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Cloning, Nucleotide Sequence, and Expression in *Escherichia coli* of the Gene for Poly(3-Hydroxybutyrate) Depolymerase from *Alcaligenes faecalis*

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The extracellular poly(3-hydroxybutyrate) depolymerase gene from *Alcaligenes faecalis* T1 was cloned into *Escherichia coli* DH1 by using the plasmid pUC8. An *A. faecalis* T1 genomic library was prepared in *E. coli* from a partial *Sau*3AI digest and screened with antibody against the depolymerase. Of the 29 antibody-positive clones, 1 (pDP14), containing about 4 kilobase pairs of *A. faecalis* T1 DNA, caused expression of a high level of depolymerase activity in *E. coli*. The enzyme purified from *E. coli* was not significantly different from the depolymerase of *A. faecalis* in molecular weight, immunological properties, peptide map, specific activity, or substrate specificity. Most of the expressed enzyme was found to be localized in the periplasmic space of *E. coli*, although about 10% of the total activity was found in the culture medium. Results of a deletion experiment with pDP14 showed that a large *Sal*I fragment of about 2 kilobase pairs was responsible for expression of the enzyme in *E. coli*. The nucleotide sequence of the large *Sal*I fragment has been determined. Comparison of the deduced amino terminus with that obtained from sequence analysis of the purified protein indicated that poly(3-hydroxybutyrate) depolymerase exists as a 488-amino-acid precursor with a signal peptide of 27 amino acids.

Poly(3-hydroxybutyrate) (PHB) is a unique intracellular reserve of organic carbon and/or chemical energy found in a wide variety of bacteria (3, 5) and is regarded as a potentially useful biodegradable natural plastic that is not derived from petroleum (13). Some bacteria secrete extracellular PHB depolymerases to degrade environmental PHB and utilize the resulting monomeric D(−)-3-hydroxybutyrate as a nutrient (2, 4, 16). We purified an extracellular PHB depolymerase from *Alcaligenes faecalis* T1, a gram-negative bacterium isolated from activated sludge that can hydrolyze not only water-insoluble PHB but also water-soluble D(−)-3-hydroxybutyrate oligomeric esters (24, 25). Since the results of inhibition and kinetic studies indicated the presence of a specific domain on the enzyme molecule (24), we investigated the mechanism of hydrolysis of these substrates through elucidation of the primary structure of this enzyme. Here, we describe the cloning and sequence analysis of the *A. faecalis* T1 gene for PHB depolymerase and its expression in *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains and plasmid. The plasmid used for cloning was pUC8, described by Vieira and Messing (27). The bacterial strains used as hosts for the plasmid were *E. coli* DH1 (F^- *endA1 hsdR17 supE44 thi-1* λ^- *recA1 gyrA96 relA1*) and *E. coli* JM103 [*thi strA supE endA sbcB hsdR* Δ(*lac-pro*) F′ *traD36 proAB lacI*^{q}Z M15]. *A. faecalis* T1 was used as the source of PHB depolymerase (25) and chromosomal DNA.

E. coli was routinely cultured in L broth (1% tryptone, 0.5% yeast extract, 1% NaCl) or on solidified L broth containing 1.5% agar. Bacteria containing pUC8 and recombinant derivatives of this plasmid were maintained in L broth containing ampicillin (50 μg/ml). *A. faecalis* T1 was grown in modified Stinson and Merrick succinate medium (25) for preparation for chromosomal DNA and in the same medium containing glucose (0.15%) instead of succinate for production of PHB depolymerase.

Isolation and cloning of the *A. faecalis* T1 PHB depolymerase gene. Chromosomal DNA was prepared from *A. faecalis* T1 by the method of Saito and Miura (22), modified by the addition of achromopeptidase (TBL-1; 1.5 mg/ml) (12) to the lysozyme-EDTA solution to lyse the organism. Plasmid DNA was isolated from a chloramphenicol-amplified culture (18) of *E. coli* by the method of Birnboim and Doly (1) and then purified by gel filtration. *A. faecalis* T1 DNA was partially digested with *Sau*3AI, and fragments of 4 to 9 kilobase pairs (kbp) in size were isolated from the agarose gel by the glass powder method (28). The size-fractionated DNAs were ligated into *Bam*HI-digested and alkaline phosphatase-treated pUC8, using T4 ligase. *E. coli* DH1 was transformed with recombinant plasmid DNA by the calcium chloride method of Mandel and Higa (17), and ampicillin-resistant (Ap^r) transformants were selected and immunologically screened (9) with anti-PHB depolymerase immunoglobulin G raised in a rabbit.

Restriction mapping and subcloning. Mapping of restriction sites was performed by the standard procedure (18). Deletion derivatives were constructed by digesting plasmids with a single restriction enzyme, followed by ligation of the products. Subcloning was performed by isolating DNA fragments from 0.8% agarose gels by electroelution onto DEAE-paper (6). These fragments were then ligated into pUC8 that had been cut with appropriate restriction enzymes. pUC8 with subcloned DNA fragments was introduced into the *E. coli* JM103 recipient by transformation, and white colonies containing recombinant plasmids were

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selected on plates in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (8).

Detection of PHB depolymerase-producing colonies on plates. Screening of colonies from transformed *E. coli* was carried out immunologically, using rabbit anti-PHB depolymerase immunoglobulin G. PHB depolymerase secreted by transformed *E. coli* was also detected on M9 plates containing purified PHB powder (2 mg/ml) by the formation of clear zones around the colonies.

DNA sequence analysis. One of the subcloned DNAs, pDP17, was cut with *Sal*I, and a DNA fragment of about 2 kbp was isolated by electrophoresis. The DNA fragment was filled in and inserted into the *Sma*I site of M13mp18. Deleted mutants were then made with a deletion kit (Takara Shuzo Co., Kyoto, Japan) according to the methods of Henikoff (10) and Yanisch-Perron et al. (29). The sequence of the deleted DNA fragments was determined by the dideoxy-chain termination method (23).

Enzyme assays. PHB depolymerase activity was assayed by measuring changes in turbidity of a PHB suspension as described previously (25). Oligomer hydrolase activity of the depolymerase was assayed by using the trimeric ester of D-(−)-3-hydroxybutyric acid (trimer) as the substrate (24, 25). Alkaline phosphatase (11) and alcohol dehydrogenase (21) were assayed as described elsewhere.

Localization of PHB depolymerase in *E. coli*. *E. coli* cells (1 g [wet weight]) grown in L broth were subjected to osmotic shock by the procedure of Neu and Heppel (20) to release periplasmic proteins, followed by centrifugation at 20,000 × *g* for 15 min; the precipitate was then suspended in 50 ml of 50 mM Tris hydrochloride (pH 7.6). The culture medium, shock fluid, and supernatant fraction from the sonic extract were concentrated to 5 ml in a dialysis sac by using a water adsorbent (Sumikagel N-100; Sumitomo Chemical Co., Osaka, Japan).

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by the procedure of Laemmli (14). Protein was stained with Coomassie brilliant blue R-250. Electroimmunoblotting of proteins was performed by using nitrocellulose membranes according to the method of Towbin et al. (26).

Peptide mapping. Samples (30 μg each) of the purified enzyme preparation in 0.125 M Tris hydrochloride (pH 6.8) containing 0.5% SDS and 10% glycerol (final volume of the mixture, 0.1 ml) were heated at 100°C for 2 min. To 20-μl portions of the reaction mixture were added various amounts of α-chymotrypsin (in 1 μl). After incubation at 37°C for 30 min, an equal volume of a 4% SDS solution was added, and the mixture was boiled for 2 min to stop proteolysis. Discontinuous electrophoresis in polyacrylamide gel containing SDS was performed as described by Laemmli (14).

Amino acid and sequence analysis. Protein samples (10 to 30 μg) were hydrolyzed with 5.7 N HCl for 22 or 72 h at 110°C and analyzed for amino acid compositions on a Hitachi 835 amino acid analyzer. Tryptophan was determined by the method of Matsubara and Sasaki (19). Values for valine and isoleucine were obtained by 72-h hydrolysis. Destruction of threonine and serine during hydrolysis was corrected for. The amino acid sequence of the enzyme was determined by using a sequenator (model 470A; Applied Biosystems, Inc., Foster City, Calif.). Duplicate analysis was performed.

Protein measurement. Protein was determined by the method of Lowry et al. (15).

Materials. Chemicals used and sources were as follows: tryptone and yeast extract, Difco Laboratories (Detroit, Mich.); chloramphenicol, lysozyme, and RNase A, Sigma Chemical Co. (St. Louis, Mo.); ampicillin, Meiji Seika Co. (Tokyo, Japan); achromopeptidase (TBL-1) and agarose, Wako Pure Chemicals (Tokyo, Japan); restriction enzymes, T4 DNA ligase, Klenow fragment of *E. coli* DNA polymerase I, and DNA of M13mp18, Takara Shuzo Co.; DEAE-cellulose paper (DE81), Whatman Ltd. (Kent, England); and IPTG and X-Gal, Nakarai Chemical Co. (Kyoto, Japan). Other chemicals of reagent grade were obtained commercially.

TABLE 1. Purification of PHB depolymerase from *E. coli* DH1 carrying pDP14[a]

Step	Total protein (mg)	Sp act (U/mg of protein)		Recovery (%)
		PHB	Trimer	
Crude extract	992		0.18	100
Butyl-Toyopearl	6.72	69.5	11.9	40
Triethylaminoethyl-cellulose	2.72	77.2	16.5	22

[a] Purification was carried out with 1 liter of culture medium (L broth). Recovery was calculated from trimer-hydrolyzing activity.

RESULTS

Cloning of the *A. faecalis* T1 PHB depolymerase gene. An *A. faecalis* T1 genomic DNA library was constructed in *E. coli* DH1 by using plasmid pUC8 from a partial *Sau*3AI digest, and about 80,000 Ap^r recombinant colonies were screened with antibody against the PHB depolymerase. Twenty-nine positive colonies were isolated. Preliminary restriction mapping revealed that all 29 transformants contained portions of a common region of the *A. faecalis* T1 genome of about 4 kbp in size. A colony containing a plasmid (pDP14) was chosen for further examination.

Purification and characterization of PHB depolymerase from transformed *E. coli*. PHB depolymerase was purified from transformed *E. coli* DH1(pDP14) grown in L broth by the procedure used for purification of the same enzyme from *A. faecalis* T1 (24) (Table 1). Although enzyme activity was not detectable in the crude extract, probably because of the nonspecific interaction between PHB and the large amount of contaminating proteins, the enzyme was efficiently purified about 50-fold at the step of butyl-Toyopearl (Tosoh, Tokyo, Japan) column chromatography (24) as calculated from the D-(−)-3-hydroxybutyrate oligomer hydrolase activity. The enzyme was further purified, and a single protein band was obtained by SDS-polyacrylamide gel electrophoresis (Fig. 1), with 22% recovery from *E. coli*. The purified PHB depolymerase from *E. coli* exhibited the same electrophoretic mobility as did the enzyme from *A. faecalis* T1, which has a molecular weight of about 50,000 (25) (Fig. 1). Upon Ouchterlony double diffusion, the anti-PHB depolymerase immunoglobulin G raised in a rabbit gave only a single continuous precipitin line with the *E. coli* and *A. faecalis* PHB depolymerases and the crude extract of *E. coli* carrying pDP14 (data not shown). In addition, the peptide maps of the two enzymes showed similar overall patterns (Fig. 2). These results indicate that the two enzymes have similar or identical structures.

Since the PHB depolymerase from *A. faecalis* T1 was demonstrated to have a few cystine bonds, one of which is essential for enzyme activity (24), the time courses of inactivation of the two enzymes with dithiothreitol and reactivation by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were compared. Both enzymes showed the same inactiva-

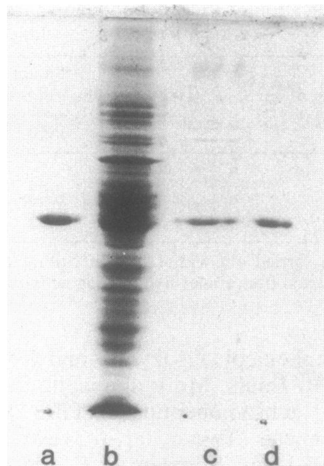

FIG. 1. SDS-polyacrylamide gel electrophoresis of PHB depolymerases obtained at various purification steps from *A. faecalis* T1 (4 μg) (lane a) and from *E. coli* DH1(pDP14) (lane b, crude extract [31 μg]; lane c, at the butyl-Toyopearl step [2.5 μg]; lane d, at the triethylaminoethyl-cellulose step [3.0 μg]).

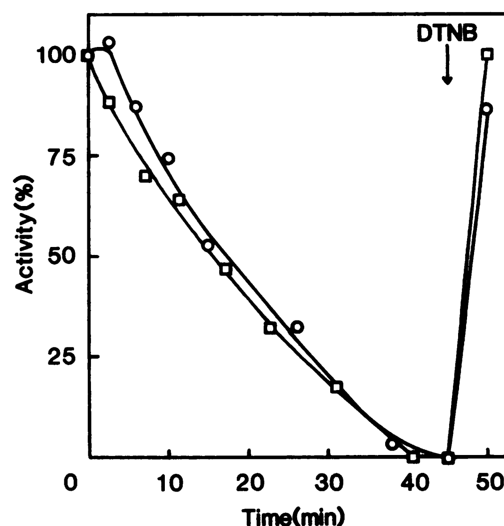

FIG. 3. Effects of dithiothreitol and DTNB on PHB depolymerases purified from 10 μg each of *A. faecalis* T1 (○) and *E. coli*(pDP14) (□). The enzymes in 0.1 ml of 0.1 M potassium phosphate (pH 7.0) containing 4 mM dithiothreitol were incubated at 30°C and then assayed for remaining activity at the times indicated. At 45 min, 20 μl of DTNB (100 mM) was added to each mixture, followed by incubation for 5 min and assay of enzyme activity.

tion and reactivation profiles for dithiothreitol and DTNB treatments (Fig. 3). Furthermore, both enzymes were similar in specific activity, K_m value for PHB, optimal pH, and substrate specificity (25) (data not shown).

Localization of PHB depolymerase in *E. coli*. In the case of *A. faecalis* T1 grown in medium containing PHB or glucose, most of the enzyme activity was excreted into culture medium (25); therefore, the enzyme distribution in *E. coli* JM103 carrying pDP14 was examined. Although about 60% of total PHB depolymerase activity, together with the activity of alkaline phosphatase (the marker enzyme of this fraction), was detected in the periplasmic fraction, about 10% of the activity was found in the culture medium (Table 2).

Deletion derivatives of pDP14. Figure 4A shows the restriction map of the inserted DNA of pDP14 and its deletion derivatives (pDP15, pDP16, and pDP17). Deletion of the *Kpn*I fragment (about 1 kbp) from pDP14 had no effect on the expression of PHB depolymerase in *E. coli* JM103, as judged from the formation of halos around streaks of bacteria on M9 agar plates containing PHB powder (Fig. 4B). On the other hand, deletion of the *Sal*I fragment (about 2.6 kbp from the 3′ end) prevented excretion of PHB depolymerase on M9 (PHB) plates (Fig. 4B, sample c). *E. coli* carrying pDP17 which was deleted by about 2.2 kbp from the 5′ end retained PHB depolymerase activity (Fig. 4B, sample d). Expression of PHB depolymerase in bacterial cells was also examined by immunodetection on nitrocellulose membranes (Fig. 4C). An immunostained protein band corresponding to the purified PHB depolymerase of *A. faecalis* was detected for the crude extracts of *E. coli* carrying pDP14, pDP15, and pDP17. These results indicate that the structural gene for PHB depolymerase is located in the *Sal*I fragment, about 2 kbp from the 3′ end of the inserted DNA of pDP14.

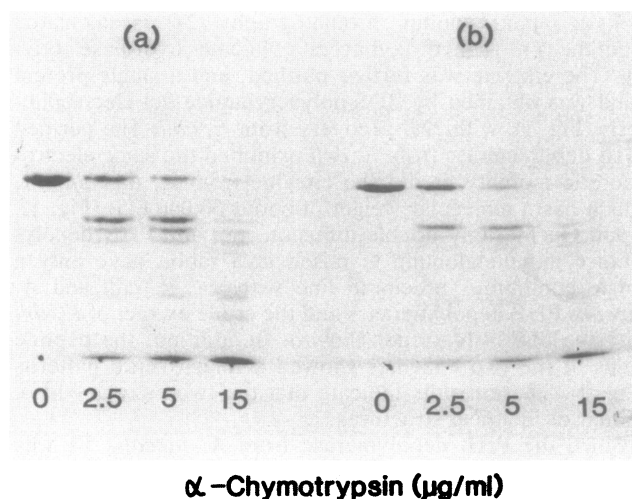

FIG. 2. Peptide maps of PHB depolymerases purified from *A. faecalis* T1 (a) and *E. coli* DH1(pDP14) (b).

Nucleotide sequence of DNA and amino acid sequence of PHB depolymerase. The nucleotide sequence of the PHB depolymerase gene was analyzed with pDP17. A strategy for sequencing is shown in Fig. 5A. A 2-kbp *Sal*I fragment in pDP17 was inserted at the *Sma*I site of M13mp18 and then, using exonuclease III and mung bean nuclease (10, 29), recombinants of various sizes were constructed. The single-stranded DNAs obtained from these recombinants were sequenced by the method of Sanger et al. (23). The DNA sequence of the PHB depolymerase gene and the deduced amino acid sequence are shown in Fig. 5B. The first 20

TABLE 2. Localization of PHB depolymerase in *E. coli* DH1

Fraction	Activity (%) found in each fraction		
	PHB depolymerase[a]	Alkaline phosphatase	Alcohol dehydrogenase
Culture supernatant	9.5	10.1	0
Shock fluid	59.4	58.1	4
Cytosol	24.6	20.3	96
Membrane	6.5	11.5	0

[a] Determined by the quantitative dot immunosorbent method.

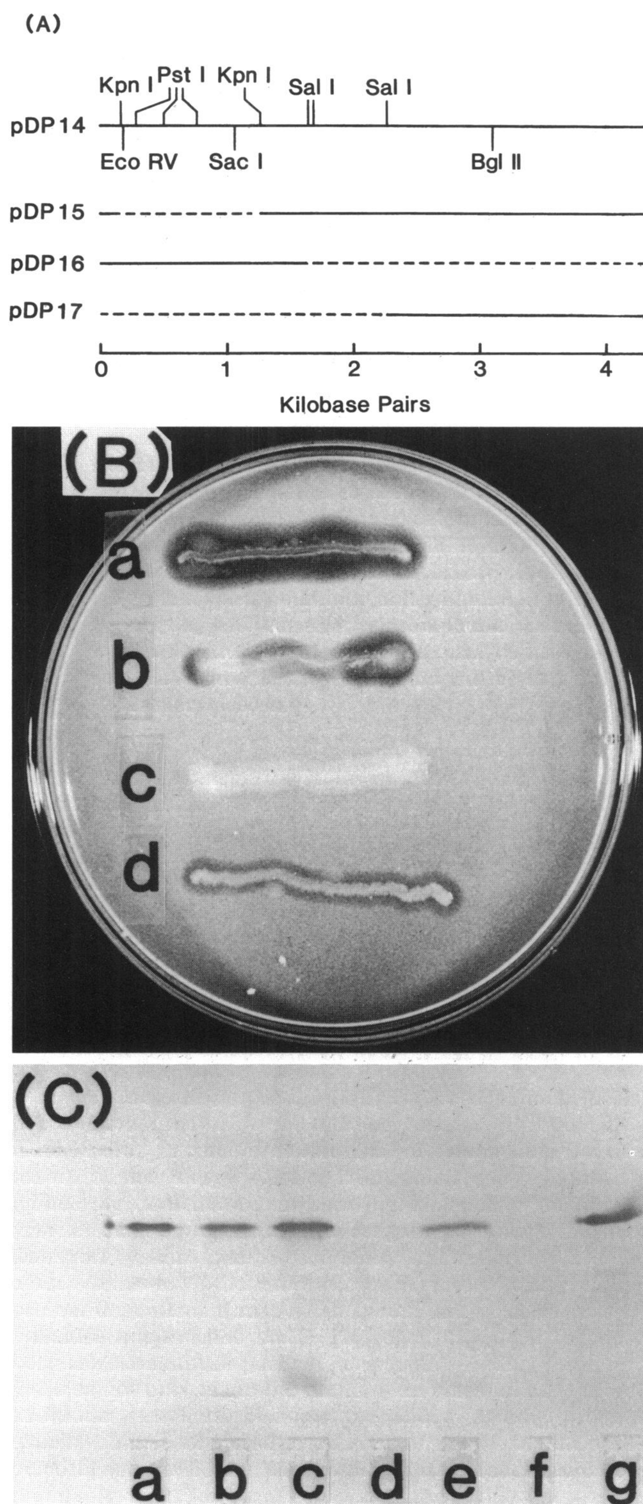

FIG. 4. Expression and excretion of PHB depolymerase in *E. coli* carrying pDP14 and its derivatives. (A) Restriction maps of the insert DNA of pDP14 and its derivatives. ---, Regions deleted. (B) Excretion of PHB depolymerase by transformed *E. coli*. *E. coli* JM103 was transformed with pDP14 and its derivatives, and excretion of PHB depolymerase was determined on M9 medium containing PHB powder (2 mg/ml). Shown are JM103 carrying pDP14 (a), pDP15 (b), pDP16 (c), and pDP17 (d). (C) Immunoblotting of crude extracts of transformed *E. coli* JM103. Purified PHB depolymerase from *A. faecalis* T1 and crude extracts from *E. coli* were subjected to SDS-polyacrylamide gel electrophoresis, followed by blotting onto a nitrocellulose sheet. PHB depolymerase was detected by immunostaining. Shown are results for PHB depolymerase purified from *A. faecalis* T1 (lanes a and g, 76 ng each), crude extracts of JM103(pDP14) (lane b, 1.6 μg), JM103(pDP15) (lane c, 1.2 μg), JM103(pDP16) (lane d, 10 μg), JM103(pDP17) (lane e, 10 μg), and JM103(pUC8) (lane f, 10 μg).

amino acids of the deduced mature coding sequence (position 28 through 47) correspond fully to the amino-terminal sequence of the purified protein as determined by automated Edman degradation. The ATG, which is considered to be the initiation codon, is located 78 bp upstream of the mature coding sequence and begins the code for a larger precursor containing a presumed signal peptide of 26 amino acids. A terminal codon (TGA) was found at 1,464 bp from the initiation (ATG) in the sequence. This reading frame translated into a protein with a molecular weight of 49,934. The deduced molecular weight of the mature protein was 46,858 (461 amino acids). The amino acid composition deduced from the nucleotide sequence agrees with that determined by amino acid analysis of the purified PHB depolymerase (Table 3).

DISCUSSION

We cloned the structural gene for the extracellular PHB depolymerase from *A. faecalis* T1 into a vector plasmid, pUC8, confirmed expression of the gene in *E. coli*, and sequenced the gene for PHB depolymerase. The cloned PHB depolymerase was expressed in *E. coli* as a fully active protein. It is possible that the promoter sequence derived from *A. faecalis* T1 is involved in expression of the PHB depolymerase gene in *E. coli*, since the level of expression did not change in the presence of IPTG (data not shown).

It is interesting that *E. coli* carrying the PHB depolymerase gene from *A. faecalis* T1 excreted a fraction of the enzyme activity into the culture medium (Table 2). This excretion seems not to have been due to rupture of the *E. coli* cell membrane, because alcohol dehydrogenase activity was not detected in the medium. It is not clear whether there is a specific gene for enzyme excretion besides the structural gene for PHB depolymerase from *A. faecalis* T1. However, since the PHB depolymerase activity excreted constituted about 10% of the total, which was about same as the level of alkaline phosphatase activity found in the medium, PHB depolymerase may not be excreted via a specific excretion system in *E. coli* but via some structural changes in the outer membrane of *E. coli* caused by expression of PHB depolymerase protein.

The molecular weight of the mature PHB depolymerase (46,858) deduced from sequencing of the DNA fragment is compatible with published values, which range from 48,000 (determined by Sephadex G-100 gel filtration) to 50,000 (determined by polyacrylamide gel electrophoresis in the presence of SDS) (25). In addition to the results of expression experiments, agreement of the deduced amino acid composition with that determined biochemically (Table 3) strongly supports the identity of the DNA segment as the PHB depolymerase gene. The PHB depolymerase has a characteristic amino acid composition of very few charged amino acids. Most of the glutamic and aspartic acids were found as the amide forms.

Recently, we found that PHB depolymerase from *A. faecalis* T1 lost hydrolytic activity toward PHB after mild

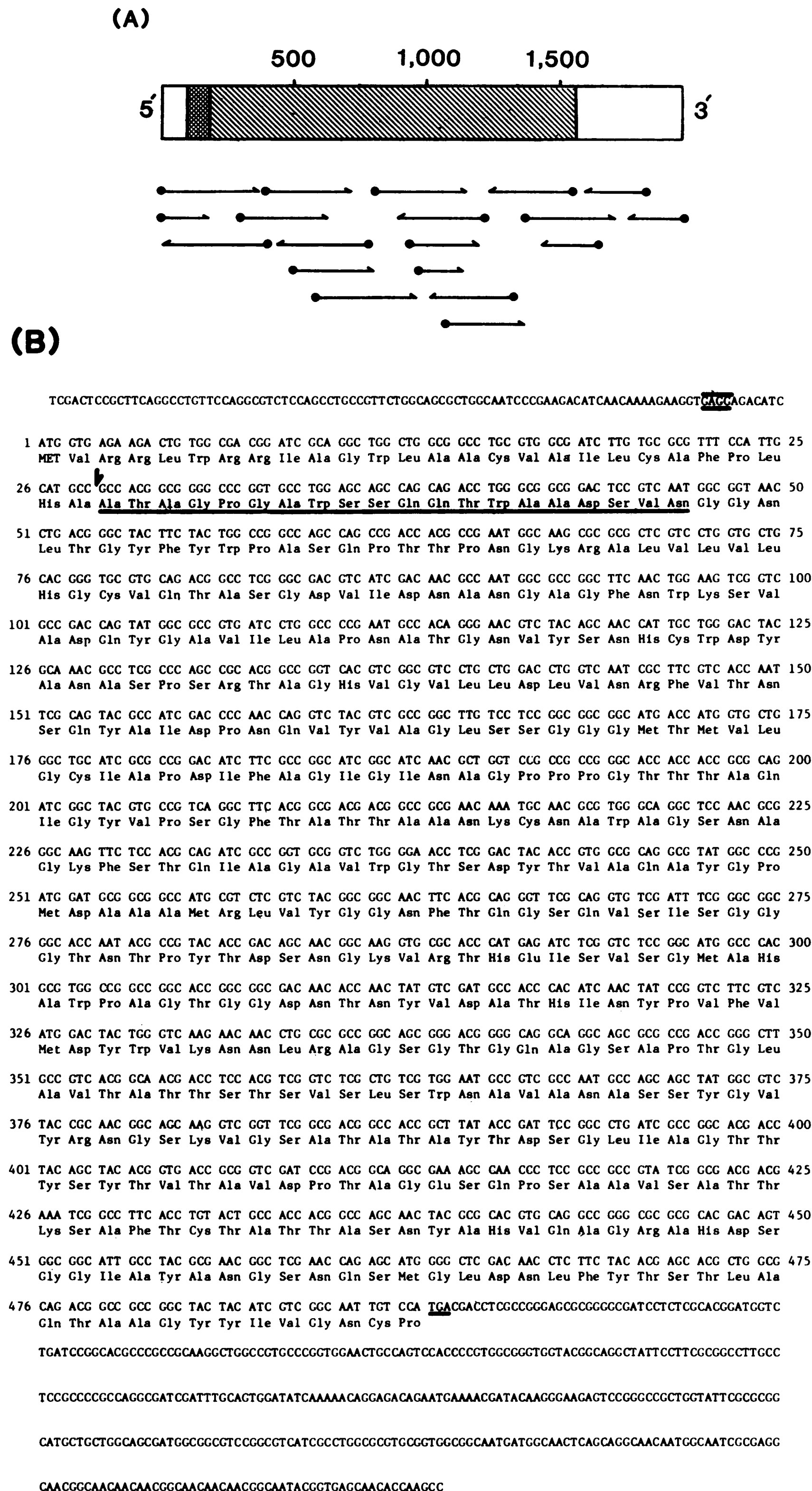

FIG. 5. Nucleotide sequence of the PHB depolymerase gene. (A) Strategy for sequence determination of the *Sal*I fragment containing the PHB depolymerase gene. Symbols: ▧, coding region for the postulated mature PHB depolymerase; ▩, signal peptide. A *Sal*I DNA fragment from pDP17 was inserted into the *Sma*I site of M13mp18, deleted, and then sequenced as described in Materials and Methods. Arrows indicate the direction and extent of sequence determination. (B) Nucleotide sequence of PHB depolymerase DNA. The line below the sequence of amino acids indicates the portion determined by an automated protein sequencer. The putative signal peptide is represented by amino acid residues 1 through 27. The arrow indicates the position of cleavage of the signal peptide. The double-lined sequence is the postulated Shine-Dalgarno region. Numbers refer to amino acid positions.

TABLE 3. Amino acid composition of PHB depolymerase of *A. faecalis* T1

Amino acid	Composition derived from:	
	Nucleotide sequence	Analysis of purified protein[a]
Asp	18	
Asn	33	
Asp + Asn	51	53
Thr	50	51
Ser	43	43
Glu	2	
Gln	17	
Glu + Gln	19	22
Pro	20	16
Gly	59	59
Ala	68	68
Cys	6	7
Met	7	7
Ile	15	14
Tyr	25	25
Phe	10	10
Lys	8	9
His	8	8
Trp	10	6
Arg	8	8

[a] Values were obtained by calculations based on a relative molecular weight of 46,858, derived from the nucleotide sequence.

trypsin treatment but retained activity toward water-soluble oligomers of D-(−)-3-hydroxybutyrate (7). The trypsin-treated enzyme, which was about 6 kilodaltons smaller than that of the native enzyme, showed no ability to bind to PHB, to which the native enzyme bound tightly (7). Clarification of the primary structure of PHB depolymerase may reveal the mechanism of hydrolysis of PHB by PHB depolymerase.

A. faecalis T1 is an unusual gram-negative bacterium that releases proteins into the culture medium. Because of the paucity of gram-negative bacteria that exhibit this activity, the mechanism of excretion of proteins into the medium by gram-negative bacteria is not yet clear. Our study may help to solve this problem.

ADDENDUM IN PROOF

The nucleotide sequence data reported in this paper have been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence data bases and assigned the accession number JO4223.

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