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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(3-hydroxyvalerate) (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 *Eco*RI 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_r, 44,906). The PHV depolymerase gene (*phaZ4*) was subcloned, and the nucleotide sequence of a 3,109-bp *Bam*HI 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 (IlfE) of enterobacteria. ORF4 (1,712 bp) was identified as the precursor of a PHV depolymerase (567 amino acids; M_r, 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^{2+} 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-hydroxybutyric 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

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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_1 (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* PHA-hydrolyzing 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 · $6H_2O$. *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 μg 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 osmotic 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 $CaCl_2$ 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°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 α-lactalbumin (M_r, 14,400) were used as molecular mass standard proteins. After electrophoresis, proteins were silver stained (4). For glycoprotein staining, sucrose 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 phenythiohydantoin 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-hydroxyoctanoic acid, 72 mol% 3-hydroxydecanoic 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 μg of polymer granules (60 μl of a 0.3% [wt/vol] suspension in water) in 50 mM Tris-HCl (pH 8.0)–1 mM $CaCl_2$ at 37°C. The apparent extinction coefficients for PHB and PHV were 3.6 and 3.0 μl ng^{-1} cm^{-1}, 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 μg 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 $MgCl_2$ 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*RI or with *Pst*I and separated by agarose gel electrophoresis. The DNA was vacuum blotted onto positively charged nylon membranes and hybridized with a ^{32}P-labeled oligonucleotide mixture [5′-TC(A/C/G/T)GC(A/C/G/T)GCCCA(A/G/C/T)GT(C/T)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^- (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-D-galactopyrano-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference(s)
Strains		
A. eutrophus H16	Source of PHB, PHB^{-a}	DSM428, ATCC 17699
A. eutrophus H16(pSN649)	pVK100::1.7 kbp *Hin*dIII::*phaZ1*, PHB$^+$	This study
A. eutrophus JMP222	PHB$^-$	16
A. eutrophus JMP222(pSN649)	pVK100::1.7-kbp *Hin*dIII::*phaZ1*, PHB$^+$	This study
Chromobacterium violaceum	Source of PHV	DSM30191
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* Δ(*lac-proAB*) *rpsL* (Sm^r) *thi-1* ϕ80*lacZ*Δ15	51
E. coli JM83(pSN480)	PHB$^+$ pUC9::5.4-kbp *Mbo*I::*phaZ1*	27
E. coli JM83(pSN654)	PHB$^+$ pUC9-2::1.7-kbp *Hin*dIII::*phaZ1*	27
E. coli JM83(pSN625)	PHB$^+$ pUC9-1::2.1-kbp *Mbo*I::*phaZ2*	7, 27
E. coli JM83(pSN487)	PHB$^+$ pUC9-1::2.5-kbp *Mbo*I::*phaZ3*	7, 27
E. coli JM83(pSN484)	PHB$^+$ pUC9-2::13.5-kbp *Mbo*I::*phaZ4*	27
E. coli JM83(pSN612)	PHB$^+$ pUC9-2::3.1-kbp *Bam*HI::*phaZ4*	This study
E. coli XL1 blue	*recA1 endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relA1* λ$^-$ *lac* [F′ *proAB lac*I^{q}ZΔM15 Tn*10*(Tc^r)]	9
E. coli XL1 blue(pSN874)	PHB$^+$ pBluescriptSK$^-$::2.5-kbp *Hin*dIII-*Bam*HI::*phaZ4*	This study
E. coli XL1 blue(pSN790)	PHB$^+$ pBluescriptSK$^-$::3.7-kbp *Pst*I::*phaZ5*	This study
E. coli XL1 blue(pSN792)	PHB$^+$ pBluescriptSK$^-$::3.7-kbp *Eco*RI::*phaZ5*	This study
E. coli XL1 blue(pSN885)	PHB$^+$ pBluescriptSK$^-$::1.85-kbp *Ssp*I-*Xba*I::*phaZ5*	This study
E. coli 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 *Hin*dIII::*phaZ1*, orientation A, PHB$^-$	This study
E. coli S17-1(pSN649)	pVK100::1.7-kbp *Hin*dIII::*phaZ1*, orientation B, PHB$^+$	This study
Plasmids		
pUC9, pUC9-1, pUC9-2	Ap^r, *lacPOZ′*	21, 59
pBluescript SK$^-$	Ap^r, *lacPOZ′*; T7 and T3 promoters	Stratagene, San Diego, Calif.
pVK100	Tc^r, Km^r	28

[a] PHB$^-$, no extracellular hydrolysis of PHB.

side-containing LB medium. Recombinant clones were picked onto LB-ampicillin master plates, and colony hybridization (20) with the ^{32}P-labeled *phaZ5*-specific oligonucleotide was performed.

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 [^{35}S]dATP[αS]. 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 *Pst*I fragment of *P. lemoignei* hybridized with the probe.

About 1,000 and 350 recombinant strains of *E. coli* XL1 blue harboring *Pst*I- 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 *Pst*I fragment and a 3.7-kbp *Eco*RI fragment in the hybrid plasmid. When Southern blots of the *Pst*I- 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.

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

PHA depolymerase gene, protein, isoenzyme	Length (amino acids)		M_r		Glycosylation	Activity[a]					Sp act (10^3 U/mg)		Optimal temp (°C)
	Preprotein	Signal peptide	Deduced mature	By SDS-PAGE		PHB	P(4HB)	PHV	PHD, P (HO-*co*-HD)	PCL, PL	PHB	PHV	
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 depolymerase 1	567	25	57,459	65,500	−	+++	+	+	−	−	22.7	0.72	50
phaZ5, PhaZ5, A	433	26	42,221	49,000	−	+++	−	−	−	−	22.3	ND	51

[a] −, 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).
[b] 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 DNA-deduced 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_{26} (Fig. 1). The calculated M_r for the mature protein was 42,221. The primary sequence contained a single pentapeptide (G-L-S_{138}-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-amino-acid-long sequence (A_{308} 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 *Bam*HI 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 *Bam*HI 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 ribosome-binding 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 (IlfE) 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-

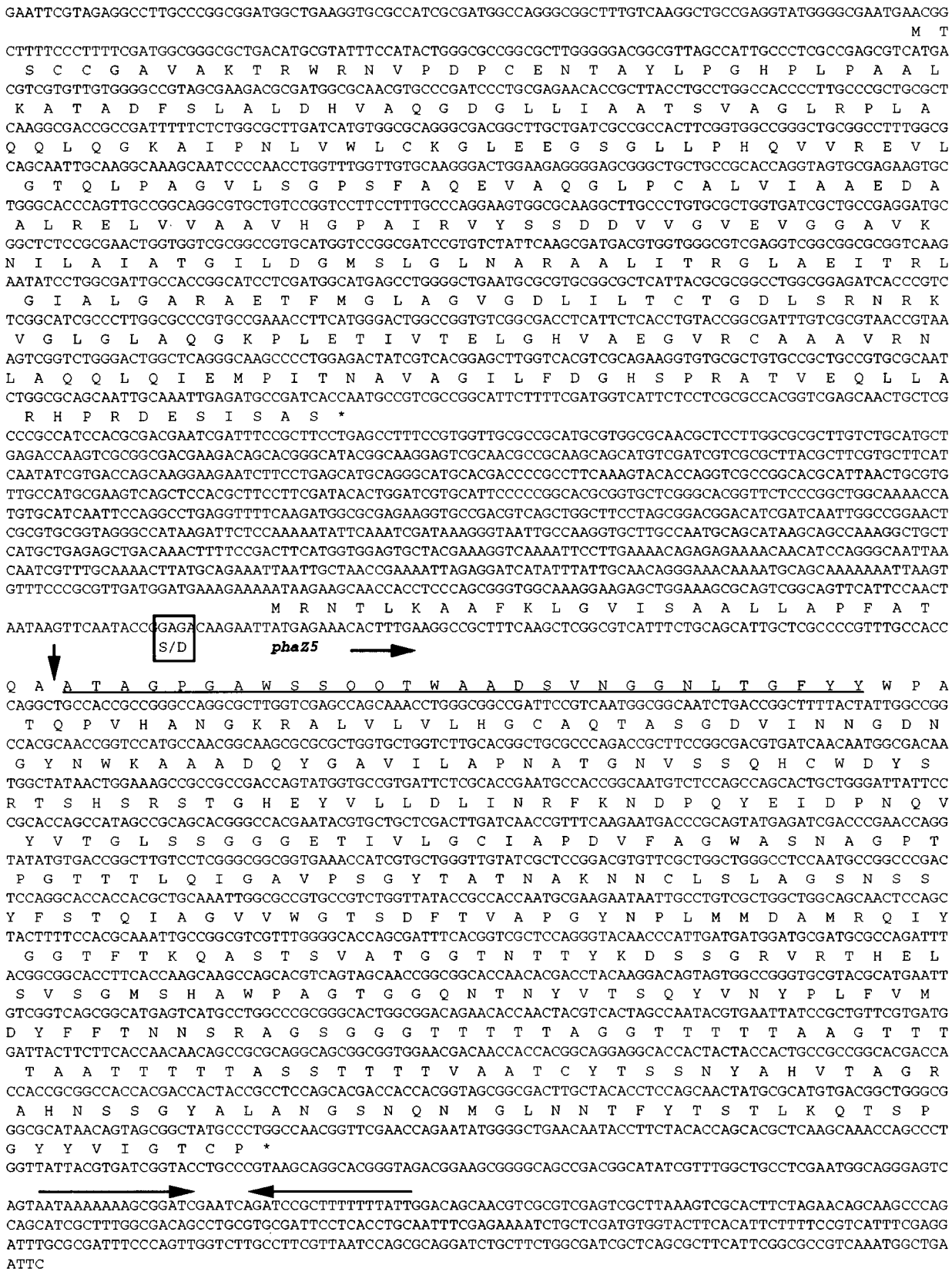

FIG. 1. Nucleotide sequence of *phaZ5* of *P. lemoignei* and of adjacent regions. Only the coding strand for *phaZ5* is shown. Amino acids are written above the first nucleotide of the corresponding codon. A putative ribosome-binding (Shine-Dalgarno [S/D]) site is boxed. Amino acids confirmed by Edman 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.

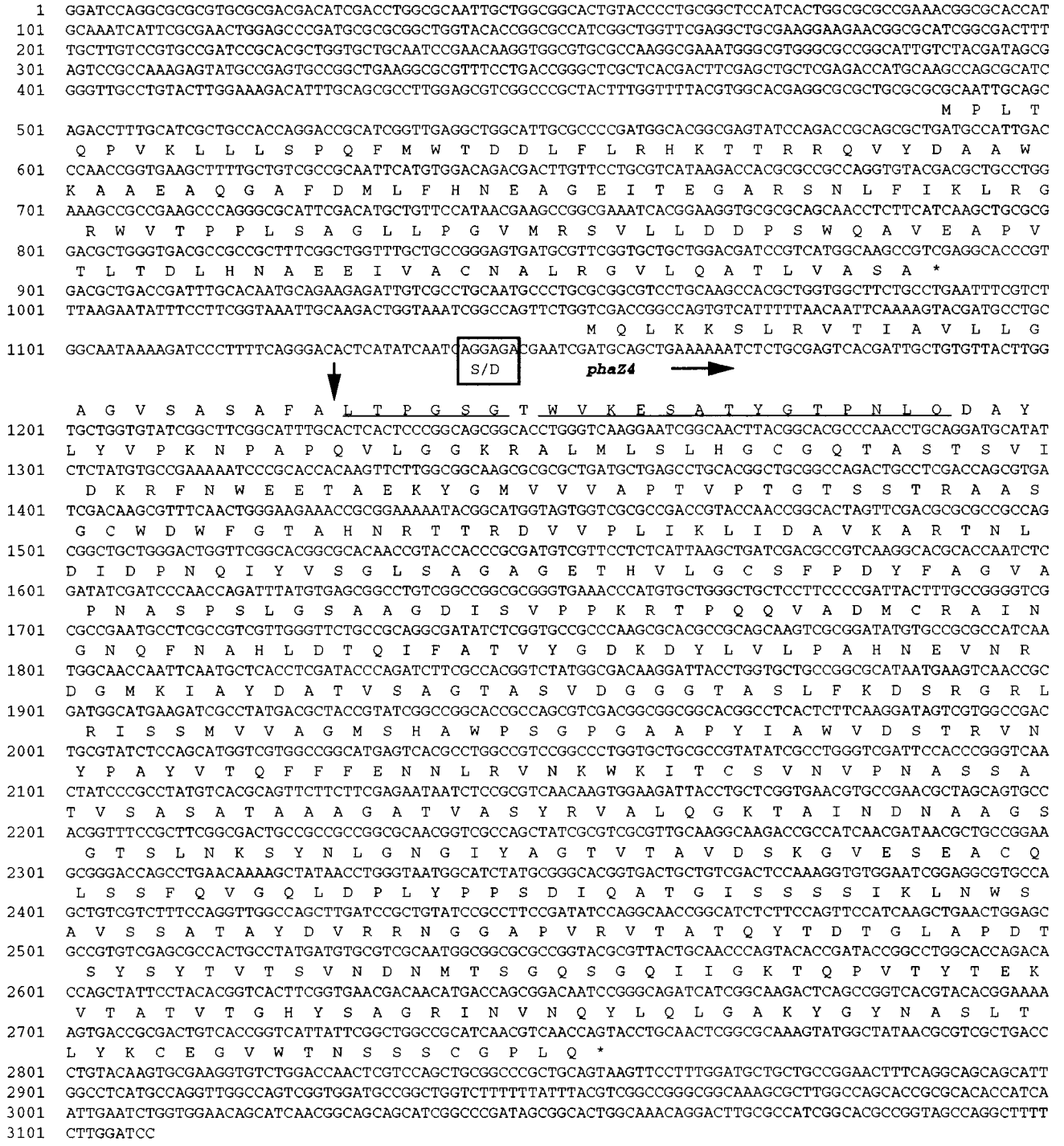

FIG. 2. Nucleotide sequence of *phaZ4* of *P. lemoignei* and of adjacent regions. Only the coding strand for *phaZ4* is shown. Amino acids are written above the first nucleotide of the corresponding codon. A putative ribosome-binding (Shine-Dalgarno [S/D]) site is boxed. Amino acids confirmed by Edman 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_{137}-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* ($PhaZ_{Afa}$) (42) and *P. pickettii* ($PhaZ_{Ppi}$) (29) (83% identical amino acids), between $PhaZ1_{Ple}$, $PhaZ2_{Ple}$, and $PhaZ3_{Ple}$ (68 to 72%), and between $PhaZ5_{Ple}$ and $PhaZ_{Afa}$ (65%). Interestingly, the first 26 amino acids of the mature depolymerases $PhaZ5_{Ple}$ and $PhaZ_{Afa}$ 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 *Hin*dIII fragment of pSN654, which harbored the PHB depolymerase C structural gene (*phaZ1*), was cloned into the *Hin*dIII 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 *Hin*dIII 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-3-hydroxybutyrate, 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 μg 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 ± 1,500, 46,500 ± 1,500, 44,000 ± 1,500, and 65,500 ± 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 11×10^3 to 23×10^3 U/mg. PHV could be hydrolyzed only by recombinant PhaZ4 (0.72×10^3 U/mg, corresponding to 3.2% of the PHB depolymerase activity) and by PHB depolymerase C (PhaZ1; 3.0×10^3 U/mg, corresponding to 26% of the PHB depolymerase activity). P(4HB) was hydrolyzed only by PhaZ4. All other polymers [PHO, P(HO-*co*-HD) PCL, and PL] were not hydrolyzed by any of the proteins.

The temperature maxima for PHB hydrolysis amounted to 61.5 ± 1, 54.5 ± 2.5, 50 ± 2, and 51 ± 1°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^{2+} 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

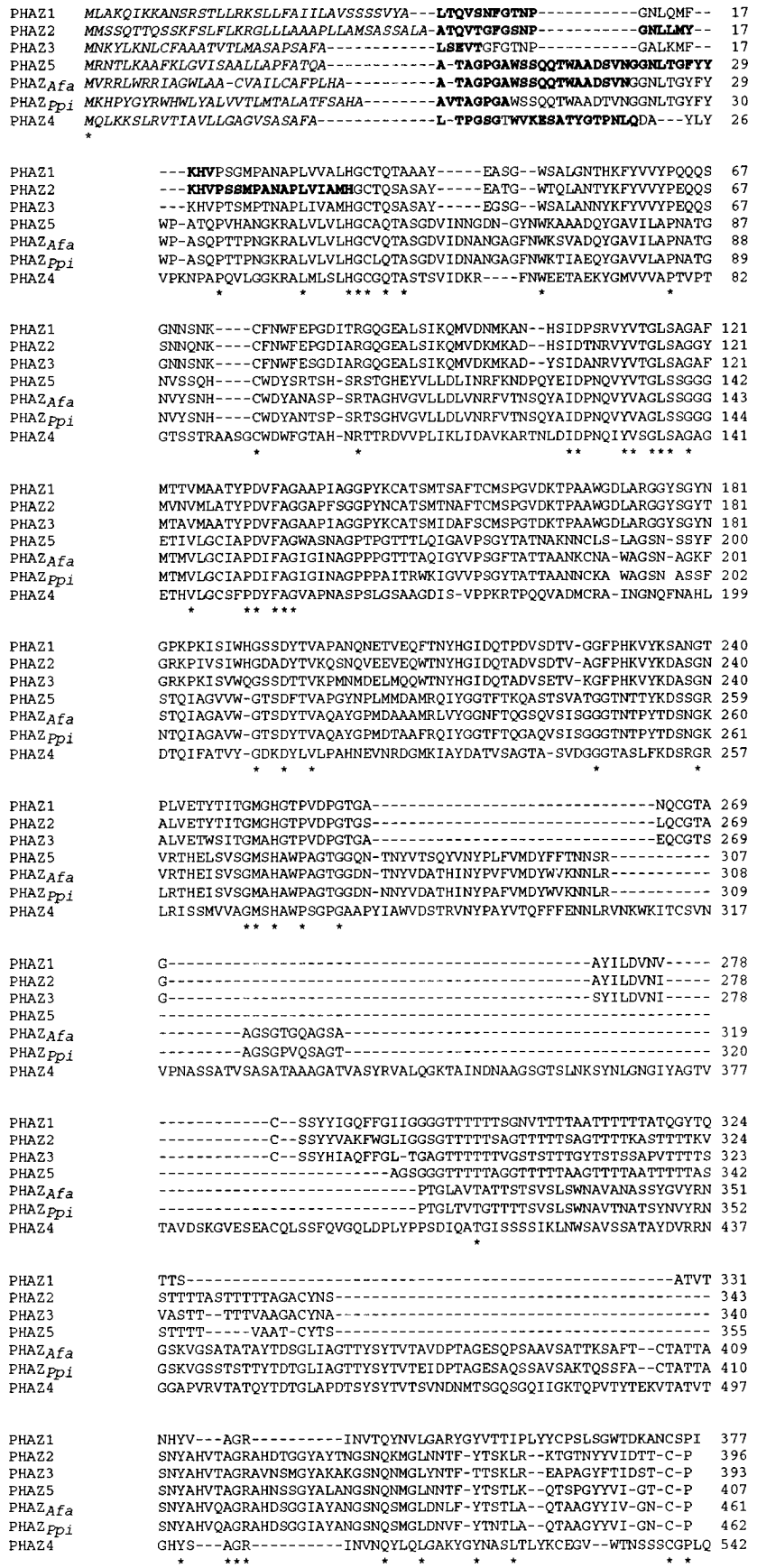

FIG. 3. Alignment of deduced amino acid sequences of PHA depolymerases. The sequences of PhaZ1 to PhaZ5 of *P. lemoignei* and of the depolymerases of *A. faecalis* ($PhaZ_{Afa}$) (42) and *P. pickettii* ($PhaZ_{Ppi}$) (30) are aligned. The signal peptides are written in italics, and amino acids that 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	pos.	Serine	pos.	Aspartate	pos.	Histidine	pos.	Oxyanion hole
		S		**D**		**H**		**H**
PhaZ1$_{Ple}$	117	IDPSRVYVTGL**S**AGAFMT	195	IWHGSS**D**YTV	253	GMG**H**GTPVDPG	35	LVVAL**H**GCDQTAA
PhaZ2$_{Ple}$	117	IDTNRVYVTGL**S**AGGYMV	195	IWHGDA**D**YTV	253	GMG**H**GTPVDPG	35	LVIAM**H**GCTQSAS
PhaZ3$_{Ple}$	117	IDANRVYVTGL**S**AGAFMT	195	VWQGSS**D**TTV	253	GMA**H**GTPVDPG	35	LIVAM**H**GCTQSAS
PhaZ4$_{Ple}$	137	IDPNQIYVSGL**S**AGAGET	212	VY-GDK**D**YLV	270	GMS**H**AWPSGPG	47	LMLSL**H**GCGQTAS
PhaZ5$_{Ple}$	138	IDPNQVYVTGL**S**SGGGET	213	VW-GTS**D**FTV	272	GMS**H**AWPAGTG	49	LVLVL**H**GCAQTAS
PhaZ$_{Afa}$	139	IDPNQVYVAGL**S**SGGGMT	214	VW-GTS**D**YTV	273	GMA**H**AWPAGTG	49	LVLVL**H**GCVQTAS
PhaZ$_{Ppi}$	140	IDPNQVYVAGL**S**SGGGMT	215	VW-GTS**D**YTV	274	GMA**H**AWPAGTG	50	LVLVL**H**GCLQTAS
CONSENSUS		ID-n-vYV-GL**S**-G+--t		vw-G-s**D**yTV		GM-**H**--P---G		L***l**H**GC-QtAs
PhaZ$_{Pfl}$	139	LNAQRQYATGI**S**SGGYNT	193	FLHGFV**D**AVV	227	LGG**H**EWFAASP	78	QNLLD**H**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$_{Ple}$), 7 (PhaZ2$_{Ple}$ and PhaZ3$_{Ple}$), 42 (PhaZ$_{Afa}$), 29 (PhaZ$_{Ppi}$), and 45 (PhaZ$_{Pfl}$).

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 diisopropylfluorophosphate 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$_{Ple}$ 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_{117}, D_{132} or D_{195}, and H_{35} or H_{253} (numbers according to the PhaZ1 sequence in Fig. 3). It is remarkable that the sequence around H_{35} is very similar to the sequences around the oxyanion hole of many lipases (24a, 25). Therefore, it is possible that the region around H_{35} represents an oxyanion hole, and H_{253} might be the active-site histidine. Recently, S_{196} of the (mature) *A. faecalis* depolymerase has been shown to be

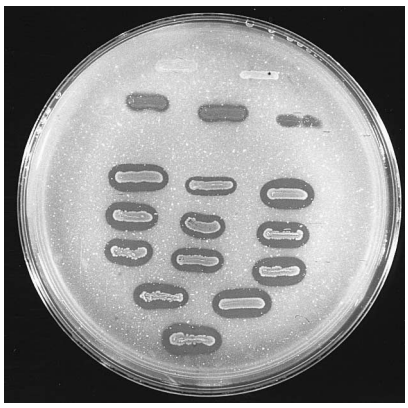

FIG. 5. Growth of transconjugants of *A. eutrophus* JMP22 harboring *phaZ1* on PHB. *A. eutrophus* H16 and *A. eutrophus* JMP222 served as negative controls and *Comamonas* sp., *P. lemoignei*, and *E. coli* JM83(pSN654) served as positive controls in the first and second rows, respectively; 12 different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

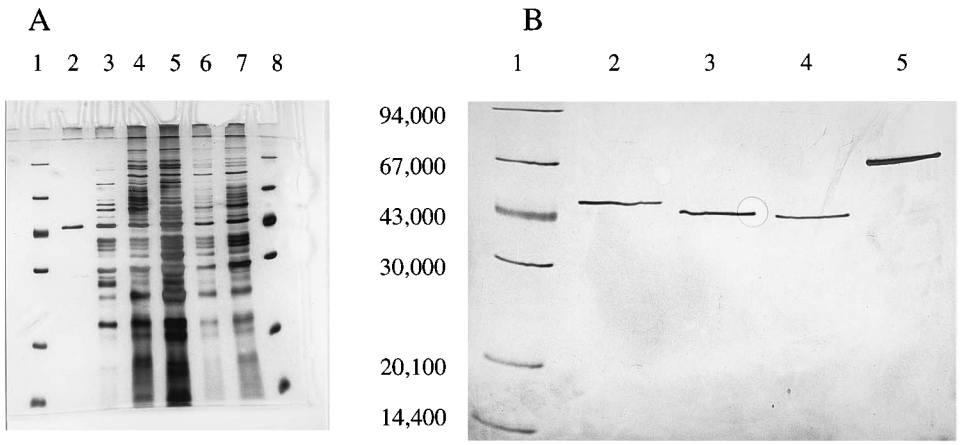

FIG. 6. Purification of PHA depolymerases from recombinant *E. coli*. (A) PhaZ2 at various steps of purification. Proteins were separated in denaturing SDS–12% polyacrylamide gel and silver stained. Lanes 1 and 8, molecular mass standard proteins (masses are indicated in daltons); lane 2, 0.18 μg of 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 combined fractions of the concentrated culture fluid and of the concentrated periplasmic fraction; lane 6, 1.9 μg of the concentrated periplasmic fraction; lane 7, 1.9 μg of the concentrated culture fluid. (B) Purification of recombinant PhaZ1, PhaZ2, 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 $PhaZ5_{Ple}$, not in $PhaZ1_{Ple}$, $PhaZ2_{Ple}$, $PhaZ3_{Ple}$, and $PhaZ4_{Ple}$ (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_{37}, C_{74}, and C_{374}) 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, serine-rich 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 $PhaZ2_{Ple}$, $PhaZ3_{Ple}$, $PhaZ5_{Ple}$, $PhaZ_{Afa}$, and $PhaZ_{Ppi}$ 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 substrate specificity to PHV in addition to PHB, the C-terminal regions of $PhaZ1_{Ple}$ and $PhaZ4_{Ple}$ might be PHV (and PHB)-specific substrate-binding sites (PHV type in Fig. 7), while the C-terminal regions of the other depolymerases represent PHB-specific 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, $PhaZ4_{Ple}$ and $PhaZ_{Afa}$ did not contain any threonine-rich region but contained an approximately 90-amino-acid-long fragment which has the consensus sequence of Fn3 (5, 22). Fn3 sequences have been found in extracellular matrix proteins of eucaryotes 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 $PhaZ4_{Ple}$ and $PhaZ_{Afa}$. 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 $PhaZ1_{Ple}$, $PhaZ2_{Ple}$, $PhaZ4_{Ple}$, and $PhaZ5_{Ple}$ 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 tran-

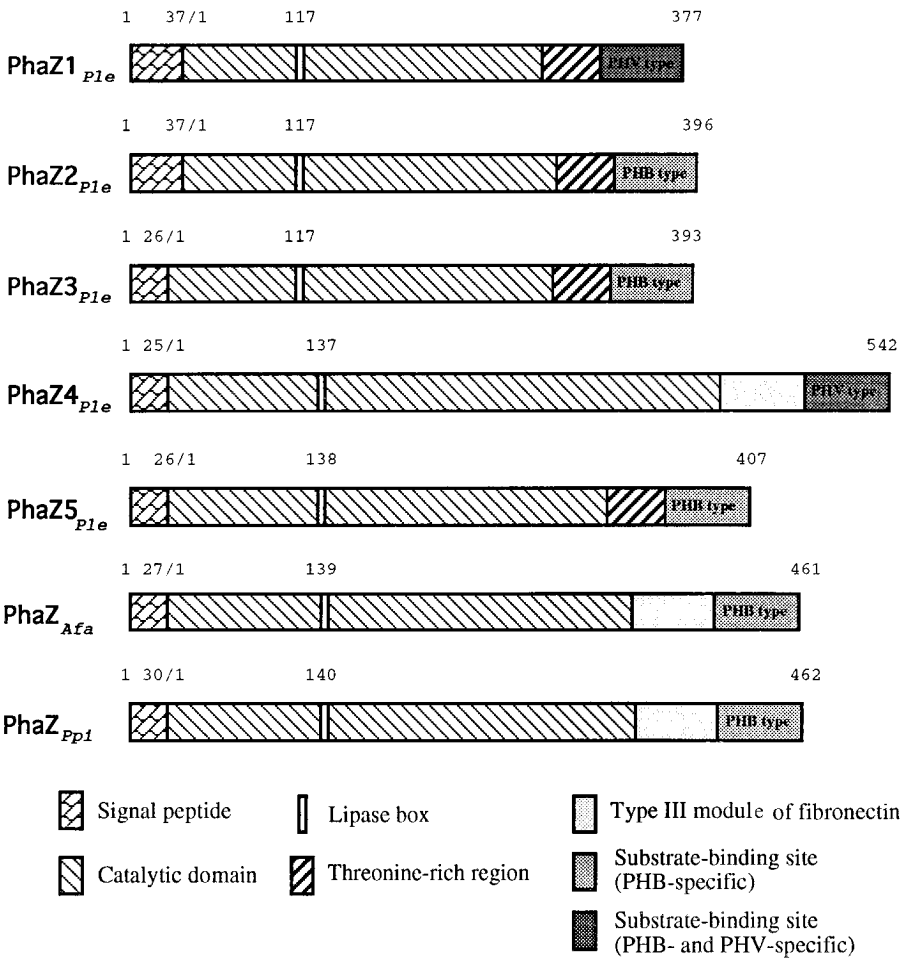

FIG. 7. Domain structures of PHA depolymerases. The numbers of the first and last amino acids of the prepeptides and of the mature proteins are given, and positions of the lipase boxes (serine) are indicated. For references, see the legend to Fig. 4).

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 σ^{70}-like promoter as a monocistronic message of about 1 kbp (45).

ACKNOWLEDGMENTS

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