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Article

Degradation of 3-Methyl-4-nitrophenol, a Main Product of the Insecticide Fenitrothion, by *Burkholderia* sp. SH-1 Isolated from Earthworm (*Eisenia fetida*) Intestine

Seon Hwa Kim[1], Myung Ryeol Park[1], Songih Han[2], Kyungsook Whang[2], Jae Han Shim[1] and In Seon Kim[1,*]

[1]*Division of Applied Bioscience and Biotechnology, Institute of Agricultural Science and Technology, College of Agriculture and Life Sciences, Chonnam National University, Gwangju 500-757, Korea*

[2]*Department of Microbiology, Mokwon University, Daejeon 302-729, Korea*

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Microorganisms were isolated from earthworm intestine and examined for their ability to degrade 3-methyl-4-nitrophenol (MNP), a main degradation product of the insecticide fenitrothion. An isolate that showed the best degradation of MNP was selected for further study. The 16S rRNA analysis showed that the isolate belongs to the genus of *Burkholderia*, close to phenanthrene-degrading *Burkholderia* sp. S4.9, and is named *Burkholderia* sp. SH-1. When time-course degradation of MNP by SH-1 was examined by high performance liquid chromatographic analysis, almost complete degradation of MNP was observed within 26 h. Colony forming unit value assays indicated that the isolate SH-1 was capable of utilizing MNP as a sole carbon source. SH-1 could also degrade *p*-nitrophenol (PNP) but could not degrade *ortho*-substituted nitroaromatics such as 2,4-, 2,6- and 2,5-dinitrophenol. Catechol was detected as the main degration product of MNP and PNP. SH-1 was also found in the soil from which earthworms were obtained. These results suggest that the dispersal of *Burkholderia* sp. SH-1 into different environment with the aid of earthworms is likely to play a role in bioremediation of the soil contaminated with MNP.

Key words: *biodegradation, earthworm, fenitrothion, 3-methyl-4-nitrophenol, MNP*

3-Methyl-4-nitrophenol (MNP) is one of the major degradation products of the insecticide fenitrothion [*O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl) thiophosphate]. Under aerobic environmental conditions, fenitrothion degradation to MNP proceeds rapidly in days to weeks [Takimoto *et al.*, 1976], suggesting a possibility that MNP may be found in the environment at a concentration higher than that of fenitrothion. MNP is now of an environmental concern as this compound exhibited estrogenic activity in recombinant yeast strain assays [Furuta *et al.*, 2004]. Although the distribution of MNP remains unknown, MNP may accumulate in soil as a result of degradation of fenitrothion. Considering the potential adverse effects of MNP on human and animals as an endocrine-disrupting chemical, studies on the degradation of MNP in the environment are required.

*Corresponding author
Phone: +82-62-530-2131; Fax: +82-62-530-2139
E-mail: mindzero@chonnam.ac.kr

Soil microorganisms and invertebrates can mediate biotic degradation of environmental contaminants in soil. Earthworms are one of the most important soil invertebrates. Their feeding activities contribute to the breakdown and incorporation of macro particles such as mineral soils and organic matters [Piearce, 1978]. The involvement of earthworms in degradation of organic contaminants has suggested their important roles in environmental bioremediation [Renoux *et al.*, 2000; Saavedra *et al.*, 2006]. A number of studies have demonstrated that earthworms can function as the delivery of beneficial bacteria into the environment [Danne *et al.*, 1996, 1997; Danne and Haggblom, 1999].

The present study was performed in an effort to understand the roles of earthworm intestinal microorganisms for degradation of MNP. Microorganisms capable of degrading MNP were isolated from earthworm intestine. Earthworms were obtained from an agricultural soil with the history of fenitrothion application. MNP degradation was examined by high performance liquid

chromatography (HPLC) analysis of MNP and its degradation products. The degradation of other nitroaromatic compounds was also investigated.

Materials and Methods

Chemicals. 3-Methyl-4-nitrophenol (purity 98%), *p*-nitrophenol (purity 98%), 2,4-dinitrophenol (purity 97%), 2,5-dinitrophenol (purity 97%), 2,6-dinitrophenol (purity 97%) and catechol (purity 99%) were purchased from Aldrich (Milwaukee, WI). Authentic standards such as catechol (purity 99%), methylhydroxyquinone (purity 98%), hydroxyquinone (purity 99%), 4-nitrocatechol (purity 97%), 1,2,4-benzentriol (purity 99%) were purchased from Sigma (St. Louis, MO). The solvents used in this study were of HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals were of analytical grade and commercially available, unless otherwise stated.

Microorganism isolation and media. Microorganisms capable of degrading 3-methyl-4-nitrophenol (MNP) were isolated by an enrichment culture technique as described elsewhere (Kim *et al.*, 2004; Shin *et al.*, 2005). Earthworms (*Eisenia fetida*) were obtained from an agriculture soil with a history of fenitrothion application for at least five years. The earthworms were washed with distilled water to remove soil on their surface followed by washing them twice in 70% (v/v) ethanol for 30 seconds to remove microorganisms on their surface. Earthworm intestines were then taken carefully and cut into small pieces by using sterilized scissors in 100 mL of brain heart infusion (BHI) media and incubated on a shaking incubator at 150 rpm at 27°C for 24 h. The BHI cultures were inoculated at 1% (v/v) into 250 mL flasks containing 100 mL of mineral salt medium (MSM) with 0.40 mmol/L of MNP as the sole carbon source and then incubated for 5 days as described above. The MSM consisted of the following constituents (g/L, pH 7.2): K_2HPO_4 2.4; KH_2PO_4 1.2; NH_4NO_3 1.0; $MgSO_4 \cdot 7H_2O$ 0.2; $CaCl_2 \cdot 2H_2O$ 0.025; 10 mL of trace elements containing 20 mg of $Na_2MoO_4 \cdot 2H_2O$, 50 mg of H_2BO_3, 30 mg of $ZnCl_2$, 10 mg of $CuCl_2$ and 20 mg of $FeCl_3$ per liter. When MNP was used as sources of carbon and nitrogen, the cultures were incubated in the absence of NH_4NO_3. After four transfers to fresh MSM containing MNP as the sole carbon source, the cultures were diluted serially with the MSM and plated onto MNP-MSM agar plates. MNP-MSM agar plates were prepared by adding MNP dissolved in dimethyl sulfoxide (DMSO, 1 mL/L) into the MSM that had been previously autoclaved and cooled to 50°C. Only DMSO was added to the MSM for control plates. Following bacterial colonies, visible colonies were obtained and tested for MNP degradation. MNP-degrading microorganisms were also isolated from the same soil from which the earthworms were obtained. For this, a 2 g amount of soil samples was suspended in 100 mL of the MSM containing 0.40 mmol/L of MNP as the sole carbon source and then incubated for 5 days as described above. After four transfers, bacterial colonies were obtained and examined for MNP degradation as described above.

Identification of MNP-degrader. Bacterial identification was carried out by 16S rRNA sequence analyses. The 16S rRNA genes were amplified by PCR using universal primers of 27f and 1492r as described previously (Kim *et al.*, 2004). PCR products containing 1,375-base segment of 16S rRNA genes were purified for automated sequencing. The 16S rRNA sequence analysis of the isolate was performed by comparing it with other 16S rRNA sequences available from a BLAST search of the DDBJ database.

MNP degradation and bacterial growth. Time-course MNP degradation and bacterial growth were investigated concurrently in the same culture flasks. For MNP degradation, bacterial cells grown overnight on BHI media were centrifuged at 8,000 × g and washed twice with sterilized MSM. The washed cells were resuspended in the MSM and inoculated at 1% (v/v) into 250 mL flasks, in triplicate, containing 50 mL of the MSM with 0.40 mmol/L of MNP as the sole carbon source. The cultures were then filtered through 0.2 μm sterilized-membrane filters. For determination of the concentration of MNP, the filtrates were diluted properly, and a 10 mL aliquot was measured by HPLC. The HPLC was a Dionex model P680 pump (Dionex, USA) equipped with a Dionex model PDA-100 photodiode array detector at 270 nm. The HPLC column was a ZORBAX C_{18} analytical stainless column (2.5 mm i.d. × 250 mm length, 5 μm film thickness). The mobile phase was 40% (v/v) acetonitrile in water adjusted to pH 2 by addition of trifluoroacetic acid and eluted at 1.0 mL/min. The concentrations of MNP were determined by HPLC on the basis of its standard calibration curve. The culture filtrates were directly used for determination of MNP and its degradation products due to their rapid degradation during the incubation. The degradation products of MNP in the cultures were investigated by HPLC analysis and comparing their retention times to those of authentic standards. For bacterial growth assays, the growth of SH-1 was measured by plating an aliquot (100 μL) of the cultures onto MNP-MSM agar plates. The colonies that appeared after incubation for 3 days were counted. Nitrite ion was determined by a Dionex model DX600 ion chromatography. In some experiments, the degradation of *p*-nitrophenol (PNP), 2,4-dinitrophenol, 2,5-dinitrophenol and 2,6-dinitrophenol was also investigated as described above.

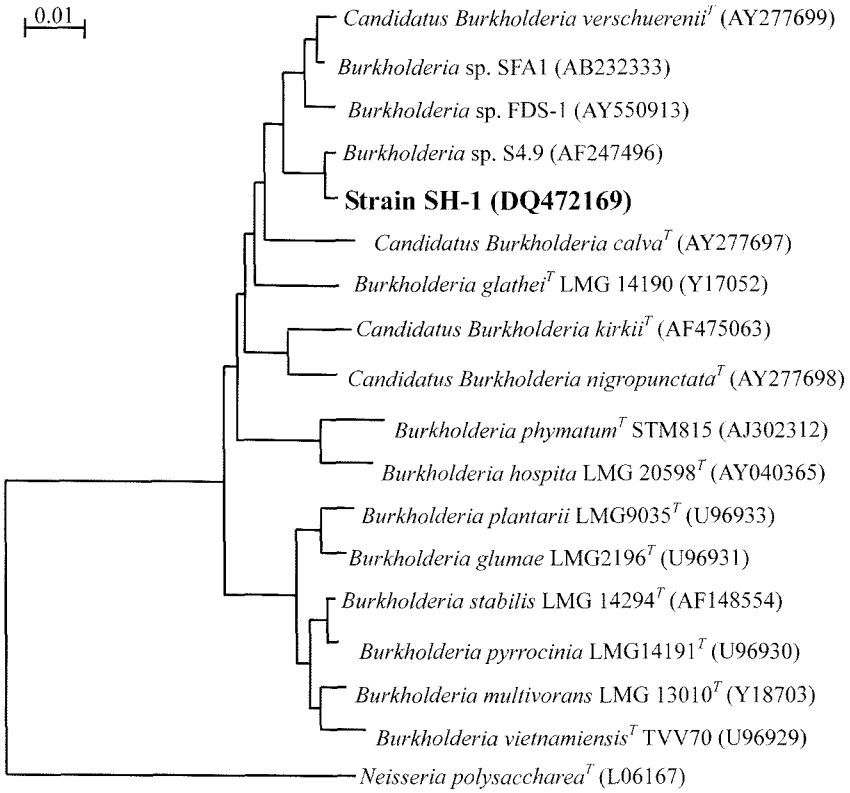

Fig. 1. Phylogenetic tree of *Burkholderia* sp. SH-1 based on 16S rRNA analyses.

Results

Bacterial identification. Five microorganisms capable of utilizing MNP as the sole carbon source were isolated from earthworm intestine. Among them, an isolate that showed the best degradation of MNP was designated as SH-1 and selected for further study. SH-1 was found not only in earthworm intestine, but also in the soil from which earthworms were obtained. The highest degree of similarity found, 99.49%, was obtained with the 16S rRNA genes of a phenanthrene- degrader *Burkholderia* sp. S4.9 (Friedrich *et al.*, 2000) with accession number AY247496 in the Genbank database (Fig. 1). SH-1 showed similarities of 98.18% to fenitrothion degraders *Burkholderia* sp. FDS-1 (Zhang *et al.*, 2006) and *Burkholderia* sp. SFA1 (Tago *et al.*, 2006). These observations indicate that SH-1 is a MNP degrader placed in the genus of *Burkholderia* and deposited in the Genbank database under accession number DQ472169.

Bacterial growth and MNP degradation. MNP degradation and bacterial growth were investigated concurrently in the same culture flasks during the experiment. Figure 2 shows the data for time-course MNP concentrations found in the cultures and growth of SH-1 during the incubation. When SH-1 was incubated with MNP as the sole carbon source, almost complete

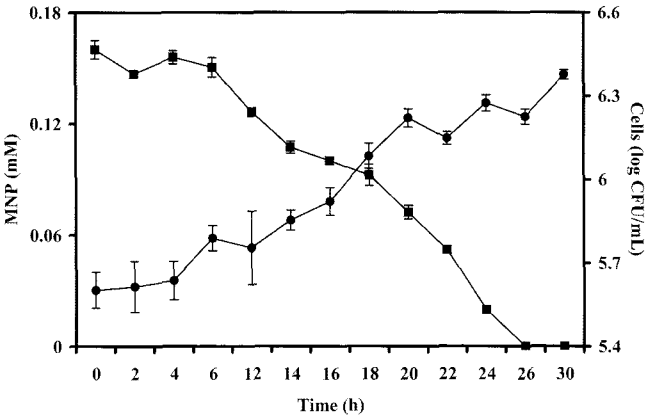

Fig. 2. Degradation of 3-methyl-4-nitrophenol by *Burkholderia* sp. SH-1 (■) and bacterial growth based on colony forming unit counting (●). The data given are the means of three measurements ± SD.

degradation of the added MNP was observed after 26 h of incubation. The yellow color of MNP gradually disappeared with the degradation of MNP. The disappearance of the yellow color was faster in cells incubated with MNP as sources of nitrogen and carbon than in cells incubated with MNP as the sole carbon source, suggesting that the removal of a nitro group from the chemical structure of MNP was correlated with the disappearance of the yellow

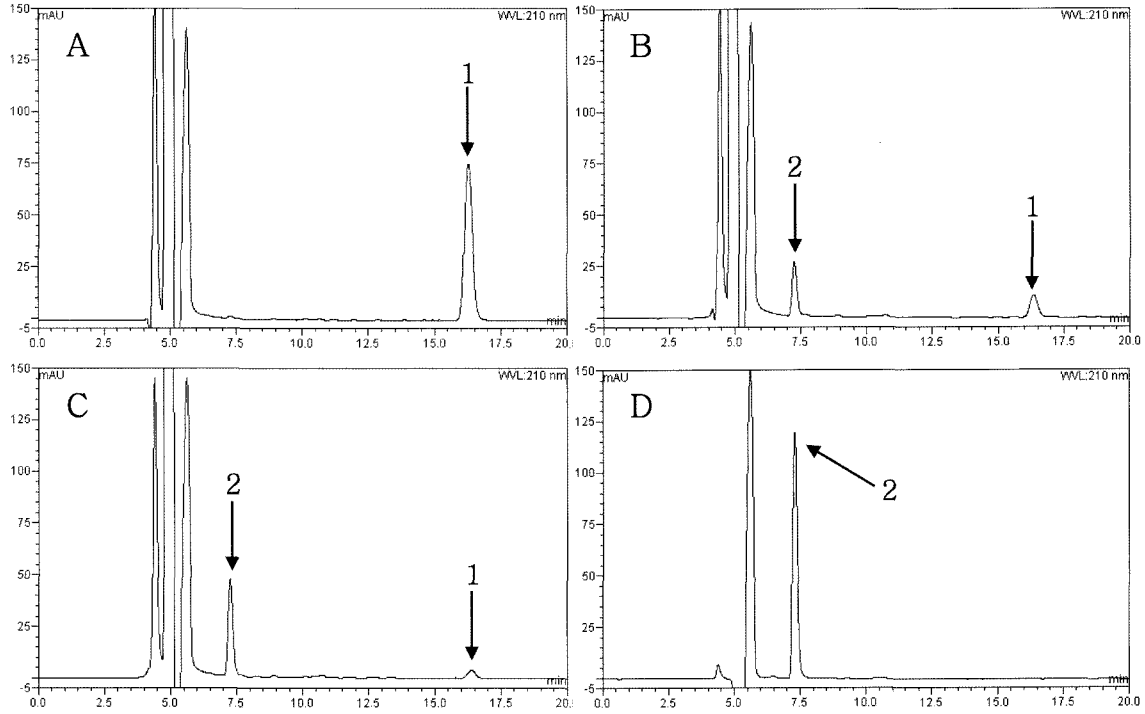

Fig. 3. Typical HPLC chromatograms of control samples (A) and the cultures incubated with 3-methyl-4-nitrophenol (MNP) as the sole carbon source for 24 h (B), the cultures incubated with MNP as sources of nitrogen and carbon for 24 h (C), and catechol authentic standard (D). The peaks 1 and 2 represent MNP and catechol, respectively.

color. The concentration of nitrite ion increased to maximum in 12 h of incubation and decreased steadily afterward (data not shown). MNP concentrations in the control samples without SH-1 remained constant. Negligible changes in the pH of the cultures were observed throughout the experiment. These observations indicate that abiotic degradation of MNP was negligible during the experiment. Colony forming unit (CFU) value assays demonstrated that SH-1 was capable of utilizing MNP as a carbon source. The growth of SH-1 was accompanied by the disappearance of MNP, giving a maximum growth in 20 h of incubation.

MNP was detected at retention time of 16.5 min on HPLC column (Fig. 3A). A peak at retention time of 7.3 min was detected in the cultures incubated with MNP (Fig. 3B and 3C), accompanying decrease of MNP peak at retention time of 16.5 min. The peak at retention time of 7.3 min was identified as catechol on the basis of HPLC analysis of catechol authentic standard (Fig. 3D). The data of Fig. 3C is for HPLC chromatogram of cells incubated with MNP as sources of nitrogen and carbon, which indicates SH-capable of utilizing MNP as a nitrogen source. Catechol started appearing after 6 h of incubation (0.01 mM), and after 8 h of incubation approximately 0.02 mM of catechol was detected. After 16 h of incubation, catechol increased up to a maximum of 0.07 mM with corresponding 50% degradation of the added MNP. Catechol concentration did not increase significantly after 16 h of incubation, giving 0.06 mM after 20 h of incubation. Catechol was not detected in the cultures incubated after 24 h of incubation, suggesting that SH-1 utilized catechol to grow.

Degradation of other nitroaromatics. Figure 4B and 4C show the data for SH-1 capable of degrading PNP when it is incubated with PNP as the nitrogen and carbon sources. PNP was detected in the cultures at retention time of 13.0 min. Catechol was detected as a major degradation product of PNP, as observed in the MNP degradation. The appearance of catechol was faster in cells incubated with PNP as the sources of nitrogen and carbon than in cells incubated with PNP as the sole carbon source. Negligible degradation was observed in cells incubated with *ortho*-substituted nitroaromatics such as 2,4-dinitrophenol, 2,5-dinitrophenol and 2,6-dinitrophenol (Table 1). These suggest a possibility that 4-monooxygeanse would involve in the degradation of MNP and PNP as demonstrated in other studies (Spain and Gibson, 1991; Oppenheim *et al.*, 2001). Based on the data presented in this study, the degradation pathway of MNP by *Burkholderia* sp. SH-1 is suggested as shown in Fig. 5.

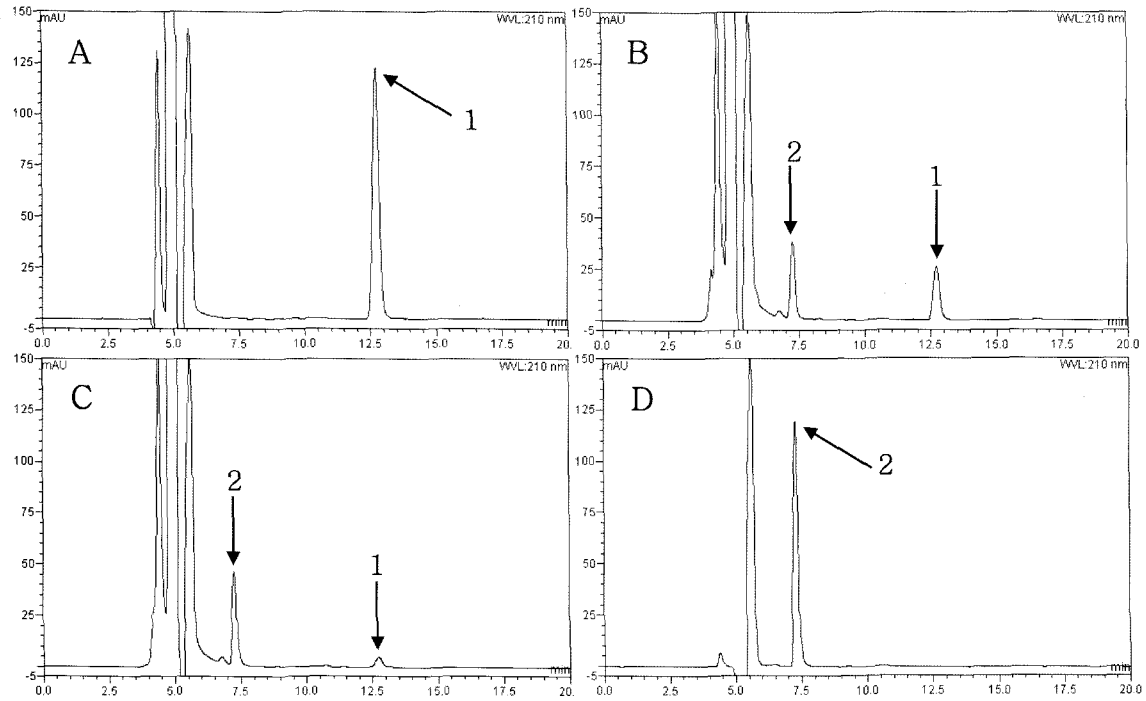

Fig. 4. Typical HPLC chromatograms of control samples (A) and the cultures incubated with *p*-nitrophenol (PNP) as the sole carbon source for 24 h (B), the cultures incubated with PNP as sources of nitrogen and carbon for 24 h (C), and catechol authentic standard (D). The peaks 1 and 2 represent PNP and catechol, respectively.

Table 1. Degradation of aromtics by *Burkholderia* sp. SH-1

Aromatics (0.4 mmol/L)	Degradation
3-Methyl-4-nitrophenol	Yes*
p-Nitrophenol	Yes
Catechol	Yes
Fenitrothion	No**
2,4-Dinitrophenol	No
2,5-Dinitrophenol	No
2,6-Dinitrophenol	No

*Yes: >90% degradation of the amount added.
**No: <5% degradation of the amount added.

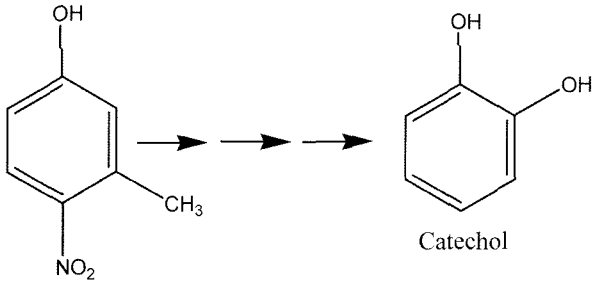

Fig. 5. Proposed degradation pathway of 3-methyl-4-nitrophenol by *Burkholderia* sp. SH-1.

Discussion

Fenitrothion is an organophosphorus insecticide that has been used extensively throughout world for controlling a wide range of pest insects since 1959. The extensive use of fenitrothion is contributing to soil and water contamination. In the soil treated with fenitrothion, the soil contamination is likely due to its degradation products rather than fenitrothion as this pesticide is degraded rapidly (Spillnet *et al.*, 1979; Mikami *et al.*, 1985). MNP is one of main degradation products of fenitrothion. Considering the possible adverse effects of MNP as an estrogenic chemical, studies related to the environmental fate of MNP are necessary. In the present study, we examined the degradation of MNP by *Burkholderia* sp. SH-1 isolated from earthworm intestine.

SH-1 belongs to the genus of *Burkholderia*, grouping with phenanthrene- and fenitrothion-degrading microorganisms (Fig. 1). A number of studies on microbial degradation of fenitrothion have demonstrated that fenitrothion-degrading *Burkholderia* strains could degrade MNP to methylhydroquinone (Hayatsu *et al.*, 2000; Zhang *et al.*, 2006). In the present study, however, methylhydroquinone was not detected as a degradation product of MNP, and instead, catechol was detected on the basis of data of HPLC analysis (Fig. 3). *Ralstonia* sp. SJ98 has been reported to degrade MNP via the

formation of catechol (Bhushan *et al.*, 2000) but the strain SJ98 is out of the phylogenetic tree of SH-1 (Fig. 1). SH-1 could also degrade PNP to catechol (Fig. 4). Most PNP-degrading microorganisms have been shown to degrade PNP to hydroquinone or 4-nitrocatechol (Spain and Gibson, 1991; Hanne *et al.*, 1993; Jain *et al.*, 1994; Leung *et al.*, 1997; Wan *et al.*, 2007) but these degradation products were not detected in our study. A study on fungal degradation of MNP showed that MNP was converted to hydroxylated metabolites and an aromatic amine condensation metabolite (Kanaly *et al.*, 2005) but these products were not detected in the present study. These data indicate that the isolate SH-1 is a new MNP degrader different from known fenitrothion degrader.

SH-1 could grow on MNP as sources of nitrogen and carbon. The removal of nitro group would be the first step in the degradation of MNP as nitrite ion was detected during the experiment. SH-1 would remove nitro and methyl groups to give catechol as the main degradation product. Bacterial mechanism for direct removal of methyl group from aromatics still remains unknown. Bhushan *et al.* (2000) has reported the direct removal of methyl group from MNP in the degradation of MNP. We performed TLC analyses, as described earlier [Spain and Gibson, 1991], to investigate whether the main degradation product detected in this study was catechol or not. However, it was not successful as Rf values of the authentic standards on TLC plate were not significantly different between them. We also further performed GC/MS analysis of cultural extracts to identify catechol. However, it was also not successful, since the concentration of degradation product found in the cultures was too low to detect due to its rapid degradation during the incubation. We do not rule out the possibility that other degradation products would be observed in the degradation of MNP. However, based on our limited data together with other study, SH-1 was suggested to degrade MNP via the formation of catechol as the main degradation product. Further study is required to characterize the degradation product in more detail.

Aromatics have been known to become bound residues when they are exposed to soil organics (Gevao *et al.*, 2000), which results in their limited biodegradation. MNP may be less accessible to biological degradation once it becomes bound residues. In such an environmental condition, earthworms can consume the MNP-bound soil organics, resulting in biodegradable product. A number of studies have demonstrated that the bound residues of environmental contaminants are bioavailable to earthworms (Morrison *et al.*, 2000; Gevao *et al.*, 2001). In the present study, SH-1 was found not only in the earthworm guts, but also in the fenitrothion-contaminated soil. This suggests that SH-1 may be dispersed into different soil environments with the aid of earthworms. The dispersal of MNP-degrading SH-1 is suggested to play a role in bioremediation of the soil contaminated with MNP.

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