Biodegradation (2006) 17: 465–472
DOI 10.1007/s10532-005-9018-6

Cloning of the organophosphorus pesticide hydrolase gene clusters of seven degradative bacteria isolated from a methyl parathion contaminated site and evidence of their horizontal gene transfer

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Accepted 2 September 2005

Key words: horizontal gene transfer, organophosphorus hydrolase gene cluster, signal peptide, degradation

Abstract

Seven organophosphorus pesticide-degrading bacteria harboring the methyl parathion degrading (*mpd*) gene were isolated from a methyl parathion contaminated site. In this study, the 4.7 kb *mpd* gene cluster, conserved in all seven bacteria capable of degrading methyl parathion, was cloned and further analysis revealed that this cluster contained five ORFs and the *mpd* gene was associated with a mobile element, IS6100. In addition to *mpd* gene ORF and *tnp*A ORF, three other ORFs showed high homology to the permease component of ABC-type transport system, the general secretion pathway protein B, and the RNA polymerase sigma 70 factor, respectively. The *mpd* genes of these 7 strains were subcloned and expressed in *E. coli*, SDS-PAGE and zymogram analysis showed that two expression products of *mpd* genes in *E. coli* were found, but the one without signal peptide showed the hydrolytic activities. Our evidences collectively suggest that *mpd* gene cluster may be disseminated through horizontal gene transfer based on phylogenetic analysis of the cluster and their host bacterial strains, and comparisons of GC content of the cluster and respective host's chromosome.

Introduction

Organophosphorus pesticides such as parathion and methyl parathion are used worldwide to control major insect pests. These insecticides are potent acetylcholinesterase (AchE) inhibitors. Since AchE is also present in all vertebrates, the potential damage by this class of insecticides to non-target organisms is extremely high, and such pesticides are now being banned in developed countries. However, they remain the major insecticides in agricultural pest management of the developing countries.

Bacterial enzymatic detoxification of organophosphorus pesticide has become the focus of many studies, because it is economical and effective. A number of soil bacteria, belong to the genera of *Flavobacterium* (Sethunathan & Yoshida 1973), *Pseudomonas* (Serdar et al. 1982), *Agrobacterium* (Horne et al. 2002), have been found capable of degrading parathion, methyl parathion and related organophosphorus pesticides. These bacteria were isolated from diverse geographical locations, but all of them synthesized organophosphorus pesticide hydrolases (OPH), which were encoded by the organophosphorus pesticide degrading (*opd*) gene responsible for the first step reaction in the degradation of parathion or methyl parathion prior to the formation of *p*-nitrophenol (McDaniel et al. 1988). The *opd* genes from *P. diminuta* GM and

Flavobacterium sp. strain ATCC 27551 were identical and that of the Indian isolate *Flavobacterium balustinum* showed 98% similarity (Mulbry & Karns 1989; Serdar et al. 1989; Somara et al. 2002). Chaudhry et al. (1988) isolated a methyl parathion-degrading bacterium, a *Pseudomonas* sp., which possessed DNA homologous to the *opd* gene from *Flavobacterium* sp. strain ATCC 27551, as revealed by Southern blot analysis. Since both methyl parathion and parathion have very similar chemical structures, it seems reasonable that the enzymes responsible for degrading these chemicals may have homology. Previous research showed that some methyl parathion degrading-bacteria possess DNA homologous to the *opd* gene (Chaudhry et al. 1988). *Agrobacterium radiobacter* P230, capable of hydrolyzing a wide range of organophosphorus insecticides, was isolated and its hydrolase gene (called *opd*A) sequence was 88.4% similarity to the *opd* gene (Horne et al. 2002).

Studies carried out on two of the plasmids, pPDL2 from *Flavobacterium* sp. strain ATCC 27551 and pCMS1 from *P. diminuta* strain MG, by restriction analysis and hybridization experiments, indicated that the *opd* gene was located in a highly conserved region that was estimated to extend approximately 2.6 kb upstream and 1.7 kb downstream of the *opd* gene (Mulbry et al. 1986, 1987). No homology was evident between the two plasmids outside this region (Mulbry et al. 1987). Furthermore, the *opd* gene cluster was confirmed to have a transposon-like organization, suggesting a potential mechanism at the molecular level for the dispersion of the *opd* gene to other soil bacteria (Siddavattam et al. 2003).

A methyl parathion-degrading bacterium *Plesiomonas* sp. strain M6 was isolated in China and its hydrolase gene (called *mpd*, GenBank accession number AF338729) showed no homology to the *opd* gene, though strain M6 was also capable of degrading parathion and other related organophosphorus pesticide, and the *mpd* gene was located on the chromosome (Cui et al. 2001). Two other methyl parathion-degrading strains, *Pseudomonas* sp. WBC-3 and *Pseudomonas putida*, were also isolated in China, and their hydrolase genes (AY251554 and AY029773) had 99% similarity to *mpd* gene of *Plesiomonas* sp. strain M6. Alignment of these three *mpd* gene sequences revealed that partial sequences upstream and downstream the *mpd* gene were conserved in these methyl parathion-degrading strains, provided physical evidence that the *mpd* gene and its flanking regions constitute a conserved cluster. Because of the very limited information available beyond either side of the *mpd* gene, an extensive analysis of this possible gene cluster could not be made at that time. In this work, we cloned the *mpd* gene clusters from seven previously isolated methyl parathion-degrading bacterial strains, subcloned the *mpd* gene ORFs and expressed them in *E. coli* strain.

Materials and methods

Bacterial strains and plasmids

Seven organophosphorus pesticide-degrading bacteria were isolated from a methyl parathion contaminated soil by this research group and they were designated as from mp-1 to mp-7. They belonged to the genera of *Pseudaminobacter* sp., *Achromobacter* sp., *Brucella* sp. and *Ochrobactrum* sp. Their 16S rRNA gene sequences were deposited in the NCBI database under accession no. AY331575 to AY331581. The strains and plasmids used in this study are listed in Table 1.

Chemicals and culture media

Bacteria were grown at 30 °C or 37 °C in Luria-Bertani medium. When necessary, ampicillin (100 μg/ml), kanamycin (50 μg g/ml), or methyl parathion (100 μg/ml) was added to the culture medium. Enzymes and reagents for gene manipulation and expression were purchased from New England Biolabs (Beverly, MA), Promega (Madison, WI), Sigma (St. Louis, MO) and Takara (Dalian, China). Oligonucleotides were synthesized by Takara Biotechnology Co. Ltd (Dalian, China). PCR products and plasmid purification kits were from Takara Biotechnology Co. Ltd (Dalian, China).

Shotgun library construction and gene cloning

To clone the organophosphorus pesticide hydrolase genes from the seven organophosphorus pesticide-degrading bacteria, gene libraries were constructed by using shotgun-cloning technique from each of the seven bacteria. The genomic DNA of these bacteria was extracted using the

Table 1. Strains and plasmids used in this study

Strain or plasmids	Genotype or phenotype	Sources
Strains		
E. coli DH5α	ΔlacU169(ø80lacZΔM15)	Novagen
E. coli BL21(DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm (DE3)*, λDE3 lysogen	Novagen
Plasmids		
pUC19	Amp^r	Takara
pET29a	Kan^r, T7 promoter	Novagen
pZT1	Amp^r, pUC19 vector with foreign fragment from strain mp-1	This study
pZT2	Amp^r, pUC19 vector with foreign fragment from strain mp-2	This study
pZT3	Amp^r, pUC19 vector with foreign fragment from strain mp-3	This study
pZT4	Amp^r, pUC19 vector with foreign fragment from strain mp-4	This study
pZT5	Amp^r, pUC19 vector with foreign fragment from strain mp-5	This study
pZT6	Amp^r, pUC19 vector with foreign fragment from strain mp-6	This study
pZT7	Amp^r, pUC19 vector with foreign fragment from strain mp-7	This study
pET29a-*mpd*1	Kan^r, pET29a vector with *mpd* gene from strain mp-1	This study
pET29a-*mpd*2	Kan^r, pET29a vector with *mpd* gene from strain mp-2	This study
pET29a-*mpd*3	Kan^r, pET29a vector with *mpd* gene from strain mp-3	This study
pET29a-*mpd*4	Kan^r, pET29a vector with *mpd* gene from strain mp-4	This study
pET29a-*mpd*5	Kan^r, pET29a vector with *mpd* gene from strain mp-5	This study
pET29a-*mpd*6	Kan^r, pET29a vector with *mpd* gene from strain mp-6	This study
pET29a-*mpd*7	Kan^r, pET29a vector with *mpd* gene from strain mp-7	This study

high-salt-concentration precipitation method (Miller et al. 1988). *Sau*3AI was employed to obtain randomly digested chromosomal fragments. The 6–9-kb fragments were recovered using a cleanup kit (Takara Biotechnology Co. Ltd, Dalian, China) and ligated into pUC19 digested with *Bam*HI and treated with calf intestinal alkaline phosphatase (Promega). The ligation products were transformed into *E. coli* DH5α competent cells. The bacteria were then spread on LB agar plates containing 100 μg/ml ampicillin and 100 μg/ml methyl parathion as indicator and incubated at 37 °C for no more than 12 h. Positive clones that produced yellow transparent halos were picked from these gene libraries.

DNA sequencing and analysis

The positive recombinant plasmids of each degrading bacterial strain were extracted. To obtain more sequence information of the hydrolase gene flanking region, the positive recombinant plasmids containing the largest insert fragment were submitted for sequencing. The inserted fragments were sequenced with an ABI Prism model 377XL DNA sequencer (Perkin-Elmer Applied BioSystems, Foster City, California), initially using pUC19 plasmid vector specific primers, subsequently the internal primers for both strands sequenced. Computer analysis of the nucleotide sequences was performed with BioEdit software (Hall 1999), ORFs were identified using the National Center for Biotechnology Information (NCBI) ORF finder tool and subsequently subjected to database searches using the BlastX (version 2.2.3) programs (Altschul et al. 1997). Promoter prediction was achieved online (http://www.fruitfly.org/seq_tools/promoter.html). The 5′ regions of the deduced organophosphorus pesticides hydrolase genes were then checked for the presence of ribosome binding sites as described by Stormo et al. (1982). The N-terminal sequences of 60 amino acids of the deduced organophosphorus pesticides hydrolases were analyzed with SignalP-NN V2.0 program (http://www.cbs.dtu.dk/services/signalP-2.0/#submission) (Nielsen et al. 1997). Multiple-sequence alignments were carried out with Clustal X (Thompson et al. 1997).

Subcloning and expression of organophosphorus pesticide hydrolase genes

Based on the deduced open reading frame (ORF) of *mpd* genes, oligonucleotide primers were

synthesized to obtain the structural genes by PCR amplification. The forward primer (5′-CGCCATATGCCCCTGAAGAACCGC-3′) overlapped an *Nde*I site (underlined) at the initiation site for organophosphorus pesticide hydrolase gene, the reverse primer 5′-CGCCTCGAGTCATCACTTGGGGTTGACGACCGA-3′ overlapped an *Xho*I site (underlined) after the termination cordon.

The PCR reaction mixtures consisted of 50 ng of each of the recombinant plasmids of these positive clones as the template, 50 pmol each of forward and reverse primers, 1× Pfu DNA polymerase reaction buffer (contain 1.5 mmol/l $MgCl_2$), 0.2 mmol/l dNTP (deoxynucleotide triphosphate), and 2.5 U of Pfu DNA polymerase for a final volume of 50 μl. The PCR reaction was performed in a Perkin-Elmer PE9600 thermocycler with the following cycling profile: initial denaturation at 96 °C for 2 min, 35 cycles of denaturation at 96 °C for 1 min, annealing at 65 °C for 1 min, and extension at 72 °C for 2 min, final extension at 72 °C for 7 min. The amplified fragment was about 1.0 kb in length, purified by PCR purification kit, digested with *Nde*I and *Xho*I, ligated with *Nde*I–*Xho*I-digested pET29a, and then transformed into *E. coli* BL21 (DE3) competent cells. The recombinant plasmids were designated pET29a-mpd.

A stock culture of *E. coli* BL21 (DE3) harboring pET29a-mpd was grown in LB-kanamycin at 37 °C overnight and 2% of stock culture was inoculated into fresh-prepared LB-kanamycin medium. After 4 h growth and the induction with 0.5mmol/l IPTG (isopropyl-β-D-thiogalactopyranoside) for 1 h, the expressed products were determined by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel eletrophoresis).

Zymogram

Zymogram analysis was performed according to the procedures described (Granelli-Piperno & Reich 1978; Vassalli et al. 1984; Li et al. 2001; Malone et al. 2002). The cell pellet from 1 ml cultures was resuspended in 100 μl 1× SDS-loading buffer without β-mercaptoethanol, heated for 5 min at 100 °C, and then subjected to 12% SDS-PAGE. After electrophoresis, the proteins were renatured to detect MPH activity. The polyacrylamide gel was washed with 2.5% Triton-100 twice (each for 30 min) to remove SDS, and then with 0.1 mol/l Tris–HCl (pH 8.0) twice (each for 30 min) to remove Triton-100. The gel was evenly laid on the LB agar plate containing 100 mg of methyl parathion/l. The plate was incubated at room temperature (24 ± 1 °C). Photograph was taken when transparent hydrolytic bands clearly appeared.

Nucleotide sequences accession numbers

The *mpd* gene cluster nucleotide sequences of the seven bacteria obtained in this study were deposited in the NCBI database under accession no. AY627033 to AY627039. The 16S rRNA gene sequences of the seven degrading bacteria are available in the NCBI database under accession no. AY331575 to AY331581.

Results

Cloning and analysis of organophosphorus pesticide hydrolase gene clusters

Sequence alignments of the largest insert fragment of the positive clones in each strain's gene library revealed that a 4777 bp region was conserved in all seven bacteria used in this investigation. Computer analysis showed that this region contained a total of five ORFs (Figure 1), two ORFs on one strand, and the other three on the complementary one. BLAST searches revealed that an ORF containing 996 base pairs had sequence similarity to the *mpd* gene. Promoter prediction indicated that a promoter-like sequence was located upstream of the *mpd* ORF and it had a typical *E. coli* promoter structure of TTGCAA-17 nucleotides-TATACT. A ribosome-binding site (AGGAAA) was also located 5 nucleotides upstream of the start code. Considering that the regulation element of the *mpd* gene could be recognized by *E. coli* DH5α

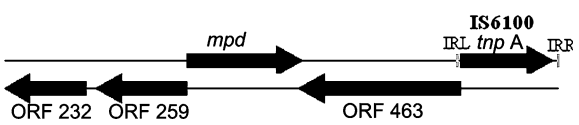

Figure 1. Gene organization map of the conserved 4777 bp *mpd* gene cluster. Both strands were shown. The black solid arrows indicate the ORFs and their transcription direction, grey rectangle indicate the inverted repeats of IS 6100 element, IRL, left inverted repeat; IRR, right inverted repeat.

transcription and translation mechanisms, it was deduced that the promoter region and 996 bp ORF was the complete sequence of organophosphorus pesticide hydrolase gene. The predicted organophosphorus pesticide hydrolase polypeptide contained 331 amino acids. When compared at the gene level, the seven hydrolases genes could be divided into five groups, the *mpd* genes of strain mp-2 and mp-7, and strain mp-5 and mp-6 were identical. The *mpd* gene sequences of the five groups contained eleven difference nucleotide sites. At the amino acids level, the seven hydrolases could be divided into four groups, hydrolases of strain mp-1, mp-5 and mp-6 were identical. There were 9 sites with different amino acids and the similarities among them were 97% (Table 2). Results of degrading experiments showed that very little differences were observed in term of the types of organophosphorus pesticides degraded and mp-4 was the only one capable of hydrolyzing trizophos among all seven (Zhang et al. 2004).

Evidence of horizontal transfer of mpd gene cluster

Detailed analysis of the conserved *mpd* gene cluster revealed that about 1.3 kb downstream of the *mpd* ORF was a perfect insertion sequence (IS6100) which has 880 nucleotides. The IS6100 element contains only one ORF of a transposase (Tpase A), and flanks by 14 base pairs terminal inverted repeat sequences (IR). The left inverted repeat sequence is 5′-GGCTCTGTTGCAAA-3′, and the right one is as follow: 5′-TTTGCAACAGAGCC-3′. In this conserved *mpd* gene cluster, the *mpd* gene is associated with an insertion sequence, providing evidence that the *mpd* gene cluster is a catabolic transposon. BLAST searches of the other three ORFs show that ORF 232 has 50% identity and 62% similarity over 232 amino acids to a permease component of ABC-type transport system (GenBank accession number, ZP 00360730) while ORF 259 has 25% identity and 43% similarity over 218 amino acids to *exe* B gene product, a general secretion pathway protein B (EMBL accession number, X81473), which may have role contributing to the secretion of the organophosphorus pesticide hydrolase. However, further research is needed to confirm this. ORF 463 has 70% identity and 79% similarity over 292 amino acids to the RNA polymerase sigma 70 factor (GenBank accession number, YP105080).

When spread of the *mpd* gene has occurred among these organophosphorus pesticide-degrading isolates, one would expect the lineage of the transferred gene to be phylogenetically less diverged, i.e., more closely related, than that of the host cells. This assumes, as expected, that the mutation rate of the 16S rRNA gene is lower than that of *mpd*. Thus, sequence divergence of the 16S rRNA gene, coupled with sequence conservation in the *mpd* gene cluster, would constitute strong evidences for *in situ* horizontal transfer of *mpd* among bacteria indigenous to the study site.

GC content of the 4777 bp fragment and the chromosomes of their host were also compared. Strains mp-1, mp-2 and mp-7 were *Pseudaminobacter* sp., *Achromobacter* sp. and *Brucella* sp., and the average GC content of their chromosomes was 63%, 65% and 57%, respectively (Kampfer et al. 1999; Coenye et al. 2003; Halling et al. 2005), the remaining strains were *Ochrobactrum* sp. and the average GC content of its chromosome was

Table 2. Comparison of differences in amino acid sequence of organophosphorus pesticide hydrolases from the seven bacterial strains used in this study

Strain	Selective position and the corresponding amino acid								
	34	72	271	274	275	277	280	301	304
mp-1	H	R	N	D	I	G	A	S	P
mp-2	H	L	I	V	S	S	A	A	S
mp-3	H	R	I	D	S	S	A	T	S
mp-4	R	R	T	D	S	S	V	S	S
mp-5	H	R	N	D	I	G	A	S	P
mp-6	H	R	N	D	I	G	A	S	P
mp-7	H	L	I	V	S	S	A	A	S

57.7–58.3% (Velasco et al. 1998). The GC content of the conserved *mpd* gene cluster was 69.63%, which was distinctively different from and higher than that of their host chromosomes, providing another evidence that this insertion fragment was acquired through horizontal transfer.

Subcloning and expression of organophosphorus pesticide hydrolase genes and zymogram analysis of the functional organophosphorus pesticide hydrolases

Based on the deduced *mpd* gene ORFs, their *mpd* genes were amplified using PCR method and then subcloned into expression vector pET29a. SDS-PAGE analysis of the *E. coli* BL21 (DE3) harboring pET29a-mpd showed that two new protein bands of about 35 and 32 kDa appeared compared with the control harboring pET29a only (Figure 2a). The 35 kDa protein agreed well with the molecular weight of organophosphorus pesticide hydrolase deduced from its coding sequence, but the 32 kDa protein was slightly smaller than the deduced molecular weight. The N-terminal sequences of 60 amino acids of the deduced organophosphorus pesticide hydrolases was analyzed with SignalP-NN V2.0 program (Nielsen et al. 1997). The statistical results (data not shown) showed that there were possibilities greater than 99% that the first 35-terminal amino acid peptides were signal peptides, and the most likely cleavage site is between position 35 and 36 (AHA-AA). The smaller protein in SDS-PAGE gel may represent another form of *mpd* gene expression products, which were processed from their precursors by removing the terminal fragments consisting of the first 35 amino acids, this phenomenon was confirmed with the *mpd* gene from *Plesiomonas* sp. strain M6 (Fu et al. 2004).

Zymogram was used to detect organophosphorus pesticide hydrolase activity with methyl parathion as a substrate. Only the band of the smaller protein was associated with hydrolytic activity, producing the single hydrolytic band corresponding to 32 kDa. From these results, it is feasible that two forms of *mpd* gene expression products in *E. coli* BL21 (DE3) were formed, the organophosphorus pesticide hydrolase precursor showing no hydrolytic activity and the active hydrolase with N-terminal signal peptide removed.

Discussion

Seven bacteria isolated from contaminated sites were capable of organophosphorus pesticide degradation. Southern blot analysis by using *mpd* as probe demonstrated that they were similar to *mpd* of *Plesiomonas* sp. strain M6 and were located on the chromosome (Zhang et al. 2004). Analysis of the *mpd* gene cluster revealed that it constituted a catabolic transposon.

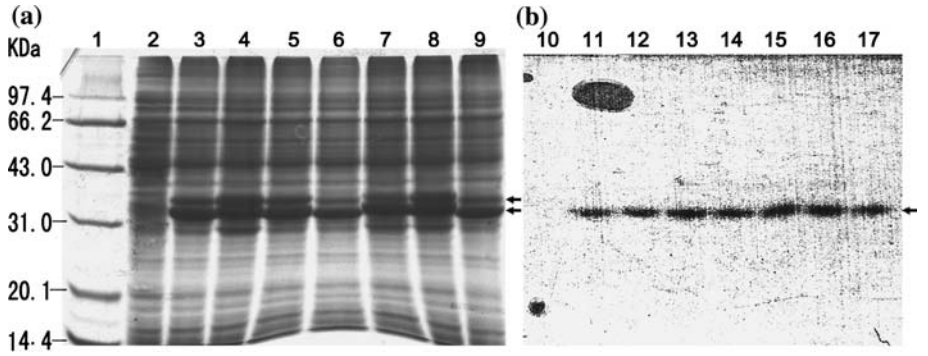

Figure 2. SDS-PAGE (a) and zymogram (b) analysis of *mpd* gene products in *E. coli* BL21(DE3) harboring pET29a and pET29a-*mpd*. Cell pellets were dissolved in 1× SDS-loading buffer without β-mercaptoethanol, subjected to 12% SDS-PAGE and zymogram analysis. Lane 1, molecular mass markers with the sizes shown aside; lanes 2 and 10, *E. coli* BL21(DE3) harboring pET29a [this served as the negative control]; lanes 3 and 11, *E. coli* BL21(DE3) harboring pET29a-*mpd*1; lanes 4 and 12, *E. coli* BL21(DE3) harboring pET29a-*mpd*2; lanes 5 and 13, *E. coli* BL21(DE3) harboring pET29a-*mpd*3; lanes 6 and 14, *E. coli* BL21(DE3) harboring pET29a-*mpd*4; lanes 7 and 15, *E. coli* BL21(DE3) harboring pET29a-*mpd*5; lanes 8 and 16, *E. coli* BL21(DE3) harboring pET29a-*mpd*6; lanes 9 and 17, *E. coli* BL21(DE3) harboring pET29a-*mpd*7. The two arrows showed the protein bands (a) while the single one indicated the hydrolytic bands (b).

In this study, the *mpd* gene clusters of the seven organophosphorus pesticide degrading bacteria were sequenced and the deduced organophosphorus hydrolases could be assigned into four groups distinguished at nine amino acid sites. This variation contributes to slight changes in the specificity between hydrolase and substrates as indicated in the organophosphorus hydrolase of strain mp-4 to hydrolyze triazophos (Zhang et al. 2004). Cho et al. (2004) have reported the altering of substrate specificity of the *opd* gene encoded organophosphorus hydrolase by directed evolution. The generated organophosphorus hydrolase variant, which has only several amino acid substitutions can improve 700-fold the hydrolysis of an otherwise poorly hydrolyzable substrate, chlorpyrifos (1200-fold less efficient than paraoxon). The *mpd* genes sequences of this research provided valuable information for elucidating the functional and structural relationship of the organophosphorus hydrolase.

The results presented here suggest that horizontal transfer of *mpd* gene cluster may play a very important role in the adaptation of bacterial populations to methyl parathion contamination. This compound may provide selective pressure for genetic adaptation of bacteria in field sites because availability of carbon source is the major limiting factor for microbial growth. The occurrence of catabolic transposons is now well documented. For example, catabolic genes for toluene, naphthalene, chlorobenzene, benzene and many other xenobiotic chemicals are associated with transposable elements (Wyndham et al. 1994; Tan 1999). These transposons include both class I composite elements, in which catabolic genes are flanked by IS elements, and class II transposons that are all ancestrally related to the Tn3 family of transposons and are characterized by short (fewer than 50 bp) IRs and the involvement of a transposase (TnpA) and a resolvase (TnpR). It has been reported that the plasmid-borne *opd* gene cluster is on a class II transposon (Siddavattam et al. 2003), but our work suggest that only one IS6100 element was associated with *mpd* gene. We have sequenced 2 kb upstream of the 4777 bp region of strain mp-1, no other IS elements were found (data not shown).

Taken together, in this study we showed that *mpd* gene cluster constituted a catabolic transposon and it contributed to the dispersion of this gene among indigenous bacteria in the polluted soil site.

Acknowledgements

This work was supported by grants 30400014 and 680600016 from the Chinese National Natural Science Foundation, and by the Chinese National Programs for High Technology Research and Development (2003AA241150).

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