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ORIGINAL RESEARCH PAPER

Reductive transformation of parathion and methyl parathion by *Bacillus* sp.

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Abstract Based on the results of phenotypic features, phylogenetic similarity of 16S rRNA gene sequences and BIOLOG test, a soil bacterium was identified as *Bacillus* sp. DM-1. Using either growing cells or a cell-free extract, it transformed parathion and methyl parathion to amino derivatives by reducing the nitro group. Pesticide transformation by a cell-free extract was specifically inhibited by three nitroreductase inhibitors, indicating the presence of nitroreductase activity. The nitroreductase activity was NAD(P)H-dependent, O_2-insensitive, and exhibited the substrate specificity for parathion and methyl parathion. Reductive transformation significantly decreased the toxicity of pesticides.

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Introduction

Organophosphorus pesticides are widely used worldwide to control agricultural and household pests. Overall, organophosphorus compounds account for ~38% of total pesticides used globally (Singh and Walker 2006). These compounds are highly toxic because they inhibit acetylcholinesterase in the central nervous system synapses, leading to a subsequent loss of nerve function and eventual death (Singh and Walker 2006). Due to the environmental concern associated with the accumulation of these compounds in soil and groundwater, there is a great need to develop safe, convenient, and economically feasible methods for their detoxification.

Biodegradation is a reliable, cost-effective technique for pesticide removal. At present a number of microorganisms, capable of degrading organophosphorus pesticides, have been isolated and characterized. Sethunathan and Yoshida (1973) isolated the first organophosphorus-degrading bacterium, a *Flavobacterium* sp., that could degrade parathion and diazinon. Both mineralization, where parathion was used as a source of carbon (Munnecke and Hsieh 1976;

Rani and Lalithakumari 1994) or phosphorus (Rosenberg and Alexander 1979), and co-metabolic hydrolysis (Serdar et al. 1982) have been reported. A *Pseudomonas* sp. was isolated that could co-metabolically degrade methyl parathion (Chaudry et al. 1988). Rani and Lalithakumari (1994) isolated *P. putida* that could hydrolyze methyl parathion and utilize *p*-nitrophenol as a source of energy.

Although in most of the studies on microbial degradation of parathion and methyl parathion, the first reaction was hydrolysis of the phosphotriester bond, there have been reports of different degradation pathways (Barton et al. 2004; Munnecke and Hsieh 1976). In this work, we report that *Bacillus* sp. DM-1 transforms parathion and methyl parathion to amino derivatives by reducing the nitro group. Moreover, we present the identification of the enzymatic system involved in the transformation.

Materials and methods

Pesticides

Parathion (*O*,*O*-diethyl-*O*-*p*-nitrophenyl phosphorothioate), and methyl parathion (*O*,*O*-dimethyl-*O*-*p*-nitrophenyl phosphorothioate), were obtained from Institute for the Control of Agrochemicals, Ministry of Agriculture, China. Stock solutions of 20 mg ml^{-1} were prepared in methanol.

Bacterial strain and culture conditions

Strain DM-1, isolated from organophosphate-polluted soil, was grown on the mineral salt (MS) medium (pH 7.0) containing 0.2 g K_2HPO_4 l^{-1}, 0.8 g KH_2PO_4 l^{-1}, 1 g $(NH_4)_2SO_4$ l^{-1}, 0.5 g $MgSO_4{\cdot}7H_2O$ l^{-1}, 0.05 g $CaCl_2$ l^{-1} and 0.01 g $FeSO_4$ l^{-1}. The MSG medium was prepared by adding 1 g glucose l^{-1} as a source of carbon.

Taxonomic identification

Strain DM-1 was initially identified using standard methods (Holt et al. 1994). For sequencing of the 16S rRNA gene, genomic DNA was prepared from strain DM-1 by a standard phenolic extraction procedure. The 16S rRNA gene was amplified by PCR using the universal primers, 8f (5′-CACGGATCCAGACTTTGATYMTGGCTCAG-3′, forward) and 1512r (5′-GTGAAGCTTACGGYTAGCTTGTTACGACTT-3′, reverse) (Weisburg et al. 1991). The PCR reaction was performed in a Perkin-Elmer PE9600 thermocycler with the following cycling profile: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min, final extension at 72°C for 8 min. The PCR product was cloned into a pMD18-T vector (TaKaRa) and sequenced. The determined sequence was compared with those available in the GenBank database using the NCBI Blast program.

In order to test the ability of the isolate to utilize (oxidize) various carbon sources in a short time, the BIOLOG bacterial identification test kit was selected (Biolog Inc., Hayward), and the procedures were followed with commercial protocol.

Identification of products of pesticide transformation

For all experiments, 10^6 cells ml^{-1} were used and samples were incubated on MSG medium with 200 mg parathion or methyl parathion l^{-1} at 30°C on a shaker at 200 rpm unless otherwise stated. Uninoculated medium was used as a control. After incubation for 24 h, the culture was extracted thrice with equal volumes of ethyl acetate. The organic phases were pooled, diluted properly and dried over anhydrous Na_2SO_4.

The organic extract was analyzed by GC with a flame photometric detector with a 525 nm filter for specific phosphorus readout using a DB-5 capillary column (J&W Scientific, CA). The oven was at 60°C for 2 min, and then programmed to 210°C at a rate of 20°C min^{-1} and held for 2 min. Pesticide transformation was estimated as the peak disappearance and quantified using a standard curve.

The GC-MS analyses were carried out in a Agilent Technologies GC (model 6890 N) coupled to a mass spectrometry detector (model 5973 N). The analytes were ionized in EI mode and separated on a DB-5MS capillary column (J&W Scientific, CA). The oven was at 60°C for 2 min,

and then programmed to 280°C at a rate of 20°C min^{-1} and held for 2 min.

Experiments with resting cells

Fully induced cells were obtained by pre-culture with 200 mg methyl parathion or parathion l^{-1} for 24 h. The collected cells were washed twice with 50 mM phosphate buffer pH 7.0, resuspended in the same buffer, and incubated with 200 mg methyl parathion or parathion l^{-1} at 30°C for 24 h. Samples boiled for 10 min were used as controls.

Enzyme assays

Cell-free extracts were prepared by resuspending the bacterial pellets (~0.1 g wetwt/ml) in ice-cold 50 mM phosphate buffer, pH 7.0 and disrupting by sonication in an ice-water bath for 5 s with 15 s intervals, after which cell debris was removed by centrifugation at 12,000*g* for 30 min at 4°C. The supernatant was used as cell-free extract. Protein concentrations were determined according to the Bradford method (Bio-Rad) using BSA as the standard.

Nitroreductase activity was assayed using the decrease at 340 nm due to the oxidation of NAD(P)H. The reaction mixture contained 0.2 μmol methyl parathion (or parathion), 0.2 μmol NAD(P)H, 50 μmol phosphate buffer pH 7.0, and cell-free extract (0.2–0.4 mg protein) in 1 ml. Reactions were initiated by the addition of methyl parathion (or parathion). Samples boiled for 10 min were used as controls. One unit is defined as a decrease of 1 μmol NAD(P)H per min at 30°C. GC-MS analyses of the transformation products were performed as described above. Pesticide transformations by cell-free extract of strain DM-1 were assayed in the presence of nitroreductase inhibitors (50 μM benzoate, dicoumarol or diphenyliodonium).

Acetylcholinesterase inhibition

The toxicities of the pesticides and their products after transformation were estimated as the inhibition of acetylcholinesterase activity. The acetylcholinesterase activity was determined using the Michel method (Hawkins and Knittle 1972) in an assay buffer containing 20 μg acetylcholinesterase ml^{-1} with or without 1 μM pesticide. One unit is defined as 0.1 ΔpH/min at 30°C. After pesticide was completely transformed by cell-free extract, the reaction mixture was lyophilized and a portion, equivalent to 1 μM in substrate basis, was added to the acetylcholinesterase reaction mixture.

Nucleotide sequence accession number

The 16S rRNA gene sequence of strain DM-1 has been deposited in the GenBank database under Accession No. DQ201643.

Results

Characterization and identification of strain DM-1

Strain DM-1 was a Gram-positive, catalase-positive, oxidase-positive, and spore-forming rod. It was positive in tests for glucose fermentation, citrate utilization, gelatin hydrolysis, and casein hydrolysis but negative for starch hydrolysis, urea hydrolysis, H_2S production, and nitrate reduction. The sequence of 1514 bp of the 16S rRNA gene of strain DM-1 was 99% identical to that of the 16S rRNA gene of *Bacillus subtilis* YJ001 (GenBank Accession No. DQ444283), 99% similar to that of the 16S rRNA gene of *Bacillus subtilis* MA139 (GenBank Accession No. DQ415893), and 99% similar to that of the 16S rRNA gene of *Bacillus subtilis* B1144 (GenBank Accession No. AB232386). The substrate utilization of strain DM-1 was compared with that of referred strains in the BIOLOG-GP database and strain DM-1 had the greatest similarity index of 0.68 with *Bacillus subtilis*. Based on these observations, the isolate was putatively identified as *Bacillus* sp. DM-1.

Reductive transformation of parathion and methyl parathion by strain DM-1

In the cultures of strain DM-1, the chromatographic peak area corresponding to methyl parathion [retention time (RT) 20.34 min] gradually

decreased over a period of 30 h and gave rise to a new peak with RT 19.64 min. Mass spectrometric analysis identified the peak as amino-methylparathion. During incubation of growing cells with parathion, a new peak with RT 18.85 min appeared, which was identified as amino-parathion according to mass spectral properties. Similarly, parathion and methyl parathion were also reduced by resting cells of strain DM-1. Pesticide concentration in controls did not change, and no new peaks appeared.

The transformation products from cell-free extract of strain DM-1 were isolated and their structures were determined by GC-MS. Compared to the controls, samples incubated with parathion or methyl parathion gave rise to a new peak with RT 18.85 or 19.64 min, respectively, which were identified as amino derivatives of pesticides according to mass spectral properties. No such conversion was observed without NAD(P)H in the reaction mixture. Reductive transformation by cell-free extract required NADH or NADPH as a cofactor, suggesting that nitroreductase activity may be involved.

Characterization of nitroreductase activity

The nitroreductase activity was found in strain DM-1 grown on media containing parathion and methyl parathion but not when nitroaromatic compounds (e.g. *p*-nitrophenol or *p*-nitrobenzoate) were substituted for these pesticides in the media. Under anaerobic conditions, the nitroreductase activities, ranged from 64 ± 3.6 to 94 ± 2.5 U/g protein, were found using parathion and methyl parathion as substrates. In contrast, approximately 90% of the activity was observed in the presence of O_2. Cell-free extract showed almost the same activities with NADH and NADPH (Table 1).

Inhibition experiments were performed upon cell-free extract transformation of pesticides. Benzoate, dicoumarol and diphenyliodonium, all known nitroreductase inhibitors (Koder and Miller 1998), were used. Each of the three inhibitors was able to stop the transformation of pesticides by cell-free extract (Table 2), indicating the presence of nitroreductase activity.

Estimation of the toxicities of transformation products

Toxicity before and after cell-free extract transformation of pesticides was determined as the inhibition of the acetylcholinesterase activity. The cell-free extract transformation of pesticides significantly decreased their capacity to inhibit acetylcholinesterase activity (Fig. 1). The decrease in the toxicity of pesticides by reductive reactions was also reported (Sethunathan and Yoshida 1973).

Discussion

Nitro-group reduction by various microorganisms, which predominantly concentrates on transformation of nitro groups of trinitrotoluene, has been well documented (Esteve-Núñez et al. 2001; Goronzy et al. 1994; Spain 1995). Microbial degradation of parathion was shown to occur through the following three pathways (Munnecke and Hsieh 1976): firstly, formation of *p*-nitrophenol via hydrolysis of the phosphotriester bond; secondly, reduction of the nitro group under low O_2 condition and then hydrolysis to yield *p*-aminophenol; thirdly, conversion of parathion to paraoxon before hydrolysis of phosphotriester bond. More recently, aerobic reduction of the nitro group of methyl parathion was found in a cyanobacterium *Anabaena* sp. (Barton et al. 2004). Nevertheless, the process of methyl parathion transformation occurred in the light, but not in the dark. Here, we report a transformation pathway that has not been previously described: aerobic reduction of the nitro group(s) of parathion and methyl parathion by a non-phototrophic bacterium (Fig. 2). Many heterotrophic microorganisms have different systems for nitro-group reduction of nitroaromatic compounds, including nitroreductase (Goronzy et al. 1994; Spain 1995). The reduction of the nitro group(s) to a hydroxylamino or amino group(s) by nitroreductases has been addressed (Esteve-Núñez et al. 2001; Goronzy et al. 1994; Spain 1995).

Pesticide transformation by cell-free extract of strain DM-1 required the addition of NAD(P)H. Under anaerobic conditions, the decrease in the

Table 1 Alternative substrates for nitroreductase activities from strain DM-1

Substrate	Nitroreductase activity (U/g protein)[a]			
	Air		N_2	
	NADH	NADPH	NADH	NADPH
Methyl parathion	84.2 ± 3.5	82.6 ± 4.7	94.6 ± 2.5	93.4 ± 2.3
Parathion	58.1 ± 2.1	57.5 ± 4.1	66.4 ± 3.4	64.3 ± 3.6

[a] Cells were pre-cultured at 30°C for 24 h in MSG medium containing 200 mg methyl parathion l^{-1}. Then, the cells were harvested, disrupted, and the nitroreductase activities were measured by pesticides as substrates with NADH or NADPH as cofactor. No activity under any condition was observed with either *p*-nitrophenol or *p*-nitrobenzoate. The measurements under anaerobic conditions were conducted in 1 ml rubber-stoppered cuvettes flushed with N_2. Data are the mean ± SD from three independent experiments

Table 2 Effect of nitroreductase inhibitors on the nitroreductase activities from strain DM-1

Inhibitor	Nitroreductase activity (U/g protein)[a]			
	Methyl parathion		Parathion	
	NADH	NADPH	NADH	NADPH
None	92.5 ± 3.1	94.1 ± 3.4	65.6 ± 2.3	63.7 ± 2.1
Benzoate (50 μM)	2.2 ± 0.3	2.0 ± 0.2	2.1 ± 0.2	2.1 ± 0.1
Dicoumarol (50 μM)	1.2 ± 0.1	1.4 ± 0.2	1.3 ± 0.1	1.3 ± 0.2
Diphenyliodonium (50 μM)	2.4 ± 0.3	2.1 ± 0.3	2.1 ± 0.2	2.2 ± 0.3

[a] Under anaerobic conditions, the nitroreductase activities were assayed by either methyl parathion or parathion with or without nitroreductase inhibitor. Data are the mean ± SD from three independent experiments

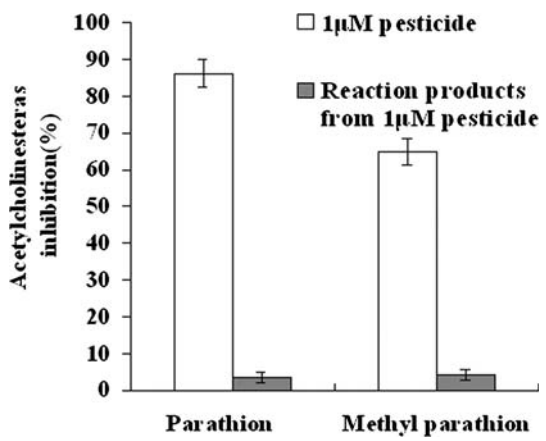

Fig. 1 Acetylcholinesterase inhibition by 1 μM pesticides and their equivalent products from cell-free extract transformation. Acetylcholinesterase activity is calculated as 8.6 U/mg without pesticides. Data are the mean ± SD from three independent experiments

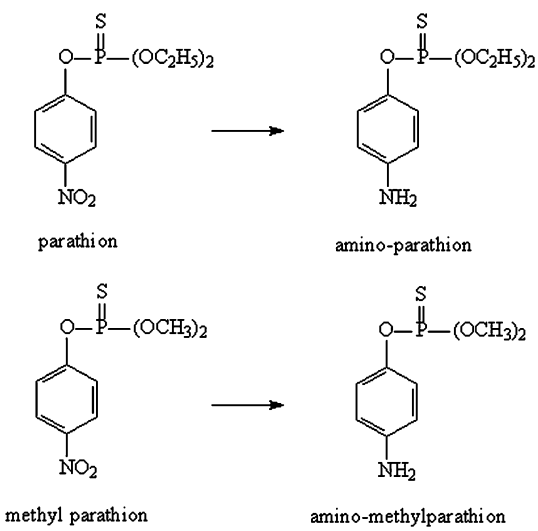

Fig. 2 Proposed pathway of the transformation of parathion and methyl parathion by *Bacillus* sp. DM-1

absorbance at 340 nm was observed, which indicated that the nitro-group reduction was coupled to NAD(P)H oxidation. NAD(P)H was able to act as a cofactor in the reaction, and no NAD(P)H oxidation was detected in the absence of pesticide. Our findings differ from previous studies with O_2-insensitive nitroreductase (NfsA) in *E. coli* utilizing NADPH (Bryant et al. 1981;

Zenno et al. 1996a) but were in accordance with the known cofactor specificity of NfsB with NADH or NADPH (Zenno et al. 1996b). Small negatively charged organic molecules such as acetate and benzoate are inhibitors of nitroreductase (Koder and Miller 1998). These compounds inhibit nitroreductases either by competing with the nitro groups for enzyme binding sites or by binding to NAD(P)H. Three nitroreductase inhibitors (benzoate, dicoumarol and diphenyliodonium) specifically inhibited the transformation of parathion and methyl parathion by cell-free extract of strain DM-1, indicating the presence of nitroreductase activity. In eukaryotic systems, nitroreductase activity has been associated with cytochrome P450 enzymatic system (Spain 1995). No inhibitory effects were observed by cytochrome P450 inhibitors (Bossche and Koymans 1998): miconazole, 1-aminobenzotriazole and metyrapone.

O_2-insensitive nitroreductases have been found and studied in various enterobacteria (e.g. *E. coli* and *Enterobacter cloacae*) (Bryant et al. 1981; Koder and Miller 1998) but also in other bacterial strains, such as *Bacillus subtilis* (Zenno et al. 1998). The evidence that approximately 90% of the activity is still retained in the presence of O_2 suggests that an O_2-insensitive nitroreductase may be involved in the nitro-group reduction of parathion and methyl parathion.

The nitroreductase activity of strain DM-1 could be induced by either parathion or methyl parathion but not in the absence of pesticide, indicating that these pesticides were metabolized by an induced enzyme system. The activity could utilize parathion and methyl parathion as substrates but not nitroaromatic compounds. However, work with purified enzyme is required to determine the correct substrate specificity and the reaction mechanisms. The ability of strain DM-1 to transform parathion and methyl parathion could be attributed to their similarity in chemical structure. Bacterial metabolism of structurally similar organophosphorus pesticides might result in the formation of common or similar metabolites.

Parathion and methyl parathion are potent acetylcholinesterase inhibitors. The risk of physiological damage in non-target organisms caused by these pesticides is extremely high since acetylcholinesterase is present in all vertebrates, including humans. Reductive reactions catalyzed by soil and water microflora have been of significance in diminishing toxicity of some organophosphorus pesticides, such as parathion (Sethunathan and Yoshida 1973). Reductive transformation of parathion and methyl parathion significantly decreased the toxicity of pesticides as estimated by their capacity to inhibit acetylcholinesterase activity. The application of this strain in bioremediation technologies is currently under investigation.

In summary, we found a reductive pathway for parathion and methyl parathion in *Bacillus* sp. DM-1 and a nitroreductase activity responsible for the transformation. Purification and characterization of the nitroreductase in strain DM-1 represent areas for further investigation.

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