

Characterization of a novel metallo- β -lactamases fold hydrolase from *Pelagibacterium halotolerans*, a marine halotolerant bacterium isolated from East China Sea

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Abstract In this study, a novel metallo- β -lactamases fold hydrolase PH-1 was identified from *Pelagibacterium halotolerans* B2^T. This novel member of the family *Hyphomicrobiaceae* was isolated from the East China Sea. *In silico* analysis demonstrated that PH-1 and its relative homologues cluster in a unique branch and constitute a new subgroup among MBLs. PH-1 was cloned and overexpressed in *Escherichia coli* BL21 in a soluble form. SDS-PAGE, MALDI-TOF/TOF-MS, and size-exclusion chromatography analysis demonstrated that the PH-1 was a monomer with molecular weight of about 29 kDa. Substrate specificity study showed PH-1 preferred penicillin type β -lactams and exhibited maximum activity toward penicillin-G. Additionally, our experiments also revealed that PH-1 was a halotolerant enzyme since it is active under 4 M NaCl. The enzyme activity of PH-1 was negatively affected by 1 mM Mn²⁺ and EDTA. These observations lay a foundation for further study of MBLs from marine bacterium.

Keywords *Pelagibacterium halotolerans* · Metallo- β -lactamase · Halotolerant · Purification

Introduction

The β -lactamase family is a group of enzymes that deactivate β -lactam antibiotics by hydrolyze the β -lactam ring, rendering them inactive (Pitout and Laupland 2008). Based on the nucleotide and amino acid sequences, β -lactamases were classified into four classes. Classes A, C, and D act by a serine-based mechanism. In contrast class B, or metallo- β -lactamases (MBLs), needs zinc for their action (Walsh et al. 2005). Over the past decades, with the global dissemination of MBLs carried on mobile genetic elements among different types of clinical pathogens, these enzymes represent one of the greatest problems in the realm of antibiotic resistance (Cornaglia et al. 2011).

The origin of β -lactamases is still not well understood. It seems reasonable to suppose that the β -lactamases originally played a key role in survival for bacteria in their natural habitat (Kristiansen et al. 2015). In lieu of this, there are increasing hypotheses that antibiotic resistance genes in clinic may have an environmental ancestor (Martinez 2008; Zheng et al. 2011a). Although identification and characterization of β -lactamase from terrestrial bacterium have already been well documented, reported identification of novel β -lactamase from marine bacterium remains a novel field.

Oceans cover more than two-thirds of the earth's surface however; there have been only few studies which investigate the β -lactamases originating from ocean (Kristiansen et al. 2015; Toth et al. 2010; Zheng et al. 2011a). Two different classes of chromosome-encoded MBLs were isolated from genus *Shewanella* (Poirel et al. 2005). Toth

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et al. reported the crystal structure of a class A β -lactamase OIH-1 from *Oceanobacillus iheyensis*, which is isolated from a depth of 1050 m in the Pacific Ocean (Toth et al. 2010). Later, this protein was further characterized by molecular-dynamics, docking, and reactivity simulations, which revealed that the active site of OIH-1 is the preferential binding pocket not only for β -lactams, but also for many kinds of other biologically active compounds in various chemical classes (Pietra 2012). Additionally, several chromosomally encoded MBLs have been identified from *Erythrobacter* species, which share low identity with known acquired MBLs (Girlich et al. 2012). These observations suggested that marine bacteria may constitute a reservoir of MBLs (Zheng et al. 2011a), and the further characterization of the β -lactamases from ocean niche is necessary for the discovery of novel biological functions.

It was interesting to search and characterize the resistance determinants responsible for resistance phenotypes in marine bacteria. In a previous work, the halotolerance property of a marine esterase from *Pelagibacterium halotolerans* B2^T was investigated. This was a novel member of the family *Hyphomicrobiaceae* from East China Sea (Jiang et al. 2012). In this study, an in silico analysis of whole genome of *P. halotolerans* B2^T identified a novel MBL fold protein PH-1, sharing a weak amino acid identity with the known acquired MBLs. This prompted us to characterize the chromosomally encoded MBL's superfamily protein from *P. halotolerans* B2^T. This may expand our knowledge of the biological function of these putative MBLs from marine life.

Materials and methods

Bacterial strains and growth conditions

P. halotolerans B2^T was isolated from East China Sea (125°59'24"E, 30°58'16"N) from a depth of 70 m, and cultured in marine 2216 broth (BD, USA) at 30 °C (Xu et al. 2011). *Escherichia coli* DH5 α and *E. coli* BL21 (DE3) (Novagen) were used for plasmid manipulation, and protein expression occurred in LB medium at 37 °C.

Bioinformatics analysis

The genomic sequence of *P. halotolerans* B2^T was obtained from NCBI GenBank under accession numbers of CP003075 (chromosome) and CP003076 (plasmid). A novel antibiotic resistance gene (*ph-1*) was found by annotation of the genome. The amino acids sequence of PH-1 was blasted to the NCBI database using a blast program. The phylogenetic tree was constructed by the Neighbor-Joining Method using Molecular Evolutionary Genetics

Analysis (MEGA) 6.0 software (Tamura et al. 2013). Amino acid sequence alignments were conducted using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Multiple sequence alignment was performed using the ClustalW algorithm of the AlignX program of the Vector NTI software (Invitrogen) according to the manufacturer's recommendations.

DNA cloning, protein expression and purification

The full-length *ph-1* gene was amplified from the genomic DNA using the primers: 5'-GGGAATTCATATGCATCATCATCATCATATGACGCAGCGCGA-3' and 5'-CCCAAGCTTCAATACCAGTCGCGCTCGACG-3'. Genomic DNA was isolated using QIAamp DNA Mini Kit according to the manufactory's instructions. The amplicon was then inserted into a pET28a vector (Invitrogen) between the NdeI and HindIII sites. *Escherichia coli* BL21 (DE3) cells (Invitrogen) were transformed with the ligated plasmid and plated on Luria Broth (LB) agar that was supplemented with kanamycin (50 μ g/ml). The identity of the DNA constructs was verified by DNA sequencing. Recombinant plasmid pET28a:PH-1 was conducted to antimicrobial susceptibility assay, and mature protein was used in analytical size-exclusion chromatography.

E. coli BL21 (DE3) cells, transformed with pET28a:PH-1, were grown in LB medium containing 100 μ g/ml ampicillin at 37 °C until an optical density of 600 nm. This was done until the range of 0.4–0.6 was reached. Expression of protein was induced by the addition of 0.2 mM IPTG to the culture, and bacteria were grown at 37 °C for 6 h. The induced cells were harvested by centrifugation and suspended in a lysis buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1 % Triton X-100, and 1 mM DTT. The cells were lysed by sonication with the resulting supernatants concentrated by ultrafiltration, and then dialyzed against a 20 mM Tris-HCl (pH 8.0). The lysate was loaded onto a Ni-NTA column and equilibrated with lysis buffer. The protein was eluted with 2 column volumes of 200 mM imidazole in lysis buffer. Subsequently, the protein was loaded onto a Superdex 200 fast protein liquid chromatography column (GE Healthcare) for further purification and characterization.

Mass spectrometry and protein identification

The overexpressed protein was subjected to Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF/MS) analyses using ABI 4800 TOF-TOF Proteomics Analyzer (Applied Biosystems). To identify the protein, a peptide mass was searched against the reference peptides in the NCBI nr database by using MASCOT software. The purified proteins were also

verified by 12 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Zheng et al. 2011b).

Analytical size-exclusion chromatography

The oligomeric status of purified PH-1 protein was determined by analytical size-exclusion chromatography (SEC) as described previously (Zheng et al. 2011a). Protein was loaded onto the column and eluted isocratically with 20 mM Tris–HCl, 50 mM NaCl, pH 8.0, and the flow rate was 1.0 mL/min. The molecular mass standards were calibrated with ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and ribonuclease (13.7 kDa), on an ÄktaPurifier system (GE healthcare).

Antimicrobial susceptibility testing

MICs for *P. halotolerans* B2^T and the recombinant *E. coli* BL21 harboring PH-1 for the following antibiotics were determined by the broth microdilution method. Penicillin, piperacillin, ampicillin, cephalosporins, imipenem, aztreonam, cefalotin, cefepime and ceftazidime, were included for study. The results were interpreted as by the standards of the Clinical and Laboratory Standards Institute (CLSI). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls.

Enzyme assay

The standard assay was performed as follows: 980 μ l sodium phosphate buffer (pH 7.0), 100 μ M penicillin-G, and the enzyme in a final volume of 1 ml. The activity of the enzyme was determined at 30 °C by measuring it under 232 nm using a Coulter DU 800 nucleic acid/protein analyzer (Beckman). All values were determined in triplicate and the blank was performed using the deactivated enzyme. The K_m and V_{max} values were determined by analyzing the slopes of the Michaelis–Menten equation using GraphPad Software (GraphPad Inc., USA) as described previously (Lamoureux et al. 2013).

Optimum conditions for enzyme activity

For the determination of substrate specificity, various antibiotic agents were added into the standard reaction mixture with final concentration of 100 μ M. The agents and wavelengths that were used were: penicillin-G (232 nm), amoxicillin (240 nm), piperacillin (235 nm), cefepime (264 nm), cefotaxime (265 nm), ceftazidime (265 nm), ceftazidime (260 nm), cefuroxime (262 nm), imipenem (297 nm),

meropenem (298 nm), aztreonam (318 nm), azithromycin (255 nm), gentamicin (245 nm), tigecycline (630 nm). The kinetic parameters (K_m and V_{max}) for PH-1 were obtained using penicillin-G as substrate at different concentrations ranging from 0 to 1 mM. The reaction was conducted using the standard method. The optimum temperature of PH-1 was examined from 10 to 60 °C. The effect of pH on the activity of PH-1 was measured over a pH range of 3.0–10.0. The buffers that were used follow: 100 mM citrate buffer (pH 3.0–6.0), 100 mM potassium phosphate buffer (pH 6.0–7.5), 100 mM Tris–HCl buffer (pH 7.5–9.0), and 100 mM sodium carbonate buffer (pH 9.0–10.0). The effect of NaCl on PH-1 activity was determined under 0–4 M NaCl.

Effect of metallic ions and EDTA on activity

The effects of metal ions (Ca^{2+} , Mg^{2+} , Zn^{2+} , Co^{2+} , Sr^{2+} , Cu^{2+} , Mn^{2+} and Ni^{2+}) were examined at final concentration of 1 mM and 100 μ M. The effect of chelating agent EDTA was examined at a final concentration of 10 mM. All the tests were performed using the standard assay.

Results

Amino acid sequence analysis

A putative MBL fold hydrolase encoding gene (WP_014132294) was identified from the genome annotation of *Pelagibacterium halotolerans* B2^T. The 693 bp *ph-1* gene encoding a protein of 230 amino acids was located in the chromosome. When analyzed with NCBI BLAST tool, the amino acid sequence showed that the protein is of MBL superfamily (Fig. 1a). Blastp showed maximum identity (46 %) to a β -lactamase from *Chelativorans* sp.BNC1. We then assessed the multiple amino acid sequence alignment of PH-1 and homologues. Sequence alignment revealed metal binding sites that were characteristic of MBLs. These included the consensus zinc binding motif HXHXDH (residues 57–63) and the critical residues for MBL activity, such as Asp150 and His197 (Fig. 1b).

To investigate the evolution of PH-1 in more detail, we have conducted phylogenetic analysis using the amino acids sequences of PH-1, PH-1 relative proteins, and three subgroups (B1, B2 and B3) of MBLs (Palzkill 2013). The result showed PH-1 and its relative proteins clustering in a unique branch that constitute a new subgroup among MBLs. This new subgroup is distinguished from the other subgroups in the tree (Fig. 2). Thus, we proposed that PH-1 and its relative proteins represent a new subgroup of MBLs based on sequence alignment and phylogenetic analysis.

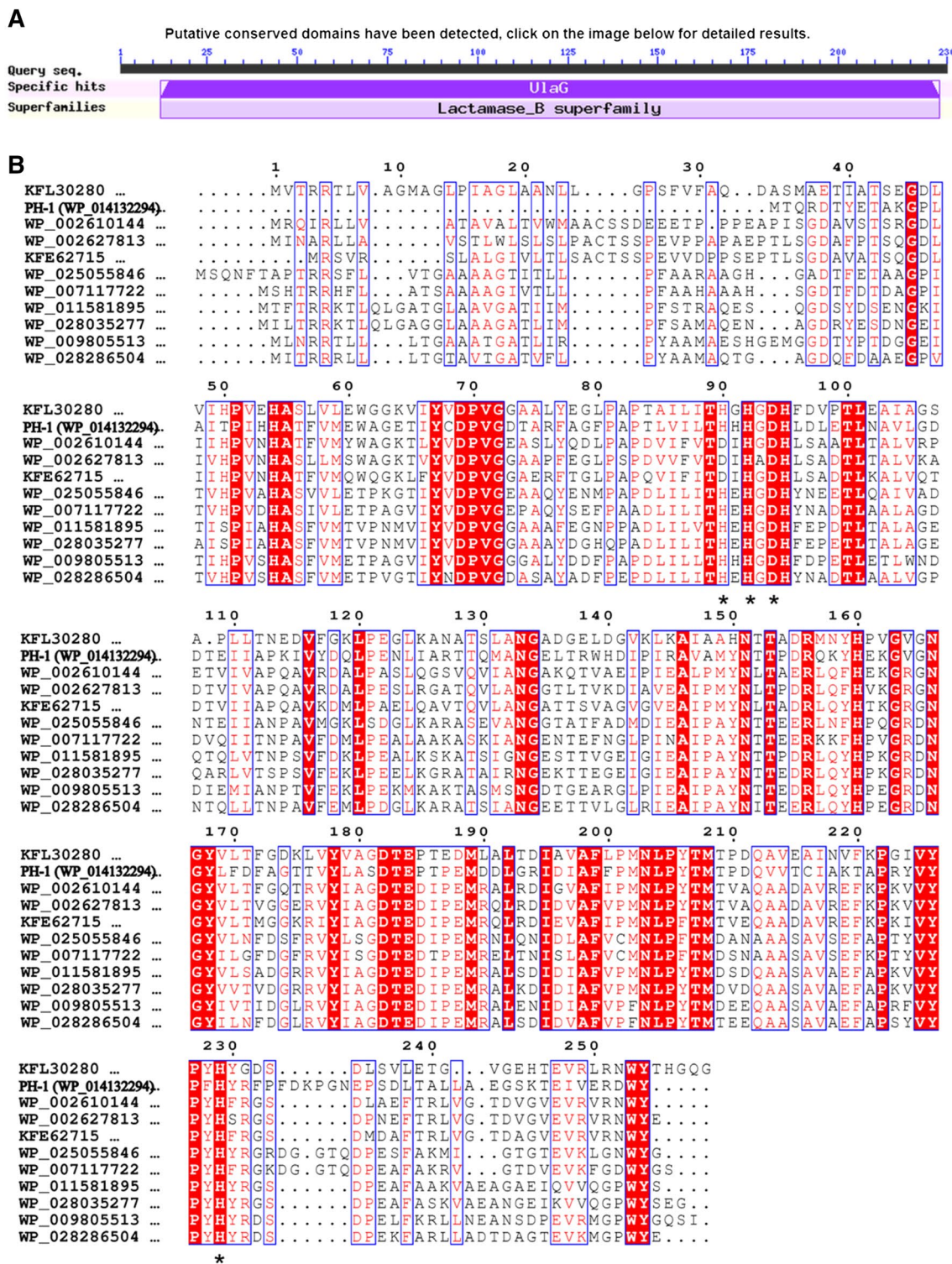
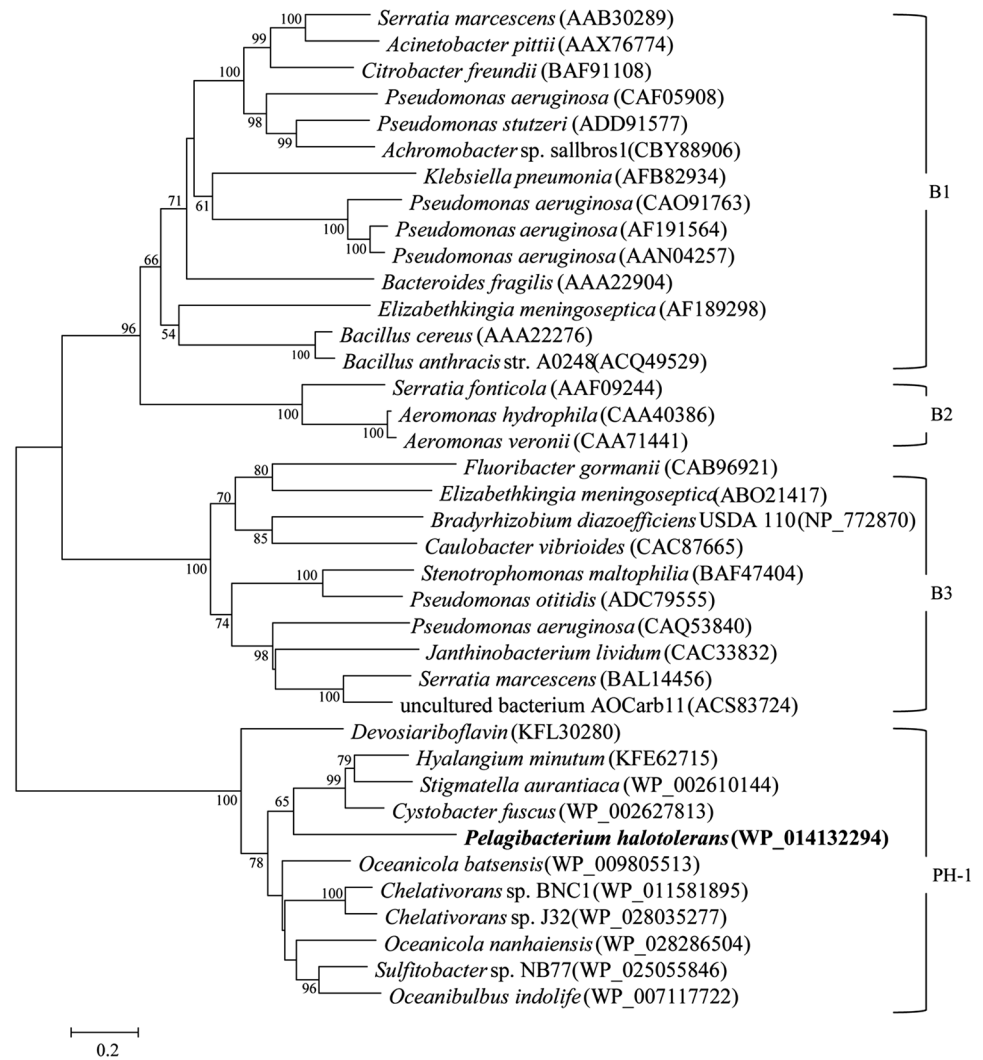


Fig. 1 Amino acid analysis of PH-1 from *Pelagibacterium halotolerans*. **a** Protein–protein BLAST of PH-1 against NCBI nr database. PH-1 belongs to the MBL superfamily. **b** Multiple amino acid sequence alignment of PH-1 (WP_014132294) and its homologues.

The identical residues are in white letters with red background, similar residues are in red letters with white background, the varied residues are in black letters, and gaps are denoted with dots. Conserved residues associated with metal binding were highlighted with asterisk

Fig. 2 Phylogenetic analysis of PH-1 (*bold type*) and other MBLs. The neighbor-joining tree was constructed by the use of MEGA6.0 software, *scale bar*, 0.2 substitutions per amino acid position



Cloning, expression and purification of PH-1

The 693 bp amplicon encoding PH-1 was ligated into NdeI/BamHI-digested pET28a plasmid with 6-residues His-tag at the end of the cloned sequence. The construct plasmid was then transformed into *E. coli* BL21 cells. After 6 h of induction under the condition of 0.1 mmol/L IPTG, high-level expression of the recombinant PH-1 was obtained. Then the crude enzyme was purified using Ni-NTA column. Subsequently an analytical fast protein liquid chromatography (FPLC) profile of Superdex 200 chromatography column demonstrated that PH-1 solution elute as a monomer (Fig. 3a). MALDI-TOF/MS confirmed the identification of PH-1 (Fig. S1). The purified proteins collected from elution fractions were separated by 12 % SDS-PAGE and the results show that PH-1 has a molecular mass of 29 kDa, which is consistent with the result of SEC (Fig. 3).

The molecular weight of PH-1 was calculated to be 26 kDa (http://www.expasy.org/tools/pi_tool.html). The increased molecular weight of the purified PH-1 was observed in SDS-PAGE and gel filtration experiments due to its fusion with the His tag.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing showed that *E. coli* BL21 strain transformed with the pET28a:PH-1 and was resistant to penicillin, ampicillin, and piperacillin. The strain was susceptible to aztreonam, ceftazidime, cefalotin, cefotaxime, ertapenem, cefepime, cefoxitin and imipenem (Table 1). Comparison of β -lactam MICs for *P. halotolerans* B2^T and *E. coli* BL21 harboring recombinant plasmid revealed no difference in MIC to the β -lactams. The high-level resistance to penicillin type antibiotics was observed in both strains.

Fig. 3 Purification and identification of protein PH-1. **a** Size-exclusion chromatography of PH-1 protein. **b** SDS-PAGE gel picture from the FPLC purification of PH-1. *M* molecular weight marker, *lane 1* soluble protein, *lanes 2–9* protein collected from the elution peak. SDS-PAGE gel concentration was 12 % and was stained by Coomassie R250

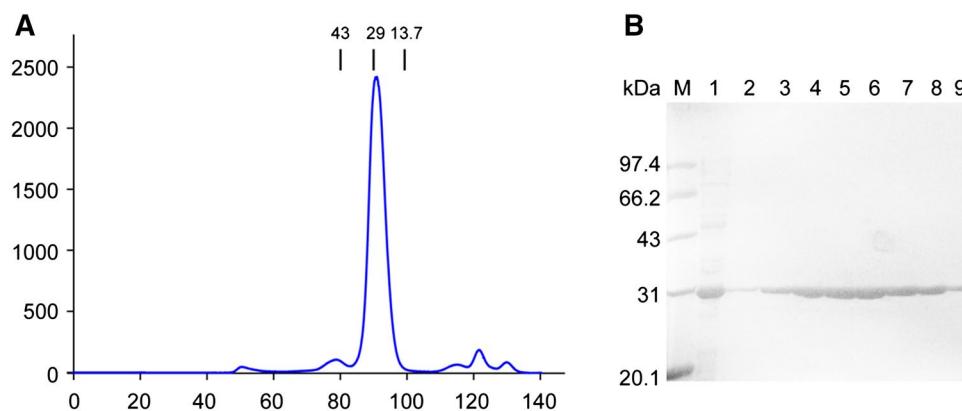


Table 1 MICs of various β -lactams for *Pelagibacterium halotolerans* B2^T and *E. coli* BL21 transformed with the pET28a:PH-1 plasmid

	MIC (mg/L) of β -lactams										
	AMP	AZT	CAZ	CEF	CTX	ETP	FEP	FOX	IPM	PEN	PIP
<i>P. halotolerans</i> B2 ^T	64	0.25	0.25	1	0.031	0.031	0.25	1	0.5	64	16
<i>E. coli</i> BL21 (PH-1)	64	0.16	1	1	0.031	0.031	0.25	1	0.25	128	32

AMP ampicillin, AZT aztreonam, CAZ ceftazidime, CEF cefalotin, CTX cefotaxime, ETP ertapenem, FEP cefepime, FOX ceftioxitin, IPM imipenem, PEN penicillin, PIP piperacillin

Biochemical characterization of PH-1

The enzymatic activity of PH-1 towards various antibiotic agents was determined by monitoring the hydrolysis of the antibiotic agents. The result showed PH-1 can hydrolyze penicillin-G, amoxicillin and piperacillin; but had no activity towards other antibiotic agents used in this study. The maximum enzymatic activity was obtained by using penicillin-G as the substrate. The K_m and V_{max} values of PH-1 for penicillin-G were $114.9 \pm 12.24 \mu\text{M}$ and $0.4736 \pm 0.01493 \mu\text{M/s}$, respectively.

The optimum activity of PH-1 was measured over a temperature range of 10–60 °C and a pH range of 3.0–10.0 with penicillin-G as the substrate. PH-1 showed highest activity at 30 °C and pH7.0 (Fig. 4). PH-1 can maintain its activity under high concentration of NaCl and most of the metal ions except high concentration of Mn^{2+} (1 mM) (Fig. 4d). Additionally, PH-1 completely loses its activity under the EDTA as other MBLs.

Discussion

The β -lactamase superfamily constitutes a diverse protein family. Members are characterized by the same folding pattern and conserved domains; however, they are involved in different biological functions (Alfredson and Korolik 2007; Daiyasu et al. 2001). *P. halotolerans* B2^T is a Gram-negative aerobic bacterium isolated from coastal seawater of the East China Sea (Xu et al. 2011). In this study, in silico

analysis of *P. halotolerans* genome revealed a novel protein of MBL superfamily; (PH-1) and then cloning, expression, purification and characterization of PH-1 were carried out.

Orthologues of PH-1 can be found in several other species; *Chelativorans*, *Cystobacter*, *Oceanicola*, *Sulfitobacter*, *Stigmatella* and *Hyalangium* were shown by BLAST searches against the nucleotide databases. Upon phylogenetic tree analysis of PH-1 and orthologues, it was found that MBL fold hydrolase from *Hyalangium minutum* (KFE62715), *Stigmatella aurantiaca* (WP_002610144), and *Cystobacter fuscus* (WP_002627813) showed high similarity to PH-1. Notably, the MBL sequence of PH-1 identified in this work significantly differed from those of acquired MBLs, such as VIM type, IMP type, and NDM type carbapenemases (Arakawa et al. 1995; Nordmann and Poirel 2002; Yong et al. 2009). PH-1 and its ten relatives classified into a unique branch in the tree. They cannot be confidently divided into three known subclasses (B1–B3) of MBL superfamily (Rasmussen and Bush 1997), but they do contain the conserved sequence motif H–X–H–X–D–H, which were well conserved in subclass B3 MBLs. To date, the functional study of PH-1 orthologues is not available.

SEC revealed that the recombinant PH-1 protein is a monomer, which is consistent with the NDM type MBLs (Zheng et al. 2011a). When expressed in *E. coli* BL21, PH-1 conferred resistance or reduced susceptibility to piperacillin, penicillin and amoxicillin. Surprisingly, MICs of carbapenems were quite low. Enzyme assays and antimicrobial susceptibility testing also confirmed that PH-1 conferred resistance or reduced susceptibility to piperacillin,

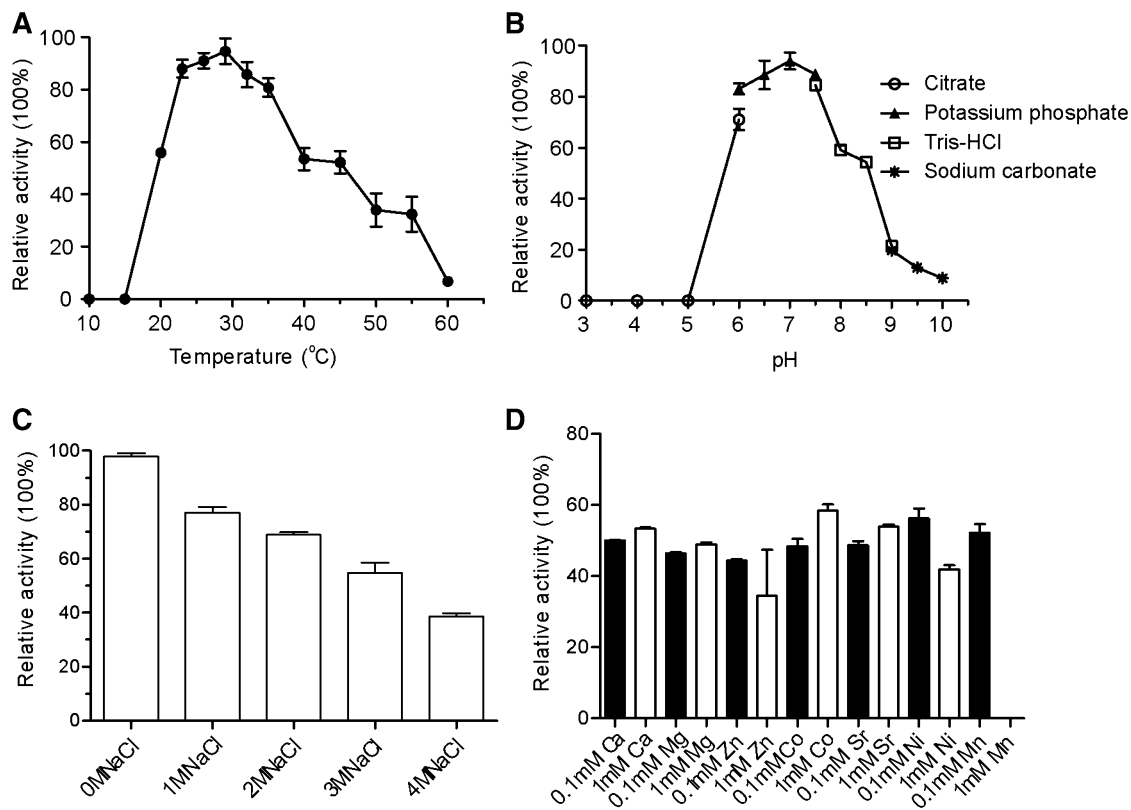


Fig. 4 Effects of temperature (a), pH (b), NaCl concentration (c), and metal ions (d) on the activity of PH-1. Enzymatic assays were performed at 30 °C, pH7.0 with penicillin-G as the substrate. The value obtained without additives in the reaction mixture was taken as 100 %

penicillin and amoxicillin; however, imipenem hydrolysis was non-detectable. According to the subgrouping of MBLs on the basis of functional characteristics, all the subgroups (3a, 3b and 3c) have the ability to hydrolyze imipenem at a readily measurable rate (Rasmussen and Bush 1997). Thus the PH-1 was also distinguished from the three subgroups of MBLs with functional analysis. In addition, metal ions are crucial to PH-1 function, as the enzyme is inactive when EDTA was added to the reaction mixture and the hydrolysis reaction stopped. This has been shown also for other MBLs (Queenan and Bush 2007). These observations suggested that some chromosome-encoded MBLs process a large diversity of carbapenemase activities while others do not (Girlich et al. 2012).

P. halotolerans B2^T can survive under high concentration of NaCl (0–2.22 M), that is to say, *P. halotolerans* B2^T has evolved to retain activity in the presence of high salt (Xu et al. 2011). Our kinetics studies of PH-1 suggest that PH-1 is active in conditions of increased salinity (Fig. 4c). It showed activity from 0 to 4 M NaCl, although it was most active in the absence of salt. This observation suggests that PH-1 is a halotolerant enzyme. In a previous study, the esterase PE10 was described as still active under 4 M NaCl, which was also cloned

from *P. halotolerans* B2^T (Jiang et al. 2012). This can be explained by the claim that the high concentrations of salt do not affect the chemical step of catalysis (Toth et al. 2010). Finally, PH-1 has a lower temperature optimum (30 °C) than acquired MBLs, which is consistent with those of MBLs from marine life (Kristiansen et al. 2015; Toth et al. 2010). These findings are expected since the marine enzyme originates from an environment with the characteristics of low temperatures and high salt concentrations.

In conclusion, our study first demonstrates overexpression and characterization of a novel MBLs protein from marine bacterium. Our data revealed that PH-1 displays a weak hydrolysis activity to β -lactams, except penicillin type β -lactams. It has a temperature optimum of 30 °C, lower than other known MBLs. It exhibits stability at high concentration of salt. PH-1 is a novel MBLs protein and might possess other favorable properties that need to be further explored.

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