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Characterization of the 3-methyl-4-nitrophenol degradation pathway and genes of *Pseudomonas* sp. strain TSN1

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3-Methyl-4-nitrophenol (3M4NP) is formed in soil as a hydrolysis product of fenitrothion, one of the major organophosphorus pesticides. A *Pseudomonas* strain was isolated as a 3M4NP degrader from a crop soil and designated TSN1. This strain utilized 3M4NP as a sole carbon and energy source. To elucidate the biodegradation pathway, we performed transposon mutagenesis with pCro2a (mini-Tn*5495*) and obtained three mutants accumulating a dark pink compound(s) from 3M4NP. Rescue cloning and sequence analysis revealed that in all mutants, the transposon disrupted an identical aromatic compound *meta*-cleaving dioxygenase gene, and a monooxygenase gene was located just downstream of the dioxygenase gene. These two genes were designated *mnpC* and *mnpB*, respectively. The gene products showed high identity with the methylhydroquinone (MHQ) monooxygenase (58%) and the 3-methylcatechol 2,3-dioxygenase (54%) of a different 3M4NP degrader *Burkholderia* sp. NF100. The transposon mutants converted 3M4NP or MHQ into two identical metabolites, one of which was identified as 2-hydroxy-5-methyl-1,4-benzoquinone (2H5MBQ) by GC/MS analysis. Furthermore, two additional genes (named *mnpA1 and mnpA2*), almost identical to the *p*-nitrophenol monooxygenase and the *p*-benzoquinone reductase genes of *Pseudomonas* sp. WBC-3, were isolated from the total DNA of strain TSN1. Disruption of *mnpA1* resulted in the complete loss of the 3M4NP degradation activity, demonstrating that *mnpA1* encodes the initial monooxygenase for 3M4NP degradation. The purified *mnpA2* gene product could efficiently reduce methyl *p*-benzoquinone (MBQ) into MHQ. These results suggest that strain TSN1 degrades 3M4NP via MBQ, MHQ, and 2H5MBQ in combination with *mnpA1A2* and *mnpCB*, existing at different loci on the genome.

[**Key words:** Organophosphorus pesticide; Fenitrothion; Nitrophenol; Degradation; Methylhydroquinone; *Pseudomonas*]

Fenitrothion (FNT) [*O*,*O*-dimethyl *O*-(4-nitro-m-tolyl) phosphorothioate] is one of the major organophosphorus pesticides that has been widely used to control infectious disease-causative pests in human and animals and plant damage-causing insects in agriculture. The use of similar pesticides, such as parathion and methylparathion, has been banned in many countries due to the strong nerve toxicity of these compounds; however, FNT is preferentially used due to its lower toxicity in higher organisms (e.g., LD_{50} of FNT = 330–800 mg kg^{-1}, LD_{50} of parathion = 13 mg kg^{-1}, orally in rats) (1,2). FNT still has strong toxicity towards aquatic organisms and animals (LC_{50} of FNT = <0.001 mg l^{-1} in water fleas, crabs, and shrimps) (3). Once FNT is introduced into soils and aquatic environments, this compound can easily be converted into 3-methyl-4-nitrophenol (3M4NP) by microbial hydrolysis and photolysis (4–6). Previously, we also demonstrated that FNT was easily degraded into 3M4NP by activated sludge and soil bacteria (7). Therefore, 3M4NP, as well as FNT, has been frequently detected in the environment (6). Notably, 3M4NP is also toxic and shows estrogenic and androsteronic activities in higher organisms (8–12). Therefore, it is important to understand the biodegradability and biodegradation mechanisms of 3M4NP.

There are several reports on bacterial 3M4NP degradation, but the information on the metabolism is limited to one bacterial genus, *Burkholderia* (13–18). *Burkholderia* sp. strain NF100 has two catabolic plasmids, pNF1 (105 kb) and pNF2 (33 kb); the former encodes a methylhydroquinone (MHQ) monooxygenase (MhqA) and an aromatic compound *meta*-cleaving dioxygenase (MhqB), while the latter encodes an FNT hydrolase (13,15,19). Purification of these enzymes and analysis of the metabolites from FNT or 3M4NP revealed that FNT is hydrolyzed into 3M4NP by the FNT hydrolase, which is further converted into MHQ. Subsequently, MHQ is oxidized by MhqA, followed by *meta*-cleavage of the aromatic-ring by MhqB (15) (Fig. 1). Although MhqA had previously been purified, the products of MHQ oxidation (by MhqA) have not yet been identified. In addition, the enzymes that convert 3M4NP into MHQ remain unknown. In the degradation of 3M4NP by *Burkholderia* sp. strain SJ98 (formerly *Ralstonia* sp. SJ98), catechol was first detected as the metabolite (14). However, recent studies on this strain have revealed that SJ98 degrades 3M4NP via MHQ using a known *p*-nitrophenol (PNP) degradation gene cluster, *pnpABA1CDEFG* (Fig. 1) (20).

In the present study, to understand 3M4NP biodegradation mechanisms in more detail, we newly isolated a 3M4NP-degrading

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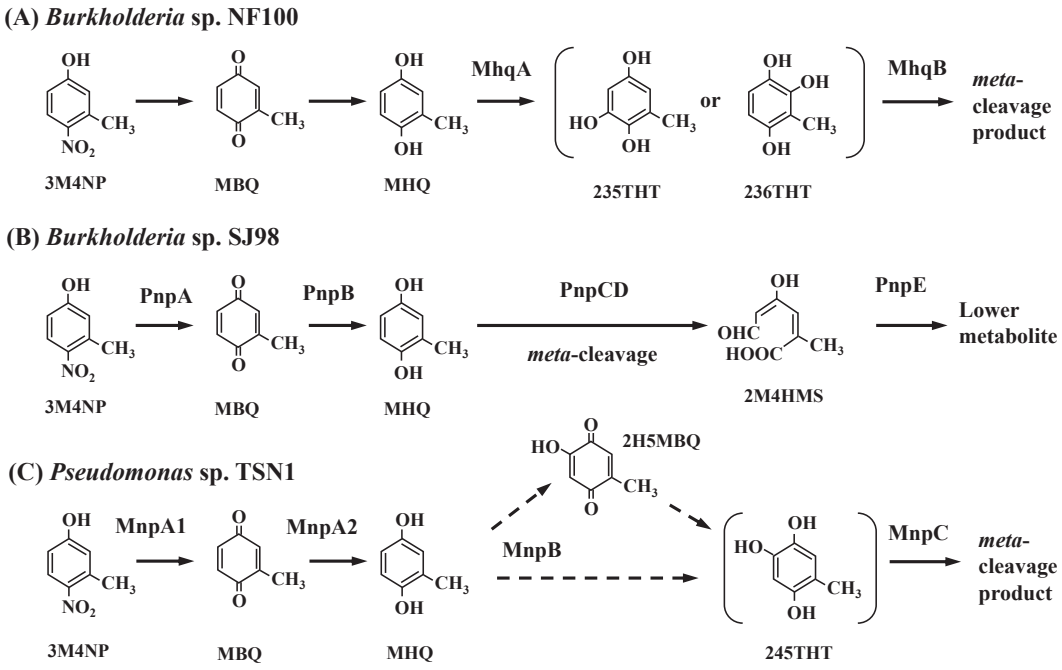

FIG. 1. The proposed 3M4NP degradation pathways of *Burkholderia* sp. NF100 (A), *Burkholderia* sp. SJ98 (B), and *Pseudomonas* sp. strain TSN1 (C). 3M4NP, 3-methyl-4-nitrophenol; MBQ, methyl-*p*-benzoquinone, MHQ, methylhydroquinone; 235THT, 2,3,5-trihyroxytoluene; 236THT, 2,3,6-trihyroxytoluene; 2M4HMS, 2-methyl-4-hydroxymuconate semialdehyde; 2H5MBQ, 2-hydroxy-5-methyl-1,4-benzoquinone.

Pseudomonas strain from soil and characterized the 3M4NP degradation pathway through chemical analysis of the metabolites and gene cloning. The identified pathway was very similar to that of strain NF100, however, we identified genes that encode the initial 3M4NP oxidation and determined the structure of a metabolite from MHQ for the first time.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, primers, and culture conditions The bacterial strains and plasmids used in the present study are listed in Table 1. *Pseudomonas* strains were cultured in MSB medium (7) or LB medium (21) at 30°C on a rotary shaker at 120 rpm, while *Escherichia coli* strains were cultured in LB medium at 37°C and 120 rpm, unless otherwise mentioned. Ampicillin (Amp), kanamycin (Km), and chloramphenicol (Cm) were added to the media at 100 mg l^{-1}, 50 mg l^{-1}, and 20 mg l^{-1}, respectively, when necessary. Primers to amplify the specific genes were custom-made by and purchased from GeneDesign, Inc. (Osaka, Japan) (Table 2).

Enrichment and isolation of 3M4NP-degrading bacteria Soil samples (0.5 g) from more than 20 places including crop fields, riversides, mountains, and forests near University of Hyogo (Himeji, Japan) were suspended with 1 ml of MSB medium and stood for several min. Then, 0.5 ml of the suspension was inoculated into 10 ml of MSB medium containing 100 mg l^{-1} of Bacto Yeast Extract (BD, Tokyo, Japan) (MSBY) and 100 mg l^{-1} of 3M4NP. The culture was shaken at 30°C on a rotary shaker at 120 rpm. After one week, 0.1 ml of the culture was transferred to the same fresh medium and incubated in the same manner. After three repeated cultivations, 0.2 ml of the culture was spread onto the same agar plates (1.5% w/v) and the plates were incubated at 30°C for several days. Colonies decolorizing the yellow color of 3M4NP on the plates were selected as the candidates for 3M4NP-degrading bacterium.

For the brief identification of the isolate, the 16S rRNA gene sequence was determined as previously described (7). An API20NE identification kit (SYSMEX bioMerieux, Tokyo, Japan) was also used to help the identification according to the manufacturer's protocol.

Polymerase chain reaction, hybridization, electroporation, and other molecular techniques Polymerase chain reaction (PCR) was conducted by using *ExTaq* polymerase (Takara Bio, Kyoto, Japan) as previously described (22). Total bacterial DNA was extracted from bacterial cells using the Illustra Bacteria Genomic Prep Mini Spin Kit (GE Healthcare Japan, Tokyo, Japan) according to the manufacturer's protocol. Southern and colony hybridizations were performed using a nylon membrane Hybond-N (GE Healthcare Japan), and a DIG High Prime DNA Labeling and Detection Kit (Roche Diagnostics Japan, Tokyo, Japan) according to the instructions of the kit. Electroporation was conducted using the BioRad Gene Pulsar II electroporator (BioRad Laboratories, Tokyo, Japan) under the following conditions: field strength 2.0 kV cm^{-1}, capacitor 25 μF, and resistor 700 Ω. Other molecular techniques, such as restriction digestion and ligation, were conducted according to the protocols provided from the suppliers of the enzymes and kits or a laboratory manual (21).

Degradation tests using cell suspensions Strain TSN1 and its mutants were inoculated into LB medium and incubated overnight. After centrifugation (6000 rpm, 4°C, 5 min) for harvest, the cells were washed with MSB medium and suspended with the same medium to adjust the optical density at 600 nm (OD_{600}) to 1.0. Next, 3M4NP or MHQ was added to the cell suspensions to obtain an appropriate concentration (the final standard concentration is 0.3 mM). Degradation tests were performed by shaking the cell suspension at 30°C on a rotary shaker at 120 rpm. The samples were withdrawn at specific intervals and subjected to HPLC analysis after removal of the cells by centrifugation (15,000 rpm, 4°C, 2 min). HPLC analysis was performed by using the Shimadzu HPLC system (CBM-20A, 2 × LC-10AD, SPD-10A, CTO-10AC, SIL-10A, Shimadzu, Kyoto, Japan) equipped with a Mightysil RP-18GP Aqua column (150 mm × 4.6 mm ID, Kanto Kagaku Kogyo, Tokyo, Japan) under the following conditions: column temperature, 40°C; detection wavelength, 290 nm; flow rate, 1 ml min^{-1}; and injection volume, 20 μl. The mobile phase used was an isocratic mixture of solution A (CH_3OH:H_2O:CH_3COOH = 50:950:1) and solution B (CH_3OH:H_2O:CH_3COOH = 950:50:1) at 6:4. The metabolites detected were fractionated by using the same system with a semi-preparative column (Mightysil RP-18GP Aqua, 150 mm × 10 mm ID) and a fraction collector FRC-10A (Shimadzu). The flow rate was set at 2.5 ml min^{-1}. The separated metabolites were analyzed by GC/MS using an Automass 150 system II (JEOL, Tokyo, Japan) under the following conditions: injection method, split less; sample volume, 1 μl; injection temperature, 250°C; interface temperature, 250°C; source temperature, 200°C; ionization method, EI; ionization potential, 70 eV; ionization current, 300 μA; carrier gas, helium 99.9999%; and flow rate, 25 ml min^{-1}. The following thermal program was used: initial temperature of 60°C for 2 min; increased at 10°C min^{-1} to 250°C; and maintained at 250°C for 2 min. The total analytical time was 23 min.

Preparation of *E. coli* cell extracts, purification of proteins, and enzymatic reactions Recombinant *E. coli* JM109 harboring pUmB was incubated at 30°C and at 120 rpm for 24 h in LB medium containing Amp and 1 mM IPTG. Then, the cells were harvested by centrifugation (6000 rpm, 4°C, 10 min), and washed with 50 mM sodium phosphate buffer (pH 7.0), and resuspended with 5 ml of the same buffer. The suspension was subjected to ultrasonication (output 6, 30 s x 10 on ice) using a TOMY UD-200 ultrasonic disruptor (TOMY, Tokyo, Japan) to disrupt the cells. The cell debris was removed by centrifugation (15,000 rpm, 4°C, 20 min), and the supernatant was used as a crude cell extract for the conversion of MHQ. The

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