (Fig. 5). Except for I. mustard shoot biomass, inoculation of SynComs led to a significant increase in the root and shoot biomasses ($p < 0.05$; Fig. 5a-c). Apart from *S. alfredii*, the inoculation of SynComs enhanced root and shoot Cd concentration compared to only Cd treatment ($p < 0.05$; Fig. 5d, e). Moreover, except for *S. nigrum*, SynComs also reduced the concentrations of H_2O_2 and superoxide anion in roots ($p < 0.05$; Fig. 5f, g). Overall, these results demonstrate that the core rhizosphere microbiota of HM-accumulating plants play an important role in host performance and fitness.

Discussion

We show that the core microbes of heavy metal-accumulating plants comprise members that are persistently maintained in the rhizospheres. This corresponds with our careful selection of the core microbiota [13, 28]. Differing from previous studies that mainly identified core microbial members using a single criterion [11, 18, 29, 30], our comprehensive approach found that all of the core ASVs observed using a 75% representation and enrichment in the core taxa analysis completely overlapped phylogenetically with those identified with the indicator species analysis, suggesting the composition of core rhizosphere microbiota observed here is robust to different analytical methods used [28]. Maintenance of the core cohorts in the rhizosphere of diverse host plants highlights that these core taxa might have adapted to the conserved host features, such as host metabolic traits and cell wall components, and thus can overcome the context dependency of microbiome composition and contribute to the fitness and health of 'holobiont' [13, 17]. Moreover, a large proportion of the core ASVs (~65%) have a genus-level classification, indicating that many of the core microorganisms of Cd accumulating plants have been isolated and/or characterized previously, providing a list of potential targets for application in metal phytoremediation research.

Microorganisms co-occurring in the rhizosphere can form highly complex and interconnected networks [31]. Keystone taxa can exert strong direct and indirect effects on microbiome structure and mediate plant-microbiome interplays, and thus are critical for plant performance [16, 32]. Among the keystone taxa identified here, members of *Sphingomonas*, *Rhizobium*, *Mucilaginibacter*, *Udaeobacter* and *Burkholderia* were also part of the core rhizosphere microbiota. These results are consistent with a recent report that found members of *Rhizobium* and *Burkholderia* as keystone taxa across studies [16], suggesting that the close association between these two lineages and their adaptability to a wide array of host plants. The contribution of keystone taxa observed here may be particularly important as they are also part of the core microbiome [16], highlighting a possibility of utilizing such taxa as the pioneer microbes in the remediation of metal-disturbed soils.

It has been proposed that the ability of community members to metabolize root exudates and microbe-microbe interactions might be the basis of the development of core root microbiota [33]. However, these ideas were postulated based on literature and needed experimental validation. By employing RNA-SIP, we

provide the empirical evidence for root exudate-driven assembly of the abundant core taxa, including *Burkholderiaceae* (β-proteobacteria), *Sphingomonas* (α-proteoabcteria) and *Mucilaginibacter* (*Bacteroidetes*), highlighting the important role that host metabolism plays in the selection and assembly of the core microbiome. The active rhizosphere bacteria (^{13}C-labelled) was overrepresented by *Proteobacteria*, *Bacteroidetes* and *Firmicutes* (Fig. 4c). Members of these phyla, especially the α-proteoabcteria and β-proteobacteria, are known to be copiotrophs, that is, they compete successfully in the rhizosphere zone enriched with root exudates [34]. Dominance of the active rhizosphere microbiome by these copiotrophs reflects their preference for specific plant metabolites [10]. Moreover, the increasing number of negative associations from unplanted soil to the rhizosphere networks coupled with the reduced rhizospheric α-diversity provides evidence of the stronger microbial competition and selection near the root zone. The microbe-microbe interactions can subsequently stabilize the composition of core communities acquired, and thus contributing to their persistence in a given habitat [35].

The core root microbiome might have evolved with their host plants, and their persistent occurrence and functions can ensure plant growth and health [14, 17]. Some of the core taxa identified here overlapped with those detected in previous studies on *Arabidopsis* [30], sugarcane [11], cooloola [17] and citrus [19], suggesting the existence of a conserved microbiome assembly across different soils. Of the core cohorts detected, particularly the shared ones among Cd-accumulating plants, are known to promote HM-accumulating plant growth and improve phytoextraction efficiency [36]. For example, *Sphingomonas*, *Burkholderiaceae*, and *Rhizobium* are well-known root-inhabiting bacteria and have been found to carry traits enhancing plant growth and fitness either through producing indole acetic acid or nitrogen fixation [37–39]. Specifically, *Sphingomonas* was the main constituent of the root-associated bacterial assemblages that undergo phytoextraction of metals [40], and its superior ability to facilitate Cd/Zn absorption and root-to-shoot transport, and reduce plant metal toxicity has made it an plausible candidate for developing a microbiome-based phytoremediation strategy [41, 42]. Members of *Burkholderia* have been shown to promote metal accumulation by increasing plant growth and HM availability [43]. *Rhizobium* lineages are best known as root-nodulating bacteria, and supply their hosts with biologically fixed nitrogen [44], whereas *Mucilaginibacter* has been shown to alleviate the salt stress experienced by *Arabidopsis thaliana* [45]. In addition to reporting beneficial traits from previous studies, the role of core microbiota in metal accumulation was also confirmed by Random Forest and linear regression analyses (Fig. S11-14).

The identification of the co-occurring core microbes provides a useful starting point for future studies to build a core synthetic community (SynCom) to manipulate plant-microbe interactions for improved phytoextraction performance and the restoration of disturbed ecosystems. By using the SynCom approach, we provided preliminary evidence supporting the beneficial effect of the core microbiota on Cd accumulation and host fitness. The *S. alfredii*-enriched SynComs consisting of the shared core rhizosphere bacteria affiliated with *Burkholderia* and *Sphingomonas* resulted in the increased Cd accumulation and root Cd stress resistance. Along with these results, we show that despite measurable edaphic differences between soils and the interspecies rhizosphere microbiota diversification in

composition, diversity and network architecture, the Cd-accumulating plants recruit a core set of bacterial taxa that could be functionally important for plant performance and fitness. This finding could, at least in part, support the notion that the core microbial functions are coded by functional genes (replicators) distributed among key microbial taxa that can vary across soils [14], so that a functional core microbiota may be driven by the traits of the particular hosts regardless of the soil. Future studies will provide a mechanistic understanding of how plant traits mediate the recruitment of beneficial replicators, and strategies for incorporating the core microbiome for ecosystem restoration and sustainable agriculture.

Our study provides an insight into the influence of major biotic (e.g. rhizosphere and plant species) and abiotic (e.g. soil type and soil pollution) factors shaping the rhizosphere microbiota of Cd-accumulating plants. Altogether, these factors explained 60% of the community compositional variation, which represents a higher explanatory power compared to previous observations [11, 35, 46, 47]. As reported in other rhizosphere microbiome studies, soil type emerged as the strongest determinant of microbial variations [30, 33, 48, 49]. Soil is not only the predominant microbial inoculum for rhizobiome, but also defines the nutrient status for plants, which in turn affects plant growth, root architecture, exudation, and microbiome assembly [50, 51]. In addition, plant species was the second most important factor that overwhelms rhizocompartment that explained 12% of the rhizobiomes variation. Notably, our finding contrasts with previous studies in *Arabidopsis thaliana*, its relatives, rice, barley and Cd-accumulating plant *S. alfredii*, where bacterial root communities were strongly affected by the edaphic properties, but host genotype or cultivar had a limited effect [30, 33, 35, 48]; specifically, the species effect size was clearly higher than those reported for corn (5-8%; *Zea mays*), barley (~6%; *Hordeum vulgare*), rice (3-5%; *Oryza sativa*) and *S. alfredii* (~4.7%). These observations suggest that host phylogeny was the major driver of the interspecies root microbiota diversity in Cd-accumulating plants. This discrepancy in the influence of host plants on rhizobiomes between different plants could be attributed to the smaller differences in the host effect at the genotype or ecotype level within *Arabidopsis*, rice and barley than at the species level in our study [30, 35, 48, 50]. Thus, it is possible that host species effects on microbial community structure may be stronger between more distantly related plant species [52, 53]. As found in the RNA-SIP experiment, the similar active rhizosphere microbiota observed in HP and SP soils suggest that *S. alfredii* recruited a subset of persistent rhizobiomes across different soils through root exudation independent of soil types (Fig. 4). Hence, the notable plant species effect on the rhizosphere microbiota could be closely linked to the quantity and quality of root exudates, i.e., chemically diverse constituents consistently enrich specific microorganisms by stimulating their growth and/or by inducing or repressing specific microbes that can facilitate host fitness and performance [51]. A deeper insight into how host plant variation affects root microbiota composition will require characterization of root metabolites and exudates.

Plant-associated microbiota is thought to play an important role in promoting host growth and fitness, but the potential of harnessing these microbes is largely impeded by the challenge of identifying the consistently important members such as the core microbiota and keystone taxa. Using HM accumulating plants as a model, our study offers the first evidence of how the functional core microbiota can be harnessed to improve host performance in metal contaminated soils. The findings of this study can be

applicable to many types of land plants, e.g. model and crop plants, and would inform future efforts to identify the metabolic cues that assemble the core microbiota in the rhizosphere, which could help address the problem of low phytoremediation efficiency [3] and accelerate the restoration of contaminated soil ecosystems [8].

Declarations

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Author's contributions

JPL and TQL conceived and designed the research. JPL, XYG and YKL performed the experiments and collected the data. JPL, SHG and SB were responsible for the bioinformatics processing and data analysis. JPL, SHG, SB, QT, YCL and TQL wrote the manuscript with contribution of all of the coauthors.

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Availability of data and materials

Sequencing reads of samples from the Cd-accumulating plants core rhizosphere microbiome experiments have been deposited in the National Center for Biotechnology Information Search database (NCBI) Sequence Read Archive under Bioproject PRJNA691442 and BioSample accession numbers from SAMN17293202 to SAMN17293206, SAMN17293212 to SAMN17293216, SAMN17293222 to SAMN17293226, SAMN17293232 to SAMN17293236, SAMN17293242 to SAMN17293246, SAMN17293252 SAMN17293256, SAMN17293262 to SAMN17293266, SAMN17293272 to SAMN17293276, SAMN17293282 to SAMN17293286, SAMN17293292 to SAMN17293336.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

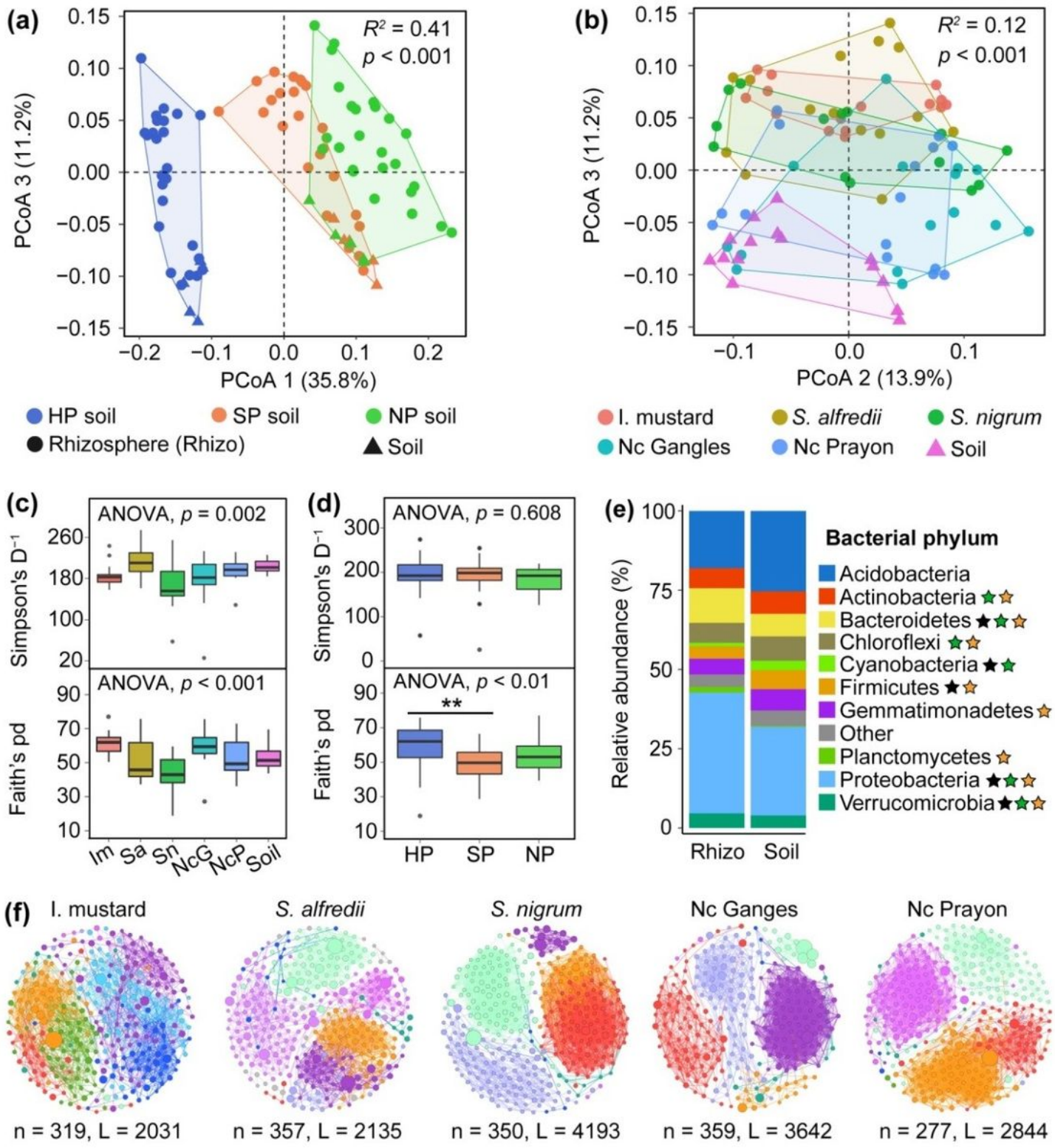

Figure 1

Bacterial composition, diversity and co-occurrence network of the rhizosphere microbiota of divergent Cd-accumulating plants as affected by soils and host species. Principal Coordinate Analysis (PCoA) ordination based on the weighted UniFrac distance highlights that soil variation (soil's geographic origin and soil pollution level) (a) and plant species (b) are important factors structuring bacterial communities. The R squared and p values represent the significance of variation in soils and plant species, as

evaluated by PERMANOVA. Bacterial α-diversity differed among plant species (c), soil types, and pollution (HP and SP) levels (d). Statistical comparison in α-diversity between HP and SP soil was determined by Wilcoxon test and are indicated with asterisks, ** $p < 0.01$. (e) The abundance of bacterial phyla were significantly (GLM: $p < 0.05$) differed between rhizosphere and unplanted soil (black star), and were affected plant species (green star) and soil effect (orange star). (f) Rhizosphere co-occurrence networks were affected by host species; N and L indicate nodes and edges, respectively. Node colors indicate modules.

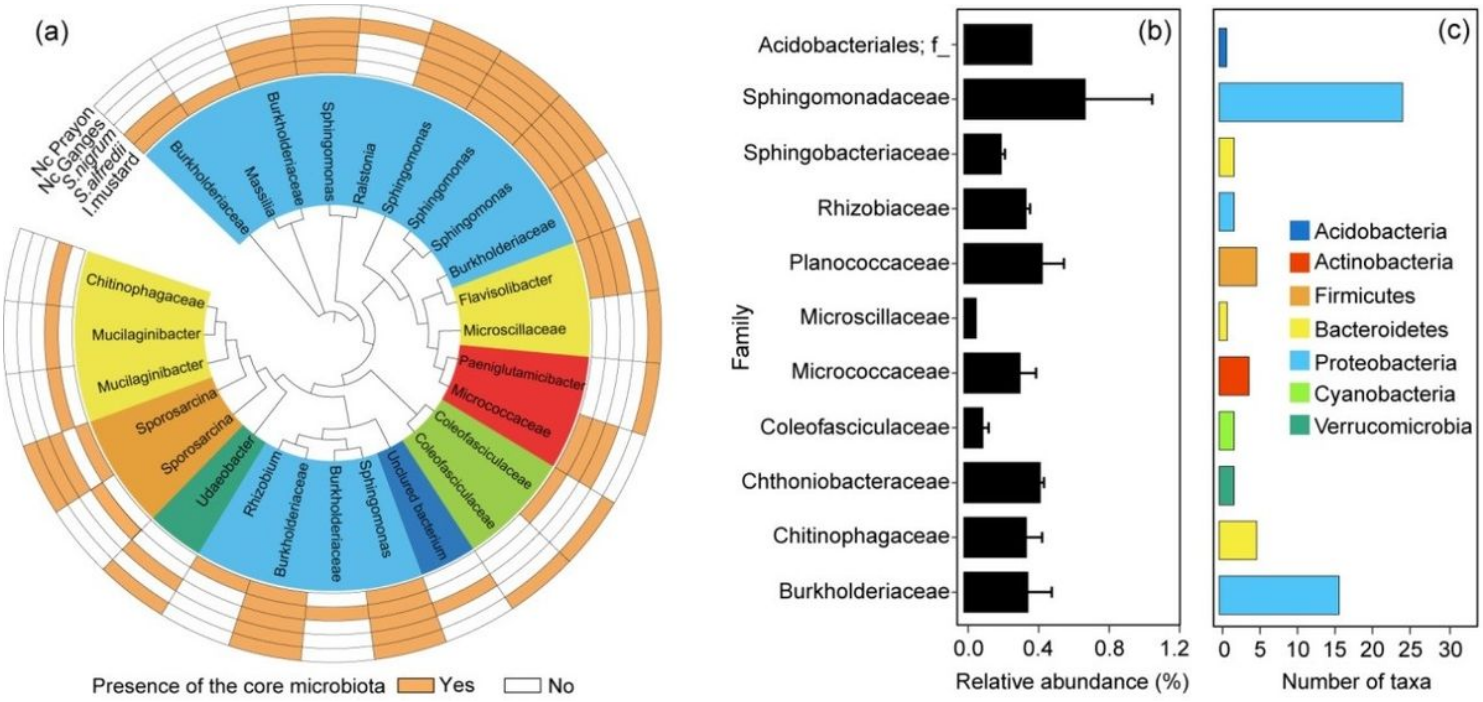

Figure 2

A core set of bacterial assemblages consistently detected in the rhizosphere of five Cd-accumulating plants across diverse soils. Cladogram showing the presence of the core bacterial ASVs in the rhizosphere of different Cd-accumulating plants (a). The abundance of these taxa at the family level is represented as bar plots with standard deviation (b), grouped by phylum and number of taxa therein (c).

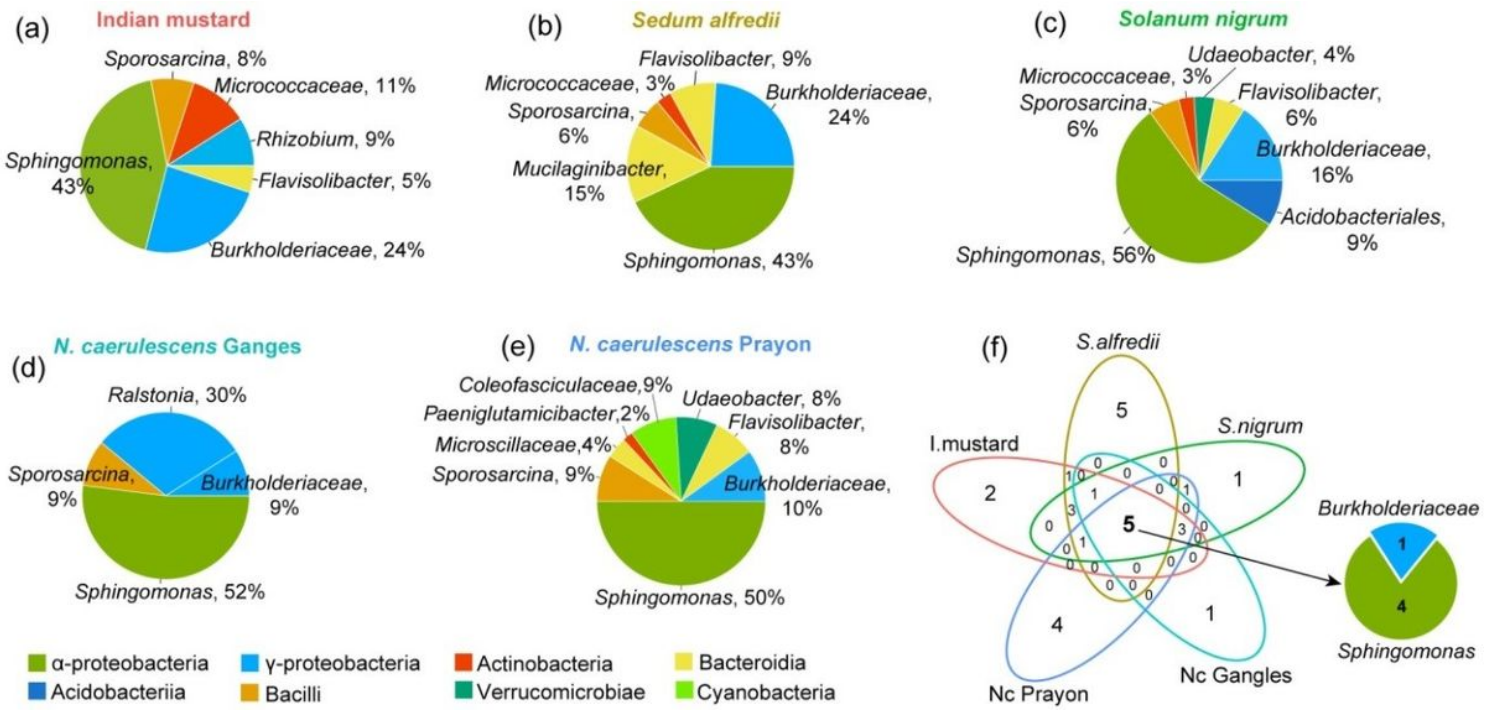

Figure 3

Core microbiome analysis of phylogenetically divergent Cd-accumulating plants and the shared core taxa among these plants. Core rhizosphere microbiome of Indian mustard (a), Sedum alfredii (b), Solanum nigrum (c), and Ganges (d) and Prayon (e) ecotypes of Noccaea caerulescens. The core rhizosphere ASVs shared among the five plants, as depicted by the Venn diagram (f). The relative abundance of the core ASVs within the core microbiome is shown in the pie charts.

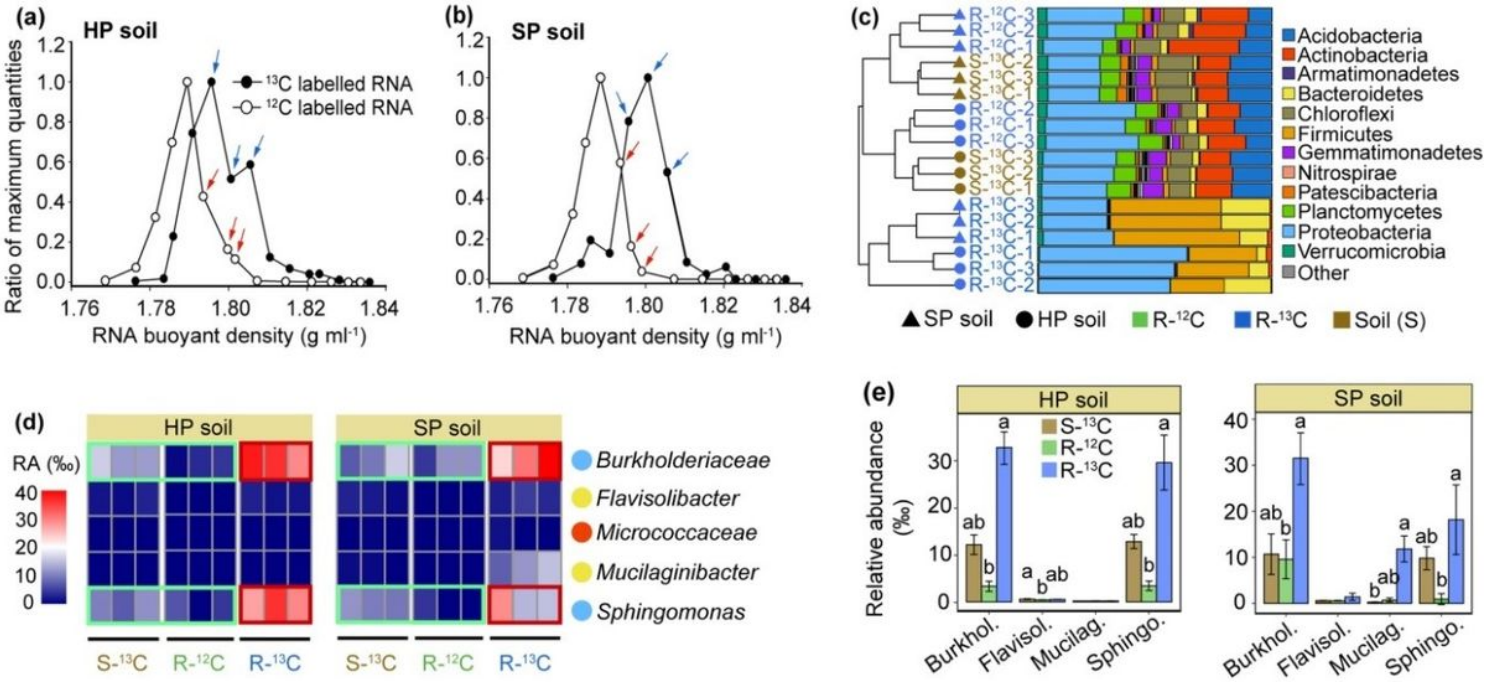

Figure 4

Bacterial communities in 13C-labelled and 12C-labelled rhizospheres and unplanted soil of Sedum alfredii. Quantitative distribution of density-resolved bacterial 16S rRNA gene from HP (a) and SP soils (b). The blue and red arrows indicate 13C-labelled and 12C-labelled RNA fractions used to 16S rRNA gene profiling. Weighted UniFrac clustering of the overall samples and the corresponding phyla abundances are displayed (c). The relative abundance of S. alfredii core rhizosphere ASVs present in CO2 labeling rhizosphere and soil samples (d), and significant differences in their relative abundances between different samples were determined by Kruskal-Wallis test with post hoc by Dunn test (e). Abbreviations: R, rhizosphere; S, unplanted soil; 12C, 12CO2 labeling sample; 13C, 13CO2 labeling sample.

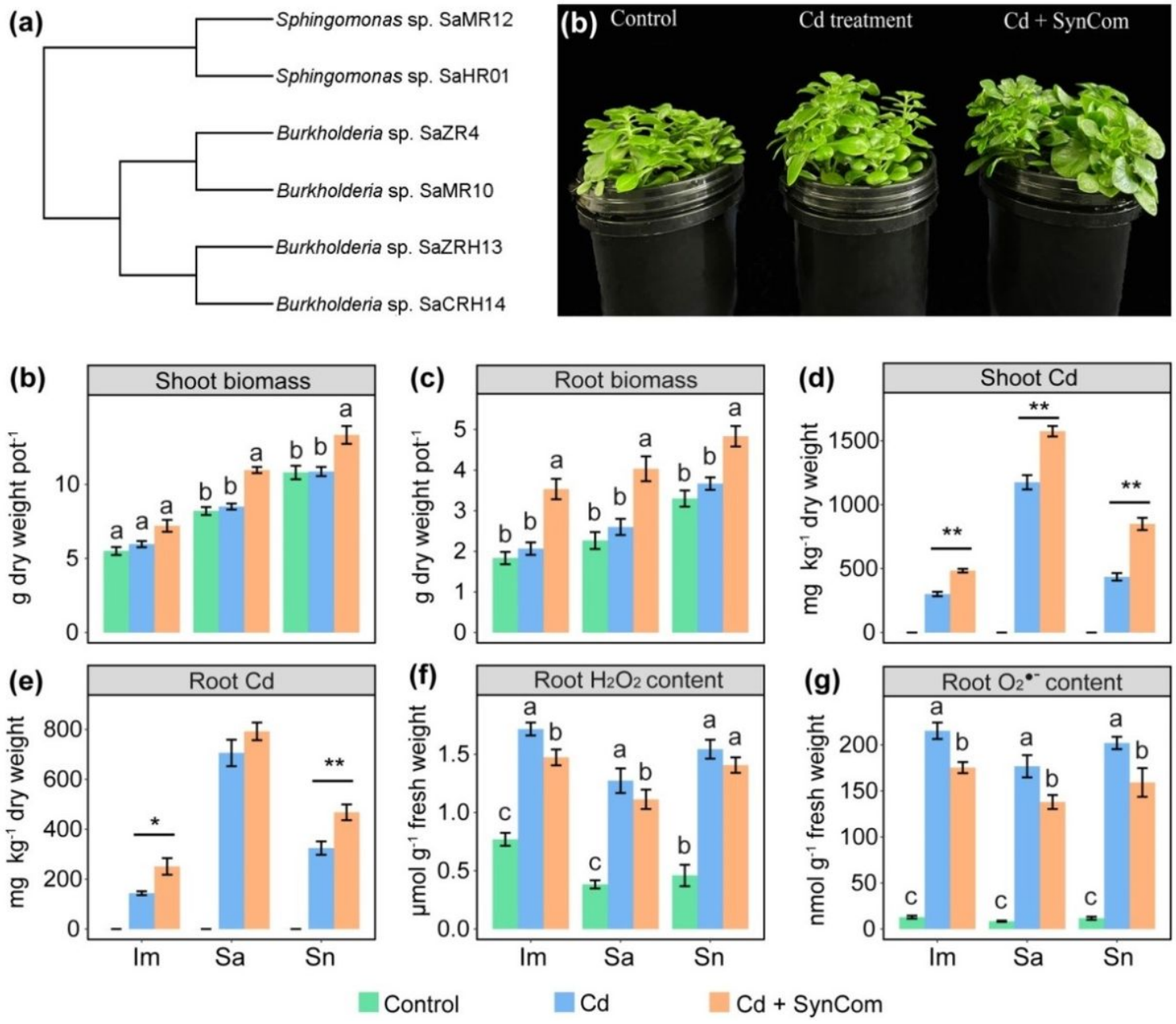

Figure 5

Characterization of the core rhizosphere SynComs enhancing Cd-accumulating plant fitness and Cd accumulation. (a) Phylogenetic tree of the core bacterial strains (Sphingomonas sp. and Burkholderia

sp.) based on the distance values calculated from average nucleotide identity of their full-length16S rRNA gene. (b) Growth status of S. alfredii cultivated in different treatments. Effects of Cd and SynComs on root dry weight (c), (d) shoot dry weight, concentration of Cd in root (e) and shoot (f), and concentration of H2O2 (g) and superoxide anion (h) of different Cd-accumulating plants. Values are mean± s.d. (shown as error bars; n = 5). Letters above bars indicate significant difference according to one-way analysis of variance (ANOVA) with Tukey's HSD test ($P < 0.05$). The asterisks *, and ** represent significant difference according to t-test at $P < 0.05$ and $P < 0.01$, respectively. Im, Indian mustard; Sa, Sedum alfredii; Sn, Solanum nigrum. ND, not detected.

Supplementary Files

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- SupplementaryMaterials.docx