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The environment shapes microbial enzymes: five cold-active and salt-resistant carboxylesterases from marine metagenomes

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Abstract Most of the Earth's biosphere is cold and is populated by cold-adapted microorganisms. To explore the natural enzyme diversity of these environments and identify new carboxylesterases, we have screened three marine metagenome gene libraries for esterase activity. The screens identified 23 unique active clones, from which five highly active esterases were selected for biochemical characterization. The purified metagenomic esterases exhibited high activity against α-naphthyl and *p*-nitrophenyl esters with different chain lengths. All five esterases retained high activity at 5 °C indicating that they are cold-adapted enzymes. The activity of MGS0010 increased more than two times in the presence of up to 3.5 M NaCl or KCl, whereas the other four metagenomic esterases were inhibited to various degrees by these salts. The purified enzymes showed different sensitivities to inhibition by solvents and detergents, and the activities of MGS0010, MGS0105 and MGS0109 were stimulated three to five times by the addition of glycerol. Screening of purified esterases against 89 monoester substrates revealed broad substrate profiles with a preference for different esters. The metagenomic esterases also hydrolyzed several polyester substrates including polylactic acid suggesting that they can be used for polyester depolymerization. Thus, esterases from marine metagenomes are cold-adapted enzymes exhibiting broad biochemical diversity reflecting the environmental conditions where they evolved.

Keywords Marine metagenome · Metagenomic library · Esterase · Cold adaptation · Salt resistance · Polyester hydrolysis

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Introduction

On Earth, microbial life can be found in all environments including various extreme conditions with high or low temperature, pH and salt concentrations. It is estimated that 90 % of the biosphere exists at temperatures below 10 °C and that the largest proportion of biomass is produced at low temperatures (Feller and Gerday 2003; Siddiqui and Cavicchioli 2006; Wilkins et al. 2013). The vast majority of environmental microorganisms have never been grown and characterized in the laboratory (Rappe and Giovannoni 2003). They represent an enormous reserve of biodiversity for fundamental knowledge and biotechnology. Although the uncultured microorganisms cannot be studied using traditional microbiological and biochemical methods, their DNA can be extracted directly from environmental samples (metagenomic DNA) and cloned for sequencing or expression in *Escherichia coli* or other

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surrogate hosts (Handelsman et al. 1998). Metagenomics has emerged as a strategic approach to study microbial communities, without culturing individual organisms, through the analysis of their DNA using both bioinformatic and experimental methods such as sequence analysis, DNA hybridization, gene expression, proteomics, metabolomics, and enzyme activity assays (Handelsman 2004; Lorenz et al. 2002; Uchiyama and Miyazaki 2009; Vieites et al. 2009). The metagenomic studies of soil and marine communities have already revealed many new species and proteins including bacteriorhodopsin and numerous unknown protein families (Beja et al. 2000; Gilbert and Dupont 2011; Tyson et al. 2004; Venter et al. 2004; Yooseph et al. 2007).

The vast sequence diversity in environmental metagenomes suggests a similar magnitude of metabolic and biochemical diversity (Dinsdale et al. 2008; Yooseph et al. 2007), but the latter is impossible to comprehensively describe based only on sequence analysis due to the presence of the large number of unknown or poorly characterized genes. This necessitates the development of experimental approaches for metagenome research including new cultivation technologies, meta-transcriptomics, meta-proteomics and activity-based screening methods (Ferrer et al. 2007; Giovannoni and Stingl 2007; Ram et al. 2005; Simon and Daniel 2011; Uchiyama and Miyazaki 2009). Agar plate-based screening of metagenomic gene libraries for enzymatic activity represents a simple and direct approach to identify metagenomic enzymes. This approach has been applied to screen different metagenomic gene libraries for esterase, lipase, cellulase, protease, laccase and other activities (Ferrer et al. 2009; Lorenz and Eck 2005; Steele et al. 2009). Screening of metagenomic gene libraries and purified proteins has greatly expanded the scope of enzyme applications and the number of enzymes useful for biocatalysis. These studies have identified more than 130 new nitrilases and many other enzymes including cellulases, lipases, carboxyl esterases and laccases (Beloqui et al. 2006; Chow et al. 2012; Ferrer et al. 2005a; Hess et al. 2011; Kourist et al. 2007; Robertson et al. 2004; Schmeisser et al. 2007).

Lipolytic enzymes are classified into eight families (Arpigny and Jaeger 1999; Hausmann and Jaeger 2010) with a large group of carboxyl esterases (EC 3.1.1.1) which hydrolyze various carboxylic esters and produce the corresponding alcohols and organic acids as products (Arpigny and Jaeger 1999; Bornscheuer 2002; Satoh and Hosokawa 2006). Most known esterases are α/β-hydrolases with the Ser-His-Asp catalytic triad, and these enzymes are widely distributed in microorganisms, plants and animals. Recently, the erythromycin esterase family of α/β-hydrolases has been identified and is proposed to use a His-Glu pair for the catalysis (Morar et al. 2012). Moreover, several active esterases have also been identified in the β-lactamase superfamily, which have a different structural fold and use the Ser residue of the β-lactamase sequence motif Ser-x-x-Lys as a catalytic nucleophile (Wagner et al. 2002). Many esterases have a broad substrate range suggesting that they have evolved to function in carbon utilization and catabolic pathways. Together with lipases (EC 3.1.1.3), esterases are the most frequently used hydrolases in biocatalysis (Bornscheuer and Kazlauskas 2006). In addition, these enzymes also show high regiospecificity and stereospecificity, which makes them useful biocatalysts in organic synthesis, especially for the production of enantiopure secondary alcohols and for the resolution of primary alcohols and carboxylic acids (Baumann et al. 2000; Bornscheuer and Kazlauskas 2006; Bornscheuer et al. 2005). Many esterases are also stable and active in organic solvents, where they catalyze synthesis reactions including synthesis of enantiopure building blocks, flavours and fragrances (Bornscheuer and Kazlauskas 2006).

Here, we present the results of enzymatic screening of the three marine metagenomic libraries for esterase activity and the biochemical characterization of five selected carboxyl esterases. The biochemical properties of these enzymes were compared with the well characterized esterase from pig liver (PLE), which is an important industrial enzyme due to its high versatility (Hummel et al. 2007; Toone et al. 1990; Zhu and Tedford 1990). Our results indicate that the metagenomic esterases identified are active against a broad range of substrates including polymeric esters and exhibit high catalytic activities and resistance to low temperatures, salts, solvents and detergents.

Materials and methods

Metagenome library screening and sequencing Lambda-ZAP metagenomic libraries were prepared as previously described (Alcaide et al. 2013; Ferrer et al. 2005b). *E. coli* XL1-Blue MRF' cells were cultured to mid-log phase in Luria Broth (LB) with 10 mM $MgSO_4$ and 0.2 % maltose. Cells were infected with an appropriate number of lambda-ZAP phage (approximately 1,000 plaque-forming units per 10-cm diameter screening plate) from the corresponding libraries, and 300 μl of infected cells were aliquoted per 4 ml of soft LB agar (0.7 %) containing 10 mM $MgSO_4$, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), 0.5 % gum arabic and 1 % of emulsified tributyrin, at 48 °C. Upon mixing, the soft agar and cells were immediately layered onto LB agar plates containing 1 mM IPTG, allowed to cool and solidify, and the plates were then incubated overnight at 37 °C. Phage plaques were examined for ability to clear tributyrin over the following 3 to 4 days. Phages positive for esterase activity were isolated from plates, and the pBK-CMV phagemids containing the cloned metagenomic segments were excised from phage

DNA according to the Stratagene (now Agilent Technologies) Lambda-ZAP protocol. All metagenomic DNA fragments were sequenced by gene walking, after which open reading frames were predicted using NCBI's ORF Finder or Geneious version 6.0.6. Translated protein sequences were analyzed by BLAST and Pfam to assign possible activities. Multiple sequence alignments were generated in ClustalX 2.1 using the BLOSUM scoring matrix, and the phylogenetic tree was calculated using the neighbour-joining method. Bootstrap values represent 100 resamplings (Fig. 1).

Gene cloning, protein purification and mutagenesis Genes predicted to have hydrolase or esterase activity were amplified by PCR from excised plasmids and cloned into the pET15b-TvL expression vector containing an N-terminal 6His tag. The sequences of the cloned genes were verified by DNA sequencing. The enzymes were expressed in the *E. coli* BL21(DE3) strain and purified by nickel affinity chromatography (Gonzalez et al. 2006). To confirm the presence of esterase activity in the cloned proteins and also check them for lipase activity, approximately 5–10 μg of the purified enzymes were spotted on LB agar plates containing either emulsified 1 % tributyrin and 0.5 % gum Arabic, or emulsified 3 % olive oil and 0.001 % Rhodamine B, respectively, and incubated overnight at 30 or 37 °C. Esterase activity was indicated by the formation of a clear halo, whereas lipase activity was identified under UV light as orange fluorescence (due to the interaction of Rhodamine B with hydrolyzed free fatty acids)

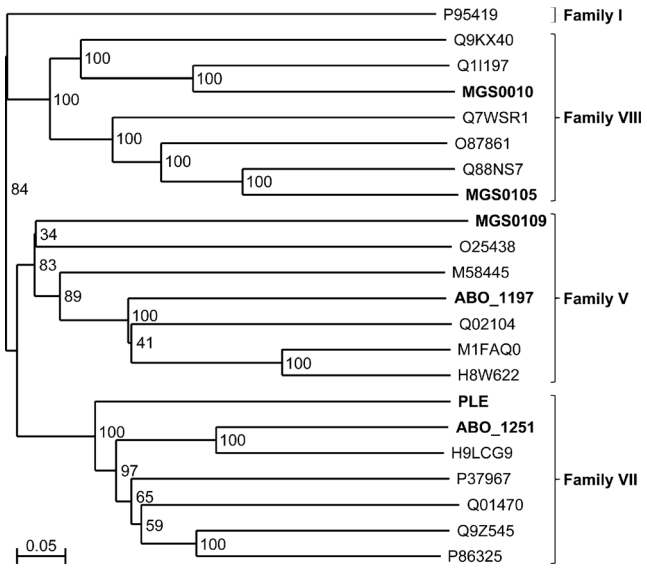

Fig. 1 Sequence diversity of five metagenomic esterases. Phylogenetic tree representing relationships between the six characterized esterases (*bolded*), related homologues and reference lypolytic enzymes representing the eight esterase families described by Arpigny and Jaeger (1999). The reference enzymes for the esterase families V, VII and VIII were Q02104, P37967 and O87861, respectively. Bootstrap values are indicated at the nodes

(Kouker and Jaeger 1987). Site-directed mutagenesis of MGS0105 was performed based on the QuikChange® site-directed mutagenesis kit (Agilent) using the MGS0105 plasmid DNA as a template for mutagenesis. All mutations were verified by DNA sequencing, and the mutant proteins were overexpressed and purified as the wild-type MGS0105 protein. PLE was purchased from Sigma (esterase from porcine liver, catalogue no. E3019).

Enzymatic assays Carboxyl esterase activity of purified proteins against model esterase substrates was measured spectrophotometrically using *p*-nitrophenyl or α-naphthyl esters of various fatty acids in a reaction mixture (200 μl) containing 50 mM buffer (Tris-HCl, pH 8.0 or CHES, 9.0–10.0), 0.25–2.0 mM substrate and 0.01–0.1 μg of enzyme. The reaction mixtures were incubated for 10 min at the indicated temperature (25 to 37 °C), and the activity was monitored at 310 nm (for α-naphthyl substrates) or 410 nm (for *p*-nitrophenyl substrates) (Lemak et al. 2012). The effect of NaCl and other compounds (KCl, glycerol, solvents and detergents) on esterase activity of purified proteins was measured by including the indicated concentrations of these compounds in the reaction mixtures. Hydrolytic activity against the 89 ester library substrates (Table S4) was determined spectrophotometrically in 96-well microplates in a reaction mixture (150 μl) containing 2 mM BES buffer (pH 7.2), 2 mM ester substrate, 0.45 mM *p*-nitrophenol and 0.1–0.5 μg of enzyme. The plates were incubated for 10 min at 25 °C, and the absorbance of *p*-nitrophenol was measured at 404 nm (ε=17.3 mM^{-1} cm^{-1}) (Janes et al. 1998). All enzyme assays were performed in triplicate. For determination of the K_m and k_{cat} values, esterase activity was determined over a range of substrate concentrations (0.01–5.0 mM). Kinetic parameters were calculated by non-linear regression analysis of raw data fit to the Michaelis-Menten function using GraphPad Prism software (version 4.00 for Windows).

Polylactic acid (PLA) depolymerization activity of purified proteins was measured using 1.5 % agarose plates containing analytical grade poly (DL-lactide) (average M.w. 2,000) purchased from PolySciTech (Akina Inc., West Lafayette, IN, USA). The polymer emulsion was prepared using 50 mM Tris-HCl buffer (pH 8.0) as aqueous phase essentially as described previously (Teeraphatpornchai et al. 2003). Briefly, 2 g of poly (DL-lactide) was dissolved in 40 ml of dichloromethane, mixed with 1 l of 50 mM Tris-HCl (pH 8.0) containing 0.1 g of Triton X-100 as surfactant and subjected to sonication (10 min). The stable emulsion was heated at 80 °C for 2 h to remove the solvent, mixed with 15 g of agarose and poured into Petri plates. After cooling to room temperature, the wells were punched manually in the solidified agarose and loaded with different enzymes. The sealed plates were incubated at 30 °C for 1–3 days, and the presence of the PLA

degrading activity was indicated by the formation of a clear halo around the wells.

Protein thermodenaturation The thermostability of purified proteins was measured as temperature-dependent aggregation using static light scattering on a StarGazer instrument as described previously (Vedadi et al. 2006). The protein samples (50 μl; 0.4 mg/ml) were incubated in a clear-bottom 384-well plate (Nunc) within the temperature range from 27 to 80 °C changing at a rate of 1 °C per min. Protein precipitation was monitored by measuring the intensity of the scattered light every 30 s with a CCD camera. The pixel intensities in each well were integrated, plotted against temperature and fitted to the Boltzman equation by non-linear regression. The resulting point of inflection of each curve was defined as the temperature of aggregation T_{agg} (Vedadi et al. 2006).

Nucleotide sequence accession numbers The sequences of MGS0010, MGS0105 and MGS0109 have been submitted to GenBank with the accession numbers KF801579, KC986400 and KC986401, respectively.

Results

Screening of marine metagenomic libraries for esterase activity

The three marine metagenomic libraries used in this work were prepared from crude oil enrichment cultures established using seawater samples collected from oil-contaminated areas in the Barents Sea (port of Murmansk, 68.98 N 33.066 E, and Kolguev Island, 68.45 N 49.2 E) and the phenanthrene + pyrene amended seawater samples from the Mediterranean Sea (near Messina, 38.198 N; 15.569 E) (Table 1). Before DNA extraction, the samples were incubated for 1 month under the same temperature conditions (3–5 °C for the Barents Sea samples and 15 °C for the Messina sample) (Table 1). The environmental DNA fragments (4 to 7 kb) were cloned into a lambda-ZAP vector, and they were packaged and maintained in lambda bacteriophage (100,000 phage clones for each Kolguev and Murmansk libraries, and 60,000 clones for the Messina library). For screening, these phage libraries were amplified to 10^7–10^9 pfu/ml (plaque-forming units) (Alcaide et al. 2013; Ferrer et al. 2005b).

Using tributyrin agar plates, we have screened 295,100 lambda-ZAP clones from these three libraries and identified 95 esterase-positive clones (hit rate 1:3,106), many of which were overlapping resulting in 23 unique clones encoding distinct genomic fragments (Table 1). Furthermore, five clones from the Kolguev library were identical to or shared high sequence identity (>97 %) with five Murmansk clones which is consistent with their close geographic origin (the west and east sides of the Barents Sea) and a similar enrichment procedure (Table S1). Sequence analysis of the 18 esterase-positive clones (containing a total of 103 genes) revealed the presence of genes homologous to proteins known to be associated with carboxyl esterase activity including α/β hydrolases (13 clones; PF07859 and PF12697), β-lactamase-like genes (2 clones; PF00144), lipase-like sequences (1 clone; PF12262) and two proteins of unknown function (DUF3089/PF11288 and DUF523/PF04463) (Tables S2 and S3). Thus, screening of metagenome gene libraries for esterase activity revealed a broad diversity of sequences of new esterases.

For detailed biochemical characterization, we selected five metagenomic esterases which could be produced as soluble proteins when expressed in *E. coli* (Table 1). The sequences of two proteins from the Murmansk library were found to be identical to the putative carboxyl esterases ABO_1197 and ABO_1251 from the ubiquitous moderately halophilic (3–10 % NaCl) marine γ-proteobacterium *Alcanivorax borkumensis*, which is the major member of microbial communities in oil-polluted ocean waters (Schneiker et al. 2006; Yakimov et al. 1998). The other three esterases shared 30 to

Table 1 Marine metagenomic libraries and esterase screening results

Metagenome location[a]	Water temperature, °C	Enrichment conditions[a]	Clones screened[b]	Positive clones[c]	Selected esterase
Messina harbor (Italy), Mediterranean	15 °C	Phenanthrene + pyrene (500 mg/L), 15 °C	24,000	18 (8)	MGS0010
Kolguev Island coastal water, Barents Sea (Russia)	3 °C	Crude oil, 4 °C	142,000	34 (5)	MGS0105 MGS0109
Port of Murmansk, Barents Sea (Russia)	5 °C	Crude oil, 4 °C	108,000	43 (10)	ABO_1197, ABO_1251

[a] All sampling sites and treatment conditions have the water salinity 3.1–3.8 %

[b] All clones are lambda-ZAP based clones

[c] Number of unique clones is shown in brackets

47 % sequence identity to predicted hydrolases from different sequenced microbial genomes and appear to belong to uncharacterized metagenomic bacteria (Fig. S1). Based on sequence and phylogenetic analyses, MGS0010 and MGS0105 belong to the esterase family VIII, which includes β-lactamase-like enzymes (Fig. 1). ABO_1251 is related to family VII esterases, whereas MGS0109 and ABO_1197 appear to be associated with the esterase family V (Fig. 1). The five metagenomic esterases showed low sequence similarity to each other (20 to 30 % sequence identity), but their sequences revealed the presence of the recognizable Ser-His-Asp catalytic triads in ABO_1197, ABO_1251 and MGS0109 (e.g. Ser143, His295 and Asp267 in ABO_1197) or the β-lactamase-like active site residues in MGS0010 (Ser76, Lys79, Tyr215 and His366) and MGS0105 (Ser68, Lys71, Tyr157 and His340) (Fig. S1).

Activity of purified metagenomic proteins against the model esterase substrates

The esterase activities of purified metagenomic enzymes were characterized using a set of 11 model esterase substrates, which include both α-naphthyl and *p*-nitrophenyl (*p*NP) esters with different acyl chain lengths (Fig. 2). At 30 °C, the purified metagenomic esterases were active within a broad range of pH with the highest activity at pH 8.0–10.0 (data not shown). The activity of metagenomic proteins was compared with the well-characterized carboxyl esterase from PLE

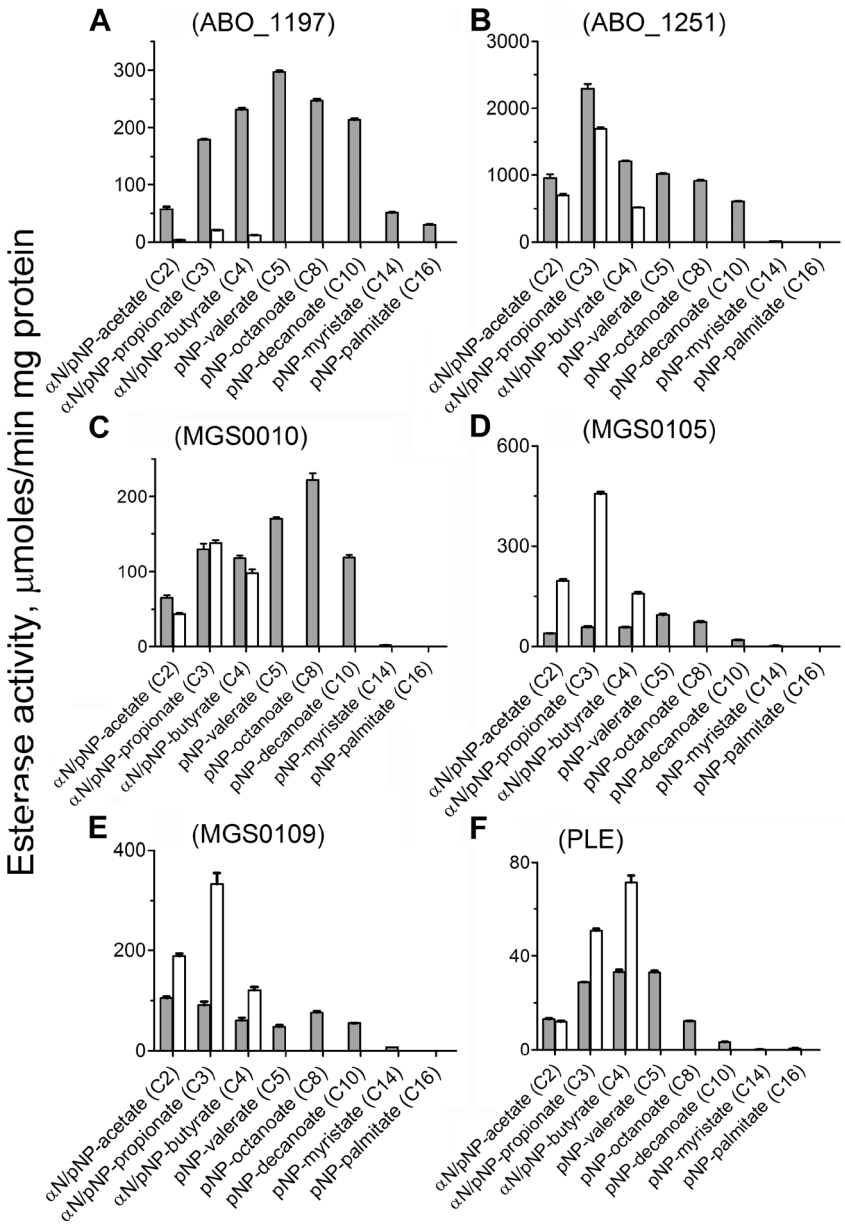

Fig. 2 Esterase activity of purified proteins against model esterase substrates. Activity profiles of the indicated proteins towards the α-naphthyl (αN; C2 to C4) and *p*-nitrophenyl (*p*NP; C2 to C16) ester substrates with different chain lengths. The *white bars* indicate activity against α-naphthyl ester substrates, whereas the *grey bars* show activity against *p*NP esters. Experimental conditions were as described in "Materials and methods". The reaction mixtures (200 μl, pH 9.0) contained the indicated substrate (1 mM) and purified proteins: 0.01 μg (MGS0105 and MGS0109), 0.03 μg (ABO_1251 and MGS0010) or 0.1 μg (ABO_1197 and PLE), and they were incubated at 30 °C (metagenomic enzymes) or 37 °C (PLE)

Table 2 Kinetic parameters of the purified metagenomic esterases and PLE

Protein	Variable substrate	K_m, mM	k_{cat}, s^{-1}	k_{cat}/K_m, M^{-1} s^{-1}
ABO_1197	αN-propionate (C3)[a]	0.78±0.07	16.7±0.5	2.1×10^4
	*p*NP-propionate (C3)	0.71±0.04	389±11	5.5×10^5
	*p*NP-valerate (C5)	0.17±0.01	330±6	1.9×10^6
	*p*NP-decanoate	0.12±0.01	301±4	2.5×10^6
	*p*NP-palmitate	0.16±0.02	19.3±1.0	1.2×10^5
	vinyl benzoate	2.57±0.22	4.2±0.2	0.2×10^4
ABO_1251	αN-acetate (C2)	0.013±0.001	669±17	5.1×10^7
	αN-propionate (C3)	0.02±0.002	1612±27	8.1×10^7
	αN-butyrate (C4)	0.005±0.0005	491±9	9.8×10^7
	*p*NP-acetate (C2)	0.16±0.017	911±51	5.6×10^6
	*p*NP-propionate (C3)	0.21±0.01	3287±94	1.6×10^7
	*p*NP-butyrate (C4)	0.06±0.003	1153±15	1.9×10^7
	*p*NP-valerate (C5)	0.04±0.002	968±15	2.4×10^7
	*p*NP-octanoate (C8)	0.06±0.003	871±17	1.5×10^7
	*p*NP-decanoate (C10)	0.18±0.003	580±5	3.2×10^6
	*p*NP-myristate (C14)	0.10±0.009	14.9±0.8	1.5×10^5
	phenyl acetate	0.16±0.01	38.1±0.7	2.4×10^5
	tripropionine	0.66±0.06	108±4	1.6×10^5
MGS0010	αN-acetate (C2)	0.021±0.001	35.4±1.0	1.7×10^6
	αN-acetate (C2) + glycerol[b]	0.16±0.01	151±5	0.9×10^6
	αN-propionate (C3)	0.012±0.001	112±3	0.9×10^7
	αN-propionate (C3) + glycerol	0.082±0.004	231±6	2.8×10^6
	αN-butyrate (C4)	0.015±0.002	79.9±4	5.3×10^6
	αN-butyrate (C4) + glycerol	0.073±0.006	140±6	1.9×10^6
	*p*NP-acetate (C2)	0.011±0.001	53.0±2.8	4.8×10^6
	*p*NP-propionate (C3)	0.004±0.0004	106±7	2.7×10^7
	*p*NP-butyrate (C4)	0.007±0.0003	95.9±2.4	1.4×10^7
	*p*NP-valerate (C5)	0.011±0.0002	138±2	1.3×10^7
	*p*NP-octanoate (C8)	0.036±0.003	181±7	5.0×10^6
	*p*NP-decanoate (C10)	0.046±0.003	96.7±2.4	2.1×10^6
	tripropionine	0.30±0.08	25.4±1.5	0.9×10^5
MGS0105	αN-acetate (C2)	0.12±0.01	196±3	1.6×10^6
	αN-propionate (C3)	0.10±0.02	369±9	3.7×10^6
	αN-propionate (C3) + glycerol	0.63±0.05	1095±43	1.7×10^6
	*p*NP-valerate (C5)	0.51±0.07	78.9±3.9	1.5×10^5
	ethyl bromoacetate	0.60±0.16	85.0±6.5	1.4×10^5
	tripropionine	0.47±0.1	31.4±2.6	6.7×10^4
MGS0109	αN-propionate (C3)	0.19±0.03	182±6.6	0.9×10^6
	*p*NP-acetate (C2)	0.44±0.11	81.4±6.5	1.9×10^5
	*p*NP-propionate (C3)	0.76±0.14	64.3±6.2	0.9×10^5
	*p*NP-octanoate (C8)	0.047±0.009	51.3±0.9	1.1×10^6
	ethyl bromoacetate	0.67±0.03	51.2±0.9	0.8×10^5
PLE	αN-propionate (C3)	0.03±0.01	42.3±1.6	1.4×10^6
	αN-butyrate (C4)	0.53±0.04	78.9±2.0	1.5×10^5
	*p*NP-propionate	1.84±0.24	57.4±4.5	3.1×10^4
	*p*NP-butyrate	0.42±0.06	56.8±2.1	1.4×10^5
	phenyl acetate	0.83±0.19	5.3±0.4	0.6×10^4

[a] αN-propionate, α-naphthyl propionate; αN-acetate, α-naphthyl acetate; αN-butyrate, α-naphthyl butyrate

[b] 35 % glycerol

(Lange et al. 2001). Based on sequence, this enzyme belongs to esterase family VII (Fig. 1), and it was most active against α-naphthyl-butyrate (Fig. 2). The metagenomic esterases were active against both types of substrates with ABO_1197, ABO_1251 and MGS0010 showing higher activity against *p*NP-esters, whereas the other three enzymes being more active towards α-naphthyl esters (Fig. 2). These results suggest that the active sites of these esterases have differences in the alcohol-binding pockets. Previously, it was found that some esterases show higher activity against phenyl esters, whereas others preferentially degrade *p*-nitrophenyl esters (Bornscheuer et al. 2005). The five metagenomic esterases are highly active enzymes with specific activities that range from 200 to over 2,000 μmoles/min/mg protein (Fig. 2). With *p*-NP esters, the metagenomic esterases exhibited different preferences for acyl chain lengths from the C2 substrate *p*NP-acetate (MGS0109) to the C8 substrate *p*NP-octanoate (MGS0010) (Fig. 2). ABO_1197 revealed a broad substrate range with the highest activity towards *p*NP-valerate (C5), but it also showed significant activity against *p*NP-palmitate (C16; up to 30 μmoles/min/mg protein), which is a representative substrate for lipases (Fig. 2). However, all purified proteins were inactive in the lipase assay with olive oil, which contains mostly oleic acid (18:1 cis-9, 50–80 %). Under the assay conditions used, the metagenomic esterases exhibited three to five times higher activity than the commercial esterase PLE (Fig. 2).

With the model esterase substrates, the purified metagenomic esterases exhibited saturation kinetics and high catalytic efficiencies with low micromolar K_m values (Table 2). ABO_1251 was the most efficient esterase based on the k_{cat}/K_m values, which were both high for α-naphthyl and *p*NP substrates. The commercial PLE preparation was less active than the metagenomic esterases (Table 2), and lower catalytic efficiencies (k_{cat}/K_m) were reported for the individual recombinant PLE isoforms (3.0 to 9.1×10^5) (Hummel et al. 2007). The functional significance of the β-lactamase-like active site residues of MGS0105 was confirmed using site-directed mutagenesis (alanine replacement). The known catalytic mechanism of β-lactamases includes two steps (acylation and deacylation) and involves a catalytic Ser nucleophile, as well as conserved Lys and Tyr residues acting as a general base and a proton acceptor, respectively (Strynadka et al. 1992). According with this, analysis of esterase activity of purified MGS0105 mutant proteins revealed the complete loss of activity in the S68A, K71A and Y157A mutant proteins, whereas other mutant proteins retained from 10 to 100 % of wild-type activity (Fig. S2). Thus, the purified metagenomic

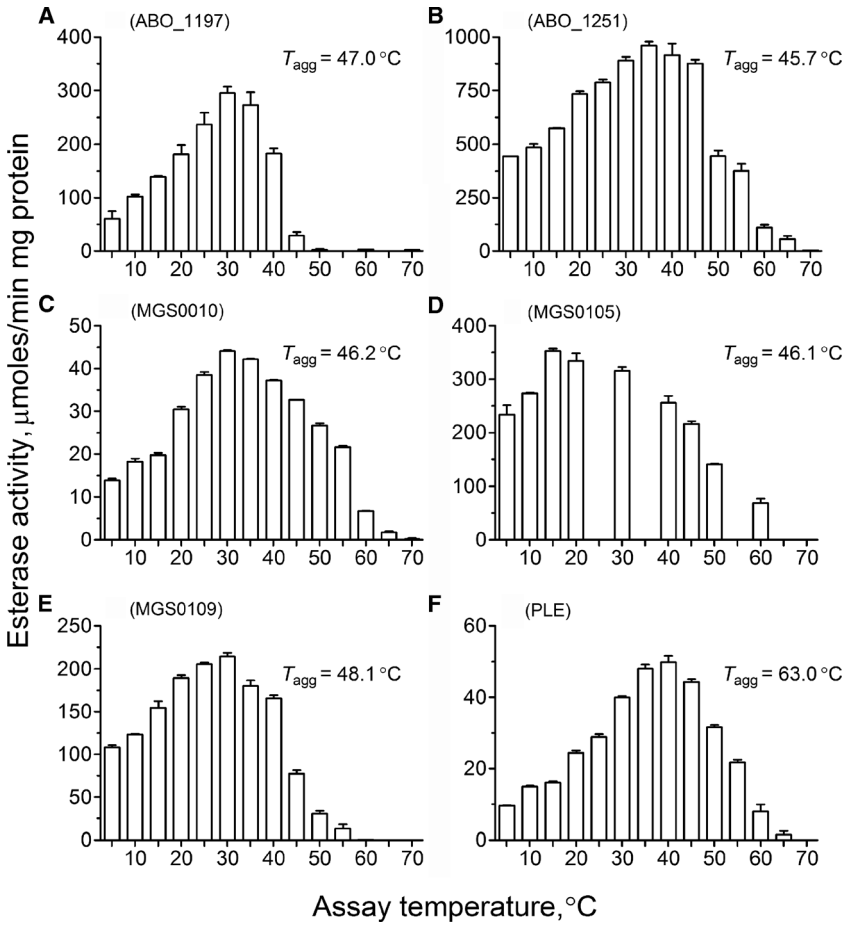

Fig. 3 Effect of assay temperature on esterase activity of purified proteins. Esterase activity of the purified proteins was measured at the indicated temperatures using 0.6 mM *p*NP-valerate (for ABO_1197) or 1.5 mM α-naphthyl propionate (for other proteins) in 50 mM CHES buffer (pH 9.0) containing 0.02–0.2 μg of enzyme. The *panels* also show the temperature of aggregation (T_{agg}) of corresponding proteins determined by measuring protein thermodenaturation (aggregation) at different temperatures

esterases revealed high enzymatic activities against the model esterase substrates with a broad range of preferences for the substrate chain length from C2 to C8.

Effect of temperature on stability and activity of metagenomic esterases

Since the metagenomic libraries used in this work were prepared from relatively cold marine metagenomes (3 to 15 °C) (Table 1), we analyzed the thermostability of the purified metagenomic esterases, as well as the effect of assay temperature on their activities. The protein thermodenaturation (aggregation) profiles (T_{agg}) of these proteins were determined using a static light scattering method (see "Materials and methods") and compared with that of PLE. In line with the ambient environmental temperatures, the protein thermodenaturation assays revealed a lower thermostability of the five metagenomic esterases (T_{agg} from 45.7 to 48.1 °C) compared to PLE (T_{agg} 63.0 °C) (Fig. 3). These results are comparable to the thermostability of the cold-adapted esterase OLEI01171 from the psychrophilic marine bacterium *Oleispira antarctica* (T_{agg} 45.7 °C) and several esterases from mesophilic bacteria (T_{agg} from 51 to 60 °C) (Lemak et al. 2012). Thus, the overall thermostability of the metagenomic esterases studied in this work correlates with the respective environmental temperatures. Similarly, the temperature profiles for hydrolysis of α-naphthyl propionate, determined with purified enzymes in the range of 5 to 70 °C, revealed different optimal temperatures. The metagenomic esterases were most active at 15 °C (MGS0105) and 30 °C (the other esterases), whereas PLE was most active at 40 °C (Fig. 3). However, ABO_1251, MGS0010, MGS0105 and PLE retained significant activities at elevated assay temperatures (55–60 °C), whereas ABO_1197 and MGS0109 were almost inactive at these temperatures (Fig. 3). On the other hand, all metagenomic esterases were highly active at 5 °C (38 to 70 %), whereas PLE showed just 18 % of maximal activity at this temperature (Fig. 3). Thus, the metagenomic esterases identified in this work are cold-adapted enzymes with high activity in the temperature range from 5 to 40 °C.

Effect of NaCl and KCl on enzymatic activity of metagenomic esterases

In contrast to the environmental temperature (which is the same both inside and outside of microbial cells), the intracellular concentrations of NaCl in marine bacteria can differ from the external salinity and can reach molar values in halophilic

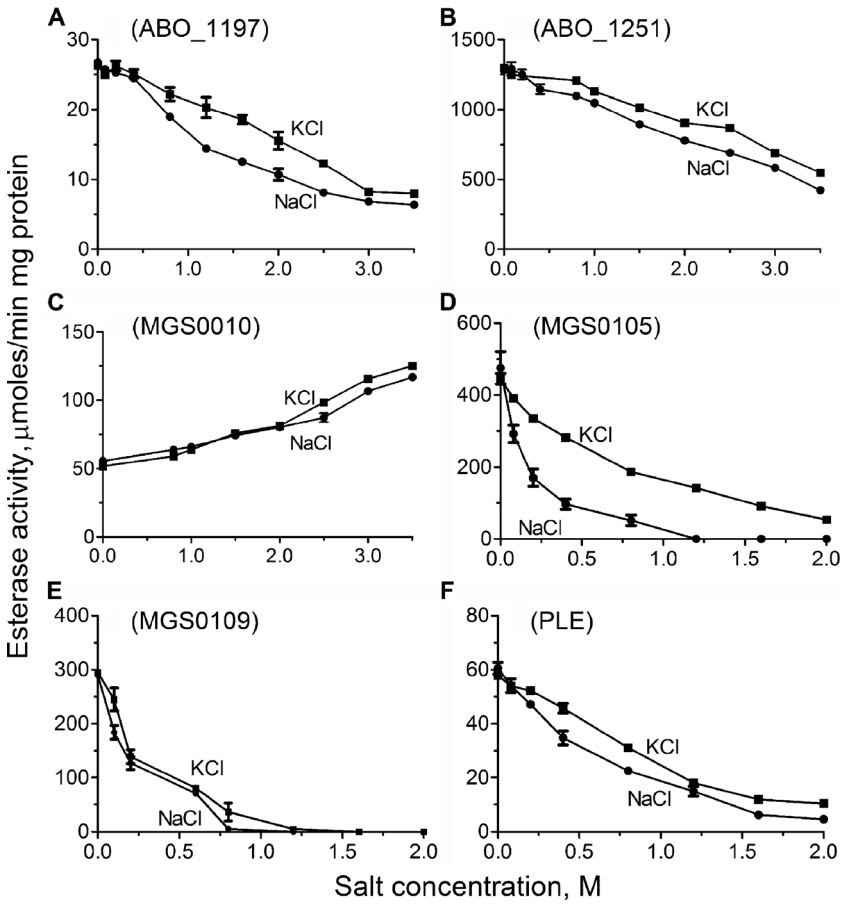

Fig. 4 Effect of NaCl and KCl on esterase activity of metagenomic esterases. The reaction mixtures (200 μl) contained the indicated concentration of KCl (*rectangles*) or NaCl (*balls*), 1.5 mM α-naphthyl propionate, 50 mM CHES buffer (pH 9.0) and 0.002–0.1 μg of enzyme; incubation temperature 30 °C (metagenomic esterases) or 37 °C (PLE)

archaea (Oren 2002). Depending on the species, bacteria can have intracellular Na^+ concentrations higher than, equal to, or lower than the levels in the external medium (Christian and Waltho 1961, 1962). Halotolerant bacteria accumulate low molecular weight organic solutes for osmoregulation, whereas both archaea and bacteria have been shown to possess energy-linked mechanisms for extruding Na^+ and accumulating K^+ against concentration gradients ("salt-in" strategy) (Danson and Hough 1997; Oren 1999; Schultz and Solomon 1961). Therefore, we have determined the effect of NaCl and KCl on the hydrolysis of the model esterase substrate α-naphthyl propionate by purified metagenomic esterases. As shown in Fig. 4, three enzymes (MGS0105, MGS0109 and PLE) showed similar sensitivity to increasing salt concentrations and were strongly inhibited or inactive at 1–2 M NaCl or KCl, whereas ABO_1197 and ABO_1251 retained ~30 % of maximal activity at much higher salt concentrations (3.5 M). Interestingly, both *A. borkumensis* esterases exhibited similar sensitivity to NaCl and KCl as might be expected for enzymes from the same organism. In contrast, MGS0010 showed no inhibition by salt at all, rather its activity increased almost two times at 3.5 M NaCl or KCl (Fig. 4). These results also indicate the absence of a direct correlation between the adaptation of metagenomic esterases to low temperatures and salt resistance. Different sensitivities to NaCl were also reported for the seven metagenomic esterases isolated from Lake Arreo (Martinez-Martinez et al. 2013). Thus, in contrast to the effects of temperature, the purified metagenomic esterases exhibited different sensitivities to high salt concentrations suggesting that these enzymes originated from organisms with different intracellular concentrations of NaCl and KCl.

Effect of glycerol, solvents and detergents on metagenomic esterases

Marine and halophilic microorganisms can tolerate widely fluctuating salt concentrations and low temperatures (Wilkins et al. 2013). In living organisms, one of the mechanisms of osmoregulation and cryoprotection is based on the formation and degradation of glycerol (Ben-Amotz and Avron 1973; Izawa et al. 2004; Lewis et al. 1995; Managbanag and Torzilli 2002; Nass and Rao 1999). Therefore, we examined the effects of glycerol on the enzymatic activity of purified metagenomic esterases. As shown in Fig. 5, the addition of low concentrations of glycerol (5–30 %) induced either no (ABO_1251) or only a small increase (ABO_1197 and PLE) in esterase activity, but these three enzymes tolerated high concentrations of glycerol (up to 50 %). In contrast, the activities of MGS0010, MGS0105 and MGS0109 were strongly stimulated by the addition of glycerol (4–5 times), and their activities reached maximal levels (1,500–2,000 μmoles/min/mg protein) at 20–30 % glycerol (Fig. 5). In MGS0010 and MGS0105, the addition of glycerol to the reaction mixture (15–35 %) increased k_{cat} 3 to 5 times, but this

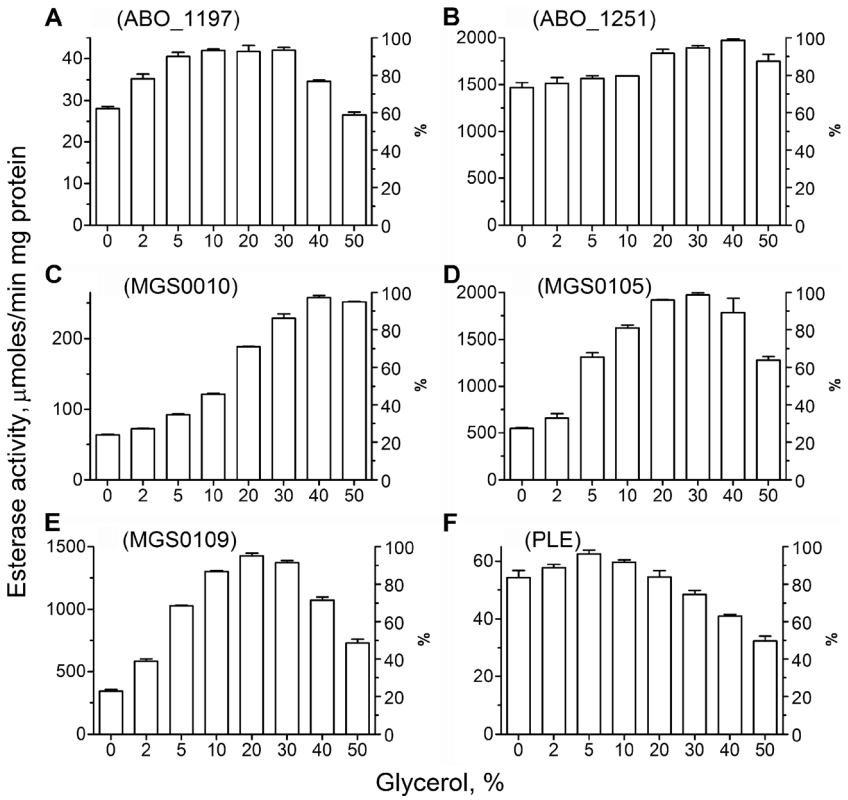

Fig. 5 Effect of glycerol on esterase activity of metagenomic esterases. The reaction mixtures contained 1.5 mM α-naphthyl propionate, 0.02–0.1 μg of protein and glycerol (as indicated); incubation temperature 30 °C (metagenomic esterases) or 37 °C (PLE)

also raised K_m (4 to 10 times) thereby reducing the catalytic efficiency k_{cat}/K_m of these enzymes 2–3 times (Table 2). Glycerol addition also affected the temperature profiles of esterase activity of MGS0105 and MGS0109 further increasing their activity at low temperatures (to almost 1,000 μmoles/min/mg protein of MGS0105 at 5 °C) and also increasing their resistance to high temperatures (40–60 °C) (Fig. S3).

We also determined the effects of organic solvents and detergents on the activity of the purified metagenomic esterases. As shown in Fig. S4, ABO_1197 activity was inhibited by low concentrations of acetonitrile and DMSO (10–20 %), whereas the other four metagenomic enzymes and PLE were more resistant to acetonitrile and were even stimulated by the addition of DMSO. When tested for resistance to the detergents Tween-20 and Triton X-100, ABO_1251 was the most resistant enzyme followed by MGS0010, MGS0105 and MGS0109, whereas ABO_1197 and PLE showed the strongest inhibition (Fig. S5). Thus, the metagenomic enzymes identified in our work exhibit different responses to the addition of glycerol and different sensitivities to organic solvents and detergents.

Activity of metagenomic esterases against monoester and polyester substrates

To characterize the substrate range and preference of the purified metagenomic esterases, these proteins were screened against a set of 89 ester substrates including various alkyl and aryl esters (Fig. 6, Table S4). With these substrates, ABO_1251 showed a clear preference for phenyl acetate with lower activity against triacetin, tripropionin and many halogenated alkyl esters (Fig. 6). Both MGS0105 and MGS0109 exhibited their highest activities with ethyl bromoacetate but also showed high hydrolytic activities towards a broad range of alkyl and aryl esters with a preference for vinyl esters. ABO_1197 also has a broad substrate range, but it was active only against alkyl esters (Fig. 6). The catalytic efficiencies (K_m/k_{cat}) of metagenomic esterases with ester substrates were

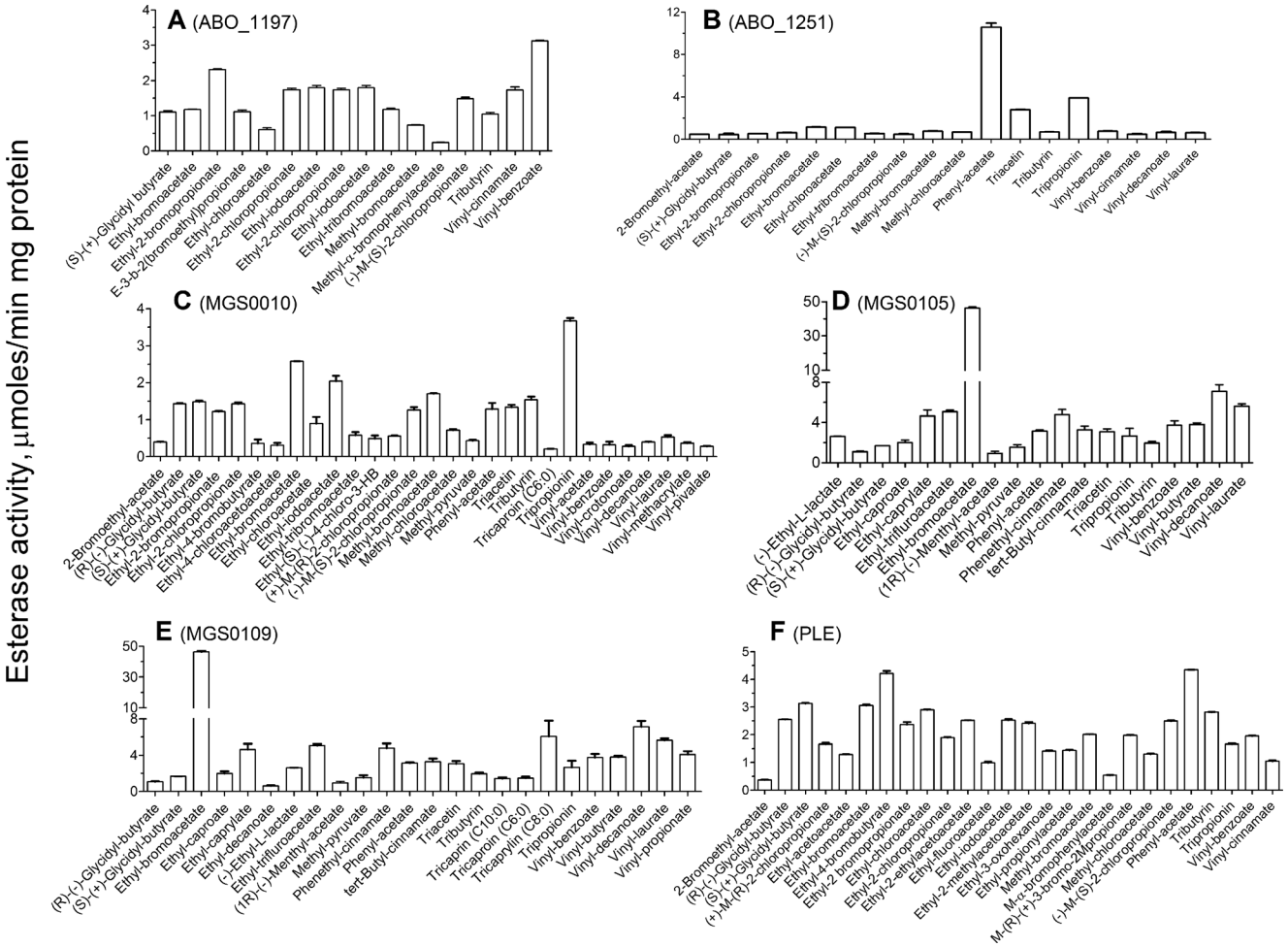

Fig. 6 Substrate profiles of metagenomic esterases. The activity of purified enzymes was assayed against a library of 89 ester substrates (only substrates supporting significant esterase activity are shown). Experimental conditions were as described in "Materials and methods". The reaction mixtures (200 μl) contained 2 mM BES buffer (pH 7.2), 2 mM substrate, 0.45 mM *p*-nitrophenol and 0.1 μg of enzyme; incubation temperature 30 °C (metagenomic esterases) or 37 °C (PLE)

somewhat lower than those for the model esterase substrates, but close to that of PLE (Table 2). The enantiopreference of the metagenomic esterases was analyzed using glycidyl butyrate and methyl-2-chloropropionate, for which both enantiomers are commercially available. With these substrates, both ABO_1197 and ABO_1251 showed activity only against (*S*)-(+)-glycidyl butyrate and (−)-methyl-(*S*)-2-chloropropionate indicating that these enzymes prefer the (*S*)-enantiomers (Fig. 6). The other three metagenomic esterases and PLE showed no apparent enantiopreference and hydrolyzed both enantiomers of glycidyl butyrate (Fig. 6). Thus, the metagenomic esterases exhibit different substrate preferences and broad substrate profiles comparable to that of PLE.

Purified metagenomic esterases and PLE were also screened for hydrolytic activity against polymeric ester substrates including polyhydroxybutyrate (PHB), PLA (PLA2, PLA10, PLA18, PLA70, PLA4032, PLA6400), polycaprolactones (PCL10, PCL45, PCL70) and polybutylene-succinate-co-adipate (17 substrates total). PLE was inactive against all polyester substrates tested, whereas the five metagenomic esterases showed high hydrolytic activities against PLA2 (poly-D,L-lactide; 2,000 Da) (Fig. 7). In addition, ABO_1197 and MGS0109 also hydrolyzed polycaprolactones (PCL10, PCL45, PCL70) and polybutylene-succinate-co-adipate (PBSA3001 and PBSA3020), whereas ABO_1251 was active with PBSA3001. Previously, several extracellular microbial lipases have been shown to be able to hydrolyze polyesters containing side chains in the polymer backbone, such as poly(4-hydroxybutyrate) and poly(6-hydroxyhexanoate) (or polycaprolactone, PCL), but not PLA (Jaeger et al. 1995). However, the lipase-like proteins (esterase family I) PlaM4, PlaM7 and PlaM9 from a compost metagenome, as well as PlaA from *Paenibacillus amylolyticus*, were found to exhibit degradation activities towards PLA and several other polyesters including PCL, PBSA, poly(butylene succinate) (PBS) and poly(ethylene succinate) (PES) (Akutsu-Shigeno et al. 2003; Mayumi et al. 2008). Our work has revealed that in addition to the previously identified lipases and proteases (Jarerat et al. 2006; Matsuda et al. 2005), proteins from several esterase families including the β-lactamase-like enzymes (family VIII) show high hydrolytic activity against PLA and other polyester substrates. Thus, the metagenomic esterases exhibit different substrate preferences and show activity against a broad range of monoester and polyester substrates including PLA.

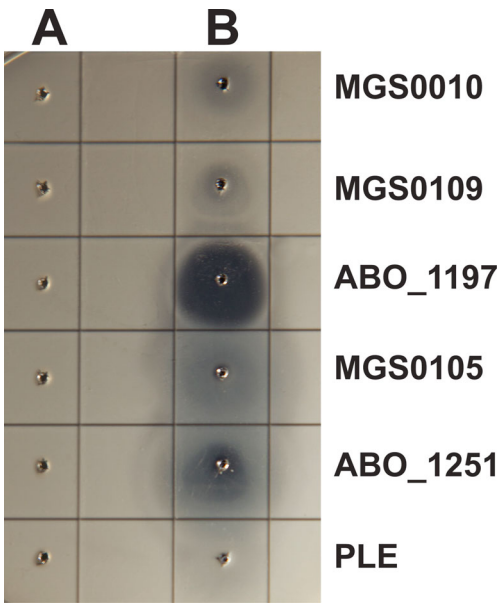

Fig. 7 Screening of purified metagenomic esterases for hydrolytic activity against polylactic acid: poly (DL-lactide). Purified proteins were loaded into the wells (50 μg/well) in 1.5 % agarose containing 0.2 % (w/v) poly (DL-lactide) (average M.w. 2,000) in 50 mM Tris-HCl (pH 8.0) and incubated at 30 °C for 24 h. The presence of hydrolytic activity against polylactide is indicated by the formation of a clear zone around the wells (shown against a *dark background*). The wells in column A show the controls without enzyme addition (buffer only), whereas the wells in column B contained the indicated enzyme

Discussion

The activities of enzymes in marine microorganisms are mainly affected by two environmental factors, namely, temperature and osmolarity. The five esterases we have cloned from marine metagenomes, over-expressed and subsequently purified retained high enzymatic activities at low temperatures (in contrast to the mesophilic PLE enzyme). The high specific activities and low overall protein thermostabilities of the five metagenomic esterases suggest that they are typical cold-adapted enzymes with an activity-stability trade-off (Siddiqui and Cavicchioli 2006). Similarly, the screening of the Lake Arreo metagenome (the ambient temperature 6.9 °C) revealed two esterases (LAE5, LAE6) with high residual activity (40–50 %) at 4 °C (Martinez-Martinez et al. 2013). However, the five different enzymes (including two esterases) from the psychrophilic and moderately halophilic marine bacterium *O. antarctica* RB-8 exhibited various sensitivities to low and high temperatures, as well as to elevated NaCl concentrations (Kube et al. 2013; Lemak et al. 2012). Similarly, the two metagenomic esterases Est_p1 and EstF isolated from marine sediments of the South China Sea showed 20–22 % of residual activity at 5 °C, whereas the CHA3 esterase from Antarctic desert soil retained less than 20 % activity at 5 °C (Fu et al. 2011; Heath et al. 2009; Peng et al. 2011). In contrast, the two metagenomic esterases EstA3 and EstCE1, which belong to esterase family VIII with similarity to β-lactamases (like MGS0105), showed no activity at 10 °C and had a temperature optimum at 50 °C (Elend et al. 2006).

On the contrary, the metagenomic esterases demonstrated different responses to high concentrations of salt or glycerol. The activity of MGS0010 was increased almost two times in the presence of 3.5 M KCl, whereas the other four metagenomic esterases exhibited different resistance to high salt concentrations. The two esterases from *A. borkumensis* (ABO_1197 and ABO_1251) showed similar resistance to high concentrations of KCl and NaCl and retained significant activity at 2–3.5 M salts, whereas both MGS0105 and MGS0109 exhibited low remaining activity in the presence of 1 M NaCl (Fig. 4). Therefore, like various thermophilic enzymes (Jabbour et al. 2013; Tan et al. 2008), some cold-adapted esterases can also exhibit significant halotolerance (Srimathi et al. 2007). In addition, these results suggest that salt resistance of marine metagenomic organisms can be based on different strategies including the development of various Na^+ export mechanisms or the evolution of salt-resistant enzymes.

Glycerol addition increased the activity of MGS0010, MGS0105 and MGS0109 but had little effect on ABO_1197 and ABO_1251. It has been reported that purified microbial and metagenomic esterases exhibit different sensitivities to organic solvents and detergents (Elend et al. 2006; Fu et al. 2011; Jiang et al. 2012; Kang et al. 2011). For example, the activity of the EstA esterase from *Arthrobacter nitroguajacolicus* was slightly stimulated (10–20 %) by the addition of DMSO (30 %) or acetonitrile (30 %), whereas lower concentrations (10 %) of these solvents reduced (20 to 90 %) the activity of esterases from *Sulfolobus solfataricus* (EST1) and *Archaeoglobus fulgidus* (Est-AF) (Kim et al. 2008; Schutte and Fetzner 2007; Sehgal et al. 2002). Similarly, several metagenomic esterases have been reported to be inhibited, unaffected or stimulated by the addition of DMSO (10–30 %), whereas all these enzymes were sensitive to acetonitrile and detergents (Triton X-100 and Tween-20) (Elend et al. 2006; Fu et al. 2011; Jiang et al. 2012; Kang et al. 2011). Since the metagenomic enzymes cannot be protected from the effect of low temperatures, the evolution of cold-adapted enzymes is the main mechanism of cold resistance in marine microbes. Thus, the biochemical characterization of novel esterases from marine metagenomes revealed a considerable biochemical diversity of these enzymes including high catalytic activity, broad substrate profiles, with activity against polyester substrates and resistance to harsh experimental conditions.

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