

# Purification of an Extracellular D-(-)-3-Hydroxybutyrate Oligomer Hydrolase from *Pseudomonas* sp. Strain A1 and Cloning and Sequencing of Its Gene

KAI ZHANG, MARI SHIRAKI, AND TERUMI SAITO\*

Department of Biological Sciences, Faculty of Science, Kanagawa University, Hiratsuka, Japan

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**An extracellular D-(-)-3-hydroxybutyrate oligomer hydrolase was purified from a poly(3-hydroxybutyrate)-degrading bacterium, *Pseudomonas* sp. strain A1. The purified enzyme hydrolyzed the D-(-)-3-hydroxybutyrate dimer and trimer at similar rates. The enzyme activity was inhibited by a low concentration of diisopropylfluorophosphate. The molecular weight of the hydrolase was estimated to be about 70,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A 10-kbp DNA fragment of A1 was detected by hybridization with the gene (2 kbp) of an extracellular poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*. Subsequent subcloning showed that a *Sma*I-*Kpn*I fragment (2.8 kbp) was responsible for expression of the hydrolase in *Escherichia coli* and an in vitro transcription-translation system. The expressed protein detected by immunostaining had the same molecular weight as the purified enzyme from A1. The protein band detected in the in vitro transcription-translation system had a molecular size of 72 kDa. The nucleotide sequence of the *Sma*I-*Kpn*I fragment was determined, and one open reading frame (2,112 nucleotides) was found. It specifies a protein with a deduced molecular weight of 72,876 (704 amino acids). In this sequence, the consensus sequence of serine-dependent hydrolysis, G-X-S-X-G, did not exist.**

Poly-3-hydroxybutyrate (PHB) is a unique intracellular reserve of organic carbon and/or chemical energy found in a wide variety of bacteria (6, 9) and is regarded as a potential biodegradable thermoplastic not derived from petroleum (12). Recently similar bacterial polyesters have been found, and these polyesters including PHB were called "polyhydroxyalkanoates" (PHAs) (7). Extracellular PHB metabolism is important in the application of these bacterial polyesters as biodegradable plastics. Many microorganisms that are capable of degrading PHB exist in the environment, and they secrete PHB depolymerases to degrade this polymer. There have been many studies on the extracellular PHB depolymerases from several bacteria and fungi (2, 3, 4, 11, 15, 22, 26, 29). Genes for some enzymes were also cloned and sequenced (10, 19, 21). *Alcaligenes faecalis* T1, a PHB-degrading bacterium, secretes a D-(-)-3-hydroxybutyric acid oligomer hydrolase (3HB-oligomer hydrolase) (EC 3.1.1.22) (23) besides a PHB depolymerase. Although the physiological role of the 3HB-oligomer hydrolase is not clear, it may be important to study this hydrolase in relation to extracellular degradation of PHB. Similar enzymes were found within the bacterial cells (8, 16, 25). *Pseudomonas* sp. strain A1 was isolated from activated sludge as a PHB-degrading bacterium (22) and was found to have an extracellular 3HB-oligomer hydrolase besides a PHB depolymerase. In this communication, we describe the purification and characterization of the 3HB-oligomer hydrolase and cloning and sequencing of its gene.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture.** *Pseudomonas* sp. strain A1 was isolated from activated sludge of the sewage treatment facility of Kanagawa University (22). The bacteria were cultured in the basic salt medium (0.1% [wt/vol]

$\text{NH}_4\text{Cl}$ , 0.05% [wt/vol]  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01% [wt/vol]  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.0005% [wt/vol]  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and 66 mM  $\text{KH}_2\text{PO}_4$ - $\text{Na}_2\text{HPO}_4$  [pH 6.8]) containing 0.15% (wt/vol) PHB powder at 30°C. The plasmids used for cloning were charomid 9-36 (Nippon Gene Co., Toyama, Japan) (18) and pUC18 and -19 (Takara Shuzo, Kyoto, Japan). The bacterial strains used as hosts for the plasmid were *E. coli* DH5 (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) and *E. coli* JM109 [*recA1 hsdR17 supE Δ(lac-proAB) endA1 gyrA96 relA1 thi /F' traD36 proAB lacI<sup>q</sup> ZΔM 15*].

**Enzyme assay.** PHB depolymerase activity was assayed by measuring the changes in turbidity of a PHB suspension as described previously (26). In brief, the reaction mixture (1 ml) comprised purified PHB granules (to make the final absorbance at 650 nm of the reaction mixture about 1.3) and 100 mM Tris-HCl (pH 7.5). The reaction was initiated by the addition of the enzyme, and then the decrease in turbidity due to insoluble PHB granules was monitored at 650 nm at 30°C with a Shimadzu recording spectrophotometer, model UV-1200. One unit of the enzyme was defined as the amount of protein required to decrease the  $A_{650}$  by 1 absorbance unit per min. 3HB-oligomer hydrolase activity was measured routinely with D-(-)-3-hydroxybutyric acid dimeric ester (dimer) as a substrate. The reaction mixture (0.4 ml) contained 100 mM Tris-HCl (pH 8.0), dimer (1.25 mM), and enzyme. The reaction was started by the addition of the enzyme. After a 15-min incubation at 37°C, the reaction was stopped by heating at 80°C for 3 min, and the reaction mixture was centrifuged at  $10,000 \times g$  for 5 min. The concentration of D-(-)-3-hydroxybutyrate was determined by using D-(-)-3-hydroxybutyrate dehydrogenase as described previously (23). One unit of enzyme catalyzes the formation of 1  $\mu\text{mol}$  of D-(-)-3-hydroxybutyric acid per min under the assay conditions. Hydrolytic activity to *p*-nitrophenyl esters was determined spectrophotometrically. Briefly, the reaction mixture (1 ml) contained 10 mM Tris-HCl (pH 7.4), either *p*-nitrophenyl acetate (0.4 mM), *p*-nitrophenyl butyrate (0.4 mM), or *p*-nitrophenyl octanoate (0.04 mM), and enzyme. The absorption at 400 nm was monitored with a spectrophotometer at 30°C. Lipase activity was assayed by titration of the released acid from olive oil. The reaction mixture (10 ml) contained 5.0 ml of olive oil emulsion and 4.0 ml of 0.1 M phosphate buffer (pH 7.0) and enzyme. After incubation at 37°C, the reaction was stopped with 10 ml of acetone-ethanol (1:1), and the released acid was titrated.

**Purification of a 3HB-oligomer hydrolase from A1.** Bacteria were inoculated into four flasks containing 400 ml of the basic salt medium with 0.15% (wt/vol) of PHB powder and incubated for 60 h at 30°C on a rotary shaker. The culture was centrifuged, and  $(\text{NH}_4)_2\text{SO}_4$  (0.3 M) was added to the supernatant fraction. The supernatant was applied to a hydrophobic adsorbent, butyl-Toyopearl column (2.5 by 6 cm) equilibrated with 10 mM phosphate (pH 7.5) containing 0.3 M  $(\text{NH}_4)_2\text{SO}_4$ . The column was washed with 500 ml of the same buffer, and the enzyme activity was eluted with a linear gradient of 0.3 to 0 M  $(\text{NH}_4)_2\text{SO}_4$  (total, 2 liters). The fractions with enzyme activity eluted at low ionic strength were pooled and centrifuged at  $15,000 \times g$ , and the supernatant was concentrated by an Amicon concentrator with a PM10 membrane. The concentrated enzyme

\* Corresponding author. Mailing address: Department of Biological Sciences, Faculty of Science, Kanagawa University, 2946 Tsuchiya, Hiratsuka, Kanagawa 259-12, Japan. Phone: 81-463-59-4111. Fax: 81-463-58-9684. E-mail: 43saito@educ.info.kanagawa-u.ac.jp.

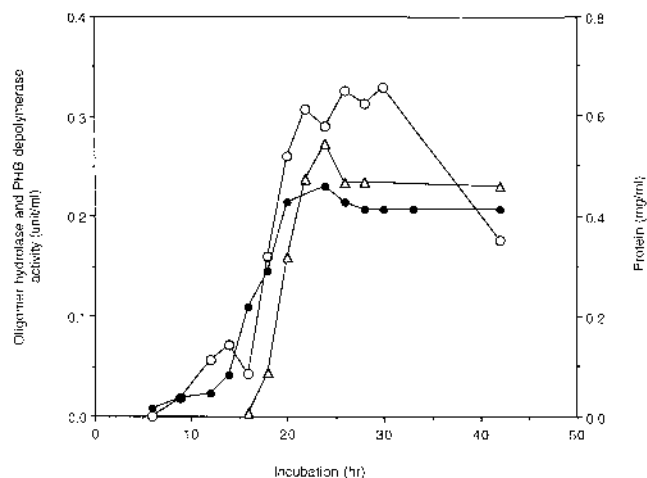


FIG. 1. Bacterial growth and excretion of 3HB-oligomer hydrolase and PHB depolymerase. *Pseudomonas* sp. strain A1 was grown at 30°C in a liquid culture medium containing 0.15% PHB powder as a sole carbon source. At intervals, after centrifugation at  $10,000 \times g$  for 10 min, 3HB-oligomer hydrolase activity ( $\circ$ ), PHB depolymerase activity ( $\Delta$ ) in the supernatant, and the protein ( $\bullet$ ) in the pellet (cells) were assayed as described in the text.

fraction was dialyzed against 10 mM Tris-HCl (pH 9.0) overnight and then applied on a triethylaminoethyl (TEAE)-cellulose column (0.75 by 15 cm) equilibrated with 10 mM Tris-HCl (pH 9.0). The enzyme was eluted with a linear gradient of 0 to 0.5 M NaCl (total, 800 ml). The fractions with high specific activity were pooled, dialyzed against 10 mM Tris-HCl (pH 7.5), and concentrated as described above.

**Isolation and cloning of the A1 3HB-oligomer hydrolase gene.** High-molecular-weight chromosomal DNA was prepared from A1 by the method of Saito and Miura (17), modified by the addition of achromopeptidase (TBL-1; 1.5 mg/ml) (19) to the lysozyme-EDTA solution to lyse the organism. Ligation with T4 DNA ligase, restriction analysis, and genomic library construction were performed with standard techniques (20). Southern and colony blots were prepared according to the standard technique with a nylon membrane (Biodyne; Pall BioSupport, East Hills, N.Y.) (20).

**Restriction mapping and subcloning.** Mapping of restriction sites was performed by the standard procedure (20). Deletion derivatives were constructed by digesting plasmids with a single restriction enzyme and ligating the products. Subcloning was performed by isolation of DNA fragments and subsequent introduction of the fragments into *E. coli* JM109 by transformation. White colonies containing recombinant plasmids were selected on plates in the presence of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) (0.6 mg per plate) and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) (2 mg per plate).

**DNA sequence analysis.** Nucleotide sequences were determined by the dideoxy nucleotide chain termination method (20) with single-strand templates by using the Auto Read sequencing kit (Pharmacia Biotech, Tokyo, Japan). A series of ordered deletions was made on the M13mp19 vector by using the Kilo-Sequence deletion kit (Takara Shuzo Co., Kyoto, Japan). Sequence information was analyzed with GENETYX-MAC software (Software Development Co., Tokyo, Japan).

**In vitro transcription-translation.** The *E. coli* S30-based coupled transcription-translation system was performed according to the technical bulletin of the producer (Promega). Briefly, the purified plasmid DNA was mixed with amino acids containing [ $^{14}$ C]leucine (11 GBq/mmol, 18.5 kBq; Amersham) and S30 extract. The mixture was incubated at 37°C for 1 h. After the reaction had been completed, a 5- $\mu$ l aliquot was removed, dried, treated with sodium dodecyl sulfate (SDS), and applied on an 12.5% SDS-polyacrylamide gel. After electrophoresis, the gel was soaked in 10% acetic acid and 30% methanol for 1 h and then in an enhancer reagent (ENLIGHTNING; Dupont/NEN Research) for 30 min. The treated gel was dried, and the radioactivity on the gel was detected by using an overnight exposure to X-ray film (Kodak X-Omat AR).

**Preparation of antibody.** The rabbits used were 3 months old, male, and about 1.8 kg. After 2 weeks of growing in good condition, 1 ml of the purified enzyme (0.62 mg) and 1 ml of Freund's complete adjuvant (Wako Pure Chemical Industries, Tokyo, Japan) were mixed and injected in the center of the back by subcutaneous injection. The same amount of protein was added in a helix vein 2 weeks after the first injection. Finally, some blood was obtained by puncturing an aorta. The blood was placed at room temperature for 1 h and centrifuged, and the supernatant was preserved in  $-20^\circ\text{C}$ .

**SDS-polyacrylamide gel electrophoresis.** SDS-polyacrylamide gel electrophoresis was performed by the procedure of Laemmli (13). Protein was stained with Coomassie brilliant blue R-250. Electrophoretograms of proteins was performed by using nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) according to the method of Towbin et al. (27).

**Protein measurement and amino acid sequence analysis.** Protein was measured by the method of Lowry et al. (14). The amino acid sequences of peptides were determined by using a sequenator (model 4774; Applied Biosystems, Inc., Foster City, Calif.).

**Chemicals.** Chemicals were obtained as follows: butyl-Toyopearl, Tosoh (Tokyo, Japan) TEAE-cellulose, Serva Feinbiochemica. Dimeric and trimeric esters of D-(−)-3-hydroxybutyric acid were prepared as described previously (25). Purified PHB granules for the assay of PHB depolymerase were prepared as described by Smibert and Krieg (24). PHB powder for bacterial cultivation was donated by Zeneca. Molecular weight markers were purchased from Pharmacia (LMW electrophoresis calibration kit), Gibco BRL (prestained protein molecular weight standards), and Amersham ( $^{14}$ C methylated protein mixture, high-molecular-weight range).

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D85373.

## RESULTS

**Enzyme purification.** A 3HB-oligomer hydrolase was isolated from the PHB-degrading bacterium *Pseudomonas* sp. strain A1, which also secretes a PHB depolymerase. During

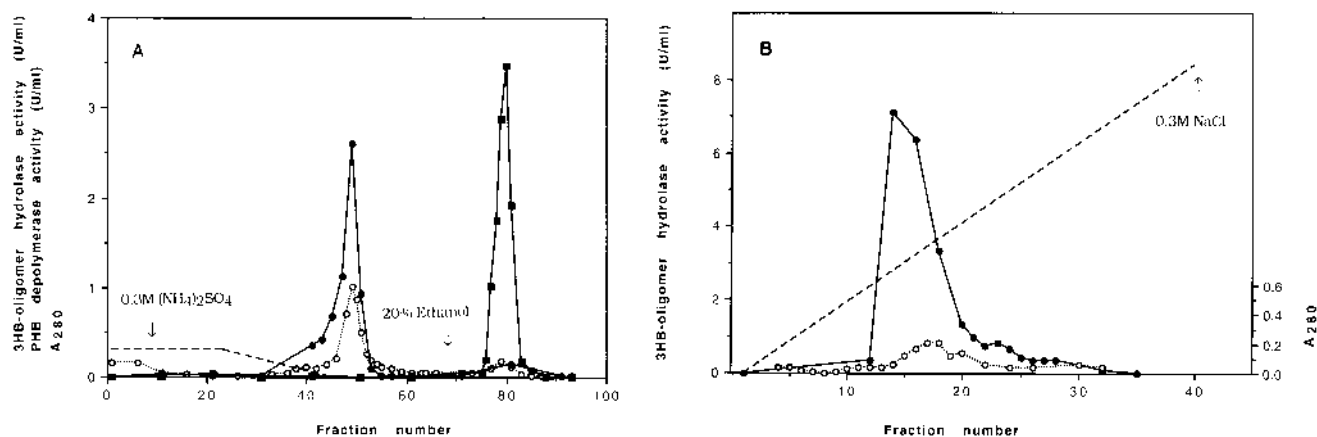


FIG. 2. (A) Elution profile of 3HB-oligomer hydrolase from a butyl-Toyopearl column. The supernatant from culture was applied to a butyl-Toyopearl column (2.5 by 6 cm). Fractions 41 to 53 (220 ml) were pooled.  $\bullet$ , 3HB-oligomer hydrolase activity;  $\blacksquare$ , PHB depolymerase activity;  $\circ$ ,  $A_{280}$ . (B) Elution profile of 3HB-oligomer hydrolase from a TEAE-cellulose column. The concentrated enzyme solution from the butyl-Toyopearl step was applied to a TEAE-cellulose column (0.75 by 15 cm). Fractions 13 to 20 (31 ml) were pooled.  $\bullet$ , 3HB-oligomer hydrolase activity;  $\circ$ ,  $A_{280}$ .

TABLE 1. Purification of 3HB-oligomer hydrolase from *Pseudomonas* sp. strain A1<sup>a</sup>

Step	Amt of protein (mg)	Activity (U)	Sp act (U/mg)	Yield (%)
Crude extract	100	720	7.2	100
Butyl-Toyopearl	14	180	13	25
TEAE-cellulose	5.4	120	22	17

<sup>a</sup> Enzyme was purified from a 1.6-liter culture as described in the text.

growth, A1 produced 3HB-oligomer hydrolase and PHB depolymerase, secreting them into the culture medium in 2 to 3 days (Fig. 1). The enzyme was purified by butyl-Toyopearl and TEAE-cellulose column chromatography. With a butyl-Toyopearl column, 3HB-oligomer hydrolase was separated from PHB depolymerase which was eluted with a buffer containing 10% ethanol (Fig. 2A). The fractions with 3HB-oligomer hydrolase activity were pooled. The pooled fraction was turbid probably due to a substance produced during PHB digestion. Most of turbidity was removed by centrifugation at  $15,000 \times g$  for 10 min. The resulting fraction was further purified on a TEAE-cellulose column, and the enzyme activity was eluted at about 0.1 M NaCl (Fig. 2B) with 17% yield (Table 1). The purified enzyme showed apparent homogeneity on SDS-polyacrylamide gel electrophoresis (Fig. 3A). The purified preparation (0.2 to 0.9 mg/ml) was stable at  $-20^{\circ}\text{C}$  for at least 6 months.

**Enzyme properties.** The molecular mass of the purified enzyme was estimated to be about 70,000 Da by SDS-polyacrylamide gel electrophoresis (Fig. 3A).  $K_m$  and  $V_{max}$  values for the D(-)-3-hydroxybutyrate dimer were 0.18 mM and 25  $\mu\text{mol}/\text{min}/\text{mg}$ , respectively (Table 2).  $K_m$  and  $V_{max}$  values for the trimer were almost the same as those for the dimer. The purified enzyme hydrolyzed methyl-D(-)-3-hydroxybutyrate at a much lower efficiency (Table 2). The purified enzyme hydro-

TABLE 2. Kinetic properties of 3HB-oligomer hydrolases from *Pseudomonas* sp. strain A1 and *E. coli*

Substrate	$K_m$ (mM)	$V_{max}$ (U/mg)	$V_{max}/K_m$
A1			
Dimer	0.18	25	138
Trimer	0.17	30	176
methyl-D(-)-3-Hydroxybutyrate	100	32	0.32
<i>E. coli</i> <sup>a</sup> dimer	0.11		

<sup>a</sup> The crude extract from *E. coli* with pUC carrying the 2.8-kbp fragment was used.

lyzed *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate at the rate of 0.94 and 0.27  $\mu\text{mol}/\text{min}/\text{mg}$ , respectively, but not *p*-nitrophenyl octanoate. Lipase activity with olive oil as a substrate in the purified preparation was under the limit of detection ( $<0.1 \mu\text{mol}/\text{min}/\text{mg}$ ). The purified preparation did not hydrolyze PHB. Diisopropylfluorophosphate (DFP) (13  $\mu\text{M}$ ) inhibited enzyme activity completely. Phenylmethylsulfonyl phosphate (5 mM), Triton X-100 (1%, vol/vol), and dithiothreitol (10 mM), which are inhibitors of PHB depolymerases (26), did not affect hydrolase activity. The purified enzyme showed maximum activity at pH 7 to 8.5.

**Cloning of the A1 3HB-oligomer hydrolase gene.** A1 genomic DNA was digested completely with *Hind*III. The resulting fragments were separated on a 1% agarose gel and transferred onto a nylon membrane. The DNA fixed on the nylon membrane was hybridized with <sup>32</sup>P-labeled 2-kbp probe DNA prepared from the PHB depolymerase gene of *A. faecalis* T1. The DNA corresponding to the positive signal that was about 10 kbp was extracted from the agarose gels, ligated to *Hind*III-digested charomid 9-36, and introduced into *E. coli* DH5 by transfection. From about 20,000 ampicillin-resistant recombinant colonies, 11 positive colonies were selected by colony hybridization. Among positive colonies, a colony having the strongest 3HB-oligomer hydrolase activity was selected for further analyses. This colony was found to have about 10 kbp of foreign DNA. Two *Eco*RI fragments, 1.0 and 2.5 kbp, and an *Eco*RI-*Hind*III fragment, 6.0 kbp, were isolated and ligated

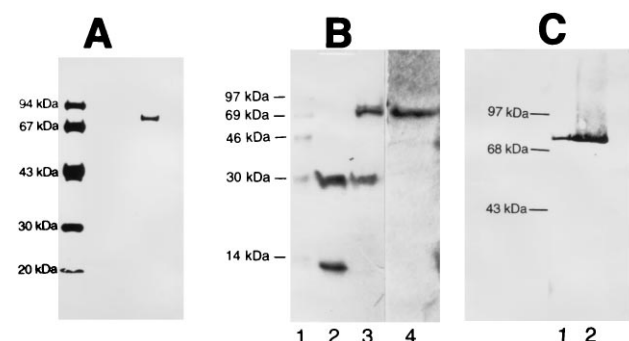


FIG. 3. (A) SDS-polyacrylamide gel electrophoresis of the purified 3HB-oligomer hydrolase. The purified 3HB-oligomer hydrolase (1  $\mu\text{g}$ ) (right) and molecular weight markers (left) were subjected to electrophoresis on 10% acrylamide gels. Proteins were stained with Coomassie brilliant blue R-250. (B) In vitro transcription-translation of the 2.8-kbp DNA fragment from A1 cloned in pUC19. The products of DNA cloned in pUC19 were analyzed by the *E. coli* S30 coupled translation system. <sup>14</sup>C-labeled translation products were analyzed on an SDS-polyacrylamide gel (12.5%) and then detected by using an overnight exposure to X-ray film. Lanes: 1, <sup>14</sup>C-labeled molecular size marker; 2, pUC19 (4  $\mu\text{g}$ ); 3, pUC19 containing the 2.8-kbp fragment (*Sma*I-*Kpn*I, 3.3  $\mu\text{g}$ ); 4, purified 3HB-oligomer hydrolase (1  $\mu\text{g}$ ). The bands at around 14 and 30 kDa were  $\alpha$  peptide of  $\beta$ -galactosidase and  $\beta$ -lactamase expressed by the pUC vector, respectively. Bands in lanes 1 to 3 were detected by autoradiography, and the purified protein in lane 4 was stained with Coomassie brilliant blue R-250. (C) Western blotting and immunostaining of the expressed 3HB-oligomer hydrolase in *E. coli*. Lanes: 1, purified 3HB-oligomer hydrolase from A1 (0.1  $\mu\text{g}$ ); 2, crude extract of *E. coli* with a pUC19 containing the 2.8-kbp *Sma*I-*Kpn*I fragment (1.5  $\mu\text{g}$ ).

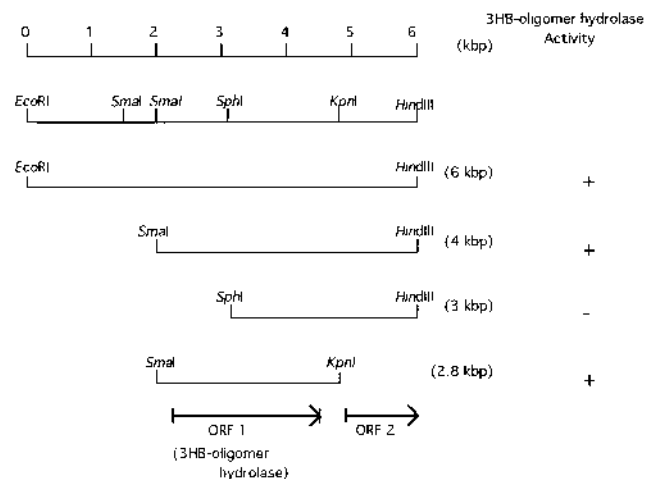


FIG. 4. Restriction endonuclease map of the inserted DNA (6 kbp) in pUC19 and the ability of recombinant plasmids to express 3HB-oligomer hydrolase activity in *E. coli* JM109. ORF1 and ORF2 represent the 3HB-oligomer hydrolase gene and PHB depolymerase gene, respectively.

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T V A N A T A P T A A E L R R L A I Y N N Y R A L V D T N A
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K G G Y G T L Y G P N V D A S G N V T S G S G M V P G V E Y
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T A L A T F S A H A A V T A G P G A W S S Q Q T W A A D T V

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FIG. 5. Nucleotide sequence carrying the 3HB-oligomer hydrolase gene. The doubly underlined sequence is the postulated Shine-Dalgarno region. The putative signal peptide is represented by amino acid residues 1 through 22. The arrow indicates the position of cleavage of the signal peptide. The common sequence (A-X-S-X-G) is underlined. ORF1 and ORF2 represent the 3HB-oligomer hydrolase gene and PHB depolymerase gene, respectively. Dashed underlining indicates the N-terminal amino acid sequence of PHB depolymerase from A1 determined by a protein sequencer (22).

with pUC19. Only the cells with pUC19 that contained the 6.0-kbp fragment showed strong 3HB-oligomer hydrolase activity.

**Subcloning and *in vitro* expression of the hydrolase gene.** Figure 4 shows a restriction map of the 6-kbp *EcoRI-HindIII* fragment. According to this map, *SmaI-HindIII* (4 kbp), *SphI-HindIII* (3 kbp), and *SmaI-KpnI* (2.8 kbp) fragments were obtained. Among these fragments, *SmaI-HindIII* and *SmaI-*

*KpnI* fragments contained the region responsible for expression of the hydrolase activity. The 2.8-kbp *SmaI-KpnI* fragment expressed *in vitro* an about 72-kDa protein band that is similar in size to the purified hydrolase (Fig. 3B).

**Properties of the expressed 3HB-oligomer hydrolase in *E. coli*.** The crude extracts from *E. coli* with pUC19 carrying the 2.8-kbp fragment (*SmaI-KpnI*) were analyzed by immunodetection on nitrocellulose membranes after SDS-polyacrylamide

gel electrophoresis (Fig. 3C). An immunostained protein band corresponding to the purified hydrolase of A1 was detected in JM109 cells carrying the 2.8-kbp fragment. The enzyme from *E. coli* showed a similar  $K_m$  value for the dimer (Table 2) and a similar pH optimum (data not shown) relative to those of the native enzyme.

**DNA sequence analysis.** The nucleotide sequence of the 3HB-oligomer hydrolase gene was analyzed with the 2.8-kbp fragment. Within the 2.8-kbp sequence, one open reading frame (2,112 nucleotides) was found (Fig. 5, ORF1). It specifies a protein with a deduced molecular weight of 72,876 (704 amino acids). The initiation codon is preceded by a putative Shine-Dalgarno sequence. Since 3HB-oligomer hydrolase is secreted from cells, a signal sequence probably exists at the N terminus. We examined the N-terminal amino acid sequence of the purified protein twice but failed. The 3HB-oligomer hydrolase gene in A1 has about 47 and 20% identity to the PHB depolymerase gene in *A. faecalis* in nucleotide and amino acid sequences, respectively. Downstream of the 3HB-oligomer hydrolase in the 6-kbp fragment, we found another open reading frame. In this open reading frame (Fig. 5, ORF2) the sequence corresponding to the N-terminal amino acid sequence of the PHB depolymerase purified from A1 (A-V-T-A-G-P-G-A-W-S-) (22) was found. Therefore, it is highly possible that the 3HB-oligomer hydrolase gene and PHB depolymerase gene are arranged in tandem.

## DISCUSSION

The 3HB-oligomer hydrolase from *Pseudomonas* sp. strain A1 shows similar properties to that of *Alcaligenes faecalis* T1 (23): the molecular size is 70 kDa in the A1 enzyme and 74 kDa in the T1 enzyme, the pH optimum is 7 to 8.5 in the A1 enzyme and 8.5 in the T1 enzyme, and both enzymes are inhibited similarly with a low concentration of DFP.

We cloned the structural gene for the extracellular 3HB-oligomer hydrolase from *Pseudomonas* sp. strain A1 into a vector plasmid, pUC19, confirmed expression of the gene in *E. coli*, and sequenced the gene for this hydrolase. It is possible that the promoter sequence derived from A1 is involved in expression of the 3HB-oligomer hydrolase gene in *E. coli*, since the level of expression did not change in the presence of IPTG (data not shown). The open reading frame assigned for the hydrolase gene has 2,112 nucleotides corresponding to the deduced sequence of 704 amino acids that add up to a molecular weight of 72,876. The *in vitro* product migrated slightly slower than the purified enzyme in SDS-polyacrylamide gel electrophoresis and gave a molecular size of about 72 kDa (Fig. 3B). The expressed protein in *E. coli* detected by immunostaining had a molecular size of about 70 kDa (Fig. 3C). These results indicate that the open reading frame represents the structural gene for the hydrolase. The possibility that the PHB depolymerase gene from *A. faecalis* used as a probe hybridized to the 3HB-oligomer hydrolase gene in A1 seems low due to relatively low homology. In the DNA fragment cloned, it was found that the 3HB-oligomer hydrolase gene and PHB depolymerase gene are arranged in tandem, although sequencing of the latter has not been completed (Fig. 5). Since the deduced amino acid sequence corresponding to the N-terminal amino acid sequence of the mature PHB depolymerase from A1 (22) was almost identical to the N-terminal amino acid sequence of mature PHB depolymerase from *A. faecalis* (A-T-A-G-P-G-A-W-S-S-Q-Q-T-W-A-A-D-S-V-) (19), we assume that the PHB depolymerase gene in *A. faecalis* used as a probe probably hybridized to a portion of the PHB depolymerase gene in the 10-kbp fragment from A1.

Since the enzyme activity in the purified hydrolase was inhibited by a low concentration of DFP, we suppose that an active serine residue is involved in the catalytic mechanism of this hydrolase. Inspection of the sequence of the structural gene failed to find the presence of the conserved serine-containing pentapeptide, Gly-X-Ser-X-Gly, that plays an essential role in catalysis by lipase, protease, and esterase (1). PHA depolymerases were found to have such a conserved pentapeptide (10). Oligomer hydrolase and PHA depolymerase are similar in catalytic properties as observed for hydrolysis of ester bonds of D(-)-3-hydroxybutyric acid. It should be noted, however, that Ala-Thr-Ser-Ser-Gly (residues 176 to 180) and Ala-Ile-Ser-Thr-Gly (residues 187 to 191) are found in the deduced amino acid sequence (Fig. 5). In the lipase of *Bacillus subtilis*, the sequence of the conserved pentapeptide was reported to be Ala-His-Ser-Met-Gly (5). Furthermore, the conserved sequence Gly-X-Ser-X-Ala was found in subtilisins from various microorganisms (1). We cannot determine which sequence present in the A1 hydrolase plays a role in the catalytic function of this enzyme. Recently, a new family of lipolytic enzymes was suggested (28). In this group of enzymes including lipase/acyltransferase, lipase, thioesterase/protease, and arylesterase from various origins, active-site serine is the first in the sequence Gly-Asp-Ser-Leu-Ser(Phe). However, we cannot find such a sequence in the 3HB-oligomer hydrolase gene from A1. Although extracellular 3HB-oligomer hydrolase has a similar catalytic function to PHB depolymerase, these two hydrolases are quite different in molecular weight (oligomer hydrolase, ~70,000; PHB depolymerase, 45,000 to 50,000 [22]) and amino acid sequence including the sequence around the active-site serine.

The physiological role of extracellular 3HB-oligomer hydrolase is not fully clear. This type of enzyme was found only in *A. faecalis* T1 (23), *Pseudomonas* sp. strain A1 in this study, and *Pseudomonas pickettii* K1 (unpublished data), as a member of the extracellular PHB depolymerase system. PHB depolymerases purified from these bacteria show relatively low activity for hydrolysis of the D(-)-3-hydroxybutyrate dimer compared to their PHB depolymerization activity (22). It is possible that these bacteria secrete 3HB-oligomer hydrolase besides PHB depolymerase to compensate the relatively low hydrolysis activity of the PHB depolymerase for the dimer.

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## REFERENCES

- Brenner, S. 1988. The molecular evolution of genes and proteins: a tale of two serines. *Nature* (London) **331**:528-530.
- Briese, B. H., B. Schmidt, and D. Jendrosseck. 1994. *Pseudomonas lemoignei* has five poly(hydroxyalkanoic acid) (PHA) depolymerase genes: a comparative study of bacterial and eukaryotic PHA depolymerases. *J. Environ. Polymer Degrad.* **2**:75-87.
- Bruccato, C. L., and S. S. Wang. 1991. Extracellular poly(3-hydroxybutyrate) depolymerase from *Penicillium funiculosum*: general characteristics and active site studies. *Arch. Biochem. Biophys.* **290**:497-502.
- Chowdhury, A. A. 1963. Poly- $\beta$ -hydroxybuttersäure abbauende Bakterien und Exoenzym. *Arch. Mikrobiol.* **47**:169-200.
- Dartois, V., A. Baulard, K. Schanck, and C. Coisaon. 1992. Cloning, nucleotide sequence and expression in *Escherichia coli* of a lipase gene from *Bacillus subtilis*. *Biochim. Biophys. Acta* **1131**:253-260.
- Dawes, E. A. 1964. Some aspects of the endogenous metabolism of bacteria. *Bacteriol. Rev.* **28**:126-149.
- Dawes, E. A. 1990. Novel microbial polymers: an introductory overview, p. 3-16. In E. A. Dawes (ed.), *Novel biodegradable microbial polymers*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Delafield, F. P., K. E. Cooksey, and M. Doudoroff. 1965.  $\beta$ -Hydroxybutyric dehydrogenase and dimer hydrolase of *Pseudomonas lemoignei*. *J. Biol. Chem.* **240**:4023-4028.

9. **Doudoroff, M.** 1966. Metabolism of poly- $\beta$ -hydroxybutyrate in bacteria, p. 385–400. In N. O. Kaplan and E. P. Kennedy (ed.), Current aspects of biochemical energetics. Academic Press, Inc., New York, N.Y.
10. **Jendrossek, D., A. Frisse, A. Behrends, M. Andermann, H. D. Krazin, T. Stanislawski, and H. G. Schlegel.** 1995. Biochemical and molecular characterization of the *Pseudomonas lemoignei* polyhydroxyalkanoate depolymerase system. *J. Bacteriol.* **177**:596–607.
11. **Jendrossek, D., I. Knoke, R. B. Habibian, A. Steinbüchel, and H. G. Schlegel.** 1993. Degradation of poly(3-hydroxybutyrate), PHB, by bacteria and purification of a novel PHB depolymerase from *Comamonas* sp. *J. Environ. Polymer Degrad.* **1**:53–63.
12. **King, P. P.** 1982. Biotechnology. An industrial view. *J. Chem. Tech. Biotechnol.* **32**:2–8.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
15. **Lusty, C. J., and M. Doudoroff.** 1966. Poly- $\beta$ -hydroxybutyrate depolymerase of *Pseudomonas lemoignei*. *Proc. Natl. Acad. Sci. USA* **56**:960–965.
16. **Merrick, J. M., and C. I. Yu.** 1966. Purification and properties of a D(-)- $\beta$ -hydroxybutyric dimer hydrolase from *Rhodospirillum rubrum*. *Biochemistry* **4**:3563–3568.
17. **Saito, H., and K. Miura.** 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim. Biophys. Acta* **72**:617–629.
18. **Saito, I., and G. R. Stark.** 1986. Charomids: cosmid vectors for efficient cloning and mapping of large or small restriction fragments. *Proc. Natl. Acad. Sci. USA* **83**:8664–8668.
19. **Saito, T., K. Suzuki, J. Yamamoto, T. Fukui, K. Miwa, K. Tomita, S. Nakanishi, S. Odani, J. Suzuki, and K. Ishikawa.** 1989. Cloning, nucleotide sequence, and expression in *Escherichia coli* of the gene for poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*. *J. Bacteriol.* **171**:184–189.
20. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Schirmer, A. D., and D. Jendrossek.** 1994. Molecular characterization of the extracellular poly(3-hydroxyoctanoic acid) [P(3HO)] depolymerase gene of *Pseudomonas fluorescens* GK13 and of its gene product. *J. Bacteriol.* **176**:7065–7073.
22. **Shiraki, M., T. Shimada, M. Tatsumichi, and T. Saito.** 1995. Purification and characterization of extracellular poly(3-hydroxybutyrate) depolymerases. *J. Environ. Polymer Degrad.* **3**:13–21.
23. **Shirakura, Y., T. Fukui, T. Tanio, K. Nakayama, R. Matsuno, and T. Tomita.** 1983. An extracellular D(-)-3-hydroxybutyrate oligomer hydrolase from *Alcaligenes faecalis*. *Biochim. Biophys. Acta* **784**:331–339.
24. **Smibert, R. M., and N. R. Krieg.** 1994. Phenotypic characterization, p. 650. In P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
25. **Tanaka, Y., T. Saito, T. Fukui, T. Tanio, and K. Tomita.** 1981. Purification and properties of D(-)-3-hydroxybutyrate-dimer hydrolase from *Zoogloea ramigera* I-16-M. *Eur. J. Biochem.* **118**:177–182.
26. **Tanio, T., T. Fukui, T. Shirakura, T. Saito, K. Tomita, T. Kaiho, and S. Masamune.** 1982. An extracellular poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*. *Eur. J. Biochem.* **124**:71–77.
27. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
28. **Upton, C., and J. T. Buckley.** 1995. A new family of lipolytic enzymes? *Trends Biochem. Sci. Lett.* **20**:178–179.
29. **Yamada, K., K. Mukai, and Y. Doi.** 1993. Enzymatic degradation of poly(hydroxyalkanoates) by *Pseudomonas pickettii*. *Int. J. Biol. Macromol.* **15**:215–220.