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**A lesson from PBS plastispheres to discovery of novel PBS degrading enzyme genes from marine vibrios**

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**Running title:**

Lessons from plastispheres for PBSase gene discovery

**Originality-Significance Statement:**

Plastic pollution has become major global issue affecting all of us. Even among biodegradable plastics (BDP), there are some types which have been proven to be non-biodegradable in marine environments, and polybutylene succinate (PBS) is one such example. We set up a PBS plastisphere formation *in vitro*, in which a vibrio bloom was observed. Further molecular biological studies identified the gene responsible for a PBS degrading enzyme (PBSase) in *Vibrio ruber*. We reported the first characterization of the vibrio PBSase, which is unique in being active at lower temperatures and also being relatively affected by NaCl. This is only the second case of prokaryotic PBSase identification, and the first case in Gram-negative typical marine bacteria. The vibrio enzyme activity

expressed in *Escherichia coli* cells was likely to be higher than that of *Acidovorax delafieldii* PbsA. These results could contribute to further developments in plastic recycling leading to a more sustainable society, and towards advanced uses of PBS in marine environments.

## Abstract

Polybutylene succinate (PBS) is an ecofriendly green plastic. However, PBS was shown to be resistant in biodegradation under marine environments, and up until now, only a limited number of PBS degrading marine microbes have been discovered. We first set up *in vitro* PBS- and PBSA-plastispheres to characterize novel PBS-degrading marine microbes. Microbial growth and oxygen consumption were observed in both PBS- and PBSA-plastispheres enriched with natural seawater collected from Usujiri, Hokkaido, Japan, and *Vibrionaceae* and *Pseudoalteromonadaceae* were significantly enriched on these films. Further gene identification indicated that vibrios belonging to the Gazogenes clade possess genes related to a PBS degrading enzyme (PBSase). The PBS degradation assay for six Gazogenes clade vibrios identified *Vibrio ruber*, *Vibrio rhizosphaerae*, and *Vibrio spartinae* as being capable of degrading PBS. We further identified the gene responsible for PBSase from the type strain of *V. ruber*, and the purified recombinant vibrio PBSase was found to have low-temperature adaptation and was apparently active under high NaCl concentrations. We also provided docking models between the vibrio PBSase and PBS and PBSA units to show how vibrio PBSase interacts with each substrate compared to the *Acidovorax* PBSase. These results could contribute to a more sustainable society through further utilization of PBS in marine environments and plastic recycling.

**Keywords:** plastisphere, marine, plastic, PBS, PBSase gene, vibrios

## INTRODUCTION

Plastics play an essential role in the modern world. However, their negative impacts on pollution in marine ecosystems due to resistance to biodegradability and the subsequent formation of microplastics, have become a significant global issue, which must be resolved in the future (Mato et al., 2001; Barnes et al., 2009; Fendall and Sewell., 2009; Gall and Thompson et al., 2015; Li et al., 2016; Amaral-Zettler et al., 2020, Sobhani et al., 2020; Zhang et al., 2020). As a remedy for such plastics pollution, biodegradable plastics (BDP) such as polyhydroxybutyrate (PHB), poly-L-lactic acid (PLA), polycaprolactone (PCL), polybutylene succinate (PBS), and polybutylene succinate adipate (PBSA) have been actively developed and thoroughly tested for biodegradability in terrestrial environments (Shah et al., 2014; Urbanek et al., 2018). Among them, PBS is commercially available as BioPBS™ for an agricultural film product ([https://www.m-chemical.co.jp/en/products/departments/mcc/sustainable/product/1201025\\_7964.html](https://www.m-chemical.co.jp/en/products/departments/mcc/sustainable/product/1201025_7964.html)), and it is well known that fungi, e.g. *Aspergillus oryzae*, degrade PBS (Shah et al., 2014). However, *Acidovorax delafieldii* strain BS-3 is the only prokaryote in which the gene *pbsA* responsible for PBS and PBSA degradation has been identified (Uchida et al. 2002; Uchida et al., 2003). Some PBS degrading Gram-positive spore-forming bacteria affiliated to *Paenibacillus* sp. and *Terribacillus* sp. have been isolated from marine sands and coastal sediments, respectively (Lee et al., 2013; Kim et al., 2022), however, unexpectedly, no apparent abundant members of Gram-negative typical marine and/or halophilic bacteria capable of degrading PBS have been found yet, which gives rise to the perception that PBS is less biodegradable in the ocean (Urbanek et al., 2020). This situation also indicates that discarded PBS might not be so different from other more typical plastics in the ocean.

After the discovery of a novel PET-assimilating microbial catalyst, *Ideonella sakaiensis*, from a sediment sample in a PET recycling plant (Yoshida et al., 2016), attempts to identify related genes

69 in publicly available metagenome pools and assessments of biological consortia around plastics,  
70 which are defined as *Plastisphere* (Amaral-Zettler et al., 2020), have been actively undertaken  
71 (Wright et al., 2020; Dey et al., 2022; Du et al., 2022), and a number of comprehensive plastic  
72 degrading microbes and/or genes database have been established recently (Gan and Zhang, 2019;  
73 Buchholz et al., 2022; Gambarini et al., 2022). As *I. sakaiensis* cells adhere to the PET film playing  
74 significant roles in PET degradation (Yoshida et al., 2016), so the bacterium is one of the key  
75 members of the *PET-Plastisphere*. Early plastisphere studies were started indicating that diatoms and  
76 bacteria belonging to *Rhodobacteriaceae*, *Rhodospirillaceae*, and *Flavobacteriaceae* are major  
77 members on the surface of high-density polyethylene (HDPE) strips after 16 weeks incubation on site  
78 in Woods Hole, USA, and that vibrio blooms on polyethylene- and polypropylene-plastispheres in  
79 North Sea coastal sediment and in the open seas of the North Atlantic (Kirstein et al., 2019; Amaral-  
80 Zettler et al., 2020). Currently, various taxonomic groups of diatoms, cyanobacteria,  
81 photoheterotrophic bacteria, heterotrophic bacteria and fungi are determined to be members of  
82 marine plastispheres (Dey et al., 2022; Du et al., 2022). Metagenomic analyses of these plastispheres  
83 revealed genes responsible for not only plastic degradation and assimilation but also for attachment  
84 and biofilm formation on plastic surfaces through exo-polysaccharide, and metal metabolism  
85 (Amaral-Zettler et al., 2020; Dey et al. 2022; Du et al., 2022). Considering the abundance, ubiquity,  
86 and huge microbial diversity of plastispheres, they represent a potential gold mine for bioresources  
87 and/or gene discovery related to effective plastic recycling (Viljakainen and Hug, 2021). However,  
88 the number of plastisphere studies of marine resistant BDPs are behind those of typical plastics. In  
89 other words, plastispheres of marine resistant BDPs could open new windows to gene discoveries to  
90 aid plastic recycling and new material designs.

91 Studies on marine PBS plastispheres could provide further insights into how PBS degrades in  
92 marine environments, but unfortunately, neither the key microbial community of marine PBS and  
93 PBSA plastispheres nor the biochemical, molecular biological and structural characterization of  
94 PBSase from marine bacteria have been fully studied until now. Therefore, the aim of this study is to  
95 characterize *in vitro* marine PBS and PBSA plastispheres, to find novel PBS-degrading marine  
96 microbes, to identify the genes encoding highly active PBSases, and to characterize those gene  
97 products. Unexpectedly, vibrio blooms were observed in the PBS plastisphere, and these findings  
98 opened the way to the discovery of Gazogenes group of marine vibrios, which is a rich genetic  
99 resource of both PBS and PBSA degrading enzymes. Here, we show a paradigm of gene discovery  
100 from PBS-plastisphere to the highly active gene product Vr19890 from *Vibrio ruber*.

101

## 102 RESULTS

### 103 *Vibrionaceae* and *Pseudoalteromonadaceae* are Enriched in PBS and PBSA Plastispheres, 104 Respectively

105 After enrichment using PBS- and PBSA-film supplemented with the Usujiri seawater (the initial total  
106 bacterial counts were  $1.4 \times 10^7$  cells/mL) (Figure S1), microbial growth and oxygen consumption were  
107 observed in both PBS- and PBSA-film plastispheres. The PCoA plot based on unweighted UniFrac  
108 distance indicated that microbial community structures of the Usujiri seawater significantly changed  
109 after enrichment, and there were no common amplicon sequence variants (ASVs) detected between  
110 the original seawater and the enriched samples (Figure 1 AB). There were some shared ASVs  
111 between the microbiomes of PBS- and PBSA-film enrichments. The microbial community structures,  
112 however, were distinct from each other. Notably, there were significant abundances of *Vibrionaceae*  
113 and *Pseudoalteromonadaceae* in the PBS- and PBSA-film plastispheres, respectively (Figure 1 CD).

Interestingly, a further BLAST search based on deduced amino acid sequences of PBSase gene (*pbsA*) from *A. delafieldii* revealed that the top 10 hits were occupied by *Vibrionaceae* sequences (below e-value of 1.25E-23), in more detail, by Gazogenes clade *Vibrio* species such as *V. gazogenes*, *V. ruber*, *V. spartinae* with 48%-52% amino acid identity (Table S1). No *Pseudoalteromonadaceae* genes showing similarities (>50%, < e-value 5.58E-6) to PbsA were observed (Table S1).

### **Marine Vibrios Belonging to the Gazogenes Clade Maintain Functional PBSase Genes and the Distribution of Related Genes in the Metagenome Library**

Based on enrichment of *Vibrionaceae* in the PBS-plastisphere, and further PbsA-related sequences in the Gazogenes clade species, we screened Gazogenes species possessing PBS and PBSA degradation abilities using an emulsified PBS (ePBS) supplemented minimum seawater agar plate. Interestingly, a weak but apparent clear zone on the ePBS-supplemented plate was observed in *V. ruber* LMG 23124<sup>T</sup> and *V. rhizosphaerae* LMG 23790<sup>T</sup> (Figure S2A). *V. ruber* LMG 23124<sup>T</sup> and *V. rhizosphaerae* LMG 23790<sup>T</sup> also possessed PBSA degrading ability, and the ability was also observed in *V. spartinae* CECT 9026<sup>T</sup> (Figure S2B).

BLAST searches based on amino acid sequences of PbsA on the genomes of *V. ruber* LMG 23124<sup>T</sup> and *V. rhizosphaerae* LMG 23790<sup>T</sup> revealed genes that showed more than 50% amino acid sequence identity in both strains. One such gene of *V. ruber* is designated to be *vr19890*, of which the nucleotide position is from 2287661 to 2288614 on the large chromosome under GenBank accession number AP024905, and was used for further characterization of the gene product because of the observation of more clear zones in the *V. ruber* strain. The length of *vr19890* gene is 954 bp, encoding 317 amino acids with 34778.55 of the deduced molecular weight and theoretical pI 8.47

(Table S2). Basic properties resemble that of *pbsA* gene product from *A. delafieldii*. The GC% of *vr19890* is 44.4% comparing to that (46.0%) of overall *V. ruber* genome, and Tn3-type ISs on upstream and downstream of *vr19890* was predicted. Amino acid identity between Vr19890 and PbsA was 48.0% (Table S2). Primary structure comparison revealed that sequences containing the GxSxG motif, which is conserved in serine hydrolases as the active center, was conserved in both gene products (PbsA: Gly172-Trp173-Ser174-Met175-Gly176; Vr19890: Gly181-Trp182-Ser183-Met184-Gly185) (Figure S3). The tertiary structure model of Vr19890 using AlphaFold2 (Jumper et al., 2021) revealed  $\alpha/\beta$  hydrolase family proteins the same as in the PbsA of *A. delafieldii* BS-3 strain (Figures 4 and S4D). Three catalytic residues, observed in active centers of typical  $\alpha/\beta$  hydrolase family proteins (Nardini and Dijkstra, 1999), were also conserved (PbsA: Ser174, Asp220, His251; Vr19890: Ser183, Asp229, His261) (Figure S3). Interestingly, the predicted 3D structure of Vr19890 was similar to that of the PET-degrading enzyme (PETase) from *Ideonella sakaiensis* 201-F6 strain (PDB ID: 5XJH) (Joo et al., 2018), and PET6 from *V. gazogenes* (PDB ID: 7Z6B) (Weigert et al., 2022) (see the section of Tertiary Structure of Vr19890 Compared to That of PbsA and PET6 described below).

Preliminary functional cloning of *vr19890* in pGEM-T vector being transformed into *E. coli* DH5 $\alpha$  revealed apparent clear zones on both ePBS ePBSA-supplemented LB agar plate after 3-5 days incubation (Figure S4AB). A promoter prediction using SoftBerry's bprom (Solovyev and Salamov, 2011) revealed a *rpoD* type promoter upstream of the *vr19890* gene. ARNold (Naville et al., 2011) predicted a Rho factor-independent terminator region downstream of the *vr19890* gene (Figure S4). Cellular localization of the Vr19890 was unlikely to be predicted but the presence of Sec/SPI signal sequence predicted using SignalP 6.0 and AlphaFold2 suggested that A44 of Vr19890 was a cleavage site as a signal sequence (Figure S4DE). As a clear zone of Vr19890 on PBSA-

supplemented plate was clearer and larger than that on the PBS plate, PBSA degradation was characterized to evaluate levels of Vr19890's PBSase activity as an indicator.

Further PSI-BLAST search of Vr19890 in the GenBank metagenomic protein database (env\_nr) with three iterations revealed that Vr19890-related genes with >30% amino acid identity were observed in coastal surface marine waters collected by the Global Ocean Sampling project in seawater from the oxygen minimum zone in the mesopelagic equatorial Pacific Ocean, and sediment collected at 15 km north of Loki's castle hydrothermal vent in the Arctic Ocean (3,283 m depth) (Table S3).

### **The Recombinant PBSase Vr19890 is Active but less under Saline Conditions**

Not only *E. coli* Rosetta2 (DE3) but also *E. coli* BL21 (DE3) transformed by the recombinant plasmid containing *vr19890* artificial gene inserted into pET-26b (+) showed ePBSA degradation. Unexpectedly, less activities were detected in the transformant with the codon-optimized *vr19890* gene, and a clear zone was likely to be different depending on host *E. coli*. ePBSA degradation activity was likely to be higher in the BL21(DE3) based transformant (Figure S5B). The *vr19890* /pET-26b(+)/BL21(DE3) transformant produced a larger clear zone at 30°C compared to that at 20°C, and less PBSAase ability on a medium supplemented with 50% ASW and 0.5 M NaCl.

To maximize the recombinant PBSase activity for purification, we set up a plate assay to quantify specific PBSase activity (Figure S6A) and tested it using the IPTG induction approach (Figure S6BC). The transformant *vr19890* artificial gene/pET-26b (+)/BL21 (DE3) expressed more functional recombinant PBSase after 18 hours overnight culture at 25°C after 1 mM IPTG induction than those of cultures with vigorous shaking (Figure S6BC). Moreover, the transformant *vr19890*

183 artificial gene/pET-26b (+)/BL21(DE3) did not require IPTG induction for functional enzyme  
184 expression (Figure S6C).

185         MagneHis purification still generated two bands with 49.0 kDa and 29.5 kDa, however, two  
186 step purification using the MagneHis system and further HisTrap affinity chromatography generated  
187 purified recombinant PBSAase with a single 29.5 kDa band (Figure 2AB). Molecular weight of C-  
188 terminal His-tag fused rVr19890 with and without the N-terminal signal peptide (Figures S4) were  
189 estimated to be 35,601.39 and 30,079.69, respectively, using ProtParam, in which the latter  
190 corresponds to the 29.5 kDa band of the purified rVr19890. Unsurprisingly, the purified rVr19890  
191 did degrade PBS film with the specific activity of 42 mg PBS film per mg protein in a day at 30°C.  
192 The specific activity was beyond that of AoC (13 mg PBS film per mg protein in a day).

193         Due to the great loss of activity in the 2nd purification step, tests for ions requirement,  
194 temperature stability, feedback inhibition, were performed using the partially purified rV19890. The  
195 thermostability of rVr19890 showed that the activity of the sample treated at 20°C for 30 min was  
196 100%, while that of the sample treated at 40°C for 30 min decreased to 30%, and the PBSase was  
197 inactivated by treatment at 50°C for 30 min (Figure S7A). No feedback inhibition on PBSase activity  
198 by end products (succinic acid, 1,4-butanediol, and adipic acid, which are monomeric components of  
199 PBSA) was observed (Figure S7B). rVr19890 was affected by NaCl, but its activity was still  
200 detectable at 500 mM (Figure S7C). Specific activities were 6.4%, 100%, 70%, 70%, and 44% after  
201 treatment with 500 mM NaCl, 30 mM KCl, 30 mM MgSO<sub>4</sub>, 30 mM MgCl<sub>2</sub>, and 30 mM CaSO<sub>4</sub>.  
202 (Figure S7C).

203         Using the purified rVr19890 (20.9 µg/mL), the maximum activity of rVr19890 was observed  
204 at 25°C and pH 9.0 in the Tris-HCl buffer system. The specific PBSA degradation was 496.6 mg for

205 5 days under maximum conditions (Figure 3). PBSase activity was detected at a lower temperature  
206 range of around 10-15°C (Figure 3).

207

### 208 **Tertiary Structure of Vr19890 Compared to That of PbsA and PET6**

209 The amino acid sequences of Vr19890 and PbsA, excluding the signal peptides (Vr19890:  
210 Met1-Ala44; PbsA: Met1-Ala41) (Figure 4AB) were used to predict their tertiary structures using  
211 AlphaFold2 (Jumper et al., 2021). The RMSD between them was 0.552 Å (between 238 pairs of  
212 atoms) (Figure 4C). Two disulfide bonds confirmed by the crystal structure of the PETase from *I.*  
213 *sakaiensis* 201-F6 (PDB ID: 5XJH) were conserved in PbsA and Vr19890, and the position of the  
214 catalytic triad in the active center was located nearby (Figure 4DE). The inter-atomic distances of  
215 these bonds were estimated at Cys217-Cys253; 1.870 Å, Cys287-Cys303; 1.567 Å for PbsA,  
216 Cys226-Cys263; 1.658 Å, Cys297-Cys314; 1.784 Å for Vr19890 (Figure 4E).

217 The molecular docking models of Vr19890 and PbsA with the monomer and dimer units of  
218 PBS and PBSA indicated that Vr19890 was predicted to interact with all substrates with a similar  
219 distance from the oxygen atom of the Ser side chain of the active center to the carbonyl carbon of the  
220 ester bond of the substrate (Figure 5A-D). In PbsA, the distance to the dimeric unit of PBS was ca. 4  
221 Å farther than for the other substrates (Figure 6B), but it was predicted to interact with other  
222 substrates with similar distances as those between Vr19890 and substrates (Figure 6A, C, D).

223 PET6 is a recently discovered PETase from *V. gazogenes* ATCC 43942 (Weigert et al., 2022).  
224 The amino acid identity between Vr19890 and PET6 were 90%, and the RMSD between them was  
225 0.552 Å (among 253 pairs of atoms) (Figure 7A). A superposed 3D model of Vr19890 against the  
226 structure of PET6 indicated not only conservation of catalytic triads but also PET binding residues  
227 (except Leu189 in PET6 to valine in Vr19890) (Figure 7B).

## DISCUSSION

To find better solutions to tackle marine plastic pollution, two of the most urgent tasks are to establish management procedures in re-assessing long term deterioration of plastics in marine environments, and in recycling plastic waste including BDP which has previously been evaluated as not being biodegradable in the ocean (Tournier et al., 2023). The former could open new opportunities in the further uses and/or developments of plastics including biodegradable plastics, and the latter also could contribute to the reductions of 1) greenhouse gas emissions, 2) amount of plastic discarded in the environment, 3) fossil fuel dependency, and 4) feedstock by recycling plastics. These could also contribute to maintaining a more sustainable society linked to the achievements of *Sustainable Development Goals*. To establish such goals, it is important to study plastispheres to find novel microbial biocatalysts capable of plastic-degrading activities and to identify the genes responsible. In fact, after the discovery of *I. sakaiensis* 201-F6 strain, which can assimilate PET, a PET-degrading enzyme (PETase) was identified (Yoshida et al., 2016), and functional improvements have rapidly progressed using classical mutations and modern deep-learning aimed structural modifications (Urbanek et al., 2020; Austin et al., 2018; Son et al., 2019; Tournier et al., 2020; Lu et al., 2022). Here, we also show a first paradigm in a gene discovery of a highly active PBS degrading enzyme from marine microbes from studies using an *in vitro* PBS-plastispheres set up. This new enzyme discovery helps in not only enhancing the uses of PBS as long-life biomaterials but also future applications in bio-recycling and bioremediation.

### *Vibrio* blooms in *in vitro* PBS-Plastispheres

Vibrios have been recognized as key microbial taxa in various plastispheres, and the pathogenicity delivery has mainly focused on considering the roles of the plastisphere (Amaral-Zettler et al., 2020; Dey et al. 2022; Du et al., 2022). *Vibrio* blooms in a PBS-plastisphere, in particular, in primary periods (< 6 days) in forming the plastispheres, were also observed *in vitro* (Figure 1C). The gene responsible for PBSase was first identified in *V. ruber* (Figures S2 and S4), which indicates roles of vibrios in plastispheres are now expanding to the roles of degradation of PBS and/or PBSA substrates in early stages of plastisphere formation, and fostering plastisphere releasing available nutrition for the consortia.

Species-level affiliations of vibrios in the PBS-plastisphere also indicated PBS degradation potential in *Photobacterium rosenbergii* but the genes responsible for the PBS degradation have not been identified yet. An undescribed gene might be functioning in this species. Further gene mining in this group of vibrios therefore needs to be addressed.

### **Characteristics of PBSase of *Vibrio ruber***

Vr19890 was capable of degrading PBS films, and in evaluating by a plate assay including ePBS or ePBSA, the vibrio enzyme degrades PBSA more efficiently than PBS (Figure S4). The amount of PBS film and ePBSA degraded per mg of enzyme were estimated to be ca. 40 mg and ca. 100 mg per day, respectively (Figure 3). It is noteworthy that Vr19890 shows an optimum temperature at 25°C, and is active even at 10°C. Among described PBS degrading enzymes including *A. delafieldii*, an Antarctic yeast, *Pseudozyma antarctica*, and a fungus, *A. oryzae*, PBS degradation at 10°C has not been reported until in this study (Shinozaki et al., 2013; Maeda et al., 2005). Optimum pH of the Vr19890 is likely to be similar to PBSases from *P. antarctica* (pH 9.5) and *A. oryzae* (pH 9.0). In particular, temperature dependency of Vr19890 could be a unique characteristic

of the enzyme's origin from marine bacteria. However, the degradation behavior of Vr19890 is affected by seawater, or more precisely, by NaCl (Figure S7C). The activity of Vr19890 at 500 mM, which is close to the actual seawater concentration, was about 6% of that under optimum conditions (Figure S7C). Such a feature against NaCl is unexpected in a secreted enzyme originating from marine bacteria. Accumulated knowledge from discoveries of bacteria from marine environments (Lee et al., 2013; Kim et al., 2022) including Vr19890 suggested that PBS and PBSA can be degraded under marine conditions. Current biodegradability evaluation methods consider only the enzymes that biodegrade in a relatively short period of time as being able to biodegrade. It is necessary to re-evaluate the biodegradability of marine-resistant BDPs in terms of longer-term biodegradation processes in seawater, to fully gauge its impacts on marine environments and ecosystems in the future.

Ser183, Asp229, and His261 are located at the active site, suggesting that Ser183 functions as a nucleophile for the carbonyl carbon atom in the ester bond, as in other plastic degrading enzymes (Nardini and Dijkstra, 1999). Since few exo-type hydrolytic enzyme for polyesters has been reported (Kawai et al., 2019), but recent accumulated knowledge on the mode of actions for polyester hydrolases based on temporal product measurements has changed our views on enzymatic plastic degradation processes (Lee et al., 2008; Shah et al., 2014; Schubert et al., 2022). Due to the different solubilities of both plastics substrates and products generated by plastic degrading enzyme reactions, plastic degradation process must be considered to be more complex by combinations of exo- and endo-lytic reactions (Schubert et al., 2022). In the PBS film degradation using a *Pseudomonas cepacia* lipase (Amano lipase PS), exo-type surface etching mechanism in preferentially generating 4-hydroxybutyl succinate (4HBS), and then following hydrolysis to succinate and 1,4-butanediol, was proposed (Lee et al., 2008). On the contrary, using a PBSA degrading enzyme from *Roseateles*

*depolymerans* strain TB-87, the primary major product from PBSA was pentamer, and then it was degraded into smaller fragments (Shah et al., 2014), which seems to be by endo-lytic manner. We could not determine the mode of action of Vr19890 in this study, we should therefore set up temporal products measurement to elucidate how Vr19890 attacks PBS film and/or ePBS in future. We also consider the states of plastics used for substrates, e.g. rate of crystalline or amorphous regions, for elucidating the vibrio PBSase because of its rather higher reactivities on PBS film comparing to the AoC.

Regarding the metabolism of PBS and PBSA degradation products by microorganisms, a lipase from filamentous fungi was used to identify the degradation products of PBSA using PBSA sheets as substrates, and succinic acid, 1,4-butanediol, and adipic acid are major end products (Mizuno et al., 2007). These products are further utilized by microbial cells, acetyl CoA synthesis, and then are metabolized via the TCA cycle (Mizuno et al., 2007). The growth of *Terribacillus* sp. JY49, a PBS-degrading bacterium, was inhibited by the presence of high concentrations (>8 mM) of succinate, the monomer component of PBS, which prolonged the time to increase the degradation yield of the PBS film (Kim et al., 2022). In *V. ruber*, PBSase was not affected by end products.

### **Structural Similarity of Plastics Degrading Enzymes Against the *Vibrio* PBSase**

The *Vibrio* PBSase is classified as a  $\alpha/\beta$  hydrolase family protein (Nardini and Dijkstra, 1999), but there are unexpected 3D structural similarities between the *Vibrio* Vr19890 and *Acidovorax* PbsA showing 0.552 Å of RMSD using 238 pairs of atoms after the AlphaFold2 prediction (Figure 4). The catalytic triad (Ser/Asp/His) and two disulfide bonds, which have been observed in detail in  $\alpha/\beta$  hydrolase family proteins (Nardini and Dijkstra, 1999), were conserved. However, further molecular docking models of Vr19890 and PbsA with the monomer and dimer

units of PBS and PBSA indicated larger differences ( $> 4 \text{ \AA}$ ) in distance against the PBS dimer unit between the two PBSase than in those against the PBS monomer. Accessibility of high molecular PBS substrate could be a key factor in determining degradation efficiency of this group of enzymes, which have been observed in the PETase-PET substrate accessibility (Austin et al., 2018). More interestingly, preliminary analyses indicated that the 3D model of vibrio PBSase is similar to that of *Ideonella* PETase with a conserved active site, but a recombinant *Ideonella* PETase expressed *E. coli* BL21(DE3) was unlikely to show PBS and PBSA degradation activity. In addition, recently, an  $\alpha/\beta$  hydrolase family protein, PET6, from *V. gazogenes* ATCC 43942 was reported to show PET degrading activity (Weigert et al., 2022). The amino acid sequence of PET6 shared 90% similarity against that of Vr19890, and the RMSD between them was  $0.565 \text{ \AA}$ , meaning Vr19890 and PET6 structures are very similar. Not only catalytic triads but also PET binding residues (except Leu189) in PET6 were likely to be conserved in the Vr19890 (Figure 7); it is expected Vr19890 retains PET degradation activity. The PETase activity of PET6 was reported to be halophilic and thermophilic (Weigert et al., 2022). However, PBSase and PBSAase activities of Vr19890 were rather likely to be inhibited under higher NaCl concentrations and active at  $20\text{-}25^\circ\text{C}$  (Figures 3 and S7). Accumulated knowledge on halophilic and/or halotolerant lipases belonging to the  $\alpha/\beta$  hydrolase family protein suggests unique several structural properties; 1) the surfaces of such proteins are populated by negatively charged amino acid residues to increase solubility and stability under high-salt conditions, 2) the presence of more acidic residues and less Lys, and 3) a decreased number of large hydrophobic amino acid residues (Lia et al., 2017). Such features are unlikely to be in the case of Vr19890. Unfortunately, we have not succeeded in acquiring enough active recombinant proteins of PbsA. We also were unable to obtain crystal structures of both Vr19890 and PbsA. To push forward biochemical characterization of Vr19890, PbsA, and the other Vr19890-related gene products such as

PET6, further robust structural and functional information would help us to elucidate differences in enzyme kinetics, substrate specificity, and salt dependencies to gain a better understanding of protein adaptations to marine environments.

### **Toward the Efficient Expression of Recombinant *Vibrio* PBSase**

During the rVr19890 expression using *E. coli* cells, two unexpected results were observed. The first was less expression in *E. coli* harboring the codon optimized *vr19890* gene. Codon optimization has been frequently used to increase recombinant proteins, in which this step is essential in industrial innovations (Niewkoop et al., 2019). Success of the high-level recombinant protein expression is considered to be affected by transcriptional and translational levels (Niewkoop et al., 2019). Varying success rates in increasing functional proteins have been recorded, which could be explained by the formation of new secondary mRNA structures (Niewkoop et al., 2019). In addition to this effect, an unexpected toxic effect of synonymous mutations in *E. coli*, called RNA toxicity, has also been reported recently (Mittal et al., 2018). In this study, the growth of recombinant *E. coli* harboring the codon-optimized gene was unlikely to be less compared to that of the *E. coli* harboring wild *vr19890* gene, RNA toxicity might affect the growth of recombinant *E. coli* causing less activities in PBS degradation (data not shown).

The other unexpected result is that there is no effect by IPTG induction during active rVr19890 production using the *E. coli* BL21(DE3) and pET vector (Figure S6). Not adding IPTG could cause the production of active rVr19890 in the BL21(DE3) at the same level in the recombinant induced with 0.05-1.5 mM IPTG. Unsurprisingly, a related phenomenon was observed in *E. coli* BL21(DE3), in particular, in expression in membrane proteins using a T7-promoter (Zhang et al., 2015). The absence of IPTG makes the Sec-translocon capacity unsaturated, which leads to

enhanced production of membrane proteins eliminating the need to form inclusion bodies in a misfold state (Zhang et al., 2015). As Vr19890 was predicted to possess a signal sequence which is used by the Sec system (Figure S4 DE), not adding IPTG might induce a similar phenomenon, which has been observed in the case of membrane proteins reported by Zhang et al. (2015).

Optimizations of active recombinant protein expression are still at the trial and error stage (Zhang et al., 2015; Niewkoop et al., 2019). These results observed in our study, which were likely to have occurred due to overexpression of genes in *vr19890*, could provide better strategies in plastic degrading enzyme genes. Not adding IPTG could also reduce the cost of rVr19890 production in industrial uses.

### Ubiquity Of PBSase Genes in The Ocean

Like PET6 and PETase ubiquity in the ocean (Weigert et al., 2022), PBSase, in which structure is also well conserved in typical PETase like proteins, is detected from marine metagenome sequences (Table S3). Similar genes against the *Vibrio* PBSase were detected in coastal and mesopelagic waters, and deep-sea sediment samples (Table S3). Considering the salt dependency of vibrio PBSase, PBS in the ocean is likely to be resistant to rapid degradation but could be degraded beyond over a longer timeframe. Unexplored plastispheres from such marine environments could also provide further novel findings in plastics material designing to reduce marine plastic pollutions.

### CONCLUSION

In conclusion, we identified Vr19890 as a PBSase from a marine bacterium, *V. ruber*, using an *in vitro* PBS-plastisphere. Vr19890 was partially characterized by temperature and pH dependencies, and in behavior against NaCl. Temperature dependency is likely to be adapted to

marine environments but NaCl dependency is not. Further elucidation of structure-function relationships of the vibrio enzyme could provide functional improvements even under marine conditions for future applications to overcome marine plastics pollution and to aid plastic recycling.

## **EXPERIMENTAL PROCEDURES**

### **Sample Collection and Enrichment of Plastispheres**

Samples were collected at Usujiri, Hokkaido (41.935029N 140.950071E) on 12th June, 2019. The ambient water temperature was 10.6°C. These samples were fixed with filter-sterilized 25% glutaraldehyde (GTA) solution (1% GTA in final) and stored at 4°C. The fixed samples were stained with 4',6-diamidino-2-phenylindole (DAPI) solution with a final concentration of 0.5 µg/mL, and the microbial cells were captured through a 0.2 µm Isopore filter (Millipore) stained with Sudan Black. The filter was placed on a glass slide and was covered by a droplet of immersion oil and a glass cover. The number of DAPI-stained bacterial cells was measured using a fluorescence microscope (AxioPhoto, Zeiss, Oberkochen, Germany).

Plastispheres were enriched aerobically using Usujiri seawater supplemented with a plastic film as a single carbon source and/or nitrogen (N), phosphate (P), and yeast extract (YE). For the enrichment of PBS- and PBSA-plastispheres, a sterilized 10 mL GC vial (SVG-10, Nichiden-Rika Glass Co., Ltd., Kobe, Japan) was used. NH<sub>4</sub>Cl (Fuji Wako Chemicals Corp., Osaka, Japan), K<sub>2</sub>HPO<sub>4</sub> (Fuji Wako Chemicals), and/or yeast extract (BD, Franklin Lakes, USA) were used for supplementation of each enrichment. Final concentrations in PBS and PBSA enrichments were set at 1 g/L (18.7 mM) NH<sub>4</sub>Cl, 0.05 g/L (0.287 mM) K<sub>2</sub>HPO<sub>4</sub> and 0.01% yeast extract, respectively. A piece of plastic film (1-3 cm x 1.5-3 cm) disinfected by being dipped in 99.5% ethanol for 30 seconds and after being completely dried on a clean bench was prepared and used. Plastic films, PBS (FZ91)

411 and PBSA (FD92) provided by Mitsubishi Chemical Corporation (Tokyo, Japan), were used. Those  
412 vials were incubated at 25°C.

413 The turbidity of the culture medium was assessed daily by eye. When turbid microbial growth  
414 was observed, serial transfers were performed, and reproducible microbial growths were confirmed.  
415 Oxygen consumption was also measured after 5 days of the seed transfer. Using a gas tight syringe  
416 (Shimadzu GLC Ltd., Tokyo, Japan), a total of 0.5 mL headspace gas was sub-sampled and analyzed  
417 using gas chromatography (GC-2014, Shimadzu Corp., Kyoto, Japan). A tandemly triple-connected  
418 ShinCarbonST (2 m, Shinwa Kako Co., Ltd.) was used for measuring O<sub>2</sub> consumption using He as  
419 the carrier gas and a TCD detector according to the manufacturer's protocol.

420

#### 421 **16S Amplicon Sequencing of Plastispheres and Assessments of PBSase Related Genes in** 422 **Enriched Bacterial Taxa**

423 A total of <3 L seawater sample was filtrated through a 0.2 µm Isopore filter and immediately  
424 frozen at -80°C. The filtrated microbial fraction was subjected to DNA extraction using the Wizard  
425 Genomic DNA Purification Kit (Promega, Madison, USA) according to the manufacturer's protocol.  
426 Microbial DNAs from PBS- and PBSA films and the enriched media were also purified using the  
427 Promega kit. Using the purified microbial DNAs as a template, the hypervariable V1-V2 region of  
428 the 16S rRNA gene was amplified by PCR with barcoded 27Fmod and 338R primers with Illumina  
429 adaptor sequences (Yamazaki et al., 2016). PCR amplicons were purified using AMPure XP  
430 magnetic purification beads (Beckman Coulter, Brea, CA, USA), and quantified using the Quant-iT  
431 PicoGreen dsDNA Assay Kit (Life Technologies Japan). Equal amounts of each PCR amplicon were  
432 mixed and then sequenced using MiSeq Reagent Kit v3 (600-cycles) with the MiSeq Illumina

platform. Those Illumina sequences were obtained in My Metagenome Inc. (Tokyo, Japan). Based on sample-specific barcodes, obtained reads were demultiplexed.

The paired-end sequence data with quality scores (i.e., Fastq files) was analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME 2, version 2018.11) (Bolyen et al., 2019). Quality controls (e.g., trimming primers and denoising sequences, removing chimeric sequences) and merging paired-end sequences were performed using DADA2 (Callahan et al., 2016). Reads with 100% similarity constituted an amplicon sequence variance (ASV). ASVs were assigned to taxonomy using the Naive Bayes classifier and Greengenes database (DeSantos et al., 2006). Using subsampled reads, unweighted UniFrac distances as beta-diversity were calculated and visualized in PCoA plots (Lozupone et al., 2011). Using relative abundance data generated by QIIME 2, the Fisher exact test was performed to evaluate which bacterial families were significantly richer on PBS and PBSA film than each medium using Statistical Analysis of Taxonomic and Functional Profiles (STAMP) version 2.13 (Parks et al., 2014).

A BLASTp search using amino acid sequence of *pbsA* gene (GenBank accession number: AB066349 and BAB86909.1) from *A. delafieldii* BS-3 against nr database filtered with *Vibrionaceae* and *Pseudoalteromonadaceae* genome sequences was performed.

#### **Tests for PBS- and PBSA-Degradation in Marine Vibrios**

Based on the PBS- and PBSA platisphere analyses described above, Gazogenes clade species were likely to show PBS- and PBSA degrading activity. Seven Gazogenes clade *Vibrio* species including *V. ruber* LMG 23124<sup>T</sup> (Jiang et al., 2022) were used for testing PBS- and PBSA-degradation ability. These strains were maintained using ZoBell 2216E agar (Jiang et al., 2022), and

those PBS- and PBSA-degradation activities were tested using a minimum seawater medium agar plate supplemented with emulsified PBS and PBSA,  $\text{NH}_4\text{Cl}$ ,  $\text{K}_2\text{HPO}_4$ , and yeast extract at  $25^\circ\text{C}$ .

PBS- and PBSA-emulsions (ePBS and ePBSA) were prepared according to Uchida et al. (2000). A total of 1.6 g of each PBS (FZ91) or PBSA (FD92) pellet and 32 mL of chloroform were added to a 400 mL metal vessel (SMT, Tokyo, Japan), and the PBS- and PBSA pellets were completely dissolved by stirring at 10,000 rpm for 5 minutes using a process homogenizer PH91 (SMT). Then 200 mL of ultrapure water mixed with 32 mg of Plysurf A210G as a surfactant (DKS Co., Ltd., Kyoto, Japan) was added, and homogenized using the process homogenizer PH91 (SMT) at 10,000 rpm for 5 minutes. The metal container was placed on a hot stirrer (RH basic 1, IKA, Staufen im Breisgau, Germany) set at  $80^\circ\text{C}$  in a draft chamber, and chloroform was volatilized by gentle shaking and heating for 3 hours. After that, the volume of emulsion was re-adjusted to 200 mL using MilliQ water. The emulsion was transferred to a 250 mL medium bottle, and autoclaved. On a clean bench, a total of 200 mL of sterilized ePBS or ePBSA was mixed with 600 mL of autoclaved seawater supplemented with 0.8 g  $\text{NH}_4\text{Cl}$ , 0.04 g  $\text{K}_2\text{HPO}_4$ , 0.08 g yeast extract, and 12 g agar, and then the plates were prepared.

### Search For PBS- and PBSA-Degrading Enzyme Genes on *Vibrio* Genomes

A further sequence search based on PbsA amino acid sequence against the complete genome sequence of *V. ruber* LMG 23124<sup>T</sup> (Jiang et al., 2022) was performed using In Silico MolecularCloning Software (In Silico Biology, Yokohama, Japan). The best hit gene sequences were compared to that of PbsA in primary (including active center) and tertiary structures. For primary structure and basic protein features, ExPASy (Gasteiger et al., 2003) was used to predict the number of amino acid residues and theoretical isoelectric points, and ClustalW (Thompson et al., 1994) and ESPrint 3.0 (Robert &

Gouet, 2014) were used to perform multiple alignments and compare amino acid residues at the active site. AlphaFold2 (Jumper et al., 2021) was used for the tertiary structure prediction and ChimeraX (Pettersen et al., 2021) for the predicted structure visualizations. Signal peptide prediction of Vr19890 was performed using SignalP 6.0 (Teufel et al., 2022). GC% of *vr19890* and *V. ruber* genome and gene affiliation of *vr19890* gene was determined using In Silico MolecularCloning (In Silico Biology).

### **DNA Extraction, Gene Manipulation, and Functional Screening**

Bacterial DNAs were extracted using the Wizard genomic DNA purification kit (Promega, Madison, USA) following the manufacturer's instructions. Primers (*vrf*; 5'-AGTACAGCGATTCTTTCTC-3' and *vrr*; 5'-CGACATGTATGGTTGAAGTG-3') (Figure S3A) were designed using In Silico MolecularCloning (In Silico Biology) to amplify the candidate gene, which is designated to be *vr19890* (locus 19890 of *V. ruber* LMG 231424<sup>T</sup> complete genome; GenBank/ENA/DDBJ accession number GCF\_024347115.1) (Jiang et al, 2022). Primers were synthesized by Sigma-Aldrich Japan LLC (Sigma-Aldrich, Ishikari, Japan). GoTaq Green Master Mix (Promega) was used for PCR amplification under the following conditions: holding at 94°C for 5 minutes, followed by 30 cycles of thermal denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 1 minute and 30 seconds, and then kept at 4°C. The PCR products were subjected to 1.5% agarose gel electrophoresis (100 V-30 minutes) using 0.5xTBE buffer system (pH 8.0) and purified using the Wizard®SV Gel and PCR Clean-Up System (Promega) after confirming a single band. The purified PCR products were cloned into pGEM-T Easy vector (Promega), and the recombinant plasmid was transformed into *E. coli* DH5α (TOYOBO, Osaka, Japan) according to manufacturer's instructions. Primary selections of the *vr19890* gene-positive

501 recombinant *E. coli* were performed by Blue/White selection on LB agar plate (BD, Franklin Lakes,  
502 USA) supplemented with 100 µg/mL ampicillin (Amp), 20 µL of 50 mg/mL X-gal (Promega) and  
503 100 µL of 0.1 M IPTG at 37°C overnight. White colonies were further subjected to test for insertion  
504 of the target gene using GoTaq Green Master Mix (Promega) according to manufacturer's protocol.  
505 PBS and PBSA degradation of the positive *E. coli* clones harboring the *vr19890* gene tested using LB  
506 agar supplemented with ePBSA, 100 µg/mL Amp, and 100 µL of 0.1 M IPTG at 25°C (Figure S3B).  
507 In brief, 50 mL of sterilized ePBS(A) and sterilized 150 mL of LB agar were mixed at around 60°C,  
508 and then ampicillin was added to a final concentration of 100 µg/mL, mixed well, and dispensed into  
509 petri dishes. After solidification and surface drying, the plates were stored at 4°C. Before use, 100 µL  
510 of 0.1 M IPTG was spread onto this plate.

511 Promoter sequences and a Rho factor-independent terminator region of *vr19890* gene were  
512 predicted using SoftBerry's bprom (Solovyev and Salamov, 2011) and ARNold web server (Naville  
513 et al., 2011), respectively.

514

#### 515 **Artificial Gene Design and Test For PBS- and PBSA Degradation of *Escherichia coli* Clones**

516 Synthesis of the artificial genes of *vr19890* gene and cloning into the expression vector pET-26b  
517 (+) (Novagen, Madison, USA) were performed by GenScript Biotech (Piscataway, USA). Codon  
518 optimized gene of *vr19890* was also designed using OptimumGene algorithm (GeneScript Biotech).  
519 The recombinant plasmids were transformed into *E. coli* Rosetta2 (DE3) or *E. coli* BL21 (DE3)  
520 (Novagen) (Figure S5). Transformations were performed according to the Novagen pET System  
521 Manual 11th Edition ([https://kirschner.med.harvard.edu/files/protocols/Novagen\\_petsystem.pdf](https://kirschner.med.harvard.edu/files/protocols/Novagen_petsystem.pdf)),  
522 using Rosetta2 (DE3). The recombinant *E. coli* Rosetta2 (DE3) and BL21 (DE3) strains (Novagen)  
523 were selected and grown overnight at 37°C on LB plates supplemented with 30 µg/mL kanamycin

(Kan) and 34 µg/mL chloramphenicol (Cm), and 30 µg/mL Kan, respectively. To maximize the functional recombinant Vr19890 (rVr19890), the effects of host strain and IPTG induction approach were tested (see details in the section Purification of Recombinant PBSase).

## **Purification of Recombinant PBSase**

rVr19890 was purified according to Novagen pET System Manual 11th Edition with minor modifications. A single colony of *vr19890* gene positive *E. coli* BL21 (DE3) was precultured using 3 mL of LB broth supplemented with 30 µg/mL Kan at 37°C with shaking of 250 rpm using a shaker (MMS-210, EYELA, Tokyo, Japan) until the OD<sub>600</sub> reached 0.4-0.6. Subsequently, the preculture was further inoculated into 100 mL LB broth in a 300 mL volumetric triangular flask supplemented with 30 µg/mL Kan and incubated with shaking of 250 rpm at 37°C until OD<sub>600</sub> reached 0.5 to 1.0. Then, 50 mL of the culture was dispensed into 50 mL Falcon tubes and was further incubated at 25°C overnight (for 18 hours).

The culture medium was centrifuged (room temperature, 10,000 x g, 5 min) and separated into the bacteria and the culture supernatant, and the enzyme was purified in a two-step approach using the MagneHis protein purification system (Promega) and HisTrap affinity chromatography (Cytiva, Tokyo, Japan) according to manufacturer's protocol with minor modifications. The enzyme was first partially purified using the MagneHis Protein Purification System (Promega). The partially purified enzyme solution was concentrated and desalted using Amicon Ultra-15 (10,000 NMWL; Merck, Darmstadt, Germany). The partially purified enzyme solution was fully purified using affinity chromatography at 4°C using the ÄKTA-FPLC system (Cytiva). The partially purified, desalted enzyme solution was loaded into a 1 mL HisTrap HP column (0.7 x 2.5 cm, Cytiva) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 20 mM imidazole and 150 mM NaCl (Buffer A). Proteins were then eluted

using 20 mM Tris-HCl buffer (pH 8.0) containing 500 mM imidazole and 150 mM NaCl (Buffer B). The absorption and elution program for affinity chromatography was as follows: purification temperature: 4°C, flow rate: 1 mL/minute, wash: buffer A, 5 CV, elution: 0-100% buffer B, 20 CV, fraction: 1 mL/tube. After purification, the proteins were concentrated and desalted using Amicon Ultra-15 (10,000 NMWL; Merck), and samples were subjected to SDS-PAGE, and gel stained with Coomassie Brilliant Blue G-250 (Merck) to determine the molecular mass of the target using PageRuler Prestained Protein Ladder (Thermo Scientific, Waltham, USA). Protein concentrations of the enzyme solutions were determined using the TaKaRa BCA Protein Assay Kit (Takara Bio, Otsu, Japan).

To test the IPTG induction maximum, the previously mentioned cultures were incubated under various concentrations of IPTG at 25°C overnight (18 hours) or with a shaking at 130 rpm at 25°C for 2, 4, and 18 hours after induction with IPTG at a final concentration of 0.05-1.5 mM. The recombinant enzymes were partially purified using the MagneHis Protein Purification System (Promega), and their PBSAase activity was compared.

### **Characterization of PBSase**

Characterization of partially purified or purified rVr19890 was performed using a plate assay containing ePBSA (Figures 2A and S6A). The composition per assay plate (20 mL, 13 cm plate) was as follows: 0.8 g/100 mL ePBSA solution, 0.1 M Tris-HCl buffer (pH 8.0), and 3.0% agar solution in a 4:1:5 volume ratio. Each assay plate theoretically contained 64 mg of PBSA. A cork borer was used to make a hole of 7.5 mm diameter in the assay plate, and 100 µL of sample was added via the hole and incubated. The amount of ePBSA degraded per mg of enzyme was calculated from the amount of PBSA in the volume of the halo portion formed (mg) and the concentration of enzyme solution to evaluate enzyme activity. Quantitative aspect of the plate assay for PBSA

degradation was also examined (Figure S6A). The partially purified rVr19890 was diluted to 1/10, 1/4, 1/2, and 3/4 using Tris-HCl (pH 8.0) buffer.

Effects of pH and temperature of the enzyme activity, ranging from pH 6.0 to pH 9.0, and from 10°C to 30°C were tested using the assay plate for both partially purified and purified enzymes. To further examine whether enzyme activity is affected by seawater components, various seawater components (NaCl, KCl,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ ) were tested at 25°C for 3 days. NaCl was added at 50 mM, 100 mM, 250 mM, and 500 mM, and the other components at 5 mM, 10 mM, 20 mM, and 30 mM, respectively. To examine the temperature stability of the enzyme, the partially purified enzyme solution was treated at 20°C, 30°C, 40°C, 50°C, and 60°C for 30 min and then placed in ePBSA plates in Tris-HCl (pH 8.0) at 25°C for 3 days. To further examine whether the enzyme activity is subject to feedback inhibition by PBSA degradation products, succinate, 1,4-butanediol, and adipic acid, monomers of PBSA, were mixed with partially purified enzyme solution at final concentrations of 1 mM and 10 mM, and 100  $\mu\text{L}$  of the mixture was added onto ePBSA plates.

The degradability of PBS film (200  $\mu\text{m}$  thickness, 1  $\text{cm}^2$ , ca 26 mg) was also tested using the HisTrap-purified recombinant enzyme. The PBS film was added in 5 mL of 0.1 M Tris-HCl (pH 8.0) with 10  $\mu\text{g}$  of purified enzyme. The reaction mixture in a 50 mL tube (CORNING #430829, Corning, USA) was incubated at 30°C with shaking for 48 hours (120 rpm, BR53FP, TITEC, Koshigaya, Japan). The dry weight of the PBS film was measured, and the specific activity of PBS film degradation was evaluated. A purified recombinant *A. oryzae* cutinase (AoC) (GenBank accession number XM\_001817101.3) was used as a control in the PBS film degradation.

### **Tertiary Structure Analyses of the PBSase from *Vibrios***

Based on the amino acid sequences of Vr19890 and PbsA, the signal peptide was predicted using SignalP6.0 (Teufel et al., 2022) as described above. The amino acid sequence without the signal peptide was further used for tertiary structure prediction analyses using AlphaFold2 (Jumper et al., 2021). The predicted tertiary structure was visualized by ChimeraX (Pettersen et al., 2021), and the Root-mean-square deviation (RMSD), positional relationship of the three catalytic residues, and estimated bond distances of the disulfide bonds were examined. Furthermore, to estimate the degradation mode of PBS and PBSA for Vr19890 and PbsA, Cheminfo's 2D to 3D ([http://www.cheminfo.org/Chemistry/2D\\_to\\_3D/index.html](http://www.cheminfo.org/Chemistry/2D_to_3D/index.html)) and OpenBabel version 3.1.1 (O'Boyle et al., 2011) were used to create molecular models of the monomer unit of PBS, the dimer unit of PBS, the adipate unit of PBSA, and the butylene succinate unit and adipate unit of PBSA, respectively. Using these substrate models, their molecular docking models were predicted using the SwissDock web tool (Grosdidier et al., 2011). After obtaining predicted molecular docking models, the distance from the oxygen atom of the side chain of the active center Ser to the carbonyl carbon of the ester bond of the substrate was also measured using ChimeraX. Structures of PET6 (7Z6B) (Weigert et al., 2022) and IsPETase (5XJH) (Austine et al., 2018; Joo et al., 2018) were also used for comparison.

### Conflicts of Interest

YK, YF, RO, JY, and S. Mino declare no conflicts of interest. TS was involved in collaborative research with S. Misawa, S. Maruyama, YI, RM, HN, SK, and YY (Mitsubishi Chemical Corporation) under a contract approved by both Mitsubishi Chemical Corporation and Hokkaido University.

616   **Data Availability**

617   The raw sequence reads used in this study are publicly available under DDBJ/EMBL/GenBank  
618   accession number of DRA016036.

619

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824 **Figure captions (80 mm, 169 mm or 110 mm)**

825 **Figure 1.** Microbial community structure of PBS- and PBSA-plastispheres. (A) PCoA plot based on  
826 unweighted UniFrac distance of 16S amplicon sequences. Orange circle; original seawater, yellow  
827 diamond; PBS-enriched medium, yellow icosahedron; PBS film, cyan diamond; PBSA-enriched  
828 medium, cyan icosahedron; PBSA film, (B) family-level bar-plot (see color palettes for microbial  
829 taxa), (C) extended bar-plots with Fisher's exact test, blue and orange indicate PBS-enriched medium  
830 and PBS film plastisphere, respectively, (D) extended bar-plots with Fisher's exact test, green and  
831 pink indicate PBSA-enriched medium and PBSA film, respectively (microbial families showing  
832 significant differences,  $p < 0.05$ , were shown).

833 **Figure 2.** Purification of rVr19890. (A) HisTrap chromatography. PBSAase activities were observed  
834 in fractions 7-10. MP and dsMP are partially purified rVr19890 using MagneHis kit and the desalted  
835 concentrate using Amicon Ultra 10k. Wells before dsMP and after MHP were empty. (B) SDS-  
836 PAGE of the purified vVr19890. M: SDS-PAGE MW marker, lane 1: MP, 2: dsMP, 3-6: fractions 7-  
837 10.

838 **Figure 3.** pH and temperature dependency of purified rVr19890. The contour graph was made based  
839 on 20 data points of ePBSA degradation measured at various pH level and temperature conditions.  
840 Contour color gradation was set to be a 100 mg stepwise of ePBSA degradation/mg protein.

841 **Figure 4.** Three-dimensional protein structure models without signal peptide of (A) Vr19890 and (B)  
842 PbsA. Vr19890 : Leu45-Asn317, PbsA : Gln42-Asn304. N-terminal and C-terminal amino acid  
843 residues are displayed and colored green. (C) Overlaid three-dimensional model of Vr19890  
844 (magenta) and PbsA (cyan). (D) Position of the catalytic triad (PbsA: Ser174, Asp220, His251;  
845 Vr19890: Ser183, Asp229, His261). (E) Positions of disulfide bonds (PbsA: Cys217-Cys253,  
846 Cys287-Cys303; Vr19890: Cys226-Cys263, Cys297-Cys314). The two disulfide bonds are shown as  
847 magenta or cyan colored.

848 **Figure 5.** Docking models for PBS and PBSA monomer and dimer substrates with Vr19890. (A)  
849 PBS monomer unit. (B) PBS dimer unit. (C) Adipic acid unit as PBSA monomer. (D) The combined  
850 butylene succinate and adipate units as a PBSA dimer.

851 **Figure 6.** Docking models for PBS and PBSA monomer and dimer substrates with PbsA. (A) PBS  
852 monomer unit. (B) PBS dimer unit. (C) Adipic acid unit as PBSA monomer. (D) The combined  
853 butylene succinate and adipate units as a PBSA dimer.

854 **Figure 7.** Three-dimensional protein structure comparisons between Vr19890 (pink gold backbone)  
855 and PET6 (cobalt backbone). (A) A side view and (B) a top view: The catalytic triads of Vr19890  
856 (see Figure 4) and PET6 are indicated by magenta and green, respectively. PET binding residues  
857 (Tyr90, Trp162, Met164, Trp187, Leu189, Val211, and Phe242) involved in enzyme-ligand  
858 interactions determined in the crystal structure of PET6 are shown by yellow. Conserved residues in  
859 Vr19890 shown by orange, except Leu189 locus to Val, are observed.  
860

861 **Figure S1.** Enrichment of plastisphere using various plastic films as sole carbon source. Vial 1: NP-  
862 SW (Usujiri seawater (SW) supplemented with nitrate (N) and phosphate (P)), vial 2: NP-SW+PBSA

863 film, vial 3: NP-SW+PBS film, vial 4: NP-SW+PCL film, vial 5: NPYE-SW (Usujiri SW  
864 supplemented with NP and yeast extract (YE)), vial 6: NPYE-SW+PBSA film, vial 7: NPYE-  
865 SW+PBS film, and vial 8: NPYE-SW+PCL film.

866 **Figure S2.** PBS and PBSA degradations by Gazogenes clade *Vibrio*. 1: *V. spartinae* CECT 9026<sup>T</sup>, 2:  
867 *V. aerogenes* LMG 19650<sup>T</sup>, 3: *V. gazogenes* ATCC 29988<sup>T</sup>, 4: *V. ruber* LMG 23124<sup>T</sup>, 5: *V. mangrovi*  
868 CECT 7927<sup>T</sup>, 6: *V. rhizosphaerae* LMG 23790<sup>T</sup>, and 7: *V. quintilis* CECT 7734<sup>T</sup>.

869 **Figure S3.** Amino acid sequence alignment of Vr19890 and PbsA, generated using ClustalW and  
870 ESPript 3.0.

871 **Figure S4.** Structure and function of vr19890 gene product. (A-C) structure of vr19890 gene and the  
872 gene expression in pGEM-T easy vector and *E. coli* DH5a, (D) predicted three-dimensional protein  
873 structure of un-matured Vr19890 using AlphaFold2 (helix and sheet are shown in red and blue,  
874 respectively. N- and C-terminal are shown in green. Arrowhead indicates the estimated cleavage site  
875 of signal sequences, see E), (E) signal peptide prediction of Vr19890 using the SignalP 6.0. A34 is  
876 predicted to a cleavage site.

877 **Figure S5.** Expression of artificial *vr19890* gene. (A) Physical map of recombinant plasmid  
878 harboring artificial gene of *vr19890*. (B) Effects of host *E. coli* on a recombinant Vr19890 product.  
879 *E. coli* Rosetta2(DE3) ( $n=4$ ) and *E. coli* BL21(DE3) ( $n=9$ ) were compared based on halo diameter  
880 after degradation of emulsified PBSA supplemented to LB agar at 25°C for 7 days. Bayes *T*-test with  
881 independent samples was performed using the JASP version 0.17.0. Halo diameter of BL21(DE3) >  
882 that of Rosetta2(DE3) was set as an alternative hypothesis (H1). The alternative hypothesis was more  
883 likely to occur by 28 folds. The error bar indicates standard error.

884 **Figure S6.** Availability of a plate assay for PBSA degradation and increase in ePBSA degradation  
885 activity of partially purified rVR19890 using MagneHis kit. (A) Enzyme concentration dependency  
886 of ePBSA activity in the plate assay supplemented with ePBSA. Partially purified rVR19890 was  
887 dilute with elution buffer of MagneHis kit. (B) Comparison between shaking (with 2 hours, 4 hours,  
888 and 18 hours) and static culture for 18 hours (PC) after 1.0 mM IPTG induction at 25°C ( $n=1$ ). (C)  
889 Effects of IPTG concentration on ePBSA activity in static culture at 25°C for 18 hours ( $n=3$ ). Bayes  
890 ANOVA was performed using the JASP version 0.17.0. No difference in effect size of ePBSA  
891 activities in all sample was set a null hypothesis (H0). The null hypothesis was likely to occur by  
892 3.130 folds against an alternative hypothesis ( $H0 \neq H1$ ). The error bar indicates standard error.

893 **Figure S7.** Characterization of partially purified rVr19890. (A) Thermal stability of rVr19890. The  
894 residual activity is shown as 100% of the activity of the sample treated at 20°C for 30 minutes. (B)  
895 Effects of possible end products on PBSase activity. 1: succinic acid, 2: adipic acid, 3: 1,4-  
896 butanediol, and 4: no end products control. (C) Effects of seawater ingredients on PBSase activity of  
897 rVr19890.

898

899 **Table S1.** BLAST search of PbsA-related genes against *Vibrionaceae* and *Pseudoalteromonadacea*  
900 database.

901 **Table S2.** Basic properties of Vr19890 and PbsA.

902     **Table S3.** BLAST search of Vr19890 related genes against metagenome database.