Distinct Signatures for Mutator Sensitivity of *lacZ* Reversions and for the Spectrum of *lacI/lacO* Forward Mutations on the Chromosome of Nondividing *Escherichia coli*

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ABSTRACT

A conditional lethal *galE*(Ts)-based strategy was employed in *Escherichia coli*, first to eliminate all growth-associated chromosomal reversions in *lacZ* or forward mutations in *lacI/lacO* by incubation at the restrictive temperature and subsequently to recover (as papillae) spontaneous mutations that had arisen in the population of nondividing cells after shift to the permissive temperature. Data from *lacZ* reversion studies in mutator strains indicated that the products of all genes for mismatch repair (*mutHLS, dam, uvrD*), of some for oxidative damage repair (*mutMT*), and of that for polymerase proofreading (*dnaQ*) are required in dividing cells; some others for oxidative damage repair (*mutY, nth nei*) are required in both dividing and nondividing cells; and those for alkylation damage repair (*ada ogt*) are required in nondividing cells. The spectrum of *lacI/lacO* mutations in nondividing cells was distinguished both by lower frequencies of deletions and IS*1* insertions and by the unique occurrence of GC-to-AT transitions at *lacO* +5. In the second approach to study mutations that had occurred in nondividing cells, *lacI/lacO* mutants were selected as late-arising papillae from the lawn of a *galE*$^+$ strain; once again, transitions at *lacO* +5 were detected among the mutants that had been obtained from populations initially grown on poor carbon sources such as acetate, palmitate, or succinate. Our results indicate that the *lacO* +5 site is mutable only in nondividing cells, one possible mechanism for which might be that random endogenous alkylation (or oxidative) damage to DNA in these cells is efficiently corrected by the Ada Ogt (or Nth Nei) repair enzymes at most sites but not at *lacO* +5. Furthermore, the late-arising papillae from the second approach were composed almost exclusively of dominant *lacI/lacO* mutants. This finding lends support to "instantaneous gratification" models in which a spontaneous lesion, occurring at a random site in DNA of a nondividing cell, is most likely to be fixed as a mutation if it allows the cell to immediately exit the nondividing state.

SPONTANEOUS mutations are believed to arise randomly in dividing cells in a two-step process in which an incorrect nucleotide that is first incorporated in one DNA strand during replication then templates the incorporation of its complement in the other. Mutational avoidance mechanisms have accordingly evolved either (i) to reduce the probability of occurrence of the first step or (ii) to correct the mismatch before it is fixed as a mutation by the second one (Maki 2002). In *Escherichia coli*, for example, the first step probability is modulated by *dnaQ* encoding the proofreading component of DNA polymerase III, as well as by the products of genes such as *mutT, mutM, ung, mug, nth, nei, ada*, and *ogt*, which act to remove nucleotides that are damaged (by oxidation, deamination, or alkylation) and that have an increased miscoding potential. The products of genes such as *mutS, mutH, mutL, dam*, and *uvrD* (of the postreplication mismatch repair pathway) act immediately after the first step to remove misincorporated nucleotides, and the *mutY* gene product similarly acts to remove a miscoded A opposite an oxidatively damaged G nucleotide. The need and the efficiency of each of these error-avoidance and error-correction mechanisms is demonstrated by the magnitude of the mutator phenotypes exhibited by the appropriate mutant strains (reviewed in Drake 1991; Echols and Goodman 1991; Miller 1992, 1996; Friedberg *et al.* 1995; Hutchinson 1996; Rupp 1996; Maki 2002).

Spontaneous mutations have also been described in nondividing or very slowly dividing cells (that is, in the absence of overt DNA replication), but the subject is more controversial. Typically, the mutants have been identified as late-arising colonies or papillae following a nonlethal selection (for growth on a carbon source or for reversion of an auxotrophy), and for this reason they have also been called adaptive mutations (Bridges 1998; Foster 1999; Rosenberg 2001). The best-studied example is the reversion to Lac$^+$ of a frameshift *lacI-Z33* mutation on F′128 in strain FC40. In brief, the results obtained so far indicate that the high frequency

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of adaptive Lac$^+$ reversions in FC40 is a unique phenomenon that is dependent on RecA and other SOS- and homologous recombination-related reactions, as well as on the episomal location of *lacI-Z33* and on F′-related conjugational transfer functions (Foster 1999; Godoy and Fox 2000; Rosenberg 2001). Adaptive mutation in the episomal strain is also apparently associated with large arrays of tandemly amplified copies of the *lac* genes (Andersson *et al.* 1998; Hendrickson *et al.* 2002) and with an increased occurrence of mutations at unselected loci on both F and the chromosome (Foster 1999; Bridges 2001; Rosenberg 2001). Two distinct classes of models have been proposed to explain this phenomenon: (i) the amplification-mutagenesis model, which invokes *lac* amplification as an essential intermediate in the pathway to adaptive mutagenesis, with SOS induction and generalized mutagenesis being unavoidable side effects of the process (Andersson *et al.* 1998; Hendrickson *et al.* 2002; Slechta *et al.* 2002); and (ii) the hypermutable state models, in which SOS-induced mutagenesis, and not amplification, is causally related to adaptive reversion (Rosenberg 2001).

Far fewer studies have been done on adaptive mutations in chromosomal genes, and even so diverse targets and assay procedures have been employed (although most of them have scored for reversions, that is, for dominant mutations; Foster 1999; Godoy *et al.* 2000; Slechta *et al.* 2002). Some studies have also employed lethal selection (*e.g.*, for rifampicin resistance) on cells from aging colonies as a means to obtain mutations in presumptively nondividing cells (Taddei *et al.* 1995, 1997; Bjedov *et al.* 2003).

We described earlier a novel strategy for obtaining *lacZ* reversions in nondividing cells of *E. coli*, to the exclusion of those that occur in dividing cells (Reddy and Gowrishankar 1997a). The *lacZ* parental strain also carried a *galE*(Ts) mutation and was grown at 42° on lactose-supplemented plates so that the Lac$^+$ revertants arising during growth (but not the Lac$^-$ parental cells) were killed by intracellular galactose released from lactose hydrolysis. The population of Lac$^-$ cells in stationary phase was then shifted to 30° so that any new Lac$^+$ revertants arising in it would survive and be identified as papillae. With this approach, we showed that a variety of mutations (including an insertion) in chromosomal *lacZ* can revert to Lac$^+$ in the central zones of nondividing cells in colonies growing on a solid medium.

In the present study, we have adopted a modified version of the method above to characterize, in nondividing cells, the mutator sensitivity of spontaneous reversions of a chromosomal *lacZ*(Am) allele. Our results indicate that alkylation- and some oxidative-damage repair functions, but not the functions of methyl-directed mismatch repair or SOS repair, are important for genome defense in nondividing cells. We have also examined a forward mutational target, namely the *lacI/lacO* repressor/operator genes, in nondividing cells and have found that the corresponding mutational spectrum is quite distinct from that in dividing cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media: *E. coli* K-12 strains and their genotypes are described in Table 1 and in the legends to Figures 1 and 2. Routine growth media were Luria-Bertani (LB) and minimal A (MM) with 0.2% glucose (Glc), as described (Miller 1992). Plates that were incubated for extended lengths of time were enclosed in a humidified box, and the medium in them was supplemented with nystatin at 20 μg/ml to inhibit the growth of fungal contaminants. Concentrations of other antibiotics used were as described earlier (Reddy and Gowrishankar 1997a,b).

Genetic and DNA methods: The viable count of bacteria in a lawn was estimated from the number of colonies obtained (at appropriate dilutions) from the suspension of an agar plug removed from the lawn with the aid of a 1-ml micropipette tip. Transfer of mutations between strains was by P1 transduction (Gowrishankar 1985). Dominance or recessivity of chromosomal *lacI/lacO* mutations was tested by introducing an F′128 derivative (carrying *lacI*$^+$ *lacO*$^+$ and *lacZ*::Tn*10*dKan as a selective marker) from donor strain GJ2479 into the mutants by conjugation (Miller 1992) and scoring for, respectively, blue or white colony phenotypes on Glc-MM supplemented with 25 μg/ml Xgal. Mutagenesis with nitrosoguanidine was performed as described (Miller 1992). Standard protocols were followed for experiments involving recombinant DNA (Sambrook *et al.* 1989).

***galE*(Ts)-based papillation method for *lacZ* reversions in nondividing cells:** In the method previously described (Reddy and Gowrishankar 1997a), colonies of the *galE*(Ts) *lacZ* parental strain had been grown on the Lac$^+$ papillation medium at 42° and then shifted to 30°, and papillae arising in the central zones of the colonies following the temperature shift were taken to represent reversions that had occurred in nondividing cells. The parental strain had also carried (i) mutations in the galactose-transporter loci *mgl* and *galP*, to preclude bystander killing of Lac$^-$ cells following release of galactose by the lysis at 42° of Lac$^+$ revertants in their vicinity; and (ii) a multicopy Amp^r plasmid carrying the *galE*(Ts) gene, to provide sufficient GalE function at the permissive temperature for papillary growth of the Lac$^+$ revertants.

Three modifications of this method were adopted in the present study: (i) the parental strain had only the chromosomal *galE*(Ts) allele and did not carry the multicopy-Amp^r plasmid with *galE*(Ts); (ii) ~10^7 cells of the parental *lacZ galE*(Ts) *galP mgl* strain or its mutator derivatives were plated in a soft agar overlay on Lac$^+$-papillation medium [MM supplemented with 0.5% Casamino acids (CAA), 0.1% lactose, and nystatin] in a 35-mm petri plate and incubated at the restrictive temperature (42°) for 72 hr, during which time the parental strain had grown as a lawn to stationary phase and all growth-associated Lac$^+$ revertants in the population were eliminated; after which (iii) the plate was shifted to the permissive temperature of 28° (rather than 30°) at which temperature the haploid *galE*(Ts) allele conferred sufficient GalE activity for growth of the Lac$^+$ papillae. After 7–10 days at 28°, 100 μg of Xgal was added in a soft agar overlay for visualization of Lac$^+$ papillae. As controls, Lac$^+$ papillae were also visualized on plates that had been continuously incubated at 28°.

With the modified method, the need to demarcate central and peripheral zones of nondividing and dividing cells, respectively, for each colony was avoided, since the entire lawn on the temperature-shifted plate could now be treated as a population of nondividing cells; furthermore we had observed that

TABLE 1

List of *E. coli* K-12 strains

Strain	Genotype[a]
GJ2218	*ara* Δ(*gpt-lac*)*5 galE516*(Ts) *galP528 mgl-353 zbh-900*::Tn*10*dKan(Ts)*1*
GJ2219	*ara galE516*(Ts) *galP528 mgl-353 zbh-900*::Tn*10*dKan(Ts)*1*
GJ2220	GJ2219 *lacZ4525*::Tn*10*dKan
GJ2222	GJ2220 *ssb-200 zjc-204*::Tn*10*dTet
GJ2224	GJ2220 *uup-351*::Tn*10*dTet
GJ2231	GJ2219 *lacZ*(Am) *lacI3098*::Tn*10*Kan
GJ2232	GJ2231 *recA56 srl*::Tn*10*
GJ2233	GJ2231 *mutS*::Tn*10*dTet
GJ2234	GJ2231 *mutY*::Tn*10*dTet
GJ2238	Same as GJ2231 but with *lacI42*::Tn*10* instead of *lacI3098*::Tn*10*Kan
GJ2321	GJ2231 *mutL*::Tn*10*
GJ2322	GJ2231 *mutM*::Tn*10*dTet
GJ2323	GJ2231 *mutT1 zad-220*::Tn*10*
GJ2324	GJ2231 *mutA cycA*::Tn*10*
GJ2334	GJ2238 *mutH471*::Kan
GJ2335	GJ2238 Δ*uvrD228*::Kan
GJ2336	GJ2238 *dam*::Tn*9*
GJ2348	GJ2238 *ada*::Cm *ogt*::Kan
GJ2351	GJ2238 *nth*::Kan *nei*::Cm
GJ2376	GJ2238 Δ(*lacI42*::Tn*10*) *mutM*::Tn*10*dTet *mutY*::Kan
GJ2377	GJ2231 *dnaQ* (*mutD5*) *zae-502*::Tn*10*
GJ2380	GJ2218/F′128 *lacI-Z33*
GJ2381	GJ2380 *recA56 srl*::Tn*10*
GJ2382	GJ2380 *lexA3 malB*::Tn*9*
GJ2421	*ara galP528 mgl-353 lacI*^{q} *PL8 zai-911*::Tn*10*dCm
GJ2422	GJ2219 *lacI*^{q} *PL8 zai-911*::Tn*10*dCm
GJ2432	GJ2219 *lacI*^{q} *PL8 lacI204 zai*-912::Tn*10*dCm
GJ2474	GJ2432 *galE*$^+$ *Kan*^{S}
GJ2479	*ara thi trpE9777* Δ(*pro-lac*)/F′128 *lacZ4525*::Tn*10*dKan

Strains GJ2218, GJ2219, GJ2220, and GJ2231 have been described previously (Reddy and Gowrishankar 1997a).

[a] Genotype designations are as described in Berlyn (1998).

the earlier method was associated with variable spontaneous loss of the multicopy *galE*(Ts) plasmid from cells in the colonies (and consequently with loss of the ability of Lac$^+$ cells to grow as papillae), presumably because of the loss of selection for the plasmid following enzymatic inactivation of the ampicillin in the medium.

***galE*(Ts)-based papillation method for *lacI/lacO* forward mutations in nondividing cells:** The strategy involved exposure of a population of cells of a *galE*(Ts) *galP mgl* strain with an inducible *lac* operon to phenyl-β-d-galactoside (PG) at 42°, so that all mutants expressing β-galactosidase constitutively (that is, with *lacI/lacO* mutations) were killed by the Gal released from PG hydrolysis. The population was then shifted to 28° to allow for growth as papillae of new *lacI/lacO* mutants that had arisen among the nondividing cells. The parental strain had the mutations *lacI*^{q} (increased *lacI* expression) and *PL8* (reduced *lac* promoter strength), both of which served to reduce the level of leaky background expression of *lacZ* in the absence of inducer (Miller 1992). The *lacI* coding region was either wild type (WT) (*lacI*$^+$) or one (*lacI204*) in which the triple-tetranucleotide hot spot site for frameshift mutation had been destroyed by synonymous codon substitution mutations (Schaaper and Dunn 1991).

An inoculum of ~10^6 cells of the parental strain was grown as a lawn at 28° for 36 hr to stationary phase on MM supplemented with 0.2% CAA, 0.05% Glc, nystatin, and 25 μg/ml Xgal in a 35-mm petri plate (to a final cell density of ~4×10^9 cells), following which PG was added to 0.05% in a soft agar overlay and the plate was shifted to 42° for 72 hr. Reconstitution experiments were undertaken to demonstrate that all PG$^+$ mutants that arose during growth of the lawn would be eliminated during the period of incubation at 42° (data not shown). The plate was then returned to 28° and scored daily over the succeeding 10–15 days for blue PG$^+$ papillae. The *galE*(Ts) derivatives of both the *lacI*$^+$ and *lacI204* parental strains yielded around three PG$^+$ papillae per 10^9 cells on the temperature-shifted plates.

***lacI/lacO* forward mutations in dividing cells:** Independent cultures were initiated, each with an inoculum of ~100 cells, and grown at 28° to an A_{600} of 0.05 in 3 ml of LB or Glc-MM. The cells were washed in MM, plated on 0.1% PG-MM, and incubated at 28° for 48–72 hr. One randomly chosen PG$^+$ mutant from each culture was saved.

Screening for mutations at *lacI* CTGG repeat hot spot: All PG$^+$ mutants that were obtained from the *lacI*$^+$ parent were first screened for presence in *lacI* of expansion or contraction at the triple tetranucleotide repeat sequence (Farabaugh *et al.* 1978). The *lacI* region spanning the repeat was PCR amplified from each mutant with primers 5′-AGCGGGCCCATT AAGTTCT-3′ and 5′-TCGGCTGAATTTGATTGCGA-3′, and the PCR products were electrophoresed on a 20% polyacrylamide gel. It was thus possible to distinguish the PCR products of the expansion mutants (87 bp) and contraction mutants (79 bp) from those of mutants that had no size change in this region (83 bp).

Sequence analysis of *lacI/lacO* mutants: The complete *lacI/lacO* locus from each mutant (that did not have its mutation at the CTGG repeat hot spot) was PCR amplified with primers 5′-CCCGACACCATCGAATG-3′ (upstream of *lacI*, top strand) and 5′-GCCTCTTCGCTATTACGCCA-3′ (in proximal *lacZ*, bottom strand), and its sequence was determined with the aid of these two primers and the series of forward and reverse sequencing primers described earlier (Erfle *et al.* 1996). Numbering schemes for nucleotides in *lacI* and *lacO* are as in Farabaugh (1978) and Gilbert *et al.* (1973), respectively.

Statistical comparisons between *lacI/lacO* spectra: The statistical significance of differences between *lacI/lacO* mutational spectra was determined with the aid of the hypergeometric test algorithm previously described (Adams and Skopek 1987; Piegorsch and Bailer 1994). The software for performing the test is described in Cariello *et al.* (1994) and is available at http://www.ibiblio.org/dnam/des_hypg.htm. The data were subjected to 1700 Monte Carlo simulations, and the proportion of the simulated tables that exhibited a distribution more improbable than that observed was taken as the estimate of the *P* value of the observed table under the null hypothesis.

RESULTS

***galE*(Ts)-based strategy for *lacZ* reversions in nondividing cells:** A modified version of the previously described *galE*(Ts)-based strategy (Reddy and Gowrishankar 1997a) was employed to examine sensitivity to various mutators for reversion of a chromosomal *lacZ*(Am) allele in nondividing cells. The parental *lacZ galE*(Ts) strains were either grown as a lawn to stationary

phase on Lac$^+$ papillation medium at the permissive temperature (28°) and maintained at 28° or grown at the restrictive temperature for *lacZ*$^+$ revertants (42°) and then shifted to 28°. Viable count measurements (data not shown) indicated that the parental cells (both WT and mutators) attained a density of $\sim 3 \times 10^9$ cells/plate at either temperature in the stationary phase and did not change much thereafter.

For each strain, the Lac$^+$ papillation frequencies (per 10^9 viable cells) on plates continuously incubated at 28° (*A*) and on the temperature-shifted plates (*B*) were determined as averages of at least three experiments, each with duplicate cultures; the variation between the individual values was <20%. Since each papilla represents an independent mutational event, the papillation frequency itself provides a direct estimate of the mutation frequency in the strain. The first value *A* is a measure of the spontaneous Lac$^+$ reversion frequency in the strain while the second value *B* is a measure of the reversions occurring in nondividing cells. However, because of the possibility that the absolute value of *B* may be artifactually elevated (particularly in the mutator strains) even if a small fraction of the mutants arising during growth had escaped killing at the restrictive temperature (see DISCUSSION), we have chosen to use the *B*/*A* ratio as a measure of the relative proportion of reversions that had arisen in nondividing cells.

***B*/*A* ratios for F′ *lacI-Z33* and chromosomal *lacZ*::Tn*10*dKan reversions:** That the *B*/*A* ratio is a valid indicator of reversions in nondividing *galE*(Ts) cells was established by two sets of experiments. First, reversion of the F′-borne *lacI-Z33* allele was reduced by *recA* or *lexA3* mutations (that abolish SOS functions) both on the temperature-shifted plates and on plates continuously incubated at 28° (see Figure 1; papillation frequencies listed in Table 2), and also by a multicopy *mutL*$^+$ plasmid (data not shown), just as has been reported earlier for adaptive reversions in FC40 (FOSTER 1999; ROSENBERG 2001). Second, the frequency of precise excision of a Tn*10*dKan insertion in *lacZ* was increased ~10-fold in *uup* or *ssb* mutants on plates continuously incubated at 28°, but was largely unaffected by the mutations on temperature-shifted plates (Table 2); these data are consistent with previous findings that the *ssb* and *uup* mutations predominantly increase precise excision of Tn*10* derivatives in dividing cells (REDDY and GOWRISHANKAR 1997a,b).

***B*/*A* ratios for chromosomal *lacZ*(Am) reversions in mutator strains:** The chromosomal *lacZ*(Am) mutation under study here is in codon 18 of the gene and is the same as that first used by CAIRNS *et al.* (1988) to identify the phenomenon that is now referred to as adaptive mutation. The earlier study, however, employed an episomally located allele. Lac$^+$ revertants in these strains can arise by all six possible types of base substitutions at *lacZ* or the suppressor tRNA loci.

The papillation frequency values *A* and *B* (as well as the *B*/*A* ratio) for the WT chromosomal *lacZ*(Am) *galE*(Ts) strain were similar to those for its *recA* derivative (Table 2). Furthermore, as expected (DRAKE 1991; ECHOLS and GOODMAN 1991; MILLER 1992; FRIEDBERG *et al.* 1995; HUTCHINSON 1996; RUPP 1996; SLUPSKA *et al.* 1996), various mutator derivatives of the WT strain exhibited 2- to 100-fold increases in Lac$^+$ papillation frequency on plates continuously incubated at the permissive temperature (that is, in the values of *A*); however, the *B*/*A* ratios for these strains exhibited no correlation with their mutator magnitude. The papillation plates for some representative strains are shown in Figure 2, and all the papillation values are listed in Table 2. On the basis of their *B*/*A* ratios, the mutator strains could be grouped into three categories: very high (0.9; *ada ogt*), very low (0.01–0.075; *mutH/L/S*, *dam*, *uvrD*, *dnaQ* [*mutD5*], and *mutM/T*), and roughly equivalent to WT (0.27–0.45; *mutY*, *nth nei*, and *mutA*). These three categories of mutations may therefore be taken to exert their predominant mutator effects, respectively, in nondividing cells, in dividing cells, and in both dividing and nondividing cells. The *B*/*A* ratio value for the *mutYM* double mutant was intermediate between that for either single-mutant strain (Table 2). The *ung* and *miaA* mutants were modest mutators, with little or no increase in Lac$^+$ papillation frequency on the temperature-shifted plates (data not shown). The values of *A* and *B*/*A* were not significantly altered also by mutations in *lexA*, *dinB*, *recB*, *recD*, *recF*, *recG*, *recQ*, *ruvABC*, *polB*, *xthA*, *mfd*, *rep*, *dps*, *nfo*, *soxR*, or *sodB* or by overexpression of MutL and MutS from a plasmid in the *mut*$^+$ *galE*(Ts) strain (data not shown).

Three sets of control experiments (data not shown) were performed to exclude artifactual explanations, such as differences in viability of the populations under selection or in efficiency of elimination of growth-associated Lac$^+$ mutants at the restrictive temperature, for the different *B*/*A* ratios in the various mutants. First, no significant differences were observed in viability of

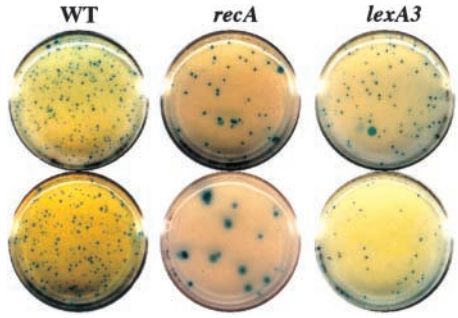

FIGURE 1.—Lac$^+$ papillation in *galE*(Ts) strain with F′128-borne *lacI-Z33* (WT) and in its *recA* and *lexA3* derivatives. Strains employed are as described in Table 2. Representative pairs of plates are shown. Plates in the top row were incubated continuously at 28°, and those in the bottom row were subjected to temperature shift from 42° to 28°.

TABLE 2

Lac$^+$ papillation frequencies in *galE*(Ts) strains

Genotype (strain no.)	Lac$^+$ papillation frequency[a] 28° (*A*)	42° → 28° (*B*)	*B/A*
With F′ *lacI-Z33*			
WT (GJ2380)	1.0	0.57	0.57
recA (GJ2381)	0.11	0.06	0.55
lexA3 (GJ2382)	0.21	0.07	0.33
With chromosomal *lacZ*::Tn*10*dKan			
WT (GJ2220)	1.0	0.07	0.07
ssb (GJ2222)	10.6	0.15	0.014
uup (GJ2224)	9.1	0.08	0.009
With chromosomal *lacZ*(Am)			
WT (GJ2231)	1.0	0.31	0.31
recA (GJ2232)	0.80	0.22	0.27
dnaQ (GJ2377)	31	0.77	0.025
mutH (GJ2334)	115	1.2	0.010
mutL (GJ2321)	123	1.7	0.014
mutS (GJ2223)	119	1.9	0.016
dam (GJ2336)	7.7	0.58	0.075
uvrD (GJ2335)	108	1.5	0.014
mutM (GJ2322)	38	0.85	0.022
mutT (GJ2323)	108	5.8	0.054
mutY (GJ2234)	46	12.3	0.027
mutY mutM (GJ2376)	115	11.5	0.1
mutA (GJ2324)	1.92	0.85	0.44
ada ogt (GJ2348)	2.23	2.0	0.90
nth nei (GJ2351)	3.0	1.35	0.45

[a] Lac$^+$ papillation frequencies *A* and *B* are defined in the text. All values are normalized to that in the cognate WT strains at 28° (taken as 1.0), for which the numbers of papillae per 10^9 cells were 117 (GJ2380), 110 (GJ2220), and 9 (GJ2231).

the populations under selection. Second, Lac$^+$ revertants of the *ada ogt* strain (which exhibited the highest *B/A* ratio) were shown in reconstitution experiments to both form papillae at 28° and be killed at 42°, just as efficiently as Lac$^+$ revertants from the WT or the *mutS* strain (which had one of the lowest values for *B/A*). Finally, when mixed cultures of differentially marked *mutH* and *mutY* strains (that are comparable in their mutator magnitude but have different *B/A* ratios) were plated on the Lac$^+$ papillation medium, each strain continued to exhibit its distinctive *B/A* ratio, indicating that the low frequency of Lac$^+$ in the *mutH* strain following temperature shift is not a consequence of massive bystander cell lethality at the restrictive temperature.

Additional support for the correlation between the different mutator mutations and their distinct *B/A* ratios came from an experiment in which we subjected the *lacZ*(Am) *galE*(Ts) strain GJ2231 to nitrosoguanidine mutagenesis, identified new mutator derivatives by screening for increased Lac$^+$ papillation in colonies continuously incubated at 28°, and then classified the mutators on the basis of their *B/A* ratios. Three mutants with a 5- to 16-fold increase in papillation and a *B/A* ratio between 0.35 and 0.46 were shown to be *mutY*, and six others with an 8- to 12-fold increase in papillation and a *B/A* ratio between 0.05 and 0.15 were mapped to *mutH* (four), *mutS* (one), and *uvrD* (one; data not shown).

***lacI/lacO* mutational spectra in nondividing cells:** Just as lactose can select for or against Lac$^+$ strains that are, respectively, *galE*$^+$ or *galE*, so too can the lactose analog PG select for or against *lacI/lacO* strains (which express β-galactosidase constitutively) that are *galE*$^+$ or *galE*. A lawn of cells of the parental *galE*(Ts) strain (with a functional *lac* repressor/operator system) was first exposed to PG at 42°, so that all *lac*-constitutive mutants in the population were eliminated and then shifted to 28°. Papillae obtained after the temperature shift were assumed to represent mutations in *lacI* or *lacO* that had arisen within the nondividing cells.

To determine the spectrum of *lacI/lacO* mutations in nondividing cells, we purified 95 PG$^+$ papillae from temperature-shifted plates of the *galE*(Ts) strain GJ2422 with the *lacI*$^+$ coding sequence; this number was composed of all papillae that arose from each of nine independently plated cultures. As a control, 130 independent PG$^+$ mutants were obtained from early exponential-phase cultures, for determination of the spectrum for dividing cells. The *lacI/lacO* lesion in each mutant from either collection was then identified as described in MATERIALS AND METHODS.

A comparison between the two mutational spectra

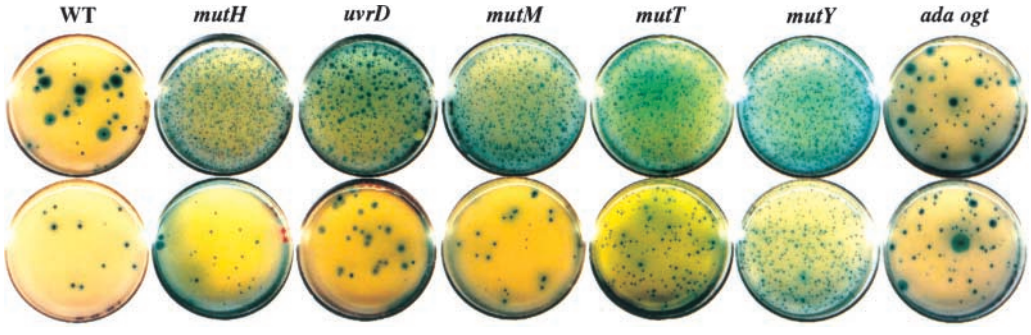

FIGURE 2.—Lac$^+$ papillation in *galE*(Ts) strain with chromosomal *lacZ*(Am) (WT) and in its indicated mutant derivatives. Strains employed are as described in Table 2. Representative pairs of plates are shown. Plates in the top row were incubated continuously at 28°, and those in the bottom row were subjected to temperature shift from 42° to 28°.

TABLE 3

***lacI*/*lacO* mutations in dividing (D) and nondividing (ND) cells of *galE*(Ts) strains**

	Mutation			
	GJ2422 (*lacI*$^+$)		GJ2432 (*lacI204*)	
	D (N = 130)[a]	ND (N = 95)	D (N = 29)	ND (N = 28)
Hot spot +CTGG	59	40	NA[b]	NA
Hot spot −CTGG	10	5	NA	NA
Frameshifts (−2, −1, +1, +2)	11	6	9	5
Deletions: Δ1108-*lacO* +4	6	0	2	0
Deletions: other	6	1	5	3
IS*1* insertions	10	2	6	1
lacO +6 (AT → GC)	12	4	2	2
lacO +5 (GC → AT)	0	11	0	4
lacI 56 (GC → AT)	0	9	0	0
Other transitions	4	7	4	5
Transversions	11	8	1	8
Other	1	2	0	0

[a] *N*, total number of mutations.
[b] NA, not applicable.

(see Table 3; details are presented in supplemental Table 4 at http://www.genetics.org/supplemental/) revealed several differences, even though the frequency of expansion (44% of total) or contraction (6% of total) of the CTGG repeat at the hot spot site in *lacI*$^+$ was similar. Statistical analysis of the data by the hypergeometric test also established that the spectra were different from one another at $P < 10^{-6}$. [The log-phase spectrum obtained in this study was more or less identical with the *lacI/lacO* spectra described earlier for WT strains (Schaaper *et al.* 1986, 1987; Halliday and Glickman 1991).]

Deletions [both at a specific hot spot site previously described (Δ1108-*lacO* +4; Halliday and Glickman 1991) and at others] as well as IS*1* insertions were underrepresented in the spectrum for nondividing cells. Two mutations (GC-to-AT transitions at positions 56 in *lacI* and +5 in *lacO*, respectively) that were prominent hot spots in nondividing cells (with each accounting for 10% of the total mutant numbers) were not represented at all among the log-phase mutants.

If one considers just the subset of *lacO* mutations, a characteristic feature of all WT spectra so far reported is a ratio of <0.05 for +5 (GC-to-AT) to +6 (AT-to-GC) transitions, and indeed all the *lacO* mutations that occurred in dividing cells were at +6. On the other hand, this ratio was 2.8 in the spectrum obtained from nondividing cells; furthermore, even apart from the *lacO* +5 site, several other positions in *lacO* recorded hits in the spectrum for nondividing cells but not in that for dividing cells (Figure 3).

In control experiments, the *lacO* +5 or the *lacI* 56 mutants, which were identified as occurring more frequently on the temperature-shifted plates, were indistinguishable from other mutants such as *lacO* +6 or *lacI* +195 with regard to three features that were tested (data not shown): (i) constitutivity of *lacZ* expression, as measured by β-galactosidase assays; (ii) sensitivity to PG at 42°; and (iii) ability to form papillae on PG-containing medium at 28°. The latter two findings were established from reconstitution experiments in which pairs of the mutants (differentially marked) that were mixed in defined ratios were plated at ~100 cells/plate along with a lawn of the Δ*lac* strain GJ2218.

It was recognized that the validity of the individual differences observed between the two spectra may be limited by the fact that multiple hypotheses were being tested. Accordingly, a second comparison of the *lacI/lacO* mutational spectra was undertaken, this time in the *galE*(Ts) *lacI204* strain GJ2432 in which the CTGG tetranucleotide repeat expansion/contraction hot spot in *lacI* has been destroyed without altering the primary structure of the encoded protein (Schaaper and Dunn 1991). (This strain was chosen to reduce the numbers of mutants for analysis, since the previous comparison had shown no difference between the two spectra for mutations at the repeat site.) Around 30 PG$^+$ mutants each from the exponential-phase cultures and from the temperature-shifted plates were studied, and several features from the first comparison were reproduced in the second (see Table 3 and Figure 3; detailed information is in supplemental Table 4): (i) a reduction in the proportion of deletions (at both Δ1108-*lacO* +4 and other sites) and of IS*1* insertions, (ii) a marked increase in the proportion of GC-to-AT transitions at +5 of *lacO*, and (iii) increased mutability at additional *lacO* positions (except +6) in the spectrum for nondividing cells relative to that for dividing cells. The ratio, in the mutants from nondividing cells, of +5 to +6 alterations in *lacO* was 2.0, whereas once again the only *lacO* mutants

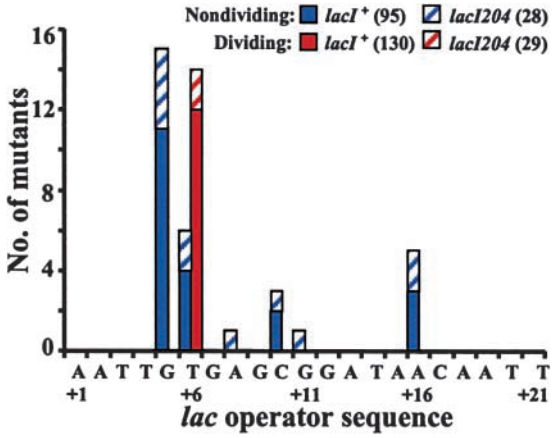

FIGURE 3.—Frequency distribution of substitution mutations in the *lacO* locus of dividing (red) and nondividing (blue) cells of *galE*(Ts) *lacI*$^+$ (GJ2422, solid bars) or *galE*(Ts) *lacI204* (GJ2432, striped bars) strains. Total numbers of *lacI/lacO* mutants obtained in the different categories are indicated in parentheses. At any individual site in *lacO*, all the substitutions were identical: +5 and +11, GC to AT; +6 and +16, AT to GC; +8, AT to CG; and +10, GC to TA.

isolated from the population of dividing cells were at +6. By the hypergeometric test, the *lacI/lacO* spectra for dividing and nondividing cells of the *lacI204* strain were different at $P = 0.005$ (95% confidence limits, 0.001–0.008).

Greatly increased frequency of dominant mutations in late-arising PG$^+$ papillae of the *galE*$^+$ strain: As mentioned above, the majority of investigators studying mutations in nondividing cells have collected mutants at late time points from populations subjected to a nonlethal selection. To compare this approach with the *galE*(Ts)-based one employed in the present work, we collected PG$^+$ papillae as a function of time from the *galE*$^+$ *lacI204* parental strain grown on CAA-Glc [that is, the same medium as that employed above for the *galE*(Ts)-based approach] and also by following the identical temperature-shift protocols. In addition, PG$^+$ papillae were collected from plates in which 0.2% acetate was substituted for CAA-Glc as the utilizable carbon source. Since in both instances the parental strain was *galE*$^+$, there was no selection against *lacI/lacO* mutants even at 42°, and the papillation frequency was $\sim 16/10^9$ cells.

Plates were examined daily for up to 14 days; papillae were marked on the days that they became visible, and all of them were purified at the end of the experiment. Since our interest was in determining the conditions, if any, in which GC-to-AT transitions at *lacI* +56 and *lacO* +5 occurred [that were the apparently characteristic features of the spectrum for nondividing cells obtained by the *galE*(Ts)-based approach], and since both mutations are dominant to *lacI*$^+$ *lacO*$^+$, we first classified the new mutants into dominant and recessive categories and then sequenced only the former.

The data for PG$^+$ mutants that were grouped into three classes are presented in Figure 4, namely as early-, intermediate-, and late-arising papillae. On lawns grown on either carbon source (CAA-Glc or acetate), the proportion of dominant mutations was low among the early-arising papillae [similar to that reported earlier for spontaneous *lacI/lacO* mutations in the *lacI204* strain (SCHAAPER and DUNN 1991)], but rose progressively to approach nearly 100% among the late-arising papillae. The preponderance of dominant *lacI/lacO* mutations among late-arising papillae was apparent even when the lawns (both on CAA-Glc and on acetate) were not subjected to temperature shift but were continuously incubated at 28°, and also when 0.2% Glc (in MM) or LB medium was used as the carbon source for growth of the lawn at 28° or 42° (data not shown).

Sequence analysis of the collection of dominant *lacI/lacO* mutants from the temperature-shifted plates with CAA-Glc or acetate revealed the occurrence of +5 *lacO* mutations (and a ratio of 2.1 for the +5 to +6 *lacO* transitions) for only the late-arising papillae in populations grown on acetate as the carbon source, but not in the other categories such as early-arising papillae on acetate or both early- and late-arising papillae on CAA-Glc (Figure 4). From the other collections, of 11 dominant mutants obtained as PG$^+$ papillae on or after day 5 on the acetate-grown lawn at 28°, 2 were again at *lacO* +5; on the other hand, none of 52 additional dominant mutants arising from the CAA-Glc-grown lawns was at this position.

The preponderance of dominant *lacI/lacO* mutations among the late-arising PG$^+$ papillae was noted also for the *galE*$^+$ *lacI*$^+$ strain GJ2421 (that is, with the triple tetranucleotide hot spot site in the *lacI* coding region). Twenty-seven of a total of 37 papillae (73%) obtained on or after day 5 on lawns of this strain grown on CAA-Glc harbored dominant mutations (but they were not sequenced).

What feature of growth on acetate is correlated with mutation at the *lacO* +5 site? The data above indicated that mutations at *lacO* +5 occur in late-arising papillae of acetate-grown but not in those of CAA-Glc-grown populations. Acetate is among the poorest of C sources for *E. coli*, whose utilization is dependent on full derepression of both the tricarboxylic acid cycle pathway and the glyoxylate bypass pathway (CLARK and CRONAN 1996). Acetate-grown cells are downregulated for the PhoP-PhoQ regulon (LESLEY and WALDBURGER 2003), and HALL (1998a) has shown that the latter regulates adaptive mutagenesis. Acetate is also known to accumulate in cultures grown on Glc with inadequate aeration (for example, to high cell densities) and to be toxic under these conditions (KLEMAN and STROHL 1994).

To determine which feature of acetate metabolism might be associated with mutations at *lacO* +5, we obtained PG$^+$ papillae of the *galE*$^+$ *lacI204* strain grown on palmitate (which feeds exclusively into the acetate

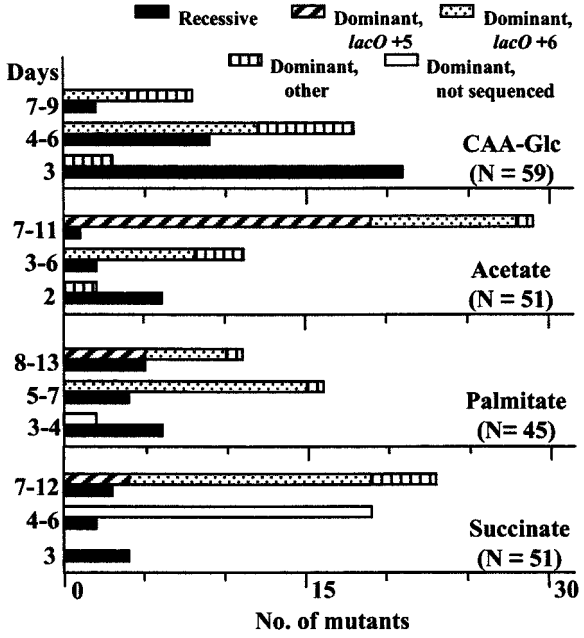

FIGURE 4.—Frequency distribution of recessive and dominant *lacI/lacO* mutations (bottom and top bars, respectively, in each pair) in PG^+ papillae from the *lacI204 galE*$^+$ strain GJ2474, as a function of their time of appearance following PG addition to plates with different utilizable carbon sources (as indicated). Dominant mutations were further classified into four categories, as per the key shown. The three ascending time intervals marked in each part correspond to the designations early, intermediate, and late, respectively, that have been used in the text. *N*, total number of mutants tested. For palmitate and succinate sections, mutants were pooled from plates that were either subjected to temperature shift or continuously incubated at 28°; for the other two sections, mutants were from temperature-shifted plates.

metabolism pathway) or on succinate (which is also a poor carbon source but whose utilization does not invoke the glyoxylate bypass). In these two cases as well, we observed that the ratio of recessive to dominant mutations was reversed in the intermediate- and late-arising papillae when compared with that in the early-arising papillae. Furthermore, transitions at *lacO* +5 were obtained in both instances (just as with the acetate-grown cultures) among the late-arising papillae (Figure 4).

DISCUSSION

We employed *galE*(Ts) strains and an incubation protocol involving temperature shift, first to prepare populations of cells in which all growth-associated *lacZ* or *lacI/lacO* mutations were eliminated and then to obtain mutations in these chromosomal target loci that had arisen in nondividing cells. Since Lac^+ reversions in the *lacZ*(Am) strain can occur only by a limited set of events, we exploited this system to study the effects in nondividing cells of the various known mutator mutations. On the other hand, since *lacI/lacO* forward mutations encompass a large variety of molecular events, we undertook a mutational spectrum analysis for this target in the nondividing cells.

In the case of adaptive Lac^+ mutation in strain FC40, Roth and coworkers have suggested that the underlying mechanism is selection for amplification of the region including the leaky *lacI-Z33* allele, resulting in slow-growing microcolonies within each of which a reversion to Lac^+ is then likely to occur (ANDERSSON *et al.* 1998; HENDRICKSON *et al.* 2002; SLECHTA *et al.* 2002). In the present study as well, the alleles under selection for Lac^+ or PG^+ may be considered to be leaky, and therefore an important question that arises is whether a similar mechanism (of growth-associated mutations within microcolonies bearing amplifications of the *lac* locus) might explain the occurrence of *lacZ* or *lacI/lacO* mutations in the ostensibly nondividing populations. However, for the following reasons we do not believe this to be a likely mechanism operating in the *galE*(Ts) strains initially grown at the restrictive temperature (although, as discussed in a subsequent section, it may indeed be so for late-arising papillae in the *galE*$^+$ strain).

First, the *galE*(Ts) mutant is Gal^- (in addition to being Gal^s) at 42° and hence cannot grow on, even if it is not killed by, the galactose that might be slowly released from PG hydrolysis at this temperature; even in the case of selection for Lac^+, ANDERSSON *et al.* (1998) have shown that *lac* locus amplification cannot permit a leaky *lacZ* mutant to form microcolonies if the strain is also Gal^-. Second, the process of chromosomal amplification is RecA dependent, whereas the Lac^+ and PG^+ papillation frequencies on temperature-shifted plates of the *galE*(Ts) strains were RecA independent. Third, *lacI/lacO* mutations that arise within a tandemly amplified region to confer a PG^+ phenotype would necessarily have to be dominant, whereas we found that a significant proportion of mutations recovered in PG^+ papillae of the *galE*(Ts) strain after temperature shift were recessive (see below). Finally, the fact that the *lacZ* reversions and *lacI/lacO* mutations exhibited, respectively, distinct mutator specificities and distinct mutational spectra also suggests that they may not have arisen in the dividing cells within microcolonies.

Need for alkylation- and oxidative-damage repair, but not for methyl-mismatch repair or SOS/recombination functions, in nondividing cells: For each *lacZ*(Am) *galE*(Ts) derivative with a mutator mutation, we determined the relative Lac^+ papillation frequency both on plates continuously incubated at the permissive temperature (which is a measure of the overall mutator magnitude, designated *A*) and on plates shifted from the restrictive to the permissive temperature (which is a measure of mutation frequency in nondividing cells, designated *B*).

As explained above, we have chosen the *B*/*A* ratio (rather than *B* itself) as an index of the relative proportion of reversions to Lac^+ that had arisen in nondividing cells. This was done to avoid the possibility of false-

positive interpretations being made from papillae observed on the temperature-shifted plates that might actually represent growth-associated mutants that had escaped killing at the restrictive temperature. The upper limit of the estimate for the fraction of such putative surviving mutants is ~0.01 (since the lowest observed *B/A* ratios were in this range).

The major interpretations arising from our data on *B/A* ratios for the various strains are that the genes involved in alkylation-damage repair (*ada ogt*) and methyl-mismatch repair are predominantly required for protection of genome integrity, respectively, in nondividing cells and in dividing cells. Mutations in all the five genes involved in mismatch repair (*mutH, -L, -S, dam*, and *uvrD*; Radman and Wagner 1986; Modrich 1991) behaved similarly in our assays. Of the genes involved in oxidative-damage repair, *mutY* and *nth nei* function in both dividing and nondividing cells whereas *mutM* is required only in dividing cells. *mutT* also appears to be a mutator predominantly in dividing cells, although in this case the values for *B* and *B/A* are sufficiently above the upper limit of false positivity to suggest that it does have a moderate mutator activity even in nondividing cells. Finally, unlike the case with adaptive reversions on the episome in the FC40 system (Foster 1999; Rosenberg 2001), recombination and SOS functions are not involved in spontaneous mutagenesis or its avoidance at chromosomal *lacZ* in nondividing cells.

We have shown that the differences in *B/A* ratios for the different mutators are not because of differences in either viability of the parental population or growth of mutants to form papillae. Residual growth of the population, following temperature shift to 28°, may also be excluded as an explanation for the papillae that arose subsequently, because in that case one would have expected a direct correlation between the values for *A* (that is, mutator magnitude) and *B/A* for the different strains.

Our finding that mutations in *ada ogt*, *mutY*, and *nth nei* promote spontaneous mutagenesis on the chromosome in nondividing cells is in accord with the results obtained by other workers using a variety of mutational targets and assay systems. For example, *ada ogt* mutants exhibit increased frequencies of mutations following prolonged starvation (Rebeck and Samson 1991; Foster and Cairns 1992; Mackay *et al.* 1994; Taverna and Sedgwick 1996; Vidal *et al.* 1998) and so too do *mutY* mutants of both *E. coli* (Bridges *et al.* 1996) and *Pseudomonas putida* (Saumaa *et al.* 2002). This implies that endogenous alkylation and oxidation damage to DNA (giving rise to mutable lesions) does occur in nondividing cells. There is evidence also that active oxygen species may contribute to spontaneous endogenous DNA lesions in nondividing cells (Benov and Fridovich 1996; Bridges and Timms 1998; Bjedov *et al.* 2003).

On the other hand, there has been less consensus in the literature on the role of mismatch repair in nondividing cells (Foster 1999; Rosenberg 2001). For example, different reports, albeit in diverse mutational assay systems, have suggested that mismatch repair functions are necessary (Foster and Cairns 1992; Jayaraman 1992; Bridges and Timms 1997; Bjedov *et al.* 2003), that they are limiting (Harris *et al.* 1997), or even that they are dispensable (Bregeon *et al.* 1999) for avoidance of mutations in the stationary phase.

Distinctive features of the *lacI/lacO* mutational spectrum in nondividing cells: In comparison with the log-phase *lacI/lacO* spectrum, that for nondividing cells exhibited (i) an unchanged frequency of expansions or contractions at the *lacI* tetranucleotide repeat, (ii) fewer deletions (at both Δ1108-*lacO* +4 and other sites) and IS*1* insertions, (iii) GC-to-AT transition hot spots at *lacO* +5 and perhaps *lacI* 56, and (iv) increased mutability in *lacO* at sites other than +6. In this context, Hall (1998b, 1999) has also reported small decreases in the proportion of IS*1* insertions among the adaptive mutations at two chromosomal loci, *bgl* and *ebgR*; and Bjedov *et al.* (2003) found that GC-to-AT transitions were increased in aging colonies at both the *rpoB* and *lacZ* loci.

The *lacO* +5 hot spot was identified in the spectra for nondividing cells (grown on CAA-Glc) from both the *lacI*$^+$ and *lacI204* parental strains, and the concomitant increase in the *lacO* +5 to +6 mutation ratio is the first reported for any of the spontaneous or induced mutational spectra in *lacO* (see, for example, Fowler *et al.* 1986; Fix *et al.* 1987; Schaaper *et al.* 1987). From an analysis of the *lacI/lacO* mutants in the *galE*$^+$ strain also grown on CAA-Glc (Figure 4), we have shown that these differences cannot be explained simply on the grounds that the mutants were obtained as papillae or from populations that had been subjected to temperature shift.

Unlike the hot spot at *lacO* +5, that at *lacI* 56 was observed in only one of the two spectra determined for nondividing cells, and even in this case all the mutations were obtained from just two of nine cultures (albeit as papillae, each of which represents an independent mutation; data not shown). Its significance for the present study is therefore unclear. Nevertheless, even if the data for this position were excluded from the comparison, the mutational spectra for dividing and nondividing cells of the *lacI*$^+$ strain were judged to be significantly different ($P = 0.002$, 95% confidence limits 0–0.004).

Dominant *lacI/lacO* mutations in late-arising PG$^+$ papillae and support for "instantaneous gratification" models of stationary-phase mutagenesis: In the second approach to studying *lacI/lacO* mutations in nondividing cells, we examined late-arising PG$^+$ papillae of the *galE*$^+$ strain grown as lawns on different carbon sources and compared them with the early-arising papillae on these plates. Once again, transition mutations at *lacO* +5 were observed among only the late-arising papillae and fur-

thermore only in cells initially grown on poor carbon sources. That growth on different carbon sources can modulate mutagenesis in aging colonies has been shown earlier (Bjedov *et al.* 2003), but the underlying mechanism is not known. Whether there is a catabolite repression effect (Ambrose and Macphee 1998), caused by Glc addition in the CAA-Glc plates, also remains to be determined. Nevertheless, the notion that *lacO* +5 is a specific target for spontaneous mutations in nondividing cells is reinforced by these data.

The other remarkable feature associated with the late-arising PG^+ papillae was that virtually all of them harbored dominant *lacI/lacO* mutations (this time, irrespective of the carbon source employed for growth of the lawn), whereas mutations in the majority of early-arising papillae were recessive (similar to that reported in previous spectra; Schaaper and Dunn 1991). The dominant mutations were distributed over a number of sites, suggesting that it is the dominance *per se* that correlates with their occurrence. To our knowledge, this is the first study in which mutations occurring in nondividing cells subjected to a nonlethal selection were sought to be classified as dominant or recessive; all other studies done previously had employed assays only for dominant reversions.

Our findings provide support to a certain class of models that we refer to as instantaneous gratification models [the earliest of which was proposed by Stahl (1988); see Foster (1992) for review], to explain the mechanisms of mutagenesis in nondividing cells. In these models, a spontaneous mutagenic lesion occurring at random on a DNA strand in a nondividing cell (with sufficient cytoplasmic concentration of *lac* repressor protein) is most likely to be fixed as a mutation if it is immediately able to confer the ability to utilize PG, either (i) by virtue of its occurrence on the template strand for transcription, whereby it can direct the synthesis of a dominant negative repressor, or (ii) by rendering the operator incapable of binding repressor. The resulting mutations will necessarily be dominant.

Absence of bias against recessive *lacI/lacO* mutations obtained by the *galE*(Ts)-based approach: The preponderance of dominant *lacI/lacO* mutations in nondividing cells was observed only when the mutants were identified as late-arising papillae from the $galE^+$ strains, and not when they were obtained by the temperature-shift strategy from the *galE*(Ts) strains. In the latter case, for example, 13 of 28 mutations in the *lacI204* background were recessive (Table 2), and the proportions did not vary with the time of appearance of papillae on the plates (data not shown). Furthermore, it is not likely that the recessive mutants were ones that actually arose during growth of the lawn but were not efficiently eliminated at 42°, since their molecular characterization suggests that the majority (if not all) of them are null for LacI function.

Although the reasons for absence of bias against recovery of recessive mutations in the *galE*(Ts)-based approach are not known, one possibility is that papillae on temperature-shifted plates of the *galE*(Ts) strain ($3/10^9$ cells) represent just a small subset of all the late-arising papillae in the $galE^+$ strain ($16/10^9$ cells). In accord with earlier suggestions (Lenski *et al.* 1989; Hendrickson *et al.* 2002), it is possible that the larger subset of the late-arising $galE^+$ papillae represented clones with two sequentially acquired mutations, the first of which was growth associated, leading to formation of microcolonies, and the second in *lacI/lacO*. As explained above, such microcolonies can occur only in the $galE^+$ strain, since their growth will not be supported in the *galE*(Ts) background at the restrictive temperature. If the first "mutation" involved amplification of the *lac* locus [which may occur by either RecA-dependent (Hendrickson *et al.* 2002) or RecA-independent (Bzymek and Lovett 2001) mechanisms], then the *lacI/lacO* mutation in the second step would necessarily have to be dominant.

Mechanism of mutation at the *lacO* +5 hot spot in nondividing cells: The mechanism by which *lacO* +5 may be rendered mutable only in nondividing cells is not known. A spontaneous GC-to-AT transition such as that at *lacO* +5 can occur following C oxidation, C deamination, G alkylation, or incorporation during replication of the incorrect nucleotide, but these lesions are ordinarily repaired in wild-type cells by, respectively, the Nth Nei glycosylases (Blaisdell *et al.* 1999; Najrana *et al.* 2000), Ung glycosylase (Coulondre *et al.* 1978; Duncan and Miller 1980), Ada Ogt methyltransferases (Mackay *et al.* 1994), and the methyl-mismatch repair system (Modrich 1991). Miller and coworkers have shown that *dcm* sites in *lacI* are hot spots for GC-to-AT mutation because deamination of the methylated C residues at these sites results in lesions that alone are blind to repair by Ung (Coulondre *et al.* 1978; Duncan and Miller 1980). Arguing by analogy, and on the basis of the *B/A* ratio data given above for *lacZ* reversions in the *ada ogt* (and *nth nei*) mutants, one could suggest that endogenous alkylation (or oxidative) damage is more or less randomly distributed on all G (or C) residues in nondividing cells and that in the wild-type strain the Ada and Ogt (or Nth and Nei) proteins might be able to repair this damage efficiently at all sites except *lacO* +5. Additionally, since the *lacO* +5 mutation is dominant, the "instantaneous gratification" models may also be relevant in explaining its occurrence.

Concluding remarks: In summary, we have found in this study that the products of the *ada ogt*, *nth nei*, and *mutY* genes are essential for avoidance of spontaneous mutations in nondividing cells, but that the genes involved in methyl-directed mismatch repair are dispensable under these conditions. In WT strains, the *lacO* +5 site is apparently mutable only in nondividing cells. Finally, we have shown for the first time that there is a substantial bias against recessive mutations in late-arising mutants, providing support to models that propose

that nucleotide lesions conferring an instantaneous gratification are likely to be fixed as mutations in nondividing cells.

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LITERATURE CITED

Adams, W. T., and T. R. Skopek, 1987 Statistical test for the comparison of samples from mutational spectra. J. Mol. Biol. **194:** 391–396.

Ambrose, M., and D. G. Macphee, 1998 Catabolite repressors are potent antimutagens in *Escherichia coli* plate incorporation assays: experiments with glucose, glucose-6-phosphate and methyl-alpha-D-glucopyranoside. Mutat. Res. **398:** 175–182.

Andersson, D. I., E. S. Slechta and J. R. Roth, 1998 Evidence that gene amplification underlies adaptive mutability of the bacterial *lac* operon. Science **282:** 1133–1135.

Benov, L., and I. Fridovich, 1996 The rate of adaptive mutagenesis in *Escherichia coli* is enhanced by oxygen (superoxide). Mutat. Res. **357:** 231–236.

Berlyn, M. K. B., 1998 Linkage map of *Escherichia coli* K-12, edition 10: the traditional map. Microbiol. Mol. Biol. Rev. **62:** 814–984.

Bjedov, I., O. Tenaillon, B. Gerard, V. Souza, E. Denamur *et al.*, 2003 Stress-induced mutagenesis in bacteria. Science **300:** 1404–1409.

Blaisdell, J. O., Z. Hatahet and S. S. Wallace, 1999 A novel role for *Escherichia coli* endonuclease VIII in prevention of spontaneous G→T transversions. J. Bacteriol. **181:** 6396–6402.

Bregeon, D., I. Matic, M. Radman and F. Taddei, 1999 Inefficient mismatch repair: genetic defects and down regulation. J. Genet. **78:** 21–28.

Bridges, B. A., 1998 The role of DNA damage in stationary phase ('adaptive') mutation. Mutat. Res. **408:** 1–9.

Bridges, B. A., 2001 Hypermutation in bacteria and other cellular systems. Philos. Trans. R. Soc. Lond. Ser. B **356:** 29–39.

Bridges, B. A., and A. R. Timms, 1997 Mutation in *Escherichia coli* under starvation conditions: a new pathway leading to small deletions in strains defective in mismatch correction. EMBO J. **16:** 3349–3356.

Bridges, B. A., and A. R. Timms, 1998 Effect of endogenous carotenoids and defective RpoS sigma factor on spontaneous mutation under starvation conditions in *Escherichia coli*: evidence for the possible involvement of singlet oxygen. Mutat. Res. **403:** 21–28.

Bridges, B. A., M. Sekiguchi and T. Tajiri, 1996 Effect of *mutY* and *mutM/fpg-1* mutations on starvation-associated mutation in *Escherichia coli*: implications for the role of 7,8-dihydro-8-oxoguanine. Mol. Gen. Genet. **251:** 352–357.

Bzymek, M., and S. T. Lovett, 2001 Instability of repetitive DNA sequences: the role of replication in multiple mechanisms. Proc. Natl. Acad. Sci. USA **98:** 8319–8325.

Cairns, J., J. Overbaugh and S. Miller, 1988 The origin of mutants. Nature **335:** 142–145.

Cariello, N. R., W. W. Piegorsch, W. T. Adams and T. R. Skopek, 1994 Computer program for the analysis of mutational spectra: application to p53 mutations. Carcinogenesis **15:** 2281–2285.

Clark, D. P., and J. E. Cronan, Jr., 1996 Two-carbon compounds and fatty acids as carbon sources, pp. 343–357 in *Escherichia coli and Salmonella: Cellular and Molecular Biology*, edited by F. C. Neidhardt, R. Curtiss, III, J. L. Ingraham, E. C. C. Lin, K. B. Low *et al.* American Society of Microbiology, Washington, DC.

Coulondre, C., J. H. Miller, P. J. Farabaugh and W. Gilbert, 1978 Molecular basis of base substitution hotspots in *Escherichia coli*. Nature **274:** 775–780.

Drake, J. W., 1991 Spontaneous mutation. Annu. Rev. Genet. **25:** 125–146.

Duncan, B. K., and J. H. Miller, 1980 Mutagenic deamination of cytosine residues in DNA. Nature **287:** 560–561.

Echols, H., and M. F. Goodman, 1991 Fidelity mechanisms in DNA replication. Annu. Rev. Biochem. **60:** 477–511.

Erfle, H. L., D. F. Walsh, J. Holcraft, N. Hague, J. G. De Boer *et al.*, 1996 An efficient laboratory protocol for the sequencing of large numbers of *lacI* mutants recovered from Big Blue transgenic animals. Environ. Mol. Mutagen. **28:** 393–396.

Farabaugh, P. J., 1978 Sequence of the *lacI* gene. Nature **274:** 765–769.

Farabaugh, P. J., U. Schmeissner, M. Hofer and J. H. Miller, 1978 Genetic studies of the *lac* repressor. VII. On the molecular nature of spontaneous hotspots in the *lacI* gene of *Escherichia coli*. J. Mol. Biol. **126:** 847–863.

Fix, D. F., P. A. Burns and B. W. Glickman, 1987 DNA sequence analysis of spontaneous mutation in a *polA1* strain of *Escherichia coli* indicates sequence-specific effects. Mol. Gen. Genet. **207:** 267–272.

Foster, P. L., 1992 Directed mutation: between unicorns and goats. J. Bacteriol. **174:** 1711–1716.

Foster, P. L., 1999 Mechanisms of stationary phase mutation: a decade of adaptive mutation. Annu. Rev. Genet. **33:** 57–88.

Foster, P. L., and J. Cairns, 1992 Mechanisms of directed mutation. Genetics **131:** 783–789.

Fowler, R. G., R. M. Schaaper and B. W. Glickman, 1986 Characterization of mutational specificity within the *lacI* gene for a *mutD5* mutator strain of *Escherichia coli* defective in 3′→ 5′ exonuclease (proofreading) activity. J. Bacteriol. **167:** 130–137.

Friedberg, E. C., G. C. Walker and W. Siede, 1995 *DNA Repair and Mutagenesis*. American Society of Microbiology, Washington, DC.

Gilbert, W., N. Maizels and A. Maxam, 1973 Sequences of controlling regions of the lactose operon. Cold Spring Harbor Symp. Quant. Biol. **38:** 845–855.

Godoy, V. G., and M. S. Fox, 2000 Transposon stability and a role for conjugational transfer in adaptive mutability. Proc. Natl. Acad. Sci. USA **97:** 7393–7398.

Godoy, V. G., F. S. Gizatullin and M. S. Fox, 2000 Some features of the mutability of bacteria during nonlethal selection. Genetics **154:** 49–59.

Gowrishankar, J., 1985 Identification of osmoresponsive genes in *Escherichia coli*: evidence for participation of potassium and proline transport systems in osmoregulation. J. Bacteriol. **164:** 434–445.

Hall, B. G., 1998a Adaptive mutagenesis at *ebgR* is regulated by PhoPQ. J. Bacteriol. **180:** 2862–2865.

Hall, B. G., 1998b Activation of the *bgl* operon by adaptive mutation. Mol. Biol. Evol. **15:** 1–5.

Hall, B. G., 1999 Spectra of spontaneous growth-dependent and adaptive mutations at *ebgR*. J. Bacteriol. **181:** 1149–1155.

Halliday, J. A., and B. W. Glickman, 1991 Mechanisms of spontaneous mutation in DNA repair-proficient *Escherichia coli*. Mutat. Res. **250:** 55–71.

Harris, R. S., G. Feng, K. J. Ross, R. Sidhu, C. Thulin *et al.*, 1997 Mismatch repair protein MutL becomes limiting during stationary-phase mutation. Genes Dev. **11:** 2426–2437.

Hendrickson, H., E. S. Slechta, U. Bergthorsson, D. I. Andersson and J. R. Roth, 2002 Amplification-mutagenesis: evidence that "directed" adaptive mutation and general hypermutability result from growth with a selected gene amplification. Proc. Natl. Acad. Sci. USA **99:** 2164–2169.

Hutchinson, F., 1996 Mutagenesis, pp. 2218–2235 in *Escherichia coli and Salmonella: Cellular and Molecular Biology*, edited by F. C. Neidhardt, R. Curtiss, III, J. L. Ingraham, E. C. C. Lin, K. B. Low *et al.* American Society of Microbiology, Washington, DC.

Jayaraman, R., 1992 Cairnsian mutagenesis in *Escherichia coli*: genetic evidence for two pathways regulated by *mutS* and *mutL* genes. J. Genet. **71:** 23–41.

Kleman, G. L., and W. R. Strohl, 1994 Acetate metabolism by *Escherichia coli* in high-cell-density fermentation. Appl. Environ. Microbiol. **60:** 3952–3958.

Lenski, R. E., M. Slatkin and F. J. Ayala, 1989 Mutation and

selection in bacterial populations: alternatives to the hypothesis of directed mutation. Proc. Natl. Acad. Sci. USA **86:** 2775–2778.

Lesley, J. A., and C. D. Waldburger, 2003 Repression of *Escherichia coli* PhoP-PhoQ signaling by acetate reveals a regulatory role for acetyl coenzyme A. J. Bacteriol. **185:** 2563–2570.

Mackay, W. J., S. Han and L. Samson, 1994 DNA alkylation repair limits spontaneous base substitution mutations in *Escherichia coli*. J. Bacteriol. **176:** 3224–3230.

Maki, H., 2002 Origins of spontaneous mutations: specificity and directionality of base-substitution, frameshift, and sequence-substitution mutageneses. Annu. Rev. Genet. **36:** 279–303.

Miller, J. H., 1992 *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Miller, J. H., 1996 Spontaneous mutators in bacteria: insights into pathways of mutagenesis and repair. Annu. Rev. Microbiol. **50:** 625–643.

Modrich, P., 1991 Mechanisms and biological effects of mismatch repair. Annu. Rev. Genet. **25:** 229–253.

Najrana, T., Y. Saito, F. Uraki, K. Kubo and K. Yamamoto, 2000 Spontaneous and osmium tetroxide-induced mutagenesis in an *Escherichia coli* strain deficient in both endonuclease III and endonuclease VIII. Mutagenesis **15:** 121–125.

Piegorsch, W. W., and A. J. Bailer, 1994 Statistical approaches for analyzing mutational spectra: some recommendations for categorical data. Genetics **136:** 403–416.

Radman, M., and R. Wagner, 1986 Mismatch repair in *Escherichia coli*. Annu. Rev. Genet. **20:** 523–538.

Rebeck, G. W., and L. Samson, 1991 Increased spontaneous mutation and alkylation sensitivity of *Escherichia coli* strains lacking the *ogt* O^6-methylguanine DNA repair methyltransferase. J. Bacteriol. **173:** 2068–2076.

Reddy, M., and J. Gowrishankar, 1997a A genetic strategy to demonstrate the occurrence of spontaneous mutations in nondividing cells within colonies of *Escherichia coli*. Genetics **147:** 991–1001.

Reddy, M., and J. Gowrishankar, 1997b Identification and characterization of *ssb* and *uup* mutants with increased frequency of precise excision of transposon Tn*10* derivatives: nucleotide sequence of *uup* in *Escherichia coli*. J. Bacteriol. **179:** 2892–2899.

Rosenberg, S. M., 2001 Evolving responsively: adaptive mutation. Nat. Rev. Genet. **2:** 504–515.

Rupp, W. D., 1996 DNA repair mechanisms, pp. 2277–2294 in *Escherichia coli and Salmonella: Cellular and Molecular Biology*, edited by F. C. Neidhardt, R. Curtiss, III, J. L. Ingraham, E. C. C. Lin, K. B. Low *et al.* American Society of Microbiology, Washington, DC.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Saumaa, S., A. Tover, L. Kasak and M. Kivisaar, 2002 Different spectra of stationary-phase mutations in early-arising versus late-arising mutants of *Pseudomonas putida*: involvement of the DNA repair enzyme MutY and the stationary-phase sigma factor RpoS. J. Bacteriol. **184:** 6957–6965.

Schaaper, R. M., and R. L. Dunn, 1991 Spontaneous mutation in the *Escherichia coli lacI* gene. Genetics **129:** 317–326.

Schaaper, R. M., B. M. Danforth and B. W. Glickman, 1986 Mechanisms of spontaneous mutagenesis: an analysis of the spectrum of spontaneous mutation in the *Escherichia coli lacI* gene. J. Mol. Biol. **189:** 273–284.

Schaaper, R. M., R. L. Dunn and B. W. Glickman, 1987 Mechanisms of ultraviolet-induced mutation. Mutational spectra in the *Escherichia coli lacI* gene for a wild-type and an excision-repair-deficient strain. J. Mol. Biol. **198:** 187–202.

Slechta, E. S., J. Harold, D. I. Andersson and J. R. Roth, 2002 The effect of genomic position on reversion of a *lac* frameshift mutation (*lacIZ33*) during non-lethal selection (adaptive mutation). Mol. Microbiol. **44:** 1017–1032.

Slupska, M. M., C. Baikalov, R. Lloyd and J. H. Miller, 1996 Mutator tRNAs are encoded by the *Escherichia coli* mutator genes *mutA* and *mutC*: a novel pathway for mutagenesis. Proc. Natl. Acad. Sci. USA **93:** 4380–4385.

Stahl, F. W., 1988 A unicorn in the garden. Nature **335:** 112–113.

Taddei, F., I. Matic and M. Radman, 1995 cAMP-dependent SOS induction and mutagenesis in resting bacterial populations. Proc. Natl. Acad. Sci. USA **92:** 11736–11740.

Taddei, F., J. A. Halliday, I. Matic and M. Radman, 1997 Genetic analysis of mutagenesis in aging *Escherichia coli* colonies. Mol. Gen. Genet. **256:** 277–281.

Taverna, P., and B. Sedgwick, 1996 Generation of an endogenous DNA-methylating agent by nitrosation in *Escherichia coli*. J. Bacteriol. **178:** 5105–5111.

Vidal, A., N. Abril and C. Pueyo, 1998 DNA sequence analysis of spontaneous *lacI*$^{-d}$ mutations in O6-alkylguanine-DNA alkyltransferase-proficient and -deficient *Escherichia coli*. Mutagenesis **13:** 367–373.

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