Review

Genetically Engineered Bacterial Cells Co-expressing Human Cytochrome P450 with NADPH-cytochrome P450 Reductase: Prediction of Metabolism and Toxicity of Drugs in Humans

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Summary: Genetically engineered bacterial cells expressing human cytochrome P450 (CYP) have been developed as new tools to predict the metabolism and toxicity of drugs in humans. There are various host cells for the heterologous expression of a form of CYP. Among them, bacterial cells such as Escherichia coli (E. coli) have advantages with regard to ease of use and high yield of protein. CYP protein could be first expressed by the modification of the N-terminal amino acid sequence in E. coli cells in 1991. Since then, many forms of human CYP have been successfully expressed in E. coli cells. Since the E. coli cells do not possess endogeneous electron transport systems to support the full catalytic activity of CYP, E. coli strains co-expressing both human CYP and NADPH-cytochrome P450 reductase (OR) have been established. Each form of CYP expressed in the E. coli cells efficiently catalyzed the oxidation of a representative substrate at an efficient rate, indicating that the OR was sufficiently expressed to support the catalytic activity of CYP. According to the studies performed so far, the modification of the N-terminal amino acid sequence of CYP did not seem to affect the catalytic properties of CYP. The human CYP expressed in the E. coli cells were applicable for studies to determine a metabolic pathway(s) of drugs and to estimate kinetic parameters of drug metabolism by human CYP. Drug-drug interactions caused by inhibition of the metabolism of drugs by human CYP could also be examined by in vitro inhibition studies with CYP expressed in the E. coli cells.

Recently, human CYP was co-expressed with the OR in Salmonella typhimurium (S. typhimurium) cells used for mutation assay (Ames test) by applying the technology for the expression of human CYP and the OR in E. coli cells, to evaluate whether chemicals including drugs are metabolically activated by human CYP and show mutagenicity.

These strains of bacteria are considered as useful tools to study the metabolism and the toxicity of drugs in humans.

Key words: Escherichia coli; Salmonella typhimurium; heterologous expression; drug metabolism

Introduction

Cytochrome P450 (CYP) is one of the phase I drug-metabolizing enzymes. It is an integral membrane-bound heme-containing enzyme. CYP catalyzes the oxidative metabolism of a wide variety of exogenous and endogenous chemicals including drugs and carcinogens. Catalyzing the bio-oxidation reactions, CYP enzymes play major roles in the detoxification and activation of chemicals to modify the actions of chemicals including drugs. Therefore, it is important to examine the properties of CYP to estimate the pharmacokinetics of drugs.

CYP superfamilies are subdivided into families and subfamilies according the homology of their amino acid sequences. Four of the families have been identified to catalyze the oxidation of foreign chemicals in humans. It has been clarified that catalytic properties are different among CYP forms. Furthermore, the catalytic properties of CYP even in the same family vary among animal species. In previous studies, rodents have been widely used to analyze drug metabolism by CYP. If drugs are metabolized by CYPs present in rodents with the same properties as humans, the results obtained by using rodents are applicable to extrapolate to humans. However, there are large species differences in the
properties of CYPs between humans and rodents. Therefore, it is difficult to extrapolate the data obtained by using rodents to humans. Thus, it is necessary to use human CYP to predict human drug metabolism that affects drug actions and toxicities. Recently, human liver specimens have been used as an alternative tool to predict human drug metabolism, while the application of these preparations is limited by several factors, including ethical reasons. Furthermore, the population of drug-metabolizing enzymes varies according to the medical background of donor patients. Another disadvantage of the use of human liver samples is the low level of drug-metabolizing enzymes in these biological materials.

Thus, there was a need to establish an alternative method(s) to overcome the species differences and to directly predict the drug metabolism in humans. We introduce herein an alternative method to predict human drug metabolism without using experimental animals and human samples. The method is based on the heterologous expression of human drug-metabolizing enzymes. As an example, the heterologous expression system of CYP using bacterial cells will be introduced.

The use of CYP preparations expressed in heterologous expression systems has become a more popular method to examine human drug metabolism, partly because the preparation possessing the same properties can be supplied constantly. Several factors should be considered to select the proper cDNA expression system. Protein yield and expense are primary concerns. Efforts made in recent years have realized the expression of several CYP forms in yeast, cultured mammalian cells, and insect cells. However, in general, the yield of the CYP protein in yeast or mammalian cells is low.

Among the heterologous expression systems, bacterial cells including *Escherichia coli* (*E. coli*) and *Salmonella typhimurium* (*S. typhimurium*) have advantages compared to other expression systems in terms of low cost to maintain, ease of use, and, and the high yield of protein with a relatively short period of culture. Although the great usefulness of the bacterial expression system to express various prokaryotic and eukaryotic proteins had been demonstrated, the use of bacterial cells for the expression of CYPs had been limited primarily to the soluble prokaryotic CYPs. Barnes et al. first expressed a eukaryotic CYP17A in *E. coli*. When the native cDNA was introduced into an expression vector, no immuno-reactive CYP17A protein was produced following the stimulation of the *tac* promoters, while the modification of cDNA coding for the amino-terminal of CYP17A led to the expression of the protein in *E. coli*. In subsequent studies, efforts have been paid to improve the modification method of the N-terminal amino acid sequence of CYPs. Many reports have appeared showing that the CYP enzyme expressed in *E. coli* after the modification of the N-terminus still possessed catalytic activities in reconstituted systems containing NADPH-cytochrome P450 reductase (OR) purified from liver microsomes from an appropriate animal. Bacterial cells have endogenous electron transport systems, while the capacity of the system to transport electrons is not sufficient to support the full catalytic activity of CYP expressed in bacterial cells. Thus, the addition of the purified preparation of the OR was needed to reconstitute the system for the efficient catalytic activity of CYP.

To make it easy to predict human drug metabolism or toxicological properties using the bacterial expression system, the co-expression of both CYP and other enzymes responsible for the transport of electrons from NADPH or NADH to CYPs was assumed to be needed. Thus, in recent studies some CYP forms were successfully expressed in *E. coli* together with the OR.

The aims of this review are to briefly summarize reports on the establishment of bacterial strains expressing CYP alone or together with an electron transport enzyme such as the OR. We will discuss the advantages and disadvantages of their application as a method to study drug metabolism and toxicology in humans. We will also mention the practical application and the perspectives in the use of bacterial cells expressing CYP.

**Properties of Bacterial Expression Systems**

Considerable amounts of knowledge concerning the *E. coli* expression system have been accumulated over the years. It has been assumed that any of the proteins can be produced in *E. coli* as long as the protein molecule is not too small, too large or too hydrophobic, and does not contain too many cysteines. These generalizations seem correct; if one wants to express Factor VIII or a complex mammalian cell surface receptor containing about 40 disulfides, *E. coli* may not be an appropriate host. However, *E. coli* is a suitable and often desirable host for a wide variety of other proteins. *E. coli* expression systems have been developed and refined for various usages such as direct protein expression, fusion protein expression and secretion.

An *E. coli* expression system is, in general, useful for the production of a heterologous protein consisting of 100 to 300 amino acids, assuming that there is not an inordinate number of cysteins. Fusion protein expression strategies often permit us to overcome the problems of protein instability. In fact, the fusion approach was a preferred method for an immunogen generation. The fusion methods also have advantages in the purification of a certain protein(s). The anaerobic environment present in *E. coli* does not permit cystein-rich proteins to form the disulfide bonds required for proper conformation. This problem can sometimes be overcome by
secretion into a more aerobic environment. However, protein secretion from \textit{E. coli} is still largely a hit-or-miss proposition and is most likely to work with a natural protein being secreted.

\textit{E. coli} cells possess proteases like yeast cells and mammalian cells.\textsuperscript{20} Lon is a major ATP-dependent protease in \textit{E. coli}, and seems to be responsible for the degradation of a number of naturally unstable proteins and many abnormal proteins.\textsuperscript{28} The Lon protease shows relatively broad substrate specificity for unfolded or misfolded proteins. \textit{E. coli} cells possessing a mutant Lon may be useful to prevent the degradation of proteins expressed in \textit{E. coli} cells to be examined.

For the purpose of gene expression in heterologous cells, proteins may be classified into three broad classes.\textsuperscript{29} The first class covers small secreted peptides, with a molecular size of less than 80 amino acids. These are most easily expressed as fusion proteins usually in \textit{E. coli}. The second class includes polypeptides that are normally secreted (e.g., enzymes, cytokines, hormones), and range in size from 80 to 500 amino acid residues. The proteins in this class are often the easiest to express in \textit{E. coli}. In particular, protein expression in \textit{E. coli} has proven to be extremely effective for the proteins in a molecular size ranging from 100 to 200 amino acids. A third class consists of very large, greater than about 500 amino acids, secreted proteins and cell surface proteins. The mammalian cell expression systems have often been used to express the proteins included in this class. CYP proteins fall into this class; however, compared to secreted proteins, much less work has been directed toward over-expression of proteins in this category.

**Expression of Human CYPs in Bacterial Cells**

In 1991, Barnes \textit{et al.}\textsuperscript{17} first expressed catalytically active mammalian CYP, bovine CYP17A, in \textit{E. coli} JM109 cells. They used the pCW vector to construct the expression plasmid. The plasmid contains two \textit{tac} promoters induced by isopropyl-\textit{b}-D-thiogalactopyranoside (IPTG) upstream of an \textit{Nde} I restriction enzyme cloning site coincident with the initiation ATG codon. The plasmid also contains a \textit{trpA} transcription terminator sequence to prevent the read-through, a phage M13 origin of DNA replication, and the \textit{laci} gene coding for the Lac repressor that prevents transcription from the \textit{tac} promoters prior to the addition of a certain inducing agent(s). The plasmid has often been employed to express various forms of CYP in bacterial cells.

To express the CYP17A, they modified the cDNA sequence encoding the N-terminal portion of the amino acid sequence to optimize the parameters, such as a codon usage preferable to \textit{E. coli} and free energy to form the secondary structure of mRNA, since they failed to express the native form of CYP17A. More specifically, the native second codon was changed from TGG (Trp) to GCT (Ala), a preferred second codons for expression of the \textit{LacZ} gene,\textsuperscript{30} and codons 4 and 5 were modified to TTA (silent mutations), since the region of the most mRNAs expressed in \textit{E. coli} has been shown to be adenosine and uridine nucleotides.\textsuperscript{32} In addition, the last nucleotide of codon 6 and 7 was changed to adenosine and thymidine (silent mutations), respectively, to minimize the formation of a secondary structure in the mRNA.\textsuperscript{33}

So far, a large number of reports on the expression of mammalian CYPs including human CYP in bacterial cells have been published, utilizing a method based on the above concept. In this chapter, the results obtained thus far on the expression for each family of human CYPs in bacterial cells will be mainly introduced. All of the bacterial expression systems for various human CYPs introduced in this review are summarized in Table 1.

**CYP1 Family**

The CYP1 family consists of two forms of CYP1A, CYP1A1 and CYP1A2, and CYP1B1 in humans.\textsuperscript{25} Guo \textit{et al.}\textsuperscript{19} established an \textit{E. coli} strain expressing human CYP1A1. They designed some CYP1A1 cDNAs containing the 5'-terminus modified by methods proposed by Barnes \textit{et al.}\textsuperscript{17} They constructed expression plasmids with pCW vector and introduced them into \textit{E. coli} DH5\textalpha{} cells, and compared the expression level of CYP1A1 holo-protein among constructions containing a different modification of the 5'-terminus of CYP1A1 cDNA. Modified Terrific Broth medium containing 100 mg/L ampicillin, 1.0 mM IPTG, 1.0 mM thiamine and trace elements\textsuperscript{34} was employed as a culture medium for the expression of CYP protein in \textit{E. coli} cells. The highest expression level of CYP1A1 protein in the membrane fractions of the established \textit{E. coli} cells was seen when the second amino acid from the start codon was modified from Leu to Ala, and a subsequent cDNA sequence encoding third to ninth amino acid residues was modified by the enhancement of the content of the AT nucleotides. All the latter modifications were silent mutations. The maximal expression level of CYP1A1 protein in the membrane fractions of the established \textit{E. coli} cells was 25 nmol/L culture. When the membrane fractions supplemented with the OR purified from rabbit were incubated with a substrate and NADPH, the catalytic activities of CYP1A1 toward benzo[a]pyrene (B[a]P) and 7-ethoxyresorufin were low, even in the presence of human cytochrome b\textsubscript{5}. On the other hand, when the purified preparations of CYP1A1 from the \textit{E. coli} cells were incubated with the OR, a substrate and NADPH, the enzyme showed catalytic activities toward B[a]P and 7-ethoxyresorufin even in the absence of cytochrome b\textsubscript{5}. A certain membrane factor(s) might inhibit the interaction of the OR and CYP1A1.
### Table 1. Summary of expression of various human CYPs in bacterial cells

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Contd.
Human CYP1A2 was first expressed in *E. coli* by Fisher *et al.* in 1992. The N-terminal region of the CYP1A2 protein was modified by the nine amino acids of the N-terminus of bovine CYP17A. The modified CYP1A2 cDNA was inserted into pCW vector. The established expression plasmid was introduced into *E. coli* DH5α cells. The maximal expression level of CYP1A2 was 700 nmol/L culture in the bacterial whole cells. Estradiol 2-hydroxylase, 7-ethoxycoumarin *O*-deethylase and 7-ethoxyresorufin *O*-deethylase activities were examined with membrane fractions of *E. coli* cells, supplemented with twice the amount of the rat OR molecule to CYP. Km values for estradiol 2-hydroxylation, 7-ethoxycoumarin *O*-deethylase and 7-ethoxyresorufin *O*-deethylation were 13, 44 and 0.01 μM, with $V_{\text{max}}$ values of 1.5, 0.36 and 2.5 nmol/min/nmol CYP, respectively. Sandhu *et al.* have also established an *E. coli* strain harboring human CYP1A2. CYP1A2 cDNAs containing the various modified 5′-terminus inserted into the pCW vector. The highest expression of CYP protein was obtained when the N-terminus of the CYP was modified according the method of Fisher *et al.* CYP1A2 expressed in the membrane fractions catalyzed the oxidations of 7-ethoxyresorufin and phenacetin. The turnover number was about 0.02 nmol/min/nmol CYP in the presence of the rabbit OR. The catalytic activity of CYP1A2 purified from the *E. coli* cells toward 7-ethoxyresorufin and phenacetin was also examined in a reconstituted system containing the rabbit OR. The activities were 0.61 nmol/min/nmol CYP for 7-ethoxyresorufin and 1.38 nmol/min/nmol CYP for phenacetin. It may be possible that a certain factor(s) of membrane inhibits the access of the OR to CYP1A2. Two independent groups have established the tester strains of *E. coli* expressing human CYP1A2 for mutation assays as mentioned below. Kranendonk *et al.* established a new strain of *E. coli* expressing human CYP1A2 to detect the mutagenicity of various promutagens activated by the CYP. They applied the expression plasmid of human CYP1A2 developed by Fisher *et al.* and introduced the plasmid carrying human CYP1A2 together with another plasmid pACYC177 carrying the *mucAB* operon essential for the mutagenicity test into *E. coli* MX100 cells, designated as BMX100/h1A2. MX100 was used as host, since no CYP activity was detectable. The mutagenicity of 2-aminoanthracene (2-AA) and aflatoxin B$_1$ (AFB$_1$) was detectable with the established *E. coli* cells. Chun transformed a plasmid carrying human CYP1A2 into *E. coli* WP2 *uvrA* cells to establish a new mutagenicity tester strain. The construction of the expression plasmid was completely the same as that reported by Sandhu *et al.* The maximal expression was observed at 48 h after the IPTG addition. The level of CYP1A2 in the membrane fraction of the established *E. coli* cells was about 300 nmol/L culture. They detected the mutagenicity of 2-AA and 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) using the *E. coli* cells. In these two systems, the electrons might be transported to CYP1A2 via a bacterial endogenous electron transport system.

Human CYP1B1 was first expressed in *E. coli* DH5α cells in 1998. The N-terminus of the CYP1B1 cDNA was modified by removal of codons from 2 to 4 and replacement of the nucleotide sequence of the resulting N-terminal seven codons to enrich the content of AT nucleotides, since the region of the most mRNAs expressed in *E. coli* has been shown to be adenosine and uridine nucleotides. The modified CYP1B1 cDNA was inserted into pCW expression vector. The expression level of the enzyme was 200 nmol/L culture. The catalytic activity of CYP1B1 purified from the *E. coli* cells was examined in a reconstituted system with the purified

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### Table 1. Continued

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rabbit OR. 7-Ethoxyresorufin O-deethylase activity was about 2.0 nmol/min/nmol CYP in the presence of twice the amount of the purified rabbit OR on a molar basis.

**CYP2 Family**

The CYP2 family was subdivided into ten subfamilies, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, CYP2F, CYP2G, CYP2J, CYP2R and CYP2S, in humans. CYP2A subfamily includes two catalytically active forms, CYP2A6 and CYP2A13. There are two members in the CYP2B subfamily, namely CYP2B6 and CYP2B7. CYP2B7 was known to be incapable of catalyzing any oxidations of chemicals. The CYP2C subfamily consists of 4 members and is regarded as a major one present in human livers. These forms are responsible for the metabolism of clinically important drugs. CYP2D6, CYP2D7 and CYP2D8 are the members of the CYP2D subfamily. Only CYP2D6 possesses catalytic activity toward foreign chemicals. CYP2E1 is a sole form belonging to the CYP2E subfamily in humans.

We expressed human CYP2A6 in *E. coli* DH5α.\(^{27}\) *S. typhimurium* YG7108 and *S. typhimurium* TA1538 cells together with the human OR. The 5'-terminal cDNA sequence of CYP2A6 encoding amino acid residues from 2 to 24 was truncated and the modified cDNA was inserted into the pCW vector. The details are described in the chapter called ‘Co-expression of CYP with the OR’ in this review. Pritchard *et al.*\(^{38}\) established a strategy whereby unmodified CYP could be expressed at a high level in *E. coli* by a fusion of the N-terminal amino acids with bacterial leader sequences. They fused the 5'-terminus of CYP2A6 cDNA with cDNA encoding outer membrane protein A (*ompA*) and inserted it into the pCW vector. *E. coli* JM109 cells were transfected with the plasmid. They also modified the 5'-terminus of native CYP2A6 cDNA by the replacement of the cDNA coding for the first eight amino acids of CYP2A6 with the cDNA encoding amino acid sequence MALLLAVF. The expression levels in the whole cells were 193 nmol/L for the former and 455 nmol/L culture for the latter.

The signal peptide bound to the bacterial protein is known to be removed and rapidly digested during the translocation of the protein across the inner membrane, to leave the native protein.\(^{39}\) Unexpectedly, most of the signal peptides were retained. Coumarin 7-hydroxylase activity was determined with membrane fractions prepared from these genetically engineered *E. coli* cells in the presence of 100 μM cumene hydroperoxide. The activities were 0.34 nmol/min/nmol CYP for the *ompA* fusion CYP2A6 and 0.32 nmol/min/nmol CYP for the MALLLAVF fusion CYP2A6. These values were similar to those obtained by the reconstituted system containing CYP2A6 purified from human livers and by the microsomes isolated from SF9 cells infected with a recombinant baculovirus carrying CYP2A6 cDNA.\(^{40}\)

Recently, CYP2A13 was expressed in bacterial cells by us with the same manner to express CYP2A6 adopted by Iwata *et al.*\(^{27}\)

There are many reports on the expression of animal CYPs belonging to the CYP2B subfamily in *E. coli*.\(^{41–45}\) We have established *E. coli* strain expressing human CYP2B6 (unpublished data). The N-terminal 20 amino acid sequence of CYP2B6 was replaced by MAR tripeptide. AT nucleotides were enriched in the first 6 codons. The expression level of the recombinant CYP2B6 was 137 nmol/L culture. We also succeeded in expressing CYP2B6 together with the OR by the method of Iwata *et al.*\(^{27}\) as described below. Recently, Hanna *et al.*\(^{46}\) have developed *E. coli* cells expressing human CYP2B6.

CYP2C9 was expressed in *E. coli* by Sandhu *et al.*\(^{15}\) and Richardson *et al.*\(^{24}\) Sandhu *et al.*\(^{18}\) constructed expression plasmids carrying CYP2C9 cDNA with modifications in the 5'-terminal region by using pCW vector and introduced them into *E. coli* DH5α cells. When the first eight amino acid residues were replaced by MALLLAVF motif or when twenty amino acid residues of the N-terminus were replaced by MA residues, detectable amounts of CYP2C9 holo-protein were generated. The expression level of CYP2C9 was 5 to 11 nmol/L culture for the former construct and 9 to 19 nmol/L culture for the latter construct. The catalytic activities of CYP2C9 purified from the strains of the *E. coli* toward tolbutamide were similar to those obtained with CYP2C9 purified from liver in the presence of human cytochrome b\(_s\). In a subsequent study performed by Richardson *et al.*,\(^{24}\) CYP2C8, CYP2C9, CYP2C18 and CYP2C19 were generated in *E. coli* XL-1 blue cells. They replaced CYP2C cDNAs at the 5'-terminal region coding for initial 18 amino acids with a cDNA encoding a universal amino acid sequence MALLLAVF, followed by LGLSCLLLLLS. The resultant cDNAs were ligated into the pCW vector. The expression levels were 1500 nmol/L culture for CYP2C8, 500 nmol/L culture for CYP2C9, 450 nmol/L culture for CYP2C18 and 700 nmol/L culture for CYP2C19. They partially purified CYP2C proteins and reconstituted with the OR. The catalytic activities of the modified human CYP2Cs expressed in *E. coli* cells were examined with representative substrates including (S)-methylenedioxyamphetamine and tetrahydrocannabinol (THC) for CYP2C9 and taxol for CYP2C8. The (S)-mephenytoin 4'-hydroxylase activity of CYP2C19 at a concentration of 0.4 mM was 3.3 nmol/min/nmol CYP. The activities of CYP2C9 in the oxidation of tolbutamide and THC were 1.4 nmol/min/nmol CYP (at 1.0 mM) and 16.2 nmol/min/nmol CYP (at 0.13 mM), respectively. CYP2C8 expressed in the *E. coli* cells catalyzed the hydroxylation of taxol with a turnover number...
of 3.1 nmol/min/nmol CYP (at 0.1 mM).

Gillam et al. first established the genetically engineered E. coli DH5α cells expressing human CYP2D6. They prepared eleven CYP2D6 cDNAs with different modifications at the 5'-terminus, expecting the high level of expression in E. coli with the pCW vector. The expression level of holo-protein varied considerably depending on the constructions. The highest yield was obtained with a construction in which the hydrophobic 21 amino acids of the N-terminus were truncated and the following amino acid sequence was replaced with MARQVHSSWNL. The application of MALLLAVF to modify the N-terminal amino acid sequence of CYP2D6 was not optimal. The holo-protein was produced depending on the presence of δ-aminolevulinic acid and FeCl3 in the culture. The optimal concentrations of δ-aminolevulinic acid and FeCl3 in the culture were 0.5 mM. The expression level of the CYP holo-protein in the whole cells was 90 nmol/L culture. They purified CYP2D6 and examined the catalytic activity of the enzyme in a reconstituted system with a five-fold excess amount of the rabbit OR. The catalytic activities of CYP2D6 toward debrisoquine 4-hydroxylation and bufuralol 1'-hydroxylation were almost similar to those obtained by the preparations of CYP2D6 purified from human liver microsomes. Kempf et al. introduced human CYP2D6 into E. coli JM109. They improved an expression plasmid to produce a large amount of CYP2D6 protein in a form suitable for purification. The hydrophobic 25 amino acids of CYP2E1 from MSALGVTV to MAALGVTV. Human CYP2E1 was first expressed in E. coli by Winters et al. They changed the first seven amino acids of CYP2E1 from MSALGVTV to MAALGVTV. Furthermore, the AT nucleotide content was enriched. The pSE420 vector, a derivative of pKK233-2, was adopted to construct the expression plasmid. pSE420 contains a trp/lac fusion trc promoter. This is repressed by the lac repressor and is induced by IPTG. The plasmid thus constructed was transformed into E. coli DH5α cells. They confirmed the expression of CYP2E1 by immunoblot analysis and CO-difference spectra with membrane fractions prepared from the established E. coli cells. The expression level of a CYP2E1 apo-protein determined by the immunoblot analysis increased 2- to 2.5-fold by the modification of the N-terminus of CYP2E1 amino acid sequence. The partially purified preparation of the recombinant CYP showed activity for the demethylation of N-nitrosodimethylamine (NDMA), when reconstituted with the OR purified from rat liver microsomes. Another approach was carried out by Gillam et al. They also established a genetically engineered E. coli DH5α harboring human CYP2E1. Variants of CYP2E1 cDNA with differently modified 5'-terminus were constructed and inserted into the pCW vector. The highest expression was achieved when the first 21 amino acids were deleted from the native CYP2E1 sequence and the resulting second amino acid Trp was changed to Ala. The expression level of the CYP was 160 nmol/L culture in the whole cells and 40 nmol/L culture in the membrane fractions. The activity of CYP2E1 purified from the E. coli cells was determined in a system reconstituted with the rabbit OR and human cytochrome b5. Chlorzoxazone 6-hydroxylase activity was detectable with a Vmax value of 5.56 nmol/min/nmol CYP and a Km value of 36 μM. Prichard et al. also developed E. coli expressing human CYP2E1 by the method employed to express CYP2A6. They fused a cDNA coding for the bacterial signal sequence ompA with the 5'-terminus of CYP2E1 cDNA, and introduced it into E. coli JM109 cells. They compared the expression level of the CYP2E1 holo-protein obtained by using the fused construction with that obtained by the construction in which the CYP2E1 cDNA encoding the first eight amino acids was replaced with cDNA coding for the MALLLAVF amino acid sequence. The expression levels in whole cells were 174 nmol/L culture for CYP2E1 fused to the ompA, and 68 nmol/L culture for CYP2E1 replaced with the MALLLAVF. The most signal peptides were retained as seen in the case with CYP2A6. Chlorzoxazone 6-hydroxylase activity was determined with membrane fractions from both strains of E. coli cells in the presence of 100 μM cumene hydroperoxide. The activity was similar to that seen with a reconstituted system containing human CYP2E1 purified from E. coli and the rabbit OR. It is of interest to analyze the location of CYP protein expressed in E. coli cells. Laeson et al. reported the expression of rabbit CYP2E1 in E. coli MV1304 cells. They examined the effect of the truncation of the N-terminal amino acid residues on the localization of the protein in E. coli cells. Approximately 75% of the CYP2E1 protein with a native N-terminus...
was located at the inner membrane of *E. coli*. The rest was present in cytosol. When the N-terminal amino acid residues from 3 to 29 were truncated, 60% of the CYP2E1 protein was still located in the inner membrane of *E. coli* cells.

**CYP3 Family**

The CYP3 family consists of only the CYP3A subfamily. CYP3A enzymes are abundantly expressed in the liver of humans. In humans, CYP3A4, CYP3A5 and CYP3A7 had been known to be forms belonging to the CYP3A subfamily. Recently, CYP3A43 was found by Gillam et al.\(^51\) Gillam et al.\(^51\) first developed the *E. coli* DH5α strain harboring human CYP3A4. CYP3A4 cDNAs containing the various modified 5′-terminus were constructed and inserted into pCW vector. When they replaced the N-terminal 18 amino acid residues with the amino acid motif MALLLAVFL, the highest expression of the recombinant CYP3A4 was achieved. The expression level in whole cells ranged from 200 to 370 nmol/L culture. CYP3A4 thus obtained was purified and reconstituted with the purified rabbit OR and human cytochrome b5, as was the case with CYP2E1. They demonstrated that the recombinant CYP3A4 in the reconstituted system showed catalytic activities of nifedipine oxidation, testosterone 6β-hydroxylation and AFB1 8,9-epoxidation. Prichard et al.\(^38\) also established the *E. coli* JM109 cells expressing human CYP3A4 in the same manner as that previously adopted to express both CYP2A6 and CYP2E1. The cDNA encoding the bacterial signal sequence ompA or pelB was fused to the N-terminus of CYP3A4 cDNA. The modified CYP3A4 cDNA was inserted into the pCW plasmid. The expression levels of the cytochrome in whole cells were 143 nmol/L culture for the recombinant CYP3A4 fused with pelB and 502 nmol/L culture for the recombinant CYP3A4 fused with ompA. Testosterone 6β-hydroxylase and nifedipine oxidation activities were detectable with the membrane fractions of both strains of *E. coli* cells in the presence of 100 μM cumene hydroperoxide. Interestingly, signal peptides were being retained. Only in the case with CYP3A4 fused to pelB leader peptide yielded the native CYP3A4. This method may be suitable to produce the native CYP in bacterial cells.

*E. coli* DH5α strain expressing human CYP3A5 was established by Gillam et al. using pCW vector.\(^23\) They changed the 18 N-terminal amino acids to MALLLAVFL. The expression level in the membrane fractions of the *E. coli* cells was 260 nmol/L culture. They purified the recombinant CYP3A5 from *E. coli* cells and reconstituted it with the purified rabbit OR and human liver cytochrome b5. The purified preparations of CYP3A5 from the *E. coli* cells were capable of catalyzing nifedipine oxidation, testosterone 6β-hydroxylation, AFB1 8,9-epoxidation, N-ethylmorphine N-demethylation, erythromycin N-demethylation and d-benzphetamine N-demethylation. Interestingly, nifedipine oxidase, testosterone 6β-hydroxylase and AFB1 8,9-epoxidase activities depended on the presence of cytochrome b5 and divalent cations.

CYP3A7 mainly expressed in the human fetal liver was successfully generated in *E. coli* DH5α cells by Gillam et al.\(^23\). They constructed more than ten CYP3A7 cDNAs with a differently modified 5′-terminus to achieve the high level of expression in *E. coli*. However, the expression levels of CYP3A7 holo-protein were 3 to 20-fold lower than those obtained for all other CYPs, under the same culture conditions. When the N-terminus of the CYP3A7 was modified by the replacement with MALLLAVFL motif adopted for the expression of bovine CYP17A, the maximal expression of CYP3A7, 43 nmol/L culture, was observed. They partially purified the recombinant CYP3A7. The role of CYP3A7 in the activation of some promutagens including 6-aminochorysene, AFB1, sterigmatocystin, 2-AA, 2-aminofluorene were examined and compared with those of CYP3A4. In a recent study, Inoue et al.\(^39\) revealed that holo-CYP3A7 was expressed efficiently when CYP3A7 was co-expressed with molecular chaperone GroEL, known to assist the correct folding of unfolded proteins.\(^55\) The expression level of the holo-CYP3A7 protein was over 100 nmol/L culture. Dehydroepiandrosterone 16α-hydroxylation was catalyzed by CYP3A7 expressed in the presence of GroEL.

The novel CYP3A, namely CYP3A43, was successfully expressed in *E. coli* DH5α cells.\(^56\) For efficient expression in *E. coli*, PCR was used to modify the N-terminus of CYP3A43. Amino acid residues 2 to 12 were deleted and residues 13 to 18 were modified to coincide with the corresponding sequence described by Barnes et al. for CYP17A.\(^17\) The modified cDNA was inserted into pSE380 expression vector.

**CYP4 Family**

In humans, the CYP4 family includes five subfamilies, CYP4A, CYP4B, CYP4F, CYP4V and CYP4X. There are two reports on the expression of CYPs belonging to the CYP4 family in a heterologous expression system. One is on the expression of rabbit CYP4A4 in *E. coli* JM109 cells and the other is on the expression of rat CYP4F4 and CYP4F5 in *E. coli* DH5α cells.\(^57,58\) To our knowledge, there is no report on the expression of human CYPs in the CYP4 family in any bacterial cells.

The expression of eukaryotic CYP has also been summarized in the twenty-second chapter of a book titled “Cytochrome P450 Protocols.”\(^59\)

Recently, Kusano et al.\(^60\) reported that the antibiotics chloramphenicol, tetracycline and erythromycin, which inhibit bacterial protein synthesis and are known to in-
duce cold shock response, unexpectedly stimulate the expression of CYP in E. coli cells. A sublethal dose of chloramphenicol (1 mg/L) efficiently enhanced the expression of bovine CYP17A over a range of two-to-three-fold. In contrast, antibiotics inducing heat shock response in E. coli such as puromycin, streptomycin and kanamycin suppressed the expression of CYP17A. The effects of antibiotics inducing cold shock response on the expression of human CYP in bacterial cells need to be further examined. The addition of such chemicals into culture of E. coli cells harboring human CYP may be useful to the high-level expression of CYP in E. coli cells. Interestingly, the expression of mitochondrial CYP11A in E. coli was not increased by the addition of a sublethal dose of antibiotics causing the cold shock response in E. coli cells, but was induced by the addition of ethanol, an inducer of heat shock response in E. coli cells.

Expression of Enzyme Which Transfers Electrons to CYP in E. coli

The use of bacterial cells such as E. coli and S. typhimurium made it possible to achieve the high level of expression of the various CYPs. However, the bacterial enzyme(s) capable of transferring electrons from electron donor to CYPs was not sufficient to support their full catalytic activities. Thus, the addition of the purified preparation of the electron transfer partner was needed to reconstitute a system of CYPs. To produce the electron transport proteins such as OR and cytochrome b₅ in bacterial cells, many efforts have been made. In recent studies, some CYP forms were expressed in E. coli together with the OR. In this chapter, the expression of an electron transport protein alone or together with CYP in bacterial cells will be mentioned.

Expression of OR in E. coli

The OR is a flavoprotein with a molecular mass of 7.8 kDa, which is bound to endoplasmic reticulum of eukaryotic cells and is involved in the electron transfer from NADPH to CYPs. One molecule of the enzyme contains one each of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Virtually, no species difference in the function of the OR has been noted: the OR purified from the rat liver can transfer electrons to human CYP. There are two reports from a group on the expression of the rat OR in bacterial cells, while there is no report on the expression of the human OR alone.

Porter et al. first succeeded in expressing the rat OR in E. coli to identify the functional amino acid residues involved in the binding to FMN, FAD and NADPH. They adopted a pCQV2 expression vector, developed by Queen et al., which carried the promoter of λ bacteriophage and the ribosomal binding site and the translation initiation site of the cro gene for the expression of foreign proteins. The plasmid also contained the temperature-sensitive cI857 repressor gene, allowing temperature-dependent regulation and induction of the gene expression. Insertion of the coding nucleotide sequence for the rat OR into this vector allowed expressing as a functional protein. Various strains of E. coli cells were transfected with the constructed plasmid pCQR. Among them, E. coli C-1A cells showed the highest expression of the OR in the cell lysates. The expression level of the flavoprotein was approximately 0.1% of the total cellular protein. The purified preparations of the OR catalyzed a cytochrome c reduction at a rate about 35 μmol/min/mg of OR. On the other hand, the OR preparations purified from the rat liver catalyzed the reaction at a rate about 50 μmol/min/mg of OR. Moreover, the OR expressed in the bacterial cells supported the B[a]P metabolism in the reconstitution system containing rat CYP1A1 at a rate about half as effective as the enzyme purified from the rat liver. Porter et al. thought that the OR expressed in the E. coli cells was located inside of the E. coli membrane and was degraded by the protease of E. coli expressed in the cytoplasm. Thus, Shen, who was a colleague of Porter, expressed the rat OR in the same strain of E. coli using a vector pIN-III-ompA3 with which the generated protein could be secreted outside of the cell membrane. The pIN-III-ompA3 vector contained a bacterial lpp promoter and a signal peptide, outer membrane protein A (ompA), to direct the transport of the expressed protein out of the cytoplasm, namely into the periplasmic space, where proteolytic activity is reduced. Even when the plasmid was employed, the OR was still mainly presented in the membrane, probably because the hydrophobic amino-terminal sequence of the OR might anchor the protein to the inner membrane to prevent its release from the membrane. The apparent molecular mass of the OR was about 80 kDa. The OR expressed in the bacterial cells catalyzed cytochrome c reduction at a rate of 51.5 μmol/min/mg protein, almost similar to that seen with the rat liver enzyme (53.3 μmol/min/mg protein). The human OR was first expressed in E. coli in a co-expression manner. The details will be described in the chapter mentioned below on the co-expression of CYP together with the OR.

Expression of cytochrome b₅

Cytochrome b₅ is a membrane-bound heme protein found in many mammalian species. The hemoprotein is required for the function of a number of reactions catalyzed by CYPs. In addition, a soluble form of cytochrome b₅ is found in erythrocytes, where it serves as an electron carrier for the NADPH-dependent reduction of methemoglobin to hemoglobin. The
membrane-bound form of cytochrome \( b_5 \) is associated with the endoplasmic reticulum; it has a molecular mass of 16.7 kDa consisting of 134 amino acid residues. The amino acid sequences of hepatic cytochrome \( b_5 \) from several animal species have been determined. Their primary structures are highly conserved. This cytochrome is composed of three domains: a hydrophilic heme-containing catalytic domain of about 98 amino acids; a membrane-binding hydrophobic domain containing about 30 amino acids at the carboxy-terminus of the molecule;\(^7\) and a membrane-targeting region represented by the 10-amino-acid sequence located at the C-terminus of the membrane-binding domain.\(^6\)

The three-dimensional crystal structure of the bovine liver cytochrome \( b_5 \) has been determined by X-ray diffraction to a resolution of 2 Å.\(^7\) A multitude of techniques has been applied to elucidate the structure and the function of this cytochrome, including an interaction with electron transfer partners. The information of the molecule of cytochrome \( b_5 \) makes the enzyme an ideal target for site-directed mutagenesis experiments designed to investigate the molecular mechanisms of electron transfer, the control of heme protein redox potential, the specificity of protein-protein interactions, and the dynamics of heme protein folding. These endeavors would be greatly aided by an efficient expression of mammalian cytochrome \( b_5 \) in a microbial system.

Thus, efforts were made to heterologously express cytochrome \( b_5 \) in \( E. coli \), as the first example of the expression of a membrane-binding heme-containing protein in bacterial cells.

There are some reports on the expression of the mammalian cytochrome \( b_5 \) in \( E. coli \). Von Bodman et al.\(^6\) first expressed rat cytochrome \( b_5 \) in \( E. coli \). The pUC13 vector was employed to express native cytochrome \( b_5 \) containing the membrane anchor domain. It was previously shown that the plasmid favored the high-level expression of \( Pseudomonas putida \) cytochrome CYP101 in \( E. coli \).\(^6\) The constructed plasmid was introduced into \( E. coli \) TB-1 cells. The cytochrome \( b_5 \) protein produced in the \( E. coli \) cells constituted about 0.8% of the total protein. The cytochrome \( b_5 \) expressed in the bacteria had the same physical properties as the protein isolated from hepatic microsomes, as to the Soret band of an oxidized protein at 410 nm with an extinction coefficient to 130 mM\(^{-1}\) cm\(^{-1}\), Soret band of a reduced protein at 423 nm with an extinction coefficient of 195 mM\(^{-1}\) cm\(^{-1}\), and visible bands at 555 and 527 nm, respectively. Funk et al.\(^6\) expressed a fragment of bovine liver cytochrome \( b_5 \), which could be obtained by solubilization with lipase, in \( E. coli \) JM83 cells to examine the functionally essential amino acid residues. The pUC19 was adopted as an expression vector. The expression level of the recombinant protein in bacteria was up to 15% of the total cellular protein. Ladokhin et al.\(^5\) reported the expression of rabbit cytochrome \( b_5 \) in \( E. coli \) XL-1 cells with the pKK223-3. Holmans et al.\(^6\) first expressed human cytochrome \( b_5 \) in \( E. coli \). The pT7-7 vector containing the IPTG inducible T7 promoter was employed to generate human cytochrome \( b_5 \). To make it easy to isolate and purify these proteins, a histidine-rich domain was fused to the recombinant protein for affinity binding to a nickel-chelate column. The plasmid pT7-7 containing modified cDNAs encoding the histidine-tagged human cytochrome \( b_5 \) was transformed into BL21(DE3)F\(^T\) cells. The recombinant human hemoprotein purified from the established \( E. coli \) cells showed the same optical characteristics as those of preparations purified from rat liver microsomes. The recombinant cytochrome \( b_5 \) could be reduced by NADH in the presence of a small amount of NADH-cytochrome \( b_5 \) reductase purified from pig testis. The ability of the purified recombinant cytochrome \( b_5 \) protein was shown to stimulate the rate of 6\(\beta\)-hydroxylation of testosterone catalyzed by human CYP3A4. The recombinant human cytochrome \( b_5 \) purified from the established \( E. coli \) cells induced a slightly higher level of the catalytic activities of CYP3A4 than did cytochrome \( b_5 \) purified from rat liver microsomes.

**Co-expression of CYP with the OR**

In recent studies, CYP was successfully expressed in \( E. coli \) together with the OR.\(^2\) Four methods or strategies to co-express CYP with the OR were developed: (I) Expression of the two proteins as a fusion protein; (II) Expression of the two proteins independently using a bi-cistronic expression plasmid; (III) Expression using a plasmid carrying two independent promoters for each gene; and (IV) Expression independently using two independent expression plasmids for both proteins. In this chapter, these four strategies to express CYP along with the OR will be described.

**Expression of CYP Together with the OR as a Fusion Protein**

The strategy to construct the fusion protein of CYP and the OR was first introduced. Miura and Fulco\(^2\) demonstrated the presence of a native fusion protein, i.e., a single protein containing both the heme domain of CYP and a flavoprotein domain corresponding to the microsomal FAD- and FMN-containing OR. They isolated and purified this soluble CYP102, from \( Bacillus megaterium \) and characterized it as an \( \omega \)-hydroxylase activity toward fatty acids. CYP102 is enzymatically the most active form among any known CYPs (turnover number > 1500/min). Murakami et al.\(^2\) fused the cDNA of rat liver CYP1A1 with the cDNA of the rat OR to construct a CYP and OR fusion protein to express in yeast. Subsequent studies by them extended their knowledge and experience to express the fusion
protein of bovine adrenal CYP17A involved in the steroid metabolism with the yeast flavoprotein OR. Following the successful expression of bovine CYP17A in E. coli, Fisher et al. applied the method to the expression of bovine CYP17A or rat CYP4A1 with the rat OR as fusion proteins in E. coli. They employed the pCW vector carrying bovine CYP17A and the pUC19 vector carrying rat liver CYP4A1. Each CYP cDNA was fused to the rat liver OR by the PCR mutagenesis method. The mutagenesis was used to modify a coding sequence for the C-terminus of CYPs and a coding sequence for the N-terminus of the rat OR to allow the fusion of these sequences with a dipeptide linker, Ser-Thr. The idea was similar to that described by Murakami et al. The membrane fraction possessed an NADPH-dependent 17α-hydroxylase activity of progesterone and pregnenolone in the absence of an externally added OR. They also showed that the fusion protein of rat CYP4A1 with the rat OR catalyzed the ω-and ω-1 hydroxylation of lauric acid.

Shet et al. first established an E. coli strain expressing human CYP protein as a fusion protein with the rat OR in the same manner as employed for bovine CYP17A. The purified CYP3A4 fusion protein had little or no metabolic activity toward testosterone or nifedipine. However, when the purified rat cytochrome b3 and a sonicated suspension of a lipid extract prepared from rat liver microsomes were added to the reaction mixture, the fusion protein efficiently catalyzed the oxidation of testosterone and nifedipine. These results differed from that seen with the fusion protein of bovine CYP17A or rat CYP4A1. Thus, it seems likely that the effects of the addition of the purified OR or cytochrome b3 appear dependent on the CYP form. In the case of bovine CYP17A and human CYP3A4, the OR level might not be sufficient to support full catalytic activity. It might also be possible that the OR expressed in the E. coli as a fusion protein did not work well.

Human CYP1A1 was also co-expressed with the rat OR as a fusion protein in E. coli DH5α cells. The purified fusion protein catalyzed the B[a]P 3-hydroxylation, 7-ethoxyresorcin O-deethylation, and zoxazolamine 6-hydroxylation. Catalytic activity was not enhanced by the addition of the purified preparations of the rabbit OR and rabbit cytochrome b5.

Expression of CYP Together with the OR Independently Using a Bi-cistronic Expression Plasmid

CYP was also expressed together with the OR separately in E. coli cells. Dong et al. first introduced a method to construct a bi-cistronic expression plasmid pJL2 to express CYP together with the OR. The bi-cistronic plasmid carries one promoter followed by two ribosome binding sites for each coding sequence of the enzyme protein to be expressed. The human CYP2E1 cDNA was linked to the down stream of a tac promoter and the first ribosome binding site. A DNA sequence including the second ribosome binding site and the signal sequence of outer membrane protein A (ompA) was inserted between the end of the CYP2E1 cDNA and the start codon of the rat OR cDNA. The established plasmid pJL2E1/OR was transformed into E. coli XL-1 blue cells. The 5'-terminus of the CYP2E1 cDNA was modified by the replacement of the second amino acid Ser with Ala to generate an Neo I site to construct the expression plasmid. The expression of CYP and the OR protein in the membrane fractions of the established E. coli cells was examined by immunoblot analysis. The expression levels of CYP and the OR proteins were 0.8 nmol/L culture and 4.7 nmol/L culture, respectively. The content of the OR was determined by the rate of cytochrome c reduction, assuming that 1 nmol of reductase reduced 3 μmol of cytochrome c reductase per min. The expression level of CYP2E1 in the whole bacterial cells was below the detection limit. CYP2E1 expressed in the membrane fraction of the established E. coli cells showed aniline hydroxylase, p-nitrophenol hydroxylase and NDMA demethylase activities. While, they could not detect the catalytic activity of CYP2E1 with the whole cells of E. coli, probably because the expression level of CYP2E1 in whole cells was too low to produce enough metabolites. Shet et al. also established the co-expression system of bovine CYP17A and the rat OR. The cDNA of bovine CYP17A was linked to the down stream of the tac promoter and the first ribosome binding site of the pCW vector. They linked the second ribosome binding site to the 5'-terminus of the rat OR cDNA and inserted it into the plasmid at the end of the coding region of CYP17A cDNA. They did not use the ompA signal sequence for the expression of the OR. The expression level of the CYP ranged from 150 to 200 nmol/L culture. The catalytic activity for the 17α-hydroxylation of progesterone appeared with whole cells of bacteria. Parikh in Guengerich’s group, established six E. coli strains co-expressing human CYP and the human OR using the bi-cistronic method. They constructed co-expression plasmids for human CYP1A1, CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 with the pCW vector. The bi-cistronic plasmid consists of the human CYP cDNA as the first cistron and the human OR cDNA as the second cistron. E. coli DH5α, JM109 and BL21 cells were transfected with the expression plasmid. Since the highest expression level of the CYP protein was obtained in the DH5α cells, all subsequent studies were conducted with the same strain of E. coli. The expression level of both CYP and the OR depended on the form of CYP expressed. Ratios of the amount of CYP to the OR ranged from 3:1 to 1:1. These ratios were substantially higher than that seen in human liver microsomes, implying that electron trans-
port might not be a rate-limiting factor in these systems. They examined the catalytic activities for all CYPs with their typical substrates. The activities were almost equal to or somewhat higher than that obtained with preparations purified from human liver microsomes. Gillam et al. 39 also established an E. coli strain harboring the human CYP3A7 together with the OR by the method introduced by Parikh et al. 38 The maximal expression level of CYP3A7 was 15 nmol/L culture. Recombinant CYP3A7 co-expressed with the OR in bacterial membranes showed catalytic activities toward erthyromycin and ethylmorphine, similar to CYP3A4 expressed in the same system. The system to express human CYP1B1 with the human OR was developed by Shimada et al. 37 using the same method as reported by Parikh et al. 26 The expression level of CYP1B1 was 200 nmol/L culture, a level similar to that expressed alone. 7-Ethoxyresorufin O-deethylase activity of CYP1B1 co-expressed with the OR in the membrane fraction was almost similar to that of CYP1B1 purified from E. coli cells expressing CYP1B1 alone and reconstituted with the rabbit OR.

**Expression of CYP Together with the OR Using a Plasmid Carrying Two Promoters for Each Gene**

Blake et al. 37 adopted another method to express human CYP3A4 together with the human OR. The human CYP3A4 and the human OR cDNAs were linked to respective tac promoters and inserted into the pCW vector tandemly. The bacterial pelB leader sequence was fused to the 5′-terminus of the OR cDNA. The plasmid was introduced into E. coli JM109 cells. The expression level of CYP3A4 holo-protein in bacterial cells was 200 nmol/L culture. CYP3A4 expressed in the whole cells of bacteria catalyzed testosterone 6β-hydroxylation and nifedipine oxidation at the substrate concentration of 200 µM. The turnover numbers of the CYP3A4 for testosterone 6β-hydroxylation and nifedipine oxidation were 17.3 and 25.5 nmol/min/nmol CYP, respectively. When the membrane fraction prepared from the E. coli cells was employed, the activity was slightly lower for testosterone 6β-hydroxylation and slightly higher for nifedipine oxidation than those obtained by using whole cells of the bacteria.

Nine forms of human CYPs such as CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 were expressed alone or together with the human OR in E. coli. 38 cDNAs for a CYP and the OR combined to tac promoters and a terminator were introduced into the pCW vector, and were linked tandemly. Each expression plasmid was transformed into E. coli DH5α cells. When CYP was expressed alone in E. coli, the expression level of holo-CYP ranged from 310 to 1620 nmol/L culture. The expression level of the holo-CYP was decreased by co-expression with the OR, and the level ranged from 66 to 381 nmol/L culture. The expression level of the OR varied depending on the forms of CYP co-expressed, and ranged from 204 to 937 units/L culture. The catalytic activities of CYP expressed in E. coli cells were examined after the cells were disrupted by freeze-thaw. Each form of CYP co-expressed in the E. coli cells with the OR catalyzed the oxidation of a representative substrate at efficient rates (Fig. 1). The rates were apparently comparable to the reported activities of CYP reconstituted with purified preparations of CYP, the OR and other necessary components. To clarify the mutagen-producing capacities of human CYP1A2, Suzuki et al. 39 established a S. typhimurium TA1538 strain co-expressing human CYP1A2, the human OR and S. typhimurium O-acetyltransferase. The details of the results of the mutation assay using the established S. typhimurium cells with heterocyclic amines will be described in the chapter below on the applications and perspectives of bacterial cells expressing human CYP.

**Expression of CYP Together with the OR Independently Using Two Expression Plasmids for Both Proteins**

Pritchard et al. 40 adopted a method to introduce simultaneously two independent plasmids carrying respective human CYP2D6 cDNA and the human OR cDNA into E. coli JM109 cells. They used the pCW vector to insert CYP2D6 cDNA and the pACYC184 plasmid to ligate the OR cDNA, since the pACYC184 plasmid possesses a different replication origin from the pCW. These two expression plasmids can simultaneously exist in the same E. coli cell. They modified the 5′-terminus of CYP2D6 cDNA by two methods. One was the method employed by Barnes et al. 17. The other was to use the ompA signal sequence linking to the 5′ end of CYP2D6 cDNA. The expression levels of CYP2D6 in E. coli whole cells determined by CO-difference spectra were 381 nmol/L culture for E. coli cells transfected with the plasmid constructed by the method of Barnes et al. 17 and 365 nmol/L culture for E. coli cells transfected with plasmids carrying the ompA signal sequence. The catalytic activity of CYP2D6 expressed in E. coli whole cells toward bufuralol was measured for both strains of E. coli. The activity seen with the CYP2D6 cDNA modified by Barnes et al. 17 was 1.4 nmol/min/nmol CYP at a substrate concentration of 50 µM. This activity was lower than that seen with the construction with ompA (4.6 nmol/min/nmol CYP). The reason why the high catalytic activity was seen in the latter construction using the ompA has not yet been clarified. When the membrane fraction prepared from E. coli cells was applied to the bufuralol hydroxylation assay, the activity was slightly higher than that obtained by using E. coli whole cells.
Co-expression of CYP, the OR and Cytochrome b₅

As mentioned above, there is strong evidence from such reconstitution experiments that, when cytochrome b₅ is included, the turnover of the metabolism of some substrates with certain CYPs such as CYP3A4 is increased. Voice et al. demonstrated that allowing CYP3A4, human OR and cytochrome b₅ to associate in an in vivo-like system by co-expressing all three proteins together in E. coli for the first time. The cytochrome b₅ cDNA was first introduced into pCW vector. Then,
the DNA fragment carrying cytochrome b5 cDNA, promoter and transcriptional terminator was removed from the expression plasmid and inserted into pACYC184 expression vector, possessing a different selection marker (chloramphenicol) and an alternative origin of replication to allow co-transfection of cells with the expression plasmid carrying CYP3A4 and the OR as mentioned above. The turnover number for the oxidation of both nifedipine and testosterone by CYP3A4 was increased in the presence of cytochrome b5. The turnover of testosterone was increased by 166% in whole cells and by 167% in preparations of bacterial membranes.

Applications of Bacterial Cells Expressing Human CYP to Further Studies

As mentioned above, efforts have been made to establish bacterial strains expressing human CYP alone and together with the OR. The catalytic activities of CYP were examined. The results have indicated that CYP expressed in the bacterial cells showed efficient catalytic activities toward representative substrates. These studies were important to prove whether or not the CYP enzyme expressed in the bacterial cells showed the same substrate specificity as the CYP enzyme present in or purified from human liver microsomes.

We established E. coli strains each co-expressing CYP3A5 or CYP1B1 with the human OR, in addition to the nine strains of E. coli transformed with a plasmid carrying human CYP and the OR, following the methods of Gillam et al.23 or Shimada et al.37 Substrate specificity of CYP expressed in E. coli cells was investigated with representative substrates such as testosterone, 4-nitrophenol, midazolam, cumarin and taxol. Testosterone 6β-hydroxylation, a known representative metabolic reaction of CYP3A subfamily, was catalyzed by CYP3A4, CYP3A5 and also CYP1A1. The metabolic clearances seen with CYP3A5 and CYP1A1 were about one-eighth and one-fourteenth as that seen with CYP3A4, respectively. 4-Nitrophenol, a known substrate for both CYP2A6 and CYP2E1, was metabolized by these two CYPs rather specifically. The metabolic clearance seen with CYP2A6 was 1.7-times higher than that seen with CYP2E1. Midazolam, a representative substrate of CYP3A, was metabolized by CYP3A4 and CYP3A5. Coumarin is known to be a specific substrate of CYP2A6. As was expected, only CYP2A6 expressed in E. coli cells was capable of catalyzing the coumarin 7-hydroxylation. Taxol 6α-hydroxylation was found to be catalyzed solely by CYP2C8. These results were consistent with those obtained in previous studies with human liver microsomes or human CYPs expressed in other heterologous systems.23,40,91 Thus, the substrate specificity of CYPs expressed in E. coli cells was confirmed. The established E. coli strains seem to be useful tools to predict metabolic pathways of various chemicals including drugs in humans.

Using bacterial cells, the large amounts of CYP are expected to be obtained in a relatively short period of culture. The large amounts of enzyme thus prepared can be applied to a bio-reactor to produce a huge amount of a metabolite of a certain drug. In a preliminary study performed by us demonstrated CYP2A6 expressed in the whole cells of E. coli continuously produced the metabolite, 7-hydroxycoumarin, for about 12 h. The production of a large amount of metabolite of a new drug, currently under development, makes it easy to identify the structure of the metabolite and hopefully to analyze the pharmacological and toxicological actions of the metabolite.

Drug-drug interactions caused by inhibition of the metabolism of drugs catalyzed by CYP have been reported. For example, the metabolism of terfenadine catalyzed by human CYP3A is inhibited by azole antifungal drugs, resulting in a remarkable increase of the drug concentration in plasma to induce severe side effects of terfenadine in humans.92–96 Thus, it is important to predict whether or not the metabolism of a certain drug catalyzed by human CYPs is inhibited by other drugs. We applied the established genetically engineered E. coli strains as a tool to predict drug-drug interactions in humans. For example, the inhibition of taxol 6α-hydroxylase activity of human CYP2C8 by various drugs was examined with the established E. coli cells co-expressing CYP2C8 together with the OR. Taxol is an anticancer drug used for ovary cancer and breast cancer. We found that dihydropyridine derivatives of calcium antagonists strongly inhibited taxol 6α-hydroxylase activity. Among the dihydropyridine derivatives of calcium blockers, nicardipine was the strongest inhibitor of the taxol 6α-hydroxylase activity of CYP2C8 with Ki value of 48 nM (Fig. 2), followed by nifedipine and nifedipine with Ki values of 2.2 μM and 15 μM, respectively. All of these drugs were found to inhibit the taxol 6α-hydroxylation in a competitive manner. The bacterial expression system of CYP which allowed us to produce a large amount of enzyme may lead to screening a wide variety of drugs on the possibility of drug-drug interactions via CYP in a relatively short period, namely high throughput screening.

The genetic polymorphism of the CYP gene has been noted as a factor modifying its role in vivo. In some cases, the CYP activities are decreased by the genetic polymorphism, resulting in the high plasma concentration of a certain drug because of a low metabolic clearance. The high concentration of the drug may cause unexpected adverse side effects. Therefore, it seems essential to examine whether or not a genetic polymorphism of CYP alters the catalytic properties of the enzyme, leading to the prediction of the change of phar-
Bacterial Cells Co-expressing Human Cytochrome P450

macokinetics of a certain drug. The bacterial expression system of CYP was expected to be applicable to predict the change of the CYP properties by a genetic polymorphism. The variant gene of CYP with a mutation(s) can be easily produced by the PCR-based site-directed mutagenesis method and inserted into expression vector. Kawajiri et al.\(^97\) have reported that there was a relationship between the CYP1A1-Val462 mutant and the risk of lung cancer. Thus, Zhang et al.\(^98\) established an E. coli strain expressing a variant CYP1A1 protein by the modification of the plasmid constructed by Guo et al.,\(^19\) and compared the activity of the variant protein in the metabolic activation of B[a]P with that of native CYP1A1-Ile\(^{62}\). However, CYP1A1-Ile\(^{62}\) reconstituted with epoxide hydrolase produced 7,8- and 9,10-dihydrodiols at rates comparable to that seen with CYP1A1-Val\(^{62}\). Recently, we found a novel genetic polymorphism of CYP2A6 gene in Japanese, namely CYP2A6*7.\(^99\) A single nucleotide polymorphism (SNP) of T1412C resulted in Ile471Thr substitution. The variant protein of CYP2A6 was expressed in E. coli DH5\(^{a}\) cells along with the OR\(^99\) and the enzymatic properties of the mutant protein were investigated. Interestingly, this variant enzyme almost lacked nicotin C-oxidase activity, although coumarin 7-hydroxylase activity was still observed. A SNP resulting in a substitution from Gln to His was also found in exon 4 of the CYP2B6 gene in Japanese.\(^100\) The mutant- and the wild-type enzymes were co-expressed with OR in E. coli according to the method described above, and the effects of the single amino acid substitution on the catalytic activity of CYP were examined by investigating the kinetic profiles of 7-ethoxyresorufin O-deethylation activity. The wild-type enzyme showed typical Michaelis-Menten kinetics, while the mutant-type enzyme represented the sigmoidal kinetics with a higher \(V_{\text{max}}\) value compared to that of the wild-type enzyme. This is the first evidence demonstrating that only one amino acid substitution, Gln172His, caused by natural SNP enhances the catalytic activity of CYP by obtaining the character of homotropic cooperativity. Such a study may provide information on the relationship between the genotype and the phenotype of CYP and on the risk of cancer or the adverse effects of drugs.

To clarify the toxicological roles of CYP in humans, CYP has been expressed in the S. typhimurium cells used for Ames mutation assay. Suzuki et al.\(^88\) established genetically engineered S. typhimurium strains transfected with a plasmid carrying human CYP1A2 and the human OR cDNAs, to examine the mutagenic activation of heterocyclic amines present in cooked foods which require metabolic activation by CYP1A2 to exert their genotoxicity. They adopted a S. typhimurium TA1538 strain as a host, since the heterocyclic amines induce the frame shift type of mutations in this strain of S. typhimurium after undergoing metabolic activation via N-hydroxylation by CYP1A2. The N-hydroxylated heterocyclic amines have been known to be further activated by O-esterification catalyzed by O-acetyltransferase.\(^101\) Thus, they introduced an expression plasmid (p1A2OR) carrying human CYP1A2 and the human OR cDNAs and an expression plasmid (pOAT) carrying S. typhimurium O-acetyltransferase, a derivative of pACYC184 vector, to S. typhimurium TA1538 cells to yield the TA1538/ARO strain. The TA1538/ARO strain was proven to express the enzymes as indicated by the high activities of 7-ethoxyresorufin O-deethylase and isoniazid N-acetylase. The TA1538/ARO strain showed a high sensitivity to mutagenic heterocyclic amines, MeIQ, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and showed a somewhat higher sensitivity to 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) compared to the parental Ames tester strain TA1538 (Fig. 3). The mutagenic activation of MeIQ, IQ, MeIQx and PhIP was seen from the concentrations at around

![Dixon plots for the inhibition of taxol 6a-hydroxyloase activity of CYP2C8 by nicardipine.](image-url)
Fig. 3. Sensitivity of genetically engineered *S. typhimurium* to heterocyclic amines. S. typhimurium cells were treated with MelIQ (A), IQ (B), MeIQx (C), or PhIP (D). ○, TA1538/ARO (TA1538 cells transfected with p1A2OR and pOAT); ●, TA1538/AR (TA1538 cells transfected with p1A2OR); ▼, TA1538/O (TA1538 cells transfected with pOAT); ▽, TA1538/C, (TA1538 cells transfected with the control plasmid).

0.3 pM, 3 pM, 30 pM and 1000 pM, respectively. When the membrane and cytosol fractions prepared from TA1538/ARO were added to a mixture containing the parental TA1538, the sensitivity of TA1538 to IQ was much lower than that seen with TA1538/ARO. These results indicate that the intracellular expression of drug-metabolizing enzymes makes the established strain of *S. typhimurium* highly sensitive to mutagenic heterocyclic amines, since the reactive intermediates formed inside the bacterial cells may efficiently bind to the DNA of the bacteria, inducing the high level of gene mutation.

To clarify the mechanism of mutagenesis or carcinogenesis, it is of importance to determine the CYP form(s) responsible for the activation. Thus, we established nine other strains of *S. typhimurium* TA1538 each co-expressing a form of CYP (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5), together with the OR. The mutagenic activation of AFB1, B[a]P, 2-acetylamino-fluorene, PhIP was detectable with these strains of *S. typhimurium* TA1538 (unpublished data). In addition to these strains of *S. typhimurium*, eleven strains of *S. typhimurium* YG7108 each co-expressing a form of CYP (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5) along with the OR have also been developed. The *S. typhimurium* YG7108 strain is a derivative of a *S. typhimurium* TA1535 strain, and is applied to detect the point mutations induced by alkylating agents including N-nitrosamines. Since the *S. typhimurium* YG7108 strain lacks *O*6-methylguanine DNA methyltransferase ada and ogt genes, the strain shows high sensitivity toward alkylating agents. Actually, we could detect the mutagenicity of N-nitrosamines with the established *S. typhimurium* YG7108 cells at concentrations lower than μM level. The application of both *S. typhimurium* TA1538 and YG7108 strains led us to examine the mutagenicity of promutagens not only inducing frame shift mutations but also causing point mutations to the bacterial gene.

Examples of the results of mutation assays with the genetically engineered *S. typhimurium* YG7108 strains
each co-expressing a form of CYP together with the OR are shown below. The roles of human CYP in the metabolic activation of N-nitrosamines were examined by us. The relationship between the structure of N-alkyl-nitrosamines and CYP form(s) involved in the activation was evaluated. We found that N-alkyl-nitrosamines with relatively short alkyl chains such as NDMA and N-nitrosomethylethylamine were primarily activated by CYP2E1 as judged by mutagen-producing capacity. With the increase of the number of the carbon atoms of the alkyl chains, the contribution of CYP2A6 increased. CYP2A6 played major roles in the activation of N-nitrosodiethylamine, N-nitrosodipropylamine, N-nitrosomethylpropylamine, N-nitrosomethylbutylamine and N-nitrosoethylbutylamine. Interestingly, CYP1A1 plays a major role in the metabolic activation of N-nitrosodibutylamine. In another study, we clarified that CYP2A6 was the main form responsible for the activation of various N-nitrosamines contained in tobacco smoke such as NNK, N'-nitrosonornicotine, N-nitrosopyrrolidine N-nitrosoanabasine and N-nitrososanatabine.

Other toxicological studies using bacterial cells expressing human CYP have been reported by Shimada et al. They applied the E. coli cells expressing human CYP as a source of the enzyme to examine the genotoxicity of chemicals including heterocyclic amines. The induction of the SOS response in the S. typhimurium NM2009, which contained a umuC regulatory sequence attached to the lacZ reporter gene, was employed as a detection marker. They added the membrane fraction of E. coli cells expressing CYP or the purified preparation of CYP from E. coli cells and the purified preparation of rabbit OR to the reaction mixture as an activation system. The involvement of human CYP1A1, CYP1A2 and CYP3A4 in the metabolic activation of various heterocyclic amines was clarified. In a subsequent study, Hammons et al. also investigated the metabolism of chemicals including heterocyclic amines and aryl amines by human CYP expressed in the E. coli cells. The roles of human CYP1A1, CYP1A2 and CYP3A4 on the metabolic activation of IQ, MeIQx, PhIP, 4-amino-biphenyl were clarified. These promutagens were mainly activated metabolically by CYP1A2. PhIP was also metabolized by CYP1A1. Josephy et al. introduced the expression plasmid carrying human CYP1A2 into S. typhimurium YG1019 cells to detect the mutagenicity of heterocyclic amines and aryl amines. The mutagenicity of 2-AA and 2-aminoflourene was detectable with this system.

CYP proteins expressed in the bacterial strains can also be applied to examine the structure-function relationship of the enzyme. Application of the site-directed mutagenesis technique allows us to clarify the key amino acid residues for catalytic activity and substrate binding. Porter established an E. coli strain harboring rabbit CYP2E1. He clarified that the Phe-426, a conserved residue over animal species, of rabbit CYP2E1 is an important residue for heme incorporation and catalytic activity using the site-directed mutagenesis method. Recently, many reports showing the results of structure-function relationship examined by using genetically engineered bacterial cells expressing human CYP as an enzyme source, have appeared.

Since the breakthrough by Barnes et al. to the successful expression of bovine CYP17A in E. coli cells in 1991, the great progress has been made on the bacterial expression system of mammalian CYP. Taking all of the results obtained in the last decade into account, the bacterial strains harboring human CYP seem to be a useful tool to study the significance of CYP on the metabolism of drugs and metabolic activation of chemicals in humans. In addition to CYPs, there are other enzymes involved in drug metabolism, such as phase II enzymes. Many chemicals, including drugs and carcinogens, are sequentially metabolized by the phase I and phase II enzymes to detoxify or activate them. Such systems, which contain multiple enzymes as seen in a whole body, are expected to be established in the future.

References

9. Doehmer, J., Dogra, S., Friedberg, T., Monier, S.,...


33) Schauer, B. and McCarthy, J. E. G.: The role of bases upstream of the Shine-Dalgarno region and in the


57) Nishimoto, M., Clark, J. E. and Masters, B. S. S.:


106) Fujita, K. and Kamataki, T.: Role of human cytochrome P450 (CYP) in the metabolic activation of N-alkylnitrosamines: application of genetically en-


