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Screening and Characterization of Novel Polyesterases from Environmental Metagenomes with High Hydrolytic Activity against Synthetic Polyesters

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Environmental Science and Technology

Peer reviewed version

Cyswllt i'r cyhoeddiad / Link to publication

11. May. 2021

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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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

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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 CO_2, 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 polyesterases 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

6

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 polyesterases 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 0.2×10^4), PLA70 (M_w $7.0 \times$

145 10^4), and poly (L-lactide) PLLA40 (M_w 4.0×10^4), that were obtained from PolySciTech

146 (Akina Inc., West Lafayette, IN, USA). Commercial-grade PLA polymers (Ingeo^{TM}

147 4032D, and Ingeo^{TM} 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

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 µg

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 µl, 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 µg 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 µg) 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

9

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 μl) 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 μg/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 μl of the selenomethionine substituted protein (12 mg/ml)

208 with 1 μl 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 transfered 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 HKL3000[45] (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 COOT[47] and

218   refined with PHENIX. Analysis and validation of structures were performed using

219   MOLPROBITY[48] and COOT validation tools. The final model was refined to

220    $R_{work}/R_{free} = 0.1532/0.19$, 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 program[51] 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

15

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 polyesterases 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 β4 strand and α8 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 (α4,

358 α10, α11, α14, α15, α16, and α18) 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 (α1,

366 α2, α10, α13, and α16) and the β1 strand (buried area 4,100 Å^2, surface area 24,590 Å^2).

367 The two MGS0156 dimers are assembled into a tetramer via interactions between the

368 α11, α15, and α18 helices (surface area 47,980 Å^2, buried area 9,400 Å^2) (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 *Staphylococcs hyicus*

378   lipase Lip (PDB code 2HIH, 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-2[55]). 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 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

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???

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722   **Table 1. Kinetic parameters of purified MGS0156 and GEN0105 with soluble mono-**

723   **ester substrates.** Results are means ± 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 ± 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

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,…

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 polyesterases. Phylogenetic tree of

741 polyesterases 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 package[66] 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[67], 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 polyesterases against soluble monoester

749 substrates of varying acyl chain length. Reaction mixtures contained 0.5 mM p-

750 nitrophenyl (pNP)- or 1.5 mM α-naphthyl (αN) 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 pNP- substrates.

753

754   **Figure 4.** Production of lactic acid during incubation of solid PLA10 with purified

755   metagenomic polyesterases: 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 ± 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 µg 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 ± 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 µg protein/assay, white bars) and polyesterase activity

782   against solid PLA10 measured using LDH assay (??? µg protein/assay, gray bars).

783