

Bioactivation of Aromatic Amines by Recombinant Human Cytochrome P4501A2 Expressed in Ames Tester Strain Bacteria: A Substitute for Activation by Mammalian Tissue Preparations¹

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ABSTRACT

The most widely used bioassay in genetic toxicology is the Ames test, which combines a bacterial mutagenicity assay (reversion of *Salmonella typhimurium* histidine-auxotrophic tester strains) with an exogenous bioactivation system (hepatic postmitochondrial supernatant or "S9"). The enzymatic activities of S9 prepared from the tissues of experimental animals are difficult to control. We show that the requirement for S9 can be obviated by the engineered expression of enzymes of bioactivation within the bacterial cell. With this strategy, reactive metabolites are produced inside the bacterial cell, proximate to the genetic target. Species boundaries can be crossed, and chimeric or mutant enzymes can be studied. We have constructed an Ames tester strain, expressing both aromatic amine *N*-acetyltransferase and human cytochrome P4501A2, which detects aromatic amine mutagenicity in the absence of S9.

INTRODUCTION

Carcinogenic aromatic amines are bioactivated in two enzymatic steps: *N*-hydroxylation (catalyzed by P450³) to give *N*-hydroxyarylamines and subsequent acetyl-CoA-dependent *O*-acetylation. NAT (1) catalyzes both the *N*-acetylation of aromatic amines and the *O*-acetylation of hydroxylamines. The *N*-acetoxy esters formed by acetylation of hydroxylamines are reactive electrophiles and give rise to covalent DNA adducts, probably via the loss of acetate anion, which yields, formally, a nitrenium ion (RN^+H):



Many aromatic amines and nitroaromatic compounds are potent mutagens in the Ames test (2). 1,8-DNP-resistant mutants of Ames tester strains such as TA98 1,8-DNP₆ are devoid of NAT/OAT activity due to a chromosomal *nat*⁻ mutation, and have greatly reduced sensitivity to the mutagenicity of nitroaromatic compounds and S9-activated aromatic amines (3). This result demonstrates that bacterial NAT/OAT activity is critical to aromatic amine mutagenicity in the Ames test. *Salmonella typhimurium* strains carrying the genes for bacterial or mammalian NAT enzymes on multicopy plasmids express very high levels of activity and are several orders of magnitude more

sensitive than conventional Ames tester strains such as TA98 (3–5). The construction of NAT-overproducing strains was an important step in the development of mutagenicity tester strains with engineered capacities for bioactivation of carcinogens.

Another mammalian enzyme of biotransformation, GST, has recently been expressed in Ames test strains (6, 7). The resulting strains are sensitive to the mutagenicity of dihaloalkanes, which are activated by glutathione conjugation.

These recent advances show that the replacement of mammalian tissue preparations by the engineered bacterial expression of recombinant genes is a realistic goal. Successful introduction of a bioactivation enzyme requires that several conditions be met: (a) the gene encoding the enzyme protein must be cloned and introduced to the bacterial tester strain; (b) the expressed protein must be enzymatically active; and (c) the bioactivation step of interest must be carried out within the intact bacterial cell. The last requirement usually depends on the availability of a second substrate, such as acetyl-CoA (in the case of NAT/OAT) or glutathione.

A key step toward replacement of S9 activation is the expression of P-450, the most important enzyme catalyzing "Phase I" biotransformation of xenobiotics (8). The cDNA for P-450 enzyme 1A2 (P4501A2), which catalyzes aromatic amine *N*-oxidation, has been expressed in *Escherichia coli* following introduction of modifications in the amino-terminal coding region (9). Electron transfer from NADPH to P-450 enzymes in the endoplasmic reticulum is normally mediated by the flavoprotein accessory enzyme NADPH-P450 oxidoreductase. Nevertheless, monooxygenase activity can be detected in intact *E. coli* cells expressing mammalian P-450, even in the absence of oxidoreductase. The *E. coli* enzymes flavodoxin and NADPH-flavodoxin reductase (10) can substitute for the accessory enzyme.

We describe the construction of a *S. typhimurium* strain which actively expresses the genes for human P4501A2 and bacterial NAT. This new strain yields a sensitive mutagenic response to aromatic amines in the absence of S9.

MATERIALS AND METHODS

Strain Construction. *S. typhimurium* strain YG1019 (11) (TA1538/1, 8-DNP pYG219) was graciously provided by Dr. T. Nohmi (National Institute of Health Sciences, Tokyo, Japan). This strain, and all other strains discussed here, bear the chromosomal *hisD3052* allele (also found in strains TA1538 and TA98) and the chromosomal *nat*⁻ mutation. Plasmid pYG219 carries the *S. typhimurium nat* gene, and YG1019 produces very high levels of NAT activity. A plasmid [construct 1024 (9)] bearing the modified coding sequence for human P4501A2 was introduced into a restriction-minus strain of *S. typhimurium* as described previously (12). The modified plasmid DNA was used to transform YG1019 (electroporation), and transformants were selected by growth on nutrient agar plates containing ampicillin (25 μg/ml) and tetracycline (6 μg/ml) to select for the plasmids bearing the genes for P4501A2 and NAT, respectively. Several transformants produced a strong mutagenic response to MeIQ, a carcinogenic and potentially mutagenic heterocyclic aromatic amine formed by pyrolysis of foods, including grilled meat (13). One such

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³ The abbreviations used are: P-450, cytochrome P-450 monooxygenase; NAT, aromatic amine *N*-acetyltransferase; OAT, arylhydroxylamine *O*-acetyltransferase; 1,8-DNP, 1,8-dinitropyrene; IPTG, isopropyl α-D-thiogalactoside; GST, glutathione S-transferase; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-f]quinoline; 2-AF, 2-aminofluorene; EROD, 7-ethoxyresorufin O-deethylase.

isolate was selected; P-450 protein expression was confirmed by immunoblotting analysis (see below). The new strain was designated DJ4501A2. An analogous strain was constructed from TA1538/1,8-DNP, which has no NAT activity (5). This strain, designated DJ4501A2.1, also carries the P-450 expression plasmid 1024 and produces P-450 protein (by immunoblotting). An additional control strain, YG1019 pCW, was constructed by transforming YG1019 with pCW, the vector from which construct 1024 was derived.

Analysis of P-450 Expression. P-450 expression in DJ4501A2 and related strains was studied by spectroscopic and biochemical analyses. Spectroscopic methods for determination of P-450 (difference spectrum, reduced-carbon monoxide versus reduced) were as described elsewhere (9). For immunoblotting, bacterial membrane preparations were obtained by ultrasonic disruption of cultures (9). Proteins were separated by SDS-PAGE (10%; Mini-Protein system; Bio-Rad, Richmond, CA) and transferred to nitrocellulose membranes (Trans-Blot, Bio-Rad). The immunoblots were probed with goat polyclonal antibody to rabbit P4501A2 (Oxford Biomedical Research, Inc., Oxford, MI) and visualized by alkaline phosphatase/5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium development. In each case, 50 μ g of protein were loaded, except as noted otherwise. All strains were induced with IPTG, except as noted.

Mutation Assays. Reversion assays were performed as described (2). Bacterial cultures were grown overnight in Oxoid Nutrient Broth No. 2 [with addition of ampicillin (100 μ g/ml) and tetracycline (6 μ g/ml) as appropriate] at 37°C with shaking. Overnight cultures were washed with buffer, diluted 10-fold into fresh broth containing antibiotics and 1 mM IPTG, and grown for about 5 h to an OD_{650 nm} of approximately 0.9. S9 fraction from Aroclor 1254-induced male Sprague-Dawley rats (used for the experiment shown in Fig. 3) was purchased from Molecular Toxicology, Ltd. [(Annapolis, MD) lot no. 0487; EROD specific activity, 1.42×10^4 pmol product/min/mg protein]. The preincubation assay technique (incubation of buffer or S9, bacteria, and mutagen at 37°C for 30 min before plating) was used, and experiments were performed in triplicate. Revertant colonies were counted after 3 days incubation at 37°C.

RESULTS AND DISCUSSION

P-450 Protein Is Expressed and Is Enzymatically Active in *S. typhimurium*. P-450 protein expression in *S. typhimurium* strain DJ4501A2 was detected by optical difference spectroscopy (Fig. 1) and immunoblotting (14) (Fig. 2) of membrane preparations. P-450 monooxygenase activity was confirmed by measurement of EROD activity (a marker reaction for P4501A2) catalyzed by a reconstituted system containing the membrane preparation and purified rabbit NADPH-P-450 oxidoreductase (9); specific enzyme activities (pmol product/min/mg protein) were: strain DJ4501A2, 2.55; strain YG1019, not detectable (<0.5).

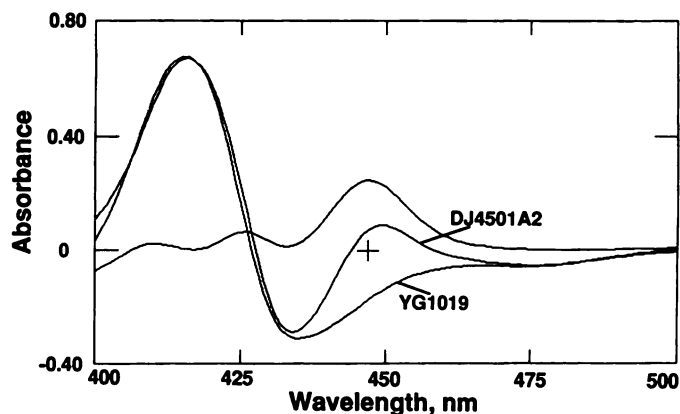


Fig. 1. Fe^{2+} -CO versus Fe^{2+} difference spectra of membrane fractions prepared from strains DJ4501A2 and YG1019 (baseline corrected). The difference between the two difference spectra (i.e., the YG1019 spectrum subtracted from the DJ4501A2 spectrum) is also shown. +, wavelength maximum of this spectrum (447.0 nm). The spectra indicate expression of 170 nmol P-450/liter of culture.

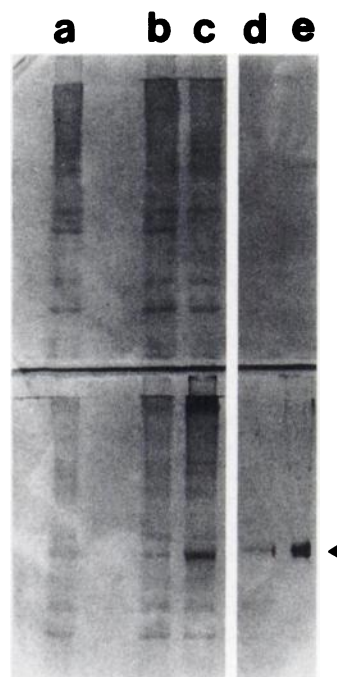


Fig. 2. Immunoblot ("Western") analysis of P4501A2 expression in bacterial membrane fractions. Top, preimmune serum; bottom, immune serum. a, YG1019; b, DJ4501A2 (minus IPTG); c, DJ4501A2; d, purified recombinant human P4501A2 (2 pmol); e, *E. coli* DH5 α P4501A2 (2 μ g). Arrowhead indicates P4501A2.

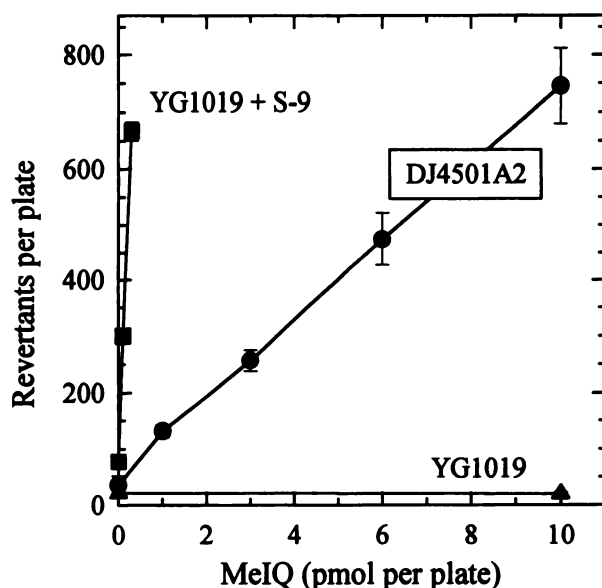


Fig. 3. Mutagenicity of MeIQ in an Ames tester strain expressing P4501A2. ●, DJ4501A2; ▲, YG1019 (data points at 1, 3, and 6 pmol omitted for clarity); ■, YG1019 with aroclor 1254-induced rat hepatic S9 activation (1 mg S9 protein/plate). Points, mean of three triplicate experiments; bars, SE. Strain YG1019 pCW also gave no significant mutagenic response in the absence of S9 activation (data not shown). Experiments performed with growth of bacterial cultures or incubation of plates at 30°C gave similar results.

The P-450-expressing Strain Detects Aromatic Amine Mutagenicity. MeIQ produced a dose-dependent increase in mutagenicity in strain DJ4501A2 (Fig. 3) in the absence of S9. Neither YG1019 nor DJ4501A2.1 yielded a mutagenic response. Thus, both P-450 and NAT enzyme activities are required for mutagenicity. When S9 activation was used, both strains YG1019 (Fig. 3) and DJ4501A2 (data not shown) were very sensitive to MeIQ, as expected.

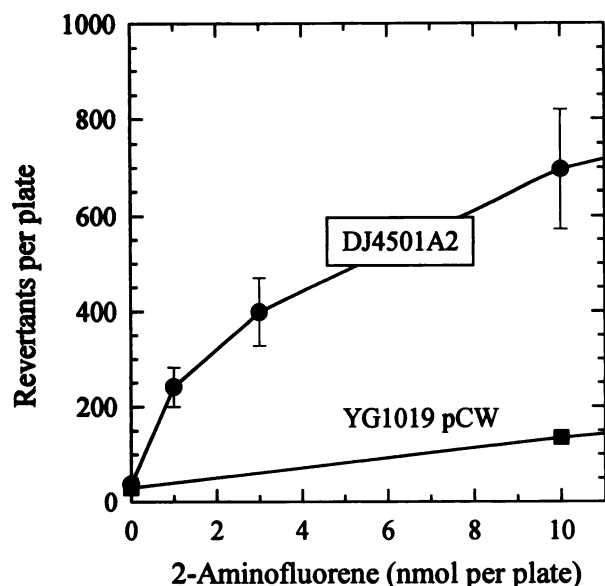


Fig. 4. Mutagenicity of 2-AF in an Ames tester strain expressing P4501A2. ●, DJ4501A2; ■, YG1019 pCW. Points, mean of three triplicate experiments; bars, SE.

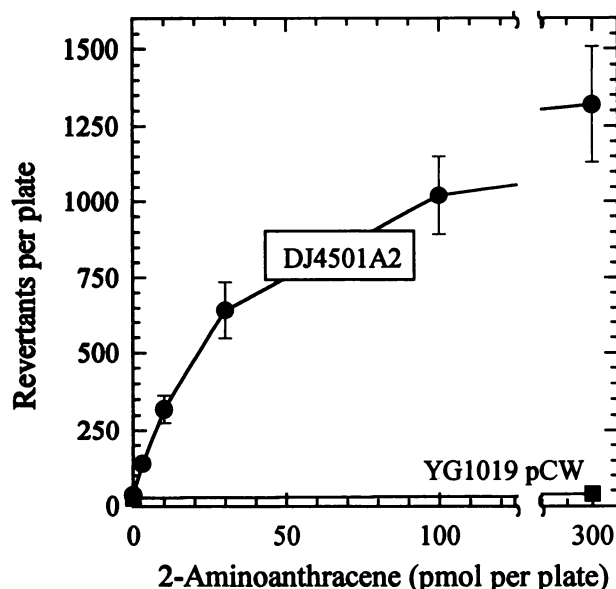


Fig. 5. Mutagenicity of 2-aminoanthracene in an Ames tester strain expressing P4501A2. ●, DJ4501A2; ■, YG1019 pCW (data points at 3 and 30 pmol omitted for clarity). Points, mean of three triplicate experiments; bars, SE.

We also tested 2-AF (Fig. 4) and 2-aminoanthracene (Fig. 5). Again, mutagenic responses were observed in strain DJ4501A2 in the absence of S9. In these experiments, we also tested the control strain YG1019 pCW, which expresses NAT but not P-450. No significant response to 2-aminoanthracene was observed in this strain. In the case of 2-AF, mutagenicity was observed, although at much lower levels than in DJ4501A2 (Fig. 4). Weak "direct acting" mutagenicity of aromatic amines [e.g., *N*-acetylbenzidine (15)] has been observed previously in NAT-overexpressing Ames tester strains.

The S9 activation system, with strain YG1019, still provides greater sensitivity than does heterologous expression of P-450 in strain DJ4501A2 (Fig. 3). However, the P-450 total activity present in the S9 fraction used in the Ames assay is more than 1 million-fold higher than the activity expressed in the bacteria, based on the EROD assay data. Mutagenicity of 2-AF without S9 in strain DJ4501A2 is greater

than that reported for either of the standard tester strains, TA1538 (16) or TA98 (17), with hepatic S9 activation. We are confident that the sensitivity of the engineered system can be improved further (e.g., by expression of NADPH-P-450 oxidoreductase).

P-450 protein expression, under control of *lac* repressor, was induced by IPTG (Fig. 2). However, MeIQ-induced mutagenicity in strain DJ4501A2 was observed even in the absence of IPTG, as noted previously for the expression of GST in *S. typhimurium* (6). This suggests that factors other than P-450 activity are rate limiting for mutagenicity. Simultaneous addition of 10 nmol α -naphthoflavone, an inhibitor of P-450 activity, reduced MeIQ mutagenicity in strain DJ4501A2 to background levels (Fig. 6).

In summary, P-450 protein expressed heterologously in *S. typhimurium* resembles the human enzyme in its spectroscopic, immunological, electrophoretic, and catalytic properties. Strain DJ4501A2 detects the mutagenicity of aromatic amines in the absence of S9 activation, and this mutagenicity is dependent on the presence of both the P-450- and NAT-bearing plasmids.

Other alternatives to the use of mammalian tissue preparations in mutagenicity testing have been developed recently. Microsomes prepared from engineered yeast, expressing human P-450, can replace S9 as an activation system in the Ames test (18, 19). However, this method cannot overcome the requirement for transport of reactive species into the bacterial cell. Mammalian cell lines expressing P-450 and NAT are also highly sensitive to the toxicity and mutagenicity of aromatic amines (20).

The functional replacement of S9 by heterologous bioactivation enzymes expressed in *S. typhimurium* fulfills the promise of the Ames assay as an alternative to the use of experimental animals in genotoxicity testing and should also provide a powerful tool for studying bioactivation mechanisms. The expression of other P-450 enzymes in the Ames test system should permit detection of additional classes of mutagens dependent on P-450 bioactivation, such as polycyclic aromatic hydrocarbons and nitrosamines.

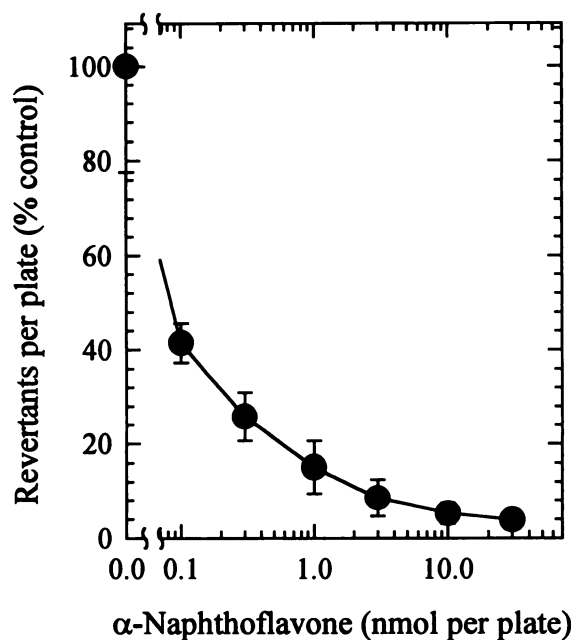


Fig. 6. α -Naphthoflavone inhibition of MeIQ mutagenicity in strain DJ4501A2. Data are represented as percentage of control response (20 pmol MeIQ; 806 revertants/plate) in the absence of inhibitor. Inhibitor and bacteria were incubated at 37°C for 15 min before addition of MeIQ. Points, mean of two triplicate experiments; bars, SE.

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