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Biodegradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol by *Cupriavidus* sp. DT-1

Peng Lu [a,1], Qinfen Li [b,1], Hongming Liu [a], Zhaozhong Feng [a], Xin Yan [a], Qing Hong [a,c,*], Shunpeng Li [a]

[a] Key Laboratory of Agricultural Environmental Microbiology, Ministry of Agriculture, College of Life Science, Nanjing Agriculture University, Nanjing 210095, People's Republic of China
[b] Environment and Plant Protection Institute, Chinese Academy of Tropical Agricultural Sciences, Danzhou 571737, People's Republic of China
[c] State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, People's Republic of China

HIGHLIGHTS

- A chlorpyrifos- and 3,5,6-trichloro-2-pyridinol (TCP)-degrading strain (DT-1) belonging to the genus *Cupriavidus* was isolated.
- The biodegradation pathway of TCP was examined.
- The *mpd* gene encoding an organophosphorus hydrolase was cloned.
- Strain DT-1 promotes the degradation of chlorpyrifos and TCP in soil.
- Strain DT-1 is a good candidate for studying the degradation mechanism of TCP.

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ABSTRACT

A bacterial strain, *Cupriavidus* sp. DT-1, capable of degrading chlorpyrifos and 3,5,6-trichloro-2-pyridinol (TCP) and using these compounds as sole carbon source was isolated and characterized. Investigation of the degradation pathway showed that chlorpyrifos was first hydrolyzed to TCP, successively dechlorinated to 2-pyridinol, and then subjected to the cleavage of the pyridine ring and further degradation. The *mpd* gene, encoding the enzyme responsible for chlorpyrifos hydrolysis to TCP, was cloned and expressed in *Escherichia coli* BL21. Inoculation of chlorpyrifos-contaminated soil with strain DT-1 resulted in a degradation rate of chlorpyrifos and TCP of 100% and 94.3%, respectively as compared to a rate of 28.2% and 19.9% in uninoculated soil. This finding suggests that strain DT-1 has potential for use in bioremediation of chlorpyrifos-contaminated environments.

1. Introduction

Chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is a broad-spectrum, moderately toxic organophosphate insecticide that is increasingly being used in agriculture because of the restrictions imposed on highly toxic organophosphate compounds (Tomlin and Council, 1994; McConnell et al., 1997). The insecticide inhibits acetyl cholinesterase in an irreversible manner and causes insect death (Karalliedde and Senanayake, 1989). The potential damage by chlorpyrifos to non-target organisms is high because acetyl cholinesterase is present in all vertebrates (Sogorb et al., 2004). The half-life of chlorpyrifos in soil depends on soil type, climate, and other environmental factors. It is generally between 60 and 120 days, but can range from 2 weeks to over 1 year (Howard, 1991). Initially, it was observed to be hydrolyzed to TCP under alkaline conditions (Racke et al., 1996), but later, the involvement of microorganisms in the hydrolysis of chlorpyrifos was described (Singh et al., 2003).

TCP, a charged molecule at neutral pH, is mobile in soil as well as leachable into groundwater and surface water and thus can widely contaminate soil and aquatic environments (Manclus and Montoya, 1995). TCP is listed as a persistent and mobile pollutant by the US Environmental Protection Agency (Armbrust, 2001). TCP shows relatively high antimicrobial effects on microorganisms (Feng et al., 1997; Cáceres et al., 2007), which prevents its own degradation by microorganisms and also limits chlorpyrifos degradation.

Bioremediation has received increasing attention as a reliable and cost-effective approach to cleaning up polluted environments.

* Corresponding author at: Key Laboratory of Microbiological Agricultural Environment, Ministry of Agriculture, College of Life Science, Nanjing Agriculture University, Nanjing 210095, People's Republic of China. Tel.: +86 25 84396685; fax: +86 25 84396314.
E-mail address: hongqing@njau.edu.cn (Q. Hong).
[1] These authors are contributed equally to this work.

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To date, several microorganisms capable of degrading chlorpyrifos have been isolated from different genera. Such microorganisms include *Enterobacter* strain B-14 (Singh et al., 2004), *Alcaligenes faecalis* DSP3 (Yang et al., 2005), *Stenotrophomonas* sp. YC-1 (Yang et al., 2006), *Sphingomonas* sp. Dsp-2 (Li et al., 2007), *Paracoccus* sp. TRP (Xu et al., 2008), *Bacillus pumilus* C2A1 (Anwar et al., 2009), *Synechocystis* sp. Strain PUPCCC 64 (Singh et al., 2011), *Pseudomonas* Iso 1–4, *Agrobacterium* Iso 5–6, and *Bacillus* Iso 7 (Maya et al., 2011). Except for *Enterobacter* strain B-14, *Stenotrophomonas* sp. YC-1 and *Synechocystis* sp. strain PUPCCC 64, these strains can hydrolyze chlorpyrifos to TCP and further degrade it. However, the degrading pathway of TCP has not been elucidated. In the present study, *Cupriavidus* sp. DT-1 capable of degrading chlorpyrifos and TCP was isolated. The degradation pathway of the strain and the related gene were investigated. A pilot study on the application of strain DT-1 for bioremediation was also carried out.

2. Methods

2.1. Chemicals and media

Chlorpyrifos (98% purity) and TCP (99% purity) were purchased from the Pesticide Research Institute (Shenyang, China). 5, 6-Dichloro-2-pyridinol (98% purity), 6-chloro-2-pyridinol (98% purity) and 2-pyridinol (99% purity) were purchased from J&K Scientific Ltd. (Shanghai, China). All other reagents were of the highest analytical-reagent grade and obtained from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China).

The mineral salts medium (MSM) contained (g/L): NH_4NO_3, 1.0; K_2HPO_4, 1.5; KH_2PO_4, 0.5; NaCl, 0.5; $MgSO_4$, 0.2; pH 7.0. Concentrated stock solutions of chlorpyrifos (10 g/L) and TCP (10 g/L) were prepared in dimethyl sulfoxide and sterilized by membrane filtration using a pore size of 0.22 μm. The solutions were added to sterilized MSM and used as the carbon source when required. Strain DT-1 was not capable of utilizing dimethyl sulfoxide as sole carbon source for growth in MSM medium.

Luria–Bertani (LB) medium contained (g/L): tryptone 10.0, yeast extract 5.0 and NaCl 10.0, pH 7.0.

2.2. Isolation and characterization of chlorpyrifos-degradingstrain

A 5-g sludge sample collected from a chlorpyrifos manufacturing site in Changzhou, Jiangsu Province, China (E119°58′ N31°47′) was inoculated into MSM medium amended with 100 mg/L chlorpyrifos. The culture was incubated at 30 °C at 180 rpm for 5 d. One milliliter of enrichment culture was subcultured into fresh MSM amended with 100 mg/L of chlorpyrifos for 5 d. High-performance liquid chromatography (HPLC) was used to determine chlorpyrifos and confirm degradation. The enrichment culture was serially diluted and spread on MSM plates containing 100 mg/L chlorpyrifos. After 3 d of incubation at 30 °C, colonies with clear haloes were purified and tested for their ability to degrade chlorpyrifos. Out of the two isolates, strain DT-1 was selected for further study due to its ability to degrade both chlorpyrifos and TCP.

Strain DT-1 was identified according to the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and sequence analysis of its 16S rRNA gene. Genomic DNA was extracted by the high salt concentration precipitation method (Miller et al., 1988). The 16S rRNA gene was amplified by PCR using standard procedures (Lane, 1991). The PCR product was ligated into vector pMD18-T (TaKaRa Biotechnology, Dalian, China) and transformed into *Escherichia coli* DH5α. An automatic sequencer (Applied Biosystems; 3730) was used to determine the 16S rRNA gene sequence. The 1490-bp 16S rRNA gene sequence was deposited in GenBank under Accession No. JQ750642. Alignment of 16S rRNA gene sequences from the GenBank database was performed using ClustalX 1.8.3 with default settings (Thompson et al., 1997). Phylogenesis was analyzed by MEGA, version 3.0. Distances were calculated using the Kimura two-parameter distance model. An unrooted tree was built by the neighbor joining method. The dataset was bootstrapped 1000 times (Weisburg et al., 1991).

2.3. Inoculum preparation for degradation studies

Strain DT-1 was cultured in LB medium to exponential phase, and then collected by centrifugation at 5000*g* for 5 min at room temperature. The cell pellets were washed twice with sterilized MSM and adjusted to approximately 2×10^8 CFU/mL. For the degradation experiments, the cells were inoculated to approximately 2×10^6 CFU/mL and incubated at 30 °C on a shaker (180 rpm) unless otherwise stated.

2.4. Degradation of chlorpyrifos and TCP by strain DT-1 in liquid culture

Degradation of chlorpyrifos was carried out in 100 mL MSM containing 100 mg/L chlorpyrifos. Different initial concentrations of TCP (50–100 mg/L) in MSM were used to investigate the effect of concentration on degradation rate and cell growth. Cultures were regularly sampled for the determination of the concentrations of chlorpyrifos and TCP and cell growth.

2.5. Degradation of chlorpyrifos and TCP by strain DT-1 in soil

Soil was collected from the campus of the Nanjing Agricultural University, Nanjing, China. The soil was sandy loam with 65% sand, 11% silt, and 16% clay. The soil contained 2.27% organic matter, 0.128% nitrogen, 0.095% phosphorus, 2.23 cmol/kg Ca^{2+}, 2.04 cmol/kg Mg^{2+}, 0.13 cmol/kg K^{2+}, and 0.17 cmol/kg Na^{2+}, and had a pH of 6.7.The soil was air dried, sieved to 2 mm, and homogenized. Glass beaker (200 mL) microcosms, each containing 100 g soil, were spiked with chlorpyrifos (100 mg/kg soil). For the set of non-inoculated beakers, MSM medium (4.0 mL, 50% of the water holding capacity of the soil) was added. For the inoculated set of beakers (inoculated), MSM medium (4.0 mL) containing strain DT-1 was added. The final concentration was 10^6 cells/g of soil. Each soil microcosm was incubated at 30 °C under sterile conditions. Soil samples (5 g) were collected for the analysis of chlorpyrifos and TCP concentration every 5 d for 30 d.

2.6. Cloning and expression of the organophosphorus hydrolase-encoding gene, mpd, from strain DT-1

The organophosphorus hydrolase encoding gene was cloned using a PCR-based technique. The primers, designed based on the sequence of known *mpd* genes (Li et al., 2007; Cui et al., 2001), were F1 (5′-CATATGCCCCTGAAGAACCGCTTGCTGG-3′) and R1 (5′-CTCGAGCTTGGGGTTGACGACCGAGTAG-3′). The PCR products were cloned and sequenced. To express the *mpd* gene, the *mpd* encoding region, minus the signal peptide, was amplified by PCR with primer pair F2 (5′-GAATTCATATGGCCGCACCGCAGGTGCGCACCTCG-3′; *Nde*I) and R2 (5′-GAATTCTCGAGCTTGGGGTTGACGACCG-3′; *Xho*I). The PCR products were digested with *Nde*I and *Xho*I, and inserted into pET-29a(+). The recombinant plasmid, pET-*mpd*, was transformed into *E. coli* BL21(DE3). Transformants were subcultured in 50 mL of LB medium and allowed to grow until the culture density reached 0.5 (OD600 nm). Subsequently, expression of *mpd* gene was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mmol/L. The induced cells were collected by centrifugation at 5000*g* for 20 min. These cells were suspended in phosphate-buffered saline (PBS; 200 mmol/L, pH 7.4)