

Cloning and Expression of a Parathion Hydrolase Gene from a Soil Bacterium, *Burkholderia* sp. JBA3

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Abstract A bacterium, *Burkholderia* sp. JBA3, which can mineralize the pesticide parathion, was isolated from an agricultural soil. The strain JBA3 hydrolyzed parathion to *p*-nitrophenol, which was further utilized as the carbon and energy sources. The parathion hydrolase was encoded by a gene on a plasmid that strain JBA3 harbored, and it was cloned into pUC19 as a 3.7-kbp *Sau*3AI fragment. The ORF2 (*ophB*) in the cloned fragment encoded the parathion hydrolase composed of 526 amino acids, which was expressed in *E. coli* DH10B. The *ophB* gene showed no significant sequence similarity to most of other reported parathion hydrolase genes.

Keywords: Parathion hydrolase gene, *Burkholderia* sp. JBA3, biodegradation

At present, the most widely used pesticides belong to the organophosphorus group [17]. Organophosphorus insecticides inhibit the normal activity of the acetylcholine esterase, resulting in accumulation of acetylcholine at the synapses and finally convulsion, paralysis, and death for insects and mammals [14]. Among the organophosphorus compounds, parathion (*O,O*-diethyl-*O*-4-nitrophenyl phosphorothioate) is among the most highly toxic chemicals registered in the Environmental Protection Agency [5]. In Korea, a massive amount of parathion has been produced and used for rice farming and horticulture [11]. Parathion residues released into the environments through agricultural applications and nonpoint-source discharges could be hazardous to wild life. Therefore, detoxification of parathion is important to protect nontarget organisms in terrestrial and aquatic

environments. Since hydrolysis of the phosphoester bond of parathion substantially reduces its toxicity, the OPH (organophosphorus hydrolase) genes of bacteria have received considerable attention for use in bioremediation [2, 4].

We isolated several bacterial strains capable of degrading parathion through enrichment culture using the pesticide as the sole carbon source. One of the isolates, identified as a *Burkholderia* species, was observed to have no sequence homology in the parathion hydrolase gene with most of other previously reported bacterial strains hydrolyzing organophosphates. In this study, we report the isolation of a parathion-degrading soil bacterium and the cloning of its parathion hydrolase gene, which was successfully expressed in an *Escherichia coli* strain.

Isolation of a Parathion-Degrading Strain *Burkholderia* sp. JBA3

Soil samples, which were obtained from rice fields throughout the country, were treated with parathion (Cheil Chemicals, Seoul, Korea) dissolved in dichloromethane to a final concentration of 100 µg/g soil and thoroughly mixed. The treated soil was incubated with periodic mixing at room temperature. Five weeks after parathion application, a 1 g soil sample was transferred into a tube containing 3 ml of mineral medium [13] containing parathion (100 µg/ml) and incubated at 28°C with shaking. After several transfers of parathion-degrading cultures into fresh medium, an appropriate diluent was spread onto a PTYG agar medium [1, 13] and each colony with distinct morphology was then tested for parathion degradation in fresh medium before strain purification. Among the isolates, strain JBA3 could rapidly utilize parathion as a sole source of carbon and energy and, furthermore, showed no sequence homology

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when analyzed with various PCR primers targeting the parathion hydrolase gene of previously reported isolates [6, 12, 15]. The nearly full sequence of the 16S rDNA of the isolate (GenBank Accession No. EF495209) was determined as described previously [9, 10]. The strain JBA3 was most closely related to *Burkholderia glathei* LMG 14190 (U96935) among type strains with a similarity of 98%. JBA3 is a rod-shaped Gram-negative bacterium and exhibited catalase/oxidase positive reactions. The organism was able to assimilate glucose, D-mannose, D-mannitol, N-acetyl-glucosamine, gluconate, and malate, and showed positive reaction for Voges-Proskauer (VP) test and nitrate reduction. The strain JBA3 produced acids from D-mannitol, inositol, D-sorbitol, L-rhamnose, and D-sucrose. Based on the 16S rDNA sequence identity, the strain was named as *Burkholderia* sp. JBA3.

Degradation of Parathion

After growth in PTYG medium, cells were harvested, washed, and resuspended in mineral medium. Aliquots of suspended cells were inoculated into duplicate flasks containing 250 ml of mineral medium containing 433 μM of parathion (Riedel-de Haën, Seelze, Germany) at a final density of $\text{OD}_{600}=0.005$. All cultures were incubated at 28°C with shaking. One ml of the culture was regularly removed and used to determine cell growth and the concentrations of parathion and *p*-nitrophenol. Cell growth was determined at optical density 600 nm. For the quantification of parathion and *p*-nitrophenol, 1 ml of acetonitrile was added to 1 ml of the culture, mixed thoroughly, and centrifuged. The upper portion was used for the measurement at optical densities 270 nm (for parathion)

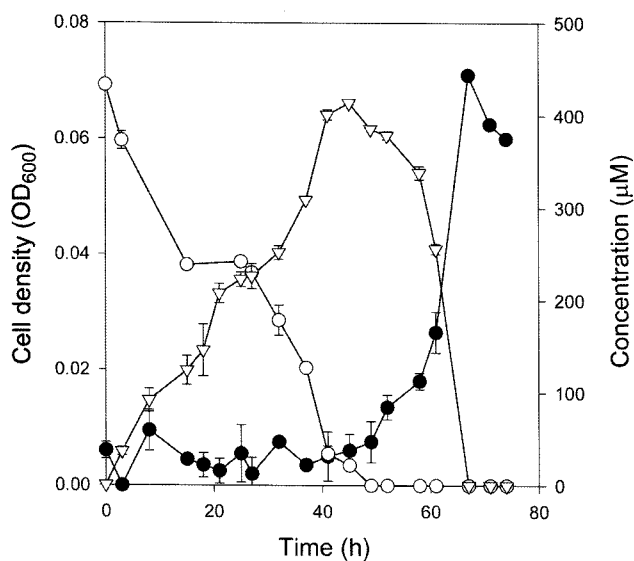


Fig. 1. Utilization of parathion by *Burkholderia* sp. JBA3. Symbols: ●, cell density; ○, parathion; ▽, *p*-nitrophenol. The error bars indicate standard deviations for duplicates.

and 410 nm (for *p*-nitrophenol) using spectrophotometry and reverse-phase HPLC (Waters, Milford, U.S.A.). The concentrations of parathion and *p*-nitrophenol were calculated using standard curves prepared from the known concentrations of parathion and *p*-nitrophenol in the same medium. The strain JBA3 completely hydrolyzed parathion to *p*-nitrophenol (Fig. 1). When most of the parathion residues were hydrolyzed, the accumulated *p*-nitrophenol began to be utilized by the strain JBA3 with concomitant cell growth. At 67 h, *p*-nitrophenol had been completely utilized and cell density started to decrease. This result indicates that the strain JBA3 first hydrolyzes most of the parathion before it utilizes its hydrolysis product, *p*-nitrophenol, as a source of carbon and energy.

Cloning of Parathion Hydrolase Gene

Total genomic DNA of the strain JBA3 was extracted with a Wizard Genomic DNA Purification Kit (Promega, Madison, U.S.A.) and partially digested with *Sau*3AI. The 2–4-kbp fragments were recovered with the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and ligated into pUC19, which had been digested with *Bam*HI and treated with calf intestinal alkaline phosphatase (Promega, Madison, U.S.A.). The ligation product was transformed into *E. coli* DH10B cells. The bacteria were then spread onto LB medium (pH 7.5) containing 100 $\mu\text{g}/\text{ml}$ of ampicillin, and the surface of the agar plate was completely covered with a 1PS filter (Whatman, Maidstone, U.K.), which had been soaked into 9 ml of acetone containing 10 μl of parathion and dried completely. The plate was incubated at 37°C for one day, and colonies producing yellow color of *p*-nitrophenol were picked and examined for the ability to hydrolyze parathion in parathion mineral medium as described above. The inserts from two transformants showing parathion hydrolysis activity were PCR-amplified with the primers M13-F and M13-R (Bioneer, Daejeon, Korea) and the PCR products were completely digested with *Sau*3AI. Because the two restriction-enzyme digests showed the same patterns when electrophoresed on an agarose gel, only one insert was sequenced by primer extension. Sequences were obtained at the National Instrumentation Center for Environmental Management (Seoul, Korea), using an ABI 3730 sequencer. The complete length of the insert was 3,668 bp (Genbank Accession No. EF495210). The 3.7-kbp fragment contained one complete ORF (ORF2) and two truncated ORFs (ORF1 and ORF3) (Fig. 2). The ORF2 (1,578 bp) encodes a protein product composed of 526 amino acids and the ORF3 (1,288 bp) encodes an incomplete protein product composed of 429 amino acids.

To know whether the ORF2 alone hydrolyzes parathion to *p*-nitrophenol, the DNA fragment containing only the ORF2 was PCR-amplified from the 3.7-kbp fragment using the primers BP/f (5'-CTGGAAATCAAGGAAATCCG)

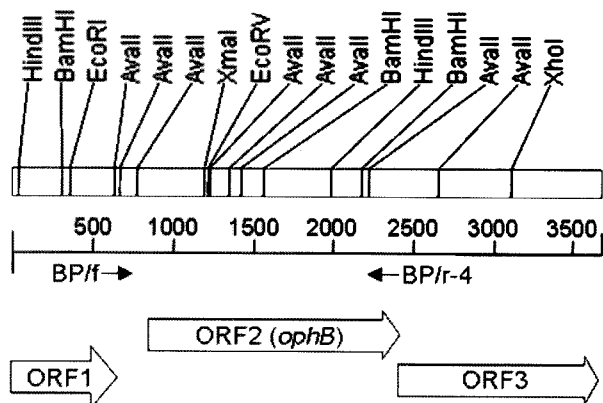


Fig. 2. Restriction map of the 3.7-kbp fragment cloned from *Burkholderia* sp. JBA3.

The figures represent the length of DNA in bp. The white arrows correspond to (from left to right) ORF1, ORF2, and ORF3, respectively. The black arrows represent primers used for PCR amplification of ORF2.

and BP/r-4 (5'-TTACTGTTGCAGAGCAGATG) (Fig. 2). The PCR product was cloned into the pGEM-T easy vector (Promega, MA, U.S.A.) and transformed into *E. coli* DH10B. The plasmid extracted was used as a template in a PCR using the primers M13-f and M13-r. The gel-purified PCR product was digested with EcoRI and ligated into pUC19, which had been digested with EcoRI and treated with calf intestinal alkaline phosphatase. The ligation product was transformed into *E. coli* DH10B cells. The ORF2 was in the opposite orientation to the *lacZ* promoter both in the pUC19 containing the complete 3.7-kbp fragment and in the pUC19 containing only the ORF2. Cultures of *Burkholderia* sp. JBA3, *E. coli* DH10B/pUC19-3.7 (*E. coli* DH10B with pUC19 containing the complete 3.7-kbp fragment), and *E. coli* DH10B/pUC19-ORF2 (*E. coli* DH10B with pUC19 containing only the ORF2) were inoculated into mineral medium containing 75 μ M of parathion at OD_{600} =0.036–0.045 and incubated at 28°C with shaking. Strain *E. coli* DH10B/pUC19-ORF2 was able to hydrolyze parathion to *p*-nitrophenol completely, although its hydrolysis activity was relatively slow compared with *Burkholderia* sp. JBA3 (Fig. 3). It took about 5 h for strain JBA3 to completely hydrolyze parathion to *p*-nitrophenol, while strains *E. coli* DH10B/pUC19-3.7 and *E. coli* DH10B/pUC19-ORF2 required about 16 h. This may be due to a slow expression level of the cloned genes in the *E. coli* DH10B strain or it may indicate that the complete product of the ORF3 or other unknown proteins is needed for fast hydrolysis.

The strain *E. coli* DH10B/pUC19-ORF2 was tested for the ability to hydrolyze several organophosphorus insecticides: EPN (*o*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate), fenitrothion (*O,O*-dimethyl *O*-*p*-nitro-*m*-tolyl phosphorothioate), and methyl parathion (*O,O*-dimethyl *O*-*p*-nitrophenyl phosphorothioate). The hydrolysis activity was determined

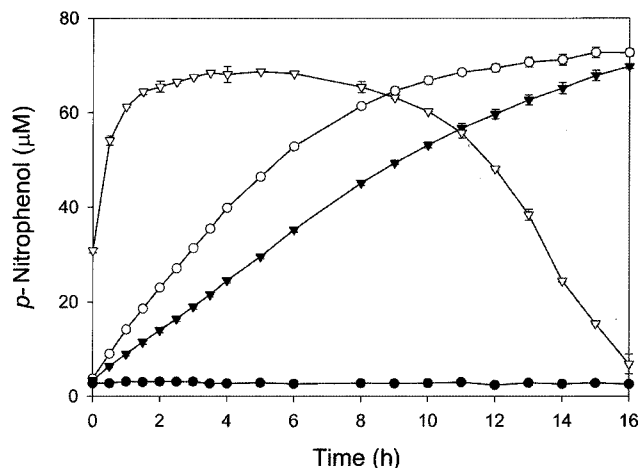


Fig. 3. The hydrolysis activities of *Burkholderia* sp. JBA3 (∇), *E. coli* DH10B/pUC19-3.7 (\circ), *E. coli* DH10B/pUC19-ORF2 (\blacktriangledown), and *E. coli* DH10B/pUC19 (\bullet).

The error bars indicate standard deviations for duplicates.

from the appearance of yellow color due to the formation of *p*-nitrophenol or 3-methyl-4-nitrophenol. Strain *E. coli* DH10B/pUC19-ORF2 could hydrolyze fenitrothion, methyl parathion, and to a lesser extent, EPN. Thus, the ORF2 was named *ophB* (organophosphorus hydrolase from *Burkholderia* sp.). The genes encoding proteins exhibiting organophosphate-hydrolyzing activity have been isolated and characterized from several bacteria. Among them, the two *opd* genes cloned from The Philippines and USA isolates [12, 15] were 100% identical to each other in the nucleotide sequences and showed 88% sequence similarity to the *opdA* gene of an Australia isolate [5]. The *ophB* gene of this study exhibited no significant sequence similarity to other previously reported bacterial isolates capable of hydrolyzing parathion [6, 7, 12, 15], but showed 96% sequence identity at the amino acid level to *fedA*, a fenitrothion hydrolase gene obtained from a fenitrothion-degrading bacteria [19]. The ORF1 showed 79% sequence identity at the amino acid level to a transposase, IS4 from *Burkholderia vietnamiensis* G4 (GenBank Accession No. EAM31871), when examined using Basic Local Alignment Search. This indicates that the *ophB* gene may be part of a transposon like other organophosphorus hydrolase genes [8, 16]. The strain JBA3 was observed to harbor seven different plasmids (data not shown). Through curing experiments using the sodium dodecyl sulfate method [3], we obtained a strain that has lost the fifth largest plasmid. The cured strain was able to grow rapidly on glucose and succinate as its wild-type strain, but it could no longer hydrolyze parathion to *p*-nitrophenol and thus could not utilize parathion as a carbon source. When each plasmid DNA excised and purified from agarose gel was used as a template for PCR amplification with specific primers selected from the internal sequence of the *ophB* gene, an amplified DNA band of the expected

size was obtained from the fifth largest plasmid. The result suggested that the *ophB* gene of strain JBA3 was on the plasmid DNA like some other organophosphate-hydrolyzing bacteria [15, 18]. Different pathways of parathion degradation were proposed but all include a hydrolysis step [17]. The hydrolysis of organophosphorus compounds leads to a decrease in mammalian toxicity by several orders of magnitude and therefore this step is very useful for detoxification of parathion. The strain JBA3 rapidly hydrolyzes parathion to *p*-nitrophenol, leading to its complete mineralization and detoxification. Its parathion hydrolase gene encodes an OPH enzyme that has a broader substrate range for insecticidal organophosphates, which could give additional advantages to its application for bioremediation.

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