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ORIGINAL ARTICLE

Application of Quantitative Real-Time Polymerase Chain Reaction on the Assessment of Organophosphorus Compound Degradation in *in situ* Soil

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Abstract Quantitative real-time PCR (qPCR) method was applied to quantify the functional gene encoding organophosphorus hydrolase for assessing the degradation efficacy by bacterial strains on an organophosphorus compound in *in situ* soil. The specific primers targeting the organophosphorus hydrolase were designed and tested on reference bacterial strains and in DNA samples extracted from *in situ* soil samples contaminated by an organophosphorus compound. The established qPCR assay is a practical method for the analysis of *in situ* soil samples undergoing bioremediation of organophosphorus compounds.

Keywords biodegradation · *opd* gene · organophosphorus hydrolase · quantitative real-time polymerase chain reaction

Introduction

Synthetic organophosphorus compounds are widely used as pesticides for controlling agricultural and household pests, and account for ~38% of the total pesticides used globally (Singh and Walker, 2006). Their mechanism of efficacy is based on their inhibition of acetylcholinesterase (AChE; EC 3.1.1.7) in the nervous system of the target pests (Sogorb et al., 2004). Due to accidents or misuse, the World Health Organization estimates pesticides poison more than 3,000,000 people annually with approximately 200,000 deaths related to exposures to organophosphorus compounds (Bird et al., 2008). Synthetic organophosphorus compounds possess high toxicity toward non-target organisms such as mammals. Environments contaminated by excessive application of organophosphorus compounds require remediation (Singh and Walker, 2006).

Biodegradation of organophosphorus compounds using bacterial enzymes provides an efficient, convenient, and cost-effective technique for detoxification of the environment (Zhang et al., 2008). Organophosphorus hydrolase (OPH, also referred to as phosphotriesterase or parathion hydrolase; EC 3.1.8.1) is the most extensively studied microbial enzyme shown to degrade organophosphorus compounds. Organophosphorus compounds are ester or thiol derivatives of phosphoric, phosphonic or phosphoramidic acids. OPH reactions include the hydrolysis of ester bonds, and aliphatic, aromatic or heterocyclic groups are released from the phosphorus atom as the leaving groups (Singh and Walker, 2006).

In *Flavobacterium* sp. ATCC 27551 (Sethunathan and Yoshida, 1973) and *Brevundimonas diminuta* (formerly *Pseudomonas diminuta* MG; (Serdar et al., 1982)), the native OPH has been identified as a membrane-bound homodimeric metalloenzyme containing zinc ions and having broad substrate specificity with hydrolyzing ability against various phosphorus containing bonds such as P-O, P-CN, P-F, and P-S (Grimsley et al., 1997; Singh and Walker, 2006; Theriot and Grunden, 2011). The organophosphorus degradation (*opd*) gene is located in the highly conserved region of each indigenous plasmid: pPDL2 (43 kb) in *Flavobacterium* sp. ATCC 27551 and pCMS1 (66 kb) in *B. diminuta* (Mulbry et al., 1986; Mulbry et al., 1987; Harper et al., 1988; Siddavattam et al., 2003).

Quantitative real-time polymerase chain reaction (qPCR) is a process used to detect amplified PCR products while the PCR reaction is occurring. The qPCR data collection is based on the

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detection of the intensity of fluorescent reporter molecules, which increase with the accumulation of PCR products during each amplification cycle (Higuchi et al., 1993). Because of its high sensitivity and ability to produce results with small amounts of target DNA, this technique is an ideal method for the quantitative analyses of a particular gene in DNA extracted from an environmental sample (Stubner, 2004; Steinberg and Regan, 2009).

The qPCR method has been widely applied to environmental studies. In addition to its use in the detection of microbial abundance, this method is useful for the determination of gene copy number of functional genes that serve as indicators associated with the *in situ* degradation of pollutants (Di Gennaro et al., 2009). This technique has been applied to the measurement of a subunit of the benzylsuccinate synthase gene (*bssA*) for use in monitoring of hydrocarbon-degrading bacteria activity (Beller et al., 2002), assessment of a subunit of the biphenyl dioxygenase gene (*bphA1*) associated with the degradation of polychlorinated biphenyl (PCBs) (Correa et al., 2010), and quantification of aromatic oxygenase genes associated with the bioremediation of petroleum-hydrocarbons (Correa et al., 2010). The bioremediation of polycyclic aromatic hydrocarbons (PAHs), which are highly persistent organic soil pollutants, has been studied using the quantification of dioxygenase genes, *nahAc*, *phnAc* and *nidA* (Cébron et al., 2008; Di Gennaro et al., 2009; Kumar and Khanna, 2010).

Previous inquiries on pesticide bioremediation assessment using qPCR were mainly focused on herbicides and included detection of the α-ketoglutarate-dependent dioxygenase gene (*tfdA*) associated with the degradation of mecoprop-p ((R)-2-(4-chloro-2-methylphenoxy) propanoic acid) (Rodríguez-Cruz et al., 2010), and the measurement of the *atzABC* genes involved in *s*-triazine mineralization for use in detection of simazine (2-chloro-4,6-bis(ethylamine)-*s*-triazine) degrading bacteria (Fajardo et al., 2012). The ecological risks posed by soil contamination with the organophosphorus insecticide, profenofos (PFF), was assessed using the measured abundance of ammonia-oxidizers as determined using qPCR and primers targeting the ammonia monooxygenase gene (*amoA*) (Liu et al., 2012). For assessment of the degradation of the organophosphorus insecticide, chloropyrifos (*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate), the presence of the *opd* gene in soil was measured using general PCR; however, no amplified PCR product was obtained (Singh et al., 2003).

In the present study, we describe a qPCR assay targeting OPH for the assessment of the degradation efficacy of an organophosphorus compound from *in situ* soil samples.

Materials and Methods

Bacterial strains and culture conditions. The reference bacterial strains used for testing the specificity of the primer design were prepared by American Type Culture Collection (USA), Korean Agricultural Culture Collection (Korea), Korean Collection for Type Cultures (Korea), and our laboratory bacterial strain collection. *E. coli* strains DH5α and BL21(DE3) (F^-, *ompT*, $hsdS_B(r_B^- m_B^-)$, *gal*, *dcm*) (Novagen Inc., USA) were used as hosts for recombinant plasmid and T7 expression, respectively. The bacterial strains were cultured in tryptic soy broth (TSB; Difco Laboratories Inc., USA), except for the *E. coli* strain, which was cultured in Luria-Bertani medium (LB; 1.0% tryptone, 0.5% yeast extract, and 0.5% NaCl) supplemented with 100 μg/mL of ampicillin (Sigma Chemical Co., USA). All bacterial strains were cultured at 30°C with shaking at 220 rpm.

Primer design. The nucleotide sequences used for primer design were retrieved from the GenBank database at NCBI (http://www.ncbi.nlm.nih.gov/genbank/). The conserved nucleotide sequence region was selected with sequence alignment using multiple alignment program ClustalW2 at EBI (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Specific primers for the *opd* gene were constructed based on a conserved nucleotide sequence region using Primer Express Software, version 2.0 (Applied Biosystems Inc., USA). All primers were synthesized by Genotech Corp. (Korea).

Conventional PCR and recombinant plasmid construction. As standard plasmids used for the creation of the qPCR calibration curve, recombinant plasmids containing the *opd* or 16S rRNA genes were constructed using genomic DNA from *Flavobacterium* sp. ATCC 27551 as a template. DNA fragments containing each target gene were amplified using the specific constructed PCR primer set (Table 1), inserted into T-vectors using the SolGent™ T-Blunt PCR Cloning kit (Solgent Co., Korea) and designated as pOPD or p16S. General PCR reaction was performed under the following conditions: initial cycle at 97°C for 1 min, 30 cycles of 97°C for 1 min, 56°C for 30 sec, and 72°C for 2 min using *Pfu* DNA polymerase (Stratagene Co., USA). The DNA sequences inserted into all recombinant plasmids were confirmed using DNA sequencing analysis (Solgent Co.).

DNA extraction and quantification. Genomic DNA from the bacterial strains was extracted using the method described by Ausubel et al. (1999) with modifications. Briefly, the bacterial cells were collected by centrifugation at 17,700 × g for 5 min, and resuspended in lysis buffer (25 mM Tris·HCl (pH 8.0), 10 mM EDTA, 50 mM glucose). After adding both 5% sodium dodecyl sulfate (SDS, Sigma Chemical Co.) and 0.1 mg/mL of proteinase K (Sigma Chemical Co.), the cell suspension was incubated at 37°C for 1 h, treated with cetyltrimethylammonium bromide (CTAB, Sigma Chemical Co.; 10% CTAB in 0.7 M NaCl), incubated at 65°C for 10 min, and then extracted with phenol:chloroform:isoamyl alcohol solution (25:24:1, Sigma Chemical Co.). The genomic DNA was precipitated using a 0.9 volume of isopropanol (Burdick & Jackson, USA) and dissolved in TE buffer (10 mM Tris·HCl (pH 8.0), 1 mM EDTA). The soil DNA was extracted using the MoBio Power Soil DNA isolation kit (MoBio Laboratories, Inc., USA) as recommended by the manufacturer. The quantity and quality of the extracted DNA were determined by the ratio of

$A_{260\,nm}$ and $A_{280\,nm}$ as measured using a NanoPhotometer (Implen GmbH, Germany).

Soil microcosm. The soil microcosm was prepared using *in situ* soil collected from a ginseng cultivation field (Yeongju, Korea). For each soil microcosm, 30 g of *in situ* soil was transferred into a 250-mL glass pot and treated with 20 mg/L of tolclofos-methyl (stock solution, 2×10^4 mg/L in acetone; Dongbang Agro Co., Korea). As a negative control group, 30 g of *in situ* soil was transferred into a glass pot, autoclaved at 121°C for 90 min, and then treated with 20 mg/L of tolclofos-methyl (stock solution, 2×10^4 mg/L in acetone; Dongbang Agro Co.). The prepared soil microcosms were placed under a darkened condition with 50% relative humidity at 28°C for 4 weeks. Soil samples were taken from each soil microcosm at one week intervals and stored in a freezer at −20°C until analysis.

The soil properties were analyzed following the methods of soil and crop plant analysis (NIAST, 2000). Briefly, the soil was dried at room temperature and sieved to 2 mm prior to analysis. The soil pH was measured using an Orion 520A pH meter (Orion Research Inc., USA) in a soil:deionized water mixture (1:5, w/v). Electrical conductivity (EC) was assessed using an Orion EC meter (Orion Research Inc.). The organic matter content was measured by the Tyurin method (Schollenberger, 1927). Soil texture was determined using the percentage of soil particles which passed through sieves of mesh sizes 2, 0.425, and 0.075 mm, and then evaluated using the USDA soil textural triangle.

Degradation of tolclofos-methyl. Soil sample (0.5 g) was mixed thoroughly with 1 mL acetonitrile (Burdick & Jackson) by vortexing, centrifuged at 17,700 × g for 1 min, and then the upper 1/3 of the fluid phase was taken. The obtained phase was filtered through a 0.45 μm polyvinylidene fluoride (PVDF) syringe filter (Millipore Co., USA) and analyzed using an high-performance liquid chromatography (HPLC) (Waters Co., USA) equipped with a UV-8010 detector (TOSOH Co., Japan) and ZORBAX Eclipse XRD-C18 column (4.6 mm × 150 mm; Agilent Technologies Inc., USA). A solution of acetonitrile-water (70:30, v/v) was used as the mobile phase at a flow rate of 2 mL/min. Standard tolclofos-methyl was detected at elution times of ~1.45 min at 200 nm, and determined using maximum absorption wavelength (λmax) scanning from 200 to 400 nm.

Quantitative Real-Time PCR. qPCR was carried out using a Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, Inc., USA). The reaction mixture was prepared using SsoFast™ EvaGreen Supermix (Bio-Rad Laboratories, Inc.). Amplification of the target gene was performed under the following conditions including 40 cycles at 97°C for 10 s, 56°C for 10 s, and 72°C for 30 s. For all of the reactions, non-template control (NTC) containing distilled water instead of template DNA was used for the detection of primer-dimer formation. At the end of the PCR reaction, a melting curve was constructed using 10 s temperature increases of 0.5°C in the range 65 to 95°C for verification of specific amplification of the target gene. qPCR was performed for three independent experiments with at least two replicates, and all data was analyzed using the Bio-Rad CFX Manager Software version 1.6 (Bio-Rad Laboratories Inc.). The gene copy number was calculated using the equation derived from the calibration curve of the target gene under the assumption that the average molecular weight of a single DNA base pair is 650 Da.

Organophosphorus hydrolase activity assay. The bacterial strains were cultured in TSB at 30°C for 48 h, and centrifuged at 17,700 × g for 2 min. Obtained cell pellet from 1 mL culture solution was suspended in 50 mM Tris·HCl (pH 8.0). The enzyme assay mixture consisting of 100 μL of whole cell suspension and 50 mM Tris · HCl (pH 8.0) containing 3% methanol was prepared and 172 μM parathion (stock solution, 10 mg/mL in methanol; Sigma Chemical Co.) was added as a substrate. At 25°C, yellow color development due to the production of *p*-nitrophenol was evaluated.

Statistical analysis. Statistical significance was evaluated using independent samples *t*-test ($p<0.05$, $p<0.1$) or one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($p<0.05$) using SPSS 19.0 (SPSS Inc., USA).

Results and Discussion

Specific primer design for OPH. In order to apply qPCR for assessment of the degradation efficacy of the organophosphorus compound, the *opd* gene encoding OPH, which facilitates the hydrolytic reaction of the ester bond in the organophosphorus compound, was evaluated as the target gene. The appropriate specific primer set for the *opd* gene was designed. The nucleotide sequences of the *opd* gene used were as follows (presented with accession no. and homology to the *opd* gene of *Flavobacterium* sp. MTCC 2495): *Flavobacterium* sp. MTCC 2495 organophosphorus hydrolase (AY766084, 100%), *Sphingomonas* sp. JK1 organophosphorus hydrolase (EU709764, 98%), *Flavobacterium* sp. ATCC 27551 parathion hydrolase (AJ421424, 100%), *Pseudomonas diminuta* phosphodiesterase (M20392, 98%), *Flavobacterium balustinum* parathion hydrolase (AJ426431, 100%) and *Agrobacterium tumefaciens* phosphotriesterase (AY043245, 100%) (Fig. 1). Based on the conserved regions, the nucleotide sequence regions were selected in order to achieve minimal probability for the formation of secondary structures and primer dimers, and to amplify less than 200 bp fragment. The specific primers designed, OPDF and OPDR, their expected DNA amplicon sizes were approximately 192 bp, corresponding to the *Flavobacterium* sp. ATCC 27551 *opd* gene positions from 152 to 343.

In addition, the universal bacterial primers, 338F (Land, 1991) and 518R (Muyzer et al., 1993), corresponding to the V3 region of 16S rRNA gene in *E. coli,* were used for specific amplification of the 16S rRNA gene in all bacterial groups with the expected DNA amplicon size being approximately 180 bp (Fierer et al., 2005) (Table 1).

Standard curve for qPCR. For quantification of the target gene,

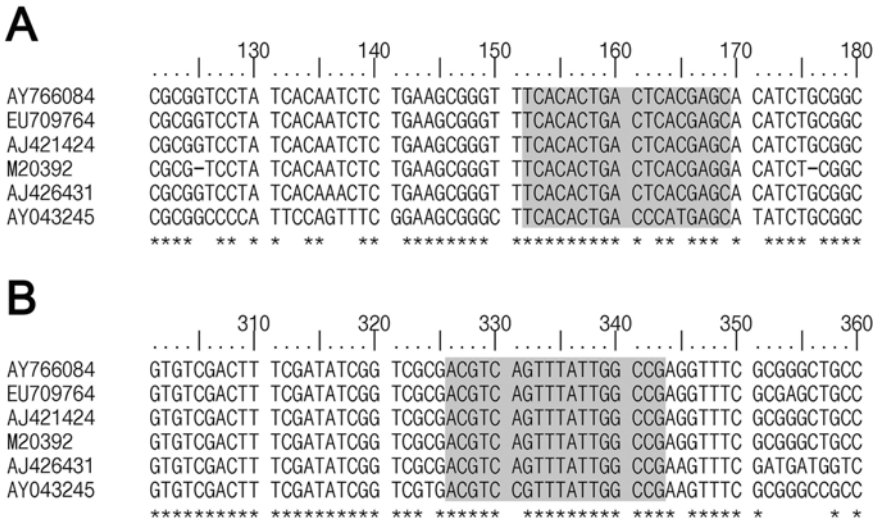

Fig. 1 Nucleotide sequence alignment for the design of the *opd* gene-specific primers. Nucleotide sequences were aligned using ClustalW. Reference bacterial strains for each nucleotide sequences are indicated by the GenBank accession number as follows: AY766084, *Flavobacterium* sp. MTCC 2495 organophosphorus hydrolase (*opd*) gene; EU709764, *Sphingomonas* sp. JK1 organophosphorus hydrolase (*opd*) gene; AJ421424, *Flavobacterium* sp. ATCC 27551 parathion hydrolase (*opd*) gene; M20392, *Pseudomonas diminuta* plasmid pCMS1 phosphodiesterase (*opd*) gene; AJ426431, *Flavobacterium balustinum* plasmid pBC9 parathion hydrolase (*opd*) gene; AY043245, *Agrobacterium tumefaciens* phosphotriesterase (*opdA*) gene. Shaded areas indicate the conserved nucleotide sequence region used for the construction of the *opd* gene-specific forward (A) and reverse primers (B).

Table 1 Primer set used for quantitative real-time PCR

Target gene	Primer sequence (5'−3')	Reference
opd	Forward primer OPDF (tcacactgactcacgagc)	This study
	Reverse primer OPDR (cggccaataaactgacgt)	This study
16S rRNA	Forward primer 338F (actcctacgggaggcagcag)	(Land, 1991)
	Reverse primer 518R (attaccgcggctgctgg)	(Muyzer et al., 1993)

a calculation curve was developed using recombinant plasmid p16S containing the 16S rRNA gene or pOPD containing the *opd* gene from *Flavobacterium* sp. ATCC 27551. Serially diluted plasmid, from 0.32 pg to 16.1 ng μL^{-1} for p16S and from 14.5 pg to 5.8 ng μL^{-1} for pOPD, were used as templates for the qPCR reaction. The standard curve for each target gene was obtained within the range of 10^5 to 10^9 gene copies for the 16S rRNA gene and from 10^6 to 10^9 gene copies for the *opd* gene. The linear correlation coefficient value was above 0.99 for all standards (Fig. 2).

Specificity of the primers targeting the *opd* gene. The specificities of the designed primers were tested using genomic DNA from the reference bacterial strains. The bacterial strains were selected as follow: those belonging to the same class of strain previously reported as an organophosphorus compound degrader, those having the ability to degrade organochlorine compounds such as endosulfan or chlorothalonil, randomly selected unidentified strains, and *E. coli*. Strain *Flavobacterium* sp. ATCC 27551 and *E. coli* BL21(DE3) harboring the recombinant plasmid pETOPD (containing the *opd* gene from *Flavobacterium* sp. ATCC 27551 based on the plasmid vector pET21a(+), unpublished data) were used as positive strains for determining OPH activity. *E. coli* BL21(DE3) harboring recombinant plasmid vector pET21a(+) was used as a negative strain for OPH activity. As described in the materials and methods section, genomic DNA was prepared from each reference bacterial strain. A qPCR reaction for the target gene was carried out using serially diluted genomic DNA as the template.

The 16S rRNA gene using specific primers 338F and 518R was amplified in all bacterial strains. On the other hand, the amplification using qPCR with specific primers targeting the *opd* gene was detected only when both *Flavobacterium* sp. ATCC 27551 and recombinant *E. coli* harboring the plasmid pETOPD containing the *opd* gene were used (Table 2).

In addition, the specific detection of the *opd* gene by qPCR was confirmed through the OPH activity assay. Parathion (*O*,*O*-diethyl *O*-(4-nitrophenyl) phosphorothioate) is one of the most extensively used pesticides for the control of a wide range of insect species. It is known that this compound is hydrolyzed by OPH, generating *p*-nitrophenol as a by-product (Singh and Walker, 2006). Reference bacterial strains were evaluated in order to confirm whether or not they produced OPH activity when parathion was used as a substrate. Yellow color was observed only in the positive controls, *Flavobacterium* sp. ATCC 27551 and the *E. coli* BL21(DE3) harboring the recombinant plasmid pETOPD (Table 2).

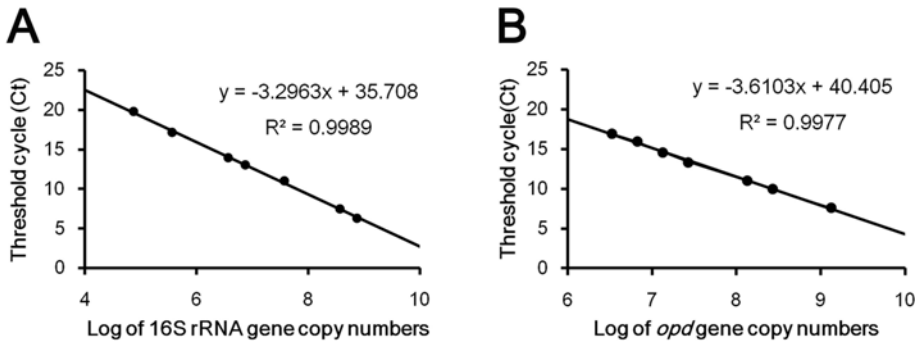

Fig. 2 Standard curve for quantitative real time PCR. qPCR was performed using a recombinant plasmid containing either a 16S rRNA gene or *opd* gene from *Flavobacterium* sp. ATCC 27551 for the template DNA. Threshold cycle (Ct) values were determined through two independent experiments. Calibration curves were derived by plotting the logarithm of each targeted gene copy number versus threshold cycle (Ct) values with (A) for the 16S rRNA gene and (B) for the *opd* gene.

Quantification of the *opd* gene from *in situ* soil using qPCR. The application of qPCR targeting the *opd* gene was confirmed using DNA samples from the *in situ* soil samples. Enhanced degradation indicates the phenomenon whereby the degradation ability of the existing soil microbes against certain pesticides in the soil can be improved with repeated administration of the same pesticide. (Singh and Walker, 2006). In order to evaluate this potential effect, the application of qPCR targeting OPH, using *in situ* soil expected to have the high exposure frequency to organophosphorus pesticides, was verified. Tolclofos-methyl ((*O*,*O*-dimethyl *O*-(2,6-dichloro-4-methylphenyl) phosphorothioate) is a widely used organophosphorus fungicide to control the soil-borne pathogen, *Rhizoctonia solani,* in ginseng growing fields in Korea (MAF and NACF, 2006). In the present study, practical assessment of qPCR assays targeting OPH were investigated using the soil microcosm from the *in situ* soil sample collected from ginseng cultivating fields applied with tolclofos-methyl.

The *in situ* soil was collected from ginseng cultivating fields in Yeongju, Korea. The soil analysis results were as follow: soil pH and EC were 5.9 and 1.8 dS m^{-1}, respectively, organic matter and soil texture were determined to be 17.1%, and the sandy loam composed of sand (65.8 %), silt (26.2%), and clay (8.0%) (Table

Table 2 Specificity of the constructed *opd* gene primer set

Bacterial strain	Strain source[a]	OPH activity[b]	Real-time PCR	
			opd gene[c]	16S rRNA gene[d]
Flavobacterium sp.	ATCC 27551	+	+	+
Pseudomonas diminuta	KACC 10191	-	-	+
Pseudomonas stutzeri	KACC 10290	-	-	+
Mycobacterium chlorophenolicum	KCTC 19089	-	-	+
Streptomyces lividans	KCTC 1163	-	-	+
Bacillus sp. E64-2	(Shin et al., 2008)	-	-	+
Ochrobacterium anthropi	(Lee et al., 2004)	-	-	+
Pseudomonas sp. Y7	Lab collection	-	-	+
Bacillus licheniformis H14	Lab collection	-	-	+
Bacillus subtilis H32	Lab collection	-	-	+
Unidentified strain 4-7	Lab collection	-	-	+
Unidentified strain 13-6	Lab collection	-	-	+
Unidentified strain 19-2	Lab collection	-	-	+
Escherichia coli K-12	Lab collection	-	-	+
E. coli BL21(DE3)/pET21a(+)	This study	-	-	NA[e]
E. coli BL21(DE3)/pETOPD	This study	+	+	NA[e]

[a] ATCC, American Type Culture Collection (USA); KACC, Korean Agricultural Culture Collection (Korea); KCTC, Korean Collection for Type Cultures (Korea); Lab collection, laboratory bacterial strains.
[b] OPH activity examined using parathion as a substrate. + and − represent the presence and absence of yellow color development resulting from the production of *p*-nitrophenol, respectively.
[c] qPCR using the *opd* gene-specific primer set; + and − represent positive and negative amplifications, respectively.
[d] qPCR using the 16S rRNA gene specific primer set; + and − represent positive and negative amplifications, respectively.
[e] NA, not assayed.

Table 3 Characterization of the soil properties

Soil texture	Sand (%)	Silt (%)	Clay (%)	Organic matter (%)	pH[a]	EC[b] (dS m^{-1})
Sandy loam	65.8	26.2	8.0	17.1	5.9	1.8

[a] Soil pH was measured in a soil:deionized water mixture (1:5, w/v)
[b] Electric conductivity

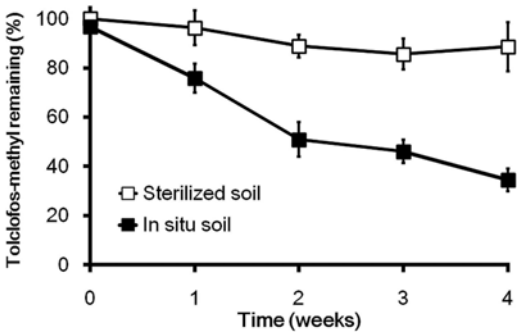

Fig. 3 Degradation of the organophosphorus fungicide, tolclofos-methyl, in *in situ* soil. The soil microcosms were prepared using *in situ* soil collected from ginseng cultivating fields. They were dosed with 20 mg/L of tolclofos-methyl and then placed under darkness with 50% relative humidity at 28ºC for 4 weeks. Soil samples were taken from each soil microcosm at 1-week intervals, and the remaining ratio of tolclofos-methyl was analyzed by HPLC. A soil microcosm containing sterilized *in situ* soil dosed with 20 mg/L of tolclofos-methyl was used as a negative control. Error bars represent the standard deviations (n=3).

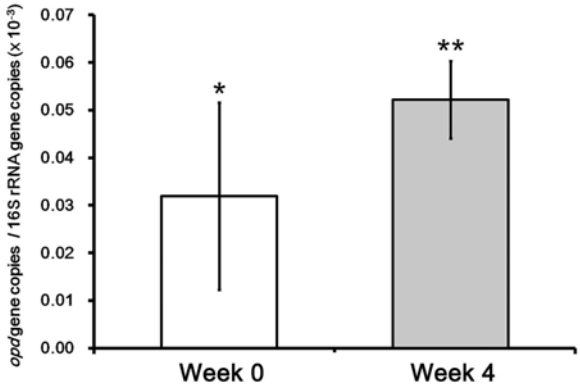

Fig. 4 Comparison of the *opd* gene copy number from *in situ* soil as determined by qPCR. Soil DNA was extracted in duplicate from the soil samples contained in each soil microcosm at weeks 0 and 4. qPCR was performed as described in materials and methods. Target gene copy number was calculated using the equation derived from the standard curve. Quantity of *opd* gene was determined as the relative ratio to 16S rRNA gene copy number in the soil DNA (g of dry soil)$^{-1}$. Error bars represent the standard deviation of three independent qPCR runs. Asterisks indicate significant difference by independent samples *t*-test (p =0.083).

3). As described in the materials and methods section, the soil microcosm was prepared to include the addition of tolclofos-methyl, and degradation of this compound was analyzed over a 4-week period. The negative control soil microcosm was prepared using sterilized *in situ* soil.

During the 4-week evaluation period, the amount of tolclofos-methyl in the soil samples gradually decreased with the remaining ratio being approximately 50% at 2 weeks and 34% at 4 weeks. In contrast, the applied tolclofos-methyl level was maintained without significant changes in value in the negative control soil microcosm (Fig. 3). The degradation pattern in the non-control soil samples was assumed to be due to the bacteria which produce OPH. Therefore, the abundance of the *opd* gene was successfully assessed using the established qPCR assay with specific target primers. The *opd* gene copy number was normalized by the 16S rRNA gene copy number in the soil DNA (g of dry soil)$^{-1}$.

During the 4-week monitoring period, the bacterial population did not change significantly. The 16S rRNA gene was measured to have 4.2×10^8 ($\pm4.0\times10^7$) copies (g of dry soil)$^{-1}$ at week 0 and 4.6×10^8 ($\pm2.5\times10^7$) copies (g of dry soil)$^{-1}$ at week 4 (data not shown). However, the quantity of *opd* gene in the soil at week 4 was 1.6 fold higher than that at week 0 with a 0.083 coefficient value (Fig. 4). The maintenance of 16S rRNA gene copies *in situ* soil can support our result of *opd* gene detection that the increases of *opd* gene copies was not derived from the reduction of bacterial population *in situ* soil, during the 4 weeks of monitoring period. In addition, this result agreed with the degradation pattern of the tolclofos-methyl in the *in situ* soil (Fig. 3), which was assumed to have resulted from the hydrolytic effects of soil microbes producing OPH against tolclofos-methyl. Based on these results, we suggest that the established qPCR assay using the specified *opd* gene primers is effective in measuring the degradation of tolclofos-methyl in the *in situ* soil.

In conclusion, the present study demonstrated the practical application of the qPCR technique on the detection of the *opd* gene concurrent to the remediation of organophosphorus compounds in the *in situ* soil. This method was successfully used to monitor the degradation efficacy against an organophosphorus compound in the *in situ* soil samples.

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