Long term effect of methylparathion contamination on soil microbial community diversity estimated by 16S rRNA gene cloning

Ruifu Zhang · Jiandong Jiang · Ji-Dong Gu · Shunpeng Li

Abstract The long-term effects of methylparathion contamination on the diversity of soil microbial community was investigated by a culture-independent approach using small subunit ribosomal RNA (SSU rRNA) gene-based cloning. Microbial DNA extracted from both the control soil sample and methylparathion contaminated soil sample was subjected to PCR amplification with primers specific for bacterial 16S rRNA gene sequences. From the PCR amplification product, clone libraries were constructed for both samples. Phylotypes were defined by performing a restriction fragment length polymorphism analysis of 16S rRNA gene sequences with the enzymes *Rsa*I and *Hha*I. A total of 603 phylotypes were identified among the 16S ribosomal DNA (rDNA) clones, the phylotype richness, frequency distribution (evenness) of the two clone libraries were compared by using a variety of diversity indices. Phylogenetic analysis of the sequences of the dominant phylotypes revealed that the bacterial communities changed noticeably. In the control soil, the dominant bacterial groups included a member of a novel bacterial division, the bacillus genus, and a member of α-proteobacteria, while in methylparathion contaminated soil, the dominant phylotypes were replaced by a member of the flexi-bactera-cytophaga-bacteroides division and two members of the γ-proteobacteria subdivision. This is the first report of the long-term effects of methylparathion (one of the major pesticides widely used in developing countries) on soil microbial community diversity and structure by a culture-independent method, and provides the evidences to assess the long-term environmental toxicological effects of methylparathion from the microbial community viewpoint.

Keywords Long-term methylparathion contamination · Microbial community · Culture-independent method · Dominant phylotype · Novel bacterial division

R. Zhang · J. Jiang · S. Li (✉)
Department of Microbiology, Key Laboratory for Microbiological Engineering of Agricultural Environment of Ministry of Agriculture, Nanjing Agricultural University, 6 Tongwei Road, Nanjing, Jiangsu 210095, People's Republic of China
e-mail: lsp@njau.edu.cn

J.-D. Gu · R. Zhang
Laboratory of Environmental Microbiology and Toxicology, Department of Ecology & Biodiversity, The University of Hong Kong, Kadoorie Biological Sciences Building, Pokfulam Road, Hong Kong SAR, People's Republic of China

Introduction

Organophosphate pesticides are a group of highly toxic agricultural chemicals that are widely used for plant protection. Up to the present, organophosphate pesticides, such as parathion, methylparathion, and methamidophos, are still extensively used worldwide especially in the developing countries despite their high toxicity. The environmental chemistry, fate, toxicology of methylparathion have been extensively studied (Clark et al. 2002; Kolosova et al. 2003; Luchini et al. 2000; Luo et al. 2003; Tsakiris et al. 2002; Wagner et al. 2003). However, the soil microbial communities exposed to long-term methylparathion contamination has not been investigated. In general, the effect of pesticide on soil microbial communities has often been

studied by conventional methods based on either cultivation techniques or metabolic activities (Somerville and Greaves 1987; Wardle and Parkinson 1990). It is well known that more than 90% of the microorganisms existing in nature are refractory to selective enrichment cultures (Ward et al. 1990). Another limitation of these approaches is that any departure from the original environmental parameters during cultivation can alter the result of community structure due to the introduction of new selective pressure. To overcome these drawbacks, molecular biological techniques are increasingly employed in the analysis of microorganisms in their natural habitats. In this context, analysis of the 16S rDNA is by far the most widely used approach in the last decade (Amann et al. 1995).

In this paper, we described the microbial community diversity of a soil that has been contaminated by methylparathion for more than 20 years. A culture-independent approach based on the PCR amplification of 16S rDNA was used in the present study.

Materials and methods

Soil sampling and characterization

The contaminated soil (CS) was sampled in the May 2003 from the field site near a methylparathion-producing plant in Shan Dong province, P. R. China. This field site had received the production wastewater for more than 20 years. About 500 g of soil was sampled from 0 to 10 cm depth and the weed and visible roots were removed as much as possible. Another soil (S) sampled from a nearby field with similar vegetation but without contamination was used as the Control. The soil was frozen and stored at –70°C prior to DNA extraction. Selected properties of these two soil samples are shown in Table 1. Both soil are sandy loam with similar parameters except that higher contents of total phosphorus and available phosphorus were observed in the contaminated soil.

Extraction of nucleic acids from soil

Nucleic acids were extracted by the procedures modified from those described by Zhou and Tiedje (1996).

Five gram of soil and 13.5 ml of DNA extraction buffer (100 mM Tris–HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB) and 100 μl of proteinase K (10 mg ml^{-1}) were mixed in a tube at 37°C by horizontal shaking at 225 rpm. After 30 min of shaking, 1.5 ml of 20% SDS was added, and the mixture was incubated at 65°C in a water bath for 2 h with gentle end-over-end inversions every 15–20 min, then the samples were submitted to three times freezing at –70°C and thawing at 65°C to lyse some Gram-positive bacteria. After centrifugation of the sample at 6,000g for 10 min, the supernatants were collected and transferred into a 50-ml centrifuge tube. The soil pellets were extracted two more times by adding 4.5 ml of the extraction buffer and 0.5 ml of 20% SDS, vortexing for 10 s, incubating at 65°C for 10 min, and centrifugating as before. Supernatants from the three cycles of extractions were combined and mixed with an equal volume of chloroform–isoamyl alcohol (24:1,v/v). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1 h. After centrifugation at 16,000g for 20 min at room temperature, the pellet was washed with cold 70% ethanol, and resuspended in sterile deionized water, to give a final volume of 500 μl. The crude DNA was purified by dialysis bag electric elution. The DNA yield was estimated by using ethidium bromide-stained agarose gels with lambda DNA as a calibration standard.

PCR amplification of small-subunit rRNA genes

Small-subunit rRNA genes were amplified from the purified DNA by primers conserved among all known bacteria. The primers used to initiate PCR were forward primer pA(5′-AGAGTTTGATCCTGGCT-CAG-3′; *Escherichia coli* bases 8–27) (Edwards et al. 1989) and reverse primer PC5B(5′-TACCTTGT-TACGACTT-3′; *E. coli* bases 1507–1492) (Wilson et al. 1990). Amplified reaction mixtures used for library generation contained 5 μl deoxynucleoside triphosphate mixture (20 mM), 5 μl 10 × Taq DNA polymerase buffer, 6 μl MgCl$_2$ (25 mM), 2 μl of each primer (25 pmol μl^{-1}), 3 μl of soil DNA template

Table 1 Selected characteristics of the soil samples

Soil samples	Parameter								
	Moisture content (%)	pH	Organic matter (g kg^{-1})	Total N (g kg^{-1})	Available N (mg kg^{-1})	Total P (g kg^{-1})	Available P (mg kg^{-1})	Total K (g kg^{-1})	Available K (mg kg^{-1})
S	20.0	7.91	17.28	0.98	82.39	0.20	4.04	18.17	97.50
CS	17.4	7.20	20.74	1.30	88.01	1.03	57.46	16.39	87.50

(20 ng μl^{-1}), 0.5 μl Taq polymerase (5 U μl^{-1}) in a final reaction volume of 50 μl. PCR was conducted with a Perkin-Elmer 9600 thermal cycler as follows: 2 min of denaturation at 94°C, followed by 30 cycles of 30 s at 94°C (denaturation), 30 s at 50°C (annealing), and 60 s at 72°C (extension), with a final 5 min 72°C extension step after cycling was complete. For each sample, 10 replicate reactions were conducted and then pooled to neutralize the effects of bias in any single thermal cycling reaction.

The 1.5-kb fragment was purified from primers by electrophoretically separating the fragment on 0.75% agarose gels in 1 × Tris–acetate–EDTA buffer. The desired fragment was visualized by staining with ethidium bromide and excised from the gel, and DNA was purified using a cleanup kit (TaKaRa Biotechnology (Dalian) Co. Ltd) as recommended by the manufacturer.

Small-subunit rRNA gene libraries

Clone libraries of small-subunit rRNA gene copies from both samples were generated. The 1.5-kb PCR product was ligated into the pMD 18-T plasmid vector (TaKaRa Biotechnology (Dalian) Co. Ltd) by using T4 DNA ligase and incubated at 16°C overnight. Recombinant plasmids were transformed into *E. coli* DH5α competent cells, and colonies containing plasmids with insert were identified by blue–white color selection on agar plates. Four hundred fifty clones for each sample were recovered and stored as glycerol stocks at –70°C. Each clone was designated as S 1–450 or CS 1–450 for the control or contaminated soil, respectively.

RFLP analysis of 16S rDNA

16S rDNA sequences of 16S rDNA clones were reamplified directly from glycerol stock preparations with pMD 18-T plasmid vector specific primers, BcaBEST primer RV-M (5′-GAGCGGATAACAATTTCACA CAGG-3′), BcaBEST PrimerM13-47 (5′-CGCCAGGG TTTTCCCAGTCACGAC-3′). Amplified reaction mixtures contained 5 μl deoxynucleoside triphosphate mixture (20 mM), 5 μl 10 × Taq DNA polymerase buffer, 6 μl MgCl$_2$ (25 mM), 1 μl of each primer (25 pmol μl^{-1}), 2 μl of glycerol stock preparations, 5 μl Tween 20 (10%), 0.5 μl Taq polymerase (5 U μl^{-1}) in a final reaction volume of 50 μl. PCR was conducted with a Perkin-Elmer 9600 thermal cycler as follows: 5 min of denaturation at 95°C, followed by 30 cycles of 30 s at 94°C (denaturation), 30 s at 58°C (annealing) and 60 s at 72°C (extension), with a final 5 min 72°C extension step after cycling was complete.

Follow PCR amplification, 10 μl of 16S rDNA from each of the clones was digested separately with 5 U of *Hha* (GCG'C) and 4 U of *Rsa* (GT'AC) (TaKaRa Biotechnology (Dalian) Co. Ltd) as recommended by the manufacturer. The digests were electrophoresed in 3% Metaphor (FMC Corporation) gels with TBE buffer (Sambrook et al. 1989). The gels were stained with ethidium bromide and then photographed under UV light.

DNA fragment sizes were determined by using TANON gel analysis software. Digitized restriction patterns were sorted with Microsoft Excel, version 5.1 (Microsoft Corp), and compared to group similar patterns. Groups of indistinguishable patterns were recorded. Each phylotype was defined as a group of sequences that had indistinguishable *Hha* and *Rsa* restriction patterns.

DNA sequencing

Recombinant plasmids having 1.5-kb inserts were purified using PCR cleanup kit (TaKaRa Biotechnology (Dalian) Co. Ltd) as recommended by the manufacturer. Sequences were determined at Takara Biotechnology (Dalian) Co. Ltd.

DNA distance analysis

To determine the average level of 16S rDNA sequence similarity of organisms that produced the same RFLP pattern, partial 16S rDNA sequences of three representatives of a RFLP group were compared. Similarity values were calculated by using the DNADIST program in PHYLIP (version 3.6; distributed by J. Felsenstein, University of Washington, Seattle). Each sequence was submitted to the CHECK-CHIMERA program of the Ribosomal Database Project (RDP-II) (Cole et al. 2003) to detect the presence of possible chimeric artifacts (Kopczynski et al. 1994; Qui et al. 2001; Wang and Wang 1996; 1997). Sequences were initially analyzed by using BLAST (National Center for Biotechnology Information) and SIMILARITY_RANK (RDP) (Cole et al. 2003) searches to determine the closest available database sequences. In addition, sequences representative of those major bacterial divisions were already included in the data set. Alignment of the final data set was accomplished by using Clustal X (Thompson et al. 1997). Neighbor-joining distance matrix analysis of sequences was performed by using the DNADIST and NEIGHBOR programs of the PHYLIP package (version 3.6; distributed by J. Felsenstein, University of Washington, Seattle). Phylogenetic trees were constructed by the

neighbor-joining method. Bootstrap analysis was performed with 100 resampled data sets.

Nucleotide sequences accession numbers

The nucleotide sequences determined in this study have been deposited in the NCBI database under accession no. AY382609 to AY382614.

Results

DNA extraction and PCR amplification from soil samples.

DNA extracted from the two soil samples was predominantly of high molecular weight (Fig. 1) and was suitable for use as PCR template, with minimal chance of introducing chimeric fragments due to sheared DNA. Yields of crude DNA from the S and CS samples were 13.01 μg g^{-1} dry soil and 12.23 μg g^{-1} dry soil, respectively. The 1.5-kb 16S rDNA fragments were amplified from both sets of samples.

RFLP phylotypes

Each phylotype from the clone libraries was defined by RFLP patterns obtained from Hha- and Rsa-digested 16S rDNA. A total of 603 phylotypes were identified through the comparison of the Hha–Rsa RFLP

Fig. 1 DNA isolated from S and CS soil samples. Lane A, Lamda DNA/Hind III marker, Lane B, S soil, Lane C, CS Soil

patterns of 823 clones (Table 2). Phylotypes defined by RFLPs obtained with these two tetrameric enzymes have a median sequence similarity value of 95.6% (Moyer et al. 1996). We determined the phylogenetic discrimination of the Hha–Rsa phylotypes in our collections by comparing partial 16S rDNA sequences of three representatives from one randomly selected RFLP patterns, the average level of similarity of 16S rDNA sequences having identical RFLP patterns was 97.0%. A total of 232 unique patterns were identified from 277 clones in S sample, representing 74.4% of the total patterns and 69.4% of the total clones, respectively. While 211 unique patterns were identified from 285 clones in CS sample, representing 72.5% of the total patterns and 67.2% of the total clones, respectively.

Phylotype richness and distribution

The number of phylotypes (richness) and the frequency distribution of the phylotype (evenness) in each of the clone libraries were evaluated by using a variety of standard diversity indices. As shown in Table 3, the phylotype richness in the methylparathion polluted soil sample was lower than that of control, the Margalef indices (R) in S and CS sample were 51.929 and 47.936, respectively. When either the Shannon–Weiner index (H) or Simpson's index (D) was used, the methylparathion polluted soil sample showed lower diversity values than the control soil sample.

Only 80 phylotypes were found in both clone libraries, representing 25.6 and 27.5% of the total phylotypes of S clone library and CS clone library, respectively. As a result, the similarity value ([(2 × common types)/(total types)] × 100) of the two libraries was very low (26.5%).

Comparison of the main phylotypes in these two soils

Nearly full length sequences of the representatives of the three mainly phylotypes in each clone library were sequenced. Phylogenetic analysis showed that the bacterial communities changed noticeably. In the control soil, the dominant bacterial group may be a member of a novel bacterial division, which still has no cultivated representatives. Similarities between this sequence and those of the most closely related cultivated organisms were less than 80%. This level of similarity is comparable to that between the presently recognized bacterial divisions. The second dominant phylotype belonged to the bacillus genus, a member of the Gram-positive division. Another dominant phylotype appear to be a new genus of the α-proteobacteria,
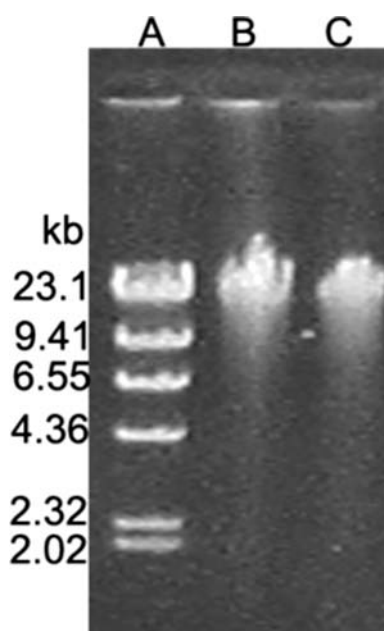
Table 2 Number of *Hha–Rsa* RFLP phylotypes in 16S rDNA clone libraries from two soils

Clone libraries	Parameter				
	Total no. of patterns	No. of unique patterns[a]	% of unique patterns	No. of rare phylotypes[b]	No. of common phylotype[c]
S	312 (399)[d]	232 (277)	74.4	255	80 (122)
CS	291 (424)	211 (285)	72.5	207	80 (139)

[a] Unique patterns were found in only one clone library

[b] Rare phylotypes were phylotypes that were found only one clone

[c] Common phylotypes were phylotypes that were found in both clone library

[d] The values in parentheses are the numbers of clones

Table 3 Diversity indices based on *Hha–Rsa* RFLP phylotypes in 16S rDNA clone libraries from two soils

Clone libraries	Diversity indices			
	Richness (R)[a]	Shannon–Weiner index (H)[b]	Evenness (E)[c]	Simpson index (D)[d]
S	51.929	8.142	0.983	0.995
CS	47.936	7.811	0.943	0.951

[a] R was calculated as follows: $d = (S–1)/\ln N$ where S is the total number of phylotypes, N is the total number of clones

[b] H was calculated as follows: $H = -\Sigma(p_i)(\log_2 p_i)$ where p_i is the proportion of the ith phylotype

[c] Evenness (E) was calculated from H as follows: $E = H/H_{max}$, where $H_{max} = \log_2(S)$ and S is the total number of phylotypes

[d] D was calculated as follows: $D = 1–\Sigma(p_i)^2$

because similarity between this sequence and the most closely related cultivated organism (*Sphingomonas asaccharolytica*) was only 94%. Clones closely related to this clone of this division have been isolated from different regions in the word (Fig. 2). While in methylparathion-contaminated soil, the dominant phylotypes were replaced by a member of the flexibactera-cytophaga-bacteroides division and two members of the γ-proteobacteria subdivision. These twoγ-proteobacteria subdivision phylotypes were closely related to the *Pseudomonas stutzeri* and *Pseudomonas putida*, the similarities were 99 and 97%, respectively. The distribution frequencies of these dominant phylotypes in the two libraries were shown in Table 4.

Discussion

Due to the bias of PCR, the community composition, richness, and structure may be distorted. We assumed that the bias operated uniformly for our two soil samples and that therefore the soil samples could be compared.

The similarity value of S clone library and CS clone library was very low (26.5%), the effect of methylparathion on the soil bacterial community structure was one possible reason, however, the low value obtained from standard similarity comparisons may have been largely the result of both libraries containing a large proportion of rare phylotypes that had low probability of being detected. Indeed, if we calculated the similarity value of the two clone libraries with the total clone number contained by the common types, the similarity value will increase to 31.7%.

In the present study, one of the dominant phylotypes in the control soil belongs to a novel division, which has no cultivated representative. It may distantly relate to the recently recognized bacterial division *Acidobacterium*. Studies from other soils of different regions also confirmed the presence of this novel division (Fig. 2). Cheryl (1997) performed a phylogenetic survey of microbial species present in two soils from northern Arizona of America, phylogenetic analysis of the sequences from the 16S rDNA clone libraries revealed that 46% of the analyzed sequences fell into this novel division. Ludwig (1997) constructed 16S rDNA libraries from soil samples taken periodically from a field with genetically modified plants. Sequence analyses of the cloned rDNAs indicated that 140 of them clustered apart from known bacterial phyla. Based on 31 full sequences this new division could be defined. Macrae et al. (2000) studied the bacterial diversity recovered from the rhizosphere of oilseed rape (*Brassica napus*), Phylogenetic analysis of the sequences revealed that 37% of the clones fell within this division. Ellis et al. (2003) investigated the bacterial community structure of the metal contamination soil in English with a variety of approaches and

Fig. 2 The phylogenetic tree of dominant bacterial rDNA clones from the two soil communities. Sequences from soil clones were analyzed with the most closely related sequences (include soil clones from other studies deposited in the database) obtained from the database, other representatives of the major bacterial groups. The phylogenetic tree was constructed by neighbor-joining method with PHYLIP package and *Bacteroides acidofaciens* (AB21156) was located as the root. Bootstrap analysis was performed with 100 resampled data sets, and bootstrap values more than 50 were indicated. Single asterisk represented soil clones in the database from other studies, clones from this study designated with double asterisks
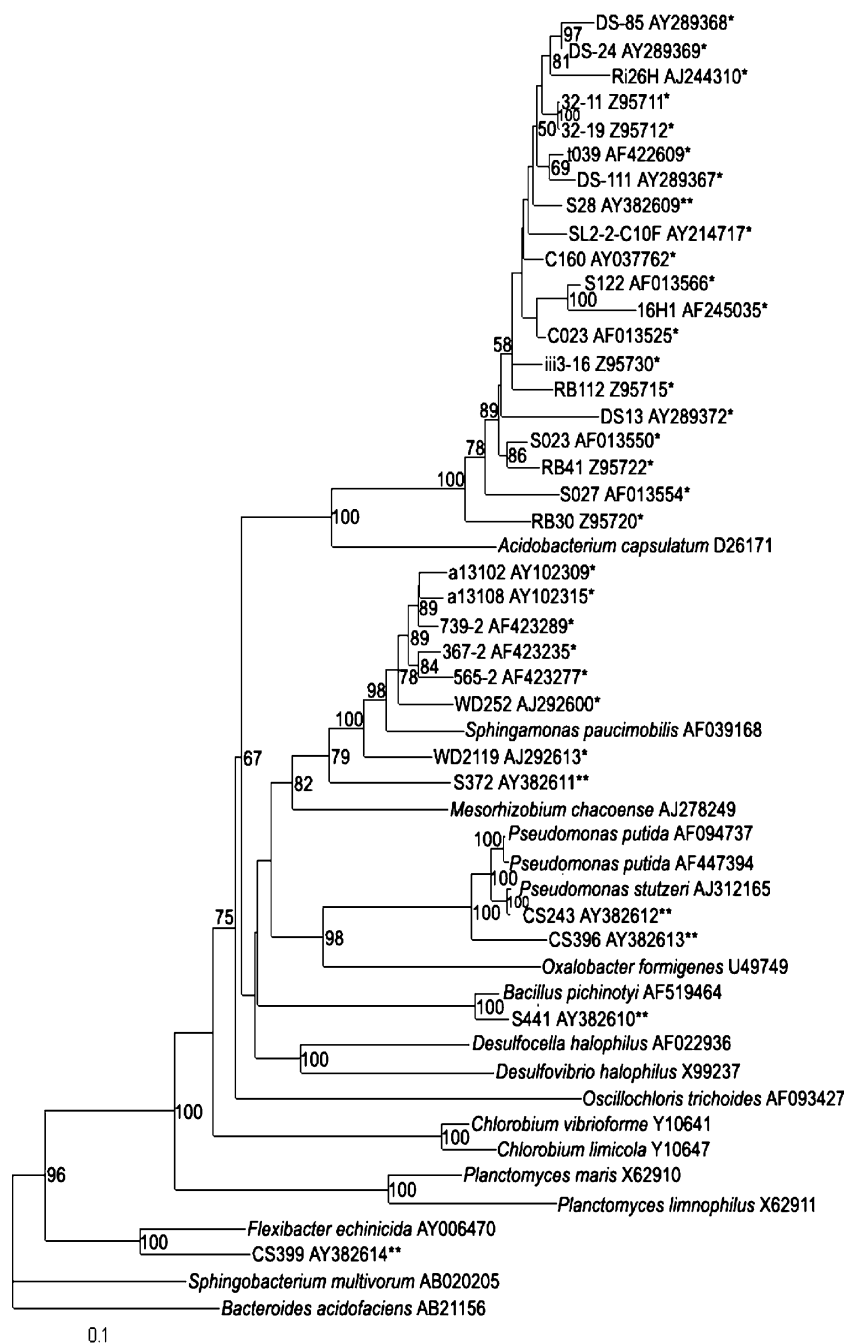
Table 4 Distribution frequency of the dominant phylotypes in the two libraries

Clone	Phylogenetic groups	Number of clones in library	% of total clones in library
S28	Uncultured novel division	10	2.5
S441	Gram-positive	6	1.5
S372	α-Proteobacteria	4	1.0
CS399	Flexibactera-cytophaga-bacteroides	7	1.7
CS396	γ-Proteobacteria	6	1.4
CS243	γ-Proteobacteria	5	1.2

confirmed the present of this division. Zhou et al. (2003) assessed the bacterial phylogenetic diversity of two humid regions sandy surface soils in Delaware (USA) and confirmed this novel division. Therefore, this division appears to be widely distributed in natural environments.

In methylparathion-polluted soil, the dominant phylotype included two members of γ-proteobacteria, which were closely related to the *Pseudomonas stutzeri* and *Pseudomonas putida*, respectively. This could be attributed to the powerful capability of *Pseudomonas* of degrading organic pollutants. To date, *Pseudomonas* constituted a great proportion of the degrading micro-organisms, López et al. (2005) isolated bacteria strains with pesticide removal capacity from an oligotrophic lake water sample amended with nine pesticides (include mathylparathion), The genetic identification of these strains showed their affiliation to *Pseudomonas pseudoalcaligenes*, *Micrococcus luteus*, *Bacillus* sp. and *Exiguobacterium aurantiacum*. Our previous investigation of the methylparathion-degrading bacteria involved the genus of *Pseudaminobacter*, *Achromobacter*, *Brucella* and *Ochrobactrum* (Zhang et al. 2005). It is possible that methylparathion degrading bacteria of *Pseudomonas* can be isolated from the contaminated soil with extensive isolation and identification.

References

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Cheryl RK, Susan MB, Joseph DB (1997) Diverse uncultivated bacterial groups from soils of the arid southwestern united states that are present in many geographic regions. Appl Environ Microbiol 63:3614–3621

Clark JM, Bing-Canar J, Renninger S, Dollhopf R, El-Zein J, Star D, Zimmerman D, Anisuzzaman A, Boylan K, Tomaszewski T, Pearce K, Yacovac R, Erlwein B, Ward J (2002) Methyl parathion in residential properties: relocation and decontamination methodology. Environ Health Perspect 6(Suppl):1061–1070

Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q, Kulam SA, Chandra S, McGarrell DM, Schmidt TM, Garrity GM, Tiedje JM (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 31:442–443

Edwards U, Rogall T, Blocker H, Emde M, Bottger EC (1989) Isolation and direct complete determination of entire genes. Nucleic Acids Res 17:7843–7853

Ellis RJ, Morgan P, Weightman AJ, Fry JC (2003) Cultivation-dependent and -independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. Appl Environ Microbiol 69:3223–3230

Kolosova AY, Park JH, Eremin SA, Kang SJ, Chung DH (2003) Fluorescence polarization immunoassay based on a monoclonal antibody for the detection of the organophosphorus pesticide parathion-methyl. J Agric Food Chem 51:1107–1114

Kopczynski ED, Bateson MM, Ward DM (1994) Recognition of chimeric small-subunit ribosomal DNAs composed of genes from uncultivated microorganisms. Appl Environ Microbiol 60:746–748

López L, Pozo C, Rodelas B, Calvo C, Juárez B, Martínez-Toledo MV, González-López J (2005) Identification of bacteria isolated from an oligotrophic lake with pesticide removal capacities. Ecotoxicology 14:299–312

Luchini LC, Peres TB, de Andrea MM (2000) Monitoring of pesticide residues in a cotton crop soil. J Environ Sci Health 35:51–59

Ludwig W, Bauer SH, Bauer M, Held I, Kirchhof G, Schulze R, Huber I, Spring S, Hartmann A, Schleifer KH (1997) Detection and in situ identification of representatives of a widely distributed new bacterial phylum. FEMS Microbiol Lett 153:181–190

Luo YC, Zeng QR, Wu G, Luan ZK, Liao BH (2003) Effect of beta-cyclodextrin compounds on the solubilization of three selected pesticides and their toxicity with methyl-parathion to Rana tigrina tadpoles. Bull Environ Contam Toxicol 70:998–1005

Macrae A, Rimmer DL, O'Donnell AG (2000) Novel bacterial diversity recovered from the rhizosphere of oil-seed rape (*Brassica napus*) determined by the analysis of 16S ribosomal DNA. Antonie Van Leeuwenhoek 78:13–21

Moyer CL, Tiedje JM, Dobbs FC, Karl DM (1996) A computer-simulated restriction fragment length polymorphism analysis of bacterial small-subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. Appl Environ Microbiol 62:2501–2507

Qui X, Wu L, Huang H, McDonel PE, Palumbo AV, Tiedje JM, Zhou J (2001) Evaluation of PCR-generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning. Appl Environ Microbiol 67:880–887

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Somerville L, Greaves MP (1987) Pesticide effects on soil microflora. Taylor and Francis, New York, NY

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882

Tsakiris IN, Toutoudaki M, Nikitovic DP, Danis TG, Stratis IA, Tsatsakis AM (2002) Field study for degradation of methyl parathion in apples cultivated with integrated crop management system. Bull Environ Contam Toxicol 69:771–778

Wagner ED, Marengo MS, Plewa MJ (2003) Modulation of the mutagenicity of heterocyclic amines by organophosphate insecticides and their metabolites. Mutat Res 36:103–115

Wang GC-Y, Wang Y (1996) The frequency of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from different bacterial species. Microbiology 142:1107–1114

Wang GC-Y, Wang Y (1997) Frequency of formation of chimeric molecules as a consequence of PCR coamplification of 16S rRNA genes from mixed bacterial genomes. Appl Environ Microbiol 63:4645–4650

Ward DM, Weller R, Bateson MM (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature 344:63–65

Wardle DA, Parkinson D (1990) Effects of three herbicides on soil microbial biomass and activity. Plant Soil 122:21–28

Wilson KH, Blitchington RB, Green RC (1990) Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. J Clin Microbiol 28:1942–1946

Zhang RF, Cui ZL, Jiang JD, He J, Gu XY, Li SP (2005) Diversity of organophosphorus pesticide-degrading bacteria in a polluted soil and conservation of their organophosphorus hydrase genes. Can J Microbiol 51:337–343

Zhou JZ, Bruns MA, Tiedje JM (1996) DNA recovery from soils of diverse composition. Appl Environ Microbiol 62:316–322

Zhou JZ, Xia BC, Huang HS, David ST, Loren JH, Richard JM, Anthony VP, Tiedje JM (2003) Bacterial phylogenetic diversity and a novel candidate division of two humid region, sandy surface soils. Soil Biol Biochem 35:915–924