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Pseudomonas lemoignei Has Five Poly(hydroxyalkanoic Acid) (PHA) Depolymerase Genes: A Comparative Study of Bacterial and Eukaryotic PHA Depolymerases*

Bernd Holger Briese,[1] Bernhard Schmidt,[2] and Dieter Jendrossek[1,3]

Four polyhydroxyalkanoate (PHA) depolymerases were purified from the culture fluid of *Pseudomonas lemoignei:* poly(3-hydroxybutyrate) (PHB), depolymerase A (M_r, 55,000), and PHB depolymerase B (M_r, 67,000) were specific for PHB and copolymers of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) as substrates. The third depolymerase additionally hydrolyzed poly(3-hydroxyvalerate) (PHV) at high rates (PHV depolymerase; M_r, 54,000). The N-terminal amino acid sequences of the three purified proteins, of a fourth partially purified depolymerase (PHB depolymerase C), and of the PHB depolymerases of *Comamonas* sp. were determined. Four PHA depolymerase genes of *P. lemoignei* (*phaZ1*, *phaZ2*, *phaZ3*, and *phaZ4*) have been cloned in *Escherichia coli*, and the nucleotide sequence of *phaZ1* has been determined recently (D. Jendrossek, B. Müller, and H. G. Schlegel, *Eur. J. Biochem.* **218,** 701-710, 1993). In this study the nucleotide sequences of *phaZ2* and *phaZ3* were determined. *PhaZ1*, *phaZ2*, and *phaZ4* were identified to encode PHB depolymerase C, PHB depolymerase B, and PHV depolymerase, respectively. *PhaZ3* coded for a novel PHB depolymerase of *P. lemoignei*, named PHB depolymerase D. None of the four genes harbored the PHB depolymerase A gene, which is predicted to be encoded by a fifth depolymerase gene of *P. lemoignei* (*phaZ5*) and which has not been cloned yet. The deduced amino acid sequences of *phaZ1-phaZ3* revealed high homologies to each other (68-72%) and medium homologies to the PHB depolymerase gene of *Alcaligenes faecalis* T_1 (25-34%). Typical leader peptide amino acid sequences, lipase consensus sequences (Gly-Xaa-Ser-Xaa-Gly), and unusually high proportions of threonine near the C terminus were found in PhaZ1, PhaZ2, and PhaZ3. Considering the biochemical data of the purified proteins and the amino acid sequences, PHA depolymerases of *P. lemoignei* are most probably serine hydrolases containing a catalytical triad of Asp, His, and Ser similar to that of lipases. A comparison of biochemical and genetic data of various eubacterial and one eukaryotic PHA depolymerases is provided also.

KEY WORDS: Biodegradable polymer; poly(3-hydroxybutyric acid); poly(3-hydroxyvaleric acid); polyhydroxyalkanoate depolymerase; *Pseudomonas lemoignei*.

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[1]Institut für Mikrobiologie der Georg-August-Universität Göttingen, Grisebachstraße 8, 37077 Göttingen, Germany.

[2]Institut für Biochemie II der Georg-August-Universität Göttingen, Göttingen, Germany.

[3]To whom correspondence should be addressed.

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INTRODUCTION

Polyhydroxyalkanoates, (PHA)[4] are polyesters of hydroxyalkanoic acids which are synthesized and accumulated intracellularly during unbalanced growth by a large variety of bacteria. They are deposited as inclusion bodies and can amount to 90% of the cell dry weight [13, 55, 57]. The monomeric composition of PHA depends on the species and the applied carbon sources (reviewed in Refs. 1, 9, 62, and 63).

The ability to degrade extracellular PHA and to use the degradation products as sources of carbon and energy depends on the secretion of specific PHA depolymerases which hydrolyze the polymer to water-soluble products [6, 7]. Aerobic and anaerobic PHB-degrading bacteria and fungi are widely distributed and have been isolated from various ecosystems such as soil, compost, aerobic and anaerobic sewage sludge, lake and marine water, estuarine sediment, and air [3a, 4–7, 25, 26, 34, 35, 37, 42, 66, 76]. Most PHA-degrading bacteria synthesize only one depolymerase. However, *Pseudomonas lemoignei* was found to have at least five PHA depolymerases: two PHB depolymerases (A and B) were described about 30 years ago [7, 33], each of which could be separated into two isoenzymes [43]. A third depolymerase (PHV depolymerase) was isolated by Müller and Jendrossek recently [41]. Genetic experiments provided evidence for the presence of two additional PHB depolymerases in *P. lemoignei* (PHB depolymerases C and D) [27].

All PHA-degrading organisms analyzed so far are restricted to the degradation of PHA, with only a little variation in the length of the carbon chain of the monomer. This specificity of the bacteria toward the polymer is attributed to the substrate specificity of their PHA depolymerases: the PHB depolymerases of *Alcaligenes faecalis*, *P. lemoignei*, and *Comamonas* sp. are specific for short-chain length hydroxyalkanoic acids (SCL-HA), such as PHB and copolymers of 3-hydroxybutyrate (3HB), 3-hydroxypropionate, and 4-hydroxybutyrate, and could not hydrolyze PHA consisting of medium-chain length hydroxyalkanoic acids (MCL-HA). The specific activity of the depolymerases decreased as the proportion of 3-hydroxyvalerate (3HV) increased in copolymers of 3HB and 3HV, and it was rather low for the homopolyester PHV [10, 26]. Only the PHV depolymerase of *P. lemoignei* hydrolyzed both homo- and copolyesters of 3HB and 3HV at high rates [41]. Recently, bacteria with the ability to degrade PHO and other MCL-PHA have been isolated [54]. The purified PHO depolymerase of *P. fluorescens* GK13 is specific for MCL-HA and does not hydrolyze PHB or similar SCL-HA.

Extracellular PHA depolymerases usually consist of a single polypeptide of relatively low molecular weight (M_r, 36,000 to 67,000). Only the PHO depolymerase of *P. fluorescens* GK13 is composed of two identical polypeptides (M_r, 26,000) [54]. The pH optima of most bacterial depolymerases are in the alkaline range, at 7.5 (*A. faecalis* [66]), 8.0 (PHB depolymerases A and B and PHV depolymerase of *P. lemoignei* [41, 43]), 9.4 (*Comamonas*, sp. [26]), and 9.5 (*C. testosteroni* [42]). Interestingly, the pH optima of eukaryotic depolymerases were in the neutral or acid range, at 7.0 (mold isolate DM2 [35]) and 6.0 (*penicillium funiculosum* [4]), and the PHB depolymerase of *P. pickettii* was found to be most active, at 5.5 [76].

Little is known about the genes and the protein structures of extracellular PHA depolymerases. Recently, 11 recombinant strains of *Escherichia coli* were isolated from a genomic library of *P. lemoignei* which expressed PHB depolymerase activity. Four clones harbored different DNA loci [27]. One of the genes (*phaZ1*) and the PHB depolymerase gene of *A. faecalis* T_1 [49] have been sequenced [27]. Both deduced amino acid sequences contain leader peptides and reveal an amino acid identity of 25% to each other. Inhibitor studies with paramethylsulfonylfluoride (PMSF) and diisopropylfluorylphosphate (DFP) have shown that both depolymerases might be serine hydrolases [33, 41, 66]. A typical lipase consensus sequence with the central serine (Gly–Xaa–Ser–Xaa–Gly) is also present in both sequences and may represent the active center of the enzyme. However, recent studies on the active center of the *A. faecalis* depolymerase by DFP inhibitor studies predict the involvement of another serine residue [51].

In this contribution we extended our genetic studies on the depolymerase genes of *P. lemoignei*. We analyzed three PHA depolymerase genes and provide evidence that the depolymerases studied belong to the serine hydrolase family.

[4] Abbreviations used: MCL-HA, medium-chain length hydroxyalkanoic acids; SCL-HA, short-chain length hydroxyalkanoic acids; 3HB, 3-hydroxybutyric acid; 3HV, 3-hydroxyvaleric acid; PAS, periodic acid–Schiff reagent; PHA, poly(hydroxyalkanoic acid); PHB, poly(3-hydroxybutyric acid); PHV, poly(3-hydroxyvaleric acid); PHO, poly(3-hydroxyoctanoic acid); PNPB, paranitrophenylbutyrate; PMSF, paramethylsulfonylfluoride; DFP, diisopropylfluorylphosphate, LB, Luria–Bertani broth; NB, nutrient broth; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Growth Conditions

All strains and plasmids used in this study are listed in Table I. Bacteria were grown in mineral salts medium [56] or a complex medium such as Luria-Bertani broth (LB) [52] or nutrient broth (NB; 0.8%, w/v) at 30 or 37°C. For accumulation of PHA the concentration of NH_4Cl was reduced to 0.05% (w/v). Carbon sources (PHB and sodium salts of gluconate, valerate, and 3HB) were applied from stock solutions of 3 to 20% (w/v). Solid media were prepared by the addition of 1.4% (w/v) agar.

Table I. Bacterial Strains and Plasmids Used in this Study

Strain or plasmid	Relevant characteristic	Source or reference
Alcaligenes eutrophus H16	Source of PHB	DSM428, ATCC17699
A. faecalis T_1	Growth on PHB	[66]
Chromobacterium violaceum	Source of PHV	DSM30191 [64]
Comamonas sp.	Growth on PHB	DSM6781 [26]
Pseudomonas lemoignei	Growth on PHB and PHV	LMG2207 [7]
Escherichia coli JM83 (pSK479)	*phaZ2*	This study, [27]
Escherichia coli JM83 (pSK480)	*phaZ1*	This study, [27]
Escherichia coli JM83 (pSK487)	*phaZ3*	This study, [27]
Escherichia coli JM83 (pSK612)	*phaZ4*	This study, [27]

Preparation of PHA Granules

The homopolyesters PHB and PHV were isolated from gluconate-grown cells of *A. eutrophus* H16 and valerate-grown cells of *Chromobacterium violaceum*, respectively, by sodium hypochlorite treatment and purification by extraction with acetone/diethyl ether as described previously [26, 41, 64].

Assays of PHB and PHV Depolymerase

A quick and simple procedure for estimating the activities of PHB depolymerase and PHV depolymerase was performed by a spot test on indicator plates: 3 ml hot liquid medium containing 0.2–0.6% (w/v) sonicated PHB or PHV granules and 1.4% (w/v) agar in 100 m*M* Tris–HCl, pH 8.0, was poured onto prewarmed (37°C) glass slides (76 × 26 mm). After solidification of the agar 2–4 μl enzyme solution was dropped onto the surface and incubated at 45°C for several hours. The diameters of the resulting cleared zones indicated semiquantitatively the activities of the depolymerases.

DNA Sequence Analysis

DNA sequencing was performed by the dideoxy-chain termination method [53] using alkaline-denatured double-stranded plasmid DNA. A T7-polymerase sequencing kit from Pharmacia-LKB (Uppsala, Sweden) was used with [^{35}S]dATP[αS]. (G + C)-rich regions were additionally sequenced using 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) [8] using sequence databases of Gene Bank, EMBL, and Swissport (release: spring 1993).

Hybridization Experiments

Southern blots of agarose-gel electrophoretically separated DNA restriction fragments on positively charged Nylon membranes (Pall Filtrationstechnik, Dreieich, Germany) were hybridized with a ^{32}P-labeled oligonucleotide mixture [5′-(AG)TT(AGCT)GG(AGCT)GT(AGCT)CC(AG)TA-3′] synthesized in a Gene Assembler Plus apparatus (Pharmacia-LKB) according to the protocol of the manufacturer [52].

Purification of Proteins

The purification of PHA depolymerases from *P. lemoignei* was performed from the culture fluid of (i) succinate-growth cells and (ii) valerate-grown cells as described recently [41]. The purification of the PHB depolymerase from *Comamonas* sp. was performed from the culture fluid of P(3HB)-grown cells according to the method of Jendrossek *et al.* [26].

For analysis of recombinant *E. coli* clones in liquid culture, bacteria were grown at 37°C in 300-ml flasks containing 50 ml mineral medium, 0.5% (v/v) glycerol, 0.01% (w/v) proline, 0.005% (w/v) thiamine, and 50 μg ampicillin/ml. At the end of exponential growth cells were centrifugated (10 min, 5000*g*, 4°C), and the supernatant was concentrated about 20-fold in dialysis bags

against solid sucrose and dialyzed against 50 m*M* Tris-HCl buffer, pH 7.5.

Determination of N-Terminal Amino Acid Sequences

Forty to one hundred micrograms of purified depolymerase protein was separated by denaturing SDS-PAGE, blotted to PVDF membranes (Millipore, Eschborn, Germany), and stained with Coomassie blue. Areas of deep color were cut out and subjected to an automated Edman degradation using a 477A pulsed liquid phase protein/peptide sequencer and a 120A online PTA amino acid analyzer (both from Applied Biosystems, Waiterstadt, Germany) according to the manufacturer's instructions.

Gel Electrophoresis

Proteins were separated by native PAGE [61] or by denaturing SDS-PAGE [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 α-lactalbumin (M_r, 14,400) were used as molecular mass standard proteins. After electrophoresis, proteins were silver-stained [3] or subjected to glycoprotein staining. For glycoprotein staining sucrose and glycerol of the loading solution were replaced by urea (80%, w/v).

Glycoprotein Staining

Staining for glycoproteins was performed using three methods. (i) Proteins were separated gel electrophoretically, and the gel was subsequently subjected to periodic acid–Schiff reagent (PAS) staining, according to the method described by Segrest and Jackson [59]. Thyroglobulin and ovalbumin served as positive control, and bovine serum albumin (BSA) as negative control. (ii) Proteins were separated gel electrophoretically and Western-blotted on PVDF membranes [69]. Detection of glycoproteins was performed using a DIG glycan detection kit (Boehringer, Mannheim, Germany) according to the manufacturer's instructions, with transferrin and creatinase as positive and negative controls, respectively. (iii) Proteins were subjected to Ouchterlony double-diffusion tests according to the method described by Oakley [44] using concanavalin A as the antibody-like component. Thyroglobulin and ovalbumin served as positive controls, and BSA as negative control.

RESULTS

N-Terminal Amino Acid Sequences of PHA Depolymerases from *Pseudomonas lemoignei*

The N-terminal amino acid sequences of various PHA depolymerases were determined by Edman degradation of the purified proteins. (i) PHB depolymerase A of *Pseudomonas lemoignei* was purified from succinate-grown cells. Interestingly, the 29 identified amino acids of the amino end of PHB depolymerase A matched the corresponding region of the PHB depolymerase of *Alcaligenes faecalis* [49] except Phe_{27} and Tyr_{28}, which were changed to Tyr_{27} and Phe_{28} in *A. faecalis* (Fig. 1). (ii) The PHV depolymerase of *P. lemoignei* was purified from valerate-grown cells as described previously [41]. The N-terminal amino acid sequence revealed no homologies to any other depolymerase listed in Fig. 1. (iii) While the synthesis of PHB depolymerase A is induced during growth on succinate and that of PHV depolymerase is induced by valerate, the synthesis of PHB depolymerase B occurs during growth on both substrates [41]. Since two very similar isoenzymes of PHB depolymerase B have been described [43], we isolated PHB depolymerase B from both succinate-grown cells and valerate-grown cells and determined the N-terminal amino acid sequences to elucidate whether both proteins were related or not. The 36 identified amino acids of both PHB depolymerase B proteins were identical but were clearly different from the N-terminal sequences of the other depolymerases (Fig. 1). We assumed that both preparations of PHB depolymerase represent PHB depolymerase B of *P. lemoignei*. (iv) However, when we determined the N-terminal sequence of a contaminating protein of partially purified PHB depolymerase B, 14 of 20 amino acids of this protein (70%) were identical to the sequence of PHB depolymerase B, and no significant homologies were found to any of the other depolymerases. We assumed that this protein represents a PHB depolymerase related to PHB depolymerase B. This partially purified protein was tentatively designated PHB depolymerase C. (v) The N-terminal amino acid sequence of the PHB depolymerase purified from *Comamonas* sp. [26] was not related to any of the other depolymerases listed in Fig. 1.

Analysis of PHB Depolymerase Genes of *P. lemoignei* and Determination of the Nucleotide Sequence

Recently, we described the cloning of four PHA depolymerase genes from *P. lemoignei* [27]. The nu-

	1 . 10 . 20 . 30 36
P. lemoignei PHB depolymerase A	ATAGPGAWSSQQTWAADS?NGGNLTGFYY
A. faecalis PHB depolymerase	ATAGPGAWSSQQTWAADSVNGGNLTGYFY
P. lemoignei PHB depolymerase B (from succinate-grown cells)	ATQVTGFGSNPGNLLMYKHVPSSMPANAPLVI?MHG
P. lemoignei PHB Depolymerase B (from valerate-grown cells)	ATQVTGFGSNPGNLLMYKHVPSSMPANAPLVIAMHG
P. lemoignei PHB depolymerase C	LTQVSNFGTNPGNLQMFKHV
P. lemoignei PHV Depolymerase	LTPGSGAWVKESATYGTPNLQ
Comamonas sp. PHB depolymerase	AVPLGQYNIATDQI(S)V(G)GL

Fig. 1. N-terminal amino acid sequences of PHA depolymerases. The amino acid sequences were determined by automated Edman degradation of the purified proteins. Unidentified amino acids are given in parentheses or by question marks. The sequence from *A. faecalis* enzyme was taken from Saito *et al.* [49].

cleotide sequence of one of these genes, *phaZ1*, was determined. When we compared the amino acid sequence deduced from the nucleotide sequence of *phaZ1* to the N-terminal amino acid sequences of the proteins described above, we found complete identity of amino acid Leu_{38} to Val_{58} to the 20 N-terminal amino acids of partially purified PHB depolymerase C. Therefore, *phaZ1* is the structural gene of PHB depolymerase C. In addition, this result confirmed the accuracy of the proposed leader peptide cleavage site for the prepeptide of *phaZ1* [27].

In this study, two additional PHB depolymerase genes, *phaZ2* and *phaZ3*, were analyzed. Both genes were expressed in *E. coli*, and PHB depolymerase activity was found in the culture fluid as indicated by the drop test on PHB indicator slides (Fig. 2). Separation of the extracellular proteins of the concentrated culture fluid of recombinant *E. coli* harboring *phaZ2* or *phaZ3*, respectively, by SDS-PAGE revealed the presence of an additional band of M_r 41,000 ± 3000 compared to the control of both clones. The nucleotide sequences of *phaZ2* and *phaZ3* were determined (Figs. 3A and B). Two open reading frames, of 1302 and 1260 bp, were identified for *phaZ2* and *phaZ3*, respectively, which (i) exhibited a bias for G or C in the third codon position, (ii) revealed a codon usage similar to that of *phaZ1* [27], and (iii) were preceded by typical Shine–Dalgarno sequences (Figs. 3A and B). The regions 200 bp upstream of *phaZ2* and *phaZ3* are very rich in (A + T) and contain sequences similar to σ^{70}-dependent promoters of *E. coli* or the *alg* promoters of *P. aeruginosa* [29]. Downstream of *phaZ2* and *phaZ3* potential hairpin structures of the deduced transcribed RNA were identified which contain seven or six uracil residues at their 3′ ends and may represent factor-independent termination signals [47] (Figs. 3A and B).

PhaZ2 and *phaZ3* code for putative proteins of 433 amino acids, M_r 45,600, or 419 amino acids, M_r 43,900, respectively. The N termini of the deduced amino acid sequence contain three (*phaZ2*) or two (*phaZ3*) positively charged amino acids followed by a series of mainly hydrophobic amino acids. This pattern is typical for signal peptides, and a leader peptidase cleavage site is predicted between Ala_{37} and Ala_{38} (PhaZ2) and between Ala_{37} and Leu_{38} (PhaZ3) [48, 72]. The M_r of the putative mature proteins was 41,800 (PhaZ2) and 41,200 (PhaZ3). All 36 identified amino acids of the N terminus of PHB depolymerase B were identical to the deduced amino acids Ala_{38} to Gly_{73} of *phaZ2* (Fig. 4). This indicated that *phaZ2* is the PHB depolymerase B structural gene of *P. lemoignei* and confirmed the accuracy of the predicted leader peptidase cleavage site (Fig. 4). No identity of the deduced amino acid sequence of *phaZ3* with the N-terminal sequences of other PHA depolymerases listed in Table II was found. However, PhaZ1, PhaZ2, and PhaZ3 revealed a high amino acid identity, 68 to 72%, to each other and a medium identity, 25 to 34%, to the depolymerase of *A. faecalis*.

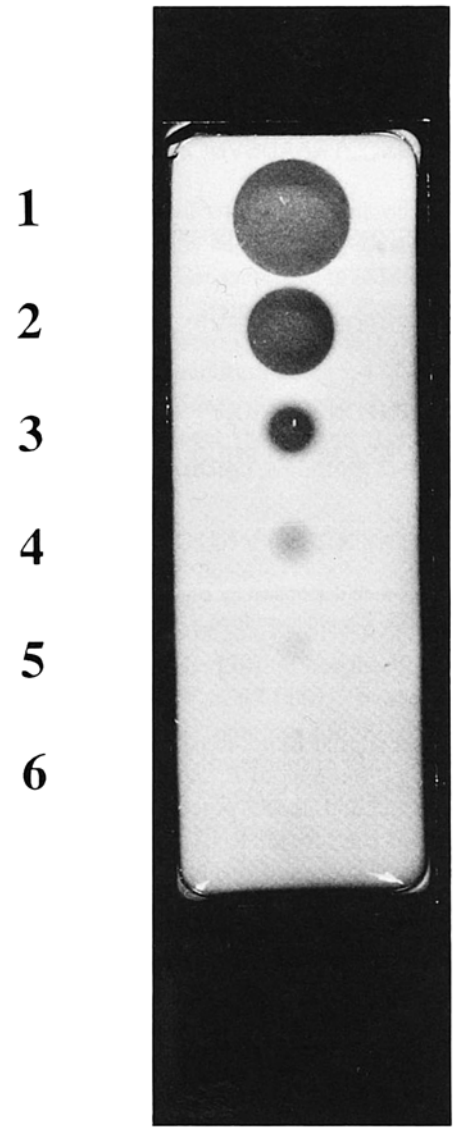

Fig. 2. PHB depolymerase drop test of the *phaZ3* gene product of *E. coli*. Two microliters of the concentrated culture fluid of *E. coli* (pSK487) (1) and 2 μl of five subsequent 1 to 2 dilutions (2 to 6) (in 10 m*M* Tris-HCl, pH 8) were dropped on the surface of a PHB depolymerase indicator slide and incubated overnight at 45°C.

We concluded that *phaZ3* encodes a PHB depolymerase, which is closely related to PHB depolymerases B and C of *P. lemoignei*. This depolymerase is tentatively designated PHB depolymerase D.

Southern blots of four recombinant plasmids harboring *phaZ1*, *phaZ2*, *phaZ3*, or *phaZ4* were hybridized with a ^{32}P-labeled oligonucleotide probe which was deduced from Tyr_{15} to Asn_{19} of the N terminus of the PHV depolymerase (noncoding strand). Only the plasmid harboring *phaZ4* hybridized with the probe and indicated that *phaZ4* is the PHV depolymerase structural gene. Sequencing of *phaZ4* is in progress.

As has been described for PhaZ1 [27], the amino acid compositions of the putative mature proteins of PhaZ2 (M_r, 41,800) and PhaZ3 (M_r, 41,200) also contain an unusual high portion of 60 and 49 threonine molecules, respectively (corresponding to 15 and 13 mol%, respectively), 27 (PhaZ2) or 22 (PhaZ3) of which are clustered near the C terminus in four repetitions of four to six threonine molecules (Fig. 4). These threonine residues are part of an approximately 40-amino acid-long sequence consisting of amino acids with characteristic side chains: They are all uncharged (with the exception of two lysine residues in PhaZ2), are unbranched, and either are hydroxylated, such as threonine, serine, and tyrosine, or are very small, such as glycine and alanine, or are hydrophobic, such as valine and phenylalanine. Other interesting features of the primary structures of PhaZ2 and PhaZ3 are (i) the presence of the same lipase consensus sequences (Gly-Leu-Ser-Ala-Gly [24, 46, 58, 73, 74]); (ii) the low fraction of charged amino acids such as Arg, Asp, Glu, Lys, and His (57 amino acids corresponding to 14 mol% and 57 amino acids corresponding to 15 mol%, respectively); and (iii) the high fractions of hydrophobic amino acids such as Ala, Ile Met, Leu, Phe, Pro, Trp, and Val (123 amino acids corresponding to 31 mol% and 134 amino acids corresponding to 34 mol%, respectively) and of amino acids in the amide form (Asn and Gln; 37 amino acids corresponding to 9 mol% and 32 corresponding to 8 mol%, respectively). The distribution of hydrophilic and hydrophobic amino acids results in a relatively hydrophobic protein with three major hydrophilic parts.

Glycoprotein Staining of Purified PHA Depolymerases from *Pseudomonas lemoignei*

PhaZ2 was identified to encode the PHB depolymerase B gene of *P. lemoignei*. The relative molecular mass, M_r, of the deduced mature protein amounts to 41,800 and is significantly lower than that which had been determined by SDS gel electrophoresis of the purified protein (67,000 [41]). We supposed that additional components could be bound to the polypeptide. Since glycosylation has been described for many extracellular proteins of eukaryotes (recently reviewed by Lis and Sharon [32]) and some extracellular proteins of bacteria (reviewed by Lechner and Wieland [31]) including the PHB depolymerase of *Penicillium funiculosum* [4], we subjected the purified PHB depolymerases A and B and

A GATCACAGTACGCAGCCGGTTTTCAATCATAACCTCAATATCCGTGAGGTGAAAGATTGTGGCGGTATTGCAACAAGATTTTACAAGAGCGAAAAGCCA 100
S/D *phaZ2*
ACCCTTAAAAGTAGATCACATAAAAAATATTC**AGGAGA**CATTT**ATG**ATGTCAAGTCAGACCACACAATCTTCGAAATTCTCGCTGTTCCTCAAGCGCGGG 200
CTTCTCCTTGCGGCAGCACCCTTGCTGGCAATGAGCGCATCCTCGGCTCTCGCTGCGACCCAGGTAACCGGCTTCGGCTCGAACCCCGGCAATCTTCTGA 300
TGTACAAGCATGTGCCGTCCAGCATGCCGGCCAATGCGCCGCTGGTCATCGCCATGCATGGTTGCACCCAGAGCGCCTCTGCCTATGAAGCCACCGGCTG 400
GACCCAGCTCGCCAACACCTACAAGTTCTATGTGGTGTATCCGGAACAGCAAAGCAGCAATAATCAGAACAAGTGCTTCAACTGGTTCGAGCCCGGCGAC 500
ATCGCGCGCGGCCAGGGCGAAGCCCTGTCGATCAAGCAAATGGTCGACAAGATGAAGGCAGACCACTCGATCGATACGAATCGTGTGTATGTCACAGGCC 600
TGTCGGCGGGCGGTTACATGGTCAACGTCATGTTGGCTACTTATCCCGACGTCTTTGCCGGCGGCGCGCCTTTTTCGGGCGGTCCGTACAACTGCGCGAC 700
TTCGATGACCAATGCGTTCACCTGCATGAGCCCGGGCGTGGATAAAACTCCGGCTGCCTGGGGCGACCTGGCGCGCGGCGGCTACAGCGGCTATACCGGC 800
CGTAAGCCGATCGTATCGATCTGGCACGGCGATGCTGACTATACCGTCAAGCAATCCAATCAGGTCGAAGAAGTGGAGCAGTGGACCAACTACCATGGCA 900
TCGACCAAACCGCAGACGTAAGCGACACGGTGGCCGGCTTCCCGCACAAGGTGTACAAGGATGCCAGCGGTAATGCGCTCGTGGAAACCTATACCATCAC 1000
CGGCATGGGTCATGGCACTCCGGTTGATCCGGGCACCGGCTCCCTGCAATGCGGCACCGCTGGCGCCTATATCCTCGATGTGAATATCTGCTCGAGCTAT 1100
TACGTTGCCAAGTTCTGGGGATTGATCGGCGGTAGCGGCACCACCACGACCACGTCGGCTGGCACCACTACGACTACGTCGGCAGGAACCACCACGACTA 1200
AGGCTTCAACCACGACCACCAAGGTTTCCACTACAACTACTGCATCCACCACGACGACAACTGCAGGCGCTTGCTACAACTCGAGCAACTACGCGCACGT 1300
GACGGCTGGTCGTGCTCATGACACCGGTGGCTATGCATATACCAACGGTTCGAACCAGAAGATGGGGCTGAACAACACCTTCTACACCAGCAAGCTGCGT 1400
AAGACCGGTACCAATTACTACGTCATCGACACTACTTGCCCG**TAA**TAGGCAACATGGAGCGCGCTGTTGCGGCGCGCACCAAAGCAAAGCGGATCCAATG 1500
GATCCGCTTTTTTATGCCAAGTTACCGGTGGTATGGGGCGACAACACTTTAAAAGTGGCCCGGCTATCTGTCTGCCACTCGATAAAGCTTTATGGTAGT 1600
TAAATAGTTTTGCTAGCCGTGATGTCGTGTTGAGTTGTGATAGCCGGCGCAGGCAAAATTTCACCGACCAGGAATTCGGGGCGGCCTTTGATTTCATCGA 1700
AGATCTGCGCCAGATACGCGCCCAGGATACCGATGCCAAGCAGGTTGGCGGAACCGAAGAACAGCACGACCACAGTCATCGATGTCCATCCCGACACGGC 1800
GACGTGCGTAAACCACGAGTAGACCGCCTGCAGTAATAGCAGGAAAGCAGCCAGCATGCCGAACAGGCCGAGACGGAACACGATCGACAGCGGCTTGGCG 1900
CTATAGGAAGTAATCGCCGTGACGGCTAGGCGCATCAGCTGCGCTGTGCTCCACTGGCTGCTGCCGGCGATCCGTGGCGCCACATCGAAGGCCAAGGGCA 2000
CCGTGGTAAATCCCGTCCAAACGGTCATGCCGCGGAAGAAGCGAATCTTTTCCGGGAAAGCCAGCAGCGTATCGACCACGCGCCGGTCCAACAGCCGAAA 2100
ATCGGAGGCGCCTTCGAGATC 2121

B GATCCTGCGGCAATCCATTGTCGCGCGCAAAATAGTTGAATGTACCCTTGGGAATGACGCCGAATGGCAGGCCGGTACTGATGACTTGTTGCGCGACGGT 100
GCGGATCGTGCCATCGCCGCCTGCCGCGACGATGACGCCGTTATGGGAGCCGGCAAGTTCGACCGCGCGCCTGATCAGGGAACTCAAGTCCCTGCTTTTC 200
CGGCACAGGAGTATTTCACCGGGATGGCCTGCTTCGCGAAGCTGTCGGTCGATTGCGTCTCGCGCGGCATCGGCGTTTCTGCGGCCTGAGCCGGCATTCA 300
TGACTATGAACAGCGGGCGGTGCTCTTCTCCGGCAGAGGCAATGGTTTCCCCGGGGTGGAAGCCTGATGCCTGAACCTCATTCCGGTCAGAAGATGTCGC 400
CATTGAAATCCTTTAGAGAAATTTCCAACCAGCTCATACATTGCCGGGTTGGCAAACAACGTCGGCTTACTTGGATTGGAGTATTTTTGGCAATAGAAAA 500
TTCCATTCGTTACTGTCAAAAAATACAGCCAATACCGAAGGGACTAAGCACGGTAAAAATTGTTAATTTTTTTAAAGTTTGACTGGCCGAAATGCCAGT 600
TTTCCGGCTGGCAACTAGTCGCTAATATTGCTCCAATTTCTTCGCAGCAACACAGCATGAACGTATATAAATGAGCAGCGAAGAATCAACCGAACGCCCA 700
S/D *phaZ3*
TT**GGGGGAGA**C**ATG**AATAAATATCTGAAAAATCTTTGCTTTGCCGCTGCAACCGTAACCCTGATGGCCTCCGCACCTTCGGCCTTCGCGCTGAGCGAAGT 800
GACCGGCTTCGGCACCAATCCTGGTGCGCTGAAGATGTTCAAGCATGTGCCGACCAGCATGCCGACCAATGCGCCGCTGATCGTGGCGATGCACGGCTGC 900
ACCCAATCGGCGTCGGCGTATGAAGGCAGCGGCTGGAGCGCGCTGGCCAACAATTACAAGTTCTACGTCGTCTATCCGGAGCAGCAAAGCGGCAACAATT 1000
CCAACAAGTGCTTCAACTGGTTCGAATCGGGTGATATCGCGCGCGGCCAGGGCGAAGCCTTGTCGATCAAGCAAATGGTCGACAAGATGAAGGCGGATTA 1100
CAGCATCGATGCCAATCGCGTCTACGTTACCGGCTTGTCGGCCGGCGCGTTCATGACGGCAGTCATGGCGGCGACTTATCCGGATGTGTTTGCCGGCGCC 1200
GCGCCGATTGCCGGCGGGCCCTACAAGTGCGCTACCTCGATGATCGATGCATTCAGCTGCATGAGCCCGGGTACCGACAAGACTCCTGCTGCCTGGGGCG 1300
ATCTGGCGCGCGGCGGTTATTCAGGTTACAACGGCCGCAAGCCGAAGATCTCGGTCTGGCAGGGCTCGTCCGATACCACGGTCAAGCCGATGAACATGGA 1400
TGAGTTGATGCAGCAGTGGACCAACTACCATGGCATCGACCAGACCGCCGACGTCAGCGAGACCGTCAAGGGCTTCCCGCACAAGGTTTACAAGGATGCA 1500
TCCGGTAACGCCCTGGTGGAAACCTGGTCGATCACCGGCATGGCGCATGGCACGCCGGTCGATCCGGGCACCGGTGCCGAACAGTGCGGTACCTCCGGTT 1600
CCTACATCCTCGATGTGAACATCTGCTCCAGCTACCACATCGCCCAGTTCTTCGGCCTGACCGGCGCCGGCACCACGACCACCACGACCGTGGGTTCGAC 1700
CAGCACCACCACCGGCTACACCAGCACGTCCAGCGCGCCGGTAACCACGACGACAAGCGTGGCCAGCACGACAACTACGACGGTGGCAGCCGGTGCTTGC 1800
TACAACGCCAGCAACTATGCCCACGTGACCGCCGGCCGTGCAGTCAACAGCATGGGGTATGCGAAGGCCAAAGGCTCGAACCAGAACATGGGCTTGTATA 1900
ACACCTTCACGACGTCCAAGCTGCGTGAAGCGCCGGCAGGCTACTTCACCATCGACAGTACCTGCCCG**TAA**CCTTATAACAGGCTCGAATCGCGGGAGAC 2000
GTTCCCGCCTTTACAACGGACCTTCGGGTCCGTTTTTATGGAAACGACTTACTTATGAATTTTTTGTTGCTGATTCGCTTTATCGCTCCCTGAAAGTGA 2100
TGACAAATCCTTGCGCATCTTCTTCAGGTAGGCGAGTATCGACTGTATAACGATAGGCCCGGCAGGAGTGCATCGAATGCGTCTGCAGGCAGCAAGCGTA 2200
AAACAACAATGGCAGGAGAGGGACGATGTATAACGTTCAGCAGGAAGTCGACGAGCGCACGCGGCTGGTCGGTCAGAACAAGTTTGAATTGATGCTGTTC 2300
AGGCTCGGTAATGCGGGGAGCACCGGGCAGAGCGTGCTCCATGGGATCAACGTTTTCAAGATCCGCGAACTGCTCGACATGGCAAGCGTGCAGGTCACGC 2400
CCATCGCCGGTTCGCCGCCTTTCATCATGGGCGTGGTGAACGTGCGCGGCCAGATC 2456

Fig. 3. Nucleotide sequences of *phaZ2*(A) and *phaZ3*(B). Putative Shine Dalgarno sequences and the assumed start codons are given in boldface letters, and putative termination signals of translation are indicated by arrows.

the PHV depolymerase of *P. lemoignei* to staining tests for glycoproteins: None of the three purified proteins reacted with concanavalin A. Since concanavalin A has a pronounced preference for α-D-mannopyranoses and α-D-glucopyranoses (reviewed by Goldstein and Hayes [18]), the depolymerases apparently contain other compounds. Therefore, a less specific staining with PAS was used. When 7 to 15 μg of purified protein of the three

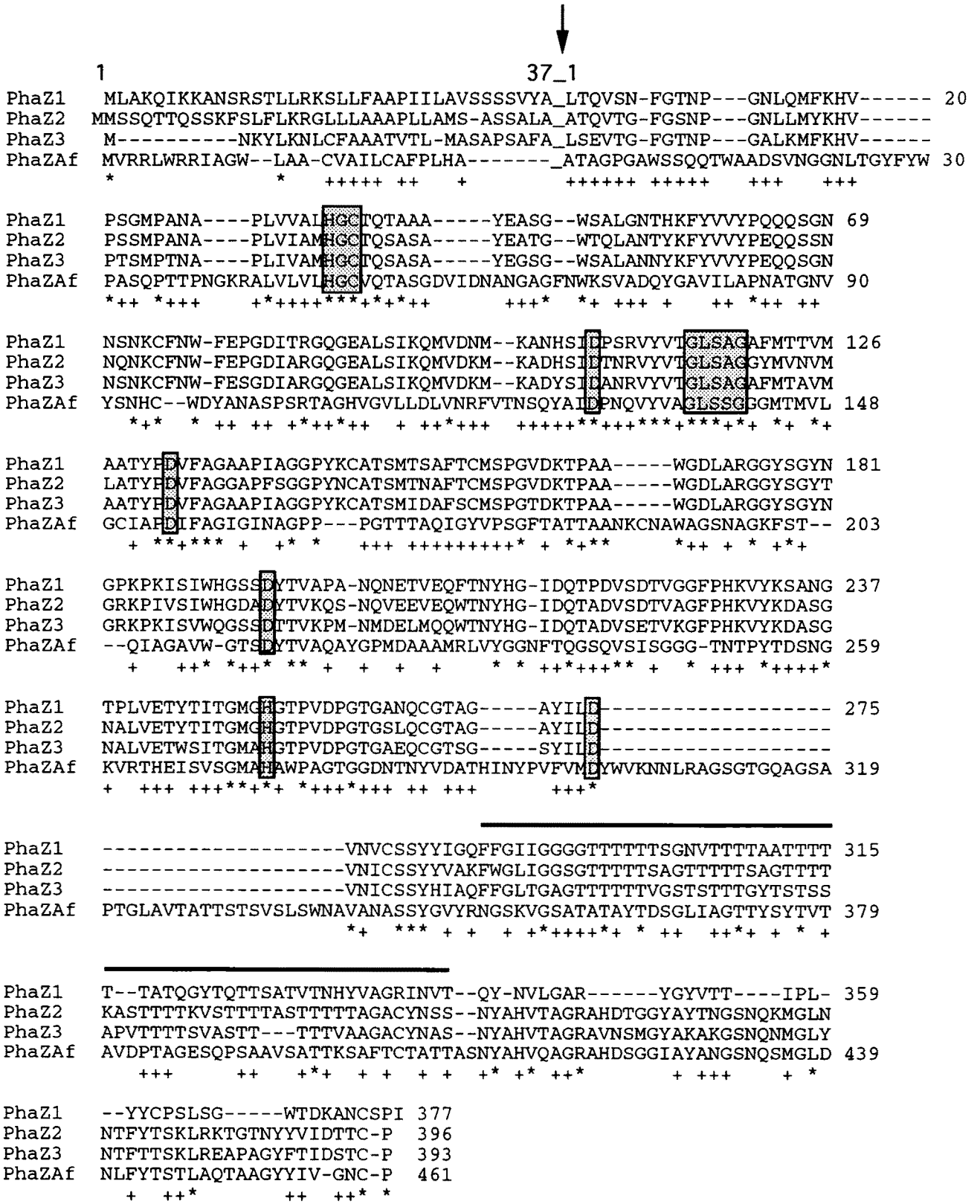

Fig. 4. Alignment of the deduced amino acid sequences of the PHB depolymerases. PhaZ1 [27], PhaZ2, and PhaZ3 of *P. lemoignei* and of the PHB depolymerase of *A. faecalis* ($PhaZ_{Af}$) [49]. The predicted leader peptidase cleavage sites are indicated by an arrow. Amino acids of PHB depolymerase B (PhaZ2) and of PHB depolymerase C, which have been determined by Edman degradation, are marked by boldface letters. The positions of amino acids conserved in all sequences and those of similar amino acids are marked by * and +, respectively. Lipase boxes and other conserved amino acids that might constitute a catalytic triad are boxed. The position of the threonine-rich regions is indicated by a black bar.

Table II. Characteristics of PHA Depolymerases[a]

	PHB depolymerase$_E$						PHV depolymerase$_E$	PHB depolymerase$_E$		PHO depolymerase$_E$	PHO depolymerase$_I$	
	Comamonas sp.	*C. testosteroni*	A, *P. lemoignei*	B, *P. lemoignei*	C, *P. lemoignei*	D, *P. lemoignei*	*P. lemoignei*	*A. faecalis*	*P. funiculosum*	*P. fluorescens*	*P. oleovorans*	*P. aerigunosa*
Reference(s)	[26]	[42]	[33, 41, 43]	[33, 41, 43]	[27]	[27]	[41]	[49, 51, 49]	[4]	[54]	[23]	[68]
Protein	$PhaZ_{Csp}$	$PhaZ_{Cte}$	$PhaZ5_{Ple}$	$PhaZ2_{Ple}$	$PhaZ1_{Ple}$	$PhaZ3_{Ple}$	$PhaZ4_{Ple}$	$PhaZ_{Afa}$	$PhaZ_{Pfu}$	$PhaZ_{Pfl}$	$PhaZ_{Pol}$	$PhaZ_{Pae}$
Regulation	+	nd	+	+	nd	nd	+	+	nd	+	nd	nd
M_r (native protein)	45,000	nd	55,000	58,000	nd	nd	53,500	48,000	38,000	48,000	nd	nd
M_r (SDS-PAGE)	44,000	50,000	54,000	67,000	nd	nd	54,000	50,000	36,000	25,000	nd	nd
Glycosylation	−	nd	+	+	nd	nd	±	nd	+	±	nd	nd
Relative activity												
PHB	100	100	100	100	100[b]	nd	100	100	nd	<2	nd	nd
PHV	2	nd	2	6	15[b]	nd	30	nd	nd	<2	nd	nd
P(3HB-*co*-3HV) (19% 3HV)	nd	≈30	84		160[b]	nd	150	<100	nd	<2	nd	nd
PHO	<2	nd	<2	<2	<2[b]	nd	<2	nd	nd	100	nd	nd
Esterase activity	−	nd	±	nd	+[b]	nd	+	nd	nd	++	nd	nd
Lipase activity	−	nd	−	nd	nd	nd	−	nd	nd	−	nd	nd
T opt(°C)	31	nd	≈65	≈50	≈62[b]	nd	≈55	nd	nd	30–37 (45)[c]	nd	nd
pH opt	9.4	9.5	8.0	8.0	nd	nd	8.0	7.5	6.0	8.6	nd	nd
Products	M	nd	M, D, T	M, D, T	nd	nd	M, O	D	nd	M, D	nd	nd
Inhibitor												
PMSF	−	+	+	nd	±[b]	nd	±	++	±	−	nd	nd
DFP	nd	+	nd	nd	nd	nd	nd	++	++	nd	nd	nd
DTT	++	+	++	++	nd	nd	++	nd	++	−	nd	nd
Gene	$phaZ_{Csp}$	$phaZ_{Cte}$	$phaZ5_{Ple}$	$phaZ2_{Ple}$	$phaZ1_{Ple}$	$phaZ3_{Ple}$	$phaZ4_{Ple}$	$phaZ_{Afa}$	$phaZ_{Pfu}$	$PhaZ_{Pfl}$	$phaZ_{Pol}$	$phaZ_{Pae}$
Cloning	+	nd	−	+	+	+	+	+	−	+	+	+
Sequence (bp)	nd	nd	nd	1299	1242	1257	nd	1383	nd	nd	849	855
Deduced protein												
Length (aa)	nd	nd	nd	433	414	419	nd	488	nd	nd	283	285
Leader peptide (amino acids)	nd	nd	nd	37	37	26	nd	27	nd		−	−
M_r (deduced mature protein)	nd	nd	nd	41,800	39,500	41,200	nd	47,000	nd	nd	31,500	31,400
Lipase box (G-x-S-x-G)	nd	nd	nd	G-L-S-A-G	G-L-S-A-G	G-L-S-A-G	nd	G-L-S-S-G	nd	nd	G-V-S-W-G	G-V-S-W-G
Threonine-rich region	nd	nd	nd	+	+	+	nd	±	nd	nd	−	−

[a] Not determined (nd); present (+); not present (−); uncertain (±); monomer (M); dimer (D); oligomer (O); intracellular (subscript I); extracellular (subscript E); optimum (opt); base pairs (bp).

[b] Values from protein of recombinant *E. coli*.

[c] Measured with paranitrophenyloctanoate.

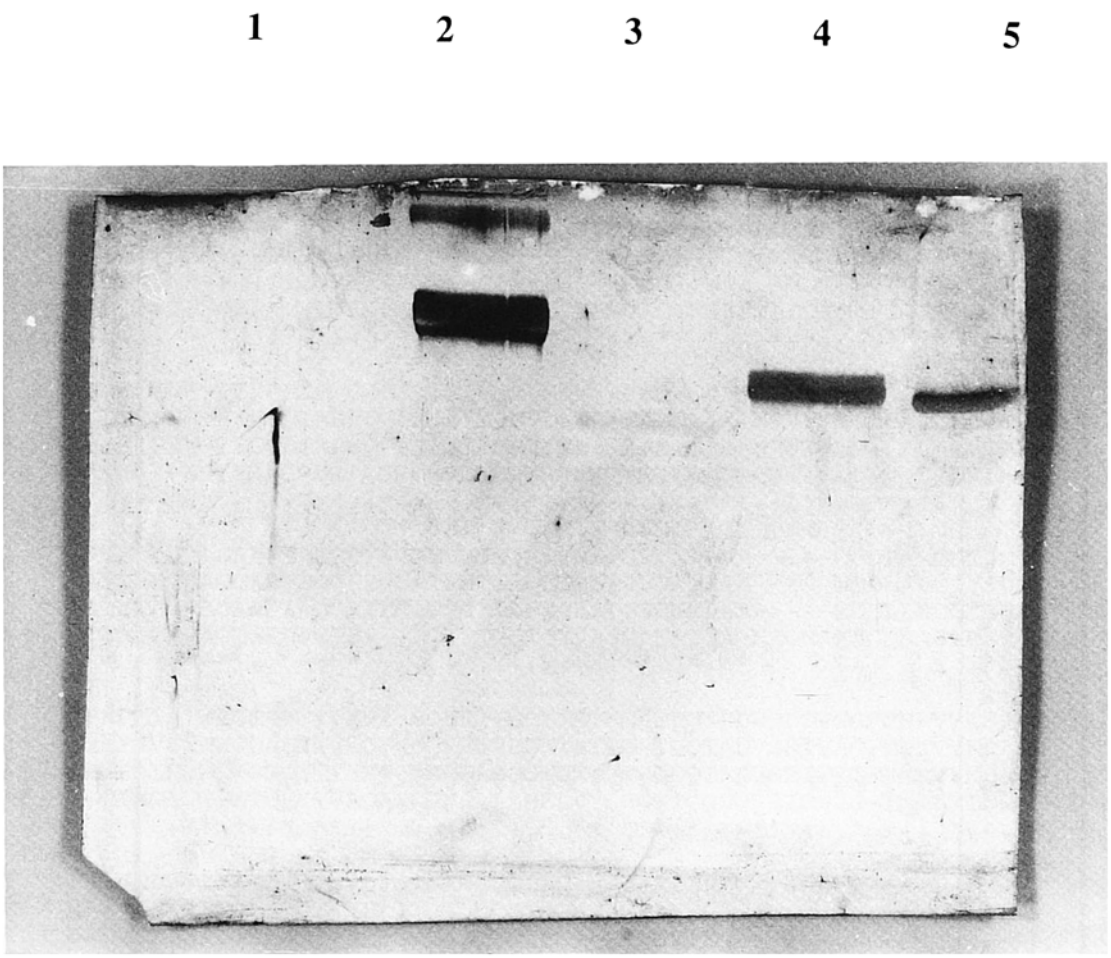

Fig. 5. Glycoprotein staining of PHB depolymerases. The purified proteins (≈ 5 μg) were separated by SDS-PAGE and were Western-blotted onto PVDF membranes. The proteins were oxidized by periodic acid and subjected to a coupled digoxygenin immunostaining. Creatinase (lane 1) and transferrin (lane 2) served as negative and positive controls, respectively. PHV depolymerase (lane 3); PHB depolymerase B (lane 4); PHB depolymerase A (lane 5). Although most of the signals appeared during the first 5 min of the visualization reaction, the staining was performed for 1 h to allow the detection of weak signals (lane 3).

depolymerases was PAS-stained in SDS-PAGE gels, they all gave a positive reaction. However, high amounts (50 μg) of the negative control, BSA, also gave a weak positive reaction. The PAS staining was then repeated with Western blots of the proteins separated by SDS-PAGE by using the more sensitive DIG detection kit. PHB depolymerases A and B as well as transferrin as the positive control gave a positive reaction within seconds (Fig. 5). The reaction of the PHV depolymerase was less pronounced: a faint band appeared after 1 h of incubation. Creatinase, the negative control, was negative even after a prolonged incubation time. We concluded that PHB depolymerases A and B contain compounds with vicinal hydroxyl groups. These compounds are most probably carbohydrates and are assumed to be responsible for the difference between the predicted M_r (41,800) and the experimentally determined value (67,000). Whether or not the PHV depolymerase is modified remained uncertain.

DISCUSSION

Four PHA depolymerase genes, *phaZ1* to *phaZ4*, have been cloned from *Pseudomonas lemoignei*, and *phaZ1* as well as its gene product has been characterized in a previous study [27]. In this study the *P. lemoignei* PHA depolymerase proteins and the depolymerase genes

phaZ2, *phaZ3*, and *phaZ4* were analyzed. The nucleotide sequences of *phaZ2* and *phaZ3* were determined, and *phaZ2* and *phaZ4* were identified to be the structural genes of PHB depolymerase B and the PHV depolymerase, respectively. The gene product of *phaZ1* (PHB depolymerase C) and the putative gene product of *phaZ3* (PHB depolymerase D) were related to PHB depolymerase B and may represent isoenzymes of PHB depolymerase B as has been described by Nakayama and co-workers [43]. An interesting result was obtained by comparison of the N-terminal amino acid sequences determined from the purified proteins: The N termini of PHB depolymerase A of *P. lemoignei* and of the PHB depolymerase of *Alcaligenes faecalis* T_1 were (nearly) identical (Fig. 1). Unfortunately, the PHB depolymerase A gene, which is named *phaZ5*, has not been cloned, although 11 clones expressing PHB depolymerase activity have been obtained [27]. It will be interesting to elucidate whether or not the sequence identity of both genes and deduced proteins covers the whole sequence. Identity could indicate horizontal gene transfer. Plasmid analysis of *P. lemoignei* and *A. faecalis* T_1 revealed the absence of linear plasmids in both bacteria and the presence of a 9-kb circular plasmid in *A. faecalis* T_1 [unpublished results]. However, the function of the *A. faecalis* plasmid is not known.

The deduced amino acid sequences of *phaZ1* to *phaZ3* revealed high homologies to each other and medium homologies to the PHB depolymerase of *A. faecalis* T_1. Alignment of the amino acid sequences of the three mature PHB depolymerases PhaZ1, PhaZ2, and PhaZ3 of *P. lemoignei* and the PHB depolymerase of *A. faecalis* T_1 revealed that 78 amino acids were conserved in all four sequences (Fig. 4). The highest degree of homology was found in the neighborhood of His_{35} (4 of 5 amino acids identical), His_{253} (4 of 7), Ser_{117} (9 of 14), Asp_{107} (9 of 14), Asp_{132} (5 of 6), and Asp_{195} (5 of 9). Histidine, serine, and glutamate or aspartate are known to form a catalytic triad in many lipases and other serine hydrolases [24, 46, 58, 73, 74]. The serine residue serves as the nucleophile, which attacks the ester bond. Usually the serine is localized in the center of a lipase box, which is characterized by a few hydrophobic amino acids followed by the pentasequence Gly-Xaa-Ser-Xaa-Gly. A perfect lipase consensus sequence is present around Ser_{117} in the three PHB depolymerases of *P. lemoignei* and around Ser_{139} in the *A. faecalis* protein (Fig. 4). Interestingly, all PHB depolymerases contain leucine at the second position of the lipase-box pentapeptide, whereas in most lipases histidine was found [24]. We assume that these PHB depolymerases represent serine hydrolases with a catalytic triad as the active center. This assumption is supported by the inhibition of the depolymerases by the serine inhibitors PMSF and DFP [33, 41, 66]. However, inhibitor-mapping studies with radioactively labeled DFP and the *A. faecalis* depolymerase favor Ser_{195}, which is not part of a lipase box, to represent the active center [51]. It will be necessary to analyze the effect of *in vitro*-induced mutations of putatively important amino acids on the enzymatic activity to determine the true active center. Another difference between the *P. lemoignei* depolymerases and the *A. faecalis* enzymes is the absence of an ~95-amino acid-long fragment, which in *A. faecalis* is homologous to the type III homology unit of fibronectin and some bacterial chitinases and cellulases [2, 36, 51, 71]. The function of the type III homology unit of fibronectin in bacterial extracellular enzymes is unknown.

The most striking feature of the amino acid sequence of the three PHB depolymerases of *P. lemoignei* was the frequent incidence of threonine or other uncharged amino acids with hydroxylated, small, or hydrophobic side chains such as Ser, Val, Ala, and Gly near the C terminus. Regions enriched in Thr, Ser, Pro, and Gly residues have been found in various extracellular cellulases, xylanases, and glucanases {e.g., *Clostridium thermocellum* [19, 75], *Cellulomonas fimi* [36], *Fibrinobacter succinogenes* [45], *Thermoanaerobacterium thermosulfurigenes* (H. Bahl, personal communication), and *Trichoderma reesei* [60, 67]; reviewed by Gilkes *et al.* [17]}, as well as in the chitinases of *Bacillus circulans* [71], *Streptomyces olivaceoviridis* [2], and *Rhizopus oligosporus* [77]. A region extremely enriched in serine residues has been described for the endoglucanase of *P. fluorescens* subsp. *cellulosa* [21]. It has been proposed that these sequences represent flexible linkers between different domains of the enzymes (reviewed by Gilkes *et al.* [17]) or may play a role in binding of the enzyme to the insoluble substrate [67, 70]. The structural similarity of threonine and the monomer of the PHB depolymerase substrate, 3-hydroxybutyrate, is astonishing and suggests the involvement of the threonine-rich region in substrate binding. However, deletion analysis of the serine-rich region of the endoglucanase of *P. fluorescens* subsp. *cellulosa* has shown that it was not essential for enzymatic activity [15]. The PHB-binding site of the *A. faecalis* enzyme was attributed to the 5-kDa fragment of the C terminus which had been cleaved off by treatment with trypsin. The major cleavage product still had 3HB-oligomer hydrolase activity but could not hydrolyze PHB [13a]. Similar results were obtained by partial proteolysis of cellobiohydrolase I from *T. reesei* [70]. We assume that the C-terminal regions of PhaZ1, PhaZ2, and

PhaZ3 of *P. lemoignei* are involved in binding the substrate PHB. Whether or not the threonine-rich region is involved in substrate binding or represents a linker between the catalytic domain and a (small) substrate-binding domain remains to be clarified.

PHB depolymerases A and B of *P. lemoignei* were identified to contain vicinal hydroxyl groups. We assume that carbohydrates were bound to the peptides. Glycosylation of the depolymerases would explain the difference between the relative molecular mass of the PHB depolymerase B measured for the purified protein (67,000) and that derived from the nucleotide sequence (41,800). The PHB depolymerase of *Penicillium funiculosum* has also been shown to be glycosylated [4]. The composition and the function of the carbohydrate moiety are unknown. In addition, the cellulases of *T. reesii* and *Cellulomonas fimi* are known to be glycosylated in the proline/threonine-rich region [11, 16, 20], and an extracellular glucoamylase from *Aspergillus niger* is also glycosylated in the C-terminal region of the protein [65]. A functional role of the carbohydrate moiety in binding the substrate of cellulases has been proposed, also [67, 70]. However, carbohydrates could also protect the extracellular protein from hydrolysis by proteases.

Little is known of the degradation of PHA consisting of monomers with more than four carbon atoms. The degradation of polycaprolacton by *Fusarium solani* and *Pullularia pullulans* was described about 20 years ago [12, 28]. Recently, several PHV- and PHO-degrading bacteria were isolated and characterized [26, 54]. The purified PHV depolymerase of *P. lemoignei* turned out to hydrolyze PHB, PHV, and copolymers of 3HB and 3HV, but were not able to hydrolyze PHO [41]. The purified PHO depolymerase of *P. fluorescens* GK13 is inactive with PHB and other SCL-HA as substrates but hydrolyzed PHO and other MCL-HA and has a nonspecific esterase activity.

All PHA depolymerases mentioned above represent extracellular enzymes and, as far as tested, are specific for "denatured" PHA; native PHA granula are not hydrolyzed, indicating a structural difference between intracellular (native) and extracellular (denatured) polymer [38, 40]. Intracellular PHA depolymerases have been described or were predicted for *Bacillus megaterium*, *Rhodospirillum rubrum*, *A. eutrophus*, *P. oleovorans*, and *P. aeruginosa* [22, 39]. The genes of the putative intracellular PHO depolymerase of *P. oleovorans* and *P. aeruginosa* have been found to be located between two copies of very similar PHO synthase genes [23, 68]. The deduced amino acid sequences revealed high homologies to each other, and the putative relative molecular masses of both proteins were similar to that of the purified extracellular PHO depolymerase of *P. fluorescens* GK13 (Table II). The intracellular localization of these PHO depolymerases presumably is on the surface of native PHO granules: Proteins of the predicted size have been found by purifying native PHO granules of *P. oleovorans* and analyzing the protein content of the surfaces [14]. Recently, the identity of an extracellular oligomer hydrolase of *A. faecalis* with its intracellular PHB depolymerase has been proposed [50]. Table II summarizes all available biochemical and genetic data on bacterial and eukaryotic PHA depolymerases.

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REFERENCES

1. A. J. Anderson and E. A. Dawes (1990) *Microbiol. Rev.* **54,** 450–472.
2. H. Blaak, J. Schnellmann, S. Walter, B. Henrissat, and H. Schrempf (1993) *Eur. J. Biochem.* **214,** 659–669.
3. H. Blum, H. Beier, and H. J. Gross (1987) *Electrophoresis* **8,** 93–99.

3a. B. H. Briese, D. Jendrossek, and H. G. Schlegel (1994) *FEMS Microbiol. Lett.* **117,** 107–112.

4. C. L. Brucato and S. S. Wong (1991) *Arch. Biochem. Biophys.* **290,** 497–502.
5. K. P. Budwill, M. Fedorak, and W. J. Page (1992) *Appl. Environ. Microbiol.* **58,** 1398–1401.
6. A. A. Chowdhury (1963) *Arch. Mikrobiol.* **47,** 167–200.
7. F. P. Delafield, K. E. Cooksey, and M. Doudoroff (1965) *J. Biol. Chem.* **240,** 4023–4028.
8. J. Devereux, P. Haeberli, and O. Smithies (1984) *Nucleic Acids Res.* **12,** 387–395.
9. Y. Doi (1990) *Microbial Polyesters*, VHC, New York.
10. Y. Doi, Y. Kanesawa, M. Kunioka, and T. Saito (1990) *Macromolecules* **23,** 26–31.
11. L. F. Fägerstam, G. Pettersson, and J. Å. Engström (1984) *FEBS Lett.* **167,** 309–315.
12. R. D. Fields, F. Rodriguez, and R. K. Finn (1974) *J. Appl. Polym. Sci.* **18,** 3571–3579.
13. W. G. C. Forsyth, A. C. Hayward, and R. B. Roberts (1958) *Nature (London)* **182,** 800–801.

13a. T. Fukui, K. Narikawa, Y. Miwa, T. Shirakura, T. Saito, and K. Tomita (1988) *Biochim. Biophys. Acta* **952,** 164–171.

14. R. C. Fuller, J. P. O'Donnell, J. Saulnier, T. E. Redlinger, J. Forster, and R. W. Lenz (1992) *FEMS Microbiol. Rev.* **103,** 279–288.
15. H. J. Gilbert, J. Hall, G. P. Hazlewood, and L. M. A. Ferreira (1990) *Mol. Microbiol.* **4,** 759–767.
16. N. R. Gilkes, M. L. Langsford, D. G. Kilburn, R. V. Miller, Jr., and R. A. J. Warren (1984) *J. Biol. Chem.* **259,** 10455–10459.

17. N. R. Gilkes, B. Henrissat, D. G. Kilburn, R. C. Miller, Jr., and R. A. J. Warren (1991) *Microbiol. Rev.* **55,** 303–315.
18. I. J. Goldstein and C. E. Hayes (1978) *Adv. Carbohydr. Chem. Biochem.* **35,** 127–140.
19. O. Grépinet, M.-C. Chebrou, and P. Béguin (1988) *J. Bacteriol.* **170,** 4582–4588.
20. E. K. Gum and R. D. Brown (1976) *Biochim. Biophys. Acta* **446,** 371–386.
21. J. Hall, G. P. Hazlewood, N. S. Huskisson, A. J. Durrant, and H. J. Gilbert (1989) *Mol. Microbiol.* **3,** 1211–1219.
22. H. Hippe and H. G. Schlegel (1967) *Arch. Mikrobiol.* **56,** 278–299.
23. G. W. Huisman, E. Wonink, R. Meima, B. Kazemier, P. Terpstra, and B. Witholt (1991) *J. Biol. Chem.* **266,** 2191–2198.
24. K. E. Jäger, S. Ransac, H. B. Koch, F. Ferrato, and B. W. Dijkstra (1993) *FEBS Lett.* **332,** 143–149.
25. P. H. Janssen and C. G. Harfoot (1990) *Arch. Microbiol.* **154,** 253–259.
26. D. Jendrossek, I. Knoke, R. B. Habibian, A. Steinbüchel, and H. G. Schlegel (1993) *J. Environ. Polym. Degrad.* **1,** 53–63.
27. D. Jendrossek, B. Müller, and H. G. Schlegel (1993) *Eur. J. Biochem.* **218,** 701–710.
28. R. Kavelman and B. Kendrick (1978) *Mycologia* **70,** 87–103.
29. W. M. Konyecsni and V. Deretic (1990) *J. Bacteriol.* **172,** 2511–2520.
30. U. K. Laemmli (1970) *Nature (London)* **227,** 680–685.
31. J. Lechner and F. Wieland (1989) *Annu. Rev. Biochem.* **58,** 173–194.
32. H. Lis and N. Sharon (1993) *Eur. J. Biochem.* **218,** 1–27.
33. C. J. Lusty and M. Doudoroff (1966) *Proc. Natl. Acad. Sci. USA* **56,** 960–965.
34. M. Matavulj and H. P. Molitoris (1992) *FEMS Microbiol. Rev.* **103,** 323–331.
35. D. W. McLellan and P. J. Halling (1988) *FEMS Microbiol. Lett.* **52,** 215–218.
36. A. Meinke, C. Braun, N. R. Gilkes, D. G. Kilburn, R. C. Miller, Jr., and R. A. J. Warren (1991) *J. Bacteriol.* **173,** 308–314.
37. J. Mergaert, C. Anderson, A. Wouters, J. Swings, and K. Kersters (1992) *FEMS Microbiol. Rev.* **103,** 317–321.
38. J. M. Merrick (1965) *J. Bacteriol.* **90,** 965–969.
39. J. M. Merrick and M. Doudoroff (1964) *J. Bacteriol.* **88,** 60–71.
40. J. M. Merrick, G. Lundgren, and R. M. Pfister (1965) *J. Bacteriol.* **89,** 234–239.
41. B. Müller and D. Jendrossek (1993) *Appl. Microbiol. Biotechnol.* **38,** 487–492.
42. K. Mukai and Y. Doi (1993) in H. G. Schlegel and A. Steinbüchel (Eds.), *Proceedings of the International Symposium on Bacterial Polyhydroxyalkanoates*, Goltze Druck, Göttingen, pp. 457–458.
43. K. Nakayama, T. Saito, T. Fukui, Y. Shirakura, and K. Tomita (1985) *Biochim. Biophys. Acta* **827,** 63–72.
44. C. L. Oakley (1971) *Meth. Microbiol.* **5A,** 173–218.
45. F. W. Paradis, H. Zhu, P. J. Krell, J. P. Phillips, and C. W. Forsberg (1993) *J. Bacteriol.* **175,** 7666–7672.
46. B. Persson, G. Bentsson-Olivecrona, S. Enerback, T. Olivecrona, and H. Jornvall (1989) *Eur. J. Biochem.* **179,** 39–45.
47. T. Platt (1986) *Annu. Rev. Biochem.* **55,** 339–372.
48. A. P. Pugsley and M. Schwartz (1985) *FEMS Microbiol. Rev.* **32,** 3–38.
49. T. Saito, K. Suzuki, J. Yamamoto, T. Fukui, K. Miwa, K. Tomita, S. Nakanishi, S. Odani, J.-I. Suzuki, and K. Ishikawa (1989) *J. Bacteriol.* **171,** 184–189.
50. T. Saito, H. Saegusa, Y. Miyata, and T. Fukui (1992) *FEMS Microbiol. Rev.* **103,** 333–338.
51. T. Saito, A. Iwata, and T. Watanabe (1993) *J. Environm. Polym. Degrad.* **1,** 99–105.
52. J. Sambrook, E. F. Fritsch, and T. Maniatis (1989) *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
53. F. Sanger, S. Nicklen, and A. R. Coulson (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
54. A. Schirmer, D. Jendrossek, and H. G. Schlegel (1993) *Appl. Environ. Microbiol.* **59,** 1220–1227.
55. H. G. Schlegel, G. Gottschalk, and R. v. Bartha (1961) *Nature (London)* **191,** 463–465.
56. H. G. Schlegel, H. Kaltwasser, and G. Gottschalk (1961) *Arch. Mikrobiol.* **117,** 475–481.
57. H. G. Schlegel and G. Gottschalk (1962) *Angewandte Chem.* **74,** 342–347.
58. J. D. Schrag, L. Yunge, S. Wu, and M. Cygler (1991) *Nature* **351,** 761–764.
59. J. P. Segrest and R. L. Jackson (1972) *Methods Enzymol.* **28,** 54–63.
60. S. Shoemaker, V. Schweickart, M. Ladner, D. Gelfand, S. Kwok, K. Myambo, and M. Innis (1983) *Bio/Technology* **1,** 691–696.
61. H. Stegemann, H. Francksen, and V. Macko (1973) *Z. Naturforsch.* **28c,** 722–732.
62. A. Steinbüchel (1991) in D. Byrom (Ed.), *Biomaterials*, Macmillan Press, London, pp. 123–213.
63. A. Steinbüchel, E. Hustede, M. Liebergesell, U. Pieper, A. Timm, and H. Valentin (1992) *FEMS Microbiol. Rev.* **103,** 217–230.
64. A. Steinbüchel, E.-M. Debzi, R. H. Marchessault, and A. Timm (1993) *Appl. Microbiol. Biotechnol.* **39,** 443–449.
65. B. Svensson, K. Larsen, I. Svendsen, and E. Boel (1983) *Carsberg Res. Commun.* **48,** 529–544.
66. T. Tanio, T. Fukui, Y. Shirakura, T. Saito, K. Tomita, T. Kaiho, and S. Masamune (1982) *Eur. J. Biochem.* **124,** 71–77.
67. T. T. Teeri, P. Lehtovaara, S. Kauppinnen, I. Salovuori, and J. Knowles (1987) *Gene* **51,** 43–52.
68. A. Timm and A. Steinbüchel (1992) *Eur. J. Biochem.* **209,** 15–30.
69. H. Towbin, T. Staehelin, and J. Gordon (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
70. H. Van Tilbeurgh, P. Tomme, M. Claeyssens, R. Bhikhabhai, and G. Pettersson (1986) *FEBS Lett.* **204,** 223–227.
71. T. Watanabe, K. Suzuki, W. Oyanagi, K. Ohnishi, and H. Tanaka (1990) *J. Biol. Chem.* **265,** 15659–15665.
72. M. E. E. Watson (1984) *Nucl. Acids Res.* **12,** 5145–5164.
73. F. K. Winkler, A. D'Acry, and W. Hunziker (1990) *Nature* **343,** 771–774.
74. S. Wohlfarth, C. Hoesche, C. Strunk, and U. K. Winkler (1992) *J. Gen. Microbiol.* **138,** 1325–1335.
75. E. Yagüe, P. Béguin, and J.-A. Aubert (1990) *Gene* **89,** 61–67.
76. K. Yamada, K. Mukai, and Y. Doi (1993) *Int. J. Biol. Macromol.* **15,** 215–220.
77. K. Yanai, N. Takaya, N. Kojima, H. Horiuchi, H. Ohta, and M. Takagi (1992) *J. Bacteriol.* **174,** 7398–7406.