

Contents lists available at [SciVerse ScienceDirect](http://SciVerse.Sciencedirect.com)

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

High-level heterologous expression of fungal cytochrome P450s in *Escherichia coli*

Hirofumi Ichinose^{a,*}, Hiroyuki Wariishi^{a,b}^a Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan^b Faculty of Arts and Science, Kyushu University, 744 Motoooka, Nishi-ku, Fukuoka 819-0395, Japan

ARTICLE INFO

Article history:

Received 5 July 2013

Available online 23 July 2013

Keywords:

Eukaryotic cytochrome P450
 High-level expression
Escherichia coli
 Large-scale screening
 Heterologous expression
 Wood-rotting basidiomycetes

ABSTRACT

A thorough understanding of the sequence–structure–function relationships of cytochrome P450 (P450) is necessary to better understand the metabolic diversity of living organisms. Significant amounts of pure enzymes are sometimes required for biochemical studies, and their acquisition often relies on the possibility of their heterologous expression. In this study, we performed extensive heterologous expression of fungal P450s in *Escherichia coli* using 304 P450 isoforms. Using large-scale screening, we confirmed that at least 27 P450s could be expressed with/without simple sequence deletion at the 5' end of cDNAs, which encode the N-terminal hydrophobic domain of the enzyme. Moreover, we identified N-terminal amino acid sequences that can potentially be used to construct chimeric P450s, which could dramatically improve their expression levels even when the expression of the wild-type sequence was unpromising. These findings will help increase the chance of heterologous expression of a variety of fungal and other eukaryotic membrane-bound P450s in *E. coli*.

© 2013 The Authors. Published by Elsevier Inc. Open access under [CC BY-NC-ND license](http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Cytochromes P450 (P450s) constitute a large superfamily of heme-containing monooxygenases that are distributed in a wide variety of organisms [1–3]. The large-scale molecular variations among the P450 in different species imply a dynamic evolutionary trajectory in which a common ancestor branched extensively into various organisms. Thus, the vast variety of P450s has emerged over the course of evolution. Within the last few years, a series of genome projects have accelerated sequence compilation of P450s. Accordingly, the large-scale divergence of fungal P450s is currently being explored [2,4–7]. Because P450s contribute to the metabolic diversity of fungi, it will be of great interest to explore the biological functions of the numerous fungal P450s.

A thorough understanding of the sequence–structure–function relationships of P450s is a challenge that can now be addressed in the post-genomic era. For biochemical studies such as mechanistic and structural investigations, significant amounts of pure en-

zymes are usually required and their availability often relies on the possibility of their heterologous expression. Yeast and bacteria have been used for the heterologous expression of recombinant enzymes including the eukaryotic membrane-bound P450s. Yeast expression systems can readily express membrane-bound P450s without tedious experimental procedures such as sequence modification [5,6,8]. However, the limited level of expression in yeast cells and the cumbersome purification processes that are required might hinder downstream applications. Conversely, the bacterial expression system using *Escherichia coli* is a powerful experimental tool that can produce high levels of recombinant proteins [9–12]. However, the heterologous expression of membrane-bound eukaryotic P450s in *E. coli* is still a challenge because the optimal modification of each individual isoform sequence is usually required. Many researchers have attempted to overexpress membrane-bound eukaryotic P450s in *E. coli*, particularly mammalian P450s, but only a few studies have overexpressed P450s from fungi [12,13]. In these investigations, the conventional strategy of modification of the 5' end of the cDNA (for example, deletion and/or replacement), which encodes the N-terminal hydrophobic domain of P450, was used to increase the chance of expression of the membrane-bound eukaryotic P450s in *E. coli* [9–12]. However, heterologous expression is still a trial-and-error process because it is difficult to theoretically and systematically adapt the experimental procedures. This may explain why only a few P450 species, especially from mammalian, have successfully been produced by heterologous expression. It is, therefore, of great interest and importance to explore heterologous expression using a wide

Abbreviations: EDTA, ethylenediaminetetraacetic acid; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; IPTG, isopropyl-1-thio- β -D-galactopyranoside; P450, cytochrome P450 monooxygenase; PMSF, phenylmethylsulfonyl fluoride; TMD, transmembrane domain.

* Corresponding author. Fax: +81 92 642 2994.

E-mail address: ichinose@agr.kyushu-u.ac.jp (H. Ichinose).

variety of P450 species, which may provide/improve expression strategies for eukaryotic P450s in *E. coli*.

Recently, we reported the genome-wide identification and isolation of 304 full-length cDNAs of the P450s from the white-rot basidiomycetes *Phanerochaete chrysosporium* [5] and the brown-rot basidiomycetes *Postia placenta* [6]. To help promote the biochemical and structural investigations of fungal P450s, in the present study, we aimed to overexpress fungal P450s using an *E. coli* expression system. Here, the heterologous expression of the 304 P450 isoform species from the wood-rotting basidiomycetes was investigated comprehensively. To the best of our knowledge, this is the first and largest study in which large-scale screening of the heterologous expression of eukaryotic P450s in *E. coli* has been conducted. The findings reported here should help advance the study and application of fungal P450s. Moreover, the fundamental information will contribute to the development/improvement of experimental strategies for the heterologous expression of eukaryotic membrane-bound P450s.

2. Materials and methods

2.1. 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).

2.2. Construction of the expression plasmids

The construction strategy used for the expression plasmids is outlined in Fig. S1. Briefly, the commercially obtained plasmids pET22 and pET19 (Novagen, Madison, WI, USA) were modified to introduce additional *NheI* and *SpeI* restriction sites. The resultant plasmids were named pET22z and pET19z, respectively. The expression plasmid was digested with different combinations of *NdeI*, *NcoI*, *NheI*, *SpeI*, *HindIII*, and *XhoI*. The truncated open reading frames of the P450s were amplified from a cDNA library using Phusion DNA polymerase (Thermo Fisher Scientific, Rockford, IL, USA) with combinations of the gene-specific forward primers (see Table S1) and the universal reverse primer for the template plasmid pGYR (5'-TCTAGACGATGATAAGCTGTCAAACATGAG-3'). Hydrophobic transmembrane domains (TMDs) in the open reading frames of the P450s were predicted using SOSUI [14]. The amplified gene fragments were subcloned into pBluescript II SK (+), digested with restriction enzymes (see Table S1), purified using a QIAquick Gel Extraction Kit (Qiagen, Tokyo, Japan), and ligated with the linearized pET22z or pET19z. The resultant expression plasmids were confirmed to contain the appropriate open reading frame.

2.3. Evaluation of heterologous expression of recombinant P450s

E. coli C41 (DE3) harboring the pGro7 plasmid (TaKaRa, Otsu, Japan) 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 (4500g), 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 zirconia beads using Beads Crusher μ T-12 (TAITEC). After removing cell debris by centrifugation (18000g), 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 $\Delta A_{450-490}$ [15].

2.4. Preparation of chimeric P450s

The experimental procedure used for the construction of chimeric P450s is outlined in Fig. S2. Briefly, chimeric P450s were generated by inverse PCR and seamless cloning of the target genes. For the inverse PCR, the gene segments between the *XbaI/NotI* and *XbaI/XhoI* sites were first transferred from the pET-based expression vector to pLW01 (step-1 in Fig. S2), developed by Mulrooney and Waskell [16]. The resultant pLW01-based plasmid containing a P450 gene (origin of the N-terminal domain) was inversely amplified to remove the catalytic domain (step-2 in Fig. S2). Meanwhile, the catalytic domain of the target P450 was amplified by PCR from the pET-based expression plasmid (step-3 in Fig. S2) and then fused with the inversely amplified plasmid (step-4 in Fig. S2) using an In-Fusion[®] HD Cloning Kit (Clontech Laboratories, Inc., Mountain View, CA, USA). The primer sequences used to construct the chimeric P450s are listed in Table S2. Although the resultant pLW01-based plasmids were potentially useful for protein expression, the gene segments between the *XbaI/NotI* and *XbaI/XhoI* sites were re-transferred from the pLW-based plasmid to pET22 (step-5 in Fig. S2) so that the expression levels could be determined under the same conditions as those used for the non-chimeric P450 expressed from the pET-based plasmid. The resultant expression plasmids were confirmed to contain the appropriate open reading frame.

3. Results and discussion

Eukaryotic membrane-bound P450s contain a helical hydrophobic TMD consisting of 20–30 amino acid residues at their N-terminal ends. A series of membrane-bound P450s have been expressed with sequence modifications such as TMD truncation and/or N-terminal replacement [9–12]. Although N-terminal modification is one of the conventional strategies that are widely used for heterologous expression of eukaryotic P450s, it is still questionable whether replacement of the N-terminal domain with the sequences reported previously can increase the chance of expression of the various P450s equally well. Previously, we demonstrated the overexpression of a membrane-bound fungal P450, CYP5150A2, from *P. chrysosporium* using the combination of a pET expression

plasmid and the *E. coli* C41 (DE3) strain [12]. A partial truncation but not the complete deletion of the TMD sequence was effective for heterologous expression of CYP5150A2. In general, TMD-truncated or TMD-modified eukaryotic P450s are still expressed in the membrane fraction [12,17,18], suggesting that well-balanced hydrophobicity at the N-terminal domain as well as non-toxicity may be required for accumulation of the P450 protein in *E. coli*. Therefore, in the present study, we explored the heterologous expression of TMD-truncated variants of fungal P450s (Table S1). Heterologous expression was examined without sequence modification for the P450s that lacked a distinctive TMD (Table S1). In addition, the expression plasmids of fungal P450s were co-transformed with pGro7, which conditionally overexpresses GroEL/ES.

3.1. Large-scale screening of heterologous expression in *E. coli*

We performed large-scale screening of heterologous expression using 304 fungal P450s and identified 27 species, including a pair of allelic variants (CYP5137A4v1 and CYP5137A4v2), that could be expressed with/without the simple deletion of the sequence encoding the native TMD (Table 1). When protein expression was induced with 0.5 mM IPTG at $OD_{600nm} = 0.8$, accumulation of active P450 in the bacterial cells showed a similar time-dependent profile and the maximum expression level was observed 48–72 h after incubation with IPTG (data not shown). After 48 h incubation, expression levels for some P450 species were over 1000 nmol (liter culture)⁻¹, similar to levels reported earlier using eukaryotic P450s [10,19–22].

Among the 27 species, CYP505D6, CYP505D8v1, CYP5137A4v1, and CYP5137A4v2 (see Table 1) were expressed without sequence modifications. The P450s in the CYP505 family lack a distinctive TMD and are loosely bound to the membrane in fungal cells [23,24]. Previously, it was reported that the P450 from *Fusarium*

oxysporum (P450foxy), which belongs to the CYP505 family, was expressed in the soluble fraction of *E. coli* [25]. Thus, some P450s in the CYP505 family may be expressed in the cytosolic fraction of *E. coli*, and consequently sequence modification was not essential for CYP505D6 and CYP505D8v1 to be expressed. In contrast, the N-terminal sequences of the allelic variants CYP5137A4v1 and CYP5137A4v2 were rich in hydrophobic amino acid residues despite their lack of a distinctive helical TMD. The hydrophobic segments of CYP5137A4v1 and CYP5137A4v2 could be topologically aligned to the helical TMD that is present in the P450s categorized into the CYP5137 family. Thus, the unique native sequences in these variants might be less toxic and, therefore, suitable for expression.

Interestingly, the expression levels of CYP5137A4v1 and CYP5137A4v2 were significantly different although the amino acid sequences that they encode were strikingly similar (97% shared sequence identity). Furthermore, CYP5148B4v2, CYP5037E1v1, and CYP5037E5v2, but not their allelic variants, were expressed at significant levels. Although the mechanisms behind the observations should be further characterized, it is clear that very small differences in the sequence can affect the expression levels of the P450s. The expression level of CYP5137A4v2 increased slightly (1.7-fold) when the amino acid residue at the second position in the sequence was changed from glycine to serine, the same as the residue at that position of CYP5137A4v1, even though its expression remained lower than that of CYP5137A4v1. In contrast, the expression level of CYP5137A4v2 was decreased significantly (0.2-fold) when an alanine residue replaced the glycine at the second position even though it has been reported that alanine encoded by GCT can increase the expression level of some P450s [9,26]. Our result, however, suggests that an alanine residue at the second position can sometimes have a negative effect on heterologous expression.

Table 1
Fungal P450s heterologously expressed in *E. coli*.

P450	Amino acid deletion	N-terminal sequence ^a	Expression level (nmol/L culture) ^b	Origin ^c	GenBank Accession No.
CYP51F1 ^d	2–37	MILSVMCNVIYQL↓LPKDKSLPPVVW	1255 ± 93	Pc	AB597796
CYP53C2	2–22	MAIVAHILVWLLDP↓HGIRSYPGPLL	219 ± 46	Pc	AB597795
CYP61A1	2–36	MTTAAILLSLLVI↓EQSVVRYKKRHL	761 ± 58	Pc	AB597875
CYP505D6	None	MATSTIPTPPSIPFLGHVASIEREVP	665 ± 22	Pc	AB597812
CYP505D8v1	None	MTNPIPCPPSPFLGHVTHIEKVPPL	1333 ± 75	Pp	AB573395
CYP512E1	2–8	MAYIFISLATLAYL↓KRLWLPDRQQ	973 ± 56	Pc	AB597904
CYP5037B3	2–13	MALALIVKAFSL↓RTRRQGLYPPGP	156 ± 14	Pc	AB597793
CYP5037B4	2–12	MAILTVVLRITAI↓RRKRWARLPPG	472 ± 63	Pc	AB597792
CYP5037E1v1	2–13	MVLICAVGYAVAK↓QRAKAPLPPGPA	228 ± 18	Pp	AB573357
CYP5037E5v2	2–11	MASLLVLAARLL↓KRSSHLPMPGP	88 ± 10	Pp	AB573401
CYP5136A1	2–16	MLIRFYRWLFHHS↓ISYLRGPVADSF	328 ± 21	Pc	AB597902
CYP5137A2	2–6	MAVLLGALLWIV↓RRILSRSSIRDIC	70 ± 17	Pc	AB597801
CYP5137A4v1	None	MSTLSSAALLTLACIYVAIRRLRRT	1820 ± 71	Pp	AB573266
CYP5137A4v2	None	MGTLSSAALLTLACIYVAIRRLRRT	355 ± 8	Pp	AB573300
CYP5139A1	2–14	MALVALIVYVSGPT↓VWHVLTSPLRH	366 ± 16	Pc	AB597853
CYP5139D7v1	2–13	MAFSCWKLLKISG↓LLQPYMSPLGDI	1230 ± 26	Pc	AB573402
CYP5141A4	2–12	MLFYCVQKYLEF↓RAVVRVSIHDPGF	319 ± 33	Pc	AB597909
CYP5141A6	2–5	MVLFVSLLAGAL↓KKHLDFAAVDK	207 ± 3	Pp	AB573323
CYP5144C1	2–7	MSLLAATLFLH↓SRQKRYPLPPGPK	163 ± 29	Pc	AB597883
CYP5146A1	2–12	MALAMMHVLRTRCVR↓TRLPPYPPGPE	380 ± 8	Pc	AB597863
CYP5147B1	2–8	MLIAALFVLSGLLNS↓RRRNMHVPPG	1310 ± 154	Pc	AB597857
CYP5148A1	2–15	MGILVLIICLLRV↓VRRNTKRRLEQI	129 ± 13	Pc	AB597876
CYP5148A2	2–26	MAGILYIVLPFF↓RKNLVDKNGNSI	380 ± 13	Pc	AB597854
CYP5148B4v2	2–24	MASLLGILWLLPFLSG↓HSYDVFGR	43 ± 7	Pp	AB573261
CYP5149A1	2–12	MTLLSIGIAPLFTL↓WRRKRTSEAYL	332 ± 20	Pc	AB597869
CYP5150A2 ^e	2–14	MAIHWKLFQGYLV↓KSLDNIPGPER	1020 ± 22	Pc	AB597898
CYP5150E1v2	2–9	MVLLALVLWALVK↓RLTATKARAIDI	120 ± 3	Pp	AB573373

^a N-terminal amino acid sequence of the TMD-truncated variant. Arrow indicates the edge of the TMD in the native sequence. Amino acid residues in italics are from the restriction sites of the plasmid.

^b Experiments were performed in triplicate. The data is the mean ± SE.

^c Pc and Pp indicate *P. chrysosporium* and *P. placenta*, respectively.

^d Heterologous expression was reported previously [13].

^e Heterologous expression was reported previously [12].

3.2. Chimeric constructs for improvement of P450 expression levels

The vast majority of P450s possess a proline-rich region after the positively charged residues neighboring the TMD. The proline-rich region can be considered as the edge of the catalytic domain because: (i) the proline residues in this region are highly conserved; (ii) the proline-rich region is important for protein maturation [27–29]; (iii) mutations in the proline-rich region can decrease enzymatic activity [30–32]; and (iv) the N-terminal TMD serves mainly as a membrane anchor while the positively charged amino acid residues that follow halt the translocation of the P450s into the endoplasmic reticulum membranes in eukaryotic cells. Therefore, we prepared a number of chimeric variants in which the chimeric junction was fixed at the position before the proline-rich region of the target P450 and after the N-terminal domain of another P450 (Fig. 1).

The heterologous expression of chimeric CYP5037E1v1, CYP5148A1, and CYP5150E1v2 was investigated intensively as model species for which the wild-type expression levels are relatively low. The N-terminal domains of the model P450s were replaced with the corresponding domains of 25 of the 27 fungal P450s whose expressions were confirmed in this study (the two CYP505 P450s, CYP505D6 and CYP505D8v1, were excluded) and an N-terminally modified human CYP3A4 (Fig. 1) [33]. A number of chimeric variants of CYP5037E1v1 were expressed at a higher level than the non-chimeric sequence (Fig. 2A). A chimeric CYP5037E1v1 modified with the N-terminal of CYP5144C1 was expressed at the highest level (2300 nmol/L culture), 10-times

higher than the non-chimeric sequence. These results implied that it was the N-terminal domain of CYP5037E1v1 that was less suitable for translation and/or protein accumulation in *E. coli* and not maturation of the catalytic domain that hindered high-level expression. In contrast, chimeric variants of CYP5148A1 either accumulated at expression levels that were similar to those for the non-chimeric variant or lost expression after chimerization (Fig. 2B). The limited expression level of CYP5148A1 can presumably be attributed to molecular aspects of the catalytic domain. Conversely, the heterologous expression of CYP5150E1v2 was improved significantly when the N-terminal domain was replaced with the sequence of CYP5139D7v1, even though most of the chimeric variants resulted in loss of expression (Fig. 2C). Interestingly, the relative expression levels of chimeric CYP5037E1v1, CYP5148A1, and CYP5150E1v2 showed dissimilar profiles; for instance, the expression level of CYP5037E1v1 was increased by chimerization with the N-terminal domains of both CYP5144C1 and CYP5139D7v1, but only CYP5139D7v1 was an effective chimeric partner for CYP5150E1v2. Apparently, it is the combination of the N-terminal region and the catalytic domain that is critical for high-level expression of the fungal P450s in *E. coli*.

The N-terminal domains of both CYP5144C1 and CYP5139D7v1 could be flexible chimeric partners to promote the high-level expression of membrane-bound P450s in *E. coli*, at least for several species (Fig. 2). The N-terminal domain of CYP5144C1 was then used to construct chimeric variants of the P450s that contained a Pro-Pro-Gly-Pro sequence in their proline-rich regions and tested for heterologous expression. The expression levels of the various

(A) Amino acid sequence around N-terminal and proline-rich region

		proline-rich region
CYP51F1		M I L S V M C N V I Y Q L L P K D K S L P P V V W H W F P P
CYP53C2		M A I V A H I L V W L L D P H G I R S Y P G P L L A K F S
CYP61A1		M T T A A I L L S L L V I E Q S V Y R Y K K R H L P G D K W T I P L
CYP512E1	M A Y I F I S L A T L A Y L K R L L W P D R Q Q L E H I P A I G P T A P I L S	
CYP5037B3		M A L A L I I V K A F L S R T R R Q G L Y P P G P K G L P I V
CYP5037B4		M A I L T V V L I R T A I A R R K R W A R L P P G P K G L P I V
CYP5037E1v1		M V L I C A V G Y A V A K Q R A K A P L P P G P A R L P I L
CYP5037E5v2		M A S L L V L V A A R L L G K R S S H L P M P P G P R G L P V L
CYP5136A1		M L I R F Y R W L F H H S I S Y L R G P V A D S F I
CYP5137A2		M A V L L G A L L W I V R R I L S R S S I R D I C G P E P E S F W
CYP5137A4v1	M S T L S S A A L L L T L A C I Y V A I R R L R R T S V S G I P G P Q P E S F L	
CYP5137A4v2	M G T L S S A A L L L T I A C I Y V A I R R L R R T S V S G I P G P Q P E S F L	
CYP5139A1		M A L V A L I V Y S V G P T V W H V L T S P L R H L P G P P N D S L L
CYP5139D7v1		M A F S C W K L L K I S G L L Q P Y M S P L G D I P G P P S P S F L
CYP5141A4		M L F Y C V Q K Y L E F R A V V R S I H D H P G F R T L L P P
CYP5141A6		M V L F V S L L A L G A L K K H L D F R A A V D K I H N Y P G L R F I F G V
CYP5144C1		M S L L L A A T L F L H S R Q K R Y P L P P G P K G L P I I
CYP5146A1		M A L A M H V L T R C V R T R L P Y P P G P P E D P I I
CYP5147B1		M L I A A L F V L S G L L N S R R R N M H V P P G P K A L P L L
CYP5148A1		M G I L V L I C L L L R V V R R N T K R R L E Q I P P G P I G L P I L
CYP5148A2		M A G I L Y I V L P F F R K N L V D K N G N S I P P G P L L R L P Y
CYP5148B4v2	M A S L I L G I L W L L P F L S G H S Y D V F G R R P R D K D S L S I P D G P L G L P I V	
CYP5149A1		M T L S I G I A P L F T L W R R K R T S E A Y L P P G P P A L P F V
CYP5150A2		M A I I W K L F Q G Y L V K S P L D N I P G P E R S S F W
CYP5150E1v2		M V L L A L V L W A L V K R L T A T K A R A I D I A G P E T D H W L
CYP3A4		M A L L L A V F L L V L L Y L Y G T H S H G L F K K L G I P G P T P L P P L

(B) Schematic diagram of chimeric P450

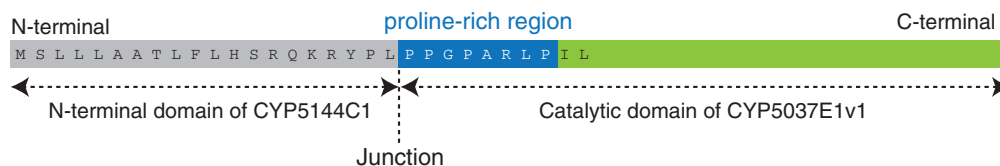


Fig. 1. N-terminal amino acid sequence and schematic diagram of chimeric P450. (A) Amino acid sequences of the N-terminal domain (gray), proposed proline-rich region (blue), and the following catalytic domain (green) of chimeric P450s. (B) Diagram of chimeric CYP5037E1v1 modified with the N-terminal domain of CYP5144C1. The catalytic domain (blue and green) of the target P450 was fused to the N-terminal domain (gray) of different P450 species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

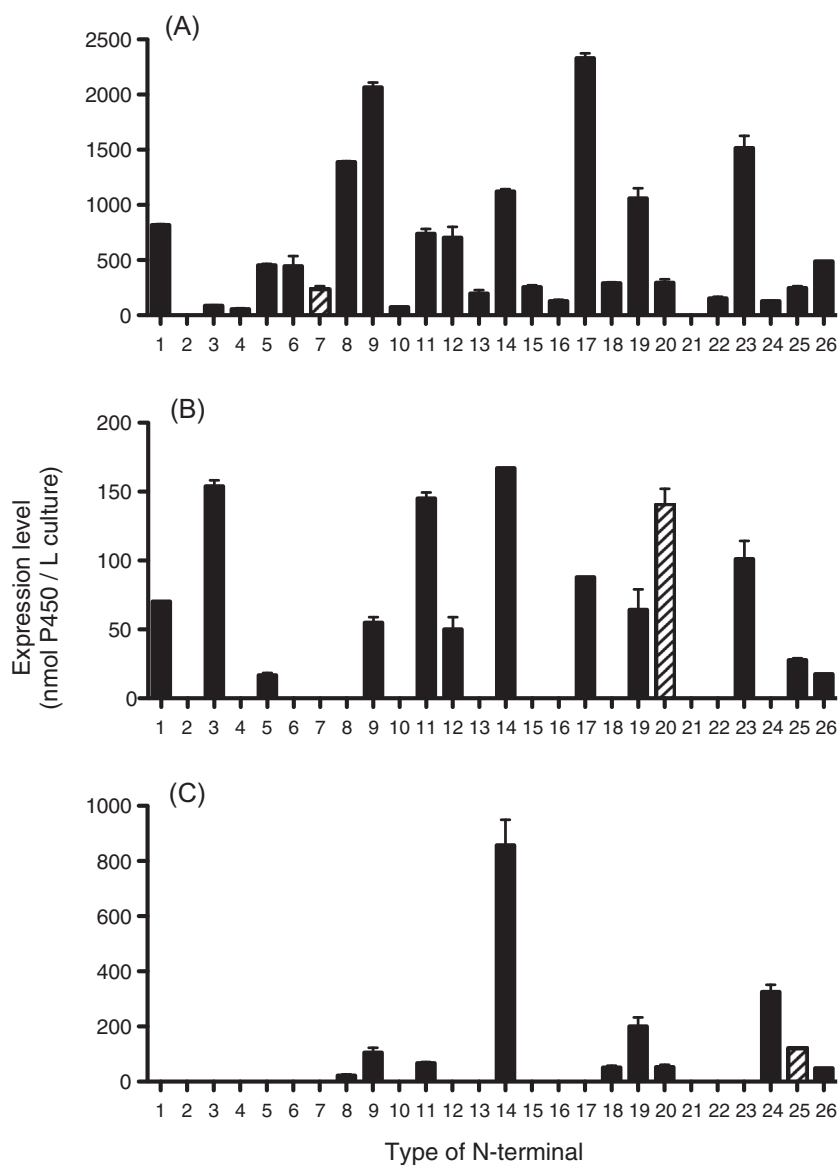


Fig. 2. Expression levels of chimeric CYP5037E1v1 (A), CYP5148A1 (B), and CYP5150E1v2 (C). The target P450s were modified with the N-terminal domains of (1) CYP51F, (2) CYP53C2, (3) CYP61A1, (4) CYP512E1, (5) CYP5037B3, (6) CYP5037B4, (7) CYP5037E1v1, (8) CYP5037E5v2, (9) CYP5136A1, (10) CYP5137A2, (11) CYP5137A4v1, (12) CYP5137A4v2, (13) CYP5139A1, (14) CYP5139D7v1, (15) CYP5141A4, (16) CYP5141A6, (17) CYP5144C1, (18) CYP5146A1, (19) CYP5147B1, (20) CYP5148A1, (21) CYP5148A2, (22) CYP5148B4v2, (23) CYP5149A1, (24) CYP5150A2, (25) CYP5150E1v2, and (26) CYP3A4. Data for the non-chimeric form of P450 are represented by the striped bar (see Table 1).

chimeric P450s were improved dramatically by the N-terminal replacements, although not all of the constructs responded equally well (Table 2). The N-terminal domain of CYP5139D7v1 also potentially improved the chance of heterologous expression even when the expression of the wild-type sequence looked unpromising (Fig. 3). Up to now, we have successfully expressed CYP5139D1, CYP5139D3v1, CYP5139D3v2, and CYP5139D8 after chimerization with the N-terminal domain of CYP5139D7v1.

In summary, we conducted a large-scale screening of the heterologous expression of fungal P450s in *E. coli*. This comprehensive approach resulted in the identification of a number of P450s that were readily expressed with/without the simple sequence deletion of the N-terminal TMD. We also showed that N-terminal domain of CYP5144C1 and CYP5139D7v1 could be flexible chimeric partners that can promote the high-level expression of membrane-bound P450s in *E. coli*. Moreover, this study revealed that the choice of combinations of N-terminal and catalytic domains is critical for high-level expression because the expression level of a chimeric

P450 could change dramatically depending on the N-terminal domain. Therefore, the variety of N-terminal sequences identified in this study may increase the chance of heterologous expression of

Table 2

Expression level of chimeric P450 modified with the N-terminal domain of CYP5144C1.

P450	Expression level (nmol/L culture)	Relative change (fold increased)
CYP5037B3	1213 ± 53	7.8
CYP5037B4	0	0
CYP5037E1v1	2330 ± 44	10.2
CYP5037E5v2	0	0
CYP5146A1	1041 ± 118	2.7
CYP5147B1	531 ± 4	0.4
CYP5148A2	44 ± 9	0.1
CYP5149A1	2172 ± 62	6.5

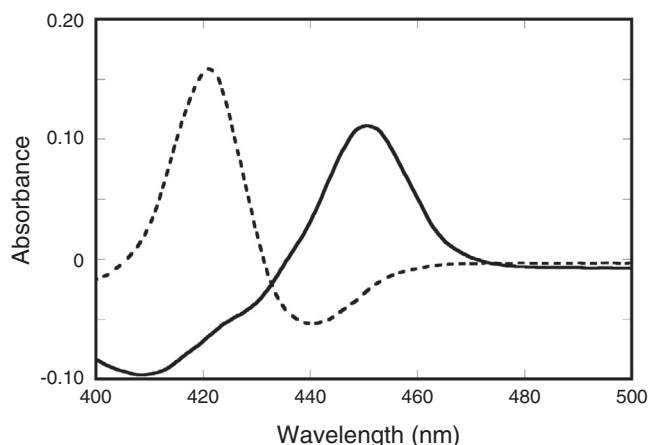


Fig. 3. CO difference spectra for CYP5139D8. Solid line, chimeric CYP5139D8 modified with CYP5139D7v1; broken line, non-chimeric CYP5139D8.

a series of membrane-bound P450 from fungi as well as from other animals and plants.

Acknowledgments

This research was supported in part by a Grant-in-Aid (No. 24688034) for Young Scientists (A) (to H.I.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2013.07.057>.

References

- [1] D.R. Nelson, A world of cytochrome P450s, in: *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 368 (2013) 20120430.
- [2] D.R. Nelson, The cytochrome p450 homepage, *Hum. Genomics* 4 (2009) 59–65.
- [3] P.R. Ortiz de Montellano, *Cytochrome P450: Structure, Mechanism, and Biochemistry*, third ed., Kluwer Academic/Plenum Publishers, New York, 2005.
- [4] S.L. Kelly, D.E. Kelly, Microbial cytochromes P450: biodiversity and biotechnology. Where do cytochromes P450 come from, what do they do and what can they do for us?, in: *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 368 (2013) 20120476.
- [5] S. Hirosue, M. Tazaki, N. Hiratsuka, S. Yanai, H. Kabumoto, R. Shinkyo, A. Arisawa, T. Sakaki, H. Tsunekawa, O. Johdo, H. Ichinose, H. Wariishi, Insight into functional diversity of cytochrome P450 in the white-rot basidiomycete *Phanerochaete chrysosporium*: involvement of versatile monooxygenase, *Biochem. Biophys. Res. Commun.* 407 (2011) 118–123.
- [6] M. Ide, H. Ichinose, H. Wariishi, Molecular identification and functional characterization of cytochrome P450 monooxygenases from the brown-rot basidiomycete *Postia placenta*, *Arch. Microbiol.* 194 (2012) 243–253.
- [7] K.H.M.N.H. Nazir, H. Ichinose, H. Wariishi, Molecular characterization and isolation of cytochrome P450 genes from the filamentous fungus *Aspergillus oryzae*, *Arch. Microbiol.* 192 (2010) 395–408.
- [8] K.H.M.N.H. Nazir, H. Ichinose, H. Wariishi, Construction and application of a functional library of cytochrome P450 monooxygenases from the filamentous fungus *Aspergillus oryzae*, *Appl. Environ. Microbiol.* 77 (2011) 3147–3150.
- [9] H.J. Barnes, M.P. Arlotto, M.R. Waterman, Expression and enzymatic activity of recombinant cytochrome P450 17 alpha-hydroxylase in *Escherichia coli*, *Proc. Natl. Acad. Sci. USA* 88 (1991) 5597–5601.
- [10] D. Cheng, R.W. Kelley, G.F. Cawley, W.L. Backes, High-level expression of recombinant rabbit cytochrome P450 2E1 in *Escherichia coli* C41 and its purification, *Protein Expr. Purif.* 33 (2004) 66–71.
- [11] C.H. Yun, S.K. Yim, D.H. Kim, T. Ahn, Functional expression of human cytochrome P450 enzymes in *Escherichia coli*, *Curr. Drug. Metab.* 7 (2006) 411–429.
- [12] H. Ichinose, H. Wariishi, Heterologous expression and mechanistic investigation of a fungal cytochrome P450 (CYP5150A2): involvement of alternative redox partners, *Arch. Biochem. Biophys.* 518 (2012) 8–15.
- [13] A. Warrilow, C. Ugochukwu, D. Lamb, D. Kelly, S. Kelly, Expression and characterization of CYP51, the ancient sterol 14-demethylase activity for cytochromes P450 (CYP), in the white-rot fungus *Phanerochaete chrysosporium*, *Lipids* 43 (2008) 1143–1153.
- [14] T. Hirokawa, S. Boon-Chieng, S. Mitaku, SOSUI: classification and secondary structure prediction system for membrane proteins, *Bioinformatics* 14 (1998) 378–379.
- [15] T. Omura, R. Sato, The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties, *J. Biol. Chem.* 239 (1964) 2379–2385.
- [16] S.B. Mulrooney, L. Waskell, High-level expression in *Escherichia coli* and purification of the membrane-bound form of cytochrome b(5), *Protein Expr. Purif.* 19 (2000) 173–178.
- [17] S.J. Pernecky, J.R. Larson, R.M. Philpot, M.J. Coon, Expression of truncated forms of liver microsomal P450 cytochromes 2B4 and 2E1 in *Escherichia coli*: influence of NH₂-terminal region on localization in cytosol and membranes, *Proc. Natl. Acad. Sci. USA* 90 (1993) 2651–2655.
- [18] A. Shukla, W. Huang, I.M. Depaz, E.M. Gillam, Membrane integration of recombinant human P450 forms, *Xenobiotica* 39 (2009) 495–507.
- [19] T. Ahn, S. Yang, C.H. Yun, High-level expression of human cytochrome P450 1A2 by co-expression with human molecular chaperone HDJ-1(Hsp40), *Protein Expr. Purif.* 36 (2004) 48–52.
- [20] C. von Wachenfeldt, T.H. Richardson, J. Cosme, E.F. Johnson, Microsomal P450 2C3 is expressed as a soluble dimer in *Escherichia coli* following modification of its N-terminus, *Arch. Biochem. Biophys.* 339 (1997) 107–114.
- [21] H. Iwata, K. Fujita, H. Kushida, A. Suzuki, Y. Konno, K. Nakamura, A. Fujino, T. Kamataki, High catalytic activity of human cytochrome P450 co-expressed with human NADPH-cytochrome P450 reductase in *Escherichia coli*, *Biochem. Pharmacol.* 55 (1998) 1315–1325.
- [22] E. Licad-Coles, K. He, H. Yin, M.A. Correia, Cytochrome P450 2C11: *Escherichia coli* expression, purification, functional characterization, and mechanism-based inactivation of the enzyme, *Arch. Biochem. Biophys.* 338 (1997) 35–42.
- [23] T. Kitazume, N. Takaya, N. Nakayama, H. Shoun, *Fusarium oxysporum* fatty-acid subterminal hydroxylase (CYP505) is a membrane-bound eukaryotic counterpart of *Bacillus megaterium* cytochrome P450BM3, *J. Biol. Chem.* 275 (2000) 39734–39740.
- [24] N. Nakayama, A. Takemae, H. Shoun, Cytochrome P450foxy, a catalytically self-sufficient fatty acid hydroxylase of the *Fusarium oxysporum*, *J. Biochem.* 119 (1996) 435–440.
- [25] T. Kitazume, A. Tanaka, N. Takaya, A. Nakamura, S. Matsuyama, T. Suzuki, H. Shoun, Kinetic analysis of hydroxylation of saturated fatty acids by recombinant P450foxy produced by an *Escherichia coli* expression system, *Eur. J. Biochem.* 269 (2002) 2075–2082.
- [26] Z. Guo, E.M. Gillam, S. Ohmori, R.H. Tukey, F.P. Guengerich, Expression of modified human cytochrome P450 1A1 in *Escherichia coli*: effects of 5' substitution, stabilization, purification, spectral characterization, and catalytic properties, *Arch. Biochem. Biophys.* 312 (1994) 436–446.
- [27] K. Kusano, M. Sakaguchi, N. Kagawa, M.R. Waterman, T. Omura, Microsomal p450s use specific proline-rich sequences for efficient folding, but not for maintenance of the folded structure, *J. Biochem.* 129 (2001) 259–269.
- [28] K. Kusano, N. Kagawa, M. Sakaguchi, T. Omura, M.R. Waterman, Importance of a proline-rich sequence in the amino-terminal region for correct folding of mitochondrial and soluble microbial p450s, *J. Biochem.* 129 (2001) 271–277.
- [29] B. Kemper, Structural basis for the role in protein folding of conserved proline-rich regions in cytochromes P450, *Toxicol. Appl. Pharmacol.* 199 (2004) 305–315.
- [30] E. Szczesna-Skorupa, P. Straub, B. Kemper, Deletion of a conserved tetrapeptide, PPGP, in P450 2C2 results in loss of enzymatic activity without a change in its cellular location, *Arch. Biochem. Biophys.* 304 (1993) 170–175.
- [31] C.D. Chen, B. Kemper, Different structural requirements at specific proline residue positions in the conserved proline-rich region of cytochrome P450 2C2, *J. Biol. Chem.* 271 (1996) 28607–28611.
- [32] C.D. Chen, B. Doray, B. Kemper, A conserved proline-rich sequence between the N-terminal signal-anchor and catalytic domains is required for assembly of functional cytochrome P450 2C2, *Arch. Biochem. Biophys.* 350 (1998) 233–238.
- [33] E.M. Gillam, T. Baba, B.R. Kim, S. Ohmori, F.P. Guengerich, Expression of modified human cytochrome P450 3A4 in *Escherichia coli* and purification and reconstitution of the enzyme, *Arch. Biochem. Biophys.* 305 (1993) 123–131.