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Genetic and Phenotypic Diversity of Fenitrothion-Degrading Bacteria Isolated from Soils

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Twenty-seven fenitrothion-degrading bacteria were isolated from different soils, and their genetic and phenotypic characteristics were investigated. Analysis of the 16S rDNA sequence showed that the isolates were related to members of the genera *Burkholderia, Pseudomonas, Sphingomonas, Cupriavidus, Corynebacterium,* and *Arthrobacter.* Among the 27 isolates, 12 different chromosomal DNA fingerprinting patterns were obtained by polymerase chain reaction (PCR) amplification of repetitive extragenic palindromic (REP) sequences. The isolates were able to utilize fenitrothion as a sole source of carbon and energy, producing 3-methyl-4-nitrophenol as the intermediate metabolite during the complete degradation of fenitrothion. Twenty-two of 27 isolates were able to degrade parathion, methyl-parathion, and *p*-nitrophenol, but only strain BS2 could degrade EPN (O-ethyl-O-*p*-nitrophenyl phenylphosphorothioate) as a sole source of carbon and energy for growth. Eighteen of the 27 isolates had plasmids. When analyzed with PCR amplification and dot-blotting hybridization using various specific primers targeted to the organophosphorus pesticide hydrolase genes of the previously reported isolates, none of the isolates showed positive signals, suggesting that the corresponding genes of our isolates had no significant sequence homology with those of the previously isolated organophosphate pesticide-degrading bacteria.

Keywords: Fenitrothion-degrading bacteria, biodegradation, diversity, organophosphorus insecticide

Organophosphorus pesticides have been widely used to control a variety of pests throughout the world [29]. These pesticides, such as fenitrothion (O,O-dimethyl-O-[*p*-nitro-m-tolyl] phosphorothioate), methyl-parathion (O,O-dimethyl-O-*p*-nitrophenyl phosphorothioate), and parathion (O,O-diethyl-O-*p*-nitrophenyl phosphorothioate), inhibit the normal activity of the acetylcholine esterase, resulting in accumulation of acetylcholine at the synapses. This inhibition causes convulsion, paralysis, and finally death for insects and mammals [26]. Excessive use of these pesticides thus may result in hazardous effects in non-target organisms and impact human health due to extreme toxicity with ease of exposure [17]. Therefore, the persistence and fate of these pesticides in the environment have received much attention.

Biodegradation by microorganisms is primarily responsible for elimination of the organophosphate insecticides released to the environment [29]. The bacterial populations were observed to increase proportionally with increasing concentrations of the organophosphate in soils [20]. In fact, many bacterial strains capable of degrading completely or partially organophosphorus pesticides have been isolated from soils [6, 7, 12, 25, 30]. The enzymatic hydrolysis of the phosphoester bond of the organophosphate insecticide substantially reduced its toxicity [29]. Among the organophosphorous pesticides, parathion has been most extensively studied regarding its environmental fate, metabolizing microorganisms, degradation pathways, and degradative genes and enzymes [3, 12, 18–20]. Another organophosphate insecticide, fenitrothion, is also widely used to control pests in agriculture and on golf courses. Fenitrothion is a nitrophenolic pesticide. Under aerobic condition, the major hydrolysis metabolite of fenitrothion is 3-methyl-4-nitrophenol, which is also toxic to many living organisms [32]. However, unlike parathion, microorganisms able to degrade fenitrothion have not been extensively studied, and thus relatively little information is available on the metabolic, genetic, and physiological properties of fenitrothion-degrading microorganisms.

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In this study, we isolated 27 fenitrothion-degrading bacteria from agricultural and golf-course soils, which had been routinely treated with the pesticide for several years. We investigated species diversity by 16S rDNA sequence analysis and REP-PCR patterns of chromosomes, and describe physiological and genetic properties of the isolates on fenitrothion biodegradation.

Materials and Methods

Media and Culture Conditions

All isolated bacteria were cultivated on mineral medium [22] containing fenitrothion at a concentration of 100 ppm (μg/ml). Peptone-tryptone-yeast extract-glucose (PTYG) medium containing (per liter) 0.25 g of peptone (Difco Laboratories, Detroit, MI, U.S.A.), 0.25 g of tryptone (Difco), 0.5 g of yeast extract (Difco), 0.5 g of glucose, 0.03 g of magnesium sulfate, and 0.003 g of calcium chloride was used for strain isolation, and colony production for the repetitive extragenic palindromic PCR (REP-PCR). All cultures were incubated at 28°C, and liquid cultures were aerated by shaking at 150 rpm on a rotary shaker (Vision Co., Bucheon, Korea).

Chemicals

Analytical grade fenitrothion (O,O-dimethyl-O-[*p*-nitro-m-tolyl] phosphorothioate), parathion (O,O-diethyl-O-*p*-nitrophenyl phosphorothioate), 3-methyl-4-nitrophenol, EPN (O-ethyl O-*p*-nitrophenyl phenylphosphorothioate), *p*-nitrophenol, malathion (S-[1.2-dicarbethoxyethyl]-O,O-dimethyl dithiophosphate), chlorpyrifos (O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate), and methyl-parathion (O,O-dimethyl-O-*p*-nitrophenyl phosphorothioate) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Soil Sampling and Isolation of Bacterial Strains

Since 2005, 122 soil samples have been taken from agricultural fields and golf courses at various locations in South Korea. The agricultural and golf-course soils have been routinely treated with fenitrothion for several years. Samples from the top 15 cm of soil were taken, sieved through a 2-mm pore-size sieve, and kept at 4°C prior to use. A 20-g amount of each soil sample was transferred to each 50-ml sterile beaker, treated with fenitrothion dissolved in dichloromethane to a final concentration of 100 μg/g soil and thoroughly mixed. The treated soil was incubated with periodic mixing at room temperature. Five weeks after fenitrothion application, a 1-g soil sample from each beaker was homogenized with 95 ml of a sterile 0.85% saline solution by shaking the preparation at 150 rpm on a rotary shaker. Samples (0.1 ml) of appropriate 10-fold dilutions were inoculated into test tubes containing 3 ml of fenitrothion medium (100 μg/ml) [23]. The tubes were incubated at 28°C for 4 weeks, and degradation of fenitrothion was analyzed by spectrophotometry and reverse-phase HPLC on a μBondapack C18 column (3.9 by 300 nm; Waters, Milford, MA, U.S.A.) and a UV detector set at 270 nm; methanol: 0.1% phosphoric acid (60:40) was used as the eluant. The culture of the terminal positive tube showing substantial cell growth, and less than 20% of the fenitrothion remaining was enriched by two additional transfers into fresh medium. Each enriched culture was streaked onto PTYG agar medium and single colonies were then tested for fenitrothion degradation in fresh fenitrothion medium before strain isolation.

Identification by 16S rRNA Gene Sequence Analysis

Total genomic DNA was extracted from the isolates, and PCR amplification of 16S rRNA genes was performed with 27 mf and 1492r as previously described [11, 13, 14]. The amplified 16S rRNA genes were sequenced using a ABI Prism BigDye Terminator Cycle Sequencing Ready Kit according to the manufacturer's instruction (Perkin-Elmer) with the sequencing primers 27 mf and 519r [1, 14]. Approximately 700 unambiguous nucleotide positions were used for comparison with the data in GenBank using the Basic Local Alignment Search Tool (BLAST) [2]. Sequences from nearest relatives were identified from the Ribosome Database Project (RDP) using the SIMILARITY-RANK program of the RDP [16].

Colony REP-PCR

Colony REP-PCR was performed using BOXA1R as a primer, as described previously [4]. Each isolate was grown on the PTYG agar medium for 24 to 48 h, and a small amount of cells was resuspended in 25 μl of PCR mixture. The cycles used were as follows: 1 cycle as 95°C for 7 min; 35 cycles at 92°C for 1 min, 52°C for 1 min, and 65°C for 8 min; 1 cycle at 65°C for 16 min; and a final soak at 4°C. After the reactions, PCR products were separated by electrophoresis on 1.2% agarose gels. After electrophoresis, the image was photographed with UV transillumination (306 nm).

Degradative Phenotype Analysis

Each strain was grown in PTYG medium. Cells were then harvested by centrifugation 10,000 ×*g* for 10 min at 4°C, washed, and resuspended in 0.85% NaCl solution. Aliquots of suspended cells were inoculated into 15-ml culture tubes, each of which contained 3 ml of mineral medium supplemented with one of the structural analogs at a concentration of 100 μg/ml. The tubes were cultured by reciprocal shaking at 150 rpm at 28°C for 4 weeks, after which the optical density at 600 nm was determined. To determine the degradation of organophosphorus insecticides, 3 ml of acetonitrile was added to a 3-ml culture of the tube, mixed thoroughly, and filtered by Minisart SRP 25. After filtering, the culture was used for the measurement of optical densities at 270 nm (fenitrothion), 274 nm (EPN), 275 nm (methyl-parathion), 283 nm (parathion), and 410 nm (3-methyl-4-nitrophenol).

Axenic Culture Experiment

After growth in PTYG medium, cells were harvested, washed, and resuspended in mineral medium. Aliquots of cells were inoculated into 15-ml tubes containing 3 ml of mineral medium supplemented with 0.3 μl fenitrothion (100 μg/ml) as the sole carbon source at a final density of OD_{600}=0.005. All cultures were incubated at 28°C in the dark on a rotary shaker (150 rpm) for 4 weeks. At specific intervals, two replicate tubes were taken out and used to determine cell growth and the concentrations of fenitrothion and 3-methyl-4-nitrophenol. Cell growths were determined at optical density 600 nm. For the quantification of fenitrothion and 3-methyl-4-nitrophenol, 3 ml of acetonitrile was added to a 3-ml culture of the tube, mixed thoroughly, and filtered by Minisart SRP 25. After filtering, the culture was used for the measurement of optical densities at 270 nm (for fenitrothion) and at 410 nm (for 3-methyl-4-nitrophenol) using spectrophotometry and reverse-phase HPLC. The concentrations of fenitrothion and 3-methyl-4-nitrophenol were calculated using standard curves prepared from the known concentrations of fenitrothion and 3-methyl-4-nitrophenol in the same medium.

Plasmid Detection

For detection of plasmid DNA, cells were lysed using a modified form [9] of the procedure previously published [10]. Plasmid curing of the isolated bacteria was performed using a sodium dodecyl sulfate (SDS) plate and elevated incubation temperature method [5]. A portion of overnight bacterial culture in PTYG liquid medium was subcultured three times into PTYG medium supplemented with SDS from 1% to 0.001% and grown at 37°C for 3 days. The culture was properly diluted and spread onto PTYG plates. Single colonies were checked for fenitrothion degradation ability.

PCR Amplification of the Organophosphate Hydrolysis Gene

The partial gene sequences specific to the fenitrothion degradation pathway were amplified by PCR with specific primers targeted for the *opd* [27, 28], *opd*A [8], *oph* [21], and *opd*B [12] genes. The primers for the *opd* gene were designed based on the conserved gene sequence found in parathion hydrolase genes of *P. diminuta* [27] and *Flavobacterium* sp. [28]: *opd*-f primer, 5'-GCTCTAGCG-GAAAAGGCTG-3' and *opd*-r primer 5'-GTCGATGAGCGCCT-TGT-3'. The primer sequences for the *opd*A gene have been reported in *Agrobacterium radiobacter.* [8]: *opd*A-f primer, 5'-GATCGT-CTGCAGCCAATCGGTACAGGCGATCTG-3' and *opd*A-r primer, 5'-GATCGTAAGCTTTCATCGTTCTGGTATCTTGACGGGGAAT-3'. The primers for *oph* were designed based on the respective gene sequences found in *Arthrobactor* sp. B-5 [21]: *oph*-f primer, 5'-TGGCATCAC-TGTTGTTTCGT-3' and *oph*-r primer, 5'-CAGTCG-ACCGCAGTTC-CAA-3'. The primer sequences for the *opd*B gene have been reported in *Burkholderia* sp. JBA3 [12]: BP/f-1 primer, 5'-CAGCTTCTGGCTTCAGCGC-3' and BP/r-3 primer, 5'-TGCA-CGCTGACACTAGCTGT-3'. The amplification of the *opd*, *opd*A, *oph*, *opd*B genes with the corresponding primers was expected to produce a 641 bp, 1,000 bp, 998 bp, and 944 bp DNA fragment, respectively.

DNA Labeling and Dot-Blot Hybridization

The fenitrothion hydrolase gene probe was PCR-amplified with the previously reported primers (BP/f-1 and BP/r-1) from *Burkholderia* sp. JBA3 [12]. The gene probe was labeled with DIG using DIG-High Prime (Roche Diagnostics, Mannheim, Germany) according to the maunfacturer's instructions. For making denatured DNA, all of the isolates were boiled at 100°C and immediately chilled on ice for 5 min. The denatured DNA fragments were spotted onto a positive charged nylon membrane (BIO-RAD, Hercules, U.S.A.). The DNA on the membrane was blotted by a Bio-DOT Microfiltration Apparatus (BIO-RAD) according to the supplier's instructions. The inverted dot-blot hybridization was analyzed using the DIG hybridization system (Roche Diagnostics).

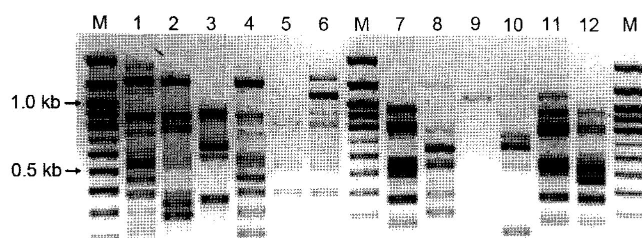

Fig. 1. REP-PCR band patterns of the representative isolates.
Lanes: 1, YI1; 2, KW10; 3, YI10; 4, YI13; 5, YI23; 6, SK2; 7, GS1; 8, GS4; 9, BS2; 10, GJ1; 11, AD1; 12, JH1; M, DNA size marker.

RESULTS

Isolation of Fenitrothion-Degrading Bacteria

Twenty-seven fenitrothion-degrading bacteria, which were capable of utilizing fenitrothion as a sole source of carbon and energy, were isolated through enrichment processes from different agricultural and golf-course soils (Table 1). Among 122 soil samples tested, 95 samples apparently did not show any detectable degradation of fenitrothion during

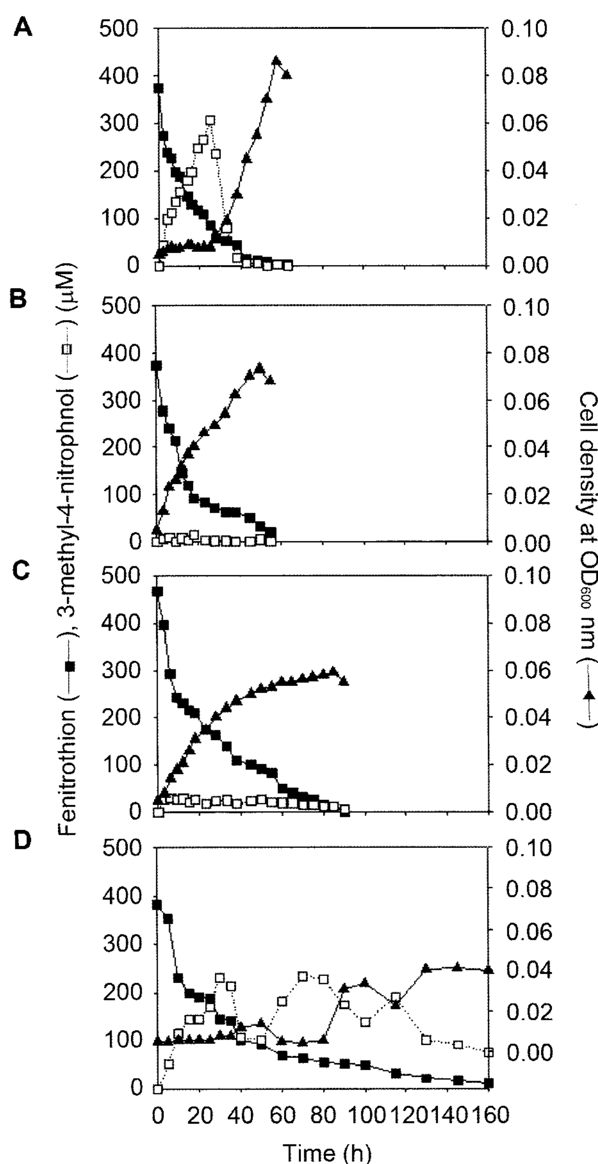

Fig. 2. Degradation of fenitrothion and growth of the representative isolates on fenitrothion.
Disappearance of fenitrothion and growth by the Group **A**, Group **B**, Group **C**, and Group **D** isolates.

Table 1. Nearest relatives of the fenitrothion-degrading isolates based upon 16S rDNA sequence.

Isolate	Growth pattern	GenBank accession no.	Soil sites[a]	GPS sites	Nearest relative	% Similarity (no. of bases compared)
YI1	A	EU483599	Yongin, Kyonggi-do (GC)	37 17 32N/ 127 11 16E	*Burkholderia* sp. PAMU-2.6	99 (727)
KW10	B	EU483611	Gunwi, Kyongsangbuk-do (AF)	36 07 26N/ 128 34 39E	*Burkholderia* sp. SH-1	99 (721)
KW11	B	EU483612	Gunwi, Kyongsangbuk-do (AF)	36 07 28N/ 128 34 39E	*Burkholderia* sp. SH-1	99 (720)
YI10	C	EU483601	Yongin, Kyonggi-do (GC)	37 17 15N/ 127 05 03E	*Pseudomonas* sp. MY1408	99 (723)
HS17	C	EU483603	Hwaseong, Kyongi-do (GC)	37 13 05N/ 127 08 11E	*Pseudomonas sp. MY1408*	98 (724)
AS32	C	EU483605	Ansan, kyonggi-do (GC)	37 19 47N/ 126 52 04E	*Pseudomonas* sp. MY1408	99 (723)
KW3	C	EU483610	Gunwi, Gyeongsangbuk-do (AF)	36 07 25N / 128 34 40E	*Pseudomonas* sp. MY1408	99 (721)
YI13	A	EU483602	Yongin, Kyongggi-do (GC)	37 13 05N/ 127 08 11E	*Burkholderia* sp. TFA1	99 (722)
YI23	A	EU483604	Yongin, Kyongggi-do (GC)	37 08 04N/ 127 08 08E	*Burkholderia* sp. SFA1	99 (721)
SK2	B	EU483613	Saengkuk, chungchongbuk-do (AF)	37 05 35N/ 127 37 45E	*Burkholderia* sp. SJ98	99 (722)
GS1	A	EU483606	Goesan, Chungchongbuk-do (AF)	36 47 24N/ 127 39 29E	*Sphingomonas* sp. I602	100 (722)
GS2	A	EU483614	Goesan, Chungchongbuk-do (AF)	36 47 24N/ 127 39 30E	*Sphingomonas* sp. I602	99 (721)
MW3	A	EU483608	Miwon, chungchongbuk-do (AF)	36 37 48N/ 127 39 29E	*Sphingomonas* sp. I602	99 (721)
MW4	A	EU483609	Miwon, chungchongbuk-do (AF)	36 37 48N/ 127 39 27E	*Sphingomonas* sp. I602	99 (722)
YI7	A	EU483600	Yongin, Kyonggi-do (GC)	36 16 41N/ 127 05 03E	*Sphingomonas* sp. I602	97 (723)
GS4	C	EU483607	Goesan, Chungchongbuk-do (AF)	36 47 24N/ 127 39 28E	*Cupriavidus* sp. WBF7	100 (721)
BS2	E	EU483615	Boseong, Chollanam-do (AF)	34 44 47N/ 127 04 20E	*Corynebacterium cyclohexanicum*	99 (721)
GJ1	E	EU483616	Gwangju, Chollanam-do (AF)	35 05 24N/ 126 51 51E	*Arthrobacter* sp. PAMU-1.14	99 (721)
AD1	D	EU483617	Andong, Gyeongsangnam-do (AF)	36 29 49N/ 128 39 52E	*Sphingomonas* sp. M5-VN5-4W	98 (720)
SC1	D	EU483618	Sancheong, Gyeongsangnam-do (AF)	35 20 30N/ 128 40 52E	*Sphingomonas* sp. M5-VN5-4W	98 (718)
JH1	D	EU483619	Jangheung, Chollanam-do (AF)	34 43 03N/ 126 54 02E	*Sphingomonas* sp. F15	97 (721)
JH2	D	EU483620	Jangheung, Chollanam-do (AF)	34 43 03N/ 126 54 03E	*Sphingomonas* sp. F15	97 (721)
BS1	D	EU483621	Boseong, Chollanam-do (AF)	34 44 47N/ 127 04 20E	*Sphingomonas* sp. F15	97 (719)
BS3	D	EU483622	Boseong, Chollanam-do (AF)	34 44 48N/ 127 04 20E	*Sphingomonas* sp. F15	97 (719)
JH3	D	EU483623	Jangheung, Chollanam-do (AF)	34 43 04N/ 126 54 03E	*Sphingomonas* sp. F15	97 (719)
DY1	D	EU483624	Damyang. Challanam-do (AF)	35 20 53N/ 126 58 26E	*Sphingomonas* sp. F15	97 (723)
DY2	D	EU483625	Damyang. Challanam-do (AF)	35 20 54N/ 126 58 26E	*Sphingomonas* sp. F15	97 (721)

[a](GC),Golf course; (AF), Agricultural field.

5 weeks of the enrichment incubation. The other 27 soil samples exhibited positive degradation of the pesticide, and thus 27 fenitrothion-degrading bacteria were isolated from the 27 different soils (Table 1).

16S rDNA Sequence and REP-PCR Analyses

When analyzed by 16S rDNA sequences, the isolates were found to be related to the genera *Burkholderia, Pseudomonas, Sphingomonas, Cupriavidus, Corynebacterium,* and *Arthrobacter* (Table 1). Most of the isolates were Gram-negative and belonged to the alpha, beta, and gamma subgroups of the Proteobacteria. However, two strains, BS2 and GJ1, were identified as a *Corynebacterium* and *Arthrobacter* species, respectively, which were Gram-positive and belonged to the Actinobacteria. These Gram-positive strains have not been reported as fenitrothion-degrading bacteria in previous studies [6, 24, 30]. Although the fenitrothion-degrading bacteria were isolated from different locations, some isolates were closely related to the same species, such as *Burkholderia* sp. SH-1 (2 isolates), *Sphingomonas* sp. I602 (5 isolates), *Sphingomonas* sp. M5-VN5-4W (2 isolates), *Sphingomonas* sp. F15 (7 isolates), and *Pseudomonas* sp. MY1408 (4 isolates). To investigate the genomic relatedness among the closely related isolates by 16S rDNA sequence analysis, REP-PCR experiment was performed by PCR amplification with the BOXA1R primer [4]. It was revealed that the 27 isolates produced 12 different DNA fingerprint patterns (Fig. 1). Identical REP-PCR patterns were observed for strains KW10 and KW11; for strains YI10, HS17, AS32, and KW3; for strains GS1, GS2, MW3, MW4, and YI7; for strains AD1 and SC1; and for strains JH1, JH2, BS1, BS3, JH3, DY1, and DY2. Since all of the strains were isolated from the different soils, their detection frequencies reflect their ubiquity in the soils examined.

Growth Pattern and Degradation Phenotype of the Isolates on Fenitrothion

To understand the growth patterns of the fenitrothion-degrading isolates, each strain was grown on PTYG and inoculated into fenitrothion minimal medium. The growth and fenitrothion degradation curves of representative strains are shown in Fig. 2. The Gram-negative isolates were classified into four groups according to their growth characteristics on fenitrothion. Group A contained strains YI1, YI13, YI23, and GS1, which degraded and grew rapidly after an initial lag period of about 1 day (Fig. 2A). During the lag period, most of the fenitrothion was converted to 3-methyl-4-nitrophenol, which is a yellow hydrolysis product of fenitrothion. When most of the fenitrothion residues were hydrolyzed, the accumulated 3-methyl-4-nitrophenol began to be utilized by the isolates with concomitant cell growth (Fig. 2A). At about 60 h, 3-methyl-4-nitrophenol had been completely utilized and cell density started to decrease. Group B contained strains KW10 and SK2, which also could degrade fenitrothion rapidly, biodegrading most of the pesticide residues within about 60 h (Fig. 2B). However, the group B strains did not show any lag phase and did not accumulate any intermediate product during degradation of fenitrothion. Group C contained strains YI10 and GS4 (Fig. 2C). The group C strains also did not show any lag phase and did not accumulate any intermediate product substantially, but they degraded and grew some more slowly, compared to the group B strains, taking about 90 h to completely degrade the fenitrothion. Group D consisted of strains AD1 and JH1. The group D strains exhibited intermittent accumulation of the intermediate product, 3-methyl-4-nitrophenol, and took more than 160 h to completely degrade the pesticide (Fig. 2D). The other two Gram-positive isolates, BS2 and GJ1, exhibited somewhat different growth patterns compared with the Gram-negative isolates, and thus are grouped into group E. These two actinobacteria degraded fenitrothion very slowly, taking more than one week for the lag period and more than one month for complete degradation of the pesticide (data not shown). The two actinobacteria did not accumulate any intermediate product substantially during degradation of fenitrothion and their final cell densities were about 0.03 at OD_{600} nm.

Degradative Diversity Analysis

The isolates were grown on PTYG medium, and then examined for their ability to degrade other organophosphates structurally

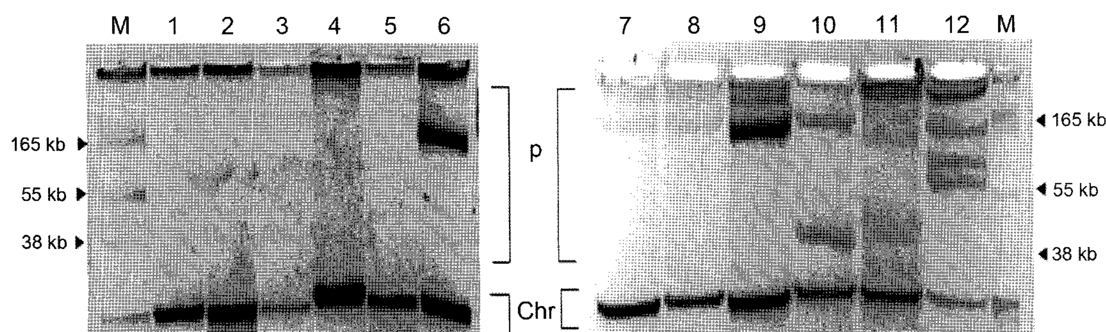

Fig. 3. Plasmid profiles of the isolates.
Lanes: 1, YI1; 2, YI10; 3, YI13; 4, BS2; 5, GS4; 6, GS1; 7, YI 23; 8, SK2; 9, GJ1; 10, KW10; 11, JH1; 12, AD1. P, plasmid band; Chr, chromosomal band.

related with fenitrothion. The substrate utilization abilities of the representative isolates of each REP-PCR group are shown in Table 2. Many of the isolates were able to utilize parathion and methyl parathion, in addition to fenitrothion, but none of them could degrade chlorpyrifos. Among the isolates, strain BS2, which was tentatively identified as *Corynebacterium cyclohexanicum,* was able to degrade and grow on EPN vigorously. Strain YI1, which could degrade only fenitrothion, was the most restrictive in substrate utilization.

Plasmid Detection and Its Relationship to Fenitrothion Degradation Phenotype

When the isolates were subjected to Kado's plasmid detection procedure [10], 7 of the 12 representative isolates exhibited one to three plasmid DNA bands (Fig. 3). These plasmids were stably maintained in cells cultivated for four months, with repeated transfers into Luria-Bertani medium.

To investigate whether the fenitrothion degradative genes are on the plasmid, the isolates containing plasmid were subjected to plasmid curing procedures (Fig. 4). We were able to obtain cured strains from three strains (JH1, KW10, and YI23) among the seven representative isolates. The cured strain of JH1 (CJH1) appeared to have lost both of its two plasmids (Fig. 4, lanes 1 and 2). Strain CJH1 was unable to degrade fenitrothion, and in addition, it exhibited a sudden loss of degradation capabilities of pesticides and pesticide intermediates, such as 3-methyl-4-nitrophenol, methyl parathion, parathion, and *p*-nitrophenol. In the case of strain KW10, the second plasmid was eliminated through the curing process (Fig. 4, lanes 3 and 4), and the resultant cured strain could not degrade fenitrothion. The single plasmid was eliminated from strain YI23 (Fig. 4, lanes 5 and 6), and its cured strain (CYI23) lost all of its degradation capabilities for insecticides including fenitrothion. The results suggested that plasmid DNAs contained the essential genes involved in degradation of fenitrothion and other organophosphate insecticides in these isolates.

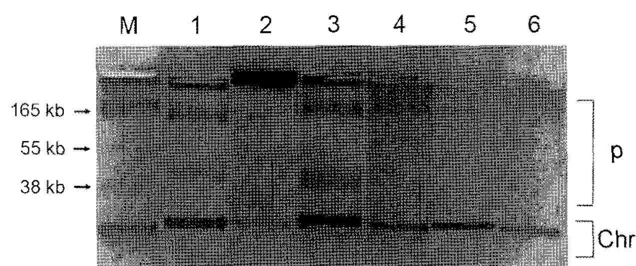

Fig. 4. Plasmid profiles of the representative isolates and their cured strains.
Lanes: 1, JH1; 2, cured strain of JH1; 3, KW10; 4, cured strain of KW10; 5, YI23; 6, cured strain of YI23. P, plasmid band; Chr, chromosomal band.

Genetic Diversity Analysis by PCR Amplification and Dot-Blotting

Since most of the isolates were able to hydrolyze other organophosphorous pesticides structurally related to fenitrothion, we investigated whether the isolates had any sequence homology with organophosphorus hydrolase genes that were previously reported in other bacterial strains. When PCR amplification was performed with various primers targeting for the previously reported genes, such as the *opd* [28, 29], *opd*A [8], *oph* [21], and *opd*B [12] genes, none of the isolates showed any positive DNA bands (data not shown). The absence of homology with the previously reported bacterial strains of fenitrothion hydrolase genes of our isolates was confirmed by the dot-blotting procedure. When dot-blotting was performed for each isolate using

Table 2. Substrate utilization patterns by the fenitrothion-degrading isolates.

Isolates[a]	Substrates[b]						
	Fenitrothion	3-Methy-4-nitrophenol	Methyl parathion	EPN	Malathion	Parathion	*p*-Nitrophenol
YI1	+	+	-	-	-	-	-
KW10	++	+	++	-	-	+	+
YI10	+	+	-	-	-	-	-
YI13	+	+	+	-	-	-	+
YI23	++	+	++	-	-	+	+
SK2	++	+	++	-	-	+	+
GS1	+	+	+	-	-	+	+
GS4	+	+	-	-	-	-	+
BS2	+	+	+	++	-	-	+
GJ1	+	+	+	-	-	+	+
AD1	++	+	++	-	-	+	+
JH1	++	+	+	-	-	+	+

[a]The isolates were grown on PTYG before the test of substrate utilization.
[b]++, Over 95% reduction in peak height as determined by UV scanning and substantial growth (OD600>0.05); +, over 80% reduction in peak height as determined by UV scanning and substantial growth (OD600>0.025); -, below 10% reduction in peak height and scant growth (OD600<0.01).

the specific hydrolase gene probe obtained from the internal sequence of the *oph*B gene, which originated from the strain *Burkholderia* sp.JBA3 [12], none of the isolates showed any detectable signals (data not shown). The results suggested that the hydrolase genes involved in fenitrothion degradation in the isolates might be different from those of the known bacterial strains able to degrade the organophosphate pesticides.

DISCUSSION

We isolated dominant fenitrothion-degrading bacteria from various soils and analyzed their diversity and properties on fenitrothion degradation by using phylogenic, phenotypic, and genotypic analyses. Species identification by 16S rDNA sequence analyses revealed that our isolates were related to members of the genera *Burkholderia, Pseudomonas, Sphingomonas, Cupriavidus, Corynebacterium*, and *Arthrobacter* (Table 1). Although several bacterial strains have been isolated as organophosphorus pesticide-degrading microorganisms in previous studies [6, 7, 12, 15, 24, 26, 31], little information is available on the genetic and phenotypic properties of fenitrothion-degrading bacteria. Tago *et al.* [30] investigated the diversity of fenitrothion-degrading bacteria, but in their study, all of the isolates were assigned to the species of the Gram-negative bacteria, such as *Rhizobium*, *Burkholderia*, *Cupriavidus*, and *Pseudomonas*. Thus, the Gram-positive strains of *Corynebacterium* and *Arthrobacter* species isolated in our study are new types of microorganisms observed to be able to degrade fenitrothion for the first time.

The Gram-negative isolates were classified into four groups based on their degradation and growth characteristics (Fig. 1). The strains of Group A, which belonged to the genera *Burkholderia* and *Sphingomonas*, first hydrolyzed most of the fenitrothion before they utilized the intermediate product 3-methyl-4-nitrophenol as the carbon and energy source. Many of the Group A strains contained a plasmid and showed moderate versatility in substrate utilization. The Group B strains, which belonged to the genus *Burkholderia*, rapidly degraded fenitrothion without accumulating the intermediate metabolite 3-methyl-4-nitrophenol. All of the Group B strains contained a plasmid and exhibited relatively high versatility in substrate utilization. The Group C strains, which belonged to the genera *Pseudomonas* and *Cupriavidus*, degraded and grew on fenitrothion at a moderate rate without accumulating the intermediate metabolite. All of the Group C strains had no plasmid DNA and exhibited restrictive versatility in substrate utilization. The Group D strains, which belonged to the genus *Sphingomonas*, degraded fenitrothion relatively slowly and showed intermittent accumulation of the intermediate metabolite 3-methyl-4-nitrophenol during their growth on the pesticide. All of the Group D strains contained plasmid DNA and exhibited relatively high versatility in substrate utilization. The other two Gram-positive strains, which belonged to the genera *Corynebacterium* and *Arthrobacter*, formed the fifth group E. These strains did not have plasmids and grew on fenitrothion more slowly than any other Gram-negative isolates without accumulating the intermediate metabolite. *Corynebacterium cyclohexanicum* strain BS2 showed more vigorous growth on EPN than fenitrothion, and thus further investigation on its EPN degradation might be useful for understanding the catabolic properties of this Gram-positive strain.

The organophosphorus insecticides are known to be highly toxic to mammals and wild animals [29]. Therefore, degradation and detoxification of organophosphates extensively used in agriculture are very important to protect non-target organisms in the environments. The rapid degradation property of the Group A and B strains would be useful for rapid breakdown of fenitrothion residues contaminated in soil. It was reported that although the phosphorous insecticides had the same phosphorothionate bond, the side chains had a significant effect on the degradation of the organophosphates [15]. Thus, in this study, none of the fenitrothion-degrading isolates could degrade malathion, which contains an unusual phosphorodithionate bond, and chlorpyrifos, which has a pyridinol ring, because of their different chemical structures to fenitrothion or its metabolites. It is of note that the elimination of plasmid DNAs results in the complete loss of the degradation capabilities of the organophosphate insecticides in the cured isolates. This suggests that the plasmids contain the essential genes involved in degradation of the organophosphates, and the same or similar degradative genes are commonly engaged in the catabolic reactions of the closely related organophosphorus insecticides. Since hydrolysis of the phosphoester bond of organophosphates substantially reduces their toxicity, diverse organophosphorus hydrolase genes have been studied and isolated from several catabolic microorganisms [8, 9, 12, 27, 28]. All of our isolates also had organophosphorus hydrolase activities, which converted fenitrothion to 3-methyl-4-nitrophenol, a yellow intermediate produced during degradation of fenitrothion by the isolates. However, when analyzed with PCR amplification and dot-blotting, none of our isolates exhibited any significant homology with the previously reported bacterial strains able to degrade various organophosphorus insecticides. The results suggested that the isolates of this study possibly have new types of organophosphorus hydrolase genes. Further investigation on the new hydrolase genes would reveal divergence of sequences of organophosphorus hydrolase genes among the fenitrothion-degrading bacteria.

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