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ORIGINAL PAPER

A novel, extremely alkaliphilic and cold-active esterase from Antarctic desert soil

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Abstract A novel, cold-active and highly alkaliphilic esterase was isolated from an Antarctic desert soil metagenomic library by functional screening. The 1,044 bp gene sequence contained several conserved regions common to lipases/esterases, but lacked clear classification based on sequence analysis alone. Moderate (<40%) amino acid sequence similarity to known esterases was apparent (the closest neighbour being a hypothetical protein from *Chitinophaga pinensis*), despite phylogenetic distance to many of the lipolytic "families". The enzyme functionally demonstrated activity towards shorter chain *p*-nitrophenyl esters with the optimal activity recorded towards *p*-nitrophenyl propionate (C3). The enzyme possessed an apparent T_{opt} at 20°C and a pH optimum at pH 11. Esterases possessing such extreme alkaliphily are rare and so this enzyme represents an intriguing novel locus in protein sequence space. A metagenomic approach has been shown, in this case, to yield an enzyme with quite different sequential/structural properties to known lipases. It serves as an excellent candidate for analysis of the molecular mechanisms responsible for both cold and alkaline activity and novel structure–function relationships of esterase activity.

Keywords Metagenomic · Esterase · Cold active · Psychrophilic · Alkaliphilic

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Introduction

The cold deserts of the McMurdo Dry Valleys, South Victoria Land, Eastern Antarctica experience an extremely harsh climate and are one of the most hostile environments on Earth (Cameron et al. 1970; Convey 1996; Wynn-Williams 1990). Despite this, the soils have proven to be unexpectedly high in both microbial biomass (Cowan et al. 2002) and phylogenetic diversity (Aislabie et al. 2006; Niederberger et al. 2008; Smith et al. 2006; Yergeau et al. 2007), with the presence of numerous novel taxa reported. We recently showed that the soils were a valuable source of novel genetic material of possible biotechnological value, wherein the screening of a metagenomic library from these soils resulted in the isolation of a novel esterase (Heath et al. 2009).

Esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) principally catalyse the hydrolysis and synthesis of ester compounds (Park et al. 2007), but typically differ in their ability to hydrolyse substrates of variable chain length. It is widely recognized that, typically, lipases act on water-insoluble (chain length of >10 carbon atoms) triglycerides, while esterases hydrolyse short-chain (chain length of <10 carbon atoms) esters (Rhee et al. 2004). However, although common in most organisms, little is known of their true in vivo function (Arpigny and Jaeger 1999). Nevertheless, both esterases and lipases attract industrial attention because they serve as useful biocatalysts in the detergent and food industries for the production of fine chemicals and in bioremediation processes (Aurilia et al. 2008). Cold-adapted esterolytic enzymes could be of further value, with respect to possible savings in energy input, as compared to their mesophilic counterparts (resulting from high catalytic activities at low temperatures). Furthermore, they can often be easily inactivated by a relatively small elevation of temperature.

Esterases and lipases from extreme environments, including alkaliphilic (Park et al. 2007), halotolerant (Xu et al. 2008), thermophilic (Rhee et al. 2005) and psychrophilic (Elend et al. 2007; Hardeman and Sjoling 2007) habitats, have been reported. These enzymes are commonly identified in metagenomic libraries because the assay is simple and inexpensive, exploiting the fact that the hydrolysis of tributyrin can be visualized as a distinct halo on agar plates. The metagenomic approach possesses two major advantages: (1) the circumvention of cultivation (technically more challenging when dealing with extremophiles) and (2) the possibility of uncovering novel structure–function relationships due to the reliance on functional screening and the high and generally unknown diversity of the genetic source material.

Esterases demonstrating extreme alkaliphily (which we hereafter define as possessing a pH optimum >10) are rare, with examples reported from *Bacillus circulans* (Kademi et al. 2000), a deep sea sediment metagenome (Park et al. 2007) and a compost metagenomic library (Kim et al. 2010). To the authors knowledge, only one other report exists of a cold-active and extremely alkaliphilic esterase (Park et al. 2007). Here, we report a cold-active enzyme with esterolytic activity isolated from an Antarctic desert mineral soil metagenomic library. Furthermore, the enzyme was shown to possess an extremely alkaliphilic pH optimum. Sequence analyses generally supported functional observations, but it was clear that neither sequence analysis nor current functional assays were definitive in the classification of this enzyme.

Experimental procedures

Metagenomic library construction and screening

The construction and screening of Antarctic mineral soil metagenomic libraries have been described previously (Heath et al. 2009). Briefly, following DNA extraction, a large insert DNA library (average insert size was determined to be approximately 30 kb; Heath et al. 2009) was generated using the CopyControl Fosmid Library Production Kit (Epicentre Biotechnologies, WI, USA). The library was screened for clones displaying lipolytic/esterolytic activity by tributyrin hydrolysis on LB tributyrin agar plates (Heath et al. 2009).

Lipolytic gene, CHA2, was identified through disruption of the gene by random Tn5 insertional mutagenesis, using the NEB GPS-M mutagenesis system (New England Biolabs, UK) and screening for functionally inactive mutants. Both the complete gene sequence and the flanking sequences were determined by primer walking, using transposon-specific primers supplied with the mutagenesis kit. The open reading frame of CHA2 was identified using the GeneMark gene prediction tool (http://exon.gatech.edu/GeneMark/) and the amino acid sequence determined using the Expasy TRANSLATE DNA translation tool (http://ca.expasy.org/). Global alignments of amino acid sequences and domain annotation were performed using EMBOSS, clustalW2 (http://www.ebi.ac.uk) and Swissmodel (www.swissmodel.expasy.org). Phylogenetic analysis of the conserved sequence motifs in bacterial lipolytic enzymes and in CHA2 was performed using the CLC genomics workbench software (CLC bio, Cambridge, USA) and the neighbour-joining method (Saitou and Nei 1987). Protein sequences of comparative lipolytic enzymes were retrieved from GeneBank (http://www.ncbi.nlm.nih.gov/).

CHA2 subcloning

CHA2 was amplified using primers 5′-CTGGGATCCATGAAAAAGATATTGTTTATTTTAATTTTAGC-3′ and 3′-GCTCGAGTTACGGGCAAGGCGAACC-3′, introducing *Bam*HI and *Xho*I sites (underlined) at the 5′ and 3′ ends, respectively. The PCR cycling conditions were: an initial denaturing step of 98°C for 30 s, followed by 30 cycles of 10 s at 98°C, 30 s annealing at 60°C and 30 s elongation at 72°C using Phusion polymerase (Fermentas). The amplicon was ligated into the GST-tag expression vector pGEX-6P-2 (GE Healthcare, UK) as a *Bam*HI–*Xho*I fragment.

Expression of CHA2 and refolding of inclusion body protein

CHA2 was expressed in *E. coli* Rosetta™ (Novagen). Cells were grown in 1 L LB (with 50 μg ml^{-1} carbenicillin and 34 μg ml^{-1} chloramphenicol) at 37°C until an OD_{600} of 0.6. The culture was cooled to room temperature, induced with 1 mM IPTG and incubated for a further 6 h at 22°C, 180 rpm.

Purification of protein via the GST fusion tag was attempted, but the expressed protein was consistently found not to bind to the column. An alternative method was used for partial purification of the enzyme. Cells were harvested by centrifugation at 6,000×*g* and lysed in BugBuster Extraction Reagent (Novagen, NJ, USA) supplemented with Benzonase Nuclease (Novagen) according to the manufacturer's recommendations. Inclusion bodies were collected by centrifugation at 10,000×*g* and washed twice in 0.1× culture volume of wash buffer (20 mM Tris–HCl, pH 7.5, 10 mM EDTA, 1% Triton X-100). Protein was resolubilized according to the Protein Refolding Kit protocol (Novagen) in CAPS buffer (pH 11.0) with 6 M urea and dialysed twice against 20 mM Tris–HCl, pH 8.5, with 0.1 mM DTT and twice against 20 mM Tris–HCl, pH 8.5, with 10% glycerol, each for at least 3 h at 4°C.

Determination of kinetic parameters and substrate specificity for CHA2

Short-chain *p*-NP esters rapidly autohydrolyse at pH levels above 7.5, and all *p*-NP ester substrates are unstable at temperatures above 25°C. Therefore, a standard assay was used to assess esterase activity. Assays were performed for 3 min under standard, predefined conditions with deviations described in text [standard conditions defined herein as: 22°C, pH 7.5 and in 100 mM sodium phosphate (pH 7.5), 100 mM NaCl, 1% acetonitrile and 0.01% (v/v) Triton X-100]. Activity was routinely assessed against 0.5 mM *p*-nitrophenyl decanoate, unless otherwise stated, due to the instability issues discussed above (Heath et al. 2009), but V_{max}, K_m and k_{cat} were determined via the measurement of the rate of hydrolysis of *p*-NP propionate and over a substrate concentration range of 0.01–1 mM. The Lineweaver–Burk plot was used to determine these kinetic parameters. Production of *p*-nitrophenol was followed at 405 nm in a Cary 50 Bio spectrophotometer (Varian, CA, USA). An extinction coefficient of 13,900 M^{-1} cm^{-1} was determined for *p*-nitrophenol under the standard assay conditions. One unit of activity is defined as the amount of enzyme required to liberate 1 µmole of *p*-nitrophenol per minute. Substrates were dissolved in acetonitrile, except *p*-NP-C16, which was dissolved in acetonitrile:isopropanol (1:1) to improve solubility. One unit of enzyme activity (U) is defined as 1 µmol of *p*-nitrophenol released per min at 22°C. All assays were carried out in triplicate. CHA2 activity towards *p*-nitrophenyl acetate (C2), propionate (C3), octanoate (C8), decanoate (C10), laurate (C12) and palmitate (C16) was determined on 0.5 mM of the *p*-NP ester substrate. CHA2 activity was determined over a temperature range of 5–60°C and over a pH range of 5–12, under standard conditions. To cover the full pH range, several buffers were used: 100 mM Tris–HCl (pH 7.0–9.0), 100 mM CAPS (pH 9.0–11.0) and 100 mM sodium phosphate buffer (pH 11.0–12.1).

Results

Sequence analysis

CHA2 was identified by functional screening of an Antarctic desert soil metagenomic library. The encoding ORF was 1,044 nucleotides in length and the translated polypeptide had a predicted molecular mass of 34.7 kDa and an isoelectric point of 9.21. The GC content of the gene was determined to be 49%. Putative upstream promoter regions and ribosome binding sites were also identified. With regard to the contextual location of the gene, CHA2 was shown to be flanked upstream by a gene encoding a UTP-glucose-1-phosphate uridyl transferase and downstream by a seryl-tRNA synthetase. The sequence of CHA2 was deposited with Genbank under the accession number EU874394.

The deduced amino acid sequence contained conserved residues common to esterases/lipases. An alignment of CHA2 with related lipolytic/esterolytic sequences obtained from the NCBI database (Fig. 1) demonstrated that the common lipolytic sequence motif GXSXG that forms part of a pentapeptide signature 'elbow' near the active site (Brady et al. 1990) was present in CHA2 as GHSYG. The serine residue within this motif has been identified as contributing to the catalytic triad, which in other lipolytic/esterolytic enzymes also includes a conserved histidine residue and either a glutamate or aspartate (Brady et al. 1990; Jaeger et al. 1994). A conserved aspartate residue (residue 332 in Fig. 1) was identified in the amino acid sequence of CHA2 and is as a putative further participating residue in the catalytic triad (Fig. 1). Typically, a histidine residue forms the third member of the triad, but from the alignment no appropriate histidine residue aligned with those known to perform this function in the sequences used for comparison. In a separate alignment of CHA2 (not shown) with those sequences identified as most similar (BlastP and including ZP_03852712.1 and ZP_04360640.1), conserved histidine residues were identified but not in the typical locale associated with lipase sequence alignments and the catalytic triad. Other lipolytic sequence motifs identified included a putative oxyanion binding region, PVXXXXHG (PVVFFAHG in CHA2).

Phylogentic alignments demonstrated clearly that CHA2 was distantly related to two new families of bacterial lipases which have recently been reported: Family A (Lee et al. 2006) and Family B (Tuffin et al. 2009). From phylogenetic analyses, CHA2 is phylogenetically very deeply rooted in relation to both families A and B (Fig. 2). Simple BlastP analyses of CHA2 show that it shares moderate similarity to current known esterolytic/lipolytic sequences (37% and 35% amino acid identity to esterases/lipases from *Chitinophaga pinensis* and *Chryseobacterium gleum*, respectively). No global model could be generated via Swissmodel with a suitable significance score (QMEAN score >0.5), although a conserved alpha beta hydrolase fold common to lipases/esterases was found to be contained within a domain unusually possessing significant similarity (E value < 0.01) to a related fold in chlorophyllase, which catalyses ester hydrolysis as part of chlorophyll degradation. For protein purification, CHA2 was cloned into pGEX-6P-2, which expresses an N-terminal glutathione-S-transferase (GST) fusion tag. Expression was successful under these conditions, but the majority of protein was in an insoluble form. Refolding from inclusion bodies yielded high amounts of active protein, which was purified to near homogeneity (Fig. 3).

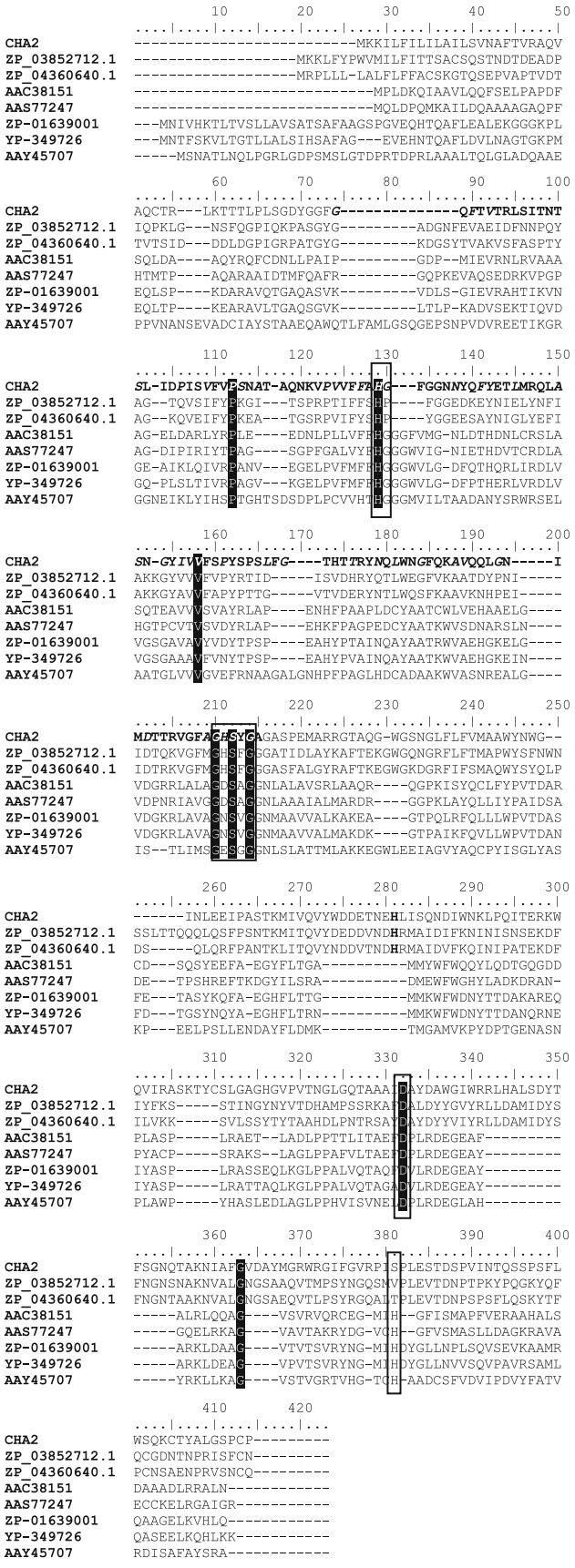

Fig. 1 A sequence alignment of CHA2 with a putative protein from *C. pinensis* (ZP_04360640.1), a lipase from *C. gleum* ATCC 35910 (ZP_03852712.1) and a number of Family I type of lipases [*Pseudomonas sp. B11-1* (AAC38151), uncultured bacterium (AAS77247), *Pseudomonas putida W619* (ZP_01639001), *Pseudomonas fluorescens pfO-1* (YP_349726) and uncultured bacterium (AAY45707)]. Alignments were made using ClustalW (http://www.ebi.ac.uk). The conserved G–X–S–X–G motif (containing the catalytic serine residue) and the catalytic Asp residue are indicated (positions 210–214 and 332, respectively). The position of the partially conserved oxyanion hole is also indicated (positions 129–130) as is the likely position of the common third conserved catalytic residue (position 381). At this locus in CHA2, no conserved histidine is present. At position 281 (shown in *bold*), there is a histidine residue that is conserved in CHA2 and the two closest sequences as identified by Blastp analysis, possibly representing a novel conserved motif or loop involved in the catalytic site. Other conserved residues are also highlighted including (*bold* in the CHA2 sequence) the region identified as similar to a chlorophyllase domain (*italic* residues are those which aligned with regions of homology in the conserved domain PLN00021 as identified by BlastP analysis; NCBI)

Biochemical analysis of CHA2

The empirical determination of the extinction coefficient of *p*-nitrophenol under the predefined conditions stated above was in accordance with previously reported values (Karadzic et al. 2006; Elend et al. 2006) The kinetic parameters determined for CHA2 on pNPC3 were: K_m of 0.44 mM and V_{max} of 0.45 U mg^{-1} (a unit is defined as 1 µmole of p-nitrophenyl released per min). The k_{cat} for CHA2 under these conditions was determined to be 0.23 s^{-1}. CHA2 was active with a range of pNP esterases [acetate (C2) to *p*-NP laurate (C12)], with the greatest activity towards *p*-NP propionate (C3). The hydrolytic activity decreased significantly as the chain length increased above C8, with no activity towards *p*-NP palmitate (C16), suggesting that CHA2 was an esterase and not a true lipase (Fig. 4). CHA2 was active over a temperature range of 5–40°C, with an optimum temperature for activity of 20°C (Fig. 5). No activity could be detected at <5°C, and the activity detected at 40°C remained approximately 40% of the maximum activity detected. Assays of CHA2 over a pH range of 5–12 showed optimum activity at pH 11 and relatively little activity between pH 5 and 9, suggesting that the enzyme was a highly alkaliphilic esterase (Fig. 6a). Even at pH 12, up to 60% of the maximal activity still remained. The auto-hydrolysis of *p*-NP decanoate is shown in Fig. 6b (insert) to demonstrate that at such extremes of pH, even longer chain *p*-NP esters are instable. The differences between enzymatic and non-enzymatic hydrolysis are accounted for in the data presented in Fig. 6a.

Discussion

Using a metagenomic approach, a novel protein with lipolytic activity was isolated from an Antarctic Dry Valley

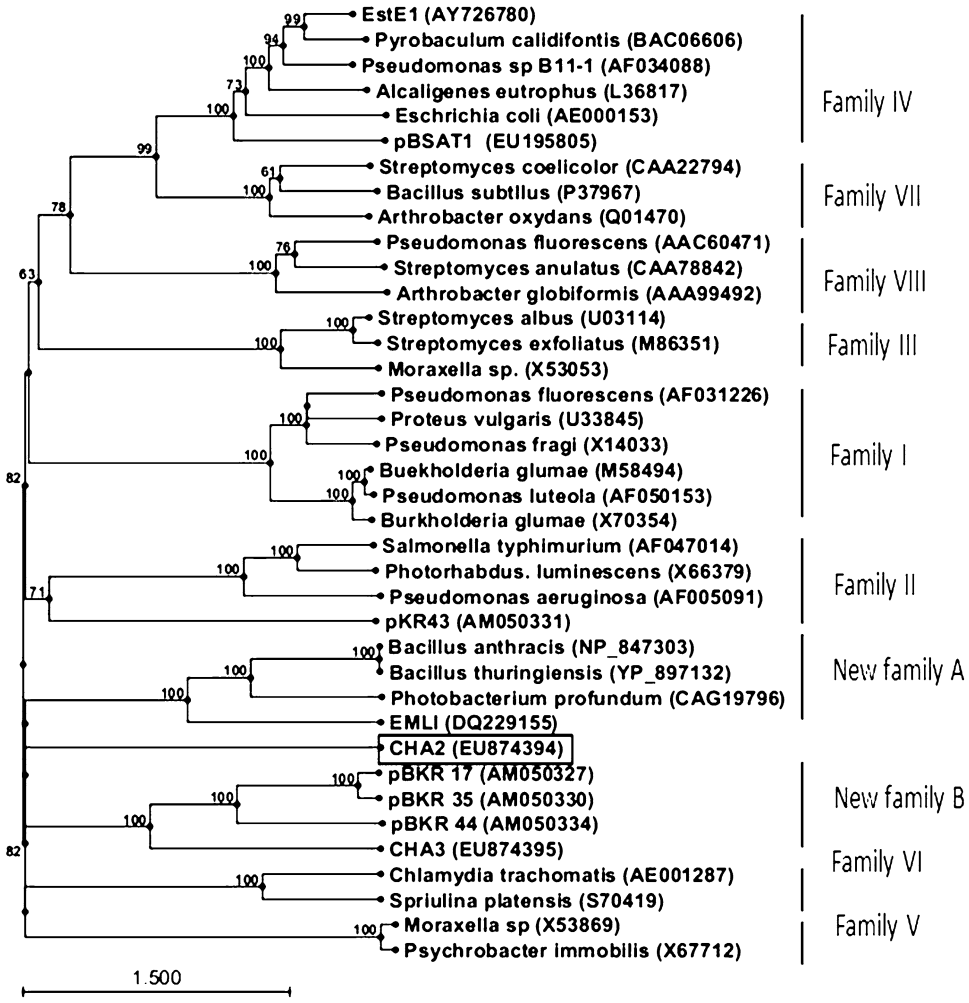

Fig. 2 A phylogenetic analysis of CHA2 (indicated) and selected lipolytic sequences discovered by functional screening of metagenomic libraries based on conserved sequence motifs of bacterial lipolytic enzymes. Phylogenetic analyses were performed using the neighbour–joining method and on the software CLC genomics workbench software (CLC Bio). Protein sequences were retrieved from GeneBank (http://www.ncbi.nlm.nih.gov/). *Bar* indicates bootstrap values with values <50 excluded (1 substitution per amino acid site)

soil fosmid library. It was found to possess entirely different kinetic properties and nucleic acid sequence to CHA2, an esterase isolated from the same library and previously reported (Heath et al. 2009). This extremely cold desert environment has been shown to contain biomass at an estimated 4×10^8 cells g^{-1}, as measured by ATP analysis, around four orders of magnitude higher than originally expected (Cowan et al. 2002). These soils harbour a rich pool of unidentified microbial species (Smith et al. 2006) and are proving to be an excellent source of novel genetic material for both fundamental and applied studies.

Antarctic soil metagenomes can be screened with relative ease; the mineral soil contains very little humic acid, making extraction and library construction simple. The low frequency of higher eukaryotes in this habitat also greatly reduces eukaryotic DNA contamination of libraries. Screening of 10,000 clones from this metagenomic library resulted in identification of a number of clones demonstrating lipolytic activity of which one was chosen for this study and denoted CHA2. This hit rate (approximately, 1 gene per 100 MB DNA) was comparable with those from agricultural (Rondon et al. 2000), forest (Lee et al. 2004) and geothermal soil metagenomic libraries (Rhee et al. 2005), but low considering that lipolytic/esterolytic genes are widespread across bacterial genera and that some prokaryotic genomes possess multiple esterase/lipase genes (Gupta et al. 2004). This perhaps illustrates the limitations of the recognition of the intrinsic promoter by the host strain, choice of host strain (*E. coli* as opposed to a psychrophilic host) and an absence of barriers for heterologous expression (Gustafsson et al. 2004, Knietsch et al. 2003).

From sequence analyses, both the putative catalytic serine residue (in the GHSYG motif) and a conserved catalytic aspartate residue were identified in sequence alignments with CHA2, but the known catalytic histidines of the Family I lipases (indicated in Fig. 1) did not correlate with any histidine residue in CHA2 in this alignment. Furthermore, due to the dissimilarity of the sequence of CHA2 to any of the available solved structures of lipolytic enzymes, the identification of the catalytic site by comparative modelling was not feasible. It is apparent that,

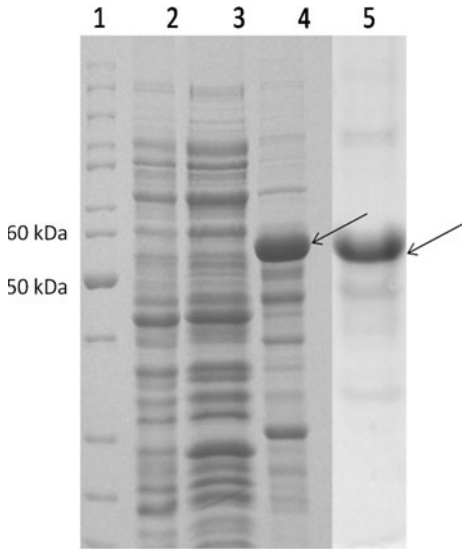

Fig. 3 SDS-PAGE analysis of CHA2 expression and purification. *Lane 1* molecular weight marker; *lane 2* uninduced total protein extract; *lane 3* soluble fraction after 6 h induction with IPTG; *lane 4* insoluble fraction after 6 h induction with IPTG (CHA2 represents 47% of the total insoluble protein); *lane 5* soluble, refolded CHA2–GST fusion protein (CHA2 represents 85% of the total protein present in this fraction). The purified CHA2–GST fusion protein is indicated (*arrow*)

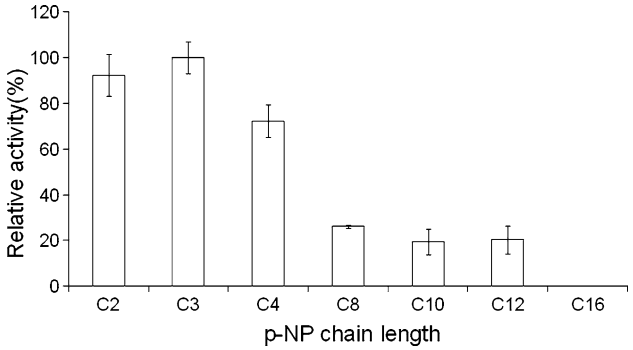

Fig. 4 Activity of CHA2 towards p-nitrophenyl esters of varying chain lengths (C2, acetate; C3, propionate; C4, butyrate; C8, octanoate; C10, decanoate; C12, laurate; C16, palmitate). Activity against *p*-NP-C3 was taken as 100%. *Error bars* represent the standard deviation. A specific activity of 0.12 U/mg was recorded as 100% on C3

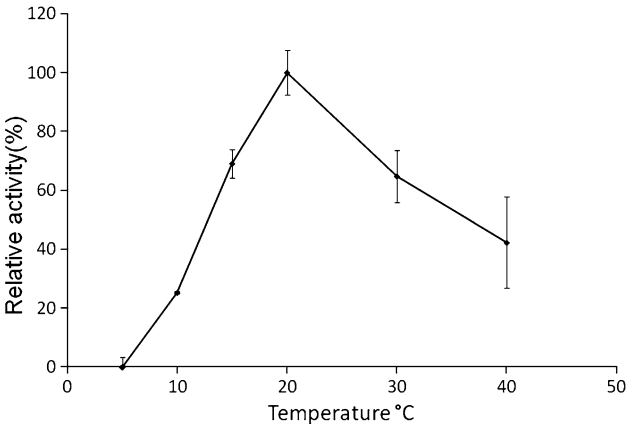

Fig. 5 Effect of temperature on CHA2 activity towards *p*-NP-decanoate. Activity at 20°C was taken as 100%. *Error bars* represent the standard deviation. A specific activity of 0.05 U/mg was recorded as 100% at 20°C

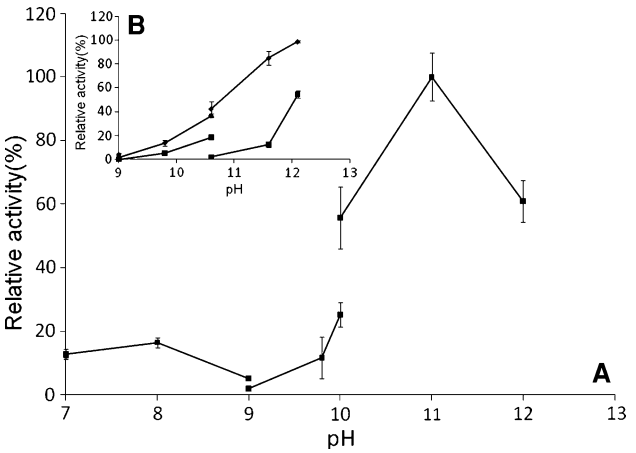

Fig. 6 **a** pH activity profile for CHA2 esterase on *p*-NP decanoate. Buffer systems were Tris–HCl (7.0–9.0), CAPS (9.0–10.0) and sodium phosphate (11.0–12.1). **b** (*insert*) Initial rate data for esterase catalysed (*diamond* data points) and non-enzymatic (*square* data points) hydrolysis of *p*-NP decanoate in a pH range of 9.0–12.1. The on-enzymatic hydrolysis has been accounted for in the data presented in 6A. *Error bars* represent the standard deviation. A specific activity of 0.4 U/mg was recorded as 100% at pH 11

although classification of esterases and lipases relies, in part, on sequence analyses, no definitive rules are applied in the identification of motifs. For example, catalytic dyads have been reported, such as in a novel esterase of *Streptomyces scabies* (Wei et al. 1995), which possesses only a serine and histidine residue in the catalytic site, the latter residue stabilized by a Trp amino acid found 3 positions upstream. Such an arrangement was not apparent in CHA2, but is used to indicate that currently our knowledge of the lipase/esterase families and their various catalytic mechanisms is incomplete. In separate alignments of CHA2 with sequences identified as most similar (Blastp analysis), conserved histidine residues were identified, but not in the expected locus (an example of such a conserved histidine is shown at nucleotide position 281 in Fig. 1). It is feasible therefore that a degree of flexibility exists in the catalytic site of lipases/esterases, which would account for this observation, or that a novel structural fold is apparent in CHA2 that would place a hitherto unreported conserved histidine within the active site. The novelty of folding in CHA2 is further highlighted in the identification of similarity between the alpha beta hydrolase fold of CHA2 and that found in chlorophyllase of higher organisms.

Certainly, such similarities between bacterial lipases/esterase have been reported before (Bornscheuer 2002) and usually for enzymes derived from metagenomic libraries wherein distinct sequence-structure novelty has also been seen.

Cloning in pGEX-6P-2 resulted in the expression of CHA2 with an N-terminal glutathione-S-transferase (GST) fusion tag. The level of CHA2 expression in *E. coli* in this construct was high, but the protein was almost exclusively found in inclusion bodies. Attempts to express CHA2 in the alternative *E. coli* host system Arctic Express (Stratagene, CA, USA), which co-expresses chaperones Cpn60 and Cpn10 from *Oleispira antarctica* to facilitate folding, did not improve the yield of soluble protein. There are some advantages in purifying recombinant protein from inclusion bodies, most notably that the protein is protected from proteolytic degradation. Furthermore, inclusion bodies usually comprise the expressed recombinant protein in a near-homogenous form and so effectively introduce a good purification step (Park et al. 2007).

After refolding from inclusion bodies, high quantities of active protein were obtained. However, attempts to remove the GST fusion tag from the active protein with PreScission Protease (GE Healthcare) were unsuccessful. It is assumed that the folding of the CHA2 protein and the GST fusion tag sterically hindered the cleavage site preventing access by the protease. It had previously been shown with a refolded esterase-GST fusion tag complex (CHA3) that the GST fusion tag possessed no esterolytic activity and did not affect the kinetic parameters of the enzyme (Heath et al. 2009; Heath, personal communication).

From kinetic analyses, it was clear that the pNP esters represented a range of substrates on which CHA2 demonstrated measurable activity, albeit slow, and therefore these substrates/assay were convenient for further study. The low optimum temperature for activity (Fig. 4) indicates that CHA2 is a cold-active enzyme as might be expected from its source and this value is certainly lower than other reported cold-active lipolytic/esterolytic enzymes such as the esterase PsyEst from *Psychrobacter* sp. Ant300 (Kulakova et al. 2004) and CHA3 (Heath et al. 2009), both from Antarctic soil, and lipase KB-Lip from a psychrotrophic *Pseudomonas* sp. strain KB700A (Rashid et al. 2001). All of these enzymes showed optimal activities between 35 and 40°C, indicative that, often, enzymes do not necessarily have to function optimally at the temperature of their environment but merely function to a degree that gives a physiological advantage. CHA2 was strongly alkaliphilic and although Antarctic soils have a pH of between 8 and 9 (Vishniac 1993), the pH optimum for activity of 11 is surprisingly high. Enzymes have certainly been reported with pH optima around pH 11 including a number of alkaline proteases (Horikoshi 1999), endoglucanases (Aygan and Arikan 2008) and lipases (Rathi et al. 2000; Watanabe et al. 1977; Karadzic et al. 2006; Ghanem et al. 2000). Very few esterases possessing an extremely alkaline pH optimum have been reported (Park et al. 2007; Kim et al. 2010; Kademi et al. 2000). Neither the molecular basis nor physiological purpose of such an extremely high pH optimum of this enzyme is immediately apparent. However, it has been reported previously that esterases from soil metagenomes have shown significant activities and stabilities under alkaline conditions that are not directly linked to their environment (Elend et al. 2006; Park et al. 2007).

In conclusion, functional screening of an Antarctic derived metagenomic library resulted in the isolation of the esterase CHA2. At the amino acid level, low identity (<40%) was found to known lipases/esterases and so this activity was confirmed via enzyme assay and characterization. Only two of the three catalytic residues of a presumptive triad could be reasonably identified and so the catalytic mechanism remains unclear. Functionally, CHA2 was found to be highly alkaliphilic (optimum at pH 11) and cold active (optimum at 20°C), a somewhat unique trait for this enzyme. It is therefore reasonable to assume that CHA2 originated from a psychrotolerant and alkaliphilic organism. Kinetic studies of CHA2 were only briefly explored since it was clear pNP esters were poor homologues of the native substrate (low apparent V_{max} on C3) and no further evidence was apparent in order to rationalize a better substrate homologue.

Collectively, this data serves to validate the metagenomic approach as a means to identify novel sequence–structure–function relationships. It is perhaps unsurprising, considering the approach chosen that certain ambiguities remain as to sequence annotation and substrate choice for functional characterization. The novelty of the CHA2 polypeptide backbone, the absence of solved protein structures on which to model it, poly-extremism and a possibly novel structure–function relationship render it an interesting candidate for crystallography studies.

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