Purification and Properties of an *Arthrobacter oxydans* P52 Carbamate Hydrolase Specific for the Herbicide Phenmedipham and Nucleotide Sequence of the Corresponding Gene

HANS-DIETER POHLENZ,¹* WERNER BOIDOL,² IRIS SCHÜTTKE,¹
AND WOLFGANG R. STREBER¹

Schering AG, Agrochemical Research,¹ and Institute for Cellular and
Molecular Biology,² P.O. Box 65 03 11, 1000 Berlin 65, Germany

Received 6 March 1992/Accepted 30 July 1992

Arthrobacter oxydans P52 isolated from soil samples was found to degrade the phenylcarbamate herbicides phenmedipham and desmedipham cometabolically by hydrolyzing their central carbamate linkages. The phenylcarbamate hydrolase (phenmedipham hydrolase) responsible for the degradative reaction was purified to homogeneity. The enzyme was shown to be a monomer with a molecular weight of 55,000. A 41-kb wild-type plasmid (pHP52) was identified in *A. oxydans* P52, but not in a derivative of this strain that had spontaneously lost the ability to hydrolyze phenylcarbamates, indicating that the gene for phenylcarbamate degradation (*pcd*) is plasmid encoded. Determination of two partial amino acid sequences allowed the localization of the coding sequence of the *pcd* gene on a 3.3-kb *Pst*I restriction fragment within pHP52 DNA by hybridization with synthetic oligonucleotides. The phenylcarbamate hydrolase was functionally expressed in *Escherichia coli* under control of the *lacZ* promoter after the 3.3-kb *Pst*I fragment was subcloned into the vector pUC19. A stretch of 1,864 bases within the cloned *Pst*I fragment was sequenced. Sequence analysis revealed an open reading frame of 1,479 bases containing the amino acid partial sequences determined for the purified enzyme. Sequence comparisons revealed significant homology between the *pcd* gene product and the amino acid sequences of esterases of eukaryotic origin. Subsequently, it was demonstrated that the esterase substrate *p*-nitrophenylbutyrate is hydrolyzed by phenmedipham hydrolase.

During the last 20 years, much attention has focused on the molecular mechanisms involved in the microbial degradation of herbicides and pesticides. For many extensively used compounds, the metabolic pathways involved in the degradation process are well characterized, and in many cases, the genes for the respective enzymes were shown to be plasmid encoded. The dissemination of biodegradative genes by bacterial plasmids has led to the rapid evolution of certain specialized strains capable of using these xenobiotic compounds as substrates. Besides this fact, one has to be aware that most pesticides are degraded by cometabolism (14). For organophosphate, carbamate, acylanilide, and phenylurea pesticides, hydrolases are important for the initial steps of degradation (14). Our interest has focused on the biodegradation of the phenylcarbamate herbicide phenmedipham [methyl(3-methylcarbaniloyloxy)carbanilate], which is commercially used in sugar beet (1). Besides some reports on the isolation of hydrolytic enzymes from microbial sources cleaving carbamate linkages (15, 16, 22, 24, 39, 40), little is known about the genes encoding these enzymes.

In this communication, the purification to homogeneity of a carbamate hydrolase from *Arthrobacter oxydans* P52, a physical map of the wild-type plasmid pHP52 harboring the gene, and the isolation as well as the nucleotide sequence of the *pcd* gene encoding the enzyme are reported. Some data on the properties of the enzyme are also presented. The sequence homology of the phenmedipham hydrolase (PMPH) with esterases of eukaryotic and prokaryotic origin is discussed. The isolation and characterization of a carbamate

hydrolase gene would make possible the genetic engineering of bacteria with enhanced or novel detoxification properties. Upon transfer of the gene to plants, the gene would then potentially be able to specify herbicide resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and growth conditions. Minimal medium (MM medium) used for the screening of soil bacteria and for the growth of *A. oxydans* P52 contained NH₄Cl (1.0 g/liter), MgSO₄ · 7H₂O (0.25 g/liter), KH₂PO₄ (3.0 g/liter), Na₂HPO₄ · 2H₂O (7.0 g/liter), NaCl (0.5 g/liter), glucose (2.0 g/liter), H₃BO₃ (0.5 µg/liter), CuSO₄ · 5H₂O (40 ng/liter), FeCl₃ · 6H₂O (0.2 µg/liter), MnSO₄ · 7H₂O (0.4 µg/liter), ZnCl₂ (0.4 µg/liter), and (NH₄)₆Mo₇O₂₄ · 7H₂O (0.2 µg/liter). Solid media (MM plates) were prepared with the addition of 1.5% agar (Difco). *A. oxydans* P52 was grown with aeration at 28°C. *Escherichia coli* DH5 (GIBCO-BRL) was used for subcloning experiments with the pUC19 vector (42). *E. coli* DH5 F' (GIBCO-BRL) was used for cloning in M13mp18 and M13mp19 bacteriophages. *E. coli* strains were grown in LB medium (25). LB medium was supplemented with ampicillin (100 µg/ml) when appropriate. All incubations were at 37°C.

Enzyme assays. For monitoring the enzyme activity of the phenylcarbamate hydrolase during purification, a semiquantitative radial plate-diffusion assay was developed. Agarose test gels containing 10 mM sodium phosphate (pH 6.8), 1 mM dithiothreitol, 0.5% (wt/vol) agarose (DNA grade; Bio-Rad), and 0.5 mg of phenmedipham per ml were poured into standard petri dishes (15 ml per dish). The water-insoluble

* Corresponding author.

herbicide was applied to the molten agarose as a microsuspension prepared by sonification. Sample wells were punched into the agar matrix, the protein solutions (5 to 30 μ l) were applied to the sample wells, and the test plates were subsequently incubated for 4 h at 30°C. Fractions containing the PMPH activity showed clear lysis zones around the sample wells against the turbid background of the test plate. This phenomenon is due to the very limited water solubility of the substrate phenmedipham and the good water solubility of the cleavage products. For substrate specificity analysis, various herbicidal active compounds carrying carbamate or amide linkages were incubated with 10 μ g of pure enzyme in a total volume of 1 ml of sodium phosphate buffer (pH 6.8) for 1 h at 37°C. The concentration of the herbicides in the assay was 3 to 20 μ g/liter depending on the water solubility of the compounds. At the end of the reaction, the enzyme was removed by ultrafiltration with a microconcentrator (Centricon 10; Amicon), and the herbicides and their hydrolysis products were analyzed by high-performance liquid chromatography (HPLC) on an ODS Hypersil column (5- μ m particle size, 4.6 mm by 12.0 cm; Shandon). The mobile phase, 0.25% (wt/vol) 1-decanesulfonic acid (sodium salt), 15% ammonium formate buffer (0.4 M) acidified to pH 3.4, 32% (vol/vol) methanol, and 6% (vol/vol) acetonitrile in HPLC-grade water, was delivered at a rate of 1.5 ml/min. Anilines were detected at 240 nm and identified by using possible hydrolysis products as standards.

Purification of phenylcarbamate hydrolase and protein sequencing. Cultures (6 liters) of *A. oxydans* P52 were grown in MM medium and were harvested in the late log phase by centrifugation at $7,000 \times g$ for 20 min. The cell pellet was suspended in 40 ml of 10 mM sodium phosphate (pH 6.8) containing 1 mM dithiothreitol, and the cells were homogenized by sonic disruption. The homogenate was then centrifuged at $40,000 \times g$ for 45 min to remove the cell debris. The supernatant was diluted 10-fold with buffer A (100 mM Tris hydrochloride [pH 7.2], 100 mM NaCl, 1 mM dithiothreitol) and applied to a DEAE-Sephacel column (20.5 by 2.6 cm) pre-equilibrated with buffer A. Subsequently, the column was washed with buffer A to remove unbound material. The PMPH was eluted with a linear 500-ml gradient to 500 mM NaCl. Enzymatically active fractions were pooled, and ammonium sulfate was added to 33% saturation. After stirring on ice for 1 h, the precipitated protein was collected by centrifugation ($20,000 \times g$, 30 min), dissolved in 1 ml of buffer A containing 10% (wt/vol) sucrose, and loaded on a Sephacryl S-300 column (95 by 2.6 cm) equilibrated with buffer A. The enzyme was eluted with buffer A at a flow rate of 2.5 cm h⁻¹. The fractions containing enzyme of the highest specific activity were pooled and applied to a Pharmacia Mono Q HR 5/5 fast protein liquid chromatography (FPLC) column (anion exchanger) equilibrated with buffer A. After removal of unbound material, the PMPH was eluted with a 20-ml linear gradient to 300 mM NaCl at a flow rate of 0.5 ml/min. The enzymatically active pool was concentrated to 0.1 ml (Centricon 10 concentrator; Amicon) and loaded on an FPLC gel filtration column (Superose 6 HR 10/30; Pharmacia) equilibrated with buffer A. The PMPH was eluted with buffer A at a flow rate of 0.2 ml/min. All operations except the FPLC steps were done at 4°C. The purified enzyme can be stored at -70°C in buffer A containing 20% (vol/vol) glycerol for at least 6 months without significant loss of activity.

Protein concentrations were determined by the procedure of Bradford (3), using bovine gamma globulin as the standard. Active column fractions were analyzed for purity by

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using the buffer system of Laemmli (17). After electrophoresis, the gels were stained with silver by the procedure of Morrissey (26) or with Coomassie brilliant blue R-250 (Serva) by the method of Diezel et al. (8). After purification, the protein was cleaved with cyanogen bromide essentially as described by Gross and Witkop (10). Cleavage peptides were separated on SDS-polyacrylamide gels by the method of Schagger and von Jagow (30) and electroblotted on Immobilon polyvinylidene difluoride membrane filters (Millipore). Amino acid sequencing of two immobilized protein fragments (peptides PI and PII) was done by the method of Matsudaira (23) with an Applied Biosystems 477A protein sequencer equipped with a 120A amino acid analyzer.

DNA isolation. The wild-type plasmid pHP52 harbored by *A. oxydans* P52 was extracted by the alkaline lysis method of Birnboim and Doly (2) with a modification described by Brandsch and Decker (4). The clear lysate obtained from a 6-liter bacterial culture grown to an optical density at 550 nm of 1.4 was further purified by centrifugation to equilibrium in a cesium chloride-ethidium bromide gradient (21). Recombinant plasmid DNAs were isolated by the method of Birnboim and Doly (2).

Recombinant DNA techniques. DNA digestions, electrophoresis of DNA, and other DNA manipulations were performed as described by Maniatis et al. (21).

Oligonucleotide probes. Two oligonucleotides of defined sequence, derived from the two partial amino acid sequences of peptides PI and PII, were synthesized as gene probes. At positions of ambiguity, guanosine or cytosine nucleotides were incorporated. The sequence of each oligonucleotide was complementary to the coding sequence. Thus, oligonucleotide I had the sequence 5'-GGGGTCCAGGCCGTCCA CGAACGGCTTGCCGGTCCAGCGGTC-3' derived from peptide PI, and for oligonucleotide II, the sequence 5'-TT CGTCGAAGACCGGGTAGAACGC-3' derived from peptide PII was chosen. The oligonucleotides were synthesized by the β -cyanoethyl phosphoramidite method with an Applied Biosystems 381A synthesizer. Oligonucleotides were 5' end labelled with [γ -³²P]ATP and T4 polynucleotide kinase (Boehringer Mannheim) as described by Maniatis et al. (21).

Southern blot analysis and hybridization. Restriction digests of pHP52 plasmid DNA were separated in 0.8% agarose gels and blotted onto GeneScreen Plus membranes (NEN Research Products) according to the manufacturer's recommendation. The blots were hybridized to the radiolabelled oligonucleotides I and II (10 ng/ml) at 40°C in 6 $\times$ SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7.2), 10 $\times$ Denhardt's solution (15), 0.5% (wt/vol) SDS, and 100 μ g of tRNA per ml from baker's yeast (Boehringer Mannheim). Unbound probe was removed by four subsequent washes at 40°C in 6 $\times$ SSC-0.5% SDS.

DNA subcloning. A 3.3-kb *Pst*I restriction fragment which was shown to hybridize with oligonucleotides I and II was separated by preparative low-melting-temperature agarose gel electrophoresis and isolated from the gel matrix (41). The fragment was subsequently ligated to *Pst*I-cleaved, dephosphorylated pUC19 DNA and then transformed into *E. coli* DH5 competent cells (GIBCO-BRL). The resulting recombinant plasmids pp52Pst and pp52Pst(inv) contained the insert in opposite orientations.

DNA sequencing. Plasmid pp52Pst carrying the carbamate hydrolase gene was purified by cesium chloride-ethidium bromide gradient centrifugation. Overlapping restriction

FIG. 1. Hydrolytic cleavage of the herbicides phenmedipham and desmedipham catalyzed by the phenylcarbamate hydrolase of *A. oxydans* P52.

fragments of the insert covering the whole coding region of the gene were isolated after electrophoresis and cloned into M13mp18 and M13mp19 phage vectors. The single-stranded phage DNAs were sequenced by the dideoxy-chain termination method (29) with the Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) and α - ^{35}S -thio-dATP (800 Ci/mmol; Amersham) according to the user instructions provided with the kit. The products of the sequence reactions were separated on 6% polyacrylamide–8 M urea gels.

Computer analysis of sequence data. Sequence assembly, data base searches, comparisons, and alignments were done with the GCG SEQUENCE ANALYSIS SOFTWARE PACKAGE version 6.2 (7) and the protein data base NBRF-Protein (release 25.0, June 1990) and SwissProt (release 15.0, August 1990) obtained from Genetics Computer Group, Inc. (University Research Park, Madison, Wis.).

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number is M94965.

RESULTS

Isolation and characterization of soil bacteria. A collection of 112 bacterial strains derived from various samples of soils treated with phenmedipham was tested for the ability to metabolize the herbicide by hydrolyzing the central carbamate linkage as depicted in Fig. 1. For the identification of bacteria performing the desired cleavage reaction, the bacterial suspensions from the soil samples were first plated on MM plates (see below) containing the herbicide in a concentration of 1 g/liter. Because of the poor water solubility of phenmedipham (4.7 mg/liter) and the good solubility of the hydrolysis products, the colonies of the bacteria that degrade phenmedipham were surrounded by clear lysis zones beyond the turbid background of the agar plates. The bacteria showing this lysis effect were grown in liquid MM medium supplemented with the herbicide. The identity of the cleavage products was then confirmed by HPLC analysis of the culture supernatants (data not shown).

Among the various isolates cometabolizing phenmedipham, strain P52 was chosen for further analysis. This strain was characterized by Gram staining, light microscopy, peptidoglycan analysis, characterization of cell wall sugars, and evaluation of various metabolic parameters. The bacteria were shown to be gram-positive, immobile, nonsporulating, strictly aerobic, katalase-positive rods of the corynebacterial type. The peptidoglycan was of the type A3 α (Lys-Ser-Thr-Ala), and galactose and rhamnose were identified as cell wall

FIG. 2. Purification of the phenylcarbamate hydrolase. SDS-PAGE of enzymatically active protein fractions. The internal protein standards marked 1 to 4 in lanes M were phosphorylase *b* (band 1, 97,400), bovine serum albumin (band 2, 66,200), ovalbumin (band 3, 42,700), carbonic anhydrase (band 4, 31,000). Lanes: A, sonic extract of *A. oxydans* P52 after centrifugation; B, DEAE-Sephacel pool; C, ammonium sulfate fraction (33 to 45% saturation); lane D, Sephacryl S-300 pool; lane E, Mono Q pool; lane F, Superose 6 pool. The gel was stained with Coomassie brilliant blue R250 as described in Materials and Methods.

sugars. The strain was able to utilize starch but not to produce acid from glucose. According to this investigation, strain P52 has to be classified as *A. oxydans*. *A. oxydans* P52 is deposited in the German Type Culture Collection under the code number DSM 4044.

Purification, partial amino acid sequencing, and properties of phenylcarbamate hydrolase of *A. oxydans* P52. Starting from a liquid culture of *A. oxydans* P52 in MM medium, a method for the purification of carbamate hydrolase to electrophoretic homogeneity was developed. The purification method involved standard chromatographic procedures such as anion-exchange chromatography on DEAE-Sephacel and Mono Q columns (Pharmacia), two gel filtration steps, and ammonium sulfate fractionations as described above. The material from the last purification step typically showed a single band when analyzed by SDS-PAGE (Fig. 2). By comparing the electrophoretic mobility of the purified carbamate hydrolase with several standard proteins, an apparent molecular weight of 53,000 was determined by SDS-PAGE. The molecular weight of the native enzyme was determined by gel filtration on an FPLC system with a Superose 6 HR 10/30 column. The column was calibrated with RNase A (13,000), chymotrypsinogen A (25,000), ovalbumin (43,000), bovine serum albumin (67,000), and aldolase (153,000). A native molecular weight of 55,000 ($\pm 1,500$) was determined for phenylcarbamate hydrolase in five independent chromatographic runs. From these data, it was concluded that the enzyme is a monomer.

Because the protein was N-terminally blocked after the first amino acid sequencing attempt, partial amino acid sequences of two cyanogen bromide cleavage peptides were determined by automatic Edman degradation. For peptides PI and PII, H_2N -Phe-Ala-Asn-Leu-Asp-Arg-Trp-Thr-Gly-Lys-Pro-Phe-Val-Asp-Gly-Leu-Asp-Pro-COOH and H_2N -Glu-His-Thr-Lys-Phe-Gly-Glu-X-Pro-Leu-Ala-Phe-Tyr-Pro-Val-Phe-Asp-Glu-COOH, respectively, could be determined as the partial sequences. Amino acid position 8 of peptide PII (marked with an X) could not be identified unequivocally.

Phenylcarbamate hydrolase of *A. oxydans* P52 is plasmid encoded. (i) **Isolation and restriction mapping of the wild-type plasmid pAO52.** When bacteria originating from a single

FIG. 3. Cloning of the *pcd* gene. The upper part shows a map of the wild-type plasmid pHP52. The restriction map of the subcloned 3.3-kb *Pst*I fragment harboring the *pcd* gene is shown in the lower part of the figure. The positions of the GTG start codon and the TGA stop codon are indicated. Fragments hybridizing to the oligonucleotides are shown as solid boxes. The vector parts of the pUC19 plasmids containing the insert are indicated by open boxes; the transcriptional orientation of the *lac* promoter (p_{lac}) is indicated by an arrow. The sequencing strategy is shown by arrows indicating the extent of nucleotides sequenced starting from the single-stranded DNA of the M13 subclones A to P. Abbreviations: Bg, *Bgl*II; Bm, *Bam*HI; Bs, *Bst*EII; Cl, *Cla*I; EV, *Eco*RV; Hd, *Hind*III; Hn, *Hind*II; Kp, *Kpn*I; Ml, *Mlu*I; Nc, *Nco*I; Nd, *Nde*I; Nh, *Nhe*I; Ps, *Pst*I; Pu, *Pvu*II; Pv, *Pvu*I; Sc, *Sca*I; Se, *Spe*I; Sp, *Sph*I; St, *Stu*I; Xb, *Xba*I; Xh, *Xho*I.

hydrolase-positive colony were replated on phenmedipham-containing agar plates, about 1 colony in 500 had lost the ability to hydrolyze phenmedipham. From this phenotypic variant (*A. oxydans* P52PMPH⁻), a protein extract was prepared which showed no hydrolase activity. There was no obvious difference in the protein pattern of *A. oxydans* P52 and P52PMPH⁻ as judged by SDS-PAGE. Thus, it was then necessary to investigate whether the phenotypic change was due to the loss of a plasmid encoding the carbamate hydrolase. Starting from five individual colonies of *A. oxydans* P52 and *A. oxydans* P52PMPH⁻, respectively, small-scale plasmid lysates (2) were prepared. The nucleic acid extracts were cleaved with *Bam*HI and electrophoretically separated in an 0.8% agarose gel. In the extracts from the hydrolase-positive strains, five distinct bands (>15, 3.9, 3.4, 1.0, and 0.9 kb) were clearly visible, whereas no such bands could be detected in the hydrolase-negative strains. This result was further confirmed by cleavage with other restriction en-

zymes and by electrophoretic analysis of the uncleaved extracts: plasmid bands belonging to the supercoiled, open circular, and linear forms were only visible in the extracts of the hydrolase-positive strains. The plasmid pHP52 was isolated on a preparative scale as described above and mapped with restriction enzymes by single and double digestions. A plasmid map of pHP52, which is 41 kb in size, is shown in Fig. 3. It was striking that the plasmid was very frequently cleaved by restriction endonucleases containing G+C-rich recognition sequences. As an example, the restriction endonuclease *Not*I with the 8-bp recognition sequence 5'-GCGGCCGC-3' recognizes 12 cleavage sites in pHP52 DNA (data not shown).

(ii) **Localization of phenylcarbamate hydrolase gene *pcd* on plasmid pHP52 by oligonucleotide hybridization.** Deduced from the partial sequences of cyanogen bromide peptides I and II, two oligonucleotides complementary to the coding sequences were synthesized (oligonucleotides I and II, see

TABLE 1. Substrate specificity of phenylcarbamate hydrolase

Substrate ^a	Relative activity (%)
Phenmedipham [methyl(3-methylcarbaniloxyloxy)carbanilate].....	100 ^b
Desmedipham (ethyl 3'-phenylcarbamoyloxy-carbanilate).....	42
Propanil [N-(3,4 dichlorophenyl)propanamide]	1.3
CIPC [isopropyl N-(3-chlorophenyl)carbamate]	<1.0
Phenisopham (propyl N-ethyl 3'-phenylcarbamoyloxy-carbanilate)	0
Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea]	0

^a Samples were incubated at a final concentration of 15 μ M.

^b The conversion of 0.17 μ mol of phenmedipham per min per mg of protein was taken as 100%.

above). Because of the high G+C content of the pHP52 plasmid DNA, guanosine or cytosine nucleotides were incorporated at the wobble positions. The electrophoretically separated restriction fragments of pHP52 used for the mapping studies were blotted to filter membranes and hybridized to oligonucleotides I and II. Under the hybridization conditions used, a specific signal was obtained with both oligonucleotides which clearly maps to the central part of a 3.3-kb *Pst*I restriction fragment (Fig. 3).

Cloning of *pcd* gene and its functional expression in *E. coli*. The 3.3-kb *Pst*I restriction fragment of pHP52 was isolated by preparative gel electrophoresis and ligated to *Pst*I-cleaved dephosphorylated pUC19 DNA. After transformation into *E. coli* DH5, the subclones pp52Pst and pp52Pst(inv) were obtained which carried the insert in opposite orientations. To investigate whether the *pcd* gene was functionally expressed under the control of its own promoter, we prepared crude protein extracts of pp52Pst and pp52Pst(inv) grown in the presence or absence of the *lac* promoter inducer IPTG (isopropyl- β -D-thiogalactopyranoside). Using the radial diffusion assay, phenylcarbamate hydrolase activity could only be detected in the extract of subclone pp52Pst grown in the presence of IPTG. This suggested that the *lac* promoter of the vector pUC19 drove the expression of the *pcd* gene in *E. coli* and that the *A. oxydans* promoter was not active.

Substrate specificity of phenylcarbamate hydrolase. The enzyme expressed in *E. coli* was purified and used for substrate specificity analysis of various pesticidal active compounds containing carbamate or amide linkages. Compounds were incubated with the enzyme, and the release of the respective anilines was measured by HPLC as described above. As depicted in Table 1, the enzyme exhibited a rather narrow substrate specificity. The diphenylcarbamates phenmedipham and desmedipham (a close analog of phenmedipham) were hydrolyzed at comparable rates, whereas phenisopham as a compound with an additional alkyl substitution at the carbamate nitrogen was not hydrolyzed at a detectable rate. Chlorpropham (CIPC), which contains a carbamate group with aryl and alkyl substitutions, and the acylanilide propanil were hydrolyzed about 100 times slower than phenmedipham. The herbicide diuron was not attacked by the carbamate hydrolase. The striking sequence homology of the carbamate hydrolase with esterases led us to investigate whether the enzyme is active on the typical esterase substrate, *p*-nitrophenylbutyrate (NPB). Indeed, NPB was readily cleaved in the presence of the carbamate

hydrolase. The K_m of the carbamate hydrolase for NPB was 25 μ M.

Nucleotide sequence of *pcd* gene. A sequence of 1,867 bases within the cloned 3.3-kb *Pst*I fragment was determined in both directions. For this purpose, various subfragments of this region were cloned in M13 as depicted in Fig. 3. The reading frame of the gene was clearly identified after formal translation of the DNA sequence by the two partial amino acid sequences obtained from the purified protein (Fig. 4). It ends with a TGA stop codon at base 1789. The only open reading frame that potentially can code for a protein with a molecular weight in the range of 55,000 begins with a GTG start codon at base 310. The putative coding region of the carbamate hydrolase gene is preceded by the sequence AGG, a probable ribosome binding site (33, 37) located 8 bp upstream from the start codon (Fig. 4). Furthermore, the reading frame was recognized as 100% coding in a computer analysis using the program TESTCODE, which is based on the algorithm of Fickett (9).

Phenylcarbamate hydrolase of *A. oxydans* shows significant sequence homology to esterases of eukaryotic origin. The amino acid sequence of PMPH deduced from the nucleotide sequence of the *pcd* gene was used as a query sequence in the data base-searching procedure FASTA. This program performs a Pearson and Lipman search for similarity between a query sequence and any group of sequences and lists the best matches according to their scores. The search revealed that the query sequence, which was not present in the data base itself, showed significant homology (>50% similarity, >25% identity) to a set of eukaryotic carboxylesterases (Table 2). Of the 13 best-matching sequences, 5 esterases of different types and origins were chosen to make sequence alignments (Fig. 5). Regions of high homology were found in the N-terminal and the central parts of the proteins. A putative active-site serine was identified in all aligned sequences by the consensus sequence Gly-X-Ser-X-Gly (20). Besides the active center, the aligned sequences share homology boxes which are highly conserved within several types of carboxylesterases (Fig. 5). This finding is consistent with the PMPH activity on NPB as a substrate and suggests that phenmedipham carbamate hydrolase can be classified as a carboxylesterase and thus be assigned to the EC sub-sub-group 3.1.1.

DISCUSSION

In a microbial screening starting from soil samples of phenmedipham-treated fields, we isolated *A. oxydans* P52 as a strain capable of hydrolyzing the central carbamate linkage of the herbicide. A monomeric phenylcarbamate hydrolase of about 55,000 molecular weight was subsequently purified from the bacterium to homogeneity. Plasmid curing experiments revealed that the *pcd* gene coding for the enzyme resides on the degradative plasmid pHP52 found in *A. oxydans* P52. Oligonucleotides derived from two partial amino acid sequences of the enzyme were used to localize the *pcd* gene on a 3.3-kb *Pst*I restriction fragment of plasmid pHP52. Cloning of this fragment with pUC19 as a vector showed that PMPH was only expressed in *E. coli* when transcribed under the control of the *lacZ* promoter. This result may indicate that the *Arthrobacter* promoter for *pcd* is inactive in *E. coli*. This is in agreement with a report by Roberts et al. (28), who demonstrated that the *ermA* gene of an *Arthrobacter* sp. coding for erythromycin resistance is not expressed in *E. coli*. A report of Brandsch et al. (5) is in contrast to these findings. Their results indicate that the gene

10 30 50 70 90 110
 GCTTCACAGCCGCGACCACTAACCTTCGCGCGCAGTGCAGCTGACACAGTTATCAGCCGGTCTGGACATCCTTGCCAGTCACGGCACCCGACCCCGGAAG
 130 150 170 190 210 230
 TGGCACCTGCTCGGGACATCGGTGCGAAGCTTCCTCTGATTCGATGCCAACTGCTTGACGGCGGTGACACATATGTAGCATAGTCGCTAGCATGGACCCGACGACACCTGCTGT
 250 270 290 310 330 350
 CGGCTCCCGCGCTATCCCGACAGCGCGGTACGGGTAGTCTCTGAGAGGACACAGAACGACACGGCGCACTGTCCCGCAACACGGCGGTATAACCCACCGGGTCCGCGCCG
 370 390 410 430 450 470
 AGCTAGTTCTGGCTCAACCATAAGGAAACTTCGTGATTACACAGCCGATCGCCACACACCGCTGGGGACCTCGGCGGTGCTTGAAGACGGCTGTACGTGTCCGAGGAGTGGCG
 MetIleThrArgProIleAlaHisThrThrAlaGlyAspLeuGlyGlyCysLeuGluAspGlyLeuTyrValPheArgGlyValPro
 S/D
 490 510 530 550 570 590
 TACGCCGAGCCGCGTGGCGACCTCGGTGGCGGGCGCGCGCGCGCGCGCTGGACCGGCTCGCGGATGCTCCCTCGCGCGCGCAACCCGCTGGAGCTGGC
 TyrAlaGluProProValGlyAspLeuArgTrpArgAlaAlaArgProHisAlaGlyTrpThrGlyValArgAspAlaSerAlaTyrGlyProSerAlaProGlnProValGluProGly
 610 630 650 670 690 710
 GGCTCGCCGATCCTTGGGACACCGGCGACCTCCGTTTGACGAGGACTGCCTGACTCTCAATCTTTGGACCCGAACTCGACGGCGGTAGCCGCGGTCTCGTCTGGATCCATGGT
 GlySerProIleLeuGlyThrHisGlyAspProPheAspGluAspCysLeuThrLeuAsnLeuTrpThrProAsnLeuAspGlyGlySerArgProValLeuValTrpIleHisGly
 730 750 770 790 810 830
 GGGGCGCTACTAACCGCTCGGGAACTTACCTACGCGACCGATACCTTCGCGCGCGAGCGGCACTTGGTAGGTATCTCAATCAATTACCGGCTCGGCGCTCTGGATTCTCGCA
 GlyGlyLeuLeuThrGlySerGlyAsnLeuProAsnTyrAlaThrAspThrPheAlaArgAspGlyAspLeuValGlyIleSerIleAsnTyrArgLeuGlyProLeuGlyPheLeuAla
 850 870 890 910 930 950
 GGAAATGGCGCAGAGAATGTCTGGCTACCGATCAGGTAGAGGCACTGCGCTGGATTGCAGATAACCTTGGCTTGGTGGAGACCCGATCACTCTCGTCTCAATCAGGC
 GlyMetGlyAspGluAsnValTrpLeuThrAspGlnValGluAlaLeuArgTrpIleAlaAspAsnValAlaAlaPheGlyGlyAspProAsnArgIleThrLeuValGlyGlnSerGly
 970 990 1010 1030 1050 1070
 GGGGCATCTCGATCGACGCGCTCCCAACACCGCTCGCCGTCAGCTGTCCACCGCGCATCTTACAAAGCCCACTTCGGGATGCAACCCCATACAGTTGAAGAATCGACGGCA
 GlyAlaTyrSerIleAlaAlaLeuAlaGlnHisProValAlaArgGlnLeuPheHisArgAlaIleLeuGlnSerProProPheGlyMetGlnProHisThrValGluGluSerThrAla
 1090 1110 1130 1150 1170 1190
 AGGACGAAGCCCTGGCCCGCATCTCGGCGCAGTACATCGAGGCGCTCGCCATGAGCCGTTGGGAGAGGCTGATTCAAGGCACGATAGGCGTCTGATGGAACACCAAAATTTGGC
 ArgThrIysAlaLeuAlaArgHisLeuGlyHisAspAspIleGluAlaLeuArgHisGluProTrpGluArgLeuIleGlnGlyThrIleGlyValLeuMetGluHisThrLysPheGly
 1210 1230 1250 1270 1290 1310
 GAATGGCCCTGGCATTCTATCCGGTGTTCGATGAGGCAACGATACCTCGCCATCCGATTGAGTCCATTATCGATTCCGACATCGAAATCATCATCGGCTGGACACGCGACGGGCACT
 GluTrpProLeuAlaPheTyrProGlnValThrIleProArgHisProIleGluSerIleIleAspSerAspIleGluIleIleIleGlyTrpThrArgAspGluGlyThr
 1330 1350 1370 1390 1410 1430
 TTTCCGTTTGGCTTCGACCCCTCAGGTTTCACAGGCGGATCGCGATCAGGTGAGTATGTTGCAGAGCGTTTCGGAGACCAACCGCCCTCGGCTACGAGGCTCAGCGCGCGACGGA
 PheProPheAlaPheAspProGlnValSerGlnAlaAspArgAspGlnValGluSerTrpLeuGlnLysArgPheGlyAspHisAlaAlaSerAlaTyrGluAlaHisAlaGlyAspGly
 1450 1470 1490 1510 1530 1550
 ACCAGTCTTTGACCGTTATCGCAACGTTGTGGCGCAGAGCTCTTTCACAGCGCTGGGTACCGGTGCGGACGAACGGGCAACGCGCAGCGGTACGGGCTATCAGTTTCGACGTA
 ThrSerProTrpThrValIleAlaAsnValValGlyAspGluLeuPheHisSerAlaGlyTyrArgValAlaAspGluArgAlaThrArgArgProValArgAlaTyrGlnPheAspVal
 1570 1590 1610 1630 1650 1670
 GTCTCGCCCTTGTGGAGGAGCCCTCGGCGCGGTCCATGCTGCAATGCGGTTTGCATTCGACCGTGGACGGGAGCCGTTCTGTTGGACGCGCTGGATCCAGACGTG
 ValSerProLeuSerAspGlyAlaLeuGlyAlaValHisCysIleGluMetProPheThrPheAlaAsnLeuAspArgTrpThrGlyLysProPheValAspGlyLeuAspProAspVal
 1690 1710 1730 1750 1770 1790
 GTGGCTCGGCTGACCAACGTTGTCATCAGGCTGATCGCATTCGTAACGAGAGACCCACGACGACAGTTCGCGGTGTGGCCCAACGTTCCGAGCGGACGACGCGGTGTG
 ValAlaArgValThrAsnValLeuHisGlnAlaTrpIleAlaPheValArgThrGlyAspProThrHisAspGlnLeuProValTrpProThrPheArgAlaAspAspProAlaValLeu
 1810 1830 1850 1870 1890 1910
 GTCGTGCGCGACGAGGAGCAGAGGTGGCGCGGATCTAGCGCGCCCGGACACGCTACGCGTTCGACCTATGAGGTGCGCGGTGCGCGGGTCTTGAGGCGGAGGGCTCGCGTAT
 ValValGlyAspGluGlyAlaGluValAlaAlaArgAspLeuAlaArgProAspHisValSerValArgThrLeu***
 1930
 GCAGTGATTCTGGATCACCAGGCGAGT

FIG. 4. Nucleotide sequence of *pcd* gene. The coding strand of the DNA is presented in the 5'-to-3' direction. Codon-specified amino acids are displayed below the sequence. A putative Shine-Dalgarno sequence (S/D) upstream is underlined. The two underlined amino acid sequence stretches correspond to the sequences determined by sequence analysis of cleavage peptides as described in the Results.

for 6-hydroxy-D-nicotine oxidase harbored by plasmid pAO1 of *A. oxydans* may be expressed from its own promoter in *E. coli*. From our data, it cannot be strictly ruled out, however, that the *pcd* gene may be part of a polycistronic transcription unit, so there may be no *Arthrobacter* promoter on the pUC subclones we tested.

Phenmedipham is a xenobiotic compound which has no equivalent in nature. The question is raised, what could be the natural substrate for the enzyme? The fact that compounds with a carbamate linkage are rarely found in a natural environment renders it unlikely that the enzyme's activity is

restricted to cleavage of carbamate groups. Many hydrolases are known to act on carbamates, esters, and amides; cholinesterases of different origins exhibit additional hydrolytic activity on peptide bonds (35). A degradative enzyme from a *Pseudomonas* sp. hydrolyzing the phenylcarbamate isopropyl *N*-(3-chlorophenyl)carbamate (CIPC) isolated by Kearney and Kaufman (15, 16) was shown to be also active on esters and acylanilides. A similar broad substrate specificity was described for a hydrolytic enzyme isolated from *Pseudomonas alcaligenes* (22) degrading a wide range of phenylcarbamate herbicides like CIPC but also acylanilides

TABLE 2. Comparisons of amino acid sequence between *A. oxydans* phenylcarbamate hydrolase and esterase from various organisms

Enzyme	Code	Species	% Identity	% Similarity	Reference
Acetylcholinesterase	EC 3.1.1.7.	<i>Torpedo californica</i>	31.8	54.0	31
		<i>Torpedo marmorata</i>	30.5	53.6	34
		<i>Drosophila melanogaster</i>	32.2	54.3	11
Cholinesterase	EC 3.1.1.8.	<i>Homo sapiens</i>	30.5	54.6	18
Carboxylesterase	EC 3.1.1.1.	<i>Rattus norvegicus</i>	30.0	52.3	19
		<i>Drosophila melanogaster</i>	25.4	51.0	27
Triacylglycerol lipase	EC 3.1.1.3.	<i>Geotrichum candidum</i>	31.5	53.8	32
Lysophospholipase	EC 3.1.1.5.	<i>Rattus norvegicus</i>	28.6	51.5	12
Juvenile hormone esterase	EC 3.1.1.59.	<i>Heliothis virescens</i>	26.2	49.3	13

FIG. 5. Alignment of the amino acid sequence of PMPH (PMPH Art) with five esterase sequences of different origin. Abbreviations: TGL Geo, triacylglycerol lipase from *Geotrichum candidum* (32); CaE Rat, carboxylesterase from *Rattus norvegicus* (19); CaE Dro, carboxylesterase from *Drosophila melanogaster* (27); LPL Rat, lysophospholipase from *R. norvegicus* (12); CheE Man, cholinesterase from *Homo sapiens* (18). The position of the putative active-site serine residues is marked with an asterisk.

Isolation of the *pcd* gene is a prerequisite for the introduction and expression of this bacterial gene in phenmedipham-sensitive crop plants to confer resistance. The general approach of introducing bacterial genes for herbicide-metabolizing enzymes into plants to enhance their herbicide tolerance has already been successful in a number of cases (6, 36, 38). Recent experiments in our laboratory (37a) indicate that the expression of the *pcd* gene in tobacco confers resistance to the herbicides phenmedipham and desmedipham.

We are grateful to U. Eder, G. Siewert, C. von Szczepanski, and L. Willmitzer for helpful advice, encouragement, and support, to J.

REFERENCES

1. **Arndt, F., and C. Kötter.** 1968. Selectivity of phenmedipham as a post emergence herbicide in sugar beet. *Weed Res.* **8**:259-271.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513-1523.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-254.
4. **Brandsch, R., and K. Decker.** 1984. Isolation and partial characterization of plasmid DNA from *Arthrobacter oxydans*. *Arch. Microbiol.* **138**:15-17.
5. **Brandsch, R., W. Faller, and K. Schneider.** 1986. Plasmid pAO1 of *Arthrobacter oxydans* encodes 6-hydroxy-D-nicotine oxidase: cloning and expression of the gene in *Escherichia coli*. *Mol. Gen. Genet.* **202**:96-101.
6. **De Block, M., J. Botterman, M. Vandewiele, J. Dockx, C. Thoen, V. Gossele, N. Rao Movva, C. Thompson, M. Van Montagu, and J. Leemans.** 1987. Engineering herbicide resistance in plants by expression of a bacterial detoxifying enzyme. *EMBO J.* **6**:2513-2518.
7. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387-395.
8. **Diezel, W., G. Koppenschläger, and F. Hoffman.** 1972. An improved procedure for protein staining in polyacrylamide gels with a new type of Coomassie brilliant blue. *Anal. Biochem.* **48**:617-620.
9. **Fickett, J. W.** 1982. Recognition of protein coding regions in DNA sequences. *Nucleic Acids Res.* **10**:5303-5318.
10. **Gross, E., and B. Witkop.** 1961. Selective cleavage of the methionyl peptide bonds in ribonuclease with cyanogen bromide. *J. Am. Chem. Soc.* **83**:1510-1511.
11. **Hall, L. M., and P. Spierer.** 1986. The *Ace* locus of *Drosophila melanogaster*: structural gene for acetylcholinesterase with an unusual 5' leader. *EMBO J.* **5**:2949-2954.
12. **Han, J. H., C. Stratowa, and W. J. Rutter.** 1987. Isolation of full-length putative rat lysophospholipase cDNA using improved methods for mRNA isolation and cDNA cloning. *Biochemistry* **26**:1617-1625.
13. **Hanzlik, T. N., A. I. A.-A. Yehia, L. G. Harshman, and B. D. Hammock.** 1989. Isolation and sequencing of cDNA coding for juvenile hormone esterase from *Heliothis virescens*. *J. Biol. Chem.* **264**:12419-12425.
14. **Johnson, L. M., and H. W. Talbot.** 1983. Detoxification of pesticides by microbial enzymes. *Experientia* **39**:1236-1246.
15. **Kearney, P. C.** 1965. Purification and properties of an enzyme responsible for hydrolyzing phenylcarbamates. *J. Agric. Food Chem.* **13**:561-564.
16. **Kearney, P. C., and D. D. Kaufman.** 1965. Enzyme from soil bacterium hydrolyzes phenylcarbamate herbicides. *Science* **147**:740-741.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
18. **Lockridge, O., C. F. Bartels, T. A. Vaughan, C. K. Wong, S. E. Norton, and L. L. Johnson.** 1987. Complete amino acid sequence of human serum cholinesterase. *J. Biol. Chem.* **262**:549-557.
19. **Long, R. M., H. Satoh, B. M. Martin, S. Kimura, F. J. Gonzalez, and L. R. Pohl.** 1988. Rat liver carboxylesterase: cDNA cloning, sequencing, and evidence for a multigene family. *Biochem. Biophys. Res. Commun.* **156**:866-873.
20. **MacPhee-Quigley, K., P. Taylor, and S. Taylor.** 1985. Primary structures of the catalytic subunits from two molecular forms of acetylcholinesterase. A comparison of NH₂-terminal and active center sequences. *J. Biol. Chem.* **260**:12185-12189.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Marty, J. L., and J. Vouges.** 1987. Purification and properties of

- a phenylcarbamate herbicide degrading enzyme of *Pseudomonas alcaligenes* isolated from soil. Agric. Biol. Chem. 51:3287-3292.
23. Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. 262:10035-10038.
24. McClure, G. W. 1974. Degradation of anilide herbicides by protham adapted microorganisms. Weed Sci. 22:323-329.
25. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. Morrissey, J. H. 1981. Silver stain from protein in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. 117:307-310.
27. Oakeshott, J. G., C. Collett, R. W. Phillis, K. M. Nielsen, R. J. Russell, G. K. Chambers, V. Ross, and R. C. Richmond. 1987. Molecular cloning and characterization of esterase-6, a serine hydrolase of *Drosophila*. Proc. Natl. Acad. Sci. USA 84:3359-3363.
28. Roberts, A. N., L. Barnett, and S. Brenner. 1987. Transformation of *Arthrobacter* and studies on the transcription of the *Arthrobacter ermA* gene in *Streptomyces lividans* and *Escherichia coli*. Biochem. J. 243:431-436.
29. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.
30. Schagger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166:368-379.
31. Schumacher, M., S. Camp, Y. Maulet, M. Newton, K. MacPhee-Quigley, S. S. Taylor, T. Friedmann, and P. Taylor. 1986. Primary structure of *Torpedo californica* acetylcholinesterase deduced from its cDNA sequence. Nature (London) 319:407-409.
32. Shimada, Y., A. Sugihara, Y. Tominaga, T. Iizumi, and S. Tsunasawa. 1989. cDNA molecular cloning of *Geotrichum candidum* lipase. J. Biochem. 106:383-388.
33. Shine, J., and L. Dalgarno. 1974. The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to non-sense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA 71:1342-1346.
34. Sikorav, J.-L., E. Krejci, and J. Massoulie. 1987. cDNA sequences of *Torpedo marmorata* acetylcholinesterase: primary structure of the precursor of a catalytic subunit; existence of multiple 5'-untranslated regions. EMBO J. 6:1865-1873.
35. Small, D. H. 1990. Non-cholinergic actions of acetylcholinesterases: proteases regulating cell growth and development? Trends Biochem. Sci. 15:213-216.
36. Stalker, D. M., K. E. McBride, and L. D. Malyj. 1988. Herbicide resistance in transgenic plants expressing a bacterial detoxification gene. Science 242:419-423.
37. Stormo, G. D., T. D. Schneider, and L. Gold. 1982. Characterization of translational initiation starts in *Escherichia coli*. Nucleic Acids Res. 10:2971-2996.
- 37a. Streber, W. R., U. Kutschka, and H.-D. Pohlenz. Unpublished data.
38. Streber, W. R., and L. Willmitzer. 1989. Transgenic tobacco plants expressing a bacterial detoxifying enzyme are resistant to 2,4-D. Bio/Technology 7:811-816.
39. Tomasek, P. H., and J. S. Karns. 1989. Cloning of a carbofuran hydrolase gene from *Achromobacter* sp. strain WM111 and its expression in gram-negative bacteria. J. Bacteriol. 171:4038-4044.
40. Westmacott, D., and S. J. L. Wright. 1975. Studies on the breakdown of *p*-chlorophenyl methylcarbamate. II. In cultures of a soil *Arthrobacter* sp. Pestic. Sci. 6:61-68.
41. Wieslander, L. 1979. A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. Anal. Biochem. 98:305-309.
42. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103-119.