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Identification and Molecular Modeling of a Novel Lipase from an Antarctic Soil Metagenomic Library

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Abstract

In this work, we present the construction of a metagenomic library in *Escherichia coli* using pUC19 vector and environmental DNA directly isolated from Antarctic topsoil and screened for lipolytic enzymes. Screening on agar supplemented with olive oil and rhodamine B revealed one clone with lipolytic activity (Lip1) out of 11000 *E. coli* clones. This clone harbored a plasmid, pLip1, which has an insert of 4722 bp that was completely sequenced from both directions. Further analysis of the insert showed three open reading frames (ORFs). ORF2 encoded a protein (Lip1) of 469 amino acids with 93% identity to the uncultured *Pseudomonas* sp. lipase LipJ03. Amino acid sequence comparison and phylogenetic analysis indicated that Lip1 lipase was closely related to family I subfamily 3. Furthermore, we present a three-dimensional model of lipase Lip1 which was generated based on the two known structures of mesophilic lipases from *Pseudomonas* sp. MIS 38 (PML lipase, PDB; 2Z8X) and *Serratia marcescens* (SML lipase, PDB: 2QUB). Finally, we report the results of comparisons between lipase Lip1 and mesophilic lipases and point out similarities and differences in the catalytic site and in other parts of the analyzed structures.

Key words: cold-adapted lipase, metagenomic library, molecular modeling

Introduction

Metagenomics, the study of genomic content DNA isolated from environmental samples, has proved particularly useful for the analysis of uncultured bacteria because greater than 99% of microbes found in natural environments cannot be cultured with currently available technologies (Schloss and Handelsman, 2003; Streit *et al.*, 2004; Daniel, 2004). Over the last few years, the metagenome-based strategy has been commonly used to explore some novel enzymes and molecules for biotechnological and pharmaceutical applications, such as lipases (Wei *et al.*, 2008; Henne *et al*., 2000; Lee *et al*., 2004; Elend *et al.*, 2007; Jeon *et al*., 2009), esterases (Jeon *et al.*, 2008), cellulases (Kim *et al.*, 2008), xylanases (Hu *et al.*, 2008) and novel antibiotics turbomycin A and B (Gillespie *et al.*, 2002) from different environments.

One approach in the search for novel biocatalysts is to generate the metagenomic library from soils that are known to harbor a high level of microbial diversity and thus, potentially a wide diversity of biocatalysts (Curtis and Sloan, 2005). A further development of this approach is to create the metagenomic library from an environment that has been subjected to extreme conditions in the likelihood that enzymes from such an environment will be able to function under those extreme conditions (Torsvik and Øvreås, 2002). Among the extreme environments, antarctic soil constitutes an attractive biocatalytic resource because of its abundance and broad diversity of cold-adapted microorganisms. Such microorganisms have developed adaptive mechanisms to perform their metabolic functions at low temperatures by incorporating unique features mainly in their proteins. Compared to proteins from mesophiles, cold-adapted proteins show decreased ionic interactions and hydrogen bonds, possess fewer hydrophobic groups and more charged groups ion on their surface as well as longer surface loops. Due to these modifications, at low temperatures psychrophilic proteins lose their rigidity and gain increased structural flexibility for enhanced catalytic

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function. The adaptive properties of psychrophilic enzymes are their high specific activity at low temperatures, a relatively low apparent temperature optimum for activity and a rather relatively high thermolability (Hoyoux *et al*., 2004; Georlette *et al*., 2004; D'Amico *et al*., 2006; Siddiqui and Cavicchioli, 2006). This unique ability of extremophilic biocatalysts to catalyze reactions at low or moderate temperatures offers great industrial and biotechnological potential (Gerday *et al*., 2000; Cavicchioli *et al*., 2002). One of the methods for prospecting these molecules with high potential for downstream application is metagenome-based approaches (Elend *et al*., 2007).

Recently, increasing attention has focused on a search for cold-adapted lipolytic enzymes. These enzymes are expected to be applied as additives to detergents used at low temperatures and biocatalysts for biotransformation of labile compounds at low temperature. Their molecular flexibility is higher relative to enzymes from mesophilic organisms and makes them particularly useful in stereoselective reactions, carried out in organic solvents and at low temperatures and yielding almost exclusively the isomer produced through conversion having the lower activation energy (Cavicchioli *et al*., 2002).

In the present study, we constructed a library of environmental DNA from soil sample collected from *Adelie penguin* rookery, which is located in the neighborhood of Henryk Arctowski Polish Antarctic Station at King George Island. The screening for novel lipolytic enzymes from uncultured soil microorganisms revealed one clone, Lip1, with a strong lipolytic activity. Further analysis revealed that the Lip1 clone harbored a novel *lip1* gene encoded lipase belonging to family I subfamily 3. The three-dimensional structure of this lipase has not yet been determined but we have modeled the 3D structure of metagenomic enzyme (Lip1) on the basis of the sequence of its gene. We analyzed the amino acid composition of lipase in comparison to mesophilic counterparts and characterized the architecture of the catalytic site The features potentially responsible for cold-adaptation and stabilization at low temperatures were also identified.

Experimental

Materials and Methods

Bacterial strains, plasmids and growth media. *E. coli* TOP10F' (Invitrogen) was used as the host strains for metagenomic DNA library construction and screening for lipolytic active clones. Plasmid pUC19 (Invitrogen) was used as DNA vector for metagenomic DNA library construction. The *E. coli* strain was grown on LB medium (Sambrook and Russel, 2001), supplemented with 100 µg/ml of ampicillin, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 2% (vol/vol) olive oil and 0.001% (wt/vol) rhodamine B (the fluorescent dye). Colonies were incubated for 1 day at 37°C, followed by incubation for 2 days at 25°C. Orange fluorescent halos around lipase-positive *E. coli* strains could be seen when these plates were exposed to UV light of 312 nm (Kouker and Jaeger, 1987). Ampicillin, IPTG and rhodamine B were purchased from Sigma-Aldrich (USA).

Sampling. The analyzed soil sample was collected from Adelie penguin rookery (S 62°09'46'', W 58°27'42'') which is located in the neighborhood of Henryk Arctowski Polish Antarctic Station at King George Island. Sampling depth was 5 cm. Next, the soil sample was sieved to remove penguin dung debris and particulate matter larger than 2 mm and was kept without disturbance at 4°C until analysis.

General DNA manipulations. Restriction enzymes, T4 DNA ligase were purchased from Fermentas (Lithuania). Restriction enzymes and other DNA-modifying enzymes were used according to the manufacturer's recommendations. The reagents for PCR and various oligodeoxynucleotides were purchased from DNA-Gdansk II (Poland).

Metagenomic DNA isolation from Antarctic soil sample. Soil samples of 5 g were mixed with 13.5 ml of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB) and 25 µl of lysozyme (10 mg/ml, A&A Biotechnology, Poland) in 15 ml Falcon tubes by horizontal shaking at 225 rpm for 30 min at 37°C. Next, 50 µl of proteinase K (20 mg/ml, A&A Biotechnology, Poland) was added to the sample and incubated again at the same conditions as described above. After the shaking treatment, 1.5 ml of 20% SDS was added, and the samples were incubated in water bath at 65°C for 2 h with gentle end-over-end inversions every 15 to 20 min. The supernatants were collected after centrifugation at 6 000 × g for 10 min at room temperature and transferred into 50-ml centrifuge tubes. The soil pellets were extracted two more times by adding 4.5 ml of extraction buffer and 0.5 ml of 20% SDS, vortexing for 10 s, incubating at 65°C for 10 min, and centrifuging as before. Supernatants from the three cycles of extractions were combined and mixed with an equal volume of chloroform: isoamyl alcohol (24:1, vol/vol). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for about 24 h. The pellet of crude nucleic acids was obtained by centrifugation at 16 000 × g for 20 min at room temperature, washed with cold 70% ethanol, and resuspended in sterile deionized water, to give a final volume of 500 µl. The extracted DNA was further purified by Genomic Mini kit (A&A Biotechnology, Poland), followed by final purification with Genomic AX Bacteria kit (A&A

Biotechnology, Poland). The pure DNA samples were stored frozen at –20°C until it was required.

Metagenomic library construction and screening for lipolytic activity. The pUC19 library of the purified DNA was constructed by the following procedures. DNA samples from Antarctic soil were used to construct metagenomic DNA library following partial digestion with HindIII and BamHI, optimized to maximize fragments in the 1–5 kbp size range. The fragments of 1–5 kbp resolved in an agarose gel were excised and concentrated. The BamHI- and HindIII-digested pUC19 DNA was overhanged using T4 DNA ligase (Epicentre, USA) to genomic DNA library digested with the same restriction endonucleases, and *E. coli* TOP10F' (Invitrogen, USA) were transformed with the ligation products. The lipase activities of the transformants were tested on Luria-Bertani agar containing: 100 μg/ml of ampicillin, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 2% (vol/vol) olive oil and 0.001% (wt/vol) rhodamine B (the fluorescent dye) designed as LB-lipRB medium.

Analysis of the lipolityc active *E. coli* clone. The *E. coli* clone showing orange halos was inoculated into a 250 ml flask containing 50 ml of LB medium (100 μg/ml ampicillin). After overnight incubation at 37°C, the cells were harvested by centrifugation at 5 000 × g for 15 min and washed twice with sterile distilled water. Next, the plasmid DNA was isolated using Plasmid Mini isolation kit (A&A Biotechnology, Poland), retransformed into *E. coli* TOP10F' and the new clones were examined on the LB-lipRB medium for lipase activity. The plasmid DNA from the one of the positive clones (pink fluorescence at UV light 312 nm) was isolated as described above and designed as pLip1. Next, a few samples with plasmid pLip1 DNA were digested with selected restriction enzymes to create restriction maps of examined plasmid. The nucleotide sequence of the DNA insert from pPINK-UV plasmid was determined using an ABI 3730 xl/ABI 3700 sequencing technology (Genomed, Poland). The open reading frames were detected using the ORF search tool provided by the National Center for Biotechnology Information (NCBI). The predicted function of ORFs was annotated using BlastX search toward the NCBI nonredundant protein database (Altschul *et al*., 1997).

Sequence data analysis and molecular modeling. GenBank was scanned for related sequences by the BLAST algorithm. Nucleotide and amino-acid sequences were aligned by using the computer program ESPript (Gouet *et al*., 1999).

Protein structure prediction was carried out *via* the GenSilico MetaServer (http://gensilico.pl/meta/, Kurowski and Bujnicki, 2003), which is a convenient gateway to a number of publicly available online services for secondary structure prediction.

The alignment between the sequences of metagenomic lipase Lip1 and its mesophilic counterparts from the database was the first step of homology modelling. The set of alternative models was generated using both MODELLER (Sali *et al*., 1995) and SWISS-MODEL (Guex and Peitsch, 1997). The models were scored using MetaMQAPII metaserver (Pawłowski *et al*., 2008). The final model was obtained using the 'Frankenstein's Monster' approach (Kosinski *et al*., 2003), which comprises cyclic realignment in poorly scored regions and merging the best scoring fragments. The sequence-structure fit in the final model was also evaluated using MetaMQAPII and ProQ server (Pawłowski *et al*., 2008), while the stereochemical parameters were assessed with PROCHECK (Laskowski *et al*., 1993).

Modeling of the lipase Lip1 was performed using templates selected based on the results of the Genesilico MetaServer. The templates used for the modeling were the crystal structures of two lipases from *Pseudomonas* sp. MIS 38 (Angkawidjaja *et al*., 2007) and *Serratia marcescens* (Meier *et al*., 2007).

Nucleotide sequence accession number. The sequences of the *lip1* gene was deposited in the GenBank database with accession number GQ352455.

Results and Discussion

Construction and screening of a metagenomic library. A metagenomic library consisting of ~11,000 clones was constructed using DNA isolated from soil sample collected from *Adelie penguin* rookery which is located in the neighborhood of Henryk Arctowski Polish Antarctic Station at King George Island. To screen for lipase-producing clones, the pUC19-derivaite clones were plated onto LB-lipRB medium. One clone with lipolytic activity was detected and designated as LIP1.

Molecular analysis of pLIP1. The insert of the recombinant plasmid recovered from Lip1 clone was sequenced. The nucleotide sequence analysis revealed the presence of 3 ORFs longer than 100 amino acid residues that exhibited similarities to genes annotated with predicted functions (Table I). The ORF's sequence analysis with using BlastX search toward the NCBI nonredundant protein database revealed that ORF2 encoded protein with high sequence similarity (93% identity) with lipase LipJ03 from uncultured *Pseudomonas* sp. strain. The Lip1 protein consisted of 469 amino acids with a deduced molecular mass (Mw) of 49 372 Da. Further analysis of lipase Lip1 sequence revealed that the enzyme belonged to family I subfamily 3 of lipases.

The program BLAST which was used to screen for sequences of lipolytic enzymes deposited in NCBI

Table I
Sequence analysis of the ORFs encoded in metagenomic insert of pLip1

ORF #	Length (amino acids)	Putative function (most similar homologue)	Putative source organism	Accession number	% Identity/ similarity	E value
1	235	hypothetical protein PFLU3139	*Pseudomonas fluorescens* SBW25	YP_002872714	99/99	$3e^{-126}$
2	469	lipase	uncultured *Pseudomonas* sp.	AAU12351	93/97	0.0
3	442	outer membrane protein	*Pseudomonas fluorescens*	BAA88494	57/78	$8e^{-88}$

showed that the metagenomic antarctic lipase displayed the highest sequence identity to the lipase from the non-culturable bacterium *Pseudomonas* (93% sequence identity) (Jiang *et al.*, 2006).

Molecular modeling. Bioinformatic analysis of metagenomic lipase Lip 1 molecule revealed the similarity in its sequence to other lipases with known structures, isolated from culturable bacteria. Among the proteins of known crystallographic structure, Lip1 showed the maximum sequence identity to PML *Pseudomonas* sp. MIS 38 lipase (72%) and SML lipase from *Serratia marcescens* (63%) (Fig. 1) that served as templates for modeling of the metagenomic lipase structure using the "Frankenstein monster" approach.

The antarctic lipase Lip1 consists of 469 amino acid residues. In contrast to PML and SML enzymes, Lip 1

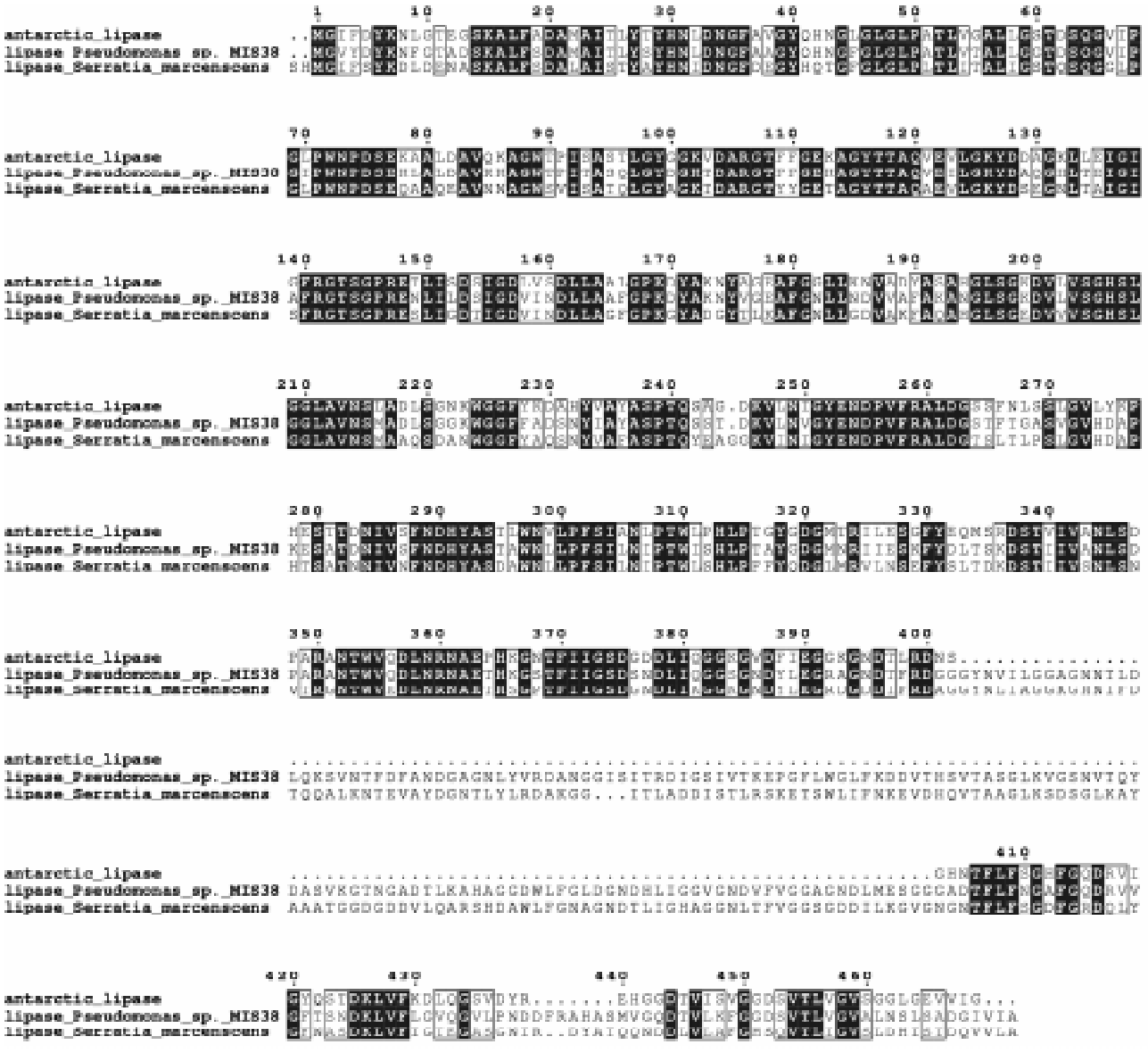

Fig. 1. Sequence alignment of the metagenomic Antarctic lipase and lipases from *Pseudomonas* sp. MIS38 (PML lipase, PDB; 2Z8X) and *Serratia marcescens* (SML lipase, PDB: 2QUB) that were templates used for structure modeling (conserved sequences are marked).

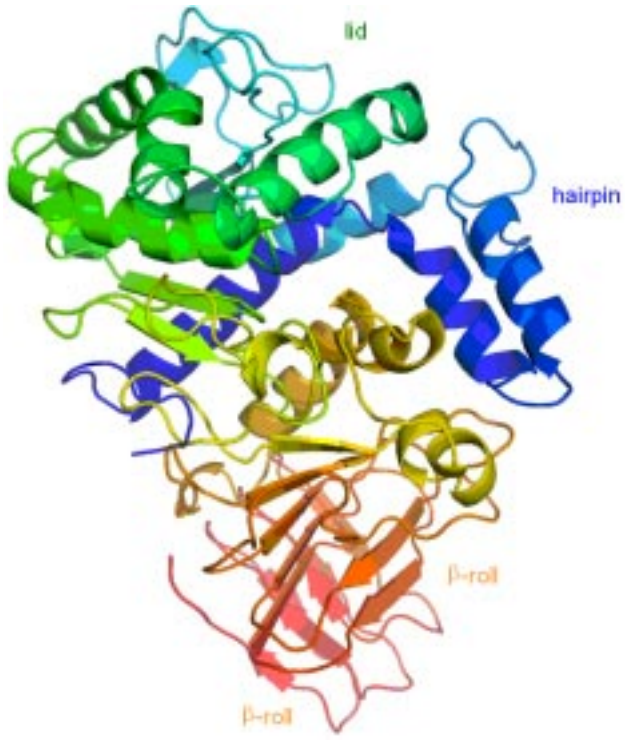

Fig. 2. Model of Lip1 lipase (the successive fragments of the molecule with defined secondary structure are marked in different colors: dark blue from the N-terminus and red at the C-terminus; the domains formed by these fragments are also shown in the model).

Fig. 3. The model structure of Lip1 lipase (clusters of Gly residues in C-terminal domain are marked in dark blue).

is characterized by a lack of 138 amino acids in the polypeptide chain. The evidence that the lack of a fragment of the polypeptide chain is possible was provided by an earlier experiment of Jiang *et al*. (2006) who isolated from the non-culturable bacterium *Pseudomonas* the gene of the homologous enzyme (by using the genome-walking approach), expressed it in the yeast *Pichia pastoris* and obtained the biologically active protein. This means that the lacking structural element in lipase Lip1 and lipase LipJ03 from non-culturable *Pseudomonas* strain is not characteristic of all lipases belonging to family I.3 and it is not essential for retaining their activity.

The Lip1 lipase consists of two domains (Fig. 2). The N-terminal domain shows a modified α/β hydrolase fold and it is rich in α-helices, while C-domain contains two β-roll motifs, laterally stacked together forming the so-called sandwich, similar to that of SML and PML. In the C-terminal part of C-domain Lip1 there are several repeats of the RTX motif – GGXG XDX(U)X (U, hydrophobic amino acids), which are responsible for activity and folding of this group of lipolytic enzymes (Fig. 3). This repeated motif of PML and SML has been proposed to function as an intramolecular chaperone, because deletion or mutation of this motif generates inactive proteins, which are incompletely folded (Meier *et al.*, 2007; Angkawidjaja *et al*., 2007).

The active site of lipases is defined by a canonical catalytic triad, which in Lip1 consists of Ser^{207}, Asp^{255}, and His ^{313}. These residues superimpose quite well with the corresponding amino acids from other lipases, *e.g.* lipases from *Pseudomonas* sp. MIS 38 or *Serratia marcescens* (Fig. 4).

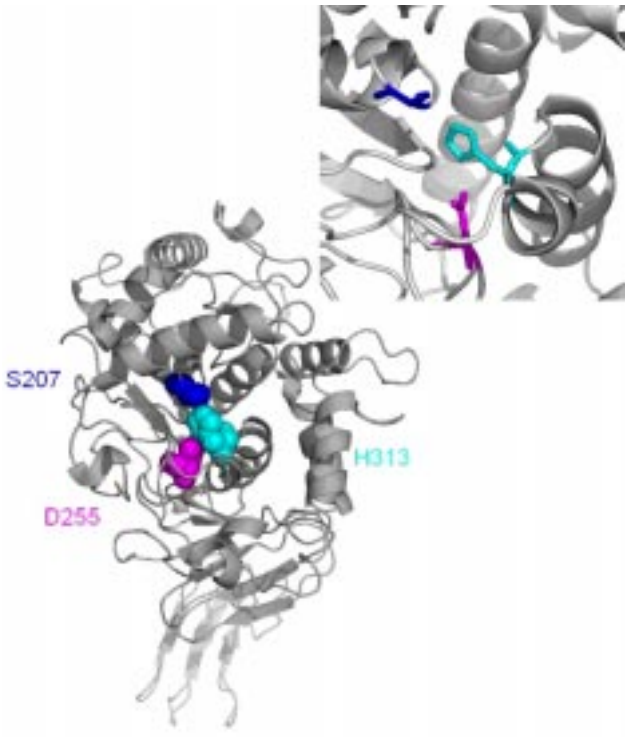

Fig. 4. Active center of the metagenomic Lip1 lipase (catalytic amino acid residues are marked with colors).

Conclusions. In this paper we present the identification, isolation of a new lipase *lip1* gene from Antarctic soil metagenomic library and analysis of the model *in silica* of lipase encoded by *lip1* gene. The results indicate that lipase encoded by gene *lip1* showed a lack of 138 amino acids in the polypeptide chain in comparison to the structures of well characterized mesophilic analogs: PML lipase from *Pseudomonas* sp. MIS 38 and SML lipase from *Serratia marcescens*. On the other hand, the analyzed lipase showed high sequence similarity (93%) to cold-adapted LipJ03 lipase from non-culturable *Pseudomonas* strain with optimum temperature activity 35°C and pH 8.0, respectively (Jiang *et al*., 2006). The results presented in this paper encourage us to further study the lip1 gene product. To this end we intend to construct a recombinant *E. coli* strain for Lip1 lipase production and purify the recombinant enzyme for further characterization.

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