

# Some Features of the Mutability of Bacteria During Nonlethal Selection

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## ABSTRACT

We describe the mutability of the Trp<sup>-</sup> chromosomal +1 frameshift mutation *trpE7999* during nonlethal selection, finding that the appearance of Trp<sup>+</sup> revertants behaves similarly to that of episomal Lac<sup>+</sup> revertants. In addition, we show that a feature of the Lac<sup>+</sup> and Trp<sup>+</sup> mutability is the accumulation of Trp<sup>+</sup> and Lac<sup>+</sup> revertants with additional unselected mutations, most of which are not due to heritable mutators. The cells undergoing nonlethal selection apparently experience an epigenetic change resulting in a subset of bacteria with elevated mutability that often remain hypermutable for the duration of selection. The epigenetic change provoked by nonlethal selection appears to be mediated by a unique function provided by the F'128 episome.

THE results of inquiry into the reversion of the Lac<sup>-</sup> frameshift mutation residing in the episome of *Escherichia coli* FC40 have been both provocative and confusing. The episomal substrate of these mutation studies is a modification of episome F'128 (Holloway and Low 1996), carrying *lacI33* (Miller 1992), a leaky *lac* allele with a +1 frameshift mutation. The *lac-pro* deleted strain of *E. coli* P90C (Miller 1992) harboring this episome-borne *lac* allele (*lacI33*) is able to mutate, regaining the ability to metabolize lactose, in a time-dependent manner during nonlethal selection in minimal lactose medium (Cairns and Foster 1991; Foster and Cairns 1992; Foster 1993, 1998; Harris *et al.* 1994; Rosenberg *et al.* 1994, 1998).

Recently, reports from two laboratories have helped clarify earlier observations (Foster 1997; Torkelson *et al.* 1997). They have shown that within the population of Lac<sup>-</sup> bacteria not only do Lac<sup>+</sup> mutations occur, but mutations also occur at other unrelated sites. One group reported a high frequency of unselected mutations among late appearing Lac<sup>+</sup> revertants on the chromosome, plasmid, and episome sites (Torkelson *et al.* 1997). These mutants are multiple and most of the time are clonal, *i.e.*, with more than one mutation in the same bacterium, but rarely do these mutants display enhanced mutability after isolation. Moreover, neither the nonselected parent cells nor the Lac<sup>-</sup> bacteria that had been subjected to prolonged lactose selection appear to display enhanced mutability. In addition, Foster (1997) has reported that mutations that accumulate during lactose selection include mutations in other episome-borne genes that do not involve lactose metabolism. These observations have led to the conclusion of

a transient hypermutability in a small fraction of the population experiencing selection (Foster 1997; Torkelson *et al.* 1997; Rosche *et al.* 1999). Like the reversion to Lac<sup>+</sup> (Cairns and Foster 1991), this genome-wide mutability was shown to be dependent on host recombination functions (Torkelson *et al.* 1997).

We have been encouraged to inquire further into the features of this elevated mutability. We investigated the reversion of a chromosomal +1 frameshift allele in the *trpE* gene of *E. coli* (*trpE7999* allele; Bronson and Yanofsky 1974; Cairns and Foster 1991) in a strain isogenic with FC40, the strain in which the *lacI33* reversion has been studied. As with the *lacI33* reversion, Trp<sup>+</sup> revertants accumulate on selection plates, although at a substantially lower rate. In addition, unselected Lac<sup>+</sup> mutants accumulate during Trp<sup>+</sup> selection and late appearing Trp<sup>+</sup> revertants also harbor additional mutations. However, we found that pure colonies of Lac<sup>+</sup> or Trp<sup>+</sup> revertants harboring additional mutations are much less frequent among the revertants that accumulate early on the selective plates than among late appearing revertants. This observation suggests that hypermutability, appearing soon after the onset of selection in a subset of bacteria under nonlethal selection, often persists in those same bacteria for the duration of selection. Our findings also show that much of the mutability observed at both loci is the result of some as-yet-uncharacterized attribute of the episome carrying the *lacI33* allele. This attribute does not appear to be a feature of other independently isolated episomes, including episomes that cover the same portion of the *E. coli* chromosome that is included in F'128.

## MATERIALS AND METHODS

**Strains:** Relevant strains and episomes used in this work are shown in Table 1; they are all isogenic strains of *E. coli* P90C

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TABLE 1  
*E. coli* strains and episomes

<i>E. coli</i>	Relevant genotype	Reference
<b>Strains</b>		
P90C	$\Delta(lac-proB)_{XIII} ara thiA$	Cairns and Foster (1991)
FC40	As P90C but Rif <sup>R</sup> /F'128 <i>lacI33proA</i> <sup>+</sup> <i>B</i> <sup>+</sup>	Cairns and Foster (1991)
FC29 Sm <sup>R</sup>	As P90C but Sm <sup>R</sup> /F'128 $\Delta lacZ proA$ <sup>+</sup> <i>B</i> <sup>+</sup>	Radice lla <i>et al.</i> (1995)
FC82	As P90C but <i>trpE7999</i> /F'128 <i>lacI33proA</i> <sup>+</sup> <i>B</i> <sup>+</sup>	Cairns and Foster (1991)
GG82	As FC82 but F <sup>-</sup>	This work
CC104	As P90C/F'128 <i>lacZ</i> <sup>-</sup> "CGC mutation"	Cupples and Miller (1989)
MG4792	As P90C but <i>lacI33</i> on chromosome	M. Marinus
FC329	As P90C but $\Delta trpE$ /F'128 $\Delta lacZ proA$ <sup>+</sup> <i>B</i> <sup>+</sup>	P. L. Foster
FC82 <i>recA</i>	As FC82 but Cm <sup>R</sup> linked to $\Delta recA$ gene	This work
AB1157	<i>thr-1 leuB6 proA hisG4 thi-1 argE3 lacY1 galK2 ara-14 xylA5 mtl1 tsx33</i> Sm <sup>R</sup> /F <sup>-</sup>	Lab collection
FC40	As FC40 but with Tn5 inserted into the <i>mutL</i> gene	This work
<b><i>mutL</i>::Tn5</b>		
FC40 <i>mutS</i> ::Tn5	As FC40 but with Tn5 inserted into the <i>mutS</i> gene	This work
<b>Episomes<sup>a</sup></b>		
F'128	<i>proA</i> <sup>+</sup> <i>B</i> <sup>+</sup> , <i>lacI</i> <sup>+</sup> <i>Z</i> <sup>+</sup> <i>Y</i> <sup>+</sup> <i>A</i> <sup>+</sup>	CGSC4288, E. Signer
CC104	As F'128 but with revertible mutation in codon GCG of <i>lacZ</i> gene	Cupples and Miller (1989)
F'FC29Kan	As F'128 but <i>proAB</i> ::Tn10 Kan, $\Delta lacZ$	Lab collection
F'597	<i>proA</i> <sup>+</sup> <i>B</i> <sup>+</sup> , <i>argF</i> <sup>+</sup> , <i>lacI</i> <sup>+</sup> <i>Z</i> <sup>+</sup> <i>Y</i> <sup>+</sup> <i>A</i> <sup>+</sup> , <i>proC</i> <sup>+</sup>	GCSC5886, E. A. Birge
F'144	<i>leu</i> <sup>+</sup> , <i>proA</i> <sup>+</sup> <i>B</i> <sup>+</sup> , <i>argF</i> <sup>+</sup> , <i>lacI</i> <sup>+</sup> <i>Z</i> <sup>+</sup> <i>Y</i> <sup>+</sup> <i>A</i> <sup>+</sup> , <i>proC</i> <sup>+</sup> , <i>purE</i> <sup>+</sup>	CGSC4266, Low (1972)
F'13	<i>proA</i> <sup>+</sup> <i>B</i> <sup>+</sup> , <i>argF</i> <sup>+</sup> , <i>lacI</i> <sup>+</sup> <i>Z</i> <sup>+</sup> <i>Y</i> <sup>+</sup> <i>A</i> <sup>+</sup> , <i>proC</i> <sup>+</sup> , <i>purE</i> <sup>+</sup>	CGSC5490, S. Schlesinger
F'104	<i>leu</i> <sup>+</sup> , <i>proA</i> <sup>+</sup> <i>B</i> <sup>+</sup> , <i>argF</i> <sup>+</sup>	CGSC4251, Low (1972)
pCJ100	pOX38 Cm <sup>R</sup> , <i>polA</i> <sup>+</sup>	Joyce and Grindley (1984)

<sup>a</sup> The episomes listed were obtained from the *E. coli* Genetic Stock Center, CGSC, Department of Biology, Yale University, unless noted otherwise. The number assigned to each strain containing these episomes by the CGSC is shown in the reference column. The *E. coli* chromosome wild-type genes present on each episome are listed on the basis of the information given by the CGSC. Cm<sup>R</sup>, chloramphenicol resistance.

(Miller 1992). The recombination-deficient and mutator derivatives of FC82 and FC40 were constructed by P1 transduction using the deletion *recA* allele, from the strain GW5180, linked to Tn10 Cm<sup>R</sup> (obtained from G. Walker lab), and the mutator alleles present in KY 232 (*mutL*::Tn5, lab collection) and PR114 (M182 *mutS*::Tn5, lab collection), respectively. GG82 is a P90C *trpE7999* episome-free derivative of FC82 (Table 1), obtained by spontaneous episomal loss after growth in Luria-Bertani (LB) medium (Miller 1992). In rich medium (LB) the episome is lost frequently ( $\sim 1 \times 10^{-3}$ ) due to the lack of selective pressure for the *pro* marker present on the episome of FC40. The episome-free derivative was no longer pale blue when plated in LB-X-Gal medium and is a *pro* auxotroph. Derivatives of MG4792 (Table 1) and GG82 carrying episomes were constructed by conjugation.

**Selection of Trp<sup>+</sup> revertants:** FC82 and its derivatives were grown from inocula of 10<sup>5</sup> bacteria as independent cultures in minimal glucose medium M9 (Miller 1992) containing 0.1% glucose and 40  $\mu$ g/ml of tryptophane. Saturated bacterial cultures (10<sup>9</sup>/ml) were washed in M9 buffer, spread at 10<sup>9</sup> bacteria/plate on the surface of minimal glucose medium plates, and incubated at 37° for up to 5 days. The bacterial cell number deposited on the surface of the plates was kept constant from experiment to experiment. Many of the Trp<sup>+</sup> reversion experiments were carried out by depositing washed bacteria on a medium containing, in addition to glucose, 0.3% casein acid-hydrolysate, an amino acid mixture lacking tryptophane (Calbiochem, San Diego and Sigma, St. Louis) and incubating at 30° for up to 5 days.

To compare the yield of Trp<sup>+</sup> mutants and Lac<sup>+</sup> mutants in the same culture, FC82 was grown as indicated above and plated on glucose (10<sup>9</sup> bacteria spread/plate) and lactose (10<sup>8</sup> bacteria in soft agar/plate) minimal plates (supplemented with tryptophane at 40  $\mu$ g/ml) along with  $1 \times 10^9$  FC29 Sm<sup>R</sup> (Table 1) per plate as scavenger. To test the influence of sodium dodecyl sulfate (SDS) in Trp<sup>+</sup> reversion, bacterial cultures were grown and plated as described above but in the presence of SDS at 0.01% (Radice lla *et al.* 1995). Cell viability on these plates was determined by taking plugs from a series of independent plates as previously described (Rosenberg *et al.* 1995).

**Lac<sup>+</sup> revertants during Trp<sup>+</sup> selection:** To determine whether Lac<sup>+</sup> revertants accumulated under conditions selective for Trp<sup>+</sup> reversion, independent cultures were grown in 0.1% glucose minimal medium to saturation, as indicated above, and 1 ml of each culture was filtered through a 90-mm, 0.45- $\mu$ m filter (Millipore Co., Bedford, MA). The filters were deposited on glucose minimal plates previously overlaid with 5 ml of soft M9 agar containing  $3 \times 10^9$  cells of FC329, an FC29 derivative with a *trpE* deletion (see Table 1) used as scavenger. At time zero, some filters were directly deposited on minimal lactose plates (with tryptophane, 40  $\mu$ g/ml) with scavenger cells as above. On days 2 and 4 a set of filters was transferred to minimal plates lacking a carbon source for 1 hr, to remove any glucose that may have been carried over with the filters. The filters were then transferred to lactose minimal plates with tryptophane (40  $\mu$ g/ml) and a layer of scavenger cells. Newly arising Lac<sup>+</sup> colonies were counted after 2 days

of incubation at 37°. Since there is the possibility of microcolonies of Trp<sup>+</sup> revertants present on the filters before transfer to lactose minimal plates, 50 colonies were picked at random from the filters on lactose plates incubated for 2 days after filter transfer. They were patched onto minimal glucose and lactose (with tryptophane) plates. None of the colonies were found to be double mutants as would be expected if the Lac<sup>+</sup> had arisen within Trp<sup>+</sup> microcolonies.

**Lac<sup>-</sup> reversion assays:** The strains FC40 and MG4792 (Table 1) and their derivatives were used as previously described in Lac<sup>-</sup> reversion tests (Radicella *et al.* 1995) with FC29 Sm<sup>R</sup> as scavenger when using fewer than 10<sup>9</sup> indicator bacteria per plate. The strain CC104 (Table 1) was also used for Lac<sup>+</sup> reversion tests. This strain is similar to FC40 but carries an episome with a regulated *lac* operon and a point mutation in the triplet encoding the active site of  $\beta$ -galactosidase.

**Detection of additional mutations:** We screened the Lac<sup>+</sup> or Trp<sup>+</sup> mutants that appear early or late after depositing bacteria on the selective plates for loss-of-function mutations in a large mutational target. The selective plates were incubated at 30°, and the revertant colonies were tested for ability to grow at 42°. We also tested the Trp<sup>+</sup> revertants for absolute auxotrophic mutations. The Lac<sup>+</sup> or Trp<sup>+</sup> revertants were screened for absence of growth under nonpermissive conditions; therefore, any Trp<sup>+</sup> or Lac<sup>+</sup> revertant carrying an additional mutation is present on the selective plates as a pure colony. This colony is the product of a mutational event that must have occurred either before or at the same time as the Lac<sup>-</sup> or Trp<sup>-</sup> bacterium became Lac<sup>+</sup> or Trp<sup>+</sup>.

Temperature-sensitive (Ts) mutants from Lac<sup>+</sup> colonies of FC40, CC104, MG4792, and their derivatives were screened by picking the Lac<sup>+</sup> colonies and patching them on lactose minimal medium incubated at 30° and 42°. We picked Lac<sup>+</sup> colonies that appeared on day 2, on day 3, and on days 7–8 after depositing Lac<sup>-</sup> cells on selective plates. The first revertant colonies, appearing on day 2, are often regarded as the product of mutation occurring during the growth of the culture (*i.e.*, before depositing cells on the selective plates). We have assumed that revertant colonies not present on day 2 and appearing on day 3 are products of early mutations occurring during selection. All Lac<sup>+</sup> colonies that failed to grow at 42° were further purified on LB medium with X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside; Diagnostic Chemicals Limited, Oxford, CT) at 30°, the permissive temperature. Three purified colonies from each isolate were further tested on LB, lactose, or glucose minimal medium and incubated at 30° and 42°. The mutants were classified into three groups based on their growth phenotypes at 42°. A Lac<sup>+</sup> Ts auxotroph grew on LB plates but did not grow on minimal glucose or lactose plates. Failure to grow on any growth medium was classified as a Ts mutation in an essential gene. Lac<sup>+</sup> colonies that grew on LB and minimal glucose plates but failed to grow on minimal lactose plates were classified as Lac Ts.

Auxotrophic and temperature-sensitive mutants were identified among Trp<sup>+</sup> revertant colonies appearing on day 1, on day 2, and on days 4–5 of selection on minimal glucose-casein plates incubated at 30°. The mutants appearing on day 2 are regarded as early products of mutations occurring during selection. Trp<sup>+</sup> colonies were screened for auxotrophy by patching colonies onto minimal glucose plus proline (40  $\mu$ g/ml) and glucose-casein plates and incubating at 30°. Proline was added to the medium to include Trp<sup>+</sup> mutants that may have lost the episome during selection in the glucose-casein medium. Temperature-sensitive auxotrophs were screened in the same manner by incubating one set of plates at 42°. Candidate clones were further purified in minimal glucose-casein plates and tested again for auxotrophy. Trp<sup>+</sup> mutants were classified as having an additional auxotrophic mutation when

the colony failed to grow on minimal glucose-proline plates at 30° or 42°. In addition, a colony Ts in the *trpE* gene (Trp Ts) is one that grows on glucose-proline-tryptophane plates at 42°.

To investigate whether the bacteria in the lawn of unreverted Lac<sup>-</sup> or Trp<sup>-</sup> cells contained unselected additional mutations, plugs were taken from areas with no visible colonies as for testing viability (see *Selection of Trp<sup>+</sup> revertants*). The plugs, containing a sample from the lawn, were resuspended in 1  $\times$  M9 buffer and plated in LB medium. Colonies appearing from the day 7 lawn of unreverted Lac<sup>-</sup> cells grown in LB plates were replica-plated on two sets of minimal glucose and LB plates and were incubated at 30° and 42°. Colonies derived from a 5-day lawn of unreverted Trp<sup>-</sup> bacteria were grown on LB plates, were replica-plated onto minimal glucose and LB plates, and were incubated at 30° and 42°.

**Characterization of multiple mutants:** The auxotrophic requirements of Lac<sup>+</sup> revertants and Trp<sup>+</sup> revertants with additional mutations were identified by the pools method described elsewhere (Holliday 1956).

We also assessed 35 Lac<sup>+</sup> revertants harboring additional mutations for a mutator phenotype (Miller 1996). These clones were grown in LB and five drops of 0.02 ml of each revertant were deposited on the appropriate plates supplemented with the antibiotics nalidixic acid (50  $\mu$ g/ml, Nal) or streptomycin (200  $\mu$ g/ml, Sm). Any candidate that showed an elevated number of mutants on both antibiotics was further tested by growing six independent cultures of each isolate on LB broth and plated again on the two antibiotics. The median frequency of mutation of FC40 for Nal<sup>R</sup> is 1  $\times$  10<sup>-8</sup> and for Sm<sup>R</sup>  $\sim$ 1  $\times$  10<sup>-9</sup>–1  $\times$  10<sup>-10</sup>. FC40 harboring either the *mutL* or *mutS* allele were used as a control (Table 1) since their frequency of mutation for these markers is elevated by  $\sim$ 100-fold. Resistance to rifampicin (Fluka Chemie AG, Buchs, Switzerland; 100  $\mu$ g/ml, Rif) was used to test the Trp<sup>+</sup> revertants with the FC40 *mutL* derivative as a control. The median frequency of mutation of FC82 to Rif<sup>R</sup> is 1  $\times$  10<sup>-8</sup>.

To show that the Lac Ts phenotype is linked to the episome, the episomes from 17 FC40 Lac Ts colonies were conjugated with P90C Sm<sup>R</sup> (lab collection), selecting for Sm<sup>R</sup> (200  $\mu$ g/ml) colonies on minimal glucose plates. Products were purified on LB medium with X-Gal and two colonies from each isolate were tested for growth at 30° and 42° on minimal lactose plates.

## RESULTS

**Reversion of a chromosomal mutation during nonlethal selection:** We used the chromosomal *trpE7999* +1 frameshift mutation in *E. coli* FC82 (see Table 1) for this analysis. When bacteria with this allele are deposited on glucose minimal agar to select for Trp<sup>+</sup> revertants, new mutants accumulate with time. Certain features of this accumulation are shared with those of the accumulation of Lac<sup>+</sup> mutants of *lacI33*, in which the frameshift allele is episome borne.

As FC82 also carries the episome with the *lacI33* allele, we can look for unselected Lac<sup>+</sup> mutants under conditions of Trp<sup>+</sup> selection, and we can ask for Lac<sup>+</sup> revertants and Trp<sup>+</sup> revertants in the same culture. To demonstrate that unselected Lac<sup>+</sup> mutants accumulate during Trp<sup>+</sup> selection, indicator cells (Trp<sup>-</sup> and Lac<sup>-</sup>) were deposited and incubated on filters on glucose minimal medium (*i.e.*, conditions of Trp<sup>+</sup> selection). Trans-

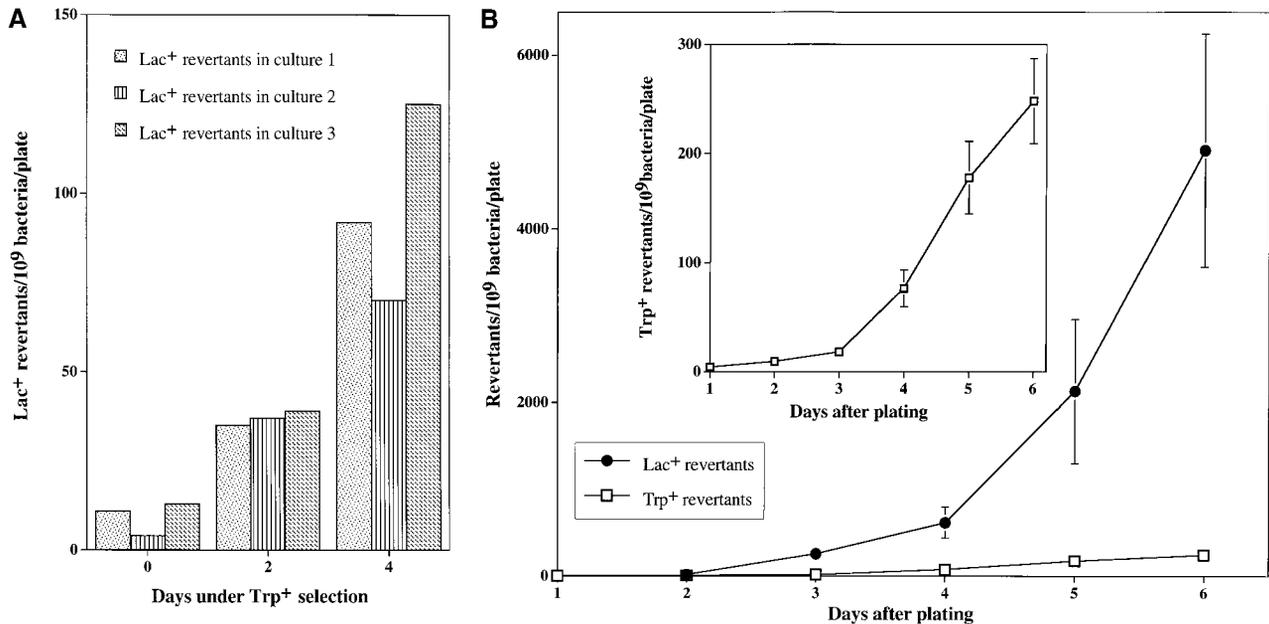


Figure 1.—Reversion of the FC82 strain bearing the +1 chromosomal frameshift allele *trpE7999*. (A) Trp<sup>+</sup> selection results in accumulation of unselected Lac<sup>+</sup> revertants. Graph shows the number of Lac<sup>+</sup> revertants present after a given number of days on Trp<sup>+</sup> selection. Independent cultures are represented by bars with different shades. (B) Trp<sup>+</sup> and Lac<sup>+</sup> revertants accumulate in cultures of FC82. The values of the accumulation shown are the average of seven independent cultures deposited on Lac<sup>+</sup> or Trp<sup>+</sup> minimal medium selection; error bars represent one standard deviation of the mean. The inset shows the accumulation of Trp<sup>+</sup> revertants in the same experiment.

ferring the filters to lactose minimal plates supplemented with tryptophane (40  $\mu$ g/ml) permitted us to detect Lac<sup>+</sup> mutants. We found that Lac<sup>+</sup> mutant colonies appeared on these filters after 2 days of incubation at 37°, and their number increased with time spent under Trp<sup>+</sup> selection (Figure 1A). Among 50 of the colonies appearing on the filters 2 days after transfer to lactose minimal plates containing tryptophane, none were double revertants, Trp<sup>+</sup> Lac<sup>+</sup>.

We obtained the number of Lac<sup>+</sup> and Trp<sup>+</sup> revertants present in the same culture by depositing aliquots of an FC82 culture on lactose minimal plates containing tryptophane and on minimal glucose plates without tryptophane. Viability with  $1 \times 10^9$  bacteria/plate is maintained in minimal glucose plates for at least the first 5 or 6 days ( $5 \times 10^8$ – $1 \times 10^9$  total viable bacteria/plate at day 6 postplating). The first Lac<sup>+</sup> revertant colonies appear on day 2 (40 hr) of incubation, and Trp<sup>+</sup> revertants appear after 1 day (24 hr). Figure 1B shows the accumulation of both types of mutants in a set of seven independent cultures. The curves describing the accumulation of Trp<sup>+</sup> revertants (Figure 1B, inset) appear to be quadratic, and the rate is substantially lower than that for the accumulation of Lac<sup>+</sup> revertants in the same strain background.

As with reversion to Lac<sup>+</sup> of the episome-borne *lacI33* allele (Cairns and Foster 1991), we observed fewer Trp<sup>+</sup> mutants accumulating in the recombination-deficient  $\Delta$ *recA*::Cm<sup>R</sup> derivative of FC82 (data not shown). However, the observed decrease could be due to loss

of viability of the recombination-deficient derivative of FC82 since we found an about threefold reduction in the number of viable cells present on the selective plates after 5 days of selection ( $9 \times 10^8$ – $3 \times 10^8$  total bacteria viable/plate). In contrast to what has been reported for the episomal Lac<sup>+</sup> reversion (Radicella *et al.* 1995), addition of 0.01% SDS to the selective plates did not decrease the yield of Trp<sup>+</sup> mutants significantly (data not shown). Trp<sup>+</sup> revertants also accumulate in a minimal glucose medium supplemented with 0.3% acid-hydrolyzed casein, a richer medium that lacks tryptophane, at both 30° and at 37°, but by the fifth day there is some loss of viability evident at the higher temperature (data not shown). Selection for Trp<sup>+</sup> revertants in this medium allows screening for the presence of additional auxotrophic mutations among the Trp<sup>+</sup> revertants.

FC40 with an episomal *lacI33* allele and MG4792 with a *lacI33* allele on the chromosome reverted to Lac<sup>+</sup> at 30° with similar rates to those obtained at 37° in each case (data not shown). Further experiments were carried out at 30° since incubation at this temperature permits us to screen for the presence of additional temperature-sensitive mutations among the Lac<sup>+</sup> revertants.

**Screening for Lac<sup>+</sup> or Trp<sup>+</sup> revertants bearing additional mutations:** The early revertants that were examined were the Trp<sup>+</sup> colonies that appeared on days 1 and 2 and Lac<sup>+</sup> colonies appearing on days 2 and 3. Early Lac<sup>+</sup> or Trp<sup>+</sup> revertants were analyzed for two consecutive days because of the likelihood that the first appearing mutants were a mixture. The very first ap-

**TABLE 2**  
**Additional mutations among Lac<sup>+</sup> and Trp<sup>+</sup> revertants**

Strain	Days 2-3				Day 3				Days 7-8			
	Lac <sup>+</sup> <sup>a</sup>	Lac Ts <sup>b</sup>	Aux <sup>c</sup>	Ess <sup>d</sup>	Lac <sup>+</sup>	Lac Ts	Aux	Ess	Lac <sup>+</sup>	Lac Ts	Aux	Ess
FC40	1863	9	1	1	1800	38	5	3	1900	36	30	47
MG4792	1413	36	N <sup>e</sup>	N					703	94	N	N
MG4792A <sup>f</sup>	1043	8	N	N					2430	3	3	1
MG4792B <sup>g</sup>	1127	34	N	N					848	27	N	N

Strain	Days 1-2				Day 2				Days 4-5			
	Trp <sup>+</sup> <sup>a</sup>	Trp Ts <sup>h</sup>	Ts <sup>i</sup>	Aux <sup>j</sup>	Trp <sup>+</sup>	Trp Ts	Ts	Aux	Trp <sup>+</sup>	Trp Ts	Ts	Aux
FC82	1712	N	2	4	1002	4	N	N	2200	N	8	40
GG82	695	1	N	4					1122	9	N	N

<sup>a</sup> Total number of Lac<sup>+</sup> or Trp<sup>+</sup> revertant colonies tested on the given day.

<sup>b</sup> Lac Ts, temperature-sensitive mutation in the LacI-Z fusion protein.

<sup>c</sup> Aux, Lac<sup>+</sup> revertants with an auxotrophic temperature-sensitive mutation.

<sup>d</sup> Ess, Lac<sup>+</sup> revertants with temperature-sensitive mutation in an essential gene.

<sup>e</sup> N, no colonies found with the given phenotype.

<sup>f</sup> MG4792A, strain MG4792 bearing the FC29Kan episome (Table 1).

<sup>g</sup> MG4792B, strain MG4792 bearing the pCJ100 episome (Table 1).

<sup>h</sup> Trp Ts, temperature-sensitive mutants in the TrpE protein.

<sup>i</sup> Ts, Trp<sup>+</sup> revertants with an auxotrophic temperature-sensitive mutation.

<sup>j</sup> Aux, Trp<sup>+</sup> revertants with an auxotrophic mutation. This class of mutants is different from the auxotrophs in *i*.

pearing Lac<sup>+</sup> mutants (Foster and Trimarchi 1994; Rosenberg *et al.* 1994) are different from those that accumulate during selection in the sequence changes involved in the reversion of the +1 frameshift. Thus, only Lac<sup>+</sup> and Trp<sup>+</sup> mutant colonies appearing after the first crop are considered the product of true postplating mutations. Those early mutants were compared to the Lac<sup>+</sup> and Trp<sup>+</sup> colonies that appeared after more prolonged incubation on the selection plates.

Table 2 shows that Ts mutants are found among Lac<sup>+</sup> revertants isolated early or much more frequently late after plating. We found Ts mutants in both biosynthetic and essential genes at high frequency, and these mutants accumulate with time.

For the Trp<sup>+</sup> revertants appearing at 30° on plates supplemented with casein acid-hydrolysate, some were Ts and others were auxotrophs at either temperature as seen in Table 2. The frequency of Trp<sup>+</sup> revertants with additional mutations increases with time as well, much like their Lac<sup>+</sup> counterparts.

The mutants that have a Lac Ts phenotype in the FC40 or Trp Ts phenotype in the FC82 strains are not being considered as having additional mutations, since they are likely to reflect changes in the hybrid repressor-β-galactosidase protein or in the TrpE protein. Transfer of the episomes from 15 Lac<sup>+</sup> Ts mutants to P90C Sm<sup>R</sup> showed that the Ts phenotype was linked to the episomes in each case.

Background cells present on the selective plates that had not reverted by days 6-7 to Lac<sup>+</sup> or by days 4-5 to

Trp<sup>+</sup> were also analyzed for additional mutations. If all bacteria maintained under selective conditions were hypermutable, then replating these bacteria for single colonies on complete medium and testing them would be expected to reveal the presence of secondary mutations like those present in the late appearing Lac<sup>+</sup> and Trp<sup>+</sup> revertants. Analysis of 2940 Lac<sup>-</sup> colonies and 1000 Trp<sup>-</sup> colonies isolated from the lawns of nonreverted cells after prolonged incubation on selective plates showed that, at this level of detection, neither the Lac<sup>-</sup> nor Trp<sup>-</sup> cells appear to accumulate other mutations during nonlethal selection.

By testing the double mutants for the presence of a stable mutator phenotype, we were able to identify the fraction of revertants that also carried such mutations. We found 2/35 late appearing Ts clones of FC40 Lac<sup>+</sup> and 1/25 FC82 Trp<sup>+</sup> auxotrophs with a recognizable mutator phenotype. Only these clones display a frequency of resistance to one or more antibiotics similar to the mutator *mutS* or *mutL* controls, and 100-fold higher than the frequency found with the wild-type parent (see materials and methods for the median frequencies to Nal<sup>R</sup>, Sm<sup>R</sup>, and Rif<sup>R</sup>). The remaining colonies with additional mutations showed no significant difference in mutability from the parent. This frequency of genetic mutators is consistent with that reported by Miller (1996). Genetic mutability is a small contributor to the cause of the high mutability that is evident.

**Influence of the episome:** The accumulation of revertants of the frameshift alleles *trpE7999* and *lacI33*

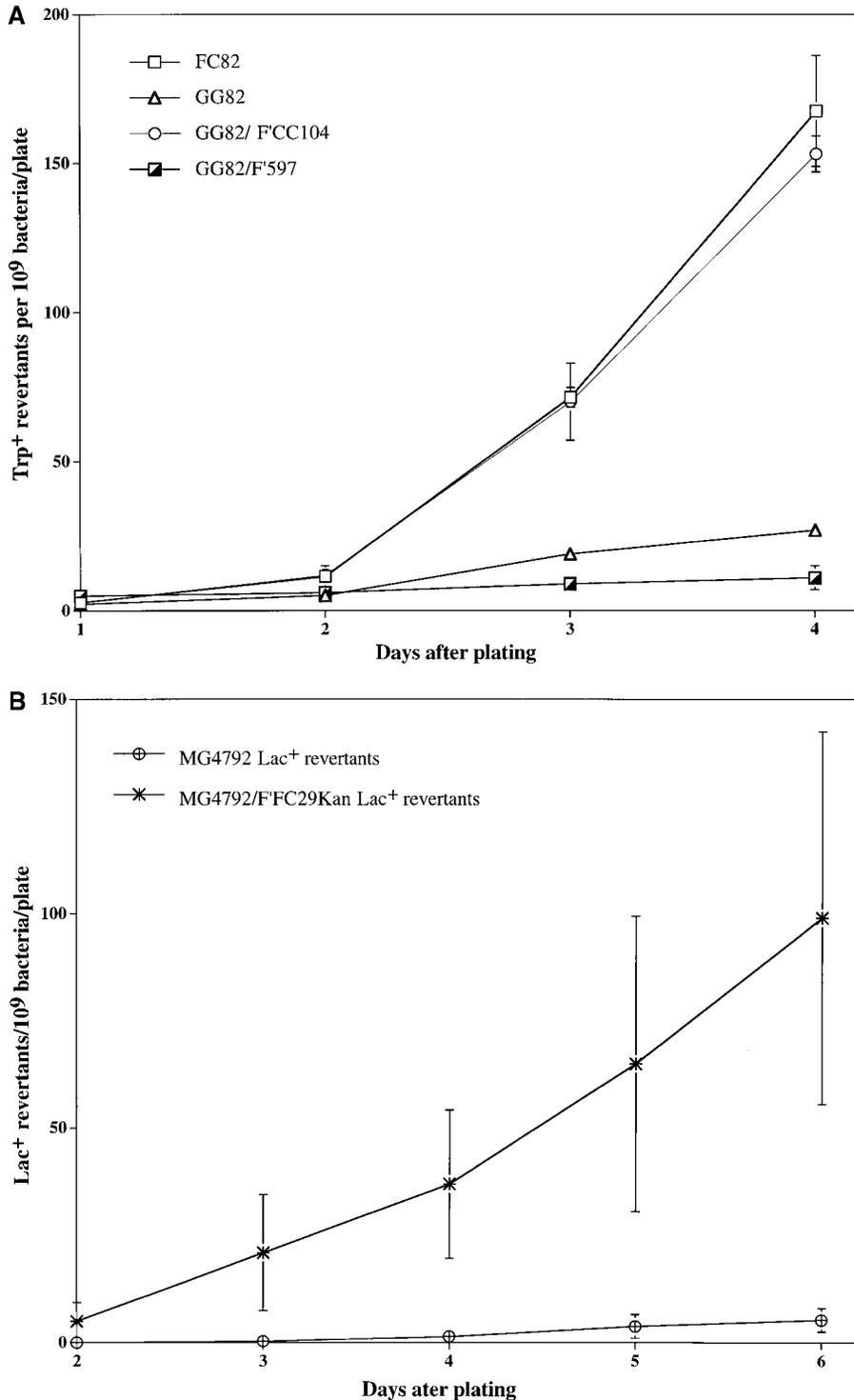


Figure 2.—Accumulation of revertants in derivatives with different episomes. (A) Accumulation of Trp<sup>+</sup> revertants measured on casein-glucose medium for FC82 is compared to that of GG82, the episome-free strain, and GG82 harboring F'CC104 (F'128 derivative) and F'597 (independently isolated) episomes. Other independently isolated episomes gave similar results to that of GG82/F'597 (see results). (B) FC29Kan episome, a derivative of F'128, provides for the accumulation of chromosomal Lac<sup>+</sup> revertants. Accumulation of Lac<sup>+</sup> revertants in MG4792 is compared in the presence and absence of the F'128 episome. Values shown are the average of 10 cultures; error bars represent one standard deviation of the mean.

showed similar characteristics, although the Trp<sup>+</sup> accumulation was quantitatively lower than the accumulation of Lac<sup>+</sup>. Both kinds of late appearing revertants display frequent additional mutations, although the *trpE7999* allele is located on the chromosome of the strain FC82 and *lacI33* is on the episome, a derivative of F'128 (Miller 1992). We asked whether an episome-free derivative of FC82 would revert and accumulate Trp<sup>+</sup> mutants. We found that GG82, the Trp<sup>-</sup> episome-free derivative, gave rise to early (day 1) Trp<sup>+</sup> revertants

with a frequency similar to FC82 when plated in minimal glucose medium with acid-hydrolyzed casein. However, subsequent accumulation of Trp<sup>+</sup> revertants (Figure 2A) was slow or absent. By day 5 there are ~10-fold fewer Trp<sup>+</sup> mutants accumulated in GG82 compared to FC82, even though the number of viable cells remains constant for both strains during the selective incubation ( $1 \times 10^9$ – $5 \times 10^8$  viable bacterial cells/plate). The fact that some of the late appearing revertants are in fact slow growers could account for their late appearance.

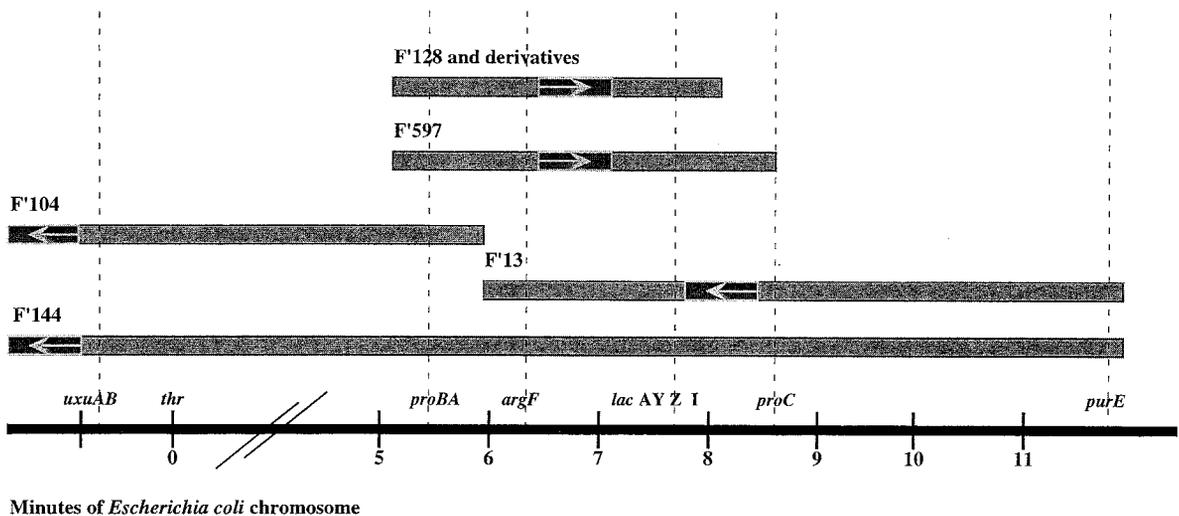


Figure 3.—Linear representation of the episomes used in this report. The map is not drawn to scale. The thick line at the bottom of the figure represents the linear sequence of the *E. coli* map, shown in minutes. Each minute corresponds to  $\sim 46$  kb. The episomes are represented by the horizontal bars. Each bar is sized approximately depending on the sequences of the *E. coli* chromosome carried by a given episome. The darker bar with an arrow represents the F portion of the episomes, but it is not clear where the F factor limits are in these episomes. The ends of each episome are drawn based on the genetic information given by CGSC and Holloway and Low (1996, and references therein). The origins of the other episomes are as follows: F'597 episome originates from P4X, F'144 from PO1 of Hfr Hayes, F'13 from PO57 of W3213, F'104 from PO1 of Hfr Hayes, and F'128 from PO65 of P804 (Holloway and Low 1996).

Due to its chromosomal location, the reversion or accumulation of Lac<sup>+</sup> of *lacI33* in MG4792 occurs at a much lower frequency than in the case of the same mutation allele located on the episome (Foster and Trimarchi 1995). Thus, we expected the chromosomal *lacI33* Lac<sup>-</sup> allele of MG4792 might display a higher reversion frequency to Lac<sup>+</sup> when the episome from FC40 was present. Figure 2B displays an experiment in which the accumulations of Lac<sup>+</sup> revertants of MG4792 strains with and without the episome are compared. The evidence shows that the presence of the FC29 *proAB*:TnKan episome (F'FC29Kan, a *lac*-deleted derivative of the episome present in FC40, F'128; see Table 1) increases the rate of accumulation of mutations of the chromosomal allele.

To learn which episome or regions of the episome are important to this process, a series of episome-containing derivatives of GG82 was constructed. The first episomes analyzed were all related to the FC40 episome, episome F'128, present in the strain CC104 and FC29Kan (see Table 1). We found that GG82 containing any of these episomes accumulates Trp<sup>+</sup> mutants in a manner similar to that of the parental FC82 strain. The results of one of these experiments are displayed in Figure 2A for GG82/F'CC104 (open circles). Similar results were observed with the derivatives of GG82 harboring the FC29Kan episome (not shown). We then investigated whether specific regions present in F'128 derivatives are responsible for the elevated rate of accumulation of mutations to Lac<sup>+</sup> or to Trp<sup>+</sup>. To address this question we constructed, by conjugation, derivatives of GG82 and/or MG4792 carrying the pCJ100 episome (Table 1)

and other independently isolated episomes harboring portions of the chromosomal region present in F'128. These strains were tested for reversion and accumulation of Trp<sup>+</sup> and/or Lac<sup>+</sup> mutants (pCJ100, F'13, F'104, F'597, F'144; see Table 1 and Figure 3 for chromosomal content, except for pCJ100 which carries the *polA* gene, not shown in Figure 3). The initial frequencies of Trp<sup>+</sup> mutants in the derivative strains of GG82 bearing these episomes and the episome free-GG82 strain are not distinguishable, and there is little or no subsequent accumulation of revertants (see Figure 2A, half-solid squares, for strain GG82/F'597). The data on the accumulation of additional mutations in these strains are in Tables 2 and 3. Table 2 shows that additional mutations were not found in the Lac<sup>+</sup> revertants of MG4792, bearing the chromosomal *lacI33* allele. The same holds true for MG4792/pCJ100 Lac<sup>+</sup> revertants (MG4792B in Table 2) and GG82 Trp<sup>+</sup> revertants (Table 2). In contrast, the data in Table 3 show that GG82/F'FC29Kan and GG82/F'CC104 Trp<sup>+</sup> revertants that accumulate late do harbor additional mutations. In Table 2 only a few additional mutations are also evident in MG4792/F'FC29Kan bacteria (see MG4792A). We do not understand why the frequency of secondary mutations is so low in this strain, which carries an F'128 episome. A limited investigation with other episomes containing the same chromosomal region present in F'128 (see Table 3) showed few or no secondary mutations, in contrast to the yield evident with F'128 derivatives.

CC104, a strain similar to FC40, contains an F'128 episome (Miller 1992) derivative with a regulated *lac* operon and with a point mutation at the codon for the

**TABLE 3**  
**Additional mutations among Trp<sup>+</sup> revertants harboring different episomes**

Strain	Days 1-2				Days 4-5			
	Trp <sup>+</sup> tested	Trp Ts	Ts	Aux	Trp <sup>+</sup> tested	Trp Ts	Ts	Aux
GG82/F'CC104 <sup>a</sup>	1195	N	N	N	960	1	6	18
GG82/F'FC29Kan <sup>a</sup>	953	N	N	N	900	5	N	19
GG82/F'128	385	N	N	1	450	N	6	6
GG82/F'597	1001	N	N	N	600	N	1	N
GG82/F'144	666	N	N	N	492	1	N	N
GG82/F'104	1288	N	N	N	891	3	N	N
GG82/F'13	1233	4	N	N	811	5	N	3

<sup>a</sup> Derivatives of F'128 (Table 1 and Figure 3). Headings are as in Table 2. Episome descriptions are in Table 1 and Figure 3.

amino acid at the active site of  $\beta$ -galactosidase, in which only a C:G to A:T transversion at that specific site (Cupples and Miller 1989) permits a Lac<sup>+</sup> phenotype. Although it would seem that the target for reversion in the *lacI33* allele is much larger than the one in CC104, the frequency of revertants appearing on the second day of incubation is approximately the same for CC104 and FC40 (approximately seven to nine Lac<sup>+</sup> mutants in 10<sup>9</sup> bacteria). CC104 gives rise to Lac<sup>+</sup> revertants that appear on day 2 (early) but accumulates few revertants (see Figure 4). We tested for additional mutations in 1745 Lac<sup>+</sup> colonies that were present on day 2 and in 504 of the rare Lac<sup>+</sup> revertants that appeared on day 7, finding none with additional mutations. Therefore, it seems that the specific transversion required for the reversion of CC104, which does not seem to accumulate during selection, does not appear to occur among the

hypermutable cells that are evident during lactose selection of FC40.

It would appear that the F'128-derived episomes present in FC40 or in FC82 play a role in the accumulation of Trp<sup>+</sup> or Lac<sup>+</sup> revertants in those strains.

**Characterization of Lac<sup>+</sup> and of Trp<sup>+</sup> multiple mutants:** The auxotrophic requirements have been identified for a number of multiple mutants that are either Lac<sup>+</sup> or Trp<sup>+</sup>. Out of 35 FC40 Ts and Lac<sup>+</sup> mutants analyzed, 3 had a single requirement for methionine, 4 had a double requirement (arginine and ornithine; cysteine and methionine; tryptophane and PABA [*p*-aminobenzoic acid]; riboflavin and nicotinic acid), and 1 had multiple requirements (serine, cysteine, PABA, methionine). The Lac<sup>+</sup> revertants of MG4792/F'FC29Kan that were Ts had multiple requirements.

In the case of those Trp<sup>+</sup> revertants that were ana-

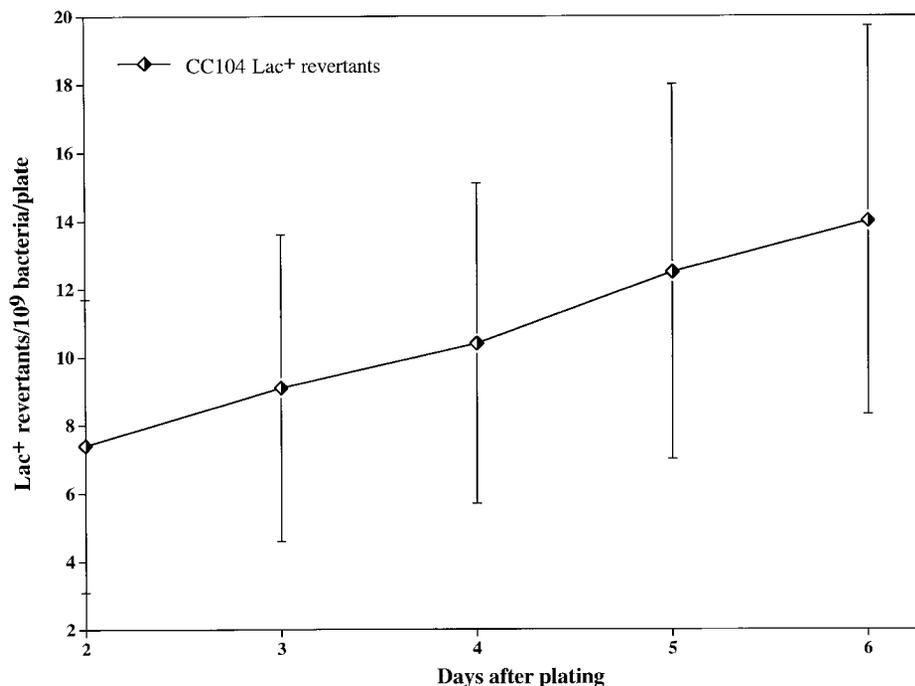


Figure 4.—CC104 accumulates Lac<sup>+</sup> mutants poorly. Graph representing the accumulation of Lac<sup>+</sup> mutants in CC104. The values of the accumulation data shown are the average of 85 independent cultures; error bars represent one standard deviation of the mean.

lyzed, we found that 11 clones out of 25 FC82 Trp<sup>+</sup> auxotrophs (Ts and non-Ts) have a single requirement (3 histidine, 2 leucine, 2 threonine, 1 serine, 1 tyrosine, 1 cysteine, 1 isoleucine). For GG82/F'CC104 Trp<sup>+</sup> 7 clones out of 18 have a single requirement (3 leucine, 2 methionine, 1 histidine, 1 threonine). The rest of the Lac<sup>+</sup> or Trp<sup>+</sup> clones with additional mutations have multiple requirements. We did not pursue further characterization of these mutants.

## DISCUSSION

One of the well-studied systems in which mutations occur during nonlethal selection is the reversion of the episomal +1 frameshift allele *lacI33* in *E. coli* FC40 (Cairns and Foster 1991). Roles for transfer functions, recombination, DNA repair, and, recently, DNA amplification underlying this phenomenon have been reported (Foster and Trimarchi 1995; Foster *et al.* 1996; Peters and Benson 1995; Radicella *et al.* 1995; Harris *et al.* 1996, 1997a,b; Rosenberg *et al.* 1996; Anderson *et al.* 1998).

Here we have described the reversion of the *E. coli* chromosomal allele *trpE7999* during nonlethal selection, and we have shown some features that are similar to those of reversion of *lacI33* in FC40. These include the ability to give rise to both selected and unselected revertants under nonlethal selection (Foster 1997), the presence of additional mutations (Hall 1990; Torkelson *et al.* 1997; Rosche *et al.* 1999) in late appearing Trp<sup>+</sup> mutants, and the dependence of reversion on the presence of the episome F'128. One difference that appears in the reversion of this chromosomal allele is that treatment expected to disable episome transfer functions, 0.01% SDS (Radicella *et al.* 1995), does not interfere with reversion (data not shown). This suggests that, although there is a role for the episome, there may be no role for episomal transfer function in the Trp<sup>+</sup> mutability.

Torkelson *et al.* (1997) showed that, among late appearing FC40 Lac<sup>+</sup> mutants containing additional mutations, in most cases (74%) the additional mutation was present in all the bacteria of the revertant colonies, *i.e.*, in pure colonies. In the remaining cases, only half or one-fourth of the Lac<sup>+</sup> mutants were mutants at another site. Mutations were detected in genes throughout the genome (Torkelson *et al.* 1997; Rosenberg *et al.* 1998). These observations suggest that the additional mutation occurred temporally earlier than or at the same time as the Lac<sup>+</sup> reversion, so that when a bacterium containing a mutation became Lac<sup>+</sup>, it was often clonal for both mutations. Since most of the double mutants did not have a heritable mutator phenotype and Lac<sup>-</sup> bacteria from the selection plates did not show additional mutations, they concluded that a small subpopulation of *E. coli* was experiencing hypermutability that affected the whole genome.

We have examined Lac<sup>+</sup> or Trp<sup>+</sup> clones for additional unselected mutations at early or at late times after plating to ask whether the bacteria that are hypermutable persist in their hypermutability. We observed that the fraction of Lac<sup>+</sup> or Trp<sup>+</sup> colonies with additional unselected mutations is much larger for revertants that appear late, day 5 for Trp<sup>+</sup> and day 7 for Lac<sup>+</sup>, than for early revertants. If the hypermutable cells were the product of new cells, in the selected population, passing through a hypermutable state in cycles, the fraction of Lac<sup>+</sup> or Trp<sup>+</sup> revertant colonies with additional unselected mutations would not be expected to increase.

Our comparison included new Trp<sup>+</sup> mutants appearing early, on day 2, and the new Lac<sup>+</sup> mutants appearing on day 3, with mutants appearing late, on the fifth day after plating in the case of Trp reversion and on the sixth or seventh day for Lac reversion. We have assumed that those Trp<sup>+</sup> mutants appearing on day 2 or later and Lac<sup>+</sup> mutants appearing on day 3 or later were the products of mutations occurring after selection had been initiated.

The additional unselected mutations screened for were loss-of-function mutations, that is, temperature-sensitive and auxotrophic mutations, which constitute large mutational targets. This choice limited our search to Trp<sup>+</sup> or Lac<sup>+</sup> colonies that were pure for those additional mutations. Thus, early and late Lac<sup>+</sup> and Trp<sup>+</sup> colonies were screened for failure to grow under nonpermissive conditions. We show that revertants appearing early during selection are much less frequently mutant at other sites. Furthermore, we find no mutations in the population of Trp<sup>-</sup> or Lac<sup>-</sup> bacteria that had experienced 1 week of selection; thus, it is indeed only a small fraction of bacteria experiencing this elevated mutability (Rosenberg *et al.* 1998; Rosche *et al.* 1999). In agreement with previous reports (Torkelson *et al.* 1997; Rosenberg *et al.* 1998), only a small fraction of the double mutants harbored heritable mutator phenotype. Therefore, the presence of additional mutations appears to be due to hypermutability in a fraction of the selected population during selection. We suggest that a subset of bacteria experiences an epigenetic change, becoming hypermutable when deposited on the nonlethal selection medium. They remain so for the duration of the selection. Thus, bacteria that experience reversion after prolonged selection often have experienced other mutations by the time the reversion that allows growth occurs. This mechanism would account for the increase in the likelihood of additional mutations among the bacteria that had undergone longer and longer periods of selection (see Tables 2 and 3). It would also account for the fact that clones of revertants with additional, recognizable mutations are so frequently pure. Since one of the criteria that we have used for additional mutations is temperature sensitivity, it seems that the mutability is not likely to be confined exclusively to frameshift mutations.

If the revertants that occur during selection are among bacteria whose mutability is established soon after the selection is imposed and persists, we suggest the establishment of an altered epigenetic mutable state that disappears when a mutation that allows growth occurs. Such an epigenetic state would be characteristic of selection conditions that are not tight; that is, some  $\beta$ -galactosidase is synthesized during  $\text{Lac}^+$  selection, and presumably some TrpE gene product is synthesized during  $\text{Trp}^+$  selection. We assume that this leakiness is not sufficient to allow bacterial multiplication. Such an epigenetic state for which, at least in this case, F'128 appears to play a role operates on the chromosome and on the episome of the bacteria. The capacity to conjugate, important for the accumulation of episomal mutations, may reflect an additional process, superimposed on the process that operates everywhere in the cell.

This behavior is consistent with the notion proposed by Ninio (1991), in which the mutability is the consequence of a small fraction of bacteria with a flawed protein involved in DNA replication or DNA repair that is present as a result of a rare failure in the fidelity of transcription or translation.

The mechanism that mediates this hypermutability in bacteria under nonlethal selection is poorly understood, although a number of models involving recombination functions and mismatch repair have been advanced (Foster 1998; Rosenberg *et al.* 1998). We have found no additional mutations among 1000  $\text{Trp}^+$  revertants that appear to have accumulated in a *recA* FC82 strain (data not shown), suggesting again that reversion of the chromosomal *trpE7999* frameshift shares some features with the reversion of the episomal *lacI33*, and they may be both mediated, at least in part, by the same mechanism. It also suggests that the ability to accumulate revertants and hypermutability may be connected. Interestingly, one prominent, perhaps essential, role in this process may be played by the F'128 episome. Its absence in GG82 or MG4792 results in a decrease of both the rate of accumulation of  $\text{Lac}^+$  or  $\text{Trp}^+$  mutants as well as the accumulation of additional mutations in the  $\text{Lac}^+$  or  $\text{Trp}^+$  clones (Tables 2 and 3). Furthermore, episomes carrying the same chromosomal regions present in F'128 (F'13, F'104, F'144, F'597) are not interchangeable with F'128 (Figure 3). There is little or no accumulation of  $\text{Trp}^+$  mutants in the presence of these episomes (Figure 2A), and additional mutations in late appearing clones of  $\text{Trp}^+$  revertants are rare when these other episomes are present (Table 3). The *dinB* gene, which has been implicated in elevated mutagenesis (Brotcorne-Lannoye and Maenhaut-Michel 1986; Kim *et al.* 1997) and for which the F'128 episome holds a copy (V. G. Godoy, unpublished results), may be a good candidate for a gene with some special feature on F'128 that could be responsible for the hypermutability. It is also possible that the amount of F factor sequences

present on the episome is responsible for the observed differences among episomes. For the case of the F'128 episome that bears the CC104 transversion mutation, there is evidence of very little, if any, accumulation of  $\text{Lac}^+$  reversion mutations during selection (Figure 4). However, the presence of this episome permits accumulation of revertants at the *trpE7999* chromosomal allele (Figure 2A) as well as additional mutations in these revertants (Table 3).

CC104 reverts to  $\text{Lac}^+$  by a specific change of a C:G to A:T, a single base pair transversion in a triplet encoding the active center of  $\beta$ -galactosidase (Cupples and Miller 1989). Therefore, it would appear that mutability that is manifested during the selection process does not apply to this class of transversion mutations, C:G to A:T, required to revert CC104. Perhaps similarly, Foster (1994) failed to find accumulation of rifampicin-resistant mutants, *rpoB*, during lactose selection. In this case only 17 specific base pair changes, affecting 14 amino acids, in the *rpoB* gene lead to  $\text{Rif}^R$  (Jin and Gross 1988). These mutations appear to be insensitive to the particular hypermutability mechanism discussed here that is manifested during nonlethal selection, causing mutation by a different mechanism.

An interesting implication of these experiments derives from the recent report of a mutator phenotype, present in mammalian cells under conditions of contact inhibition, and apparently absent during their growth (Loeb 1997; Richards *et al.* 1997; Meuth *et al.* 1999). They are also reminiscent of the observations of Kennedy *et al.* (1980) in which C3H 10T 1/2 mouse cells appear to spawn transformed foci at or after reaching contact inhibition. If a subset of mammalian cells were to experience hypermutability when they had ceased dividing, the appearance of the multiple mutations, a general feature of cancer cells, could become possible.

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