EXPRESSION OF RECOMBINANT HUMAN CYTOCHROME P450 IN Ames Test Strains for Mutagenicity Testing

A Thesis

Presented to

the Faculty of Graduate Studies

of

The University of Guelph

by

TRACEY HENRY

In partial fulfilment of the requirements for the degree

Master of Science

June 1997

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0-612-24476-8
ABSTRACT

EXPRESSION OF RECOMBINANT HUMAN CYTOCHROME P4501A1 IN AMES TEST STRAINS FOR MUTAGENICITY TESTING

Tracey L. Henry
University of Guelph, 1997

Dr. P. David Josephy
Advisor

Heterologous expression of mammalian bioactivation enzymes in bacteria can obviate the requirement for the addition of exogenous enzymes in bacterial genotoxicity testing. Ames tester strains bearing a plasmid which carries the cDNA of human P450 1A1 were constructed. It was determined that both NADPH-cytochrome P450 reductase and plasmid pKM101, which carries the genes for error-prone DNA repair (mucAB), were likely required for the activation of P450 1A1-dependent mutagens. Subsequently, tester strains bearing plasmids coding for a P450 1A1:NADPH-cytochrome P450 reductase fusion protein and the MucAB proteins were constructed. Strain testing indicated that functional MucAB proteins were produced. However, functional P450 1A1 was not detected in any of the strains. Immunoblot analysis indicated that the level of P450 1A1 expression was low in strains bearing both plasmids. The presence of the plasmid bearing the mucAB genes may have affected the level of P450 1A1 expression.
For Noni, Nana, and Papa
Acknowledgments

There are many people who have made significant contributions to my thesis work. My advisor, David Josephy, whose advice, support, encouragement, and humour were invaluable. Dr. A. Mellors and Dr. C. Whitfield, my advisory committee members, who provided many useful suggestions.

Thanks to Lillian DeBruin for many helpful discussions, and to Heather Lord and James Oak for keeping excellent lab notes.

I also wish to express my gratitude to my family in Niagara Falls, who have been a constant source of support and encouragement.

And finally, thanks to my sisters and brothers at C.U.P.E. 3913, the Central Student Association, and the Graduate Student Association who contributed to my informal education in labour studies, sociology, and politics.
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List of Abbreviations:

2-AA 2-aminoanthracene
AF-2 furylfuramide
δ-ALA δ-aminolevulinic acid
ampR ampicillin-resistant
ampS ampicillin-sensitive
B[α]P benzo[α]pyrene
BCA bicinecinonic acid
BP 7,8-diol 7,8-dihydroxy-7,8-dihydrobenzo[α]pyrene
BP 7,8-diol-9,10-epoxide 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[α]pyrene
BP 7,8-epoxide 7,8-epoxy-7,8-dihydrobenzo[α]pyrene
CmR chloramphenicol-resistant
DMSO dimethyl sulfoxide
EDTA disodium ethylenediamine tetraacetate
EROD 7-ethoxyresoru6n O-deethylase
FAD flavin adenine dinucleotide
FMN flavin mononucleotide
GAPDH glyceradehyde-3-phosphate dehydrogenase
HMG-CoA β-hydroxy-β-methylglutaryl-coenzyme A
HPLC high-performance liquid chromatography
kanR kanamycin-resistant
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>MeIQ</td>
<td>2-amino-3,5-dimethylimidazo[4,5-β]quinoline</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MNNG</td>
<td>N-methyl-N′-nitro-N-nitrosoguanidine</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NAT</td>
<td>N-acetyltransferase</td>
</tr>
<tr>
<td>4-NQO</td>
<td>4-nitroquinoline-1-oxide</td>
</tr>
<tr>
<td>2-NF</td>
<td>2-nitrosofluorene</td>
</tr>
<tr>
<td>OD&lt;sub&gt;650&lt;/sub&gt;</td>
<td>optical density measured at 650 nm</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PhIP</td>
<td>2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>S-9</td>
<td>hepatic 9000 x g supernatant</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>2,4,5-TMA</td>
<td>2,4,5-trimethylaniline</td>
</tr>
<tr>
<td>tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>tetracycline-resistant</td>
</tr>
<tr>
<td>Trp-P-1</td>
<td>3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole</td>
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1. **Introduction**

1.0 **Perspective**

The Ames test is a bacterial (*Salmonella typhimurium*) mutagenicity assay which is used widely to screen potentially mutagenic and carcinogenic chemicals. Many compounds require metabolic activation by mammalian enzymes to exert their carcinogenic effects. Since bacteria do not carry out the ‘bioactivation’ process, an exogenous source of activating enzymes is frequently required to observe chemical mutagenicity in bacterial assays. Mammalian liver homogenate is commonly used as the source of activation enzymes in bacterial mutagenicity testing. Heterologous expression of enzymes of bioactivation in *Salmonella* can obviate this requirement. In addition to the obvious advantage of reducing the use of animals in mutagenicity experiments, heterologous expression of bioactivation enzymes provides increased specificity and sensitivity in bacterial mutagenicity tests.

Cytochrome P450 monooxygenases are among the most significant biotransformation enzymes. Metabolic activation of a vast number of chemical carcinogens proceeds through P450-catalysed oxidations. The focus of the present study is the development of Ames tester strain bacteria which express recombinant human P450s and can detect P450-dependent mutagens, in the absence of mammalian liver homogenate.
1.1 Cytochrome P450

Cytochrome P450s are a super-family of enzymes which metabolize a diverse range of endogenous and xenobiotic compounds. Multiple isoforms of cytochrome P450 are found in mammals, insects, plants, yeast, and bacteria (Groves and Han, 1995). P450s have been identified in many mammalian tissues, including liver, kidney, lung, adrenal cortex, and intestine (Groves and Han, 1995). Eukaryotic P450s are membrane proteins, with the exception of the fungal P450 55A1 (Shoun et al., 1989). The number of substrates of cytochrome P450s is well into the thousands, due to multiple isoforms and the ability of many isoforms to metabolize a broad range of substrates. Reactions catalysed by cytochrome P450s include hydroxylation, epoxidation, dealkylation, sulfoxidation, and dehalogenation (Ortiz de Montellano, 1995). P450s oxidize nonpolar xenobiotics, producing compounds of increased polarity which are more easily excreted or detoxified. However, some P450 metabolites are far more reactive than their precursors and can pose a threat to the integrity of susceptible nucleophiles, such as DNA.

1.2 P450 analytical techniques

1.2.1 Fe²⁺-CO vs Fe⁺² optical difference spectroscopy

Many ferrous heme proteins, including cytochrome P450, form a characteristic carbon monoxide complex in the presence of CO gas and a reducing agent. This ferrous-CO complex can be detected using optical difference spectroscopy, which records the difference in absorbance between CO-bound and CO-free P450 samples. P450 was first
identified, and so named, because of its characteristic absorption maximum at 450 nm (Omura and Sato, 1964). Since the iron must be in a reduced state to bind CO, a reducing agent is added to the samples prior to the addition of CO gas. $\text{Fe}^{2+}$-CO vs $\text{Fe}^{2+}$ difference spectroscopy is routinely used to determine P450 holoenzyme content in microsomes (artificial vesicles produced though homogenation of membranous structures, especially endoplasmic reticulum) and bacterial membrane preparations.

1.2.2. Enzyme assays

The catalytic activity of a P450 sample is measured using functional markers which are P450 substrates. A vast number of P450 isozyme-specific enzyme activity assays have been developed (Correia, 1995). 7-Ethoxyresorufin O-deethylase (EROD) is a functional marker substrate commonly used to measure P450 1A1 catalytic activity. The product formed in a P450-catalysed deethylation of the substrate has a fluorescence emission maximum at 590 nm. Measurement of fluorescence intensity at this wavelength provides a simple measurement of P450 1A1 activity. Other common P450 assays may use colourimetric or HPLC analysis of the products (Josephy, 1997).

1.2.3. Cytochrome c reductase activity

NADPH cytochrome P450-reductase (P450-reductase) is an essential component of P450-catalysed xenobiotic metabolism in mammalian species (see 1.6.; Strobel et al., 1995). P450-reductase activity is commonly assayed by measuring its associated cytochrome c reductase activity. This indirect measurement is much simpler than direct
determination of P450-reductase activity, which requires anaerobic conditions and rapid reaction techniques (Guengerich, 1989). Reduction of cytochrome c can be measured by recording the change in absorbance at 550 nm, upon the addition of NADPH to a sample containing cytochrome c and P450-reductase (Guengerich, 1989). Cytochrome c reductase activity is an accurate and convenient method for assaying P450-reductase activity.

1.3. Cytochrome P450 mechanism

The general mechanism of the P450 catalytic cycle is well established (Groves and Han, 1995). The common features of the P450 active site are an iron protoporphyrin IX (heme), with an axial cysteine and a coordination site for oxygen (figure 1). The catalytic cycle of cytochrome P450 is outlined by the following paradigm: (1) substrate binding; (2) reduction of ferric heme to the ferrous state; (3) binding of molecular oxygen to give the Fe\(^{2+}\)(O\(_2\)) complex; (4) transfer of a second electron to produce a peroxo-iron(III) complex; (5) protonation of the complex and cleavage of the O-O bond to give
water and a highly reactive iron-oxo species; (6) transfer of the oxygen atom from the iron-oxo complex to the substrate; and (7) release of the product (figure 2).

![Proposed catalytic cycle of cytochrome P450.](image)

The widely accepted mechanism of cytochrome P450-catalysed hydroxylation of alkane hydrocarbons entails the transfer of a hydrogen atom from the substrate to the oxo-iron complex to produce a metal bound hydroxyl radical and an alkyl radical (Ortiz de Montellano, 1995). Subsequent transfer of the metal bound hydroxyl radical to the alkyl radical intermediate produces the hydroxylated product. An alternative mechanism, proposed by Newcomb et al., suggests that the hydroxylation reaction must be a
concerted step and that the radical species generated is a transition state, as opposed to an intermediate (1995). Further investigation is required to determine conclusively the details of this complicated mechanism.

The hydroxylation of an aromatic substrate by P450 commonly proceeds through a mechanism distinct from that of alkane hydroxylation. P450-catalysed insertion of a hydroxyl group into an aromatic ring entails epoxidation, followed by opening of the epoxide ring, and migration of the hydride to the adjacent carbon (the "NIH-shift"; see 1.11). Subsequent tautomerisation of the ketone produces the hydroxylated product (see figure 6). Formation of the epoxide can occur via concerted formation of the two carbon-oxygen bonds of the epoxide or addition of the oxygen to one carbon of the π
bond, followed by ring closure of the epoxide (Ortiz de Montellano, 1995).

Alternatively, aromatic substrates with strong electron-donating groups may circumvent the formation of the epoxide through the formation of a single carbon-oxygen bond, followed by electron transfer from the substrate to the P450 heme (figure 3; Josephy, 1996). The evidence for the proposed mechanisms of hydroxylation of aromatic π bonds is ambiguous, and further studies are required to establish firmly the pathway(s) of polycyclic aromatic hydrocarbon oxidation.

1.4. Cytochrome P4501A1

Cytochrome P450 1A1 (P4501A1) is an extrahepatic enzyme which has generated considerable interest because of its potential role in smoking-induced lung cancer. P4501A1 is strongly induced by cigarette smoke in lung, placenta, and lymphocytes (Fujino et al., 1982). The constitutive level of P450 1A1 expression in lymphocytes has been associated with lung cancer risk in smokers (Kouri et al., 1982).

Cytochrome P450 1A1 metabolizes many potentially carcinogenic substrates, including benzo[a]pyrene and other PAHs. Benzo[a]pyrene is metabolized by P450 1A1 to numerous hydroxy, dihydrodiol, and quinone products (Hall et al., 1989). In addition to polycyclic aromatic hydrocarbons, P450 1A1 has been shown to activate a variety of aromatic amines, including MeIQ, Trp-P-1, and PhIP (Shimada et al., 1996). Other procancerogens, such as aflatoxin B₁ and 2-nitropyrene, may also be activated by P450 1A1 (Shimada et al., 1996).
P4501A1 is a polymorphic gene in humans. A Japanese study has shown a correlation between a restriction fragment length polymorphism (RFLP) in human P450 1A1 and incidence of lung cancer in Asian populations (Kawajiri et al., 1990). Digestion of lymphocyte DNA with MspI reveals a clear DNA polymorphism. Genetic frequencies of the predominant homozygous (0.49), heterozygous (0.40), and rare homozygous alleles (0.11) were determined in a healthy population. The frequency of the rare allele was 3-fold higher in lung cancer patients. In a subsequent study, Hayashi et al. demonstrated a genetic linkage between the MspI polymorphism and an amino acid replacement (Ile→Val) in the heme-binding region of human P4501A1 (1991). In a study of foundry workers who were exposed to PAHs, individuals with both the rare homozygous MspI allele and the isoleucine-to-valine amino acid replacement were shown to have an increased level of leukocyte DNA adducts (Hemminki et al., 1997). The increased frequency of the MspI polymorphism in lung cancer patients, and the association of the polymorphism with an increased level of DNA adducts, suggest that P450 1A1 genotypes may contribute to lung cancer risk.

1.5. Bacterial expression of P450s

Heterologous expression of cytochrome P450s in bacterial systems has been the focus of extensive research. Interest in recombinant expression of cytochrome P450s stems from the potential of such systems to provide substantial quantities of catalytically-active protein. Such systems are particularly useful in the study of those proteins, which cannot be easily purified, or obtained in sufficient quantities, from natural sources.
Purification of mammalian P450s from natural sources is confounded by contamination with closely related P450 isoforms. Studies of human P450s are further limited by lack of availability of human tissue samples. Bacterial expression systems circumvent these problems and provide additional advantages, including ease of manipulation and cost-effectiveness.

Initial attempts to express full-length, unaltered, mammalian cytochrome P450s, using standard vectors, were unsuccessful (Barnes et al., 1991). Several modifications of the P450 cDNA sequence were required to achieve high-level protein expression. Various modifications of the nucleotide sequence encoding the N-terminal region of the polypeptide enhanced the expression of mammalian P450s in E. coli. These modifications included truncation of the hydrophobic segment (Larson et al., 1991; Li et al., 1991); replacement of the second codon with GCT, which occurs frequently in this position in E. coli open reading frames; introduction of silent mutations which increase the levels of A and T in mRNA transcripts, to resemble more closely the composition of this region in E. coli mRNAs; and introduction of silent mutations which minimize mRNA-secondary structure formation (Barnes et al., 1991). The N-terminal region was targeted for alterations because it functions as a signal-anchor sequence, directing insertion of the protein into the endoplasmic reticulum and inner mitochondrial membrane, as opposed to playing a direct role in catalysis (Wachenfeldt and Johnson, 1995).

Barnes et al. were successful in expressing bovine 17α-hydroxylase (P450 17A) in E. coli, with the N-terminal modifications described above (1991). Rabbit P450 2E1
<table>
<thead>
<tr>
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<th>Modification*</th>
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<tr>
<td>Human</td>
<td></td>
<td></td>
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<tr>
<td>1A1</td>
<td>Ala; min-fold</td>
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</tr>
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<td>1A2</td>
<td>17A terminus</td>
<td>Sandhu et al., 1994</td>
</tr>
<tr>
<td>2C8, 2C9, 2C18, 2C19</td>
<td>17A terminus, 2C primer</td>
<td>Richardson et al., 1995</td>
</tr>
<tr>
<td>2D6</td>
<td>truncation, Ala, AT, min-fold</td>
<td>Gillam et al., 1995a</td>
</tr>
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<td>2E1</td>
<td>truncation, Ala, AT, min-fold</td>
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<tr>
<td>3A4</td>
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<td>17A terminus, 2C primer</td>
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<tr>
<td>7</td>
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<td>Li and Chiang, 1991</td>
</tr>
<tr>
<td>4A1</td>
<td>truncation</td>
<td>Chaurasia et al., 1995</td>
</tr>
</tbody>
</table>

* Ala, replacement of the second codon with GCT; min-fold, modification of bases in the 5' terminus to reduce the formation of secondary structure in the mRNA; 17A terminus, replacement of the N-terminus with that used for recombinant bovine P450 17A; 2C primer, introduction of a consensus sequence for P450 2C directly following the modified P450 17A terminal sequence; truncation, removal of a portion of the N-terminus; AT, optimized the AT content of the 5' terminal codons.
(Larson et al., 1991) and rat 7α-hydroxylase (P450 7; Li and Chiang, 1991) were expressed in E. coli with a truncated N-terminal sequence. Subsequently, many other mammalian P450s have been expressed in E. coli using the previously described approaches (see Table 1; Guengerich et al., 1996).

Recombinant, N-terminal-modified P450s are predominantly located in the bacterial inner membrane (Pernecky et al., 1995; Guengerich et al., 1996). This result was unexpected, since the hydrophobic N-terminal region functions as a membrane anchor. Pernecky et al. (1995) found that the N-terminal region of modified P450 2E1 (Δ3-29) had a large number of uncharged, hydrophobic amino acids, which could facilitate the translocation of proteins into the inner membrane. Replacement of the first 17 N-terminal amino acids of P450 2E1 (Δ3-29) with the first 19 of the soluble bacterial P450_{BM-3} (BM-3:2E1) resulted in 80% of the expressed protein being localized in the cytosol. These results suggest that the N-terminal region of modified P450 2E1 is primarily responsible for the membrane insertion of the protein. (However, the existence of the 20% of BM-3:2E1 which remains membrane bound indicates that peptides other than those in the N-terminal region are also involved in membrane localization of P450 2E1.)

The N-terminal region appears to be of greater importance to membrane binding in P450 2B4 than in P450 2E1. The N-terminal modified P450 2B4 (Δ2-29) is predominantly cytosolic, unlike the full-length protein, which is membrane-bound. Substitution of the P450_{BM-3} N-terminal sequence in P450 2B4 (Δ2-29; BM-3:2B4) resulted in only a modest decrease in the proportion of the protein which was membrane-
bound. These results further suggest that the N-terminal region is primarily responsible for insertion of recombinant P450s into the membrane.

The activities of purified, reconstituted recombinant mammalian P450s, relative to their native forms, vary with the isoform. Purified recombinant human P450 2E1 activity is comparable to that of P450 2E1 purified from human tissues, with turnover numbers for chlorzoxazone 6-hydroxylation of 5.6 min⁻¹ and 5.5 min⁻¹, respectively (Gillam et al., 1994; Peter et al., 1990). In contrast, the N-demethylase activity of purified recombinant rabbit P450 2B4 with benzphetamine was found to be only 41% that of purified rabbit P450 2B4 (Pernecky et al., 1995). The reported activities of purified recombinant human P450 3A4 and 3A5 are also substantially lower than of the analogous native proteins (Gillam et al., 1995; Wrighton et al., 1990). It has been proposed that the N-terminal modifications may influence activity through interaction with NADPH-cytochrome P450 reductase (Pernecky et al., 1995). However, preliminary experiments in this area have been inconclusive. Differences in activities of native and recombinant P450s may also be attributable to the difficulties in optimizing the reconstitution conditions for the purified proteins (Guengerich et al., 1996).

Typically, the activities of recombinant human P450s in intact cells or isolated bacterial membranes have been significantly lower than those observed in reconstituted systems (Guengerich et al., 1996). Human P450 1A1 or 1A2 activity in isolated membranes was less than 10% that of the corresponding purified recombinant enzyme (Guo et al., 1994; Sandhu et al., 1994). Similarly, purified reconstituted P450 2E1 activity was five times greater than that observed in intact membranes (Gillam et al.,...
The diminished P450 activity in membrane preparations may be due to inaccessibility of the NADPH-P450 reductase (see 1.6. and 1.7.; Guengerich et al., 1996) or the presence of an inhibitor in the membrane preparation (Sandhu et al., 1994). The level of P450 catalytic activity in intact cells is relevant to the present investigation because we are developing a system which relies on activity of the recombinant P450s in whole bacterial cells.

Blake et al. (1996) reported very high levels of human P450 3A4 activity, in both membranes and intact E. coli cells, when human NADPH-cytochrome P450 reductase was coexpressed with the P450. Intact cells and isolated membranes catalysed testosterone 6β-hydroxylation with turnover rates of 17.3 min⁻¹ and 25.5 min⁻¹, respectively. These rates exceed the turnover rate of 6 min⁻¹ reported for P450 3A4 activity in human liver microsomes (Yamazaki et al., 1996). Hydroxylase activity was not detectable in cells or membranes which lacked either P450 3A4 or P450-NADPH reductase (Blake et al., 1996). These results highlight the functional importance of ensuring facile electron transport to the P450 protein.

1.6. Electron transport to cytochrome P450: NADPH cytochrome P450 reductase

Cytochrome P450-catalysed oxidations require electron transfer from reduced pyridine nucleotides to the P450. Electron transfer to microsomal cytochrome P450s from NADPH is mediated by NADPH-cytochrome P450 reductase (P450 reductase). The reductase is anchored to the endoplasmic reticulum by a single transmembrane helix, with the bulk of the protein exposed to the cytosol (Black and Coon, 1982). P450
reductase is widely distributed in mammalian species and tissues in association with
cytochrome P450 (Wachenfeldt and Johnson, 1995). Unlike cytochrome P450, only one
form of cytochrome P450 reductase has been identified (Wachenfeldt and Johnson,
1995).

Purified P450 reductase contains equimolar amounts of the flavins FMN and FAD
(Dignam and Strobel, 1977), which mediate electron transfer from the two-electron
donor, NADPH, to the single-electron acceptor, cytochrome P450. Studies of an FMN-
depleted reductase, which was able to oxidize NADPH, but unable to reduce cytochrome
P450, suggested that electrons flow from NADPH to reductase-bound FAD (Vermilion
and Coon, 1978). Furthermore, the FMN-depleted reductase was unable to produce an
air-stable semiquinone, as observed in the holoenzyme (Strobel et al., 1995). The
formation of the semiquinone is required for direct electron transfer to the cytochrome
P450 heme iron. Thus, the hypothesized pathway for the flow of electrons is:

\[ \text{NADPH} \rightarrow \text{FAD} \rightarrow \text{FMN} \rightarrow \text{acceptor} \]

(Vermilion and Coon, 1978). Subsequent $^{31}$P-NMR studies demonstrated that the air-
stable semiquinone detected in the reductase is formed exclusively on FMN
(Narayanasami et al., 1992). Kurzban and Strobel (1986) produced an FAD-depleted
reductase which could not accept electrons from NADPH, further confirming the
pathway of electron flow in NADPH-cytochrome P450 reductase proposed by Vermilion
and Coon.

Bacterial and mitochondrial systems rely on a flavoprotein/iron-sulfur protein
system to transfer electrons from reduced pyridine nucleotides to cytochrome P450
Mitochondrial cytochrome P450 enzymes are supported by adrenodoxin reductase, an FAD-containing flavoprotein, and adrenodoxin, an iron-sulphur protein (Lambeth et al., 1982). The analogous electron transfer system for the soluble bacterial cytochrome P450 \textsubscript{cam} consists of putidaredoxin reductase and putidaredoxin (Lambeth et al., 1982).

Soluble flavoproteins have been shown to support electron transport to recombinant eukaryotic cytochrome P450s, expressed in bacterial strains which do not contain endogenous P450s (Jenkins and Waterman, 1994; Yamazaki et al., 1995). The flavoproteins were first identified after recombinant cytochrome P450 17A hydroxylase activity was observed in \textit{E. coli}, in the absence of an exogenous source of NADPH-cytochrome P450 reductase (Barnes et al., 1991). Flavodoxin and NADPH-flavodoxin reductase were identified as the \textit{E. coli} proteins supporting the activity of recombinant bovine P450 17A hydroxylase (Jenkins and Waterman, 1994). \textit{E. coli} flavodoxin binds P450 17A directly, with an apparent $K_\text{m}$ of ~0.2 $\mu$M. Furthermore, amino acid sequence alignment revealed significant sequence similarity between the \textit{E. coli} flavoproteins and homologous domains in rat microsomal P450 reductase. These results suggested that the flavoproteins were responsible for the cytochrome P450 activities, in reconstituted, recombinant \textit{E. coli} systems. However, purified flavodoxin and NADPH-flavodoxin reductase were shown to be ten-fold less efficient than purified, recombinant rat P450 reductase in supporting P450 17A hydroxylase activity (Jenkins and Waterman, 1994).
The significantly lower enzyme activity of P450 17A hydroxylase when supported by endogenous bacterial flavoenzymes suggests that the \textit{in vivo} catalytic activity of recombinant P450s could be improved if P450 reductase were also present in the bacterial cell.

1.7. 

Recombinant cytochrome P450 reductase

Coexpression of recombinant NADPH-cytochrome P450 reductase and cytochrome P450 is a significant step towards the development of systems that can perform cytochrome P450-dependent oxidations in live bacteria. Several approaches are possible, including: (i) coexpression of NADPH-P450 reductase on separate plasmids; (ii) expression of cytochrome P450 and NADPH-P450 reductase from a single plasmid, in a poly-cistronic manner; and (iii) generation of a cytochrome P450:NADPH-P450 fusion protein (Guengerich \textit{et al.}, 1996). The first approach is undesirable, due to the frequent instability of systems containing more than one plasmid. However, the two latter possibilities, which entail expression of both proteins from a single plasmid, are the focus of current research in bacterial P450 expression systems (Guengerich \textit{et al.}, 1996).

A single polypeptide which is functionally analogous to the microsomal monooxygenase system has been identified in \textit{Bacillus megaterium} (Nahri and Fulco, 1986). Cytochrome P450 102 is a catalytically self-sufficient protein containing heme, FAD, and FMN, in a 1:1:1 ratio. Upon trypsinolysis, the protein is cleaved into two domains: a substrate-binding hemoprotein, and a flavoprotein domain capable of NADPH-dependent cytochrome c reduction. Similar artificial fusion proteins have been
developed for recombinant P450 expression systems in yeast (Murakami et al., 1987) and *E. coli* (Guengerich et al., 1996). Reductase fusion proteins of several isoforms of human P450 have been constructed, including P450 1A1 (Chun et al., 1996), P450 3A4 (Shet et al., 1993), P450 1A2, and P450 3A5 (Chun and Guengerich, unpublished results).

As mentioned above, a human cytochrome P4501A1:NADPH-cytochrome P450 reductase (P4501A1-reductase) fusion protein has been constructed and expressed in *E. coli* (Chun et al., 1996). The complete cDNA sequence coding for human cytochrome P450 1A1 was fused to the sequence coding for the soluble portion of rat NADPH-cytochrome P450 reductase, and sub-cloned into the expression vector, pCW. The plasmid was transformed into *E. coli* strain DH5α, and expression of the fusion protein was optimized through isopropyl-β-D-thiogalactoside (IPTG) induction, and incubation with δ-aminolevulinic acid (δ-ALA), a heme precursor. The human P450 1A1-reductase was isolated from bacterial membranes and characterized. The molecular weight of the fusion protein was 129 kDa, as expected. The NADPH-cytochrome c reductase activity (see 1.2.3.) of the purified fusion protein (20 μmol/min/mg protein) was only about one-half of the expected activity of purified, endogenous rat NADPH-P450 reductase (Yasukochi and Masters, 1976). This may be due to a tighter interaction between the reductase and the P450, which does not favour electron transfer to exogenous electron acceptors such as cytochrome c. Alternatively, the reductase moiety could be inherently less stable than the free reductase. The $V_{max}$ and $K_m$ values for cytochrome P450-dependent 7-ethoxyresorufin O-deethylation and B[α]P 3-hydroxylation were 0.65 and
1.04 nmol product/min/nmol enzyme and 0.34 and 19 μM, respectively. These rates are comparable to those achieved in previous studies of purified recombinant human P450 1A1 activity in reconstituted systems, with an exogenous source of NADPH-cytochrome P450 reductase (Guo et al., 1994). However, the rates of catalytic activity achieved with either the recombinant human cytochrome P450 1A1 with an exogenous source of reductase, or the P4501A1:rat reductase fusion protein are considerably lower than those reported for purified rat liver cytochrome P450 1A1 (e.g. B[a]P 3-hydroxylation rates of 10-25 nmol product/min/nmol enzyme; Chun et al., 1996).

An alternative approach to the construction of fusion proteins, for the production of catalytically active, recombinant cytochrome P450 systems in bacteria, is the development of polycistronic expression vectors. Polycistronic gene organization was employed to develop a prokaryotic system which expressed various eukaryotic proteins, including cyclophilin, FK506-binding protein, and a domain of an MHC class Iα molecule at detectable levels (Ito and Kurosawa, 1992). The expression of the various eukaryotic proteins was achieved through the construction of a vector that placed the eukaryotic genes downstream from a well-expressed gene and incorporated a ribosome-binding site (RBS; see figure 4; Ito and Kurosawa, 1992).

Figure 4. Schematic representation of a polycistronic gene construct. GST; glutathione transferase. GST, a well-expressed gene, is placed upstream from the foreign gene.
Polycistronic gene organization has been useful in the construction of vectors which coexpress human cytochrome P450s and P450-reductase in *E. coli* (Dong and Porter, 1996; Blake *et al.*, 1996). An expression vector bearing the cDNAs for human cytochrome P450 2E1 and rat NADPH-P450 reductase, arranged in tandem, was constructed and transformed into *E. coli* (Dong and Porter, 1996). Immunoblot analysis confirmed the production of two distinct polypeptides, with the expected molecular weights. The reported rates of aniline and *p*-nitrophenol hydroxylation, by solubilized membranes from the transformed *E. coli* cells, were 1.8 and 2.0 nmol/min/nmol P450, respectively. However, P450 activity was not demonstrated in intact *E. coli*.

As mentioned above, a polycistronic-cytochrome P450 monooxygenase system with very high levels of catalytic activity in *E. coli* has been reported by Blake *et al.* (1996). A vector bearing the cDNA for human cytochrome P450 3A4 and human cytochrome P450 reductase, organized in tandem, was constructed. The bacterial PelB signal sequence was translationally fused to the P450 reductase. The PelB leader sequence was isolated from the phytopathogenic enterobacterium *Erwinia chrysanthemi*, and directs the transport of the reductase into the inner bacterial membrane (Anderson *et al.*, 1994). The cytochrome P450 3A4 yield obtained was relatively high: 200 nmol/l culture vs 0.8 nmol/l culture in the P450 2E1 polycistronic construct (Dong and Porter, 1996). Rates of 6β-hydroxylation of testosterone and oxidation of nifedipine, by whole cells, were 15.2 and 17.3 nmol/min/nmol P450, respectively. These turnover rates are higher than those previously reported for both reconstituted recombinant cytochrome P450 3A4 membranes (Yamazaki *et al.*, 1995) and human liver microsomes (Yamazaki *et
The levels of catalytic activity reported by Blake et al. (1996) far surpass the activities reported in previous studies of recombinant cytochrome P450-bacterial expression systems. This success may be due to one or more of the following: (i) increased human cytochrome P450 activity when coupled to human P450 reductase, as opposed to rat reductase; (ii) incorporation of the bacterial PelB signal sequence into the recombinant reductase protein; and (iii) more sensitive methods for detection of oxidized products.

1.8. Expression of recombinant human enzymes in *Salmonella typhimurium*

Systems expressing recombinant enzymes in *S. typhimurium* have proven useful in the development of improved bacterial mutagenicity assays (see 1.13). A system was developed in *S. typhimurium* to investigate the mutagenic activation of aromatic amines by human enzymes (Grant et al., 1992). Subsequently, Ames tester strains which activate aromatic amines in the absence of an exogenous source of bioactivation enzymes were developed (Josephy et al., 1995; Josephy et al., 1997). In these studies, plasmid vectors developed for *E. coli* systems were used for expression in *S. typhimurium*.

Activation of aromatic amines requires both *N*-hydroxylation and *N*-acetylation, catalyzed by P450 1A2 and NAT, respectively:

\[
\text{Ar-NH}_2 \xrightarrow{\text{P450 1A2}} \text{Ar-NHOH} \xrightarrow{\text{NAT}} \text{Ar-NHOAc}
\]
Ames tester strains bearing plasmids coding for either human NAT 1 or NAT 2, yielded functional enzymes which supported mutagenic activation of aromatic amines in mutagenicity assays (Grant et al., 1992). An exogenous source of P450 1A2 (mammalian liver homogenate or S9) was provided to catalyze the initial hydroxylation of aromatic amines to N-hydroxyarylamines. Subsequent N-acetylation, catalyzed by NAT, gives rise to N-acetoxy esters. Spontaneous heterolysis of the highly reactive N-acetoxy esters produces the ultimate DNA reactive species, presumably a nitrenium ion (RN'\textsc{H}). Both strains expressing recombinant human NAT activated aromatic amines to mutagenic species in Ames assays. The NAT-overexpressing strains demonstrated greatly enhanced sensitivity to arylamines, compared to the parental strain which was NAT. These strains provide tools for studying the role of human NAT enzymes in the metabolic activation of aromatic amines to genotoxic products.

The NAT over-expressing Ames tester strains were further developed by Josephy et al. (1995). The strains were transformed with a plasmid encoding human P450 1A2 (DJ4501A2) and were shown to be sensitive to aromatic amine mutagenicity in the absence of an exogenous source of activating enzymes. In modified Ames tests, mutagenicity of 2-aminofluorene was greater in DJ4501A2, in the absence of S9, than in either of the standard tester strains with S9 activation. The expressed P450 protein demonstrated the spectroscopic, immunological, electrophoretic, and catalytic properties of the human protein. However, the level of expression of the recombinant P450 1A2 in S. typhimurium was only one-quarter of that observed in E. coli. The activity of proteases in Ames tester strains may account for the decreased level of expression.
observed in *S. typhimurium* (Guengerich *et al.*, 1996), although many other factors may be contributing to this difference.

There are several advantages to the engineered expression of bioactivation enzymes in Ames tester strains: (i) reduction in the requirement for mammalian tissue preparations; (ii) generation of reactive metabolites inside the bacterial cell, as opposed to their production outside the cell; (iii) the ability to study enzymes from specific tissues or species, in the absence of competing isoforms; and (iv) the ability to study chimeric or mutant enzymes (Josephy *et al.*, 1995). The goal of the current research project is to engineer the expression of human P450 1A1 in an Ames tester background and to detect activation of P450 1A1-dependent mutagens in the absence of an exogenous source of bioactivation enzymes.

1.9. **P450 expression in *Saccharomyces cerevisiae***

Expression of recombinant human P450s has been studied extensively in eukaryotic *Saccharomyces cerevisiae*. Yeast and bacterial systems are similar in that they both offer simple, cost-effective production and purification of large quantities of proteins. An additional advantage of the yeast system is the presence of endoplasmic reticulum and mitochondria. However, expression levels in recombinant yeast cells vary considerably (Bellamine *et al.*, 1994) and the presence of endogenous P450s can complicate the development of heterologous expression systems.

Human P450 1A1 has been successfully expressed in *S. cerevisiae*, with P450 1A1-specific activity detected in isolated microsomal fractions (Eugster *et al.*, 1990).
The observed P450 1A1 activity was supported by a heterologous electron donor, presumably yeast oxidoreductase. In a subsequent study, human P450 1A1 activity in *S. cerevisiae* was increased sixteen-fold when the P450 1A1 was coexpressed with human NADPH-P450 reductase (Eugster *et al.*, 1992). EROD activity (see 1.2.2.) in whole cells expressing the reductase and P450 1A1 was 45.5 pmol product/OD₆₀₀ units/min. A concomitant decrease in the amount of immunoreactive P450 1A1 was observed in the coexpression system, suggesting that the increased activity was a result of more efficient coupling between the human oxidoreductase and the human P450 1A1.

In further studies, which parallel approaches to bacterial P450 expression systems, human P450 1A1-human NADPH-P450 reductase fusion proteins have been constructed and expressed in *S. cerevisiae* (Wittekindt *et al.*, 1995). The cDNA encoding the entire human P450 reductase sequence was fused to the coding region of human P450 1A1 and expressed under the control of the constitutive *S. cerevisiae GAPDH* promoter. In a second construct, the amino terminus of the reductase was replaced by the membrane-anchor domain of yeast HMG-CoA. CO-difference spectroscopy indicated that the holoenzyme content of microsomes containing the fusion proteins was approximately 0.1 nmol/mg, which is comparable to levels observed in a strain containing only P450 1A1. Western blot analysis of whole cells indicated the presence of intact fusion constructs. However, immunoblots of microsomes revealed degradation of the fusion proteins. Whole cell EROD activities of the full-length reductase fusion and the amino terminal-modified reductase fusion proteins were found to be 15% and 8%, respectively, of those observed in cells expressing only P450 1A1.
The turnover number for EROD activity in microsomes isolated from oxidoreductase-deficient yeast cells expressing the full-length reductase fusion protein was 0.3 pmoles product/pmole enzyme/min. These results suggest that, while the level of fusion protein production in this system may be adequate, the stability of the protein and the ability of the fused enzymes to metabolize their substrates are limited.

In addition to heterologous expression of proteins in yeast, there has been some interest in the development of mutation assay systems in *S. cerevisiae*. Forward mutation to cycloheximide resistance, L-canavanine resistance, and DL-α-aminoacid acid resistance in a wild-type *S. cerevisiae* strain was used to test for sensitivity to polycyclic and heterocyclic compounds (Mitchell and Gilbert, 1991). While mutations were detected, the doses required were up to 100-fold higher than those detected by the Ames assay (see 1.13). Furthermore, cell counting was impeded by heavy background growth and cell clumping. Mutagenicity assays have yet to be developed in *S. cerevisiae* which are comparable to the Ames test in sensitivity and simplicity.

1.10. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) have long been implicated in certain types of cancer. Over 200 years ago, it was observed that chimney sweeps had a high incidence of scrotal cancer, a disease which is very rare among the general population. In the early 1900s, it was demonstrated experimentally that cancer could be induced in rodents which were exposed to coal tar. Further studies focused on identifying one or more individual chemicals that were the cancer-causing agents in coal-tar. In 1933,
more than 150 years after the first description of an occupational cancer, a polycyclic aromatic hydrocarbon (PAH), benzo[a]pyrene (B[a]P), was identified as an active coal-tar carcinogen.

The simplest PAHs are naphthalene and anthracene, which are composed of two or three six-membered rings, respectively (Figure 5). More complex PAHs include pyrene (four fused rings) and various substituted pyrenes and anthracenes. Unlike benzene, the carbon-carbon bond lengths in anthracene and other PAHs are not equivalent. This can be accounted for by considering the resonance structures for a given PAH.

![Fig. 5. Structures of various polycyclic aromatic hydrocarbons.](image)

Analysis of bond length data reveals the type of carbon-carbon bond, at a given position, which contributes most prominently to a resonance hybrid. For example, the length of the 1,2 carbon bond of naphthalene is 1.36Å, compared with 1.39Å in benzene. Since two of the three resonance structures of naphthalene shows an olefinic bond in this position, it is shorter than a typical aromatic carbon-carbon bond. The 1,2; 4,5; 7,8; 9,10; and 11,12 bonds of B[a]P are activated relative to benzene, and contribute to the biological reactivity of PAHs.
1.11. Polycyclic aromatic hydrocarbon metabolism

Metabolic activation of benzo[a]pyrene proceeds through an arene oxide intermediate. The first evidence which suggested that an arene oxide intermediate is formed during enzymatic hydroxylations was reported in 1967 (Guroff et al.). They unexpectedly observed the migration, to an adjacent position on an aromatic ring, of the group displaced by hydroxide during the P450-catalyzed hydroxylation of acetanilide (Figure 6). The migration of the hydrogen atom at the position of hydroxylation was coined the “NIH-shift” because it was discovered in the laboratories of the National Institute of Health, Bethesda, MD. This observation indicated that an arene oxide intermediate may be formed, since an electron-deficient carbon can arise from the opening of an epoxide. Subsequent migration of an adjacent group, to the nascent carbenium ion results in the observed shift. Further studies, which trapped radiolabelled naphthalene 1,2-oxide as a metabolite of naphthalene, provided direct evidence that epoxide intermediates are formed during the metabolism of aromatic hydrocarbons (Jerina and Daly, 1974).
Epoxides are highly reactive, due to their strained three-membered ring structure. The major reaction pathways of arene oxides include spontaneous isomerization to phenols and quinones, hydrolysis (catalyzed by epoxide hydrolase), and conversion to glutathione conjugates (catalyzed by cytosolic glutathione S-transferase). Depending upon which ring position is initially oxidized, and which metabolizing enzymes are present, one or more of these pathways may occur.

Isomerization to phenols and quinones is a major pathway for transformation of arene oxides produced during primary metabolism of arene oxides. $B[a]P \, 2,3-,\, 4,5-,\, 7,8-$
and 9,10-oxides can potentially isomerize to their respective phenols. 3-Hydroxy-B[a]P and 9-hydroxy-B[a]P were reported as two major B[a]P metabolites formed in the presence of rat liver microsomes (Waterfall and Sims, 1972). 1- and 7- hydroxy-B[a]P have also been identified as B[a]P metabolites (Thakker et al., 1985). The formation of phenols from epoxides is energetically favourable, since it restores aromaticity to the molecule. This is confirmed by the fact that isomerization proceeds in the absence of functional enzymes (Waterfall and Sims, 1972). Phenols may be further oxidized to quinones by rat liver microsomal enzymes. Indeed, 1,6-, 3,6-, and 6,12-B[a]P-quinones have been identified as products of BP metabolism (Thakker et al., 1985).

Metabolism of arene oxides to dihydrodiols is a crucial step in the bioactivation of PAHs. In the presence of rat liver homogenates, 7,8-dihydro-7,8-dihydroxy B[a]P (BP 7,8-diol) and 9,10-dihydro-9,10-dihydroxy B[a]P are formed (Waterfall and Sims, 1972). The hydrolysis of arene oxides to dihydrodiols is catalyzed by microsomal epoxide hydrolase (Josephy, 1997). The 7,8-, and 9, 10-dihydrodiol products are not directly reactive. However, these species possess an olefinic bond in the terminal ring, which can be further metabolized to the direct-acting dihydrodiol-epoxide (Figure 7).
In 1973, Sims et al. identified 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene 9,10-oxide (BP 7,8-diol-9,10-epoxide) as the major B[a]P metabolite which reacts directly with DNA. Their experiments compared DNA hydrolysates from hamster embryo cells that had been incubated with [³H]-BP 7,8-diol-9,10-epoxide and with [¹⁴C]-benzo[a]pyrene. The adducts formed from cellular metabolism of B[a]P were shown to be identical to the BP 7,8-diol-9,10-epoxide-derived adducts. The high chemical and
biological reactivity of this molecule is attributed to the presence of an epoxide group on
the saturated angular benzo ring in the bay region of the molecule (Thakker et al., 1985). Bay region epoxides are very reactive molecules which are not scavenged effectively by
detoxication enzymes. Many studies have indicated that these molecules are very poor
substrates for epoxide hydrolase (Thakker et al., 1985 and refs. therein). The generation
of reactive electrophilic molecules, in the absence of effective biological scavengers,
poses a threat to the integrity of susceptible nucleophiles, such as DNA.

1.12. Aflatoxin B₁

Aflatoxin B₁ is a fungal toxin produced by certain strains of Aspergillus flavus
and related molds. Aspergillus flavus is a ubiquitous mold which grows on a variety of
agricultural products, when adequate moisture and heat are available. Under such
conditions, foodstuffs can become contaminated with toxins and pose a serious health
risk to both animals and humans. Aflatoxin B₁ is acutely hepatotoxic and carcinogenic.
In South Asian and African countries, where food supplies have been heavily
contaminated with aflatoxin B₁, liver cancer is one of the most frequently observed
malignancies (Hayes and Campbell, 1986). Epidemiological studies have revealed a
significant increase in respiratory cancers in workers who are occupationally exposed to
dusts from aflatoxin B₁-contaminated products (Hayes et al., 1984). Further evidence
has suggested that lung tissue, in addition to liver, is also exposed to aflatoxin B₁ as a
result of ingestion (Harrison and Garner, 1991).
Aflatoxin B₁ is metabolically activated to aflatoxin B₁-8,9-epoxide, the ultimate DNA binding species (Miller, 1978). Two stereoisomers of the epoxide, an endo- and exo-epoxide, have been identified as products of the microsomal bioactivation of aflatoxin B₁ (see figure 8; Raney et al., 1992a). The exo-epoxide was shown to be 500-fold more mutagenic than the endo-isomer, in S. typhimurium reversion assays (Iyer et al., 1994). Mechanistic studies revealed that, upon intercalation of the epoxides into DNA, only the exo-epoxide is correctly positioned for a backside S₂2 attack on the N⁷ position of guanine (Iyer et al., 1994). While both isomers are formed upon bioactivation of aflatoxin B₁, the exo-epoxide is the principal DNA-binding metabolite.

Aflatoxin B₁ is a substrate for several of the cytochrome P450 isozymes. P450 1A1 and P450 1A2 were shown to activate aflatoxin B₁, in S. typhimurium mutagenicity.
assays (Shimada et al., 1996). In a reconstituted system, E. coli membranes from a strain expressing human P450 3A4 preferentially activated aflatoxin B1 to the exo-epoxide, with no endo-epoxide detected by HPLC analysis (Ueng et al., 1995). P450 3A4 is the predominant P450 isoform identified in human liver tissues, and is primarily responsible for aflatoxin B1 metabolism in human liver microsomes (Guengerich, 1995). In rabbit lung tissue, P450 2B4 and P450 4B1 are the principle isoforms which catalyze aflatoxin B1 bioactivation (Massey, 1996). These isoforms are preferentially expressed, and have higher catalytic activity than other P450s in rabbit lung (Daniels et al., 1990).

Aflatoxin B1 metabolism is complex: the endo- and exo-epoxides comprise only a small fraction of the total aflatoxin B1 metabolites. Hydroxylation and O-dealkylation, catalyzed by various P450 isoforms, both occur during the initial metabolism of aflatoxin B1 (Massey, 1996). The 3α-hydroxylation product, aflatoxin Q1, is the predominant aflatoxin B1 metabolite produced by P450 3A4-catalyzed metabolism (Ueng et al., 1995). P450 1A2 preferentially metabolizes aflatoxin B1 to aflatoxin FM1, via hydroxylation in the 9α position (see Figure 8; Ueng et al., 1995). Hydroxylation at position 8 and O-dealkylation can also occur, for a total of four initial aflatoxin B1 metabolites, in addition to the two epoxides. Since the non-epoxide metabolites are not DNA-binding species, hydroxylation and O-dealkylation are considered deactivating pathways.

The primary detoxication pathway for aflatoxin B1 is glutathione S-transferase (GST)-catalyzed conjugation of aflatoxin B1-8,9-epoxide (Massey, 1996). GST conjugates of both the endo- and exo- epoxide are formed (Raney et al., 1992b). GSTs are a superfamily of enzymes, which vary considerably in their substrate specificity and
distribution. Two classes of GSTs, Alpha and Mu, have demonstrated high activity toward activated aflatoxin B₁ in vitro (Stewart et al., 1996).

1.13. Mutagenicity testing

The Ames test is a mutagenicity assay developed in the laboratory of Dr. Bruce N. Ames (Ames et al., 1973). Due to its simplicity and sensitivity, the Ames test is currently one of the most widely used mutagenicity assays. The Ames test detects mutagenicity through the reversion of histidine-auxotrophic mutants to the prototrophic phenotype. The S. typhimurium tester strains developed by Ames and colleagues each bear a mutation in one of the genes of the histidine biosynthesis operon. Exposure of these strains to mutagenic compounds results in reversion of the non-functional allele to the wild-type. This phenotypic change is easily detectable by plating on minimal agar with trace amounts of histidine (to support the first few cell divisions and allow expression of the reversion mutation). The number of colonies which revert to the His⁺ phenotype in the presence of a mutagen is a measure of its potency. As a routine control, the number of colonies which revert to His⁺ spontaneously is also determined, in each experiment.

The Ames tester strains bear several mutations which make them highly sensitive to chemical mutagens. Galactose (gal) and deep rough (rfa) mutations were introduced into the

S. typhimurium tester strains to disrupt the polysaccharide side-chain of the lipopolysaccharide (LPS) which coats the bacterial surface (Ames et al., 1973). These
mutations increase the cell's permeability to chemicals. A deletion in the *gal* operon eliminates galactose synthesis from the cell, resulting in the disruption of O-antigen biosynthesis. The *rfa* mutation inhibits synthesis of polysaccharides distal to the ketodeoxyoctanoate core. A deletion through the *uvrB* region of the bacterial chromosome, which eliminates the DNA nucleotide excision repair system, was also introduced. In the absence of excision repair, pyrimidine dimers and other bulky pre-mutagenic adducts are not repaired by the UvrABC system, resulting in a higher frequency of mutagenesis.

Some details of the mutational specificity of a mutagen can be elucidated using the Ames assay. Two of the Ames tester strains (TA1537 and TA1538) were derived by treatment with the frameshift mutagen ICR364-OH (Ames *et al.*, 1972). As a result, TA1538 contains a frameshift mutation in the *hisD* gene, which codes for histidinol dehydrogenase. In an Ames assay, reversion of this strain to the *his*<sup>+</sup> phenotype indicates that a particular chemical is a frameshift mutagen. Similarly, TA1537 is a "frameshift" strain with a mutation in the *hisC* gene, which codes for histidinol-phosphate aminotransferase. TA1535 was derived from a spontaneous mutant of LT-2, the wild-type strain. This strain has a single nucleotide substitution in the gene coding for ATP phosphoribosyltransferase. Mutagens give characteristic patterns of responses when tested in different Ames strains. For example, 2-nitrosofluorene (2-NF) is mutagenic in the frameshift strains TA1537 and TA1538, but not in TA1535, indicating that 2-NF is a frameshift mutagen.
Many mutagens are only detected in the Ames assay in the presence of an exogenous source of bioactivation enzymes. Incubation of the test compound and the bacteria with hepatic post-mitochondrial supernatant (S9) is a standard method in the Ames assay. S9 is rich in cytochrome P450s, the most significant enzymes of xenobiotic bioactivation. The addition of S9 in the Ames assay approximates the \textit{in vivo} conditions under which procarcinogenic compounds are metabolized to DNA-reactive species.

1.14. SOS DNA repair

The sensitivity and scope of the Ames test are significantly increased by the incorporation of pKM101, an R-factor derivative carrying the genes for SOS DNA repair, to the original Ames tester strains (McCann \etal, 1975). SOS DNA repair is a highly mutagenic process which allows DNA replication to proceed past non-instructive DNA lesions. TA100 and TA98 are pKM101-bearing analogs of TA1535 and TA1538, respectively. The pKM101-bearing strains are more sensitive to a number of mutagens, including 4-nitroquinoline-1-oxide (4-NQO), \textit{N}-methyl-\textit{N}'-nitro-\textit{N}-nitrosoguanidine (MNNG), aflatoxin B\textsubscript{1}, benzo[\textit{a}]pyrene, and niridazole. Indeed, several mutagens previously undetectable in the Ames test, such as furylfuramide (AF-2), mitomycin C, and 1-phenyl-1-(3,4-xylyl)-2-propynyl-\textit{N}-cyclohexylcarbamate, are mutagenic in TA100 and TA98. Plasmid pKM101 enhances mutagenesis, while providing resistance to killing by UV and chemicals. The increase in susceptibility to mutagenesis is observed only in \textit{RecA}\textsuperscript{+} and \textit{LexA}\textsuperscript{+} strains (Walker, 1977).
Walker et al. sub-cloned the region of plasmid pKM101 required for mutation enhancement. Two plasmid-encoded proteins, MucA and MucB, are responsible for pKM101-mediated mutagenesis (Perry and Walker, 1982). The muc region of pKM101 (about 1.6 kb) codes for the 16 kDa MucA and 45 kDa MucB proteins. The mucAB genes are organized in an operon which is regulated by lexA (Elledge and Walker, 1983). Furthermore, the mucAB operon is homologous to the umuDC operon (Perry et al., 1985), which encodes chromosomal genes required for mutagenic DNA repair in E. coli (Shinagawa et al., 1983) and S. typhimurium (Smith et al., 1990; Thomas et al., 1990). The E. coli umuDC operon codes for a 16 kDa and a 45 kDa protein, and the mucAB genes can restore induced mutability to E. coli umuDC mutants (Walker and Dobson, 1979).

LexA, RecA, UmuD, and UmuC are essential elements of the SOS regulon, which facilitates DNA replication past a chemical- or UV-induced lesion (Walker, 1995). The SOS response is highly mutagenic, since it often results in the insertion of an incorrect nucleotide opposite a noncoding or miscoding lesion. LexA functions as a repressor of umuD, umuC (Elledge and Walker, 1983), and recA (Walker, 1984). In response to DNA damage, RecA binds to regions of single-stranded DNA in the presence of a nucleoside triphosphate to form a nucleoprotein filament (Walker, 1995). In this tripartite complex, RecA is activated, this form is referred to as RecA*. LexA molecules diffuse to RecA*, where they undergo autodigestion, which inactivates LexA as a repressor (Silatry et al., 1986). Upon derepression, umuD and umuC are transcribed. UmuD, which shares significant homology with the carboxy-terminal domain of LexA
(Perry et al., 1985), also undergoes RecA*-mediated cleavage to produce an activated polypeptide, UmuD' (Woodgate and Sedgewick, 1992). UmuD' exists as homodimer and associates with one molecule of UmuC to produce an active trimer. The exact mechanism of translesion synthesis has not been elucidated, although studies have concluded that DNA polymerase III, UmuD', UmuC, and RecA are required for the process (Rajagopalan et al., 1992).

*S. typhimurium* LT2 and its derivatives carry a 60-MDa plasmid (the cryptic plasmid) which codes for *samAB*, a *umuDC*-like operon (Nohmi et al., 1991). LT2 and TA1538 contain both the *umuDC*<sub>ST</sub> and *samAB* operons. However, *S. typhimurium* demonstrates a weak mutagenic response to UV and many chemicals in the absence of pKM101 (McCann et al., 1975). Nohmi et al. demonstrated that the *samAB* operon, when subcloned onto a high-copy number vector, could restore significant levels of UV mutability to UmuD and UmuC deficient *E. coli* strains. Poor expression of the *umuDC*<sub>ST</sub> and *samAB* operons, when they are in the single-copy state, the absence of a factor which is required for operon function, and/or the presence of an inhibitor of the operon, are factors which may contribute to the suppression of the operons in *S. typhimurium* (Nohmi et al., 1991).

In summary, the MucAB proteins significantly enhance the scope and sensitivity of the Ames test. Many P450-dependent mutagens require the presence of pKM101 for their detection in the Ames test. The present study focuses on the development of Ames tester strain bacteria which can activate and detect P450-dependent mutagens in the absence of exogenous activation enzymes.
2. Materials and Methods

2.1 Chemicals

The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): alkaline phosphatase-conjugated anti-rabbit immunoglobulin, aflatoxin B₁, 2-AA, 2-AF, benzo[a]pyrene, MNNG, δ-ALA, ampicillin, kanamycin, and IPTG. MeIQ and Trp-P-1 were obtained from Chemsyn Science Laboratories (Lenexa, KA). BP 7,8-diol was supplied by Midwest Research Institute (Kansas City, MO). Lysozyme and tetracycline were purchased from Boehringer-Mannheim Ltd. (Laval, Que.). Protein molecular weight markers and Bsu36I were obtained from New England Biolabs Ltd. (Mississauga, Ontario). Chloramphenicol was purchased from Aldrich Chemical Co. (Milwaukee, WI). 2,4,5-TMA was supplied by Pfaltz and Bauer Research Chemicals (Waterbury, CT). An aroclor-induced rat liver S-9 preparation was obtained from Molecular Toxicology, Inc. (Annapolis, MD). Rabbit anti-human P450 1A1 antibody was provided by Dr. F. Guengerich. All other chemicals were reagent grade or better.

2.2 Media

Nutrient broth and agar were prepared using Oxoid Nutrient Broth No. 2 (Oxoid Canada Ltd., Nepean, Ont.) and supplemented with ampicillin (100 μg/mL), tetracycline (8 μg/mL), kanamycin (50 μg/mL), chloramphenicol (15 μg/mL), IPTG (1.0 mM), or δ-ALA (0.3 mM), as required. Minimal glucose medium containing Vogel-Bonner salts, glucose, histidine, and biotin was prepared as described (Maron and Ames, 1983).
### Table 2.
**Bacterial strains and plasmids**

<table>
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<th>Strain</th>
<th>Genotype</th>
<th>Source or Refer</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>TA1538</td>
<td>hisD3052 Δ(chl bio uvrB) rfa</td>
<td>B. Ames (via D. Br)</td>
</tr>
<tr>
<td>TA1535</td>
<td>hisG46 Δ(chl bio uvrB) rfa</td>
<td>B. Ames (via D. Br)</td>
</tr>
<tr>
<td>YG7104</td>
<td>hisG46 Δ(chl bio uvrB) rfa Δogf&lt;sub&gt;ST&lt;/sub&gt;::Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Yamada et al., 1994</td>
</tr>
<tr>
<td>TA100</td>
<td>hisG46 Δ(chl bio uvrB) rfa pKM101</td>
<td>McCann et al., 1997</td>
</tr>
<tr>
<td>LB5000</td>
<td>metaA22 metE551 trpC2 ilv452 H1-b H2-e,n,x flap66 rpsL120 xyl404 leu hsd L6 hsdA29 hsd SB</td>
<td>Bullas and Ryu, 1998 E. Daub</td>
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<tr>
<td><em>E. coli</em></td>
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</tr>
<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 φ80 lacZΔM15 hsdR17 recA1 endA1 gryA96 thi-1 relA1</td>
<td>J. Wood</td>
</tr>
<tr>
<td>TK610</td>
<td>thr1 ara14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 glnV44 galK2 LAM-umuC36 rac-O hisG4 rpsL31xyLA5 mtl1 thi1 uvrA6 ilv-325</td>
<td>G. Walker</td>
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</table>

**Plasmids**

<table>
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<th>Relevant Phenotype</th>
<th>Source or Refer</th>
</tr>
</thead>
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<td>pP4501A</td>
<td>human P450 1A1, amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>F. Guengerich</td>
</tr>
<tr>
<td>pP4501A1-reductase</td>
<td>human P450 1A1:rat P450 reductase fusion, amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>F. Guengerich</td>
</tr>
<tr>
<td>pGW249</td>
<td>mucA&lt;sup&gt;+&lt;/sup&gt;, mucB&lt;sup&gt;+&lt;/sup&gt;, kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>G. Walker</td>
</tr>
</tbody>
</table>
2.4. General Procedures

2.4.1. Reisolation and storage of bacterial strains

Bacterial colonies were isolated by streaking out freshly thawed permanent bacterial culture onto a nutrient agar plate containing the appropriate antibiotics, and incubating overnight at 37°C. A single bacterial colony was picked from the plate and inoculated into nutrient broth (75 mL) containing the proper antibiotics, and grown overnight at 37°C, with gentle shaking. Spectral grade DMSO (0.9 mL) was added to freshly grown overnight culture (10 mL) and aliquots (1 mL) were transferred to cryovials. The cryovials were placed on a bed of crushed dry ice and then transferred to a -70°C freezer.

2.4.2. Bacterial growth conditions

Bacterial cultures were grown in screw-top Erlenmeyer flasks (250 mL) containing nutrient broth (75 mL) and the appropriate antibiotics. Flasks were inoculated with thawed stock bacterial culture (0.1 mL) and incubated overnight, at 37°C, with gentle shaking. Cultures were grown until the desired OD₆₅₀ was reached.

2.4.3. Plasmid mini-prep

Small-scale preparations of plasmid DNA were obtained by an alkaline lysis method, as described (Sambrook et al., 1989). The optional phenol:chloroform extraction step was performed only when the DNA preparation was to be analysed by
restriction enzyme analysis. Isolated DNA was dissolved in Tris-EDTA buffer (50 µL), pH 8.0 and stored at -20°C.

2.4.4. Electrotransformation of bacteria

Overnight bacterial culture (75 mL) was washed twice with cold sterile water, then with 10% glycerol. Cells were resuspended in the residual 10% glycerol. Cell suspension (48 µL) and plasmid DNA (600 ng) were mixed in a 1 mm-gap electroporation cuvette and placed on ice for one minute. The chilled cuvette was then transferred to a BTX Transporator Plus electroporator (San Diego, CA) and pulsed once at 1.4 kV. Nutrient broth (1 mL, 20-fold dilution) was immediately added to the cuvette. Cells were then transferred to a sterile tube and incubated for one hour, at 37°C. Aliquots of the diluted electroporated cells (10 µL, 100 µL, and 500 µL) were plated onto selective agar plates and incubated overnight at 37°C. Well-isolated single colonies were streaked onto selective media and incubated overnight. Single colonies were chosen from these plates and used to prepare bacterial stocks.

2.4.5. Bacterial Conjugation

2.4.5.1. Liquid incubation

As indicated in section 3.2.1., the liquid incubation method described by McCann et al. (1975) was followed. Briefly, aliquots of fully grown cultures of the donor strain (0.1 mL) and the recipient strain (1.0 mL) were diluted into 10 mL of nutrient broth and
incubated at 37°C for 20 hours. Aliquots of the incubation mixture (10 μL, 100 μL, 500 μL) were plated onto selective medium.

2.4.5.2. Plate incubation

The bacterial conjugation method described by Kunter and Galinski (1995) was performed as indicated. Donor and recipient strains were grown overnight at 37°C, with gentle shaking. Both strains were sub-cultured and grown until the OD₆₅₀ reading of the donor strain and the recipient strain reached 0.5 and 1.0, respectively. Both the donor and recipient strain (0.5 mL of each) cultures were microcentrifuged and resuspended in nutrient broth (0.1 mL). The conjugation mixture was pipetted onto a Millipore filter disk (type HA, 45 μM) that had been placed on the surface of a nutrient agar plate. After incubation overnight at 37°C, the filter disk was removed from the plate and the cells were resuspended in 1 mL of phosphate buffered saline. Dilutions of the cells (10¹-, 10²-, 10³-, 10⁴-, and 10⁵-fold) were plated onto selective media.

2.4.6. Phenotypic confirmation of *S. typhimurium* strains

The following methods for the phenotypic confirmation of Ames tester strains were described by Maron and Ames (1983).

2.4.6.1. Histidine requirement (His⁺ auxotrophic Ames tester strains)

Histidine (0.1 mL of 0.1 M) and biotin (0.1 mL of 0.5 mM) were applied to a minimal glucose plate. Samples were streaked across the plate using a cotton swab, and plates were incubated overnight at 37°C. Control plates containing biotin but no
histidine were prepared. The His" phenotype was confirmed by the inability of the cells to
grow in the absence of histidine.

2.4.6.2.  rfa mutation (deep-rough phenotype)

Bacterial culture (0.1 mL) was overlaid onto nutrient agar plates. A BBL filter
disk (Becton-Dickinson, Cockeysville, MD) was placed in the middle of the plate and
crystal violet solution (10 µL of 10 mg/mL) was pipetted onto the disk. Plates were
incubated overnight at 37°C. The presence of the rfa mutation, which makes the cells
permeable to toxins such as crystal violet, was confirmed by a 14 mm zone of inhibition
of growth around the disk.

2.4.6.3.  ΔuvrB mutation

Bacterial cultures were streaked onto nutrient agar plates using a cotton swab.
Half of the plate was irradiated, with ultra-violet light (plates were held at 20 cm from a
30 Watt UV light source), for 10 seconds. Plates were incubated overnight at 37°C.
The presence of the ΔuvrB mutation was confirmed by growth only on the non-
irradiated side of the plate.

2.4.6.4.  Spontaneous mutations

Bacterial culture (0.1 mL) was overlaid onto a minimal glucose plate. Plates
were incubated at 37°C, for 2-3 days, as required for colonies to grow to an approximate
diameter of 3-5 mm.
2.4.6.5. Antibiotic resistance

Bacterial culture (0.1 mL) was overlaid onto a nutrient agar plate. A BBL filter disk was placed in the middle of the plate, and ampicillin (1 mg), kanamycin (0.5 mg), or tetracycline (0.8 mg; in a 10 μL volume) was pipetted onto the disk. Plates were incubated overnight at 37°C. Antibiotic resistance was demonstrated by growth to the edge of the disk (amp<sup>R</sup>) or with a ≤10 mm zone of inhibition (kan<sup>R</sup>).

2.4.7. Restriction enzyme analysis of plasmid DNA

Plasmid DNA was heated at 70°C for 15 minutes. Upon cooling, Bsu36I (30 units) was added to the plasmid DNA, and incubated at 37°C, for one hour. RNase A (0.5 mg) was added to the digested DNA, and the entire sample was loaded onto a 1% agarose gel. Bands were visualized by staining with ethidium bromide and fluorescence.

2.4.8. Isolation of bacterial membranes

The following was adapted from a method for the isolation of bacterial membranes from *E. coli* (Sandhu et al., 1994). Bacterial cultures were supplemented with thiamine (1 mM), antibiotics, trace elements, δ-ALA (0.3 mM as indicated), and IPTG (1 mM, as required), and grown for 24-36 hours, at 37°C or 30°C as noted, with vigorous shaking. Cultures were chilled (-20°C) for 1 hour, and all subsequent steps were performed at 4°C. Cells were centrifuged at 4000 x g, resuspended in Tris-acetate buffer containing lysozyme (2 mg/mL), and gently shaken for 30 minutes. The resulting spheroplasts were centrifuged at 4000 x g, resuspended in potassium phosphate buffer.
containing DTT (0.1 mM), and frozen at -70°C. Protease inhibitors, leupeptin (2.0 μM), PMSF (1.0 mM), and aprotinin (0.04 U/mL) were added upon thawing. Thawed spheroplasts were lysed with three 15 second bursts (at 60% power) from a Microson (HL-SX2005) sonicator (Mandel Scientific Company Ltd.). The lysate was centrifuged for 10 minutes at 10 000 x g. The resulting cell debris was collected as a pellet and discarded. The supernatant was centrifuged at 180 000 x g for 35 minutes. Membrane pellets were resuspended in Tris-acetate buffer (1-2 mL).

2.4.9. Pre-adsorption of antisera to remove bacterial antibodies

The removal of antibacterial antibodies from antisera was performed as described by Gruber and Zingales (1995). Bacterial culture (150 mL) was grown overnight, at 37°C, with vigorous shaking. Half the culture was autoclaved at 121°C, for 1 hour. Formaldehyde was added to the remainder (0.5% wt/vol) and the culture was incubated for 2 hours at 37°C. The treated bacterial suspensions were mixed, washed twice with phosphate buffered saline, aliquoted into ten centrifuge tubes, centrifuged, and stored at -20 °C. The bacterial pellets were resuspended in serum (1.5 mL of 1:50 dilution) and incubated at room temperature under mild agitation. After centrifugation, the serum was transferred to another bacterial pellet and the absorption cycle was repeated. Following eight serial absorption cycles, the serum was filter-sterilized (0.22 μM Millipore membrane, Bedford, MA) and stored at -20 °C.
2.4.10. Western Immunoblotting

Western immunoblotting as described by Sambrook *et al.* (1989) was used to analyse bacterial samples for P4501A1 expression. Briefly, bacterial membrane proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel, using a Bio-Rad Mini-Protean II system and electrophoretically transferred to a nitrocellulose membrane. The nitrocellulose membrane was incubated in blocking solution (5% skim milk powder) for 8 hours, and then transferred to the primary antibody solution (1:1000 dilution of pre-adsorbed rabbit anti-human antiserum). After a 2 hour incubation with the primary antiserum, the nitrocellulose membrane was washed three times with phosphate-buffered saline, followed by a final wash in phosphate-free, azide-free buffer (50 mM Tris-Cl, 150 mM NaCl). The membrane was then incubated with alkaline phosphatase-conjugated anti-rabbit immunoglobulin (1:5000 dilution), for 1 hour. The blot was developed by the addition of nitro blue tetrazolium (0.1 mL) and 5-bromo-4-chloro-3-indolyl phosphate (0.1 mL).

2.4.11. Mutagenicity assays

Mutagenicity assays were based on an adaptation of the method of Maron and Ames (1983). Cultures of the appropriate *S. typhimurium* strains were grown overnight at 37°C with gentle shaking, until an OD₆₅₀ reading of 1.0 was reached. The required amount of test compound and bacteria (0.1 mL) were incubated at 37°C for 30 minutes with gentle shaking. At the end of the incubation period, molten top agar (2 mL containing 50 μM histidine, 50 μM biotin) was added to each tube, and the contents
were overlaid onto minimal glucose plates. After incubation at 37°C for 2-3 days, the number of revertant colonies on each plate was counted.

2.4.12. Mutagen spot tests

Cultures of the appropriate S. typhimurium strains were grown overnight at 37°C with gentle shaking, until an OD₆₅₀ of 1.0 was reached. Bacterial culture (0.1 mL) in molten top agar (2 mL containing 50 μM histidine and 50 μM biotin) was overlaid onto minimal glucose plates. A sample of the test compound (DMSO solution, 10 μL volume) was pipetted directly onto the plate. After incubation at 37°C for 2-3 days, the size of the zone of inhibition and the distribution of revertant colonies was determined.

2.4.13. Preparation of benzo[a]pyrene-7,8-dihydrodiol stock solution

A 5 mg sample of benzo[a]pyrene-7,8-dihydrodiol was dissolved 95% ethanol (5 mL). The approximately 1 mg/mL solution was diluted 1000-fold and the absorbance at 368 nm (λₘₐₓ) was measured using a Hewlett-Packard diode array spectrophotometer (Corvallis, OR). The concentration of the solution was calculated using the Beer-Lambert equation: A=εcl where A is the absorbance measured at a given wavelength, ε is the compound’s molar extinction coefficient, c is the concentration of the sample, and l is the length of the light path through the sample. The molar extinction coefficient used for the calculation of benzo[a]pyrene-7,8-dihydrodiol stock solution concentration was ε₃₆₈ = 3.69 x 10⁴ L mol⁻¹ cm⁻¹ (NCI Chemical Carcinogen Reference Standard Repository, data sheet NCI No. L0113).
2.4.14. \textit{Fe}^{2+}/\textit{Fe}^{2+}-\text{CO} \text{ difference spectroscopy}

The presence of P450 holoprotein was determined as described by Omura and Sato (1964). An aliquot of thawed bacterial membranes (2 mg total protein) was incubated on ice for 30-60 minutes, after the addition of Na$_2$S$_2$O$_4$ (5-10 mg). The membranes were divided into two cuvettes, sample and reference, and placed in an SLM-Aminco DW-2C spectrophotometer (Rochester, NY). Approximately 20 CO bubbles were delivered into the sample cuvette, and the difference spectrum from 370 nm to 520 nm was measured. A baseline difference spectrum was recorded prior to the addition of CO.
3. Results and Discussion

3.1. hisD3052 Strains

3.1.1. Strain construction

The Ames test strain TA1538 (hisD3052) was chosen as the background strain for the expression of P450 1A1, in our first series of experiments. In standard S9-mediated Ames tests, TA1538 was more sensitive to benzo[a]pyrene mutagenicity than was TA1535 (hisG46; McCann et al., 1975). S9 was required to observe the mutagenic effects of benzo[a]pyrene in all Ames tester strains. The expression of P450 1A1 in TA1538 was our first strategy for mutagenic activation of PAHs, in the absence of an exogenous source of enzymes.

A plasmid containing the full-length cDNA of the human P450 1A1 (pP4501A1) was obtained from the laboratory of Dr. F. Peter Guengerich. Since plasmid pP4501A1 had been isolated from E. coli, it was moved into an S. typhimurium strain which lacks restriction enzyme functions but can still methylate DNA (LB5000; Bullas and Ryu, 1983; McGowan-Jordan and Josephy, 1990). This step was required to circumvent the S. typhimurium restriction enzymes present in TA1538, which may have cleaved plasmid DNA which was isolated directly from E. coli. Plasmid pP4501A1 was isolated from LB5000 and used to transform TA1538 by electroporation. Transformants were detected by growth on selective medium (100 μg/mL ampicillin). Typical transformation yields were 300-500 colonies per μg of plasmid DNA.

Six isolates of TA1538/pP4501A1 were selected for strain testing (see 2.4.6.). The testing confirmed the presence of the plasmid (ampicillin resistance) and the Ames
tester strain phenotype (histidine requirement, UV-sensitivity, crystal-violet sensitivity). The spontaneous reversion rate was 14 to 17 colonies per plate, which is typical of TA1538.

3.1.2. Fe$^{2+}$/Fe$^{2+}$-CO difference spectroscopy

After the construction of the TA1538/pP4501A1, bacterial membranes were harvested and the presence of P450 1A1 holoprotein was assayed using Fe$^{2+}$/Fe$^{2+}$-CO difference spectroscopy. A trial run of the membrane isolation technique described by Sandhu et al. (1994) was performed and followed at each step, using a standard Pierce bicinchoninic acid (BCA) assay kit (Rockford, IL; see table 3). Previous studies have detected the presence of P450, using Fe$^{2+}$/Fe$^{2+}$-CO difference spectroscopy, with sample concentrations of 1 mg/mL (Josephy et al., 1995). Results of the BCA assay indicated that the technique recovered a sufficient amount of protein. DH5α pP4501A2 was chosen as a positive control for the spectral analysis because this strain was shown to express P450 1A2 in previous studies (Sandhu et al., 1994; see figure 9).
<table>
<thead>
<tr>
<th>Major steps in the isolation of bacterial membranes</th>
<th>Description of the sample</th>
<th>Total volume (mL)</th>
<th>Protein concentration (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grow bacteria overnight</td>
<td>Bacterial cultures</td>
<td>150.0</td>
<td></td>
</tr>
<tr>
<td>Centrifuge and resuspend cells (4 000g)</td>
<td>Supernatant (nutrient broth)</td>
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<tr>
<td></td>
<td>Resuspended cells</td>
<td>50.0</td>
<td></td>
</tr>
<tr>
<td>Centrifuge and resuspend spheroplasts (4 000g)</td>
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<tr>
<td></td>
<td>Resuspended spheroplasts</td>
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<tr>
<td>Sonicate spheroplasts and centrifuge (10 000g)</td>
<td>10 000g supernatant</td>
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<td></td>
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<tr>
<td></td>
<td>Centrifuged cell debris (resuspended)</td>
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</tr>
<tr>
<td>Centrifuge 10000g supernatant (180 000g)</td>
<td>180 000g supernatant</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bacterial membranes (resuspended)</td>
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</tr>
</tbody>
</table>

Note. Protein concentrations were determined using the Pierce bicinchoninic acid (BCA) assay kit. Bolded lines represent samples which were retained and used in subsequent steps.
Figure 9. Fe$^{2+}$-CO versus Fe$^{2+}$ difference spectrum of membranes prepared from DH5α pP4501A2 (baseline corrected). The spectrum indicates a level of expression of approximately 10 nmol P450/ liter of culture.
P4501A1 holoenzyme was not detected in bacterial membranes isolated from TA1538/pP4501A1 using $\text{Fe}^{2+}/\text{Fe}^{2+}$-CO difference spectroscopy. Initially, membranes were isolated from cultures grown as described in section 2.4.8., at 37°C with a 12 hour period of IPTG induction. Subsequent trials focused on attempting to increase the level of P4501A1 production through modifying bacterial growth conditions. δ-ALA, a heme precursor which has been shown to increase levels of heterologous P450 production, was added to bacterial cultures (Gillam et al., 1995a). The length of IPTG induction time was increased to 24 hours and the growth temperature was lowered to 30°C, to match the conditions used for expression of pP4501A1-encoded proteins in E. coli (Guo et al., 1994). In all trials, the P450 peak was not observed in the $\text{Fe}^{2+}/\text{Fe}^{2+}$-CO difference spectrum of the samples.

3.1.3. Immunoblot analysis of TA1538/pP4501A1

Since the presence of the recombinant protein had not been spectrally detected, immunoblot (Western blot) analysis was performed to confirm that the recombinant P4501A1 protein was being produced in the S. typhimurium strain. Rabbit anti-human P4501A1 antiserum was obtained from Dr. F. Peter Guengerich. When screening bacterial expression systems, a common problem is the presence of cross-reactivity arising from the antibacterial antibodies contained in the sera from immunized animals. To circumvent this undesirable reactivity, antibacterial antibodies were removed from the primary antiserum by serial adsorption on formalin-fixed, autoclaved S. typhimurium pellets (Gruber and Zingales, 1995). This proved to be very successful, as judged by the
Figure 10. Immunoblot analysis of human P4501A1 expression in *S. typhimurium* cells. Cultures were induced with 1 mM IPTG (unless otherwise noted). In each case 75 µg of bacterial membrane protein was used for SDS-PAGE. a, molecular weight markers; b, TA1538 pP4501A1-1 (uninduced); c, TA1538; d, TA1538 PP4501A1-4; e, TA1538 PP4501A1-1.

difference in levels of background reactivity between blots probed with pre-adsorbed vs unadsorbed antisera. Bands which correspond to the expression of P4501A1 were observed within the molecular weight range of the protein (58 kDa; Figure 10).

Figure 10 also indicates that human P4501A1 is constitutively expressed in TA1538 (see lanes b, d, and e). This result was unexpected, since the vector bears a *tac* promoter upstream from the P4501A1 gene and the *lacF* gene, which produces a repressor of the *tac* promoter. In the absence of an inducer, such as IPTG, the expression of the gene under the control of the *tac* promoter should be repressed. When plasmid pP4501A1 is expressed in *E. coli*, the expression of the gene can be controlled
by IPTG-induction (Guo et al., 1994). However, the results in figure 10 indicate that the expression of human P450 1A1 in *S. typhimurium* is constitutive. Similar results have been observed in the laboratories of both Dr. P. D. Josephy (unpublished data) and Dr. F. P. Guengerich (Thier et al., 1993). The constitutive expression of P4501A1 in *S. typhimurium* may be due to degradation of the repressor protein by *S. typhimurium* proteases.

3.1.4. Mutagenicity assays in TA1538/pP4501A1

Mutagenicity assays were performed in TA1538/pP4501A1, using BP 7,8-diol as the substrate. As discussed earlier, the activation of benzo[a]pyrene requires epoxide hydrolase to catalyze the production of BP 7,8-diol from BP 7,8-epoxide (see figure 7). Since epoxide hydrolase was not present in our system, BP 7,8-diol was chosen as a substrate for P4501A1. The standard Ames reversion assay protocol was followed, in the absence of S9. BP 7,8-diol was not activated by TA1538/pP4501A1. Doses of BP 7,8-diol ranging from 0 to 150 nmol of substrate per plate were investigated. This range was established from previous experiments performed in the presence of S9 (McCann et al., 1975). The data were collected in two experiments, with each of six dose points performed in triplicate.

A second mutagen, aflatoxin B1, was tested as a possible substrate for the recombinant P450 1A1. In mutagenicity assays lacking S9, activation of aflatoxin B1 was not observed. The assays were repeated three times, with each of four dose points
performed in duplicate. The dose range of aflatoxin B₁ used in these experiments was from 0 to 250 nmol per plate.

3.1.5. Human P4501A1-NADPH-cytochrome P450 reductase fusion protein

The absence of P450-reductase in TA1538 pP4501A1 may have contributed to inability of the strain to activate mutagens. In *E. coli*, endogenous flavodoxin and NADPH-flavodoxin reductase have been suggested as a probable source of electrons for heterologous P450s (Jenkins and Waterman, 1994). However, the catalytic activities of P450s are significantly higher in the presence of NADPH-P450 reductase (Yamazaki *et al*., 1995). To enhance P450 activity in the Ames tester strains, TA1538/pP4501A1-reductase was constructed. A plasmid containing the full-length cDNA coding for a human P4501A1-rat liver NADPH-cytochrome P450 oxidoreductase fusion protein (pP4501A1-reductase) was obtained from Dr. F. Peter Guengerich. The plasmid was moved into LB5000, followed by introduction into TA1538. A preliminary mutagenicity assay indicated that the strain was unable to activate BP 7,8-dihydrodiol.

An obvious problem with this system was the absence of the plasmid pKM101, which increases the susceptibility of *E. coli* and *S. typhimurium* to chemical and UV mutagenesis. Plasmid pKM101 encodes the MucAB proteins, which are required for error-prone bypass of replication blocking DNA lesions (Friedberg *et al*., 1995). The introduction of pKM101 into TA1538 and TA1535 greatly increased the sensitivity of the Ames assay and the number of mutagens detected (McCann *et al*., 1975).
Subsequent experiments in the present study focused on the construction of Ames tester strains expressing both P4501A1- P450 reductase and the MucAB proteins.

3.2. hisG46 Strains

3.2.1. Strain construction

The his G46 Ames tester strain TA1535 was selected as a suitable background for coexpressing P4501A1-P450 reductase and MucAB. TA1535 was not chosen initially because benzo[a]pyrene and aflatoxin B₁ mutagenesis in this strain are completely dependent upon pKM101 (McCann et al., 1975). However, since TA100 (TA1535 pKM101) is more sensitive to mutagenesis by benzo[a]pyrene and aflatoxin B₁ than TA98 (TA1538 pKM101), TA1535 was the appropriate background for our second series of experiments.

In addition to the MucAB proteins, pKM101 codes for a β-lactamase and the proteins required for conjugal transfer (Langer et al., 1981). Since both pKM101 and pP4501A1-reductase are ampicillin-resistant, antibiotic selection of cells bearing both plasmids was not possible. To circumvent this problem, a kanamycin resistant derivative of pKM101 was obtained from Dr. Graham C. Walker. pKM101bla-455::Tn5 (pGW249) has lost its ampicillin resistance, while retaining the MucAB and conjugal transfer functions of pKM101.

Our first strategy for the construction of a hisG46 strain bearing both pGW249 and pP4501A1-reductase is outlined in figure 11. Since pGW249 was provided in an E. coli host strain (TK610), the plasmid had to be moved into LB5000, to circumvent
transformation
with pP4501A1-reductase

TA1535 (hisG46)  TK610 pGW249

conjugation
with tetR derivative of LB5000

TA1535 pP4501A1-reductase  LB5000 (tetR) pGW249

conjugation:
select ampR and kanR

TA1535 pP4501A1-reductase pGW249

Figure 11. Construction of TA1535 pP4501A1-reductase pGW249

digestion by S. typhimurium restriction enzymes. However, it was necessary first to transform LB5000 with a tetracycline-resistance encoding plasmid (pBR322 (tetR), a pBR322 derivative which confers resistance to tetracycline but not to ampicillin), to provide an antibiotic resistant phenotype which could be exploited in the selection of transformants following the conjugal transfer of pGW249 (see section 2.4.5.1.). LB5000 (tetR) pGW249 transformants were selected on medium containing kanamycin (50 µg/mL) and tetracycline (10 µg/mL). TA1535 was electrotransformed with pP4501A1-reductase and transformants were selected by growth on selective medium (100 µg/mL amp). To complete the construction, pGW249 had to be transferred from LB5000 (tetR) pGW249 to TA1535 pP4501A1-reductase.
In three independent attempts, the final step of the construction failed to produce transformants. This conjugation may have been unsuccessful due to the presence of incompatible plasmids in both the donor and recipient strains. The plasmid which conferred tetracycline resistance to LB5000 is a derivative of pBR322, as is plasmid pP4501A1-reductase. These two plasmids are incompatible, since they both bear the pMB1 replicon. pMB1 is the replicon which is carried by pBR322 and its derivatives. The presence of incompatible plasmids in both strains may have resulted in some instability during the conjugation. Alternatively, pBR322-amp* could have accompanied pGW249 into TA1535 pP4501A1-reductase, producing a non-viable strain due to the presence of too many plasmids in a single cell.

To achieve the objective of constructing a hisG46 strain bearing both pGW249 and pP4501A1-reductase, a second strategy was developed. Figure 12 describes an alternative strain construction strategy which circumvents the problem of conjugal transfer between donor and recipient strains which both bear multiple plasmids. The strain YG7104 (hisG46ΔogtST::CmR) was obtained from Dr. Takehiko Nohmi (National Institute of Health Sciences, Tokyo, Japan). The S. typhimurium ogtST gene codes for DNA methyl transferase activity and plays a major role in protecting against the mutagenic effects of alkylating agents (Yamada et al., 1995). YG7104 is a ogtST- null derivative of TA1535 which carries the gene for chloramphenicol resistance. This strain was chosen for its antibiotic resistance, not the Ogt- phenotype. The presence of an antibiotic resistance gene located on the chromosome allowed for selection against the
donor strain during conjugal transfer of pGW249, while avoiding the complications of introducing selectable markers via plasmids.

The construction of the strain YG7104 pGW249 required two bacterial conjugation steps. As mentioned above, pGW249 was moved by bacterial conjugation into LB5000(tet^R) to facilitate its transfer from *E. coli* to *S. typhimurium*. LB5000(tet^R) pGW249 was the donor strain in a second conjugation with YG7104. Conjugants were selected on agar plates containing kanamycin (50 μg/mL) and chloramphenicol (10 μg/mL).
Construction of pGW249 donor strain:

Recipient: YG7104

donor strain: tet\textsuperscript{R} derivative of 
\textit{S. typhimurium} LB5000
(restriction carrier strain)

Donor: \textit{E. coli} host carrying 
pGW249 (G. Walker lab.), a kan\textsuperscript{R} derivative of 
R-factor pKM101; carries error-prone 
DNA repair genes

conjugation: 
select kan\textsuperscript{R} and tet\textsuperscript{R}

expression vectors for 
human P4501A1 and 
P4501A1-P450 reductase; 
pCW ori derivatives (amp\textsuperscript{R}); 
transform into LB5000

conjugation: 
select cm\textsuperscript{R} and kan\textsuperscript{R}

YG7104 pGW249 
(comparable to TA100)

mini-prep. DNA

electrotransformations

YG7104 pGW249 
P4501A1

YG7104 pGW249 
P4501A1-P450 reductase

Figure 12. Construction of YG7104 pGW pP4501A1 and YG7104 pGW pP450 1A1-reductase.

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The presence of functional MucAB proteins was confirmed by performing spot tests (see 2.4.12) with the MucAB-dependent mutagens niridazole, 4-NQO, MNNG, and AF-2. The zones of inhibition and the distributions of colonies on the plate were recorded (see figure 13 and table 4). Within the zone of inhibition, the concentration of mutagen was high enough to kill most of the bacterial lawn. The ring of revertants bordering the zone of inhibition are a result of the mutagen diffusing to a concentration which permitted cell survival and mutagenesis. In the niridazole, 4-NQO, and AF-2 spot tests, the density of revertants outside the zone of inhibition on the YG7104 pGW249 plates was significantly higher than the YG7104 plates (see table 4). These spot tests demonstrated that pGW249 greatly enhanced chemical mutagenesis in YG7104, confirming the presence of functional MucAB proteins. MNNG mutagenicity was also tested in YG7104 pGW249. Since YG7104 is deficient in DNA methyltransferase activity, it is hypersensitive to methylating agents such as MNNG. A further increase in the sensitivity to MNNG was not observed in the presence of pGW249.

A final electrotransformation was required to complete the construction of the Ames tester strains expressing the MucAB proteins and human P450s. YG7104 pGW249 was electrotransformed with pP4501A1 and pP4501A1-reductase. Transformants were selected on medium containing kanamycin (50 μg/mL) and ampicillin (100 μg/mL). Stock cultures of several isolates of YG7104 pGW249 pP4501A1 (YG pGW1A1) and YG7104 pGW249 pP4501A1-reductase (YG pGW1A1-reductase) were prepared and used for strain testing.
Figure 13. Mutagen spot test results. A. YG7104 pGW249 with 0.2 μg and 2.0 μg niridazole spotted directly onto plate. B. YG7104 pGW249 with 0.5 μg and 5.0 μg 4-NQO spotted directly onto plate.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Compound</th>
<th>Dose (per plate)</th>
<th>Zone of Inhibition</th>
<th>Description of plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>YG7104</td>
<td>Niridazole</td>
<td>0.2 µg</td>
<td>no zone</td>
<td>13 colonies on plate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0 µg</td>
<td>no zone</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-NQO</td>
<td>0.5 µg</td>
<td>20 mm</td>
<td>38 colonies on plate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0 µg</td>
<td>30 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AF-2</td>
<td>0.02 µg</td>
<td>15 mm</td>
<td>13 colonies on plate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2 µg</td>
<td>28 mm</td>
<td></td>
</tr>
<tr>
<td>MNNG</td>
<td></td>
<td>2.0 µg</td>
<td>22 mm</td>
<td>-very high density of colonies around ring -&gt; 500 colonies</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 µg</td>
<td>34 mm</td>
<td>- too numerous to count</td>
</tr>
<tr>
<td>YG7104 pGW249</td>
<td>Niridazole</td>
<td>0.2 µg</td>
<td>20 mm</td>
<td>- high density of colonies around ring -&gt; 500 colonies</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0 µg</td>
<td>40 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-NQO</td>
<td>0.5 µg</td>
<td>20 mm</td>
<td>- high density of colonies around ring -&gt; 500 colonies</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0 µg</td>
<td>35 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AF-2</td>
<td>0.02 µg</td>
<td>15 mm</td>
<td>- high density of colonies around ring -&gt; 500 colonies</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2 µg</td>
<td>25 mm</td>
<td></td>
</tr>
<tr>
<td>MNNG</td>
<td></td>
<td>2.0 µg</td>
<td>30 mm</td>
<td>- very high density of colonies around ring -&gt; 500 colonies</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 µg</td>
<td>45 mm</td>
<td>- too numerous to count</td>
</tr>
</tbody>
</table>

*Note.* Both high and low doses of each compound were spotted onto a single plate.
3.2.2. Strain testing of YG pGW1A1 and YG pGW1A1-reductase

Strain testing indicated that YG pGW1A1 and YG pGW1A1-reductase retained the phenotypic traits of Ames tester strains bearing the *mucAB* genes. Both strains required histidine to grow and were sensitive to crystal violet. The spontaneous reversion rate and resistance to killing by UV were higher in YG pGW1A1 and YG pGW1A1-reductase than in TA1535 pP4501A1. The *mucAB* operon is responsible for enhanced cell survival upon exposure to DNA-damaging UV radiation (McCann *et al*., 1975). The spontaneous mutator phenotype observed in YG pGW1A1 and YG pGW1A1-reductase is also conferred by the *mucAB* operon (McCann *et al*., 1975).

Eisenstadt *et al.* (1989) determined the mutational spectrum of spontaneous revertants in *hisG46* and found that pKM101 enhanced the spontaneous frequency of most of the spontaneous base pair substitutions. Transversions (purine replaced by a pyrimidine, or vice versa) were observed to a greater extent than transitions (purine replaced a purine, or pyrimidine replaced by pyrimidine). It was suggested that the presence of pKM101 increases the tolerance of the replication machinery for mismatch combinations, particularly those that yield transversions. The spontaneous mutator phenotype and the increased resistance to killing by UV confirm the presence of functional MucAB proteins in YG pGW1A1 and YG pGW1A1-reductase.
3.2.3. Restriction enzyme analysis of plasmids

The plasmids pP4501Al and pP4501Al-reductase were isolated from YG pGW1Al and YG pGW1Al-reductase (see 2.4.3.), respectively, and digested with restriction enzymes (see 2.4.7). Both plasmids produced four characteristic fragments when digested with the restriction enzyme Bsu36I. Agarose gel electrophoresis of digested plasmid samples verified that pP4501Al and pP4501Al-reductase were present in YG pGW1Al and YG pGW1Al-reductase, respectively (see figure 14). Two isolates of each strain were tested. The isolate YG pGW1Al-16 was discarded because it did not produce the expected fragments (see figure 14, lane 3).

3.2.4. Mutagen spot test

Mutagen spot tests were useful for screening a number of compounds which were potentially mutagenic in our system. YG pGW1Al and YG pGW1Al-reductase were treated with six different mutagens to determine if a mutagenic response could be demonstrated. Aflatoxin B1, BP 7,8-diol, 2-AA, MeIQ, Trp-P-1, and 2,4,5-TMA were used as substrates for the mutagen spot tests (see 2.4.2.12.). Activation of 2-AA, MeIQ, Trp-P-1 by purified recombinant P4501Al (Shimada et al., 1996) and P4501Al-1A1 reductase fusion protein (Chun et al., 1996) has been demonstrated. As indicated in table 5, YG pGW1Al and YG pGW1Al-reductase did not activate any of the mutagens that were tested. Since the spot tests failed to detect activation of any of the test compounds, it was decided that any further mutagenicity testing would remain focused on aflatoxin B1 and BP 7,8-diol.
Figure 14. A. Maps of plasmid pP4501A1, showing Bsu36I sites. B. Map of plasmid pP4501A1-reductase, showing Bsu36I sites. C. Agarose gel electrophoresis of Bsu36I-digested plasmid mini-prep DNA isolated from the following strains: lane 1, molecular weight markers; lane 2, YG7104 pGW249; lane 3, YG pGW1A1-16; lane 4, YG pGW1A1-18; lane 5, YG pGW1A1-red-20; lane 6, YG pGW1A1-red-21.
### Table 5

**Results of spot tests with P4501A1-dependent mutagens**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Compound</th>
<th>nmol/plate</th>
<th># revertants on plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>YG7104 pGW249</td>
<td>2-AA</td>
<td>50</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>aflatoxin B₁</td>
<td>25</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>MeIQ</td>
<td>1</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>Trp-P-1</td>
<td>10</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>2,4,5-TMA</td>
<td>5000</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td>BP 7,8-diol</td>
<td>30</td>
<td>130</td>
</tr>
<tr>
<td>YG pGW1A1-18</td>
<td>2-AA</td>
<td>50</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>aflatoxin B₁</td>
<td>25</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>MeIQ</td>
<td>1</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>Trp-P-1</td>
<td>10</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>2,4,5-TMA</td>
<td>5000</td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>BP 7,8-diol</td>
<td>30</td>
<td>124</td>
</tr>
<tr>
<td>YG pGW1A1-red-21</td>
<td>2-AA</td>
<td>50</td>
<td>191</td>
</tr>
<tr>
<td></td>
<td>aflatoxin B₁</td>
<td>25</td>
<td>211</td>
</tr>
<tr>
<td></td>
<td>MeIQ</td>
<td>1</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>Trp-P-1</td>
<td>10</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td>2,4,5-TMA</td>
<td>5000</td>
<td>183</td>
</tr>
<tr>
<td></td>
<td>BP 7,8-diol</td>
<td>30</td>
<td>114</td>
</tr>
</tbody>
</table>

*Note.* This experiment was repeated and similar results were obtained (data not shown.)
3.2.5. Aflatoxin B<sub>1</sub> mutagenicity assay

A complete aflatoxin dose-response mutagenicity assay was performed in YG pGW1A1 and YG pGW1A1-reductase. The strain YG7104 pGW249 was included as a control. Five dose points ranging from 0 to 2.5 mM aflatoxin B<sub>1</sub> were tested, in duplicate. YG pGW1A1 and YG pGW1A1-reductase did not activate aflatoxin B<sub>1</sub>.

3.2.6. BP 7,8-diol + S9 mutagenicity assay

A standard Ames assay, including the addition of mammalian liver homogenate, was performed in YG7104 pGW1A1 and YG pGW1A1-reductase using BP 7,8-diol as the substrate. Aroclor 1254-induced rat liver 9000 x g supernatant (S9) was used as a source of P450 activity. The results of the mutagenicity assay are shown in figure 15. In the presence of S9, mutagenic activation of BP 7,8-diol was observed in YG7104 pGW249, YG7104 pGW1A1 and YG7104 pGW1A1-reductase. This control confirmed that the strains had retained their ability to detect chemical mutagens. Since YG7104 pGW1A1 and YG7104 pGW1A1-reductase were unable to support the activation of mutagenic compounds, in the absence of S9, further analysis of P450 expression in the strains was required.
Figure 15. Mutagenicity of BP-7,8-diol in YG7104 pGW249, YG7104 pGW249 pP4501A1, and YG7104 pGW249 pP4501A1-reductase, with activation by Aroclor 1254-induced rat liver S9.

3.2.7. Immunoblot (Western) analysis

Immunoblot analysis of bacterial membranes isolated from YG pGW249, YG pGW1A1 and YG pGW1A1-reductase was performed, using pre-absorbed polyclonal rabbit antiserum raised against human cytochrome P450 1A1. Figure 16 demonstrates that P4501A1 and P4501A1-reductase were expressed in our S. typhimurium
YG pGW1A1 and YG pGW1A1-reductase produced an immunoreactive protein with an electrophoretic mobility that corresponds to the molecular weight of the P4501A1-reductase fusion protein (129 000 kDa; see lanes b and c). As expected, YG7104 and YG7104pGW1A1 did not produce an immunoreactive protein at this position (lanes a and d). The bands present directly below the P4501A1-reductase suggest that proteolysis of the fusion protein may have occurred, although this was not shown conclusively in the present study. Proteolysis of recombinant P450 reductase has been reported by other groups (Dong and Porter, 1996; Parikh et al., 1997).

A band with the expected electrophoretic mobility of P4501A1 was detected in the membranes isolated from YG pGW1A1. The apparent molecular weight of the protein in this band (61 kDa), determined by its electrophoretic mobility relative to the marker proteins, is slightly higher than the actual molecular weight of P4501A1 (58.2 kDa). However, this discrepancy is well within the 5 to 10% accuracy range of protein molecular weight determination using SDS-PAGE (Voet and Voet, 1990).
Figure 16. Immunoblot analysis of human P4501A1 expression in *S. typhimurium*. In each case 50 µg of bacterial membrane protein was used for SDS-PAGE analysis. *a*, YG pGW249; *b*, YG pGW1A1-red-21; *c*, YG pGW1A1-red-20; *d*, YG pGW1A1-18; *e*, molecular weight markers.

Figure 16 indicates that P4501A1 and P4501A1-reductase are expressed in YG pGW1A1 and YG pGW1A1-reductase. However, the immunoblot analysis suggests that the level of protein expression in these strains is not very high. The intensity of the bands is very low compared to that of the bands detected in the Western blot analysis of TA1538 pP4501A1 (see figure 10). Although this comparison is not direct, the difference in the intensity of the bands was greater than that which would likely be attributed to the variance introduced by performing two separate analyses. The presence
of pGW249 may be inhibiting the expression of P4501A1 and the P4501A1-reductase fusion protein. Plasmid pGW249 is a 35 kb plasmid which codes for several genes other than mucAB. Approximately 40% of the pGW249 DNA encodes the genes required for conjugal transfer (tra). The tra gene products may have a significant impact on the membrane, and affect on the incorporation of P450 1A1 or P4501A1-reductase into the membrane. Furthermore, the gene products encoded on pGW249 may be preferentially expressed over those encoded by pP4501A1 and pP4501A1-reductase. Low levels of P4501A1 and P4501A1-reductase expression are likely contributing to the inability of YG pGW1A1 and YG pGW1A1-reductase to activate P450-dependent mutagens.

Another factor which may be contributing to the low levels of recombinant P450 expression in our system is the use of an S. typhimurium background strain, as opposed to an E. coli background. The pP4501A1 and pP4501A1-reductase plasmids were designed for expression in E. coli. In the case of recombinant human P450 1A2, a plasmid was constructed using the same expression vector as pP4501A1 and pP4501A1-reductase. This vector was used to express P450 1A2 in both S. typhimurium and E. coli (Sandhu et al., 1994; Josephy et al., 1995). The level of expression of human P450 1A2, as judged by spectrally detectable P450 hemoprotein, was four times higher in E. coli than in S. typhimurium. Although there are no obvious reasons for this discrepancy, expression levels of recombinant proteins may be intrinsically lower in S. typhimurium than in E. coli.

Immunoblot analysis was used to directly compare levels of expression of recombinant P450 proteins in YG pGW249 and an E. coli background. The E. coli
strain DH5α was transformed with pP4501A1 and pP4501A1-reductase. Membranes were isolated from DH5α, DH5α pP4501A1, and DH5α pP4501A1-reductase. Figure 17 shows immunoblot analysis of membrane protein isolated from *S. typhimurium* and DH5α strains bearing the pP4501A1 and pP4501A1-reductase. The level of P4501A1-reductase fusion protein expression in DH5α pP4501A1-reductase (lane e) is significantly higher than in YG pGW249-reductase (lane f). These results further suggest that YG pGW249 is not an optimal background for expression of recombinant human P450s.

![Immunoblot analysis of human P4501A1 expression in bacterial membranes.](image)

Figure 17. Immunoblot analysis of human P4501A1 expression in bacterial membranes. *a*, DH5α; *b*, YG7104; *c*, DH5α pP4501A1; *d*, YG pGW1A1; *e*, DH5α pP4501A1-reductase; *f*, YG pGW1A1-reductase. In each case 50 μg of bacterial membranes was used for SDS-PAGE.
The comparison between YG pGW1A1 (lane c) and DH5α pP4501A1 (lane d) was inconclusive due to the apparent cross-reactivity of a DH5α protein with the same molecular weight as P4501A1 (lane 1). Immunoblot analysis was repeated on a separate batch of DH5α membranes and an identical band was observed (data not shown). Some anti-\textit{E. coli} immunoglobulins may have been present because the antisera was preabsorbed with YG7104, and not DH5α.

The immunoblot analysis indicated that P4501A1 and P4501A1-reductase fusion are not being expressed at high levels in YG pGW1A1 and YG pGW1A1-reductase, respectively. Higher levels of P450 1A1 and P450 1A1-reductase expression have been demonstrated using Western blot analysis of \textit{S. typhimurium} and \textit{E. coli} strains lacking pGW249. While an \textit{S. typhimurium} background may not be optimal for production of recombinant human P450s, levels of expression which can support activation of promutagens have been demonstrated in an Ames tester strain (Josephy \textit{et al.}, 1995). Clearly, the levels of recombinant P450 expression in the YG pGW249 background are not high enough to support the activation P450-dependent mutagens. Based on the results of figures 10, 16, and 17, the presence of pGW249 appears to be having a significant effect on the levels of recombinant human P450 expression.
4. Conclusions

The development of Ames tester strains which detect chemical mutagens in the absence of an exogenous source of bioactivation enzymes requires the generation of active monooxygenase systems in living bacteria. The first strain constructed in this project was an Ames tester strain (TA1538; hisD3052) bearing a plasmid encoding the cDNA of P4501A1. Immunoblot analysis revealed that P4501A1 was expressed in TA1538 pP4501A1. However, mutagenic activation of BP 7,8-diol and aflatoxin B₁ was not detected in this strain. The level of P450 1A1 activity in this strain may have been low, as judged by its inability to activate P4501A1-dependent mutagens, because electron transfer from endogenous bacterial flavodoxins was unable to effectively catalyse the reduction of P450 1A1 by NADPH. Another obvious problem with this strain was the absence of plasmid pKM101, which greatly increases the sensitivity of Ames tester strains to chemical mutagenesis.

Subsequent experiments focused on the development of Ames tester strains which expressed mammalian P450-reductase and the MucAB proteins of plasmid pKM101. For the construction of strains bearing the pKM101 derivative, pGW249, a hisG46 Ames tester strain (YG7104) was selected because this background is more sensitive to mutagens than hisD3032 in the presence of the MucAB proteins. The strain YG7104 pGW249 was shown to be sensitive to MucAB-dependent mutagens. YG7104 pGW249 was transformed with a plasmid bearing the cDNA of a fusion protein between P4501A1 and P450-reductase. The analogous strain bearing plasmid pP4501A1 was also constructed.
Strain testing indicated that YG pGW1A1 and YG pGW1A1-reductase retained the phenotypic traits of Ames tester strains. The presence of functional MucAB proteins in YG pGW1A1 and YG pGW1A1-reductase was also demonstrated. Plasmids isolated from YG pGW1A1 and YG pGW1A1-reductase produced characteristic fragments when digested with restriction enzymes. These confirmed that the strain construction was successful.

Mutagenic activation of aflatoxin B₁, BP 7,8-diol, 2-aminoanthracene, MeIQ, Trp-P-1, and 2,4,5-trimethylaniline were tested in YG pGW1A1 and YG pGW1A1-reductase. Mutagen spot tests failed to detect activation of any of the test compounds. A standard Ames assay, including the addition S9, was performed in YG pGW249, YG pGW1A1, and YG pGW1A1-reductase. Mutagenic activation of BP 7,8-diol was detected in all of the strains. This control confirmed that the strains had retained their ability to detect chemical mutagens.

Immunoblot analysis of membrane proteins isolated from YG pGW1A1 and YG pGW1A1-reductase indicated that P4501A1 and P4501A1-reductase were expressed in these strains. However, comparison with immunoblots using membranes isolated from TA1538 pP4501A1 and DH5α pP4501A1-reductase indicated that the level of P4501A1 and P4501A1-reductase expression was lower in the presence of pGW249. Plasmid pGW249 is a 35 kb plasmid which codes for many genes other than MucAB. Approximately 40% of the pGW249 DNA encodes the genes required for conjugal transfer (tra). The tra gene products may have a significant impact on the membrane, and thus an affect on the incorporation of P450 1A1 or P4501A1-reductase into the
membrane. Furthermore, the gene products encoded on pKM101 may be preferentially expressed over those encoded by pP4501A1 and pP4501A1-reductase. Low levels of P4501A1 and P4501A1-reductase expression were likely contributing to the inability of YG pGW1A1 and YG pGW1A1-reductase to activate P450-dependent mutagens.

Immunoblot analysis also indicated that proteolysis of the P4501A1-reductase fusion protein may have occurred. Proteolysis of the reductase could significantly reduce the activity of recombinant P4501A1 in the bacterial membrane. Reduced P4501A1 activity, due to proteolysis of the reductase, may have contributed to the inability of YG pGW1A1-reductase to activate P450-dependent mutagens.

In summary, it is likely that both P450-reductase and the MucAB proteins are required for the mutagenic activation of P4501A1-dependent mutagens in Ames tester strains. In strains bearing pP4501A1 or pP4501A1-reductase and pGW249, functional MucAB proteins were produced. However, the presence of pGW249 may have lowered the levels of P450 expression in these strains.
5. Future Studies

The development of an active P450 1A1 monooxygenase system in Ames tester strains requires further studies. Many of the complications which arose during the strain construction, and with the expression of P450 1A1 in the presence of pGW249 can be avoided by engineering the genes of interest onto a single plasmid. Two-plasmid systems are unstable and prone to poor growth. To avoid such problems, the mucAB genes (1.9 kb) can be amplified by the PCR and ligated into a suitable site in the P450 1A1 vector.

The system could be further improved by the use of a recently developed bicistronic construct, which independently co-expresses P450 1A1 and P450-reductase (Parikh et al., 1997). P450 1A1 EROD activity was observed in whole E. coli cells transformed with the bicistronic construct. High levels of P450 1A1 activity in whole cells have not been previously reported. Furthermore, the whole cell activity was higher than that previously reported for purified recombinant P450 1A1, reconstituted with purified P450-reductase. The pCW vector bearing the bicistronic construct contains PvuI and BstEII restriction enzyme sites, which would facilitate the insertion of the PCR-amplified mucAB genes. The construction of a single vector bearing the bicistronic P450 1A1/P450-reductase and the mucAB genes circumvents the problematic multiple-plasmid approach. This strategy also incorporates a P450 1A1 expression vector which has been shown to generate substantial levels of P450 1A1 activity in live bacterial cells. With these improvements, we expect that the levels of catalytically active P450 1A1 will be high enough to support the activation of P450-dependent mutagens in Ames tester strains, in the absence of an exogenous source of activation enzymes.
6. References


Chun, Y.-C., and Guengerich, F.P., unpublished results.


