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ABSTRACT OF DISSERTATION

Matthew Troy Cooper

The Graduate School
University of Kentucky
2002

CREATION OF A BACTERIAL MUTAGENICITY ASSAY
HIGHLY SENSITIVE TO DIALKYL NITROSAMINES

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
Requirements for the degree of Doctor of Philosophy in
The Graduate School
at the University of Kentucky

By

Matthew Troy Cooper

Lexington, Kentucky

Director: Dr. Todd D. Porter, Associate Professor, Pharmaceutical Sciences

Lexington, Kentucky

2002

ABSTRACT OF DISSERTATION

CREATION OF A BACTERIAL MUTAGENICITY ASSAY HIGHLY SENSITIVE TO DIALKYLNITROSAMINES

Although dialkylnitrosamines are environmentally significant carcinogens, the use of short-term bioassays to assess the mutagenic potential of these compounds remains problematic. The Ames test, a mutagenicity assay based on the reversion of *Salmonella typhimurium* histidine auxotrophs, is the most widely used bioassay in genetic toxicology, but the traditional Ames tester strains are largely insensitive to dialkylnitrosamine mutagenicity. I have constructed several mutagenicity tester strains that co-express combinations of full-length human cytochrome P450 2E1, rat cytochrome P450 reductase, and human cytochrome b₅ in *S. typhimurium* lacking *ogt* and *ada* methyltransferases (YG7104ER, *ogt*-; and YG7108ER, *ogt*-, *ada*-).

These new strains are susceptible to dialkylnitrosamine mutagenicity in the absence of an exogenous metabolic activating system (S9 fraction). Mutagenicity is dependent upon the coexpression of P450 2E1 with P450 reductase and is similar or greater than that obtained with the parental strains in the presence of S9 fraction from ethanol-induced rat liver. Coexpressing human cytochrome b₅ with cytochrome P450 2E1 and cytochrome P450 reductase potentiates the mutagenicity observed with dialkylnitrosamines. These strains were sensitive to nitrosamines with varying alkyl side chains, including dimethylnitrosamine, diethylnitrosamine, dipropylnitrosamine, and dibutylnitrosamine. Mutagenicity decreased with alkyl chain length, consistent with the stringency of the *ada*-encoded enzyme for methyl and ethyl DNA adducts. These new strains may prove useful in the evaluation of nitrosamine contamination of food and environmental samples,

and may serve as useful tools in investigating the molecular properties of proteins in the cytochrome P450 monooxygenase system.

KEYWORDS: Salmonella typhimurium, nitrosamines, mutagenicity, cytochrome P450

Matthew Troy Cooper

July 22, 2002

CREATION OF A BACTERIAL MUTAGENICITY ASSAY
HIGHLY SENSITIVE TO DIALKYL NITROSAMINES

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DISSERTATION

Matthew Troy Cooper

The Graduate School
University of Kentucky
2002

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CHAPTER ONE

Background

The Ames Assay

One of the greatest challenges facing modern science is developing diagnostic and predictive assays to determine the safety of substances in humans. Issues including accuracy, sensitivity, selectivity, and reproducibility must be carefully weighed when evaluating or developing appropriate models for human safety. Technical ease of use, economics, and easily quantifiable results can also have an influence on the adoption of methodologies. Each of these factors has played a role in the wide acceptance of bacterial-based mutagenicity assays.

The earliest useful bacterial mutagenicity assays were reversion or forward mutation assays developed using *Escherichia coli*. Initially, there was great excitement concerning the use of these assays in predicting human carcinogenicity. However, as scientists began to further understand the process of chemical mutagenicity and carcinogenicity the limitations of these bacterial assays soon became apparent.

In the late 1960s and early 1970s, Bruce Ames, Ph.D., applied his knowledge of the histidine operon in the *Salmonella typhimurium* LT-2 strain to the development of the first widely-accepted, *in vitro*, bacterial-based mutagenicity assay. The Ames test, as it became known, utilized the chemically induced reversion of histidine auxotrophs as an indicator of mutagenic potential. Ames and his colleagues produced a number of *S. typhimurium* *his*⁻ mutants that could be reverted to *his*⁺ prototrophy by either frameshift or base pair mutations. The genotypes of these strains are listed in Table 1. The frameshift strains are derivatives of an acridine dye-induced -1 base pair frameshift mutation, *hisD3052* [1]. The base pair substitution strains are derived from the *hisG428* and *hisG46* mutants [1]. In order to increase the sensitivity of these *S. typhimurium* strains to chemical mutagens, several other genes were altered including *uvrB*, *gal*, and *rfa*. The *uvrB* deletion eliminated the endogenous excision repair system, thereby increasing the susceptibility of these strains to mutagens. The *gal* and *rfa* mutations

removed the polysaccharide side chain of the lipopolysaccharide (LPS) cell wall, resulting in a nonpathogenic bacterial cell more permeable to test compounds.

Further modification of some tester strains included introduction of plasmid pKM101, which contains the *mucAB* operon. The *mucAB* (*m*utagenesis, *U*V, and *c*hemical) genes encode two proteins of the SOS response responsible for mutagenesis promotion [2]. The SOS response describes an adaptive reaction to DNA damage in prokaryotes mediated by more than 20 genes, most of which are involved in functions that promote the survival of DNA-damaged cells [3]. The MucA (16 kDa) and MucB (46 kDa) members of the SOS family are proteins which increase mutagenesis by interacting with the DNA replication apparatus and enable the conversion of lesions into mutations via replicative bypass. Replication bypass allows the cell to finish chromosome replication despite severe, replication-blocking DNA damage that would otherwise be lethal. Two of the most commonly used Ames test *S. typhimurium* strains, TA98 (which detects frameshift mutations) and TA100 (which detects base-substitution mutations), contain pKM101.

Another important component of the Ames test was the addition of rat liver homogenates, particularly from animals treated with substances that induce cytochrome P450 (CYP) enzymes, to the assay mix. These homogenates provided a source of metabolic enzymes that activated promutagens to their reactive form, thus making the assay more relevant to *in vivo* mammalian models of carcinogenicity. Later modifications included the use of rat or human microsomes [4, 5], S9 fraction (postmitochondrial supernatant remaining after centrifugation at 9,000 g) [4, 5], cell extracts from human CYP-expressing cell lines [6-8], or purified human liver enzymes [9].

The traditional Ames test (detailed in Figure 1) gained wide acceptance in the 1970s and has become the most widely used screening tool for mutagenicity in the chemical and pharmaceutical industries. The primary force behind the extensive use and popularity of the Ames test and other bacterial mutagenicity assays (Table 2) has been their ease of use, low costs, and their initial promise of qualitative predictability for cancer in rodents and, by extension, humans. Over the past few decades, a tremendous number of

compounds have been examined using the Ames test. Depending upon the number and nature of substances examined, the positive predictivity of carcinogenicity using the Ames test can range from 69-100%, while the concordance of these compounds to cancer in rodents can vary from 61-66% (detailed in Table 3) [10].

The difficulty of the Ames test, as it is traditionally performed, to accurately predict human mutagenicity and carcinogenicity highlights some of the significant problems with this assay. Of greatest relevance to human risk assessment is the use of laboratory rats as a source of the activating enzyme preparation. A rapidly expanding body of evidence indicates that rodent CYPs and other activating enzymes are poor models for the corresponding human enzymes [11]. Examples of species differences in CYP-catalyzed metabolism include the activation of the 7,8-diol of benzo(α)pyrene by CYP3A enzymes in humans and CYP1A1 in the rat [12]; the inactivation of dinitropyrenes by CYP3A enzymes in humans and CYP1A enzymes in the rat [13]; and the inability of human CYP1A2 to activate the pneumotoxin 3-methylindole, in contrast to the high activity obtained with mouse CYP1A2 [14]. To circumvent these species-specific differences, the S9 fraction can be prepared from human liver, but for many investigators this is not feasible due to the difficulty of human tissue procurement. Moreover, the CYP content is usually low and is not amenable to experimental induction in notable contrast to rats.

A second disadvantage to this system is the undefined nature of the S9 fraction. This high-speed supernate of a liver homogenate contains the cytosolic and microsomal (endoplasmic reticular) fractions, from which nuclei and mitochondria have been removed by sedimentation. The enzyme and cofactor composition of this crude preparation is only poorly understood. Consequently, the use of this "black box" mixture tells the investigator nothing about which enzymes are responsible for the activation of a given test compound, nor about the chemical modification that converts the substance to an active mutagen. In an effort to more narrowly delineate the enzymes involved in the activation of many mutagens, some groups have substituted the microsomal fraction for the S9 preparation [15-18]. However, the endoplasmic reticulum remains a complex mixture of enzymes, including multiple forms of CYP, and has not assisted investigators in better defining complex biotransformations and sequela. Purified liver enzymes have also been used in systems reconstituted with coenzymes and cofactors to activate mutagens [9, 19, 20]. This method has the

advantage of defining which liver enzymes are responsible for activation of a given chemical; however, the purification of human enzymes is labor intensive, limited by the availability of human liver tissue, and limited by the ability to purify the active form of a given human enzyme. Thus, although this approach is valuable, it is not generally appropriate for routine testing of chemicals by most laboratories.

A third disadvantage to the traditional Ames test is the obligate metabolic activation of mutagens outside the bacterial cell. This activation must be followed by the transport or diffusion of these highly reactive, short-lived intermediates across the bacterial cell wall, the periplasmic space, and finally the cell membrane in order to react with the bacterial DNA inside the cell. This arrangement of barriers to cellular entry is a dubious model for other mammalian cells such as hepatocytes or epithelial cells, although the DNA in eukaryotic cells is also compartmentalized within the nucleus. For instance, it has been found that addition of benzo(α)pyrene-7,8-diol-9,10-epoxide, the ultimate mutagen of benzo(α)pyrene, to the culture media of a DNA repair-deficient mammalian cell line expressing CYP1A1 was considerably less cytotoxic than the addition of the 7,8-diol, which is metabolized to the diol-epoxide by CYP1A1 [21]. This argues that the endogenously produced alkylating species are substantially more toxic than those produced outside the cell. Of particular relevance to this dissertation, the mutagenicity of nitrosamines is greatly enhanced in mammalian cells expressing either CYP2A3 or CYP2E1 [22, 23], as compared to the relatively weak mutagenicity obtained with these compounds in the traditional Ames test [24-32]. This exogenous activation also requires larger doses of mutagen to achieve an effect, which can be problematic in estimating true genotoxic doses [33]. Exposure time to the mutagen is also limited by the “metabolic lifetime” of the S9 fraction, which is determined by the supply of cofactors and the maintenance of active enzymes.

Cytochrome P450 Metabolism

As mentioned in the text above, the cytochrome P450 enzymes can be considered a confounding variable in the predictability of the Ames test. CYP enzymes constitute a superfamily of heme monooxygenase enzymes found in a number of species from bacteria to humans [34]. CYPs were named after their ability to bind carbon monoxide, which resulted in a unique absorbance spectrum with a maximum absorption at 450 nm

[35]. Individual CYPs are named by the abbreviation CYP followed by the family number of the gene which encodes the enzyme (e.g. CYP17, CYP21) [36]. Families, by definition, are CYP gene sequences with >40% identity. This family number can be followed by a subfamily designation indicated by a capital letter (e.g. CYP3A, CYP2E). Subfamily members have >55% sequence identity. An Arabic numeral following the subfamily designation (e.g. CYP3A4, CYP2E1) denotes individual members of a subfamily. Members must differ in their sequences by more than 3%.

P450s are involved in the metabolism of many drugs, endogenous compounds, and xenobiotics. They are capable of catalyzing a number of different reactions including hydroxylation, peroxidation, epoxidation, deamination, *N*-, *O*- and *S*-dealkylation, sulfoxidation, desulfuration, dehalogenation, and *N*-oxide reduction. Their substrates are as diverse as their catalytic ability. Endogenous substrates include steroids, fatty acids, and prostaglandins, but P450s also commonly metabolize foreign substances such as organic solvents, drugs, ethanol, pesticides, and alkyl and aryl hydrocarbons. P450-mediated reactions typically follow this basic formula: $RH + O_2 + 2e \rightarrow R-OH + H_2O$.

Driving the catalytic cycle of CYP is an important protein named NADPH-cytochrome P450 reductase (CPR). CPR is an integral membrane flavoprotein that catalyzes the transfer of electrons from NADPH to P450 and is essential to the function of the microsomal CYP system. CPR is unique in that it is one of only three mammalian enzymes known to contain both flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), the other being various forms of nitric oxide synthase and methionine synthase reductase. In addition to CYPs, CPR is also capable of transferring reducing equivalents to several other heme proteins, such as cytochrome *c*, heme oxygenase, and cytochrome *b*₅. CPR was first observed in whole liver in 1950 and in microsomes by Strittmater and Velic in 1956 [37, 38]. CPR has been identified in a number of species and exhibits a high degree of sequence identity across species.

Detailed studies of the mechanism of electron flow through CPR have demonstrated that electrons from NADPH are transferred first to FAD and then to FMN before being

transferred one by one to the heme of CYP. These electrons can be used at two points in the reaction cycle: once early in the cycle reducing the ferric (3+) iron atom to a ferrous (2+) state, and later in the cycle by reducing the oxy complex to a peroxy complex. CYPs and CPR work in concert to oxidize substrates, such as nitrosamines, into their mutagenic forms.

Human Enzymes in Bacterial Systems

In an effort to better emulate human metabolism and increase the relevancy of results in bacterial mutagenicity assays, investigators have expressed human xenobiotic-metabolizing enzymes in bacterial tester strains (summarized in Table 4). Indeed, there are now numerous examples of enhanced cytotoxicity and mutagenicity in test systems in which the activating enzymes are expressed within the target cell [11, 33, 39-41]. A large number of mammalian biotransformation enzymes have been expressed in bacteria, including those responsible for phase I and phase II type metabolism [40]. The expression of these enzymes in the appropriate tester strain has greatly increased the utility of these assays. Even non-bacterial systems that combine the expression or “over-expression” of a particular mammalian biotransforming enzyme with a specific genetic target are becoming increasingly popular tools in genotoxicity assessment.

Bacterial assays have the advantages of being easy to perform, a good vehicle for heterologous enzyme expression, low in cost, amenable to high-throughput technology, and benefit from a large, extensively validated, historical database. Bacterial assays suffer from issues such as the need for expression of coenzymes and the relevance of the bacterial DNA target to human risk assessment. Mammalian cell-based systems, on the other hand, address these limitations, but can be problematic to culture, difficult to maintain enzyme expression, and require extensive validation prior to accepting the data as reliable.

N-nitrosamines in the Ames Test

N-nitrosamines are a sizeable and varied family of natural and synthetic compounds having the general formula $(R_1)(R_2)N-N=O$. Characteristically liquids, oils, or volatile solids, nitrosamines and their precursors are widely distributed in the environment.

Humans are commonly exposed to these compounds via a variety of sources including air, soil, water, and diet.

The potency of nitrosamines in causing acute tissue injury and death varies significantly. Structure and molecular weight play a large role in determining the acute toxicity of nitrosamines. Small molecular weight dialkylnitrosamines tend to be the most potent of this chemical class in terms of acute lethality, while the larger molecular weight cyclic nitrosamines are the most well tolerated at acute doses. The single-dose, oral LD₅₀ of nitrosamines in rats can range from 20 to greater than 5000 mg/kg, with most compounds having an LD₅₀ of between 150 and 500 mg/kg [42]. The liver is the chief organ damaged upon exposure to low molecular weight nitrosamines, commonly causing centrilobular necrosis with hemorrhage [43].

Despite the disturbing effects of acute nitrosamine exposure, chronic exposure remains the most studied and most relevant to human risk assessment. As with the acute toxicity of nitrosamines, the impact of structure and molecular weight on the chronic potency of nitrosamines can be profound. The low molecular weight dialkylnitrosamines tend to be more potent mutagens and carcinogens than the complexly branched, high molecular weight nitrosamines. Chronic exposure of nitrosamines has similar effects on a wide variety of species. Approximately 90% of 300 tested nitrosamines caused tumor formation in laboratory test animals [44, 45]. Animals studied included mammals, birds, fish, and amphibians. Of the approximately 40 animal species tested, none were resistant to nitrosamine carcinogenicity.

Many dialkylnitrosamines are potent mutagens and carcinogens in rodents as well as powerful alkylating agents in humans [46-48]. Humans are exposed to dialkylnitrosamines from a variety of sources including tobacco products, preserved meats, alcoholic beverages, and industrial solvents [49, 50]. Nitrosamines may also contribute to human cancer and there is particular concern that tobacco specific nitrosamines are causative agents in human lung, esophageal, and oral cancer [51]. Nitrosamine effects have been demonstrated in 29 organs. The tissue affected appears to

be dependent upon structure of the compound, the dosage, and route of administration. The predominant sites of tumor formation include the liver, kidney, esophagus, pancreas, stomach, gut, urinary bladder, nasal cavities, brain and nervous system, oral cavity, lungs, heart, skin, and hematopoietic system.

Despite the well-documented mutagenicity and carcinogenicity of dialkylnitrosamines in mammals, the exact mechanism of nitrosamine activation and resulting carcinogenicity remains controversial. Despite the debate concerning the precise sequence of electronic and molecular rearrangements and the exact nature of the final mutagenic species, there is strong evidence in the literature for the basic nature of the reaction (Figure 2) [52]. The metabolism of small molecular weight dialkylnitrosamines initially involves the enzymatic hydroxylation of the carbon atom immediately adjacent to the *N*-nitroso group (α -carbon) by members of the cytochrome P450 family (namely CYP2E1 or CYP2A6) [53-55]. This oxidation results in an unstable product (α -hydroxydialkylnitrosamine) that rapidly decomposes to an aldehyde and a diazohydroxide. The latter dissociates to a diazonium hydroxide and ultimately to a carbonium ion and molecular nitrogen. The diazohydroxide and subsequent intermediates are highly electrophilic. Their major reaction is with water yielding an alcohol, but they also react with DNA to produce a variety of alkylated DNA bases. Detoxification by denitrosation competes with this metabolic activation process [56]. The denitrosation is also catalyzed by P450 and results ultimately in the production of nitrite, an aldehyde, and a primary amine [57, 58]. It is generally believed that the highly reactive carbonium ion is the ultimate alkylating agent and mutagen, preferentially reacting with DNA at the O^6 position of guanine residues [59]. The methylated DNA damage is then misrepaired resulting in a G:C \rightarrow A:T transition and, ultimately, carcinogenicity [60].

Members of the cytochrome P450 family of enzymes were first associated with nitrosamine metabolism in 1973 when it was demonstrated that carbon monoxide and monochromatic light at a wavelength of 450 nm could severely inhibit the demethylation of dimethylnitrosamine (DMN) by mouse liver microsomes [15]. Tu, *et al.*, was the first to implicate CYP2E1 (then known as P450 LM3a, P450_{et}, or ethanol-inducible P450) as

the chief enzyme responsible for metabolizing DMN by examining the rate of DMN demethylation in purified liver microsomes isolated from ethanol-treated rats [57]. This observation was also seen in rabbits [54] and humans [61]. More recently, CYP2A6 has also been associated with the metabolism of nitrosamines in humans [62].

Historically, the utilization of bacteria-based assays to predict the mammalian mutagenicity of nitroso compounds, particularly N-nitrosamines, has proven difficult [24, 25, 27-32, 63, 64]. N-nitrosamines, known carcinogens in rats and suspected carcinogens in humans, commonly yield negative results in the traditional Ames test. Indeed, this problem was apparent as early as 1977 when Meselson and Russell found that the correlation between compounds found to be mutagenic in the Ames test to those which are known to be carcinogenic in rats dropped from 0.94 to 0.36 when nitroso compounds were considered [65]. These data highlight the problematic nature of using *in vitro* models to predict *in vivo* mutagenicity and carcinogenicity.

Overview of Rationale and Objectives

The efficacy of a predictive *in vitro* bacterial model for nitrosamine mutagenicity is reliant on many variables including: (a) the homologous metabolism of the promutagen to its active, mutagenic form, (b) the concentration of the mutagenic species in the test system, (c) the fraction of the mutagenic species which permeates into the tester strain, (d) the fraction of the mutagenic species which produces DNA adducts, (e) the fraction of the DNA adducts that are not repaired, (f) the efficiency of each DNA adduct at generating mutations, and, (g) the probability that the mutation will occur at a site which leads to a phenotypic change [32]. Issues with components of each of these factors have plagued the efforts of investigators in utilizing bacterial-based systems to predict the mutagenicity of nitrosamines. I will attempt to identify the chief confounding factor(s) involved in this process and propose a new test system which alleviates the obstacles to a bacterial-based mutagenicity assay that is sensitive to nitrosamine mutagenicity.

Given variables above, careful experimentation is required to establish the role and impact of each factor or series of factors on the process of nitrosamine mutagenicity in

bacterial assays. Each variable outlined above plays a critical role. A test system devoid of any one of these factors would be insensitive to the mutagen. As described in the preceding pages, the traditional Ames test is a relatively poor predictor of the carcinogenic potential of nitrosamines in humans, even in the presence of mammalian liver S9 fractions or microsomes. Traditional means of inducing rat liver CYP enzymes prior to the Ames test, such as administration of Aroclor 1254 (a complex mixture of polychlorinated biphenyls) or a mixture of phenobarbital and beta-naphthoflavone, typically fail to increase the level of CYP2E1 in rat liver microsomes or S9 [18]. In fact, uninduced rats characteristically have higher levels (on a percentage basis) of CYP2E1 present in the liver [66]. Even when high levels of mammalian CYP2E1 are present in the assay mix, the evaluation of nitrosamine mutagenicity remains problematic [66, 67]. This observation leads one to believe that the factors of homologous metabolism and mutagenic species concentration mentioned above are essential, however, their impact is minimal on DMN mutagenicity in the bacterial test system.

It is known that the variables of mutagenic species permeation and the nature of the ultimate mutagen mentioned above also have an impact on nitrosamine mutagenicity due to the increased mutagenicity seen with DMN in acidic media. Early *in vitro* studies also revealed that the half-life of the α -hydroxydimethylnitrosamine could be extended from several seconds to several minutes by lowering the pH of the surrounding medium from 7.4 to 6.5 [68]. This observation was applied to the Ames test using nitrosamines with an increase in mutagenicity when the assay was performed at a pH of 6.5 [69]. It is thought that increasing the lifetime of intermediates increases their chance of transportation or diffusion into the cells. Another approach to increasing the amount of active metabolite within a cell is to express the activating enzymes within the target cell itself. Prior to metabolism, nitrosamines are comparatively non-reactive and can more easily cross the cell wall and membrane. If CYP2E1 were expressed within the target cell membrane, nitrosamines would be metabolized within the cell in close proximity to the target DNA without the need for transportation or diffusion into the cell. Prokaryotic organisms inherently lack the metabolic machinery necessary to metabolize nitrosamines.

Nitrosamines are not mutagenic, *per se*, but must be activated by liver enzymes such as CYP2E1 and CYP2A6, in order to react with DNA [62].

The fraction of DNA adducts that are repaired during the mutagenic process has not been fully investigated despite early work recognizing the role of repair in nitrosamine mutagenicity. Indeed, as early as 1977 Pegg documented the importance of O^6 -demethylase activity in rat liver after DMN treatment [70]. In that study, the alkylation of liver DNA was examined after administration of DMN to Sprague-Dawley rats. The amounts of O^6 -methylguanine and 7-methylguanine were measured in liver DNA at 4 and 24 hours after treatment. Pegg observed a linear relationship between 7-methylguanine levels and dose of the nitrosamine at both timepoints. In contrast, the corresponding levels of O^6 -methylguanine were not directly proportional to dosage but were less than expected. Evidence was obtained to support the hypothesis that these results were due to an enzymatic removal of O^6 -methylguanine from liver DNA. More recently, Yamada, *et al.*, constructed two bacterial strains derived from the popular *S. typhimurium* TA1535 Ames tester strain that lack the *ada* and *ogt* genes [71]. The *ada* and *ogt* genes code for two O^6 -methylguanine DNA methyltransferases that repair alkylation damage in DNA. These new strains give investigators an opportunity to explore the roles DNA repair plays in nitrosamine mutagenicity. The new strains also reveal that the efficiency of each DNA adduct at generating mutations is not a rate-limiting factor in determining nitrosamine mutagenicity given that methylating agents that require no metabolic activation, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), elicit a strong mutagenic response in the bacteria.

Insuring that a mutation will occur at a site that leads to a phenotypic change also plays a crucial role in nitrosamine mutagenicity; however, the currently available bacterial tester strains have already been optimized to insure that mutations will occur at such “hot spots” [72, 73]. It is doubtful that any further alterations in the bacterial genome would result in more sensitive strains in this regard.

It should also be noted that the enzymatic activity of CYP2E1 could be augmented by cytochrome b₅, a heme-containing coenzyme capable of providing reducing electrons to the heme of CYP enzymes. The interactions between CYP2E1 and cytochrome b₅, and the effects of cytochrome b₅ on the CYP2E1-mediated metabolism and associated mutagenicity of nitrosamines are only poorly understood.

In order to produce an assay that optimizes nitrosamine mutagenicity by addressing the aforementioned variables, three objectives are proposed for the studies described herein.

Objective #1: Express human CYP2E1 and CPR in an appropriate bacterial tester strain for nitrosamine mutagenicity assessment.

Objective #2: Determine the mutagenic potency of dimethylnitrosamine, diethylnitrosamine, dipropylnitrosamine and dibutylnitrosamine in the CYP2E1/CPR coexpressing tester strains.

Objective #3: Examine the effect of cytochrome b₅ coexpression on nitrosamine mutagenicity in the CYP2E1/CPR coexpressing tester strains.

Table 1: Genotypes of common Ames tester strains						
Histidine Mutation				LPS	Repair	R-factor
<i>his</i> D6610	<i>his</i> D3052	<i>his</i> G46	<i>his</i> G428			
TA90	TA1538	TA1535		<i>rfa</i>	<i>uvrB</i>	- R
TA97	TA98	TA100		<i>rfa</i>	<i>uvrB</i>	+ R
	TA1978	TA1975		<i>rfa</i>	+	- R
TA110	TA94	TA92		+	+	+ R
	TA1934	TA1950		+	<i>uvrB</i>	- R
		TA2410		+	<i>uvrB</i>	+ R
TA89	TA1964	TA1530		<i>gal</i>	<i>uvrB</i>	- R
	TA2641	TA2631		<i>gal</i>	<i>uvrB</i>	+ R
			TA102	<i>rfa</i>	+	+ R

Adapted from Maron and Ames, 1983 [73].

Table 2: Common Bacterial Mutagenicity Assays							
Assay Name	Bacterium	Mutation Type	LPS	Excision Repair	Genetic Target	Mutator Plasmid	Reference
Ames test	<i>S. typhimurium</i>	reversion	<i>rfa</i>	<i>uvrB/uvr+</i>	<i>his</i>	pKM101	Maron, et al., 1983
WP2 test	<i>E. coli</i>	reversion	<i>rfa, rfa+</i>	<i>uvrA</i>	<i>trp</i>	pKM101	Venitt, et al., 1984
K12/343/112	<i>E. coli</i>	reversion/forward	<i>rfa, rfa+</i>	<i>uvrB</i>	several	pKM101	Mohn, et al., 1984
AraR test	<i>S. typhimurium</i>	forward	<i>rfa</i>	<i>uvrB</i>	arabinoseS	pKM101	Ruiz-Rubio, et al., 1985
SOS Chromotest	<i>E. coli</i>	induction	<i>rfa</i>	<i>uvrA</i>	SOS response	None	Quillardet, et al., 1985
Umu test	<i>S. typhimurium</i>	induction	<i>rfa</i>	<i>uvrB</i>	SOS response	None	Oda, et al., 1985
MX100	<i>E. coli</i>	reversion	<i>rfa</i>	<i>uvrA</i>	<i>arg</i>	pKR11	Kranendonk, et al., 1996

Adapted from Kranendonk, *et al.*, 2000 [40].

Table 3: Comparison of group statistics between chemical databases and within their combination	73 chemicals	42 chemicals	115 chemicals
Ames test positives (%)	33	29	32
Carcinogenic in rodents (%)	60	56	59
Positive predictivity (%)	83	100	89
Negative predictivity (%)	51	62.00	55
Sensitivity (%)	45	52	48
Specificity (%)	86	100	91
Concordance with rodent cancer (%)	62	73	66
Significance of association (<i>P</i> value)	0.004	<0.001	<0.001

Taken from Fetterman, et al, 1997 [10].

Table 4: Mammalian biotransformation enzymes expressed in bacterial mutagenicity tester strains				
Enzyme Form	Species	Bacterium	Genetic Target	Ref
Glutathione-S-transferase				
GSTT5-5	rat	<i>S. typh.</i>	<i>his</i> reversion	Their, et al., 1993
GSTT5-5	rat	<i>S. typh.</i>	<i>umu</i> induction	Oda, et al., 1996
GSTT5-5	rat	<i>S. typh.</i>	<i>his</i> reversion	Their, et al., 1995
GSTT5-5	rat	<i>S. typh.</i>	<i>umu</i> induction	Shimada, et al., 1996
GSTA1-1	human	<i>S. typh.</i>	<i>his</i> reversion	Simula, et al., 1993
GSTP1-1	human	<i>S. typh.</i>	<i>his</i> reversion	Simula, et al., 1993
GSTT1-1	human	<i>S. typh.</i>	<i>his</i> reversion	Their, et al., 1996
N-acetyl transferase				
NAT1	human	<i>S. typh.</i>	<i>his</i> reversion	Grant, et al., 1992
NAT2	human	<i>S. typh.</i>	<i>his</i> reversion	Wild, et al., 1995
OAT	bacterial	<i>S. typh.</i>	<i>umu</i> induction	Oda, et al., 2001
Cytochrome P-450				
CYP1A1	human	<i>E. coli</i>	<i>arg</i> reversion	Kranendonk, et al., 1999
CYP1A1	human	<i>S. typh.</i>	<i>umu</i> induction	Oda, et al., 2001
CYP1A2	human	<i>S. typh.</i>	<i>his</i> reversion	Josephy, et al., 1995
CYP1A2	human	<i>E. coli</i>	<i>lac</i> reversion	Kranendonk, et al., 1999
CYP1A2	human	<i>S. typh.</i>	<i>umu</i> induction	Oda, et al., 2001
CYP1A2	human	<i>E. coli</i>	<i>arg</i> reversion	Kranendonk, et al., 1998
CYP2A6	human	<i>S. typh.</i>	<i>his</i> reversion	Kushida, et al., 2000
CYP2A6	human	<i>S. typh.</i>	<i>his</i> reversion	Kamataki, et al., 2000
CYP3A4	human	<i>S. typh.</i>	<i>his</i> reversion	Kranendonk, et al., 1999
CYP3A4	human	<i>S. typh.</i>	<i>umu</i> induction	Oda, et al., 2001
CYP3A5	human	<i>S. typh.</i>	<i>his</i> reversion	Kranendonk, et al., 1999
CYP1B1	human	<i>S. typh.</i>	<i>umu</i> induction	Oda, et al., 2001
CYP2C9	human	<i>S. typh.</i>	<i>umu</i> induction	Oda, et al., 2001
CYP2D6	human	<i>S. typh.</i>	<i>umu</i> induction	Oda, et al., 2001
CYP2E1	human	<i>S. typh.</i>	<i>his</i> reversion	Cooper, et al., 2000
CYP2E1	human	<i>S. typh.</i>	<i>his</i> reversion	Kamataki, et al., 2000
CYP2E1	human	<i>S. typh.</i>	<i>umu</i> induction	Oda, et al., 2001
Sulfotransferase				
HSTa	rat	<i>S. typh.</i>	<i>his</i> reversion	Glatt, et al., 1998
PSTIV	rat	<i>S. typh.</i>	<i>his</i> reversion	Glatt, et al., 1998
HSST	human	<i>S. typh.</i>	<i>his</i> reversion	Glatt, et al., 1998
HP-PST	human	<i>S. typh.</i>	<i>his</i> reversion	Glatt, et al., 1998
M-PST	human	<i>S. typh.</i>	<i>his</i> reversion	Glatt, et al., 1998
EST	human	<i>S. typh.</i>	<i>his</i> reversion	Glatt, et al., 1998

Adapted from Kranendonk, *et al.*, 2000 [40].

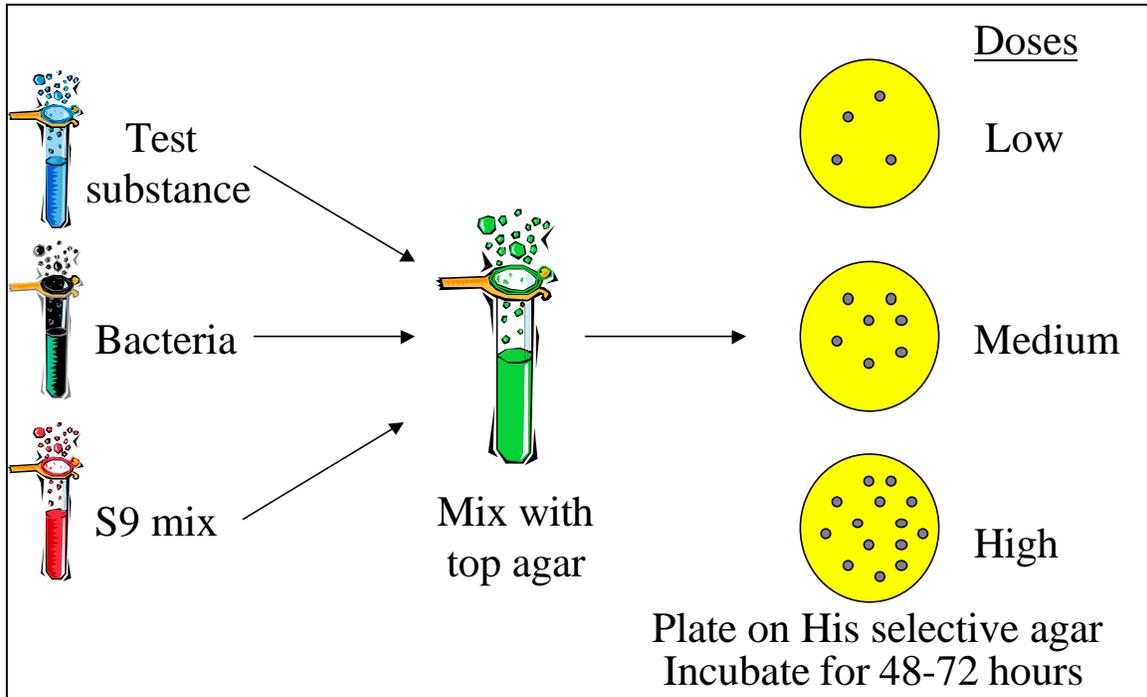


Figure 1: The Ames test procedure. The appropriate bacterial strain is mixed with the test substance and S9 fraction, then allowed to incubate at 37°C for 30 minutes. Molten top agar is then added to the mixture and spread on histidine-deficient agar plates. Colonies are counted after the plates are allowed to incubate for 48-72 hours at 37°C. The number of colonies that form is directly proportional to the mutagenic potential of the test substance.

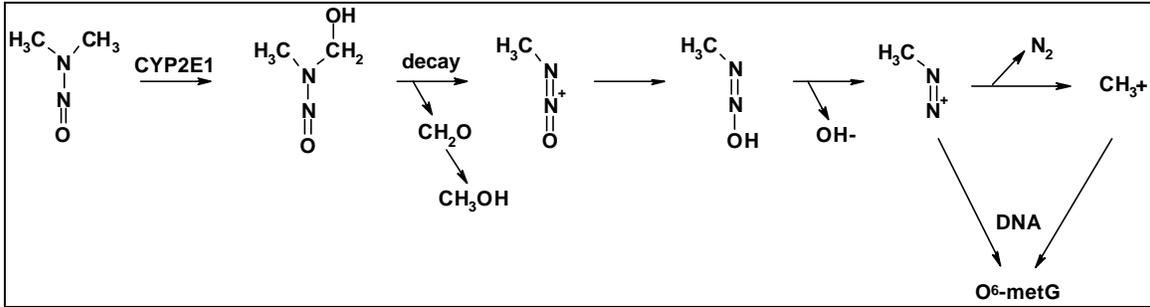


Figure 2: Mechanism of dimethylnitrosamine (DMN) metabolism by CYP2E1.

CHAPTER TWO

Construction of a Nitrosamine-Responsive Salmonella Test System Expressing CYP2E1 and P450 Reductase

RATIONALE

Nitrosamines are potent mutagens and carcinogens in rodents as well as powerful alkylating agents in humans [46-48]. Nitrosamines are not mutagenic, *per se*, but must be activated by liver enzymes such as CYP2E1 and CYP2A6, in order to react with DNA at the O^6 position of guanine residues [59, 62]. The methylated DNA damage is then misrepaired resulting in a G:C \rightarrow A:T transition resulting in, ultimately, carcinogenicity [60].

In order to better evaluate the carcinogenic risk associated with nitrosamines, I have created a bacterial reversion mutagenicity assay based on the Ames test [5, 73]. Commonly used Ames test strains, such as TA1535 and TA100, are only weakly sensitive to dialkylnitrosamine mutagenicity [5, 32, 74]. As mentioned previously in this work, many factors may contribute to this weak response. One overlooked cause of dialkylnitrosamine insensitivity in the traditional Ames assay may be the robust DNA repair enzymes present in the standard Ames strains. Most tester strains contain the genes *ogt* and *ada* that code for methyltransferase proteins capable of repairing the alkylation damage to DNA created by dialkylnitrosamines. The *ogt* gene encodes a constitutive, 19 kDa methyltransferase capable of repairing alkylation damage to the O^6 position of guanine and the O^4 position of thiamine [75]. The product of *ada* is an inducible, 39 kDa methyltransferase which demethylates O^6 -methylguanine, O^4 -methylthiamine and methylphosphotriester [76]. Consequently, the use of bacterial strains with intact bacterial DNA repair systems could yield misleading results in mutagenicity assays given the differing DNA repair characteristics and activities of these bacterial enzymes in comparison to their mammalian counterparts. Of particular interest to this work, standard Ames strains, including TA1535, are proficient at repair of methylated DNA and thus resistant to nitrosamine mutagenicity. The repair of these types of mutations appears less efficient in mammalian cells [77].

The following describes the construction of two *S. typhimurium* strains that express the full-length genes for human CYP2E1 and its electron-transfer partner, CPR. CYP2E1 and CPR were expressed in several of the YG71XX strains created by Yamada, *et al.*, which lack the *ogt* and/or *ada* methyltransferases involved in DNA repair [71]. By coexpressing enzymatically active CYP2E1 and CPR in methyltransferase-deficient tester strains, I am able to demonstrate sensitivity to dialkylnitrosamine mutagenicity even when used in the absence of an exogenous activating system (S9 fraction).

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this chapter are described in Table 5. Dr. T. Nohmi (National Institute of Health Sciences, Tokyo, Japan) graciously provided *S. typhimurium* strains YG7104 (*ogt*-) and YG7108 (*ogt*-, *ada*-). Strain TA1535 was obtained from Dr. Bruce Ames (Department of Molecular and Cell Biology, University of California, Berkeley, CA). Plasmids for human CYP2E1 and rat CPR expression were created as described elsewhere [78]. Plasmids were introduced into the bacterial strains via electroporation. Fresh transformants were selected for each mutagenicity assay by growth on Luria-Bertani nutrient agar plates containing 100 µg ampicillin/mL.

Analysis of Enzyme Expression

Bacterial membranes were prepared as previously described [79]. CYP2E1 and CPR expression was confirmed by spectroscopic and biochemical analyses. Proteins were separated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The immunoblots were probed with sheep polyclonal antibody to rabbit CYP2E1 and sheep polyclonal antibody to rabbit CPR (kindly provided by Dr. Minor Coon, Department of Biological Chemistry, University of Michigan). P450 expression was also verified via CO difference spectrum; P450 reductase expression was quantified by means of cytochrome *c* reduction (assuming specific activity of 3 µmol/min/mL = 1

nmol CPR/mL) [80]. Enzymatic activity could not be reliably quantified in whole cells or membrane isolates (Appendix A).

Mutation Assays

Reversion assays were performed as described [81]. Freshly transformed single bacterial colonies were picked and grown overnight in Oxoid Nutrient Broth No. 2 with 100 µg/mL ampicillin at 37°C and shaking at 250 rpm. Growth at lower temperatures had no effect on the mutagenicity of dialkylnitrosamines (data not shown). Cultures were induced with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) at 10 hours post-inoculation. S9 fraction from Aroclor 1254- and ethanol-induced male Sprague-Dawley rats was purchased from Molecular Toxicology (Annapolis, MD). The preincubation assay technique (30 min) was used in all experiments requiring S9. Preincubation time, temperature and pH had no effect on dialkylnitrosamine mutagenicity with assays utilizing CYP2E1 and CPR coexpressing strains (data not shown). All assays were performed in triplicate. Revertant colonies were counted after 72 hours. See Appendix A for additional details on development of this assay.

RESULTS

Coexpression of CYP2E1 and P450 reductase in S. typhimurium

CYP2E1 expression in the three *S. typhimurium* strains was between 17 and 20 nmoles/liter culture as determined from isolated membrane preparations (Table 6) and was similar to levels obtained previously with the pINIIIompA3 vector [78]. CPR was expressed at a ratio of 1:2 CYP:CPR. Immunoblots confirmed the expression of each protein in these strains (Figure 3).

P450-Expressing Strains are Sensitive to Dialkylnitrosamine Mutagenicity

Dimethylnitrosamine (DMN) was unable to elicit a mutagenic response in strains TA1535, YG7104 and YG7108 in either the presence or absence of 4% Aroclor1254-induced S9 (data not shown). Increasing the concentration of Aroclor1254-induced S9 to

10% had no effect on DMN mutagenicity, but did increase the number of spontaneous revertants in each strain. The use of 4% ethanol-induced S9 did yield a mutagenic response to DMN in YG7104 and YG7108 strains, although the level of spontaneous revertants increased in these strains to 50 and 250 revertants/plate, respectively (Figure 4). Strain TA1535 remained insensitive to DMN. Increasing the concentration of ethanol-induced S9 to 10% dramatically increased the number of spontaneous revertants in YG7104 and YG7108 to unacceptable levels (over 500 per plate). TA1535 exhibited a satisfactory spontaneous background (approximately 25 revertants/plate) but remained insensitive to DMN mutagenicity.

DMN produced a dose-dependent increase in mutagenicity in both strains YG7104ER and YG7108ER in the absence of S9 (Figure 5) indicating that the coexpression of CYP2E1 and CPR could substitute for an exogenous activating system in these strains. In contrast to the methyltransferase-deficient strains, the coexpression of CYP2E1 and CPR in TA1535 revealed no increase in sensitivity to DMN mutagenicity. The level of spontaneous revertants in the ER strains is only slightly higher than that seen in the native strains (Table 5) and well below that seen in experiments with the native strains and ethanol-induced S9. As shown in Figure 6, the mutagenicity of DMN was dependent upon the simultaneous coexpression of CYP2E1 and CPR. Strains expressing CYP2E1 alone were only slightly more sensitive than the CYP2E1 non-expressing strains YG7108, YG7108V and YG7108R. The mutagenicity of DMN in YG7104ER and YG7108ER was inhibited in a dose-dependent manner by the addition of ethanol, a potent inhibitor of CYP2E1 (Figure 7).

Diethylnitrosamine (DEN) mutagenicity closely resembled that of DMN with YG7108ER being the most sensitive and TA1535ER being non-responsive to the mutagen (Figure 8). While cytotoxicity was observed with DEN in strain YG7108ER at concentrations above 10 $\mu\text{mol}/\text{plate}$, strains YG7104ER and TA1535ER were viable up to 100 μmol of DEN. The sensitivity of strains YG7104ER and YG7108ER to dipropylnitrosamine (DPN) (Figure 9) and dibutylnitrosamine (DBN) (Figure 10) mutagenicity was very similar. No mutagenic response was seen in strain TA1535ER for

either compound. DPN was cytotoxic at concentrations above 50 $\mu\text{mol}/\text{plate}$ in strain YG7108ER and 100 $\mu\text{mol}/\text{plate}$ with strains YG7104ER and TA1535ER, whereas DBN exhibited this effect at concentrations above 10 $\mu\text{mol}/\text{plate}$ for YG7108ER and 50 $\mu\text{mol}/\text{plate}$ for strains YG7104ER and TA1535ER.

DISCUSSION

The creation of short-term, *in vitro* genotoxicity assays utilizing heterologously expressed human xenobiotic metabolizing enzymes has been of great interest in recent years [39]. Various systems have been constructed using CYPs, CPR, CYP/CPR fusion enzymes and acetyltransferases expressed separately or in combination in many different genetic backgrounds [82-90]. Each of these systems has shown sensitivity to test compounds without the need for S9.

This chapter shows the establishment of a reliable, easy-to-use, bacterial reversion assay that is sensitive to dialkylnitrosamines. In the past, the Ames test had to be heavily modified in order to elicit a mild mutagenic response to dialkylnitrosamines. These alterations included such modifications as changing the pH of the assay [91], preincubating with the mutagen [24], using uninduced or alternatively induced S9 [28], using the S9 from different mammalian species [28], using concentrated microsomes instead of S9 [69], pretreatment with sub-threshold concentrations of nitrosamines [92], or a combination of these modifications [69]. This heterologously expressed human enzyme bacterial reversion assay offers greater sensitivity to dialkylnitrosamines than previous assays without the need for extensive procedural modifications.

This chapter has shown that *S. typhimurium* strains YG7104ER and YG7108ER express CYP2E1 and CPR simultaneously and that this concurrent expression is critical for nitrosamine mutagenicity. The mutagenicity of all nitrosamines investigated occurred in a dose-dependent fashion without the addition of S9. Moreover, this mutagenicity was inhibited by ethanol, a potent inhibitor of CYP2E1. Thus, these results support the view

that intracellular CYP2E1 and CPR can metabolically activate dialkyl nitrosamines and cause mutation within the cell.

The genotoxic potency of the nitrosamines in both YG7104ER (*ogt*-) and YG7108ER (*ogt*- and *ada*-) was DMN>DEN>>DPN>DBN. This order is repeated when examining the specificity of the *ogt* and *ada* enzymes for the repair of alkylated DNA adducts [93]. Given the insensitivity of the TA1535ER strain, it is evident that the *ogt* and *ada* methyltransferases are highly efficient in the repair of dialkyl nitrosamine-induced DNA damage and thereby prevent the incorporation of heritable mutations. Furthermore, it is clear that even relatively low expression of the appropriate activating enzymes is sufficient to give a robust mutagenic response when expressed in a suitable genetic background.

The difference in sensitivity between TA1535ER and YG7104ER reveals the role *ogt* plays in repairing DNA damage. *ogt* demonstrated an ability to repair a broad range of alkylation damage with a preference for methyl and ethyl adducts. This agrees with past characterizations of the *S. typhimurium ogt* enzyme where it was shown that *ogt*- mutants were most sensitive to treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine, which are methylating and ethylating agents, respectively [71]. In these experiments, there was no increased sensitivity to propyl or butyl adducts in YG7108ER when compared to YG7104ER, indicating that the *ada* methyltransferase has a stringent preference for methyl and ethyl adducts. Thus, the repair of longer chain adducts, as generated with DPN and DBN, appears to be largely dependent on the activity of the *ogt* methyltransferase, consistent with its reported role [76]. A recent study from Kamataki *et al* [87] similarly reported enhanced mutagenicity of short chain nitrosamines in strain YG7108 expressing a truncated human CYP2E1 with CPR. This group did not examine mutagenic activation with strain YG7104 and thus could not differentiate the roles of the two methyltransferases (*ogt* and *ada*) in nitrosamine adduct DNA repair *in vivo*.

This study is complementary to a growing body of evidence that demonstrates that recombinant Ames strains are viable alternatives to exogenous mammalian enzyme preparations for mutagen activation [82, 88, 89, 94]. Although many of these past studies have focused chiefly on the CYP1A2-mediated mutagenicity of heterocyclic amines, they reveal the great potential of coexpressing CYPs with CPR in mutagenicity tester strains possessing the appropriate DNA repair background.

In conclusion, I have demonstrated that sensitivity to dialkylnitrosamine mutagenicity can be achieved by coexpressing human CYP2E1 and CPR in methyltransferase-deficient *S. typhimurium* strains YG7104 and YG7108, thereby eliminating the need for mammalian tissue homogenates (S9) for nitrosamine activation.

TABLE 5. Bacterial strains and plasmids used in this study			
Strain or Plasmid	Characteristics	Spontaneous Revertants	Reference
Strains			
<i>S. typhimurium</i>			
TA1535	<i>hisG46 rfa uvrB</i>	20-25	[73]
TA1535ER	TA1535 with plasmid pIN3ER	20-25	this chapter
YG7104	<i>hisG46 rfa uvrB ogt_{ST}::Cm^r</i>	30-40	[71]
YG7104ER	YG1704 with plasmid pIN3ER	30-40	this chapter
YG7108	<i>hisG46 rfa uvrB ogt_{ST}::Cm^r ada_{ST}::Km^r</i>	40-45	[71]
YG7108V	YG7108 with plasmid pIN3	40-50	this chapter
YG7108R	YG7108 with plasmid pIN3R	40-50	this chapter
YG7108E	YG7108 with plasmid pIN3E	40-50	this chapter
YG7108ER	YG7108 with plasmid pIN3ER	45-55	this chapter
Plasmids			
pIN3	pINIIIompA3; a pBR322-based expression vector with cleavable ompA signal peptide upstream of the cloning site		[95]
pIN3R	pIN3 vector expressing CPR		[78]
pIN3E	pIN3 vector expressing CYP2E1		[78]
pIN3ER	pIN3 vector expressing CYP2E1 and CPR		[78]

<u>Strain</u>	<u>CYP2E1 Content^a</u>	<u>CPR Content^a</u>
TA1535ER	20 ± 2	41 ± 2
YG7104ER	18 ± 4	37 ± 2
YG7108ER	17 ± 3	36 ± 2

^aUnits are nmol/L, means ± standard deviations; n = 3.

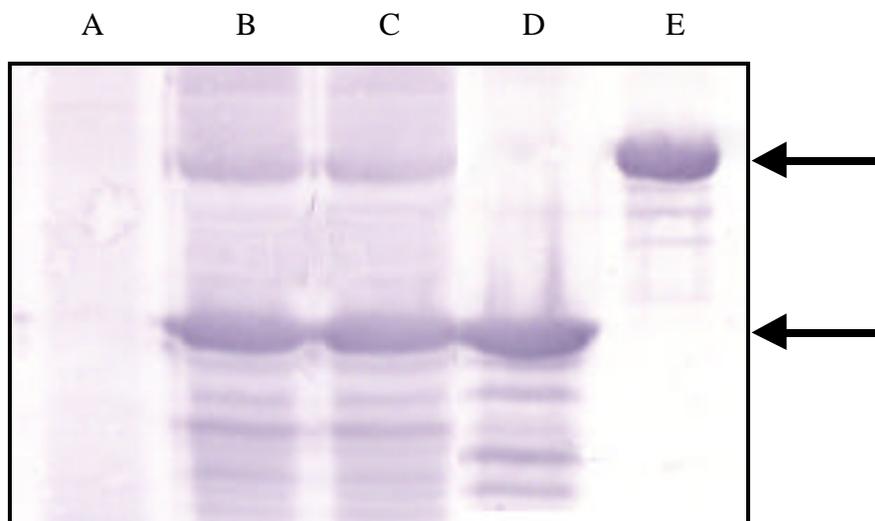


Figure 3: Immunoblot of CYP2E1 (lower arrow) and P450 reductase (upper arrow) expression in bacterial membrane fractions of YG7104 and YG7108. A, YG7104; B, YG7104ER; C, YG7108ER; D, purified CYP2E1; E, purified CPR. Lanes containing membrane fraction were loaded at 50 μ g of total protein per lane.

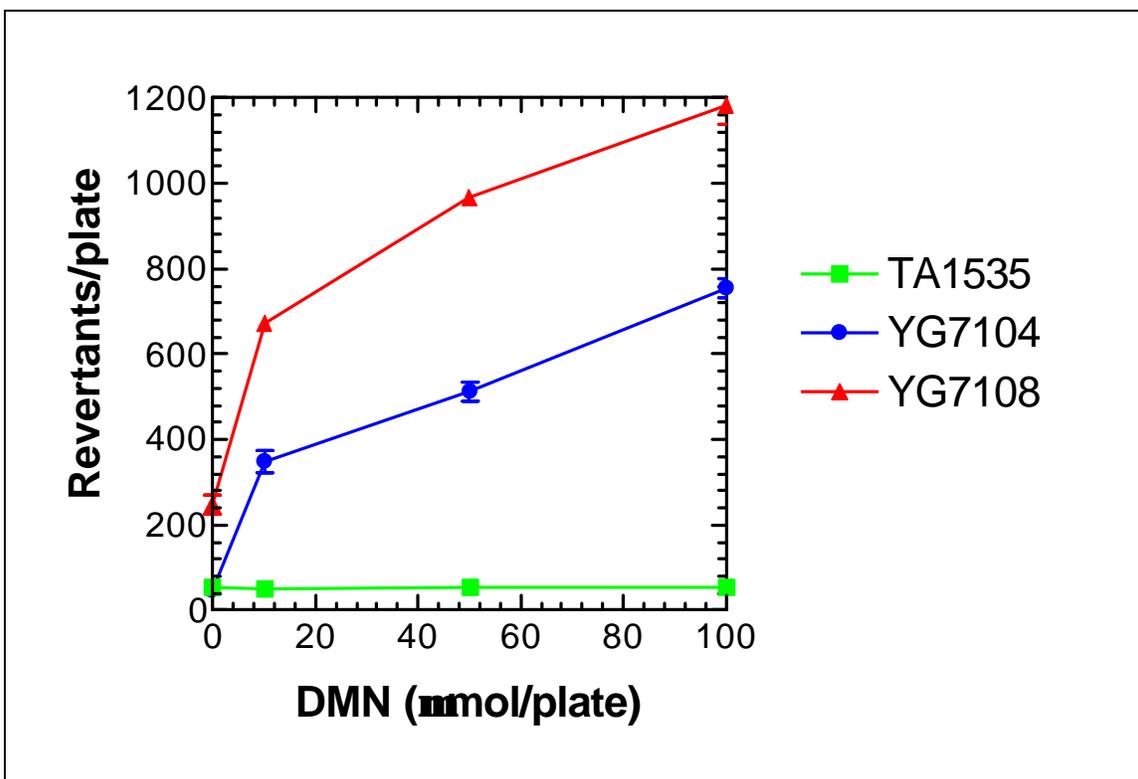


Figure 4: Mutagenicity of DMN in native tester strains with 4% ethanol-induced S9. Squares, TA1535; circles, YG7104; triangles, YG7108. Data points are the mean of three triplicate experiments; error bars indicate standard error. These strains gave no mutagenic response in the presence of 4% and 10% Aroclor-induced S9 (data not shown).

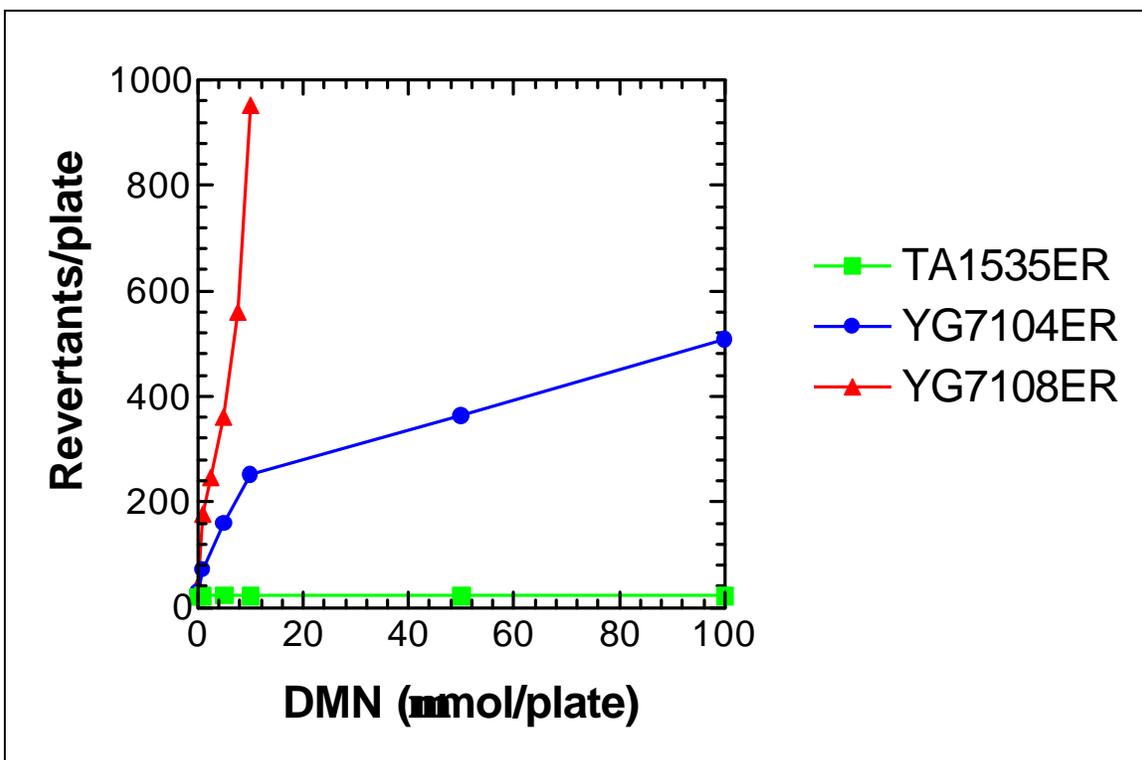


Figure 5: DMN mutagenicity in CYP2E1 and P450 reductase coexpressing strains. Squares, TA1535ER; circles, YG7104ER; triangles, YG7108ER. Data points are the mean of three triplicate experiments; error bars indicate standard error.

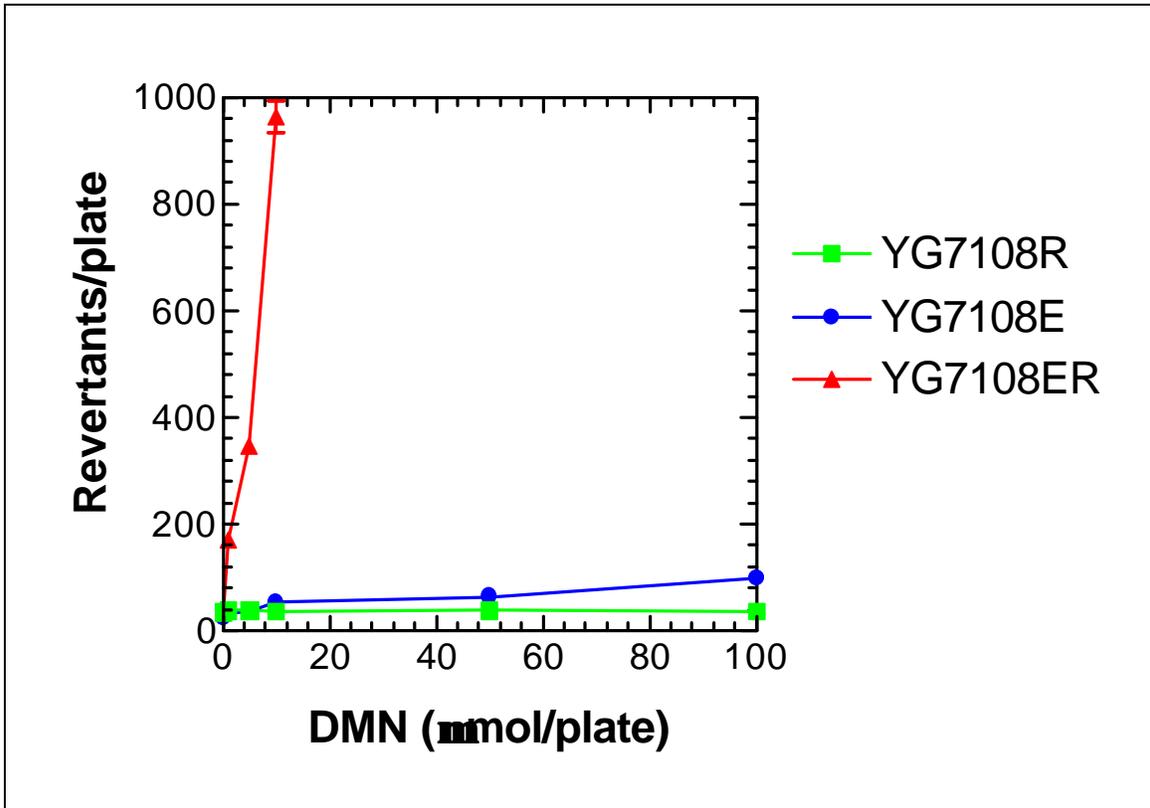


Figure 6: Both CYP2E1 and P450 reductase must be present simultaneously in order for YG7108 to be sensitive to DMN mutagenicity. Squares, YG7108R; circles, YG7108E; triangles, YG7108ER. Data points are the mean of three triplicate experiments; error bars indicate standard error. Like YG7108R, strains YG7108 and YG7108V (not shown) were also insensitive to DMN mutagenicity.

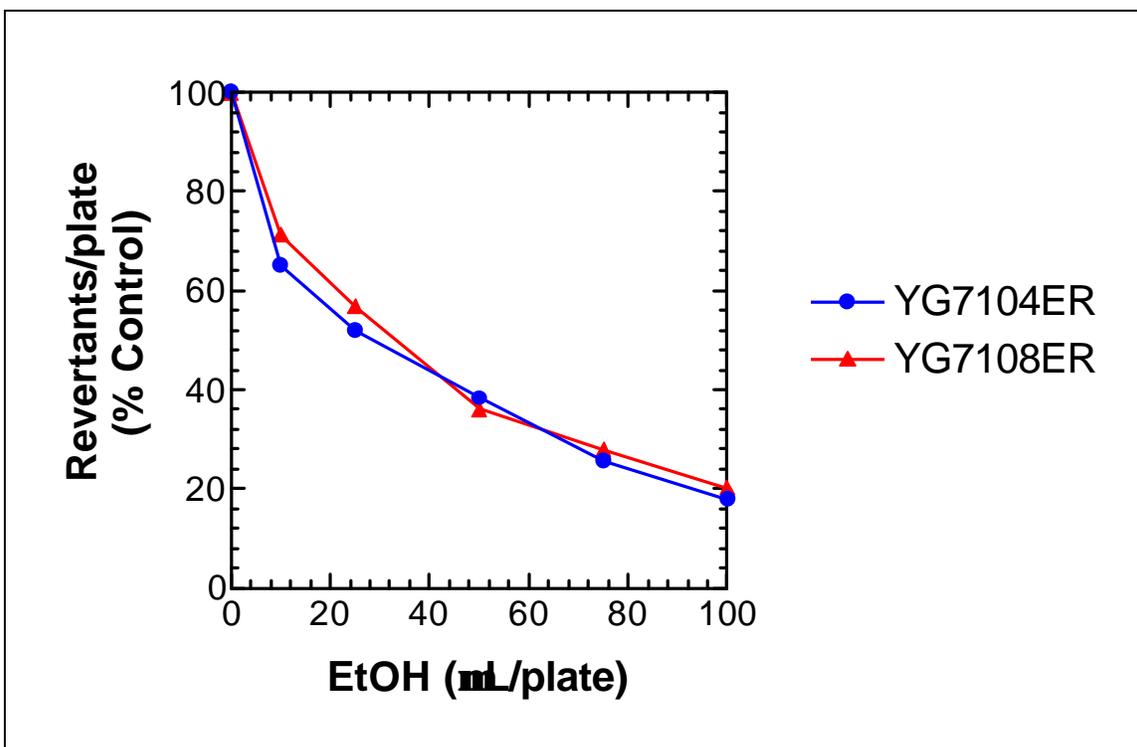


Figure 7: Ethanol inhibition of DMN mutagenicity in CYP2E1 and P450 Reductase coexpressing strains. Squares, YG7104ER; triangles, YG7108ER. Data points are the mean of three triplicate experiments; error bars indicate standard error. Data are represented as percentage of control response (50 μ mol DMN for YG7104ER, 363 revertants/plate; 5 μ mol DMN for YG7108ER, 355 revertants/plate) in the absence of ethanol. Bacteria were incubated for 15 minutes with ethanol at 37° C prior to addition of DMN.

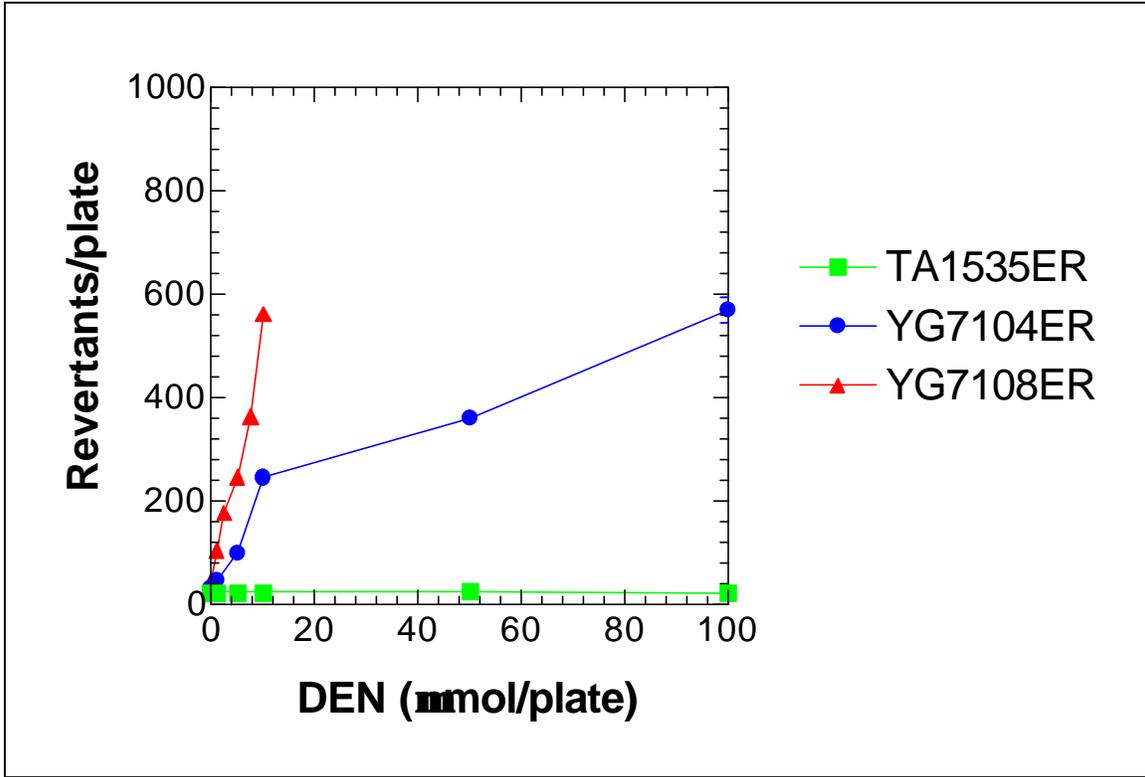


Figure 8: DEN mutagenicity in CYP2E1 and P450 reductase coexpressing strains. Squares, TA1535ER; circles, YG7104ER; triangles, YG7108ER. Data points are the mean of three triplicate experiments; error bars indicate standard error.

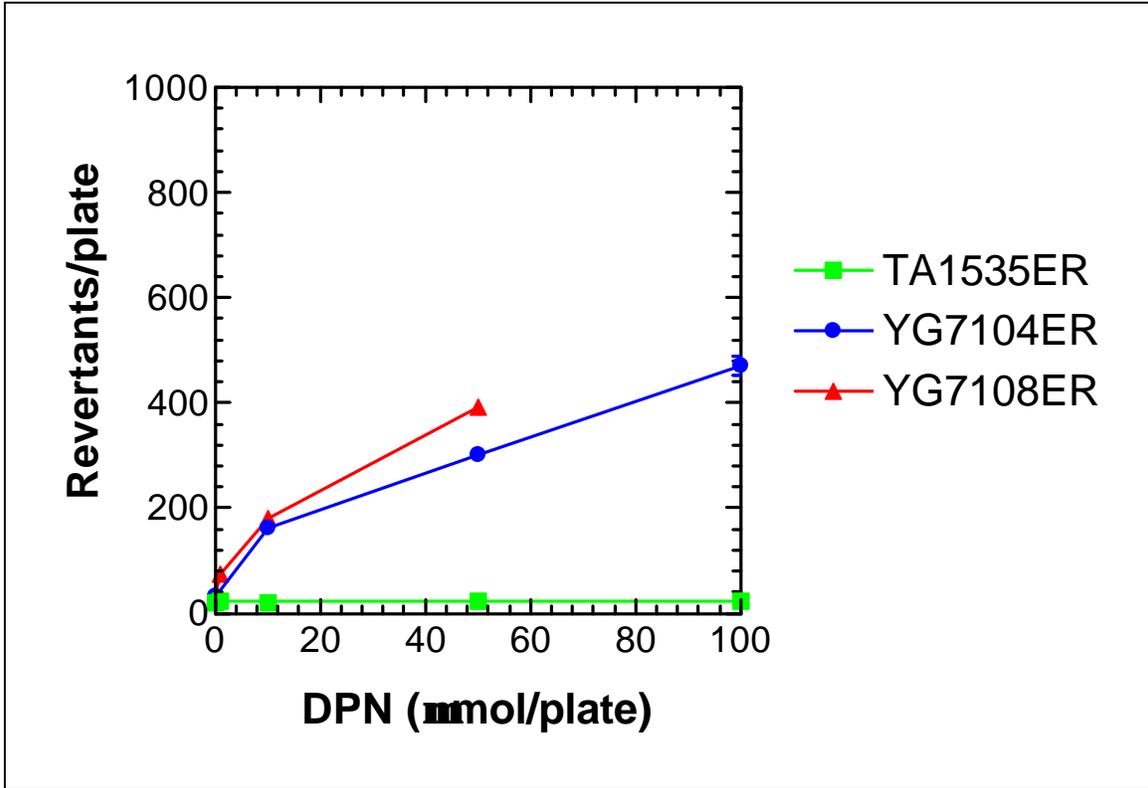


Figure 9: DPN mutagenicity in CYP2E1 and P450 reductase coexpressing strains. Squares, TA1535ER; circles, YG7104ER; triangles, YG7108ER. Data points are the mean of three triplicate experiments; error bars indicate standard error.

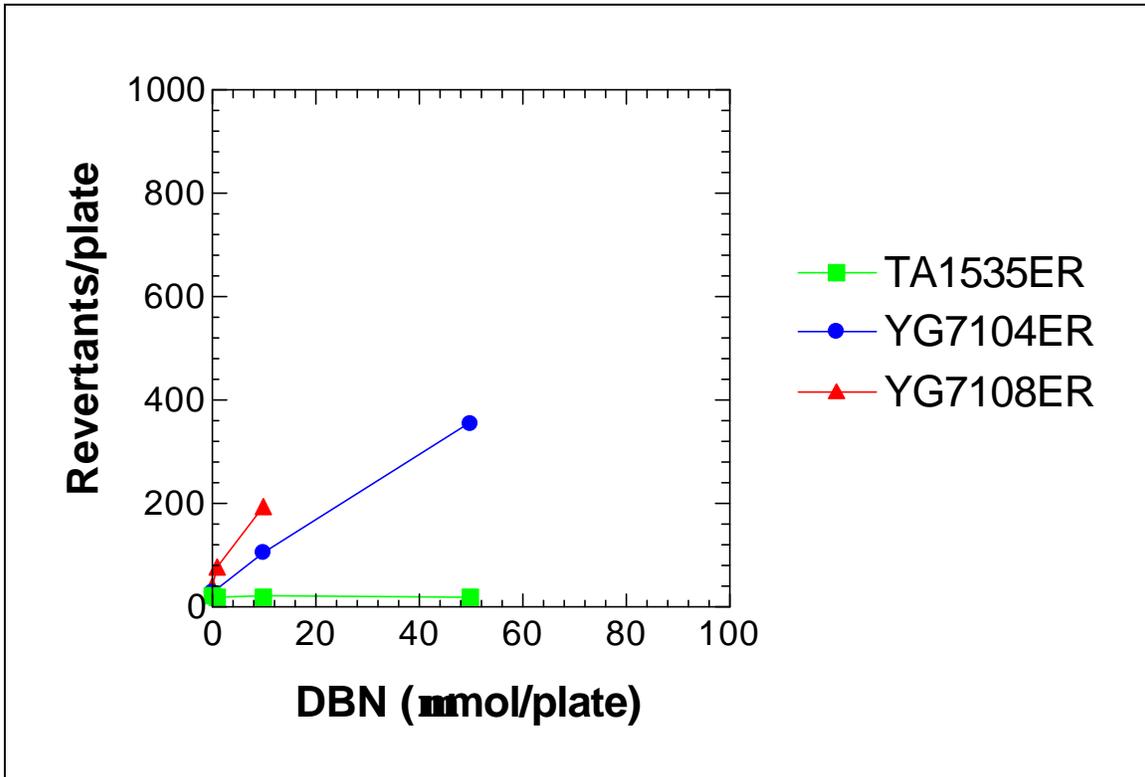


Figure 10: DBN mutagenicity in CYP2E1 and P450 reductase coexpressing strains. Squares, TA1535ER; circles, YG7104ER; triangles, YG7108ER. Data points are the mean of three triplicate experiments; error bars indicate standard error.

CHAPTER THREE

Influence of Cytochrome b₅ on the CYP2E1-Mediated Mutagenicity of Nitrosamines

RATIONALE

Cytochrome b₅ is a ubiquitous, 17 kDa electron transfer protein capable of accepting and transferring a single electron at a redox potential of 20 mV. Cytochrome b₅ is found in all tissues, except erythrocytes, in a membrane-bound form. It provides electron-reducing equivalents that drive a number of reactions including fatty acid desaturation, fatty acid elongation, plasmalogen biosynthesis, cholesterol biosynthesis, and CYP monooxygenations [96].

Despite years of research, the exact role of cytochrome b₅ in CYP reactions is only poorly understood. It has been shown that cytochrome b₅ can stimulate, inhibit, or have no effect on CYP substrate metabolism and that these activities depend on the specific isozyme, the substrate, and the assay conditions for any given reaction. It is thought that cytochrome b₅ stimulates most CYP reactions via donation of the second electron needed to drive the CYP monooxygenase cycle [97].

The previous chapter addressed the coexpression of CYP2E1 and CPR in methyltransferase-deficient strains of *Salmonella typhimurium*, resulting in two bacterial strains sensitive to the mutagenicity of small molecular weight nitrosamines [98]. Given the often-ambiguous role of cytochrome b₅ in CYP metabolism, the newly developed human CYP2E1/CPR coexpressing bacterial strains presented a unique opportunity to examine the influence of cytochrome b₅ on a specific CYP2E1-mediated reaction in a physiological system.

The CYP2E1-mediated metabolism of dialkylnitrosamines is of particular interest given the ubiquitous presence and widespread exposure of nitrosamines to the human population [49, 50]. Nitrosamines are not intrinsically mutagenic but must be activated by liver enzymes such as CYP2E1 or CYP2A6 in order to react with DNA [62]. Small

molecular weight, symmetric dialkylnitrosamines are commonly metabolized in this manner [52].

This chapter describes the construction of two methyltransferase-deficient *S. typhimurium* strains that express the full-length genes for human CYP2E1 and its electron-transfer partners, CPR and cytochrome b₅. By coexpressing cytochrome b₅ with CYP2E1 and CPR, I was able to demonstrate dialkylnitrosamine mutagenicity in a system even more sensitive than previous CYP2E1/CPR coexpressing systems or assays that employ exogenous activating enzymes (S9 fraction).

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study are described in Table 7. Dr. T. Nohmi (National Institute of Health Sciences, Tokyo, Japan) graciously provided *S. typhimurium* strains YG7104 (*ogt*-) and YG7108 (*ogt*-, *ada*-). The pIN3ER plasmid for human CYP2E1 and rat CPR expression was created as described elsewhere [78]. Human cytochrome b₅ cDNA was kindly provided by A. W. Steggle (Northeastern Ohio university). pIN3b₅ was created via insertion into the pIN3 vector multiple cloning site of a human cytochrome b₅ PCR fragment with XbaI and HindIII sites engineered upstream and downstream of cytochrome b₅ cDNA, respectively. This resulted in the removal of the ompA signal peptide and introduction of a full-length, human cytochrome b₅ cDNA immediately downstream of the *lpp-lac* promoter. pIN3b₅ER (Figure 11) was created by PCR amplification of the CPR cDNA from pIN3ER with a forward primer which introduced a HindIII site upstream of the CPR cDNA. This fragment was then digested with HindIII and inserted into the HindIII site immediately upstream of the cytochrome b₅ cDNA in pIN3b₅. The newly formed pIN3Rb₅ was then linearized with XbaI. The CYP2E1 cDNA was then removed from pIN3ER with XbaI and inserted upstream of the CPR and cytochrome b₅ cDNAs in the linearized pIN3Rb₅. pIN3ERb₅ (Figure 11) was created by amplifying the cytochrome b₅ cDNA from pIN3b₅ via overlap

extension PCR in the presence of CPR cDNA. The PCR fragment was then digested with NheI and HindIII and inserted into the pIN3ER vector linearized with NheI and HindIII.

Plasmids were introduced into the bacterial strains via electroporation. Fresh transformants were selected for each mutagenicity assay by growth on Luria-Bertani nutrient agar plates containing 100 µg ampicillin/mL.

Analysis of Enzyme Expression

Bacterial membranes were prepared as previously described [79]. Immunoblots confirmed the expression of CYP2E1 and CPR in YG7108ER (Figure 3), YG7108b5ER (Figure 12), and YG7108ERb5 (Figure 12). Cytochrome b₅ expression was confirmed in strains YG7108b5ER and YG7108ERb5 via immunoblotting as well (Figure 13). Proteins were separated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The immunoblots were probed with sheep polyclonal antibody to rabbit CYP2E1, sheep polyclonal antibody to rabbit CPR (kindly provided by Dr. Minor Coon, Department of Biological Chemistry, University of Michigan) and goat polyclonal antibody to rabbit cytochrome b₅ (Oxford Biomedical Research, Oxford, MI). CPR expression was also quantified by means of cytochrome *c* reduction (assuming specific activity of 3 µmol/min/mL = 1 nmol cytochrome P450 reductase/mL) [80]. Values are given in Table 8. Spectral quantification and enzymatic activity of CYP2E1 and cytochrome b₅ could not be reliably quantified in whole cells or membrane isolates (Appendix A).

Mutation Assays

Reversion assays were performed as described [81]. Freshly transformed single bacterial colonies were picked and grown overnight in Oxoid Nutrient Broth No. 2 with 100 µg/mL ampicillin at 37°C and shaking at 250 rpm. Growth at lower temperatures had no effect on dialkylnitrosamine mutagenicity (data not shown). Cultures were induced with 1 mM IPTG at 10 hours post-inoculation. Preincubation time, temperature and pH had

no effect on mutagenicity in assays that did not utilize S9 (data not shown). All assays were performed in triplicate. Revertant colonies were counted after 48 hours. See Appendix A for additional details on development of this assay.

RESULTS

Coexpression of CYP2E1 and CPR in *S. typhimurium*

Unfortunately, CYP2E1 expression was unable to be spectrophotometrically quantified in the YG7108ER, YG7108b5ER, and YG7108ERb5 strains (Appendix A). Spectrophotometric determination of cytochrome b₅ was also inconclusive given the high background absorbance of other bacterial cytochromes. Immunoblots, however, confirmed the expression of CYP2E1 and CPR in strains YG7108ER (Figure 3), YG7108b5ER (Figure 12), and YG7108ERb5 (Figure 12). Cytochrome b₅ expression was also confirmed via immunoblotting in strains pIN3b5ER and pIN3ERb5 (Figure 13).

Cytochrome b₅-Expressing Strains are More Sensitive to Dialkylnitrosamine Mutagenicity

As described in Chapter Two, DMN was able to elicit a dose-dependent mutagenic response in the CYP2E1 and CPR coexpressing strain YG7108ER in the absence of an exogenous metabolic system [98]. The sensitivity to DMN dramatically increased when human cytochrome b₅ was expressed via insertion of the b₅ cDNA upstream of the ER fragment (Figure 14). When the b₅ cDNA was inserted downstream of the ER fragment, DMN mutagenicity increased only slightly.

DEN mutagenicity closely resembled that of DMN with YG7108b5ER being the most sensitive and YG7108ERb5 being only slightly more sensitive to the mutagen (Figure 15). Strain YG7108b5ER was also more sensitive than YG7108ERb5 to DPN (Figure 16) and DBN (Fig. 17) mutagenicity, with both b₅-expressing strains remaining more sensitive than the non-b₅-expressing strains.

DISCUSSION

There have been many recent advances in the creation of short-term, *in vitro* genotoxicity assays utilizing heterologously expressed human xenobiotic metabolizing enzymes [39]. Many bacterial-based systems have been created using expressed human biotransformation enzymes [82-90]. Each of these systems has shown sensitivity to test compounds without the need for S9.

In this chapter, the effect on nitrosamine mutagenicity of coexpressing human cytochrome b₅ with human CYP2E1 and rat CPR was examined. The coexpression of human cytochrome b₅ into the established YG7108 strains expressing human CYP2E1 and rat CPR presented ideal opportunity to examine the effect of this enzyme *in vivo* on nitrosamine metabolism. Most studies scrutinized the role of cytochrome b₅ in *in vitro* models. For instance, purified rat liver cytochrome b₅ added to human TK-143 microsomes coexpressing human CYP2E1 and CPR has been shown to lower the K_m of DMN demethylation from 31 μM to 22 μM and increase V_{max} 2.2-fold [99]. Complementary studies have shown that K_m values are increased and V_{max} values are decreased for 7-ethoxycoumarin O-deethylation when b₅ is omitted from the NADPH-supported CYP2E1-reconstituted systems [100]. This effect is also observed with DMN and DEN in reconstituted rat CYP2E1 systems supplemented with rabbit cytochrome b₅ [67].

This chapter has demonstrated that *S. typhimurium* strains YG7108b5ER and YG7108ERb5 express cytochrome P450 2E1, CPR, and cytochrome b₅ simultaneously and that the presence of cytochrome b₅ greatly increases nitrosamine mutagenicity in these cells. The mutagenicity of all nitrosamines investigated occurred in a dose-dependent fashion without the addition of S9. Moreover, this mutagenicity was inhibited by ethanol, a potent inhibitor of CYP2E1. Thus, these results support the view that addition of intracellular cytochrome b₅ can increase the CYP2E1-mediated metabolism of dialkyl nitrosamines *in vivo* and cause mutation within the cell. Nearly identical results were obtained when coexpressing cytochrome b₅ with CYP2E1 and CPR in the YG7104 (*ogt*-) tester strain (data not shown).

The genotoxic potency of the nitrosamines in all strains was DMN>DEN>>DPN>DBN. The magnitude of the cytochrome b₅ mutagenic enhancement decreased with increasing alkyl chain length. The augmentation of CYP2E1-mediated nitrosamine metabolism has been shown *in vitro* to diminish with longer alkyl side chains [101].

This chapter is complementary to other recent work coexpressing cytochrome b₅ with human P450s and P450 reductase [102]. The development of these coexpression systems is a valuable tool in investigating the role of cytochrome b₅ *in vivo*. In addition, the creation of the bacterial tester strains used in this study provides researchers with extremely sensitive tools for studying CYP2E1-mediated mutagenicity and adds to a growing body of evidence that demonstrates that recombinant Ames strains are viable alternatives to exogenous mammalian enzyme preparations for mutagen activation [82, 88, 89, 94].

In conclusion, I have demonstrated that human cytochrome b₅ dramatically increases the sensitivity to dialkylnitrosamine mutagenicity of YG tester strains by coexpressing human CYP2E1, CPR and cytochrome b₅ simultaneously within the cell.

TABLE 7. Bacterial strains and plasmids used in this study				
Strain or Plasmid	<i>Characteristics</i>	Spontaneous Revertants	<i>Reference</i>	
<i>S. typhimurium</i>				
YG7108	<i>hisG46 rfa uvrB ogt_{ST}::Cm^r ada_{ST}::Km^r</i>	40-45	[71]	
YG7108ER	YG7108 with plasmid pIN3ER	45-55	[98]	
YG7108b ₅	YG7108 with plasmid pIN3b ₅	35-45	[103]	
YG7108b ₅ ER	YG7108 with plasmid pIN3b ₅ ER	45-55	[103]	
YG7108ERb ₅	YG7108 with plasmid pIN3ERb ₅	45-55	[103]	
Plasmids				
pIN3	PINIIIompA3; a pBR322-based expression vector with cleavable ompA signal peptide upstream of the cloning site		[95]	
pIN3ER	PINIIIompA3-based expression vector with cleavable ompA signal peptide upstream of the human CYP2E1 and rat CPR cDNAs		[98]	
PIN3b ₅	pINIIIompA3-based expression vector, with the ompA signal peptide removed, containing the human cytochrome b ₅ cDNA		[103]	
PIN3b ₅ ER	pIN3ER-based plasmid with human cytochrome b ₅ cDNA inserted upstream of the CYP2E1 and CPR cDNAs		[103]	
PIN3ERb ₅	pIN3ER-based plasmid with human cytochrome b ₅ cDNA inserted downstream of the CYP2E1 and CPR cDNAs		[103]	

TABLE 8. CPR Quantitation	
<u>Strain</u>	<u>P450 Reductase Content^a</u>
YG7108ER	38 ± 3
YG7108b ₅ ER	45 ± 2
YG7108ERb ₅	40 ± 3

^aUnits are nmol/L, means ± standard deviations; n = 3.

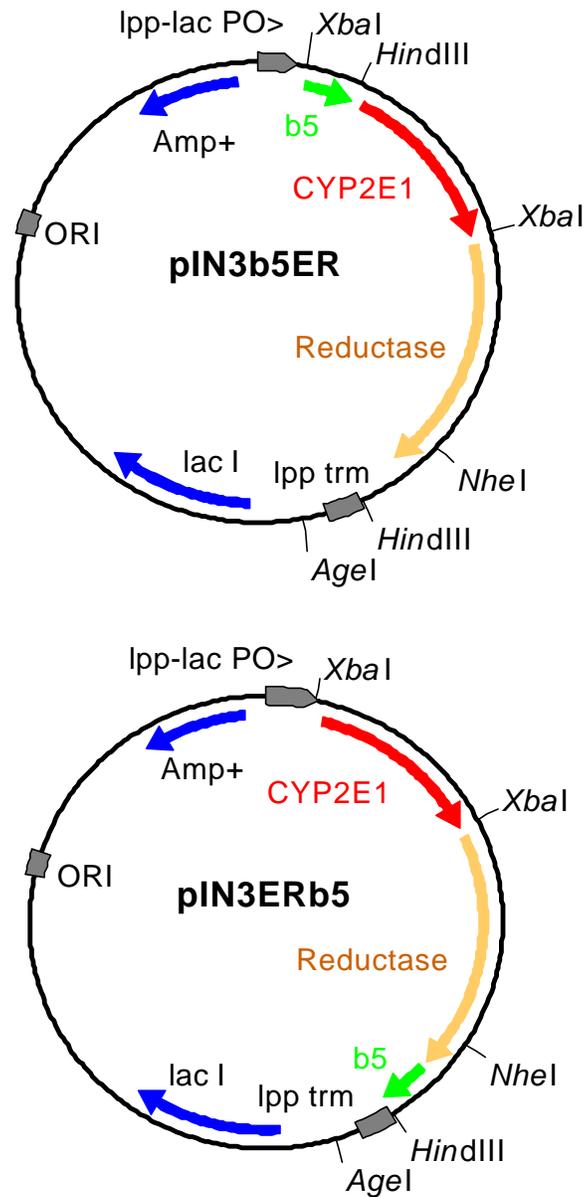


Figure 11: Plasmid maps of pIN3b5ER and pIN3ERb5. These 11-kb plasmids contain cDNAs for human cytochrome b5, human CYP2E1, and rat CPR on the pINIIIompA3 expression vector.[95] Expression is driven by the *lpp-lac* fusion promoter (*lpp-lac PO>*), under control of the *lacI* repressor protein, and thus is inducible by IPTG. *Amp⁺* encodes β -lactamase for ampicillin-resistance; *ORI* represents the plasmid origin of replication; and *lpp trm* is the transcription terminator sequence from the *lpp* gene.

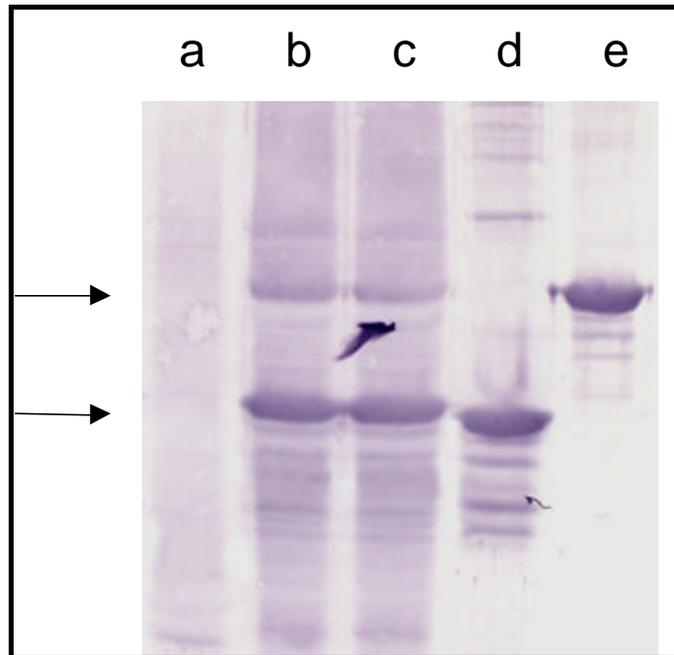


Figure 12: Immunoblot of CYP2E1 (lower arrow) and CPR (upper arrow) expression in bacterial membrane fractions of the YG7108 strains. (a) YG7108; (b) YG7108b5ER; (c) YG7108ERb5; (d) purified CYP2E1; (e) purified CPR. Lanes containing membrane fraction were loaded at 100 μ g of total protein per lane.

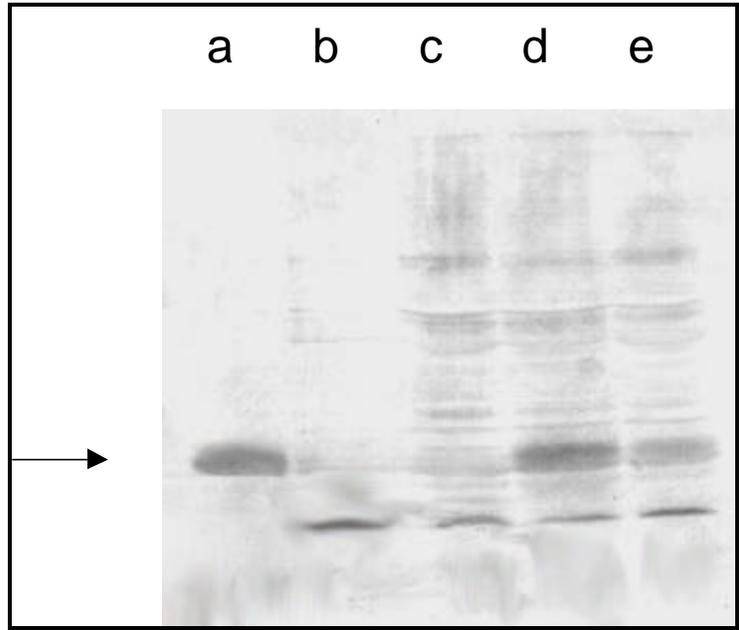


Figure 13: Immunoblot of cytochrome b5 expression (arrow) in bacterial membrane fractions of the YG7108 strains. (a) Purified cytochrome b5; (b) YG7108; (c) YG7108ER; (d) YG7108b5ER; (e) YG7108ERb5. Lanes containing membrane fraction were loaded at 120 μ g of total protein per lane.

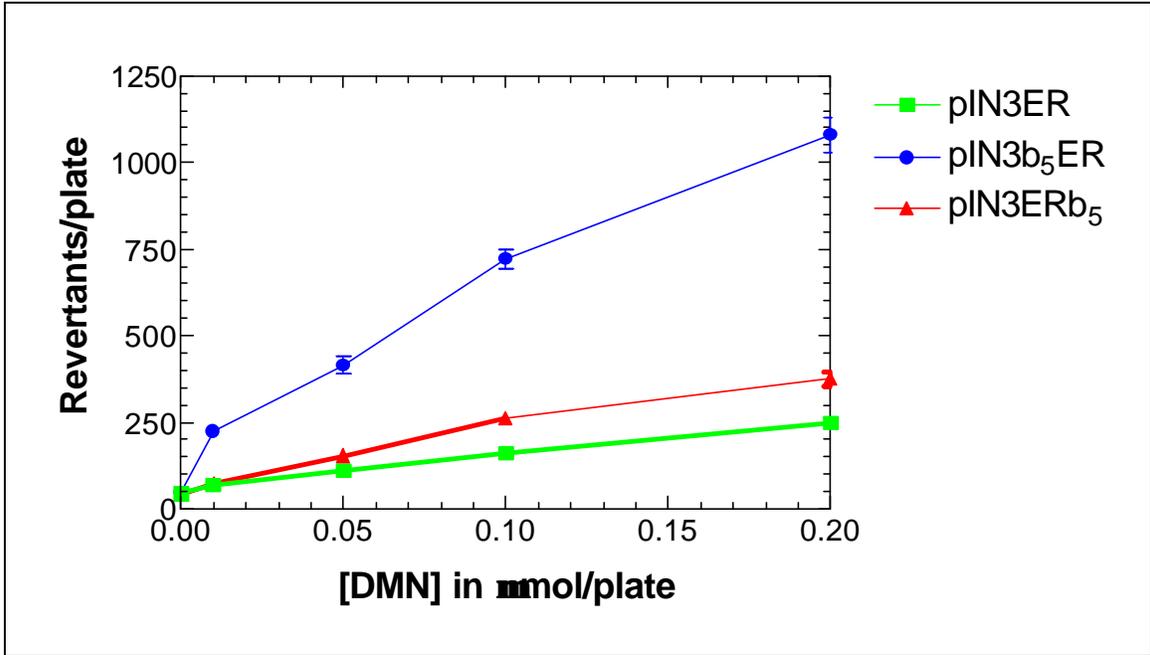


Figure 14: Effect of cytochrome b5 expression on DMN mutagenicity in YG7108 tester strains. Squares, pIN3ER; circles, pIN3b₅ER; triangles, pIN3ERb₅. Data points are the mean of three triplicate experiments; error bars indicate standard error.

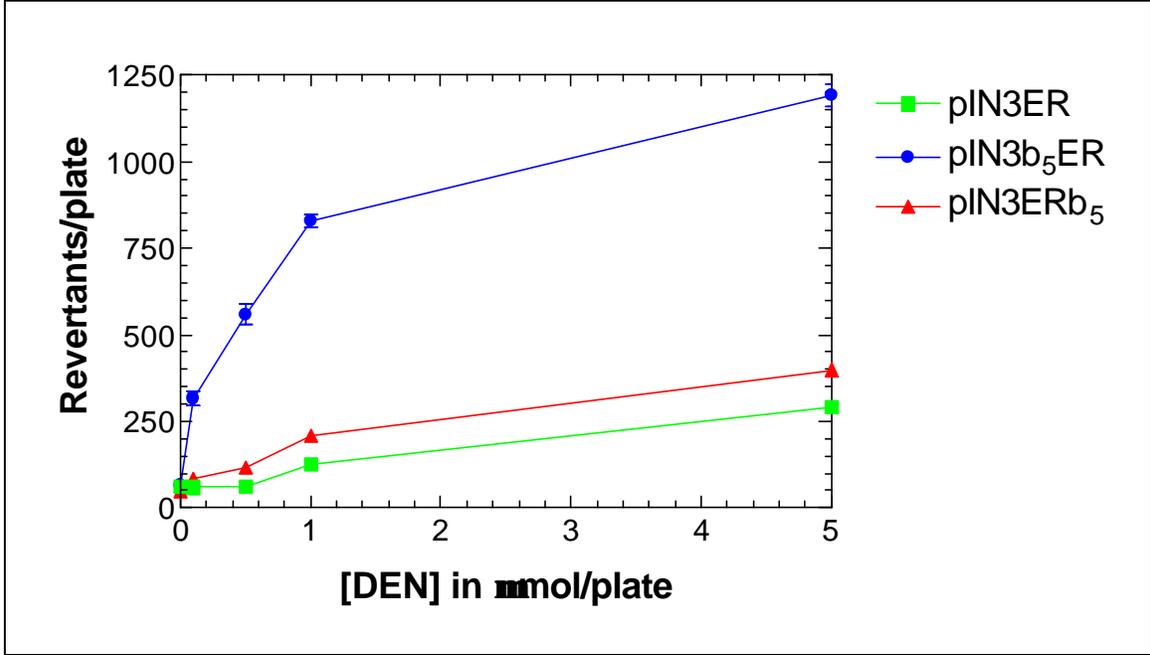


Figure 15: Effect of cytochrome b5 expression on DEN mutagenicity in YG7108 tester strains. Squares, pIN3ER; circles, pIN3b₅ER; triangles, pIN3ERb₅. Data points are the mean of three triplicate experiments; error bars indicate standard error.

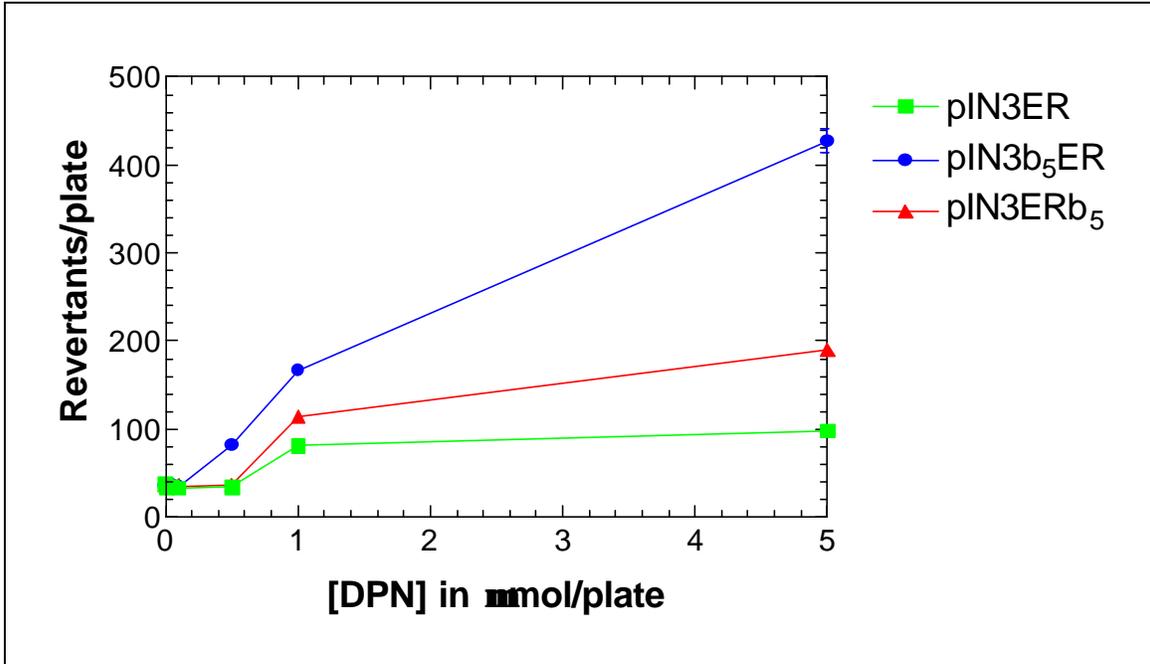


Figure 16: Effect of cytochrome b5 expression on DPN mutagenicity in YG7108 tester strains. Squares, pIN3ER; circles, pIN3b₅ER; triangles, pIN3ERb₅. Data points are the mean of three triplicate experiments; error bars indicate standard error.

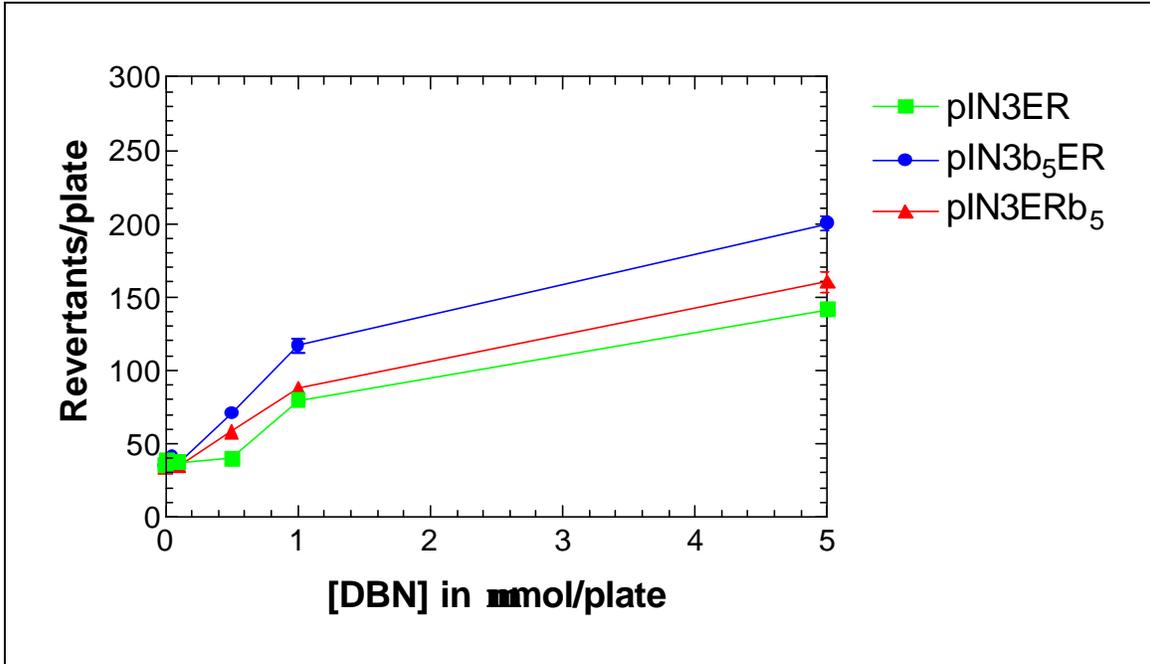


Figure 17: Effect of cytochrome b5 expression on DBN mutagenicity in YG7108 tester strains. Squares, pIN3ER; circles, pIN3b₅ER; triangles, pIN3ERb₅. Data points are the mean of three triplicate experiments; error bars indicate standard error.

CHAPTER FOUR

Summary and Conclusions

The research presented in this dissertation has focused on three particular objectives:

- 1. Express human CYP2E1 and CPR in an appropriate bacterial tester strain for nitrosamine mutagenicity assessment.**
- 2. Determine the mutagenic potency of dimethylnitrosamine, diethylnitrosamine, dipropylnitrosamine and dibutylnitrosamine in the CYP2E1/CPR coexpressing tester strains.**
- 3. Examine the effect of cytochrome b₅ coexpression on nitrosamine mutagenicity in the CYP2E1/CPR coexpressing tester strains.**

Objective #1: Express human CYP2E1 and CPR in an appropriate bacterial tester strain for nitrosamine mutagenicity assessment.

Summary and Conclusion. The difficulty in assessing the mutagenic risk of nitrosamines in humans using bacterial-based assays can be chiefly attributed to many issues, chief among them: (a) the lack of nitrosamine-metabolizing enzymes homologous to human CYP2E1 in bacterial tester strains; and (b) the lack of bacterial tester strains responsive to the ultimate mutagen in nitrosamine metabolism. The simultaneous expression of full-length human CYP2E1 with full-length rat CPR in methyltransferase-null bacterial tester strains created a system in which the mutagenic potential of nitrosamines could be assessed and quantified. The expression of CYP2E1 and CPR was detected in membrane fractions by immunoblot. The immuno-detected proteins migrated in SDS-PAGE at the same rate as their respective purified standards. The enzymatic activity of CYP2E1 in recovered membranes was shown to be 18 and 17 nanomoles per liter of cells in strains YG7104ER and YG7108ER, respectively. The amount of

cytochrome c reduction in YG7104ER membranes corresponded to 37 nanomoles of CPR per liter of cells, while YG7108ER revealed 36 nanomoles of CPR per liter, confirming the expression of enzymatically active, heterologous CPR within the tester strains.

The mutagenicity of DMN was dependent upon the presence of both CYP2E1 and CPR (either at high levels in ethanol-induced rat liver S9, or expressed within the cell) in the methyltransferase-deficient bacterial tester strains. Native tester strains (*ogt+/ada+*) alone or in the presence of Aroclor 1254-induced S9, or tester strains with vector only or CPR only were not sensitive to DMN mutagenicity. Methyltransferase-null tester strains expressing CYP2E1 only were weakly responsive. Furthermore, the mutagenicity observed with DMN in strains YG7104ER and YG7108ER was inhibited by ethanol, a potent inhibitor of CYP2E1. These observations not only strongly support the requirement for methyltransferase-deficient tester strains for assessing nitrosamine mutagenicity, but also show that nitrosamine mutagenicity can be sensitively assessed in strains simultaneously expressing of both CYP2E1 and CPR without the need for an exogenous metabolic activating system.

Objective #2: Determine the mutagenic potency of dimethylnitrosamine, diethylnitrosamine, dipropylnitrosamine and dibutylnitrosamine in the CYP2E1/CPR coexpressing tester strains.

Summary and Conclusion. The double-deletion mutant YG7108ER (*ogt-* and *ada-*) was approximately ten-fold more sensitive to DMN mutagenicity than the single-deletion mutant YG7104ER (*ogt-*), reaching roughly 350 revertants/plate at 5 micromoles and 50 micromoles of DMN, respectively. Similar sensitivity was shown with nitrosamines of increasing alkyl chain length. Overall, the genotoxic potency of the nitrosamines in both YG7104ER and YG7108ER was DMN > DEN >> DPN > DBN. Not surprisingly, this is the same order (methyl > ethyl >> propyl > butyl) observed when examining the specificity and efficiency of the *ogt* and *ada* enzymes for the repair of alkylated DNA

adducts [71, 104]. This work reinforces the need for methyltransferase-deficient tester strains for the highly sensitive assessment of the mutagenicity of alkylating agents.

Objective #3: Examine the effect of cytochrome b₅ coexpression on nitrosamine mutagenicity in the CYP2E1/CPR coexpressing tester strains.

Summary and Conclusion. I have demonstrated that expressing cytochrome b₅ in YG7104ER and YG7108ER increases the sensitivity of these strains to nitrosamine mutagenicity. It has been shown *in vitro* elsewhere that cytochrome b₅ can decrease the K_m of CYP2E1-mediated DMN demethylation from 31 micromolar to 22 micromolar, while increasing V_{max} 2.2-fold [99]. Figure 18 graphically demonstrates the impact of cytochrome b₅ expression on DMN mutagenicity in the YG7108 methyltransferase-null *S. typhimurium* tester strain. Strain YG7108b5ER is approximately 100-fold more sensitive to nitrosamine mutagenicity than its cytochrome b₅-deficient YG7108ER counterpart. Strains YG7108ER and 7108b5ER also have a much lower spontaneous reversion rate than their YG7108 parent strain in the presence of ethanol-induced S9, imparting a much higher degree of resolution in assays utilizing these strains. This high degree of resolution is essential when examining low doses or low potency mutagens. The findings in chapter three provide further *in vivo* evidence of the significant impact of cytochrome b₅ on CYP2E1-mediated DMN metabolism using a physiological outcome.

Significance of this Work

The creation of short-term, *in vitro* genotoxicity assays utilizing heterologously expressed human xenobiotic metabolizing enzymes has been of great interest in recent years. With the advent of genomics and synthetic combinatorial chemistry, the number of new chemical entities being created is increasing at an amazing rate. Each of these substances will need to be evaluated for safety in order to be approved for use in humans. To effectively assess the risk associated with these new entities, reliable predictive assays are essential. The low cost, speed, reproducibility, flexibility, robustness, and sensitivity of bacterial-based assays make them ideally suited for this task. As the use of these assays

increase, the need for test systems not reliant upon exogenous sources of mammalian liver preparations becomes imperative. This dissertation describes an assay that can assist in fulfilling this need. The assays described herein accurately and sensitively predict the mutagenic potency of a traditional problematic class of compounds in a highly reproducible fashion.

Although the most obvious impact of this new assay is its utility in establishing the mutagenic potential of chemical entities, its other uses may result in a more profound impact on science.

Potential Uses for the Described Assay

Metabolism studies. While the work described herein was limited to some small molecular weight dialkyl nitrosamines, its use could easily extend to the mutagenicity and cytotoxicity assessment of other CYP2E1 substrates. CYP2E1 substrates comprise a myriad of small molecular weight substances. Such compounds include acetaminophen, acetaldehyde, benzene, styrene, 1,1,1-trichloroethane, 1,2-dichloropropane, carbon tetrachloride, chloroform, ethylene dibromide, ethylene dichloride, halothane, methylchloride, methylene dichloride, vinylchloride, and trichloroethylene, most of which are hepatotoxic [105].

One benefit of this assay is its ability to measure *in vivo* metabolism rates using a quantifiable physiologic outcome (bacterial mutation frequencies). Given the high resolution observed with the CYP2E1/CPR/Cyt b₅ assay described earlier, it is reasonable to assume investigators could apply this established system to examine other cytochromes P450 that are effected by the presence of cytochrome b₅ (*e.g.* 1A2 and 3A4). The exact manner in which cytochrome b₅ effects the different P450 enzymes remains unclear [97]. There is some dispute concerning whether the assistance afforded by cytochrome b₅ is purely via allosteric effects on CYP and CPR protein stability, or if the electron-transferring capabilities of cytochrome b₅ play a role. Examining CYP/CPR/holo b₅ versus CYP/CPR/apo b₅ strains would enable an investigator to address the nature of these interactions in more detail.

The role of cytochrome b₅ could also be further examined by coexpressing a P450 enzyme in combinations with CPR, cytochrome b₅, and cytochrome b₅ reductase. Cytochrome b₅ reductase catalyzes the reduction of two molecules of cytochrome b₅ using NADH as the physiological electron donor. A strain coexpressing CYP, cytochrome b₅, and cytochrome b₅ reductase would metabolize substrates in a purely NADH-driven manner in contrast to the NADPH-driven metabolism seen in the CYP/CPR/cyt b₅ strains. Comparing the mutation rates of two such tester strains at a given mutagen concentration would provide insight into the contribution of each of these nucleotide reductants.

An investigator could also use the described assay as a positive selection mechanism in targeted random mutagenesis studies of the CYP/CPR/cytochrome b₅/cytochrome b₅ reductase enzymatic system. Targeted random mutagenesis (*id est*, the use of ambiguously synthesized oligonucleotide sequences to generate random amino acid sequences in proteins), or “directed evolution,” is an increasingly valuable tool in molecular biology [106, 107]. However, the sheer number of possible individual proteins that can be produced even when randomizing small oligonucleotide sequences can severely limit the practicality of this potentially powerful tool of molecular biology. In order to limit the number of useful clones generated by this process, an investigator requires effective positive and negative selection pressures. The assay described herein could be useful in such investigations since the bacteria require a metabolic event resulting in chemical mutagenicity in order to proliferate. Negative selection pressure exists in the form of histidine-deficient media, *id est*, metabolically competent enzymes are required in the assay to activate mutagens and revert the bacteria to histidine prototrophy. The promutagen provides the positive selection pressure. By adjusting the levels of promutagen present in the assay, one can select for clones expressing enzymes with increased metabolic rates.

For example, an investigator would first choose a section of the CYP (or CPR, or cytochrome b₅, or cytochrome b₅ reductase) enzyme to be randomized. Targeted

randomized mutagenesis followed by whole plasmid amplification would create a large number of mutant plasmids each with an individual, random sequence. Most mutations, presumably, would result in a random protein with diminished functionality (when compared to the native sequence) or a total loss of function. However, a small number of the random proteins may have increased metabolic activity. These clones could be selected via transfection of the randomized plasmids into the bacterial tester strain followed by treatment with relatively low concentrations of promutagen. If the investigator exposed the bacteria with the highest possible no observable effect dose and the assay yielded more revertants than native enzyme-expressing controls at a similar dose, one could assume that the revertants contained random forms of the enzyme capable of metabolizing the mutagen more readily than its native form.

Similarly, an investigator could use this methodology as a restoration-of-function assay. For instance, if a known alteration in the nucleotide sequence of a protein knocks-out enzyme activity, one could randomize the sequence in the effected region, generate random plasmids as outlined above, transfect the tester strains with the random plasmids, and then dose the strains at a known mutagenic concentration of a chemical mutagen. The mutagen provides positive selection pressure on the system and only those mutations that restore function will metabolize the mutagen, thus growing in the histidine-selective agar.

Characterization of polymorphisms. A number of specific CYP enzymes, including CYP2E1, are expressed in several different forms in the population [108]. Some forms of a given enzyme often vary from the native nucleotide sequence at a single allele. Single nucleotide polymorphisms (SNPs) are capable of exhibiting vastly different pharmacokinetic characteristics than the native form. Using this assay, the metabolic differences of these different polymorphic forms could be examined and quantified in a physiological system. Indeed, this technique has already been utilized to examine the effects of CYP2A6 polymorphisms on the susceptibility of Japanese populations to nitrosamine-mediated cancer susceptibility [87].

Characterization of antioxidants. In addition to determining the mutagenic potential of CYP-metabolized compounds, the assays described in this work could be used to assess and quantify the protective effects of antioxidants or other inhibitors of the mutagenicity process. Of particular interest to this work, ascorbic acid, and green tea, which contains many uncharacterized polyphenolic antioxidants, have been shown to inhibit the mutagenic and carcinogenic potential of nitrosamines [109-111].

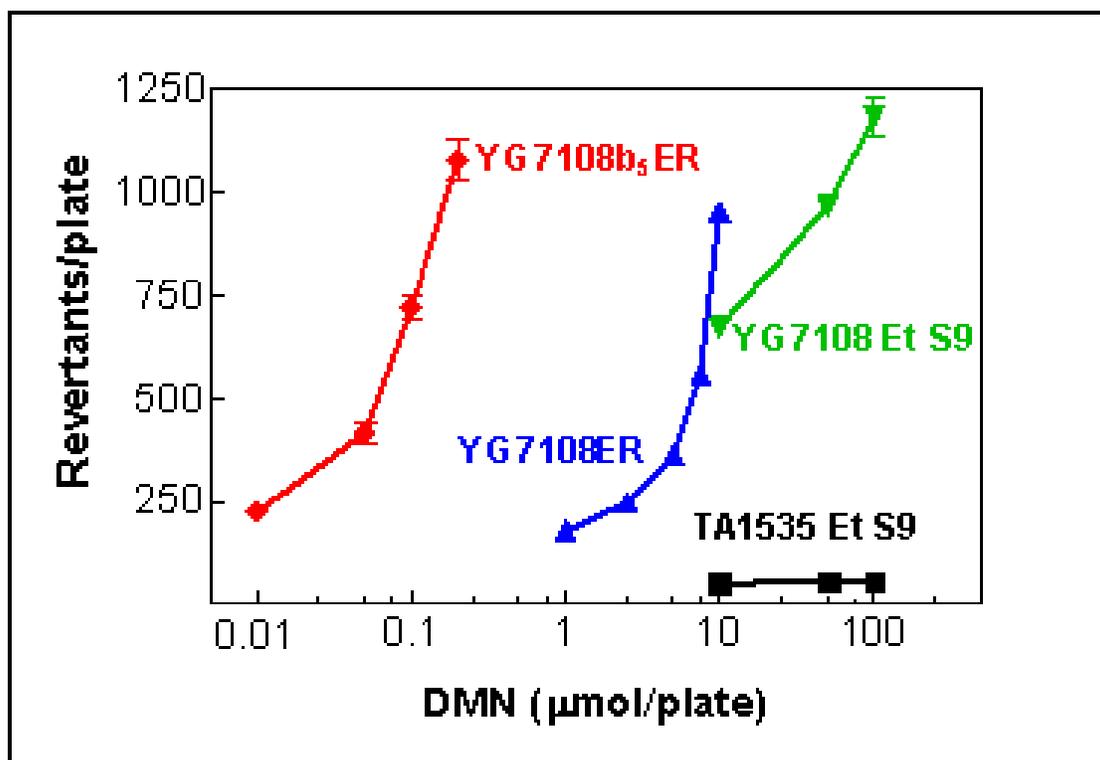


Figure 18: The effect of different exogenous and heterologously expressed metabolic activating systems on DMN mutagenicity in YG7108 tester strains. Squares, TA1535 with ethanol-induced S9; inverted triangles, YG7108 with ethanol-induced S9; triangles, YG7108ER with no exogenous metabolic activating system; diamonds, YG7108 with no exogenous metabolic activating system. Data points are the mean of three triplicate experiments; error bars indicate standard error.

Appendix A

There is often a great deal of “unsuccessful” experimental results that go unreported or underreported in scientific research. This phenomenon has seemingly increased in modern times due to two factors: 1) high impact research journals place a premium on reporting data that supports a hypothesis, rather than a null hypothesis; and, 2) the current competitive nature of scientific research strongly encourages investigators to publish their data in such journals. It is important to note that the lack of reporting these data in scientific journals does not lessen their potential impact on science. Indeed, sharing such data could only improve scientific endeavors by increasing research efficiency. If such information were readily available, “failed” experiments would be less likely to be repeated, thus saving valuable resources.

In an effort to give the reader a better understanding and appreciation of the assay development process required to produce the this body of work, I would like to give a brief overview of the plasmids, bacterial tester strains, and miscellaneous assay protocol variations which yielded bacterial assays not sensitive to nitrosamine mutagenicity.

Plasmids

Early in the development of a nitrosamine-sensitive bacterial-based mutagenicity assay, it was thought that the levels of CYP2E1 and CPR were rate-limiting within the cell. In order to address this issue, a number of plasmids (Table 9) were examined in widely used bacterial tester strains (Table 10) for their ability to express human CYP2E1 and rat CPR.

Strains utilizing the pJL2 vector were first examined for nitrosamine mutagenicity. The pJL2 vector is a high copy number plasmid derived from pKK223-3. pJL2 was created by insertion of a translational enhancer element and ribosome binding site between the *EcoR1* and *Pst1* sites of pKK223-3 and substitution of the pBR322 origin of replication with that from the pUC series of plasmids, to increase plasmid copy number [112]. The enhancer element is based on that of the bacteriophage T7 gene 10 element. The vector uses the *tac* promoter and thus expression is IPTG-inducible. Strains utilizing the pJL2

vector for CYP2E1 and CPR expression were not sensitive to nitrosamine mutagenicity. Additional efforts were made to increase CYP2E1 expression since, at the time, it was thought that CYP2E1 expression was rate-limiting in nitrosamine mutagenicity.

In addition to the vector above, a number of different expression plasmids were created using the pIN3 vector. pIN3 is a pBR322-derived plasmid that uses the *lpp-lac* promoter and is thus IPTG-inducible. pIN3 was created by introducing the outer membrane protein A (*ompA*) coding region into the cloning site [95]. Translation is highly efficient due to the use of the *ompA* translation initiation region (*ompA* is one of the most abundant proteins in bacteria) [113]. There are three cloning sites at end of the *ompA* signal peptide that can be used for cDNA insertion; however, the cDNA coding sequence must be in-frame with the signal peptide sequence. pIN4 is a high-copy version of pIN3 in which the pBR322 origin of replication is replaced by the origin from the pUC series of plasmids [79]. The expression of CYP2E1 and CPR in *E. coli* and *S. typhimurium* strains using these plasmid vectors is described elsewhere [79]: while expression of CYP2E1 and CPR was higher in the pIN4 strains (28 and 83 nmol/L, respectively) when compared to the pIN3 strains (17 and 48 nmol/L, respectively), pIN4-containing strains demonstrated a dramatic decrease in viability in *S. typhimurium* and were thus not suitable for use in mutagenicity assays. Bacterial strains must be able to reliably produce a viable “bacterial lawn” (a hazy bacterial film that is visibly present on selective media which indicates cell viability) in the assay in order to yield meaningful results. The integrity of this “lawn” is important to demonstrate that the cells are viable and thus able to mutate in response mutagen exposure.

After development of the YG tester strains described in Chapters 2 and 3, it was observed that the pIN3 expression vector yielded optimal results in the nitrosamine mutagenicity assays. The bacterial cells were viable, had a low spontaneous reversion rate, and were sensitive to nitrosamine mutagenicity.

Nitrosamine Insensitive Bacterial Tester Strains

Since the advent of the Ames test, a number of bacterial tester strains have been developed differing in species, genotype, and plasmid content (reviewed in Tables 1 and 2). Each strain exhibits its own specificity and selectivity towards mutagens. This is largely dependent upon the number and type of DNA repair enzymes that were deleted from their genome in an effort to create more sensitive tester strains. During the early development of the assay described in Chapter Two, a number of these strains were examined (Table 10). None of the strains listed in Table 9 were sensitive to nitrosamine mutagenicity in the presence of Aroclor 1254-induced S9 or when transfected with any of the plasmids described in Tables 5 and 7.

The insensitivity of the strains in Table 10 to nitrosamine mutagenicity emphasizes the critical role the *ogt* and *ada* methyltransferases have in nitrosamine mutagenicity. The strains listed in Table 10 are diverse in the number and type of genomic DNA repair enzyme deletions. The traditional Ames TA tester strains all carry the *uvrB* deletion. The *uvrB* deletion effects nucleotide excision repair by eliminating the protein which interacts with *uvrA* to form a dimer capable of detecting alterations in the DNA helix caused by DNA damage. *uvrB* also has endonuclease activity essential for excising the damaged DNA from the genome.

The *E. coli* WP2 strains also eliminate the endogenous excision DNA repair process via deletion of the *uvrA* protein, resulting in strains similar to the traditional Ames *S. typhimurium* TA strains. The WP2*hcr* strain has an additional deletion of *lon11*, an ATP-dependent serine protease capable of degrading proteins *umuD* and *umuC* which contribute to error-prone DNA repair in *E. coli*. WP67 varies from its WP2 parent by having an additional deletion of *polA1*. *polA1* codes for DNA polymerase A1 which has 5'-3' exonuclease proof-reading capability. The IC strains were also derived from WP2. The IC203 strain is similar to WP2, but lacks the *OxyR* gene. *OxyR* codes for a DNA binding transcription factor that activates the expression of antioxidant enzymes such as catalase, glutathione reductase, and alkyl hydroperoxidase. IC204 was derived from IC203 and has the *umuDC* deletion. IC206 was derived from IC204 and contains all of

its genomic deletions plus the deletion of *MutY* which codes for a glycosylase capable of repairing 8-oxoguanine lesions. Many of the aforementioned strains also carry the pKM101 plasmid, described earlier in Chapter One.

Despite the many genomic deletions of miscellaneous DNA repair enzymes, none of these strains were sensitive to nitrosamine mutagenicity, even when harboring the pIN3ER expression plasmid. This observation highlights the significance of the *ogt* and *ada* methyltransferases.

Assay Manipulations

As described in the Materials and Methods section of Chapters One and Two, the assay described in this work was unresponsive to several different assay manipulations. As is the case with most assay development, many different conditions and reagents were investigated during assay optimization.

One of the first difficulties encountered in developing the assays described in Chapters Two and Three, was the inefficient transformation of the tester strains. Several methodologies of chemical transformation utilizing calcium chloride and rubidium chloride were ineffectual in yielding transformants. Electroporation was necessary for transfecting the plasmids used in this work. The problems encountered with transforming the cells appeared to be cell line dependent. Typical results from electroporating 1 μ L of pIN3ER (10 ng DNA/mL) into 25 μ L of electrocompetent bacterial stock using a Gibco BRL Cell-Porator *E. coli* Pulser set on “medium” (2.5KV) would be 160 colonies/plate for the YG7104 strain and 80 colonies/plate for the YG7108 strain. It is also important to note that only freshly electroporated transformants behave well in the assay. Transformants that are cultured then frozen yield spurious results in the mutagenicity assay and lose their viability (i.e. they do not reliably produce a “bacterial lawn”). These observations are clearly linked to freezing the strains. Cultures that are continuously maintained (i.e. never frozen, but freshly inoculated each day into new media) experience upward drift in their spontaneous reversion rates in addition to losing

sensitivity to nitrosamines in the mutagenicity assay. These observations require freshly electroporated cells for each mutagenicity experiment.

It has been shown elsewhere that heterologous CYP expression in bacteria can be increased by lowering the culture temperature from 37° C to 30° C, extending culture times from 24 hours to 48 hours, and lowering the shaking speed during orbital incubation from 250 rpm to 150 rpm [114]. Reducing the temperature of the cultured YG pIN3ER cells to increase CYP2E1 expression had no impact on the sensitivity of these cells to nitrosamines, suggesting that CYP2E1 levels were not rate-limiting for mutagenesis. Extending culture times prior to the assay also had no impact on nitrosamine sensitivity; however, it resulted in an increase in the number of spontaneous revertants for each strain. Lowering the rotation rate of the cultures resulted in noticeably lower bacterial cell titers. Combining all three alterations to culture conditions resulted in cultures not appropriate for mutagenicity assays due to low cell titers and high than acceptable (2x normal) spontaneous revertant rates.

Other efforts to increase CYP2E1 and CPR expression in the YG tester strains proved to be detrimental to the integrity of the assay detailed in Chapters Two and Three. For instance, growing tester strains in liquid media other than Oxoid Nutrient Broth No. 2, including Luria-Bertani broth or Terrific broth, resulted in unacceptable spontaneous revertant rates. Increasing the concentration of antibiotics in the culture medium in an effort to select for cells with well-expressed plasmids had a negative effect on cell viability and did not increase the sensitivity of the YG tester strains to nitrosamine mutagenicity. Also, the addition of delta-aminolevulinic acid (ALA), a heme precursor, has been shown to increase the expression of some CYP enzymes in bacteria [115]; however, its addition to the culture media had no effect on nitrosamine mutagenicity.

It was observed that the optimal nitrosamine mutagenicity from the YG pIN3 tester strains was obtained from cultures that were induced with 1 mM IPTG 12 ± 2 hours after inoculation into Oxoid Nutrient Broth No. 2. Cultures induced prior to this time point

generally did not reach acceptable cell concentrations for the assay. Cultures induced after this period were less sensitive to nitrosamine mutagenicity.

Other investigators, especially those who use liver S9 fractions, have shown that reducing the pH of the preincubation mix or increasing the preincubation time increases nitrosamine mutagenicity in bacterial assays. Changes in the pH of the preincubation (from 5.0 to 9.0) mix of the assay described in Chapters Two and Three had no effect on the sensitivity of the strains to nitrosamine mutagenicity. In fact, preincubation is not necessary in the described assay. These observations are not unexpected given that the assay does not rely on exogenous activation of the promutagens.

It should also be noted that preincubating the VB agar plates prior to the addition of the bacteria/mutagen/top agar mix to room temperature or 37° C is critical to the integrity of the agar plates for scoring. Molten top agar mix is maintained at 45° C and its addition to refrigerated agar plates can cause uneven top agar distribution and results in plates that are difficult to score due to uneven colony distribution.

In the traditional Ames test, plates were normally scored 24 hours after addition of the top agar; however, this is not the ideal time point for scoring colonies produced in this assay. Due to slower growth of the YG pIN3 strains (presumably due to the metabolic load of expressing heterologous proteins) it is ideal to wait 48 - 60 hours before counting colonies. Not all colonies are reliably noticeable at the 24 or 36 hour time point. Allowing colonies to form for over 60 hours may result in the colonies becoming too large (i.e., the colonies may grow together and make individual colony distinction difficult).

Considerable effort was expended in attempting to obtain CO difference spectra for the bacterial strains with plasmids pIN3b5ER and pIN3ERb5; however, all attempts to reliably produce a CO difference spectrum on whole YG cells or membrane fractions were unsuccessful. Obtaining meaningful metabolism data attributable to the expressed CYP2E1 in whole YG cells or isolated membranes also proved difficult. Methodologies

detecting the metabolism of *p*-nitrophenol and aniline colorimetrically, and chlorzoxazone electrochemically were included in these efforts. It also proved difficult to obtain spectra from whole cells or membrane fraction demonstrating the expression of cytochrome b_5 in these strains. It is thought that low protein expression, coupled with interference from other bacterial heme proteins including cytochromes *d* and *o*, contributed to this observation. Attempts to utilize differing concentrations of potassium chloride to selectively subtract the absorbance due to bacterial cytochromes were also unsuccessful.

Table 9: Plasmid vectors used in assay development.

<u>Plasmid</u>	<u>Features</u>	<u>Reference</u>
pJL2	High copy vector derived from pKK223-3	[116]
pIN3	Low copy vector derived from pBR322	[79]
pIN4	High copy number vector derived from pIN3	[79]

Table 10: Bacterial tester strains used in assay development.

<u>Strain</u>	<u>Organism</u>	<u>Genotype</u>	<u>Plasmid</u>	<u>Reference</u>
TA1535	<i>S. typhimurium</i>	<i>hisG46, rfa, uvrB</i>	none	[1]
TA100	<i>S. typhimurium</i>	<i>hisG46, rfa, uvrB</i>	pKM101	[1]
TA7001	<i>S. typhimurium</i>	<i>hisG1775, rfa, uvrB</i>	pKM101	[117]
WP2	<i>E. coli</i>	<i>uvrA, trp-65</i>	none	[118]
WP2hcr	<i>E. coli</i>	<i>lon11, uvrA, trp-65</i>	none	[118]
WP67	<i>E. coli</i>	<i>polA1, uvrA, trp-65</i>	none	[118]
IC203	<i>E. coli</i>	<i>OxyR, uvrA, trp-65</i>	pKM101	[119]
IC204	<i>E. coli</i>	<i>umuDC, OxyR, uvrA, trp-65</i>	pKM101	[119]
IC206	<i>E. coli</i>	<i>MutY, umuDC, OxyR, uvrA, trp-65</i>	pKM101	[119]

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VITA

Name: Matthew Troy Cooper
Date of Birth: June 15, 1972
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EDUCATION

1995-2002 Graduate Student, University of Kentucky Graduate Center for Toxicology, Lexington, KY.

1990-1995 B.S., Chemistry, University of Tulsa, Tulsa, OK.

PROFESSIONAL EXPERIENCE

RESEARCH EXPERIENCE

1995-2001 Research Assistant, Graduate Center for Toxicology, University of Kentucky, Lexington, Kentucky.
Advisor: Todd D. Porter, Ph.D.

Summer 1998 Summer Intern, Merrill Lynch, Lexington, KY.
Advisor: Travis Musgrave

Summer 1997 Summer Intern, Dow Corning Corporation, Midland, MI.
Advisor: Jim McKim, Ph.D.

Summer 1996 Summer Intern, Procter & Gamble Company, Cincinnati, OH.
Advisor: Marilyn Aardema, Ph.D.

- 1994-1995 Research Assistant, Department of Biological Sciences, College of Arts and Sciences, University of Tulsa, Tulsa, OK.
Advisor: Kathleen Duncan, Ph.D.
- 1992- 1995 Toxicology Consultant, Cooper Consulting, Tulsa, OK.
Advisor: Self-employed.
- 1991-1993 Research Chemist, Baker Performance Chemicals, Sand Springs, OK.
Advisor: Ron Matherly, Houston, TX.
- 1991-1992 Research Assistant, Department of Chemistry, University of Tulsa, Tulsa, OK.
Advisor: Dale Teeters, Ph.D.

CONSULTING EXPERIENCE

- 1993-1995 Amoco Production Company, Tulsa, OK.
- 1995 Permafix Environmental Laboratories, Tulsa, OK.
- 1994 Dowell Schlumberger, Tulsa, OK.
- 1994 Sun Oil, Tulsa, OK.

TEACHING EXPERIENCE

- Spring 1998 PHR 647, University of Kentucky, Introduction to Molecular Pharmacotherapeutics – 3-hour lecture to class of 21 graduate students entitled “Utilization of Computers and the Internet in Molecular Pharmacology”

Spring 1996 PHR 647, University of Kentucky, Introduction to Molecular Pharmacotherapeutics – 3-hour lecture to class of 27 graduate students entitled “Utilization of Computers and the Internet in Molecular Pharmacology”

Spring 1995 BIOL 3053, University of Tulsa, Microbiology - 1-hour lecture to 45 undergraduate students entitled “Microbiology in Industry.”

1990-1992 CHEM 1011, University of Tulsa, General Chemistry Laboratory - 6 hours/week lectured on basic chemistry and laboratory skills; assisted in lab supervision of 60 undergraduate students

SPECIAL TRAINING AND CERTIFICATIONS

1997 Ames II Assay, Xenometrics, Boulder, CO.

1995 Good Laboratory Practices (GLP), Procter & Gamble, Cincinnati, OH.

1991 Microtox and Mutatox Toxicity Assays, Azur Environmental, Carlsbad, CA.

HONORS

Mar. 2000 University of Kentucky Graduate School Travel Award, Lexington, KY.

Mar. 2000 University of Kentucky Graduate Center for Toxicology Research Challenge Travel Award, Lexington, KY.

Nov. 1999 Second Place, Student Poster Competition, Ohio Valley Society of Toxicology Regional Meeting, Louisville, KY.

1998-2002 National Research Service Award, National Institute of Health, Bethesda, MD.

1997-1998 Lymon T. Johnson Research Fellow, University of Kentucky, Lexington, KY.

1993-1995 Tri-Beta Biological Sciences Honor Society, University of Tulsa, Tulsa, OK.

1991-1992 Lantern Honor Society, University of Tulsa, Tulsa, OK.

GRANT SUPPORT

"Ames Mutagenicity Testing with Recombinant Human P450s," National Institute of Health, F32 ES-05852, PI: Matthew Cooper, Co-PI: Todd Porter, 12/98 - 12/02, \$60,000.

"Lymon T. Johnson Research Assistantship," University of Kentucky, PI: Matthew Cooper, Co-PI: Todd Porter, 12/97-12/98, \$6,196.

"Ames Mutagenicity Testing with Recombinant Human P450s," National Institute of Health, R01 ES-06420, PI: Todd Porter, 6/96-4/98, \$20,042.

"Cooperative Bioremediation of Naphthalene," University of Tulsa Research Grant, PI: Matthew Cooper, Co-PI: Kathleen Duncan, 1/95 – 5/95, \$350.

"Cooperative Bioremediation of Naphthalene," National Science Foundation, PI: Matthew Cooper, Co-PI: Kathleen Duncan, 5/94 – 8/94, \$9,000.

MEMBERSHIPS AND PROFESSIONAL ACTIVITIES

MEMBERSHIPS

1996 – 2001 Member, Society of Toxicology

1996 – 2001 Member, International Society for the Study of Xenobiotics

1996 – 1998 Member, American Association of Pharmaceutical Scientists

1990 – 1995 Member, American Chemical Society

PROFESSIONAL ACTIVITIES

1996 – 1997 Secretary, Toxicology Student Forum, University of Kentucky, Lexington, KY.

1993 – 1994 Vice President, American Chemical Society Student Affiliates, University of Tulsa, Tulsa, OK.

1992 – 1993 Secretary, American Chemical Society Student Affiliates, University of Tulsa, Tulsa, OK.

PROFESSIONAL PRODUCTIVITY

PUBLICATIONS

Cooper, MT and Porter, TD. (2001) Cytochrome b5 Coexpression Increases Nitrosamine Mutagenicity in Human CYP2E1 and NADPH-Cytochrome P450 Reductase Expressing Salmonella typhimurium YG7104 and YG7108 Tester Strains. Mutation Res, 484, 61-8.

Cooper, MT and Porter, TD. (2000) Metabolic Activation and Mutagenicity of Nitrosamines by Coexpressed Human CYP2E1 and NADPH-Cytochrome P450 Reductase in Salmonella typhimurium YG7104 and YG7108 Tester Strains. Mutation Res, 454, 45-52.

ABSTRACTS

Cooper MT and Porter TD. (2000) Cytochrome b5 Coexpression Increases Nitrosamine Mutagenicity in Human CYP2E1 and NADPH-Cytochrome P450 Reductase Expressing Salmonella typhimurium YG7104 and YG7108 Tester Strains. International Society for the Study of Xenobiotics National Meeting, Indianapolis, IN.

Cooper MT and Porter TD. (2000) The Coexpression of Human CYP2E1, P450 Reductase and Cytochrome b5 as an Alternative to Mammalian Tissue Preparations (S9) in the Ames Assay. Ohio Valley Society of Toxicology Annual Meeting, Cincinnati, OH.

Cooper MT and Porter TD. (2000) S. typhimurium YG7104 and YG7108 Coexpressing Human CYP2E1 and P450 Reductase are Sensitive to Nitrosamines in the Ames Assay. 13th Annual Microsomes and Drug Oxidations International Meeting, Stresa-Lago Maggiore, Italy.

Cooper MT and Porter TD. (2000) Salmonella typhimurium YG7104 and YG7108 Coexpressing Human CYP2E1 and P450 Reductase are Sensitive to Promutagenic CYP2E1 Substrates in the Ames Assay. Society of Toxicology National Meeting, Philadelphia, PA.

Cooper MT and Porter TD. (1999) Salmonella typhimurium YG7104 and YG7108 Coexpressing Human CYP2E1 and P450 Reductase are Sensitive to Nitrosamine Mutagenicity. Ohio Valley Society of Toxicology Regional Meeting, Louisville, KY.

Cooper MT and Porter TD. (1999) The Influence of Methyltransferases in Dimethylnitrosamine Mutagenicity with Ames Tester Strains Coexpressing Human CYP2E1 and P450 Reductase. International Society for the Study of Xenobiotics National Meeting, Nashville, TN.

Shinyu C, Cooper MT and Porter TD. (1996) Coexpression of P450-2E1 and P450 reductase in Escherichia coli and Salmonella typhimurium. Society of Toxicology National Meeting, Cincinnati, OH.

Cooper MT, Shinyu C and Porter TD. (1995) Towards replacement of the S9 fraction: Coexpression of P450-2E1 and P450 reductase in Escherichia coli. Ohio Valley Society of Toxicology Regional Meeting, Cincinnati, OH.

Cooper MT, Shinyu C and Porter TD. (1995) Coexpression of human P450-2E1 and rat NADPH-P450 reductase in Escherichia coli for utilization in mutagenicity testing. University of Kentucky Graduate Student Research Day, Lexington, KY.

Cooper, MT and Duncan, KE. (1995) Cooperative bioremediation: utilization of surfactant-producing and bio-degradative bacteria in naphthalene bioremediation. American Society of Microbiology National Meeting, Denver, CO.

Cooper, MT. (1994) The utility of bioluminescent bacteria in toxicity screening and petrochemical product development. Baker Performance Chemicals Technical Conference, Houston, TX.

Cooper, MT. (1993) Efficacy of the Microtox bioassay in predicting EPA compliance for petrochemicals. Baker Performance Chemicals Technical Conference, Houston, TX.

Cooper, MT. (1992) New methods for predicting the toxicity of water insoluble and water dispersible chemicals utilizing bioluminescent bacteria. American Chemical Society National Meeting, Denver, CO.

Cooper, MT and Teeters, D. (1992) Polyethylene dichroism measurement utilizing FT-IR spectroscopy. American Chemical Society National Meeting, Denver, CO.

Cooper, MT and Teeters, D. (1991) Hydrocarbon soil contamination measurement utilizing thermogravimetric/differential scanning calorimetry. University of Tulsa Student Research Day, Tulsa, OK.

PRESENTATIONS

"The Expression of Human P450 Enzymes as an Alternative to Mammalian Tissue Preparations in the Ames Assay," Seminar, Graduate Center for Toxicology, University of Kentucky, Lexington, KY. January 2000.

"Alternatives to Mammalian Tissue Preparations in the Ames Assay," Seminar, Division of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY. January 2000.

"The Importance of Addressing Toxicity Issues During Product Development," invited presentation, Sun Oil Company, Tulsa, OK. June 1994.

"The Importance of Addressing Toxicity Issues During Product Development," invited presentation, Baker Performance Chemicals, Houston, TX. April 1994.

"Utilization of the Microtox Assay in Product Risk Assessment," invited presentation, Dowell Schlumberger, Tulsa, OK. January 1994.

"Utilization of the Microtox Assay in Product Risk Assessment," invited presentation, Amoco Production Company, Tulsa, OK. November 1993.

"Utilization of the Microtox Assay in Product Risk Assessment," invited presentation, Baker Performance Chemicals, Houston, TX. July 1993.