

1 Finding needles in haystacks: identification of novel conserved 2 PETase enzymes in *Streptomyces*.

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7 Abstract

8 The rising use of plastic results in an appalling amount of waste which scatters into the environment
9 affecting environmental, animal, and human health. One of these plastics is PET which is mainly used
10 for bottles and textiles. In this research, we investigate the PET degrading ability of the *IsPETase*
11 homolog *ScLipA* from *Streptomyces coelicolor*. Of 96 different *Streptomyces* strains screened, 18 %
12 were able to degrade the model substrate BHET. Three different variants of lipase A, named *ScLipA*,
13 *S2LipA* and *S92LipA* were identified and analyzed in detail. The *lipA* gene was deleted from *S.*
14 *coelicolor* M145 using CRISPR/Cas9, resulting in reduced BHET degradation. *LipA* overexpression in
15 the knock-out background significantly enhanced BHET degradation. All three enzymes were
16 expressed in *E. coli* BL21 for protein purification and biochemical analysis, showing that enzymatic
17 activity most likely resides in a dimeric form of the enzyme. The optimum pH and temperature were
18 determined to be pH 7 and 25 °C for all three variants. Using these conditions, the activity on BHET
19 and amorphous PET film was investigated. *S2LipA* efficiently degraded BHET and caused roughening
20 and small indents on the surface of PET films, consistent with PET-degrading activity. The frequent
21 occurrence of the *S2LipA* variant in *Streptomyces* suggests an environmental advantage towards the
22 degradation of more hydrophobic substrates such as these polluting plastics in the environment.

23

24

25 Introduction

26 Petroleum-derived plastics are among the most useful and widespread synthetic polymers in the
27 modern world. They are relatively easy and cheap to produce, extremely versatile and durable
28 materials, which makes them remarkably attractive for a wide range of applications [1], [2]. This has
29 caused the demand and production of plastics to rise steadily to an estimated 9.2 billion metric tons
30 (Mt) produced as of 2023 [1], [3], [4]. Of all plastic produced, 75 % (6.9 billion Mt) has been
31 mismanaged or landfilled [4]. Durability and resistance to degradation of these polymers, combined
32 with the increasing output of waste generated by humans, makes plastic accumulation and pollution a
33 major global environmental concern [5]. In fact, commonly used plastics are generally not
34 biodegradable, accumulate in landfills or leak into terrestrial and/or aquatic environments, where they
35 disintegrate into micro- and nano-plastics, thereby posing a serious threat to many ecosystems [5].

36 Polyethylene terephthalate (PET) is extensively used to produce bottles, food packaging, clothing and
37 films. The high demand for PET makes it one of the most common plastics that we encounter in
38 everyday life with 18.8 million tons being produced worldwide in 2015 alone [6]. PET waste
39 management in most countries today consists mainly of incineration and landfilling. Both practices are
40 causing detrimental effects to the environment, such as leaching and the release of toxic compounds
41 [7]. On the other hand, an increasing number of countries have recycling systems in place to prevent
42 environmental accumulation and make renewed use of the PET waste produced. Despite such efforts,
43 the recovery ratio is far less than 50 % and only a minor fraction is used to manufacture new products,
44 with substantial amounts of PET still entering the environment [8].

45 With plastic waste accumulating in the environment, investigating the adaptation and response of soil
46 and aquatic microorganisms to plastic exposure has proven eminent. Microbial response and
47 adaptation mechanisms are likely to help battle plastic pollution and initiate remediation and recycling
48 options. Enzymatic degradation already has proven to enable natural routes to depolymerize these
49 recalcitrant polymeric materials. Several PET-hydrolyzing enzymes (PHEs) have been identified,
50 initiating biodegradation of this polymer. Predominantly, these are esterases, lipases, cutinases and
51 cutinase-like enzymes exhibiting hydrolysis of the ester bond between terephthalic acid (TPA) and
52 ethylene glycol (EG) moieties within the PET polymer [9]–[14]. It was not until 2016 that Yoshida and
53 colleagues reported the discovery of a novel bacterium, *Ideonella sakaiensis* 201-F6 (hereafter *I.*
54 *sakaiensis*), which had evolved towards the degradation and utilization of PET as a sole carbon

55 source [15]. The enzyme identified as responsible for the first step in the catabolism of PET is the
56 *IsPETase* (ISF6_4831), classified as an extracellular esterase. This enzyme hydrolyses PET into its
57 monomer mono-(2-hydroxyethyl) terephthalic acid (MHET), with only trace amounts of bis(2-
58 hydroxyethyl) terephthalate (BHET) and terephthalic acid (TPA) being observed. To complete the
59 breakdown of PET, *I. sakaiensis* possesses another enzyme, the *IsMHETase* (ISF6_0224), which is
60 capable of hydrolyzing MHET into TPA and ethylene glycol (EG), common starting chemicals to
61 polymerize PET [15]. Compared to other PHEs which have a broader range of substrates, the
62 *IsPETase* displays significantly higher activity against BHET, PET films, and commercial bottle-derived
63 PET at 40 °C [15]. Currently, all PETase-like enzymes are described in the PAZY-database [16].

64 Many genera of soil and aquatic bacteria are exposed to accumulated plastics. An important phylum of
65 bacteria remaining to be investigated is the *Actinobacteria*. This phylum consists of various complex
66 genera such as *Thermobifida*, *Rhodococcus*, *Streptomyces* and many more. Several enzymes of
67 actinobacterial origin such as the LCC, *TfCut2* and many more have previously been identified and
68 characterized to exhibit PET degrading abilities earning their place in the PAZY database [9]–[11],
69 [13], [16]–[19]. However, for various other genera including *Streptomyces* only limited information is
70 available [20]–[25]. Only one *Streptomyces* enzyme, SM14est has been described [21].

71 *Streptomyces* are filamentous growing soil-dwelling bacteria with a characteristic lifecycle. They are
72 renowned for their capability to produce antibiotics but are also known to excrete enzymes to degrade
73 complex natural polymers, including plant biomass [26]–[28]. Depending on the soil pollution rate and
74 the distance to the polluted area, it is possible to recover $7,5 \times 10^4$ to $3,0 \times 10^4$ plastic microparticles
75 per kilogram (kg) of soil [29]. This equates to 2-20 milligrams (mg) of recoverable plastic particles per
76 kg of soil [29]. Hence, the rhizosphere and its associated microbes, such as *Streptomyces* are
77 extensively exposed to plastics. This exposure to plastics and their ability to degrade complex biomass
78 with hydrolytic enzymes makes them a promising genus to explore for novel PHEs [20]–[25], [30], [31].
79 These possible PHEs are expected to be secreted enzymes. Expression of enzymes in *Streptomyces*
80 is a highly regulated process and hence, such regulation will likely play an important role in putative
81 PET degrading activity [32].

82 In this research, we identified Lipase A from *Streptomyces coelicolor* (ScLipA) as a homolog to the
83 *IsPETase*. Consequently, we investigated the ability of *S. coelicolor* to degrade the PET model

84 compound BHET. Conservation of this BHET degrading ability was investigated by screening a
85 collection of 96 previously isolated *Actinobacteria* (predominantly *Streptomyces*) under various
86 conditions [33]. This yielded insight into the optimal conditions needed to induce BHET degradation as
87 well as in the abundance of strains exhibiting BHET-degrading capability. In one or more of the
88 provided conditions, 44 % of the strains could degrade BHET, 18 % of the evaluated strains were able
89 to degrade BHET individually confirming a wider distribution of this ability in nature. The presence of
90 LipA in these strains was explored and the corresponding genes were sequenced. The enzyme is
91 highly conserved and can be classified into three different variants. The *S. coelicolor* variant further
92 named ScLipA, the MBT92 variant (S92LipA) which is only present in MBT92, and a conserved variant
93 present in all other active strains (S2LipA). The functionality of all three LipA variants in the
94 degradation ability of BHET and amorphous PET in *Streptomyces* species was investigated. A knock-
95 out of the *lipA* gene was constructed in *S. coelicolor* M145, and overexpression constructs of the three
96 variants were expressed in the knock-out to study the effect of the genes *in vivo*. The individual
97 enzymes were expressed in *Escherichia coli* and purified for the analysis of substrate specificity,
98 thermostability, and other properties. Enzyme characteristics were examined using enzyme assays
99 and liquid chromatography-mass spectrometry (LC-MS). Additionally, the strains, as well as the
100 purified enzymes, were incubated on amorphous PET films to investigate their true ability to adhere to
101 and degrade PET.

102 In conclusion, we present a full comprehensive identification and characterization of a novel,
103 conserved PET-degrading enzyme family of *Streptomyces*. Structural characteristics and *in vivo* and *in*
104 *vitro* activity provide insight into the evolutionary and ecological adaptation of soil bacteria towards
105 plastics in their environment.

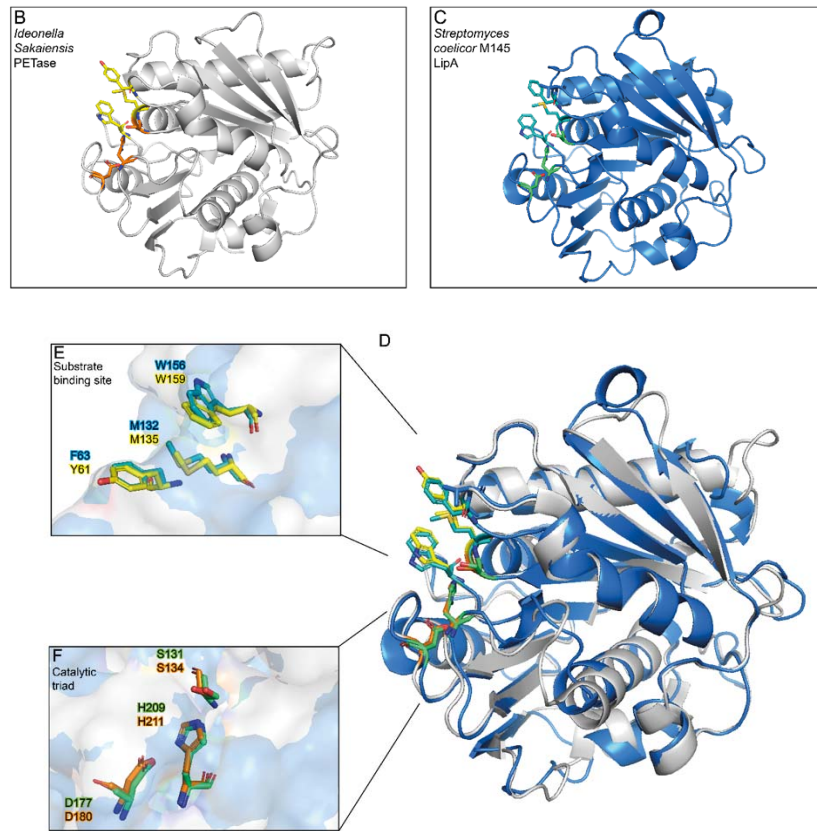
106 Results

107 Scavenging the *Streptomyces* genome for candidate IsPETase homologs.

108 Homologs of the IsPETase (Accession number A0A0K8P6T7, [34]) encoded by the genomes of
109 *Streptomyces coelicolor*, *Streptomyces scabies* and *Streptomyces avermitilis* were identified using
110 protein BLAST. The most promising enzyme was a *S. coelicolor* enzyme, annotated as a putatively
111 secreted lipase A (LipA, accession number Q9L2J6, [35]), showed a query coverage of 91 % and an
112 identity of 48 % with the IsPETase (Fig. 1A) [36]. The 3D structure of the IsPETase (Fig. 1B) and the
113 AlphaFold model of ScLipA (Fig. 1C) were superimposed, to compare the three-dimensional structures

114 of the enzymes [37]. When overlaid, the overall structures looked very similar (Fig. 1D). The catalytic
 115 triad was conserved, as well as two of the three residues of the previously described substrate binding
 116 site (Fig. 1E and F) [36]. The difference in the binding site is a phenylalanine in LipA instead of a
 117 tyrosine in *Is*PETase, as shown in figure 1E. The high similarity and the presence of the important
 118 residues for PET degradation hint towards the putative PET degrading activity of the *Streptomyces*
 119 *coelicolor* lipase A (ScLipA). On the N-terminal end of the protein, a twin-arginine translocation (TAT)
 120 signal is present indicating that the natural substrate of this enzyme is located outside of the cell [38].
 121 Indeed, for plastics-degrading enzymes, extracellular activity appears to be essential.

A	<i>Is</i> PETase	<QTNPYARGPNPTAASLEASAGPFTVRSFTVS--RPSGYGAGTVYYPTN-AGGTVGAI	57
	ScM145 LipA	<ADNPFYERGPAPTESSIEALRGPYSVADTSVSSSLAVIGFGGGTIYYPTSDGTFGAVVI	59
		*** ** * * : * : * * * : * : * * : * : * : * : * : * : * : * : * : * : * : *	
	<i>Is</i> PETase	VPGYTARQSSIKWVGPRLASHGFFVITIDTNTSLDQPSRSSQMMALRQVSLNGTSSS	117
	ScM145 LipA	APGFYAYQSSIAWLGPRLASQGFVFTIDTNTSLDQPSRGRQLAALDYLT----GRS	114
		. * : *	
	<i>Is</i> PETase	PIYGKVDIARMGVMSMGGGSLISAANNPSLKAAPQAPWDSSTNFSVTVPTLIFAC	177
	ScM145 LipA	SVRGRIDSGRLGVMGHSMGGGTLEAAKSRPSLQAAIPLTPWNLKSWPEVSTPTLVVGA	174
		: * : * : * : *	
	<i>Is</i> PETase	ENDSIAPVNSSALPIYDSMSRN-AKQFLEINGGSHSCANSNGNSNQALIGKGVAMMKRFM	236
	ScM145 LipA	DGDTIAPVASHAEPFYSLPSSDRAYLELNATHFSPNT--SNTTIAKYSISWLKRFI	231
		. : * : * * * * * * * * : : : * * : * : * * * * * * * * : : : * * : * : * : * * * * *	
	<i>Is</i> PETase	DNDTRYSTFACENPNSTRVSDERTANCS*---264	
	ScM145 LipA	DDDTRYEQFLCPLPRPSLTIEEYRGNCPHCS*262	
		* : * * * * * * * * * * * * : : : *	



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Figure 1: Sequence and structure comparison of *Is*PETase and *Sc*LipA.
 A) sequence comparison of the *Is*PETase and *Sc*LipA indicating the binding sites with yellow asterisks

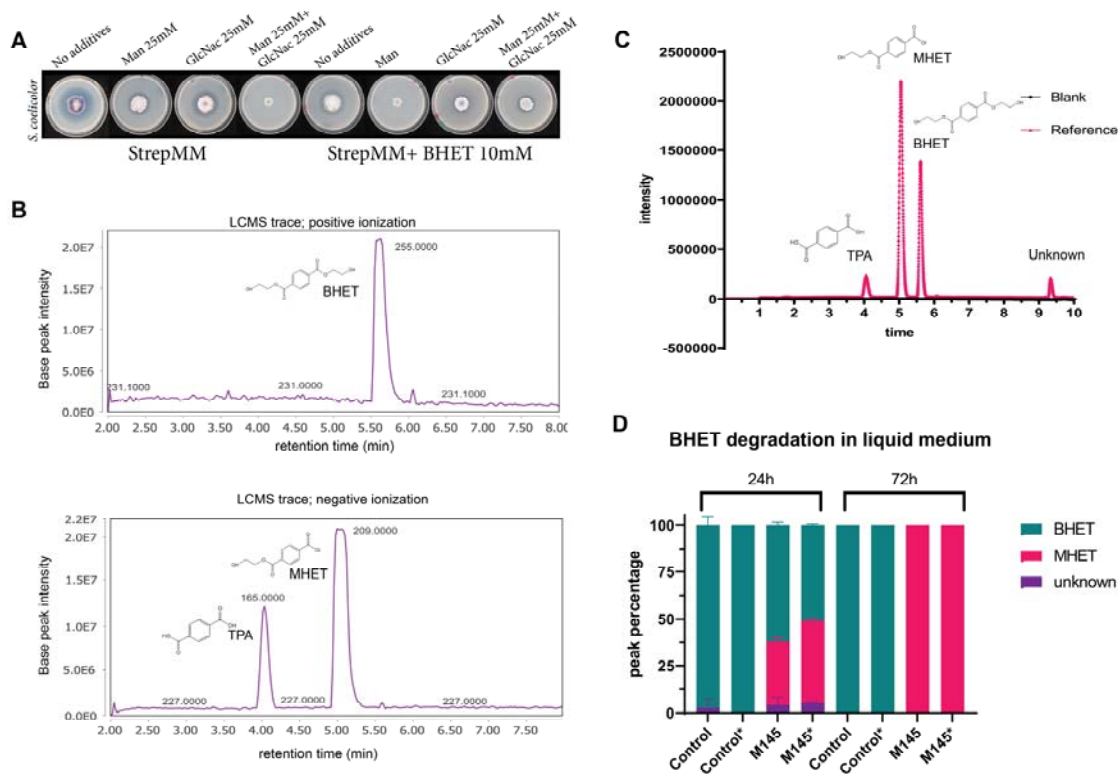
126 and the catalytic triad with orange asterisks. B) the structure of the IsPETase as provided by Uniprot
127 (5XJH). The catalytic triad is displayed in orange, and the binding site is in yellow. C) Predicted model
128 of the structure of ScLipA constructed with AlphaFold. The catalytic triad is shown in green and the
129 binding domains are displayed in cyan. D) An overlay of the IsPETase and ScLipA. E) overlay of the
130 binding domains, the tyrosine on position 63 of the IsPETase overlaps with a phenylalanine on position
131 61 if the ScLipA. F) overlay of the catalytic triads.

132 BHET degrading activity of *S. coelicolor*

133 To further investigate the activity of *S. coelicolor* M145 on BHET, the strain was grown on
134 *Streptomyces* minimal medium (StrepMM) agar plates containing BHET. Due to its limited solubility,
135 the addition of BHET causes the plates to become turbid. When BHET is converted to MHET and/or
136 TPA, a halo of clearance appears. The secreted enzyme activity was expected to be tightly regulated
137 in response to environmental growth conditions [32]. Therefore, we tested *N*-acetyl glucosamine
138 (GlcNAc) as a possible inducer of secreted enzyme activity. GlcNAc is a monomer of chitin and
139 peptidoglycan, which under poor nutritional conditions induces development and antibiotic production
140 in *Streptomyces coelicolor* [39], [40], whereas in nutrient-rich environments these responses are
141 blocked [35]. We anticipated that GlcNAc may act as an inducer for the production of extracellular
142 enzymes such as lipases, esterases and possibly BHET-degrading enzymes. Hence, *S. coelicolor*
143 M145 was grown in the presence and absence of BHET [10 mM], mannitol [25 mM] and *N*-acetyl
144 glucosamine [25 mM] in all possible combinations.

145 Interestingly, a clearance halo was observed at 18 days of growth as shown in figure 2A. Notably,
146 figure 2A displays that the addition of GlcNAc as inducer seemed to induce the degradation of BHET.
147 Additionally, *S. coelicolor* produces agarase, which resulted in 'sinking' halos which you can see in the
148 controls without BHET (Fig. 2A, StrepMM) [41]. These 'sinking halos' looked very similar to clearance
149 halos. Therefore, the ability *S. coelicolor* to degrade BHET in liquid culture was investigated using
150 liquid chromatography-mass spectrometry (LC-MS). For the liquid analysis, strain M145 was
151 precultured and inoculated in minimal liquid medium without polyethylene glycol (NMM) with and
152 without BHET and GlcNAc. Samples were taken after 24 h and 72 h and analyzed using LC-MS. In the
153 negative ionization mode, the mass of TPA (166 g/mol) could be observed around 4 min retention time
154 showing a small peak on the UV spectrum, MHET (210 g/mol) appeared around 5.2 min with a strong
155 signal on both the MS as well as the UV. Finally, BHET (255 g/mol) could be observed in the positive
156 ionization mode between 5.5 and 6 min with a strong signal at 240 nm (Fig. 2B and 2C). Around 9.5
157 min, impurity was observed, which was present in the BHET stock (Fig. 2C). When grown in NMM
158 medium containing BHET, 30-50 % of the BHET was degraded within 24 h, after 72 h, all BHET was

159 degraded and only MHET was present. Indeed, the addition of GlcNAc enhanced the degradation of
 160 BHET (Fig. 2D).



161

162 **Figure 2: The ability of *S. coelicolor* M145 to degrade BHET.**

163 A) Degradation of BHET by *S. coelicolor* after 18 days of growth on Strep MM Difco agar with and
 164 without Mannitol, BHET and GlcNAc B) LC-MS trace of BHET in positive ionization, MHET and TPA in
 165 the negative ionization. C) The UV spectrum at 240 nm of TPA, MHET and BHET with corresponding
 166 retention times. D) The control samples are displayed as control and only contain NMM with BHET [10
 167 mM]. The addition of GlcNAc [25 mM] to the cultures is indicated with an asterisk. Peak intensity and
 168 area percentage were calculated using GraphPad. The areas are presented in percentage compound
 169 present in culture. BHET is indicated in turquoise and MHET is indicated in magenta. Some impurities
 170 are always present as a peak around 9.5 min retention time, this compound is called unknown and
 171 presented in purple.

172 Bulk screen and individual screening of *Actinobacteria* strain collection

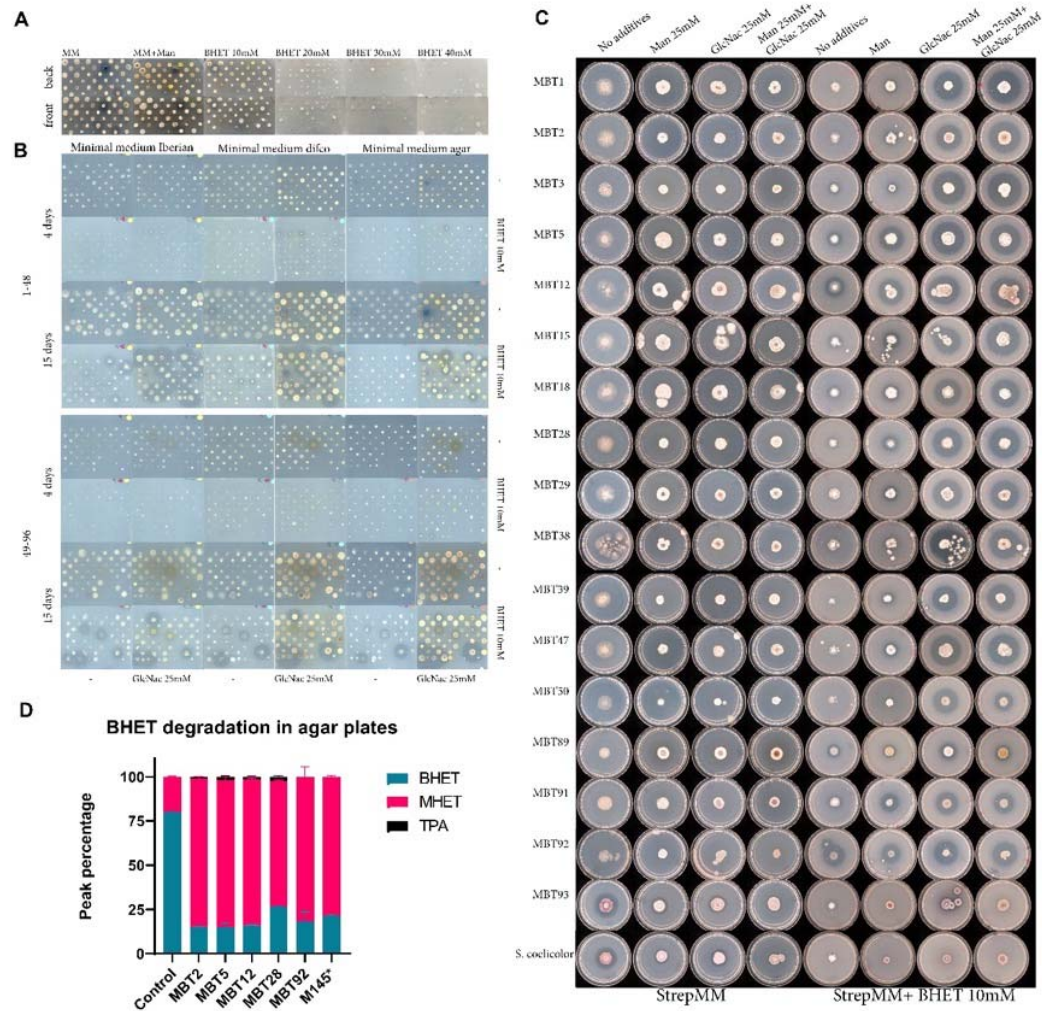
173 With *S. coelicolor* M145 exhibiting a clear but modest BHET degrading activity on plates, we set out to
 174 screen a diverse collection of 96 *Actinobacteria* of our strain collection [42] to investigate the spread of
 175 this characteristic and identify additional BHET degrading bacteria. 36 strains were randomly chosen
 176 from this collection to perform an initial BHET toxicity test. Growth of these strains appeared to be
 177 delayed with 10 mM BHET, and impaired with 20 mM BHET and higher concentrations. Hence, a
 178 concentration of 10 mM of BHET was chosen for screening, with all tested strains able to grow and
 179 halos readily observed (Fig. 3A).

180

181 A bulk screen for BHET degradation was performed with all 96 strains split over two 96-well plates to
182 provide them with enough space for development. Seven strains did not grow on the plates and were
183 taken out of the screen. Spore suspensions were stamped onto plates containing StrepMM with
184 different agar brands containing combinations of BHET [10 mM], Mannitol [25 mM] and GlcNAc [25
185 mM]. The type of agar used clearly impacted the observed degradation patterns. The agar brand
186 seems to have a clear influence on the degradation pattern (Supplement 1). Difco agar was chosen as
187 the agar source for all further experiments since most strains showed growth on Difco agar. The
188 addition of GlcNAc resulted in a different halo pattern and more predominant halos during the bulk
189 screen and induced BHET degradation in most strains (Fig 3B, Supplement 1).

190 *Actinobacteria*, especially *Streptomyces*, depend on environmental cues and interspecies interactions
191 for the induction of enzymes and secondary metabolites [43]. Consequently, it was important to
192 investigate their individual BHET degrading activity on separate plates. All strains showing halos in the
193 bulk BHET screen were grown on individual plates (Supplement 2). Of 38 active strains, only 17
194 strains exhibited individual activity on BHET after 10 days of growth (Fig. 3C). Interestingly, most
195 strains required the addition of GlcNAc to express the BHET degrading enzyme. Only 5 strains, MBT5,
196 12, 15, 91 and 92 degraded BHET in the absence of GlcNAc. MBT92 appeared to exhibit constitutive
197 expression showing a similar-sized halo in all conditions. To substantiate a good correlation between
198 halo size and biochemical degradation, strains MBT2, 5, 12, 28, 92 and *S. coelicolor* M145 were
199 incubated with approximately 2×10^6 spores on StrepMM Difco plates containing BHET and GlcNAc.
200 Samples of agar were taken after 15 days using an agar excision tool to cut out part of the halo. Since
201 *S. coelicolor* M145 displayed no activity after 15 days (see also above), halos were excised after 18
202 days. Samples were spun down using nylon spin columns, separating all liquids from the agar, and
203 prepped for LC-MS analysis (Fig. 3D). In all strains, clear conversion from BHET to MHET was
204 observed. MBT2, 5, 12, and 28 show traces of TPA suggesting further conversion of MHET.

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Figure 3: Actinobacteria screens on BHET.

209 A) Toxicity screen of 36 Actinobacteria on BHET concentrations ranging from 0 to 40mM of BHET. B)

210 Bulk screens of 96 Actinobacteria on StrepMM Iberian agar, Difco agar and agar agar with and without

211 N-acetyl glucosamine as inducer at 4 and 15 days of growth. C) Individual screen of all active strains

212 on Strep MM Difco agar with and without Mannitol, BHET and GlcNAc after 10 days of growth. D)

213 Analysis of BHET degradation in agar plugs after 15 days of growth using LC-MS. The peak

214 percentage was calculated using GraphPad. *The agar plug of M145 was taken after 18 days.

215

Identification of LipA in BHET-degrading strains

216

A PCR with degenerative primers was performed to investigate if *lipA* is present in the strains showing

217

activity in the individual screens displayed in figure 3C. The PCR results are displayed in supplemental

218

data S4. The PCR fragments were cloned into a linearized pJET2.1 and sequenced. Sequence

219

alignment demonstrated substantial divergence within the signal sequence while presenting only minor

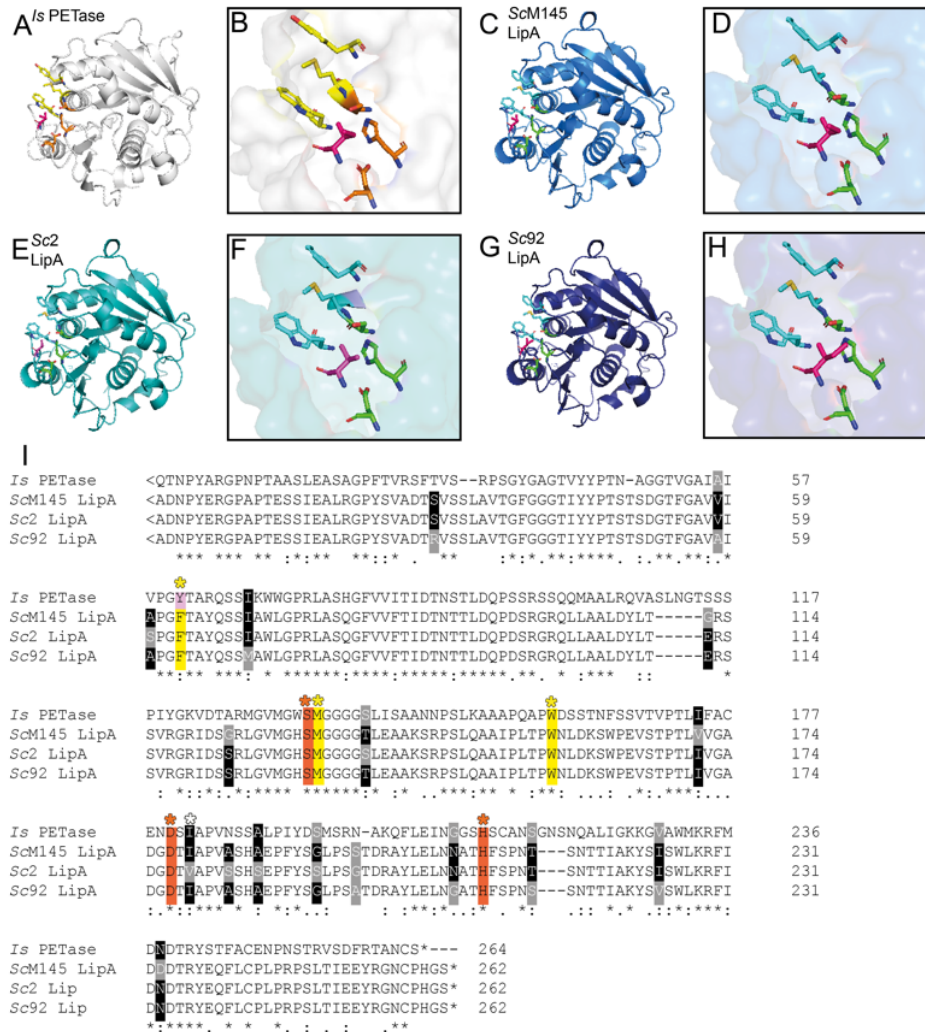
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variations in the overall enzyme coding region (S5). The enzyme sequences were aligned and could

221

be clustered into three variants the *S. coelicolor* variant (ScLipA), the MBT92 variant (S92LipA) which

222 is only present in MBT92, and a conserved variant present in all other active strains (S2LipA). (S6).
 223 The enzyme structures predicted with AlphaFold showed minor changes in the enzyme structure with
 224 minor influence on the overall structure (Fig. 4A-H) [37]. The amino acid alignment showed some
 225 variations between the proteins especially surrounding the catalytic triad (Fig. 4I). The signal peptides,
 226 identified with SignalP 5.0, contained several differences [44]. The alignment containing the signal
 227 sequence is provided in the supplementary data (S6).

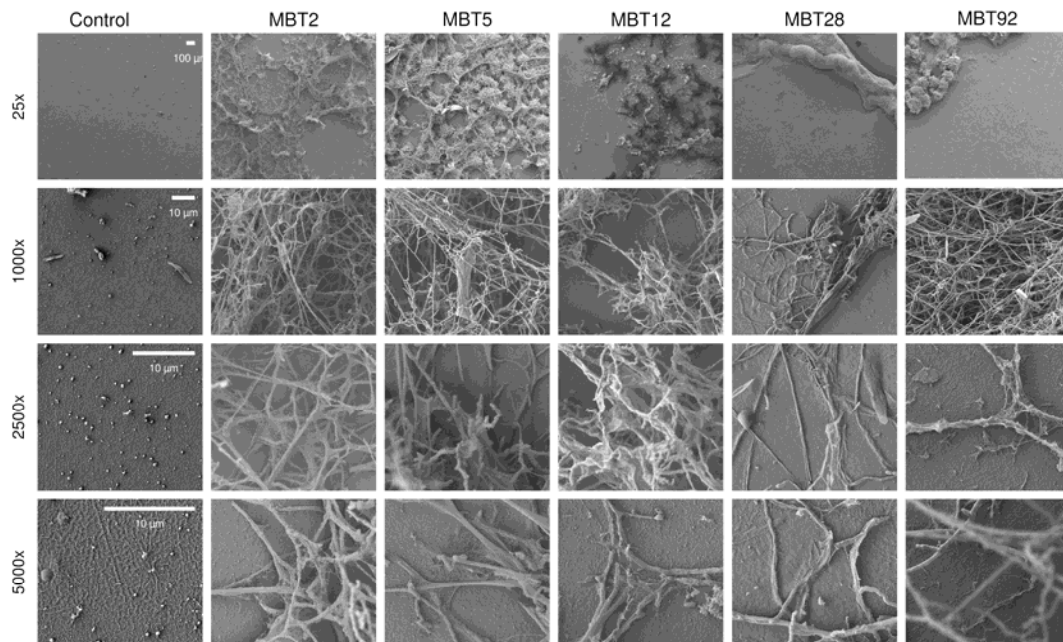


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229 **Figure 4: Comparison of Lipase A variants to the IsPETase.**

230 A) the structure of the IsPETase as provided by AlphaFold (5XJH) indicating the binding residues in
 231 yellow and the catalytic triad in orange, the pink residue that might influence binding. B-D) Predicted
 232 model of the structure of ScLipA (B), S2LipA (C) and S92LipA (D) constructed with AlphaFold. E-H)
 233 Enlarged image of the catalytic triad and binding site from IsPETase (E), ScLipA (F), S2LipA (G) and
 234 S92LipA (H). I) sequence comparison of the IsPETase and ScLipA, S2LipA and S92LipA indicating the
 235 binding residues with yellow asterisks/highlight and the catalytic triad with orange asterisks/highlight.
 236 The white asterisk shows a residue that might influence binding. Sequence differences from the
 237 different lipases are highlighted in grey, when only one sequence differs the conserved amino acids
 238 are highlighted in black.

239 Scanning electron microscopy imaging of *Streptomyces* species and enzymes on amorphous PET film



240

241 **Figure 5: Scanning electron micrographs of MBT2, 5, 12, 28 and 92 on amorphous PET film.**
242 PET films incubated for 2 weeks at 30 °C in NMM with 0.05 % [w/v] glucose with 10^7 spores and
243 inoculated for 2 weeks at 30 °C. From left to right: control, strain MBT2, MBT5, MBT12, MBT28 and
244 MBT92. From top to bottom the magnifications 25 x, 1000 x, 2500 x and 500 0x.

245 MBT2, 5, 12, 28 and 92 were incubated in NMM with and without GlcNAc. All strains form a biofilm
246 surrounding the edges of the plastic films, thereby fixing them to the 12 wells plates. After fixation, the
247 samples were sputter-coated and visualized with scanning electron microscopy (SEM). All strains
248 appeared to adhere well to the plastic films, forming hyphal structures colonizing the film (Fig. 5).
249 Some areas were heavily overgrown and did not allow for visualization of the film. The less cultivated
250 areas did not show significant damage over the control film suggesting that either more biomass is
251 needed to observe degradation, or no PET degradation was achieved.

252 **Knock-out and overexpression of the three *lipA* variants in *S. coelicolor***

253 To obtain a better understanding of the role and function of LipA *in vivo*, a *lipA* knock-out mutant was
254 constructed in *S. coelicolor* M145. The knock-out was validated via diagnostic PCR and sequencing (S
255 7.1).

256 Additionally, strains were constructed expressing one of the three different *lipA* variants under the
257 control of the pGAP promoter (S 7.2). pGAP is a semi-constitutive promoter that is induced by simple
258 sugar molecules [45]. To avoid interference of the native ScLipA and other regulatory elements, all

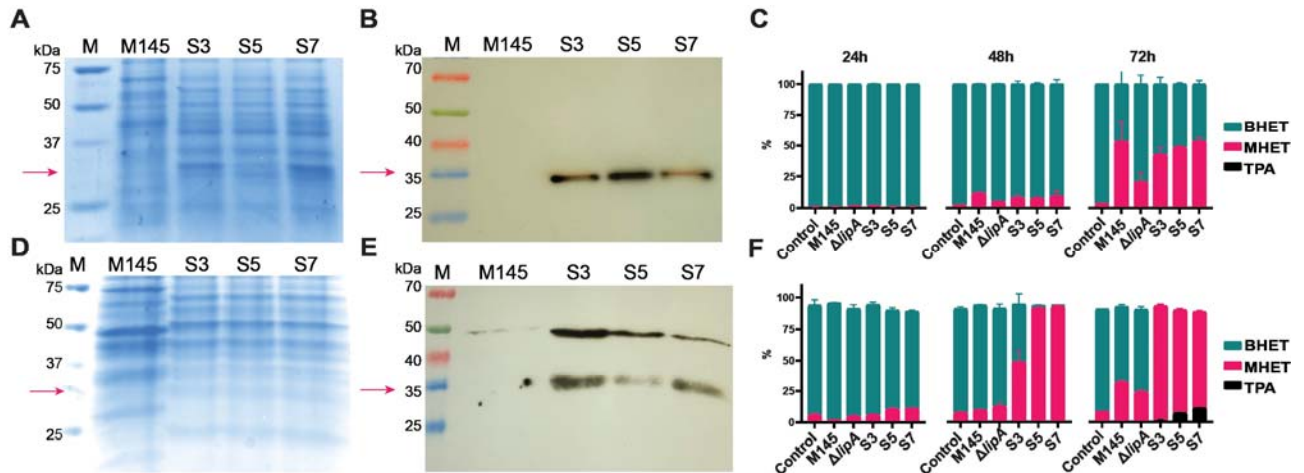
259 over-expression constructs were cloned in strain $\Delta lipA$. This resulted in strain S3 expressing *SclipA*,
260 S5 expressing *S2lipA* and S7 expressing *S92lipA*. Two expression media were chosen namely NMM
261 as minimal medium with a defined carbon source and rich medium tryptic soy broth sucrose (TSBS)
262 which is a protein expression medium. Expression was validated on both media using SDS page and
263 Western blot (Fig. 6A-B and 6D-E). Both NMM with 5 % (w/v) glucose and TSBS show clear signal on
264 western blot indicating that the enzyme is expressed.

265 BHET degradation was examined *in vivo* by inoculating $1,0 \times 10^8$ spores in 50 ml of NMM with glucose
266 [5 % (v/v)] and BHET or TSBS containing BHET. Samples were taken at 24, 48 and 72 h and analyzed
267 using LC-MS. The percentages BHET, MHET and TPA were calculated over the total peak area. After
268 24 h, while the spores are still germinating, limited activity is observed. After 48 h, on NMM, the knock-
269 out shows significantly less activity on BHET than the wildtype strain. After 72 h, there is a clear
270 difference in the degradation pattern of the knock-out compared to the wildtype strain. The
271 overexpression constructs show 40-50 % degradation, which is similar to the wildtype strain, although
272 it is significantly higher than in the knock-out strain (parental strain) (Fig. 6C). Some residual activity,
273 not related to LipA, was still observed in the knock-out strain.

274 After 48 h on TSBS, there was no difference observed in the BHET degrading ability of the wildtype
275 strain compared to the knock-out strain; After 72 h, the wildtype strain degraded significantly more
276 BHET than the knock-out strain. Unlike minimal medium, the strains containing the overexpressed *lipA*
277 variants showed drastic BHET degradation on TSBS after 48 h, with some variation between the
278 different enzyme variants (Fig. 6F). After 72 h, the knock-out converted approximately 23 % of the
279 BHET to MHET, whereas the wild-type strain converted approximately 32 %, the overexpression
280 strains degraded all BHET after 72 h and TPA could be observed in the medium. The knock-out still
281 showed some activity suggesting the presence of at least one other enzyme with BHET degrading
282 activity. A two-way ANOVA clearly indicated the statistical significance of the results (S8).

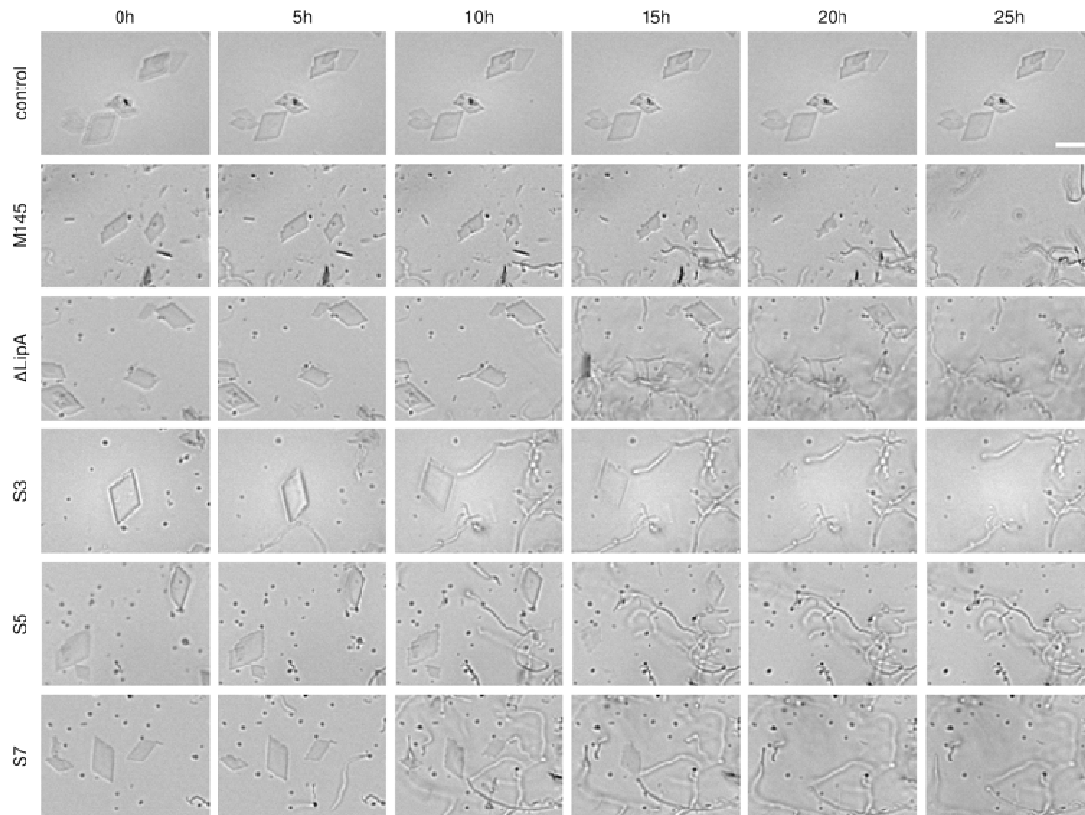
283 To observe physical changes within the cultures, automated time-lapse microscopy imaging was used
284 to follow and visualize the development of the strains and degradation of the BHET. This microscope
285 can make brightfield images at time intervals resulting in a timelapse video (S9). It takes all strains
286 around 10 h to start developing (Fig. 7). Initially, no or only slight changes were observed in the
287 presence and shape of the BHET crystals but over time the BHET crystals roughened and
288 disappeared. The overexpression strains degraded the BHET crystals between 15 and 20 h, where

289 the edges of the crystals started to roughen until the entire crystal fell apart (Fig. 7). The knock-out
290 strain also degraded crystals, but complete degradation was only observed after 25 h. The wildtype
291 strain degraded the BHET in approximately 23 h. For time-lapse videos, see supplementary data (S9).



292
293

294 **Figure 6: Expression of the LipA variants in *S. coelicolor* M145 Δ lipA in TSBS medium.**
295 A) SDS-PAGE of concentrated samples on NMM medium, faint band around ~32 kDa in the
296 overexpression strains. B) Western blot of NMM samples showing clear signal around 32 kDa C)
297 Analysis of BHET degradation in NMM of the wildtype strain, Δ lipA, S3, S5 and S7 using LC-MS.
298 Samples were taken at 24 h, 48 h and 72 h. The percentage BHET is presented in turquoise, the
299 percentage MHET in magenta and the concentration TPA in black. The area percentage was
300 calculated using GraphPad. D) SDS-PAGE of concentrated samples on TSBS medium, faint band
301 around ~32 kDa in the overexpression strains. B) Western blot of TSBS samples showing clear signal
302 around 32 kDa C) Analysis of BHET degradation in TSBS of the wildtype strain, Δ lipA, S3, S5 and S7
303 using LC-MS. Samples were taken at 24 h, 48 h and 72 h. The percentage BHET is presented in
304 turquoise, the percentage MHET in magenta and the concentration TPA in black.



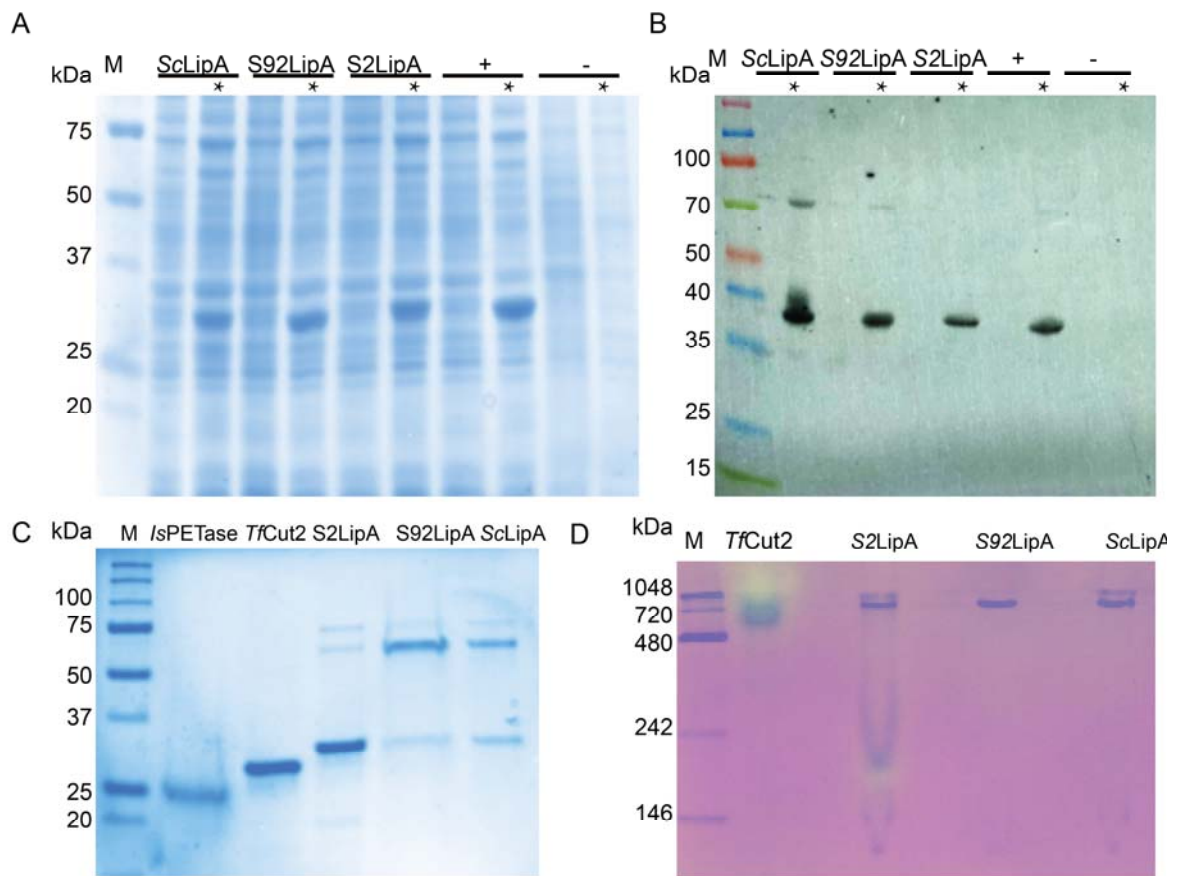
305

306 **Figure 7: Time-lapse images of BHET degradation by *S. coelicolor* M145, Δ lipA, S3, S5 and S7.**
307 Time-lapse images at 5-hour intervals of wild type and Δ lipA mutant and overexpression strains S3, S5
308 and S7 in the presence of BHET particles (diamond-shaped). A negative control lacking bacterial
309 inoculation was used to demonstrate that the BHET particles do not undergo natural degradation over
310 time. The scale bar is 10 μ m.

311 Expression and in vitro characterization of Lipase A variants

312 For extensive *in vitro* analysis of the enzymes, the corresponding gene sequences were trimmed to
313 remove their signal peptide, codon-optimized, synthesized, cloned into pET16b (adding an N-terminal
314 His-tag to the protein) and expressed in *E. coli* BL21 A-I. As positive controls, the genes encoding for
315 *IsPETase* and *TfCut2* were synthesized and expressed in the same way. Both *IsPETase* and *TfCut2*
316 have PET degrading activity, however, their substrate preference, efficiency, optimal conditions, and
317 stability vary. *IsPETase* has PET as the preferred substrate whereas *TfCut2* is a cutinase that also
318 displays high activity on cutinase-like substrates such as the para-nitrophenyl substrates and lipase-
319 like substrates as tributyrin. Clear bands around 32 kDa indicated the presence of the corresponding
320 enzymes (Fig. 8A), which was confirmed by Western blot (Fig. 8B). His-tag purification resulted in
321 samples containing three bands for the LipA enzymes (Fig. 8C). The higher band (~60 kDa) could also
322 be observed in the western blot and most likely corresponded to a dimeric form or aggregate of the
323 enzyme (Fig. 8C). Native zymogram analysis clearly showed that enzyme activity resided with the ~60

324 kDa band for S2LipA, further indicating the dimeric form of the LipA enzymes as being active (Fig 8D).
325 TfCut2 did not migrate through the gel, which might be due to the difference in isoelectric point.



326
327

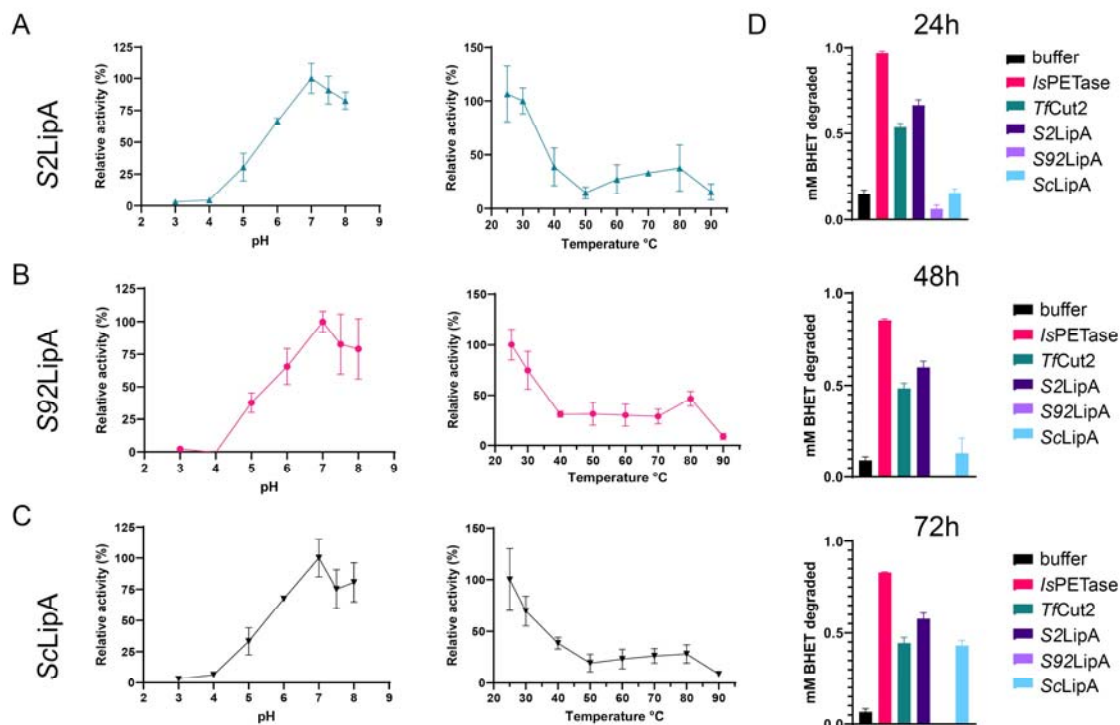
328 **Figure 8: Expression and purification of Lipase A enzymes in *E. coli*.**
329 A); SDS-PAGE of uninduced and induced BL21 cultures. The Lipase A variants have a molecular
330 weight of 28 kDa. The lanes with induced samples are indicated with an asterisk (*). The successful
331 induction is evident in the induced samples, as indicated by the presence of thick bands around 28
332 kDa. 1: ladder, Biorad precision plus; 2&3: ScLipA; 4&5: S92LipA; 6&7: S2LipA; 8&9: Positive control
333 pET16b with insert; 10&11: pET16b without insert. (B); Western blot was conducted to identify the
334 induced proteins as the Lipase_A variants. In the induced samples clear black bands are visible
335 around 38kDa, signifying the presence of the His-tagged Lipase A variants. 1: ladder, Spectra
336 multicolor broad range protein marker; 2 till 11: same samples as for the SDS-PAGE. C); SDS-PAGE
337 gel of the purified Lipase_A variants, along with the IsPETase and TfCut2; 1: Ladder, Biorad precision
338 plus; 2: IsPETase sample; 3: TfCut2 sample; 4: S2LipA sample; 5: S92LipA; 6: ScLipA. Expected
339 bands of the PETase is 25 kDa, TfCut2 (28 kDa), and the Lipase A variants 30 kDa. The Lipase
340 variants show three bands one around 60 kDa and one around 70 kDa. D) Zymogram of TfCut2 and
341 LipA variants on 1% tributyrin. TfCut2 and S2LipA show degradation of tributyrin (yellow spots) in one
342 of the bands. Protein contents of other LipA variants it to low to observe activity. NativeMarker used as
343 marker.

344

345 The optimal pH and temperature for enzyme activity were determined using paranitrophenyl
346 dodecanoate as substrate according to the method of Altammar and colleagues [46]. A pH range from
347 pH 3 to pH 8, was tested for one hour at 30 °C showing pH 7.0 as the optimal pH (Fig. 9A-C). A similar

348 approach was taken to determine the optimal temperature. Enzymes were incubated using a
349 temperature range of 25 °C-90 °C for 1 h. Thus, the optimal temperature appeared to be 25 °C (Fig. 9
350 A-C).

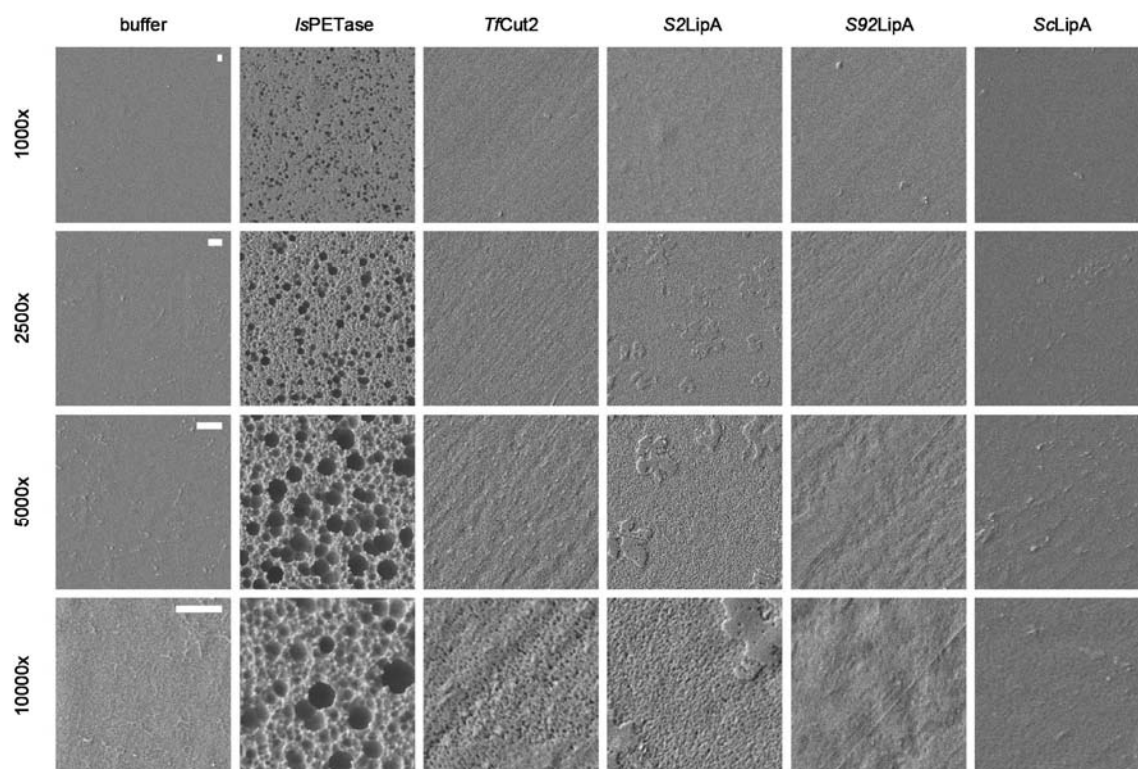
351 These conditions were then used to examine BHET degradation using a colorimetric assay. 2,5 ng/ml
352 of enzyme was incubated with 1 mM of BHET for 24, 48 and 72 h. The positive controls *IsPETase* and
353 *TfCut2* show 50-100 % decrease in BHET (Fig. 9D). *S2LipA*, under these conditions, shows
354 significantly more BHET degradation than *TfCut2* after 24 h. *ScLipA* shows delayed activity, after 72 h,
355 BHET degrading activity is observed. The statistical analysis is provided in the supplement (S10).
356 Simultaneously, amorphous PET films were incubated in pH 7.0 at 25 °C with 15 µg/ml of enzyme for
357 7 days and visualized using the SEM. *IsPETase* and *TfCut2* were used as positive controls, whereas
358 buffer without enzyme was used as negative control. The samples only incubated with buffer show a
359 smooth clean surface. PET films incubated with the *IsPETase* show clear damage in the form of dents
360 and cavities (Fig. 10). The *TfCut2* showed roughening of the surface and at 10.000 x magnification
361 small indents. *S2LipA* showed a similar pattern as *TfCut2* only here it appeared that the top layer of
362 the plastic was not completely degraded (2500 x-10.000 x), in places where this layer is degraded
363 similar damage is observed as shown in *TfCut2*. Both *S92LipA* and *ScLipA* show very limited to no
364 activity (Fig. 10).



365

366 **Figure 9: Determination of optimal enzyme conditions and BHET degradation under optimum**
 367 **conditions.**

368 A-C) relative activity on paranitrophenol dodecanoate at different pH for S2LipA (A), S92LipA(B) and
 369 ScLipA (C). D) Enzymatic BHET degradation using colorimetric assay after 24, 48 and 72 h. The
 370 activity of buffer control (black) as negative control, the IsPETase (magenta) and TfCut2 (turquoise) as
 371 positive controls and the samples S2LipA (dark purple), S92LipA (lavender) and ScLipA (light blue) is
 372 displayed as the concentration BHET degraded in mM.
 373



374

375 **Figure 10: Effect of IsPETase, TfCut2, S2LipA, S92LipA and ScLipA on amorphous PET film.**
376 Amorphous PET films after 7 days of incubation with 15 µg/ml enzyme at 25 °C pH7 at 1000 – 10.000
377 x magnification. The scalebar is equal to 1 µm and applies to all images of the same magnification.
378 Each row displays a different magnification whereas the columns show a different enzyme.

379

380 Discussion

381 Plastics are very useful materials, simplifying everyday life. Unfortunately, poor waste management of
382 plastics leads to polluting leakage into the environment. Understanding and harnessing nature's
383 response to this abundant plastics pollution may help find new solutions for sustainable plastics
384 depolymerization and recycling. However, and importantly, the abundance of micro-organisms that
385 have adapted to utilization and degradation of plastics is not known, and evolution and conservation of
386 genes and proteins involved remains largely unexplored.

387 In this research, we have investigated plastics degrading activity of an IsPETase homolog Lipase A
388 (LipA) from *Streptomyces coelicolor*. Of 96 *Streptomyces* strains 44 % was able to degrade BHET in at
389 least one of the tested conditions, 18 % of the strains showed degrading activity when tested
390 individually. Clearly, regulation of the induction of BHET-degrading activity by GlcNAc presented an
391 important factor, however, the exact mechanism is still unknown. When strains MBT2, 5, 12, 38 and 92

392 were grown on amorphous PET films, they clearly showed adherence to the plastic while no
393 immediate damage was observed.

394 We have shown the presence of *lipA* in 15 different *Streptomyces* strains as a highly conserved gene
395 with some variations. The three variants *ScLipA* (present in *S. coelicolor*), *S92LipA* (present in MBT92
396 and *S2LipA* (present all other active strains) were further investigated. The *in vivo* activity of *ScLipA*
397 was investigated by comparing a knock-out strain with the wildtype strain, in both rich and minimal
398 medium; the knock-out exhibited a significant decrease in its BHET degrading activity. Since some
399 remaining BHET degradation was still observed in the knock-out strain, at least one other enzyme is
400 likely to be involved in BHET degradation. However, this activity most likely resides with another type
401 of enzyme since no other *IsPETase* homolog was found during the homology search. All three *LipA*
402 variants, when expressed in the knock-out background under a semi-constitutive promoter, exhibited
403 significantly enhanced degradation of BHET compared to the wildtype, on both rich and minimal
404 medium. The variant enzymes were expressed in *E. coli*, purified and subjected to enzyme assays.
405 Interestingly, after purification, two clear bands are visible at ~30 kDa and ~60 kDa. Native enzyme
406 analysis via a zymogram indicated that activity only resided with the higher band, suggesting that the
407 active form was a dimer. Indeed, 3D-structure prediction with AlphaFold-multimer suggested the
408 formation of a dimeric enzyme with a probability of 90 % (S11) [47].

409 All enzyme variants were found to have an optimal pH of 7 and optimal temperature of 25 °C.
410 Colorimetric BHET assays confirmed BHET degrading activity of *S2LipA* and *ScLipA* under optimal
411 conditions. Additionally, incubation of amorphous PET film with purified *S2LipA* caused the formation
412 of indents and roughening of the surface, similar to the activity of PET-degrading cutinase *TfCut2*,
413 further confirming *PETase*-like activity.

414 Our data suggests that PET is not the main substrate for these enzymes and that PET degradation
415 merely appears as a moonlighting effect of the lipase activity, similar to the moonlighting effect
416 described for *TfCut2*. *S92LipA* and *ScLipA* showed less activity than *S2LipA* which most probably is
417 due to structural variations in the enzymes caused by the various amino acid substitutions. For
418 example, *S2LipA* contains a region rich in serines close to important substrate binding residues and
419 the substrate binding cleft (Fig. 4I) as opposed to *S92LipA* and *ScLipA*. This may result in enhanced
420 formation of hydrogen bonds with the substrate and/or enhanced enzyme stability [48]–[50].

421 Additionally, serines have previously been found to make an enzyme more hydrophobic in neutral and
422 alkaline conditions which is preferable for PET binding [51], [52]. Since all of the strains in our study
423 are environmental isolates, we hypothesize that the abundance of S2LipA in these strains is initially
424 caused by evolution towards a more stable and hydrophobic lipase and not toward plastic degradation
425 per se. Yet, current pollution rates may push environmental evolution of those enzymes towards
426 enhanced PET affinity. The current abundance of PET-degrading enzymes in nature indicates that
427 nature can indeed take on the challenge of degradation and bioremediation. By further investigating
428 and understanding this phenomenon, the microbial and enzyme characteristics involved, and applying
429 those features, we may ultimately move towards less pollution and a more sustainable future.

430

431 Materials and methods

432 Strains and culturing conditions

433 For the initial tests, *Streptomyces coelicolor* M145 [28] and Actinobacteria from the MBT strain
434 collection of the Institute of Biology Leiden were used for screening. These strains were isolated from
435 the Himalaya mountains, Qinling mountains, Cheverny and Grenouillere France and the Netherlands
436 [42]. The *Streptomyces* plates were incubated at 30 °C for 10 days or longer before investigating the
437 BHET degradation. *E. coli* was grown on Luria-Burtani (LB) agar or in LB cultures overnight at 37 °C.
438 Routine *Streptomyces* manipulation, growth and preparation of spore stocks was performed according
439 to the *Streptomyces* manual [53]. Spore suspensions were obtained by growing the strains on Soy
440 Flour Mannitol agar (SFM [54]) until sporulation.

441

442 Homology search

443 Potential homologs of the PETase *Ideonella sakaiensis* (Uniprot A0A0K8P6T7 (PETH_IDESA)) in the
444 Actinobacterial genomes were identified by performing a BLASTp for the genome of *Streptomyces*
445 *coelicolor* (NCBI 100226) [35]. BLASTp was run in the default setting. The hits were ranked based on
446 their BLAST score, their query cover, their percent identity and the presence of the catalytic triad and
447 substrate binding site.

448

449 Bulk screens

450 The strains are inoculated on the plates using the stamping method. A stamp fitting a 96-wells plate
451 was autoclaved, placed in the stamping device and placed in the 96 wells plate, containing 100 µl of
452 each spore stock in separate wells, and stamped on the plates of interest. Each spot contained
453 approximately 2 µl of spore solution. The plates were grown for 10 days at 30 °C. the spore plates
454 were stored at -20 °C.

455

456 Plate assays

457 Plate assays were performed using minimal medium plates with different types of agars. Upon media
458 selection, *Streptomyces* minimal medium (StrepMM [53]) Difco agar was chosen as standard condition
459 [55]. Plates and additives are described in table 1. Bis(2-hydroxyethyl) terephthalate (BHET) provided

460 by Sigma (Cas: 959-26-2, PN 465151), N-acetyl glucosamine (GlcNAc) provided by Sigma (Cas 7512-
461 17-6, PN A4106).

462 **Table 1: Plate compositions for screening**

Experiment	Medium	Agar	Addition
Toxicity screen	StrepMM	Iberian Agar	Mannitol 25 mM + BHET 0, 10 mM, 20 mM, 30 mM and 40 mM
Bulk screens	StrepMM	Iberian Agar Difco agar Agar-Agar	+/- BHET 10 mM +/- GlcNAc 25 mM
Individual screens <i>S. coelicolor</i> and active strains	StrepMM	Difco Agar	+/- Mannitol 25 mM +/- BHET 10 mM +/- GlcNAc 25 mM

463

464

465 Individual screens

466 For the individual screens 2 μ l of uncorrected spore solution was spotted on 5cm agar plates. And
467 grown at 30 °C for 10 days.

468

469 Agar excision agar samples

470 *S. coelicolor* M145, and *Streptomyces* species MBT2, MBT5, MBT12, MBT38 and MBT92 with 2×10^6
471 spores were inoculated on StrepMM Difco with GlcNAc [25 mM] and BHET [10 mM]. After 14 days of
472 growth, a part of the halo was excised using an agar excision tool. The agar and liquids were
473 separated using clear spin-filtered microtubes (Sorenson BioSciences) spun at 10.000 rpm for 15 min.
474 The flow-through was stored at -20 °C and prepared for LC-MS analysis according to the sample
475 preparation protocol.

476

477 Identification variants Strep strains

478 For the identification of the Lipase A variants in the *Actinobacteria* collection genomic DNA of the
479 active strains was isolated. A PCR was performed using degenerative primers (Table 2) based on the
480 sequence of the *2lipA* gene. PCR was performed with Phusion according to the Phusion protocol
481 provide by Thermofischer scientific (F531S). The blunt-end PCR products were cloned in pJET2.1
482 using the CloneJET PCR cloning kit from Thermofischer scientific (K1231).

483 **Table 2: Primers for amplification of Lipase A variants**

Primer name	Sequence	Purpose	source
JA_G_F1_MBT2/12_SCO0713	GCGTCAGGAGCCGTGCG	PCR LipA	This work
JA_R1_MBT2/12_SCO0713	GTGCAGCAGAACCCACAC	PCR LipA	This work
pJET2.1_F	CGACTCACTATAGGGAGAGCGGC	pJET2.1	Thermofisher

		Sequencing	CloneJET PCR Kit K1231
pJET2.1_R	AAGAACATCGATTTTCCATGGCAG	pJET2.1 Sequencing	ThermoFisher CloneJET PCR Kit K1231

484

485 **CRISPR/Cas knock-out**

486 To obtain a LipA knock-out strain, the CRISPR-Cas9-based pCRISPomyces was used [56], [57]. In
 487 this system, the CRISPR-Cas9 mediated double stranded break will be repaired via homology-directed
 488 repair. Two homologous arms of approximately 1000 bp with a 40 nt overlap at both sides of *lipA* have
 489 been amplified via PCR on genomic DNA of M145. These homologous arms can be digested into the
 490 pCRISPomyces-2 via three-piece Gibson Assembly using the XbaI site. Additionally, a sgRNA will be
 491 annealed into the plasmid using Golden Gate cloning. Primers for these homologous arms bordering
 492 LipA and sgRNA were made using the protocol of Cobb and colleagues. Assembled plasmids were
 493 confirmed via sequencing. Assembled plasmids were transferred to *Streptomyces coelicolor* M145 via
 494 conjugation with *E. coli* ET12567 harboring the pUZ8002 plasmid [58], [59]. Single colonies were
 495 streaked on SFM with apramycin [50 µg/ml] to check for true apramycin resistance. Resistant colonies
 496 were picked to TSBS for gDNA isolation. Correct gene knock-outs were confirmed via diagnostic PCR
 497 and sequencing. Plasmid loss was achieved by restreaking strains for several generations on SFM
 498 agar without antibiotic pressure at 37 °C and checking for the loss of apramycin resistance [50], [51].

499 **Table 3: sgRNA and primers for homologous arms and diagnostic PCR**

Name	Sequence	Length (nt)	Tm
sgRNA1-F	ACGCTCCAGCATCGAAGCCCTGCG	24	80,7
sgRNA1-R	AAACCGCAGGGCTTCGATGCTGGA	24	78,2
JA_MC_F_HA1	TGCCGCCGGGCGTTTTTATGGTCACCGGCC AGGACGA	38	92,2
JA_MC_F_HA2	CGTGCGGGCAGGTGTGGGGTTCTGCTG	28	86,8
JA_MC_R_HA1	CCCCACACCTGCCCGCACGGTTCCTGA	28	87,6
JA_MC_R_HA2	CTTTTACGGTTCCTGGCCTCGTGAGGCTGAG CGTGAG	38	84,8
JA_MRJ_F_KO_S CO0713	GTGCACCGTTCGACGGACGA	20	69.8
JA_R1_MBT2/12_ SCO0713	GTGCAGCAGAACCCCCACAC	20	68.8

500

501 Overexpression strains were obtained by using the pSET152 integrative plasmid [60]. To create
 502 overexpression strains for LipA and its variants, the genes were transferred to a pSET152 integrative
 503 plasmid. Genomic DNA of *S. coelicolor* M145, *Streptomyces* sp. MBT2 and *Streptomyces* sp. MBT92

527 and 260 nm. The following solvent system, at a flow rate of 0.5 ml/min, was used: solvent A, 0.1 %
528 formic acid in water; solvent B, acetonitrile. Gradient elution was as follows: 80:20 (A/B) for 1 min,
529 80:20 to 45:55 (A/B) over 6 min, 45:55 to 0:100 (A/B) over 1 min, 0:100 (A/B) for 2 min, then reversion
530 back to 80:20 (A/B) over 1 min and 80:20 (A/B) for 2 min. This system was connected to a Shimadzu
531 8040 triple quadrupole mass spectrometer (ESI ionisation).

532

533 The reference chromatogram used for the identification of BHET and its monomers [~ 1 mg/ml each].
534 The area percentage was calculated using GraphPad Prism. The statistical analysis consists of a two-
535 way ANOVA using the default setting of GraphPad Prism ($p=0,05$). A multiple comparison was
536 performed providing insights into the significance of each compound present in each culture. The
537 outcomes of the statistical analysis are provided in Supplement S8.

538 [Microscopy](#)

539 The Lionheart FX automated microscope (BioTek) was used for the time-lapse imaging of BHET
540 particle degradation. In total, $3.5 \cdot 10^4$ spores were precultured in TSBS + nalidixic acid [50 $\mu\text{g/ml}$] for
541 48 h. Spores were precultured in TSBS containing nalidixic acid [50 $\mu\text{g/ml}$] for 48 h. Of this solution, 5
542 μl was inoculated in 200 μl TSBS containing ampicillin [50 $\mu\text{g/ml}$] and BHET [0.25 mM]. This solution
543 was added to a Greiner Bio-One SensiPlate 96-well, non-treated black plate with a clear bottom and
544 spun down to settle the mixture. Bright-field pictures were taken every 15 min over the course of 60 h
545 on a 40 x magnification at 30 °C.

546 [Expression in *E. coli*](#)

547 The used S2LipA, S92LipA and ScLipA were codon optimized for *E. coli* and ordered with GeneART
548 from ThermoFisher Scientific (Netherlands). The genes arrived in the ThermoFisher plasmid pMA
549 containing an ampicillin resistance.

550 The plasmids were transformed to chemical competent *E. coli* DH5 α cells for amplification and
551 isolated using the "GeneJET plasmid miniprep Kit" (ThermoFischer, K0503). After plasmid isolation, the
552 *lipA* variants were cloned into the pET16b plasmid using restriction/ligation with restriction enzymes
553 BamHI and NdeI [10 U/ μl] (ThermoFischer Scientific). BamHI was added half an hour after incubation
554 with NdeI to achieve higher efficiency. Following restriction, the restriction enzymes were inactivated
555 by incubating the samples for 20 min at 80 °C in a water bath. The *lipA* genes and the linearized

556 pET16b plasmids were extracted from gel using the “GeneJET GEL extraction Kit” (ThermoFischer,
557 K0692). T4 ligase was used according to the user manual of the manufacturer. See Table 6 for an
558 overview of the used plasmids in this study.

559 *Table 6: Plasmids for expression Lip A variants in E. coli*

Plasmids	Characteristics
pMA_LipA_MBT2	Amp ^R , LipA_MBT2
pMA_LipA_MBT92	Amp ^R , LipA_MBT92
pMA_LipA_M145	Amp ^R , LipA_M145
pET16b_LipA_MBT2	Amp ^R , His-tag, LipA_MBT2
pET16b_LipA_MBT92	Amp ^R , His-tag, LipA_MBT92
pET16b_LipA_M145	amp ^r , His-tag, LipA_M145

560

561 *Table 7: E. coli strains used in this study.*

Strain	Plasmid	Purpose
<i>E. coli</i> BL21	pET16b	Negative control
	pET16b_PETase	Positive control PETase activity
	pET16b_TfCut2	Positive control Cutinase activity
	pET16b_LipA M145	
	pET16b_LipA MBT2	
	pET16b_LipA MBT92	

562

563 Transformed *E. coli* BL21 A-I (Invitrogen C607003) strains were grown on agar plates with ampicillin
564 [100 µg/mL] at 37 °C or in LB liquid medium at 37 °C on 200 rpm.

565 Enzyme purification

566 For protein production, 20 ml precultures with ampicillin (100 µg/ml) of the transformed *E. coli* BL21 A-I
567 were made following the standard culture conditions. 5 ml of these precultures was transferred to 500
568 ml TB containing ampicillin. When an OD value between 0.6-0.8 was reached, the cultures were
569 induced using of arabinose [0.2 % (w/v)] and IPTG [0.5 mM]. The induced cultures were incubated
570 overnight along with a 1 ml non-induced sample at 16 °C with 200 rpm shaking.

571 The pellet was harvested from 1 ml of the induced and non-induced samples, by spinning down for 5
572 min at 13000 rpm and removing the supernatant. Subsequently, this pellet was resuspended in the
573 300 µl of MilliQ water. The samples were sonicated (Bandelin, Sonopuls) for 2x 30 sec at 15 %

574 Amplitude with the MS73 probe to release to proteins. From the cell lysate, 3 µl was loaded on SDS-
575 PAGE to evaluate if the induction was successful.

576 Pellet the 500 ml cultures were harvested by ultracentrifugation (Himac) for 30 min, 6000 rpm at 4 °C.
577 The harvested pellet was transferred to 50 ml falcon tubes and frozen in liquid nitrogen. The frozen
578 pellets were stored at -20 °C.

579 Next, defrosted pellet was resuspended in 15 ml Buffer A (table 8). These solutions were sonicated
580 (Bandelin, Sonopuls) 3 times for 30 sec at 15 % Amplitude. Subsequently, to remove the insoluble
581 faction's ultracentrifugation (Himac) took place for 60 min, 10.000 rpm at 4 °C. The Sample was
582 filtered through a 0.2 µm filter (Filtropur S 0.2 µm, Sarstedt).

583 To obtain purified Lipase A variants, His – affinity purification on an AKTA system was performed,
584 following the AKTA start manual. In the purification process, two additional buffers were employed
585 (Tabel 8).

586 *Table 8: Buffers for the His – affinity purification*

Buffer	Type of Buffer	Composition
Buffer A	Equilibration buffer	25mM Tris-HCl and 300 mM NaCl pH 7.5
Buffer B	Elution Buffer	25mM Tris-HCl, 300 mM NaCl and 500 mM imidazole pH 7.5
Buffer C	Desalting Buffer	25 mM Tris-HCl and 150 mM NaCl pH 7.5

587 During the purification, buffer B was used to first wash the non-binding proteins (15 % buffer B) and
588 then to elute the Lipase A variants from the column (100 % buffer B). After purification, the fractions
589 were desalted using buffer C desalting columns (Cytiva, PD-10) according to the protocol of Cytiva
590 columns. The resulting purified Lipase A variants were stored at -20 °C in a 1:1 mixture of Buffer with
591 40 % glycerol.

592 [SDS-PAGE, Western blot and Zymogram](#)

593 For SDS and Western-Blot, 50 ml of TSBS / NMM + 5 % glucose (v/v) were inoculated with 50 µl
594 dense spore prep and incubated for 24 h. Cultures were centrifuged at 4000 g for 10 min and
595 supernatant was filtered through a 0.2 µm filter. The supernatant was concentrated using a Viaspin
596 column (Sartorius 5 kDas). Protein concentrations were estimated and normalized to 200 ng/µl by
597 performing a Bradford assay.

598 Overall, all SDS pages contained 12 % acrylamide and were run for 20 min at 70 V to stack the
599 proteins on the gels. Further, the gel was run at 150 V until the loading dye reached the bottom of the
600 gel. SDS-page gels were stained with Coomassie-blue staining. For western blot, the gels were
601 transferred using a BioRad Trans-blot Turbo and the corresponding transfer packs (1704157EDU)
602 according to the mixed gel protocol of BioRad. Gel was washed using Tris buffered saline (TBS) buffer
603 and blocked using Tris buffered saline with 0.5 % Tween 20 (TBST) buffer containing 1 % Elk milk.
604 The blot was blocked between 60-90 min. His-antibody was added to a final concentration of 1 µg/ml
605 and incubated overnight (K953-01). The blot was rinsed with water and washed 4 times with TBST, the
606 blot is then incubated with luminol for 1 min, (product number) dried, and developed on X-ray film.

607 For the zymogram, an 8 % native gel, was run for 30 min at 70 V and 2 h at 150 V. The gel was rinsed
608 with demi water and washed with demi water and equilibrated by incubating 3 times for 30 min with 25
609 mM NaCl. After, the gel is placed on a 1 % tributyrin, 0.5 % agarose plate and incubated at 30 °C for
610 1.5 h [61].

611 Enzyme assays

612 Standard enzyme assays

613 The concentration of enzyme was estimated using the Bradford method (ref and company). The
614 esterase/cutinase activity was tested using para-nitrophenyl dodecanoate (sigma) The protocol
615 followed was based on the protocol of Altammar and colleagues with minor adjustments [46]. For the
616 optimal pH test, 50 mM citrate buffers ranging from pH 3 to pH 7 were used, for pH 7.5 and 8 50 mM
617 Tris-HCl buffer was used. The incubation step of 10 min was prolonged to 1 h. The reaction was
618 terminated using 0.1 M sodium carbonate [46]

619 Colorimetric assay BHET degradation

620 The colorimetric assay was performed according to the methods of Beech and colleagues [62]. 0.5 ng
621 of enzyme was incubated with 1mM of BHET for 24, 48 and 72 h and measured at 615 nm in the
622 Tecan M Spark. A reference line was made by adding BHET in the concentrations of 1 mM to 0 mM in
623 steps of 0.1 mM an excess of *TfCut2* was added to convert all BHET to MHET.

624 The amount of BHET degraded was calculated with GraphPad using the above-mentioned reference.
625 The statistical analysis consists of a one-way ANOVA using the default setting of GraphPad Prism ($p =$
626 0.05). A comparison was made between the means of each enzyme treatment providing insights into

627 the significance of the BHET degrading activity of each enzyme. The outcomes of the statistical
628 analysis are provided in Supplemental data S10.

629 SEM

630 The effect of *Streptomyces* on amorphous plastic films was investigated by incubating 10⁷ spores in 3
631 ml of NMM with amorphous PET for two weeks at 30 °C. The samples were fixed with 1.5 %
632 glutaraldehyde (30 min). Subsequently, samples were dehydrated using series of increasing ethanol
633 percentages (70 %, 80 %, 90 %, 96 % and 100 %, each step 30 minutes) and critical point dried
634 (Baltec CPD-030). Hereafter the samples were coated with 10 nm Platinum palladium using a sputter
635 coater, and directly imaged using a JEOL JSM6700F. When investigating enzymes on PET film, 15
636 µg/ml of enzyme was incubated for 7 days, the films were washed with water and 70 % ethanol, air
637 dried, sputter coated with 10 nm Platinum palladium and visualized using a JEOL JSM6700F.

638 Contributions

639
640 JAV: conceptualization, data acquisition (including LC-MS, SEM and Lionheart) data analysis, figure
641 design and writing. MC: Data acquisition (including LC-MS), data analysis and writing; SL homology
642 search, 3D-structure modeling, PyMOL analysis and figure design; AM: Data acquisition; CB: Data
643 acquisition; PI: development LC-MS methods and acquisition LC-MS data; JW: development SEM
644 methods and acquiring SEM images; MEC: development Lionheart methods and acquisition Lionheart
645 videos and pictures. GvW providing *Streptomyces* strains and proofreading; AR: conceptualization,
646 supervision and reviewing. JdW: conceptualization, supervision, writing and reviewing.

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656 **References**

- 657 [1] R. Geyer, J. R. Jambeck, and K. L. Law, "Production, use, and fate of all plastics ever made," *Sci*
658 *Adv*, vol. 3, no. 7, p. e1700782, Jul. 2017, doi: 10.1126/sciadv.1700782.
- 659 [2] J.-A. Verschoor, H. Kusumawardhani, A. F. J. Ram, and J. H. de Winde, "Toward Microbial
660 Recycling and Upcycling of Plastics: Prospects and Challenges," *Front Microbiol*, vol. 0, p. 701,
661 Mar. 2022, doi: 10.3389/FMICB.2022.821629.
- 662 [3] A. L. Brooks, S. Wang, and J. R. Jambeck, "The Chinese import ban and its impact on global
663 plastic waste trade," *Sci Adv*, vol. 4, no. 6, Jun. 2018, doi: 10.1126/SCIADV.AAT0131.
- 664 [4] T. R. Walker and L. Fequet, "Current trends of unsustainable plastic production and
665 micro(nano) plastic pollution," 2023, doi: 10.1016/j.trac.2023.116984.
- 666 [5] S. Bahl, J. Dolma, J. J. Singh, and S. Sehgal, "Biodegradation of plastics: A state of the art
667 review", doi: 10.1016/j.matpr.2020.06.096.
- 668 [6] I. Taniguchi, S. Yoshida, K. Hiraga, K. Miyamoto, Y. Kimura, and K. Oda, "Biodegradation of PET:
669 Current Status and Application Aspects," 2019, doi: 10.1021/acscatal.8b05171.
- 670 [7] A. A. Adeniran and W. Shakantu, "The Health and Environmental Impact of Plastic Waste
671 Disposal in South African Townships: A Review," *Int J Environ Res Public Health*, vol. 19, no. 2,
672 p. 779, Jan. 2022, doi: 10.3390/ijerph19020779.
- 673 [8] F. Kawai, T. Kawabata, and M. Oda, "Current knowledge on enzymatic PET degradation and its
674 possible application to waste stream management and other fields," *Applied Microbiology and*
675 *Biotechnology*, vol. 103, no. 11. Springer Verlag, pp. 4253–4268, Jun. 04, 2019. doi:
676 10.1007/s00253-019-09717-y.
- 677 [9] E. Herrero Acero *et al.*, "Surface engineering of a cutinase from *Thermobifida cellulolytica* for
678 improved polyester hydrolysis," *Biotechnol Bioeng*, vol. 110, no. 10, pp. 2581–2590, 2013, doi:
679 10.1002/BIT.24930.
- 680 [10] E. Herrero Acero *et al.*, "Enzymatic surface hydrolysis of PET: Effect of structural diversity on
681 kinetic properties of cutinases from *Thermobifida*," *Macromolecules*, vol. 44, no. 12, pp. 4632–
682 4640, 2011, doi: 10.1021/ma200949p.
- 683 [11] K. Kitadokoro *et al.*, "Structural insights into the unique polylactate-degrading mechanism of
684 *Thermobifida alba* cutinase," *FEBS Journal*, vol. 286, no. 11, pp. 2087–2098, Jun. 2019, doi:
685 10.1111/febs.14781.
- 686 [12] S. Chen, L. Su, S. Billig, W. Zimmermann, J. Chen, and J. Wu, "Biochemical characterization of
687 the cutinases from *Thermobifida fusca*," *J Mol Catal B Enzym*, vol. 63, no. 3–4, pp. 121–127,
688 May 2010, doi: 10.1016/J.MOLCATB.2010.01.001.
- 689 [13] D. Ribitsch *et al.*, "Characterization of a new cutinase from *Thermobifida alba* for PET-surface
690 hydrolysis," *Biocatal Biotransformation*, vol. 30, no. 1, pp. 2–9, Feb. 2012, doi:
691 10.3109/10242422.2012.644435.
- 692 [14] S. Anbalagan *et al.*, "Hydrolytic Degradation of Polyethylene Terephthalate by Cutinase
693 Enzyme Derived from Fungal Biomass-Molecular Characterization," vol. 12, no. 1, pp. 653–667,
694 2022, doi: 10.33263/BRIAC121.653667.

- 695 [15] S. Yoshida *et al.*, "A bacterium that degrades and assimilates poly(ethylene terephthalate),"
696 *Science (1979)*, vol. 351, no. 6278, pp. 1196–1199, Mar. 2016, doi: 10.1126/science.aad6359.
- 697 [16] P. C. F. Buchholz *et al.*, "Plastics degradation by hydrolytic enzymes: The plastics-active
698 enzymes database—PAZy," *Proteins: Structure, Function, and Bioinformatics*, vol. 90, no. 7, pp.
699 1443–1456, Jul. 2022, doi: 10.1002/PROT.26325.
- 700 [17] D. Ribitsch *et al.*, "A New Esterase from *Thermobifida halotolerans* Hydrolyses Polyethylene
701 Terephthalate (PET) and Polylactic Acid (PLA)," *Polymers (Basel)*, vol. 4, no. 1, pp. 617–629,
702 Feb. 2012, doi: 10.3390/polym4010617.
- 703 [18] C. Roth *et al.*, "Structural and functional studies on a thermostable polyethylene terephthalate
704 degrading hydrolase from *Thermobifida fusca*," *Appl Microbiol Biotechnol*, vol. 98, no. 18, pp.
705 7815–7823, Sep. 2014, doi: 10.1007/S00253-014-5672-0.
- 706 [19] M. Furukawa, N. Kawakami, A. Tomizawa, and K. Miyamoto, "Efficient Degradation of
707 Poly(ethylene terephthalate) with *Thermobifida fusca* Cutinase Exhibiting Improved Catalytic
708 Activity Generated using Mutagenesis and Additive-based Approaches," *Sci Rep*, vol. 9, no. 1,
709 pp. 1–9, Dec. 2019, doi: 10.1038/s41598-019-52379-z.
- 710 [20] B. Godden, T. Legon, P. Helvenstein, and M. Penninckx, "Regulation of the production of
711 hemicellulolytic and cellulolytic enzymes by a *Streptomyces* sp. growing on lignocellulose," *J*
712 *Gen Microbiol*, vol. 135, no. 2, pp. 285–292, 1989, doi: 10.1099/00221287-135-2-285.
- 713 [21] E. L. Almeida, A. F. C. Rincón, S. A. Jackson, and A. D. W. Dobson, "In silico Screening and
714 Heterologous Expression of a Polyethylene Terephthalate Hydrolase (PETase)-Like Enzyme
715 (SM14est) With Polycaprolactone (PCL)-Degrading Activity, From the Marine Sponge-Derived
716 Strain *Streptomyces* sp. SM14," *Front Microbiol*, vol. 10, 2019, doi: 10.3389/fmicb.2019.02187.
- 717 [22] H. A. El-Shafei, N. H. Abd El-Nasser, A. L. Kansoh, and A. M. Ali, "Biodegradation of disposable
718 polyethylene by fungi and *Streptomyces* species," *Polym Degrad Stab*, vol. 62, no. 2, pp. 361–
719 365, 1998, doi: 10.1016/S0141-3910(98)00019-6.
- 720 [23] T.-K. Chua, M. Tseng, and M.-K. Yang, "Degradation of Poly(ϵ -caprolactone) by thermophilic
721 *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* 76T-2," *AMB Express*, vol. 3, no. 1, p.
722 8, 2013, doi: 10.1186/2191-0855-3-8.
- 723 [24] A. L. Pometto, B. Lee, and K. E. Johnson, "Production of an extracellular polyethylene-
724 degrading enzyme(s) by *Streptomyces* species.," *Appl Environ Microbiol*, vol. 58, no. 2, p. 731,
725 1992, doi: 10.1128/aem.58.2.731-733.1992.
- 726 [25] K. J. Hsu, M. Tseng, T. M. Don, and M. K. Yang, "Biodegradation of poly(β -hydroxybutyrate) by
727 a novel isolate of *streptomyces bangladeshensis* 77T-4," *Bot Stud*, vol. 53, no. 3, pp. 307–313,
728 2012.
- 729 [26] M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt, *The Prokaryotes*.
730 2006.
- 731 [27] E. A. Barka *et al.*, "Taxonomy, Physiology, and Natural Products of Actinobacteria,"
732 *Microbiology and Molecular Biology Reviews*, vol. 80, no. 1, pp. 1–43, Mar. 2016, doi:
733 10.1128/MMBR.00019-15/ASSET/408FB6C5-97FF-4452-9C36-
734 736B06EB1413/ASSETS/GRAPHIC/ZMR0041524040006.JPEG.

- 735 [28] P. A. Hoskisson and G. P. van Wezel, "Streptomyces coelicolor," *Trends Microbiol*, vol. 27, no. 5,
736 pp. 468–469, May 2019, doi: 10.1016/J.TIM.2018.12.008.
- 737 [29] G. Grause, Y. Kuniyasu, M. F. Chien, and C. Inoue, "Separation of microplastic from soil by
738 centrifugation and its application to agricultural soil," *Chemosphere*, vol. 288, 2022, doi:
739 10.1016/j.chemosphere.2021.132654.
- 740 [30] T.-K. Chua, M. Tseng, and M.-K. Yang, "Degradation of Poly(ϵ -caprolactone) by thermophilic
741 *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* 76T-2," *AMB Express*, vol. 3, no. 1, p.
742 8, 2013, doi: 10.1186/2191-0855-3-8.
- 743 [31] E. A. Barka *et al.*, "Taxonomy, Physiology, and Natural Products of Actinobacteria,"
744 *Microbiology and Molecular Biology Reviews*, vol. 80, no. 1, pp. 1–43, Mar. 2016, doi:
745 10.1128/MMBR.00019-15/ASSET/408FB6C5-97FF-4452-9C36-
746 736B06EB1413/ASSETS/GRAPHIC/ZMR0041524040006.JPEG.
- 747 [32] J. R. McCormick and K. Flärdh, "Signals and regulators that govern *Streptomyces*
748 development," *FEMS Microbiol Rev*, vol. 36, no. 1, pp. 206–231, Jan. 2012, doi:
749 10.1111/J.1574-6976.2011.00317.X.
- 750 [33] H. Zhu *et al.*, "Eliciting antibiotics active against the ESKAPE pathogens in a collection of
751 actinomycetes isolated from mountain soils," *Microbiology (United Kingdom)*, vol. 160, no.
752 PART 8, pp. 1714–1726, Aug. 2014, doi: 10.1099/MIC.0.078295-0/CITE/REFWORKS.
- 753 [34] S. Yoshida *et al.*, "A bacterium that degrades and assimilates poly(ethylene terephthalate),"
754 *Science (1979)*, vol. 351, no. 6278, pp. 1196–1199, Mar. 2016, doi: 10.1126/science.aad6359.
- 755 [35] S. D. Bentley *et al.*, "Complete genome sequence of the model actinomycete *Streptomyces*
756 *coelicolor* A3(2)," *Nature*, vol. 417, no. 6885, pp. 141–147, May 2002, doi: 10.1038/417141A.
- 757 [36] X. Han *et al.*, "Structural insight into catalytic mechanism of PET hydrolase," *Nat Commun*, vol.
758 8, no. 1, p. 2106, 2017, doi: 10.1038/s41467-017-02255-z.
- 759 [37] J. Jumper *et al.*, "Highly accurate protein structure prediction with AlphaFold," *Nature* 2021
760 596:7873, vol. 596, no. 7873, pp. 583–589, Jul. 2021, doi: 10.1038/S41586-021-03819-2.
- 761 [38] L.-F. Wu, B. Ize, A. Chanal, Y. Quentin, and G. Fichant, "Bacterial Twin-Arginine Signal Peptide-
762 Dependent Protein Translocation Pathway: Evolution and Mechanism JMMB Minireview,"
763 2000. [Online]. Available: www.caister.com/bacteria-plant
- 764 [39] M. A. Świątek, M. Urem, E. Tenconi, S. Rigali, and G. P. van Wezel, "Engineering of N-
765 acetylglucosamine metabolism for improved antibiotic production in *Streptomyces coelicolor*
766 A3(2) and an unsuspected role of NagA in glucosamine metabolism," *Bioengineered*, vol. 3, no.
767 5, p. 280, Sep. 2012, doi: 10.4161/BIOE.21371.
- 768 [40] Ś. Magdalena, E. Tenconi, S. Rigali, and G. P. Van Wezel, "Functional Analysis of the N-
769 Acetylglucosamine Metabolic Genes of *Streptomyces coelicolor* and Role in Control of
770 Development and Antibiotic Production," *J Bacteriol*, vol. 194, no. 5, p. 1136, Mar. 2012, doi:
771 10.1128/JB.06370-11.
- 772 [41] M. J. Bibb, G. H. Jones, R. Joseph, M. J. Buttner, and J. M. Ward, "The agarase gene (*dag A*) of
773 *Streptomyces coelicolor* A3(2): affinity purification and characterization of the cloned gene
774 product.," *J Gen Microbiol*, vol. 133, no. 8, pp. 2089–2096, Aug. 1987, doi: 10.1099/00221287-
775 133-8-2089/CITE/REFWORKS.

- 776 [42] H. Zhu, S. K. Sandiford, V. C. Da Cunha, J. M. Raaijmakers, and G. P. Van Wezel, "A New
777 Collection of Actinomycetes Isolated from Himalaya and Qinling Mountain Soils as Source of
778 Novel Bioactive Compounds."
- 779 [43] M. F. Traxler, J. D. Watrous, T. Alexandrov, P. C. Dorrestein, and R. Kolter, "Interspecies
780 interactions stimulate diversification of the *Streptomyces coelicolor* secreted metabolome,"
781 *mBio*, vol. 4, no. 4, Aug. 2013, doi: 10.1128/MBIO.00459-13/FORMAT/EPUB.
- 782 [44] J. J. Almagro Armenteros *et al.*, "SignalP 5.0 improves signal peptide predictions using deep
783 neural networks," *Nature Biotechnology* 2019 37:4, vol. 37, no. 4, pp. 420–423, Feb. 2019, doi:
784 10.1038/S41587-019-0036-Z.
- 785 [45] A. L. Zhang *et al.*, "Recent advances on the GAP promoter derived expression system of *Pichia*
786 *pastoris*," *Mol Biol Rep*, vol. 36, no. 6, pp. 1611–1619, Jul. 2009, doi: 10.1007/S11033-008-
787 9359-4.
- 788 [46] K. A. Altammar *et al.*, "Characterization of AnCUT3, a plastic-degrading paucimannose
789 cutinase from *Aspergillus niger* expressed in *Pichia pastoris*," *Int J Biol Macromol*, vol. 222, no.
790 PB, pp. 2353–2367, 2022, doi: 10.1016/j.ijbiomac.2022.10.022.
- 791 [47] R. Evans *et al.*, "Protein complex prediction with AlphaFold-Multimer," 2022, doi:
792 10.1101/2021.10.04.463034.
- 793 [48] C. N. Pace *et al.*, "Contribution of hydrogen bonds to protein stability," *Protein Science*, vol. 23,
794 no. 5, pp. 652–661, May 2014, doi: 10.1002/PRO.2449.
- 795 [49] A. C. C. Carlsson *et al.*, "Increasing Enzyme Stability and Activity through Hydrogen Bond-
796 Enhanced Halogen Bonds," *Biochemistry*, vol. 57, no. 28, pp. 4135–4147, Jul. 2018, doi:
797 10.1021/ACS.BIOCHEM.8B00603/SUPPL_FILE/BI8B00603_LIVESLIDES.MP4.
- 798 [50] T. M. Gray and B. W. Matthews, "Intrahelical hydrogen bonding of serine, threonine and
799 cysteine residues within alpha-helices and its relevance to membrane-bound proteins," *J Mol*
800 *Biol*, vol. 175, no. 1, pp. 75–81, May 1984, doi: 10.1016/0022-2836(84)90446-7.
- 801 [51] A. S. Panja, S. Maiti, and B. Bandyopadhyay, "Protein stability governed by its structural
802 plasticity is inferred by physicochemical factors and salt bridges," *Sci Rep*, vol. 10, no. 1, p.
803 1822, Feb. 2020, doi: 10.1038/S41598-020-58825-7.
- 804 [52] R. Graham *et al.*, "The role of binding modules in enzymatic poly(ethylene terephthalate)
805 hydrolysis at high-solids loadings," *Chem Catalysis*, vol. 2, no. 10, pp. 2644–2657, Oct. 2022,
806 doi: 10.1016/J.CHECAT.2022.07.018.
- 807 [53] Kieser Tobias, "Practical *Streptomyces* Genetics," 2000.
- 808 [54] G. Hobbs, C. M. Frazer, D. C. J. Gardner, J. A. Cullum, and S. G. Oliver, "Dispersed growth of
809 *Streptomyces* in liquid culture," *Appl Microbiol Biotechnol*, vol. 31, no. 3, pp. 272–277, Sep.
810 1989, doi: 10.1007/BF00258408/METRICS.
- 811 [55] D. A. Hopwood, "Phase-contrast observations on *Streptomyces coelicolor*," *J Gen Microbiol*,
812 vol. 22, no. 1, pp. 295–302, Feb. 1960, doi: 10.1099/00221287-22-1-295/CITE/REFWORKS.
- 813 [56] R. E. Cobb, Y. Wang, and H. Zhao, "High-Efficiency Multiplex Genome Editing of *Streptomyces*
814 Species Using an Engineered CRISPR/Cas System," *ACS Synth Biol*, vol. 4, no. 6, pp. 723–728,
815 Jun. 2015, doi: 10.1021/sb500351f.

- 816 [57] Y. Tong, P. Charusanti, L. Zhang, T. Weber, and S. Y. Lee, "CRISPR-Cas9 Based Engineering of
817 Actinomycetal Genomes," *ACS Synth Biol*, vol. 4, no. 9, pp. 1020–1029, Sep. 2015, doi:
818 10.1021/acssynbio.5b00038.
- 819 [58] X. Zhong, L. Zhang, G. P. van Wezel, E. Vijgenboom, and D. Claessen, "Role for a Lytic
820 Polysaccharide Monooxygenase in Cell Wall Remodeling in *Streptomyces coelicolor*," *mBio*,
821 vol. 13, no. 2, Apr. 2022, doi: 10.1128/MBIO.00456-22/ASSET/72DB623E-AFC0-484E-B66D-
822 B7D40E776338/ASSETS/IMAGES/MEDIUM/MBIO.00456-22-F006.GIF.
- 823 [59] F. Flett', V. Mersinias, and C. P. Smith, "High efficiency intergeneric conjugal transfer of plasmid
824 DNA from *Escherichia coli* to methyl DNA-restricting streptomycetes," *FEMS Microbiol Lett*,
825 vol. 155, no. 2, pp. 223–229, Oct. 1997, doi: 10.1111/J.1574-6968.1997.TB13882.X.
- 826 [60] M. Bierman, R. Logan, K. O'Brien, E. T. Seno, R. Nagaraja Rao, and B. E. Schoner, "Plasmid
827 cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp.,"
828 *Gene*, vol. 116, no. 1, pp. 43–49, Jul. 1992, doi: 10.1016/0378-1119(92)90627-2.
- 829 [61] A. M. J. Ng, H. Zhang, and G. K. T. Nguyen, "Zymography for Picogram Detection of Lipase and
830 Esterase Activities," *Molecules 2021, Vol. 26, Page 1542*, vol. 26, no. 6, p. 1542, Mar. 2021, doi:
831 10.3390/MOLECULES26061542.
- 832 [62] J. Lusty Beech *et al.*, "A flexible kinetic assay efficiently sorts prospective biocatalysts for PET
833 plastic subunit hydrolysis," *RSC Adv*, vol. 12, no. 13, pp. 8119–8130, Mar. 2022, doi:
834 10.1039/D2RA00612J.
- 835