Journal of Bioscience and Bioengineering
VOL. 120 No. 3, 268–274, 2015

ELSEVIER

www.elsevier.com/locate/jbiosc

Sequence modifications and heterologous expression of eukaryotic cytochromes P450 in *Escherichia coli*

Hirofumi Ichinose,* Mayumi Hatakeyama, and Yukiyo Yamauchi

Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

Received 10 November 2014; accepted 26 January 2015
Available online 25 February 2015

The development of a heterologous expression system is often a principal step in biochemical and biotechnological studies on cytochromes P450 (P450s). However, heterologous expression of eukaryotic membrane-bound P450s in *Escherichia coli* is still a trial-and-error process because theoretical and systematical experimental procedures have not yet been established. In this study, we generated a series of chimeric variants of fungal P450s by replacing their N-terminal domains with the N-terminal domains of other P450s and explored their potentials for heterologous expression in *E. coli*. Large-scale screening demonstrated that a number of fungal P450s could be expressed when their N-terminal amino acid sequences were replaced with the corresponding domain of CYP5144C1, even when the expression of the non-chimeric sequence was unpromising. Furthermore, a comprehensive screening resulted in the identification of 64 different types of chimeric partners whose N-terminal domains could potentially be used to increase the expression levels of various P450s. These findings will help to elaborate experimental strategies for high-level heterologous expression of a variety of eukaryotic membrane-bound P450s in *E. coli*.

[**Key words:** Eukaryotic cytochrome P450; *Escherichia coli*; Heterologous expression; Chimerization; Sequence modification; N-terminal domain]

Cytochromes P450 (P450s) constitute a large superfamily of heme-containing monooxygenases that are distributed in a wide variety of organisms (1–3). In recent years, genome projects have accelerated the compilation of P450 sequences and, as a result, the large-scale divergence of P450s in living organisms has been reported (3–7). P450s contribute to various secondary metabolic systems such as detoxification of xenobiotics and biosynthesis of metabolites (8,9); therefore, understanding of the sequence–structure–function relationships of the P450s is likely to provide new insights into their biochemistry and biotechnology. In addition to their biological importance, the use of P450s in the biotechnology sector is of great interest (10–14). For example, the possible applications of the biocatalytic regio- and stereo-specific oxidation activity of the P450 enzymes attract much attention because (i) biocatalysts can help reduce the number of tedious steps such as blocking and deblocking that are commonly required in conventional chemical syntheses, (ii) biocatalysts produce a single product with no by-product formation, which reduces the cost of downstream purification steps, and (iii) biocatalysts produce enantiomeric mixtures (rather than racemic mixtures) that are strictly required in some fields, such as the pharmaceutical industry.

Heterologous expression is a principal step for both basic and application studies. A suitable quantity and quality of recombinant enzymes are often required for the studies and their availability often relies on the possibility of heterologous expression. As the bacterial expression system using *Escherichia coli* is a powerful experimental tool that can produce high expression levels of recombinant proteins, many researchers have attempted to overexpress P450s in *E. coli* (15–23). As a result, efficient techniques such as sequence modification, vector selection, chaperon coexpression, codon optimization, and combinations of these have been emerged. Now, it has become a conventional strategy to introduce sequence modifications such as deletion and/or replacement of the hydrophobic N-terminal domains (NTDs) of the P450s to help increase the chance of expression of the membrane-bound eukaryotic P450s in *E. coli* (15–23). However, it remains difficult to rationally design experimental procedures because each P450 often requires different optimal sequence modification.

NTD-modified eukaryotic P450s are known to be still expressed in membrane fraction in *E. coli* because of their indelible hydrophobic topologies (18–20). Eukaryotic P450s expressed in *E. coli* might be more stable in the membrane than in the cytosol. Since hydrophobic NTDs of membrane-bound P450s serve mainly as membrane anchors to endoplasmic reticulum in eukaryotic cells, wild type sequences of NTDs seemed to be obstacles to express eukaryotic P450s in *E. coli*. Thus, topology optimization by sequence modification of NTD would be the first important step for heterologous expression of membrane-bound P450s in *E. coli*. Recently, we performed extensive heterologous expression of fungal P450s in *E. coli* using 304 P450 isoforms, and demonstrated that at least 27 P450s could be expressed with/without simple sequence deletion of NTD (21). The study also demonstrated that expression levels of several P450s (e.g., CYP5037B3, CYP5037E1v1, CYP5146A1,

* Corresponding author. Tel./fax: +81 92 642 2994.
E-mail address: ichinose@agr.kyushu-u.ac.jp (H. Ichinose).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.01.019

CYP5149A1) could be dramatically improved by chimerization with NTD of CYP5144C1. This result made us wonder to what extent the NTD of CYP5144C1 is effective as a chimeric partner for eukaryotic P450s. In the present study, we employed the NTD of CYP5144C1 as a chimeric partner for 141 fungal P450s, whose heterologous expressions were not achieved only by simple deletion of wild-type NTDs (21), and explored its efficiency of heterologous expression. In addition, we gathered sequence information of NTDs that can be useful as chimeric partners because the information would be a great help to design suitable NTD sequences for high-level heterologous expression in *E. coli*. In this study, a large-scale screening of heterologous expression of a model P450, CYP5037E1v1, was performed after chimerization with 99 different NTDs to identify potential chimeric partners. To the best of our knowledge, this is the largest screening of potential chimeric partners to develop experimental strategies for high-level heterologous expression of membrane-bound P450s in *E. coli*. Furthermore, a comparative study on heterologous expression using various types of P450s was conducted to gain insight into the relationships among expression level, NTD and overall sequence similarity of catalytic domain. This study will help to elaborate experimental strategies for the high-level expression of a variety of eukaryotic membrane-bound P450s in *E. coli*.

MATERIALS AND METHODS

Chemicals The L-arabinose, 5-aminolevulinic acid, carbenicillin, chloramphenicol, ethylenediaminetetraacetic acid (EDTA), and phenylmethylsulfonyl fluoride (PMSF) used in this study were purchased from Wako Pure Chemicals (Osaka, Japan). The 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) was purchased from Dojindo Laboratories (Kumamoto, Japan), isopropyl-1-thio-β-D-galactopyranoside (IPTG) was purchased from Takara Bio (Otsu, Japan), and dithiothreitol, tryptone, and yeast extract were purchased from Nakarai Tesque, Inc. (Kyoto, Japan). Custom synthesized oligonucleotide primers were obtained from Life Technologies Japan Ltd. (Tokyo, Japan). All the other chemicals were reagent grade. Deionized water was obtained from the Milli Q System (Millipore, Tokyo, Japan).

Enzymes and gene templates used for PCR amplification Gene amplification by PCR was carried out using Phusion DNA polymerase (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions. We used the pET22-based plasmids that harbor partially truncated (at the N-terminal) fungal P450s that were constructed previously in our laboratory (21) as gene templates for the PCR amplification. Custom synthesized oligonucleotide primers were obtained from Life Technologies Japan Ltd.

Construction of chimeric P450s paired with CYP5144C1 NTD As an experimental strategy, we constructed the plasmids of chiemric P450s in which NTDs were replaced with the corresponding domain of CYP5144C1 (Fig. S1). Briefly, the expression plasmids were generated by seamless cloning of the PCR-amplified gene fragments and enzymatically digested pET22 plasmid using an In-Fusion HD Cloning Kit (Clontech Laboratories, Inc., Mountain View, CA, USA). Then, a gene fragment containing the NTD of CYP5144C1 was amplified by PCR using a combination of universal forward primer (primer-petF, 5′-TAGAGGATCGAGATCTCGATCCCGCGA-3′) and gene-specific reverse primer (primer-1, 5′-CAGCGGATAACGCTTCTGC-3′). The resultant PCR fragment was designated as fragment-1. For amplification of gene fragments containing the catalytic domains of target P450s, PCR was carried out with a combination of universal reverse primer (primer-petR, 5′-GCTAGTTATTGCTCAGCGGTGGCAGCAG-3′) and gene-specific forward primer (primer-2). The resultant PCR fragment was designated as fragment-2. The primer sequences are shown in Table S1. Meanwhile, a commercially obtained pET22 plasmid (Novagen, Madison, WI, USA) was digested enzymatically by *Bgl*II and *Bpu*1102I and the linearized plasmid was designated as fragment-P. Expression plasmids of the chimeric P450s were then generated by recyclization of the gene fragments (fragment-1, -2, -P) using an In-Fusion HD Cloning Kit (Clontech Laboratories, Inc.). In these constructs, the P-P-G-P amino acid sequences in their proline-rich regions were encoded by the original codons of the target P450s.

Chimerization of CYP5037E1v1 with various N-terminal domains Fig. S2 shows our experimental strategy on plasmid construction in which the catalytic domain of CYP5037E1v1 was combined with various NTDs from other chimeric partners. Briefly, the NTD of CYP5037E1v1 was replaced with the corresponding domains of 99 different P450s with distinctive conserved L-P-P-G-P or Y-P-P-G-P amino acid sequences in their proline-rich regions. To amplify the gene fragments containing the NTD of a chimeric partner, PCRs were carried out with a combination of universal forward primer (primer-petF) and gene-specific reverse primer (primer-3). The resultant gene fragment was designated as fragment-3. To amplify the gene fragment containing the catalytic domains of CYP5037E1v1, two different gene-specific forward primers (primer-4L, 5′-CTGCCGCCGGGTCCGGCGCGACTACCGAT-3′ and primer-4Y, 5′-TATCCGCCGGGTCCGGCGCGACTACCGAT-3′) were designed to possess codons for the L-P-P-G-P or Y-P-P-G-P sequences respectively. Gene fragments of CYP5037E1v1 were then amplified by PCR with a combination of universal reverse primer (primer-petR) and gene-specific forward primer (primer-4L or primer-4Y). The resultant gene fragments were designated as fragment-4L or -4Y, respectively. The primer sequences are shown in Table S2. Expression plasmids were generated by recyclization of fragment-3, fragment-P, and either fragment-4L or -4Y. In these constructs, the L-P-P-G-P and Y-P-P-G-P sequences in the proline-rich region were universally encoded by CTG-CCG-CCG-GGT-CCG and TAT-CCG-CCG-GGT-CCG, respectively.

Silent mutations of proline residues in the proline-rich region Silent mutations of the nucleotides encoding the proline residues were introduced for the proline-rich region of chimeric CYP5037E1v1 modified with the CYP5144C1 NTD. Each isoform of the chimeric CYP5037E1v1 encoded the P-P-G-P amino acid sequence using different nucleotide codons, namely CCT-CCT-GGT-CCT, CCG-CCG-GGT-CCG, CCC-CCC-GGT-CCC, or CCA-CCA-GGT-CCA. To introduce silent mutations, the NTD of CYP5144C1 was amplified using a combination of universal forward primer (primer-petF) and gene-specific primer (primer-5). The resultant gene fragment was designated as fragment-5. The gene fragment of the catalytic domain of CYP5037E1v1 was amplified with a combination of gene-specific primer (primer-6) and universal reverse primer (primer-petR). The resultant fragment was designated fragment-6. The primer sequences are shown in Table S3. Plasmid construction was carried out by seamless cloning of fragment-5, -6, and -P.

Expression profiles of chimeric P450s The catalytic domains of 19 target P450s were chimerized with the NTDs of five different chimeric partners. The 19 target P450s were CYP5027B6v1, CYP5027B6v2, CYP5037B2, CYP5037B5v2, CYP5037D1v1, CYP5037D1v2, CYP5037E1v2, CYP5037E2v1, CYP5037E3v1, CYP5144A6, CYP5144A7, CYP5144A13, CYP5144C2, CYP5144D1, CYP5147A3, CYP5147A4, CYP5348E1v2, CYP5348J6, and CYP5348L1v2. The five chimeric partners were CYP5348N1, CYP5348T3P, CYP5348L1v2, CYP5144C1, and CYP5144C8. Gene fragments of the NTDs of the chimeric partners were amplified using a combination of universal forward primer (primer-petF) and gene-specific reverse primer (primer-7). The resultant gene fragment was designated as fragment-7. The gene fragments of the catalytic domains of the target P450s were amplified using a primer combination of gene-specific forward primer (primer-8) and universal reverse primer (priemr-petR). The resultant gene fragment was designated as fragment-8. The primer sequences are shown in Table S4. Plasmid construction was carried out by seamless cloning of fragment-7, -8, and -P.

Heterologous expression of P450s *E. coli* C41(DE3) harboring the pGro7 plasmid (Takara) was transformed with the expression plasmids. The transformants harboring the expression plasmid were selected on Luria-Bertani (LB) agar plates containing carbenicillin (100 mg/L) and chloramphenicol (20 mg/L). A fresh transformant was inoculated into 1 mL of LB medium supplemented with carbenicillin (100 mg/L) and chloramphenicol (20 mg/L) and grown overnight at 37°C on a 96-deep-well plate with shaking. After the preincubation, 0.3 mL of the growth culture was seeded into 30 mL of Terrific Broth medium supplemented with carbenicillin (100 mg/L), chloramphenicol (20 mg/L), and 5-aminolevulinic acid (0.5 mM) in a 100-mL Erlenmeyer flask. Cells were grown at 37°C for 2.5 h with shaking (140 rpm), typically resulting in OD_{600} = 0.8–1.0. The culture was then cooled to 24°C and supplemented with IPTG (0.5 mM) and L-arabinose (2.0 g/L) and incubated with shaking (140 rpm) for 48 h. The *E. coli* cells from 25 mL culture were then harvested by centrifugation (4500 ×*g*), washed with 10 mL of 50 mM HEPES buffer (pH 7.4), and resuspended in 5.0 mL of lysis buffer consisting of 50 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, and 20% glycerol. The bacterial cells were disrupted by bead beating with 0.2 mm zilconia beads using Beads Crusher μT-12 (Taitec) (21). After removing cell debris by centrifugation (18,000 ×*g*), carbon monoxide (CO) difference spectra of the supernatants were recorded on a UV–vis spectrophotometer equipped with a head-on photomultiplier (Hitachi; U3900H). The recombinant P450 concentration was calculated based on the CO difference spectrum with an extinction coefficient of 91 mM cm^{-1} for $\Delta A_{450-490}$ (24).

RESULTS AND DISCUSSION

Heterologous expression of P450s chimerized with CYP5144C1 NTD Recently, we conducted a large-scale screening of heterologous expression using 304 fungal P450s and demonstrated 27 species could be expressed with/without the simple deletion of the sequences encoding native transmembrane domains (21). The study also suggested the presence of useful chimeric partners with which expression levels of various P450s could be improved by chimerization. Consequently, here we have designed an experimental procedure for high-level heterologous