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Improved metagenome screening efficiency by random insertion of T7 promoters

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ABSTRACT

Metagenomes constitute a major source for the identification of novel enzymes for industrial applications. However, current functional screening methods are hindered by the limited transcription efficiency of foreign metagenomic genes. To overcome this constraint, we introduced the 'Enforced Transcription' technique, which involves the random insertion of the bi-directional T7 promoter into a metagenomic fosmid library. Then the effect of enforced transcription was quantitatively assessed by screening for metagenomic lipolytic genes encoding enzymes whose catalytic activity forms halos on tributyrin agar plates. The metagenomic library containing the enforced transcription system yielded a significantly increased number of screening hits with lipolytic activity compared to the library without random T7 promoter insertions. Additional sequence analysis revealed that the hits from the enforced transcription library had greater genetic diversity than those from the original metagenome library. Enhancing heterologous expression using the T7 promoter should enable the identification of greater numbers of diverse novel biocatalysts from the metagenome than possible using conventional metagenome screening approaches.

1. Introduction

The demand for enzymes with novel and specific properties for the field of industrial biocatalysis is continuously expanding. Metagenomes constitute one of the best natural sources for novel molecules (Lorenz and Eck, 2005; Schmeisser et al., 2007); however, it has been estimated that >99% of the microorganisms in the environment cannot be cultivated and that their enzyme catalytic activities have yet to be explored (Amann et al., 1995; Rappé and Giovannoni, 2003). One approach for identifying novel enzymes involves cloning the DNA extracted from metagenomes into expression vector systems and then using functional screening methods. However, few novel enzymes have been identified using these techniques, since only approximately 7–73% of the enzymatic activity of a metagenomic library is thought to be expressed in a heterologous host strain, such as *Escherichia coli*. Moreover, the external promoters in a large-size insert may have even less chance to be recognized in the host system (Gabor et al., 2004).

Previously, it was reported that the expression level of metagenomic genes can be enhanced by inserting bi-directional arabinose-inducible or T7 promoters (Grant et al., 2001; Leggewie et al., 2006) into metagenomic DNA. This technique, MuExpress, was validated by demonstrating that the expression of green fluorescence protein (GFP) and putative lipase genes was increased post transposition of the T7 promoter and by the identification of a novel esterase with lipolytic activity from a metagenomic library. However, the value of this technique is still in question because of the lack of supporting examples and concerns that the reintroduction of T7 promoters into a library could lead to the reduction of genetic diversity (Lussier et al., 2011). Therefore, more quantitative evidences will be required to confirm that random insertion of the T7 promoter improves metagenomic screening efficiency by circumventing the problem of low expression of metagenomic genes.

In this study, we quantitatively analyzed the effect of random insertion of the bi-directional T7 promoter into a metagenomic

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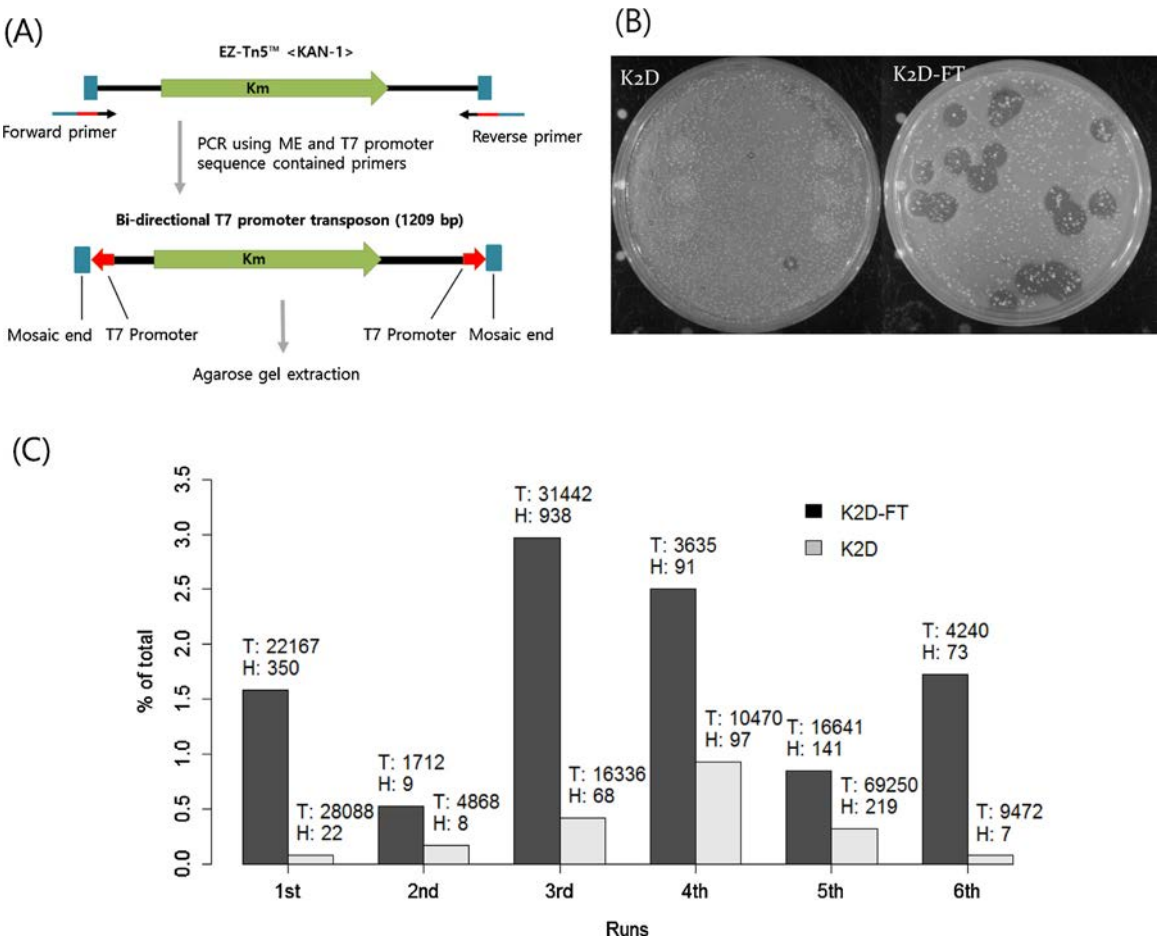

Fig. 1. Metagenome screening for lipolytic genes from two metagenomic libraries, K2D-FT and K2D. (A) Construction of bi-directional T7 promoter transposon (B) The zone of clearance was observed due to lipolytic activity on the tributyrin plates after 4 days of incubation at 37 °C. More positive hits were identified on the K2D-FT plates than those of K2D library. (C) Six rounds of screenings were conducted for finding metagenomic lipolytic genes using tributyrin LB agar plates. The positive hit ratio (% of total) was obtained by dividing the number of positive hits (H) by the total number of colonies (T) in each run. In all six cases, K2D-FT showed constantly higher screening efficiency than K2D.

Table 1
Summary of tributyrin plate based metagenomic screening results. K2D-FT library was constructed with EPI300(DE3) strain while K2D library used EPI300. The screening efficiency (% of total) of K2D-FT library (2%) was 6.6-fold higher than that of K2D library (0.3%). The library size was determined by counting the number of colonies in square plates after the DNA library transformed into the host strains, EPI300 and EPI300(DE3).

Metagenome	Strain	Library diversity	Total no. of colonies	No. of positive colonies	% of total
K2D-FT	EPI300(DE3)	8×10^4	79,837	1597	2.00%
K2D	EPI300	1.3×10^5	138,484	421	0.30%

fosmid library, designated as the 'enforced transcription (FT)' system. Metagenomic DNAs were obtained from tidal flat sediments in Taean, South Korea and its constructed library diversity was approximately 1.3×10^5. Putative lipolytic genes were screened for by detecting halos on tributyrin agar plates. Our results indicate that, in spite of the additional library modification step, the screening efficiency of T7 promoter inserted library increased approximately 6.7-fold compared to that without the T7 promoter. Thanks to the recent developments of the next generation sequencing (NGS) technique that enables to evaluate diversity of microbial communities, the actual genetic diversity of our screened positive clones were examined by the Metaphlan software, an established taxonomic binning method that utilizes clade-specific marker genes representing each of the taxons (Segata et al., 2012). Interestingly, community analysis of the screened colonies revealed that the T7 promoter inserted library had significantly greater genetic diversity compared to the original metagenome library.

2. Material and methods

2.1. Reagents

E. coli strain EPI 300 was purchased from Epicentre (Madison, WI, USA) and *E.coli* strain EPI 300 (DE3) was constructed by inserting T7 RNA polymerase into the host using a DE3 lysogenization kit (Novagen, Madison, WI, USA), according to the manufacturer's protocol. Tn5 transposase, gum arabic, glyceryl tributyrate, and antibiotics (kanamycin and ampicillin) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lysogeny broth (LB) was purchased from BD (Franklin Lakes, NJ, USA). All other reagents were purchased from commercial sources and were of analytic grade.

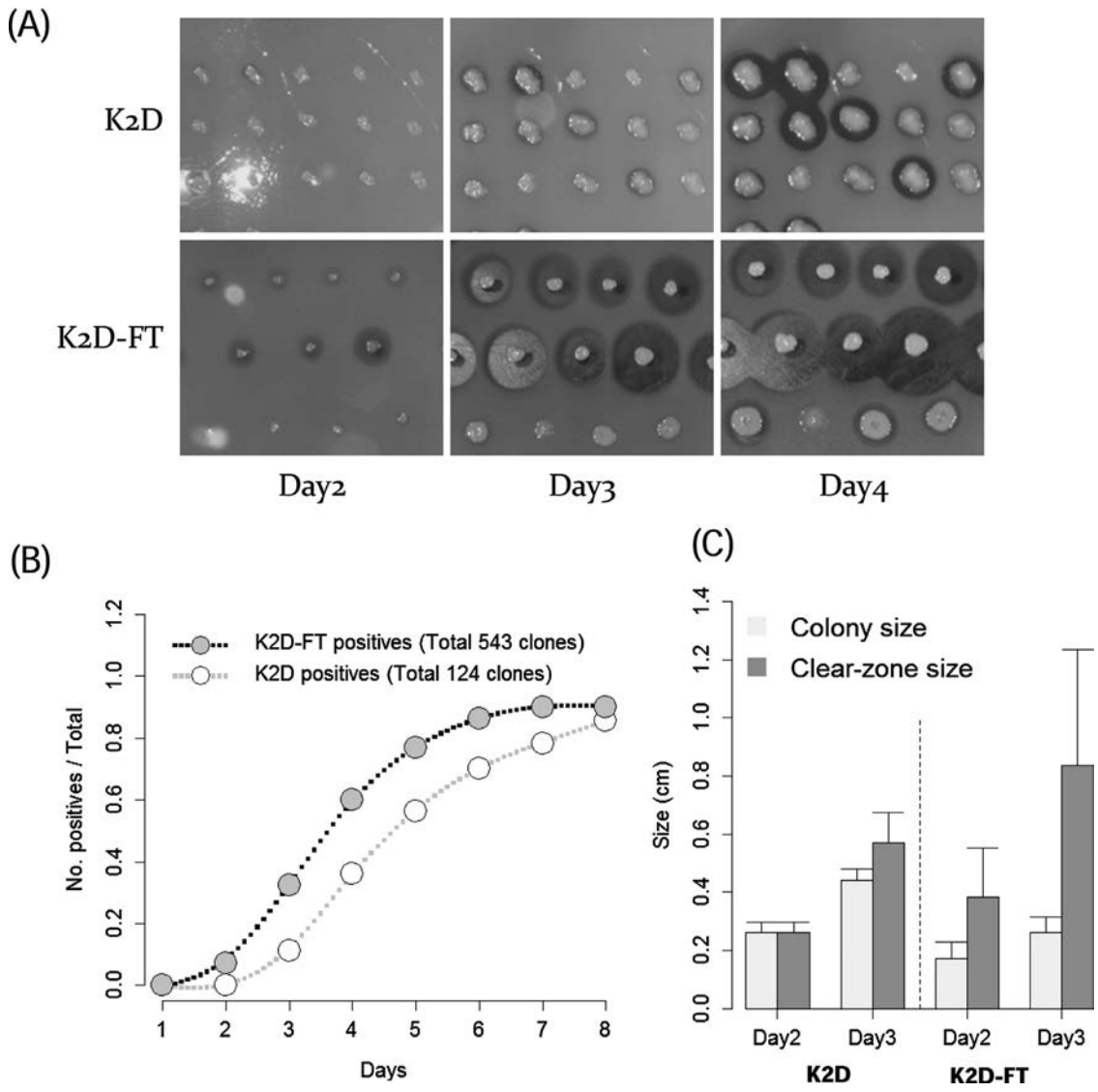

Fig. 2. Effect of the enforced transcription system on metagenomic screening. 543 and 124 positive clones from K2D-FT and K2D, respectively, were transferred into new tributyrin plates and their lipolytic activities were observed for 8 days. (A) Clear zone forming images of K2D and K2D-FT positive clones on tributyrin plates on day 2, 3, and 4. (B) Positive clones from K2D-FT showed faster lipolytic activity than those of K2D. (C) Comparison of colony size and clear zone diameter (*cm*) between K2D and K2D-FT positive clones. K2D-FT clones showed approximately 2.78 times larger clear zone ratio (a clear zone diameter divided by its colony size) than those of K2D on day 3. The error bars represent standard deviations.

2.2. Construction of the bi-directional T7 transposon

EZ-Tn5^{TM} <KAN-1> Transposon (Epicentre, USA) containing the mosaic end (ME) and kanamycin (Km) resistance gene was used to obtain the bi-directional T7 transposon by performing polymerase chain reaction (PCR) with the forward (5′-CTG TCT CTT GTA CAC ATC TCC CTA TAG TGA GTC GTA TTA GCT AGC ATC ATG AAC AAT AAA ACT GTC-3′) and reverse (5′-CTG TCT CTT GTA CAC ATC TCC CTA TAG TGA GTC GTA TTA GCT AGC TGA AGC TTG CAT GC-3′) primers (Fig. 1A). The amplified bi-directional T7 promoter transposon was purified by agarose gel extraction.

2.3. Random transposon insertion into the metagenome library

A soil-derived metagenomic library was constructed in *E. coli* EPI300(DE3) containing the pCC1FOS vector using a CopyControl^{TM} Fosmid Library Production Kit (Epicentre), according to the manufacturer's protocol, and library plasmids were purified using a midiprep kit (QIAGEN, Hilden, Germany). Next, 200 ng of the metagenomics DNA library, 137.06 ng of the purified bi-directional T7 promoter transposon, 1 unit of the EZ-Tn5, transposase and the Ez-Tn5 reaction buffer were mixed and incubated for 2 h at 37 °C. The transposition reaction was stopped by adding 0.1% Sodium dodecyl sulfate (SDS) and incubating at 70 °C for 10 min. The reaction mixture was then purified using a DNA purification kit (QIAGEN, Hilden, Germany). The transposed plasmid was concentrated by ethanol precipitation (Pellet-Paint, Merck-Millipore, Darmstadt, Germany), resuspended in 5 μl of distilled water, transformed into *E. coli* EPI300(DE3) electroporation-competent cells (Invitrogen, Carlsbad, CA, USA), and plated on LB agar plates containing chloramphenicol (12.5 μg/ml) and kanamycin (10 μg/ml). Plates were incubated for 16 h at 37 °C. Evolved colonies were harvested in storage medium (2YT medium containing 15% glycerol and 2% glucose) and stored at −80 °C.

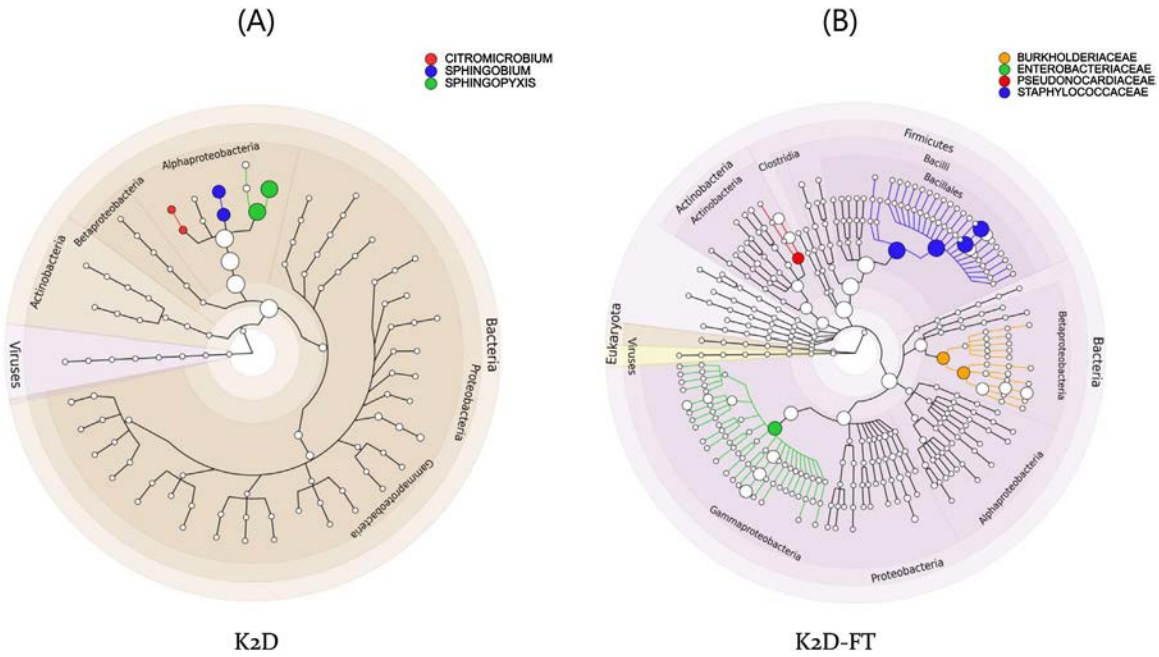

Fig. 3. Community analysis of the positive hit sequences. (A) Metagenomic fosmid sequences of the total 14 positive K2D clones were analyzed. Most of the sequences originated from *Alphaproteobacteria* class strains mainly containing *Citrobicrobium*, *Sphingobium*, and *Sphingopyxis* genus. (B) The sequences of 113 K2D-FT positive clones showed much wider diversity than that of K2D. Major sources of the K2D-FT sequences were belonging to *Basilli* and *Gammaproteobacteria* classes. Details of the percentages are shown in Table 2.

2.4. Tributyrin (TBN) plate based metagenomic library screening for lipolytic genes

Lipolytic activity was detected on LB agar plates containing 1% tributyrin. Plates were prepared by mixing 50 ml of 10 × gum arabic solution, 5 ml of glyceryl tributyrate, and 445 ml of LB medium in a blender (time interval: 2 min on, 30 s off, 4 cycles), then adding 7.5 g of agar to the mixture, and autoclaving. The mixture was cooled to approximately 55 °C prior to the addition of chloramphenicol (34 μg/ml) and 0.5 mM Isopropyl β-*D*-1-thiogalactopyranoside (IPTG) and then dispensed into petri dishes. The frozen metagenomic library cells containing the T7-Km transposon (5×10^8 cells) were grown in 1 ml of LB broth with chloramphenicol (34 μg/ml) at 37 °C for 1 h for recovery. Next, 1000-fold serially diluted cells were plated onto LB agar plates containing tributyrin and chloramphenicol and incubated at 37 °C. Colonies with lipolytic activity were detected by the formation of a zone of clearance; positive colonies (zone of clearance) and negative colonies were enumerated for 7 days.

2.5. NGS based sequence analysis

A 454 system (Roche, Basel, Switzerland) was used to sequence the DNA of the 14 and 113 screening hits obtained from the tidal flat sediments originated metagenomic library (K2D) without FT and the K2D with FT library, respectively. Because of the large number of positive hit colonies, DNA samples of similar concentrations were mixed, thus reducing the number of samples to 11 pooled DNA samples. All 25 DNA samples were multiplexed into a single 454 run. Contigs were estimated with the GS FLX Assembler software and CLC Genomics workbench (v7.5) was used to identify ORFs from the contigs. MetaPhlAn was used to analyze the microbial community composition in the two groups of DNA samples (Segata et al., 2012) and GraPhlAn software was used for graphical representation of community composition (https://bitbucket.org/nsegata/graphlan/wiki/Home).

3. Results

3.1. Metagenomic library construction and function based screening

Metagenomic DNAs were extracted from tidal flat sediments in Taean, South Korea. The DNA segments ranging from 30 to 50 kb were cloned into the fosmid vector, pCC1FOS, to construct a metagenome library (K2D) approximately 1.3×10^5 in size. The FT system was constructed by inserting the T7 promoter sequence in EZ-Tn5 Transposon containing the mosaic end (ME) and Km resistance gene (Fig. 1A). The metagenomic DNA library and the purified T7-Km transposon with the EZ-Tn5 transposase were combined in order to create a metagenomic DNA library with bi-directional T7 promoter transposon (K2D-FT). Both DNA libraries were transformed into *E. coli* EPI300(DE3). K2D-FT library diversity decreased by 0.625-fold (8×10^4) compared to the genetic diversity of the original library due to the additional transformation step. Both the K2D and K2D-FT libraries were screened for lipolytic genes by selecting clones that formed a zone of clearance when grown on plates containing tributyrin. Fig. 1B shows the plate of each library after 4 days of incubation. K2D-FT library clearly provided a higher number of positive hits clones and larger size of clearance zone than K2D. Table 1 compares the screening results from the two metagenome libraries. The total numbers of colonies after six runs were 79,837 and 138,484 for K2D and K2D-FT, respectively.

3.2. Effect of enforced transcription on screening efficiency

Prior to conducting the comparison, we defined screening efficiency as the ratio of the number of hits (clones selected as screen-positive) divided by the total number of colonies. In Table 1, K2D-FT exhibited 2.0% screening efficiency (two positive clones among 100 metagenomic clones); while the efficiency of K2D was 6.6-fold lower (0.3%). Screening was run six times (Fig. 1C) and K2D-FT outperformed K2D in all experiments though there was some variance in screening efficiency. To further analyze the hits

obtained from multiple screenings, a total of 543 K2D-FT and 124 K2D clones exhibiting zones of clearance were re-grown on fresh tributyrin plates and clearance zone formation was observed and compared between the colonies of the two libraries for 8 days. Tributyrin plates images of day 2, day 3, and day 4 of incubation revealed that K2D-FT colonies showed faster expression and larger zones of clearance than the K2D colonies (Fig. 2A). More specifically, in Fig. 2B, K2D-FT colonies began to form zones of clearance on the plates on day 2 while no lipolytic enzyme activity was observed for the K2D colonies on the same day. The lipolytic activity of the K2D-FT hits was nearly saturated after 6 days of observation while the K2D hits continued to increase linearly until the end of the experiment (8 days). Moreover, a comparison of zone of clearance size, which is indicative of the strength of enzyme expression, showed that on average, the K2D-FT hits were clearly larger in size than the K2D hits (Fig. 2C). These results suggested that FT enhances the expression of metagenomics lipolytic genes owing to the inserted bi-directional T7 promoters.

3.3. Sequencing based characterization of strain diversity

In order to confirm the effect of FT on metagenomic gene expression, it was necessary to determine whether the hits of both libraries were distinctly independent genes; this consequently revealed wider genetic diversity among the K2D-FT colonies than the K2D colonies. DNAs were extracted from freshly transformed colonies of 14 K2D and 113 K2D-FT colonies showing lipolytic activity on day 3 (Fig. 2B) and then sequenced using a 454 system with multiplexing tags. The results suggested that the DNAs of the 113 K2D-FT samples originate from a much wider range of strains than the K2D hits (Fig. 3, Table 2). Sequences from K2D were mainly originated from *Bacteria* kingdom especially *Alphaproteobacteria* class strains which include *Citrobicrobium*, *Sphingobium*, and *Sphingopyxis* genus. On the other hand, *Bacilli* and *Gammaproteobacteria* classes were major of the K2D-FT clones. In particular, Ascomycota (Eukaryota kingdom) and Firmicutes phylum strains shown in K2D-FT library were not detected in K2D colonies. *Staphylococcus* derived lipolytic genes were the most highly identified among the K2D-FT hits, which corresponds with previous studies describing novel esterase/lipase enzymes isolated from *Staphylococcus* strains (de Abreu et al., 2014; Talon et al., 1996; van Kampen et al., 2001). To further characterize the hit sequences, 35,197 lipolytic genes were collected from the BRENDA database by searching for enzymes that use “tributyrin” as a substrate and a customized BLAST database was constructed. Following contig assembly and ORF estimation for the short reads of both K2D and K2D-FT hits, all ORF sequences were blasted against the customized lipolytic gene database to obtain their functional inference. 208 K2D ORFs and 1646 K2D-FT ORFs were highly matched (e-value < 10E-15) to sequences in the lipolytic gene database (Table 3). Approximately 8 times more lipolytic gene products were found from K2D-FT ORFs than those of K2D. In Table 4, most ORFs were annotated as hypothetical proteins. Lysophospholipase and arylesterase acting on carboxylic ester bonds were the major gene products in both libraries but K2D-FT showed additional unique annotations as a part of specific proteins such as SarR (Transcriptional regulator), BioH (bioin biosynthesis protein), YcfP (esterase), and SecG (protein export membrane protein). ORFs with the highest scores were identified as lysophospholipase from *Staphylococcus aureus*, which is comparable to our previous community analysis in Fig. 3B.

Table 2
Summary of community analysis of the positive clones. Sequences from K2D mainly originated from *Bacteria* kingdom, especially from the *Alphaproteobacteria* class while *Bacilli* and *Gammaproteobacteria* classes were major sources of the K2D-FT clones.

Metagenome	Clade division (class level)	% of total
K2D	Bacteria	99.989
	Proteobacteria	99.987
	Alphaproteobacteria	97.169
	Gammaproteobacteria	2.816
	Betaproteobacteria	0.001
	Actinobacteria	0.002
	Viruses	0.01
	Viruses_no name	0.01
K2D-FT	Bacteria	99.952
	Firmicutes	53.655
	Bacilli	53.595
	Clostridia	0.049
	Negativicutes	0.01
	Proteobacteria	35.437
	Gammaproteobacteria	21.264
	Alphaproteobacteria	13.037
	Betaproteobacteria	1.129
	Deltaproteobacteria	0.006
	Actinobacteria	10.688
	Bacteroidetes	0.107
	Sphingobacteriia	0.103
	Bacteroidia	0.003
	Fusobacteria	0.053
	Thermotogae	0.008
	Cyanobacteria	0.002
	Gloeobacteria	0.002
	Eukaryota	0.029
	Ascomycota	0.029
	Viruses	0.017
	Viruses_noname	0.017

4. Discussion

There is a large discrepancy between the diversity of microorganisms that exist within the environment and those that can be cultured in the laboratory. The vast majority of bacteria remain

Table 3
Summary of BLAST results. The total estimated ORFs from the sequences from K2D and K2D-FT were 2405 and 28,003, respectively (allowing for multiple ORFs in an actual ORF). From the customized BLAST database containing 35,197 enzymes that use tributyrin as substrate, 208 K2D ORFs were matched to 20 enzymes with less than 10E-15 evalue while 1646 ORFs of K2D-FT were found to be similar with 162 enzymes.

Metagenome	Summary of BLAST output		
K2D	Total estimated ORFs	2405	
	Average length (bp)		649.45
	No. of aligned ORFs	208	
	Average aligned length (bp)		322.36
	Average evalue		10E-15
	No. of hit subjects (lipolytic genes)		20
K2D-FT	Total estimated ORFs	28003	
	Average length (bp)		750.49
	No. of aligned ORFs	1646	
	Average aligned length (bp)		488.7
	Average evalue		10E-15
	No. of hit subjects (lipolytic genes)		162

Table 4
Major annotated gene products of BLAST analysis. Annotations of the 208 (K2D) and 1646 (K2D-FT) ORFs were summarized. Despite the enzymatic functions of the most ORFs have not identified yet (hypothetical proteins), lysophospholipase and arylesterase acting on carboxylic ester bonds were the major gene products in the both libraries. However, K2D-FT showed additional unique genes including SarR (Transcriptional regulator), BioH (bioin biosynthesis protein), YcfP (esterase), and SecG (protein export membrane protein), which requires further evaluation of their enzymatic functions.

Metagenome	Annotated gene products	No. of aligned ORFs
K2D	hypothetical protein | transposase	114
	lysophospholipase family protein | putative transposase	60
	hypothetical protein | pimeloyl-[acyl-carrier protein] methyl ester	6
	putative oxidoreductase	5
	sugar ABC transporter permease	4
	arylesterase | oxidoreductase	3
	hypothetical protein | oxidoreductase | multifunctional acyl-CoA thioesterase I	2
	hypothetical protein | short-chain dehydrogenase/reductase | arylesterase	2
	arylesterase | short-chain dehydrogenase/reductase | hypothetical protein	2
	ABC transporter ATP-binding protein | arylesterase | oxidoreductase	2
	Putative oxidoreductase |Arylesterase precursor	1
	lipase/esterase	1
	Others	6
K2D-FT	hypothetical protein | transposase	135
	lysophospholipase family protein | putative transposase	75
	transposase for insertion sequence-like element | hypothetical protein	60
	lysophospholipase	57
	hypothetical protein | lysophospholipase	57
	lysophospholipase | transcriptional regulator	55
	membrane protein | lysophospholipase | transcriptional regulator	50
	hypothetical protein	41
	lysophospholipase | proline dehydrogenase	40
	transposase | transposase | lysophospholipase	38
	acyl-CoA thioesterase I	30
	alpha/beta hydrolase | transcriptional regulator sarA	30
	transposase for insertion sequence-like element | lysophospholipase	30
	hypothetical protein | lysophospholipase | hypothetical protein	30
	transcriptional regulator | lysophospholipase	30
	biotin biosynthesis protein BioH	20
	transcriptional regulator | lysophospholipase | membrane protein	20
	hypothetical protein | IS431mec, transposase family protein	19
	lysophospholipase | transposase | transposase	19
	Beta N-acetyl-glucosaminidase | YcfP protein: probably an esterase	18
	enolase | hypothetical protein | SecG subunit | carboxylesterase	18
	proline dehydrogenase | lysophospholipase	18
	ABC transporter permease | arylesterase | hypothetical protein	16
	lysophospholipase | hypothetical protein	16
	Others	724

unexplored and only 1% of species can be cultured using standard laboratory techniques (McGarvey et al., 2012). Here, we demonstrated that forced-transcription using a T7-transposon system significantly enhanced the heterologous expression of foreign metagenomic genes in *E. coli*. The bi-directional T7-Km transposon was randomly inserted into metagenomic DNA, and genes encoding lipolytic enzymes were screened for by the presence of zones of clearance on tributyrin plates. Our results showed that 6-fold more colonies with lipolytic activity were identified in K2D-FT, the FT metagenomic library and that the average zone of clearance size of these colonies was significantly larger than that of the K2D hits. NGS analysis revealed that the K2D-FT library exhibited more extensive genetic diversity than the K2D library. Blast analysis successfully matched the screened candidate ORFs to diverse lipolytic genes such as lipases, esterases, and carboxylesterases. Although the additional step required for the construction of FT libraries may decrease the genetic diversity of an original metagenomic library, the FT technique can significantly increase the metagenomic screening efficiency both quantitatively and qualitatively.

Most of the novel enzymes have been identified using function-based screening methods which can directly evaluate the enzyme activities (Simon and Daniel, 2011). These function-based screening methods including flow cytometry-based high-throughput screening techniques (Choi et al., 2013; Uchiyama et al., 2005; Uchiyama and Miyazaki, 2010) can easily be used with the FT technique. In particular, the fluorescent reporter based genetic enzyme screening system (GESS) (Choi et al., 2013) has a great potential for the use of the FT system since the broad applicability of GESS will provide a synergistic effect for novel industrial purpose screenings.

Our results clearly showed that FT technology can effectively identify the range of biocatalysts of interest without any technical difficulties though further characterization of the lipolytic gene candidates will be necessary. We believe that the FT technique has the potential to broaden our knowledge of unknown microbial species by expanding the range of detectable enzyme activities.

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