

Isolation of Methyl Parathion-Degrading Strain M6 and Cloning of the Methyl Parathion Hydrolase Gene

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A degradative bacterium, M6, was isolated and presumptively identified as *Plesiomonas* sp. strain M6 was able to hydrolyze methyl parathion to *p*-nitrophenol. A novel organophosphate hydrolase gene designated *mpd* was selected from its genomic library prepared by shotgun cloning. The nucleotide sequence of the *mpd* gene was determined. The gene could be effectively expressed in *Escherichia coli*.

Organophosphate pesticides are a group of highly toxic agricultural chemicals widely used in plant protection. Up to the present, organophosphate pesticides, such as parathion, methyl parathion, and methamidophos, are still extensively used worldwide despite their high toxicity. The microbial degradation of parathion has been intensively researched. Two parathion-degrading bacteria, *Pseudomonas diminuta* GM and *Flavobacterium* sp. strain ATCC 27551 (16, 22) have been isolated in the Philippines and the United States. Both of them possess parathion hydrolase activity, and the hydrolase from *Flavobacterium* sp. strain ATCC 27551 was purified (14). The *opd* genes encoding parathion hydrolases from *P. diminuta* GM and *Flavobacterium* sp. strain ATCC 27551 were cloned and sequenced (9, 11, 14, 21). The two genes show high homology (17). Another three bacteria that were able to degrade parathion which showed no homology on the protein level to the those found in *Pseudomonas* and *Flavobacterium* parathion hydrolases were isolated (15). Studies on the degradation of methyl parathion were also made. The pathway that *P. putida* uses to degrade methyl parathion was studied by Rani and Lalithakumari (19). They found that *P. putida* could hydrolyze methyl parathion and use *p*-nitrophenol as sole carbon source. However, no further research on the nucleic acid level was reported. Chaudhry and colleagues isolated a methyl parathion-degrading bacterium, a *Pseudomonas* sp. (3), which possessed DNA homologous to the parathion hydrolase gene from *Flavobacterium* sp. strain ATCC 27551, as revealed by Southern blot analysis of its total DNA against a *Pst*I fragment containing the *opd* gene (3). Methyl parathion and parathion have similar chemical structures. So, it seems reasonable that the enzymes degrading these chemicals may have homology. Previous research indeed showed that some methyl parathion degrading-bacteria possess DNA homologous to the *opd* gene (3). Here, we report a novel gene from *Plesiomonas* sp. strain M6 that is absolutely different from *opd* genes encoding parathion hydrolases (Table 1).

A methyl parathion-degrading bacterial strain, M6, was isolated. M6 hydrolyzed methyl parathion to dimethyl phospho-

rothinate and *p*-nitrophenol but could not degrade *p*-nitrophenol further. However, it was able to use benzoic acid and phenylacetic acid as sole carbon and energy source. M6 is a rod-shaped gram-negative bacterium with polar flagella. It oxidized and fermented glucose to produce acid, but no gas formed. It could not use starch and molecular nitrogen as carbon and nitrogen sources. Catalase and oxidase reactions of M6 were positive. M6 was sensitive to vibriostatic agent O/129. It was presumptively identified as a *Plesiomonas* sp. based on its biochemical characteristics.

UV scanning was used to measure the hydrolysis of methyl parathion. The scanning results are shown in Fig. 1a. The results showed that M6 had strong methyl parathion-degrading ability. When 200 mg of methyl parathion/ml was added to the liquid medium, it was decomposed into *p*-nitrophenol in less than 15 min (Fig. 1). The maximum absorption peak of methyl parathion was recorded at 273 nm. A new absorption peak at 310 nm appeared, which was caused by the degradation end product, *p*-nitrophenol (with maximum absorption at 310 nm). The methyl parathion hydrolase of M6 was an endoenzyme, since no catalytic activity was observed in the supernatant (Fig. 1).

To clone the methyl parathion hydrolase gene, a gene library was constructed from genomic DNA of M6 by the shotgun technique. M6 genomic DNA was extracted by the method of high-salt-concentration precipitation (12). *Sau*3AI was employed to obtain randomly digested chromosomal fragments. The 2- to 4-kb fragments were recovered by a glassmilk purification kit (Biostar, Canada) and ligated into pUC19 digested with *Bam*HI and treated with calf intestinal alkaline phosphatase (Promega). The ligation product was transformed into *E. coli* DH5 α cells. The bacteria were then plated on Luria-Bertani medium containing 100 mg of methyl parathion/ml as indicator and incubated at 37°C for no more than 12 h. Two positives that produced yellow transparent halos were selected from the gene library. The recombinant plasmids were subsequently designated as pMT1 and pMT2. According to the physical maps of the two positive recombinant plasmids, there were 2.2- and 4.5-kb inserts, respectively, and the 2.2-kb fragment was included in the 4.5-kb fragment (Fig. 2). The cloned methyl parathion hydrolase gene was named *mpd*. To verify the origination of the inserts, a 1.4-kb *Sma*I fragment from pMT1 was used as probe to hybridize against pMT1,

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TABLE 1. Bacteria and plasmids used in the study

Strains or plasmids	Relevant phenotype	Source or reference
<i>E. coli</i> DH5 α	<i>recA1 endA1 hsdR17 D lacU169</i> (ϕ 80 <i>lacZ</i> Δ <i>M15</i>)	20
<i>E. coli</i> BL21	<i>hsdS gal</i> (λ <i>imm434 clts b2 red Dam Sam</i> (λ))	20
M6M	M6 strain deficient in methyl parathion hydrolase	This work
pUC19	Amp ^r	20
pET-29	Amp ^r	Novagen
pMT1	Amp ^r , pUC19 vector with 2.2-kb foreign fragment	This work
pMT2	Amp ^r , pUC19 vector with 4.5-kb foreign fragment	This work
pETM	Amp ^r , pET-29 vector with 1.0-kb foreign fragment	This work

pMT2, *Hind*III-digested M6 chromosomal DNA, and *Hind*III-digested M6M chromosomal DNA. The probe was labeled by [α -³²P]dCTP with a Takara random primer DNA labeling kit, version 2 (Takara). The activity of the labeled probe was nearly 10⁹ dpm/ μ g of DNA. Hybridization result showed that the probe gave strong signals for pMT1 and pMT2 DNA, and a weak band also appeared on the track of M6 chromosomal (Fig. 3, lanes B, C, and D). The result verified that the inserts originated from strain M6 chromosomal DNA. The probe did not give a signal against M6M total DNA (Fig. 3, lane A). The results indicated that the hydrolase gene in M6M was deleted by unknown reasons.

The nucleotide sequence of the 2.2 kb of pMT1 was determined by primer extension. The sequencing primers were

R-primer (5'-GAGCGGATAACAATTTTCACACAGG-3'), NJS 215P1 (5'-GCCACTGCCAGTCACTT-3'), NJS215P3-02 (5'-AATCGGCTTCTTTCTGGTCC-3') and NJS215P4 (5'-CGGTTCITCAGGGGCATGGTGA-3'). Sequence was determined at Takara Biotechnology (Dalian) Co. Ltd. The complete length of the fragment was 2,191 bp.

The *mpd* gene could be expressed in *E. coli* DH5 α without induction of IPTG (isopropyl- β -D-thiogalactopyranoside) as shown in the process of gene cloning. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (9) analysis of the *E. coli* DH5 α cells harboring *mpd*(pMT1) showed that a new protein band at about 35 kDa appeared compared with the control of harboring pUC19 vector (Fig. 4). The methyl parathion hydrolase activities of the crude cell-free extracts of

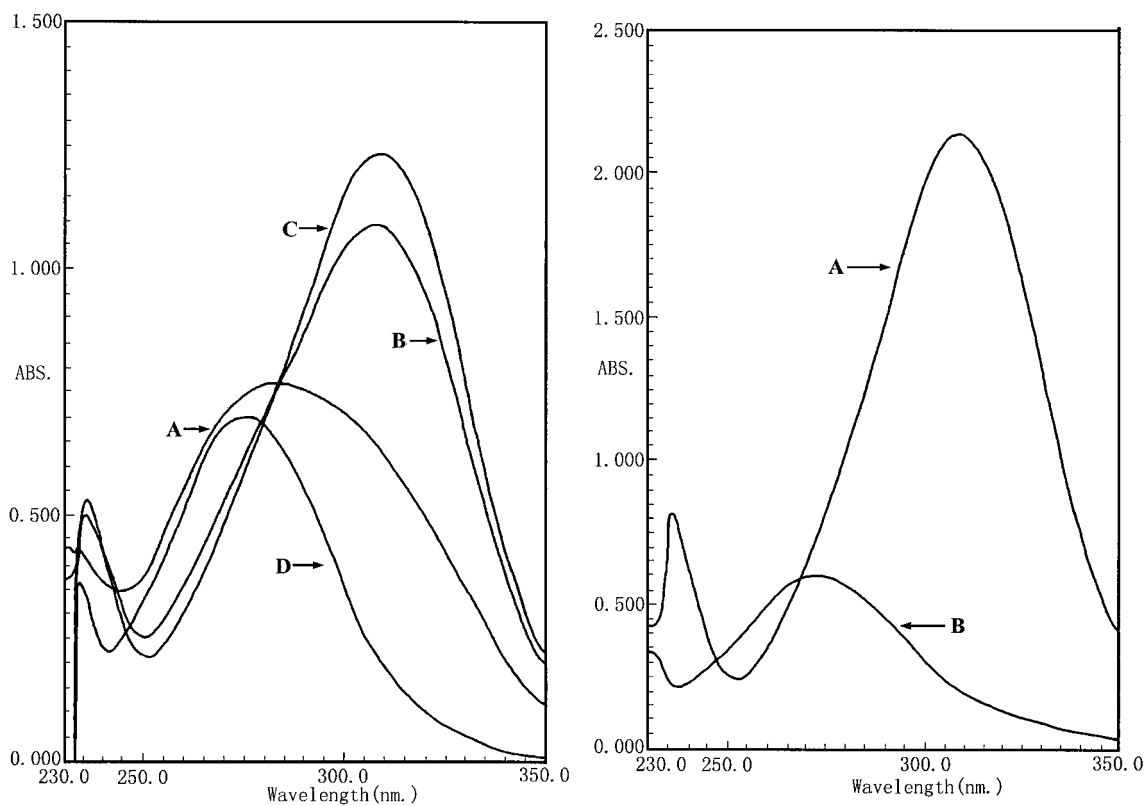


FIG. 1. (Left) Determination of degradation of methyl parathion by strain M6 at different times by UV scanning. Curves: A, methyl parathion treated with M6 for 5 min; B, treated for 10 min; C, treated for 15 min; D, methyl parathion control. (Right) Localization of the methyl parathion hydrolase enzyme in the cell. Curves: A, methyl parathion treated with the M6 cells; B, methyl parathion treated with supernatant of the culture media. No hydrolase activity was measured in the supernatant.

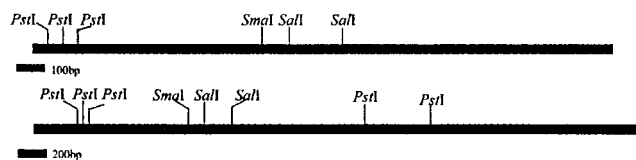


FIG. 2. Restriction endonuclease maps of the cloned fragments containing the *mpd* gene from pMT1 (above) and pMT2 (below).

E. coli DH5 α , M6, and *E. coli* DH5 α containing pMT1 were measured. Total protein of the crude extracts was detected by the Bradford method (1). The activity was expressed by the formation of *p*-nitrophenol in 1 min per microgram of protein. Catalytic activities (micromoles/microgram of protein/minute) ($\mu\text{mol}/\mu\text{g}\cdot\text{protein}/\text{min}$) of the crude cell-free extracts were as follows: *E. coli* DH5 α , 0; M6, 2.6×10^{-5} ; *E. coli* DH5 α containing pMT1, 1.4×10^{-5} ; and *E. coli* BL21 containing pETM, 1.5×10^{-4} .

The sequence was analyzed with the software Bioedit (18). There were eight open reading frames (ORFs) that encoded proteins larger than 100 amino acids on the strand and an incomplete large ORF on the reverse. The eight ORFs started from positions 570, 654, 699, 723, 870, 1164, 1191, and 1323 and all ended at TGA (position 1718). Promoter prediction (http://www.fruitfly.org/seq_tools/promoter.html) revealed that there was a promoter-like sequence located at position 651 to 696. It had a typical *E. coli* promoter structure of TTGCAA-17 nucleotides-TATACT. There was a ribosome binding site (AGGA) 5 nucleotides upstream of the start code at 711 to 716. Considering that the regulation element of the *mpd* gene could be recognized by *E. coli* DH5 α translation mechanisms and the molecular size of the gene product was about 35 kDa, it was deduced that the region ranging from 657 to 1718 was the complete sequence of the methyl parathion hydrolase gene. Primers 5'-GAATTCATATGCCCTGAAGAAC-3'

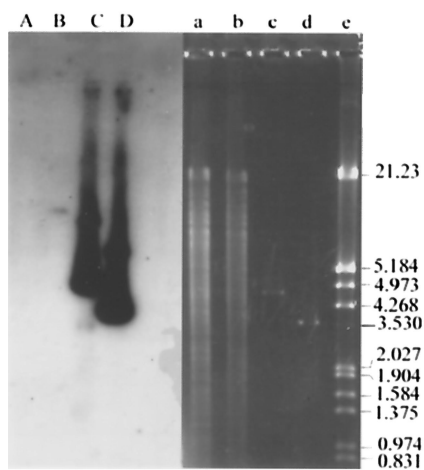


FIG. 3. Southern blot analysis of chromosomal DNA from M6 and M6M and recombinant plasmids pMT1 and pMT2 by using a 1.4-kb *Sma*I fragment as probe. Lanes A to D are the blots while restriction digests are shown in lanes a to d. Lanes: a and b, *Hind*III-digested M6M and M6 chromosomal DNA; c and d, recombinant plasmids pMT2 and pMT1; e, the marker, *Hind*III-digested lambda DNA, with molecular sizes of bands given in kilobases on the right.

(forward) and 5'-CGGAATTCTCACTTGGGGTTGAC-3' (reverse) were synthesized on the basis of the nucleotides sequence of the 2.2-kb fragment. They were designed to amplify DNA containing the entire ORF of the *mpd* gene with an *Nde*I site at the 5' end and an *Eco*RI site at the 3' end. PCR was carried out with rTaq DNA polymerase (Takara). The reaction mixture contained 0.5 μM concentrations of each forward and reverse primer, 0.2 mM deoxynucleoside triphosphate, 2.0 mM MgSO_4 , $1 \times$ PCR buffer, 50 ng of pMT1 DNA, and 2.5 U of rTaq DNA polymerase per 50 μl . The mixture was subjected to 30 cycles of the following treatment: denaturation for 1 min at 94°C, annealing for 1 min at 52°C, and a final extension for 1 min at 72°C, with a final 5-min 72°C extension step after cycling was complete. The amplified DNA fragment of 1.0 kb was cloned into *Nde*I-*Bam*HI sites of the vectors pET-29 (Novagen) to yield pETM. pETM was transformed into *E. coli* BL21 cells (Novagen). Recombinant harboring pETM showed remarkable expression of *mpd* by SDS-PAGE (Fig. 4). The molecular mass of the enzyme coincided with that of *mpd* expressed in DH5 α cells.

The *mpd* nucleotide sequence and predicted protein sequence were compared with those in the GenBank database by an online alignment search (<http://www.ncbi.nlm.nih.gov/BLAST/>). No regions of extensive DNA homology could be detected. The predicted protein had 31% identity in a 307-amino-acid overlap with a hypothetical beta-lactamase SCJ21.1 (CAB52357) from *Streptomyces coelicolor* and also 27 to 29% identity with beta-lactamase (CAB39710 and CAB73577) from other sources, indicating that the hydrolase might be a member of the lactamase superfamily.

Biodegradation of organophosphate agents was widely studied for its high toxicity to the environment and animals (3, 9, 10, 18, 19, 22). Two organophosphate pesticides hydrolase genes (*opd*; GenBank accession numbers, M29593 and M20392) from *P. diminuta* GM and *Flavobacterium* sp. strain ATCC 27551 have been cloned and sequenced. The two genes shared 86.3% identity on the nucleic acid level at the coding region. Southern blot analysis of a methyl parathion-degrading strain of a *Pseudomonas* sp. against a α - ^{32}P -labeled 1.2-kb probe containing an *opd* gene from *P. diminuta* GM showed that its methyl parathion hydrolase gene had homology to the *opd* gene (3). The restriction map of the *mpd* gene we cloned was completely different from that of the *opd* gene. Sequence comparison also revealed that the sequence of the *mpd* gene shared no intensive homology with the *opd* genes. A comparison of the coding regions of the three sequences was made (data not shown).

Organophosphorus hydrolases play important roles in the decontamination of organophosphate pesticides and will be useful in the bioremediation of pollution caused by these pesticides (5). Recombination expression of these enzymes can supply enzyme products at low cost for environment protection.

Nucleotide sequence accession number. The nucleotide sequence of the *mpd* gene has been assigned the GenBank accession number AF338729.

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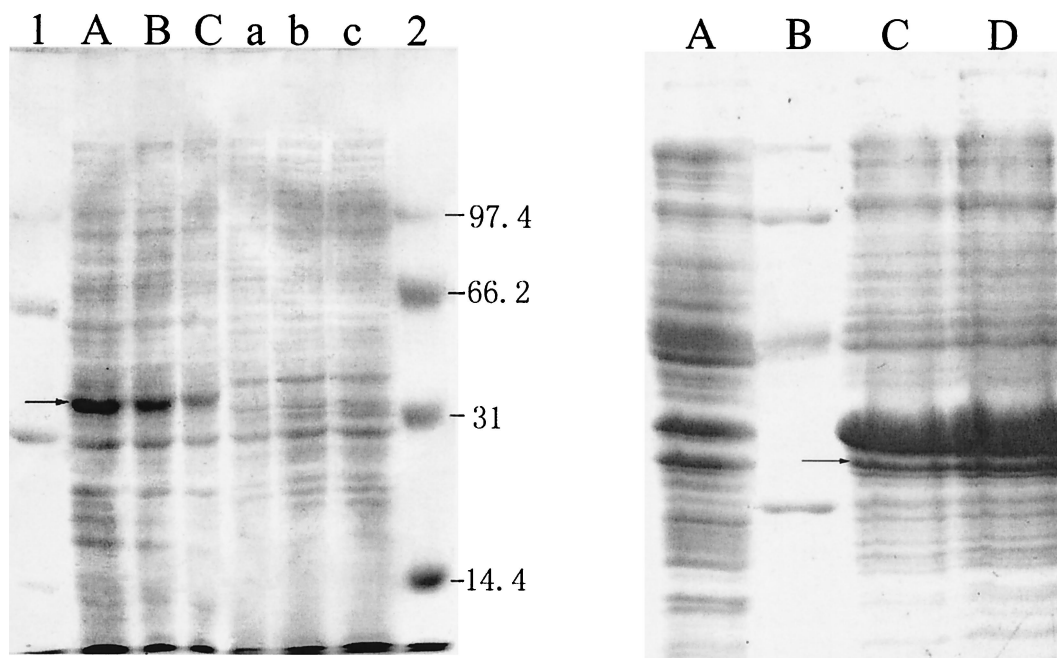


FIG. 4. (Left) SDS-PAGE gel displaying new protein band in *E. coli* DH5 α cells containing pMT1. Lanes A to C, samples of *E. coli* DH5 α containing plasmid pMT1; lanes a to c, samples of *E. coli* DH5 α containing vector plasmid pUC19. Lanes A, B, and C were loaded with 20, 10, and 5 μ l of loading sample, respectively. Lanes a, b, and c were treated the same as lanes A, B, and C. Lanes 1 and 2 were the protein molecular markers with molecular sizes of bands given in kilodaltons on the right. (Right) Expression of the amplified *mpd* gene in *E. coli* BL21. Lane A, 10 μ l of sample of the control, *E. coli* BL21; lane B, protein molecular marker with the sizes 97.4, 66.2, 42.7, and 31.0 kDa (top to bottom); lanes C and D, samples of *E. coli* BL21 harboring pETM after inducing with IPTG for 4 h. Lanes C and D were loaded with 10- and 20- μ l samples, respectively. Arrows showed the new protein bands expressed in the recombinant *E. coli* strains.

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