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ORIGINAL PAPER

Isolation of the *opdE* gene that encodes for a new hydrolase of *Enterobacter* sp. capable of degrading organophosphorus pesticides

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Abstract Microbial enzymes that can hydrolyze organophosphorus compounds have been isolated, identified and characterized from different microbial species in order to use them in biodegradation of organophosphorus compounds. We isolated a bacterial strain Cons002 from an agricultural soil bacterial consortium, which can hydrolyze methyl-parathion (MP) and other organophosphate pesticides. HPLC analysis showed that strain Cons002 is capable of degrading pesticides MP, parathion and phorate. Pulsed-field gel electrophoresis and 16S rRNA amplification were performed for strain characterization and identification, respectively, showing that the strain Cons002 is related to the genus *Enterobacter* sp. which has a single chromosome of 4.6 Mb and has no plasmids. Genomic library was constructed from DNA of *Enterobacter* sp. Cons002. A gene called *opdE* (Organophosphate Degradation from *Enterobacter*) consists of 753 bp and encodes a protein of 25 kDa, which was isolated using activity methods. This gene *opdE* had no similarity to any genes reported to degrade organophosphates. When kanamycin-resistance cassette was placed in the gene *opdE*, hydrolase activity was suppressed and *Enterobacter* sp. Cons002 had no growth with MP as a nutrients source.

Keywords Methyl-parathion · Organophosphorus pesticides · *Enterobacter* sp. Cons002 · *opd*E Gene · Degradation

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Introduction

Synthetic organophosphate (OP) compounds have been widely used as pesticides and have played an important role in raising agricultural productivity and controlling pests. However, these compounds are often toxic in non-target organisms, and their transformation products are frequently found in the environment (Van and Pletschke 2010).

These compounds irreversibly inhibit acetylcholinesterase (AchE), a key enzyme in the central nervous systems of insects and non-target organisms, including humans (Sogorb and Vilanova 2002; Zhang et al.

2006). Effective methods for degrading organophosphates are needed to ensure that the health of neither humans nor the environment will be compromised. Previous forms of disposal for OP compounds have consisted of chemical or physical treatments (Chen et al. 2000), but they produce toxic residues; therefore, other environmentally friendly technologies, like the use of microorganisms or their enzymes, are now being considering for eliminating organophosphorus residues.

Several enzymes called phosphotriesterases (PTE) isolated from different microorganisms have been found to hydrolyze and detoxify OPs. This reduces OP toxicity by decreasing the ability of OPs to inactivate AchE (Ghanem and Raushel 2005; Singh and Walker 2006; Porzio et al. 2007; Theriot and Grunden 2010; Shen et al. 2010). These enzymes specifically hydrolyze phosphoester bonds, such as P–O, P–F, P–NC, and P–S, and the hydrolysis mechanism involves a water molecule at the phosphorus center (Ortiz-Hernández et al. 2003). Different microbial enzymes with the capacity to hydrolyze MP have been identified, such as organophosphorus hydrolase (OPH; encoded by the *opd* gene), methyl-parathion hydrolase (MPH; encoded by the *mpd* gene), and hydrolysis of coroxon (HOCA; encoded by the *hoc*A gene), which were isolated from *Flavobacterium* sp. (Sethunathan and Yoshida 1973), *Plesimonas* sp. strain M6 (Cui et al. 2001) and *Pseudomonas moteilli* (Horne et al. 2002), respectively.

We have previously described the characterization of a bacterial consortium that had been isolated from agricultural soils with a history of pesticide application (Yáñez-Ocampo et al. 2009). The isolated consortium was adapted for several weeks to a mineral medium without an additional carbon source. The mineral medium contained untreated OP pesticide wastes generated from livestock-dipping operations that used an OP pesticide mixture. The consortium was subcultured and continuously enriched throughout the application of selective pressure. The consortium consisted of 14 bacterial strains, 12 of which were capable of degrading MP. Out of the 12, strain Cons002 showed the greatest ability to hydrolyze MP and was selected for further research (Specific whole cell activity = 2.03 ± 0.18 mU/mg protein).

In the present study, the strain Cons002 was characterized. The *opdE* gene was isolated, sequenced, and effectively cloned into *Escherichia coli*. We found that the *opdE* gene is responsible for the degradation of organophosphorus pesticides. A phylogenetic analysis permitted the study of the evolution of the *opdE* gene.

Materials and methods

Reagents

MP (*O*,*O*-dimethyl *O*-4 nitrophenyl phosphorothioate), parathion (*O*,*O*-diethyl-*O*-*p*-nitrophenyl phosphorothioate), phorate (*O*,*O*-diethyl *S*-[(ethylthio) methyl] phosphorodithioate), cadusafos (*S*,*S*-di-*sec*-butyl *O*-ethyl phosphorothioate) and butiphos (*S*,*S*,*S*-tributyl phosphorotrithioate) were purchased from Chemservice (99% purity). All of the other chemicals were of reagent grade and were obtained from J. T. Baker, Mexico City. Restriction enzymes were acquired from New England Biolabs.

Bacterial strains

The bacterial strains used were *E. coli* XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17* [r_κ^- m_κ^+] *supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔ*M15 Tn*10* Tet^r]); *E. coli* DH5α*supE*44 D*lacU*169 (f80*lacZ* DM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 (Sambrook and Russell 2001); strain Cons002 (Wild-type strain, MP^r, Ap^r) (Yáñez-Ocampo et al. 2009); Hydro1257 (DH5pKS + *opdE*), *Enterobacter* opdE- (*Enterobacter opdE*::Km^r) and *Enterobacter* pKSopdEGM (*Enterobacter opdE*::Km^r pKS + *opdE*) (this study); and BL21 (DE3) (hsdS *gal* [λcIts857 *ind*1 *Sam*7 nin5 *lac*UV5-T7]). The plasmids pJET (sequencing vector Ap^r [Fermentas]), *pBluescript II K*$^+$ (*pKS* +) (*oriR101*, *repA101* (ts); *araBp-gam-bet-exo* [Stratagene]), pKD78 (Red helper plasmid, Ts; Cm^r), pKD4 (*oriR*γ; Km^r, Ap^r) (Datsenko and Wanner 2000) and pET-22b (+) (Novagen) were used.

Culture medium composition

For the cultivation of the Cons002 strain, minimal medium (MM) was modified from a recipe described by Brown (1980) and had the following composition (g/l): 2.92 KH_2PO_4, 2.74 K_2HPO_4, 0.20 $MgSO_4 \cdot 7H_2O$, 2.0 KNO_3, and 0.99 $(NH_4)_2SO_4$. It also contained glucose at concentration of 50 mg/l and 2 ml/l of a trace element solution composed of (g/l)

2.8 H_3BO_3, 2.55 $MnSO_4 \cdot H_2O$, 0.20 $CuSO_4 \cdot {}_5H_2O$, 2.43 $CoCl_2 \cdot {}_6H_2O$, and 0.25 $ZnSO_4 \cdot 7H_2O$ at pH 7 ± 0.05 and 30°C. The MP was dissolved in methanol and added to the media to a final concentration of 20 mg/l. *Escherichia coli* was grown at 37°C in LB medium with ampicillin, kanamycin, chloramphenicol and gentamicin at concentrations of 100, 60, 30 and 15 μg/ml, respectively. When necessary, the Cons002 strain was grown in Tripticasein soy (TS) broth (Bioxon, Becton–Dickinson, Mexico).

Degradation of MP by Cons002

The Cons002 strain was cultured overnight in 3 ml of LB medium containing 100 μg/ml ampicillin. The culture was then used to inoculate 50 ml of MM containing 50 mg/l glucose and 20 mg/l MP with an initial OD_{600} of 0.05. Samples (1 ml) were collected at 0, 4, 8, 12, 16, 20, 24 and 28 h after inoculation for viable count analysis (CFU/ml). Chemical analyses of residual MP and *p*-nitrophenol (PNP) concentrations were carried out by HPLC using the Waters 2487600 ultraviolet (UV) detector, a Dual Absorbance Detector, and an EC 250/4 Nucleosil 120-5 C18 HPLC column. The mobile phase was methanol/water (60/40, v/v), and a flow rate of 0.8 ml/min was used. MP and PNP were detected at 275 nm. Simultaneously, the glucose concentrations in the culture supernatants were measured using an YSI 2700 SELECT biochemistry analyzer (YSI Inc., Yellow Springs, OH) equipped with a glucose oxidase membrane (YSI 2365). To determine the concentration of glucose in each culture supernatant, a 1 ml sample was taken every 4 h. The sample was centrifuged for 1 min, and the supernatants were used to determine the concentration of glucose. The same medium without inoculant was used as a control.

Utilization of other OP pesticides as substrates

The Cons002 strain was cultured in MM with 50 mg/l glucose and 20 mg/l of the parathion, cadusafos, butiphos or phorate pesticides. The cultures were incubated under previously described conditions; non-inoculated media were also used as controls. After 28 h of incubation, the residual concentration of pesticides was analyzed by HPLC under the same conditions described above. Parathion was detected at 275 nm, while phorate, cadusafos and butiphos pesticides were determined at 230 nm.

Enzyme assay

Cells were grown in MM with 20 mg/l of MP for 48 h at 28°C and agitated at 100 rpm. After being harvested, the cells were suspended in 2 ml of distilled water and sonicated at 25% efficiency for 75 s (3 pulses of 25 s each) in a sonicator (Branson Sonifier 450). After sonication, the solution was centrifuged (17,000×*g* for 10 min), and the intracellular fractions were tested for hydrolase activity. The protein concentration was determined, using bovine serum as standard, by the method developed by Lowry et al. (1951).

Hydrolysis of MP was measured spectrophotometrically by monitoring the production of PNP at 405 nm ($\varepsilon_{max} = 17{,}000\ M^{-1}\ cm^{-1}$) for 6 h at 28°C; assays were performed in 20 mM Tris–HCl (pH 9). Enzyme activity was defined as the amount of enzyme required to release 1 μmol of PNP per min at 37°C. The data are reported as specific activity (mU/mg of protein).

Bacterial identification

Chromosomal DNA was extracted by the method developed by Miller et al. (1988) and used for PCR amplification of the 16S rRNA gene. The 16S rRNA gene of the bacterial strain Cons002 was amplified with the universal primers 63Fw (5′-CAGGCCTAACACATGCAAGTC-3′) and L1401 Rv (5′-CGGTGTGTACAAGACCC-3′) (Lopez et al. 2003). The amplified products were purified, and the 1.4 kb fragments were ligated into the pJET plasmid. The recombinant plasmid was transformed into competent *E. coli* DH5α cells. The inserted fragments were sequenced with the primers 63Fw, L1401 and U968 (internal primer; 5′-AACGCGAAGAACCTTAC-3′) by the Sequence Unit of Biotechnology Institute UNAM, Mexico. The sequence data were analyzed using the BioEdit sequence analysis software (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

A modified protocol for pulsed-field gel electrophoresis (PFGE) (Smith and Cantor 1987) was used for bacterial identification. The sample plugs were placed in 10 ml of fresh lysis solution (10 mM Tris–HCl, pH 7.2, 50 mM NaCl, 100 mM EDTA, 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine, 20 μg/ml RNase

(DNase-free) and 1 mg/ml lysozyme) to disrupt the cells. The DNA fragments were separated by electrophoresis on a 0.6% agarose gel (Bio-Rad) using a 0.9× TAE buffer (20 mM NaCl, 50 mM EDTA, and 10 mM Tris–HCl, pH 7.2) in a contour-clamped homogeneous electric field device (CHEF-DR II) from Bio-Rad; the pulse time was increased from 2,600 s to 85 h at 1.8 V, 14°C and 6 V/cm. The electrophoresis was performed using alternating pulses at a 106° angle, with a 5–20 s pulse time gradient over a period of 85 h.

Isolation and sequence of the *opdE* gene

A gene library was constructed from genomic DNA. A partial digestion was performed with the enzyme *Sau*3AI to obtain randomly digested chromosomal DNA. The 1–5 kb fragments were recovered using a cleanup kit (High Pure PCR-Roche) and ligated into a pKS^+ vector that had been digested with *Bam*HI and treated with calf intestinal alkaline phosphatase (Roche). The ligation products were transformed into *E. coli* XL1-Blue competent cells, and the bacteria were plated on LB agar plates containing 100 μg/ml ampicillin for selection. The library was screened for clones harboring hydrolase activity towards MP. Positive clones have been sequenced in both directions with the primers T7 (5′-AATACGACTCACTATAG-3′) and T3 (5′-ATTAACCCTCACTAAAG-3′) (Stratagene) and the internal primers opdEFw (5′-TTTACCCGCT CACGCGGTCGTCC-3′) and opdERV (5′-GCTCGA GATCATTGCCGCACCAGAATAC-3′) to ensure the absence of sequencing errors.

Construction of a defective *opdE* mutant

A deletion of the *opdE* gene was made in the Cons002 strain using Red-mediated recombination (Datsenko and Wanner 2000). The *opdE* deletion was constructed using the kanamycin resistance *cassette* generated by PCR from the pKD4 template using primers opdEH1P1 (5′-TCACCCAGGAAGGTGTTTATGAGCAAAAAA ACGCAGCATTTCTGTAGGCTGGAGCTGCTTCG-3′) and opdEH2P2 (5′-TCGCCATGAACTTTTCATTAAG TGGATCTCCGCGCTGAGTGGCATATGAATATC CTCCTTAG-3′). The strain mutant was selected for kanamycin resistance, and its specific activity was determined. The mutant was confirmed by PCR using genomic DNA from the mutant strain as a template. The primers opdEH1P1 and opdEH2P2 amplified the predicted 1,500 bp fragment, while internal primers for the *opdE* gene, XHyFw (5′-TGCTCGAGATGAGCA AAAAAACGCAGCATTTCTCG-3′) and BHyRv (5′-AGGATCCTCATCATAAGTGGATCTCGGC GC-3′), could not be used to amplify products (data not shown).

Nucleotide sequence accession number

The nucleotide sequence of the *opdE* gene was deposited into the DDBJ database with accession number JF837335.

Construction and plasmid complementation of the *Enterobacter* strain lacking the *opdE* gene

To construct the plasmid pKSopdE, the *opdE* gene was amplified using PCR. The upstream primer, opdERBS (5′-AACTCGAGAAG*AAGGAG*ATATCAT**ATG**AG CAAAAAAACG-3′), contained an *Xho*I restriction site (underlined bases) and a strong RBS (in italic) at the *opdE* start codon (in bold), while the downstream primer, opdERV (5′-AAGCTTCATCA**TAA**GTGG ATCTCCGCGCT-3′), contained a *Hin*dIII restriction site (underline bases) at the stop codon (in bold). The 0.783 kb fragments were recovered using a clean-up kit (High Pure PCR-Roche) and ligated into pKS^+ digested with *Xho*I and *Hin*dIII. The ligation products were transformed into *E. coli* DH5α competent cells, and the bacteria were plated on LB agar plates containing 100 μg/ml ampicillin. The gentamicin-resistance *cassette* was obtained from the pBSL142 plasmid (Alexeyev et al. 1995) using the *Xba*I restriction site, and the resulting 1.5 kb fragment was recovered using a clean-up kit (High Pure PCR-Roche) and ligated into pKSopdE digested with *Xba*I to obtain pKSopdEGM. The ligation products were transformed into *E. coli* DH5α competent cells, and the bacteria were plated on LB agar plates containing 15 μg/ml gentamicin. The *Enterobacter* opdE-strain was transformed with pKSopdEGM to obtain *Enterobacter* pKSopdEGM; the presence of *opdE* was confirmed by PCR, and the specific activity of the strain was determined.

Expression and purification

Once the complete sequence of the *opdE* gene was obtained, the primers opdEFW-(5′-TGGGATCC**AT GAGCAAAAAAACGCAGC**-3′) (sense, the *Bam*HI site is underlined and the start codon is marked in bold) and opdERV-5′-GTCGAC**TAA**GTGGATCTCCGC GCTGAG-3′ (antisense, the *Sal*I site is underlined and the termination codon is marked in bold) were designed to amplify the open reading frame using the bacterial genomic DNA as a template. The amplified DNA fragment, *opdE*, was digested and ligated into the pJET vector and subcloned into the pET-22b (+) vector (Novagen), which encodes a C-terminal hexahistidine tag for affinity purification. The recombinant plasmids were transformed into *E. coli* BL21 (DE3), and positive transformants were cultured in 100 ml LB medium with 100 μg/ml ampicillin at 30°C with shaking. When the cell density had reached OD_{600} = 0.5, IPTG was added to the culture at a final concentration of 1 mM. After a 4-h induction, the cells were harvested by centrifugation at 12,000×*g*. The expression level of the protein was assessed by analysis on a 12% SDS-PAGE gel.

The periplasmic protein from *E. coli* was first extracted by previously described methods (Dresler et al. 2006). The culture pellet was obtained by centrifugation (10,000×*g*, 5 min, 4°C) and then exposed to osmotic shock by suspension in 1 ml of ice-cold 20% sucrose in 30 mM Tris–HCl, pH 8. Following 20 min incubation at 37°C, the spheroplasts were pelleted (10,000×*g*, 10 min, 4°C), resuspended in 1 ml of ice-cold 5 mM $MgSO_4$ and incubated on ice for 10 min. After centrifugation (10,000×*g*, 10 min, 4°C), the supernatant (total periplasmic proteins [PP]) was stored at 4°C for subsequent purification. The total PP was passed through 4 ml of Ni-NTA His-Bind Resin (Novagen), which had been equilibrated with 1× Ni-NTA Bind buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 10 mM imidazole). The column was washed with 1× Ni-NTA Wash buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 20 mM imidazole) and then the target protein was eluted in four steps by the addition of 0.5 ml 1× Ni–NTA Elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 250 mM imidazole). The eluted fractions were collected separately and analyzed on an SDS-PAGE gel.

Results

Cell growth and degradation of MP in liquid cultures by the Cons002 strain

The cell growth of the Cons002 strain, expressed in viable counts (CFU/ml), with and without MP is shown in Fig. 1. After the first 20 h of incubation, the Cos002 strain grew in the presence of the MP pesticide but not in the absence of the pesticide. This result suggests that Cons002 likely requires a pesticide as an alternative nutrient source.

The degradation kinetics of MP and glucose, as well as the production of PNP, were assessed simultaneously. The ability of Cons002 to degrade MP was measured by HPLC analysis during bacterial growth. Both glucose and MP levels decreased from the initial levels, while PNP, the degradation product of MP, increased proportionally during the first 20 h until the PNP could not be degraded further (Fig. 2). However, the results suggest that other metabolites like dimethylthiophosphoric acid could be use as nutrients sources.

Removal of other OP pesticides in liquid culture

The Cons002 strain removed OPs, MP, parathion and phorate in 28 h. It is interesting to note that Cons002 can significantly degrade MP (71%, 14.2 mg/l), but it only slightly removes parathion (17%, 3.5 mg/l) and phorate (21%, 4.1 mg/l) (Fig. 3). Cadusafos and butiphos could not be removed from liquid medium. These results show that Cons002 probably has the

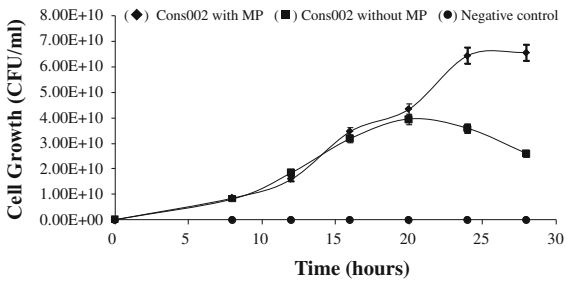

Fig. 1 Cell growth of Cons002 in the presence of MP (20 mg/l) (*filled diamond*) or in the absence of MP (*filled square*). Control MM containing 50 mg/l glucose without cells (*filled circle*). The samples were incubated at 30°C for 28 h as described in the "Materials and methods" section. Each value is the mean of three replicates with *error bars* representing the standard deviation of the mean

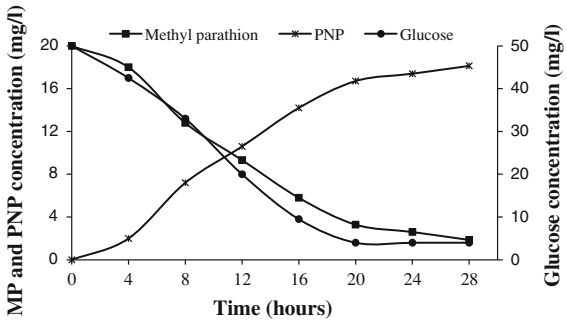

Fig. 2 Loss of MP (*filled diamond*), loss of glucose (*filled circle*), and production of PNP (*asterisks*) throughout the growth (for 28 h at 30°C) of the Cons002 strain cultivated in MM supplemented with 50 mg/l glucose and MP (20 mg/l) at an initial OD_{600} of 0.05

ability to break down the P–S bond of some organophosphate pesticides but not the P–O bond.

Bacterial identification

The Cons002 isolate was circular, smooth and yellow-white. It is a gram-negative bacteria strain. When the partial 16S rRNA gene of Cons002 was compared to previously published sequences in the EMLB database, the sequence displayed the highest identity (99%) with the 16S rRNA gene of an *Enterobacter* sp. (NCBI database, EF471901.1). PFGE was performed to identify the number of replications and the genome size in the *Enterobacter* sp. The genome size was estimated using the genomes of *Mesorhizobium loti*, *Schizosaccharomyces pombe* and *Hansenula wingei* as molecular weight markers for comparison to the *Enterobacter* sp. strain Cons002 genome (4.87 Mb) (data not shown).

Construction of a genomic library

The first genes known to be responsible for degrading MP have been isolated from bacterial plasmids, but some bacterial genes, including those from *P. monteilii*, *Plesimonas* sp. strain M6, *Burkholderia* sp. strain NF100, *Pseudomonas putida* and *Stenotrophomonas* sp. strain SMSP-1 (Singh and Walker 2006; Horne et al. 2002; Tago et al. 2006; Seo et al. 2007), have been cloned from genomic DNA. The genomic DNA extracted from Cons002 strain was used to generate a library consisting of 5000 clones. Considering a genome size of ∼4.87 Mb for the Cons002 strain and an average *Enterobacter* gene size of ∼1 kb, the theoretical genome coverage of this library is ∼3×.

Clones were assayed for MP hydrolysis activity, yielding one positive clone (Hydro1257) that demonstrated the ability to degrade MP (Specific activity = 1.061 ± 0.022 mU/mg protein). The control *E. coli* XL1-Blue strain, with or without pKS+, showed no detectable activity towards MP (Fig. 4). A molecular analysis showed that the Hydro1257 clone contains a fragment of 1156 bp, which was sequenced in both directions.

The OpdE, a novel bacterial Opd-related enzyme

The clone Hydro1257 contains one open reading frame of 759 bp that is 95% identical, at the amino

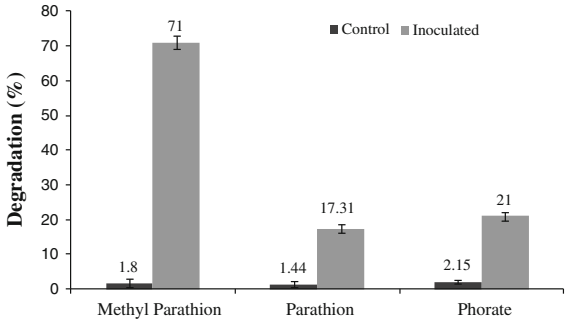

Fig. 3 Biodegradation of OP pesticides by Cons002 in liquid culture. All pesticides were added at an initial concentration of 20 mg/l. The residual concentration was measured by HPLC at 48 h as described in the "Materials and methods" section. Uninoculated media were run as controls. The data are the means ± standard deviation of three replications

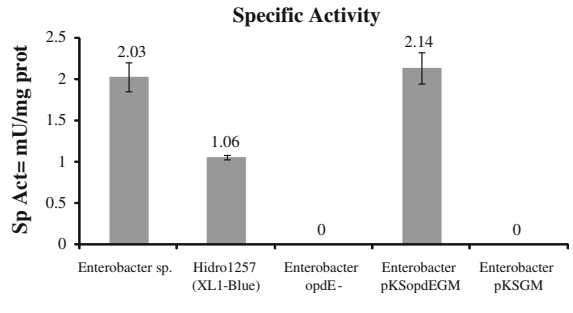

Fig. 4 Specific activity of *Enterobacter* sp. Cons002 (2.03 ± 0.18 mU/mg protein), clone Hydro1257 strain XL1-Blue (1.061 ± 0.022 mU/mg protein) and *Enterobacter* pKSopdEGM (2.14 ± 0.185 mU/mg protein) cultures grown in MM for 48 h at 30°C. Controls: *Escherichia coli* XL1-Blue, *Enterobacter* opdE- and *Enterobacter* pKSGM. Data are the means ± standard deviations of three replications

acid level, to an endonuclease/exonuclease/phosphatase of *Enterobacter cloacae* subsp. cloacae ATCC 13047 (GenBank Accession no. YP_003613430). The gene is called *opdE* (Organophosphates Degrading from *Enterobacter*) and encodes a hydrolase of organophosphates (OpdE).

Through sequence comparison with bacterial hydrolase enzymes retrieved from GenBank http://www.ncbi.nlm.nih.gov, we identified the conserved motifs, including the putative COG3568 metal hydrolase motif, which is found in a large number of functionally diverse enzymes, mainly from prokaryotes. The COG3568 motifs is related to pfam03372, a motif found in a family of Mg^{2+}-dependent bacterial endonuclease/exonuclease/phosphatase enzymes, and to COG0708 XthA, a motif found in exonuclease III family.

At the amino acid level, the OpdE protein shows slight similarity with proteins reported to degrade organophosphate pesticides, such as OPH protein encoded by *opd*, *opch2* and *ophB* genes (10, 8 and 4%, respectively); the MPH protein encoded by *mpd* gene (5%) and the OPAA protein encoded by *opaA* gene (10%) (Data not show). Furthermore, to clarify the phylogenetic relationship of the OpdE with other enzymes that degrading organophosphates, a neighbor joining phylogenetic tree was constructed using the amino acid sequences of the OPH from *B. diminuta* and *Flavobacterium* spp., OP-degrading enzyme (OPDA) from *Agrobacterium radiobacter*, amidohydrolase superfamily, MPH from the *Pseudomonas* sp. WBC-3, and organophosphorus acid anhydrolase (OPAA) from *Alteromonas undina* and *Alteromonas haloplanktis*. As shown in Fig. 5, OpdE formed a distinct group, which is located closest to the branch of putative OPAA (accession number U29240 and U56398) of *Alteromonas* and also, OPDA (accession number FJ550130) from *Lactobacillus brevis*. Together, our results on hydrolysis activity, protein domains, and phylogenetic analysis, clearly indicate that OpdE is a new group of enzyme family that degrading organophosphates.

A defective *opdE* mutant

Enterobacter sp. Cons002 that contains a kanamycin-resistance *cassette* insertion in *opdE* showed no specific activity towards a MP substrate (Fig. 4), and it was unable to grow with MP as a nutrient source (Fig. 6). This confirms that OpdE is the protein required for *Enterobacter* sp. Cons002 to grow with MP.

Complementation of *Enterobacter* opdE-mutant strain

To confirm the role of the *opdE* gene in MP degradation, the *Enterobacter* opdE-mutant strain was complemented with the construct pKSopdEGM. The transformed strain (called *Enterobacter* pKSopdEGM) was selected with gentamicin. Functionality of the *opdE* gene was confirmed because the complemented mutant strain demonstrated hydrolysis activity. The *Enterobacter* pKSopdEGM strain was tested by measuring the hydrolysis activity and a specific activity $= 2.14 \pm 0.185$ mU/mg protein was obtained (Fig. 4).

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

To display the gene product and to confirm the molecular weight of OpdE, the expression of the *opdE* gene in *E. coli* BL21 (DE3) cells was analyzed by SDS-PAGE (Fig. 7). The molecular weight of the induced band was $\sim$25 kDa, which is consistent with the amino acid sequence of the *opdE* gene. The specific activity of the crude enzyme extracts of *E. coli* BL21 (DE3) carrying the *opdE* gene was 8.32 mU/mg protein.

Discussion

Biodegradation by microorganisms is the primary means for the elimination of organophosphate insecticides released to the environment, and several bacterial strains are capable of completely or partially degrading organophosphorus. Some bacteria have been isolated from soils contaminated with organophosphate compound pesticides (Singh and Walker 2006; Kim et al. 2009; Wang et al. 2010). Bacteria capable of degrading MP have been obtained from agricultural soils by the enrichment culture technique (Yáñez-Ocampo et al. 2009). Species identification by 16S rDNA sequence analysis revealed that the isolated strain is related to genus *Enterobacter*. Some species of this genus have been reported to degrade different

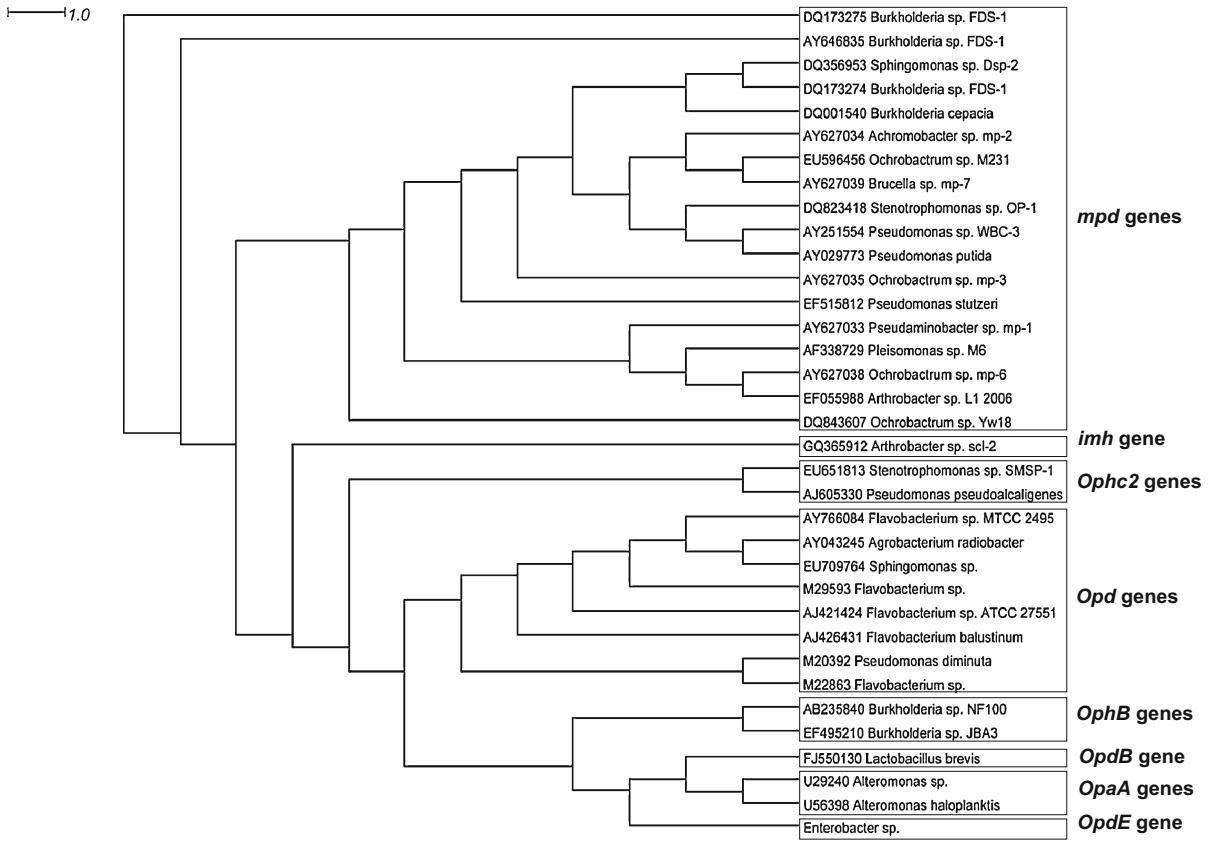

Fig. 5 Phylogenetic reconstruction of the organophosphorus-degrading enzymes family. Phylogenetic tree was constructed from a single multiple alignment using the Phylyp package V 3.57. The *boxes* indicate distinct clusters of organophosphorus-degrading enzymes. For each sequence, NCBI accession number and species are shown

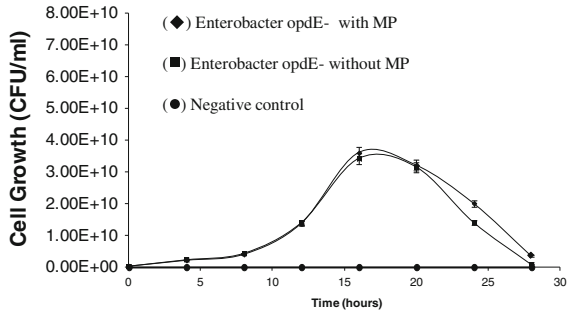

Fig. 6 Cell growth of *Enterobacter* opdE- in the presence of MP (20 mg/l) (*filled diamond*) or the absence of MP (*filled square*). MM containing 50 mg/l glucose without cells was used as a control (*filled circle*). The samples were incubated at 30°C for 28 h as described in the "Materials and methods" section. Each value is the mean of three replicates, and error bars represent the standard deviation of the mean

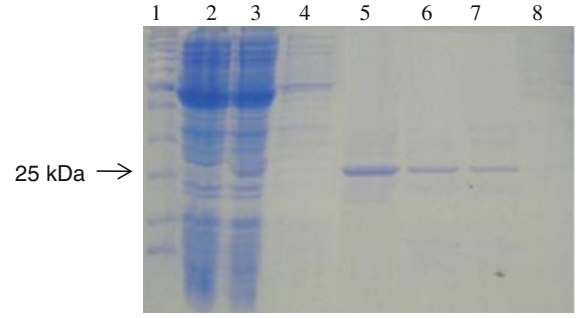

Fig. 7 SDS-PAGE gel electrophoresis of the purified protein. *Lane 1* protein marker (Fermentas), *lane 2 E. coli* transformed with the pET-22b (+) plasmid, *lane 3* PP with expressed OpdE, *lane 4* Ni-NTA wash, *lanes 5, 6, 7* and *8* Ni-NTA elutions

contaminants, such as chlorpyrifos (Singh et al. 2004); 2,4,6-trinitrotoluene (Robertson and Jjemba 2005); di- and trichlorobenzenes (Adebusoyea et al. 2007); products of the highly toxic organophosphate nerve agents sarin, soman, GF, VX and rVX (Ghanem et al. 2007) and petroleum (Kaoa et al. 2010).

Enzymes that can hydrolyze OP compounds have been identified and characterized after isolation from different microbial species (Singh and Walker 2006). The *opdE* gene is different from genes encoding organophosphorus hydrolases deposited in GenBank, which indicates that the enzyme is a novel organophosphorus hydrolase. The use of enzymes for the detection and decontamination of OPs and for medical applications is of great interest (Sogorb and Vilanova 2002; Singh 2009; Theriot and Grunden 2010; Van and Pletschke 2010).

No natural substrates have been identified to OPs hydrolases (Afriat et al. 2006), and by the other side, there is a rich literature addressing the evolutionary potential by promiscuous activities (Seibert and Raushel 2005). It is widely accepted that many enzymes evolved via gene duplication (Hurles 2004; Thoden et al. 2004) and that some of they are capable of catalyzing other reactions, in addition to the ones for which they are physiologically specialized. Additionally, some publications showed that mutations increase the efficiency of a promiscuous activity by changes in the catalytic site or genetic expression. This fact allows to bacteria some physiologically and evolutionary advantages (O'Brien and Herschlag 1999; DePristo 2007; Khersonsky and Tawfik 2010). By the other hand, several groups have suggested that OP hydrolases diverged from one pre-existing progenitor protein (DePristo 2007; Khersonsky and Tawfik 2010).

The OPH enzymes encoded by the *opd* and *mpd* genes are members of the amidohydrolase superfamily, a group of enzymes sharing the same $(\beta/\alpha)_8$-barrel structural fold that catalyzes the hydrolysis of amide or ester functional groups at carbon and phosphorus centers (Seibert and Raushel 2005). These centers might have evolved from PTE-like lactonase (PLL) enzymes, which hydrolyze lactones and have low activity towards OPs (Afriat et al. 2006). Another possibility is that they evolved from the phosphotriesterase homology protein (PHP) of *E. coli*, which is a metalloenzyme with a $(\beta/\alpha)_8$-barrel structure (Scanlan and Reid 1995).

Because these families of proteins are different (Fig. 5) in regard to scaffolding, catalytic mechanisms and the chemical structures of target lactones and OPs, it was proposed that a promiscuous phosphotriesterase of lactonases emerged from the overlap between the transition states of lactones and OPs during hydrolysis (Afriat et al. 2006). Other hydrolases with similar transition states, such as esterases or prolidases (like OPAA), also have promiscuous phosphotriesterase activity, supporting this hypothesis (Elias et al. 2008).

Several studies of proteins reveal families of enzymes; non-homologous enzymes evolve in separate biological contexts to catalyse the same or similar biochemical transformation. Often such enzymes have nothing in common beyond their function (Gherardini et al. 2007, Pratt and McLeish 2010). As shown in the phylogenetic tree, there are several groups of proteins that have, probably, different ancestors, but can degrade OP compounds. OpdE protein has endonuclease and exonuclease domains and too degrade OPs, suggesting that evolution is according to their function. These studies are the beginning to find many more progenitors for others degrading OPs.

Some bacteria, such as *Flavobacterium* sp. ATCC 27551 (diazinon), *Burkholderia* sp. NF100 (fenitrothion), *Arthrobacter* sp. scl-2 (isocarbophos), and *Enterobacter* sp. strain B14 (chlorpyrifos) (Sethunathan and Yoshida 1973; Tago et al. 2006; Rong et al. 2009; Singh et al. 2004) have been reported to utilize OPs as a nutrients source. However, when *Enterobacter* sp. Cons002 was cultured in the presence of MP without glucose, the bacteria did not show any growth (data not shown).

Data of this work show that *Enterobacter* sp. Cons002 uses MP as a nutrient source, probably the dimethyl thiophosphoric acid, as growth was observed despite the depletion of glucose. In contrast, *Enterobacter* opdE-could not degrade MP, and therefore growth was affected when glucose in the culture medium was depleted (Fig. 6). Additionally, *Enterobacter* sp. Cons002 was not able to grow after the depletion of glucose without MP (Fig. 1). These results suggest that MP is degraded in a cometabolism process.

The *opdE* gene from Cons002 was cloned and expressed in *E. coli*, and this is the first report of the recombinant expression of a gene from the genus *Enterobacter* that encodes a protein that degrades OPs.

In conclusion, we isolated *Enterobacter* sp. Cons002, which is able to degrade the pesticides MP, parathion and phorate and probably uses these pesticides as a nutrient source. We characterized the *opdE* gene, which encodes a hydrolase enzyme with the capacity to hydrolyze MP. The phylogenetic analysis suggests that the organophosphorus-degrading functions of OpdE might have evolved from

different progenitors than those previously reported. This kind of enzyme is thought to play an important role in the degradation of organophosphate compounds and has many promising applications, including the bioremediation of materials polluted by OPs, the development of biosensors for OPs and therapies for OP poisoning.

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