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ABSTRACT

The continuous growth of global plastics production for more than 50 years has resulted in elevated levels of pollution and serious environmental problems. Enzymatic depolymerization of synthetic polyesters represents an attractive approach for plastics recycling and effective use of carbon resources. In this study, screening of over 200 purified uncharacterized hydrolases from environmental metagenomes and sequenced microbial genomes identified 27 proteins with detectable activity and at least 10 proteins with high hydrolytic activity against synthetic polyesters. The metagenomic esterases GEN0105 and MGS0156 were active against a broad range of synthetic polyesters including polylactic acid, polycaprolactone, and bis(benzoyloxyethyl)-terephthalate. With solid polylactic acid as substrate, both enzymes produced a mixture of lactic acid monomers, dimers, and higher oligomers. The crystal structure of MGS0156 was determined at 1.95 Å resolution and revealed a modified α/β hydrolase fold, with a highly hydrophobic active site and lid domain. Mutational studies of MGS0156 identified the residues critical for hydrolytic activity against both monoester and polyester substrates, and demonstrated a two-times higher polyesterase activity in the L169A mutant protein. Thus, environmental metagenomes contain diverse polyesterases with high hydrolytic activity against a broad range of synthetic polyesters with potential applications in plastics recycling.

Introduction

Over the last 50 years, global production of plastics has continuously increased, reaching 322 million tons in 2015.[1] Synthetic polymers have become indispensable to our lives, with numerous applications in industry and everyday life.[2, 3] The six types of plastics accounting for approximately 90% of the total demand include polyethylene (PE, low-density and high-density PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyethylene terephthalate (PET), and polyurethane (PUR).[1, 2] The majority of plastics are made from petroleum and represent short-lived products (e.g. packaging materials), which are disposed of within one year after manufacture. For packaging plastics, it is estimated that only 28% of materials are collected for recycling/incineration, while 30-40% are land filled, and the rest (30-40%) appears to escape the collection system.[1, 4] Most petroleum-based plastics have been considered to be remarkably resistant to biological degradation.[5, 6] A tremendous increase in production of synthetic polymers and their persistence in the environment resulted in elevated levels of pollution and serious environmental problems.[1, 6] Therefore, production of biodegradable plastics from renewable feedstocks represents a promising solution and has become a focus of research.

Biodegradable synthetic polymers combine beneficial physical properties of polymers with biodegradability, determined by the presence of hydrolysable backbones such as polyesters, polycarbonates, polyurethanes and polyamides.[6-8] In contrast, biodegradation of PE, PP, PS and PVC is obstructed by the lack of hydrolysable bonds in their backbones. Among synthetic polymers, aliphatic polyesters such as polylactic acid (PLA) and polycaprolactone (PCL) are generally known to be susceptible to biological

degradation, whereas aromatic polyesters (like polyethylene terephthalate (PET)) have better mechanical properties, but are more resistant to microbial or enzymatic attack.[4, 9] Therefore, there is high interest in the development of different co-polyesters, including aliphatic-aromatic co-polyesters, which combine excellent mechanical properties with biodegradability, e.g. poly(butylene succinate-*co*-adipate (PBSA) and poly(butylene adipate-*co*-terephthalate (PBAT).[10] In general, biodegradability of synthetic polymers has been determined by their hydrophobicity, degree of crystallinity, surface topography and molecular size.[6, 7, 11] Presently, PLA and starch-based polymers represent the two most important commercial, biodegradable plastics accounting for about 47% and 41%, respectively.[12]

The most sustainable option for plastics waste treatment is a closed-loop recycling process based on the recovery of chemical feedstocks and their reuse for the synthesis of novel polymers (a circular economy) (Andersen MS. Sustainability Science, 2007, 2: 133-140; Kubo).[13, 14] For the most effective use of carbon resources, it is ideal that discarded plastic waste be restored to original raw materials using physical, chemical, or enzymatic recycling.[15] Compared to physical, thermal, and chemical plastics depolymerization, biocatalytic (microbial or enzyme-based) recycling has several advantages including low energy consumption, mild reaction conditions, and the possibility for stereospecific degradation and enzymatic repolymerization.[15, 16] In contrast to complete plastics biodegradation to CO_2, biocatalytic plastics recycling is aimed at reusing the products generated by enzymatic treatment.

Various bacteria and fungi have been reported to degrade plastic materials in diverse environmental conditions such as soils, sludges, composts, and marine water.[10] Many

aliphatic polyesters, including PLA and PCL, were found to be degraded by *Aspergillus, Penicillium, Pullularia, Trichoderma,* and other fungal strains isolated from environmental samples.[17, 18] Among bacteria, different strains of *Bacillus, Pseudomonas, Leptothrix, Roseateles, Corynebacterium, Streptomyces,* and *Enterobacter* can efficiently degrade both aliphatic and aliphatic-aromatic co-polyesters (e.g. PBAT).[10, 19] Most of the biodegradable polyesters are degraded by serine-dependent hydrolases such as lipases, esterases, proteases, and cutinases.[10] Several polyester degrading lipases and esterases have been characterized biochemically, including *Paenibacillus amylolyticus* PlaA, *Thermobifida fusca* TfH, ABO1197 and ABO1251 from *Alcanivorax borkumensis*, several clostridial esterases (Chath_Est1, Cbotu_EstA, Cbotu_EstB), and the metagenomics polyesterases PlaM4, EstB3, and EstC7.[20-25] Cutinases comprise a family of serine hydrolases produced by bacteria, fungi, and plants, whose natural substrate is the biopolyester cutin (a major component of plant cuticle).[26] Several purified bacterial (*Thermobifida*), fungal (*Humicola, Aspegillus, Fusarium*), and metagenomic cutinases have been shown to hydrolyze synthetic polyesters including PET and polyurethane.[4, 27-30] Crystal structures have been determined for the thermophilic fungus *Humicola insolens* cutinase HiC, metagenomic LC-cutinase from leaf-branch compost, as well as for the polyester degrading esterases from *Rhodopseudomonas palustris* (RPA1511) and *Clostridium hathewayi* (Chath_Est1).[23, 31-33] In contrast to lipases, polyesterase structures revealed a wide-open active site directly accessible to polymeric substrates as shown by the structure of RPA1511 in complex with polyethylene glycol bound close to the catalytic triad.[33] In addition, mutagenesis and protein engineering experiments with the

Thermobifida cellulosilytica cutinases Thc_Cut1 and Thc_Cut2 demonstrated an important role of enzyme surface and hydrophobic interactions for polyester hydrolysis.[34]

Although recent studies have identified a number of polyester degrading enzymes, the continuously growing global demand for plastics and novel polymers has also stimulated the interest in novel enzymes and biocatalytic approaches for polymer synthesis and recycling technologies. The discovery of novel polymer degrading enzymes and engineering of more active enzyme variants, as well as understanding of the molecular mechanisms of these enzymes represent the key challenges for the development of biocatalytic strategies for polymer hydrolysis and synthesis.[1] In this work, we have identified over 30 active metagenomic polyesterases through enzymatic screening, and biochemically characterized MGS0156 and GEN0105, which showed high hydrolytic activity against a broad range of polyesters (PLA, PCL, PET, PBSA, and PES). The crystal structure of MGS0156 revealed an open active site with hydrophobic surface, whereas structure-based mutagenesis studies identified amino acid residues critical for enzymatic activity.

MATERIALS AND METHODS

Reagents. All chemicals and substrates used in this study were of analytical grade unless otherwise stated. Polymeric substrates were purchased from Sigma-Aldrich (St. Louis, MO, USA) except poly (D,L-lactide) PLA2 (M_w 0.2 × 10^4), PLA70 (M_w 7.0 × 10^4), and poly (L-lactide) PLLA40 (M_w 4.0 × 10^4), that were obtained from PolySciTech (Akina Inc., West Lafayette, IN, USA). Commercial-grade PLA polymers (Ingeo™ 4032D, and Ingeo™ 6400D) were products of NatureWorks LLC (NE, USA), poly (D-

148 lactide) PURASORB^{TM} PD 24 of Corbion Purac (Amsterdam, The Netherlands), whereas
149 polybutylene succinate (PBS) (Bionolle^{TM} 1001MD, and Bionolle^{TM} 1020MD) and
150 polybutylene succinate-co-adipate (PBSA) (Bionolle^{TM} 3001MD, and Bionolle^{TM}
151 3020MD) were purchased from Showa Denko K.K., Japan. The surfactant Plysurf
152 A210G was obtained from Dai-ichi Kogyo Seiyaku Co. (Tokyo, Japan) and used to
153 emulsify the polymers.
154 **Gene cloning, protein purification, and mutagenesis.** For recombinant expression,
155 the coding sequences of selected hydrolase genes were PCR amplified and cloned into a
156 modified pET15b (Novagen) vector containing an N-terminal 6His tag as described
157 previously.[35] Since full length MGS0156 (1-421 aa) showed low expression in *E. coli*, a
158 truncated variant of this protein (75-421 aa) with the N-terminal signal peptide removed
159 was used. Recombinant proteins were overexpressed in *Escherichia coli* BL21 (DE3)
160 Codon-Plus strain (Stratagene) and purified to near homogeneity (>95%) using metal-
161 chelate affinity chromatography on Ni-NTA Superflow (Ni^{2+}-nitrilotriacetate) resin
162 (Qiagen). Size exclusion chromatography was performed using a HiLoad 16/60 Superdex
163 200 column (GE Healthcare) equilibrated with 10 mM HEPES (pH 7.5), 0.25 M NaCl
164 and 1 mM TCEP [tris-(2-carboxyethyl)phosphine].[36] The L-lactate dehydrogenase
165 (PfLDH) from *Plasmodium falciparum*[37] and the D-lactate dehydrogenase (D-LDH3)
166 from *Lactobacillus jensenii*[38] (used in lactate assays) were heterologously expressed in *E.*
167 *coli* and affinity purified to near homogeneity. Site-directed mutagenesis of
168 metagenomics esterases was performed using a QuickChange® kit (Stratagene) according
169 to the manufacturer's protocol. Wild-type MGS0156 and GEN0105 were used as the
170 templates, and mutations were verified via DNA sequencing. The selected residues were

mutated to Ala or Gly (for Ala replacements producing insoluble proteins). Mutant proteins were overexpressed and purified in the same manner as described for the wild-type proteins. Multiple sequence alignment was conducted by Clustal Omega v1.2.1 through EMBL-EBI server, whereas phylogenetic analysis was performed by MEGA v7.0 using the neighbor-joining method.[39, 40]

Esterase assays with soluble substrates. Carboxylesterase activity was measured spectrophotometrically as described previously.[35] Purified enzymes (0.05-10.0 μg protein/reaction) were assayed against α-naphthyl or *p*-nitrophenyl (*p*NP) esters of different fatty acids (0.25-2.0 mM) as substrates in a reaction mixture containing 50 mM HEPES-K buffer (pH 8.0).[35] Reaction mixtures (200 μl, in triplicate) were incubated at 30 °C in a 96-well plate format. Enzyme kinetics were determined by substrate saturation curve fitting (non-linear regression) using GraphPad Prism software (version 7.0 for Mac, GraphPad Software, CA, USA).

Polyester degradation (polyesterase) screens. Emulsified polyester substrates were prepared in 50 mM Tris-HCl buffer (pH 8.0), containing agarose (1.5%, w/v), and plate polyesterase assays were performed using 50-100 μg of purified protein/well (30 °C) as described previously.[33, 41] The presence of polyesterase activity was inferred from the formation of a clear halo around the wells with purified proteins.[33, 41]

Analysis of the reaction products of solid PLA depolymerization. Purified enzymes (50 μg) were incubated with PLA10 powder (10-12 mg) in a reaction mixture (1 ml) containing 0.4 M Tris-HCl buffer (pH 8.0) for 18 hr at 30 °C with shaking. Supernatant fractions were collected at different time points, clarified using centrifugal filters (MWCO 10 kDa), and the produced lactic acid was measured using lactate

dehydrogenase (LDH) as described previously.[33, 42] For the analysis of oligomeric PLA products in supernatant fractions (passed through 10 kDa filters), the flow-through aliquots (90 µl) were treated for 5 min at 95 °C with 1 M NaOH (final concentration) to convert oligomeric PLA products to lactic acid monomers before lactate measurements using both L- and D-LDHs (the data were corrected for the presence of monomeric lactic acid before the alkaline treatment). Both LDH enzymes were added to the reaction mixture in excess (total 500 µg/ml, 50/50) to maintain the reaction rate in the first order with lactate concentration. To identify the water-soluble products of PLA hydrolysis, the filtered supernatant fractions from solid PLA reactions were analysed using reverse phase liquid chromatography,[43] coupled with mass spectrometry (LC-MS). The platform configuration and methodology were as described previously.[33]

Protein crystallization and crystal structure determination of MGS0156. Purified MGS0156 (75-421 aa) was crystallized at room temperature using the sitting drop vapor diffusion method by mixing 1 µl of the selenomethionine substituted protein (12 mg/ml) with 1 µl of crystallization solution containing 30 % (w/v) PEG 4k, 0.2 M ammonium acetate, 0.1 M sodium citrate (pH 5.6), and 1/70 chymotrypsin. Crystals were harvested using mounted cryo-loops and transfered into the cryo-protectant (Paratone-N) prior to flash-freezing in liquid nitrogen. Data collections were carried out at the beamlines 19-ID of the Structural Biology Center, Advanced Photon Source, Argonne National Laboratory.[44] The data set was collected from a single crystal to 1.95 Å at the wavelength of 0.9794 Å and processed using the program HKL3000[45] (Table S1). The structure of MGS0156 was determined by the Se-methionine SAD phasing, density modification, and initial model building as implemented in the PHENIX suite of programs.[46] The initial

models (~90% complete) were further built manually using the program COOT[47] and refined with PHENIX. Analysis and validation of structures were performed using MOLPROBITY[48] and COOT validation tools. The final model was refined to R_{work}/R_{free} = 0.1532/0.19, and it shows good geometry with no outliers in the Ramachandran plot. Data collection and refinement statistics are summarized in Table S1. Surface electrostatic charge analysis was performed using the APBS tool in Pymol on a model generated by the PDB2PQR server.[49, 50] The topology diagram of MGS0156 was generated by HERA program[51] through PDBsum server.[52] The atomic coordinates have been deposited in the Protein Data Bank, with accession code 5D8M.

RESULTS AND DISCUSSION

Screening of purified microbial hydrolases for polyesterase activity. To discover novel polyesterases, 213 purified uncharacterized hydrolases (Table S2) from environmental metagenomes and sequenced microbial genomes were screened for hydrolytic activity against emulsified PLA10 [poly (DL-lactide); M_w 10K], PLLA40 [poly(L-lactide); M_w 40K], polycaprolactone PCL10 (M_w 10K), and bis(benzoyloxyethyl) terephthalate (3PET) using agarose-based screens. These screens revealed the presence of detectable polyesterase activity in 37 proteins, mostly from the α/β hydrolase superfamily (Table S3). Most of these proteins were active against PLA10? (22 proteins), 3PET (13 proteins), and PCL (11 proteins), whereas nine proteins exhibited activity toward poly(L-lactide) (PLLA40). Thus, a significant number of microbial and metagenomic hydrolases exhibit hydrolytic activity against synthetic polyesters.

The present work is focused on the biochemical characterization of the metagenomic polyesterases GEN0105 and MGS0156, which showed high hydrolytic activity against PLA10, PCL10, and 3PET (Figure 1). Carboxyl esterase activity of these enzymes was initially identified using tributyrin-based esterase screens of the metagenomic gene libraries from an anaerobic urban waste degrading facility (GEN0105) or paper mill waste degrading microbial community (MGS0156).[53]

The MGS0156 gene encodes a protein comprised of 421 amino acids with a potential N-terminal signal peptide (1-75 aa), whereas the GEN0105 sequence (322 aa) appears to lack an obvious signal peptide (Figure S1). Based on sequence analysis, both GEN0105 and MGS0156 belong to serine dependent α/β hydrolases, but share low sequence identity to each other (21.1%). Both enzymes represent metagenomic proteins as GEN0105 shares 61% sequence identity with the predicted esterase B0L3I1_9BACT from an uncultured bacterium, whereas the closest homologue of MGS0156 (DesfrDRAFT_2296 from *Desulfovibrio fructosivorans*) shows 71% sequence identity to this protein (Figure S1). Phylogenetic analysis revealed that GEN0105 is associated with esterase family IV, which also includes the cutinase-like polyesterase CLE from *Cryptococcus* sp. strain S-2 (Figure 2).[54, 55] In contrast, MGS0156, as well as MGS0084 and GEN0160 showed no clustering with known families of lipolytic enzymes, suggesting that these proteins represent new esterase families (Figure 2). Thus, the type II (lipase/cutinase type) polyesterases, including PLA depolymerases, exhibit broad phylogenetic diversity and are associated with esterase families I, III, IV, V as well as with new esterase families.

Carboxyl esterase activity of GEN0105 and MGS0156 against soluble monoester substrates. The acyl chain length preferences of purified recombinant GEN0105 and MGS0156 (75-421 aa) were characterized using spectrophotometric assays with α-naphthyl and *p*-nitrophenyl (*p*NP) monoesters (Figure 3). For these substrates, GEN0105 was most active against α-naphthyl butyrate, *p*NP -butyrate and *p*NP -valerate (C4 and C5 substrates). Compared to GEN0105, the specific activity of MGS0156 was an order of magnitude greater with a preference for longer (C8-C10) substrates (Figure 3). MGS0156 also exhibited significant hydrolytic activity against *p*NP-palmitate (C16) (Figure 3), which is in line with the lipolytic activity of this protein against olive oil observed in agar-based screens (data not shown), indicating that it is a lipase-like enzyme. With monoester substrates, both enzymes demonstrated saturation kinetics with MGS0156 showing high catalytic efficiencies with low K_m values toward a broad range of substrates (Table 1).

Based on temperature profiles of esterase activity, both GEN0105 and MGS0156 are mesophilic esterases showing maximal activity between 35-40°C and retained approximately 20% of maximal activity at 5°C (Figure S2). This is similar to the mesophilic esterase BioH from *E. coli*, whereas the cold-resistant esterase OLEI01171 from *Oleispira antarctica* was most active at 20°C and retained 82% of its maximal activity at 5°C.[36] In addition, GEN0105 and MGS0156 showed similar sensitivity to inhibition by detergents (Triton X-100 and Tween 20), whereas MGS0156 retained higher residual activity (25 - 75%) in the presence of salts (0.5 – 2.5 M NaCl or KCl) (Figure S2). Thus, with monoester substrates, GEN0105 and MGS0156 exhibit different

acyl chain length preferences and salt resistances, but similar sensitivities to temperature and detergents.

Hydrolytic activity of metagenomics polyesterases against 22 polyester substrates. The polyester substrate ranges of purified GEN0105 and MGS0156 were determined using agarose-based assays with 22 emulsified synthetic polyesters, including PLA and PCL, with different molecular weights and compositions, as well as their copolymers and 3PET (Table 2). Polyesterase activity of these enzymes was compared with the activity of the recently identified metagenomic esterases GEN0160 and MGS0084.[53] As shown in Figure 1, the four metagenomic esterases exhibited polyesterase activity against emulsified PCL10, which was higher or comparable to that of the previously identified polyesterase PlaM4 from compost.[24] When screened against 22 emulsified polyesters, GEN0105 and MGS0156 degraded 17 and 13 substrates, respectively, including PLA, PLGA (full name?), PCL, PBSA, and 3PET (Table 2). Both enzymes hydrolyzed the majority of the tested PLA polymers, with GEN0105 displaying activity against poly(L-lactide) and neither enzyme displaying activity against poly(D-lactide). Previously, it has been shown that type I (protease) PLA depolymerases are specific toward poly(L-lactide), as opposed to type II (cutinase/lipase) PLA depolymerases, which show preference for poly(DL-lactide).[56, 57] Besides GEN0105, only the cutinase-like type II enzyme CLE from *Cryptococcus* sp. strain S-2 has been shown to be able to hydrolyze poly(L-lactide).[54, 56] PLA substrates with the acid end protected by the addition of an ester group were also hydrolyzed by GEN0105 and MGS0156, suggesting that these polyesterases can exhibit endo-type hydrolysis. In contrast, GEN0160 and MGS0084

showed no polyesterase activity against PLA substrates (except for MGS0084 toward PLA2) and 3PET (Table 2). Finally, the four metagenomic esterases showed no hydrolytic activity toward poly(D-lactide), PHB and PBS. Thus, GEN0105 appears to be the most versatile polyesterase from the four tested enzymes, being able to hydrolyze a copolymer of hydroxybutyric acid and hydroxyvaleric acid (PHBV), as well as the commercial polymer Ingeo™ PLA6400 from NatureWorks (Table 2).

Analysis of the reaction products of solid PLA hydrolysis. To demonstrate hydrolytic activity of the identified metagenomic polyesterases against solid PLA substrates, purified MGS0156 and GEN0105 were incubated with solid poly(DL-lactide) (PLA10???) powder suspended in 0.4 M Tris-HCl buffer. At indicated time points (Figure 4), reaction mixture aliquots were cleared using centrifugal filters (MWCO 10 kDa), and the production of monomeric and oligomeric lactic acid products was analyzed using L- and D-lactate dehydrogenases (as described in Materials and Methods). After 6 hours of incubation at 30 °C, MGS0156 hydrolyzed approximately 80% of the solid PLA substrate producing a mixture of oligomeric and monomeric products (Figure 4). The proportion of monomeric lactic acid product increased with longer incubation times resulting in almost full (95%?) conversion of solid PLA substrate (monomeric + oligomeric products) after overnight incubation (Figure 4). GEN0105 degraded ~70% of solid PLA after overnight incubation, but was able to produce significant amounts of lactic acid within the first 30 min of incubation (Figure 4). The presence of significant amounts of oligomeric products during incubation of MGS0156 and GEN0105 with solid PLA (Figure 4) also suggests that they can catalyze both endo- and exo-esterase cleavage

of polyester substrates. Liquid chromatography-mass spectrometry (LC-MS) was used for direct analysis of water-soluble reaction products from solid PLA hydrolysis by MGS0156 and GEN0105 (Figure 5). The soluble reaction products were separated using a C18 column and analyzed using mass spectrometry. These analyses revealed that both enzymes produced mixtures of lactic acid monomers and oligomers with different chain lengths (Figure 5 and Table S4). In line with the results of LDH-based assays, GEN0105 showed a higher degree of monomeric products compared to lactic acid oligomers, suggesting that it may preferentially hydrolyze short chain substrates (Figure 4).

Recently, we have found that the purified polyesterase ABO2449 from *Alcanivorax borkumensis* required the addition of detergents (e.g. 0.1% Plysurf A210G) for solid PLA hydrolysis, suggesting that detergents can facilitate protein binding to solid PLA.[33] However, in this work detergents (0.1% Plysurf A210G or Triton X-100) significantly reduced hydrolytic activity of MGS0156 against solid PLA, and had no effect on polyesterase activity of GEN0105 (data not shown). With monoester substrates, GEN0105 retained significant catalytic activity in the presence of up to 20% detergent, whereas MGS0156 was much more sensitive to detergents (Figure S2). Thus, metagenomic polyesterases show different kinds of responses to detergents.

Crystal structure and active site of MGS0156. Purified metagenomic esterases (GEN0105, GEN0160, MGS0084, and MGS0156) were submitted for crystallization trials, with only MGS0156 (75-421 aa) producing diffracting crystals (Materials and Methods). The crystal structure of the seleno-methionine-substituted MGS0156 was solved at 1.95 Å resolution (Table S1), and revealed a protomer with an α/β-hydrolase

fold comprised of a slightly twisted central β-sheet with seven parallel β-strands (-5x, -1x, 2x, $(1x)_3$) and 19 α-helices (Figure 6A and Figure S3). The predicted catalytic nucleophile Ser232 is positioned on a short sharp turn (the nucleophilic elbow) between the β4 strand and α8 helix. It is located at the bottom of the MGS0156 active site, which is partially covered by a ring-shaped lid domain formed by seven short α-helices (α4, α10, α11, α14, α15, α16, and α18) connected by flexible loops (Figure 6A).

Analysis of the MGS0156 crystal contacts using the quaternary prediction server PISA suggested that this protein may form tetramers in solution through dimerization of dimers (Figures 6B, C). The tetrameric state of MGS0156 is consistent with the results of size-exclusion chromatography, which revealed a predominance for MGS0156 tetramers (70%), as well as the presence of some octomeric (25%) and monomeric (5%) forms (151 kDa, 296 kDa, and 40 kDa; predicted Mw 39 kDa). The tightly packed MGS0156 dimer is created through multiple interactions between residues located on several α-helices (α1, α2, α10, α13, and α16) and the β1 strand (buried area 4,100 $Å^2$, surface area 24,590 $Å^2$). The two MGS0156 dimers are assembled into a tetramer via interactions between the α11, α15, and α18 helices (surface area 47,980 $Å^2$, buried area 9,400 $Å^2$) (Figure 6C). In the MGS0156 tetramer, the four active sites are not adjacent to each other and are separated from the monomer interfaces with the two active site cavities open on the wide sides of the oligomeric assembly (Figure 6C).

A structural homology search of the DALI and PDBeFold databases revealed hundreds of structurally homologous proteins, mostly lipases and carboxylesterases with low overall sequence similarity to MGS0156 (<20% sequence identity). The top structural homologues include the LipA lipases from *Pseudomonas aeruginosa* (PA2862) (PDB

code 1EX9, Z-score 24.3, rmsd 2.5 Å, 17% identity) and *Burkholderia cepacia* (PDB code 1OIL, Z-score 24.2, rmsd 2.6 Å, 16% identity), as well as the *Staphylococcs hyicus* lipase Lip (PDB code 2HIH, Z-score 23.2, RMSD 1.89 Å, 13% identity). This Dali search also identified structurally homologous polyesterases from *Clostridium botulinum*[58] (PDB code 5AH1, Z-score 22.3, rmsd 2.6 Å, 15% identity) and *Pelosinus fermentans*[59] (PDB code 5AH0, Z-score 21.4, rmsd 2.5 Å, 18% identity).

The lid domain of MGS0156 contains many hydrophobic residues creating a hydrophobic surface extending to the catalytic site cavity (Figure S4). The lid domain is additionally stabilized by a disulfide bond between the Cys173 and Cys287 (Figure 7). Disulfide bonds are not very common in esterase-type polyester hydrolases, with just a few reports restricted to fungal cutinases (from *A. oryzae*[60], *F. solani*[61], and *Cryptococcus* sp. strain S-2[55]). However, in cutinases the disulfide bond is involved in the stabilization of the protein core domain.

The MGS0156 structure revealed two conformations for the catalytic Ser232 side chain, one of which is hydrogen bonded to the Nε2 atom of the catalytic His373 (3.2 Å), whereas the other one is a bit further away (3.9 Å) and appears to be H-bonded to the backbone amide of Lys233 (2.7 Å) (Figure 7). This is similar to the recently reported two conformations for the catalytic Ser130 of the naproxen esterase from *Bacillus subtilis*, representing the resting and acting states of the active site.[62] Like in known α/β hydrolases, the catalytic His373 of MGS0156 is supposed to act as a base, deprotonating the Ser232 side chain to generate a nucleophilic alkoxide group. The MGS0156 structure also indicates that the third member of its catalytic triad is Asp350 (2.8 Å to His373), whereas its oxyanione hole appears to include the main chain NH groups of Lys233 and

399 Leu169 (2.7 Å and 3.8 Å to Ser232, respectively) (Figure 7). The composition of the
400 MGS0156 catalytic triad (Ser232, His373, and Asp350) was confirmed using site-
401 directed mutagenesis, demonstrating that alanine replacement of these residues produced
402 catalytically inactive proteins (Figure 8). Like other biochemically characterized carboxyl
403 esterases,[23, 33, 36, 63] MGS0156 has a hydrophobic acyl-binding pocket formed by the side
404 chains of Leu169, Phe271, Leu275, Phe278, Leu299, Phe338, and Val353 (Figure 7).
405 The alcohol-binding pocket of the MGS0156 active site is located near the catalytic
406 Ser232 and is also filled mostly with hydrophobic residues, including Leu170, Val174,
407 Ile334, Met378, Phe380, and Ile391 (Figure 7).
408 Since GEN0105 failed to produce diffracting crystals, a structural model of this protein
409 was generated using the Phyre2 server[64] and was used as a guide to identify its catalytic
410 residues (Figure S5). The structural model of GEN0105 revealed a classical α/β hydrolase
411 fold for this protein, with Ser168 as the nucleophilic serine in a conserved GXSXG motif
412 (Figure S5). The other two residues of the GEN0105 catalytic triad are His292 (3.1 Å
413 from Ser168) and Glu262 (2.7 Å from His292). The catalytic role of these residues in
414 GEN0105 activity was confirmed using site-directed-mutagenesis (data not shown).
415
416 **Structure-based site-directed mutagenesis of MGS0156.** To identify the residues of
417 MGS0156 important for polyesterase activity, 30 active site residues were mutated to Ala
418 or Gly using site-directed mutagenesis. Hydrolytic activities of purified mutant proteins
419 were compared against wild-type protein activity using assays with α-naphthyl acetate,
420 emulsified PCL10, and solid PLA10 as substrates (Figure 8). As expected, these assays
421 revealed a critical role of the MGS0156 catalytic triad (Ser232, His373, and Asp350) for

hydrolysis of all tested substrates (Figure 8). These assays also demonstrated the importance of three residues adjacent to the catalytic Ser232 (His231 and Lys233) and His373 (Asp372) (3.7 – 5.0 Å), which show strong sequence conservation (Figure 8 and Figure S1). The side chains of conserved Cys173 and Cys287 form a disulfide bridge stabilizing the protein lid domain, with alanine replacement of these residues reducing the hydrolytic activity of MGS0156 toward all substrates (Figures 7 and 8). In addition, enzymatic activity of MGS0156 against both mono- and polyesters was found to be significantly reduced in the L299G, L335A, and M378G mutant proteins, which are located in the active site cleft, likely contributing to substrate binding (Figures 7 and 8). Reduced monoesterase activity was also observed in the L169A, L170G, E172G, V174G, S265A, L352G, and F380G mutant proteins (Figure 8). The polyesterase activity of these mutant proteins appeared to be unaffected based on agarose screens with emulsified PCL10, but was reduced (except for L169A and S265A) in LDH-coupled assays with solid PLA10 (Figure 8). These results suggest that the LDH-coupled polyesterase assay is more sensitive than the agarose-based screen. In addition, the LDH-coupled assay with solid PLA10 revealed a greatly diminished polyesterase activity in E330A, L335A, F338G and V353A mutant proteins, whereas their activity toward α-naphthyl acetate was close to that of the wild-type protein or slightly reduced (Figure 8B). Finally, the polyesterase and monoesterase activities of MGS0156 were not significantly effected in the mutant proteins S175G, L179G, L197G, R199G, F271G, R277G, or E280G, suggesting that these residues are not essential for substrate binding or enzymatic activity.

Interestingly, LDH-based assays with solid PLA10 revealed a two-fold increase in polyesterase activity of L169A, whereas its monoesterase activity was reduced to approximately 20% of the wild-type protein (Figure 8). As shown in Figure 4C, following three hours of incubation with solid PLA10 the L169A mutant protein demonstrated at least 90% substrate conversion to monomeric and oligomeric products, whereas the wild-type enzyme hydrolyzed only 50% of substrate. In the MGS0156 active site, the side chain of L169 is located close to the catalytic Ser232 (6.4 Å) and can potentially contribute to substrate binding/coordination (Figure Active site). Furthermore, the L169G mutant protein showed lower polyesterase activity against PLA10 and PCL10 compared to L169A, both in LDH- and agarose-based assays (data not shown). Therefore, we propose that hydrophobic interactions with polyester substrates at the position of Leu169 are important for polyesterase activity, with the Ala side chain providing better environment (reduced steric hindrance) for polyester binding) compared to Leu.

Recently, we have determined the crystal structure and identified eight residues critical for PLA hydrolysis by the *R. palustris* polyesterase RPA1511, which belongs to esterase family V (Figure 2).[33] However, structural superposition of this protein with MGS0156 revealed only two apparently homologous residues in MGS0156: Leu296 (Leu212 in RPA1511) and Leu299 (Leu220 in RPA1511). While mutagenesis of Leu299 (to Gly) abolished both polyesterase and monoesterase activities of MGS0156, replacement of Leu296 (by Gly) had no significant effect on either activity (Figure 8). Thus, our results indicate that although polyesterases from different esterase families have distinct binding

modes for polyesters, their active sites contain a significant number of hydrophobic residues which play an important role in substrate hydrolysis.

In summary, enzymatic screening of purified hydrolases and carboxyl esterases from environmental metagenomes and microbial genomes revealed a large number of enzymes with hydrolytic activity against various synthetic polyesters. These enzymes are adapted to function under different experimental conditions reflecting the corresponding environmental conditions of microbial communities. The biochemical and structural characterization of novel polyesterases from environmental metagenomes advances our understanding of enzymatic hydrolysis of synthetic polyesters and contributes to the development of enzyme-based plastic recycling.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: The Supplemental file includes Table S1-S??? and Figures S1-S???

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Table 1. Kinetic parameters of purified MGS0156 and GEN0105 with soluble mono-ester substrates. Results are means ± SD from at least two independent determinations.

Protein	Variable substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
MGS0156	α-Naphthyl acetate (C2)	0.16 ± 0.02	130 ± 5	8.1×10^5
	α-Naphthyl propionate (C3)	0.050 ± 0.005	155 ± 4	3.1×10^6
	α-Naphthyl butyrate (C4)	0.065 ± 0.004	115 ± 7	1.8×10^6
	*p*NP-acetate (C2)	0.161 ± 0.018	450 ± 21	2.8×10^6
	*p*NP-propionate (C3)	0.055 ± 0.005	419 ± 11	7.6×10^6
	*p*NP-butyrate (C4)	0.058 ± 0.006	635 ± 24	1.1×10^7
	*p*NP-valerate (C5)	0.036 ± 0.002	474 ± 8	1.3×10^7
	*p*NP-octanoate (C8)	0.145 ± 0.009	1101 ± 26	7.6×10^6
	*p*NP-decanoate (C10)	0.32 ± 0.03	775 ± 33	2.4×10^6
	*p*NP-laurate (C12)	0.13 ± 0.01	116 ± 5	0.9×10^6
	*p*NP-myristate (C14)	0.31 ± 0.17	68 ± 3	0.2×10^6
	*p*NP-palmitate (C16)	0.108 ± 0.004	31.0 ± 0.5	0.3×10^6
GEN0105	α-Naphthyl propionate (C3)	0.602 ± 0.09	88.79 ± 5	1.5×10^5

Table 2. Hydrolytic activity of purified metagenomic polyesterases against different polyesters. The presence of polyesterase activity was analyzed using agarose-based assays with the indicated emulsified polyesters.

Polyesters	**GEN0105**	**GEN0160**	**MGS0084**	**MGS0156**
1. PLA (D,L); M_w 2K	+	–	+	+
2. PLA (D,L); M_w 10K	+	–	–	+
3. PLA (D,L); M_w 10K, ET[a]	+	–	–	+
4. PLA (D,L); M_w 18K	+	–	–	+
5. PLA (D,L); M_w 70K	+	–	–	+
6. PLA (L); M_w 40K	+	–	–	–
7. PLA (L); ester term	+	–	–	–
8. PLA (D); M_w 124K	–	–	–	–
9. Ingeo™ PLA6400	+	–	–	–
10. Ingeo™ PLA4032	–	–	–	–
11. PLGA[b]	+	–	+	+
12. PHB	–	–	–	–
13. PHBV	+	–	–	–
14. PCL; M_w 10K	+	+	+	+
15. PCL; M_w 45K	+	+	+	+
16. PCL; M_w 70K	+	+	+	+
17. Bionolle™ PBS 1001MD	–	–	–	–
18. Bionolle™ PBS 1020MD	–	–	–	–
19. Bionolle™ PBSA 3001MD	+	+	+	+
20. Bionolle™ PBSA 3020MD	+	+	+	+
21. PES	+	+	+	+
22. 3PET	+	–	–	+

[a] ET, ester terminated.

[b] PLGA,…

Figure Legends

Figure 1. Polyesterase activity of purified metagenomic carboxylesterases. Agarose-based screen of purified proteins for the presence of polyesterase activity against emulsified PCL10. The presence of polyesterase activity is indicated by the formation of a clear zone around the wells containing purified proteins (50 μg of protein/well, 72 hours at 30 °C). Agarose (1.5%) plates contained 0.2% emulsified PCL10 in 50 mM Tris-HCl (pH 8.0) buffer. PlaM4, a previously characterized polyester hydrolase (ref?), and porcine liver esterase (PLE) were used as positive and negative controls, respectively.

Figure 2. Phylogenetic analysis of metagenomic polyesterases. Phylogenetic tree of polyesterases showing their relatedness to known esterase families (I – VIII, based on Arpigny and Jaeger, 1999).[65] The phylogenetic tree was generated by the MEGA7 software package[66] using the neighbor-joining method. The numbers on the nodes correspond to the percent recovery from 1,000 bootstrap resamplings. Evolutionary distances were calculated using the Poisson correction method[67], and are in the units of the number of amino acid substitutions per site. GenBank accession numbers or Uniprot IDs are shown in parentheses.

Figure 3. Esterase activity of metagenomic polyesterases against soluble monoester substrates of varying acyl chain length. Reaction mixtures contained 0.5 mM *p*-nitrophenyl (*p*NP)- or 1.5 mM α-naphthyl (αN) esters of varying chain lengths, and 0.01 μg of purified MGS0156 (A) or GEN0105 (B). The white bars show activity against α-naphthyl esters, whereas the gray bars represent activity against *p*NP- substrates.

Figure 4. Production of lactic acid during incubation of solid PLA10 with purified metagenomic polyesterases: wild-type MGS0156 (A), GEN0105 (B) and MGS0156 L169A (C). Monomeric and oligomeric lactic acid products were measured using D- and L-lactate dehydrogenases as described in Materials and Methods. Results are means ± SD from at least two independent determinations.

Figure 5. LC-MS analysis of reaction products for solid PLA hydrolysis by purified MGS0156 and GEN0105. Reaction mixtures (1.0 ml) contained 12 mg of solid PLA10 and 50 µg of purified enzyme in 0.4 M Tris-HCl (pH 8.0). Samples were collected after O/N incubation at 30 °C, filtered by centrifugation and analysed by LC-MS as described in Materials and Methods. Each peak is labelled with a number representing the oligomeric state of the polyester species. Results are means ± SD from at least two independent determinations.

Figure 6. Crystal structure of MGS0156. (A) Overall fold of the MGS0156 protomer shown in three views related by a 90° rotation. The protein core β-sheet is shown in cyan with α-helices colored in grey, and the lid domain in magenta. The position of the active site is indicated by the side chain of the catalytic Ser232. (B) Two views of the MGS0156 dimer related by a 90° rotation. The two protomers are colored in cyan and magenta. (C) Two surface presentation of the protein tetramer shown in two views related by 90° rotation. The protomers are shown in different colors, and the active site openings are indicated by arrows.

Figure 7. Close-up view of the MGS0156 active site. The protein ribbon is colored in gray with amino acid side chains shown as sticks and carbon atoms colored in green.

Only the side chains of catalytic triad and residues potentially involved in substrate binding are shown.

Figure 8. Mutational analysis of MGS0156: hydrolytic activity of purified mutant proteins against mono- and polyester substrates. (A), Agarose-based screen showing polyesterase activity against emulsified PCL10. (B), Monoesterase activity against α-naphthyl acetate (2 mM, 0.02 µg protein/assay, white bars) and polyesterase activity against solid PLA10 measured using LDH assay (??? µg protein/assay, gray bars).