Stable-isotope probing enabled cultivation of the indigenous strain Ralstonia sp. M1 capable of degrading phenanthrene and biphenyl in industrial wastewater

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BIODEGRADATION

Stable-Isotope Probing-Enabled Cultivation of the Indigenous Bacterium *Ralstonia* sp. Strain M1, Capable of Degrading Phenanthrene and Biphenyl in Industrial Wastewater

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ABSTRACT To identify and obtain the indigenous degraders metabolizing phenanthrene (PHE) and biphenyl (BP) from the complex microbial community within industrial wastewater, DNA-based stable-isotope probing (DNA-SIP) and cultivation-based methods were applied in the present study. DNA-SIP results showed that two bacterial taxa (*Vogesella* and *Alicyclobacillus*) were considered the key biodegraders responsible for PHE biodegradation only, whereas *Bacillus* and *Cupriavidus* were involved in BP degradation. *Vogesella* and *Alicyclobacillus* have not been linked with PHE degradation previously. Additionally, DNA-SIP helped reveal the taxonomic identity of *Ralstonia*-like degraders involved in both PHE and BP degradation. To target the separation of functional *Ralstonia*-like degraders from the wastewater, we modified the traditional cultivation medium and culture conditions. Finally, an indigenous PHE- and BP-degrading strain, *Ralstonia pickettii* M1, was isolated via a cultivation-dependent method, and its role in PHE and BP degradation was confirmed by enrichment of the 16S rRNA gene and distinctive dioxygenase genes in the DNA-SIP experiment. Our study has successfully established a program for the application of DNA-SIP in the isolation of the active functional degraders from an environment. It also deepens our insight into the diversity of indigenous PHE- and BP-degrading communities.

IMPORTANCE The comprehensive treatment of wastewater in industrial parks suffers from the presence of multiple persistent organic pollutants (POPs), such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), which reduce the activity of activated sludge and are difficult to eliminate. Characterizing and applying active bacterial degraders metabolizing multiple POPs therefore helps to reveal the mechanisms of synergistic metabolism and to improve wastewater treatment efficiency in industrial parks. To date, SIP studies have successfully investigated the biodegradation of PAHs or PCBs in real-world habitats. DNA-SIP facilitates the isolation of target microorganisms that pose environmental concerns. Here, an indigenous phenanthrene (PHE)- and biphenyl (BP)-degrading strain in wastewater, *Ralstonia pickettii* M1, was isolated via a cultivation-dependent method, and its role in PHE and BP degradation was confirmed by DNA-SIP. Our study provides a routine protocol for the application of DNA-SIP in the isolation of the active functional degraders from an environment.

KEYWORDS stable-isotope probing (SIP), active degraders, biphenyl, distinctive dioxygenase genes, phenanthrene

With the rapid development of modern industrial processes, pollution caused by industrial wastewater is becoming increasingly serious (1). Various persistent organic pollutants (POPs), such as polycyclic aromatic hydrocarbons (PAHs) and poly-

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chlorinated biphenyls (PCBs), have been found in industrial wastewaters (2–5). Particularly in industrial parks, the cooccurrence of multiple POPs in wastewater challenges the effectiveness of wastewater treatment plants (WWTPs). Typically, PAHs and PCBs coexist in industrial wastewater (6). PAHs are a class of hydrophobic organic compounds with two or more fused aromatic rings and are ubiquitous by-products of the burning of fossil fuels (coal, petroleum), waste incineration, and wood processing (7). PCBs are a class of chlorinated organic compounds formed by the addition of chlorine to biphenyl (BP), with 209 different PCB congeners, and have been largely used in industries as heat transfer fluids, lubricants, and flame retardants (8). The extensive use and multiple sources of PAHs and PCBs make them ubiquitous in the environment and sometimes bioaccumulative (6). In industrial wastewater, PAHs and PCBs are normally present at levels that pose risks to ecosystems and public health, and remediation technologies (e.g., physicochemical methods and bioremediation) are therefore required to reduce their concentrations to acceptable levels or minimize exposure to receptors (9–12).

The bioremediation or microbial degradation of PAHs and PCBs involves the use of microorganisms to remove the pollutants from wastewater or to neutralize them by conversion to carbon dioxide, water, and other by-products (13, 14). This is considered to be a cost-effective and ecofriendly remediation option and is therefore used as the dominant treatment strategy in industrial WWTPs (15, 16). Microbial degradation or mineralization depends on the metabolic potential of microorganisms to detoxify or transform these pollutants. Several enzyme-catalyzed metabolic reactions in the biodegradation process include the addition of oxygen to double bonds, dehalogenation, ring cleavage, and the addition of hydroxyl groups to the benzene ring (17–19). Some functional genes have been identified and linked to the initial metabolic process of PAH and PCB degradation, including PAH ring-hydroxylating dioxygenase (PAH-RHD) (20) and biphenyl dioxygenase (*bphA*) genes (11). These genes are usually used as biomarkers to quantify the bacterial populations capable of degrading PAHs and PCBs, respectively. Additionally, the downstream metabolism of PAHs and PCBs, from catechols to other intermediates via *meta* or *ortho* fission, is similar and is shared by many bacterial lineages (21, 22). It is therefore of great importance to improve wastewater treatment efficiency in WWTPs by isolating microorganisms possessing both the metabolic pathways and the ability to degrade these pollutants.

To explore the fate of PAHs and PCBs in the environment, a number of studies based on cultivation have attempted to identify and isolate microorganisms that are able to degrade them individually. To date, many PAH and PCB degraders have been isolated, e.g., bacteria belonging to the genera *Paenibacillus* (23, 24), *Burkholderia* (25, 26), and *Pseudomonas* (27, 28). Such cultivation-dependent methods provide clues to determining the phenotypes and metabolic characteristics of these degraders. However, only a few microorganisms responsible for pollutant degradation in real-world habitats could be isolated (29), and whether the cultivable degraders are actually functional in their natural habitats is still questionable. The majority of microorganisms present in the natural environment are uncultivable (30), and it is difficult to reveal the ecological functions of uncultivable indigenous microorganisms using cultivation-dependent approaches. In contrast, cultivation-independent methods can circumvent the requirement of culturing microorganisms in order to assess their metabolic responses and can directly link their identities to functions in their natural habitats (31). One such technique, DNA stable-isotope probing (DNA-SIP), has been used successfully on environmental samples by introducing substrates labeled with stable isotopes, such as ^{13}C or ^{15}N, in order to monitor their incorporation into microbial DNA and identify the target microorganisms, particularly functional yet uncultivable species (32). Additionally, SIP results can suggest the physiological features of the functional degraders and enable the modification of cultivation media or conditions to specifically enrich or separate the targeted degraders that are inert under conventional cultivation conditions.

Phenanthrene (PHE) and BP have been used as model compounds for the study of

PAH and PCB degradation (32, 33). Stable-isotope probing studies have successfully explored the biodegradation of PAHs or PCBs in real-world habitats, such as industrial wastewater (16, 34), seawater (35), and soils or sediments (36, 37). However, previous studies have only identified indigenous degraders of PAHs or PCBs individually, whereas degraders of both PAHs and PCBs have not been reported. There is also a lack of successful case studies in which these degraders have been identified via cultivation-dependent methods (29). Therefore, it has not been possible to interrogate their nutritional profiles and apply them in bioaugmentation schemes to enhance the efficiency of WWTPs. In the present study, DNA-SIP coupled with high-throughput sequencing was used to identify active degraders metabolizing both PHE and BP in industrial wastewater. In addition, an indigenous degrading bacterium responsible for both PHE and BP degradation was successfully isolated using cultivation-dependent methods and was found to possess distinctive PAH-RHD and *bphA* genes, which were enriched in the ^{13}C-labeled DNA fraction. This study proved that some indigenous bacterial degraders can metabolize multiple POPs in industrial wastewater, providing valuable information for the management of WWTPs in industrial parks.

RESULTS

PHE and BP biodegradation in wastewater. The residual percentages of [^{12}C]PHE, [^{13}C]PHE, [^{12}C]BP, and [^{13}C]BP in wastewater are shown in Fig. 1a. PHE and BP concentrations decreased only slightly in the sterile control in contrast with the decreased concentrations for the biotic treatments. After a 3-day incubation, residual PHE percentages were 27.5 to 30.6% and 26.7 to 31.4% in the ^{12}C- and ^{13}C-labeled PHE microcosms, and residual BP percentages were 22.5 to 25.7% and 21.9 to 26.1% in the ^{12}C- and ^{13}C-labeled BP microcosms, respectively. The results suggested the presence of significant biodegradation in the biotic microcosms. There was no significant difference ($P > 0.05$) between the [^{12}C]PHE and [^{13}C]PHE treatments or between the [^{12}C]BP and [^{13}C]BP treatments.

PHE and BP degraders as revealed by DNA-SIP. The abundance of bacterial 16S rRNA genes quantified by quantitative PCR (qPCR) from each fraction of each treatment is shown in Fig. 1b. In both the PHE- and BP-amended microcosms, bacterial 16S rRNA levels in fractions with buoyant density (BD) values of 1.7551 to 1.7556 g/ml (heavy DNA fractions) were higher for ^{13}C-labeled samples than for ^{12}C-labeled samples (Fig. 1b, shading). The indigenous microorganisms responsible for ^{13}C-labeled substrate assimilation were identified by comparing the relative abundances of specific operational taxonomic units (OTUs) in each fraction from the ^{13}C- and ^{12}C-labeled treatments. In the PHE-amended treatments (see Fig. S1a in the supplemental material), three bacterial lineages, represented by OTU_11, OTU_16, and OTU_70, were enriched in the heavy DNA fractions of the [^{13}C]PHE microcosms, but not in the [^{12}C]PHE microcosms, indicating their involvement in PHE biodegradation. Similarly, for the BP-amended samples, the relative abundances of OTU_11, OTU_18, and OTU_27 were significantly higher only in the heavy DNA fractions of the [^{13}C]BP treatments (Fig. S1b), indicating that the microorganisms represented by these OTUs played a primary role in BP metabolism. Among them, only OTU_11 was enriched in both the [^{13}C]PHE- and [^{13}C]BP-amended treatments, suggesting that it plays a vital role in both PHE and BP degradation.

The representative sequences of the five SIP-identified OTUs were aligned to the sequences of different genera on the basis of a BLAST search in GenBank. A neighbor-joining tree based on partial 16S rRNA gene sequences showed the phylogenetic positions of these active degraders and other related taxa (see Fig. S2 in the supplemental material). OTU_11 was assigned to the genus *Ralstonia* (phylum *Proteobacteria*, class *Betaproteobacteria*, order *Burkholderiales*, family *Ralstoniaceae*). It shared 100% similarity with *Ralstonia pickettii* ATCC 27511 (GenBank accession number JOVL01000020) and formed a subclade with a high bootstrap value of 90. OTU_27 also belonged to the family *Burkholderiaceae* but was characterized as belonging to the genus *Cupriavidus* and shared 100% similarity with *Cupriavidus taiwanensis* LMG 19424

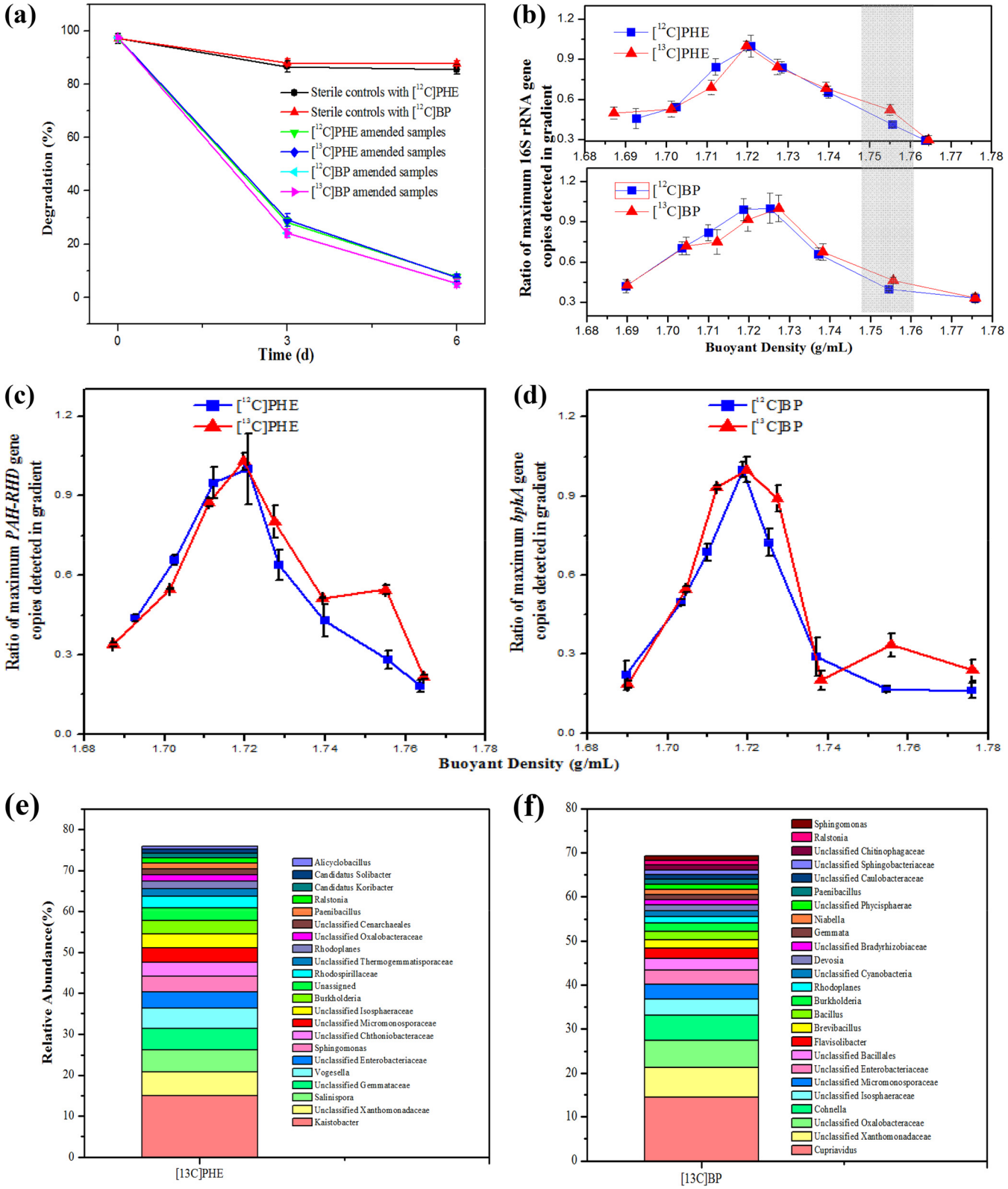

FIG 1 (a) Degradation of phenanthrene (PHE) and biphenyl (BP) in wastewater after 3- and 6-day incubations. (b) Quantitative distribution of density-resolved bacterial 16S rRNA genes obtained from wastewater samples in the [^{12}C]PHE and [^{13}C]PHE treatments (top) and the [^{12}C]BP and [^{13}C]BP treatments (bottom). The heavy DNA fractions are shown against a shaded background. The bacterial template distribution within each buoyant density gradient fraction was quantified by qPCR. The relative abundances of bacterial 16S rRNA genes detected in the gradient (*y* axis) were calculated as the ratio of 16S rRNA gene copies in each buoyant density fraction to those with the highest abundance. Data are means ± standard deviations; $n = 3$. (c and d) Quantitative distribution of PAH-RHD (c) and *bphA* (d) genes obtained from wastewater samples in [^{12}C]PHE and [^{13}C]PHE treatments (c) and in [^{12}C]BP and [^{13}C]BP treatments (d). The

(Continued on next page)

(accession number CU633749). OTU_16 was assigned to the genus *Vogesella* (phylum *Proteobacteria*, class *Betaproteobacteria*, order *Neisseriales*, family *Chromobacteriaceae*). OTU_18 and OTU_70 were classified in the genera *Bacillus* and *Alicyclobacillus*, respectively, within the same order, *Bacillales* (phylum *Firmicutes*). OTU_18 shared 100% similarity with many strains in this order, including *Bhargavaea ginsengi* ge14 (EF371375), *Bacillus jeddahensis* JCE (HG931339), and *Bacillus cucumis* AP-6 (KT895286), and formed a subclade with a high bootstrap value of 100.

Isolation and characterization of PHE- and BP-degrading strains. In total, 20 strains were isolated as cultivable PHE or BP degraders. Among them, only one representative that shared 100% similarity with the OTU_11 sequence, named *Ralstonia* sp. strain M1 (GenBank accession number MH844635), could metabolize both PHE and BP in industrial wastewater. Strain M1 is aerobic, Gram-negative, and rod-shaped, has no flagella, and measures 0.3 to 0.6 by 0.8 to 1.4 μm (Fig. 2a and b). Colonies growing on minimal medium agar plates supplemented with 100 mg/liter PHE (MM-P) or BP (MM-B) at 30°C for 48 h were circular, smooth, convex, and white-pigmented, with a colony diameter of 1.0 to 2.5 mm. This strain maintained satisfactory growth across a broad range of conditions, including salinity (0 to 4%, wt/vol; optimum, 1%), pH (4.0 to 9.0; optimum, pH 7.0), and temperature (15 to 45°C; optimum, 30°C) (see Fig. S3 in the supplemental material). The phenotypic characteristics of strain M1 are listed in Table S4 in the supplemental material. High 16S rRNA gene sequence similarity (100%) was found between strain M1 and *R. pickettii* ATCC 27511 (GenBank accession number JOVL01000020). In the neighbor-joining phylogenetic dendrograms based on 16S rRNA gene sequences (Fig. 3a), strain M1 belonged to the genus *Ralstonia* and formed a subclade together with *R. pickettii* ATCC 27511 and *Ralstonia*-like organisms represented by OTU_11.

As shown in Fig. 2c and d, strain M1 grew well in both the MM-P and MM-B media, and more than 60% of the PHE or BP was degraded within 3 days. Because it was enriched in the heavy DNA fractions of both the [^{13}C]PHE and [^{13}C]BP samples, *Ralstonia* sp. strain M1 was confirmed to be the major active indigenous PHE and BP degrader in wastewater.

Functional genes involved in PHE and BP metabolism. To further confirm the environmental significance of strain M1, we focused on the PAH-RHD and *bphA* genes involved in PHE and BP metabolism. One type of PAH-RHD gene and one type of *bphA* gene were detected in strain M1; these had 84% similarity with the naphthalene 1,2-dioxygenase (NDO) gene of *Cupriavidus* sp. strain HPC(L) (GenBank accession number ESJ26713.1) and 97% similarity with the *bphA* gene of *Cupriavidus necator* (NCBI reference sequence WP_013952432.1), respectively (Fig. 3b and c). The PAH-RHD and *bphA* genes in the PHE- and BP-amended treatments were quantified against each density-resolved fraction. A remarkable enrichment of PAH-RHD and *bphA* genes in the heavy DNA fractions was observed in the [^{13}C]PHE and [^{13}C]BP treatments (Fig. 1c and d), respectively, indicating that these functional genes were labeled with the assimilated ^{13}C. In contrast, no such enrichment or similar trend was detected in the [^{12}C]PHE and [^{12}C]BP treatments. Accordingly, the genes detected in the heavy fractions of the ^{13}C-labeled microcosms were associated with PHE and BP metabolism. The same primer sets were also used to amplify, clone, and sequence the active PHE- and BP-degrading genes in the heavy DNA fractions of the ^{13}C-labeled samples, and two genes matching those of strain M1 were successfully obtained (7 of the 20 PAH-RHD gene clones and 9 of the 21 *bphA* clones). This result suggested that strain M1 was the host of these two distinctive dioxygenase genes that were involved in PHE and BP degradation.

FIG 1 Legend (Continued)
relative abundances of these genes detected in the gradient (*y* axis) were calculated as the ratio of PAH-RHD or *bphA* gene copies in each buoyant density fraction to those with the highest abundance. (e and f) Relative abundances of 16S rRNA-defined taxa by genus in the heavy DNA fractions of the [^{13}C]PHE (e) and [^{13}C]BP (f) microcosms. The selected taxa have a minimal relative abundance of >1%.

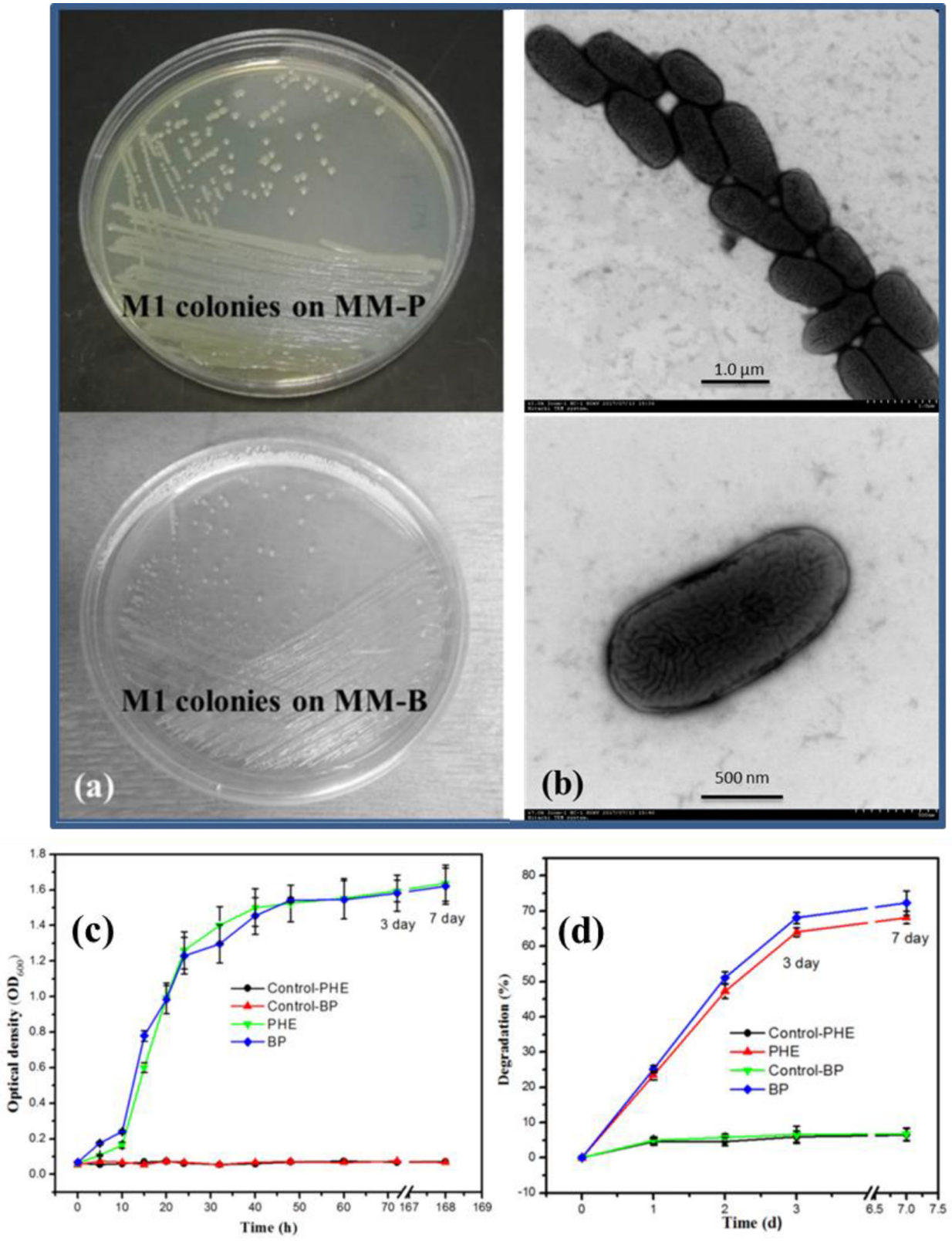

FIG 2 (a) Colonies of *Ralstonia* sp. M1 cultivated on minimal medium agar plates supplemented with 100 mg/liter PHE (MM-P) or BP (MM-B). (b) Transmission electron micrograph of *Ralstonia* sp. M1 cells. Bars indicate 1,000 nm (top) and 500 nm (bottom). (c and d) Growth curves of *Ralstonia* sp. M1 (c) and percentages of degradation of PHE and BP by *Ralstonia* sp. M1 (d). Controls without cells are referred to as Control-PHE or Control-BP.

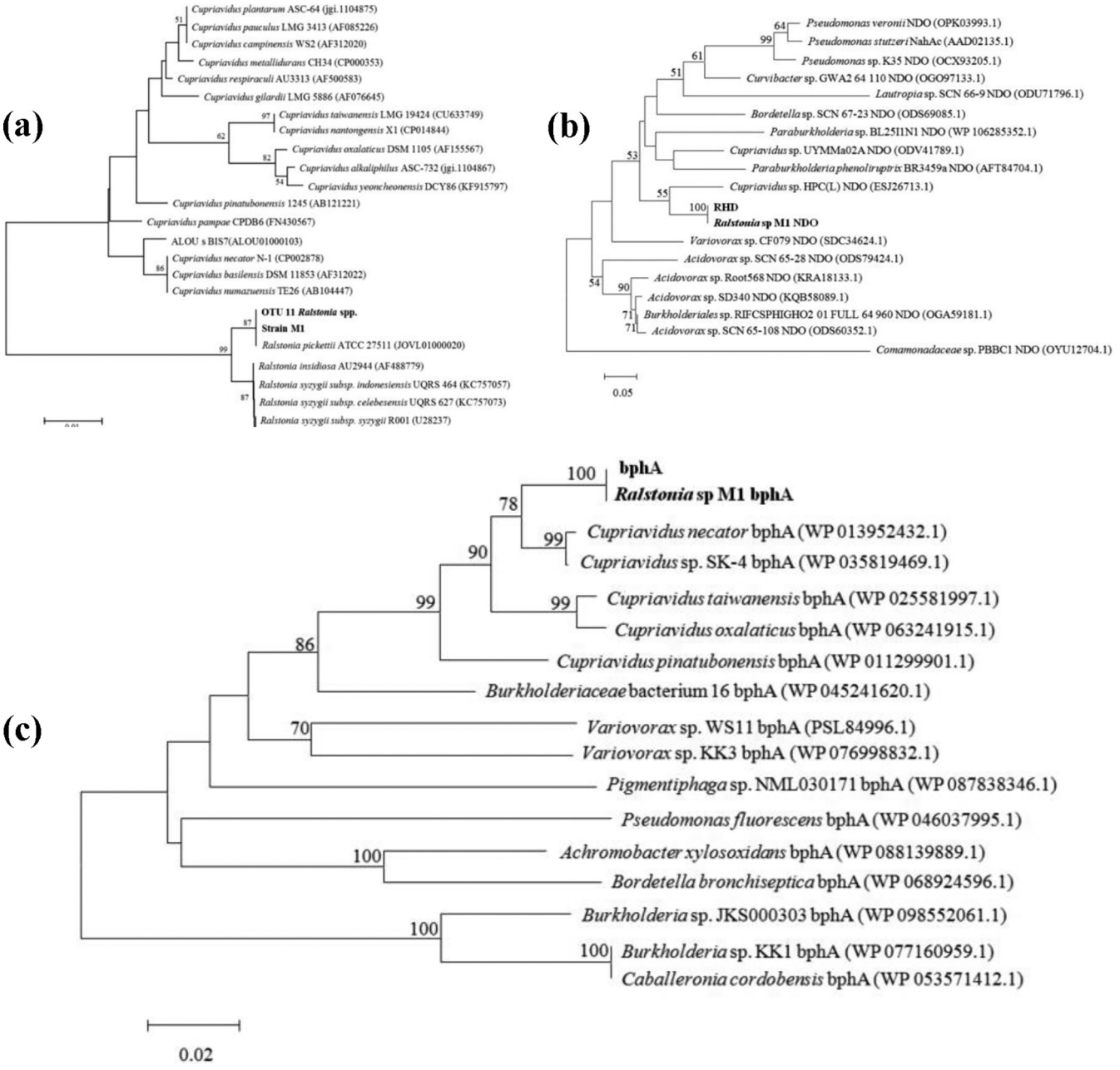

FIG 3 (a) Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of *Ralstonia* sp. M1 and representatives of other, related taxa. Bootstrap values of >50% are shown at the branch points. The bar indicates 0.005 substitution per nucleotide position. (b and c) Phylogenetic tree-based PAH-RHD (b) and *bphA* (c) gene sequences from strain M1. Bootstrap values of >50% are shown at the branch points. Bars indicate 0.05 and 0.02 substitution per nucleotide position, respectively.

DISCUSSION

WWTPs in industrial parks suffer from the complexity and toxicity of pollutants such as PAHs and PCBs. Their coexistence poses unacceptable risks to the environment and public health, challenging the efficiency of WWTPs (38, 39). Conventional biological treatment is considered to be a cost-effective and ecofriendly approach for eliminating PAHs and PCBs from industrial wastewater (12, 34, 40). However, a major drawback of biological treatment is the fact that the majority of indigenous microbes responsible for degradation are not cultivable (16, 41), and the active indigenous PAH or PCB degraders are difficult to isolate and study. DNA-SIP is a cultivation-independent technique that provides the opportunity to link the identities of microorganisms to their *in situ*

functions (31, 42). It has been successfully applied to identify individual microorganisms native to field sites that are capable of PAH or PCB biodegradation (32, 33). However, the indigenous bacterial degraders responsible for the metabolism of these two pollutants have not been reported. Our DNA-SIP results identified the active microbes directly participating in the biodegradation of PHE or BP in wastewater. Of the five bacterial lineages, *Vogesella* and *Alicyclobacillus* were the predominant genera deriving carbon from PHE, whereas *Bacillus* and *Cupriavidus* were responsible for BP degradation. *Ralstonia* was the only active degrader of both PHE and BP in wastewater. Here, only some rare species were enriched in the heavy DNA fractions, indicating the low abundance of functional PHE and BP degraders in wastewater samples. Nevertheless, the SIP results confirmed their role in metabolizing PHE and BP and provided strong evidence for their primary role in PHE and BP metabolism. It should be noted that the majority of the abundant bacteria, such as members of the genus *Kaistobacter* and unclassified *Xanthomonadaceae*, were not directly linked to PHE or BP degradation (Fig. 1e and f), because many other organic carbons were present in the wastewater, and PHE and BP were the only two carbons promoting the rare PHE- and BP-degrading species.

Among the PHE degraders, the genus *Vogesella* belongs to the class *Betaproteobacteria* and includes recognized heterotrophic strains such as *Vogesella indigofera* ATCC 19706, *Vogesella perlucida* LMG 24214, and *Vogesella lacus* LMG 24504 (43). Some *Vogesella* strains have been isolated from various sources, including water and sediments, and members of this genus are capable of degrading low-molecular-weight organic compounds, such as peptidoglycan and several polymeric substances (44). The genus *Alicyclobacillus* was initially described in 1992 and was characterized as moderately thermophilic, acidophilic, and strictly aerobic (45). Strains of this genus are able to degrade lignocellulosic and phenolic compounds (46). Until now, only one study indicated that microorganisms belonging to the genus *Alicyclobacillus* had a relationship to PAH degradation, which was evidenced by the enrichment of indigenous bacterial taxa such as *Sphingobium*, *Phenylobacterium*, *Alicyclobacillus*, and *Arthrobacter* during the degradation of crude oil (47). However, the PHE degradation capabilities of *Vogesella* and *Alicyclobacillus* have not been reported previously; thus, the mechanisms controlling their metabolism of PHEs in wastewater remain unknown. Our results have expanded the available knowledge regarding these genera and provide strong evidence that members of the genera *Vogesella* and *Alicyclobacillus* are the primary microorganisms responsible for PHE degradation in industrial wastewater.

Bacillus and *Cupriavidus* were identified as active biphenyl-degrading bacteria. *Bacillus* species are ubiquitous in nature and occupy diverse ecological niches in microbiomes across soil, water, plants, and humans. Some *Bacillus* strains play increasingly important roles in microbial remediation and the fermentation industry (48). Considerable numbers of *Bacillus* strains are potential degraders of various aromatic compounds, such as BP and naphthalene (49, 50). The genus *Cupriavidus* belongs to the order *Burkholderiales*. Some *Cupriavidus* strains could efficiently degrade BP or PCBs and contained the gene encoding BP dioxygenase (51, 52). However, no previous studies using the SIP method have confirmed the BP degradation capabilities of indigenous *Bacillus* and *Cupriavidus* strains in wastewater. Our results provide unequivocal evidence that some microorganisms in these two taxa are actively involved in BP degradation in industrial wastewater.

Ralstonia was the only bacterial lineage capable of degrading both PHE and BP in industrial wastewater (see Fig. S4 in the supplemental material). Members of the genus *Ralstonia* have been recognized as potent microorganisms possessing versatile metabolic capabilities, such as pathways for PAH and PCB degradation (53–55). Sequence analysis of the SIP-identified OTU_11 suggested its close relationship to the species *R. pickettii* ATCC 27511 (Fig. S2). *R. pickettii*, formerly named *Pseudomonas pickettii*, has been isolated from various terrestrial and aquatic habitats (56). *R. pickettii* strains have been reported to possess a remarkable ability to degrade toxic organic compounds, such as biaryl chemicals (BP and 4-chlorobiphenyl) (57) and PAHs (anthracene and

naphthalene) (58). To the best of our knowledge, PHE degradation by *R. pickettii* has not been reported prior to this study. Drawing together the results from the DNA-SIP and cultivation-dependent methods showed a dominance of 100% similarity with *R. pickettii* strain ATCC 27511 from the heavy DNA fractions and the ability of *Ralstonia* sp. M1 to degrade both PHE and BP. It was therefore clear that this strain was linked to the metabolism of PHE and BP in industrial wastewater. These findings were further supported by the occurrence and enrichment of the distinctive PAH-RHD and *bphA* genes in both heavy DNA fractions in SIP microcosms and the DNA of strain M1. The increases in PHE or BP degradation efficiency with strain M1 were not significantly different between days 3 and 7 (Fig. 2d), a finding consistent with those of our previous study (12). This was possibly due to adverse conditions, such as toxic intermediates on the isolate, in the latter stage of incubation. The finding that strain M1 has PHE-degrading ability equivalent to that of the species *R. pickettii* is very promising. Additionally, our findings provide reference data for the application of strain M1 in the treatment of industrial wastewater containing PAHs, PCBs, or other organic pollutants.

It should be noted that the distinctive PAH-RHD and *bphA* genes of *Ralstonia* sp. M1 are related to the genes of *Cupriavidus* species, which are also *Burkholderiales* strains (Fig. 3b and c), hinting at the possible horizontal transfer of these genes between species in wastewater. Horizontal gene transfer (HGT) has been reported for many bacterial genera, including *Acinetobacter* and *Ralstonia* (59, 60). Some of the *bphA* genes originating from *Ralstonia* might have been shared via HGT, because they are found in various bacterial species (40). HGT also plays an important role in organic compound degradation. The removal of PHE and BP from the industrial wastewater hinted at the occurrence of HGT, which is consistent with the fact that organic pollutants, such as PAHs, could promote HGT events and further enhance degradation efficiency (40, 61). Based on cultivation-dependent methods, strain M1, which is responsible for both PHE and BP biodegradation, was successfully isolated from wastewater and was found to possess the distinctive PAH-RHD and *bphA* genes present in the heavy DNA fraction in SIP microcosms, further confirming their involvement in the degradation of PHE and BP in wastewater.

Although various cultivable PAH and PCB degraders have been isolated from contaminated sites (23, 24, 62), their application for bioaugmentation in soil remediation and wastewater treatment is subject to certain restrictions, because their survival and degradation ability are highly dependent on environmental conditions (16). Autochthonous bioaugmentation has been proposed as an alternative approach to overcome these difficulties but suffers from the fact that the active indigenous degraders are scarcely cultivable (15, 41). Some studies have attempted to identify the active indigenous bacteria responsible for pollutant degradation via cultivation-independent methods (15, 32, 41). However, work remains ongoing, and it remains a great challenge to routinely isolate the active degraders of target pollutants, which would be of benefit in wastewater treatment (32). In the present study, the *R. pickettii* strain M1 was found to be responsible for the metabolism of PHE and BP and was identified via cultivation-dependent isolation from the wastewater microbial community. Its ability to degrade PHE and BP in wastewater was confirmed by a cultivation-independent SIP approach, offering a routine protocol for identifying and isolating the active indigenous microbes responsible for the degradation of multiple POPs without prior knowledge about which microorganisms are involved in pollutant degradation.

Conclusion. In this study, DNA-SIP was applied to identify bacterial taxa metabolizing PHE and BP in industrial wastewater. The results showed that two indigenous bacterial taxa (*Vogesella* and *Alicyclobacillus*) were responsible for PHE biodegradation only. Another two bacterial taxa (*Bacillus* and *Cupriavidus*) were involved in BP degradation. *Ralstonia* was the only bacterial group capable of degrading both PHE and BP in industrial wastewater. DNA-SIP helps reveal the taxonomic identities of functional degraders involved in PHE and BP degradation. To target the separation of functional *Ralstonia*-like PHE degraders from the wastewater, we modified the traditional cultiva-

TABLE 1 Primers for the amplification of 16S rRNA, PAH-RHD, and *bphA* genes

Target	Primer	Sequence (5′–3′)
16S rRNA	515f	GTGCCAGCMGCCGCGGTAA
	806r	AACGCACGCTAGCCGGACTACVSGGGTATCTAAT
	27f	CA GCMGCCGCGGTAANWC
	1492r	CCGTCAATTCMTTTRAGTT
	Bac519F	CA GCMGCCGCGGTAANWC
	Bac907R	CCGTCAATTCMTTTRAGTT
PAH-RHD	RHDf	GACGACTGAAACCTGGATCG
	RHDr	TCGATCCGCACCGGGTAG
bphA	bpf	ACCAGCAAGGACGTGTATGA
	bpr	GCGGATTCGGAGATCAGGGA

tion medium and culture conditions. Finally, an indigenous *R. pickettii* M1 strain was successfully isolated and found to be responsible for the metabolism of PHE and BP. It possessed distinctive PAH-RHD and *bphA* genes, which might have been shared by HGT. Our study reports that *R. pickettii* is able to degrade PHE, and our findings provide reference data for wastewater management in WWTPs receiving industrial wastewater containing multiple POPs. This work expands our knowledge of microorganisms possessing the ability to degrade PHE and BP and provides a routine protocol for identifying and isolating the active indigenous microbes responsible for the degradation of multiple POPs without prior knowledge about which microorganisms are involved in pollutant degradation.

MATERIALS AND METHODS

Sample collection. Untreated industrial wastewater samples were collected from an electronic-waste (e-waste) recycling plant (23°35′N, 113°2′E; altitude, 3.1 m) located in Qingyuan, Guangdong Province, China. In this recycling area, e-wastes are processed to recover valuable metals and other useful components using simple and primitive methods, such as extracting metals with strong acids and combusting or roasting circuit boards on open fires in workshops. These crude e-waste recycling activities release large amounts of carcinogenic and hazardous chemicals, such as PAHs, PCBs, and polybrominated diphenyl ethers, into the surrounding water, air, and soil during the recycling process, threatening local ecosystems and human health (63–66). After being transferred to the laboratory, the wastewater samples were immediately stored at 4°C before degradation and SIP experiments. The contents of PAHs and of total PCBs (∑PCBs) were determined by following the procedures adopted in our previous studies (67, 68) and are listed in Table S1 in the supplemental material.

SIP microcosms. SIP microcosms were constructed individually for the two substrates (PHE and BP) in a 150-ml serum bottle containing 50 ml of the original wastewater. Unlabeled or ^{13}C-labeled substrates (99 atom% ^{13}C [Cambridge Isotope Laboratories Inc., Tewksbury, MA, USA] dissolved in acetone) were added to the bottles, which were then sealed with a rubber stopper and an aluminum cap using a gas-tight syringe at a final concentration of 5 mg/liter for each substrate. Four biotic treatments were designated as follows: [^{12}C]PHE (original wastewater supplied with [^{12}C]PHE), [^{12}C]BP (original wastewater supplied with [^{12}C]BP), [^{13}C]PHE (original wastewater supplied with [^{13}C]PHE), and [^{13}C]BP (original wastewater supplied with [^{13}C]BP). Microcosms without substrates (blank controls) and treatments with filter-sterilized wastewater (sterile controls) were also established. Each treatment was conducted in triplicate. All microcosms were incubated in the dark with shaking at 120 rpm and room temperature (~25°C) as described previously (12). According to the analysis of residual PHE and BP, degradation efficiency was >60% on day 3 and >90% on day 6 (Fig. 1a). Because cross-feeding normally occurs at the end of the degradation process (69), all samples for DNA extraction and fractionation were taken on day 3 from the start of each treatment.

DNA extraction, ultracentrifugation, high-throughput sequencing, and computational analyses. DNA was extracted from the cell pellets by centrifuging 50 ml of each sample from all treatments and was quantified using an ND-2000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA ultracentrifugation was conducted as described previously (16). The hypervariable V4 region of bacterial 16S rRNA gene fragments was amplified for DNA extracted from each fraction derived from the [^{12}C]PHE, [^{12}C]BP, [^{13}C]PHE, and [^{13}C]BP microcosms using primer set 515f/806r (Table 1), as described previously (12). PCR was performed using the method described by Song et al. (70). Sequencing was conducted using 2 × 250 bp paired-end (PE) technology on an Illumina MiSeq sequencer in a standard pipeline. The qualified sequences were analyzed using the mothur software package (71, 72) and were assigned using an OTU-based method to generate microbiome profiles (73–75). In the SIP microcosms, the active PHE and BP degraders were identified by OTUs enriched in the [^{13}C]DNA fractions from the [^{13}C]PHE and [^{13}C]BP microcosms, respectively, through comparison with the corresponding ^{12}C-labeled substrate treatments. Finally, indigenous PHE degraders represented by OTU_11, OTU_16,

and OTU_70, and BP degraders represented by OTU_11, OTU_18, and OTU_27, were selected and identified as the active degraders. Details of the nucleic acid extraction, ultracentrifugation, high-throughput sequencing, and computational analyses are provided in the supplemental material. The resulting partial 16S rRNA gene sequences were made available in GenBank.

Isolation and cultivation of PHE and BP degraders. The phyla identified by SIP provided clues for our improvement in cultivation conditions benefiting and specifically enriching the target functional PHE and BP degraders. According to the work of Wongwongsee et al. in which *Ralstonia* strains were cultivable with 100 mg/liter PAHs and 30°C (76), we modified the traditional cultivation medium, used 100 mg/liter PHE or BP, and conducted isolation at 30°C to target the separation of functional *Ralstonia*-like PHE degraders from the wastewater. Briefly, wastewater (5 ml) was added to 50 ml of modified minimal medium (pH 7.0) (see Table S2 in the supplemental material), with 100 mg/liter PHE (MM-P) or BP (MM-B) as the carbon source. After incubation at 25°C for 7 days, 5 ml of the culture was subcultured in 50 ml of the corresponding fresh MM-P and MM-B, respectively, and was incubated under the same conditions for another 7 days. After three sequential rounds of enrichment, these two enriched populations were serially diluted and were spread on corresponding agar plates, which were incubated at 25°C for 4 days. The colonies were repeatedly transferred to agar medium in order to isolate and purify the microorganisms capable of growing in the presence of PHE or BP. The degradation efficiencies of the isolated strains were evaluated in MM-P and MM-B, in the dark, for 7 days on a 180-rpm shaking plate at 30°C. PHE and BP degradation was determined using the method described under "Chemical analysis" below. The genomic DNAs of these isolates were extracted, and their near-full-length 16S rRNA gene sequences were PCR amplified using two bacterial universal primers, 27f and 1492r (Table 1) (77). Following gel purification with a gel extraction kit (D2500-01; Omega Bio-tek, Norcross, GA, USA), the PCR products were cloned and sequenced. In addition, the morphological and physiological characteristics were studied using a method described previously (78).

The growth conditions (i.e., pH, temperature, and salinity) were established by following previously reported methods (78). To confirm PHE and BP utilization by the strains isolated, their optimal growth curves and degrading efficiencies were investigated as described previously (12). At the beginning of the experiment, cell counts were adjusted to approximately 1×10^8 CFU/ml (79). Cell growth was evaluated by measuring the increase in the optical density at 600 nm (OD_{600}). Controls without cells (referred to as Control-PHE and Control-BP) were also established. These tests were conducted in triplicate.

Detection of PAH-RHD and *bphA* genes. The PAH-RHD and *bphA* genes in the corresponding heavy DNA fractions or isolated degraders were amplified by primer pairs RHDf/RHDr and bpf/bpr, respectively, as listed in Table 1. These specific primers were designed based on the published sequences of *Ralstonia pickettii* DTP0602 (*Betaproteobacteria*; GenBank assembly accession number GCA_000471925.1) using Primer Premier 5.0 software. PCR mixtures contained 1 μl of template DNA, 0.5 μl of each primer (100 nM), 12.5 μl of rTaq premix buffer (TaKaRa Bio Inc., Shiga, Japan), and 10.5 μl of deionized water. The PCR program for the RHDf/RHDr primer set was as follows: 5 min at 95°C (1 cycle); 95°C for 30 s, 52°C for 30 s, and 72°C for 20 s (32 cycles); and a final extension at 72°C for 5 min (1 cycle). For the bpf/bpr primer set, amplification was conducted as follows: 5 min at 95°C (1 cycle); 95°C for 30 s, 55°C for 30 s, and 72°C for 70 s (32 cycles); and a final extension at 72°C for 5 min (1 cycle). The PCR products were gel purified, cloned, sequenced, and analyzed as described previously (32, 80). Phylogenetic dendrograms were prepared using the method described above.

qPCR. The abundances of the bacterial 16S rRNA, PAH-RHD, and *bphA* genes in each fraction were determined by quantitative PCR (qPCR) using the universal bacterial primer pair Bac519F/Bac907R and primer pairs RHDf/RHDr and bpf/bpr, respectively (Table 1). The 20-μl PCR mixture contained 10 μl of SYBR green PCR Premix *Ex Taq* II (TaKaRa), 0.2 μl of each primer (10 μM), and 1 μl of the DNA template. The PCR was performed using an ABI 7500 real-time PCR system (Applied Biosciences, Waltham, MA, USA). Tenfold serial dilutions of known copy numbers of the plasmid with 16S rRNA, PAH-RHD, and *bphA* genes extracted from *Escherichia coli* were generated to produce standard curves, respectively. The reactions were conducted as follows: initial denaturation at 94°C for 10 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 15 s. The SYBR green signal was measured after a 20-s step at 72°C in each cycle.

Chemical analysis. The analysis of PHE and BP was conducted by gas chromatography (model 7890; Agilent, Santa Clara, CA, USA) using a capillary column (DB-5ms; length, 30 m; inside diameter [i.d.], 0.25 mm; film thickness, 0.25 μm) and a mass spectrometric detector (MSD) (model 5975; Agilent) as described previously (32). Briefly, the water samples were spiked with 1,000 ng deuterated PAHs (PHE treatments) or [^{13}C]BP (BP treatments) as a recovery standard and were then extracted twice with dichloromethane. Following concentration and purification with a silica gel–alumina column (i.d., 8 mm), the eluent was concentrated to approximately 50 μl using a gentle stream of N_2, and 1,000 ng hexamethylbenzene was added as an internal standard to all samples prior to instrumental analysis. The components and concentrations of the deuterated PAHs and [^{13}C]BP standards and the internal standard are listed in Table S3 in the supplemental material. The recovery rates of PHE and BP during the extraction procedure were 75 to 85% and 79 to 87%, respectively.

Statistical analysis. Data are expressed as means ± standard deviations. Statistical analyses were performed using Origin 8.0 (OriginLab Corporation, Northampton, MA, USA). The phylogenetic information from the sequences was analyzed using the basic local alignment search tool (BLAST) algorithm (National Center for Biotechnology Information, Bethesda, MD, USA) and MEGA, version 4.0. The least significant difference test was used to determine differences at a P level of 0.05.

Accession number(s). The partial 16S rRNA gene sequences of the PHE and BP degraders identified in this study have been deposited in GenBank with the following accession numbers: MH815094 for

OTU_11, MH815095 for OTU_70, MH815096 for OTU_16, MH815097 for OTU_18, and MH815098 for OTU_27. The GenBank accession numbers for the partial PAH-RHD and *bphA* gene sequences are MH844633 and MH844634, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00511-19.

SUPPLEMENTAL FILE S1, PDF file, 0.9 MB.

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