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1 **Screening and characterization of novel polyesterases from environmental**  
2 **metagenomes with high hydrolytic activity against synthetic polyesters**

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34 **ABSTRACT**

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

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## 57 **Introduction**

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

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

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

90 The most sustainable option for plastics waste treatment is a closed-loop recycling  
91 process based on the recovery of chemical feedstocks and their reuse for the synthesis of  
92 novel polymers (a circular economy) (Andersen MS. Sustainability Science, 2007, 2:  
93 133-140; Kubo).<sup>13,14</sup> For the most effective use of carbon resources, it is ideal that  
94 discarded plastic waste be restored to original raw materials using physical, chemical, or  
95 enzymatic recycling.<sup>15</sup> Compared to physical, thermal, and chemical plastics  
96 depolymerization, biocatalytic (microbial or enzyme-based) recycling has several  
97 advantages including low energy consumption, mild reaction conditions, and the  
98 possibility for stereospecific degradation and enzymatic repolymerization.<sup>15,16</sup> In contrast  
99 to complete plastics biodegradation to CO<sub>2</sub>, biocatalytic plastics recycling is aimed at  
100 reusing the products generated by enzymatic treatment.

101 Various bacteria and fungi have been reported to degrade plastic materials in diverse  
102 environmental conditions such as soils, sludges, composts, and marine water.<sup>10</sup> Many

103 aliphatic polyesters, including PLA and PCL, were found to be degraded by *Aspergillus*,  
104 *Penicillium*, *Pullularia*, *Trichoderma*, and other fungal strains isolated from  
105 environmental samples.<sup>17, 18</sup> Among bacteria, different strains of *Bacillus*, *Pseudomonas*,  
106 *Leptothrix*, *Roseateles*, *Corynebacterium*, *Streptomyces*, and *Enterobacter* can efficiently  
107 degrade both aliphatic and aliphatic-aromatic co-polyesters (e.g. PBAT).<sup>10, 19</sup> Most of the  
108 biodegradable polyesters are degraded by serine-dependent hydrolases such as lipases,  
109 esterases, proteases, and cutinases.<sup>10</sup> Several polyester degrading lipases and esterases  
110 have been characterized biochemically, including *Paenibacillus amylolyticus* PlaA,  
111 *Thermobifida fusca* TfH, ABO1197 and ABO1251 from *Alcanivorax borkumensis*,  
112 several clostridial esterases (Chath\_Est1, Cbotu\_EstA, Cbotu\_EstB), and the  
113 metagenomics polyesterases PlaM4, EstB3, and EstC7.<sup>20-25</sup> Cutinases comprise a family  
114 of serine hydrolases produced by bacteria, fungi, and plants, whose natural substrate is  
115 the biopolyester cutin (a major component of plant cuticle).<sup>26</sup> Several purified bacterial  
116 (*Thermobifida*), fungal (*Humicola*, *Aspegillus*, *Fusarium*), and metagenomic cutinases  
117 have been shown to hydrolyze synthetic polyesters including PET and polyurethane.<sup>4, 27-</sup>  
118 <sup>30</sup> Crystal structures have been determined for the thermophilic fungus *Humicola insolens*  
119 cutinase HiC, metagenomic LC-cutinase from leaf-branch compost, as well as for the  
120 polyester degrading esterases from *Rhodopseudomonas palustris* (RPA1511) and  
121 *Clostridium hathewayi* (Chath\_Est1).<sup>23, 31-33</sup> In contrast to lipases, polyesterase structures  
122 revealed a wide-open active site directly accessible to polymeric substrates as shown by  
123 the structure of RPA1511 in complex with polyethylene glycol bound close to the  
124 catalytic triad.<sup>33</sup> In addition, mutagenesis and protein engineering experiments with the



125 *Thermobifida cellulosilytica* cutinases Thc\_Cut1 and Thc\_Cut2 demonstrated an  
126 important role of enzyme surface and hydrophobic interactions for polyester hydrolysis.<sup>34</sup>

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

140

## 141 **MATERIALS AND METHODS**

142 **Reagents.** All chemicals and substrates used in this study were of analytical grade  
143 unless otherwise stated. Polymeric substrates were purchased from Sigma-Aldrich (St.  
144 Louis, MO, USA) except poly (D,L-lactide) PLA2 ( $M_w 0.2 \times 10^4$ ), PLA70 ( $M_w 7.0 \times$   
145  $10^4$ ), and poly (L-lactide) PLLA40 ( $M_w 4.0 \times 10^4$ ), that were obtained from PolySciTech  
146 (Akina Inc., West Lafayette, IN, USA). Commercial-grade PLA polymers (Ingeo<sup>TM</sup>  
147 4032D, and Ingeo<sup>TM</sup> 6400D) were products of NatureWorks LLC (NE, USA), poly (D-

148 lactide) PURASORB™ PD 24 of Corbion Purac (Amsterdam, The Netherlands), whereas  
149 polybutylene succinate (PBS) (Bionolle™ 1001MD, and Bionolle™ 1020MD) and  
150 polybutylene succinate-co-adipate (PBSA) (Bionolle™ 3001MD, and Bionolle™  
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.<sup>35</sup> 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<sup>2+</sup>-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].<sup>36</sup> The L-lactate dehydrogenase  
165 (PfLDH) from *Plasmodium falciparum*<sup>37</sup> and the D-lactate dehydrogenase (D-LDH3)  
166 from *Lactobacillus jensenii*<sup>38</sup> (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

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

176 **Esterase assays with soluble substrates.** Carboxylesterase activity was measured  
177 spectrophotometrically as described previously.<sup>35</sup> Purified enzymes (0.05-10.0  $\mu$ g  
178 protein/reaction) were assayed against  $\alpha$ -naphthyl or *p*-nitrophenyl (*p*NP) esters of  
179 different fatty acids (0.25-2.0 mM) as substrates in a reaction mixture containing 50 mM  
180 HEPES-K buffer (pH 8.0).<sup>35</sup> Reaction mixtures (200  $\mu$ l, in triplicate) were incubated at  
181 30 °C in a 96-well plate format. Enzyme kinetics were determined by substrate saturation  
182 curve fitting (non-linear regression) using GraphPad Prism software (version 7.0 for Mac,  
183 GraphPad Software, CA, USA).

184 **Polyester degradation (polyesterase) screens.** Emulsified polyester substrates were  
185 prepared in 50 mM Tris-HCl buffer (pH 8.0), containing agarose (1.5%, w/v), and plate  
186 polyesterase assays were performed using 50-100  $\mu$ g of purified protein/well (30 °C) as  
187 described previously.<sup>33, 41</sup> The presence of polyesterase activity was inferred from the  
188 formation of a clear halo around the wells with purified proteins.<sup>33, 41</sup>

189 **Analysis of the reaction products of solid PLA depolymerization.** Purified enzymes  
190 (50  $\mu$ g) were incubated with PLA10 powder (10-12 mg) in a reaction mixture (1 ml)  
191 containing 0.4 M Tris-HCl buffer (pH 8.0) for 18 hr at 30 °C with shaking. Supernatant  
192 fractions were collected at different time points, clarified using centrifugal filters  
193 (MWCO 10 kDa), and the produced lactic acid was measured using lactate

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

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

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

226

## 227 **RESULTS AND DISCUSSION**

228 **Screening of purified microbial hydrolases for polyesterase activity.** To discover  
229 novel polyesterases, 213 purified uncharacterized hydrolases (Table S2) from  
230 environmental metagenomes and sequenced microbial genomes were screened for  
231 hydrolytic activity against emulsified PLA10 [poly (DL-lactide);  $M_w$  10K], PLLA40  
232 [poly(L-lactide);  $M_w$  40K], polycaprolactone PCL10 ( $M_w$  10K), and  
233 bis(benzoyloxyethyl) terephthalate (3PET) using agarose-based screens. These screens  
234 revealed the presence of detectable polyesterase activity in 37 proteins, mostly from the  
235  $\alpha/\beta$  hydrolase superfamily (Table S3). Most of these proteins were active against PLA10?  
236 (22 proteins), 3PET (13 proteins), and PCL (11 proteins), whereas nine proteins exhibited  
237 activity toward poly(L-lactide) (PLLA40). Thus, a significant number of microbial and  
238 metagenomic hydrolases exhibit hydrolytic activity against synthetic polyesters.

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

245 The MGS0156 gene encodes a protein comprised of 421 amino acids with a potential  
246 N-terminal signal peptide (1-75 aa), whereas the GEN0105 sequence (322 aa) appears to  
247 lack an obvious signal peptide (Figure S1). Based on sequence analysis, both GEN0105  
248 and MGS0156 belong to serine dependent  $\alpha/\beta$  hydrolases, but share low sequence  
249 identity to each other (21.1%). Both enzymes represent metagenomic proteins as  
250 GEN0105 shares 61% sequence identity with the predicted esterase B0L3I1\_9BACT  
251 from an uncultured bacterium, whereas the closest homologue of MGS0156  
252 (DesfrDRAFT\_2296 from *Desulfovibrio fructosivorans*) shows 71% sequence identity to  
253 this protein (Figure S1). Phylogenetic analysis revealed that GEN0105 is associated with  
254 esterase family IV, which also includes the cutinase-like polyesterase CLE from  
255 *Cryptococcus* sp. strain S-2 (Figure 2).<sup>54,55</sup> In contrast, MGS0156, as well as MGS0084  
256 and GEN0160 showed no clustering with known families of lipolytic enzymes,  
257 suggesting that these proteins represent new esterase families (Figure 2). Thus, the type II  
258 (lipase/cutinase type) polyesterases, including PLA depolymerases, exhibit broad  
259 phylogenetic diversity and are associated with esterase families I, III, IV, V as well as  
260 with new esterase families.

261

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

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

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

286

287 **Hydrolytic activity of metagenomics polyesterases against 22 polyester substrates.**

288 The polyester substrate ranges of purified GEN0105 and MGS0156 were determined  
289 using agarose-based assays with 22 emulsified synthetic polyesters, including PLA and  
290 PCL, with different molecular weights and compositions, as well as their copolymers and  
291 3PET (Table 2). Polyesterase activity of these enzymes was compared with the activity of  
292 the recently identified metagenomic esterases GEN0160 and MGS0084.<sup>53</sup> As shown in  
293 Figure 1, the four metagenomic esterases exhibited polyesterase activity against  
294 emulsified PCL10, which was higher or comparable to that of the previously identified  
295 polyesterase PlaM4 from compost.<sup>24</sup> When screened against 22 emulsified polyesters,  
296 GEN0105 and MGS0156 degraded 17 and 13 substrates, respectively, including PLA,  
297 PLGA (full name?), PCL, PBSA, and 3PET (Table 2). Both enzymes hydrolyzed the  
298 majority of the tested PLA polymers, with GEN0105 displaying activity against poly(L-  
299 lactide) and neither enzyme displaying activity against poly(D-lactide). Previously, it has  
300 been shown that type I (protease) PLA depolymerases are specific toward poly(L-  
301 lactide), as opposed to type II (cutinase/lipase) PLA depolymerases, which show  
302 preference for poly(DL-lactide).<sup>56, 57</sup> Besides GEN0105, only the cutinase-like type II  
303 enzyme CLE from *Cryptococcus* sp. strain S-2 has been shown to be able to hydrolyze  
304 poly(L-lactide).<sup>54, 56</sup> PLA substrates with the acid end protected by the addition of an  
305 ester group were also hydrolyzed by GEN0105 and MGS0156, suggesting that these  
306 polyesterases can exhibit endo-type hydrolysis. In contrast, GEN0160 and MGS0084



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

313

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

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

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

347

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

353 fold comprised of a slightly twisted central  $\beta$ -sheet with seven parallel  $\beta$ -strands (-5x, -  
354 1x, 2x, (1x)<sub>3</sub>) and 19  $\alpha$ -helices (Figure 6A and Figure S3). The predicted catalytic  
355 nucleophile Ser232 is positioned on a short sharp turn (the nucleophilic elbow) between  
356 the  $\beta$ 4 strand and  $\alpha$ 8 helix. It is located at the bottom of the MGS0156 active site, which  
357 is partially covered by a ring-shaped lid domain formed by seven short  $\alpha$ -helices ( $\alpha$ 4,  
358  $\alpha$ 10,  $\alpha$ 11,  $\alpha$ 14,  $\alpha$ 15,  $\alpha$ 16, and  $\alpha$ 18) connected by flexible loops (Figure 6A).

359 Analysis of the MGS0156 crystal contacts using the quaternary prediction server PISA  
360 suggested that this protein may form tetramers in solution through dimerization of dimers  
361 (Figures 6B, C). The tetrameric state of MGS0156 is consistent with the results of size-  
362 exclusion chromatography, which revealed a predominance for MGS0156 tetramers  
363 (70%), as well as the presence of some octomeric (25%) and monomeric (5%) forms (151  
364 kDa, 296 kDa, and 40 kDa; predicted Mw 39 kDa). The tightly packed MGS0156 dimer  
365 is created through multiple interactions between residues located on several  $\alpha$ -helices ( $\alpha$ 1,  
366  $\alpha$ 2,  $\alpha$ 10,  $\alpha$ 13, and  $\alpha$ 16) and the  $\beta$ 1 strand (buried area 4,100 Å<sup>2</sup>, surface area 24,590 Å<sup>2</sup>).  
367 The two MGS0156 dimers are assembled into a tetramer via interactions between the  
368  $\alpha$ 11,  $\alpha$ 15, and  $\alpha$ 18 helices (surface area 47,980 Å<sup>2</sup>, buried area 9,400 Å<sup>2</sup>) (Figure 6C). In  
369 the MGS0156 tetramer, the four active sites are not adjacent to each other and are  
370 separated from the monomer interfaces with the two active site cavities open on the wide  
371 sides of the oligomeric assembly (Figure 6C).

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

376 code 1EX9, Z-score 24.3, rmsd 2.5 Å, 17% identity) and *Burkholderia cepacia* (PDB  
377 code 1OIL, Z-score 24.2, rmsd 2.6 Å, 16% identity), as well as the *Staphylococcus hyicus*  
378 lipase Lip (PDB code 2HHH, Z-score 23.2, RMSD 1.89 Å, 13% identity). This Dali  
379 search also identified structurally homologous polyesterases from *Clostridium*  
380 *botulinum*<sup>58</sup> (PDB code 5AH1, Z-score 22.3, rmsd 2.6 Å, 15% identity) and *Pelosinus*  
381 *fermentans*<sup>59</sup> (PDB code 5AH0, Z-score 21.4, rmsd 2.5 Å, 18% identity).

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

389 The MGS0156 structure revealed two conformations for the catalytic Ser232 side  
390 chain, one of which is hydrogen bonded to the Nε2 atom of the catalytic His373 (3.2 Å),  
391 whereas the other one is a bit further away (3.9 Å) and appears to be H-bonded to the  
392 backbone amide of Lys233 (2.7 Å) (Figure 7). This is similar to the recently reported two  
393 conformations for the catalytic Ser130 of the naproxen esterase from *Bacillus subtilis*,  
394 representing the resting and acting states of the active site.<sup>62</sup> Like in known α/β  
395 hydrolases, the catalytic His373 of MGS0156 is supposed to act as a base, deprotonating  
396 the Ser232 side chain to generate a nucleophilic alkoxide group. The MGS0156 structure  
397 also indicates that the third member of its catalytic triad is Asp350 (2.8 Å to His373),  
398 whereas its oxyanion 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,<sup>23, 33, 36, 63</sup> 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<sup>64</sup> and was used as a guide to identify its catalytic  
410 residues (Figure S5). The structural model of GEN0105 revealed a classical  $\alpha/\beta$  hydrolase  
411 fold for this protein, with Ser168 as the nucleophilic serine in a conserved GX SXG 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  $\alpha$ -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

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

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

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

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

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

476

## 477 **ASSOCIATED CONTENT**

### 478 **Supporting Information**

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

481

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722 **Table 1. Kinetic parameters of purified MGS0156 and GEN0105 with soluble mono-**  
723 **ester substrates.** Results are means  $\pm$  SD from at least two independent determinations.

724

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

725

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

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

729

730 <sup>a</sup> ET, ester terminated.

731 <sup>b</sup> PLGA,...

732 **Figure Legends**

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

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

748 **Figure 3.** Esterase activity of metagenomic polyesterses against soluble monoester  
749 substrates of varying acyl chain length. Reaction mixtures contained 0.5 mM *p*-  
750 nitrophenyl (*p*NP)- or 1.5 mM  $\alpha$ -naphthyl ( $\alpha$ N) esters of varying chain lengths, and 0.01  
751 µg of purified MGS0156 (A) or GEN0105 (B). The white bars show activity against  $\alpha$ -  
752 naphthyl esters, whereas the gray bars represent activity against *p*NP- substrates.

753



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

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

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

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

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

778 **Figure 8.** Mutational analysis of MGS0156: hydrolytic activity of purified mutant  
779 proteins against mono- and polyester substrates. (A), Agarose-based screen showing  
780 polyesterase activity against emulsified PCL10. (B), Monoesterase activity against  $\alpha$ -  
781 naphthyl acetate (2 mM, 0.02  $\mu$ g protein/assay, white bars) and polyesterase activity  
782 against solid PLA10 measured using LDH assay (??  $\mu$ g protein/assay, gray bars).  
783