Short Communication

A Novel Organophosphorus Pesticide Hydrolase Gene Encoded on a Plasmid in *Burkholderia* sp. Strain NF100

KANAKO TAGO¹, SUMIKO YONEZAWA², TOSHIHIDE OHKOUCHI², TOMOYUKI NINOMIYA²,
MASAYUKI HASHIMOTO³ and MASAHITO HAYATSU^{2*}

¹ United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu, Gifu 501-1193, Japan

² Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka, Shizuoka 422-8529, Japan

³ Division of Gene Research, Department of Life Science, Research Center for Human and Environmental Sciences, Shinshu University, 3-15-1 Tokida, Ueda, Nagano 386-8567, Japan

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The fenitrothion hydrolase gene encoded on the plasmid pNF2 in *Burkholderia* sp. strain NF100 was cloned and sequenced. The 6.5-kbp *Eco*RI fragment contained three ORFs (ORF1 to 3) the deduced amino acid sequences of which shared no significant homology with any sequence present in databases. The results of frameshift mutation showed that ORF1 and ORF2 were essential for full enzymatic activity, and ORF3 was not involved in the degradation of fenitrothion. Fenitrothion hydrolase was located in the cell membrane fraction. The DNA region containing the fenitrothion hydrolase gene was spontaneously lost from pNF2.

Key words: *Burkholderia*, organophosphorus pesticides, hydrolase, plasmid

Organophosphorus pesticides, such as fenitrothion (*O,O*-dimethyl *O*-(4-nitro-*m*-tolyl) phosphorothioate) and parathion (*O,O*-diethyl *O*-4-nitrophenyl phosphorothioate), are widely used to control various pests in agriculture and public health protection. These pesticides are potent acetylcholinesterase inhibitors, and thus various hazardous effects in humans and non-target organisms can occur due to their toxicity. On the other hand, microorganisms play a major role in the degradation of these pesticides. In general, hydrolysis of one of the phosphoester bonds reduces the toxicity of the pesticides, and in the case of parathion, a 100-fold reduction in toxicity occurs. Thus bacterial organophosphorus pesticide hydrolases have received considerable attention, and several have been purified and characterized^{3,8,13,16}. Two types of bacterial phosphotriesterases and their genes have been studied extensively: the

prolidase-type (OPAA-1 and OPAA-2) from *Alteromonas* spp. isolated in the USA³ and organophosphorus hydrolase (OPH) from *Flavobacterium* sp. strain ATCC 27551 and *Brevundimonas diminuta* strain MG isolated in the Philippines and the USA, respectively^{15,16,19}. *Alteromonas* sp. strain JD6.5 was found to contain OPAA-2 in the cytoplasm, whereas OPH is constitutively produced as a membrane-associated protein. The *opd* gene encoding OPH was found to be located on distinct large plasmids, pPDL2 in strain ATCC 27551 and pCMS in strain MG⁵. Sequencing of the *opd* genes showed that the nucleotide sequences of the Philippines and USA isolates were 100% identical^{15,19}. Recently, *opd*-type genes have been identified in several bacteria isolated in the USA and Australia^{2,9}. The gene, called *opdA*, was identified in an *Agrobacterium* isolate in Australia⁹. The *opdA* gene was approximately 88% identical at the nucleotide level to *opd*. On the other hand, an organophosphorus pesticide hydrolase and its gene have been isolated from *Arthrobacter* sp. strain B-5 in Japan and

* Corresponding author; E-mail: hayatsubi@yahoo.co.jp, Tel & Fax: +81-54-238-4875

characterized¹⁷). The deduced amino acid sequence of the hydrolase from strain B-5 showed 58.1% amino acid identity with the arylalkylphosphatase (AdpB) from *Nocardia* sp. strain B-1¹⁴). Both the hydrolases from strain B-1 and strain B-5 are located in the cytoplasm of cells. We previously reported the isolation and characterization of *Burkholderia* sp. strain NF100, which mineralizes fenitrothion through methylhydroquinone⁶). Enzymes responsible for the metabolism of fenitrothion are encoded on two plasmids, pNF1 and pNF2, in strain NF100. In the present study, we characterized the gene encoding organophosphorus pesticide hydrolase (fenitrothion hydrolase) on pNF2.

Burkholderia sp. strains NF100, NF103 and NF1033 (Str^r, Rif^r) have been described previously⁶). *Escherichia coli* strains HB101¹⁸) and JM109¹⁸) and the plasmid pBluescript II SK(+)¹⁸), helper plasmid pRK2013⁴) and broad-host-range plasmid pBI101¹⁰) have been used for cloning experiments. The alkaline-SDS plasmid extraction method¹⁸) was used to extract pNF2 encoding a fenitrothion hydrolase from strain NF103. The plasmid was digested with *Eco*RI, and the DNA fragments were cloned into an *Eco*RI site in pBI101. After transformation of pBI101 containing an *Eco*RI fragment into *E. coli* strain HB101, the plasmids were transferred to strain NF1033, which did not have pNF2 and could not hydrolyze fenitrothion, by triparental matings using pRK2013 as a helper plasmid. Transconjugants that had been able to utilize fenitrothion were selected on a mineral salts agar plate containing 0.4 mg of yeast extract and 0.8 mg of a fenitrothion emulsion consisting of 50% fenitrothion (FNT medium)⁶) and 20 µg each of kanamycin and streptomycin per ml. The selected transconjugant contained a plasmid (designated pFED) having a 6.5-kbp DNA fragment in the *Eco*RI site of pBI101.

An assay for the fenitrothion-hydrolyzing activity was carried out in 50 mM Tris-HCl buffer (pH 8.5) containing 0.2 mM fenitrothion and 0.1% (wt/vol) Triton X-100. The fenitrothion-hydrolyzing activity was determined by measuring the formation of 3-methyl-4-nitrophenol at 400 nm. To determine the DNA sequence of the cloned gene, pFED was subjected to restriction enzyme digestion with some endonucleases. The products were ligated to pBluescript II SK(+) and transformed into *E. coli* strain JM109. The DNA sequence of each clone was determined by Texas Red dye-primer cycle sequencing on an automated DNA sequencer using a Thermo sequenaseTM premixed cycle sequencing kit (Amersham Biosciences Corp., Piscataway, NJ, USA).

The transconjugant strain NF1033(pFED) had the ability to hydrolyze fenitrothion. The fenitrothion-hydrolyzing activity in crude extracts of strain NF1033(pFED) was 70% of that of wild-type strain NF100. Loss of the activity could be due to the copy number of pBI101 or the absence of the genes in the rest of pNF2. Further investigations are needed to elucidate the potential mechanism causing the loss of the activity. A restriction map of pNF2 was constructed by single and multiple enzyme digestion (Fig. 1A). The cloned 6.5-kbp DNA fragment was confirmed to be located on pNF2 by Southern hybridization and comparison of restriction maps of pNF2 and the cloned DNA fragment (Fig. 1). These results indicate that the cloned fragment from pNF2 encoded the fenitrothion-hydrolyzing enzyme. Sequence analysis revealed the presence of three ORFs in the cloned DNA fragment (Fig. 1B). ORF1 (1,593 bp) encoded a protein product of 55.2 kDa composed of 531 amino acids. ORF2 (1,506 bp) encoded a protein product of 55.2 kDa composed of 502 amino acids. ORF3 (1,362 bp) encoded a protein product of 50.0 kDa composed of 454 amino acids.

Fig. 1. Restriction maps of (A) pNF2 and (B) the 6.5-kbp *Eco*RI fragment from pNF2 containing the fenitrothion hydrolase gene. Restriction sites are: A, *Ava*II; B, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; H, *Hind*III; S, *Sac*I; Xh, *Xho*I; Xm, *Xma*I. (A) The black line indicates the 6.5-kbp *Eco*RI fragment and the gray line indicates the 13-kbp DNA region that is lacking in pNF2. (B) The top line represents the length of DNA in kb. The arrows correspond to (from left to right) ORF1 (designated *fedA*), ORF2 (*fedB*) and ORF3 (*orf3*), respectively.

The sequence similarity of the deduced amino acid sequences presumed from the DNA sequences of the three ORFs was examined using the BLAST program and several protein databases. The deduced amino acid sequence of ORF1 demonstrated no significant similarity to any of the previously identified organophosphorus hydrolyzing enzymes, Opd (accession number M20392), OpdA (AY043245), OPAA-2 (U29240) or OpaB (M91040). Some sequence similarity was observed with a putative protein from *Nocardia farcinica* strain IFM 10152 that was identified in the genome sequencing project (AP006618). This putative protein showed 36% sequence identity at the amino acid level to ORF1 over the entire protein. The function of this putative protein is not known. The deduced amino acid sequences of ORF2 and ORF3 showed no significant sequence similarity to any other protein.

To provide genetic evidence that each polypeptide encoded by the 6.5-kbp DNA fragment was involved in the hydrolysis of fenitrothion, a frameshift mutation was introduced into one of the three ORFs, and its effect on fenitrothion hydrolase activity was examined. A frameshift mutation was introduced into ORF1 by elimination of the *Xma*I restriction site in ORF1 on pFED. The resulting plasmid was designated pFED-1. The plasmids pFED-2 and pFED-3 were obtained by elimination of the *Xho*I site in ORF2 and the *Avr*II site in ORF3, respectively. The frameshift mutation caused a stop codon and was expected to inactivate the products encoded by each ORF. The plasmids were transferred to strain NF1033 by triparental matings as described above. Fenitrothion hydrolase activity was measured using crude extracts of the transconjugant prepared by sonic disruption (Table 1). The mutation in ORF1 led to a complete loss of fenitrothion hydrolase activity. The mutation in ORF2 caused a 91.2% reduction in enzymatic activity compared with that of strain NF1033(pFED). The knockout of ORF3 did not result in a loss of the activity. Therefore, ORF1 and ORF2 were shown to be essential for

the full enzymatic activity, and ORF3 might not be involved in the degradation of fenitrothion. The three ORFs in the 6.5-kbp DNA fragment were designated *fedA*, *fedB* and *orf3*, respectively.

To estimate whether FedA and FedB were membrane or soluble proteins, their secondary structures were predicted based on the physicochemical properties of their amino acid sequences such as hydrophobicity and charges. According to the SOSUI system⁷⁾, a tool for predicting the secondary structure of membrane proteins from a protein sequence, FedA was a soluble protein. Similarly, the Kyte-Doolittle hydrophilicity profile of FedA showed only short hydrophobic regions. This is unlike the case for an integral membrane protein, which would be expected to possess membrane-spanning regions. On the other hand, the SOSUI system indicated that the deduced amino acid sequence of FedB contained 12 transmembrane helices. The predicted gene product of *fedB* was also shown to be very hydrophobic, rich in noncharged residues, by the Kyte-Doolittle hydrophilicity profile. The *fedB* gene is thought to be involved in the membrane-association of fenitrothion hydrolase. The *fedB* gene partially overlapped the *fedA* gene, suggesting they could be cotranscribed. Therefore, it is assumed that *fedA* is a gene encoding a catalytic protein of the fenitrothion-hydrolyzing enzyme, and the *fedB* gene product is also an essential component of the enzyme and involved in membrane localization. Thus, we investigated the membrane localization of the fenitrothion hydrolase. One liter of culture was harvested by centrifugation, and the resulting cell paste was suspended in 50 mM sodium phosphate buffer (pH 7.0). Cells were disrupted by sonication (model VP-15S, Taitec Co., Saitama, Japan). The cell debris was removed by centrifugation at 10,000×g for 10 min. The lysate was subjected to centrifugation at 170,000×g for 1 h at 4°C. The supernatant was decanted, and the pellet consisting of cell walls and membranes was uniformly suspended in the buffer. The cell wall-membrane suspension was extracted with the buffer containing 0.1% Triton X-100. Detergent-solubilized protein was separated by centrifugation at 170,000×g for 1 h at 4°C. After centrifugation of the lysate at 170,000×g for 1 h, 63.1% of the fenitrothion-hydrolyzing activity in the cell lysate was associated with the cell wall-membrane fraction. When the cell wall-membrane fraction was treated with the buffer containing 0.1% Triton X-100, 90% of the hydrolyzing activity in the fraction was released into the soluble fraction. The results indicated that the fenitrothion hydrolase from strain NF100 was located in the cell membrane as was OPH.

We previously reported that about 2% of cells treated

Table 1. Effect of frameshift mutation in three ORFs on fenitrothion hydrolase activity in *Burkholderia* sp. strain NF100

Plasmid	Destroyed gene (deleted site)	FNT ^a hydrolase activity (U mg protein ⁻¹)	Relative activity (%)
pFED	—	0.0432	100
pFED-1	ORF1 (<i>Xma</i> I)	n.d. ^b	n.d. ^b
pFED-2	ORF2 (<i>Xho</i> I)	0.0038	8.8
pFED-3	ORF3 (<i>Avr</i> II)	0.0519	120.1

^a FNT is an abbreviation for fenitrothion.

^b not detected.

with mitomycin C lost their fenitrothion-hydrolyzing activity and that cells unable to hydrolyze fenitrothion either had lost pNF2 or were carrying a smaller plasmid termed pNFD2⁶). The ability of strain NF100 to hydrolyze fenitrothion was found to be unstable and was lost irreversibly under non-stress conditions. About 0.13% of the strain spontaneously lost fenitrothion hydrolase activity after repeated subculture on FNT agar medium. We collected hydrolase activity-deficient colonies and constructed a restriction map of pNFD2. Comparison of the maps of pNF2 and pNFD2 revealed that an approximately 13-kbp DNA fragment containing the 6.5-kbp DNA fragment encoding three ORFs was deleted from pNF2 (Fig. 1A). Plasmid stability during starvation has been reported with other bacterial species. It has been shown that in *Aeromonas salmonicida*¹²) and *Lactococcus lactis*¹¹), the number of plasmids possessed by the cells decreased during starvation. However, in some cases, plasmids were stably maintained for long periods of starvation¹). We investigated the population dynamics of strain NF100 and stability of pNF2 and the 6.5-kbp DNA fragment encoding the three ORFs under conditions of carbon starvation, which is similar to the environment in soil. Triplicate sand microcosms were prepared by mixing 50 g of sterilized sand and 10 ml of mineral salts medium containing 5×10^8 cells in Erlenmeyer flasks. The microcosms were incubated at 25°C for 30 days and the total number of strain NF100 was counted regularly by the spread-plate method using FNT medium. A halo around a colony on the FNT medium was taken as evidence that the colony exhibited fenitrothion hydrolase activity. Plasmid profiles of the mutants were analyzed by agarose gel electrophoresis. There was no significant change during 30 days in the number of strain NF100 when the initial inoculum was 1.0×10^7 CFU g⁻¹ sand (data not shown). The mutants always presented as a small population, generally constituting 0.04 to 0.09% of the cells in the microcosms. The mutants harbored a plasmid of similar size to pNFD2 or had lost the entire pNF2. These results showed that pNF2 was stably maintained in strain NF100 in a carbon-starved condition as in the soil environment.

We cloned a novel organophosphorus pesticide hydrolase gene that appears to encode a catalytic protein and a membrane-associated protein. The sequence information of *fed* could be used to provide a DNA probe to study the prevalence and diversity of this gene in soil microbial populations. This will provide a useful tool to study changes in bacterial populations and horizontal gene transfer during the development of enhanced fenitrothion degradation in soil.

The nucleotide sequence of the 6.5-kbp DNA fragment

was deposited in the DDBJ database with accession number AB235840.

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