Cloning and Sequencing of a Plasmid-Borne Gene (opd) Encoding a Phosphotriesterase

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Plasmid pCMS1 was isolated from *Pseudomonas diminuta* MG, a strain which constitutively hydrolyzes a broad spectrum of organophosphorus compounds. The native plasmid was restricted with *PstI*, and individual DNA fragments were subcloned into pBR322. A recombinant plasmid transformed into *Escherichia coli* possessed weak hydrolytic activity, and Southern blotting with the native plasmid DNA verified that the DNA sequence originated from pCMS1. When the cloned 1.3-kilobase fragment was placed behind the lacZ' promoter of M13mp10 and retransformed into *E. coli*, clear-plaque isolates with correctly sized inserts exhibited isopropyl-β-D-thiogalactopyranoside-inducible whole-cell activity. Sequence determination of the M13 constructions identified an open reading frame of 975 bases preceded by a putative ribosome-binding site appropriately positioned upstream of the first ATG codon in the open reading frame. An intragenic fusion of the opd gene with the lacZ gene produced a hybrid polypeptide which was purified by β-galactosidase immunoaffinity chromatography and used to confirm the open reading frame of opd. The gene product, an organophosphorus phosphotriesterase, would have a molecular weight of 35,418 if the presumed start site is correct. Eighty to ninety percent of the enzymatic activity was associated with the pseudomonad membrane fractions. When dissociated by treatment with 0.1% Triton and 1 M NaCl, the enzymatic activity was associated with a molecular weight of approximately 65,000, suggesting that the active enzyme was dimeric.

Synthetic organophosphorus neurotoxins are used extensively as agricultural and domestic pesticides including insecticides, fungicides, and herbicides. Naturally occurring bacterial isolates capable of metabolizing this class of compounds have received considerable attention (20, 25) since they provide the possibility of both environmental and in situ detoxification (reviewed in reference 18). *Pseudomonas putida* MG and *Flavobacterium* spp. have been shown to possess the ability to degrade an extremely broad spectrum of organophosphorus phosphotriesters as well as thiol esters (4, 6). Recently, certain mammalian neurotoxins, such as diisopropylphosphonofluoridate (1) and Soman (1,2,2-trimethylpropyl-methylphosphonofluoridate; J. DeFrank, personal communication), have been shown to be hydrolyzed by selected bacteria. Several of the bacterial strains possess constitutively expressed phosphotriesterases with broad substrate ranges including many commonly used organophosphorus pesticides (4, 6). None of these strains has shown the ability to utilize these neurotoxins as sole nutrient or energy sources, thus making mutant selection difficult (C. S. McDaniel and J. R. Wild, Arch. Environ. Contam. Toxicol., in press). The hydrolysis of organophosphorus compounds by the pseudomonad phosphotriesterase has been shown to proceed via nucleophilic addition of a molecule of water across the acid anhydride bond (V. E. Lewis, W. J. Donarski, J. R. Wild, and F. M. Raushel, Biochemistry, in press). (The class of enzymes EC 3.1.3 [which includes diisopropylphosphorofluoridase and somanase] to which the opd gene product belongs was recently renamed "organophosphorus acid anhydrolase" at the 1987 DFPase Workshop held at Woods Hole Marine Biological Laboratories, Woods Hole, Mass. Synonyms which have been used include phosphotriesterase, parathion hydrolase, paraxoxynase, and parathion aryl esterase.) In addition, applications of enzymatic hydrolysis have been limited due to lack of economical fermentations of the native soil bacteria (19).

Two bacterial strains from the closely related genera *Pseudomonas* and *Flavobacterium* encode organophosphorus-degrading genes (opd) on large plasmids (40 to 65 kilobases [kb]) (15, 23, 24), while the locations of the degradative genes are unknown in other bacteria (13, 22). In the present study, the opd gene from *Pseudomonas diminuta* was sequenced and its membrane-associated gene product was expressed in heterologous genetic backgrounds from several promoter systems. The native enzyme has been partially purified, allowing molecular weight estimation, and the open reading frame has been verified by direct amino acid sequence of a purified β-galactosidase fusion polypeptide.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. diminuta* MG is the original host of pCMS1 and was obtained from the laboratory of D. Gibson, *Escherichia coli* strains HB101-4442 (auxotrophic for uracil and proline; 10) and JM103 were used as host cells for the cloning vectors, pBR322 (3) and phage M13mp10 (14), respectively. The recombinant plasmid pBR322-038 contained a 1.3-kb *PstI* fragment of pCMS1 cloned into the ampicillin resistance gene of pBR322. M13mp-038/008 and M13mp10-038/004 were oppositely oriented phage constructions which were enzymatically active or inactive, depending upon the orientation of the 1.3-kb fragment of pBR322-038. Hybrid gene fusions were produced in plasmid pMC1403 and expressed in *E. coli* CQ4 (28).

Media and growth conditions. Cultures were grown at 32°C (*P. diminuta*) or 37°C (*E. coli*). Nutrient medium consisted of 10 g of tryptone (Difco Laboratories), 5 g of yeast extract (Difco), and 5 g of NaCl per liter (TYE). TF minimal medium (17) was used for *E. coli* strains and was supplemented with uracil (50 μg/ml), proline (25 μg/ml), vitamin B₁ (0.01%),...
Casamino Acids (0.1%), glucose (0.2%), and antibiotics (25 to 50 μg/ml) as required.

Isolation of plasmid DNA. Standard protocols for the isolation of DNA from E. coli for plasmid (7) orophage (14) have been previously described. Isolation of predominantly covalently closed circular plasmid DNA from P. diminuta was accomplished via a mild lysis procedure modified from that of Berns and Thomas (2).

Cloning and sequencing of opd from the native plasmid. The PstI restriction fragments of pCMS1 were inserted into pBR322, inactivating the ampicillin gene (Focus 5:3, Bethesda Research Laboratories [BRL], Gaithersburg, Md., 1983). The resulting recombinant plasmids were used to transform competent HB101-4442, and tetracycline-resistant (Tc') colonies were selected and evaluated for ampicillin sensitivity (Ap'). The plasmid structure of selected Tc' Ap' transformants was determined, and clones representing the different inserts were analyzed for activity.

The 1.3-kb Pstl insert of pBR322-038 was excised from its vector, purified by preparative agarose gel electrophoresis using a modified freeze-squeeze phenol procedure (S. A. Benson, Biotechniques March/April:66-67, 1984), and subsequently introduced into the multiple cloning site of M13mp10. The resulting recombinant molecules were transformed into competent E. coli JM103 cells, and clear-plaque isolates were selected. All subsequent manipulations of viral recombinant DNAs were performed according to the methodology of the BRL “M13 Cloning/Dideoxy Sequencing Manual.” A variety of 5' and 3' deletions of pBR322-038 were constructed, using various restriction sites surrounding the opd gene (BamHI, AvaI, NruI, SalI, SphiI). In addition, 3' exonuclease III deletions were utilized to identify gene boundaries.

Dideoxy sequencing was accomplished by the method of Sanger as detailed in the BRL “M13 Cloning/Dideoxy Sequencing Manual.” In cases where GC compaction was evident, reverse transcriptase as well as the Klenow fragment of DNA polymerase was used (BRL, manufacturer’s protocols). Oligonucleotide primers were synthesized using phosphoramidite chemistry with an Applied Biosystems Synthesizer according to the manufacturer’s recommendations.

The 5' region of the opd gene was subcloned into the β-galactosidase gene for the purposes of producing a lacZ fusion polypeptide. The 1.3-kb opd fragment was restricted with AvaI (see Fig. 3); the staggered restriction fragment was end-filled with DNA polymerase (Klenow fragment) and blunt-end ligated into the 5' Smal cloning site of the lacZ fragment of pMC1403 (28). This hybrid genetic construction was transformed into E. coli CQ4 (5).

Production of opd probes and Southern DNA hybridization. Various constructions containing the opd gene sequence (pCMS1, pBR322-038, M13mp10-038/008, and the inactive M13mp10-038/004) were evaluated for hybridization with the opd-containing fragment. Undigested controls and corresponding Pstl-digested samples were electrophoresed on a 0.7% agarose-TBE gel (89 mM Tris base, 89 mM borate, and 2.5 mM sodium EDTA). After photography, the gels were transferred (26) onto nitrocellulose paper and probed with 32P-labeled nick-translated pBR322-038 DNA.

Phosphotriesterase assay. Routine analysis of parathion hydrolysis in whole cells was accomplished by suspending cultures in 10 mM Tris hydrochloride (pH 8.0) containing 1.0 mM sodium EDTA (TE buffer). Cell-free lysates were assayed using sonicated extracts as described previously (10) in 0.5 ml of TE buffer. The suspended cells or cell extracts were incubated with 10 μl of substrate (100 μg of parathion in 10% methanol), and p-nitrophenol production was monitored at a wavelength of 400 nm. To induce the gene under lac control, 1.0 μmol of isopropyl-β-D-thiogalactopyranoside (Sigma) per ml was added to the culture media.

Column chromatography, affinity chromatography, and protein sequencing. P. diminuta cells from a 200-liter fermentation (grown in the National Institutes of Health-Dephartment of Energy-sponsored fermentation facility of the Department of Biochemistry and Biophysics, Texas A&M University) were harvested by a continuous-flow centrifuge and suspended in 2.0 liters of 1.0 M NaCl. Samples of this suspension were agitated in a Waring blender for 30 s, and the resulting suspension was centrifuged at 400 × g for 10 min. Portions of this suspension (5.0 ml) were sonicated, treated with 0.1% Triton X-100, and stirred at room temperature for 2 h before chromatography.

The molecular weight of the native enzyme was determined by ascending Sephadex G-200 chromatography in the presence of 50 mM CHES buffer [2-(N-cyclohexyl-amino)ethanesulfonic acid (pH 9.0)] at 4°C. Enzymatic activity was located by introducing 50-μl aliquots of column fractions (2.0 ml) into a reaction volume of 0.8 ml containing 0.2 mM paraaxon and 50 mM CHES buffer (pH 9.0).

Purification of hybrid β-galactosidase proteins encoding the 5' region of the opd gene was achieved by immunoaffinity chromatography (28) and preparative gel electrophoresis. Gas-phase sequencing of the purified fusion polypeptide (Applied Biosystems 470A Sequencer, Applied Biosystems 120A On-line-PTH Analyzer, TAE Bioscience Support Laboratory) was accomplished by the methods of Hewick et al. (12).

RESULTS

Partial purification and molecular weight estimation. Upon cellular disruption of the native P. diminuta strain by sonication or French pressure cell disruption, 80 to 90% of the activity was associated with the particulate fraction. It was possible to release activity from the particulate complex by treatment with 0.1% Triton X-100 or 0.2% Tween 20 without significant loss of activity. When these enzyme preparations were analyzed by Sephadex G-200 column chromatography, the molecular weight of the enzymatically active fractions was 60,000 to 65,000.

Cloning of pCMS1 into pBR322. The entire DNA from the degradative plasmid was digested with PstI (generating fragments of approximately 18.5, 17.3, 5.3, 4.3, 1.7, 1.6, 1.3, and 0.8 kb) and was subcloned into pBR322 within that vector's ampicillin gene. Cell-free lysates of Ap' clones selected from the Tc' transformants of E. coli HB101-4442 were tested for activity. One single-colony isolate was selected for its ability to hydrolyze parathion, and the expected phenotype (Tc' Ap'; auxotrophy for uracil and proline; parathion hydrolysis) was verified. A 5.6-kb, CsCl-purified plasmid was isolated from this strain and used to transform competent HB101-4442 cells, regenerating the phenotype and demonstrating that the hydrolytic activity was mediated by the recombinant plasmid. Other isolates with a similarly sized insert but lacking the hydrolytic activity were subsequently shown to have an orientation opposite to that of the active clone (data not shown). This observation demonstrated that the orientation of the opd-containing fragment within the pBR322 vector was critical to heterologous expression. Thus, it appeared that the expression of the 1.3-kb fragment (approximately 1 to 2% of the
The data were consistent with directional information that the active fragments contained the opd gene in the same orientation and length of coding sequence (Fig. 1). An open reading frame of approximately 1,038 nucleotides, with an initiation codon ATG, was located in all cases. The open reading frame was disrupted by several Rho-dependent terminator structures beginning at the 17.6-kb position (Fig. 2). The 1.3-kb PstI fragment, which contained the opd gene, was used to probe the Southern blots (Fig. 1). The results demonstrated that the native plasmid encoded a plasmid-mediated, parathion-degrading activity on a 1.3-kb PstI fragment.

**Nucleotide sequencing.** Dideoxy sequencing along both strands of the opd gene revealed a potential translational reading frame of 975 base pairs, and the DNA sequence verified the known restriction pattern for the opd-encoding fragment (Fig. 2). Five oligonucleotide primers were constructed for the purposes of sequencing regions lacking convenient restriction sites. In all cases, these primers efficiently promoted DNA synthesis.

The open reading frame (CTC-GGC-ACC) began 12 base pairs from the 5' PstI site and continued to a position at 1,038 base pairs before encountering a pair of closely spaced TGA stops (Fig. 3). A potential start site (ATG) was located 17 codons into the open reading frame. This codon appeared to be a candidate for the translational start since it is preceded by an AAGCAA sequence 15 base pairs upstream; the sequence and spacing are in good agreement with known *Pseudomonas* ribosomal binding sites (11, 15). In addition, several potential rho-dependent terminator structures ranging in free energy of association from -12.6 to -15.4 kcal/mol (ca. -52.7 to -64.4 kJ/mol) were located 3' of the open reading frame (data not shown).

This predicted amino acid sequence would give rise to a protein of 35,418 daltons before posttranslational modifications, if any. However, there are other potential start sites further into the sequence which would give rise to slightly smaller proteins (Fig. 3). In particular, valine 7 represents a possible start site after GTG (formylmethionyl) codons are known in *Pseudomonas* spp. (8) and since a potential ribosomal binding site was located for this start site. However, the insertion of a BamHI linker into the *SpI*I site (Fig. 2) disrupted the first putative ATG translational start site, and these genetic constructions possessed no enzymatic activity.

**Amino acid sequencing of fusion polypeptides.** When a fusion protein was constructed between the 5' region of the *opd* gene and the *lacZ* gene at the *Aval-Smal* site, a hybrid polypeptide was recovered, purified, and subjected to amino acid sequencing. Amino acid sequencing confirmed the

![FIG. 1. Southern hybridization of opd probe (pBR322-038; pBR322 plus the 1.3-kb opd fragment) with the native plasmid and subclones. (A) Agarose gel electrophoresis of opd-containing DNAs. Lanes 6, 8, 11, and 15 contain PstI-digested M13mp10-038/004 (inactive orientation), M13mp10-038/008 (active orientation), pBR322-038, and pCMS1, respectively. The lanes correspond to those described for panel A.](http://jb.asm.org/)

![FIG. 2. Restriction map and DNA sequence strategy for the 1.3-kb fragment containing the opd gene. The direction and length of sequence determinations are shown with arrows (kilobase scale at bottom). The placement of the various restriction sites, exonuclease-generated subclones, and synthetic DNA primers used in the sequencing is shown along the fragment. The putative coding region for the opd gene is shaded.](http://jb.asm.org/)
predicted reading frame for 16 amino acids 5' of the fusion junction (Fig. 3). The sequence is 168 amino acids away from the presumed translational start for the opd gene product; however, truncated polypeptides are typical of fusions of membrane proteins with \( \beta \)-galactosidase (28), and proteolysis in the heterologous background may have produced a posttranslationally modified polypeptide.

Subcloning regional deletions. Figure 4 summarizes results obtained with various subclones of the 1.3-kb fragment containing the opd gene. Deletions outside the putative coding region remained active when the sequence was properly oriented for expression from the lacZ promoter. If the orientation was reversed or if deletions were made within the putative coding region, activity was eliminated.

**DISCUSSION**

The gene (opd) encoding a broad-substrate-range phosphotriesterase of *P. diminuta* MG has been shown to be encoded on a 50- to 60-kb plasmid (15, 23, 24; C. S. McDaniel, Ph.D. dissertation, Texas A&M University, College Station, 1985). The plasmid-borne gene was contained within a 1.3-kb restriction fragment and was transferred into a variety of plasmid and phage vectors and expressed in *E. coli*. The 1.3-kb fragment encoding the opd gene was sequenced, and its proper reading frame was confirmed by

<table>
<thead>
<tr>
<th>Nucleotide Number</th>
<th>Amino Acid Number</th>
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<tbody>
<tr>
<td>5'</td>
<td>CT GCA GCC TGA CTC GCC ACC AGT GCC TGC</td>
</tr>
<tr>
<td>60-119</td>
<td>AGG ATG CAA ACC AGA AGG GTG TGT CTC AAG</td>
</tr>
<tr>
<td>120-179</td>
<td>Met Gin Thr Arg Val Val Lys</td>
</tr>
<tr>
<td>180-239</td>
<td>COG CCT TCA AAT CCA ATC TCT GAA GGC GGT TCC Arg Pro Ile Thr Ile Ser Gla Ala Gly Phe</td>
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<tr>
<td>240-299</td>
<td>Arg Gin Asp Ser Cys Val Leu Gly Gly Ser</td>
</tr>
<tr>
<td>300-359</td>
<td>GGC TGT GAG ATT GCC CAG AGC GGC Gly Cys Ile Ala Ser Val Leu Thr Tyr Gly Gln</td>
</tr>
<tr>
<td>360-419</td>
<td>GCC GAT TAT CCC GCA GTG CAG TAT ATC Arg Phe Tyr Arg Ser Arg Gin Phe Ile</td>
</tr>
<tr>
<td>420-479</td>
<td>TAT CTG GGC GCC ACC GCC TGG TGG TGC GAC</td>
</tr>
<tr>
<td>480-539</td>
<td>Tyr Leu Ala Ala Thr Gly Tyr Phe Asp</td>
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<tr>
<td>540-599</td>
<td>ATT ACC GGC GCC ATT ATG AAT GGC GCG GGC</td>
</tr>
<tr>
<td>600-659</td>
<td>Ile Arg Ala Gly Ile Ile Lys Val Ala Thr</td>
</tr>
<tr>
<td>660-719</td>
<td>GCC CCA AGT CAG CCG GAT GGG GAG CAA GGC AAG</td>
</tr>
<tr>
<td>720-779</td>
<td>Arg Ser Gin Arg Asp Gly Gly Arg Gly</td>
</tr>
<tr>
<td>780-839</td>
<td>GTC GCG GCC GCC TAT CTC GAC CAC ACC TGG</td>
</tr>
<tr>
<td>840-899</td>
<td>Arg Thr Asp Gin Asp Gin CTA CCC CTC CTG GGC</td>
</tr>
<tr>
<td>900-959</td>
<td>CAC ATT GAG GCA TCA CCG CTC GCG GGC</td>
</tr>
<tr>
<td>960-1019</td>
<td>ACG AAG GGC CTC GAT CAC GAC CAC ACC TAC</td>
</tr>
<tr>
<td>1020-1079</td>
<td>Leu Lys Ala Leu Leu Asp Gin Gly Gly Tyr</td>
</tr>
<tr>
<td>1080-1139</td>
<td>GAC GGC GCC ACC ATC TGC CAG TGG CAC TGG</td>
</tr>
<tr>
<td>1140-1199</td>
<td>Met Gin Thr Arg Val Val Lys</td>
</tr>
<tr>
<td>1200-1259</td>
<td>TTG AGG TCG GCC ACC ATC CAG TGA GGG GGC</td>
</tr>
<tr>
<td>1260-1322</td>
<td>CAG 3'</td>
</tr>
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FIG. 3. Nucleotide sequence of the opd gene fragment. The amino acid sequence corresponding to the open reading frame beginning with the first ATG codon is identified below the sequence. Primers used in the sequencing are shown above the nucleotide sequence by overlining. The 3' stop codon is indicated with a period. The amino acids confirmed by protein sequencing are underlined.
implications relative to the loss of efficacy of these biolabile pesticides. The potential mobility of these plasmid-borne genes may be analogous to the reduction of antibiotic efficacy in clinical and agricultural situations by plasmid-borne resistance factors (29).

It is clear that many soil bacteria possess degradative, plasmid-borne genes which could be readily transferred and expressed among a variety of bacterial and viral hosts. This phenomenon is not limited to organophosphorus neurotoxins, since plasmid-borne genes for degradative enzymes of herbicides have been well documented (9, 27). In the case of the opd genes, a wide range of pesticides sharing a common chemical structure are degraded (6), providing the potential for rapid evolution of genes to degrade a variety of pesticides and challenging the agrochemical rationale of substituting pesticides of similar chemical structure or increasing application rates for extended pest control. Rapid mutational adaptation in an enriched soil bacterial population could render ineffective any subsequent applications of a similar chemical.

LITERATURE CITED