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Isolation and Characterization of Bacteria Capable of Degrading Polycyclic Aromatic Hydrocarbons (PAHs) and Organophosphorus Pesticides from PAH-Contaminated Soil in Hilo, Hawaii

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Nineteen bacterial strains were isolated from petroleum-contaminated soil in Hilo, HI, and characterized by two different spray-plated methods, turbidity test in liquid medium, and 16S rRNA gene sequence analysis. Analysis of the soil showed 13 polycyclic aromatic hydrocarbons (PAHs) in a range from 0.6 to 30 mg/kg of dry weight each and 12 PAH metabolites. Five distinct bacterial strains (C3, C4, P1-1, JS14, and JS19b1) selected from preliminary plating and turbidity tests were further tested for PAH degradation through single PAH degradation assay. Strains C3, C4, and P1-1 degraded phenanthrene (40 mg/L) completely during 7 days of incubation. Strain JS14 degraded fluoranthene (40 mg/L) completely during 10 days of incubation. Strain JS19b1 degraded 100% of phenanthrene (40 mg/L) in 7 days, 77% of fluorene (40 mg/L) in 14 days, 97% of fluoranthene (40 mg/L) in 10 days, and 100% of pyrene (40 mg/L) in 14 days. Turbidity tests showed that strains P1-1, JS14, and JS19b1 utilized several organophosphorus pesticides as growth substrate. P1-1 can degrade carbofenothion, chlorfenvinphos, diazinon, fonofos, and pirimiphos-methyl. JS14 can transform chlorfenvinphos and diazinon. JS19b1 can break down diazinon, pirimiphos-methyl, and temephos.

KEYWORDS: Biodegradation; *Arthrobacter*; *Burkholderia*; *Sinorhizobium*; *Mycobacterium*; organophosphorus pesticides; polycyclic aromatic hydrocarbons

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are an important group of environmental pollutants. PAHs are introduced into the environment from various sources such as natural oil seeps, refinery and oil storage wastes, accidental spills from oil tankers, petrochemical industrial effluents, coal tar processing wastes, combustion processes, and wood preservative wastes (*1*−*3*). Some PAHs are hydrophobic, persistent, toxic, mutagenic, and carcinogenic (*4*, *5*). The fate of PAHs in the environment is associated with both abiotic and biotic factors including volatilization, photooxidation, adsorption to soil particles, bioaccumulation, chemical oxidation, and biotransformation (*1*, *5*).

Organophosphorus pesticides (OPs) have been widely used to control a wide range of insects and possess high mammalian toxicity. As a result of growing awareness over OP pollution and toxicity, research efforts have been made toward degrading OPs with microorganisms (*6*). Microbial degradation of OPs is a major factor determining the fate of OPs in the environment.

Microbial degradation is the most significant and influential process in the mineralization of persistent organic pollutants. Many bacterial strains have been isolated and characterized for biodegradation of PAHs varying from two-ring PAHs to five-ring PAHs (*7*−*14*). Different bacterial genera are involved in the mineralization of PAHs. These include *Burkholderia* (*8*), *Pseudomonas* (*9*), *Sphingomonas* (*10*, *15*), and *Stenotrophomonas* (*12*) as Gram-negative and *Mycobacterium* (*2*−*4*, *7*, *14*) and *Rhodococcus* (*16*) as Gram-positive. There have been also isolated different bacterial genera, for example, *Arthrobacter* (*17*, *18*), *Agrobacterium* (*19*), *Burkholderia* (*20*), *Enterobacter* (*21*), *Plesiomonas* (*22*), and *Pseudomonas* (*23*, *24*), capable of degrading various OPs such as chlorpyrifos (*21*, *24*), coumaphos (*21*), diazinon (*21*), fenitrothion (*20*), monocrotophos (*17*), and parathion (*21*, *22*).

The objectives of the present work were to isolate PAH- and OP-degrading bacteria from a petroleum-contaminated soil and to study degradation potential. It is also hoped that PAH degradation capabilities of certain bacterial genera can be exploited for the development of effective bioremediation technologies for environmental cleanup as well as an understanding of natural transformation processes of PAHs in the environment.

MATERIALS AND METHODS

Chemicals. Fluorene, phenanthrene, fluoranthene, pyrene, benzo[*a*]pyrene, and *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide were pur-

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chased from Aldrich (Milwaukee, WI), and the purity was >98%. All chemicals used for media were of at least reagent grade. All organic solvents used were of high-performance liquid chromatographic (HPLC) grade and purchased from Fisher Scientific (Pittsburgh, PA). A mixture of 16 PAH standards in methanol/methylene chloride (1:1, v/v) was purchased from Supelco (Bellefonte, PA). All of the organophosphorus pesticides were purchased from AccuStandard (New Haven, CT) except temephos, ethoprophos, pirimiphos-methyl, and crotoxyfos, which were from Aldrich. Individual PAH stock solutions were 1000 mg/L in acetone. The PAH metabolites acenaphthenone, diphenic acid, 1-hydroxy-2-naphthoic acid, 2-hydroxy-1-naphthoic acid, naphthalene-1,2-dicarboxylic acid anhydride, phthalic acid, protocatechuic acid, and 9-fluorenone-1-carboxylic acid were purchased from Aldrich. Gentisic acid and 3-hydroxy-2-naphthoic acid were purchased from TCI America (Portland, OR). Naphthalene-1,2-dicarboxylic acid was prepared by basic hydrolysis of naphthalene-1,2-dicarboxylic acid anhydride. Phenanthrene-4,5-dicarboxylic acid was synthesized according to the method of Young and Funk (*25*). These metabolite standards were methylated into corresponding methyl esters or ethers with diazomethane that was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide in a diazomethane generator (Aldrich).

Soil. Soil samples were taken from a former oil gasification company site located in Hilo, HI (19° 49′ 20″ N latitude, 155° 05′ 01″ W longitude). The company started approximately in 1918 and ceased its operations in 1965 as a result of tsunami inundation. The operation steam stripped crude oil to produce cooking gas. The waste product generated was a tar rich in PAHs. A tank containing the tar was buried at the site after the company was closed. In 1996, the tank bottom was excavated during construction of a flood control channel, and approximately 3000 m^3 of tar and contaminated soil were stored in a plastic sheet in a berm on the site. Heavy flooding in November 2000 swept PAH-contaminated silt from the channel scour pond onto the surface of five Hawaii County Park soccer fields. Surface soils were grab-sampled, well mixed, and placed in a 20-L plastic container and stored at 4 °C until used. The fresh soil that had 26.8% moisture was passed through a 2 mm sieve and then used for bacterial isolation. The soil samples were air-dried and passed through a 2 mm sieve for chemical analysis.

Analyses of PAHs and PAH Metabolites. PAHs (20 g dried soil) were extracted from the soil samples with a mixture of chloroform and methanol (2:1, v/v) by ultrasonication (*26*). The PAHs in the extracts were separated and quantified with a gas chromatograph (GC) (5890II, Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector (FID) and a ZB-1 column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, Phenomenex, Torrance, CA). The injector and detector temperatures were 250 and 300 °C, respectively. The oven temperature was initially at 80 °C for 1 min, programmed to 280 °C at a rate of 10 °C/min, and then held at 280 °C for 10 min. The PAHs were identified by comparison of the retention times with those of PAH standards.

To extract PAH metabolites from the soil samples, 400 g of the sieved soil was treated with 100 mL of 1 N hydrochloric acid (HCl) and 500 mL of acetone. After 2 h of vigorous shaking, soil was filtered out. After acetone was removed with a rotary evaporator, 400 mL of distilled water was added to the extract solution and extracted with ethyl acetate (3 × 250 mL). The combined ethyl acetate extract (i.e., organic phase) was washed three times with 250 mL of aqueous sodium hydroxide (10 mM) (i.e., aqueous phase). The organic phase was dried over anhydrous sodium sulfate and then concentrated to 5 mL of ethyl acetate (neutral fraction). After the aqueous phase was adjusted to pH 2−2.5 with 6 N HCl, it was extracted three times with 250 mL of ethyl acetate. After solvent removal, the residue was dissolved in 5 mL of ethyl acetate (acidic fraction). Metabolites in each fraction were derivatized with diazomethane.

GC−mass spectrometry (GC-MS) analysis was performed with a Varian QP-5000 GC with a Saturn-2000 mass spectrometer, equipped with a ZB-1 column (60 m, 0.25 μm film thickness) and helium as carrier gas. The column temperature started at 120 °C for 2 min, was raised to 280 °C at a rate of 2 °C/min, and was held at 280 °C for 10 min. Injector and analyzer temperatures were 270 and 280 °C, respectively. Mass spectra were recorded in electron impact (EI) mode at 70 eV and compared with those of the metabolite authentic standards.

Soil Enrichment and Isolation of PAH-Degrading Bacteria. Bacterial strains in the soil were grown in minimal medium (MM) (1 g of soil per 25 mL of MM) to which solid fluorene, phenanthrene, fluoranthene, pyrene, or benzo[*a*]pyrene were added at a level of 1 mg/mL. A MM formula was adapted from Bastiaens et al. (*27*) in this study. Specifically, the minimal medium contained (per liter) 8.8 g of $Na_2HPO_4 \cdot 2H_2O$, 3.0 g of KH_2PO_4, 1.0 g of NH_4Cl, 0.5 g of NaCl, 1.0 mL of 1 M $MgSO_4$, and 2.5 mL of a trace element solution [(per liter) 23 mg of $MnCl_2 \cdot 2H_2O$, 30 mg of $MnCl_4 \cdot H_2O$, 31 mg of H_3BO_3, 36 mg of $CoCl_2 \cdot 6H_2O$, 10 mg of $CuCl_2 \cdot 2H_2O$, 20 mg of $NiCl_2 \cdot 6H_2O$, 30 mg of $Na_2MoO_4 \cdot 2H_2O$, and 50 mg of $ZnCl_2$] (pH 7). Each suspension of 2 g of soil, 50 mg of a PAH, and 50 mL of MM was incubated at 28 °C while shaking at 150 rpm in the dark. After 3 months of incubation, the cultures were shaken vigorously with a model 25 Controlled Environment Incubator Shaker (New Brunswick Scientific Co., Inc., Edison, NJ) for 3 h to loosen bacteria from the soil particles and then were allowed to sit for 3 h to precipitate the soil particles. The aqueous phase (1 mL of the upper aqueous phase) was used to inoculate liquid enrichment cultures in 50 mL of MM fortified with fluorene, phenanthrene, fluoranthene, pyrene, or benzo[*a*]pyrene as the sole carbon and energy source. The cultures were incubated at 28 °C while shaking at 150 rpm in the dark. When turbidity was observed, an aliquot of the culture was serially diluted and was transferred onto nutrient agar plates for determination of colony numbers and strain isolation. The nutrient agar plates were prepared with 15 g of agar and 8 g of nutrient broth (Difco) per liter, and the medium was autoclaved prior to use.

Biodegradation Screening. The isolated pure strains were tested with two different spray-plated methods and a liquid medium turbidity test for five individual PAHs (fluorene, phenanthrene, fluoranthene, pyrene, and benzo[*a*]pyrene) to determine the PAH degradation potential of the strains. The first spray-plated method (SPM1) was that isolates were pregrown for 2−5 days, in general, to have colonies on yeast extract−polypeptone−glucose (YEPG) agar plates, which were then sprayed with a 1% PAH acetone solution (*28*). After solvent vaporization, the plates were incubated at 28 °C and observed daily. The second spray-plated method (SPM2) was to spray a 1% PAH acetone solution on the MM agar plates. After solvent vaporization, isolates were spotted on the plates and incubated at 28 °C. Isolates producing significantly clear zones by utilization of the PAH were scored as positive. The turbidity test (TBT) was that pregrown cells on the nutrient agar plate were inoculated into sterilized culture tubes containing 10 mL of MM and 400 μg of PAH. The culture tube was incubated at 28 °C while shaking at 150 rpm. When turbidity occurred, cultures were scored as positive and marked as PAH-degrader candidates. In addition to PAHs, 20 organophosphorus pesticides were screened by the above turbidity test for degradation by the four strains, C3, P1-1, JS14, and JS19b1.

Analysis of 16S rDNA Sequence. Genomic DNA was extracted with a mixture of phenol and chloroform (*29*). The 16S rRNA gene was amplified from the genomic DNA (100−200 ng/μL) by Polymerase Chain Reaction (PCR) with *Taq* DNA polymerase (Takara Mirus Bio, Inc.) and primers 27F and 1492R (*30*). Amplification was performed with a Techne thermal cycler (Techne, Inc., Burlington, NJ) at 95 °C for 4 min followed by 30 cycles of 95 °C for 1 min, 55 °C for 50 s, and 72 °C for 1.5 min, and a final elongation step at 72 °C for 7 min. The PCR product was purified using the Ultraclean PCR purification kit (Mo Bio Lab, Inc., Carlsbad, CA) and sequenced in both directions using an Applied Biosystems 377XL DNA sequencer. 16S rDNA sequences were manually edited and assembled in Sequencher and Seqman (DNASTAR). Assembled sequences each in excess of 1300 bases were compared with those in the public domain through a BLASTn search (*31*). A phylogenetic tree was constructed with the MegAlign (DNASTAR) based on complete or nearly complete 16S rDNA sequences aligned by Clustal method.

PAH Biodegradation Assays. Bacterial cells were pregrown in PAH-supplemented MM to an optical density of 0.3 at 580 nm; specifically, phenanthrene was supplemented for strains C3, C4, P1-1, and JS19b1, fluoranthene for JS19b1 and JS14, pyrene for JS19b1, and fluorene for JS19b1. A 200 μL aliquot of each PAH stock solution

Table 1. Concentrations of 16 Priority PAHs in Hilo Soil

PAH	concentration[a] (mg/kg of dry soil)
naphthalene	ND[b]
acenaphthylene	3.1
acenaphthene	0.6
fluorene	ND
phenanthrene	3.4
anthracene	0.9
fluoranthene	20
pyrene	30
benzo[*a*]anthracene	7.0
chrysene	9.7
benzo[*b*]fluoranthene	20
benzo[*k*]fluoranthene	5.0
benzo[*a*]pyrene	20
indeno[1,2,3-*cd*]pyrene	22
dibenz[*a,h*]anthracene	ND
benzo[*g,h,i*]perylene	24

[a] Mean of duplicate samples. [b] Not detected.

was placed into a sterilized culture tube. After the solvent was evaporated with nitrogen gas, the MM (4 mL) and the pregrown cells (1 mL) were added. The culture tubes were incubated (28 °C, 150 rpm in the dark) and extracted, at various time intervals, with ethyl acetate (5 mL) for GC determination of PAH degradation. Cultures inoculated with boiled dead cells were used as controls. All experiments were carried out with triplicate.

Nucleotide Sequence Accession Numbers. The 16S rRNA gene sequences of all strains have been deposited in GenBank under the following accession numbers: C3 (AY943387), C4 (AY943388), C6 (AY943389), P1-1 (AY943390), JS1-1 (DQ104979), JS1-2 (DQ104980), JS2 (DQ104981), JS6 (DQ104991), JS7 (DQ104982), JS8 (DQ104983), JS9 (DQ104984), JS11 (DQ104985), JS12 (DQ104986), JS13 (DQ104987), JS14 (AY943385), JS15 (DQ104988), JS18 (DQ104989), JS19b1 (AY943386), JS23b (DQ104990).

RESULTS

Determination of PAHs and Their Metabolites in Soil Samples. PAH analysis was focused on the 16 priority PAHs listed by the U.S. EPA. Naphthalene, fluorene, and dibenz[*a*,*h*]-anthracene were not detected, whereas the concentrations of each of the 13 other PAHs in the soil samples varied from 0.6 to 30 mg/kg of dry weight (**Table 1**). Pyrene had the highest concentration among the 16 PAHs. **Table 1** shows that high molecular weight PAHs dominated in the soil, which was probably due to their persistence, low water solubility, low volatility, and high octanol/water partitioning coefficients (K_{ow}) (*32*). Twelve different PAH metabolites were also detected and identified in a methylated form except acenaphthenone (**I**)

Table 2. GC-MS Identification of PAH Metabolites in the Soil Samples Collected from Hilo, Hawaii

ID	metabolite[a]	fragment *m/z* (molecular ion, % relative abundance)	possible parent
I	acenaphthenone	168 (M^+, 89), 140 (100)	acenaphthene, fluoranthene
II	phthalic acid (diMe)	194 (M^+, 1), 163 (100), 149 (7), 133 (19)	several PAHs
III	gentisic acid (diMe)	182 (M^+, 63), 151 (100), 123 (28), 115 (92)	several PAHs
IV	protocatechuic acid (diMe)	182 (M^+, 48), 151 (100), 123 (34), 105 (66)	several PAHs
V	1-hydroxy-2-naphthoic acid (Me)	202 (M^+, 55), 170 (100), 142 (15), 114 (62)	phenanthrene
VI	2-hydroxy-1-naphthoic acid (diMe)	216 (M^+, 37), 202 (19), 170 (100), 142 (32), 114 (58)	phenanthrene
VII	3-hydroxy-2-naphthoic acid (Me)	202 (M^+, 63), 170 (100), 142 (85), 114 (53)	anthracene
VIII	2,2′-diphenic acid (diMe)	270 (M^+, 0), 239 (6), 211 (100), 196 (18), 180 (7), 168 (7)	phenanthrene, pyrene
IX	naphthalene-2,3-dicarboxylic acid (diMe)	244 (M^+, 16), 213 (1), 170 (100), 149 (41), 142 (21), 114 (47)	anthracene
X	naphthalene-1,2-dicarboxylic acid (diMe)	244 (M^+, 25), 213 (4), 170 (100), 149 (53), 142 (14), 114 (37)	phenanthrene
XI	9-fluorenone-1-carboxylic acid (Me)	238 (M^+, 100), 223 (2), 207 (99), 179 (48), 151 (58)	fluoranthene
XII	phenanthrene-4,5-dicarboxylic acid (diMe)	294 (M^+, 0), 263 (4), 235 (100), 220 (47), 204 (4), 192 (8), 176 (8)	pyrene

[a] diMe, dimethyl ester/ether; Me, methyl ester.

Table 3. Bacterial Identification by 16S rRNA Gene Sequences and Results of Screening Tests for Degradation of Five PAHs

isolate ID	best match in GenBank: strain	accession no.	% identity (matched base)	screening test (SPM1[a]/SPM2[b]/TBT[c]): FLE[d]	PHE[e]	FLA[f]	PYR[g]	BAP[h]
JS1-2	*Achromobacter xylosoxidans*	AY946288	99 (1418/1422)	–/–/–	–/–/–	–/–/–	–/–/–	n[i]/–/–
JS1-1	*Achromobacter xylosoxidans* strain NFRI-A1	AB161691	99 (1434/1435)	–/–/–[j]	–/–/–	–/–/–	–/–/–	n/–/–
P1-1	*Arthrobacter* sp. CAB1	AB039736	96 (1391/1437)	–/–/–	+/+/+[k]	–/–/–	–/–/–	n/+/–
JS18	*Bacillus cereus* biovar *toyoi*	AJ310100	99 (1460/1466)	–/–/–	–/–/–	–/–/–	–/–/–	n/–/–
JS23b	*Bacillus* sp. CJ11076	AF500212	100 (1464/1464)	–/–/–	–/–/–	–/–/–	–/–/–	n/–/–
JS15	*Bacillus* sp. DU	AJ842963	99 (1423/1433)	–/–/+	–/–/–	–/–/–	–/–/–	n/–/–
JS13	*Bacillus thuringiensis*	D16281	99 (1462/1466)	–/–/–	–/–/–	–/–/–	–/–/–	n/–/–
JS2	*Bosea* sp. MN 51a	AJ313022	99 (1364/1373)	–/–/–	–/+/–	–/–/–	–/–/–	n/–/–
JS11	*Bosea thiooxidans*	AJ250796	99 (1379/1390)	–/–/–	–/–/–	–/–/–	–/–/–	n/–/–
C3	*Burkholderia* sp. 56	AY177370	99 (1406/1413)	–/–/–	+/+/+	–/–/–	–/–/–	n/–/–
JS12	*Mesorhizobium loti* MAFF303099	BA000012	97 (1356/1386)	–/–/–	–/–/–	–/–/–	–/–/–	n/–/–
JS6	*Mesorhizobium plurifarium*	Y14161	97 (1353/1388)	–/–/+	–/+/–	–/–/–	–/–/–	n/–/–
JS9	*Mesorhizobium plurifarium* subsp. *americium* strain Ls29	AF516881	97 (1341/1370)	–/–/–	–/–/–	–/–/–	–/–/–	n/–/–
JS7	*Mesorhizibium* sp. ORS3165	AY875973	97 (1353/1389)	–/–/–	–/–/–	–/–/–	–/–/–	n/–/–
JS14	*Mycobacterium* sp. PX3.15	AY337605	99 (1287/1297)	–/–/–	–/+/–	+/+/+	–/–/–	n/–/–
JS19b1	*Mycobacterium* sp. RJGII.135	U30661	99 (1312/1315)	–/–/+	+/+/+	–/–/+	–/+/+	n/–/–
C4	*Sinorhizobium* sp. HF6	AB195269	99 (1389/1398)	–/–/–	–/+/+	–/–/–	–/–/–	n/–/–
C6	*Stenotrophomonas maltophilia*	AB008509	99 (1444/1449)	–/–/–	–/–/+	–/–/–	–/–/–	n/–/–
JS8	uncultured *Alcaligenaceae bacterium*	AB114615	99 (1436/1442)	–/–/–	–/–/–	–/–/–	–/–/–	n/–/–

[a–c] Described under Materials and Methods. [d] Fluorene. [e] Phenanthrene. [f] Fluoranthene. [g] Pyrene. [h] Benzo[*a*]pyrene. [i] Not tested. [j] "–" indicates no degradation. [k] "+" indicates positive degradation.

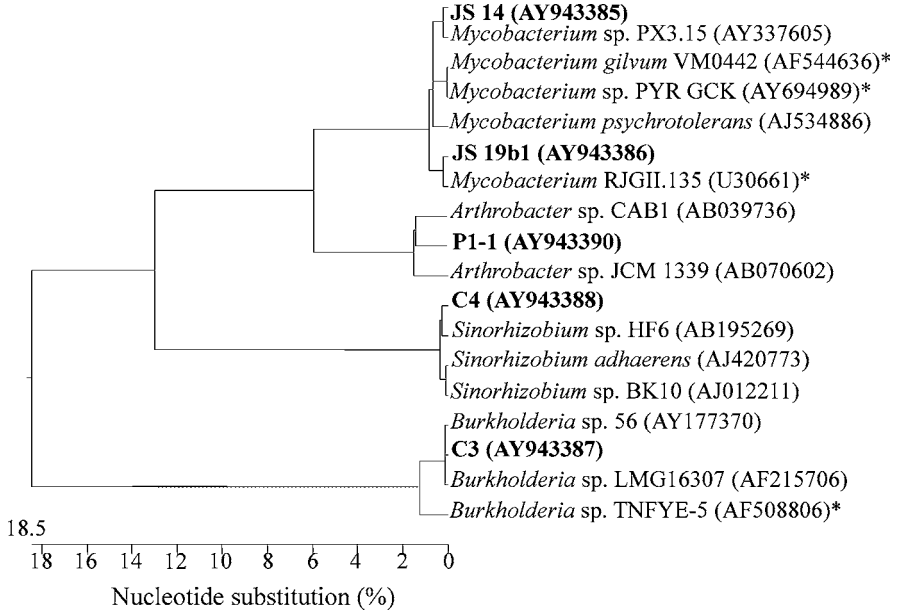

Figure 1. Phylogenetic positions of the five PAH-degraders based on complete or nearly complete 16S rDNA sequences aligned by Clustal method. Strains marked with an asterisk are well-known as PAH-degraders. GenBank accession numbers are in parentheses.

Table 4. Screening Test Results for Degradation of Organophosphorus Pesticides

	DT_{50}[a] (days)			strains[b]			
chemical	pH 5	pH 7	pH 9	C3	P1-1	JS14	JS19b1
temephos	ST	ST	US	–	–	–	+
famphur	ND	ND	ND	–	–	–	–
diazinon	ND	185	6	–	+	+	+
methidathion	ST	ST	ND	–	–	–	–
azinphos-methyl	ND	50	4	–	–	–	–
coumaphos	ND	185	6	–	–	–	–
chlorpyrifos	ND	100	0.15	–	–	–	–
parathion-methyl	68	40	33	–	–	–	–
fenamiphos	365	3000	100	–	–	–	–
bensulide	RS	RS	RS	–	–	–	–
sulprofos	26	151	56	–	–	–	–
chlorfenvinphos	RS	RS	ND	–	+	+	–
fonofos	101	127	1.4	–	+	–	–
ethoprophos	ST	ST	ND	–	–	–	–
dioxathion	ND	ND	ND	–	–	–	–
carbofenothion	ND	ND	ND	–	+	–	–
parathion	272	260	130	–	–	–	–
pirimiphos-methyl	ND	7.2	35	–	+	–	+
dichlorvos	31.9	2.9	2	–	–	–	–
crotoxyfos	ND	ND	ND	–	–	–	–

[a] DT_{50}, degradation time for 50% of the concentration; ND, no data available; RS, relatively stable; ST, stable; US, unstable (no numeric data available); all DT_{50} values were cited from "*The Pesticide Manual*" (*40*). [b] "–" indicates no growth; "+" indicates growth.

(**Table 2**). Metabolite **I** was detected in the neutral fraction, and the others were in the acidic fraction. Metabolites **II**, **III**, and **IV** may be derived from several parent PAHs (**Table 2**).

Isolation of PAH-Degrading Bacteria. A total of 19 bacterial species were isolated and phenotypically studied for their PAH degradation potential (**Table 3**). Five PAHs (fluorene, phenanthrene, fluoranthene, pyrene, and benzo[*a*]pyrene) were used to screen PAH-degrading bacteria. All isolates were screened with the two PAH spray-plated methods and turbidity test in liquid MM. Results of the two spray-plated methods showed seven strains (C3, C4, P1-1, JS2, JS6, JS14, and JS19b1) positive for one or more than one of the five PAHs (phenanthrene, fluoranthene, pyrene, fluorene, and benzo[*a*]pyrene). Results of the liquid MM turbidity test showed eight strains (C3, C4, C6, P1-1, JS6, JS14, JS15, and JS19b1) positive for fluorene, phenanthrene, fluoranthene, and/or pyrene. Strains C3, C4, P1-1, and JS19b1 were marked as the most effective (≥ +/+) for phenanthrene degradation, and JS14 was the most effective for fluoranthene (**Table 3**). Five strains (C3, C4, P1-1, JS14, and JS19b1) were selected for further studies because of their high PAH degradation abilities (≥ +/+).

Screening Test of OP Pesticides. The results of the turbidity test showed that strains P1-1, JS14, and JS19b1 utilized diazinon as growth substrate (**Table 4**). Also, strain P1-1 degraded chlorfenvinphos, fonofos, carbophenothion, and pirimiphos-methyl (**Table 4**). Strain JS14 could grow in liquid MM supplemented with chlorfenvinphos. The turbidity was increased by growth of strain JS19b1 utilizing temephos and pirimiphos-methyl (**Table 4**). Strain P1-1 was the most capable of degrading OPs.

Analysis of 16S rDNA Sequence. Isolated bacteria were classified according to the best match of their 16S rDNA sequences with the GenBank database (**Table 3**). Isolates belonging to the α-proteobacteria had the best match of the sequences to *Sinorhizobium* sp. HF6 (C4), *Bosea* sp. MN51a (JS2), *Mesorhizobium plurifarium* (JS6 and JS9), *Mesorhizobium* sp. ORS3165 (JS7), *Bosea thiooxidans* (JS11), and *Mesorhizobium loti* (JS12). The sequence similarities of isolated *β*-proteobacteria were 99% for *Burkholderia* sp. 56 (C3), *Achromobacter xylosoxidans* (JS1-1 and JS1-2), and uncultured *Alcaligenaceae bacterium* (JS8). One isolated *γ*-proteobacteria was the closest to *Stenotrophomonas maltophilia* (C6). Seven Gram-positive bacteria isolated include three actinobacteria and four firmicutes. Isolates belonging to the actinobacteria had the best similarity to *Arthrobacter* sp. CAB1 (P1-1), *Mycobacterium* sp. PX3.15 (JS14), and *Mycobacterium* sp. RJGII.135 (JS19b1). Isolates belonging to the firmicutes matched well with *Bacillus thuringiensis* (JS13), *Bacillus* sp. (JS15 and JS23b), and *Bacillus cereus* biovar *toyoi* (JS18). Phylogenetic positions of the five PAH-degrading strains (C3, C4, P1-1, JS14, and JS19b1) were placed according to the best similarity with the sequences of the 16S rRNA in the GenBank database (**Figure 1**).

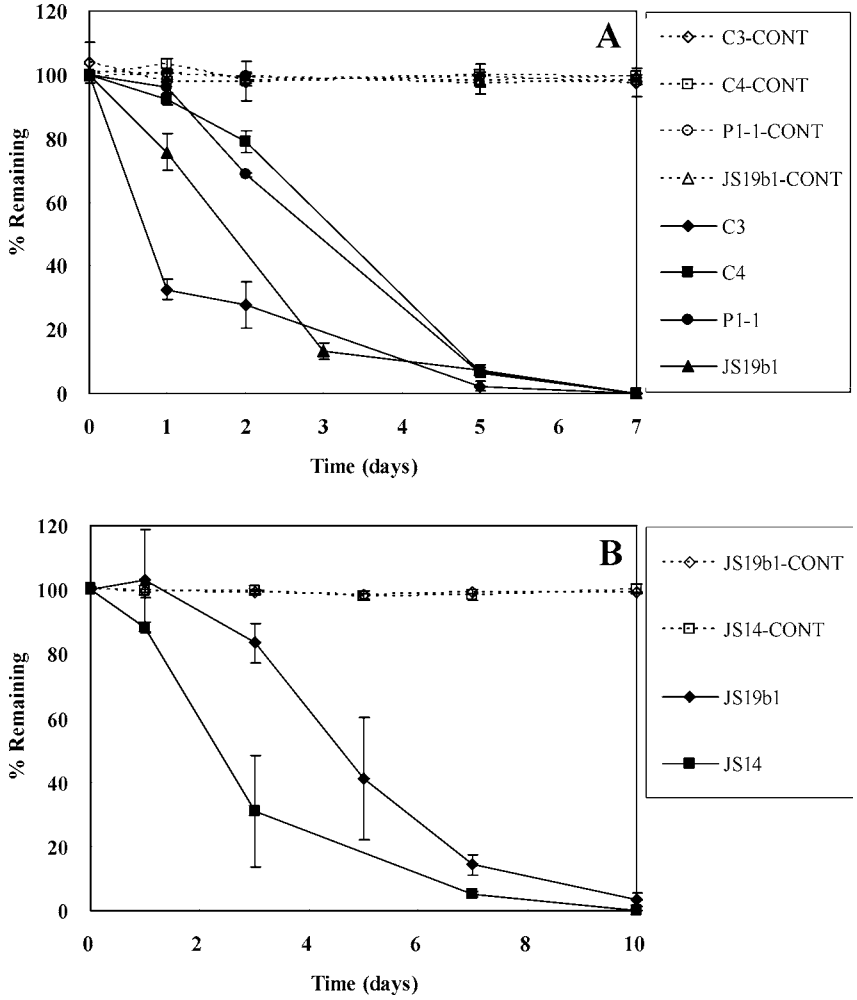

Figure 2. Degradation kinetics of phenanthrene (**A**) and fluoranthene (**B**). Error bars represent the standard deviations of triplicates. CONT stands for control (i.e., boiled dead cells).

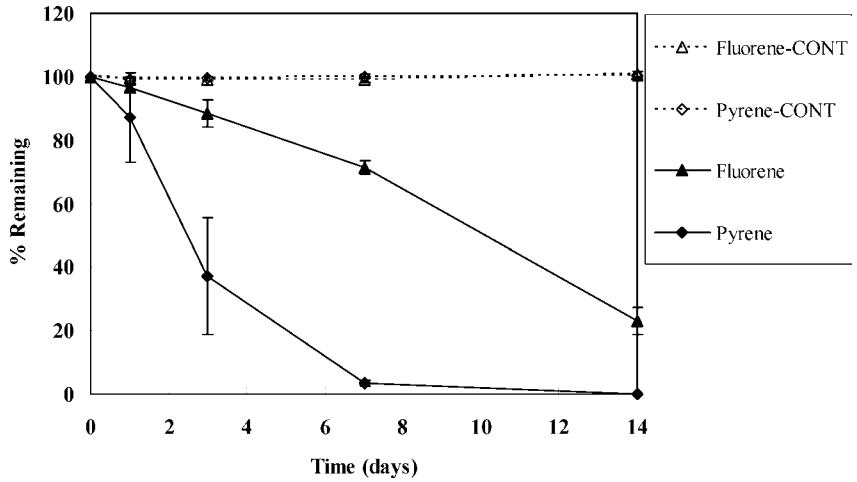

Figure 3. Degradation kinetics of fluorene and pyrene by strain JS19b1. Error bars represent the standard deviations of triplicates. CONT stands for control (i.e., boiled dead cells).

PAH Biodegradation. The degradation of several PAHs was performed with the five strains (C3, C4, P1-1, JS14, and JS19b1) that showed significant PAH-degrading activities. Phenanthrene (40 mg/L) was completely degraded by strains C3, C4, P1-1, and JS19b1 (**Figure 2A**) during 7 days of incubation. The degradation rate of phenanthrene by strain C3 was the fastest in the first day, whereas that of the other strains increased after 1 day of incubation. Strains JS14 and JS19b1 degraded fluoranthene rapidly (**Figure 2B**). The rate of fluoranthene degradation by JS14 was faster than that of JS19b1. Fluoranthene (40 mg/L) was completely degraded by JS14 during 10 days of incubation, but in the case of JS19b1, approximately

3% of fluoranthene remained in 10 days. JS19b1 degraded pyrene (40 mg/L) completely and degraded approximately 77% of fluorene in 14 days (**Figure 3**). JS19b1 utilized fluorene, phenanthrene, fluoranthene, and pyrene as the sole carbon and energy source (**Table 3** and **Figures 2** and **3**).

DISCUSSION

The 12 metabolites detected are probably produced from various PAHs including acenaphthene (**I**), anthracene (**VII**), phenanthrene (**V**, **VI**, **VIII**, and **X**), fluoranthene (**I** and **XI**), and pyrene (**VIII** and **XII**). Metabolites **I** and **XI** are observed in the pathway of fluoranthene degradation by *Mycobacterium* sp. PYR-1 (*33*). Metabolites **II**−**IV** are known to be produced from several PAHs including phenanthrene, fluoranthene, and pyrene (*3*, *33*, *34*). In general, phenanthrene is initially dioxygenated at 1,2-C, 3,4-C, and/or 9,10-C positions. Metabolites **V** and **VI** are probably produced from 3,4- and 1,2-dioxygenations of phenanthrene, respectively (*34*). Recently, Kim et al. (*35*) reported that the K-region dihydrodiols of phenanthrene and pyrene, phenanthrene-9,10-dihydrodiol, and pyrene-4,5-dihydrodiol are oxidized to catechol, which is cleaved by an ortho-ring cleavage dioxygenase to form dicarboxylic acid, for example, 2,2′-diphenic acid (**VIII**) and phenanthrene-4,5-dicarboxylic acid (**XII**). Metabolite **X** may be produced through an intracleavage of 3,4-dihydroxyphenanthrene (*35*). The presence of metabolites **VII** and **IX** in soil indicates microbial biodegradation of anthracene (*36*, *37*).

In this study, a total of 19 bacterial strains (7 α-, 4 β-, and 1 γ-proteobacteria and 7 Gram-positive) were isolated and screened for PAH degradation potential. Five isolates (C3, C4, P1-1, JS14, and JS19b1) degraded PAHs effectively, and their phylogenetic positions are shown in **Figure 1**, which was constructed by the analysis of 16S rRNA gene sequences. It has been reported that PAH degradation in soil is dominated by a limited number of taxonomic groups of bacterial strains such as *Sphingomonas*, *Burkholderia*, *Pseudomonas*, and *Mycobacterium* (*38*). Many *Mycobacterium* species apparently degrade low and high molecular weight PAHs (*3*, *14*, *36*). However, to our knowledge, no *Mycobacterium* species has been reported for the degradation of OPs. *Mycobacterium* strain JS19b1 showed potential to degrade temephos, diazinon, and pirimiphos-methyl (**Table 4**) and showed degradation of phenanthrene, fluoranthene (**Figure 2**), fluorene, and pyrene (**Figure 3**). Despite a similar phylogenetic position by 16S rRNA gene sequence (**Figure 1**), strain JS14 has PAH degradation activities different from those of JS19b1. This result indicates that the genus *Mycobacterium* exhibits broad substrate specificity.

A number of *Arthrobacter* species are known to be OP-degraders (*6*, *17*, *18*) similar to strain P1-1 isolated in this study. Samanta et al. (*39*) reported that the strain *Arthrobacter sulphurreus* RKJ4 degraded phenanthrene through the formation of *o*-phthalic acid and protocatechuic acid. However, little is known about PAH degradation by the *Arthrobacter* and *Sinorhizobium* species. Strains P1-1 and C4 isolated in this study, which are the closest to *Arthrobacter* sp. CAB1 and *Sinorhizobium* sp. HF6, respectively, are able to utilize phenanthrene as the sole carbon and energy source (**Figure 2A**). They are potentially useful in PAH bioremediation in terms of expanding PAH-degraders. Low identity match (96%) of 16S rRNA gene sequences between *Arthrobacter* sp. CAB1 and P1-1 suggests that *Arthrobacter* sp. P1-1 may be a new species (**Table 3**). One *Burkholderia* species is known as a PAH degrader (*8*). Strain C3 degrades approximately 65% of phenanthrene in just 1 day, which suggests an efficient PAH uptake system during the initial exponential growth phase (*38*) and an effective dioxygenase system.

In conclusion, five distinct strains (C3, C4, P1-1, JS14, and JS19b1) isolated and characterized are powerful PAH-degraders. Four isolates (C3, C4, P1-1, and JS19b1) utilize phenanthrene as a sole carbon and energy source. JS14 and JS19b1 degrade fluoranthene. Interestingly, JS19b1 is capable of degrading fluorene, phenanthrene, fluoranthene, and pyrene. Detection of 12 probable PAH metabolites suggests that the isolated bacteria are PAH-degraders in the field. P1-1, JS14, and JS19b1 are able to degrade several OPs. P1-1 can degrade carbofenothion, chlorfenvinphos, diazinon, fonofos, and pirimiphos-methyl. JS14 can transform chlorfenvinphos and diazinon. JS19b1 can degrade diazinon, pirimiphos-methyl, and temephos. These isolates can be potentially useful in PAH and pesticide bioremediation.

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