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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 α/β 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 2015.¹ Synthetic polymers have become indispensable to our
60 lives, with numerous applications in industry and everyday life.^{2,3} 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).^{1,2} 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.^{1,4} Most petroleum-based plastics have been considered to
69 be remarkably resistant to biological degradation.^{5,6} 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.^{1,6} 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.⁶⁻⁸ 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.^{4,9}
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)).¹⁰ In general, biodegradability of synthetic polymers has
86 been determined by their hydrophobicity, degree of crystallinity, surface topography and
87 molecular size.^{6,7,11} Presently, PLA and starch-based polymers represent the two most
88 important commercial, biodegradable plastics accounting for about 47% and 41%,
89 respectively.¹²

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).^{13,14} 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.¹⁵ 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.^{15,16} In contrast
99 to complete plastics biodegradation to CO₂, 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.¹⁰ 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.^{17, 18} 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).^{10, 19} Most of the
108 biodegradable polyesters are degraded by serine-dependent hydrolases such as lipases,
109 esterases, proteases, and cutinases.¹⁰ 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 polyesterses PlaM4, EstB3, and EstC7.²⁰⁻²⁵ 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).²⁶ Several purified bacterial
116 (*Thermobifida*), fungal (*Humicola*, *Aspegillus*, *Fusarium*), and metagenomic cutinases
117 have been shown to hydrolyze synthetic polyesters including PET and polyurethane.^{4, 27-}
118 ³⁰ 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).^{23, 31-33} 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.³³ 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.³⁴

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.¹ In this work, we have
134 identified over 30 active metagenomic polyesterses 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 (IngeoTM
147 4032D, and IngeoTM 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.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 (Ni²⁺-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

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.^{39, 40}

176 **Esterase assays with soluble substrates.** Carboxylesterase activity was measured
177 spectrophotometrically as described previously.³⁵ Purified enzymes (0.05-10.0 μ g
178 protein/reaction) were assayed against α -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).³⁵ Reaction mixtures (200 μ 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 μ g of purified protein/well (30 °C) as
187 described previously.^{33, 41} The presence of polyesterase activity was inferred from the
188 formation of a clear halo around the wells with purified proteins.^{33, 41}

189 **Analysis of the reaction products of solid PLA depolymerization.** Purified enzymes
190 (50 μ 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.^{33,42} For the analysis of oligomeric PLA
195 products in supernatant fractions (passed through 10 kDa filters), the flow-through
196 aliquots (90 μ 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 μ 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,⁴³ coupled with mass spectrometry (LC-MS). The platform
204 configuration and methodology were as described previously.³³

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 μ l of the selenomethionine substituted protein (12 mg/ml)
208 with 1 μ 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.⁴⁴ 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 HKL3000⁴⁵ (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.⁴⁶ The initial

217 models (~90% complete) were further built manually using the program COOT⁴⁷ and
218 refined with PHENIX. Analysis and validation of structures were performed using
219 MOLPROBITY⁴⁸ 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.^{49, 50} The topology diagram of MGS0156 was
224 generated by HERA program⁵¹ through PDBsum server.⁵² 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 α/β 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).⁵³

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 α/β 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).^{54,55} 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 α -
265 naphthyl and *p*-nitrophenyl (*p*NP) monoesters (Figure 3). For these substrates, GEN0105
266 was most active against α -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.³⁶ 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 MGS0084.⁵³ 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.²⁴ 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).^{56, 57} 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).^{54, 56} 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.³³
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 α/β -hydrolase

353 fold comprised of a slightly twisted central β -sheet with seven parallel β -strands (-5x, -
354 1x, 2x, (1x)₃) and 19 α -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 β 4 strand and α 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 α -helices (α 4,
358 α 10, α 11, α 14, α 15, α 16, and α 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 α -helices (α 1,
366 α 2, α 10, α 13, and α 16) and the β 1 strand (buried area 4,100 Å², surface area 24,590 Å²).
367 The two MGS0156 dimers are assembled into a tetramer via interactions between the
368 α 11, α 15, and α 18 helices (surface area 47,980 Å², buried area 9,400 Å²) (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*⁵⁸ (PDB code 5AH1, Z-score 22.3, rmsd 2.6 Å, 15% identity) and *Pelosinus*
381 *fermentans*⁵⁹ (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*⁶⁰, *F. solani*⁶¹, and
387 *Cryptococcus* sp. strain S-2⁵⁵). 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.⁶² 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,^{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 server⁶⁴ 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 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 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

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 α -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).³³ 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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720

721

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	α -Naphthyl acetate (C2)	0.16 \pm 0.02	130 \pm 5	8.1 $\times 10^5$
	α -Naphthyl propionate (C3)	0.050 \pm 0.005	155 \pm 4	3.1 $\times 10^6$
	α -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	α -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.

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	+	-	-	+

729

730 ^a ET, ester terminated.

731 ^b 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).⁶⁵ The phylogenetic tree was generated by the MEGA7
743 software package⁶⁶ 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 method⁶⁷, 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 α -naphthyl (α N) esters of varying chain lengths, and 0.01
751 µg of purified MGS0156 (A) or GEN0105 (B). The white bars show activity against α -
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 μ 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 β -sheet is shown in cyan
768 with α -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 α -
781 naphthyl acetate (2 mM, 0.02 μ g protein/assay, white bars) and polyesterase activity
782 against solid PLA10 measured using LDH assay (??? μ g protein/assay, gray bars).
783