JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Degradation of Cyhalofop-butyl (CyB) by *Pseudomonas azotoformans* Strain QDZ-1 and Cloning of a Novel Gene Encoding CyB-Hydrolyzing Esterase

Zhi-Juan Nie,[‖] Bao-Jian Hang,[‖] Shu Cai, Xiang-Ting Xie, Jian He,* and Shun-Peng Li

Key Laboratory of Microbiological Engineering of Agricultural Environment, Ministry of Agriculture, Life Sciences College of Nanjing Agricultural University, Nanjing, Jiangsu 210095, People's Republic of China

ABSTRACT: Cyhalofop-butyl (CyB) is a widely used aryloxyphenoxy propanoate (AOPP) herbicide for control of grasses in rice fields. Five CyB-degrading strains were isolated from rice field soil and identified as *Agromyces* sp., *Stenotrophomonas* sp., *Aquamicrobium* sp., *Microbacterium* sp., and *Pseudomonas azotoformans*; the results revealed high biodiversity of CyB-degrading bacteria in rice soil. One strain, *P. azotoformans* QDZ-1, degraded 84.5% of 100 mg L^{-1} CyB in 5 days of incubation in a flask and utilized CyB as carbon source for growth. Strain QDZ-1 could also degrade a wide range of other AOPP herbicides. An esterase gene, *chbH*, which hydrolyzes CyB to cyhalofop acid (CyA), was cloned from strain QDZ-1 and functionally expressed. A *chbH*-disrupted mutant dchbH was constructed by insertion mutation. Mutant dchbH could not degrade and utilize CyB, suggesting that *chbH* was the only esterase gene responsible for CyB degradation in strain QDZ-1. ChbH hydrolyzed all AOPP herbicides tested as well as permethrin. The catalytic efficiency of ChbH toward different AOPP herbicides followed the order quizalofop-P-ethyl ≈ fenoxaprop-P-ethyl > CyB ≈ fluazifop-P-butyl > diclofop-methyl ≈ haloxyfop-P-methyl; the results indicated that the chain length of the alcohol moiety strongly affected the biodegradability of the AOPP herbicides, whereas the substitutions in the aromatic ring had only a slight influence.

KEYWORDS: cyhalofop-butyl (CyB), microbial degradation, *Pseudomonas azotoformans* strain QDZ-1, CyB-hydrolyzing esterase gene, molecular structure and biodegradability

INTRODUCTION

Cyhalofop-butyl (CyB), 2-[4-(4-cyano-2-fluorophenoxy) phenoxy]propanoic acid, butyl ester (R), is a widely used aryloxyphenoxy propanoate (AOPP) herbicide for the postemergence control of grasses in rice fields.[2] Although herbicides are typically applied to the crop and weed canopy, substantial quantities of the active ingredient penetrate to the soil surface. Therefore, great concerns have been raised about the occurrence and fate of the herbicides in the environment.

In general, CyB and other AOPP analogues in the environment are degraded by both abiotic and biotic pathways, including photooxidation, and chemical and microbial degradation.[9] Laboratory and field studies have demonstrated that microbial metabolism is the most important factor in the fate of AOPP herbicides in soil, water, and sediment. Up to now, several AOPP herbicide degrading strains have been described. Smith-Greenier and Adkins[15] isolated six diclofop-methyl-degrading strains from Manitoba soil. Liang et al.[7] reported that *Brevundimonas* sp. LY-2, isolated from a soil sample that had been exposed to lactofen for many years, could metabolize lactofen to 1-(carboxy)ethyl-5-(2-chloro-4-(trifluoromethyl)phenoxy)-2-nitrobenzoate. Jackson and Douglas[9] demonstrated that the initial degradation of CyB in soil and water was due to a combination of microbial and chemical processes but that subsequent degradation was predominantly a microbial process. The metabolic pathway of CyB in soil and sediment/water systems was also studied; CyB was rapidly degraded to CyA through cleavage of the ester linkage. The acid was further transformed to cyhalofop amide (CyAA) and cyhalofop diacid (CyD) by sequential oxidation of the cyano group.[10,12] However, up to now, no report was available in the literature regarding the microbial degradation of CyB by a pure bacterial culture. Some important questions, such as which kinds of microorganisms are involved in CyB residue degradation in the environment, the microbial metabolic pathway, and the relationship between chemical hydrolysis and microbial degradation, were still unanswered.

In the present study, five CyB-degrading strains were isolated and identified. A novel esterase gene, *chbH*, with responsibility for the cleavage of the ester linkage of CyB was cloned and functionally expressed. The influence of the molecular structure of the AOPP herbicides on their biodegradability was also analyzed.

MATERIALS AND METHODS

Chemicals and Media. CyB, diclofop-methyl, haloxyfop-P-methyl, quizalofop-P-ethyl, fenoxaprop-P-ethyl, and fluazifop-P-butyl were obtained from Sigma-Aldrich Chemical Co. (Shanghai, China). Permethrin, bifenthrin, and malathion were obtained from Yangnong Chemical Group Co., Ltd., Jiangsu province, China. All of the above chemicals were of analytical grade. The Luria−Bertani (LB) medium consisted of the following components (in g L^{-1}): 10.0 tryptone, 5.0 yeast extract, and 5.0 NaCl. Mineral salts medium (MSM) consisted of the following components (in g L^{-1}): 1.0 $(NH_4)_2SO_4$, 1.0 NaCl, 1.5 K_2HPO_4, 0.5 KH_2PO_4, and 0.2 $MgSO_4 \cdot 7H_2O$, pH 7.0. For solid medium, 15.0 g of

Received: January 28, 2011
Revised: April 27, 2011
Accepted: May 2, 2011
Published: May 02, 2011

agar was added per liter. Medium was sterilized at 121 °C for 30 min. The stock solutions of the above herbicides or insecticides (1%, w/v) were prepared in methanol and sterilized by membrane filtration (pore size = 0.22 μm).

Isolation and Identification of CyB-Degrading Bacteria. The soil sample used as initial inoculants was collected from the surface layer (0−10 cm) of a rice field, which had been exposed to CyB for many years, in Jiangsu province, China. About 1.0 g of the soil sample was added to a 150 mL Erlenmeyer flask containing 50 mL of MSM supplemented with 100 mg L^{-1} CyB as carbon sources; CyB was first dissolved in methanol and then transferred into the medium. The culture was incubated at 30 °C on a rotary shaker at 150 rpm for about 5 days. Five milliliters of the enrichment culture was transferred into another 50 mL of fresh enrichment medium for another 5 days. Then the enrichment culture was spread on MSM agar plates supplemented with 100 mg L^{-1} CyB. Bacterial colonies producing a visible transparent halo due to CyB degradation were picked out, purified by repeated streaking, and tested for their degrading capabilities.

The isolates were characterized and identified by morphological, physiological, and biochemical characteristics and 16S rRNA gene analysis. The morphological and physiological and biochemical characterizations were carried out according to the diagnostic tables of bacteria proposed by Cowan and Steel.[4] Genomic DNA was extracted by a high-salt precipitation method.[14] The 16S rRNA gene sequence was amplified by PCR as described by Wang et al.[19] using a set of universal primers, 5′-AGAGTTTGATCCTGGCTCAG-3′ (*Escherichia coli* bases 8−27) and 5′-TACCTTGTTACGACTT-3′ (*E. coli* bases 1507−1492), originally presented by Lane.[6] The PCR product was purified by PCR purification kit (Axygen) and ligated into the vector pMD18-T (TaKaRa Biotechnology, Dalian, China) and then transformed into *E. coli* DH5α. An automatic sequencer (Applied Biosystem, model 3730) was used to determine the 16S rRNA gene sequence. Pairwise sequence similarity was calculated by using a global alignment algorithm, implemented at the EzTaxon server.[3] Phylogenetic analysis was performed by using the software package MEGA version 4.1[16] after multiple alignment of the sequence data with CLUSTAL_X.[17] The G+C content of the genomic DNA was determined by thermal denaturation[11] using *E. coli* K-12 DNA as reference. DNA−DNA hybridizations were performed according to the method of Ezaki et al.[5]

Growth and Degradation Experiments. The isolate growing in LB medium for about 12 h was harvested by centrifugation (5000 rpm, 10 min) and washed twice with fresh MSM. After the cell density had been adjusted to about 1.0×10^8 cfu mL^{-1}, an aliquot (1%, v/v) was inoculated into 20 mL of MSM supplemented with 100 mg L^{-1} CyB as carbon source in a 50 mL Erlenmeyer flask. The cultures were incubated at 30 °C and 150 rpm on a rotary shaker. At an interval of 24 h, three culture flasks were removed from incubation. Bacterial growth was monitored by measuring the colony forming units (CFU mL^{-1}), and concentrations of CyB were determined by HPLC analysis as described below. Each treatment was performed in three replicates, and the control experiments without inoculation and without substrate were carried out under the same conditions.

Degradation of other AOPP herbercides (diclofop-methyl, haloxyfop-P-methyl, quizalofop-P-ethyl, fenoxaprop-P-ethyl, and fluazifop-P-butyl) by the strain was also studied at the same conditions.

Cloning of the *chbH* Gene Encoding CyB-Hydrolyzing Esterase. DNA manipulation was carried out as described by Sambrook et al.[14] The genomic DNA library of strain QDZ-1 was constructed according to the method of Wang et al.[18] with some modification. The insertion fragments in the transforms were about 4−6 kb. Colonies that produced clear transparent halos on LB plates supplemented with 100 mg L^{-1} CyB were screened and further tested for degrading capabilities. Nucleotide and deduced amino acid sequence analyses were performed using Omiga software 2.0. BlastN and BlastP were used for the nucleotide sequence and deduced amino acid identity searches (www.ncbi.nlm.nih.gov/Blast), respectively.

Gene Expression and Purification of the Recombinant ChbH. The open reading frame (ORF) of *chbH* without its translation stop codon was amplified by PCR with one primer pair [chbF1, ATGCGGTACCATGGCTGTCGAATGGGTTGTCG (*Kpn*I, corresponding to sites 1−22 of the *chbH* gene), and chbR1, ATGCCTCGAGCTGGCTCGCCAGGGCGACAGG (*Xho*I, corresponding to sites 976−996 of the *chbH* gene)] and inserted into the *Kpn*I−*Xho*I site of pET29a(+) to generate the recombinant plasmid pet-chbH. The recombinant plasmid pet-chbH then transformed into *E. coli* BL21-(DE3). The induction and purification of the recombinant ChbH were carried out according to the methods described by Wang et al.[18] The protein concentration was quantified by the Bradford method using bovine serum albumin as the standard.[1]

Enzyme Assay. Enzymatic activities toward CyB and other AOPP herbercides were performed in 50 mM sodium phosphate buffer (pH 7.5) at 37 °C. One microliter of chemical solution was added to 3 mL of the preincubated enzyme solution. The enzyme mixture was incubated for 10 min. No more than 10% of the substrate was hydrolyzed during the assay. The esterase activities against *p*-nitrophenyl acetate, permethrin, bifenthrin, and malathion were performed as described by Liang et al.[8] One activity unit was defined as the amount of enzyme required to catalyze the formation or hydrolysis of 1 μmol of product or substrate per minute. For kinetic studies, a stock solution of substrate was appropriately diluted in methanol into at least five different concentrations around the K_m values. Kinetic values were obtained from Lineweaver−Burk plots against various substrate concentrations.

Construction of *chbH*-Disrupted Mutant. A 524 bp fragment (corresponding to sites 198−721 of the *chbH* gene) used for the homologous recombination directing sequence was amplified from the genomic DNA of strain QDZ-1 with one primer pair [chbF2, ACCTGGATCCGGGCAGTAGTCGCAACCTGACCTATGC (*Bam*HI, corresponding to sites 198−224 of the *chbH* gene), and chbR2, GCTTCTGCAGGGTCGTTGACCCGGTTGATCTCG (*Pst*I, corresponding to sites 699−721 of the *chbH* gene)] and inserted into the *Bam*HI−*Pst*I site of the suicide plasmid pJQ200SK (Gm resistant, Quandt and Hynes[13]) to yield pJQdchbH. Then plasmid pJQdchbH was introduced into strain QDZ-1 (Ap resistant) by triparental conjugation using pRK2013 as the helper plasmid. A mutant with single recombination events was selected on the basis of resistance to Ap and Gm on LB plates. One of the selected mutant strains was designated dchbH. The *chbH* gene of the mutant was divided into two separate parts by the integrated plasmid pJQ200SK.[13] The single recombination event was checked by PCR using two primer pairs: chbF1 and chbR1; and chbF1 and R3 (GCGAGTCAGTGAGCGAGGAA, corresponding to sites 497−477 downstream of *Pst*I in the plasmid pJQ200SK). Theoretically, when chbF1 and chbR1 were used as primers, a fragment of 1016 bp would be yielded for wild type and no fragment would be yielded for mutant dchbH; when chbF1 and R3 were used as primers, a fragment of 1228 bp would be yielded for mutant dchbH, and no fragment would be yielded for wild type.

Two methods were used to determine the CyB-degrading activities of wild-type strain QDZ-1 and mutant dchbH. In method I, the wild-type strain QDZ-1 and mutant dchbH were inoculated on MSM plate supplemented with 200 mg L^{-1} CyB and incubated at 30 °C for 3 days and then investigated as to whether the growth and transparent halo occurred. In method II, the wild-type strain QDZ-1 and mutant dchbH were cultivated in 20 mL of LB medium for about 12 h, harvested by centrifugation, and inoculated into 20 mL of MSM supplemented with 100 mg L^{-1} CyB. After incubation at 30 °C on a rotary shaker for 2 days, the CyB concentrations of the cultures were determined by HPLC analysis as described below. The control experiments without inoculation were carried out under the same conditions.

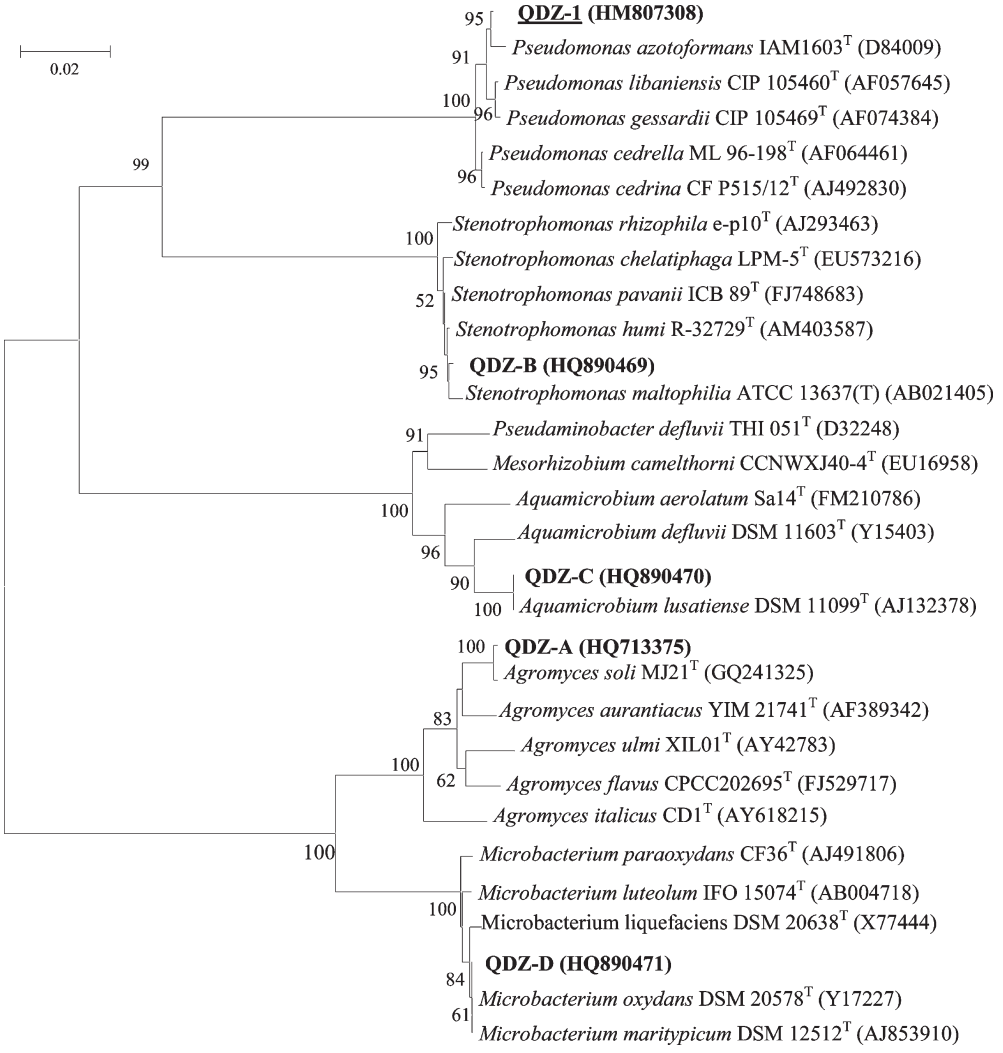

Figure 1. Phylogenetic tree based on the 16S rRNA gene sequences of the CyB-degrading strains and related species. The GenBank accession number for each microorganism used in the analysis is shown after the species name. The scale bar indicates 0.02 substitution per nucleotide position. Bootstrap values obtained with 1000 resamplings are indicated as percentages at all branches.

Chemical Analysis. CyB and other AOPP herbercides in cultures were extracted by the addition of an equal volume of dichloromethane followed by 1 h of shaking using a reciprocal shaker. The dichloromethane phase was dried over anhydrous Na_2SO_4, and then the dichloromethane was removed using a stream of nitrogen at room temperature. The residues were dissolved in 200 μL of methanol. The chemical concentrations in samples were analyzed by HPLC equipped with a Zorbax C-18 ODS Spherex column (250 mm × 4.6 mm). The mobile phase was methanol/water (85:15, v/v), and the flow rate was 1.0 mL min^{-1}. Column elutions were monitored by measuring at 230 nm with a Waters 2487 wavelength absorbance detector, and the injection volume was 20 μL. The recovery rates of CyB from liquid culture at concentrations of 0.1, 1.0, 10.0, and 100.0 mg L^{-1} were determined to be 88.5 ± 5.1, 92.7 ± 3.9, 98.1 ± 3.3, and 96.4 ± 2.1%, which indicated that the procedure was efficient in extracting CyB from liquid culture.

Identification of the Metabolite of CyB Degradation by ChbH. The enzyme mixture was supplemented with 50 mg/L CyB and incubated for 2 h; control experiments with heat-inactivated enzyme were carried out under the same conditions. The solution mixture was extracted as described above. The metabolite was identified by LC-MS as described by Liang et al.[7]

Nucleotide Sequence Accession Numbers. The nucleotide sequences of the 16S rRNA genes of strains QDZ-A, QDZ-B, QDZ-C, QDZ-D, and QDZ-1 were deposited in the GenBank database under accession numbers HQ713375, HQ890469, HQ890470, HQ890471, and HM807308, respectively. The accession number of the *chbH* gene was HQ733860.

RESULTS AND DISCUSSION

Strain Isolation and Identification. CyB is an ester with low water solubility (0.44 mg L^{-1} at pH 7), whereas its degradation product, CyA, is more soluble (251 mg L^{-1} at pH 7). An agar plate supplemented with 100 mg L^{-1} CyB appeared opaque due to the poor solubility of CyB. When a CyB-degrading strain was inoculated on this agar plate, a visible transparent halo around the colony would be produced due to the fact that CyB was transformed to CyA. In this study, we used this transparent halo as a CyB-degrading activity indicator to isolate CyB-degrading strains and to screen the CyB-hydrolyzing esterase gene from the genomic library. Five pure cultures, designated QDZ-A, QDZ-B, QDZ-C, QDZ-D, and QDZ-1, were isolated from the

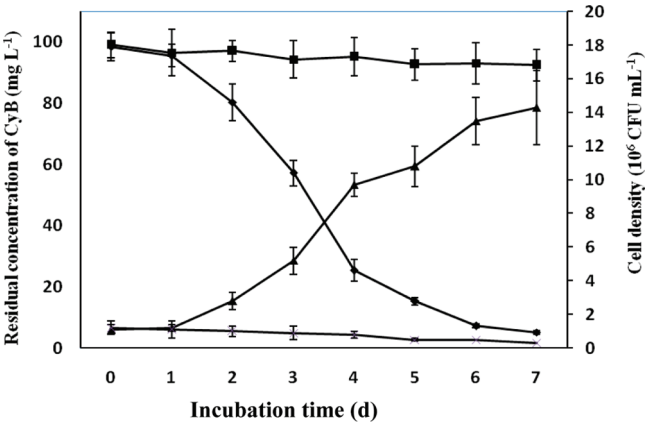

Figure 2. Degradation and utilization of CyB during growth of strain QDZ-1 in MSM: (■) CyB control; (□) CyB inoculated; (▲, ×) cell density of strain QDZ-1 with the addition of CyB and without addition in MSM medium, respectively. Error bars represent the standard error of three replicates.

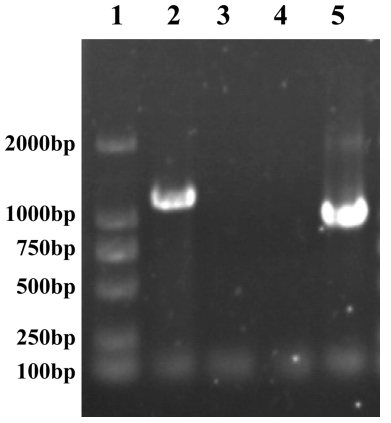

Figure 3. PCR analysis of the single recombination event. Lanes: 1, DL2000 marker; 2, amplified fragment by primer piar chbF1 and R3 using mutant dchbH DNA as template; 3, amplified fragment by primer pair chbF1 and chbR1 using mutant dchbH DNA as template; 4, amplified fragment by primer pair chbF1 and R3 using wild-type strain QDZ-1 DNA as template; 5, amplified fragment by primer pair chbF1 and chbR1 using wild-type strain QDZ-1 DNA as template.

enrichment culture. On the basis of the results of morphological and physiological characteristics and phylogenetic analysis of the 16S rDNA sequence (Figure 1), the five strains were identified preliminarily as *Agromyces* sp. (strain QDZ-A), *Stenotrophomonas* sp. (strain QDZ-B), *Aquamicrobium* sp. (strain QDZ-C), *Microbacterium* sp. (strain QDZ-D), and *Pseudomonas* sp. (strain QDZ-1). When inoculated into MSM supplemented with 100 mg L^{-1} CyB and incubated at 30 °C for 5 days, strains QDZ-A, QDZ-B, QDZ-C QDZ-D, and QDZ-1 were able to degrade about 61.7, 72.4, 44.5, 67.8, and 85.3% of the initially added CyB, respectively. The results gave the most direct evidence that indigenous microorganisms were involved in the CyB degradation in soil and also revealed the high biodiversity of CyB-degrading bacteria in rice soil. Strain QDZ-1 was selected for further study due to its relatively high degrading efficiency and the fact that isolates in the genus *Pseudomonas* play a very important role in the degradation of a wide variety of xenobiotic pollutants.

Figure 4. Wild-type strain QDZ-1 (A) and mutant dchbH (B) grown on MSM plate supplemented with 200 mg L^{-1} CyB.

Table 1. Degradation of CyB by Strain QDZ-1 and Mutant dchbH

	control	strain QDZ-1	mutant dchbH
residual concentration of CyB[a] (mg L^{-1})	98.5 ± 4.1 a	24.3 ± 2.1 b	96.7 ± 5.5 a

[a] Different letters indicate significant differences ($p < 0.05$, LSD test).

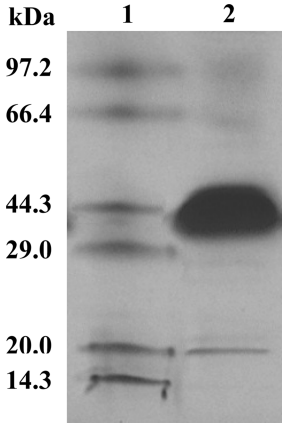

Figure 5. SDS-PAGE analysis of the purified ChbH (lane 2) and protein markers (lane 1) stained with Coomassie brilliant blue G250.

Strain QDZ-1 is non-spore-forming, Gram-negative, short rod-shaped (approximately 1.5−2.2 μm in length and 0.6−0.8 μm in width), and motile with polar flagella. Colonies grown on LB agar for 1−2 days are circular, convex with entire margins, and pale yellow. Fluorescent pigment is produced after 2 days of incubation. Growth is observed over a temperature range of 15−37 °C (optimum 25−30 °C), a salinity range of 0−3.5% NaCl (optimum 0.5% NaCl), and a pH range of 5.5−10.0 (optimum 7.0). The strain is positive for cytochrome oxidase, gelatin liquefaction, nitrate reduction. The DNA G+C content is 62.5 mol %.

An almost-complete 16S rRNA gene sequence (1372 nts) was determined for strain QDZ-1. Phylogenetic analysis of the 16S rDNA gene sequences revealed that strain QDZ-1 grouped among *Pseudomonas* species and formed a subclade with *Pseudomonas azotoformans* IAM1603^T (similarity = 99.6%) with a high bootstrap value of 95% (Figure 1). Strain QDZ-1 showed a relatively high DNA−DNA relatedness to *P. azotoformans*

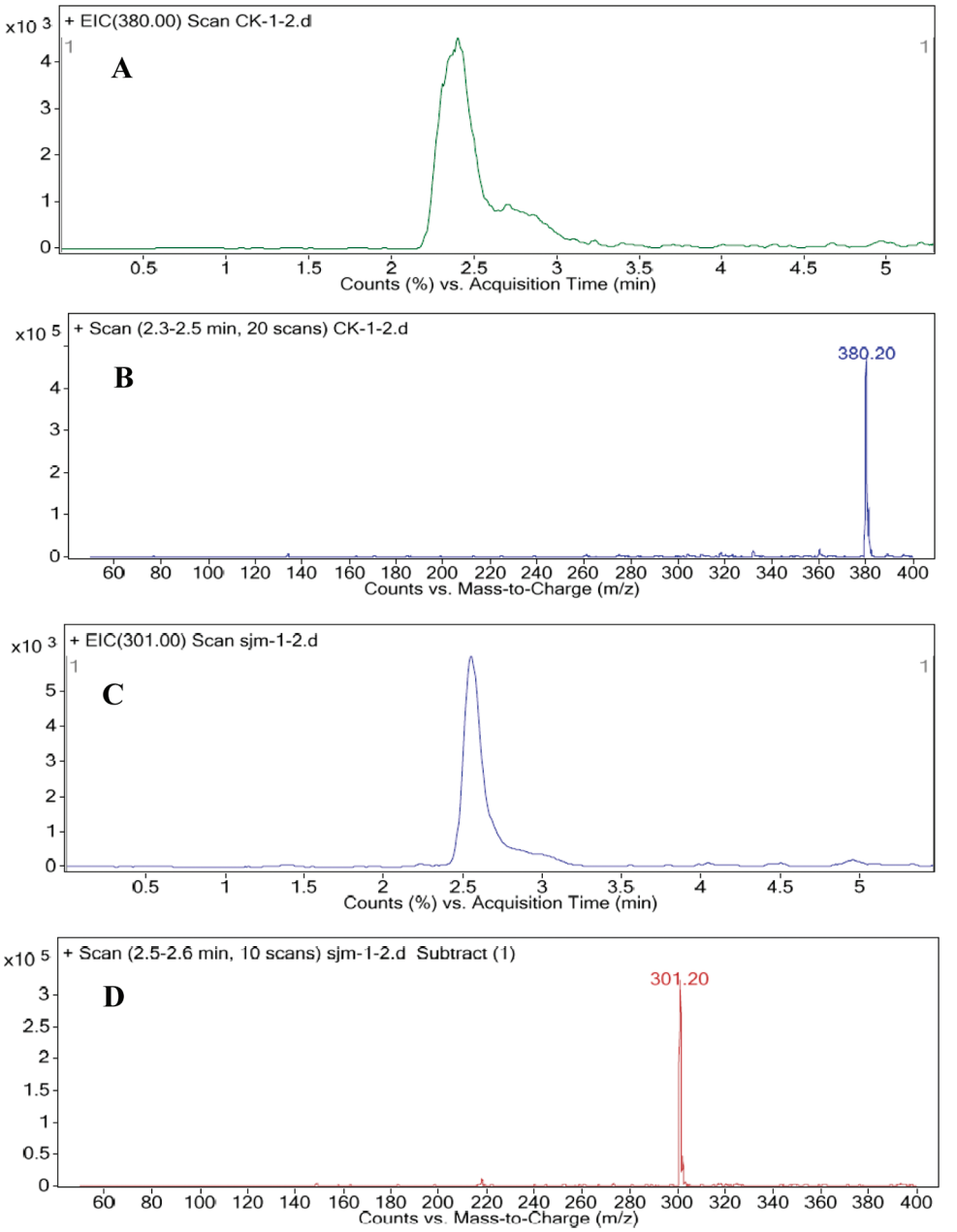

Figure 6. LC-MS spectrum of the dichloromethane extracts from the control with added CyB and heat-inactivated ChbH (A, B) and from the treatment with added CyB and ChbH (C, D).

IAM1603^T (76.4%; reciprocal, 81.2%), the value was above the threshold of 70% recommended for the delineation of bacterial species.[20] Thus, on the basis of the above characteistics, strain QDZ-1 was identified as *P. azotoformans*.

Degradation of CyB and Other AOPP Herbercides by Strain QDZ-1. The growth of strain QDZ-1 on liquid MSM supplemented with 100 mg L^{-1} CyB and its ability to degrade CyB are shown in Figure 2. The growth curve showed a steady increase in bacterial population after a short lag phase (about 24 h). Simultaneously, HPLC analysis showed a substantial reduction in the concentrations of CyB. After incubation for 5 days, about 84.5% of the 100 mg L^{-1} CyB initially added was degraded by strain QDZ-1; correspondingly, the cell densities were increased from about 1.3×10^6 to 1.4×10^7 cfu mL^{-1}. No significant change in CyB concentration was observed in cultures that were not inoculated with strain QDZ-1, and no growth was observed for strain QDZ-1 when it was inoculated into the culture without the addition of CyB. Thus, we concluded that strain QDZ-1 was able to degrade CyB and utilize it as carbon source for growth. HPLC-MS analysis demonstrated that CyB was degraded to CyA through cleavage of the ester linkage in strain QDZ-1 (data not shown).

When other AOPP herbicides were used as substrates, strain QDZ-1 could degrade 68.9% diclofop-methyl, 64.6% haloxyfop-P-methyl, 92.6% quizalofop-P-ethyl, 90.8% fenoxaprop-P-ethyl, and 79.6% of fluazifop-P-butyl and used these AOPP herbicides as carbon sources for growth.

Cloning of the *chb*H Gene Encoding CyB-Hydrolyzing Esterase. A positive clone that produced a transparent halo

Table 2. Kinetic Constants for Hydrolysis of Various Chemicals

substrate	specific activity (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})
CyB	2.76 ± 0.24	1.53 ± 0.13	0.41 ± 0.04	3.73
fluazifop-P-butyl	2.45 ± 0.12	1.27 ± 0.06	0.35 ± 0.02	3.63
fenoxaprop-P-ethyl	3.95 ± 0.21	2.19 ± 0.12	0.36 ± 0.03	6.08
quizalofop-P-ethyl	4.12 ± 0.25	2.43 ± 0.12	0.37 ± 0.02	6.57
diclofop-methyl	2.15 ± 0.11	1.21 ± 0.04	0.59 ± 0.03	2.05
haloxyfop-P-methyl	2.02 ± 0.12	1.15 ± 0.03	0.61 ± 0.04	1.89
permethrin	0.11 ± 0.01	0.05 ± 0.01	0.17 ± 0.02	0.29
bifenthrin	0	0	NM[a]	NM[a]
malathion	0	0	NM[a]	NM[a]
p-nitrophenyl acetate	0	0	NM[a]	NM[a]

[a] NM, not measurable. K_m and k_{cat}/K_m could not be calculated due to no specific activity data available.

around the colony was screened from approximately 5000 transformants. The inserted fragment in the transformant was 5560 bp and contained four complete ORFs. The four ORFs were then subcloned into the linear vector pMD18-T and transformed into competent *E. coli* DH5α. One ORF was confirmed to be the target gene encoding the CyB-hydrolyzing esterase. This gene was designated *chbH*. Sequence analysis indicated that the *chbH* gene consisted of 996 bp encoding a protein of 332 amino acids. The deduced protein was compared with the known enzymes available from the Protein Data Bank (NCBI database). *ChbH* showed the highest similarity with several hypothetical proteins (esterase or hydrolase), for example, a lactone-specific esterase from *Pseudomonas fluorescens* (82% identity), a hydrolase or acyltransferase from *Ralstonia eutropha* H16 (50% identity), and an α/ hydrolase from *Burkholderia multivorans* ATCC 17616 (48% identity). Esterases are a group of related enzymes that catalyze the hydrolysis of a wide range of ester-containing endogenous and xenobiotic compounds. They are involved in the degradation or detoxification of numerous toxic pesticides and drugs in microbes, animals, and plants. Some esterase genes responsible for the deesterification of organophosphates, pyrethroids, and carbamates have been cloned from pesticide-degrading bacteria.[18] To our knowledge, *chbH* is the first identified esterase for AOPP herbicide hydrolysis in microorganisms.

Functional Confirmation of *chbH* Gene in Strain QDZ-1. To address the function of *chbH* gene in strain QDZ-1, a *chbH*-disrupted mutant dchbH was constructed by insertion of a suicide vector pJQ200SK into the *chbH* gene. The single recombination event was confirmed by PCR analysis (Figure 3).

The CyB-degrading activities were investigated using wild-type strain QDZ-1 and mutant dchbH. Wild-type strain QDZ-1 was able to grow on a MSM plate supplemented with 200 mg L^{-1} CyB and produced a visible transparent halo due to CyB degradation around the colony, whereas mutant dchbH could not, indicating that mutant dchbH lost the CyB-degrading ability (Figure 4). When wild-type strain QDZ-1 and mutant dchbH were inoculated on liquid MSM medium supplemented with 100 mg L^{-1} CyB, about 75.3% of 100 mg L^{-1} CyB initially added was degraded by strain QDZ-1 within 2 days, whereas no obvious CyB degradation was observed for mutant dchbH (Table 1). The results indicated that *chbH* was the only esterase gene responsible for the degradation of CyB in strain QDZ-1.

Gene Expression and Purification of the Recombinant ChbH. The recombinant ChbH was produced in *E. coli* BL21-(DE3) and purified from the crude extract using Ni−nitrilotriacetic acid affinity chromatography. The purified enzyme gave a single band on SDS-PAGE (Figure 5). The molecular mass of the denatured enzyme was approximately 36 kDa, which was in good agreement with the molecular mass deduced from the amino acid sequence. HPLC-MS analysis demonstrated that ChbH catalyzed the hydrolysis of CyB to butyl alcohol and cyhalofop acid (Figure 6).

The enzyme was strongly inhibited by many metal ions (Ni^{2+}, Cu^{2+}, Hg^{2+}, and Zn^{2+}; 0.5 mM) and surfactants SDS and Tween 80 (10 mM). Chelating agents EDTA and 1,10-phenanthroline had little effect on the enzyme activity, indicating that ChbH apparently had no requirement for metal ions. The optimal pH was observed to be approximately 7.0. The enzyme was very stable at pH 6.0−9.5 and temperature up to 50 °C.

Kinetic Analysis of the Enzyme. The substrate specificities of the enzyme were tested with various AOPP herbicides, *p*-nitrophenyl acetate, malathion, bifenthin, and permethrin, as the substrates (Table 2). The results indicated that ChbH was able to hydrolyze all of the AOPP herbicides tested with different hydrolysis rates, indicating that the enzyme has potential applications in biotransformation and in situ bioremediation of diphenyl ester herbicide residues, where they cause environmental contamination problems. Quizalofop-P-ethyl was hydrolyzed most rapidly; the catalytic efficiency value (k_{cat}/K_m) of fenoxaprop-P-ethyl was a little lower than that of quizalofop-P-ethyl, but was about 1.7-fold higher than that of CyB and fluazifop-P-butyl and about 3.0-fold higher than that of diclofop-methyl and haloxyfop-P-methyl. The results indicated that the chain length of the alcohol moiety strongly affected the biodegradability of the AOPP herbicides. However, to our surprise, different AOPP herbicides with the same alcohol moiety have about the same degradation efficiencies, suggesting that the substitutions in the aromatic ring have only slight influence on the degradation efficiency. ChbH showed weak activity to permethrin (a kind of pyrethroid pesticide). No hydrolysis activities were observed when *p*-nitrophenyl acetate, malathion, and bifenthin were used as substrates. AOPP herbicides and permethrin have an aryloxyphenoxy group, whereas *p*-nitrophenyl acetate, malathion, and bifenthrin have no such structure. These results suggested that maybe the aryloxyphenoxy group was necessary for the enzyme−substrate interaction.

AUTHOR INFORMATION

Corresponding Author

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

Author Contributions

‖ Both authors contributed equally to this work.

Funding Sources

This work was supported by the National Natural Science Foundation of China (30830001, 30970099) and the Transformation Fund for Agricultural Science and Technology Achievements (2009GB23600516).

REFERENCES

(1) 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.

(2) Buendía, J.; Barotti, R.; Fullick, K.; Thompson, A.; Velilla, J. Cyhalofop, a new postemergence herbicide for grass control in rice. In *6th EWRS Mediterranean Symposium*, Montpellier, France, 1998.

(3) Chun, J.; Lee, J.-H.; Jung, Y.; Kim, M.; Kim, S.; Kim, B. K.; Lim, Y. W. EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int. J. Syst. Evol. Microbiol.* **2007**, *57*, 2259–2261.

(4) Cowan, S. T.; Steel, K. J. *Manual for the Identification of Medical Bacteria*; Cambridge University Press: London, U.K., 1965.

(5) Ezaki, T.; Hashimoto, Y.; Yabuuchi, E. Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int. J. Syst. Bacteriol.* **1989**, *39*, 224–229.

(6) Lane, D. J. 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*; Stackebrandt, E., Goodfellow, M., Eds.; Wiley: Chichester, U.K., 1991; pp 115−175.

(7) Lang, B.; Zhao, Y. K.; Lu, P.; Li, S. P.; Huang, X. Biotransformation of the diphenyl ether herbicide lactofen and purification of a lactofen esterase from *Brevundimonas* sp. LY-2. *J. Agric. Food Chem.* **2010**, *58*, 9711–9715.

(8) Liang, W. Q.; Wang, Z. Y.; Li, H.; Wu, P. C.; Hu, J. M.; Luo, N.; Cao, L. X.; Liu, Y. H. Purification and characterization of a novel pyrethroid hydrolase from *Aspergillus niger* ZD11. *J. Agric. Food Chem.* **2005**, *53*, 7415–7420.

(9) Jackson, R.; Douglas, M. An aquatic risk assessment for cyhalofop-buthyl: a new herbicide for control of barnyard grass in rice. *XI Symposium Pesticide Chemistry: Human and Environmental Exposure to Xenobiotics*, Cremona, Italy, 1999; pp 345−354.

(10) Jilisa, K. M.; Zhu, G. N.; Zhao, L.; Chen, Y. X. Degradation of cyhalofop-butyl in soil. *Acta Agric. Zhejiangensis* **2002**, *14* (4), 205–209.

(11) Mandel, M.; Marmur, J. Use of ultraviolet absorbance−temperature profile for determining the guanine plus cytosine content of DNA. *Methods Enzymol.* **1968**, *12B*, 195–206.

(12) Maria, V. P.; Ilaria, B.; Sonia, B.; Carlo, E. G.; Alba, P. Hydrolysis and adsorption of cyhalofop-butyl and cyhalofop-acid on soil colloids. *J. Agric. Food Chem.* **2008**, *56* (13), 5273–5277.

(13) Quandt, J.; Hynes, M. F. Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. *Gene* **1993**, *127*, 15–21.

(14) Sambrook, J.; Russell, D. W. *Molecular Cloning: a Laboratory Manual*, 3rd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 2001.

(15) Smith-Grenier, L. L.; Adkins, A. Isolation and characterization of soil microorganisms capable of utilizing the herbicide diclofop-methyl as a sole source of carbon and energy. *Can. J. Microbiol.* **1996**, *42* (3), 227–233.

(16) Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. MEGA4: molecular evolutionary genetic analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **2007**, *24*, 1596–1599.

(17) Thompson, J. D.; Gibson, T. J.; Plewniak, F.; Jeanmougin, F.; Higgins, D. G. The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **1997**, *25*, 4876–4882.

(18) 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.

(19) Wang, B. Z.; Guo, P.; Zheng, J. W.; Hang, B. J.; Li, L.; He, J.; Li, S. P. *Sphingobium wenxiniae* sp. nov., a synthetic pyrethroids-degrading bacterium isolated from activated sludge of a SPs-manufacturing wastewater treatment facility. *Int. J. Syst. Evol. Microbiol.* **2010**, doi:10.1099/ijs.0.023309-0.

(20) Wayne, L. G.; Brenner, D. J.; Colwell, R. R. International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* **1987**, *37*, 463−464.