JOURNAL OF BACTERIOLOGY, Oct. 1968, p. 975-980

Vol. 96, No. 4
Printed in U.S.A.

Selection of a Mutant of *Escherichia coli* Which Has High Mutation Rates

ROBERT B. HELLING
Department of Botany, University of Michigan, Ann Arbor, Michigan 48104

Received for publication 15 June 1968

A mutation which causes high mutation rates in all other loci tested was induced with nitrosoguanidine and was selected through the ability of the progeny of such mutant cells to mutate to streptomycin resistance at a higher rate than the wild-type cells. This mutation (*mut-2*) and the Treffers' mutation (*mutT1*) mapped at approximately the same position to the right of *leu*. Specificity studies showed that the two mutations differ in rates of mutation produced.

The control of mutation rate is of interest both at the molecular level and at the population level. Obviously, any finite mutation rate produces a detrimental load in populations; on the other hand, mutations must occur in order to allow populations to adapt to and survive in changing environments. A particularly illustrative example of this is shown by the response of a growing culture of *Escherichia coli* to the addition of a high concentration of streptomycin. Only rare mutant variants survive, while the bulk of the population is killed. If the mutation rate to streptomycin resistance was infinitely low, this entire population would have perished. On the other hand, streptomycin resistance is not normally advantageous to the cell. Such mutants have functionally altered ribosomes (9, 26), may be dependent on the continuing presence of streptomycin for viability (streptomycin dependent), and frequently have a lower growth rate than wild-type cells (3, 8); thus, a very high mutation rate to streptomycin resistance might be a severe load on the population. When such considerations are amplified to include all of the genes of a cell, it becomes clear that mutation rates are subject to selection, just as selection of any other trait occurs. Experimental evidence supporting such a supposition has been reported (31).

One can imagine that mutation rates must be higher for populations experiencing rapid environmental change than for relatively stable populations in a stable environment. This immediately suggests a procedure by which one can select for new mutants which show higher mutation rates than their parents. By exposing a small population of haploid cells to a lethal agent such as streptomycin, most "normal" cells (which have a low wild-type mutation rate) can be rapidly eliminated, leaving a few resistant survivors. A much higher proportion of the progeny of a mutant exhibiting high mutation rates should be resistant to the streptomycin; thus, if one starts with a mixed population, the frequency of cells with high mutation rates should be considerably increased after streptomycin treatment.

This paper describes the first mutant selected by such a procedure.

MATERIALS AND METHODS

Media. Minimal medium (7), LB, and LA (2) have been described. TB10 is TB [containing, per liter, 10 g of tryptone (Difco) plus 5 g of NaCl (*p*H 7.2)] diluted 10-fold with water. TAS is TB plus 1.1% agar and 200 μg of streptomycin sulfate per ml.

Transductions. Transductions, construction of multiple-mutant strains, lysogeny tests, and production of bacteriophage P1bt were as previously described (12).

Bacterial strains. E. coli B/r and its arabinose-nonutilizing (*ara*) and leucine-requiring (*leu*) mutants have been described (6, 11, 12). A map illustrating the order of the mutations is shown in Fig. 1. *E. coli* Bs-1 is a radiation-sensitive derivative of *E. coli* B (13), received through Amikam Cohen from the collection of H. Adler. I received *mutT1* in a K-12 strain, Hfr *Mu trp*, from Eli C. Siegel (24); it was transduced into a *leuB1* mutant of B/r by selecting for Leu$^+$ and by looking for the unselected cotransfer of *mutT1*.

Induction of mutations. About 5×10^8 cells of Bs-1, harvested from logarithmic growth in LB, were resuspended in 0.85% saline containing 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) per ml, and were incubated at 30 C for 20 min. After the treated cells were centrifuged and washed twice with 10^{-2} M $MgSO_4$, they were resuspended in 100 ml of LB and were incubated at 37 C overnight. (About 95% of the cells were killed by the treatment with NTG.)

Mutation rates. Samples (1.0 ml) of TB10 containing about 100 cells/ml were distributed to a series of tubes with a Cornwall syringe-type pipetting device. After incubation overnight at 37 C, 4 ml of melted

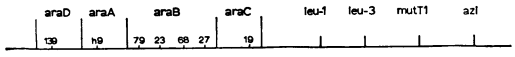

FIG. 1. *Genetic linkage map showing location of mutT1.*

agar medium was added to each tube, and the contents were poured into sterile petri dishes. After hardening, another 10 to 12 ml of agar medium was poured on top and the plates were incubated for 2 days before counting. For determining the frequency of streptomycin-resistant mutants, melted TAS was used; for determining the frequency of reversion to Ara$^+$, minimal medium containing 0.4% L-arabinose as sole carbon source was used. Forty tubes were used to determine each mutation rate, and the final population size (n) was estimated from the average viable count of five additional tubes treated in the same way.

Two methods were used for calculating the average number of mutations per tube (m). The P_0 method was used for low mutation rates (mutants were not present in every tube). From the Poisson distribution, P_0 (proportion of tubes containing no mutants) = e^{-m}. For the high mutation rates (most or all of the tubes contained mutants, generally in the range of 20 to 600 per tube), the median method for determining m (based on the median number of mutants per tube) was used. By dividing m by the total number of cells per tube, n, the mutation rate α, mutations per cell division, was determined. Derivations of these and other methods for determining mutation rate and analyses of their limitations are presented by Lea and Coulson (17) and Ryan (22).

RESULTS

The steps involved in obtaining the mutator gene mutation *mut-2* may be outlined as follows. After overnight growth to allow the survivors of the treatment with the mutagen to recover and to allow mutations to be expressed, 10^4 cells were transferred into 200 ml of fresh medium and were allowed to increase to a density of 5×10^7 cells/ml. Then 200 μg of streptomycin per ml was added, and incubation continued until only mutant cells which were streptomycin resistant survived. The streptomycin-resistant mutations could have been induced by the NTG, or they could have occurred spontaneously in the progeny of cells which had a high mutation rate as a result of some other mutation induced by NTG. Few spontaneous mutations in cells with a normal mutation rate were expected because of the low frequency of mutation to streptomycin resistance in such cells.

A lysate of transducing phage P1 was made from the streptomycin-resistant cells, and the lysate was used to transduce Leu$^-$ (*leu-3*) recipient cells to Leu$^+$. It was known that mutator mutations could be obtained in the *leu* region because the Treffers' mutation (*mutT1*) had previously been reported to cotransduce with *leu* (24). When Leu$^+$ transductants were replicated to streptomycin-containing TAS plates, about 0.5 to 2% of the colonies in one experiment gave streptomycin-resistant survivors. No resistant survivors were obtained among the Leu$^+$ transductants resulting from phage grown on an unmutated Bs-1 host, or from phage grown on streptomycin-resistant survivors of a second independent experiment carried out in parallel.

One of the transductant colonies which gave streptomycin-resistant survivors on the replica plate was picked and, after restreaking to obtain a clone, was tested and shown to mutate at high frequency to streptomycin resistance. Phage was grown on this strain and was used to transduce a *leu-3* recipient to Leu$^+$. This time, about 35% of the transductants contained the *mut-2* mutation as shown by the frequency of streptomycin-resistant colonies produced after replication. One of these transductant colonies was purified and designated *mut-2* B/r; this colony or its derivative was used for all further work.

Mutation rates. The rate of mutation to streptomycin resistance was measured in several derivatives of *mut-2* and *mutT1* (Table 1). The rate of mutation in cells containing the *mut-2* mutation was relatively constant and approximated 3×10^{-8} mutations per cell division, as calculated by the median method. This is about 35% of the rate of mutation in *mutT1* strains but much higher than the normal rate, which is generally on the order of 10^{-10} to 10×10^{-10} mutations per cell division (8, 20, 22; Table 1). The rate difference between *mut-2* and *mutT1* has been noted consistently in all derivative strains containing one or the other of the mutations. This difference is large enough so that the range of mutation rates among different strains carrying one of the *mut* mutations does not overlap the range of mutation rates in strains with the other *mut* mutation (Table 1). In Table 1 (line 7), it is shown that *mutT1* Bs-1 had approximately the same mutation rate to streptomycin resistance as other *mutT1* derivatives of strain B/r.

It was found that *mut-2* and *mutT1* not only have high rates of mutation to streptomycin resistance, but also have high mutation rates to resistance to azide, erythromycin, cycloserine, and phage T4. Thus, the mutant genes probably are capable of producing other mutations in any gene.

The different rates of mutation to streptomycin resistance showed that *mut-2* and *mutT1* are not identical. Further studies of their specificity were done by coupling them with various *ara* mutations and studying rates of reversion to Ara$^+$. It was found (as expected) that neither mutation caused the appearance of revertants of *araB27*, *araC19*, or *leuB1*. These are probably multisite mutations

TABLE 1. *Frequency of mutation to streptomycin resistance in mutT1 and mut-2 strains*

Strain[a]	Mutation rate (mutations per cell division) calculated by	
	P_0 method	Median[b] method
araB23 mut$^+$	6.0×10^{-10}	
araB14 mutT1		5.7×10^{-8}
araB23 mutT1		7.1×10^{-8}
araB68 mutT1		9.6×10^{-8}
araC19 mutT1		9.1×10^{-8}
mean (*mutT1*)		$\bar{\alpha} = 7.9 \times 10^{-8}$
		$s = 1.8 \times 10^{-8}$
		$5.2 \times 10^{-8} < \alpha^c < 10.6 \times 10^{-8}$
ara$^+$ *mutT1* Bs-1[d]		9.0×10^{-8}
ara$^+$ *mut-2*		2.3×10^{-8}
araB14 mut-2		2.1×10^{-8}
araB23 mut-2		2.4×10^{-8}
araB68 mut-2		4.3×10^{-8}
mean (*mut-2*)		$\bar{\alpha} = 2.8 \times 10^{-8}$
		$s = 10^{-8}$
		$0.8 \times 10^{-8} < \alpha^c < 4.8 \times 10^{-8}$

[a] All strains are derivatives of *E. coli* B/r with the exception of *ara*$^+$ *mutT1* Bs-1.
[b] Mean mutation rate, $\bar{\alpha}$; standard deviation, s.
[c] With 95% confidence, the true value of the mutation rate, α, is to be found in the interval shown, calculated by $\bar{\alpha} \pm 1.96\ s$.
[d] In this experiment, 80 tubes (1 ml) were used instead of 40 tubes.

since spontaneous revertants have not been detected (5, 12). Neither mutation affected the rate of reversion of *araB68* (Table 2, lines 1 to 3). On the other hand, the rates of reversion of two other *ara* mutations were greatly increased by both *mut-2* and *mutT1*. The rate of reversion of *araB79* was increased to about the same extent by the two (Table 2, lines 4 to 6). The rate of reversion of *araB23* was increased to a greater extent by *mutT1* than by *mut-2*. Revertants of both *araB23* and *araB79* were heterogeneous in colony size and morphology, indicating that many were partial revertants which probably had two mutations, the second partially reversing the effect of the primary *araB23* or *araB79* mutation.

Mapping. Three-point crosses were carried out to locate both *mutT1* and *mut-2* more precisely. The first set of experiments shown in Table 3 (first four lines) indicates that both mutations fall to the right of *leuB1*, on the side away from the arabinose genes. Leu$^+$ transductants jointly receiving the *ara* marker from the transducing phage are *less* likely to receive the *mut* gene from the phage than are Leu$^+$ transductants which did *not* receive the *ara* marker from the phage, and vice versa. This indicates that the *ara* and *mut* genes are probably on opposite sides of *leu*, since both are more closely linked to *leu* than to each other.

This conclusion is reinforced by the results of the last two crosses listed in Table 3. It was known

TABLE 2. *Frequency of ara reversion in mutT1- and mut-2-containing strains*

Strain	Mutation rate (mutations per cell division) calculated by	
	P_0 method	Median method
araB68 mut$^+$	1.5×10^{-9}	
araB68 mutT1	1.5×10^{-9}	
araB68 mut-2	1.4×10^{-9}	
araB79 mut$^+$	7.3×10^{-10}	
araB79 mutT1		7.6×10^{-7}
araB79 mut-2		9.4×10^{-7}
araB23 mut$^+$	1.7×10^{-9}	
araB23 mutT1		7.3×10^{-7}
araB23 mut-2		2.9×10^{-7}

that *leu-3* maps to the right of *leuB1*, and thus between *leuB1* and *mutT1* if my previous results were interpreted correctly. Crossovers between *leuB1* and *leu-3* were obtained by selecting for Leu$^+$ transductants. In the first case (Table 3, line 5), the great majority of the Leu$^+$ transductants contained the *mut* allele from the recipient (*leu*$^+$ *mutT1*) and resulted from a double crossover wherein a single segment of genes from the phage (including the *leu*$^+$ gene but not the *mut*$^+$ gene) was inserted into the recipient chromosome. To obtain the other class of recombinants (*leu*$^+$ *mut*$^+$),

TABLE 3. *Three-point crosses showing that mut-2 and mutT1 map to the right of the leu operon*

Donor	Recipient	No. of Leu+ transductants that score as[a]			
		Mut+		Mut-	
		Ara+ Mut+	Ara- Mut+	Ara+ Mut-	Ara- Mut-
mutT1	*araB27 leuB1*	13	21	12	14
araAh9	*leuB1 mutT1*	17	6	19	18
mut-2	*araB27 leuB1*	15	13	14	18
araAh9	*leuB1 mut-2*	21	6	22	11
leu-3	*leuB1 mutT1*	5		112	
leuB1 mutT1	*leu-3*	28		11	

[a] In every case, the selected phenotype was Leu+. Only mutant alleles are designated. To identify the unselected markers, the transductant colonies were isolated in pure culture and then were picked into 1 ml of TB. The tubes were incubated for 24 hr at 37 C; then 0.1 ml was plated on TAS to detect the *mut* alleles (appearance of streptomycin-resistant colonies). Cells from the same tubes were tested to see whether they were Ara- or Ara+.

two separate genetic fragments from the donor must be incorporated into the recipient chromosome by a quadruple crossover wherein the recipient retains its original *leu-3*+ locus; this class is expected to be much less frequent. In the reciprocal cross (Table 3, last line), a considerable number of Leu+ transductants contained the *mut* allele from the donor, the frequency in this case not being dependent on an additional set of crossovers but only on the distance separating *leu-3* and *mutT1*. If *mutT1* were actually to the left of *leu*, the expected ratios would be quite different. The results unequivocally indicate that *mutT1* is to the right of the *leu* operon.

The cotransduction frequency of the *mut* genes with *leuB1* is a measure of the genetic distance from *leuB1*. Pooled frequencies of the top four sets of data in Table 3 showed that the *mutT1* locus cotransduces with the *leuB1* locus 41% of the time (49 of 120 Leu+ transductants), and that *mut-2* cotransduces with *leuB1* 49% of the time (59 of 120 Leu+ transductants). These results showed that the two *mut* mutations map very close to each other and to the *leu* operon.

Presumably, *mut-2* and *mutT1* lie between *leu* and the locus for azide resistance (*azi*) which is also cotransducible at low frequency with *leu* (18, 27). On the basis of the results of Arima and Oka (1), such resistance probably results from the appearance, under aerobic conditions, of increased quantities of a cytochrome oxidase relatively resistant to azide (such as cytochrome a_2). Neither the *azi* nor the *leu* genes appear to have any functional relationship to the *mut* gene(s).

DISCUSSION

Three strains of *E. coli* exhibiting high mutation rates as a result of a single mutation have been reported. These strains map at widely different positions. The Treffers' mutator (*mutT1*) maps at about 1 min on the *E. coli* chromosome and cotransduces with *leu* (24, 27, and this paper). A second mutator gene, *ast*, probably maps between 3 and 8 min (between *tonA* and *proA;* 27, 30), whereas *mutS1* maps at about 53 min (24, 27). A mutant gene (*mut*) causing high mutation rates in *Salmonella typhimurium* LT7 has been shown to cotransduce with *purA* (15). If the homology between *Salmonella* and *E. coli* is complete in this region, *E. coli* has a gene mapping at about 88 min which can mutate to give high mutation rates (23, 27).

High mutation rates in *mut* mutants must result from errors in replication or from uncorrected damage to DNA which has already been made. Errors in replication may be caused in any one of a number of ways. (i) One of the macromolecular components normally involved in deoxyribonucleic acid (DNA) replication may be defective. The most obvious example would be a mutant DNA polymerase which makes mistakes more frequently than does the wild-type enzyme. A mutant DNA polymerase in phage T4 has been shown to increase mutation rates (25). (ii) Some other cell component, e.g., a nuclease, which is not usually involved in replication, may acquire an increased affinity for one of the normal components of replication and thus interfere with replication. (iii) An analogue of one of the small molecules required for DNA synthesis may accumulate in the mutant cell (but not in the wild-type cell) and lead to mistakes in replication; e.g., by interference with incorporation of the normal nucleotides or by incorporation in place of a normal nucleotide. This is thought to be the way the *Salmonella* mutator gene leads to high muta-

tion rates (14). (iv) Interference with the supply of nucleoside triphosphates or other substrates or of cofactors or enzymes involved in replication may be mutagenic. Thymine starvation is known to be mutagenic (4, 28), but this is a complex phenomenon and there is no evidence that starvation for other bases is mutagenic. (v) The ionic strength, *p*H, cation concentrations, etc., of the interior of the cell may be altered, thus affecting replication.

Mutation may result from a breakdown or alteration of the DNA already made. In the normal cell, such damage does not occur extensively *or*, if it occurs, the damage is repaired. If the high rate of mutation in *mutT1* cells results from alteration of old DNA, the processes normally involved in repair of irradiation damage are unlikely to be involved in either the alteration or its repair, since a *mutT1* derivative of strain Bs-1 which lacks at least two of these repair functions (10, 19) showed the same mutation rate to streptomycin resistance as strain B/r which had these functions.

Yanofsky, Cox, and Horn have shown that the *mutT1* mutation causes the replacement of adenine plus thymine (AT) base pairs by guanine plus cytosine (CG) base pairs (transversion; 29). It seems unlikely that such high specificity results from the presence of a base analogue, gross damage to the DNA, general interference with replication, etc.; thus, it is possible that a component of DNA polymerase itself is defective. This hypothesis is supported by the observation that viruses which have their own DNA polymerase exhibit normal mutation rates when grown in a *mutT1* host, whereas (presumably) other viruses dependent on the host polymerase show high mutation rates when grown on a *mutT1* host (cited in 16). On the other hand, this hypothesis is contradicted by the results of Pierce (21), who found that phage T4 (which is known to be dependent for replication on a polymerase coded in the phage genome) could be mutated by *mutT1*. Thus, on the basis of Pierce's results it is reasonable to assume that *mutT1* does *not* directly affect the structure of the polymerase.

It is worth noting that it may be possible to obtain two important classes of mutants when reliable selective methods are developed. One such class would have high mutation rates at 30 C and show lethality at 42 C. It should be possible to identify the enzymatic lesion leading to high mutation rates in such strains.

It should also be possible to isolate a series of *mut* mutants, each with a unique specificity in the mutational change it produces. It is known that *mutT1* causes AT to CG transversions. Other mutations may cause the reverse transversion, and still others may cause transitions, base addition, or deletion. Although its specificity has not yet been shown to differ, *mut-2* is clearly not identical to *mutT1*. A spectrum of mutants which cause specific base pair changes would be quite useful because chemical mutagens generally lack such high specificity.

ACKNOWLEDGMENTS

I thank A. Cohen and E. C. Siegel for contributing strains.

This investigation was supported by grant GB-5737 from the National Science Foundation.

LITERATURE CITED

1. Arima, K., and T. Oka. 1965. Cyanide resistance in *Achromobacter*. I. Induced formation of cytochrome a_2 and its role in cyanide-resistant respiration. J. Bacteriol. **90**:734–743.
2. Bertani, G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. **62**:293–300.
3. Cavalli-Sforza, L. L., and J. Lederberg. 1956. Isolation of pre-adaptive mutants in bacteria by sib selection. Genetics **41**:367–381.
4. Coughlin, C. A., and E. A. Adelberg. 1956. Bacterial mutation induced by thymine starvation. Nature **178**:531–532.
5. Cribbs, R. M. 1965. Reversions of the L-ribulokinase structural gene of *Escherichia coli*. Genetics **52**:99–106.
6. Cribbs, R., and E. Engelsberg. 1964. L-Arabinose negative mutants of the L-ribulokinase structural gene affecting the levels of L-arabinose isomerase in *Escherichia coli*. Genetics **49**:95–108.
7. Davis, B. D., and E. S. Mingioli. 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17–28.
8. Demerec, M. 1951. Studies of the streptomycin-resistance system of mutations in *Escherichia coli*. Genetics **36**:585–597.
9. Flaks, J. G., E. C. Cox, M. L. Whitting, and J. R. White. 1962. Polypeptide synthesis with ribosomes from streptomycin-resistant and dependent *E. coli*. Biochem. Biophys. Res. Commun. **7**:390–393.
10. Greenberg, J. 1967. Loci for radiation sensitivity in *Escherichia coli* strain Bs-1. Genetics **55**:193–201.
11. Gross, J., and E. Englesberg. 1959. Determination of the order of mutational sites governing L-arabinose utilization in *Escherichia coli* B/r by transduction with phage Plbt. Virology **9**:314–331.
12. Helling, R. 1967. The effect of arabinose-specific enzyme synthesis on recombination in the arabinose genes of *Escherichia coli*. Genetics **57**:665–675.
13. Hill, R., and E. Simson. 1961. A study of radiation sensitive and resistant mutants of *E. coli* B. J. Gen. Microbiol. **24**:1–14.
14. Kirchner, C. E. J. 1960. The effects of the mutator gene on molecular change and mutation in *Salmonella typhimurium*. J. Mol. Biol. **2**:331–338.
15. Kirchner, C. E. J., and M. J. Rudden. 1966.

Location of a mutator gene in *Salmonella typhimurium* by cotransduction. J. Bacteriol. **92**:1453–1456.
16. Kornberg, A. 1967. Biosynthesis of DNA, p. 22–38. *In* V. V. Koningsberger and L. Bosch (ed.), Regulation of nucleic acid and protein biosynthesis. Elsevier, Amsterdam.
17. Lea, D. W., and C. A. Coulson. 1948. The distribution of the numbers of mutants in bacterial populations. J. Genet. **49**:264–285.
18. Lennox, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
19. Mattern, I. E., H. Zwenk, and A. Rörsch. 1966. The genetic constitution of the radiation-sensitive mutant *Escherichia coli* Bs-1. Mutation Res. **3**:374–380.
20. Newcombe, H. B., and R. Hawirko. 1949. Spontaneous mutation to streptomycin resistance and dependence in *Escherichia coli*. J. Bacteriol. **57**:565–572.
21. Pierce, B. L. S. 1966. The effect of a bacterial mutator gene upon mutation rates in bacteriophage T4. Genetics **54**:657–662.
22. Ryan, F. J. 1963. Mutation and population genetics. *In* W. J. Burdette (ed.), Methodology in basic genetics. Holden-Day, San Francisco.
23. Sanderson, K. E. 1967. Revised linkage map of *Salmonella typhimurium*. Bacteriol. Rev. **31**: 354–372.
24. Siegel, E. C., and V. Bryson. 1967. Mutator gene of *Escherichia coli* B. J. Bacteriol. **94**:38–47.
25. Speyer, J. F. 1965. Mutagenic DNA polymerase. Biochem. Biophys. Res. Commun. **21**:6–8.
26. Speyer, J. F., P. Lengyel, and C. Basilio. 1962. Ribosomal localization of streptomycin sensitivity. Proc. Natl. Acad. Sci. U.S. **48**:684–686.
27. Taylor, A. L., and C. D. Trotter. 1967. Revised linkage map of *Escherichia coli*. Bacteriol. Rev. **31**:332–353.
28. Weinberg, R., and A. B. Latham. 1956. Apparent mutagenic effect of thymine deficiency for a thymine-requiring strain of *Escherichia coli*. J. Bacteriol. **72**:570–572.
29. Yanofsky, C., E. C. Cox, and V. Horn. 1966. The unusual mutagenic specificity of an *E. coli* mutator gene. Proc. Natl. Acad. Sci. U.S. **55**:274–281.
30. Zamenhof, P. J. 1966. A genetic locus responsible for generalized high mutability in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. **56**:845–852.
31. Zamenhof, S., L. H. Heldenmuth, and P. J. Zamenhof. 1966. Studies on mechanisms for the maintenance of constant mutability: mutability and the resistance to mutagens. Proc. Natl. Acad. Sci. U.S. **55**:50–58.