

Supplemental Material

Development of a targeted gene disruption system in the PET-degrading bacterium *Ideonella sakaiensis* and its applications to PETase and MHETase genes

Running title: Targeted gene disruption system in *Ideonella sakaiensis*.

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Table S1. Primers used in this study.

No.	Primer name	Sequence (5'-3')	Purpose	
1	tetR'_IF-F	<u>GGCACCGTGTATGAAGAGCAATAACGCCCTCATCGT</u>	pT'18 <i>mobsacB</i> construction	Amplify <i>tetR'</i>
2	tetR'_IF-R	<u>CGGCTTCCATTACGTCGAGGTCGCACG</u>		
3	inv-tetR'_IF-F	<u>CTCGACGTGAATGGAAGCCGGCGGCA</u>		Remove <i>tetR</i> from pT'18 <i>mobsacB</i>
4	inv-tetR'_IF-R	<u>TGCTCTTCATACACGGTGCCTGACTGCGTTA</u>		
5	sacB'_IF-F	<u>GACATGAACGATGAATATCAAGAAGTTCGCCAAACAGGC</u>	pT'18 <i>mobsacB'</i> construction	Amplify <i>sacB'</i>
6	sacB'_IF-R	<u>TTTTGCGTTTTTACTTGTGACGGTGAGCTGGC</u>		
7	inv-sacB'_IF-F	<u>CAACAAGTAAAAACGCAAAAGAAAATGCCGATGGTA</u>		Remove <i>sacB</i> from pT'18 <i>mobsacB</i>
8	inv-sacB'_IF-R	<u>TGATATTCATCGTTTATGTCTCCTTTTTATGTACTGTGTTAGC</u>		
9	pyrF'_IF-F	<u>TTTAACCCATCGCGGGCCCGCGGGCGCGGTTCTACAAT</u>	pT'18 <i>mobpyrF</i> construction	Amplify the region including <i>pyrF</i> and its promoter region
10	pyrF'_IF-R	<u>TTTTGCGTTTTTTCAGCGCCGCGCCGCGTTGA</u>		
11	inv-pyrF'_IF-F	<u>GCGGCGCTGAAAACGCAAAAGAAAATGCCGATGGG</u>		Remove <i>sacB</i> and its promoter region from pT'18 <i>mobsacB</i>
12	inv-pyrF'_IF-R	<u>CGGGCCCGCGATGGGTTAAAAAGGATCGATCCTCTAGCG</u>		
13	dpyrF1'_IF-F	<u>GAATTCGAGCTCGGTACCCCGGCCACGACTGGGAGGGCTT</u>	pT' <i>msB'</i> D <i>pyrF</i> construction	Amplify 5'-709 bp of <i>pyrF</i>
14	dpyrF1'_IF-R	<u>GCCGCGTTGATCAGCCCAGGCGCCGCGCCAGG</u>		
15	dpyrF2'_IF-F	<u>CCTGGGCTGATCAACGCGGCGCGGCGCTGAG</u>		Amplify 3'-770 bp of <i>pyrF</i>
16	dpyrF2'_IF-R	<u>TCGACTCTAGAGGATCCCCAGGGCCTGTTTCGACAGCCCCTACGCC</u>		
17	dpetase'_IF-F	<u>TCGAGCTCGGTACCCGTCGAAGAAGGCGTTTCATC</u>	pT' <i>mpFDpetase</i> construction	Amplify the region including <i>petase</i> and its flanking regions
18	dpetase'_IF-R	<u>CTCTAGAGGATCCCCGTAAGCTGCTCGTGACAG</u>		
19	inv-dpetase-F	<u>GTCTCACCGTTTCCAATCAGGCGTG</u>		Remove <i>petase</i> from the cloning plasmid by inverse PCR
20	inv-dpetase-R	<u>GTTGTCTCCTGTTGGTGTAGGTGTAC</u>		
21	dmhetase1'_IF-F	<u>AATTCGAGCTCGGTACCCATGCTTCTGGGCGACGATGTGG</u>	pT' <i>mpFDmhetase</i> construction	Amplify 5'-648 bp of <i>mhetase</i>
22	dmhetase1'_IF-R	<u>GTGGCCCCTACTTATGTCTCCTTCGACTGGTTCCG</u>		
23	dmhetase2'_IF-F	<u>GAGACATAAGTAGGGGCCACGTAGCGTGCC</u>		Amplify 3'-641 bp of <i>mhetase</i>
24	dmhetase2'_IF-R	<u>TCGACTCTAGAGGATCCCCATCAGCTCCAGCACGGGGC</u>		
25	M13F	<u>GTA AACGACGGCCAGT</u>	Pop-in check in gene disruption	Anneal to the outside of the multi cloning site in pUC-type plasmid
26	M13R	<u>CAGGAAACAGCTATGAC</u>		
27	dpyrF'_out-f	<u>TCGGCAACTACAACGCCACCTCTC</u>	Pop-in and disruption check for Δ <i>pyrF</i>	Anneal to the outside of <i>pyrF</i>
28	dpyrF'_out-r	<u>CGAGGAGGACATGACGCACGACGTCTA</u>		
29	dpyrF'_in-f	<u>CGCCGCCGAACGACGCCACGACT</u>		Anneal to <i>pyrF</i>
30	dpyrF'_in-r	<u>AGCACCGCGCGGAGGAGCTGAC</u>		

31	dpetase_out-f	AAATGGCGCTGCTTGATCGCCTCCATCT	Pop-in and disruption check for Δ <i>petase</i>	Anneal to the outside of <i>petase</i>
32	dpetase_out-r	CCTGATGACGCTCTACCTCACCGACAACCT		Anneal to <i>petase</i>
33	dpetase_in-f	CACCGTGACTACCCCAACACGC		
34	dpetase_in-r	TTCTCGCAGGCGAAGGTGGAGTAGC		
35	dmhetase_out-f	TGATCGCGCACGCCTATCAGTTC	Pop-in and disruption check for Δ <i>mheta</i>	Anneal to the outside of <i>mheta</i>
36	dmhetase_out-r	TACAGCCAGTGTCTCTGAGTTGAG		Anneal to <i>mheta</i>
37	dmhetase_in-f	ATGCGAGGCTCTGAAGGACGGAAATG		
38	dmhetase_in-r	AAGTTCGCTTCGGTGTGATGTCTCC		

Nucleotides shared with the vector for In-Fusion cloning are underlined.

A

ATGAAGAGCAATAACGCCCTCATCGTGATCCTCGGTACCGTGACGCTTGACGCGGTGGGTATCGGTCTGGTC
ATGCCGGTGCTCCCCGGCCTTCTGCGCGACATCGTGCATTCCGACAGCATCGCCAGCCATTACGGCGTCCTG
CTGGCCCTTTATGCCCTGATGCAGTTCCTGTGTGCGCCCGTGTGGGCGCCCTGAGCGACCGCTTTGGCCGC
CGCCCGGTGCTCCTGGCCAGCCTCCTGGGTGCCACGATCGATTACGCCATTATGGCCACCACCCCGTCTG
TGGATCCTGTATGCGGGCCGGATCGTGGCCGGGATTACGGGCGCCACGGGCGCCGTGGCGGGCGCCTACAT
CGCCGATATCACGGACGGTGAAGATCGCGCCCGGCACCTTGGCCTGATGTCCGCGTGCTTTGGCGTGGGTAT
GGTGGCGGGCCCCGTTGCCGGCGGGCTCCTGGGCGCCATCAGCCTGCATGCCCGTTTCTCGCGGCCGCC
GTTCTGAACGGCCTTAACCTGCTGCTGGGGTGTTCCTGATGCAGGAAAGCCACAAAGGCGAGCGCCGCCCC
ATGCCGCTTCGGGCCTTTAACCCGGTTAGCTCCTTCCGCTGGGCCCGCGGCATGACGATCGTCGCGGCACTT
ATGACCGTCTTCTTATTATGCAGCTTGTGCGCCAGGTGCCCGCAGCCCTGTGGGTCAATTTTCGGTGAGGATC
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CCTTCGTGACCGGGCCGGCCACCAAGCGCTTCGGGGAGAAGCAAGCGATCATTGCAGGCATGGCGGGCCGAC
GCCCTGGGGTACGTCCTCCTCGCGTTCGCAACCCGCGGGTGGATGGCATTCCCCATCATGATCCTGCTGGCG
TCCGGCGGCATCGGCATGCCGGCGCTGCAGGCAATGCTGTCCCGTCAGGTGACGACGACCACCAAGGCCA
GCTGCAGGGCTCCCTCGCAGCGCTGACCTCGCTGACCTCGATCACCGGCCCCCTGATCGTCACCGCGATCTA
CGCGGCGTCCGCATCGACCTGGAACGGCCTCGCATGGATCGTGGCGCGGCACTGTACCTCGTCTGCCTCC
CGGCGCTGCGTCTGGCGCGTGGTTCGCGTGGCAGCTCGACGTGA

B

ATGAATATCAAGAAGTTCGCCAAACAGGCAACGGTTCTTACGTTTACCACCGCCCTCCTTGCCGGTGGCGCCA
CCCAGGCCTTTGCCAAGGAAACCAATCAGAAACCGTACAAAGAGACCTACGGGATCTCCCATATCACCCGTCA
CGACATGCTGCAAATCCCGGAACAGCAAAAAGTAAAAGTACCAAGTGTCCGAATTCGATTCCAGCACCAT
AAGAACATCTCGAGCGCAAAGGGTCTCGATGTGTGGGATAGCTGGCCGCTGCAAAACGCCGACGGTACGGTG
GCCAACTATCACGGCTATCACATCGTCTTCCGCCCTTGGCCGGTATCCGAAGAATGCAGATGACACGAGCATCT
ACATGTTCTACAAAAGGTGGGCGAAACGAGCATTGATTTCGTGGAAAAATGCCGGCCGTGTGTTAAGGACAG
CGACAAATTTGATGCCAATGACAGCATTCTGAAGGACCAGACCCAGGAGTGGTGGGCTCCGCCACCTTCAC
CTCCGATGGGAAAATTCGCCTCTTCTACACCGATTTTCAGCGGCAACATTATGGTAAACAAACCTCACCACGG
CCCAGGTGAACGTTTCCGCAAGCGATAGCAGCCTTAATATTAACGGCGTTCGAGGACTACAAGAGCATCTTCGA
CGGTGATGGTAAGACGTACCAGAACGTGCAACAGTTTATCGACGAGGGCAACTATAGCAGCGGCGACAACCA
TACCCTGCGGGACCCCCATTACGTGGAAGACAAAGGCCACAAGTATCTGGTGTTTCGAGGCGAACACGGGCAC
CGAAGACGGGTATCAGGGCGAGGAGAGCCTGTTCAACAAGGCGTACTACGGCAAATCCACCTCCTTCTTTTCG
CCAGGAGTCCAGAACTGCTCCAGTCCGACAAGAAGCGCACCCGCGGAGCTCGCGAACGGGGCGCTGGGCA
TGATCGAGCTGAACGACGACTACACGCTGAAGAAGGTGATGAAGCCGCTGATCGCGTCCAACACGGTGACCG
ACGAGATCGAGCGCGAACGTGTTCAAGATGAACGGCAAGTGGTACCTGTTACGGACTCGCGCGGGTCCA
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CGTACAAGCCCCTGAACAAGACCGGCCTGGTCTGAAGATGGACCTGGACCCCAACGACGTACGTTACCT
ACTCGCACTTCGCGGTCCCCAGGCGAAGGGCAACAACGTCGTCATCACCTCGTACATGACCAACCGCGGCT
TCTACGCGGACAAGCAGTCGACCTTTGCACCCTCGTTCCTGCTGAACATCAAGGGCAAGAAGACCTCGGTCTG
CAAGGACTCGATCCTCGAGCAGGGCCAGCTCACCGTCAACAAGTAA

Figure S1 DNA sequences of *tetR'* (A) and *sacB'* (B). Marker genes (*tetR* and *sacB*) in pT18*mobsacB* were codon-optimized for the suitable expression in the genus *Ideonella*, resulting in *tetR'* (A) and *sacB'* (B), respectively.

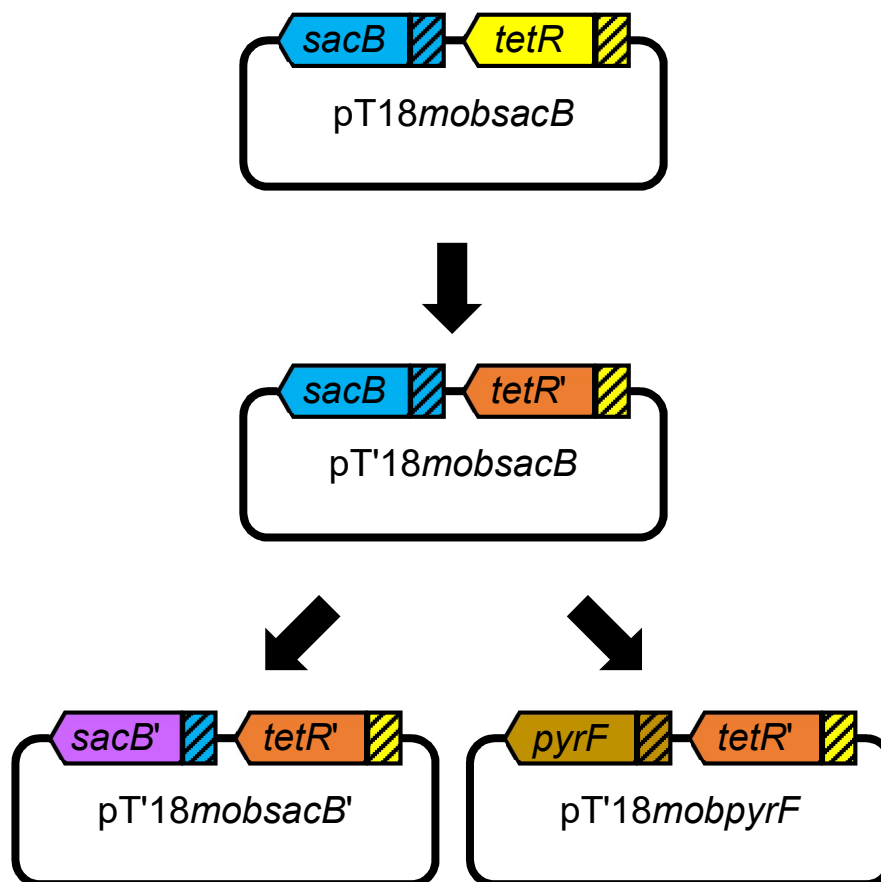


Figure S2 Schematic diagrams for the construction of *pT'18mobsacB* and *pT'18mobpyrF*. Yellow and light blue boxes with slant lines indicate original promoters of *tetR* and *sacB* in *pT18mobsacB* plasmid, respectively. Brown box with slant lines denotes original promoter of *pyrF* in *I. sakaiensis* genome. *pyrF*, orotidine 5'-phosphate decarboxylase gene; *sacB*, levansucrase gene; *sacB'*, levansucrase gene with codon optimization for expression in genus *Ideonella*; *tetR'*, tetracycline repressor protein gene with codon optimization for expression in genus *Ideonella*.

gcgagcaggtgctgctgcccgcgtggacggcctgctggccacgctggcgcgatggcgacgcatggccgaggtgccgatgctcagccgc
accacggccagaccgcccagcccaccctgctggcaaggaggtggcgaacgtcgggcccggtcagcgccgcgcgcgcatcgc
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CCGCTGCTGATCCCCGGCATCGGCGCCCAGGGCGGCGATGCCGAGGCCACGGTGCGGGCCGGC
TGCGCGGCACGGCCGACGCCACCACCGGCCCGGTGATCGTCAGCTCCTCGCGCGCGGTGCTC
TACGCGAGCGCCGGCGCGGACCATGCCACGGCCCGCCGCGCCGCGGCGCTCGCCACCCGCGAC
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gccctggcactcctgcgcatgcgaccgctg

Figure S3 DNA sequence of *pyrF* locus in *I. sakaiensis*. The *pyrF* (ISF6_5168, upper case) and its flanking 1,000 bps (lower case). ISF6_5169 and ISF6_5167 are shown by blue and green letters. Regions integrated into the disruption vector (pT'*msB*'D*pyrF*) for homologous recombination are shaded with gray. Predicted -35 and -10 boxes of *pyrF* are underlined with red. The region between red arrows was amplified and replaced with the region of *sacB* and its promoter in pT'*18mobsacB* to produce pT'*18mobpyrF* (Fig. S2).

A

accagccgcccggccccggcgccctcaagacgccggtgagacggctcggggatcagcgagcgtgatcgggggagcttggcgcgagctcgtcagccg
ctgcttctctgctcagcgtgagcgacgggtcctggtcgacaacatgcctccatcgacacccgcgatgcatgctggccgggccaagagcgcctgggagggtggcg
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B

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CCGCCCGAACCAGTGCCTATGACGCAGGTTGCCGCGCGGATGATGAAATTCGATTCGACATCGATCCGTTGAAGAT
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Figure S4 DNA sequences of *petase* and *mhetase* loci in *I. sakaiensis*. (A) The *petase* (ISF6_4831, upper case) and its flanking 1,000 bps (lower case). ISF6_4830 and ISF6_4832 are shown by blue and green letters. Regions integrated into the disruption vector (pT'*mpFDpetase*) for homologous recombination are shaded with gray. (B) The *mhetase* (ISF6_0224, upper case) and its flanking 1,000 bps (lower case). ISF6_0225 and ISF6_0223 are shown by blue and green letters. Regions integrated into the disruption vector (pT'*mpFDmhetase*) for homologous recombination are shaded with gray.

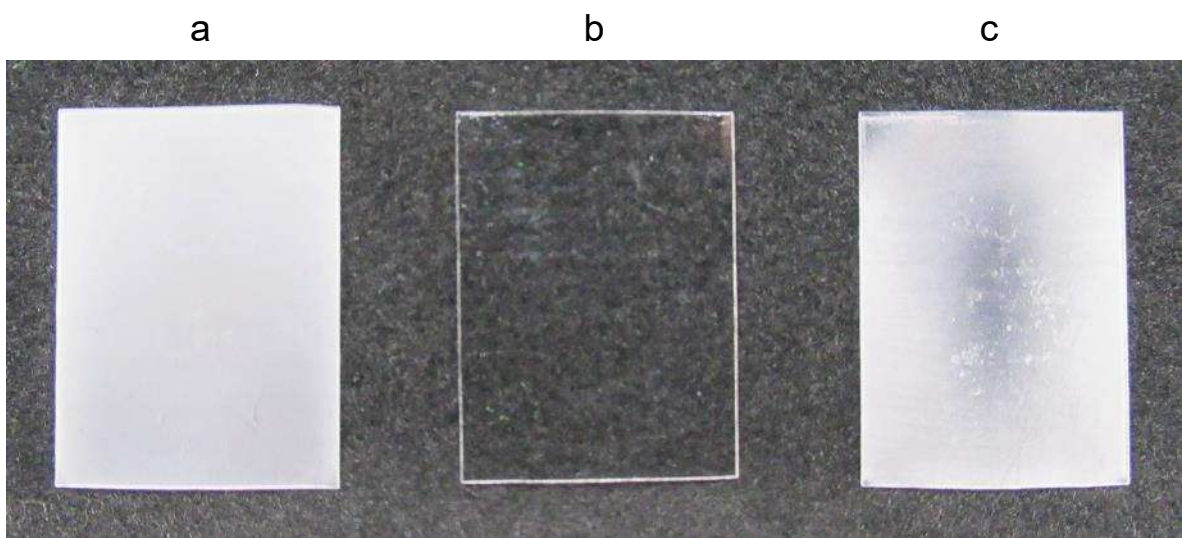


Figure S5 Visual images of PET film cultured with $\Delta pyrF$ (host), $\Delta petase$, and $\Delta mhetase$ strains. The parent ($\Delta pyrF$) (a), $\Delta petase$ (b), and $\Delta mhetase$ (c) strains were cultured with PET film in SV-Ura-PET at 30°C for 10 days. After cultivation, PET film was washed and photographed. A black cloth was used as a background. SEM images of these samples are shown in Fig. 4.

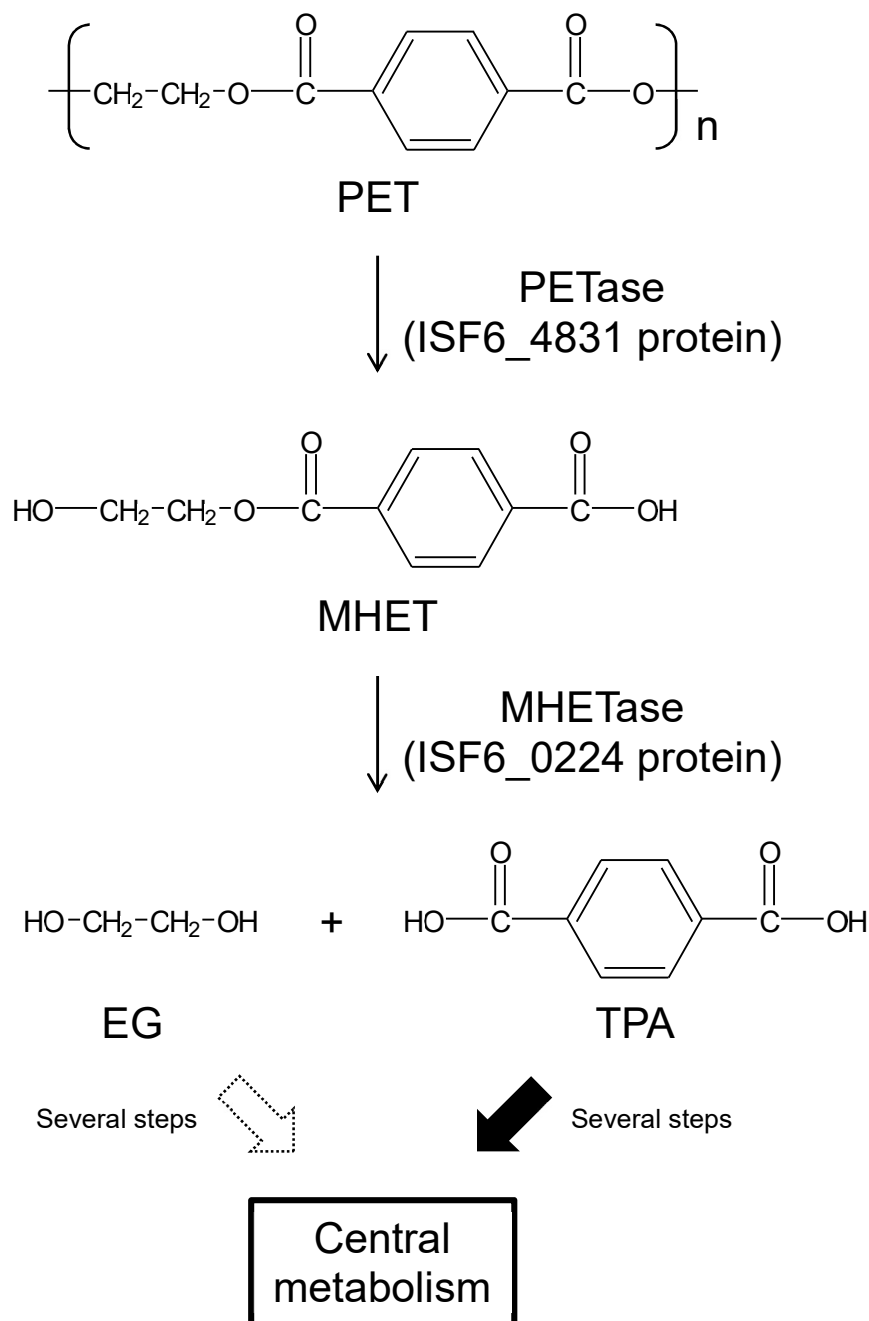


Figure S6 Proposed PET metabolism in *I. sakaiensis*. PET, poly(ethylene terephthalate); MHET, mono(2-hydroxyethyl) terephthalic acid; EG, ethylene glycol; TPA, terephthalic acid; PETase, PET hydrolase; MHETase, MHET hydrolase.