APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2002, p. 3371–3376 Vol. 68, No. 7
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.7.3371–3376.2002

Identification of an *opd* (Organophosphate Degradation) Gene in an *Agrobacterium* Isolate

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Received 12 December 2001/Accepted 4 April 2002

We isolated a bacterial strain, *Agrobacterium radiobacter* P230, which can hydrolyze a wide range of organophosphate (OP) insecticides. A gene encoding a protein involved in OP hydrolysis was cloned from *A. radiobacter* P230 and sequenced. This gene (called *opdA*) had sequence similarity to *opd*, a gene previously shown to encode an OP-hydrolyzing enzyme in *Flavobacterium* sp. strain ATCC 27551 and *Brevundimonas diminuta* MG. Insertional mutation of the *opdA* gene produced a strain lacking the ability to hydrolyze OPs, suggesting that this is the only gene encoding an OP-hydrolyzing enzyme in *A. radiobacter* P230. The OPH and OpdA proteins, encoded by *opd* and *opdA*, respectively, were overexpressed and purified as maltose-binding proteins, and the maltose-binding protein moiety was cleaved and removed. Neither protein was able to hydrolyze the aliphatic OP malathion. The kinetics of the two proteins for diethyl OPs were comparable. For dimethyl OPs, OpdA had a higher k_{cat} than OPH. It was also capable of hydrolyzing the dimethyl OPs phosmet and fenthion, which were not hydrolyzed at detectable levels by OPH.

Synthetic organophosphates (OPs) are used widely as insecticides in agriculture. OPs contain three phosphoester linkages and are hence often termed phosphotriesters. The phosphorus is also linked by a double bond to either an oxygen (P═O) in oxon OPs or a sulfur (P═S) in thion OPs. These insecticides are potent acetylcholinesterase (AchE) inhibitors, and various clinical effects can occur due to OP poisoning in humans. In general, hydrolysis of one of the phosphoester bonds reduces the toxicity of an OP, and in the case of parathion (*O*,*O*-diethyl *p*-nitrophenyl phosphorothioate), a 100-fold reduction in toxicity occurs (37, 38).

Enzymatic detoxification of OPs has become the focus of many studies because other means of removing OP residues are impractical or costly or are themselves environmentally hazardous. Enzymes from insect species resistant to OPs have been identified and considered for use in bioremediation (27). However, these enzymes are capable of hydrolyzing only oxon OPs and function at rates several orders of magnitude below the diffusion-limited maximum rate (28). Bacterial enzymes have also received considerable attention and may have advantages in terms of broader substrate specificities (both oxon and thion OPs) and superior kinetics (10, 13). The most widely studied bacterial enzyme is the OPH (organophosphorus-hydrolyzing) protein.

OPH is a zinc-containing homodimeric protein found in the membrane of *Flavobacterium* sp. strain ATCC 27551 and *Brevundimonas diminuta* MG (5, 13). OPH is capable of hydrolyzing a wide range of oxon and thion OPs (13) and hydrolyzes paraoxon at a rate approaching the diffusion limits (6, 35). The OPH enzyme is encoded by the *opd* gene. Other *opd*-containing organisms (for example, a *Pseudomonas* strain) have been identified by using this gene in Southern hybridization analysis (8), while other OP-hydrolyzing organisms clearly do not contain the *opd* gene (10, 11). *Flavobacterium* sp. strain ATCC 27551 and *B. diminuta* MG contain identical *opd* genes, but it is not clear how this has occurred as the genes are on very different plasmids (17, 25). Another class of OPs are the prolidase-type enzymes identified in *Alteromonas* spp. These enzymes are better at detoxifying the nerve gas reagents sarin and soman than the insecticidal OPs (10), and therefore OPH and homologues of this enzyme are better suited to bioremediation of insecticidal OPs.

We previously isolated a bacterium capable of hydrolyzing coumaphos (3-chloro-7-diethoxy phosphino thioloxy-4-methyl coumarin) from an enrichment culture containing OPs as the sole phosphorus source (16). In this paper we describe identification of this organism and characterization of the gene-enzyme system responsible for its OP-hydrolyzing activity. We also demonstrate that the protein produced by this strain is well suited for bioremediation of dimethyl OPs.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown on a modified Luria-Bertani medium (16). *Escherichia coli* was grown at 37°C, and *Agrobacterium* species were grown at 28°C. When used in the medium for *E. coli*, ampicillin, kanamycin, and tetracycline were used at concentrations of 100, 25, and 10 μg/ml, respectively. When included in the medium for growth of *Agrobacterium*, rifampin, kanamycin, and tetracycline were included at concentrations of 100, 25, and 1 μg/ml, respectively.

Bacterial identification. Isolate P230 is a gram-negative, catalase-positive, oxidase-positive, rod-shaped bacterium (tests were performed by using standard methods [19, 20]). Its 16S rRNA gene was PCR amplified from chromosomal DNA (extracted by the method of Rainey et al. [32]) by using the 27f and 1492r universal primers (23). Sequencing of PCR fragments was performed by using the 27f, 530f, 1100r, and 1492r universal primers (23) after purification of the PCR products with a QIAquick PCR purification kit (Qiagen).

DNA manipulation. Routine DNA manipulations were carried out as described by Sambrook et al. (34). Isolation of chromosomal DNA from *Agrobacterium radiobacter* P230 was performed as described by Gardiner et al. (14). A size-fractionated genomic library was constructed from 10- to 15-kb DNA frag-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
E. coli strains		
DH10β	Host for α-complementation cloning vectors	Gibco BRL
S17-1	Mobilizing strain; carries chromosomally integrated derivative of RP4	40
JM109 λ*pir*	Host for α-complementation cloning vectors; lysogenized with phage lambda carrying the *pir* gene; allows replication of R6K-based suicide vectors	29
S17-1 λ*pir*	S17-1 lysogenized with λ*pir*	30
Agrobacterium strains		
A. tumefaciens C58	Wild type, OP^- Rif^r	44
A. radiobacter P230	Wild type, OP^+ Rif^r	16
A. radiobacter Par^-	$OpdA^-$	This study
Plasmids		
pBluescript KS+ (pBS)	Cloning vector, Ap^r	Stratagene
pJP5603	R6K-based suicide vector	29
p65	12-kb *Sau*3AI partial fragment of *A. radiobacter* P230 genome in pBS	This study
pH2	4-kb *Hind*III fragment of p65 in pBS	This study
pB1	8-kb *Bam*HI fragment of p65 in pBS	This study
pP1	6-kb *Pst*I fragment of pB1 in pBS	This study
pJPH2	4-kb *Hind*III fragment from pH2 in pJP5603	This study
pJPH2Sp	pJPH2 with *Not*I Ω Sp^r from pUI1188Sp in *Not*I site in *opdA*	This study
pUI1188Sp	ΩSp^r in pBS	A. Suwanto
pMAL-c	Overexpression vector for MBP fusion proteins	New England Biolabs
pMAL-c2X	As for pMAL-c, with different cloning sites	New England Biolabs
pmal-opdA	*malE*::*opdA* translational fusion in pMAL-c	This study
pFmal	*malE*::*opd* translational fusion in pMAL-c2X	This study
pR751::Tn*813*	Conjugative, cointegrative plasmid	3

[a] Ap, ampicillin; Rif, rifampin; ΩSp^r, spectinomycin-resistant cassette bordered by transcription terminators.

ments (obtained from a partial *Sau*3AI digest) cloned into the *Bam*HI site of plasmid pBluescript KS+ (pBS) and transformed into *E. coli* DH10β. Triparental matings of the library clones were performed by using the method of Bonnett et al. (2) with the conjugative cointegrative plasmid pR751::Tn*813*. Insertional mutants were detected by PCR by using *opdA*-specific primers listed below with extracted chromosomal DNA (32). The GenBank accession number for the *opdA* gene is AY043245.

Assays and biochemical techniques. Cell extracts were prepared as previously described (16). Protein concentrations were determined by the method of Bradford (4) by using bovine serum albumin as a standard. For more accurate determinations of purified protein concentrations, the method of Gill and von Hippel (15) was employed. Hydrolysis of coumaphos, hydrolysis of coroxon, and hydrolysis of *O*,*O*-dimethyl 4-methyl umbelliferyl phosphate (dMUP) were measured by monitoring the formation of fluorescent products (16, 33; A. L. Devonshire, R. Heidari, K. L. Bell, P. M. Campbell, B. E. Campbell, W. A. Odgers, J. G. Oakeshott, and R. J. Russell, submitted for publication). Hydrolysis of parathion, hydrolysis of methyl parathion, and hydrolysis of paraoxon were measured spectrophotometrically by monitoring the production of *p*-nitrophenol at 405 nm (13). Hydrolysis of fenthion was measured spectrophotometrically by determining the loss of fenthion (at 252 nm) (18), while hydrolysis of phosmet and hydrolysis of malathion was measured by quantifying the formation of thiol groups produced during hydrolysis by using Ellman's reagent (22). Hydrolysis of diazinon and hydrolysis of chlorfenvinphos (CVP) were measured by using the radiometric partition assay of Campbell et al. (7). All assays were performed in 50 mM Tris-HCl (pH 8.0) at 25°C. Sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (ratio of acrylamide to bisacrylamide, 30:1) was performed by the method of Laemmli (21).

Construction of plasmids for OPH and OpdA overexpression and purification. OPH and OpdA were overexpressed in *E. coli* DH10β by using the pMAL protein fusion and purification system, which results in expression of a maltose-binding protein (MBP) fusion protein (New England Biolabs). To construct an MBP-OPH overexpression plasmid, the *opd* gene (26) without the signal peptide domain was amplified by a PCR. The upstream and downstream oligonucleotide primers, 5′GATCGTGGATCCTCGATCGGCACAGGCGATCGG and 5′GATCGTAAGCTTTCATGACGCCCGCAAGGTCGG, respectively, were designed to contain a *Bam*HI restriction site at the *opd* start codon and a *Hin*dIII restriction site at the stop codon (underlined bases). The PCR fragment was subsequently cloned into the *Bam*HI-*Hin*dIII restriction sites of pMAL-c2X (New England Biolabs) to generate the recombinant plasmid pFmal. An MBP-OpdA overexpression plasmid was constructed in a similar way. The *opdA* gene was amplified by PCR by using upstream and downstream primers, 5′GATCGTCTGCAGCCAATCGGTACAGGCGATCTG and 5′GATCGTAAGCTTTCATCGTTCGGTATCTTGACGGGGAAT, respectively, with a *Pst*I site at the start codon and a *Hin*dIII site at the stop codon (underlined bases). The PCR fragment was subsequently cloned into the *Pst*I-*Hin*dIII sites of pMAL-c (New England Biolabs) to generate the recombinant plasmid pmal-opdA.

Optimal production of MBP fusion proteins was obtained when mid-log-phase cells (optical density at 600 nm, 0.6) were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 5 h at 37°C. Harvested cells were disrupted by sonication, and the soluble fraction was loaded onto an amylose resin (New England Biolabs) equilibrated with 50 mM Tris-HCl (pH 7.5). MBP fusion proteins were eluted with 10 mM maltose in 50 mM Tris-HCl (pH 7.5). Fractions containing coumaphos-hydrolyzing activity were pooled and cleaved with Xa protease (10 μg/ml; New England Biolabs) for 5 h. The cleaved fractions were then placed on a DEAE-Sepharose ion-exchange column. Cleaved OPH and OpdA proteins were not bound to the resin and eluted with the void volume. This purification process removed the Xa protease, as well as the MBP moiety. Fractions from the collected sample appeared to be homogeneous, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (data not shown). Approximately 35 μg of purified protein was obtained from 500 ml of culture.

Chemicals. Radiolabeled (specific activity, 306.5 MBq/mmol) CVP {2-chloro-1-(2,4-dichlorophenyl)vinyl di-[^{14}C]ethyl phosphate} was obtained from International Isotope München. Fenthion {*O*,*O*-dimethyl *O*-[3-methyl 4-(methylthio)phenyl] phosphorothioate} was a gift from G. W. Levot, Department of Agriculture, New South Wales, Australia. Malathion [*O*,*O*-dimethyl *S*-(1,2-dicarbethoxyethyl) phosphorodithioate], methyl parathion (*O*,*O*-dimethyl *O*-*p*-nitrophenyl phosphorothioate), and parathion (*O*,*O*-diethyl *p*-nitrophenyl phosphorothioate) were obtained from Riedel-de Haan AG, Seelze, Germany. Coumaphos (3-chloro-4-methyl-7-coumarinyl diethyl phosphorothioate) was a gift from Bayer, Bayerwerk, Germany. Coroxon (3-chloro-4-methyl-7-coumarinyl diethyl phosphate), radiolabeled (14.8 MBq/mmol) diazinon {*O*,*O*-di-[^{14}C]ethyl-*O*-(2-isopropyl-4-methyl-6-pyrimidinyl)-phosphorothioate}, and phosmet {*S*-[(1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)methyl] *O*,*O*-dimethyl phosphorodithioate} were purchased from Alltech. dMUP was synthesized by Alan Devonshire (unpublished data).

RESULTS AND DISCUSSION

Identification of isolate P230. The sequence of approximately 1,320 bp of the 16S rRNA gene of P230 was 100% identical to that of the 16S rRNA gene of *A. radiobacter* LMG383 (GenBank accession no. ara130719), 99.7% similar to that of the 16S rRNA gene of *Agrobacterium* sp. strain LMG11936 (GenBank accession no. asp130721), 99.5% similar to that of the 16S rRNA gene of *Agrobacterium* sp. strain MSMC211 (GenBank accession no. asaj4859), and 99.3% similar to that of the 16S rRNA gene of *Agrobacterium* sp. strain LMG11915 (GenBank accession no. asp130720) (calculated by using the FASTA algorithm [29]). Use of the Biolog system (Oxoid) showed that isolate P230 was capable of using glucose, sucrose, and ornithine as carbon sources, suggesting that it was most similar to either *Agrobacterium tumefaciens* biovar 1 or *A. radiobacter* biovar 1, depending on the presence of a tumor-inducing plasmid. Attempts to isolate a plasmid(s) from isolate P230 were not successful. Tumor-inducing ability was also tested on tomato seedlings by using the method of Lippincott and Heberlein (24), and there was no evidence of tumors after a period of 4 weeks. Isolate P230 was therefore designated a strain of *A. radiobacter* biovar 1.

Functional cloning of the gene encoding an OP-hydrolyzing enzyme. As it was not known whether the gene of interest would be expressed in *E. coli*, a library of *A. radiobacter* P230 genomic DNA fragments was constructed in pBS in *E. coli* and transferred via the conjugative cointegrative plasmid pR751::Tn*813* (contained in *E. coli* JM109 λ*pir*) into *A. tumefaciens* strain C58, which possessed negligible parathion- and coumaphos-hydrolyzing activity. Approximately 350 transformants from the genomic library of *A. radiobacter* P230 were transferred by using this triparental mating procedure. The mating mixtures were then assayed for coumaphos-hydrolyzing activity. One transformant (p65) exhibited coumaphos-hydrolyzing activity in the mating mixture. This clone also conferred coumaphos-hydrolyzing activity on *E. coli* DH10β; the activity was 3.30 ± 0.07 nmol of chlorferon produced/min/mg of protein (mean ± standard error), compared with 0.78 ± 0.04 nmol of chlorferon produced/min/mg of protein in control extracts of *E. coli* DH10β(pBS). While clone p65 contained approximately 12 kb of P230 chromosomal DNA, coumaphos-hydrolyzing activity was confined to a 4-kb *Hin*dIII fragment (Fig. 1). The activity associated with the 4-kb *Hin*dIII fragment in *E. coli* depended on the orientation of the fragment in pBS (Fig. 1), suggesting that it was dependent on the *lacZ* promoter in pBS for expression. This fragment was sequenced.

Sequence of the gene encoding the OP-hydrolyzing enzyme. One open reading frame in the 4-kb *Hin*dIII fragment was identified that was 88.4% identical at the nucleotide level to the *opd* phosphotriesterase gene previously identified in *Flavobacterium* sp. strain ATCC 27551 and *B. diminuta* MG (26). The amino acid sequence of its putative translation product was 94.2% similar and 90.0% identical to the OPH sequence (26). This gene was termed *opdA* (*opd* from *Agrobacterium*), and an alignment of its inferred amino acid sequence with that of the *Flavobacterium-B. diminuta* OPH is shown in Fig. 2A. Some notable differences were observed between the *Flavobacterium-B. diminuta* OPH sequence and the sequence of OpdA from *A. radiobacter* P230. The first difference is that

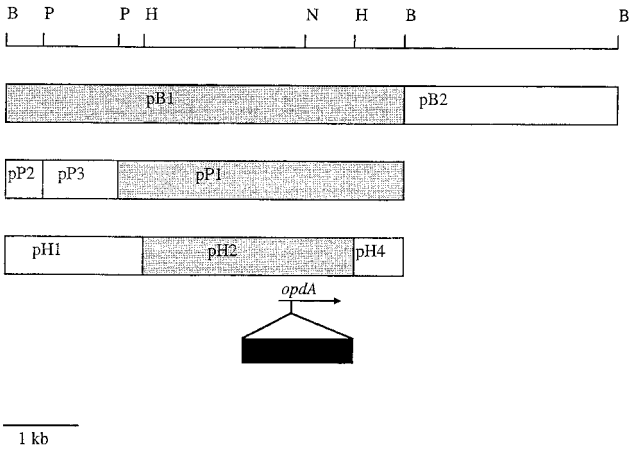

FIG. 1. Subcloning of the *opdA* gene and insertional inactivation. The positions of restriction enzyme sites used for subcloning are shown at the top, and the boxes represent fragments generated by the restriction enzymes. The shaded boxes represent fragments that conferred OP-hydrolyzing activity on *E. coli* when they were cloned into pBluescript; the left side is closest to the T7 sequencing primer in pBluescript. The arrow represents the *opdA* gene and indicates its direction of transcription. The solid box represents the spectinomycin resistance cassette placed into the *Not*I site in *opdA* in pJPH2Sp. B, *Bam*HI; P, *Pst*I; N, *Not*I; H, *Hin*dIII.

there is one less amino acid in the putative signal sequence of OpdA. The second is that there is a frameshift near the 3′ end of the *opdA* gene (Fig. 2B), resulting in an additional 16 amino acids in OpdA. This region has been sequenced five times in

A.

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OPH    1  MQTRRVVLKSAAAAGTLLGGLAGCASVAGSIGTGDRINTVRGPITISEAG 50
          |||||  ||||||  ||||||||||:|  |||||  |||||||| :||||
OpdA   1  MQTRRDALKSAAAI.TLLGGLAGCASMARPIGTGDLINTVRGPIPVSEAG 49

OPH   51  FTLTHEHICGSSAGFLRAWPEFFGSRKALAEKAVRGLRRARAAGVRTIVD 100
          |||||||||||||||||||||||||||||||||||||:|| ||| ||||
OpdA  50  FTLTHEHICGSSAGFLRAWPEFFGSRKALAEKAVRGLRHARSAGVQTIVD 99

OPH  101  VSTFDIGRDVSLLAEVSRAADVHIVAATGLWFDPPLSMRLRSVEELTQFF 150
          |||||||||| ||||||||||||||||||||||||||:||||||||||
OpdA 100  VSTFDIGRDVRLLAEVSRAADVHIVAATGLWFDPPLSMRMRSVEELTQFF 149

OPH  151  LREIQYGIEDTGIRAGIIKVATTGKATPFQELVLKAAARASLATGVPVTT 200
          |||||  |||||||||||||||||||||||||||||||||||||||||
OpdA 150  LREIQHGIEDTGIRAGIIKVATTGKATPFQELVLKAAARASLATGVPVTT 199

OPH  201  HTAASQRDGEQQAAIFESEGLSPSRVCIGHSDDTDDLSYLTALAARGYLI 250
          ||  |||||||||||||||||||||||||||||||||||||:|||||||:
OpdA 200  HTSASQRDGEQQAAIFESEGLSPSRVCIGHSDDTDDLSYLTGLAARGYLV 249

OPH  251  GLDHIPHSAIGLEDNASASALLGIRSWQTRALLIKALIDQGYMKQILVSN 300
          |||::| ||||||:|||| ||:| ||||||||||||||| ||   ||||:
OpdA 250  GLDRMPYSAIGLEGNASALALFGTRSWQTRALLIKALIDRGYKDRILVSH 299

OPH  301  DWLFGFSSYVTNIMDVMDRVNPDGMAFIPLRVIPFLREKGVPQETLAGIT 350
          |||||||||||||||||:|||||||:|||||||||||||||| |||||:|
OpdA 300  DWLFGFSSYVTNIMDVMDRINPDGMAFVPLRVIPFLREKGVPPETLAGVT 349

OPH  351  VTNPARFLSPTLRAS 365
          | |||||||||:||
OpdA 350  VANPARFLSPTVRAVVTRSETSRPAAPIPRQDTER 384
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B.

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opdA 1085 GGCCGTCGTGACACGATCTGAAACTTCCCGCCCTGCCGCGCCTATTCCCCGTC
               ||| ||| |||
opd  1089 GGC-GTCATGA

opdA 1138 AAGATACCGAACGATGA
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FIG. 2. (A) Alignment of the amino acid sequences of OPH and OpdA. The secretion signals are indicated by boldface type. The vertical lines indicate identical residues, while the colons indicate similar residues. (B) Alignment of DNA sequences at the C termini of the two genes.

both directions with multiple sequencing primers to ensure that the extra base in *opdA* is not a sequencing error. Our data thus suggest that OpdA is a 384-amino-acid protein with a molecular mass of approximately 35 kDa when it is cleaved from its signal peptide.

One obvious sequence difference between OPH and OpdA is an extended C terminus. The C terminus of OPH was previously shown not to interact with the substrate analogue 4-methylbenzylphosphonate in a crystallized structure of OPH, but rather to lie on the outside of the protein structure (43). It therefore seems unlikely that the extended C terminus in OpdA contributes directly to differences in substrate selectivity. It may, however; confer different physical properties on OpdA.

Plasmid pJPH2 (4-kb *Hin*dIII fragment in pJP5603) conferred coumaphos-hydrolyzing activity of 3.7 ± 0.4 nmol/min/mg on *E. coli* JM109 λ*pir*. Insertion of the spectinomycin-resistant cassette into the *Not*I site of *opdA* (Fig. 1) reduced the OP-hydrolyzing activity to that seen for the vector-only control (0.031 ± 0.003 nmol/min/mg). Note that this activity is lower than the background phosphotriesterase activity for *E. coli* strain DH10β described above; we and others have observed variable activities in different *E. coli* strains (37), but the values are well within the range of variation for background phosphotriesterase activity. The *opdA* gene is at one end of the 4-kb *Hin*dIII fragment, and no open reading frames have been identified downstream of *opdA*. We therefore interpret the loss of activity as the result of an insertion in *opdA* and not as the result of a loss of expression of any other gene. The construct was then used to inactivate *opdA* in *A. radiobacter* P230, to create *A. radiobacter* Par$^-$. This mutant also exhibited reduced parathion-, methyl parathion-, coumaphos-, coroxon-, and paraoxon-hydrolyzing activities (approximately 0.42% ± 0.07% of the wild-type activities). This demonstrates that the *opdA* gene is the only gene in *A. radiobacter* P230 encoding an enzyme capable of hydrolyzing OPs.

The *opd* and *opdA* genes have been identified in organisms isolated from different geographic locations, from the Philippines (39) to the United States (8, 36) and Australia (16). Also, they have been found in four taxonomically distinct organisms. Presumably, these genes were acquired from the same ancestral organism. Since distantly related *opd*-like genes have been identified in organisms in genome-sequencing projects (1, 31), the native host of the *opd* and *opdA* genes may well be a ubiquitous soil organism. Although no plasmids could be identified in *A. radiobacter* P230 and the *opdA* gene appears to be chromosomally located, the inability of *A. tumefaciens* C58 to hydrolyze OPs and the lack of hybridization of an *opd* probe to *A. tumefaciens* C58 bulk DNA suggest that *A. radiobacter* P230 acquired *opdA* by a lateral gene transfer mechanism. This is not uncommon, as other plasmid-borne genes (for example, the TOL genes involved in toluene catabolism [41]) have also been found chromosomally. Movement of the TOL genes through soil populations has been suggested to occur via transposition (42). This may be the means by which the *opd* genes having the same DNA sequence and encoding the same protein sequence were acquired by *B. diminuta* MG and *Flavobacterium* sp. strain ATCC 27551 (17), as well as the means by which a related gene was acquired by *A. radiobacter* P230.

Analysis of the substrate specificity of OpdA and comparison with OPH. To assess the substrate range of OpdA, the enzyme was purified and analyzed in parallel with its counterpart from *Flavobacterium* sp. strain ATCC 27551, OPH. The substrates tested were paraoxon, parathion, methyl parathion, malathion, CVP, phosmet, diazinon, fenthion, coumaphos, coroxon, and dMUP (Fig. 3). Note that the k_{cat} values for OPH reported here are much lower than those reported previously (12, 13), probably because we did not add metals to the assay mixtures or during growth, both of which can increase the specific activity (12). No activity against malathion or CVP could be detected for either OPH or OpdA. In general, OpdA appeared to have a slightly higher K_m for the diethyl substrates, but the k_{cat} values of the two enzymes against the diethyl substrates were comparable. All four dimethyl substrates tested were hydrolyzed by OpdA, while OPH hydrolyzed methyl parathion and dMUP but apparently did not hydrolyze fenthion or phosmet. The inability of OPH to hydrolyze fenthion has been reported previously (5). Furthermore, the k_{cat} values for OpdA against methyl parathion and dMUP were significantly higher than those for OPH; in the case of dMUP the values were approximately four times higher, and in the case of methyl parathion the values were almost 20 times higher. If the ratio for phosmet was similar to the latter value, only 7.2 mol of phosmet per mol of OPH would be hydrolyzed in a 24-h period. Given the amounts of enzyme used in assays in this study (in the nanomolar to micromolar range), this amount of hydrolysis would not be observed by our methods.

Chen-Goodspeed et al. (9) identified three essential substrate binding sites in OPH. These were termed the small and large subsites, which bound the diethyl or dimethyl moieties of the substrate, and the leaving group subsite, which bound the aromatic leaving group. OpdA retains all the amino acid residues important in substrate binding in OPH in the small subsite and the leaving group subsite. However, OpdA differs in three of the four residues in the large subsite. These residues are H254R, H254Y, and L271F. It is conceivable that the change of histidine to the much larger residue tyrosine and the change of leucine to the larger residue phenylalanine might close in the binding site, enhancing the activity of OpdA with dimethyl substrates compared to the activity of OPH. The kinetics of an H254R mutation of OPH made by diSioudi and coworkers are consistent with this possibility (12). The OPH mutant had an almost 20-fold decrease in the ability to hydrolyze diisopropyl fluorophosphate (DFP) (12). The leaving group of DFP contains two isopropyl groups and is considerably larger than the dimethyl leaving group of methyl parathion. It could be anticipated that OpdA also has a reduced capacity to hydrolyze DFP as a result of a smaller large subsite. By inference, OpdA may not be as effective in degrading nerve agents as OPH since these agents contain bulkier alkyl groups (e.g., isopropyl chains in the case of soman) than the insecticidal OPs suitable for detoxification by OpdA.

Constriction of the large subsite in OpdA compared to the OPH large subsite might also affect the stereospecificity of the enzyme. While most insecticidal OPs have either dimethyl or diethyl substituents, a few have a mixture of methyl and ethyl or, in exceptional cases, propyl groups. The stereospecificity of OPH for these asymmetric OPs has been attributed to the size difference between the small and large subsites (9). This spec-

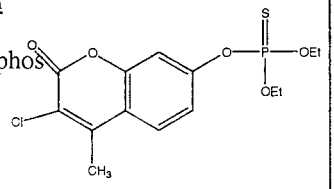
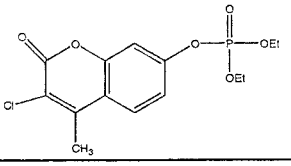
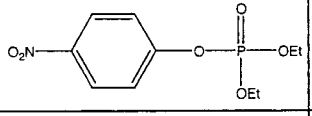
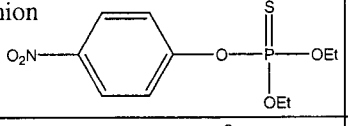
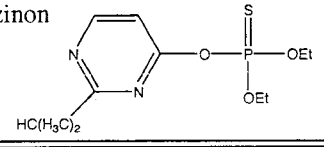
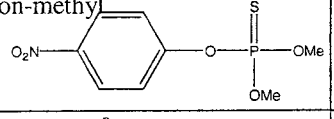
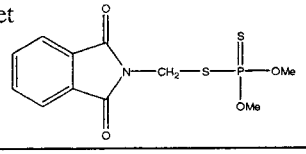
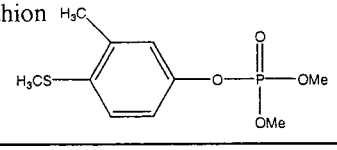
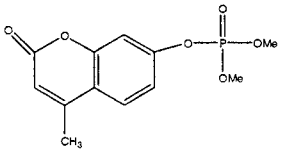
Substrate/Structure	K_m (μM)		k_{cat} (min^{-1})	
	OpdA	OPH	OpdA	OPH
diethyl				
coumaphos 	8.3±1.8	21.4±6.0	12.4±0.6	14.1±2.6
coroxon 	15.9±1.9	25.3±1.3	22.7±0.1	39.5±5.3
paraoxon 	242±61	225±14	33.5±0.5	46.0±0.4
parathion 	92.6±6.4	50.6±12.2	21.9±2.0	23.5±0.2
diazinon 	51.9±4.5	54.2±5.4	65.2±6.7	56.5±2.9
dimethyl				
parathion-methyl 	61.2±2.3	32.9±1.7	94.2±0.8	5.46±0.05
phosmet 	208.3±13.2	nd	0.100±0.002	nd
fenthion 	148.6±17.2	nd	1.63±0.01	nd
dMUP 	66.0±9.1	46.7±4.2	81.7±9.1	20.5±2.3

FIG. 3. Kinetic constants for hydrolysis of OPs by OPH and OpdA. nd, not detected.

ificity might be reduced in OpdA because of the proposed constriction in its large subsite.

Conclusions. OPH is believed to be an ideal enzyme for bioremediation of insecticidal OPs because of its ability to hydrolyze the compounds at a rate approaching the diffusion limits (35), and it is by far the best enzyme of the phosphotriesterases identified (10, 24). In this study we isolated a naturally occurring variant of OPH that has a broader substrate range for insecticidal OPs and kinetics superior to those of OPH for some substrates. OpdA is capable of hydrolyzing

dimethyl substrates at a higher rate than OPH, and the rate of hydrolysis of diethyl OPs is not affected; therefore, OpdA is a better enzyme for practical bioremediation of insecticidal OPs.

ACKNOWLEDGMENTS

We thank Susan Dorrian and Michelle Williams for their technical assistance.

This research was supported by Orica Australia Ltd. and the Australian Horticulture Research and Development Corporation.

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