SUPPLEMENTARY MATERIAL for

Biodegradation of PET plastic by a marine strain *Rhodococcus pyridinivorans* P23 with a membrane anchoring PET esterase in a biofilm model

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This supplementary materials includes supplementary tables, figures and amino acid sequence, nucleotide sequence and codon optimized nucleotide sequence of PET esterase (OQN32_06240).

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Supplementary Tables and Figures

Supplementary Table 1 Potential PET hydrolase candidates in *R. pyridinovorans* P23

gene	gene _description	Super family	Number of TM domain	Outside (a.a.)	BHET degradation activity
OQN32_06240	Esterase	Abhydrolase_3; Acetyl esterase/lipase; acetyl esterase	1	36-340	Yes
OQN32_01115	Cutinase	Abhydrolase; Cutinase	1	32-319	No
OQN32_18580	Alpha/beta-hydrolase	Abhydrolase_1; Pimeloyl-ACP methyl ester carboxylesterase	1	28-345	No
OQN32_15860	Alpha/beta-hydrolase	Abhydrolase_9	4	155-434	No
OQN32_04705	Alpha/beta-hydrolase	Abhydrolase_9	4	164-527	No
OQN32_03920	Alpha/beta-hydrolase	Abhydrolase_9	5	218-591	No
OQN32_07550	Alpha/beta-hydrolase	Abhydrolase_9	5	198-577	No
OQN32_07555	Alpha/beta-hydrolase	Abhydrolase_9	5	199-573	No

Supplementary Table 2 Putative *R. pyridinovorans* P23 proteins involved in TPA degradation

ORF# (gene name)	Homolog(s) in *Ideonella sakaiensis* 201-F6 and *Rhodococcus jostii* RHA1				
	Gene	Identity (%)[a]	Gene_description	Source	Reference
OQN32_25270 (*pcaR*)	ISF6_0225	14.00%	pca regulon regulatory protein PcaR	*I. sakaiensis* 201-F6	1
OQN32_25275 (*pcaA*)	ISF6_0227	67.61%	Large subunit of the oxygenase component of TPA 1,2-dioxygenase (TPADO)	*I. sakaiensis* 201-F6	1
OQN32_25280 (*pcaC*)	ISF6_0228	55.19%	Small subunit of the oxygenase component of TPA 1,2-dioxygenase (TPADO)	*I. sakaiensis* 201-F6	1
OQN32_25285 (*pcaF*)	ISF6_0229	47.95%	1,2-dihydroxy-3,5-cyclohexadiene-1,4-dicarboxylate (DCD) dehydrogenase	*I. sakaiensis* 201-F6	1
OQN32_25290 (*pcaD*)	ISF6_0230	45.75%	Reductase component of TPA 1,2-dioxygenase (TPADO)	*I. sakaiensis* 201-F6	1
OQN32_25295 (*pcaK*)	ISF6_0226	33.91%	Probable terephthalate transporter, MFS superfamily	*I. sakaiensis* 201-F6	2
OQN32_09320 (*pcaG*)	ISF6_0626	58.52%	protocatechuate 3,4-dioxygenase, alpha chain	*I. sakaiensis* 201-F6	1
OQN32_09325 (*pcaH*)	ISF6_0627	61.18%	protocatechuate 3,4-dioxygenase, beta chain	*I. sakaiensis* 201-F6	1
OQN32_22690 (*scoB*)	-	62.10%	succinyl-CoA-3-ketoacid-CoA transferase subunit B	*I. sakaiensis* 201-F6 (GAP37024.1)	1
OQN32_22695 (*scoA*)	-	56.13%	succinyl-CoA-3-ketoacid-CoA transferase subunit A	*I. sakaiensis* 201-F6 (GAP37023.1)	1
OQN32_22700 (*pcaB*)	*pcaB*	57.02%	3-carboxy-cis, cis-muconate cycloisomerase	*Rhodococcus jostii* RHA (ABG93161)	3
OQN32_22705 (*pcaL*)	*pcaL*	61.94%	3-Oxoadipate enol-lactone hydrolase/ 4-carboxymuconolactone decarboxylase	*Rhodococcus jostii* RHA1 (ABG93162)	3
OQN32_22710 (*pcaR*)	*pcaR*	65.93	IclR family transcriptional regulator	*Rhodococcus jostii* RHA1 (ABG93163)	3
OQN32_22715 (*atoB*)	*pcaF*	80.20%	acetyl-CoA acetyltransferase	*Rhodococcus jostii* RHA1 (ABG93164)	3

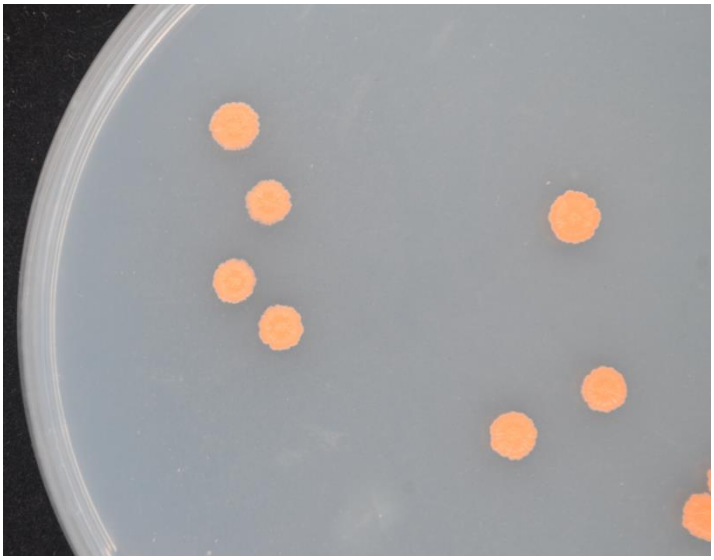
[a] Identity with the corresponding *R. pyridinovorans* P23 protein was determined by their alignment using DNAMAN program.

Supplementary Table 3 Results of RNA-Seq read mapping onto *R. pyridinovorans* P23 genome

Culture medium	Biological replicate #	Raw reads	Raw Bases (bp)	Clean Reads	Clean Bases (bp)	Uniq Mapped Reads (bp) (Ratio(%))
PET film	1	53,131,308	8,022,827,508	52,313,712	6,168,633,222	36,868,905 (70.48%)
	2	48,086,358	7,261,040,058	47,180,464	5,657,426,189	27,554,953 (58.4%)
Disodium terephthalate ($TPA\text{-}Na_2$)	1	50,738,140	7,661,459,140	49,875,730	6,128,877,174	34,189,433 (68.55%)
	2	49,914,608	7,537,105,808	49,092,838	5,942,975,926	33,002,160 (67.22%)
2216E	1	31,011,330	4,682,710,830	30,498,234	4,235,556,482	29,386,103 (96.35%)
	2	29,206,832	4,410,231,632	28,677,856	4,051,191,962	27,733,459 (96.71%)

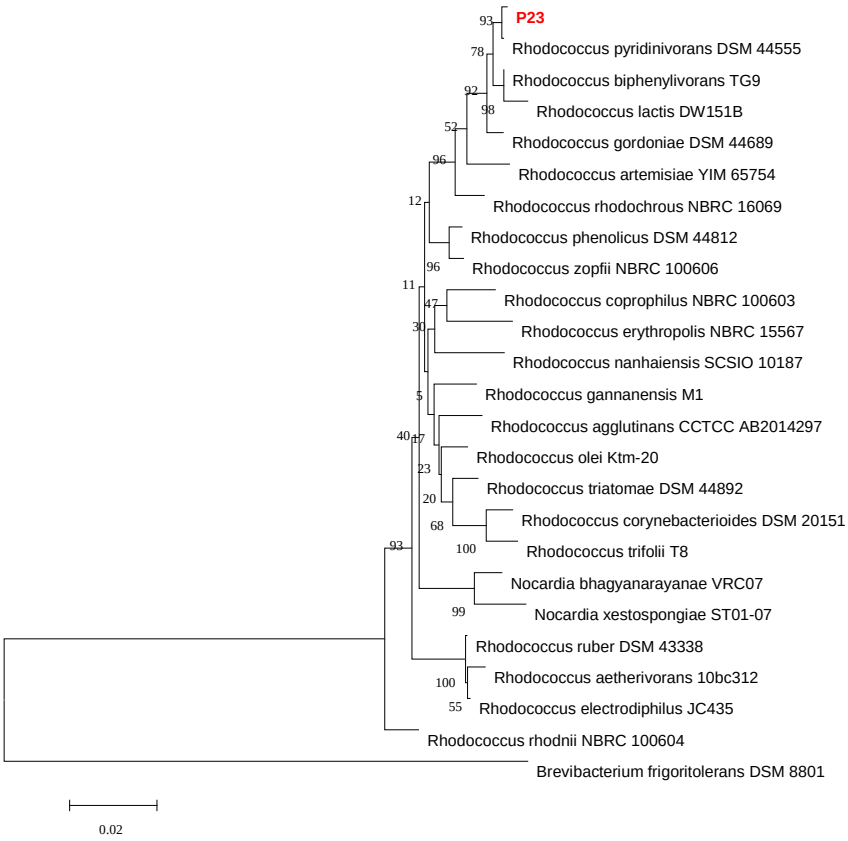
Supplementary Figure 1 Colony morphology of *Rhodococcus pyridinovorans* P23 cultured on 2216 marine agar plate

Rhodococcus pyridinovorans P23 was cultivated on 2216 marine agar medium in a 9 cm petri dish for 5- 10 days. The colonies were red with irregular edges, 3- 5 mm diameter.



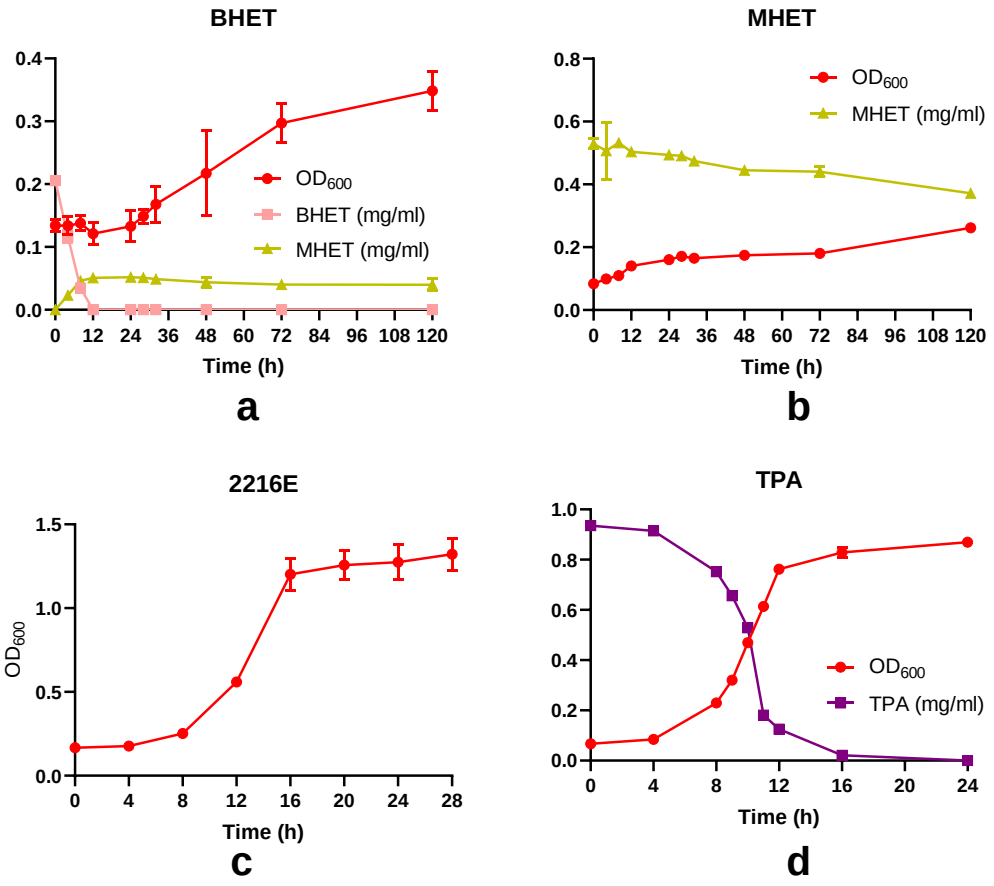
Supplementary Figure 2 Neighbour-joining phylogenetic dendrogram, based on 16S rRNA gene sequences, for strain P23 and related species

Bar, 2% sequence divergence. Out group, *Brevibacterium frigoritolerans* DSM 8801.



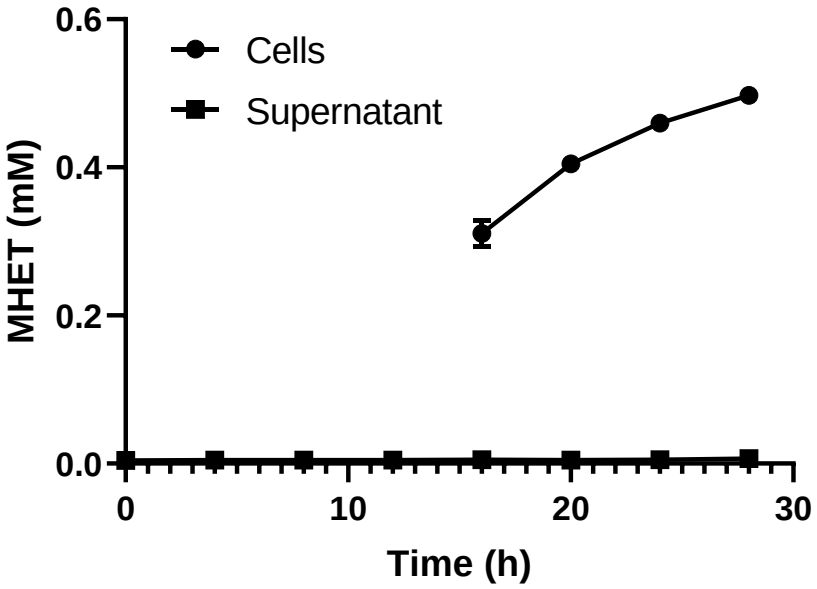
Supplementary Figure 3 Growth curves for *R. pyridinovorans* P23 grown on either bis(hydroxyethyl) terephthalate (BHET) (a), mono(hydroxyethyl) terephthalate (MHET) (b), 2216 marine broth medium (c) or disodium terephthalate (TPA-Na_2) (d)

R. pyridinovorans P23 was cultured in 150 ml 2216 marine broth medium or 150 ml of mineral salt medium with BHET (0.02%, w/v), MHET (0.05%, w/v), or TPA-Na_2 (0.1%, w/v), and the optical density at 600 nm of the fluid was monitored. Two independent cultures (biological replicate) were performed for each culture.



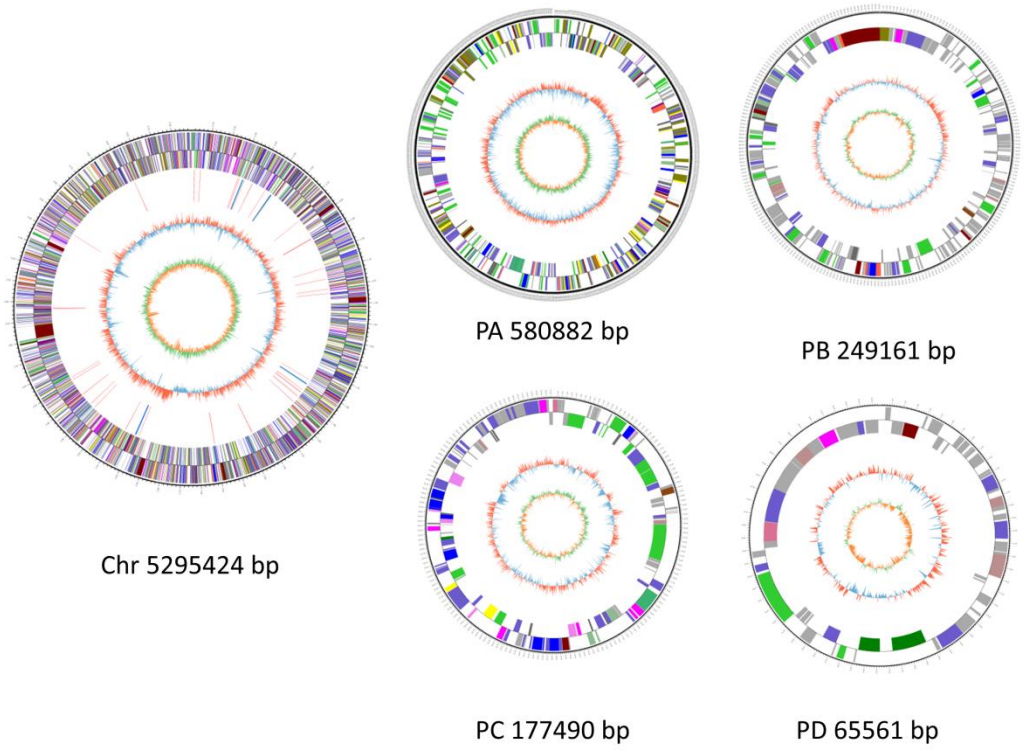
Supplementary Figure 4 BHET hydrolase activity tracking of *R. pyridinovorans* P23 cultured in 2216 marine broth medium

After cultivation of *R. pyridinovorans* P23 in 2216 marine broth medium, cells were separated from supernatant by centrifugation. Then cells and supernatant were tested for their BHET hydrolase activity, respectively. Result showed that the MHET accumulation increased from 0.31 mM to 0.50 mM in 16 to 28 h cultured cells due to the increase in the number of cells sampled, and no MHET production was detected in the supernatant sample. Obviously, the BHET hydrolase (PET hydrolase) locates on the cell membrane of *R. pyridinovorans* P23 exhibited definite BHET hydrolase activity while the supernatant not.



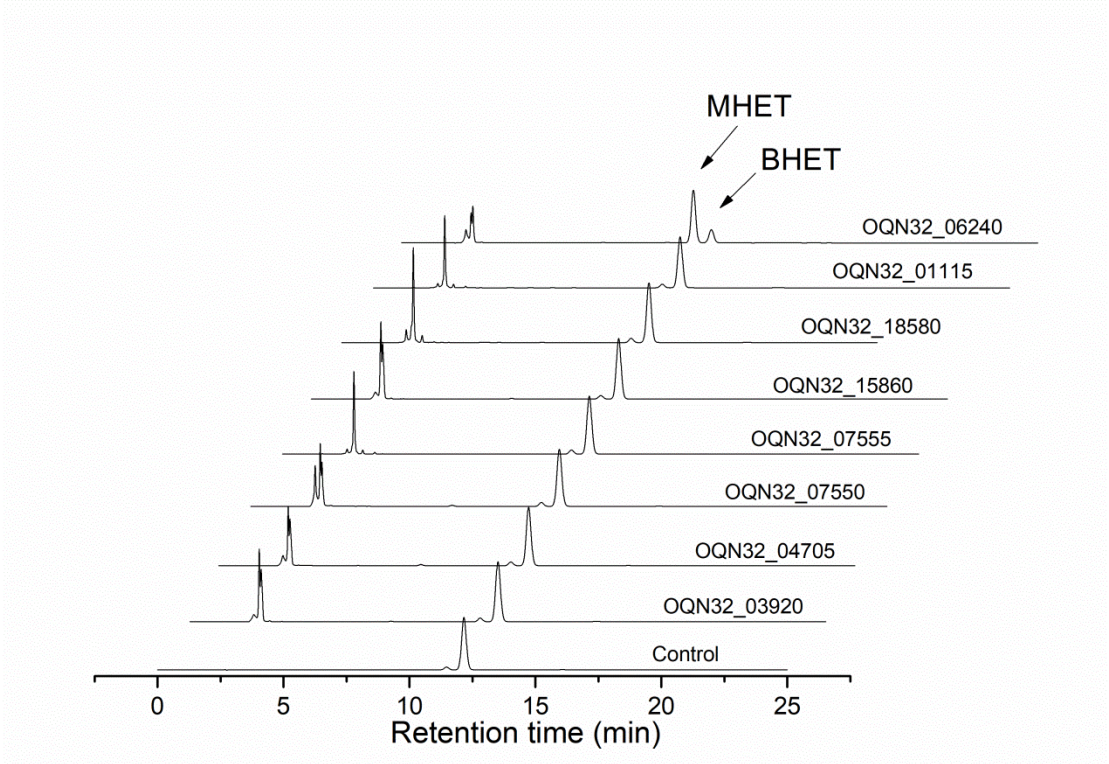
Supplementary Figure 5 Circular representation of *R. pyridinovorans* P23 genome

R. pyridinovorans P23 contains a circle chromosome of 5,295,424 bp with 68.20% GC content and 4 plasmids of 580,882 bp (plasmid A, 64.00% GC content), 249,161 bp (plasmid B, 63.37% GC content), 177,490 bp (plasmid C, 65.96% GC content), and 65,561 bp (plasmid D, 64.78% GC content). From the outside to the center: label of genome size, CDSs on the forward strand (colored by COG categories), CDSs on the reverse strand (colored by COG categories), rRNA and tRNA genes, G+ C content (peaks in red/blue indicate values higher or lower than average G+ C content, respectively), GC Skew. CDSs are depicted in different colors according to COG categories.



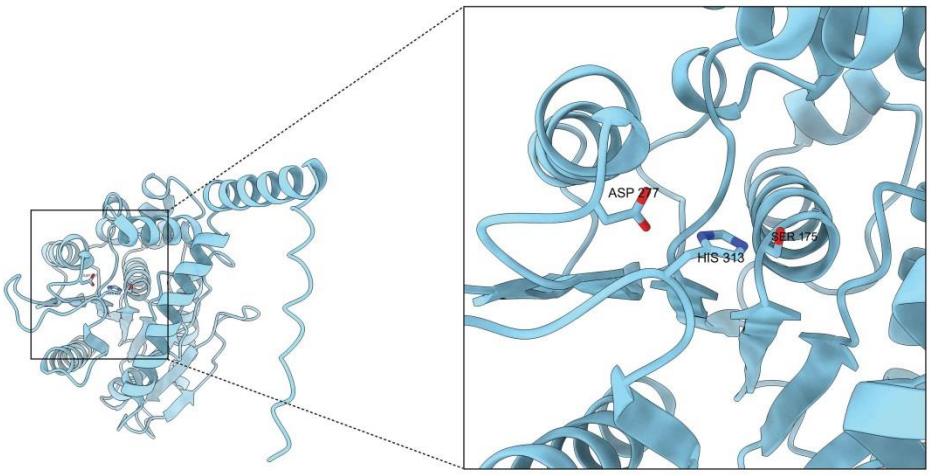
Supplementary Figure 6 HPLC spectrum of BHET after enzymatic degradation with cell lysate of potential PET hydrolases

After the transmembrane domains were removed, the genes for potential PET hydrolases (see Table S1) were commercially synthesized with codon optimization for expression in *Escherichia coli* BL21 (DE3) (Sangon, Shanghai, China). The potential PET hydrolases were expressed in *E. coli* BL21 (DE3) by 0.5 mM IPTG induction at 20 ℃. The *E. coli* cells were harvested by centrifugation (4,000× g, 10 min, 4 ℃), resuspended in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 0.1% Triton X-100, pH 8.0) and disrupted by sonication on ice. After centrifugation (12,000× g, 15 min, 4 ℃), insoluble debris was removed, and the supernatant containing the soluble protein was subjected to BHET hydrolysis. Results showing that only the cell lysate of OQN32_06340 could degrade BHET into MHET.



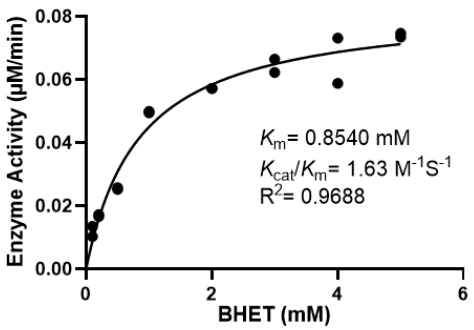
Supplementary Figure 7 The predicted three-dimensional structure of PET esterase (OQN32_06240) protein

The three-dimensional structure of PET esterase (OQN32_06240) was predicted with AlphaFold2, and the amino acid residues $S_{175}D_{277}H_{313}$ forming a catalytic triad were identified.

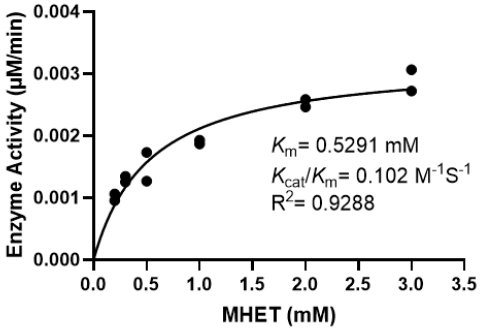


Supplementary Figure 8 Non-linear fit curves of PET esterase (OQN32_06240) towards BHET (a) and MHET (b) measured at pH 4.5, 30 ℃

Two replicates were performed in each substrate concentration.



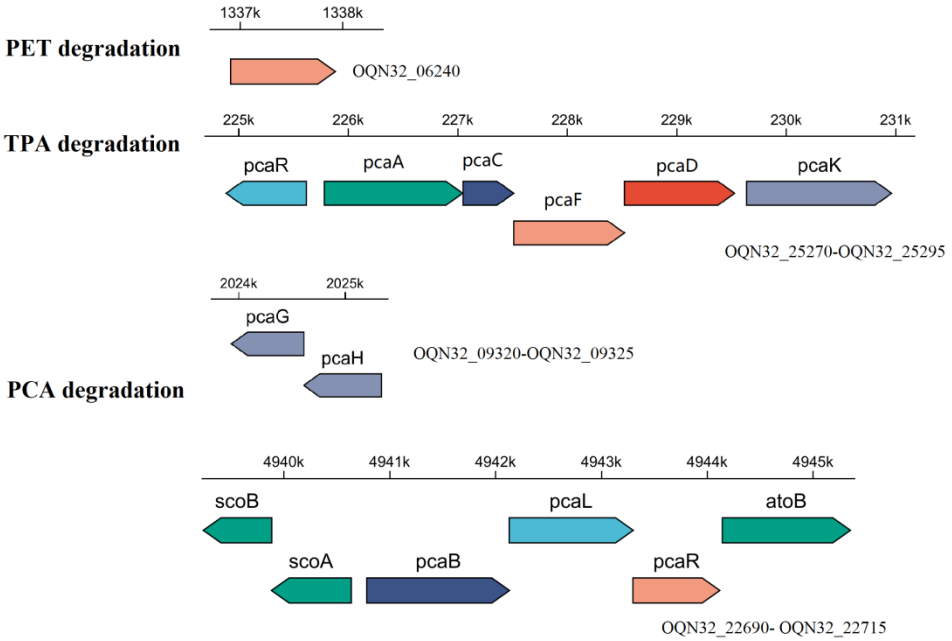
a



b

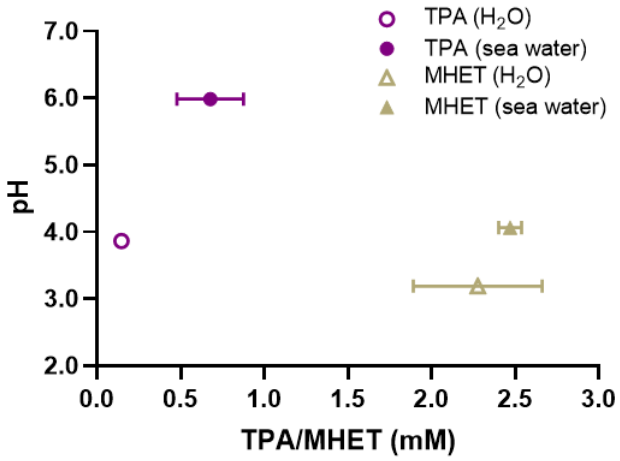
Supplementary Figure 9 Gene organization of PET degradation related gene clusters from *R. pyridinovorans* P23

PET degradation related gene clusters of *R. pyridinovorans* P23 includes PET degradation esterase (OQN_06240), TPA degradation operon in pA plasmid, and PCA degradation operons. The scale of gene size was also present to indicate the positions of genes in genome and pA plasmid.



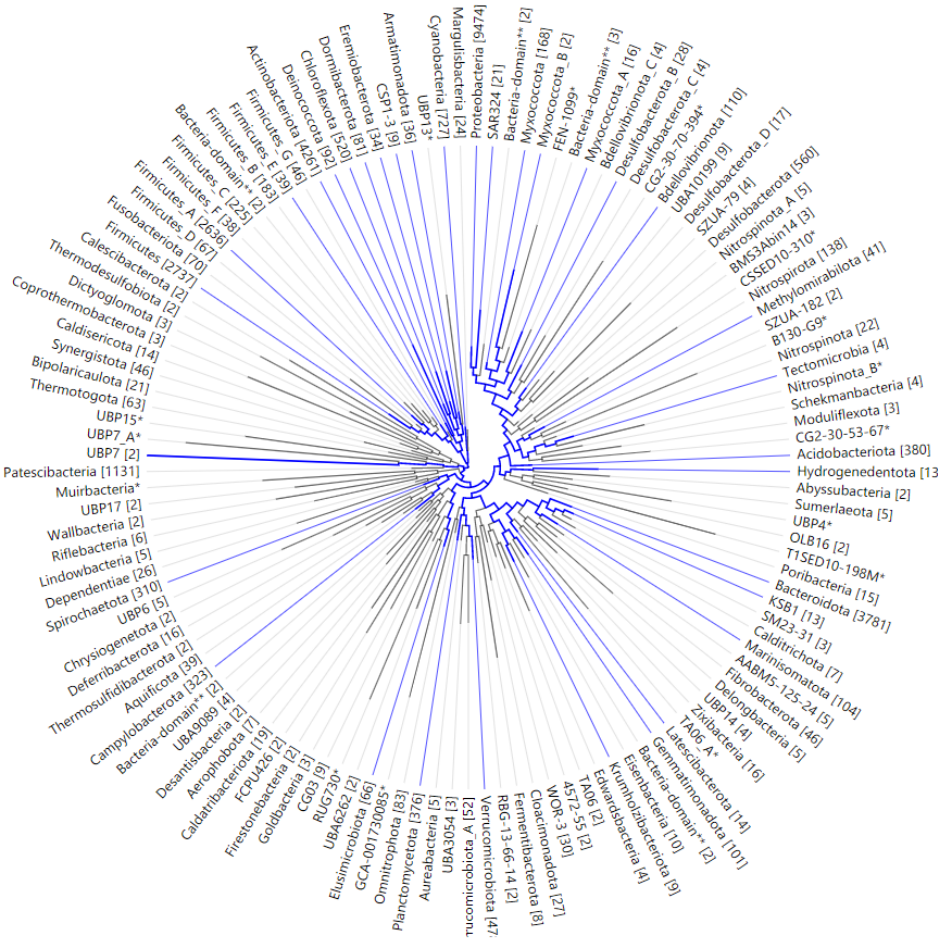
Supplementary Figure 10 The pH decreased with the concentrations of mono(hydroxyethyl) terephthalate (MHET) (a) and terephthalate (TPA) (b) increased

TPA and MHET were dissolved in distilled water (H_2O) and sea water (collected from Xiamen Bay, China) respectively. In each experiment, two replicates were performed. When TPA/MHET solution reached a certain pH, the concentration of TPA/MHET was determined by HPLC. Results showed that both TPA and MHET could make the distilled water (H_2O) and sea water acid, and TPA was more powerful as it possessed two –COOH groups.

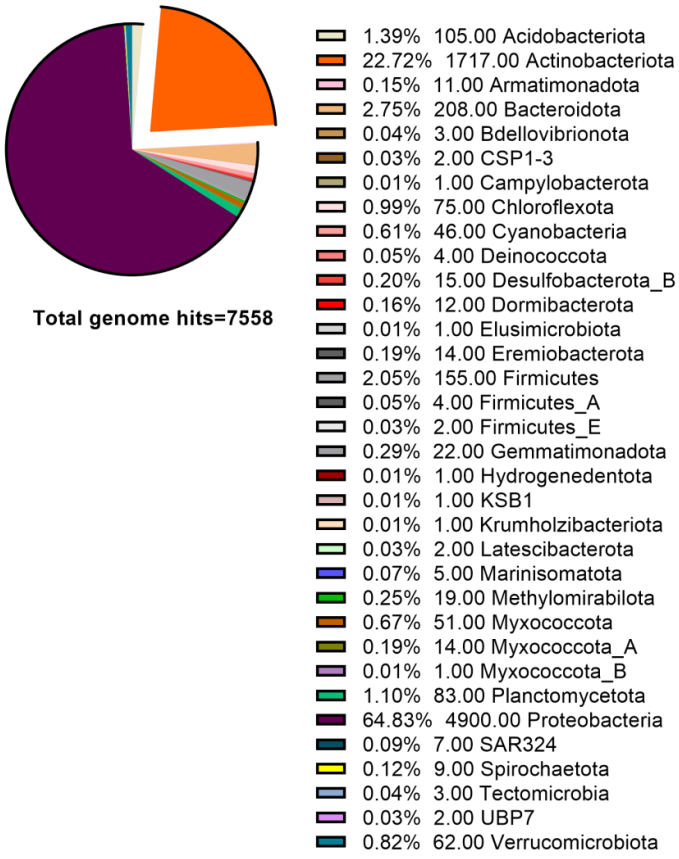


Supplementary Figure 11 Biodiversity of PET degrading bacteria in the environment possessing the same PET degradation pathway with *R. pyridinovorans* P23 using pfam search pattern

Genome hits with the coexistence of PET esterase (PF07859) and large subunit of the oxygenase component of TPA 1,2-dioxygenase (TPADO) (PF00848) in the phylum level showing *Proteobacteria* and *Actinobacteriota* as the main compositions. a, Blue lines in indicate genome hits of bacteria in the phylum level. Numbers in square brackets shows the totally genomes in the database. b, Bacteria composition of genome hits in the phylum level showing *Proteobacteria* and *Actinobacteriota* as the main compositions.



a


Total genome hits=7558
1.39% 105.00 Acidobacteriota
22.72% 1717.00 Actinobacteriota
0.15% 11.00 Armatimonadota
2.75% 208.00 Bacteroidota
0.04% 3.00 Bdellovibrionota
0.03% 2.00 CSP1-3
0.01% 1.00 Campylobacterota
0.99% 75.00 Chloroflexota
0.61% 46.00 Cyanobacteria
0.05% 4.00 Deinococcota
0.20% 15.00 Desulfobacterota_B
0.16% 12.00 Dormibacterota
0.01% 1.00 Elusimicrobiota
0.19% 14.00 Eremiobacterota
2.05% 155.00 Firmicutes
0.05% 4.00 Firmicutes_A
0.03% 2.00 Firmicutes_E
0.29% 22.00 Gemmatimonadota
0.01% 1.00 Hydrogenedentota
0.01% 1.00 KSB1
0.01% 1.00 Krumholzibacteriota
0.03% 2.00 Latescibacterota
0.07% 5.00 Marinisomatota
0.25% 19.00 Methylomirabilota
0.67% 51.00 Myxococcota
0.19% 14.00 Myxococcota_A
0.01% 1.00 Myxococcota_B
1.10% 83.00 Planctomycetota
64.83% 4900.00 Proteobacteria
0.09% 7.00 SAR324
0.12% 9.00 Spirochaetota
0.04% 3.00 Tectomicrobia
0.03% 2.00 UBP7
0.82% 62.00 Verrucomicrobiota

b

Amino acid sequences of PET esterase (OQN32_06240):

12- 35 a.a. shaded in green is the transmembrane domain. The first 35 a.a. underlined would be removed in heterologous expression in *Escherichia coli* BL21(DE3).

> OQN32_06240
MAEDVTESRPRRPIGPRVVLVLAFVVVVTAAAFVLTPVPGSLVVRKVFERDAR
EQTEKLAVDAPETDFVADLHYREDDPDAYLDVYTPPGTTEALPTIVWTHGGA
WLSGNRTNYAGYYRRLAAAGFTVVSVGYSLAPGHRYPTPVRQLVDAQRYLL
EHADELHIDTERIVLAGDSAGAQLSAQIAAAVTDPDYAALLGVDPAFTPENVR
GVVLNCGIYDVSAIGGSGGLIGWGVEQAMWAYTGAREFATSDAAGQMSVLN
SVTENFPATYISGGNADPLTATQSERLAQRLTGLGVQVDALFYPDDHTPELAH
EYQFDLSTPDARAALERTIDFVRKVTT

Nucleotide sequence of coding gene of PET esterase (OQN32_06240):

The initiation and termination codons are shaded in red. The first 35 codons underlined would be removed.

> OQN32_06240
TTGGCCGAAGACGTCACCGAGAGCAGGCCTCGTCGCCCGATCGGACCCCG
GGTCGTGCTCGTTCTGGCATTCGTCGTGGTGGTCACGGCCGCCGCATTCGT
CCTCACCCCCGTGCCGGGGTCACTGGTGGTGCGCAAGGTGTTCGAACGCG
ACGCGCGGGAGCAGACCGAGAAGCTCGCGGTGGACGCTCCGGAGACGGA
CTTCGTCGCCGACCTCCACTACCGGGAGGACGACCCGGACGCCTACCTCG
ACGTGTACACACCGCCAGGGACCACCGAGGCGCTGCCGACGATCGTGTGG
ACGCACGGCGGCGCATGGTTGTCGGGTAATCGCACCAACTACGCGGGTTAC
TACAGGCGTCTCGCGGCCGCCGGATTCACTGTGGTGTCGGTCGGCTACTCG
CTCGCACCGGGCCATCGCTATCCGACCCCGGTGCGGCAACTCGTCGATGCA
CAGCGCTACCTGCTCGAGCACGCCGACGAACTGCACATCGACACCGAGCG
GATCGTGCTCGCCGGTGACTCGGCCGGGGCGCAACTGTCCGCGCAGATCG
CCGCGGCGGTCACCGACCCCGACTACGCCGCGCTCCTCGGTGTCGACCCG
GCGTTCACACCGGAGAACGTGCGCGGTGTCGTGCTCAACTGCGGCATCTA
CGACGTGTCCGCGATCGGTGGGAGCGGCGGGCTGATCGGATGGGGCGTCG
AGCAGGCGATGTGGGCCTACACCGGCGCCCGGGAATTCGCCACTTCGGAT
GCGGCGGGCCAGATGTCGGTGTTGAACTCGGTCACCGAGAACTTCCCGGC
CACCTACATTTCCGGTGGCAACGCCGATCCGCTCACCGCCACCCAGTCCGA
GAGGCTCGCGCAGCGACTCACCGGACTGGGTGTGCAGGTGGATGCGTTGT
TCTATCCGGACGATCACACACCGGAACTGGCACACGAGTACCAGTTCGATC
TGTCCACGCCGGATGCGCGTGCCGCGCTCGAGCGGACGATCGATTTCGTCC
GCAAGGTCACGACGTAG

Codon optimized nucleotide sequence of coding gene of PET esterase

(OQN32_06240):

The *Nde*I (5′ end) and *Xho*I (3′ end) sites are shaded in yellow.

> OQN32_06240 (codon optimized)
CATATGACCCCAGTACCTGGTAGCCTGGTAGTGCGTAAGGTGTTCGAGCGT
GACGCACGTGAGCAAACCGAGAAACTGGCGGTGGACGCTCCTGAAACGG
ACTTCGTCGCAGACCTGCATTACCGCGAGGATGACCCAGACGCATACCTGG
ACGTGTACACGCCACCGGGTACTACTGAAGCGCTGCCGACTATCGTGTGGA
CTCACGGTGGTGCATGGCTGAGCGGTAACCGTACTAACTACGCGGGTTACT
ACCGCCGTCTGGCAGCTGCTGGTTTCACCGTCGTGAGCGTCGGTTACAGCC
TGGCACCGGGTCATCGTTACCCGACTCCGGTTCGTCAACTGGTTGATGCAC
AGCGTTACCTGCTGGAACACGCAGATGAACTGCACATCGACACCGAACGC
ATCGTCCTGGCAGGTGATTCCGCTGGTGCTCAGCTGTCCGCTCAGATCGCT
GCCGCCGTTACTGACCCGGATTACGCTGCTCTGCTGGGTGTGGATCCGGCT
TTCACTCCGGAAAACGTTCGCGGTGTTGTTCTGAACTGCGGCATCTATGAC
GTTTCCGCGATCGGCGGCTCCGGTGGCCTGATTGGCTGGGGCGTTGAACAG
GCTATGTGGGCATATACCGGCGCGCGTGAATTCGCCACGTCTGATGCCGCG
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GCGCAGCGTCTGACCGGCCTGGGCGTTCAGGTTGATGCCCTGTTTTATCCG
GATGATCACACCCCGGAACTGGCCCACGAATATCAGTTTGATCTGTCTACG
CCGGACGCGCGTGCGGCGCTGGAACGCACCATTGACTTTGTACGCAAAGT
AACCACCCTCGAG

References:

1. Yoshida, S. et al. A bacterium that degrades and assimilates poly(ethylene terephthalate). *Science* **351**, 1196-1199 (2016).
2. Nichols, N. N.＆Harwood, C. S. PcaK, a high-affinity permease for the aromatic compounds 4-hydroxybenzoate and protocatechuate from Pseudomonas putida. *J Bacteriol* **179**, 5056-5061 (1997).
3. Hara, H., Eltis, L. D., Davies, J. E.＆Mohn, W. W. Transcriptomic analysis reveals a bifurcated terephthalate degradation pathway in Rhodococcus sp. strain RHA1. *J Bacteriol* **189**, 1641-1647 (2007).