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Novel Biphenyl-Oxidizing Bacteria and Dioxygenase Genes from a Korean Tidal Mudflat▽†

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Gene-targeted FLX titanium pyrosequencing integrated with stable isotope probing (SIP) using [^{13}C]biphenyl substrate revealed that tidal mudflat sediments harbor novel aromatic ring hydroxylating dioxygenases (ARHD). More than 80% of the detected ARHD genes comprise four clades (0.5 distance) with 49 to 70% amino acid identity to sequences in public databases. The 16S rRNA sequences enriched in the ^{13}C fraction were from the *Betaproteobacteria*, bacilli (primarily *Paenibacillus*-like), and unclassified phyla.

Stable isotope probing (SIP) was used in previous studies of aerobic biphenyl metabolism to determine the active members of biphenyl-metabolizing soil microbial communities and their aromatic ring hydroxylating dioxygenase (ARHD) genes (15, 20, 21). We extended this approach to marine tidal flats since this is a very different environment: salty, high in sulfate, with regular water exchange due to tidal cycles, and often anaerobic with depth. These systems have a remarkable remineralization capacity with a high microbial diversity (3, 10, 16, 22), and some, including the one studied, receive pollutants from industrial and urban sources (12). Since biphenyl is commonly produced from oil combustion and vehicle exhaust, it would be expected to reach these tidal flats. In this study, we characterized ARHD genes and the coenriched bacterial populations from a tidal mudflat metagenome using gene-targeted amplicon FLX titanium pyrosequencing of DNA fractionated by SIP.

Sediments were sampled from a previously characterized site of the Yeochari tidal flat, Kangwha, South Korea (37°36′30″N, 126°22′58″E). The Korean tidal flats are the fifth largest in area in the world. Three replicate microcosms of 5 g each were amended with ^{13}C-labeled biphenyl and incubated for 14 days. After incubation, DNA was extracted from the three microcosms and combined to obtain a sufficient amount of DNA for SIP. [^{13}C]DNA was separated from [^{12}C]DNA by CsCl density gradient ultracentrifugation, and each band was carefully extracted with a syringe needle from ethidium bromide (EtBr)-containing gradients (17). 16S rRNA genes were PCR amplified from [^{12}C]DNA and [^{13}C]DNA fractions by using a 27F and 518R primer set (14). ARHD genes were amplified with a published 888F and 300R primer set (11) after multiple displacement amplification (MDA) of the limited DNA recovered from the [^{13}C]DNA fractions. Gene-targeted pyrosequencing was performed by Macrogen, Inc. (Seoul, South Korea), using a 454/Roche GS-FLX titanium instrument (Roche, Nutley, NJ). The microbial community structure was analyzed with the pyrosequencing pipeline provided by the Ribosomal Database Project (RDP) (2, 5). Operational taxonomic units (OTUs) were clustered at 97% sequence identity, and the median sequence of the cluster was used to determine taxonomy and percent identity to the nearest neighbor in Greengenes (6). Sequences from the [^{12}C]DNA that were also found in the [^{13}C]DNA were assumed to be contaminating and removed. The ARHD sequences were subjected to BLASTX to identify the closest sequences in the nonredundant protein sequence (nr) database. The phylogenetic tree of ARHD amino acid sequences was constructed after alignment by MUSCLE (7) with MEGA4 (13). In order to compare the conserved region with the well-known dioxygenases from Pfam, an amino acid conservation analysis was carried out using ARHD sequences (9, 23). The experimental details are described in the supplemental material.

We analyzed 3,490 and 7,699 16S rRNA gene reads produced from the [^{12}C]DNA and [^{13}C]DNA fractions, respectively. The retained sequences passed a quality filter that allowed up to two mismatches in the forward primer, a minimum average exponential quality score of 20, no ambiguous bases, and a required length longer than 250 bp. The Shannon index values for OTUs in the [^{12}C]DNA and [^{13}C]DNA were 5.56 and 3.88, respectively, suggesting a reduced bacterial diversity in [^{13}C]DNA. The rarefaction curve (see Fig. S1 in the supplemental material) for the [^{13}C]DNA demonstrates that the richness of the biphenyl-metabolizing populations (97% clustering) was reduced from that of the background community ([^{12}C]DNA) such that its OTU richness was captured by using SIP-integrated deep sequencing.

Bacteria that were significantly enriched in the [^{13}C]DNA, compared to the [^{12}C]DNA, belonged to *Betaproteobacteria*, bacilli, and unclassified phyla ($P = 0.0097$) (Fig. 1). A relatively high abundance of the unclassified group with the [^{13}C]DNA indicated the possibility of novel biphenyl-degrading bacteria in this mudflat community. The dominant enriched members

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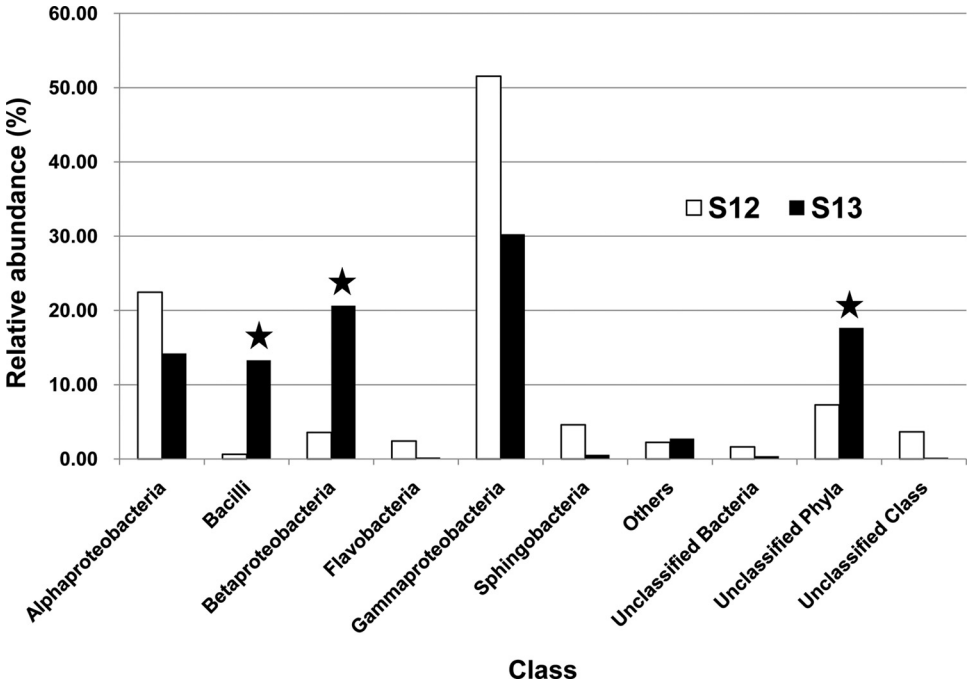

FIG. 1. Relative abundances of [^{12}C]DNA (S12) and [^{13}C]DNA (S13) in microbial community members. RDP Classifier was used at the class level with a 50% bootstrap threshold (4). Solid stars indicate the class members that exhibited significant enrichment in S13 compared to S12.

were affiliated with the genera *Paenibacillus* (28.6% of the total [^{13}C]DNA), *Pusillimonas* (14.1%), and *Alcaligenes* (4.4%) (Table 1). Polychlorinated biphenyl (PCB) degradation capabilities have previously been reported for *Paenibacillus* and *Alcaligenes* isolated from PCB-contaminated soil (1, 18), whereas *Pusillimonas* isolated from polycyclic aromatic hydrocarbon (PAH)-contaminated soil has been reported to degrade pyrene (8). Notably, the dominant biphenyl-metabolizing populations found in this study were different from those in rhizosphere soils (15, 21) or river sediment (20) where the biphenyl-SIP approach was also used. Only *Variovorax* and *Pseudomonas*, detected by [^{13}C]DNA with low abundances (0.56% and 0.10%, respectively), were found in those previous studies. These data suggest that the biphenyl-metabolizing bacteria

TABLE 1. Phylogenetic classification of 16S rRNA genes detected only in the [^{13}C]DNA (S13) fraction[a]

S13 (%)	Closest relative by Greengenes	Accession no.	Class	Identity (%)
6.25	*Pusillimonas terrae*	DQ466075	*Betaproteobacteria*	98.74
5.65	*Paenibacillus* sp. strain KO_CM21	GQ497919	Bacilli	93.75
5.12	*Pusillimonas terrae*	DQ466075	*Betaproteobacteria*	96.14
4.99	*Paenibacillus* sp. Eur1 9.9	DQ444978	Bacilli	87.93
4.92	*Paenibacillus* sp. KCTC 13564	GQ303568	Bacilli	88.10
1.92	*Pusillimonas terrae*	DQ466075	*Betaproteobacteria*	97.54
1.90	*Paenibacillus favisporus* strain GMP03	AY308758	Bacilli	85.85
1.68	*Alcaligenes* sp. strain L6	X92415	*Betaproteobacteria*	97.03
1.68	*Paenibacillus* sp. strain P-3	AM411970	Bacilli	87.52
1.58	*Paenibacillus* sp. strain KBC101	AB186915	Bacilli	87.16
1.40	*Paenibacillus ruminocola* strain CA8	DQ085278	Bacilli	82.20
1.27	*Paenibacillus* sp. strain KO_CM21	GQ497919	Bacilli	92.25
0.82	*Paenibacillus* sp. KCTC 13564	GQ303568	Bacilli	88.70
0.78	*Paenibacillus validus* strain SB 3263	GU191921	Bacilli	99.74
0.70	*Paenibacillus koreensis* strain YC300	AF130254	Bacilli	88.16
0.68	*Alcaligenes* sp. strain L6	X92415	*Betaproteobacteria*	96.01
0.64	*Alcaligenes* sp. strain L6	X92415	*Betaproteobacteria*	97.16
0.64	*Paenibacillus koreensis* strain YC300	AF130254	Bacilli	87.02
0.56	*Variovorax paradoxus* strain S110	CP001635	*Betaproteobacteria*	100.00
0.55	*Bacillus mucilaginosus* strain HSCC 1605T	AB045091	Bacilli	93.65
0.53	*Alcaligenes* sp. strain L6	X92415	*Betaproteobacteria*	95.24
0.53	*Paenibacillus ehimensis* strain IFO 15659	AB021184	Bacilli	86.05
0.51	*Pusillimonas terrae*	DQ466075	*Betaproteobacteria*	96.80

[a] Only taxa of >0.5% abundance are shown.

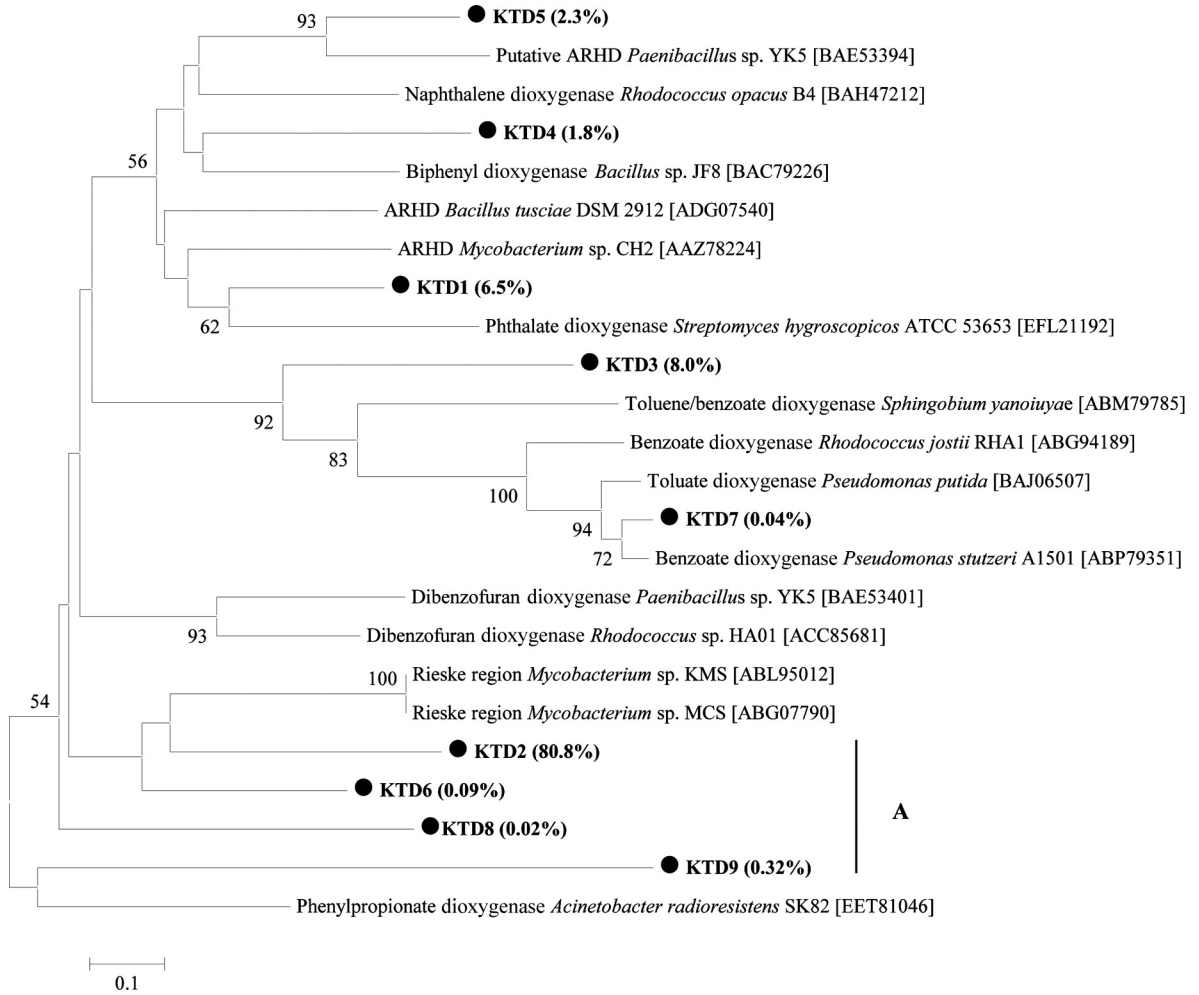

FIG. 2. Neighbor-joining phylogenetic tree (1,000 bootstraps) based on approximately 100-amino-acid sequences of ARHD genes. The Korean tidal flat dioxygenase (KTD) sequences are marked by dark circles. The number in the parentheses after each KTD sequence indicates the percentage of sequences in that cluster.

from the tidal mudflat may contain a PCB degradative capability with a phylogenetic signature distinctly different from that of the terrestrial biphenyl-metabolizing populations.

We also PCR amplified from the [^{13}C]DNA and pyrosequenced ARHD gene fragments that contain coding regions for the Rieske-type [2Fe-2S] cluster. Using a strict quality filtering procedure described in the supplemental material, 4,924 ARHD gene reads (60.3%) were retained from a total of 8,169 reads. Most of these sequences (99.8%) coded for the conserved Rieske domain, which is essential for function and indicates that the retained sequences were for the correct protein. A BLASTX search against the nr database revealed best matches to other ARHD genes but that the obtained genes were diverse and different from those known. More than 80% of the detected ARHD genes were in clade A and had only 49 to 70% amino acid identity to known ARHD genes (Fig. 2). The clade represented by KTD2, which had 80.8% of the sequences, also contains considerable amino acid diversity (40 to 60% amino acid identity) since the clade was clustered at a distance of 0.5. A few of the sequences matched to a putative ring hydroxylating dioxygenase (2.3% of 4,664 reads) of *Paenibacillus*, which was a dominant genus in the 16S rRNA analysis. This suggests that *Paenibacillus*-like bacteria are capable of biphenyl metabolism in the tidal mudflat community.

ARHD sequences were compared with 467 representative sequences (see the supplemental material) from the Pfam protein family database by using the amino acid conservation analysis method of Iwai et al. (9). Conservation analysis identified several conserved amino acids in the ARHD genes beyond those known to be important for function. They were located between the end of the Rieske [2Fe-2S] domain and the beginning of the ring hydroxylating alpha subunit (catalytic domain) (Fig. 3). Because even a single amino acid substitution in a functionally important, conserved region can enhance the rate of the aromatic oxygenation by biphenyl dioxygenase (19), these findings suggest that the host strains may exhibit different degradation kinetics and/or PCB congener specificities compared to the known ARHD.

Using the SIP-pyrosequencing method, we discovered apparently novel biphenyl-metabolizing bacteria and ARHD genes from a tidal mudflat. Bacteria similar to *Paenibacillus*, which are known to be a terrestrial PCB-degrading population

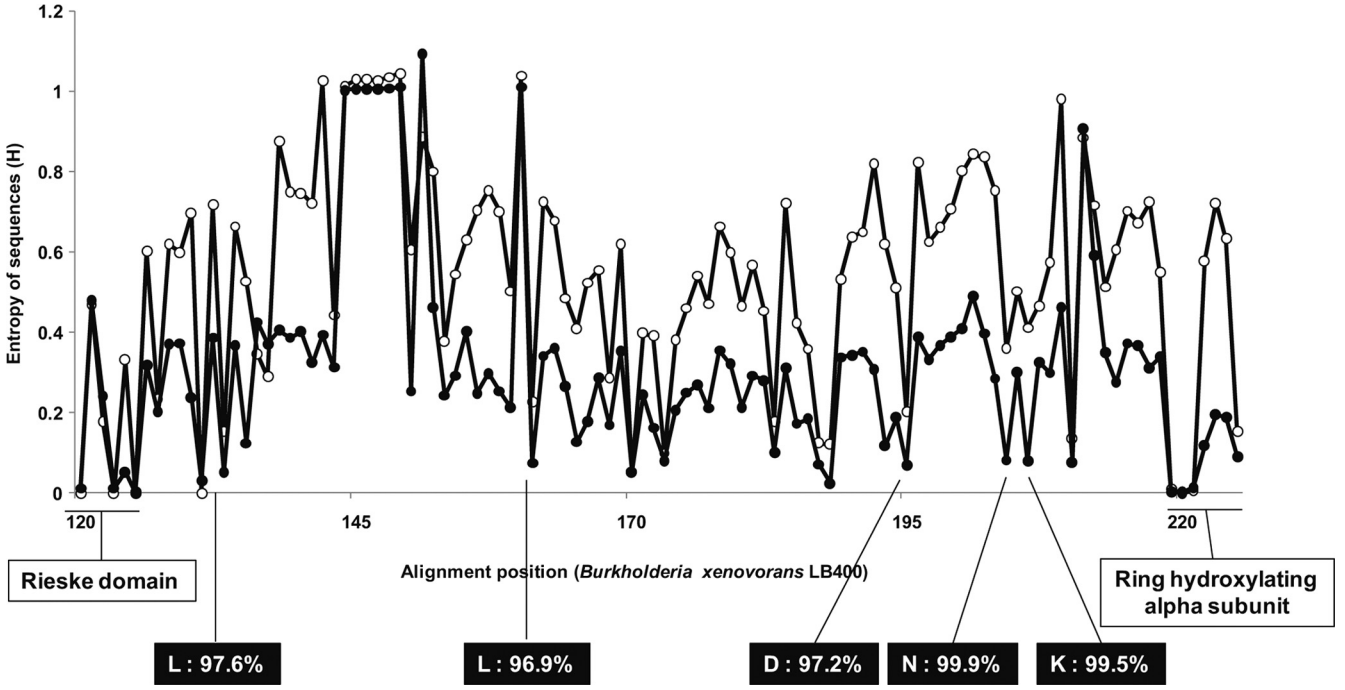

FIG. 3. Shannon entropy (H′) at each alignment position and conserved residues among the obtained and reference ARHD sequences. Open circles indicate the entropy of reference sequences, and solid circles indicate the entropy of the SIP-enriched (obtained) sequences. Known domains, amino acids, and their percentages of the total obtained sequences are shown in boxes.

(18), were identified as one of the dominant biphenyl-utilizing populations at this marine site.

Nucleotide sequence accession numbers. All sequences have been deposited in the Short Read Archive database at NCBI (accession no. SRA028415, SRA028416, and SRA028424).

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