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Proliferation of Mutators in A Cell Population

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A Lac$^-$ strain of *Escherichia coli* that reverts by the addition of a G to a G-G-G-G-G-G sequence was used to study the proliferation of mutators in a bacterial culture. Selection for the Lac$^+$ phenotype, which is greatly stimulated in mismatch repair-deficient strains, results in an increase in the percentage of mutators in the selected population from less than 1 per 100,000 cells to 1 per 200 cells. All the mutators detected were deficient in the mismatch repair system. Mutagenesis results in a similar increase in the percentage of mutators. Mutagenesis combined with a single selection can result in a population of more than 50% mutators when a sample of several thousand cells is grown out and selected. Mutagenesis combined with two or more successive selections can generate a population that is 100% mutator. These experiments are discussed in relation to ideas that an early step in carcinogenesis is the creation of a mutator phenotype.

Although cells with higher spontaneous mutation rates, or mutators, have been studied for many years, the recent finding that the inherited form of human nonpolyposis colon cancer results from a defect in the human counterpart to the bacterial mismatch repair system and that this defect creates a mutator phenotype (1, 7, 18, 33) has generated new excitement in the field of repair systems in general and focused renewed attention on mutators in particular. Loeb has suggested (21, 22) that tumor progression may require the creation of a mutator cell, since the multistage process of carcinogenesis (32), which probably occurs by genetic instability and clonal selection (8), depends on a series of mutations. The spontaneous mutation rate cannot account for the multiple mutations observed in human tumors, but if creation of a mutator occurs early in tumor progression, then the multiple mutations could be generated (21, 22). Can mutators arise and proliferate rapidly enough in a cell population to play a role in carcinogenesis? The first mutators discovered in bacteria were found in existing laboratory strains (27, 37). Early experiments on detecting mutators dealt with screening isolates in the wild (12, 16) and also involved mutagenized populations of bacteria, sometimes coupled to different selections (6, 13, 14, 19). For example, Jyssum (16) found that 4 of 110 natural isolates of *Escherichia coli* from patients were mutators, while Gross and Siegel (12) detected mutator activity in 1 of 408 natural *E. coli* isolates. Such findings suggested that natural populations might be undergoing constant selection, since competition experiments in chemostats showed that under certain conditions mutators have increased fitness in a population (2, 4, 11, 29). Siegel and Bryson (35, 36) discovered the *mutS* gene after observing the mutator phenotype of a strain selected to be azaserine resistant. Also, Helling (13) mutagenized cells and selected for streptomycin-resistant cells and then screened for mutator activity and detected examples of mutators carrying *mutT* defects, and Hoess and Herman utilized a multiple auxotroph to select for reversion of frameshift mutations in a mutagenized population after six successive selections (14).

In the work reported here, we focus on how easily a population can be converted to a high percentage of mutators. We show how selection, even in the absence of mutagenesis, can greatly increase the percentage of mutators in a population and how this percentage can be enhanced further with the use of mutagens and additional selections. In some cases, virtually 50 to 100% of the survivors of mutagenized cell populations that are subjected to a single selection are mutators.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strain CC107 (see Fig. 1) carries an F′*lacpro* episome in the P90C (5, 25) strain background, *ara* Δ(*gpt-lac*)*5*. The *lac* region on the F factor carries a *lacI* mutation and also a frameshift in the *lacZ* gene that reverts by the addition of a GC base pair to a monotonous run of GC base pairs. This strain is described by Cupples et al. (5). Strain EM100 carries an F′*lacpro* episome which is *lacIZ* in the P90C background. The *lacZ* gene on the episome carries an amber mutation at coding position 17. All other strains and bacterial genetic methods, such as determination of rifampin resistance (Rif^r), nalidixic acid resistance (Nal^r), and trimethoprim resistance, which results in ThyA$^-$ strains, are described by Miller (25). For control experiments, a *mutH* derivative of CC107, in which a miniTn*10* had integrated into the *mutH* gene, was used (26a).

Preparation of cultures. Unless otherwise stated, all cultures for the experiments reported here were prepared by inoculating a portion of a single colony into Luria-Bertani medium (LB) or other medium and growing overnight. A different single colony was used for each culture. Therefore, all mutants occurring in different cultures are of independent origin, since each single colony is derived from a single isolated cell. A 5-ml culture was inoculated in the experiments in which Lac$^+$ revertants of CC107 were used.

Mutagenesis. All methods of mutagenesis are exactly as described by Miller (25). UV-light mutagenesis was performed at different doses, and mutants were measured at doses (90-s exposure to a germicidal lamp at a distance of 37 cm) that gave between 1 and 3% survival. Control experiments showed that there was no detectable difference in survival after UV between mismatch repair-deficient derivatives of CC107 (*mutH*) and wild-type CC107 (data not shown.) Ethyl methanesulfonate (EMS) was used at doses (45 s of exposure to 0.03 ml of EMS added to 2 ml of resuspended cells) that gave no detectable killing of either CC107 or *mutH* derivatives of CC107. 2-Aminopurine (2AP) was used at concentrations of 700 μg/ml. Cultures were prepared by subculturing 10^4 to 10^5 cells into 4 ml of LB with 2AP, and these were then grown for 12 to 16 generations in LB with 2AP before plating for mutants.

Spontaneous mutators in CC107 were detected by direct visualization on glucose minimal medium with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and P-Gal (phenyl-β-D-galactoside) (30). On this medium, mutator colonies have a greatly increased number of blue papillae (30). We failed to detect any mutators among over 100,000 colonies from 150 different cultures. From the Poisson distribution it can be calculated that if the frequency of mutators were 3×10^{-5}, then the probability of finding no mutators would be $P = 0.05$. So, we can say that the frequency of mutators is equal to or less than 3×10^{-5} with a confidence level of 95%.

Competition experiments. In order to test whether under the conditions of the experiments reported here mutators deficient in the mismatch repair system had a growth advantage or growth disadvantage, different ratios of CC107 and the

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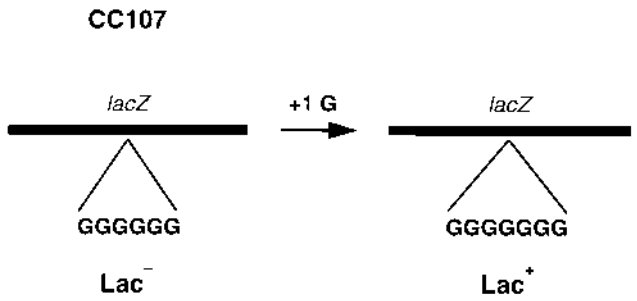

FIG. 1. Lac$^+$ reversion in CC107. The strain CC107 reverts from Lac$^-$ to Lac$^+$ by a frameshift at a run of six G's (2). The addition of a seventh G restores the Lac$^+$ character.

mutH derivative described above were mixed either in LB or in LB containing 2AP and grown for 40 generations, by subculturing 10^7 cells from successive overnight cultures into fresh 4-ml cultures. At different intervals, samples were plated on LB and LB-plus-tetracycline plates. In some cases, colonies growing on LB were then replicated onto LB-plus-tetracycline plates. Since the *mutH* derivative carries a miniTn*10* in the *mutH* gene, this allows us to determine whether a colony is the wild type or the CC107 derivative. We used ratios of wild-type to *mutH* of 25:1 and 100,000:1. The latter ratio simulates the situation that occurs when the first mutator arises spontaneously in a wild-type culture. Approximately 12 generations occur between the first mutator arising and plating of the culture, so examining competition for 20 generations or more should be sufficient to determine whether there is a relevant growth advantage. In any case, for both ratios, no change in the ratio was observed after 12, 20, 27, 35, or 42 generations in either LB or LB with 2AP.

Successive selections. Overnight cultures (5 ml) inoculated from a single colony were transferred to 200 ml of LB in 500-ml flasks and grown to saturation overnight. These were subcultured by transferring 2 ml of each culture to 200 ml of minimal medium with 1.5 g of P-Gal per liter in 500-ml flasks. After 40 h of growth, a second transfer of 2 ml of each culture to 200 ml of P-Gal minimal medium was carried out, and these cultures were grown to saturation. The two transfers ensured that a large sample size of cells was transferred and that enough generations in P-Gal occurred to result in greater than 95% of the cells being Lac$^+$. This could be tested by plating samples on lactose MacConkey indicator medium and observing the proportion of red Lac$^+$ colonies to white Lac$^-$ colonies. In several additional cases, a fivefold-larger sample of cells was transferred to a fivefold-larger volume of medium in a larger flask. This did not affect the results of the experiment.

RESULTS

Spontaneous mutators. To examine the origin and proliferation of mutators in bacterial cell populations, we used a strain of *E. coli*, CC107 (5), which reverts from Lac$^-$ to Lac$^+$ by a frameshift at a monotonous run of 6 GC base pairs. The Lac$^-$ strain contains the sequence GGGGGG in the *lacZ* gene, and the Lac$^+$ revertant contains the sequence GGGGGGG (Fig. 1). CC107 is a good probe for mutators in a population, because mutants with defects in the mismatch repair genes, *mutH*, *mutL*, *mutS*, and *uvrD*, stimulate this frameshift significantly (5), raising the Lac$^+$ mutant frequency from the spontaneous level of approximately 2×10^{-7} to 3×10^{-7} to near 10^{-4}. We expect these mutators to represent the bulk of mutators detected with CC107, since although numerous genes which result in the mutator phenotype have been identified in *E. coli* (for reviews, see references 3 and 26), only a limited set results in strong mutators that can induce frameshifts. In addition to mutants with defects in one of the four mismatch repair genes, *mutD* strains lacking the editing subunit of DNA polymerase III also produce frameshifts (9), although this latter mutator often displays a growth disadvantage and should be underrepresented among spontaneous mutators.

In the absence of any selection, the fraction of cells in a population of 10^{10} cells grown from a single cell with a defect in any one of these strong mutator genes is on the order of several times 10^{-6}, the approximate fraction of cells with a

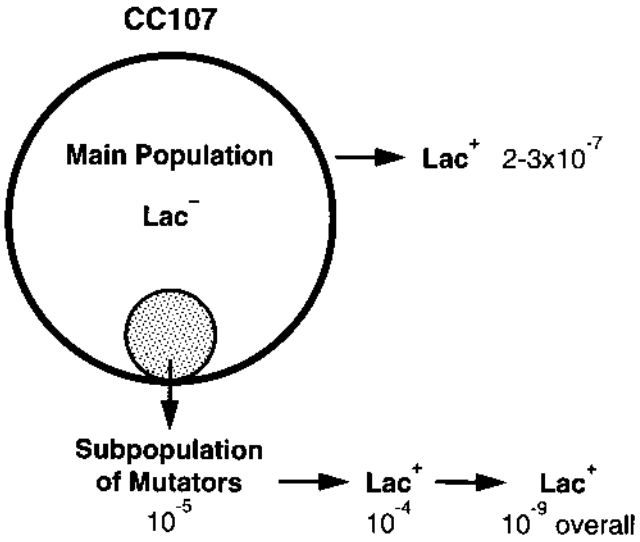

FIG. 2. Reversion frequencies in a population of Lac$^-$ cells. The main population of Lac$^-$ cells gives rise to single-step Lac$^+$ revertants, which appear at approximately 2×10^{-7} to 3×10^{-7} in the population. A small subpopulation of mutators arises at close to 10^{-5}, which then gives rise to single-step revertants at a higher rate, approximately 10^{-4}. The frequency of Lac$^+$ revertants in the overall population due to the subpopulation of mutators is thus close to 10^{-9} and represents about 0.5% of the total Lac$^+$ revertants seen.

defect in any average-size gene in *E. coli* that leads to loss of function. The total fraction with defects in one of the four strong mutator genes that lead to frameshifts (*mutH*, *mutL*, *mutS*, and *uvrD*) is thus expected to be on the order of 10^{-5} in a population of wild-type cells. Although it is possible that mutators may have increased or decreased fitness under certain conditions after many generations of growth (2, 4, 11, 29), control competition experiments with wild-type and *mutH* derivatives of strain CC107 demonstrate that after up to 40 generations of growth in LB under the conditions employed here, there is no growth advantage or disadvantage of the *mutH* derivative over the wild type (see Materials and Methods). To verify that spontaneous mutators are not seen at frequencies greater than 10^{-5} in 4-ml LB cultures of CC107 initiated from a single colony and to establish a background for our experiments, we examined over 100,000 colonies from CC107 (see Table 1 and Materials and Methods) by plating approximately 700 cells from each of 150 independent cultures on minimal glucose plates with glucose, P-Gal, and X-Gal and screening for mutators that increase the number of Lac$^+$ papillae. Reconstruction experiments show that we can easily detect a mutator among as many as 1,000 colonies on these plates. We failed to detect a single mutator among the 100,000 colonies, showing that the frequency of spontaneous mutators that can generate reversion to Lac$^+$ in a population of CC107 is less than 3×10^{-5} (see Materials and Methods).

Mutators in a population undergoing selection. We tested 320 independent cultures by plating a sample of approximately 10^8 cells from each culture on lactose minimal plates to select for Lac$^+$ revertants. Revertants can occur via a single-step pathway by a simple frameshift. Alternatively, they can also be generated in two steps by first having a mutator arise by mutation and then having the mutator cell induce the frameshift. Thus, each population of cells will have a rare subpopulation that generates mutations at a higher rate (Fig. 2). By selecting for Lac$^+$ revertants, we may be able to isolate members of that subpopulation by screening the Lac$^+$ revertants for those with

TABLE 1. Frequency of mutators in wild-type cultures of CC107 and among Lac^+ revertants of CC107[a]

No. of cultures of CC107	Total colonies screened	Total Lac^+ revertants screened	Mutators detected	Frequency
150	>100,000		0	$\leq 10^{-5}$
320		1,600	8	5×10^{-3}

[a] 150 LB cultures of CC107 initiated from different single colonies were grown overnight and plated on glucose minimal plates with X-Gal and P-Gal to detect mutators by an elevated number of blue papillae (30). 320 LB cultures of CC107 were plated on lactose minimal plates, and five Lac^+ colonies from each culture were screened for mutator activity by monitoring the frequency of Rif^r colonies in replicated patches and then testing more quantitatively in liquid broth cultures.

TABLE 2. Frequency of mutators among mutagenized populations of CC107[a]

Culture treatment	% Mutators	No. of mutators with defect in gene:			
		mutS	*mutH*	*uvrD*	*mutL*
None	<0.001				
2AP	0.6–1.0	2	2	0	5
EMS	1.0–2.0				
UV	0.25				

[a] Mutagenized cells were grown overnight to allow expression of the mutator phenotype and then plated on minimal glucose X-Gal–P-Gal plates. Colonies with increased numbers of blue papillae were picked and tested further. All nine mutators derived by 2AP mutagenesis were found to have defects in one of the mismatch repair genes.

mutator activity. We tested five Lac^+ colonies from each of the 320 cultures (1,600 colonies) for their frequency of mutating to rifampin resistance (Rif^r). Those that showed an increased level were repurified, and several new colonies were retested for increased Rif^r and Nal^r. From the 1,600 tested colonies, 8 were found to be mutators (see Table 1). The eight were from seven different cultures and, thus, represented at least seven independent mutants. Six of these were mapped with P1 lysates prepared on strains containing tetracycline resistance (Tet^r) markers near each of the mismatch repair genes. These tests showed that three of the mutators resulted from mutations in the *mutH* gene and three resulted from mutations in the *mutS* gene.

The results shown in Table 1 establish that selecting for Lac^+ colonies in a population of Lac^- cells in CC107 results in an increase from less than 1 in 100,000 mutators to 1 in 200 (8/1,600 = 0.5%). This 500-fold increase is striking and allows us to state the following principle.

(i) Selection for a mutant phenotype in a population can greatly increase the proportion of mutators in the selected population.

The extent of the increase will depend on the nature of the mutation that results in the selected phenotype. In the case of CC107, the Lac^+ reversion is greatly stimulated by several mutators that can arise in the population. However, in some cases the stimulation is more modest. This is true for the strain EM100 (see Materials and Methods), which carries a different F′*lacpro* episome in the same strain background as CC107. Here the *lacZ* gene contains an amber mutation at coding position 17 that can revert by any one of a number of base substitutions at the site of the amber mutation itself or by the creation of one of several nonsense suppressor mutations. The stimulation of these changes by most of the mutators that can arise in *E. coli* is smaller than the stimulation of the frameshift in CC107. We examined 270 cultures of strain EM100, plating samples on lactose minimal plates. Five revertants per plate (1,350) colonies were screened for mutator activity by their ability to generate Rif^r mutants. Only one mutator was found among the 1,350 colonies, in this case.

The effect of mutagenesis on the proportion of mutators in a population. We grew CC107 in cultures with 2AP (see Materials and Methods) and plated them on glucose minimal plates with X-Gal and P-Gal to visualize mutators in the mutagenized population. Although 2AP is a mutagen of only moderate strength, 2AP-treated cultures of CC107 had frequent mutators in each independent culture, with an average from 12 cultures of slightly more than one mutator per 200 cells (Table 2). Competition experiments showed that 2AP did not confer a growth advantage or disadvantage to *mutH* mutator derivatives of CC107 relative to the wild-type CC107 (see Materials and Methods). Examples of these mutators were analyzed further by characterizing their elevated mutation rate for rifampin resistance and by genetic mapping, and we verified that they carry mutations in *uvrD*, *mutL*, *mutS* or *mutH*, or *mutD*. All nine mutators tested had a defect in one of the mismatch repair genes. Similar results were obtained with EMS and UV light, an environmental mutagen, as is also shown in Table 2. Since mutagens increase the frequency of mutators from 1/100,000 to 1/200, we can thus state the obvious.

(ii) Exposure to a mutagen significantly increases the proportion of mutators in a cell population.

Successive selections. We transferred 12 cultures of CC107 grown overnight to saturation in broth (see Materials and Methods) to liquid minimal medium with P-Gal as the sole carbon source to allow growth of only the Lac^+ revertants in the overnight population. They were grown to saturation before being transferred again to P-Gal medium. The cultures, which now contained greater than 95% Lac^+ colonies, were then transferred to broth and grown overnight to allow expression of mutant phenotypes that have a significant phenotypic lag (such as Rif^r) before being plated on medium with rifampin. This procedure represents successive selections for Lac^+ and then Rif^r. The presumption is that the percentage of mutators is increased by selecting for Lac^+, as shown in Table 1, and then further increased by selecting for Rif^r among the Lac^+ revertants. We tested 50 Lac^+ Rif^r colonies from each culture for mutator activity by monitoring the increase in mutants resistant to nalidixic acid (Nal^r) after replica plating patches. Wild-type cells typically have no Nal^r colonies growing out of a replicated patch of cells, whereas mutants lacking the mismatch repair system (*mutH*, *mutL*, *mutS*, or *uvrD*) have various amounts of Nal^r colonies. Reconstruction experiments showed a reproducible and easy-to-recognize phenotype for increased frequency of Nal^r by mutators. More quantitative tests in liquid culture verified the mutator phenotype. (The mutator nature of colonies was also verified by monitoring an increased frequency of trimethoprim resistance.) It was found that 3 of 12 Lac^+ cultures yielded 25% or more mutators among the Rif^r mutants.

As an additional control experiment, we plated 50 cultures of CC107 grown overnight in 5 ml of LB broth on plates selecting for either Lac^+ or Rif^r, and we tested 50 Lac^+ or Rif^r colonies from each culture. We found that 0 of 50 cultures yielded more than 4% mutators when plated only for Lac^+, and 0 of 50 cultures yielded more than 2% mutators when plated only for Rif^r. Thus, the percentage of mutators, which

TABLE 3. Frequency of mutators among mutagenized populations of CC107 following subculture and selection[a]

Culture treatment	% Mutators after subculture among:	
	Lac^+	Rif^r
None	0.5	<0.1
2AP	96	36

[a] Mutagenized cultures were subcultured by transferring between 1,000 and 10,000 cells to fresh LB medium (25) and growing to saturation before plating on medium selecting either for Lac^+ revertants or Rif^r mutants. The surviving colonies were then screened for mutator activity by replicating patches and testing for increased Nal^r and either increased Rif^r or Lac^+. One hundred colonies from each selection from each of several 2AP-treated cultures were tested, and 1,600 colonies from each selection from over 300 untreated cultures were selected.

increased from less than 0.001% in the spontaneous population to 0.5% in the Lac^+ population (Table 1), has now gone as high as 40% in some cases. However, this is true in only some of the cultures (3 of 12), and even when a very large cell population is involved (see Materials and Methods), many of the cultures do not show this significant increase.

Mutation followed by selection. It would appear that the near 1% of the mutators present in 2AP-mutagenized cultures of CC107 could be increased easily by selection. Therefore, we plated eight different 2AP-grown cultures of CC107 on lactose minimal medium, tested 50 to 100 Lac^+ colonies for mutator activity by replicating patches onto medium testing for an increased frequency of Rif^r mutants, and found that the percentage of mutators in the selected population was no higher than the starting 1%. We attribute this to the fact that the Lac^+ phenotype was also increased by the 2AP mutagenesis, so selecting for this phenotype does not increase the proportion of mutators in the selected population.

After mutagenesis, a cell population of as few as several hundred should contain a mutator, whereas it would take a population of 10,000 to 100,000 to contain a Lac^+ revertant cell induced by the mutagenic treatment. Therefore, in a large population of mutagenized cells, a single selection may not greatly increase the proportion of mutator phenotypes. However, if a subpopulation of such cells is grown up and subjected to a selection, then the proportion of mutators should increase greatly, since the small population contains a mutator, but not an induced Lac^+ mutant (in this case). Whereas the percentage of mutators might be near 0.5%, the Lac^+ colonies the mutators induce at 10^{-4} would then represent 0.5×10^{-6} of the population, compared with the spontaneous (nonmutator-induced) background of 0.2×10^{-6} to 0.3×10^{-6} Lac^+. In other words, we would predict that greater than 50% of the Lac^+ colonies would now be mutators. To demonstrate this, we subcultured several thousand cells and then plated for Lac^+ or Rif^r mutants. The resulting Lac^+ colonies were initially tested for mutator activity by the increased frequency of Rif^r, and the Rif^r colonies were tested for increased frequency of Lac^+ and Nal^r. Table 3 shows the results. It can be seen that more than 95% of the Lac^+ colonies are mutators, and 36% of the Rif^r colonies are mutators. This striking result leads to the following conclusion.

(iii) Exposure to a mutagen followed by growth of a small population can result in almost 100% of the surviving cells becoming mutators after a single selection.

Mutation followed by successive selections. We can best demonstrate the effects of successive selections by transferring

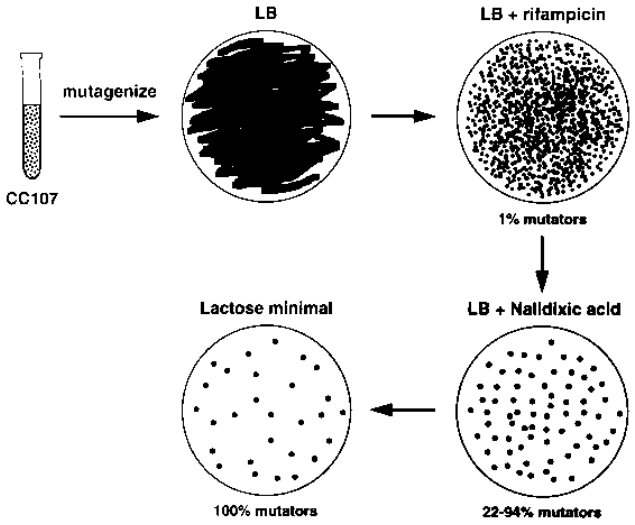

FIG. 3. The increase in the percentage of mutators in a population of cells undergoing selection.

cells to different media via replica plating, as depicted in Fig. 3. We spread approximately 10^8 mutagenized CC107 cells on plates containing rich medium (LB plates; see Materials and Methods). After overnight growth, the plates are confluent with cells. These are then replicated onto rich medium containing rifampin to select for Rif^r colonies. Over 1,000 Rif^r colonies emerge from the background. When samples of these are tested, approximately 1% of them are mutators. The plates with rifampin are replicated in turn onto rich medium with nalidixic acid to select for Nal^r colonies. Now only 20 to 100 colonies grow up overnight. However, between 20 and 95% of these are mutators (see Table 4), as determined by their increased reversion to Lac^+. If these are further replicated onto lactose minimal medium, then 100% of the colonies that grow are mutators, which can be demonstrated by higher rates of generating trimethoprim-resistant colonies.

We can also demonstrate the effect of two successive selections on mutagenized cells by first growing mutagenized cells in LB medium containing rifampin, which selects for the Rif^r cells, and then plating on medium with nalidixic acid. Here, the 2AP-mutagenized cultures showed a high frequency (100%) of mutators among the Nal^r colonies, whereas the EMS-mutagenized cultures displayed a lower proportion (data not shown). Therefore, we can state the following.

(iv) Exposure to a mutagen followed by successive selections can result in close to 100% of the remaining cells in a population being mutators.

DISCUSSION

Loeb (21, 22) has compiled estimates of somatic and germ cell mutation rates and concluded that spontaneous mutations cannot account for most cancer cell lines that require more than two mutations for their generation, and certainly not for cells that may require five or six mutations in order to become full-fledged tumor cells. However, if the creation of a mutator cell were an early step in carcinogenesis, then the increased mutation rates could explain tumor formation and progression (21). The finding that various tumor lines display mutator phenotypes supports this notion. For instance, inherited susceptibility to colon cancer is due to a defective copy of one of

the genes involved in mismatch repair (1, 7, 18, 33). When a somatic cell loses the other copy of the gene, the resulting cell that lacks mismatch repair is a mutator and leads to more frequent colon cancer.

How do mutator cells arise and overtake a population? Some of the first mutators observed were detected among stocks of laboratory strains of bacteria (27, 37), and approximately 1% of natural isolates (4 of 128 in one study [16] and 1 of 408 in another study [12]) have been shown to be mutators, leading to the assumption that populations exposed to continuous selective forces will have an increased proportion of mutators. This has been addressed in several ways. Competition experiments in chemostats have shown that after 60 to 100 generations, mutators in a population have an increased fitness under certain conditions (2, 4, 11, 29). The role of selection in increasing the percentage mutators has also been exploited in experiments aimed at isolating new mutator strains (6, 13, 14, 19, 35, 36). For instance, Hoess and Herman (14) reported that after six successive selections for reversion of different frameshift mutations, the resulting colonies were mutators deficient in the mismatch repair system.

The study reported here focuses on the ease with which mutator cells can proliferate in a population undergoing selection. Selecting for Lac^+ cells from a Lac^- population carrying a frameshift mutation (Fig. 1) that reverts by replication errors can increase the frequency of mutators in the population from less than 1/100,000 to 1/200 (Table 1). This is because, in the case used here, Lac^+ cells can be generated either as a single step at about 2×10^{-7} to 3×10^{-7} or as a two-step process (Fig. 2) in which the subpopulation of mutators of about 10^{-5} generates Lac^+ cells at the much higher frequency of 10^{-4}. This results in a frequency of Lac^+ cells of 10^{-9}, contributed by the mutator subpopulation. This 10^{-9} fraction represents about 1 in 200 of the Lac^+ revertants created by a single step in the overall nonmutator population, which occur at close to 2×10^{-7}. The actual observed fraction of mutants contributed by mutators in any selection will vary, depending on several factors, including the nature of the mutation being reverted, as shown here. Ninio has also estimated the fraction of mutants contributed by mutators (31).

Mutators are also generated by mutagens, and the percentage of mutators in the population can be increased to as high as 1% (Table 2). The combination of mutagenesis plus a single selection at first may not result in an increased percentage of mutators much above the 1% level, since mutagenesis also increases the selected phenotype among nonmutators. This has also been found in previous studies (see, for instance, reference 19). However, additional steps can result in the majority of the population being converted to mutators. We can see this by either taking mutagenized cells and subculturing a population of 1,000 to 5,000 cells and then selecting for Lac^+ (Table 3) or submitting mutagenized cells to two or more successive selections (Fig. 3; Table 4).

The work described in this paper can be considered in light of the finding that many mutator tumor cell lines that lack mismatch repair have mutations in the *RII* gene (23), encoding a receptor for transforming growth factor β, an inhibitor of epithelial cell growth (20, 28, 38). Loss of this inhibition is believed to be involved in tumor development (10, 15, 34, 39). The mutations are frameshifts at either a run of 10 A's or a GTGTGT sequence, not unlike the GGGGGG→GGGGGGG frameshift involved in the Lac^+ reversion employed here, and are thus a target for frequent mutational events. Mutator cells in a population undergoing a growth selection to overcome the effects of transforming growth factor β will be enhanced in the same way that selection for Lac^+ cells enhances mutator frequencies in bacterial cultures of strain CC107. Also, many tumor lines have to overcome the normal action of different tumor suppressors (for reviews, see references 17 and 24). For example, close to half of all tumor cell lines for many types of cancer have mutations in the gene encoding the p53 tumor suppressor. Successive mutations can result from the action of the mutator and successive selections to overcome the tumor suppressors, in further analogy to the successive-selection experiments reported here (Fig. 3; Table 4) in which the surviving population is completely mutator.

TABLE 4. Frequency of mutators in mutagenized populations of CC107 after successive selections by replica plating[a]

Cell treatment	% Mutators after successive selection by replication for:			
	None	Rif^r	Rif^r Nal^r	Rif^r Nal^r Lac^+
2AP	0.5	1	62;94	100
EMS	0.5	1	22;32	100

[a] Figure 3 diagrams this experiment, which is described in the text. The two different numbers for the percentage of mutators among the Rif^r Nal^r colonies represent two different experiments. The mutator phenotype of the Rif^r Nal^r Lac^+ cells was determined by testing for an increased rate of generation of $ThyA^-$ mutants, by selecting for trimethoprim resistance (see Materials and Methods).

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