# Biochemical and Molecular Characterization of the *Pseudomonas lemoignei* Polyhydroxyalkanoate Depolymerase System

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Pseudomonas lemoignei has five different polyhydroxyalkanoate (PHA) depolymerase genes (phaZ1 to phaZ5), which encode the extracellularly localized poly(3-hydroxybutyrate) (PHB) depolymerases C, B, and D, poly(3hydroxyvalerate) (PHV) depolymerase, and PHB depolymerase A, respectively. Four of the five genes (phaZ1 to phaZ4) have been cloned, and one of them (phaZ1) was studied in detail earlier (D. Jendrossek, B. Müller, and H. G. Schlegel, Eur. J. Biochem. 218:701-710, 1993). The fifth PHA depolymerase gene (phaZ5) was identified by colony hybridization of recombinant Escherichia coli clones with a phaZ5-specific oligonucleotide. The nucleotide sequence of a 3,704-bp EcoRI fragment was determined and found to contain two large open reading frames (ORFs) which coded for a polypeptide with significant similarities to glycerol-3-phosphate dehydrogenases of various sources (313 amino acids;  $M_r$ , 32,193) and for the precursor of PHB depolymerase A (PhaZ5; 433 amino acids; M<sub>r</sub>, 44,906). The PHV depolymerase gene (phaZ4) was subcloned, and the nucleotide sequence of a 3,109-bp BamHI fragment was determined. Two large ORFs (ORF3 and ORF4) that represent putative coding regions were identified. The deduced amino acid sequence of ORF3 (134 amino acids;  $M_{r}$ , 14,686) revealed significant similarities to the branched-chain amino acid aminotransferase (IIfE) of enterobacteria. ORF4 (1,712 bp) was identified as the precursor of a PHV depolymerase (567 amino acids; M,, 59,947). Analysis of primary structures of the five PHA depolymerases of P. lemoignei and of the PHB depolymerases of Alcaligenes faecalis and Pseudomonas pickettii revealed homologies of 25 to 83% to each other and a domain structure: at their N termini, they have typical signal peptides of exoenzymes. The adjacent catalytic domains are characterized by several conserved amino acids that constitute putative catalytic triads which consist of the consensus sequence of serine-dependent hydrolases including the pentapeptide G-X-S-X-G, a conserved histidine and aspartate, and a conserved region resembling the oxyanion hole of lipases. C terminal of the catalytic domain an approximately 40-amino-acid-long threonine-rich region (22 to 27 threonine residues) is present in PhaZ1, PhaZ2, PhaZ3, and PhaZ5. Instead of the threonine-rich region PhaZ4 and the PHB depolymerases of A. faecalis and P. pickettii contain an approximately 90-amino-acid-long sequence resembling the fibronectin type III module of eucaryotic extracellular matrix proteins. The function of the fibronectin type III module in PHA depolymerases remains obscure. Two types of C-terminal sequences apparently represent substrate-binding sites; the PHB type is present in the PHB depolymerases of A. faecalis and P. pickettii and in PhaZ2, PhaZ3, and PhaZ5, and the PHV type is present in the PHV-hydrolyzing depolymerases (PhaZ4 and PhaZ1). phaZ1 was transferred to A. eutrophus H16 and JMP222. All transconjugants of both strains were able to grow with extracellular PHB as a carbon source and produced translucent halos on PHB-containing solid media. PhaZ1, PhaZ2, PhaZ4, and PhaZ5 were purified from P. lemoignei and from recombinant E. coli; the processing sites of the precursors in E. coli were the same as in P. lemoignei, and similar substrate specificities were determined for the wild-type and the recombinant proteins. All PHA depolymerases hydrolyzed PHB at high specific activities. PhaZ1 and PhaZ4 additionally cleaved PHV, and PhaZ4 hydrolyzed poly(4-hydroxybutyrate). None of the depolymerases was able to hydrolyze polylactide or PHA consisting of monomers with more than five carbon atoms. While the wild-type depolymerase proteins were glycosylated and found to contain glucose and N-acetylglucosamine, none of the recombinant proteins was glycosylated. PHB hydrolysis was dependent on divalent cations such as Ca<sup>2+</sup> and was inhibited by the presence of EDTA.

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters which are synthesized and accumulated intracellularly during unbalanced growth by a large variety of bacteria. They are deposited in form of inclusion bodies and can amount to more than 90% of the dry cell mass (17, 46a). Beside poly(3-hydroxy-

\* Corresponding author. Mailing address: Institut für Mikrobiologie der Georg-August-Universität Göttingen, Grisebachstraße 8, 37077 Göttingen, Germany. Phone: 49-551-39-3777. Fax: 49-551-39-3793. butyric acid) (PHB), polymers consisting of short-chain-length hydroxyalkanoic acids (SCL-HAs; 3 to 5 carbon atoms) or medium-chain-length hydroxyalkanoic acids (MCL-HAs; 6 to 14 carbon atoms) have been detected (reviewed in references 1, 14, 36a, 49, 50, and 52). The monomeric composition of PHA depends on the bacterial species as well as on the carbon sources supplied (23, 56). Because of their thermoplastic properties and synthesis from renewable resources, PHAs are of biotechnological interest, and PHB and its copolymers with 3-hydroxyvalerate (3HV) have been commercialized as BIOPOL.

The ability to degrade extracellular PHA depends on the secretion of specific PHA depolymerases which hydrolyze the polymer to water-soluble products (10, 12) and is widely distributed among bacteria and fungi. Aerobic and anaerobic PHA-degrading bacteria were isolated from various ecosystems such as soil, compost, aerobic and anaerobic sewage sludge, fresh and marine water, estuarine sediment, and air (references 6, 15, 26, and 46 and references cited therein). The PHA depolymerases of Alcaligenes faecalis T<sub>1</sub> (55), Comamonas sp. (26), Comamonas testosteroni (35), Pseudomonas lemoignei (31, 36, 37), Pseudomonas pickettii (61), and fungi (8, 32, 34) were purified, and most of them are well characterized. The organisms mentioned are restricted to the degradation of SCL-PHAs, and their corresponding enzymes degrade only SCL-PHAs such as PHB or poly(3-hydroxyvaleric acid) (PHV) as substrates. Recently, we isolated bacteria capable of growing with MCL-PHA as the sole source of carbon and energy (46). We purified the PHA depolymerase of one strain (Pseudomonas fluorescens GK13) and characterized its extracellular PHA depolymerase. The purified enzyme [poly(3-hydroxyoctanoate) (PHO) depolymerase] was specific for MCL-HA but could not hydrolyze PHB and PHV. Recently, physical modification of MCL-PHA by electron beam-induced cross-linking was described (11). Interestingly, the rubber-like product remained biodegradable.

One of the most interesting PHB-degrading bacteria is P. lemoignei. It is specialized to the utilization of SCL-PHAs, their hydrolysis products, and very few organic acids, and it is unable to grow on sugars, amino acids, or other complex media (12). While most PHA-degrading bacteria apparently synthesize only one depolymerase, P. lemoignei was found to have at least five PHA depolymerases; two PHB depolymerases (A and B) were described about 30 years ago (12, 31), each of which could be separated into two isoenzymes (37). Both enzymes are specific for PHB and copolyesters of 3-hydroxybutyrate (3HB) and 3HV. A fifth depolymerase, which was specifically synthesized during growth on PHV or valerate and which hydrolyzed PHV in addition to PHB and copolyesters of 3HB and 3HV (PHV depolymerase), recently was isolated by Müller and Jendrossek (36). Genetic experiments also provided evidence for the presence of at least five PHA depolymerase genes in P. lemoignei (27). Four of the corresponding genes (phaZ1 to phaZ4) have been cloned, and the nucleotide sequences of three of them have been determined (7, 27). In this study, we continued our study on the P. lemoignei PHAhydrolyzing system.

## MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. *P. lemoignei* was grown in a mineral medium described by Stinson and Merrick (53), supplemented with 0.5% (wt/vol) sodium succinate  $\cdot$  6H<sub>2</sub>O. *Alcaligenes eutrophus* was grown in mineral medium (47) with 0.5% (wt/vol) sodium gluconate. Media for transconjugants harboring pVK100-derived hybrid plasmids additionally contained 15 µg of tetracycline per ml. *Escherichia coli* was usually grown on Luria-Bertani broth (LB) (43) in the presence of ampicillin (50 µg/ml) and/or tetracycline (15 µg/ml), depending on the strain and plasmid.

**Purification of PHA depolymerases from recombinant** *E. coli*. Recombinant strains of *E. coli* were grown (37°C) in batch cultures in 2-liter flasks filled with 500 ml of M9 mineral salts medium (43) supplemented with 0.1% (wt/vol) glucose, 0.5% (vol/vol) glycerol (87%), 0.01% (wt/vol) proline, 0.005% (wt/vol) thiamine, and 50  $\mu$ g of ampicillin per ml for 24 h after inoculation with 0.04 volume of a glucose-grown seed culture. After growth for 10 h, 0.2 mM isopropylthiogalactoside (IPTG) was added. Cells were harvested by centrifugation (30 min, 13,000 × g), and periplasmatic proteins were released by comotic shock (38). The culture supernatant and the periplasmic fraction were combined and concentrated by ultrafiltration through YM10 membranes (Amicon, Witten,

Germany). All subsequent steps were performed at 0 to 4°C, and buffers additionally contained 1 mM CaCl<sub>2</sub> and 5% (vol/vol) glycerol. Ammonium sulfate precipitation (75%), chromatography on DEAE-Sephacel, and chromatography on Sepharose CL-6B were performed as described in detail by Nakayama et al. (37). Finally, the purified PHA depolymerase was dialyzed (10 mM Tris-HCl [pH 7.4]), concentrated (Microsep 30 concentrator; Filtron, Karlstein, Germany), and stored at  $-20^{\circ}$ C.

Gel electrophoresis. Proteins were separated by denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (30). Phosphorylase b ( $M_r$ , 94,000), albumin ( $M_r$ , 67,000), ovalbumin ( $M_r$ , 43,000), carbonic anhydrase ( $M_r$ , 30,000), trypsin inhibitor ( $M_r$ , 20,100), and  $\alpha$ -lactalbumin ( $M_r$ , 14,400) were used as molecular mass standard proteins. After electrophoresis, proteins were silver stained (4). For glycoprotein staining, succose and glycerol in the loading solution were replaced by urea (80%, wt/vol).

Amino acid analysis. N-terminal amino acid sequences of the blotted proteins were determined on an automated gas-phase sequencer with on-line phenythio-hydantoin detection (models 470A and 120A; Applied Biosystems, Weiterstadt, Germany).

**Glycoprotein detection.** The protein of interest was separated by SDS-PAGE and Western blotted (immunoblotted) on polyvinylidene difluoride membranes. Detection of glycoproteins was performed with a digoxigenin (DIG) glycan detection kit (Boehringer, Mannheim, Germany) according to the manufacturer's instructions.

**Preparation of polymer suspensions.** The homopolyesters PHB and PHV were isolated from sodium gluconate-grown cells of *A. eutrophus* H16 and sodium valerate-grown cells of *Chromobacterium violaceum* (51), respectively, by the sodium hypochlorite method as described previously (26, 36). PHO was isolated from *Pseudomonas oleovorans* grown in mineral medium with sodium octanoate (0.75%, wt/vol) as the carbon source, and poly(3-hydroxybutyrate-*co*-3-hydroxydecanoate) [P(HO-*co*-HD)] was isolated from sodium gluconate-grown cells (1.5%, wt/vol) of *P. putida* KT2440, as described by Timm and Steinbüchel (56). The monomeric compositions of the polyesters were determined gas chromatographically under conditions described in detail earlier (56). PHO contained about 92 mol% 3-hydroxyoctanoic acid and 8 mol% 3-hydroxyhexanoic acid. P(HO-*co*-HD) consisted of 3 mol% 3-hydroxyhexanoic acid, 20 mol% 3-hydroxydoceanoic acid, and 5 mol% 3-hydroxydodecanoic acid.

MCL-PHA suspensions were prepared as described by Marchessault (31a). MCL-PHA was dissolved in acetone at a final concentration of 2 g/liter. Four volumes of MCL-PHA-acetone solution was added slowly under stirring into 1 volume of cool, distilled water. Then the organic solvent was removed by using a rotary evaporator, and a milky MCL-PHA suspension in water (5 to 8 g/liter) was obtained. Suspensions of polycaprolactone (PCL; Union Carbide, Bound Brook, N.J.) were prepared by the same procedure. However, it was necessary to heat all solutions to 50 to 60°C. Solution-cast films of poly(4-hydroxybutyrate) [P(4HB)] were prepared from a 2.5% (wt/vol) solution of P(4HB) in chloroform to which traces of Sudan red had been added (46). Polylactide (PL; Boehringer Ingelheim, Germany) was suspended in water (3%, wt/vol) and sonicated before use.

Assays of PHB and PHV depolymerase. PHB and PHV depolymerase activities were measured photometrically by the decrease of the optical density at 650 nm in 1-ml cuvettes containing 180  $\mu$ g of polymer granules (60  $\mu$ l of a 0.3% [wt/vol] suspension in water) in 50 mM Tris-HCl (pH 8.0)–1 mM CaCl<sub>2</sub> at 37°C. The apparent extinction coefficients for PHB and PHV were 3.6 and 3.0  $\mu$ l ng<sup>-1</sup> cm<sup>-1</sup>, respectively. Since the apparent extinction coefficient was different for each polymer preparation, all measurements were performed with one preparation. One unit of depolymerase activity was defined as the hydrolysis of 1  $\mu$ g of polyester per min. Alternatively, the activity was estimated from the diameter of clearing zones in drop tests on glass slides which were covered with a thin agar layer containing 0.2% (wt/vol) polymer granules in 50 mM Tris-HCl (pH 8.0) (for details, see references 7 and 27).

**Isolation, transformation, and analysis of DNA.** Most steps were performed as described by Sambrook et al. (43). *E. coli* was grown aerobically in LB medium supplemented with 20 mM MgCl<sub>2</sub> and transformed by the calcium chloride procedure. Total genomic DNA of *P. lemoignei* was isolated from cells grown on sodium 3-hydroxybutyrate (0.4%, wt/vol) (2). Plasmid DNA from *E. coli* was prepared by the alkaline lysis method and analyzed by separating restriction fragments in agarose gels.

**Identification and cloning of** *phaZ5.* Genomic DNA of *P. lemoignei* was digested with *Eco*R1 or with *Pst*I and separated by agarose gel electrophoresis. The DNA was vacuum blotted onto positively charged nylon membranes and hybridized with a <sup>32</sup>P-labeled oligonucleotide mixture [5'-TC(A/C/G/T)GC(A/C/G/T)GC(A/G/C/T)TG(T/C)TG(T/C)TG-3'] which was derived from the amino acids at positions 11 to 17 ( $Q_{11}$  to  $D_{17}$ ) of the noncoding strand of the N-terminal amino acid sequence of the purified PHB depolymerase A (ATAG PGAWSSQQTWAADSVNGGNLTGFYY [7]). *Eco*RI and *Pst*I-restricted genomic DNAs with sizes of about 3 to 4.5 kbp were electroeluted from agarose gels for 2 h in dialysis bags at 100 V, precipitated with ethanol, purified by chromatography on Elutip-d columns (Schleicher & Schüll, Dassel, Germany), and finally ligated to *Eco*RI- or *Pst*I-linearized pBluescript SK<sup>-</sup> (4 h, 26°C). After transformation of *E. coli* XL1 blue, colonies of recombinant clones were identified by their white appearance on 5-bromo-4-chloro-3-indolyl-o-galactopyrano-

Strain or plasmid	Relevant characteristics	Source or reference(s)		
Strains				
A. eutrophus H16	Source of PHB, $PHB^{-a}$	DSM428, ATCC 17699		
A. eutrophus	pVK100::1.7 kbp <i>Hin</i> dIII:: <i>phaZ1</i> , PHB <sup>+</sup>	This study		
H16(pSN649)		-		
A. eutrophus JMP222	PHB <sup>-</sup>	16		
A. eutrophus	pVK100::1.7-kbp <i>Hin</i> dIII:: <i>phaZ1</i> , PHB <sup>+</sup>	This study		
JMP222(pSN649)		-		
Chromobacterium	Source of PHV	DSM30191		
violaceum				
P. oleovorans	Source of PHO	ATCC 29347		
P. putida KT2440	Source of P(HO-co-HD)	60		
P. lemoignei	Growth on PHB and PHV	LMG2207		
Comamonas sp.	Growth on PHB	DSM6781		
E. coli JM83	ara $\Delta(lac-proAB)$ rpsL (Sm <sup>r</sup> ) thi-1 $\phi$ 80lacZ $\Delta$ 15	51		
E. coli JM83(pSN480)	PHB <sup>+</sup> pUC9::5.4-kbp <i>Mbo</i> I:: <i>phaZ1</i>	27		
E. coli JM83(pSN654)	PHB <sup>+</sup> pUC9-2::1.7-kbp <i>Hin</i> dIII:: <i>phaZ1</i>	27		
<i>E. coli</i> JM83(pSN625)	PHB <sup>+</sup> pUC9-1::2.1-kbp <i>Mbo</i> I:: <i>phaZ2</i>	7, 27		
<i>E. coli</i> JM83(pSN487)	PHB <sup>+</sup> pUC9-1::2.5-kbp <i>Mbo</i> I:: <i>phaZ3</i>	7, 27		
<i>E. coli</i> JM83(pSN484)	PHB <sup>+</sup> pUC9-2::13.5-kbp <i>Mbo</i> I:: <i>phaZ4</i>	27		
E. coli JM83(pSN612)	PHB <sup>+</sup> pUC9-2::3.1-kbp BamHI::phaZ4	This study		
E. coli XL1 blue	recA1 endA1 gyrA96 thi hsdR17 ( $r_{K}^{-}m_{K}^{+}$ ) supE44 relA1 $\lambda^{-}$ lac [F' proAB lacI $^{q}Z\Delta M15 Tn10(Tc^{r})$ ]	9		
E. coli XL1	PHB <sup>+</sup> pBluescriptSK <sup>-</sup> ::2.5-kbp <i>Hin</i> dIII- <i>Bam</i> HI::phaZ4	This study		
blue(pSN874)		-		
E. coli XL1	PHB <sup>+</sup> pBluescriptSK <sup>-</sup> ::3.7-kbp <i>Pst</i> I:: <i>phaZ5</i>	This study		
blue(pSN790)				
E. coli XL1	PHB <sup>+</sup> pBluescriptSK <sup>-</sup> ::3.7-kbp <i>Eco</i> RI:: <i>phaZ5</i>	This study		
blue(pSN792)				
E. coli XL1	PHB <sup>+</sup> pBluescriptSK <sup>-</sup> ::1.85-kbp <i>SspI-XbaI</i> :: <i>phaZ5</i>	This study		
blue(pSN885)				
<i>E. coli</i> S17-1	recA proA thi-1; harbors the tra genes of plasmid RP4 in the chromosome	48a		
E. coli S17-1(pSN648)	pVK100::1.7-kbp <i>Hin</i> dIII:: <i>phaZ1</i> , orientation A, PHB <sup>-</sup>	This study		
E. coli S17-1(pSN649)	pVK100::1.7-kbp <i>Hin</i> dIII:: <i>phaZ1</i> , orientation B, PHB <sup>+</sup>	This study		
Plasmids				
pUC9, pUC9-1, pUC9-2	Ap <sup>r</sup> , <i>lacPOZ</i> ′	21, 59		
pBluescript SK <sup>-</sup>	Ap <sup>r</sup> , <i>lacPOZ</i> '; T7 and T3 promoters	Stratagene, San Diego, Calif		
pVK100	Tc <sup>i</sup> , Km <sup>i</sup>	28		

TABLE 1. Bacterial strains and plasmids used in this study

<sup>a</sup> PHB<sup>-</sup>, no extracellular hydrolysis of PHB.

side-containing LB medium. Recombinant clones were picked onto LB-ampicillin master plates, and colony hybridization (20) with the <sup>32</sup>P-labeled *phaZ5*specific oligonucleotide was performed. **DNA sequence analysis.** DNA sequencing was performed by the dideoxy-chain

DNA sequence analysis. DNA sequencing was performed by the dideoxy-chain termination method (44) with alkaline-denatured double-stranded plasmid DNA and synthetic oligonucleotides as primers. The T7 polymerase sequencing kit of Pharmacia-LKB (Uppsala, Sweden) was used with [<sup>35</sup>S]dATP[aS]. The DNA was additionally sequenced in the presence of 7-deazaguanosine-5'-triphosphate instead of dGTP. DNA sequence data and deduced amino acid sequences were analyzed with the sequence analysis package (version 6.2, June 1990) described by Devereux et al. (13), using sequence databases of GenBank, EMBL, and Swissprot (release spring 1994).

Nucleotide sequence accession numbers. The sequences of phaZ4 and phaZ5 are available from GenBank under accession numbers U12976 and U12977, respectively.

## RESULTS

Identification and cloning of the PHB depolymerase A gene (*phaZ5*). *P. lemoignei* has at least five PHA depolymerases (PHB depolymerases A, B, C, and D, and PHV depolymerase [27, 31, 36, 37]). In previous studies, we described the cloning of four of the corresponding genes (*phaZ1* [PHB depolymerase C], *phaZ2* [PHB depolymerase B], *phaZ3* [PHB depolymerase D], and *phaZ4* [PHV depolymerase]) of *P. lemoignei* in *E. coli* (Table 2), and the nucleotide sequences of *phaZ1*, *phaZ2*, and *phaZ3* were determined (7, 27). Unfortunately, the PHB depolymerase A gene (*phaZ5*) was not detected by screening of about 7,500 recombinant clones for halo formation on opaque

PHB-containing media (27). An additional screening was performed, but again *phaZ5* could not be identified. Only *phaZ1*, *phaZ2*, or *phaZ3* was identified in 13 different clones. The reason for the frequent detection of *phaZ1* is unknown, and further screening for *phaZ5* by this method did not appear promising. Therefore, Southern hybridization of chromosomal DNA of *P. lemoignei* with a *phaZ5*-specific oligonucleotide, which was derived from  $Q_{11}$  to  $D_{17}$  of the N-terminal amino acid sequence of the purified PHB depolymerase A (7), was performed. A 3.7-kbp *Eco*RI fragment and a 3.7-kbp *PstI* fragment of *P. lemoignei* hybridized with the probe.

About 1,000 and 350 recombinant strains of *E. coli* XL1 blue harboring *PstI*- and *Eco*RI-restricted and size-fractionated (3.0- to 4.5-kbp) genomic DNA of *P. lemoignei* in pBluescript, respectively, were screened by colony hybridization with the *phaZ5*-specific oligonucleotide. One clone each specifically hybridized with the probe and contained a 3.7-kbp *PstI* fragment and a 3.7-kbp *Eco*RI fragment in the hybrid plasmid. When Southern blots of the *PstI*- and *Eco*RI-restricted plasmid DNA of both clones were hybridized with the oligonucleotide, a single signal corresponding to the 3.7-kbp fragments appeared in both clones and indicated that the 5' region of *phaZ5* had been cloned. Since both clones expressed PHB depolymerase activity in *E. coli* and produced translucent halos on PHB-containing solid medium, they apparently contained the complete structural PHB depolymerase A gene.

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PUA depolymerese	Leng (amino a	th acids)	$M_{1}$	r				Act	ivity <sup>a</sup>		Sp (10 <sup>3</sup> U	act U/mg)	) Optimal		
gene, protein, isoenzyme	Preprotein	Signal peptide	Deduced mature	By SDS- PAGE	Glycosylation	PHB	P(4HB)	PHV	PHD, P (HO-co-HD)	PCL, PL	PHB	PHV	temp (°C)		
phaZ1, PhaZ1, C	414	37	39,510	44,000	_	+++	_	++	_	_	11.3	2.97	61.5		
phaZ2, PhaZ2, B	433	37	41,793	46,500	_	+ + +	_	_	_	_	14.1	ND	54.5		
phaZ3, PhaZ3, D	419	26	41,162	$ND^b$	ND	ND	ND	ND	ND	ND	ND	ND	ND		
phaZ4, PhaZ4, PHV	567	25	57,459	65,500	_	+ + +	+	+	_	_	22.7	0.72	50		
depolymerase 1															
phaZ5, PhaZ5, A	433	26	42.221	49.000	_	+ + +	_	_	_	_	22.3	ND	51		

TABLE 2. Properties of PHA depolymerases of P. lemoignei purified from recombinant E. coli

<sup>*a*</sup> -, no PHA depolymerase activity detectable (no haloformation); +, low PHA depolymerase activity (small halo); ++, medium PHA depolymerase activity (medium halo); +++, high PHA depolymerase activity (large halo).

<sup>b</sup> ND, not determined.

Nucleotide sequence of the PHB depolymerase A gene (*phaZ5*). The nucleotide sequence of a 3,704-bp *Eco*RI fragment was determined for both strands (Fig. 1), and two colinearly oriented open reading frames (ORFs) with codon usage similar to that of other genes of *P. lemoignei* were found (ORF1, positions 197 to 1138; ORF2, positions 2029 to 3330). Both ORFs were preceded by potential ribosome-binding sites (ORF1, 5'-GGGGCGAA-3'; ORF2, 5'-GGAGA-3') 3 and 8 bp upstream of the ATG start codons, respectively. A 17-bp inverted repeat, which might constitute a termination site of transcription, was found downstream of ORF2 between positions 3404 and 3443 (Fig. 1).

Amino acid sequences of ORF1 and ORF2 (*phaZ5*). ORF1 coded for a putative protein of 313 amino acids ( $M_r$ , 32,193). The deduced amino acid sequence of ORF1 was compared with other sequences of databanks (release spring 1994), using the TBLASTN and BLASTP programs. High homology scores were obtained with the glycerol-3-phosphate dehydrogenases of *E. coli* (GenBank accession number [GB] U00039; 50% identity in a 165-amino-acid overlap), of *Drosophila melanogaster* (GB J04567), and of various other eucaryotic species and with a translated nucleotide sequence of *Salmonella typhimurium* (GB X59594). We assume that ORF1 is the structural gene related to a glycerol-3-phosphate dehydrogenase.

ORF2 was identified to be the structural gene of the PHB depolymerase A of P. lemoignei (phaZ5). It coded for a protein of 433 amino acids ( $M_r$ , 44,906). Twenty-nine N-terminal amino acids of the purified PHB depolymerase A, which had been determined earlier (7), were identical with the DNAdeduced amino acid sequence from residue 27 onwards. The first 26 deduced amino acids showed the characteristics of signal peptides of secretory precursors (40): three positively charged amino acids such as arginine at position 2 and lysine at position 6 and 10 were followed by a core of mainly hydrophobic amino acids, a helix-breaking proline, and a signal peptidase cleavage site C-terminal of the sequence T-Q-A<sub>26</sub> (Fig. 1). The calculated  $M_r$  for the mature protein was 42,221. The primary sequence contained a single pentapeptide (G-L-S<sub>138</sub>-S-G) which resembles the fingerprints of many lipases, esterases, proteases, and other serine-dependent hydrolases (G-X-S-X-G) and which is present in all PHA depolymerases known so far. A second interesting feature of PhaZ5 is the presence of a threonine-rich region in the C-terminal region: 24 threonine residues are clustered in five repetitions of four to five threonine residues. This threonine-rich region is part of a 44-aminoacid-long sequence  $(A_{308} \text{ to } T_{351})$  consisting of amino acids with characteristic side chains: they are all uncharged, are unbranched, are hydroxylated such as threonine (24 residues)

or serine (3 residues), are relatively small such as glycine (7 residues) and alanine (9 residues), or are hydrophobic such as valine (1 residue). Very similar threonine-rich regions were described for PhaZ1, PhaZ2, and PhaZ3 of *P. lemoignei* (7, 27). At the C terminus, the dipeptide cysteine-proline was found, which is also present in all other known PHB depolymerases.

Nucleotide sequence of the PHV depolymerase gene (phaZ4). The PHV depolymerase gene (phaZ4) of P. lemoignei was recently identified on a 13.5-kbp insert of the hybrid plasmid pSN484 by hybridization with a phaZ4-specific oligonucleotide (7). A subclone of pSN484, which harbored a 3.1-kbp BamHI fragment in pUC9-2, was isolated. This strain [E. coli JM83(pSN612)] expressed the depolymerase, as revealed by halo formation on opaque polymer granule-containing solid medium, and was used for further analysis. The nucleotide sequence of the 3,109-bp BamHI fragment of pSN612 was determined for both strands. Two ORFs (ORF3, positions 590 to 991; ORF4, positions 1154 to 2858 [Fig. 2]) were identified and found to have codon usage similar to that of other genes of P. lemoignei. ORF4 was preceded by a putative ribosomebinding site 7 bp upstream of the ATG start codon (5'-AG GAGA-3'). In addition, the 3' and 5' ends of two other ORFs were found upstream of ORF3 (positions 2 to 298) and downstream of ORF4 (position 2929 to the end), respectively.

Amino acid sequence of ORF3 and of ORF4 (*phaZ4*). The deduced amino acid sequences of ORF3 and ORF4 coded for polypeptides of 134 amino acids ( $M_r$ , 14,686) and 567 amino acids ( $M_r$ , 59,947), respectively. The deduced amino acid sequence of ORF3 was compared with other sequences in databanks (release spring 1994), using the TBLASTN and BLASTP programs. Significant homology scores were obtained with a 38-amino-acid-long fragment of the branched-chain amino acid aminotransferases (IIfE) of *E. coli* and *S. typhimurium* (GB X02413 and Swissprot accession number [SP] P15168). In addition, a low degree of similarity was found to a 35-amino-acid-long fragment of the 4-amino-4-deoxychorismate lyases (PabC) of *E. coli* (SP P28305) and *Bacillus subtilis* (SP P28821).

ORF4 was identified as a PHA depolymerase structural gene: the N-terminal amino acid sequence of the ORF4 gene product had the characteristics of signal peptides of secretory precursors, and a putative signal peptidase cleavage site was predicted between  $A_{25}$  and  $L_{26}$  (Fig. 2). The calculated  $M_r$  for the mature protein was 57,459. Twenty-one N-terminal amino acids of the PHV depolymerase had been determined earlier (7); the sequence was in agreement with the DNA-deduced amino acid sequence of ORF4 from residue 26 onwards and confirmed the assumed signal peptidase cleavage site. How-

1	GAATTCGTAGAGGCCTTGCCCGGCGGATGGCTGAAGGTGCGCCATCGCGATGGCCAGGGCGGCTTTGTCAAGGCTGCCGAGGTATGGGGCGAATGAACGG
101	CTTTTCCCTTTTCGATGGCGGGGGCGCTGACATGCGTATTTCCATACTGGGCGCGCGGCGCTGGGGGGCGTAGCCATGGCCTCGCCGAGCGTCATGA
201	S C C G A V A K T R W R N V P D P C E N T A Y L P G H P L P A A L CGTCGTGTTGTGGGGCCGATGGCGATGGCGCAACGTGCCCGATGCCCGACGACACCCCGCTGCCCTGCCCTGCCCTGCCCTGCCCTGCCCGCCC
301	K A T A D F S L A L D H V A Q G D G L L I A A T S V A G L R P L A CAAGGCGACCGCCGATTTTTTCTCTGGCGCTTGATCATGTGGCGCAGGGCGACGGCTTGCTGATCGCCGCCACTTCGGTGGCCGGCGTGCGGCCTTTGGCG
401	Q Q L Q G K A I P N L V W L C K G L E E G S G L L P H Q V V R E V L CAGCAATTGCAAGGCAAAGCCAAAGCCACCTGGTTTGGTTGTGCAAGGGCAAGGGGAGGGGGGCTGCCGCGCCCCAGGTAGTGCGAAGAGGGC
501	G T Q L P A G V L S G P S F A Q E V A Q G L P C A L V I A A E D A TGGGCACCCAGTTGCCGGCAGGCGTGCCGGCGGCGGCGCGGGGAGGATGC
601	A L R E L V V A A V H G P A I R V Y S S D D V V G V E V G G A V K GGCTCTCCGCGAACTGGTGGTCGCGCCGTGCATGGTCCGGCGTCCGGTGTCTATTCAAGCGATGACGTGGTGGGGCGTCGAGGTCGGCGCGCGGCGGCGACAA
701	N I L A I A T G I L D G M S L G L N A R A A L I T R G L A E I T R L AATATCCTGGCATGCCACCGCATCGCATGGCATGGCCTGATGGCCTGGCGGGCG
801	G I A L G A R A E T F M G L A G V G D L I L T C T G D L S R N R R TCGGCATCGCCTTGGCGCCGTAACCTTCATGGGACTGGCGACCTCATTCTCACCGGCGCGTTTGTCGCGCTAACCGTAA
901	N G L G L A Q G K P L E T I V T E L G H V A E G V R C A A A V R N AGTCGGTCGGGACTGGGCAGGCCCCTGGAGACTATCGTCACGGAGCTTGGTCACGGAGAAGGTGGCGCGTGGCCGTGCCCGTGCGCAAT
1001	L A Q Q L Q I E M P I T N A V A G I L F D G H S P R A T V E Q L L A CTGGCGCAGCAATTGCAAATTGAGATGCCGATCACCAATGCCGTCGCCGGCATTCTTTTCGATGGTCATTCTCCTCGCGCCACGGTCGAGCAACTGCTCG R H P R D E S I S A S *
1101	${\tt cccgccatccacgcgacgaatccgattccgcttccctgagcctttccgtggttgcgccgcatgcgtgccgcaacgctccttggcgcgcttgtctgcatgcttgcctgcatgctgtctgcgcgcaacgctcttggcgcgcttgtctgcatgcttgtctgcatgcttgtctgtc$
1201	GAGACCAAGTCGCGGCGACGAAGACAGCACGGGCATACGGCAAGGAGTCGCAACGCCGCAAGCAGCATGTCGATCGTCGCGCTTACGCTTCGTGCTTCAT
1301	CAATATCGTGACCAGGAAGGAAGAATCTTCCTGAGCATGCAGGGCATGCACGACCCCGCCTTCAAAGTACACCGGGCGCGCGC
1401	TTGCCATGCGAAGTCAGCTCCACGCTTCCTTCGATACACTGGATCGTGCATTCCCCCGGCACGCGGTGCTCGGGCACGGTTCTCCCCGGCTGGCAAAACCA
1501	TGTGCATCAATTCCAGGCCTGAGGTTTTCAAGATGGCGCGAGAAGGTGCCGACGTCAGCTGGCTTCCTAGCGGACGGA
1601	CGCGTGCGGTAGGGCCATAAGATTCTCCAAAAATATTCAAATCGATAAAGGGTAATTGCCAAGGTGCTTGCCAATGCAGCATAAGCAGCCAAAGGCTGCT
1701	CATGCTGAGAGCTGACAAACTTTTCCGACTTCATGGTGGAGTGCTACGAAAGGTCAAAATTCCTTGAAAACAGAGAGAAAACAACATCCAGGGCAATTAA
1801	CAATCGTTTGCAAAACTTATGCAGAAATTAATTGCTAACCGAAAATTAGAGGATCATATTTATT
1901	GTTTCCCGCGTTGATGGATGAAAGAAAAATAAGAAGCAACCACCTCCCAGCGGGTGGCAAAGGAAGAGCTGGAAAGCGCAGTCGGCAGTTCATTCCAACT M R N T L K A A F K L G V I S A A L L A P F A T
2001	
2001	AATAAGTTCAATACCUGAGACAAGAATTATGAGAAACACTTTGAAGGCCGCTTTCAAGCTCGGCGTCATTTCTGCAGCATTGCTCGCCCCGTTTGCCACC
2001	
2001	$\begin{array}{c} AATAAGTTCAATACCTGAGAGACAAGAATTATGAGAAAACACTTTGAAGGCCGCTTTCAAGCTCGGCGTCATTTCTGCAGCATTGCTCGCCCCGTTTGCCACCS/D pbaZ5 \longrightarrowQ A A T A G P G A W S S O O T W A A D S V N G G N L T G F Y Y W P ACAGGCTGCCACCGCCGGGCCAGGCGGTCGGTCGAGCCAGCAAACCTGGGCGGCGATTCCGTCAATGGCGGCAATCTGACCGGCTTTTACTATTGGCCGG$
2001 2101 2201	AATAAGTTCAATACCTGAGACAAGAATTATGAGAAAACACTTTGAAGGCCGCTTTCAAGCTCGGCGTCATTTCTGCAGGCATTGCTCGCCCCGTTTGCCACC y $pbaZ5$
2001 2101 2201 2301	AATAAGTTCAATACCGGAGACAAGAATTATGAGAAACACTTTGAAGGCCGCTTTCAAGCTCGGCGTCATTTCTGCAGGCATTGCTCGCCCCGTTTGCCACC S/D $pbaZ5$
2001 2101 2201 2301 2401	AATAAGTTCAATACCGAGACAAGAATTATGAGAAACACTTTGAAGGCCGCTTTCAAGCTCGGCGTCATTTCTGCAGCATTGCTCGCCCCGTTTGCCACC S/D $phaZ5$ $p$
2001 2101 2201 2301 2401 2501	AATAAGTTCAATACCGAGACAAGAATTATGAGAAACACTTTGAAGGCCGCTTTCAAGCTCGGCGTCATTTCTGCAGCATTGCTCGCCCCGTTTGCCACC S/D $phaZ5$ $p$
2101 2201 2301 2401 2501 2601	AATAAGTTCAATACCGAGACAAGAATTATGAGAAACACTTTGAAGGCCGCTTTCAAGCTCGGCGTCATTTCTGCAGCATTGCTCGCCCCGTTTGCCACC S/D $phaZ5Q$ A A T A G P G A W S S O O T W A A D S V N G G N L T G F Y Y W P A CAGGCTGCCACGCGCGGGCCAGGCGTTGGTCGAGCCAGCAAACCTGGGCGGCCGATTCGGTCATTGGCGGCGAATCTGACCGGCTTTACTATTGGCCGG T Q P V H A N G K R A L V L V L H G C A Q T A S G D V I N N G D N CCACGCAACCGGTCCATGCCAACGGCAGCGCGGGGGGGGG
2101 2201 2301 2401 2501 2601 2701	AATAAGTTCAATACCGAGACAAGAATTATGAGAAACACTTTGAAGGCCGCTTTCAAGCTCGGCGTCATTTCTGCAGCATTGCTCGCCCCGTTTGCCACC S/D phaZ5 Q A A T A G P G A W S S O O T W A A D S V N G G N L T G F Y Y W P A CAGGCTGCCACGCGCGGGCCAGGCGTTGGTCGAGCCAGCAAACCTGGGCGGCCGATTCGGTCAATGGCGGCAATCTGACCGGCTTTACTATTGGCCGG T Q P V H A N G K R A L V L V L H G C A Q T A S G D V I N N G D N CCACGCAACCGGTCCATGCCAACGGCAGCGCGGGCGGTGGTGGTGGGCCGGGCCCAGACCGGTCGGCGGCGGACGGGGCGACAACAATGGCGACAA G Y N W K A A A D Q Y G A V I L A P N A T G N V S S Q H C W D Y S TGGCTATAACTGGAAAGCCGCCGCGCGGCGGGGGCGGGGCGGGGTGGATCTCCGCACCGGCAATGTCTCCAGCAGCACTGCTGGGGATTATTCC R T S H S R S T G H E Y V L L D L I N R F K N D P Q Y E I D P N Q V CGCACCAGCCATAGCCGCAGCACGGGCCACGAATACGTGCTGGCGCGGACTTGATCAACGGTTTCAAGAATGACCGGCAGCAGCACCAGG Y V T G L S S G G G E T I V L G C I A P D V F A G W A S N A G P T TATATGTGACCGGCTTGTCCTCGGGCGGGGGGGAAACCATCGTGCTGGGTGTTGATCGCTCCGGACGTGGCGCGCGGCGCGCGC
2101 2201 2301 2401 2501 2601 2701 2801	AATAAGTTCAATACCGGGG CAGGATTATGGGAAACACTTTGAAGGCCGCTTTCAAGCTCGGCGTCATTTCTGCAGCATTGCTCGCCCCGTTTGCCCCCGTTTGCCCCCGTTTGCCCCGCCG
2101 2201 2301 2401 2501 2601 2701 2801 2901	ATTAGETTCATTACCEGAGACATTATTGCAGACACTETTGCAGGCCETTTCAAGCTCGGCGTCATTTCTGCAGCATTGCTCGCCCCCGTTTGCCACC S/D $pha25Q$ A A T A G P G A W S S O O T W A A D S V N G G N L T G F Y Y W P A CAGGCTGCCACCGCCGGGCCAGGCGCTTGGTCGAGCCAGCAACCTGGGCGGCGATTCGTCAATGGCGGCGAATCTGACCGGCTTTACTATTGGCCGG T Q P V H A N G K R A L V L V L H G C A Q T A S G D V I N N G D N CCACGCAACCGGTCCATGCCAGGCGAAGCGGCGGCGGTGGTGGTGGTGTGGCGGCGCGCGGCGCGGGGGG
2101 2201 2301 2401 2501 2601 2701 2801 2901 3001	ATTACTIVE ATTACCEGAGE CAAGAATTATIGAGAACACTTIGAGGECGCTTTEAAGETCGGEGETATTTETGEGGECATTGETCGECCCGCTTGGECGCCCGGTTGGECGAGECGA
2101 2201 2301 2401 2501 2601 2701 2801 2901 3001 3101	ATTAGTICATACCIGACAACAAGAACAAGAACAAGAACAACTITIGAAGGCCGCCTTTCAAGCTCGGCGCTCATTTCTGCGCGCGC
2101 2201 2301 2401 2501 2601 2701 2801 2901 3001 3101 3201	ATAAGTICATACCUGAGACAAGAATTATGGGGAAACACTTTGAAGGCCGCTTTCAAGCTCUGGCTCATTTCUGCAGCATTUCTCCAGCATTUCTCCAGCATTUCTCCAGCATTUCTCCAGCATTUCTCCAGCATTUCTCCAGCATTUCTCCAGCATTUCTCCAGCATTUCTCCAGCATTUCTCCCGTCATUGCCCCCCUGTTTGCCCCGTTUGGCCGGCCCCGCCCGCC
2101 2201 2301 2401 2501 2601 2701 2801 2901 3001 3101 3201 3301	ATTAGET CAATACCE CAAGAATTATEGGAAACACTETTGAAGCCGCETTTCAAGCTCGGCGTCATTTCTGCGCGCCATTGCTCGCCCGTTTGCTGCCCCCTTTGCCAACCC $\begin{bmatrix} S/D \\ pha25 \\ \hline \\ S/D \\ pha25 \\ \hline \\ CACGCCGCCACCGCCGGCCAGGCGCAGCCAGCCAACCTGGGCGGACTCCGTCAATGCGGCGGCATTGCCTCTTTGCCTGTCTTTGCCCGGCTTTTGCCTGGCGGACCCACTGCCAATGCGCGCCGGCCTTTGCCTTTGCCTGTCTTGCCGGGCGCAATGCGCGGCGCGCGGCGGCGGCGGCGGCGGCGGCGGCGGCG$
2101 2201 2301 2401 2501 2601 2701 2801 2901 3001 3101 3201 3301 3401	ATTAGET CAATACCEGAAGGAATTATGAGAAACACTTETGAAGCCGCTTTGAAGCCGGCTGATTCTCGGGGGTATTTCTGCGGCCGGTGCCCGCCGCCGCCGCCGCCGCGGGAAGCAATTGGCGGGCG
2001 2101 2201 2301 2401 2501 2601 2701 2801 2901 3001 3101 3201 3301 3501	$ \begin{array}{c c c c c c c c c c c c c c c c c c c $

3701 ATTC

FIG. 1. Nucleotide sequence of *phaZ5* of *P. lemoignei* and of adjacent regions. Only the coding strand for *phaZ5* is shown. Amino acids are written above the first nucleotide of the corresponding codon. A putative ribosome-binding (Shine-Dalgarno [S/D]) site is boxed. Amino acids confirmed by Edman degradation are underlined, and the processing site of the depolymerase precursor is marked by a vertical arrow. The orientation of transcription and a putative termination signal of transcription are indicated by arrows.

1	GGATCCAGGCGCGCGTGCGCGACGACATCGACCTGGCGCAATTGCTGGCGGCACTGTACCCCTGCGGCTCCATCACTGGCGCGCCGAAACGGCGCACCAT
101	GCAAATCATTCGCGAACTGGAGCCCGATGCGCGCGCGGCTGGTACACCGGCGCCATCGGCTGGTTCGAGGCGCAGGAAGAACGGCGCGCATCGGCGACTTT
201	TGCTTGTCCGTGCCGATCCGCACGCTGGTGCTGCAATCCGAACAAGGTGGCGTGCGCCAAGGCGAAATGGGCGTGGGCGCCGGCATTGTCTACGATAGCG
301	AGTCCGCCAAAGAGTATGCCGAGTGCCGGCTGAAGGCGCGTTTCCTGACCGGGCTCGCTC
401	GGGTTGCCTGTACTTGGAAAGACATTTGCAGCGCCTTGGAGCGTCGGCCCGCTACTTTGGTTTTACGTGGCACGAGGCGCGCGC
501	AGACCTTTGCATCGCTGCCACCAGGACCGCATCGGTTGAGGCTGGCATTGCGCCCCGATGGCACGGCGAGTATCCAGACCGCGCGCG
001	O P V K L L L S P O F M W T D D L F L R H K T T R R O V Y D A A W
601	CCAACCGGTGAAGCTTTTGCTGTCGCCGCAATTCATGTGGACAGACGACTTGTTCCTGCGTCATAAGACCACGCGCCGCCAGGTGTACGACGCTGCCTGG
	KAAEAOGAFDMLFHNEAGEITEGARSNLFIKLRG
701	AAAGCCGCCGAAGCCCABGGGGCATTCGACATGCTGTTCCATAACGACGCGGCGACATCACGGCGACGCGCGCG
	R W V T P P L S A G L L P G V M R S V L L D D P S W O A V E A P V
801	GACGCTGGGTGACGCCGCCGCTTTCGGCTGGTTTGCTGCCGGGAGTGATGCGTTCGGTGCTGCTGGACGATCCGTCATGGCAAGCCGTCGAGGCACCCGT
	TLTDLHNAEEIVACNALRGVLOATLVASA*
901	GACGCTGACCGATTTGCACAATGCAGAAGAGATTGTCGCCTGCAATGCCCTGCGCGCGC
1001	TTAAGAATATTTCCTTCGGTAAATTGCAAGACTGGTAAATCGGCCAGTTCTGGTCGACCGGCCAGTGTCATTTTTAACAATTCAAAAAGTACGATGCCTGC
	MQLKKSLRVTIAVLLG
1101	ggcaataaaagatcccttttcagggacactcatatcaatqaggagagagatcgatgcagctgaaaaaaaaatctctgggagtcacgattgctgtgttacttgg
	S/D phaza
	A G V S A S A F A <u>L T P G S G</u> T <u>W V K E S A T Y G T P N L O</u> D A Y
1201	TGCTGGTGTATCGGCTTCGGCATTTGCACTCACTCCCCGGCAGCGGCACCTGGGTCAAGGAATCGGCAACTTACGGCACGCCCCAACCTGCAGGATGCATAT
	L Y V P K N P A P Q V L G G K R A L M L S L H G C G Q T A S T S V I
1301	CTCTATGTGCCGAAAAAATCCCGCACCACAAGTTCTTGGCGGCAAGCGCGCGC
	D K R F N W E E T A E K Y G M V V V A P T V P T G T S S T R A A S
1401	TCGACAAGCGTTTCAACTGGGAAGAAGAACCGCGGGAAAAATACGGCATGGTAGTGGTCGCCGCCGCCGCACCAGCGCACTAGTTCGACGCGCCGCCAG
	G C W D W F G T A H N R T T R D V V P L I K L I D A V K A R T N L
1501	CGGCTGCTGGGACTGGTTCGGCACGGCGCACCAACCGTACCACCGCGATGTCGTTCCTCTCATTAAGCTGATCGACGCCGTCAAGGCACGACCACCAATCTC
	D I D P N Q I Y V S G L S A G A G E T H V L G C S F P D Y F A G V A
1601	GATATCGATCCCAACCAGATTTATGTGAGCGGCCTGTCGGCCGGC
1701	P N A S P S L G S A A G D I S V P P K R T P Q Q V A D M C R A I N
1/01	CGCCGAATGCCTCGCCGTCGTTCGGCGCGCGCCGCCGCCGCCGCCGC
1001	
1901	
1001	
1901	GAIGGAAIGAAAIGGCCIAIGAGGCIACGIIAIGGGCCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGACGGCCGACGCCIACICHICAAGAAIAGICGIGGCCGA
2001	
2001	V D X V V T O F F F F N N L R V N K W K T T C S V N V D N A S S A
2101	CTATCCCCCCCCTATGTCACGAGATTCTTCTTCGACGAGATAATCTCCCCCCCC
	T V S A S A T A A A G A T V A S Y R V A L O G K T A I N D N A A G S
2201	AG9DDDTDDAATADDADACTADDDDDAAAADDTDDDDTATDDADDDTATDDAADDDDDD
2201	G T S L N K S Y N L G N G I Y A G T V T A V D S K G V E S E A C O
2301	GCGGGACCAGCCTGAACAAAAGCTATAACCTGGGTAATGGCATCTATGCGGGCACGGTGACTGCTGTCGACTCCAAAGGTGTGGAATCGGAGGCGTGCCA
	L S S F O V G O L D P L Y P P S D I O A T G I S S S S I K L N W S
2401	GCTGTCGTCTTTCCAGGTTGGCCAGCTTGATCCGCTGTATCCGCCTTCCGATATCCAGGCAACCGGCATCTCTCCAGGTTCCATCAAGCTGAACTGGAGC
	A V S S A T A Y D V R R N G G A P V R V T A T Q Y T D T G L A P D T
2501	GCCGTGTCGAGCGCCACTGCCTATGATGTGCGTCGCAATGGCGGCGCGCGGGTACGCGTTACTGCAACCCAGTACACCGGTAGCACCAGACA
	SY SY T V T S V N D N M T S G Q S G Q I I G K T Q P V T Y T E K
2601	CCAGCTATTCCTACACGGTCACTTCGGTGAACGACAACATGACCAGCGGACAATCCGGGCAGATCATCGGCAAGACTCAGCCGGTCACGGAACAA
	V T A T V T G H Y S A G R I N V N Q Y L Q L G A K Y G Y N A S L T
2701	AGTGACCGCGACTGTCACCGGTCATTATTCGGCTGGCCGCATCAACGTCAACCAGTACCTGCAACTCGGCGCAAAGTATGGCTATAACGCGTCGCTGACC
	LYKCEGVWTNSSSCGPLQ*
2801	CTGTACAAGTGCGAAGGTGTCTGGACCAACTCGTCCAGCTGCGGCCCGCTGCAGTAAGTTCCTTTGGATGCTGCCGGCAGCAACTTTCAGGCAGCAGCATT
2901	GGCCTCATGCCAGGTTGGCCAGTCGGTGGATGCCGGCTGGTCTTTTTTATTTA
3001	ATTGAATCTGGTGGAACAGCATCAACGGCAGCAGCAGCACCGGCCCGATAGCGGCACTGGCAAACAGGACTTGCGCCATCGGCACGCCGGTAGCCAGGCTTTT

3101 CTTGGATCC

FIG. 2. Nucleotide sequence of *phaZ4* of *P. lemoignei* and of adjacent regions. Only the coding strand for *phaZ4* is shown. Amino acids are written above the first nucleotide of the corresponding codon. A putative ribosome-binding (Shine-Dalgarno [S/D]) site is boxed. Amino acids confirmed by Edman degradation are underlined, and the processing site of the PHB depolymerase precursor is marked by a vertical arrow. The orientation of transcription is indicated by an arrow.

ever, one mismatch was found between the deduced amino acid sequence ( $T_{32}$  of the precursor) and the N-terminal PHV depolymerase sequence, which had an alanine ( $A_7$ ) at the corresponding position (Fig. 2). Repeated amino acid sequencing of the purified protein and nucleotide sequencing of both strands of the gene confirmed the results. The reason for this disagreement is unknown. As was found for PhaZ5 and all other known PHA depolymerases, PhaZ4 contains the typical lipase box pentapeptide of serine hydrolases (G-X-S<sub>137</sub>-X-G). In addition, a cysteine and a proline are also present near the C terminus. However, in PhaZ4, a threonine-rich region is absent. At the position of the threonine-rich region of PhaZ1, PhaZ2, PhaZ3, and PhaZ5, the depolymerase PhaZ4 has a fibronectin type III module (Fn3) fingerprint (5, 22). When the PhaZ4 sequence was compared with other sequences in databanks (release spring 1994), high homology scores were obtained with the *A. faecalis* PHB depolymerase (one Fn3 sequence [41]) and with many other proteins containing one to several Fn3 repeats such as bacterial chitinases, cellulases, amylases, and eucaryotic proteins of the extracellular matrix such as tenascin, titin, undulin, and fibronectin itself.

Comparison of PhaZ4 and PhaZ5 with other PHA depolymerases. The deduced amino acid sequences of all known PHA depolymerases were compared. They varied from 25 to 83% identical amino acids. The highest degrees of homologies were found between the depolymerases of A. faecalis (PhaZ<sub>Afa</sub>) (42) and *P. pickettii* (PhaZ<sub>Ppi</sub>) (29) (83% identical amino acids), between PhaZ1<sub>Ple</sub>, PhaZ2<sub>Ple</sub>, and PhaZ3<sub>Ple</sub> (68 to 72%), and between PhaZ5<sub>Ple</sub> and PhaZ<sub>Afa</sub> (65%). Interestingly, the first 26 amino acids of the mature depolymerases PhaZ5<sub>Ple</sub> and PhaZ<sub>Afa</sub> were identical. However, the degree of homology decreased in the C-terminal parts of the proteins. When the primary sequences of all PHA depolymerases were aligned, regions containing several conserved amino acids were found (Fig. 3). These regions include two histidine residues, two aspartate residues, and one serine residue (Fig. 4). In addition, several single amino acids, including three cysteine residues, were present in all primary structures. No extended homologies were found when the primary structure of the recently sequenced PHO depolymerase gene of P. fluorescens GK13 was compared with sequences of the PHA depolymerases mentioned above (45).

Expression of PHB depolymerase genes in A. eutrophus. To study the expression of PHA depolymerase genes in other bacteria, one PHB depolymerase gene was transferred to A. eutrophus. The 1.7-kbp HindIII fragment of pSN654, which harbored the PHB depolymerase C structural gene (phaZ1), was cloned into the HindIII site of the broad-host-range vector pVK100-encoded kanamycin resistance gene and was transformed to E. coli S17-1. Recombinant strains were sensitive to kanamycin but were still resistent to tetracycline. Two hybrid plasmids (pSN648 and pSN649) which differed with respect to the orientation of the 1.7-kbp HindIII fragment were obtained. Only the strain harboring pSN649 expressed PHB depolymerase activity and produced translucent halos on PHB-containing solid media. Plasmid pSN649 was transferred from E. coli S17-1 to A. eutrophus H16 and to A. eutrophus JMP222 by conjugation. Twelve tetracycline-resistant transconjugants each were tested for the ability to hydrolyze PHB by streaking the colonies on (i) mineral medium supplemented with 0.2% (wt/vol) PHB granules, (ii) mineral medium supplemented with 0.2% (wt/vol) PHB granules and 0.05% (wt/vol) DL-3hydroxybutyrate, and (iii) mineral medium supplemented with 0.2% (wt/vol) PHB granules and 0.5% (wt/vol) yeast extract. A. eutrophus H16 and JMP222 (both with no halo formation on PHB) and P. lemoignei, Comamonas sp., and E. coli S17-1 (pSN649) (all with halo formation on PHB) served as controls. All transconjugants of strain H16 and strain JMP222 grew and produced halos on PHB-containing media and exhibited functional expression and secretion of PhaZ1 (Fig. 5). The diameters of the halos of H16 transconjugants on mineral medium plus PHB but without any additional soluble carbon source were very small, and the halos appeared only after prolonged incubation (1 week).

Purification and characterization of PHA depolymerases from recombinant *E. coli*. The recombinant depolymerase proteins PhaZ1, PhaZ2, PhaZ4, and PhaZ5 were purified from the combined fractions of the periplasmic proteins and the concentrated culture fluid of *E. coli* JM83(pSN654), *E. coli*  JM83(pSN625), E. coli XL1 blue(pSN874), and E. coli XL1 blue(pSN885), respectively. All proteins were purified by the same procedure, which included ammonium sulfate precipitation and subsequent chromatography on DEAE-Sephacel and Sepharose CL-6B. However, an additional purification step (chromatography on Superdex 200 HR 10/30) was necessary for PhaZ4. The purification process is shown for PhaZ2 in Fig. 6A. The purified proteins appeared gel electrophoretically homogeneous (Fig. 6B). However, two additional but very faint bands appeared when 2  $\mu g$  of purified protein or more was used for electrophoresis. Five N-terminal amino acids of PhaZ1, PhaZ2, and PhaZ5 were determined by Edman degradation and were in complete agreement to the corresponding nucleotide sequences. In addition, the signal peptidase cleavage sites were the same as determined for the wild-type proteins. For PhaZ4, 12 amino acids of the N terminus were identified. Again, the same mismatch to the wild-type sequence was found, and a threonine was detected in position 7 (see above). All other amino acids were the same as the wild-type PHV depolymerase.

The apparent molecular masses of the purified recombinant proteins were calculated from their migration in SDS-PAGE (Fig. 6B) and found to be  $49,000 \pm 1,500, 46,500 \pm 1,500$ ,  $44,000 \pm 1,500$ , and  $65,500 \pm 2,500$  for the PhaZ5, PhaZ2, PhaZ1, and PhaZ4, respectively. Table 2 summarizes the data for the purified recombinant depolymerases. All experimentally determined values were significantly higher than the values deduced from the nucleotide sequence. The specific activities and the specificities of the purified proteins to various PHAs were tested photometrically and by a drop test on solid polymer media, respectively. The specific activities of all four proteins with PHB as the substrate were very high and amounted to  $11 \times 10^3$  to  $23 \times 10^3$  U/mg. PHV could be hydrolyzed only by recombinant PhaZ4 ( $0.72 \times 10^3$  U/mg, corresponding to 3.2% of the PHB depolymerase activity) and by PHB depolymerase C (PhaZ1;  $3.0 \times 10^3$  U/mg, corresponding to 26% of the PHB depolymerase activity). P(4HB) was hydrolyzed only by PhaZ4. All other polymers [PHO, P(HOco-HD) PCL, and PL] were not hydrolyzed by any of the proteins.

The temperature maxima for PHB hydrolysis amounted to  $61.5 \pm 1$ ,  $54.5 \pm 2.5$ ,  $50 \pm 2$ , and  $51 \pm 1^{\circ}$ C for recombinant PhaZ1, PhaZ2, PhaZ4, and PhaZ5, respectively (Table 2). Above these temperatures, the polypeptides were inactivated within minutes. These relatively high temperature maxima were obtained only in the presence of divalent cations such as  $Ca^{2+}$  (1 mM). When the four recombinant proteins were preincubated (50°C) with 3 mM EDTA for 5 min before the reaction was started by the addition of polymer granules, the activity was zero. The activity was also zero if EDTA was added to an ongoing reaction. At lower temperatures (37°C), the effect of EDTA was less pronounced: the activity continuously decreased to zero within 5 min after the addition of EDTA. No inhibition was observed if the concentration of Ca<sup>2+</sup> was higher than that of EDTA. Similar results have been described for the partially purified PHB depolymerases of the wild type (12) and were confirmed for the purified PHB depolymerases A and B of the wild type in this study (data not shown).

**Detection of glycoproteins.** The PHB depolymerases A and B of the wild type recently was shown to be glycosylated by reaction with periodic acid-Schiff's reagent (7). The glycoprotein staining was repeated with the purified PHB depolymerases A, B, and C and the PHV depolymerase from *P. lemoignei*. The PHB depolymerases A, B, and C gave strong signals, while the reaction of the PHV depolymerase was only weakly positive. When the experiment was performed with the

PHAZ1	MLAKOIKKANSRSTLLRKSLLFAIILAVSSSSVYA	-LTOVSNFGTNPGNLOMF	17
PHAZ2	MMSSOTTOSSKESLELKRGLLLAAAPLLAMSASSALA	-ATOVTGFGSNPGNLLMY	17
DUN72	MINUT ANT CENT ANTIME MACA DOA EN	I ON WE-	17
PRAZ5	MINK ILKNLCFAAATVILMASAPSAFA		20
PHA25	MRNTLKAAFKLGVISAALLAPFATQA	-A-TAGPGAWSSQQTWAADSVNGGNLTGFII	29
PHAZAfa	MVRRLWRRIAGWLAA-CVAILCAFPLHA	-A-TAGPGAWSSQQTWAADSVNGGNLTGYFY	29
PHAZ Pro i	MKHPYGYRWHWLYALVVTLMTALATFSAHA	-AVTAGPGAWSSQQTWAADTVNGGNLTGYFY	30
PHAZ4	MOLKKSLRVTIAVLLGAGVSASAFA	-L-TPGSGTWVKESATYGTPNLODAYLY	26
	*		
DUX 71		VFA SCWEALCNTHEFYW/VPOOOS	67
PRAGI			67
PHAZ2	KHVPSBMPANAPLVIAMHGCTQSASA	YEATGWTQLANTYKFYVVYPEQQS	67
PHAZ3	KHVPTSMPTNAPLIVAMHGCTQSASA	YEGSGWSALANNYKFYVVYPEQQS	67
PHAZ5	WP-ATQPVHANGKRALVLVLHGCAQTASG	DVINNGDN-GYNWKAAADQYGAVILAPNATG	87
PHAZAfa	WP-ASQPTTPNGKRALVLVLHGCVQTASG	DVIDNANGAGFNWKSVADQYGAVILAPNATG	88
PHAZ pm	WP-ASOPTTPNGKRALVLVLHGCLOTASG	DVIDNANGAGENWKTIAEOYGAVVLAPNATG	89
DUNZA	VORNON DOVI CORDALMI, SLHCCCOTA ST	CUT DKD FNWFETA FKYCMUUVA DTUDT	82
PRAZ4		* *	02
			101
PHAZI	GNNSNKCFNWFEPGDITRGQGEALS	IROMVDNMKANHSIDPSRVIVTGLSAGAF	121
PHAZ2	SNNQNKCFNWFEPGDIARGQGEALS	IKQMVDKMKADHSIDTNRVYVTGLSAGGY	121
PHAZ3	GNNSNKCFNWFESGDIARGQGEALS	IKQMVDKMKADYSIDANRVYVTGLSAGAF	121
PHAZ5	NVSSQHCWDYSRTSH-SRSTGHEYV	LLDLINRFKNDPQYEIDPNQVYVTGLSSGGG	142
PHAZ AFA	NVYSNHCWDYANASP-SBTAGHVGV	LLDLVNRFVTNSOYATDPNOVYVAGLSSGGG	143
DUNG			1 / /
PHALPpi	NVISNACWDIANISP-SRISGRVGV	LUDBVIKF VINSQIAIDENQVIVAGUSSGGG	144
PHAZ4	GTSSTRAASGCWDWFGTAH-NR'ITRDVVP	LIKLIDAVKARTNLDIDPNQIYVSGLSAGAG	141
	* *	** ** *** *	
PHAZ1	MTTVMAATYPDVFAGAAPIAGGPYKCATS	MTSAFTCMSPGVDKTPAAWGDLARGGYSGYN	181
DHA72	MUNUMLATYPDVFACCAPESCCPYNCATS	MTNAFTCMSPGVDKTPAAWGDLARGGYSGYT	181
FILAD2	MUNUMERTIPOVIAGONI POGLINCATO		101
PHALS	MTAVMAATIPDVFAGAAPIAGGPIKCAIS	MIDAF SCHOPEGIDK IPAAWGDLARGGISGIN	101
PHAZ5	ETIVLGCIAPDVFAGWASNAGPTPGTTTL	QIGAVPSGITATNAKNNCLS-LAGSN-SSIF	200
PHAZAfa	MTMVLGCIAPDIFAGIGINAGPPPGTTTA	QIGYVPSGFTATTAANKCNA-WAGSN-AGKF	201
PHAZ Pro i	MTMVLGCIAPDIFAGIGINAGPPPAITRW	KIGVVPSGYTATTAANNCKA WAGSN ASSF	202
PHAZA	ETHVIGCSEPDVFAGVAPNASPSLGSAAG	DIS-VPPKRTPOOVADMCRA-INGNOFNAHL	199
110101	* ** ***		
5-113 F 4	CONDUCT OF LUCCODY IN DANONE TO C	THE REPORT OF THE PARTY OF THE	240
PHAZ1	GPKPKISIWHGSSDYTVAPANQNETVEQF	TNYHG1DQTPDVSDTV-GGFPHKVYKSANGT	240
PHAZ2	GRKPIVSIWHGDADYTVKQSNQVEEVEQW	TNYHGIDQTADVSDTV-AGFPHKVYKDASGN	240
PHAZ3	GRKPKISVWQGSSDTTVKPMNMDELMQQW	TNYHGIDQTADVSETV-KGFPHKVYKDASGN	240
PHAZ5	STOIAGVVW-GTSDFTVAPGYNPLMMDAM	ROIYGGTFTKOASTSVATGGTNTTYKDSSGR	259
PHAZAFA	STOTAGAVW-GTSDYTVAOAYGPMDAAAM	RLVYGGNFTOGSOVSISGGGTNTPYTDSNGK	260
DUNZ-		DATVOOTETOON OVET COOOTNEEDVED CNOK	261
PRALPp1	NIQIAGAVW-GISDIIVAQAIGEMDIAAF	KQ1166TFTQ6AQV31566GTNTFTTD5NGK	201
PHAZ4	DTQ1FATVY-GDKDYLVLPAHNEVNRDGM	KIAYDATVSAGTA-SVDGGGTASLFKDSRGR	257
	* * *	* *	
PHAZ1	PLVETYTITGMGHGTPVDPGTGA	NQCGTA	269
PHAZ2	ALVETYTITGMGHGTPVDPGTGS	LQCGTA	269
PHA73	ALVETWSTTGMAHGTPVDPGTGA	EOCGTS	269
DHA75	VETHELSVSCMSHAWDAGTCCON-TNVVT	SOYVNY PLEVMDYFFTNNSR	307
PHAZJ	VETHELSVSGMSHAWFAGTGGQN HNVD	ATHIN VOVEVMOVEVMINI P	308
FRALATA	VRINEISVSSMANAWFAGIGGDN INIVD		200
PHALPpi	LRINEISVSGMANAWPAGIGGDN-NNIVD		309
PHAZ4	LRISSMVVAGMSHAWPSGPGAAPYIAWVD	STRVNYPAYVTQFFFENNLRVNKWKITCSVN	317
	** * * *		
PHAZ1	G	AYILDVNV	278
PHAZ2	G	AYILDVNI	278
PHAZ3	G	SYTLDVNT	278
DHA75	~		
DUNZ	2000002020		210
PRAGATA	+AG3G1GQAG3A		313
PHAZ Ppi	AGSGPVQSAGT		320
PHAZ4	VPNASSATVSASATAAAGATVASYRVALQ	GKTAINDNAAGSGTSLNKSYNLGNGIYAGTV	377
PHAZ1	CSSYYIGQFFGIIGG	GGTTTTTTSGNVTTTTAATTTTTTATQGYTQ	324
PHAZ2	CSSYYVAKFWGLIGG	SGTTTTTSAGTTTTTSAGTTTTKASTTTTKV	324
DHA73	SSYHTAOFFGL-TG	AGTTTTTTVGSTSTTTGYTSTSSAPVTTTTS	323
DUA75			342
PIAZJ	action	DOL AVER CECTOL CONTACT AND CONTROL	261
PHALAfa			221
PHAZPpi	p	TGLTVTGTTTTSVSLSWNAVTNATSYNVYRN	352
PHAZ4	TAVDSKGVESEACQLSSFQVGQLDPLYPP	SDIQATGISSSSIKLNWSAVSSATAYDVRRN	437
		*	
PHAZ1	TTS	ATVT	331
PHA72	STTTTASTTTAGACYNS		343
DHA73	VA CTTTTTV/ ACA CVNIA		340
DUNCE			250
FIR4D	31111VAAI+CI13		100
PHAZAfa	GSKVGSATATAYTDSGLIAGTTYSYTVTÄ	VUPIAGESQPSAAVSATTKSAFTCTATTA	409
PHAZ PDI	GSKVGSSTSTTYTDTGLIAGTTYSYTVTE	IDPTAGESAQSSAVSAKTQSSFACTATTA	410
PHAZ4	GGAPVRVTATQYTDTGLAPDTSYSYTVTS	VNDNMTSGQSGQIIGKTQPVTYTEKVTATVT	497
	•·····		
PHAZ1	NHYVAGRINVTOYNVL	GARYGYVTTIPLYYCPSLSGWTDKANCSPI	377
PHA72	SNYAHVTAGRAHDTGGYAYTNGSNOKMGL	NNTF-YTSKLRKTGTNYYVIDTT-C-P	396
DHA73	CNVA HUTADDAVNICMOVA VA VOCINOMO	WTF-TTSKLRFADACVETTOCT-C-D	393
- 11GG3	CNVA LIUTAONA INDIG IANANGONQNIGL		107
PRAZO	SINIARV TAGRAHNSSGYALANGSNQNMGL	NNIF-IISIDA-QISPGIIVI-GT-C-P	401
гнаzAfa	SNYAHVQAGKAHDSGGIAYANGSNQSMGL	DNLF - ITSTLA - QTAAGYYIV-GN-C-P	401
PHAZ <sub>PP</sub> i	SNYAHVQAGRAHDSGGIAYANGSNQSMGL	DNVF-YTNTLAQTAAGYYVI-GN-C-P	462
PHAZ4	GHYSAGRINVNQYLQL	GAKYGYNASLTLYKCEGVWTNSSSCGPLQ	542

FIG. 3. Alignment of deduced amino acid sequences of PHA depolymerases. The sequences of PhaZ1 to PhaZ5 of *P. lemoignei* and of the depolymerases of *A. faecalis* (Pha $Z_{Afa}$ ) (42) and *P. pickettii* (Pha $Z_{Ppi}$ ) (30) are aligned. The signal peptides are written in italics, and amino acids that have been determined by Edman degradation are marked by boldface letters. The positions of amino acids conserved in all sequences are marked by asterisks. The numbers of amino acids of the mature proteins are given at the right.

Peptide		Serine		Aspartate		Histidine		Oxyanion hole
	pos.	S	pos.	D	pos.	н	pos.	н
		······································						
PhaZ1 <sub>Ple</sub>	117	IDPSRVYVTGL <b>S</b> AGAFMT	195	IWHGSSDYTV	253	GMG <b>H</b> GTPVDPG	35	LVVAL <b>H</b> GCDQTAA
PhaZ2 <sub>Ple</sub>	117	IDTNRVYVTGLSAGGYMV	195	IWHGDA $\mathbf{D}$ YTV	253	GMG <b>H</b> GTPVDPG	35	LVIAM <b>H</b> GCTQSAS
PhaZ3 <sub>Ple</sub>	117	IDANRVYVTGLSAGAFMT	195	VWQGSSDTTV	253	GMA <b>H</b> GTPVDPG	35	LIVAM <b>H</b> GCTQSAS
PhaZ4 <sub>Ple</sub>	137	IDPNQIYVSGL <b>S</b> AGAGET	212	$VY-GDK\mathbf{D}YLV$	270	GMS <b>H</b> AWPSGPG	47	LMLSL <b>H</b> GCGQTAS
PhaZ5 <sub>Ple</sub>	138	IDPNQVYVTGLSGGGET	213	$VW-GTS\mathbf{D}FTV$	272	GMS <b>H</b> AWPAGTG	49	LVLVL <b>H</b> GCAQTAS
PhaZ <sub>Afa</sub>	139	IDPNQVYVAGLSSGGGMT	214	$VW-GTS\mathbf{D}YTV$	273	GMA <b>H</b> AWPAGTG	49	LVLVL <b>H</b> GCVQTAS
PhaZ <sub>Ppi</sub>	140	IDPNQVYVAGLSSGGGMT	215	VW-GTS <b>D</b> YTV	274	GMA <b>H</b> AWPAGTG	50	LVLVL <b>H</b> GCLQTAS
CONSENSUS		ID-n-vYV-GL <b>S</b> -G+t		vw-G-s <b>D</b> yTV		GM- <b>H</b> PG		L***1 <b>H</b> GC-QtAs
PhaZ <sub>Pf1</sub>	139	LNAQRQYATGI <b>S</b> SGGYNT	193	$FLHGFV\mathbf{D}AVV$	227	LGG <b>H</b> EWFAASP	78	QNLLD <b>H</b> GYAVIAP

FIG. 4. Alignment of amino acid sequences of PHA depolymerases in the regions surrounding putative active sites. The amino acids serine (S), aspartate (D), and histidine (H) of the catalytic triad and histidine of the putative oxyanion hole are given in boldface letters, and their positions (pos.) in the mature proteins are indicated. A consensus sequence is written below. Amino acids present in all sequences are written in capital letters, and lowercase letters are used for amino acids present in at least six sequences. Positions of amino acids with hydrophobic or small side chains are indicated by \* or +, respectively. At the bottom, the corresponding sequence of the PHO depolymerase of *P. fluorescens* GK13 is given. Data are from references 27 (PhaZ1<sub>Ple</sub>), 7 (PhaZ2<sub>Ple</sub> and PhaZ3<sub>Ple</sub>), 42 (PhaZ<sub>Afa</sub>), 29 (PhaZ<sub>Ppl</sub>), and 45 (PhaZ<sub>Pnl</sub>).

corresponding recombinant proteins, none of the proteins reacted, which indicated that the recombinant proteins were not glycosylated. To analyze the composition of the sugar components, the purified wild-type PHB depolymerases A, B, and C and the PHV depolymerase were acetylated and subjected to an alditolactate analysis. Glucose and *N*-acetylglucosamine were identified in all proteins in ratios of 0.4:1 (PHB depolymerases A, B, and C) and 0.5:1 (PHV depolymerase).

### DISCUSSION

*P. lemoignei* has at least five PHA depolymerase genes (*phaZ1* to *phaZ5*), which code for PHB depolymerases C, B, and D, PHV depolymerase, and PHB depolymerase A, respectively. All five genes were sequenced, and all depolymerases except PHB depolymerase D were purified and characterized (references 12, 31, 36, and 37 and this study). All proteins were specific for PHB and copolymers of 3HB and 3HV and did not hydrolyze PHA with more than five carbon atoms per monomer. Additionally, the PHV depolymerase and PHB depolymerase C had significant activity with PHV homopolyester as the substrate. All depolymerases are more or less inhibited by serine inhibitors such as phenylmethylsulfonyl fluoride or disopropylfluorophosphate and are sensitive to reducing agents such as dithioerythritol.

The corresponding structural genes (*phaZ1* to *phaZ5*) have been cloned and sequenced in this and previous studies (7, 27). The N-terminal amino acid sequences of the purified recombinant proteins were identical to the DNA-deduced sequences of *phaZ1*, *phaZ2*, and *phaZ5*. However, one mismatch in position 7 of the mature PhaZ4 protein ( $T_7$ ) to the DNA-deduced sequence of *phaZ4* ( $A_{32}$ ) was found. Because of this difference and because the quotient of the PHV depolymerase and PHB depolymerase activities of purified PhaZ4<sub>Ple</sub> was drastically lower (3.2%) than that for the purified wild-type PHV depolymerase (30%) (36), PhaZ4 might represent an isoenzyme (PHV depolymerase 1) of the true PHV depolymerase (PHV depolymerase 2). In that case, a sixth depolymerase gene (*phaZ6*) of *P. lemoignei* has to be postulated.

The alignment of all sequences revealed clusters of three to eight conserved amino acids around  $H_{35}$  (numbers according to the PhaZ1 sequence in Fig. 3),  $S_{117}$ ,  $D_{132}$ ,  $D_{195}$ , and  $H_{253}$ and indicated the importance of these regions.  $S_{117}$  is part of a lipase box fingerprint of serine-dependent hydrolases,  $G-X_1$ -S- $X_2$ -G (24, 39). Whereas in most lipases  $X_1$  has a polar character such as histidine or tyrosine (24a, 25),  $X_1$  of PHA depolymerases is hydrophobic such as isoleucine (PHO depolymerase of *P. fluorescens* GK13 [45]), valine (putative intracellular PHO depolymerases of *P. oleovorans* and *P. aeruginosa* [24, 57]), or leucine (all other PHA depolymerases). In addition to the lipase box, three of the four amino acids C terminal of the pentapeptide are also conserved (V-Y-V-X; Fig. 3 and 4).

In all known serine hydrolases, the active-site serine forms a catalytic triad with an aspartate (or glutamate) and a histidine residue, in which the hydroxyl group of serine serves as a nucleophile that attacks the ester bond (24a, 48, 54). A transient tetrahedral intermediate of the carbonyl carbon is formed, and the negatively charged oxygen atom is stabilized by two main-chain NH groups (oxyanion hole). We assume that PHA depolymerases probably also contain a catalytic triad consisting of S<sub>117</sub>, D<sub>132</sub> or D<sub>195</sub>, and H<sub>35</sub> or H<sub>253</sub> (numbers according to the PhaZ1 sequence in Fig. 3). It is remarkable that the sequence around H<sub>35</sub> is very similar to the sequences around the oxyanion hole of many lipases (24a, 25). Therefore, it is possible that the region around H<sub>35</sub> represents an oxyanion hole, and H<sub>253</sub> might be the active-site histidine. Recently, S<sub>196</sub> of the (mature) *A. faecalis* depolymerase has been shown to be



FIG. 5. Growth of transconjugants of *A. eutrophus* JMP22 harboring *phaZ1* on PHB. *A. eutrophus* H16 and *A. eutrophus* JMP222 served as negative controls and *Comamonas* sp., *P. lemoignei*, and *E. coli* JM83(pSN654) served as positive controls in the first and second rows, respectively; 12 different transconjugants were tested in the five bottom rows.



FIG. 6. Purification of PHA depolymerases from recombinant *E. coli*. (A) PhaZ2 at various steps of purification. Proteins were separated in denaturing SDS-12% polyacrylamide gel and silver stained. Lanes 1 and 8, molecular mass standard proteins (masses are indicated in daltons); lane 2, 0.18 µg of purified PhaZ2 after CM Sepharose chromatography; lane 3, 0.98 µg of partially purified PhaZ2 after DEAE-Sephacel chromatography; lane 4, 2.90 µg of partially purified PhaZ2 after ammonium sulfate precipitation; lane 5, 2.0 µg of concentrated reactions of the concentrated culture fluid and of the concentrated periplasmic fraction; lane 6, 1.9 µg of the concentrated culture fluid. (B) Purification of recombinant PhaZ1, PhaZ4, PhaZ4, and PhaZ5. Lane 1, molecular mass standard proteins; lanes 2 to 5, 0.3 to 0.4 µg of purified proteins (PhaZ5 [lane 2], PhaZ2 [lane 3], PhaZ1 [lane 4], PhaZ4 [lane 5]).

the binding site of diisopropylfluorophosphate (41). However, this serine is not part of a lipase box and is present only in the depolymerases of *A. faecalis* and *P. pickettii* and in PhaZ5<sub>Ple</sub>, not in PhaZ1<sub>Ple</sub>, PhaZ2<sub>Ple</sub>, PhaZ3<sub>Ple</sub>, and PhaZ4<sub>Ple</sub> (Fig. 3). Further studies are necessary to determine the true active center of PHA depolymerases. All known PHB depolymerases contain six to nine cysteine residues, three of which (C<sub>37</sub>, C<sub>74</sub>, and C<sub>374</sub>) are conserved in all seven PHA depolymerases. Since the purified proteins are sensitive to reducing agents such as dithioerythritol, we assume that at least two of the cysteine residues form an essential disulfide bridge.

Among the most astonishing features of the depolymerase primary structures are the threonine-rich regions of PhaZ1, PhaZ2, PhaZ3, and PhaZ5: 22 to 27 threonine residues of an approximately 40-amino-acid-long sequence are clustered in four to five repetitions of 4 to 6 threonine residues. Most of the remaining amino acids in the threonine-rich region are also hydroxylated (e.g., serine or tyrosine) or are small (e.g., alanine and glycine). To our knowledge, similar threonine-rich regions have not been described for other proteins. However, serinerich regions, regions enriched in serine, threonine, glycine, and proline, or alanine and proline-rich regions have been described for proteins which consist of two or more domains. These regions are assumed to function as flexible linkers between the domains (reviewed by Gilkes et al. [19]). If the threonine-rich regions of PHA depolymerases function as linkers, what do they link together? The region N terminal of the threonine-rich region most probably represents the catalytic domain (see above). Interestingly, the C-terminal regions of PhaZ2<sub>Ple</sub>, PhaZ3<sub>Ple</sub>, PhaZ5<sub>Ple</sub>, PhaZ<sub>Afa</sub>, and PhaZ<sub>Ppi</sub> are very similar but are different from the corresponding regions of  $PhaZ1_{Ple}$  and  $PhaZ4_{Ple}$ . Since  $PhaZ1_{Ple}$  and  $PhaZ4_{Ple}$  are the only known PHA depolymerases which have a significant sub-strate specificity to PHV in addition to PHB, the C-terminal regions of PhaZ1<sub>Ple</sub> and PhaZ4<sub>Ple</sub> might be PHV (and PHB)specific substrate-binding sites (PHV type in Fig. 7), while the C-terminal regions of the other depolymerases represent PHBspecific substrate-binding sites (PHB type in Fig. 7). For the A. faecalis PHB depolymerase, it was shown that a trypsin-generated 5-kDa C-terminal fragment was necessary for binding the polymeric substrate (substrate-binding domain), while the remaining major polypeptide fragment still hydrolyzed oligomeric esters (catalytic domain) (18). A similar domain structure was described for the cellobiohydrolase I from Trichoderma reesei (58). The domain structures of the currently known PHA depolymerases are summarized in Fig. 7. Interestingly,  $\text{PhaZ4}_{\text{Ple}}$  and  $\text{PhaZ}_{\text{Afa}}$  did not contain any threoninerich region but contained an approximately 90-amino-acidlong fragment which has the consensus sequence of Fn3 (5, 22). Fn3 sequences have been found in extracellular matrix proteins of eucarvotes such as fibronectin, tenascin, titin, and udulin and in procaryotic extracellular proteins that hydrolyze polymeric compounds such as cellulose, chitin, xylose, or amylose (references 3 and 33 and references cited therein). All six strictly conserved amino acids of Fn3 (22) are also present in PhaZ4<sub>Ple</sub> and PhaZ<sub>Afa</sub>. These amino acids (two tyrosines, two valines, one tryptophan, and one leucine) all have hydrophobic side chains. However, neither the function of these strictly conserved amino acids nor the overall function of Fn3 is known for any of the proteins. Because of the strictly conserved amino acids, function as a linker between two domains is unlikely.

The depolymerases of *P. lemoignei* are glycosylated and contain glucose and *N*-acetylglucosamine. The PHB depolymerase of *Comamonas* sp. is not glycosylated (26), but the PHB depolymerase of *Penicillium funiculosum* is and contains mannose, galactose, and glucose as main components (8). The glycosylation of other PHA depolymerases has not been tested. Since the depolymerases PhaZ1<sub>Ple</sub>, PhaZ2<sub>Ple</sub>, PhaZ4<sub>Ple</sub>, and PhaZ5<sub>Ple</sub> synthesized by recombinant *E. coli* strains were not glycosylated but were still active, the glycoside chain is not essential for activity. The physiological function of the glycosylation might be the protection against extracellular proteases, which are secreted by a variety of bacteria.

Nothing is known about the sequence and position of PHA depolymerase promoters of *P. lemoignei* and the regulation of transcription. When the 5'-upstream sequences of the PHA depolymerase genes of *P. lemoignei* were compared, a 16-bp sequence (5'-ACAAGAGCGAAAAGCC-3') 45 bp upstream of *phaZ1* was identical to the 5'-upstream sequence 45 bp upstream of *phaZ2*. Other identities of such size were not found between any of the 5'-upstream regions of the other genes. It will be necessary to determine the position of trans-



FIG. 7. Domain structures of PHA depolymerases. The numbers of the first and last amino acids of the prepeptides and of the mature proteins are given, and positions of the lipase boxes (serine) are indicated. For references, see the legend to Fig. 4).

scription initiation and the length of the transcripts for the PHA depolymerase genes in the near future. Recently, RNA analysis of the PHO depolymerase gene (*phaZ*) of *P. fluorescens* GK13 has shown that the gene is transcribed from a  $\sigma^{70}$ -like promoter as a monocistronic message of about 1 kbp (45).

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#### REFERENCES

- Anderson, A. J., and E. A. Dawes. 1990. Occurrence, metabolism, metabolic role, and industrial use of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54:450–472.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
- Blaak, H., J. Schnellmann, S. Walter, B. Henrissat, and H. Schrempf. 1993. Characteristics of an exochitinase from *Streptomyces olivaceoviridis*, its corresponding gene, putative protein domains and relationship to other chitinases. Eur. J. Biochem. 214:659–669.
- Blum, H., H. Beier, and H. J. Gross. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8:93–99.
- 5. Bork, P., and R. F. Doolittle. 1992. Proposed acquisition of an animal protein

domain by bacteria. Proc. Natl. Acad. Sci. USA 89:8990-8994.

- Briese, B. H., D. Jendrossek, and H. G. Schlegel. 1994. Degradation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by aerobic sewage sludge. FEMS Microbiol. Lett. 117:107–112.
- Briese, B. H., B. Schmidt, and D. Jendrossek. 1994. Pseudomonas lemoignei has five different PHA depolymerase genes. A comparative study of bacterial and eucaryotic PHA depolymerases. J. Environ. Polym. Degrad. 2:75–87.
- Brucato, C. L., and S. S. Wong. 1991. Extracellular poly(3-hydroxybutyrate) depolymerase from *Penicillium funiculosum*: general characteristics and active site studies. Arch. Biochem. Biophys. 290:497–502.
- Bullock, W. O., J. M. Fernandez, and J. M. Stuart. 1987. XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques 5:376–379.
- Chowdhury, A. A. 1963. Poly-β-hydroxybuttersäure abbauende Bakterien und Exoenzym. Arch. Mikrobiol. 47:167–200.
- De Koning, G. J. M., H. M. M. van Bilsen, P. J. Lemstra, W. Hazenberg, B. Witholt, H. Preusting, J. G. van der Galien, A. Schirmer, and D. Jendrossek. 1994. A biodegradable rubber by crosslinking poly(hydroxyalkanoate) from *Pseudomonas oleovorans*. Polymer 35:2090–2097.
- Delafield, F. P., M. Doudoroff, N. J. Palleroni, C. J. Lusty, and R. Contopoulos. 1965. Decomposition of poly-β-hydroxybutyrate by pseudomonads. J. Bacteriol. 90:1455–1466.
- Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12:387–395.
- 14. Doi, Y. 1990. Microbial polyesters. VHC, New York.
- Doi, Y., Y. Kanesawa, and N. Tanahashi. 1992. Biodegradation of microbial polyesters in the marine environment. Polym. Degrad. Stab. 36:173–177.
- Don, R. A., and J. M. Pemperton. 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J. Bacteriol. 145:681–686.
- 17. Forsyth, W. G. C., A. C. Hayward, and R. B. Roberts. 1958. Occurrence of

poly-β-hydroxybutyric acid in aerobic Gram-negative bacteria. Nature (London) 182:800-801.

- Fukui, T., T. Narikawa, K. Miwa, Y. Shirakura, T. Saito, and K. Tomita. 1988. Effect of limited tryptic modification of a bacterial poly(3-hydroxybutyrate) depolymerase on its catalytic activity. Biochim. Biophys. Acta 952: 164–171.
- Gilkes, N. R., B. Henrissat, D. G. Kilburn, R. C. Miller, Jr., and R. A. J. Warren. 1991. Domains in microbial β-1,4-glycanases: sequence conservation, function, and enzyme families. Microbiol. Rev. 55:303–315.
- Grunstein, M., and J. Wallis. 1979. Colony hybridization. Methods Enzymol. 68:379–389.
- Hanna, Z., C. Fregeau, G. Prefontaine, and R. Brousseau. 1984. Construction of a family of universal expression plasmid vectors. Gene 30:247–250.
- Hansen, C. K. 1992. Fibronectin type III-like sequences and a new domain type in prokaryotic depolymerases with insoluble substrates. FEBS 305:91– 96
- Haywood, G. W., A. J. Anderson, D. F. Ewing, and E. A. Dawes. 1990. Accumulation of polyhydroxyalkanoate containing primarily 3-hydroxydecanoate from simple carbohydrate substrates by *Pseudomonas* sp. strain NCIMB 40135. Appl. Environ. Microbiol. 56:3354–3359.
- Huisman, G. W., E. Wonink, R. Meima, B. Terpstra, and B. Witholt. 1991. Metabolism of poly(3-hydroxyalkanoates) (PHA) by *Pseudomonas oleo*vorans. J. Biol. Chem. 266:2191–2198.
- 24a.Jaeger, K. E., S. Ransac, B. W. Dijkstra, C. Colson, M. van Heuvel, and O. Misset. 1994. Bacterial lipases. FEMS Microbiol. Rev. 15:29–63.
- Jaeger, K. E., S. Ransac, H. B. Koch, F. Ferrato, and B. W. Dijkstra. 1993. Topological characterization and modeling of the 3D structure of lipase from *Pseudomonas aeruginosa*. FEBS Lett. 332:143–149.
- Jendrossek, D., I. Knoke, R. H. Habibian, A. Steinbüchel, and H. G. Schlegel. 1993. Degradation of poly(3-hydroxybutyrate), PHB, by bacteria and purification of a novel PHB depolymerase of *Comamonas* sp. J. Environ. Polym. Degrad. 1:53–63.
- Jendrossek, D., B. Müller, and H. G. Schlegel. 1993. Cloning and characterization of the poly(hydroxyalkanoic acid)-depolymerase gene locus, *phaZ1*, of *Pseudomonas lemoignei* and its gene product. Eur. J. Biochem. 218:701-710.
- Knauf, V. C., and E. W. Nester. 1982. Wide host range cloning vectors: a cosmid clone bank of an Agrobacterium T<sub>i</sub> plasmid. Plasmid 8:45–54.
- Kuruso, Y., K. Kohama, Y. Uchida, T. Saito, and H. Yukawa. 1994. Cloning and nucleotide sequencing of the poly(3-hydroxybutyrate) depolymerase gene from *Pseudomonas pickettii*, p. 357–361. *In* Y. Doi and K. Fukuda (ed.), Biodegradable plastics and polymers. Elsevier Science B.V., Amsterdam.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
- Lusty, C. J., and M. Doudoroff. 1966. Poly-β-hydroxybutyrate depolymerases of *Pseudomonas lemoignei*. Biochemistry 56:960–965.
- 31a.Marchessault, R. H.
- Matavulj, M., and H. P. Molitoris. 1992. Fungal degradation of polyhydroxyalkanoates and a semiquantitative assay for screening their degradation by terrestrial fungi. FEMS Microbiol. Rev. 103:323–332.
- Matuschek, M., G. Burchardt, K. Sahm, and H. Bahl. 1994. Pullulanase of *Thermoanaerobacterium thermosulfurigenes* EM1 (*Clostridium thermosulfurigenes*): molecular analysis of the gene, composite structure of the enzyme, and a common model for its attachment to the cell surface. J. Bacteriol. 176:3295–3302.
- McLellan, D. W., and P. J. Halling. 1988. Acid-tolerant poly(3-hydroxybutyrate) hydrolases from moulds. FEMS Microbiol. Lett. 52:215–218.
- Mukai, K., K. Yamada, and Y. Doi. 1993. Enzymatic degradation of poly-(hydroxyalkanoates) by a marine bacterium. Polym. Degrad. Stab. 41:85–91.
- Müller, B., and D. Jendrossek. 1993. Purification and properties of poly(3hydroxyvaleric acid) depolymerase from *Pseudomonas lemoignei*. Appl. Microbiol. Biotechnol. 38:487–492.
- 36a.Müller, H. M., and D. Seebach. 1993. Poly(hydroxyfettsäureester), eine fünfte Klasse von physiologisch bedeutsamen organischen Biopolymeren. Angew. Chem. 105:483–509.
- Nakayama, K., T. Saito, T. Fukui, Y. Shirakura, and K. Tomita. 1985. Purification and properties of extracellular poly(3-hydroxybutyrate) depolymerases from *Pseudomonas lemoignei*. Biochim. Biophys. Acta 827:63–72.
- Neu, H. C., and L. A. Heppel. 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. 240:3685–3692.
- Persson, B., G. Bentsson-Olivecrona, S. Enerback, T. Olivecrona, and H. Jornvall. 1989. Structural features of lipoprotein lipase: lipase family rela-

tionships, binding interactions, non-equivalence of lipase cofactors, vitellogenin similarities and functional subdivision of lipoprotein lipase. Eur. J. Biochem. **179**:39–45.

- Pugsley, A. P. 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. 57:50–108.
- Saito, T., A. Iwata, and T. Watanabe. 1993. Molecular structure of extracellular poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis* T1. J. Environ. Polym. Degrad. 1:99–105.
- 42. Saito, T., K. Suzuki, J. Yamamoto, T. Fukui, K. Miwa, K. Tomita, S. Nakanishi, S. Odani, J. I. 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.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Sanger, F., S. Nicklen, and A. R. Coulsen. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.
- Schirmer, A., 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.
- Schirmer, A., D. Jendrossek, and H. G. Schlegel. 1993. Degradation of poly(3-hydroxyoctanoic acid) [P(3HO)] by bacteria: purification and properties of a P(3HO) depolymerase from *Pseudomonas fluorescens* GK13. Appl. Environ. Microbiol. 59:1220–1227.
- 46a.Schlegel, H. G., G. Gottschalk, and R. von Bartha. 1961. Formation and utilization of poly-β-hydroxybutyric acid by knallgas bacteria (*Hydrogenomo-nas*). Nature (London) 191:463–465.
- Schlegel, H. G., H. Kaltwasser, and G. Gottschalk. 1961. Ein Submersverfahren zur Kultur wasserstoffoxidierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch. Mikrobiol. 38:209–222.
- Schrag, J. D., Y. Li, S. Wu, and M. Cygler. 1991. Ser-His-Glu triad forms the catalytic site of lipase from *Geotrichum candidum*. Nature (London) 351: 761–764.
- 48a.Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gramnegative bacteria. Bio/Technology 1:784–791.
- Steinbüchel, A. 1991. Polyhydroxyalkanoic acids, p. 123–213. In D. Byrom (ed.), Biomaterials. Macmillan Press, London.
- Steinbüchel, A. 1993. Thermoplastisch verformbare und biologisch abbaubare Polyester aus Bakterien: Geeignete neue Werkstoffe aus nachwachsenden Rohstoffen? CLB Chem. Labor Biotech. 44:378–384.
- Steinbüchel, A., E.-M. Debzi, R. H. Marchessault, and A. Timm. 1993. Synthesis and production of poly(3-hydroxyvaleric acid) homopolyester by *Chromobacterium violaceum*. Appl. Microbiol. Biotechnol. 39:443–449.
- Chromobacterium violaceum. Appl. Microbiol. Biotechnol. 39:443-449.
  52. Steinbüchel, A., E. Hustede, M. Liebergesell, U. Pieper, A. Timm, and H. E. Valentin. 1992. Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. FEMS Microbiol. Rev. 103:217–230.
- Stinson, M. W., and J. M. Merrick. 1974. Extracellular enzyme secretion by Pseudomonas lemoignei. J. Bacteriol. 119:152–161.
- Stroud, R. M. 1974. A family of protein cutting proteins. Sci. Am. 231:74–88.
   Tanio, T., T. Fukui, Y. 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.
- Timm, A., and A. Steinbüchel. 1990. Formation of polyesters of mediumchain-length 3-hydroxyalkanoic acids from gluconate by *Pseudomonas aeruginosa* and other fluorescent pseudomonads. Appl. Environ. Microbiol. 56:3360–3367.
- Timm, A., and A. Steinbüchel. 1992. Cloning and molecular analysis of the poly(3-hydroxyalkanoic acid) gene locus of *Pseudomonas aeruginosa* PAO1. Eur. J. Biochem. 209:15–30.
- van Tilbeurgh, H., P. Tomme, M. Claeyssens, R. Bhikhabhai, and G. Pettersson. 1986. Limited proteolysis of the cellobiohydrolase I from *Trichoderma reesei*. FEBS Lett. 204:223–227.
- Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259–268.
- Worsey, M. J., and P. A. Williams. 1975. Metabolism of toluene and the xylenes by *Pseudomonas putida (arvilla)* mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. 124:7–13.
- Yamada, K., K. Mukai, and Y. Doi. 1993. Enzymatic degradation of poly-(hydroxyalkanoates) by *Pseudomonas pickettii*. Int. J. Biol. Macromol. 15: 215–220.