Enrichment and Elimination of *mutY* Mutators in *Escherichia coli* Populations

Lucinda Notley-McRobb, Shona Seeto and Thomas Ferenci

School of Molecular and Microbial Biosciences, University of Sydney, Sydney, New South Wales 2006, Australia

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ABSTRACT

The kinetics of mutator sweeps was followed in two independent populations of *Escherichia coli* grown for up to 350 generations in glucose-limited continuous culture. A rapid elevation of mutation rates was observed in both populations within 120–150 generations, as was apparent from major increases in the proportion of the populations with unselected mutations in *fluA*. The increase in mutation rates was due to sweeps by *mutY* mutators. In both cultures, the enrichment of mutators resulted from hitchhiking with identified beneficial mutations increasing fitness under glucose limitation; *mutY* hitchhiked with *mgl* mutations in one culture and *ptsG* in the other. In both cases, mutators were enriched to constitute close to 100% of the population before a periodic selection event reduced the frequency of unselected mutations and mutators in the cultures. The high proportion of mutators persisted for 150 generations in one population but began to be eliminated within 50 generations in the other. The persistence of mutator, as well as experimental data showing that *mutY* bacteria were as fit as near-isogenic *mutY*+ bacteria in competition experiments, suggest that mutator load by deleterious mutations did not explain the rapidly diminishing proportion of mutators in the populations. The nonmutators sweeping out mutators were also unlikely to have arisen by reversion or antimutator mutations; the *mutY* mutations were major deletions in each case and the bacteria sweeping out mutators contained intact *mutY*. By following *mgl* allele frequencies in one population, we discovered that mutators were outcompeted by bacteria that had rare *mgl* mutations previously as well as additional beneficial mutation(s). The pattern of appearance of *mutY*, but not its elimination, conforms to current models of mutator sweeps in bacterial populations. A mutator with a narrow mutational spectrum like *mutY* may be lost if the requirement for beneficial mutations is for changes other than GC → TA transversions. Alternatively, epistatic interactions between mutator mutation and beneficial mutations need to be postulated to explain mutator elimination.

The frequency of DNA repair mutants is relatively high in many bacterial populations, and ~1–2% of *Escherichia coli* and *Salmonella enterica* isolates are reported to exhibit mutator activity (Leclerc et al. 1996). The proportion was even higher in urinary tract isolates (Denamur et al. 2002). These high values are not restricted to enteric organisms: Pseudomonas populations involved in cystic fibrosis contained ~20% mutators (Oliver et al. 2000). There is increasing interest in explaining the frequency of mutators in bacterial populations. Their enrichment is explained as a result of second-order selection (Taddei et al. 1997; Rainey 1999; Radman et al. 2000) and, experimentally, mutators are co-enriched through selection for beneficial mutations. Three repeated rounds of selection for different mutations are sufficient to convert a nonmutator population into a predominantly mutator one (Mao et al. 1997). Population parameters affecting mutator spread have also been extensively modeled (Tenaillon et al. 1999). These approaches all suggest that any environment needing mutations for increased fitness would result in enrichment of mutators, particularly if multiple rounds of mutation are involved.

The adaptation of *E. coli* to prolonged nutrient limitation is one situation where multiple mutations are essential (Notley-McRobb and Ferenci 1999a,b, 2000a). Adaptation under glucose limitation is associated with beneficial mutations in at least five genes within 200 generations of continuous culture. Given the secondary enrichment of mutators discussed above, it should follow that mutators should be enriched under extended nutrient-limited conditions. Early competition experiments already showed that mutators outcompete nonmutators when inoculated into glucose-limited chemostats (Chao and Cox 1983; Trobner and Piechocki 1984a) and, indeed, recent results suggest that mutators appear in glucose-limited populations even without seeding with mutators (Notley-McRobb et al. 2002b). The adaptation of *E. coli* recycled in batch cultures for thousands of generations has also revealed that mutators became enriched in experimental populations subject to continued selection (Sniegowski et al. 1997). Despite these examples, the trajectories of mutator appearance and disappearance have not been well documented in experimental populations. In this study, we provide detailed...
population changes involving mutY mutator sweeps, identify the molecular basis of hitchhiking of mutY, and describe its eventual elimination from two populations.

In contrast to the appearance of mutators, their disappearance from populations is less well investigated. Given that high mutation rates are detrimental to bacteria in terms of random lethal and disadvantageous changes (TROBNER and PIECHOCKI 1984b), and given presumed evolutionary selection against high mutation rates (KIMURA 1967), there are good reasons for eliminating mutator mutations from bacterial populations. According to DRAKE (1991), a nearly invariant microbial mutation rate appears to be determined by deep general forces, perhaps by a balance between the usually deleterious effects of mutation and the physiological costs of further reducing mutation rates. The load of detrimental mutations could indeed be a reason why mutators are outcompeted in experimental populations. This notion is readily tested in E. coli populations adapting to glucose limitation. The transience of mutator sweeps was indeed observed in two populations reported here, and we analyze the events leading to the lack of persistence of mutY mutators. Attempts have been made to model the transience of mutator sweeps (TADDEI et al. 1997; TENAILLON et al. 1999) but few data are available to compare modeling results with genuine experimental data.

The mutator gene studied, mutY, is responsible for preventing the GC → TA transversions due to G oxidation in aerobic environments (NGHiem et al. 1988). First, the preponderance of these particular base changes in several glucose-limited populations of E. coli, cultured for prolonged periods in chemostats (NOTLEY-McROBB and FERENC 1999a,b, 2000a), led to the identification of mutY mutators in these populations (NOTLEY-McROBB et al. 2002b). The analysis of mutY sweeps now permits a description of the kinetics of mutator enrichment and the comparison of the pattern to models of population changes (TADDEI et al. 1997; TENAILLON et al. 1999). Second, the question of the secondary enrichment of mutators could be directly tested by identification of mutations beneficial under glucose limitation linked to mutator hitchhiking. Finally, the elimination of mutators is considered in light of the observed transient nature of mutY sweeps.

MATERIALS AND METHODS

Bacterial strains: All bacterial strains used in this study are derivatives of E. coli K12. The two populations described here were inoculated with strains BW2952 (F− araD139 Δ(argF-lac) U169 rpsL150 deoC1 relA1 thiA lacZΔ25 fhuA1 phoB5301 [mgl(lacZ)Δ]) and BW3468, respectively (BW2952 and BW3468 were obtained by spreading 250 μl of the overnight cultures into 4 ml Luria broth (LB). After overnight growth at 37°C, cultures were harvested during mid-exponential growth. TA plates were incubated overnight at 37°C. Second, the question of the secondary enrichment of mutators can be directly tested by identification of mutations beneficial under glucose limitation linked to mutator hitchhiking. Finally, the elimination of mutators is considered in light of the observed transient nature of mutY sweeps.

Culture conditions: The basal salts medium used in all experiments was minimal medium A (MILLER 1972) supplemented with glucose, lactate, or glycerol as specified for each experiment. Batch cultures contained 0.4% (w/v) sugar, unless otherwise specified, and were harvested at mid-exponential growth. TA plates contained 5 g/liter NaCl, 10 g/liter tryptone, and 1.5% (w/v) agar.

Eighty-milliliter glucose-limited chemostats were set up as previously described (DEATH et al. 1993). Glucose-limiting chemostats had 0.02% (w/v) glucose in the feed medium and were run at dilution rates of 0.5 hr−1. Population 21 was started with strain BW2952 and population L3 was started with strain BW3143. The chemostats were maintained for several weeks and populations were sampled regularly for 250 generations and 350 generations, respectively, at which point the cultures were terminated by technical breakdowns. During this period, 10 ml of culture was collected aseptically from the overflow port and was directly subject both to the T5 resistance assay and a portion stored in 40% (w/v) glycerol at −70°C for later use. Samples were streaked on nonselective nutrient agar and randomly separated colonies were purified and numbered (e.g., sampling day 1, A; day 2, B) for further testing. At least eight random colonies were tested for the phenotypic properties below.

Competition studies between mutY+ and mutY− strains (BW3490 and BW3468, respectively) were performed by mixing in chemostats as described in NOTLEY-McROBB and FERENC (1999a).

T5 phage resistance assays: The frequency of T5-resistant mutants in a population in the flhA gene was determined by the method of HELLING et al. (1987). Unseparated samples from the chemostat population were mixed directly with a suspension of bacteriophage T5 (kindly supplied by K. Heller) at a multiplicity of 50 per cell. Generally, 100 μl of chemostat sample (2–3 × 107 bacteria) was mixed directly with 100 μl of T5 phage (≥106 pfu), except when the proportion of T5-resistant mutants rose, in which case a 10-fold dilution of the chemostat sample was required. The mixed suspensions were held on ice for 15 min to allow absorption, before spreading 50-μl aliquots in duplicate onto TA plates containing 5 mM CaCl2 and incubating overnight at 37°C.

Mutator assay: For initial qualitative screening of elevated mutation frequencies in isolates, a single colony of each isolate to be tested was inoculated into 4 ml Luria broth (LB). After overnight growth at 37°C, cultures were centrifuged for 10 min at 4000 × g and resuspended in 0.5 ml LB. A total of 10 μl of the suspension (~105 bacteria) was spotted (nine spots per plate) on LB agar containing rifampicin at 100 μg/ml. The plates were incubated overnight at 37°C and the number of rifampicin-resistant colonies in each spot was counted. Isolates producing <10 rifampicin-resistant colonies per spot were considered to have wild-type mutation rates, while >50 colonies were indicative of a mutator phenotype. The latter isolates as well as those showing intermediate numbers were restested at least twice to exclude the possibility of random jackpot events. A more quantitative estimate of mutation frequency was obtained by spreading 250 μl of the overnight cultures of individual isolates (or less in the case of high mutation rates) onto rifampicin plates as well as onto nutrient agar plates at the appropriate dilutions to determine total counts.

Phenotypic tests for mgl, psOG, mlc, and mutT mutations: To detect mgl-con mutations, the initial rate of uptake of 1 μM [U-14C]galactose by glycerol-grown chemostat isolates was determined using bacteria resuspended to an A660 of 0.2 as described previously (DEATH et al. 1993).

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For *ptsG* and *mlc* changes, the increased sensitivities to the *PtsG* and *PtsM* substrates, methyl-a-glucoside and 2-deoxyglucose, respectively, were determined by plating lactate-grown isolates onto glycerol minimal agar plates (Curits and Epstein 1975) overlaid with a 6 mm disc containing 10 μl 20% w/v methyl-a-glucoside or 20% w/v 2-deoxyglucose. Zones of inhibition were measured after overnight incubation at 37°C.

For *mutY*, β-galactosidase activity of the *mutGlacZ* fusion was measured on glycerol-grown isolates treated with chloroform and SDS by the method of Miller (1972).

**Mutation analysis:** Sequencing of the 1431-bp *ptsG* sequence, 318 bp of its upstream regulatory region, the *mglD/gadB* sequence, and the *mgl* operator was performed using the methods previously described (Manche et al. 1999; Notley-McRobb and Ferenci 1999a). The deletion in *mutY* was monitored by PCR analysis using the primers F5 and R5, which generate a 4.22-kb fragment including *mutY* in wild-type bacteria (Notley-McRobb et al. 2002b).

**RESULTS**

**Enrichment of mutators in *E. coli* chemostat populations:** In an earlier study, we demonstrated that the elevation of mutation frequencies was due to major (>7 kb) deletions in bacteria in four separate glucose-limited populations (Notley-McRobb et al. 2002b). The deletion in each case completely eliminated the *mutY* gene. To follow the population changes leading to the widespread appearance of *mutY* mutations in two of these populations, we fully analyzed the periodic selection events and the time course of accumulation of mutators and their eventual elimination, as shown in Figure 1. An obvious symptom of mutator activity in both populations was the elevation of the proportion of bacteria with unselected mutations. In cultures with a low proportion of mutators, as in the first 100 generations (Figure 1), spontaneous resistance to T5 arises in bacteria at a frequency under $5 \times 10^{-6}$. The *fhuA* mutations causing T5 resistance are selectively neutral and so do not overrun the populations; the frequency is determined by the rate of appearance of mutations balanced by periodic selection events reducing T5 resistance through spread of T5-resistant bacteria with beneficial mutations. In both chemostats L3 and 21, the frequency of T5 resistance dramatically increased to 3 or $4 \times 10^{-4}$ at points K and N in Figure 1, A and B, respectively. This elevation of the proportion of T5-resistant mutants was due to increased mutation rates in the populations; it coincided with mutator appearance, as evidenced by the screening of isolates for mutators in each population using the rifampicin resistance assay (Figure 1, C and D). Consistent with the spread of mutators, mutation frequencies of isolates to Rif resistance were all $>2 \pm 1 \times 10^{-3}$ before elevation of T5 resistance but 30- to 50-fold higher in isolates obtained at points O and Q in populations L3 and 21, respectively. At these points, all the tested members of these populations were mutators as shown in Figure 1, C and D. The increase and decrease in the distribution of T5 resistance was mirrored by the increase and decrease in the proportion of mutators in both populations.

The enrichment of mutators is generally ascribed to secondary selection in bacterial populations (Mao et al. 1997; Radman et al. 2000). The coselection of mutators with beneficial mutations was tested by screening of isolates for mutations favored under glucose limitation, namely, in *mgl*, *mutT*, *mlc*, *ptsG*, and *rpoS* (Manche et al. 1999; Notley-McRobb and Ferenci 1999a,b, 2002a). As shown in Figure 1, C and D, the mutator spread was associated with sweeps by one of these beneficial mutations in each L3 and 21 population. In L3, the enrichment of *ptsG* mutants closely followed the mutator appearance. The *ptsG* mutation was sequenced in representative isolates and found to be a transversion leading to either V12F or G13C substitutions in the *PtsG* protein (Figure 2). These structural mutations are commonly selected because they lead to elevated glucose transport (Manche et al. 1999; Notley-McRobb and Ferenci 2000b). The DNA change in both types of *ptsG* mutant was consistent with *mutY*-dependent elevation of transversions. The simplest explanation of these data is that a spontaneous mutator mutation led to the appearance of the rare *ptsG* point mutations and subsequent hitchhiking of the mutator with the beneficial mutation under continued glucose limitation. One complication was that a minority of the *ptsG* mutations was not associated with mutator at points M and N (25 and 15%, respectively). These mutations are largely supplanted by mutator-*ptsG* isolates at points O–S, but may be the progenitors of the later isolates at c–e that lead to elimination of mutator. Presumably, the predominance of mutators by point O was the result of further, unidentified beneficial mutations generated in the mutator isolates, which allowed mutators to outcompete nonmutators with *ptsG* mutations by point O.

In the second population, the increased proportion of mutators occurred simultaneously with an *mgl* sweep. As shown in Figure 1B, close to 100% of tested members of the population were both a mutator and a carrier of *mglD* or *mgl* operator mutations at points Q and R. Hence most genomes carried both a mutator and a beneficial mutation. But, as above, not all *mgl* mutations were associated with a mutator at points O and P and the *mgl* frequency at point P exceeded the mutator frequency. Hence the population contained a coexistence of several *mgl* alleles, some associated with mutator, some not. As shown in Figure 3, at point O, the nonmutators carried *mgl* mutations (a G → A substitution and a ΔG frameshift) not obviously consistent with a *mutY*-related spectrum. Later, at point R, three common *mgl* mutations were found by sequencing (H122Q, A296E, and A11D), each with a C → A base change and linked to a *mutY* defect. The G → A substitution and a ΔG frameshift coexisted with the *mutY*-associated changes in isolates at point R. Given that both mutator and nonmutator *mgl* mutations coexisted at point O and P,
what led to the predominance of mutators at points Q–R in Figure 1, B and D. Probably, as in L3 with \( \text{ptsG} \) mutations, it is necessary to postulate that a further beneficial mutation besides \( \text{mgl} \) occurred to help the mutator-\( \text{mgl} \) combination overhaul the nonmutator \( \text{mgl} \) isolates. If so, this other beneficial change was not in \( \text{malT}, \text{mlc}, \text{rpoS}, \text{or ptsG} \) in the P–R samples (results not shown). Despite this complexity, the chromosomal linkage of the beneficial mutation in \( \text{mgl} \) and the mutator in the majority of isolates at point Q is consistent with the notion that mutator enrichment is a secondary selection based on hitchhiking with beneficial mutation(s). 

**The loss of mutators from chemostat populations:** An interesting property of both studied populations is that the mutator abundance was not maintained permanently. Once acquired, the \( \text{mgl} \) mutation frequency in population 21 remained at close to 100% and was still at 100% after the proportion of mutators began to drop.
at points R–V (Figure 1B). Likewise, as is obvious from isolates at point e in the L3 population in Figure 1A, the ptsG mutations became unlinked from mutators. Possible reasons for the shift to mgl mutY+ or ptsG mutY+ bacteria are enrichment of mutY revertants or suppressor mutations. Reversion of mutY or lateral acquisition of wild-type mutY seems unlikely, given the size of the deletions in these strains (Figure 4 and Notley-McRobb et al. 2002b) and the lack of known gene transfer mechanisms in these F− bacteria. As shown in the PCR analysis in Figure 4, the mutY deletions prevalent in population 21 at point Q were replaced at point V by isolates with a wild-type pattern around mutY, so phenotypic suppression of the deletion was also unlikely to be the explanation for elimination of mutator.

A frequently mentioned factor in the prevalence of mutators is mutator load of deleterious mutations (Kimura 1967). An overload of deleterious mutations may drive selections to loss of mutator. To test whether the load resulting from the large mutY deletion was itself a factor in the elimination of mutY mutations, competition experiments were carried out between mutY− bacteria extracted at point Q and derivatives made mutY+ by transduction with a linked transposon transferred from a mutY+ strain. As shown in Figure 5, the mutY mutation was not eliminated over a period corresponding to the interval between points R and V in Figure 1B. Hence mutator/deletion load is unlikely to explain the reduction in the proportion of mutator isolates.

A remaining explanation for the reduction in mutator frequency was a periodic selection event due to sweeps by mgl and ptsG mutants originally in the minority, nonmutator part of the population at point R in 21 or Y in L3. The mgl allelic shifts observed in Figure 3 at different points in population 21 support this notion. The fitter bacteria that sweep out mutators between R and V contained A252E and Y151 frameshift mutations in mglD not present before point R that are nonmutators. Isolates with mutator-associated mgl mutations (A296E, A11D, and H122Q), which are in the majority at point R, were largely eliminated by point V. The A11D and A296E mutations also constituted five of seven clones sequenced from point Q (results not shown). The A252E and Y151 alleles of mutY must have been enriched

**Figure 3.**—Sequence changes in mgl in population 21. At each time point indicated, corresponding to the samples in Figure 1B, 9–12 isolates were sequenced for the mgl mutations in population 21 by methods previously described (Manche et al. 1999; Notley-McRobb and Ferenci 1999a). The mutations were either in the operator (mglO) or in substitutions in the repressor mglD. The mgl mutations underlined were associated with mutator backgrounds. Isolates not underlined had a normal mutY band as shown in Figure 4.

**Figure 4.**—The nature of the mutY mutation during the progress of population 21. PCR analysis using two pairs of primers, F5 and R5 (Notley-McRobb et al. 2002b), was carried out to detect changes in the mutY region in isolates obtained at the points corresponding to those shown in Figure 1B.
known to require polygenic mutations for increased fitness (Notley-McRobb and Ferenci 1999b) and indeed provided an ideal situation to observe the enrichment of mutator. The mutY sweeps studied here led to a near-total spread in the populations, as predicted for weak or intermediate-strength mutators like mutY (Taddei et al. 1997).

The results of mutY appearance and elimination can be considered in terms of an evolutionary adaptive landscape. The fitness peaks appearing with the beneficial mutations at points O and R in L3 and 21, respectively, were mainly due to DNA point mutations facilitated by mutator activity. Once widespread in population L3, the mutator persisted through several periodic selection events, indeed over >100 generations. The fitness peaks in this interval, revealed by periodic selection events in L3, were reached either through further mutY-stimulated mutations or through mutations with other mutational spectra; these changes were not identified. Given that the majority of bacteria were altered in mutY, new beneficial mutations were highly likely to occur in this mutY background, whether with the mutY spectrum or not. Also, competition experiments suggested that the mutY deletion was not a negative fitness load on these bacteria, so there was no major selection for loss of mutator.

A further conclusion is that the intermediate fitness peaks associated with mutator were cut off from the final fitness peaks in these experiments. The elimination of mutators on the way to the final fitness peak in populations L3 and 21 is caused by organisms coexisting, but in the minority, during the earlier mutator-inspired peaks. The sweep leading to elimination of the mutator was most likely through acquisition of a new, unidentified mutation leading to the final periodic selection events shown in Figure 1. The final sweep was by organisms that obtained the beneficial mutations in mgl or ptsG, but through nonmutator spontaneous mutations. These arose before or during the earlier, mutator-inspired peaks but reached the final peak and became a majority through acquisition of new, unidentified mutation(s). The tempting conclusion is that the last peak cannot be reached through a mutY-inspired transversion event; otherwise it would have preferentially occurred in the mutY majority. The new mutation may require other base changes or deletions, amplifications, or frameshifts. The requirement for these other classes of mutations may provide an explanation for the elimination of mutY. Indeed, for any mutator with a narrow mutational spectrum, elimination by fitter bacteria with other types of mutations is a distinct possibility.

An alternative explanation for elimination of mutY mutations may be that some mutations appearing easily in the mutY backgrounds are incompatible with other less frequent but more beneficial mutations. This explanation would mean that the mutY mutator population is stuck in a local adaptive peak due to epistatic interac-

**DISCUSSION**

The secondary enrichment of mutators is closely associated with selection for multiple mutations (Mao et al. 1997). The nutrient-limited chemostat environment was....
tions between different mutations. Given that we do not know all the mutations on the path to fitness, the possibility of epistatic interactions cannot be excluded.

How do these results conform to modeling predictions of the fixation of mutators in bacterial populations (Taddei et al. 1997; Tenaillon et al. 1999)? It needs to be noted that the constant population size in our experiments was $1.6 \times 10^{10}$ bacteria. The population size is a parameter predicted to have a major influence on mutator spread (Taddei et al. 1997; Tenaillon et al. 1999). Also, the mutator strength is an important factor, which for mutY is $\sim 40$-fold (Notley-McRobb et al. 2002b).

Integrating these parameters into published models permits a comparison with the real data in Figure 1.

The frequency of the fixation of the mutator allele was predicted to occur in 20–80% of populations containing $10^{10}$ bacteria and with mutator strengths of 10 and 100, respectively (Tenaillon et al. 1999). Experimentally, the mutY fixation was found not only in the two populations described here in detail, but also in 4 of 11 populations initially screened (Notley-McRobb et al. 2002b). Hence the model accurately predicted the likely occurrence of mutators, which was in 36% of cultures with mutY with the intermediate 40-fold mutator strength.

The mutator frequency approached 100% in our two populations, both in $< 200$ generations. The prediction of 100% fixation of mutators was indeed made for 10- and 100-fold mutators (Taddei et al. 1997) but the time of fixation was highly variable in repeat runs of the model (Tenaillon et al. 1999). We do not have enough data sets to make firm conclusions about the time of fixation of mutators, but the Tenaillon model still fits with our results in this respect.

Less consistent with the predictions was the elimination of mutator in our populations. With 10-fold mutators, the prediction was that mutators would be maintained in high proportion over thousands of generations (Taddei et al. 1997). The elimination of mutator was predicted for 100-fold mutators, but only after $\sim 600$ generations. In reality, the elimination of mutY mutators was much faster than predicted and began within 50–150 generations. In our case, mutator load or reversion or antimutator mutations could all be eliminated as driving forces for mutY elimination. Also relevant is that the elimination of the mutS mutation from one glucose-limited population also occurred much more quickly than was modeled and was within 20 generations (Notley-McRobb and Ferenci 2000a).

The experimental results suggest that the assumptions built into the model need to be reviewed before accurate prediction of in vivo mutator elimination is possible. The nature of the adaptive landscape is probably more complex than that of the one modeled so far in the study of mutators. The multiplicity of adaptive peaks, the spectrum of mutations, and interactions between beneficial mutations could have a strong effect on the fate of mutators in large populations. Certainly, bacterial populations contain enough genetic heterogeneity to offer rare combinations of beneficial mutations the chance to rapidly sweep and eliminate mutators. The multiplicity of mgl alleles, the coexistence of mutators and nonmutators, and the possibility of multiple mutators in one population all suggest the existence of a complex pool of alternative solutions to overcoming adaptive hurdles.

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