Environmental Science & Technology

pubs.acs.org/est

Article

In Situ Discrimination and Cultivation of Active Degraders in Soils by Genome-Directed Cultivation Assisted by SIP-Raman-Activated Cell Sorting

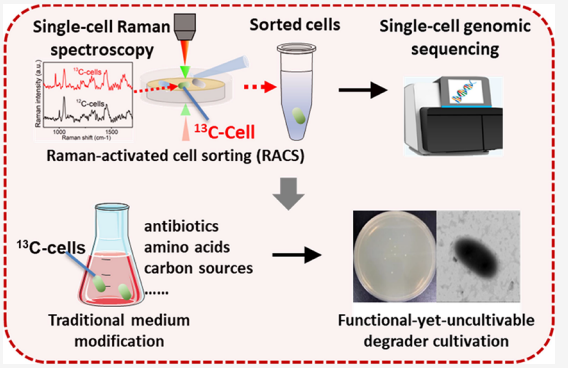
Jibing Li, Dayi Zhang, Chunling Luo,* Bei Li, and Gan Zhang

Cite This: *Environ. Sci. Technol.* 2023, 57, 17087−17098

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: The identification and *in situ* cultivation of functional yet uncultivable microorganisms are important to confirm inferences regarding their ecological functions. Here, we developed a new method that couples Raman-activated cell sorting (RACS), stable-isotope probing (SIP), and genome-directed cultivation (GDC)—namely, RACS-SIP-GDC—to identify, sort, and cultivate the active toluene degraders from a complex microbial community in petroleum-contaminated soil. Using SIP, we successfully identified the active toluene degrader *Pigmentiphaga*, the single cells of which were subsequently sorted and isolated by RACS. We further successfully assembled the genome of *Pigmentiphaga* based on the metagenomic sequencing of ^{13}C-DNA and genomic sequencing of sorted cells, which was confirmed by *gyrB* gene comparison and average nucleotide identity determination. Additionally, the genotypes and phenotypes of this degrader were directly linked at the single-cell level, and its complete toluene metabolic pathways in petroleum-contaminated soil were reconstructed. Based on its unique metabolic properties uncovered by genome sequencing, we modified the traditional cultivation medium with antibiotics, amino acids, carbon sources, and growth factors (e.g., vitamins and metals), achieving the successful cultivation of RACS-sorted active degrader *Pigmentiphaga* sp. Our results implied that RACS-SIP-GDC is a state-of-the-art approach for the precise identification, targeted isolation, and cultivation of functional microbes from complex communities in natural habitats. RACS-SIP-GDC can be used to explore specific and targeted organic-pollution-degrading microorganisms at the single-cell level and provide new insights into their biodegradation mechanisms.



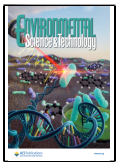
KEYWORDS: *Raman-activated cell sorting, stable-isotope probing, genome-directed cultivation, active toluene degrader, genomic sequencing*

1. INTRODUCTION

Microorganisms are the most abundant and widely distributed lifeforms on Earth, and they play critical roles in the biogeochemical cycling of organic compounds in ecosystems.[1,2] Microbial degradation of organic pollutants is the most important and sustainable process for contaminant decomposition, and active microorganisms act as degraders that convert contaminants to nontoxic compounds. Thus, investigation of the identities and metabolic characteristics of the *in situ* active degraders is critical to understanding their biodegradation mechanisms and improving their organic pollutant removal efficiencies.[3,4] Nevertheless, it remains challenging to identify, isolate, and cultivate the *in situ* active microorganisms and analyze their metabolic features. In recent years, many efficient contaminant-degrading bacteria have been isolated by cultivation-dependent methods, and their pathways have been explored to elucidate their biodegradation mechanisms.[5,6] However, these cultivation-dependent approaches may bias our view of the diversity of microorganisms, especially functional-yet-uncultivable microbes, since the vast majority (over 99%) of microbes in nature have not been cultivated.[3,7] Additionally, although the metabolic pathways of isolated microbes have been thoroughly investigated under laboratory conditions, they might not be responsible for biodegradation in natural ecosystems.[8,9]

Various cultivation-independent methods have been developed to discover and explore functional, yet uncultivable microbes in the natural environment, especially their unique ecological functions and metabolic pathways. Among them, stable-isotope probing (SIP) is a powerful approach that is used to identify the functional-yet-uncultivable microbes responsible for biodegradation and assimilation of organic

Received: June 4, 2023
Revised: September 21, 2023
Accepted: October 2, 2023
Published: October 12, 2023



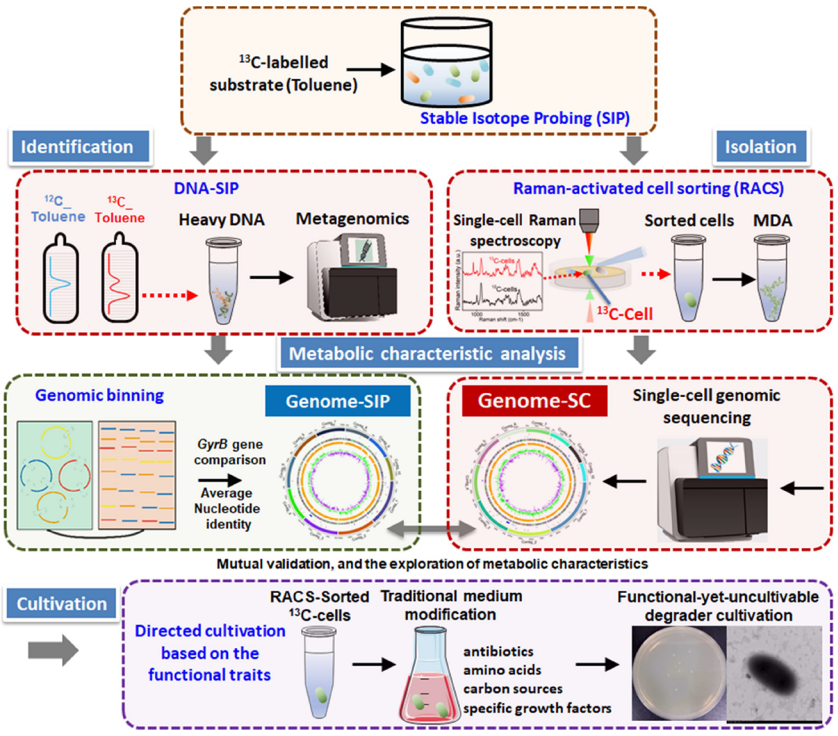


Figure 1. Schematic of protocols for identifying, sorting, and cultivating the active-yet-uncultivable microbes from the whole microbiota via RACS-SIP-GDC.

pollutants [e.g., benzene, toluene, ethylbenzene, and xylene (BTEX), polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs)] in complex microbial communities,[9−13] linking their identities to functions without the requirement of cultivation.[10,14] Nevertheless, SIP coupling with traditional molecular means such as TRFLP, high-throughput sequencing, and metagenomics can only identify the active microbes involved in biodegradation and assimilation of organic pollutants at the community level, not able to designate functional genes to their genotypes at the single-cell level[8,15] or isolate and cultivate them.[16−18] Thus, researchers are still unable to obtain the living bacterial cells responsible for *in situ* degradation of organic pollutants to explore their genetic and metabolic information further. Consequently, new techniques are urgently needed to improve studies at the single-cell level.

Raman-activated cell sorting (RACS) is an emerging technology for microbial cell isolation.[19,20] By characterizing the peaks of intrinsic biomolecules, e.g., proteins (1500−1700 cm^{-1}), carbohydrates (470−1200 cm^{-1}), phosphate groups of DNA (980, 1080, and 1240 cm^{-1}), single-cell Raman spectroscopy (SCRS) can effectively distinguish cell types or functions at single-cell level in an attractive, nondestructive manner.[21−23] When microorganisms assimilate substrates labeled with stable isotopes (such as ^{13}C, ^{15}N, or ^{2}H), the shift of Raman bands of isotopically labeled biomolecules enables the identification of functional-yet-uncultivable microorganisms by SCRS.[22−24] Accordingly, RACS combined with SIP (RACS-SIP) enables *in situ* recognition of metabolic incorporation of specific isotopic substrates into individual cells. Meanwhile, RACS can separate individual cells through a sorting system based on the SCRS properties of the identified active microorganisms.[21,25] Therefore, RACS-SIP can detect and sort functional yet uncultivable microorganisms in complex environments at the single-cell level. Combined with single-cell genomic sequencing, the functions of individual microbial cells in complex microbial communities can be linked directly to their underlying genotypes.[21,26] To date, RACS-SIP has been successfully applied to identify and sort specific functional-yet-uncultivable degraders, such as carbon-fixing bacteria and antibiotic-resistant microorganisms, from water and the human gut microbiota.[21,25] However, no studies have attempted to use this technology to explore the functional, yet uncultivable microbes responsible for the degradation of soil organic pollutants. Once introduced into the field of organically polluted soil remediation, it will be possible to identify and isolate functional-yet-uncultivable microorganisms capable of *in situ* degradation of organic pollutants at the single-cell level and reveal their genetic metabolic activities, providing the opportunity to explore the microbial degradation mechanism of soil organic pollutants.

Although RACS can sort microbial cells of specific functional-yet-uncultivable microbes from complex microbial communities, their cultivation remains challenging due to the lack of understanding of the cultivation conditions required. Nevertheless, functional and metabolic annotation of genomes allows genome-level analysis of functional-yet-uncultivable microbes, and even inferences of their cultivation requirements, possibly enabling their cultivation.[27,28] Accordingly, SIP-RACS combined with omics analyses could achieve the rapid identification, isolation, characterization, and targeted cultivation of functional yet uncultivable microbes. To our knowledge, no studies have investigated the biodegradation mechanisms of soil organic pollutants using this integrated technology or attempted to customize special culture media or modify existing media to achieve the cultivation of functional degraders sorted by RACS based on their genetic and metabolic properties.

https://doi.org/10.1021/acs.est.3c04247
Environ. Sci. Technol. 2023, 57, 17087−17098

BTEX compounds are important organic pollutants and components of petroleum hydrocarbon pollution.[10] These compounds can easily enter the environment through various pathways, causing serious water and soil pollution.[29,30] BTEX have been blacklisted as priority pollutants by many countries due to their strong toxic and carcinogenic effects on humans and soil organisms.[31] Thus, in the present study, we used toluene as a model organic pollutant due to its biodegradation diversity and environmental relevance.[32,33] We introduced a novel approach coupling RACS-SIP and genome-directed cultivation (RACS-SIP-GDC) to identify, isolate, and cultivate active toluene-degrading bacterial cells from a complex microbial community in petroleum-contaminated soil (Figure 1). RACS-SIP was successfully applied to identify the active toluene degraders and further isolate their single cells based on their spectral shifts. By application of subsequent metagenomic sequencing of ^{13}C-DNA from SIP incubations and genomic sequencing of sorted cells by RACS, we reconstructed the complete metabolic pathways of the active degraders in petroleum-contaminated soil and linked their identities to functions at the single-cell level. Based on their metabolic properties, we successfully cultivated microbes sorted by RACS by modifying the culture medium. Our results imply that RACS-SIP-GDC is a powerful approach for single-cell-level identification, isolation, and cultivation of the active but previously uncultured degraders responsible for BTEX assimilation in complex communities. Our approach will be useful for the exploration of other specific and targeted organic pollution-degrading microorganisms in soil environments and provide new insights into their biodegradation mechanisms.

2. MATERIALS AND METHODS

2.1. Sample Collection. Petroleum-contaminated soil was collected from depths of 0−20 cm in the Shengli oil field in China (37°28′N, 118°39′E). After transport to the laboratory, some soil samples were stored at −80 °C for initial DNA extraction. The remaining samples were immediately stored at 4 °C for the SIP and RACS experiments. The petroleum contents and soil characteristics of the samples are listed in Table S1.

2.2. SIP Microcosms. Microcosms were performed in 150 mL serum bottles containing 5 g of soil and 20 mL of phosphate-buffered mineral medium (Table S5), as described previously.[34] After sealing the bottles with rubber plugs and aluminum caps, unlabeled toluene (1 μL, ≥ 99%, Shanghai Aladdin Biochemical Technology, Shanghai, China) or ^{13}C-labeled toluene (1 μL, $^{13}C_6$, 99%, Cambridge Isotope Laboratories, Tewksbury, MA, USA) was added as the carbon source at a final concentration of 35 mg/L. The addition was performed using a syringe, following the methodology established in our previous study.[35] Two biotic treatments including soil with ^{12}C-toluene (^{12}C_Toluene) and soil with ^{13}C-toluene (^{13}C_Toluene) were established as well as a sterile control using sterilized soils amended with unlabeled toluene. For each treatment, six replicates were used. Each bottle was incubated at room temperature (28 °C) for 6 days and stirred at 180 rpm. Toluene in soil from each sample (days 0, 3, and 6 after incubation) was analyzed by a gas chromatograph (Agilent, Santa Clara, CA, USA) equipped with a CTC Analytics CombiPAL autosampler and a DB-5 column (30 m × 0.32 mm × 0.25 μm). The detector temperature was set at 200 °C. A standard curve was constructed with a correlation coefficient of 0.997, and the sample recovery rate was 78.2−85.6%. As almost all of the toluene was degraded by day 6 (residual toluene = 0.1−0.5%, Table S2), to avoid cross-feeding, soil samples were collected on day 3 for DNA extraction.

2.3. DNA Ultracentrifugation, Real-Time Quantitative PCR, 16S rRNA Gene Amplicon Sequencing, and Analysis. DNA was extracted from each sample using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA), according to the manufacturer's instructions. After detection with a NanoDrop 2000 spectrophotometer, DNA from the ^{12}C_Toluene and ^{13}C_Toluene microcosms was used for the CsCl gradient ultracentrifugation as previously described.[9,36] Briefly, approximately 5 μg of DNA was mixed with tris EDTA/CsCl solution at a buoyancy density (BD) value of ∼1.77 g/mL and transferred to the Quick-Seal polyallomer tubes (13 × 51 mm, 5.1 mL, Beckman Coulter, Pasadena, CA, USA). Ultracentrifugation was conducted in a Beckman Coulter L-100XP ultracentrifuge at 47,500 rpm for 48 h at 20 °C. Subsequently, 14 fractions were separated from each tube using a fraction recovery system (Beckman Coulter). After the BD values were determined with an AR200 digital refractometer, the separated DNA samples were purified through glycogen-assisted ethanol precipitation, and the bacterial 16S rRNA genes in all DNA fractions from the microcosms were amplified. The PCR reactions were conducted in a 20 μL mixture comprising 10 μL of SYBR Green PCR Premix Ex *Taq*II (TaKaRa, Japan), 0.2 μL of each primer (10 μM), and 1 μL of DNA template, utilizing the ABI 7500 real-time PCR system (Applied Biosciences, USA). Based on the relationships between the buoyant density (BD) and 16S rRNA gene abundance of each fraction (Figure S2), the DNA fraction with a BD value of 1.7415 g/mL was identified as the "heavy" DNA (^{13}C-DNA) fraction of the ^{13}C_Toluene microcosms.

For amplicon sequencing, the hypervariable V4 region of the 16S rRNA gene was amplified from total DNA extracted from all treatments, and DNA was separated from the SIP treatments using the primer set 515*F*/806R (Table S3). After sequencing on an Illumina MiSeq sequencer, sequences were processed and analyzed using the software of Quantitative Insights into Microbial Ecology (QIIME).[37,38] Subsequently, the qualified sequences were assigned to operational taxonomic units (OTU) based on 97% sequence identity with the UPARSE algorithms.[39,40] Potential chimeras in representative sequences were removed by the Uchime algorithm. The SILVA (release_132) database and Uclust classifier with default parameters were used for taxonomic assignment.[41] Details for this section are provided in the Supporting Information.

To detect the active toluene-assimilating organisms, the relative abundance of each OTU in all the DNA fractions from the ^{12}C_Toluene and ^{13}C_Toluene microcosms was calculated, and the top 100 OTUs were selected for further analysis. Based on our previous methods, the toluene-assimilating organisms characterized by OTUs were significantly enriched in the heavy DNA (^{13}C-DNA) fractions from ^{13}C_toluene microcosms compared to those from ^{12}C_toluene microcosms.[4,5]

2.4. Metagenomic Assembly and Binning of Toluene Degraders from ^{13}C-DNA. Illumina library preparation and sequencing were performed using the Illumina NovaSeq-PE250 at Personal Biotechnology Co. (Shanghai, China). Approximately 4 Gb of sequencing data (24,030,270 reads) was generated for the ^{13}C-DNA of the ^{13}C_Toluene micro-

https://doi.org/10.1021/acs.est.3c04247
Environ. Sci. Technol. 2023, 57, 17087−17098

cosms. After removing the sequencing adapters, the reads were trimmed using Trimmomatic (version 0.33).[42] Using cutadapt, reads with either an average quality score <25 or length <80 bps were removed. Human contamination (Blastn E-value threshold $\leq 10^{-5}$, bitscore ≥50, percent identity ≥75%) was further excluded by aligning reads to the human reference genome (build 37) using bowtie2 (version 2.1.0).[43] Short sequences were assembled into contigs using MEGAHIT v1.2.9 in the MetaWRAP v1.3.2 toolkit,[44,45] evaluated by QUAST v5.0.2.[46] Contigs <1000 nt were filtered using a custom Python script. A total of 435,592 scaffolds were generated from ^{13}C-DNA, the longest reaching 965,041 bp. To explore the toluene metabolic pathways of functional microorganisms, the assembled metagenomes were binned using MaxBin2 and metaBAT2,[45] evaluated by CheckM[46] (comp ≥80%, cont ≤10%) to generate high-quality bins. Assembled contigs of the target bins related to toluene degradation were used for protein sequence prediction and annotation using Prokka v1.1, while KEGG pathways were inferred using HUMAnN2.[36] As 16S rRNA genes are difficult to assemble into the metagenome-assembled genomes, the *gyrB* gene that encodes the B subunit protein of DNA gyrase (topoisomerase type II) and has a single copy was chosen as a molecular classification marker.[47] Furthermore, we confirmed whether the assembled bins represented the genomes of the SIP-identified active degraders using whole-genome comparisons based on average nucleotide identities (ANI).[48] From the ^{13}C-DNA of the ^{13}C_Toluene microcosms, three high-quality metagenomic bins were assembled (Table S4) and compared to the identities of active degraders.

2.5. Spectral Acquisition and Analysis in RACS. To sort the active degrading bacterial cells, samples of the ^{13}C_Toluene microcosms were used for RACS, and those in ^{12}C_Toluene microcosms were used as controls. Bacterial cells in the original samples at t = 0 days were used to establish an RACS benchmarking experiment. First, 3 mL of the sample was transferred in a centrifuge tube and placed in an ice block, followed by sonication at 300 mW for 1 min with a 1-s pause for every 1 s of sonication. To remove impurities that might have interfered with Raman spectral acquisition, the samples were centrifuged at 2000*g* for 5 min. To collect and clean bacterial cells, the supernatants were then collected, centrifuged at 5000*g* for 5 min, and washed four times with deionized water. The microbial cell pellet was then resuspended in deionized water, and 2 μL of cell suspension was spotted onto a sorting chip (metal-coated ejection chip, HOOKE Instruments Ltd., Changchun, China) and air-dried at room temperature prior to Raman spectral acquisition.[24] SCRS was performed using a 532 nm neodymium-doped yttrium aluminum garnet laser (Laser Quantum, Bedford, MA, USA) with a 300-grooves/mm diffraction grating. Raman spectra were acquired in the range 400−2000 cm^{-1} with a laser power of 5 mW and an acquisition time of 5 s. The measured spectral data were preprocessed using LabSpec 6 software (Horiba) for baseline correction and vector normalization.[25] When bacterial cells utilize isotopically labeled substrates and synthesize macromolecular markers such as phenylalanine, purine compounds (guanine and adenine), and unsaturated lipids, the heavier isotopic atoms replace the lighter atoms, changing their bond vibrations and shifting their wavenumbers to lower positions.[22,49]

According to the previous study, the positions of the Raman bands of phenylalanine, purines (guanine and adenine), and unsaturated lipids in unlabeled bacterial cells are 1001−1003, 1578, and 1663 cm^{-1} respectively. For functional bacterial cells incorporating ^{13}C, these Raman bands would be significantly red-shifted, with extents of −37, − 47, and −35 cm^{-1}, respectively.[49] Accordingly, the positions of the Raman bands detected via SCRS from the functional bacterial cells incorporating ^{13}C-toluene were determined and analyzed to establish a relationship between the red shift and ^{13}C assimilation in the ^{13}C_Toluene microcosms.

2.6. Isolation of ^{13}C-Cells by RACS and Single-Cell Genomic Sequencing. Single cells of the active microbes with a ^{13}C-shift were then sorted using the single-cell ejection technique of PRECI SCS.[25] Briefly, after determining the location of active bacterial cells from the cell coating chip, they were sorted one by one into the cell receiver (HOOKE Instruments Ltd., Changchun, China) loaded with cell lysis buffer (2 μL) using a laser beam. In total, 11 individual cells were ultimately isolated and lysed, and the whole procedure of Raman band identification, ^{13}C-shift analysis and sorting took less than 1 h for each chip to ensure minimal change of biological traits. Their genomic DNA was amplified by multiple displacement amplification (MDA),[21] and verified by PCR using the primer set 27*F*/1492R (Table S3) to confirm whether the sorted cells were successfully amplified. Genomic sequencing was performed using the PE150 strategy of the Illumina HiSeq X-ten platform (Illumina, USA).[25] In total, 2 Gb of sequencing data were acquired and filtered to remove short reads with nucleotides (nt) <80 and mean quality scores <25. Short sequences were then assembled into contigs using the MEGAHIT assembler;[44] 384 assembled contigs >1000 bp in length were obtained from the single-cell samples. Subsequently, the assembled contig data were functionally predicted and annotated using Prokka v1.12.31.[50] The genomes of the functional microbial cells were assembled and obtained using the binning tools of MetaWRAP.[45] To further confirm the identities of the sorted cells and investigate their functional genes and metabolic characteristics, *gyrB* gene comparison, ANI methods, and the KEGG database were applied. In addition, the AromaDeg database was used to detect functional genes that might be involved in aerobic degradation of aromatic compounds from the sorted degraders.[51] To align the functional gene sequences related to the AromaDeg enzyme families, the MAFFT and L-INS-i strategies were used with the parameters E-value >0.00001, identity >50%, query coverage >50%, and topic coverage >30%.

2.7. Cultivation of Active Toluene Degraders by Modifying the Culture Medium. To cultivate the active but previously uncultured microorganisms sorted by RACS from petroleum-contaminated soil, we attempted to modify the traditional cultivation medium based on the metabolic and genetic information revealed by RACS, coupled with the genome binning of toluene degraders from genomic sequencing. This method is capable of directly cultivating the targeted active microorganisms using the information obtained by genomic analysis.[27,52] Here, we took into consideration that genes involved in the metabolism of vitamins (e.g., vitamins A, B1, B2, B3, B6, B7, B12, and lipoic acid) were identified, and those associated with trace metals conducive to microbial growth (e.g., Mg, Ca, Cu, Zn, Fe, Co, Na, Mo, and Ni) were also detected in the assembled genome (Figure 4). The degraders contained leucine and lactate dehydrogenase, and were possibly resistant to aminoglycoside antibiotics due to the

https://doi.org/10.1021/acs.est.3c04247
Environ. Sci. Technol. 2023, 57, 17087−17098

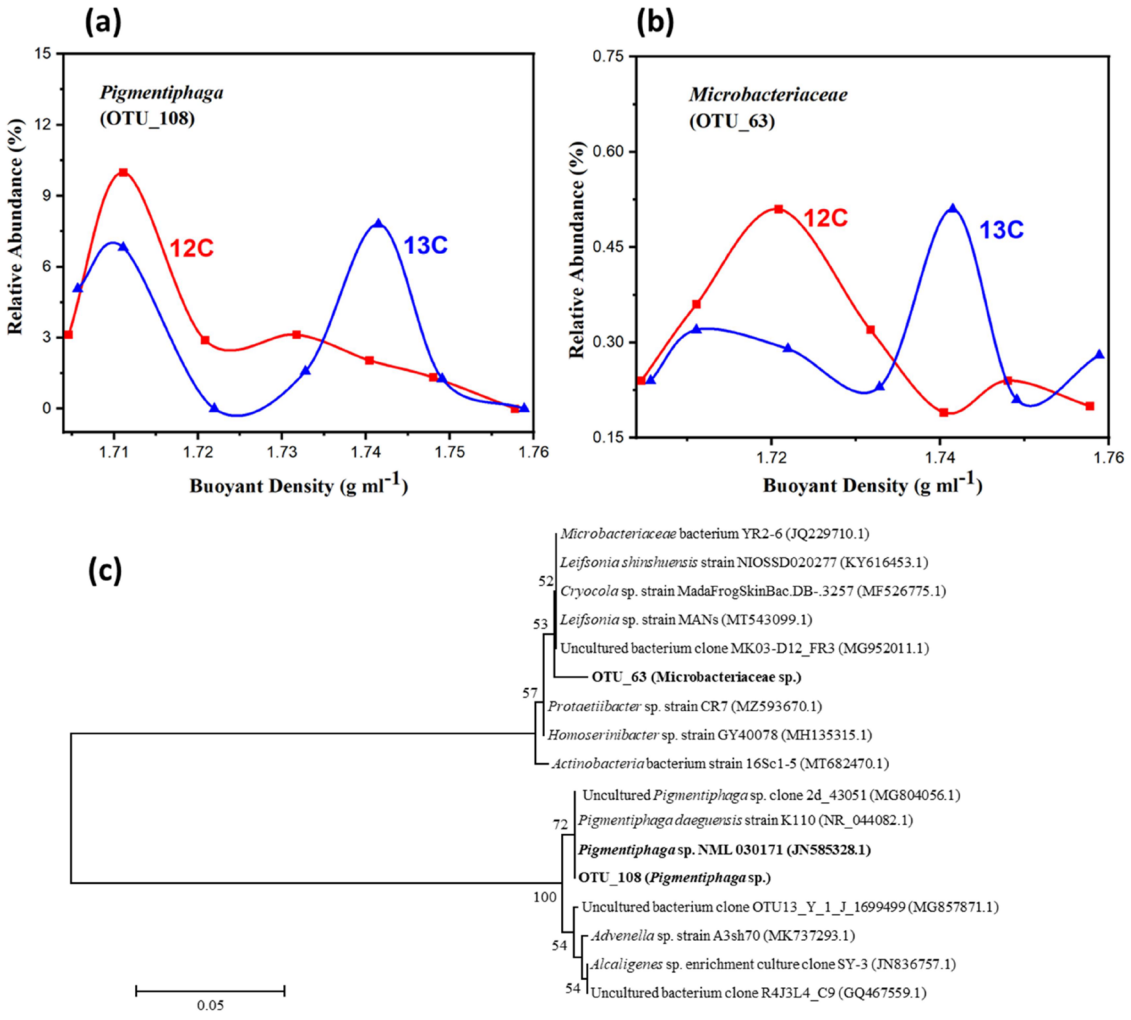


Figure 2. Relative abundances of (a) OTU_108 and (b) OTU_63 fragments over a range of buoyant densities in DNA extracted from petroleum-contaminated soil amended with either ^{12}C-toluene or ^{13}C-toluene. 12C and 13C represent the ^{12}C_Toluene and ^{13}C_Toluene treatments, respectively. (c) Phylogenetic tree of SIP-identified OTUs based on their 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1200 replications) > 50% are shown at the branch points. Bar: 0.05 substitutions per nucleotide position.

presence of an aminoglycoside antibiotic resistance gene in the genome. Thus, we modified the traditional cultivation medium (phosphate-buffered mineral medium; Table S5) by supplementation with 10 mL/L vitamin and mineral stock solutions, as well as 5 mg/L streptomycin (an aminoglycoside antibiotic), and 50 mM lactate and leucine to target the cultivation of active toluene degraders from contaminated soil (Table S5). In traditional cultivation medium, only lactate was added as a source of carbon and energy.

To realize the cultivation of active degraders, the laser pulse acts on the cell coating chip to transfer each cell to an individual cell receiver containing 4 μL of traditional cultivation (TC) or modified minimal (MM) media during the sorting process. Totally, 20 single cells were sorted individually by RACS and swiftly transferred to TC medium with lactate as well as MM medium supplemented with lactate and leucine. After incubation at room temperature for 7 days, the culture solutions were spread on the corresponding solid media and maintained under the same culture conditions for 4 days to obtain single, pure bacterial isolates. Pure cultures were passaged a few times to check for purity. Subsequently, the genomic DNAs of the cultivated bacteria were extracted, and their 16S rRNA gene sequences were amplified using the bacterial universal primers of 27F and 1492R (Table S3) and sequenced to determine their identities. Cell and colony morphology were explored using a transmission electron microscope (JEM-1400; Jeol) as described previously.[53,54] In addition, toluene degradation experiments were performed in 150 mL brown glass bottles containing 20 mL of MM medium, and bacterial cells were adjusted to approximately 5×10^6 colony-forming units/mL with the dilution plate counting method.[5,55] After the bottles were sealed with rubber stoppers and aluminum seals, 1 μL of unlabeled toluene was added into the bottles as a carbon source. Sterile controls without bacterial cells were also established. All tests were performed in triplicate, using the same standard and incubation conditions as those used for the SIP microcosms.

2.8. Statistical Analysis. Data were expressed as means ± the standard deviation (SD). Statistical analyses were performed using analysis of variance (ANOVA) in SPSS (version 24.0; SPSS Inc., USA) and Origin 8.0 (OriginLab Corporation, Northampton, MA, USA) software. p-Values of less than 0.05 ($p < 0.05$) and 0.001 ($p < 0.001$) were considered to indicate statistical significance. Phylogenetic information regarding the isolated strain, active degrading bacteria and functional genes was determined by BLAST

https://doi.org/10.1021/acs.est.3c04247
Environ. Sci. Technol. 2023, 57, 17087−17098

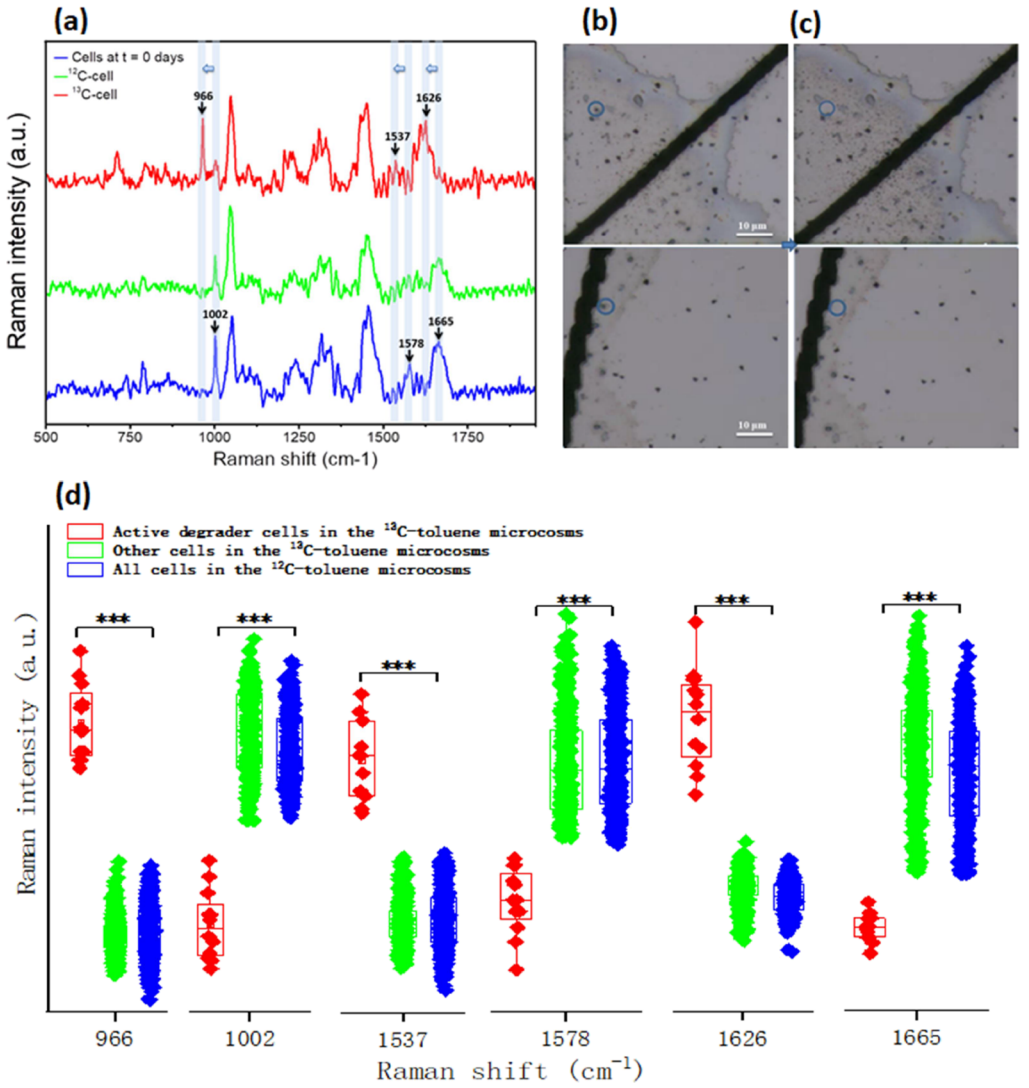


Figure 3. RACS of the active toluene degraders in soil. (a) Single-cell Raman spectra of cells after cultivation with ^{12}C-toluene and ^{13}C-toluene, tagged as ^{12}C-cell and ^{13}C-cell, respectively. Cells at t = 0 days indicate the Raman spectra of cells treated with ^{13}C-toluene at time t = 0 days. Each spectrum represents an average of SCRS from detected cells (red, 11 SCRS of ^{13}C-cells from the ^{13}C-toluene microcosms, green, 489 SCRS of ^{12}C-cells from the ^{13}C-toluene microcosms; 500 SCRS of ^{12}C-cells from the ^{12}C-toluene microcosms). (b) Active bacterial cells on the sorting chip were identified by SCRS from the ^{13}C-toluene microcosms. (c) Cells were ejected off the sorting chip by RACS. (d) Intensities of Raman bands at 966, 1002, 1537, 1578, 1624, and 1660 cm^{-1} based on the active cells detected in the ^{13}C-toluene microcosms, unlabeled cells in the ^{13}C-toluene microcosms, and cells in the ^{12}C-toluene treatments. All comparisons denoted by asterisks (***) and a black bar are significantly different (one-way ANOVA, p < 0.001).

(National Center for Biotechnology Information, Bethesda, MD, USA) and MEGA version 5.0 after multiple alignments.[56] Clustering was performed with the neighbor-joining method, and the phylogenetic tree was determined using bootstrap analysis based on 1200 resamplings.

3. RESULTS

3.1. Active Toluene Degraders Identified by SIP and their Metagenomic Assembly and Binning from ^{13}C-DNA. Details of the toluene biodegradation and soil microbial community structures, as well as the active toluene degraders, are provided in the Supporting Information. Based on the correlations between 16S rRNA gene abundance and BD values (Figure S2), DNA in the fraction with a BD value of 1.7415 g/mL was selected as the heavy DNA fraction for the SIP microcosms, and microbes represented by two OTUs (OTU_108 and OTU_63) were the active degraders (Figure 2). Based on the phylogenetic analysis, OTU_63 was assigned to the unclassified Microbacteriaceae (order Micrococcales); OTU_108 belongs to the genus *Pigmentiphaga* of Alcaligenaceae (order Burkholderiales) and shares 100% identity with *Pigmentiphaga* sp. NML 030171 (JN585328.1) (Figure 2c).

To investigate further the genetic and metabolic information on the active *Pigmentiphaga* sp. (OTU_108; with an abundance of 7.8% in the heavy DNA fraction) and unclassified Microbacteriaceae (OTU_63; 0.51%), metagenome binning was applied to the ^{13}C-DNA metagenomes and three high-quality assembled bins were generated (Table S4). Among them, bin 3 with a completeness of 98.2% was associated with the active degrader *Pigmentiphaga* sp. (OTU_108; Figure S3). Based on the phylogenetic analysis, the sequence of the *gryB* gene was 100% identical to that of *Pigmentiphaga* sp. NML 030171. Furthermore, the ANI value between bin 3 and strain NML 030171 was 98.5% (Figure S4),

https://doi.org/10.1021/acs.est.3c04247
Environ. Sci. Technol. 2023, 57, 17087−17098

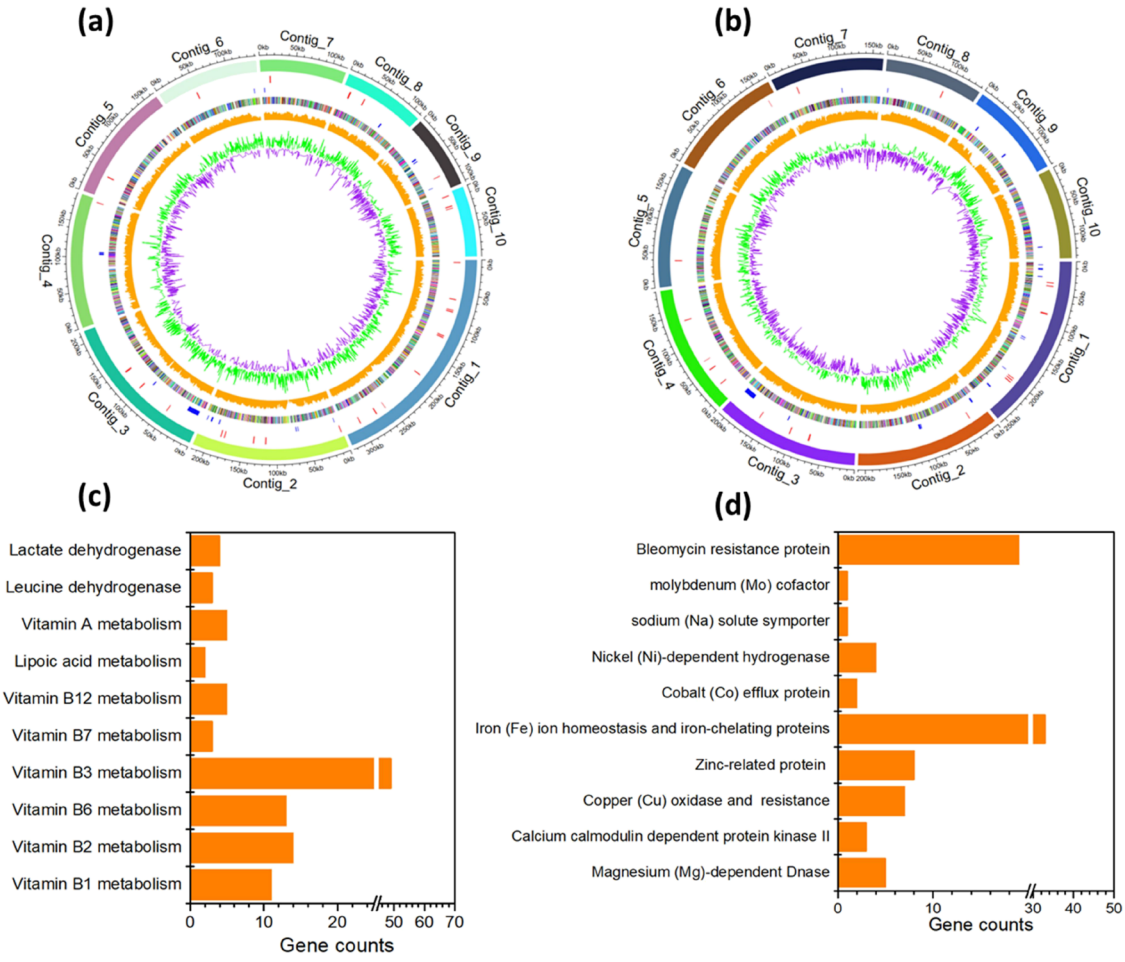


Figure 4. Circular representations of genome-SC from sorted cells (a) and genome-SIP from ^{13}C-DNA (b). From inside out: GC skew (Green >0, Purple <0), GC percent, peaks out/inside the circle indicate values higher or lower than the average G+C content, regions in minus and plus strands (colored by COG functional categories), and genes annotated by the CAZy database. Contigs are highlighted in different colors in the outermost circle. (c) Counts of genes involved in the metabolism of cofactors, vitamins, leucine, and lactate in the genome of *Pigmentiphaga* sp. (d) Counts of genes associated with aminoglycoside antibiotic resistance and metal transport, efflux, oxidase, resistance, and metalloprotease in the *Pigmentiphaga* sp. genome.

which was higher than the intra- and interspecies threshold value (95%), indicating that the microorganism containing the genome assembled from ^{13}C-DNA (referred to as genome-SIP) and strain NML030171 were the same species. Thus, bin 3 was speculated to be the genome of the *Pigmentiphaga* sp. (OTU_108). Regrettably, we did not obtain the genome of the microorganisms represented by OTU_63, probably due to their low abundance in the heavy DNA fraction.

3.2. Identification and Sorting of Toluene-Degrading Bacterial Cells by RACS. In each of the ^{12}C-toluene and ^{13}C-toluene microcosms, 500 bacterial cells were examined by SCRS. Among them, 11 cells from the ^{13}C-toluene microcosms exhibited Raman bands significantly shifting compared to those in the ^{12}C-toluene microcosms (Figure 3a), explained by ^{13}C incorporation. All cells from the ^{12}C-toluene microcosms exhibited three identical Raman bands as biomarkers for unlabeled cells,[49] while active degraders assimilated ^{13}C in the ^{13}C-toluene microcosms, showing significant red shifts from 1002 to 966 cm^{-1}, 1578 to 1537 cm^{-1}, and 1665 to 1626 cm^{-1}, consistent with previous studies;[49,57] however, no bacterial cells with ^{13}C red shifts were observed in the ^{12}C-toluene treatments. Accordingly, the bacterial cells with a ^{13}C-shift could be identified, and the three bands were selected as biomarkers to sort the active cells. Further analysis on Raman intensities illustrated that the ^{13}C-assimilating cells in the ^{13}C-toluene microcosms exhibited remarkably higher intensities at 966, 1537, and 1624 cm^{-1} but lower intensities at 1002, 1578, and 1660 cm^{-1} ($p < 0.001$) (Figure 3d). These results indicated that RACS-SIP effectively recognized microbial cells actively assimilating toluene in the complex microbial community.

In this study, 11 single cells with identical ^{13}C-shifted bands were sorted using RACS (Figure 3b,c). After amplification, MDA products were subjected to genome sequencing. Based on the sequence analysis, one assembled genome with a high completeness of 65.8% was generated from the sorted cells assimilating ^{13}C, designated as genome-SC. In this genome, the 16S rRNA gene was not detected; however, the *gyrB* gene was identified and was exactly the same as that of genome-SIP; its phylogenetic tree showed that the *gyrB* gene of genome-SC was most closely related to *Pigmentiphaga* sp. NML030171 (WP 087839044.1) with 100% identity, indicating that the sorted cells were the active degrading microorganism represented by OTU_108 (Figure S3). Additionally, the ANI value between genome-SC and genome-SIP was 100%, indicating that these two genomes were derived from the

https://doi.org/10.1021/acs.est.3c04247
Environ. Sci. Technol. 2023, 57, 17087−17098

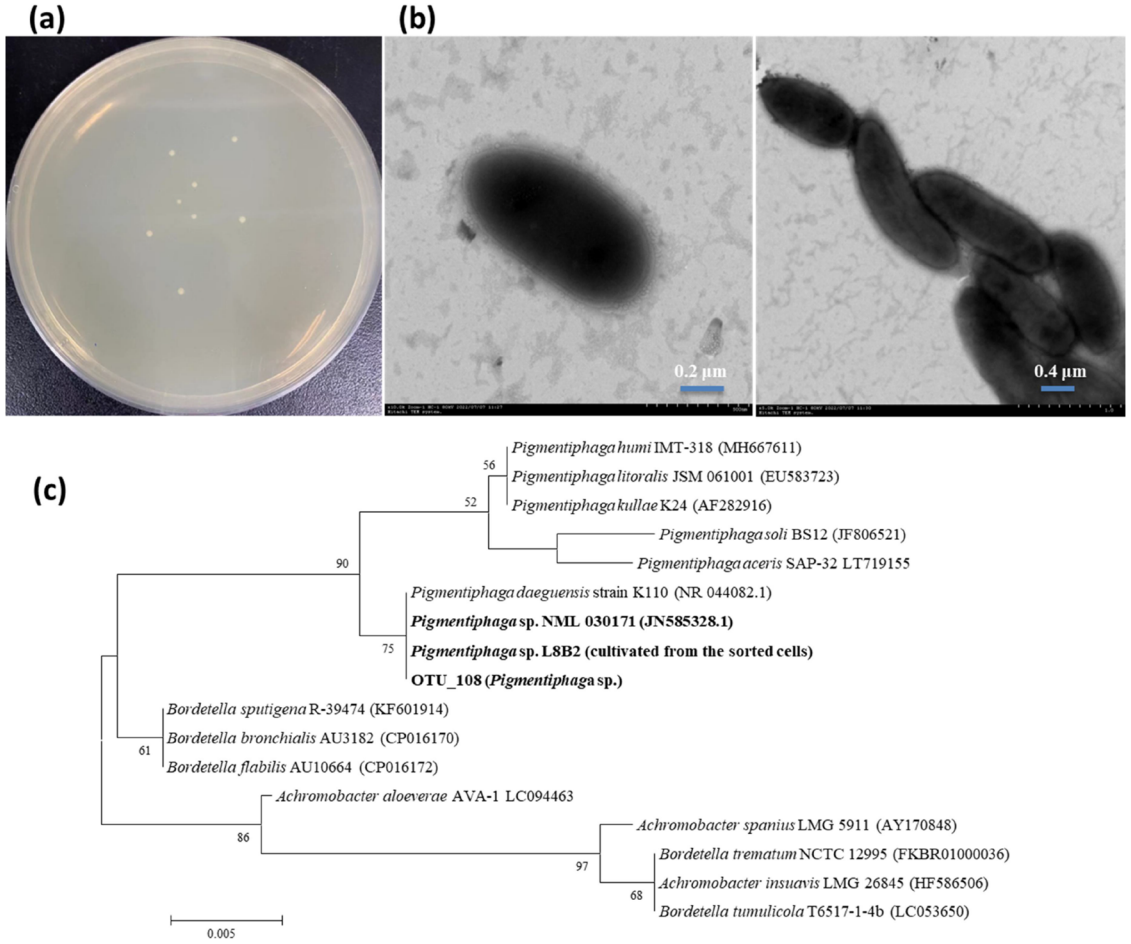


Figure 5. (a) Colonies of *Pigmentiphaga* sp. L8B2 cultivated on MM medium agar plates. (b) Transmission electron micrographs of the bacterial cells of strain L8B2. Bars indicate 0.2 μm (left) and 0.4 μm (right). (c) Phylogenetic tree of the isolated active toluene degrader strain L8B2. Neighbor-joining tree based on 16S rRNA (rRNA) gene sequences showing the phylogenetic position of this strain and representatives of other related taxa. Bootstrap values (expressed as percentages of 1200 replications) > 50% are shown at the branch points. The bar indicates 0.005 substitutions per nucleotide position.

same bacterium and further supporting the above results (Figure S4).

3.3. Toluene Biodegradation Pathway and Metabolic Characteristics of *Pigmentiphaga* sp. (OTU_108). To confirm further the involvement of the sorted *Pigmentiphaga* in toluene degradation and explore its metabolic characteristics, we evaluated its functional genes based on the KEGG database (Figures 4 and S5). In genome-SC, several genes involved in the toluene biodegradation pathway (toluene monooxygenase, phenol hydroxylase, aryl-alcohol dehydrogenases, benzoate 1,2-dioxygenase, catechol 1,2-dioxygenase, and 2,3-dihydroxytoluene dioxygenase) were detected, and a nearly complete metabolic pathway was reconstructed (Figure S5). Notably, an additional toluene-degradation-related gene (dihydroxycyclohexadiene carboxylate dehydrogenase, *benD*) was identified in genome-SIP, due to its higher completeness (98.2%) than genome-SC (65.8%) (Figure 4). This result indicated that *Pigmentiphaga* sp. could metabolize toluene through various pathways. We also analyzed the functional genes related to the AromaDeg enzyme families in the sorted cells (Table S6), and a variety of genes were detected, including aromatic ring-hydroxylating and ring-opening dioxygenases, biphenyl and naphthalene oxygenases, and protocatechuate 3,4-dioxygenase. These results hinted at the potential of *Pigmentiphaga* sp. for the degradation of aromatic compounds, such as PAHs and PCBs.

We chose genome-SC to analyze the metabolic properties of the sorted cells to explore clues for their cultivation. Genes were screened for those involved in the metabolism of cofactors, vitamins, amino acids, and organic compounds, as well as those related to metal transport, efflux, oxidase, resistance, and metalloproteases, which are important for microbial cultivation. As shown in Figure 4, genes involved in the metabolism of vitamins (e.g., vitamins A, B1, B2, B3, B6, B7, B12, and lipoic acid) were identified, and those associated with trace metals conducive to microbial growth (e.g., Mg, Ca, Cu, Zn, Fe, Co, Na, Mo, and Ni)[58] were also detected in genome-SC. Additionally, *Pigmentiphaga* sp. (OTU_108) contained the aminoglycoside antibiotic resistance protein, as well as leucine and lactate dehydrogenase (Figure 4). Based on these metabolic features, a specific MM medium was developed to cultivate the active *Pigmentiphaga* strains sorted by RACS.

3.4. Cultivation of an Active Toluene-Degrading Bacterium Sorted by RACS. Based on the metabolic characteristics of *Pigmentiphaga* sp. and the optimization of the cultivation medium, a significant proliferation of active microbial cells was observed, resulting in noticeable turbidity

https://doi.org/10.1021/acs.est.3c04247
Environ. Sci. Technol. 2023, 57, 17087−17098

(OD_{600} = 0.78) in the MM medium after 7 days of incubation. Following purification, a single bacterial strain was successfully cultivated from the sorted cells and identified as *Pigmentiphaga* sp. L8B2. This strain was named *Pigmentiphaga* sp. L8B2, which is a Gram-negative, rod-shaped, nonspore-forming, and 0.34−0.46 μm × 0.85−1.57 μm bacterium (Figure 5). Colonies on MM agar plates after 7 days of incubation at room temperature were smooth, convex, white in color, and 1.0−2.0 mm in diameter. Based on the 16S rRNA gene sequence analysis, strain L8B2 shares 100% identity with the strains of OTU_108 and *Pigmentiphaga* strain NML 030171. In the neighbor-joining phylogenetic dendrogram (Figure 5c), this strain belongs to the genus *Pigmentiphaga* and formed a subclade together with the strains of OTU_108 and NML 030171. Meanwhile, strain L8B2 was able to grow in MM medium with toluene as the sole carbon source, and more than 90% of the toluene was biodegraded after 3 days of incubation, whereas only <10% of toluene was removed in sterile control (Figure S6). Accordingly, strain L8B2 was identified as the active toluene degrader, represented by OTU_108. However, upon the introduction of active cells into the TC medium, the medium retained its optical clarity (OD_{600} = 0), and no cultivable microbes were found, hinting at the advantages of cultivation medium optimization in targeted cultivation of the active degraders. Our results indicated the feasibility of RACS-SIP combined with genome binning analysis for the identification, isolation, and characterization of the active degraders at the single-cell level from complex microbial communities, which provides inferences for the modification of cultivation medium for target microbes based on their metabolic characteristics, enabling successful cultivation of the active but previously uncultured degrading microorganisms.

4. DISCUSSION

In this study, we introduced a method called RACS-SIP-GDC (Figure 1), which integrates RACS, SIP, genome analysis, and directed cultivation to characterize, isolate, and cultivate toluene-assimilating organisms from petroleum-contaminated soils. In general, SIP can trace microorganisms that assimilate stable isotope-labeled substrates, linking their identities and functions.[59] RACS-SIP can detect and sort the active degraders based on their Raman shift;[49] genome binning analysis is able to assemble microbial genomes from sorted single cells and explore their genetic characteristics and metabolic potential.[51] The combination of genome binning and RACS-SIP therefore provides an opportunity to obtain the genomes of the active degraders in the environment, revealing their genetic and metabolic characteristics. However, accurately determining the links between the identified active degraders and their assembled genomes remains a great challenge. Here, we used *gyrB* gene comparison and ANI determination to confirm their association.[60,61] Using the above-integrated techniques, we successfully obtained the genome of an active degrader represented by OTU_108 identified by SIP (genome-SIP) and of ^{13}C-incorporating cells sorted by RACS (genome-SC), and determined their relationship.

Functional and metabolic annotation of single-cell genomes and metagenomes allowed us to infer physiological or ecological roles for species in a complex community, which could be used to capture specific targeted as-yet-uncultured microbes from complex communities into pure cultures.[18,58] Accordingly, the genetic and metabolic characteristics of an active degrader identified by RACS-SIP can be used to elucidate its preferred cultivation conditions. Several genes involved in the metabolism of cofactors and vitamins, amino acids, and organic compounds, as well as genes associated with trace metals and aminoglycoside antibiotic resistance, were detected in the genome-SC of the active degrader *Pigmentiphaga* sp. (Figure 4). These cofactors and vitamins, amino acids, organic compounds, and trace-metal elements contribute to the growth and cultivation of the identified degraders.[58,62] In addition, the application of antibiotics such as streptomycin could effectively screen microorganisms with specific resistances (*Pigmentiphaga* sp. in this study) and avoid contamination from other microorganisms, which is conducive to the cultivation of the active functional-yet-uncultivable degraders.[58,63] Based on the above results, we modified the composition of the traditional medium (TC) by adding the substances and elements detailed above to prepare a new medium (MM), and successfully cultivated the sorted target cells of *Pigmentiphaga* sp. represented by OTU_108 in contaminated soil. In contrast, they remained uncultured on the TC medium, evidencing that some previously uncultured microbes were only cultivable under specifically tailored conditions instead of a well-accepted artificial cultivation medium. The results confirmed the feasibility of RACS-SIP-GDC to identify, isolate, and cultivate previously uncultured microorganisms in complex communities based on their genetic and metabolic characteristics. This approach can also provide technical guidance for screening functional but previously uncultured microbes responsible for toluene degradation.

Using RACS-SIP-GDC, we successfully cultivated the BTEX degrader *Pigmentiphaga* sp. L8B2 from microbial cells sorted by RACS using a modified cultivation medium. Sequencing analysis of the *gyrB* gene revealed that the sorted bacterial cells shared 100% identity with *Pigmentiphaga* sp. strain NML030171; additionally, the ANI between genome-SIP and strain NML030171 was >95%, (Figure S4), indicating that strains L8B2 and NML030171 are the same species of *Pigmentiphaga*, but may be different subspecies. Strain NML030171 was recovered from clinical materials, which was able to reduce nitrate and was more susceptible to a number of antibiotics, including aztreonam and tobramycin.[64] However, its functions associated with the degradation of organic pollutants such as BTEX, PAHs, and PCBs have not been reported. For strain L8B2, we used genome-SC to reconstruct a nearly complete toluene metabolic pathway and identified several genes responsible for toluene biodegradation, especially the toluene monooxygenase encoding the key metabolic enzyme catalyzing the first step in aerobic toluene degradation,[65] confirming its involvement in toluene degradation. Additionally, several genes related to the degradation of aromatic compounds, such as aromatic ring-hydroxylating and ring-opening dioxygenases, biphenyl and naphthalene oxygenases, and protocatechuate 3,4-dioxygenase, were identified in the sorted single cells of *Pigmentiphaga* sp., indicating its potential to metabolize multiple aromatic compounds in oil-contaminated soils, including PAHs and PCBs. Accordingly, our study provides a novel method to clarify the molecular mechanisms underlying organic-pollutant bioremediation by specific microbes in complex microbial communities at the single-cell level.

In conclusion, our study proposed a RACS-SIP-GDC approach that integrates multiple techniques including

https://doi.org/10.1021/acs.est.3c04247
Environ. Sci. Technol. 2023, 57, 17087−17098

RACS, SIP, genome analysis, comparative *gyrB* gene sequence analysis, ANI, and directed cultivation to identify, isolate, and cultivate specific functional, yet uncultivable microorganisms in complex microbial communities in natural habitats. Using this technique, we successfully identified and sorted microbial cells of previously uncultured toluene degraders in complex communities and achieved their targeted cultivation after analyzing their genetic and metabolic characteristics to tailor the cultivation medium. The overall process comprises the identification of previously uncultured degraders by SIP, simultaneously detecting and sorting the microbial cells of target bacteria via RACS, exploring their genetic characteristics and metabolic potential through genome binning analysis, and modifying the specific cultivation medium to match their metabolic traits. This study provides a standard identification−sorting−cultivation process to recognize, isolate, and cultivate functional yet uncultivable microbes in complex microbial communities.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.3c04247.

Relative abundance of 16S rRNA defined bacterial genera in the ^{12}C_Toluene (^{12}C) and ^{13}C_Toluene (^{13}C) microcosms; correlation between 16S rRNA gene abundance and buoyant density (BD, g/mL) in DNA extracted from the ^{12}C_Toluene and ^{13}C_Toluene microcosms; phylogenetic tree of the assembled genome-SIP and genome-SC associated with the active toluene degrader identified by RACS-SIP based on the neighbor-joining method using gyrB gene sequences; ANI values between the genome-SIP and genome-SC and strain NML030171; reconstructed toluene metabolic pathway of the sorted cells incorporating ^{13}C-toluene characterized by RACS-SIP (*Pigmentiphaga* sp.); toluene-degrading efficiency of *Pigmentiphaga* sp. L8B2 in MM medium supplied with toluene as carbon source; concentrations of petroleum content and soil characteristics; residual toluene percentage in soil; primers used for the PCR of 16S rRNA genes; the information on the assembled bins from ^{13}C-DNA fraction; components of the traditional cultivation medium (a), modified minimal medium (b), vitamin stock solution (c), and mineral stock solution (d); the count of functional genes encoding enzymes for aerobic bacterial degradation of aromatics in the sorted bacterial cells (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Chunling Luo − *State Key Laboratory of Organic Geochemistry and Guangdong-Hong Kong-Macao Joint Laboratory for Environmental Pollution and Control, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China; CAS Center for Excellence in Deep Earth Science, Guangzhou 510640, China; University of Chinese Academy of Sciences, Beijing 100039, China;* orcid.org/0000-0003-2359-4246; Phone: +86-20-85290290; Email: clluo@gig.ac.cn; Fax: +86-20-85290706

Authors

Jibing Li − *State Key Laboratory of Organic Geochemistry and Guangdong-Hong Kong-Macao Joint Laboratory for Environmental Pollution and Control, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China; CAS Center for Excellence in Deep Earth Science, Guangzhou 510640, China; University of Chinese Academy of Sciences, Beijing 100039, China;* orcid.org/0000-0003-3115-9017

Dayi Zhang − *Key Laboratory of Groundwater Resources and Environment, Ministry of Education, Jilin University, Changchun 130012, China; College of New Energy and Environment, Jilin University, Changchun 130021, China;* orcid.org/0000-0002-4175-5982

Bei Li − *State Key Lab of Applied Optics, Changchun Institute of Optics, Fine Mechanics and Physics, Chinese Academy of Sciences, 130033 Changchun, China; HOOKE Instruments Ltd., 130033 Changchun, China*

Gan Zhang − *State Key Laboratory of Organic Geochemistry and Guangdong-Hong Kong-Macao Joint Laboratory for Environmental Pollution and Control, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China; CAS Center for Excellence in Deep Earth Science, Guangzhou 510640, China;* orcid.org/0000-0002-9010-8140

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.3c04247

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support was provided by the National Natural Science Foundation of China (Nos. 32061133003 and 42277210), the Key-Area Research and Development Program of Guangdong Province (2020B1111530003), the Natural Science Foundation of Guangdong Province, China (2023B1515020038), and the Youth Innovation Promotion Association CAS (2023368). We acknowledge the cooperation between China and the EU through the EiCLaR project (European Union's Horizon 2020, N° 965945).

■ REFERENCES

(1) Zhou, Z. C.; Liu, Y.; Pan, J.; Cron, B. R.; Toner, B. M.; Anantharaman, K.; Breier, J. A.; Dick, G. J.; Li, M. Gammaproteobacteria mediating utilization of methyl-, sulfur- and petroleum organic compounds in deep ocean hydrothermal plumes. *ISME J.* **2020**, *14* (12), 3136−3148.

(2) Whitman, W. B.; Coleman, D. C.; Wiebe, W. J. Prokaryotes: The unseen majority. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95* (12), 6578−6583.

(3) Dombrowski, N.; Donaho, J. A.; Gutierrez, T.; Seitz, K. W.; Teske, A. P.; Baker, B. J. Reconstructing metabolic pathways of hydrocarbon-degrading bacteria from the Deepwater Horizon oil spill. *Nat. Microbiol.* **2016**, *1* (7), 16057.

(4) Li, J.; Luo, C.; Zhang, D.; Song, M.; Cai, X.; Jiang, L.; 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.

(5) Li, J.; Luo, C.; Song, M.; Dai, Q.; Jiang, L.; Zhang, D.; 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.
(6) Moreno-Forero, S. K.; van der Meer, J. R. Genome-wide analysis of Sphingomonas wittichii RW1 behaviour during inoculation and growth in contaminated sand. *ISME J.* **2015**, *9* (1), 150−165.
(7) Rappé, M. S.; Giovannoni, S. J. The Uncultured Microbial Majority. *Annu. Rev. Microbiol.* **2003**, *57* (1), 369−394.
(8) Jiang, L.; Song, M.; Luo, C.; Zhang, D.; Zhang, G. Novel Phenanthrene-Degrading Bacteria Identified by DNA-Stable Isotope Probing. *PLoS One* **2015**, *10* (6), No. e0130846.
(9) Li, J.; Zhang, D.; Song, M.; Jiang, L.; Wang, Y.; Luo, C.; 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.
(10) Winderl, C.; Penning, H.; von Netzer, F.; Meckenstock, R. U.; Lueders, T. DNA-SIP identifies sulfate-reducing Clostridia as important toluene degraders in tar-oil-contaminated aquifer sediment. *ISME J.* **2010**, *4* (10), 1314−1325.
(11) Wang, S.; Li, J.; Jiang, L.; Wang, S.; Zhao, X.; Dai, Y.; Luo, C.; Zhang, G. The influence of anaerobic dechlorination on the aerobic degradation of PCBs in e-waste-contaminated soils in an anaerobic-aerobic two-stage treatment. *Sci. Total Environ.* **2022**, *844*, 157195−157195.
(12) Luo, C.; Zhao, X.; Zhang, D.; Dai, Y.; Li, Q.; Wang, S.; Lin, H.; Zhang, G.; Li, J. Toward a More Comprehensive Understanding of Autochthonous Bioaugmentation (ABA): Cases of ABA for Phenanthrene and Biphenyl by Ralstonia sp. *M1 in Industrial Wastewater* ACS ES&T Water, 2021, *1*, 1390−1400, DOI: 10.1021/acsestwater.0c00257.
(13) Li, J.; Peng, K.; Zhang, D.; Luo, C.; Cai, X.; Wang, Y.; Zhang, G. Autochthonous bioaugmentation with non-direct degraders: A new strategy to enhance wastewater bioremediation performance. *Environ. Int.* **2020**, *136*, No. 105473.
(14) Li, J.; Luo, C.; Zhang, D.; Cai, X.; Jiang, L.; Zhang, G. Stable-isotope probing enabled cultivation of the indigenous strain Ralstonia sp. M1 capable of degrading phenanthrene and biphenyl in industrial wastewater. *Appl. Environ. Microbiol.* **2019**, *85* (14), No. AEM.00511−19.
(15) Li, J.; Luo, C.; Zhang, G.; Zhang, D. Coupling magnetic-nanoparticle mediated isolation (MMI) and stable isotope probing (SIP) for identifying and isolating the active microbes involved in phenanthrene degradation in wastewater with higher resolution and accuracy. *Water Res.* **2018**, *144*, 226−234.
(16) Chen, Y.; Murrell, J. C. When metagenomics meets stable-isotope probing: progress and perspectives. *Trends Microbiol.* **2010**, *18* (4), 157−163.
(17) Castaneda, L. E.; Barbosa, O. Metagenomic analysis exploring taxonomic and functional diversity of soil microbial communities in Chilean vineyards and surrounding native forests. *PeerJ* **2017**, *5*, No. e3098.
(18) Cai, X. X.; Li, J. B.; Guan, F. Y.; Luo, X. S.; Yuan, Y. Unveiling metabolic characteristics of an uncultured Gammaproteobacterium responsible for *in situ* PAH biodegradation in petroleum polluted soil. *Environ. Microbiol.* **2021**, *23* (11), 7093−7104.
(19) Pereira, F. C.; Wasmund, K.; Cobankovic, I.; Jehmlich, N.; Herbold, C. W.; Lee, K. S.; Sziranyi, B.; Vesely, C.; Decker, T.; Stocker, R.; Warth, B.; von Bergen, M.; Wagner, M.; Berry, D. Rational design of a microbial consortium of mucosal sugar utilizers reduces Clostridiodes difficile colonization. *Nat. Commun.* **2020**, *11* (1), 5104.
(20) Lee, K. S.; Landry, Z.; Pereira, F. C.; Wagner, M.; Berry, D.; Huang, W. E.; Taylor, G. T.; Kneipp, J.; Popp, J.; Zhang, M.; Cheng, J.-X.; Stocker, R. Raman microspectroscopy for microbiology. *Nat. Rev. Methods Primers* **2021**, *1* (1), 80.
(21) Jing, X. Y.; Gou, H. L.; Gong, Y. H.; Su, X. L.; Xu, L.; Ji, Y. T.; Song, Y. Z.; Thompson, I. P.; Xu, J.; Huang, W. E. Raman-activated cell sorting and metagenomic sequencing revealing carbon-fixing bacteria in the ocean. *Environ. Microbiol.* **2018**, *20* (6), 2241−2255.
(22) Song, Y. Z.; Yin, H. B.; Huang, W. E. Raman activated cell sorting. *Curr. Opin. Chem. Biol.* **2016**, *33*, 1−8.
(23) Jin, N.; Yang, K.; Li, J.; Song, Y.; Ding, A.; Sun, Y.; Li, G.; Zhang, D. Toxicity Characterization of Environment-Related Pollutants Using a Biospectroscopy−Bioreporter-Coupling Approach: Potential for Real-World Toxicity Determination and Source Apportionment of Multiple Pollutants. *Anal. Chem.* **2023**, *95* (9), 4291−4300.
(24) Cui, L.; Yang, K.; Li, H.-Z.; Zhang, H.; Su, J.-Q.; Paraskevaidi, M.; Martin, F. L.; Ren, B.; Zhu, Y.-G. Functional Single-Cell Approach to Probing Nitrogen-Fixing Bacteria in Soil Communities by Resonance Raman Spectroscopy with N-15(2) Labeling. *Anal. Chem.* **2018**, *90* (8), 5082−5089.
(25) Wang, Y.; Xu, J.; Kong, L.; Li, B.; Li, H.; Huang, W. E.; Zheng, C. Raman-activated sorting of antibiotic-resistant bacteria in human gut microbiota. *Environ. Microbiol.* **2020**, *22* (7), 2613−2624.
(26) Xu, T.; Gong, Y. H.; Su, X. L.; Zhu, P. F.; Dai, J.; Xu, J.; Ma, B. Phenome-Genome Profiling of Single Bacterial Cell by Raman-Activated Gravity-Driven Encapsulation and Sequencing. *Small* **2020**, *16* (30), No. 2001172.
(27) Carini, P. A "Cultural" Renaissance: Genomics Breathes New Life into an Old Craft. *mSystems* **2019**, *4* (3), 5.
(28) Cross, K. L.; Campbell, J. H.; Balachandran, M.; Campbell, A. G.; Cooper, S. J.; Griffen, A.; Heaton, M.; Joshi, S.; Klingeman, D.; Leys, E.; Yang, Z. M.; Parks, J. M.; Podar, M. Targeted isolation and cultivation of uncultivated bacteria by reverse genomics. *Nat. Biotechnol.* **2019**, *37* (11), 1314−1321.
(29) Li, Y. F.; Li, F. Y.; Ho, C. L.; Liao, V. H. C. Construction and comparison of fluorescence and bioluminescence bacterial biosensors for the detection of bioavailable toluene and related compounds. *Environ. Pollut.* **2008**, *152* (1), 123−129.
(30) Mukherjee, A. K.; Bordoloi, N. K. Biodegradation of benzene, toluene, and xylene (BTX) in liquid culture and in soil by Bacillus subtilis and Pseudomonas aeruginosa strains and a formulated bacterial consortium. *Environ. Sci. Pollut. Res.* **2012**, *19* (8), 3380−3388.
(31) Fayemiwo, O. M.; Daramola, M. O.; Moothi, K. BTEX compounds in water - future trends and directions for water treatment. *Water Sa* **2017**, *43* (4), 602−613.
(32) Berlendis, S.; Cayol, J.-L.; Verhé, F.; Laveau, S.; Tholozan, J.-L.; Ollivier, B.; Auria, R. First Evidence of Aerobic Biodegradation of BTEX Compounds by Pure Cultures of Marinobacter. *Appl. Biochem. Biotechnol.* **2010**, *160* (7), 1992−1999.
(33) Meckenstock, R. U.; Morasch, B.; Kästner, M.; Vieth, A.; Richnow, H. H. Assessment of Bacterial Degradation of Aromatic Hydrocarbons in the Environment by Analysis of Stable Carbon Isotope Fractionation. *Water, Air, Soil Pollut.: Focus* **2002**, *2* (3), 141−152.
(34) Li, Q.; Li, J.; Jiang, L.; Sun, Y.; Luo, C.; Zhang, G. Diversity and structure of phenanthrene degrading bacterial communities associated with fungal bioremediation in petroleum contaminated soil. *J. Hazard. Mater.* **2021**, *403*, No. 123895.
(35) Luo, C.; Xie, S.; Sun, W.; Li, X.; Cupples, A. M. Identification of a Novel Toluene-Degrading Bacterium from the Candidate Phylum TM7, as Determined by DNA Stable Isotope Probing. *Appl. Environ. Microbiol.* **2009**, *75* (13), 4644−4647.
(36) Li, J.; Luo, C.; Zhang, D.; Zhao, X.; Dai, Y.; Cai, X.; Zhang, G. The catabolic pathways of *in situ* rhizosphere PAH degraders and the main factors driving PAH rhizoremediation in oil-contaminated soil. *Environ. Microbiol.* **2021**, *23* (11), 7042−7055.
(37) Caporaso, J. G.; Kuczynski, J.; Stombaugh, J. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **2010**, *7* (5), 335−336.
(38) Hou, R.; Gan, L.; Guan, F. Y.; Wang, Y.; Li, J. B.; Zhou, S. G.; Yuan, Y. Bioelectrochemically enhanced degradation of bisphenol S: mechanistic insights from stable isotope-assisted investigations. *Iscience* **2021**, *24* (1), No. 102014.
(39) Edgar, R. C. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* **2013**, *10* (10), 996−998.

(40) Li, J.; Luo, C.; Zhang, D.; Cai, X.; Jiang, L.; 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.
(41) Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glockner, F. O. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* **2013**, *41* (Database issue), D590−D596.
(42) Bolger, A. M.; Lohse, M.; Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30* (15), 2114−2120.
(43) Langmead, B.; Trapnell, C.; Pop, M.; Salzberg, S. L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* **2009**, *10* (3), R25.
(44) Li, D.; Liu, C.-M.; Luo, R.; 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* (10), 1674−1676.
(45) Uritskiy, G. V.; DiRuggiero, J.; Taylor, J. MetaWRAP-a flexible pipeline for genome-resolved metagenomic data analysis. *Microbiome* **2018**, *6* (1), 158.
(46) Parks, D. H.; Imelfort, M.; Skennerton, C. T.; Hugenholtz, P.; Tyson, G. W. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res.* **2015**, *25* (7), 1043−55.
(47) Yamamoto, S.; Harayama, S. Phylogenetic relationships of Pseudomonas putida strains deduced from the nucleotide sequences of gyrB, ropD and 16S rRNA genes. *Int. J. Syst. Bacteriol.* **1998**, *48*, 813−819.
(48) Jain, C.; Rodriguez-R, L. M.; Phillippy, A. M.; Konstantinidis, K. T.; Aluru, S. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat. Commun.* **2018**, *9*, 5114.
(49) Wang, Y.; Huang, W. E.; Cui, L.; Wagner, M. Single cell stable isotope probing in microbiology using Raman microspectroscopy. *Curr. Opin. Biotechnol.* **2016**, *41*, 34−42.
(50) Seemann, T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **2014**, *30* (14), 2068−2069.
(51) Thomas, F.; Corre, E.; Cebron, A. Stable isotope probing and metagenomics highlight the effect of plants on uncultured phenanthrene-degrading bacterial consortium in polluted soil. *ISME J.* **2019**, *13* (7), 1814−1830.
(52) Droge, J.; McHardy, A. C. Taxonomic binning of metagenome samples generated by next-generation sequencing technologies. *Briefings Bioinf.* **2012**, *13* (6), 646−655.
(53) Li, J.; Lu, Q.; Liu, T.; Zhou, S.; Yang, G.; Zhao, Y. Paenibacillus guangzhouensis sp. nov., an Fe (III)-and humus-reducing bacterium from a forest soil. *Int. J. Syst. Evol. Microbiol.* **2014**, *64* (11), 3891−3896.
(54) Li, J.; Yang, G.; Wu, M.; Zhao, Y.; Zhou, S. Bacillus huizhouensis sp. nov., isolated from a paddy field soil. *Antonie Van Leeuwenhoek* **2014**, *106* (2), 357−363.
(55) Li, J.; Lin, T.; Lu, Q.; Wang, J. J.; Liao, C.; Pan, Y.; Zhao, Y. Changes in physicochemical properties and bactericidal efficiency of acidic electrolyzed water ice and available chlorine decay kinetics during storage. *LWT-Food Sci. Technol.* **2014**, *59* (1), 43−48.
(56) Li, J.; Yang, G.; Lu, Q.; Zhao, Y.; Zhou, S. Bacillus haikouensis sp. nov., a facultatively anaerobic halotolerant bacterium isolated from a paddy soil. *Antonie Van Leeuwenhoek* **2014**, *106* (4), 789−794.
(57) Hu, W. C.; Liang, J. S.; Ju, F.; Wang, Q. J.; Liu, R. P.; Bai, Y. H.; Liu, H. J.; Qu, J. H. Metagenomics Unravels Differential Microbiome Composition and Metabolic Potential in Rapid Sand Filters Purifying Surface Water Versus Groundwater. *Environ. Sci. Technol.* **2020**, *54* (8), 5197−5206.
(58) Lewis, W. H.; Tahon, G.; Geesink, P.; Sousa, D. Z.; Ettema, T. J. G. Innovations to culturing the uncultured microbial majority. *Nat. Rev. Microbiol.* **2021**, *19* (4), 225−240.
(59) Neufeld, J. D.; Vohra, J.; Dumont, M. G.; Lueders, T.; Manefield, M.; Friedrich, M. W.; Murrell, J. C. DNA stable-isotope probing. *Nat. Protoc.* **2007**, *2* (4), 860−866.
(60) Govil, T.; Paste, M.; Samanta, D.; David, A.; Goh, K. M.; Li, X. K.; Salem, D. R.; Sani, R. K. Metagenomics and Culture Dependent Insights into the Distribution of Firmicutes across Two Different Sample Types Located in the Black Hills Region of South Dakota, USA. *Microorganisms* **2021**, *9* (1), 113.
(61) Zou, Y. Q.; Xue, W. B.; Lin, X. Q.; Lv, M.; Luo, G. W.; Dai, Y.; Sun, H. P.; Liu, S. W.; Sun, C. H.; Hu, T. Y.; Xiao, L. Butyribacter intestini gen. nov., sp. nov., a butyric acid-producing bacterium of the family Lachnospiraceae isolated from human faeces, and reclassification of Acetivibrio ethanolgignens as Acetanaerobacter ethanolgignens gen. nov., comb. nov. *Syst. Appl. Microbiol.* **2021**, *44* (3), No. 126201.
(62) Kenters, N.; Henderson, G.; Jeyanathan, J.; Kittelmann, S.; Janssen, P. H. Isolation of previously uncultured rumen bacteria by dilution to extinction using a new liquid culture medium. *J. Microbiol. Methods* **2011**, *84* (1), 52−60.
(63) Aslam, F.; Yasmin, A.; Thomas, T. Essential Gene Clusters Identified in Stenotrophomonas MB339 for Multiple Metal/Antibiotic Resistance and Xenobiotic Degradation. *Curr. Microbiol.* **2018**, *75* (11), 1484−1492.
(64) Bridger, N.; Drews, S.; Burdz, T.; Wiebe, D.; Pacheco, A. L.; Ng, B.; Bernard, K. Isolation and characterization of Pigmentiphaga-like isolates from human clinical material. *J. Med. Microbiol.* **2013**, *62* (5), 708−711.
(65) Arenghi, F. L. G.; Pinti, M.; Galli, E.; Barbieri, P. Identification of the Pseudomonas stutzeri OX1 toluene-o-xylene monooxygenase regulatory gene (touR) and of its cognate promoter. *Appl. Environ. Microbiol.* **1999**, *65* (9), 4057−4063.

https://doi.org/10.1021/acs.est.3c04247
Environ. Sci. Technol. 2023, 57, 17087−17098