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Isolation and characterization of cold-active family VIII esterases from an arctic soil metagenome

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Abstract Functional screening for lipolytic enzymes at low temperatures resulted in the isolation of the novel cold-active esterases, EstM-N1 and EstM-N2, from a metagenomic DNA library of arctic soil samples. EstM-N1 and EstM-N2 were 395 and 407 amino acids in length, respectively, and showed the highest similarity to class C β-lactamases. However, they shared a relatively low level of sequence similarity (30%) with each other. Phylogenetic analysis of bacterial lipolytic enzymes confirmed that EstM-N1 and EstM-N2 belonged to family VIII of bacterial esterases/lipases. The $(His)_6$-tagged esterases were purified to about 99% homogeneity from the soluble fraction of recombinant *Escherichia coli* cultures. The purified EstM-N1 and EstM-N2 retained more than 50% of maximal activity in the temperature range of 0–35°C, with optimal temperatures of 20°C and 30°C, respectively. Both enzymes preferred the short acyl chains of *p*-nitrophenyl esters and exhibited very narrow substrate specificity, indicating that they are typical esterases. The β-lactamase activity of EstM-N1 and EstM-N2 was also detected and reached about 31% and 13% of the positive control enzyme, *Bacillus cereus* β-lactamase, respectively. These first cold-active esterases belonging to family VIII are expected to be useful for potential biotechnological applications as interesting biocatalysts.

Keywords Esterase · Metagenome · Family VIII · Cold-active enzyme

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Introduction

Bacteria produce different classes of lipolytic enzymes, including carboxylesterases (EC 3.1.1.1) and true lipases (EC 3.1.1.3). Esterases that hydrolyze small ester-containing molecules at least partly soluble in water and lipases that display maximal activity towards water-insoluble long-chain triglycerides are fundamentally carboxylic ester hydrolases (EC 3.1.1). Bacterial esterases and lipases are grouped into eight different families; Jaeger and others have previously reported the extensive classification of bacterial lipolytic enzymes based mainly on a comparison of their amino acid sequences and some fundamental biological properties (Arpigny and Jaeger 1999). Lipolytic enzymes such as esterases and lipases are currently attracting enormous attention because of their considerable industrial potential (Jaeger and Eggert 2002; Panda and Gowrishankar 2005). These enzymes are used in organic chemical synthesis, chiral resolution of racemic compounds, and modification of lipids for use in nutraceuticals, cosmetics, and pharmaceuticals. Therefore, additional microbial esterases/lipases with different characteristics are being explored.

The metagenome includes the genomes from micro-organisms that have not been cultured or cultivated thus far and plays an important role in revealing novel sequences, genes, and biological pathways (Simon and Daniel 2009). On the basis of the functional and sequence-based search of

the collective microbial genomes in a given habitat (Uchiyama and Miyazaki 2009), metagenomics has been used to discover novel, potentially important enzymes (Schmeisser et al. 2007; Steele et al. 2009). To date, several genes encoding esterases and lipases have been identified in metagenomic DNA libraries prepared from a wide range of environmental samples such as sediments/soils and seawater. Distinguished features such as cold-adapted activity (Hårdeman and Sjöling 2007), thermostability (Tirawongsaroj et al. 2008; Rhee et al. 2005), and alkalophilic activity (Meilleur et al. 2009) have been found in the metagenomic lipolytic enzymes. The phylogenetically new groups of esterases/lipases were also uncovered (Lee et al. 2006).

In this paper, we report the isolation, expression, and characterization of two novel metagenome-derived esterase genes that belong to family VIII esterases/lipases. An enzymatic activity-based screening strategy under low temperatures was used to isolate enzymes, and thus, both purified esterases displayed distinctly cold-adapted activity profiles.

Materials and methods

Bacterial strains and metagenomic library

Escherichia coli DH5α [F^-(*80d* Δ*lacZM15*) Δ(*lacZYA-argF*) *U169 deoR hsdR17*(r^- m^+) *recA1 endA1 phoa supE44* λ^- *thi-1 gyrA96 relA1*] (RBC, Taiwan) and JM109 [e14$^-$(McrA$^-$) *recA1 endA1 gyrA96 thi-1 hsdR17* (rK^- mK^+) *supE44 relA1* Δ(*lac-proAB*) [F' *traD36 proAB* $lacI^qZ\Delta M15$]] (RBC) were used for the cloning and expression of the esterase genes. The metagenomic DNA library, constructed using soil samples of the Arctic region (the Dasan Station at Ny-Alesund (78°55′N, 11°56′E)), was supplied by the research program of Microbial Genomics and Applications Center in Korea (http://www.microbank.re.kr). As described previously (Jeon et al. 2009a), the fosmid vector (CopyControl™ pCC1FOS or pEpiFOS™-5 fosmid library production kit; Epicentre Biotechnologies, USA) was used to construct the library. The library screened in this study comprised 24 clone pools with ~2,500 fosmid clones per pool.

Library screening for cold-active lipolytic enzymes

The arctic soil metagenome library was screened for fosmid clones showing lipolytic activity on LB-agar plates containing 0.8% (*w*/*v*) tributyrin. The plates were first incubated at 37°C until the host *E. coli* had formed colonies and then further incubated at 4°C for up to 2 days. The lipolytic clones were selected based on their ability to produce clear halo around the colony. The lipolytic activity of clones was confirmed by streaking cells repeatedly on the LB-tributyrin-agar plates. After the lipolytic *E. coli* clones were grown in LB medium at 30°C, the cells were harvested by centrifugation, washed with phosphate-buffered saline, and resuspended in lysis buffer containing 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, and 0.05% Tween 20. The suspension was incubated on ice for 30 min and then sonicated six times for 3 s. After the cell lysate was centrifuged for 20 min at 4°C and 25,000×*g*, the supernatant fractions were used to assay for esterase activity using *p*-nitrophenyl butyrate at both 10°C and 40°C.

Subcloning and DNA sequencing

Fosmid DNAs were isolated from positive colonies using the FosmidMAX DNA purification kit (Epicentre Biotechnologies) and digested with *Bam*HI or *Eco*RI. The size of extracted DNA was examined by pulsed-field gel electrophoresis and fractions containing DNA fragments of 1–15 kb were ligated into the corresponding sites of pUC19. The ligated DNA was transformed into *E. coli* DH5a, and the transformants were examined for lipolytic activity as mentioned above. The DNA sequence of metagenomic inserts in the subcloned plasmids was determined on an ABI automatic sequencer using the primer-walking technique at Macrogen Inc. (Daejeon, Korea). DNA sequence analysis, database search, and gene structure characterization were carried out at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) using web-based programs and resources such as the open reading frame (ORF) finder and the BLAST search algorithms.

Heterologous expression and purification of the recombinant esterases

Restriction enzyme sites (underlined in the primer sequence below) allowing the cloning of *estM-N1* and *estM-N2* into the expression vector pQE30 (Qiagen) were introduced in the primers. The primers estMN1-F (5′-ggatccatcatgaacaatt taaacacacgc-3′) and estMN1-R (5′-gagctccatactatgcccgag catcgcatag-3′) were used to amplify *estM-N1*; estMN2-F (5′-ggatccatgttggagcttgtatcgccg-3′) and estMN2-R (5′-ggatcctattcacgcagcgctgcgtagat-3′) were used to amplify *estM-N2*. After a standard PCR reaction was performed, the PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen) and then sequenced to ensure a correct gene amplification. Restriction-digested fragments (*estM-N1*; *Bam*HI/*Sac*I, *estM-N2*; *Bam*HI) were ligated into pQE30 linearized with the same enzymes to produce the expression plasmids pQE30-EstM-N1 and pQE30-EstM-N2. These

plasmids were transformed *E. coli* JM109 cells by electroporation (Gene Pulser Xcell; Bio-Rad, USA).

E. coli cells were grown at 37°C in 100 ml of LB medium supplemented with ampicillin (100 μg/ml) until an OD_{600} of 0.6 and then induced with different concentrations of isopropyl β-D-1-thiogalactopyranoside (IPTG). After 16 h of cultivation at 18°C, the cells were harvested by centrifugation at 9,800×*g* for 15 min and resuspended in Native IMAC lysis buffer (300 mM KCl, 50 mM KH_2PO_4, 5 mM imidazole, pH 8.0, Bio-Rad). The resuspended cells were lysed with sonication (VCX 750; Sonics & Materials Inc., USA) and centrifuged at 12,000×*g* for 30 min at 4°C. The supernatants were filtered through a 0.45 μm filter and used as a source for intracellular soluble proteins.

The histidine-tagged EstM-N1 and EstM-N2 enzymes were purified by immobilized metal affinity chromatography (IMAC) using the Profinia protein purification system (Bio-Rad) according to the manufacturer's instructions. The purification procedure using the Bio-Scale Mini Profinity IMAC cartridge (1 ml, Bio-Rad) and the Bio-Scale Mini Bio-Gel1 P-6 desalting cartridge (10 ml, Bio-Rad) were adopted. The enzyme purity was estimated by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in eluted fractions using 10% polyacrylamide gels. The protein concentrations were determined using the Bradford assay kit (Bio-Rad) with bovine serum albumin as a standard. The purity of the sample was shown on SDS-PAGE and quantified by the microfluidics-based electrophoresis system (Experion and Quantity One software; Bio-Rad).

Enzyme assays

Esterase activity was measured by a spectrophotometric method using *p*-nitrophenyl esters, as described previously (Kwon et al. 2009a). The assay mixture was made by mixing 10 mM substrate resolved in acetonitrile, ethanol, and Tris–HCl to a final ratio of 1:4:95. The reaction was started by adding a quantified amount of enzyme, and at once, the absorbance of the liberated *p*-nitrophenol was continuously monitored at 405 nm with a microplate reader (Bio-Rad). The esterase activity was calculated by comparing the sample values to a standard curve drawn with different concentrations of *p*-nitrophenol. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute from *p*-nitrophenyl butyrate.

The pH stat titration method was also used to measure the enzyme activity according to protocols previously described (Kwon et al. 2009b). Briefly, fatty acids liberated from triacylglycerides were automatically titrated with 0.01 N NaOH to maintain a constant pH of 8.0 at 45°C (TitraLab 870; Radiometer Analytical, France). One unit (U) of enzyme activity was defined as the amount of enzyme releasing 1 μmol of fatty acid per minute from triacylglycerides.

The β-lactam hydrolytic activity was determined spectrophotometrically using Nitrocefin (3-(2,4-dinitrostyryl)-(6*R*,7*R*)-7-(2-thienylacetamido)-ceph-3-*em*-4-carboxylic acid, E-isomer) as a substrate (Calbiochem, USA). The enzyme was incubated in assay buffer (100 mM phosphate, pH 7.0) containing 1 mM nitrocefin at 30°C, and the absorbance change at 486 nm was monitored. The molar extinction coefficient of hydrolyzed nitrocefin under these conditions was 20,500 M^{-1} cm^{-1}. The same amount of *Bacillus cereus* β-lactamase (penicillinase) enzyme (Sigma) was used as a positive control.

Biochemical properties

The same amount of purified enzymes was used to analyze biochemical properties, with all experiments performed in triplicate. Substrate preference was examined by assaying the hydrolytic activities for *p*-nitrophenyl esters (C2:0, C4:0, C6:0, C8:0, C12:0, C14:0, and C16:0) and triglycerides (C2:0, C4:0, C8:0, C16:0, and C18:1) of varying acyl chain lengths from C2 to C18, respectively. The kinetic parameters of EstM-N1 and EstM-N2 were determined by measuring the initial velocity of hydrolysis at various concentrations of *p*-nitrophenyl butyrate (0.04–0.6 mM).

The optimum temperature was determined by assaying the hydrolytic activity for *p*-nitrophenyl butyrate at different temperatures ranging from 0–60°C. Thermostability was analyzed by measuring the residual activity after pre-incubating the enzymes at different temperatures ranging between 0°C and 50°C. The pre-incubation period of enzymes was varied from 30 to 150 min. For determining the optimum pH of each enzyme, the pH of the reaction mixtures was varied from 6.0–10.0 using different pH buffers (50 mM sodium phosphate for pH values of 6.0, 7.0, and 8.0; 50 mM Tris–HCl for pH values of 7.0, 8.0, 9.0, and 10.0). In this case, esterase activity was calculated with each *p*-nitrophenol standard curve prepared using each corresponding pH buffer. The effects of divalent ions ($CaCl_2$, $CoCl_2$, $MgSO_4$, $ZnSO_4$, $NiSO_4$, $FeSO_4$, $MnSO_4$, $CuSO_4$) and chelating agent ethylenediaminetetraacetic acid (EDTA) on enzyme activity were determined by adding them to the assay solution at final concentrations of 1 and 5 mM, respectively.

Nucleotide sequence accession numbers

The nucleotide sequences obtained have been deposited in the GenBank database under accession numbers HQ154132 (*estM-N1*) and HQ154133 (*estM-N2*).

Results

Screening and identification of cold-active esterases

Cold-active esterases were isolated from a metagenomic fosmid library generated from arctic sediment. Expression screening of the fosmid library for cold-active lipolytic enzymes was based on the halo-forming activity of the *E. coli* host colonies when incubated on tributyrin agar plates at a low temperature. The first screening resulted in 121 positive clones out of ~60,000 different clones, as indicated by a transparent halo surrounding the colony. Because the size of the halo did not always reflect the degree of enzymatic activity, the ratio of *p*-nitrophenyl ester-hydrolyzing activity at 10°C relative to that at 40°C was examined using the crude extracts of positive *E. coli* cultures. Finally, two clones showing far stronger lipolytic activity at cold temperatures than the others were identified after the repeated tests and selected for further investigation. After fosmid DNA was isolated from the two positive clones (Fos-EstM-N1 and Fos-EstM-N2) and digested with restriction enzymes, the resulting metagenome DNA fragments were cloned into pUC19. The subclones that expressed extracellular lipolytic activity were isolated from each subclone library, and the insert DNAs were sequenced from both ends.

Sequence analysis

An ORF of 1,188 bp encoding a putative esterase (EstM-N1) of 395 amino acids was identified from the metagenomic DNA sequences (12,095 bp) from the positive clone Fos-EstM-N1. An additional seven ORFs longer than 100 amino acids were also revealed (Electronic supplementary material, Table S1). Another ORF (1,224 bp) encoding a 407-amino acid putative esterase (EstM-N2) was identified from the 4,242 bp sequenced from the second positive clone. No signal peptide was predicted for either esterase by the prediction program SignalP (http://www.cbs.dtu.dk/services/SignalP/), on condition that each gene was derived from either Gram-negative or Gram-positive bacteria.

The two esterases shared a relatively low level of sequence similarity (30%) with each other (Electronic supplementary material, Fig. S1). EstM-N1 showed a high degree of sequence similarity to a large number of putative and experimentally proven class C β-lactamases such as those from *Geodermatophilus obscurus* DSM 43160 (57%; YP_003408918), *Roseiflexus* sp. RS-1 (54%; YP_001276807), *Truepera radiovictrix* DSM 17093 (56%; YP_003705818), and *Ralstonia pickettii* 12 J (50%; YP_001893331), ranging from 48–57% with query coverage of more than 90%. The other esterase, EstM-N2, also showed a high degree of sequence similarity to bacterial β-lactamases, ranging from 61% to 75% with query coverage of more than 93%.

Esterases showing significant similarities to class C β-lactamases have been grouped into family VIII of lipolytic enzymes. EstM-N1 and EstM-N2 contained the Ser-X-X-Lys motif characteristic of β-lactamases within their N-terminal region but lacked the conserved pentapeptide motif (Gly-X-Ser-X-Gly) of esterases and lipases. In addition to the family VIII esterases sequenced to date, the bacterial lipolytic enzymes representing eight different families, as classified by Arpigny and Jaeger (1999), were retrieved for phylogenetic analysis. As shown Fig. 1, EstM-N1 and EstM-N2 clearly clustered with the family VIII esterases identified from bacteria and various metagenomes. Meanwhile, the primary structures of both esterases showed relatively modest similarities to other family VIII esterases. EstM-N1 was most similar to LR1 esterase from a pond water metagenome, with 37% similarity and 53% identity. EstM-N2 showed 40% similarity and 57% identity to Est2K esterase and 38% similarity and 56% identity to EstC esterase.

Heterologous expression and purification of the recombinant esterases

The esterase-encoding genes were amplified by PCR, subcloned into a pQE30 vector where a $(His)_6$ tag was placed in-frame to the N terminus and expressed in *E. coli* strain JM109. Both esterases were expressed at extremely high levels, showing band sizes consistent with the molecular weight of 44.0 and 45.5 kDa deduced from both amino acid sequences, as shown by SDS-PAGE analysis (Fig. 2). The densitometric amount reached to almost 50% of the total cellular protein. Both esterases were found mainly in the insoluble fraction, even under a variety of culture conditions in which temperature, induction stage, and IPTG concentration were varied (data not shown). However, after a single stage of the purification procedure was carried out with the soluble fraction under non-denaturing conditions, the enzymes were purified with overall enzyme yields around 33% and were visualized as each strong single band with the densitometric purity reaching almost 99% (Fig. 2).

Temperature apparent optimum and thermostability

EstM-N1 and EstM-N2 showed the highest activity at 30°C and 20°C, respectively, while activity decreased rapidly at temperatures >40°C (Fig. 3). Both esterases were highly active at the low-temperature range between 0°C and 35°C, retaining a minimum of 50% relative activity. Above all, they showed remarkably high activity (>95%) at temperatures of 20–30°C. Thermostability analysis showed that esterases

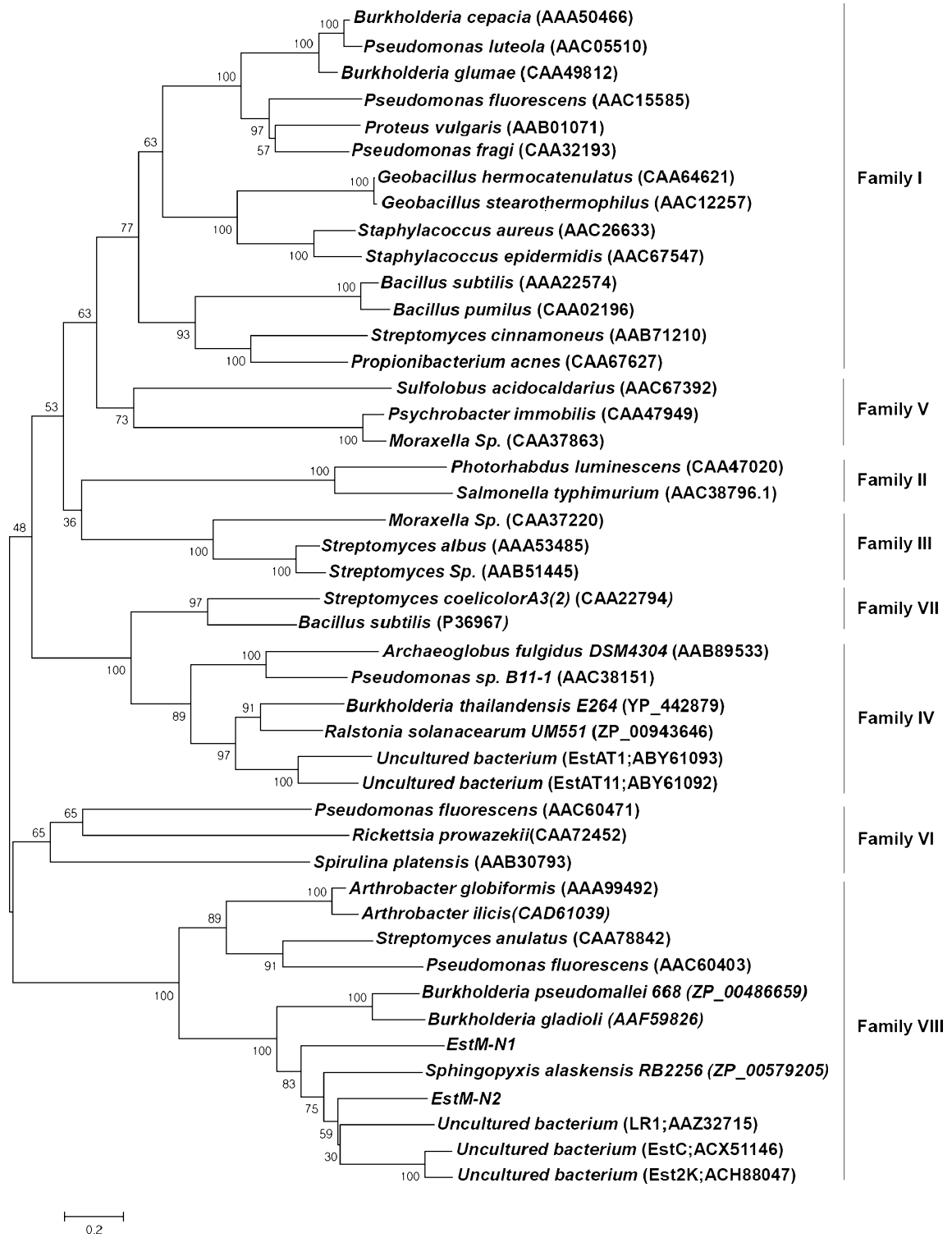

Fig. 1 Phylogenetic analysis of EstM-N1, EstM-N2, and closely related proteins. The phylogenetic tree was generated using the neighbor-joining method (MEGA4.0 software). The protein sequences for previously bacterial lipolytic enzymes were retrieved from GenBank. The numbers at the nodes indicate the bootstrap percentages of 1,000 replicates

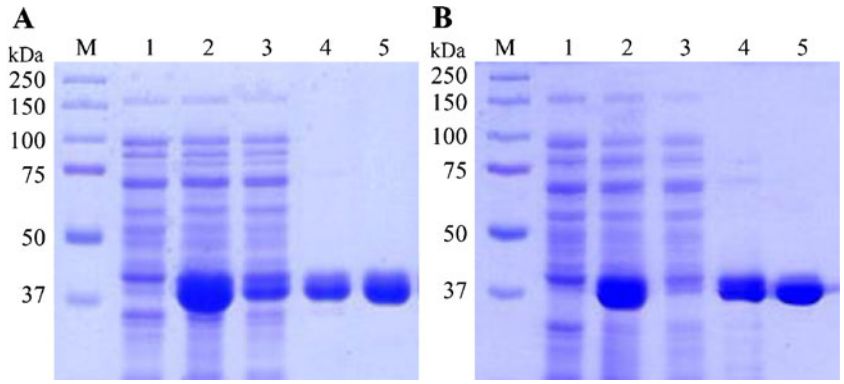

Fig. 2 SDS-PAGE of the purified EstM-N1 (**a**) and EstM-N2 (**b**). *Lane M* protein standards (Bio-rad), *1* total cellular extracts before induction, *2* total cellular extracts after induction, *3* soluble fraction, *4* insoluble fraction, *5* purified enzyme

remained highly stable at temperatures <20°C but were extremely thermolabile at temperatures >40°C (Fig. 4).

Biochemical properties

Hydrolytic activity toward *p*-nitrophenyl esters and triglycerides of various fatty acids was examined. Both esterases, EstM-N1 and EstM-N2, showed the highest activity with *p*-nitrophenyl butyrate (37.8 and 7.5 U/mg). When the kinetic parameters were determined for *p*-nitrophenyl butyrate, the initial reaction velocities of the enzymes obeyed Michaelis–Menten kinetics. Their K_m values were essentially similar, while the k_{cat} value of EstM-N1 was about 7.7-fold higher than that of EstM-N2, providing an explanation for the relatively lower specific activity of EstM-N2. As shown in Fig. 5, the overall hydrolytic pattern showed a strong preference for a narrow range of short-chain acyl substrates. Medium- and long-chain acyl substrates were poor substrates; both esterases showed no or less than 5% activity against *p*-nitrophenyl ester substrates with acyl chains longer than C12 or against long-chain triglycerides such as tripalmitin and triolein.

It is well known that nitrocefin is sensitive to hydrolysis by all known lactamases produced by Gram-positive and Gram-negative bacteria but not by most of the family VIII esterases. Interestingly, the lactamase activity of EstM-N1 and EstM-N2 was 31% and 13%, respectively, of that of the positive control enzyme, *B. cereus* β-lactamase. These levels of relative specific activity were higher than the 11% of EstC, the first member of family VIII esterases shown to exhibit a detectable β-lactam hydrolyzing activity (Rashamuse et al. 2009).

The optimal pH of EstM-N1 and EstM-N2 was tested under buffered conditions over the range of pH 6–10. Both enzymes showed similar pH-activity curves; maximal activity was observed at pH 9.0 and nearly 50% of maximal activity was still observed at pH 10.0. Both enzymes completely lost activity at pH 6.0.

When the effect of metal ions on esterase activity was tested (Table 1), all divalent ions inhibited the esterase activity to different extents; EstM-N1 was more severely affected than EstM-N2. No divalent ions promoted the activity of both esterases. The chelating agent EDTA inhibited EstM-N1 to ~50% at 1 and 5 mM but exhibited

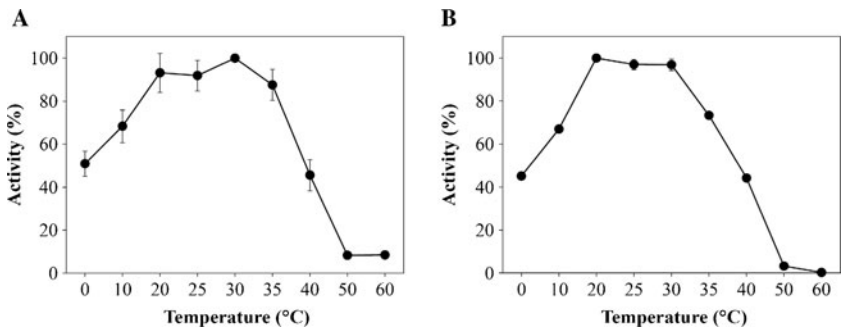

Fig. 3 Effect of temperature on the activities of the EstM-N1 (**a**) and EstM-N2 (**b**). In **a** and **b**, the values obtained at 30°C or 20°C, respectively, were taken as 100%

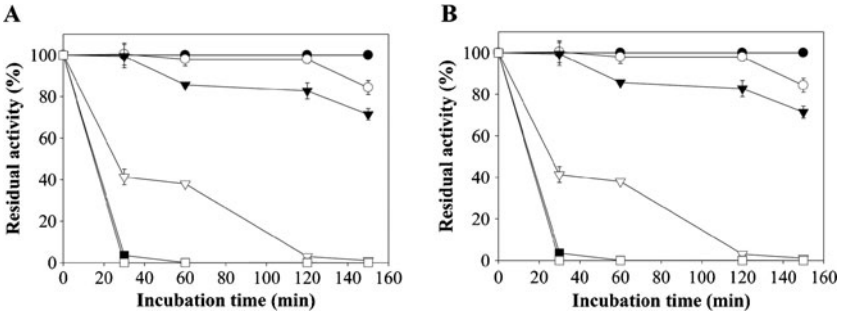

Fig. 4 Thermostability profile of the EstM-N1 (**a**) and EstM-N2 (**b**). Incubation temperatures were 0°C (*filled circle*), 10°C (*empty circle*), 20°C (*filled inverted triangle*), 30°C (*empty inverted triangle*), 40°C (*filled square*), and 50°C (*empty square*)

no statistically significant reduction in the esterase activity of EstM-N2. Therefore, it was obvious that EstM-N2 did not require a divalent cation, although EstM-N1 was not definite about the requirement of divalent cations.

Discussion

In this study, we have isolated and characterized two novel cold-active esterases from an arctic soil metagenome. Phylogenetic analysis revealed that these metagenomic esterases belonged to family VIII of esterases/lipases. Among the many lipolytic enzymes derived from metagenomes, both esterases in this study were the first to show cold-adapted catalytic properties and also to belong to the lipolytic enzyme family VIII sharing the sequence characteristics of class C β-lactamases and penicillin binding proteins.

The cold-adapted enzymes are generally regarded to be originated from psychrophiles and in most cases show a high specific activity and a low thermal stability (Georlette et al. 2004). Due to their catalytic properties distinguishable from mesophilic or thermophilic counterparts, the enzymes with cold-adapted activity constitute a tremendous potential for biotechnological applications. To date, the metagenomic approach has produced several cold-active esterases and lipases, including EML1 from a deep-sea sediment metagenome (Jeon et al. 2009b), h1Lip1 from a sea sediment metagenome (Hårdeman and Sjöling 2007), LipCE (family I.3) from an oil-contaminated soil metagenome (Elend et al. 2007), EstAT1 and EstAT11 from an arctic sediment metagenome (Jeon et al. 2009a), and Lipo1 from an activated sludge metagenome (Roh and Villatte 2008). These enzymes typically showed relatively higher specific activity in the temperature range of 0–30°C, whereas the catalytic activities of most other microbial lipolytic enzymes were severely reduced or almost undetectable at 0°C. Therefore, in addition to the prolonged incubation of *E. coli* cells containing the metagenomic DNA library to detect halo-forming cells at a low temperature (4°C), the ratio of specific activities at low (10°C) and high temperatures (40°C) were measured. In this manner, the *E. coli* cells harboring *estM-N1* and *estM-N2* were isolated by showing a ratio of more than 1.2; and actually, the purified EstM-N1 and EstM-N2 displayed ~1.5-fold higher activity at 10°C than at 40°C. In addition, over a low temperature range (0–30°C), EstM-N1 and EstM-N2 retained more than 50% of the activity observed at their optimum temperatures of 30°C and 20°C, respectively. On the basis of these results, we concluded that EstM-N1 and EstM-N2 are cold-active

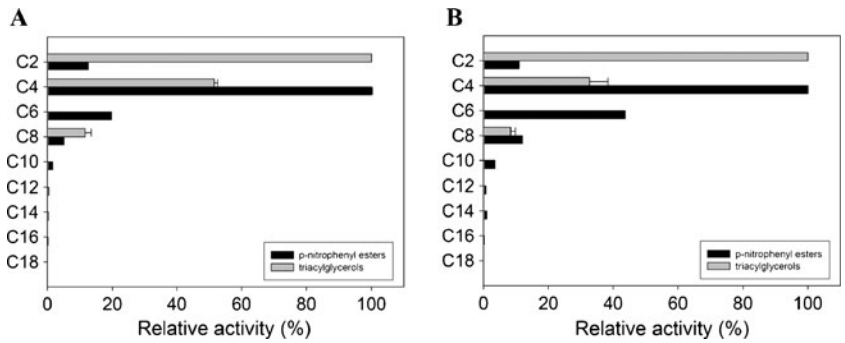

Fig. 5 Substrate preference of EstM-N1 (**a**) and EstM-N2 (**b**) toward *p*-nitrophenyl esters and triacylglycerides

Table 1 Effect of cations and EDTA on the activity of EstM-N1 and EstM-N2

Compounds	Relative activity (%)[a]			
	EstM-N1		EstM-N2	
	1 mM	5 mM	1 mM	5 mM
$CaCl_2$	32.8±1.8	25.2±1.6	90.7±6.1	89.9±4.0
$CoCl_2$	23.4±0.8	72.6±2.2	80.5±6.8	73.2±3.1
$MgSO_4$	54.2±2.0	33.3±9.5	68.1±6.7	61.5±1.9
$ZnSO_4$	21.8±0.2	13.1±0.4	67.8±2.6	41.6±0.5
$NiSO_4$	60.2±2.3	13.4±0.4	49.5±5.8	50.6±0.3
$FeSO_4$	104.3±3.1	45.0±6.8	83.6±12.1	20.4±5.7
$MnSO_4$	68.2±4.8	23.0±0.5	89.9±3.1	82.5±5.6
$CuSO_4$	12.5±1.2	0.7±0.1	87.0±1.5	24.7±2.0
EDTA	58.6±2.4	49.9±2.4	102.0±5.1	103.9±2.8

[a] The relative activities are given as a percentage of the activity in the absence of cation

esterases and thus may be promising candidates for low-temperature applications such as food processing, detergent, and synthesis for heat-sensitive materials.

Although the catalytic activities depend on the substrate and the assay conditions which the researchers used, we tried to compare the catalytic activity of cold-active esterases with those of previously reported esterases, especially family VIII esterases. The specific activities of EstM-N1 and EstM-N2 were similar to EstCE1 (Elend et al. 2006) with an optimal temperature of 47°C and Est2K (Kim et al. 2010) with an optimal temperature of 50°C, but were far lower than those of EstC (~776 U/mg; Rashamuse et al. 2009) and EstA3 (~514 U/mg; Elend et al. 2006) with optimal temperatures of 50°C. Because high activity of an enzyme would be advantageous to practical applications, the cold-active esterases in this study are needed to be improved with regard to the level of maximal catalytic activity at their optimal temperatures.

The deduced amino acid sequences of EstM-N1 and EstM-N2 showed significant similarities to β-lactamases. Both had the conserved β-lactamase Ser-x-x-Lys motif (EstM-N1, Ser63-Met64-Thr65-Lys66; EstM-N2, Ser74-Met75-Ser76-Lys77) but lacked the classical Gly-x-Ser-x-Gly motif; the catalytic serine for all the family VIII esterases is known to be part of the Ser-X-X-Lys motif (Wagner et al. 2002). However, both the motifs had been found simultaneously in many other family VIII esterases such as those from *Streptomyces chrysomallus* (EstA; Berger et al. 1998), *Burkholderia gladioli* (EstB; Petersen et al. 2001), and *Arthrobacter nitroguajacolicus* (EstA; Schütte and Fetzner 2007). Interestingly, the family VIII esterases such as Est2K from a metagenome of completely fermented compost (Kim et al. 2010) and EstC from a metagenome of aqueous acidic leachate (Rashamuse et al. 2009) were also found to lack the Gly-x-Ser-x-Gly motif.

In terms of the substrate specificity, esterases preferentially break the ester bonds of shorter chain acyl substrates, whereas lipases show a much broader substrate range (Fojan et al. 2000). EstM-N1 and EstM-N2 displayed an extremely narrow substrate range, with the highest activity for *p*-nitrophenyl butyrate (C4). Indeed, they showed no detectable activity toward *p*-nitrophenyl ester substrates with an acyl chain longer than C12 (Fig. 5) or toward a variety of vegetable oils such as tripalmitin, soybean oil, and high-oleic sunflower oil. The differences in substrate preference may be due to the physical state of the substrate; esterase activity is generally found to be significantly higher toward more water-soluble substrates than toward poorly soluble substrates like triglyceride emulsions (Fojan et al. 2000). Therefore, in addition to the sequence analyses and temperature-activity profiles, the experimental results on substrate specificity confirmed that both of the metagenome-derived enzymes were esterases, especially cold-active type VIII esterases.

Most of the family VIII esterases shows no or negligible amide-hydrolyzing activity of β-lactam ring substrates. It has been reported that the lack of β-lactam hydrolyzing activity could be due to steric hindrances observed in the substrate binding pocket of family VIII esterases (Wagner et al. 2002). However, in addition to the obvious ester-hydrolyzing activity, EstM-N1 and EstM-N2 exhibited lactamase activity. EstC which showed only 27% and 38% similarity to EstM-N1 and EstM-N2, respectively, was the first family VIII esterase reported to have β-lactamase activity toward nitrocefin (Rashamuse et al. 2009). However, Est2K showed no β-lactamase activity on nitrocefin, though Est2K showed the highest identity to EstC (Kim et al. 2010). Meanwhile, the lactamase activities of EstM-N1 and EstM-N2 were higher than that of EstC, even if the values could not be compared directly. Together, these results suggested that the promiscuous β-lactamase activity of three metagenomic esterases could have been maintained as family VIII esterases evolved from class C β-lactamases and vice versa, and that EstM-N1 and EstM-N2 possibly hold closer evolutionary relationships to class C β-lactamases than EstC.

In conclusion, we have discovered two novel cold-active family VIII esterases with only moderate sequence similarity to all of the known lactamases and esterases. The characterization of both purified esterases revealed their substrate specificity, temperature and pH optimum, and the presence of lactamase activity, which will be useful for developing them further for biotechnological applications. This study also showed that activity-based screening, when appropriately designed to search for desired properties, can be used to

identify novel adapted enzymes from the metagenome, the untapped microbial genome.

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