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Isolation of an Organophosphorus-degrading Strain *Pseudomonas* sp. Strain YF-5 and Cloning of *mpd* Gene from this Strain

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Organophosphorus compounds (OP compounds) are toxic, because they can inhibit the acetylcholine esterase in central nervous system of human and animals. OP compounds are extensively used for insecticides control. Water, soil and agricultural products have been contaminated seriously by OP compounds in some regions. It is urgent to find effective methods to remove OP compounds contamination. In this study, an organophosphorus-degrading bacterium (strain YF-5) was isolated from sludge. Based on the results of phenotypic features and phylogenetic similarity of 16S rRNA gene sequences, strain YF-5 was identified as *Pseudomonas* sp. In liquid culture, 100 mg l^{-1} methyl parathion and chlorpyrifos were decomposed completely by strain YF-5 (10^6 cells mL^{-1}) within 8 h and 20 h, respectively. The methyl parathion degrading (*mpd*) gene was cloned from strain YF-5. Result of sequence BLAST indicated that this gene has 99% similarity to *mpd* of *Plesiomonas* sp. M6.

Key words: Isolation, *Pseudomonas* sp, Organophosphorus, Degrading, *mpd*.

Organophosphorus compounds (OP compounds) are the most widely used insecticides, accounting for about 34% of the world-wide applied insecticides[1].The large-scale use of OP compounds and the low decomposition rates of OP compounds in the environment cause the accumulation of these compounds in the soils. The undegraded OP compounds in contaminated soil favor their ingress into water and agricultural products for human consumption. Recent studies indicated severe contamination of OP compounds existed in water, soil, fruits and vegetables[2-4]. The toxic effect of OP compounds has a cumulative nature and causes various degenerative disorders of the nervous system[5]. Additionally, OP compounds are strong mutagens causing multiple chromosomal aberrations and carcinogenesis[6,7]. Thus, it is extremely urgent to search effective and environment-friendly techniques for eliminating OP compounds.

Biodegradation is an effective method to degrade OP compounds. Organophosphorus hydrolase (OPH) isolated from *Pseudomonas diminuta* MG[8] has attracted considerable interest because of its ability to degrade a broad range of OP compounds[9]. Methyl parathion hydrolase (MPH, E.C.3.1.8.1), which can hydrolyze a broad spectrum of OP compounds such as parathion, methyl parathion,, malathion, fenitrothion, chlorpyrifos [10], is also an important enzyme in hydrolyzing OP compounds. MPH was first found in *Plesiomonas* sp. strain M6[11], and then the MPH coding gene *mpd* was also cloned from *Stenotrophomonas* sp.[12] and *Sphingomonas* sp.[13], subsequently.

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In this study, we isolated an organophosphorus-degrading strain *Pseudomonas* sp. YF-5. The *mpd* gene encoding MPH which is capable of hydrolyzing a broad spectrum of OP compounds[10,14], was cloned from this strain. Result of sequence BLAST indicated that this gene has 99% similarities to *mpd* of *Plesiomonas* sp. M6. The *mpd* gene cloned from *Pseudomonas* sp. YF-5 was successfully expressed in *E. coli*. Moreover, the degradation of OP compounds in liquid culture by this strain was also evaluated. This work contributed to finding effective method to remove OP compounds contamination.

MATERIALS AND METHODS

Chemical

Two OP compounds chlorpyrifos and methyl parathion (99.0% pure analytical grade) were supplied by shenyang chemical industry research institute (Shenyang, China).

Strain isolation and culture conditions

Organophosphorus-degrading strains were isolated from soil samples by using the methods of Yang *et al*. with some modifications[12]. A mineral salt medium (MSM, pH 7.0) containing (g l^{-1}) KH_2PO_4, 1.0; K_2HPO4, 2.0; $(NH_4)_2SO_4$, 1.0; NaCl, 0.5; $MgSO_4$, 0.2; $FeSO_4$, 0.02 and $CaCl_2$, 0.05 was used. About 1 g of sludge was added to an Erlenmeyer flask (500 mL) containing 100mL MSM with 50 mg l^{-1} chlorpyrifos as the carbon source and incubated at 28 °C on a shaker at 160 rpm for 7 days. The culture then was inoculated (inoculation amount: 2%) into a fresh MSM containing 100 mg l^{-1} chlorpyrifos as the carbon source and incubated at 28 °C, 160 rpm for another 7 days, and then the culture was spread on MSM agar plates containing 100 mg l^{-1} chlorpyrifos, incubated at 28 °C. After 3 days incubation, visible colonies were formed, and their degrading ability was tested by inoculation in MSM with 100 mg l^{-1} chlorpyrifos. A 5 mL of the liquid culture was taken for pesticide concentration determination. The residual pesticide in MSM was extracted with 25 mL acetonitrile under sonication for 30 min followed by filtration, drying, redissolution by acetone, and finally detected by gas chromatography (GC). A Shimadzu GC-2014C Series gas chromatograph was used for analysis of the pesticides. Flame photometric detector (FPD) and fused silica capillary column (length: 30 m, internal diameter: 0.53 mm, and film thickness: 1 ¼m; RESTEX, USA) were used in this study. The air and hydrogen flow rates were 81.8 and 3.2 mL min^{-1}, respectively. Nitrogen was used as the carrier gas at a flow rate of 1 mL min^{-1}. The temperatures of injector, column, and detector were set at 250, 150, and 250 °C, respectively. The column temperature was initially set at 150 °C for 3 min, then heated to 250 °C at a rate of 8 °C min^{-1}, and finally maintained for 8 min.

Taxonomic identification

Strain YF-5 was identified with reference to Bergey's Manual of Determinative Bacteriology[15]. Total genomic DNA of strain YF-5 was prepared by a standard phenolic extraction procedure[16]. The 16S rRNA gene was amplified by PCR using the universal primers[17], 8f (5'-CACGGATCCAGACTTT GATYMTGGCTCAG-3',forward) and 1512r (5'-GTGAAGCTTACGGYTAGCTTGTTACGACTT-3', reverse). The PCR reaction was performed in a Bio-Rad Peltier thermal cycler and the PCR conditions were as follows: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1.5 min, 30 cycles and the final extension at 72 °C for 8 min. The PCR product was inserted into a pMD18-T vector (Takara, Dalian, China) and sequenced. The 16S rRNA gene of strain YF-5 was compared with those available in the GenBank database using the NCBI BLAST program. Multiple alignments of sequences was accomplished using CLUSTAL X program[18], and the MEGA 4.0 software (www.megasoftware.net) was used to construct a neighbor-joining phylogenetic tree. Kimura 2-parameter model, and a bootstrap analysis for evaluation of the phylogenetic topology were used.

Cloning of *mpd* gene from strain YF-5

The *mpd* gene of strain YF-5 was amplified by PCR with a pair of primers (Cui *et al*. 2001). The PCR primers were as follows: forward, 5'-GAATTCATATGCCCCTGAAGAAC-3', and reverse, 5'-GAATTCTCGAGCTTGGGG TTGACGACCG-3' (*Nde*I and *Xho*I restriction sites, respectively, are underlined). PCR was performed in a Bio-Rad Peltier thermal cycler. The PCR conditions were as follows: denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, elongation at 72 °C for 1 min, 30 cycles. The 1.0 kb PCR

products were purified by using a QIAquick PCR Purification Kit (QIAGEN, Dusseldorf, Germany), inserted into the pMD18-T plasmid, and then transformed into *E. coli* DH5a competent cells. The recombinant plasmid pMD18-T containing *mpd* gene was extracted from *E. coli* DH5a by the alkali lysis method[16] ,and sent to TaKaRa for sequencing.

For MPH expression and purification, the recombinant plasmid pMD18-T containing *mpd* gene was digested with *Nde*I-*Xho*I and the resulting fragment (*mpd*) was subcloned into *Nde*I-*Xho*I sites of the expression vector pET30a (Novagen) to yield recombinant pET30a vector containing *mpd* gene which allowed the intracellular expression of *mpd* gene as a C-terminal fusion to a 6×His tag. The recombinant pET30a vector was transformed into *E. coli* BL21 (DE3) competent cells as the host. After IPTG introduction, the expression product was purified by Ni-NTA Agarose (QIAGEN) and analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Cell growth and degradation of OP compounds in MSM

Strain YF-5 was precultured in MSM with 100 mg L^{-1} chlorpyrifos, and incubated overnight at 28 °C on a shaker at 160 rpm in the dark. The cell pellets were obtained by centrifugation, washed twice with fresh MSM and quantified by dilution plate count method. For all experiments, 10^6 cells mL^{-1} were adopted and samples were incubated on a shaker at 160 rpm, 28 °C unless otherwise stated. OP compound degradation by strain YF-5 was studied in MSM containing 100 mg L^{-1} chlorpyrifos and 100 mg L^{-1} methyl parathion at 28°C,160 rpm in the dark. Bacterial growth of strain YF-5 was measured by the changes in the absorbance in 600 nm. The residual pesticide content in the culture was detected by gas chromatography, as described above. The MSM culture without strain YF-5 inoculation was used as the control (100%). All samples were run in triplicate to ensure the accuracy.

RESULTS AND DISCUSSION

Strain isolation and identification

After plate screening, five OP organophosphorus-degrading bacteria were isolated by the enrichment procedure using chlorpyrifos as the sole carbon source. One strain named YF-5 exhibited the highest hydrolyzing capability and was selected for further study. Strain YF-5 was gram negative, strictly aerobic, motile, and rod shaped. Colonies of strain YF-5 on nutrient agar plate were circular, smooth and brown color. Strain YF-5 showed catalase, oxidase, urease, nitrate reductase and starch hydrolysis activities. The tests for gelatin hydrolysis, indole production, H_2S production, phenylalanine deaminase and pigment production of train YF-5 were negative. The phylogenetic tree based on the 16S rRNA gene of strain YF-5 (Fig. 1) indicates the close

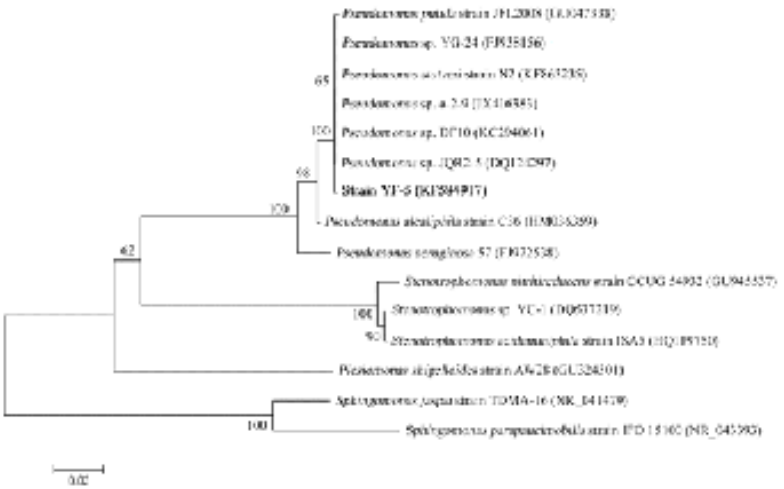

Fig. 1. Phylogenetic tree of strain YF-5 based on 16S rRNA gene sequence analysis. Bootstrap values obtained with 1000 repetitions were indicated as percentages at all branches. GenBank accession numbers are given in brackets

relationship of strain YF-5 with *Pseudomonas*. Based on the phenotypic features and phylogenetic similarity of 16S rRNA gene sequences, strain YF-5 was identified as *Pseudomonas*.

Cloning of *mpd* gene and its expression in *E. coli*

The MPH encoding gene *mpd* was cloned from strain YF-5. The PCR primers were designed according to the known *mpd* gene of *Plesiomonas* sp.M6 (Cui *et al*. 2001). The result of sequence BLAST showed that this *mpd* gene was 99% similar to *mpd* gene of *Plesiomonas* sp. M6 (GenBank accession no. AF338729), 99% similar to *mpd* gene of *Stenotrophomonas* sp. YC-1 (GenBank accession no. DQ677027) and 99% similar to *mpd* gene of *Ochrobactrum* sp. mp-4 (GenBank accession no. AY627036) at the nucleotide level.

The *mpd* gene cloned from strain YF-5 was inserted into PET-30a, and expressed in *E. coli* BL21 (DE3), under the induction of IPTG, a clear band migrated with a molecular weight of 35 kDa was observed in SDS-PAGE (Fig. 2). The molecular weight of the expression product was identical to the calculated weight of MPH. These results indicated that the *mpd* gene was successfully expressed *E. coli*. MPH is an important enzyme in degrading OP compound, it exhibits high catalytic activities and wide substrate specificities against OP compounds such as parathion, methyl parathion, chlorpyrifos, malathion, DDVP, andfenitrothion[10,14]. MPH was first found in *Plesiomonas* sp. strain M6 [11] , and then the MPH encoding gene *mpd* was also cloned from *Pseudomonas* sp. strain WBC-3[19], *Stenotrophomonas* sp. [12] and *Sphingomonas* sp.[13], subsequently.

Cell growth and OP compounds degradation in liquid culture

The degradation of OP compounds and the growth of strain YF-5 in mineral salt medium (MSM) were shown in Fig. 3 and Fig. 4. In MSM culture, 100 mg L^{-1} methyl parathion and 100 mg L^{-1} chlorpyrifos was decomposed completely by strain YF-5 (10^6 cells mL^{-1}). The degradation of OP compounds was accompanied by the cell growth of strain YF-5. Degradation of OP compounds was negligible in –culture samples without strain YF-5 and no cell growth was detected. As shown in Fig. 3, 100 mg L^{-1} of methyl parathion was decomposed completely by strain YF-5 within 8 h. In contrast,

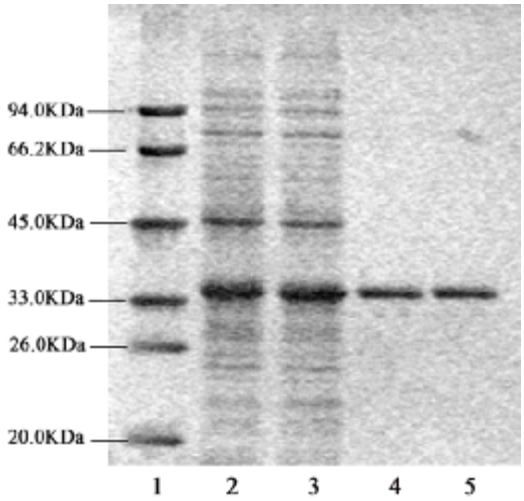

Fig. 2. SDS-PAGE analysis (gel, 12% polyacrylamide; stain, CBB-R250): lane 1, protein marker; lane 2 and 3, *E. coli* cell lysis after IPTG induction; lane 4 and 5, purified MPH

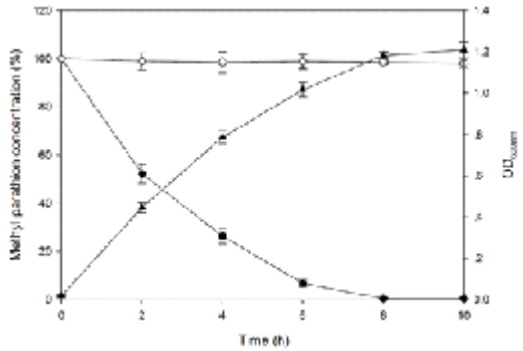

Fig. 3. Methyl parathion degradation in MSM by strain YF-5 and cell growth. (○) uninoculated; (●) Strain YF-5 inoculated; (▲) Growth of strain YF-5.

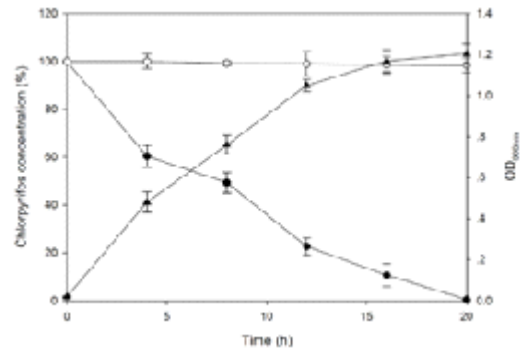

Fig. 4. Chlorpyrifos degradation in MSM by strain YF-5 and cell growth. (○) uninoculated; (●) Strain YF-5 inoculated; (▲) Growth of strain YF-5

the degradation efficiency of chlorpyrifos, relative to that of methyl parathion, by strain YF-5, was much lower, as shown in Fig. 4, 100 mg L^{-1} of chlorpyrifos was decomposed completely by strain YF-5 within 20 h. The hydrolyzing efficiency of chlorpyrifos in liquid culture by strain YF-5 was similar to that of *Stenotrophomonas* SP. strain YC-1 which was isolated by Yang *et al*.[12] .

Nucleotide sequence accession number

The nucleotide sequences of the 16S rRNA gene and *mpd* gene of strain YF-5 have been deposited in the GenBank database under accession no. KF584917 and KF584916.

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