Cloning of *mpd* gene from a chlorpyrifos-degrading bacterium and use of this strain in bioremediation of contaminated soil

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Abstract

An effective chlorpyrifos-degrading bacterium (named strain YC-1) was isolated from the sludge of the wastewater treating system of an organophosphorus pesticides manufacturer. Based on the results of phenotypic features, phylogenetic similarity of 16S rRNA gene sequences and BIOLOG test, strain YC-1 was identified as the genus *Stenotrophomonas*. The isolate utilized chlorpyrifos as the sole source of carbon and phosphorus for its growth and hydrolyzed chlorpyrifos to 3,5,6-trichloro-2-pyridinol. Parathion, methyl parathion, and fenitrothion also could be degraded by strain YC-1 when provided as the sole source of carbon and phosphorus. The gene encoding the organophosphorus hydrolase was cloned using a PCR cloning strategy based on the known methyl parathion degrading (*mpd*) gene of *Plesiomonas* sp. M6. Sequence BLAST result indicated this gene has 99% similar to *mpd*. The inoculation of strain YC-1 (10^6 cells g^{-1}) to soil treated with 100 mg kg^{-1} chlorpyrifos resulted in a higher degradation rate than in noninoculated soils. Theses results highlight the potential of this bacterium to be used in the cleanup of contaminated pesticide waste in the environment.

Introduction

Organophosphorus pesticides are widely used in agriculture to control major insect pests. These compounds have been implicated in several nerve and muscular diseases in human beings. Organophosphorus compound poisoning is a worldwide health problem with around 3 million poisonings and 200 000 deaths annually (Karalliedde & Senanayake, 1999; Sogorb *et al.*, 2004). Chlorpyrifos (*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate) may be P–O–C linkage as is found in other organophosphorus pesticides, such as parathion, methyl parathion and fenitrothion. Chlorpyrifos is one of the most widely used pesticides in the US and accounts for 11% of total pesticide use (EPA, 2004).

Biodegradation is considered to be a reliable cost-effective technique for pesticide removal. For some organophosphates such as parathion, it has been relatively easy to isolate degrading bacteria: two different strains, *Flavobacterium* sp. ATCC 27551 and *Pseudomonas diminuta* MG, have been isolated from soils in the Philippines and United States, respectively (Sethunathan & Yoshida, 1973; Serdar *et al.*, 1982). In addition, studies on the degradation of methyl parathion also were made (Chaudhry *et al.*, 1988; Rani & Lalithakumari, 1994). The environmental fate of chlorpyrifos has been studied extensively. Degradation in soil involves both chemical hydrolysis and microbial activity. In most cases, the aerobic bacteria tend to transform chlorpyrifos by hydrolysis to produce diethylthiophosphoric acid (DETP) and 3,5,6-trichloro-2-pyridinol (TCP). Unlike other organophosphorus compounds, chlorpyrifos has been reported to be resistant to enhanced degradation (Racke *et al.*, 1990). It was suggested that the accumulation of TCP, which has antimicrobial properties, prevents the proliferation of chlorpyrifos-degrading microorganisms in soil (Racke *et al.*, 1990). Chlorpyrifos has been reported to be degraded cometabolically in liquid media by *Flavobacterium* sp. and *P. diminuta*, which were initially isolated from a diazinon-treated field and by parathion enrichment, respectively (Sethunathan & Yoshida, 1973; Serdar *et al.*, 1982). However, these microorganisms do not utilize chlorpyrifos as a source of carbon. Attempts to isolate chlorpyrifos-degrading bacteria from chlorpyrifos-treated soil have not been successful until recently when Singh *et al.* (2003) isolated six chlorpyrifos-degrading bacteria from an Australian soil showing enhanced degradation of chlorpyrifos.

Yang *et al.* (2005) isolated *Alcaligenes faecalis* DSP3, which is capable of degrading both chlorpyrifos and TCP. Although the microbial degradation of chlorpyrifos has been researched, the existing papers lack the information on the genetic and enzymatic aspects involved in the degradation of chlorpyrifos.

Currently, various gene/enzyme systems involved in degradation of certain organophosphates have been identified. An organophosphate-degrading gene (*opd*) was isolated from various organisms and diverse geographic locations (Serdar *et al.*, 1982; Mulbry *et al.*, 1986; Horne *et al.*, 2002). The organophosphorus hydrolase (OPH), which is encoded by the *opd* gene, exhibits broad substrate specificities and high hydrolytic activities against organophosphates. However, chlorpyrifos is hydrolyzed by the OPH almost 1200-fold slower than the preferred substrate paraoxon (Cho *et al.*, 2004). In 2001, an gene (called *mpd*) encoding an organophosphate degrading protein was isolated from a parathion-methyl degrading *Plesiomonas* sp., but showed no homology to the known *opd* genes (Cui *et al.*, 2001).

In this paper, we describe the isolation and identification of a chlorpyrifos-degrading bacterium. The *mpd* gene encoding the OPH was cloned from this strain and expressed in *Escherichia coli*. Moreover, the degradation of chlorpyrifos in soil by bacterial inoculation also was investigated.

Materials and methods

Chemical

Samples of chlorpyrifos, TCP, parathion, methyl parathion and fenitrothion (99.0% pure analytical grade) were obtained from Institute for the Control of Agrochemicals, Ministry of Agriculture, China. All other chemicals were of the highest purity commercially available (Sigma-Aldrich, St Louis, MO).

Isolation and culture conditions

A mineral salt medium (MSM, pH 7.0) containing ($g\,L^{-1}$) K_2HPO_4, 1.5; KH_2PO_4, 0.5; $(NH_4)_2SO_4$, 0.5; NaCl, 0.5; $MgSO_4$, 0.2; $CaCl_2$, 0.05 and $FeSO_4$, 0.02 was used in this study. A modified medium (MSM minus P) was prepared as described by Yang *et al.* (2005). About 1 g of sludge was added to an Erlenmeyer flask (250 mL) containing 100 mL MSM with chlorpyrifos ($50\,mg\,L^{-1}$) as the sole carbon source and incubated at 30 °C on a rotary shaker at 200 r.p.m. for 3 days. The bacteria then were washed and inoculated into fresh MSM containing $100\,mg\,L^{-1}$ chlorpyrifos and incubated for 3 days. Samples from these cultures were spread on MSM agar plates containing $100\,mg\,L^{-1}$ chlorpyrifos. After 3 days incubation at 30 °C, microbial colonies became visible. We selected and purified these colonies and tested their degrading capability by inoculation in liquid medium. Pesticide residues were measured by high performance liquid chromatography (HPLC) according to the method of Singh *et al.* (2002).

Taxonomic identification

Strain YC-1 was identified with reference to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). For sequencing of the 16S rRNA gene, total genomic DNA was prepared from strain YC-1 by a standard phenolic extraction procedure (Sambrook *et al.*, 1989). The 16S rRNA gene was amplified by PCR using the universal primers, 8f (5′-CACGGATCCAGACTTTGATYMTGGCTCAG-3′, forward) and 1512r (5′-GTGAAGCTTACGGYTAGCTTGTTAC GACTT-3′, reverse) (Weisburg *et al.*, 1991). The PCR reaction was performed in a Perkin-Elmer PE9600 thermocycler with the following cycling profile: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1.5 min, final extension at 72 °C for 8 min. The PCR product was cloned into a pMD18-T vector (Takara) and sequenced. The determined sequence was compared with those available in the GenBank database using the NCBI BLAST program. Multiple alignments of sequences, construction of a neighbor-joining phylogenetic tree with the Kimura 2-parameter model, and a bootstrap analysis for evaluation of the phylogenetic topology were accomplished using CLUSTAL X program (Thompson *et al.*, 1997) and MEGA 3.1 software (www.megasoftware.net).

In order to test the ability of the isolate to rapidly utilize (oxidize) various carbon sources in a short time, the BIOLOG bacterial identification test kit was selected (Biolog Inc., Hayward), and the procedures were followed with commercial protocol.

Inoculum preparation for degradation studies

Strain YC-1 was precultured in flasks containing MSM with $100\,mg\,L^{-1}$ chlorpyrifos. Flasks were incubated overnight at 30 °C on a shaker at 200 r.p.m. in the dark. The cultures were centrifuged and the cell pellets were washed twice with fresh medium and quantified by the dilution plate count technique. For all experiments, 10^6 cells mL^{-1} were used and samples were incubated at 30 °C on a shaker at 200 r.p.m. unless otherwise stated.

Growth and degradation of chlorpyrifos

Degradation studies were carried out at 30 °C in MSM containing $100\,mg\,L^{-1}$ chlorpyrifos. Cultures were regularly checked for bacterial growth, the degradation of chlorpyrifos and accumulation of TCP. Cultures were run in triplicate to ensure accuracy. The medium without inoculation

was used as controls. Effects of extra carbon sources, incubation temperature, pesticide concentration, inoculum density and medium pH also were studied.

Substrate range

Crossed-feeding studies with other organophosphorus pesticides also were performed. The liquid medium was supplemented with parathion, parathion-methyl, or fenitrothion at 100 mg L^{-1}. The pesticide residues were measured by GC-ECD (Lan *et al.*, 2005).

Degradation of chlorpyrifos by isolate YC-1 in soil

The soils used were from the campus of Tsinghua University, Beijing, China and were never exposed to any organophosphorus compound before. The soil had a pH of 6.8 and organic matter content of 2.62%. Soil samples (5 kg) were sterilized by fumigation as described by Singh *et al.* (2004). Subsamples (100 g) of the fumigated and nonfumigated soil were treated under aseptic condition with chlorpyrifos (100 mg kg^{-1}), respectively. Three sets of fumigated soil and nonfumigated soil were inoculated with strain YC-1 (10^6 cells g^{-1}), and another set without inoculation was kept as controls. The inoculum was thoroughly mixed into the soils under sterile condition. The soil moisture was adjusted by the addition of distilled water to 50% of its water-holding capacity. The soils were incubated at 30 °C in the dark. Details for chlorpyrifos extraction and analysis are described by Singh *et al.* (2002). Four soils with different pHs from various locations were used to study the effects of soil pH on chlorpyrifos degradation.

Cloning and expression the *mpd* gene from isolate YC-1

The entire *mpd* functional gene of strain YC-1 was amplified by PCR with a pair of primers (Cui *et al.*, 2001). The following primers were used: forward, 5′-GAATTCATATG CCCCTGAAGAAC-3′, and reverse, 5′-GAATTCTCGAG CTTGGGGTTGACGACCG-3′ (*Nde*I and *Xho*I restriction sites, respectively, are underlined). PCR was performed under 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and elongation at 72 °C for 1 min. The amplified fragment was about 1.0 kb in length, purified by PCR purification kit, ligated into the vector pMD18-T, and then transformed into *E. coli* DH5α competent cells. White transformants were selected on Luria Bertani (LB) agar plates containing 100 μg mL^{-1} ampicillin and prespread with 20 μL of 20 mg mL^{-1} X-Gal and 40 μL of 100 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Plasmid DNA was extracted from the transformant by the alkali lysis method (Sambrook *et al.*, 1989). The purified plasmid was digested with *Nde*I and *Xho*I, and then run on 0.7% (w/v) agarose gel to confirm the size of the inserted fragment.

The inserted fragment from positive recombinant plasmid (pMDQ) was sequenced, on both strands, using an ABI 377XL DNA sequencer (PE Applied BioSystems) and a Taq FS Dye Terminator Sequencing Kit (ABI) with universal primers M13F and M13R. BLASTN was used for the nucleotide sequence identity search and BLASTP for the deduced amino acid identity search (www.ncbi.nlm.nih.gov/blast).

The recombinant plasmid pMDQ 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 pETQ. The recombinant plasmid pETQ was transformed into *E. coli* BL21 (DE3). To achieve high-level expression of *mpd* gene, mid-log-phase cells (absorbance of 0.6 at 600 nm) were induced with 1 mM IPTG for 3 h at 30 °C. The expressed products were determined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Nucleotide sequence accession number

The nucleotide sequences of the 16S rRNA gene and *mpd* gene of strain YC-1 have been deposited in the GenBank database under accession no. DQ537219 and DQ677027.

Results

Strain isolation and identification

From the sludge samples, six chlorpyrifos-degrading bacteria were isolated using chlorpyrifos as the sole carbon source by the enrichment procedure. One of them was named strain YC-1; it showed the highest degrading capability and was selected for further study. Strain YC-1 was a gram-negative, strictly aerobic, motile, and straight rod with polar flagellum. It was positive in tests for oxidase, catalase, citrate utilization, and H_2S production, but negative for glucose fermentation, starch hydrolysis, gelatin liquefaction, and nitrate reduction. The phylogenetic tree based on the 16S rRNA gene of strain YC-1 (Fig. 1) depicts the position of strain YC-1 within the genus *Stenotrophomonas*. The substrate utilization of strain YC-1 was compared with that of referred strains in the BIOLOG-GN database and strain YC-1 had the greatest similarity index of 0.82 with *Stenotrophomonas maltophilia*. Based on these observations, the isolate was putatively identified as the genus *Stenotrophomonas*. To date, no chlorpyrifos-degrading bacterium has been reported from the genus.

Cell growth and degradation in liquid culture

The degradation patterns of chlorpyrifos and the growth response of strain YC-1 in MSM are shown in Fig. 2.

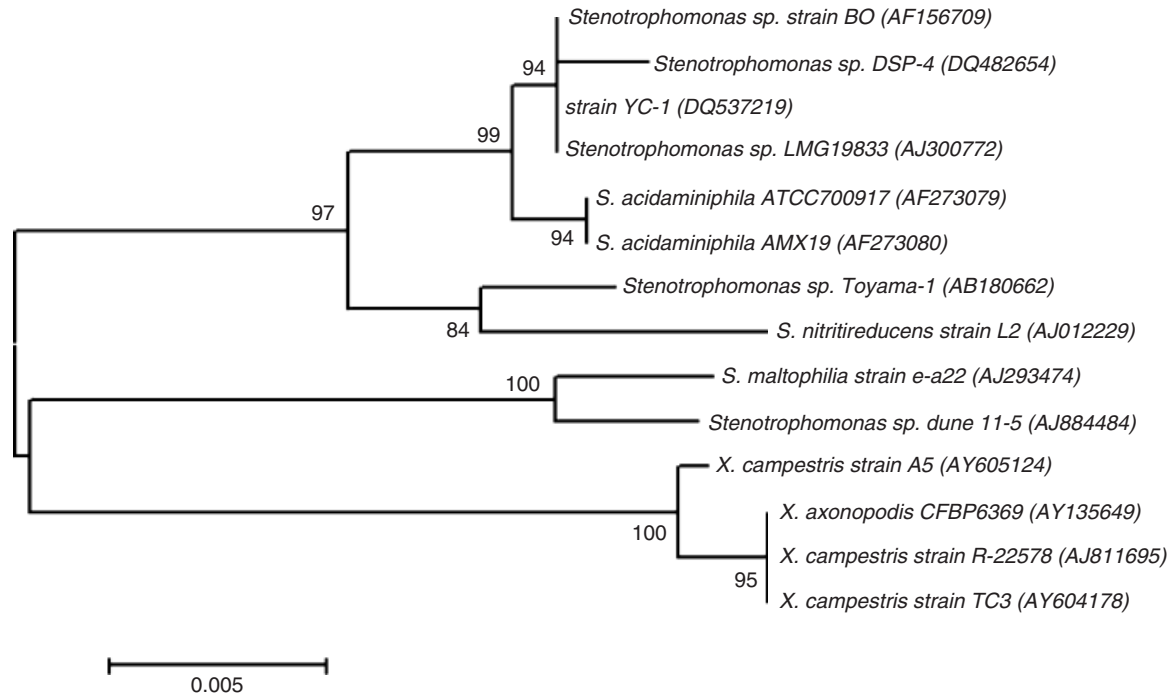

Fig. 1. Phylogenetic tree of strain YC-1 based on 16S rRNA gene sequence analysis. Bootstrap values obtained with 1000 repetitions were indicated as percentages at all branches. GenBank accession numbers are given in brackets.

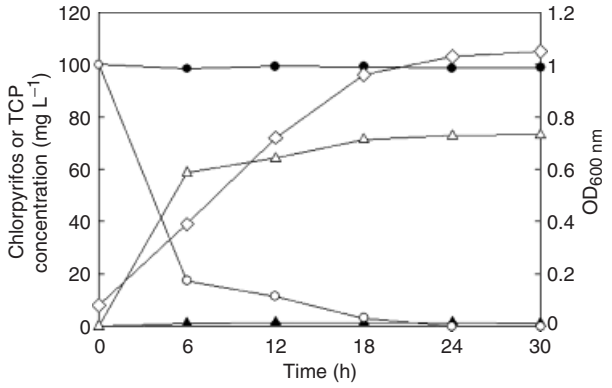

Fig. 2. Utilization of chlorpyrifos as a sole source of carbon for growth by strain YC-1. ●, chlorpyrifos control; ○, chlorpyrifos inoculated; □, $OD_{600\,nm}$; ▲, TCP control; △, TCP inoculated. Bacterial growth was monitored by measuring OD at 600 nm. The standard errors were within 5% of the mean.

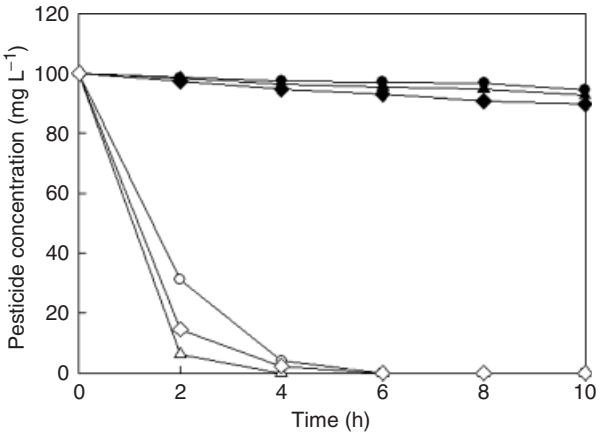

Fig. 3. Degradation of different organophosphorus pesticides by strain YC-1. ●, parathion control; ○, parathion inoculated; ▲, parathion-methyl control; △, parathion-methyl inoculated; ■, fenitrothion control; □, fenitrothion inoculated. The standard errors were within 5% of the mean.

100 mg L^{-1} of chlorpyrifos was degraded completely by strain YC-1 within 24 h and degradation of chlorpyrifos was accompanied by bacterial growth. This bacterium degraded chlorpyrifos to DETP and TCP and utilized DETP as a source of carbon and phosphorus, but it did not degrade TCP. The degradation patterns of chlorpyrifos in MSM and MSM minus P were very similar and were not affected by the absence of a phosphorus supplement (data not shown). Degradation of chlorpyrifos was negligible in uninoculated samples and no bacterial growth was observed.

All organophosphorus pesticides tested in the crossfeeding experiment were degraded by strain YC-1 (Fig. 3). 100 mg L^{-1} of parathion, methyl parathion and fenitrothion were completely degraded via hydrolysis of the phosphotriester bond by strain YC-1 within 10 h.

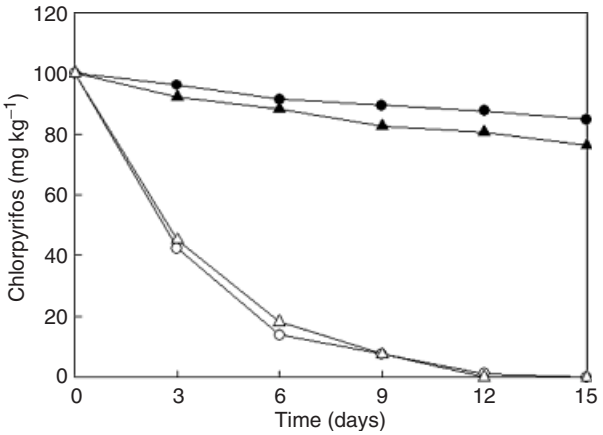

Fig. 4. Degradation of chlorpyrifos in soils inoculated with strain YC-1 at the rate of 10^6 cells g^{-1}. ●, fumigated soil, uninoculated; ○, fumigated soil inoculated; ▲, nonfumigated soil, uninoculated; △, nonfumigated soil inoculated. The standard errors were within 5% of the mean.

Degradation of chlorpyrifos by isolate YC-1 in soil

The addition of strain YC-1 to fumigated and nonfumigated soils resulted in a more rapid rate of chlorpyrifos degradation than that of uninoculated controls (Fig. 4). One hundred milligrams per kilogram of chlorpyrifos was degraded completely within 15 days. Degradation of chlorpyrifos in control nonfumigated soils (without inoculation) was lower where less than 24% of the applied concentration was degraded in 15-day incubation studies (Fig. 4). Degradation patterns of chlorpyrifos in fumigated soils with inoculation were similar to those of nonfumigated soils. After incubation for 15 days, strain YC-1 inoculated could be isolated from the nonfumigated soils, which indicated that the organism could be responsible for the observed degradation. The rate of degradation in inoculated increased with increasing soil pH from 4.3 to 7.0 but there was no significant difference in degradation rate in soil with pH 7.0–8.4 (data not shown). The degradation rate of chlorpyrifos in acidic soils was slower than in neutral and alkaline soils.

mpd gene cloning and its expression in *E. coli*

No plasmid was detected in strain YC-1 by the alkali lysis method, which indicated that the *mpd* gene was located on the chromosome. The primers in the cloning strategy were designed according to the known *mpd* gene of *Plesiomonas* sp. M6 (Cui *et al.*, 2001). The amplified fragment is shown in Fig. 5. Sequence BLAST result showed that the gene was 99% similar to *mpd* gene of *Plesimonas* sp. M6 (GenBank accession no. AF338729), 99% similar to *mpd* gene of *Pseudomonas putida* (GenBank accession no. AY029773) and 99% similar to *mpd* gene of *Ochrobactrum* sp. mp-4

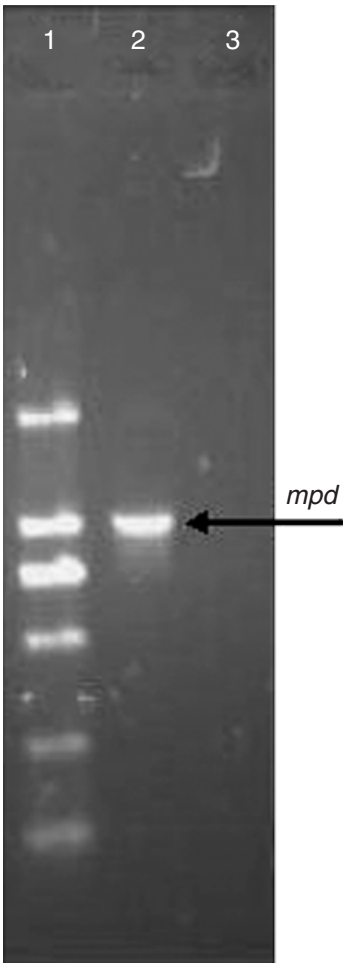

Fig. 5. PCR amplification of *mpd* gene. Lane 1, D2000 marker; lane 2, strain YC-1; lane 3, *Escherichia coli* DH5α (as control). *mpd* gene is indicated by arrow.

(GenBank accession no. AY627036) at the nucleotide level. The recombinant plasmid pMDQ was transformed into *E. coli* DH5α cells. The bacteria were then spread on LB agar plates containing 100 μg mL^{-1} ampicillin and incubated at 37 °C. HPLC analysis showed the recombinants completely degraded 100 mg L^{-1} of chlorpyrifos in liquid medium within 24 h. After the recombinant plasmid pETQ was expressed in *E. coli* BL21 (DE3), a clear IPTG inducible band corresponding to about 35 kDa was observed in SDS-PAGE (Fig. 6). The molecular mass of the expression product was identical to the calculated mass of MPH. These results indicated successful expression of the *mpd* gene in *E. coli*.

Discussion

In this study, strain YC-1 was isolated from an organophosphate-polluted wastewater and showed greatest similarity

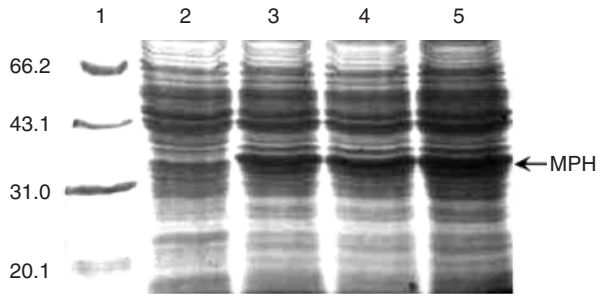

Fig. 6. Expression of *mpd* gene in *Escherichia coli*. Lane 1, protein markers; lane 2, total proteins from *E. coli* BL21 (DE3) cells harboring pETQ without IPTG induction; lane 3–5, total proteins from the same cells induced by IPTG for 1, 2 and 3 h, respectively. Expression of *mpd* is indicated by arrow.

to members of the genus *Stenotrophomonas*. Recent interest on *Stenotrophomonas* species has focused their capacity to degrade xenobiotic compounds (Binks *et al*., 1995). The bacterium isolated in this study had very strong OPH activity and the activity was induced by organophosphorus pesticides. The isolate hydrolyzed 100 mg L^{-1} of chlorpyrifos within 24 h when inoculated with 10^6 cells mL^{-1}. To our knowledge, strain YC-1 has the fastest degrading rate among the chlorpyrifos-degrading bacteria reported. It was unusual that all organophosphorus pesticides tested were used for the supply of carbon and phosphorus for strain YC-1. Other species of bacteria will utilize organophosphates as a source of carbon or phosphorus (Sethunathan & Yoshida, 1973; Rosenberg & Alexander, 1979). Our findings that addition of glucose or succinate had no effect on the degradation pattern of chlorpyrifos differ from the observed inhibitory effect of other carbon sources on degradation with *Enterobacter* sp. B-14 (Singh *et al*., 2004). The observed growth inhibition in the presence of chlorpyrifos greater than 200 mg L^{-1} could be due in part to antimicrobial activity of one of the degradation products, TCP (Racke *et al*., 1990). At the high inoculum density ($> 10^4$ cells mL^{-1}), chlorpyrifos was degraded completely within 24 h. However, degradation was slow initially at lower cell density ($< 10^4$ cells mL^{-1}), but the lag phase was followed by rapid degradation. Chlorpyrifos degradation by the isolate was very rapid at temperatures ranging from 15 to 37 °C, with the most rapid degradation rates at 30 °C. The isolate rapidly degraded chlorpyrifos in MSM at pHs ranging from 6.3 to 8.4, but degradation of chlorpyrifos was very slow at pH 5 or below. The isolate exhibited versatility in utilizing dimethyl compounds such as methyl parathion and fenitrothion, and diethyl compounds such as chlorpyrifos and parathion as its carbon source. They have diethyl (or dimethyl) phosphorothionate side chains, and there was a phosphotriester bond in all compounds, which may explain the reason for their degradation. Due to its broad specificity against a range of organophosphorus compounds, strain YC-1 possesses a great potential to be used for the remediation of highly toxic organophosphate nerve agents.

The *opd* genes from *P. diminuta* MG and *Flavobacterium* sp. ATCC 27551 were plasmid borne (Serdar *et al*., 1982; Mulbry *et al*., 1987). Horne *et al*. (2002) isolated a chromosome-based *opd* gene from *Agrobacterium radiobacter*. This gene, called *opd*A, was *c*. 88% identical at the nucleotide level to *opd* (Horne *et al*., 2002). Another gene with identical function is *mpd*, first isolated from *Plesiomonas* sp. M6 (Cui *et al*., 2001). To data the *mpd* genes detected in organophosphate degrading bacteria from Chinese soils were highly conserved although they were present in bacteria from different genera (Liu *et al*., 2005; Zhang *et al*., 2005). Here, *mpd* genes were detected in all the isolates and were chromosome based. The *mpd* and *opd* genes appear to have evolved from different sources; they have little sequence similarity and *mpd* genes have a chromosomal location, as we found for strain YC-1. An exception is the plasmid location of *mpd* in *Pseudomonas* sp. WBC-3 (Liu *et al*., 2005).

The 996 bp *mpd* gene of strain YC-1 encoded a protein with high similarity to the hydrolase from *Plesiomonas* sp. M6 (Cui *et al*., 2001); variation in amino acid composition may reflect differences in substrate specificity (Zhang *et al*., 2006). Cho *et al*. (2004) have reported minor substitutions in amino acids improved the hydrolysis of chlorpyrifos by a wild-type OPH 700-fold. OPDA has about 10-fold higher activity for dimethyl OPs than OPH (Horne *et al*., 2002). Horne *et al*. (2006) reported the difference in kinetics is largely explained by just two amino acid differences between the two proteins. The *mpd* gene sequence of strain YC-1 provided valuable information for elucidating the structural and functional relationship of the OPH.

OPH is remarkable for its extremely broad substrate profile. It can catalyze the hydrolysis of many nerve agents and organophosphorus pesticides (Dumas *et al*., 1989). MPH also might share the broad substrate range of OPH. Hydrolysis of organophosphorus compounds by OPH or MPH reduces mammalian toxicity by several orders of magnitude. Recombinant expression of genes with potential use in bioremediation in another host such as *E. coli* may be valuable for low cost production facilitating their environmental and health related uses as well as to obtain more information on substrate specificity and protein structure-activity association.

Bioremediation, which involves the use of microorganisms to detoxify and degrade pollutants, has received increased attention as an effective biotechnological approach to clean up polluted environments. Several chemicals have been successfully removed from soil and aquatic environments using degrading microorganisms such as parathion (Barles *et al*., 1979), coumaphos (Mulbry *et al*.,

1996, 1998) and atrazine (Struthers *et al.*, 1998). The addition of strain YC-1 (10^6 cells g^{-1}) to soil with a low indigenous population of chlorpyrifos-degrading bacteria treated with 100 mg of chlorpyrifos kg^{-1} resulted in a higher degradation rate than was observed in noninoculated soils. The results confirmed that the newly isolated chlorpyrifos-degrading bacterium can be successfully used for bioremediation of contaminated soils.

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