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Toward a Generalized and High-throughput Enzyme Screening System Based on Artificial Genetic Circuits

Su-Lim Choi,[†,∥,∇,○] Eugene Rha,[†,○] Sang Jun Lee,[‡] Haseong Kim,[†] Kilkoang Kwon,[†] Young-Su Jeong,[§] Young Ha Rhee,[∥] Jae Jun Song,[⊥] Hak-Sung Kim,[§] and Seung-Goo Lee*[,#,†]

[†]Biochemicals and Synthetic Biology Research Center, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahak-ro, Yuseong-gu, Daejeon, South Korea

[‡]Infection and Immunity Research Center, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahak-ro, Yuseong-gu, Daejeon, South Korea

[§]Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 291 Daehak-ro, Yuseong-gu, Daejeon, South Korea

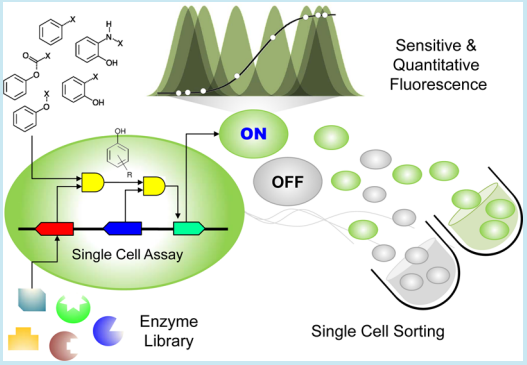
[∥]Department of Bioscience and Biotechnology, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon, South Korea

[⊥]Applied Microbiology Research Center, Korea Research Institute of Bioscience and Biotechnology, 181 lpsin-gil, Jeongeup-si, South Korea

[#]Biosystems and Bioengineering Program, University of Science and Technology, 217 Gajung-ro, Yuseong-gu, Daejeon, South Korea

Supporting Information

ABSTRACT: Large-scale screening of enzyme libraries is essential for the development of cost-effective biological processes, which will be indispensable for the production of sustainable biobased chemicals. Here, we introduce a genetic circuit termed the Genetic Enzyme Screening System that is highly useful for high-throughput enzyme screening from diverse microbial metagenomes. The circuit consists of two AND logics. The first AND logic, the two inputs of which are the target enzyme and its substrate, is responsible for the accumulation of a phenol compound in cell. Then, the phenol compound and its inducible transcription factor, whose activation turns on the expression of a reporter gene, interact in the other logic gate. We confirmed that an individual cell harboring this genetic circuit can present approximately a 100-fold higher cellular fluorescence than the negative control and can be easily quantified by flow cytometry depending on the amounts of phenolic derivatives. The high sensitivity of the genetic circuit enables the rapid discovery of novel enzymes from metagenomic libraries, even for genes that show marginal activities in a host system. The crucial feature of this approach is that this single system can be used to screen a variety of enzymes that produce a phenol compound from respective synthetic phenyl-substrates, including cellulase, lipase, alkaline phosphatase, tyrosine phenol-lyase, and methyl parathion hydrolase. Consequently, the highly sensitive and quantitative nature of this genetic circuit along with flow cytometry techniques could provide a widely applicable toolkit for discovering and engineering novel enzymes at a single cell level.



KEYWORDS: *genetic circuit, high-throughput screening, metagenome, enzyme screening, DmpR, phenolic compounds*

Novel enzymes are essential to develop new bioprocesses such as cost-effective biorefineries and bioenergy resources, which will be indispensable for a sustainable bioera.[1] The metagenome is one of the richest resources of enzyme, as it contains a variety of uncharacterized and untapped enzyme-encoded genes from uncultivated environmental bacteria.[2] Traditionally, novel enzymes have been identified by screening their activities on microtiter plates or on solid media, or by mining DNA sequence databases. However, these conventional approaches have not been able to satisfy the high-throughput needs of large-scale screening of metagenomic libraries. Moreover, metagenomic genes tend to be expressed at lower levels in host systems due to compatibility issue associated with recombinant expression.

In order to address these needs, new approaches have been attempted by converting enzyme function into the transcription activation of reporter proteins in microbial host systems.[3−6] A phenol-degrading operon from *Ralstonia eutropha* E2 has been isolated using substrate-induced genetic expression screening (SIGEX) method, where the substrate activates the expression of a reporter protein.[4] Although the catabolic operon is cloned together with the regulatory element, the catalytic capability of the isolated genes needs to be re-examined because the method

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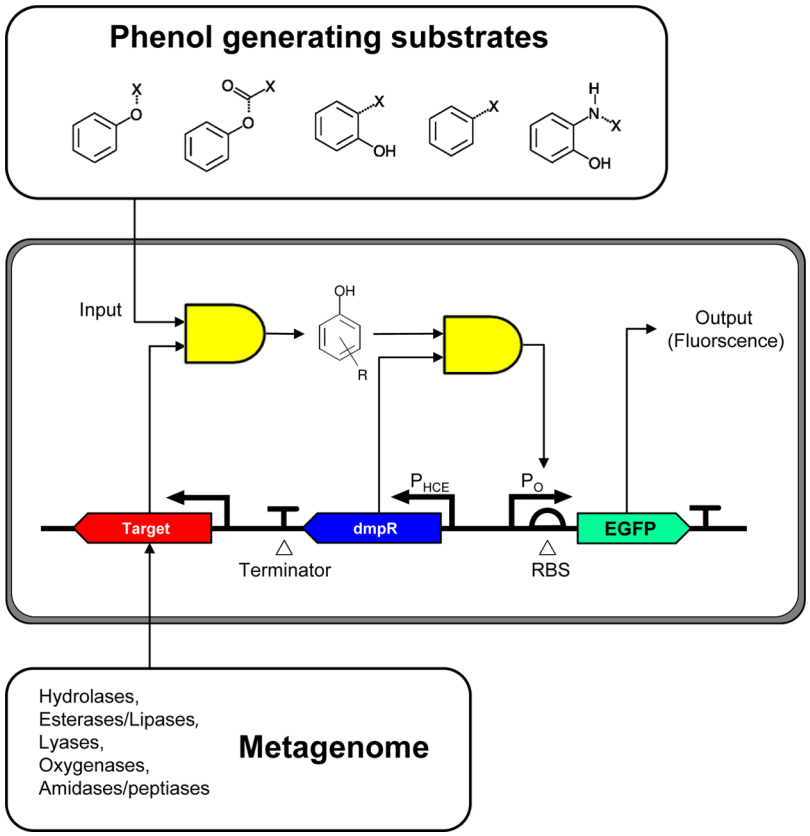

Figure 1. Schematic representation of GESS with two AND logic gates. Intracellular phenolic compounds are generated by various enzymes within the cells and visualized through EGFP whose expression activated by the phenol−DmpR complex. X groups can be α/β-glycosidaes, phosphate, alkyl, amine, amino acids, or halogens. R groups represent different substituents on the aromatic ring. Names and activities of the compounds tested are shown in Figure 5. Genes for target enzymes such as hydrolase, esterase/lipase, lyase, oxygenase, and amidase/peptidase are selected from genetic libraries based on fluorescence signals.

does not directly detect enzyme activity. A product-induced screening technique with a quorum molecule-dependent transcription activator in a bacterial cell was reported to detect an acyl-homoserine-lactone synthase.[5] Additionally, several new screening approaches for the direct detection of enzyme products using transcriptional activators have been introduced; some notable examples include amidase screening using the benzoate-responsive transcriptional activator BenR,[6] screening for dehydrochlorinase activity on γ-hexachlorocyclohexane with an optimized mutant of XylR,[7] and benzaldehyde dehydrogenase screening using benzoate-responsive NahR.[8]

However, a drawback of these screening methods is that they require specific metabolite-responsive or product-induced transcriptional systems. Because of this, these methods cannot be applied generally, which is a necessary quality for a metagenomic enzyme screening system. Accordingly, we drew our attention to phenol-dependent transcription activators because a vast amount of natural enzymatic reactions can produce phenols from phenolic compounds. For example, in the BRENDA database (http://www.brenda-enzymes.info), over 300 enzyme species have been reported to generate phenols or phenyl compounds as byproducts of their catalytic reactions. In our previous work,[9] a screening system to detect organophosphate (OP)-degrading enzymes was developed by incorporating DmpR (dimethylphenol regulatory protein) that responds to the phenolic compounds.[10] Although this screening system demonstrated that more than 50% of widely used OP-based pesticides release a phenolic group, the low sensitivity of the system prevented its use as a metagenomic enzyme screening method.

In this study, we have dramatically improved the sensitivity of our previous screening system by redesigning the genetic components of the circuit, and demonstrated its practical use for metagenomic screening. This circuit, termed the Genetic Enzyme Screening System (GESS), is a single cell-based genetic reporter system with wide applicability for diverse enzyme screenings from metagenomic or mutation libraries. Individual cells harboring GESS showed quantitative responses to phenolic compounds and were characterized in various conditions by switching its genetic parts and using different growth media. The genetic circuit was also assessed by detecting the *in vivo* catalytic activity of various enzymes (tyrosine phenol-lyase, lipase, cellulase, and methyl parathion hydrolase) that generate phenolic compounds. To evaluate the applicability of GESS, we performed high-throughput enzyme screening using a metagenomic library derived from sea tidal flat sediments. Using fluorescence-activated cell sorting (FACS), which was able to analyze approximately 10^7 cells per hour, GESS rapidly identified a novel phosphatase gene whose amino acid sequence shares 59% homology with *Sphingomonas* alkaline phosphatase.

RESULTS AND DISCUSSION

Design and Optimization of the Genetic Circuit. Diverse phenol-tagged compounds, which can be degraded by various enzymes (e.g., hydrolases, esterases/lipases, lyases,

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oxygenases, and amidases/peptidases) to release phenol, are commercially available or can be synthesized. Intracellular phenol is specifically recognized by DmpR, an NtrC family transcriptional regulator of the (methyl) phenol catabolic operon.[11,12] Figure 1 depicts the GESS where DmpR is constitutively expressed under the control of its P_{HCE} promoter from pHCEIIB. DmpR recognizes phenolic compounds derived from enzymatic reactions on various substrates. EGFP, the reporter of the target enzyme reaction, was obtained from the plasmid pMGFP[13] and is regulated by the DmpR-responsive P_o promoter (*Pseudomonas putida* KCTC 1452).

Our previously constructed screening system,[9] which employed a DmpR variant harboring the E135K mutation (DmpR(E135K)), was not applicable to metagenomic enzyme screening due to its low sensitivity. Note that mutant DmpR(E135K) recognizes both 4-nitrophenol and phenol, whose details will be discussed in later sections. In order to improve the sensitivity, we introduced three genetic parts: a T7 ribosome-binding site (RBS) optimized for *E. coli*, a transcriptional terminator (rrnBT1T2 from pHCEIIB) and the P_{HCE} promoter. Figure 2A depicts six different versions of GESS plasmids (pGESS) including pUEGFP-DmpR, which was described in our previous study.[9] pUEGFP-DmpR contained DmpR(E135K) and P_{HCE} while pGESSv1 was constructed with wild-type DmpR along with its own promoter, P_X, from the genomic DNA of *Pseudomonas putida* KCTC 1452. pGESSv2 has only P_{HCE} promoter instead of P_X and, in pGESSv3, an optimized T7 RBS sequence was inserted at N-terminal end of EGFP. The pGESSv4 vector employed rrnBT1T2 terminator, at the both ends of the dmpR and EGFP in pGESSv3. pGESS(E135K) harbors DmpR(E135K) instead of wild-type DmpR in pGESSv4. Figure 2B depicts the fluorescence intensity of the six versions of GESS. Of these, pGESS(E135K) showed the strongest EGFP expression level, which was 13.49 fold stronger than that of pUEGFP-DmpR. Notably, in flow cytometry analysis, the mean fluorescence of *E. coli* cells with pGESSv4 was approximately >90 fold higher than that of cells grown without phenol (Supporting Information Figure S1). These results indicated that the addition of the optimized T7 RBS and terminators was crucial for improving GESS sensitivity, allowing its application to metagenomic enzyme screening.

Phenol acts as a gratuitous or nonmetabolizable inducer of DmpR transcription in *E. coli*. Because *E. coli* cannot use phenol as a carbon source for growth, the response of cells to this compound can be quantified.[14] Before testing this, the cytotoxicity of phenol in *E. coli* was investigated with various phenol concentrations ranging from 0 to 10 mM. The phenol cytotoxicity for the host was estimated not to be significant at concentrations up to 2 mM, indicating that the viability of *E. coli* cells was not affected by the presence of phenol (Supporting Information Figure S2). However, the colony numbers were reduced starting at 5 mM phenol there were no colonies in the 10 mM phenol M9 plate. Similar results have been previously described.[15]

To test the quantitative response of GESS to phenol, we measured fluorescent signals from *E. coli* DH5α cells harboring pGESSv4 at different phenol concentrations (up to 100 μM) in M9-glucose and LB media. Strong fluorescent signals were observed in colonies grown in the presence, but not in the absence, of phenol (Figure 3). In M9-glucose media, the fluorescence intensity was directly proportional to the concentration of phenol in the range 1−20 μM and was far

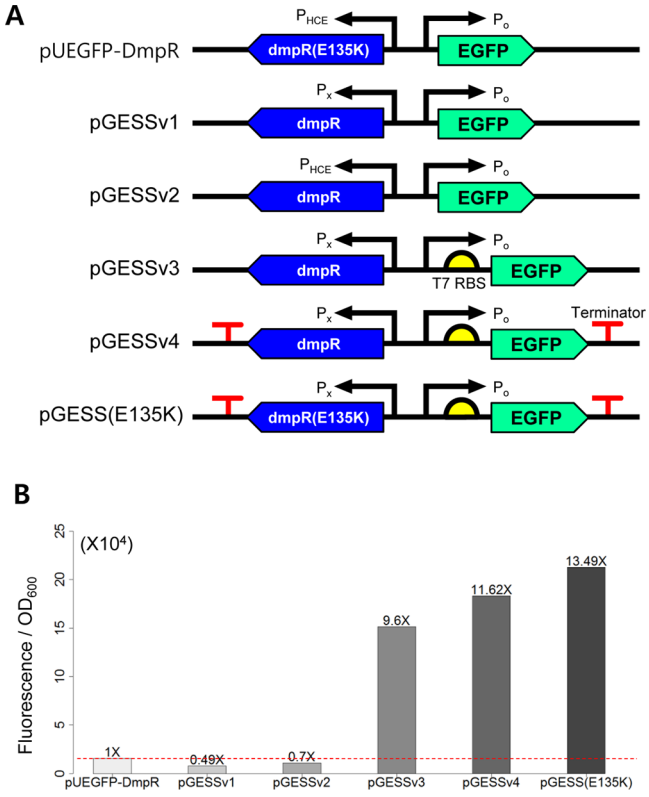

Figure 2. Different versions of pGESS plasmids. (A) Plasmid pUEGFP-DmpR, in which mutant dmpR(E135K) is under the control of HCE promoter PHCE, was used in our previous work.[10] pGESSv1 contains wild-type DmpR with its own promoter P_X and the DmpR-regulated σ54 dependent promoter P_o. pGESSv2 has PHCE instead of P_X. pGESSv3 has an optimized T7 RBS sequence at the N-terminus of EGFP. pGESSv4 and pGESS(E135K) employed a transcriptional terminator sequence (rrnBT1T2 from pHCEIIB) at both ends of the *dmpR* and EGFP genes in pGESSv3. (B) Fluorescence intensity analysis of the six different versions of GESS. Each sample was cultured for 12 h in M9 glucose media and treated with 1 mM phenol. Sample intensity was obtained by subtracting the negative control that the fluorescence level without the phenol treatment and being divided by the Optical Density (OD_{600}) of the samples. On the basis of our previous work with pUEGFP-DmpR,[10] the intensities of pGESSv4 and pGESS(E135K) increased approximately 11.6 and 13.5 fold, respectively.

higher than that in LB medium (Figure 3). This difference might be due to the high levels of cellular (p)ppGpp,[16] a nutritional alarmone present at higher levels during the stationary growth phase in minimal medium, which positively regulates DmpR-mediated transcription.[17]

Application of the Genetic Circuit for Cell-based Enzyme Assay. In order to investigate whether GESS could actually detect a phenol-generating enzyme activity, tyrosine phenol-lyase (TPL), which catalyzes the cleavage of tyrosine to produce phenol, pyruvate, and ammonium though the α,β-elimination reaction, was used as the test enzyme (Figure 4A). *E. coli* cells that carried both the pGESSv4 and *tpl* gene (on the high copy plasmid pHCEIIB-TPL) emitted strong fluorescent signals, while the same cells lacking *tpl* produced no signal under the same conditions (Figure 4B). In Figure 4C, the fluorescence intensity of cells with *tpl* in M9 medium was 7-fold higher than that in LB medium, which is consistent with the comparison depicted in Figure 3. The generation of phenol by the TPL enzyme was confirmed by the detection of clear

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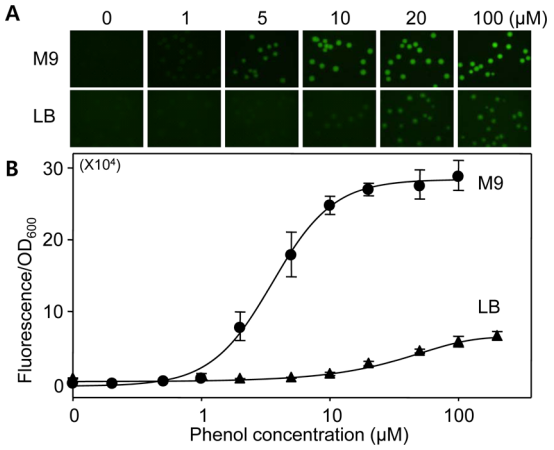

Figure 3. Quantitative response of GESS to phenol. (A) GESS performance is more enhanced in M9-glucose compared to LB medium. Fluorescence microscopy images of cells grown in M9-glucose and LB agar media containing different concentrations of phenol. (B) Specific fluorescence intensity of cells harboring pGESS grown in M9-glucose (closed circles) or LB (closed triangles). Data are the mean ± standard deviation (SD) from three experiments. Note that GESS shows a quantitative response to phenol concentration.

phenol peak using high performance liquid chromatography (HPLC; Figure 4D).

Next, we examined whether GESS could be practically used to identify an enzyme activity from a low copy number genomic library. We attempted to detect the expression of *tpl* from a single copy fosmid library of *Citrobacter freundii*,[18] which contains genomic DNA fragments of approximately 30 kb. The fosmid library cells were transformed with pGESSv4 and spread on LB agar containing 1 mM tyrosine. After incubation for 48 h at 30 °C, several colonies showing strong fluorescence were observed (Figure 4E). Selected candidate colonies were confirmed by PCR of the *tpl* structural gene (Supporting Information Figure S3A). Additionally, fluorescence generated by the selected clones carrying both pGESS and *tpl* in the fosmid was analyzed using flow cytometry (Figure 4F). Fluorescence emission clearly increased compared with that of cells carrying the empty fosmid vector (negative in Figure 4F). Again, the catalytic activity of a selected single clone was confirmed by identifying phenol by HPLC (Supporting Information Figure S3B), indicating that GESS successfully detected the extremely low catalytic activity of enzymes expressed in *E. coli*.

Testing the Genetic Circuit as Generalized Enzyme Screening System. Since DmpR in GESS can be activated by a wide range of phenolic derivatives,[10] we investigated the

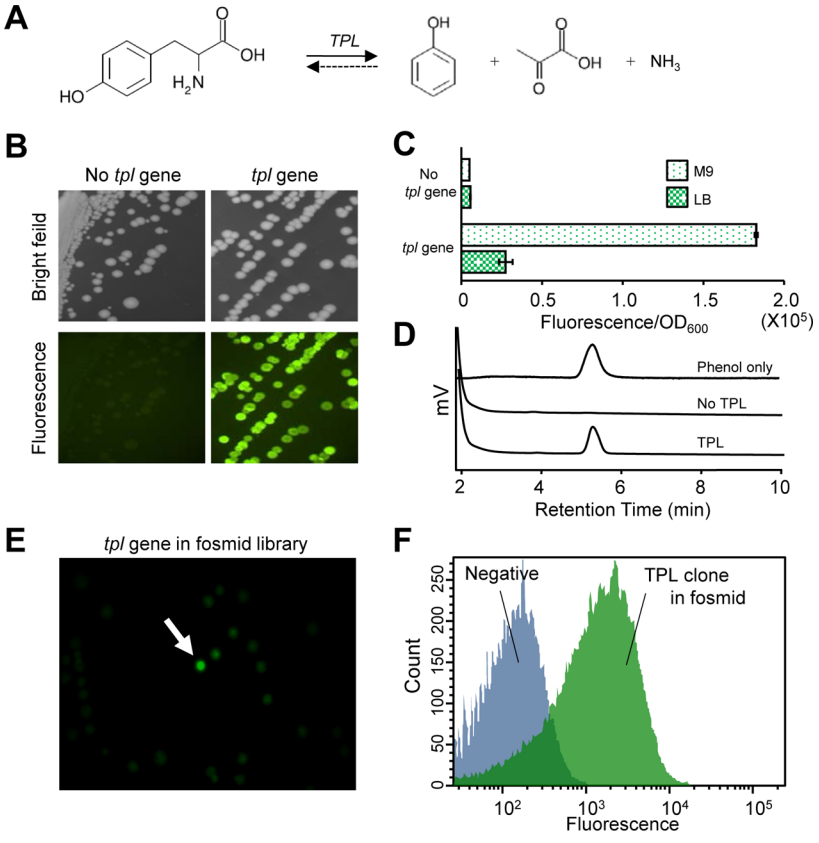

Figure 4. Detection of TPL activity using the GESS system. (A) Reactions catalyzed by TPL in which tyrosine has been degraded to phenol, pyruvate, and ammonium. (B) Fluorescence generated by pSHCE-TPL in cells containing pGESS assessed by fluorescence microscopy. "No *tpl*" indicates cells transfected with pGESS and an empty pSHCE vector. (C) Quantitative fluorescence measurement of cells transfected with pGESS and pSHCE-TPL and grown in M9-glucose or LB medium containing tyrosine (1 mM). Data are the mean ± SD from 3 experiments. (D) HPLC profiles of phenol produced by enzymatic reactions in cell extracts containing tyrosine and TPL. Phenol (10 μM) was injected as a positive control for HPLC analysis. (E) Selection of fluorescent cells from a fosmid library of *Citrobacter freundii* in LB agar medium containing tyrosine. (F) Flow cytometric analysis of a selected *tpl* fosmid clone from a *C. freundii* genomic library using GESS. Cells were grown in M9-glucose containing tyrosine. "Negative" indicates cells harboring pGESS and an empty fosmid.

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ability of 20 different chemicals to induce DmpR transcription (Figure 5A). Among these, 15 phenolic chemicals (not

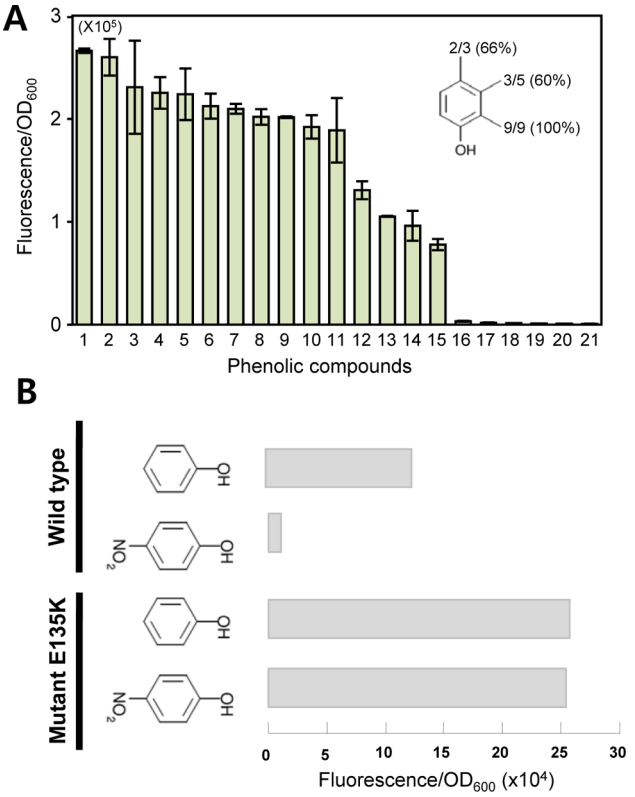

Figure 5. Ligand specificity of the GESS system. (A) Different phenolic compounds detectable by GESS: 1, 2-chlorophenol; 2, phenol; 3, 2-iodophenol; 4, 2-fluorophenol; 5, 2-methylphenol; 6, 2-ethylphenol; 7, 3-methylphenol; 8, 2-nitrophenol; 9, 2-hydroxyphenol; 10, 2-methoxyphenol; 11, 2-aminophenol; 12, 3-chlorophenol; 13, 3-nitrophenol; 14, 4-chlorophenol; 15, 4-methylphenol; 16, 3-hydroxyphenol; 17, 3-ethylphenol; 18, 4-nitrophenol; 19, 2-hydroxybenzoic acid; 20, benzene; or 21, none. Each compound was at 100 μM concentration. All the values represent mean ± SD of three replicate experiments. Numbers marked on phenol structures indicate the counts of phenolic compounds that generate fluorescence among the tested side chain derivatives. (B) Fluorescence intensity of wild type DmpR and mutant DmpR(E135K) treated with 1 mM phenol and 4-nitrophenol. Wild-type DmpR is activated only by phenol, whereas mutant DmpR(E135K) shows strong fluorescence levels in detecting both phenol and 4-nitrophenol. This aspect is different from the DmpR(E135K) reported by Pavel et al. in that their DmpR(E135K) did not show high fluorescence level in phenol detection as much as that of 4-nitrophenol reaction.

including 3-hydroxyphenol, 3-ethylphenol, 4-nitrophenol, 2-hydroxybenzoic acid, or benzene) generated fluorescence in the cells. In particular, phenolic compounds with an *ortho*-substitution induced stronger fluorescence than those with either *meta*- or *para*-substitutions. A variety of 2-nitrophenol- and 4-nitrophenol-tagged substrates are widely available for classical chromogenic enzyme assays. Therefore, these substrates could be used to discover a range of enzymes if GESS was developed to detect both 2- and 4-nitrophenol. As mentioned above, *para*-substituted phenols hardly detected by wild-type DmpR (Figure 5A). However, Pavel et al. reported that the E135K mutant of *Pseudomonas sp.* CF600 DmpR recognized 4-methyl-phenol and other derivatives.[19] Accordingly, we constructed a new plasmid, pGESS(E135K), containing mutant DmpR(E135K) instead of wild-type DmpR, and tested the fluorescence intensity of *E. coli* cells in the presence of 4-nitrophenol or phenol. As shown in Figure 5B, mutant DmpR(E135K) showed a similar intensity of fluorescence with both phenol and 4-nitrophenol.

Using pGESS(E135K), three other enzymes were tested with 4-nitrophenol-generating substrates: a lipase from a soil metagenome, a Cex cellulase from *Cellulomonas fimi*, and a methyl parathion hydrolase (MPH) from a *Pseudomonas* species (Figure 6). The cex gene, encodes β-1,4-exoglycanase, which is capable of hydrolyzing cellulose and xylan as well as a range of soluble aryl glycosides. Therefore, the presence of this enzyme generates *p*-nitrophenol from *p*-nitrophenyl-cellobioside as a substrate. Lipase and MPH were also assumed to generate 4-nitrophenol as a common product from 4-nitrophenyl butyrate and methyl parathion, respectively (Figure 6A). In all the cases, the fluorescence levels of cells expressing these enzymes increased compared to the cells without the enzyme. These results demonstrated that different enzymes could be visualized via the fluorescence of pGESS(E135K) in the presence of 4-nitrophenol-generating substrates (Figure 6B). In addition to the *in vivo* GFP detection, changes in levels of 4-nitrophenol, an enzymatic product measured using traditional spectrophotometry, were similar to those obtained using GESS (Figure 6C). However, in terms of detection sensitivity, GESS showed higher performance than the spectrophotometric assay. For example, detection of *p*-nitrophenol by *E. coli* cells with pGESS(E135K) was nearly 100 fold more sensitive than the direct detection of *p*-nitrophenol in 96-microtiter plates using VICTOR V (Supporting Information Figure S4). These findings suggested that diverse enzyme genes can be screened using mutant DmpR(E135K) with the 4-nitrophenol-tagged substrates with high sensitivity.

High-throughput Screening from a Metagenome Library. A metagenome is a potentially huge reservoir of valuable enzymes that could be targeted by GESS. In order to construct a metagenome library, our samples were taken from tidal flat sediments that included diverse ecosystems, and phosphatases that could be employed in molecular cloning and for immunological detection were chosen as target enzymes for the GESS application. Cells harboring the metagenomic fosmid library were transformed with pGESSv4. To remove false positives, 10^6 nonfluorescent cells cultured in LB medium without the addition of any phenolic substrate were collected using a FACSAria flow cytometer (Figure 7A). Collected cells were then regrown in LB medium containing phenyl phosphate. Note that the M9-glucose medium was not used for alkaline phosphatase screening, since, in the presence of phenyl phosphate, cells generates strong background fluorescence that result in false positive signals (Supporting Information Figure S5). This is possibly because the *E. coli phoA* gene encodes an alkaline phosphatase that is highly expressed under starvation and stationary phase conditions.[21] As shown in Supporting Information Figure S5, a phoA knockout strain of *E. coli* was not applicable likely due to the presence of other phosphatase genes such as *aphA* and *umpG* in the *E. coli* genome.[20]

Of the 10^6 cells grown for 16 h in LB medium containing phenyl phosphate, 47 highly fluorescent cells were sorted using FACSAria (Figure 7B) and spread on LB agar medium containing phenyl phosphate. After incubation for 36 h at 30 °C, colonies showing high levels of fluorescence were selected by fluorescence microscopic analysis (indicated by the arrow in

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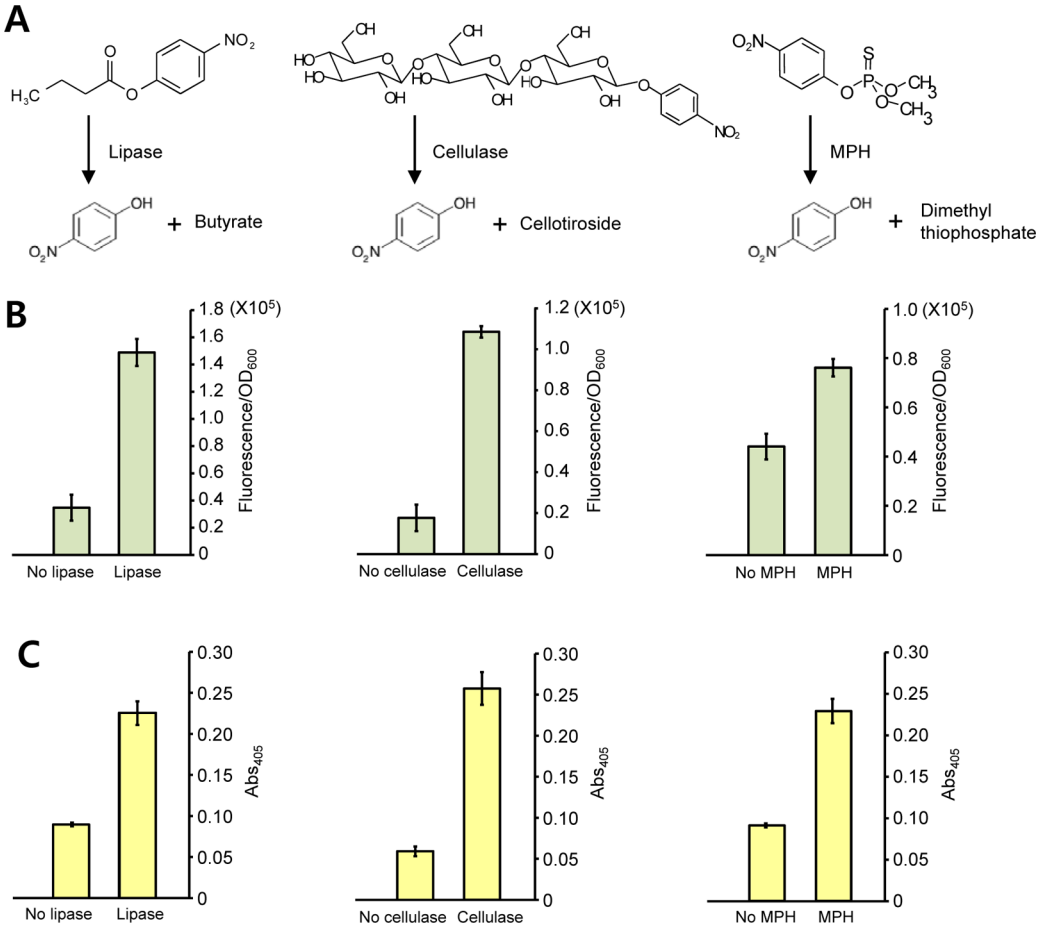

Figure 6. Quantitative detection of different enzyme activities using the GESS system. (A) Enzymatic reactions of lipase, cellulase, and methyl parathion hydrolase (MPH) generating 4-nitrophenol as a common product in the presence of 4-nitrophenol-tagged substrates (4-nitrophenyl butyrate, 4-nitrophenyl cellotrioside, and methyl parathion). (B) Quantitation of the fluorescence generated by cells harboring pGESS(E135K) and lipase, cellulase, or MPH genes grown in M9-glucose containing 4-nitrophenol-tagged substrates. "No lipase", "No cellulase", or "No MPH" indicates fluorescence generated by cells harboring pGESS(E135K) and empty vector under the same conditions. Values from three replicates are shown as mean ± SD (C) Spectrophotometric measurement of 4-nitrophenol generated from 4-nitrophenol-tagged substrates in cell extracts obtained from cells carrying pGESS(E135K) and the lipase, cellulase, or MPH genes and grown in LB broth. Values represent mean ± SD of triplicates.

Figure 7C). Flow cytometric analysis also revealed that the selected cells showed stronger fluorescence signals than cells carrying the empty fosmid vector (Figure 7C).

To confirm phosphatase activity, a shotgun library (37 kb insert length) of the fosmid was constructed as described in Methods section and reintroduced into *E. coli* DH10B cells with pGESS. Several selected fluorescent colonies grown on LB agar medium containing phenyl phosphate were streaked on LB agar medium containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as a colorimetric substrate. Blue colonies indicated that the selected cells contain metagenome genes encoding a phosphatase within a plasmid vector, pSTV28 (Figure 7D). DNA sequencing resulted in the identification of a phosphatase gene comprising 1689 nucleotides and encoding 563 amino acids. Sequence homology searches against the non-redundant NCBI database (http://www.ncbi.nlm.nih.gov) using BLASTP showed that the isolated phosphatase was closely related (59% homologous at the amino acid level) to an alkaline phosphatase from *Sphingomonas sp.* (Supporting Information Figure S6). Thus, we confirmed that GESS allows the rapid identification of novel enzyme genes from metagenomes.

Perspectives for a Generalized and High-throughput Screening System. Transcriptional regulator-based genetic screening methods have received much attention as novel enzyme selection systems, because they enable the rapid screening of libraries comprising innumerable genetic variants.[22] However, there is a limitation in that only a few of the specific enzymes corresponding to the regulators are detectable. In this regard, the GESS used in this study along with the phenol-responsive transcription regulator DmpR and its mutant DmpR(E135K) suggests a system that could be applied to a wide range of natural enzymatic reactions that produce phenolic compounds. Phenol is neither synthesized nor metabolized in a native *E. coli* cell.[14] Therefore, we anticipated that GESS would quantitatively and sensitively respond to phenol *in vivo*. Indeed, this system detected low concentrations of phenol in the range 1−20 μM in M9 medium (Figure 3), suggesting that GESS could detect very low levels of enzyme activity at the single cell level. For instance, an enzyme with low catalytic activity (e.g., *C. freundii* TPL, $k_cat < 2s^{-1}$)[23] was detected in a genomic fosmid library of *C. freundii* (Figure 4E, F). Interestingly, we also observed that several colonies surrounding a single *tpl*-positive colony showed marginal levels of fluorescence (Figure 4E), possibly caused by the diffusion of phenol in the agar media. This phenomenon suggests that the

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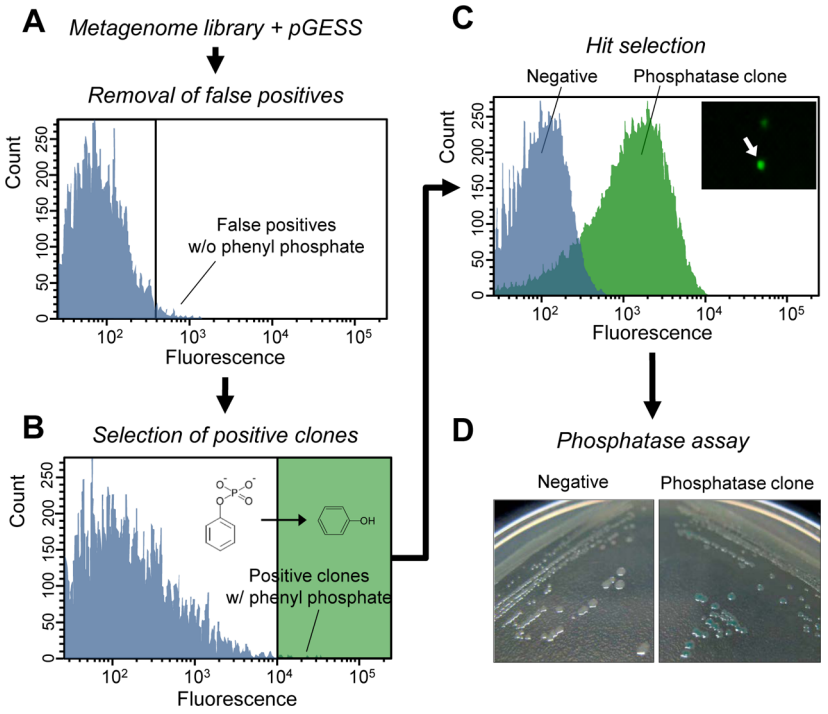

Figure 7. High-throughput screening of phosphatases from metagenome library. (A) Removal of false-positive cells harboring a metagenome fosmid library and pGESS showing fluorescence in LB broth without phenyl phosphate as the phenol-generating substrate. (B) Sorting of positive clones showing high fluorescence intensity (green). Cells were grown in LB broth in the presence of phenyl phosphate (1 mM). (C) Selection of a strongly fluorescent colony on LB agar containing phenyl phosphate and a flow cytometric histogram generated for this clone. "Negative" indicates cells harboring pGESS and an empty fosmid. (D) Phosphatase assay of cells harboring pGESS and a phosphatase gene in a plasmid vector (pSTV28) on LB agar containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the chromogenic substrate.

GESS system could be used to design a cell-to-cell signaling circuit based on the diffusion of enzyme products.

To utilize this system more widely, we constructed the plasmid pGESS(E135K), which sensitively responded to 4-nitrophenol-tagged compounds. As shown in Figure 6, the activities of various enzymes (i.e., lipase, cellulose, and MPH) that were not detectable using wild-type DmpR were detected with DmpR(E135K). Moreover, the insertion of optimized RBS and terminator sequences into pGESS dramatically improved the sensitivity of the system. Taken together, these results indicated that GESS could be engineered further to detect diverse array of enzymes and could be expanded to include more synthetic substrates.

It is important to note that our new screening platform depends on expensive FACS analysis and there is still a clear need for the use of colorimetric colony screening assays, due to their simplicity and cost effectiveness. However, such colorimetric colony screenings are only applicable to enzymes with a known chromogenic substrate. Furthermore, quantitative analysis tools in these assays are quite limited. By comparison, our system has a wide applicability toward a variety of enzymes lacking chromogenic substrates and is capable of high-throughput screening assays with high sensitivity and straightforward quantification. As well, by replacing EGFP with alternative reporters such as antibiotic resistance genes or chromogenic enzymes, it should be possible to use GESS without the need for expensive FACS analysis.

In conclusion, we have developed a practical and useful genetic circuit that turns enzyme activity into *in vivo* fluorescence intensity. The exceptional sensitivity, high-throughput, and analytical properties of GESS enabled us to detect diverse enzymes from the metagenomes. We envision that the quantitative sorting of fluorescent cells along with mathematical/statistical analyses will further allow the development of a plug-and-play platform for the evolutionary engineering of important industrial enzymes in the future.

METHODS

Materials. All chemicals were purchased from Sigma-Aldrich (Saint Louis, MO), with the exception of 4-nitrophenyl cellotrioside, which was purchased from Santa Cruz Biotechnology (Paso Robles, CA). The *gfp* gene was obtained from the plasmid pMGFP[13] by digestion with *Eco*RI and *Hind* III and ligated into pUC19 (Novagen, CA) to yield pUC19-gfp. DNA fragments containing the DmpR gene (encoding a phenol-binding transcriptional activator) and a σ54-dependent promoter (P_o) were amplified by PCR using the genomic DNA of *Pseudomonas putida* KCTC 1452 as a template and primers 5′-CCGGAATTCGAGCTGATCGAAAGTCGG and 5′-CCGGAATTCCTAGCCTTCGATGCCGAT (*Eco*RI site underlined). PCR fragments were cloned into the *Eco*RI site of pUC19-gfp to yield the plasmid pGESS. The E135K mutant of *dmpR* was derived using the QuickChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) and primers 5′-GATCGACTCCTTCGAGGTGAAAATCTGCCAGACC-GACCTG and 5′-CAGGTCGGTCTGGCAGATTTTT-CACCTCGAAGGAGTCGATC (nucleotide sequences for K135 underlined) to yield plasmid pGESS(E135K).

Tyrosine Phenol-lyases. The *tpl* gene from *Symbiobacterium toebii* was digested from pHCEIIB-TPL[24] and ligated into the *Hin*dIII and *Nde*I sites of pSHCE to yield pSHCE-TPL. A constitutive HCE promoter and the multicloning sites of pHCEIIB (Takara, Japan) were digested and ligated into the

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*Cla*I and *Tth111*I sites of pSTV28 to yield pSHCE. *E. coli* DH5α cells carrying both pGESS and pSHCE-TPL were grown in LB agar medium containing tyrosine (1 mM) and pyridoxal-5-phosphate (PLP; 10 μM) at 30 °C for 18 h. Forsmid cloning of the *tpl* gene of *Citrobacter freundii* was done using the CopyControl Fosmid Library Production Kit (Epicenter Biotechnologies, Madison, WI). The diversity of the fosmid library was estimated to be 6.5×10^3 clones. pGESS was transformed into *E. coli* EPI300 cells carrying the genomic fosmid library by electroporation and transformants were plated onto LB agar medium containing tyrosine (1 mM) and PLP (10 μM) at 30 °C for 48 h. Strong fluorescent colonies were selected using an AZ100 M fluorescence multizoom microscope (Nikon, Tokyo, Japan) equipped with a GFP filter (455−485 nm excitation, 500−545 nm emission). To confirm whether the *tpl* gene was present in candidate cells, colony PCR was performed using the *tpl* gene-specific primers 5′-GATCCATATGAATTATCCGGCAGAACCC and 5′-GATCAAGCTTTTAGATATAGTCA AAGCGTGC.

Lipase. A lipase gene in a CopyControl fosmid was isolated from a soil metagenome using a tributyrin plate assay. To measure the cellular fluorescence generated in response to lipase activity, *E. coli* EPI300 cells harboring pGESS(E135K) and a fosmid carrying a lipase gene were grown in M9-glucose broth containing CopyControl induction solution, and 4-nitrophenyl butyrate (100 μM) at 30 °C for 16 h.

Cellulase. The *cex* gene encoding a cellulase was amplified from the genomic DNA of *Cellulomonas fimi* KCTC 9143 using primers 5′-CCCCATATGGCGACCACGCTCAAGGAGGC and 5′-CCGCTCGAGGCTCGCGCCGAAGG CCTC (*Nde* I and *Xho* I sites underlined) and inserted into the *Nde* I and *Xho* I sites of pET21a (Invitrogen, CA) to yield pET-Cex. *E. coil* BL21(DE3) cells carrying pET-Cex and pGESS(E135K) were grown in M9-glucose broth containing IPTG (0.5 mM) and 4-nitrophenyl cellotrioside (100 μM) at 30 °C for 16 h and the cellular fluorescence generated by cellulase activity was measured in a fluorescence plate reader.

MPH. Plasmid pPRO-MPH, which contains the *mph* gene encoding methyl parathion hydrolase cloned in plasmid pPROLar (Invitrogen, Carlsbad, CA), was a kind gift from Prof. Hak-Sung Kim, KAIST, Deajeon, Korea. *E. coli* DH5α cells harboring pPRO-MPH and pGESS(E135K) were grown in M9-glucose broth containing IPTG (0.5 mM) and methyl parathion (100 μM) at 30 °C for 16 h. Cellular fluorescence generated by MPH activity was measured using a fluorescence plate reader (Victor V, Perkin-Elmer, Waltham, MA) with an EGFP filter set (488 nm excitation, 530/20 nm emission).

Metagenomic Screening. Metagenomic DNAs (approximately 30 kb) from tidal flat sediments in Taean, Korea were obtained using a hydroshear machine (GeneMachines, Taunton, MA). A metagenomic DNA fosmid library was constructed in *E. coli* EPI300 cells using the CopyControl Fosmid Library Production Kit. The diversity of the metagenomic library was estimated to be 2×10^4 clones. pGESS was transformed into *E.coli* EPI300 cells carrying the metagenomic library by electroporation. The resulting library cells were grown in LB broth containing ampicillin (50 μg/mL), chloramphenicol (12.5 μg/mL) and CopyControl induction solution at 30 °C for 12 h prior to FACS. False-positive cells were removed from the library using a FACSAria (BD Bioscience, Franklin Lakes, NJ). A blue laser (488 nm) and a BP filter (530/30 nm) were used to analyze EGFP fluorescence. Cell culture broth was diluted 1/100 with PBS buffer for FACS analysis. Forward and side scatter were used to exclude debris and dead cells. Nonfluorescent cells (10^6 cells) were collected and grown in LB broth containing phenyl phosphate (1 mM) at 30 °C for 16 h. Strongly fluorescent cells were sorted and spread onto LB agar plates containing phenyl phosphate. After incubation at 30 °C for 36 h, a single colony showing strong green fluorescence was selected on the LB agar plates. Finally, a fosmid (about 30 kb) extracted from the selected phosphatase clone was digested with *Bam*HI and inserted into the pSTV28 vector to yield a shot-gun library in *E. coli* DH10B cells carrying pGESS. The shotgun library cells were streaked and grown on LB agar containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 20 μg/mL) as the chromogenic substrate at 37 °C for 18 h to confirm cellular phosphatase activity. Then the colonies exhibiting blue color were selected and confirmed by DNA sequencing. The nucleotide sequence of a novel phosphatase from the metagenome was deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession number GQ250428. Multiple amino acid sequence alignment were performed using ClustalW (DNASTAR, Madison, WI).

Analysis. *Fluorescence Microscope.* *E. coli* DH5α cells harboring pGESS were grown in M9 plus glucose (0.4%) or LB agar medium containing different concentrations of phenol (1, 5, 10, 20, and 100 μM) and ampicillin (50 μg/mL) at 30 °C for 36 h. Colonies were observed by fluorescence microscope as described above.

Fluorescence Plate Reader. Fluorescent cells cultured in broth at 30 °C for 16 h were analyzed using a fluorescence plate reader Victor V. For the calculation of the specific cellular fluorescence, the optical density was obtained by measuring the absorbance at 600 nm in a UV/visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

High Pressure Liquid Chromatography (HPLC). To measure tyrosine phenol-lyase (TPL) activity, *E. coli* DH5α cells carrying pSHCE-TPL and pGESS were grown in LB broth at 37 °C for 18 h, harvested by centrifugation at 5000 rpm for 10 min, and resuspended in PBS (pH 7.4). Cell extracts (1 mg protein/mL) were prepared by sonication and subsequently mixed with a reaction buffer containing 100 mM potassium phosphate (pH 8.0), 1 mM tyrosine, and 100 μM PLP (pyridoxal-5-phosphate) at 37 °C for 24 h. Phenol formed in the reaction mixture was analyzed using an HPLC instrument (SCL-10A vp, Shimadzu, Japan) fitted with a C_{18} reverse column (C/N.18R03, Chemco Pak, Japan), with a mobile phase comprising a mixture of acetonitrile and water (50:50) at room temperature. In addition, the TPL activity of a single clone selected from the genomic library of *C. freundii* was also analyzed by HPLC.

pNP Colorimetric Assay. *E. coli* cells expressing lipase, cellulase or MPH were grown in LB broth containing CopyControl induction solution or IPTG (0.5 mM) at 37 °C for 18 h. Cells were harvested by centrifugation at 5000 rpm for 10 min and resuspended in PBS (pH 7.4). Individual cell extracts were prepared by sonication. The catalytic activity of lipase (0.012 mg), cellulase (0.18 mg) and MPH (0.13 mg) in each cell extract was determined based on the amount of 4-nitrophenol released from 4-nitrophenyl butyrate (500 μM), 4-nitrophenyl cellotrioside (200 μM), and methyl parathion (200 μM), respectively. The enzyme reaction was carried out at 37 °C for 2 h in 0.2 mL of 100 mM potassium phosphate buffer (pH 8.0) and terminated by adding Na_2CO_3 (0.5 mM).

Absorbance at 405 nm was read using a Victor V microplate reader.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures as described in the text. This material is available free of charge *via* the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: sglee@kribb.re.kr.

Present Address

[∇]Amicogen, 694-4 Sangchon-dong, Jinju-si, South Korea

Author Contributions

[○]S.L.C. and E.R. contributed equally to this work.

Author Contributions

S.L.C. and E.R. are cofirst authors. S.L.C., E.R., and S.J.L. designed and performed experiments and wrote the manuscript. H.K. revised the manuscript and prepared it for submission. G.K. and Y.S.J. performed HPLC and imaging experiments and analyzed data. Y.H.R., J.J.S., and H.S.K. conceived the research and assisted in research design and data interpretation. S.G.L. conceived research, analyzed data, and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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