A Genetic Strategy to Demonstrate the Occurrence of Spontaneous Mutations in Nondividing Cells Within Colonies of *Escherichia coli*

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ABSTRACT

A genetic strategy was designed to examine the occurrence of mutations in stationary-phase populations. In this strategy, a parental population of cells is able to survive under both permissive and restrictive conditions whereas mutants at a particular target locus exhibit a conditional-lethal phenotype. Thus, by growing the population to stationary phase under restrictive conditions and then shifting it to permissive conditions, mutations that had arisen in stationary phase can be studied without confounding effects caused by the occurrence of similar mutations during growth of the population. In two different applications of this strategy, we have studied the reversion to Lac^+ in stationary phase of several Lac^- mutations in *Escherichia coli*. Our results indicate that a variety of spontaneous point mutations and deletions, particularly those that are sensitive to the mechanisms of replication slippage (for their generation) and methyl-directed mismatch repair (for their correction), can arise in nondividing populations of cells within a colony. The frequency of their occurrence was also elevated in *mutS* strains, which are defective in such mismatch repair. These data have relevance to the ongoing debate on adaptive or directed mutations in bacteria.

SPONTANEOUS mutations are thought to arise as errors in replication of the genetic material when cells (or organelles or viruses) increase their numbers, with the errors that either are blind to or escape the correction mechanisms becoming then fixed in the genome. Can spontaneous mutations also arise in nondividing cells? An early study by RYAN (1955) suggested that His^+ revertants could arise in populations of nondividing His^- cells (of *Escherichia coli*) starved for the amino acid. Recent interest in this question was revived by claims, in both bacteria and yeast, that directed mutations (also variously referred to as adaptive or Cairnsian or selection-induced or postplating mutations) can occur in starving cell populations that are subjected to nonlethal selections (reviewed in FOSTER 1993). It has been speculated that endogenous DNA lesions caused by base alkylation (MACKAY *et al.* 1994) or oxidative stress (BRIDGES 1996; BRIDGES *et al.* 1996), as also by stationary-phase induction of the SOS response (TADDEI *et al.* 1995), may contribute to the occurrence of these mutations. However, the claims for directed mutation have also generated much contention and controversy. The criticism has focused on the validity of the assumptions that the mutants were not preexisting in the population and that the population itself was truly nondividing, as also on the evidence that the mutations were "directed" by the environmental selection conditions (reviewed in SMITH 1992; LENSKI and MITTLER 1993; SNIEGOWSKI and LENSKI 1995; MACPHEE and AMBROSE 1996).

One example of directed mutation that has been intensively studied is the reversion to Lac^+ of a frameshift mutation *lacI33::lacZ* present on F′ *lac pro* in *E. coli* strain FC40 (reviewed in ROSENBERG *et al.* 1995). The mutational spectrum of "adaptive" reversion events of this allele is different from that of the "random" reversion events that occur during exponential growth, and it is only the former that are also RecA- and RecBCD-dependent. It has been suggested that stationary-phase cells are physiologically deficient in DNA mismatch repair and that this deficiency accounts for the distinct spectrum of adaptive reversions in strain FC40 (FOSTER *et al.* 1995; LONGERICH *et al.* 1995; FENG *et al.* 1996). On the other hand, it is now known that adaptive reversions of *lacI33::lacZ* occur only when the mutation is carried on a conjugation-proficient F′ (reviewed in ROSENBERG *et al.* 1995); redundant homosexual transfer of the F′ between individual cells in the population has been demonstrated under these conditions (RADICELLA *et al.* 1995; PETERS *et al.* 1996). Whether the findings with FC40 bear relevance to the occurrence of other categories of adaptive mutation or at other (nonepisomal) locations therefore remains uncertain (*e.g.*, see GALITSKI and ROTH 1996; PRIVAL and CEBULA 1996; HALL 1997).

The principal difficulty in studying, and perhaps even defining the existence of, stationary-phase mutations (including directed mutations) is that the same muta-

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TABLE 1

List of *Escherichia coli* K-12 strains

Strain	Genotype	Reference/source
CSH115	*ara* Δ(*gpt-lac*)*5 rpsL mutS*::Tn*10*dTet	MILLER (1992)
CSH117	*ara* Δ(*gpt-lac*)*5 rpsL mutY*::Tn*10*dTet	MILLER (1992)
CSH142	*ara* Δ(*gpt-lac*)*5*	MILLER (1992)
CSH143	*ara* Δ(*gpt-lac*)*5 gyrA* /F′[*proAB*$^+$ *lacI lacZ*(Am)]	MILLER (1992)
FC82	*ara* Δ(*gpt-lac*)*5 trpE9777 thi* /F′[*proAB*$^+$ *lacI33*::*lacZ*]	P. L. FOSTER
JM101	*supE thi* Δ(*gpt-lac*)*5* /F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	MILLER (1992)
KL226	HfrC(P02A) *tonA22 ompF relA pit-10* T2^r	B. J. BACHMANN
MG1655	Wild-type	B. J. BACHMANN
GJ1242	*araD139* Δ(*argF-lac*)*U169 ptsF25 relA1 deoC1 rbsR flbB5301 rpsL150 zhc-3168*::Tn*10*Kan	From MC4100 (GOWRISHANKAR 1985) by Kan^r transduction
GJ1823	*zbh-900*::Tn*10*dKan(Ts)*1 lacZ4525*::Tn*10*dKan	This work
GJ1826L$^+$	*zbh-900*::Tn*10*dKan(Ts)*1 recA56 srl-3000*::Tn*10*	This work
GJ1886	*ara zbh-900*::Tn*10*dKan(Ts)*1 lacZ4525*::Tn*10*dKan *uup-351*::Tn*10*dTet	REDDY and GOWRISHANKAR (1997)
GJ2202	CSH143 *galE28 zbh-900*::Tn*10*dKan(Ts)*1*	This work
GJ2203	GJ2202 *galE516*(Ts)	This work
GJ2207	GJ2202 *galE516*(Ts) *galP526 mgl-351*	This work
GJ2214	FC82 *galE516*(Ts) *zbh-900*::Tn*10*dKan(Ts)*1 galP527 mgl-352*	This work
GJ2218	CSH142 *galE516*(Ts) *zbh-900*::Tn*10*dKan(Ts)*1 galP528 mgl-353*	This work
GJ2219	*ara galE516*(Ts) *zbh-900*::Tn*10*dKan(Ts)*1 galP528 mgl-353*	This work
GJ2220	GJ2219 *lacZ4525*::Tn*10*dKan	This work
GJ2231	GJ2219 *lacZ*(Am) *lacI3098*::Tn*10*Kan	This work
GJ2235	GJ2219 *lacI33*::*lacZ zaj-3099*::Tn*10*Kan	This work

Genotype designations are as in BERLYN *et al.* (1996). All strains are F$^-$ unless otherwise specified. In the strains listed, the following mutations were transduced from strains described earlier: *zhc-3168*::Tn*10*Kan, *lacI3098*::Tn*10* and *zaj-3099*::Tn*10*Kan from CAG18605, CAG18420 and CAG18594, respectively (SINGER *et al.* 1989; BERLYN *et al.* 1996); *galE28* from PL2 (*E. Coli* Genetic Stock Center); and *recA56* and *srl-3000*::Tn*10* from GJ971 (SAROJA and GOWRISHANKAR 1996).

tions can also occur in dividing cells so that it is only by the time of appearance of the mutants that the two classes can ostensibly be separated. We describe below an alternative approach that uses a spatial rather than temporal distinction between the two classes of mutants to address this question. In this approach, a novel conditional-lethal strategy was devised to selectively eliminate revertants for a particular mutation that arose during the phase of exponential growth of a population, so that any revertants that arose in the population in stationary phase could then be analyzed. Our results indicate that a variety of mutations, especially those that are particularly sensitive to the mechanisms of replication slippage and methyl-directed mismatch repair, can occur in nondividing cells.

MATERIALS AND METHODS

Bacterial strains and media: *E. coli* K-12 strains used in the study are listed in Table 1. Growth media (MILLER 1992) included LB or minimal A supplemented with carbon sources (as indicated) and auxotrophic requirements (as appropriate). Tetracycline (Tet), ampicillin (Amp), streptomycin (Str), chloramphenicol (Cm), Kanamycin (Kan) and Xgal were used at 15, 50, 100, 25, 40 and 25 μg/ml, respectively. Casamino acids (Difco) were used at 0.5% supplementation.

Obtaining Kan^r(Ts) mutation by P1 localized mutagenesis and construction of GJ1823: A preparation of P1 phage grown on a strain carrying the *zbh-900*::Tn*10*dKan insertion 50% cotransducible with the *galETK* operon at 17 min (J. Gowrishankar, unpublished results) was mutagenized *in vitro* with hydroxylamine as described (CUNNINGHAM-RUNDLES and MAAS 1975) and then used in transduction experiments. Kan^r transductants selected at 30° were scored for a Kan^s phenotype at 42°, and in this manner one temperature-sensitive Kan^r mutation was identified and designated *zbh-900*::Tn*10*dKan(Ts)*1*. Strain GJ1823 was constructed from MG1655 in two successive steps of P1 transduction, the first involving the transfer of the Kan^r(Ts) mutation (with Kan^r selection at 30°) and the second involving the transfer (with Kan^r selection at 42°) of a *lacZ4525*::Tn*10*dKan insertion obtained earlier in this laboratory (J. Gowrishankar, unpublished results).

Isolation of *galE*(Ts) mutant and construction of multicopy galE(Ts) plasmid: Spontaneous Gal$^+$ mutants of strain GJ2202 [carrying the *lacZ*(Am) and missense *galE28* mutations] were selected at 30° and were screened for a Gal$^-$ Gal^s phenotype at 42°. A putative temperature-sensitive *galE*$^+$ pseudorevertant [*galE516*(Ts)] that was so identified was designated GJ2203. On Lac$^+$ papillation medium at 30°, GJ2203 colonies exhibited very small-sized papillae, presumably because the *galE*(Ts) allele was not sufficiently Gal$^+$ at the permissive temperature to allow rapid growth of the papillae. To overcome this problem, the *galE516*(Ts) mutation was transferred on to a multicopy plasmid as follows. Plasmid pAA102

(which is a pBR322 derivative carrying the wild-type *galETK* operon and has a unique *Bst*EII site toward the 5′ end of *galE* and two *Eco*RV sites located respectively toward the 3′ end of *galE* and in *galT*; BUSBY *et al.* 1982) was first digested completely with *Bst*EII and partially with *Eco*RV; the former end was "blunted" with Klenow DNA polymerase and the molecule was recircularized; this resulted in the deletion of a 1.1-kilobase *Bst*EII-*Eco*RV segment internal to the *galE* gene, and the resultant $\Delta galET^{+}K^{+}$ plasmid was designated pHYD601. In the second step, a phage P1 lysate prepared on a GJ2203/pHYD601 transformant was used to transduce GJ2202 to Amp^{r} Gal^{+} at 30°, so as to identify plasmid derivatives into which the *galE516*(Ts) mutation had been transferred by homologous recombination from the chromosome of GJ2203. One such plasmid was designated pHYD603. GJ2203/pHYD603 derivatives exhibited satisfactory Lac^{+} papillation at 30° and continued to be Gal^{-} Gal^{s} at 42°.

Isolation of *galP mgl* mutants: *galE*(Ts) strains were rendered mutant successively in *galP* and then *mgl* [the two loci encoding galactose-uptake systems in *E. coli* (BERLYN *et al.* 1996)] by selection, respectively, for resistance to 1 mM 2-deoxygalactose (NAGELKERKE and POSTMA 1978) and resistance at 42° to 0.1% D-galactose on minimal A medium supplemented with Casamino acids. The first selection yielded exclusively *galP* mutants, while in the second selection the desired category of *galP mgl* mutants was distinguished from others carrying mutations in the *galETK* operon (that conferred Gal^{r}) by the fact that the former continued to be sensitive to internally generated galactose (from lactose hydrolysis) at 42°.

Construction of chromosomal lac mutant derivatives of *galE*(Ts) strains: GJ2218, a *galE*(Ts) *galP mgl* derivative of the Δ*lac-pro* strain CSH142, was first made Lac^{+} Pro^{+} by conjugation with a HfrC donor strain KL226, so as to construct GJ2219. Subsequently, the frameshift, amber and Kan^{r} insertion mutations in *lac* were each introduced into GJ2219 by P1 transduction (the first two in linkage, respectively, with the *zaj-3099*::Tn*10*Kan and *lacI3098*::Tn*10*Kan insertions, with selection in all instances for Kan^{r} at 42°).

Construction of F′ *traD* derivatives: Random insertions of Tn*10*dCm were generated in the F′ *lac pro* strain CSH143 following infection with phage λ1324, as described (MILLER 1992). The pool of Cm^{r} cells was used as donor in conjugation with CSH142, and selection for Cm^{r} transconjugants permitted the enrichment for Tn*10*dCm insertions that had occurred on the F′. A P1 lysate prepared on this second pool of colonies was used to transduce JM101 (which carries a *traD* mutation on F′ *lac pro*) to Cm^{r}, and individual transductants were screened that had now become proficient in conjugal transfer of the F′. In this manner, a Cm^{r} insertion (designated *zzf-902*::Tn*10*dCm) 60% linked to the *traD* locus was identified and was used to transduce the *traD36* mutation from JM101 into *galE*(Ts) derivatives of CSH143 and FC82.

Lac^{+} papillation assays: Lac^{+} papillation assays were done by methods modified from that described (MILLER 1992). One of two different Lac^{+} papillation media were used. For experiments with the Kan^{r}(Ts) strains, cultures were spread at dilutions sufficient to give ~25 colonies per plate (of 85 mm diameter) on LB medium supplemented with Kan, Xgal and 0.1% lactose. In the case of *galE*(Ts) *galP mgl* strains carrying plasmid pHYD603, a 3-μl volume of suspension containing $\sim 10^{4}$ cells was spotted on the surface of minimal A plates supplemented with ampicillin, Casamino acids (plus tryptophan in the case of FC82 derivatives) and 0.1% lactose, at a density not exceeding 20 spots per plate; Lac^{+} papillae that had grown on each spot were visualized by the gentle addition of 20 μl of Xgal solution (0.5 mg/ml) followed (once sufficient blue coloration had developed) by 20 μl of 1 M sodium carbonate.

Quantitative analysis of distribution of papillae in different colony zones: As explained below, we have devised a strategy in which a Lac^{-} colony is grown on Lac^{+} papillation medium initially at 42° and then at 30°, but any Lac^{+} mutants that may arise can survive and form papillae only at the latter temperature. The ratio of colony diameter at the time of temperature downshift to that at the end of the incubation was ~0.65. On photographs, each colony was therefore demarcated into a peripheral zone and a central zone by concentric circles drawn to the same ratio, and the number of papillae in each zone was counted. The summed values for several individual colonies were then used to calculate the ratio of peripheral to central zone papillation for a strain.

Other procedures: Methods for phage P1 transduction (GOWRISHANKAR 1985), conjugation (MILLER 1992) and *in vitro* DNA manipulations (SAMBROOK *et al.* 1989) were as described. Unless otherwise indicated, the *ssb-200*, *recA56* and *galE* mutations were introduced into strains by linked transduction, respectively, with the *zjc-904*::Tn*10*dTet (REDDY and GOWRISHANKAR 1997), *srl-3000*::Tn*10* and *zbh-900*::Tn*10*dKan(Ts)*1* insertions. Strains were rendered *uup*, *mutS* or *mutY* by Tet^{r} transduction from GJ1886, CSH115 and CSH117, respectively.

RESULTS

A genetic strategy to study stationary-phase mutagenesis: We have designed a conditional-lethal selection strategy in which a parental population of cells is able to survive and grow under both restrictive and permissive conditions whereas mutants at a particular locus arising in that population are killed under the former condition and survive under the latter. Thus, if the parental population is grown to stationary phase under the restrictive condition, all mutants of the class under study that arose during the phase of exponential growth will be killed. If the population is then subjected to selection under permissive conditions, the only mutants that would survive and be scored are those which arose after shift to the latter condition, *i.e.*, in the stationary phase.

We have applied this strategy in two different ways to examine the occurrence of stationary-phase mutations in *E. coli*. Both involve Lac^{-} parental populations of cells grown as colonies on agar plates, and the mutations under study are reversions to Lac^{+}, with independent revertants being scored as papillae on the surface of the colonies (MILLER 1992). In the first, the target reversion is the precise excision of a transposition-defective Kan^{r} element inserted in the *lacZ* gene; conditional lethality of Lac^{+} revertants (on Kan-containing medium) was achieved with the presence of a Kan^{r}(Ts) allele at another site so that the Lac^{+} revertants are Kan^{S} at 42° and Kan^{r} at 30° (while the Lac^{-} population is Kan^{r} at both temperatures). In the second example, we have obtained a *galE*(Ts) mutation to study the stationary-phase reversion of a variety of Lac^{-} mutations (including point mutations) to Lac^{+}; it is known that Lac^{+} *galE*, but not Lac^{-} *galE*, strains are killed upon

exposure to lactose (because of the generation by hydrolysis from lactose of galactose in the former, to which *galE* mutants are sensitive; BECKWITH 1978), and the temperature-sensitive *galE* allele has then enabled us to adapt this property to the conditional-lethal strategy outlined above.

Isolation of Kan^r(Ts) mutant to study stationary-phase excision of *lacZ*::Kan^r: A Kan^r(Ts) mutation was identified following phage P1-mediated localized mutagenesis of a Tn*10*dKan element, as described in MATERIALS AND METHODS. The data shown in Figure 1A indicate that a strain bearing the Kan^r(Ts) insertion is rapidly killed upon addition of Kan to a culture growing in LB at 42° or upon temperature shift to 42° of a culture growing in LB-Kan at 30°.

In furtherance of the strategy outlined above, we constructed a strain carrying the Kan^r(Ts) insertion as well as a second *lacZ*::Tn*10*dKan insertion. We then performed the following reconstitution experiment in broth in order to simulate the papillation assay conditions that we wished to use with this strain. A population of Δ*lac* Kan^r cells was grown to stationary phase in LB-lactose Kan medium (so that lactose alone remained as the available carbon source), and the population was then seeded with a small proportion of presumptive Lac$^+$ revertant cells of the Kan^r(Ts) strain. Viable count measurements as a function of the time of incubation at 42° confirmed that the Lac$^+$ "revertants" were indeed eliminated rapidly from the population (Figure 1B).

On Kan-containing Lac$^+$ papillation medium, the frequency of Lac$^+$ papillae in colonies of the Kan^r(Ts) *LacZ*::Kan^r strain GJ1823 at 42° was <1% that at 30° and also <1% that at 42° itself in the absence of Kan (data not shown). These values provide an estimate of the relative proportion of cells in the population that bear a Kan^r(Ts) to Kan^r reversion, by either mutation or gene conversion, or a duplication of *lacZ*::Kan^r.

Use of *tex* mutations to demarcate zones of dividing and nondividing cells in colonies: The kinetics of growth of colonies on a solid surface have been described earlier (PIRT 1967; COOPER *et al.* 1968; WIMPENNY 1979). Unlike those in broth, cells in a colony do not exhibit a homogeneous stationary phase; instead, the central zone is comprised predominantly of nondividing cells following local exhaustion of nutrients in the substratum, while the periphery represents a zone of actively dividing cells. To quantitate the relative proportions of dividing and nondividing cells in different zones of any colony incubated at 30° (after initial growth at 42°), we used Lac$^+$ papillation itself as a marker, by including in the strain *tex* mutator mutations (LUNDBLAD and KLECKNER 1985), which increase the frequency of *lac*::Kan^r precise excisions during exponential growth. None of the previously described *tex* mutations tested had a sufficiently strong mutator phenotype for our purpose (data not shown), and we therefore isolated new *tex* mutants that exhibited a five- to 12-fold increase in the *lacZ*::Tn*10*dKan excision frequency in log-phase cultures (REDDY and GOWRIS-

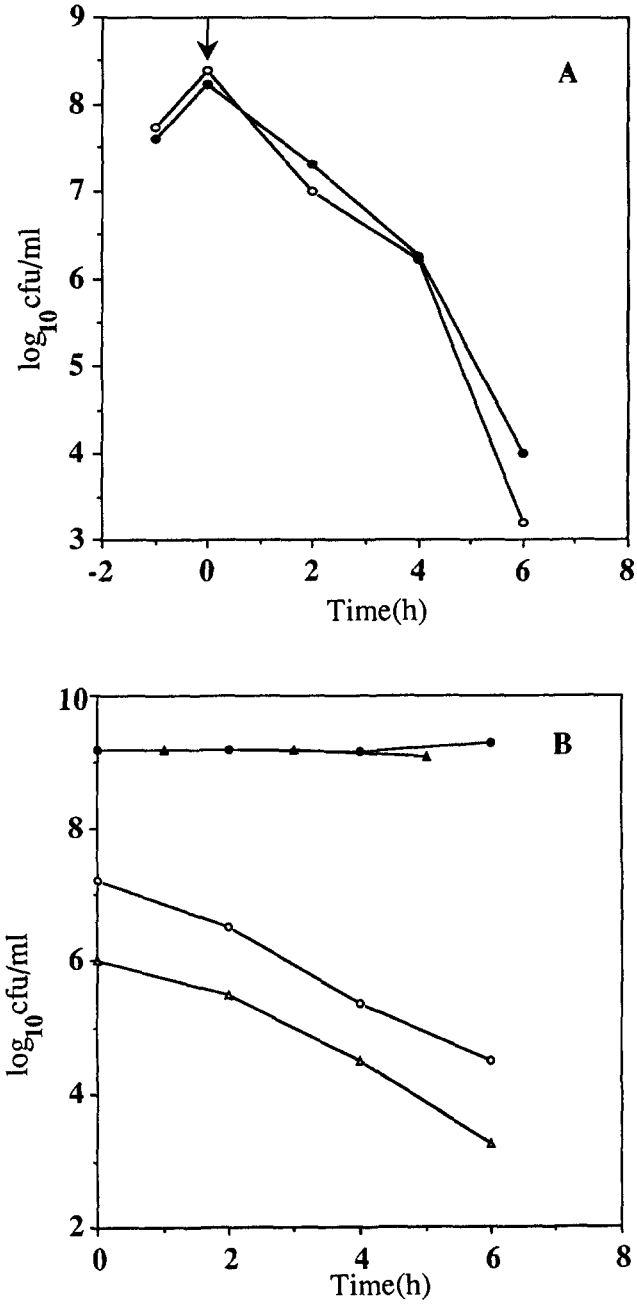

FIGURE 1.—Conditional-lethal phenotype of Kan^r(Ts) mutant. (A) Viable count [colony forming units (cfu)/ml] of a Kan^r(Ts) strain (GJ1826L$^+$) as a function of time after addition of Kan to a culture growing in LB at 42° (○) or shift from 30° to 42° of a culture growing in LB-Kan (●). The time of intervention in each instance is marked by the arrow. (B) Viable count of GJ1826L$^+$ [lac$^+$ Kan^r(Ts) Tet^r Str^s] determined on LB-Tet plates as a function of time of incubation at 42° after its addition, in 1:100 (○) or 1:1000 (△) ratio, to a culture of GJ1242 (Δ*lac* Kan^r Str^r Tet^s) grown to stationary phase in LB-lactose Kan medium. Viable count values for GJ1242 in the two mixtures, determined on LB-Str plates, are indicated by ● and ▲, respectively.

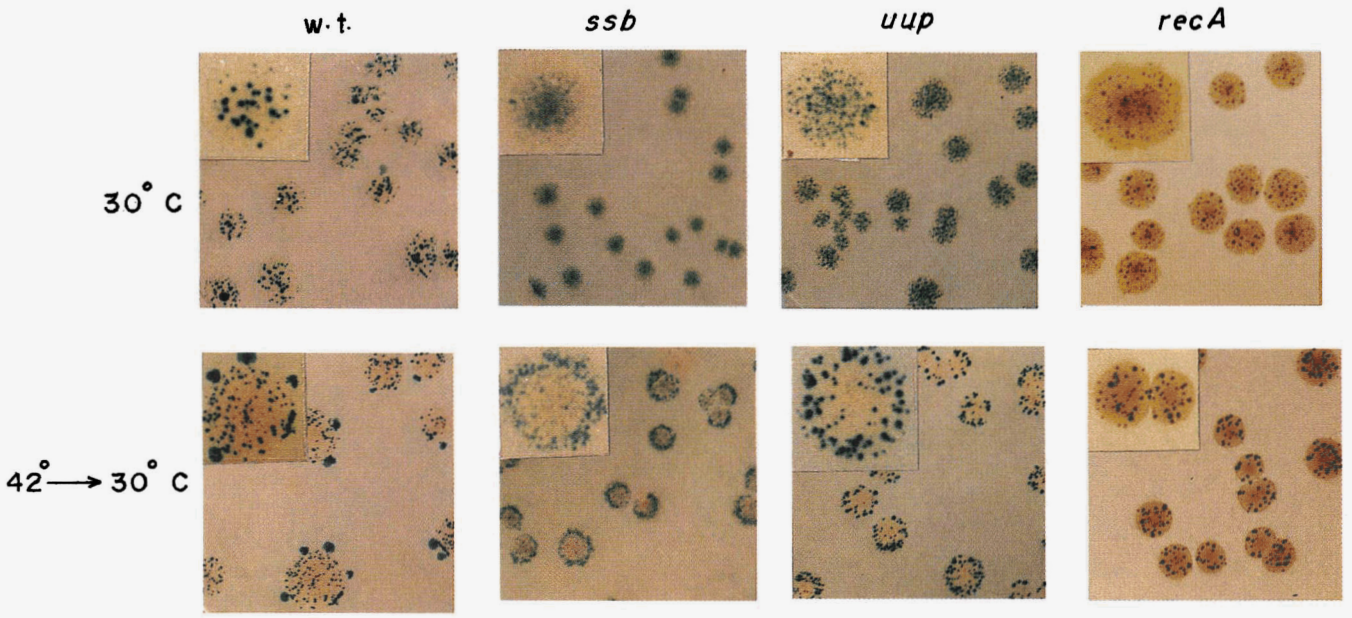

FIGURE 2.—Lac$^+$ papillation patterns in the Kan^r(Ts) *lacZ*::Kan^r strain GJ1823 (wild-type, w.t.) and its *ssb-200* (*ssb*), *uup-351*::Tn*10*dTet (*uup*) or *recA56* (*recA*) derivatives. Strains were plated on Lac$^+$ papillation medium and incubated either continuously at 30° (top row) or for 2–3 days at 42° followed by 5–7 days at 30° (bottom row). Inset for each panel shows the papillation pattern at higher magnification.

HANKAR 1997). One of them (*ssb-200*) is in the gene encoding single-stranded DNA binding protein and creates a glycine-to-aspartate substitution at position 4 in the SSB protein, and another is an insertion mutation in the *uup* gene (*uup-351*::Tn*10*dTet).

Demonstration of *lacZ*::Kan^r excisions in nondividing cells: Lac$^+$ papillation patterns in colonies of the Kan^r(Ts) *lacZ*::Kan^r strain with or without the *ssb*/*uup* mutations were examined after continuous incubation at 30° or after temperature downshift from 42° to 30° (Figure 2). As expected, papillae in colonies of the *tex* strains continuously grown at 30° occurred in much larger numbers than in the *tex*$^+$ counterpart, and they were distributed uniformly over the colonies. This pattern was altered to that of an annulus or ring in the mutator colonies that were grown initially at 42° and then shifted to 30°. This pattern change itself provides a visual validation of the conditional-lethal phenotype in the Lac$^+$ revertants (with consequent loss of papillae in the central zones of colonies on the temperature-shifted plates).

The ratio of the number of papillae in the peripheral zone to that in the central zone of the temperature-shifted colonies for each strain was calculated as described in MATERIALS AND METHODS, and the values are given in Table 2. If one assumes that the vast majority of papillae in the *tex* strains represent mutations that have occurred in growing cells, the distribution ratios for these strains suggest that at least 80% of all cell divisions in colonies following the temperature shift have occurred in the peripheral zone. This conclusion is in conformity with the results from earlier studies on colony growth kinetics (PIRT 1967; WIMPENNY 1979).

If one further assumes that all *lacZ*::Kan^r precise excisions even in the *tex*$^+$ parent occur only in dividing cells, one expects that the ratio of distribution of papillae would be similar for both *tex*$^+$ and *tex* isogenic strains, although the number of papillae would be much higher in the latter. However, as shown in Figure 2, colonies of the *tex*$^+$ strain that were incubated first at 42° and then at 30° did not exhibit an annular distribution pattern of papillae, and the results in Table 2 indicate that the distribution ratio of peripheral to central zone papillation for this strain was only about one-sixth that for the *ssb* strain. Control experiments (with colonies grown on medium without lactose supplementation) indicated that there was no difference in viability of the stationary-phase populations between the *tex*$^+$ and *tex* mutant strains that could possibly have accounted for the difference in the distribution of papillae on the temperature-shifted plates (data not shown).

Thus, as much as 85% of the central zone papillation in the *tex*$^+$ colonies may represent an excess over the numbers expected from the excision frequency for dividing cells and from the number of dividing cells in this zone. We interpret this excess to signify the occurrence of excision events in nondividing cells of the central zone; at the very least, they represent reversions that are blind to the mutator effects of two different *tex* gene mutations. Introduction of the *recA56*(Def) mutation did not alter the papillation frequency or pattern in either the *tex*$^+$ strain (Figure 2; Table 2) or the *ssb-*

TABLE 2

Ratio of papillae in peripheral zone to central zone for temperature-shifted colonies of *lacZ*::Kan^r strains

Mutation	Conditional-lethal strategy	
	Kan^r(Ts)	*galE*(Ts)
Nil (wild type)	1.7	1.3
ssb-200	9.0	7.5
uup-351	4.8	ND[a]
recA56	1.3	ND
ssb-200 recA56[b]	8.2	ND

Strains (other than the *ssb-200 recA56* double mutant) are those described in the legends to Figure 2 [Kan^r(Ts)] and Figure 4 [*galE*(Ts)]. Ratios were calculated as described in MATERIALS AND METHODS.

[a] ND, not determined.

[b] The *ssb-200 recA56* double mutant was constructed by transduction of the *recA56* allele, with the aid of a *zgb-905*::Tn*10*dCm insertion 60% linked to the locus (M. REDDY, unpublished results), into the *ssb-200 zjc-904*::Tn*10*dTet derivative of GJ1823.

200 derivative (Table 2). In all cases examined, >99% of papillae purified from the temperature-shifted plates were Kan^r(Ts) (data not shown), indicating that they had arisen at 30°.

In the arguments above, we have not presumed that all excisions in the two *tex* strains are growth associated. We have only assumed that these strains exhibit a substantial increase, compared to *tex*$^+$, in the log-phase excision frequency without alteration in the frequency (if any) of excisions in nondividing cells.

Obtaining a *galE*(Ts) mutation and its use in combination with galP and *mgl* mutations to study stationary-phase mutations: In the second approach for application of the conditional-lethal strategy, we obtained a *galE*(Ts) mutant (see MATERIALS AND METHODS). The data in Figure 3 demonstrate that the *galE*(Ts) mutation was effective in mediating galactose-induced lysis at 42°. This mutation was then used in the papillation studies below, but the following additional manipulations (as described in MATERIALS AND METHODS) were necessary for the purpose:

1. The *galE*(Ts) mutation was cloned as part of the entire *galETK* operon on a multicopy plasmid pHYD603, so as to provide sufficient GalE activity at 30° for Lac$^+$ papillae to be visualized within a reasonable period.
2. We found that even the Lac$^-$ *galE*(Ts) cells in colonies grown on lactose-supplemented medium were killed at 42° by galactose released following lysis of Lac$^+$ mutants in the population (*i.e.*, a bystander effect). To circumvent this problem, we introduced mutations into the genes *galP* and *mgl*, which encode the two known galactose transporters in *E. coli*, so that the strains were rendered cell-autonomous for galactose-mediated lysis; that is, the strains were now resistant to exogenous galactose but remained sensitive to endogenously released galactose from lactose hydrolysis.

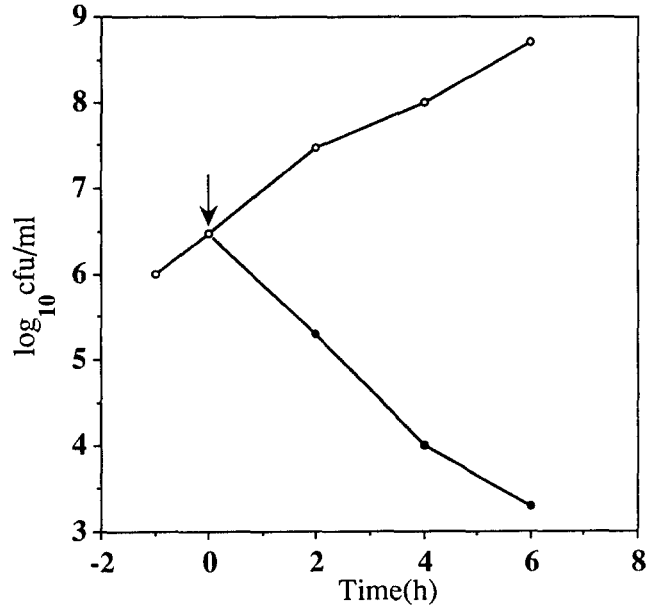

FIGURE 3.—Conditional-lethal phenotype of *galE516*(Ts) mutant. The strain used was GJ2203/pHYD603, which carries the *galE*(Ts) mutation both on the chromosome and on a multicopy plasmid bearing the *galETK* operon. Viable counts (cfu/ml) were determined as a function of time after addition of 0.1% D-galactose to a culture of the strain growing in minimal A-Casamino acids at 30° and that was then immediately split into two halves: one-half continuing to be incubated at 30° (○) and the other shifted to 42° (●). The time of intervention is marked by the arrow.

Although Lac$^+$ papillation efficiency in *galE*(Ts) strains was still only 50% of that with the Kan^r(Ts) approach, we were now able to extend our studies to the analysis of reversions of *lac* frameshift and base-substitution mutations.

Lac$^+$ papillation patterns in *galE*(Ts) derivatives: Derivatives of *galE*(Ts) strains were constructed carrying one of the following three mutations: the *lacZ*::Tn*10*d-Kan insertion, the *lacI33*::*lacZ* frameshift mutation or an amber mutation at codon 17 of *lacZ*. Strains bearing the lacZ(Am) allele can revert to Lac$^+$ by base changes either at codon 17 itself or at amber suppressor loci that together represent all the six possible transitions and transversions (MILLER 1992).

Colonies of these strains were examined for their Lac$^+$ papillation patterns either after continuous growth at 30° or following temperature-shift from 42° to 30° (Figures 4–6). We also compared the effects on papillation of (1) the location of the point mutations (on the chromosome *vs.* on an F′); (2) mutations in mutator loci [the *ssb-200* mutation for chromosomal *lacZ*::Kan^r, *mutS* for *lacI33*::*lacZ* and both *mutS* and *mutY*

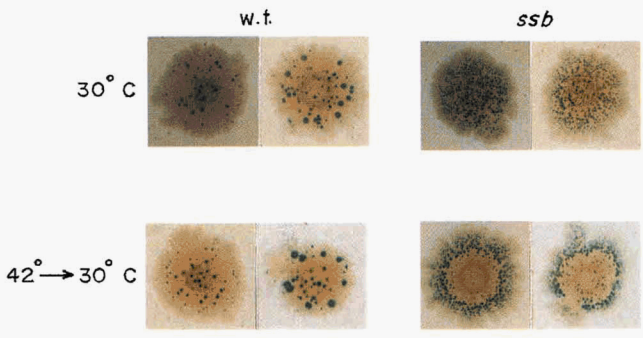

FIGURE 4.—Lac$^+$ papillation patterns in the *galE*(Ts) *lacZ*::Kan^r strain (w.t.) and its *ssb-200* (*ssb*) derivative. Strains were spotted on Lac$^+$ papillation medium and incubated as described in the legend to Figure 3. Both strains carried the multicopy *galE*(Ts) plasmid pHYD603.

for *lacZ*(Am)]; (3) the *recA56* mutation; and (4) a *traD* mutation (for strains bearing the *lac* point mutations on the F′). The following conclusions could be drawn from the Lac$^+$ papillation patterns in the *galE*(Ts) strains.

1. An annular papillation pattern was observed following temperature shift for the *ssb lacZ*::Kan^r strain (Figure 4) as well as for the *mutY* derivatives of *lacZ*(Am) [on both F′ (Figure 5) and chromosome (Figure 6)]. The calculated ratios of peripheral to central zone papillae were also high for these colonies (Tables 2 and 3). These results confirm the efficacy of the *galE*(Ts) mutation in eliminating Lac$^+$ revertants at the restrictive temperature.
2. The observations made earlier for wild-type chromosomal *lacZ*::Kan^r excision [in the Kan^r(Ts) strain] were essentially reproduced also in the *galE*(Ts) derivative (Figure 4 and Table 2), thereby reinforcing the conclusion that such excisions are able to occur in nondividing cells.
3. The F′ derivative (without mutator mutation) carrying *lacI33::lacZ* yielded very large numbers of papillae on continuous incubation at 30° and marked central-zone papillation on the temperature-shifted plates (Figure 5). The Lac$^+$ papillation pattern for the episomal *lacZ*(Am) mutation also resembled that for the frameshift allele, but the absolute numbers of papillae were smaller (Figure 5). In both cases, the calculated ratios of peripheral to central zone papillae in the temperature-shifted colonies (Table 3) were low and similar to the values reported in Table 2 for chromosomal *lacZ*::Kan^r excision, indicative therefore of reversions occurring in nondividing cells.
4. In their chromosomal location, the frameshift and the amber *lac* mutations reverted to Lac$^+$ at a low frequency, but examination of sufficient numbers of temperature-shifted colonies clearly established the occurrence of central-zone papillation (Figure 6), and an associated low zonal disribution ratio of papillae (Table 3), for both of them.
5. Mutations in *recA* or *traD* were associated with a considerable decrease in reversion frequencies for either point mutation on the F′ (Figure 5). On the other hand, the *recA* mutation did not significantly affect the chromosomal reversion frequencies (data not shown).
6. Unlike *mutY* and *ssb*, which gave annular papillation on the temperature-shifted plates of *lacZ*(Am) and

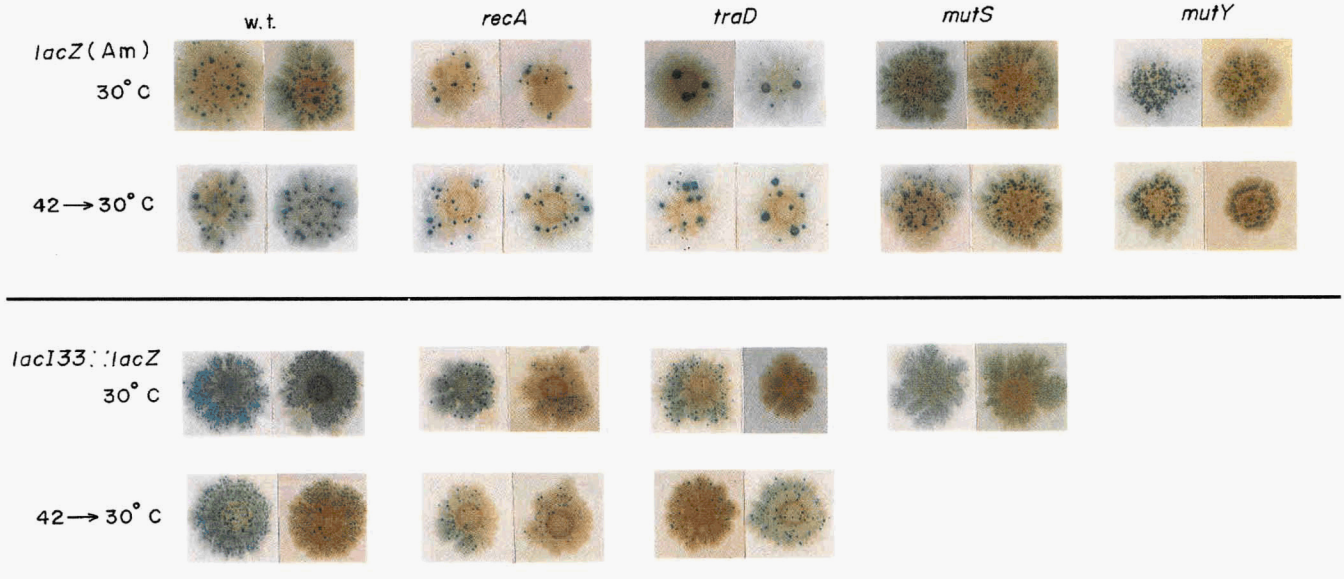

FIGURE 5.—Lac$^+$ papillation in *galE*(Ts) strains carrying *lac* point mutations on F′. Strains GJ2207 and GJ2214 [bearing, respectively, the *lacZ*(Am) and *lacI33::lacZ* frameshift mutations on F′*lac pro*, w.t.] and their *recA56* (*recA*), *traD36* (*traD*), *mutS*::Tn*10*dTet (*mutS*) or *mutY*::Tn*10*dTet (*mutY*) derivatives were spotted on Lac$^+$ papillation medium and incubated as described in the legend to Figure 3. All strain derivatives carried the multicopy *galE*(Ts) plasmid pHYD603.

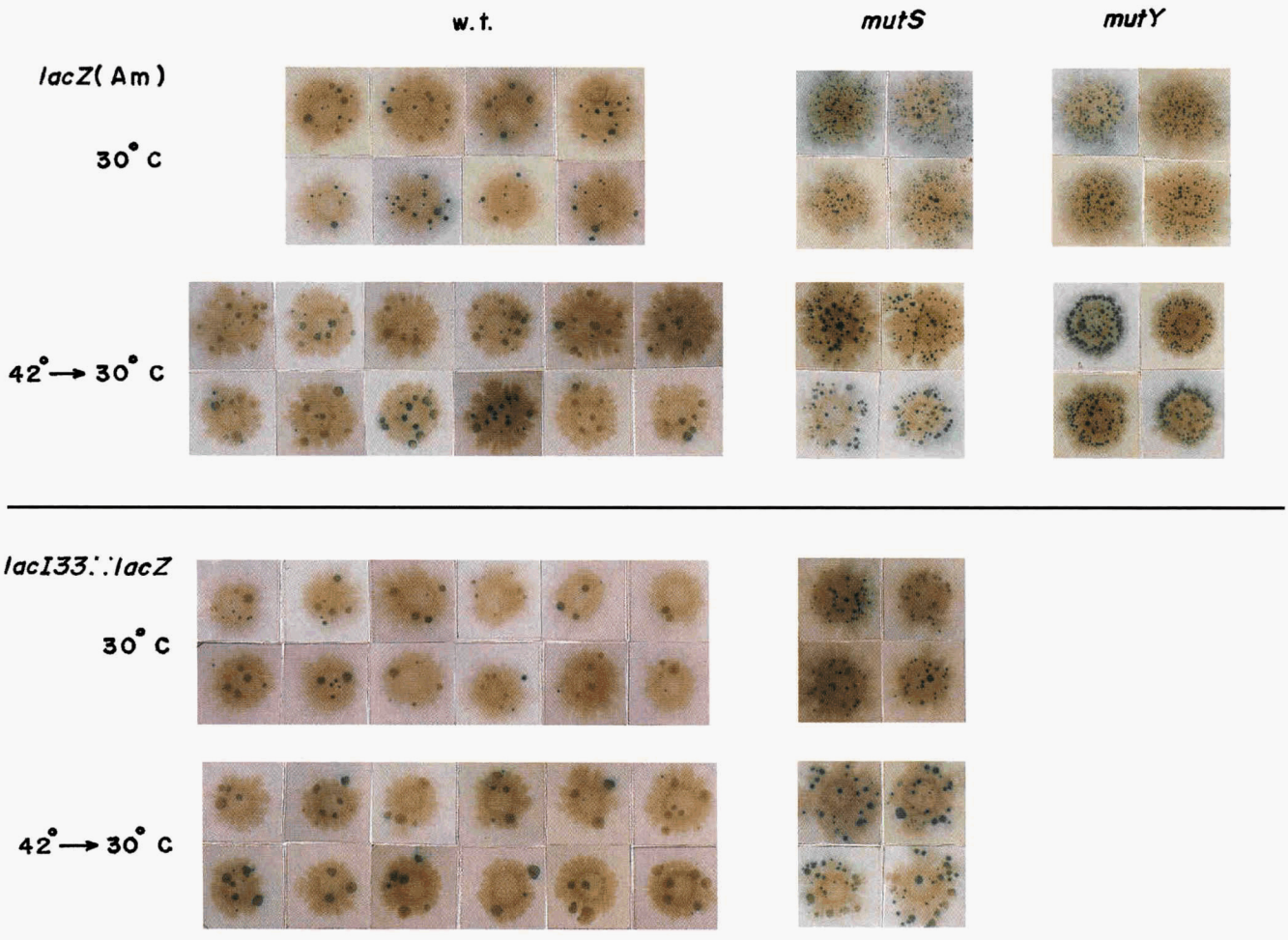

FIGURE 6.—Lac$^+$ papillation in *galE*(Ts) strains carrying *lac* point mutations on chromosome. Strains GJ2231 and GJ2235 [bearing, respectively, the *lacZ*(Am) and *lacI33*::*lacZ* frameshift mutations on the chromosome; w.t.] and their *mutS*::Tn*10*dTet (*mutS*) or *mutY*::Tn*10*dTet (*mutY*) derivatives were spotted on Lac$^+$ papillation medium and incubated as described in the legend to Figure 3. All strain derivatives carried the multicopy *galE*(Ts) plasmid pHYD603.

lacZ::Kan^r derivatives, respectively, the *mutS* mutation gave increased central-zone papillation for both the *lacZ*(Am) and *lacI33*::*lacZ* targets after temperature-shift (Figures 5 and 6). This was reflected also in the low ratio of peripheral to central zone papillae in the temperature-shifted *mutS* colonies (Table 3). The data therefore suggest that the reversion frequency for each of these mutations in nondividing cells may be elevated in the absence of methyl-directed mismatch repair.

The *galE*(Ts) strain carrying both the *lacI33*::*lacZ* mutation on the F′ and the mutator *mutS* allele grew very poorly on lactose-supplemented plates at 42°. Our interpretation is that under these conditions the frequency of reversion to Lac$^+$ (and consequent release of galactose by lysis) during growth at 42° is so high that bystander killing occurs despite the presence of *galP* and *mgl* mutations. Thus, the effect of the mutator mutation may be additive to the F′ position effect on increase in the reversion frequency of the frameshift *lac* allele.

DISCUSSION

Chromosomal and F′ mutations occur in nondividing cells: We have used a conditional-lethal strategy for elimination of log-phase reversions in a population, in combination with a means to quantitate the relative distribution of dividing cells in different zones of a colony at the permissive temperature, to demonstrate that reversion to Lac$^+$ of a chromosomal *lac*::Kan^r insertion and of two other *lac* mutations both on the chromosome and on an F′ can occur in nondividing cells. Our argument for their occurrence is based on spatial patterns of Lac$^+$ papillation in colonies and is primarily statistical.

We emphasize that our observations support the idea that the mutations above have arisen in *nondividing* cells and not merely in *stationary-phase* cells, on the ground that the central-zone reversions are blind to the effects of several mutator mutations that increase the reversion frequency at the target loci during exponential growth. The distinction may be important, because stationary-

TABLE 3

Ratio of papillae in peripheral zone to central zone for temperature-shifted colonies of strains carrying *lacZ*(Am) or *lacI33*::*lacZ* frameshift mutations[a]

Mutation	*lacZ*(Am) on		*lacI33*::*lacZ* on	
	F′	Chromosome	F′	Chromosome
Nil (wild type)	1.2	1.6	1.2	1.8
mutS::Tn*10*dTet	1.9	1.4	ND[b]	1.5
mutY::Tn*10*dTet	4.0	3.6	ND	ND

[a] Strains are those described in the legends to Figure 5 [*lacZ*(Am) and *lacI33*::*lacZ* mutations on F′ *lac pro*] and Figure 6 (same mutations on chromosome). Ratios were calculated as described in MATERIALS AND METHODS.

[b] ND, not determined.

phase broth cultures have been shown to exhibit considerable cell turnover, with "killer" mutants growing at the expense of the rest of the population (ZAMBRANO *et al.* 1993; ZAMBRANO and KOLTER 1996).

Deletions of the transposon prophage Mu (reviewed in SNIEGOWSKI and LENSKI 1995) and transposition of IS*30* (NAAS *et al.* 1994) are known to be stimulated in stationary-phase cells. The Tn*10*dKan element used in our work is also derived from a transposon, but there are important mechanistic differences between the former set of phenomena and precise excision of the latter. Tn*10*dKan is incapable of autonomous transposition, and precise excision is entirely dependent on host-encoded functions (EGNER and BERG 1981; FOSTER *et al.* 1981); on the other hand, the former events are transposition related (SHAPIRO 1993; NAAS *et al.* 1994). As shown for Tn*10*dKan in this study, precise excisions of Tn*3* and IS*150* have also been reported to be stimulated in the stationary phase (BOE 1990; MITTLER and LENSKI 1992).

A common mechanism for mutations in nondividing cells: Of the various instances examined, we found that the frequency of stationary-phase reversion to Lac$^+$ was most pronounced for the chromosomal *lacZ*::Kan^r and for the F′ frameshift *lac* mutations. Based on the lines of reasoning below, we suggest that these two events represent particularly sensitive assay systems for the phenomena of "replication slippage" (STREISINGER *et al.* 1966) and methyl-directed mismatch repair.

Our finding that the reversion in nondividing cells of the F′ *lacI33*::*lacZ* frameshift allele is both RecA- and Tra-dependent establishes considerable parallels between this study and those cited in the Introduction on "adaptive" reversion of this mutation. On minimal lactose selection plates at the permissive temperature, our strain also displayed a progressive time-dependent increase in the yield of Lac$^+$ mutants (data not shown), exactly as described in the earlier studies. The spectrum of mutations generated under these conditions is similar to that expected from replication slippage in cells deficient in mismatch repair (ROSENBERG *et al.* 1995).

Similarly, precise excision of chromosomal *lacZ*::Kan^r is representative of several other mutations that have been postulated to occur by replication slippage between short direct repeats. These include nearly precise excision of Tn*10* and its derivatives (FOSTER *et al.* 1981; D'ALENCON *et al.* 1994), spontaneous deletions between microhomologies (ALBERTINI *et al.* 1982; CHEDIN *et al.* 1994) and instability of dinucleotide and trinucleotide repeats in eukaryotes (CASKEY *et al.* 1992; DE LA CHAPELLE and PELTOMAKI 1995). The frequency of their occurrence is often greatly increased by the presence of inverted repeats immediately internal to the directly repeated segments (EGNER and BERG 1981; FOSTER *et al.* 1981; TRINH and SINDEN 1993; CANCEILL and EHRLICH 1996), and this frequency is also sensitive to methyl-directed mismatch repair (LUNDBLAD and KLECKNER 1985; LOVETT and FESCHENKO 1996).

We therefore conclude that the occurrence of mutations in nondividing cells is most prominent in two entirely different instances, which have in common the property of being sensitive to replication slippage errors and/or correction by mismatch repair. It is likely that the reversion of the chromosomal *lac* frameshift and amber mutations occurs through the same mechanism(s), albeit at a lower frequency.

Our data also suggest that a mutation in *mutS* increases central-zone papillation on the temperature-shifted plates for both *lacI33*::*lacZ* and *lacZ*(Am) derivatives. This finding is consistent with earlier work (STAHL 1988; BOE 1990; FOSTER and CAIRNS 1992; JAYARAMAN 1992), and supports our hypothesis that methyl-directed mismatch repair is an important error-correction mechanism in nondividing cells. Nevertheless, the same observation leads one to the inference that the postulated deficiency of mismatch repair in wild-type cells in stationary phase (FOSTER *et al.* 1995; LONGERICH *et al.* 1995; FENG *et al.* 1996) may not be absolute.

Are mutations in nondividing cells "adaptive"? In the present experiments, lactose (the selective agent) was always present in the medium. Consequently, we have not directly addressed the question whether the mutations that occur in nondividing cells are adaptive, that is, whether they are "directed" by the conditions of selection. Our study differs also from those earlier on adaptive mutation in that we have not examined the temporal course of occurrence of Lac$^+$ papillae in the temperature-shifted colonies. Recent sugestions that variant sequences are generated randomly during the process of DNA turnover in nondividing cells and that selection operates to fix the "beneficial" variant as a mutant, appear to provide a reasonable basis for explaining our results, without invoking the controversies associated with the directed mutation hypothesis

(STAHL 1988; FOSTER 1993; ROSENBERG *et al.* 1995; MACPHEE and AMBROSE 1996; BRIDGES 1997).

Our strategy will now also permit the screening for mutants with altered central-zone papillation, and thus will facilitate a genetic dissection of mechanisms that underlie spontaneous mutagenesis in nondividing cells.

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