JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Biodegradation of Butachlor by *Rhodococcus* sp. Strain B1 and Purification of Its Hydrolase (ChlH) Responsible for N-Dealkylation of Chloroacetamide Herbicides

Hong-Ming Liu, Li Cao, Peng Lu, Haiyan Ni, Yun-Xiang Li, Xin Yan, Qing Hong,* and Shun-Peng Li

Key Laboratory of Agricultural Environmental Microbiology, Ministry of Agriculture, College of Life Science, Nanjing Agriculture University, Nanjing 210095, People's Republic of China

Supporting Information

ABSTRACT: *Rhodococcus* sp. strain B1 could degrade 100 mg/L butachlor within 5 days. Butachlor was first hydrolyzed by strain B1 through N-dealkylation, which resulted in the production of butoxymethanol and 2-chloro-*N*-(2,6-dimethylphenyl)-acetamide. Butoxymethanol could be further degraded and utilized as the carbon source for the growth of strain B1, whereas 2-chloro-*N*-(2,6-dimethylphenyl)acetamide could not be degraded further. The hydrolase designated ChlH, responsible for the N-dealkylation of the side chain of butachlor, was purified 185.1-fold to homogeneity with 16.1% recovery. The optimal pH and temperature of ChlH were observed to be 7.0−7.5 and 30 °C, respectively. This enzyme was also able to catalyze the N-dealkylation of other chloroacetamide herbicides; the catalytic efficiency followed the order alachlor > acetochlor >butachlor > pretilachlor, which indicated that the alkyl chain length influenced the N-dealkylation of the chloroacetamide herbicides. This is the first report on the biodegradation of chloroacetamide herbicides at the enzyme level.

KEYWORDS: *butachlor, biodegradation, Rhodococcus sp. B1, protein purification, N-dealkylation*

INTRODUCTION

Chloroacetamide herbicides are a class of important herbicides used worldwide for controlling weeds in plantings of rice, corn, soybean, and other crops. They are often used as pre-emergence or early postemergence herbicides.[1,2] These herbicides inhibit the synthesis of lipid, protein, and lignin of the plant.[3] Alachlor, acetochlor pretilachlor, and butachlor are commonly used chloroacetamide herbicides (Figure 1).

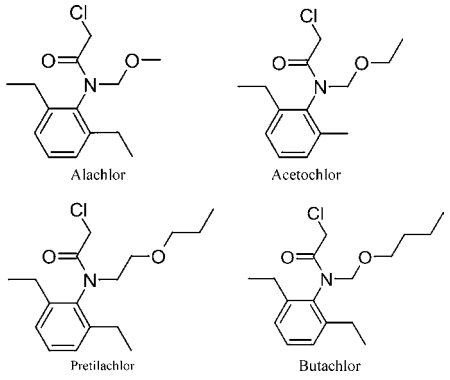

Figure 1. Chemical structural formulas of four chloroacetamide herbicides.

Butachlor is now one of the top three herbicides applied in China.[2] The excessive and frequent application of butachlor may result in high levels of butachlor residues, which have been detected in ground and surface waters.[4] The butachlor residues could also be accumulated in agricultural crops. Thus, they pose a potential health hazard to consumers. Butachlor is known to exert genotoxic effects on amphibians and to induce apoptosis in mammalian cells;[5,6] it can also cause toxicity to earthworms in soil.[7] Great concern and interest have been raised regarding the environmental behavior and degradation mechanism of butachlor residues in soil.

Generally, butachlor in the environment is degraded by both abiotic and biotic pathways including photo-oxidation, chemical hydrolysis, and biodegradation,[8,9] but biodegradation plays a major role in the degradation of butachlor. To date, several microorganisms capable of degrading butachlor have been isolated and characterized.[10−12] The degradation pathway was studied in strain FLY-8, in which butachlor was initially degraded to alachlor by partial C-dealkylation, then converted to 2-chloro-*N*-(2,6-dimethylphenyl)acetamide by N-dealkylation, and then transformed to 2,6-diethylaniline, which was further degraded via the aniline degradation pathway.[13] Zheng et al. identified five metabolites produced during the degradation of butachlor by strain DCA-1 and proposed a different degradation pathway of butachlor.[14] However, all of the evidence for the degradation of butachlor has come from metabolite identification; the enzymatic basis of the pathway has not been described.

The objective of this study was to describe the degradation of butachlor by a newly isolated *Rhodococcus* sp. strain B1 together with identification of the metabolites and purification of its hydrolase (ChlH) responsible for the N-dealkylation of chloroacetamide herbicides.

Received: September 16, 2012
Revised: November 22, 2012
Accepted: November 27, 2012
Published: November 27, 2012

ACS Publications © 2012 American Chemical Society

MATERIALS AND METHODS

Chemicals and Media. Butachlor (purity of 96.5%), alachlor (purity of 95.5%), acetochlor (purity of 95.5%), and pretilachlor (purity of 96.5%) were provided by Binnong Technology Co., Ltd., Shandong Province, China. HPLC grade acetonitrile was purchased from Sigma-Aldrich (St. Louis, MO, USA). Q-Sepharose (fast flow) and DEAE-Sepharose (fast flow) were purchased from GE Healthcare (USA). All other reagents used were of the highest analytical reagent grade and obtained from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China).

The mineral salts medium (MSM) consisted of the following components (in g/L): $(NH_4)_2SO_4$ (1.0), NaCl (1.0), K_2HPO_4 (1.5), KH_2PO_4 (0.5), and $MgSO_4{\cdot}7H_2O$ (0.2) (pH 7.0). Butachlor was added into the medium as the carbon source. Stock solutions of different chloroacetamide herbicides with dimethyl sulfoxide or methanol in 10 g/L were prepared by membrane filtration with a pore size of 0.22 μm. The solutions were added to the sterilized MSM or the reaction solution for determining the activity of ChlH when required. Strain B1 was not capable of utilizing dimethyl sulfoxide as the sole carbon source for growth in MSM (data not shown).

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

Isolation and Identification of Butachlor-Degrading Strain. To isolate butachlor-degrading bacteria, the enrichment culture was carried out according to the method of Wang et al.[15] Soil samples used for enrichment were collected from a rice field in Changzhou, Jiangsu province, China (E 119° 58′, N 31° 47′), which has been subjected to the application of butachlor for many years. About 2.0 g of the soil sample was added to a 250 mL flask containing 100 mL of MSM with the addition of 100 mg/L butachlor as the carbon source, which was incubated at 30 °C on a rotary shaker at 180 rpm for 5 days, then 3 mL of the enrichment culture was subcultured into another 100 mL of fresh enrichment medium and incubated for 5 days. After six transfers, HPLC was used to determine the concentration of butachlor and confirm its degradation. Then, the enrichment culture was serially diluted and spread on MSM agar plates containing 100 mg/L butachlor. Colonies that grew on the plates were purified and tested for the capabilities to degrade butachlor. Strain B1 was selected for its ability to degrade butachlor.

Identification of strain B1 was carried out according to *Bergey's Manual of Determinative Bacteriology*.[16] The 16S rRNA gene was amplified by PCR using standard procedures,[17] and its sequence was aligned with the known sequences in the GenBank database by BLAST. Phylogenesis was analyzed by MEGA version 3.0 software. Distances were calculated using the Kimura two-parameter distance model.[18] An unrooted tree was built by the neighbor-joining method. The data set was bootstrapped 1000 times.

Degradation of Butachlor by Strain B1 in Liquid Culture. The cells of strain B1 were collected by centrifugation at 6000*g* for 5 min at 4 °C after it was precultured in LB medium at 30 °C on a shaker at 180 rpm until the later exponential phase. Cell pellets were washed twice with MSM and adjusted to 1.0×10^8 CFU/mL. For all experiments, the cells were inoculated to approximately 1.4×10^6 CFU/mL and incubated at 30 °C and 180 rpm on a rotary shaker. At regular time intervals, 5 mL samples were collected, and the concentration of the herbicide was determined by high-performance liquid chromatography (HPLC). Each treatment was performed in three replicates, and the control experiment without the inoculation of strain B1 was carried out under the same conditions.

Preparation of Crude Extract. The cells of strain B1 precultured in LB liquid medium were harvested by centrifugation at 6000*g* for 5 min at 4 °C, washed twice with Tris-HCl buffer (pH 7.2), and resuspended in the same buffer. The cells were ruptured by 3 s pulsed sonication for 20 min, the disrupted cell suspension was centrifuged at 12000*g* for 10 min at 4 °C, and supernatant was passed through a cellulose acetate filter with a pore size of 0.2 mm. The supernatant obtained was referred to as crude extract. The protein concentration was calculated according to the method of Bradford.[19]

Protein Purification. All of the following experiments were performed at 4 °C unless otherwise stated. The crude extract of strain B1 can produce a transparent halo on the potassium phosphate buffer (pH 7.5) solid plates containing 100 mg/L butachlor with 0.75% agarose within 20 min of incubation at 30 °C, which was due to the N-dealkylation of butachlor. This phenomenon was used as an indicator to monitor enzyme activities, which facilitated the purification procedure.

Anion-Exchange Chromatography on DEAE-Sepharose Fast Flow. The anion-exchange column (bed volume, 20 mL) was equilibrated with Tris-HCl (pH 7.2). After the column was washed with 200 mL of the same buffer, the crude extract was eluted with 600 mL of a linear concentration gradient from 0 to 0.5 M NaCl. The active part was eluted at 0.3 M NaCl. The gradient elute was collected and stored at −20 °C.[20]

Anion-Exchange Chromatography on Q-Sepharose Fast Flow. The above-mentioned active part was loaded onto a Q-Sepharose column (20 mL) pre-equilibrated with Tris-HCl buffer (pH 7.2), and the matrix was washed with the same buffer to remove unbound proteins. The enzyme was eluted with an increasing linear gradient from 0 to 0.5 M NaCl in buffer. Fractions containing the enzyme activity were collected in 0.4 M NaCl.[21]

Rcovery of Bands from Native Polyacrylamide Gel Electrophoresis (PAGE). The above-mentioned fractions were condensed to 1 mL by ultrafiltration and subjected to PAGE analysis.[22] When the PAGE was completed, the polyacrylamide gel was excised into 10 bands and separately recovered in buffer; these fractions were recovered using a D-tube Electroelution Accessory Kit (Novagen, Germany), and the fractions containing the enzyme activity were subjected to condensation and analysis of enzyme activity. Finally, the purified enzyme was obtained and stored at −20 °C.

Determination of Enzyme Activity. The enzymatic activities toward various chloroacetamide herbicides were assayed in 1 mL of 50 mM potassium phosphate buffer (pH 7.5) at 30 °C for 20 min. The reactions were initiated by the addition of purified ChlH (35 μg/mL to the final concentration of 0.1 μg/mL). Reactions were stopped by adding 2 mL of dichloromethane, and the substrate was extracted and analyzed by HPLC. One unit (U) of enzyme activity was defined as the amount of enzyme that converted 1 μmol of each chloroacetamide herbicide to its products. Control samples without enzyme were analyzed in parallel.

For kinetic studies, the chloroacetamide herbicides were appropriately diluted into at least six different concentrations, from 10 to 150 μM around the dissociation constant (K_m) value. Kinetic values were obtained from the Hanes−Woolf equation.

Biochemical Characterization. The molecular weight of the denatured protein was determined by discontinuous sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE).[23] The molecular mass of the native protein was determined by gel filtration.[24] To determine the optimum pH, the activities were measured using 10 mM citric acid−NaOH buffer at pH 4.0−5.5, 10 mM phosphate buffer at pH 5.0−8.0, 10 mM Tris-HCl buffer at pH 7.2−9.0, and 10 mM glycine−NaOH buffer at pH 8.5−10.0. The optimum temperature was determined with a constant pH of 7.2 and different temperatures from 10 to 50 °C. The effects of potential inhibitors or activators on the ChlH activities were determined by adding various metal salts (Ni^{2+}, Hg^{2+}, Cu^{2+}, Fe^{2+}, Ba^{2+}, Zn^{2+}, Cr^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, and Co^{2+}) and chemical agents (1,10-phenanthroline, SDS, Triton X-100, and PMSF) into the enzyme solution, which was incubated for 10 min at 30 °C. The activity was then assayed as described above and expressed as a percentage of the activity obtained in the absence of the added compound.

Chemical Analysis. Butachlor and other chloroacetamide herbicides in the liquid samples were analyzed according to the following procedures. The samples were centrifuged at 12000*g* for 5 min, and then the supernatant was collected and extracted with the double volume of dichloromethane and dried over anhydrous sodium sulfate; the organic phase was evaporated in a water bath. The residues were redissolved in methanol and subjected to HPLC analysis (600 Controller, Rheodyne 7725i Manual Injector, and 247 Dual λ

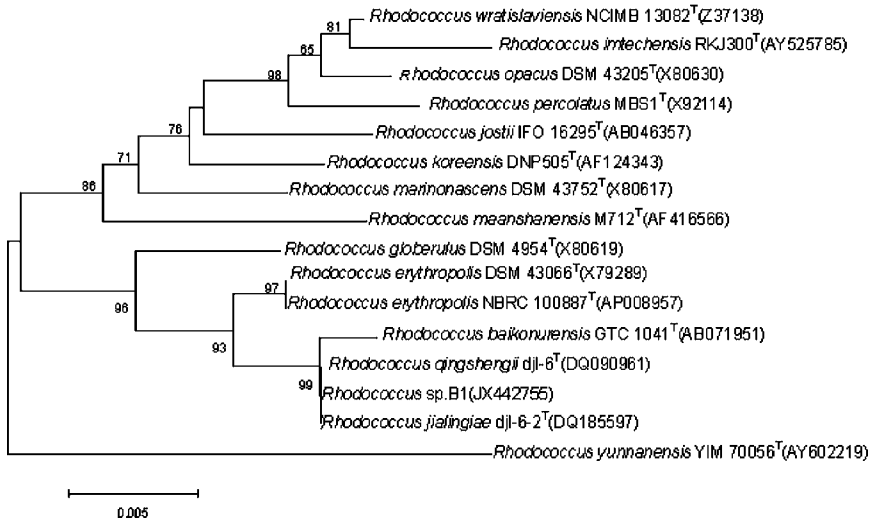

Figure 2. Neighbor-joining tree showing the phylogenetic relationship between *Rhodococcus* sp. strain B1 and related species based on the 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) >50% are given at the nodes. The scale bar represents an evolutionary distance (k_{nuc}) of 0.005. The accession numbers are in parentheses.

Absorbance Detector; Waters Co., Milford, MA, USA). The separation column (4.6 mm × 250 mm × 5 μm) for the HPLC was filled with Kromasil 100-5C18. The mobile phase containing acetonitrile/water (80:20, v/v) was delivered at a flow rate of 0.8 mL/min. The wavelength for the detection of butachlor and other chloroacetamide herbicides was 215 nm. The injection volume was 20 μL.

To identify the metabolites of butachlor, strain B1 was inoculated in MSM containing 100 mg/L butachlor as the sole carbon source and incubated at 30 °C at 180 rpm; then the cultures were centrifuged at 12000*g* for 5 min, and the supernatant was filtered through a 0.45 μm fiber filter and freeze-dried, redissolved in 1 mL of methanol, and identified by HPLC-MS (LC-MSD-Trap-SL, Agilent, USA). The HPLC system was implemented as described above.

RESULTS AND DISCUSSION

Isolation and Identification of Butachlor-Degrading Strain. Bacterial strain B1 capable of degrading butachlor was obtained from the enrichment. It was a non-spore-forming, Gram-positive, nonmotile bacterium. Colonies grown on LB agar were opaque, dry, convex, and red. The optimum growth condition was at pH 7.5−8.0 and 30 °C. Strain B1 was positive for urease, catalase, and Voges−Proskauer test but negative for starch hydrolysis, oxidase, and nitrate reduction. Its 16S rRNA gene sequence was compared with those available sequences in GenBank. Strain B1 showed high similarity with the strain from the genus of *Rhodococcus* species, and the highest similarity of 100% was with strains *Rhodococcus qingshengii* djl-6 and *Rhodococcus jialingiae* djl-6-2. A phylogenetic tree based on known representatives of *Rhodococcus* species and other species is presented in Figure 2. On the basis of the above characters, strain B1 was preliminarily identified as *Rhodococcus* sp.

Degradation of Butachlor by Strain B1. The degradation of butachlor and the growth of strain B1 were investigated simultaneously (Figure 3). The results showed that strain B1 could degrade 100 mg/L butachlor within 5 days. Along with the degradation of butachlor, the cell mass of the strain increased from $1.4 \times 10^6$to 1.3×10^7 CFU/mL, which meant that strain B1 could use butachlor as the carbon source to support its growth. The genus *Rhodococcus* is an unusual group of bacteria known for extraordinary ability to degrade recalcitrant environmental pollutants. *Rhodococcus* sp. strain DN22 could degrade hexahydro-1,3,5-trinitro-1,3,5-triazine

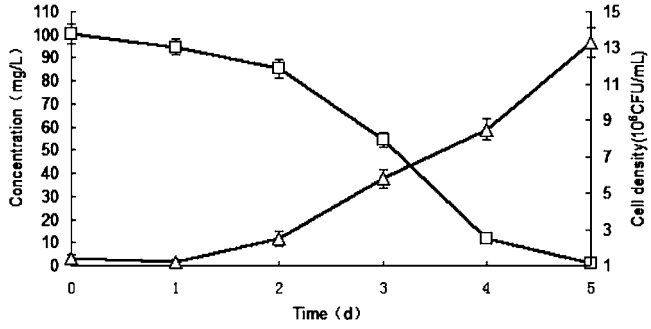

Figure 3. Utilization of butachlor as sole carbon source for growth and degradation by *Rhodococcus* sp. strain B1: (□) concentration of butachlor; (△) growth of strain B1.

(RDX) aerobically.[25] *Rhodococcus* sp. EC1 was capable of mineralizing hexane.[26] *Rhodococcus* sp. djl-6 and djl-6-2 could degrade carbendazim,[27,28] and *Rhodococcus* sp. strain IGTS8 could remove covalently bound sulfur from dibenzothiophene (DBT).[29] *Rhodococcus* sp. strain RHA1 is a polychlorinated biphenyl (PCB) degrader that can degrade 10 mg/L PCB48 (equivalent to Aroclor1248) in a few days.[30] *Rhodococcus* strain TE1 metabolized atrazine under aerobic conditions to produce deethyl- and deisopropylatrazine;[31] *Rhodococcus chlorophenolicus* could degrade not only PCP but also TeCH and 10 other chlorophenols.[32] However, strain B1 was the first strain from the genus *Rhodococcus* that was able to degrade butachlor.

To date, several studies of the biodegradation of chloroacetamide herbicides have been reported. Xu et al. isolated one strain named *Pseudomonas oleovorans* LCa2, which was able to degrade 98.0% of 7.6 mg/L acetochlor after a week of incubation.[33] *Paracoccus* sp. strain FLY-8 could mineralize butachlor and degrade 65.2% of 100 mg/L butachlor in MSM medium within 5 days of incubation.[13] *Catellibacteriumcaeni* sp. nov DCA-1^T could degrade 81.2% of 50 mg/L butachlor in 84 h.[14] Strain B1 was able to completely degrade 100 mg/L butachlor within 5 days, which was observed to be the highest degradation efficiency.

Identification of the Metabolites of Butachlor. The degradation metabolites of butachlor were identified by HPLC-

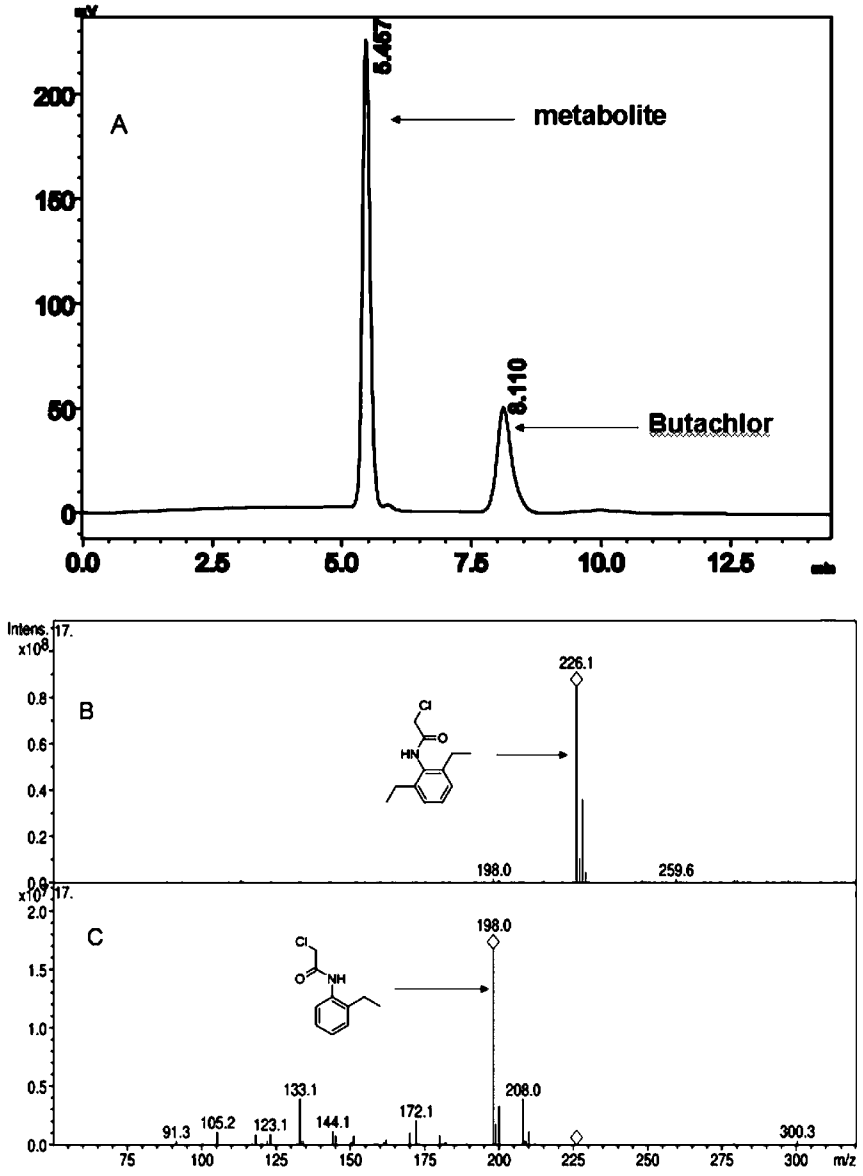

Figure 4. HPLC-MS profile of the metabolite produced during butachlor degradation by *Rhodococcus* sp. strain B1: (A) HPLC spectrum of butachlor and its metabolite; HPLC spectra of the extract obtained from the culture at 72 h, (B, C) positively charged ions mass spectra for metabolite (5.46 min).

MS/MS. One metabolite appeared in HPLC with the retention time of 5.46 min. In positive-ion chemical ionization, this product showed a protonated molecular ion at m/z 226.00 $[M + H]^+$ and characteristic fragment ion peaks at m/z 198.0 $[M - CH_2 - CH_2]$ (Figure 4). On the basis of these results, we deduced that this metabolite was 2-chloro-*N*-(2,6-dimethylphenyl)acetamide; the other metabolite is butoxymethanol, which was formed through cleavage of the C−N bond (Figure 5). This N-dealkylation reaction was also reported in the degradation of butachlor by strains FLY-8[13] and DCA-1.[14] Strain B1 could degrade butoxymethanol further and utilize it as the carbon source for growth, whereas it could not degrade 2-chloro-*N*-(2,6-dimethylphenyl)acetamide (data not shown). In the following research, the hydrolase ChlH involved in this reaction was purified and characterized.

Protein Purification. The enzyme was purified 185.1-fold with a 16.1% yield after the final step with a specific activity of 114.8 U/mg of protein (Table 1). From SDS-PAGE the molecular mass of enzyme was determined to be approximately 45 kDa (Figure 6). Gel filtration indicated a molecular mass of about 90 kDa, which suggested that the enzyme was a homologous dimer.

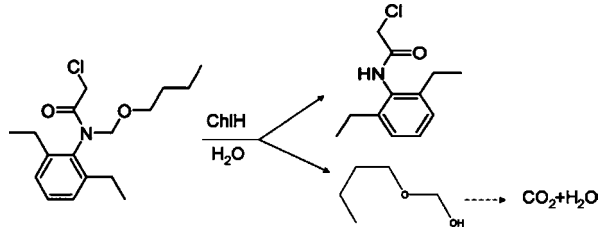

Figure 5. Inferred metabolic pathway of butachlor by *Rhodococcus* sp. strain B1.

Substrate Specificity of the Enzyme. The substrate specificities of the enzyme were investigated against four chloroacetamide herbicides, which were widely used in the

dx.doi.org/10.1021/jf303936j | *J. Agric. Food Chem.* 2012, 60, 12238−12244

Table 1. Purification of ChlH

fraction	total protein (mg)	total activity (U)	specific activity (U/mg)	purification *x*-fold	yield (%)
crude extract	2190.2	1357.5	0.62	1.0	100.0
DEAE-Sepharose anion-exchange	311.2	893.1	2.9	4.6	65.8
Q-Sepharose anion-exchange	41.6	570.4	13.7	22.1	42.0
PAGE gel electronic elution	1.9	218.1	114.8	185.1	16.1

Table 2. Substrate Specificity and Apparent Kinetic Constant of ChlH

substrate	specific activity (μmol min^{-1} mg^{-1})	K_m (mM)	K_{cat} (s^{-1})	catalytic efficiency, k_{cat}/K_m (mM^{-1} s^{-1})
alachlor	44.8 ± 4.5	0.0831 ± 0.006	33.6 ± 3.4	404.7 ± 14.0
acetochlor	46.1 ± 1.0	0.0880 ± 0.001	34.6 ± 0.8	393.0 ± 12.7
butachlor	11.6 ± 1.6	0.0767 ± 0.011	8.7 ± 1.3	113.1 ± 0.5
pretilachlor	11.2 ± 1.2	0.0912 ± 0.0071	8.4 ± 0.7	92.0 ± 1.0

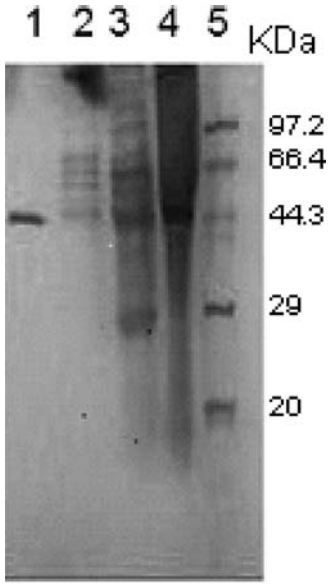

Figure 6. SDS-PAGE analysis of the purified enzyme from *Rhodococcus* sp. strain B1. Lanes: 1, after PAGE gel electronic elution; 2, after Q-Sepharose anion-exchange chromatography; 3, after DEAE-Sepharose anion-exchange chromatography; 4, crude extract; 5, protein markers.

control of weeds. The HPLC-MS/MS results demonstrated that ChlH could catalyze the N-dealkylation of these chloroacetamide herbicides with a C−N bond, resulting in the production of the corresponding metabolites. The hydrolysis efficiency descended as follows: alachlor > acetochlor > butachlor > pretilachlor (Table 2), indicating that the hydrolysis activities of the ChlH decreased with the increase of the alkyl chain length.

Characteristics of ChlH. The optimal pH of ChlH was observed to be 7.0−7.5. The enzyme was stable in the pH range of 6.0−8.0, retaining approximately 80% of the original activity after preincubation in the buffer at that pH range for 1 h. The activity of ChlH was maximal at 30 °C. The enzyme was fairly stable up to 40 °C, retaining >80% of its activity at 40 °C for 1 h, had 25% residual activity at 50 °C for 1 h, and was completely inactivated at 60 °C for 30 min (data not shown). Most of the metal ions tested had no noticeable inhibitory effect on the ChlH activity; however, Hg^{2+}, Ca^{2+}, and Ni^{2+} (1 mM) were able to severely inhibit its activity, and the addition of Mg^{2+} increased the enzymatic activity by 1.1-fold. The inhibitor of serine hydrolases PMSF, chelating agents EDTA, and 1,10-phenanthroline (10 mM) were only able to show a 15−25% inhibition of enzyme activity The thiol reagents pCMB and iodoacetamide (1 mM) and the surfactants SDS, Tween-80, and Triton X-100 all showed a 40−70% inhibition of ChlH activity (Table3).

Table 3. Effects of Metal Ions and Chemical Agents on ChlH Activity

reagent	concentration	relative activity (%)
no addition	0 mM	100
Ba^{2+}	1 mM	85.4 ± 2.5
Co^{2+}	1 mM	53.5 ± 1.5
Ca^{2+}	1 mM	10.9 ± 0.6
Cu^{2+}	1 mM	75.7 ± 1.4
Cr^{2+}	1 mM	52.7 ± 0.8
Hg^{2+}	1 mM	0
Mg^{2+}	1 mM	110.5 ± 0.8
Mn^{2+}	1 mM	83.8 ± 2.1
Ni^{2+}	1 mM	13.5 ± 0.3
Zn^{2+}	1 mM	85.5 ± 1.2
Fe^{2+}	1 mM	28.2 ± 1.5
EDTA	10 mM	75.9 ± 1.1
PMSF	10 mM	87.5 ± 2.1
SDS	2 mg/mL	56.8 ± 0.6
Triton X-100	20 mg/mL	48.5 ± 0.7
Tween-80	20 mg/mL	74.1 ± 1.5
iodoacetamide	1 mM	58.2 ± 1.2
pCMB	1 mM	56.2 ± 1.3
1,10-phenanthroline	10 mM	85.5 ± 1.3

Many enzymes that can hydrolyze pesticides and herbicides have been reported. The organophosphate pesticide hydrolase from *Flavobacterium* sp. (ATCC27551)[34] and *Plesiomonas* sp. M6[35] were able to hydrolyze organophosphate insecticides. A hydrolase converting carbaryl to 1-naphthol was purified from *Pseudomonas aeruginosa*.[36] PytH from *Sphingobium* sp. strain JZ-1 was a pyrethroid-hydrolyzing carboxylesterase.[15] SulE was a hydrolase from *Hansschlegelia zhihuaiae* S113, responsible for sulfonylurea herbicide de-esterification.[37]

To the best of our knowledge, ChlH is the first enzyme from pure culture that could hydrolyze butachlor through the cleavage of the C−N bond of the side chain. It could also hydrolyze other chloroacetamide herbicides with a C−N bond. The cleavage of C−N could be catalyzed by amidases or demethylases in the degradation of pesticides. One amidase, AmdA, purified from *Rhodococcus* strain had activity on several 2-aryl propionamides.[38] LibA from *Variovorax* sp. strain SRS16

was a monomeric linuron hydrolase, which could hydrolyze linuron to 3,4-dichloroaniline and *N*,*O*-dimethylhydroxylamine.[39] The *ampA* gene cloned from *Paracoccus* sp. strain FLN-7 encoding a novel arylamidase catalyzes amide bond cleavage in the amide pesticides.[40] PamH protein belonged to the amidase signature enzyme family from *Paracoccus* sp. M-1 that was highly active on aromatic and short-chain aliphatic amides (benzamide and propionamide).[41] A novel *N*-demethylase (Ndm) with broad substrate specificity was purified from CBB5. Ndm displayed activity toward caffeine, theobromine, theophylline, and 3-methylxanthine.[42] The *N*-demethylase genes of CBB5 enabled bacteria to live on caffeine.[43] The present study showed that ChlH could carry out the N-dealkylation of butachlor and other chloroacetamide herbicides, and it could be used in the bioremediation of chloroacetamide herbicide-contaminated environments.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +86 25 84396685. Fax: +86 25 84396314. E-mail: hongqing@njau.edu.cn.

Funding

This work was supported by grants from the Chinese National Natural Science Foundation (31070099 and 31000060), the National Science and Technology Project in Rural Areas of "the twelfth five-year-plan" (2012AA101403-4), and the Project for Science and Technology of Jiangsu Province (BE201270608).

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Dwivedi, S.; Singh, B. R.; Al-Khedhairy, A. A.; Alarifi, S.; Musarrat, J. Isolation and characterization of butachlor-catabolizing bacterial strain *Stenotrophomonas acidaminiphila* JS-1 from soil and assessment of its biodegradation potential. *Lett. Appl. Microbiol.* **2010**, *51*, 54−60.

(2) Yu, Y. L.; Chen, Y. X.; Luo, Y. M.; Pan, X. D.; He, Y. F.; Wong, M. H. Rapid degradation of butachlor in wheat rhizosphere soil. *Chemosphere* **2003**, *50*, 771−774.

(3) Michel, C.; Jochen, S.; Peter, B. A specific and sensitive assay to quantify the herbicidal activity of chloroacetamides. *Pestic. Sci.* **1998**, *52*, 381−387.

(4) Chiang, H. C.; Duh, J. R.; Wang, Y. S. Butachlor, thiobencarb, and chlomethoxyfen movement in subtropical soils. *Bull. Environ. Contam. Toxicol.* **2001**, *66*, 1−8.

(5) Panneerselvam, N.; Sinha, S.; Shanmugam, G. Butachlor is cytotoxic and clastogenic and induces apoptosis in mammalian cells. *Indian J. Exp. Biol.* **1999**, *37*, 888−892.

(6) Geng, B. R.; Yao, D.; Xue, Q. Acute toxicity of the pesticide dichlorvos and the herbicide butachlor to tadpoles of four anuran species. *Bull. Environ. Contam. Toxicol.* **2005**, *75*, 343−349.

(7) Muthukaruppan, G.; Janardhanan, S.; Vijayalakshmi, G. Sublethal toxicity of the herbicide butachlor on the earthworm *Perionyx sansibaricus* and its histological changes. *J. Soils Sediments* **2005**, *5*, 82−86.

(8) Lin, Y. J.; Lin, C.; Yeh, K. J.; Lee, A. Photodegradation of the herbicides butachlor and ronstar using natural sunlight and diethylamine. *Bull. Environ. Contam. Toxicol.* **2000**, *64*, 780−785.

(9) Zheng, H.; Ye, C. Photodegradation of acetochlor and butachlor in waters containing humic acid and inorganic ion. *Bull. Environ. Contam. Toxicol.* **2001**, *67*, 601−608.

(10) Chen, Y. L.; Chen, J. S. Degradation and dissipation of herbicide butachlor in paddy fields. *J. Pestic. Sci.* **1979**, *4*, 431−438.

(11) Chakraborty, S. K.; Bhattacharyya, A. Degradation of butachlor by two soil fungi. *Chemosphere* **1991**, *23*, 99−105.

(12) Wang, J. H.; Lu, Y. T.; Chen, Y. Y. Comparative proteome analysis of butachlor-degrading bacteria. *Environ. Geol.* **2008**, *53*, 1339−1344.

(13) Zhang, J.; Zheng, J. W.; Liang, B.; Wang, C.; Cai, S.; Ni, Y. Y.; He, J.; Li, S. P. Biodegradation of chloroacetamide herbicides by *Paracoccus* sp. FLY-8 in vitro. *J. Agric. Food Chem.* **2011**, *59*, 4614−4621.

(14) Zheng, J. W.; Li, R.; Zhu, J. C.; Zhang, J.; He, J.; Li, S. P.; Jiang, J. D. Degradation of the chloroacetamide herbicide butachlor by *Catellibacteriumcaeni* sp. nov DCA-1^T. *Int. Biodeter. Biodegr.* **2012**, *73*, 16−22.

(15) Wang, B. Z.; Guo, P.; Hang, B. J.; Li, L.; He., J.; Li, S. P. Cloning of a novel pyrethroid-hydrolyzing carboxylesterase gene from *Sphingobium* sp. strain JZ-1 and characterization of the gene product. *Appl. Environ. Microbiol.* **2009**, *75*, 5496−5500.

(16) Holt, J. G.; Krieg, N. R.; Sneath, P. H. A.; Staley, J. T.; Williams, S. T. *Bergey's Manual of Determinative Bacteriology*, 9th ed.; Williams and Wilkins: Baltimore, MD, 1994.

(17) Lane, D. J. 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematic*; Stackebrandt, E., Goodfellow, M., Eds.; Wiley: Chichester, UK, 1991; pp 371−375.

(18) Kumar, B.; Tamura, K.; Jakobsen, I. B.; Nei, M. MEGA. Molecular Evolutionary Genetics Analysis Software,Arizona State University: Tempe, AZ, 2001.

(19) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(20) Wieser, M.; Wagner, B.; Eberspächer, J.; Lingens, F. Purification and characterization of 2,4,6-trichlorophenol-4-monooxygenase,a dehalogenating enzyme from *Azotobacter* sp. strain GP1. *J. Bacteriol.* **1997**, *179*, 202−208.

(21) Swetha, V. P.; Basu, A.; Phale, P. S. Purification and characterization of 1-naphthol-2-hydroxylase from carbaryl-degrading *Pseudomonas* srain C4. *J. Bacteriol.* **2007**, *189*, 2660−2666.

(22) Waterborg, J. H.; Matthews, H. R. The electrophoretic elution of proteins from polyacrylamide gels. *Methods Mol. Biol.* **1994**, *32*, 169−175.

(23) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(24) Wu, P. C.; Liu, Y. H .; Wang, Z. Y.; Zhang, X. Y.; Li, H.; Liang, W. Q.; Luo, N.; Hu, J. M.; Lu, J. Q.; Luan, T. G.; Cao, L. X. Molecular cloning, purification, and biochemical characterization of a novel pyrethroid-hydrolyzing esterase from *Klebsiella* sp. strain ZD112. *J. Agric. Food Chem.* **2006**, *54*, 836−842.

(25) Fournier, D .; Halasz, A.; Spain, J.; Fiurasek, P.; Hawari, J. Determination of key metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine with *Rhodococcus* sp. srain DN22. *Appl. Environ. Microbiol.* **2002**, 166−172.

(26) Lee, E. H.; Cho, K. S. Characterization of cyclohexane and hexane degradation by *Rhodococcus* sp. EC1. *Chemosphere* **2008**, *71*, 1738−1744.

(27) Wang, Z. C.; Wang, Y. Y.; Gong, F. F.; Zhang, J.; Hong, Q.; Li, S. P. Biodegradation of carbendazim by a novel actinobacterium *Rhodococcus jialingiae* djl-6-2. *Chemosphere* **2010**, *81*, 639−644.

(28) Xu, J. L.; Gu, X. Y.; Shen, B.; Wang, Z. C.; Wang, K.; Li, S. P. Isolation and characterization of a carbendazim-degrading *Rhodococcus* sp. djl-6. *Curr. Microbiol.* **2006**, *53*, 72−76.

(29) Denome, S. A.; Oldfield, C.; Nash, L. J.; Young, K. D. Characterization of the desulfurization genes from *Rhodococcus* sp. strain IGTS8. *J. Bacteriol.* **1994**, *176*, 6707−6716.

(30) Masai, E.; Yamada, A.; Healy, J. M.; Hatta, T.; Kimbara, K.; Fukuda, M.; Yano, K. Characterization of biphenyl catabolic genes of Gram-positive polychlorinated biphenyl degrader *Rhodococcus* sp. strain RHA1. *Appl. Environ. Microbiol.* **1995**, *61*, 2079−2085.
(31) Behki, R.; Topp, E.; Dick, W.; Germon, P. Metabolism of the herbicide atrazine by *Rhodococcus* strains. *Appl. Environ. Microbiol.* **1993**, *59*, 1955−1959.
(32) Apajalahti, J. H. A.; Salkinoja-Salonen, M. S. Degradation of polychlorinated phenols by *Rhodococcus chlorophenolicus*. *Appl. Microbiol. Biotechnol.* **1986**, *25*, 62−67.
(33) Xu, J.; Qiu, X. H.; Dai, J. Y.; Cao, H.; Yang, M .; Zhang, J.; Xu, M. Q. Isolation and characterization of a *Pseudomonas oleovorans* degrading the chloroacetamide herbicide acetochlor. *Biodegradation* **2006**, *17*, 219−225.
(34) Mulbry, W. W.; Karns, J. S .; Kearney, P. C .; Nelson, J. O.; McDaniel, C. S.; Wild, J. R. Identification of a plasmid-borne parathion hydrolase gene from *Flavobacterium* sp.by southern hybridization with *opd* from *Pseudomonas diminutat*. *Appl. Environ. Microbiol.* **1986**, *51*, 926−930.
(35) Cui, Z. L.; Li, S. P.; Fu, G. P. Isolation of methyl parathion-degrading strain M6 and cloning of the methyl parathion hydrolase gene. *Appl. Environ. Microbiol.* **2001**, *67*, 4922−4925.
(36) Chapalmadugu, S.; Chaudhry, G. R. Isolation of a constitutively expressed enzyme for hydrolysis of carbaryl in *Pseudomonas aeruginosa*. *J. Bacteriol.* **1993**, *175*, 6711−6716.
(37) Hang, B. J.; Hong, Q.; Xie, X. T.; Huang, X .; Wang, C. H .; He, J.; Li, S. P. SulE, a sulfonylurea herbicide de-esterification esterase from *Hansschlegelia zhihuaiae* S113. *Appl. Environ. Microbiol.* **2012**, *78*, 1962−1968.
(38) Mayaux, J. F.; Cerbelaud, E.; Soubrier, F.; Yeh, P.; Blanche, F.; Pétré, D. Purification, cloning, and primary structure of a new enantiomer-selective amidase from a *Rhodococcus* strain: structural evidence for a conserved genetic coupling with nitrile hydratase. *J. Bacteriol.* **1991**, *173*, 6694−6704.
(39) Bers, K.; Leroy, B.; Breugelmans, P.; Albers, P.; Lavigne, R.; Sørensen, S. R .; Aamand, J.; Mot, R. D.; Wattiez, R.; Springael, D. A novel hydrolase identified by genomic-proteomic analysis of phenylurea herbicide mineralization by *Variovorax* sp. strain SRS16. *Appl. Environ. Microbiol.* **2011**, *77*, 8754−8764.
(40) Zhang, J.; Yin, J. G.; Hang, B. J.; Cai, S.; He, J.; Zhou, S. G.; Li, S. P. Cloning of a novel arylamidase gene from *Paracoccus* sp. strain FLN-7 that hydrolyzes amide pesticides. *Appl. Environ. Microbiol.* **2012**, *78*, 4848−4855.
(41) Shen, W. L.; Chen, H. H.; Jia, K. Z.; Ni, J.; Yan, X.; Li, S. P. Cloning and characterization of a novel amidase from *Paracoccus* sp. M-1, showing aryl acylamidase and acyl transferase activities. *Appl. Microbiol. Biotechnol.* **2012**, *94*, 1007−1018.
(42) Summers, R. M.; Louie, T. M.; Yu, C. L.; Subramanian, M. Characterization of a broad-specificity non-haem iron N-demethylase from *Pseudomonas putida* CBB5 capable of utilizing several purine alkaloids as sole carbon and nitrogen. *Microbiology* **2011**, *157*, 583−592.
(43) Summers, R. M.; Louie, T. M.; Yu, C. L.; Gakhar, L.; Louie, K. C.; Subramanian, M. Novel, highly specific N-demethylases enable bacteria to live on caffeine and related purine alkaloids. *J. Bacteriol.* **2012**, *194*, 2041−2049.