APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1989, p. 289–293
0099-2240/89/020289-05$02.00/0
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Vol. 55, No. 2

Purification and Characterization of Three Parathion Hydrolases from Gram-Negative Bacterial Strains

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Received 2 September 1988/Accepted 14 November 1988

Three unique parathion hydrolases were purified from gram-negative bacterial isolates and characterized. All three purified enzymes had roughly comparable affinities for ethyl parathion and had broad temperature optima at ca. 40°C. The membrane-bound hydrolase of *Flavobacterium* sp. strain ATCC 27551 was composed of a single subunit of approximately 35,000 daltons (Da) and was inhibited by sulfhydryl reagents such as dithiothreitol (DTT) and by metal salts such as $CuCl_2$. The cytosolic hydrolase of strain B-1 was composed of a single subunit of approximately 43,000 Da and was stimulated by DTT and inhibited by $CuCl_2$. The membrane-bound hydrolase of strain SC was composed of four identical subunits of 67,000 Da and was inhibited by DTT and stimulated by $CuCl_2$. The substrate ranges of the three enzymes also differed, as evidenced by their relative affinities for parathion and the related organophosphate insecticide *O*-ethyl-*O*-4-nitrophenyl phenylphosphonothioate (EPN). The B-1 hydrolase displayed equal affinity for both compounds, the *Flavobacterium* enzyme showed twofold-lower affinity for EPN than for parathion, and the SC hydrolase displayed no activity toward EPN. The range in characteristics of these three enzymes can be exploited in different waste disposal strategies.

Organophosphate compounds such as parathion (*O*,*O*-diethyl-*O*-4-nitrophenyl phosphorothioate) constitute the largest class of insecticides currently used in the industrialized countries. The relatively low persistence of organophosphates has been attributed to their susceptibility to hydrolysis by soil microbial enzymes. Interest in these hydrolytic enzymes has increased because of their effect on the fate of pesticides in the environment as well as their potential use in pesticide waste detoxification.

Parathion hydrolase activity (which yields diethylthiophosphoric acid and *p*-nitrophenol as products of parathion hydrolysis) has been reported for a variety of bacterial isolates (for a review, see reference 11). However, detailed studies have focused on the activities of only two bacterial isolates, *Flavobacterium* sp. strain ATCC 27551 and *Pseudomonas diminuta* MG (2, 10, 13). Although the reported hydrolase activities of these two strains differed in several respects, the genes responsible (termed *opd* genes for organophosphate degradation) have been cloned from both organisms and shown to be closely related by Southern hybridization (8, 9, 12). More recently, yet another bacterial isolate possessing parathion hydrolase activity has been shown by Southern hybridization to contain DNA homologous to the *Flavobacterium opd* gene (3).

Parathion hydrolases encoded by genes related to the *opd* gene of *Flavobacterium* sp. strain ATCC 27551 have a variety of characteristics which make them attractive for use in waste disposal strategies. All are constitutively produced in their native hosts, have broad pH and temperature optima, and have relatively broad substrate ranges for structurally similar organophosphates (2, 10). Indeed, the hydrolase-producing *Flavobacterium* strain has already been used in a pilot-scale system to detoxify waste containing high concentrations of the organophosphate insecticide coumaphos [*O*,*O*-diethyl-*O*-(3-chloro-4-methyl-2-oxo-2*H*-1-benzopyran-7-yl)phosphorothioate] (6). However, there are situations in which these enzymes are not suitable, and no alternatives were available. Our laboratory has been particularly interested in characterizing parathion hydrolases unrelated to those encoded by the *opd* gene, with the ultimate goal of extending the range of pesticide wastes that can be biodegraded. On a more basic level, characterization and comparison of different hydrolases may help us understand how these enzymatic activities have evolved within the soil microbial community. In this report we describe the purification and characterization of three unique parathion hydrolases, two of which have not been described previously.

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MATERIALS AND METHODS

Bacterial Strains. *Flavobacterium* sp. strain ATCC 27551 has been described previously (8, 13). Strain B-1 was isolated from an enrichment culture with the organophosphate insecticide coumaphos as a carbon source and material from cattle dipping vats as the inoculum (14). Strain SC was isolated from an enrichment culture with parathion as a carbon source and sludge from Columbia, S.C., as the inoculum. Strain SC was a gram-negative, oxidase-negative, strictly aerobic, nonmotile rod. It was capable of growth on glucose, xylose, ribose, mannitol, ribitol, guanine, cytosine, and *p*-nitrophenol. All three strains were grown on LB medium (Gibco, Madison, Wis.) with shaking, strain B-1 at 37°C and strains SC and the *Flavobacterium* sp. at 30°C.

Purification of hydrolases. (i) Flavobacterium sp. Cells from 4 liters of *Flavobacterium* culture which had been grown for 48 h were pelleted by centrifugation (4,000 × *g*, 10 min, 4°C). The cell pellets were weighed and suspended with 2 ml of ice-cold 50 mM potassium phosphate (KP_i) (pH 7.2) per g of wet cells. This cell suspension was passed twice through a chilled French pressure cell (15,000 lb/in^2) and then subjected to ultracentrifugation (105,000 × *g*, 2 h, 4°C). The 105,000 × *g* pellet was weighed and suspended in 8 ml of ice-cold 20 mM KP_i (pH 7.2)–0.4% Triton X-100 per g of pellet (wet weight). After incubation at 4°C for 16 h, the suspension was subjected to ultracentrifugation as described

above, and the supernatant from this treatment (crude Triton extract) was removed and used as source of parathion hydrolase for further purification.

Triton X-100 was removed from the crude Triton extract by passing the extract through a column containing Extracti-Gel D (Pierce Chemical Co. Rockford, Ill.) which had been equilibrated with 50 mM KP_i (pH 7.2). The treated material was pumped at 4 ml/min directly onto a TSK-DEAE column (2.15 by 15 cm) (Waters Associates, Milford, Mass.) that had been equilibrated with 20 mM KP_i (pH 7.2) (buffer A). The column was washed with buffer A at 4 ml/min, and fractions containing the unbound protein were collected. Fractions containing parathion hydrolase activity were pooled and used for further purification.

A portion of the pooled TSK-DEAE fractions containing parathion hydrolase activity was brought to 0.5 M $(NH_4)_2SO_4$ by the addition of 1 volume of buffer A containing 1 M $(NH_4)_2SO_4$ and pumped at 4 ml/min onto a TSK-phenyl column (2.15 by 15 cm) (HP-Genenchem, South San Francisco, Calif.) that had been equilibrated with 20 mM KP_i (pH 7.2)–0.5 M $(NH_4)_2SO_4$ (buffer B). The column was washed with the equilibration buffer until all unbound material was eluted and a stable baseline was obtained. A linear gradient of 0.5 to 0 M $(NH_4)_2SO_4$ and 0 to 10% dimethyl sulfoxide (DMSO) in buffer A was then run to elute the bound material from the column. Peak active fractions were pooled.

Size exclusion chromatography of pooled phenyl fractions was carried out with a Protein-Pak 300SW column (7.8 by 300 mm) (Waters) equilibrated and run with buffer A at 1 ml/min. Pooled phenyl fractions were concentrated by vacuum dialysis to approximately 1 ml, and 0.1 ml of this concentrate was injected onto the column. Fractions containing hydrolase activity were pooled.

(ii) Strain SC hydrolase. Crude Triton extracts were prepared from 2 liters of 16-h cultures in the same manner as for the *Flavobacterium* sp. except that $CuCl_2$ was added to all buffers to a final concentration of 1 mM.

Triton was removed from the SC crude Triton extract as described above, and the treated material was loaded onto a TSK-DEAE column (2.15 by 15 cm) that had been equilibrated with 20 mM KP_i (pH 7.2)–0.01 mM $CuCl_2$ (buffer C). The column was washed with buffer C until all unbound protein was eluted and a stable baseline was obtained. A linear gradient of 0 to 1.0 M NaCl in buffer C was then run (5 ml/min) to elute bound material from the column. Fractions containing parathion hydrolase activity were pooled, dialyzed against buffer C, reapplied to the TSK-DEAE column, and eluted in the same manner as for the first chromatography step. This step was repeated a third time in the same manner except that a linear gradient of 0 to 0.5 M NaCl was used to elute bound material from the column. Fractions containing parathion hydrolase activity were pooled.

(iii) Strain B-1 hydrolase. Cells from 4 liters of a 48-h culture were pelleted as above, weighed, and suspended in 2 ml of 50 mM KP_i (pH 7.2)–1 mM dithiothreitol (DTT)–0.1 mM EDTA per g of wet cells. The cells were disrupted as above, and whole cells and large debris were removed by centrifugation (12,000 × *g*, 10 min, 4°C). The supernatant was subjected to ultracentrifugation as above, and the supernatant from this treatment (crude soluble fraction) was removed and used as a source of parathion hydrolase for further purification.

A portion of the crude soluble fraction was pumped onto a TSK-DEAE column (2.15 by 15 cm) that had been equilibrated with 20 mM KP_i (pH 7.2)–1 mM DTT–0.1 mM EDTA (buffer D). The column was washed with buffer D until all unbound material was eluted and a stable baseline was obtained. A linear gradient of 0 to 1.0 M NaCl in buffer D was then run (5 ml/min) to elute bound material from the column. Fractions containing parathion hydrolase activity were pooled and used for further purification.

Pooled B-1 TSK-DEAE fractions were subjected to chromatography on a TSK-phenyl column as described above for the *Flavobacterium* sp. hydrolase except that all buffers contained 1 mM DTT and 0.1 mM EDTA, and bound material was eluted with a linear gradient of 0.5 to 0 M $(NH_4)_2SO_4$ (i.e., DMSO was omitted). Active fractions were pooled, dialyzed overnight at 4°C against 100 volumes of buffer D, and used for further purification.

Pooled B-1 TSK-phenyl fractions were subjected to a second round of chromatography on a TSK-DEAE column in the same manner as before except that the bound material was eluted with a linear gradient of 0 to 0.5 M NaCl in buffer D. Active fractions were pooled.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed by the method of Laemmli (7). The 1.5-mm-thick slab gels were 12% acrylamide resolving gels with 4% acrylamide stacking gels. The proteins in the gels were fixed and stained in a solution of 0.25% Coomassie brilliant blue R in 40% methanol–7% acetic acid. The gels were destained in 40% methanol–7% acetic acid.

Determination of native molecular weights. Enzyme native molecular weights were estimated by size exclusion chromatography with a Protein-Pak 300SW column equilibrated and run in buffer A. The column was calibrated with freshly prepared protein standards (Combithek Calibration Proteins II; Boerhinger Mannheim, Indianapolis, Ind.) run in buffer A.

Protein determinations. Protein concentrations in crude extracts were determined by the method of Bradford (1) with the Bio-Rad protein assay kit with immunoglobulin G as the standard. The concentration of protein in partially purified and purified enzyme preparations was determined by the spectrophotometric method of Kalb and Bernlohr (5).

Enzyme assays. Hydrolysis of ethyl parathion and EPN (*O*-ethyl-*O*-4-nitrophenyl phenylphosphonothioate) was measured by monitoring the appearance of *p*-nitrophenol at 405 nm. Routine hydrolase assay mixtures contained 0.6 ml of 50 mM Tris hydrochloride (pH 8.5), 0.1% Triton X-100, and 50 μg (final concentration of 172 μM for ethyl parathion) of the pesticide per ml (added from a 10-mg/ml methanol stock solution), to which 10 μl of partially purified or purified hydrolase extracts was added. Unless otherwise noted, all assays were run at 30°C for 1 min. The temperature optima of the three hydrolases were determined in assay mixes containing 10 μg of ethyl parathion per ml. The K_m constants of the three enzymes were determined by using EPN and ethyl parathion concentrations between 13 and 55 μM and 17 and 69 μM, respectively.

RESULTS

Enzyme purification. Initial experiments with crude extracts of the *Flavobacterium* sp., strain B-1, and strain SC revealed that in each case extracts made in solutions buffered by potassium phosphate were considerably higher in hydrolase activity and were more stable than extracts made in solutions buffered by Tris hydrochloride or MOPS (3-[*N*-morpholino]propanesulfonic acid).

The presence of 1 mM DTT and 0.1 mM EDTA in the lysis buffer stabilized and increased hydrolase activity in extracts

TABLE 1. Purification of parathion hydrolases from a *Flavobacterium* sp., strain SC, and strain B-1

Organism	Purification step	Total protein (mg)	Total activity (IU)	Sp act (IU/mg of protein)	Recovery (%)	Purification (fold)
Strain B-1	Crude soluble	532.5	1.117	0.002	100	
	TSK-DEAE 1	43.5	0.718	0.016	64.3	7.6
	TSK-phenyl	1.60	0.681	0.425	60.9	202.0
	TSK-DEAE 2	0.23	0.152	0.655	13.6	311.0
Strain SC	Triton extract	57.5	187.2	3.25	100	
	TSK-DEAE 1	9.6	33.5	3.49	17.9	1.1
	TSK-DEAE 2	3.0	15.6	5.20	8.3	1.6
	TSK-DEAE 3	0.36	2.6	7.22	1.4	2.2
Flavobacterium sp.	Triton extract	215.9	726.4	3.36	100	
	TSK-DEAE	10.83	295.0	27.2	40.6	8.1
	TSK-phenyl	0.05	150.9	3,018	20.7	898
	300SW	0.002	3.6	1,605	—[a]	478

[a] Only a small portion of the TSK-phenyl-purified material was subjected to 300SW chromatography. Of the portion which was run, the recovery of hydrolase activity was 62%.

of strain B-1 (activity half-life was approximately 3 days at 4°C). After ultracentrifugation of strain B-1 crude extracts, >95% of hydrolase activity was associated with the soluble fraction. Purification of the B-1 hydrolase was accomplished by chromatography of the crude soluble fraction on a TSK-DEAE column, followed by chromatography of active fractions on a TSK-phenyl column. Final chromatography of the pooled TSK-phenyl fractions on the TSK-DEAE column only slightly purified the B-1 protein but was included as a concentration step (Table 1). The final preparation had a specific activity over 300 times that of the starting material and yielded a single band of approximately 43,000 daltons (Da) when subjected to SDS-PAGE (Fig. 1).

In contrast to B-1, hydrolase activity in SC extracts was enhanced and stabilized by the addition of $CuCl_2$, $CoSO_4$, or $ZnSO_4$ to the lysis buffer. Other metal salts were also tested but only increased activity slightly ($NiSO_4$, CuCl, $MnCl_2$), had no effect ($FeCl_3$, $MgCl_2$, RbCl), or were inhibitory ($CaCl_2$, $SnCl_4$) compared with extracts to which metals were not added. Approximately 75% of the activity in crude extracts was associated with the membrane fraction after ultracentrifugation. Extraction of the membrane pellet with buffer containing 0.4% Triton X-100 solubilized greater than 90% of the membrane-associated activity. Partial purification of the solubilized SC hydrolase was accomplished by three chromatographic runs on a TSK-DEAE column. Attempts to purify the SC hydrolase further by TSK-phenyl chromatography were unsuccessful. However, after multiple rounds of DEAE chromatography, a 67,000-Da band in SDS protein gels was associated with SC hydrolase activity (Fig. 2). Although purification of this enzyme was apparent from SDS-PAGE of extracts taken at different stages of purification (Fig. 2), Table 1 shows that the specific activity of SC extracts did not increase significantly during the purification steps.

Approximately 90% of the hydrolase activity in *Flavobacterium* crude extracts was associated with the membrane fraction after ultracentrifugation. A single extraction of the membrane pellet with buffer containing 0.4% Triton X-100 solubilized approximately 30% of the membrane-associated activity. Purification of the *Flavobacterium* hydrolase to a

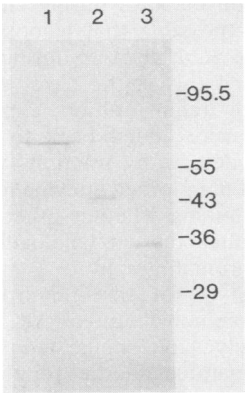

FIG. 1. Comparison of the subunit molecular masses of the parathion hydrolases from a *Flavobacterium* sp., strain SC, and strain B-1. Purified proteins from each strain were subjected to SDS-PAGE as described in the text. Molecular mass values of protein standards are expressed in kilodaltons. Lanes: 1, SC hydrolase; 2, B-1 hydrolase; 3, *Flavobacterium* sp. hydrolase.

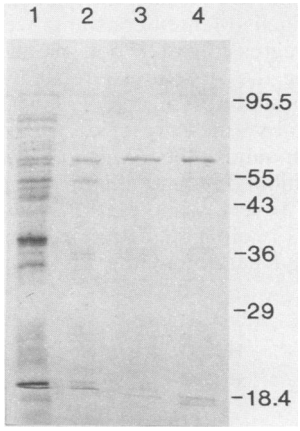

FIG. 2. Partial purification of the parathion hydrolase from strain SC. Samples were taken at different stages of SC hydrolase purification and subjected to SDS-PAGE as described in the text. Molecular mass values of protein standards (mid-range kit; Diversified Biotech, Newton Centre, Mass.) are expressed in kilodaltons. Lanes: 1, crude Triton extract; 2, pooled fractions after first DEAE step; 3, pooled fractions after second DEAE step; 4, pooled fractions after third DEAE step.

TABLE 2. Comparison of hydrolases from a *Flavobacterium* sp., strain SC, and strain B-1

Hydrolase	Mass (Da)		Temp optimum (°C)	K_m (μM) of[a]:		Cellular location	Relative hydrolysis (% of control)[b] with:	
	Native	Subunit		Ethyl parathion	EPN		DTT	$CuCl_2$
Flavobacterium sp.	30,500	35,000	40	91	211	Membrane	<1	53
Strain SC	270,000	67,000	40	106	—[c]	Membrane	<1	288
Strain B-1	40,000	43,000	40	25	26	Cytoplasm	244	18

[a] The chemical structures of ethyl parathion and EPN are shown in Fig. 3.
[b] Calculated as relative rates of ethyl parathion hydrolysis by extracts prepared in buffers containing either 1 mM DTT or 1 mM $CuCl_2$ versus the rate of hydrolysis by extracts made with buffer containing no additions (control).
[c] The rate of hydrolysis of EPN was <1% of the rate of hydrolysis of ethyl parathion at similar substrate concentrations, and the kinetics of EPN hydrolysis did not conform to the equation of Michaelis and Menton.

single band of approximately 35,000 Da on SDS gels was accomplished by the chromatographic methods used (Fig. 1). The specific activity of the purified extracts increased nearly 900-fold during this procedure (Table 1). Size exclusion chromatography of pooled TSK-phenyl fractions containing *Flavobacterium* hydrolase activity was necessary to remove minor high-molecular-weight proteins in the preparation (data not shown). However, the specific activity of the extract decreased approximately twofold as a consequence of this step (Table 1).

Enzyme characterization. Native molecular weights of the three hydrolases were estimated by chromatography of purified extracts on a 300SW column. Subunit molecular weights of these proteins were determined by SDS-PAGE (Table 2, Fig. 3). For the B-1 hydrolase, our estimates for its native and subunit molecular masses were comparable (40,000 and 43,000 Da, respectively). Therefore, the enzyme is probably composed of a single subunit of approximately 41,500 Da. Similarly, the comparable native and subunit molecular masses of the *Flavobacterium* enzyme (30,500 and 35,000 Da, respectively) suggest that this enzyme is also composed of a single subunit somewhat smaller than that of the B-1 hydrolase. In contrast, our data suggest that the hydrolase of strain SC is a much larger enzyme (native molecular mass of 270,000 Da) composed of four identical subunits of 67,000 Da each (Table 2, Fig. 3).

In order to compare the substrate specificities of the three enzymes, Michaelis-Menten constants were estimated from least-squares regression of Woolf plots for the three enzymes by using the structurally related organophosphates ethyl parathion and EPN (Table 2, Fig. 3). Taking the K_m values as a relative measure of each enzyme's affinity for these two compounds, the *Flavobacterium* and strain SC hydrolases exhibited equal affinities for ethyl parathion (Table 2). However, while the *Flavobacterium* enzyme showed about a twofold reduction in affinity for EPN compared with ethyl parathion, the SC hydrolase showed no detectable activity toward EPN (thus, no K_m could be determined for the SC enzyme with EPN). In contrast, the hydrolase of strain B-1 demonstrated equal affinity for both substrates (Table 2).

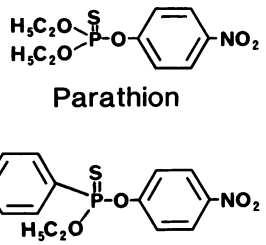

FIG. 3. Chemical structures of parathion and EPN.

Temperature optima for the three enzymes were determined by kinetic experiments with ethyl parathion. All three enzymes displayed relatively broad optima at about 40°C.

DISCUSSION

Although a variety of bacterial isolates possessing parathion hydrolase activity have been reported in the literature (3, 10) and the genes responsible have been cloned from two isolates, purification of these proteins has never been reported. By using high-pressure liquid chromatography, we have been able to purify three distinct hydrolases from soil bacteria. In our hands these methods were rapid and resulted in reasonable yields of highly purified enzyme.

During the isolation procedures for the *Flavobacterium* and strain B-1 hydrolases, enzyme purification was evidenced both by increases in specific activities and by SDS-PAGE of fractions taken at different stages of the procedures. However, although partial purification of the strain SC hydrolase could be shown by SDS-PAGE, the specific activity of the SC hydrolase preparation did not rise concurrently. Experiments designed to determine why strain SC extracts did not increase in specific activity after DEAE chromatography showed that the enzyme's specific activity was not affected by any of the following: its extraction from the membrane with Triton X-100, removal of Triton from the extract, or addition of NaCl during chromatography (data not shown). It is possible that a loosely bound factor is removed from the SC hydrolase during DEAE chromatography. However, if this is the case, the loss is probably irreversible, since reconstitution experiments in which DEAE fractions were added back to the DEAE-purified enzyme failed to detect any fractions which enhanced the specific activity of the purified enzyme (data not shown). We are currently examining whether other cofactors could be involved or whether the enzyme suffers conformational changes during chromatography.

Characterization of the three hydrolases revealed that they had some common features. All appeared to be produced constitutively, have similar temperature optima, and have roughly comparable affinities for ethyl parathion. However, the three enzymes are quite distinct in their sizes (and the sizes of their subunits), their relative affinities for the structurally related organophosphates ethyl parathion and EPN, their cellular locations, and their stimulation or inhibition by divalent cations and DTT. The substrate ranges of the three enzymes are of particular interest, not only from

the standpoint of their potential use in pesticide waste disposal, but also because such information may provide insight into how their active sites function. Most pesticide hydrolases examined to date seem to have rather relaxed substrate specificities, being able to hydrolyze almost any compound within the same general class of pesticide (11). Both the *Flavobacterium* and B-1 hydrolases seemed to follow this pattern, as they both hydrolyzed EPN as well as parathion. Preliminary experiments measuring the ability of these enzymes to hydrolyze other pesticides over a 24-h period indicate that they also hydrolyze coumaphos (in which the *p*-nitrophenol of parathion is replaced by a coumarin moiety) (data not shown). The hydrolase from strain SC is unique in that it lacks the ability to hydrolyze either EPN or coumaphos. Thus, substitution on either side of the phosphate ester of parathion renders a compound completely resistant to hydrolysis by the SC enzyme. Additional substrate range experiments with the SC hydrolase are under way to determine whether steric hindrance at the active site or some other factor is responsible for its inability to hydrolyze substrates other than parathion.

Previous analyses of parathion hydrolases from soil bacteria have focused primarily on two bacterial isolates, *Pseudomonas diminuta* MG and *Flavobacterium* sp. strain ATCC 27551. The plasmid-borne *opd* genes responsible for these enzymes have both been cloned and shown to be closely related by Southern hybridization experiments. In contrast, Southern hybridization experiments which used DNA containing the *Flavobacterium* sp. *opd* gene to probe DNA from strains SC and B-1 failed to detect DNA from these two strains with homology to *opd* (data not shown). In light of the significant differences in the properties of these three enzymes, it is not surprising that their genes have little or no homology.

Investigations of degradation enzymes and the genes which encode them have shown that genetically related degradative enzymes can be produced by geographically and taxonomically diverse groups of bacterial isolates (4). Thus, in many cases, the widespread occurrence of bacteria with degradative abilities may be due to the spread of a relatively few ancestral degradation genes among the soil microflora (4). However, our results suggest that this is not the case for bacterial parathion hydrolase activities, of which there are at least three classes. Indeed, preliminary characterizations of parathion hydrolases from other bacterial isolates in our laboratory collection suggest that two other distinct classes of these enzymes exist. This diversity of enzymes, while making the ecology of organophosphate biodegradation complex, offers great potential for pesticide waste disposal technology, in which the different characteristics of different wastes preclude the use of a single enzyme or organism for all situations.

ACKNOWLEDGMENTS

We gratefully acknowledge Daniel Shelton for the gift of strain B-1 and for his help in the isolation of strain SC and Vicki Wolff for excellent technical assistance.

This work was supported in part by a grant to W.M. from the Maryland Agricultural Biotechnology Center.

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