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Molecular Characterization of Extracellular Medium-chain-length Poly(3-hydroxyalkanoate) Depolymerase Genes from *Pseudomonas alcaligenes* Strains

Do Young Kim, Hyun Chul Kim, Sun Young Kim and Young Ha Rhee*

Department of Microbiology, School of Bioscience and Biotechnology, Chungnam National University, Daejeon 305-764, Republic of Korea

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A bacterial strain M4-7 capable of degrading various polyesters, such as poly(ε-caprolactone), poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), poly(3-hydroxyoctanoate), and poly(3-hydroxy-5-phenylvalerate), was isolated from a marine environment and identified as *Pseudomonas alcaligenes*. The relative molecular mass of a purified extracellular medium-chain-length poly(3-hydroxyalkanoate) (MCL-PHA) depolymerase ($PhaZ_{PalM4-7}$) from *P. alcaligenes* M4-7 was 28.0 kDa, as determined by SDS-PAGE. The $PhaZ_{PalM4-7}$ was most active in 50 mM glycine-NaOH buffer (pH 9.0) at 35°C. It was insensitive to dithiothreitol, sodium azide, and iodoacetamide, but susceptible to *p*-hydroxymercuribenzoic acid, *N*-bromosuccinimide, acetic anhydride, EDTA, diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, Tween 80, and Triton X-100. In this study, the genes encoding MCL-PHA depolymerase were cloned, sequenced, and characterized from a soil bacterium, *P. alcaligenes* LB19 (Kim *et al.*, 2002, *Biomacromolecules* 3, 291-296) as well as *P. alcaligenes* M4-7. The structural gene ($phaZ_{PalLB19}$) of MCL-PHA depolymerase of *P. alcaligenes* LB19 consisted of an 837 bp open reading frame (ORF) encoding a protein of 278 amino acids with a deduced M_r of 30,188 Da. However, the MCL-PHA depolymerase gene ($phaZ_{PalM4-7}$) of *P. alcaligenes* M4-7 was composed of an 834 bp ORF encoding a protein of 277 amino acids with a deduced M_r of 30,323 Da. Amino acid sequence analyses showed that, in the two different polypeptides, a substrate-binding domain and a catalytic domain are located in the *N*-terminus and in the *C*-terminus, respectively. The $PhaZ_{PalLB19}$ and the $PhaZ_{PalM4-7}$ commonly share the lipase box, GISSG, in their catalytic domains, and utilize ^{111}Asn and ^{110}Ser residues, respectively, as oxyanions that play an important role in transition-state stabilization of hydrolytic reactions.

Key words: Medium-chain-length polyhydroxyalkanoate, polyhydroxyalkanoate depolymerase, *Pseudomonas alcaligenes*

Microbial polyhydroxyalkanoates (PHAs) comprise a wide variety of different polyesters, with more than 150 (*R*)-hydroxyalkanoates identified as known constituents, which are synthesized intracellularly by numerous prokaryotes as carbon and energy storage compounds under unbalanced growth conditions (Steinbüchel and Valentin, 1995). Extensive studies have shown that these polymers can easily be degraded by microorganisms, including bacteria, filamentous fungi, and yeasts which are widely distributed in ecosystems (Jendrossek, 2001; Kim and Rhee, 2003). The ability of microorganisms to degrade PHAs is dependent on the secretion of specific PHA depolymerases, a family of serine hydrolases.

Until now, more than 80 extracellular PHA depolymerases have been purified and characterized from prokaryotic and eukaryotic microorganisms, but most of the purified enzymes are specific to short-chain-length PHAs like poly (3-hydroxybutyrate) and/or its copolyesters with 3-hydroxyvalerate (Jendrossek, 2001). Accordingly, although some bacterial decomposition of medium-chain-length (MCL)-PHAs has been pronounced (Ramsay *et al.*, 1994; Foster *et al.*, 1995; Quinteros *et al.*, 1999; Park *et al.*, 2001), a limited number of reports have demonstrated biochemical characteristics of MCL-PHA depolymerases that are specific to polymers consisting of 3-hydroxyalkanoates with six or more carbon atoms. The poly (3-hydroxyoctanoate) (PHO) depolymerase ($PhaZ_{PflGK13}$) from *Pseudomonas fluorescens* GK13 (Schirmer *et al.*, 1993), the poly(3-hydroxyphenylvalerate) (PHPV) depolymerase ($PhaZ_{XspJS02}$) from *Xanthomonas* sp. JS02 (Kim *et al.*, 2000b), the MCL-PHA depolymerase ($PhaZ_{PspRY-1}$) from *Pseudomonas* sp. RY-1 (Kim *et al.*, 2000a), the MCL-PHA depolymerase ($PhaZ_{PalLB19}$) from *P. alcaligenes* LB19 (Kim *et al.*, 2002), the MCL-PHA depolymerase ($PhaZ_{PinK2}$) from *P. indica* K2 (Elbanna *et al.*, 2004), and the MCL-PHA depolymerase

* To whom correspondence should be addressed.
(Tel) 82-42-821-6413; (Fax) 82-42-822-7367
(E-mail) yhrhee@cnu.ac.kr

($PhaZ_{SspKJ-72}$) from *Streptomyces* sp. KJ-72 (Kim *et al.*, 2003) are examples of purified MCL-PHA depolymerases. Although the biochemical properties of some MCL-PHA depolymerases are well documented, the molecular characteristics of the genes encoding MCL-PHA depolymerases are unclear, as $PhaZ_{PflGK13}$ is the only MCL-PHA depolymerase which has been studied to a molecular level thus far (Schirmer and Jendrossek, 1994).

In this study, we describe some properties of a novel extracellular MCL-PHA depolymerase from a marine isolate, *P. alcaligenes* M4-7, and discuss the molecular structure of its MCL-PHA depolymerase gene. In addition, we provide common and distinctive characteristics of the MCL-PHA depolymerase gene from a soil bacterium, *P. alcaligenes* LB19 (Kim *et al.*, 2002), in comparison with the PHO depolymerase gene from *P. fluorescens* GK13 (Schirmer and Jendrossek, 1994).

Materials and Methods

Preparation of polyesters and their latex suspensions

MCL-PHAs were produced by culturing *P. oleovorans* ATCC 29347 in a mineral salt medium containing carboxylic acids with corresponding chemical structures as described elsewhere (Lageveen *et al.*, 1988). Synthesized PHAs were isolated and purified from lyophilized cells by extraction with hot chloroform using a Soxhlet apparatus as previously described (Kim *et al.*, 2000c). Latex suspensions of MCL-PHAs were prepared according to the method described by Ramsay *et al.* (1994) with minor modifications. Poly(3-hydroxybutyrate) (PHB) was produced by *Ralstonia eutropha* KHB 8862 from glucose (Chung *et al.*, 2001). Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) copolyesters were synthesized by a threonine-overproducing mutant of *Alcaligenes* sp. SH-69 (Choi *et al.*, 2003). A suspension of PHB was made by the ultrasonic dispersion of PHB granules in distilled water. Polycaprolactone (PCL; number average molecular weight, 80,000) was purchased from the Aldrich Chemical (USA).

Isolation and identification of a MCL-PHA-degrading microorganism

An MCL-PHA-degrading bacterial strain, M4-7, was isolated from a seawater sample by enrichment using an artificial seawater agar medium containing 0.2% (wt/v) poly (3-hydroxyoctanoate) (PHO) as the sole carbon source. Each liter of basal medium was prepared by dissolving 1.0 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4$, 0.05 g NaCl, 0.05 g $CaCl_2 \cdot 2H_2O$, and 20 g agar in one liter of artificial sea water. The artificial sea water contained 30 g NaCl, 10.8 g $MgCl_2 \cdot 6H_2O$, 5.4 g $MgSO_4$, 0.7 g KCl, 0.001 g $CaCl_2 \cdot 2H_2O$, 0.001 g $FeSO_4 \cdot 7H_2O$, and 0.001 g $MnSO_4 \cdot 5H_2O$ per liter of distilled water. Sequence analysis of the 16S rRNA gene of the isolate was performed as previously described (Kim *et al.*, 2002). Using the PHYDIT program, the 16S rDNA sequences were aligned with those from strains of the genus *Pseudomonas* on the basis of similarities in the primary and secondary RNA structures (Chun, 1995). Phylogenetic analysis of the nucleotide sequences of the 16S rDNA showed that the M4-7 strain was closely linked with *P. alcaligenes* LMG 1224^T, with the highest sequence similarity of 99.3%. Based on these results, the isolated strain M4-7 was identified as *P. alcaligenes*.

Production and purification of MCL-PHA depolymerase

In order to obtain MCL-PHA depolymerase, the M4-7 strain was first grown by shaking on a rotary shaker at 200 rpm for 18 h at 30°C using six 2 L Erlenmeyer flasks, each containing 500 ml of marine broth (Difco 279110, USA). Cells were harvested by centrifugation and transferred to a fermentor containing an artificial seawater medium supplemented with 0.2% (wt/v) colloidal PHO. The fermentation experiments were conducted in a 5 L jar fermentor (Korea Fermentor, Korea) with a working volume of 3 L for 48 h as described elsewhere (Kim *et al.*, 2002). The temperature and pH were automatically controlled at optimal values of 30°C and pH 8.0, respectively. The total protein level in the culture supernatant was first concentrated about 100-fold by ultrafiltration using a PM10 membrane. After separating the obtained total proteins on a 8% polyacrylamide gel according to the method described by Dunn (1996), MCL-PHA depolymerase was purified by electroelution. Zymography analysis was performed using a 0.8% agarose membrane containing 0.1% PHO at 37°C. MCL-PHA depolymerase activity was routinely assayed by measuring decrease in turbidity at 650 nm of a PHO latex as previously described (Kim *et al.*, 2002). The relative molecular mass (M*r*) of the denatured MCL-PHA depolymerase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel according to the method described by Laemmli (1970). Protein concentrations were measured according to the method described by Bradford (1976) using bovine serum albumin as the standard. The effects of protein inhibitors on enzyme activity were determined using the reaction mixture (1.97 ml) containing enzymes 25 μl (15 mU) of the enzyme solution, inhibitor (1 - 10 mM), and 50 mM glycine-NaOH buffer (pH 8.0) (Kim *et al.*, 2002). After preincubation of the reaction mixture for 1 h at 37°C, the enzymatic reaction was initiated by the addition of 30 μl of a 10 mM *p*-nitrophenyloctanoate solution. The concentration of *p*-nitrophenol formed was then colorimetrically determined.

Bacterial strains and culture conditions

In order to clone the MCL-PHA depolymerase genes from *P. alcaligenes* M4-7 and *P. alcaligenes* LB19, they were first grown aerobically in Luria-Bertani (LB) broth

(1% Bactotryptone, 0.5% Bacto-yeast extract, and 0.5% NaCl, pH 7.0). The *E. coli* DH5α [relevant characteristics: *supE44 ΔlacU169*(ϕ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was grown at 37°C in an LB broth in the presence of ampicillin (50 μg/ml), when necessary. In this study, the plasmid, pBluescript II KS^+ (Stratagene, USA), was used for cloning and expression.

N-Terminal amino acid sequencing and proteolytic digestion of MCL-PHA depolymerases

The *N*-terminal amino acid sequences of the two purified $PhaZ_{Pal/LB19}$ polypeptides (Kim *et al.*, 2002) and the $PhaZ_{Pal/M4\text{-}7}$ were determined by automated Edman degradation of the peptides using a sequencer (Model 4774, Applied Biosystems, USA). The proteolytic digestion of the $PhaZ_{Pal/LB19}$ polypeptides was accomplished by treatment with 8 μg of *Staphylococcus aureus* V8 protease in the presence of 0.01% (wt/vol) SDS in 50 mM phosphate buffer (pH 7.8) for 5 h at 37°C. The resulting peptides were separated on an SDS-16% polyacrylamide gel.

Preparation of DNA probe

In this study, it was found that the *N*-terminal amino acid sequences of two $PhaZ_{Pal/LB19}$ polypeptides (M_r 26.5 kDa and M_r 27.5 kDa) (Kim *et al.*, 2002) and the proteolytic pattern of the polypeptides by V8 protease were similar to the results obtained for $PhaZ_{Pfl/GK13}$ (Schirmer and Jendrossek, 1994). Therefore, in order to amplify a MCL-PHA depolymerase DNA fragment from *P. alcaligenes* LB19 genomic DNA by PCR, 5' and 3' primers were designed on the basis of oligonucleotide primers, which were used to clone $PhaZ_{Pfl/GK13}$ (Schirmer and Jendrossek, 1994). The upstream primer (FLB) sequence used was 5'-CGCCCTGCCGAAGT-CAGCTGT-3', and the downstream primer (RLB) sequence was 5'-TTGCCNAGRAARTCRTARTC-3'.

The PCR mixture (100 μl) contained a PCR buffer, 100 pmol of each primer, 25 mM of each dNTP, 20 ng of template DNA, and 2.5 units of ExTaq DNA polymerase (Takara, Japan). A DNA thermal cycler (Perkin-Elmer Applied Biosystems, USA) was used to amplify the gene. The initial template denaturation was carried out for 5 min at 94°C. The profile, 40 sec at 94°C, 40 sec at 57°C, and 40 sec at 72°C, was subsequently repeated for 30 cycles. The amplified DNA was separated on a 0.8% agarose gel and purified using a gel extraction kit (NucleoGen, Korea). The purified PCR products were cloned into a pGEM-T easy vector (Promega, USA), and the nucleotide sequence of the target fragment was analyzed. When the oligonucleotides, FLB and RLB, were used as primers, a 341 bp fragment was amplified by PCR using the genomic DNA of the LB19 strain as the template. This fragment contained sequences coinciding with the *N*-terminal and the internal amino acid sequences at each end. A digoxigenin (DIG) DNA labeling and detection kit (Roche, Germany) was used to label the fragment.

***Cloning of the* $phaZ_{Pal/LB19}$ *and the* $phaZ_{Pal/M4\text{-}7}$**

Chromosomal DNA from the LB19 and M4-7 strains were isolated according to the method described by Syn and Swarup (2000). The genomic DNA of the LB19 strain was partially digested with the restriction endonucleases, *Pst*I, *Sma*I, and *Xho*I, and separated by agarose gel electrophoresis. The DNA was blotted onto a positively charged nylon membrane (Schleicher & Schuell, Germany) and hybridized with a 341 bp DIG-labeled DNA probe. Southern hybridization was performed at 68°C for 12 h. The positive DNA fragments were subcloned into the pBluescript II KS^+ and transformed into the *E. coli* DH5α. The genomic DNA of the M4-7 strain was partially digested with various restriction endonucleases, such as *Eco*RI, *Xba*I, *Hin*dIII, and *Xho*I, and separated by agarose gel electrophoresis. The DNA was blotted onto a positively charged nylon membrane (Schleicher & Schuell, Germany) and hybridized with a 0.5 kbp DIG-labeled DNA probe prepared with a 0.5 kbp *Pst*I fragment from the LB19 strain which was hybridized with the 341 bp DIG-labeled DNA probe.

Isolation of MCL-PHA depolymerase-expressing transformants

Approximately 580 white transformants with the 3.5 - 5.5 kbp of *Sma*I fragments from the LB19 strain and 600 white transformants with the 2.5 - 3.5 kbp of *Eco*RI fragments from the M4-7 strain, which were grown on LB agar media containing ampicillin (50 μg/ml) and pre-spread with 30 μl of X-Gal (50 mg/ml) and 50 μl of 100 mM IPTG, were transferred to the PHO-containing agar plates where 50 μl of 100 mM IPTG was added as an inducer. The transformants were incubated for 3 days at 37°C, and, in each case, one PHO-degrading clone was selected as a candidate with the full $phaZ_{Pal/LB19}$ or $phaZ_{Pal/M4\text{-}7}$.

DNA sequencing and sequence analysis

DNA sequencing was performed by a dideoxy chain-termination method using an automated DNA sequencer with *Taq* dye terminator and *Taq* dye primer cycle sequencing kits (Applied Biosystems, Model 373A, USA). Computer analysis of the resulting nucleotide sequence was performed using the DNASIS DNA and protein sequence analysis program (Hitachi Software Engineering Co., Japan).

Nucleotide sequence accession numbers

The 16S rDNA sequence of the M4-7 strain has been deposited in GenBank under accession no. AY835998. The $phaZ_{Pal/LB19}$ and $phaZ_{Pal/M4\text{-}7}$ have been deposited in GenBank under accession no. AY232717 and AY370932, respectively.

Results and Discussion

Purification of MCL-PHA depolymerase from P. alcaligenes M4-7

Because we observed high depolymerase activity of the

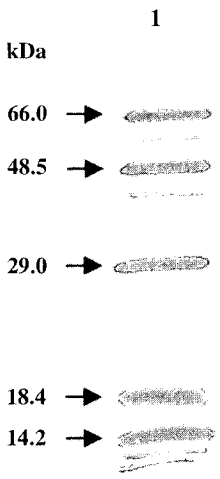

Fig. 1. SDS-PAGE of the purified MCL-PHA depolymerase from *P. alcaligenes* M4-7. Proteins were separated on an SDS-12% polyacrylamide gel and revealed by Coomassie brilliant blue R-250 staining. The numbers on the left show the molecular weight (kDa) of the standard proteins. Lanes: 1, molecular mass standards; 2, purified enzyme (4 μg).

$PhaZ_{PalM4-7}$ to PHO, even in a reaction mixture containing 40% ethanol, clearly indicating that the enzyme was not influenced by ethanol, the first attempt to purify $PhaZ_{PalM4-7}$ was achieved by hydrophobic interaction column chromatography (HIC) using Octyl-Sepharose CL-4B resins. It was found that $PhaZ_{PalM4-7}$ could strongly bind to hydrophobic materials, as do many PHA depolymerases (Schirmer *et al.*, 1993; Kim *et al.*, 2002). However, when the enzymes which had adsorbed to the resins in the column were eluted with a gradient of 0 - 50% ethanol, a significant reduction in enzyme activity occurred, suggesting that this strategy may not be suitable for the purification of $PhaZ_{PalM4-7}$. It is presumed that the decrease in enzyme activity during HIC is closely related to the modification of $PhaZ_{PalM4-7}$, such as partial cleavage of a polypeptide, as demonstrated in the case of the PHB depolymerase of *Marinobacter* sp. NK-1 (Kasuya *et al.*, 2000). Therefore, after separating the whole protein of the culture supernatant on an 8% polyacrylamide gel, we purified $PhaZ_{PalM4-7}$ by electroelution. The homogeneity of the purified $PhaZ_{PalM4-7}$ was confirmed by SDS-PAGE (Fig. 1).

Properties of MCL-PHA depolymerase from P. alcaligenes M4-7

Biochemical characteristics of the purified $PhaZ_{PalM4-7}$ are listed in Table 1. The relative molecular mass (M_r) of the enzyme was estimated to be 28 kDa, as determined by SDS-PAGE. This M_r of $PhaZ_{PalM4-7}$ was similar to that (25 - 30 kDa) of most MCL-PHA depolymerases, as

Table 1. Biochemical properties of various MCL-PHA depolymerases

Characteristics	$PhaZ_{PflGK13}$	$PhaZ_{PspRY-1}$	$PhaZ_{XspJS02}$	$PhaZ_{PalLB19}$	$PhaZ_{SspKJ-72}$	$PhaZ_{PinK2}$	$PhaZ_{PalM4-7}$
Quaternary structure	dimer	tetramer	monomer	monomer	monomer	ND	ND
M_r (SDS-PAGE) (kDa)	25.0	28.0	41.7	27.6	27.1	28.0	28.0
pI	5.7[a]	5.9	ND[b]	5.7	4.7	ND	5.9[a]
Carbohydrate content	-	ND	ND	ND	-	ND	ND
Optimum pH	8.5	8.5	8.5	9.0	8.7	8.5	9.0
Optimum temp. (°C)	45	35	60	45	50	35	35
Sensitivity to							
DTT	-	-	ND	-	-	-	-
DFP	ND	ND	ND	+	+	+	+
PMSF	-	-	ND	+	-	+	+
PHMB	-	ND	ND	+	-	+	+
Sodium azide	-	-	ND	+	-	+	-
Iodoacetamide	ND	ND	ND	+	-	ND	-
NEM	ND	ND	ND	+	ND	ND	+
NBSI	ND	ND	ND	+	+	ND	+
Acetic anhydride	ND	ND	ND	+	+	ND	+
EDTA	-	+	ND	+	-	+	+
Triton X-100	+	+	ND	+	+	+	+
Tween 80	+	+	ND	+	+	+	+
Main hydrolysis product of MCL-PHA	dimer	ND	ND	monomer	dimer	ND	ND
Reference	Schirmer *et al.*, 1993	Kim *et al.*, 2000a	Kim *et al.*, 2000b	Kim *et al.*, 2002	Kim *et al.*, 2003	Elbanna *et al.*, 2004	This study

[a]Theoretical value calculated from amino acid sequences. [b]Not determined. DTT, dithiothreitol; DFP, diisopropylfluoro phosphate; PMSF, phenylmethylsulfonyl fluoride; PHMB, *p*-hydroxymercuribenzoic acid; NEM, *N*-ethylmaleimide; NBSI, *N*-bromosuccinimide.

shown in Table 1, but was smaller than that of $PhaZ_{XspJS02}$ (M_r, 41.7 kDa) (Kim *et al.*, 2000b). Meanwhile, it has been reported that most SCL-PHA depolymerases have bigger M_r (40 - 50 kDa) compared to MCL-PHA depolymerases (Jendrossek, 2001). The $PhaZ_{PalM4-7}$ showed over 60% of its maximum activity in the pH range 7.0 to 9.5, and was drastically inactivated at pH values above 10.5. The highest activity of the enzyme was obtained in 50 mM glycine-NaOH buffer, pH 9.0. Similar observations of enzymes with optimum alkaline pH have also been made from all MCL-PHA depolymerases reported thus far (Table 1). These results suggest that MCL-PHA depolymerases are alkaline enzymes exhibiting maximum activity in the range of pH 8.5 - 9.0. In contrast, some bacterial and fungal SCL-PHA depolymerases were most active at acidic or neutral pH ranges (Jendrossek, 2001; Kim and Rhee, 2003). $PhaZ_{PalM4-7}$ was fairly stable at pH values between 7.0 and 10.0, retaining more than 95% of its original activity even after 1 h pre-incubation at these pH values. The optimum temperature of $PhaZ_{PalM4-7}$ was 35°C. It was relatively stable for 1 h below 40°C, but was significantly inactivated to less than 10% of its original activity within 10 min when exposed to temperatures above 52°C. The optimum temperature (35°C) of this enzyme is also identical to those of $PhaZ_{PspRY-1}$ (Kim *et al.*, 2000a) and $PhaZ_{PinK2}$ (Elbanna *et al.*, 2004), while it is much lower than those of $PhaZ_{XspJS02}$ (60°C) (Kim *et al.*, 2000b), $PhaZ_{SspKJ-72}$ (50°C) (Kim *et al.*, 2003), $PhaZ_{PflGK13}$ (45°C) (Schirmer *et al.*, 1993), and $PhaZ_{PalLB19}$ (45°C) (Kim *et al.*, 2002).

The inhibitory effects of various types of protein inhibitors on $PhaZ_{PalM4-7}$ activity are also listed in Table 1. Dithiothreitol, which reduces disulfide bonds, had no effect on the enzyme activity after 1 h of pre-incubation at 35°C. It should be noted that all known MCL-PHA depolymerases were not inhibited by dithiothreitol, in contrast to the inhibition of most SCL-PHA depolymerases by dithiothreitol. These results suggest that disulfide linkages in the active sites of MCL-PHA depolymerases may either be absent or are not essential. $PhaZ_{PalM4-7}$ was significantly inhibited by *p*-hydroxymercuribenzoic acid and *N*-ethylmaleimide at a concentration of 10 mM, but was insensitive to other sulfhydryl reagents, such as sodium azide and iodoacetamide. Inhibition of enzyme activity by *N*-bromosuccinimide and acetic anhydride indicated that tryptophan and lysine residues play key roles in the catalytic domain of the enzyme. Sensitivity of $PhaZ_{PalM4-7}$ to EDTA reveals that this enzyme is a metalloprotein which requires metal ions as a cofactor for catalytic activity. The inactivation of the enzyme by both diisopropyl fluorophosphates (DFP) and phenylmethylsulfonyl fluoride (PMSF) strongly suggested the presence of serine residues in its active site. However, although greatly inhibited by DFP, $PhaZ_{PflGK13}$ (Schirmer *et al.*, 1993), $PhaZ_{PspRY-1}$ (Kim *et al.*, 2000a), and $PhaZ_{SspKJ-72}$ (Kim *et al.*, 2003) were not susceptible to

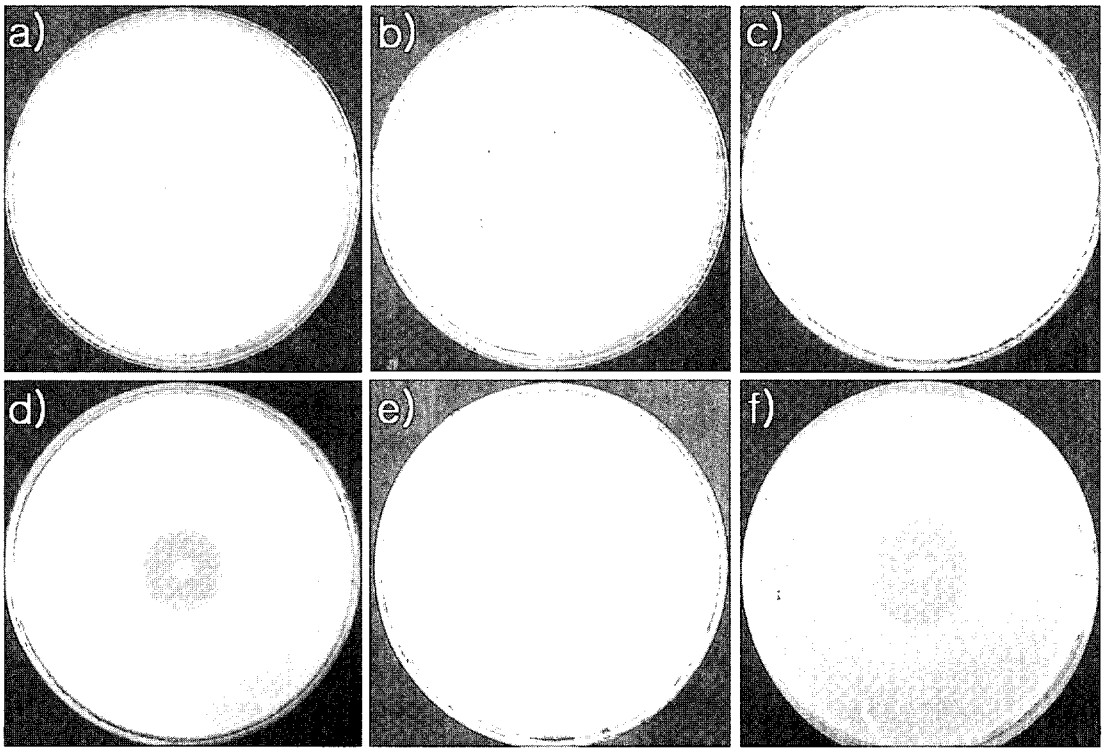

Fig. 2. Biodegradation of various polyesters by *P. alcaligenes* M4-7 grown on an artificial seawater mineral medium containing respective polymer as the sole carbon source. The experiments were done at 30°C for 3 days. a) PHB; b) PHBV with 25 mol% 3HV; c) PHBV with 60 mol% 3HV; d) PCL; e) PHO; f) PHPV.

PMSF. We believe that the resistance of the enzymes to PMSF is due to their compact architecture, which probably makes the active serine fairly inaccessible to PMSF, with its bulky aromatic moiety. It appears that $PhaZ_{PalM4-7}$ was completely inhibited by nonionic detergents, such as 0.1% Tween 80 and 0.05% Triton X-100, as shown in the other PHA depolymerases reported so far.

In the present study, it was found that *P. alcaligenes* M4-7 could efficiently degrade various polyesters, including PHBV with 25 and 60 mol% 3HV, PCL, PHO, PHN, and PHPV (Fig. 2). However, its ability to degrade PHB homopolymer was very weak, implying that SCL-PHA depolymerase from *P. alcaligenes* M4-7 is a distinct enzyme with different substrate specificities compared to the general PHB depolymerases. The purified $PhaZ_{PalM4-7}$ could hydrolyze PHO, PHN, and PHPV, but not PHB, PHBV, and PCL. This suggests that *P. alcaligenes* M4-7 has at least three different extracellular hydrolases, including SCL-PHA depolymerase, MCL-PHA depolymerase, and PCL depolymerase. It has also been reported that *Streptomyces* sp. KJ-72 (Kim *et al.*, 2003), *S. exfoliatus* (Klingbeil *et al.*, 1996), *P. indica* K2 (Elbanna *et al.*, 2004), and *Xanthomonas* sp. JS02 (Kim *et al.*, 2000b) can decompose both SCL-PHA and MCL-PHA, depending on the PHA depolymerase produced for the respective polymer.

On the basis of the biochemical properties of MCL-PHA depolymerases shown in Table 1, we propose that MCL-PHA depolymerases share some characteristics, described as follows: (1) M_r (25.0 - 30.0 kDa) of most MCL-PHA depolymerases is smaller than those (40.0 - 50.0 kDa) of SCL-PHA depolymerases, although the M_r of the $PhaZ_{XspJS02}$ (Kim *et al.*, 2000b) was 41.7 kDa; (2) pI value is in the acidic range; (3) They are most active at alkaline pH value (8.0 - 9.0); (4) In contrast to SCL-PHA depolymerases, MCL-PHA depolymerases are insensitive to DTT; (5) They are inactivated by either PMSF or DFP, as well as by detergents, such as Tween 80 or Triton X-100.

N-Terminal amino acid sequences

The *N*-terminal amino acid sequences of the purified $PhaZ_{PalLB19}$ that was separated on a SDS-polyacrylamide gel into two different polypeptides with a M_r of 26.5 kDa and 27.5 kDa (Kim *et al.*, 2002), respectively, were determined by automated Edman degradation. The amino acid sequence in the *N*-terminus of the larger polypeptide was ASRXSERPRTLLRPAEV, while the shorter polypeptide contained a sequence of RPAEVSXSYQSTWLDSG in the *N*-terminal region. These *N*-terminal amino acid sequences of the polypeptides were nearly identical to the sequences in the *N*-terminal region of the premature $PhaZ_{PflGK13}$ from *P. fluorescens* GK13 (Schirmer and Jendrossek, 1994). When the denatured $PhaZ_{PalLB19}$ was partially digested with V8 protease, an approximately 14 kDa polypeptide fragment was generated as the major product. This proteolytic pattern of $PhaZ_{PalLB19}$ was also similar to that of $PhaZ_{PflGK13}$. Therefore, the *N*-terminal amino acid sequence of the fragment was not determined. The amino acid sequence in the *N*-terminus of the $PhaZ_{PalM4-7}$ was DSRXSERAKTLLLPA. This was also similar to the sequences in the *N*-terminal region of the larger $PhaZ_{PalLB19}$ polypeptide and the premature $PhaZ_{PflGK13}$ (Schirmer and Jendrossek, 1994).

Cloning of $PhaZ_{PalLB19}$ *and* $PhaZ_{PalM4-7}$ *genes*

Southern blots of *Pst*I, *Sma*I, and *Xho*I-digested genomic DNA of *P. alcaligenes* LB19 were hybridized with a 341 bp DIG-labeled DNA probe and, as a result, only one signal appeared in all cases. The DNA probe hybridized with a 0.5 kbp *Pst*I fragment, while it combined with a 4.7 kbp *Sma*I fragment and a 5.6 kbp *Xho*I fragment. From these results, it was concluded that the 0.5 kbp *Pst*I fragment does not contain the complete $PhaZ_{PalLB19}$ gene, but the *Sma*I and *Xho*I fragments may have a full open reading frame encoding $PhaZ_{PalLB19}$. Meanwhile, the 0.5 kbp DIG-labeled *Pst*I fragment hybridized with a 2.8 kbp fragment of *Eco*RI-digested genomic DNA from *P. alcaligenes* M4-7, which may contain a full open reading frame encoding $PhaZ_{PalM4-7}$.

Approximately 580 recombinant strains of *E. coli* DH5α harboring partially *Sma*I-digested genomic DNA (3.5 - 5.5 kbp) from *P. alcaligenes* LB19 in pBluescript II KS^+ were screened for expression of MCL-PHA depolymerase activity. One clone produced a large translucent halo on the M9 solid medium containing PHO as the sole carbon source, suggesting that it contains the complete structural gene of $PhaZ_{PalLB19}$. An analysis of the recombinant plasmid of the clone revealed the presence of a 4.7 kbp *Sma*I fragment. To minimize the region encoding the full $PhaZ_{PaLB19l}$ gene, a cloned 4.7 kbp *Sma*I fragment was digested with several endonucleases. The resulting fragments were subcloned into the pBluescript II KS^+ vector and their halo-forming abilities were evaluated in *E. coli*. As a result, a 1373 bp *Kpn*I-*Sma*I fragment was found to confer the halo-forming ability in the smallest size. Approximately 600 recombinant strains of *E. coli* DH5α harboring partially *Eco*RI-digested genomic DNA (2.5 - 3.5 kbp) from *P. alcaligenes* M4-7 in pBluescript II KS^+ were also screened for expression of MCL-PHA depolymerase activity. As a result, one clone with a 2.8 kbp *Eco*RI fragment was isolated as a candidate capable of degrading PHO, indicating that it possesses the full open reading frame of the $PhaZ_{PalM4-7}$ gene.

Nucleotide and deduced amino acid sequences of the $\mathbf{phaZ_{PalLB19}}$ *and the* $\mathbf{phaZ_{PalM4-7}}$

The nucleotide sequence of a 1373 bp *Kpn*I-*Sma*I fragment determined for both strands is shown in Fig. 3. From the results, one 837 bp open reading frame was identified and assigned to the structural gene of $PhaZ_{PalLB19}$. The

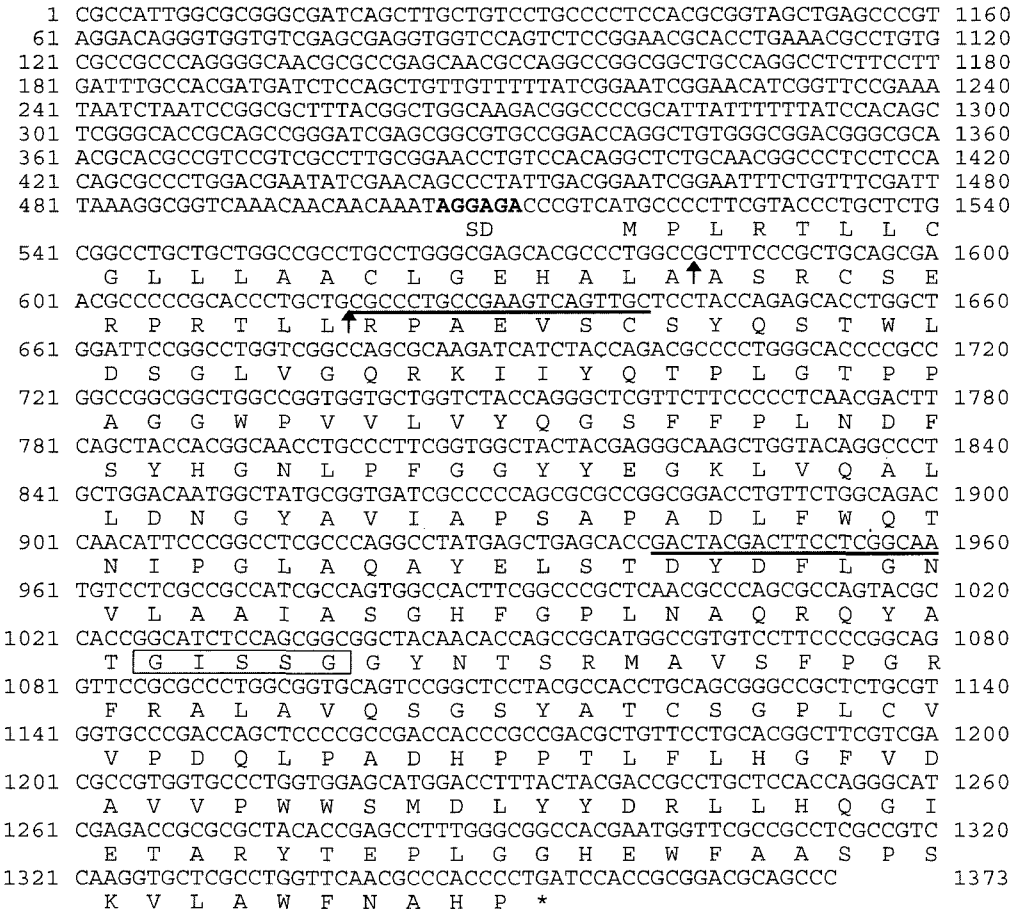

Fig. 3. Nucleotide sequence of a 1373 bp *Kpn*I-*Sma*I fragment containing the MCL-PHA depolymerase gene from *P. alcaligenes* LB19 and the deduced amino acid sequence. The putative ribosome binding site is in boldface. The cleavage sites of the pre-protein are indicated by arrows and the lipase box is shown as a box. Underlines indicate the binding positions of a sense (FLB) or an anti-sense (RLB) primer for PCR. A possible terminator sequence is indicated by an asterisk.

assumed coding sequence began with the initiation codon, ATG, at position 518. This was preceded by a potential ribosomal binding site located from position 508 to position 513, which extended to the stop codon, TGA, at position 1354. The encoded polypeptide is a protein of 278 amino acids and has a deduced M_r of 30,188 Da. Interestingly, the molecular structure of the premature PhaZ$_{Pal\text{LB19}}$ was quite similar to that of the premature PhaZ$_{Pf\text{GK13}}$ (Schirmer and Jendrossek, 1994). A 97% (270/278) amino acid sequence homology was observed between the two proteins, although the size (278 amino acids) of the two polypeptides was identical.

Fig. 4 shows that the PhaZ$_{Pal\text{M4-7}}$ gene consists of an 834 bp open reading frame encoding a protein of 277 amino acids with a deduced M_r of 30,323 Da. PhaZ$_{Pal\text{M4-7}}$ exhibited a high homology percentage (87%) with PhaZ$_{Pal\text{LB19}}$ or PhaZ$_{Pf\text{GK13}}$.

The amino acid residues, 35 to 51, of the deduced gene product of *phaZ*$_{\text{Pal}LB19}$ were identical to the *N*-terminal amino acid sequence determined for the mature PhaZ$_{Pal\text{LB19}}$ polypeptide with an M_r of 26.5 kDa measured by SDS-PAGE. Furthermore, the amino acid residues, 23 to 39, of the deduced gene product corresponded to the *N*-terminal amino acid sequence determined for the mature PhaZ$_{Pal\text{LB19}}$ polypeptide with an M_r of 27.5 kDa. The deduced molecular masses (26,555 Da and 27,926 Da) of the two PhaZ$_{Pal\text{LB19}}$ polypeptides were in good agreement with the molecular masses (26.5 kDa and 27.5 kDa) of the purified PhaZ$_{Pal\text{LB19}}$ polypeptides determined by SDS-PAGE. The calculated pI values of the larger polypeptide and the smaller polypeptide were 5.94 and 5.53, respectively. Based on these results, it was concluded that the secretion of the two PhaZ$_{Pal\text{LB19}}$ polypeptides by *P. alcaligenes* LB19 was due to the presence of two different processing sites (^{22}Ala–^{23}Ala, ^{34}Leu–^{35}Arg) for the signal sequence in the *N*-terminus region of the premature protein. A similar phenomenon was also observed in the case of PhaZ$_{Pf\text{GK13}}$ (Schirmer and Jendrossek, 1994). However, the post-translational modification of the premature PhaZ$_{Pf\text{GK13}}$ resulted in the formation of two polypeptides with an M_r of 26,573 and an M_r of 26,687, respectively. This is due to the occurrence of proteolytic cleavages at

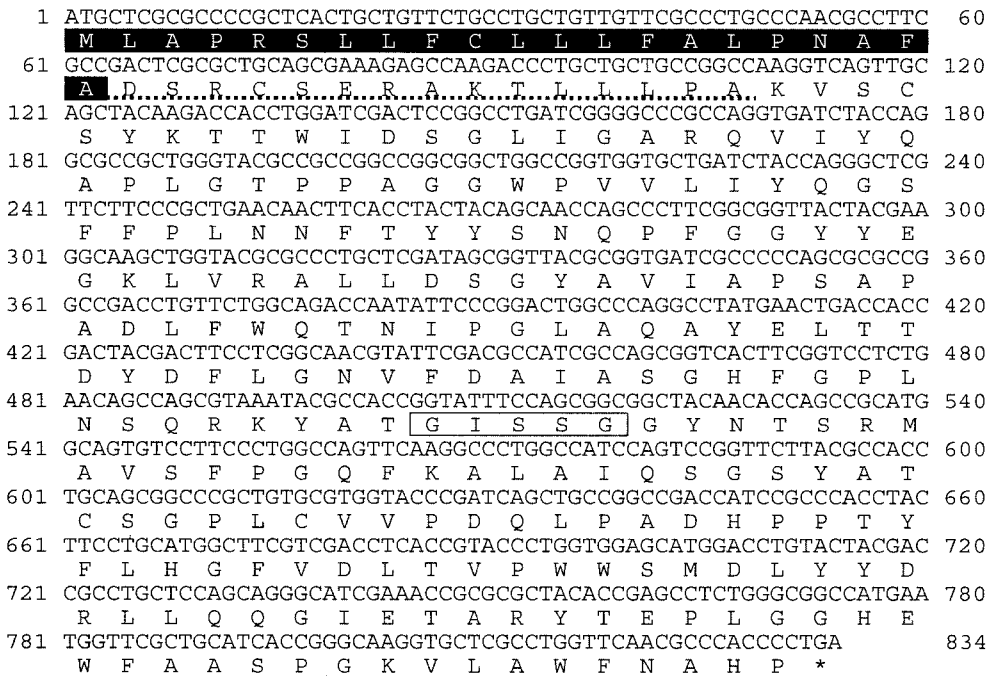

Fig. 4. Nucleotide sequence of the MCL-PHA depolymerase gene from *P. alcaligenes* M4-7 and the deduced amino acid sequence. The signal peptide is marked in black and the lipase box is shown as a box. The broken line indicates the sequence determined by the Edman degradation method. A possible terminator sequence is indicated by an asterisk.

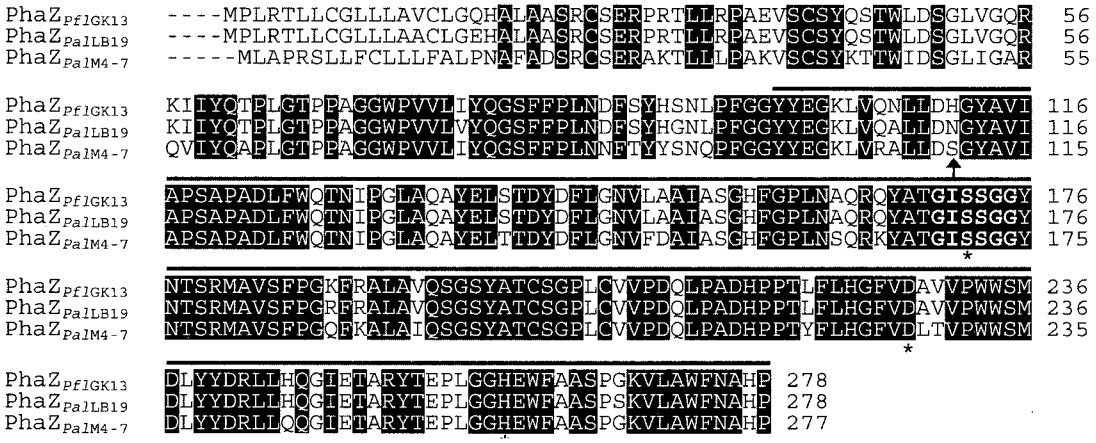

Fig. 5. Multiple alignments of the deduced amino acid sequences of the extracellular MCL-PHA depolymerases. The positions of amino acids conserved in all sequences are marked in black. Lipase boxes are in boldface and other conserved amino acids that might constitute a catalytic triad are indicated by asterisks. The oxyanion is indicated by an arrow. Catalytic domains are indicated by a black bar. The proteins are indicated in abbreviations. Their accession numbers and origins are: $PhaZ_{PflGK13}$ (U10470, *P. fluorescens* GK13), $PhaZ_{PalLB19}$ (AY232717, *P. alcaligenes* LB19), and $PhaZ_{PalM4-7}$ (AY370932, *P. alcaligenes* M4-7).

two different positions, ^{33}Leu−^{34}Leu and ^{34}Leu−^{35}Arg, in the *N*-terminal region of the premature $PhaZ_{PflGK13}$.

As shown in Fig. 5, the *N*-terminal amino acid sequences (DSRXSERAKTLLLPA) of the mature $PhaZ_{PalM4-7}$ were identical to the amino acid residues, 22 to 36, of the deduced gene product of $phaZ_{PalM4-7}$. The calculated molecular mass (28,019 Da) of the mature $PhaZ_{PalM4-7}$ was in agreement with the molecular mass (28 kDa) of $PhaZ_{PalM4-7}$ determined by SDS-PAGE. The deduced pI value of the enzyme was 5.9.

Fig. 5 illustrates that the *N*-terminal regions of the mature $PhaZ_{PalLB19}$ and $PhaZ_{PalM4-7}$ contained many hydrophobic amino acids, such as proline, valine, leucine, and serine, suggesting that the substrate-binding domain is located in this region. Previous studies using PCR-based mutagenesis analysis showed that the putative substrate-binding domain of $PhaZ_{PflGK13}$ is located in the *N*-terminal region of the premature protein (amino acid ^{34}Leu-^{98}Gly) (Jendrossek *et al.*, 1997). In contrast to all SCL-PHA depolymerases, a catalytic triad of the premature

$PhaZ_{PalLB19}$ (^{172}Ser, ^{228}Asp, ^{262}His) and the premature $PhaZ_{PalM4-7}$ (^{171}Ser, ^{227}Asp, ^{261}His) existed in the *C*-terminus region, which is similar to that (^{172}Ser, ^{228}Asp, ^{262}His) of the premature $PhaZ_{PflGK13}$. This indicates the presence of a catalytic domain in the *C*-terminus region. Based on these data, it is strongly proposed that extracellular MCL-PHA depolymerases probably share the type IV depolymerase of the composite structure offered by Klingbeil *et al.* (1996).

Three MCL-PHA depolymerases from *P. alcaligenes* LB19, *P. alcaligenes* M4-7, and *P. fluorescens* GK13 (Schirmer and Jendrossek, 1994) commonly share a lipase consensus sequence, GISSG (Fig. 5). However, it is of note that a putative His residue (oxyanion), which is found in most extracellular PHA depolymerases and lipases (Jendrossek, 2001), was replaced by ^{111}Asn in the premature $PhaZ_{PalLB19}$ and by ^{110}Ser in the premature $PhaZ_{PalM4-7}$. Considering that the highly conserved oxyanion (His) might participate in the transient state stabilization of the hydrolysis reaction by allowing the formation of a hydrogen bond to the negatively-charged oxygen atom of the active site Ser (Jendrossek, 2001), it is strongly believed that ^{111}Asn in the premature $PhaZ_{PalLB19}$ and ^{110}Ser in the premature $PhaZ_{PalM4-7}$ might also participate in hydrolytic reactions as another form of oxyanion (His). A previous study clearly showed that ^{221}Ser and ^{155}Asn in a subtilisin play key roles as potential hydrogen-bond donors in transition-state stabilization of hydrolytic reactions (Roberts *et al.*, 1972).

In conclusion, the present results demonstrate that $PhaZ_{PalLB19}$ and $PhaZ_{PflGK13}$ have significant similarities in terms of molecular structure, although, as previously described, some of their biochemical properties are significantly different from each other (Schirmer *et al.*, 1993; Kim *et al.*, 2002). Therefore, the apparent differences in the enzymatic characteristics of the two MCL-PHA depolymerases, such as their quaternary structure, sensitivity to some inhibitors (PMSF, sodium azide, *p*-hydroxymercuribenzoic acid, and EDTA), and the main hydrolysis product of a MCL-PHA, are explained by the small differences in the amino acid sequence of the mature proteins influencing the three-dimensional structure. Despite the fact that the composite structure of mature $PhaZ_{PalM4-7}$ is very similar to that of $PhaZ_{PflGK13}$ (Schirmer and Jendrossek, 1994) and $PhaZ_{PalLB19}$, some distinct characteristics (optimum temperature and sensitivities to protein inhibitors) of $PhaZ_{PalM4-7}$ demonstrated in this study are also consistent with the explanation above that substitutions of amino acids in the mature protein probably resulted in the formation of a protein with a specific quaternary structure. From an evolutionary perspective, the high sequence similarities of molecular structure among $PhaZ_{PalM4-7}$, $PhaZ_{PalLB19}$, and $PhaZ_{PflGK13}$ suggest the possibility of horizontal transfer of the MCL-PHA depolymerase gene in the *Pseudomonas* strains.

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