Structure of the plastic-degrading *Ideonella sakaiensis* MHETase bound to a substrate

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The extreme durability of polyethylene terephthalate (PET) debris has rendered it a long-term environmental burden. At the same time, current recycling efforts still lack sustainability. Two recently discovered bacterial enzymes that specifically degrade PET represent a promising solution. First, *Ideonella sakaiensis* PETase, a structurally well-characterized consensus \( \alpha/\beta \)-hydrolase fold enzyme, converts PET to mono-(2-hydroxyethyl) terephthalate (MHET). MHETase, the second key enzyme, hydrolyzes MHET to the PET educts terephthalate and ethylene glycol. Here, we report the crystal structures of active ligand-free MHETase and MHETase bound to a nonhydrolyzable MHET analog. MHETase, which is reminiscent of feruloyl esterases, possesses a classic \( \alpha/\beta \)-hydrolase domain and a lid domain conferring substrate specificity. In the light of structure-based mapping of the active site, activity assays, mutagenesis studies and a first structure-guided alteration of substrate specificity towards bis-(2-hydroxyethyl) terephthalate (BHET) reported here, we anticipate MHETase to be a valuable resource to further advance enzymatic plastic degradation.
Appreciating its simple synthesis, robustness and durability, industrial production of PET was launched soon after its discovery and has been gradually increasing, projected to be over 70 million tons in 2020. One of the biggest advantages of PET is its chemical inertness due to the hydrophobicity of the terephthalic acid (TPA) moiety, rendering it nearly resistant to environmental degradation. Although PET and other synthetic polymer plastics are considered nontoxic, larger particles and micro granules thereof are durable, omnipresent in marine or terrestrial habitats and accumulate in living organisms. Often, they are also the carriers of potentially toxic colorants and additives. Current recycling efforts cover only a fraction of PET waste and yield downgraded lower value products. They depend on the addition of large quantities of virgin polymer and significant consumption of energy. Alternatively, several enzymes have been identified that can hydrolyze PET to TPA and ethylene glycol at elevated temperatures, albeit with low activity. Enzyme optimization by biotechnology has been successful to some degree, but has so far not led to enzymes, which can fully penetrate and degrade a thick layer of highly crystalline PET in a cost-effective and environmentally friendly manner.

Recently, the bacterial strain *Ideonella sakaiensis* 201-F6 was discovered and shown to grow on low-crystallinity PET films. Two α/β-hydrolase fold enzymes (α/β-hydrolases), PETase and MHETase, work together to degrade PET in two steps via MHET, yielding TPA and ethylene glycol—the building blocks required for a new round of PET synthesis (Fig. 1a). Recent crystal structures of PETase bound to ligands confirmed the predicted α/β-hydrolase fold, elucidated substrate binding, mode of catalysis and even permitted the enhancement of catalytic properties or alteration of substrate specificity. Compared to known

![Diagram of PET degradation](image_url)

**Fig. 1** The structure of *I. sakaiensis* MHETase displays a bipartite domain architecture. **a** *I. sakaiensis* PETase and MHETase degrade PET to terephthalic acid and ethylene glycol. Side products are not shown. **b** MHETase structure with the α/β-hydrolase domain (MHETase_{hyd}) colored in salmon and the lid domain (MHETase_{lid}) in light blue. Disulfide bonds are shown as sticks. **c** Close-up view of the MHETase catalytic triad, oxygen hole and the water molecules in the substrate-binding site. **d** *A. oryzae* FaeB (PDB-ID: 3WMT), α/β-hydrolase domain (AoFaeB_{hyd}) in crimson red, lid domain (AoFaeB_{lid}) in cyan. **e** Close-up view of the AoFaeB catalytic triad, oxygen hole and the water molecules in the substrate-binding site. Dashed lines indicate hydrogen bonds, rotation angles relate to the overview. Interacting residues are shown as sticks and colored by atom type. Carbon—as given for the respective molecule; nitrogen—blue; oxygen—red; sulfur—yellow. Water oxygens are shown as green spheres. Calcium is shown as purple sphere.
PET-degrading esterases, PETase from *I. sakaiensis* shows higher activity at ambient temperature and on highly crystalline PET.

In contrast, the structure of *I. sakaiensis* MHETase, the second enzyme—and crucial for full PET degradation—is still unknown. MHETase was initially assigned to the tannase enzyme family, which belongs to Block X of the α/β-hydrolase fold enzymes classified in the ESTHER database (2.87 Å RMSD for 194 out of 282 residues aligned; Supplementary Fig. 3c). This family includes fungal and bacterial tannases and feruloyl esterases. Other significantly different bacterial tannases can be found in a distinct Block H (Tannases_bact) in this database. Consistently, MHETase was shown to exclusively hydrolyze MHET but not BHET, PET, p-nitrophenyl (pNP) aliphatic esters or aromatic ester compounds such as ethyl gallate and ethyl ferulate which are converted by other enzymes from the tannase family, indicating a highly restricted substrate specificity. All plastic-degrading enzymes known so far display an α/β-hydrolase fold. MHETase, however, is likely to possess a scaffold unprecedented for plastic-degrading enzymes. This may be exploited in order to improve catalysis and to expand substrate specificity and thus significantly advance enzymatic plastic polymer degradation.

Here, we present the crystal structures of *I. sakaiensis* PETase, MHETase and MHETase bound to a nonhydrolyzable analog (MHETA) or to benzoic acid. A structure-based mapping of the active site by mutations and binding studies with different substrates was used to determine the molecular basis for product inhibition and guided the development of MHETase variants with enhanced activity towards MHET or even an altered substrate specificity towards BHET. We anticipate our data to significantly advance the current understanding of enzymes degrading synthetic polymers.

**Results**

**Structure and phylogeny of *I. sakaiensis* MHETase.** We have determined crystal structures of recombinantly expressed and purified *I. sakaiensis* MHETase in its ligand-free form (2.0 Å resolution), MHETase bound to a nonhydrolyzable mono-(2-hydroxyethyl) terephthalamide (MHETA, 2.1 Å resolution) or to benzoic acid (BA, 2.2 Å resolution) as well as ligand-free PETase (2.0 Å resolution) (Supplementary Figs. 1, 2a–f, Supplementary Table 1). The structure of PETase was solved by molecular replacement (MR) employing the structural coordinates of *T. fusca* cutinase TICut2 (PDB entry 4CGJ11,23; see Methods). The structure of MHETase was solved by an MR pipeline employing a recent feruloyl esterase structure (PDB entry 6G21; see Methods). The overall domain architecture of the 65 kDa MHETase resembles that of feruloyl esterases, with a lid domain inserted between β-strand 7 and α-helix 15 of the α/β-hydrolase fold (Fig. 1b, Supplementary Fig. 1).

As previously observed for feruloyl esterases, the presence of a structural calcium-binding site was confirmed by X-ray fluorescence spectroscopy for MHETase (Fig. 1b, Supplementary Fig. 2b). Likewise, one of five disulfide bonds is flanking a catalytic triad (formed by S225, H528, D492) and the oxoanion hole comprising the backbone amide nitrogen atoms of G132 and E226 (Fig. 1c, d). In the ligand-free structure of MHETase, several water molecules are maintained by a hydrogen bond network at the substrate-binding site (Fig. 1c). While the α/β-hydrolase domain superimposes well with the closest structurally characterized feruloyl esterase homologo FaEB from *A. oryzae* (1.60 Å RMSD for 280 out of 342 residues aligned, 32.5% amino acid identity), the lid domain of MHETase contains several additional loops that markedly differ from FaEB (2.33 Å RMSD for 148 out of 215 residues aligned, 18.9% identity) (Fig. 1b, d). The overall structures of MHETase and FaEB are structurally similar (2.04 Å RMSD for 421 out of 559 residues aligned) despite a relatively low number of amino acid identities (27.5%). When comparing MHETase with known tannase structures, e.g. tannin acyl α/β-hydrolase from *Lactobacillus plantarum* (LptE), it is evident that only the overall fold of the α/β-hydrolase domain is similar (2.77 Å RMSD for 195 out of 282 residues aligned, 13.8% identity), while very large differences (5.24 Å RMSD) are observed for the lid domain (Supplementary Fig. 3a, b). PETase and MHETase only share the α/β-hydrolase fold (2.87 Å RMSD for 184 out of 262 residues aligned; Supplementary Fig. 3c).

A phylogenetic analysis groups MHETase with the feruloyl esterases and tannases of *Block X* in the ESTHER database. It is located in a branch with no other structures (Supplementary Fig. 4). The structures of the closest MHETase relatives are 3WMT and 6G21, two feruloyl esterases of *Aspergillus oryzae*. With them, MHETase shares not only the catalytic triad S225-H528-D492 (3WMT: S203-H457-D417, 6G21: S169-H421-D381) but also G132 (G125, G91) as part of the oxoanion hole and C224-C529 (C202-C528, C168-C422), whose disulfide bond holds the catalytic residues Ser and His together. All these residues in the catalytic domain of the disulfide bond is typical (>80% conservation) for the tannase family in *Block X* of α/β-hydrolases according to the ESTHER database (Fig. 1b–e).

The lid domain of the feruloyl esterases displays the same α-helical fold, but the amino acid sequences cannot be aligned without structural information. A binding pocket—like that for MHET in MHETase—also exists in feruloyl esterases, but not a single one of the lining residues is conserved. However, a comparison of the MHETase active site to that of FaEB reveals several residues around the catalytic triad that may contribute to substrate positioning in a similar fashion (e.g. L235, F354 and L245, F415 in FaEB and MHETase, respectively) (Fig. 1c, e). Most likely, it is the alteration in the substrates, especially the carboxylic acid group of MHET vs. the phenolic (methyl ether) groups and the elongating double bond of ferulates that has evoked this difference. The substrates of tannases, e.g. gallates, are more similar at least with respect to their size to MHET. The only tannase structure available in complex with ethyl gallate is from *Lactobacillus plantarum* (4JOK), which belongs to the bacterial tannase family in *Block H* of α/β-hydrolases according to the ESTHER database. The tannase catalytic domains are sufficiently conserved, such that the catalytic triad superimposes well. However, the sequences differ strongly, the disulfide bond is missing and the lid domain has a markedly different fold.

**Structure of MHETase bound to a nonhydrolyzable ligand.** The main chain conformation in the MHETase-MHETA complex structure is nearly identical to that of MHETase without substrate (RMSD 0.54 Å) and sheds light on the positioning of MHET for catalysis. While the catalytic triad and oxoanion hole residues are part of the α/β-hydrolase domain, substrate specificity is almost exclusively conferred by the lid domain (Fig. 2a, b). Hydrophobic contacts between the phenyl ring of MHETA and the α/β-hydrolase domain are restricted to primarily F495, and to a lesser extent G132 and A494. Strikingly, MHETase is tightly bound by the lid domain residues F415, L254 and W397 surrounding nearly the entire MHET phenyl moiety. The two oxygens of the free carboxylic make contacts to R411, which is held in place by S416, S419 and the backbone amide of G258, which maintain a hydrogen bond network involving three water molecules.

Despite their overall high similarity, a detailed comparison of MHETase structures in the absence and presence of the substrate reveals an induced-fit mechanism upon MHETA binding (Fig. 2c, d). In the ligand-free structure, F415 points away from
the active site and thus opens it for substrate binding. The association of MHETase then triggers a near 180° rotation of the F415 side chain around $\chi_1$, closing the active site and consolidating the interaction.

Lastly, unlike PETase, MHETase binds to its substrate very tightly with a $K_m$ of 7.3 µM\textsuperscript{17}. A comparison of the active-site molecular surfaces of LptE, PETase and MHETase in their substrate-bound states illustrates a higher solvent accessibility of LptE and PETase, which is partially related to the induced-fit mechanism observed for MHETase and the number of residues contacting the respective substrate (Fig. 2c, d, Supplementary Figure 5 a–d).

The positioning of the substrate in the active site of MHETase is reminiscent of the tannin acyl $\alpha/\beta$-hydrolase from \textit{L. plantarum} (PDB-ID: 4J0K)\textsuperscript{25} bound to ethyl gallate (EthGal, yellow), superimposed on helix a5 of MHETase (not shown). $\alpha/\beta$-Hydrolase domain (LptE\textsuperscript{Hyd}) in olive, lid domain (LptELid) in dark blue. Rotation symbols indicate views relative to a. Color scheme for interacting residues and water oxygens as in Fig. 1. Calcium is shown as magenta spheres.

MHETase ligand spectrum and implications for the active site.

The substrate-binding position of PETase fundamentally differs from that of MHETase as shown for the PETase-1-(2-hydroxyethyl) 4-methyl terephthalate (HEMT) and p-nitrophenol (pNP) co-structures (Supplementary Figure 5 a-c). In particular for PETase, the absence of a lid domain limits the number of residues involved in immediate substrate recognition down to four.

Primarily the phenyl moiety of HEMT and also pNP is bound by hydrophobic contacts of Y85, M132, W156 and I179, but the methyl ester or hydroxyl group in the 4-position of HEMT or pNP, respectively, is fully exposed to the bulk solvent (Supplementary Figure 5 a–c). In summary, the complexity of substrate recognition by MHETase clearly distinguishes it from other enzymes, such as tannases or even PETase.

Apart from the entire substrate, even substrate sub-structures and analogs such as benzoic acid or nicotinic acid are able to bind tightly to MHETase as observed in the respective co-structure and in differential scanning fluorimetry (DSF) measurements (Supplementary Figure 6a, Fig. 3). It is again mainly lid domain residues, which establish the contacts to benzoic acid and place it in an identical position as MHETA (Supplementary Figure 6b).

A comparison of potential MHETase ligands with limited variations by DSF confirms which functional groups are recognized by the MHETase binding site (for ligand quality control, see Supplementary Figure 7a–f). Our structural analysis suggests that R411 enforces a strictly required negative charge in the 4-position to the hydrolyzed ester bond, which clearly
explains why diesters and diamides show no binding to MHETase. BHET binding is thus excluded and MHET will only bind in the proper orientation but not with the hydroxyethyl group buried in the substrate pocket (Fig. 2b). Nitro groups can bind weakly when partially charged as a resonance structure with a phenolate or thiophenolate group. Negatively charged tetrahedral groups (sp³) as in sulfonic and arsenic acids can bind to MHETase but are clearly outperformed by the planar (sp²) group of the carboxylate (Fig. 3). Consequently, the loss of the positive charge in the MHETase R411Q and R411A variants leads to strongly reduced substrate binding and decreased inhibition by benzoate in mono-4-nitrophenyl terephthalate (MpNPT) hydrolysis (Supplementary Figure 8, Supplementary Table 2).

Effect of MHETase mutants and generation of BHETase activity. The central role of R411 in coordinating the carboxylic acid function of the substrate was additionally confirmed by activity assays with the variants R411A and R411Q. These mutants show a strong increase of \( K_m \) and some decrease in turnover rate against MpNPT (Fig. 4a). Furthermore, R411A and R411Q mutations almost completely abolish the conversion of the natural substrate MHET (Fig. 4b). If the hydrogen bond of the substrate carboxylate to S416 or S419 is also abolished in double mutants, \( K_m \) further increases to about 1000-fold over the wild-type level (Fig. 4a). Thus, substrate recognition strongly relies on the aromatic ring as well as the carboxylate function of MHET both guiding its positioning for hydrolysis. Inhibitor experiments with benzoate derivatives and R411 and S416 mutants also demonstrate the importance of the interaction between the carboxylate moiety of the ligand and R411 together with hydrogen bonding for tight binding (Supplementary Table 2).

The high affinity for compounds with a benzoate substructure is expected to lead to product inhibition by formed TPA when...
higher concentrations of MHET are hydrolyzed in vitro. This is demonstrated by the decreasing reaction rate for MHET hydrolysis over time (Supplementary Figure 9a). The effect of product inhibition is likely less pronounced in the natural environment where formed TPA is metabolized by the bacterium. In the natural environment where low substrate concentrations are expected, it is advantageous for biotechnological applications running at substrate concentrations about 105-fold higher than $K_m$—which is in the 100 mM range.

The structural reasons for a high MHETase activity towards MHET ($k_{cat} 11.1 \pm 1.4 \text{ s}^{-1}$) and a very low activity towards BHET ($k_{cat} 0.0011 \pm 0.0002 \text{ s}^{-1}$) have not been explained before (Fig. 4b, c)\(^\text{10}\). In the light of our structural data, we anticipated that modifications in the distal part of the binding pocket that mediates electrostatic interactions with the MHET carboxylate may confer activity towards BHET. Strikingly, the S416A and S419G mutants retain MHETase activity and permit the conversion of BHET to TPA which may be explained by the increased flexibility of R411 in these mutants allowing BHET binding (Fig. 4c). Also, providing more space in the inner active site and introduction of potential hydrogen bonding partners conferred by the variants F424Q and F424N significantly increases the turnover of BHET by MHETase (Figs. 2b, 4c). The removal of positive charge in the variants R411A and R411Q also allows a significantly higher turnover of BHET. When these

While this mutation could be a disadvantage for the bacterium in the natural environment where low substrate concentrations are expected, it is advantageous for biotechnological applications running at substrate concentrations about 105-fold higher than $K_m$—which is in the 100 mM range.

While residues of the catalytic triad were verified by activity assays with the respective alanine mutants, an unaltered high turnover of H488A rules out the presence of a catalytic tetrad in MHETase (Figs. 2b, 4a, b)\(^\text{24}\). The importance of F495 for substrate binding is underlined by significant decreases in turnover rates of the natural substrate MHET and the chromogenic substrate MpNPT by the respective alanine mutant (Fig. 4a, b). The activity at high substrate concentrations is increased with the W397A variant at the expense of a lower substrate affinity (Fig. 4a, b).
mutations are further combined with mutations at S416 and S419, the turnover of BHET can be increased 120-fold compared to wild type (Fig. 4c).

Lastly, wild-type MHETase and variants S416A F424N and R411A S419G F424N, which have BHETase activity, were also examined for activity towards coumaric acid methyl ester, caffeic acid methyl ester, chlorogenic acid and p-hydroxy benzoic acid methyl ester, substrates for feruloyl and chlorogenate esterases. No activity above background (no enzyme) could be detected.

Discussion
The microbial degradation and metabolism of PET—and its degradation intermediate MHET—as a carbon and energy source has only come up recently in the environment. To understand the evolutionary origin of MHETase is therefore highly relevant for enzymatic plastic degradation in general. Phylogenetic analysis groups MHETase in the tannase family of the α/β-hydrolase fold enzymes (Supplementary Figure 4). Its closest relatives act on the larger substrate ferulate whereas the more similar gallate is the substrate of more distantly related tannases. Whether MHETase derived from a feruloyl esterase or tannase esterase cannot be answered yet. Typical tannase substrates like hydroxy cinnamates and hydroxy benzoates are neither converted by MHETase (as shown already) nor by the MHETase variants with BHETase activity which we engineered and presented here.

Our structural results on MHETase identified the lid domain as the major difference to the closely related tannase and feruloyl esterases (Fig. 1b-e). Interestingly, it was already pointed out that major switches in enzyme function might occur during natural evolution through loop insertion, deletion or recombination. To this end, our results indicate that MHETase might originate indeed from a loop modification in the lid domain leading to the reported activity towards MHET hydrolysis although no homologous tannase or feruloyl esterase sequence could be identified in the I. sakaiensis genome. Notably, these loops confer a crucial specificity for the para-carboxy group of the substrate (Fig. 3). This specialization for the natural substrate MHET also explains the very low activity of MHETase towards the intermediate BHET in the wild-type enzyme. In the natural environment, missing activity towards BHET is not critical as the upstream enzyme, PETase, already hydrolyzes BHET to MHET.

The structure of MHETase represents a key step in understanding the process of microbial PET degradation in I. sakaiensis. Our structural and mutational analyses shed light on the substrate recognition using an induced-fit mechanism and enabled first structure-guided alterations of substrate specificity of MHETase. We were thus successful in generating an MHETase variant, which hydrolyzes the PETase products MHET and BHET down to the very building blocks, which are required for a sustainable re-synthesis of the polyethylene terephthalate polymer. Contrasting consensus α/β-hydrolases, the bipartite architecture of MHETase parts catalysis from substrate recognition—a scenario where we envision the lid domain as a tunable platform to enhance catalytic properties (e.g. alleviating substrate release) or alter substrate specificity (as shown initially for the S416A, R411Q or F424N mutants reported here).

With the structures of MHETase available, our detailed insights into its mechanism and in particular the generation of a BHETase with altered substrate specificity, it will now be possible to rationally create even more efficient MHETase variants cleaving other partial degradation products from related polymers. Replacing TPA in PET by thiophen-, furan- or pyridine-dicarboxylic acid has long been described. Exchanging the carboxylic esters by sulfonic esters in polymers is also possible. Polymers of 2,5-furandicarboxylate with ethylene glycol (PEF) or other alcohols are suitable to replace PET in bottles. This new plastic PET can be degraded by PETase. The product hydroxyethyl-2,5-furandicarboxylate is similar enough to MHET to envision structure-guided mutagenesis of MHETase to evolve a “MHEFase” for the full cycle from renewable carbohydrates to PEF and back to polymer building blocks by green chemistry.

The potential use of MHETase in recycling of these alternative polyesters underlines the need to understand and customize binding to different substrates. We thus anticipate that our extensive structural characterization and initial rational modulation of MHETase substrate specificity provides an excellent starting point for the development of tailor-made, enzymatic PET degradation systems based on a MHETase scaffold and in combination with PETase.

Methods
Reagents. PET was obtained from commercial bottles. All other chemicals were purchased at the highest purity from Sigma-Aldrich, Carl Roth, Alfa Aesar or Acros if not stated otherwise.

Synthesis of ligands and substrates. Identity and purity of all synthesized compounds was verified by NMR. 1H spectra were measured in DMSO-d6 on a Bruker Avance II 300 equipped with a 5 mm PABBO BB-1H/D Z-GRD Z10475/0398 probehead at 25–28 °C (Fig. S7a-f). For the calibration of the measurements transcinnamylaldehyde was used.

**Buhydroyxethyl terephthalate (BHET):** BHET was synthesized from a PET bottle by alcoholysis with ethylene glycol. Twenty grams PET and 0.2 g anhydrous sodium acetate were refluxed in 120 mL ethylene glycol for 8 h and afterwards cooled overnight. 120 mL H2O was added and filtration was performed at 4 °C. The product was washed with 20 mL cold H2O and extracted several times with hot H2O. BHET appeared as white needles (18 g (68%), Mp 210–212 °C).

**Buhydroyxethyl terephthalic acid amide (BHETA):** BHETA was synthesized from PET by amonolysis with 2-amino ethanol. Twenty grams PET and 0.2 g anhydrous sodium acetate were refluxed in 120 mL ethanolamine for 8 h and afterwards cooled overnight. 120 mL H2O was added and filtration was performed at 4 °C. The product was washed with 20 mL cold H2O and recrystallized twice with 100 mL hot H2O. BHETA appeared as lightly rose needles (20 g (76%), Mp 240–245 °C).

**Dimethyl terephthalate (DMT):** DMT was synthesized by esterification of terephthaloyl chloride with methanol. 25 mmol terephthaloyl chloride was reacted with 30 mL methanol at RT and then refluxed for 3 h. After distilling the methanol and drying at 60 °C, 4.08 g was obtained (Mp 144–148 °C). Washing with 0.5 M KOH and water did not change the melting point.

**Mono(4-nitrophenoxy) terephthalate (MNPT):** MNPT was synthesized from BHET by partial hydrolysis with KOH. 8.7 mmol BHET was reacted with 8.4 mmol KOH in 18 mL MgSO4-dried ethylene glycol at 110–130 °C for 2.5 h. Thirty milliliters H2O was added and the mixture was extracted three times with 5 mL CHCl3. The aqueous phase was adjusted to pH 3 with 25% HCl and filtered at 4 °C. Two extraction steps with 30 mL hot H2O and filtration at 4 °C, the precipitate was dried at 60 °C (0.56 g (30%), Mp 185–190 °C).

**Monohydroyxethyl terephthalic acid amide (MHETA):** MHETA was synthesized by partial amidation of terephthaloyl chloride with ethanolamine. 150 mmol NaOH and 50 mmol ethanolamine in 50 mL H2O were added dropwise within 1 h to 50 mmol terephthaloyl chloride in 50 mL H2O at 0 °C. The reaction was performed for another 2 h at 0 °C and 2 h under reflux, followed by hot filtration. The pH was adjusted to 3 with 25% HCl and filtered at 4 °C. After two extraction steps with 30 mL hot H2O and filtration at 4 °C, the precipitate was dried at 60 °C (0.56 g (30%), Mp 205–212 °C).

**Mono-4-nitrophenoxy terephthalate (MnPPT):** MnPPT was synthesized by esterification of terephthaloyl chloride with 4-nitrophenol. 50 mmol terephthaloyl chloride and 50 mmol sodium 4-nitrophenolate were suspended in 50 mL diethyl ether and reacted for 2 h at 0 °C, then at RT overnight. 2.5 g Na2CO3 and 4.5 g NaHCO3 in 50 mL H2O were added and reacted at RT for 10 h. The pH was adjusted to 8.5 with NaOH. The insoluble fraction was further extracted with a total of 2.5 g Na2CO3 and 2.5 g NaHCO3 in 100 mL H2O and then washed until a neutral pH. MnPPT was precipitated with HCl at pH 3 and washed twice with 50 mL 0.1 M HCl and then until a neutral pH. MnPPT was separated from contaminating bis-4-nitrophenoxy terephthalate by extraction with 100 mM NaPi pH 7.4 and acid precipitation. The very faint yellow slurry was dried at 60 °C (Mp 202 °C).

**Purification as well as crystallization and structure solution.** I. sakaiensis PETase (amino acid residues 28–290) was ordered from Genscript (Piscataway,
USA) as a codon-optimized synthetic gene containing a C-terminal His$_{6}$-tag subcloned into pET-21b. A codon-optimized DNA fragment encoding E. coli Shuf$_{1}$ MHETase (amino acid residues 20–200) cloned in a pUC19 vector was ordered from GenScript and later subcloned into a pColdIII expression plasmid with a N-terminal His$_{6}$-tag (TAKARA BIO, Inc., Otsu, Shiga, Japan) by FastCloning (Supplementary Figure 11).

For protein expression, E. coli Shuffle T7 express cells (New England Biolabs, Frankfurt, Germany) were transformed with the plasmids and selected on lysogeny broth (LB) agar plates containing 100 µg mL$^{-1}$ ampicillin. After growth overnight at 30 °C, overnight cultures were inoculated for overexpression. For overexpression, 1 L baffled Erlenmeyer flasks containing 200 mL LB medium supplemented with 100 µg mL$^{-1}$ ampicillin were incubated at 30 °C and 160 rpm shaking velocity to an optical density at 600 nm (OD$_{600}$) of 1 before 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. HMETase crystal was cryo-protected with 0.1 M sodium acetate, pH 5.0, 10% (v/v) PEG8000 and 0.1 M zinc acetate (space group P2$_1$2$_1$2$_1$). E. coli Shuf$_{1}$ MHTase crystals grown with MDP were cryo-cooled in their reservoir solution. The PETase crystal was cryo-protected with 0.1 M sodium acetate, pH 5.0, 10% (v/v) PEG8000, 0.1% (v/v) PEG400 and 0.5 M lithium sulfate. MHTase crystals were grown in reservoir containing 0.1 M MES, pH 6.5, 10% (v/v) PEG8000 and 0.1 M zinc acetate (space group P21). PETase crystals grown with MFD were cryo-cooled in their reservoir solution. For denaturation, the temperature was held for 20 min and stored at −16 °C for mutant W397A). Purity of the enzyme was measured for the substrates MHET and BHET to TPA which was quantified via HPLC. The experiment was repeated three times.

MnNPT stock solutions were prepared in DMSO at concentrations of 10, 0.1, and 0.1 µM. Substrate concentrations were 0.1–1200 µM in 100 mM TRIS pH 7.5 or 100 mM NaPi pH 7.5. Kinetic parameters are the same in both buffers. The MnNPT concentration was kept below 10 µM to avoid potential self-assembly.

For measurement of feruloyl and chlorogenate esterase activity four substates were tested: coumaric acid methyl ester (CouM-ME), caffeic acid methyl ester (Caff-ME), chlorogenic acid (Chlorogen) and p-hydroxy-hydroxy benzoic acid methyl ester (pHB-ME). UV–Vis spectra using 10 µM of the ester and the free acid were measured and the Tm calculated (Supplementary Fig. 10). Hydrolysis was measured as for MnNPT, but with 10–35 nM enzyme, 100 µM substrate and at 335 nm (CouM-ME, $\Delta$ε$_{335}$ = −6100 M$^{-1}$ cm$^{-1}$), 350 nm (Caff-ME, $\Delta$ε$_{350}$ = −5700 M$^{-1}$ cm$^{-1}$), 350 nm (Chlorogen, $\Delta$ε$_{350}$ = −7400 M$^{-1}$ cm$^{-1}$) and 280 nm (pHB-ME, $\Delta$ε$_{280}$ = −3900 M$^{-1}$ cm$^{-1}$).

Differential scanning fluorimetry. For the analysis of ligand binding to MHTase, DSF was used. Experiments were conducted with a Promethitus NT.48 (NanoTemper, Munich, Germany). The device has a fixed excitation wavelength of 285 nm and emission wavelengths of 330 and 350 nm. MHTase (wt) was always used at 100 µM in the final solution. Final buffer concentrations were 100 mM TRIS pH 7.5, 150 mM NaCl with or without 20% DMSO. High sensitivity capillaries as provided by NanoTemper were used. The temperature range 20 to 80 °C was scanned at 0.5 K per min. Ligands were prepared in 21.7 mM stock solutions and diluted to 10 mM final concentration. The saturated solution (with or without 42.5% DMSO) was used as stock, if the compounds did not fully dissolve. For compounds providing reliable measurements by absorption (4-nitrophenol, 4-nitrophenol, 2-hydroxybenzoic acid) or fluorescence (BHET) 1 mM final concentrations were also tested. Tm values are reported as provided by the Promethitus software.
(maximum of the slope for the $f_{30 \text{mm}}/f_{30 \text{mm}}$ ratio). The experiment was performed as a single measurement.

**Sequence alignment and phylogenetic tree.** Protein homology searches for MHEtase-like proteins were performed with the NCBI basic local alignment search tool (BLAST) in the ESTHER database (http://bioweb.ensam.inra.fr/ESTHER/general what=blast) using the block_X.pep database. Multiple sequence alignment was performed by Muscle alignment using MEGA7.20 The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model.31 The tree with the highest log likelihood ($-1956.87$) is shown. Initial trees (from the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The analysis involved $32$ amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of $376$ positions in the final dataset. Evolutionary analyses were conducted in MEGA7.20

**Data availability**

Structure coordinates and diffraction data were deposited with the Protein Data Bank (http://www.pdb.org) under accession codes 6QG9 (MHEtase), 6QGA (MHEtase MHeta), 6QCB (MHEtase BA), and 6QGC (PETase). The source data underlying Figs. 3, 4a-c, and Supplementary Figures 2a-b, 4, 8, 9a-c and 10 is provided as a Source Data file. Other data are available from the corresponding authors upon reasonable request.

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**Author contributions**

U.T.B and G.W. initiated the study and directed the project. M.C.W., L.B., L.R., E.A.P.M. and H.M. cloned, expressed and purified PETase, MHEtase and mutants, conducted binding studies and activity assays under the supervision of D.B. and G.J.P. E.A.P.M. and G.J.P. conducted MHEtase and PETase crystallographic and computational analyses. G.J.P. M.S.W. and E.A.P.M. prepared the manuscript, which was revised and approved by all authors. This work was supported by a startup funding grant from the University of Greifswald to G.W.
