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Lipase Diversity in Glacier Soil Based on Analysis of Metagenomic DNA Fragments and Cell Culture

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Lipase diversity in glacier soil was assessed by culture-independent metagenomic DNA fragment screening and confirmed by cell culture experiments. A set of degenerate PCR primers specific for lipases of the hormone-sensitive lipase family was designed based on conserved motifs and used to directly PCR amplify metagenomic DNA from glacier soil. These products were used to construct a lipase fragment clone library. Among the 300 clones sequenced for the analysis, 201 clones encoding partial lipases shared 51–82% identity to known lipases in GenBank. Based on a phylogenetic analysis, five divergent clusters were established, one of which may represent a previously unidentified lipase subfamily. In the culture study, 11 lipase-producing bacteria were selectively isolated and characterized by 16S rDNA sequences. Using the above-mentioned degenerate primers, seven lipase gene fragments were cloned, but not all of them could be accounted for by the clones in the library. Two full-length lipase genes obtained by TAIL–PCR were expressed in *Pichia pastoris* and characterized. Both were authentic lipases with optimum temperatures of ≤40°C. Our study indicates the abundant lipase diversity in glacier soil as well as the feasibility of sequence-based screening in discovering new lipase genes from complex environmental samples.

Keywords: Lipase, gene diversity, metagenomic library, sequence-based screening, glacier soil

Lipases (triacylglycerol hydrolases; E.C. 3.1.1.3) are a class of enzymes that catalyze triacylglycerol hydrolysis, ester synthesis, and transesterification [18]. Because of their functional versatility, such as stability in organic solvents, broad substrate specificity, and high regio- and/or stereo-selectivity in catalysis [13], microbial lipases have recently emerged as an important group of biotechnologically relevant enzymes that are critical for many industrial applications [4, 13].

Hormone sensitive lipase (HSL) is a multifunctional superfamily of lipases. Based on amino acid sequences alignment and analysis, considerable cold-adapted lipases, such as lipase 2 from *Moraxella* TA144 and lipP from *Pseudomonas* sp. [7, 9], have been classified into the HSL family. Comparative study of the HSL family would therefore be very valuable for the comprehensive understanding of the low temperature lipases.

Culture-dependent methods traditionally have been used to isolate new lipases from microorganisms. However, current estimates indicate that less than 1% of these organisms are readily culturable with known cultivation techniques [3]. This problem can be solved by using a culture-independent method, namely direct isolation and analysis of metagenomic DNA from the environment, thereby preserving the major genetic information of microbial communities [3, 16]. Metagenomic library screening has become a powerful tool to exploit novel biocatalysts from microbial communities [24, 34, 35]. Two broad screening strategies — one based on function, and the other based on sequence — are generally used in metagenomic studies. Both of these, however, have advantages and disadvantages individually. Functional screening may possibly capture entirely novel protein families. Several novel lipases have been identified by function-based screening from metagenomic libraries [26, 35]. However, certain issues, such as recognition of expression signals and codon usage, proper protein folding, post-translational modifications, and the presence of introns, may prevent functional expression of genes in a heterologous background. Thus, only a small

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amount of genes have been isolated using this method [10, 14]. A sequence-based method can obtain more genes from the environment. The obtained genes showing genetic diversity may have new enzymatic characteristics and potential values in application, even though the genes do not belong to a novel protein family. Abundant gene sequences can be obtained quickly and conveniently, thereby allowing a comprehensive analysis of genes from most of the organisms in the environment sample. Indeed, several new lipases have been screened from complex biomasses using this strategy [5].

Among the extreme environments where biological samples can be acquired, glacier soil constitutes an attractive biocatalytic resource because of its abundance and broad diversity of low-temperature microorganisms [38]. Moreover, the glacier soil environment represents a relatively closed and cold ecosystem with a profuse biotic community, containing abundant microorganisms [8].

MATERIALS AND METHODS

Strains, Plasmids, Media, and Chemicals

Escherichia coli TOP10 (TransGen, China) and *Pichia pastoris* GS115 (Invitrogen, U.S.A.) were used as the hosts for the recombinant plasmid in the cloning and expression phases, respectively. Plasmid pEASY-T3 (TransGen, China) was used as the TA cloning vector for PCR products, and pPIC9K (Invitrogen) was the expression vector. Media used during the expression phase, including BMGY, BMMY, and RDB, were prepared according to the manual of the *Pichia* expression kit (Invitrogen). The lipase-inducing medium contained 5 g/l yeast extract, 5 g/l $NaNO_3$, 5 g/l K_2HPO_4, 0.3 g/l $MgSO_4 \cdot 7H_2O$, 0.01 g/l $CaCl_2$, and 0.01 g/l $FeSO_4 \cdot 7H_2O$. After supplementation with 10 g/l olive oil, the medium was emulsified entirely by sonication 20 times for 5 s [11], adjusted to pH 7.5, and autoclaved at 121°C for 20 min. The substrate *p*-nitrophenyl palmitate (*p*NPP, C_{16}) was purchased from Sigma (U.S.A.). All other chemicals were of analytical grade and commercially available.

Isolation of Metagenomic DNA from Glacier Soil

Samples of glacier soil were collected from China No. 1 glacier in Xinjiang Province. Metagenomic DNA was isolated using the direct lysis method with modifications, as previously described [6]. Briefly, the soil sample was passed through a fine mesh screen. Then, 20 ml of sterile sodium phosphate buffer (0.1 M, pH 7.2) was added to 10 g of soil, and the mixture was stirred vigorously for 10 min. The slurry was centrifuged at 1,000 ×*g* for 5 min to remove clay and sand. Then, 20 ml of the supernatant was added to a 50-ml centrifuge tube, and 20 ml of the preheated (70°C) lysis buffer [100 mM Tris-HCl, 100 mM Na_2EDTA, 1.5 M NaCl, 1% (w/v) cetyl trimethyl ammonium bromide, 2% (w/v) SDS, pH 8.0] was added. The mixture was incubated at 70°C for 2 h in a water bath. The crude soil "lysate" was centrifuged at 3,500 ×*g* for 10 min at 4°C twice, and 0.7 volume of isopropanol was added to the supernatant. The mixture was mixed gently by inversion and then incubated at room temperature for 30 min. The resultant precipitate was pelleted at 6,000 ×*g* for 30 min at 4°C. The pellet was then dried in air for 2–3 h at room temperature. The crude metagenomic DNA was suspended in 200 µl of TE and purified with the Cycle-Pure DNA kit (Omega, U.S.A.).

Design of Primers Specific for Lipases

Considering the extremophilic environment of glacier soil, a set of degenerate primers was designed to amplify lipase genes based on published cold-related lipases belonging to the HSL family obtained by an Entrez search at NCBI (http://www.ncbi.nlm.nih.gov/Entrez/). The potential consensus regions of lipase amino acid sequences were identified by sequence alignment using ClustalW [29]. To test the specificity and universality of the primers, an iterative test was used to examine them by comparison with available lipase sequences using BLASTx (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The primer sequences were then modified to maximize the number of lipase genes amplified. After repeating the process several times, the primers complementary to the selected consensus regions were designed (Table 1).

Construction of Lipase Gene Fragment Clone Library

The purified DNA from glacier soil was amplified by touchdown PCR with minor modification [32]. To amplify the lipase genes present in the soil metagenomic DNA, the degenerate primers CLF and CLR (Table 1) were added at a final concentration of 5 µM each, along with approximately 200 ng of the metagenomic DNA as the template. The 50-µl reactions also included 1× GC PCR buffer (TaKaRa, Japan), 0.4 mM dNTPs, and 0.25 U *Taq* polymerase (TaKaRa). PCR was carried out under the following conditions: 94°C for 5 min, followed by 10 cycles of 94°C for 30 s, 55°C for 30 s with a gradual decrease of 1°C per cycle, and 72°C for 30 s, and then followed by 30 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 30 s. The final extension step was at 72°C for 5 min. PCR products were visualized on an agarose gel and purified using a gel extraction kit (Omega). To construct the lipase gene fragment clone library, the purified PCR products were cloned into pEASY-T3 vector and transformed into *E. coli* TOP10 by electroporation (Bio-Rad, U.S.A.). The positive white clones were randomly selected from the LB agar plates containing 0.8 mg/ml X-gal, 3 mM IPTG, and 100 µg/ml ampicillin and sequenced with vector-derived M13F primer.

Isolation and Characterization of Lipase-Producing Microorganisms

A dilution method with lipase-inducing medium was used to isolate microorganisms from the glacier soil. Colonies were repeatedly picked and streaked on LB agar plates to achieve homogeneity. The homogeneous microorganisms were incubated in 100-ml flasks containing 20 ml of the lipase-inducing medium in a rotary shaker at 180 rpm at 15°C. After 5 days, the culture was homogenized by sonication 10 times for 5 sec each and centrifuged at 10,000 ×*g* for 5 min. The supernatant containing lipase was subject to a lipase activity assay using a spectrophotometric method as described below. Each strain showing hydrolysis activity against *p*NPP in the culture was defined as a lipase-producing microorganism. To identify the lipase-producing microorganisms, partial 16S rDNA sequences were amplified with the primers 27F and 1492R (Table 1) and sequenced using the same primers to authenticate the isolates.

Cloning of the Lipase Genes from Lipase-Producing Microorganisms

Standard procedures for DNA extraction, plasmid isolation, restriction enzyme digestion, and ligation were performed as previously described

Table 1. Primers used in this study.

Primers	Sequence (5'→3')[a, b]
CLF	GTGGTGTAYTTYCAYGGBGG
CLR	CAGGTTGCCRCCSGCRCTRTCNCC
27F	AGAGTTTGATCCTGGCTCAG
1492R	TACCTTGTTACGACTT
Lip3Adsp1	GCAGCCTCGACAGTCACGACGCG
Lip3Adsp2	GCGGCGCTCGGAAATTTTGCCCTGC
Lip3Adsp3	CTGCAGGACGGCGCCAACCATGG
Lip3Ausp1	CCGCGAGGCATCCAGCCCATGG
Lip3Ausp2	CCAGTTCACTGCATCGCAAGCGT-CATGC
Lip3Ausp3	GCCAATCGTCGACACAGCGCGTCG
LipXDdsp1	CGGCAGCCTGGATTCCCATGATGCG
LipXDdsp2	ATTGACCGTGGATTATCGACTGGCCCCG
LipXDdsp3	AACTGGCTGGTGCGCGAAGGCGC
LipXDusp1	GCGCCACCCGATTCGCATCCAGC
LipXDusp2	ACCAGCCAGTTGCCGGCATCGCAG
LipXDusp3	AGGCCAGTCGCCGGCACAGCG
LipDB5dsp1	CGGAGCACCCGTACCCCGCG
LipDB5dsp2	CGCGGGTCTCGAGGACTGCTACC
LipDB5dsp3	CAGCGACTGCTGGATGAACCGGAGC
LipDB5usp1	CGCCCACCAGGACGATCCGATGC
LipDB5usp2	ATGCGGATCGTCGAGCTGGGCCAG
LipDB5usp3	CAGCAGTCGCTGCACGATCTGGTAGC
LipDB8dsp1	GCAGCTCTCGACGACGCGGTCTCC
LipDB8dsp2	CCGCCTTCATGCAGTTGGTCACCGAG
LipDB8dsp3	GACTATTCGGAAGACCGCGTTGC-CATCG
LipDB8usp1	GATGGCAACGCGGTCTTCCGAAT-AGTCG
LipDB8usp2	GAACTCGGTGACCAACTGCATGAAG-GCG
LipDB8usp3	GAGACCGCGTCGTCGAGAGCTGCG
AD1	TGWGNAGWANCASAGA
AD2	AGWGNAGWANCAWAGG
AD3	CAWCGICNGAIASGAA
AD4	TCSTICGNACITWGGA
picL3A-F	GTG***GAATTC***AGTGAGTTTGAG-CAAAGCGCC
picL3A-R	GTT***GCGGCCGC***CTAATTCCCCAGT-TGAGC
picLXD-F	GGTT***GAATTC***ATGGCCGTATTGCCA-GAACTTG
picLXD-R	GTA***GCGGCCGC***TCAAACCCGCGAACG-CAC

[a]B: C, G or T; N: A, C, G or T; R: A or G; S: G or C; W: A or T; Y: C or T; I: inosine.
[b]Restriction sites are labeled in bold and italic font.

[33]. The lipase gene fragments were amplified using bacterial genomic DNA as the template following the same procedure as library construction. To obtain the full-length lipase genes, genome walking thermal asymmetric interlaced PCR (TAIL-PCR) was performed as previously described [27] using genomic DNA of the lipase-producing microorganism as template. The nested insertion-specific primers (Table 1) for TAIL-PCR were named "up special primer (usp) 1-3" and "down special primer (dsp) 1-3." TAIL-PCR was preformed using the Genome Walking kit (TaKaRa) according to the manufacturer's instructions.

Sequence Analysis

Sequences from the lipase fragment clone library were compared with the known lipases in GenBank using BLASTx [2]. To better understand the diversity and relationship of different lipase genes in glacier soil, the deduced amino acid sequences of lipase fragments were processed for phylogenetic analyses using MEGA 3.1 [23]. The reference group comprised equivalent regions of HSL genes available in GenBank.

The lipase gene sequences from lipase-producing microorganisms were assembled using the ContigExpress program of the Vector NTI suite 7.0 (InforMax, U.S.A.). Open reading frames in the assembled sequence were analyzed by the ORF finder tool [36], and the amino acid sequence of each identified ORF was used to find the closest match according to BLAST. The potential signal peptide and hydrolytic domain of the identified lipase genes were predicted using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP). Multiple alignments between protein sequences were performed using ClustalW1.83.

Enzyme Assay

The lipase assay was performed as described elsewhere [14, 37]. Briefly, the substrate solution was prepared with one volume of isopropanol containing 0.08 mM of *p*NPP and nine volumes of 50 mM sodium phosphate (pH 8.0) containing 0.2% of sodium deoxycholate and 0.1% of gum Arabic. After pre-incubation at 37°C for 3 min, 2.4 ml of the freshly prepared substrate solution was added to 0.1 ml of crude enzyme and incubated at 37°C for 15 min. The absorbance at 410 nm was measured with an Ultrospec 2000 spectrophotometer (Amersham Pharmacia, Sweden).

Expression of Lipase Genes in *P. pastoris*

Two sets of primers, picL3A-F and picL3A-R, and picLXD-F and picLXD-R, containing EcoRI and NotI restriction sites (Table 1), were used to amplify the lipase genes of Lip3A and LipXD directly from the genomic DNA lacking signal peptide sequences, respectively. PCR reactions were carried out with 32 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 2 min. The PCR products encoding the mature proteins Lip3A and LipXD were digested with EcoRI and NotI, respectively, and cloned into the corresponding sites of vector pPIC9K to generate recombinant plasmids. The recombinant plasmids were linearized and transformed into *P. pastoris* GS115 competent cells by electroporation (Bio-Rad). The transformed cells were plated on RDB plates and incubated at 30°C to screen for *His*$^+$ transformants, which were then transferred into 3 ml of BMGY medium and cultivated at 30°C for 48 h. The cells were pelleted by centrifugation, suspended in 1 ml of BMMY containing 0.5% methanol, and then induced for expression at 30°C for 48 h. The culture was centrifuged, and the supernatant was collected as the crude enzyme. Protein expression in the culture supernatant was assessed by enzyme assay and SDS-PAGE.

Optimum Temperature and Thermostability of the Recombinant Lipases

The temperature versus activity profiles of these crude enzymes were assessed in 50 mM sodium phosphate (pH 8.0) at temperatures

of 10–60°C. To examine the thermostability, the enzymes were incubated in 50 mM sodium phosphate (pH 8.0) at 40, 50, or 60°C, each for 5, 10, and 30 min. The remaining lipase activity was measured at 37°C and pH 8.0.

Nucleotide Sequence Accession Numbers

The sequences of partial lipase genes from the lipase fragment clone library were deposited into GenBank under accession numbers EU569045–EU569207. The full-length sequences of lipases from isolates were given accession numbers EU580534–EU580537.

RESULTS

Degenerate Primers Specific for Lipases

Two consensus regions, H-G and G-X-S-X-G (X represents any amino acid), were found in most of the lipases at the consensus regions of the oxyanion hole and the active site, respectively [5, 20, 22]. Certain primers were designed previously and used to amplify unknown lipases based on these two consensus regions [5]. However, it was difficult using these sites to design degenerate primers because of the low sequence similarity and shortness of the conserved sequences. Our strategy was to classify the lipase sequences into different groups and focus on the lipases belonging to the HSL family. Based on the amino acid sequence alignment of six HSL cold-related lipases, two conserved regions [V/L]-[F/Y/D]-[F/I]-H-G-G-[G/A] and G-[D/V]-S-[A/V]-G-G-[N/C]-[L/M/I] were found around the oxyanion hole and active site, respectively (Fig. 1). A set of degenerate primers, CLF and CLR, were then designed without considering the codon usage because the aim of our PCR strategy was to amplify lipases from biomass containing unknown organisms. The validity of these primers was established by comparison with the available lipase gene sequences in GenBank. After several rounds of modification, the primers were optimized to maximize the number of lipases amplified (Table 1).

Lipase Fragment Clone Library

The yield of DNA from the soil was approximately 2–3 μg/g soil. The purified metagenomic DNA of 20–30 kb was extracted from the glacier soil and used to construct a lipase clone library. Lipase metagenomic DNA fragments, generally 200–280 bp, were amplified using primers CLF and CLR, ligated into pEASY-T3, and transformed into *E. coli* TOP10. Collectively, the constructs constituted a lipase fragment clone library named GCLP (Glacier Soil Lipase), comprising >2,000 clones. To evaluate the validity

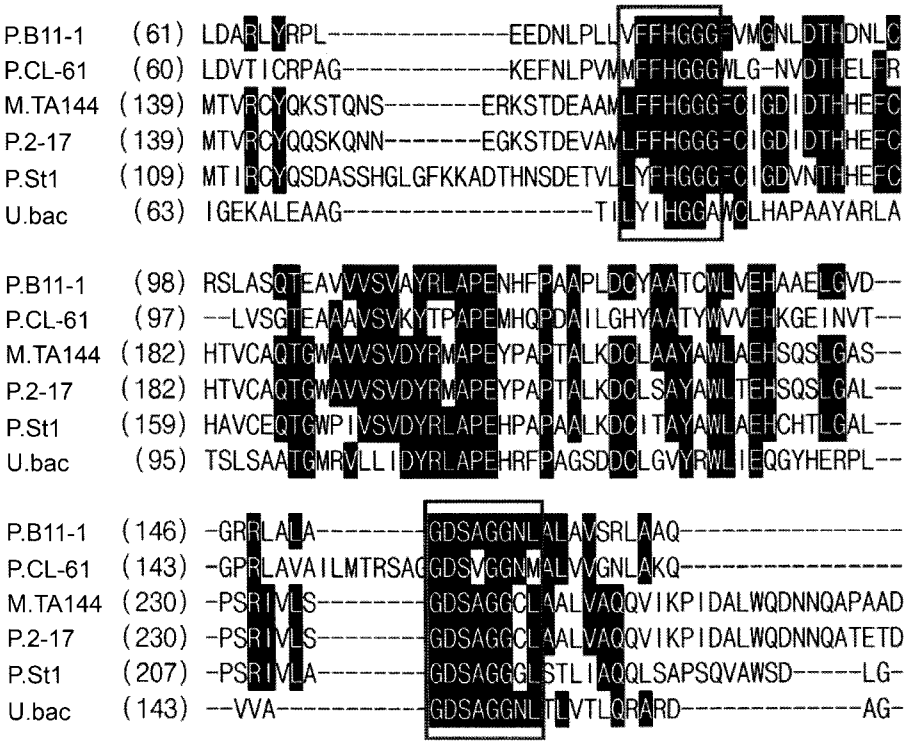

Fig. 1. Amino acid sequence alignment of cold-related lipases from the hormone-sensitive lipase (HSL) group in GenBank (NCBI).
The sequences displayed in the alignments are as follows: P. B11-1: cold-adapted lipase from *Pseudomonas* sp. B11-1 (AAC38151); P. CL-61: cold-adapted lipase from *Pseudomonas* sp. CL-61 (ABC25547); M. TA144: lipase from the Antarctic psychrotroph *Moraxella* sp. TA144 (CAA37862); P. 2-17: cold-active lipase of *Psychrobacter* sp. 2-17 isolated from the marine Antarctic (ABR12515); P. St1: cold-active lipase from the psychrophilic bacterium *Psychrobacter* sp. St1 (AAF70342); U. bac: cold-active lipase from an uncultured bacterium of marine sediment (AAZ67909). Conserved amino acids are highlighted. The two consensus regions are boxed.

Table 2. Similarity in amino acid sequences for lipases from lipase-producing microorganisms and their closest homolog[a].

Lipase	Source	Clone	ORF[b]	GenBank Accession No.	Closest homologous lipase (Accession No.)	Identity (%)
Lip3A	*Pseudomonas syringae*	GCLP08	320	EU580534	*Pseudomonas* lipase (YP_608777)	53
LipXD	*Pseudomonas* sp.	GCLP111	318	EU580535	*Pseudomonas* lipase (YP_608777)	51
LipDB5	*Brachybacterium tyrofermentans*	GCLP84	310	EU580536	*Brevibacterium* lipase (ZP_00379884)	70
LipDB8	*Rhodococcus erythropolis*	GCLP162	313	EU580537	*Rhodococcus* esterase (YP_705941)	71

[a]The corresponding protein, source, and database accession number are given for the closest homologs based on a BLAST search at NCBI.
[b]Predicted length of the amino acid residues in the ORF.

of the library, 20 positive clones were selected for plasmid extraction and analysis using restriction enzyme digestion. About 95% of these 20 clones had a recombinant plasmid with an insert size similar to those of lipase fragments.

Among the 300 positive clones from the GCLP library that were sequenced for analysis, 201 sequences showed the highest identity (51–82%) to lipase genes based on a GenBank search, indicating that many of the lipase sequences from the GCLP library might be novel.

Isolation of Lipase-Producing Microorganisms

A total of 11 lipase-producing bacteria were isolated by using lipase-inducing medium and classified into seven genera based on 16S rDNA sequence, including *Pseudomonas* (five strains) and *Brachybacterium* (one), *Flavobacterium* (one), *Janthinobacterium* (one), *Proteobacterium* (one), *Rhizobium* (one), and *Rhodococcus* (one). Many lipases have been cloned and isolated from *Pseudomonas* [1, 17], and lipase sequences from *Flavobacterium*, *Janthinobacterium*, *Proteobacterium*, *Rhizobium*, and *Rhodococcus* have also been reported in GenBank. This is the first report, however, of the *Brachybacterium* lipase.

Cloning Lipase Genes from Lipase-Producing Microorganisms

Genomic DNA was extracted from each of the 11 lipase-producing bacteria. A total of seven amplicons of ~250 bp were amplified using primers CLF and CLR. These products were ligated into pEASY-T3 and transformed into *E. coli* TOP10. The positive clones sequenced with primer M13F showed highest identities to lipase genes in GenBank. The seven lipase fragments were aligned with sequences from the GCLP library, and four were identical to clones GCLP08, 84, 111, and 162, respectively (Table 2).

The full-length lipase genes identical to the GCLP library clones were obtained using TAIL–PCR. Among these, two lipase genes, *Lip3A* and *LipXD*, were selected for expression in *P. pastoris*. *Lip3A* from *Pseudomonas syringae*, encoding 320 amino acids, had the highest identity (53%) to a lipase from *Pseudomonas entomophila* L48 (GenBank Accession No. YP_608777) with over 315 amino acids. The deduced amino acid sequence indicated a 35-residue signal peptide, predicted using signalP. *LipXD* from *Pseudomonas* sp., encoding 318 amino acids, had the highest identity (51%) to a lipase from *Pseudomonas entomophila* L48 (GenBank Accession No. YP_608777) with over 318 amino acids (Table 2).

Sequence Analysis

The 201 clones similar to lipase genes were subjected to phylogenetic analysis (Fig. 2). To better understand the

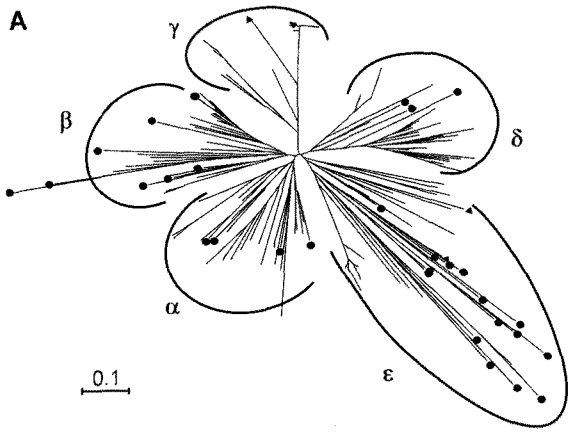

Fig. 2. Phylogenetic tree showing the relationship of partial amino acid sequences of lipases from the GCLP (Glacier Soil Lipase) library to certain HSL lipases and four closely related lipases cloned in this study.

The tree was constructed by the neighbor-joining method using MEGA 3.1. The length of the branches indicates the divergence among the amino acid sequences. The scale bar at the bottom of the tree represents substitutions per base position. **A**. Overview of the whole phylogenetic tree. α, β, γ, δ, and ε refer to clusters of the phylogenetic tree that are described in the text. The reference HSL sequences are marked with a closed circle (●), and the full-length lipases cloned in this study are marked with a closed triangle (▲). **B**. Subtree of the clusters α, β, and δ. **C**. Subtree of the clusters γ and ε. The sequences having >95% similarity with each other are compressed into one trapezoid. Values and percentages on the trapezoids indicate the number of sequences in the group and the range of identity to the known lipases in GenBank, respectively. All the reference sequences were derived from lipases of the HSL family.

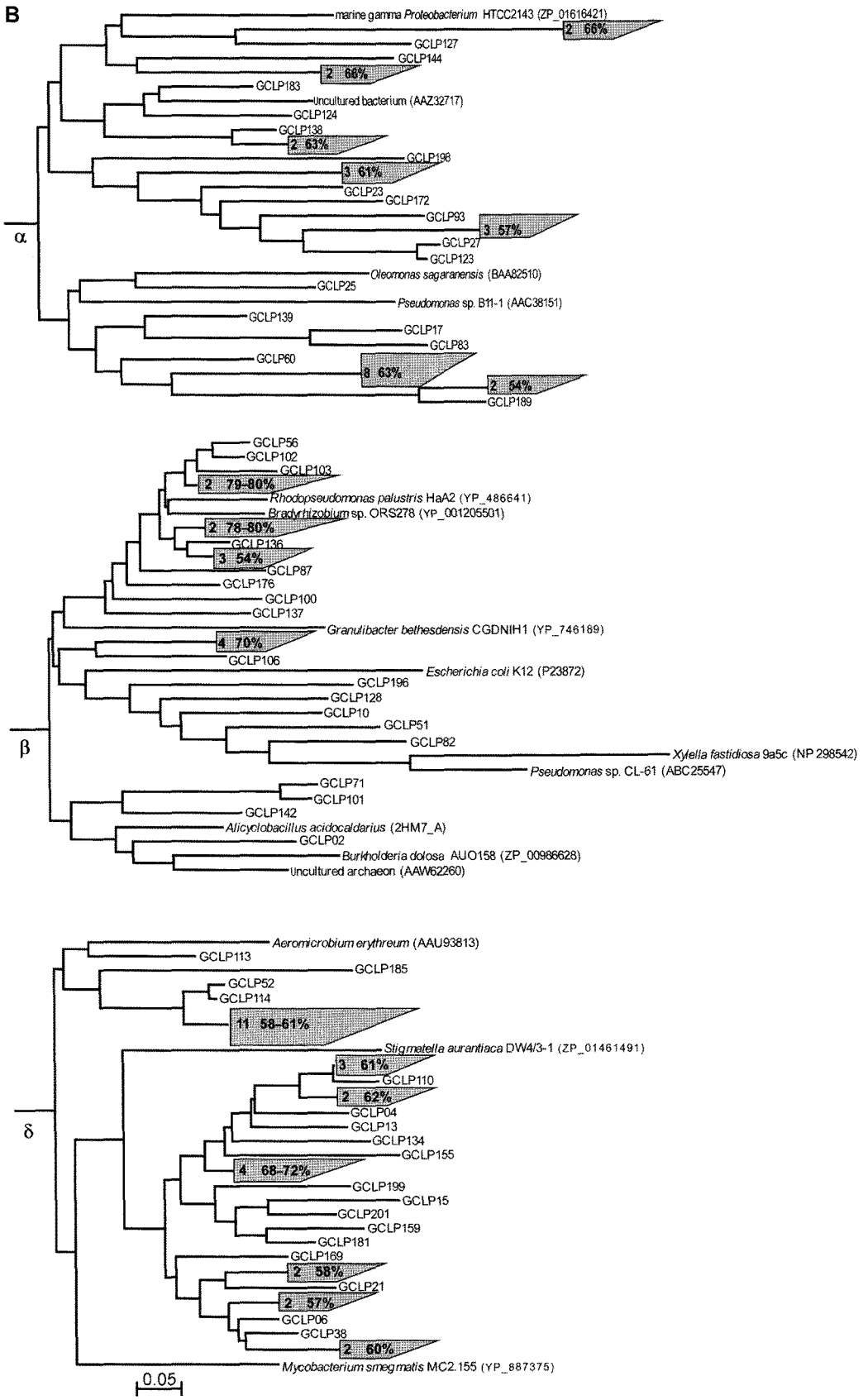

Fig. 2. Continued.

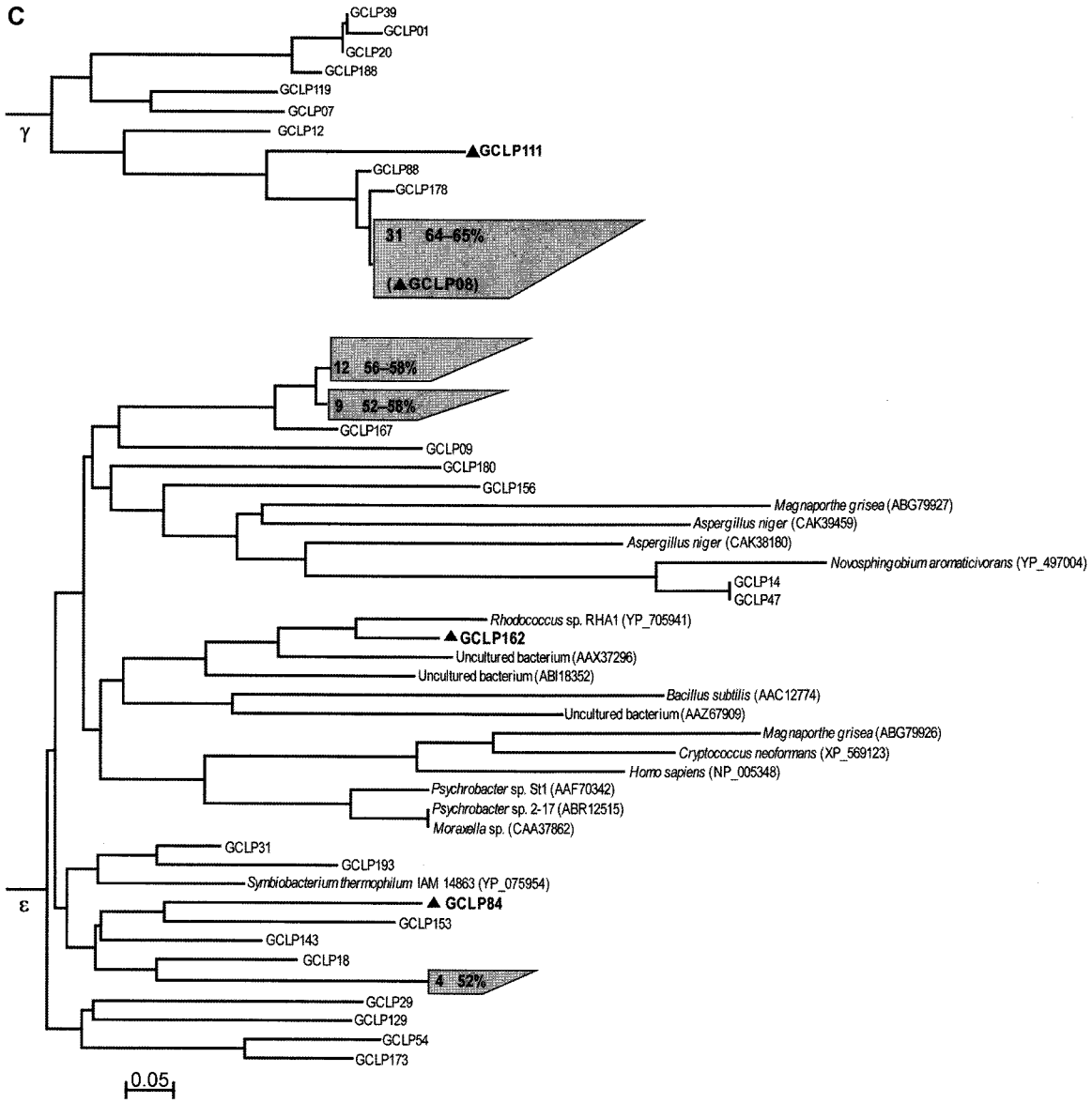

Fig. 2. Continued.

gene diversity of the GCLP lipases and their relationship to the characterized HSL family members, a phylogenetic tree was constructed based on the 201 sequences from the GCLP library using 32 HSL lipases derived from animal, fungi, and bacteria as references. The phylogenetic tree showed that all of the sequences formed five divergent clusters, denoted α, β, γ, δ, and ε, indicating the substantial diversity of lipases in glacier soil even within a family derived from the same PCR primers (Fig. 2A). The reference lipases were distributed into clusters α, β, δ, and ε; no known lipases distributed into cluster γ, suggesting that the lipase sequences in this cluster might constitute a novel HSL subfamily.

There were 117 sequences in clusters α, β, and δ, and this also was the case for most of the bacterial HSL lipases that were similar to the GCLP sequences (Fig. 2B), including the lipases from *Aeromicrobium erythreum*, *Bradyrhizobium* sp., *E. coli*, *Granulibacter* sp., *Mycobacterium* sp., *Oleomonas sagaranensis*, *Proteobacterium* sp., *Pseudomonas* sp., *Rhodopseudomonas* sp., *Xylella fastidiosa,* and many uncultured bacteria. Among them, two lipases from *Pseudomonas* spp. CL61 and B11-1 [7] had been characterized as cold-related lipases. These results suggested that there are potentially abundant cold-related lipases in glacier soil. Four clones, GCLP02, GCLP71, GCLP101, and GCLP142, showed similarity with a carboxylesterase (Accession No. AAW62260) from uncultured archaeon in cluster β.

The four full-length lipase genes from the lipase-producing bacteria in this study, *Lip3A* and *LipXD,* and *LipDB5* and *LipDB8*, accounted for the clones in clusters

γ and ε, respectively (Fig. 2C). In cluster ε, the lipase fragments were closely related to lipases from eukaryotes such as *Homo sapiens*, *Aspergillus niger*, *Cryptococcus neoformans*, and *Magnaporthe grisea* [15, 28, 31]. Furthermore, three cold-related lipases, CAA37862, ABR12515, and AAZ67909, from *Moraxella* sp., *Psychrobacter* sp. 2-17, and an uncultured bacterium [9, 12, 30], respectively, showed high similarity to the human HSL in cluster ε, corresponding to a previous report [25]. Additionally, only two clones, GCLP14 and GCLP47 (EU569058), showed high similarity to a carboxylesterase (YP_497004) from *Novosphingobium aromaticivorans*, although the similarities were much lower compared with other HSL lipases and lipase fragments of GCLP.

There were 41 lipase sequences in cluster γ, and approximately 75% of them were identical, indicating that the microorganisms containing these genes might be abundant in glacier soil. As we mentioned above, the sequences in cluster γ were unknown based on a GenBank search. Two full-length lipase genes, *Lip3A* and *LipXD*, corresponding to clones GCLP08 and GCLP111 in cluster γ, respectively, were selected for further expression and characterization.

Expression and Characterization of Lipase Genes in *P. pastoris*

The two lipase genes *Lip3A* and *LipXD* were expressed in *P. pastoris*. After 48 h induction with methanol, the lipase activity of Lip3A and LipXD were 3.4 and 0.57 unit/ml culture, respectively. The recombinant enzymes were then visualized by SDS–PAGE, and the molecular masses were estimated to be 31 and 33 kDa, respectively (data not shown). These apparent molecular mass values were consistent with those of calculated values, indicating that the lipase genes were expressed successfully in *P. pastoris*.

The optimum temperatures for lipase activity of Lip3A and LipXD were 40 and 37°C, respectively (Fig. 3A). At 10°C, Lip3A retained 17% activity and LipXD had 33% activity with reference to 100% activity at optimum temperatures. In a thermostability test, Lip3A retained approximately 13% of its activity after incubation at 50°C for 10 min, and LipXD retained 26% of its activity after incubation at 50°C for 30 min (Figs. 3B and 3C). These activity data indicated that Lip3A and LipXD had some low-temperature lipase characteristics, although they are not typical cold-active lipases.

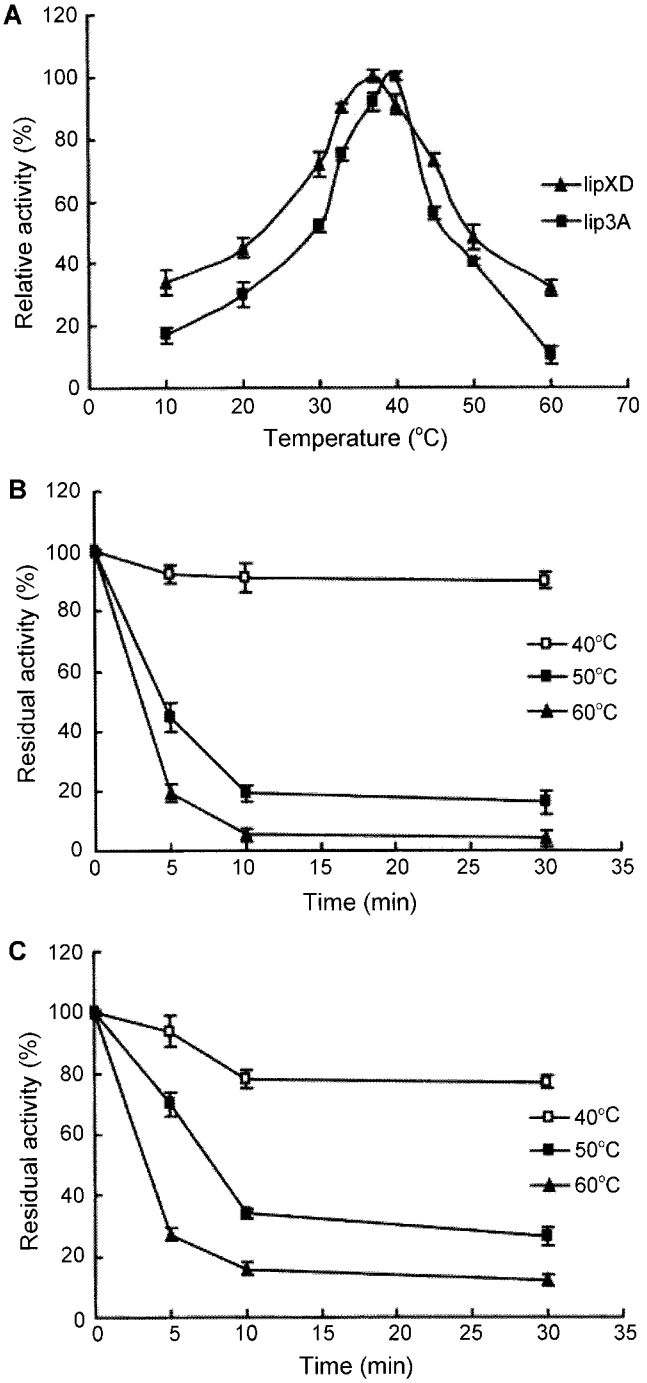

Fig. 3. Effect of temperature on the activity of recombinant Lip3A and LipXD.
A. Temperature versus activity profiles of Lip3A and LipXD. **B.** Thermal stability of Lip3A. **C.** Thermal stability of LipXD.

DISCUSSION

No primers are omnipotent for broad-based amplification of lipase gene sequences owing to the low similarity and different consensus motifs among different lipase families [4]. In this study, a set of degenerate primers specific for HSL lipases was used, but HSL lipase genes could not be amplified from genomic DNA of four lipase-producing bacteria, including one strain of each *Janthinobacterium* sp. and β-*Proteobacterium* sp. and two of *Pseudomonas* sp. These stains may produce lipases of another family. This problem could be solved by designing more sets of degenerate primers specific for different lipase families.

The aim of our study, however, was to estimate HSL lipase diversity by using a more efficient lipase screening strategy that was applied to a sample from a low-temperature environment, such that lipases from other families were not considered.

Microbial lipases have become important enzymes in industry mainly because of their versatility with regard to various practical processes. Their multifunctional properties are based on their diverse structures, which of course are a consequence of differences in protein sequence [13, 19, 21]. Thus, prospecting for novel lipase genes with various characteristics is of interest from both industrial and academic standpoints. In our present study, a large number of lipase gene fragments (more than 2,000 clones) were cloned from glacier soil, thus providing a chance to assess the lipase diversity of an extremophilic low-temperature environment. The sequence and phylogenetic analyses showed that all of the lipase sequences in the GCLP library had relatively low similarity to known lipases from multifarious organic resources, including human, fungi, bacterium, and even archaea, in GenBank, although all of them belong to the HSL family. Some of the lipase fragments from the GCLP library were similar to true lipases, esterases, and carboxylesterases. Collectively, these results support our conclusion that even a low-temperature environment like glacier soil exhibits a great diversity of lipase genes.

The phylogenetic tree indicated a cluster that is distinct from the other lipases in the GCLP library and known lipases. The majority of the sequences in this cluster were similar to each other. Expression and characterization of two lipases, Lip3A and LipXD, in this cluster confirmed that lipases corresponding to the sequences in the GCLP have *de facto* lipase activity. Therefore, this cluster might represent a novel subfamily of the HSL family.

There are 38 redundant sequences among the 201 lipase gene fragments of the GCLP library. Three lipase sequences from lipase-producing bacteria could not be accounted for in the sequenced clones in the library. This limited number of sequences (300 clones) is unlikely to fully reflect all the information on lipase genes in the library. This deficit could be ameliorated by increasing the number of clones for sequencing. In future studies, sequence-based methods would be used to obtain full-length lipase sequences directly from metagenomic cosmid or bacterial artificial chromosome libraries of glacier soil. Such a comprehensive approach will yield a less biased estimate of lipase diversity in glacier soil.

To our knowledge, the present study constitutes the first investigation of microbial lipase gene diversity in glacier soil using a culture-independent strategy. Our study demonstrates the feasibility and validity of the sequence-based screening approach for identifying novel functional lipases directly from DNA isolated from complex environmental samples without the need for laboratory culturing of microorganisms. This work also provides a methodological foundation for the rapid and effective discovery of novel lipase genes from metagenomic DNA.

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References

1. Alquati, C., L. De Gioia, G. Santarossa, L. Alberghina, P. Fantucci, and M. Lotti. 2002. The cold-active lipase of *Pseudomonas fragi*, heterologous expression, biochemical characterization and molecular modeling. *Eur. J. Biochem.* **269:** 3321–3328.
2. Altschul, S. F., T. L. Madden, A. A. Schaffeer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **25:** 3389–3402.
3. Amann, R. I., W. Ludwig, and K. H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59:** 143–169.
4. Arpigny, J. L. and K. E. Jaeger. 1999. Bacterial lipolytic enzymes: Classification and properties. *Biochem. J.* **343:** 177–183.
5. Bell, P. J. L., A. Sunna, M. D. Gibbs, N. C. Curach, H. Nevalainen, and P. L. Bergquist. 2002. Prospecting for novel lipase genes using PCR. *Microbiology* **148:** 2283–2291.
6. Brady, S. F. 2007. Construction of soil environmental DNA cosmid libraries and screening for clones that produce biologically active small molecules. *Nat. Protoc.* **2:** 1297–1305.
7. Choo, D. W., T. Kurihara, T. Suzuki, K. Soda, and N. Esaki. 1998. A cold-adapted lipase of an Alaskan psychrotroph, *Pseudomonas* sp. strain B11-1: Gene cloning and enzyme purification and characterization. *Appl. Environ. Microbiol.* **64:** 486–491.
8. Christner, B. C., E. M. Thompson, L. G. Thompson, V. Zagorodnov, K. Sandman, and J. N. Reeve. 2000. Recovery and identification of viable bacteria immured in glacial ice. *Icarus* **144:** 479–485.
9. Feller, G., M. Thiry, and C. Gerday. 1991. Nucleotide sequence of the lipase gene *lip2* from the Antarctic psychrotroph *Moraxella* TA144 and site-specific mutagenesis of the conserved serine and histidine residues. *DNA Cell Biol.* **10:** 381–388.
10. Gabor, E. M., W. B. Alkema, and D. B. Janssen. 2004. Quantifying the accessibility of the metagenome by random expression cloning techniques. *Environ. Microbiol.* **6:** 879–886.
11. Haba, E., O. Bresco, C. Ferrer, A. Marques, M. Busquets, and A. Manresa. 2000. Isolation of lipase-secreting bacteria by deploying used frying oil as selective substrate. *Enzyme Microb. Tech.* **26:** 40–44.
12. Hardeman, F. and S. Sjoling. 2007. Metagenomic approach for the isolation of a novel low-temperature-active lipase from

uncultured bacteria of marine sediment. *FEMS Microbiol. Ecol.* **59:** 524–534.
13. Hasan, F., A. A. Shah, and A. Hameed. 2006. Industrial applications of microbial lipases. *Enzyme Microb. Tech.* **39:** 235–251.
14. Henne, A., R. A. Schmitz, M. Bomeke, G. Gottschalk, and R. Daniel. 2000. Screening of environmental DNA libraries for the presence of genes conferring lipolytic activity on *Escherichia coli*. *Appl. Environ. Microbiol.* **66:** 3113–3116.
15. Holm, C., T. G. Kirchgessner, K. L. Svenson, G. Fredrikson, S. Nilsson, C. G. Miller, *et al.* 1988. Hormone-sensitive lipase: Sequence, expression, and chromosomal localization to 19 cent-q13.3. *Science* **241:** 1503–1506.
16. Hugenholtz, P. and N. R. Pace. 1996. Identifying microbial diversity in the natural environment: A molecular phylogenetic approach. *Trends Biotechnol.* **14:** 190–197.
17. Iizumi, T., K. Nakamura, Y. Shimada, A. Sugihara, Y. Tominaga, and T. Fukase. 1991. Cloning, nucleotide sequencing, and expression in *Escherichia coli* of a lipase and its activator genes from *Pseudomonas* sp. KWI-56. *Agric. Biol. Chem.* **55:** 2349–2357.
18. Jaeger, K. E., B. W. Dijkstra, and M. T. Reetz. 1999. Bacterial biocatalysts: Molecular biology, three-dimensional structures and biotechnological applications of lipases. *Annu. Rev. Microbiol.* **53:** 315–351
19. Jaeger, K. E. and T. Eggert. 2002. Lipases for biotechnology. *Curr. Opin. Biotech.* **13:** 390–397.
20. Jaeger, K. E., S. Ransacb, B. W. Dijkstra, C. Colson, M. v. Heuvel, and O. Misset. 1994. Bacterial lipases. *FEMS Microbiol. Rev.* **15:** 29–63.
21. Jaeger, K. E. and M. T. Reetz. 1998. Microbial lipases form versatile tools for biotechnology. *Trends Biotechnol.* **16:** 396–403.
22. Kim, H. K., Y. J. Jung, W. C. Choi, H. S. Ryu, T. K. Oh, and J. K. Lee. 2004. Sequence-based approach to finding functional lipases from microbial genome databases. *FEMS Microbiol. Lett.* **235:** 349–355.
23. Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform.* **5:** 150–163.
24. Lammle, K., H. Zipper, M. Breuer, B. Hauer, C. Buta, H. Brunner, and S. Rupp. 2007. Identification of novel enzymes with different hydrolytic activities by metagenome expression cloning. *J. Biotechnol.* **127:** 575–592.
25. Langin, D., H. Laurell, L. S. Holst, P. Belfrage, and C. Holm. 1993. Gene organization and primary structure of human hormone sensitive lipase: Possible significance of a sequence homology with a lipase of *Moraxella* TA144, an Antarctic bacterium. *Proc. Natl. Acad. Sci. U.S.A.* **90:** 4897–4901.
26. Lee, S. W., K. Won, H. K. Lim, J. C. Kim, G. J. Choi, and K. Y. Cho. 2004. Screening for novel lipolytic enzymes from uncultured soil microorganisms. *Appl. Microbiol. Biotech.* **65:** 720–726.
27. Liu, Y. G. and R. F. Whittier. 1995. Thermal asymmetric interlaced PCR: Automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. *Genomics* **25:** 674–681.
28. Loftus, B. J., E. Fung, P. Roncaglia, D. Rowley, P. Amedeo, and D. Bruno. 2005. The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans*. *Science* **307:** 1321–1324.
29. Park, J. M., N. S. Han, and T. J. Kim. 2007. Rapid detection and isolation of known and putative α-L-arabinofuranosidase genes using degenerate PCR primers. *J. Microbiol. Biotechnol.* **17:** 481–489.
30. Parra, L. P., F. Reyes, J. P. Acevedo, O. Salazar, B. A. Andrews, and J. A. Asenjo. 2008. Cloning and fusion expression of a cold-active lipase from marine Antarctic origin. *Enzyme Microb. Tech.* **42:** 371–377.
31. Pel, H. J., J. H. Winde, D. B. Archer, P. S. Dyer, G. Hofmann, and P. J. Schaap. 2007. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat. Biotechnol.* **25:** 221–231.
32. Piraee, M. and L. C. Vining. 2002. Use of degenerate primers and touchdown PCR to amplify a halogenase gene fragment from *Streptomyces venezuelae* ISP5230. *J. Ind. Microbiol. Biotech.* **29:** 1–5.
33. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NewYork.
34. Schmeisser, C., H. Steele, and W. R. Streit. 2007. Metagenomics, biotechnology with non-culturable microbes. *Appl. Microbiol. Biotech.* **75:** 955–962.
35. Voget, S., C. Leggewie, A. Uesbeck, C. Raasch, K. E. Jaeger, and W. R. Streit. 2003. Prospecting for novel biocatalysts in a soil metagenome. *Appl. Environ. Microbiol.* **69:** 6235–6242.
36. Wheeler, D. L., D. M. Church, S. Federhen, A. E. Lash, T. L. Madden, J. U. Pontius, *et al.* 2003. Database resources of the National Center for Biotechnology. *Nucleic Acids Res.* **31:** 28–33.
37. Winkler, U. K. and M. Stuckmann. 1979. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *J. Bacteriol.* **138:** 663–670.
38. Zhu, F., S. Wang, and P. Zhou. 2003. *Flavobacterium xinjiangense* sp. nov. and *Flavobacterium omnivorum* sp. nov., novel psychrophiles from the China No. 1 glacier. *Int. J. Syst. Evol. Microbiol.* **53:** 853–857.