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Systematic screening of *Escherichia coli* single-gene knockout mutants for improving recombinant whole-cell biocatalysts

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Abstract Systematic screening of single-gene knockout collection of *Escherichia coli* BW25113 (the Keio collection) was performed to select mutants that could enhance the deethylation of 7-ethoxycoumarin catalyzed by CYP154A1. After 96-well plate high-throughput screening followed by test tube assays, four mutants (Δ*cpxA*, Δ*gcvR*, Δ*glnL*, and an unknown-gene-deleted one (Δ*uk*)) were able to increase the CYP154A1 activity by approximately 1.4–1.7 times compared with that of the control strain. When new mutants were constructed by disrupting individually the *cpxA, gcvR, glnL,* and *uk* genes in *E. coli* BW25113, three of them (Δ*cpxA*, Δ*gcvR*, and Δ*glnL*) showed high levels of CYP154A1 activity. However, the *uk*-disruptant failed to enhance the CYP154A1 activity, suggesting that the high CYP154A1 activity of the Δ*uk* mutant in the Keio collection was due to a spontaneous mutation in the chromosome. In-frame deletion mutants of Δ*cpxA*, Δ*gcvR*, and Δ*glnL* also exhibited high enzyme activity, and complementation of these mutations could decrease CYP154A1 activity. These results indicated that the enhancement of the enzyme activity was not caused by polar effects on their neighbor genes. To our knowledge, this is the first report on a genome-wide screening of the genes for deletion to improve the activity of a recombinant whole-cell biocatalyst.

Keywords Systematic screening · Keio collection · Whole-cell biocatalysis · Cytochrome P450 monooxygenase

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Introduction

Biocatalysis has matured to a standard technology in the fine chemicals industry, which is reflected by the number of biotransformation processes running on a commercial scale (Straathof et al. 2002). Both isolated enzymes and whole cells have been used as industrial catalysts in commercial biotransformations. Compared with isolated enzymes, whole-cell biocatalysts can be cost-effectively prepared. Whole-cell biocatalysis is particularly advantageous for bioproductions in which cofactors for oxidative and reductive reactions are required, since metabolically active cells can recycle cofactors with existing machinery (Cirino and Sun 2008; Duetz et al. 2001; Goldberg et al. 2007; Ishige et al. 2005). In addition, recombinant DNA techniques have allowed the overproduction of a desired enzyme in various heterologous hosts and the use of cells as "bags stuffed with catalysts".

The activity of a whole-cell catalyst may be affected by cellular components involved in substrate transport, product accumulation, energy metabolism, and cofactor regeneration. Fujii et al. (2009) have found that disruptions in the AcrB-Tol efflux pump system of *Escherichia coli* strains could increase the intracellular amounts of substrates and the concentrations of active enzymes. By using *E. coli* strains with *tolC acrAB* mutations expressing cytochrome P450 monooxygenase (P450) genes, the production levels of pravastatin, 25-hydroxy vitamin D_3, and 25-hydroxy 4-cholesten 3-one were enhanced substantially. Ni and Chen (2005) have reported that a lipoprotein mutation altered the outer membrane structure of *E. coli* and enhanced the membrane permeability to hydrophobic substrates. When toluene dioxygenase was expressed in this mutant, the bioconversion of toluene to *o*-cresol was more effective than in the wild-type strain. Xie et al. (2007) have identified that

BioH, a carboxylesterase involved in the biotin biosynthesis, could hydrolyze dimethylbutyryl-*S*-methyl mercaptopropionate during the production of simvastatin acid using *E. coli* as a whole-cell catalyst. A Δ*bioH* strain was effective in eliminating the degradation of dimethylbutyryl-*S*-methyl mercaptopropionate and significantly increased the product yield.

Recently, the collection of single-gene knockout (SGK) mutants of *E. coli* K-12 (the Keio collection) has been constructed (Baba et al. 2006) and used for identifying unknown gene functions (Melnick et al. 2004) and for analyzing the dynamics of metabolic pathways (Hua et al. 2003; Yang et al. 2003; Zhao et al. 2004). The Keio collection has also been successfully used to substantiate the value of systematic approaches for the understanding of the linkage between different cellular systems (Eydallin et al. 2007; Inoue et al. 2007; Ito et al. 2005; Niba et al. 2008; Perrenoud and Sauer 2005; Tenorio et al. 2003). However, to the best of our knowledge, there has been no report on systematic screening of SGK mutants to identify cellular components that affect the activity of a recombinant whole-cell catalyst.

In this study, we designed a method to systematically screen SGK mutants that can enhance the activity of a recombinant whole-cell catalyst using the Keio collection. The deethylation of 7-ethoxycoumarin, a model reaction used in numerous P450 studies, was employed. The oxidative *O*-dealkylation of coumarins can be catalyzed by a number of mammalian and bacterial P450 enzymes. Here, CYP154A1, a P450 derived from *Streptomyces coelicolor* A3(2) was used. Most prokaryotic P450s require specific redox partners, ferredoxin and ferredoxin reductase, for electron transfer, and NAD(P)H as a cofactor. Therefore, P450s are usually used in metabolically active cells for industrial purpose and are likely the most suitable model enzymes for exploring cellular components that affect the activity of a recombinant whole-cell catalyst.

In this article, systematic screening of SGK collection of *E. coli* BW25113 transformed with a plasmid harboring the CYP154A1 gene with respect to their activity towards 7-ethoxycoumarin was performed. Three *E. coli* mutants, including Δ*cpxA*, Δ*gcvR*, and Δ*glnL*, exhibited 1.4–1.7 times higher biotransformation activity compared with that of the wild-type *E. coli* strain. We excluded the polar effect of the introduced kanamycin resistance gene cassette on the expression of downstream genes by construction of in-frame deletion mutants of Δ*cpxA*, Δ*gcvR*, and Δ*glnL*. We also confirmed that the observed enhanced activities truly depends on the respective deleted genes by construction of new deletion mutations and observing complementation by wild-type genes on a low copy vector. The systematic approach described here would enable the rapid identification of genes responsible for the desired phenotypes and contribute to the breeding of improved microbial catalysts.

Materials and methods

Materials, strains, and plasmids

E. coli BW25113, the Keio collection, and helper plasmids (pKD46 and pCP20) were described previously (Baba et al. 2006). 7-Ethoxycoumarin, 7-hydroxycoumarin, and 5-aminolevulinic acid were purchased from Wako Pure Chemical (Osaka, Japan). DNA polymerase, restriction enzymes, and other DNA-modifying enzymes were purchased from Takara Bio (Shiga, Japan). All other reagents are commercially available and of analytical grade. A P450 expression vector pET-*cyp154a1-camAB*, carrying the *S. coelicolor* A3(2) *cyp154a1* and the *Pseudomonas putida camA* and *camB*, was described previously (Agematsu et al. 2006). A single-copy vector, pBAC-Lac (Asakawa et al. 1997), was a gift from Dr. S. Asakawa of Keio University.

Construction of CYP154A1 expression vector

The *cyp154a1* and *camAB* were amplified from pET-*cyp154a1-camAB* with the primers CYP-P1 and CYP-P2 shown in Table 1. The PCR product was gel-purified, digested with *Eco*RI, and ligated to the corresponding site of the expression vector pKK223-3 (Brosius and Holy 1984). The nucleotide sequence was analyzed to confirm the direction of inserted genes and to verify the absence of PCR mutations. The resulting plasmid was designated as pKK-CYP-camAB.

Transformation of SGK mutants

The transformation of 3,985 individual SGK mutants with pKK-CYP-camAB was carried out in 96-well plates (U96 DeepWell™, Nunc International, Tokyo, Japan) in accordance with the method described by Hanahan (1983). SGK mutants, which had been kept as glycerol stocks at −80°C, were inoculated to 500 μl of Luria-Bertani (LB) medium containing kanamycin (20 μg/ml) in a 96-well plate. The plate was sealed with a Breathe-EASIER™ sealing film (Diversified Biotech, Boston, MA) for cultivation. The cells were precultured aerobically at 30°C for 24 h with orbital shaking (1,300 rpm) on a plate shaker (Mix-EVR, TAITEC, Tokyo, Japan). An aliquot (10 μl) of the fully grown culture was then inoculated to 500 μl of SOB medium (Hanahan 1983) in a 96-well plate and incubated at 30 °C with shaking to the mid-log phase of growth. The cells were collected by centrifugation at 3,000×*g* for 10 min at 4°C. After removing the supernatant, the cells were resuspended

Table 1 PCR primers used in this study

Primer	Sequence (5′ to 3′)
CYP-P1	TTGAATTC [a] ATGGCGACCCAGCAGCCCGC
CYP-P2	GGGAATTC [a] TTACCATTGCCTATCGGGAA
cpxA-SD-P1	AGCTAGC[b]GGAGTGCATGCATGATAGGCAGCTTAACCGC
cpxA-SD-P2	TAAGCTT[c]TTAACTCCGCTTATACAGCGG
gcvR-SD-P1	AGCTAGC[b]GGAGTGCATGCATGACACTGTCATCGCAACATTAT
gcvR-SD-P2	TAAGCTT[c]TTACTTAACTCCATCCTGTTCATC
glnL-SD-P1	AGCTAGC[b]GGAGTGCATGCATGGCAACAGGCACGCAG
glnL-SD-P2	TAAGCTT[c]TTATTTCCTGATAGGCAGGTAAAC
cpxA-P1	CTCTATTTGCTGGCACAGCATC
cpxA-P2	GTTATCGAAAAGCTGGACGC
gcvR-P1	CAATCGCGACAATACTTCCCGTGAA
gcvR-P2	ATCCATGTTATCGCGTAAGCCGCAG
glnL-P1	TACACGGTGATGATGTGGTAGGC
glnL-P2	ATATCTGAAAGCAGCACATCCGGCG
cpxA-F	ACACACCAGACGCCTGTCATTAT
gcvR-F	ACAAACCTTCTTGCGACGGA
glnL-F	AGAAGCGAAAGAGATCCCACAG
Km-R	AGTTCATTCAGGGCACCG
Km-F	TCCTGCCGAGAAAGTATCCATC
cpxA-R	ATGGGCGATATTTTCCGCTG
gcvR-R	TTCTTTCAGCCAGTTCAGCAC
glnL-R	TGACGGCAGCATCCAGAT

[a] *Eco*RI restriction sites are underlined

[b] *Nhe*I restriction sites are underlined

[c] *Hin*dIII restriction sites are underlined

in 170 μl of ice-chilled transformation buffer (TB) (Hanahan 1983) and kept on ice for 15 min. The cells were then collected by centrifugation and resuspended in 40 μl of TB buffer containing 50 pg of pKK-CYP-camAB. The plate was kept on ice for 30 min and soaked in a waterbath at 42°C for 1 min. Then, 960 μl of fresh SOC medium (Hanahan 1983) was added to each well, and the plate was incubated at 37°C for 1 h. An aliquot of the mixture (10 μl) was transferred to 400 μl of LB medium supplemented with ampicillin (50 μg/ml) and kanamycin (20 μg/ml). The culture was incubated overnight at 30°C with shaking. The overnight-grown cultures were mixed with an equal volume of glycerol and stored at −80°C.

High-throughput screening

An autoinduction medium was prepared using the Overnight Express™ Auto Induction System (Novagen, Madison, WI) in accordance with the manufacturer's protocol. The glycerol stock of a transformant was inoculated with a sterilized toothpick to 400 μl of LB medium containing ampicillin (50 μg/ml) and kanamycin (20 μg/ml). Cultivation was carried out in a 96-well plate with orbital shaking (1,300 rpm) for 24 h at 30°C. An 8-μl aliquot of the preculture was then inoculated to 400 μl of the autoinduction medium containing 5-aminolevulinic acid (80 μg/ml), ampicillin (50 μg/ml), and kanamycin (20 μg/ml) in a 96-well plate. Further cultivation was carried out at 30°C with shaking for 24 h. The cells were harvested by centrifugation (3,000×*g*, 10 min, 4°C) and suspended in 400 μl of a reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.4), 2% (*w*/*v*) glycerol, 50 μg/ml ampicillin, 0.1 mM IPTG, and 100 μM 7-ethoxycoumarin. The reaction was carried out at 30°C with shaking (1,300 rpm) on the plate shaker. After 24 h of incubation, the reaction mixture was centrifuged to remove bacterial cells. The supernatant was subjected to high performance liquid chromatography (HPLC) analysis on a COSMOSIL $5C_{18}$–AR-II Waters column (4.6 mm×50 mm, Nacalai Tesque, Japan). The column was eluted by a linear gradient of methanol (30–100 vol.%) in 0.1 vol.% orthophosphate at a flow rate of 1 ml/min. The eluates were detected using an SPD-20A UV/VIS detector (Shimadzu, Kyoto, Japan) at 325 nm. *E. coli* BW25113 (pKK-CYP-camAB) and *E. coli* BW25113 (pKK223-3) were used as positive and negative controls, respectively.

Test tube CYP154A1 assay

An overnight-grown culture of an *E. coli* transformant (40 μl) was inoculated into a test tube (180×18 mm) containing 2 ml of the autoinduction medium supplemented with 5-aminolevulinic acid (80 μg/ml) and adequate antibiotics (50 μg/ml ampicillin, 20 μg/ml kanamycin, and/or 20 μg/ml chloramphenicol). The cells were grown at 30°C with

reciprocal shaking (200 rpm) for 12 h. After the incubation, 1 ml of the culture was sampled from the test tube. The cells were harvested by centrifugation, transferred to a test tube containing 2 ml of the reaction mixture for enzyme assay, and incubated at 30°C with reciprocal shaking (200 rpm). After 24 h of incubation, an aliquot of the reaction mixture was centrifuged to obtain the supernatant for HPLC analysis. The test tube culture was also adequately diluted with 50 mM potassium phosphate buffer, and the optical density at 600 nm (OD_{600}) was measured using a UV150-02 spectrophotometer (Shimadzu). An OD_{600} of 1.0 corresponded to 0.61 mg dry cells per milliliter. Total enzyme activity was expressed as picomoles of 7-hydroxycoumarin produced per hour per milliliter of the culture. Specific activity was calculated from total activity divided by milligram dry cells of 1-ml culture. At least three independent cultures were subjected to CYP154A1 assay.

Gene disruption

The genes encoding CpxA, GcvR, GlnL, and an unknown (*uk*) gene were disrupted in accordance with the method described by Datsenko and Wanner (2000). The *uk* gene was located at a nucleotide position ranging from 1,079,456 to 1,079,647 on the chromosomal DNA of *E. coli* K-12 W3110 (Riley et al. 2006). Although this gene was formerly named *yneE* encoding a possible protein, it has been eliminated from the list of ORFs in the re-annotation published in 2006 (Riley et al. 2006). The chromosomal DNA of the respective disruptant in the Keio collection was used as the PCR template to amplify a kanamycin resistance (km^r) gene, which was flanked by Flippase (FLP) recognition target sites and approximately 200 bp of the adjacent chromosomal sequences. An adequate set of primers listed in Table 1 (P1 and P2 primers) were used as the PCR primers. The PCR product was introduced by electroporation into *E. coli* BW25113 carrying a Red helper plasmid pKD46, and km^r transformants were selected. The Red helper plasmid was removed by growing the transformant at 43°C. Replacement of a target gene with the km^r-gene was confirmed by colony PCR. Primers designed according to the km^r-gene cassette were used as common primers. Two PCRs were conducted using locus-specific primers approximately 500 bp upstream and downstream of the target gene (F and R primers in Table 1) with the respective common primers.

Excision of the km^r-gene cassette was performed using a helper plasmid pCP20, as described by Datsenko and Wanner (2000), to generate an in-frame deletion mutant. Excision of the cassette was confirmed by PCR using respective F and R primers (Table 1).

Double-knockout strains were constructed by disrupting the second gene of the in-frame deletion strain of the first gene disruptant using the abovementioned method.

Construction of complementary plasmids

The *araC*-P_{araBAD}-MCS region of the plasmid pBAD33 (Guzman et al. 1995) was prepared by digestion with *Cla*I and *Hin*dIII. This DNA fragment was inserted into the corresponding sites of the pBAC-Lac plasmid to construct pBAC-CM. The genes encoding CpxA, GcvR, and GlnL were amplified from the genomic DNA of *E. coli* strain BW25113 by PCR using primers SD-P1 and SD-P2 in Table 1. The amplified DNA fragments were first cloned into the *Eco*RV site of the pBlueScriptII SK(+) plasmid and sequenced. After digesting the resulting plasmids with *Nhe*I and *Hin*dIII, the individual cloned genes were inserted into pBAC-CM to make expression vectors. The expression vectors were then transformed into in-frame deletion mutants. For complementary tests, 1 mM L-arabinose and 12 μg/ml chloramphenicol were added to the autoinduction medium.

Results

SGK mutants with enhanced CYP154A1 activity

When 96 randomly chosen mutants were inoculated to a 96-well plate, approximately 70–80% of the mutants could be simultaneously transformed with pKK-CYP-camAB. Untransformed mutants were subjected to another trial. By repeating this process, 3,978 SGK mutants could be transformed with pKK-CYP-camAB. Seven SGK mutants failed to be transformed, since they hardly grew in SOB medium at 30°C.

The 3,978 mutants were examined for their ability to produce 7-hydroxycoumarin in 96-well plates. Deethylation of 7-ethoxycoumarin has been widely employed as a model P450 reaction in various studies, since 7-hydroxycoumarin could be easily quantified by fluorescence measurement. However, since background fluorescence was not negligible in the 96-well plate assay, the reaction product was quantified by HPLC. The total activity of the wild-type strain harboring pKK-CYP-camAB (positive control) reached a maximum at approximately 24 h on the 96-well plate, while no significant activity was detected with that harboring pKK223-3 (data not shown). After 24 h of incubation, 81 SGK mutants were found to exhibit total enzyme activities of more than two-fold greater than that of the wild-type (Fig. 1). It was also observed that some mutants had a distinctly low CYP154A1 activity.

The 81 SGK mutants were subjected to test tube assays. Aside from enzyme activity, bacterial growth was also monitored in test tube assay, and mutants were selected based on the criterion that a certain strain could

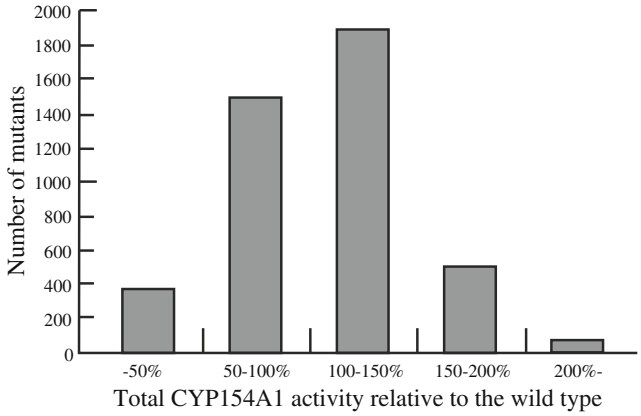

Fig. 1 Total CYP154A1 activity relative to that detected with wild-type in 96-well plate assays

exhibit not only an enzyme activity which is appreciably higher than that of the wild-type, but also a growth rate similar to or higher than that of the wild-type. The positive control exhibited a total and specific enzyme activity of 2.12±0.20 nmol/h/ml of the culture and 0.27± 0.03 nmol/h/mg dry cells, respectively. Many mutants showed CYP154A1 activity at levels similar to that of the wild-type (Fig. 2). However, four mutants, Δ*cpxA*, Δ*gcvR*, Δ*glnL*, and Δ*uk*, exhibited a total activity of more than 1.4-fold greater than that of the wild-type. The Δ*cpxA* mutant showed growth similar to that of the positive control (Fig. 3). The growth rates of the Δ*gcvR*, Δ*glnL*, and Δ*uk* mutants were somewhat slower than that of the positive control. This indicated that these mutants had specific CYP154A1 activity higher than that of the positive control in test tube assays. Figure 3 also shows that all of the four strains reached stationary phase after

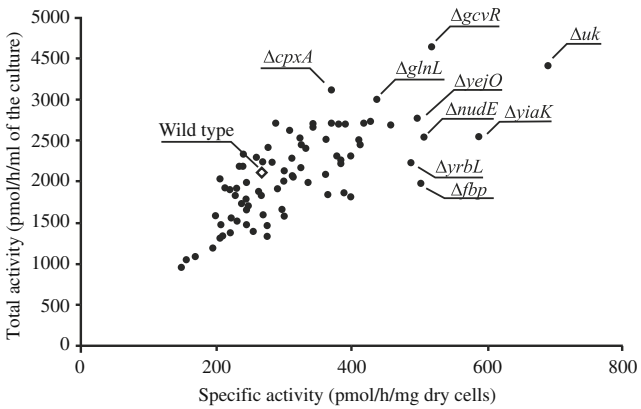

Fig. 2 Total and specific CYP154A1 activities detected with test tube cultures. CYP154A1 activity was assessed with the wild-type (*open diamond*) and SGK mutants (*black circles*) of *E. coli* BW25113. All experiments were conducted at least in triplicate. Mean values are shown in this figure. The standard deviations are within ±11.2% of their respective means

12 h of incubation, and their highest enzyme activity appeared around 12 h. SGK mutants, including Δ*fbp*, Δ*nudE*, Δ*yejO*, Δ*yiaK*, and Δ*yrbL*, showed a specific enzyme activity of approximately two-fold greater than that of the wild-type strain (Fig. 2). However, since these mutants showed poor growth in the autoinduction medium, they were not used for further studies.

Genes responsible for enhancing CYP154A1 activity

To further confirm if the deletion of the selected genes was responsible for the increased CYP154A1 activity, km^r-gene replacement mutants were constructed from *E. coli* BW25113 in the same manner as that used for the Keio collection. Km^r-gene replacement mutants in the *cpxA*, *gcvR*, and *glnL* genes showed CYP154A1 activity at levels similar to those detected with the corresponding SGK mutants in the Keio collection (Table 2). By contrast, the km^r-gene replacement mutant of *uk* failed to show high enzyme activity. This suggests that an unexpected spontaneous mutation was responsible for the enhancement of the CYP154A1 activity in the Δ*uk* mutant in the Keio collection. The Δ*cpxA*, Δ*gcvR*, and Δ*glnL* mutants were used for further studies.

Complementation analysis of the in-frame deletion mutants was performed using single-copy expression plasmids. The *araC*-P_{araBAD}-MCS region of the pBAD33 was introduced into pBAC-Lac to construct a single-copy expression vector, pBAC-CM. The *cpxA*, *gcvR*, or *glnL* gene was then placed under the control of P_{araBAD}. When the resulting plasmid was transformed into the in-frame deletion mutants, Δ*glnL* and Δ*cpxA* mutants showed CYP154A1 activity at a level similar to that of the wild-type (Table 2). The introduction of pBAC-CM without the *gcvR* gene to Δ*gcvR* mutant significantly decreased CYP154A1 activity. However, when Δ*gcvR* mutant was transformed with pBAC-CM carrying the *gcvR* gene, the CYP154A1 activity was further decreased to 37% of the positive control. Although the reason remains unknown, the constitutive expression of the *gcvR* gene is likely to reduce the CYP154A1 enzyme activity.

Discussion

CYP154A1 activity of double-knockout strains

To pursue synergistic effects between each gene disruption, double-knockout strains were constructed. The combination of *cpxA*- and *gcvR*-deficient mutant could not be generated by the method used in this study. This combination might have a lethal effect on *E. coli* BW25113. The enzyme activities of Δ*cpxA*/Δ*glnL* and

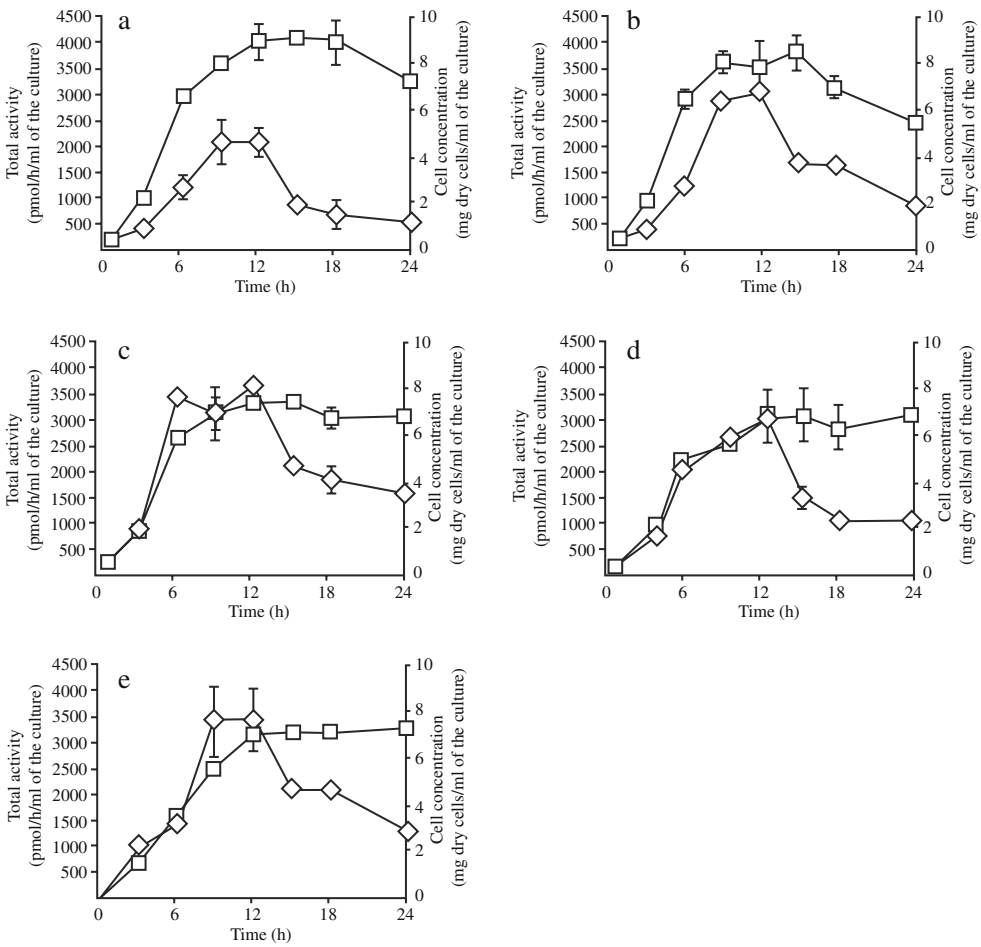

Fig. 3 Time course of growth (*empty squares*) and total CYP154A1 activity (*empty diamonds*) of the wild-type strain and SGK mutants carrying pKK-CYP-camAB in test tube assays. The wild-type (**a**) and Δ*cpxA* (**b**), Δ*gcvR* (**c**), Δ*glnL* (**d**), and Δ*uk* (**e**) mutants of *E. coli* BW25113 were cultivated in test tubes containing 2 ml of autoinduction medium. All experiments were conducted at least in triplicate. Mean values with the standard deviations (*error bars*) are depicted

Δ*gcvR*/Δ*glnL* mutants were tested by test tube assay (Table 3). The Δ*cpxA*/Δ*glnL* mutant showed 1.52 and 1.47 times higher specific activity compared with Δ*cpxA* and Δ*glnL* SGK mutants, respectively. Similarly, the specific activity of Δ*gcvR*/Δ*glnL* mutant was 1.47 and 1.75 times higher than that of Δ*gcvR* and Δ*glnL* SGK mutants, respectively. However, due to their poor growth, the total activities of both double-knockout mutants were modest.

In the present study, among the 81 mutants selected through the first screening at 96-well plate scale, three mutants were finally confirmed to exhibit significantly

Table 2 Relative total activities

Strain[a]	Deleted gene			
	Δ*cpxA*	Δ*gcvR*	Δ*glnL*	Δ*uk*
SGK mutants[b]	147±2.3	172±3.4	142±3.7	161±18.7
Reconstructed mutants[c]	162±14.6	163±16.9	131±12.1	61.9±8.3
In-frame deletion mutants[d]	141±4.5	162±4.0	139±2.0	nd
In-frame deletion mutants with pBAC-CM	141±4.9	75.7±10.9	140±1.9	nd
In-frame deletion mutants with pBAC-CM carrying the deleted genes	99.1±6.0	37.7±2.3	105±4.4	nd

[a] Total activity was expressed as the percentage of total activity of the wild-type. All experiments were conducted at least in triplicate. Values are means±SD

[b] SGK mutants from the Keio collection

[c] New mutants constructed by km^r-gene replacement in the present study

[d] SGK mutants from which the km^r-gene cassette was removed

nd not determined

Table 3 Enzyme activities of the *E. coli* single and double-knockout strains carrying pKK-CYP-camAB relative to that of the wild-type

	Δ*cpxA*	Δ*gcvR*	Δ*glnL*	Δ*cpxA*/ ΔgcvR	Δ*cpxA*/ Δ*glnL*	Δ*gcvR*/ Δ*glnL*
Relative total activity (%)	141±4.5	162±4.0	131±12.1	–[a]	168±5.1	186±5.9
Relative specific activity (%)	147±4.4	181±3.8	152±10.4	–[a]	224±6.3	266±7.1

The Δ*cpxA*/Δ*glnL* is the in-frame deletion mutant of *cpxA* from which the *glnL* is replaced by a kanamycin cassette. The Δ*gcvR*/Δ*glnL* is the in-frame deletion mutant of *gcvR* from which the *glnL* is replaced by a kanamycin cassette

All experiments were conducted at least in triplicate. Values are means±SD

[a] not applicable

higher total enzyme activities than that of the wild-type. Compared with the high-throughput assay in the 96-well plate, the test tube assay is a more reliable screening method. Furthermore, it is of practical importance to reproduce the high activities at larger scales. In fact, when the selected mutants and the wild-type harboring pKK-CYP-camAB were cultivated in a 500-ml Erlenmeyer flask containing 100 ml of the autoinduction medium, the maximum total CYP154A1 activities of Δ*cpxA*, Δ*gcvR*, and Δ*glnL* were 1.65, 1.56, and 1.28 times higher than that of the wild-type, respectively.

Although 33 mutants showed both relatively higher total and specific activity compared to that of the wild-type, the rest of them showed total activities lower than or similar to that of the wild-type (Fig. 2). This might be attributed to insufficient aeration in 96-well plates, unexpected changes caused by sample preparation and manipulation at a small scale (400 μl of the reaction mixture), changes in the expression profile of a specific enzymatic system under different culture conditions, etc.

Replacement of a target gene with a selectable marker gene often causes a polar effect on the expression of downstream genes, since the selectable marker gene has its own terminator which prevents downstream genes in the same operon from being transcribed (Tunca et al. 2007; Zhang et al. 2009). In SGK mutants of the Keio collection, the km^r-gene cassette was flanked by FLP recognition target sites. The excision of the km^r-gene cassette with FLP recombinase could create an in-frame deletion of the respective genes (Baba et al. 2006). To examine polar effect, in-frame deletion mutants of Δ*cpxA*, Δ*gcvR*, and Δ*glnL* were constructed by the method described previously (Datsenko and Wanner 2000). After transformation with pKK-CYP-camAB, the total CYP154A1 activity of the resulting mutants was examined using test tube assays. All the in-frame deletion mutants exhibited P450 activity at levels similar to those detected with the SGK mutants of the Keio collection (Table 2). This result implies that the enhanced enzyme activity was not caused by a polar effect.

In order to further clarify the underlying mechanisms for the improved CYP154A1 activity, the concentration of active CYP154A1 in the recombinant cells was determined by CO differential spectral analysis (Omura and Sato 1963). However, the concentration was too low for accurate quantification (≈35pmol/mg dry cells). Although we also tried to quantify the mRNA expression level of CYP154A1 by quantitative RT-PCR, no statistically significant difference was found between the mutants and the wild-type (data not shown).

CpxA is a membrane-bound sensory histidine kinase in a two-component regulatory system with the CpxR cognate response regulator in *E. coli* (Wulf et al. 2002). The physiological functions associated with the Cpx system (e.g., resistance to hostile conditions, mobility, adherence factors, and metabolism) are reported to be markedly diverse and complex (Dorel et al. 2006; Wulf et al. 1999; Zhou et al. 2003). It is reported that constitutive activation of *E. coli* Cpx pathway significantly depressed the active uptake of the lactose analog thiomethyl-β-D-galactopyranoside (Mileykovskaya and Dowhan 1997). Until now, it has only been identified that the Cpx system could be activated by either overexpression of the outer membrane lipoprotein NlpE (Gupta et al. 1995; Snyder et al. 1995) or mutational activation of CpxA (Danese et al. 1995; Snyder et al. 1995). Suppose that the Cpx system of *E. coli* BW25113 could be activated under the experimental conditions used in this study. This will lead to a difference in the uptake of lactose, which is an inducer for the promoter used in this study, between the wild-type strain and the *cpxA*-deficient mutant expressing CYP154A1.

Both *gcvR* and *glnL* are involved in the regulation of amino acid metabolism in *E. coli*. GcvR is described as a negative regulator of the glycine cleavage enzyme system, which is induced in the presence of glycine and repressed in the presence of purines (Ghrist and Stauffer 1995). Ghrist and Stauffer (1995) reported that the inactivation of *gcvR* led to a high-level constitutive expression of a glycine cleavage enzyme. This might cause changes in the metabolic balance, and thus the cellular energy and redox status might shift to that suitable for a CYP154A1-catalyzed reaction. In the present study, the CYP154A1 activity of the Δ*gcvR* mutant was significantly decreased by the introduction of pBAC-CM. In general, plasmids can

impose a metabolic burden on the host cell. The introduction of pBAC-CM into the Δ*gcvR* cells might deteriorate the suitable cellular status for CYP154A1. GlnL is responsible for the regulation of *glnA* encoding glutamine synthetase and other genes involved in the assimilation of a wide range of nitrogen sources (Ginsburg and Stadtman 1973). Disruption of *glnL* directly or indirectly causes changes in the expression levels of these genes (Atkinson and Ninfa 1992; Chen et al. 1982). Enhancement of the CYP154A1 activity in the Δ*glnL* mutant might also be attributed to an alteration of the cellular energy and redox status resulting from metabolic abnormalities. Prospective studies will be required to reveal the detailed mechanisms for the enhanced CYP154A1 activities in the selected mutants.

In the field of white biotechnology, the breeding of production strains has been traditionally depended on repeated random mutation and selection (Ikeda and Nakagawa 2003; Sakuradani et al. 2009). By stepwise assembly of beneficial phenotypes with the use of mutagenic approach, many commercially potent producers have been developed. However, the classical method based on random mutation often results in the accumulation of detrimental mutations in the genome. The systematic screening of SGK mutants can avoid introducing unnecessary mutations and minimize the number of mutant strains to be investigated without losing the comprehensiveness. Furthermore, the reverse genetic approach enables rapid identification of genes responsible for the desired phenotypes. Although the present work has been done exclusively with one enzyme, other enzymes could be addressed using the same method as shown in this work.

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