Polar Research

Cloning and heterologous expression of two cold-active lipases from the Antarctic bacterium *Psychrobacter* sp. G

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Keywords
Antarctic; cold-active lipase; gene cloning; gene expression; *Psychrobacter*.

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doi:10.1111/j.1751-8369.2010.00189.x

Abstract

Antarctic bacteria producing extracellular lipolytic enzymes with activity at low temperature were isolated, and the most promising strain, named G, was identified as a *Psychrobacter* species based on *16S* rDNA sequence alignment. The genomic DNA of this bacterium was used to construct its plasmid genomic library into pUC118 plasmid vectors, and to screen the cold-active lipolytic enzyme genes. Two genes encoding for cold-active lipolytic enzymes, *Lip-1452* (with an open reading frame of 1452 bp in length) and *Lip-948* (with an open reading frame of 948 bp in length), were screened. The primary structure of the two lipases deduced from the nucleotide sequence showed a consensus pentapeptide containing the active serine (*Lip-1452*, GDSAG, and *Lip-948*, GNSMG) and a conserved His-Gly dipeptide in the N-terminal part of the enzyme. Protein sequence alignment and conserved regions analysis indicated that the two lipases probably belonged to family IV and family V of the bacterial lipolytic enzymes, respectively. The upstream and downstream sequences of the two lipolytic lipases were also obtained. The two lipase genes were cloned into the expression vector pCold III and integrated into *Escherichia coli* BL21 (DE3). The functional expression of both lipase genes by *E. coli* BL21 (DE3) cells was observed as the formation of clear haloes around colonies on a 1% (vol/vol) tributyrin plate upon induction with isopropyl-β-D-thiogalactopyranoside at 5°C. A lipase activity assay showed that the specific activity of the pCold III+*Lip-948* expression system was up to 3.7 U ml^{-1}, whereas that of pCold III+*Lip-1452* was very low.

Enzymes from psychrotrophic and psychrophilic microorganisms have received increasing attention because of their relevance for both basic and applied research. Research efforts have been stimulated by the recognition that cold-adapted enzymes might offer novel opportunities for biotechnological exploitation, based on their high catalytic activity at low temperatures, low thermostability and unusual specificities. Cold-active enzymes with huge biotechnological potentials include protease, lipase, amylase and cellulase in the detergent industry, β-galactosidase in the dairy industry, dehydrogenase as a biosensor in environmental protection, oxidase in bioremediation, and many enzymes, e.g., methylase and aminotransferase, in biotransformation (Alquati et al. 2002; Cieśliński et al. 2005; Parra et al. 2008).

Bacteria produce different classes of lipolytic enzymes, including carboxylesterases (EC 3.1.1.1) that hydrolyse water-soluble esters and lipases (EC 3.1.1.3) that hydrolyse long-chain triacylglycerol substances, catalysing both the hydrolysis and the synthesis of acylglycerides and other fatty acid esters (Rosenau et al. 2000; Lee et al. 2004).

Lipolytic enzymes are important biocatalysts for various industrial applications (e.g., ester synthesis, optical resolution, transesterification and washing) because, among other characteristics, they lack requirements for cofactors, they are remarkably stable in organic solvents, they have broad substrate specificity, and they have region- and stereo-selectivity. In the past decade, increasing attention has been drawn to potential applications of cold-adapted lipolytic enzymes from microorganisms populating permanently cold environments (Pfeffer et al. 2007; Długołecka et al. 2008). These enzymes are characterized by higher k_{cat} and physiological

efficiency (k_{cat}/k_m), and by a lower and rather constant k_m at temperatures close to 0°C (Yang et al. 2008). Several recent papers reviewed cold-active microbial lipases (Joseph et al. 2007; Joseph et al. 2008). A dozen genes encoding cold-active lipolytic enzymes have been cloned from different species and environmental metagenomes (Choo et al. 1998; Quyen et al. 1999; Alquati et al. 2002; Ryu et al. 2006; Zhang et al. 2007; Długołecka et al. 2008; Parra et al. 2008; Yang et al. 2008; Jeon et al. 2009).

From water samples collected off King George Island, Antarctica, we isolated dozens of cold-adapted microorganisms that can degrade lipids at low temperature (5°C). One of the promising lipolytic strains, named "G", was identified as a *Psychrobacter* species by molecular identification based on *16S* rDNA. In this study, we report the cloning and sequencing of two lipase genes from the strain *Psychrobacter* sp. G., and their heterologous expression in *Escherichia coli* BL21 (DE3).

Material and methods

Samples and isolation of lipolytic enzyme-producing strains

Surface (0–20 cm) water samples were collected at 62°12′39″S, 58°54′41″W, on 9 January 2008 in the waters off south-western King George Island, one of the South Shetland Islands, using a water sampler (model WB-PM, Beijing Purity Instrument Co., Beijing, China). The temperature and salinity of the samples were 1.9–2.5°C and 33.1–33.8, respectively. The screening medium comprised 5 g of peptone, 1 g of yeast extract, 10 ml of tributyrin and 1000 ml of seawater. The strains producing lipolytic enzymes were detected by the formation of clear haloes around the colony at 5°C. The lipolytic enzyme activity assay was performed with a substrate of olive oil at 35°C, as described by Yang et al. (2004).

Strains and vectors

Of the lypolytic strains detetcted, one (G) was selected for further analysis on account of its higher extracellular lipase activity at low temperature, and its low enzymatic optimum temperature. The strain was identified as *Psychrobacter* sp. by molecular identification based on *16S* rDNA alignment, as described by Yang et al. (2004).

Escherichia coli DH5α and BL21 (DE3) were used as host strains for DNA manipulation and gene expression, respectively. Plasmid vectors pUC118 *Hinc*II/BAP (code D3322; Takara Biotechnology Co., Otsu, Japan) and pCold III (code 3363; Takara Biotechnology) were used as vectors for gene cloning and heterologous expression, respectively.

Cloning of the lipolytic enzyme genes

The chromosomal DNA from *Psychrobacter* sp. G was isolated using a Genomic DNA Prep Kit (model number DP302-02; Tiangen Biotech, Beijing, China), following the instructions in the manufacturer's manual. The chromosomal DNA was partially digested with *Sau*3AI and 2–6-kb fragments were purified from a 0.8% agarose gel using an Agarose Gel DNA Purification Kit v2.0 (model number DP209-02; Tiangen Biotech, Beijing, China). These DNA fragments were ligated into pUC118 *Hinc*II/BAP with a DNA Ligation Kit v2.0 (model number D6022; Takara Biotechnology Co.).

The resultant plasmids were introduced into *E. coli* DH5α, providing a genomic library containing 2.1×10^5 cfu/ml recombinant *E. coli* clones. The colonies producing cold-active lipolytic enzymes were detected by the formation of clear haloes around the colonies on lysogeny broth (LB) agar plates supplemented with ampicillin (50 μg ml^{-1}), 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 1% (vol/vol) tributyrin at low temperature (5°C) (Yang et al. 2008). The recombinant plasmids from the lipolytic enzyme positive clone were extracted and sequenced.

Construction of the expression plasmid of *Lip-1452* and *Lip-948*

An *E. coli* expression vector, pCold III, was used to express the lipolytic enzymes in *E. coli*. Based on the upstream and downstream sequences of the known lipolytic enzyme gene sequences, the specific primers for polymerase chain reaction (PCR) amplification were designed and synthesized as follows: primer F1 5′-CTGTAG**GAGCTC**ATGTCTAACTCAACAGTACTAT-3′, primer R1 5′-CATAAA**TCTAGA**ATCAATC TTACAGGTACCAA-3′ for *Lip-1452*, and primer F2 5′-CTGTAG**GAGCTC**ATGC TATTAAAACGCC T-3′ and primer R2 5′-CATAAA **TCTAGA**TTACGCCTTAAAACATCA-3′ for *Lip-948* were used to generate a PCR product carrying a *Sac*I restriction site at its 5′ end and an *Xba*I restriction site at its 3′ end.

Lipase gene expression in *E. coli*

Escherichia coli BL21 (DE3) transformed with the expression plasmid pCold III+*Lip-1452* or pCold III+*Lip-948* was grown overnight with shaking (200 revolutions per min) in 10 ml LB. Expression was performed in LB medium (supplemented with 0.2% glucose) with the cold-shock vectors, in accordance with the manufacturer's instructions. When OD_{600} was 0.5, the culture solution was refrigerated at 15°C and left to stand at the same temperature for 30 min. After adding IPTG to a final

concentration of 0.5 mmol L^{-1}, cells were cultured at 15°C for 24 h. Cells were harvested and ruptured by ultrasonic lysis in 25 mmol L^{-1} sodium phosphate buffer. The cell debris (insoluble fraction) was removed by centrifugation at 12 000 × g for 20 min, and the supernatant was used for lipolytic enzyme assay and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Lipolytic enzyme assay and SDS-PAGE analysis

The lipolytic enzyme assay was performed with tributyrin as substrate at 35°C, as described by Pfeffer et al. (2007). One unit (U) of enzymatic activity was defined as the quantity of enzyme that liberates 1 μmol of fatty acids per minute.

SDS-PAGE was run essentially as described by Sambrook & Russel (2001). The samples were dissolved in Laemmli's sample buffer and were heated at 95°C for 5 min and cooled before being applied to a gel. The SDS gel was composed of a 5% (weight/vol) stacking gel and a 15% (weight/vol) resolving gel. The samples were subjected to electrophoresis first at 80 V on the stacking gel and then 120 V on the resolving gel. After electrophoresis, the gel was fixed and stained with 0.1% (weight/vol) Coomassie brilliant blue R-250.

Results

Isolation and identification of psychrotrophic strains with cold-active lipolytic activity

From nearly 100 strains, 26 strains producing lipolytic enzymes were detected by the formation of clear haloes around the colonies. Strain G was selected as the most promising producer of lipolytic enzymes because it had the highest lipolytic activity (12.8 U ml^{-1} culture) among the 26 strains above when they were cultured at 5°C for 72 h in Zobell 2216E medium (YSI China Ltd, Beijing, China), supplemented with 1% Tween 80 (ICI America, Bridgewater, NJ, USA). The optimal temperature of the lipolytic enzyme was about 35°C. The strain was identified as a *Psychrobacter* species based on *16S* rDNA alignment. The almost-complete *16S* rDNA sequence of strain G (FJ386502), which consisted of 1406 bp, was analysed using the nucleotide–nucleotide basic algorithm alignment search tool (BLASTN) program for comparing DNA sequences. It was found to be more than 99% identical to hundreds of different species *Psychrobacter*, such as *Psychrobacter cryohalolentis* K5 (AY660685), *Psychrobacter* sp. DVS12b (AY864464) and *Psychrobacter* sp. ice-oil-471 (DQ521392).

Cloning of the lipolytic enzyme genes

Among approximately 20 000 recombinant colonies from the genomic library of *Psychrobacter* sp. G, 10 lipolytic enzyme positive transformants were identified on the basis of the formation of clear haloes around the colony on a tributyrin plate at 5°C, which indicated that they might encode cold-active lipolytic enzymes. From these 10 colonies, two different lipolytic genes that contained an open reading frame of 1452 bp (*Lip-1452*, GU247898; Fig. 1) and 948 bp (*Lip-948*, GU247897; Fig. 2) were obtained. They encoded 315 and 483 amino acids, giving calculated molecular weights of about 53 and 34 kDa, respectively.

Sequence analysis

The protein–protein basic algorithm alignment search tool (BLASTP) was used to search for homologies to other amino acid sequences deposited in the National Center for Biotechnology Information database (accessible at http://www.ncbi.nlm.nih.gov). The highest homology (98.9%) of *Lip-1452* was achieved with the α/β-hydrolase fold-3 from *P. cryohalolentis* K5 (YP_581719). It was 65.3% identical to the cold-active esterase from *Pseudomomas* sp. St1 (AF260707), and 54.5% identical to the lipase from *Psychrobacter* sp. 2–17 (ABR12515). The two conserved regions, HGGGF and GDSAG, were identified in *Lip-1452* (Figs. 1, 3).

Based on the conserved catalytic triad and overall identity of the amino acid sequences, *Lip-1452* was identified as a member of the hormone-sensitive lipase (HSL) group, namely family IV of the bacterial lipases (Fig. 3). *Lip-1452* probably had a catalytic triad (Ser-His-Asp), and contained an oxyanion hole to stabilize the tetrahedral intermediate (Parra et al. 2008). Based on alignment using MEGALIGN 5.0 (DNAStar, Madison, WI, USA), the two conserved regions, HGGGF and GDSAG, could be identified in the amino acid sequences of *Lip-1452*. The primary structure of the pentapeptide GDSAG started at Gly297, and Ser299 was probably also a member of the triad. Perhaps Asp414 and His444 were the second and third members of the triad, respectively. The conserved Asp was in a consensus sequence LDXL, and the His constituting the triad was preceded by a Pro and followed by a Gly. All these characters indicated that *Lip-1452* was a member of the HSL group of bacterial lipases (Arpigny et al. 1999; Parra et al. 2008).

Dozens of homologues of *Lip-948* were obtained through BLASTP analysis. The highest homology (97.8%) was the α/β hydrolase fold from *P. cryohalolentis* K5 (YP_579291). It was 82.5% identical to the triacylglycerol lipase from *Moraxella* sp. (AF260707). It was also

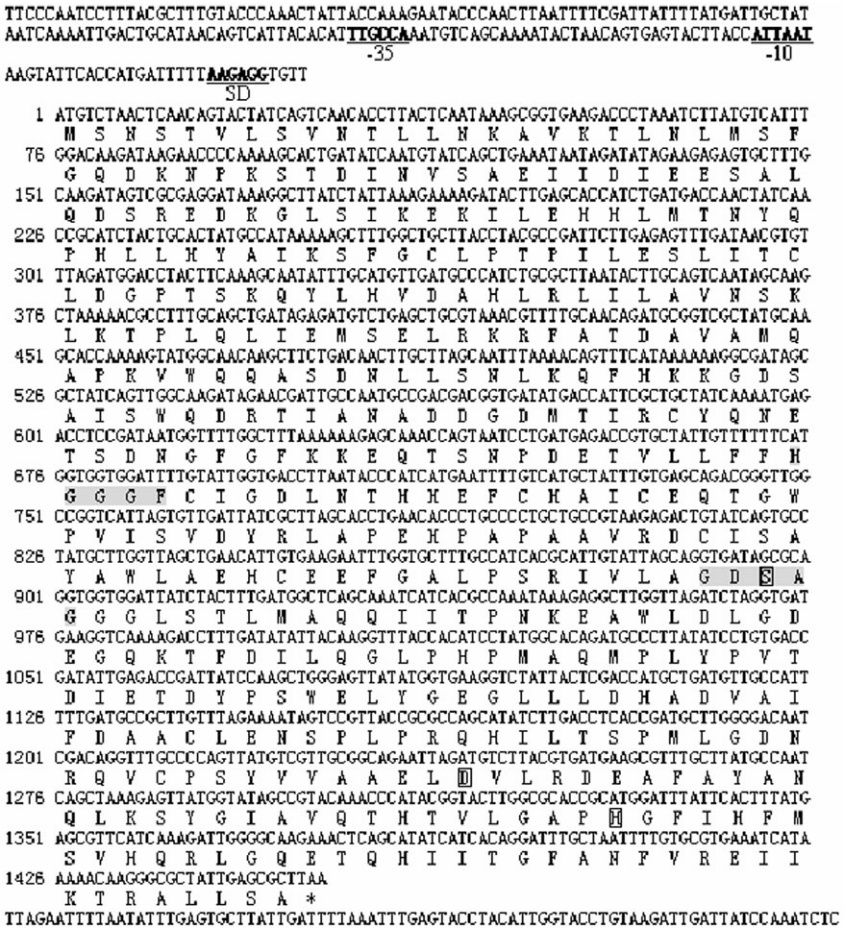

Fig. 1 Nucleotide sequence of *Lip-1452* from *Psychrobacter* sp. G, and its deduced amino acid sequence below.

very similar to the triacylglycerol lipase from *Psychrobacter* sp. 7195 (80.6%, CAJ76164) and *Psychrobacter immobilis* (79.6%, Q92104). The two conserved regions, HGFGG and GNSMG, were identified in *Lip-948* and are shown in Figs. 2 and 4.

Lip-948 probably belonged to family V, based on sequence alignment, especially for the two conserved regions HGFGG and GNSMG. Perhaps Ser142, Asp264 and His291 composed the catalytic triad (Figs. 2, 4). It probably contained a signal peptide (Met1-Ala27), which indicated that *Lip-948* might be a secreted protein.

Lipase expression in *E. coli*

The *Lip-1452* and *Lip-948* sequences were separately inserted into the expression vector pCold III, and recombinant plasmid was constructed. *E. coli* BL21 (DE3) cells were then transformed with the recombinant plasmid and induced with 0.1 mM IPTG at 15°C. The formation of clear haloes around both *Lip-1452* and *Lip-948* colonies on an LB tributyrin plate indicated that there were functional expression of lipolytic enzymes in the expression system above.

Table 1 Summary of enzyme activity of lipase gene expressed in different systems.

Vector	Halo formation on LB tributyrin plate	Specific activity (U ml^{-1})
pCold III	No	None detected
pCold III+*Lip-1452*	Yes	None detected
pCold III+*Lip-948*	Yes	3.7

The total proteins from the fermentation culture medium were analysed using SDS-PAGE (Figs. 5, 6). New protein bands with molecular masses of approximately 53 and 34 kDa, which were consistent with the molecular mass deduced from the nucleotide sequence, were produced by recombinant *E. coli* harbouring pCold III+*Lip-1452* and pCold III+*Lip-948*, respectively. Although lipase activity of the two expression systems was detected on a tributyrin plate, when the specific activity was measured with the method used by Pfeffer et al. (2007), the enzyme activity of recombinant *E. coli* harbouring pCold III+*Lip-1452* was relatively low, whereas the specific activity of the expression system of pCold III+*Lip-948* was 3.7 U ml^{-1} culture (Table 1).

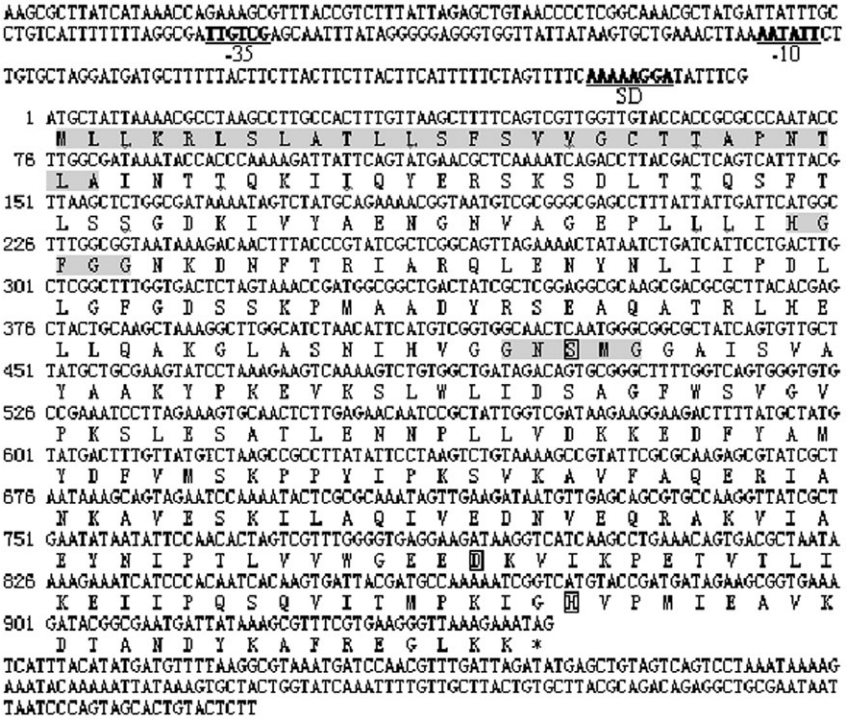

Fig. 2 Nucleotide sequence of *Lip-948* from *Psychrobacter* sp. G, and its deduced amino acid sequence below.

Discussion

Our work describes two lipolytic enzyme genes from the plasmid genomic library of *Psychrobacter* sp. G, collected from seawater in Antarctica. Simple overexpression in *E. coli* was used to determine the enzymatic activity of subclones generated from an original active pUC118 clone. The strain was identified as a *Psychrobacter* species for its close similarity at the *16S* rDNA level to hundreds of species of the *Psychrobacter* genus. The specific lipolytic activity was 12.8 U ml^{-1}, with a substrate of olive oil emulsion, when it was cultured in Zobell 2216E medium supplemented with 1% Tween 80.

The genomic library of *Psychrobacter* sp. G was constructed in pUC118 *Hinc*II/BAP, and was introduced into *E. coli* DH5α. Among 10 colonies forming clear haloes on a tributyrin agar plate, two lipase genes with different opening reading frames were identified. One open reading frame consisted of 1452 (*Lip-1452*) nucleotides that encoded 483 amino acids with a molecular mass of approximately 53 kDa and a pI of 5.4. The other open reading frame consisted of 948 (*Lip-948*) nucleotides that encoded 315 amino acids with molecular weights of 34 kDa and a pI of 8.0.

Arpigny et al. (1999) were the first to classify bacterial lipolytic enzymes into eight families based on differences in amino acid sequences and biological properties. Family I, the largest group, was further divided into seven subfamilies (I.1–I.7; Angkawidjaja & Kanaya 2006). The primary structure of *Lip-1452* and *Lip-948* indicated that they were probably members of families IV and V, respectively, of the bacterial lipolytic enzymes. The amino acid sequence alignment of *Lip-1452* indicated the two conserved regions, HGGGF and GDSAG, were identified in this sequence. Perhaps its catalytic triad consisted of Ser238, Asp414 and His444 (Fig. 3). The primary structure of this protein showed that it was a member of family IV, namely the HSL group (Arpigny et al. 1999).

Evidence points to *Lip-1452* and *Lip-948* belonging to a large superfamily called the "α/β hydrolase" superfamily. The members of this superfamily share the "nucleophilic elbow", which is a characteristic sequence motif, Gly-Xaa-Ser-Xaa-Gly, for most esterases and lipases. The Ser residue in this motif constitutes a "catalytic triad" with Asp and His residues that are placed in the specific order serine-aspartic acid-histidine in the polypeptide (Suzuki et al. 2003). *Lip-1452* was characterized by the motif GDSAG and *Lip-948* was characterized by GNSMG. *Lip-1452* had the catalytic triad in the specific order Ser299-Asp414-His444, and *Lip-948* had the catalytic triad Ser142-Asp264-His291.

The activity of the recombinant lipase produced by the expression system was relatively low, consistent with

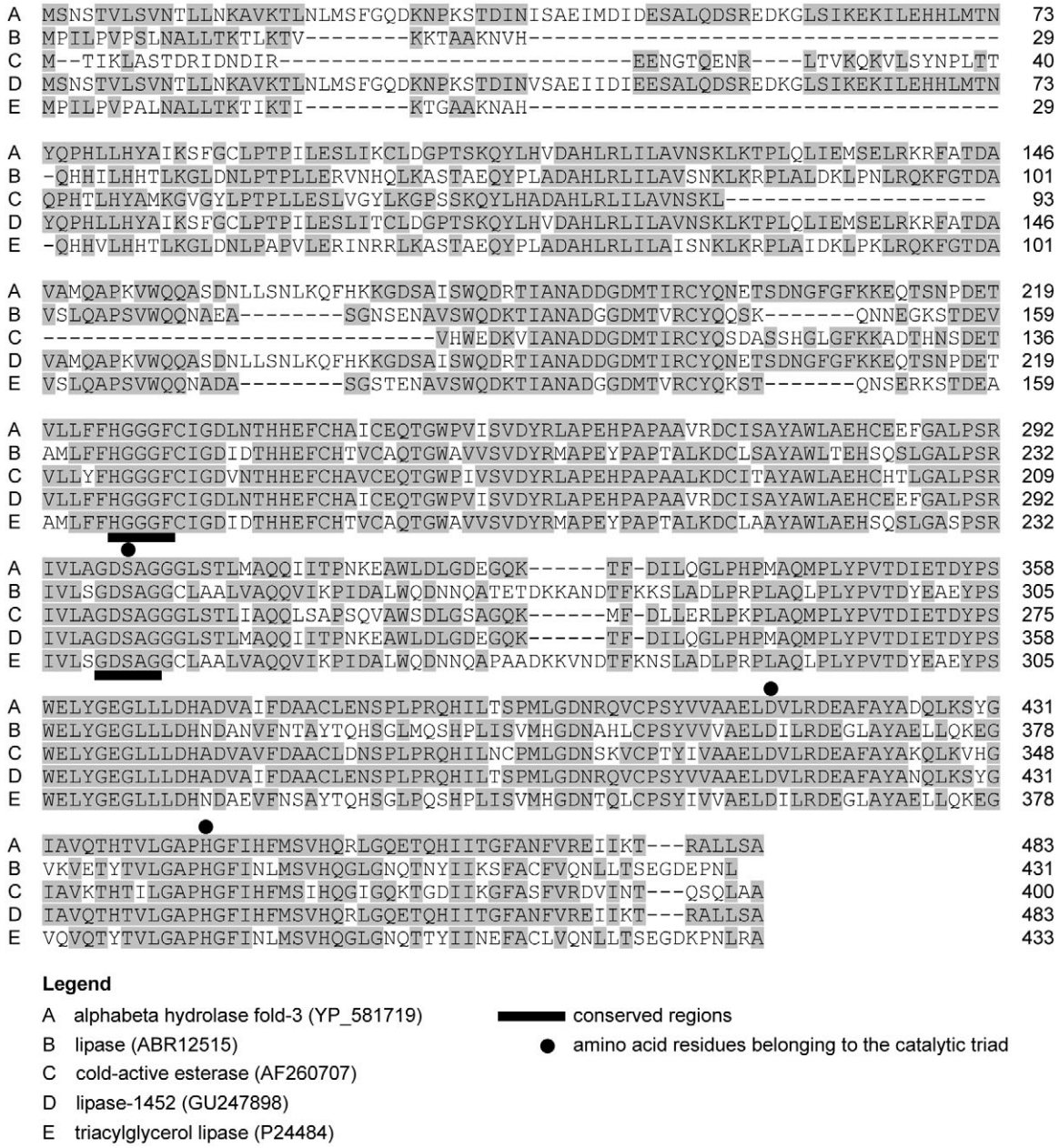

Fig. 3 Alignment of amino acid sequences of *Lip-1452* with homologous lipases. YP_581719, α/β-hydrolase fold-3 from *Psychrobacter cryohalolentis* K5; AF260707, cold-active esterase from *Pseudomonas* sp. St1; ABR12515, lipase from *Pseudomonas* sp. 2–17; P24484, lipase from *Moraxella* sp.

most previous findings (Suzuki et al. 2003; Pfeffer et al. 2007; Długołecka et al. 2008; Parra et al. 2008). Most of the lipase expressed in *E. coli* seemed to exist as an inclusion body. There are reports that lipases are not fully processed by some species of *E. coli,* and lipases aggregate as a result of their own hydrophobicity (Yang et al. 2008). Both *Lip-1452* and *Lip-948* were expressed in *E. coli;* however, functional heterologous expression was low, especially for *Lip-1452*. Because the heterologous expression of lipase in *E. coli* cells yielded inclusion bodies with no catalytic activity, renaturation studies had to be executed to obtain a catalytically active form of the enzyme (Suzuki et al. 2003). However, the expression in an active form of lipase belonging to sub-

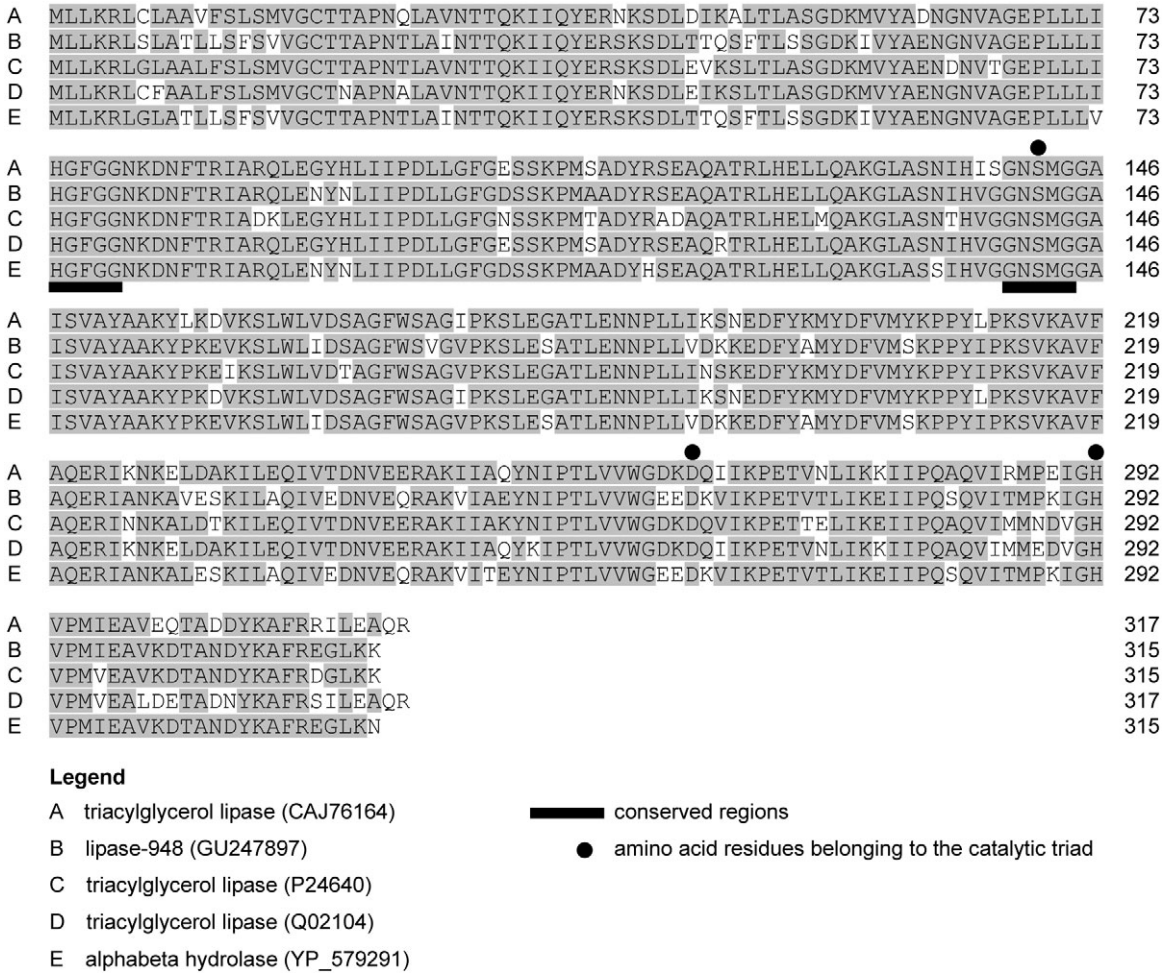

Fig. 4 Alignment of amino acid sequences of *Lip-948* with homologous lipases. YP_579291, α/β-hydrolase fold from *Psychrobacter cryohalolentis* K5; P24640, triacylglycerol lipase from *Moraxella* sp.; CAJ76164, triacylglycerol lipase from *Psychrobacter* sp. 7195; Q02104, triacylglycerol lipase from *Psychrobacter immobilis*.

families I.1 and I.2 depends on a chaperone protein named lipase-specific foldase, the gene of which is located downstream of the lipase gene for the efficient secretion and folding of active lipase (Arpigny et al. 1999; Quyen et al. 1999), whereas the folding of subfamily I.3 lipases does not require the assistance of any molecular chaperone (Angkawidjaja & Kanaya 2006). For example, a lipase belonging to subfamily I.1 or I.2 was heterologously expressed in *E. coli*, and the chemical refolding of inactive lipase in the absence of its chaperone yielded only 25 U mg^{-1}, whereas in a simple and rapid in vitro refolding procedure with its modified and truncated chaperone, functionally active lipase was obtained with a specific activity of up to 4850 U mg^{-1} and a yield of 31 400 U g^{-1} of *E. coli* wet cells (Quyen et al. 1999). Iizumi et al. (1991) reported that the activator gene (*act*), existing downstream of the lipase (*lip*) of *Pseudomonas fragi*, enhanced the heterologous expression of *lip* in *E. coli*. When the *lip* was expressed in *E. coli* using *lac* promoter on the pUC plasmid vector, the lipase activity of *E. coli* carrying both the *lip* and *act* was 200 times greater than that carrying only *lip*.

Acknowledgements

This work was financially supported by China's National High-Tech R & D Program (2007AA091905) and the Basic Scientific Research Operation Fund of China (GY02-2007-T11).

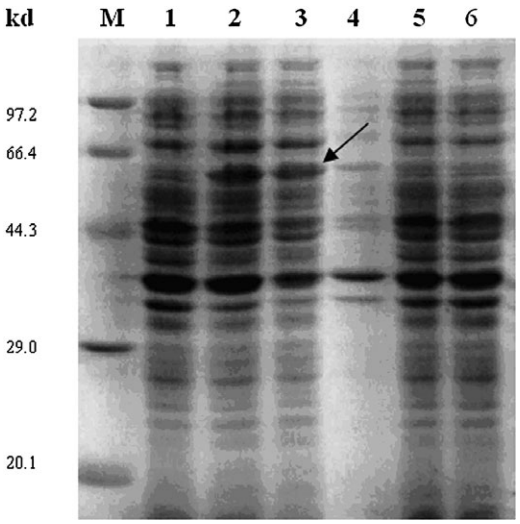

Fig. 5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of pCold III+*Lip-1452* in *Escherichia coli* BL21 (DE3); M, marker; lane 1, uninduced cell of pCold III+*Lip-948*; lane 2, total protein of pCold III+*Lip-1452* after 24-h induction; lane 3, supernatant after supersonic lysis; lane 4, pellet; lane 5, total protein of pCold III in BL21 after induction; lane 6, uninduced cell of pCold III. The arrow points to the predicted protein.

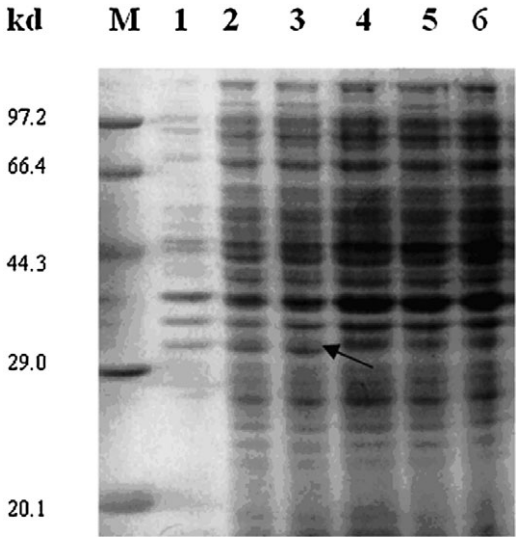

Fig. 6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of pCold III+*Lip-948* in *Escherichia coli* BL21 (DE3); M, marker; lane 1, pellet; lane 2, supernatant after supersonic lysis; lane 3, total protein of pCold III+*Lip-1452* after 24-h induction; lane 4, uninduced cell of pCold III+Lip-*948*; lane 5, total protein of pCold III in BL21 after induction; lane 6, uninduced cell of pCold III. The arrow points to the predicted protein.

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