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Parathion Hydrolase Specified by the *Flavobacterium opd* Gene: Relationship between the Gene and Protein

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The sequence of a 1,693-base-pair plasmid DNA fragment from *Flavobacterium* sp. strain ATCC 27551 containing the parathion hydrolase gene (*opd*) was determined. Within this sequence, there is only one open reading frame large enough to encode the 35,000-dalton membrane-associated hydrolase protein purified from *Flavobacterium* extracts. Amino-terminal sequence analysis of the purified *Flavobacterium* hydrolase demonstrated that serine is the amino-terminal residue of the hydrolase protein. The amino-terminal serine corresponds to a TCG codon located 87 base pairs downstream of the presumptive ATG initiation codon in the nucleotide sequence. The amino acid composition of the purified protein agrees well with that predicted from the nucleotide sequence, using serine as the amino-terminal residue. These data suggest that the parathion hydrolase protein is processed at its amino terminus in *Flavobacterium* sp. Construction in *Escherichia coli* of a *lacZ-opd* gene fusion in which the first 33 amino-terminal residues of *opd* were replaced by the first 5 residues of *lacZ* resulted in the production of an active hydrolase identical in molecular mass to the hydrolase isolated from *Flavobacterium* sp. *E. coli* cells containing the *lacZ-opd* fusion showed higher levels of hydrolase activity than did cells containing the parent plasmid.

The microbial degradation of hazardous waste offers a promising strategy by which such some wastes may be economically and safely detoxified. For selected situations, microbial processes have considerable advantages over other technologies in that microbial processes can yield precise products, function at low concentrations of solute, and require relatively low levels of technology for construction and maintenance. However, there are relatively few instances in which microbial processes are being actively used to control hazardous wastes.

Organophosphate compounds such as the insecticide parathion (*O,O*-diethyl-*O*-4-nitrophenyl phosphorothioate) are susceptible to microbial hydrolysis by bacterial parathion hydrolases. In fact, such soil hydrolases are thought to play a role in the relatively low persistence of these compounds. Since organophosphates constitute a large fraction of the insecticides used in the industrialized countries, there is a need for economical and reliable methods to detoxify organophosphate wastes (such as residual pesticide concentrates in their original containers, contaminated stock solutions, and dilute pesticide solutions resulting from the washing of spraying equipment). This need has stimulated recent research on parathion hydrolases.

Parathion hydrolase activities have been described in a variety of bacterial isolates and are characterized by broad substrate ranges for compounds structurally related to parathion, broad temperature and pH optima, and high stability (1, 10, 13, 14). Of the parathion hydrolases that have been characterized in detail, the enzymes encoded by the related *opd* genes of *Flavobacterium* sp. strain ATCC 27551 and *Pseudomonas diminuta* MG are noteworthy because of their high specific activities (10, 16). The hydrolase-producing *Flavobacterium* strain has 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-2H-1-benzopyran-7-yl) phosphorothioate] (6).

Our laboratory has been particularly interested in modifying the *Flavobacterium opd* gene in order to maximize parathion hydrolase activity in other bacterial hosts. Our specific goals in this study were to (i) determine the nucleotide sequence of the *Flavobacterium opd* gene; (ii) determine the amino-terminal sequence and total amino acid composition of the hydrolase protein isolated from *Flavobacterium* extracts; and (iii) characterize the hydrolase proteins produced in *Escherichia coli* strains when the *opd* gene (and mutagenized *opd* genes) is activated by an *E. coli* promoter. While this work was in progress, the nucleotide sequences of the *Flavobacterium* and *P. diminuta opd* genes were reported (4, 9). We discuss the differences between our nucleotide sequence and those sequences.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Flavobacterium* sp. strain ATCC 27551 and the conditions for its culture have been described previously (10, 17). *E. coli* DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used as the host for plasmids pWM513 and pJK33 (described below) for hydrolase purification. pWM44 is a hybrid plasmid in which a 7.3-kilobase-pair (kb) *Eco*RI fragment from the *Flavobacterium* plasmid pPDL2 was inserted into pBR325 (11). pWM513 is a subclone of pWM44 in which a 1.3-kb *Pst*I fragment containing the *opd* gene was inserted into pUC19 (21).

DNA sequencing. The sequencing strategy is shown in Fig. 1. Subfragments of pWM44 and pWM513 were ligated into M13 vectors mp18 and mp19 (21) and sequenced by the dideoxy method of Sanger et al. (15), using a Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) and a Macrophore sequencing apparatus (LKB Instruments, Inc., Rockville, Md.). The DNA sequences of several subfragments were independently determined by using a model 370A sequencer (Applied Biosystems, Inc., Foster City, Calif.),

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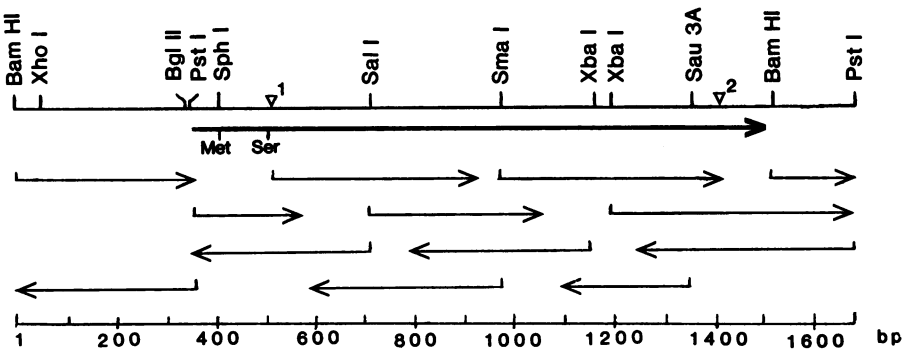

FIG. 1. Strategy for sequencing the 1,693-bp DNA fragment from the *Flavobacterium* plasmid pPDL2 containing the *opd* gene. Light arrows indicate DNA sequences from M13 subclones that were combined to yield the entire DNA sequence. Heavy arrow shows the ORF for the *opd* gene. Positions of the putative translation initiation site and actual amino terminus are marked Met and Ser, respectively. Restriction sites used to generate M13 subclones are shown (only one of many *Sau*3A sites is shown). The site of fusion between the *lacZ* and *opd* genes in plasmid pJK33 is marked ∇^1. The linker insertion site used to generate plasmid pJK33∇Dde is marked ∇^2. The length of the sequence in base pairs is shown.

with 7-deaza-dGTP and Sequenase used as described by the manufacturer.

Protein purification, amino-terminal sequencing, and amino acid composition analysis. Purification of the membrane-bound *Flavobacterium* hydrolase has been described elsewhere (10). The hydrolase proteins specified by pWM513 and pJK33 were purified from *E. coli* extracts, using essentially the procedure used for the *Flavobacterium* hydrolase except that the pWM513 and pJK33 hydrolases were isolated from the soluble fraction of cell extracts rather than from the membrane fraction. Before amino-terminal analysis and amino acid composition analysis, protein samples were further purified by reverse-phase chromatography, using a Brownlee RP300 column (Applied Biosystems) and a gradient of 0 to 67% acetonitrile–33% *n*-propanol in 0.1% trifluoroacetic acid. Amino-terminal residues of protein samples were determined by using 10-μg samples and an Applied Biosystems model 477A peptide sequencer. The amino acid composition of a 3-μg sample of the *Flavobacterium* hydrolase was determined by high-performance liquid chromatography separation of the amino acids from a 24-h hydrolysate in 6 N HCl at 108°C.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed by the method of Laemmli (8), using 12% resolving gels and 4% 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.

Construction of the *lacZ-opd* fusion plasmid pJK33. To generate a series of *lacZ-opd* gene fusions, the 1.2-kb *Sph*I-*Pst*I fragment from pWM513 was digested with exonuclease S1 (3 mg/ml of DNA, 15,000 U/ml of enzyme, 37°C, 30 min). These digestion conditions allowed the digestion of both single- and double-stranded DNA (see Results). After extraction with phenol-chloroform and precipitation with ethanol, the DNA was subjected to blunt-end ligation with an excess of the *Eco*RI linker oligonucleotide 5′-GGAAT TCC-3′ (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and then digested with *Bam*HI and *Eco*RI. After extraction with phenol-chloroform and precipitation with ethanol, the DNA was ligated to pUC19 DNA that had been digested with *Bam*HI and *Eco*RI and used to transform *E. coli* DH5α cells. Ampicillin-resistant transformants were screened for parathion hydrolase activity (10). DNA sequencing was used to determine the *lacZ-opd* fusion sites in the recombinant plasmids that were generated.

Mutagenesis of pJK33. pJK33 is an active *lacZ-opd* gene fusion plasmid (its construction is described above). The 910-base-pair (bp) *Eco*RI-*Dde*I DNA fragment from pJK33 was isolated and ligated to a *Dde*I-digested *Dde*I-*Hin*dIII linker oligonucleotide, 5′-TGAGGTAGAAGCTT-3′ (Synthecell Corp., Rockville, Md.). After ligation, the DNA was digested with *Hin*dIII, ligated to pUC19 DNA that had been digested with *Eco*RI and *Hin*dIII, and used to transform *E. coli* DH5α cells. Insertion of the oligonucleotide was verified by DNA sequencing the recombinant plasmid (pJK33∇Dde) that was generated.

RESULTS

Nucleotide sequence of the *Flavobacterium* plasmid fragment containing the *opd* gene. Figure 2 shows the nucleotide sequence of the 1,693-bp *Bam*HI-*Pst*I DNA fragment from the 39-kb *Flavobacterium* plasmid pPDL2. Previous studies have demonstrated the orientation and approximate location of the *opd* gene within the 1,168-bp region bounded by the *Pst*I site at position 357 and the *Bam*HI site at position 1525 (11, 12, 16). Within this region, there is only one open reading frame (ORF) large enough to encode the 35,000-dalton (Da) hydrolase protein (10). Upstream of this reading frame, a possible promoter was identified on the basis of its homology to the *E. coli* promoter consensus sequence at positions −35 and −10 (5). A possible Shine-Dalgarno sequence, AAGG, is located 7 bp upstream of the first ATG codon that occurs in the reading frame (19).

Amino-terminal analysis and amino acid composition of the *Flavobacterium* hydrolase. By using the first ATG codon (position 419) within the ORF as the presumptive translation initiation site of the *opd* gene, a protein of 39,000 Da is predicted from the nucleotide sequence. This value is considerably larger than the estimate of 35,000 Da obtained from chromatography and electrophoresis of the purified protein (10). One explanation for the discrepancy between the predicted and observed protein masses is that the hydrolase protein is translated as a larger polypeptide that is then subjected to posttranslational cleavage to yield a smaller, mature species. Indeed, the posttranslational processing of this membrane-bound protein may play an integral role in determining its ultimate cellular location. To test this hy-

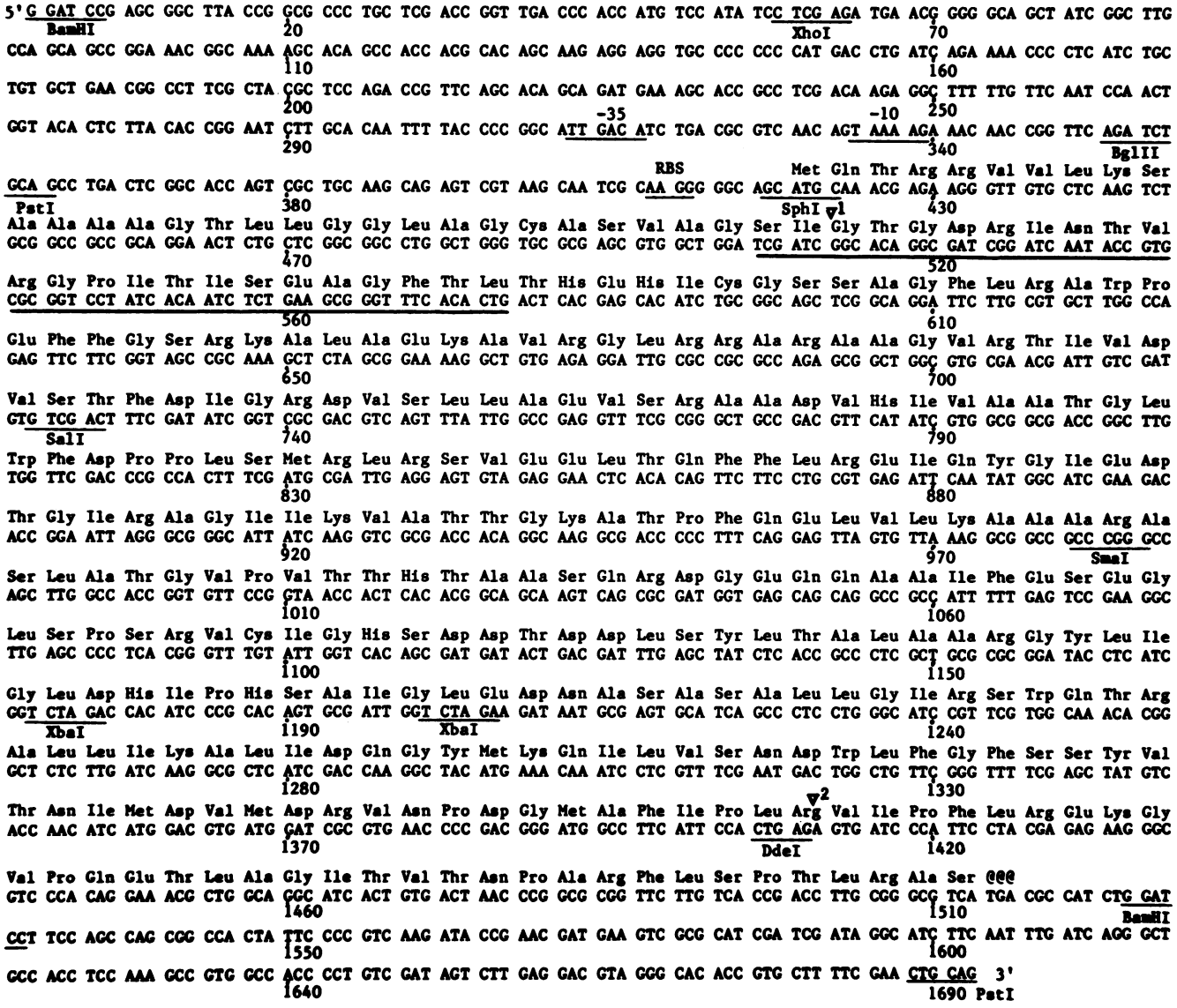

FIG. 2. Nucleotide and deduced amino acid sequences of the *Flavobacterium* parathion hydrolase gene *opd*. Putative promoter sequences (−35 and −10), a Shine-Dalgarno sequence (RBS), and sites of oligonucleotide insertion for constructing pJK33 (∇^1) and pJK33∇Dde (∇^2) are shown above the appropriate locations. The amino-terminal residues of the hydrolase protein that was purified from *Flavobacterium* extracts are underlined by a thick line. Restriction sites used in the DNA sequencing are underlined by thin lines.

pothesis, the hydrolase protein was purified from *Flavobacterium* extracts, and the first 24 amino-terminal residues were determined (Fig. 2). The sequence of the amino-terminal residues of the *Flavobacterium* hydrolase revealed that serine is the amino-terminal residue, corresponding to a TCG codon (position 506) 87 bp downstream of the presumptive ATG initiation codon in the DNA sequence (Fig. 2). Comparison of the amino acid composition of the purified protein with the composition predicted by the DNA sequence (using serine at position 506 as the amino terminus) showed close agreement between the two (Table 1). Thus, although we have no direct evidence of a precursor polypeptide in *Flavobacterium* extracts, the amino terminus of the mature protein indicates that it is the product of proteolytic cleavage. A plot of the hydropathic character of the polypeptide predicted from the DNA sequence revealed the hydrophobic nature of the putative leader peptide (Fig. 3).

Construction of *lacZ-opd* fusions. Even within constructions in which an exogenous promoter (such as the *lacZ* promoter in pWM513) was inserted upstream of *opd*, parathion hydrolase activity was markedly lower in *E. coli* extracts than in extracts from wild-type *Flavobacterium* cells (Table 2). It is possible that the parathion hydrolase encoded by *opd* is incorrectly processed from its larger precursor in *E. coli* and that proper processing is a prerequisite for optimal enzyme activity. This proposal is supported by comparison of the hydrolase protein isolated from *E. coli* cells containing pWM513 (in which the 1.3-kb *Pst*I fragment containing the *opd* gene is inserted into pUC19) with the hydrolase protein from *Flavobacterium* extracts. First, unlike membrane-bound enzyme in *Flavobacterium* sp., the pWM513 enzyme from *E. coli* extracts was primarily located in the cytosol (Table 2). Second, the pWM513 enzyme was larger than that isolated from *Flavobacterium*

TABLE 1. Comparison of the experimental and predicted amino acid compositions of the *Flavobacterium* sp. hydrolase protein

Amino acid	Molar composition (%)		Predicted no. of residues[a]
	Experimental	Predicted[a]	
Asn + Asp	7.61	7.44	6 + 19 = 25
Gln + Glu	8.39	7.74	10 + 16 = 26
Ser	7.65	7.74	26
Gly	8.66	8.33	28
His	2.01	2.08	7
Arg	7.81	7.74	26
Thr	7.63	7.44	25
Ala	12.60	11.61	39
Pro	4.65	4.17	14
Tyr	1.57	1.49	5
Val	6.11	6.55	22
Met	1.65	1.49	5
Cys	0.44[b]	0.60	2
Ile	5.86	7.74	26
Leu	10.17	9.82	33
Phe	4.51	4.46	15
Lys	2.68	2.38	8
Trp	—[c]	1.20	4

[a] Predicted from the DNA sequence, using serine as the amino-terminal residue.

[b] Recovery of Cys by this method is typically quite low relative to levels of other residues and therefore is of little comparative value.

[c] Trp is destroyed during hydrolysis and therefore cannot be quantitated.

TABLE 2. Comparison of parathion hydrolase activities of extracts of *Flavobacterium* sp. and *E. coli* containing *opd* plasmids[a]

Host strain	Plasmid	Sp act (μmol·min^{-1}·mg of protein^{-1})[b]	% of total activity
Flavobacterium sp.	pPDL2		
Crude extract		6.35	
Soluble fraction		3.10	36
Membrane fraction		17.35	64
E. coli DH5α	pWM513		
Crude extract		0.10	
Soluble fraction		0.11	82
Membrane fraction		0.04	18
E. coli DH5α	pJK33		
Crude extract		0.28	
Soluble fraction		0.33	91
Membrane fraction		0.11	9
E. coli DH5α, crude extract	pJK33∇Dde	ND	

[a] Cell extracts from 500-ml cultures of *Flavobacterium* sp. and *E. coli* were prepared as previously described (10).

[b] Detection limit of the assay is <0.00002 μmol · min^{-1} · mg of protein^{-1}. ND, No detectable activity.

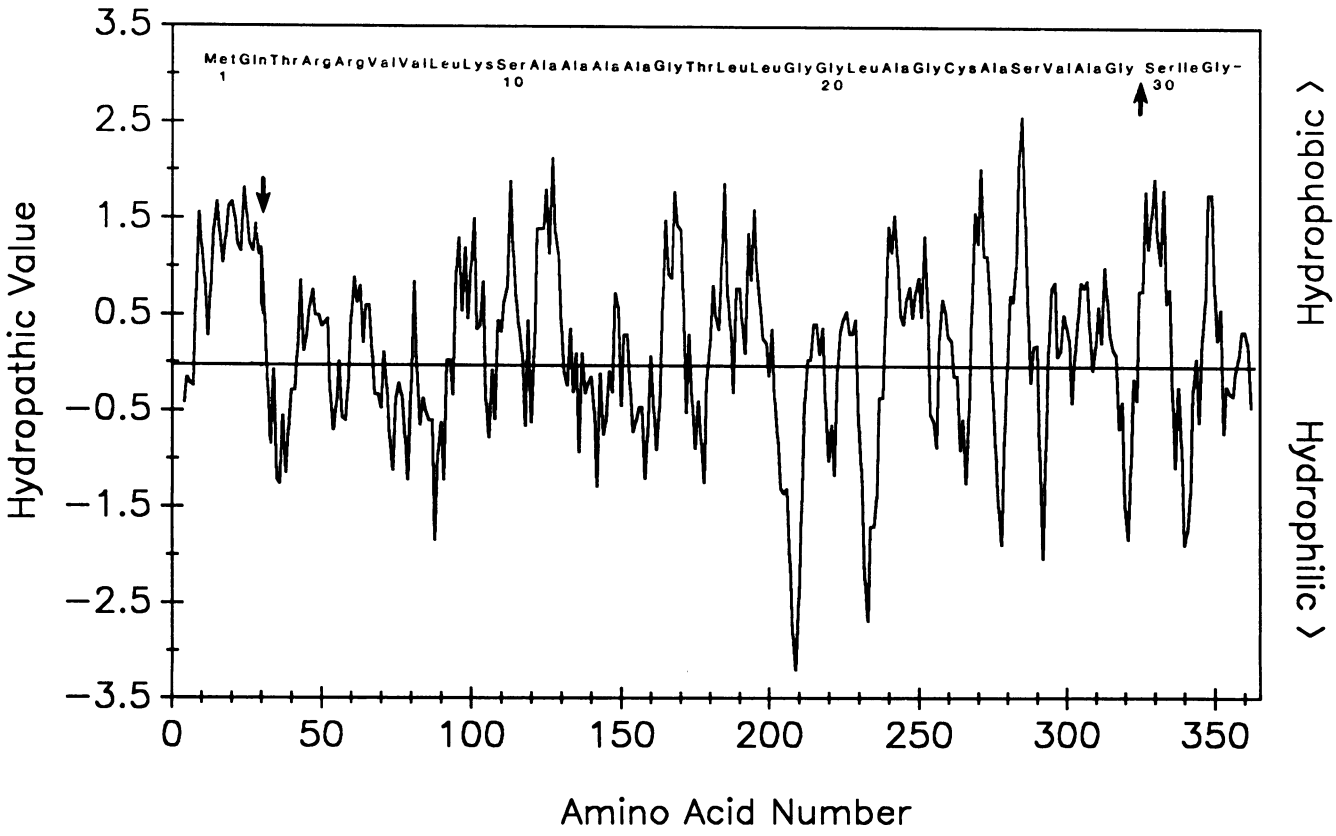

FIG. 3. Plot of the hydrophobic character of the proposed parathion hydrolase precursor polypeptide. Values were calculated for amino acids deduced from the DNA sequence, using an averaging length of seven residues (7). The deduced amino acid sequence of the first 32 residues of the precursor polypeptide is shown above the plot. The leader peptide cleavage site is marked by a vertical arrow.

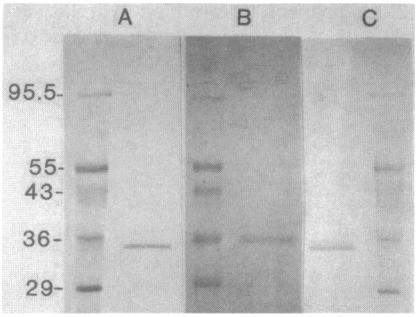

FIG. 4. Comparison of the parathion hydrolase protein from *Flavobacterium* sp. strain ATCC 27551 containing pPDL2 with the hydrolase proteins produced in *E. coli* strains containing pWM513 and pJK33. Purified proteins from each strain were subjected to SDS-PAGE as described in the text. Molecular mass values of protein standards are expressed in kilodaltons. (A) *Flavobacterium* sp.(pPDL2) hydrolase; B, *E. coli*(pWM513) hydrolase; (C) *E. coli* (pJK33) hydrolase.

sp. (Fig. 4). Although it is likely that the pWM513 enzyme corresponds to the predicted hydrolase precursor protein, we have been unable to directly test this hypothesis by sequencing the amino-terminal residues of the pWM513 protein.

If improper (or lack of) processing of the hydrolase in *E. coli* is the basis of the low activity in cells containing pWM513, then deletion of the coding region for the signal peptide could lead to increased parathion hydrolase activity in *E. coli* extracts. To test this hypothesis, *lacZ-opd* fusions were constructed in which the 5′ end of the *opd* gene was partially digested and fused to the first five codons of *lacZ* in pUC19. In one of the resulting hybrid plasmids (pJK33), the first five codons of *lacZ* were fused to the *opd* codon GGC (Gly) at position 518 (Fig. 2), thus deleting the 29-residue signal peptide along with the first four residues of the native mature protein. Amino-terminal sequencing of the hydrolase protein from extracts of cells containing pJK33 confirmed that this fusion protein contained the first five *lacZ* residues (Met-Ile-Thr-Asn-Ser-) followed by hydrolase residues (-Gly-Asp-Arg-). Extracts of *E. coli* cells containing pJK33 showed levels of hydrolase twofold higher than those of cells containing the parent plasmid pWM513 (Table 2). Moreover, the pJK33 *lacZ-opd* fusion protein was identical in mass to the *Flavobacterium* protein, as judged by SDS-PAGE (Fig. 4). That the two proteins had the same mass suggests that the *Flavobacterium* hydrolase is not processed further after cleavage of the leader peptide.

Mutagenesis of the carboxyl region of the *Flavobacterium* hydrolase. The results of our nucleotide-sequencing experiments on the *Flavobacterium opd* gene showed 50 differences from the results of a previous study (Fig. 5) (4). One result of the many differences between the two sequences is that they predict different translation termination points for the hydrolase protein. To test whether the translation of the *Flavobacterium* hydrolase terminates before the point predicted by our nucleotide sequence, a mutant of the *lacZ-opd* fusion gene in pJK33 was constructed by inserting a TAG nonsense codon 3′ to the *opd Dde*I site at position 1406 (Fig. 2). Consistent with the predictions of our sequence, *E. coli* cells containing the mutant plasmid (pJK33-∇Dde) displayed no hydrolase activity (Table 2).

DISCUSSION

This study presents the DNA sequence of the *opd* gene from the 39-kb *Flavobacterium* plasmid pPDL2. Although the sequence we determined varies substantially from a previously reported sequence (4), our sequence is fully consistent with the experimentally determined amino-terminal sequence and amino acid composition of the purified protein. We give evidence that the *Flavobacterium* enzyme is processed at its amino terminus. In addition, we show that deletion of the nucleotide sequence encoding the putative leader peptide increases activity of the gene in *E. coli* extracts.

Examination of the *opd* gene DNA sequence reveals one ORF capable of encoding the 35,000-Da hydrolase protein. A search of the DNA sequence upstream of the putative *opd* coding region revealed putative promoter and Shine-Dalgarno sequences with appropriate spacing. In fact, the sequences found are a perfect match for the −35 consensus sequence (GGTACA) and a less favorable match at −10 (TAAAAG). If these sequences are used in *Flavobacterium* sp. as an RNA polymerase promoter, then it is unclear why they do not function in *E. coli*. Nevertheless, expression of the *opd* gene in *E. coli* is entirely dependent on exogenous promoters (6; W. Mulbry, Ph.D. dissertation, University of Maryland, College Park, 1987). It is possible that there is a transcription termination sequence downstream of this pro-

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455      463....493   497...523   527...533          544...585          596...642  645...657  660...681       689...788   792
  GCCGCAGGA         GAGCG        TCGGA        ACCGTGCGCGGT        AGCACATCTGCG        GCCG        CGGA        GCCGCGCCA        ATCGT
    * * *             *           **            *      *            *      *            *           *           *  **            *
  GC G A GA         GA CG        TGCGA        AT GTGCGCG T        AGGACATCT CG        GC G        CGCA        GC GC  CA        ATC T
 99       104....134   137...163   167...173          182...223          233...279  281...293  296...317       322...421   424

840   844...859   862...872    876...895   898...1048      1053...1075   1079...1145          1158...1225    1230...1402  1405
  GGAGT        AC AG        CG TGA        AG AC        GC AG CA         AGGCT         TCGCTGCGCGCGG         AGCCCT         TCCA
    **            *            *            *            * *             *              *    **              ***            *
  GGTAT        ACTAG        CGGTGA        AGTAC        GCGAGGCA         AG CT         T GCTG  CGCGG         ACCGCT         T CA
472   476...491   495...505    510...529   533....683      690.....712   715.....781          790.....857    862....1034  1036

1422  1425...1489   1492..1500          1512...1535   1539...1605  1608..1633  1635...1659                                1687
  TCCT         CT TG        CCTTGCGGGCGT C         AGCCA         ATTT        CG T        AGG ACG TAGGGCACACCGTGCTTTTC  GAA
    *            *            *   ***   *          **            *           *           *   *   *  ***            **
  TC T         CTGTG        C TTGC   CGTGC         ACGCA         ATGC        CGGT        AGGGACGGTAGCGACGACCGTGCTTTTCGTGAA
1053  1055...1119   1123..1131          1140...1163   1167...1233  1236..1261  1264...1288                                1320
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FIG. 5. Differences between the *Flavobacterium* nucleotide sequence determined in this study and that reported by Wild and co-workers (4). The upper nucleotide sequence and numbering system correspond to the sequence determined in this study; the lower nucleotide sequence and numbering system correspond to the sequence reported by Wild and co-workers (4). Positions where the two sequences differ are marked (*).

moter sequence that is recognized in *E. coli* but not in *Flavobacterium* sp. However, this appears unlikely, since insertion of an exogenous promoter upstream of these sequences (at the *Bam*HI site [position 1]; 16) activates the *opd* gene at levels roughly equivalent to those of constructions in which the promoter is inserted downstream of these sequences (at the *Pst*I site [position 357]). Recently, the 1.5-kb *Bam*HI fragment that contains the *Flavobacterium opd* gene has been shown to function in the gram-positive organism *Streptomyces lividans* in the absence of exogenous promoter sequences (18). The responsible promoter sequences were localized by a deletion experiment to the region that contains our putative promoter sequences.

Wild and co-workers (4) have noted the presence of a sequence 5′ to the *opd* coding region that bears some resemblance to a *nif*-type promoter sequence (3). This sequence is also present in the nucleotide sequence we have determined. We have cloned a 7.3-kb *Eco*RI fragment that encompasses nearly the entire region conserved between the *opd*-containing plasmids (12) in the broad-host-range vector pVDZ′2 (2). When this construct was introduced into *Pseudomonas putida*, the cells showed little or no parathion hydrolase expression (J. S. Karns, unpublished observations). Thus, if the promoter postulated by Wild and co-workers is responsible for initiating transcription of the *opd* gene in *Flavobacterium* sp., it does not function in *P. putida*. The activity of such promoters is frequently positively regulated, and the lack of a positive regulatory element may explain the lack of parathion hydrolase expression in gram-negative hosts other than the native *Flavobacterium* sp. and *P. diminuta*. However, since the conserved region between the two plasmids was cloned, it seems that any positive regulator unique to *opd* expression would also have been cloned. The lack of expression in *P. putida* combined with the apparently constitutive expression of *opd* in *Flavobacterium* sp. and *P. diminuta* argue against any such regulated promoter.

The putative Shine-Dalgarno sequence AAGG is located 7 bp upstream of the first ATG codon that occurs in the *opd* ORF. However, amino-terminal sequencing of the hydrolase protein shows that the amino-terminal residue is a serine located 29 residues downstream of this Met codon. The putative 29-residue leader peptide, like other leader peptides (20), is hydrophobic and has basic residues near its amino terminus. However, the cleavage site used by *Flavobacterium* cells would not be predicted by current models (there would be several other preferred sites) (20). The isolation from *E. coli* extracts of a hydrolase protein that is larger than that from *Flavobacterium* extracts suggests that the signal peptide is not cleaved in *E. coli*. In contrast, the signal peptide may be functional in gram-positive organisms—when the 1.5-kb *Bam*HI fragment containing the *Flavobacterium opd* gene is cloned in *S. lividans*, parathion hydrolase is secreted into the culture medium (18).

The predicted mass of the processed *opd* hydrolase (36,000 Da) differs slightly from the observed mass of the protein from *Flavobacterium* extracts (35,000 Da). Despite this difference, the veracity of the nucleotide sequence is supported by agreement of the predicted and experimental amino acid compositions, confirmation of amino-terminal sequence, and results of a deletion cloning experiment (in which expression was eliminated by the insertion of an in-frame nonsense codon about 100 bp upstream of the predicted carboxyl terminus). Therefore, it is likely that the discrepancy between predicted and observed mass is probably due to the aberrant mobility of the hydrolase during electrophoresis and gel filtration chromatography.

Relatively low expression of the *Flavobacterium opd* gene even in the presence of exogenous *E. coli* promoters lead to an examination of the *opd* protein produced in *E. coli*. The *E. coli* protein is larger than the protein isolated from *Flavobacterium* extracts and is therefore either an unprocessed or a misprocessed form of the precursor protein. Moreover, in contrast to the membrane-associated *Flavobacterium* protein, the *E. coli* protein is primarly partitioned in the cytosol. Extracts of *E. coli* cells containing a *lacZ-opd* translational fusion in which the leader peptide was deleted and replaced by five amino-terminal residues of *lacZ* displayed greater hydrolase activity than did cells that contained the entire *opd* gene.

Wild and co-workers previously reported the sequence of the 1.3-kb *Pst*I fragment from the *Flavobacterium* plasmid pPDL2 (erroneously termed pSM55 in their work) (4). Although their sequence (corresponding to positions 357 to 1693 in Fig. 2) agrees in large part with the sequence we determined, there are 50 base differences between the two in the region that they overlap. The reason for these substantial differences (which lead to significantly different predicted hydrolase proteins) is unclear. It is unlikely that these changes stemmed from cloning from different original sources, since M13 subclones used for both studies were derived from pWM44. The predictions of our nucleotide sequence agree well with experimentally determined values for amino-terminal sequence, amino acid composition, and approximate site of carboxyl terminus. In contrast, the predictions from the nucleotide sequence of Wild and co-workers are not consistent with any of these experimental values.

Southern hybridization experiments as well as restriction mapping of the *Flavobacterium opd* plasmid pPDL2 and *P. diminuta opd* plasmid pCMS1 have shown that the two *opd* genes are related if not identical (11, 12). More recently, Wild and co-workers reported the nucleotide sequence of the *Pseudomonas opd* gene (9). This sequence was identical to the sequence they later determined for the *Flavobacterium* gene with the exception of a single-base change (4, 9). Although we have no independent sequence information for the *Pseudomonas opd* gene or any protein work to test their sequence, the two genes are probably identical. Therefore, it appears likely that the *Pseudomonas opd* nucleotide sequence of Wild and co-workers is in error in precisely the same regions as is their *Flavobacterium opd* nucleotide sequence. A more thorough analysis of the *Pseudomonas opd* nucleotide sequence will undoubtably resolve this question.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Through personal communication we have learned that Cüneyt Serdar and his colleagues at AMGEN have determined the correct nucleotide sequence of the *Pseudomonas diminuta opd* gene. Their work (Bio/Technology, in press)

shows that within the protein-coding region, the nucleotide sequence of the *P. diminuta opd* gene is identical to the *Flavobacterium* sequence we have presented here.

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