The Consequences of Growth of a Mutator Strain of *Escherichia coli* as Measured by Loss of Function Among Multiple Gene Targets and Loss of Fitness

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ABSTRACT

We have examined the composition of members of mutator populations of *Escherichia coli* by employing an extensive set of phenotypic screens that allow us to monitor the function of >700 genes, constituting ~15% of the genome. We looked at mismatch repair deficient cells after repeated cycles of single colony isolation on rich medium to generate lineages that are forced through severe bottlenecks, and compared the results to those for wild-type strains. The mutator lineages continued to accumulate mutations rapidly with each increasing cycle of colony isolation. By the end of the 40th cycle, after ~1000 generations, most of the lineages had reduced colony size, 4% had died out, 55% had auxotrophic requirements (increasing to 80% after 60 cycles), and 70% had defects in at least one sugar or catabolic pathway. In addition, 33% had a defect in cell motility, and 26% were either temperature-sensitive or cold-sensitive lethals. On the other hand, only 3% of the wild-type lineages had detectable mutations of any type after 40 cycles. By the 60th cycle, the typical mutator cell carried 4–5 inactive genes among the 15% of the genome being monitored, indicating that the average cell carried at least 24–30 inactivated genes distributed throughout the genome. Remarkably, 30% of the lineages had lost the ability to utilize xylose as a carbon source. DNA sequencing revealed that most of the Xyl^- mutants had a frameshift in a run of eight G's (GGGGGGGG) in the *xylB* gene, either adding or deleting one -G-. Further analysis indicated that rendering *E. coli* deficient in mismatch repair unmasks hypermutable sites in certain genes or intergenic regions. Growth curves and competition tests on lineages that passed through 90 cycles of single colony isolation showed that all lineages suffered reduced fitness. We discuss these results in terms of the value of mutators in cellular evolution.

WHY have free-living organisms rejected a permanent mutator (high mutation rate) lifestyle as a way of generating diversity? Populations of free-living organisms, and certainly microorganisms, derive great benefit from having diverse phenotypes to help withstand a variety of environmental challenges. For instance, pathogenic organisms need to vary surface antigens to evade host immune responses (for reviews see Moxon *et al.* 1994; Seifert 1996; Deitsch *et al.* 1997). Yet, excluding viruses, most organisms studied to date maintain low mutation rates, having evolved extensive and intricate mechanisms to repair replication errors and DNA damage (for review see Friedberg *et al.* 1995). Instead, diversity is achieved by a variety of gene-specific mechanisms (Moxon *et al.* 1994; Deitsch *et al.* 1997) and by amplification of certain subpopulations in response to environmental stress and selective pressures (Anderson and Roth 1978; Edlund *et al.* 1979; Mekalanos 1983; Tlsty *et al.* 1984; Albertini and Galizzi 1985; Deretic *et al.* 1986).

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Mutators also increase in populations under severe (Mao *et al.* 1997) or prolonged selection (Gibson *et al.* 1970; Nestman and Hill 1973; Cox and Gibson 1974; Chao and Cox 1983; Sniegowski *et al.* 1997). This may be the underlying reason why several percent of natural isolates of *Escherichia coli* and *Salmonella typhimurium* are found to be mutators (Jyssum 1960; Gross and Siegel 1981; LeClerc *et al.* 1996; Matic *et al.* 1997). And yet, populations with high mutation rates are seen as accumulating deleterious mutations at a higher rate than advantageous mutations (see, for instance, Kimura 1967; Leigh 1973). Moreover, Muller (1964) postulated that, in the absence of sexual recombination that can correct mutations, deleterious mutations will accumulate more rapidly, due to the effects of the resulting ratchet or of forcing change in basically only one direction. This effect is amplified in small populations that exhibit high genetic drift. The ratchet effect has been shown in a number of experiments dealing with RNA phage that showed a loss of fitness when propagated through bottlenecks (Chao 1990; Chao *et al.* 1992; Escarmis *et al.* 1996), in protozoa (Bell 1988), and in bacteria (Andersson and Hughes 1996; Kibota and Lynch 1996). Andersson and Hughes (1996) passaged

444 lineages of wild-type *S. typhimurium* through a bottleneck by streaking colonies derived from a single cell for 60 cycles. This resulted in 1% of the lineages suffering a significant loss of fitness and in 3.5% becoming auxotrophs. Also, Kibota and Lynch (1996) examined 50 initially identical lineages of *E. coli* maintained for 300 cycles of single colony streaking. They found that the mean fitness decreased linearly over time.

How high a price does a cell pay for having a high mutation rate? In other words, how extensive is the accumulation of mutations in cells in a mutator population? To answer this question, we examined the distribution of mutations that appear during the growth of a mutator population of *E. coli* carrying a defect in the mismatch repair system, and compared it with the distribution in a wild-type strain. We compiled a series of phenotypic screens to identify inactivated genes among a large set of targets that encompasses close to 700 genes, or ~15% of the *E. coli* genome, and we used these tests to examine members of cell populations before and after passing through single cell bottlenecks that increased the genetic drift. We found that, in contrast to wild-type populations, populations of mutator cells accumulate mutations that inactivate genes very rapidly. Thus, after only 10 cycles of restreaking colonies, loss of specific functions could be detected in close to 40% of the lineages. Continued cycles of passaging colonies through single cell bottlenecks resulted in increased mutations. The increase was approximately linear through 60 cycles. By the 60th cycle of streaking, the typical cell had 4–5 genes inactivated among the targets screened, suggesting that as many as 24–30 genes are impaired in a typical mutator cell after 60 single colony isolations. The lineages also accumulated mutations deleterious to growth on rich medium. Measurements after 90 cycles showed that all lineages exhibited a loss of fitness.

Several hotspots in the genome are revealed by this work, such as a site in the *xyl* operon that results from a run of eight G's on one strand in the *xylB* gene (-GGGGGGGG-). After 60 cycles of single colony isolation, close to 30% of the lineages have a mutation at this hypermutable site. Further sequencing experiments revealed additional genetic instability at an intergenic sequence of 10 consecutive G's. We discuss the overall consequences of a mutator phenotype in terms of the evolution and maintenance of the entire genome.

MATERIALS AND METHODS

Bacterial strains: The starting strain, J93, is derived from strain G90, a gift from Walter Gilbert. It carries a deletion of the *lac* genes (deletion *RV*) and appears wild type for all other markers. A nonreverting *mutS* derivative of this strain was constructed by P1 transduction (for all genetic techniques, see Miller 1992) of a *mutS*::miniTn*10* that we detected and characterized. Tetracycline-resistant transductants were verified to have the properties of *mutS* strains by their high rate of generating Rif^r mutants.

Genetic tests: Auxotrophs were determined by replica plating onto glucose minimal medium, as well as rich (LB) medium. Typically, double replications were used. Colonies were gridded onto an LB plate, grown overnight, and then replicated onto a velvet. The velvet was subsequently replicated onto a second, sterile plate, and this second plate was then replicated onto a second, sterile velvet. This second velvet was then used for replicating onto a fresh minimal plate. This method reduces the material transferred by the velvet, and allows a cleaner interpretation of results. Auxotrophic requirements were identified by adding supplements in groups of three and then trying individual supplements. In cases where the supplement could be identified, the appropriate supplement was added for the respective mutant in subsequent tests to identify additional auxotrophic requirements that appeared as a second, or third, independent defect.

Sugar-negative mutants were identified both by failure to grow on minimal medium containing the relevant sugar as a carbon source and by color reactions on MacConkey plates. Because the strain is deleted for the *lac* genes, we used lactose MacConkey plates supplemented with the appropriate sugar. All plates were incubated overnight at 37°, but in the case of melibiose, a second set of plates was also incubated at 39.5°. Both plates were analyzed. Because the *bgl* operon is normally silenced, requiring a mutation to activate it, all mutant strains were initially negative on MacConkey plates supplemented with salicin, a β-glucoside. However, the *mutS* nature of the strain background generated Sal^+ papillae after 1–2 days in all but a few colonies. Some of these colonies were mutants defective in the *bgl* operon, and some were simply defective in papillation. Therefore, we examined the putative Sal^- mutants for reversion on salicin minimal medium, using the starting strain as a control. The starting *mutS* strain gave easily recognizable Sal^+ derivatives. Half of the Sal^- nonpapillators behaved like the starting strain, indicating that they had a normal *bgl* operon but were blocked in papillation. The other half failed to give any Sal^+ mutants, indicating that they indeed possessed a mutation in the *bgl* operon.

The Biolog ES microplates were used according to the protocols provided by the manufacturer (Biolog, Inc., Hayward, CA). Briefly, fresh colonies on a tryptone plate (10 g tryptone, 5 g NaCl/liter) were suspended in saline, and the OD_{600} was adjusted to read between 0.25 and 0.28. Then, the suspension was micropipetted into the wells of the Biolog ES microtiter plate, one strain per plate. The plates were incubated overnight and read the following day. A wild-type control was used for comparison. A purple reaction indicated a positive result, and a completely colorless reaction indicated a negative result. Some reactions were typically intermediate, and each mutant that yielded negative results was retested at least once. Five mutant strains gave very poor indication for all of the reactions, and these strains were not included in the tabulation for this test.

Motility tests: These tests were carried out on plates containing 10 g tryptone, 5 g NaCl, and 3 g agar/liter, poured the same day onto petri dishes with plastic dividers separating the plates into four sectors. One fresh colony was toothpicked into the center of one sector, and after overnight growth the spreading of the partial cell lawn was compared to standards (supplied by Dr. John S. Parkinson) that were chemotaxis or motility deficient, as well as wild-type standards and the starting strain.

Phage resistance: This was tested with high-titer lysates of phage T6 and P1*vir*. The lysate (0.2 ml) was spread onto a tryptone plate, and grids of mutants were replicated onto the plate. After overnight incubation, replicated patches that

appeared to indicate resistance were noted, and those strains tested more thoroughly by spot tests and cross streaking.

Tests for *mutT*: We examined the level of streptomycin-resistant mutants in patches or cultures of each mutant. Although the starting strain is *mutS*, the level of Str^r mutants induced by this mutator is very low, whereas that induced by *mutT* is significantly higher. In fact, the presence of high levels of Str^r mutants is diagnostic for defects in *mutT*. We detected two strains that clearly had a reproducibly high level (50- to 100-fold over the control *mutS* strain) of Str^r mutants in independent cultures. The *mutT* locus is ~50% linked to the *leu* genes. We verified that the mutation responsible for this higher rate of Str^r was linked to *leu* by making a P1vir lysate on one of the strains and then transducing a Leu^- strain (AS210; Miller *et al.* 1998) to Leu^+, showing 50% linkage of the new mutator. (The second strain was itself Leu^-, so it could not be tested in this manner.)

Competition experiments: The strain to be tested (for instance, *mutS* lineage no. 22 after 90 cycles of single colony isolation) was inoculated into 5 ml LB supplemented with 50 μg/ml thymidine and grown overnight without aeration, as was the control strain (the starting *mutS* strain). Each culture was subcultured the following morning into fresh medium. The strains to be tested were diluted 1:50, and the control was diluted 1:100. These cultures were incubated at 37° in a water bath for ~3 hr to generate exponentially growing cultures. From these cultures, mixtures were prepared by diluting into 5 ml of fresh medium. This mixture was titered to determine the starting ratio. Typically, $\sim 2 \times 10^5$ cells of the control were mixed with 8×10^5 cells of the strain being tested. The cultures were grown overnight, the titer was determined again, and the final ratio was calculated.

Number of cells in a colony: Single colonies from the starting strain and several different lineages were picked after 24 hr of growth at 37° on LB plates. Each colony was transferred to 1 ml LB broth and was titered immediately on LB plates. The number of cells varied from 1.6×10^7 for the lineages yielding smaller colonies to 1.1×10^8 for the starting strain. The lineages were tested after 90 passages of single colony isolation. This corresponds to 24 generations for the slower growing strains and 27 generations for the faster growing strains, such as the starting strain.

Growth rates: A fresh overnight culture was prepared by inoculating a single colony into LB medium and incubating without shaking at 37°. Typically, two or three strains were tested with the starting strain as a control. The following day, the starting strain was diluted 1:200, and the strains to be tested were diluted 1:50 into 20 ml LB in a 125-ml flask. After incubating at 37° without shaking, the flasks were shaken at 200 rpm on a New Brunswick Scientific G24 environmental incubator shaker. After 30 min, 2-ml samples were withdrawn, and the optical density was determined in glass cuvettes at 600 nm with a Turner spectrophotometer (SP-830). Points were taken every 15 min for ~2 hr. The readings were plotted on semilog paper, and the growth rate was determined from the slope of the straight line drawn through the points during exponential growth. The doubling time of each lineage tested was compared to the starting strain control on each day. (Duplicate samples on the same day gave identical results.)

Sequencing: DNA was amplified using primers synthesized to generate a 2-kb amplification product. Freshly growing colonies of mutants to be sequenced were picked and added directly to a PCR mixture (GIBCO, Grand Island, NY). The DNA was amplified with the HROMHOT program. The program was initiated with a hot start at 94°, followed by an 80° step at which the enzyme was added. The mixture was then put through 26 cycles on a PTC thermocycler at the following temperatures and times: 94° for 30 sec, 55° for 30 sec, 72° for 5 min. The cycles were followed by a 10-min incubation at 72°, and the program was terminated at 4°. The product was applied to a low-melting-point agarose gel and the pure separated PCR product was cut from the gel, digested with gelase, and purified (QIAGEN). The product was used for sequencing with an Epicentre kit, using primers prepared to allow sequencing on both strands. The following primers were used for sequencing.

For the 8G repeat in *xylB*:

Primer 1: 5′ TTTCTCGTCGTGGCTGATAAG 3′. The 5′ end is 150 bases upstream of the 5′ end of the 8G repeat.
Primer 2: 5′ GTTATGCGCTGGCAGATGGCATGGA 3′. The 5′ end is 146 bases downstream of the 3′ end of the 8G repeat.

For the 10G repeat in *xylB*:

Primer 1: 5′ GGGCATTTCGCACCTCATCATCT 3′. The 5′ end is 136 bases upstream of the 5′ end of the 10G repeat.
Primer 2: 5′ CTGACGGCAGGTAAAGTGTGGTA 3′. The 5′ end is 133 bases downstream of the 3′ end of the 10G repeat.

RESULTS

Experimental system: We started with a wild-type strain of *E. coli*, J93, that is deleted for the *lac* genes. We prepared a *mutS* derivative of J93 by transducing into it a miniTn*10* integrated into the *mutS* gene. A purified colony was inoculated into 5 ml of broth and grown overnight before plating dilutions onto LB plates supplemented with 50 μg/ml thymidine. Of the resulting single colonies, 100 were used to begin individual lineages that were propagated by streaking single colonies onto broth plates each day. A similar set of 100 colonies was prepared from the starting J93 strain. Each colony is derived from a single cell. Typically, 8 colonies were streaked per plate and incubated at 37°. To avoid bias in picking, the lowest colony toward the center in each sector was picked for the next cycle of streaking.

Table 1 lists the phenotypic tests we used, along with the number of genes being monitored. The goal was to score for gene inactivations, as measured by a specific altered phenotype. We also looked for conditional lethals at both high and low temperature, although in these cases we do not know which gene is affected and

TABLE 1

Phenotypic tests for loss of gene function

Tests	No. of genes
Auxotrophs	200
Biolog ES	350–400
Motility	40–50
Osmolarity	30–50?
Sugar fermentation	50
Phage resistance	5
Specialized	~10
Conditional lethal (24°; 42°)	?

how many gene targets are involved. The tests we applied comprise several categories.

1. Auxotrophs. Auxotrophs are unable to grow on unsupplemented glucose minimal medium. Mutations in genes encoding biosynthetic pathways for amino acids, purines and pyrimidines, and certain vitamins are among those that result in auxotrophs. Approximately 200 genes are involved (Riley 1993).
2. Biolog ES plates. These plates, obtained from Biolog, Inc., consist of a microtiter plate with 96 wells. Each well contains tetrazoleum to measure the oxidation of different catabolites. About 83 of these reactions are pertinent to the strain of *E. coli* we are using. A positive reaction turns the well blue, and a negative reaction leaves the well colorless. It is estimated that ~400 genes are involved in the pathways monitored by the Biolog ES plate (B. Bochner, personal communication).
3. Metabolism of different carbon sources. We prepared MacConkey indicator media for a series of 14 different sugars (arabinose, fructose, fucose, galactose, gluconic acid, maltose, mannitol, mannose, melibiose, rhamnose, salicin, sorbitol, xylose, and dulcitol), and the corresponding minimal medium plates to test for mutants unable to grow on these sugars as a carbon source. (Lactose would represent an additional, 15th sugar, although the starting strain we used is deleted for the *lac* genes.) In principle, these plates might be expected to overlap with the Biolog ES plate. Instead, they complement it, since there are many mutants that give a negative reaction on MacConkey plates and are unable to grow on the various sugars on minimal medium, and yet still test positive on the Biolog plate. The Biolog plate measures oxidation reactions in a cell suspension, whereas MacConkey medium measures acid production generated by metabolism of growing cells, and the minimal medium measures the ability to grow on the specific sugar. Certain mutants may lack permeases and escape detection in the Biolog plates, while others may have some residual activity that scores as a positive in the Biolog plate but not in the MacConkey or minimal plate test. About 50–60 genes are monitored by these plates.
4. Cell motility. "Swim agar" plates, with low concentrations of agar, can be used to detect loss of motility or chemotaxis functions. About 50 genes are involved in cell motility and chemotaxis. Defects in either of these can be seen easily by this test (see materials and methods), particularly when compared with known mutants in these pathways.
5. Phage resistance. Resistance to phage, such as the T phage, lambdoid phage, the P series, and others occurs by inactivating different genes. As many as 20 genes can be scored with the appropriate set of phage, although only 4 or 5 such genes were monitored here.
6. Sensitivity or resistance to chemical agents. Certain recombination- (*e.g.*, *recA*) and repair-deficient strains (*e.g.*, *polA*) are sensitive to methyl methanesulfonate (MMS), while outer-membrane-deficient mutants are sensitive to crystal violet, deoxychlolate, high salt, and in many cases MacConkey medium. High-salt-sensitive mutants are scored using plates with 0.5 m NaCl and with 0.7 m NaCl. These add another 20–30 genes to the set being scored.
7. Special cases. A set of specific genes can be monitored with special tests. For instance, the *ebgR* gene can be detected by a weak blue reaction on Xgal indicator plates in a *lacZ* strain (B. Hall, personal communication). Also, the defects in the *mutT* gene can be monitored by the greatly increased frequency of streptomycin-resistant mutants above the low background in a *mutS* strain. See materials and methods for a complete description of the tests used here.
8. Conditional lethals. It is not known how many genes are required for growth on enriched medium, but the number is probably between 1000 and 1500 genes, based on the smallest genome sizes of free-living microorganisms. In any case, only conditional mutants can be scored in these genes, since complete inactivation would be lethal under the conditions used here. We tested for failure to form colonies on complex medium at both low (24°) and high temperatures (42°).

Detection of mutants in mutator populations: We tested both the wild-type and *mutS* lineages after 10, 20, 30, and 40 cycles of single colony isolation, and we also monitored the selected gene sets, eliminating the Biolog tests, in the case of the *mutS* strains after 50, 60, 80, and 90 cycles. Each single colony of the wild-type and starting *mutS* strain represents ~27 generations of growth, based on direct measurements of the number of cells in a colony (see materials and methods). Thus, each 10 cycles of purification represents 270 generations. For the *mutS* lineages, the colonies grow more slowly after many cycles of streaking, so that by 60 and 90 cycles, some colonies have grown for only 24 generations. Therefore, the number of cycles of single colony streaking is an approximation rather than a precise measure of the number of generations that each lineage has undergone. We compared the results of passing the *mutS* lineages through single colony isolations with those of the starting set.

The wild-type lineages showed very few mutations. Even by the 40th cycle, there was only one auxotroph from the 100 wild-type lineages, and only 2 other lineages with a single sugar pathway defect in each case. However, the *mutS* lineages accumulated mutations at a rapid rate compared with the wild type. Tables 2–4 and Figures 1 and 2 depict the results for the *mutS*

TABLE 2

Increase in mutations in *mutS* lineages after successive generations

	No. of *mutS* lineages with the following (*x*) number of mutations												
Cycle	0	1	2	3	4	5	6	7	8	9	10	11	12
0	97	3	0	0	0	0	0	0	0	0	0	0	0
10	62	28	9	0	0	1	0	0	0	0	0	0	0
20	33	35	17	6	1	3	2	0	0	1	0	0	0
30	17	22	24	13	10	1	4	4	0	2	1	0	0
40	7	16	15	14	17	11	7	5	1	2	2	1	0
50[a]	3	11	14	10	13	17	12	5	6	1	2	2	0
60[b]	1	8	15	7	12	20	16	5	5	3	2	1	1

The mutations shown in Figures 1 and 2 have been used to generate this table.
[a] No additional mutations revealed after 40 cycles of streaking by the Biolog test.
[b] No additional mutations revealed after 50 cycles of streaking by the motility test.

lineages. After the 10th cycle alone, we could identify 38% of the lineages as having more than one inactivated gene (Table 2). The number of mutations increased with increasing cycles, as shown in Table 2 and Figures 1 and 2. By 40 cycles, 93% of the lineages displayed at least one inactive gene among the targets tested (Table 2). By the 60th cycle, the typical cell had four to five of the target genes inactivated by mutation.

By the 60th cycle, 80% of the lineages had become auxotrophs, and a significant number of these had become multiple auxotrophs. We could detect these by first identifying the initial auxotrophic requirement. For instance, by the 10th cycle, one lineage required cysteine. By the 20th cycle, this Cys$^-$ auxotroph had picked up a second auxotrophic marker. In several cases, we identified both auxotrophic requirements, and, in one case, we could document the accumulation of three different auxotrophic requirements in the same lineage. However, we should note that the ability to detect second and third auxotrophic mutations diminishes as mutations accumulate, since in a significant fraction of cases we could not identify the required nutrient for either the first or second auxotrophic mutation (see below). Thus, after a certain point, we recognized fewer and fewer of the mutations that were really there.

Figure 1 plots the accumulation of mutations with increasing cycles of single colony streaking, for each type of mutation (excluding several rare categories described below). The mutations plotted in Figure 1 are totaled in Figure 2. These curves show continued increases, and, until the diminishing ability to distinguish new mutations sets in, are almost linear.

Distribution of mutations: Tables 3 and 4 show the distribution of mutations for the *mutS* lineages in terms of the functions lost. Table 3 shows the auxotrophic requirements detected after 60 cycles. About 40% of the auxotrophic requirements could be pinpointed. The mutations causing these requirements are distributed clearly among many different genes. Some of the sugar metabolism pathways are affected in a significant fraction of lineages, for example xylose in 30% of them (see below), while other pathways are affected in only a few lineages, such as galactose and arabinose, that are affected in 1 and 4% of the lineages, respectively. Table 4 shows the defects identified by the Biolog plates after 40 cycles of single colony isolation. Again, some functions are deficient in a significant fraction of the strains, such as glycolic acid catabolism, and in 11% of the lineages, whereas other functions are inactivated in only

TABLE 3

Phenotypes detected among *mutS* lineages

Auxotrophs		Sugar utilization	
ade	4	Arabinose	4
arg	1	Fructose	5
cys	2	Fucose	15
his	2	Galactose	1
ile	1	Gluconic acid	4
leu	3	Maltose	14
lys	2	Mannitol	5
met	5	Mannose	12
pro	2	Melibiose	29
ser	2	Rhamnose	22
thre	2	Salicin	12
thy	1	Sorbitol	11
trp	4	Xylose	30
ala + cys	1	Dulcitol	12
glu + gln	1		
his + trp	1		
ile + leu	1		
uri + arg	3		
val + ile	3		
val + ile + leu	1		
cys or met	2		
met or ile	2		
uri or gua	1		
Unknown	69		

The data are shown for 60 cycles of single colony isolation. For auxotrophs, the requirements, when known, are indicated. For sugar utilization defectives, the sugar affected is shown.

TABLE 4

Catabolic defects in *mutS* lineages revealed by Biolog plates

Carbon source	Catabolic defects in *mutS* lineages
Glycolic acid	11
l-Threonine	10
d-Saccharic acid	5
l-Aspartic acid	5
d-Galactonic acid 8-lactone	5
Tween 20, 40, 80	5
l-Glutamine	5
l-Fucose	5
Glycl-l-glutamic acid	4
Formic acid	3
l-Asparagine	3
Mucic acid	3
l-Arabinose	2
d-Alanine	2
Dulcitol	3
l-Rhramnose	2
d-Melibiose	2
l-Alanine	2
l-Galactonic acid 8-lactone	2
d-Sorbitol	1
d-Xylose	1
Acetic acid	1
Maltose	1
Thymidine	1
α-keto glutaric acid	1
α-hydroxy butyric acid	1
2′-Deoxy adenosine	1
Adenosine	1
Glycyl-l-aspartic acid	1
Fumaric acid	1
Bromo succinic acid	1
Inosine	1
Mono-methyl succinate	1
d-Malic acid	1
Glycyl-l-proline	1
Glucuronamide	1
d-Galacturonic acid	1

one or even none of the lineages. Several mutant types are not shown in the tables. After 60 cycles, 22 of the lineages failed to grow on rich medium at 30° (cold sensitive), and 4 lineages failed to grow at 42° (temperature sensitive). Additional mutant types include four phage-resistant mutants (two resistant to P1 and two resistant to T6) and three mutants sensitive to MMS. Also, one mutant acquired the ability to oxidize lactulose, as indicated by the Biolog tests.

Loss of fitness: By 90 cycles of single colony isolation, all lineages had reduced colony size on rich medium, and five lineages had died out. We tested for loss of fitness of each surviving lineage in two ways: by measuring growth rates in rich medium and by measuring the ability to compete with the starting strain in mixed cultures in rich medium. (Details of the experiments are given in materials and methods.) Typically, we determined the growth curves for two or three strains and also for the control (the starting strain). We tabulated the ratio of the doubling time of the starting strain control to that of each strain, measured on the same day. Each of the 95 remaining lineages grew more slowly than the starting strain. Figure 3 depicts the results, allowing one to see the range of values, which varied from 0.92 to 0.52 (growth rates relative to the starting strain).

In some cases we could document a successive lowering of the growth rate with successive generations. Table 5 shows the growth rate, relative to the starting strain, of mutant lineage 57, after 40, 60, and 90 cycles of single colony isolation. The doubling time increases at each stage. The same is true for lineage 92, which shows a decrease in the growth rate at 60 cycles, and fails to grow at all after 90 cycles (data not shown). These examples demonstrate a stepwise loss of fitness.

In a second set of experiments, we prepared co-cultures, each containing a mixture of the starting strain and one of the lineages from 90 cycles of streaking for single colonies. The co-cultures were initiated by diluting growing cultures of each strain into rich medium and then allowing the mixture to grow overnight, for ~15–20 generations. Samples of the co-cultures were examined on different indicator plates to allow the determination of the titer of each strain and of the wild type, taking advantage of markers that were present in each lineage. For instance, if a lineage had become Mal^-, the co-culture was titered before and after overnight growth on maltose MacConkey plates. In this case, the starting strain yielded red colonies, and the mutant lineage yielded white colonies. Since 85 of the remaining 95 lineages carried at least one easily determined phenotypic difference from the starting strain, it was straightforward to determine the ratio of the cells in the mutant lineage to those of the wild type. (See materials and methods for further details.) Table 6 shows typical results for several lineages and also summarizes the results for all of the lineages. Here we tabulated the starting and final ratios for each experiment and then calculated the ratio change. The ratio change indicates the extent to which the starting strain out-competed each lineage. The data in Table 6 show that the starting strain out-competed each of the 85 lineages tested, in one-on-one tests in rich medium.

Growth in absence of severe bottlenecks: We analyzed two independent cultures of the starting *mutS* strain grown with much less severe bottlenecks. We grew the cultures for 280 generations. Each day, $\sim 5 \times 10^7$ cells, representing a 1:1000 dilution of a 20-ml culture, were transferred to fresh LB medium and grown for 10 generations. The cells were then plated, and 100 colonies from each culture were examined with most of the screens described above. Table 7 displays the results. It can be seen that even mutators growing without passing

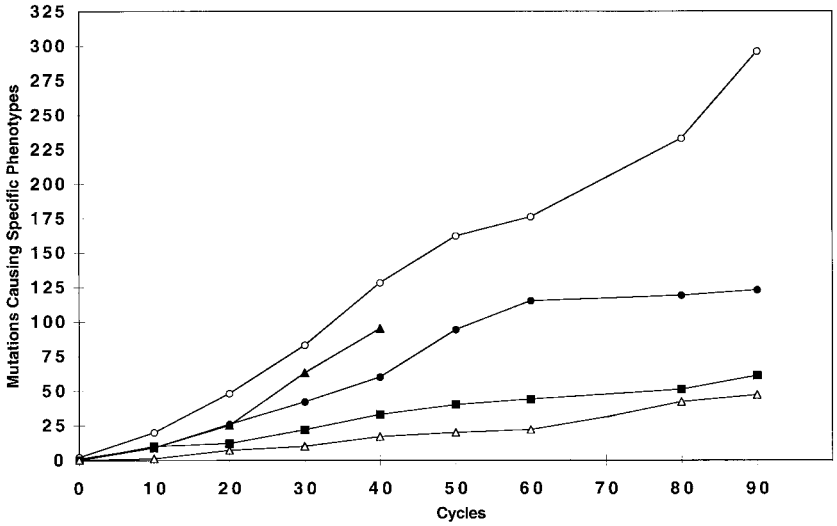

Figure 1.—The increase in mutations that cause loss of gene function among 100 lineages. The results are plotted for each of five different assays. The open circles (○) represent mutations that affect one of the sugar utilization pathways. The solid circles (●) represent mutations resulting in auxotrophy, the solid squares (■) represent mutations causing loss of motility, the solid triangles (▲) represent mutations identified by Biolog plates causing loss of a catabolic pathway, and the open triangles (△) represent mutations resulting in high salt sensitivity.

through severe bottlenecks still accumulate mutations rapidly. The results from Table 7 provide evidence for numerous independent mutations. For instance, in one culture, five different sugar requirements were detected, and at least six different mutations leading to auxotrophy were detected. In the second culture, seven different sugar requirements were noted. The totals of 25 and 14% auxotrophs, and 7 and 51% sugar-pathway-deficient mutants in the two cultures can be compared to the values of 9% auxotrophs and 21% sugar-deficient mutants found in the lineages streaked for 10 cycles (~250 generations; Table 3). Interestingly, again the Xyl^- phenotype occurs frequently among the cells grown continuously in culture.

Genomic hotspots: As Table 3 shows, as many as 30% of the lineages passed through severe bottlenecks have become Xyl^- after 60 cycles of streaking. (This increases to 45% after 90 cycles.) Also, the two cultures analyzed after 280 generations in continuous culture showed 3 and 25% Xyl^- mutants, respectively. A closer analysis indicated that the vast majority of the mutants displayed a characteristic phenotype on xylose MacConkey plates, throwing off numerous red Xyl^+ streaks and sectors after 1–2 days of growth. These mutants revert at very high frequencies, since overnight cultures yielded close to 1% Xyl^+ revertants. This suggested that a hypermutable sequence, or hotspot, might be present in the *xyl* operon, which is unstable in a mismatch repair deficient strain. We scanned the DNA sequence of the *xyl* genes, and we found that the *xylB* gene contains a stretch of eight consecutive G's (-GGGGGGGG-) on one strand. Such repeat tract sequences generate frequent frameshifts in mismatch repair deficient strains in *E. coli* (Cupples *et al.* 1990), as well as in yeast (Strand *et al.* 1993). Therefore, we sequenced the region of the *xylB* gene surrounding this sequence in 13 examples of the rapidly sectoring Xyl^- mutants described above. In all 13 cases examined, we indeed detected a frameshift mutation at the sequence of eight consecutive G's. In 4 cases, the eight G's lost a base to become seven G's, and in 9 cases

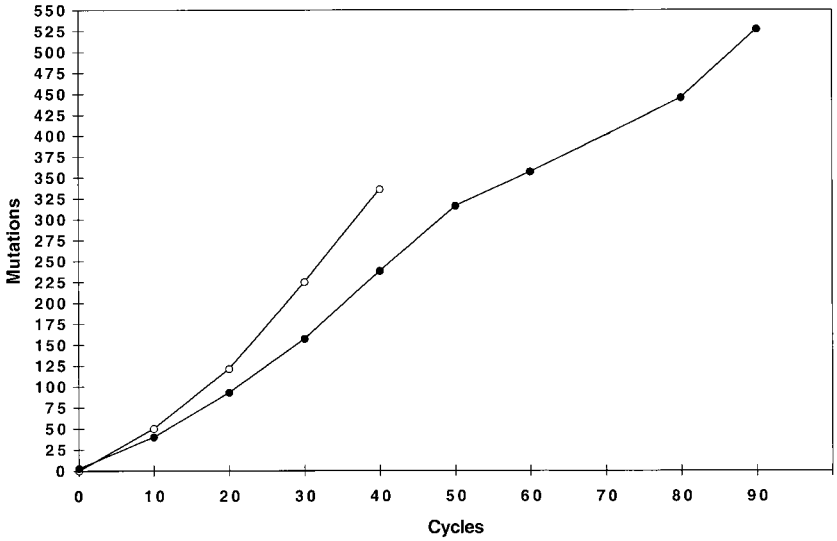

Figure 2.—The combined increase in mutations that cause loss of gene function among 100 lineages. The open circles represent the combined totals for mutations leading to auxotrophy, loss of sugar utilization, loss of catabolic pathways (from Biolog results), loss of motility, and sensitivity to high salt. The solid circles represent the same combined totals, excluding the Biolog results (not determined after the 40th cycle).

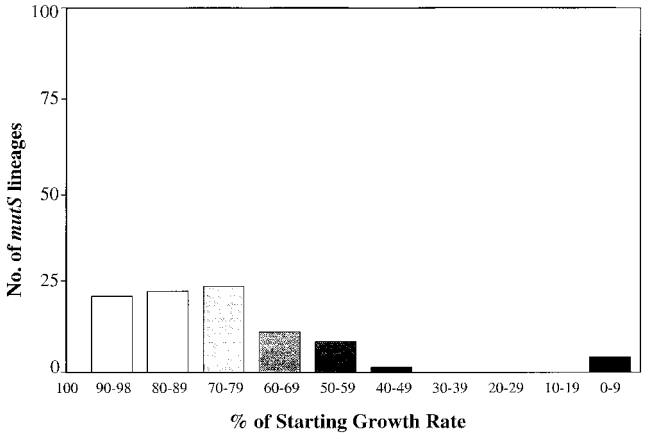

Figure 3.—Loss of fitness among *mutS* lineages passaged through single colony isolation. The percentage of the growth rate of the starting strain is shown for the 95 surviving *mutS* lineages after 90 cycles of single colony isolation.

they gained a base to become nine G's. Xyl^+ controls and a Xyl^- mutant that did not throw off Xyl^+ sectors on MacConkey indicator medium all showed eight G's at the *xylB* hotspot (Table 8). Of the 30 Xyl^- mutants, 26 gave the identical rapid sectoring phenotype. Since all 13 examples that we sequenced from this group showed a frameshift at the sequence of eight G's, we assume that most or all of the remaining 13 from this group also resulted from a frameshift at the same sequence.

We scanned the entire genomic sequence of *E. coli* for the largest runs of mono- and dinucleotides that would be subject to frameshifts in coding sequences, as well as runs of bases in noncoding sequences. Table 9 displays these sequences. It can be seen that the run of 8 G's in the *xylB* gene represents one of only six such runs (for either G's or C's) in coding sequences. The largest run of G's (C's) in a noncoding sequence is a stretch of 10. We sequenced this region in 14 of the *mutS* lineages grown for 60 cycles, and we found that in 10 cases the 10 G's had mutated to either 9, 11, or 12 G's (Table 10). After an additional 30 cycles, 8 of the 14 lineages had again changed the number of G's in the repeat. In all seven wild-type examples sequenced, after 60 cycles, we found 10 G's at the hotspot (data not shown).

DISCUSSION

Mutations in mutators: What does a mutator population actually look like? We decided to answer this question by gathering a large number of phenotypic screens that reflect gene inactivations and applying them to members of a mutator (*mutS*) population after multiple generations of growth in rich medium. As many as 700 genes can be monitored for inactivation by the assays used. This amounts to ~15% of all the genes in the cell. In addition, essential genes, which probably number 1000–1500, can be scored with conditional lethals. When passaged through the severe bottleneck produced by continued single colony isolations, mutants lacking different functions appeared frequently compared with wild-type cells treated in the same manner. Mutations that inactivate genes continued to accumulate with increasing generations, in a near linear fashion, so that, after 60 cycles of single colony isolation, almost every cell had at least one phenotype resulting from an inactivated gene (Table 2), and the typical cell had 4–5 detectable genes inactivated, and some members of the population had >10 genes inactivated, among the genes scored. A rough extrapolation from the ~15% of the gene targets we could examine (not including conditional lethals) predicts that the typical cell had as many as 20–30 genes inactivated, considering the entire genome. Moreover, all of the lineages displayed reduced fitness. After 90 cycles of single colony isolation, 5 lineages failed to grow on rich medium, and all of the remaining 95 lineages showed reduced growth rates (Figure 3). In all 85 cases tested, there was reduced ability to compete with wild type in rich medium (Table 6).

TABLE 5

Incremental loss of fitness in a *mutS* lineage: Doubling time (minutes) after 0, 40, 60, and 90 cycles of single colony isolation

	Starting	40 cycles	60 cycles	90 cycles
mutS lineage 57	24	35	41	55

Experiments in chemostats (Gibson *et al.* 1970; Nestman and Hill 1973; Cox and Gibson 1974; Chao and Cox 1983) or continuous cultures (Sniegowski *et al.* 1997), and theoretical arguments (Taddei *et al.* 1997) show that mutators can out-compete wild-type cells under certain conditions. Other experiments have demonstrated that mutators can emerge after continued selective pressure (Helling 1968; Liberfarb and Bryson 1970; Hoess and Herman 1975; Mao *et al.* 1997) and are found at several percent of the wild-type isolates of *E. coli* and *S. typhimurium* (Jyssum 1960; Gross and Siegel 1981; LeClerc *et al.* 1996; Matic *et al.* 1997). In fact, we have defined specific media that actually select for the growth of only mutator colonies (Miller *et al.* 1998). So, why have microorganisms not evolved to be mutators? Would it not be helpful during evolution to be able to create more diverse variants? The traditional answer is that mutators will generate deleterious mutations more rapidly than advantageous mutations. The work reported here demonstrates directly that there is an extensive accumulation of mutations in mutator populations. This accumulation occurs for auxiliary pathways both when bottlenecks occur and also in the absence of severe bottlenecks. However, in the presence

TABLE 6

Competition between starting *mutS* and subsequent lineages

mutS lineage no.	Starting ratio 0 cycle:90 cycle	Final ratio 0 cycle:90 cycle	Ratio change
29	7:7	264:25	10.6
55	15:84	245:15	91.5
81	10:26	409:5	213
Ratio change:	2.5–10	10–100	>100
No. of *mutS* lineages:	13	33	39

Mixed cultures were prepared and grown overnight, and the titer was determined as described in materials and methods. The ratio of the starting strain (0 cycles) to the 90-cycle lineage is shown, as is the ratio change.

of severe bottlenecks, mutator lineages also accumulate mutations that decrease fitness (Figure 3 and Tables 5 and 6). Clearly, cells pay a heavy price for having an increased mutation rate. Continued growth of mutator lineages can lead to the elimination of the evolved pathways for biosynthesis, catabolism, and motility, among other functions not selected for during growth of the mutator strain. For this reason, virtually all free-growing organisms described so far have evolved elaborate repair mechanisms to keep mutation rates low. Instead, they appear to rely on specific hypermutable genes (for reviews see Moxon *et al.* 1994; Deitsch *et al.* 1997; see below) and on the ability to amplify rare subpopulations (Anderson and Roth 1978; Mekalanos 1983; Tlsty *et al.* 1984) to generate needed diversity in response to environmental stress. When mutator subpopulations are amplified in response to environmental challenges, the mutator allele hitchhikes along with the mutation conferring better survival (for example, Mao *et al.* 1997; LeClerc *et al.* 1998), but when continued selection abates, the mutator allele ultimately can be disadvantageous, as shown here. The consequences of continued growth of a cell with a high mutation rate support the growing body of work (for review see Lawrence and Ochman 1998) that implicates lateral or horizontal transfer and recombinational reshuffling as a prime force in bacterial speciation, rather than stepwise mutations. For instance, Lawrence and Ochman (1998) analyzed the sequenced genome of *E. coli* and determined that 755 of the 4288 open reading frames (ORFs) in *E. coli* were introduced by lateral transfer events in the 100 million years since *E. coli* diverged from Salmonella. They also concluded that none of the phenotypic traits that distinguish *E. coli* from Salmonella arose by stepwise mutation.

TABLE 7

Mutations affecting different phenotypes in *mutS* lineages grown without severe bottleneck

	Per 100 colonies			
Culture	Auxotrophs	Sugar utilization	Motility	cs/ts
1	26 (at least 6 different types)	7 3 Xyl$^-$ 1 Gluc$^-$ 1 Gal$^-$ 1 Fru$^-$ 1 Sor$^-$	2	4
2	14 (types not determined)	48 25 Xyl$^-$ 14 Mel$^-$ 2 Sal$^-$ 4 Mal$^-$ 1 Ara$^-$ 1 Gluc$^-$ 1 Mtl$^-$	0	0

Cultures were grown for 280 generations and plated on LB medium. A total of 100 colonies were tested for auxotrophy, sugar utilization defects, motility, and temperature- (ts) and cold-sensitive (cs) phenotypes.

Detection systems: It is interesting to evaluate the different phenotypic screens for utility, considering the ease of use, expense, and results. By these criteria, the set of MacConkey plates testing for 15 sugars proved by far the most useful. Although monitoring as few as 50–60 genes, these plate tests, which can be done by simple replica plating, yield more mutants than testing for auxotrophy, even though ~200 genes are targets for auxotrophic mutations.

The MacConkey plate array, either as individual plates or as a 16-well microtiter plate (see materials and methods), proved almost as valuable as the Biolog plate test. However, the Biolog plates do allow the scoring of many mutant phenotypes not easily accessible by other assays, although these plates have more ambiguities, requiring some repetition. Given the increased expense of the Biolog plates, they are perhaps best used for typing strains rather than for identifying mutations among hundreds of candidate strains. On the other hand, the swim agar plates for scoring loss of cell motility are very useful, with the sole drawback being that one cannot identify without detailed additional experiments which of the motility or chemotaxis genes are inactivated by the mutation causing the loss of motility.

TABLE 8

Sequence changes in the *xylB* gene

No. of examples sequenced	Sequence at hotspot	Change from wild type
6 Xyl^+	6 with $(G)_8$	None
13 Xyl^- rapidly sectoring (unstable)	9 with $(G)_9$	+1 (G)
	4 with $(G)_7$	−1 (G)
1 Xyl^- nonsectoring (stable)	$(G)_8$	None

The DNA sequence was determined at a hotspot in the *xylB* gene for 13 rapidly reverting Xyl^- clones, 1 Xyl^- that does not revert rapidly, and 6 Xyl^+ clones. $(G)_7$ signifies seven consecutive G's, $(G)_8$ signifies eight consecutive G's, and $(G)_9$ signifies nine consecutive G's. The wild type has eight G's.

In this study, we used only a few of the possible special plates, such as MMS-containing medium to test for genes altered in recombination or repair, or T6 and P1 to test for specific phage resistance. In principle, a complete set of bacteriophage that reveal phage resistance genes would be very useful and could monitor as many as 20–30 genes. The drawback here is that a set of such phage is no longer readily available. It would be of considerable value to prepare such a kit and also to compile a set of additional tests based on resistance to specific chemicals. It is possible to envision tests that would soon allow the rapid screening for as many as 1000 specific gene functions, and perhaps, in time, a majority of the genes on the *E. coli* chromosome and those of similar bacteria.

Conditional lethals, such as cold- or temperature-sensitive mutants, are valuable for scoring essential genes, although which gene is involved is not revealed without additional study. Also, inactivating mutations such as nonsense mutations or frameshifts, or large deletions and insertions, cannot be involved in creating a conditional lethal. One is relying solely on specific base substitutions, limiting somewhat the frequency of appearance of the respective mutants.

Genomic hotspots: The detection of specific hotspots in this experiment raises some interesting questions. Certain bacteria have hypermutable sequences in genes with a variable expression in response to different environmental conditions. These have been termed "contingency loci," and they often involve repetitive sequence units (for reviews see Moxon *et al.* 1994; Deitsch *et al.* 1997). For instance, in *Haemophilus influenzae*, the intergenic region between the *hifA* and *hifB* genes, which encode the fimbriae proteins, has 10 repeats of the -TA- sequence in the promoter. When the sequence mutates to yield 11 repeats, the transcription of the gene is lowered, and when it mutates to 9 repeats, transcription is abolished altogether. Other genes in *H. influenzae* have 16 copies of the tetranucleotide -CAAT- within the coding sequence of the *lic2* gene, required for synthesis of the lipolysaccharide core. When this changes to 15 repeats, the reading frame is altered, abolishing the activity of the gene product. In each of these and other cases, the number of repeats is so high that even in a

TABLE 9

Distribution of mononucleotide repeats of eight bases or more in the genome of *E. coli*

Repeats	No. in coding sequence	No. in noncoding sequence	Locus of coding sequence
$(A)_9$	4	2	*wbbJ* (Acetyl CoA acetyl transferase) PID: g510256
			yibA (hypothetical protein) PID: g1790021
			yghB (hypothetical protein) PID: g1789384
			creC (creC sensor protein) PID: g1790861
$(T)_9$	1	10	286-aa ORF (hypothetical ribitol dehydrogenase family protein encoded by *B. subtillus kduD* gene) PID: g1789135
$(C)_9$	0	1	None
$(C)_8$	2	3	Hypothetical 529-aa ABC transporter protein PID: g1788945
			leuT tRNA
$(G)_{10}$	0	1	None (46 bp after *orf257*; PID: g1786555)
$(G)_8$	4	0	tRNA *leu*
			yfiH (hypothetical 26.3-kD protein in clpB 5′ region) PID: g1788945
			ydeK (hypothetical protein in hipA 5′ region) PID: g1787788
			xylB (xylulokinase) PID: g1789987

TABLE 10

Sequence change at mononucleotide run of 10 G's in 14 mutants

	No. of G repeats after 0, 60, and 90 cycles		
Lineage no.	0	60	90
1	10	11	10
2	10	11	12
3	10	10	9
4	10	12	11
5	10	12	11
6	10	10	10
7	10	10	10
8	10	9	9
9	10	11	11
10	10	11	9
11	10	11	11
12	10	9	9
13	10	11	10
14	10	10	11

Colonies were chosen at random from 14 *mutS* lineages before and after 60 and 90 cycles of single colony isolation, and the region including a 10-G mononucleotide run was sequenced.

wild-type strain there is enough mutability to produce 0.1–1% variants in the population. *E. coli* does not have any simple repeat sequences with that level of variability under normal conditions. However, growing cells continually in the absence of mismatch repair, as done here in the *mutS* background, unmasks "conditional" contingency loci, such as that found in the *xylB* gene. The hypermutability of the -GGGGGGGG- sequence in *xylB* in the *mutS* background is such that growth of a culture overnight is sufficient time for 1% Xyl^- mutants to arise, and growth of the Xyl^- mutants overnight is sufficient time for 1% Xyl^+ revertants to arise. Given the examples of repeats from Table 9 that should be similarly active, as is the sequence of 10 G's in a noncoding region (Table 10), one might ask whether these hypermutable sites are the last remaining remnants of sequences that were once hypermutable in wild-type strains, or whether there is a set of contingency loci dependent on the cell becoming mismatch repair deficient under certain conditions. Also, this sequence of 10 G's might prove useful as a marker for determining whether natural isolates of *E. coli* have passed through a hypermutable stage resulting from the loss of mismatch repair.

Conclusions: Inactivating the mismatch repair system in *E. coli* destabilizes the genome. Repeat tract sequences are particularly unstable, as is the case in higher cells. Although the increase in mutation rate may confer an initial advantage on such mutator cells when new phenotypes are being continually selected for, the continued accumulation of mutations in unselected genes leads to multiple loss of function that can be very costly in other environments. When passaged through severe bottlenecks, mutator lineages also accumulate mutations that confer loss of fitness.

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