Salmonella typhimurium Strains Expressing Human Arylamine N-Acetyltransferases: Metabolism and Mutagenic Activation of Aromatic Amines

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ABSTRACT

Epidemiological studies have established the carcinogenic risk of occupational exposure to aromatic amines such as benzidine, N-naphthylamine, and 4-aminobiphenyl. Metabolic activation of these chemicals to reactive, genotoxic electrophiles, via enzymatic N-oxidation and subsequent conjugation reactions, is necessary for their carcinogenic potential to be realized. Many aromatic amines are mutagenic in prokaryotic test systems, in the presence of exogenous mammalian activating enzymes such as those contained in hepatic 9000 × g supernatant. However, in the Ames (Salmonella typhimurium) assay, induction of mutations by aromatic amines and nitroarenes is also almost completely dependent upon the activity of the endogenous bacterial enzyme, N-acetyltransferase/O-acetyltransferase. The relevance of this assay to the prediction of the carcinogenic potential of aromatic amines in humans is thus restricted by the likelihood that the bacterial and human enzymes possess different substrate specificities. In this paper we report the construction and use of new tester strains of S. typhimurium that express high levels of functional human arylamine N-acetyltransferases, NAT1 and NAT2, retaining characteristic arylamine substrate specificities that are distinct from those of the bacterial enzyme. These new strains support the mutagenic activation of benzidine, 2-amino-3,4-dimethylimidazole, and 4-fluorophenol in the Ames test and may provide a new tool for evaluating the carcinogenic potential of aromatic amines.

INTRODUCTION

NAT3 (EC 2.3.1.5) is involved in the biotransformation of several drugs and toxicologically important chemicals bearing primary aromatic amino or hydrazino functional groups. In humans, the genes encoding two isozymes of NAT have been cloned (1). These isoforms, NAT1 and NAT2, are closely related in sequence but display very different substrate specificity patterns (2). The NAT2 gene locus is the site of the human acetylation polymorphism, since allelic variations in this gene (3, 4) ultimately result in the production of markedly reduced amounts of NAT2 enzyme protein in the livers of roughly 60% of the individuals in Caucasian populations (5). A substantial body of epidemiological evidence has suggested that the “slow acetylator” phenotype, determined by the presence of such allelic variants at the NAT2 gene locus, may be a risk factor for predisposition to bladder cancer from occupational exposure to aromatic amines (reviewed in Ref. 6). Population variation in NAT1 function and its possible consequences for xenobiotic toxicity, on the other hand, have only recently begun to be investigated.

Several lines of experimental evidence suggest that aromatic amine carcinogens, such as 2-aminofluorene and benzidine, require metabolic activation by both acetylation and by N-oxidation via cytochromes P-450 or prostaglandin H synthase before their carcinogenic potential can be manifested. For instance, the presence of such enzyme systems has been shown to be necessary for 2-aminofluorene and benzidine to elicit a positive mutagenic response in the Ames (Salmonella typhimurium) test (7, 8), which is generally considered to be a relevant predictor of carcinogenicity. In particular, Ames tester strains which over-express the gene encoding S. typhimurium NAT are considerably more sensitive to the mutagenic effects of many aromatic amino and nitro compounds (9). This sensitivity is probably a consequence of the associated O-acetyltransferase activity of the bacterial enzyme, which catalyzes the conversion of hydroxylamines to highly reactive acetoxy esters (10). However, the implications of this phenomenon for the human toxicology of aromatic amines are not clear, since the human NAT enzymes may not have the same catalytic properties as the form naturally present in S. typhimurium.

In the present work, we show that the genes for human NAT1 and NAT2 can be expressed in S. typhimurium to yield fully functional enzymes retaining their characteristic specificities, which are distinct from that of the S. typhimurium NAT. Both NAT1 and NAT2 support the mutagenic activation of aromatic amines in the Ames assay, although with different specificities. These new tester strains may provide a more relevant means of predicting the toxic potential of aromatic amines in humans.

MATERIALS AND METHODS

Construction of S. typhimurium Strains Expressing Human NAT1 and NAT2. The tac promoter-based expression vectors pNAT1 and pNAT2 were constructed by amplification of the NAT1 and NAT2 coding exons (1) via expression-cassette polymerase chain reaction (11) and subcloning of the resulting expression cassettes into the phagemid vector pKEN2 (kindly provided by Dr. G. Verdine, Harvard University, Cambridge, MA). [Full details of this construction and of the expression of the human NAT1 and NAT2 proteins in Escherichia coli strains DMG100 and DMG200 (X90/pNAT1 and X90/pNAT2, respectively) are presented elsewhere (12).] The NAT-deficient (NAT−) S. typhimurium strain TA1538/1,8-DNP (13, 14) was transformed with each plasmid by electroporation. The parent strain is devoid of bacterial NAT activity (see Table 1), and therefore much less sensitive to aromatic amine mutagenicity than is the isogenic NAT-proficient strain, TA1538 (14, 15). The transformants were initially selected for ampicillin resistance, and isolates were routinely tested for characteristic phenotypic traits (antibiotic resistance, sensitivity to UV light, histidine growth factor requirement, and crystal violet sensitivity). The resulting strains were designated DJ400 (TA1538/1,8-DNP pNAT1) and DJ460 (TA1538/1,8-DNP pNAT2).

In Vitro Enzyme Activity Measurements. Freshly plated overnight colonies of each S. typhimurium strain were grown in liquid culture (typically 3 ml) for 6 h in Luria-Bertani medium with or without ampicillin (25 μg/ml) before harvesting; E. coli cultures were grown in Luria-Bertani-ampicillin for 3 h, isopropyl β-D-thiogalactopyranoside was
human NAT genes closely paralleled the data for the analogous E. coli strains (Table 1), for the human genes expressed in mammalian cell culture (2) and for partially purified enzyme preparations from human liver cytosol (2).

We also performed immunoblot analysis of extracts from various bacterial strains (Fig. 1), using a polyclonal rabbit antiserum against human liver NAT2 (16). This antibody preparation cross-reacts with human NAT1 and with both of the mammalian enzymes, even under conditions such that the absolute yields of the mammalian enzymes were similar. This further implies that the human and bacterial enzymes have substantially different kinetic characteristics. In general, the results obtained with the S. typhimurium strains bearing human NAT genes closely paralleled the data for the analogous E. coli strains (Table 1), for the human genes expressed in mammalian cell culture (2) and for partially purified enzyme preparations from human liver cytosol (2).

We also performed immunoblot analysis of extracts from various bacterial strains (Fig. 1), using a polyclonal rabbit antiserum against human liver NAT2 (16). This antibody preparation cross-reacts with human NAT1 and with both of the rabbit liver NAT isozymes. Strains DJ400 and DJ460 produced immunoreactive proteins with identical electrophoretic mobilities to human NAT1 and NAT2 expressed in E. coli (Fig. 1) or observed in human liver (1, 2). As expected, TA1538/1,8-DNP had no detectable NAT activity, as expected. YG1012, the strain which overproduces the bacterial NAT to activity levels over 900-fold higher than the parental strain TA1538 (18), showed a stronger preference for the more hydrophobic (and carcinogenic) substrates 2-aminofluorene and benzidine, and relatively little activity for aromatic amine drugs (p-aminobenzoic acid, p-aminosalicylic acid, sulfamethazine, procainamide) which are known to be substrates for either human NAT1 or NAT2 (2). Under the conditions of the assay, benzidine was metabolized to both monoacetylbenezidine and diacetylbenezidine, as observed previously (19). Strain DJ400 yielded very high levels of activity characteristic of human NAT1 (2): high turnover of p-aminobenzoic acid and p-aminosalicylic acid; much less activity for sulfamethazine and procainamide; and high activity for the carcinogens 2-aminofluorene and benzidine. In marked contrast, DJ460 showed higher activities for sulfamethazine and procainamide than for p-aminobenzoic acid and p-aminosalicylic acid; again, this reflects the pattern of human NAT2 (2). DJ460 also metabolized 2-aminofluorene and benzidine at an appreciable rate relative to the other substrates tested, but far more slowly than did DJ400. When compared with YG1012, the strains expressing human NAT enzymes produced less diacetylbenezidine relative to monoacetylbenezidine, even under conditions such that the absolute yields of the monoacetylated metabolite were similar.

Results and Discussion

Table 1 shows the results of enzyme assays of cell extracts prepared from the following strains: strain TA1538/1,8-DNP; strain YG1012 [also denoted TA1538/1, 8-DNP pYG213, provided by M. Watanabe, National Institutes of Hygienic Sciences, Tokyo, Japan; this is a strain bearing the bacterial NAT gene on a pBR322 derivative (18)]; E. coli strains DMG100 and DMG200, bearing pNAT1 and pNAT2, respectively (12); and the corresponding S. typhimurium strains, DJ400 and DJ460, constructed for the present work. The NAT strain TA1538/1,8-DNP had no detectable NAT activity, as expected. YG1012, the strain which overproduces the bacterial NAT to activity levels over 900-fold higher than the parental strain TA1538 (18), showed a strong preference for the more hydrophobic (and carcinogenic) substrates 2-aminofluorene and benzidine, and relatively little activity for aromatic amine drugs (p-aminobenzoic acid, p-aminosalicylic acid, sulfamethazine, procainamide) which are known to be substrates for either human NAT1 or NAT2 (2). Under the conditions of the assay, benzidine was metabolized to both monoacetylbenezidine and diacetylbenezidine, as observed previously (19). Strain DJ400 yielded very high levels of activity characteristic of human NAT1 (2): high turnover of p-aminobenzoic acid and p-aminosalicylic acid; much less activity for sulfamethazine and procainamide; and high activity for the carcinogens 2-aminofluorene and benzidine. In marked contrast, DJ460 showed higher activities for sulfamethazine and procainamide than for p-aminobenzoic acid and p-aminosalicylic acid; again, this reflects the pattern of human NAT2 (2). DJ460 also metabolized 2-aminofluorene and benzidine at an appreciable rate relative to the other substrates tested, but far more slowly than did DJ400. When compared with YG1012, the strains expressing human NAT enzymes produced less diacetylbenezidine relative to monoacetylbenezidine, even under conditions such that the absolute yields of the monoacetylated metabolite were similar.

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The mutation *hisD3052*, used in many Ames mutagenicity tester strains, including TA1538, provides a sensitive target for reversion by arylamines. Sensitivity to arylamines is greatly enhanced in bacterial NAT-overexpressing derivative strains, such as YG1012 (18, 20); this suggests that acetylation plays an important role in the bioactivation process. Therefore, it is imperative to determine whether the human NAT enzymes play a similar role. Such a capacity would have important implications for aromatic amine-induced genotoxicity in humans. We tested this hypothesis in our newly constructed *hisD3052* strains which are devoid of bacterial NAT and express high levels of human NAT1 or NAT2.

The results of mutagenicity assays performed with these strains and selected aromatic amine carcinogens are given in Fig. 2. Aroclor 1254-induced rat liver 9000 x g supernatant fraction was used as a source of cytochrome P-450 activity. The background ("spontaneous") revertant yields (Table 2) were low enough to allow use of these strains for mutagenicity testing.

2-Aminofluorene mutagenicity is almost completely dependent on NAT activity; NAT-deficient strains are far less sensitive, and bacterial NAT-overproducing strains far more sensitive, compared to the NAT wild-type parent strain TA1538 (or TA98) (9, 18). Both strains DJ400 (human NAT1) and DJ460 (human NAT2) showed a mutagenic response to 2-aminofluorene at much lower doses than did the NAT-deficient parent strain (Fig. 2a). The response of DJ400 declined above 0.3 nmol/plate. This effect is due to 2-aminofluorene-induced loss of the plasmid encoding NAT1, as was previously observed with the bacterial *nat* gene (15): at doses above 10 nmol/plate, the response increased again, following the dose-response curve of the NAT-deficient parent strain (data not shown).

The activating effect of bacterial NAT on benzidine mutagenicity has been noted previously (15, 20). As observed with 2-aminofluorene, DJ400 and DJ460 both activated benzidine; in this case, DJ460 was the most active strain at all doses tested. No response was detected with TA1538/1.8-DNP (Fig. 2b).

We also examined the heterocyclic food pyrolysis product 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (21, 22). This compound is highly mutagenic in strain DJ460 (note doses in the pmol range). In contrast, the response of strain DJ460 was only slightly above that of TA1538/1.8-DNP (Fig. 2c).

For each of the aromatic amines examined here, the new strains expressing human NAT isozymes are more sensitive than the NAT-deficient parent strain TA1538/1.8-DNP. Mutagenic responses are observed over similar concentration ranges to those reported for YG1012 (20). Thus, both human NAT isozymes are capable of activating aromatic amines to mutagenic species. Since DJ400 has much higher NAT activity for typical aromatic amine mutagens than does DJ460 (Table 1), one might expect this strain to be the more sensitive. However, the relative sensitivities of strains expressing the two isozymes cannot be directly related to the NAT activities presented in Table 1 for several reasons: (a) *O*-acetylation rather than *N*-acetylation is probably the critical mutagenic activation step. The relationship between OAT and NAT activities for these isozymes is presently under study; preliminary data indicate that both human isozymes possess N-hydroxy-2-aminofluorene OAT activity (23); (b) kinetic properties must be considered. The activities reported in Table 1 are measured at a...
Table 2 Spontaneous revertant yields

<table>
<thead>
<tr>
<th>Condition</th>
<th>Revertants/plate ± SE (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA1538/1,8-DNP</td>
<td></td>
</tr>
<tr>
<td>DJ400</td>
<td>28±1.7(13)</td>
</tr>
<tr>
<td>DJ460</td>
<td>20±1.1(25)</td>
</tr>
<tr>
<td>S9</td>
<td>33±2.0(11)</td>
</tr>
<tr>
<td>S9 + DMSO</td>
<td>14±1.1(18)</td>
</tr>
<tr>
<td>S9 + DMSO</td>
<td>45±2.5(23)</td>
</tr>
</tbody>
</table>

* S9, 9000 × g supernatant from Aroclor 1254-induced rat liver; DMSO, dimethyl sulfoxide.

In summary, the S. typhimurium strains we have developed provide valuable new tools for studying the genotoxic potential of aromatic amines and the role of human NAT enzymes in the etiology of chemically induced cancers.

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REFERENCES


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