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Research Article

Enhanced Mutagenesis of *Salmonella* Tester Strains Due to Deletion of Genes Other Than *uvrB*

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The standard *Salmonella* mutagenicity (Ames) tester strains are missing 15–119 genes due to the extended Δ(*gal-bio-uvrB*) mutations that render the strains excision-repair deficient (Δ*uvrB*). We constructed strains of *Salmonella* that are homologous to tester strains TA98 and TA100 except that in place of the *uvrB* deletion, they contain single-gene defects in either *uvrB, moaA, moeA,* or both *uvrB* and *moeA*. We then tested the following mutagens in these strains: 2-acetylaminofluorene, Glu-P-1, 4-aminobiphenyl, benzo[*a*]pyrene, MX, 1-nitropyrene, 6-hydroxylaminopurine (HAP), and 2-amino-6-hydroxylaminopurine (AHAP). We confirmed in *Salmonella* a previous finding in *Escherichia coli* that the enhanced mutagenicity of the purine analogues HAP and AHAP is not due to the deletion of the *uvrB* gene but due to the deletion of *moeA* and/or *moaA*, which are involved in molybdenum cofactor biosynthesis. The spontaneous mutant frequency and induced mutagenic potency of mutagens due to the extended Δ*uvrB* mutation are due largely to the deletion of *uvrB* and to some extent of *moeA/moaA* at the frameshift *hisD3052* allele of TA98 but involve other genes in addition to *uvrB* and *moeA/moaA* at the base-substitution *hisG46* allele of TA100. The extended Δ*uvrB* mutation does not prevent the detection of mutagens that would have been detected in a strain containing a single *uvrB* defect. Because of the deletion of *moeA/moaA*, the extended *uvrB* deletion generally enhanced spontaneous and induced mutagenicity, especially at the base-substitution allele. This enhanced sensitivity may underlay the severe health effects in humans who have mutations in molybdenum cofactor biosynthesis genes. Environ. Mol. Mutagen. 48:694–705, 2007. Published 2007 Wiley-Liss, Inc.

Key words: Ames test; mutagenic potency; Δ*uvrB*; molybdenum cofactor; *moeA*; *moaA*

INTRODUCTION

The *Salmonella* (Ames) mutagenicity assay, introduced more than 35 years ago [Ames, 1971], is still the most widely used assay for evaluating drugs, industrial chemicals, and environmental samples for mutagenic activity [Mortelmans and Zeiger, 2000]. The *S. enterica* Typhimurium strains used in the assay contain various histidine alleles, and mutagenesis is measured by reversion to histidine independence [Maron and Ames, 1983]. In addition, the tester strains also contain various mutations and genetic factors that enhance the sensitivity of the bacteria to mutagens.

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The first of these mutations to be added was a Δ*uvrB* allele, which eliminated nucleotide excision repair and conferred enhanced sensitivity to many mutagens [Ames, 1971]. Five different Δ*uvrB* alleles, formally called *chl* (*bio uvrB gal*), were isolated independently for inclusion in the main tester strains [Busch et al., 1986; Popkin et al., 1989]. Because of the methods used to introduce these and other mutations, as well as the pKM101 plasmid, strains containing the same histidine allele are not isogenic [Popkin et al., 1989].

Each Δ*uvrB* allele was isolated as a mutation conferring anaerobic resistance to chlorate, which is a consequence of the loss of one or more genes involved in molybdenum cofactor biosynthesis [Shanmugam et al., 1992; Rajagopalan, 1996]. Three of the five operons required for molybdenum cofactor biosynthesis (*mod, moa, and moe*) are located near the *uvrB* gene in *Escherichia coli* and *Salmonella*, providing a convenient means by which to eliminate nucleotide excision repair in these bacteria [Shanmugan et al., 1992; Rajagopalan, 1996].

When the Δ*uvrB* alleles were introduced into what would eventually become the Ames tester strains, all that was known was that each mutation resulted in a single deletion through at least the galactose operon, biotin operon, *uvrB* gene, and chlorate-resistance genes, making the Ames tester strains double auxotrophs for both histidine and biotin [Ames, 1971]. For the next 30 years, nothing more was known about the size of these deletions or other genes that were missing until we used genomic hybridization to show that the Ames strains are missing 15-119 genes due to the Δ*uvrB* mutations [Porwollik et al., 2001]. In particular, strain TA98 is missing 119 genes (125 kb of DNA or 2.6% of the genome), and TA100 is missing 47 genes (50 kb of DNA or 1.9% of the genome). Among the questions raised by this finding is whether the enhanced sensitivity of the Ames strains to various mutagens is due to the elimination of the nucleotide excision repair system alone or to the deletion of other genes.

Although similarly derived Δ*uvrB* mutations in *E. coli* have not been fully characterized with regard to the extent of the deletions, studies by one of us (RMS) revealed that such *E. coli* Δ(*uvrB-bio*) strains were hypersensitive (both in terms of killing and mutagenesis) to the base analogues 6-hydroxylaminopurine (HAP) and 2-amino-6-hydroxylaminopurine (AHAP) [Pavlov et al., 1996; Kozmin et al., 2000]. In contrast, strains containing a point mutation in either *uvrA* or *uvrB* were not hypersensitive, indicating that the hypersensitivity does not result from the lack of nucleotide excision repair [Pavlov et al., 1996; Kozmin et al., 2000]. Instead, hypersensitivity was shown to result directly from the loss of the genes for molybdenum cofactor biosynthesis [Kozmin et al., 2000].

To explore this observation in *Salmonella* and to expand the analysis to mutagens other than base analogues, we constructed homologues of the two main Ames tester strains, the frameshift strain TA98 and the base-substitution strain TA100, but carrying single-gene mutations in either *uvrB*, *moeA*, or *moaA*, or in both *uvrB* and *moeA*, instead of their respective Δ(*gal-bio-uvrB*) mutations. In addition to HAP and AHAP, we also tested the nitroarene 1-nitropyrene (1-NP), the heterocyclic amine Glu-P-1, the aromatic amines 2-acetylaminofluorene (2-AAF) and 4-aminobiphenyl (4-AB), the polycyclic aromatic hydrocarbon benzo[*a*]pyrene (BaP), and the chlorinated organic 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX), which is the primary mutagen in chlorinated drinking water. Four are direct-acting mutagens (HAP, AHAP, 1-NP, and MX), and four require metabolic activation (Glu-P-1, 2-AAF, 4-AB, and B[*a*]P).

The results were analyzed to assess the influence of the various mutations on spontaneous mutant frequency and the mutagenic potencies of the various mutagens relative to the corresponding TA98 and TA100 tester strains. We assessed whether the missing genes in the Ames Δ*uvrB* strains actually enhanced the mutagenic sensitivity of these strains relative to those containing a mutation in only the *uvrB* gene. Finally, we identified some human homologues among the 119 genes deleted by Δ*uvrB* in TA98 and discuss the possible role that mutations in these genes may play in human disease.

MATERIALS AND METHODS

Chemicals

Glu-P-1 was obtained from Toronto Research Chemicals (North York, ON, CA), and HAP was obtained from MP Biomedicals (Solon, OH). 1-NP, 2-AAF, 4-AB, and BaP were purchased from Sigma (St. Louis, MO). MX was a gift from R. Franzén, Tampere University of Technology, Tampere, Finland. AHAP was a gift from I. Kuchuk, Indiana University. All of the mutagens were dissolved in dimethyl sulfoxide (DMSO, Burdick and Jackson, Muskegon, MI).

Strains

Strain TA100 [*hisG46 chl-1005* (*bio uvrB gal*) *rfa-1001* pKM101$^+$ Fels-1$^+$ Fels-2$^+$ Gifsy-1$^+$ Gifsy-2$^+$] and TA98 [*hisD3053 chl-1008* (*bio uvrB gal*) *rfa-1004* pKM101$^+$ Fels-1$^+$ Fels-2$^+$ Gifsy-1$^+$ Gifsy-2$^+$] were obtained from B.N. Ames, Oakland Children's Hospital, Oakland, CA. All of the other strains had the same genotype as either TA98 or TA100 except that Δ*uvrB* mutation (*bio uvrB gal*) was replaced with a single-gene mutation in either the *uvrB, moeA,* or *moaA* genes or in both the *uvrB* and *moeA* genes (Table I).

Strain Construction

All new tester strains were constructed starting from *S. typhimurium* LT2 strains HisG46 and HisD3052 provided by Dr. T. Cebula (US FDA). These are the original parents of the TA100 and TA98 tester strains and carry the *hisG46* and *hisD3052* markers, respectively [Ames, 1971; Popkin et al., 1989]. Phage P22 HT 12/4 *int* used for transductions of *Salmonella* strains was obtained from Dr. R. Maurer (Case Western Reserve University). P22 transductions were performed as described in

TABLE I. Strains Constructed for Use in This Study

TA98 homologues[a]		TA100 homologues[b]	
Strain	Relevant defect	Strain	Relevant defect
SN13504	*moeA*	SN13497	*moeA*
SN13506	*moaA*	SN13499	*moaA*
SN15944	*uvrB*	SN15939	*uvrB*
SN15948	*uvrB moeA*	SN15942	*uvrB moeA*

[a]The genotype of frameshift strain TA98 is *hisD3053 chl-1008* (*bio uvrB gal*) *rfa-1004* pKM101$^+$ Fels-1$^+$ Fels-2$^+$ Gifsy-1$^+$ Gifsy-2$^+$. The TA98 homologues have the noted single-gene mutations instead of the *chl-1008* (*bio uvrB gal*) mutation, which is the Δ*uvrB* mutation.

[b]The genotype of base-substitution strain TA100 is *hisG46 chl-1005* (*bio uvrB gal*) *rfa-1001* pKM101$^+$ Fels-1$^+$ Fels-2$^+$ Gifsy-1$^+$ Gifsy-2$^+$. The TA100 homologues have the noted single mutations instead of the *chl-1005* (*bio uvrB gal*) mutation, which is the Δ*uvrB* mutation.

Sternberg and Maurer [1991]. Transductions in *E. coli* and in rough (*galE*) strains of *Salmonella* were performed using a laboratory stock of P1*virA*. Phage C21, used for selecting deep-rough (*waa*, previously called *rfa*) variants, was purchased from the NCCB (Netherlands Culture Collection of Bacteria, Utrecht, The Netherlands). *Salmonella* strains TT4799 (*bio-101*::Tn*10*, *galE*) and TT22971 (*metA22 metE551 trpD2 ilv-452 leu pro hsdLT6 hsdSA29 hsdB strA120* [pKD46]) were obtained from Dr. J. Roth (University of California at Davis). *Salmonella* strains RM2721 (*galE496*, *metE55*, *rpsL120*, *xyl-404*, *H1-b*, *H2-e,n,x*, *fliB*, *ilv452*, *hdsL6*(r$^-$m$^+$), *hsdSA29*(r$^-$m$^+$), Fels-2$^-$) and RM2725 (as RM2721, but *mutL*::Tn*10*) were obtained from Dr. R. Maurer (Case Western Reserve University). *Salmonella* strain TAC22 (*trpB223*, pKM101) was obtained from Dr. T. Cebula (US FDA). *E. coli moeA* or *moaA* donor strains NR12383 (*moeA121*::mini-Tn*10cam*) [Kozmin et al., 2000] and NR13488 (*moaA130*::mini-Tn*10cam*) [unpublished data] were derived in the author's (RMS) laboratory.

The Δ*uvrB 501*::*kan* allele was created in *Salmonella* strain TT22972 using the PCR-based gene replacement method of Datsenko and Wanner [2000]. The PCR primers used contained 50-nt extensions complementary to the left or right end of the gene to be deleted as well as a 20-nt priming 3′ end complementary to the kanamycin-resistance (Kan^r) of plasmid pKD13 [Datsenko and Wanner, 2000]. The genomic base-pair coordinates of the left and right endpoints of the deletion were 864,663 and 866,297, respectively, according to the published sequence of the *Salmonella typhimurium* genome [McClelland et al., 2001, GenBank record NC_003197] and correspond to deletion of residues 76-622 of the UvrB protein. Selection was for kanamycin resistance. The presence of the deletion in new isolates was confirmed by PCR followed by testing for sensitivity to UV light. The new *uvrB* allele, designated Δ*uvrB 501*::*kan*, was then transferred by transduction using phage P22 into HisG46 and HisD3052 yielding SN15820 (*hisG46* Δ*uvrB*) and SN15821 (*hisD3052* Δ*uvrB*). The latter two strains were then used as donors to introduce this Δ*uvrB* allele into the new tester strains, as described later.

First, HisG46 and HisD3052 were converted to their *bio* derivatives by P22-mediated transduction using *Salmonella* donor strain TT4799 (*bio-101*::Tn*10*, *galE*) using selection for tetracycline resistance. These strains were then converted to corresponding *bio*$^+$ *galE496* derivatives by P22-mediated transduction using as RM2721 as donor, selecting for growth on minimal medium lacking biotin. The new *galE* derivatives were named SN13394 (*hisG46 galE496*) and SN13396 (*hisD3052 galE486*). The *galE* strains displayed the expected rough phenotype as evidenced by their acquired sensitivity to bacteriophage P1 when grown in LB medium and their retained sensitivity to P22 when grown in the presence of galactose. The *galE* derivatives were used to introduce the *moaA* and *moeA* alleles present in *E. coli* strains NR13488 and NR12383, respectively, by P1 transduction. First, to overcome the restriction and homeology barriers between *E. coli* and *Salmonella*, the *moeA* and *moaA* alleles were transferred by P1 transduction into RM2725, in which the cross-species transductions proceeded with good efficiency [Rayssiguier et al., 1989]. The presence of the *moa* or *moe* allele was confirmed by sensitivity to the base analog HAP resulting from their lack of molybdenum cofactor [Kozmin et al., 2000; Kozmin and Schaaper, 2007]. To minimize retention of linked *E. coli* sequences, the *moaA* and *moeA* alleles were cycled through RM2721 or RM2725 for an additional four or five times by repeated, sequential P1 transductions.

P1-mediated transfer of the *moaA* or *moeA* alleles into SN13394 (*hisG46*) or SN13396 (*hisD3052*) then yielded the following strains: SN13465 (*hisG46 moaA*), SN13470 (*hisG46 moeA*), SN13466 (*hisD3052 moaA*), and SN13474 (*hisD3052 moeA*). These strains and their parents were then made Δ*uvrB* by P22-mediated transduction using SN15820 and SN15821 as donors. Selection was for kanamycin resistance; the new transductants were confirmed by their UV sensitivity. In this manner we created the following strains: SN15916 (*hisG46* Δ*uvrB*), SN15919 (*hisG46 moaA* Δ*uvrB*), SN15920 (*hisG46 moeA* Δ*uvrB*), SN15922 (*hisD3052* Δ*uvrB*), SN15924 (*hisD3052 moaA* Δ*uvrB*), and SN15926 (*hisD3052 moeA* Δ*uvrB*).

Deep-rough (*waa*, formerly called *rfa*) variants of the various strains were selected on the basis of their resistance to phage C21. LB plates were spread with 0.1 ml (~10^8) C21 phage. After the plates had dried thoroughly, 0.1 ml of an overnight LB culture of a bacterial strain was spread on top of the phage. Plates were incubated overnight at 37°C. Typically, the next day these plates contained 2–50 phage-resistant colonies. Several of these were re-streaked on a C21-containing LB plate, and well-growing colonies were purified one more time on an LB plate without C21. Deep-rough mutants among them were then identified based on their sensitivity to crystal violet. The crystal violet test was performed by adding 10 ml of a crystal violet solution (10 mg/ml) to a small filter disk placed in the center of an LB plate on which the strain of interest had been plated in a soft-agar layer. Under these conditions, *waa* mutants show a clear zone of killing or nongrowth, whereas *waa*$^+$ strains are not significantly inhibited. In this manner we obtained the following strains: SN13439 (*hisG46 waa-117*), SN13419 (*hisG46 moaA waa-101*), SN13454 (*hisG46 moeA waa-125*), SN15928 (*hisG46* Δ*uvrB waa-141*), SN15930 (*hisG46 moaA* Δ*uvrB waa-145*), SN15931 (*hisG46 moeA* Δ*uvrB waa-149*), SN13441 (*hisD3052 waa-121*), SN13421 (*hisD3052 moaA waa-109*), SN13458 (*hisD3052 moeA waa-133*), SN15933 (*hisD3052* Δ*uvrB waa-153*), SN15935 (*hisD3052 moaA* Δ*uvrB waa-157*), and SN15937 (*hisD3052 moeA* Δ*uvrB waa-161*).

Finally, plasmid pKM101 was added to these constructs by mating with plasmid pKM101 donor strain TAC22 and selecting conjugants on minimal-medium plates containing histidine and ampicillin (100 mg/ml). In this manner, we obtained the following strains: SN13495 (*hisG46 waa-117* pKM101), SN13499 (*hisG46 moaA waa-101* pKM101), SN13497 (*hisG46 moeA waa-125* pKM101), SN15939 (*hisG46* Δ*uvrB waa-141* pKM101), SN15941 (*hisG46 moaA* Δ*uvrB waa-145* pKM101), SN15942 (*hisG46 moeA* Δ*uvrB waa-149* pKM101), SN13502 (*hisD3052 waa-121* pKM101), SN13506 (*hisD3052 moaA waa-109* pKM101), SN13504 (*hisD3052 moeA waa-133* pKM101), SN15944 (*hisD3052* Δ*uvrB waa-153* pKM101), SN15946 (*hisD3052 moaA* Δ*uvrB waa-157* pKM101), and SN15948 (*hisD3052 moeA* Δ*uvrB waa-161* pKM101).

Mutagenesis

The strains were grown overnight (16 h) in Oxoid nutrient broth No. 2 and used with or without S9 mix (Aroclor-induced rat liver S9 from Moltox, Boone, NC) in the standard plate-incorporation assay [Maron and Ames, 1983]. Each compound was tested at least twice (and sometimes up to four times) per strain at 2 plates per dose. Three plates were used for all DMSO controls. Positive controls were omitted in a formal sense because most of the mutagens used are positive controls or are well-established mutagens in TA98 and TA100. All experimental runs

TABLE II. Spontaneous Mutant Yields (Mutant Frequencies) and *P*-Values Comparing Strains for +S9 (Top and Above Diagonal) and −S9 (Left and Below Diagonal)

		Strain (mutation)				
		TA98 (Δ*uvrB*)	SN13504 (*moeA*)	SN13506 (*moaA*)	SN15944 (*uvrB*)	SN15941 (*uvrB moeA*)
Frameshift	Mean ± SE[a]	36 ± 2	26 ± 3	30 ± 3	34 ± 4	36 ± 5
TA98 [Δ(*gal-bio-uvrB*)]	25 ± 2	– – – –	0.003	0.07	0.61	0.97
SN13504 (*moeA*)	16 ± 3	0.005	– – – –	0.19	0.12	0.07
SN13506 (*moaA*)	16 ± 3	0.006	0.99	– – – –	0.47	0.30
SN15944 (*uvrB*)	24 ± 3	0.62	0.07	0.07	– – – –	0.73
SN15948-1 (*uvrB moeA*)	18 ± 4	0.06	0.67	0.68	0.20	– – – –
		TA100 (Δ*uvrB*)	SN13497 (*moeA*)	SN13499 (*moaA*)	SN15939 (*uvrB*)	SN15942 (*uvrB moeA*)
Base substitution	Mean ± SE[a]	111 ± 3	18 ± 3	32 ± 3	83 ± 5	94 ± 6
TA100 [Δ(*gal-bio-uvrB*)]	103 ± 2	– – – –	<0.0001	<0.0001	<0.0001	0.007
SN13497 (*moeA*)	14 ± 3	<0.0001	– – – –	0.0005	<0.0001	<0.0001
SN13499 (*moaA*)	33 ± 3	<0.0001	<0.0001	– – – –	<0.0001	<0.0001
SN15939 (*uvrB*)	87 ± 4	0.0004	<0.0001	<0.0001	– – – –	0.14
SN15948-2 (*uvrB moeA*)	76 ± 4	<0.0001	<0.0001	<0.0001	0.05	– – – –

[a]Mean revertants/plate ± standard error from mixed model: means to the right correspond to +S9; means below to −S9. These spontaneous mutant yields are the same as the spontaneous mutant frequencies, where the units would be revertants/10^8 survivors. For comparison, the spontaneous mutant frequencies (average of +S9 and −S9) of the homologous strains having only *his* and *rfa* mutations and the pKM101 plasmid are 19 for the TA98 homologue (UTH8413) and 33 for the TA100 homologue (TA92) [DeMarini, 2000].

used a parental strain (TA98 and TA100) and their respective homologues. Plates were incubated for 3 days, and revertants (rev) were counted on an AccuCount 1000 automated colony counter (BioLogics, Manassas, VA). 1-NP, MX, HAP, and AHAP were tested without S9 mix, whereas BaP, 4-AB, 2-AAF, and Glu-P-1 were tested with S9 mix.

Statistical Analysis

We conducted a separate analysis for each mutagen. Each analysis used data from all 10 strains and multiple experimental runs (8 each for AHAP, 4-AB, and MX; 9 each for 2-AAF and Glu-P-1; and 10 for the remaining compounds) conducted on different dates. Each experimental run involved several, though possibly different, dose levels and only a subset of the strains. Each compound-strain-run-dose combination used 2 or 3 replicate plates. We modeled the counts from each plate using generalized linear mixed models assuming a Poisson error distribution with the identity link function. We modeled the expected response for each plate as a linear function of dose with a separate intercept and slope for each strain (after omitting data from high dose levels where the response leveled off). We considered run, any interactions involving run, and replicate plates as possible sources of extra-Poisson variation.

The strain-specific slope parameters in each analysis represent the strain-specific potencies of the respective compound (rev/μg). Our modeling approach appropriately integrates all the individual experimental runs with possibly different numbers of dose levels and different subsets of strains to provide a single potency estimate for each strain for each compound. The variance reported for the potency estimates takes account of all sources of extra-Poisson variation mentioned earlier. We used SAS software (SAS Institute, Cary, NC), in particular the GLIMMIX macro, to fit these models <http://support.sas.com/ctx/samples/index.jsp?sid=536>.

The spontaneous mutant yields (rev/plate) for all of the strains were calculated from all the control (DMSO) plates from all of the experiments, using a Poisson mixed model. We modeled the expected response for each plate using a factorial analysis-of-variance approach: main effects for strain (10 levels) and S9 (2 levels) as well as interaction terms. As before, we considered run, any interactions involving run, and replicate plates as possible sources of extra-Poisson variation.

Identification of Human Homologues of Deleted *Salmonella* Genes

We identified the human genes that are homologous to the 119 genes deleted in TA98 by comparing the amino acid sequences of the resulting proteins available in databases maintained by the National Center for Biotechnology Information at the National Institutes of Health. Thus, the GeneID number for each of the 119 genes missing from TA98 was obtained from the *S. typhimurium* LT2 complete genome protein table at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genome&cmd=Retrieve&dopt=Protein+Table&list_uids=202. The protein reference sequences keyed to the GeneID numbers for the identified genes were then submitted to protein-protein BLAST (http://www.ncbi.nlm.nih.gov/BLAST/beta/Blast.cgi?PAGE=Proteins&PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on). The protein sequence was used to search the swissprot database (see above URL) for human homologues. Human homologues with *e*-values $<10^{-25}$ were examined in Online Mendelian Inheritance in Man (OMIM) for human disorders associated with nonfunctional genes (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM).

RESULTS

Spontaneous Mutant Yields (Mutant Frequencies)

The raw data are available online at the following URL (http://www.interscience.wiley.com/jpages/0893-6692/suppmat). Table II shows the estimated mean of the spontaneous mutant yields (rev/plate), which are also mutant frequencies (rev/10^8 treated cells or 10^8 survivors), for all of the strains. *P*-values compare the spontaneous mutant yields among strains of one allele either with or without the addition of S9 mix. Strain-to-strain differences are more common at the base substitution allele than at the frameshift allele, regardless of the presence or absence of S9.

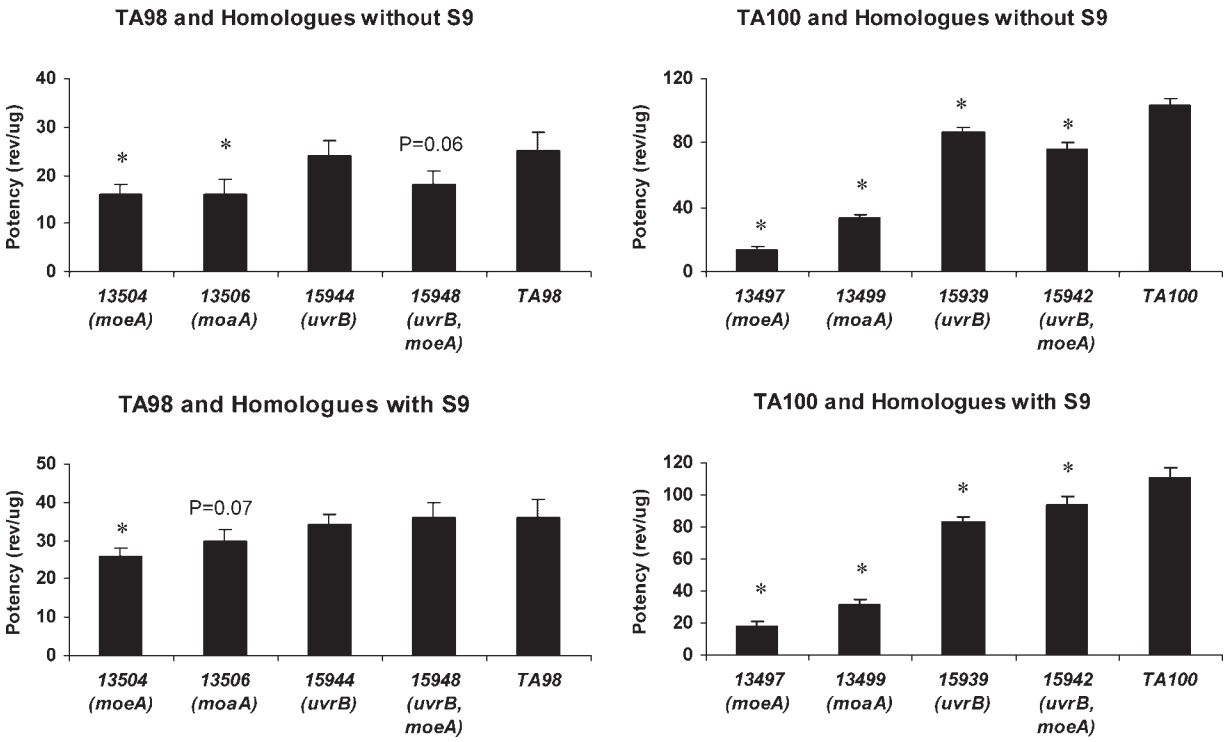

Fig. 1. Average (± SE) spontaneous mutation frequencies of TA98 and TA100 with and without S9 as estimated by a Poisson mixed model. *, significantly different from Ames homologues at $P < 0.05$.

Moreover, the addition of S9 increased significantly ($P < 0.05$) the spontaneous mutant frequencies of TA98 and all of its homologues as well as those of TA100 and one of its homologues, SN15942 (*uvrB moeA*) (statistics not presented).

In the absence or presence of S9, the spontaneous mutant frequency of TA98 was not significantly different from that of a strain with the single *uvrB* mutation or the strain with the combined single *uvrB* and *moeA* mutations (Table II). This observation suggests that the absence of nucleotide excision repair alone accounts for essentially all of the spontaneous mutant frequency of TA98 due to the extended Δ*uvrB* mutation, whereas absence of the remaining genes may contribute little to the spontaneous mutant frequency of this strain. Single defects in either *moeA* or *moaA* produced significantly lowered spontaneous mutant frequencies relative to TA98 in the absence of S9, as did *moeA* in the presence of S9.

In contrast, the spontaneous mutant frequency of the base-substitution strain TA100 differed significantly from all of its homologues containing single-gene defects (Table II). Thus, a single defect in *moeA, moaA,* or *uvrB,* or in *uvrB* and *moeA* together, significantly lowered the spontaneous mutant frequency relative to TA100. These results indicate that at the base-substitution allele, the spontaneous mutant frequency observed in TA100 is due not only to the deletion of *uvrB* but also to the absence of molybdenum cofactor biosynthesis, as well as possibly other genes in the extended Δ*uvrB* mutation, which involves the deletion of 47 genes.

Considering all possible strain comparisons at the base-substitution allele, all comparisons with one exception (*uvrB* vs. *uvrB moeA* in the presence of S9) are significantly different from each other. This pattern clearly indicates the important contribution that the deletion of each gene (*uvrB, moeA,* or *moaA*) plays in the spontaneous mutant frequency at the base-substitution allele. In contrast, at the frameshift allele, strains carrying mutations in only the molybdenum cofactor biosynthesis genes have significantly lower spontaneous frequencies: TA98 vs. *moeA* or *moaA* (-S9) and TA98 vs. *moeA* (+S9). Therefore, as long as the *uvrB* mutation is present, the spontaneous mutant frequency is not significantly different from TA98.

A tendency exists, however, for the spontaneous mutant frequency to be lower in the double mutant (*uvrB moeA*) than in either the single *uvrB* or Δ(*gal-bio-uvrB*) strain for both the frameshift and the base-substitution alleles when S9 is not used. With the addition of S9, the spontaneous mutant frequency of the double mutant increased and became identical (frameshift allele) or similar (base-substitution allele) to the Δ(*gal-bio-uvrB*) strain (Fig. 1). Thus, *moeA* defects tend, overall, to lower the spontaneous mutant frequency, even when combined with a *uvrB* defect, and this tendency is reversed somewhat with the addition of S9, especially at the frameshift allele. As a

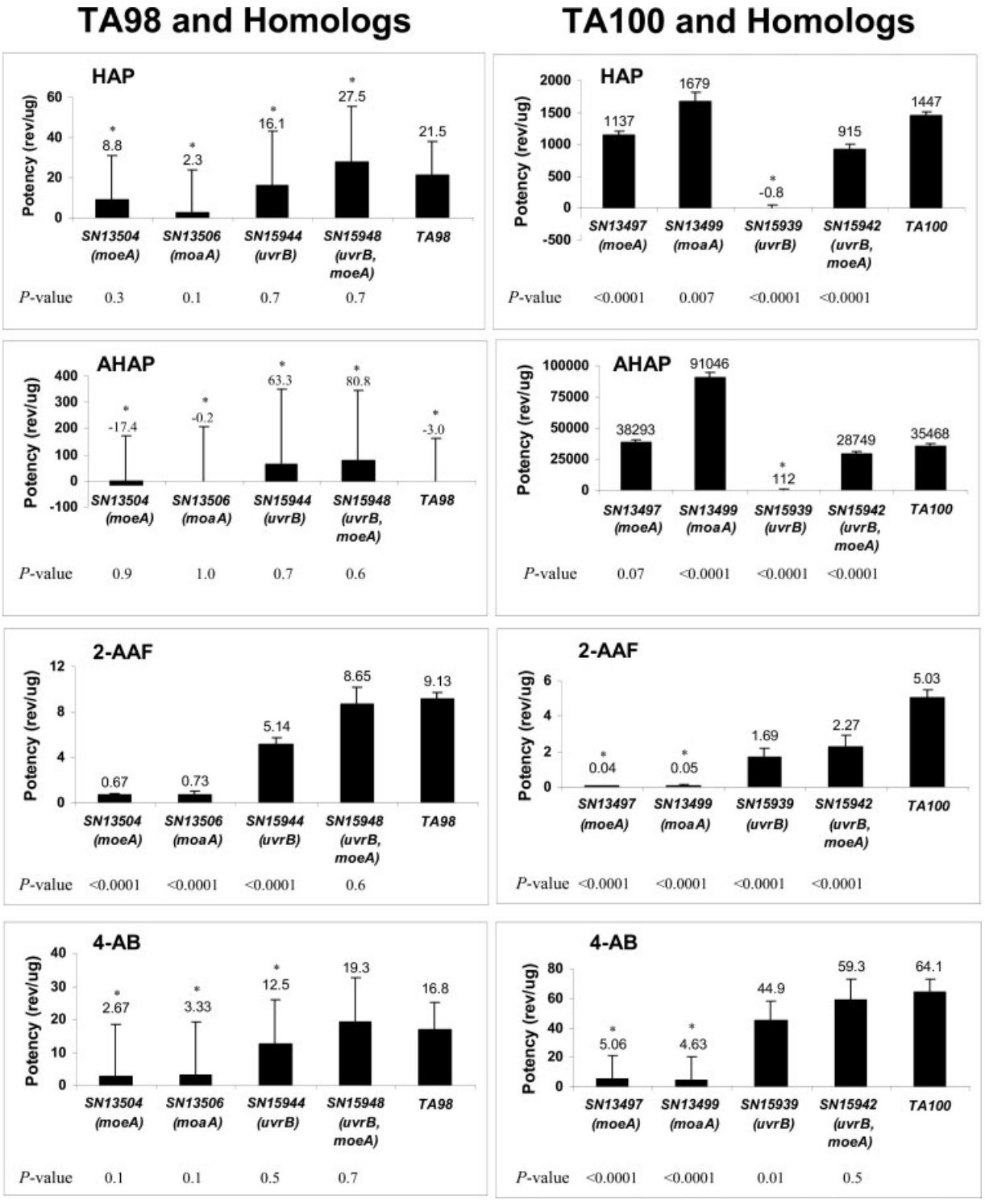

Fig. 2. Average mutagenic potencies (rev/μg) of all mutagens tested in all strains as estimated by a Poisson mixed model. Error bars show the upper 95% confidence limit for each mean. *P*-values refer to comparisons of an Ames strain to each of its homologues; *, not significantly different from zero (i.e., not mutagenic) at $P < 0.05$. The vertical scales differ among the panels for illustrative purposes. (*Continued on next page*)

result, molybdenum cofactor biosynthesis genes may play a role in determining mutagenic potency even in TA98 and/or may be a factor in the increased spontaneous mutant frequency seen with the use of S9.

Mutagenic Potencies of Base Analogues

The potencies of HAP and AHAP in TA98 and its homologues tended to vary considerably about the mean

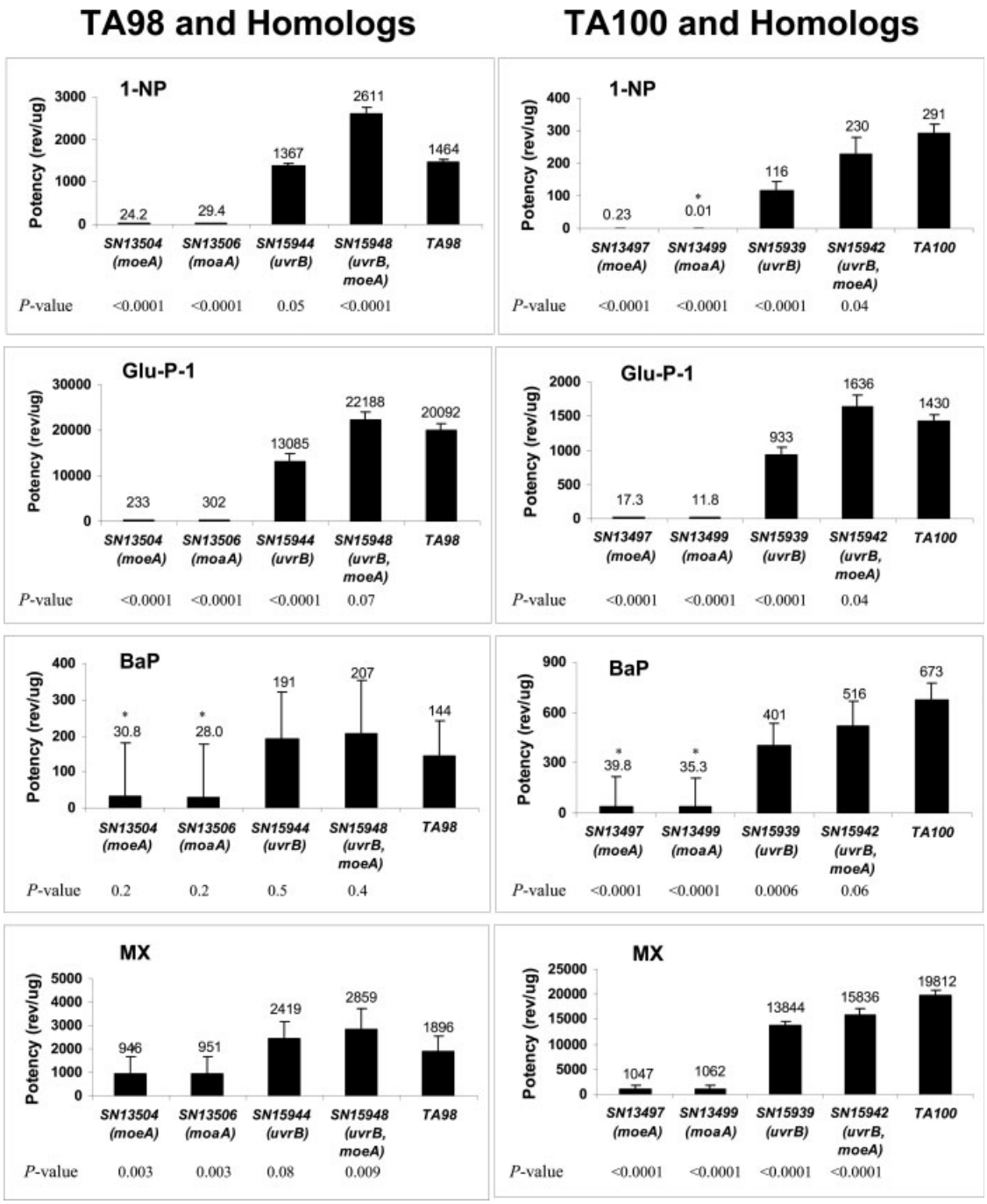

Fig. 2. (*Continued*)

(Fig. 2). Nonetheless, the base analogues HAP and AHAP were generally not mutagenic in strains containing the frameshift allele.

At the base-substitution allele, HAP and AHAP produced results similar to those obtained by Kozmin et al. [2000] in *E. coli*. Both compounds were mutagenic only when a molybdenum cofactor biosynthesis gene (*moeA* or *moaA*) was mutated, and both compounds produced their highest mutagenic potencies in a strain containing a single defect in only the *moaA* gene (Fig. 2). Neither compound was mutagenic in a strain containing a mutation in only the *uvrB* gene (Fig. 2). These results indicate that the absence of the 47 genes in TA100 reduces the mutagenic potency of the base analogues compared to their potency in a strain mutant in only the molybdenum operons. Thus, as in *E. coli*, these base analogues are mutagenic in a

TABLE III. Relative Influence of Single-Gene Defects on Mutagenic Potencies Compared to Δ(*gal-bio-uvrB*)

	TA98 [Δ(*gal-bio-uvrB*)]			TA100 [Δ(*gal-bio-uvrB*)]		
Mutagen	*moeA* or *moaA*	*uvrB*	*uvrB, moeA*	*moeA* or *moaA*	*uvrB*	*uvrB, moeA*
HAP	NM[a]	NM	NM	↑	↓	↓
AHAP	NM	NM	NM	↑	↓	↓
1-NP	↓	↓	↑	↓	↓	↓
MX	↓	=	↑	↓	↓	↓
BaP	=	=	=	↓	↓	↓
4-AB	=	=	=	↓	↓	=
2-AAF	↓	↓	=	↓	↓	↓
Glu-P-1	↓	↓	=	↓	↓	↑

[a]NM, not mutagenic.

Δ*uvrB* strain of *Salmonella* not because of the absence of nucleotide repair but because of the absence of molybdenum cofactor biosynthesis.

Mutagenic Potencies of Nonbase Analogues

The mutagenic potencies of the nonbase analogues varied widely across frameshift strains (Fig. 2), but most nonbase analogues were mutagenic in every strain. 4-AB and BaP were exceptions: both were nonmutagenic ($P > 0.67$) in strains *moeA* (SN13504) and *moaA* (SN13506), and 4-AB was nonmutagenic ($P = 0.06$) in strain *uvrB* (SN15944). The latter is the only instance in which a nonbase analog was statistically not mutagenic in a strain containing a deletion in the *uvrB* gene and was most likely due to the high variability about the mean. Even with this high variability, the *P*-value approached significance; therefore, it is likely that 4-AB is weakly mutagenic in SN15944.

At the frameshift allele, 2-AAF, 1-NP, and Glu-P-1 were significantly less mutagenic in the *uvrB* strain (SN15944) relative to TA98 (Fig. 2). Thus, the deletion of genes in addition to those involved in nucleotide excision repair accounted for the potencies of these compounds in TA98. In contrast, 4-AB, BaP, and MX had mutagenic potencies that were not significantly different in the *uvrB* strain (SN15944) relative to TA98, suggesting that the deletion of *uvrB* alone may account for the potencies of these compounds in TA98. 2-AAF, 1-NP, Glu-P-1, and MX were less mutagenic in strains containing a defect in *moeA* (SN13504) or *moaA* (SN13506) relative to TA98. In contrast, 4-AB and BaP had mutagenic potencies in *moeA* or *moaA* strains that were not significantly different from those in TA98. However, this may be due to the presence of high variability about the mean because the trend is for much lower potencies in *moeA* or *moaA* strains compared to *uvrB* strains even for these compounds.

At the frameshift allele, nonbase analogues tended to exhibit higher mutagenic potencies in the double mutant (*uvrB moeA*) compared with TA98. However, this was significant only in the direct-acting (those requiring no metabolic activation) compounds 1-NP and MX. The indirect acting (those requiring metabolic activation with S9) compounds 2-AAF, 4-AB, Glu-P-1, and BaP, did not have significantly different potencies in the double mutant than in TA98. This suggests that the deletion of one or more of the 119 genes of the Δ(*gal-bio-uvrB*) in TA98 may actually interfere with the ability of these compounds to exhibit their full mutagenic potency at the frameshift allele and that the interference is lessened with the addition of S9.

The mutagenic potencies of the nonbase analogues varied widely across base-substitution strains (Fig. 2), but only Glu-P-1 and MX were mutagenic in every strain. 2-AAF, 4-AB and BaP were nonmutagenic ($P > 0.07$) in the strains *moeA* (SN13504) and *moaA* (SN13506), and 1-NP was not mutagenic in strain *moaA* ($P = 0.75$).

At the base-substitution allele, 2-AAF, 1-NP, BaP, and MX were significantly less mutagenic in the single *uvrB* mutant strain (SN15939) than in TA100, suggesting that the deletion of other genes in conjunction with *uvrB* accounts for the potency of these compounds in TA100. Also at the base-substitution allele, the mutagenic potencies of all of the nonbase analogues were less in strains with single defects in *moeA* (SN13497) or *moaA* (SN13499) compared with TA100. This observation indicates that the absence of the molybdenum cofactor synthesis genes alone does not account for the mutagenic potencies of the nonbase analogues in TA100. The mutagenic potencies of 2-AAF, 1-NP, BaP, and MX were also less in a strain having mutations in both *moeA* and *uvrB* (SN15942) relative to TA100. Thus, the deletion of additional genes beyond those involved in nucleotide excision repair and molybdenum cofactor synthesis accounts for the mutagenic potencies of these compounds in TA100. In contrast, the mutagenic potency of 4-AB in strain SN15942 was not significantly different than in TA100, and Glu-P-1 was more mutagenic in strain SN15942 than in TA100. The relative influence of the single-gene defects on the mutagenic potencies of all of the mutagens compared to the Δ(*gal-bio-uvrB*) strains is shown in Table III.

Human Homologues of Deleted *Salmonella* Genes

At least 17 of the 119 genes deleted in TA98 are homologous to human genes (Table IV). Among these are genes involved in molybdenum cofactor biosynthesis, whose deletion clearly increased some induced mutagenicity in *Salmonella*. As discussed later, deficiency in this activity also is associated with severe human disease [Lee et al., 2002].

DISCUSSION

Spontaneous Mutation

At the base-substitution allele, none of the single-gene mutation strains had spontaneous mutant frequencies as high as TA100. Thus, deletion of additional genes plays a role in the spontaneous mutant frequency of TA100. In contrast, the *uvrB* frameshift strain had a spontaneous mutant frequency that was not significantly different from that of TA98. Thus, deletion of *uvrB* alone appears to be sufficient to account for the effect of the Δ(*gal-bio-uvrB*) mutation at the frameshift allele. The situation is likely more complex, with the deletion of various genes (119 in TA98) having a net influence on the spontaneous mutant frequency.

As discussed below under mechanistic considerations, the absence of some of the genes deleted by Δ(*gal-bio-uvrB*) in TA98 and TA100 may reduce DNA repair and replication fidelity as well as detoxification of DNA-reactive agents. Consequently, the loss of such mechanisms would likely increase the spontaneous mutant frequency as well as induced mutagenic sensitivity. The differential effects of the deletions on spontaneous mutant frequencies at the frameshift vs. base-substitution alleles are unclear, especially considering the many other deleted genes whose effects we have not yet explored systematically. Nonetheless, our results clearly show that genes other than *uvrB* influence the spontaneous mutant frequency of the Δ*uvrB* strains of the Ames *Salmonella* tester set.

Base Analogues

Elimination of genes in the molybdenum cofactor biosynthesis pathway is both necessary and sufficient to account for the mutagenicity of the base analogs HAP and AHAP in TA100. These compounds are not mutagenic in a strain containing a single *uvrB* defect. This result confirms previous observations in *E. coli* [Kozmin et al., 2000]. Thus, the mutagenic activity of these compounds in TA100 is not due to the deletion of *uvrB* but to the deletion of *moeA* or *moaA* in the Δ(*gal-bio-uvrB*) mutation of TA100.

Of the two molybdenum cofactor biosynthesis pathway genes examined, the absence of *moaA* appears to play the major role in enhancing mutagenic potency. Absence of *moaA* resulted in a significant increase in potency over that of TA100, especially for AHAP, whereas the absence of *moeA* resulted in potency values that were either not different or, in fact, significantly lower than that of TA100. The *moa* operon codes for the synthesis of molybdopterin, a key protein in molybdenum cofactors of all organisms, whereas genes in the *moe* operon appear to be involved in the transfer of sulfur groups to molybdenum or other components of the cofactor complex [Rajagopalan, 1996]. The molybdoenzymes play a general role in protecting against *N*-hydroxylated base analogs and related compounds; thus, their deletion removes this protection, enhancing mutagenesis by these agents.

Nonbase Analogues

As noted by Rosenkranz and Mermelstein [1983], *N*-hydroxyl compounds (hydroxylamines) are the active intermediates for the mutagenicity of both aromatic nitro compounds (by reduction) and aromatic amines (by oxidation). Our results with compounds in these chemical classes, which are HAP, AHAP, 1-NP, Glu-P-1, 2-AAF, and 4-AB, support this idea. In contrast, the mutagenic potencies of the other compounds were much less in strains having single *moeA* or *moaA* defects than in TA98 or TA100. In general, the mutagenic potencies of these compounds were similar in each Δ(*gal-bio-uvrB*) strain and its homologue with a single *uvrB* defect. Potencies were increased further by the additional mutation in a molybdenum cofactor biosynthesis gene. Thus, the elimination of the nucleotide excision repair pathway appears to play a significant role in the enhanced mutagenic potency of most compounds in strains containing the Δ(*gal-bio-uvrB*) mutation.

Mutagenic potencies of the compounds other than HAP and AHAP in strains containing mutations in both *uvrB* and *moeA* were higher than in strains with single defects in either *uvr*B or *moeA* alone. In TA98 homologues, the absence of both *uvrB* and *moeA* often resulted in potency enhancement relative to the Δ(*gal-bio-uvrB*) homologue (TA98), indicating that there is some interference with mutagenic enhancement caused by some of the other genes deleted by the Δ(*gal-bio-uvrB*) mutation. This interference does not, however, diminish the ability of TA98 to identify mutagens. Conversely, although the same potency enhancement by *uvrB* and *moeA* occurs in TA100 homologues, the potency levels do not reach those attained in TA100 itself, indicating that the absence of additional genes is required to produce the mutagenic potency observed in TA100.

Mechanistic Considerations

It may seem counter-intuitive that the deletion of many genes would enhance the mutagenic sensitivity of an organism. How might missing genes enhance mutagenic sensitivity beyond the deletion of *uvrB*? Ames [1971] selected strains that were the most sensitive to a variety

TABLE IV. Homologous Human Genes among the 119 Genes Deleted by Δ*uvrB* in Strain TA98 of *Salmonella*

Salmonella gene identifier	Score (bits)	*E*-value	*Salmonella* gene symbol	*Salmonella* NP accession number	Human gene name	Disease due to nonfunctional gene	Human accession number
STM0858	577	5.30 E−163		459835	Electron-transferring-flavoprotein dehydrogenase	glutaric aciduria II	Q16134
STM0776[a]	368	1.00 E−101	*galE*	459755	UDP-glucose 4-epimerase	galactose epimerase deficiency	Q14376
STM0791[a]	362	2.00 E−99	*hutH*	459769	Histidine ammonia-lyase	histidinemia	P42357
STM0820[a]	262	2.00 E−69	*rhlE*	459798	ATP-dependent RNA helicase (*DDX3X*)		O00571
STM0838	253	9.00 E−67	*ybiT*	459815	ATP-binding cassette, sub-family F, member 3		Q9NUQ8
STM0855	237	3.00 E−62		459832	Electron transfer flavoprotein subunit beta (Beta-ETF).	glutaric aciduria II	P38117
STM0802[a]	219	1.00 E−56	*moaA*	459780	Molybdenum cofactor biosynthesis protein 1B (*MOCS1B*)	molybdenum cofactor deficiency	Q9NZB8
STM0846	178	4.00 E−44	*moeA*	459823	Gephyrin	neurological condition hyperplexia, molybdenum cofactor deficiency	Q9NQX3
STM0795[a]	172	1.00 E−42	*bioF*	459773	Glycine C-acetyltransferase (*GCAT*)		O75600
STM0857	172	2.00 E−42		459834	2-@Methylbutyryl-CoA dehydrogenase (*SBCAD*)	methylbutyryl glycinuria	P45954
STM0804[a]	151	8.00 E−37	*moaC*	459782	Molybdenum cofactor biosynthesis protein 1B (*MOCS1B*)	molybdenum cofactor deficiency	Q9NZB8
STM0845	149	1.00 E−35	*moeB*	459822	Molybdenum cofactor synthesis 1 (*MOCS1*)	molybdenum cofactor deficiency	O95396
STM0793[a]	147	5.00 E−35	*bioA*	459771	Similar to Ornithine aminotransferase, mitochondrial precursor	gyrate atrophy	P04181
STM0817[a]	142	4.00 E−33	*ybhF*	459795	ATP-binding cassette, sub-family A, member 8 (*ABCA8*)		O94911
STM0852	122	2.00 E−27	*yliG*	459829	CDK5 regulatory subunit associated protein 1(*CDK5RAP1*)		Q96SZ6
STM0878	117	8.00 E−26	*potG*	459855	ATP-binding cassette, sub-family A, member 12 (*ABCA12*)	Lamellar ichthyosis type 2; Harlequin ichthyosis	EAW70520
STM0847	114	5.00 E−25	*ybiK*	459824	Threonine aspartase 1		Q9H6P5

[a]Genes deleted from both TA98 and TA100; otherwise genes are missing from only TA98.

of mutagens. Although there was no knowledge of the underlying basis for the differential sensitivity of one strain compared with another, the current tester strains are a result of this selection process. Our previous [Porwollik et al., 2001] and current studies identify that the deletion of some genes accounts for the sensitivity of the selected strains.

The standard five Ames strains (TA97, TA98, TA100, TA104, and TA1537) are missing genes associated with molybdenum cofactor biosynthesis, *moaABCDE* [Porwollik et al., 2001]. Molybdoenzymes inactivate *N*-hydroxyl compounds (hydroxylamines), which are active intermediates for the mutagenic activity of both aromatic nitro-compounds and aromatic amines [Rosenkranz et al., 1983]. This helps explain the finding that mutagens in these chemical classes are among those that exhibit differences in mutagenic potency and specificity at the base-substitution or frameshift allele of *Salmonella* when comparing Δ*uvrB* versus uvr^+ strains [DeMarini, 2000]. These mutagens include aromatic amines such as 2-AAF and 4-AB, heterocyclic amines such as Glu-P-1, and nitroarenes such as 1-NP [DeMarini, 2000].

The deletion of many other genes might enhance the mutagenic potency of various mutagens. For example, the deletion of *dinG* (a *lexA*-regulated gene), *rhlE* (an RNA helicase), *dps* (a stress-response-specific DNA-binding protein), and *yliJ* (a glutathione *S*-transferase) [Porwollik et al., 2001] may reduce the ability of the Δ(*gal-bio-uvrB*) strains to repair DNA damage or to inactivate mutagens. The genes *mdfA* and *mdaA* (*nfsA*), which encode, respectively, a multi-drug translocase and a major oxygen-independent nitroreductase, are missing from TA98. The deletion of *mdaA* (*nfsA*) is especially important for mutagenesis considering the long-recognized role of nitroreductases for the activation of nitroarenes to mutagens [Rosenkranz et al., 1982]. The deletion of various membrane-transport genes and genes of unknown function [Porwollik et al., 2001] may also influence mutagenic potency.

As noted earlier, the single-gene defects studied here have a differential effect on mutagenesis at the frameshift versus the base-substitution allele (Table III). At the frameshift allele, the absence of just the nucleotide excision repair system along with the molybdenum cofactor synthesis system is generally sufficient to account for the mutagenic potency exhibited by the compounds in TA98 (Table III). Interestingly, the deletion of these two classes of genes causes 1-NP and MX to be more mutagenic than they are in TA98. The absence of additional genes among the 119 genes missing in TA98 beyond *uvrB* and the molybdenum cofactor synthesis genes probably does not increase the mutagenic potency of compounds at the frameshift allele but may somewhat decrease their potency. Moreover, S9 may reintroduce metabolic factors originally removed in the Δ(*gal-bio-uvrB*) that are necessary for full mutagenic activity of many compounds.

In contrast, deletion of these two classes of genes is insufficient to account for the mutagenic potency of the compounds at the base-substitution allele in TA100. Although only 47 genes are missing in TA100 (all of which are also missing in TA98), some of these deleted genes in addition to *uvrB* and the *moeA*/*moaA* genes clearly influence the mutagenic potencies of compounds in TA100. Thus, some of the missing genes described earlier exert a significant influence on both spontaneous and mutagen-induced base-substitution mutagenesis. At the frameshift allele, some of the additional 72 missing genes in TA98 (119 – 47 = 72) may compensate or otherwise cancel out the influence of the 47 genes missing among both TA98 and TA100.

Human Homologues of Genes Deleted in Δ(*gal-bio-uvrB*) Strains

Our finding that the absence of at least molybdenum cofactor biosynthesis enhances spontaneous and induced mutagenesis at the base-substitution allele of *Salmonella* prompted us to consider the health consequences of mutations in such genes in humans. Among the 17 genes that emerged from this analysis (Table IV), the most prevalent cellular function affected by the genes listed in Table IV is molybdenum cofactor biosynthesis. Human molybdenum cofactor deficiency is a rare and devastating autosomal-recessive disease for which no therapy is known. The absence of active sulfite oxidase, which is a molybdenum cofactor-dependent enzyme, results in neonatal seizures and early childhood death [Lee et al., 2002]. The loss of molybdenum cofactor results in a complete inactivation of the MoCo-dependent enzymes sulfite oxidase and xanthine dehydrogenase. This inactivation could lead to increased sulfite levels, which might deplete glutathione. Depleted glutathione would compromise cellular response to oxidative stress and could lead to increased levels of DNA damage [Graf et al., 1998].

The deleted *Salmonella* genes and their human homologues catalyze reactions that are important for the physiology of any cell. This importance is reflected in the conservation of amino acid sequences of these genes from bacteria to humans. The present study and previous work in *E. coli* [Kozmin et al., 2000] indicate clearly that deletion of molybdenum cofactor biosynthesis genes in bacteria enhances susceptibility to mutagenesis by certain base analogues as well as by a wide range of environmental mutagens. Our data suggest the possibility that enhanced susceptibility to mutagenesis or elevated spontaneous mutagenesis may underlay some aspects of the diseases associated with deficiency in molybdenum biosynthesis in humans.

Implications for Mutagenicity Screening

Our results show that the deletion of large numbers of genes other than *uvrB* in TA98 and TA100 does not di-

minish the ability of these strains to identify mutagens or assess mutagenic potency. In fact, at the base-substitution allele, all mutagens tested were more mutagenic in TA100 compared to the homologous strain with the single *uvrB* defect. Similarly, at the frameshift allele, all mutagens tested were either equally or more mutagenic in TA98 compared with the homologous strain with a single *uvrB* defect. Thus, the Δ(*gal-bio-uvrB*) mutation enhances the mutagenic potency of nearly all compounds tested at both alleles and permits the detection of the base analogues, which otherwise would be negative in a strain with the single *uvrB* defect. Other than the base analogues, all mutagens were mutagenic both in Δ(*gal-bio-uvrB*) and *uvrB* strains, with the exception of 4-AB in the frameshift *uvrB* strain (SN15944), which had a mutagenic potency that was almost ($P = 0.06$) significantly different from zero.

Thus, the absence of the genes due to the Δ(*gal-bio-uvrB*) mutation does not cause mutagens to be missed that would otherwise have been detected in a *uvrB* strain. In fact, Δ(*gal-bio-uvrB*) actually enhanced the ability to detect most mutagens, especially at the base-substitution allele. These findings demonstrate for the first time that the inclusion of the Δ(*gal-bio-uvrB*) mutations into the Ames tester strains of *Salmonella* generally enhances the mutagenic sensitivity of the strains beyond that conferred by *uvrB* alone. Given that the means to produce a mutation in only *uvrB* was not available in the late 1960s when the strains were first developed, it is perhaps fortuitous that a Δ(*gal-bio-uvrB*) mutation was used.

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