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ORIGINAL PAPER

Isolation of a methyl parathion-degrading strain *Stenotrophomonas* sp. SMSP-1 and cloning of the *ophc2* gene

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Abstract A rod-shaped, gram-negative bacterium *Stenotrophomonas* sp. SMSP-1 was isolated from the sludge of a wastewater treating system of a pesticide manufacturer. Strain SMSP-1 could hydrolyze methyl parathion to *p*-nitrophenol (PNP) and dimethyl phosphorothioate but could not degrade PNP further. Strain SMSP-1 was able to hydrolyze other organophosphate pesticides, including fenitrothion, ethyl parathion, fenthion, and phoxim, but not chlorpyrifos. A 4395-bp DNA fragment, including an organophosphorus hydrolase encoding gene *ophc2*, was cloned from the chromosome of strain SMSP-1 using the shotgun technique. Its sequence analysis showed that *ophc2* was associated with a typical mobile element IS*Ppu12* consisting of *tnpA* (encoding a transposase), *lspA* (encoding a lipoprotein signal peptidase), and *orf1* (encoding a CDF family heavy metal/H^+ antiporter). The *ophc2* gene was effectively expressed in *E. coli*. This is the second report of cloning the *ophc2* gene and the first report of this gene from the genus of *Stenotrophomonas.*

Keywords Biodegradation · Methyl parathion · *Stenotrophomonas* · *ophc2*

Introduction

Organophosphate (OP) pesticides such as ethyl parathion (*O*,*O*-diethyl *O*-*p*-nitrophenylphosphorothioate) and methyl parathion (*O*,*O*-dimethyl *O*-*p*-nitrophenylphosphorothioate; MP) have been used extensively for the control of a wide range of insect species all over the world. These insecticides inhibit acetyl cholinesterase in an irreversible manner and cause insect death (Karalliedde and Senanayake 1989). The potential for damage by this class of insecticides to nontarget organisms is very high because acetyl cholinesterase is present in all vertebrates (Dumas et al. 1989; Sogorb et al. 2004).

Organophosphates contain three phosphoester linkages, and the hydrolysis of one phosphoester bond can reduce their toxicity significantly. Therefore, it is considered to be an effective way to reduce the toxicity of OPs. Bioremediation is considered a major method for degrading contaminants in the environment and takes various forms. Investigations of microbial degradation are useful in the development of strategies for the detoxification of the insecticides by microorganisms. Many researchers have isolated strains that can hydrolyze organophosphorus pesticides. Two parathion-degrading bacteria, *Pseudomonas diminuta* MG and *Flavobacteriun* sp.

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ATCC27551, were isolated in the Philippines and the United States (Serdar et al. 1982; Mulbry et al. 1986). *Agrobacterium radiobacter* P230, which could hydrolyze a wide range of organophosphorus pesticides, was isolated in Australia (Horne et al. 2002). An MP-degrading bacterium M6 (Cui et al. 2001) and an OP-degrading bacterium *Pseudomonas pseudoalcaligenes* C2-1 (Wu et al. 2004) were isolated in China. These bacteria all possessed OP pesticide hydrolase (OPH) activity. Three OPH encoded genes (*opd*, *mpd*, and *ophc2*) have been reported to date. The *opd* gene was found in *P. diminuta* MG (GenBank Accession No. M20392; McDaniel et al. 1988), *Flavobacteriun* sp. ATCC27551 (GenBank Accession No. M22863; Harper et al. 1988), and *A. radiobacter* P230 (GenBank Accession No. AY043245; Horne et al. 2002). The *mpd* gene was found in *Plesiomonas* sp. M6 (GenBank Accession No. AF338729; Cui et al. 2001) and *Burkholderia* sp. FDS-1 (GenBank Accession No. AY646835; Zhang et al. 2006a). However, *ophc2* has only been found in *P. pseudoalcaligenes* C2-1 (GenBank Accession No. AJ605330; Wu et al. 2004).

In the present study, an MP-degrading strain SMSP-1 was isolated from the genus of *Stenotrophomonas*. Its OPH encoding gene was cloned, sequenced, and effectively expressed in *E. coli*. This gene sequence has 98% homology with gene *ophc*2. This is the second report of cloning the *ophc*2 gene and the first report of this gene from the genus of *Stenotrophomonas.*

Materials and methods

Medium

Mineral salts medium (MM; pH 7.0) contained (g l^{-1}) NaCl 1.0, NH_4NO_3 1.0, KH_2PO_4 0.5, K_2HPO_4 1.5, and $MgSO_4{\cdot}7H_2O$ 0.1. MPMM was MM supplemented with 20 mg l^{-1} MP. Luria–Bertani medium (LB; pH 7.0) contained the following constituents (g l^{-1}): tryptone 10.0, yeast extract 5.0, and NaCl 10.0.

Chemicals and analysis method

Methyl parathion (purity of 99%), chlorpyrifos (purity of 98%), phoxim (purity of 99%), fenitrothion (purity of 98%), ethyl parathion (purity of 99%), and fenthion (purity of 99%) were obtained from Beijing Kang-Lin Science & Technology Co. Ltd., China. All other chemicals used were of analytical grade, and purchased from Shanghai Chemical Reagent Co. Ltd., China.

For the determination of OP pesticides, all samples were analyzed as follows: 2 ml aliquot of the sample was extracted with 5 ml dichloromethane. The extracts were dried over anhydrous Na_2SO_4 and then evaporated under reduced pressure using a centrifugal evaporator at room temperature. The residual organic material was redissolved in 5 ml acetonitrile, and 20 µl of the resulting solution was subjected to reverse-phase high-performance liquid chromatography (HPLC; 600 Controller, Rheodyne 7725i Manual Injector and 2487 Dual λ Absorbance Detector; Waters Co., Milford, MA). The separation column for the HPLC (internal diameter, 4.6 mm; length, 25 cm) was filled with Kromasil 100-5C18. The mobile phase was acetonitrile/water (70:30, v/v), and the flow rate was 1.0 ml min^{-1}. Detection wavelengths for MP, chlorpyrifos, phoxim, fenitrothion, ethyl parathion, and fenthion were 273, 227, 274, 267, 274, and 243 nm, respectively. For the detection of PNP produced by MP hydrolysis, the sample was centrifuged at 5,000 × *g* for 15 min, and the supernatant was filtered through a 0.45 µm fiber filter to remove the cell and cell debris. The resulting solution was subjected to HPLC detection as described above, and the detection wavelength was 315 nm.

Isolation and identification of MP-degrading strain

MP-degrading strain was isolated by the enrichment culture technique. The OP-contaminated sludge was collected from a wastewater treating system of a pesticide manufacturer. Five milliliters of the sludge were added into a 250 ml flask with 100 ml MPMM. The culture was incubated at 30°C at 180 rpm for 5 days. Five milliliters of enrichment culture were then subcultured into fresh MPMM every 5 days. HPLC was used to detect MP and confirm degradation. Then, the enriched culture was serially diluted and spread on MPMM plates containing 2% (w/v) purified agar. After 3 days of incubation at 30°C, microbial colonies became visible and a clear yellow

halo appeared around colonies capable of degrading MP, which were purified and tested for their degrading capability in liquid MPMM.

Identification of strain SMSP-1 was carried out according to Bergey's Manual of Determinative Bacteriology (Holt et al. 1994) and the sequence analysis of its 16S rRNA gene. Genomic DNA was extracted by the method of high salt concentration precipitation (Miller et al. 1988). The 16S rRNA gene was amplified by PCR using standard procedures. The PCR product was ligated into the vector pMD-18T (TaKaRa Biotechnology, Dalian, China) and then transformed into *E. coli* DH5α. An automatic sequencer (Applied Biosystems; 3730) was used to determine the 16S rRNA gene sequence. The 16S rRNA gene sequence 1417 bp in length was deposited at GenBank under Accession No. EU312979. Alignment of the different 16S rRNA gene sequences from GenBank database was performed using CLUSTALX 1.8.3 (Thompson et al. 1997) with default settings. Phylogenesis was analyzed by MEGA version 3.0 software. Distances were calculated using the Kimura two-parameter distance model. Unrooted tree was built by the neighbor joining method. The dataset was bootstrapped 1,000 times (Weisburg et al. 1991).

Inoculum preparation for degradation experiments

The SMSP-1 cells were collected by centrifugation at 5000 × *g* for 5 min at room temperature after it was precultured in LB medium at 30°C on a shaker at 180 rpm until the later exponential phase. Cell pellets were washed twice with MM and adjusted to OD 1.5 at 600 nm (OD_{600nm}). For all experiments, cells were inoculated at 5% (v/v) level into a 250 ml flask containing 100 ml MPMM and then incubated at 30°C at 180 rpm unless otherwise stated.

Degradation experiments

Degradation studies were carried out at 30°C in MPMM. Cultures were regularly sampled and checked for MP degradation and PNP accumulation. The medium without inoculation was used as controls.

Cross-feeding studies with other OP pesticides were also performed. The liquid MM was supplemented with fenitrothion, chlorpyrifos, phoxim, fenthion, and ethyl parathion at 20 mg l^{-1}. Pesticide residues were determined as described above.

Cloning and expression of the *ophc2* gene from strain SMSP-1

Plasmids pUC118 and pET29a (TaKaRa Biotechnology, Dalian, China) were used as cloning and expression vectors, respectively. *E. coli* DH5α and *E. coli* BL21(DE3) were used as the host of the cloning and expression plasmids, respectively. Genomic DNA of strain SMSP-1 was extracted by the method described above and digested partially with *Sau*3AI. The 3–9 kb fragments were recovered by DNA Gel Extraction Kit (V-gene Biotechnology Ltd., Hangzhou, China) and ligated into pUC118. The ligation product was transformed into competent *E. coli* DH5α cells, which were then plated on LB plates containing 100 mg l^{-1} ampicillin and 100 mg l^{-1} MP. The plates were incubated at 37°C for 16 h and then at 10°C for 15 days. Positive clones producing clear yellow halos were selected to analyze the sequence of the inserted fragments in their recombinant plasmids.

To express the *ophc2* gene, the *ophc2* and *ophc2-n* genes (*ophc*2 gene without the predicted signal peptide encoding sequence) were amplified from the genomic DNA of strain SMSP-1 by PCR. Primers designed according to the sequence analysis results were as follows: *ophc2*-F (5′-CATATGCGTCTTTTCTCGC TGAG-3′, forward), *ophc2-n*-F (5′-CATATGGCCGC ACCGGCACAACAG-3′, forward), and *ophc2*-B/*ophc2-n*-B (5′-CTCGAGGCGGTCGCTACGGATC GG-3′, reverse). The *Nde*I and *Xho*I sites (underlined) were incorporated into primers to facilitate directional cloning of the PCR product into the *Nde*I-*Xho*I site of pET29a. The expressed protein was the natural product without S tag of the vector. The resulting plasmids were designated as pE-*ophc2* and pE-*ophc2-n*, respectively, and were transformed into *E. coli* BL21(DE3). The transformants were subcultured into 50 ml LB medium and allowed to grow until culture density reached 0.5 (OD_{600nm}). Then, *ophc* and *ophc2-n* gene expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mmol l^{-1}, and 0, 1, 2, and 3 h later, crude enzyme extracts prepared from these cultures were

analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme assay

Crude enzyme extracts of *E. coli* BL21(DE3)/pE-*ophc2* and pE-*ophc2-n* were prepared using the following method: IPTG-induced cells were collected by centrifugation at 5,000 × *g* for 20 min. These cells were suspended in phosphate-buffered saline (PBS; 200 mmol l^{-1}, pH 7.4) and sonicated at 4°C for 5 min. The homogenate was centrifuged at 15,000 × *g* for 20 min, and the supernatant was used as crude enzyme extract. The OPH activity was determined according to the PNP production released by MP hydrolysis. Crude enzyme extract samples (20 µl) were added to an assay mixture containing 974 µl PBS and 6 µl MP (10 mg ml^{-1}). One unit of OPH activity was defined as the amount of enzyme required to produce 1 µmol PNP min^{-1} at 30°C.

Results

Isolation and identification of MP-degrading strain SMSP-1

Bacterial strain SMSP-1 with the ability of degrading MP was obtained from the enrichment. It produced a yellow halo on the MPMM solid plates. UV scanning was used to determine MP hydrolysis. The scanning results are shown in Fig. 1. The maximum absorption peak of MP was recorded at 273 nm. A new absorption peak at 315 nm appeared, which was caused by the hydrolyzing product PNP (with maximum absorption at 315 nm). Taxonomic properties of strain SMSP-1 were as follows: a rod-shaped bacterium with dimensions of about 1.5 µm in length and 0.5 µm in width; gram-negative; esterase production, positive; egg yolk reaction, negative; nitrate reduction, positive; and hydrolysis of gelatin, positive. Its 16S rRNA gene sequence was compared with those available sequences in GenBank. Strain SMSP-1 showed high similarity with the *Stenotrophomonas* species. A phylogenetic tree based on known representatives of describing *Stenotrophomonas* species and other species is presented in Fig. 2. Based on the above characters, strain SMSP-1 was preliminarily identified as *Stenotrophomonas* sp.

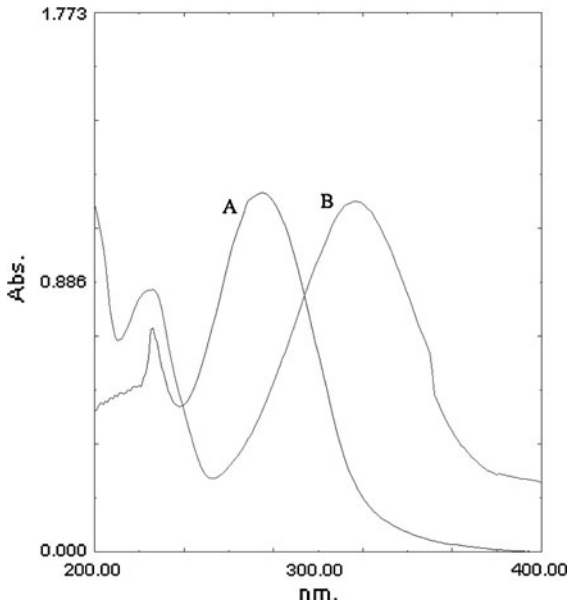

Fig. 1 Determination of MP degradation by strain SMSP-1 via UV scanning. Curves: A, MP CK (20 mg l^{-1} in MPMM); B, MP treated by strain SMSP-1 after 48 h

MP-degrading ability of strain SMSP-1

The degradation kinetics of MP and production PNP were investigated simultaneously. MP was completely hydrolyzed to PNP up to the 48th hour. In the entire degradation period, upon the disappearance of MP, PNP accumulated with approximately the same rate as the decrease of MP. PNP could not be further degraded by strain SMSP-1 (Fig. 3). Optimal temperature and initial pH of medium for degradation of MP were 30°C and 7, respectively.

The degradation capability of strain SMSP-1 toward other OP pesticides was also tested. A total of 20 mg l^{-1} fenitrothion and ethyl parathion was completely degraded via hydrolysis of the phosphoester bond within 48 h. Degradation rates of fenthion and phoxim were 37.8 and 59.7%, respectively, during 48 h, but strain SMSP-1 showed no degradation capability toward chlorpyrifos (data not shown).

Cloning and sequence analysis of the *ophc2* gene

Three positive clones that produced yellow halos were selected. The recombinant plasmids they harbored were designated as pT1, pT2, and pT3. They carried 4.8, 7.0, and 9.0 kb inserts, respectively, and

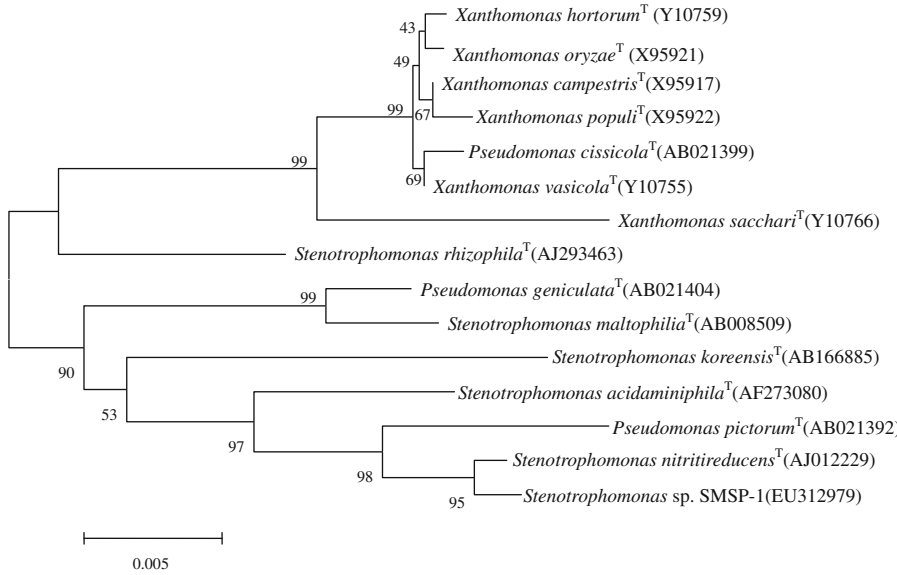

Fig. 2 Phylogenetic tree of strain SMSP-1 based on 16S rRNA gene sequence analysis. Bootstrap values obtained with 1,000 repetitions are indicated as percentages at all branches

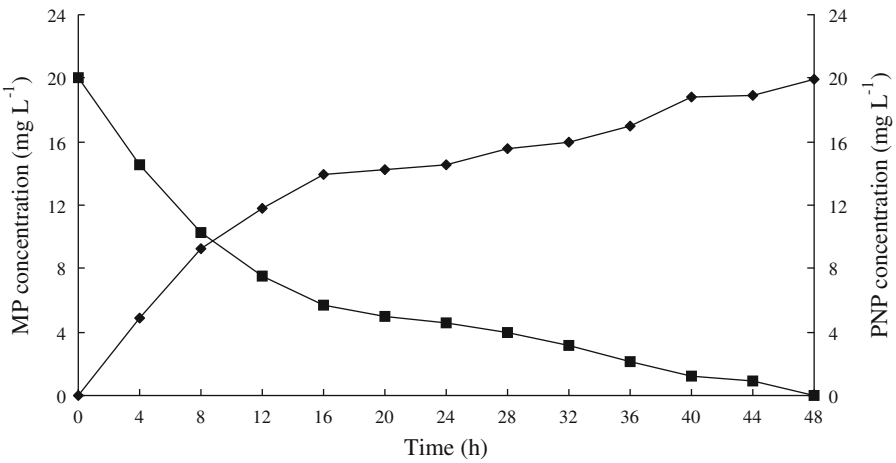

Fig. 3 MP degradation and PNP accumulation by strain SMSP-1. *filled square*, the concentration of MP; *filled diamond*, the concentration of PNP. Negative control without inoculating strain SMSP-1 shows no notable change in MP concentration. Data are means of triplicate treatments; standard error is less than 5%

restriction map analysis indicated that the 4.8 kb fragment was included in pT2 and pT3. The inserted fragment of pT1 was sequenced and analyzed. Its exact length was 4395 bp and designated as F1 (GenBank Accession No. EU651813). Sequence of the F1 fragment was analyzed by online ORF Finder and BLASTx program (www.ncbi.nlm.nih.gov). The result is shown in Fig. 4. The *ophc2* gene (334-1308) of strain SMSP-1 showed 98% similarity with the *ophc2* gene of *P. pseudoalcaligenes* C2-1 (GenBank Accession No. AJ605330), its encoding protein OPHC2 (an OPH) contained a 24-amino acid signal peptide and the predicted cleavage site was between the 24th and 25th amino acids. The *ophc2* gene of strain SMSP-1 was associated with a typical mobile element IS*Ppu12* consisting of IS*Ppu12*-IR (1473-1496), *tnpA* (encoding a transposase; complement 1531-2745), *lspA* (encoding a lipoprotein signal peptidase; complement 2842-3354), *orf1* (encoding a CDF family heavy metal/H^+ antiporter; complement (3358-4254), and IS*Ppul2* promoter (4255-4297 complement). Its sequence showed 99% similarity with IS*Ppu12* of *Pseudomonas putida* mt-2 (GenBank Accession No. AY138113; Peter et al. 2002), but the obtained IS*Ppu12* element in strain SMSP-1 was not complete and only lacked another IS*Ppu12*-IR.

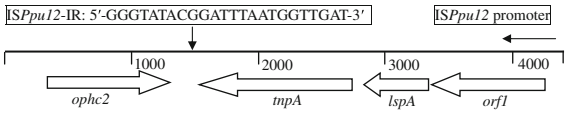

Fig. 4 The putative gene map of F1 fragment. Coding regions and transcriptional direction of genes are indicated by *broad arrows*

The deduced site might be in the upstream of IS*Ppul2* promoter region according to the sequence of *P. putida* mt-2. The mobile element IS*Ppu12* was also reported in several other bacteria generally associated with plasmids and xenobiotic degradation genes, and it had been suggested to be involved in gene silencing, activation, and horizontal transferring (Weightman et al. 2002). However, the function of the IS*Ppu12* element associated with the *ophc2* gene in strain SMSP-1 needs further study.

Expression of the *ophc2* gene in *E. coli* BL21 (DE3)

The expression of *ophc2* and *ophc2-n* in *E. coli* BL21(DE3) cells was analyzed by SDS-PAGE. Results showed that both genes were hyperexpressed in *E. coli*. There were two more protein bands compared with the control harboring only pET29a. The molecular weight of one band was about 36 kDa, and another one was about 2–3 kDa smaller (indicated by two arrows in lanes 4–6 in Fig. 5). Only one band with the same molecular weight as the former

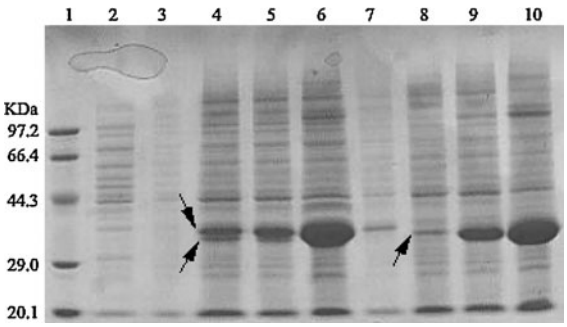

Fig. 5 Expression of *ophc2* and *ophc2-n* gene in *E. coli* BL21(DE3) Lane 1, protein molecular weight standards. Lane 2, crude enzyme extracts of *E. coli* BL21 (DE3)/pET29a as a control. Lanes 3–6, crude enzyme extracts of *E. coli* BL21 (DE3)/pE-*ophc2* after induction for 0, 1, 2, and 3 h, respectively. Lanes 7–10, crude enzyme extracts of *E. coli* BL21 (DE3)/pE-*ophc2-n* after induction for 0, 1, 2, and 3 h, respectively. The target proteins are indicated by *arrows*

smaller one appeared in lanes 8–10, which indicated that the protease cleavage site was located at the N terminal of OPHC2 and properly cleaved by proteases of *E. coli*. Molecular weight of the protein with signal peptide was a little larger than that without signal peptide (about 2–3 kDa), which equaled the molecular weight of 24 amino acids. All these results indicated that OPHC2 of strain SMSP-1 contained a 24-amino acid signal peptide, and the prediction on the cleavage site was right (Fig. 6). The activity of crude enzyme extracts of *E. coli* BL21 (DE3)/pE-*ophc2* and pE-*ophc2-n* was 4 and 2.2 units ml^{-1}, respectively, which showed the successful expression of *ophc2* and *ophc2-n* in *E. coli*. The *ophc2* gene was firstly cloned from *P. pseudoalcaligenes* C2-1 and was also expressed in *E. coli*. However, the signal peptide of OPHC2 was not cleaved in *E. coli*, so the author deduced that it was due to the fusion expression with the S tag of the expression vector pET-30a (Wu et al. 2004). In the present study, the *Nde*I and *Xho*I sites were incorporated into primers to facilitate directional cloning of the PCR product into *Nde*I-*Xho*I sites of pET29a, and the expressed OPHC2 was the natural product without S tag of the vector, which was properly cleaved by the proteases of the *E. coli*.

Discussion

The molecular basis of degradation of certain OP pesticides has been studied extensively. Three OPH encoded genes, *opd*, *mpd*, and *ophc2*, have been reported to date. The *opd* gene has been isolated from temporally, geographically, and biologically different species (McDaniel et al. 1988; Horne et al. 2002; Chaudhry et al. 1988; Siddavattam et al. 2003). The *mpd* gene was first cloned from *Plesiomonas* sp. M6 (Cui et al. 2001). Until now, the *mpd* genes found in OP pesticide-degrading bacteria from Chinese soils were highly conserved, although they existed in bacteria from different genera (Zhang et al. 2006b; Masahito et al. 2000; Yang et al. 2006; Liu et al. 2005). The *ophc2* gene was first cloned from *P. pseudoalcaligenes* C2-1 (Wu et al. 2004). The coding sequence of gene *ophc2* showed only 46% similarity with the genes encoding organophosphorus hydrolase deposited in GenBank, which indicated that OPHC2 is a novel organophosphorus hydrolase. In this

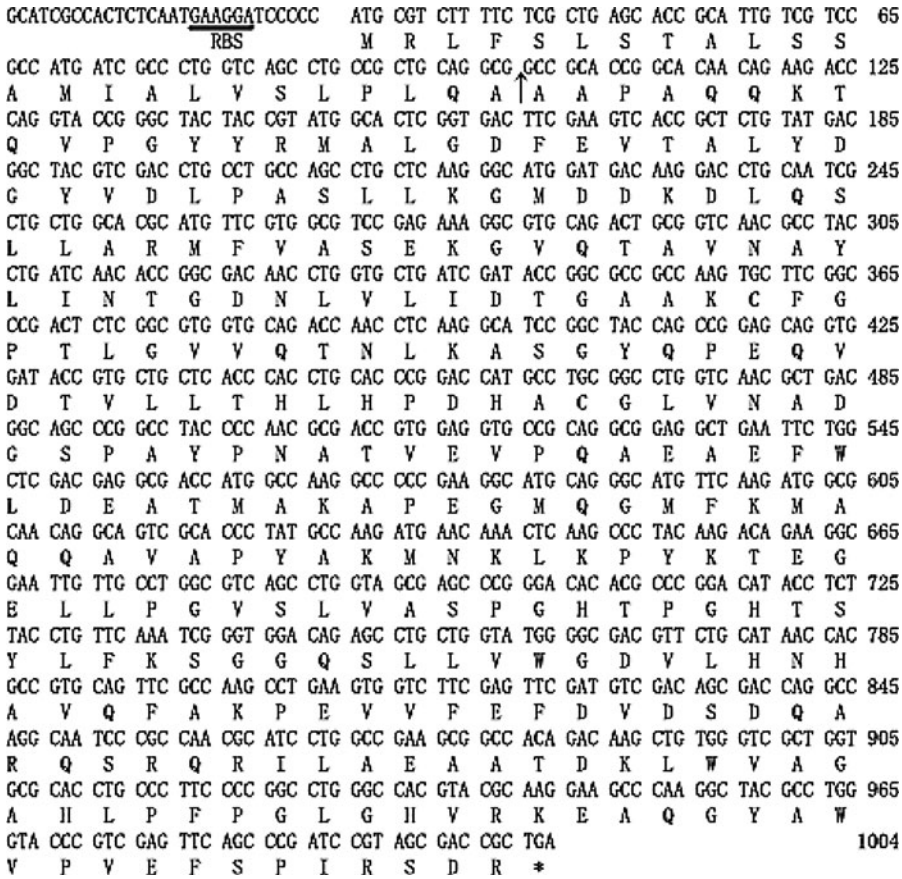

Fig. 6 Nucleotide and deduced amino acid sequences of ophc2 gene from strain SMSP-1. RBS site is *underlined*. The cleavage site of a signal peptide is indicated by an *arrow*

study, the organophosphorus hydrolase gene of strain SMSP-1 was cloned and expressed in *E. coli*, which is the second report of cloning the *ophc2* gene and the first report of this gene from the genus *Stenotrophomonas.*

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