

# Cloning, expression, and functional characterization of an organophosphates insecticides degrading gene (*opdC*) from a potential probiotic *Lactobacillus plantarum* WCP931

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# Abstract

An organophosphorus (OP) insecticides degrading strain *Lactobacillus plantarum* WCP931, harboring OP hydrolase (OpdC) gene, was isolated during *kimchi* fermentation. The strain WCP931 appeared a significant survival rate of 51 to 96% under the artificial gastric acidic condition at pH 2 to 3 after 3 h. The *opdC* gene consisting 831 bp encoding 276 amino acids was cloned from the strain WCP907. The recombinant *Escherichia coli* harboring *opdC* gene depleted 77% chlorpyrifos (CP) in M9 medium after 6 days of incubation. The OpdC enzyme represents a novel member of GHSQG family of esterolytic enzymes or new Opd group. The OpdC molecular mass was estimated to be approximately 31 kDa in SDS-PAGE and showed maximum activity at 40 °C with pH 6. However, the mutated OpdC (Ser116 → Ala116) enzyme had no activity towards OP insecticides and *p*-nitrophenol- $\beta$ -butyrate. Importantly, relative activity of OpdC against P-O bond insecticides was higher than P-S bond insecticide, which indicated its broad substrate specificity. It is suggested that *opdC* gene of strain WCP931 play role for the biodegradation of OP insecticides during *kimchi* fermentation.

## Introduction

Chlorpyrifos (CP) is one of the most extensively applied organophosphorus (OP) insecticides worldwide (Ahmed et al. 2012). However, its residues persist in the environment for unpredictable period of time (Tariq et al. 2007) and may enter vegetables cultivated on the polluted sites and pose a great threat to the human health (Huete-Soto et al. 2017). The environmental fate of CP has been extensively studied, and its half-life in soil varies from 10 to 120 days (Haque et al. 2018; Getzin 1981) resulting in 3,5,6-trichloro-2-pyridinol (TCP) as the major degradation product. Hwang and Moon (2018) reported that the levels of CP residue in Korean Cabbages were ranged between 0.12~0.75 mg/kg after 32-35 days of CP (0.24~0.72 g a.i./m<sup>2</sup>) treatment in different cabbage fields. Yu et al. (2006) reported that the CP-degrading fungal strain *Verticillium* sp. being used in detoxification of the insecticide on vegetables. In previously, Cho et al. (2009a) investigated that lactic acid bacteria (LAB), including *Leuconostoc* genus (*L. mesenteroides* WCP907) and *Lactobacillus* genus (*Lactobacillus brevis* WCP902, *Lactobacillus plantarum* WCP931, and *Lactobacillus sakei* WCP904), could utilize OP insecticides as a source of carbon and phosphorus in a defined medium and decontaminated the insecticides in *kimchi* vegetables during fermentation.

*L. plantarum*, like all probiotics, is a beneficial bacterium that can be used for improved health. It has been shown to be an effective treatment for promoting normal digestive health, irritable bowel syndrome (Ducrotte et al. 2012), Crohn's disease, and colitis. This bacterium is acid and bile salt tolerant, which allows it to survive the passage through the gastrointestinal tract of humans (Cebeci and Gurakan, 2003). It can destroy potential pathogens such as *Listeria monocytogenes*, *Escherichia coli*, *Yersinia enterocolitica*, *Enterobacter cloacae* and *Enterococcus faecalis* and preserve critical nutrients, vitamins, and antioxidants (Johansson 1998; Siroli et al. 2015). *L. plantarum* also has beneficial immuno-modulatory activity *via* an increased interleukin-10 synthesis and secretion in macrophages and T-cells derived from the inflamed colon. (Mark 2003).

Characters of fermented *kimchi* are originated by the action of LAB during fermentation. On the basis of acidity, *kimchi* fermentation was divided into five stages: initial stage, immature stage, optimum-ripening stage, over-ripening stage, and rancid stage (Cho et al. 2009b). In previous reports, *kimchi* fermentation is dominated by *Lactobacillus* sp. and *Leuconostoc* sp. (Cho et al. 2009b; Kim et al. 2012; Park et al. 2006). In fact, the population density of *L. mesenteroides* reached to its maximum during the main ripening period of *kimchi* and then declined as the pH of *kimchi* decreased, whereas that of acid tolerant *lactobacilli* such as *L. plantarum* continued to increase until the last stage of fermentation (Cho et al. 2009b). Although *L. plantarum* probiotic has involved in multiple health benefits, but its biodegradation ability to OP insecticides was unknown. We first report the role of *L. plantarum* in biodegradation of OP insecticides (Cho et al. 2009a). Zhang et al. (2014) reported relationship of phosphatase from LAB including *L. plantarum* and OP insecticides degradation in skimmed milk and Harishankar et al. (2013) reported the *in vitro* degradation of chlorpyrifos using intestinal bacterium *L. plantarum*.

However, the gene of *L. plantarum* responsible for the degradation of OP insecticides is not revealed yet. In this study, an OP insecticides hydrolase gene (named *opdC*) is cloned from potential probiotic strain *L. plantarum* WCP931 and its purified recombinant OpdC enzyme is characterized. The site directed mutagenesis and bioinformatics tools analysis predicted the potential active site of OpdC enzyme that played vital role for the degradation of nerve poisoning organophosphate insecticides. This study first time reveals the cloning and functional characterization of an *opd* gene from the potential probiotic *L. plantarum* WCP931 which augmented the diversity of *opd* gene in nature.

## Method And Materials

### Strains, plasmids, media and chemicals

The strains *E. coli* DH5 $\alpha$ , BL21 (DE3), and recombinant *E. coli* cells (expressing *opdC* gene) were cultured at 37 °C in a Luria-Bertani (LB) medium and M9 medium supplemented with the appropriate antibiotics. The Luria Beratani (LB), lactobacilli MRS (MRS), and minimal nutrient (M9) medium were purchased from Difco Co. (MD, USA). The pGEM-T easy vector (Promega Co., Madison, WI, USA) was used for cloning and sequencing of the interest genes. The vector pBluescript II SK<sup>(+)</sup> and pET32a<sup>(+)</sup> (Stratagene, CA, USA) was used for subcloning and over-expression as well as purification of the gene product. The OP insecticides such as chlorpyrifos (CP), cadusafos (CS), coumaphos (CM), diazinon (DZ), dyfonate (DF), ethoprophos (EP), fenamiphos (FA), methylparathion (MPT), and parathion (PT) and the two chemicals including 3,5,6-trichloro-2-pyridinol (TCP) and diethylthiophosphoric acid (DETP) were obtained from the Sigma-Aldrich, Inc. (Germany). To determine esterase activity, the tributyrin, *p*-nitrophenol- $\beta$ -butyrate (*p*-NPB), and *p*-nitrophenol (*p*-NP) were also purchased from Sigma-Aldrich, Inc. (Germany). The genomic and plasmid DNAs were isolated using the G-spin genomic DNA extraction and Plasmid DNA Purification Kits (iNtRON Biotechnology, South Korea). The restriction enzymes were purchased from Gibco-BRL (Thermo Fisher Scientific Co., USA) and Promega Co. (USA). The HPLC-grade water, all the other reagents were of analytical grade.

## Identification of strain WCP931 and determination of acid and artificial gastric acid tolerances

The OP insecticides degrading strain WCP931 was isolated from *mulkimchi* sample and identified as previously described by Haque et al. (2018). The acid and gastric acid tolerance activity of the strain *L. plantarum* WCP931 was further confirmed previously by Cho et al. (2008).

## Degradation of CP and substrate range test in liquid medium

The bacterial strain *L. plantarum* WCP931 grown in MRS broth was used as inoculum for the degradation of CP in liquid medium. A total of 500  $\mu$ L of bacterial culture suspension ( $10^8$  cfu/mL) was inoculated into 50 mL of 1/25 MRS medium with CP (100 mg/L). The *E. coli* DH5 $\alpha$  was cultured as the control in the similar conditions. The recombinant *E. coli* DH5 $\alpha$  (pGCY300) growth was also confirmed in the M9 medium with CP (100 mg/L). Moreover, CS, CM, DZ, DF, EP, FA, MPT, and PT cross feeding were also performed using the identical condition. At periodic intervals, an individual flask was sacrificed and its contents were used to determine the growth and degradation of OP insecticides. The cultures were run in triplicate to ensure the accuracy.

## OP insecticides degradation and esterase activity assay

The OP insecticides and its residue concentrations, after degrading by the strain WCP931, recombinant *E. coli*, and OpdC protein were determined using TLC and HPLC methods according to previously described Cho et al. (2009a). A 5 mL of supernatant was collected from the culture and 4 mL of the culture filtrate was extracted with ethyl acetate (8 mL  $\times$  3). The TLC plate was developed for the analysis of degradation of CP and TCP as described by Islam et al. (2010). However, the degradation of CP, CM, CS, DZ, DY, EP, FA, MPT, and PT was determined according to the changes of absorbance at 214 nm measured by a High press liquid chromatography (HPLC) (Perkin-Elmer 200 series, CT, USA). The 50  $\mu$ L enzyme sample was added to an assay mixture containing 700  $\mu$ L phosphate-buffer saline (200 mM, pH 6.5) and 250  $\mu$ L CP, CM, CS, DZ, DY, EP, FA, MPT, PT (200 mg/L), and was incubated at 30  $^{\circ}$ C for 12 h, respectively. After that 10  $\mu$ L of the filtrate that was mixed with methanol and passed through a 0.45- $\mu$ m PVDF filter (GmbH, Dassel, Germany) and was injected into a C18 column (250  $\times$  4.6 mm, 5  $\mu$ m, Phenomenex, CA, USA) of HPLC. The concentration of the degraded insecticides residues were determined according to the peak areas in the chromatograms against standard pesticides as described Cho et al. (2009a), Islam et al. (2010), and Haque et al (2018). The OP insecticides degradation and its estimation were repeated in three times to obtain the standard error of the experiments.

The esterase activity of OpdC and mutant OpdC enzyme was determined by a spectrophotometric method. The rate of hydrolysis of *p*NPB (100 mg/L) at 30  $^{\circ}$ C was measured in 50 mM sodium phosphate buffer (pH 7.0) by a spectrophotometer at 420 nm. One unit of esterase was defined as the amount of enzyme required to release 1  $\mu$ mol of *p*NP per minute under the assay conditions. All the experiments were conducted in triplicate to reveal the error.

## Cloning and sequencing of the *opdC* gene

The complete open reading frame of *opdC* from *L. plantarum* WCP931 genomic DNA was amplified using 5'-AAA GGA TCC TGA TTG ATC TGA CAA TGG G-3' (sense, *Bam*HI sites are indicated by underline), 5'-AAA GAA TTC CTT GCT ATA CTG ATT CGC TAG CC-3' (antisense, *Hind*III sites are indicated by underline) primers sequence based on the carboxylesterase available in the database, and cloned into pGEM-T easy vector (Promega, Madison, WI, USA). The recombinant plasmid was digested with *Bam*HI and *Hind*III and cloned into pBluscript II SK+ digested with the same restriction enzyme. The nucleotide and amino acid sequences were determined and analyzed as described by Islam et al. (2010) and Haque et al. (2018). The phylogenetic tree and alignment of conserved regions of the OpdC protein with other Opd enzymes and esterolytic enzymes amino acid sequences was analyzed using DNAMAN10.0 software package (Haque et al. 2018).

### **Expression and purification of the OpdC enzyme**

To facilitate high expression levels of OpdC, the PCR product generated with primers 5'-AAA GGA TCC TGA TTG ATC TGA CAA TGG G-3' (sense, *Bam*HI sites are indicated by underline), 5'-AAAA GA ATT CCT TGC TAT ACT GAT TCG CTA GCC-3' (antisense, *Hind*III sites are indicated by underline) was cloned into expression vector pET-32a(+) (Novagen), resulting in the addition of a C-terminal (His)<sub>6</sub> tag. The *E. coli* strain BL21 (DE3) carrying pET-32a(+)/OpdC was grown at 37 °C to mid-log phase in LB medium containing 50 µg/mL ampicillin, respectively. The pellet of recombinant *E. coli* cells was made and washed with 10mM Tris-HCl buffer (pH 7.0). Thereafter the pellet was resuspended in the same buffer and kept at -20 °C for 30 min. Next, it was mixed with 1 mg of bovine DNase I and incubated at 37 °C for 30 min. Triton X-100 was added to the suspension to set a final concentration of 2.5%. The supernatant was collected and stored at 4 °C. The solubilized recombinant OpdC with His-tag was applied on a HisTrap kit (Amershan Pharmacia Biotech). The purification of expressed His<sub>6</sub>-tagged OpdC protein was conducted and eluted with 100 mM imidazole with 0.1% Triton X-100 as described Islam et al. (2010). The molecular weight of purified OpdC was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The amount of OpdC in the solution was adjusted at 50 µg/mL and was used for activity assay towards organophosphate insecticides.

### **pH and temperature stability of OpdC enzyme**

The effect of pH and temperature on esterase activity was investigated using purified OpdC protein. 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 above mentioned protocol, to obtain values from pH 3.0 to 11.0; all of the assays were performed at 30 °C. To determine the effect of temperature on the enzymatic activity, samples were incubated at temperatures ranging from 10 to 70 °C for 1 h. Hydrolysis of CS, CP, CM, DZ, DY, EP, FA, MPT, and PT were measured by changes in absorbance at 214 nm by HPLC. Enzyme sample (50 µL) was added to an assay mixture containing 700 µL 200 mM phosphate-buffer saline (PBS, pH 6.5) and 250 µL CS, CP, CM, DZ, DY, EP, FA, MPT, and PT (100 mg/L). All assays were performed in triplicate.

## Active site prediction analyses of OpdC enzyme

The site directed mutagenesis technique and bioinformatic tools were used to confirm the potential active site of the OpdC enzyme. To confirm location of the catalytic sites of OpdC, amino acid exchanges were introduced at position 116 (serine to alanine) of OpdC was performed using oligonucleotide primer: 5'-TCTTGCCGGGTTTTCGGCTGG CGG CCACG-3' (sense), 5'-CGTGGCCGC CAGCCG AAA ACCCGGCAAGA-3' (antisense) 5'. The positions of the mutated codons are underlined. The 50 µL of reaction mixtures contained 1 µL of the pET-32a(+)/*opdC* DNA (80 ng/µL), 4 µL of 10 µmol of each primer, 5 µL of 2 mM dNTP mixture, and 5 µL of 10× *Pfu* DNA polymerase buffer, which, in turn, contained 20 mM MgSO<sub>4</sub>, and 2.5 U of *Pfu* DNA polymerase (purchased from Stratagene; La Jolla, CA, USA). The PCR products were incubated on ice for 5 min, and 1 µL of *DpnI* restriction enzyme (10 U/µL) was added. Then, the mixture was incubated for 1 h at 37 °C. The *DpnI*-treated plasmids were then transformed into *E. coli* DH5α according to the manufacturer's specifications. The site-directed mutagenesis procedure was adapted from as described Haque et al. (2018). The predictive active site of the OpdC protein was investigated using sequential application of several bioinformatic tools. At first, the modeling of the OpdC protein was built in Swiss modeling (<https://swissmodel.expasy.org/>) to create the protein data bank (pdb) file of the OpdC based on an esterase protein from the protein data bank (accession number: 4bzw). After that 4bzw was used to get an alignment with OpdC with the help of 'Clustal Omega' (<https://www.ebi.ac.uk/Tools/msa/clustalo/>), then the clustal file was selected and used to find a secondary similarity analysis between the OpdC and 4bzw using 'ESPrnt3' (<http://esprnt.ibcp.fr/ESPrnt/ESPrnt/>).

## Results

### Identification and resistance of the strain WCP931 under acidic and artificial gastric acidic conditions

The levels of 16S rRNA similarity between the WCP931 strain and reference LAB were 85.4 to 99.5% and the phylogenetic study clearly demonstrated that strain WCP931 strain was closely related to *Lactobacillus* sp.. Finally, the CP degrading strain was named *Lactobacillus plantarum* WCP931 (supplementary Fig. S1).

The survival rates of *L. plantarum* WCP931 when cultured under acidic and artificial gastric acidic conditions are shown in Fig. 1. Moderate survival rates are observed for the CP-degrading strain WCP931 with 86% (acidic condition) and 51% (artificial gastric acidic condition) at pH 2.0, 95% (acidic) and 84% (artificial gastric acidic) at pH 2.5, and 99% (acidic) and 96% (artificial gastric acidic) at pH 2.5 after 3 h, respectively.

### Degradation of CP by the strain WCP931 in liquid culture

The cell growth response and the degradation pattern of CP in the strain of WCP931 are shown in Fig. 2. The strains grown markedly increased until 1 day (OD 0.85), then decreased at 2 days, and after these grown slowly at 6 days (OD 0.94) during incubation, respectively. However, LAB strain WCP931 exhibited

an initial rapid degradation of CP of approximately 66 mg/L during first 3 days of incubation. After these CP was degraded slowly and maximum degradation was found 87 mg/L at 9 days of incubation (Fig. 2A). The strain WCP931 was able to degrade CP to DETP and TCP and utilized DETP as the sole source of carbon and phosphorus. All OP insecticides tested in the cross feeding experiment were degraded by the WCP931 strains. All tested OP insecticides have DEPT as side chains (CP, CM, DZ, MPT, and PT) and had no DEPT as side chains (CS, DF, EP, and FA). Except DF, other eight OP insecticides (including CS, CP, CM, DZ, EP, FA, MPT, and PT) hydrolyzed at a phosphoester bond by the strains of WCP931. At 9 days, the strain WCP931 attained 72 to 88% degradation of CP, CM, DZ, MPT, and PT, respectively (Fig. 2B).

### Sequence analysis of the *opdC* gene and OpdC protein

PCR amplification of the total DNA from *L. plantarum* WCP931 with specific primers produced amplification product of about 1.5 kb. After sequencing, total 1500 bp nucleotide sequence is found in the open reading frame of *opdC* of *L. plantarum* WCP931. The open reading frame of *opdC* starts with an ATG start codon and TAA Ochre stop codon (Fig. 3). The gene product is predicted to contain 276 amino acids with a predicted molecular mass of 31 kDa, respectively ([http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)). Analysis of the amino acid sequence with the program PSORT (<http://www.cbs.dtu.dk/services/SignalP/>) revealed no potential signal sequence. The calculated pI of OpdC is 5.18.

The amino acid sequence GFSAG, starting at residue 116 for the OpdC (Fig. 3 and supplementary Fig. S2), fits the Gly-X-Ser-X-Gly motif found in most bacterial and eukaryotic serine hydrolases, such as lipase, esterase, and serine proteinase as well as in  $\beta$ -lactamase (Bornscheuer 2002; Brenner 1988; Ouyang et al. 2013). A phylogenetic tree containing the esterolytic and lipolytic proteins showed that the OpdC protein does not belong to group I, II, III or IV (Fig. 4). This separation of OpdC from the known esterolytic and lipolytic proteins suggests that OpdC represent a new type of esterase.

### Degradation of CP by the clone in liquid culture

The degradation patterns of CP of the clone pGCY300 (*opdC*) are shown in Fig. 5. The CP and TCP with  $R_f$  values of 0.57 and 0.66, respectively were detected in samples drawn at 0, 1, 3, 6, and 9 days (Fig. 5A). The clone decomposed markedly until 2 days (78 mg/L), then decreased markedly at 6 days (24 mg/L), and after these grown slowly until 9 days during incubation. On the other hand, during 3 days, the clone exhibited slowly increment of TCP of approximately 32 mg/L of TCP at 3 days and after these increased markedly at 6 days (68 mg/L) (Fig. 5B). The nine OP insecticides (such as CS, CP, CM, DZ, EP, FA, MPT, and PT) were disintegrated by the recombinant *E. coli* with *opdC* gene. The recombinant cells fetched 46 to 90% degradation of CP, CM, DZ, FA, MPT, and PT at 37 °C for 9 days, respectively (Fig. 5C).

### Purification and characterization of the OpdC protein

OpdC protein was purified from *E. coli* BL21 (DE3) overproducing OpdC using column filtration techniques. Protein fractions were analyzed by SDS-PAGE, and one protein band (31 kDa) was present after the final purification step (Fig. 6A). The effect of pH on the ability of OpdC to hydrolyze  $\rho$ NPB was

determined at 40 °C with various buffers ranging from pH 3.0 to 11.0. The maximum activity of OpdC was observed at pH (Fig. 6B). The optimal temperature for OpdC hydrolysis of  $\rho$ NPB was determined at pH 6 by measuring the activity across a temperature range. The OpdC activity was observed maximum at 30 °C (Figs. 6C). In fact, nine OP insecticides were decomposed by OpdC (Fig. 6D). Except for DF, eight OP insecticides (including CS, CP, CM, DZ, EP, FA, MPT, and PT) hydrolyzed at a phosphoester bond by the OpdC protein. Especially, the higher enzyme activity was observed towards the CP, CM, DZ, MPT, and PT by OpdC protein.

### Identification of residues essential for enzyme activity of OpdC

Most lipases and carboxyl esterases have the consensus sequence motif Gly-X-Ser-X-Gly that includes the active site serine. Analysis of the deduced amino acid sequences of OpdC showed a potential serine hydrolase motif such as G-F-S116-A-G of OpdC. To determine whether Ser116 were involved in catalytic esterase action, there were replaced by site-direct mutagenesis and the mutant proteins were expressed in *E. coli* and purified. The purified OpdC enzyme showed 78% degradation rate of CP, while the mutant OpdC had no enzymatic activity towards  $\rho$ NPB and CP (Table 1). The structure of OpdC is comprised by 31.5% of  $\alpha$ -helix and 21% of  $\beta$  strand, respectively. In particular, the G-F-S-A-G motif for OpdC was found in the  $\beta$ 5 and  $\alpha$ 3 helix of the predicted structures (supplementary Fig. S2). By using the scoring option found in 'ESPrnt3' (<http://esprnt.ibcp.fr/ESPrnt/ESPrnt/>) and data found on Metapocket2.0 ([projects.biotec.tu-dresden.de/metapocket/](http://projects.biotec.tu-dresden.de/metapocket/)) the highest probable active sites was marked as seen in Fig. 7. The predicted OpdC structure revealed that the active site of this enzyme was located in the known architecture of the hydrolases (Fig. 7A). The key nucleophile, Ser116, forms part of a predicted catalytic triad with Ala116, Gly118, and Phe114 for the OpdC protein. Importantly, the OpdC enzyme had shown average 18.59% amino acid identity with Opd group (OpdA, OpdB, OpdD, and OpdE) enzymes of lactic acid bacteria. In particular, it had shared 55% homology with their nearest OpdD enzyme of *L. sakei* WCP904 (Haque et al., 2018). These results suggested that the OpdC indicates the existence of a new LAB esterase/opdase group.

## Discussion

The isolated *L. plantarum* WCP931 was screened for its ability to degrade OP insecticides. *L. plantarum* is a lactic acid bacterium found in many habitats, including meat and dairy products, plant and vegetable fermentations, and the gastrointestinal and urogenital tracts. This strain has been attributed probiotic activities in humans and animals (Cebeci and Gurakan 2003; Kim et al. 2020). The bacterium WCP931 is unusual as it has been shown to hydrolyze CP and utilizes part of the compound (DEPT) as its sole source of carbon when cultured in M9 or 1/25 MRS medium. The evidence from the studies with TLC and HPLC analysis for CP supports the proposed pathway. A higher bacterial population of *L. plantarum* WCP931 was observed in 1/25 MRS medium along with CP degradation. Moreover, the isolated bacterium WCP931 exhibited versatility in utilizing dimethyl compounds such as MPT, and diethyl compounds such as CP, CM, DZ, and PT as its carbon source, which suggests that strain WCP931 has potential role for the decontamination of the OP insecticides. The bacterial degradation and

detoxification of insecticides is mainly depending on the presence of degradative enzymes (Singh et al. 2006). Thus, the isolate WCP931 possess a great potential to provide a versatile gene or enzymes system for the remediation of highly toxic OP insecticides, since it showed broad specificity against a range of OP compounds. As consequence, we isolated OP insecticides degrading gene (*opdC*) from the *L. plantarum* WCP931. The recombinant *E. coli* harboring *opdC* gene was capable of degrading CP. It could utilize the CP as the sole carbon source when grown in M9 medium that was supplemented with CP instead of glucose. In particular, it had depleted the CP concentration to 77% in M9 medium after 6 days. In previous study, the *E. coli* harboring *opdD* gene depleted CP concentration to 73%, in M9 medium after 6 days (Haque et al. 2018), while the *E. coli* harboring *opdB* gene depleted the CP concentration to 67% in M9 medium after 6 days (Islam et al. 2010). Therefore, the CP degrading ability of *opdC* clone is stronger than those reported *opdB* (Islam et al. 2010) and *opdD* clones (Haque et al. 2018).

Three OP hydrolase genes namely *opd*, *mpd*, and *ophc2* have been reported yet. Among them, the *opd* gene is widely distributed in nature and isolated from different species (Chaudhry et al. 1988; Haque et al. 2018; Islam et al. 2010; Serdar et al. 1982; Siddavattam et al. 2003). The reported *opd* genes were belongs to chromosome (Mulbry et al. 1987; Serdar et al. 1982) or plasmid (Horne et al. 2002) of the isolated strains. However, yet no study reported the *opd* gene from the super probiotic *L. plantarum*. Therefore, the cloned and functionally expressed chromosome based *opdC* gene from the *L. plantarum* WCP931 increasing the diversity of the hosts of OP hydrolases. Despite the lack of any signal sequences in the N-terminal region of the OpdC, the *E. coli* cells secreted those enzymes in the CP mixed liquid culture, consequently, the recombinant *E. coli* harboring the *opdC* genes was capable of degrading OP insecticides.

The nonclassical secreted proteins often seem to have a cytoplasmic function as well as extracellular functional role (Bendtsen et al. 2005). In addition, carbohydrate and protein metabolism related proteins were identified as extracellular, although none of these proteins have a signal peptide (Antelmann et al., 2001; Vitikainen et al., 2004). Since insecticides and their hydrolysis products were extracted from culture medium, therefore, it is assumed that the OpdC enzymes used non classical pathways to display its extracellular activities. In fact, OP are hydrophobic in nature, thus, compounds in the culture medium are in equilibrium with compounds inside the bacterial cell. Therefore, hydrolysis could be taking place inside the cell, followed by release of the hydrolysis product into the culture medium. As a matter of fact, finding products in the culture medium does not rule out the possibility that hydrolysis takes place inside the cell.

The OpdC enzyme hydrolyzed range of OP insecticides containing a P-O bond and P-S bond, indicating that the recombinant OpdC has broad substrate specificity. A similar result was obtained by some previous reports (Li et al. 2007; Haque et al. 2018; Horne et al. 2002; Islam et al. 2010; Yang et al. 2006). However, relative activity of the OpdC enzyme against the P-O bond insecticides was much higher than P-S bond insecticides, which is consistent with the previously reported OpdB and OpdD enzymes character (Islam et al. 2010; Haque et al. 2018). However, minor variations of relative substrate activities were observed for the OpdC enzyme than those reported OpdB, OpdA, OpdE enzymes. Thus, the OpdC hydrolysis activity depends on the molecular structure of insecticides used in this study.

Temperature had influenced the OpdC activity. The optimum pH of OpdD (6.0) from *L. sakei* WCP904 (Haque et al. 2018) and OpdB from *L. brevis* WCP902 (6.0) (Islam et al. 2010) and was lower than those OpdB from *Pseudomonas* sp. BP3 (8.0) (Singh et al. 2006). The *L. plantarum* is known to be able to adapt to stressful environments such as those in the gastrointestinal tract with a low pH or high salt content. In order to survive in acidic environments, this bacterium uses the F<sub>0</sub>F<sub>1</sub>-ATPase and sodium-proton pumps to help regulate and maintain the intracellular pH (Kleerebezem, et al. 2003). In fact, *kimchi* fermentation involving LAB is conducted at acidic pH. Among the LAB, the *L. plantarum* is quite predominant when pH is lowered and responsible for acidifying of the *kimchi*. Therefore, the OpdC maximum activity obtained at acidic pH 5~6 is quite logic/consistent. However, OP insecticides are stable in mild acidic to neutral pH and easily decomposed in alkine pH (Lee and Lee 1997). As consequence, the degradation rate of CP in acidic soils was slower than in neutral and alkaline soils (Li et al. 2007; Yang et al. 2006). Importantly, optimum temperature (40 °C) for OpdC was similar with that recorded for OP hydrolase (OpdB) of *L. brevis* WCP902 (40 °C) (Islam et al. 2010), but higher than those were recorded for OpdD of *L. sakei* WCP904 (30 °C) (Haque et al. 2018).

The OpdC protein has a sequence Gly-X-Ser-X-Gly motif and catalytic active site of serine residues. Actually, this motif and active site is found in most bacterial and eukaryotic serine hydrolases, such as lipase, esterase, and serine proteinase as well as in  $\beta$ -lactamase (Bornscheuer 2002; Brenner 1988; Ouyang et al. 2013). However, the phylogenetic tree analysis of OpdC protein showed that it does not belong to the known families of esterolytic and lipolytic proteins (groups I, II, III, IV or even new group of soil metagenome), indicating the existence of a new LAB esterase/opdase group, represented by OpdC (Fig. 4). Importantly, our previous reported OpdB and OpdD enzyme had shown the Gly-X-Ser-X-Gly motif and catalytic active site of serine residues. As consequence, we are going to propose a new esterase group (LAB-opd esterase) that is summarized by OP hydrolase genes from LABs strain isolated during *kimchi* fermentation.

As seen in Fig. 5, the  $\alpha$ -helix,  $\beta$  sheet, random coil, and  $\beta$  turn were observed in both structure of OpdC enzymes and were matched with the catalytic motif G-X-S-X-G of OpdC and OpdD enzymes (Islam et al. 2010; Haque et al. 2018). When the Ser116 residue was replaced by Ala, the mutant OpdC enzymes had no enzymatic activity towards the  $p$ NPB and CP. As consequence, the mutation at Ser116 of OpdC has massively reorganized the structural conformation than the native OpdC protein respectively (Fig. 7B). The amino acid residues Ser<sup>10</sup>-Asp<sup>154</sup>-His<sup>157</sup> are involved in the catalytic active site of *E. coli* thioesterase I/protease I (Lee et al. 2006), therefore, the predicted structure of OpdC is partially shared the structure of esterase as well as new LAB-Opd hydrolase. As the results, the classification of the esterase is expanded in to LAB-Opd group (Islam et al. 2010; Haque et al. 2018) where OpdC are included in this study.

With regard to the safety of insecticide in *kimchi*, we had concluded that the fermented *kimchi* meets the minimal residue criteria for food safety due to OPs degradation by *kimchi* microorganisms such as *L. mesenteriodes*, *L. brevis*, *L. plantarum*, and *L. sakei* (Cho et al., 2009a). The present study suggested that

the genes *opdC* in *L. plantarum* WCP931 along with *opdB* in *L. bevis* WCP902 and *opdD* in *L. sakei* WCP904 play role to degrades the OP insecticides during *kimchi* fermentation.

## Abbreviations

LAB: Lactic acid bacteria; OP: Organophosphorus; CP: Chlorpyrifos; CS: cadusafos; CM; comnaphos; DZ: diazinon; DF: dyfonate; EP: ethoprophos; FA: fenamiphos; MPT: methylparathion; PT: parathion; TCP: 3,5,6-trichloro-2-pyridinol; DETP: Diethylthiophosphate; TLC: Thin layer chromatogram; HPLC: High press liquid chromatogram.

## Declarations

### Ethics approval and consent to participate

In current research, the article doesn't contain any data collected from humans or animals and also there was no involvement of human participants and doesn't belongs to any health was related outcomes and articles.

### Consent for publication

This research article entitled as "Cloning, expression, and functional characterization of an organophosphates insecticides degrading gene (*opdC*) from a potential probiotic *Lactobacillus plantarum* WCP931" an original work was carried out by authors: All authors approve of its submission to as AMB express. It is not under consideration by another journal at the same time as AMB express. I am the author responsible for the submission of this article and I accept the conditions of submission and the Springer Open Copyright and License Agreement.

### Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

### Competing interests

As a corresponding author, I confirm that I have read Springer Open's guidance on competing interests and have included a statement indicating that none of the authors have any competing interests in the paper.

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### Authors' contributions

KMC conceived and designed the experiments. KMC, MAH, and JHL interpreted the data and wrote the manuscript. HYL, DYK, and JEH performed the experiments and analyzed the data. All authors read and approved the final manuscript.

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## Table

**Table 1. Esterase and organophosphorus (OP) hydrolase activities for the hydrolysis of  $\rho$ -nitrophenyl butyrate ( $\rho$ -NPB) and chlorpyrifos (CP) by the OpdC and mutant OpdC enzyme**

Proteins	Esterase activity <sup>a</sup> (U/mg) / CP degradation degree <sup>b</sup> (%)
OpdC	397±15.88 <sup>c</sup> / 78
OpdCM	< 0.01 / 2

<sup>a</sup> Esterase activity is indicated the micromoles of  $\rho$ -NPB hydrolyzed min/mg.

<sup>b</sup> The OpdC and OpdCM activities were assayed with CP as substrate at pH 6.0 and 40 °C for 12 h, respectively.

<sup>c</sup> Values indicate the means of three replications. The standard errors were within 5% of the mean.

## Figures

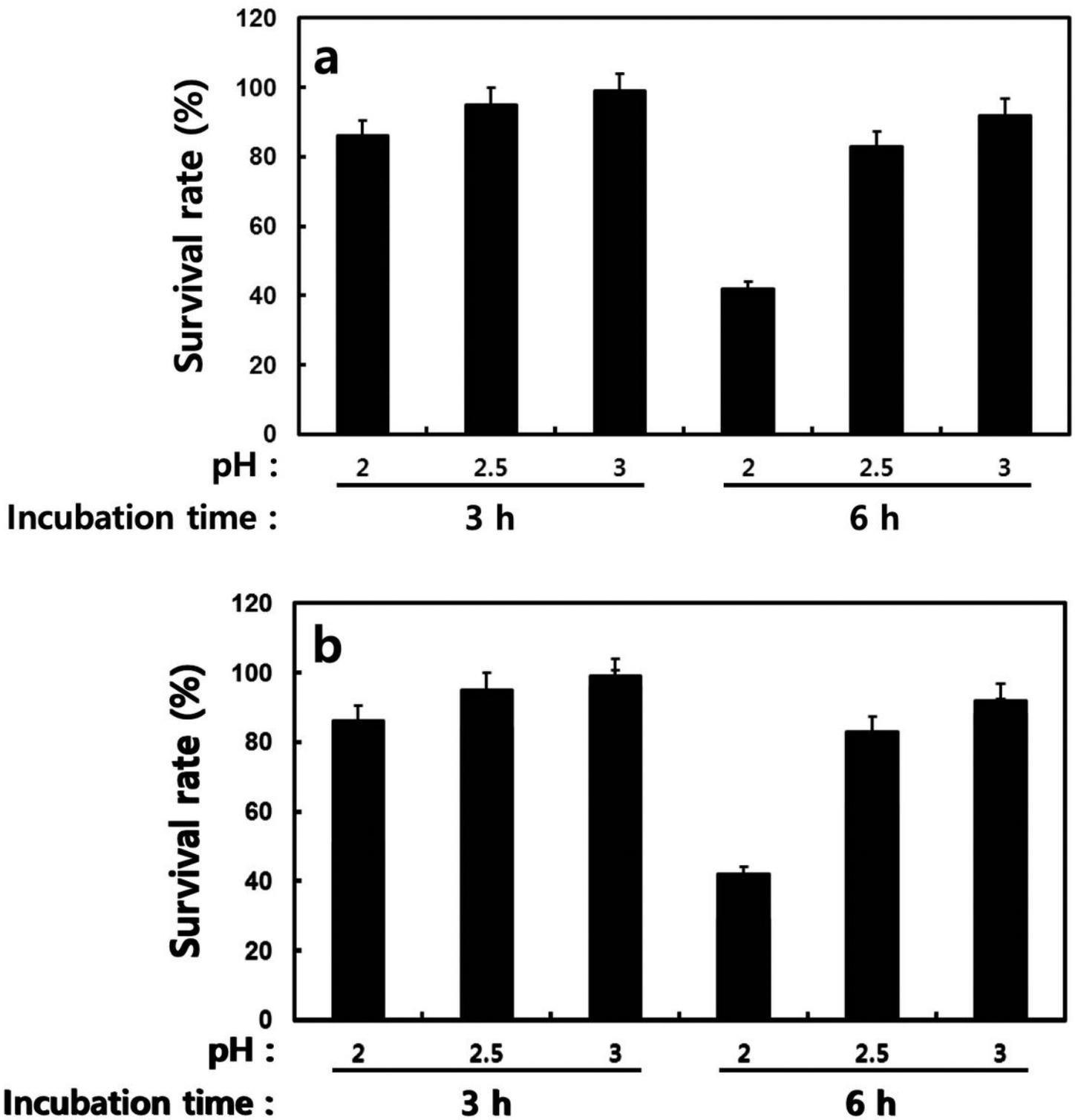


Figure 1

a Survival rates of *L. plantarum* WCP931 under acidic conditions and b survival rates of *L. plantarum* WCP931 under artificial gastric acidic conditions at pH 2.0, 2.5, and 3.0 after 3 and 6 h of incubation. *L. plantarum* WCP931 was tested in triplicate for its tolerance in acidified and artificial gastric acidified MRS. The standard errors were within 5% of the mean.

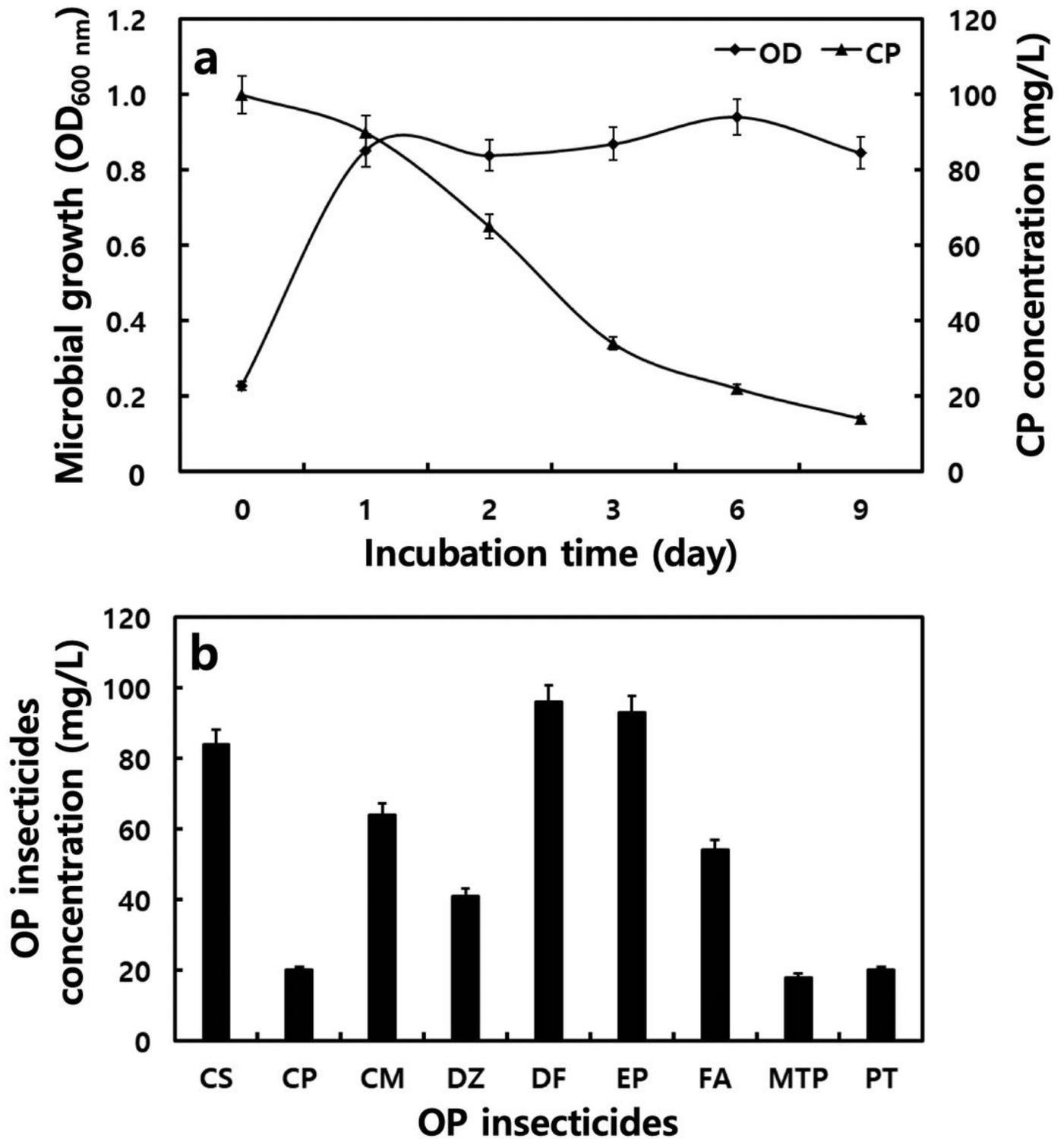


Figure 2

a Cell growth response and b CP degradation pattern of *L. plantarum* WCP931 in 1/25 MRS containing 100 mg/L of CP for 9 days. The standard errors were within 5% of the mean.

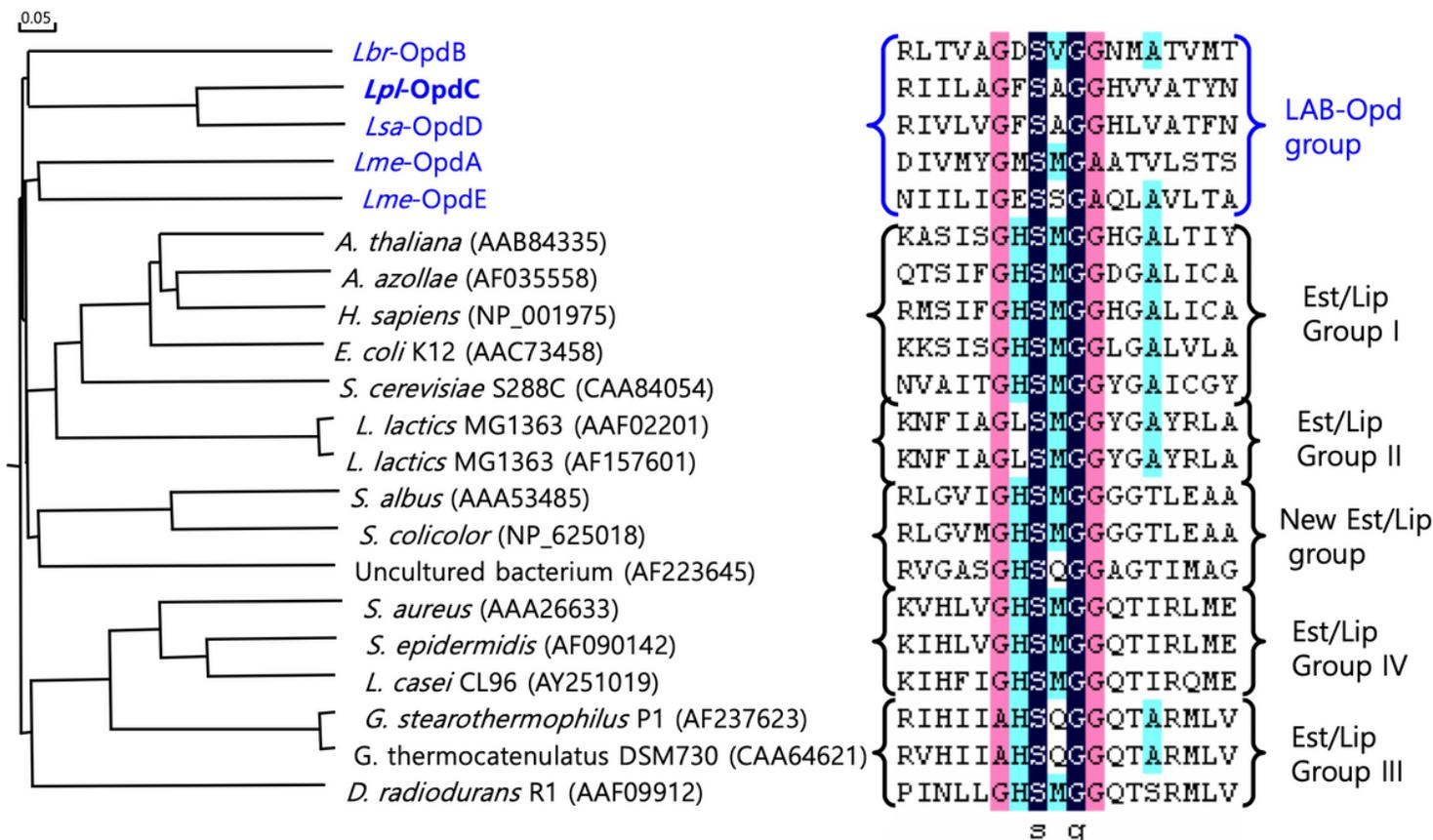
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1      CTGGGTTTGTGGCGTGTGCTCCAATAAGACCACCCCGCGCCCCTTAACGCTGTAAACTAG
61     GCCGTCTTTGGCTAGTTGTGCGATTGCCTTACGAACAGCATAGCGACTGCTATCAAATTC
121    TGTAATTAATTCGTCTTCAATGGGTAATCGCATCTTAACGCTGTAGTGTCCCGTGAGAAT
181    GCGGTCCTTTAAAACGTGATAGATGTCGCTGAATGTTTGTTCCTTCCCATTTTAATACCCCC
241    GCTCATCATATAATTAATTTAATTATAGCAAATAATAGTCATTAGCGAGTAAACAAGCGA
301    ATAAATATCGATATTTATTCGAATAAAAATTGACAAGCAATCATGTGACCTACTATAATG
361    AAAGCGAAATCAAGTCGGTAATACGGGAGTAGGAGGGGGAACATATGCAAGTTGAACAGC
1      M Q V E Q
421    GCACATTAATACTGCGGCACACCCATTTTCAGATCACGGCGTATTGGTTAGACCAAATTA
6      R T L N T A A H P F Q I T A Y W L D Q I
481    GTGATTTTCAAACAGCTGTTGATTATCCAATCATGATTATCTGCCCGGGAGGTGGCTTTA
26     S D F E T A V D Y P I M I I C P G G G F
541    CTTATCATTCTGGGCGGGAAGAAGCGCCAATTGCAACCCGGATGATGGCCGCCGGAATGC
46     T Y H S G R E E A P I A T R M M A A G M
601    ATACGGTGGTCTTGAATTACCAATTAATTGTGGGAGATCAGTCCGTCTATCCTTGGGCGT
66     H T V V L N Y Q L I V G D Q S V Y P W A
661    TGCAGCAATTAGGTGCAACAATTGATTGGATTACGACCCAGGCGTCCGCGCACCATGTTG
86     L Q Q L G A T I D W I T T Q A S A H H V
721    ATTGTCAGCGGATTATTCTTGCCGGGTTTTTCGGCTGGCGGCCACGTGGTTGCGACTTATA
106    D C Q R I I L A G F S A G G H V V A T Y
781    ATGGTGTTCGACCCAACCAGAATTGCGCACACGGTATCATTTGGATCATTATCAAGGCC
126    N G V A T Q P E L R T R Y H L D H Y Q G
841    AGCATGCTGCCATTATTTTAGGCTATCCAGTCATTGATTTGACAGCGGGGTTTCCCACGA
146    Q H A A I I L G Y P V I D L T A G F P T
901    CTTGCGGCGCGGTAATCAGATTACCACGGATGCCCGGTTGTGGGCAGCCCAGCGCCTAG
166    T S A A R N Q I T T D A R L W A A Q R L
961    TCACACCAGCTAGTAAACCCGCTTTTGTCTGGCAAACGGCGACGGATGAGTCGGTCCCGC
186    V T P A S K P A F V W Q T A T D E S V P
1021   CAATCAATTCGTTAAAATATGTGCAGGCCATGCTACAACATCAGGTCGCAACTGCGTATC
206    P I N S L K Y V Q A M L Q H Q V A T A Y
1081   ATTTATTTGGTAGTGGGATTCATGGGTTAGCGTTAGCCAATCATGTGACGCAAAAACCTG
226    H L F G S G I H G L A L A N H V T Q K P
1141   GCAAGGACAAGTATTTGAATGACCAGGCTGCAATATGGCCACAATTAGCATTGCGGTGGT
246    G K D K Y L N D Q A A I W P Q L A L R W
1201   TACAAGAACAGGGATTGTTAGCTGGTAATTATTGAAAATAAATCTGGGCGATCAGTCGTG
266    L Q E Q G L L A G N Y *
1261   CTTCACTGATCGCCGTTTTTTGTCAACGATCCTGACACCAATTAATTTTCTGCGGTATA
1321   ATAATACGAATAAGCTTATTAAGTAAGAAGGAGCGATTGAGATGGCAACAATCAACACT
1381   TGGTCATTATTTCTTAGATGCGTTAGGATTTCTGTATTTACGCGAACATCAAGCAGAAT
1441   TACCCGTTCTGAGTGGATTGATGACCGGTGGTACTTGGGTCAAGTCGGTGACAGGCATTT

```

**Figure 3**

Nucleotide and deduced amino acid sequences of *opdC* gene from *L. plantarum* WCP931. Bold letters and underlines the start codon and serine residue. The stop codon is indicated by asterisk. The consensus sequences region is indicated by yellow box.



**Figure 4**

Phylogenetic tree showing the evolutionary relatedness and levels of homology between the esterolytic and lipolytic enzyme amino acid sequences and the alignment of the conserved regions found in the primary esterolytic and lipolytic enzymes.

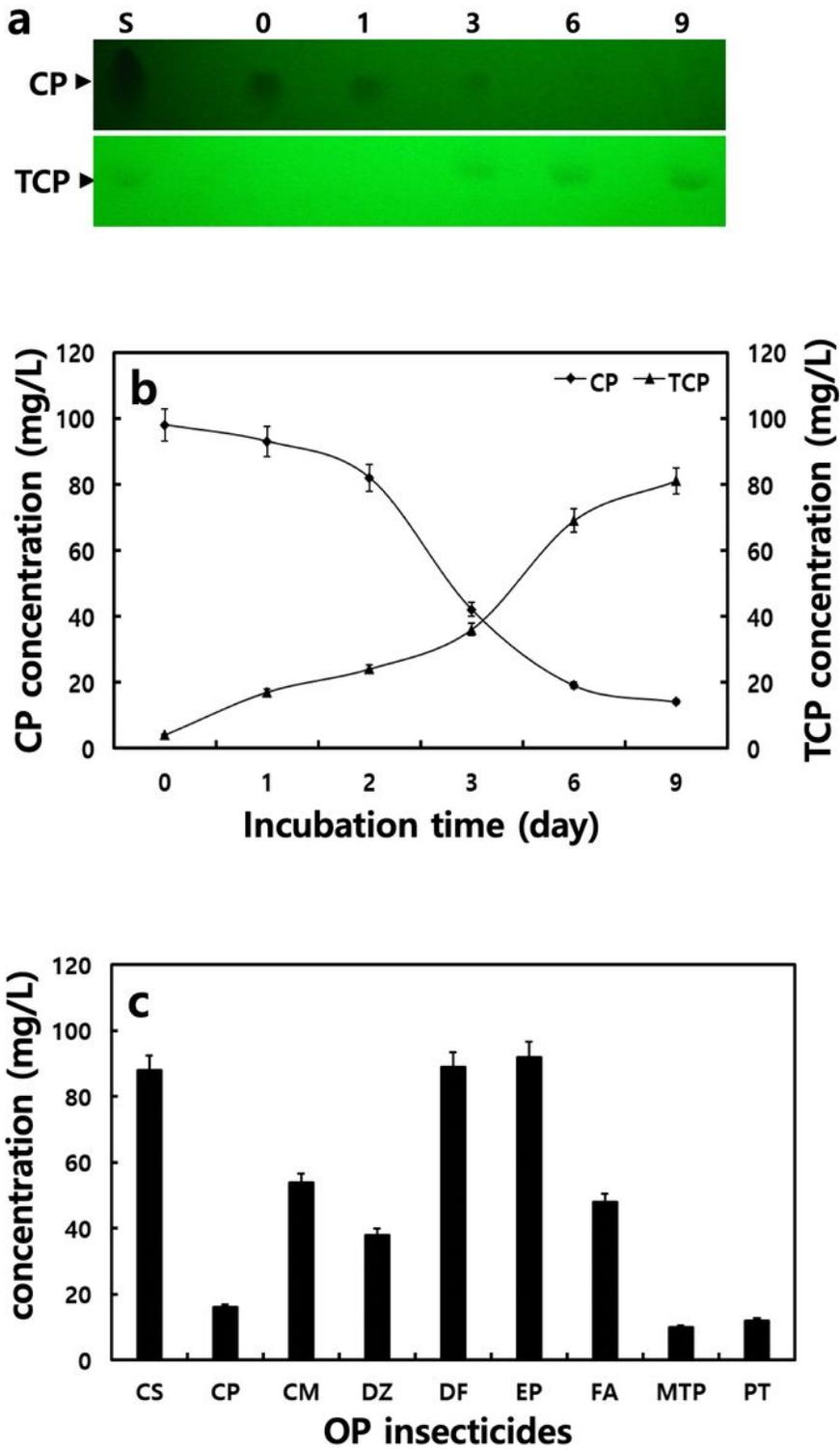
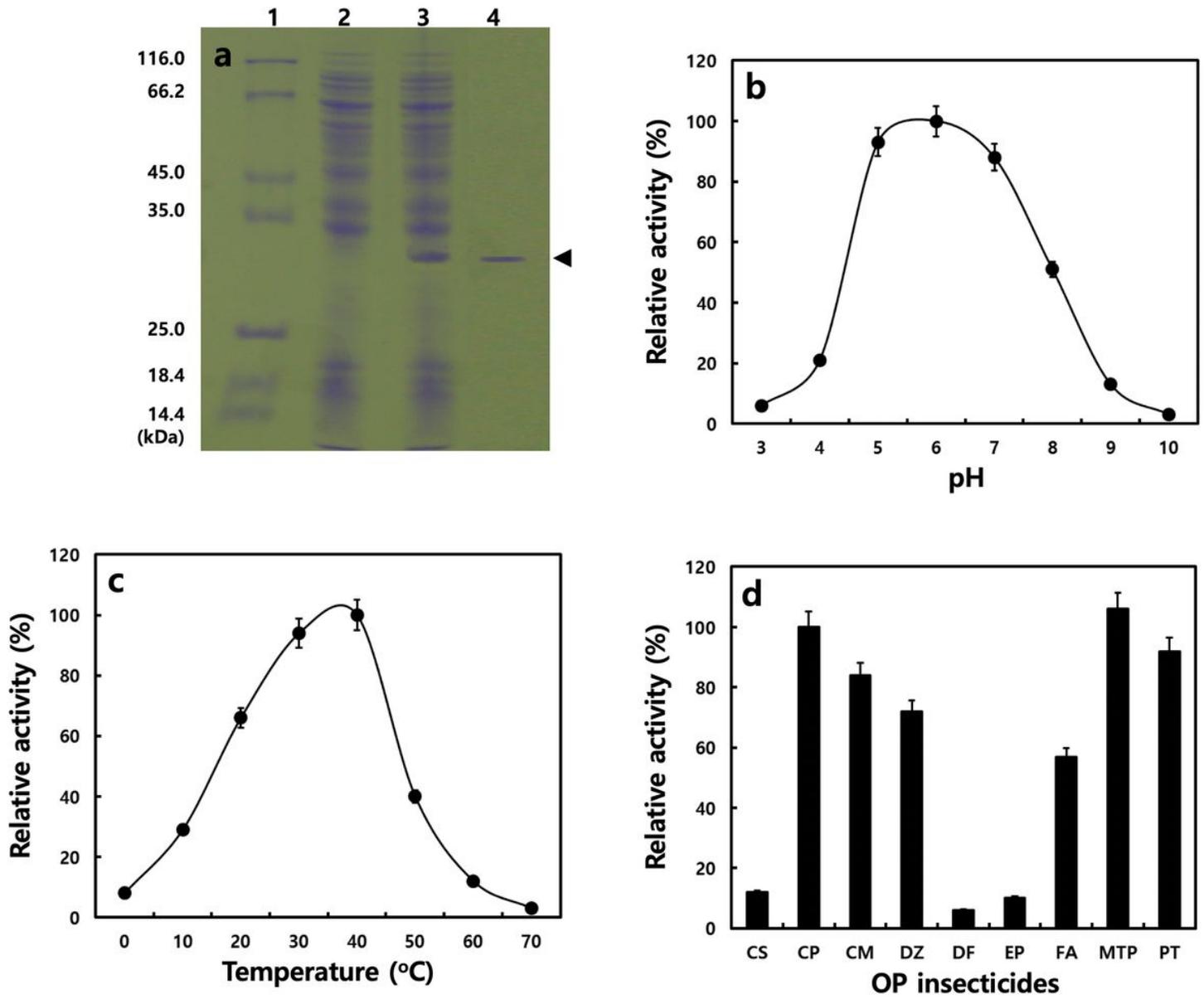


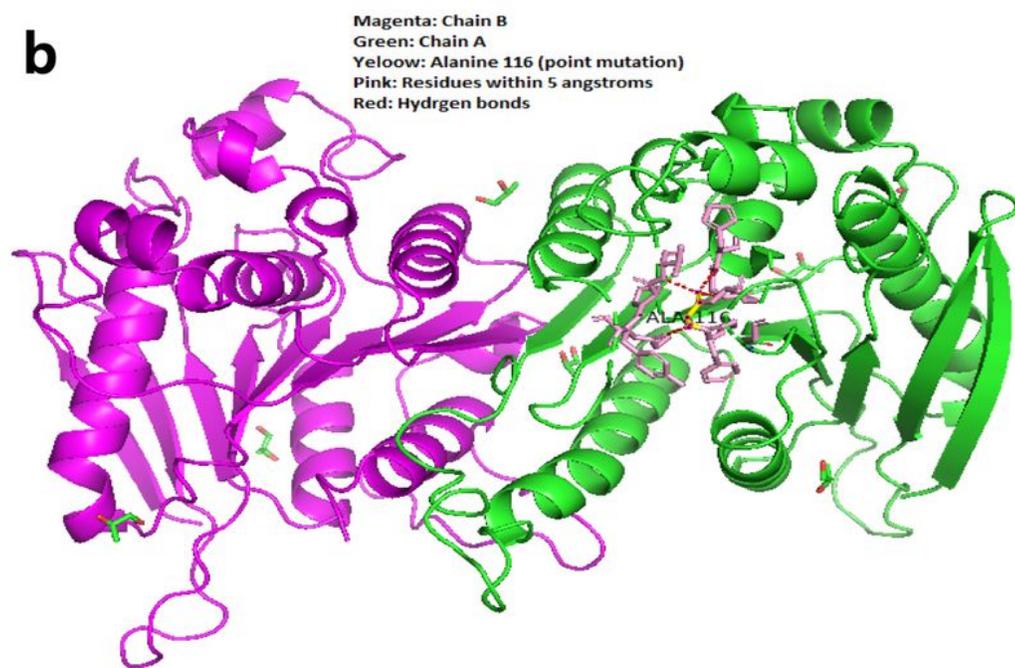
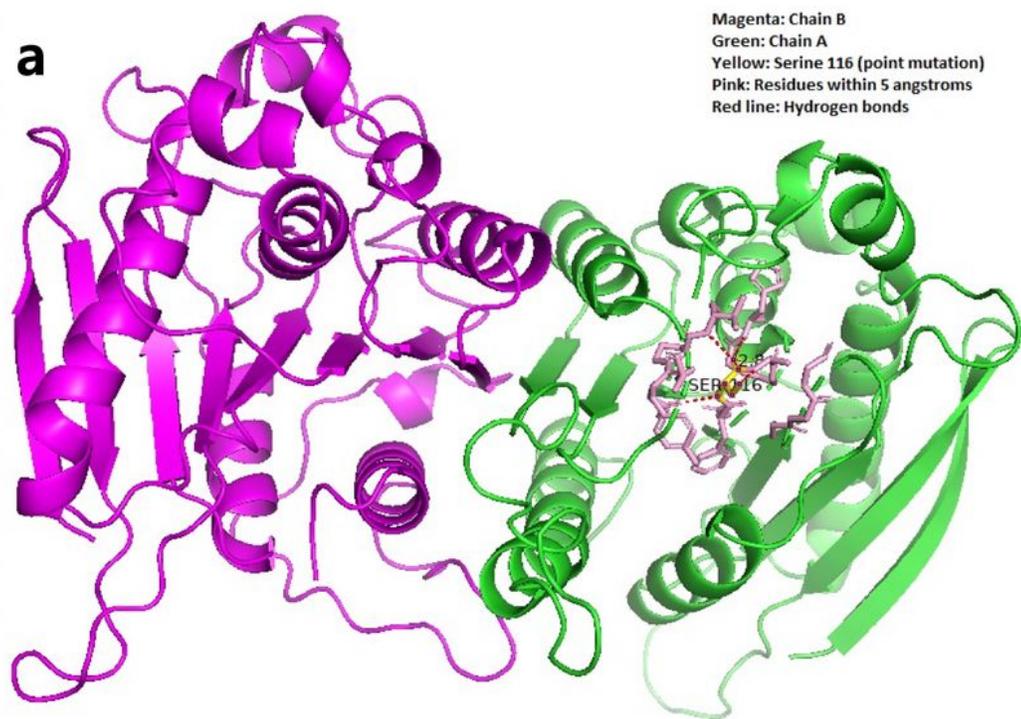
Figure 5

a TLC profile and b changes of CP and TCP concentration of the recombinant *E. coli* with *ophC* gene growing in the M9 medium containing 100 mg/L of CP for 9 days. The standard errors were within 5% of the mean.



**Figure 6**

a Electrophoretic analysis of the purified OpdC. Separation was performed on a 12.5% (w/v) SDS polyacrylamide gel and after was stained with 0.025% Coomassie blue R-250. Lane 1, standard marker; lane 2, crude extract from *E. coli* BL21 (DE3) containing pET-32(+)/OpdC; lane 3, crude extract from IPTG-induced *E. coli* BL21 (DE3) containing pET-32(+)/OpdC; lane 4, purified OpdC protein from Hi-Trap kit (Amersham). b pH effect on the relative activity of OpdC. The esterase activity of OpdC was assayed using pNPB as substrate at different pH values at 40 oC for 1 h. c Effect of temperature on the relative activity of OpdC. The esterase activity of OpdC was assayed using pNPB as substrate at different temperature values at pH 6.0 for 1 h. d Substrate relative activities of OpdC on the various organophosphorus (OP) insecticides, including cadusafos (CS), chlorpyrifos (CP), comnaphos (CM), diazinon (DZ), dyfonate (DF), ethoprophos (EP), fenamiphos (FA), methylparathion (MPT), and parathion (PT), as substrate at 40 oC at pH 6.0 for 12 h. The standard errors were within 5% of the mean.



**Figure 7**

a Predictive active site of the OpdC protein and b predictive active site of the OpC mutant. The modeling was designed based on an esterase protein from protein data bank (accession number: 4bzw). After that 4 bzw was used to get an alignment with OpdC with the help of 'Clustal Omega', then the clustal file was selected and used to find a secondary similarity analysis between the OpdC and 4bzw using 'ESPr3'.

## Supplementary Files

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