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Screening of Promoters from Metagenomic DNA and Their Use for the Construction of Expression Vectors

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This study was focused on the screening of valuable genetic resources, such as promoters from metagenome, and describes a promoter trapping system with a bidirectional probe concept, which can select promoters or operons from various biological resources including metagenomic DNA. A pair of reporters, GFP and DsRed, facing the opposite direction without promoters, is an effective system that can function regardless of the direction of inserted promoters. The feasibility of this system was tested for the isolation of constitutively expressed promoters in *E. coli* from a soil metagenome, resulting in a potential pool of various promoters for practical application. The analyses of structural organization of the trapped genes demonstrated that constitutively expressible promoters in *E. coli* were broadly distributed within the metagenome, and suggested that some promoters were useful for the construction of expression vectors. Based on these observations, three constitutive promoters were employed in the expression vector system and their potentials for practical application were evaluated in terms of expression level, protein solubility, and effects on host growth.

Keywords: Constitutive expression, dual reporter, metagenome, promoter, trap vector

An analysis of the 16S rRNA sequence of environmental genomes showed that a small proportion of microorganisms in nature can be cultured [1]. Most other strains have physiological characteristics that cannot easily be defined and are difficult to uncover. Therefore, it is important to develop new techniques to make pure cultures of such noncultivable strains or mine useful genes originating from them. Accordingly, a great deal of attention has been paid to the discovery of new enzymes and metabolites, and some have succeeded to mine novel enzymes or metabolites from unidentified strains [5, 7, 9]. For these purposes, research for total genomes (metagenome) of all strains in a habitat has been undertaken in order to overcome limitations of the presently available screening methods that are dependent on pure culture techniques [8, 16, 17].

Even though the mining of new biomaterials through metagenomic DNA brought about very interesting results from the points of exploiting and securing new resources [5, 17], researches have been focused mainly on the screening or selection of ORF encoding proteins. On the other hand, regulatory elements including promoters have not been exploited to select from metagenome to date. As a promising resource, mining of useful regulatory regions from metagenome might be an alternative route to providing promoters for the commercial production of bioresources. Currently, a few promoter systems are commercially available and effective in a limited range of hosts. However, they also require an expensive inducer. Because of these problems, research is urgently needed to identify new promoters and develop novel expression systems [18]. In these contexts, attempts to discover promoters from various strains have rapidly increased. In general, the promoter trap is performed by inserting a genomic DNA fragment into a promoter trap vector with promoterless fused reporter gene, thereby selecting the trapped promoter by the expression of the reporter. The fundamental problem, however, is that only promoters trapped in the same orientation as the reporter gene are detected [3, 6, 10, 15, 19]. Thus, a trapping method to screen promoters from various resources, including metagenomic DNA, is highly desired. However, no systematic approaches have so far been described to screen promoters from metagenome for practical application.

In order to investigate and find the potential of metagenome as a promoter resource, a promoter trapping system with a bidirectional probe concept was tested to screen valuable promoters, which can effectively be used for the construction of novel expression vectors. Although there are many microorganisms useful for evaluation of

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our trap system, the typical host *E. coli* was first employed as a trap-host because of its reliability, suitability for gene manipulation, and well-known regulatory mechanism.

MATERIALS AND METHODS

Construction of Bidirectional Reporter Vector pBGR1

To construct a plasmid that has reporter proteins working in both directions, the *dsRed* gene encoding a red fluorescent protein was amplified by PCR using a forward 5'-ATAAGCTTCTACAGGAACAG-3' and reverse 5'-AGGATTTCATGGCCTCCTCC-3' primer from pDsRed2-N1 (Clontech, U.S.A.). The amplified gene was digested with HindIII and EcoRI and inserted into pBluescript II SK(+) (Stratagene, U.S.A.) at the same sites to produce pBR1. In the case of the GFPuv coding gene, the BamHI site used for cloning into the trap vector is naturally present in the gene and needed to be removed. This was achieved by site-directed mutagenesis (GGATCC→ GGTACC), and the resulting GFPbk was then amplified by PCR using a forward 5'-AGTCTAGAATGAGTAAAGGA-3' and reverse 5'-GCTCTAGATTATTTGTAGAG-3' primer. The resulting gene was inserted into pBR1 at the XbaI site to produce pBGR1. After being confirmed by DNA sequencing, it was transformed into *E. coli* XL1-Blue. To verify that there was no expression of reporter protein without a trapped promoter, the transformed cells were checked using a fluorescent imaging analyzer (Fuji, Japan).

Purification of Soil Metagenome

The soil used for extracting the metagenome was gathered from the Ocean Ecological Park (Incheon, Korea), directly frozen in liquid nitrogen, and then kept in a deep freezer at -80°C. An aliquot of samples (1 g) was thawed at 4°C and total DNA was extracted using the FastDNA Spin Kit (Bio101, U.S.A.). Although the metagenome was basically extracted according to the supplier's protocol, the quantity of beads and the shear force were adjusted when too much DNA was fragmented.

Construction of a Metagenome Library and Promoter Screening

The metagenome was digested with Sau3AI, and the resulting fragments (0.2–3 kb) were purified though agarose gels. The collected fragments were ligated with pBGR1 and transformed into *E. coli* XL1-Blue. The resulting cells were spread onto LB agar plates containing 50 μg/ml ampicillin. The promoter-trapped fluorescent clones were primarily screened by a UV hand lamp (Vilber Lourmat, France). Under the same conditions, *E. coli* cells transformed with pDsRed2-N1 and pGFPuv were used to compare the red and green fluorescence, respectively, as controls. As for the second screening of stable promoters that had few fluctuations depending on culture media and temperature, quantitative analyses of fluorescence were performed using a microtiter fluorescence analyzer (Tecan, Switzerland). Excitation and emission wavelengths determined previously in other work were used [4, 11].

Electrophoresis and Sequence Analysis

The expressions of reporter proteins in the selected clones were analyzed by SDS-PAGE and agarose gel [2, 12]. The screened cells were cultured in LB medium (5 ml) at 37°C and 200 rpm for 12–14 h, and collected by centrifugation. Then, the process of freezing to -80°C and thawing was repeated twice. Thereafter then, the cells were suspended in 500 μl of 20 mM Tris-HCl buffer (pH 8.0) and disrupted by ultrasonication (Sonics & Materials, U.S.A.). The crude extracts were centrifuged (16,000 ×*g*) at 4°C for 30 min to collect the supernatant. An aliquot of samples (10 μl) was used for analyses of protein expression in 9% acrylamide gel. Electrophoresis was conducted at 100 V and room temperature. In order to confirm the reporter protein expression by fluorescence, the crude extract (25 μl), including 5 μl of loading dye, was also loaded onto 1% agarose gel. Electrophoresis was conducted at 150 V and room temperature, and the developed gel was analyzed by excitation wavelength of 395 nm for GFP and 488 nm for DsRed without any staining.

For the analyses of structural organization of trapped genes, DNA sequencing was carried out using *dsRed*-gene binding primer DR-FS, 5'-GTTCACGGTGCCCTCCATGC-3', and *gfp*-gene binding primer G-RS, 5'-CATCACCATCTAATTCAACA-3'. The promoter region was predicted by the method described previously [14], and multiple sequence alignment was carried out with the ClustalW program and then adjusted manually [3].

Construction and Evaluation of Expression Vector with Trapped Promoters

The predicted promoter sequence, including the upstream region, was amplified by PCR and used for the construction of new expression vectors. Three pairs of primers (G118 forward 5'-AATGCAGCCCGGGGGATC-3' and reverse; 5'-TCAAGCTTCT-GCAGGAATTCCCATGGCATGCTTAGTCTTCCTCA-3'; G196 forward 5'-AATGCAGCCCGGGGGATC-3' and reverse 5'-TC-AAGCTTCTGCAGGAATTCCCATGGCATAAATTTCCTCCAGG C-3'; and G200 forward 5'-AATGCAGCCCGGGGGATC-3' and reverse 5'-TCAAGCTTCTGCAGGAATTCCCATGGCATTTTGC-GCATTCCCTT-3') were used for PCR reaction. To facilitate gene cloning, a multicloning site was incorporated into the downstream region of the ribosome-binding site of the reverse primer sequence. The underlined bases indicate the multicloning site incorporated into each primer. After amplification, both ends of resulting fragments were converted to blunt ends and cloned into a pBluescript II SK(+) vector from which the promoter had been removed through treatment of PvuII. At these artificially incorporated multicloning sites, PCR-amplified GFP was subcloned to investigate the constitutive expression ability of new promoters selected. The protein expression driven by the novel promoters was evaluated in terms of expression level and the solubility of the reporter protein.

Nucleotide Sequence Accession Number

The insert sequences harboring screened promoters reported in this work have been deposited in the GenBank database under the following accession numbers: R2, EU122295; R60, EU122296; R97, EU122297; R107, EU122298; G52, EU122299; G110, EU122300; G118, EU122301; G121, EU122302; G160, EU122303; G196, EU122304, G200, EU122305.

RESULTS AND DISCUSSION

Construction of a Bidirectional Fluorescence Reporter System for Promoter Trapping

To construct a promoter-trap vector, pBluescript II SK(+) was used as a scaffold to provide the origin of replication

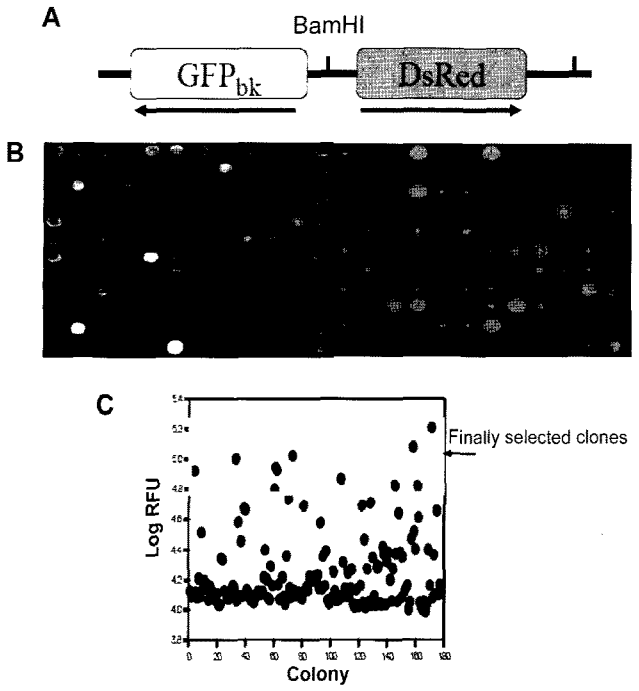


Fig. 1. Construction of pBGR1 vector and its use for promoter trapping.

A. The prototype of vectors, including the ampicillin resistance gene and the replication origin, was derived from pBluescript II SK(+). **B**. A typical recombinant *E. coli* library that was primarily screened on solid plates. Green or red fluorescence was clearly detected in *E. coli* containing pBGR1 with trapped promoters. **C**. Comparison of fluorescence level emitted by primary screened clones in liquid culture at 37°C. The 11 clones finally selected are boxed.

and a selection marker required for the basic vector system. In addition, as a reporter to check the functional promoter in the inserted genes, *dsRed* was inserted in the direction of MCS of pBluescript II SK(+), and another reporter, *gfp*, was inserted in the opposite direction of *dsRed*, facing the opposite direction without promoters (Fig. 1A). The BamHI site was located between the two genes and used as a cloning site for the DNA fragment from the metagenomic DNA. For this purpose, the BamHI site in the GFPuv gene was substituted by the site-directed mutagenesis technique. In the substituted mutant GFPbk, no notable changes in fluorescence and maturation time were found. There was also no detectable fluorescence of either reporter in pBGR1 without an insert.

Fluorescent proteins with high sensitivity as reporters were inserted in both directions into the pBGR1 vector, two probes working in either direction. Thus, it was predicted that functional promoters could be selected regardless of the direction of trapped genes. Moreover, because it is generally known that a fluorescent protein can function solely or as a fusion protein, it was possible to detect promoters in clones harboring inserts with incomplete ORFs [19]. In the case of gene fusion, the reporter works when an in-frame translational fusion is achieved. Therefore, to screen more diverse promoters, it would be advantageous for each reporter to have its own RBS. In fact, the RBS sequence was inserted into the primer during subcloning to construct a pBGR2 plasmid, which was also used in the screening of promoters. However, we analyzed the results of the promoter library using pBGR1 only.

Construction and Screening of a Promoter Library from Metagenomic DNA

As a potential source of applicable promoters, metagenome was primarily evaluated for *in vivo* distribution of a constitutive promoter of typical host *E. coli*. Thus, natural resources from a tidal flat were used as environmental samples for metagenome extraction, and the biodiversity of metagenome was assessed through RFLP and 16S rRNA analyses before employing it in the library construction. In particular the sequencing results of the PCR-amplified 16S rRNA gene (total 15 clones) from metagenome showed an independency and was classified as unidentified ones. The digested fragments of metagenomic DNA were separated in approximate sizes of 0.2–3 kb and introduced into pBGR1. The pool of recombinant genes was transformed into *E. coli* XL1-Blue, resulting in about 115,000 clones with an insert. After analyzing their clonal independency, a hand-type UV lamp and a fluorescent image analyzer were used to select 224 clones that induced green fluorescence and 178 clones that induced red fluorescence *via* the trapped promoter (Fig. 1B). An analysis by the size of the inserts from all of the selected clones showed that more than 80% were different, and that over 95% were independent clones on the restriction enzyme map and DNA sequencing. The average size of the inserts was approximately 0.5–1.2 kb.

With the 402 clones, the growing temperature (30–37°C) and medium (LB, NB, and M9) for *E. coli* cells were varied in order to further select strong promoters that work constitutively under these culture conditions. At this stage, a sensitive fluorescent image analyzer was used to finally select 7 green fluorescent clones and 4 red fluorescent clones (Fig. 1C), all of which did not fluctuate with culturing conditions and also had a negligible effect on cell growth. For the selected clones, the strength of trapped promoters was determined by the expression of reporter protein. For this purpose, each clone was cultured for about 10 h in an LB liquid medium containing no inducer. After disrupting cultured cells, the expression of the reporter was examined by polyacrylamide gels (Fig. 2). The reporters are expressed in fusion proteins or independently, depending on the organization of trapped gene fragments, thereby showing the protein bands in different positions. Meanwhile, clones G118 and 200 were expressed as fusion proteins, whereas clones G52, 110, 121, 167, and 196 were independently expressed. In the case of red fluorescence cells (R2, 60, 97, 107), it was found that the proteins except clone 97 were

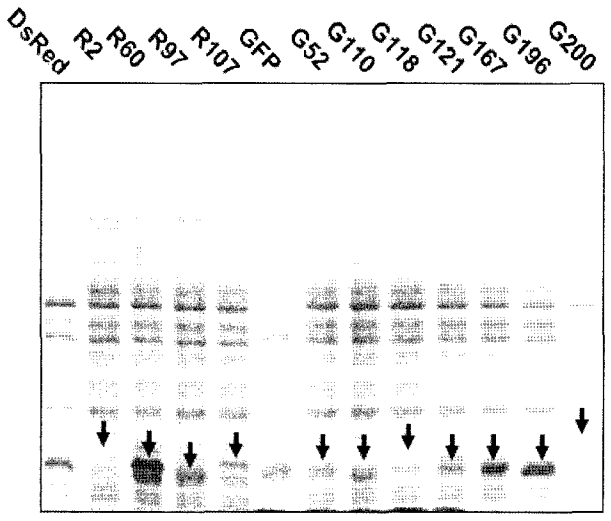


Fig. 2. Analyses of reporter protein expressed in the clone with trapped genes.
The crude extract of proteins samples (10 μg) was loaded on acrylamide gel (9%). The number indicates the clone harboring pBGR1 with a trapped insert.

expressed as fusion proteins. As controls, the *E. coli* cells harboring pGFPuv or pDsRed2-N1 were induced with 1 mM IPTG and treated in the way described above.

Analyses of Structural Organization and Putative Promoters in the Trapped Genes

The nucleotide sequences of trapped genes were determined completely in both directions and used for analyses of structural organization. Based on the nucleotide sequences, the corresponding amino acid sequences were deduced and scanned with sequences in GenBank using both BLAST N and X programs. Out of 11 clones, 8 clones had annotated ORFs and showed considerable amino acid homologies (>30%) with deposit sequences of GenBank, although incomplete ORFs were found (Fig. 3). Deduced genes from clones R2, G52, G110, and G196 function as bacterial transport machinery, such as outer membrane proteins or efflux pump. High homology with some important enzymes involved in sugar (R107, G52, and G118) and sugar acid (G167) metabolism were found in the trapped fragments. G118, 196, and 200 were predicted to have putative hypothetical proteins, and another three clones (R60, R97, and G121) had no ORF predicted. Most of them are generally known to be involved in energy generation, uptake, and metabolic capability for carbohydrates.

To predict a reasonable promoter, the sequence upstream of the ORF that was expressed with the reporter as a fusion protein was searched. In another case that had an independently

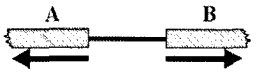
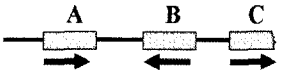
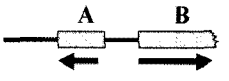
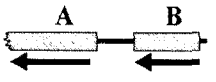
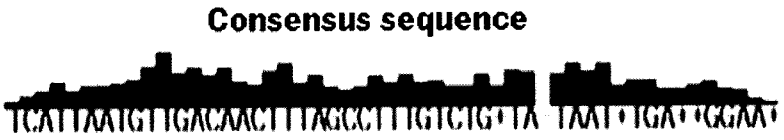
Clone	Insert size(bp)	Analyses of predicted ORF	Structural organization of the trapped fragment
R-2	632	A: Putative PQQ-binding exported phosphoesterase B: Putative outer membrane protein	
R-60	191	Not detected	
R-90	121	Not detected	
R-107	353	Putative class II aldolase/adducing family protein	
G-52	815	A: Putative ribulokinase B: Putative heavy metal efflux pump, Czc family C: Putative aspartate kinase	
G-110	263	Putative outer membrane protein	
G-118	653	A: Putative conserved hypothetical protein B: Putative glucose sorbose dehydrogenase	
G-121	725	Not detected	
G-167	242	Putative uronate isomerase	
G-196	532	A: Putative hypothetical protein B: Putative TonB-dependent outer membrane receptor	
G-200	618	Putative hypothetical protein	

Fig. 3. Description of the structural organization of putative genes in the clone expressing high-level fluorescence.
The single line represents a noncoding region, including promoters, and putative genes are boxed.



Clone		-35	Spacer	-10	+1(bolded base)
R-2	ACAAC	TTGCCA	TTACGGGCATCTACAGCA	TAAATT	GCATGATCGTCCGAA
R-60	TT	GTGATA	GAATCCGGATTAATCC	GTCAAT	ATGCTTGGTCAAAAAAAGGA
R-97	CACGA	TTGTTG	TACCCGCACTTCCCCGGA	TCGAAT	GAAATTGCTTTCTCA
R-107	ACAAA	CTTGTC	AATAAAAAATTTATTTCA	TATATT	TAAACTATTCCACGA
G-52	TGCTT	GATTGT	AACTGTTTAATGTATTGATGTA	TTCGCT	TTGTATGCCAA
G-110	CTGATG	TTGACA	GGTTTGATTTTTGTCAT	TATTAT	TTTGCTGCTCTTGCC
G-118	AAATTGGAAC	ATATTG	GTCAAGGCATTGATAAA	AGTATT	GTTCCGGATGC
G-121	GGAATT	TTAACG	CCCATATTTATAGCTT	TATAGC	CGGGGGTACGGATTC
G-167	TCTAT	TTTACA	CCCTAACTTAAACCTAGC	TAAATT	CTAATTTATTGCTAA
G-196	ATCCTGT	TTGATA	AAGTCAAATTGCAGG	TCAAAA	TCATCGTAGTACCGGT
G-200	GATGTGT	TGGATA	TGTTTAATGTGCGTTATC	TGATTA	CAACAGATCGTGC
E coli Consensus		TTGACA		TATAAT	

Fig. 4. Description of putative promoters, transcription start points (TSP), and aligned consensus sequences in the clone constitutively expressing high-level fluorescence.
All of these organizations were predicted only by computer programs, and therefore, real functions *in vivo* remain unconfirmed.

expressed reporter, the probable promoter in the upstream of the reporter protein was analyzed. It is generally known that the consensus sequence of promoter is recognized by the sigma factor and is somewhat different in each strain. Therefore, it may be difficult to find the consensus sequence of promoter in trapped genes because it is difficult to identify the strain from which the promoter came, owing to the complex nature of metagenome originating from tens of thousands of microorganisms [8, 14]. The selected clones, however, were limited to those with a promoter that was constitutively expressed in *E. coli*. Thus, the sequence and organization would be expected to be similar to those of *E. coli*. Based on this, the putative promoter sequences, including transcription start points (TSP), were deduced using the identical procedure described previously [14], and then aligned using the ClustalW program (Fig. 4). The predicted promoter was found to be partly matched with the consensus sequence of *E. coli* in regions −10 and −35. In particular, the R2 clone showed similar sequence patterns in both regions. It was found that the G110 and G196 clones were consistently conserved in region −35, and the R107 clone sequence was similar in region −10. Overall, the similarity was 50–60% in both regions, and the length of the spacer between −10 region and TSP was 2–11 bases. However, some putative spacers and/or promoters predicted by the computer program were not matched well with the typical consensus of *E. coli* sigma factor. In general, the real function of a promoter *in vivo* can be determined through footprinting, deletion analyses, or 5' RACE PCR [3]. These studies are presently in progress. As a preliminary work, the *in vivo* function of predicted promoters including RBS was tested by subcloning them into promoterless plasmids and then attempting to express protein arbitrarily selected.

Construction of Novel Expression Vectors with Putative Promoters

As a plausible approach to confirm promoter function *in vivo*, the construction of constitutive expression vectors was attempted. To produce new expression vectors using the selected promoters, three clones (G118, G196, and G200) were randomly selected, and PCR was performed using each primer set. The amplified fragments were then ligated with promoterless pBluescript II SK(+), thus yielding new expression vectors pG118, pG196, and pG200. The efficiency of these expression systems were analyzed by subcloning GFPuv into new vectors as a reporter protein. As controls, the original clones selected from the pBGR1 library and IPTG (1 mM)-induced pGFPuv were used. As shown in Fig. 5A, reporter protein GFP in novel vectors pG118, pG196, and pG200 was expressed 2–18 times more distinctly than those of the original clone (G118, 196, and

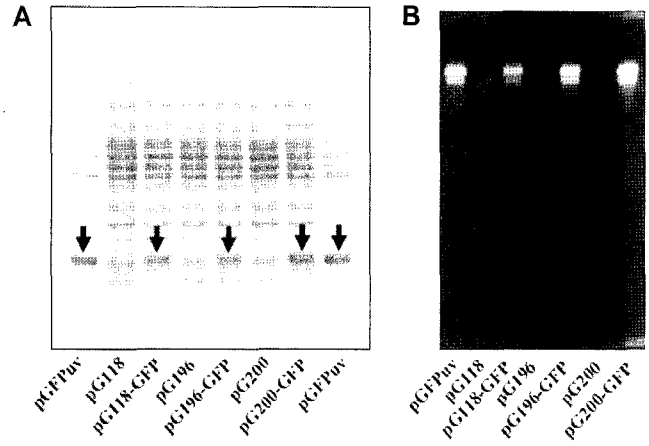


Fig. 5. SDS-PAGE and agarose gel electrophoresis for the comparison of the expression level and fluorescence of reporter protein GFPuv under the control of promoters screened from metagenomic DNA.

A. Comparison of expression level by 9 % acrylamide gel. **B.** Comparison of the fluorescence of expressed reporter by 1% agarose gel under native conditions. As positive and negative controls, IPTG-induced pGFPuv (Clontech) and empty vector were used, respectively.

200), when compared with those in Fig. 2. Specifically, the expression level of pG200 was quite similar to that of pGFPuv expressed by 1 mM IPTG under the control of the P_{lac} promoter. These results strongly suggested that the novel promoters were stable and constitutively functioned in *E. coli*. Similar results were also obtained by the analysis of the same sample on agarose gel under native conditions (Fig. 5B). As expected, the fluorescence intensities of pG200 in the gel were similar to or slightly higher than pGFPuv. There are two fluorescent bands on the gel, because the fluorescence of GFPuv appears in the monomer and dimer positions during overexpression [4]. All of them were expressed mainly in the soluble fraction (>95%).

As for further evaluation of constitutive expression vectors, the reporter proteins were expressed at 30°C. Consistent expression levels were also seen for three expression systems; however, about 3–4-folds decreases in the amount of expression were observed. These results are also comparable to but slightly lower (25–35%) than, those of constitutive expression vector reported previously in other work, when comparing the expression level at 30°C [19]. *E. coli* strains harboring each of the expression vectors did not strongly affect cell growth when compared with those of *E. coli* strains harboring pBluescript II SK-GFPuv, pGFPuv, or pTrc99A-GFPuv. A very small amount of GFPuv was detected in *E. coli* cells grown at 25°C. The retransformation of these plasmids into freshly prepared *E. coli* competent cells showed an identical result.

To our best knowledge, this is the first study that screened valuable promoters from metagenome using the dual fluorescence-based reporter system. A promoter is an essential element in the production of proteins and metabolic engineering for improvement of useful strains, because natural promoters are tightly regulated by its own metabolite or *trans*-acting elements. Therefore, this study is expected to contribute to the development of promoter-trap systems if the bidirectional probe proposed in this work can be mounted on a broad-host-spectrum-range plasmid that is maintained in various hosts. In addition, the library prepared in this work led us to understand the genetic organization of constitutive expressible promoters in *E. coli* and also provides a basis for stable protein production in *E. coli* and related bacteria when inducible systems are not suitable because of its metabolic burden, expensive inducer, and incorrect folding when expressed explosively by an inducer [3]. Consequently, the mining of highly useful promoters for various bacteria through a biotreasure metagenome has strong potentials for practical applications.

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