Detection and classification of mutagens: A set of base-specific Salmonella tester strains

(histidine operon mutations/carcinogens)

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ABSTRACT A detection and classification system for mutagens has been developed that identifies the six possible base-pair substitution mutations. A set of six Salmonella typhimurium (TA7001 to TA7006) strains has been constructed, each of which carries a unique missense mutation in the histidine biosynthetic operon. In addition to the his mutation, these strains carry different auxiliary features that enhance the mutability of the target his mutation. These include the R factor pKM101, which has the SOS-inducible mucAB system; a deletion of the uvrB component of excision repair; and rfa mutations to increase the accessibility of bulky chemicals to the bacteria. Another set of strains (TA7041 to TA7046) contain a wild-type rfa gene. Reversion via the base substitution unique to each strain was verified by sequence analyses of >800 revertants obtained from different types of mutagens. The strains have considerably lower spontaneous reversion frequencies and detect a variety of mutagens at a sensitivity comparable to the Salmonella tester strains TA100, TA102, and TA104. The low spontaneous frequency of reversion of a mixture of the six tester strains (≈10 revertants per plate) enables a single mutation assay with the mixture that is followed by classification of the type of mutation with the individual strains.

The detection of mutagens and determination of the types of mutation induced are of importance to the understanding of the etiology of cancer and other degenerative diseases that involve mutations. Point mutations in human oncogenes or tumor suppressor genes (e.g., p53) may lead to cancer, and the pattern of missense mutations can give clues as to the mutational events involved (1). Thus a simple test that detects mutagens and determines the pattern of the six possible base substitutions induced by each would be useful for providing information on mutagenic mechanisms.

The previous Salmonella mutagenicity test (2) has been used extensively over the past two decades to measure the mutagenic potential of many compounds. These strains have point mutations in the histidine biosynthetic operon that render them unable to grow in the absence of histidine; however, they are not diagnostic for the type of base-pair substitution caused by the mutagen.

Each of the six strains described here, either with (TA7001–TA7006) or without (TA7041–TA7046) the rfa mutation, reverts by only one specific base-pair substitution out of the six possible changes. Reversion of the target mutation in a gene for histidine biosynthesis restores the mutant his gene to the wild type so that the cell can grow and form a colony without histidine. The number of colonies formed is a direct measure of the mutagenic potential of the test compound. The spontaneous reversion rates of the strains described here are considerably lower than that of the previous Salmonella tester strains (2), and their sensitivity to reversion by mutagens is comparable. In addition, the strains described here have added the ability to determine the spectrum of base substitutions.

Two other systems that detect all six possible base substitutions without further genetic or biochemical analysis have been reported. The Saccharomyces cerevisiae system (3) is based on an essential Cys-22 residue in iso-1-cytochrome c encoded by the CYCl gene. The Escherichia coli system (4) has a mutational target on a plasmid in an active site glutamate residue in the \( \beta \)-galactosidase gene. Since the point mutation is extrachromosomal, it can be transferred into various backgrounds such as those differing in mismatch repair.

MATERIALS AND METHODS

Target Mutation (A-T \( \rightarrow \) G-C) in Set 1. The mutation hisG1775 (5) was recombined with a bacteriophage clone, M13mp9::his4 (6), deleted for part of the hisG gene (covering the hisG1775 mutation) and the hisD gene. Recombinant M13 phage were selected with an active hisD gene product that allowed growth on histidinol and were plaque-purified to prepare single-stranded DNA templates for diodeoxyxynucleotide sequencing. The hisG1775 mutation was identified as a G-C \( \rightarrow \) A-T transition in which the wild-type Gly-153 (GTT) was replaced by the mutant Asp-153 (GAT). No other mutations were found in hisG. The hisG1775 mutation is the basis of the TA7041 and TA7001 strains in set 1 (see Table 1).

In Vitro Mutagenesis. Target mutations for sets 2–5 were synthesized in DNA oligomers. These oligomers were used as primers to extend single-stranded M13 DNA templates (7) or were used in isommetrical polymerase chain reactions (PCRs) to fix the mutations in double-stranded DNA fragments (8), which were subcloned into appropriate M13 clones. These M13 mutant clones were used to transform competent DH5\( \alpha \) FIQ cells (GIBCO/BRL) (9) and mutant plagues were screened by diodeoxyxynucleotide sequencing using deoxyadenosine 5'-[\( \alpha \)-\( \beta \)-\( \delta \)-\( \epsilon \)]triphosphate.

Target Mutation (T-A \( \rightarrow \) A-T) for Set 2. The codon for Lys-217 (AAA) of hisC was changed to Ile-217 (ATA) by using PCR to generate a DNA fragment that was subcloned into M13mp8::hisDC1. The ile mutation was transferred to the chromosome in TA4302 (\( \Delta \)his29/F42fnp301 lac\(^{-}\)) by M13 transduction using histidinol selection (10). Transductants carrying the designated mutant allele, hisC9118, formed the basis of the TA7042 and TA7002 strains in set 2 (see Table 1).

Abbreviations: STN, streptonigrin; NO NO, 4-nitroquinoline-1-oxide; MNG, N-methyl-N'-nitro-N-nitrosoguanidine; MMS, methyl methanesulfonate; N4AC, N¹-aminocytidine; 5azaC, 5-azacytidine; ANG, angelicin.

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Target Mutations for Sets 3 (T-A → G-C), 4 (G-C → A-T), and 5 (C-G → A-T). For set 3, the site of the mutation identified for hisG1775, Gly-153 (GGG wild type), was changed to Val-153 (GTT) by in vitro site-directed mutagenesis (7). The target mutations for sets 4 and 5 were designed to substitute Gly-169 (GGG) and Ala-169 (GCC), respectively, for the wild-type Asp-169 (GAT). Both mutants revert to Glu-169 (GAG) because the wobble base was changed from thymidine to guanosine to obtain the specificity of reversion. There was no difference in growth rates of Glu-169 and Asp-169 strains. PCR was used to fix the Gly-169 and Ala-169 mutations into double-stranded DNA fragments, which were subcloned into M13mp9 (6). Each of these mutations in hisG was transferred to AZ1549 by M13 transduction (10). The Val-153 (GTT), Gly-169 (GGG), and Ala-169 (GCC) mutations were designated hisG9074 (TA7043 and TA7003 of set 3), hisG9133 (TA7044 and TA7004 of set 4), and hisG9130 (TA7045 and TA7005 of set 5), respectively (see Table 1).

Target Mutation (C-G → G-C) in Set 6. The mutation in hisG9070 (11) was found by direct PCR sequence analyses to be a GC to CG transversion in which the wild-type Gly-163 (GGA) was replaced by Arg-163 (CGA) in the hisG gene. No other mutations were found in hisG and this mutation became the basis for the TA7046 and TA7006 strains in set 6 (see Table 1).

Intermediate Bacterial Strains. To obtain a common genetic background for the tester strain, P22int4HT (12, 13) carrying each target mutation was used to transduce SB8052 to growth on histidinol. The hisD3052 mutation of SB8052 was made in a stable deletion mutant, ara-9 (14), which is present in the current Salmonella tester strains and the tester strains described here designated TA70xx. The R factor donor strain, TA4593 (argB69/pKM101), was constructed by transferring pKM101 (15) from TA94 (16) to SAA757 (argB69) by conjugation. TA4490 [hisD3052 Δara9 Δchl1004 (bio chlD uvrB chlA)galE503/pKM101] was made by mating TA2684 (11) with TA4593 and was used as recipient for strains carrying TA704x backgrounds. These strains carry the R factor and are deficient in excision repair. In addition, the complete tester strains TA700x carry rfa mutations (rfa1041-1046) that were isolated by resistance to lysis by the bacterial phage C21 (17). Strains that carry the his mutation in SB8052 background (TA701x), pKM101 (TA702x), and the uvrB deletion (TA703x) will be discussed elsewhere.

Mutagenicity Testing. The protocols detailed in Marion and Ames (2) were used, except that the glucose in the agar plates was reduced from 2.0% (wt/vol) to 0.4% to optimize cell growth. High concentrations of glucose have been shown to reduce the reversion response for some mutants (18). Enhanced sensitivity of the strains to some mutations was obtained by a preincubation of bacteria in liquid medium containing the mutagen at 37°C before top agar was poured on the plates (2).

The concentrations of mutagens used for testing were chosen from doses previously reported for Salmonella tester strains (2, 11, 16, 19) to compare the sensitivity of the base-specific strains described here directly to that of TA100, TA102, and TA104.

Mutagenic Agents. Streptonigrin (STN, Flow Laboratories), 4-nitroquinoline-1-oxide (NQNO, Sigma), N-methyl-N-nitro-N-nitrosoguanidine (MNNG, Aldrich), 4-(N-methyl-N-nitro-N-nitrosoguanidine)-1-methyl methanesulfonate (MMS, Aldrich), were dissolved in dimethyl sulfoxide (Sigma). N4-Aminocytidine (N4AC, Sigma) and 5-azacytidine (5azaC, Sigma) were dissolved in H2O. Angelicin (ANG, HRI Associates, Concord, CA) was dissolved in 95% ethanol.

Before testing ANG with UVA (320–400 nm) irradiation, the cells were removed from Oxoid broth. A 12-hr overnight culture (5.5 ml) was centrifuged at 1080 x g for 10 min. The cell pellet was washed and resuspended in 1 x VBC medium (2) to the same density as the original overnight culture as measured by absorbance at 650 nm. ANG (7.5 μl of 200 μg/ml) plus 1.5 ml of cell suspension was shaken at 37°C for 30 min, transferred to a Petri dish (60 x 15 mm), and rotated gently during the 7.5-min irradiation, 10 cm from a near-UV source (5.9 ± 0.1 J) (Gates-Raymaster from Thomas). A 100-μl aliquot of this mixture was plated according to the standard plate incorporation assay (2). Control plates contained cells exposed to UVA only or to ANG in the absence of UVA activation.

Reversion Analyses. Results from the reference mutagens are summarized in Table 1. Data are reported as revertants per plate—i.e., the difference between the number of revertants on plates with and without the mutagen (20). The mutagenic potency of a compound is also expressed as a fold mutagenicity (the ratio of induced reversion frequency to spontaneous frequency).

Sequence Analyses of Revertants. At least 10 revertants induced by the reference compounds listed in Table 1 were sequenced for each strain. In addition, >100 revertant colonies (48 hr at 37°C) by each of 16 mutagens (data not shown) other than those used as reference testing agents were sequenced from each set of strains. These mutagens included 2-aminopurine, bromodeoxyuridine, N-butyl hydroperoxide, chlorambucil, cumene hydroperoxide, 2,3-epoxy-1-propanol, ethylmethanesulfonate, formaldehyde, N4-hydroxy-cytidine, hydrogen peroxide, ICR-191, methyl glyoxal, mitomycin C, 4-nitrophenyl, 2-nitrofluorene, and styrene oxide. Another 10–20 revertants from each strain that arose after 72 and 96 hr were also analyzed.

One or two colonies were dispersed in 30 μl of deionized H2O. A 2-μl aliquot was used as template in a total reaction volume of 50 μl for asymmetrical amplification by PCR (8) using primer ratios of 100:1 to generate single-stranded DNA templates for sequencing. Excess primers and PCR buffer salts were removed by Sephadex spin columns (G-50 fine, Boehringer Mannheim). The sequencing primer was 5′-end-labeled with adenosine 5′-O-[γ-32P]triphosphate by using T4 polynucleotide kinase (United States Biochemical). This primer was used to sequence 7 μl of the PCR product by Sequenase version 2.0 using dITP mixes (United States Biochemical).

RESULTS

Specificity of Reversion in Differing Genetic Backgrounds. Strains of each set reverted only by the base change indicated in Table 1 when induced by >20 mutagenic agents. Components such as the R factor pKM101 did not change the specificity of reversion of any of the target mutations in spite of its error-prone repair characteristics nor did the uvrB or rfa genes. The uvrB mutation causes a deficiency in excision repair of bulky lesions as measured by lack of survival after UV irradiation at 254 nm. The R factor plasmid pKM101 carries the mucAB genes, which compensate for the weak SOS-mediated mutagenic activities of the two umu-like operons in Salmonella (21). Several mutations affect the lipopolysaccharide component of the cell envelope. All strains carry the galE503 mutation (22) and cannot synthesize galactose residues for the outer core of the lipopolysaccharide component. The primary tester strains (TA7001–TA7006) carry all mutations and have alterations in the core structure of the lipopolysaccharide component that increase cell permeability.

Specificity of Reversion with Various Mutagens. The sequence of revertants obtained from mutagenesis by >20 mutagens was found to change to the wild-type base (Table 1). Missense suppression may result in revertants that grow more slowly and have mutations at an alternate site. The incubation period was extended from 48 hr to 72 hr and 96 hr
to look for possible slow-growing revertant colonies due to missense suppression though <1% additional colonies were found. Of the 20 revertant colonies of TA7045 obtained after 72 hr that were sequenced, one revertant (from 5azaC) retained the original mutant sequence, presumably due to missense suppression. This revertant colony was not visible at 48 h and, therefore, would not contribute to the base substitution analyses.

Reference Mutagens: To verify the mutability of complete tester strains from all six sets, only two mutagens, STN and NQNO, are necessary since they included reversion frequencies of at least 15-fold over spontaneous frequencies (Table 1). STN induced reversions at A-T or A-T base pairs, which are target mutations in sets 1-3 (e.g., TA7001, TA7002, and TA7003, respectively) but not at G-C or C-G base pairs (Fig. 1). On the other hand, the strains of sets 4-6 (e.g., TA7004, TA7005, and TA7006, respectively) have G-C or C-G base pairs as their target mutations and respond to NQNO at least 30-fold over spontaneous (Fig. 2). Similarly, reversion of T-A or A-T targets by NQNO was not detected.

A second positive control mutagen can establish the target mutation unambiguously. Set 1 strains are reverted strongly by N4AC (Fig. 3). While set 4 strains were reverted weakly by N4AC, these strains were not reverted to any significant extent by STN (Fig. 1). Set 2 strains can be distinguished from those of set 3 although both sets were induced by MMS and ANG/UVA, because TA7002 and TA7042 are reverted more strongly by MMS (~100 revertants per plate) than TA7003 and TA7043 (~10 revertants per plate) (Fig. 4). Set 3 strains responded to ANG/UVA at 150- to 200-fold over spontaneous frequencies, while TA7002 and TA7042 were at 43- and 36-fold, respectively (Fig. 5).

Of the strains that respond to NQNO, set 4 strains are reverted weakly by N4AC and strongly by MNNG (=100-fold), whereas strains of other sets showed <10-fold by MNNG (Fig. 6). Set 5 strains were reverted by MMS at 13-fold over spontaneous compared to <3-fold for sets 4 and 6 (Fig. 4). 5azaC reverted only TA7006 and TA7046 of set 6 (Fig. 7). Eventually, a set of six mutagens, one specific for each base-pair substitution would be desirable.

Suggested Testing Strategies. To enhance detection of reversion of the strains at lower concentrations of compounds, a period of preincubation of 10-30 min in liquid medium was used. This may become a preferred procedure in the initial screening of compounds as a marked increase in sensitivity was observed for a number of reactive chemicals. For example, formaldehyde induced TA7005 ~20-fold over spontaneous reversion frequencies when this strain was preincubated with 10 μg per plate for 20 min at 37°C. Without preincubation, 50 μg per plate reverted TA7005 5-fold and 100 μg per plate was needed to revert TA102 2.5-fold (19).

For the initial screening of compounds, the six strains of similar genetic background may be mixed and the mixture may be used in testing. This is possible due to the minimal complementation and slow spontaneous reversion frequencies
of the mixture (7–13 revertants per plate). Cells from overnight cultures of TA7001–TA7006 were mixed in equal proportions to approximate the density of an overnight culture of a single strain and used to test MNNG. The number of revertants induced in this mixture was 2062 revertants per plate. Thus the number of revertants obtained by the mixture was similar to the arithmetic average of 1784 revertants per plate when tested by each individual strain (90 + 121 + 63 + 1108 + 8163 + 1156 divided by 6).

**DISCUSSION**

The six strains (Table 1) can not only indicate the mutagenic potential of a compound but also identify the base substitutions induced by a mutagen. Mutagenic potency is determined from the linear part of the dose–response curve and is expressed in two ways. The frequency of reversion per amount of mutagen is calculated by subtracting the spontaneous reversion frequency (20). The extent of the induced response per amount of mutagen also is expressed as a fold increase of the spontaneous level. The spectrum of missense mutations was obtained for seven reference mutagens: STN, NQNO, N4AC, MMS, ANG/UVa, MNNG, and 5azaC (Figs. 1–7).

Though some mutagens induce only one type of base substitution, most induce several types. For example, 5azaC-induced only strains of set 6 to revert by a G→C transversion (Fig. 7). This specificity was in agreement with the results reported in the yeast CYCl system (3), the E. coli lacZ system (4), and a previous Salmonella tester strain, TA4016 (11), although TA7006 is the most sensitive detection system. 5azaC replaces cytidine, preventing its methylation, and is bound to the DNA cytosine methylase irreversibly, thereby crosslinking the enzyme to the DNA strand and interfering in mismatch repair (23).

Another base analog, N4AC, induces A→T → G-C transitions as indicated by reversion of set 1 strains and, to a lesser extent, G→A → A-T transitions, as indicated by set 4 strains (Fig. 3). N4AC is metabolized via cytidine deaminase and/or uridine-cytidine kinase to N²-amino-deoxycytidine 5'-triphosphate, which is incorporated into DNA (24). N²-Aminodeoxycytidine (N4AdC) in the imino form (25) can be mispaired with adenosine (26). If it is not removed, a gua-

**FIG. 3. N4AC (10 μg)-induced base substitutions.**

nosine is inserted opposite the amino form of N4AdC in the next round of replication such that the original A→T base pair is replaced by a G-C base pair. This may be a simple explanation for the transitions detected by TA7001 and TA7041 of set 1. DNA polymerases also incorporate the major amino form of N4AdC opposite guanosine (24). Upon replication, an adenosine may be inserted opposite, and the original guanosine is replaced by adenosine. This results in a G→A→T transition as detected by set 4 strains (Fig. 3).

Reactive mutagens often have complex patterns of mutagenesis. STN, a bulky quinone preferentially intercalates into the DNA helix at A-T base pairs since it induced reversion in strains of sets 1–3. When it is associated with DNA, STN has the capacity to generate superoxide radicals by redox cycling with molecular oxygen (27). STN also has been found to be a potent inhibitor of topoisomerase 1 (28). There was a substantial increase in the mutagenicity induced by STN in all strains that carry rfa mutations, which enhances permeability. STN induces TA102 → 6-fold above spontaneous (19) compared to a ratio of ~600-fold for set 3 (Table 1); however, the net number of revertants for TA102 (19) is somewhat greater than for sets 1–3.

NQNO reacts with guanosine at G-C or C-G base pairs (Fig. 3) giving two major DNA adducts at the N2 and C8 positions (29). The relative proportions of these adducts appear to be dependent on the context of the guanosine. The adducts formed by NQNO can induce all three base changes as illustrated by the reversion profiles of strains from sets 4–6 (Fig. 2). The effect of a rfa mutation is minimal with NQNO (Fig. 2), which is consistent with its small size. The target guanosine in sets 4 and 5 do not share the same context since set 4 is between guanosines and set 5, by far the main target, is between cytidines on the opposite strand. The specificity for G-C base pairs is similar to that found in the yeast system (3), though the set 5 strains are more sensitive.

ANG and other monofunctional psoralens have been studied because of their antiviral properties and their activity against psoriasis. The major products of the photochemical reactions of ANG with DNA are adducts linked by a cyclobutane ring to the 5–6 position of the pyrimidines. Their preference for thymine over cytosine is reflected by the response at TA or A-T base pairs as compared to C-G or G-C.
base pairs (Fig. 5). The difference in response for sets 2 and 3, or sets 1 and 3, is presumably due to the difference in context of the target thymidine. While adducts are formed at cytidine less efficiently than at thymidine, ANG/UVA clearly induced transitions at C-G base pairs, as indicated by the response of set 5 and 6 (Fig. 5). The previous strains, TA102 and TA100, have a mutagenic ratio of <10-fold compared to ~200-fold for set 3, though the absolute number of revertants is somewhat less for set 3.

Both MMS and MNNG induce a wide spectrum of mutations by alkylating purines and pyrimidines; however, the major MNNG adduct is O4-methylguanine, which mispairs with thymidine instead of cytidine. In the next round of replication, thymidine is correctly paired with adenine instead of thymidine. The resulting G-C → A-T transition can be detected by set 4 strains (Fig. 5). Similarly, the thymine adduct, O4-methylthymine causes misincorporation of guanine on the opposite strand of DNA, resulting in a A-T → G-C transition as detected by TA7001 of set 1. The mutagenicity ratios for strains of sets 1 and 4 were ~10 times higher than for TA100.

MMS induced four out of the six possible base substitution to about the same extent (between 6-16-fold over spontaneous frequencies) as indicated in Fig. 4. Since this alkylating agent was much less specific, the absolute ratios of induction for any single type of base substitution were much lower than those found for other mutagens discussed here. The sensitivity is comparable to TA100.

The six sets of strains described here have low spontaneous reversion frequencies and high sensitivity to mutagens. Results obtained can be compared to the very large database on the previous Salmonella tester strains. The assay indicates the mutagenic potential and identifies the types of base substitution induced. Identification does not require further analysis such as DNA sequencing or hybridization, because the specificity of reversion has been verified by sequence analyses of >800 revertants. However, as the results above show, the context to the target base can affect the formation of the premutagenic lesion and also may modulate the mutagenic effectiveness of the lesion. Thus the context in which the target bases reside in these strains may influence the results obtained. With this possible caveat this system offers the potential to compare the patterns of base substitutions induced by particular environmental mutagens with patterns observed in genes such as p53.

These strains may be useful in increasing the understanding of the mechanisms involved in mutagenesis and structure–activity relationships. For a compound like ANG, which forms a stable adduct with pyrimidines in DNA upon irradiation with UVA, those strains that carried pyrimidines as the target base reverted and the reversion pattern correlated with the expected chemical yield of each type of premutagenic lesion. Thus, the system has the potential to demonstrate types of base substitutions in response to DNA lesions. Conversely, if a compound was found to revert a subset of the strains, the lesion(s) may be postulated and the significant DNA adduct might be characterized more easily.

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