Identification of an extracellular poly(3-hydroxybutyrate) depolymerase in the genus *Alteromonas* and its phylogenetic distribution among *Alteromonas* species

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ABSTRACT

Poly(3-hydroxybutyrate), P(3HB), is an aliphatic polyester that is susceptible to biodegradation even in marine environments. The high biodegradability of P(3HB) can be attributed to the presence in the environment of extracellular P(3HB) depolymerase (PhaZ), the initial enzyme involved in P(3HB) degradation. In this study, we aimed to identify the gene encoding PhaZ in the marine P(3HB)-degrading bacterium *Alteromonas* sp. D210916BOD_24, which was previously isolated. First, we conducted genome analysis of the strain, revealing that the strain possesses a PhaZ homolog. It possesses a catalytic domain with a lipase box near the center, a substrate-binding domain comprising two regions, and a fibronectin type III linker domain, which fit the marine bacterial domain pattern. Disruption of the PhaZ homolog gene caused the strain to lose its P(3HB) degradation ability. The recombinant PhaZ homolog protein exhibited significant activity toward P(3HB). The results indicated that the PhaZ homolog indeed functions as PhaZ in *Alteromonas* sp. D210916BOD_24. Furthermore, we focused on the distribution and genomic placement of PhaZ homolog genes. The PhaZ homolog was present in 4/20 *Alteromonas* strains examined, with *Alteromonas* sp. D210916BOD_24 showing substantial divergence from the other three strains in the 16S rDNA-based phylogenetic tree. The gene arrangement around the PhaZ homolog gene was clearly different between *Alteromonas* sp. D210916BOD_24 and the others. Additionally, large-scale gene locations and orientations exhibited considerable differences. These findings suggest that the horizontal transfer of PhaZ homolog genes occurred after a certain degree of species division.

(239/250)

Keywords: polyhydroxyalkanoate, poly(3-hydroxybutyrate), poly(3-hydroxybutyrate) depolymerase, PhaZ, marine bacteria, *Alteromonas*

Statements and Declarations

Conflict of Interest

The authors declare no conflicts of interest associated with this manuscript.

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INTRODUCTION

Plastics have become indispensable owing to their usefulness in terms of processability and durability. However, marine plastic pollution is a global concern, which can threaten aquatic organisms, ecosystems, and human health (MacLeod et al. 2021, Wright and Kelly 2017). For example, microplastics can change the effects of the bioaccumulation and toxicity of antibiotics on aquatic organisms (Wang et al. 2021). Plastics should be collected and recycled following their use; however, a non-negligible proportion of plastic waste enters the marine environment through rivers and other sources. A 2021 study estimated that between 0.8 and 2.7 million metric tons of plastics enter the oceans via the rivers each year globally (Meijer et al. 2021). Plastic waste may persist in marine environments for several centuries or longer owing to its durability and enzyme-resistant chemical structure (Barnes et al. 2009). Biodegradable plastics can address the problem of plastic pollution. Polyhydroxyalkanoates (PHAs), e.g., poly(3-hydroxybutyrate) [P(3HB)], are biopolymers synthesized by microorganisms that can be used as biodegradable plastics. Several kinds of PHAs, e.g., P(3HB), have been reported to undergo degradation in deep-sea environments (Omura et al. 2024). The high biodegradability of P(3HB) is due to the presence of the dedicated degrading enzyme, P(3HB) depolymerase (PhaZ).

Extracellular PhaZ is the initial attacker in the biodegradation process of environmental P(3HB) and has been well-studied. The primary structure of extracellular PhaZ is generally made up of a signal peptide (SP), a catalytic domain (CD), a linker domain (LD), and a substrate-binding domain (SBD) (Fig. 1a). The CD contains a catalytic triad of Ser, His, and Asp and has a lipase box centered on its Ser (Gly-X-Ser-X-Gly). The CD is classified into two types: Type A in which the lipase box is located near the center, and Type B in which the lipase box is located near the N-terminus (Fig. 1b) (Kasuya et al. 1999; Suzuki et al. 2021). There are three main types of LD: fibronectin type III (Fn3), cadherin-like (Cad), and threonine-rich (Thr) LDs (Fig. 1b). The SBD is composed of one or two regions (Fig. 1b). With a few exceptions (Hachisuka et al. 2023; Ma et al. 2011; Suzuki et al. 2022), the PhaZ proteins of marine bacteria generally consist of a Type A CD, an Fn3 or Cad LD, and an SBD composed of two regions (Fig. 1c) (Suzuki et al. 2021). While a PhaZ from the fungus *Penicillium funiculosum* was found to consist of only a Type B CD (Brucato and Wong 1991; Hisano et al. 2006; Miyazaki et al. 2000), no functional extracellular PhaZ consisting of only a CD has been found in any bacterial species.

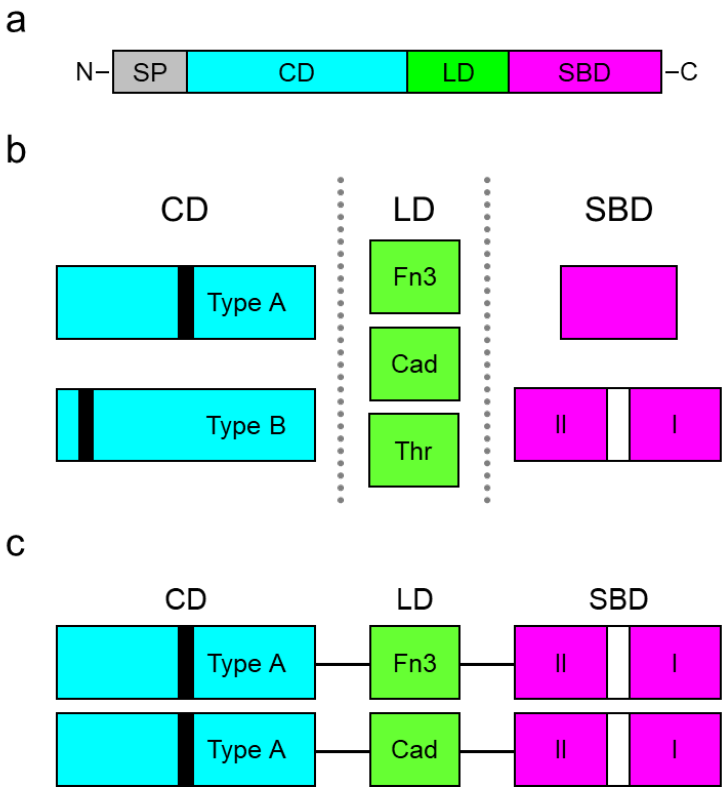


Fig. 1 Domain organization of extracellular PhaZ. The general primary structure of extracellular PhaZ (a), sequence patterns of each domain (b), and two major domain compositions in marine bacterial extracellular PhaZ (c) are shown. Gray, light blue, light green, and purple indicate signal peptide (SP), catalytic domain (CD), linker domain (LD), and substrate-binding domain (SBD), respectively. The black area in the CD symbol indicates the lipase box.

Alteromonas bacteria are aerobic marine species belonging to the family Alteromonadaceae in the order Alteromonadales and the phylum Gammaproteobacteria. A wide variety of phenotypes exist even among genetically closely related strains in this genus (Ivanova et al. 1996). In spite of the low number of reports on the isolation of marine P(3HB)-degrading bacteria (Hachisuka et al. 2023; Suzuki et al. 2021), several P(3HB)-degrading *Alteromonas* strains have been found at multiple locations (Hachisuka et al. 2023; Kato et al. 2019). However, there are no reports that identify the PhaZ protein produced and used by P(3HB)-degrading *Alteromonas* strains.

In this study, we address the identification of extracellular PhaZ in *Alteromonas* sp. D210916BOD_24, which was isolated as a P(3HB)-degrading bacterium previously (Hachisuka et al. 2023). *Alteromonas macleodii* NBRC 102226, the type strain with the highest 16S rDNA identity to the D210916BOD_24 strain, has a PhaZ homolog consisting only of a Type B CD (AFS36858.1), based on the genomic information. This homolog is

widely distributed in the genus *Alteromonas*. However, in a previous report, *A. macleodii* NBRC 102226 did not exhibit P(3HB)-degrading activity, and the prepared recombinant protein of the above homolog displayed no activity toward P(3HB) (Hachisuka et al. 2023). Thus, we suggest that P(3HB)-degrading *Alteromonas* spp. might possess a functional PhaZ with a Type B CD, similar but slightly different from the above homolog, or possess another PhaZ without a Type B CD, entirely different from the above homolog. This study first performed a genomic analysis of the D210916BOD_24 strain to identify a potential candidate gene that codes PhaZ. Subsequently, we conducted physiological and biochemical assays on the candidate gene to verify its authenticity. Additionally, we examined the distribution of this gene's homologs to discuss the differences in P(3HB) degradation ability across strains in the genus *Alteromonas*.

MATERIALS AND METHODS

Culture conditions

Lysogeny broth (LB), Terrific Broth (TB; Becton, Dickinson and Company; Franklin Lakes, NJ, USA), Marine Broth 2216 (MB; Becton, Dickinson and Company), and artificial seawater-based (ASWB) (Hachisuka et al. 2023) media were used in this study. The LB contained 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. The ASWB contained 36 g/L Daigo ASW (Shiotani M.S.; Hyogo, Japan), 1 g/L NH_4NO_3, 0.5 g/L yeast extract, 1 mL/L trace metal solution, and 1 mL/L vitamin solution. The components of the trace metal and vitamin solutions are described in a previous paper (Hachisuka et al. 2023). The pH of the ASWB was approximately 7.5. Agar powder was added at 1.5% (w/v) to make a solid plate. Ampicillin (Amp), chloramphenicol (Chl), and kanamycin (Kan) were used as necessary.

Genomic analysis

The genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega; Madison, WI, USA). The genome sequencing of *Alteromonas* sp. D210916BOD_24 was conducted using the PacBio Sequel IIe system at the Bioengineering Lab. Co., Ltd. (Kanagawa, Japan). The genome assembly and annotation analyzed using the DNA Data Bank of Japan (DDBJ) Fast Annotation and Submission Tool (DFAST) (Tanizawa et al. 2016) were recorded in the database through the DDBJ. The sequence accession number of the complete genome is AP038333.

Construction of a disruption strain

The disruption vector of the PhaZ homolog gene was constructed using the following procedure: a Kan resistance gene (*kanR*) and its promoter of the pK18*mobsacB* plasmid (Schäfer et al. 1994) were amplified using

the primers iKanR_IF-F (5′-TGC AGG TCG ACT CTA GAC AGC AAG CGA ACC GGA ATT GCC A-3′) and iKanR_IF-R (5′-CCC GGG GAT CCT CTA GTC AGA AGA ACT CGT CAA GAA GGC G-3′). The PCR fragment was inserted into the XbaI site of the pUC18 plasmid via seamless cloning, resulting in pUC18-P*kanR*. Then, the upstream-700 bp of the PhaZ homolog gene was amplified using the primers iFH_IF-F (5′-GCC AAG CTT GCA TGC CCG CAG AGA ATT ACG GTA TGG TGA-3′) and iFH_IF-R (5′-CTG TCT AGA GTC GAC CCT GAT TGC GAT CGA CCC GTT TAT TA-3′) and inserted into the PstI site of pUC18-P*kanR*, resulting in pUC18-U700-P*kanR*. Similarly, the downstream 700 bp of the PhaZ homolog gene was inserted into the SmaI site of pUC18-U700-P*kanR* using the primers iLH_IF-F (5′-TGA CTA GAG GAT CCC CCC TCC CGA GCT AAG TGG TGT AAC G-3′) and iLH_IF-R (5′-TTC GAG CTC GGT ACC CCG TCG TCT TGC CAA GTA CAA TTG C-3′), resulting in pUC18-U700-P*kanR*-D700. Finally, the U700-P*kanR*-D700 region of pUC18-U700-PkanR-D700 was amplified using the primers dPhaZ_IF-F (5′-GAA TTC GAG CTC GGT ACC CCG CAG AGA ATT ACG GTA TGG TGA-3′) and dPhaZ_IF-R (5′-CGA CTC TAG AGG ATC CCC CGT CGT CTT GCC AAG TAC AAT TGC-3′), and inserted into the SmaI site of pT18mobsacB (Wei et al. 2013).

Construction of the PhaZ homolog gene-disruption strain was performed using conjugation and homologous recombination. Conjugation from *Escherichia coli* S17-1 λpir (Simon et al. 1983) to *Alteromonas* sp. D210916BOD_24 was conducted with reference to previous studies (Hachisuka et al. 2021; Shimizu et al. 2019). *E. coli* S17-1 λpir transformed with the disruption vector was cultured in LB-Kan·nH_2SO_4 (100 μg/mL) at 37°C for approximately 20 h. *Alteromonas* sp. D210916BOD_24 was cultured in MB at 20°C for approximately 12 h. Cells were harvested from 1 mL of *E. coli* S17-1 λpir culture and 0.5 mL of *Alteromonas* sp. D210916BOD_24 culture by centrifugation (5,000 × *g*, 5 min) and individually suspended in 500 μL of LB. They were then mixed, harvested by centrifugation at 5,000 × *g* for 5 min, and suspended in 80 μL of LB again. The mixture was dropped onto a nitrocellulose membrane (Merck Millipore, Burlington, MA) on a 4/5 LB + 1/5 MB plate and incubated at 28°C for 1 day. The cells on the membrane were suspended in 1 mL of ASWB and inoculated onto a 1/5 MB-Kan·nH_2SO_4 (200 μg/mL)-NaCl [5% (w/v)] plate. The agar medium was incubated for 2 days, and single colonies were picked from the plate.

The genotype of the transformant was examined by PCR with primer sets that anneal outside the homologous regions for homologous recombination, dPhaZ_out-f (5′-GGT GCT GCA CGG ATG CAC TCA GTC G-3′) and dPhaZ_out-r (5′-CTG TTG CCA CCT CCG CTACTACACAC-3′), or within the KanR gene, dPhaZ_in-f (5′-GAA CAA GAT GGA TTG CAC GCA GGT TC-3′) and dPhaZ_in-r (5′-GGC GAT ACC GTA AAG CAC GAG GAA GC-3′). The absence of unintended mutations in the homologous regions was confirmed through DNA

158 sequencing analysis of the PCR fragment using the primer set dPhaZ_out-f/dPhaZ_out-r.

159 **Degradation assay using medium plates**

160 MB and 1/5 MB containing 2 g/L P(3HB) powder suspension was used as a medium for the degradation
161 assay. P(3HB) powder was purchased from HighChem Company Limited (Tokyo, Japan) and used as is. Wild-
162 type and disruption strains precultured on an MB plate were inoculated into MB and 1/5 MB plates with P(3HB)
163 using sterile toothpicks. The plates were then incubated at 30°C for a week.

164 **Preparation of the recombinant enzyme**

165 The PhaZ homolog gene without SP in *Alteromonas* sp. D210916BOD_24 was amplified from the genomic
166 DNA using the primer set ePhaZ_F (5′-AAA AAC ATA TGA TGG GAT TGG GTA AAG GTT TTA GG-3′) and
167 ePhaZ_R (5′-AAA AAC TCG AGT GGG CAG CTT CCT ACA CTC CAT AG-3′) with NdeI and XhoI restriction
168 sites and inserted into the NdeI-XhoI region of pET21a (+) (Merck, Darmstadt, Germany). The expression vector
169 was introduced into the *E. coli* Rosetta-gami B(DE3)pLysS strain (Merck). The transformant was cultured in TB
170 supplemented with 100 μg/mL Amp·Na and 30 μg/mL Chl at 37°C, and isopropyl β-D-1-thiogalactopyranoside
171 (IPTG; final concentration 1.0 mM) was added to the medium when the OD_{600} reached 0.5–0.6. Then, the cells
172 were further cultivated at 16°C for 24 h. Cells were harvested by centrifugation (5,000 × *g*, 10 min), resuspended
173 in 50 mM Tris-HCl buffer (pH 8.0) with 0.3 M NaCl, and disrupted by sonication (20 kHz) using an Ultrasonic
174 Disruptor UD-211 (TOMY SEIKO Co, Ltd.; Tokyo, Japan) while being chilled in ice water. Subsequently, the
175 cell-free fraction obtained from the 1-L culture was subjected to 0.25 mL of TALON Metal Affinity Resin (Takara
176 Bio Inc.; Shiga, Japan). Proteins mainly containing the His-tagged PhaZ homolog were eluted with 50 mM Tris-
177 HCl buffer (pH 8.0) containing 0.3M NaCl and 150 mM imidazole. The eluted fraction was subjected to a PD-10
178 Desalting Column (Cytiva or Danaher Corporation; Buckinghamshire, UK). The concentration of the purified
179 protein was determined by the absorbance value at 280 nm. The molar extinction coefficient of the protein was
180 calculated using the ExPASy ProtParam tool (https://web.expasy.org/protparam/).

181 **Enzyme assay**

182 The P(3HB) powders were roughly washed to remove the chemical additives before being used for the
183 enzyme assay, with reference to a previous study (Hachisuka et al. 2023). The reaction mixture contained 50 mM
184 Tris-HCl (pH 8.0), 1 mM $CaCl_2$, 400 mg/L P(3HB) suspension, 0–1.0 M NaCl and 10 μg/mL purified enzyme.
185 P(3HB) powder suspension was prepared by sonication (20 kHz, UD-211) for 10 min. The reaction was initiated
186 by adding the 10 μg of enzyme, whose activity was measured by monitoring the OD_{650} at 30°C for 10 min using
187 1 cm light path cuvettes. One unit of enzyme was defined as the amount of protein required to decrease the OD_{650}

by one/min. Values immediately after the start of the measurement were not used for the calculation due to instability.

The effects of pH and temperature on enzyme activity were examined using *p*-nitrophenyl butyrate (*p*NPB). The reaction mixture contained 10 mM each buffer, 1 mM $CaCl_2$, 0.5 mM *p*NPB, and 10 μg/mL purified enzyme. As buffers, 10 mM potassium phosphate (pH 6.0–8.0) and 10 mM Tris-HCl (pH 8.0–9.0) were tested. The esterase activity toward *p*NPB was measured by monitoring changes in absorbance at 405 nm resulting from the release of *p*-nitrophenol.

Phylogenetic tree construction and genomic information analysis

Phylogenetic trees of the 16S rDNA and PhaZ homolog amino acid sequences were constructed using the maximum likelihood method in MEGA 11 (Tamura et al. 2021). Bootstrap replications were performed 500 times. The homolog sequences from various bacteria were intermittently selected based on the results of an NCBI Protein BLAST search using the homolog from *Alteromonas* sp. D210916BOD_24 as the query. The presence or absence of homologs and the gene's location in each bacterium was examined using EzBioCloud (Chalita et al. 2024).

RESULTS

Genomic analysis of the isolated *Alteromonas* sp. D210916BOD_24

The genomic analysis of the isolated *Alteromonas* sp. D210916BOD_24 showed that this strain possesses a PhaZ homolog consisting only of a Type B CD (BFT30787.1), whose CD region shows 72.6% identity and 93.1% similarity to the CD region of *A. macleodii* NBRC 102226 (AFS36858.1). More importantly, the genomic analysis revealed the existence of another PhaZ homolog with a Type A CD in *Alteromonas* sp. D210916BOD_24 (BFT29534.1), whose homolog is absent in *A. macleodii* NBRC 102226. The PhaZ homolog consists of a Type A CD, an Fn3 LD, and an SBD composed of two regions (Fig. S1), suggesting that it is a typical marine bacterial PhaZ (Fig. 1b). Thus, we examined whether the homolog protein functions as an authentic PhaZ in *Alteromonas* sp. D210916BOD_24.

Characterization of the disruption strain

A strain with a disrupted PhaZ homolog gene was constructed by homologous recombination using the wild-type D210916BOD_24 strain as the parental strain (Fig. S2a). *kanR* and its promoter were inserted into the center of the *phaZ* homolog by double crossover (Figs. S1 and S2a), and its genotypes were confirmed by PCR analysis (Fig. S2b). The disrupted and wild-type strains were inoculated on 1/5 MB and MB agar plates containing 2 g/L P(3HB) powder suspension, and the plates were incubated at 30°C for a week (Figs. 2 and S3). We used 1/5

MB in addition to MB because a previous study had shown that clear zone formation of the wild-type strain was much faster on 1/5 MB plate than on MB plate (Hachisuka et al. 2023), which was also true for this study. On the 1/5 MB plate with P(3HB) powder suspension (Fig. 2), the wild-type strain formed a clear zone 3 days after inoculation. In contrast, the disrupted strain did not form a clear zone even a week after inoculation. Although confirmation via gene complementation and/or genome analysis of the disrupted strain is necessary for a more rigorous evaluation, these results suggest that the PhaZ homolog is involved in P(3HB) metabolism in this strain and was the essential enzyme that metabolized P(3HB), at least under the culture conditions tested.

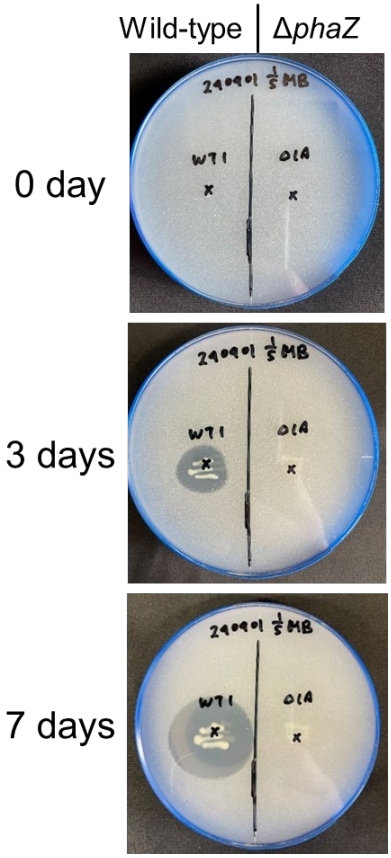


Fig. 2 P(3HB) degradation assay on a medium agar plate. Wild-type and *phaZ homolog*-disruption (Δ*phaZ*) strains of *Alteromonas* sp. D210916BOD_24 were cultured on 1/5 MB + 2 g/L P(3HB).

Degradation assay of the PhaZ homolog

The C-terminal His-tagged protein of the *phaZ homolog* was overexpressed in recombinant *E. coli* and subjected to affinity chromatography. The protein was purified to the main band, judging from the SDS-PAGE results (Fig. S4).

The enzyme activity of the His-tagged PhaZ homolog was first measured using *p*NPB as a substrate (Fig. S5). The optimal pH and temperature of the esterase activity were approximately 8.0 (Fig. S5a) and 40°C (Fig.

S5b), respectively. Subsequently, P(3HB) depolymerase activity was examined at NaCl concentrations of 0–1.0 M using P(3HB) powder suspension as a substrate (Fig. 3). The measurements were performed at pH 8.0 and 30°C, accounting for the appropriate pH and temperature and the stability of the P(3HB) suspension. The PhaZ homolog displayed significant activity with various concentrations of NaCl, suggesting that the protein indeed functions as PhaZ. In addition, the activity tended to increase with increasing NaCl concentration in the range of 0–0.8 M, with the highest activity observed at 0.8 M.

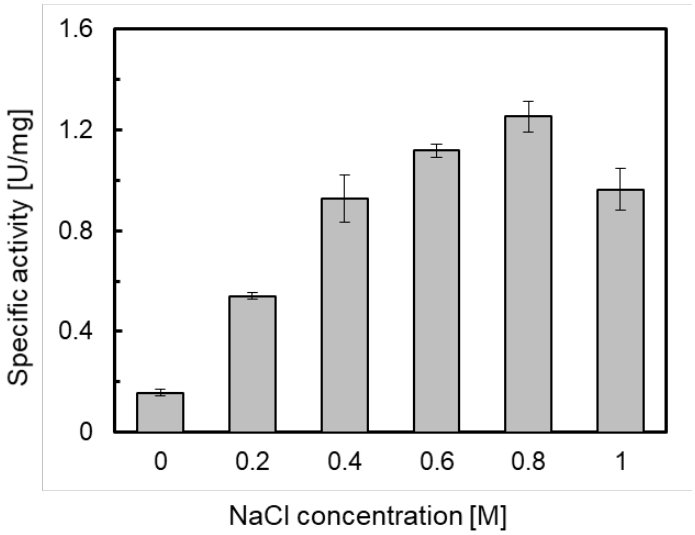


Fig. 3 Enzymatic activity assay of the PhaZ homolog toward P(3HB). The measurements were performed at various NaCl concentrations. Each value represents the mean (±SE) of three independent measurements.

Distribution of PhaZ homologs in *Alteromonas* spp.

The analyses up to this point have demonstrated that *Alteromonas* sp. D210916BOD_24 has a functional PhaZ with a Type A CD; in contrast, *A. macleodii* NBRC 102226 does not contain its homolog. We next focused on the distribution of the PhaZ homolog in *Alteromonas* spp. A phylogenetic tree based on 16S rDNA sequences was constructed for the *Alteromonas* strains with revealed genomic information (Fig. 4). The PhaZ homolog was found to be distributed in only 4/20 species. *A. lipolytica* JW12, *A. alba* 190, and *A. oceani* S35 are evolutionarily close to each other, and thus the distribution among these species may involve vertical spread. In contrast, these three strains and *Alteromonas* sp. D210916BOD_24 are not evolutionarily adjacent to each other. This distribution, at the least, cannot be attributed to vertical spread, although the existence of other undiscovered species harboring PhaZ homologs and being phylogenetically adjacent to *Alteromonas* sp. D210916BOD_24 remains plausible. There are two possible explanations: 1) the homolog gene fell out from many *Alteromonas* spp. after vertical spread; 2) the horizontal spread of the homolog gene occurred after being split into a certain number of species.

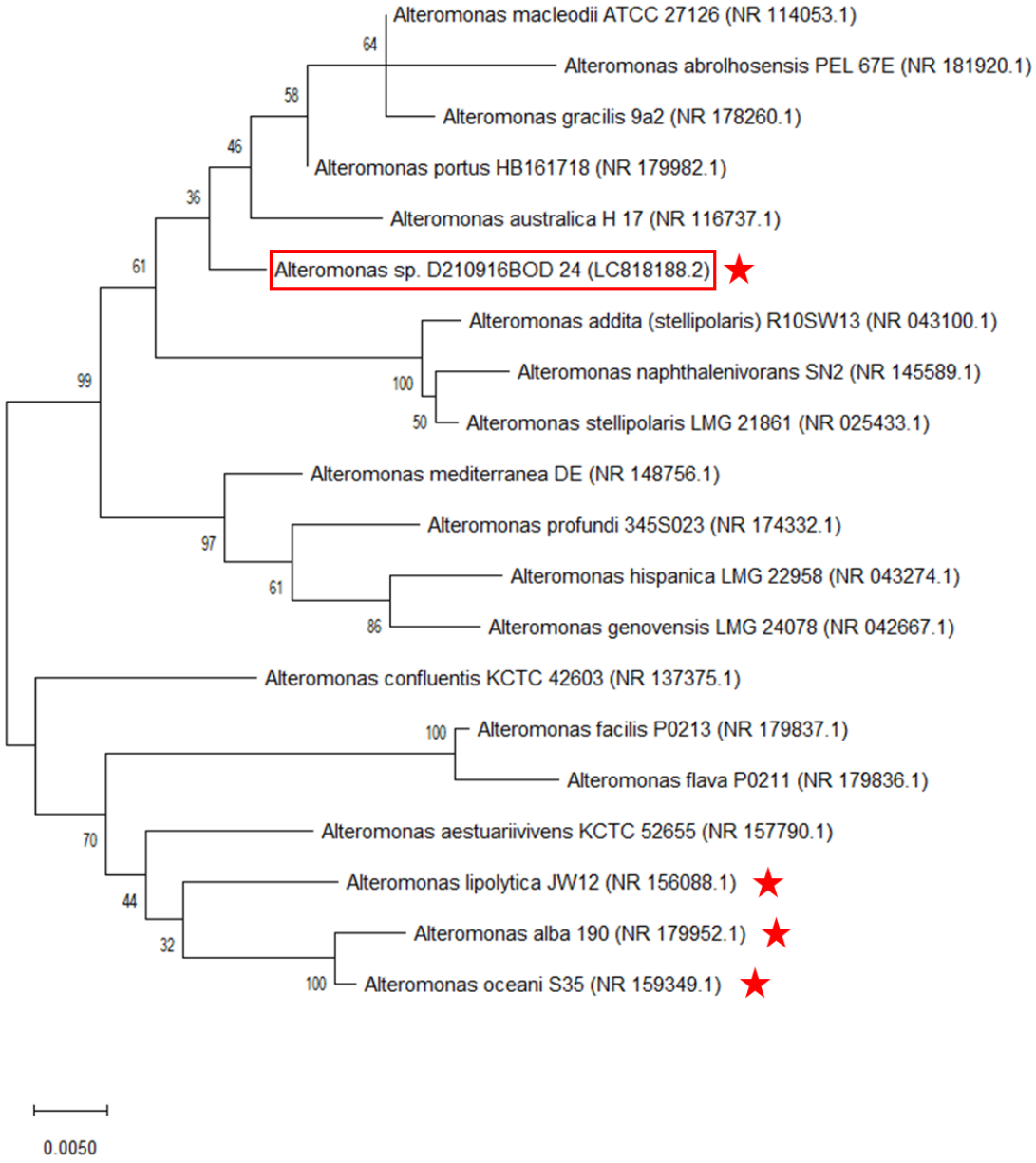


Fig. 4 Phylogenetic analysis of *Alteromonas* spp., whose genome was decoded. The tree was constructed based on the 16S rDNA sequences, and the numbers are shown in parentheses. Red stars indicate strains that possess the PhaZ homolog. *Alteromonas* sp. D210916BOD_24 is highlighted in the red square. The bootstrap values and a bar indicating sequence divergence (0.0050) are shown.

Location of PhaZ homolog genes in *Alteromonas* spp.

To verify the above hypotheses, we examined the gene location of the PhaZ homolog in the four *Alteromonas* strains that possess the PhaZ homolog (Fig. 5). The three strains that were closely related in the 16S rDNA sequence (*A. lipolytica* JW12, *A. alba* 190, and *A. oceani* S35) had a similar gene arrangement around the

phaZ homolog, which differed from the gene arrangement around the *phaZ in Alteromonas* sp. D210916BOD_24 (Fig. 5a). In *Alteromonas* sp. D210916BOD_24, the gene annotated as amidohydrolase family protein was present next to *phaZ*. There is no homolog of this gene in *Alteromonas* spp., and this gene displayed high homology to genes derived from *Microbulbifer* and *Marinobacter* spp. The location of the *phaZ homolog* was also examined on a larger scale (Fig. 5b). The distances between the Thr and Glu biosynthesis gene clusters common to the four strains and the PhaZ homolog gene were compared. The distance was in the order of 10^5 bp in *A. lipolytica* JW12, *A. alba* 190, and *A. oceani* S35, whereas it was on the order of 10^7 bp in *Alteromonas* sp. D210916BOD_24. In addition, the orientation of the phaZ homolog gene to the Thr and Glu biosynthesis gene clusters was opposite in the former three strains compared to the latter. These analyses support that the PhaZ homolog gene was not inherited by complete vertical spread from a common ancestor, but rather, propagated via some degree of horizontal spread.

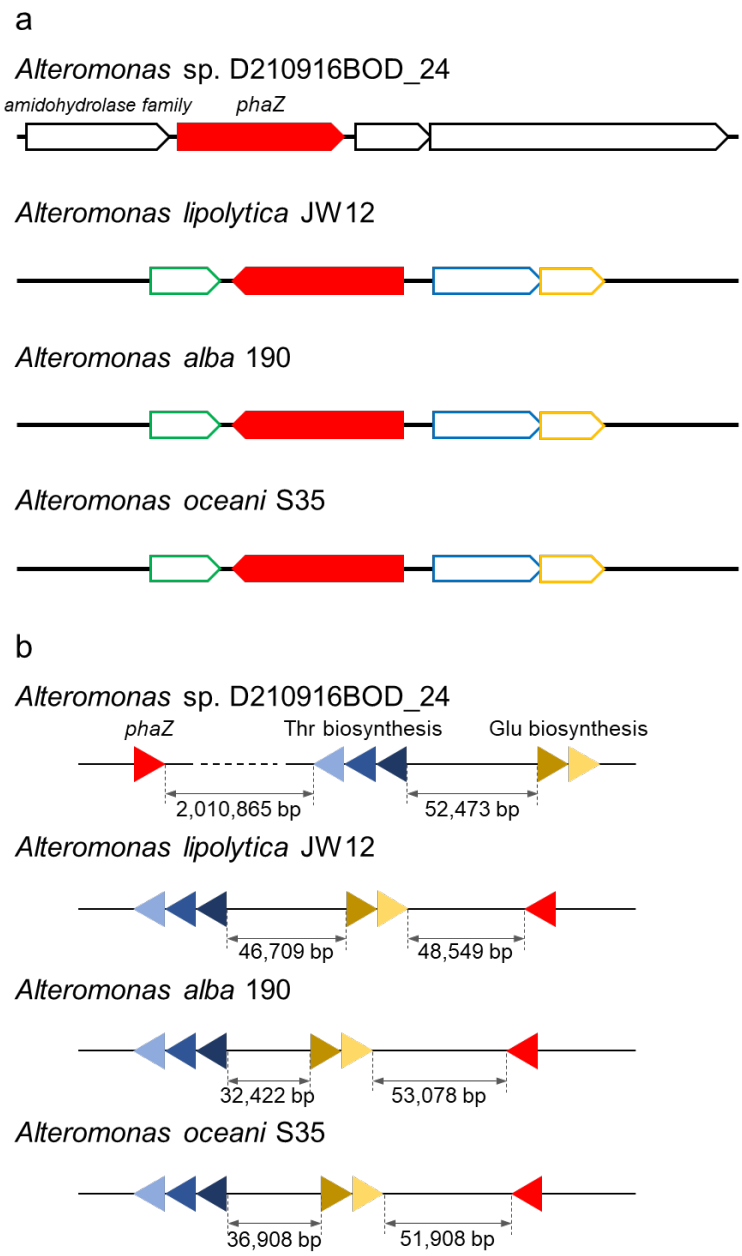


Fig. 5 Locations of the PhaZ homolog gene in the genome of *Alteromonas* spp. (a) Gene arrangements around the PhaZ homolog gene are shown. Green, blue, and yellow open arrowed boxes are homolog genes common among species. (b) The distance between the PhaZ homolog gene and the Thr and Glu biosynthesis gene clusters (*thrA*/*thrB1*/*thrC* and *gltB*/*gltD*) is shown. The length of a gene is not on the same scale as the distance between genes. The genome sequence of *Alteromonas* sp. D210916BOD_24 was analyzed in a complete and circular state (4,183,897 bp), but the genome sequences of the other strains were obtained in a scaffold state.

DISCUSSION

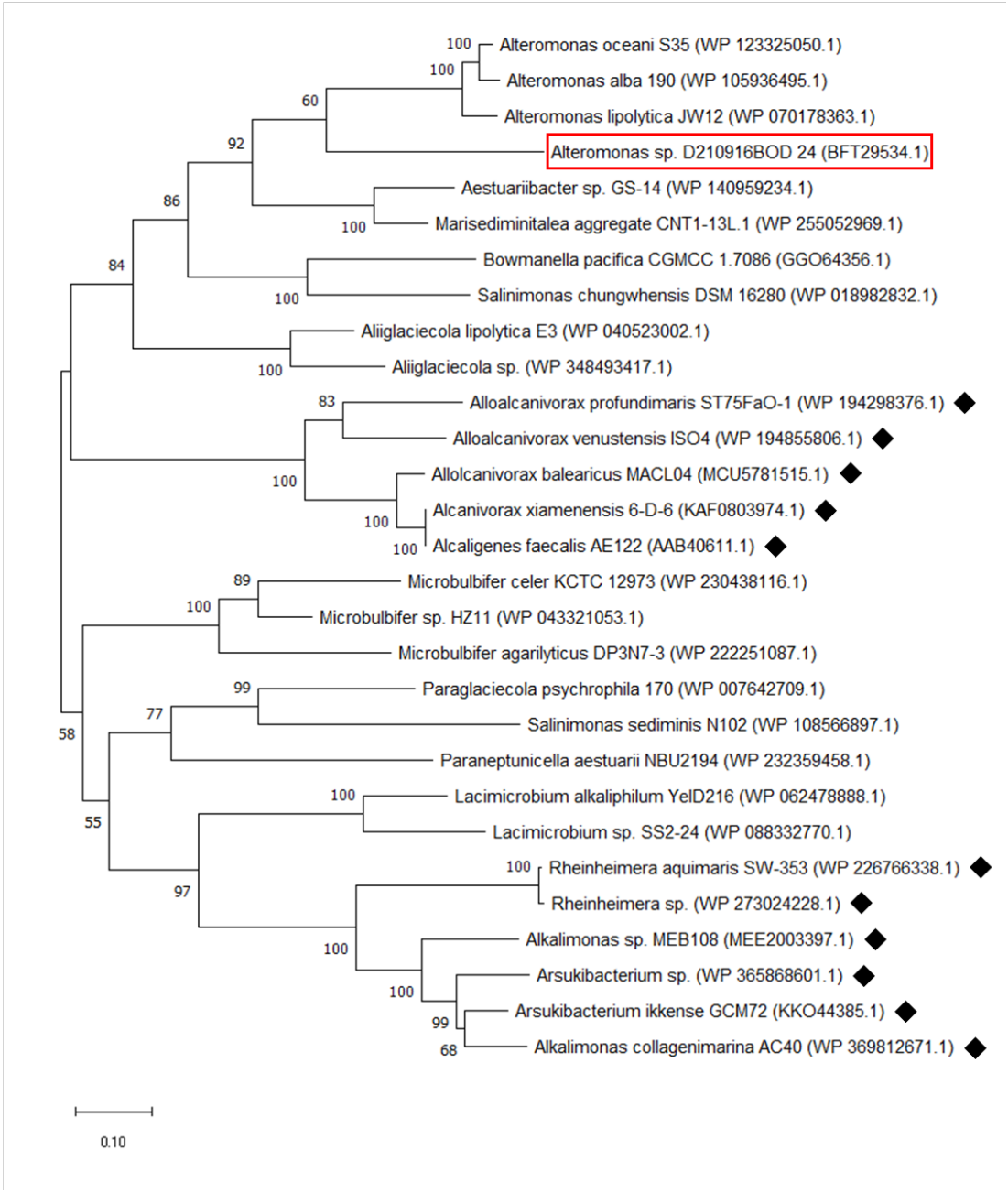
In this study, with the help of genomic analysis, we identified a PhaZ gene responsible for the degradation

ability of P(3HB) in *Alteromonas* sp. D210916BOD_24. Disruption of the PhaZ gene caused the strain to lose its ability to degrade P(3HB). The recombinant PhaZ protein displayed significant activity toward P(3HB). The only other marine PhaZs that consist of a Type A CD, an Fn3 LD, and an SBD comprising two regions and display *in vitro* activity are those derived from *Alcaligenes faecalis* AE122 (Kita et al. 1995; Kita et al. 1997) and *Shewanella* sp. AJ-6,1α (Sung et al. 2016). The sequence identities of PhaZ from *Alteromonas* sp. D210916BOD_24 toward those from *A. faecalis* AE122 (AAB40611) and *Shewanella* sp. AJ-6,1α (BAU59415) were 59.2% and 47.8%, respectively. Furthermore, the activity of PhaZ from *Alteromonas* sp. D210916BOD_24 increased with increases in the NaCl concentration in the range of 0–0.8 M. This result is entirely different from the characteristic of PhaZ from *Shewanella* sp. JKCM-AJ-6,1α in the previous study (Sung et al. 2016), where the activity of the full-length PhaZ from *Shewanella* sp. JKCM-AJ-6,1α decreased with increasing NaCl concentration. Considering the enzymes of halophilic microorganisms may help comprehend these differences in salinity tolerance. The amino acid composition of enzymes from halophiles is characterized by a relatively high acidic amino acid content and a relatively low basic amino acid content (Madern et al. 2000; Paul et al. 2008). The negative charges on the surface of proteins attract a large number of salt ions and water molecules bound to those cations, forming a hydrated salt ion network that maintains the stability and functionality of proteins in the presence of high salt concentrations. The percentages of acidic and basic amino acids are 7.6% and 5.0%, respectively, in PhaZ from *Shewanella* sp. JKCM-AJ-6,1α (BAU59415.1), while they were 9.2% and 3.8%, respectively, in PhaZ from *Alteromonas* sp. D210916BOD_24. This may be one of the reasons for the difference in the salt tolerance between these two proteins.

Distribution analysis of the PhaZ homolog gene in *Alteromonas* spp. ruled out the pure vertical spread of the gene. In addition, the location of the PhaZ homolog gene was analyzed, demonstrating that horizontal transfer of the gene occurred in *Alteromonas* spp. after some degree of species split. To examine the distribution of the PhaZ homolog gene outside *Alteromonas* spp., a phylogenetic tree was constructed using amino acid sequences with relatively high homology to the PhaZ protein from *Alteromonas* sp. D210916BOD_24 (Fig. 6a). Some bacteria belonging to a different family/order from *Alteromonas* strains (e.g., *Alloalcanivorax venustensis* ISO4, *Alloalcanivorax balearicus* MACL04) formed a clade closer to *Alteromonas* strains than some bacteria belonging to the same family/order as *Alteromonas* strains (e.g., *Paraneptunicella aestuarii* NBU2194). The corresponding bootstrap value was relatively low; however, based on the 16S rDNA sequences, *A. venustensis* ISO4 and *A. balearicus* MACL04 were located distinctly farther from *Alteromonas* spp. than *P. aestuarii* NBU2194 (Fig. 6b). In addition, a distribution survey of the PhaZ homolog gene in the genera that appeared in Fig. 6a and contained

318 a relatively large number of strains with decoded genomes revealed that the PhaZ homolog gene is present only
319 in certain strains (Tables S1 and S2). These results are unlikely to occur from pure vertical spread, suggesting that,
320 at least in part, horizontal transfer of the PhaZ homolog genes occurred even in bacteria outside of *Alteromonas*
321 spp. The PhaZ homolog of *A. faecalis* AE122, belonging to the Betaproteobacteria, was mixed with the others
322 derived from Gammaproteobacteria (Fig. 6a). The PhaZ homologs of *Salinimonas chungwhensis* DSM 16280 and
323 *Salinimonas sediminis* N102 were incorporated in a clade away from each other. These results for the *Alcaligenes*
324 and *Salinimonas* strains can be more clearly attributed to horizontal spread. One study proposed approaches to
325 identify horizontally transferred genes and estimate the time since gene transfer, using genome information, such
326 as base composition and codon usage patterns (Lawrence and Ochman 1998). This approach may allow us to
327 estimate the age at which horizontal transmission of each PhaZ homolog gene occurred, enabling a deeper
328 subsequent discussion.

a


Alteromonas oceani S35 (WP 123325050.1)
Alteromonas alba 190 (WP 105936495.1)
Alteromonas lipolytica JW12 (WP 070178363.1)
Alteromonas sp. D210916BOD 24 (BFT29534.1)
Aestuariibacter sp. GS-14 (WP 140959234.1)
Marisediminitalea aggregate CNT1-13L.1 (WP 255052969.1)
Bowmanella pacifica CGMCC 1.7086 (GGO64356.1)
Salinimonas chungwhensis DSM 16280 (WP 018982832.1)
Aliiglaciecola lipolytica E3 (WP 040523002.1)
Aliiglaciecola sp. (WP 348493417.1)
Alloalcanivorax profundimaris ST75FaO-1 (WP 194298376.1)
Alloalcanivorax venustensis ISO4 (WP 194855806.1)
Alloalcanivorax balearicus MACL04 (MCU5781515.1)
Alcanivorax xiamenensis 6-D-6 (KAF0803974.1)
Alcaligenes faecalis AE122 (AAB40611.1)
Microbulbifer celer KCTC 12973 (WP 230438116.1)
Microbulbifer sp. HZ11 (WP 043321053.1)
Microbulbifer agarilyticus DP3N7-3 (WP 222251087.1)
Paraglaciecola psychrophila 170 (WP 007642709.1)
Salinimonas sediminis N102 (WP 108566897.1)
Paraneptunicella aestuarii NBU2194 (WP 232359458.1)
Lacimicrobium alkaliphilum YelD216 (WP 062478888.1)
Lacimicrobium sp. SS2-24 (WP 088332770.1)
Rheinheimera aquimaris SW-353 (WP 226766338.1)
Rheinheimera sp. (WP 273024228.1)
Alkalimonas sp. MEB108 (MEE2003397.1)
Arsukibacterium sp. (WP 365868601.1)
Arsukibacterium ikkense GCM72 (KKO44385.1)
Alkalimonas collagenimarina AC40 (WP 369812671.1)
0.10

329

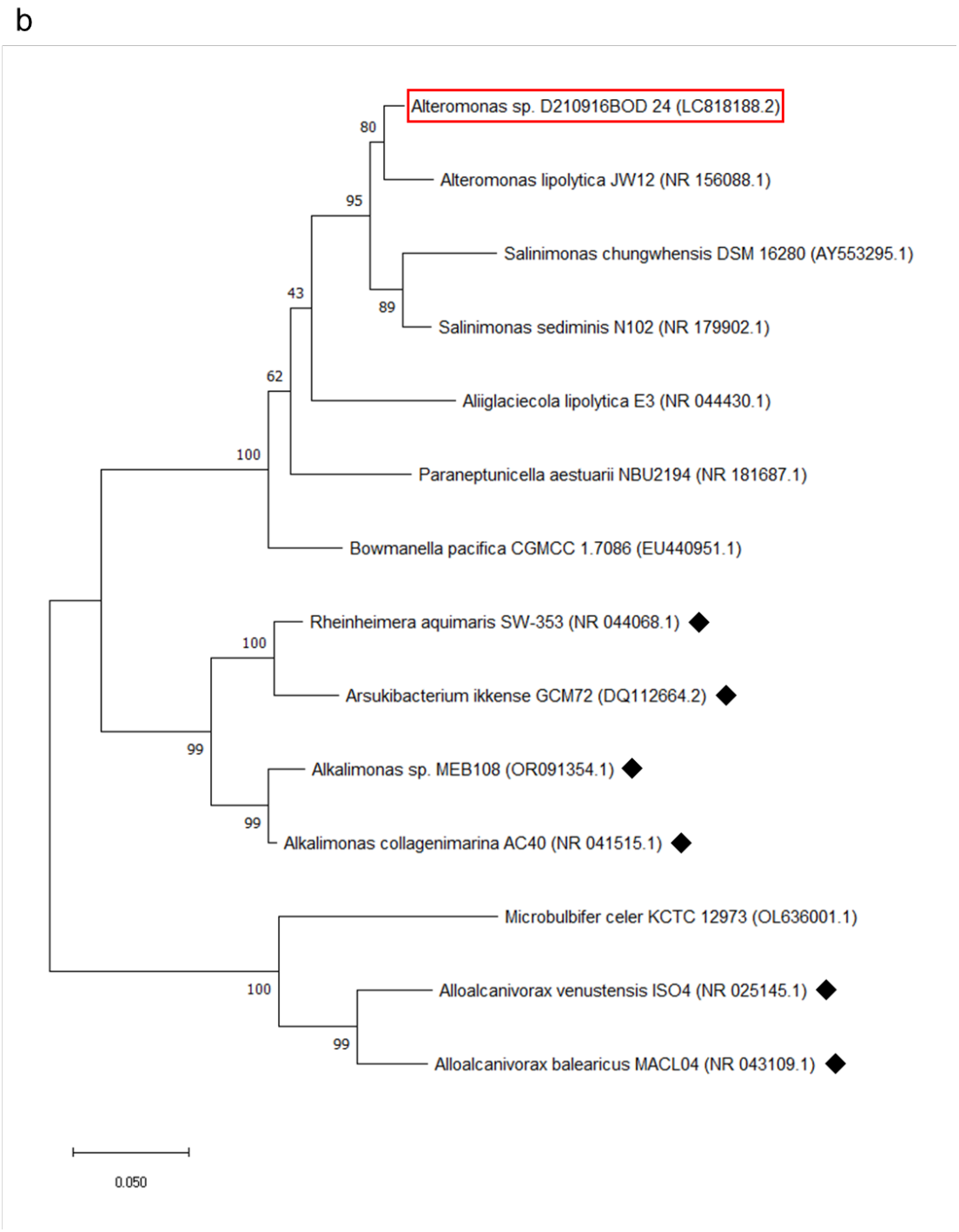


Fig. 6 Phylogenetic analysis of various bacteria that possess the PhaZ homolog. (a) The tree was constructed based on the PhaZ homolog amino acid sequences, and the numbers are shown in parentheses. These PhaZ homologs were selected from those that showed an E-value of 0.0 when the PhaZ homolog from *Alteromonas* sp. D210916BOD_24 was used as a query in NCBI Protein BLAST. They were randomly selected from various genera. (b) The tree was constructed based on the 16S rDNA sequences of some of the bacteria listed in (a), and the numbers are shown in parentheses. Black diamonds indicate strains belonging to a different family/order than the genus *Alteromonas*. The other strains belong to Alteromonadaceae (family)/Alteromonadales (order).

Alteromonas sp. D210916BOD_24 is highlighted in the red square. Bootstrap values and the bar indicating sequence divergence (a, 0.10; b, 0.050) are shown.

This study elucidated that horizontal transfer of the PhaZ gene can occur. Until now, the focus had been on the actual identification of PhaZ from isolated P(3HB)-degrading microorganisms and not on whether it is widely distributed among closely related species. Therefore, to our knowledge, this is the first such report on the spread of the PhaZ gene. A previous study reported that at least a certain number of strains without a PHA synthase gene possess a PhaZ gene (Hachisuka et al. 2023). That report is reasonable in light of the conclusion of the current study. From an evolutionary perspective, it would be interesting to determine whether the PhaZ gene is more prone to horizontal spread than other genes. We would like to address this issue in the near future.

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Supplementary Information

A separate file in a PDF document is submitted.

Author Contributions

Kyogo Iseki: formal analysis, investigation, validation, and writing–original draft. Shin-ichi Hachisuka: conceptualization, formal analysis, funding acquisition, investigation, project administration, validation, writing–original draft, and writing–review & editing. Hiroshi Kikukawa: supervision and writing–review & editing. Takeharu Tsuge: resources and writing–review & editing. Ken'ichiro Matsumoto: supervision and writing–review & editing.

Data Availability

The data on the genome analysis have been deposited in the DDBJ database. Sequence Read Archive number: DRR567972. Sequence accession number: AP038333. All other data supporting the findings of this study are included in the article and its supplementary materials.