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Genetically Engineered Bacterial Cells Expressing Human Cytochrome P450 as an Alternative Tool in the Prediction of Metabolism and Toxicity of Chemicals in Humans

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Abstract

Genetically engineered bacterial cells expressing human cytochrome P450 (P450 or CYP) have provided new tools, as an alternative method for experimental animals and human specimens. Using the bacterial system, it is possible to investigate the functions of human P450 in the detoxification or the metabolic activation of various xenobiotics and the metabolism of endogenous compounds. This review focused on the development of bacterial cells expressing human P450, and the application of this system to drug metabolism and toxicological studies. There are many kinds of host cells for the heterologous expression of a form of P450. Among them, bacterial cells including *Escherichia coli* (*E. coli*) and *Salmonella* have advantages with regard to ease of use and the high yield of protein. The modification of the N-terminal amino acid sequence of P450 allowed it to express P450 protein in bacterial cells (Barnes et al., 1991). It was an excellent breakthrough for the establishment of an expression system of P450 using bacterial cells. Since then, many isoforms of human P450 have been successfully expressed in bacterial cells. Many reports that appeared so far have indicated that the P450 enzyme expressed in *E. coli* after modification of the N-terminus showed considerable catalytic activities in systems reconstituted with NADPH-P450 reductase purified from liver microsomes from appropriate animals. Bacterial cells do not possess endogeneous electron transport systems to support the full catalytic activity of P450 expressed in bacterial cells. Thus, systems co-expressing both P450 and other electron transport enzymes from NADPH or NADH to P450s have been established. The catalytic activities were detected even if the whole cells of bacteria co-expressing P450 with the reductase were used. Recently, these

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strains of bacteria were applied to analyze the toxicological and pharmacological roles of P450 in humans. For example, we established *Salmonella* strains harboring human CYP2A6 or CYP2E1 together with the reductase, and clarified that CYP2A6 was responsible for the activation of *N*-nitrosamines with relatively long alkyl chain(s) such as NNK, NNN and NMPhA, whereas CYP2E1 was involved in the activation of *N*-nitrosamines with relatively short alkyl chain(s) such as NDMA and NDEA. These strains of bacteria may be useful to study drug metabolism and toxicology in humans, and may be an alternative method to those using experimental animals.

Keywords: *E. coli., Salmonella Typhimurium, Catalytic Activity, Substrate Specificity, Drug-drug Interaction, Mutation Assay*

Abbreviations: 2-AA, 2-aminoanthracene; 2-AAF, 2-acetylaminofluorene; AFB_1, aflatoxin B_1; B[*a*]P, benzo[*a*]pyrene; *Escherichia coli, E. coli*; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; IPTG, isopropyl-β-D-thiogalactopyranoside; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; NADH, nicotinamide adenine dinucleotide reduced form; NADPH, nicotinamide adenine dinucleotide phosphate-reduced form; NDEA, *N*-nitrosodiethylamine; NDMA, *N*-nitrosodimethyllamine; NMPhA, *N*-nitrosomethylphenylamine; NNK, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone; NNN, *N*-nitrosonornicotine; PAH, polycyclic aromatic hydrocarbon; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; P450 or CYP, cytochrome P450; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

Introduction

In general, the purpose of developing alternative methods is to reduce the use of experimental animals. The purpose of studies using experimental animals is to predict various physiological phenomena in humans without using humans. In this respect, rodents have been widely used to analyze drug metabolism in both *in vivo* and *in vitro* experiments. If drugs are metabolized by so-called drug metabolizing enzymes present in experimental animals with the same properties as humans, the results obtained by using experimental animals are applicable to extrapolate to humans. However, there are large species differences on the properties of enzymes involved in drug metabolism between humans and experimental animals. Therefore, it is difficult to extrapolate the data obtained using experimental animals to humans. Recently, human liver specimens have been adopted as an alternative tool to predict human drug metabolism, while the application of these preparations is limited by several factors, including ethical reasons. In addition, the population of drug metabolizing enzymes varies according to the medical background of donor patients. Another disadvantage of the use of human livers is the low level of drug metabolizing enzymes in these biological materials, which are, in some cases, insufficient to analyze the chemical structure of the metabolites of a new drug.

Thus, there was a need to establish alternative methods 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, we introduced the heterologous expression system of cytochrome P450 (P450 or CYP)[2] using bacterial cells.

P450 is one of the phase I drug-metabolizing enzymes. It is an integral membrane-bound heme-containing enzyme. P450 catalyzes the oxidative metabolism of a wide variety of endogenous and exogenous compounds, including steroids, fatty acids, drugs, carcinogens, and other xenobiotics (Porter et al., 1991; Guengerich et al., 1991; Guengerich et al., 1993). Catalyzing the bio-oxidation reactions, P450 enzymes play roles in the detoxification and the activation of chemicals to modify the actions of chemicals (Thakker et al., 1985). P450 superfamilies are subdivided into families and subfamilies according the identity of their amino acid sequences. Four of the families have been identified to catalyze the oxidation of foreign chemicals in humans (Nelson et al., 1996). It has been clarified that catalytic properties are different among various P450 isoforms. Furthermore, since the catalytic properties of P450 even in the same family vary among animal species, it is necessary to use human P450 to predict any human drug metabolism that will affect drug actions and toxicities.

Thus, the use of P450 preparations expressed in heterologous expression systems has become a more popular alternative method for examining 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. Yield and expense are primary concerns. Efforts made in recent years have realized the expression of several P450 isoforms in yeast (Bligh et al., 1992; Renaud et al., 1993), cultured mammalian cells (Doehmer et al., 1988; Aoyama et al., 1990a; Crespi et al., 1991; Schneider et al., 1996) and insect cells (Imaoka et al., 1993; Buters et al., 1994; Sakuma et al., 1995). However, in general, the yield of the P450 protein in yeast or mammalian cells is low.

Among the heterologous expression systems, bacterial cells including *E. coli* and *Salmonella* expression systems have advantages compared to other expression systems in terms of low cost to maintain, ease of use, and the high yield of protein with a relatively short period of incubation. Although the bacterial expression system had demonstrated great usefulness in the expression of many prokaryotic and eukaryotic proteins, the use of bacterial cells for the expression of P450s had been limited primarily to the soluble prokaryotic P450s (Unger et al., 1986). Barnes et al. first expressed an eukaryotic P450, CYP17A, in *E. coli* (Barnes et al., 1991). When the native cDNA was introduced into an expression plasmid, no immuno-reactive CYP17A protein was produced following derepression 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 were made to improve the modification method of the N-terminal amino acid sequence of P450s. Many reports have appeared showing that the P450 enzyme expressed in *E. coli* after the modification of the N-terminus still possessed catalytic activities in reconstituted systems containing NADPH-P450 reductase purified from liver microsomes from an appropriate animal (Sandhu et al., 1993; Guo et al., 1994; Sandhu et al., 1994; Gillam et al., 1994; Gillam et al., 1995a; Gillam et al., 1995b; Richardson et al., 1995 etc.). Bacterial cells have endogenous electron transport systems, while the capacity of the system to transport electrons is not sufficient to support the catalytic activity of P450 expressed in bacterial cells. (Jenkins et al., 1994). Thus, the addition of the purified preparation of the reductase was needed to reconstitute the system for the efficient catalytic activity of P450.

To make it easy to predict human drug metabolism or toxicological properties using

the bacterial expression system, the co-expression of both P450 and other enzymes playing roles to transport electrons from NADPH or NADH to P450s was assumed to be needed. Thus, in recent studies some P450 isoforms were expressed in *E. coli* together with the reductase (Parikh et al., 1997; Iwata et al., 1998).

The aims of this review are to briefly summarize reports on the establishment of bacterial strains expressing P450 alone or together with an electron transport enzyme such as the reductase. We will discuss the advantages and disadvantages of their application as a method alternative to the methods using experimental animals or human samples in studies of drug metabolism and toxicology. We will also mention the practical application and the perspectives in the use of bacterial cells expressing P450.

Properties of Bacterial Expression Systems

Considerable amounts of knowledge have been accumulated over the years concerning the *E. coli* expression system. It has been assumed that any proteins can be produced in *E. coli* as long as the protein molecule is not too small, too large and too hydrophobic, and does not contain too many cysteines (Goeddel, 1990). These generalizations are 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 (Switzer et al., 1976). However, *E. coli* is a suitable and often desirable host for a large number 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 (Goeddel, 1990).

An *E. coli* expression system is usually useful for the production of a heterologous protein consisting of 100 to 300 amino acids (Goeddel, 1990), assuming that there is not an inordinate number of cysteins. Fusion protein expression strategies often permit one to overcome the problems of protein instability (Marston, 1986). In fact, the fusion approach was a preferred method for an immunogen generation. The fusion methods also have advantages in the protein purification. 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, secretion from *E. coli* is still largely a hit-or-miss proposition and is most likely to work with a natural protein being secreted.

E. coli cells possess protease like yeast cells and mammalian cells (Goeddel, 1990). Lon is a major ATP-dependent protease in *E. coli*, and seems to be responsible for the degradation of a number of naturally unstable proteins and many abnormal proteins (Goeddel, 1990). The Lon shows relatively broad substrate specificity for unfolded or misfolded proteins. Lon mutant *E. coli* cells may be useful to prevent the degradation of proteins heterologously expressed in *E. coli* cells.

For the purpose of gene expression in heterologous cells, proteins can be grouped into three broad classes (Goeddel, 1990). 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 *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 acids. The proteins in this class are often the easiest to express. In particular, protein expression in *E. coli* has proven to be extremely effective for the proteins in the 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 are often used to express the proteins included in this class. P450 proteins fall into this class;

however, relative to secreted proteins much less work has been directed toward over-expression of proteins in this category.

Expression of Human P450s in Bacterial Cells

In 1991, Barnes et al. first expressed catalytically active mammalian P450, bovine CYP 17A, in *E. coli* JM109 cells (Barnes et al., 1991). They used the pCW plasmid to construct the expression plasmid. The plasmid contains two *tac* promoters being induced by IPTG upstream of an *Nde* I restriction enzyme cloning site coincident with the initiation ATG codon. The plasmid also contains a *trp*A transcription terminator sequence to prevent the read through, a pharge M13 origin of DNA replication, and the *lacI*^{q} gene encoding the Lac repressor that prevents transcription from the *tac* promoters prior to the addition of inducing agents. The plasmid has often been used to express various molecular forms of P450 in bacterial cells.

To express the cytochrome, 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 *E. coli* and free energy to form the secondary structure of mRNA, for bacterial expression, 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 codon for expression of the *LacZ* gene (Looman et al., 1987), and codons 4 and 5 were changed to TTA (silent mutations), since the region of the most mRNAs expressed in *E. coli* has been shown to be adenosine and uridine nucleotides (Stormo et al., 1982). In addition, the last nucleotide of codon 6 and 7 was changed to adenosine and thimidine (silent mutations), respectively, to minimize a secondary structure formation in the mRNA (Schauder et al., 1989).

Until now, a large number of reports on the expression of mammalian P450s including human P450 in bacterial cells have been published utilizing a method based on the above concept. In this chapter, the results obtained so far on the expression for each family of human P450s in bacterial cells will be mainly introduced. All of the bacterial expression systems for various human P450s introduced in this review are summarized in Table 1.

CYP1 Family

The CYP1 family contains two forms of CYP1A, CYP1A1 and CYP1A2, and CYP1B1 in humans (Nelson et al., 1996). CYP1A1 probably is not expressed constitutively in the hepatic tissues (McManus et al., 1990), while it is induced by treatment of animals with some agents. TCDD and PAHs are known inducers of CYP1A1 in animals, including rats, mice and monkeys (Komori et al., 1992). CYP1A1 is involved in the metabolic activation of promutagens including PAHs. On the other hand, CYP1A2 is presumably expressed constitutively in the liver and can be induced by xenobiotics such as aromatic amines in experimental animals. Recently, it was found that CYP1A2 was induced by piperonyl butoxide and acenaphthylene by a mechanism(s) not mediated by an aromatic hydrocarbon-responsive receptor (Ryu et al., 1996). CYP1A2 is known to activate heterocyclic amines and aromatic amines to mutagens (Ishii et al., 1980; Yamazoe et al., 1984). CYP1B1 is a relatively new isoform of P450 belonging to the CYP1 family (Sutter et al., 1994). This CYP isoform is known to be responsible for the metabolic activation of a wide variety of promutagens such as aromatic hydrocarbons, heterocyclic amines and aromatic amines (Shimada et al., 1996).

Guo et al. (Guo et al., 1994) established an *E. coli* strain expressing human CYP1A1. They designed some CYP1A1 cDNAs containing the 5' terminus modified by methods including that proposed by Barnes et al. (Barnes et al., 1991). They constructed expression plasmids with pCW and introduced them

Table 1 Summary of expression of various human P450s in bacterial cells

P450	NADPH-P450 reductase	Co-expression method	Bacterial strain	Expression plasmid	References
CYP1A1	-		*E. coli* DH5α	pCW	Guo et al. (1994)
	+	fusion	*E. coli* DH5α	pCW	Chun et al. (1996)
	+	bi-cistron	*E. coli* DH5α	pCW	Parikh et al. (1997)
	+	two promoters	*E. coli* DH5α	pCW	Iwata et al. (1998)
CYP1A2	-		*E. coli* DH5α	pCW	Fisher et al. (1992a)
	-		*E. coli* DH5α	pCW	Sandhu et al. (1994)
	-		*E. coli* MX100	pCW	Kranendonk et al. (1998)
	+	bi-cistron	*E. coli* DH5α	pCW	Parikh et al. (1997)
	+	two promoters	*E. coli* DH5α	pCW	Iwata et al. (1998)
	+	two promoters	*Salmonella* TA1538	pCW	Suzuki et al. (1998)
CYP1B1	+	bi-cistron	*E. coli* DH5α	pCW	Shimada et al. (1998)
CYP2A6	-		*E. coli* JM109	pCW	Prichard et al. (1997)
	+	two promoters	*E. coli* DH5α	pCW	Iwata et al. (1998)
CYP2C8	-		*E. coli* XL-1 blue	pCW	Richardson et al. (1995)
	+	two promoters	*E. coli* DH5α	pCW	Iwata et al. (1998)
CYP2C9	-		*E. coli* DH5α	pCW	Sandhu et al. (1993)
	-		*E. coli* XL-1 blue	pCW	Richardson et al. (1995)
	+	bi-cistron	*E. coli* DH5α	pCW	Parikh et al. (1997)
	+	two promoters	*E. coli* DH5α	pCW	Iwata et al. (1998)
CYP2C18	-		*E. coli* XL-1 blue	pCW	Richardson et al. (1995)
CYP2C19	-		*E. coli* XL-1 blue	pCW	Richardson et al. (1995)
	+	two promoters	*E. coli* DH5α	pCW	Iwata et al. (1998)
CYP2D6	-		*E. coli* DH5α	pCW	Gillam et al. (1995a)
	-		*E. coli* JM109	pDS	Kempf et al. (1995)
	+	bi-cistron	*E. coli* DH5α	pCW	Parikh et al. (1997)
	+	two promoters	*E. coli* DH5α	pCW	Iwata et al. (1998)
	+	two plasmids	*E. coli* JM109	pCW pACYC184	Prichard et al. (1997)
CYP2E1	-		*E. coli* DH5α	pSE420	Winters et al. (1992)
	-		*E. coli* DH5α	pCW	Gillam et al. (1994)
	-		*E. coli* JM109	pCW	Prichard et al. (1997)
	+	bi-cistron	*E. coli* XL-1 blue	pJL2	Dong et al. (1996)
	+	bi-cistron	*E. coli* DH5α	pCW	Parikh et al. (1997)
	+	two promoters	*E. coli* DH5α	pCW	Iwata et al. (1998)
CYP3A4	-		*E. coli* DH5α	pCW	Gillam et al. (1993)
	-		*E. coli* JM109	pCW	Prichard et al. (1997)
	+	fusion	*E. coli* DH5α	pCW	Shet et al. (1993)
	+	bi-cistron	*E. coli* DH5α	pCW	Parikh et al. (1997)
	+	two promoters	*E. coli* JM109	pCW	Blake et al. (1996)
	+	two promoters	*E. coli* DH5α	pCW	Iwata et al. (1998)
CYP3A5	-		*E. coli* DH5α	pCW	Gillam et al. (1995b)
CYP3A7	+	bi-cistron	*E. coli* DH5α	pCW	Gillam et al. (1997)

into *E. coli* DH5α cells, and compared the expression level of CYP1A1 holo-protein. Modified Terrific Broth medium containing 100 mg/L ampicillin, 1.0 mM IPTG, 1.0 mM thiamine and trace elements (Fisher et al., 1992) was employed as a culture medium for the expression of P450 protein in *E. coli* cells. The highest expression level of CYP1A1 protein in the membrane fractions of the established *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 holo-protein was 25 nmol/L culture. When the membrane fractions supplemented with the rabbit reductase were incubated with a substrate and NADPH, the catalytic activities of CYP1A1 toward B[*a*]P and 7-ethoxyresorufin were low, even in the presence of human cytochrome b_5. On the other hand, when the preparations of CYP1A1 purified from the *E. coli* cells were incubated with the rabbit reductase, a substrate and NADPH, the enzymes showed catalytic activities toward B[*a*]P and 7-ethoxyresorufin even in the absence of cytochrome b_5. The addition of α-naphthoflavone in the purification step protected P450 from denaturation.

Human CYP1A2 was first expressed in *E. coli* by Fisher et al. in 1992 (Fisher et al., 1992). The N-terminal region of the protein was modified by the alignment of nine amino acids of the N-terminus of bovine CYP17A at the 21 amino acid residue of CYP1A2. The modified CYP1A2 cDNA was inserted into pCW plasmid. The established expression plasmid was introduced into *E. coli* DH5α cells. The maximal expression level of the cytochrome was 700 nmol/L culture in the bacterial whole cells. Estradiol 2-hydroxylase, 7-ethoxycoumarin *O*-deethylase and 7-ethoxyresorufin *O*-deethylase activities were assayed with membrane fractions prepared from *E. coli* cells, supplemented with twice the amount of the rat reductase. *K*m values for estradiol 2-hydroxylation, 7-ethoxycoumarin *O*-deethylation and 7-ethoxyresorufin *O*-deethylation were 13, 44 and 0.01 μM, with *V*max values of 1.5, 0.36 and 2.5 nmol/min/nmol P450, respectively. Sandhu et al. (Sandhu et al., 1994) also established an *E. coli* strain harboring human CYP1A2. CYP1A2 cDNAs containing the different types of the modified 5'-terminus were inserted into the pCW plasmid. The highest expression of P450 protein was obtained when the N-terminus of CYP1A2 was modified according the method of Fisher et al. (Fisher et al., 1992). CYP1A2 expressed in the membrane fractions showed catalytic activities toward 7-ethoxyresorufin and phenacetin. The turnover number was about 0.02 nmol/min/nmol P450 in the presence of the rabbit reductase. 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 reductase. The activities were 0.61 nmol/min/nmol P450 toward 7-ethoxyresorufin and 1.38 nmol/min/nmol P450 toward phenacetin, respectively. It may be possible that membrane structure inhibits the access of the reductase to CYP1A2. Two independent groups established the tester strains of *E. coli* for mutation assays by the introduction of human CYP1A2 into *E. coli* cells as mentioned below. Kranendonk et al. (Kranendonk et al., 1998) established a new strain of *E. coli* expressing human CYP1A2 to detect the mutagenicity of various promutagens by metabolic activation through CYP1A2. They also applied the expression plasmid of human CYP1A2 constructed by Fisher et al. (Fisher et al., 1992). They introduced a plasmid carrying human CYP1A2 together with another plasmid pACYC177 carrying the *mucAB* operon required for the mutagenicity test into *E. coli* MX100 cells, designated as BMX100/h1A2. MX100 was used since no CYP activity could be detected. The mutagenicity of 2-AA and

AFB1 was detected by the established *E. coli* cells. Chun (Chun, 1998) introduced 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 Sandhu et al. (Sandhu et al., 1994). The maximal expression was observed at 48 h after the IPTG addition. The level of CYP1A2 in the membrane fraction from the established *E. coli* cells was about 300 nmol/L culture. They detected the mutagenicity of 2-AA and MeIQ using the *E. coli* cells. In these two systems, the electrons might be transported to CYP1A2 via a bacterial endogenous electron transport system (Jenkins et al., 1994).

Human CYP1B1 was first expressed in *E. coli* DH5α cells in 1998 (Shimada et al., 1998). The N-terminus of the CYP1B1 cDNA was modified by removal of codons 2-4 and replacement of the nucleotide sequence of the resulting N-terminal seven codons to enrich the content of AT nucleotides. The modified CYP1B1 cDNA was introduced into pCW expression plasmid. The expression level of the enzyme was 200 nmol/L culture. The catalytic activity of CYP1B1 purified from the *E. coli* cells was evaluated in a reconstituted system containing the purified rabbit reductase. 7-Ethoxyresorufin *O*-deethylase activity was about 2.0 nmol/min/nmol P450 in the presence of twice the amount of the purified rabbit reductase on a molar basis.

CYP2 Family

The CYP2 family was subdivided into seven subfamilies, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, CYP2F and CYP2J, in humans. This CYP2A subfamily includes two isoforms. CYP2A6 and CYP2A7 have been identified to be present so far in human livers. It was shown that CYP2A6 catalyzed coumarin 7-hydroxylation and SM-12502 S-oxidation (Yamano et al., 1990; Nunoya et al., 1996). The genetic polymorphism is seen in the *CYP2A6* gene (Fernandez-Salguero et al., 1995; Nunoya et al., 1998). The CYP2B subfamily includes two members, CYP2B6 and CYP2B7. Only CYP2B6 shows catalytic activity towards chemicals such as lidocaine, (*S*)-mephenytoin and toluene. Anticancer agent cyclophsphamide is activated by CYP2B6 to an active form (Chang et al., 1993). However, little is known on the role of human CYP2B6 compared to other CYPs of rodents belonging to CYP2B. The CYP2C subfamily contains 4 forms and is regarded as a major one existing in human livers. These forms are responsible for the metabolism of clinically important drugs. CYP2C8 metabolizes anticancer drug taxol. CYP2C9 catalyzes the metabolism of many drugs such as nonsteroidal anti-inflammatory drugs and tolbutamide. CYP2C19 is responsible for the metabolism of drugs including omeprazole and (*S*)-mephenitoin. There is little information on the role of CYPs of the CYP2C subfamily on the metabolic activation of promutagens. The CYP2D subfamily includes three members in humans, CYP2D6, CYP2D7 and CYP2D8. Only CYP2D6 possesses catalytic activity. CYP2D6 is involved in the metabolism of many drugs including β-blockers and some antihistaminic drugs. The genetic polymorphism in the *CYP2D6* gene is well studied so far (Gonzalez et al. 1988; Yokoi et al., 1996). CYP2E1 is a sole form belonging to the CYP2E subfamily in humans. CYP2E1 is able to activate a variety of promutagens and toxic substrates including benzene, carbon tetrachloride, vinyl chloride and *N*-alkylnitrosamines with relatively low molecular weights.

We expressed human CYP2A6 in *E. coli* DH5α (Iwata et al., 1998), *Salmonella* YG7-108 and *Salmonella* TA1538 cells together with the human reductase. The 5'-terminal cDNA sequence of CYP2A6 encoding amino acid residues from 2 to 24 was truncated and inserted into the pCW plasmid. The details were described in the chapter called 'Co-expression of P450 with the NADPH-P450 reductase' in this review. Pritchard et al.

(Pritchard et al., 1997) established a strategy whereby unmodified P450 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 plasmid. *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 for both constructions of CYP2A6 in the whole cells were 193 nmol/L culture and 455 nmol/L culture, respectively. The signal peptide bound to the bacterial protein is removed and rapidly digested during the translocation of the protein across the inner membrane, to leave the native protein (Novak et al., 1986). They anticipated the digestion of the signal peptide bound to CYP2A6. Unexpectedly, most of the signal peptides were being retained. They could not prepare the native CYP2A6 protein in this system. 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 P450 for the *ompA* fusion CYP2A6 and 0.32 nmol/min/nmol P450 for the MALLLAVF fusion CYP2A6, respectively. 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 (Liu et al., 1996).

There are many reports on the expression of animal CYPs belonging to the CYP2B subfamily in *E. coli* (Pernecky et al., 1993; John et al., 1994; Born et al., 1995; Lehnerer et al., 1995; Harlow et al., 1997). To our knowledge, there is no report on the expression of human CYP2B6 in any bacterial cells.

CYP2C9 was expressed in *E. coli* by Sandhu et al. (Sandhu et al., 1993) and Richardson et al. (Richardson et al., 1995). Sandhu et al. constructed expression plasmids carrying CYP2C9 cDNA with modifications in the 5' terminal region using pCW and introduced it into E. coli DH5α cells. When the first eight amino acid residues were replaced by MALLLAVF or when twenty amino acid residues of the N-terminus were replaced by MA, detectable amounts of CYP2C9 holo-protein were expressed. The expression level of CYP2C9 was 5 - 11 nmol/L culture for the former construct and 9 - 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 purified liver CYP2C9 in the presence of human cytochrome b_5. In a subsequent study done by Richardson et al. (Richardson et al., 1995), CYP2C8, CYP2C9, CYP2C18 and CYP2C19 were expressed in *E. coli* XL-1 blue cells. They replaced CYP2C cDNAs at the 5' terminus region coding for initial 18 amino acids with a cDNA encoding a universal amino acid sequence MALLLAVF followed by LGLSCLLLLS. The resultant cDNAs were ligated into the pCW plasmid. 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, respectively. They partially purified CYP2C proteins and reconstituted with the reductase. The catalytic activities of the modified human CYP2Cs expressed in *E. coli* cells were evaluated with representative substrates including (*S*)-mephenytoin for CYP2C19, tolbutamide and tetrahidrocannabinol (THC) for CYP2C9 and taxol for CYP2C8, respectively.

Gillam et al. (Gillam et al., 1995a) 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 high level expression in *E. coli* with the pCW

plasmid. 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 δ-aminolevulinic acid and $FeCl_3$ in the culture. The optimal concentrations of δ-aminolevulinic acid and $FeCl_3$ in the culture were 0.5 mM. The expression level of the P450 holo-protein in the whole cells was 90 nmol/L culture. They purified the CYP2D6 and examined the catalytic activity of the enzyme in the reconstituted system with a five-fold excess amount of the rabbit reductase. The catalytic activities of CYP2D6 toward debrisoquine 4-hydroxylation and bufuralol 1'-hydroxylation were similar to those obtained by the preparations of CYP2D6 purified from human liver microsomes. Kempf et al. (Kempf et al., 1995) introduced human CYP2D6 into *E. coli* JM109. They improved an expression plasmid to yield a large amount of CYP2D6 protein in a form suitable for purification. The hydrophobic 25 amino acids in the N-terminus were replaced by codons for $[His]_6$ tag to increase solubility and to allow rapid purification by Ni^{2+}-chelate affinity chromatography. They adopted the pDS9 expression plasmid (Stuber et al., 1990). The plasmid contains a synthetic ribosomal binding site (RBSII) designed for an optimal mRNA recognition and binding. It also contains the t_0 transcriptional terminator from λ-pharge, and the replication origin derived from plasmid pBR322. The CYP2D6 cDNA in pDS9 is under control of the T5 promoter and two *lac* operator sequences. The expression level of CYP2D6 was 105 nmol/L culture. The expressed enzyme was purified from the cytosolic fraction by an affinity chromatography with Ni^{2+}-nitrilotriacetate-agarose, and reconstituted with the rat reductase. Kinetic analysis revealed a *K*m value for bufuralol 1'-hydroxylation of 27 μM, which was similar to that of the native enzyme purified from human liver microsomes.

Human CYP2E1 was first expressed in *E. coli* by Winters et al. (Winters et al., 1992). They modified the first seven amino acids of CYP2E1 from MSALGVTV to MAALGVTV. Furthermore, the AT nucleotide content was enhanced. The pSE420 plasmid, 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 induced by IPTG. The plasmid thus constructed was introduced 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 apoprotein 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 P450 showed activity for the demethylation of NDMA, when reconstituted with the reductase purified from rat liver microsomes. Another approach was done by Gillam et al. (Gillam et al., 1994). 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 plasmid. 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 in the whole cells and 40 nmol/L in the membrane fractions, respectively. The catalytic activity of CYP2E1 purified from the *E. coli* cells was determined in a system reconstituted with the rabbit reductase and human cytochrome b_5. Chlorzoxazone 6-hydroxylase activity was detectable with a

Vmax value of 5.56 nmol/min/nmol P450 and a *K*m value of 36 μM. Prichard et al. (Prichard et al., 1997) also established *E. coli* harboring 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 amino acid sequence MALLLAVF. 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, respectively. The most signal peptides were retained as seen in the case with CYP2A6. Chlorzoxazone 6-hydroxylase activity was determined with membrane fractions prepared 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 reductase (Gillam et al., 1994). It is of interest to analyze the location of P450 protein expressed in *E. coli* cells. Laeson et al. (Laeson et al., 1991) reported the expression of rabbit CYP2E1 in *E. coli* MV1304 cells. They analyzed the effect of the truncation of the N-terminal amino acid residues on the localization of the protein in *E. coli* cells. About 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 the most abundantly expressed in the liver of humans. In humans, CYP3A4, CYP3A5 and CYP3A7 are members of the CYP3A subfamily. CYP3A4 is the major isoform among adult human liver CYP3A enzymes and is responsible for the metabolism of a variety of steroids and drugs that are structurally unrelated (reviewed by Li et al., 1995). In addition to these substrates, CYP3A4 metabolically activates promutagens such as AFB_1 (Hashimoto et al., 1995). CYP3A7 is a major form expressed in the human fetal liver (Kitada et al., 1987a). CYP3A7 catalyzes the hydroxylation of dehydroepiandrosterone 3-sulfate as an endogenous substrate (Kitada et al., 1987b). This CYP is also capable of activating AFB_1 (Kitada et al., 1989; Hashimoto et al., 1995). CYP3A5 is expressed in the adult liver, while the expression is polymorphically seen in only about 20% of individuals (Aoyama et al., 1989). CYP3A5 efficiently catalyzes the 1'-hydroxylation of midazolam. However, little is known on the catalytic properties of CYP3A5 (Gorski et al., 1994).

Gillam et al. (Gillam et al., 1993) first developed the *E. coli* DH5α strain harboring human CYP3A4. CYP3A4 cDNAs containing the modified 5' terminus were constructed and inserted into pCW plasmid. When they replaced the N-terminal 18 amino acid residues with the amino acid sequence MALLLAVF, the highest expression of the recombinant CYP3A4 holo-protein was observed. The expression level in whole cells was 200 to 370 nmol/L culture. CYP3A4 expressed in *E. coli* cells was purified and reconstituted with the purified rabbit reductase and human cytochrome b_5, as was the case with CYP2E1. They demonstrated that the recombinant CYP3A4 in the reconstituted system possessed catalytic activities toward nifedipine oxidation, testosterone 6β-hydroxylation and AFB_1 8,9-epoxydation. Prichard et al. (Prichard et al., 1997) also established the *E. coli* JM109 cells harboring human CYP3A4 with the same manner as that 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*, respectively. Testosterone 6β -hydroxylase and nifedipine oxidase activities were detected with membrane fractions prepared from 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 cells expressing human CYP3A5 were established by Gillam et al. using pCW plasmid. (Gillam et al., 1995b). They changed the 18 N-terminal amino acids to MALLLAVF as in the case with CYP3A4. 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 reductase and human liver cytochrome b_5 as in the case with CYP3A4. The purified preparations of CYP3A5 from the *E. coli* cells showed activities for nifedipine oxidation, testosterone 6β-hydroxylation, AFB_1 8,9-epoxidation, *N*-ethylmorphine *N*-demethylation, erythromycin *N*-demethylation and *d*-benzphetamine *N*-demethylation. Interestingly, nifedipine oxidase, testosterone 6β-hydroxylase and AFB_1 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. (Gillam et al., 1997). They tried to construct more than ten kinds of CYP3A7 cDNA 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 P450s, under the same conditions. When the N-terminus of the CYP3A7 was modified by the replacement with MALLLAVFL 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-aminochrysene, AFB_1, sterigmatocystin, 2-aminoanthracene, 2-aminofluorene were analyzed and compared with those of CYP3A4.

CYP4 Family

In humans, the CYP4 family includes three subfamilies, CYP4A, CYP4B and CYP4F. The CYP4A subfamily consists of CYP4A9 and CYP4A11. CYP4A11 is known to be responsible for the metabolism of fatty acids and prostaglandins. The CYP4B subfamily contains only CYP4B1. CYP4B1 catalyzes the activation of some promutagens including aryl amines such as bendizines.

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 (Nishimoto et al., 1993; Kawashima et al., 1997). 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 cytochrome P450 has also been summarized in twenty-second chapter of a book entitled as "Cytochrome P450 Protocoles (Jenkins et al., 1998)".

Recently, Kusano et al. (Kusano et al., 1999) have reported that the antibiotics chloramphenicol, tetracycline and erythromycin, which inhibit bacterial protein systhesis and are known to induce cold shock response,

unexpectedly enhance the expression of CYP in *E. coli* cells. A sublethal dose of chloramphenicol (1 mg/ml) efficiently increased the expression of bovine CYP17A over range of two- to threefold. In contrast, antibiotics inducing heat shock response in *E. coli* such as puromycin, streptomycin and kanamycin decreased the expression of CYP17A. The effects of antibiotics inducing cold shock response on the expression of human CYP in bacterial cells may be further examined. The addition of the 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 mitocondrial P450scc in *E. coli* was not increased by the addition of sublethal dose of antibiotics inducing 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 Protein Which Transfers Electrons to P450 in *E. coli*

The use of bacterial cells such as *E. coli* and Salmonella made it possible to achieve the high level of expression of the various P450s. However, the amounts of bacterial enzyme capable of transferring electrons to P450 were not sufficient to exert its full catalytic activities (Jenkins et al., 1994). Thus, the addition of the purified preparation of the electron transfer partner was needed to reconstitute a system of P450s. To produce the electron transport proteins such as NADPH-P450 reductase and cytochrome b_5 in bacterial cells, many efforts were made to express these enzymes (Porter et al., 1987; Shen et al., 1989; Andersen et al., 1994; Von Bodman et al., 1986; Ladokhin et al., 1991; Funk et al., 1990; Holmans et al., 1994). In recent studies, some P450 forms were expressed in *E. coli* together with the reductase (Parikh et al., 1997; Iwata et al., 1998). In this chapter, the expression of an electron transport protein alone or together with P450 in bacterial cells will be mentioned.

Expression of NADPH-P450 Reductase in E. coli

NADPH-P450 reductase is a flavoprotein with the molecular weight of 7.8 kDa, which is bound to endoplasmic reticulum of eukaryotic cells and is responsible for the electron transfer from NADPH to P450s. One molecule of the enzyme contains one each of FMN and FAD (Iyanagi et al., 1973). Virtually, no species difference in the function of this reductase has been noted: the reductase purified from the rat liver can transfer an electron to human P450. There are two reports from a group on the expression of the rat reductase in bacterial cells (Porter et al., 1987; Shen et al., 1989), while there is no report on the expression of the human reductase. The expression of the house fly reductase in bacterial cells has also been reported (Andersen et al., 1994).

Porter et al. (Porter et al., 1987) first succeeded in expressing the rat reductase in *E. coli* to identify the functional amino acid residues involved in the binding to FMN, FAD and NADPH. They adopted a pCQV2 expression plasmid, developed by Queen et al. (Queen et al., 1983), which contained 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 for temperature-dependent regulation and induction of the expression. Insertion of the coding nucleotide sequence for the rat reductase into this plasmid allowed it to express a functional protein. Various strains of *E. coli* cells were transformed with the constructed plasmid pCQOR. Among them, *E. coli* C-1A cells were applied for the highest expression of the reductase in the cell lysates. The expression level of the flavoprotein was approximately 0.1% of the total cellular protein. The purified preparations of the reductase catalyzed a cytochrome c reduction at a rate of about 35 μmol/min/mg of the reductase. On the other hand, the reductase preparations purified from

the rat liver catalyzed at a rate of about 50 μmol/min/mg of reductase. Moreover, the reductase expressed in the bacterial cells supported 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 reductase 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 (Shen et al., 1989), colleagues of Porter, also expressed the rat reductase in the same strain of *E. coli* using an expression plasmid pIN-III-ompA3 with which the expressed protein could be secreted outside of the cell membrane. The pIN-III-ompA3 plasmid contained a bacterial *lpp* promoter and a signal peptide, *ompA*, to direct the transport of the expressed protein out of the cytoplasm into the periplasmic space, where proteolytic activity is reduced. Even when the plasmid was employed, the reductase was still mainly presented in the membrane, probably because the hydrophobic amino-terminal sequence of the reductase might anchor the protein to the inner membrane to prevent its release from the membrane. The apparent molecular weight of the reductase was about 80 kDa. The reductase expressed in the bacterial cells catalyzed cytochrome *c* reduction at a rate of 51.5 μ mol/min/mg prot., similar to that seen with the rat liver enzyme (53.3 μmol/min/mg prot.). The human reductase 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 P450 together with the reductase.

In addition to mammalian reductase, the house fly reductase has been successfully expressed in *E. coli* (Andersen et al., 1994). Plasmids pSE380 and pUHHG were chosen for the expression of the reductase. The pSE380 plasmid contained the IPTG inducible *trc* promoter fused with a ribosome binding site. The host *E. coli* strain was the XL-1 Blue. The pUHHG plasmid contained the IPTG inducible *tac* promoter and the bacterial signal peptide *pelB* for the transport of the expressed protein outside of the cytoplasm into the periplasmic space. The N-terminus of the reductase cDNA was modified by a site-directed mutagenesis. A second codon AGC (Ser) was replaced with GCT (Ala). The expression plasmid was transformed into DH5α *lac* I^q cells. In the case using the construction derived from pSE380, the reductase expressed in the XL-1 Blue cells showed activity to reduce cytochrome *c*. However, the reductase did not support the activity of CYP6A1 for aldrin epoxidation. They concluded that inability to transfer electrons to CYP6A1 was caused by the production of the reductase with slightly small molecular mass. They could obtain the full molecular mass of the reductase protein using the construction with the pUHHG plasmid. The protein catalyzed the cytochrome c reduction and supported the aldrin epoxidation by CYP6A1 in the reconstitution system.

Expression of Cytochrome b_5

Cytochrome b_5 is a membrane-bound heme protein found in many mammalian species (Oshino, 1980; Peterson et al., 1986). The hemoprotein is required for the function of a number of reactions catalyzed by P450s (Hildebrandt et al., 1971; Schenkman et al., 1976; Aoyama et al., 1990b). In addition, a soluble-form of cytochrome b_5 is found in erythrocytes, where it serves as an electron carrier for the NADPH-dependent reduction of methemoglobin to hemoglobin (Hultquist et al., 1978). The membrane-bound form of cytochrome b_5 is associated with the endoplasmic reticulum; it has a molecular weight of 16.7 kDa consisting of 134 amino acids. The amino acid sequence of hepatic cytochrome b_5 from several animal species has 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 (Ozols et al., 1977); and a membrane-targeting region represented by the 10-amino-acid sequence located at the carboxy-terminus of the membrane-binding domain (Mitoma et al., 1992). The three-dimensional crystal structure of the tryptic fragment of bovine liver cytochrome b_5 has been determined by X-ray diffraction to a resolution of 2Å (Mathews et al., 1972). A multitude of techniques has been applied to clarify 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 probe 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 obtain the heterologous expression of cytochrome b_5 in *E. coli* cells, as the first example of the expression of a membrane-binding heme-containing protein in a bacterial system.

There are some reports on the expression of the mammalian cytochrome b_5 in *E. coli*. Von Bodman et al. (Von Bodman et al., 1986) first expressed rat cytochrome b_5 in *E. coli*. The pUC13 plasmid 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 $P450_{cam}$ in *E. coli* (Unger et al., 1986). 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. (Funk et al., 1990) expressed a fragment of bovine liver cytochrome b_5, which was obtainable by solubilization with lipase, in *E. coli* JM83 cells to analyze the functionally critical amino acid residues. The pUC19 plasmid was adopted as an expression plasmid. The expression level of the recombinant protein in bacteria was up to 15% of the total cellular protein. Ladokhin et al. (Ladokhin et al., 1991) reported the expression of rabbit cytochrome b_5 in *E.coli* XL-1 cells with the pKK223-3. Holmans et al. (Holmans et al., 1994) first expressed human cytochrome b_5 in *E. coli*. The pT7-7 plasmid containing the IPTG inducible T7 promoter was applied to express human cytochrome b_5. To make it easy to isolate and purify these proteins, a histidine-rich domain was introduced into 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^Q cells. The recombinant human hemoprotein purified from the established *E. coli* cells showed the same optical characteristics as that of preparations purified from rat liver microsomes. The recombinant b_5 was enzymatically 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 as the extent of stimulation for the rate of 6β-hydroxylation of testosterone catalyzed by human CYP3A4. The recombinant human cytochrome b_5 purified from the established *E. coli* cells showed a slightly higher level of the catalytic activities of CYP3A4 than did cytochrome b_5 purified from rat liver microsomes.

Co-expression of P450 with the NADPH-P450 Reductase

In recent studies, P450 was successfully expressed in *E. coli* together with the NADPH-P450 reductase (Parikh et al., 1997; Iwata et al., 1998). Four methods or strategies to co-express P450 with the reductase were employed: (1) Expression of the two proteins as a fusion protein; (2) Expression of the two proteins independently using a bi-cistronic expression plasmid; (3) Expression using a plasmid carrying two independent promoters for each gene; and (4) Expression independently using two independent expression plasmids for both proteins. In this chapter, these four strategies to express both P450 and the reductase will be described.

Expression of P450 together with the Reductase as a Fusion Protein

The strategy to construct the fusion protein was first introduced. Miura and Fulco (Miura and Fulco, 1974) demonstrated the presence of a native fusion protein-i.e., a single protein containing both the heme domain of P450 and a flavoprotein domain corresponding to the microsomal FAD- and FMN-containing NADPH-P450 reductase. They isolated and purified this soluble $P450_{BM-3}$, named CYP102, from *Bacillus megaterium* and characterized it as an ω-hydroxylase of fatty acids. CYP102 is enzymatically the most active form among any known P450s (turnover number > 1500 / min). Murakami et al. (Murakami et al., 1987) fused the cDNA of rat liver P450c (CYP1A1) with the cDNA of the rat reductase to construct a P450-reductase fusion protein to express in yeast. Subsequent studies by this group extended their knowledge and experience to express the fusion protein of bovine adrenal CYP17A involved in the steroid metabolism with the yeast flavoprotein NADPH-P450 reductase (Sakaki et al., 1989). Following the successful expression of bovine CYP17A in *E. coli* (Barnes et al., 1991), Fisher et al. (Fisher et al., 1992) applied the method to the expression of bovine CYP17A and rat CYP4A1 with the rat reductase as fusion proteins in *E. coli*. They employed the pCW plasmid carrying bovine CYP17A and the pUC19 plasmid carrying rat liver CYP4A1. Each P450 cDNA was fused to the rat liver reductase by the PCR mutagenesis method. The mutagenesis was introduced to modify a coding sequence for the C-terminus of P450s and a coding sequence for the N-terminus of the rat liver reductase to allow the fusion of these sequences with a dipeptide linker, Ser-Thr. The idea was similar to that described by Murakami et al. (Murakami et al., 1987). The coding sequence of the N-terminus of bovine CYP17A was modified according to the method described previously (Barnes et al., 1991). Twenty-two N-terminus amino acid residues of rat CYP4A1 was deleted and replaced with the 9 N-terminus amino acid residues of modified bovine CYP17A. Growth of transformed *E. coli* for 72 h at 27℃ resulted in the expression of the fusion protein at a level of about 700 nmol/L culture. The yield was similar to that obtained with *E. coli* expressing bovine CYP17A alone (Barnes et al., 1991). The membrane fraction possessed an NADPH-dependent 17α-hydroxylase activity of progesterone and pregnenolone in the absence of an externally added reductase. The activities were similar to that seen in the case using membrane-bound recombinant bovine CYP17A with a 2- or 3-fold excess of purified recombinant rat reductase (Barnes et al., 1991). They also showed that the fusion protein of rat CYP4A1 with the rat reductase catalyzed the ω-and ω-1 hydroxylation of lauric acid.

Subsequent studies for the expression of P450 together with the reductase using the method were mainly conducted by Fisher and Estabrook et al. (1993, 1994, 1995, 1996) and Guengerich et al. (1994, 1996, 1997). Shet (Shet et al., 1993) with Estabrook first established an *E. coli* strain expressing human P450 protein as a fusion protein with the rat reduc-

tase 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 b_5 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 (Fisher et al., 1992). Shet et al. (Shet et al., 1996) further analyzed the ω-hydroxylase activity of lauric acid with the purified recombinant fusion protein containing CYP4A1 and the rat reductase. It is of interest to note that the addition of a 20-fold excess of the purified rat reductase and equimolar of recombinant human cytochrome b_5 (Holmans et al., 1994) results in the 45-fold increase of the catalytic activity. Shet et al. (Shet et al., 1994) also assayed the 17α-hydroxylation of both progesterone and pregnenolone with the purified recombinant fusion protein of CYP17A and the reductase, while they found no stimulation of 17α-hydroxylation by the addition of the purified rat reductase. They also assayed the 17α-hydroxylation of progesterone with intact bacterial cells expressing the fusion protein. However, the catalytic activity was about 0.05% of that seen with the purified protein. Additional studies showed that the addition of a 4-fold excess of purified rat reductase resulted in the 10-fold stimulation of the testosterone 6β-hydroxylation (Shet et al., 1995). Thus, it seems likely that the effects of the addition of the purified reductase or cytochrome b_5 appear dependent on the P450 isoform. In the case of bovine CYP17A and human CYP3A4, the reductase level might not be sufficient to exert full activity. In a subsequent study, Chaurasia et al. (Chaurasia et al., 1995) determined the kinetic parameters of ω-hydroxylation of lauric acid using the same purified recombinant fusion protein of P450 4A1 and the rat reductase. The apparent *K*m and *V*max values were 3-4 mM and 4-5 nmol/min/nmol P450, respectively.

Human CYP1A1 was co-expressed with the rat reductase as a fusion protein in *E. coli* DH5α cells (Chun et al., 1996). They applied the pCW/1A1#17 plasmid as a source of human CYP1A1 cDNA (Guo et al., 1994). The rat reductase was fused with human CYP1A1 by a Ser-Thr linker. The expression level of CYP1A1 was about 150 nmol/L culture. The purified fusion protein catalyzed B[*a*]P 3-hydroxylation, 7-ethoxyresorfin *O*-deethylation, and zoxazolamine 6-hydroxylation. Catalytic activity was not increased by the presence of the purified preparations of the rabbit reductase and rabbit cytochrome b_5. They examined the effects of α-naphtoflavone and divalent cations on the 7-ethoxyresorfin *O*-deethylase activity (Chun et al., 1997). α-Naphtoflavone, a specific inhibitor of CYP1A, inhibited the 7-ethoxyresorfin *O*-deethylase activity of CYP1A1. The addition of the divalent cations such as Ca^{2+} and Mg^{2+} resulted in the stimulation of the CYP1A1 activity toward 7-ethoxyresorfin *O*-deethylation.

Expression of P450 together with the Reductase Independently Using a Bi-cistronic Expression Plasmid

P450 was also expressed together with the reductase separately in *E. coli* cells. Dong et al. (Dong et al., 1996) first introduced a method to construct bi-cictronic expression plasmid pJL2 to express P450 together with the reductase. The bi-cistronic plasmid carries one promoter followed by two ribosome binding sites for each coding sequence of the enzyme 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 *ompA* was inserted between the end of the CYP2E1 cDNA and the start codon of the rat reductase cDNA. The established plasmid pJL2E1/OR was introduced 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 *Nco* I site to construct the expression plasmid. The expression of P450 and the reductase protein in the membrane fractions of the established *E. coli* cells was examined by immunoblot analysis. The expression levels of both proteins were 0.8 nmol/L culture and 4.7 nmol/L culture, respectively. The content of the reductase was determined by the rate of cytochrome *c* reduction, assuming that 1 nmol of reductase reduced 3 μmol of cytochrome *c* reductase per min (Vermilion et al., 1978). The low level of the expression of CYP2E1 was below the detection limit. When assayed using membrane fraction, CYP2E1 expressed in the established *E. coli* cells showed catalytic activities toward aniline hydroxylation, *p*-nitrophenol hydroxylation and NDMA demethylation. They could not detect the catalytic activity of CYP2E1 when the whole cells of *E. coli* were employed, probably because the expression level of CYP2E1 in whole cells was too low to detect the activity. Shet et al. (Shet et al., 1997) also established the co-expression system of bovine CYP17A and the rat reductase. The cDNA of bovine CYP17A was linked to the down stream of the *tac* promoter and the first ribosome binding site of the pCW plasmid. They linked the second ribosome binding site to the 5'-terminus of the rat reductase cDNA and inserted it into the plasmid at the end of the CYP17A cDNA. They did not use the *ompA* signal sequence for the expression of the reductase. The expression level of the cytochrome was 150 to 200 nmol/L culture. The catalytic activity for the 17α-hydroxylation of progesterone appeared with whole cells of bacteria. Parikh (Parikh et al., 1997), a colleague of Guengerich, established six *E. coli* strains co-expressing human P450 and the human reductase using the bi-cistronic method. They constructed co-expression plasmids for human CYP1A1, CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 with the pCW plasmid. The bi-cistronic plasmid consists of the human P450 cDNA as the first cistron and the human reductase cDNA as the second cistron. *E. coli* DH5α, JM109 and BL21 cells were transfected with the plasmid. As the highest expression level of the P450 protein was obtained in the DH5α cells, all subsequent studies were conducted with the same strain of *E. coli*. The expression level of both P450 and the reductase depended on the form of P450. Ratios of the amount of P450 to the reductase ranged from 3:1 to 1:1. These ratios were substantially higher than that seen with human liver microsomes, implying that electron transport might not be a rate-limiting factor in this system. They assayed the catalytic activities for all P450s toward typical substrates. The activities were almost equal to or somewhat higher than that obtained with preparations purified from human liver microsomes. Gillam et al. (Gillam et al., 1997) also established an *E. coli* strain harboring the human CYP3A7 together with the reductase by the method introduced by Parikh et al. The maximal expression level of CYP3A7 was 15 nmol/L culture. Recombinant CYP3A7 co-expressed with the reductase in bacterial membranes showed catalytic activities toward erythromycin and ethylmorphine similar to CYP3A4 expressed in the same system. The system to express human CYP1B1 with the human reductase was developed by Shimada et al. (Shimada et al., 1998) by the same method (Parikh et al., 1997). 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 reductase in the membrane fraction was almost similar to that of CYP1B1 purified from in *E. coli* cells expressing CYP1B1 alone and reconstituted with the rabbit reductase.

Expression of P450 together with the Reductase Using a Plasmid Carrying Two Promoters for Each Gene

Blake et al. (Blake et al., 1996) adopted another method to express human CYP3A4 together with the human reductase. The expression plasmid included two promoters for human CYP3A4 and the human reductase cDNAs. The human CYP3A4 and the human reductase cDNAs were linked to respective *tac* promoters and inserted into the pCW plasmid tandemly. The bacterial *pel*B leader sequence was fused with the 5'-terminus of the reductase 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 P450, 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 that obtained by using whole cells of the bacteria.

Nine forms of human P450s such as CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 were expressed alone or together with the human reductase in *E. coli* (Iwata et al., 1998). cDNAs for a P450 and the reductase combined to *tac* promoters and a terminator were introduced into the pCW plasmid, and were linked tandemly. Each expression plasmid was introduced into *E. coli* DH5α cells. When P450 was expressed alone in *E. coli*, the expression level of holo-P450 ranged from 310 to 1620 nmol/L culture. The expression level of the holo-P450 was decreased by co-expression with the reductase, and the level ranged from 66 to 381 nmol/L culture. The expression level of the reductase varied depending on the forms of P450 co-expressed, and ranged from 204 to 937 units/L culture. The catalytic activities of P450 expressed in *E. coli* cells were assayed after the cells were disrupted by freeze-thaw. When co-expressed with the reductase, human P450 catalyzed the oxidation of representative substrates at efficient rates (Fig. 1). The rates were apparently comparable to the reported activities of P450 reconstituted with purified preparations of P450, the reductase and other necessary compounds. To clarify the mutagen-producing activities of human CYP1A2, Suzuki et al. (Suzuki et al., 1998) established a *Salmonella typhimurium* TA1538 strain co-expressing human CYP1A2, the human reductase and *Salmonella O*-acetyltransferase. The details of the results of the mutation assay using the *Salmonella* cells with heterocyclic amines will be described in the chapter below on the applications and perspectives of bacterial cells expressing human P450.

Expression of P450 together with the Reductase Independently Using Two Expression Plasmids for Both Proteins

Pritchard et al. (Pritchard et al., 1998) adopted a method to introduce two independent plasmids carrying human CYP2D6 and the human reductase cDNAs into *E. coli* cells. They adopted the pCW plasmid to express CYP2D6 and the pACYC184 plasmid to express the reductase, since the pACYC184 plasmid possesses a different origin from the pCW. These two plasmids can exist simultaneously 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. (Barnes et al., 1991). The other carried the *ompA* signal sequence linked to the 5' end of CYP2D6 cDNA. These plasmids were introduced into *E. coli* JM109 cells. 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 Barnes et al. (Barnes et al., 1991) and 365 nmol/L cul-

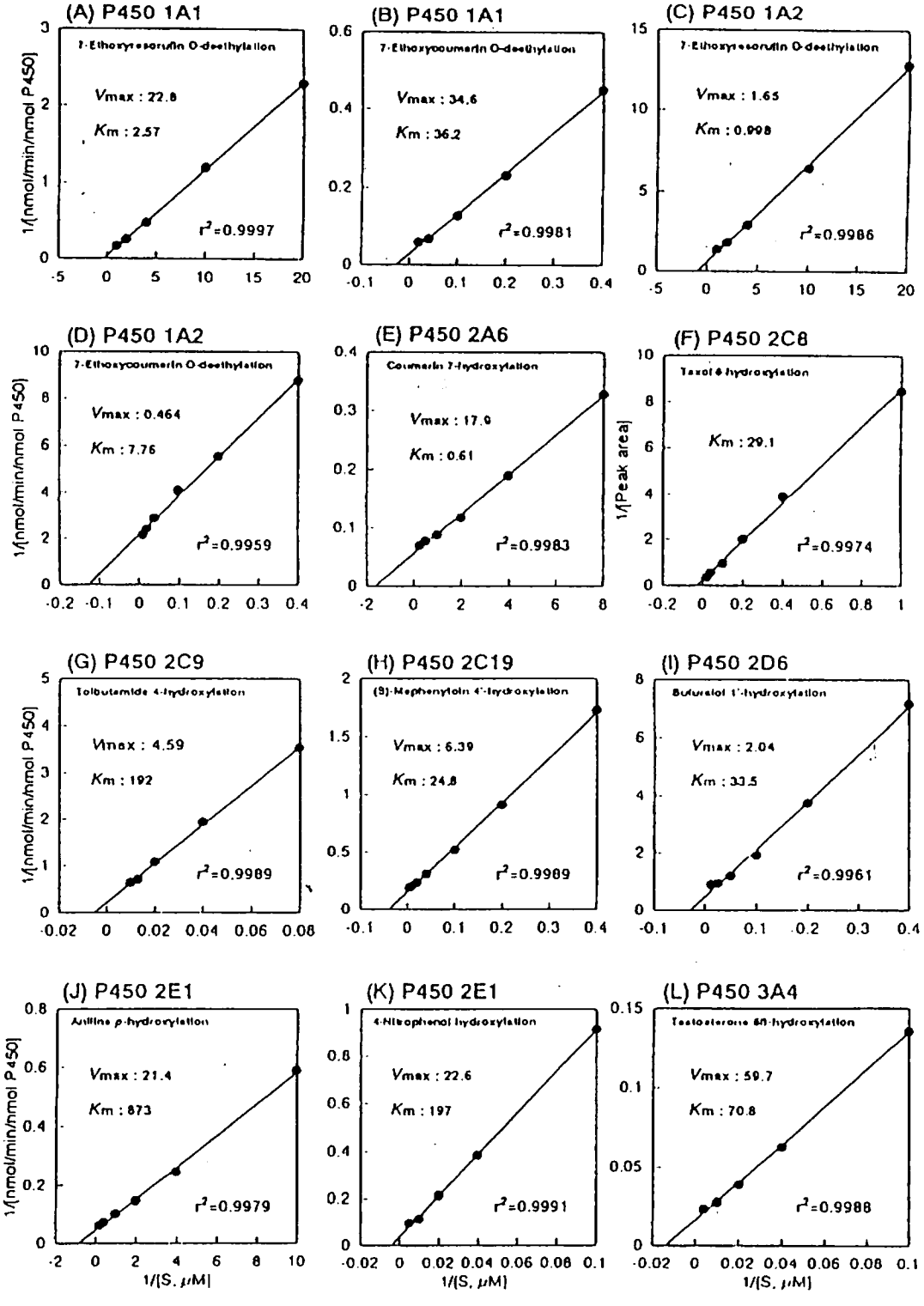

Fig. 1 Lineweaver-Burk plots for the metabolism of a representative substrate by a form of P450 expressed in *E. coli* (Iwata et al., 1998). The values are the means of duplicate determinations. The *K*m and *V*max values are expressed as μM and nmol/min/nmol P450, respectively.

ture 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. was 1.4 nmol/min/nmol P450 at a substrate concentration of 50 μM. This activity was lower than that seen with the other using *ompA* (4.6 nmol/min/nmol P450). The reason why the high catalytic activity was seen in the latter linking the *ompA* to CYP2D6 is not clear. 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 on the normal CYP2D6 basis.

Applications of Bacterial Cells Expressing Human P450 to Further Studies

As mentioned above, efforts have been made to establish bacterial strains expressing human P450 alone and together with the reductase. The catalytic activities of P450 were examined. The results indicated that P450 expressed in the bacterial cells showed efficient catalytic activities toward representative substrates. These studies were important to prove whether or not the P450 enzyme expressed in the bacterial cells showed the same substrate specificity as the P450 enzyme purified from human liver microsomes.

We established an *E. coli* strain co-expressing CYP3A5 with the human reductase, in addition to the nine strains of *E. coli* transformed with a plasmid carrying human P450 and the reductase. Substrate specificity of P450 expressed in *E. coli* cells was evaluated by applying representative substrates. The substrates tested were testosterone, 4-nitrophenol, midazolam, cumarin and taxol. Testosterone 6β-hydroxylation, a known representative metabolic reaction catalyzed by CYP3A subfamily, was metabolized by CYP3A4, CYP3A5 and also CYP1A1. The metabolic clearance seen with CYP3A5 and CYP1A1 was about one-eighth and one-fourteenth as 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 as a specific substrate for CYP2A6. Only CYP2A6 expressed in *E. coli* cells was responsible for the metabolism of coumarin. Taxol 6α-hydroxylation was found to be catalyzed solely by CYP2C8. These results were consistent with those obtained previously by using human liver microsomes or human CYPs expressed in other heterologous expression systems (Gorski et al., 1994; Liu et al., 1996; Gillam et al., 1995b)43-46). Thus, the substrate specificity of P450s 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.

It is possible to obtain large amounts of P450 using bacterial cells in a relatively short period. The large amounts of enzyme preparations can be applied to a bio-reactor to produce a sufficient amount of a metabolite of a drug. 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.

Inhibition of the metabolism of a certain drug by another drug causes a drug-drug interaction. The metabolism of terfenadine by 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. To evaluate the usefulness of the established genetically engineered *E. coli* strains as a tool to predict drug-drug interactions in humans, we analyzed the

inhibitory effects of some drugs on the metabolism by CYPs expressed in *E. coli* cells. The inhibitory effects of two taxoids, taxotere and taxol, on the midazolam hydroxylations were examined. Taxotere, mainly metabolized by CYP3A, strongly inhibited the midazolam hydroxylations, with the *Ki* value of about 5 μM. Taxol, which was mainly metabolized by CYP2C8, did not inhibit the midazolam hydroxylations. Conversely, we also analyzed the inhibitory effects of drugs on the taxol 6α-hydroxylation. Among the drugs tested, miconazole, an azole antifungal drug, strongly inhibited the taxol 6α-hydroxylation, with the *Ki* value of about 2 μM. On the other hand, drugs known as substrates for CYP2C8 such as carbamazepine and cyclophsphamide did not inhibit the taxol 6α-hydroxylation. This finding was in accordance with the fact that the *Km* values of these drugs for CYP2C8 were over 100 μM. The results suggest that the established bacterial strains may also be useful to predict drug interactions in humans.

Genetic polymorphism is seen in the metabolism of drugs catalyzed by some forms of P450. The bacterial expression system of P450 is expected to be applicable to the prediction of P450 isozyme(s) mainly involved in the metabolism of drugs. Additionally, the alteration of catalytic activity by the mutation of the P450 gene may be important to predict the change of pharmacokinetics in the drugs. The variant gene of P450 can be introduced into bacterial cells to clarify the change of the catalytic activities caused by the mutations. It was reported that there was a relationship between the CYP1A1-Val^{462} mutant and the risk of lung cancer (Kawajiri et al., 1993). Thus, Zhang et al. (Zhang et al., 1996) established an *E. coli* strain expressing a variant CYP1A1 protein by the modification of the plasmid constructed by Guo et al. (Guo et al., 1994), and compared the activity of the variant protein in the metabolic activation of B[*a*]P with that of native CYP1A1-Ile^{462}. However, CYP1A1-Ile^{462} reconstituted together with epoxide hydrolase produced 7, 8- and 9, 10-dihydrodiols at comparable rates to that seen with CYP1A1-Val^{462}. Such a study may provide information on the relationship between the genotype and the phenotype of P450 and on the risk of cancer or the adverse effects of drugs.

To clarify the toxicological roles of P450s in humans, Suzuki et al. (Suzuki et al., 1998) established genetically engineered *Salmonella typhimurium* strains transfected with a plasmid carrying human P450 and the human reductase cDNAs. To detect the mutagen-producing activities of human P450 from heterocyclic amines present in cooked foods, which require metabolic activation to exert their genotoxicity, they adopted a *Salmonella typhimurium* TA1538 strain as a host. Heterocyclic amines induce the frame shift type of mutations in this strain of *Salmonella* after undergoing metabolic activation via *N*-hydroxylation by cytochrome CYP1A2, followed by *O*-esterification by *O*-acetyltransferase (Saito et al., 1983). Thus, they introduced an expression plasmid (p1A2OR) carrying human CYP1A2 and the human reductase cDNAs and an expression plasmid (pOAT) carrying *Salmonella O*-acetyltransferase to *Salmonella* 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, IQ and MeIQx and showed a somewhat higher sensitivity to PhIP compared to the parental Ames tester strain TA1538 (Fig. 2). The minimum concentrations of MeIQ, IQ, MeIQx and PhIP giving positive results were defined by evidence that the number of colonies increased dose-dependently and rose two times higher than that obtained by vehicle alone as a control in the TA1538/ARO strain at 0.3 pM, 3 pM, 30 pM and 1000 pM, respectively. When the membrane and cytosol fractions prepared from TA1538/ARO were added

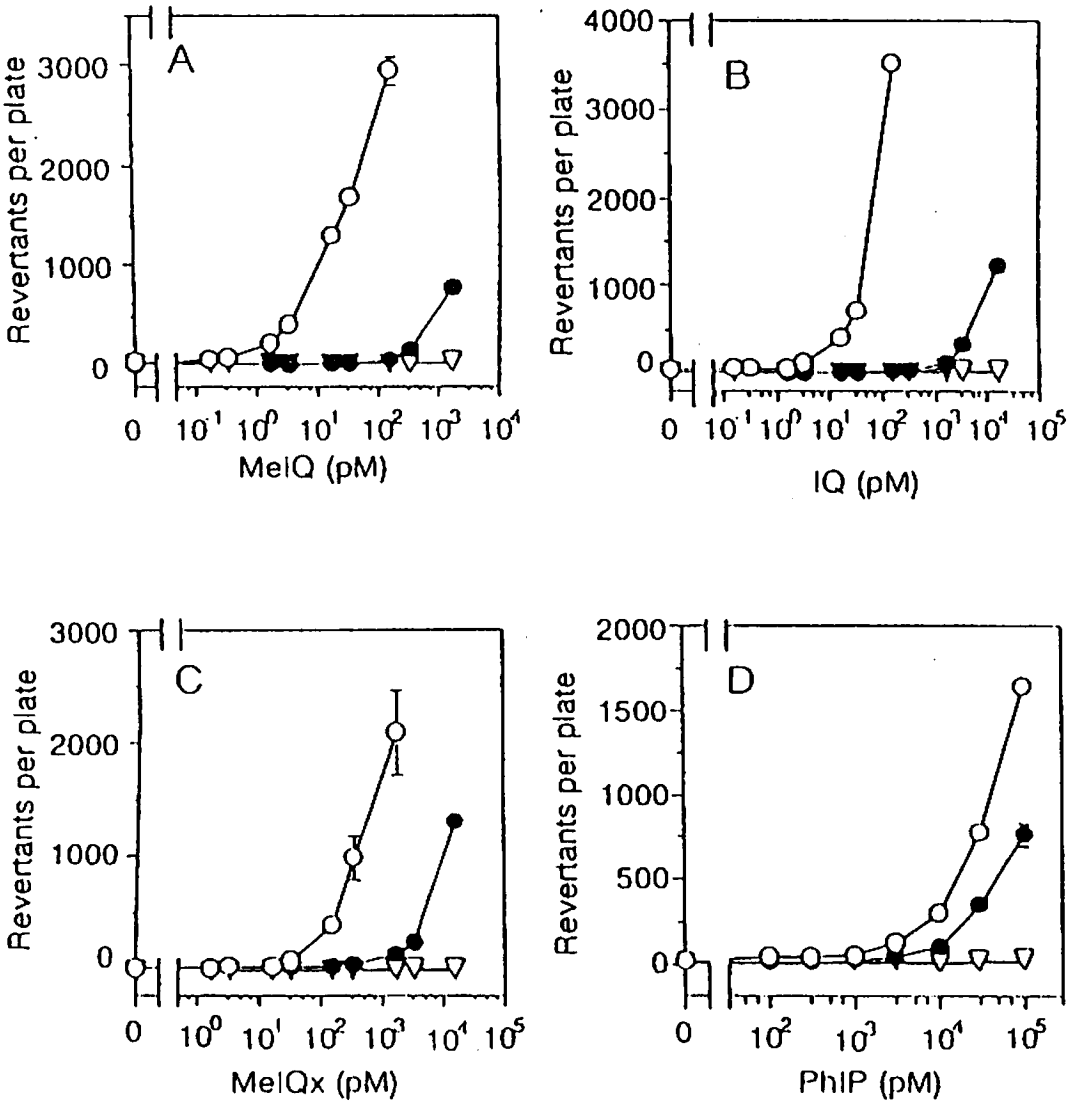

Fig. 2 Sensitivity of genetically engineered *Salmonella* to heterocyclic amines (Suzuki et al., 1998). *Salmonella* cells were treated with MeIQ (A), IQ (B), MeIQx (C), or PhIP (D). ○, TA1538/ARO (TA1538 cells transfected with p1A2OR and pOAT); ● , TA1538/AR (TA1538 cells transfected with p1A2OR); ▽ , TA1538/O (TA1538 cells transfected with pOAT); ▼, TA1538/C, (TA1538 cells transfected with the control plasmid)

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 *Salmonella* highly sensitive to mutagenic heterocyclic amines.

To clarify the mechanism of mutagenesis or carcinogenesis, it is of importance to determine the P450 isozyme(s) responsible for the metabolic activation of chemicals. Thus, we established other *Salmonella* TA1538 co-expressing each of the nine forms of P450, CYP1A1, CYP2A6, CYP2C8, CYP2C9,

Table 2 Mutagen-producing capacity of each form of CYP expressed in *Salmonella typhimurium* cells for promutagens

	Mutagen-producing capacity[b]				
	AFB1	B[*a*]P	PhIP	2-AAF	NNK[c]
CYP1A1	-	180(1)	45000(4.3)	6500(3.6)	39(1)
CYP1A2	110(0.15)[a]	8.2(10)	31000(4.3)	11000(3.6)	70(1)
CYP2A6	1.5(3.6)	-	-	-	8.0(10)
CYP2C8	1.2(3.6)	-	-	-	0.12(100)
CYP2C9	-	0.74(10)	-	-	-
CYP2C19	-	-	-	-	2.71(10)
CYP2D6	-	-	-	-	-
CYP2E1	-	-	-	-	
CYP3A4	17(0.45)	-	-	-	0.45(10)
CYP3A5	-	-	-	-	-

a; Number in parentheses represents minimal concentration (MC) (μM) of promutagens giving positive result. The results were judged as positive when the number of colonies increased in a concentration dependent manner and reached a level twice as high as that obtained with vehicle alone as a control.

b; Number of revertants/nmol promutagen (MC)/pmol P450.

c; Mutagen-producing capacity was detactable with *Salmonella* TA1538 cells expressing human CYP except for the case of NNK. Mutagen-producing capacity for NNK was detactable with *Salmonella* YG7108 cells expressing human CYP.

-; Not detectable.

CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5, together with the reductase. In addition to these *Salmonella* strains, *Salmonella* YG7108 co-expressing each of the ten forms of P450, CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5, together with the reductase was also developed. The *Salmonella* YG7108 strain is a derivative of a *Salmonella* TA1535 strain, showing O^6-methylguanine DNA methyl transferase ada_{ST}- and ogt_{ST}- deficient genotypes, and is applied to detect point mutations occurring in the genomic DNA of the *Salmonella*. We expected that the application of the TA1538 and YG7108 strains of *Salmonella* led us to detect not only promutagens inducing frame shift mutations but also promutagens causing point mutations to the *Salmonella* gene. The mutagenicity of AFB_1, B[*a*]P, 2-AAF, PhIP was detected with the ten strains of *Salmonella* TA1538 cells expressing each form of human P450 together with the reductase. However, the mutagenicity of NNK was not detected with these ten *Salmonella* TA1538 strains expressing each form of P450 with the reductase, probably because NNK usually induced the point mutations. As was expected, the mutagenicity of NNK was detectable with the ten strains of *Salmonella* YG7108 cells expressing each form of P450 with the reductase (Table 2).

The roles of CYP2A6 and CYP2E1 in the metabolic activation of *N*-alkylnitrosamines were also examined using the YG7108 cells expressing CYP2A6 or CYP2E1. CYP2A6 was responsible for the activation of *N*-nitrosamines with relatively large alkyl chain(s) such as NNK, NNN and NMPhA, while CYP2E1 was involved in the activation of *N*-nitrosamines with relatively small alkyl chain(s) such as NDMA and NDEA.

Other toxicological studies using bacterial cells expressing human P450 have been reported by Shimada et al (Shimada et al., 1994). They applied the *E. coli* cells expressing human P450 as a source of the enzyme to examine the genotoxicity of chemicals including heterocyclic amines. The induction of the SOS response in the *Salmonella 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 or the purified preparation of P450 from *E. coli* cells and the purified preparation of rabbit reductase 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. (Hammons et al., 1997) also assayed the metabolism of chemicals including heterocyclic amines and aryl amines by human P450 expressed in the *E. coli* cells. The roles of human CYP1A1, CYP1A2 and CYP3A4 on the metabolic activation of IQ, MeIQx, PhIP, 4-aminobiphenyl were clarified. IQ, MeIQx, PhIP and 4-aminobiphenyl were mainly metabolically activated by CYP1A2. PhIP was also metabolized by CYP1A1. Josephy et al. (Josephy et al., 1995) introduced the expression plasmid carrying human CYP1A2 into *Salmonella* YG1019 cells to detect the mutagenicity of heterocyclic amines and aryl amines. The mutagenicity of 2-AA and 2-aminofluorene was detectable by this system.

P450 proteins expressed in the bacterial strains can be applied to examine the structure-function relationship. Application of site-directed mutagenesis technique allows one to clarify the key amino acid residues for catalytic activity and substrate binding. Porter (Porter, 1994) established an *E. coli* strain harboring rabbit CYP2E1. He clarified that the Phe^{-429}, 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.

The bacterial strains harboring human P450 seem to be a useful tool to study the significance of P450 on the metabolism of drugs and chemicals in humans. This system may be applied as one of the alternative tools for experimental animals. In addition to cytochrome P450, 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, should be established in the future.

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