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Identification of a Novel Alkaliphilic Esterase Active at Low Temperatures by Screening a Metagenomic Library from Antarctic Desert Soil▿†

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A novel esterase was identified through functional screening of a metagenomic library in *Escherichia coli* obtained from Antarctic desert soil. The 297-amino-acid sequence had only low (<29%) similarity to a putative esterase from *Burkholderia xenovorans*. The enzyme was active over a temperature range of 7 to 54°C and at alkaline pH levels and is a potential candidate for industrial application.

The cold deserts of the McMurdo Dry Valleys, South Victoria Land, Eastern Antarctica, are widely acknowledged as having the harshest soil environments on Earth (6, 8, 26). Despite the apparent hostility of the environment, we and others have reported both unexpectedly high biomass (9) and phylogenetic diversity (1, 19, 24, 29) in Antarctic soils. The presence of numerous novel taxa suggests that these soils might prove to be valuable sources of genetic material for mining novel industrial enzymes active at low temperatures (9, 23).

Esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) catalyze the hydrolysis and synthesis of ester compounds. Their applications in industry cover a broad spectrum, including as detergent additives, in food processing, in environmental bioremediation, and in biomass and plant waste degradation for the production of useful organocompounds (3, 16).

In this study, a novel esterase was isolated from a metagenomic fosmid library obtained from Antarctic soil. The enzyme displays activity over a wide temperature range and has very low similarity (<29% amino acid identity) to esterases in the GenBank database. The study demonstrates the value of Antarctic metagenomes as a source of novel industrial products.

Metagenomic fosmid library production and screening. Soil was collected from the Miers Dry Valley in the austral summer season, 2006. The sample site was a 1-m^2 quadrant, at an altitude of 434 m on the northern slope (GPS position, 78°05.930′S, 163°48.174′E; average soil temperature, 3°C; relative humidity, 33%; C content, 0.04%; N content, 0.26%). Four surface (0- to 2-cm) mineral soil samples from each corner of the quadrant were mixed to generate a homogeneous fifth sample, of which 100 g was used for library construction.

Metagenomic DNA was extracted from the soil using a method described previously (30). A fosmid library (average insert size, 30 kb) was constructed using the CopyControl fosmid library production kit (Epicentre Biotechnologies, Madison, WI), which represented 3×10^8 bp of metagenomic DNA. The library was screened for clones displaying lipolytic/esterolytic activity by tributyrin hydrolysis on LB agar plates (1% tryptone, 1% NaCl, 0.5% yeast extract, 1.5% agar), supplemented with 1% tributyrin, 1% gum arabic, 0.01% arabinose, and chloramphenicol (12.5 μg ml^{-1}), and incubated at 22°C for 3 days and then 4°C for 7 days.

To identify the lipolytic/esterolytic open reading frame (ORF) in one active fosmid clone of the three detected, a pUC19 subclone library was generated from a partially digested AluI fosmid. The subclone library was rescreened on LB-tributyrin-carbenicillin (50 μg ml^{-1}), and the putative esterase-encoding ORF was identified from sequenced active clones using the GeneMark gene prediction tool (http://exon.gatech.edu/GeneMark). The ORF CHA3 was 894 nucleotides in length, with ATG start and TAG stop codons, and encoded a polypeptide with a molecular mass of 33.9 kDa. A putative upstream promoter region and ribosome binding site were detected. The highest identity scores in the GenBank database, from a global amino acid alignment, were with putative esterases of *Burkholderia xenovorans* LB400 (29%) (YP 553227), *Algoriphagus* sp. strain PR1 (29%) (ZP 01719709), and *Flavobacterium johnsoniae* UW101 (28%) (YP 001194687).

The deduced amino acid sequence contained conserved residues common to esterases/lipases (2, 20). A GXSXG pentapeptide that forms part of a signature "elbow" near the active site was present in CHA3 as GLSMG at positions 155 to 159. A catalytic triad was proposed to consist of Ser157, Asp247, and His276 (5, 15). A putative oxyanion binding region that might function to stabilize the oxyanion intermediate in the active site was identified as PV-HG from positions 73 to 80 in the polypeptide (4, 10, 15). (Refer to the supplemental material for amino acid sequence alignments.)

Subcloning and expression of CHA3. CHA3 was amplified by PCR using primer sequences 5′ CTGGGATCCATGAATACAGATAAACG and 3′ GCTCGAGCTATTTAATAAATTTTTCG and ligated into pGEX-6P-2 at the BamHI and XhoI sites. CHA3 was expressed in *Escherichia coli* BL21(DE3)/pLysS

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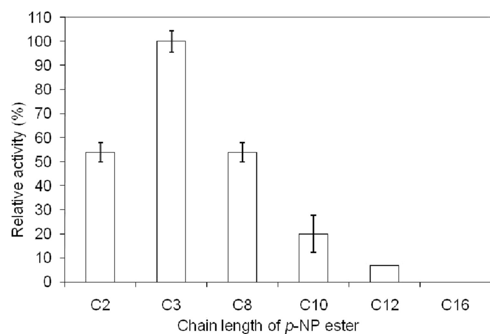

FIG. 1. Activity of CHA3 at 22°C toward *p*-NP esters of various chain lengths (C_2, acetate; C_3, propionate; C_8, octanoate; C_{10}, decanoate; C_{12}, laurate; and C_{16}, palmitate) in 1-ml reactions containing 100 mM sodium phosphate (pH 7.5), 100 mM NaCl, 1% acetonitrile, 0.01% (vol/vol) Triton X-100, and 0.5 mM *p*-NP ester substrate.

grown in 1 liter LB (50 μg ml^{-1} carbenicillin, 34 μg ml^{-1} chloramphenicol) at 37°C until reaching an optical density at 600 nm of 0.6. The culture was cooled to 25°C, induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and incubated for a further 4 h at 22°C, with 180 rpm. Cells were harvested by centrifugation at 6,000 × *g* and lysed in BugBuster extraction reagent (Novagen, NJ), supplemented with Benzonase nuclease (Novagen) (1 μl per 1 g wet cells). Inclusion bodies were collected by centrifugation at 10,000 × *g* and then refolded, according to the protein refolding kit (Novagen). The glutathione-*S*-transferase fusion tag was cleaved from the CHA3 protein in solution using PreScission protease (GE Healthcare) for 12 h at 4°C and separated from CHA3 by dialysis against 20 mM sodium phosphate, pH 7.5, and 1 M ammonium sulfate overnight at 4°C. CHA3 was purified to near homogeneity, shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.

Biochemical characterization of CHA3. Kinetic parameters were determined by fitting data to the direct linear plot of Eisenthal and Cornish-Bowden (11), and 1 unit of activity is defined as micromoles of *p*-nitrophenol (*p*-NP) released per minute. CHA3 was active with *p*-NP acetate (C_2) through to *p*-NP laurate (C_{12}), with greatest activity shown toward *p*-NP propionate (C_3) (Fig. 1). Hydrolytic activity decreased as chain length increased, with no activity toward *p*-NP palmitate (C_{16}), suggesting that CHA3 is an esterase and not a true lipase.

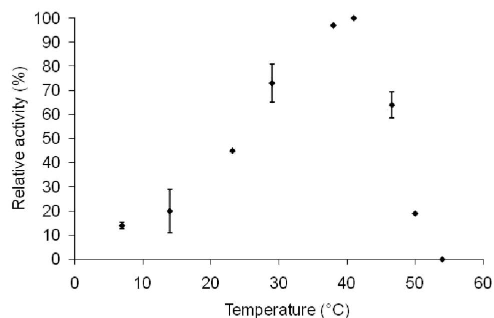

FIG. 3. Effect of temperature on activity of CHA3 toward *p*-NP decanoate.

CHA3 had a K_m value of 0.27 (±0.03) mM for *p*-NP-C_3 and 0.55 (±0.05) mM for *p*-NP-C_2 and a turnover number (k_{cat}/K_m) of 14.8 mM^{-1} s^{-1} for *p*-NP-C_3 and 3.6 mM^{-1} s^{-1} for *p*-NP-C_2. When assayed over a pH range of 4.5 to 11 (Fig. 2), optimum activity was observed at pH 9.0. Activity was 2.6-fold higher at pH 9.0 than at pH 7.0, with no activity below pH 5.5, suggesting that the enzyme is an alkaliphilic esterase.

CHA3 was active over a temperature range of 7 to 50°C (Fig. 3), with an optimum temperature of 40°C, above which it was unstable. The enzyme retained 100% of initial activity at 30°C over a 60-min period but was rapidly inactivated at 50°C, with 25% of initial activity remaining after 5 min and no activity remaining after 30 min (Fig. 4). CHA3 displayed characteristics of an enzyme active at low temperatures, comparable to the following reported cold-active enzymes: esterase PsyEst from *Psychrobacter* sp. strain Ant300 (17) and lipase KB-Lip from *Pseudomonas* sp. strain KB700A, both with optimal activity at 35°C (21); a cold-active lipase from marine sediment, with optimal activity at 35°C (13); and a cold-adapted lipolytic enzyme from activated sludge, with increased thermostability and activity at temperatures below 40°C (22). CHA3 was less thermostable and had a lower optimum temperature than a cold-adapted lipase from *Pseudomonas* sp. strain B1-11 isolated from Alaskan soil (7), which showed greatest activity at (and was unstable above) 45°C. The k_{cat}/K_m ratio of CHA3 was 30-fold lower than that of a mesophilic esterase from *Lactococcus lactis* (18), although CHA3 was assayed at suboptimal temperature and pH values because of assay limitations. It has been proposed that cold-adapted enzymes might trade off substrate affinity for catalytic velocity, seen as markedly high K_m

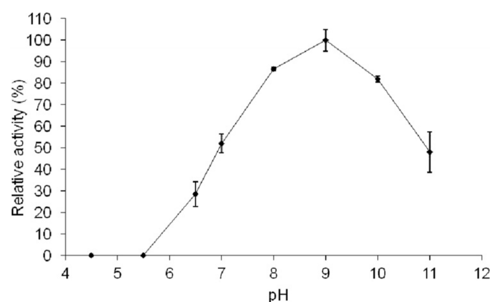

FIG. 2. Effect of pH on activity of CHA3 toward *p*-NP decanoate. Assay buffers used were 100 mM sodium acetate (pH 4.5 to 5.5), 100 mM MES (morpholineethanesulfonic acid) (pH 5.5 to 7.0), 100 mM Tris-HCl (pH 7.0 to 9.0), and 100 mM CAPS (pH 9.0 to 11.0).

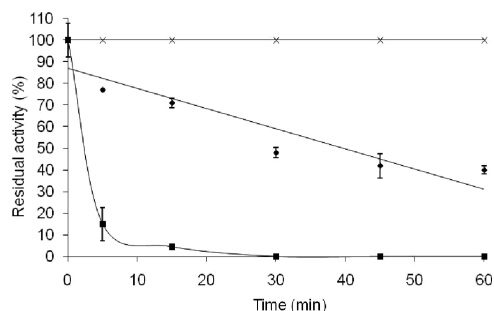

FIG. 4. Thermal inactivation profile of CHA3 at 30°C (×), 45°C (◆), and 50°C (■). Activity is expressed as a percentage of that at zero time in the standard assay at 22°C toward *p*-NP propionate.

values and lower k_{cat}/K_m ratios (27, 28). Whether CHA3 fits with this theory is the subject of subsequent studies.

The Antarctic cold deserts are ice free, and although the mean summer air temperature is around 0°C, surface temperatures during periods of direct sunlight can reach around 15°C (12). ATP analysis of the soils has revealed microbial activity and biomass of a magnitude four times higher than expected, estimated at up to 4×10^8 cells g^{-1} of wet weight (9). The soils support bacterial phyla known to produce extracellular lipases, proteases, and phosphatases (1, 14, 25), and although CHA3 cannot be reliably assigned to any source organism, the enzyme probably originates from a psychrotolerant soil bacterium that is metabolically active when microclimate conditions favor growth.

This work illustrates the advantage of function-based rather than sequence-based screening. At the nucleotide level, the esterase sequence had no identity to any GenBank nucleotide sequences, and it is unlikely that it would have been detected by low stringency PCR-based screening methods. The novelty of the polypeptide backbone, together with its low-temperature-active and alkaliphilic properties, makes it an interesting candidate for industrial application and development. This work highlights the metagenome obtained from Antarctic desert soil as a potential source of enzymes with industrial value.

Nucleotide sequence accession number. The gene sequence obtained in this study has been deposited in the GenBank database under accession number EU874395.

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