APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1996, p. 1636–1641 Vol. 62, No. 5
0099-2240/96/$04.00+0

Cloning and Expression of a Gene Encoding a Bacterial Enzyme for Decontamination of Organophosphorus Nerve Agents and Nucleotide Sequence of the Enzyme

TU-CHEN CHENG,[1]* STEVEN P. HARVEY,[1] AND GRACE L. CHEN[2]

U.S. Army Edgewood Research, Development and Engineering Center, Research and Technology Directorate, Aberdeen Proving Ground, Maryland 21010,[1] and Kennedy Krieger Institute, Baltimore, Maryland 21005[2]

Received 21 June 1995/Accepted 8 February 1996

Organophosphorus acid (OPA) anhydrolase enzymes have been found in a wide variety of prokaryotic and eukaryotic organisms. Interest in these enzymes has been prompted by their ability to catalyze the hydrolysis of toxic organophosphorus cholinesterase-inhibiting compounds, including pesticides and chemical nerve agents. The natural substrates for these enzymes are unknown. The gene (*opaA*) which encodes an OPA anhydrolase (OPAA-2) was isolated from an *Alteromonas* sp. strain JD6.5 *Eco*RI-λZAPII chromosomal library expressed in *Escherichia coli* and identified by immunodetection with anti-OPAA-2 serum. OPA anhydrolase activity expressed by the immunopositive recombinant clones was demonstrated by using diisopropylfluorophosphate (DFP) as a substrate. A comparison of the recombinant enzyme with native, purified OPAA-2 showed they had the same apparent molecular mass (60 kDa), antigenic properties, and enzyme activity against DFP and the chemical nerve agents sarin, soman, and O-cyclohexyl methylphosphonofluoridate. The gene expressing this activity was found in a 1.74-kb *Pst*I-*Hin*dIII fragment of the original 6.1-kb *Eco*RI DNA insert. The nucleotide sequence of this *Pst*I-*Hin*dIII fragment revealed an open reading frame of 1,551 nucleotides, coding for a protein of 517 amino acid residues. Amino acid sequence comparison of OPAA-2 with the protein database showed that OPAA-2 is similar to a 647-amino-acid sequence produced by an open reading frame which appears to be the *E. coli pepQ* gene. Further comparison of OPAA-2, the *E. coli* PepQ protein sequence, *E. coli* aminopeptidase P, and human prolidase showed regions of different degrees of similarity or functionally conserved amino acid substitutions. These findings, along with preliminary data confirming the presence of prolidase activity expressed by OPAA-2, suggest that the OPAA-2 enzyme may, in nature, be used in peptide metabolism.

Enzymes which catalyze the hydrolysis of organophosphate (OP) compounds have been under investigation since Mazur (22) first described the hydrolysis of diisopropylfluorophosphate (DFP) and its detoxification by crude preparations of human and rabbit tissues. These enzymes catalyze the hydrolysis of many highly toxic, acetylcholinesterase-inhibiting compounds, including chemical warfare G-type nerve agents and pesticides. This group of enzymes has been classified as organophosphorus acid (OPA) anhydrolases (OPAA; EC 3.1.8.2).

The first OP-hydrolyzing enzyme gene to be cloned, sequenced, and expressed was that for organophosphorus acid hydrolase (OPH; EC 3.1.8.1), which is encoded by the *opd* gene of *Pseudomonas diminuta* MG and *Flavobacterium* sp. strain ATCC 27551 (15, 23, 25, 30). The open reading frame (ORF) of the *opd* gene contains 975 bases which encode a polypeptide of 325 amino acid residues with a molecular mass of 35 kDa (24). OPH is a well-characterized metalloenzyme which typically contains one to two ions of zinc or cobalt (9, 27). Subsequently, an OPA anhydrolase (OPAA-2) that possesses high levels of DFP-hydrolyzing activity was purified from *Alteromonas* sp. strain JD6.5 (7). OPAA-2 (7) and OPH (2, 10) have functional similarities in that both enzymes catalyze the hydrolysis of sarin (GB; *O*-isopropyl methylphosphonofluoridate), DFP, and paraoxon. However, OPAA-2 has significant soman (GD; *O*-pinacolyl methylphosphonofluoridate) activity, which is not found in the native OPH, whereas OPH has much higher catalytic activity on paraoxon than OPAA-2.

Recently, two other *Alteromonas* enzymes, from *A. undina* (3) and *A. haloplanktis* C (20), have been purified and characterized. The OPA anhydrolases from these *Alteromonas* species are similar in their catalytic properties. In addition to degrading DFP, they have activity against sarin, soman, and GF (*O*-cyclohexyl methylphosphonofluoridate) (3, 6, 7, 20). These enzymes also exhibit various degrees of activity with chromogenic substrates for phosphodiesterase and acid or alkaline phosphatases. The antibodies derived from OPA anhydrolase of strain JD6.5 also showed cross-reactivity with proteins from these bacteria in Western immunoblot analysis experiments (3, 6, 7), suggesting that the OPA anhydrolases obtained from these sources have common structural domains. In general, they all have optimum activity at pH 7.5 to 8.5 and 40 to 55°C. All apparently comprise a single polypeptide, based on their observed migration as a single band under denaturing polyacrylamide gel electrophoresis (PAGE) conditions (3, 7, 20). They are also similarly activated by Mn^{2+} and inhibited by the DFP analog Mipafox (*N*,*N*′-diisopropyl phosphorodiamidofluoridate) and the sulfhydryl inhibitors *p*-chloromercuribenzoate (PCMB) and *N*-ethylmaleimide (NEM) (3, 7, 20).

Although OPA anhydrolases are found in a wide variety of prokaryotes and eukaryotes (6, 19), their natural function has not been elucidated. It is unlikely that such enzymes evolved to protect against anticholinesterase compounds since, in the case of prokaryotes, the enzymes are found in organisms which contain no cholinesterases. Nevertheless, their wide phyloge-

* Corresponding author. Phone: (410) 612-8632. Fax: (410) 612-8661. Electronic mail address: tccheng@cbdcom.apgea.army.mil.

netic distribution suggests that the enzymes do serve an important function.

In this study, in order to gain insight on the natural function of OPA anhydrolases, the gene for OPAA-2 (*opaA*) was cloned into *Escherichia coli* and sequenced. Cloning also permitted overexpression of active, recombinant enzyme, identified by immunodetection and enzymatic activity. The deduced amino acid sequence of recombinant OPAA-2 was then compared with other known sequences in order to determine possible evolutionary relationships. The OPAA-2 enzyme sequence was found to exhibit homology with sequences of *E. coli* aminopeptidase P (EC 3.4.11.9) and human prolidase (EC 3.4.13.9). Enzyme assays with OPAA-2 also demonstrated significant prolidase activity, suggesting that the enzyme may be a prolidase.

MATERIALS AND METHODS

Organism and cultivation. *Alteromonas* sp. strain JD6.5 was grown on salt medium (50 g of NaCl, 10 g of $MgSO_4$, 10 g of proteose peptone, 6 g of yeast extract, 5 g of Casamino Acids, and 2.5 g of HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] per liter, pH 6.5) at 37°C for 18 to 24 h. Cells were then harvested by centrifugation (7,500 × *g*) for 10 min and stored at −20°C. *E. coli* XL1-Blue (Stratagene Cloning Systems, La Jolla, Calif.) was grown on LB medium (10 g of NaCl, 10 g of tryptone, and 5 g of yeast extract, pH 7.2) as described in the manufacturer's protocol.

Enzyme assay. OPAA activity for DFP, GB, GD, and GF was assayed by monitoring released F^- with an ion-specific electrode (16, 17). Activity for methylpinacolyl *p*-nitrophenyl phosphonate (NP-GD; a chromogenic soman analog) was determined by monitoring the absorbancy at 405 nm for *p*-nitrophenol (extinction coefficient, 10,101 at pH 7.2) (3, 7). The reaction medium contained 500 mM NaCl, 50 mM bis-Tris propane (pH 7.2), 0.1 mM $MnCl_2$, 0.1 to 3 mM OP substrate, and 2 to 25 μl of enzyme sample in a total volume of 2.5 ml (7). One unit of OPA anhydrolase activity catalyzes the release of 1.0 μmol of F^- (DFP, GB, GD, or GF) or *p*-nitrophenyl group (NP-GD) per min at 25°C. Specific activity is expressed as units of enzyme activity per milligram of protein. The kinetic parameters for DFP and the chemical agents were determined by a progress curve fit with the Michaelis-Menten equation, using at least five different substrate concentrations per curve. The substrate concentrations used were between approximately 0.3 and 3 times the K_m. Other concentrations were used in initial range-finding experiments but were not included in the final calculations. Curve fitting was accomplished with the EZ-FIT software (28). The program uses the Nelder-Mead simplex and Marquardt nonlinear regression algorithms sequentially.

The Coomassie protein assay reagent (Pierce, Rockford, Ill.) was used for protein determination, with bovine serum albumin (BSA) as the standard.

Construction of a genomic library expressing OPAA-2. High-molecular-weight genomic DNA from strain JD6.5 was prepared as described by Davis et al. (5). Predigested (calf intestinal alkaline phosphatase and *Eco*RI) ZAPII Express vector, helper phage R408, Gigapack II gold packaging extract, and host strain XL1-Blue were obtained from Stratagene Cloning Systems. Briefly, the genomic DNA was partially digested with *Eco*RI, and DNA fragments of 4 to 8 kb were size fractionated by preparative agarose gel electrophoresis followed by purification from the gel with the Geneclean II kit (Bio-101, La Jolla, Calif.). The DNA fragments were ligated to *Eco*RI-digested, phosphatase-treated λZAP arms and then packaged with an in vitro lambda packaging extract. *E. coli* XL1-Blue was infected with the recombinant phage preparation and plated onto LB agar plates.

Screening of cloned OPAA-2. To verify that the genomic library contained OPAA-2 genomic DNA from strain JD6.5, it was screened for the presence of recombinant plaques with anti-OPAA-2 serum. The anti-OPAA-2 serum that was raised against purified OPAA-2 from strain JD6.5 has been described previously (7). Approximately 6×10^4 PFU were plated at a density of 1.5×10^4 PFU per 90-mm-diameter plate on *E. coli* XL1-Blue cells. After 4 h of growth at 37°C, the plates were overlaid with nitrocellulose filters saturated with 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for an additional 3 h. After cooling at 4°C for 1 h, the filters were removed from the plates and blocked with TBS (50 mM Tris [pH 7.5], 150 mM NaCl) containing 5% dry skim milk overnight at room temperature.

Immunodetection was carried out by first incubating the filters with a 1:2,000 (vol/vol) dilution of anti-OPAA-2 serum in TBS for 1 h. After three washes in TBS, the filters were incubated with goat anti-rat immunoglobulin G (IgG)-alkaline phosphatase conjugate (Fisher Scientific, Pittsburgh, Pa.). Following three washes with TBS, the bound alkaline phosphatase was assayed with the substrate 5-bromo-4-chloro-3-indolylphosphate–nitro blue tetrazolium (BCIP/NBT).

Excision of plasmid from the λZAP vector, mapping, and subcloning of the cloned gene. The excision of the plasmid from the λZAP clones was carried out as described by Short et al. (31). The restriction enzyme digestion and subcloning of various inserted DNA fragments in pBluescript were performed by standard procedures as described before (21).

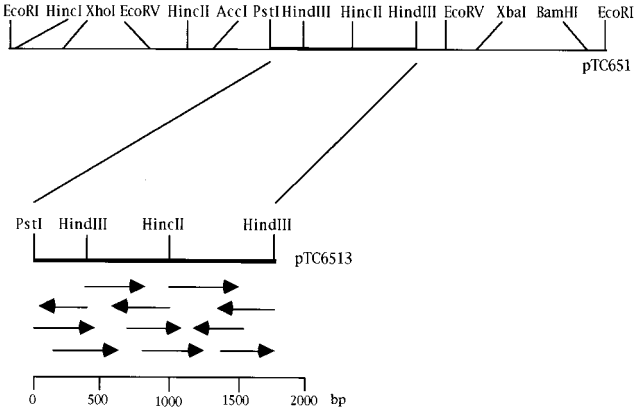

FIG. 1. Restriction map of and sequencing strategy for the *opa* gene. Plasmid pTC651 is a recombinant plasmid consisting of pBluescript and the 6.1-kb *opaA* gene of *Aeromonas* sp. strain JD6.5. The 1.74-kb *Pst*I-*Hin*dIII fragment of pTC6513 contains the *opaA* gene and encodes enzyme activity against DFP. The positions of various restriction sites, the restriction enzyme- and exonuclease-generated subclone, and synthetic primers used in the sequencing are shown along the fragment. Arrows indicate the direction and extent of sequencing (base pairs shown at the bottom).

Western analysis of OPAA. Sodium dodecyl sulfate (SDS)-PAGE was carried out by the method of Laemmli (18). After electrophoresis, the proteins in the gel were transferred to a nitrocellulose filter with the Millipore (Bedford, Mass.) Polyblot transfer system according to the manufacturer's protocol. For Western immunoblotting analysis, the same anti-OPAA-2 serum and detection kit as described for the immunodetection of recombinant clones were used.

Purification of recombinant OPAA. Frozen cells harvested from 1 liter of the recombinant *E. coli* culture in lag phase were resuspended in BM buffer (10 mM bis-Tris propane, 0.1 mM $MnCl_2$, pH 7.2) at a ratio of 3 ml of buffer per g of wet cells. The cells were sonicated for 2 min at 4°C, and the cellular debris was removed by centrifugation. Approximately 200 mg of protein from the crude lysate was then used for OPAA purification. To remove nucleic acids, 4 volumes of BPA-1000 (Tosohaas, Montgomeryville, Pa.) was added to the lysate and incubated at 4°C for 10 min. The enzyme activity was recovered in the cell extract after centrifugation at 2,000 × *g* for 5 min. Further purification of the enzyme through $(NH_4)_2SO_4$ fractionation, DEAE ion-exchange chromatography, and hydroxyapatite chromatography was then followed by procedures similar to those described previously (3, 7).

DNA sequencing. Automated DNA sequencer model 373A (Perkin Elmer, Applied Biosystems Div., Foster City, Calif.) was used to conduct sequencing. After subcloning, both strands of DNA fragments were sequenced by using fluorescently labeled T7 or T3 primer or, when sequence-specific primers were used, fluorescently labeled dideoxynucleotides, with the *Taq* cycle sequencing method, following the manufacturer's protocol (Perkin Elmer, Applied Biosystems Div.). Computer analysis of DNA was performed with the MacVector version 4.1.4 program (Kodak, New Haven, Conn.).

Nucleotide sequence accession number. The GenBank accession number for the *opaA* gene sequence is U29240.

RESULTS

Cloning of the OPA anhydrolase gene. Of 6×10^4 plaques examined, 5 positive clones were detected. All five clones produced strong signals with the anti-OPAA-2 serum. After excision and recovery of plasmids from the λZAP clones, all were found to contain a 6.1-kb *Eco*RI DNA fragment. The orientation of this DNA fragment with respect to the vector was confirmed by restriction digestion, and the plasmids were designated pTC651 (Fig. 1) and pTC652. No restriction sites for *Kpn*I, *Sma*I, *Stu*I, or *Cla*I were found in the DNA insert.

The *opaA* gene from strain JD6.5 was expressed in *E. coli*. With a non-optimized, single-batch incubation of cells in a shaker flask, the yield of OPAA-2 reached a maximum at about 24 h in the stationary phase of growth and slowly decreased

TABLE 1. Purification of OPAA-2 from recombinant cells[a]

Purification step	Total vol (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude extract	15.0	204.0	3,060	15		100
BPA-1000	60.0	144.0	3,070	21	1.4	100
$(NH_4)_2SO_4$ (45–65%)	5.2	58.2	3,250	56	3.7	106
DEAE-Sephacel	6.0	7.3	770	105	7.0	25
HA-Ultrogel	1.9	0.9	270	300	20.0	9

[a] Protein and activity values are given as means of triplicates for each enzyme preparation.

thereafter. To verify the presence of the OPAA-2 polypeptide, protein lysates of pTC651- and pTC652-carrying cells from these cultures were subjected to Western analysis with the anti-OPAA-2 serum. As expected, expression from pTC651 and pTC652 yielded a protein with a molecular mass of 60 kDa that was indistinguishable from native OPAA-2, as assessed by immunodetection (data not shown). A significant amount of a protein with the electrophoretic mobility expected for OPAA-2 was also observed in these clones by SDS-PAGE.

Expression of the OPA anhydrolase gene in *E. coli*. The crude lysate obtained from both pTC651- and pTC652-harboring clones exhibited the same level of DFP-hydrolyzing activity regardless of the presence or absence of the inducer IPTG. These results suggest that the *opaA* gene is expressed by a promoter within the cloned DNA. The total enzyme activity in these recombinant cells is approximately 100 times that of the parental strain JD6.5. The cloned OPAA-2 from pTC651-harboring cells was purified by the procedures summarized in Table 1. Following purification, the specific activity increased almost 20-fold, with a 9% recovery, suggesting that the expressed enzyme constituted about 5% of the total protein in the recombinant cells. The purified enzyme appears to be homogeneous and identical to the native enzyme, as judged by SDS-PAGE analysis (Fig. 2A) and Western blot analysis with anti-OPAA-2 serum (Fig. 2B).

The purified recombinant OPAA-2 is capable of hydrolyzing DFP and a wide variety of chemical nerve agents. The apparent K_m values for DFP, GB, GD, GF, and a chromogenic soman analog, NP-GD, were 2.99, 1.57, 2.48, 0.63, and 1.27 mM, respectively. Apparent V_{max} values against these substrates were 230, 442, 151, 652, and 52 μmol/min/mg, respectively. The recombinant enzyme also hydrolyzes paraoxon at about 2% of the rate of DFP (V_{max}; 6.11 μmol/min/mg). Just like the native enzyme (6, 7), the recombinant enzyme showed optimal activity against DFP at pH 8.5 and 50°C. The recombinant enzyme was also stimulated and stabilized by Mn^{2+} and the reducing agents dithiothreitol and β-mercaptoethanol (data not shown).

Subcloning of OPA anhydrolase gene. Clone pTC651 was chosen for subcloning. One subclone, pTC6513 (Fig. 1), was obtained by digestion with *Pst*I followed by partial digestion with *Hin*dIII, and the purified 1.74-kb *Pst*I-*Hin*dIII fragment was subcloned into pBluescript *Pst*I and *Hin*dIII sites. Five of these pTC6513 transformants that have been tested were shown to retain the complete *opaA* gene by Western analysis and DFP-hydrolyzing activity. The transformant with pTC6513, in the presence of IPTG, showed about 50 to 60% lower total enzyme activity than that with pTC651. The *Pst*I-*Hin*dIII insert of pTC6513 was thus the smallest fragment which encoded functional OPAA-2.

Nucleotide sequencing of the OPA anhydrolase gene. The nucleotide sequence of the *Pst*I-*Hin*dIII fragment carried by pTC6513 was determined in both strands. The 1,741-bp nucleotide sequence oriented from the *Pst*I site to *Hin*dIII site and the deduced amino sequence are shown in Fig. 3. Within this sequence, only one large ORF was found. This ORF consisted of a 1,551-bp nucleotide sequence capable of encoding a peptide of 517 amino acid residues. The start codon begins 35 nucleotides from the *Pst*I site of the *Pst*I-*Hin*dIII fragment and terminates at TAA (position 1587). The calculated molecular mass of the protein encoded by this region is 59 kDa, very close to the 60 kDa of native OPAA-2 determined by Western analysis (7). The deduced N-terminal amino acid sequence of the enzyme from strain JD6.5 closely resembles that of the *A. haloplanktis* C enzyme (12 matches out of 15 residues), as determined by protein sequencing (20).

Preceding the start codon is a putative ribosome-binding site (Shine-Dalgarno sequence), 5′-AGTGGGT-3′, in which five of seven nucleotides are identical to the ribosome-binding site of the *A. haloplanktis* A23 *amy* gene (5′-ATTGGAT-3′) (14) and the common feature of the *E. coli* ribosome-binding site (5′-AGGAGGT-3′) (4). Upstream from this site (22 nucleotides from the start codon) is a TATTT sequence which was tentatively identified as the −10 region. The pTC6513-harboring cells, when induced with IPTG, displayed lower enzyme activity than the pTC651-harboring cells. Loss of expression in the pTC6513 transformant might be attributable to the poor *opaA* ribosome-binding site.

Amino acid sequence analysis of strain JD6.5 OPAA-2. The amino acid sequence of OPAA-2 was used to screen the NCBI peptide database (1). The only match found was a 647-amino-acid sequence produced by an ORF which appears to be the *pepQ* gene of *E. coli* (26). A previously published report (26) noted 29% homology with a prolidase (X-Pro dipeptidase) from a human source (13) and aminopeptidase P from *E. coli* (32). Prolidase is a ubiquitous enzyme which hydrolyzes dipeptides with a prolyl residue in the carboxyl-terminal position, whereas aminopeptidase P hydrolyzes the amino acid-proline bond when proline is in the penultimate position from the amino terminus. In Fig. 4, we compare the amino acid sequences of OPAA-2 with those of *E. coli* PepQ protein, *E. coli* aminopeptidase P (a proline aminopeptidase), and human prolidase with the MacVector alignment program. These sequences were aligned with appropriate gaps to obtain maximum homology. The sequences of OPAA-2 and *E. coli* PepQ are highly conserved throughout the aligned regions, starting

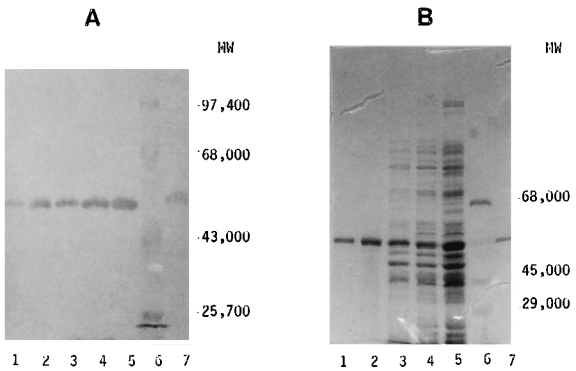

FIG. 2. Purification of cloned OPAA-2. (A) Western analysis of fractions from cloned OPAA-2 purification steps. (B) SDS-PAGE of fractions from cloned OPAA-2 purification steps. Lane 1 to 7, pooled final HA-Ultrogel fractions, pooled DEAE-Sephacel fractions, 45 to 65% ammonium sulfate fraction, BPA-1000 supernatant, crude lysate, protein molecular weight standards, and purified OPAA-2 from strain JD6.5, respectively.

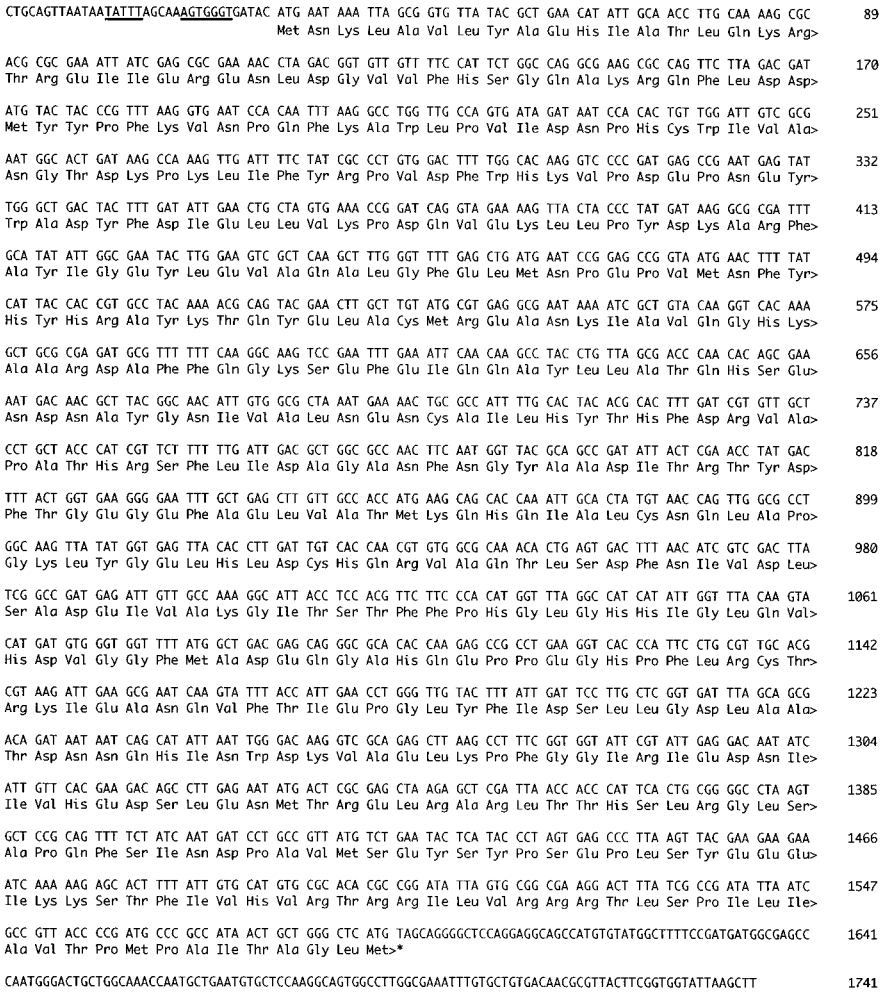

FIG. 3. Nucleotide and amino acid sequences of the OPA anhydrolase gene of strain JD6.5. The amino acid sequence corresponding to the ORF beginning with the first ATG codon begins at nucleotide 37. The 3′ stop codon is indicated (*).

from the N-terminal amino acid (methionine). The amino acid sequence of OPAA-2 (residues 1 to 500), with the introduction of several insertions and gaps, shows approximately 47% amino acid identity to that of *E. coli* PepQ (residues 1 to 497). A higher level of similarity (73%) was observed when amino acids of similar functional groups from both enzyme sequences were included in the analysis. There was no sequence match in the C-terminal region of OPAA-2 (residues 500 to 517). A segment of the OPAA-2 sequence (residues 56 to 499) also exhibited similarity to human prolidase (residues 96 to 466). The overall amino acid identity of OPAA-2 and human prolidase is 28%. A higher degree of similarity (56%) was observed when similar functional amino acids were included in the analysis. Also, 30% amino acid identity was observed between the aligned segments of OPAA-2 (residues 156 to 359) and *E. coli* aminopeptidase P (residues 174 to 377). When the comparisons included similar functional amino acids, 52% similarity was observed. In conclusion, the results suggest that OPAA-2, the human prolidase, and *E. coli* aminopeptidase P are evolutionarily related. Furthermore, *E. coli pepQ* appears to encode a protein similar to OPAA-2, based on their structural similarities.

DISCUSSION

We report here the cloning and sequencing of the gene (*opaA*) for OPAA-2 from *Aeromonas* sp. strain JD6.5. The amino acid sequence analysis provides insight into a possible natural function of OPAA-2. Significant structural similarity was found between OPAA-2 and a protein encoded by the *E. coli pepQ* gene found in the peptide database. Amino acid sequence comparison also showed significant structural similarity between these two sequences and those of human prolidase and *E. coli* aminopeptidase P (Fig. 4). In spite of the functional similarities between OPAA-2 (7) and OPH (2, 10), no sequence homology was found between these two enzymes (data not shown).

Although the native biological role of *E. coli* aminopeptidase P is not known, a human prolidase deficiency results in a syndrome causing abnormalities of the skin and other collagenous tissue (29). Affected patients excrete massive amounts of iminopeptides into the urine, and it is these peptides which function as the substrate for prolidase (11). In order to investigate the possibility that OPAA-2 may be functionally related to aminopeptidase P or prolidase, we determined the relative enzyme activities of OPAA-2 on various tripeptides and dipep-

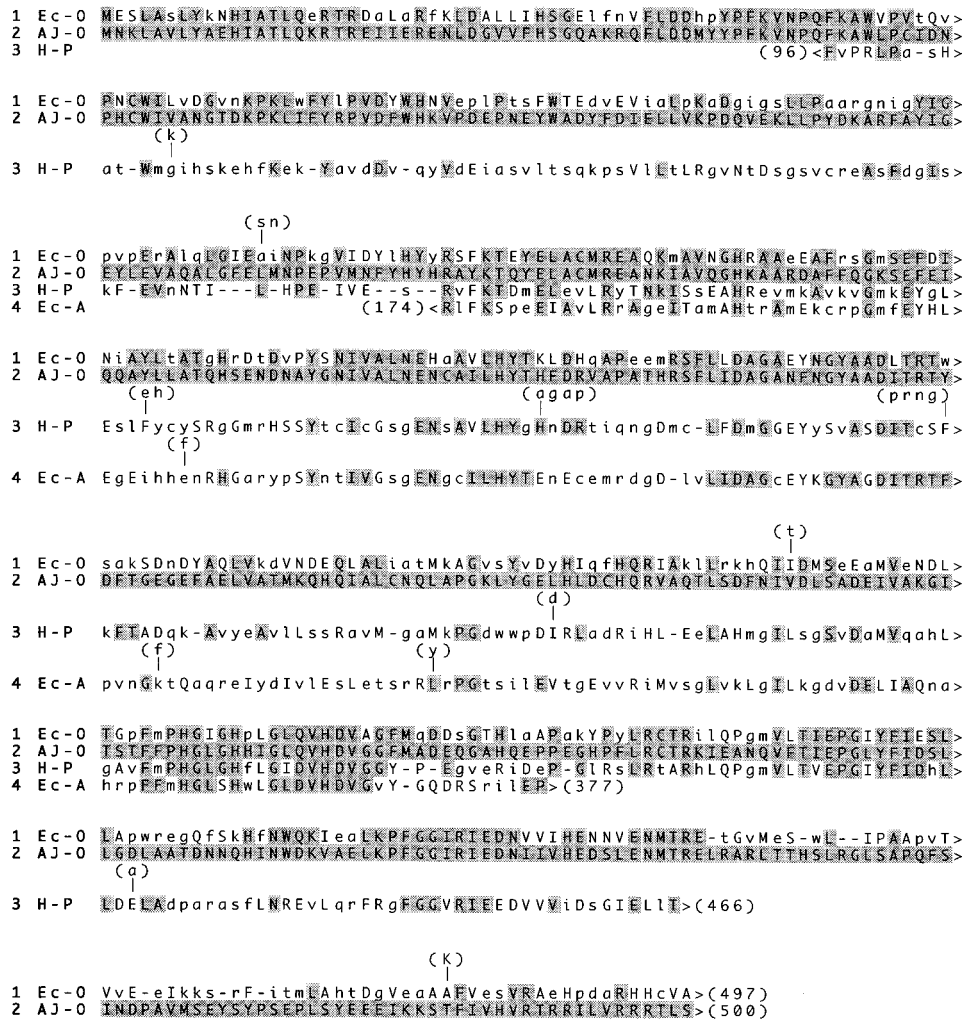

FIG. 4. Comparison of the amino acid sequences of strain JD6.5 OPA anhydrolase (AJ-O) with those of *E. coli* PepQ (Ec-O), *E. coli* aminopeptidase P (Ec-A), and human prolidase (H-P). Residues in capital letters that are identical or similar in two or more sequences are shadowed and not shadowed, respectively. Residues in lowercase letters are mismatched, and those within parentheses are insertions (left to right) following the residue with the vertical line.

tides by measuring the release of amino acids by the modified Cd-ninhydrin method (method D) (8), using 50 mM bis-Tris propane, pH 8.5, and 0.1 mM $MnCl_2$ as the reaction buffer. Preliminary results demonstrated that recombinant OPAA-2 also displayed high specific activity against Leu-Pro (~800 μmol of amino acids released per min per mg) and Ala-Pro (~650 μmol/min/mg), with no activity against Pro-Leu and Pro-Gly. The substrates for aminopeptidase P (Gly-Pro-Ala and Ala-Pro-Phe) were also not digested. Under the same conditions, the specific activity of OPAA-2 for DFP hydrolysis was ~700 μmol of F^- released per min per mg. On the basis of this evidence, OPAA-2 appears to be a prolidase rather than an aminopeptidase. The human prolidase (12) and *E. coli* aminopeptidase P (32) are composed of 492 and 440 amino acids, respectively, whereas OPAA-2 is composed of 517 amino acids. These enzymes are all activated by manganese (7, 13, 32). Although the biological significance of prolidase in bacterial metabolism is not clear, the structural homology and functional similarities suggest that OPAA-2 and prolidase may have evolved from the same ancestral gene. The significant OPAA-2 prolidase activity also suggests that in nature, the enzyme may play a role in bacterial peptide metabolism.

OPA anhydrolase is an enzyme with a broad spectrum of substrate specificity, e.g., hydrolysis of P-F and P-O bonds of various OP compounds. In contrast, prolidase catalyzes C-N bond cleavages. The functional characteristics of these enzymes suggest that the structural configuration around such bonds may play an essential role in the mechanism of hydrolysis. Further studies on the sequence homology of various *Alteromonas* OPA anhydrolases and their activities against dipeptides, DFP, and G-type chemical agents may offer further insights into the natural function of these enzymes.

ACKNOWLEDGMENTS

We thank Joseph J. DeFrank, Ilona J. Fry, and Vipin K. Rastogi for useful discussions and critical reading of the manuscript.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Attaway, H., J. O. Nilson, A. M. Baya, M. J. Voll, W. E. White, D. J. Grimes, and R. R. Colwell.** 1987. Bacterial detoxification of diisopropyl fluorophosphate. Appl. Environ. Microbiol. **53:**1685–1689.
3. **Cheng, T.-C., S. P. Harvey, and A. N. Stroup.** 1993. Purification and properties of a highly active organophosphorus acid anhydrolase from *Alteromo-*

nas undina. Appl. Environ. Microbiol. **59:**3138–3140.
4. **Crombrugghe, B., and I. Pastan.** 1978. The operon. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Davis, L. G., M. D. Dibner, and J. F. Battey.** 1986. Basic methods in molecular biology, p. 44–46. Elsevier, New York.
6. **DeFrank, J. J., W. T. Beaudry, T.-C. Cheng, S. P. Harvey, A. N. Stroup, and L. L. Szafraniec.** 1993. Screening of halophilic bacteria and *Alteromonas* species for organophosphorus hydrolyzing enzyme activity. Chem. Biol. Interact. **87:**141–148.
7. **DeFrank, J. J., and T.-C. Cheng.** 1991. Purification and properties of an organophosphorus acid anhydrolase from a halophilic bacterial isolate. J. Bacteriol. **173:**1938–1943.
8. **Doi, E., D. Shibata, and T. Matoba.** 1981. Modified colorimetric ninhydrin methods for peptidase assay. Anal. Biochem. **118:**173–184.
9. **Dumas, D. P., S. R. Caldwell, J. R. Wild, and F. R. Raushel.** 1989. Purification and properties of the phosphotriesterase from *Pseudomonas diminuta*. J. Biol. Chem. **264:**19659–19665.
10. **Dumas, D. P., H. D. Durst, W. G. Landis, F. M. Raushel, and J. R. Wild.** 1990. Inactivation of organophosphorus nerve agents by the phosphotriesterase from *Pseudomonas diminuta*. Arch. Biochem. Biophys. **277:**155–159.
11. **Endo, F., I. Matsuda, S. Tanaka, and A. Ogata.** 1982. Human erythrocyte prolidase and prolidase deficiency. Pediatr. Res. **16:**227–231.
12. **Endo, F., K. Motohara, Y. Indo, and I. Matsuda.** 1987. Immunochemical studies of human prolidase with monoclonal and polyclonal antibodies: absence of the subunit of prolidase in erythrocytes from a patient with prolidase deficiency. Pediatr. Res. **22:**627–633.
13. **Endo, F., A. Tanoue, H. Nakai, A. Hata, Y. Indo, K. Tatani, and I. Matsuda.** 1989. Primary structure and gene localization of human prolidase. J. Biol. Chem. **264:**4476–4481.
14. **Feller, G., T. Lonhienne, C. Deroanne, C. Libioulle, J. V. Beeumen, and C. Gerday.** 1992. Purification, characterization, and nucleotide sequence of the thermolabile amylase from the antarctic psychrotroph *Alteromonas haloplanktis* A23. J. Biol. Chem. **267:**5217–5221.
15. **Harper, L. L., C. S. McDaniel, C. E. Miller, and J. R. Wild.** 1988. Dissimilar plasmids isolated from *Pseudomonas diminuta* MG and a *Flavobacterium* sp. (ATCC 27551) contain identical *opd* genes. Appl. Environ. Microbiol. **44:** 246–249.
16. **Hoskin, F. C. G., M. A. Kirkish, and K. E. Steinmann.** 1984. Two enzymes for the detoxification of organophosphorus compounds—sources, similarities, and significance. Fund. Appl. Toxicol. **4:**165–172.
17. **Hoskin, F. C. G., and A. H. Roush.** 1982. Hydrolysis of nerve gas by squid type diisopropylphosphorofluoridate hydrolyzing enzyme on agarose resin. Science **215:**1255–1257.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
19. **Landis, W. G., and J. J. DeFrank.** 1990. Enzymatic hydrolysis of toxic organofluorophosphate compounds. Adv. Appl. Biotechnol. Ser. **4:**183–201.
20. **Liu, L., J. Wu, B. Wang, and D. M. Anderson.** 1995. Purification and characterization of organophosphorus acid anhydrolase from *Alteromonas haloplanktis* C and *Alteromonas* sp. M, p. 12. *In* International Workshop on Biocatalytic Degradation of Chemical Warfare Related Materials. U.S. Army Edgewood Research, Development and Engineering Center, Aberdeen Proving Ground, Md.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Mazur, A.** 1946. An enzyme in animal tissue capable of hydrolyzing the phosphorus fluorine bond of alkyl fluorophosphates. J. Biol. Chem. **164:** 271–286.
23. **McDaniel, C. S., L. L. Harper, and J. R. Wild.** 1989. Cloning and sequencing of a plasmid-borne gene (*opd*) encoding a phosphotriesterase. J. Bacteriol. **170:**2306–2311.
24. **Mulbry, W., and J. Karns.** 1989. Parathion hydrolase specified by the *Flavobacterium opd* gene: relationship between the gene and protein. J. Bacteriol. **171:**6740–6746.
25. **Mulbry, W., J. S. Karns, P. C. Kearney, J. O. Nelson, C. S. McDaniel, and J. R. Wild.** 1986. Identification of a plasmid-borne parathion hydrolase gene from *Flavobacterium* sp. by Southern hybridization with *opd* from *Pseudomonas diminuta*. Appl. Environ. Microbiol. **51:**926–930.
26. **Nakahigashi, K., and H. Inokuchi.** 1990. Nucleotide sequence between the *fadB* gene and *rrnA* operon from *Escherichia coli*. Nucleic Acids Res. **18:** 6439.
27. **Omburo, G., L. Millins, and F. Raushel.** 1993. Structural characterization of divalent cation sites of bacterial phosphotriesterase by ^{113}Cd NMR spectroscopy. Biochemistry **32:**9148–9155.
28. **Perella, F. W.** 1980. EZ-FIT: a practical curve-fitting microcomputer program for the analysis of enzyme kinetic data on IBM-PC compatible computers. Anal. Biochem. **174:**437–447.
29. **Scriver, R. C., R. J. Smith, and J. M. Phang.** 1983. Disorders of proline and hydroxyproline metabolism, p. 360–381. *In* J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown (ed.), The metabolic basis of inherited disease. McGraw-Hill Book Co., New York.
30. **Serdar, C. M., D. T. Gibson, D. M. Munnecke, and J. H. Lancaster.** 1982. Plasmid involvement in parathion hydrolysis by *Pseudomonas diminuta*. Appl. Environ. Microbiol. **44:**246–249.
31. **Short, J. M., J. M. Fernandez, J. A. Sorge, and W. D. Huse.** 1988. λZAP: a bacteriophage λ expression vector with *in vivo* excision properties. Nucleic Acids Res. **16:**7583–7589.
32. **Yoshimoto, T., H. Tone, T. Honda, K. Osatomi, R. Kobayashi, and D. Ysuru.** 1989. Sequencing and high expression of aminopeptidase P gene from *Escherichia coli* HB101. J. Biochem. **105:**412–416.