Appl Microbiol Biotechnol (2017) 101:2153–2162
DOI 10.1007/s00253-016-7991-9

ENVIRONMENTAL BIOTECHNOLOGY

Identification of alkaline phosphatase genes for utilizing a flame retardant, tris(2-chloroethyl) phosphate, in *Sphingobium* sp. strain TCM1

Shouji Takahashi[1] · **Hiroshi Katanuma**[1] · **Katsumasa Abe**[1] · **Yoshio Kera**[1]

Received: 12 July 2016 / Revised: 31 October 2016 / Accepted: 5 November 2016 / Published online: 19 November 2016

Abstract Tris(2-chloroethyl) phosphate (TCEP) is a haloalkyl phosphate flame retardant and plasticizer that has been recognized as a global environmental contaminant. *Sphingobium* sp. strain TCM1 can utilize TCEP as a phosphorus source. To identify the phosphomonoesterase involved in TCEP utilization, we identified four putative alkaline phosphatase (APase) genes, named *SbphoA*, *SbphoD1*, *SbphoD2*, and *SbphoX-II*, in the genome sequence. Following expression of these genes in *Escherichia coli*, APase activity was confirmed for the *SbphoA* and *SbphoX-II* gene products but was not clearly observed for the *SbphoD1* and *SbphoD2* gene products, owing to their accumulation in inclusion bodies. The single deletion of either *SbphoA* or *SbphoX-II* retarded the growth and reduced the APase activity of strain TCM1 cells on medium containing TCEP as the sole phosphorus source; these changes were more marked in cells with the *SbphoX-II* gene deletion. In contrast, the deletion of either *SbphoD1* or *SbphoD2* had no effect on cell growth or APase activity. The double deletion of *SbphoA* and *SbphoX-II* resulted in the complete loss of cell growth on TCEP. These results show that SbPhoA and SbPhoX-II are involved in the utilization of TCEP as a phosphorus source and that SbPhoX-II is the major phosphomonoesterase involved in TCEP utilization.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-016-7991-9) contains supplementary material, which is available to authorized users.

✉ Shouji Takahashi
shoutaka@vos.nagaokaut.ac.jp

[1] Department of Bioengineering, Nagaoka University of Technology, Nagaoka, Niigata 940-2188, Japan

Keywords *Sphingobium* sp. strain TCM1 · Alkaline phosphatase · Tris(2-chloroethyl) phosphate · *E. coli* expression · Gene disruption

Introduction

The haloalkyl phosphorus compound tris(2-chloroethyl) phosphate (TCEP) has been widely used as a flame retardant or a plasticizer in plastics and forms throughout the world. This extensive use has resulted in its leakage into the environment, and thus it is now detected in indoor and outdoor air, rivers, lakes, disposal sites, drinking water, and even wild animals (Takahashi et al. 2013). TCEP has high physicochemical and microbiological stability in the environment and may exert neurotoxicity, reproductive toxicity, and carcinogenicity in rat and mouse (Takahashi et al. 2013). It has also been shown to exhibit toxic effects in aquatic fishes. This compound thus poses a serious threat to the health of humans and ecosystems. Therefore, methods for the removal of this environmental contaminant are needed.

To develop microbial techniques for the removal of TCEP from the environment, we have isolated two bacterial strains, *Sphingobium* sp. strain TCM1 and *Sphingomonas* sp. strain TDK1, that can utilize TCEP as a phosphorus source for cell growth (Takahashi et al. 2010). Strain TCM1 has a much higher TCEP degradation ability than strain TDK1. These bacterial strains are the only TCEP-degrading microorganisms reported so far, and they can also assimilate a broad range of other organophosphorus compounds for use as phosphorous sources, including tri(haloalkyl), triaryl, and trialkyl phosphorus compounds (Takahashi et al. 2010).

The utilization of organophosphorus compounds for cell growth in microorganism has been proposed to be mediated by three types of phosphoesterases: phosphotriesterase (PTE),

phosphodiesterase (PDE), and phosphomonoesterase (PME) (Fig. 1) (Singh and Walker 2006). In sphingomonads, the involvement of PDE and PME is shown in the degradation pathway of tributyl phosphate (TBP) in *Sphingobium* sp. strain RSMS, although PTE has not identified in this strain (Rangu et al. 2014, 2016). From these findings, the metabolism of TCEP in strain TCM1 is considered to include these three types of enzymes (Fig. 1). At each step, one of the phosphate ester bonds is hydrolyzed to produce 2-chloroethanol (2-CE), and the final step also gives Pi for cell growth. Strain TCM1 cells cannot degrade 2-CE, which also exhibits some toxicity to humans and animals (National Toxicology Program 1985). Therefore, we have developed a complete microbial detoxification technique for TCEP using strain TCM1 combined with a 2-CE-degrading bacterium, *Xanthobacter autotrophicus* strain GJ10 (Takahashi et al. 2012). We have already identified a PTE of strain TCM1, named haloalkylphosphorus hydrolase (HAD), that can hydrolyze TCEP to produce bis(2-chloroethyl) phosphate (BCE) and 2-CE but not further degrade BCE (Fig. 1) (Abe et al. 2014). HAD can also hydrolyze a variety of other organophosphorus compounds, including tri(haloalkyl), trialkyl, and triaryl phosphates and some organophosphate pesticides (Abe et al. 2014). Conversely, the PDE and PME of the TCEP degradation pathway remain to be identified. The high TCEP elimination rate and low 2-CE generation rate of strain TCM1 suggest that the steps catalyzed by PDE, PME, or both might be rate limiting in the pathway (Takahashi et al. 2010). Identification of the PDE and PME involved in TCEP degradation in strain TCM1 is, therefore, important to develop an efficient and effective method for complete TCEP degradation using this microorganism.

Alkaline phosphatase (APase) is a metal-dependent PME that can hydrolyze a wide variety of monophosphate esters most efficiently under alkaline conditions. APases can be mainly grouped into three families, namely PhoA, PhoD, and PhoX (Ragot et al. 2015; Sebastian and Ammerman 2009). PhoA APases have been identified in various organisms ranging from bacteria to plants and animals and are usually referred to as typical APases (Lin et al. 2013). *Escherichia coli* APase, a representative of the PhoA family, can hydrolyze various phosphomonoesters and requires Mg^{2+} and Zn^{2+} for activity (Kim and Wyckoff 1991). The bacterial APases of the PhoA family have a classic signal peptide and are exported to the extracellular space via the Sec pathway (Pugsley 1993). PhoD gene homologs are ubiquitous in both aquatic and terrestrial ecosystems, and are more abundant in marine bacteria than genes of the other APase families (Kageyama et al. 2011; Ragot et al. 2015). The APase of *Bacillus subtilis*, an archetype of the PhoD family, has significant PDE activity, as well as PME activity, and is secreted into the extracellular medium through a twin-arginine translocation (Tat) pathway (Pop et al. 2002). This PhoD requires Ca^{2+} for activity and contains Fe^{3+} and Ca^{2+} in the active site of the crystal structure (Rodriguez et al. 2014). The enzymes of the PhoX family are further divided into two subfamilies, PhoX-I and PhoX-II, although there is likely to be no close phylogenetic linkage between them (Zaheer et al. 2009; Lee et al. 2015). The homologous genes of PhoX-I are found in various members of *Alphaproteobacteria* and *Gammaproteobacteria*, while those of PhoX-II are often found in sphingomonads of *Alphaproteobacteria* (Zaheer et al. 2009; Lee et al. 2015). The *Pseudomonas fluorescens* PhoX-I APase is activated by Ca^{2+}, and its structure shows the presence of Fe^{3+} and Ca^{2+} in the active site (Yong et al. 2014). An enzyme of the PhoX-II family (PhoK) was first characterized in *Sphingomonas* sp. strain BSAR-1, and another (mAP) was recently isolated from a metagenomic library constructed with tidal flat sediments (Nilgiriwala et al. 2008; Lee et al. 2015). PhoK requires Ca^{2+} and Zn^{2+} for catalysis but not Mg^{2+} (Bihani et al. 2011). Although PhoK displays very low PDE activity, PhoX-II APases are more similar to nucleotide phosphodiesterases than to members of the other APase families. PhoX-I enzymes have been shown to be secreted via the Tat pathway (Wu et al. 2007; Zaheer et al. 2009), while the secretion pathway of PhoX-II APases remains unclear.

It has been reported that *E. coli* APase can hydrolyze 2-fluoroethyl phosphate, a compound structurally similar to 2-chloroethyl phosphate (CEP, Fig. 1) (O'Brien and Herschlag 2002), suggesting that, in strain TCM1, APase may be involved in the utilization of TCEP as a phosphorus source. In this study, we identified four putative APase genes that belong

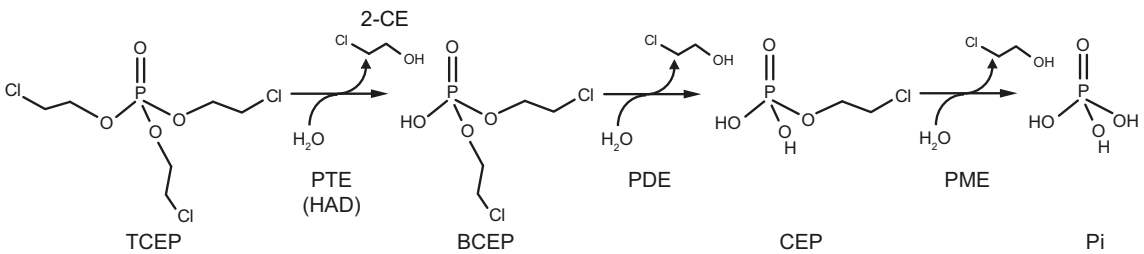


Fig. 1 Possible degradation pathway of TCEP in *Sphingobium* sp. strain TCM1. Enzymes: *PTE* phosphotriesterase, *PDE* phosphodiesterase, *PME* phosphomonoesterase, *HAD* haloalkylphosphorus hydrolase. Compounds: *TCEP* tris(2-chloroethyl) phosphate, *BCEP* bis(2-chloroethyl) phosphate, *CEP* 2-chloroethyl phosphate, *2-CE* 2-chloroethanol, *Pi* inorganic phosphate

to the PhoA, PhoD, and PhoX families in the draft genome sequence of strain TCM1. Expression of these genes in *E. coli* and characterization of gene deletion mutants of strain TCM1 showed that two APases, named SbPhoA and SbPhoX-II, are essential for utilization of TCEP as the sole phosphorus source. In particular, SbPhoX-II is the main APase involved in TCEP utilization for cell growth.

Materials and methods

Bacterial strains, plasmids, and culture conditions

Sphingobium sp. strain TCM, which has been deposited in the NITE Biological Resource Center (NBRC, Japan) under code NBRC 112374, was used as the wild-type strain (Takahashi et al. 2010). The wild-type strain and its mutants were grown at 30 °C in lysogeny broth (LB) or A-Cl minimal medium (Takahashi et al. 2010) containing 20 μM KH_2PO_4 or TCEP as a sole phosphorus source. SOC medium (20 g/l tryptone, 5 g/l yeast extract, 0.5 g/l NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$, 20 mM glucose, pH 7.0) was used for the recovery of TCM1 transformants. When necessary, 25 μg/ml kanamycin was added to the cultivation medium. *E. coli* strain DH5α was used as a host for DNA manipulation, and strain BL21(DE3) (New England Biolabs, Beverly, MA, USA) was used as a host for protein expression. *E. coli* cells were cultivated at 30 °C or 37 °C in LB or 2× YT medium (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, pH 7.0). When necessary, 100 μg/ml ampicillin was added to the cultivation medium. For solid media, 1.5% (*w*/*v*) agar was added.

Identification of APase gene homologs

The draft genome sequence of strain TCM1 (LXVX00000000) was submitted to the Rapid Annotation using Subsystem Technology (RAST) server for annotation (http://rast.nmpdr.org/rast.cgi). The predicted amino acid sequences of APase genes were further analyzed using the blastp or tblastn program against a protein or bacterial genome database (DNA Data Bank of Japan; http://ddbj.nig.ac.jp/blast/blastn?lang=ja).

Construction of expression vectors

Total DNA of strain TCM1 was prepared from overnight cultures in LB medium as described by Bickley and Owen (1995). The open reading frame of each putative APase gene (locus_tags—A7Q26_23575, A7Q26_18390, A7Q26_15460, and A7Q26_04985) was amplified by PCR using the primers listed in Table S1 and total DNA of strain TCM1 as a template with Tks Gflex DNA Polymerase or *TaKaRa Ex Taq* Hot Start Version DNA polymerase (Takara Bio, Shiga, Japan) under the following conditions: initial denaturation at 94 °C for 1 min, followed by 30 cycles at 98 °C for 10 s, 60 °C for 15 s, and 68 °C for 1 min, with a final extension at 68 °C for 10 min. The resulting PCR products were digested with *Nde*I and *Bam*HI and ligated into the same sites of pET25b to obtain their expression vectors. After sequencing analysis of the inserts, each plasmid was introduced into *E. coli* BL21(DE3).

E. coli BL21(DE3) cells harboring each APase expression vector were grown in 5 ml of LB medium containing 100 μg/ml ampicillin at 30 °C for 14 h. One milliliter of the culture was transferred into 100 ml of the same medium. When the absorbance at 600 nm reached 0.5, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to the culture medium to a final concentration of 1 mM. After cultivation for 6 h at 30 °C, the cells were harvested by centrifugation at 5000 ×*g* for 10 min at 4 °C, resuspended in 10 ml of 50 mM Tris-HCl (pH 9.0), and disrupted by sonication using an Ultrasonic Disruptor UD-201 (Tomy Seiko, Tokyo, Japan). The homogenate was centrifuged at 20,000×*g* for 30 min at 4 °C, and the supernatant and the pellet were used as a crude extract (soluble fraction) and an insoluble fraction, respectively. The proteins in these fractions were separated using SDS–12% polyacrylamide gel electrophoresis (SDS-PAGE) and then stained with Coomassie brilliant blue R-250. Protein concentration was determined by the Bradford method using a protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard.

Enzyme assays

A plate assay for APase activity of *E. coli* cells was carried out by incubating cells overnight at 37 °C on LB agar containing 100 μg/ml ampicillin, 40 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and 1 mM IPTG.

APase and PDE activities of crude extracts of *E. coli* cells were assayed by monitoring the release of *p*-nitrophenol (*p*-NP) from *p*-nitrophenyl phosphate (PNPP) and bis(*p*-nitrophenyl) phosphate (BNPP). The reaction mixture, containing 50 mM Tris-HCl (pH 9.0) and 5 mM PNPP or BNPP, was added to wells of a 96-well plate. After incubation of the mixture at 37 °C for 5 min, crude extract was added to the wells, and the release of *p*-NP was monitored spectrophotometrically at 405 nm (ε = 18,500 M/cm) at 37 °C on a microplate reader (SpectraMax; Molecular Devices, Sunnyvale, CA, USA). For analysis of the effect of metal ions, activity was measured in the presence of 5 mM Zn^{2+}, Mg^{2+}, or Ca^{2+}. Mg^{2+} and Ca^{2+} were added as chloride salts, while Zn^{2+} was added as the sulfate salt. One unit of enzyme is defined as the amount of enzyme that liberates 1 μmol of *p*-NP per minute.

APase activity of intact TCM1 cells was determined according to the method of Manoil (1991). Briefly, cells were collected from 1 ml of culture medium by centrifugation at

10,000×*g* for 10 min at 4 °C, washed once with 1 ml of ice-cold 10 mM Tris-HCl (pH 9.0), and resuspended in 1 ml of the same buffer. The OD_{600} of the cell suspension was measured, and then 100 μl of the suspension was combined with 0.9 ml of 1 M Tris-HCl (pH 9.0) and 100 μl of a permeabilization solution (50 μl of 0.1% SDS and 50 μl of chloroform) and kept at 37 °C for 5 min. After the addition of 100 μl of 0.4% PNPP, the suspension was kept at 37 °C for 5 min. The reaction was stopped by adding 120 μl of a stop solution (a 1:5 mix of 0.5 M EDTA (pH 9.0):1 M KH_2PO_4), and then the OD_{550} and OD_{420} were measured. The activity per cell (OD_{600}) was expressed in Miller units (Miller 1972), calculated as follows: $1000 \times [OD_{420} - (1.75 \times OD_{550})]/T$ (min) × *V* (ml) × OD_{600}, where *T* represents the length of reaction time and *V* represents the cell suspension volume.

Construction of gene-disruption vectors

For constructing markerless gene deletion vectors for each APase gene, approximately 1.0-kbp regions just upstream and downstream of each putative APase gene coding region were amplified by PCR using the primers listed in Table S1 and total DNA of strain TCM1 as a template with Tks Gflex DNA Polymerase (Takara Bio) under the following conditions: initial denaturation at 94 °C for 1 min, followed by 30 cycles at 98 °C for 10 s, 60 °C for 15 s, and 68 °C for 1.5 min, with a final extension at 68 °C for 10 min. The resulting PCR products were ligated into *Sma*I-digested pK18mobsacB using the In-Fusion HD Cloning Kit (Clontech, Palo Alto, CA, USA) to obtain the respective APase gene-disruption vectors.

Construction of deletion mutants

Strain TCM1 cells were grown in 10 ml of LB medium at 30 °C for 12 h with shaking at 169 rpm. One milliliter of the culture was transferred into 50 ml of LB medium and incubated at 30 °C for 8 h with shaking at 200 rpm. Cells were harvested by centrifugation at 5000×*g* for 10 min at 4 °C, washed twice in ice-cold 10% (*w*/*v*) glycerol, and resuspended in 100 μl of ice-cold 10% glycerol. The cell suspension was kept at −80 °C until use.

Five micrograms of an APase gene-disruption vector was mixed with the cell suspension and kept on ice for 1 min. The cell suspension was transferred into a precooled 2-mm-gap electroporation cuvette and subjected to a pulse of 2.5 kV in a MicroPulser electroporation apparatus (Bio-Rad). The cell suspension was then combined with 1 ml of ice-cold SOC medium and incubated at 30 °C for 6 h with shaking at 169 rpm. An aliquot of the cell suspension was plated onto LB agar medium containing 25 μg/ml kanamycin and incubated for 2 days. The cells grown on the medium were inoculated into 10 ml of LB medium and incubated for 1 day, and then 500 μl of the culture was spread onto LB agar medium containing 10% (*w*/*v*) sucrose to select gene deletion strains. The gene deletions were confirmed by PCR using the primers listed in Table S1 and total DNA of the mutants prepared using a PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). The PCR conditions were the same as those used for construction of the gene-disruption vectors except that the extension time was 2 min.

Characterization of mutants

Wild-type and mutant cells were grown in 100 ml of A-Cl medium containing 20 μM NaH_2PO_4 as the sole phosphorus source at 30 °C for 96 h with shaking at 162 rpm and collected by centrifugation at 5000×*g* for 10 min at 4 °C. Cells were washed with 100 ml of A-Cl medium lacking a phosphorus source, suspended in 100 ml of the same medium, and incubated at 30 °C for 96 h with shaking at 162 rpm to allow consumption of available phosphorus sources in the cells. The cells were then collected by centrifugation, washed once with the same medium, and resuspended in the same medium to an OD_{600} of 10. An aliquot of the cell suspension was transferred into A-Cl medium containing 20 μM NaH_2PO_4 or TCEP as the sole phosphorus source, and cell growth was monitored spectrophotometrically at 600 nm. APase activity of the cells was determined as described above.

Nucleotide sequence accession numbers

The nucleotide sequences of *SbphoA*, *SbphoD1*, *SbphoD2*, and *SbphoX-II* genes have been deposited at DDBJ/EMBL/GenBank under the accession numbers LC157910, LC157909, LC157912, and LC157911, respectively.

Sequence analysis

Multiple alignments were performed using Clustal Omega at EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic trees were generated using MEGA6.0 (http://www.megasoftware.net/) by the maximum-likelihood method with 1000 bootstrap replicates to verify tree topology. The amino-terminal signal sequence was predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) or TatP 1.0 servers (http://www.cbs.dtu.dk/services/TatP/).

Results

Identification of putative APase genes of strain TCM1

To identify APase-coding genes of *Sphingobium* sp. strain TCM1, we searched its genome sequence for APase gene homologs using the RAST server (Kera et al. 2016). The

search revealed four APase gene homologs (locus_tags—A7Q26_23575, A7Q26_18390, A7Q26_15460, and A7Q26_04985) with open reading frames of 1548, 1125, 1665, and 1584 bp, respectively, which code for proteins composed of 516, 375, 555, and 528 amino acids, respectively (Table 1). BLAST analysis of these amino acid sequences showed that the proteins coded by genes A7Q26_23575, A7Q26_18390, A7Q26_15460, and A7Q26_04985 have the highest identity to putative APases of *Sphingobium* sp. strain C100, *Sphingobium yanoikuyae* (Pho1, UniProt accession number A0A0J9CTQ7; and Pho2, A0A085K328), and an uncharacterized protein of *Sphingomonas sanxanigenens* DSM 19645 that is highly homologous to a putative APase of *Sphingobium* sp. strain SYK-6 (UniProtKB accession no. G2ILT4), respectively (Table 1). Phylogenetic analysis of the gene products classified A7Q26_18390 into the PhoA family, A7Q26_23575 and A7Q26_04985 into the PhoD family, and A7Q26_15460 into the PhoX-II family (Fig. 2). The amino acid sequences of these products include most of the functional amino acid residues involved in the binding of catalytic metal ions and Pi (Figs. S1–S3). Therefore, we named these genes *SbphoA* (A7Q26_18390), *SbphoD1* (A7Q26_23575), *SbphoD2* (A7Q26_04985), and *SbphoX-II* (A7Q26_15460). SbPhoA has 26.9% amino acid identity to *E. coli* PhoA APase. SbPhoD1 and SbPhoD2 have 37.9 and 39% identity to *B. subtilis* PhoD APase. SbPhoX-II has 85.4% identity to *Sphingomonas* sp. BSAR-1 PhoK. Analyses performed using the SignalP and TatP servers showed that SbPhoA and SbPhoX-II contain a signal sequence for the Sec pathway and that SbPhoD2 possesses a Tat signal (Figs. S1–S3). While SbPhoD1 does not have an obvious Tat signal, it does have a Tat signal-like sequence (Fig. S3).

Expression of putative APase genes in *E. coli*

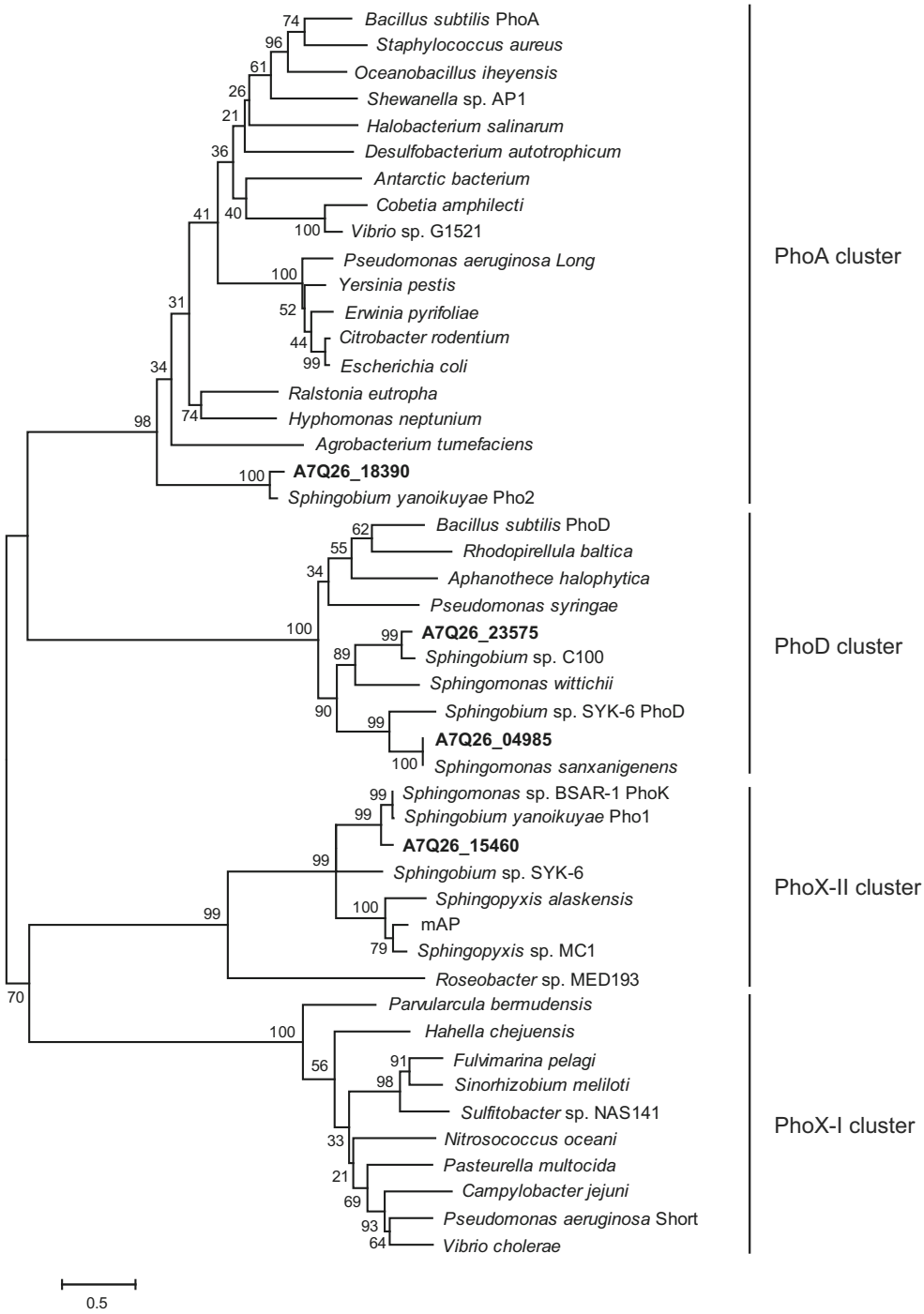
To determine whether the APase gene homologs code for active APases, we expressed them in *E. coli* BL21(DE3) and determined APase activity on an agar plate containing BCIP as a substrate (Fig. 3). APase activity was clearly observed in *E. coli* cells expressing *SbphoA* or *SbphoX-II* but not in those expressing *SbphoD1* or *SbphoD2*. The activity of SbPhoX-II appeared to be much higher than that of SbPhoA. We analyzed the activity of crude extracts of the *E. coli* cells using PNPP as the substrate (Table 2). Similar to the plate assay, analysis of crude extracts showed significant APase activity for SbPhoA and SbPhoX-II, but not for SbPhoD1 and SbPhoD2. The activity of SbPhoX-II was approximately 6500 times higher than that of SbPhoA. These results show that at least two APase gene homologs, *SbphoA* and *SbphoX-II*, encode active APases. We also determined PDE activity of the crude extracts using BNPP as the substrate (Table 2). Compared with the control extract, significant PDE activity was observed in that of SbPhoA and SbPhoX-II. The PDE activity of SbPhoA was comparable to the APase activity, while the activity of SbPhoX-II was considerably lower, suggesting much higher specificity of SbPhoX-II toward phosphomonoesters, similar to PhoK of *Sphingomonas* sp. strain BSAR-1 (Bihani et al. 2011). We confirmed expression of the gene products in *E. coli* by SDS-PAGE (Fig. 3b). In the crude extract (soluble fraction), a likely candidate gene product was observed only for the *SbphoX-II* gene, while other likely candidate gene products were found in the insoluble fractions, suggesting their accumulation in inclusion bodies.

Effect of metal ions on APase activity

To characterize the metal ion dependency of each gene product, we analyzed the effect of several divalent metal ions on the APase activity of the crude extracts (Table 2). The activity of SbPhoA was increased 6.9-fold and 1.7-fold by adding 5 mM Zn^{2+} and 5 mM Ca^{2+}, respectively, while the activity of SbPhoX-II was increased 1.5-fold by adding Mg^{2+}. Interestingly, Zn^{2+} markedly decreased the activity of SbPhoX-II to 19% of the value obtained without Zn^{2+}. In addition, significant activity (12.5 mU/mg)

Table 1 Putative APase genes of *Sphingobium* sp. strain TCM1

Gene	Length	Amino acids	Homologous gene (UniProt accession number)	Score (bit)	*E* value	Identity (%)
A7Q26_23575 (*SbphoD1*)	1548	516	*Sphingobium* sp. strain C100 Alkaline phosphatase (W1S4Q0) 517 a.a.	946	0	86
A7Q26_18390 (*SbphoA*)	1125	375	*Sphingobium yanoikuyae* Alkaline phosphatase (A0A0J9CTQ7) 376 a.a.	658	0	90
A7Q26_15460 (*SbphoX-II*)	1665	555	*Sphingobium yanoikuyae* Alkaline phosphatase (A0A085K328) 559 a.a.	946	0	85
A7Q26_04985 (*SbphoD2*)	1584	528	*Sphingomonas sanxanigenens* DSM 19645 Uncharacterized protein (W0A6N3) 528 a.a.	1083	0	99



was observed for SbPhoD1 in the presence of Zn^{2+}, suggesting that *SbphoD1* might also encode an APase. Conversely, no significant activity was obtained for SbPhoD2 with all metal ions tested, although the activities with metal ions were slightly higher than the activity of the control extract. These results suggest that SbPhoA and SbPhoD1 might require Zn^{2+} and SbPhoX-II might utilize Mg^{2+} for catalysis.

Involvement of APase genes in TCEP utilization

To determine whether the APase genes are involved in the utilization of TCEP, we created gene deletion mutants and analyzed their growth on TCEP as the sole phosphorus source (Fig. 4a, b). In a minimal medium containing 20 μM NaH_2PO_4 as the sole phosphorus source, the APase gene deletion mutants grew well, similar to the

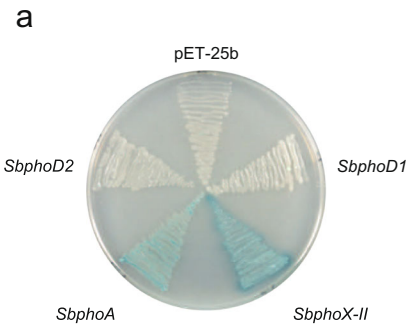
◀**Fig. 2** Phylogenetic tree of putative APases of *Sphingobium* sp. strain TCM1 and other known and putative APases. The phylogenetic analysis was performed using the maximum-likelihood method in MEGA6.0 with 1000 bootstrap trials. The *numbers* at nodes indicate bootstrap value percentages. Amino acid sequences from the bacteria listed below were used for the analysis (accession numbers are shown in *parentheses*). PhoA cluster: *Bacillus subtilis* subsp. *subtilis* str. 168 (NCBI protein accession no. NP_388822.2), *Staphylococcus aureus* subsp. *aureus* str. JKD6008 (NCBI no. WP_000953739.1), *Oceanobacillus iheyensis* (NCBI no. WP_011065431), *Shewanella* sp. AP1 (UniProtKB no. Q8RQU7), *Desulfobacterium autotrophicum* HRM2 (UniProtKB no. C0QFB4), *Antarctic bacterium* TAB5 (UniProtKB no. Q9KWY4), *Cobetia amphilecti* (UniProtKB no. Q1W622), *Vibrio* sp. G15-21 (UniProtKB no. Q93P54), *Halobacterium salinarum* (NCBI no. WP_010904149), *Erwinia pyrifoliae* DSM 12163 (UniProtKB no. D2T9N9), *Escherichia coli* str. K12 substr. W3110 (UniProtKB no. P00634), *Pseudomonas aeruginosa* PAO1 long type (NCBI no. NP_251986), *Yersinia pestis* biovar Microtus str. 91001 (NCBI no. NP_993417), *Agrobacterium tumefaciens* CCNWGS0286 (UniParc no. UPI000233382C), *Ralstonia eutropha* H16 (NCBI no. YP_726648), *Hyphomonas neptunium* ATCC15444 (UniProtKB no. Q0BWI9), and *Sphingobium yanoikuyae* Pho2 (NCBI no. WP_048939515). PhoD cluster: *Pseudomonas aeruginosa* PAO1 short type (UniProtKB no. P35482), *Pseudomonas syringae* (NCBI no. WP_011266682), *Sphingomonas sanxanigenens* DSM 19645 (UniProtKB no. W0A6N3), *Bacillus subtilis* PhoD (NCBI no. WP_009966461), *Rhodopirellula baltica* SH1 (NCBI no. NP_868602), *Sphingomonas wittichii* (NCBI no. WP_011951389), *Sphingobium* sp. C100 (NCBI no. WP_024019602), and *Aphanothece halophytica* (UniProtKB no. F5HRA4). PhoX-II cluster: *Roseobacter* sp. MED193 (NCBI no. WP_009807681), *Sphingopyxis* sp. MC1 (NCBI no. WP_003042625), *Sphingopyxis alaskensis* RB2256 (NCBI no. YP_615103), *Sphingobium* sp. SYK-6 (NCBI no. YP_004833865), *Sphingobium yanoikuyae* Pho1 (NCBI no. WP_037510231), *Citrobacter rodentium* ICC168 (NCBI no. YP_003364064), *Sphingomonas* sp. BSAR-1 (UniProtKB no. A1YYW7), and mAP (UniProtKB no. K9JA07). PhoX-I cluster: *Parvularcula bermudensis* (NCBI no. WP_013300011), *Hahella chejuensis* KCTC2396 (NCBI no. YP_437582), *Pasteurella multocida* (NCBI no. A1C3J6), *Pseudomonas fluorescens* Pf0-1 (UniProtKB no. G8Q1D5), *Vibrio cholerae* (UniProtKB no. Q6JJM1), *Campylobacter jejuni* (NCBI no. WP_021137608), *Nitrosococcus oceani* (NCBI no. WP_002811528), *Sulfitobacter* sp. NAS-14.1 (UniProtKB no. A3SXY0), *Fulvimarina pelagi* HTCC2506 (UniProtKB no. Q0G5E0), and *Sinorhizobium meliloti* 1021 (NCBI no. NP_385195)

wild-type strain, although the growth of the *SbphoA* and *SbphoX-II* deletion mutants was slightly lower (Fig. 4a). On medium with 20 μM TCEP as the sole phosphorus source, the *SbphoD1* and *SbphoD2* deletion mutants grew like the wild type, while the growth of the *SbphoX-II* and *SbphoA* deletion mutants was two thirds and one fifth lower, respectively, than that of the wild type even after 120 h of cultivation (Fig. 4b). We also analyzed the APase activity of the mutant cells during cultivation (Fig. 4c). The *SbphoD1* and *SbphoD2* mutant cells maintained a high activity, comparable to that of the wild-type cells, during cultivation, while the activities of the *SbphoA* and *SbphoX-II* mutant cells were about two thirds and one third lower, respectively, than the activity of the wild-type cells.

To further analyze the involvement of the *SbphoA* and *SbphoX-II* genes in TCEP utilization, we created an *SbphoA* and *SbphoX-II* double deletion mutant and determined its growth on TCEP (Fig. 5). The double deletion mutant grew well on 20 μM NaH_2PO_4 as the sole phosphorus source, although the growth was a little slower compared with that of the wild type (Fig. 5a). In contrast, no growth of the mutant was observed on 20 μM TCEP (Fig. 5b). These results clearly show that SbPhoA and SbPhoX-II, but not SbPhoD1 and SbPhoD2, are PMEs responsible for the utilization of TCEP as a phosphorus source, and that SbPhoX-II is the main PME needed for TCEP utilization.

Discussion

In this study, we sought to identify the PME responsible for utilization of TCEP as a phosphorus source in *Sphingobium* sp. strain TCM1. We identified four APase



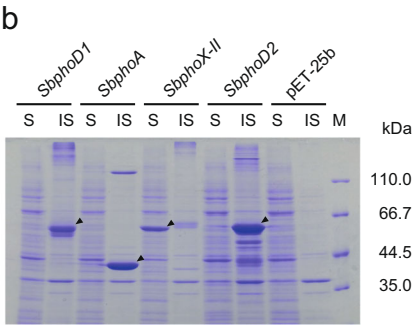


Fig. 3 Expression of putative APase genes of *Sphingobium* sp. strain TCM1 in *E. coli*. **a** Plate assay of APase activity of *E. coli* BL21(DE3) cells expressing each putative APase gene or harboring an empty vector (pET-25b). *E. coli* cells were plated on LB agar medium containing 100 μg/ml ampicillin, 40 μg/ml BCIP, and 1 mM IPTG and incubated overnight at 37 °C. **b** SDS-PAGE analysis of the expression of putative APase genes in *E. coli*. Soluble (S) and insoluble (IS) proteins of *E. coli* BL21(DE3) cells expressing each putative APase gene or harboring an empty vector (pET-25b) were separated on SDS–12% polyacrylamide gels and stained with Coomassie brilliant blue. *M* molecular size markers. *Arrowheads* indicate the positions of the likely candidate products of putative APase genes

Table 2 PME and PDE activities of crude extracts of *E. coli* cells expressing APase gene homologs from *Sphingobium* sp. strain TCM1

Metal ion	Substrate	Activity (mU/mg protein)				
		–	SbPhoA	SbPhoD1	SbPhoD2	SbPhoX-II
–	PNPP	0.3 ± 0.0	11.5 ± 4.1	0.1 ± 0.1	0.9 ± 0.1	74,000 ± 5420
Zn^{2+}		2.5 ± 2.0	79.9 ± 3.2	12.5 ± 1.0	4.9 ± 1.0	14,000 ± 2560
Mg^{2+}		0.8 ± 0.2	13.7 ± 1.5	0.4 ± 0.2	1.7 ± 0.1	113,000 ± 4010
Ca^{2+}		0.6 ± 0.2	19.5 ± 1.5	0.9 ± 0.1	1.5 ± 0.1	87,100 ± 1760
–	BNPP	5.6 ± 0.2	16.4 ± 0.5	9.0 ± 0.4	8.0 ± 1.0	15.4 ± 0.4

gene homologs belonging to three different families, PhoA, PhoD, and PhoX, and showed that two of them, *SbphoA* and *SbphoX-II*, are involved in TCEP utilization. SbPhoX-II was identified as the main PME. Because the hydrolysis of CEP or PME is suggested to be a rate-limiting step in the TCEP degradation pathway in strain TCM1, the identification of this enzyme may facilitate development of an efficient technique for detoxification of this environmental contaminant.

Although *E. coli* has only one APase gene, most bacterial strains possess more than one. For example, *P. fluorescens* has two APase genes (*phoX* and *phoD*) (Monds et al. 2006) and *B. subtilis* has three (*phoA*, *phoB*, and *phoD*) (Antelmann et al. 2000). We also found multiple APase gene homologs in the genome sequences of several sphingomonads, such as *Sphingobium* sp. C100, *Sphingobium japonicum* UT26S, *S. yanoikuyae*, and *S. sanxanigenens*. *S. yanoikuyae* and *S. sanxanigenens* have the same set of APase gene homologs found in strain TCM1, while *Sphingobium* sp. C100 lacks PhoA APase and *S. japonicum* UT26S has only PhoX-II APase, suggesting that PhoX-II APase may be the main PME in sphingomonads. These different APase compositions among sphingomonads might be caused by differences in the availability of Pi and phosphorus compounds among habitats. The different substrate preference of SbPhoA and SbPhoX-II suggests that the multiple APases of strain TCM1 could make it possible for this bacterium to utilize various organophosphorus compounds.

We have shown that two of the four APases of strain TCM1, SbPhoA and SbPhoX-II, are involved in the utilization of TCEP as a phosphorus source, and SbPhoX-II is the main APase involved in this utilization (Fig. 4). We initially hypothesized that SbPhoA is the main PME for TCEP utilization because *E. coli* PhoA APase can hydrolyze haloalkyl phosphates like CEP (O'Brien and Herschlag 2002). Our study is the first to show that PhoX-II APases can also hydrolyze a haloalkyl phosphate. It should be noted that SbPhoX-II and PhoK of *Sphingomona*s sp. strain BSAR-1, which is also a member of the PhoX-II family (Fig. 2), have different metal requirements despite their high amino acid sequence identity (85.4%): SbPhoX-II is markedly activated by Mg^{2+}, but not by Ca^{2+} or Zn^{2+} (Table 2), while PhoK of BSAR-1 requires Ca^{2+} and Zn^{2+} for its activity, but not Mg^{2+} (Bihani et al. 2011). Further studies on the enzymatic properties of SbPhoX-II and determination of its three-dimensional structure might provide insights into its substrate preference and the different metal requirement.

In the TBP-degrading bacterium *Sphingobium* sp. strain RSMS, two phosphoesterase proteins are identified as potential candidate enzymes involved in TBP

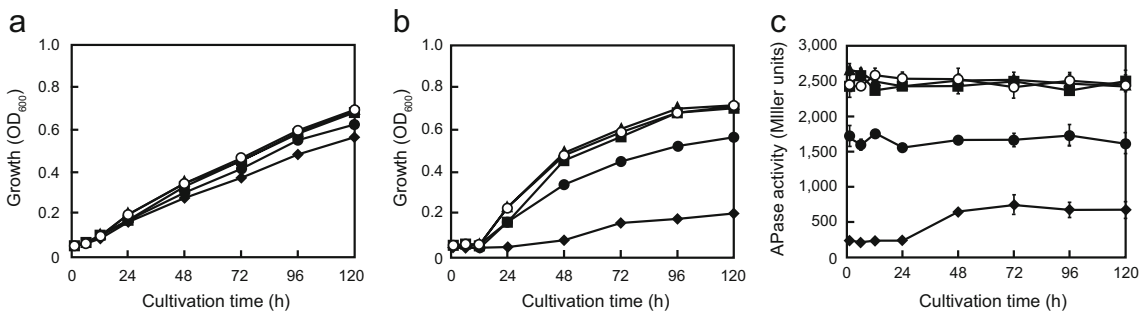


Fig. 4 Growth and APase activity of APase gene deletion mutants of *Sphingobium* sp. strain TCM1. The growth of wild-type and APase mutant cells on 20 μM NaH_2PO_4 (**a**) or TCEP (**b**) as the sole phosphorus source was determined by measuring the OD_{600}. **c** APase activity of wild-type and APase mutant cells during cultivation on 20 μM TCEP as the sole phosphorus source. *Open circles*, wild-type; *closed circles*, Δ*sbphoA*; *closed triangles*, Δ*sbphoD1*; *closed squares*, Δ*sbphoD2*; *closed diamonds*, Δ*sbphoX-II*. These data are the averages ± standard deviations (*error bars*) of at least three measurements

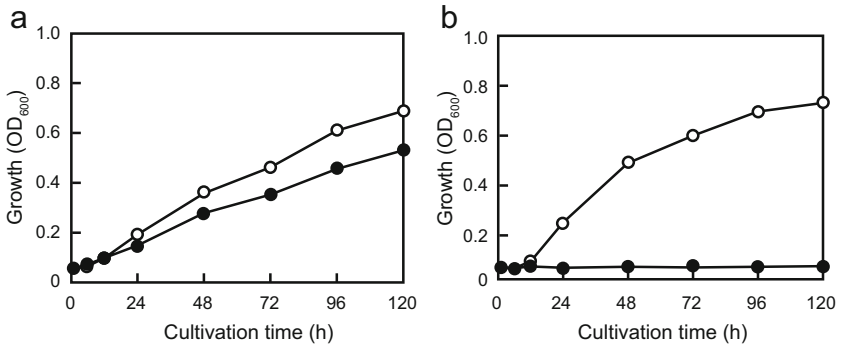


Fig. 5 Growth and APase activity of an *SbphoA* and *SbphoX-II* double deletion mutant of *Sphingobium* sp. strain TCM1. The growth of wild-type and APase double deletion mutant cells on 20 μM NaH_2PO_4 (**a**) or TCEP (**b**) as the sole phosphorus source was determined by measuring the OD_{600}. *Open circles*, wild type; *closed circles*, Δ*sbphoA*Δ*sbphoX-II*. These data are the averages ± standard deviations (*error bars*) of at least three measurements

degradation pathway (Rangu et al. 2016). These proteins are similar to the phosphohydrolase of *Aliivibrio fischeri* or exopolyphosphatase-like protein of *Marinomonas mediterranea* MMB-1 and show PME and PDE activities but not PTE activity in vitro assay. Strain TCM1 can also assimilate TBP as a phosphorus source (Takahashi et al. 2010), but we find no proteins with significant homology to the two phosphoesterases in the genome sequence, suggesting that some enzymes involved in the metabolism of TBP might be different between the strains. It is now unknown whether strain RSMS can degrade and assimilate TCEP. If possible, different types of enzymes could also be involved in their TCEP degradation pathways. Further studies are desired to reveal the degradation pathway of organophosphorus compounds in these *Sphingobium* strains.

PhoX APases are known to possess a Tat signal sequence (Wu et al. 2007; Lee et al. 2015). We predicted that PhoX-I APases have a Tat signal sequence, but we found that PhoX-II enzymes, including SbPhoX-II and the BSAR-1 PhoX-II APase, have a Sec signal instead of a Tat signal, except for the mAP enzyme derived from a metagenomic library (Fig. S5). This suggests that, in contrast to PhoX-I enzymes, most PhoX-II enzymes are likely transported to the extracellular space via the Sec pathway. This difference in transport pathways and the lack of significant homology between PhoX-I and PhoX-II APases (Fig. S 4) indicate that it may be appropriate to change the name of the PhoX-II family to a less confusable name, such as PhoK.

We have shown that the *SbphoD1* and *SbphoD2* genes are not involved in the utilization of TCEP as a phosphorus source (Figs. 4 and 5). Although it is still uncertain whether the *phoD* genes encode active APases due to their inactive expression in *E. coli*, their high homologies to known PhoD APases, the conservation of catalytic amino acid residues, and the presence of a Tat or Tat-like signal strongly indicate that they might code for active PhoD APases. In *B. subtilis*, PhoD APase is considered to be involved in the utilization of teichoic acid, a phosphodiester-linked glycopolymer found in the cell wall of gram-positive bacteria, as a phosphorus source for cell growth (Eder et al. 1996). Because *Sphingobium* sp. strain TCM1 is a gram-negative bacterium, SbPhoD1 and SbPhoD2 do not play the same role but might have other specific functions. Further analyses are needed to determine their enzymatic properties and physiological functions in strain TCM1.

Acknowledgement This work was supported in part by a Grant-in-Aid for Scientific Research (B) (24310055, to Y.K.) from the Ministry of Education, Culture, Sports, Science, and Technology (Japan).

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no competing interests.

References

Abe K, Yoshida S, Suzuki Y, Mori J, Doi Y, Takahashi S, Kera Y (2014) Haloalkylphosphorus hydrolases purified from *Sphingomonas* sp. strain TDK1 and *Sphingobium* sp. strain TCM1. Appl Environ Microbiol 80(18):5866–5873. doi:10.1128/AEM.01845-14

Antelmann H, Scharf C, Hecker M (2000) Phosphate starvation-inducible proteins of *Bacillus subtilis*: proteomics and transcriptional analysis. J Bacteriol 182(16):4478–4490. doi:10.1128/JB.182.16.4478-4490.2000

Bickley J, Owen RJ (1995) Preparation of bacterial genomic DNA. Methods Mol Biol 46:141–147. doi:10.1385/0-89603-297-3:141

Bihani SC, Das A, Nilgiriwala KS, Prashar V, Pirocchi M, Apte SK, Ferrer JL, Hosur MV (2011) X-ray structure reveals a new class and provides insight into evolution of alkaline phosphatases. PLoS One 6(7):e22767. doi:10.1371/journal.pone.0022767

Eder S, Shi L, Jensen K, Yamane K, Hulett FM (1996) A *Bacillus subtilis* secreted phosphodiesterase alkaline phosphatase is the product of a

Pho regulon gene, *phoD*. Microbiology 142(8):2041–2047. doi:10.1099/13500872-142-8-2041

Kageyama H, Tripathi K, Rai AK, Cha-Um S, Waditee-Sirisattha R, Takabe T (2011) An alkaline phosphatase/phosphodiesterase, PhoD, induced by salt stress and secreted out of the cells of *Aphanothece halophytica*, a halotolerant cyanobacterium. Appl Environ Microbiol 77(15):5178–5183. doi:10.1128/AEM.00667-11

Kera Y, Abe K, Kasai D, Fukuda M, Takahashi S (2016) Draft genome sequences of *Sphingobium* sp. strain TCM1 and *Sphingomonas* sp. strain TDK1, haloalkyl phosphate flame retardant and plasticizer-degrading bacteria. Genome Announc 4(4):e00668–e00616. doi:10.1128/genomeA.00668-16

Kim EE, Wyckoff HW (1991) Reaction mechanism of alkaline phosphatase based on crystal structures: two-metal ion catalysis. J Mol Biol 218(2):449–464. doi:10.1016/0022-2836(91)90724-K

Lee DH, Choi SL, Rha E, Kim SJ, Yeom SJ, Moon JH, Lee SG (2015) A novel psychrophilic alkaline phosphatase from the metagenome of tidal flat sediments. BMC Biotechnol 15:1. doi:10.1186/s12896-015-0115-2

Lin HY, Shih CY, Liu HC, Chang J, Chen YL, Chen YR, Lin HT, Chang YY, Hsu CH, Lin HJ (2013) Identification and characterization of an extracellular alkaline phosphatase in the marine diatom *Phaeodactylum tricornutum*. Mar Biotechnol 15(4):425–436. doi:10.1007/s10126-013-9494-3

Manoil C (1991) Analysis of membrane-protein topology using alkaline-phosphatase and β-galactosidase gene fusions. Methods Cell Biol 34:61–75. doi:10.1016/S0091-679X(08)61676-3

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Monds RD, Newell PD, Schwartzman JA, O'Toole GA (2006) Conservation of the Pho regulon in *Pseudomonas fluorescens* Pf0-1. Appl Environ Microbiol 72(3):1910–1924. doi:10.1128/AEM.72.3.1910-1924.2006

Nilgiriwala KS, Alahari A, Rao AS, Apte SK (2008) Cloning and overexpression of alkaline phosphatase PhoK from *Sphingomonas* sp. strain BSAR-1 for bioprecipitation of uranium from alkaline solutions. Appl Environ Microbiol 74(17):5516–5523. doi:10.1128/AEM.00107-08

O'Brien PJ, Herschlag D (2002) Alkaline phosphatase revisited: hydrolysis of alkyl phosphates. Biochemistry 41(9):3207–3225. doi:10.1021/bi012166y

Pop O, Martin U, Abel C, Muller JP (2002) The twin-arginine signal peptide of PhoD and the TatAd/Cd proteins of *Bacillus subtilis* form an autonomous Tat translocation system. J Biol Chem 277(5):3268–3273. doi:10.1074/jbc.M110829200

Program NT (1985) NTP toxicology and carcinogenesis studies of 2-chloroethanol (ethylene chlorohydrin) (CAS No. 107-07-3) in F344/N rats and Swiss CD-1 mice (dermal studies). Natl Toxicol Program Tech Rep Ser 275:1–194

Pugsley AP (1993) The complete general secretory pathway in gram-negative bacteria. Microbiol Rev 57(1):50–108

Ragot SA, Kertesz MA, Bunemann EK (2015) *phoD* alkaline phosphatase gene diversity in soil. Appl Environ Microbiol 81(20):7281–7289. doi:10.1128/AEM.01823-15

Rodriguez F, Lillington J, Johnson S, Timmel CR, Lea SM, Berks BC (2014) Crystal structure of the *Bacillus subtilis* phosphodiesterase PhoD reveals an iron and calcium-containing active site. J Biol Chem 289(45):30889–30899. doi:10.1074/jbc.M114.604892

Rangu SS, Basu B, Muralidharan B, Tripathi SC, Apte SK (2016) Involvement of phosphoesterases in tributyl phosphate degradation in *Sphingobium* sp. strain RSMS. Appl Microbiol Biotechnol 100(1):461–468. doi:10.1007/s00253-015-6979-1

Rangu SS, Muralidharan B, Tripathi SC, Apte SK (2014) Tributyl phosphate biodegradation to butanol and phosphate and utilization by a novel bacterial isolate, *Sphingobium* sp. strain RSMS. Appl Microbiol Biotechnol 98(5):2289–2296. doi:10.1007/s00253-013-5158-5

Sebastian M, Ammerman JW (2009) The alkaline phosphatase PhoX is more widely distributed in marine bacteria than the classical PhoA. ISME J 3(5):563–572. doi:10.1038/ismej.2009.10

Singh BK, Walker A (2006) Microbial degradation of organophosphorus compounds. FEMS Microbiol Rev 30(3):428–471. doi:10.1111/j.1574-6976.2006.00018.x

Takahashi S, Abe K, Kera Y (2013) Microbial degradation of persistent organophosphorus flame retardants. In: Petre M (ed) Environmental biotechnology—new approaches and prospective applications. InTech, Croatia, pp 91–122. doi: 10.5772/53749

Takahashi S, Miura K, Abe K, Kera Y (2012) Complete detoxification of tris(2-chloroethyl) phosphate by two bacterial strains: *Sphingobium* sp. strain TCM1 and *Xanthobacter autotrophicus* strain GJ10. J Biosci Bioeng 114(3):306–311. doi:10.1016/j.jbiosc.2012.04.010

Takahashi S, Satake I, Konuma I, Kawashima K, Kawasaki M, Mori S, Morino J, Mori J, Xu H, Abe K, Yamada RH, Kera Y (2010) Isolation and identification of persistent chlorinated organophosphorus flame retardant-degrading bacteria. Appl Environ Microbiol 76(15):5292–5296. doi:10.1128/AEM.00506-10

Wu JR, Shien JH, Shieh HK, Hu CC, Gong SR, Chen LY, Chang PC (2007) Cloning of the gene and characterization of the enzymatic properties of the monomeric alkaline phosphatase (PhoX) from *Pasteurella multocida* strain X-73. FEMS Microbiol Lett 267(1):113–120. doi:10.1111/j.1574-6968.2006.00542.x

Yong SC, Roversi P, Lillington J, Rodriguez F, Krehenbrink M, Zeldin OB, Garman EF, Lea SM, Berks BC (2014) A complex iron-calcium cofactor catalyzing phosphotransfer chemistry. Science 345(6201):1170–1173. doi:10.1126/science.1254237

Zaheer R, Morton R, Proudfoot M, Yakunin A, Finan TM (2009) Genetic and biochemical properties of an alkaline phosphatase PhoX family protein found in many bacteria. Environ Microbiol 11(6):1572–1587. doi:10.1111/j.1462-2920.2009.01885.x