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Isolation and characterization of a *Pseudomonas oleovorans* degrading the chloroacetamide herbicide acetochlor

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Abstract

To date, no pure bacterial cultures that could degrade acetochlor have been described. In this study, one strain of microorganism capable of degrading acetochlor, designated as LCa2, was isolated from acetochlor-contaminated soil. The strain LCa2 is *Pseudomonas oleovorans* according to the criteria of Bergey's manual of determinative bacteriology and sequence analysis of the partial 16S rRNA gene. Optimum growth temperature and pH were 35 °C and 8.0, respectively. The strain could degrade 98.03% of acetochlor treated at a concentration of 7.6 mg l^{-1} after 7 days of incubation and could tolerate 200 mg l^{-1} of acetochlor. When the acetochlor concentration became higher, the degradation cycle became longer. The acetochlor biodegradation products were identified by GC–MS based on mass spectral data and fragmentation patterns. The main plausible degradative pathways involved dechlorination, hydroxylation, N-dealkylation, C-dealkylation and dehydrogenation.

Introduction

Acetochlor (2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)-acetamide) is a selective preemergent herbicide used to control broadleaf weeds and annual grasses in corn. It is one of three kinds of herbicides that are the most widely used in China. The total mass used has been increasing and was already over 1.5×10^7 kg in 1996 (Wang 1999). The USEPA has classified acetochlor as a B-2 carcinogen (U.S. Environmental Protection Agency, 1994). In general, chloroacetamide herbicide's residues and metabolites are relatively common in watershed and groundwater aquifers in agricultural areas that have a history of herbicide application (Stamper & Tuovinen 1998). The prevalence of these compounds in the environment has stimulated investigations into the degradation of hazardous substances in water and contaminated soil. The biological decontamination of pesticide wastes or spills has become an increasingly important area of research. Various studies such as photochemical, microbial and degradative dissipation of acetochlor (Istvan 2000) and its analogous compounds like alachlor (Sun et al. 1990), propachlor (Martin et al. 1999), butachlor (Chakraborty & Bhattacheryya 1991) and metochlor (Pothuluri et al. 1997) were previously reported. However, to date, no pure bacterial cultures that could degrade acetochlor have been described.

The metabolism of acetochlor in soil has been reported. Feng (1991) has proposed glutathione conjugation as an initial pathway of acetochlor metabolism by soil microorganisms. In soil treated with 10 mg acetochlor kg^{-1} soil, two major

degradation products were detected upon 1 month after treatment. These were the de(ethoxymethyl) acetochlor and chloroacetyl-indoline (Istvan 2000). Until recently, little has been reported on the metabolism of acetochlor by pure cultures. The main objectives of this research were to give the first description of the isolation and characterization of a pure bacterial culture able to degrade acetochlor and to describe the metabolism of acetochlor by a pure bacterial culture isolated from acetochlor-contaminated soil.

Materials and methods

Chemicals

Acetochlor (98.6%) was purchased from Shengyang Kaifa New Technology Co., Ltd. 2-Chloro-*N*-(2-ethyl-6-methylphenyl)acetamide (CMEPA) (99.0%) was kindly provided by Dr. Randy L. Rose.

Isolation of bacteria

Soil samples were collected from the pesticide storage of a factory with a history of acetochlor production in China. Two grams of samples were inoculated in LB medium and mineral salts medium (MSM) containing acetochlor (from 50 to 200 mg l^{-1}). MSM containing (g l^{-1}, distilled water) K_2HPO_4, 1.76 g, KH_2PO_4, 0.2 g, $(NH_4)_2SO_4$, 0.5 g, KCl, 0.2 g, NaCl, 0.1 g, $MgSO_4$, 0.2 g, $FeCl_3 \cdot 6H_2O$, 0.002 g, $CaCl_2 \cdot H_2O$, 0.05 g, $ZnSO_4 \cdot 7H_2O$, 0.0002 g, $CuSO_4$, 0.001 g, Na_2MoO_4, 0.001 g, $CoCl_2$, 0.0001 g, $MnSO_4$, 0.0004 g, yeast extract, 0.05 g and 1l of water, were adjusted to pH 7.2 as described by Hone et al. (1990) and revised. After 8 weeks, cultures were plated on MSM agar plates containing acetochlor (200 mg l^{-1}). One isolate named strain LCa2 showed the best growth on MSM and was selected for further characterization. A stock of LCa2 was maintained at −80 °C in 15% glycerol (Sambrook & Russell 2001).

Bacterial identification

Strain LCa2 was identified according to the criteria of Bergey's manual of determinative bacteriology and by sequencing the partial 16S rRNA gene (Holt et al. 1994). Alignment of the partial 16S rRNA gene sequence was performed with sequences deposited in the GeneBank database.

Optimum growth temperature and pH

The pH range and optimum pH for growth of strain LCa2 were determined by monitoring the growth (OD_{600}) of cultures incubated in LB containing acetochlor (200 mg l^{-1}) with different initial pH values (4.0–9.0). The temperature range and optimum growth temperature were also determined by observing growth in acetochlor (200 mg l^{-1}) containing LB at different temperatures, i.e. 20, 25, 30, 35, 40, 45 °C.

Degradation of acetochlor

The experiments were done in 300-ml Erlenmeyer flasks containing 150 ml liquid MSM, supplemented with acetochlor at a concentration of 7.6 or 57 mg l^{-1}. The flasks were incubated at a shaking air bath at 30 °C and 180 rpm in the dark. Each experiment was carried out in triplicate and necessary control samples were included. At regular intervals of time, a 10 ml of the growth medium was processed for detecting the presence of the parent compound and its metabolites by using HPLC and GC–MS and monitoring the growth (colony forming units: CFU).

A 1 ml of the growth medium was serially diluted to plate on LB agar to determine the CFU. The plates were incubated at 30 °C for 24 h. A 1 ml of the growth medium were centrifuged at 10,000 *g* for 5 min and the supernatant was filtered with 0.45 μm membrane and analyzed by HPLC. The other 8 ml portions were partitioned with an equal volume of hexane/ethyl acetate (1:1) three times after acidification with H_2SO_4 and saturation with NaCl in a 50-ml separatory funnel. The organic phase was passed through anhydrous Na_2SO_4 and evaporated to dryness on a rotary vacuum evaporator, followed by dissolution in 1 ml of hexane/ethyl acetate (1:1). The extracted was analyzed by GC-MS.

Analytical methods

Concentration of acetochlor was measured by HPLC (Shimadzu10A, Japan), fitted with a detector (SPD-M10A) operating at an absorbance

wavelength of 210 nm. The column was Waters Spherisorb ODS2 (25 cm × 4.6 mm × 5 μm). The mobile phase contained methanol/water (75:25, v/v) and the flow rate was 1.0 ml min^{-1}. The column was operated at 40 °C.

Mass spectra of acetochlor and its metabolites were obtained on a mass selective detector (Agilent GC model 6890 series, MSD model 5973 series) using a capillary column (DB-5 MS, 60 m × 0.25 mm i.d., 0.25 μm film thickness) operated in splitless mode. Injection port was 250 °C. Detector temperature was 280 °C. Helium was used as the carrier gas (1 ml min^{-1}). The MS was operated in the full-scan mode, scanning from 30 to 350 m/z. Temperature program: 50 °C 7 min, 5 °C min^{-1} to 280 °C 5 min.

Results and discussion

Isolation and characterization of acetochlor degrading microbe

Five acetochlor-resistant pure cultures were isolated from soil samples according to their morphology. Of these isolates, the best growing strain was designated as LCa2 and was selected for subsequent studies. Strain LCa2 could not be maintained on LB agar slants at 4 °C, however preservation at −80 °C in 15% glycerol was ideal. The strain LCa2 could grow in acetochlor (200 mg l^{-1}) containing LB in the range of pH 6.0–9.0 with optimum at pH 8.0. Growth was observed within the temperature range 20–45 °C, while 30 °C was the optimum temperature (data not shown). LCa2 was sensitive to ampicillin (50 mg l^{-1}).

Taxonomy of the strain LCa2

The characteristics of the isolated LCa2 were listed in Table 1. These morphological and biochemical properties are characteristic criteria for *Pseudomonas oleovorans* (Holt et al. 1994). Upon comparison of the partial 16S rRNA gene sequence (1492 bases) obtained from strain LCa2 with sequences from the Genebank Database, the highest degree of identity (99%) was obtained with the 16S rRNA gene sequence of a *Ps. oleovorans* (genebank No.D84018). The partial 16S rRNA gene sequences have been deposited in the Genebank Database under accession No.AY623816. Analysis of the 16S rRNA gene sequence and phenotypic analysis suggested that this strain (LCa2) was a *Ps. oleovorans* strain.

Table 1. Taxonomic characteristics of bacterial isolate strain LCa2

Test item	Results	Test item	Results
G stain	G^-	O/F test	Oxidation
Cell shape	Rod	Glucose with acid production	+
Motility	+[a]	Levan production	+
Catalase	+	As sole source of carbon	
Oxidase	–[b]	Glucose	+
Pyocyanine	–	Fucose	+
Fluorescein	+	Arginine	–
Soluble-pigment (not fluorescein)	–	Gelatin liquefied	–
VP test	–	Hydrolysis of starch	+
MR test	–	Hydrolysis of casein	–
Indole test	–	Lipase (Tween80)	–
Nitrate reduction	–	Urease	+
Aerobic growth	+	Arginine dihydrolase	–
Citrate used	+	Lysine decarboxylase	–
Malonate used	–	ONPG	+
Pseudomonas oleovorans			

[a]Positive result.
[b]Negative result.
Abbreviation: VP – Vogues Proskauer; MR – Methyl Red; ONPG – *o*-nitrophenol β-galactosidase; O/F – oxidation/fermentation.

Acetochlor degradation by Ps. oleovorans *LCa2*

When the initial acetochlor concentration was 7.6 mg l^{-1}, 98.03% of the added acetochlor was degraded by *Ps. oleovorans* LCa2 and only 4.25% disappeared in the uninoculated control group after 7 days of incubation (Figure 1). When the concentration of acetochlor was raised to 57 mg l^{-1} in MSM with and without sucrose, the degradation cycle became longer (Figure 2). In the cases of MSM, MSM with 2 g sucrose l^{-1} and MSM with 10 g sucrose l^{-1}, 43.37, 58.20 and 45.03% of the added acetochlor was transformed after 3 days of incubation. And then after the 6th day, the amount of acetochlor either remained constant (in MSM and MSM with 10 g sucrose l^{-1}) or continued to decline at a slower rate (in MSM with 2 g sucrose l^{-1}). The addition of sucrose could increase the ability of *Ps. oleovorans* LCa2 to transform acetochlor, but the effect of a higher concentration of sucrose is not better than that of a lower concentration.

Acetochlor was a poor biodegradable organic compound (Qu et al. 1999). In the soil treated with 10 mg acetochlor kg^{-1} soil, 66% of the parent herbicide remained without degradation upon 1 month after treatment (Istvan 2000). Under the lower concentration of acetochlor (7.6 mg l^{-1}), it still needed 7 days that 98.03% of the added herbicide was degraded by the LCa2. However, under the higher concentration of acetochlor (57 mg l^{-1}), this herbicide was not transformed completely (only 74.3% transformed) after 20 days of incubation. The reason of incomplete transformation may be that the LCa2 needs long time to degrade the higher concentration of acetochlor, although it could tolerate this concentration. This may indicate that acetochlor is difficult to degrade, or it is possible that the transformation of acetochlor would be more efficient by a microbial community rather than an individual microbial species.

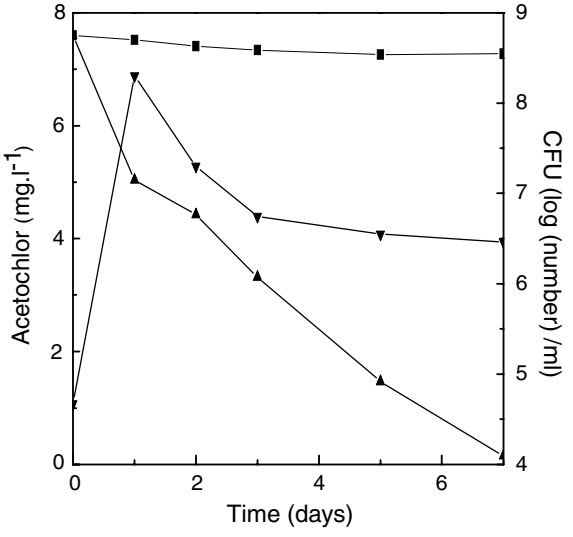

Figure 1. Degradation of acetochlor (7.6 mg l^{-1}) (▲), growth of *Ps.oleovorans* LCa2 in MSM (▼), and uninoculated control (■).

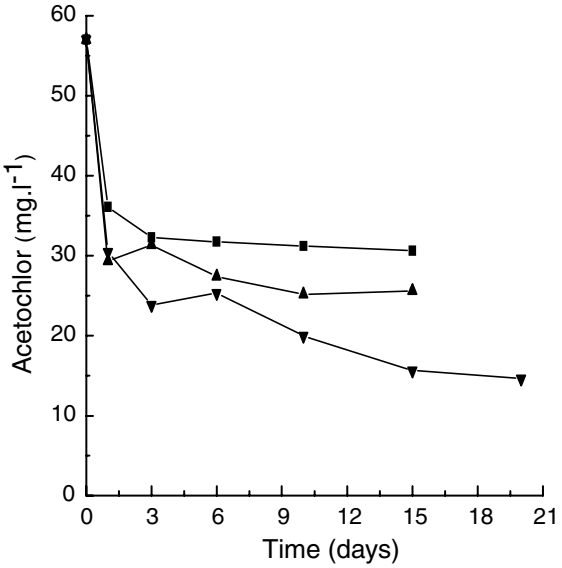

Figure 2. Degradation of acetochlor (57 mg l^{-1}) by *Ps.oleovorans* LCa2 in three different media. MSM (■), MSM with 2 g sucrose l^{-1} (▼), MSM with10 g sucrose l^{-1} (▲).

Ps. oleovorans *LCa2 produces several metabolites from acetochlor*

The acetochlor biodegradation products were identified by GC–MS based on mass spectral data and fragmentation patterns. The main products identified in our study were CMEPA (I: MW = 211), 2-chloro-*N*-(methoxymethyl)-*N*-(2–ethyl−6-methyl phenyl)acetamide (II: MW = 255), 2-hydroxy-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)acetamide (III: MW = 251), *N*-(ethoxymethyl)-*N*-(2-ethyl-6-methyl) benzoylamide (IV: MW = 221), *N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)acetamide

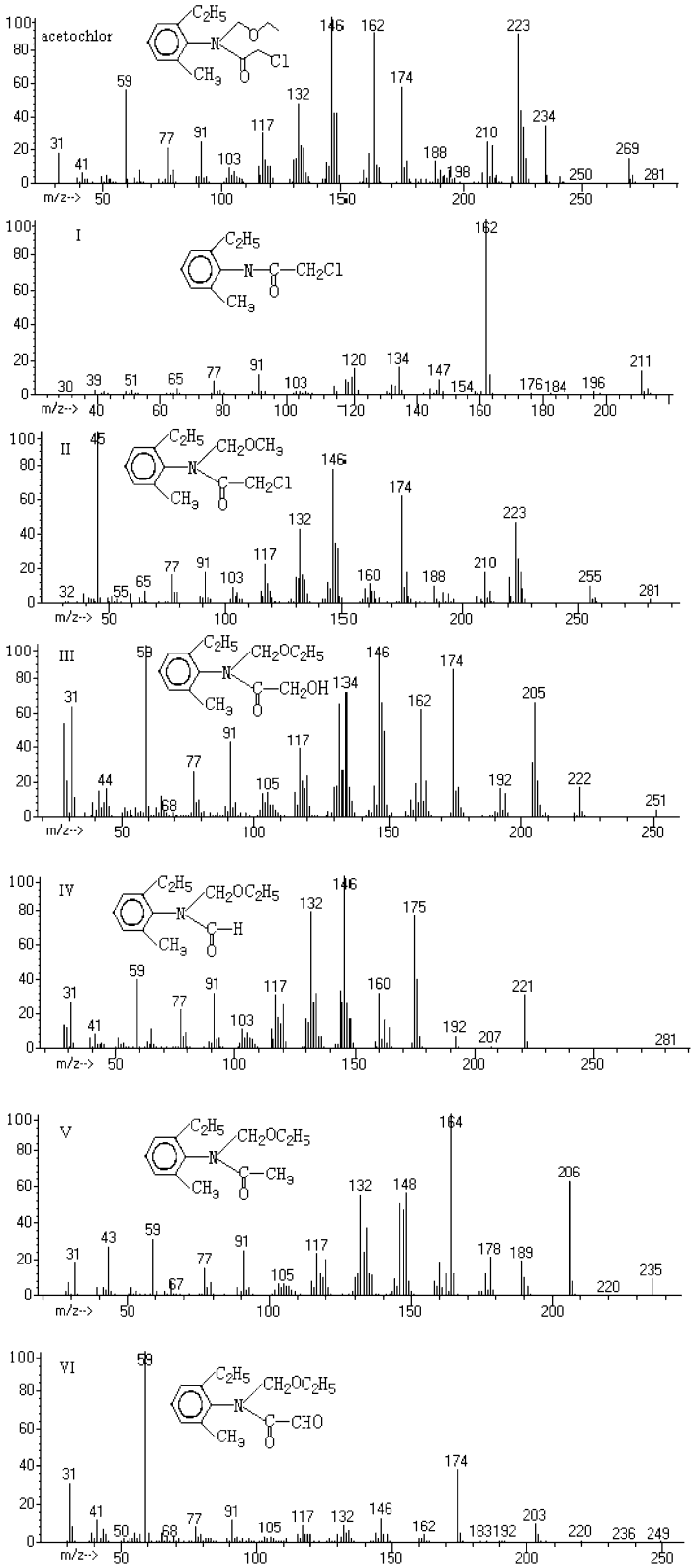

Figure 3. Mass spectra of acetochlor and metabolites shown with structures.

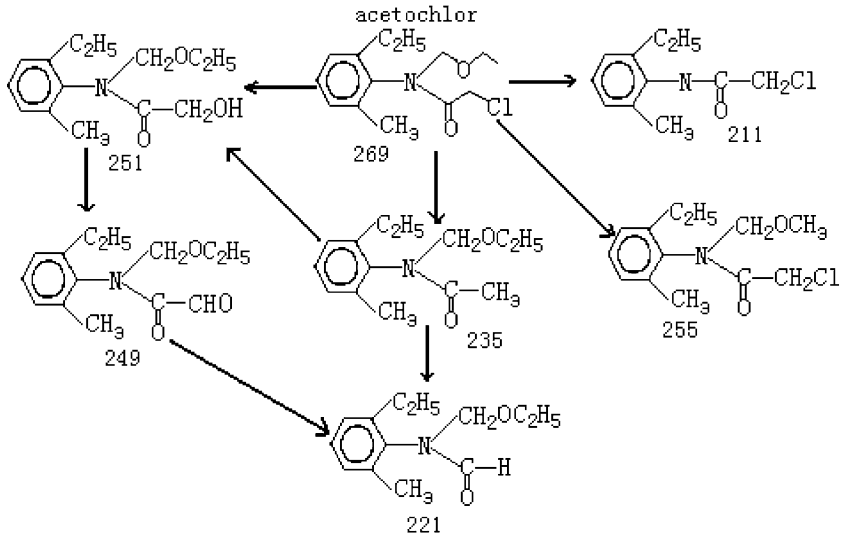

Figure 4. The plausible partial metabolic pathways of acetochlor by *Ps. oleovorans* LCa2.

(V: MW = 235) and 2-aldehyde-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl) acetamide (VI: MW = 249) (Figure 3).

The main acetochlor biodegradation products previously reported included 2-ethyl-6-methylaniline, chloroacetyl-indoline, CMEPA, 2-hydroxy-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)acetamide, oxanilic acid and ethane sulfonic acid (Istvan 2000; Dagnac et al. 2002; Ye et al. 2002). Compared with previous results, compounds II, IV, V and VI have not been mentioned before as acetochlor biodegradation products and their structure were suggested based on their mass spectra and fragmentation patterns. However, compound V has been detected in the photodegradation products of acetochlor (Sandor 1994; Istvan 2000). Compound II was formed probably through partial C-dealkylation of acetochlor. The partial C-dealkylation was also found in the biodegradation of butachlor by two soil fungi, where 2-hydroxy-*N*-(methoxymethyl)-*N*-(2-ethyl-6-methylphenyl) acetamide was detected (Chakraborty & Battacheryya 1991). Compounds I and II were also detected in previous studies. Compound I was confirmed by comparison with authentic standard. The N-dealkylation of acetochlor resulted in the formation of de(ethoxymethyl) acetochlor (CMEPA). Many studies also report the N-dealkylation and formation of de(methoxymethyl) alachlor [2-chloro-N-(2,6-diethylphenyl)acetamide] in the degradation of alachlor (Tiedje & Hagedorn 1975; Fang 1977; Pothuluri et al. 1993). A study on the metabolism of acetochlor and alachlor in human and rat liver microsomes also indicated the formation of de(ethoxymethyl) acetochlor and de(methoxymethyl) alachlor through N-dealkylation by Cytochrome P-450 enzymes (Coleman et al. 2000). Compound III was identified by comparison with the spectrum reported by Istvan (2000), and this compound was corresponds to hydroxyacetochlor that was presumed to be produced by dechlorination–hydroxylation of acetochlor (Ye et al. 2002).

On the basis of the metabolites that were tentatively identified in this study, we have proposed the plausible partial metabolic pathways of acetochlor by *Ps. oleovorans* LCa2 in Figure 4. From Figure 4, it could be seen that the main plausible degradative pathways of acetochlor involved dechlorination, hydroxylation, N-dealkylation, C-dealkylation and dehydrogenation.

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