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Article

Shifts in a Phenanthrene-Degrading Microbial Community are Driven by Carbohydrate Metabolism Selection in a Ryegrass Rhizosphere

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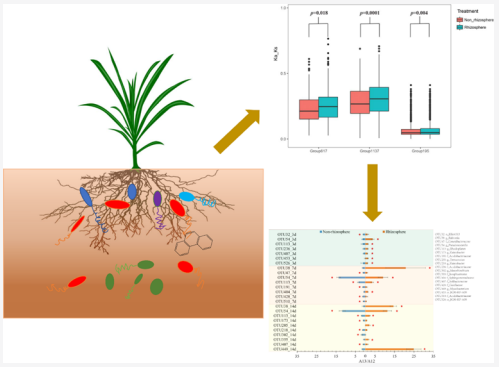
ABSTRACT: Plants usually promote pollutant bioremediation by several mechanisms including modifying the diversity of functional microbial species. However, conflicting results are reported that root exudates have no effects or negative effects on organic pollutant degradation. In this study, we investigated the roles of ryegrass in phenanthrene degradation in soils using DNA stable isotope probing (SIP) and metagenomics to reveal a potential explanation for conflicting results among phytoremediation studies. Phenanthrene biodegradation efficiency was improved by 8% after 14 days of cultivation. Twelve and ten operational taxonomic units (OTUs) were identified as active phenanthrene degraders in non-rhizosphere and rhizosphere soils, respectively. The active phenanthrene degraders exhibited higher average phylogenetic distances in rhizosphere soils (0.33) than non-rhizosphere soils (0.26). The K_a/K_s values (the ratio of nonsynonymous to synonymous substitutions) were about 10.37% higher in the rhizosphere treatment among >90% of all key carbohydrate metabolism-related genes, implying that ryegrass may be an important driver of microbial community variation in the rhizosphere by relieving the carbohydrate metabolism pressure and improving the survival ability of r-strategy microbes. Most K_a/K_s values of root-exudate-related metabolism genes exhibited little change, except for fumarate hydratase that increased 13-fold in the rhizosphere compared to that in the non-rhizosphere treatment. The K_a/K_s values of less than 50% phenanthrene-degradation-related genes were affected, 30% of which increased and 70% behaved oppositely. Genes with altered K_a/K_s values had a low percentage and followed an inconsistent changing tendency, indicating that phenanthrene and its metabolites are not major factors influencing the active degraders. These results suggested the importance of carbohydrate metabolism, especially fumaric acid, in rhizosphere community shift, and hinted at a new hypothesis that the rhizosphere effect on phenanthrene degradation efficiency depends on the existence of active degraders that have competitive advantages in carbohydrate and fumaric acid metabolism.



INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants with carcinogenic, teratogenic, and mutagenic properties, posing a serious risk to human health.[1] Bioremediation has been performed for decades, aiming for low cost and environment-friendly PAH remediation. Microbes play an important role in organic pollutant degradation owing to their high diversity, activity, and frequency in soil.[2,3] A wide variety of bacterial, fungal, and algal species have recently been reported to degrade PAHs, and their degradation pathway, gene organization, enzyme system, and genetic regulation have been explored in great detail.[4]

Plant-assisted microbial degradation is an attractive bioremediation strategy because various plants such as ryegrass, willow, and maize can significantly improve the degradation efficiency of different organic pollutants in soil.[5−9] Various root exudates such as sugars, organic acids, and proteins[10,11] can enhance PAH degradation efficiency by increasing the microbial population and the abundance and diversity of degradation genes,[12,13] as well as promoting the development of organic-pollutant-degrading enzymes.[14] Some conflicting reports document that rhizosphere has no effect or negative effect on PAH degradation.[12,15] Therefore, a better understanding of plant-assisted microbial degradation is yet to be determined; such a discovery would deepen our insights into the roles of plants and microbes in the natural environment and improve plant−microbe degradation efficiency.

Two barriers have prevented the accurate determination of rhizosphere effects on PAH degradation to date. Firstly, it is necessary to identify PAH degraders *in situ* using a cultivation-independent method because previous studies have questioned

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whether the degradation ability of isolated microbes can be reproduced in their primal environments.[16,17] DNA-stable isotope probing (DNA-SIP) directly links microbial identity to function by incorporating stable isotopes such as ^{13}C and ^{15}N from target compounds into the microbial DNA.[18] Coupled with metagenomics, SIP has been used to reveal detailed information about functional microbes, *e.g.*, genomic information in uncultivated active methanotrophs[19] and metabolic potential in iron-reducing bacteria involved in anaerobic aromatic hydrocarbon degradation.[20] For studies on organic pollutant degradation in the rhizosphere, SIP has identified organic pollutant degraders[21,22] and examined the plant effects on the diversity and composition of degraders.[12,23] Combining DNA-SIP and metagenomics would be useful to explore the underlying mechanisms of regulating the active degraders in the rhizosphere.

The second challenge in examining rhizosphere effects on PAH degraders is appropriate analytical tools revealing the mechanisms. Although previous studies focus on the relationships between functional microbes and gene abundance,[24,25] there is hardly any studies addressing the response of functional genes to the unique microenvironment in the rhizosphere. Deeper insights are suggested to address functional proteins that are constituted by amino acids corresponding to codons on genes. As different codons can transform to the same amino acids, codon changes leading to amino acid alteration are nonsynonymous (K_a), whereas the others are named synonymous (K_s). The ratio of K_a to K_s then provides more information on microbial functions and is frequently used to indicate the selective forces acting on proteins.[26] For most sequences, K_a is much smaller than K_s because selection tends to eliminate deleterious mutations, maintaining the protein in its present form and function.[26] When an environmental variable is a limiting factor and critical for microbial structure assembly, genes responsible for such a variable tend to keep an efficient function and maintain a low K_a/K_s value. On the contrary, the absence of the selection pressure from the alleviated environmental variables can drive the increase in the K_a/K_s values of the corresponding genes. The K_a/K_s value is therefore an indicator illustrating the links between the rhizosphere selection pressure and functional genes, as an alternative method to determine the major environmental variables influencing PAH degrading microbial community in the rhizosphere.

In this study, we used phenanthrene and ryegrass as a model PAH compound and plant species in phytoremediation to investigate the rhizosphere effects on PAH degraders. Both DNA-SIP and metagenomics were employed to evaluate the phenanthrene degradation performance and active degraders. By studying the structure, functions, functional genes, and metagenomes of the active phenanthrene degraders in the rhizosphere and non-rhizosphere soils, we attempted to reveal the underlying mechanisms of rhizosphere effects on phenanthrene degraders. Our results contribute to new knowledge on the relationships between plants and microbes and benefit future phytoremediation efforts.

MATERIALS AND METHODS

Plant Cultivation and Soil Sampling. Soil was collected from the South China Agricultural University farm and sieved through a 2 mm mesh. The collected soil was split into planted and unplanted groups of pots, each containing 1000 g of soil. Deionized water was used to maintain the soil moisture at 60% of its water holding capacity during 45 days of cultivation. In the planted group, 20 of 5 days ryegrass seedlings (~7 cm) were transplanted into each pot. Each group was prepared in triplicate. During harvesting, we vigorously shook the plants and collected rhizosphere (R) soils adhering to the roots, and non-rhizosphere (NR) soils were collected as a control. Both R and NR soils were prepared for further SIP experiments.

To confirm SIP experiment results that carbohydrate metabolism, especially fumaric acid metabolism, is important for microbial community construction, R soils from 10 different plants (cucumber, lettuce, maize, peanut, rice, ryegrass, rape, soybean, sunflower, and tomato) and their control (soils in NR treatment) were collected after 45 days of cultivation. These plants were cultured in pots containing 1000 g of soil with moisture at 60% by spraying deionized water. The plant sizes are shown in Figure S1. NR and R soils were harvested and subjected to DNA extraction, 16S rRNA gene sequencing, and the further analysis. All treatments were prepared in triplicate.

Root Exudate Analysis. After planting ryegrass in soils for 45 days, the ryegrasses were shaken carefully until the soils detached from roots and were cultured in water for 12 h. The water containing root exudates was freeze-dried to ~10 mL and filtered using a 0.22 μm filter membrane. One-half of the concentrated solution was further concentrated using a stream of high-purity nitrogen gas and dissolved in 500 μL of ethyl acetate. The extract was derivatized using *N*,O-bis-(trimethylsilyl)trifluoroacetamide containing 1% of trimethylchlorosilane and pyridine for 3 h at 70 °C. The silylated product (sugar) was analyzed on an Agilent 7890 gas chromatograph equipped with a DB-5MS column (30 m × 0.25 mm × 0.25 μm). The residual concentrated solution was used for organic acid analysis by high-performance liquid chromatography (HPLC) equipped with an XB-18 column (250 mm × 4.6 mm × 5 μm). The root exudate concentration unit is 1 μg root exudates per 1 g fresh weight (FW) of the whole plant for 12 h.

Microcosm Cultivation. For both R and NR soils, microcosms were cultivated with 5 g of soil, 5 mL of sterile water, and 50 μg of [U-^{13}C14]-labeled or -unlabeled phenanthrene in a 150 mL serum bottle with a final phenanthrene concentration of 10 μg/g. The amended concentration of phenanthrene is comparable to previous field studies with the same order of magnitude[27−29] where the maximal concentration was 3.4 μg/g. The three treatments in each group included sterile soils with ^{12}C-phenanthrene (negative control), original soils with ^{12}C-phenanthrene, and original soils with ^{13}C-phenanthrene. The sterile soils were prepared by sterilizing at 121 °C for 30 mins every 12 h over 24 h. Each treatment was performed in triplicate. All serum bottles were sealed with sealing films, allowing the free exchange of air. Microcosms were incubated at 25 °C with shaking at 120 rpm and then harvested on days 3, 7, and 14. After sampling, soils were stored at −80 °C until further analyses.

Phenanthrene Analyses. Phenanthrene analyses were performed as previously described.[30] Briefly, 1 g of freeze-dried, homogenized, and pulverized soil was spiked with 1000 ng of deuterated phenanthrene as surrogate standards and extracted for 48 h with dichloromethane in a Soxhlet apparatus. Active copper was applied to remove sulfur. The extract was concentrated and purified in a multilayer column filled with neutral alumina (3% deactivated), neutral silica gel

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(3% deactivated), and anhydrous Na_2SO_4. Then, we added 1000 ng of hexamethylbenzene to 0.5 mL of the concentrated extract under a gentle stream of N_2 before measurement on an Agilent 7890 gas chromatograph equipped with a DB-5MS column (30 m × 0.25 mm × 0.25 μm).

DNA Extraction and Centrifugation. On days 3, 7, and 14, DNA from each microcosm was extracted using a PowerSoil DNA Isolation Kit following the manufacturer's instructions. The DNA concentration quantification was performed using Qubit 3.0. Approximately, 5 μg of DNA was mixed with tris-ethylenediaminetetraacetic acid (EDTA) buffer and CsCl solution with a final buoyant density (BD) of 1.77 g/mL, and transferred to Quick-Seal polyallomer tubes. After sealing, tubes were centrifuged at 20 °C for 48 h at 178 000*g*. We collected 15 fractions with centrifuged gradients from each tube and measured these for BD using an AR200 digital refractometer as previously described.[16] CsCl in each fraction was removed using a MicroElute DNA-Pure Kit.

16S rRNA Gene Quantification, Amplification, and DNA Sequencing. We targeted the 16S rRNA gene using the primer pair 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Real-time quantitative polymerase chain reaction (realtime-qPCR) was performed to detect the abundance of 16S rRNA genes in each fraction. The reaction solution was a 20 μL mixture containing 10 μL of SYBR green PCR Premix Ex Taq II, 0.2 μL of each primer, 1 μL of DNA template, and 8.6 μL of H_2O. The thermal cycling program was as follows: 2 min at 95 °C for denaturation, 40 cycles of 10 s at 95 °C, 30 s at 56 °C, and 20 s at 72 °C, followed by 55−95 °C for melting curve analyses. The SYBR green signal was measured after the 72 °C step in each cycle. A recombinant plasmid containing the 16S rRNA gene fragment was serially diluted from 10^9 to 10 copies/μL as a standard curve. According to the copy number of 16S rRNA genes in each fraction, light (fractions 5−7) and heavy (fractions 9−11) DNA fractions were selected for further analyses (Figure S2).

The amplicons of 16S rRNA gene for sequencing in the light and heavy DNA fractions were obtained using the primers described above. We labeled 806R with a 12 bp barcode to separate the sequences in one sample from those in others. The reaction mixture contained 10.5 μL of H_2O, 12.5 μL of rTaq premixed buffer, 0.5 μL of each primer, and 1 μL of template DNA. Each reaction was performed in triplicate with the following thermal cycling program: 95 °C for 5 min, 28 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, followed by 72 °C for 5 min. The triplicate products were pooled and purified using a MicroElute Cycle-Pure Kit. The concentration, purity, and length of amplicons were measured using Qubit 3.0, Nanodrop 2000, and agarose gel electrophoresis, respectively. The products of each sample were combined in approximately equimolar amounts and sent to Biomarker Technologies for amplicon sequencing using the Hiseq 2500 PE250 high-throughput sequencing system.

For metagenomics sequencing, total DNA from R and NR treatments and ^{13}C-labeled DNA from the heavy DNA fractions (9−11) were sent to Biomarker Technologies using the Hiseq 2500 PE250 high-throughput sequencing system.

Processing of High-Throughput Sequencing Data. For DNA amplicon sequencing data, 16S rRNA gene raw data were assembled using Pear software.[31] The sequence data were validated, filtered, and clustered following the QIIME 2 plugin manual.[32] Briefly, we demultiplexed sequences according to the barcodes, denoised data using the dada2 plugin, and truncated each sequence at the 250 bp position.[33] Taxonomic information of representative sequences was classified based on the q2-feature-classifier plugin. Megan5 software was used for the calculation of functional microbial phylogenetic diversity. The Shannon diversity index and Unifrac distance were calculated using QIIME 2. PCoA was performed based on Unifrac distance[34] using the "vegan" package in R.

For metagenome sequencing data, unpaired reads and sequences with quality scores < 20 and length < 150 bp were removed using the Trimmomatic tool. Then, the quality of short sequence data was evaluated using the FastQC tool. About 15 200 503 reads per sample were obtained. To obtain high-quality contigs, all paired-end reads from different samples were assembled together using the MEGAHIT assembler.[35] This process yielded 1,343,862 contigs that were >500 bp in length in the metagenomics library. Prokka software was used for open reading frame identification.[36] Then, the open reading frames were annotated by HUMAnN3.[37]

Genes with different abundances between NR and R treatments were identified by R using "edgR" package, annotated by HUMAnN3 based on Uniref90, and transferred to the related KO numbers in Kyoto Encyclopedia of Genes and Genomes (KEGG) database for further analysis. To investigate the key genes affected by ryegrass rhizosphere on a proportional rather than absolute basis, we normalized gene abundance as follows

$$H = \frac{|R_{abu} - NR_{abu}|}{R_{abu} + NR_{abu}} \quad (1)$$

where H is the normalized abundance of a single gene, and R_{abu} and NR_{abu} are gene abundances in R and NR treatments, respectively.

The normalized abundance of a single module (M) is calculated as follows

$$M = \sum_{i=1}^{n} H_i \quad (2)$$

where n is the number of genes within the module.

Phenanthrene-degradation-related genes, including upstream genes encoding phenanthrene dioxygenase and downstream genes for both phthalate (phthalate-3,4-dihydrodiol dehydrogenase and phthalate-4,5-dioxygenase genes) and salicylate (salicylate hydroxylase gene) pathways were annotated based on local databases containing functional protein sequences downloaded from the National Center for Biotechnology Information (NCBI) and UniProt (Table S1). After blasting with Blast 2.5.0 software,[38] protein sequences with *e*-values < 10^{-10} and sequence lengths > 50 aa were extracted and annotated as phenanthrene-degradation-related enzymes. Their encoding genes were also extracted and used for further analyses. The other genes were extracted from annotation data based on the PAH degradation pathway (map00624) in the KEGG database.

The carbohydrate metabolism modules showed the greatest difference among all metabolism modules between R and NR treatments for both the active phenanthrene degraders and the whole microbial community. Among the top 10 metabolic modules with altered abundance, carbohydrate-metabolism-related modules were identified as citrate cycle (tricarboxylic acid (TCA) cycle), reductive citrate cycle, glycolysis

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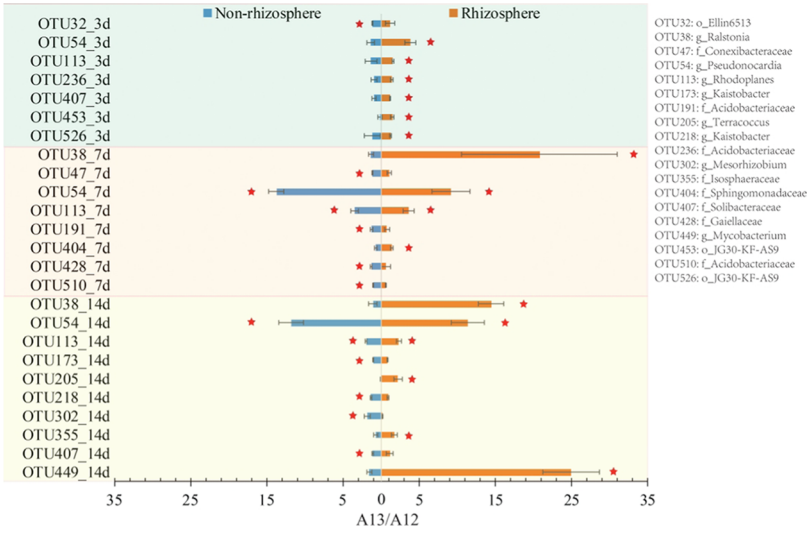

Figure 1. A13/A12 ratios of phenanthrene degraders in non-rhizosphere (NR) and rhizosphere (R) treatments. All the OTUs listed represented the active phenanthrene degraders, and the asterisk indicates their functions at specific time points or in diffeent treatments.

(Embden−Meyerhof−Parnas pathway), reductive pentose phosphate cycle, and pentose phosphate pathway. TCA, glycolysis, and pentose phosphate cycles were selected for further study because microbes often act as decomposers in the soil environment and they are suitable for analyzing the degradation process. The pace of the TCA cycle is determined by the citrate synthase reaction,[39] and the activity of 6-phosphofructokinase catalyzing the rate-limiting step is used as a glycolysis indicator in microorganisms.[40] Glucose-6-phosphate dehydrogenase is the rate-limiting enzyme of the pentose phosphate pathway and can also produce the principal intracellular reductant.[41] Thus, genes encoding citrate synthase, 6-phosphofructokinase, and glucose-6-phosphate dehydrogenase were extracted for K_a/K_s analyses.

Gene counts were obtained by mapping the high-quality reads to assembled contigs using Bowtie2 software.[42] The counts were standardized to parts per hundred million (pphm) for gene abundance comparison. The K_a/K_s values for each group were calculated using KaKs_Calculator 2.0 software[43] after gene group clustering using the OrthoMCL algorithm.[44]

Statistical Analyses. To identify the active phenanthrene degraders in both NR and R treatments, we calculated A13/A12 for each operational taxonomic unit (OTU) according to a previous protocol.[45] A13 and A12 represent the ratio of OTU abundance in the heavy DNA fraction to that in the light DNA fraction derived from ^{13}C-labeled treatments and unlabeled treatments, respectively (eq 3). Here, ABU_{12H} and ABU_{13H} represent the OTU abundance in the heavy DNA fraction from unlabeled and ^{13}C-labeled treatments; ABU_{12L} and ABU_{13L} represent the OTU abundance in the light DNA fraction from unlabeled and ^{13}C-labeled treatments, respectively. OTUs involved in phenanthrene degradation have A13/A12 values significantly higher than 1.0, as evaluated using *t*-test in the R software environment at a significance level of 0.05. Means and standard errors were calculated using the Microsoft Excel 2013 software. The segregation of microbial communities from original soil, NR, and R treatments was checked by ANOSIM.

$$\mathrm{A13/A12} = \frac{\mathrm{ABU_{13H}/ABU_{13L}}}{\mathrm{ABU_{12H}/ABU_{12L}}} \quad (3)$$

Accession Number. All the high-throughput sequencing data were submitted to NCBI (PRJAN561291 and PRJNA449235).

RESULTS

Root Exudates. Six sugars were detected and quantified in this study (Table S2) and their total concentration was 288 ± 14.17 μg/g fresh weight (FW). Glucose and sucrose were most abundant among them, accounting for 40.5 and 46.2% of the total sugar content, respectively. The concentration of total organic acids (Table S3) was 698.2 ± 26.42 μg/g FW. Oxalic acid was the most abundant organic acid and accounted for 80.16%. Though tartaric acid was the second abundant organic acid, it only made up 8.45% of the total content. Other organic acids were no more than 5% of the total organic acid content.

Microbial Community of R and NR Treatments in the SIP Experiment. Ryegrass was precultured for 45 days and then used for the SIP experiment to investigate the rhizosphere effects of fixed root exudates on microbial communities. In DNA-SIP microcosms, microbes with abundance > 1‰ at the phylum level are illustrated in Figure S3. In R treatments, the rhizosphere environment enriched *Actinobacteria*, *Chloroflexi*, and *Bacteroidetes* but decreased the relative abundance of *Proteobac*teria, *WPS-2*, *Firmicutes*, and *Crenarchaeota*. Additionally, microbial genera with abundance > 1‰ are shown in Figure S4. The relative abundance of *Kaistobacter* and *Agromyces* increased, whereas that of *Rhodanobacter*, *Burkholderia*, and *Gemmata* decreased. At the phylum and genus level, more than 79 and 48% of predominant microbes (>1‰) were enriched or inhibited by ryegrass, indicating an important role of ryegrass root exudates in the soil microbial community structure. By analyzing the whole microbial communities in original soils (CK), after 45-day ryegrass preculture (0 day), and during SIP experiment (3, 7, 14 days), we found remarkable segregation of microbial communities from the original soil, and NR and R treatments (Figure S5, p = 0.003 [CK *vs* NR], 0.004 [CK *vs* R] and 0.001 [NR *vs* R]). In addition, there were remarkable changes in the microbial Shannon index decreasing from 7.61 (original soils and pre_0 day) to 6.13 in NR treatment (p = 0.0007) and 6.09 (p = 0.0014) in R treatment (Figure S6), whereas it did not change

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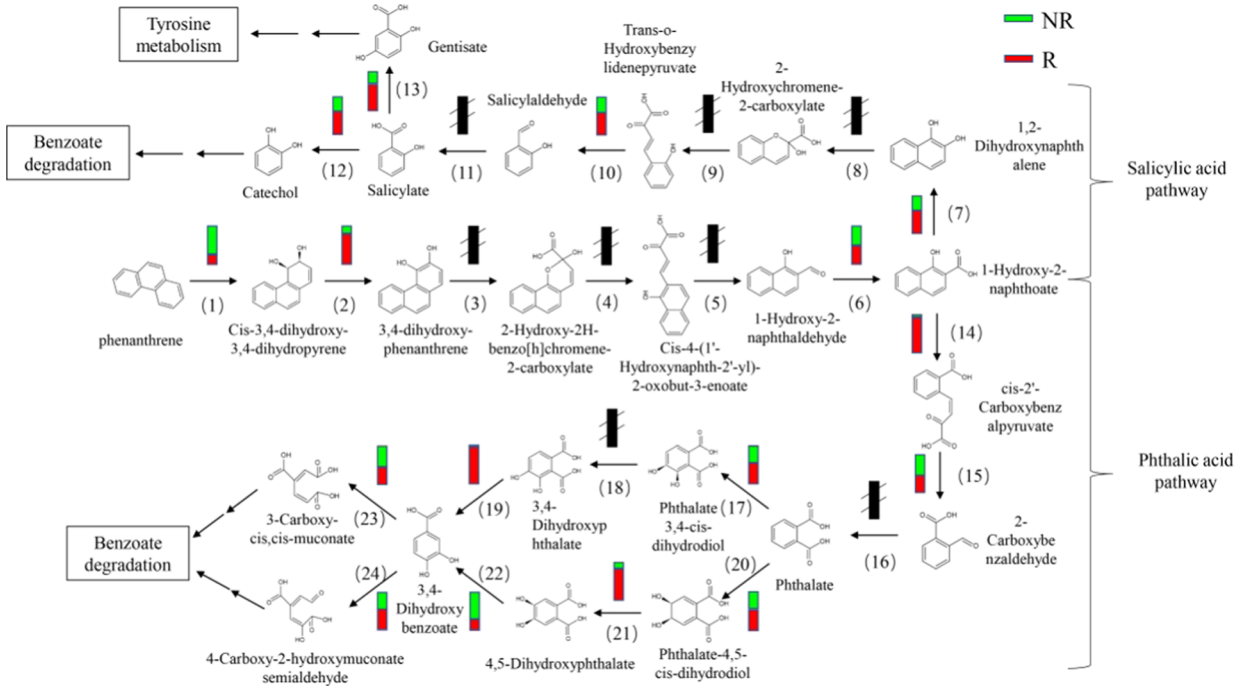

Figure 2. Phenanthrene degradation pathway. Rectangles stand for the degradation gene abundance. The black rectangle represents the undetected genes in samples. The numbers stand for the enzymes and are as follows: (1) PAH dioxygenase, (2) *cis*-3,4-dihydrophenanthrene-3,4-diol dehydrogenase, (3) extradiol dioxygenase, (4) epimerase, (5) hydratase-aldolase, (6) aldehyde dehydrogenase, (7) salicylate hydroxylase, (8) 1,2-dihydroxynaphthalene dioxygenase, (9) 2-hydroxychromene-2-carboxylate isomerase, (10) trans-*o*-hydroxybenzylidenepyruvate hydratase-aldolase, (11) salicylaldehyde dehydrogenase, (12) salicylate hydroxylase (13) salicylate 5-hydroxylase, (14) 1-hydroxy-2-naphthoate dioxygenase (15) 4-(2-carboxyphenyl)-2-oxobut-3-enoate aldolase, (16) 2-formylbenzoate dehydrogenase (17) phthalate 3,4-dioxygenase, (18) phthalate 3,4-*cis*-dihydrodiol dehydrogenase, (19) 3,4-dihydroxyphthalate decarboxylase, (20) phthalate 4,5-dioxygenase, (21) phthalate 4,5-*cis*-dihydrodiol dehydrogenase, (22) 4,5-dihydroxyphthalate decarboxylase, (23) protocatechuate 3,4-dioxygenase, (24) protocatechuate 4,5-dioxygenase.

throughout DNA-SIP experiment in both NR and R treatments ($p > 0.05$). This result hinted that 45-day preculture significantly shaped the soil microbial community structure owing to rhizosphere effects. Such effects lasted for at least 14 days in the SIP experiment after ryegrass was removed, evidenced by both grouped microbial communities in R and NR treatments and Shannon indices.

Phenanthrene Degradation. Phenanthrene degradation curves in the negative control, NR, and R treatments are illustrated in Figure S7. Residual phenanthrene in sterile soils changed little during incubation, indicating negligible abiotic degradation. Microbes played an important role in phenanthrene degradation, as phenanthrene degradation efficiency achieved was 48.3 and 56.3% after 14 days in NR and R treatments (8.0% difference, $p = 0.053$), respectively. Phenanthrene degradation efficiency increased with time; the residual phenanthrene on day 3 was 79.7 and 76.4% in NR and R treatments, respectively (3.3% difference; $p = 0.417$) and it decreased to 67.5 and 61.2% (6.3% difference; $p = 0.058$) after 7 days of incubation.

Active Phenanthrene Degraders Identified by DNA-SIP Were Affected by Ryegrass. In NR and R treatments, 12 and 10 OTUs were identified as the active phenanthrene degraders based on A13/A12 ratios, respectively (Figure 1 and Table S4). The active phenanthrene degraders in NR treatment belong to six classes (OTU113, OTU173, OTU218, and OTU302: *Alphaproteobacteria*; OTU47 and OTU428: *Thermoleophilia*; OTU54 and OTU449: *Actinobacteria*; OTU191 and OTU510: *Acidobacteriia*; OTU32: *DA052*; OTU407: *Solibacteres*). In R treatment, the active phenanthrene degraders are associated with seven classes (OTU113 and OTU404: *Alphaproteobacteria*; OTU54, OTU205, and OTU449: *Actinobacteria*; OTU236: *Acidobacteriia*; OTU38: *Betaproteobacteria*; OTU407: *Solibacteres*; OTU355: *Planctomycetes*; OTU453 and OTU526: *Ktedonobacteri*) (Figure S8A). It is worth noting that OTU54, OTU113, OTU407, and OTU449 were identified as the active phenanthrene degraders in both NR and R treatments. The average phylogenetic distance of the active phenanthrene degraders in R treatment (0.33) was significantly higher than that in NR treatment (0.26, $p < 0.01$). This result suggested a lower phylogenetic diversity of the active phenanthrene degraders in the rhizosphere (Figure S8B).

The relative abundance of the active phenanthrene degraders in NR treatment was 0.15% on day 3, increasing to 2.1% (day 7) and 7.9% (day 14) (Table S5). In R treatment, the relative abundance increased slightly from 1.3% (day 3) to 1.3% (day 7) and 1.8% (day 14) (Table S5), which was lower than that in NR treatment except for day 3. However, 16S rRNA gene copy numbers in rhizosphere soils (2.97×10^9 copies/g soil) were 12.4-fold higher than those in NR treatment (2.40×10^8 copies/g soil) on day 14 and changed slightly throughout the degradation process (Figure S9). Thus, the absolute abundance of the active phenanthrene degraders in R treatment was higher than that in NR treatment. These results further evidenced the impacts of rhizosphere on soil functional microbes and were consistent with higher phenanthrene degradation efficiency in R treatment on day 14.

Ryegrass Rhizosphere Effects on Phenanthrene Degradation Genes. Metagenomic analysis unraveled the

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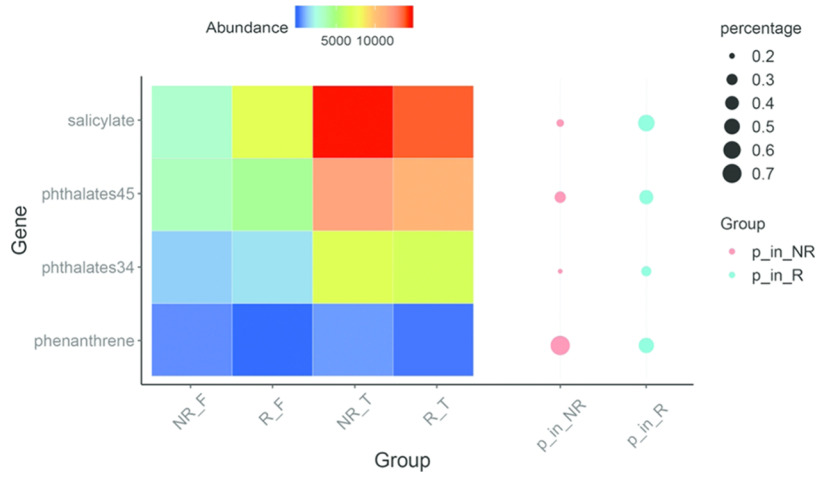

Figure 3. Heatmap showing the relative abundance of the phenanthrene dioxygenase (phenanthrene), phthalate-3,4-dihydrodiol dehydrogenase (phthalate-34), phthalate-4,5-dioxygenase (phthalate-45), and salicylate hydroxylase (salicylate) genes. Circles represent ratios of ^{13}C-labeled functional genes to the total genes in NR and R treatments. NR and R represent non-rhizosphere and rhizosphere treatments, respectively. T and F stand for gene abundance in the total and functional microbial community, respectively.

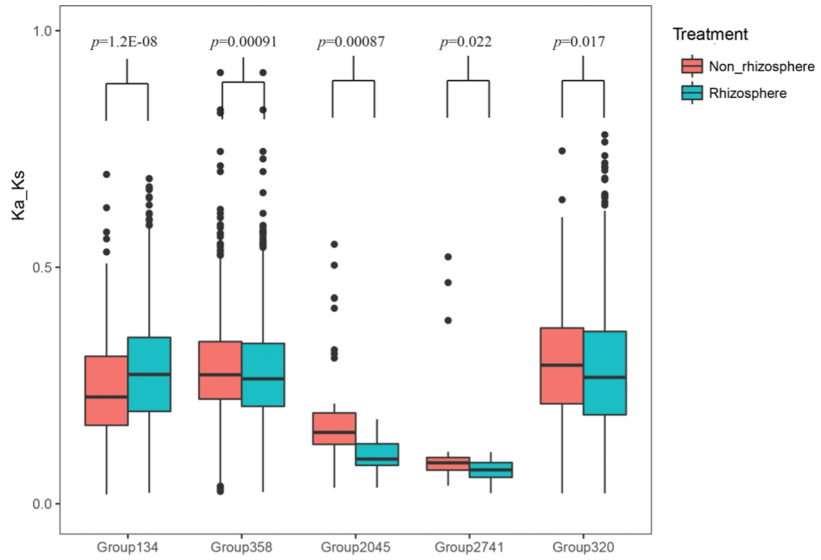

Figure 4. Significant differences in K_a/K_s values among ^{13}C-labeled phenanthrene-degradation-related genes. Groups 134 and 358 belong to the phthalate-4,5-dioxygenase gene; groups 2045, 2741, and 320 are salicylate hydroxylase genes.

phenanthrene degradation pathways and relative genes in detail (Figure 2). Phenanthrene was first transformed into 1-hydroxy-2-naphthoate and then into 1-hydroxy-2-naphthoate in the downstream salicylic acid pathway or phthalic acid pathway. In the salicylic acid pathway, 1-hydroxy-2-naphthoate was degraded to gentisic acid and entered the tyrosine metabolism pathway or transformed to catechol and metabolized through the benzoate degradation pathway. In the phthalic acid pathway, 1-hydroxy-2-naphthoate was transformed to 3-carboxy-*cis*,*cis*-muconate or 4-carboxy-2-hydroxymuconate semialdehyde, which were also metabolized through the benzoate degradation pathway. Though both salicylic acid and phthalic acid pathways existed in NR and R treatments, they exhibited different gene abundances (Figure 2). Among the 16 identified phenanthrene-degradation-related genes, 10 increased and 6 decreased in R treatment. The inconsistent tendency indicated an ambiguous role of the rhizosphere in phenanthrene degradation, which needs further investigation.

For key genes in the phenanthrene degradation pathway, the total abundance of upstream phenanthrene dioxygenase genes in NR treatment (667 pphm) was higher than that in R treatment (218 pphm) (Figure 3). Among them, 478 (71.6%, NR treatment) and 102 (47.1%, R treatment) pphm were labeled with ^{13}C and involved in phenanthrene degradation (Figure 3). The total abundance of phenanthrene downstream degradation genes in both phthalate (phthalate-3,4-dihydrodiol dehydrogenase gene and phthalate-4,5-dioxygenase gene) and salicylate (salicylate hydroxylase gene) pathways was not significantly different between NR and R treatments. However, proportions of ^{13}C-labeled genes were much higher in R treatment (1768 pphm, 26.57% for phthalate-3,4-dihydrodiol dehydrogenase gene; 4358 pphm, 40.29% for phthalate-4,5-dioxygenase gene; 7341 pphm, 53.29% for salicylate hydroxylase gene) than in NR treatment (1395 pphm, 19.82%; 3,539 pphm, 30.16%; and 3,137 pphm, 21.57%; respectively). Notably, the salicylate pathway (salicylate hydroxylase genes) was more promoted than the phthalate pathway (phthalate-

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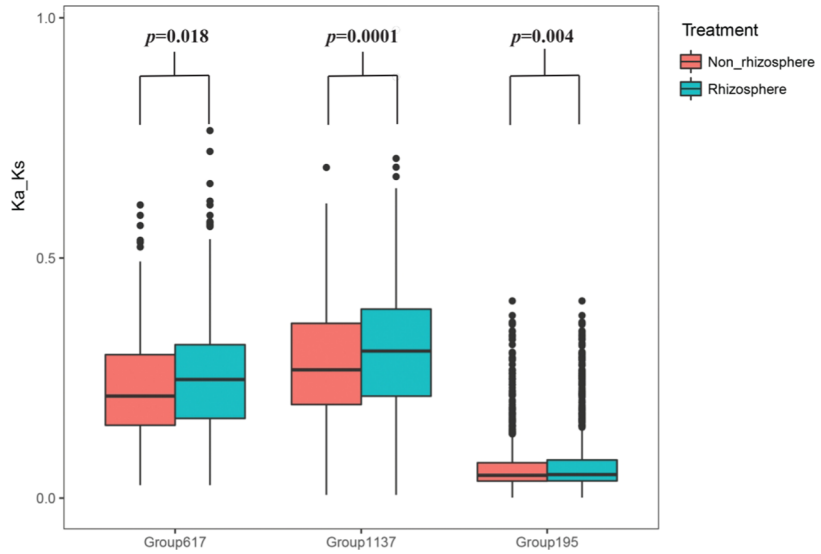

Figure 5. Significant differences in K_a/K_s values among the key genes of the Embden−Meyerhof−Parnas pathway, tricarboxylic acid cycle, and pentose phosphate pathway.

3,4-dihydrodiol dehydrogenase and phthalate-4,5-dioxygenase genes). We clustered homologous gene groups for ^{13}C-labeled genes involved in phenanthrene degradation using OrthoMCL and calculated the average K_a/K_s ratios and p values (Table S6). Only one phenanthrene upstream degradation gene group (phenanthrene dioxygenase gene) was identified. Its average K_a/K_s ratio was little influenced by the rhizosphere, indicating the limited influence of ryegrass. By contrast, the diversity of genes encoding phenanthrene downstream degradation enzymes was much higher than 12 (phthalate-3,4-dihydrodiol dehydrogenase), 9 (phthalate-4,5-dioxygenase), and 12 (salicylate hydroxylase) ^{13}C-labeled degrading gene groups that were identified. Among them, K_a/K_s ratios were significantly different for five groups (134, 358, 2045, 2741, and 320) in R treatment than those in NR treatment (Figure 4). Groups 134 and 358 belonged to the phthalate-4,5-dioxygenase gene, and groups 2045, 2741, and 320 belonged to the salicylate hydroxylase genes. Thus, ryegrass increased the average K_a/K_s ratio in group 134 but had opposite effects on other groups ($p < 0.05$).

Ryegrass Rhizosphere Effects on Microbial Carbohydrate Metabolism Genes. Genes with significantly different abundance between NR and R treatments were annotated and clustered using KEGG modules. The M values of the normalized carbohydrate-metabolism-related genes corresponding to phenanthrene degraders and the whole microbial community were 8490 and 10 923, respectively. They were the highest among all modules and indicated a critical effect of the rhizosphere on carbohydrate metabolism genes. The 10 most abundant altered submodules belonging to carbohydrate-metabolism-related modules were identified as TCA cycle, reductive citrate cycle, glycolysis cycle (Embden−Meyerhof−Parnas pathway), reductive pentose phosphate cycle, and pentose phosphate pathways (Table S7). The key genes belonging to the most altered carbohydrate metabolism modules were investigated and 3, 2, and 6 gene groups of key genes were identified in both NR and R treatments. Although ryegrass had a limited influence on the diversity of the carbohydrate metabolism gene group, it significantly altered the K_a/K_s ratio of partial genes ($p < 0.01$, Figure 5). The average K_a/K_s ratios of groups 617, 1137, and 195 increased from 0.23, 0.27, and 0.066 in NR treatment to 0.25, 0.31, and 0.073 in R treatment, respectively. The three gene groups of carbohydrate metabolism key genes accounted for more than 90% of all key genes with the same function.

Ryegrass Rhizosphere Effects on Root Exudate Metabolism Genes. Genes related to the root exudate metabolism were picked based on the KEGG database. Only the fumarate hydratase gene had a significantly increased K_a/K_s value in R treatment (0.24), which was 13-fold higher than that in NR treatment (0.016, $p = 0.0058$), indicating the importance of fumaric acid in shifting rhizosphere microbial community.

Rhizosphere Effects of Different Plants on Microbial Genes. To get more evidence, we collected 10 rhizosphere soil samples with their control (NR treatment) and analyzed their microbial functional traits. Ten modules were significantly different between NR and R treatments (Figure S10). Among them, genes encoding drug resistance, cellular community, carbohydrate metabolism, and unclassified metabolism were enriched in R treatment. Particularly, carbohydrate metabolism genes changed most except for the unclassified-metabolism genes.

The enriched root exudate-metabolism-related genes in the rhizosphere included those encoding D-lactate dehydrogenase, acetate CoA/acetoacetate CoA-transferase β subunit, acetate CoA/acetoacetate CoA-transferase α subunit, mannitol-1-/sugar-/sorbitol-6-phosphatase, L-arabinose isomerase, citrate lyase subunit alpha/citrate CoA-transferase, citrate lyase subunit γ, and fumarate hydratase (Figure S11). Among them, genes related to fumaric acid and fumarate hydratase significantly increased by 9.7% and exhibited the highest abundance.

DISCUSSION

Ryegrass is a powerful phytoremediation plant that can enhance the degradation of petroleum,[46] PAHs,[47] PCBs,[48] and other organic pollutants.[49] Our results proved that ryegrass could improve phenanthrene degradation efficiency, consistent with the previous findings. The most common interpretation

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for the positive effects of ryegrass in pollutant degradation is that root exudates can increase the soil microbial biomass and shift microbial community.[13,47,50] To explain the conflicting results reporting no effect or negative effects of rhizosphere on PAH degradation,[12,15] it is important to identify the active degraders, explore their genetic traits, and elucidate how root exudates influence PAH degradation by shaping soil microbes.

In this study, we employed DNA-SIP to identify OTUs responsible for *in situ* phenanthrene degradation, and observed 19 OTUs possessing phenanthrene degradation ability in NR and R treatments. Among them, 8 OTUs with complete taxonomic information were classified into 7 genera and the other 11 OTUs were hardly annotated at the genus level. OTU205 (*Terracoccus* genus) is rarely reported and its ecological function has not been determined to the best of our knowledge; this study is the first to report its organic pollutant degradation ability. Although OTU54 (*Pseudonocardia*) is involved in pyrene degradation,[51] it is not associated with phenanthrene metabolism until this work confirmed its phenanthrene degradation function. Phenanthrene degradation ability of the remaining genera (*Rhodoplanes*, *Kaistobacter*, *Mesorhizobium*, *Ralstonia*, and *Mycobacterium*) has been widely confirmed.[52−56] Interestingly, *Terracoccus*, *Rhodoplanes*, *Kaistobacter*, and *Pseudonocardia* linked to phenanthrene degradation were only revealed by DNA-SIP, demonstrating the high resolution of DNA-SIP in microbial degrader identification *in situ*.

Rhizosphere accelerated the activities of phenanthrene degraders shared by R and NR treatments. A rhizosphere priming effect has been revealed in previous studies. *Phragmites australis* rhizosphere accelerated the biodegradation of pyrene and benzo[a]pyrene,[57] and the root exudates of *Slender oat* and *Spirodela polyrrhiza* accelerated the biodegradation rates of phenanthrene[58] and a variety of aromatic compounds.[59] However, there is still no direct evidence for the underlying mechanisms, and the proposed ones include positive effects on pollutant solubility,[60] rich nutritional environment improving microbial activity,[10] and signal substances regulating microbial metabolism.[61] Our study raised another potential explanation that ryegrass rhizosphere could alter the active phenanthrene degrader community structure and only 4 out of 9 OTUs were shared between NR and R treatments. The plant effects on the structure and diversity of the active phenanthrene degraders have been previously reported,[13,62] and rhizosphere effects on the variety, activity, and abundance of phenanthrene degraders have been widely investigated.[58,63] Nevertheless, no study focuses on the changes in the mean phylogenetic distances of the active microbes. Our results showed that ryegrass sheltered microbes with large phylogenetic distances to play roles in phenanthrene degradation, indicating that ryegrass creates a friendly microenvironment for microbes and decreases competition because plants can provide more nutrition[6] and oxygen[64] and release structural analogues of PAHs.[65]

Some studies have suggested that genes encoding PAH-ring hydroxylating dioxygenase (PAH-RHD) are the key ones responsible for the first step in PAH degradation. Thus, PAH-RHD genes are used as biomarkers to indicate the PAH degradation ability of a microbial population.[66,67] In the present study, although the relative abundance of both total and active PAH-RHD genes was higher in NR treatment than in R treatment, it was opposite for the absolute abundance as the copy numbers of 16S rRNA genes were 12.4-fold higher in R treatment than NR treatment. These results were consistent with the reported increasing copy numbers of PAH-RHD genes in plants.[47,68] For example, tall fescue stimulated the PAH-RHD Gram-negative gene expression[69] and water-soluble celery root exudates increased the PAH-RHD gene expression by about 16−300-fold.[70] Similarly, the absolute abundance of salicylic acid and phthalic acid degradation genes was higher in R treatment than in NR treatment, suggesting the enhancement of both two pathways for phenanthrene downstream degradation, *e.g.*, salicylic pathway catalyzed by salicylate hydroxylase and phthalic acid pathway catalyzed by phthalate-3,4-dihydrodiol dehydrogenase or phthalate-4,5-dioxygenase.[71] This can be explained by the ubiquity of salicylic and phthalic acids in root exudates,[72,73] which implies the high potential of phenanthrene downstream degradation. Comparing with downstream degradation genes, PAH-RHD genes might be the limiting factor in phenanthrene degradation. Ryegrass promoted the phenanthrene remediation efficiency, as expected by removing the restricted factor, *e.g.*, autochthonous bioaugmentation.[52]

Rhizosphere effects could alter the active degraders and degradation genes; however, the underlying mechanism of these processes remains unclear. The K_a/K_s values of phenanthrene degradation genes are causally related to phenanthrene mineralization. Here, K_a/K_s values of five groups of phenanthrene-degradation-related genes were altered by the rhizosphere. About 30% of them in five groups had higher K_a/K_s values in R treatment compared to NR treatment, whereas the other 70% were opposite. The abundance of these genes accounted for ≤50% of the total functional genes. These results suggest that phenanthrene and its metabolites have limited effects on the structure of phenanthrene degrader community, although components of root exudates such as organic acids can improve the bioavailability and mobility of PAHs to increase phenanthrene utilization by microbes.[74]

According to previous studies, carbohydrates can change microbial competition,[75] alter microbial catabolic profiles,[76] shift microbial community, and influence microbial responses to heavy metals[77] and organic pollutions.[78] Specifically, the high abundance of carbohydrate catabolism genes in the phenanthrene-degrading bacterial consortium has been observed in planted soils.[79] In addition, PAH degradation is tightly related to carbohydrate metabolism gene transcripts.[80] Our results also imply an important role of carbohydrate metabolism genes driven by rhizosphere effects. Plants may change the active phenanthrene degraders by altering the microbial carbohydrate metabolism. Therefore, we used K_a/K_s values as an indicator to explore the characteristics of key carbohydrate metabolism genes. The significant increase in K_a/K_s values of >90% carbohydrate metabolism key genes in R treatment indicated that carbohydrate metabolism pressure plays an essential role in altering microbial community structure. The carbohydrate secreted by ryegrass provided a suitable microenvironment for r-strategy microbes with low carbohydrate utilization ability. Therefore, we inferred that carbohydrate metabolism selection within the ryegrass rhizosphere can cause a shift in the phenanthrene degrader community. When the change in the carbohydrate metabolism selection pressure causes side effects on the active phenanthrene degraders, phenanthrene degradation could be promoted or postponed and even decreased, explaining the conflicting results of ryegrass impacts on phenanthrene degradation.

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Besides carbohydrate metabolism, fumaric acid was identified as the key factor shifting the rhizosphere microbial community. Fumaric acid is often viewed as a potential electron shuttle,[81] terminal electron acceptor,[82] propionic acid precursor,[83] important chemotaxis inducer,[84] potential methane reducer[85] and key intermediates in phosphorus mobilization.[86] According to previous DNA-SIP studies, microbial utilization of fumaric acid was widespread among the microbial communities,[87] but its roles remain unclear for the low abundance in the environment. Although fumaric acid was less than 1.5% of total organic acids in this study, it is involved in 29 metabolism pathways from the KEGG database. These metabolism pathways include the TCA cycle, biosynthesis of several amino acids and metabolism process, carbon fixation and metabolism pathway, and aromatic compound degradation pathway. It indicates that fumaric acid is an important metabolic intermediate for microbes and possesses a key role in the microbial community construction.

Rhizosphere forms a new soil microenvironment, which can change the microbial community structure and then alter soil microbes with phenanthrene degradation abilities. The carbohydrate and fumaric acid metabolism pressure plays an important role, namely, the rhizosphere can enrich microbes with competitive advantages on carbohydrates and fumaric acid metabolism. Phenanthrene degraders with strong competitiveness (SCDs) in carbohydrates and fumaric acid metabolism can survive well in rhizosphere soils, while phenanthrene degraders with low competitiveness (LCDs) might be inhibited by the growth of other microbes. Therefore, ryegrass will promote phenanthrene degradation in soils containing SCDs, which is consistent with our results and some positive results in previous studies. However, in soils dominated by LCDs, ryegrass decreases the phenanthrene degradation efficiency, which includes some negative results of phenanthrene degradation in previous ryegrass rhizosphere studies. These results suggest an underlying mechanism for rhizosphere effects on PAH degradation and provide an explanation for contradictory findings among current studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c04951.

Protein sequences downloaded from NCBI and UniProt used for protein annotation; sugar concentration in ryegrass root exudates; organic acid concentration in ryegrass root exudates; taxonomic information of phenanthrene degraders; relative abundance of all active phenanthrene degraders in NR and R treatments; average Ka/Ks ratios of phenanthrene degradation related genes and the p values of one-way ANOVA; ten altered metabolic modules with top abundance and their corresponding M value in phenanthrene degraders and total microbial community; heights of all the cultivated plants; the copy number of 16S rRNA genes (normalized to the highest abundance) along with CsCl gradient fractions in NR and R treatments; dominant microbes (>1‰) on the phyla level in R and NR treatments; dominant microbes (>1‰) on genus level in R and NR treatments; score plot of PCoA for microbial communities from original soil, NR and R treatments; shannon index in original soil, NR and R treatments; residual phenanthrene percentage in R, NR and sterilized samples; A) phylogenetic tree of identified OTUs responsible for in situ phenanthrene degradation in NR and R treatments, B) differences in phylogenetic distance between NR and R treatments; the copy numbers of 16S rRNA genes on day 0, 3, 7 and 14 between R and NR treatments; the 10 different abundance modules between NR and R treatments; root exudates metabolism related genes enriched in R treatment (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Peng, N. N.; Li, Y.; Liu, Z. G.; Liu, T. T.; Gai, C. Emission, distribution and toxicity of polycyclic aromatic hydrocarbons (PAHs) during municipal solid waste (MSW) and coal co-combustion. *Sci. Total Environ.* **2016**, *565*, 1201−1207.

(2) Wang, C.; Li, Y. Z.; Tan, H.; Zhang, A. K.; Xie, Y. L.; Wu, B.; Xu, H. A novel microbe consortium, nano-visible light photocatalyst and

microcapsule system to degrade PAHs. *Chem. Eng. J.* **2019**, *359*, 1065−1074.
(3) Liao, X. Y.; Wu, Z. Y.; Li, Y.; Luo, J. P.; Su, C. M. Enhanced degradation of polycyclic aromatic hydrocarbons by indigenous microbes combined with chemical oxidation. *Chemosphere* **2018**, *213*, 551−558.
(4) Ghosal, D.; Ghosh, S.; Dutta, T. K.; Ahn, Y. Current State of Knowledge in Microbial Degradation of Polycyclic Aromatic Hydrocarbons (PAHs): A Review (vol 7, 1369, 2016). *Front. Microbiol.* **2016**, *7*, No. 1369.
(5) Günther, T.; Dornberger, U.; Fritsche, W. Effects of ryegrass on biodegradation of hydrocarbons in soil. *Chemosphere* **1996**, *33*, 203−215.
(6) Yoshitomi, K. J.; Shann, J. R. Corn (*Zea mays* L.) root exudates and their impact on C-14-pyrene mineralization. *Soil Biol. Biochem.* **2001**, *33*, 1769−1776.
(7) Khan, Z.; Roman, D.; Kintz, T.; delas Alas, M.; Yap, R.; Doty, S. Degradation, Phytoprotection and Phytoremediation of Phenanthrene by Endophyte Pseudomonas putida, PD1. *Environ. Sci. Technol.* **2014**, *48*, 12221−12228.
(8) Van Aken, B.; Correa, P. A.; Schnoor, J. L. Phytoremediation of Polychlorinated Biphenyls: New Trends and Promises. *Environ. Sci. Technol.* **2010**, *44*, 2767−2776.
(9) Mo, C. H.; Cai, Q. Y.; Li, H. Q.; Zeng, Q. Y.; Tang, S. R.; Zhao, Y. C. Potential of different species for use in removal of DDT from the contaminated soil. *Chemosphere* **2008**, *73*, 120−125.
(10) Bais, H. P.; Weir, T. L.; Perry, L. G.; Gilroy, S.; Vivanco, J. M. The role of root exudates in rhizosphere interations with plants and other organisms. *Annu. Rev. Plant Biol.* **2006**, *57*, 233−266.
(11) Joner, E. J.; Leyval, C. Rhizosphere gradients of polycyclic aromatic hydrocarbon (PAH) dissipation in two industrial soils and the impact of arbuscular mycorrhiza. *Environ. Sci. Technol.* **2003**, *37*, 2371−2375.
(12) Cébron, A.; Louvel, B.; Faure, P.; France-Lanord, C.; Chen, Y.; Murrell, J. C.; Leyval, C. Root exudates modify bacterial diversity of phenanthrene degraders in PAH-polluted soil but not phenanthrene degradation rates. *Environ. Microbiol.* **2011**, *13*, 722−736.
(13) Li, J. B.; Luo, C. L.; Zhang, D. Y.; Cai, X. X.; Jiang, L. F.; Zhao, X.; Zhang, G. Diversity of the active phenanthrene degraders in PAH-polluted soil is shaped by ryegrass rhizosphere and root exudates. *Soil Biol. Biochem.* **2019**, *128*, 100−110.
(14) Tejeda-Agredano, M. C.; Gallego, S.; Vila, J.; Grifoll, M.; Ortega-Calvo, J. J.; Cantos, M. Influence of the sunflower rhizosphere on the biodegradation of PAHs in soil. *Soil Biol. Biochem.* **2013**, *57*, 830−840.
(15) Rentz, J. A.; Alvarez, P. J. J.; Schnoor, J. L. Repression of Pseudomonas putida phenanthrene-degrading activity by plant root extracts and exudates. *Environ. Microbiol.* **2004**, *6*, 574−583.
(16) Song, M. K.; Jiang, L. F.; Zhang, D. Y.; Luo, C. L.; Yin, H.; Li, Y. T.; Zhang, G. Identification of biphenyl-metabolising microbes in activated biosludge using cultivation-independent and-dependent approaches. *J. Hazard. Mater.* **2018**, *353*, 534−541.
(17) Jiang, L. F.; Song, M. K.; Luo, C. L.; Zhang, D. Y.; Zhang, G. Novel Phenanthrene-Degrading Bacteria Identified by DNA-Stable Isotope Probing. *PLoS One* **2015**, *10*, No. e0130846.
(18) Radajewski, S.; Ineson, P.; Parekh, N. R.; Murrell, J. C. Stable-isotope probing as a tool in microbial ecology. *Nature* **2000**, *403*, 646−649.
(19) Chen, Y.; Dumont, M. G.; Neufeld, J. D.; Bodrossy, L.; Stralis-Pavese, N.; McNamara, N. P.; Ostle, N.; Briones, M. J. I.; Murrell, J. C. Revealing the uncultivated majority: combining DNA stable-isotope probing, multiple displacement amplification and metagenomic analyses of uncultivated Methylocystis in acidic peatlands. *Environ. Microbiol.* **2008**, *10*, 2609−2622.
(20) Kim, S. J.; Park, S. J.; Cha, I. T.; Min, D.; Kim, J. S.; Chung, W. H.; Chae, J. C.; Jeon, C. O.; Rhee, S. K. Metabolic versatility of toluene-degrading, iron-reducing bacteria in tidal flat sediment, characterized by stable isotope probing-based metagenomic analysis. *Environ. Microbiol.* **2014**, *16*, 189−204.
(21) Leigh, M. B.; Pellizari, V. H.; Uhlik, O.; Sutka, R.; Rodrigues, J.; Ostrom, N. E.; Zhou, J. H.; Tiedje, J. M. Biphenyl-utilizing bacteria and their functional genes in a pine root zone contaminated with polychlorinated biphenyls (PCBs). *ISME J.* **2007**, *1*, 134−148.
(22) Lünsmann, V.; Kappelmeyer, U.; Benndorf, R.; Martinez-Lavanchy, P. M.; Taubert, A.; Adrian, L.; Duarte, M.; Pieper, D. H.; von Bergen, M.; Muller, J. A.; Heipieper, H. J.; Jehmlich, N. In situ protein-SIP highlights Burkholderiaceae as key players degrading toluene by para ring hydroxylation in a constructed wetland model. *Environ. Microbiol.* **2016**, *18*, 1176−1186.
(23) Uhlik, O.; Jecna, K.; Mackova, M.; Vlcek, C.; Hroudova, M.; Demnerova, K.; Paces, V.; Macek, T. Biphenyl-Metabolizing Bacteria in the Rhizosphere of Horseradish and Bulk Soil Contaminated by Polychlorinated Biphenyls as Revealed by Stable Isotope Probing. *Appl. Environ. Microbiol.* **2009**, *75*, 6471−6477.
(24) Taketani, R. G.; Kavamura, V. N.; Mendes, R.; Melo, I. S. Functional congruence of rhizosphere microbial communities associated to leguminous tree from Brazilian semiarid region. *Environ. Microbiol. Rep.* **2015**, *7*, 95−101.
(25) Hamonts, K.; Clough, T. J.; Stewart, A.; Clinton, P. W.; Richardson, A. E.; Wakelin, S. A.; O'Callaghan, M.; Condron, L. M. Effect of nitrogen and waterlogging on denitrifier gene abundance, community structure and activity in the rhizosphere of wheat. *FEMS Microbiol. Ecol.* **2013**, *83*, 568−584.
(26) Hurst, L. D. The Ka/Ks ratio: diagnosing the form of sequence evolution. *Trends Genet.* **2002**, *18*, 486−487.
(27) Yu, X. Z.; Gao, Y.; Wu, S. C.; Zhang, H. B.; Cheung, K. C.; Wong, M. H. Distribution of polycyclic aromatic hydrocarbons in soils at Guiyu area of China, affected by recycling of electronic waste using primitive technologies. *Chemosphere* **2006**, *65*, 1500−1509.
(28) Wang, Y.; Tian, Z. J.; Zhu, H. L.; Cheng, Z. N.; Kang, M. L.; Luo, C. L.; Li, J.; Zhang, G. Polycyclic aromatic hydrocarbons (PAHs) in soils and vegetation near an e-waste recycling site in South China: Concentration, distribution, source, and risk assessment. *Sci. Total Environ.* **2012**, *439*, 187−193.
(29) Tang, X. J.; Shen, C. F.; Cheema, S. A.; Chen, L.; Xiao, X.; Zhang, C. K.; Liu, W. L.; Li, F.; Chen, Y. X. Levels and distributions of polycyclic aromatic hydrocarbons in agricultural soils in an emerging e-waste recycling town in Taizhou area. *J. Environ. Sci. Health, Part A* **2010**, *45*, 1076−1084.
(30) Song, M. K.; Jiang, L. F.; Zhang, D. Y.; Luo, C. L.; Wang, Y.; Yu, Z. Q.; Yin, H.; Zhang, G. Bacteria capable of degrading anthracene, phenanthrene, and fluoranthene as revealed by DNA based stable-isotope probing in a forest soil. *J. Hazard. Mater.* **2016**, *308*, 50−57.
(31) Zhang, J. J.; Kobert, K.; Flouri, T.; Stamatakis, A. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* **2014**, *30*, 614−620.
(32) Caporaso, J. G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F. D.; Costello, E. K.; Fierer, N.; Pena, A. G.; Goodrich, J. K.; Gordon, J. I.; Huttley, G. A.; Kelley, S. T.; Knights, D.; Koenig, J. E.; Ley, R. E.; Lozupone, C. A.; McDonald, D.; Muegge, B. D.; Pirrung, M.; Reeder, J.; Sevinsky, J. R.; Tumbaugh, P. J.; Walters, W. A.; Widmann, J.; Yatsunenko, T.; Zaneveld, J.; Knight, R. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **2010**, *7*, 335−336.
(33) Callahan, B. J.; McMurdie, P. J.; Rosen, M. J.; Han, A. W.; Johnson, A. J. A.; Holmes, S. P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581−583.
(34) Lozupone, C.; Knight, R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* **2005**, *71*, 8228−8235.
(35) Li, D. H.; Liu, C. M.; Luo, R. B.; Sadakane, K.; Lam, T. W. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* **2015**, *31*, 1674−1676.
(36) Seemann, T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **2014**, *30*, 2068−2069.

https://dx.doi.org/10.1021/acs.est.0c04951
Environ. Sci. Technol. 2021, 55, 962−973

(37) Franzosa, E. A.; McIver, L. J.; Rahnavard, G.; Thompson, L. R.; Schirmer, M.; Weingart, G.; Lipson, K. S.; Knight, R.; Caporaso, J. G.; Segata, N.; Huttenhower, C. Species-level functional profiling of metagenomes and metatranscriptomes. *Nat. Methods* **2018**, *15*, 962−968.
(38) Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. Basic Local Alignment Search Tool. *J. Mol. Biol.* **1990**, *215*, 403−410.
(39) Krebs, H. A. Rate control of the tricarboxylic acid cycle. *Adv. Enzyme Regul.* **1970**, *8*, 335−353.
(40) Valdez, B. C.; French, B. A.; Younathan, E. S.; Chang, S. H. Site-Directed Mutagenesis in Bacillus-Stearothermophilus Fructose-6-Phosphate 1-Kinase - Mutation at the Substrate-Binding Site Affects Allosteric Behavior. *Biochem. Biophys. Res. Commun.* **1989**, *264*, 537−542.
(41) Tian, W. N.; Braunstein, L. D.; Pang, J. D.; Stuhlmeier, K. M.; Xi, Q. C.; Tian, X. N.; Stanton, R. C. Importance of glucose-6-phosphate dehydrogenase activity for cell growth. *J. Biol. Chem.* **1998**, *273*, 10609−10617.
(42) Langmead, B.; Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **2012**, *9*, 357−359.
(43) Wang, D.; Zhang, Y.; Zhang, Z.; Zhu, J.; Yu, J. KaKs_Calculator 2.0: a toolkit incorporating gamma-series methods and sliding window strategies. *Genomics, Proteomics Bioinf.* **2010**, *8*, 77−80.
(44) Li, L.; Stoeckert, C. J.; Roos, D. S. OrthoMCL: Identification of ortholog groups for eukaryotic genomes. *Genome Res.* **2003**, *13*, 2178−2189.
(45) Jiang, L. F.; Luo, C. L.; Zhang, D. Y.; Song, M. K.; Sun, Y. T.; Zhang, G. Biphenyl-Metabolizing Microbial Community and a Functional Operon Revealed in E-Waste-Contaminated Soil. *Environ. Sci. Technol.* **2018**, *52*, 8558−8567.
(46) Khan, S.; Afzal, M.; Iqbal, S.; Khan, Q. M. Plant-bacteria partnerships for the remediation of hydrocarbon contaminated soils. *Chemosphere* **2013**, *90*, 1317−1332.
(47) Guo, M. X.; Gong, Z. Q.; Miao, R. H.; Rookes, J.; Cahill, D.; Zhuang, J. Microbial mechanisms controlling the rhizosphere effect of ryegrass on degradation of polycyclic aromatic hydrocarbons in an aged-contaminated agricultural soil. *Soil Biol. Biochem.* **2017**, *113*, 130−142.
(48) Lu, Y. F.; Lu, M.; Peng, F.; Wan, Y.; Liao, M. H. Remediation of polychlorinated biphenyl-contaminated soil by using a combination of ryegrass, arbuscular mycorrhizal fungi and earthworms. *Chemosphere* **2014**, *106*, 44−50.
(49) Wang, S.; Zhang, S. Z.; Huang, H. L.; Christie, P. Behavior of decabromodiphenyl ether (BDE-209) in soil: Effects of rhizosphere and mycorrhizal colonization of ryegrass roots. *Environ. Pollut.* **2011**, *159*, 749−753.
(50) Hamdi, H.; Benzarti, S.; Aoyama, I.; Jedidi, N. Rehabilitation of degraded soils containing aged PAHs based on phytoremediation with alfalfa (*Medicago sativa* L.). *Int. Biodeterior. Biodegrad.* **2012**, *67*, 40−47.
(51) Chen, S.-C.; Duan, G. L.; Ding, K.; Huang, F. Y.; Zhu, Y. G. DNA stable-isotope probing identifies uncultivated members of Pseudonocardia associated with biodegradation of pyrene in agricultural soil. *FEMS Microbiol. Ecol.* **2018**, *94*, No. fiy026.
(52) Li, J. B.; Luo, C. L.; Zhang, D. Y.; Song, M. K.; Cai, X. X.; Jiang, L. F.; Zhang, G. Autochthonous Bioaugmentation-Modified Bacterial Diversity of Phenanthrene Degraders in PAH-Contaminated Wastewater as Revealed by DNA-Stable Isotope Probing. *Environ. Sci. Technol.* **2018**, *52*, 2934−2944.
(53) Li, J. B.; Zhang, D. Y.; Song, M. K.; Jiang, L. F.; Wang, Y. J.; Luo, C. L.; Zhang, G. Novel bacteria capable of degrading phenanthrene in activated sludge revealed by stable-isotope probing coupled with high-throughput sequencing. *Biodegradation* **2017**, *28*, 423−436.
(54) Jamal, M. T.; Pugazhendi, A. Degradation of petroleum hydrocarbons and treatment of refinery wastewater under saline condition by a halophilic bacterial consortium enriched from marine environment (Red Sea), Jeddah, Saudi Arabia. *3 Biotech.* **2018**, *8*, No. 276.
(55) Chávez-Gómez, B.; Quintero, R.; Esparza-Garcia, F.; Mesta-Howard, A. M.; de la Serna, F. J. Z. D.; Hernandez-Rodriguez, C. H.; Gillen, T.; Poggi-Varaldo, H. M.; Barrera-Cortes, J.; Rodriguez-Vazquez, R. Removal of phenanthrene from soil by co-cultures of bacteria and fungi pregrown on sugarcane bagasse pith. *Bioresour. Technol.* **2003**, *89*, 177−183.
(56) Hennessee, C. T.; Li, Q. X. Effects of Polycyclic Aromatic Hydrocarbon Mixtures on Degradation, Gene Expression, and Metabolite Production in Four Mycobacterium Species. *Appl. Environ. Microbiol.* **2016**, *82*, 3357−3369.
(57) Toyama, T.; Furukawa, T.; Maeda, N.; Inoue, D.; Sei, K.; Mori, K.; Kikuchi, S.; Ike, M. Accelerated biodegradation of pyrene and benzo[a]pyrene in the *Phragmites australis* rhizosphere by bacteria-root exudate interactions. *Water Res.* **2011**, *45*, 1629−1638.
(58) Miya, R. K.; Firestone, M. K. Enhanced phenanthrene biodegradation in soil by slender oat root exudates and root debris. *J. Environ. Qual.* **2001**, *30*, 1911−1918.
(59) Hoang, H.; Yu, N.; Toyama, T.; Inoue, D.; Sei, K.; Ike, M. Accelerated degradation of a variety of aromatic compounds by *Spirodela polyrrhiza*-bacterial associations and contribution of root exudates released from *S. polyrrhiza*. *J. Environ. Sci.* **2010**, *22*, 494−499.
(60) Lu, J. Y.; Dijkstra, F. A.; Wang, P.; Cheng, W. X. Roots of non-woody perennials accelerated long-term soil organic matter decomposition through biological and physical mechanisms. *Soil Biol. Biochem.* **2019**, *134*, 42−53.
(61) Shaw, L. J.; Morris, P.; Hooker, J. E. Perception and modification of plant flavonoid signals by rhizosphere micro-organisms. *Environ. Microbiol.* **2006**, *8*, 1867−1880.
(62) Cébron, A.; Louvel, B.; Faure, P.; France-Lanord, C.; Chen, Y.; Murrell, J. C.; Leyval, C. Root exudates modify bacterial diversity of phenanthrene degraders in PAH-polluted soil but not phenanthrene degradation rates. *Environ. Microbiol.* **2011**, *13*, 722−736.
(63) Singer, A. C.; Crowley, D. E.; Thompson, I. P. Secondary plant metabolites in phytoremediation and biotransformation. *Trends Biotechnol.* **2003**, *21*, 123−130.
(64) Singer, A. C.; Smith, D.; Jury, W. A.; Hathuc, K.; Crowley, D. E. Impact of the plant rhizosphere and augmentation on remediation of polychlorinated biphenyl contaminated soil. *Environ. Toxicol. Chem.* **2003**, *22*, 1998−2004.
(65) Fletcher, J. S.; Hegde, R. S. Release of Phenols by Perennial Plant-Roots and Their Potential Importance in Bioremediation. *Chemosphere* **1995**, *31*, 3009−3016.
(66) Song, M.; Luo, C. L.; Jiang, L. F.; Zhang, D. Y.; Wang, Y. J.; Zhang, G. Identification of Benzo[a]pyrene-Metabolizing Bacteria in Forest Soils by Using DNA-Based Stable-Isotope Probing. *Appl. Environ. Microbiol.* **2015**, *81*, 7368−7376.
(67) Li, J. B.; Luo, C. L.; Song, M. K.; Dai, Q.; Jiang, L. F.; Zhang, D. Y.; Zhang, G. Biodegradation of Phenanthrene in Polycyclic Aromatic Hydrocarbon-Contaminated Wastewater Revealed by Coupling Cultivation-Dependent and -Independent Approaches. *Environ. Sci. Technol.* **2017**, *51*, 3391−3401.
(68) Storey, S.; Ashaari, M. M.; McCabe, G.; Harty, M.; Dempsey, R.; Doyle, O.; Clipson, N.; Doyle, E. M. Microbial community structure during fluoranthene degradation in the presence of plants. *J. Appl. Microbiol.* **2014**, *117*, 74−84.
(69) Guo, M. X.; Gong, Z. Q.; Miao, R. H.; Jia, C. Y.; Rookes, J.; Cahill, D.; Zhuang, J. Enhanced polycyclic aromatic hydrocarbons degradation in rhizosphere soil planted with tall fescue: Bacterial community and functional gene expression mechanisms. *Chemosphere* **2018**, *212*, 15−23.
(70) Meng, L. A.; Zhu, Y. G. Pyrene Biodegradation in an Industrial Soil Exposed to Simulated Rhizodeposition: How Does It Affect Functional Microbial Abundance? *Environ. Sci. Technol.* **2011**, *45*, 1579−1585.

https://dx.doi.org/10.1021/acs.est.0c04951
Environ. Sci. Technol. 2021, 55, 962−973

(71) Seo, J.-S.; Keum, Y.-S.; Li, Q. X. Bacterial Degradation of Aromatic Compounds. *Int. J. Environ. Res. Public Health* **2009**, *6*, 278−309.
(72) Yu, H. Y.; Liang, H. B.; Shen, G. M.; Sampietro, D. A.; Gao, X. X. Effects of allelochemicals from tobacco root exudates on seed germination and seedling growth of tobacco. *Allelopathy J.* **2014**, *33*, 107−119.
(73) Yuan, J.; Wu, Y. C.; Zhao, M. L.; Wen, T.; Huang, Q. W.; Shen, Q. R. Effect of phenolic acids from banana root exudates on root colonization and pathogen suppressive properties of *Bacillus amyloliquefaciens* NJN-6. *Biol. Control* **2018**, *125*, 131−137.
(74) Jia, H.; Hou, D. Y.; Dai, Y.; Lu, H. L.; Yan, C. L. Effects of root exudates on the mobility of pyrene in mangrove sediment water system. *Catena* **2018**, *162*, 396−401.
(75) Grondin, J. M.; Tamura, K.; Dejean, G.; Abbott, D. W.; Brumer, H. Polysaccharide Utilization Loci: Fueling Microbial Communities. *J. Bacteriol.* **2017**, *199*, No. 00860-16.
(76) Yu, J.; Unc, A.; Zhang, X. K.; Steinberger, Y. Responses of the soil microbial catabolic profile and diversity to vegetation rehabilitation in degraded semiarid grassland. *Appl. Soil Ecol.* **2016**, *101*, 124−131.
(77) Nakatsu, C. H.; Carmosini, N.; Baldwin, B.; Beasley, F.; Kourtev, P.; Konopka, A. Soil microbial community responses to additions of organic carbon substrates and heavy metals (Pb and Cr). *Appl. Environ. Microbiol.* **2005**, *71*, 7679−7689.
(78) Zhao, O.-Y.; Zhang, X. N.; Feng, S. D.; Zhang, L. X.; Shi, W.; Yang, Z. X.; Chena, M. M.; Fanga, X. D. Starch-enhanced degradation of HMW PAHs by Fusarium sp in an aged polluted soil from a coal mining area. *Chemosphere* **2017**, *174*, 774−780.
(79) Thomas, F.; Corre, E.; Cébron, A. Stable isotope probing and metagenomics highlight the effect of plants on uncultured phenanthrene-degrading bacterial consortium in polluted soil. *ISME J.* **2019**, *13*, 1814−1830.
(80) de Menezes, A.; Clipson, N.; Doyle, E. Comparative metatranscriptomics reveals widespread community responses during phenanthrene degradation in soil. *Environ. Microbiol.* **2012**, *14*, 2577−2588.
(81) Esteve-Núñez, A.; Sosnik, J.; Visconti, P.; Lovley, D. R. Fluorescent properties of c-type cytochromes reveal their potential role as an extracytoplasmic electron sink in Geobacter sulfurreducens. *Environ. Microbiol.* **2008**, *10*, 497−505.
(82) Islam, F. S.; Pederick, R. L.; Gault, A. G.; Adams, L. K.; Polya, D. A.; Charnock, J. M.; Lloyd, J. R. Interactions between the Fe(III)-reducing bacterium Geobacter sulfurreducens and arsenate, and capture of the metalloid by biogenic Fe(II). *Appl. Environ. Microbiol.* **2005**, *71*, 8642−8648.
(83) Remling, N.; Riede, S.; Lebzien, P.; Meyer, U.; Holtershinken, M.; Kersten, S.; Breves, G.; Flachowsky, G.; Danicke, S. Effects of fumaric acid on rumen fermentation, milk composition and metabolic parameters in lactating cows. *J. Anim. Physiol. Anim. Nutr.* **2014**, *98*, 968−981.
(84) Gupta Sood, S. Chemotactic response of plant-growth-promoting bacteria towards roots of vesicular-arbuscular mycorrhizal tomato plants. *FEMS Microbiol. Ecol.* **2003**, *45*, 219−227.
(85) Wood, T. A.; Wallace, R. J.; Rowe, A.; Price, J.; Yanez-Ruiz, D. R.; Murray, P.; Newbold, C. J. Encapsulated fumaric acid as a feed ingredient to decrease ruminal methane emissions. *Anim. Feed Sci. Technol.* **2009**, *152*, 62−71.
(86) Roelofs, R. F. R.; Rengel, Z.; Cawthray, G. R.; Dixon, K. W.; Lambers, H. Exudation of carboxylates in Australian Proteaceae: chemical composition. *Plant, Cell Environ.* **2001**, *24*, 891−903.
(87) Paterson, E.; Gebbing, T.; Abel, C.; Sim, A.; Telfer, G. Rhizodeposition shapes rhizosphere microbial community structure in organic soil. *New Phytol.* **2007**, *173*, 600−610.

https://dx.doi.org/10.1021/acs.est.0c04951
Environ. Sci. Technol. 2021, 55, 962−973