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An Improved Bacterial Test System for the Detection and Classification of Mutagens and Carcinogens

(*Salmonella typhimurium*/lipopolysaccharide/frameshift mutations)

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ABSTRACT We previously described a set of four strains of *Salmonella typhimurium* designed for detecting the various types of mutagens, and showed their utility in detecting a wide variety of carcinogens as mutagens. The lipopolysaccharide that normally coats these bacteria is a barrier to penetration of mutagens to the cell membrane. The set of tester strains has been improved by adding a mutation (*rfa:* deep rough) that results in a deficient lipopolysaccharide. The techniques for using these strains for detecting mutagens are presented and the tests are shown to be extremely sensitive and convenient. The specificity of frameshift mutagenesis is clarified. As adjuncts to the test with the four strains, we describe a test that compares mutagenic killing in deep rough strains with and without DNA excision repair, and a test using forward mutagenesis in a deep rough strain lacking excision repair.

We have described a set of four tester strains of *Salmonella typhimurium* designed for detecting and classifying mutagens (1–4). Each strain, which has been selected for its sensitivity and specificity, can be reverted back to the wild type by particular mutagens. In addition, the strains have a deleted excision repair system and, consequently, are very much more sensitive to various mutagens. These strains are very valuable for detecting carcinogens as mutagens. The present paper describes a marked improvement in sensitivity obtained with new lipopolysaccharide (LPS)-defective derivatives of the strains, additional information as to specificity of frameshift mutagenesis, and methods for handling the strains.

MATERIALS AND METHODS

Compounds. We are indebted to H. J. Creech for ICR-191 and to J. A. Miller and H. Bartsch for nitrosobiphenyl and the fluorene compounds (4), except for 2-nitrofluorene (from Aldrich), 2,7-diaminofluorene (from Sigma), and 2,7-diacetylaminofluorene (from Schuchardt). 4-Nitroquinoline-*N*-oxide was purchased from Schuchardt, 9-aminoacridine was from Sigma, and methyl-nitro-nitroso-guanidine was from Aldrich.

Bacterial Strains. Each strain (Table 1) contains one of four mutations in the histidine operon resulting in a requirement for histidine. These four mutations are discussed in detail in *Results*. The deletion through the *uvrB* region of the chromosome eliminates the excision repair system for DNA. The *gal* and *rfa* (deep rough) mutations eliminate, to different extents, the polysaccharide side chain of the LPS that coats the bacterial surface, making the bacteria more permeable and completely nonpathogenic. The TA1535 set (TA1535, TA1536, TA1537, TA1538), which is *rfa* and *uvrB*, is recommended for general testing for mutagens and carcinogens *in vitro*, as it is the most sensitive to mutagenesis. The TA1975 set is used for examining the effect of repair on mutagenesis and killing (in comparison to the TA1535 set). The TA1950 set and TA1530 set are less sensitive to mutagens *in vitro*, but may be required for use in the host-mediated assay in which *Salmonella* strains are incubated in the peritoneum of a mouse or rat (5). For good mutagenesis, especially with frameshift mutagens, there must be some bacterial multiplication in the peritoneum; this multiplication is influenced by the type of LPS. Which of the three sets to use must be determined for each type of animal. We also have constructed a set of auxotrophic derivatives of the TA1950 set of testers so that the four strains could be incubated as a set in the peritoneum of a single animal and sorted out afterward by plating on selective media.

Construction of Deep Rough Strains. The deep rough (*rfa*) derivatives of the tester strains were isolated from the TA1530 set of strains, which already carried deletions through the *gal–bio–uvrB* region of the chromosome (1, 2), by selection for bacteria resistant to C21 phage (6). Phage C21 will lyse *gal* deletion mutants, but not the deep rough strains or the wild type. The C21-resistant colonies were picked from confluent-lysis plates (nutrient agar) and purified. They were then screened for sensitivity to sodium deoxycholate (2 mg)

TABLE 1. *Genotype of the TA strains used for testing mutagens*

Additional mutations in:		Histidine mutation in strain			
LPS	Repair	*hisG46*	*hisC207*	*hisC3076*	*hisD3052*
+	+	*hisG46*	*hisC207*	*hisC3076*	*hisD3052*
+	Δ*uvrB*	TA1950	TA1951	TA1952	TA1534
Δ*gal*	Δ*uvrB*	TA1530	TA1531	RA1532	TA1964
rfa	Δ*uvrB*	TA1535	TA1536	TA1537	TA1538
rfa	+	TA1975	TA1976	TA1977	TA1978

All strains were originally derived from *S. typhimurium* LT-2. Wild-type genes are indicated by a +. The deletion (Δ) through *uvrB* also includes the nitrate reductase (*chl*) and biotin (*bio*) genes. The Δ*gal* strains (and the *rfa uvrB* strains) have a single deletion through *gal chl bio uvrB*. The *rfa*, repair$^+$ strains (last row) have a mutation in *galE*.

Abbreviation: LPS, lipopolysaccharide.

and crystal violet (10 μg), which were applied to sterile filter paper discs (6 mm) placed in the center of nutrient agar pour plates containing 2×10^8 bacteria. After overnight incubation at 37°, a zone of inhibition could be seen around each disc, and the strains (TA1535, etc.) were chosen that had maximum sensitivity: 14 mm with crystal violet and 13 mm with deoxycholate. The wild type and the strains with the *gal* deletion show no zone of inhibition. The inhibition by the two compounds can be used as a simple test to confirm the properties of the deep rough strains.

Deep rough derivatives (TA1978, etc.) were constructed from the histidine-requiring mutants (*hisD3052*, etc.), which have a normal excision repair system. Mutations were first introduced in the galactose operon by selecting for resistance to deoxygalactose. Plates containing no carbon source were spread with 0.2 ml of 40% glycerol, 0.2 ml of 0.5 mM biotin, 0.06 ml of 100 mM histidine, 0.1 ml of 40% 2-deoxygalactose, 30 μl of 4% galactose, and 0.1 ml of a nutrient broth culture of *hisD3052*. Resistant colonies appeared after incubation for 2 days at 37°; these were picked and screened for sensitivity to C21 phage. The phage-sensitive strains (about 1/250 tested) were checked to make sure they were still resistant to ultraviolet light; from these, C21-resistant derivatives that were sensitive to crystal violet were then isolated as explained above.

Construction of gal⁺ uvrB⁻ Strains. The TA1950 set of strains were constructed from the four original histidine mutants, *hisG46*, etc., by introduction of a *chl bio uvrB* deletion. Each of the histidine mutants was put through an anaerobic selection (8) on glycerol–chlorate agar pour plates (minimal medium, 2% glycerol, 0.3% $KClO_3$, 6 μmol of histidine, 0.1 μmol of biotin, and 2×10^8 bacteria). The plates were incubated overnight at 37° inside of a dessicator under an atmosphere of N_2, then incubated aerobically for 2 more days. Chlorate-resistant colonies were picked and tested for sensitivity to ultraviolet light by streaking across a nutrient broth plate and exposing half of the plate to a 15-W G.E. germicidal lamp at 33 cm for 6 sec. After overnight incubation, the sensitive strains (about 1/200) were picked; they were also Gal^+ and Bio^-.

Quantitative Testing of Mutagens. A large number of suspected mutagens can be tested very simply by placing crystals or a few μl of the compound directly on a lawn of the tester strain (see below) (1). For quantitative testing and greater sensitivity, a known amount of the mutagen is incorporated in the form of a solution into the top agar of a pour plate. In this way the mutagen is spread uniformly over the plate along with the bacteria.

Pour plates are made by adding 0.1 ml of the tester strain culture to a small sterile test tube (13 × 100 mm) that contains 2 ml of molten (45°) *top agar* (which contains a trace of histidine and excess biotin). An appropriate volume of the mutagen in solution can be added to the tube with a sterile disposable capillary pipette. The tube is then mixed well by rotation between the palms of one's hands and poured onto the surface of a minimal agar plate with Vogel–Bonner *E* medium (7) (1.5% agar, 2% glucose). The plate can be tilted quickly to cover the surface with the top agar, then it is allowed to remain on a level surface several minutes for the agar to harden. A control plate for the spontaneous reversion rate should be done for each tester strain in which the mutagen is omitted. A sterility check of the mutagen solution also should be done. All plates are incubated upside down at 37° for 2 days, after which the number of revertant colonies appearing can be counted. If too much of the compound is added so that the light lawn of the background and the spontaneous revertants are inhibited, then the experiment should be repeated with less compound.

The standard components of the pour plate assay do not have to be made each day. Bacterial cultures are grown up in nutrient broth for about 8 hr from a 1/100 inoculum and can be stored in a refrigerator for at least 1 week. Aqueous solutions of mutagen are prepared in sterile screw-cap tubes with sterile water or with dimethylsulfoxide (Schwarz–Mann, spectrophotometric grade, sterile as is). It is possible to add up to 0.5 ml of dimethylsulfoxide per plate without appreciably interfering with mutagenesis. *Top agar* (0.6% Difco agar, 0.6% NaCl) is autoclaved and stored in bottles in volumes of 100 ml at room temperature. Before use the agar is melted by placing the bottle in boiling water, and 10 ml of a sterile solution of 0.5 mM L-histidine·HCl–0.5 mM biotin is added; the bottle is mixed by gentle swirling. The agar is added to the tubes and allowed to equilibrate to 45° before addition of any bacteria.

The trace of histidine in the top agar allows all the bacteria on the plate to undergo several divisions; this growth is necessary in many cases for mutagenesis to occur. The slight background that grows up also allows any inhibition by the compound to be seen. Further increase of the histidine on the plate enhances mutagenesis, but it also gives a heavy background lawn that obscures the revertants.

The Repair Test: Comparison of Strains with and without Repair for Zones of Killing. Pour plates with a lawn of bacteria (TA1978/TA1538) are prepared as described for mutagen testing, except that an additional 3.0 μmol of L-histidine is added, thus allowing full growth of the bacterial lawn and giving more distinct zones of killing by test compounds. Up to 25 μl of the test solution is pipetted onto sterile filter paper discs (6 mm) with disposable glass capillary pipettes, and each loaded disc is placed on a plate. The plates are then inverted and placed in a 37° incubator for 24 hr, and the zone of killing is measured.

Forward Mutagenesis Test. We have discussed (1) a forward mutagenesis test using resistance to the proline analogue, azetidine carboxylic acid. The use of strain TA1538 increases the general sensitivity of this test, but with this strain an excess of histidine (3 μmol) should be added to the usual top agar, which has an excess of biotin. Addition of a trace of proline (0.02 μmol) also increases the sensitivity. The azetidine carboxylic acid can be L, or D,L (use 10 μmol of the L form per plate) and is available from Aldrich or Calbiochem. Mutagens can be added to the top agar for quantitative results or spotted for qualitative results.

RESULTS

Bacterial LPS is a barrier to mutagen penetration

We have made three sets of tester strains with different LPS coats (Table 1): TA1950, etc., have a normal LPS, TA1530, etc., have a partial LPS due to a deletion through the *gal* operon, and the newly constructed, and preferred, set, TA1535, etc., has a deep rough *rfa* mutation, which removes the LPS down to the ketodeoxyoctanoate–lipid core (6).

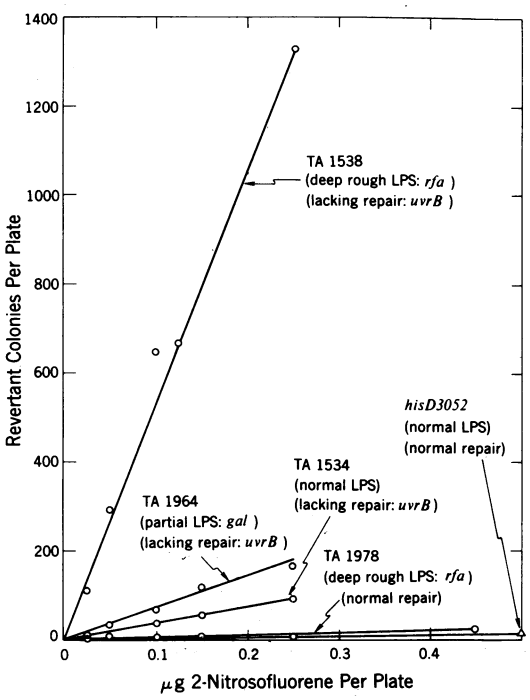

FIG. 1. Effect of repair and LPS mutations on sensitivity to mutagenesis by 2-nitrosofluorene. The spontaneous revertants, about 15 per plate, have been subtracted.

Fig. 1 shows quantitative data on the reversion by 2-nitrosofluorene of the same histidine mutation (*hisD3052*) in strains from each of these three sets that differ in their LPS. The advantage of the deep rough mutant, TA1538, is apparent. Comparison of various mutagens on strains with partial or no LPS is shown in Table 2. It can be seen that as the ring system increases in size the advantage of using the *rfa* derivatives becomes more pronounced.

Comparison of strains with and without excision repair

The difference in the number of revertant colonies between a strain with and without repair is useful in determining whether a frameshift mutagen is a simple or a reactive intercalator (1–4). This comparison was previously done with the pair TA1534/*hisD3052*, but we have now constructed a new pair, TA1538/TA1978, which is much more sensitive because both strains have the defective, deep-rough LPS. The differences between TA1538 and TA1978 or between TA1534 and *hisD3052* can be seen in Fig. 1, in which the sensitivity to reversion of the histidine mutation, *hisD3052*, by 2-nitrosofluorene, a reactive intercalator, is compared.

The new pair of deep rough strains is also much more sensitive in the *repair test*, a comparison of zones of killing by a compound. If the strain without repair (TA1538) is more sensitive to killing by a compound than the strain with repair (TA1978), then the compound is deduced to be killing because of a covalent reaction with DNA. This type of test has been studied in detail for the *polA* mutants in *E. coli* (9) and for the *rec* system in *B. subtilis* (10). We have used it for the *uvrB* excision repair system in our *Salmonella* strains (1, 2). The *repair test* for several compounds is shown in Table 3. Our *repair test* is well over 10-times more sensitive than the *polA* test, based on the reported zone of killing for 2-nitrosofluorene (9) compared to Table 3. One limitation of the *repair test* is that it does not detect simple intercalating mutagens, as shown in Table 3 for 9-aminoacridine, ethidium bromide, quinacrine, and ICR 364-OH, which are equally inhibitory to both strains. Another limitation is that the *repair test* with *uvrB* does not detect a difference with methylating agents (and ethylating agents), as has been discussed in connection with the influence of *uvrB* on mutagenesis (2). The *polA* test (9) does detect methylating agents and the *polA* deep rough derivative of *hisG46* is being constructed.

Nature and specificity of the tester strains

The specificity of the most sensitive set (deep rough and lacking repair) of the four tester strains can be seen in Table 4. Some of the mutagens are fairly specific for reverting one of the strains and not the others, and these mutagens can be used as positive controls for checking out the strains when testing new compounds.

TA1535. This strain contains the histidine mutation *hisG46*, which is a missense mutation (11). It is reverted well by a wide variety of carcinogenic mutagens that cause base-pair substitutions (1, 2), but not by mutagens that cause

TABLE 2. *Comparison of mutagens on tester strains with different LPS*

Compound added	*His*$^+$ revertants per plate		
	Normal LPS (TA1534)	*gal*Δ LPS (TA1964)	Deep-rough LPS (TA1538)
2-Ring compounds			
4-Nitroquinoline-*N*-oxide (0.5 μg)	198	255	288
4-Hydroxylaminoquinoline-*N*-oxide (8 μg)	97	106	153
4-Nitrosobiphenyl (10 μg)	52	98	250
3-Ring compounds			
2-Nitrosofluorene (0.25 μg)	91	166	1327
2,7-Bis-(acetylamino)-fluorene (25 μg)	286	457	3900
2-Nitrofluorene (50 μg)	156	267	4200
2,7-Diaminofluorene (50 μg)	9	18	141
N-Hydroxy-2-aminofluorene (1 μg)	288	1023	3800
N-Hydroxy-2-acetylaminofluorene (25 μg)	22	49	256
N-Acetoxy-2-acetylaminofluorene (10 μg)	47	58	1630
	(TA1952)	(TA1532)	(TA1537)
ICR-191 (1 μg)	31	170	955
4-Ring compounds			
ICR-312 (5 μg)	56	138	1706

The number of revertants caused by the mutagen on each plate is shown with the control subtracted: control plates never had more than 20 colonies. The mutagens were added to the top agar in dimethylsulfoxide, except for the ICR compounds, which were in sterile water, and 2-nitrosofluorene, which was in 95% ethanol. ICR-191 is a substituted acridine and ICR-312 is a substituted benzacridine (13).

frameshift mutations. It is not clear which base pair changes can revert the strain, but we suspect that most, if not all, of the six possible base pair substitutions can be detected with this strain.

TA1536. This strain contains the histidine mutation *hisC207*, a frameshift mutation (11, 12). It is reverted by various acridine half-mustards, the ICR compounds (12, 13), but not by any of the various other frameshift mutagens we have tried. This is the least useful of our tester strains, as ICR can also be detected easily with TA1537 (and TA1538) (Table 4). We are leaving it in the set as it is the most sensitive to ICR-191. It is not clear what the frameshift mutation is in this strain, but as ICR-191 is likely to react with a G, we suspect that a GC is involved. It does not appear to have the $\begin{matrix}\text{-GGGG-}\\\text{-CCCC-}\end{matrix}$ sequence present in TA1537, or the alternating G-C sequence that is present in TA1538. In an experiment analogous to that described for TA1537, we have shown that the *hisC207* mutation is not suppressible by the *sufB*, -CCCC-, frameshift suppressor (TA1951, *hisC207 uvrB*, donor; TR944 recipient). Oeschger and Hartman suggest that frameshift mutations such as *hisC207* that do not revert at all with methyl-nitro-nitrosoguanidine (Table 4) are likely to be deletions of a base pair (14).

TA1537. This strain contains the histidine frameshift mutation *hisC3076* (14). The following evidence suggests that the frameshift mutation in this strain has an added $\begin{matrix}\text{-G-}\\\text{-C-}\end{matrix}$ base pair resulting in $\begin{matrix}\text{-GGGG-}\\\text{-CCCC-}\end{matrix}$. It is suppressed by a frameshift suppressor *sufB* of Riddle and Roth (15), which they have shown to be a mutation in $tRNA^{Pro}$ that suppresses +1 frameshift mutations of the -CCCC- type. This is likely to be caused by an added G in the normal -GGG- anticodon of the proline tRNA (15). We transduced TR944 (*sufB his01242 hisΔ2236*) (15) with P22 phage grown on *hisC3076* on a plate with no histidine, and found 5000 very small transductants (presumptive suppressed strains) and 250 large transductants (presumptive wild-type recombinants). Two of the small transductants were checked out by crossing with histidine deletions. We conclude they contain a suppressor, as they still contained the original histidine mutation. A control plate using the histidine deletion without the suppressor (TA462) as the recipient showed only 114 large, presumptive wild types. Another control plate with a known suppressible mutant (15) (*hisC3737* in TR970) transduced into TA944 gave about 3500 small colonies. TA1537 is reverted weakly by methyl-nitro-nitrosoguanidine (Table 4), a finding consistent with the properties of a +1 frameshift addition in a $\begin{matrix}\text{-GGGG-}\\\text{-CCCC-}\end{matrix}$ sequence (14, 16). The TA1537 tester strain is reverted by 9-aminoacridine and ICR-191 (Table 4) and epoxides of polycyclic hydrocarbons (3), among other agents. It is reverted by various carcinogenic polycyclic hydrocarbons that have been activated by a mammalian microsomal system (manuscript in preparation).

TABLE 3. *Repair test*

Test compound	Diameter of zone of killing (mm)	
	Repair+	Repair−
Crystal violet (10 μg)	19 (11)	20 (12)
Mitomycin C (1 μg)	<6	27
2-Nitrosofluorene (1 μg)	<6 (<6)	13 (<6)
(10 μg)	9 (<6)	32 (23)
4-Nitrosobiphenyl (100 μg)	12	24
4-Nitroquinoline-*N*-oxide (20 μg)	18	29
ICR-372 (100 μg)	14	19
ICR-372-OH (100 μg)	14	13
9-Aminoacridine (100 μg)	13	14
Ethidium bromide (50 μg)	15	16
Quinacrine (20 μg)	11	12
Diethylsulfate (10 μl)	14	15
N-Methyl-*N'*-nitro-*N*-nitroso-guanidine (100 μg)	28	28
Nitrogen mustard (100 μg)	11	16

The zones of killing produced by various test compounds are compared for the deep rough tester strains with (TA1978) and without (TA1538) excision repair. The data in parentheses are for a similar pair (*hisD3052*/TA1534) with a normal LPS. Diameters less than that of the disc (6 mm) cannot be measured. For a given test compound and strain, the diameter of the zone of killing is roughly proportional to (amount of compound)^3. ICR-372 is an alkylating and ICR 372-OH a nonalkylating derivative of aza-quinacrine (13).

TABLE 4. *Mutagen specificity for the four tester strains*

	Revertant colonies per plate on tester strains			
	TA1535	TA1536	TA1537	TA1538
Control (spontaneous)	18	0	5	12
N-Methyl-*N'*-nitro-*N*-nitro-soguanidine (10 μg)	40,000	0	36	15
ICR-191 (5 μg)	32	14,000	7500	2300
9-Aminoacridine (100 μg)	48	0	3800	15
2-Nitrosofluorene (0.5 μg)	32	0	36	3400
4-Nitroquinoline-*N*-oxide (1 μg)	107	0	30	380

The number of colonies per plate is shown: the spontaneous controls have not been subtracted.

TA1538. This strain contains the *hisD3052* frameshift mutation that was first used by Hartman *et al.* (17) to show that 4-nitroquinoline-*N*-oxide and hycanthone were frameshift mutagens. We have discussed (4) the evidence of Isono and Yourno that this strain contains a $\begin{matrix}\text{-CGCGCG-}\\\text{-GCGCGC-}\end{matrix}$ sequence that is reverted by 2-nitrosofluorene with a −2 deletion of a $\begin{matrix}\text{-CG-}\\\text{-GC-}\end{matrix}$. This strain is reverted by various aromatic nitroso derivatives of amine carcinogens (4), and, in combination with a microsomal activation system, by the carcinogens aflatoxin B1 and benzpyrene and by a wide variety of aromatic amine carcinogens (manuscript in preparation).

DISCUSSION

The LPS that is normally present on the surface of *S. typhimurium* apparently acts as a partial barrier to the passage of mutagens to the membrane, as shown by the marked increase in the sensitivity to mutagens of LPS-defective strains. We first tried this approach when it was found that dibenz(*a,h*)anthracene epoxide did not work as a frameshift mutagen, although the closely related carcinogen benzanthracene epoxide did. We reasoned that the large size of the compound might prevent its entrance into the bacteria, and indeed we found that introducing the *rfa* mutation into the tester strain enabled us to detect the mutagen (3). This *rfa* mutation causing a shortened LPS has been put into all of our tester strains and gives a marked increase in sensitivity in detecting mutagens: the larger the ring system, the more pronounced the effect.

The genetics and biochemistry of the LPS of *Salmonella* have been investigated in detail (6). A deletion through the *gal* operon eliminates galactose synthesis from the cell and, therefore, the LPS loses the part of the polysaccharide chain that is distal to the first galactose unit. The *rfaF* and *rfaE* mutants (deep rough) are defective in enzymes that are responsible for the synthesis of the polysaccharide distal to the ketodeoxyoctanoate–lipid core and are particularly sensitive to deoxycholate and various antibiotics and other large compounds (6, 18). These mutations presumably cause the maximum permissible stripping of the LPS without lethality.

We have discussed the advantages of this *Salmonella* system for the detection of mutagens (1–4). We have screened many mutants to obtain those that were most sensitive to a wide variety of mutagens, then we increased the sensitivity by several orders of magnitude by introducing mutations in the repair system and the LPS. A comprehensive set of back-mutation tester strains has certain practical and theoretical advantages over a single forward mutagenesis assay (1, 2). The proof of the advantages of this set is its comprehensiveness in detecting the known classes of mutagens at very low concentrations (1–4) and its utility in making the correlation between carcinogenicity and mutagenicity (1–4, and manuscript in preparation).

Although our set of four strains appears to be able to detect almost all mutagens, it is clear that it is not yet completely comprehensive. We do not yet have a frameshift tester involving a repetition of A-T sequences. In addition, there are a few frameshift mutagens, such as activated aflatoxin B1 and activated benzpyrene that, although they work on our tester strains, are relatively inactive when compared to their activity in the *repair test*. The *repair test* compares zones of killing in strains with and without repair, and we recommend its use as an adjunct test until the back-mutation tester set is completed. The *repair test* by itself, however, can be much less sensitive than a specific back-mutation test, as can be seen with 2-nitrosofluorene (Fig. 1 versus Table 3). Another limitation of the *repair test* is that methylating agents, and simple intercalating mutagens that do not react with DNA covalently, may not show an appreciable difference in zones of inhibition.

The forward mutagenesis test described also makes a useful adjunct to the set of back-mutation testers. It is not desirable as the sole test, however, as it is less sensitive than a specific back-mutation test (because of the many spontaneous mutants of various types) and it may, in particular cases, not work at all. We have found, for example, that 2-nitrosofluorene does not cause an increase in azetidine carboxylic acid resistance, even though it clearly is a very powerful mutagen for the G-C-G-C-G-C sequence. We calculate that this sequence is a rare one in DNA (it might occur in only about 25% of the genes) and, thus, it is reasonable that it may not occur in any one particular gene.

We have combined these test systems with a mammalian liver microsomal system (manuscript in preparation), and have shown that an extremely wide range of chemical carcinogens, become powerful mutagens through metabolic activation and can be detected with great sensitivity and simplicity.

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