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Isolation of a chlorpyrifos-degrading bacterium, *Sphingomonas* sp. strain Dsp-2, and cloning of the *mpd* gene

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Abstract

A highly effective chlorpyrifos-degrading bacterium strain Dsp-2 was isolated from the polluted treatment system of a chlorpyrifos manufacturer. This strain was preliminarily identified as *Sphingomonas* sp. based on its morphological, physiological and biochemical tests as well as 16S rDNA analysis. It utilized chlorpyrifos as its sole source of carbon for growth, by hydrolyzing chlorpyrifos to 3,5,6-trichloro-2-pyridinol (TCP). It could also utilize parathion, parathion-methyl, fenitrothion and profenofos, but not phoxin and triazophos. Bioremediation of chlorpyrifos-contaminated soil was examined using Dsp-2. Dsp-2 addition to soil treated with 100 mg kg^{-1} chlorpyrifos resulted in a higher degradation rate than control soils without inoculation. The moderate pH, moisture and inoculum density could have promoted degradation. The gene encoding the chlorpyrifos hydrolytic enzyme was cloned by PCR. Although BLAST sequence search results indicated that this gene has 99% similarity to *mpd* (a gene encoding the parathion-methyl hydrolyzing enzyme in *Plesiomonas* sp. M6), its hydrolytic efficiency for chlorpyrifos was significantly greater than the wild-type *mpd* from strain M6.

Keywords: Chlorpyrifos; Biodegradation; *Sphingomonas* sp.; *mpd* gene

1. Introduction

Chlorpyrifos [*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate] has been widely used since the 1960s [5], particularly for the control of foliar insects in cotton fields, paddy fields and pastures, and on vegetable crops. Its environmental fate has been extensively studied, and its half-life in soil varies from 10 to 120 days [11,25] resulting in 3,5,6-trichloro-2-pyridinol (TCP) as the major degradation product. However, few studies have been conducted on the microbial degradation of chlorpyrifos. Although it is mid-toxic and has low-residual when applied to plants or mixed with soil, it produces hazardous environmental effects. TCP, the main degradation product of chlorpyrifos, is moderately mobile due to its greater water solubility, which results in widespread contamination of soils and aquatic environment [18]. Currently, two effective degrading strains, *Enterobacter* strain B-14 and *Alcaligenes faecalis* strain DSP3, have been isolated separately by Singh et al. [28] and Yang et al. [37], but the gene encoding the key degrading enzyme has not been reported.

Previous research had shown that the hydrolysis rates of OPH (an organophosphorus hydrolase isolated from *Pseudomonas diminuta* MG and *Flavobacterium* sp. strain ATCC27551) differ dramatically for members of the family of organophosphorus compounds, ranging from hydrolysis at the diffusion-controlled limit for paraoxon, to several orders of magnitude slower for malathion, chlorpyrifos, and VX (a nerve toxicant) [9,12]. Cho et al. [6] demonstrated that wild-type OPH had minimal degradatory activity for chlorpyrifos, and directed evolution was used to improve the hydrolysis rate of OPH toward a very poorly hydrolyzed substrate. The *mpd* gene, which is completely different from the *opd* gene,

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was cloned by Cui et al. [7]. Several organophosphorus compounds can be hydrolyzed by the *mpd*-encoded MPH enzyme, including parathion, parathion-methyl and fenitrothion. Chlorpyrifos has a similar chemical structure to parathion-methyl. Thus, it seems reasonable that the enzymes degrading these chemicals may likewise have structural homology.

In the present study, we isolated a chlorpyrifos-degrading bacterium, *Sphingomonas* sp. strain Dsp-2. Degradation of chlorpyrifos by strain Dsp-2 in liquid culture and in soils was studied. Factors that govern chlorpyrifos degradation were also examined, with the goal of elucidating a possible application in chlorpyrifos-contaminated environmental remediation. The gene encoding the chlorpyrifos hydrolytic enzyme was cloned from Dsp-2 using a PCR-based technique and used to study the difference between *mpd* from strain Dsp-2 and *Plesiomonas* sp. M6. Importantly, this is the first report of cloning the *mpd* gene from the genus *Sphingomonas*.

2. Materials and methods

2.1. Chemicals and media

Analytical-grade chlorpyrifos, 3,5,6-trichloro-2-pyridinol (TCP), parathion, parathion-methyl, triazophos, phoxim, fenitrothion and profenofos (>99% purity) were obtained from the Pesticide Research Institute, Shenyang, China. All other reagents used in this study were of analytical grade.

Luria–Bertani (LB) medium and mineral salts medium (MSM) that contained (in grams per liter) 1.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 1.5 g NH_4NO_3, were used for the isolation of bacterial strains. The initial pH was adjusted to 7.0. When necessary, chlorpyrifos (100 mg l^{-1}) dissolved in ethanol (Dsp-2 cannot use ethanol as carbon source for its growth) was added to the medium.

2.2. Isolation and characterization

A polluted water sample was collected from an industry that has manufactured chlorpyrifos for more than 10 years in Nantong, China. Dilutions of the water were plated onto mineral salt agar plates containing 100 mg l^{-1} chlorpyrifos as a carbon source. Bacterial colonies that developed transparent zones, indicating degradation of chlorpyrifos, were isolated and purified. One strain, designated Dsp-2, which possessed the highest degradation capacity, was selected for further investigation.

Strain Dsp-2 was identified based on morphological, physiological and biochemical tests with reference to *Bergey's Manual of Determinative Bacteriology* combined with 16S rDNA sequence analysis. Total genomic DNA was prepared from strain Dsp-2 by high-salt precipitation [19]. 16S rDNA was amplified with the universal primers: 5′-AGAGTTT GATCCTGGCTCAG-3′ (*Escherichia coli* bases 8–27) and 5′-TACCTTGTTACGACTT-3′ (*E. coli* bases 1507–1492). The purified PCR product was sequenced from both directions using an ABI 377 sequencer (Perkin–Elmer Applied Biosystems, Foster City, California). The nucleotide sequence coding for 16S rDNA of strain Dsp-2 (1453 bp) was deposited in the GenBank database under accession number AY994060. Alignment of different 16S rDNA sequences from GenBank was performed using Clustal X 1.8.3 [35] with default settings. Phylogeny was analyzed with MEGA version 3.0 software and distance was calculated using the Kimura 2 parameter distance model. Unrooted trees were built using the neighbor joining method [29]. Each dataset was bootstrapped 1000 times.

2.3. Chemical analysis

Remaining chlorpyrifos in the cultures was extracted with an equal volume of trichloromethane. The extracts were then dried over anhydrous Na_2SO_4 and evaporated under reduced pressure using a centrifugal evaporator at room temperature. Residual organic material was redissolved in an equal volume of methanol. Soil samples were extracted with methanol, and all samples were analyzed by high-performance liquid chromatography (HPLC, 600 Controller, Rheodyne 7725i Manual injector and 2487 Dual λ Absorbance Detector; Waters Co., Milford, MA). The separation column used for HPLC (internal diameter, 4.6 mm; length, 25 cm) was filled with Kromasil 100-5C18. The mobile phase was methanol: water (85:15, *v*:*v*), and the flow rate was 1.0 ml min^{-1}. Chlorpyrifos and TCP were detected at 230 and 320 nm, respectively.

2.4. Inoculum preparation for degradation studies

Dsp-2 cells were pre-cultured in LB medium, harvested by centrifugation at 6000×*g* for 5 min and washed three times with sterilized water. For all experiments, cells were used at a concentration of 10^8 cells ml^{-1} and samples were incubated at 30 °C on a shaker at 150 rpm unless otherwise stated.

2.5. Degradation of chlorpyrifos by Dsp-2 in cell culture and soil

MSM medium was used for growth of degrading cultures. Cultures were regularly checked for bacterial growth, degradation of chlorpyrifos and accumulation of TCP. Medium without inoculation was maintained and tested in the same manner as above as controls. A soil (pH 6.3 with 67% sand, 12% silt and 15% clay, 12% organic matter) from a cottage in Nanjing, China was used for this study. Soil samples (2 kg) were sterilized as described by Zhang et al. [38]. Subsamples (100 g) of fumigated and non-fumigated soil were treated under aseptic conditions with chlorpyrifos (100 mg kg^{-1}), respectively. Three sets of fumigated soil and non-fumigated soil were inoculated with chlorpyrifos-degrading bacterium (10^8 cells g^{-1}). Another set of soil without inoculation was kept as a control. The inoculum was thoroughly mixed into the soils under sterile conditions. The moisture of the soil was adjusted to 40% water-holding capability and incubated in the dark [30].