JOURNAL OF BACTERIOLOGY, Jan. 1975, p. 259-266

Vol. 121, No. 1
Printed in U.S.A.

Positive Selection of Mutants with Deletions of the *gal-chl* Region of the *Salmonella* Chromosome as a Screening Procedure for Mutagens That Cause Deletions

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Received for publication 30 August 1974

We have developed a convenient and specific positive selection for long deletions through the *gal* region of the chromosomes of *Salmonella typhimurium* and *Escherichia coli*. Through simultaneous selection for mutations in the two closely linked genes, *gal* and *chlA*, a variety of deletions of varying length, some extending through as much as 1 min of the chromosome, could be readily obtained. Many of these deletions resulted in the loss of a gene, which we named *dhb*, concerned with the ability of the bacterium to synthesize the iron chelating agent enterobactin. The selection was adapted for the screening of mutagens for their ability to generate long deletions in the bacterial deoxyribonucleic acid. Forty agents were screened for this capability. Nitrous acid, previously reported to be an efficient mutagen for this purpose, increased the frequency of deletion mutations 50-fold in our system. Three others, nitrogen mustard, mitomycin C, and fast neutrons, were shown to increase the frequency of long deletions between five- and eightfold. The remainder were found to be incapable of generating these deletions.

The generation of long deletions in the deoxyribonucleic acid (DNA) of bacteria has interested many workers (reviewed in 12). No mutagen has been found, however, that specifically causes deletions, although it has been reported that several agents such as nitrous acid and ultraviolet (UV) light generate deletions in addition to other types of genetic damage (11, 22).

We have devised a simple positive selection for *Salmonella typhimurium* mutants containing large deletions in the galactose region of the chromosome and have used it to screen mutagens for their ability to cause deletions. The technique relies on the simultaneous selection for inactivation of two closely linked genes in the region. One set of genes, the *gal* operon, codes for the enzymes involved in the metabolism of galactose. Growth of *gal*$^+$ *S. typhimurium* in the presence of the galactose analogue 2-deoxy-D-galactose (2DG) results in the selection of mutants with defective *gal* genes because this analogue is converted to a bacteriostatic product thought to be 2-deoxy-glucose phosphate (K. Floyd, personal communication).

The second selected gene, *chlA*, is one of a set of seven known bacterial genes (*chlA-G*) involved in the anaerobic reduction of nitrate ions by nitrate reductse. Nitrate reductase also allows conversion of chlorate ions to a toxic product thought to be chlorite (15-17, 26, 28). Growth of *S. typhimurium* anaerobically in the presence of chlorate results in the selection of mutants with defective *chl* genes. We have found that, when *S. typhimurium* is grown anaerobically in the presence of both 2-deoxygalactose and chlorate, mutants with deletions through the entire *gal-chl* region can be selected. The deleted sequence includes genes involved in biotin synthesis (*bio*) and the excision repair of UV irradiation-induced lesions (*uvrB*). As a result, the strains are hypersensitive to UV light and require biotin for growth on minimal medium.

MATERIALS AND METHODS

Bacterial strains. The wild-type strain of *S. typhimurium* LT2 was employed throughout. Strain *galE503* carries a mutation in the gene coding for uridine 5'-diphosphate-galactose epimerase. This strain, called M1 when originally isolated by H. Nikaido, was kindly provided by him.

Chemicals. 2DG was purchased from Sigma Chemical Co., St. Louis, Mo., or from Calbiochem, San Diego, Calif., as indicated. 2,3-Dihydroxybenzoic acid was purchased from Aldrich Chemical Co., Milwaukee, Wis. L-Azetidine-2-carboxylic acid was also obtained from Calbiochem. Chemicals listed in

Tables 3 and 4 were obtained from the following sources: numbers 1 to 7 from Aldrich Chemical Co.; numbers 8 and 9 from Calbiochem; numbers 10 to 12 as a gift from H. J. Creech at the Institute for Cancer Research, Philadelphia, Pa.; number 13 from Cyclo Chemical Co., Los Angeles, Calif.; number 14 from Fisher Scientific Co., Fair Lawn, N.J; numbers 15 and 16 from J. T. Baker Chemical Co., Phillipsburg, N.J.; number 17 from Sterling-Winthrop Research Institute, Renssalaer, N.Y.; numbers 18 and 19 as a gift from H. R. Mahler at the University of Indiana, Bloomington, Ind.; number 20 from Mann Research Laboratories, N.Y., N.Y.; number 21 as a gift from J. A. Miller and H. Bartsch (4); numbers 22 to 29 as gifts of J. G. Moffatt at Syntex Research Laboratories, Palo Alto, Calif. Number 30, psoralen (4,5'-8-trimethyl[furano-3',2': 6,7 coumarin]) was a product of Paul B. Elder Co., Bryan, Ohio.

Media. Nutrient medium is 0.8% nutrient broth (Difco) with 0.5% NaCl. Nutrient agar contains nutrient medium and 1.5% agar (Difco). Minimal medium E (32) was supplemented with carbon sources as indicated. Minimal glucose (or glycerol) plates contained 1.5% agar and 2% glucose (glycerol) in addition to medium E. Top agar is 0.6% agar with 0.5% NaCl. Deletion selection medium was prepared by the addition of 1.5% agar, 0.3% $KClO_3$ and 2% glycerol to minimal medium E. Plates were poured containing about 30 ml of this mixture and were supplemented with biotin (3 μM) and 2DG (0.13%) just prior to use. Addition of galactose (0.004%) was required when a batch of chromatographically pure 2DG was used (Calbiochem). This was unnecessary when a batch of galactose-contaminated 2DG was used (Sigma).

Centrifugation. Centrifugations were performed in the SS-34 rotor of a Sorvall RC2B centrifuge at 4,000 rpm for 15 min at 4 C.

Culture conditions. Cultures (in nutrient broth unless noted otherwise) were incubated with vigorous shaking at 37 C, and growth was monitored by observing the increase in absorbance at 650 nm. Cells for mutagenesis were washed in medium E except where otherwise indicated.

Selection of mutants with deletions. Samples of cultures were suspended in 2 ml of top agar and poured on selective medium plates which were then incubated upright in a small desiccator under a slight positive pressure of nitrogen. Wooden clamps (designed by A. B. Champion) secured the top. The lid of each plate was lined with filter paper, and a dish of silica gel desiccant was placed in the jar to absorb moisture released during the 37 C incubation. A Gas Pak anaerobic indicator (BBL, Cockeysville, Md.) was placed in the desiccator to monitor the absence of oxygen. The anaerobic phase of the incubation proceeded for 16 h. The plates were then removed from the desiccator and incubated aerobically at 37 C until colonies were visible, usually 36 to 48 h later.

Plate testing of mutagens. Pour plates were prepared as described above except that glucose (0.004%) was added to the medium and the plates were incubated aerobically for 8 h prior to the anaerobic growth. The trace of glucose causes a delay (by catabolite repression) of the 2DG selection. A few crystals or drops of mutagen were placed on the agar near the edge of the plate immediately after the top agar had dried. The number of colonies clustered around the spot of mutagen beyond the zone of inhibition was used as an index of the extent of mutagenesis. The number of these colonies which were sensitive to UV irradiation (*uvrB*$^-$) was used as an index of the extent of deletion-mutation generation.

UV light mutagenesis. Log-phase cells (A_{650} = 0.6) were centrifuged and resuspended at the original density in 0.9% saline. Samples (2 ml) were placed in sterile glass petri dishes which were exposed to light from a General Electric germicidal lamp G15T8 at 33 ergs per mm^2 per s. Samples of 0.01 ml were removed from the dish and suitably diluted for viable cell counts. At the same time, samples of 0.1 ml or 1.0 ml were diluted into 5 or 50 ml of nutrient broth, grown to log phase, and plated for mutation and deletion frequency studies. All tubes and dishes were wrapped in aluminum foil to minimize photoreactivation.

Nitrous acid mutagenesis. Following the procedure of Schwartz and Beckwith (22), 5 ml of an overnight nutrient broth culture was washed in an equal volume of 0.1 M acetate buffer (pH 4.6), after which 0.3 ml of 0.05 M sodium nitrite (in the acetate buffer) was added for resuspension of the pellet. The culture was then shaken as indicated at 37 C. The incubation was terminated by the addition of 10 ml of medium E (0 C) followed by centrifugation. The pellet was resuspended in 5 ml of nutrient broth, 2 ml of which was then further diluted into 100 ml of nutrient broth and grown to log phase for study. A control culture, treated identically except that the sodium nitrite was omitted, showed no significant killing.

Fast neutron mutagenesis. Stationary-phase cells (2 ml) in sealed polyethylene bottles were exposed to a 10 kW U^{235} source for 20 min. The cells were then centrifuged, resuspended, and grown in nutrient broth overnight prior to plating on selective medium.

Nitrogen mustard mutagenesis. A 40-ml volume of nutrient broth-grown, log-phase cells was washed and then resuspended in 40 ml of medium E with biotin, glucose, and nitrogen mustard [*N*-methyl bis-(2 chloroethyl)-amine] at 15 mg/ml. Samples (5 ml) were incubated at 37 C with shaking for the times indicated. At the conclusion of the incubation, each sample was washed and then resuspended in 50 ml of nutrient broth and grown overnight. Parallel cultures were treated in an identical fashion except that no mutagen was added.

Psoralen mutagenesis. Log-phase cells in 5 ml of medium E with glucose and biotin were washed and then resuspended in 1 ml of medium E with 16.5 μg of 4,5'-8-trimethyl psoralen per ml. The psoralen had been dissolved in ethanol and through dilution the cells were therefore in 5% ethanol. After a 5-min incubation period at 37 C, the suspension was irradiated for 25 min with light from a General Electric "Black Light" lamp F40BLB at 33 cm. The cells were then diluted into 50 ml of nutrient broth and grown overnight.

Mitomycin C mutagenesis. Log-phase cells grown

in nutrient broth were washed and then resuspended in 5 ml of medium E with glucose and 10 μg of mitomycin C per ml. They were then incubated for 30 min at 37 C and washed, and then 1 ml was resuspended in 5 ml of nutrient broth and grown overnight.

Efficiency of mutagenesis. Samples of mutagenized cultures and their nonmutagenized controls were spread on minimal glucose plates with either 10 μmol of L-azetidine-2-carboxylic acid, 10 μmol of HIPA, or on minimal glycerol plates with 40 mg of 2DG and galactose as discussed above. Resistant colonies were counted, and the relative numbers in the two cultures served as an indication of the extent of mutagenesis achieved. (HIPA is the histidine analogue D(+)-α-hydrazino-imidazole propionic acid [24] and was synthesized in this laboratory by Julia Lever.)

Screening for UV sensitivity. Colonies were streaked across a nutrient agar plate, and the first half of the streak was exposed to 200 ergs of light per mm^2 from the germicidal lamp. The plate was then incubated overnight. UV-sensitive strains grew only on the unirradiated half of the plate.

RESULTS

Selection of spontaneous deletion mutants. Virtually all mutants selected on the basis of their anaerobic growth on selection medium in the presence of 2-deoxy-galactose and potassium chlorate were shown to carry deletions in the *gal-chl* region of the chromosome. The gene order in this region at 33 min on the chromosome is, as reported by Stouthamer (27) and Sanderson (21), *nicA*, *aroG*, *gal*, *hut*, *bio*, *chlD*, *uvrB*, *chlA*. As shown in Table 1, chlorate-resistent strains appeared at a frequency of 10^{-5}, 2DG-resistant strains appeared at about one-fifth that rate, whereas strains resistant to both appeared at a frequency of slightly less than 10^{-7}. These doubly resistant strains contained a large deletion through both genes as they were also mutant in the unselected markers *uvrB* and *bio* which lie between *gal* and *chlA*. The expected frequency of strains with point mutations in these four genes was about 10^{-23}.

Length of deletions in mutants selected. The distribution of deletions by length, as determined by phenotype analysis, was significantly skewed towards the minimal requirement in the selection (Table 2): the class covering only the genes from *gal* to *chlA*. The longest deletions we found, in mutants selected on medium supplemented with nicotinic acid and 2,3-dihydroxybenzoic acid in addition to biotin, extended through the genes from *nicA* through *dhb* (see below), a distance estimated to be roughly 1 min of the chromosome. *aroG* is the gene for the phenylalanine repressible DAHP synthetase and thus *aroG* mutants do not require phenylalanine unless tyrosine and tryptophan are added.

Additional genes in *Escherichia coli* that have been shown to lie between *nicA* and *chlA* code for 6-phosphogluconolactonase (*pgl*) and for enzymes involved in porphyrin biosynthesis (*popB*) and photoreactivation of UV-damaged DNA (*phr*) (30).

We were unable to find any mutants with deletions extending only as far as *chlD*. All *gal*$^-$ *chl*$^-$ *uvrB*$^+$ mutants studied could be reverted to either *gal*$^+$ or *chl*$^+$ or were *bio*$^+$, indicating that they contained two point mutations (Table 1). It has been reported that *chlD* mutants retain some residual nitrate reductase activity (2, 26), and it is assumed that this is sufficient to produce toxic levels of the inhibitor under our conditions.

New requirement of 2,3-dihydroxybenzoic

TABLE 1. *Spontaneous mutants resistant to chlorate and to 2DG*[a]

Supplement	Colonies appearing (avg)	Colony phenotype[b]					Cells having phenotype shown (%)
		Chlorate	Gal	Bio	UV	Reversion	
None	10^8	S	+	+	R	NA	NA
Chlorate (aerobic)	10^8	S	+	+	R	NA	NA
Chlorate (anaerobic)	10^3	R	+	+	R	+	99 (89/90)
2DG (aerobic)	2×10^2	S	−	+	R	+	96 (81/84)
2DG + glucose (0.4%) (aerobic)	10^8	S	+	+	R	NA	NA
2DG + chlorate (anaerobic)	8	R	−	−	S	−	99 (100/101)
2DG + chlorate + 0.004% glucose (anaerobic)	20	R	−	−	S	−	100 (20/20)
2DG + chlorate + 0.012% glucose (anaerobic)	2×10^2	R	−	−	S	−	9 (9/100)
		R	+, −	+	R	+	91 (91/100)

[a] "Unsupplemented" medium contains biotin, salts, glycerol, citrate, and ammonia. ~10^8 cells plated.

[b] Symbols: chlorate (R, resistant; S, sensitive); galactose (+, utilization as carbon source; −, none); bio (−, requirement; +, not); UV (R, resistant; S, sensitive); reversion (+, >10^{-8} to either gal$^+$ or chl$^+$ [growth on nitrate as nitrogen source]; -, none; NA, not applicable).

TABLE 2. *Distribution by phenotype of spontaneous deletions selected in 23 independent selections*[a]

Genotype of deletion	No. of deletions	Representative strain
gal bio uvrB chl	14 (61%)	TA1661
gal bio uvrB chl dhb	4 (17%)	TA1665
aroG gal bio uvrB chl dhb	3 (13%)	TA1660
nicA aroG gal bio uvrB chl dhb	2 (9%)	TA1674
nicA aroG gal bio uvrB chl	0[b] (<4%)	TA1701

[a] Selection as described in Materials and Methods with addition of 3 μM nicotinic acid and 7 μM dihydroxybenzoic acid to medium. Frequency of all deletion mutants is 10^{-7} to 10^{-8}.

[b] This deletion was isolated several years ago in this laboratory by A. Liggett, and this makes it appear that *dhb* is on the *chl* side. Other data (unpublished results) indicate that the requirement for 2,3-dihydroxybenzoic acid (*dhb*) is eliminated at high frequency through the spontaneous appearance of what appears to be an unlinked suppressor. If this had in fact occurred before TA1701 was characterized, then *dhb* might instead lie between gal and *aroG*.

acid. Some deletion mutants were shown to require 2,3-dihydroxybenzoic acid for growth on our minimal medium, which contains the iron chelator citrate. It is suggested that the deletion in these strains covers a gene *dhb*, which is involved in the synthesis of the iron-sequestering agent enterobactin (18, 19). The map position of this gene was tentatively determined by deletion mapping (Table 2).

Selection of mutagen-generated deletion mutants. It was expected that addition to the selection plate of mutagens efficient in causing deletion mutants would result in an increase in the number of 2DG-chlorate-resistant colonies. This could then be used as a screening procedure for such mutagens. However, many mutagens act only on dividing cells and the procedure as developed allowed selection against nonmutants before any growth could occur. The procedure was therefore modified with the addition of a trace of glucose to the medium and the insertion of an 8-h aerobic preincubation (see Materials and Methods). Aerobic growth protected the cells from chlorate, and glucose prevented inhibition by 2DG, probably through catabolite repression or inducer exclusion (Table 1) (13). Through diauxie, the glucose was preferentially metabolized and exhausted during this preincubation period so that, during the subsequent anaerobic growth, both the chlorate and 2DG selections proceeded. The optimal level of glucose was found to be 0.004% (Table 1). Here, one or two cell divisions occurred, but not enough bacteria were present to make the probability of double mutants significant. When this medium was used to screen mutagens, the number of colonies clustered around the spot of added mutagen on the plate provided a visualization of the ability of an agent to cause deletions. The presence of a deletion mutation could then be confirmed through screening for UV light sensitivity.

Nitrous acid mutagenesis. Nitrous acid has been reported to be an efficient mutagen for the generation of deletions in both phage T4 (31) and *E. coli* (22). To verify the claim that our system could detect deletion-causing mutagens, we sought to duplicate these results in *Salmonella*. Since nitrous acid acts only at low pH, it was necessary to first mutagenize a culture and then examine the number of deletion mutants in the culture. Results of four independent runs are averaged in Table 3. Under conditions resulting in the killing of 95% of the cells, we found a 42-fold increase in mutation rate to 2DG resistance alone, whereas the average increase in deletion frequency was 56-fold. All mutagenized cultures were checked for mutation rates to resistance to either HIPA, L-azetidine-2-carboxylic acid, or 2DG in order to compare increases in deletion frequency with increases in the frequency of all mutations.

Three treatments resulted in a minor increase in the deletion frequency.

Nitrogen mustard mutagenesis. Nitrogen mustard [CH_3-N-(CH_2-CH_2Cl)$_2$] treatment resulted in an increase of up to eightfold over the spontaneous level on plates. It was then used to mutagenize a culture in liquid medium. At a killing level of 93%, nitrogen mustard did increase the deletion mutation frequency about fivefold (Table 3). There was, however, a parallel increase in HIPA-resistant mutants in a selection for both point mutants and deletions.

Fast neutron mutagenesis. Fast neutron mutagenesis, utilizing a U^{235} reactor, was slightly more effective than mustard. Maximal mutagenesis, achieved at a killing rate of 75%, resulted in an eightfold increase in deletions with a fivefold increase in all mutations. It is, however, a laborious process, not in our view justified by the marginal increase in mutants it produces.

Mutagenesis by DNA cross-linking agents. Psoralen and mitomycin C were also used to mutagenize cultures. Psoralen mutagenesis had no effect, although mitomycin C-mutagenized cultures did show a sixfold increase in deletion frequency (Table 3).

Screening of other mutagens. Thirty-five other compounds or treatments were tested for their ability to increase the frequency of deletion mutants in *Salmonella* (Tables 3 and 4). Two mutagens, *N*-methyl-*N'*-nitro-*N*-

TABLE 3. *Generation of deletions in liquid culture*

Mutagen	Time of exposure	Survival (%)	Avg no. of analogue-resistant mutants per plate	Fold increase of analogue-resistant mutants over control	Avg no. of *gal-chl* deletions per plate	Fold increase of *gal-chl* deletions over control
16. Nitrous acid	0 min	100	120 (2DG)[a]	1.0	3	1.0
	7 min	1-5	5100 (2DG)	42.5	168	56.0
	10 min	0.1-1	200 (2DG)	1.7	7	2.3
2. Nitrogen mustard	0 min	100	53 (HIPA)	1.0	10	1.0
	20 min	14	114 (HIPA)	2.2	30	3.0
	30 min	7	225 (HIPA)	4.2	51	5.1
	90 min	0.7	138 (HIPA)	2.6	37	3.7
UV light	0 s	100	54 (2DG)	1.0	12.5	1.0
	20 s	1	414 (2DG)	7.7	19	1.5
	30 s	0.1	1145 (2DG)	21.2	4	0.32
30. Psoralen	0 min	100	287 (2DG)	1.0	5.5	1.0
	25 min	5	350 (2DG)	1.2	3	0.55
8. Mitomycin C	0 min	100	8 (L-AC)	1.0	5	1.0
	30 min	1	54 (L-AC)	6.8	31	6.2
Fast neutrons	0 min	100	97 (2DG)	1.0	7	1.0
	20 min	25	487 (2DG)	5.0	57	8.1

[a] Abbreviations: 2DG, 2-deoxy-D-galactose; HIPA, D(+)-α-hydrazino-imidazolepropionic acid; L-AC, L-azetidine-2-carboxylic acid.

TABLE 4. *Compounds which fail to increase the frequency of deletion mutants by as much as fourfold over control*[a]

1. NG
3. β-Propiolactone
4. 1,3-Propane sultone
5. 1,2-Dibromoethane
6. Bis-(2-chloroethyl)amine hydrochloride
7. Tetranitromethane
9. L-Ethionine
10. ICR191
11. ICR58
12. ICR228
13. L-Ethionyl-L-alanine
14. Diethylsulfate
15. $MnCl_2 \cdot 4H_2O$
17. Nalidixic acid
18. 5α-Pregnan-3β-20α-ylenebis-(trimethylammonium iodide)[b]
19. Pregn-5-ene-3β,20α-diamine[c]
20. Quinacrine dihydrochloride
21. 2-Nitrosofluorene
22. 3'-Deoxyadenosine[d]
23. 2',3'-Dideoxyadenosine
24. 7-Deazaadenosine
25. 3'-Chloro-3'-deoxy-thymidine
26. 3'-Chlorothymidine
27. Adenosin-2'-ene
28. 3'-Deoxythymidine
29. Thymidin-2'-ene
30. Psoralen

[a] Plate test performed as described in Materials and Methods. Only colonies within a 3.5-cm radius of the spot of mutagen were counted. In no case would the ratio of colonies on treated plates to those in controls have been increased through the use of smaller or larger radius. In no case did the zone of inhibition caused by the mutagen extend beyond 1.5 cm from the spot. One- to 5-μliter amounts of liquid mutagens were used. A 5-μg amount of 2-nitrosofluorene was used. One or two crystals of NG and mitomycin C was used. Enough crystals to covers the tip of a toothpick were used for the other compounds except $MnCl_2$, which was placed down in 2-mg quantities.

[b] Malouetine.

[c] Irehdiamine A (IDA-A).

[d] Agents 22 through 29 were also tested in conjunction with UV light irradiation. Cells were exposed as described in Materials and Methods and 1.0 ml was plated on selection medium immediately after exposure. Crystals of test compounds were placed on the plates and the preincubation with glucose was begun, all plates being wrapped in aluminum foil. No increases above control levels were observed, although at survival rates of 10^{-3}, which had given the greatest increases in mutagenesis (Table 3), no increases of less than 20-fold could have been observed because of the amount of irradiated culture plated.

nitrosoguanidine (NG) and diethylsulfate gave a spurious positive result, as almost all of the colonies they produced were UV-resistant double mutants rather than deletions. This is not unexpected since each of these mutagens is known to increase the frequency of point mutations by as much as two or three orders of magnitude (1, 29). Further, NG has been reported to cause multiple, closely linked mutations (6), a result selected for here. Thus, the frequency of double mutations generated by these substances is high enough to give a positive result in our system. All of the other agents studied were shown to be ineffective in deletion mutation generation.

Growth of deletion mutants in the presence of wild-type parent. One requirement of a test system such as this is that the growth conditions do not select against either the mutants or the wild type. Since evidence is accumulating to indicate that some seemingly innocuous conditions do favor the growth of certain strains over others (5), we tested the ability of two *gal-chl* deletion mutants to grow in the presence of the wild-type parent. Although the growth rates of the three strains were identical, and the percentage of deletion mutants in a mixed culture remained constant during log phase, these mutants were selected against during stationary phase (unpublished results).

These strains have altered lipopolysaccharide layers (10) and are probably deficient in heme production (9). Either or both of these factors might be responsible for the selection against deletion mutants in dense cultures. Whatever the explanation, it is clearly necessary to stop the growth of mutagenized cultures in log phase. Several of our experiments were performed before the results of this reconstruction were known and the cultures were allowed to reach stationary phase. However, increasing the observed frequencies of deletion formation by as much as a factor of five would not make any of the mutagens tested a better potentiator of deletions than is nitrous acid.

DISCUSSION

We have developed a positive selection procedure for mutants with long deletions through the *gal* region of the chromosome of *S. typhimurium*. This technique has proved to be of value in the screening of mutagens for the ability to cause deletions. The sensitivity of the selection procedure is evident from our isolation of spontaneous and nitrous acid-induced deletions. Although the plate test for mutagen activity failed to uncover an agent which increases deletion mutation frequencies dramatically, its ability to demonstrate the generation of *gal*$^-$ *chl*$^-$ double mutants by NG and diethylsulfate indicates that, if an efficient mutagen were tested, it would be detected.

Our use of this selection for screening deletion mutagens has shown that of those tested, nitrous acid is the most effective and convenient mutagen for this purpose. This verifies earlier reports of its action (11, 22, 31). Mutagenesis with nitrogen mustard, mitomycin C, and fast neutrons also resulted in some increase in the frequency of deletion mutations but far less so than nitrous acid. The best of these three, fast neutrons, also required a rather complex procedure. The major disadvantage of all four was that they generated other types of mutations in addition to deletions (see also 11).

In contrast to our results, Schwartz and Beckwith (22) reported a 230-fold increase in deletion frequency using nitrous acid and a 500-fold increase using UV light. We found no appreciable effect of UV light. Schwartz and Beckwith suggest that most of their "deletions" might be either frameshift mutations or microdeletions covering only a small number of nucleotide pairs. Another possible explanation for this discrepancy lies in the fact that their system selects for intragenic deletions whereas ours requires the excision of several genes. Alternatively, the results could be due to inherent differences between our *Salmonella* strain and the *E. coli* strain of Schwartz and Beckwith.

Other reports (7) have claimed that introduction of a *polA* mutation into strains of *E. coli* B led to a 20-fold increase in the frequency of deletions in the *tonB-trp* region of the chromosome. Doubt has been cast on these results, however, by Berg (5), who showed that certain auxotrophs might have a selective advantage over prototrophs during growth in *pol*$^-$ backgrounds.

Our method for the selection of deletions using 2DG and chlorate should be useful for the further study of the mechanism of both spontaneous and mutagen-caused deletion generation. Other systems have been tried, but less effectively. The use of galactose with a uridine 5'-diphosphate-galactose epimerase (10) mutant was independently developed by Stouthamer (27) and by Rolfe and Onodera (20) at the same time that out work centered around the use of galactose with the epimerase mutant *galE503* (3). We found the newer method preferable because of the flexibility it allows in the use of any *gal*$^+$ strain.

Another method (23) requires the construc-

tion of a lysogen with thermo-inducible lambda phage mutant (impossible in *Salmonella*) in addition to the use of marker rescue experiments to verify the existence of the deletions in each strain selected. Further, this selection results in classes of mutations other than the desired deletions and for these reasons appears far less flexible and convenient than ours.

The *tonB-trp* deletions mentioned above have been used extensively in the study of deletions. It is now, however, a true selection. Rather, it involves the screening of colicin-resistant mutants for a tryptophan requirement. In addition, as many as 95% of the *colB*^{r} mutants of *E. coli* B are deletions which cover the entire *trp* operon and end at apparently the same point (8, 25). This casts doubt on generalizations drawn from a study of this region in this strain.

Our procedure does not detect intragenic or small intergenic deletions, but if a single mechanism is involved in formation of all deletions of more than several base pairs in length, then this limitation may not be important.

The rationale we employed is not restricted to the *gal-chl* region of *Salmonella*. Other pairs of selectable, closely linked markers with no essential genes between them should exist given the large numbers of toxic analogues, antibiotics, and bacteriophages whose action relies on bacterial products that can be inactivated through single mutations. Through this technique, deletion mutants could be isolated for the study of many other genes in which mutations are not now easily selected.

The selection of *gal-chl* deletion mutants has also served for the selection of strains with nonreverting, tight mutations in such genes as *uvrB* (4) and *hut* (14), and in the curing of strains containing episomes covering this region (L. Kier, personal communication). The method has also been shown to be applicable to *E. coli* (J. McCann, personal communication).

Finally, the existence of a requirement for 2,3-dihydroxybenzoic acid by some of our mutants is of interest in view of the significant distance between the *gal* region and other genes (*enb*) known to be involved in the biosynthesis of enterobactin (18). Although investigations of this question are not yet complete, it is suggested that *dhb* is involved in the regulation of this pathway or of the aromatic amino acid biosynthetic pathway of which 2,3-dihydroxybenzoic acid is a product.

ACKNOWLEDGMENTS

This investigation was supported by Atomic Energy Commission grant AT (04-3) PA 156 to B.N.A. M.D.A. was a Public Health Service Trainee.

We would like to thank Alex Karu for his advice on the use of psoralen and generously sharing his supply of it; Michael Syvanen for his guidance in the use of fast neutron mutagenesis; and Joyce McCann for her critical reading and discussion of this manuscript.

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