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ARTICLE

Organophosphorus Hydrolase (OpdB) of *Lactobacillus brevis* WCP902 from Kimchi Is Able To Degrade Organophosphorus Pesticides

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Lactobacillus brevis WCP902 that is capable of biodegrading chlorpyrifos was isolated from kimchi. The *opd*B gene cloned from this strain revealed 825 bp, encoding 274 aa, and an enzyme molecular weight of about 27 kDa. OpdB contains the same Gly-X-Ser-X-Gly motif found in most bacterial and eukaryotic esterase, lipase, and serine hydrolases, yet it is a novel member of the GDSVG family of esterolytic enzymes. Its conserved serine residue, Ser82, is significantly involved with enzyme activity that may have application for removing some pesticides. Optimum organophosphorus hydrolase (OpdB) activity appeared at pH 6.0 and 35 °C and during degradation of chlorpyrifos, coumaphos, diazinon, methylparathion, and parathion.

KEYWORDS: Kimchi; pesticide-degrading bacterium; *Lactobacillus brevis*; *opd*B gene; food safety

INTRODUCTION

"Kimchi" is a fermented vegetable that Koreans traditionally and frequently consume at a daily intake of ≈120 g per person (≈10% of total intake). Because kimchi is such a staple food item in Korea, consumption safety is essential for all foods, and the pesticide levels in vegetable production should be carefully monitored and managed. Furthermore, kimchi is becoming a popular food to non-Koreans, contributing to a worldwide concern for food safety, including safety of fermented foods. This means that health officials should evaluate the pesticide residues in kimchi raw materials and the fate of residues during the fermentation process (*1*).

Organophosphorus (OP) insecticides are a group of highly toxic agricultural chemicals that are widely used to control a wide range of insect pests (*2*). OP poisoning is a worldwide health problem with around 3 million poisonings and 200,000 deaths annually (*3*). OP insecticides are potent acetylchloinesterase (AchE) inhibitors that have 50% lethal doses between 4−13 and 14−24 mg kg^{-1} of mammalian body mass. Although OPs are banned in most developed countries, they are still widely applied in developing countries for the control of major agricultural pests (*4*). In particular, chlorpyrifos (CP, *O*,*O*-diethyl-*O*-3,5,6-trichloro-2-pyridyl phosphorothioate) has been commercially used since the 1960s to control foliar insects on cotton, paddy, pasture, and vegetable crops (*5*).

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Biodegradation is a reliable and cost-effective technology with the potential for removing pesticides from food products. Microbial degradation of OP pesticides is of particular interest because OP is highly toxic to mammals and is widely and extensively used in developing countries. Research has identified the various genes (*opd*) and enzymes (organophosphorus hydrolase) involved in degradation of OPs. Zhang et al. (*4*) recently isolated seven methylparathion-degrading bacteria from soil that had long-term methylparathion contamination, and they cloned and sequenced their *opd* genes. Zhang et al. (*2*) isolated a fenitrothion-degrading strain *Burkholderia* sp. FDS-1 from the sludge of a wastewater treatment system in an OP manufacturing company, and they cloned and sequenced its *opd* genes. Yang et al. (*3*) and Li et al. (*6*) also isolated the CP-degrading strains *Stenotrophomonas* sp. YC-1 and *Sphingomonas* sp. Dsp-2 from the sludge of a wastewater treatment system in an OP manufacturing company, and they cloned and sequenced the *opd* genes. Previous studies showed that OP contains phosphoric acid esters that can be hydrolyzed and detoxified by carboxylesterase and phosphotriesterase (*7*−*10*).

Lactic acid bacteria (LAB) are a functional class of microaerophilic, Gram-positive bacteria that ferment hexose sugars to primarily produce lactic acid. LAB include a variety of industrially important genera including *Lactococcus*, *Enterococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Leuconostoc*, *Lactobacillus*, and *Weissella* species (*11*). Dating back to the origins of agriculture, nearly all societies have exploited LAB in the preservation of foods and beverages due to their ability to metabolize substances (*12*). LAB are indigenous to most farm habitats, including fruit, vegetable, cereal grain, and dairy farms. LAB that contribute to kimchi fermentation include *Leuconostoc mesenteroides*, *Leuconostoc*

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citreum, *Leuconostoc gasicomitatum*, *Lactobacillus brevis*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactobacillus sakei*, *Lactococcus lactis*, *Pediococcus pentosaceus*, *Weissella confuse*, and *Weissella koreensis* (*13*−*15*).

Previously we isolated chlorpyrifos-degrading lactic acid bacteria, namely, *Lactobacillus mesenteroides* WCP907, *L. brevis* WCP902, *L. plantarum* WCP931, and *L. sakei* WCP904 during kimchi fermentation (*16*). In this study, we isolated and cloned a gene (*opd*B) encoding organophosphorus hydrolase enzyme (OpdB) from *L. brevis* WCP902 and expressed it in *Escherichia coli*. This is the first report of a gene encoding organophosphorus hydrolase enzyme from lactic acid bacteria. Moreover, OpdB enzyme has potential use for the decontamination of pesticide from food materials such as kimchi, an important food product worldwide.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media. The LAB were isolated from kimchi and cultured at 28 °C in MRS, 1/25 MRS, and mineral salt medium supplemented with nitrogen (MSMN) (*17*) medium. *E. coli* DH5α, BL21 (DE3) and recombinant *E. coli* cells were cultured at 37 °C in Luria−Bertani medium or LB medium and M9 medium supplemented with the appropriate antibiotics. M9 medium and all antibiotics were purchased from Sigma Chemical Co. (St. Louis, MO) and used at the following concentrations: ampicillin, 50 μg mL^{-1}; kanamycin, 50 μg mL^{-1}. The MRS and LB media were purchased from Difco (Becton Dickinson Co., Sparks, MD). pGEM-T easy vector (Promega, Madison, WI) was used for cloning and sequencing. pBluescript II SK (+) (Stratagene, La Jolla, CA) and pET28a (+) vector (Novagen, Washington, DC) vector were used for subcloning and high expression, respectively.

Chemicals and Regents. Cadusafos (CS; *S*,*S*-di-*sec*-butyl *O*-ethyl phosphorodithioate), chlorpyrifos (CP; *O*,*O*-diethyl-*O*-3,5,6-trichloro-2-pyridyl phosphorothioate), coumaphos (CM; *O*-3-chloro-4-methyl-2-oxo-2*H*-1-benzopyran-7-yl *O*,*O*-diethyl phosphorothioate), diazinon (DZ; *O*-3-chloro-4-methyl-2-oxo-2*H*-1-chromen-7-yl *O*,*O*-diethyl phosphorothioate), dyfonate (DF; *O*-ethyl *S*-phenyl ethylphosphonodithioate), ethoprophos (EP; *O*-ethyl *S*,*S*-dipropyl phosphorodithioate), fenamiphos (FA, *RS*-ethyl 4-methylthio-*m*-tolyl isopropylphosphoramidate), methyl-parathion (MPT; *O*,*O*-dimethyl *O*-4-nitrophenyl phosphorothioate), parathion (PT; *O*,*O*-diethyl *O*-4-nitrophenyl phosphorothioate), 3,5,6-trichloro-2-pyridinol (TCP), and diethylthiophosphoric acid (DETP) were obtained from ChemService (West Chester, PA) and Sigma Chemical Co. HPLC-grade water, methanol, acetonitrile, and glacial acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Tributyrin, *p*-nitrophenol-β-buytiric acid (p-NPB), and *p*-nitrophenol (p-NP) were purchased from Sigma Chemical Co. All other reagents were of analytical grade.

Isolation and Identification of Strain WCP902. *L. brevis* WCP902 was isolated from kimchi. To determine lactic acid bacteria, blended and diluted 100 μL kimchi samples were spread on MRS agar plates (selective media for lactic acid bacteria) and incubated at 28 °C for 48 h (*18*). The bacterial strains appearing were grouped on the basis of morphology and colony color. Strain WCP902 was identified by microscopy (figure not shown) and 16S rDNA analysis. The 16S rDNA of strain WCP02 was amplified by PCR. PCR amplification was performed as follows: 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. The PCR primers used to amplify 16S rDNA fragments were the bacterial-specific, 5′-CGG AGA GTT TGA TCC TGG-3′ (sense) and 5′-TAC GGC TAC CTT GTT ACG AC-3′ (antisense) (*19*) (**Table 1**). Amplified 16S rDNA fragments were used as sequencing templates. The 16S rDNA sequence of strain WCP902 was aligned with that of other bacterial species obtained from the GenBank database. The 16S rDNA similarity values were calculated from the alignments, and evolutionary distances were calculated.

Recombinant DNA Techniques. Standard procedures for restriction endonuclease digestions, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation, and other cloning-related techniques were followed (*20*). Nucleotide sequences were determined with the dideoxy chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer, Norwalk, CT). Assembly of the nucleotide sequences and the amino acid sequence analysis were done with the DNAMAN analysis system (Lynnon Biosoft, Quebec, Canada). DNA and amino acid sequence homology searches were performed at the National Center for Biotechnology Information with the BLAST network service and the nonredundant DNA and protein sequence databases. Nucleotide sequence data reported are available in the GenBank database under accession no. FJ480208 for the 16S rDNA and FJ550130 for *opd*B of *L. brevis* WCP902.

Table 1. Oligonucleotide Primers in This Study

name	sequence[a] (5′→3′)	object
877F	CGGAGAGTTTGATCCTGG	16S rDNA (*19*)
878R	TACGGCTACCTTGTTACGAC	
LAB-EstF	TDGCYGHVSGVWBDCAYDCRGTBGT	cloning
LAB-EstR	GYTBRAKSGGVCCTRGHCTRWYYCC	
Lbr-opd1F	TCGCCGGTGACTCTGTTGG	sequencing
Lbr-opdBF	AAAAGAATTCACTCCAAATTAACCAGCG	cloning
Lbr-opdBR	AAAAAACGTTGCTCATTGATGTTTGCC	
Plbr-opdBF	TTTTGAATTCATGCCAGTTATCTTT-TATATTCAT	expression
Plbr-opdBR	TTTTAACGTTTTGATTGTTACGTTGCAA	
OpdB-S82AF	CGGTCGCCGGTGAC**GCT**GTTGGGG-GGAACAT	site-directed mutagenesis
OpdB-S82AR	ATGTTCCCCCCAAC**AGC**GTCACCG-GCGACCG	

[a] *Bam*HI and *Hin*dIII sites are indicated by underline. Bold letters indicate the codon encoding alanine by site-directed mutagenesis.

Growth and Degradation of Chlorpyrifos. *L. brevis* WCP902 was cultured in 3 mL of MRS medium; from that medium 500 μL of bacterial culture suspension (8.0 cfu mL^{-1}) was inoculated into 50 mL of $^{1}/_{25}$ MRS with CP (0.29 mM). Finally, 100 μL of culture suspension (8.0 cfu mL^{-1}) of *L. brevis* WCP902 was inoculated into 50 mL of MSMN medium, where CP (0.29 mM) was supplemented as the only source of carbon instead of glucose. *E. coli* DH5α was also cultured as negative control in similar MSMN media. The recombinant *E. coli* cell (pSCY200) growth was also confirmed in M9 medium with CP (0.29 mM). At periodic intervals, an individual flask was sacrificed, and its contents were used to determine growth and degradation of CP. Each of the strain cultures, such as WCP902, *E. coli* DH5α, and the recombinant *E. coli* cells, was run in triplicate to ensure accuracy. Growth was monitored by spectrophotometer at 600 nm (Spectronic 2D, USA). Inoculated culture of *E. coli* DH5α with CP (0.29 mM) was used as the negative control. Residual concentration of CP was analyzed by HPLC.

Substrate Range. Degradation of other OP insecticides was also carried out using identical conditions. The liquid MSMN or M9 medium was supplemented with CS, CP, CM, DZ, DF, FA, EP, MPT, and PT; concentrations of the insecticides are given in **Table 2**. The insecticide residues were measured by HPLC. Growth of strain WCP902 and recombinant *E. coli* cells on methanol, ethanol, propanol, isopropanol, and acetone was tested by using MSMN or M9 medium with one of these compounds as the sole source of carbon.

Cloning and Sequencing of *opd* Gene. To amplify *opd*B homologues from the *L. brevis* WCP902 chromosome, degenerate oligonucleotide primers were designed on the basis of conserved amino acid sequences adjacent to the conservation region of carboxylesterase available in the database. The sense and antisense degenerate oligonucleotide primers are 5′-TDG CYG HVS GVW BDC AYD CRG TBG T-3′ (LAB-EstF, sense) and 5′-GYT BRA KSG GVC CTR GHC TRW YYC C-3′ (LAB-EstR, antisense), respectively. PCR was performed using *L. brevis* WCP902 genomic DNA, Super-Therm DNA polymerase (JMR, Side Cup, Kent, U.K.), 1.5 mM $MgCl_2$, and 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s (*21*−*23*). The anticipated product of approximately 180 bp was isolated from an agarose gel using a gel extraction kit (iNtRON Biotechnology, Suwon, Korea). PCR product was confirmed sequenced by BLAST search. From these initially

Table 2. Extent of Degradation and Relative Substrate Activity of OpdB Enzyme on Organophosphorus (OP) [a] Insecticides

OP insecticide	initial concn of OP insecticide (mM)	residual OP concn (mM)/ rel substrate activity[b] (%)
cadusafos (CS)	0.37	0.33/13
chlorpyrifos (CP)	0.29	0.06/100
coumaphos (CM)	0.28	0.12/74
diazinon (DZ)	0.33	0.18/58
dyfonate (DF)	0.41	0.38/8
ethoprophos (EP)	0.41	0.36/16
fenamiphos (FA)	0.30	0.18/49
methylparathion (MPT)	0.38	0.11/89
parathion (PT)	0.34	0.11/87

[a] The organophosphorus hydrolase (OpdB) activity was assayed with CS, CP, CM, D), DF, EP, FA, MPT, and PT as substrate at pH 6.0 and 5 °C for 12 h. [b] Values indicate the means of three replications ($n = 3$).

sequenced DNA regions we then amplified downstream "outside" DNA by primer walking using only one specific primer 5′-TCG CCG GTG ACT CTG TTG G-3′ (Lbr-opd1F). The amplified fragments were isolated for further nucleotide sequencing. The complete open reading frame (ORF, *opd*B) was amplified from genomic DNA using 5′-AAA AGA ATT CAC TCC AAA TTA ACC AGC G-3′ (Lbr-opdBF, sense) (*Bam*HI sites are indicated by underline), 5′-AAA AAA CGT TGC TCA TTG ATG TTT GCC-3′ (Lbr-opdBR, antisense) (*Hin*dIII sites are indicated by underline), and cloned into pGEM-T easy vector. The recombinant plasmid was digested with *Bam*HI and *Hin*dIII and cloned into pBluescript II SK+ digested with the same restriction enzyme. Nucleotide sequences were determined by the dideoxy chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Co., Norwalk, CT). The samples were analyzed with an automated DNA sequencer (model 3100; Applied Biosystems, Foster City, CA). Additional oligonucleotide primers were designed on the basis of available sequence information to extend and confirm the existing sequence. The BLAST program was used to find the protein coding regions.

Expression and Purification of the Enzymes. For high expression of enzymes, the PCR products generated with primers [5′-TTT TGA ATT CAT GCC AGT TAT CTT TTA TAT TCA T-3′ (sense, *Bam*HI sites are indicated by underline) and 5′-TTT TAA CGT TTT GAT TGT TAC GTT GCA A-3′ (antisense, *Hin*dIII sites are indicated by underline)] (**Table 1**) were cloned into expression vector pET-28a (+) (Novagen), resulting in the addition of a C-terminal $(His)_6$ tag. *E. coli* BL21 (DE3) carrying pET-28a (+)/*opd*B was grown at 37 °C to midlog phase in LB medium containing 50 μg mL^{-1} kanamycin. Expression was then induced by adding IPTG to a final concentration of 0.5 mM, and further growth was continued for 5 h. The cells were harvested by centrifugation (6000*g*, 10 min) and washed twice with 10 mM Tris-HCl buffer (pH 7.0). The cells were resuspended in the same buffer and stored at −20 °C. The frozen cells were mixed with 50 mM Tris-HCl buffer (pH 7.5) containing 1 mg of bovine DNase I and incubated at 37 °C for 30 min. Triton X-100 was added to the suspension to attain a final concentration of 2.5%. The supernatant was collected and stored at 4 °C. The solubilized recombinant OpdB with His-tag was applied on a HisTrap kit (Amershan Pharmacia Biotech). Purification of expressed His_6-tagged protein was carried out accordingly as previously described by Guo et al. (*24*), and protein (OpdB) was eluted with 100 mM imidazole with 0.1% Triton X-100. The purified protein sample was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined according to the method of Bradford (*25*). After estimation of the amount of protein in the protein solution, we set the concentration at 50 μg/mL, which we used in the activity assay.

Characteristics of Enzymes. The esterase activity was determined by a spectrophotometeric method using p-NPB as the substrate. The rate of hydrolysis of p-NPB at 35 °C was measured in 50 mM sodium phosphate buffer (pH 7.0) at 420 nm according to the method of Alvarez-Macarie et al. (*26*). One unit of esterase was defined as the amount of enzyme required to release 1 μmol of p-NP per minute under the assay conditions. The effects of pH and temperature on the esterase activity were examined with the purified recombinant enzyme. The effect of pH on the esterolytic activity was determined by using the protocol described above, to obtain values from pH 3.0 to 11.0; all of the assays were performed at 30 °C for 1 h. To determine the effect of temperature on the enzymatic activity, samples were incubated at temperatures ranging from 10 to 70 °C for 1 h. The relative activities of OphB affected by pH and temperature were measured by calculating highest absorbance denoted to 100% relative activity and then calculated other relative activities. Hydrolysis of CS, CP, CM, DZ, DF, FA, EP, MPT, and PT was measured by changes in absorbance at 214 nm by HPLC. OphB enzyme sample (50 μL) was added to an assay mixture containing 700 μL of 200 mM phosphate-buffered saline (PBS, pH 6.5) and 250 μL of CS, CP, CM, DZ, DF, FA, EP, MPT, and PT. All assays were performed in triplicate.

Site-Directed Mutagenesis. Site-directed mutagenesis of the *opd*B gene (pET-28a (+)/OpdB) was performed to create the mutations using the following oligonucleotide primers: 5′-CGG TCG CCG GTG AC**G CT** GTT GGG GGG AAC AT-3′ (sense) and 5′-ATG TTC CCC CCA ACA **GC**G TCA CCG GCG ACC G-3′ (antisense) (**Table 1**). The 50 μL reaction mixtures contained 1 μL of the pET-28a(+)/*opd*B DNA (80 ng μL^{-1}), 4 μL of 10 pmol of each primer, 5 μL of 2 mM dNTP mixture, 5 μL of 10× *Pfu* DNA polymerase buffer containing 20 mM $MgSO_4$, and 2.5 U of cloned *Pfu* DNA polymerase purchased from Stratagene. PCR products were incubated on ice for 5 min, and 1 μL of *Dpn*I restriction enzyme (10 U μL^{-1}) was added for 1 h of incubation at 37 °C. *Dpn*I-treated plasmids were then transformed into *E. coli* DH5α according to the manufacturer's specifications (site-directed mutagenesis kit, Stratagene).

Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC) Analysis. TLC and HPLC were performed to determine the nine OPs being degraded by the strain of WCP902 and the recombinant *E. coli* cells. Five milliliters of the culture was collected and centrifuged. Four milliliters of supernatant was extracted with ethyl acetate (8 mL × 3), and the organic layer was dried with Na_2SO_4 and concentrated under vacuum. Extracted sample was spotted on precoated silica gel aluminum plates (0.25 mm, Merck). TLC plate was developed with a chloroform and hexane (4:1, v/v) solvent system for detection of CP, whereas an ethyl acetate, isopropanol, and NH_4OH (5:3:2, v/v) system was used for detection of TCP. Finally, the target compounds were detected with UV wavelength (254 nm). In TLC, TCP was confirmed as the spot with approximately 0.66 of R_f value, and more polar CP was determined as the spot with about 0.57 of R_f value. One milliliter of supernatant was mixed with 1 mL of methanol for preparation of HPLC sample. The above mixed solution was filtered through a 0.45 μm Minipore PVDF filter (Schleicher and Schuell, GmbH, Dassel, Germany) for HPLC analysis. Injection volume was 10 μL of filtered sample. The analysis of OP and TCP was carried out on HPLC (HPLC, Perkin-Elmer 200 series) using a C18 column (250 × 4.6 mm, 5 μm, Phenomenex, Torrence, CA). The mixture of 0.5% acetic acid and methanol (1:4 v/v) was eluted with a flow rate of 1 mL min^{-1} at 30 °C. Target compounds, OP and TCP, were measured at 214 nm on a UV detector (Perkin-Elmer UV 200 series). In HPLC analysis, TCP was detected approximately at 5.5 min and OPs were detected between 4.6 and 14.0 min depending on features of each functionality. The calibration curves for nine OPs were made from the serial dilutions of the samples dissolved in 100% methanol. The linear range and the equation of linear regression were obtained sequentially at 0, 10, 25, 50, 75, and 100 μg mL^{-1}. The above serial standard solutions were filtered through a 0.45 μm Minipore PVDF filter (Schleicher and Schuell) for HPLC analysis. Injection volume was 10 μL of serial standard solutions.

RESULTS

Isolation and Identification of Chlorpyrifos-Degrading Lactic Acid Bacteria. We were able to obtain a CP-degrading strain of WCP902 from fermented kimchi. We compared the 16S rDNA sequence (FJ480208) of the WC902 strain with the sequences of all validly described LAB from related taxa. We observed the highest 16S rDNA sequence similarity between strain WCP902 and *L. brevis* ATCC 14869^T (99.4%) (data not shown).

Cell Growth and Degradation of Chlorpyrifos-Degrading Strain in Liquid Culture. The cell growth response and degradation pattern of the strain WCP902 with CP are shown in **Figure 1**. During incubation, strain WCP902 grew markedly on the first day (OD 0.82) and slightly decreased on the second day; after that, growth of strain WCP902 increased until the sixth day (OD 0.941) and

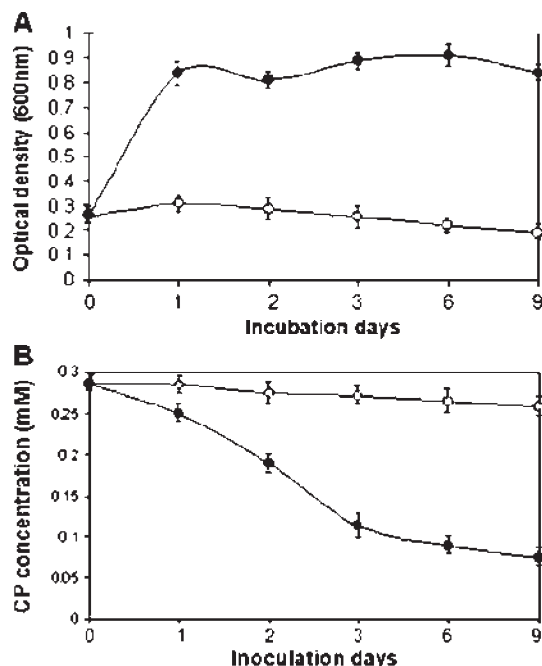

Figure 1. (**A**) Cell growth response and (**B**) chlorpyrifos (CP) degradation pattern of *Lactobacillus brevis* WC902 (●) and *Escherichia coli* DH5α as control (○) in MSMN medium containing 100 mg/L of CP for day 9. Values indicate the means of three replications ($n = 3$).

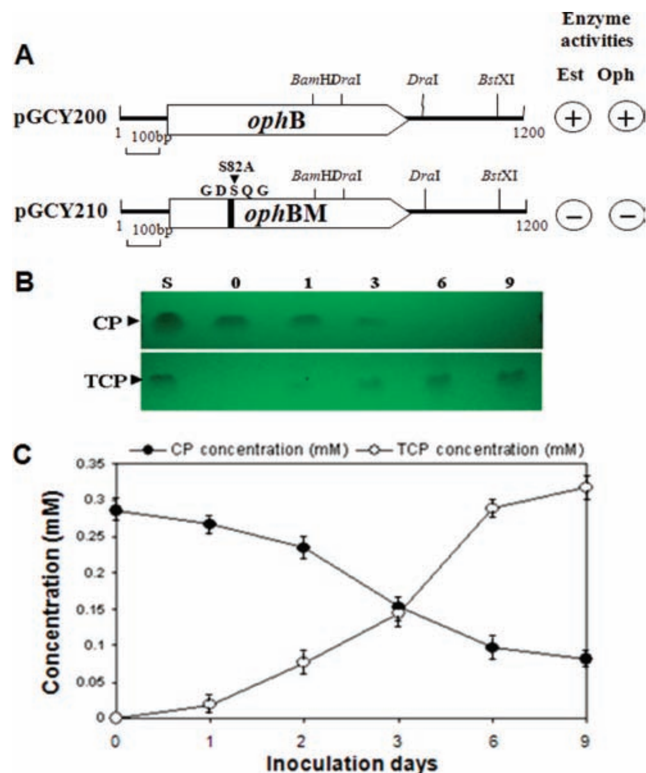

Figure 2. (**A**) Physical map of recombinant *opd*B gene from *Lactobacillus brevis* WCP902. The pSCY200 named plasmid was constructed by cloning the fragment of 1.2 kb into pBluescript II SK(+) vector. The pSCY210 named plasmid was constructed by site-directed mutagenesis (serine 80). (**B**) TLC profile and (**C**) changes of chlorpyrifos (CP) and 3,5,6-trichloro-2-pyridinol (TCP) of *opd*B clone (pSCY200) growing in the M9 medium containing 0.29 mM CP for day 9. In the *x*-axis, inoculation time (day) and curves are discontinuous. Values indicate the means of three replications ($n = 3$).

declined after 6 days (OD 0.912) (**Figure 1A**). On the other hand, CP degradation was very high from the first day to day 3 day of incubation (from 0.285 to 0.114 mM, respectively). Besides, from 3 days to 9 days of incubation CP degradation was a little slow (from 0.114 to 0.075 mM, respectively); however, maximum degradation was found at 9 days of incubation, because CP was almost completely degraded at this time (**Figure 1B**). This strain degraded all of the OPs tested in the cross-feeding experiment. Some of the tested OPs had DETP as side chains (CP, CM, DZ, MPT, and PT) and others had no DETP as side chains (CS, DF, EP, and FA). Except for DF, WCP902 hydrolyzed all of the other eight OPs at a phospho-ester bond. Most notably, WCP902 degraded CP, CM, DZ, MPT, and PT from 44 to 76% by day 9 (data not shown).

Cloning and Sequencing of the *opd* Gene. After sequencing, a total of 723 bp nucleotide sequences was found in the open reading frame of *opd*B (**Figure 2A**). ORF of *opd*B started with an ATG initiation codon and ended with a TGA opal stop codon at position 860 bp. It encoded a protein of 240 amino acids that had a predicted molecular mass of ≈27 kDa. PSORT (Compute pI/M_w tool; http://www.expasy.org/tools/pi_tool.html) revealed no potential signal sequence, and the calculated pI of OpdB was 4.84.

Degradation of Chlorpyrifos by the *opd*B Clone. The CP degradation patterns of the *opd*B clone, pSCY200 (*opd*B), are shown in **Figure 2B**,**C**, and the TCP results are shown in **Figure 2B**. We detected CP and TCP R_f values of 0.57 and 0.66, respectively, in samples drawn at 0, 1, 2, 3, 6, and 9 day intervals. During incubation, the *E. coli* DH5α (harboring the *opd*B gene) degraded CP until day 9 (from 0.285 to 0.075 mM), where degradation of CP was maximum at 2 days of inoculation. In contrast, TCP concentration gradually increased until day 9 (from 0 to 0.318 mM) (**Figure 2C**).

Amino Acid Sequence Analysis of OpdB. Similarities of OpdB amino acid sequences were 7.7−57.9% with other esterolytic protein sequences of the mentioned strains in the phylogenetic tree in **Figure 3** (percentage of similarity not shown). The amino acid sequence of GDSVG, starting at residue 82 (**Figure 2A**) fits the Gly-X-Ser-X-Gly motif found in most bacterial and eukaryotic serine hydrolases. However, a catalytic triad of Ser-Asp/Glu-His, which is highly conserved in most esterase and lipase groups, was not present in OpdB. The phylogenetic tree showed that the OpdB protein had highest similarity with the exterolytic enzyme of *Streptomyces ambofaciens* ATCC 23877 (CAJ90196), and the dark blue color shows the most conserved region among the esterolytic and lipolytic enzymes (**Figure 3**).

Purification and Characterization of OpdB Proteins. We purified the OpdB protein from *E. coli* BL21 (DE3), which was overproducing OpdB. When we analyzed protein fractions with SDS-PAGE, a single 27 kDa protein band was present after the final purification step (**Figure 4**). We tested the effects of pH and temperature on the ability of OpdB to hydrolyze p-NPB, first using various buffers of pH 3.0−11.0 at 40 °C and then across a temperature range at pH 6.0. Maximum activity occurred at pH 6.0 and 35 °C (**Figure 5**). The OpdB enzymes decomposed nine OP insecticides. Except for DF, OpdB enzymes hydrolyzed eight OP insecticides (CS, CP, CM, DZ, EP, FM, MPT, and PT). The residual concentrations of organophosphorus compounds ranged from 0.06 to 0.38 mM, and relative substrate activities ranged from 8 to 100%, whereas OpdB enzyme showed highest degradation activity on chlorpyrifos and lowest degradation activity on dyfonate (**Table 2**).

Identification of Residues Essential for OpdB Enzyme Activity. Most lipases and esterases have the consensus sequence motif Gly-X-Ser-X-Gly, in which serine is the active site. Analysis of the

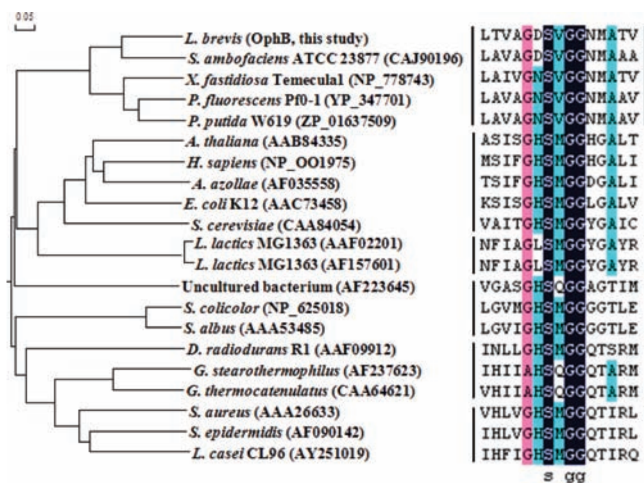

Figure 3. Phylogenetic tree showing the evolutionary relatedness and homology degree between the esterolytic and lipolytic enzymes amino acid sequences and the alignment of the conserved regions found in the primary esterolytic and lipolytic enzymes. The aligned enzymes were from *Lactobacillus brevis* WCP902, *Streptomyces ambofaciens* ATCC 23877, *Xylella fastidiosa* Temecula1, *Pseudomonas fluorescens* Pf0-1, *Pseudomonas putida* W619, *Arabidopsis thaliana*, *Homo sapiens*, *Anabaena azollae*, *Escherichia coli* K12, *Saccharomyces cerevisiae* S288C, *Lactococcus latis* subsp. *cremoris* MG1363 (*lip*), *Lactococcus lactis* subsp. *cremoris* MG1363 (*estA*), soil uncultured bacterium (*lipA*), *Streptomyces coelicolor* A3(2), *Streptomyces albus*, *Deinococcus radiodurans* R1, *Geobacillus thermocatenulatus* DSM730, *Geobacillus stearothermophilus* P1, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Lactobacillus casei* CL96. Dark blue color indicates the most conserved region, then pink and sky blue, which is marked by DNAMAN software.

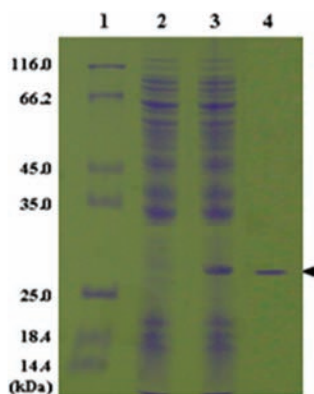

Figure 4. Electrophoretic analysis of the purified OpdB. Separation was performed on a 12.5% (w/v) SDS−polyacrylamide gel. Lanes: 1, standard marker; 2, crude extract from *E. coli* BL21 (DE3) containing pET-28a (+)/OpdB; 3, crude extract from IPTG-induced *E. coli* BL21 (DE3) containing pET-28a (+)/OpdB; 4, purified OpdB protein from Hi-Trap kit (Amersham). The gel was stained with 0.025% Coomassie blue R-250. Molecular weight makers used were β-galactosidase (116,000), bovine serum albumin (66,200), ovalbumin (45,000), lactate dehydrogenase (35,000), restriction endonuclease Bsp981 (14,400), β-lactoglobulin (18,400), and lysozyme (14,400).

deduced amino acid sequences of OpdB revealed a potential serine hydrolase motif, G-D-S82-V-G. To determine whether Ser82 was involved in catalytic esterase action, we replaced Ser82 with Ala using site-directed mutagenesis, expressed the mutant proteins in *E. coli*, and purified them. Subsequently, the mutant proteins (mutant OphB) had no activity on p-NPB and CP (**Figure 2A**).

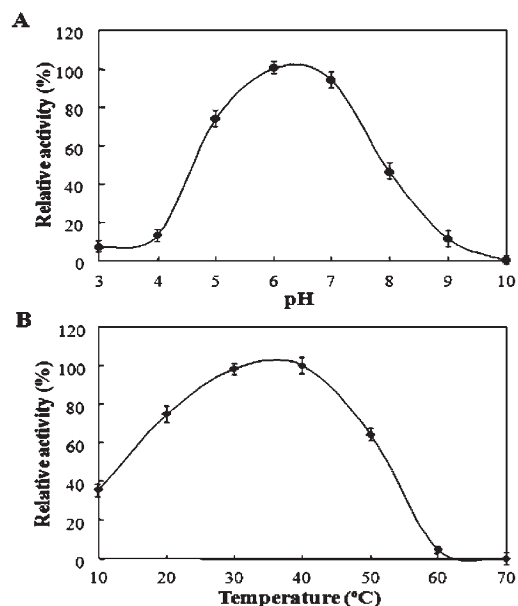

Figure 5. (**A**) Effect of pH and (**B**) temperature on the relative activity of OpdB. The enzyme activity of OpdB was assayed using *p*-nitrophenyl butyrate as substrate at the different pH values and temperature at 35 °C for 1 h. Values indicate the means of three replications (n = 3).

DISCUSSION

In this study, we isolated CP-degrading LAB from fermented kimchi. 16S rDNA characterization of the WCP902 isolate showed the highest similarity among members of the LAB, particularly *L. brevis*. This study is the first report of a gene encoding organophosphorus hydrolase enzyme of LAB species (*L. brevis* WCP902) from kimchi. *L. brevis* WCP902 was capable to degrade CP. When *L. brevis* WCP902 was cultured in MSMN medium with CP (0.29 mM), at that time *L. brevis* WCP902 was hydrolyze CP and used the hydrolyzed product as their sole source of carbon because in the MSMN medium CP was only source of carbon. High population of the strain WCP902 was found in MSMN medium and was associated with CP degradation. LAB is also involved in the production of kimchi, wine, sourdough, and a number of other indigenous foods (*13*−*15*).

Previous studies of OP-degrading microorganisms suggest that these bacteria mainly degrade compounds co-metabolically (*27*,*28*). Some isolated species of bacteria utilize OP as a source of either carbon or phosphorus (*29*) from hydrolysis products (*30*). We determined that *L. brevis* WCP902 utilized OP insecticides as carbon source, whereas *L. brevis* WCP902 were grown in MSMN media supplemented with OP insecticides, which were the only source of carbon in the MSMN medium instead of glucose. In addition, *L. brevis* WCP902 could hydrolyze five OP insecticides (e.g., CP, CM, DZ, MPT, and PT), whereas *L. brevis* WCP902 exhibited versatility in their utilization of either dimethyl compounds (e.g., MPT) or diethyl compounds (e.g., CP, CM, DZ, and PT) as carbon source. They had either diethyl or dimethyl phosphorothionate side chains, and all had a phosphotriester bond, which may contribute to their degradation. Sethunathan and Yoshida (*31*) isolated *Flavobacterium* species that could use PT as phosphorus sources but not DZ as a carbon sources; however, this *Flavobacterium* strain could not use other OPs as sources of either phosphorus or carbon. Shelton (*32*) isolated a bacteria consortium that could use diethylthiophosphoric acid as a carbon source but could not use it as a phosphorus or sulfur source. Recently, Yang et al. (*3*) and Zhang et al. (*2*) reported that CP-degrading bacteria could use CP as a source of phosphorus and

carbon. In addition, Yang et al. (*5*) isolated an *Alcaligenes* species that could use CP, PT, DZ, and even TCP as carbon source. Kertesz et al. (*33*) suggest that ideal environmental enrichment conditions are needed not only when selecting for strains with the desired degrading enzyme systems but also when selecting for specific regulation mechanisms needed for degradation pathways. Due to its broad specificity against a range of OP compounds, *L. brevis* WCP902 has great potential to provide either a versatile gene or an enzyme system that can be used in remediation of highly toxic organophosphate neurotoxic agents.

The molecular basis for degradation of specific OPs has been extensively reported (*34*−*37*). The widely distributed OP-degrading gene (*opd*) has been isolated from temporally, geographically, and biologically different species (*31*, *34*, *36*). The *opd* genes of *P. diminuta* MG and *Flavobacterium* sp. ATCC 27551 are plasmid borne (*34*, *35*). Horne et al. (*36*) isolated a chromosome-based *opd* gene from *Agrobacterium radiobacter*. Another *opd* gene (*mpd*) with identical function was first cloned from *Plesiomonas* sp. M6 (*28*). The *mpd* genes detected in OP-degrading bacteria from Chinese soils and the sludge of a wastewater treatment system of an OP manufacturer were highly conserved, although they were in bacteria from different genera (*4*, *6*, *38*). In this study, *opd*B gene of *L. brevis* WCP902 was chromosome-based. The *mpd* genes have a chromosomal location, except in *Pseudomonas* sp. WBC-3, where *mpd* has a plasmid location (*38*). This is the first report of an *opd* gene clone from the LAB group (*L. brevis*). Its complete sequence was 723 bp encoding 240 amino acids with a predicted molecular mass ≈27 kDa.

In this study, we found OpdB has a Gly-X-Ser-X-Gly sequence motif where serine is the catalytic active site (**Figure 3**). This same motif and active site are found in most bacterial and eukaryotic serine hydrolases, such as lipase, esterase, serine proteinase, and β-lactamase (*39*−*41*). We observed high levels of organophosphorus hydrolase activity in CP, CM, DZ, MPT, and PT. Several investigators have previously reported that many OPs contain esters of phosphoric acid and can, therefore, be hydrolyzed and detoxified by carboxylesterase and phosphotriesterase (*7*, *10*). OP hydrolase is remarkable for its extremely broad use of variable substrates. It can catalyze hydrolysis of many neurotoxic agents and OP insecticides (*42*). Hydrolysis of OP compounds by organophosphorus hydrolase reduces mammalian toxicity by several orders of magnitude. Recombinant gene expression has potential use in bioremediation (with other hosts such as *E. coli*), has low cost of production, has environmental and health uses, and provides information on substrate specificity and protein structure activity associations (*3*).

In conclusion, microorganisms are a major component of the ecosystem and are significantly involved in the degradation of insecticides. We cloned *opd*B gene from *L. brevis* WC902 strain and expressed it in *E. coli* DH5α. OpdB enzyme has potential use for the decontamination of raw food materials such as kimchi. Future studies are needed to determine the potential for industrial uses of OpdB, and studies on the degradation ability of DETP and TCP (CP by product) need to be conducted.

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