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ORIGINAL PAPER

The cold-active Lip1 lipase from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 is a member of a new bacterial lipolytic enzyme family

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Abstract The genome of the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 was searched for the presence of genes encoding ester-hydrolysing enzymes. Amongst the others, the gene PSHAa0051 coding for a putative secreted esterase/lipase was selected. The psychrophilic gene was cloned, functionally over-expressed in *P. haloplanktis* TAC125, and the recombinant product (after named *Ph*TAC125 Lip1) was purified. *Ph*TAC125 Lip1 was found to be associated to the outer membrane and exhibited higher enzymatic activity towards synthetic substrates with long acyl chains. A structural model was constructed using the structure of carboxylesterase Est30 from *Geobacillus stearothermophilus* as template. The model covered the central part of the protein with the exceptions of *Ph*TAC125 Lip1 N- and C-terminal regions, where the psychrophilic protein displays extra-domains. The constructed model showed a typical α/β-hydrolase fold, and confirmed the presence of a canonical catalytic triad consisting of Ser, Asp and His. The sequence analysis showed that *Ph*TAC125 Lip1 is distantly related to other lipolytic enzymes, but closely related to other putative psychrophilic esterases/lipases. The aligned proteins share common features, such as: (1) a conserved new active-site pentapeptide motif (LGG(F/L/Y)STG); (2) the likely extracytoplasmic localization, (3) the absence of a typical calcium-binding pocket, and (4) the absence of a canonical lid. These observations strongly suggest that aligned proteins constitute a novel lipase family, typical of psychrophilic marine γ-proteobacteria, and *Ph*TAC125 Lip1 could be considered the first characterised member of this family.

Keywords *Pseudoalteromonas haloplanktis* TAC125 · Psychrophilic bacterial strain · α/β Hydrolase

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Introduction

The world of bacterial lipases is rapidly growing and there is a steadily increasing demand to identify, characterise and produce lipases for a variety of biotechnological applications, with special emphasis on enantioselective biotransformations (Jaeger and Reetz 1998; Jaeger and Eggert 2002). Enzymes from psychrophilic micro-organisms have recently received increasing attention due to their relevance for both basic and applied research. Indeed, cold-adapted enzymes might offer novel opportunities in many industrial processes in which high enzymatic activity or peculiar stereospecificity at low temperature and low thermostability are required. For instance, cold-active lipases are

expected to be applicable as additives to detergents used at low temperatures and biocatalysts for biotransformation of heat-labile compounds (Margesin and Shinner 1994).

Esterases and lipases (carboxylester hydrolases of group EC 3.1.1.1) catalyse the hydrolysis (and synthesis) of an ester bond resulting in the formation of an alcohol and a carboxylic acid. Both types of enzymes belong to the family of serine hydrolases and share structural and functional characteristics, including a catalytic triad, an α/β hydrolase fold and a cofactor-independent activity. Esterases hydrolyse the ester bonds of water-soluble fatty acid esters with short-chain acyl groups ($C \leq 8$), whereas lipases (EC 3.1.1.3) hydrolyse long-chain acylglycerol ($C \geq 8$) (Jaeger et al. 1999).

The structural family of α/β hydrolases comprises a wide variety of enzymes whose activities rely mainly on a catalytic triad usually formed by seryl, histidyl, and aspartyl residues. Ser usually appears in the conserved pentapeptide G-X-S-X-G; this motif is usually located in the sharp turn between a β-strand and an α-helix, known as the nucleophilic elbow (Akoh et al. 2004). Bacterial esterases and lipases have been grouped into eight families, based on comparison of their amino-acid sequences and some fundamental biological properties (Arpigny et al. 1999). Enzymes in Family 1 are called true lipases and are further classified into six subfamilies. Enzymes belonging to Family 2–8 are esterases. However, the in silico analysis of complete bacterial genomes highlighted the presence of putative esterase/lipase encoding genes whose sequences cannot be grouped into any of the previously mentioned enzyme families. For instance, the extracellular depolymerases PhaZ7 of *Paucimonas lemoignei* was classified into the new Family 9 of esterases (Handrick et al. 2001) because of absence of significant amino acid homology, while the carboxylesterase (EstD) of *Thermotoga maritima* and related sequences have been grouped into the novel Family 10 (Levisson et al. 2007).

Recently, the genome of *Pseudoalteromonas haloplanktis* TAC125 (*P. haloplantkis* TAC125), a Gram-negative bacterium isolated from Antarctic seawater, has been elucidated (Medigue et al. 2005). It is the first completed genome from an Antarctic organism. By combining in silico genome analysis with *in vivo* functional characterisation, several molecular strategies were highlighted, accounting for the remarkable versatility and adaptation to psychrophilic lifestyle of *P. haloplantkis* TAC125.

Several genes encoding putative esterase/lipase were annotated in the genome of *P. haloplanktis* TAC125, and recently one of them was functionally characterised (Aurilia et al. 2007). The PSHAa1385 gene was expressed in *Escherichia coli* and the encoded product was identified as a cytoplasmic, dimeric feruloyl esterase.

In this paper, we focused our attention on another *P. haloplanktis* TAC125 gene, PSHAa0051, selected on the basis of the peculiar features of the encoded product that was annotated as "putative secreted esterase". The gene was over-expressed in *P. haloplanktis* recombinant cells by using the "cold" gene-expression technology we have developed in the cold-adapted bacterium (Duilio et al. 2004). The recombinant enzyme (denoted *Ph*TAC125 Lip1), found associated to the bacterial outer membrane although containing none of the described anchoring domains, was purified and functionally characterised. A homology model was built using the carboxylesterase Est30 from *Geobacillus stearothermophilus* as template. A search for the presence of canonical motifs described in esterases and lipases enzymes by Arpigny (Arpigny and Jaeger 1999) demonstrated that *Ph*TAC125 Lip1 seems to be very distantly related to other lipolytic enzymes, but closely related to several putative esterases and lipases from psychrophilic micro-organisms. Data presented in this paper support the idea that *Ph*TAC125 Lip1 belongs to a novel group of lipolytic enzymes.

Materials and methods

Bacterial strains, culture conditions and genetic manipulations

P. haloplanktis TAC125 is a Gram-negative bacterium isolated from Antarctic seawater (Médigue et al. 2005). It was grown in aerobic conditions at 4°C and 15°C in TYP broth (16 g/l yeast extract, 16 g/l bacto tryptone, 10 g/l marine mix) at pH 7.5, supplemented with 100 μg/ml ampicillin, if transformed. Antarctic bacteria transformation was achieved by intergeneric conjugation as previously reported (Duilio et al. 2004). *E. coli* DH5α (Hanahan 1983) cells were used as host for gene cloning and were routinely grown in Luria–Bertani broth (Sambrook and Russell 2001) containing 100 μg/ml ampicillin if transformed.

Computational search and identification of lipase-encoding sequences

The genome sequence of *P. haloplanktis* TAC125 was accessed through the MaGe annotation platform (https://www.genoscope.cns.fr/agc/mage/wwwpkgdb/Login/log.php?pid=7#ancreLogin) and was searched for the presence of putative lipases/esterases by using the Explore functionality. The database-search criterion was the presence of the words "lipase and/or esterase" in the annotation tags. The PSHAa0051 gene ("*PSHA*" stands for *P. haloplanktis*, "*a*" denotes chromosome I and the figure indicates the gene

position) was selected for further analysis. It contains the InterPro family motif IPR 000379, found in all lipase/esterase enzymes belonging to the α/β hydrolase superfamily.

Multiple sequence alignments

Multiple sequence alignments were carried out with the program Multalin version 5.4.1 (Corpet 1988) using the following lipase sequences: *Colwellia psychroerytrea* (*Colpsy*, A.N. Q48AN1), *Pseudoalteromonas tunicata* D2 (*Pseutun* A.N. A4CF12), the putative lipase/esterase Q2SGZ8 from *Hahella chejuensis* (Jeong et al. 2005), *G. stearothermophilus* (*Geost* Est30, PDB ID ITQH:A), and *P. haloplanktis* TAC125 (*Ph*TAC125 Lip1, A.N. Q3IF07). The alignment featuring the highest score was obtained using the Blosum 62 matrix (Henikoff and Henikoff 1992) and standard Multalin parameters.

Construction of a cold expression vector (pPM13*lip1*) for the recombinant *Ph*TAC125 Lip1 production in *P. haloplanktis* TAC125

A cold-adapted gene-expression vector was constructed by cloning the *Ph*TAC125 *lip1* gene sequence into the pPM13 expression vector (Papa et al. 2007). The *Ph*TAC125 Lip1 gene was amplified from the *P. haloplanktis* TAC125 genome by PCR to introduce *Nde*I and *Bam*HI restriction sites (primers PSHA0051fw 5′-CAATCTGCAGTGGCATATGAACAATAATAG-3′ and PSHA0051rev 5′-GCGAAACTGCAGCCAGGATCCCTTACCTTGTGC-3′) and to remove the translation stop codon. The resulting PCR product was subjected to *Bam*HI digestion, filled in and digested by *Nde*I. A gene fragment encoding the *c-myc* tag was PCR-amplified from the p*ScpelB* vector (kindly provided by Dr B. Soehling, University of Halle, Germany) by using the primers tagNofw (5′GTGGAGTAGTAA TCCACTCACTTTCGG3′) and tagErev (5′GCTTGTCGA ATTCCTATGCGGCCC3′) to introduce *Not*I and *Eco*RI restriction sites, respectively. The product was subjected to *Not*I digestion, filled in and digested by *Eco*RI. Final recombinant vector (pPM13*lip1*) was constructed by cloning the two DNA fragments into pPM13 vector, previously digested by *Nde*I and *Eco*RI. Finally, the sequence of the amplified fragments was checked to rule out the occurrence of any mutation during synthesis.

Recombinant *Ph*TAC125 Lip1 production and cellular localization

P. haloplanktis TAC125(pPM13*lip1*) recombinant cells were grown at 4 and 15°C and the *Ph*TAC125 Lip1 production and its cellular localization were evaluated at different growth phases. Cell pellets (corresponding to 6.5 mg of dry weight) were re-suspended in 2 ml of 50 mM Tris–HCl pH 7.5, 50 mM EDTA and disrupted by ultrasonic treatment consisting in six cycles of 30 s on/1 min off, on ice. The mixture was centrifuged for 15 min at 10,000×*g* at 4°C; the resulting supernatant and pellet were collected as soluble and insoluble fractions, respectively.

Total cell extract was obtained by resuspending 6.5 mg of dried cells into 400 µl of SDS loading dye, followed by 30 min treatment of at 90°C.

Proteins contained into 2 ml of extra-cellular medium were precipitated by adding ice-cold trichloroacetic acid (TCA) at a final concentration of 10% followed by overnight incubation at 4°C. The samples were centrifuged at 10,000×*g* for 30 min at 4°C. The protein content was determined with the Bio-Rad assay kit using bovine serum albumin as standard.

Inclusion body preparation was performed by low-speed centrifugation (at 2,000×*g* at 4°C, for 20 min) of protein mixture derived by ultrasound treatment described above. The pellet was re-suspended in 1 ml of 50 mM Tris–HCl pH 8, 1 mM EDTA, and centrifuged at 1,800×*g* at 4°C, for 20 min. This wash was repeated two times and the inclusion bodies were lastly dried by a centrifugation step of 5 min at 9,500×*g* at 4°C, re-suspended in 50 µl of loading buffer and analysed by Western blotting.

*Ph*TAC125 Lip1 extraction from the insoluble cellular fraction

Six hundred and fifty milligram (dry weight) of wild type *P. haloplanktis* TAC125 or pPM13*lip1* recombinant cells were resuspended in 30 ml of 50 mM sodium phosphate pH 7.1, 1 mM EDTA, 1 mM PMSF buffer, and disrupted by French press treatment (Sinstem, Limited Basic Z Model), applying a 1.6-Kbar pressure. The mixture was centrifuged at 12,000×*g* for 30 min at 4°C, and the pellet, corresponding to the insoluble fraction, was collected. Aliquots of insoluble fraction were then treated with 50 mM sodium phosphate pH 7.1 containing one of the detergents (Triton X-100, CHAPS, Tween 20, Tween 80, Na-cholate and SDS) at 1.0% (v/v) final concentration, and slowly mixed at 15°C for 1, 3, 5, 7, 17 h. After incubation, each mixture was centrifuged at 12,000×*g* for 20 min at 4°C, the supernatant being the "membrane solubilized fraction".

Protein extraction by Triton X-100 treatment was performed on intact *P. haloplanktis* TAC125-pPM13*lip1* cells, grown in TYP broth for 72 h at 4°C. The cells were then collected by centrifugation, and incubated in an equal volume of fresh broth supplemented with 0.5% Triton X-100 at 4°C. After 5 h incubation, the culture was

centrifuged at 12,000×*g* for 20 min at 4°C, and the supernatant (consisting of growth-medium containing several extracted proteins) collected as "medium solubilized fraction".

*Ph*TAC125 Lip1 purification from "medium solubilized fraction" of recombinant cells

All procedures were performed at 4°C. Medium-solubilized fraction measuring 140 ml was concentrated on an Amicon apparatus equipped with a YM1 membrane (1,000-Da cut off) and dialysed against 30 mM Tris–HCl pH 8.0 at 4°C for 16 h. Solid ammonium sulphate was then slowly added under stirring to bring the solution to 23% saturation. The suspension was incubated at 4°C under gentle agitation for 2 h and then centrifuged at 12,000×*g* at 4°C for 20 min. The pellet was dissolved in a minimal volume (20 ml) of 10 mM ethanolamine pH 10.0 (Buffer A). The enzyme solution was dialysed against the same buffer and loaded onto a Q-Sepharose FF (Amersham) column (15 × 1.5 cm) equilibrated with same buffer, and connected to a BioLogic Workstation (Bio-Rad). After extensive washing, a linear 0–1 M-NaCl gradient in Buffer A was applied to separate the proteins. Active fractions for esterase activity were eluted at approx. 800 mM NaCl, pooled, concentrated in a Savant centrifuge and dialysed against Buffer A. The presence of *Ph*TAC125 Lip1 recombinant protein was evaluated by enzyme assays and Western blotting. Table 1 summarises the purification of the recombinant *Ph*TAC125 Lip1.

Enzyme assays and effects of various compounds on the *Ph*TAC125 Lip1 activity

Esterase-catalysed hydrolysis of *p*-nitrophenyl esters was followed by monitoring the production of *p*-nitrophenol at 405 nm in 1-cm path-length cuvettes using a single-beam HP ultraviolet spectrophotometer (Manco et al. 1994). The standard assay mixture contained 40 mM sodium–phosphate buffer pH 7.1, 0.36% Triton X-100, 2% propan-2-ol, 0.2 mM *p*-nitrophenyl caproate. Stock solutions of *p*-nitrophenyl esters from C_2 to C_{10} were prepared by dissolving substrates in acetonitrile. Samples of identical composition as the assay mixture, omitting the enzyme, provided suitable blanks. One activity unit is the amount of protein releasing 1 µmol *p*-nitrophenoxide/min. The absorption coefficients were measured at each indicated temperature and ranged between 14,000 and 21,000 M^{-1} cm^{-1}. The pH variation with temperature was duly taken under consideration.

The purified fraction containing *Ph*TAC125 Lip1 was incubated at 25°C for 1 h in a 20 mM Tris–HCl buffer pH 7.9, supplemented with several potential inhibitors (Table 2) at 10 mM final concentration. The residual activity was measured with *p*-nitrophenyl caproate at the same temperature.

Electrophoresis and activity stain

Electrophoretic runs were performed at room temperature in a Bio-Rad Mini Protean II cell unit. SDS-PAGE (12%) was performed as described by Laemmli (1970). Activity staining was performed on denatured gels using a fluorogenic substrate, 4-metylumbelliferone-butyrate (MUF-butyrate), as described by Diaz et al. (1999).

Protein samples for SDS-PAGE were prepared under denaturing (1% SDS, 95°C, 20 min) and reducing conditions and separated on SDS-containing polyacrylamide (12%) gels using standard methods (Sambrook and Russell 2001). For immunoblotting, the gels were transferred to a polyvinylidene difluoride membrane (Immobilon PSQ, Millipore). The immunodetection of protein was performed using anti-*c-myc* monoclonal antiserum (Inalco-Calbiochem) prepared in blocking buffer (PBS; 5% skimmed milk). Anti-rabbit IgG peroxidase conjugate was used as secondary antibody. Proteins were detected by chemiluminescence using horseradish-peroxidase conjugate antibodies (Super Signal West Dura Pierce, USA).

Homology modelling

*Ph*TAC125 Lip1 was modelled using *G. stearothermophilus* carboxylesterase (Liu et al. 2004) (1TQH pdb entry) as template. The atomic coordinates of *Geost* Est30 in its open conformation were obtained from the Brookhaven Protein Data Bank. The template was selected according to the position of the catalytic triad (Ser, Asp,

Table 1 Purification of the recombinant *Ph*TAC125 Lip 1

Step	Total protein[a] (mg)	Total activity[b] (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Solubilised medium	220	17.5	0.079	100	1
23% Ammonium sulphate	76	10.26	0.135	59	1.7
Q-Sepharose F/F	1.4	1.36	0.95	7.8	12

[a] The amount of total proteins in all samples was determined with Bio-Rad assay kit using bovine serum albumin as standard

[b] The activity was measured with *p*-nitrophenyl caproate at 25°C

Table 2 Effect of various compounds on *Ph*TAC125 Lip 1

Compound (10 mM)	Residual activity (%)[a]
No addition	100
$HgCl_2$	11
$MgCl_2$	54
$CaCl_2$	52
$CuCl_2$	57
$CoCl_2$	36
$MnCl_2$	55
$FeCl_3$	0
2-Mercaptoethano1	43
DTT	45
EDTA	47
SDS	0.9
PMSF	0

[a] The residual activity was measured with *p*-nitrophenyl caproate at 25°C

His). To generate the 3D-model, the sequences alignment was submitted to the comparative structural modelling program MODELLER 8v2 (Marti-Renom et al. 2000), which uses sequence alignment to extract a large number of spatial restraints for homology modelling of the target protein. These spatial restraints are then expressed as 50 PDBs, combined into an objective function. The n model was selected on the basis of the lowest value of the MODELLER objective function. Hydrogen atoms were added to the model and the strain had to be removed by 100 steps of energy minimisation of the structure using GROMACS package (Berendsen et al. 1995).

The electrostatic surfaces were generated with the APBS software package (Baker et al. 2001). Images were produced with visual molecular dynamics (VMD) 1.8.5 (10) and PyMol (http://pymol.sourceforge.net).

Results

Identification, sequence analysis and multiple alignment of *P. haloplanktis* TAC125 Lip1

The genome of *P. haloplanktis* TAC125 (Medigue et al. 2005) (https://www.genoscope.cns.fr/agc/mage/wwwpkgdb/Login/log.php?pid=7#ancreLogin) was searched for the presence of putative lipase/esterase encoding genes. Amongst the several genes retrieved, PSHAa0051 was selected for further in silico analysis, since the encoded product was annotated as "putative secreted esterase". Indeed, the gene (1,368 bp long) codes for a protein of 456 residues (hereafter called *Ph*TAC125 Lip1), which displays a canonical N-terminal signal peptide for the extra-cytoplasmic addressing of the resulting protein. SignalP 3.0 program (http://www.cbs.dtv.dk/signalIP) predicted a Signal peptidase I cleavage site between positions 25 and 26 of Lip1 sequence with high probability (*e* value 0.98). Furthermore, Lip1 sequence analysis by InterProScan platform (http://www.ebi.ac.uk/interpro) highlighted the presence of the IPR 000379 motif, which is found in all lipase/esterase enzymes belonging to the α/β hydrolase superfamily (Jaeger et al. 1994), supporting the annotation accuracy. No other conserved IPR motifs could be found. A homology search was then performed at the SIB (*Swiss Institute of Bioinformatics*) using the BLAST network service (http://www.expasy.org/tools/blast/) (Altschul et al. 1997). *Ph*TAC125 Lip1 was similar to the hypothetical protein Q48AN1 (42% identity over 432 residues) from *C. psychrerythraea* (Mèthe et al. 2005), the hypothetical protein A4CF12 (40% identity over 426 residues) from *P. tunicata* D2 (Holmstrom et al. 1998), the putative lipase/esterase Q2SGZ8 (32% identity over 379 residues) from *H. chejuensis* (Jeong et al. 2005) and to several putative lipoproteins displaying lipase/esterase domains.

The three sequences were searched for the presence of a signal peptide. Canonical N-terminal signal peptide was predicted by Signal 3.0 program for both *C. psychrerythraea* and *P. tunicata* proteins (cleavage site between positions 21–22, and 22–23, respectively). The *H. chejuensis* enzyme was predicted to be a lipoprotein by LipoP 1.0 server (http://www.cbs.dtu.dk/services/LipoP/), which identified a Signal peptidase II cleavage site between positions 32 and 33, with a Gln residue in position +2. Multiple sequence alignment of *Ph*TAC125 Lip1 with the three above-mentioned proteins was constructed (Fig. 1). The alignment displays the four psychrophilic proteins in their mature form, i.e. devoid of their predicted signal peptide. Position conserved in at least three over the four sequences are highlighted in grey, and fully conserved residues in red. Amongst the conserved regions, the GXSXG motif (positions 175–179 with respect to *Ph*TAC125 Lip1 numbering) was observed. This motif is typical of α/β-hydrolases; it has been shown that Ser residue of the conserved pentapeptide is located at the active site of lipases (Arpigny and Jaeger 1999) and esterases to form a "catalytic triad" with a Asp and a His residue (indicated by open circles). In order to identify the other two active-site residues, the alignment was searched for the presence of the motif conserved in eight esterase/lipase families (Arpigny and Jaeger 1999). None of the previously described residue patterns were found.

The *Ph*TAC125 Lip1 sequence was aligned with that of Est30 carboxylesterase from *G. stearothermophilus* (Ewis et al. 2004), which appeared to be the closest bacterial enzyme with a resolved 3D structure (PDB ID

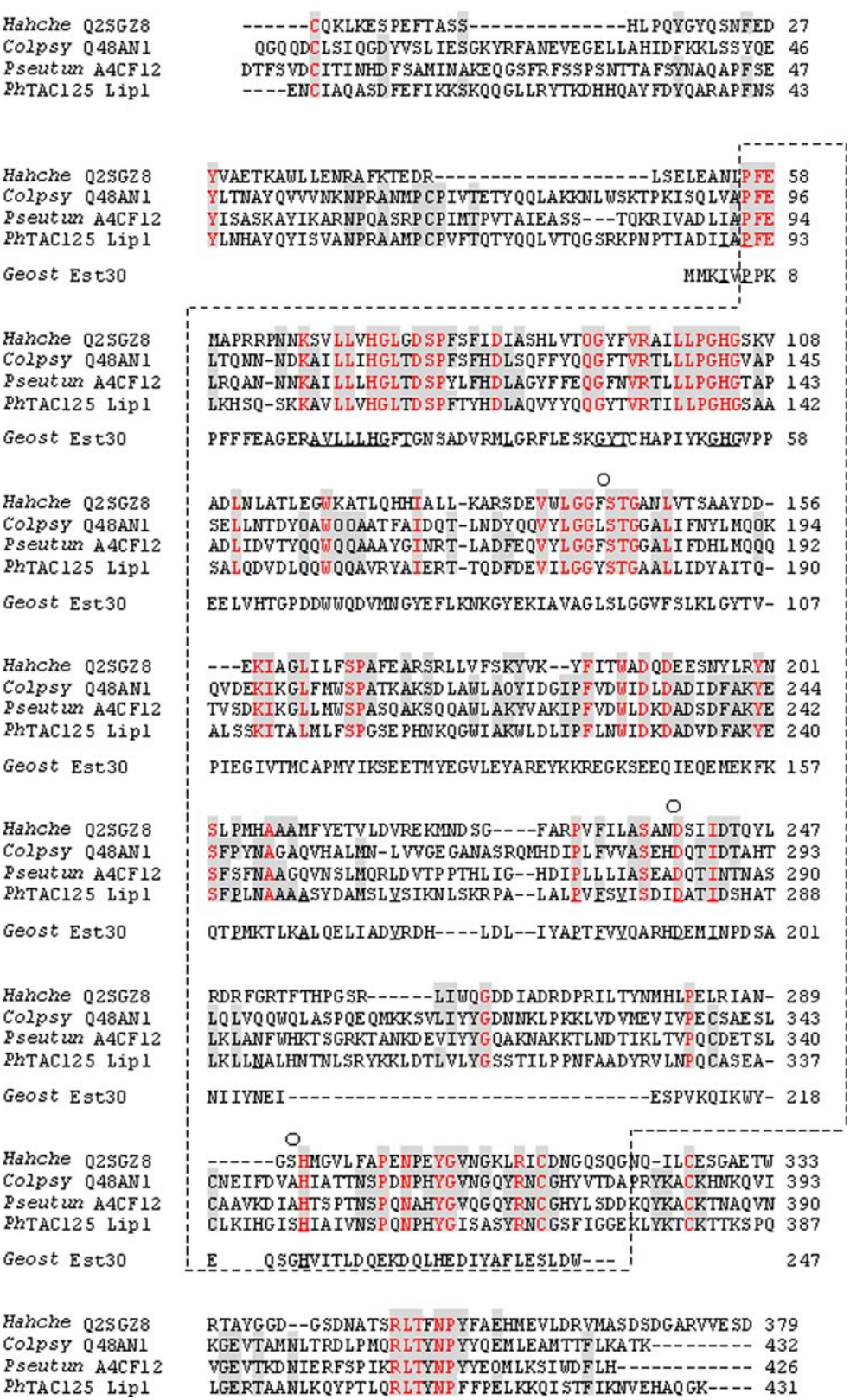

Fig. 1 Alignment of *Ph*TAC125 Lip1 sequence with those of other homologous enzymes. *Hahche* Q2SGZ8, *Hahella chejuensis* putative esterase/lipase (A.N. Q2SGZ8); *Colpsy* Q48AN1, *Colwellia psychrerythraea* hypothetical protein (A.N. Q48AN1); *Pseutun* A4CF12, *Pseudoalteromonas tunicata* hypothetical protein (A.N. A4CF12); *Geost* Est30, *Geobacillus stearothermophilus* carboxylesterase Est30 (PDB ID ITQH:A). The conserved residues in all sequences are in *red*; those conserved at least in three sequences are in *grey*; conserved residues between *Ph*TAC125 Lip1 and *Geost* Est 30 are *underlined*; *open circles* indicate the active site residues. The *dashed box* indicates the region of *Ph*TAC125 Lip1 modelled using *Geost* Est 30 as template

ITQH:A) (Liu et al. 2004). Although differing in sequence length, the two proteins share 20% identity (over 247 aligned residues underlined in Fig. 1). From this alignment it was possible to identify the *Ph*TAC125 Lip1 likely catalytic Asp and His (positions 280 and 346, respectively).

Construction of the expression vector pPM13*lip1*

The recombinant *Ph*TAC125 Lip1 protein production in *P. haloplanktis* TAC125 cells was achieved by cloning the PCR-amplified gene into the cold-adapted gene expression vectors pPM13 (Papa et al. 2007). This vector contains all the signals required for efficient replication in either *E. coli* or *P. haloplanktis* TAC125 (Duilio et al. 2004) and a strong transcriptional promoter element (PM13) isolated from *P. haloplanktis* TAC125 genome by a promoter-trap library. It also contains a *c-myc* tag encoding a peptide that was immunodetected by specific monoclonal antibodies. The amplified *Ph*TAC125 *lip1* gene was cloned in the cold-expression vector, so that the resulting tagged protein contained the *c-myc* tag at its C-terminal end.

Analysis of *Ph*TAC125 Lip1 recombinant production and cellular localization

The *P. haloplanktis* TAC125 wild-type and pPM13*lip1* recombinant cells were grown in liquid TYP medium at 4 and 15°C; cell pellets and corresponding extra-cellular medium samples were collected at different growth phases. The protein samples were analysed by Western Blotting using anti-*c-myc* antibodies and it was shown that *Ph*TAC125 Lip1 was produced in *P. haloplanktis* TAC125 recombinant cells at both temperatures (data not shown).

Figure 2a shows the immunoblotting analysis obtained loading total cell lysate, insoluble and soluble fractions (after cell-pellet lysis), and corresponding extra-cellular medium of wild-type and recombinant psychrophilic cells grown at 4°C. The *Ph*TAC125 Lip1 protein was only located in the insoluble fraction.

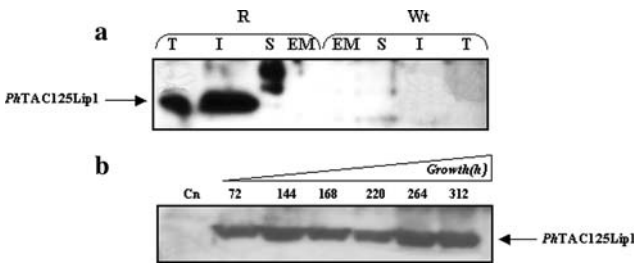

Fig. 2 **a** Production and cellular localization of *Ph*TAC125 Lip1 in recombinant *P. haloplanktis* TAC125 strain. Western-blotting analysis of (*T*) total cellular extracts; (*S*) soluble and (*I*) insoluble fractions; (*EM*) extra-cellular medium of wild type (*Wt*) and pPM13lip1 recombinant *P. haloplanktis* TAC125 strain (*R*) (grown at 4°C). **b** Kinetics of PSHAa0051 recombinant production in the extract of the insoluble part of pPM13lip1-*P. haloplanktis* TAC125 (*R*) collected during growth phase. *Cn* insoluble fraction of wild type strain. *Figure* denotes hours. Anti-*c-myc* monoclonal antiserum was used for immunodetection. Each lane contains 20 μg of protein mixture

The recombinant-protein accumulation in the insoluble fraction could be explained by two alternative hypotheses: (1) aggregation as insoluble inclusion bodies; (2) association to the membranes. The first hypothesis was excluded by analysing the inclusion bodies preparation from recombinant cellular pellets carried out following the method described in the "Materials and methods" section.

SDS-PAGE and *c-myc* immunodetection experiments demonstrated that the recombinant protein does not aggregate in inclusion bodies (data not shown). Therefore, anchoring to the cell membranes is the most likely explanation for the presence of the recombinant protein into the insoluble fraction.

Figure 2b shows the kinetics of recombinant *Ph*TAC125 Lip1 accumulation in the insoluble fraction during cell growth at 4°C. The amount of recombinant protein detected by Western blotting was almost the same over the growth; therefore, in the following experiments *P. haloplanktis* TAC125-pPM13*lip1* recombinant cells were routinely grown for 72 h at 4°C.

*Ph*TAC125 Lip1 extraction from the membranes by means of detergents

A solubilisation procedure using detergents was performed on *P. haloplanktis* TAC125-pPM13*lip* insoluble fractions in order to extract *Ph*TAC125 Lip1 from the cells as first step of purification.

Six detergents (Triton X-100, CHAPS, Tween 20, Tween 80, Na-cholate and SDS), differing in chemical structure and properties, were tested for their ability to extract the psychrophilic recombinant lipase. The screening was performed by incubating the insoluble extracts of recombinant cells in phosphate buffer supplemented with each detergent at 1.0% (v/v) final concentration. Triton X-100 was shown to be able to extract the highest protein amount. To investigate the ability of the detergent to extract the protein, a Western-blotting analysis was carried out on the insoluble fractions before and after Triton treatment. As shown in Fig. 3a, the absence of recombinant protein after the treatment demonstrated that Triton X-100 was able to fully solubilise recombinant *Ph*TAC125 Lip1 after only 5 h of incubation.

With the aim of achieving a selective *Ph*TAC125 Lip1 extraction, a novel extraction method was set up. *P. haloplanktis* TAC125 wild-type and recombinant cells were grown in liquid broth at 4°C for 72 h. The cells were recovered by mild centrifugation (1,500×*g* for 20 min), and incubated in fresh TYP broth supplemented with Triton X-100 (0.5% v/v final concentration) for 5 h at 4°C. During incubation, the growth of *P. haloplanktis* TAC125 cells was monitored and appeared that the cells were able to duplicate even in the presence of the

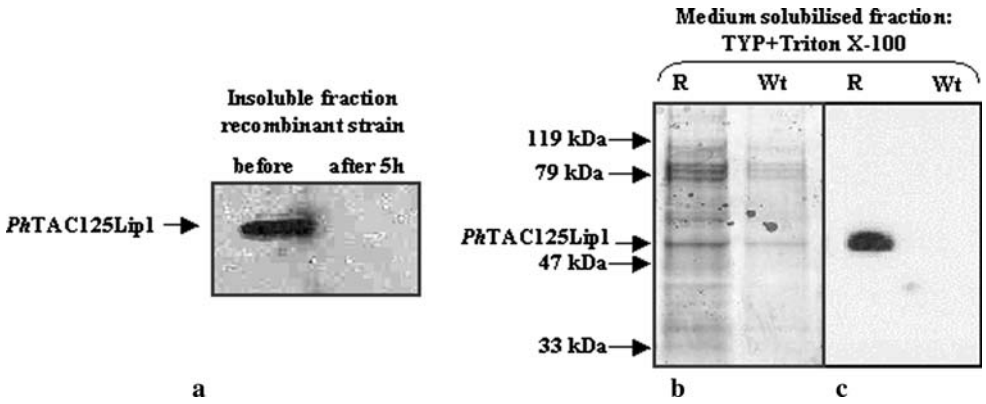

Fig. 3 Comparison of the extra-cellular patterns obtained by the solubilisation methods (see "Materials and methods") performed on *P. haloplanktis* TAC125-pPM13*lip* recombinant cells. **a** Western blotting analysis of insoluble fraction before and after the Triton X-100 detergent treatment. **b** SDS-PAGE analysis of medium solubilized fraction and **c** corresponding Western blotting anti-*c-myc* immunodetection. *R*, *P. haloplanktis* TAC125-pPM13*lip* recombinant cells, and *Wt*, *P. haloplanktis* TAC125. Each lane contains 20 μg of protein mixture

detergent (data not shown). The "medium solubilized fractions", isolated from the cells by mild centrifugation and consisting of growth medium containing several extracted proteins, were analysed by SDS-PAGE (Fig. 3b) and Western blotting (Fig. 3c). Anti-*c-myc* antibodies recognised a specific product in the growth medium from recombinant cells, which displayed elecrophoretic mobility similar to the protein observed in the broths from both recombinant and wild-type cells in SDS-PAGE. The TYP broth from wild-type cells probably contains endogenous Lip1, which cannot be revealed by Western blotting due to the lack of the epitope recognised by anti-*c-myc* antibodies.

*Ph*TAC125 Lip1 purification

Figure 4 shows the 12% SDS-PAGE analysis of medium-solubilized fractions from wild type (lane cn) and recombinant (lane 1) *Ph*TAC125 cells, followed by MUF-butyrate stain to visualise esterase/lipase activities. As expected, the wild type displays modest esterase activity, which appeared to be enhanced in the recombinant strain. The recombinant sample was subjected to the purification procedure described in Experimental procedures section, consisting of ammonium sulphate precipitation (lane 2), followed by Q-sepharose FF chromatography (lane 3). The zymography shows the enrichment of the protein associated to MUF-butyrate activity, which was also recognised by anti-*c-myc* antibodies (data not shown) and was therefore identified as *Ph*TAC125 Lip1. After each purification step, the samples were assayed for esterase activity using *p*-nitrophenyl caproate as substrate (see Table 1). The protein purification was very close to 70%.

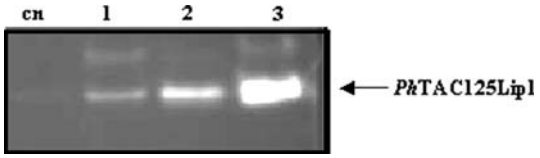

Fig. 4 Activity stain with MUF-butyrate performed on SDS-PAGE. *cn* medium of wild type; *1* detergent-treated medium; *2* fraction after ammonium sulphate precipitation; *3* fraction after Q-Sepharose chromatography. Each lane contains 20 μg of protein mixture

Effect of pH and temperature

The dependence of *Ph*TAC125 Lip1 on pH was monitored at 405 nm using an absorption coefficient of 15,500 M^{-1} cm^{-1} at 25°C (Fig. 5a). *P. haloplanktis* TAC125 Lip1 reaches the apparent maximal activity at pH values above 8.5.

The apparent optimal temperature for Lip1 activity was measured in the range 5–50°C at pH 7.1 (Fig. 5b). The activity increased up to 40°C.

Substrate specificity and effects of compounds on *Ph*TAC125 Lip1

The substrate specificity of *Ph*TAC125 Lip1 was examined at 25°C using several *p*-nitrophenyl esters with aliphatic acyl-chain length of C_2–C_{10}. The Lip1 specific activity increases in this order: $C_2 < C_5 < C_6 \leq C_8 < C_{10}$, as reported in Fig. 6 where the rates of hydrolysis are expressed as percentages relative to C_{10} hydrolysis rate. Furthermore, *Ph*TAC125 Lip1 proved to be also able to hydrolyze acylglycerols such as triolein and tricaprylin (data not shown).

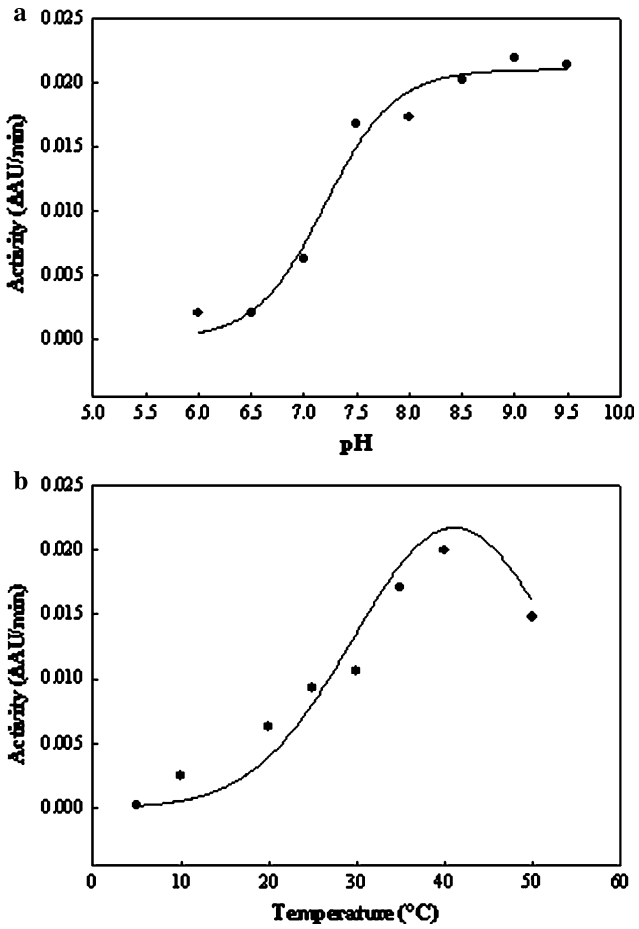

Fig. 5 Effect of **a** pH and **b** temperature on the activity of *Ph*TAC125 Lip1. Twenty millimolar sodium phosphate buffer was used in the pH range 6.0–7.5, whereas in the pH range 8.0–9.5, 20 mM Tris–Cl was used. The activity was measured using 0.2 mM *p*-nitrophenyl caproate as substrate

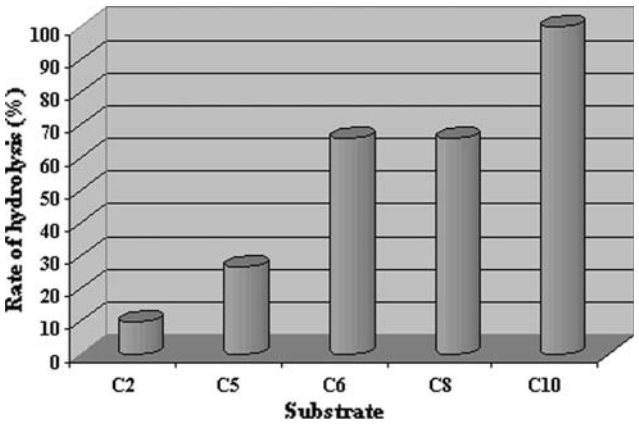

Fig. 6 Activity (%) of *Ph*TAC125 Lip1 as function of the length of the acyl chain of *p*-NP-esters

The effect of several compounds on enzyme activity was also investigated. Lip1 was incubated in the presence of each for 1 h at 25°C, and the residual activity was measured under standard conditions.

As reported in Table 2, all compounds showed an inhibitory effect on Lip1 activity at various extents. Amongst the tested metal ions, Hg^{2+} resulted to be a strong inhibitor, supporting the presence of functionally important thiol group(s) needed for enzyme catalytic activity. Inhibition by Ca^{2+} ions was also observed, in contrast with observation on other lipases, e.g. those from *Aspergillus niger* and *Humicola lanuginose*; these enzymes are in fact activated by Ca^{2+}, since the ion facilitates the removal of free fatty acids formed at the water–oil interface (Iwai et al 1964).

Homology modelling

In order to model the structure of *Ph*TAC125 Lip1, the crystal structure of *G. stearothermophilus* carboxylesterase Est30 was selected as template (PDB ID ITQH) (Liu et al. 2004), and the alignment is shown in Fig. 1. Similarity extends along all the sequences, with the exceptions of the N-terminal and C-terminal regions, where the psychrophilic protein displays extra domains. Therefore, modelling was limited to the Lip1 central region between residues 91 and 375 (see dotted box in Fig. 1).

Figure 7 shows the ribbon representations of the *Ph*TAC125 Lip1 model (panel a) and the template *Geost* Est30 structure (panel b). In both structures, the active-site residues are displayed. The most evident difference with respect to the *Geost* Est30 structure consists of a large insertion of 32 residues placed between an α-helix (αE) and the following *β*-sheet (*β*8). The sequence of 32-residues insertion of *Ph*TAC125 Lip1 were subjected to secondary structure prediction analysis by Jpred software (Cuff et al. 1998). It turned out that the first 11 residues of the insertion (i.e. the sequence HNTNLSRYKKL) display a high propensity to form α-helix. Therefore, the αE helix in *Ph*TAC125 Lip1 model resulted to be 11 residues longer with respect to the corresponding secondary structure of the thermophilic template (Fig. 7a, b). The same prediction failed to predict any propensity of the remaining 21 residues to form a secondary structure and, therefore, this region appears unstructured in the model (Fig. 7a). It is worth mentioning that the insertion is quite conserved, in both position and length, in all psychrophilic proteins aligned in Fig. 1.

As far as the size of the active-site pocket is concerned, *Ph*TAC125 Lip1 displays a wider groove (maximal height, higher than 10 Å) with respect to *Geost* Est30 (maximal height, 7.51 Å).

The electrostatic surfaces of the modelled structure (panel c) and of the template (panel d) are displayed in Fig. 7; the view is from the side of the binding-site, as in panel a and b. The *Ph*TAC125 Lip1 surface appeared more hydrophobic than that of *Geost* Est30. By using POPS

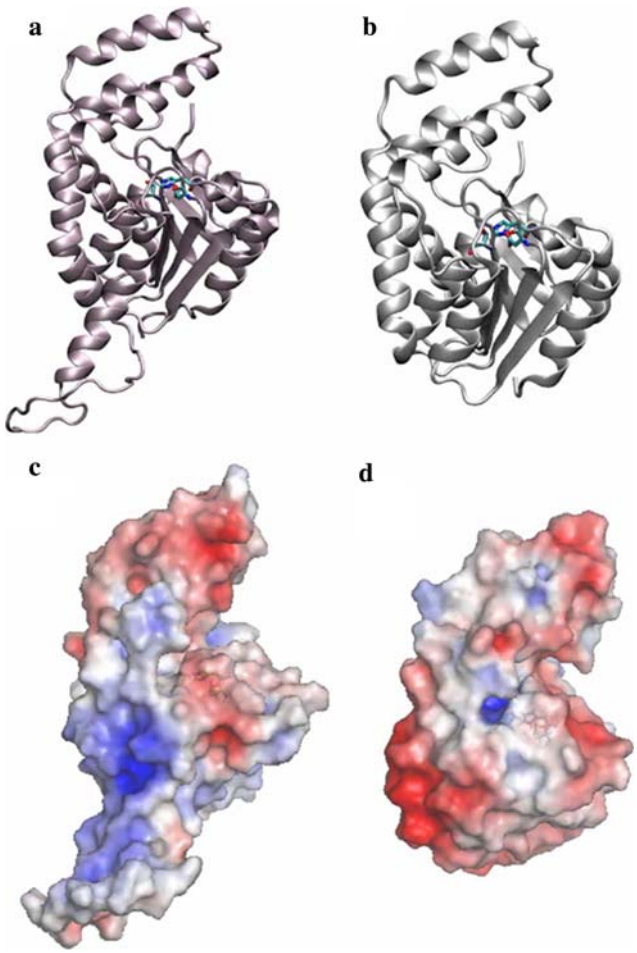

Fig. 7 Ribbon representation and electrostatic potential of *Ph*TAC125 Lip1 (**a**, **c**) and 1TQH (**b**, **d**). Each molecule is viewed from the side of the binding-site. The residues of the catalytic triad in licorice. The potential ranges from −6*k*T (*red*) to the maximal positive value +6*k*T (*blue*)

software (Fraternali and Cavallo 2002), the accessible surface areas (SASA) of both proteins were calculated. In line with the above observation, 61% of the total *Ph*TAC125 Lip1 area (16,925.2 Å^2) is hydrophobic, whereas this percentage drops to at 54% in the *Geost* Est30 exposed area (12,353.7 Å^2). The tendency to be more hydrophobic than thermophilic counterparts is also observed in the *Ph*TAC125 Lip1 active-site pocket. The *Geost* Est30 active-site groove is characterised by a positively charged surface, which has no counterparts in the model of the psychrophilic enzyme.

Discussion

The traditional way to discover novel biocatalysts used to be the screening for the desired activity. Nowadays, due to the availability of an ever-increasing number of complete genome sequences, in silico analysis has become an important means for the discovery and identification of novel industrial biocatalysts (Kwuon et al. 2004). In order to extract the maximal amount of information from the available genome sequences, several computational tools have been developed with the aim of classifying conserved genes according to their homologous relationships. The process of biocatalyst discovery by in silico genome analysis is followed by recombinant expression of the selected gene and molecular characterization of the encoded product.

We applied the above strategy to the identification of a novel cold-active lipase from the Antarctic Gram-negative bacterium *P. haloplanktis* TAC125. Its genome has been recently released, and this represents the first complete genomic information of an Antarctic bacterium (Medigue et al. 2005). Several putative esterase- or lipase-encoding genes were identified in the *Ph*TAC125 genome. Our attention was focused on PSHAa0051, whose encoded product was annotated as a "conserved hypothetical protein, putative secreted esterase". In fact, the protein (named *Ph*TAC125 Lip1) shares some features with the α/β hydrolases superfamily, and belongs to COG 1647, a conserved subfamily of serine hydrolases. Furthermore, *Ph*TAC125 Lip1 displays a canonical N-terminal signal peptide, supporting the likely extra-cytoplasmic localization of the mature enzyme.

Interestingly, a homology search in the protein databank highlighted the presence of Lip1 closer homologues in the genome of three marine psychrophilic γ-proteobacteria (i.e. *H. chejuensis*; *C. psychrerythraea*; and *P. tunicata*). The four proteins share the presence of an N-terminal signal peptide for the extra-cytoplasmatic localization and high level of identity (from 38 to 45%) all along the sequence.

The recombinant production of *Ph*TAC125 Lip1 in the mesophilic *E. coli* was attempted, but the protein was always found associated to the inclusion bodies, regardless of any changes in the experimental conditions. Refolding of *Ph*TAC125 Lip1 in the inclusion bodies was unsuccessful.

Taking advantage of the availability of an efficient gene-expression system, which uses *Ph*TAC125 as recombinant host (Duilio et al. 2004), *Ph*TAC125 Lip1 was produced in the psychrophilic cells at 4°C. Contrary to expectation, the recombinant protein did not accumulate in the extra-cellular medium, but was found in the cellular membrane fraction. We demonstrated that a detergent treatment on intact cells allowed the full solubilization of *Ph*TAC125 Lip1, without affecting cells vitality. This result is strongly suggestive of the *Ph*TAC125 Lip1 outer membrane localization. The absence of any known signal for either inner or outer

membrane localization, suggests that Lip1 is associated to the microbial outer membrane by a still uncharacterised anchoring domain.

*Ph*TAC125 Lip1 was purified from the medium solubilized fraction and subjected to functional characterization. The enzyme shows an apparent pH optimum around 8.5, and apparent maximal activity at about 40°C, similar to cold-active lipase from *Pseudomonas* sp. strain B-11-1 (35°C) (Choo et al. 1998).

Esterases and lipases can be distinguished on the basis of substrate specificity. Lipases are carboxylesterases displaying maximal activity towards water-insoluble long-chain acylglycerols ($\geq C_8$), whereas esterases hydrolyse partially water-soluble ester substrates with short-chain fatty acids ($\leq C_8$). However, lipases can also hydrolyse esterase substrates (Verger 1997; Arpigny et al. 1997; Wilheilm et al. 1999). Kinetic analysis using artificial substrates indicated that *Ph*TAC125 Lip1 showed higher activity towards long acyl-chain substrates. The psychrophilic enzyme resulted to be also able to hydrolyze long-chain acylglycerol (de Pascale, data not shown), thus suggesting that *Ph*TAC125 Lip1 can be considered as a novel psychrophilic lipase.

Bacterial esterases and lipases have been classified into eight families based on comparison of their sequences and some biological properties (Arpigny and Jaeger 1999). *Ph*TAC125 Lip1 and the other aligned psychrophilic proteins were searched for conserved motifs. The only one found is the generic GXSXG pentapeptide, that was to be LGG(F/L/Y)STG in the cold-adapted enzymes, which is different from every of the consensus motifs found in the eight reported lipolytic families (Arpigny and Jaeger 1999). The aligned sequences share other features, such as (1) presence of an N-terminus signal peptide for the extra-cytoplasmic addressing, (2) sequence length, (3) high sequence similarity, (4) lack of a canonical calcium-binding pocket (Nardini and Dijkstra 1999), and (5) absence of a canonical lid (Van Tilbeurg et al. 1993). Phylogenetic analysis (performed on a data set consisting of 17 homologous sequences showed in Table S1 supplemental material section) demonstrated that the psychrophilic proteins are strictly correlated and belong to the same clade. These observations strongly suggest that the four proteins constitute a novel lipase family, typical of psychrophilic marine γ-proteobacteria.

To gain structural information about this lipase family, a *Ph*TAC125 Lip1 model was constructed. The model production was expected to be difficult due to the lack of 3D structures of homologous lipolytic enzymes. Despite the very low sequence identity (20%), *Ph*TAC125 Lip1 was modelled using the structure of a thermophilic carboxylesterase (Est30 from *G. stearothermophilus*) as template. Modelling was carried out on the central region of *Ph*TAC125 Lip1, which also contains all the active site residues. The extra N- and C-terminal domains, conserved in this novel family, were not modelled due to the absence of any similarity with known structures. The main structural difference between the two molecules is the presence of a long insertion in *Ph*TAC125 Lip1, which protrudes opposite to the catalytic region. Considering the position of the insertion in the model and its conserved quite hydrophobic nature, this portion of the protein may interact with the conserved extra C-terminal region, possibly constituting an independent domain.

Contrary to observations in other psychrophilic enzymes, usually characterised by the presence of many hydrophilic residues exposed to the solvent (D'Amico et al. 2006), the *Ph*TAC125 Lip1 electrostatic surface revealed a large number of nonpolar residues. As a consequence, the surface is highly hydrophobic when compared to the *Geost* Est30 structure.

May data presented in this paper suggest at least which region of *Ph*TAC125 Lip1 is involved in outer membrane interaction? Some hints can be obtained by studying the *H. chejuensis* homologous protein. The in silico analysis of its sequence highlighted that the *H. chejuensis* enzyme is a lipoprotein, predicted to be located in the inner leaflet of the outer membrane due to the absence of a Asp residue in position +2, which is acknowledged as retention signal for the inner membrane localization (Bos et al. 2007). Since the main structural difference between *Ph*TAC125 Lip1 and the *H. chejuensis* lipoprotein is indeed in the extra N-terminal portion, we hypothesize that this region could contain a novel anchoring motif responsible for the outer membrane association. Further experiments will however be required to confirm our hypothesis, and to identify the structural features of the *Ph*TAC125 Lip1 N-terminal domain.

On the grounds of *Ph*TAC125 Lip1 features reported in this paper, its role in membrane-lipid metabolism could be envisaged. Membrane fluidity is a key factor in the adaptation of micro-organisms to cold (Russell 2000), and it is likely that psychrophilic bacteria may have evolved ad hoc enzymes responsible for cellular adaptation to temperature fluctuations. Since such a novel lipase family only contains enzymes found in cold-adapted bacteria, we suggest a possible role in regulating the required membrane fluidity for *Ph*TAC125 Lip1 and its homologues.

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