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Selection and Characterization of Forest Soil Metagenome Genes Encoding Lipolytic Enzymes

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Abstract A metagenome is a unique resource to search for novel microbial enzymes from the unculturable microorganisms in soil. A forest soil metagenomic library using a fosmid and soil microbial DNA from Gwangneung forest, Korea, was constructed in *Escherichia coli* and screened to select lipolytic genes. A total of seven unique lipolytic clones were selected by screening of the 31,000-member forest soil metagenome library based on tributyrin hydrolysis. The ORFs for lipolytic activity were subcloned in a high copy number plasmid by screening the secondary shortgun libraries from the seven clones. Since the lipolytic enzymes were well secreted in *E. coli* into the culture broth, the lipolytic activity of the subclones was confirmed by the hydrolysis of *p*-nitrophenyl butyrate using culture supernatant. Deduced amino acid sequence analysis of the identified ORFs for lipolytic activity revealed that 4 genes encode hormone-sensitive lipase (HSL) in lipase family IV. Phylogenetic analysis indicated that 4 proteins were clustered with HSL in the database and other metagenomic HSLs. The other 2 genes and 1 gene encode non-heme peroxidase-like enzymes of lipase family V and a GDSL family esterase/lipase in family II, respectively. The gene for the GDSL enzyme is the first description of the enzyme from metagenomic screening.

Keywords: Forest soil metagenome, lipolytic enzymes, GDSL family esterase/lipase

Microbial enzymes, especially lipases (E.C. 3.1.1.3) and esterases (E.C. 3.1.1.1), have received much attention as biocatalysts for various biotechnological applications. Both esterases and lipases have useful features such as having no requirements for cofactors, remarkable stability in organic solvents, broad substrate specificity, stereoselectivity, and positional selectivity [10, 11]. Searching for microbial enzymes has depended primarily on pure culture-based screening of microorganisms for the desired lipolytic activity. However, the microbial culture-based screening has a limitation to find novel microbial enzymes, owing to the difficulties in cultivating the majority of microorganisms from most microbial habitats where over 99% of bacteria are not culturable [2, 9]. This excludes the majority of the microorganisms from the use of their microbial genes and enzymes.

Metagenome is the total microbial genome directly isolated from natural environments, and the metagenome can be cloned in culturable bacteria such as *Escherichia coli* [4, 21] to discover novel microbial enzymes [7]. In fact, the metagenomic approach was successful in searching for novel lipolytic enzymes in varied soils [8, 14, 17, 19, 21]. The soil microbial habitat, especially forest soil, contains probably several thousand bacterial species per single gram of soil [26]. Our previous microbial community analysis revealed that phylum *Acidobacteria*, one of the major phyla with only a few cultured representatives, is the most abundant group in forest soil in Korea [18]. Therefore, there is a high probability of finding novel lipases in the uncharacterized bacteria present in forest soil. In the present study, we focused on forest soil environments to explore the microbial lipolytic enzymes of soil microbes using a metagenomic approach. We constructed a metagenomic fosmid library from forest topsoil and selected several novel lipolytic clones. The corresponding genes for lipolytic activity were identified and characterized. In this study, we report the description of a metagenome clone encoding the GDSL family of serine esterase/lipase

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions

Escherichia coli DH5α and EPI-100 cultures were grown at 37°C on Luria-Bertani (LB) agar or in LB broth supplemented with the appropriate antibiotics. The following antibiotic

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concentrations were used for *E. coli* strains: chloramphenicol, 25 μg/ml; and ampicillin, 100 μg/ml. Plasmids pEpiFOS-5 (Epicentre, Madison, WI, U.S.A.) and pUC119 were used to construct the forest soil metagenomic library and to subclone genes for lipolytic activity, respectively.

DNA Preparation from Soil

Soil samples from forest topsoil were collected from Gwangneung forest of Korea National Arboretum at Gyeonggi Province, Korea. Bulk forest topsoil was collected in November 2003 from three different sites where Serrata Oak (*Quercus serrata*) was dominant. The forest soils were used for metagenomic DNA extraction and stored at −80°C for further experiments. Soil DNA extraction was carried out as previously described using SDS and proteinase K treatment [29], and further purification of the DNA for cloning into a fosmid was performed following the methods of Rondon *et al.* [21]. The size of extracted DNA was examined by pulsed-field gel electrophoresis (Bio-Rad CHEF-DR II, 1–6 s switch, 6 V/cm, 120 fixed angle, 5-h running time), and the appropriate size of DNA ranging from 30–50 kb was isolated from the gel for library construction.

Library Construction and Screening

A metagenomic library using the extracted DNA was constructed in a commercial fosmid vector, pEpiFOS-5 (Epicentre, Madison, WI, U.S.A.). The library was constructed by DNA size fractionation, clean-up of the metagenomic DNAs, and subsequent ligation into a fosmid vector as previously described [17]. The ligation mixture was then packaged into lambda phages using MaxPlax Lambda Packaging Extracts (Epicentre, Madison, WI, U.S.A.). The packaged library was transduced into *E. coli* EPI-100, and *E. coli* transformants were selected on LB agar supplemented with chloramphenicol. The presence of recombinant plasmids and the polymorphism of the insert DNA were examined by agarose gel electrophoresis of a BamHI digestion of the purified plasmids from randomly selected *E. coli* transformants. The library clones were stored in cryotubes as clone pools, with approximately 500 clones per pool.

To screen the lipolytic activity, LB agar medium emulsified with 1% tributyrin was used as previously described [17]. Clones with hydrolytic activity of the tributyrin were selected on the emulsified tributyrin media after 2 days of incubation at 37°C. Restriction digestion analysis of the selected lipolytic fosmid clones was carried out with BamHI, and duplicates were removed after electrophoresis.

General DNA Manipulation

Plasmid preparation, restriction endonuclease digestion, DNA ligation, plasmid DNA transformation, agarose gel electrophoresis, and other standard recombinant DNA techniques were carried out by the standard methods described by Sambrook *et al.* [23]. DNA sequencing and primer synthesis were performed commercially at the DNA sequencing facility of GenoTech Corp. (Daejeon, Korea). DNA sequences were analyzed with the BLAST program provided by the National Center for Biotechnology Information.

Subcloning and Lipase Activity Assay

The selected unique fosmid clones were named pElp120E, pElp164S, pElp271, pElp286, pElp293, pElp352, and pElp353. The selected clones were purified, partially digested with Sau3AI to have 1–4 kb size DNA, and ligated into a linearized pUC119 with BamHI to generate the secondary shotgun library. The secondary library was subjected to selection of subclones showing clear zones around the colonies on the LB agar with tributyrin. The subclones with the smallest DNA size were selected and named after the initial fosmid clones as follows: pUlp120E, pUlp164S, pUlp271, pUlp286, pUlp293, pUlp352, and pUlp353. As shown by our previous study [17], lipases were secreted into the culture broth from the 7 selected clones, so we tested the lipolytic activity of the culture supernatants from *E. coli* cells carrying the individual subclones using *p*-nitrophenyl butyrate. The enzyme substrate 2 mM *p*-nitrophenyl butyrate was used at a final concentration of 0.1 mM in sodium phosphate buffer (0.1 M, pH 7.25) containing 0.1 M NaCl and 0.3% Triton X-100. *E. coli* carrying the individual subclones were grown in LB broth for 18 h at 37°C, and the culture supernatants were used directly. The enzymatic reaction was started at room temperature by adding 0.1 ml of individual culture supernatants to the above-mentioned reaction mixture. The activity was determined by measuring *p*-nitrophenol formation at 400 nm from the enzymatic hydrolysis of *p*-nitrophenyl butyrate.

Phylogenetic Analysis of Lipases

Deduced amino acids sequences of 7 lipolytic enzymes and 3 lipolytic enzymes obtained from our previous study [17] were subjected to protein phylogenetic analysis. A total of 10 sequences were aligned with the CLUSTALW program [25] and visually examined with the GENEDOC program. Terminal gaps in the alignment were removed prior to phylogenetic analysis, whereas the internal gaps were left and analysis was conducted by scoring gaps as characters. Phylogenetic trees were constructed using PRODIST with the Jones-Taylor-Thornton matrix model and NEIGHBOR with the neighbor-joining method [22] in the PHYLIP (phylogeny inference package) programs, version 3.62. We generated 1,000 bootstrapped replicate resampling data sets with SEQBOOT (PHYLIP, version 3.62). We followed the standard protocol for the default settings of the computer programs used in this procedure.

Table 1. List of lipolytic enzymes from forest soil metagenome and similar enzymes in GenBank.

Clone	Subclones	No. of a.a.	Most similar lipase (protein accession), organism	Identity (%)	References
Elp286	Ulp286	273	Alpha/beta hydrolase, BioH (BAB39459), *Kurthia* sp.	43	[16]
Elp164S	Ulp164S	298	Carboxyl esterase (NP 149260), *Clostridium acetobutylicum*	49	[20]
Elp352	Ulp352	296	Esterase (AAS77242), uncultured bacterium	98	[17]
			Esterase (1EVQA), *Alicyclobacillus acidocaldarius*	34	[27]
Elp293	Ulp293	310	Esterase (AAS77247), uncultured bacterium	56	[17]
			Lipase (BAD41110), *Symbiobacterium thermophilum*	55	[26]
Elp271	Ulp271	311	Esterase (AAS77247), uncultured bacterium	95	[17]
			Esterase/lipase (AAZ64807), *Ralstonia eutropha*	58	Unpublished
Elp353	Ulp353	226	Lysophospholipase L1 (EAT72123), *Verminephrobacter eiseniae*	66	Unpublished
Elp120E	Ulp120E	322	Esterase (BAD07370), *Pseudomonas putida*	52	[24]

Nucleotide Sequence Accession Number

The nucleotide sequences of the insert DNA of pUlp120E, pUlp164S, pUlp271, pUlp286, pUlp293, pUlp352, and pUlp353 have been deposited in the GenBank database under accession numbers EF213583 to EF213589.

RESULTS AND DISCUSSION

Forest Soil Metagenome Library

A metagenome library using soils from Gwangneung forest of Korea National Arboretum was constructed into a fosmid vector, pEpiFOS-5. The soil microbial DNA prepared from forest topsoil was approximately 2–3 μg per single gram of forest topsoil and heavily contaminated with humic substances. The DNA was size-fractionated and further purified to remove the humic substances before construction of a library. From the forest topsoil, we obtained a total of 80,500 clones and maintained them for activity-based screening. The average insert DNA size was estimated as 35–40 kb when 40 randomly picked clones were analyzed by preparative pulsed-field gel electrophoresis after BamHI digestion (data not shown). Fosmid vectors were ideal to construct various genomic DNA libraries [15] and we were routinely successful in constructing the metagenomic libraries from various soils, containing gigabase-scale genomic DNA in all from a microgram DNA and representing several microbial genome equivalents [17]. Therefore, we used this library to search for novel microbial enzymes from unculturable microorganisms.

Lipolytic Clone from Forest Soil

Approximately 31,000 clones were subjected to selection of lipolytic active clones based on tributyrin hydrolysis. A total of 7 unique lipolytic active clones were selected and they were named pElp120E, pElp164S, pElp271, pElp286, pElp293, pElp352, and pElp353. The frequency of the unique lipolytic clones from the 31,000-clone library was similar to that of our previous results from a different forest soil metagenomic library [17]. From our previous study, we obtained 8 unique lipolytic clones by screening a 33,700-member library in the same screening method. Genes encoding lipolytic activity were successfully subcloned into a high copy number plasmid, pUC119, from the 7 unique clones. The lipase activity was confirmed by the *p*-nitrophenyl butyrate hydrolysis using the culture supernatants of *E. coli* carrying the individual subclones (pUlp120E, pUlp164S, pUlp271, pUlp286, pUlp293, pUlp352, and pUlp353), whereas the culture supernatant of *E. coli* carrying pUC119 did not show the hydrolysis of *p*-nitrophenyl butyrate (data not shown).

The seven selected subclones were completely sequenced. We defined the ORFs from the metagenomic subclones and they were similar to genes encoding either esterase or lipase in the GenBank database (Table 1). Interestingly, two genes from pElp271 and pElp352 were highly identical to genes identified from our previous study (Fig. 1).

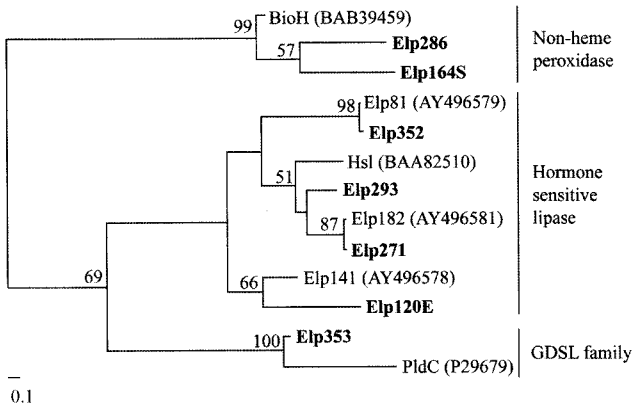

Fig. 1. Phylogenetic relationships of the lipolytic enzymes obtained from a forest soil metagenome and retrieved from a database.

The bold letters Elp followed by a clone number indicates forest soil metagenomic clones. The Elp141, Elp81, Elp182 were previously reported from other forest soil [17]. Three lipases, BioH, Hsl, and PldC, closely related with our clones, were retrieved from a database and their accession numbers are indicated. Bootstrap values are shown for each node that had >50% support in a bootstrap analysis of 1,000 replicates. The scale bar indicates 0.1 change per amino acid.

Although clones were originated from different forest soils, two forest soils may contain similar microorganisms. The highest similarity of our lipolytic enzymes was mostly found from the genes encoding putative enzymes from the genomics result. When the deduced amino acid sequences of our enzymes were compared with the published lipolytic enzymes with experimental proof, the identity values were far lower to a level of 20%–40% (data not shown). This result indicated that our metagenomic clones contain novel lipolytic enzymes. The selection of our lipolytic enzymes was fully dependent on the secreted enzyme activity in *E. coli*. Sequence-based screening [12] might be an alternative to select more clones encoding lipolytic enzymes from the library, since a large fraction of lipolytic enzymes are not efficiently secreted in *E. coli*. Library construction in an engineered *E. coli* host for efficient secretion of lipolytic enzymes would be valuable to obtain more diverse lipolytic enzymes [5].

From our previous reports, we only obtained hormone-sensitive lipases (HSL) in the lipase family IV classified by Arpigny and Jaeger [3]. Most of them were probably carboxyesterases of lipase family IV involved in the hydrolysis of short-chain esters [17]. This result has raised the possibility of the biased selection of lipolytic active clones by the tributyrin hydrolysis-based screening. However, the present study clearly showed that the tributyrin hydrolysis-based screening is not biased to select only HSLs from the metagenome library (see below).

Phylogenetic Relatedness of Lipases from Forest Soil

We analyzed the phylogenetic relatedness of the deduced amino acids from 7 genes with three known HSLs from a forest soil metagenome and three lipolytic enzymes such as a HSL (Hsl in Fig. 1) of family IV, a BioH of family V, and PldC (family II) in *E. coli* (Fig. 1). The analysis indicated that our 4 enzymes, the members of HSL in lipase family IV, were clustered with other HSLs in the database. The other 3 proteins from pElp286, pElp164S, and pElp353 were not members of lipase family IV. The deduced amino acids from the gene of pElp353 were most similar to a lysophospholipase in family II (GDSL family) by Arpigny and Jaeger. Two other genes from pElp286 and pElp164S showed high similarity to genes encoding esterase/lipase/peptidase from various bacteria in lipase family V. Our analysis adopted the lipase classification based on amino acid sequences and biological properties proposed by Arpigny and Jaeger [3].

Lipolytic enzymes such as esterases (E.C. 3.1.1.1) and lipases (E.C. 3.1.1.3) catalyze both the hydrolysis and the synthesis of acylglycerides and other fatty acid esters. Compared with carboxylesterases, the true lipases have their maximal activity on water-insoluble long-chain esters [3]. Although we obtained the more diverse esterase/lipase genes from our metagenome analysis, there was still high frequency to obtain HSL family lipase and we have not found the true lipase so far from this screening. However, the finding of lipolytic enzymes in families II and V encouraged us to continue the tributyrin-based screening to search for novel lipolytic enzymes. Since we subcloned the small piece of DNA with lipolytic activity from the original lipolytic fosmid clones, we do not know if the lipolytic genes were originated from unculturable microorganisms or not. Yet, the majority of microorganisms are not culturable from forest soils [18], and most of the seven clones were probably originated from unculturable microorganisms.

GDSL Family of Serine Esterases/Lipases

One of our lipolytic clones pElp353, contained a GDSL family of serine esterases/lipases. The deduced amino acid sequence of the gene shared strong similarity (66% identity) to the gene encoding the lysophospholipase L1-like protein of *Verminephrobacter eiseniae* (unpublished). The comparison of the amino acid sequence to the published GDSL enzymes, PldC of *E. coli* [13] and EstA of *Lactobacillus helveticus* [6], revealed a relatively low identity of 41% and 26%, respectively. Alignment of the amino acid sequences of the three genes revealed that our enzyme contained the characteristic conserved blocks of the GDSL family of esterases/lipases (Fig. 2) [1]. The GDSL family enzyme is a relatively new enzyme subclass containing a distinct GDSL sequence motif and serine, aspartate, and histidine residues as catalytic sites, whereas most lipases contained the GxSxG motif. Our GDSL enzyme contained four strictly conserved residues, Ser-Gly-Asn-His, in four conserved blocks, I, II, III, and V, respectively. Because of the four conserved residues and oxyanion structure (Fig. 2), our GDSL family enzyme is most similar to SGNH-hydrolase,

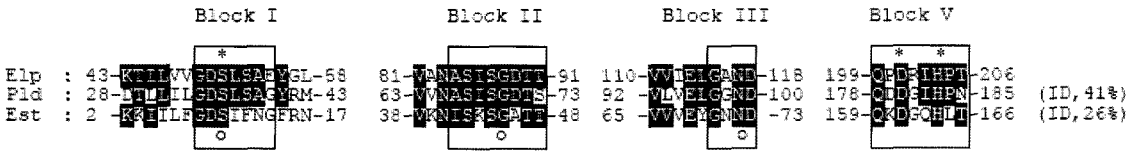

Fig. 2. Alignment of lipase Elp353 (Elp) with the GDSL family of serine esterases/lipases, PldC of *E. coli* (Pld) [13] and EstA of *Lactobacillus helveticus* (Est) [6].

Identical bases are shown as white letters on a dark background. Asterisks are the catalytic triad (serine, aspartate, and histidine). Open circles are the conserved oxyanion hole residues (serine, glycine, and asparagine). The identity (ID) of the amino acid sequences to Elp353 is indicated.

a subgroup of the GDSL family enzyme [1]. The GDSL family enzymes have interesting features such as broad substrate specificity and regioselectivity. The broad substrate specificity is due to a flexible active site that changes confirmation with the binding of the different substrates [1]. Therefore, it will be interesting to test if our GDSL enzyme from the forest soil metagenome has the broad substrate specificity as do usual GDSL family enzymes.

In this study, we identified seven lipolytic enzymes from a forest soil metagenome comprising lipase families II, IV, and V. To our best knowledge, this report is probably the first description of the GDSL family of serine esterases/lipases from metagenomic approach. The potential of the enzymes for biotechnological application is under investigation.

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