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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Identification and characterization of novel poly(DL-lactic acid) depolymerases from metagenome

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Abstract Many poly(lactic acid) (PLA)-degrading microorganisms have been isolated from the natural environment by culture-based methods, but there is no study about unculturable PLA-degrading microorganisms. In this study, we constructed a metagenomic library consisting of the DNA extracted from PLA disks buried in compost. We identified three PLA-degrading genes encoding lipase or hydrolase. The purified enzymes degraded not only PLA, but also various aliphatic polyesters, tributyrin, and *p*-nitrophenyl esters. From their substrate specificities, the PLA depolymerases were classified into an esterase rather than a lipase. Among the PLA depolymerases, PlaM4 exhibited thermophilic properties; that is, it showed the highest activity at 70 °C and was stable even after incubation for 1 h at 50 °C. PlaM4 had absorption and degradation activities for solid PLA at 60 °C, which indicates that the enzyme can effectively degrade PLA in a high-temperature environment. On the other hand, the enzyme classification based on amino acid sequences showed that the other PLA depolymerases, PlaM7 and PlaM9, were not classified into known lipases or esterases. This is the first report on the identification and characterization of PLA depolymerase from a metagenome.

Keywords Metagenome · Depolymerase · Esterase · Biodegradable plastic · Polylactide · Lipase

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Introduction

Biodegradable aliphatic polyesters such as poly(lactic acid) (PLA), poly(butylene succinate) (PBS), and poly(butylene succinate-*co*-adipate) (PBSA) are one of the most commercially promising materials. In particular, PLA is expected to become widely used as an alternative to ordinary plastics owing to its good chemical properties and ease of synthesis by conventional processes. On the other hand, PBS and PBSA have already been used as agricultural multifilms (Gross and Kalra 2002).

It has been confirmed that PLA, PBS, and PBSA are degraded by microorganisms in soil or compost (Ghorpade et al. 2001; Jarerat et al. 2002; Suyama et al. 1998), and some of these degrading microorganisms have been characterized (Kleeberg et al. 1998; Maeda et al. 2005; Pranamuda et al. 1995; Tseng et al. 2007). Pranamuda et al. (1997) were the first to isolate a PLA-degrading *Amycolatopsis* sp. strain, HT-32, and the purified PLA-degrading enzyme from this strain also showed protease activity toward other proteins such as silk (Pranamuda et al. 2001). Nakamura et al. (2001) also purified a PLA-degrading enzyme from *Amycolatopsis* sp. K104-1, which also degraded casein and fibrin. This enzyme has been cloned and identified as a type of elastase-like protease (Matsuda et al. 2005). On the other hand, some lipases or esterases have also been reported to be PLA-degrading enzymes. Sakai et al. (2001) have purified a thermophilic PLA depolymerase from *Bacillus smithii* and shown that it exhibited esterase activity. Teeraphatpornchai et al. (2003) have isolated the *Panibacillus amylolyticus* strain TB-13 that degrades various aliphatic polyesters including PLA from soil samples, and Akutsu-Shigeno et al. (2003) were the first to successfully clone a PLA depolymerase gene (*plaA*) from this bacterium. Because PlaA can degrade not

only PLA and PBSA but also triglycerides and *p*-nitrophenyl esters, the enzyme belongs to the lipase/esterase family. Recently, Masaki et al. (2005) have reported that a cutinase from the yeast *Cryptococcus* sp. strain S-2 can degrade high-molecular-weight PLA and other aliphatic polyesters.

Recently, various molecular analyses such as 16S rDNA studies have confirmed that only less than 1% of microorganisms in the natural environment can be cultured by traditional culture-based methods (Bintrim et al. 1997; Rondon et al. 1999). We strongly predict that some unculturable microorganisms may also be associated with PLA biodegradation. Unfortunately, all PLA-degrading microorganisms had been isolated by traditional culture-based method, and to the best of our knowledge, no studies of unculturable microorganisms associated with PLA biodegradation have been reported. From these viewpoints, it is important to obtain knowledge on PLA biodegradation by microorganisms including those that are unculturable.

In this study, we have constructed libraries with the metagenome extracted from PLA disks buried in compost. As a result of screening for PLA-degrading enzyme genes from their metagenomic libraries, we identified three PLA depolymerase genes, and the enzymes they encoded were characterized. This is the first report on the identification of PLA depolymerase from a metagenome.

Materials and methods

Chemicals Poly(DL-lactic acid) with weight-average molecular weights of 0.5×10^4 (PLA0005) and 2.0×10^4 (PLA0020) and poly(caprolactone) (PCL) were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Poly (L-lactic acid) with a weight-average molecular weight of 13×10^4 (PLA) was kindly supplied by Toyota Motor (Aichi, Japan). PBS (BIONOLLE 1001™; PBS1001; weight-average molecular weight, 2.6×10^5) and PBSA (BIONOLLE emulsion EM-301™ were purchased from Showa Highpolymer (Tokyo, Japan). Poly(ethylene succinate) (PES) was provided by Nippon Shokubai. Poly (3-hydroxybutyric acid) (PHB) was purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). All other compounds used were standard commercial preparations.

Bacterial strains and plasmids Escherichia coli DH10B and the pUC18 vector (Toyobo, Osaka, Japan) were used for the construction of metagenomic libraries. The *E. coli* strain BL21(DE3) and pET-24a(+) (Novagen, Madison, WI, USA) were used as the expression host and plasmid, respectively.

DNA preparation from PLA disks buried in compost Compost was supplied by Mizuho (Ibaraki, Japan). PLA disks (2 cm diameter, 4 mm thickness), which were molded from a PLA pellet, were buried in compost. After incubation for 10 days at 65 °C, PLA disks were removed, and adherent soil was gently brushed off from the PLA disks and used for DNA extraction. The method of DNA extraction from the PLA disks was based on direct lysis methods (Gabor et al. 2003). The extracted DNA was purified by electroelution (Rondon et al. 2000).

PCR amplification, cloning, and sequencing of 16S rDNA from metagenome The 16S rRNA genes were amplified by polymerase chain reaction (PCR) from the metagenome extracted from the PLA disks as a template, using Ex *Taq* polymerase (Takara Bio, Shiga, Japan), with eubacterium-specific primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1494R (5′-TGACTGACTGAGGYTACCTTGT TAC-3′). The PCR conditions were as follows: 1 cycle of 2 min at 94 °C, then 30 cycles each of 20 s at 94 °C, 10 s at 50 °C, and 2 min at 72 °C. The PCR products were cloned into the pGEM-T Easy cloning vector (Promega, Madison, WI, USA). Thirty-five clones were randomly selected from the clone libraries, and cloned 16S rDNA was sequenced using an ABI Prism 310 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The resulting sequence was compared with sequences in the National Center for Biotechnology Information GeneBank database using the BLASTn (basic local alignment search tool [BLAST]) program.

Screening for PLA-degrading clones from metagenomic libraries Purified DNA was partly digested with *Sau*3AI, and DNA fragments between 2 and 4 kb were recovered and ligated into *BamH*I-digested and dephosphorylated pUC18. The products were transfected into *E. coli* DH10B. For the detection of transformants with PLA degradation activity, emulsified PLA solution (1%) containing 1.5% agar was prepared and overlaid on Luria–Bertani (LB) agar plates (Akutsu-Shigeno et al. 2003). Colonies that showed clear zones on the indicator plates were isolated as positive clones.

DNA sequencing and analysis DNA sequencing was carried out using an ABI Prism 310 DNA sequencer. The nucleotide and amino acid sequences were analyzed using the GENETYX-MAC program, version 10 (Software Development, Tokyo, Japan), and BLAST on the National Center for Biotechnology Information. The putative signal peptides were predicted using the SignalP program, version 3.0 (Bendtsen et al. 2004). Amino acid sequences were aligned using the Clustal X program, and the phylogenetic tree was constructed using the NJprot program on the basis of the amino acid alignment (Jeanmougin et al. 1998; Fig. 1).

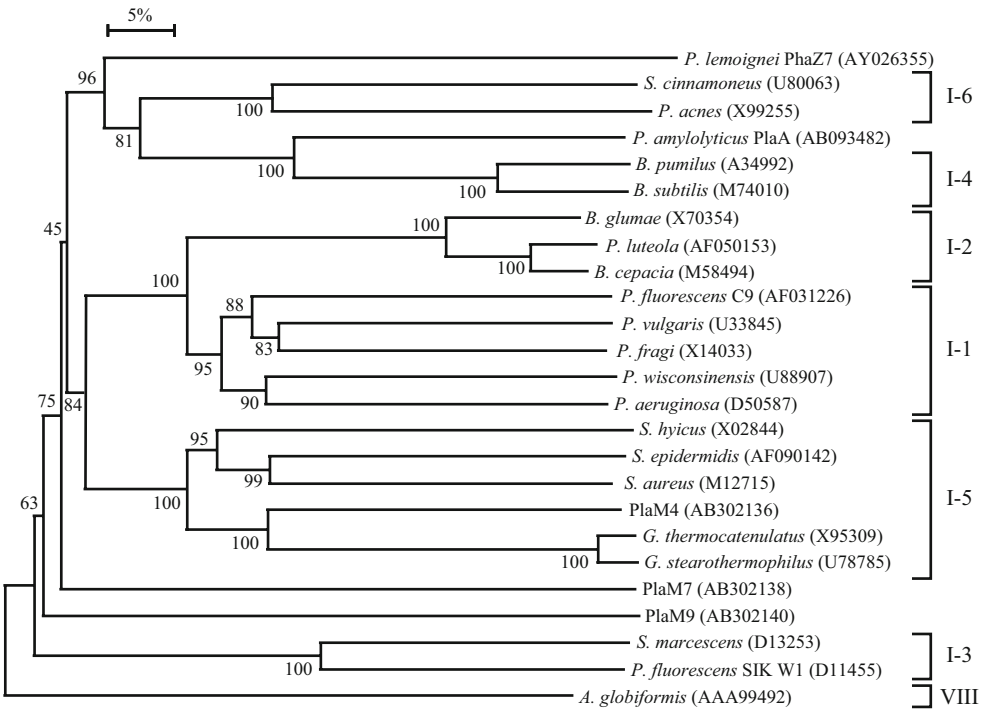

Fig. 1 Phylogenetic tree of PLA depolymerases (PlaM4, PlaM7, and PlaM9) and family I lipases. The tree includes the esterase from *Arthrobacter globiformis* belonging to family VIII as an outgroup. The *numbers at the nodes* indicate the percent recovery in 100 bootstrap resampling

Plasmid construction for expression of PLA depolymerase genes The genes encoding putative PLA depolymerase were subcloned into the vector pET21a(+) by PCR amplification. For subcloning *plaM4*, the restriction sites for *Nhe*I and *Hind*III were incorporated into the forward and reverse primer sequences, respectively. To amplify from Ser^{28} to Tyr^{431}, the following primers were used: forward, 5′-CCTAGCTAGCAGTGAAAAACATTACAAGCC-3′ and reverse, 5′-GCCCAAGCTTATAATCATCAGGCAAAGAAT-3′ (the *Nhe*I and *Hind*III restriction sites are underlined, respectively). The construct was designated pLA-NH4. For subcloning *plaM7*, the restriction sites for *Nde*I and *Xho*I were incorporated into the forward and reverse primer sequences, respectively. To amplify from Ser^{27} to Arg^{283}, the following primers were used: forward, 5′-GGAATTCCATATGTCGGAAAAACAG TTCGATCTGGTTCTC-3′ and reverse, 5′-CCG CTCGAGCCGCAAGACTTCCGCCG C-3′ (the *Nde*I and *Xho*I restriction sites are underlined, respectively). The construct was designated pLA-NX7. For subcloning *plaM9*, the restriction sites for *Nde*I and *Not*I were incorporated into the forward and reverse primer sequences, respectively. To amplify from Met^{1} to Lys^{338}, the following primers were used: forward, 5′-GGAATTCCATATGCATGAGTCGGTC CATGC-3′ and reverse, 5′-ATAAGAATGCGGCCGC TACTTCGACAAATTACGCAAAATTCCCG-3′ (the *Nde*I and *Not*I restriction sites are underlined, respectively). The construct was designated pLA-NN9.

Expression and purification of PLA depolymerases *E. coli* BL21(DE3) cells transformed with pLA-NH4, pLA-NX7, and pLA-NN9 were inoculated to 100 ml of LB medium (containing 100 μg of ampicillin/ml). After incubation for 3 h at 37 °C, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM, and the culture was further incubated for 3 h at 37 °C. Cells were harvested by centrifugation at 8,000×*g* for 10 min at 4 °C. Harvested cells were sonicated, and the crude enzyme fraction was loaded onto an Ni^{2+}-immobilized Chelating Sepharose Fast Flow (Pharmacia Biotech, Upsala, Sweden) column (1.0 by 10 cm) equilibrated with 20 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl and 60 mM imidazole. The column was eluted with 150 mM imidazole for 10 min at a flow rate of 1.0 ml/min. The eluted solution samples showing PLA degradation activity were pooled and concentrated by ultrafiltration (YM10 membrane; Millipore, Bedford, MA, USA).

Enzyme activity assay for various substrates

(1) Degradation activities toward PLA and other aliphatic polyesters. The degradation activities toward PLA and other aliphatic polyesters were determined by measuring the decrease in the turbidity of the emulsions of these substrates. Emulsified PLA and other aliphatic polyesters were prepared as described by Teeraphatpornchai et al. (2003). These emulsions were diluted with 20 mM potassium phosphate buffer (pH 7.0) to obtain an optical density (OD) 1.0 at 580 nm. The reaction was started by adding 0.05 ml of enzyme solution containing 30 μg of protein to 0.15 ml of

emulsion in a 96-well microtiter plate and incubating the mixture for 30 min at 30 °C. When the reaction was terminated, turbidity was measured at a wavelength of 580 nm.

(2) *p*-Nitrophenyl esters. Esterase activities for the *p*-nitrophenyl esters of acetate (C2), butyrate (C4), caproate (C6), caprylate (C8), caprate (C10), palmitate (C16), and stearate (C18) were assayed by incubating the enzyme with 1 mM substrates at 30 °C in 100 mM potassium phosphate buffer (pH 7.0). The reaction was measured at 410 nm, and one unit was defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol per minute.

(3) Triglycerides. Emulsions of triolein and tributyrin were prepared as described above and diluted with 20 mM potassium phosphate buffer (pH 7.0) to obtain OD 1.0 at 580 nm. The reaction mixture consisted of 2 ml of an emulsified substrate and 50 μg of the enzyme, and the mixture was incubated for 30 min at 30 °C. After incubation, turbidity was measured at a wavelength of 580 nm.

Absorption and degradation activities toward solid PLA - PlaM4 (0.3 mg) was incubated in 1 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 10 mg of solid PLA fabricated in powder form with a molecular weight of 0.5×10^4, 2×10^4, or 13×10^4 for 1 h at 60 °C. Residual powder was collected by centrifugation and washed twice with 100 mM potassium phosphate buffer (pH 7.0). The resulting supernatant and pellet were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). To measure degradation activity, total organic carbon (TOC) in the supernatant of the reaction mixture was measured using a TOC-V analyzer (Shimadzu, Kyoto, Japan).

Protein assay Protein concentration was determined as described by Lowry et al. (1951). SDS-PAGE was performed using a 12.5% separating gel as described by Laemmli (1970).

Nucleotide sequence accession numbers The nucleotide sequences of *plaM4*, *plaM7*, and *plaM9* were submitted to GenBank, and they were assigned the accession numbers AB302136, AB302138, and AB302140, respectively.

Results

Microbial diversity of expected PLA degrader in compost - Composting is a typical method for treating biodegradable plastic waste, and PLA can be efficiently degraded in compost (Ghorpade et al. 2001). Thus, PLA degraders in compost accumulate on the surface of PLA disks, and we directly extracted DNA from the surface of PLA disks buried in compost to analyze various genomes of cultured/uncultured PLA degraders. 16S rDNA sequence analysis revealed that Firmicutes (63%) was most abundant on PLA disks; this phylum comprised classes of Bacilli (37%) and Clostridia (26%). The other bacteria were classified into Bacteroidetes (17%), Proteobacteria (14%), and unclassified bacteria (6%). Actinobacteria were not detected among the clones, although some PLA degraders had been isolated from the Pseudonocardiaceae family and related genera belonging to Actinobacteria (Jarerat et al. 2002).

Screening and identification of PLA depolymerase genes To obtain the PLA depolymerase gene from metagenomic DNA, we constructed a metagenomic library containing 40,000 clones with an insert that averages 2.5 kb, which represents a total of 100 Mb and is the equivalent of 20 (5 Mb) bacterial genomes. As a result of the screening using emulsified-PLA-containing agar plates, we obtained seven positive clones. The sequences of insert DNA in these recombinant plasmids showed that they substantially overlapped each other, and three clones, designated pLA-M4, pLA-M7, and pLA-M9, were chosen for further study.

Nucleotide sequence analysis and BLASTX analysis of these clones indicated that all clones contain open reading frames (ORFs) encoding a lipase or hydrolase gene (Table 1). These ORFs were assumed to encode a putative PLA depolymerase because currently known PLA depolymerase genes are hydrolases such as proteases and esterases (Akutsu-Shigeno et al. 2003; Masaki et al. 2005; Matsuda et al. 2005). These ORFs were designated *plaM4*, *plaM7*, and *plaM9*. In only pLA-M9, the ORF encoding a Zn-dependent protease was also assumed as a putative PLA depolymerase gene; however, there was no PLA degradation activity in the subclone containing the ORF encoding the Zn-dependent protease (data not shown). *PlaM4*, *plaM7*, and *plaM9* were chosen for further study.

Molecular analysis of deduced amino acid sequences

BLASTP analysis of PlaM4 amino acid sequences showed that PlaM4 was homologous to lipase from *B. cereus* ATCC10987 (48% identity) and other *Bacillus* spp. The molecular mass of the translated protein was estimated to be 48,854 Da, and the first 27 amino acid residues were predicted to be a signal peptide cleaved between Ala^{27} and Ser^{28} in Gram-positive bacteria. BLASTP analysis of PlaM7 amino acid sequences showed that PlaM7 was poorly homologous to lipase from *B. cereus* G9241 (28%

Table 1 Annotation table of coding sequences (CDSs) predicted in PLA-degrading clones from PLA disk surface metagenome

Plasmid	Predicted CDS	AA identity/similarity (%)	Most homologous protein	Putative source organism
pLA-M4	>1…266 (direct)	50/59	Transposase	*Geobacillus kaustophilus*
2036 bp	**415…1,710 (direct)**	**48/65**	**Lipase**	***Bacillus clausii ATCC10987***
	1,849..>2,036 (reverse)	51/75	Oligopeptide transport ATP-binging protein	*Staphylococcus aureus*
pLA-M7	>1..402 (direct)	57/75	Coproporphyrinogen III oxidase	*Bacillus licheniformis*
2085 bp	**603..1,454 (direct)**	**28/45**	**Lipase**	***Bacillus cereus G9241***
	1,639..1,981 (direct)	74/87	Transcriptional regulator	*Desulfitobacterium hafniense*
pLA-M9	**54..1070 (direct)**	**42/59**	**Hydrolase**	***Treponema denticola***
2970 bp	1,120..s1,605 (direct)	67/80	Hypothetical protein	*Bacillus clausii*
	1,618..2,578 (direct)	56/71	Zn-dependent protease	*Bacillus clausii*
	2,624..>2,970 (direct)	42/56	Hydrolase	*Treponema denticola*

The fragment encoding PLA depolymerase is shown in bold type.

identity) and other *Bacillus* spp. The molecular mass of the translated protein was estimated to be 31,449 Da. The first 26 amino acid residues were predicted to be a signal peptide cleaved between Ala^{26} and Ser^{27} in Gram-positive bacteria. BLASTP analysis of PlaM9 amino acid sequences showed that PlaM9 was most homologous to hydrolase from *Treponema denticola* (42% identity). In addition, PlaM9 was homologous to hydrolase from *B. cereus* (39% identity) and lysophospholipase from *Thermoanaerobacter tengcongensis* (39% identity). The molecular mass of the translated protein was estimated to be 37,651 Da. In this gene, a signal peptide was not predicted by the SignalP program, version 3.0 (Bendtsen et al. 2004). Lipases and esterases harbor a highly conserved pentapeptide G–X–S–M–G around a catalytic serine. PlaM4 and PlaM9 exhibited the consensus sequence G–H–S–M–G at positions 159 to 163 and 134 to 138, respectively. PlaM7 exhibited the consensus sequence A–H–S–M–G at positions 126 to 130 observed specifically in various *Bacillus* lipases.

For classification of these putative PLA depolymerases, their amino acid sequences were aligned and compared with representative esterases and lipases that had been classified previously by Arpigny and Jaeger (1999). The constructed phylogenetic tree exhibited that all putative PLA depolymerases were classified into family I, which were included as true lipases, and PlaM4 was classified into family I-5 and showed 40% to 45% identity with thermophilic *Bacillus* family I-5 lipases. On the other hand, PlaM7 and PlaM9 did not belong to any branches in family I and showed ~20% identity with family I lipases.

Substrate specificity of purified PLA depolymerases To confirm the PLA degradation activities of these gene products, they were subcloned and overexpressed in *E. coli* using the pET system. These recombinant proteins were purified as described in "Materials and methods." The molecular masses of PlaM4, PlaM7, and PlaM9, as determined by SDS-PAGE, were about 48, 30, and 38 kDa, respectively, which were almost the same as those calculated from amino acid sequences (data not shown).

The degradation activities toward emulsified PLA with various molecular weights were examined using purified PlaM4, PlaM7, and PlaM9. PlaM4, PlaM7, and PlaM9 showed degradation activities toward PLA except for PLA ($MW = 13 \times 10^4$; Table 2). Furthermore, purified PlaM4 and PlaM7 showed degradation activities toward other emulsified polyesters such as PBS, PBSA, PES, PCL, and PHB, but PlaM9 could not degrade PHB (Table 2). For the triglycerides, all enzymes degraded tributyrin, but not triolein, the typical substrates of esterases and lipases, respectively. In addition, PlaM4, PlaM7, and PlaM9 showed the highest activity toward the short-chain fatty acids C_6, C_6, and C_4, respectively. The specific activities of PlaM7 and PlaM9, however, were much lower than that of PlaM4. In addition, PlaM7 and PlaM9 appeared to be a mesophilic enzyme (data not shown), regardless of the fact that the compost was treated in a high-temperature environment (65 °C) in this study. Thus, we surmised that these two enzymes might not play a primary role in PLA degradation in compost. From these findings, we characterized PlaM4 in detail.

Optimal temperature and thermostability of PlaM4 Enzyme activity was assayed at various temperatures and for various incubation periods using *p*-nitrophenyl acetate as the substrate. Purified PlaM4 showed the highest activity at 70 °C and pH 7.0 and retained 89% and 65% of initial activity after incubation for 1 h at 50 and 60 °C, respectively. These findings indicate that PlaM4 is a thermophilic enzyme and is most functional around this temperature.

Absorption and degradation activities of PlaM4 toward solid PLA For degrading a hydrophobic, insoluble substrate

Table 2 Substrate specificity of purified PLA depolymerases

Substrate	PlaM4	PlaM7	PlaM9
Polyesters[a]			
PLA (MW=13×10^4)	–	–	–
PLA (MW=2×10^4)	++	+	+
PLA (MW=0.5×10^4)	++	+	+
PBS	++	++	+
PBSA	++	+	+
PES	++	++	+
PCL	++	++	+
PHB	++	++	–
Triglycerides[a]			
Triolein	–	–	–
Tributyrin	++	++	+
p-Nitrophenyl esters[b]			
C_2	71.5	0.2	0.6
C_4	195.6	2.9	5.3
C_6	234.8	4.2	3.2
C_8	141.9	3.2	3.3
C_{10}	117.3	1.2	0.2
C_{16}	29.7	0	ND
C_{18}	4.4	ND	ND

ND not determined
[a] Decrease in turbidity of emulsion of >50% (++) or <50%(+) or no decrease (−) after incubation at 30 °C for 30 min.
[b] Specific activity (U/mg).

such as biodegradable plastics, the degrading enzyme needs to access and absorb onto the surface of the substrate. In general, enzymes have difficulty in accessing hydrophobic substrates because of their hydrophilicity. We investigated whether PlaM4 can access and absorb onto the surface of solid PLA at a high temperature. SDS-PAGE showed that PlaM4 can absorb onto the surface of solid PLA with a lower molecular weight, but absorption onto the surface of solid PLA with a high molecular weight was incomplete (Fig. 2a). In addition, from TOC assay of degraded solid PLA, PlaM4 showed degradation activity only toward PLA with a lower molecular weight (Fig. 2b). These results indicate that absorption onto the PLA surface may be important for the degradation activity of PlaM4, similarly to other plastic-degrading enzymes.

Discussion

Many PLA-degrading microorganisms have been isolated from the natural environment (Pranamuda et al. 1997; Nakamura et al. 2001; Sakai et al. 2001; Teeraphatpornchai et al. 2003; Maeda et al. 2005; Masaki et al. 2005). However, there are only few reports on PLA-degrading microorganisms in compost despite the fact that they are attracting attention as a tool for an effective waste treatment of biodegradable plastics (Ghorpade et al. 2001). Furthermore, there is no study about biodegradation of PLA by microorganisms including unculturable bacteria from metagenomics. In this study, we first procured metagenomic DNA from PLA disks buried in compost and succeeded in identifying three novel PLA-degrading genes from it.

All known PLA depolymerases are classified as hydrolases such as esterases (EC 3.1) or proteases (EC 3.4). PLA depolymerases classified as proteases are isolated from the Pseudonocardiaceae family belonging to Actinobacteria. However, in this study, the 16S rDNA sequence analysis revealed that bacteria on degraded PLA disks were occupied by Firmicutes and Proteobacteria, not Actinobacteria. This result suggests that Actinobacteria are not the main PLA degraders in compost, at least in this study. It is found that the soil bacteria belonging to Firmicutes and Proteobacteria have the ability to degrade various aliphatic

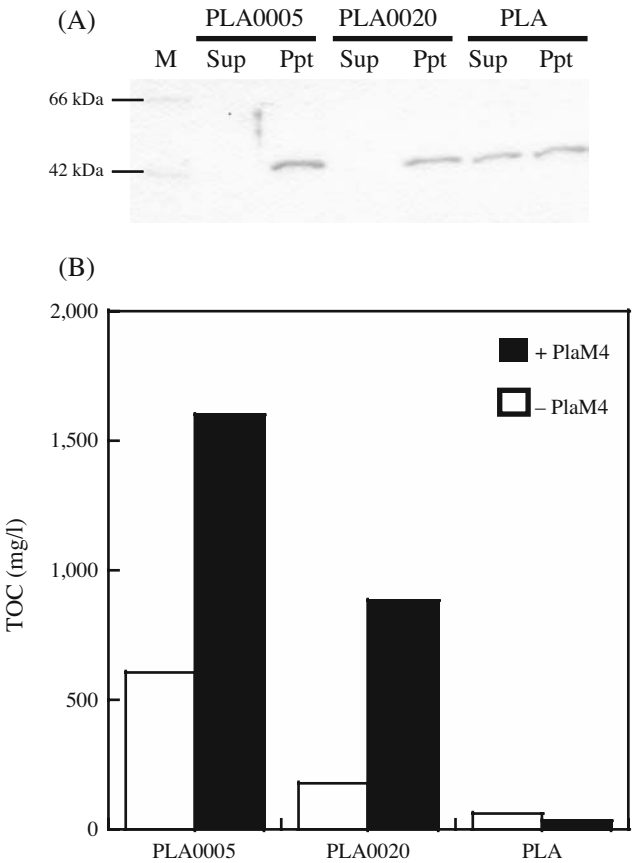

Fig. 2 Absorption (**a**) and degradation (**b**) activities of PlaM4 to solid PLA. The purified PlaM4 was incubated with PLA powder for 1 h at 60 °C as described in "Materials and methods." After centrifugation, the supernatant and pellet were eluted by SDS-PAGE (**a**). *Lane M* represents albumin from bovine serum (66.2 kDa) and aldolase from rabbit muscle (42.4 kDa) as molecular markers, respectively. TOC in the supernatant was measured as described in "Materials and methods" for degradation of PLA (**b**)

polyesters (Suyama et al. 1998). On the basis of substrate specificity, PlaM4, PlaM7, and PlaM9 are classified as carboxylesterases (EC 3.1.1.1) rather than lipases. These enzymes showed esterase activity and similar specificity for various aliphatic polyesters (Table 2). Considering these results, we assume that bacteria producing lipases and/or esterases are also distributed as degraders of PLA and various aliphatic polyesters in soil and compost.

Composting with PLA is most effective at high temperatures (50–60 °C). The PLA depolymerase from *B. smithii* isolated from a garbage fermentor showed thermophilic properties; that is, its highest activity was at 60 °C, and it was stable even after incubation at 60 °C for 10 min (Sakai et al. 2001). It means that enzyme thermostability and thermoactivity are very important in PLA biodegradation in compost. Among PlaM4, PlaM7, and PlaM9, PlaM4 exhibited thermophilic properties. Because PlaM4 is thermophilic, a high-temperature environment such as compost is suitable for degrading PLA by this enzyme. Although PlaM4 cannot degrade high-molecular-weight PLA, PLA disks with a high molecular weight were degraded approximately to one-half in compost. This may be due to the fact that PlaM4 degrades indirectly high-molecular-weight PLA in compost, because PLA is easily hydrolyzed at a high rate under high-temperature conditions. It is difficult for PLA depolymerases classified as lipases or esterases to degrade PLA with a high molecular weight (Lim et al. 2004; Tokiwa and Jarerat 2004; Tokiwa and Calabia 2006). In this study, we found that PlaM4 was able to bind to solid PLA with different molecular weights, but the binding to solid PLA with a high molecular weight appeared to be incomplete. This observation implies that the physical property of the surface of high-molecular-weight PLA may block the absorption of lipase- or esterase-type PLA depolymerases.

The metagenomic approach has led to the discovery of many novel genes owing to the remarkable gene diversity in metagenomes (Cowan et al. 2005). We also obtained esterase genes possessing unique amino acid sequences. PlaM4 classified into family I-5 showed a higher homology with *Bacillus* lipase than with *Staphylococcus* lipase; however, it did not show conserved Ala–His–Ser–Met–Gly, a distinctive feature of *Bacillus* lipase. In contrast to PlaM4, PlaM7 showed no homology with any representative *Bacillus* lipase, although it showed conserved Ala–His–Ser–Met–Gly. From the phylogenetic tree, we consider that PlaM7 and PlaM9 might be novel carboxylesterases classified into a new subfamily of family I. Although we need to determine in the future studies whether the genes encoding these two PLA depolymerases show functional expression in soil and compost, our study suggests that there are many unidentified PLA-degrading enzyme genes in the environment.

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