

Mismatch repair protein MutL becomes limiting during stationary-phase mutation

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Postsynthesis mismatch repair is an important contributor to mutation avoidance and genomic stability in bacteria, yeast, and humans. Regulation of its activity would allow organisms to regulate their ability to evolve. That mismatch repair might be down-regulated in stationary-phase *Escherichia coli* was suggested by the sequence spectrum of some stationary-phase ("adaptive") mutations and by the observations that MutS and MutH levels decline during stationary phase. We report that overproduction of MutL inhibits mutation in stationary phase but not during growth. MutS overproduction has no such effect, and MutL overproduction does not prevent stationary-phase decline of either MutS or MutH. These results imply that MutS and MutH decline to levels appropriate for the decreased DNA synthesis in stationary phase, whereas functional MutL is limiting for mismatch repair specifically during stationary phase. Modulation of mutation rate and genetic stability in response to environmental or developmental cues, such as stationary phase and stress, could be important in evolution, development, microbial pathogenicity, and the origins of cancer.

[Key Words: Mismatch repair; MutL; mutation; adaptive mutation; genetic instability; evolution; stationary phase]

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In *Escherichia coli* mismatch repair is the single largest contributor to avoidance of mutations due to DNA polymerase errors in replication (Radman 1988; Modrich 1991). Mismatch repair also promotes genetic stability by editing the fidelity of genetic recombination and transposon excision, and by the involvement of its component proteins in transcription-coupled DNA repair and very-short-patch repair (Radman 1988; Modrich 1991; Lieb and Shehnaz 1995; Mellon and Champe 1996). The mismatch repair proteins are highly conserved throughout evolution and appear to play roles in simple and complex eukaryotes similar to those that they play in bacteria (Reenan and Kolodner 1992; Modrich 1994; Baker et al. 1995, 1996; de Wind et al. 1995; Datta et al. 1996; Hunter et al. 1996; Kolodner 1996). These proteins act on incorrectly paired and unpaired bases in DNA that arise via DNA synthesis errors, recombination of diverged sequences, and DNA damage. In all of these circumstances, mismatch repair enforces genetic stability. The consequences of failing to maintain this enforcement are profound for speciation (Rayssiguier et al. 1989; Radman and Wagner 1993; Matic et al. 1995; Hunter et al. 1996; Zahrt and Maloy 1997) and for formation of cancers (Modrich 1994, 1995; Kolodner 1996).

Four proteins play critical roles in mismatch repair in *E. coli* (for review, see Modrich 1991). MutS binds to DNA base mismatches, to insertion/deletion single-strand loops of four or fewer nucleotides, which are intermediates in frameshift mutation, and probably also to sites of DNA damage (Mello et al. 1996). MutL interacts with MutS after mismatch binding and is thought to coordinate MutS with MutH. MutH endonuclease then nicks the unmethylated (new) DNA strand of a nearby hemimethylated GATC sequence. GATCs are hemimethylated during DNA replication because the new strand is transiently unmethylated. MutU helicase enters DNA at the single-strand nick and displaces the nicked strand, which may or may not be degraded during displacement (see R.S. Harris, K.J. Ross, M.-J. Lombardo, and S.M. Rosenberg, unpubl.). DNA polymerase III then resynthesizes the DNA, thus correcting the sequence. In recombination, the mismatches or small loops are caused by formation of heteroduplex DNA between non-identical sequences rather than by replication errors as just described.

Although central to maintenance of genetic stability, little is known about whether the mismatch repair system might be regulated (but see Stahl 1988; Rayssiguier et al. 1989; Hastings and Rosenberg 1992; Rosenberg 1994, 1997; Longrich et al. 1995; Rosenberg et al. 1995, 1996 for hypotheses). If mismatch repair were regulated, then cells would regulate their potential to evolve. Two lines of work from *E. coli* have converged on the first

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evidence in any organism suggesting that mismatch repair proteins might be regulated and, more specifically, have suggested the down-regulation of mismatch repair during the differentiated states of stationary phase and nutritional stress.

First, stationary-phase reversions of a *lac* +1 frameshift mutation in *E. coli* (Cairns and Foster 1991) appear to be DNA polymerase errors that escape mismatch repair. The stationary-phase reversion mechanism in the *lac* frameshift assay system is distinct from growth-dependent Lac reversion (Rosenberg 1994, 1997; Rosenberg et al. 1995, 1996) in that the former includes homologous recombination (Harris et al. 1994, 1996; Foster et al. 1996) and produces mutations with a highly distinctive DNA sequence spectrum, mostly single-base deletions in small mononucleotide repeats (Foster and Trimarchi 1994; Rosenberg et al. 1994). This mutation spectrum is different from growth-dependent reversions of the same allele (Foster and Trimarchi 1994; Rosenberg et al. 1994) but is identical to growth-dependent reversions in mismatch repair-null mutant strains (Longerich et al. 1995). Thus, depressed mismatch repair could be responsible for the unique stationary-phase mutation spectrum. Most stationary-phase mutants are not heritably mismatch repair-defective (Longerich et al. 1995) [although some are (Torkelson et al. 1997)], indicating that any loss of mismatch repair during stationary-phase mutation must be transient. Such transient loss could occur either by down-regulation of the mismatch repair system or by a block at the DNA level, for example, either by under- or overmethylation of DNA sites required for operation of this methyl-directed repair system (Longerich et al. 1995).

Second, the recent discovery that MutS and MutH mismatch repair protein levels decrease in stationary phase and starving bacterial cells appears to support the hypothesis of down-regulation of mismatch repair at the protein level (Feng et al. 1996). However, although MutS and MutH protein levels decrease during stationary phase and starvation, so does DNA replication. Therefore, it is possible that the proper ratio of these proteins to replication errors is preserved, leaving mismatch repair functional in stationary-phase, starving cells.

If MutS, MutH, or any mismatch repair protein became limiting for mismatch repair function during stationary-phase mutation, then overproduction of the limiting mismatch repair protein might restore mismatch repair function and thereby decrease stationary-phase mutation. We report that overproduction of MutL has this effect, that overproduction of MutS does not, and that overproduction of MutL does not act indirectly by preventing the stationary-phase decline of MutS or MutH protein levels. Overproduction of MutL does not depress growth-dependent Lac reversion. The data imply that functional MutL protein becomes limiting specifically during stationary-phase mutation and that the decreased levels of MutS and MutH observed in stationary phase are appropriate and not limiting for the amount of DNA synthesis in stationary phase. These results imply a loss of mismatch repair function, at the level of MutL

protein, specifically during the differentiated state of stationary phase. This provides the first evidence in any organism that mismatch repair function is not constitutive but, rather, can be modulated by cell physiology and differentiation. Environmental change could promote genetic change by this route.

Results

The term stationary-phase mutation is used here to refer to what has also been called "adaptive mutation" (for review, see Foster 1993). This process occurs in stressed, starving cells and so may reflect stress responses in general. In the assay system used here—reversion of a *lac* frameshift mutation in *E. coli*—the stationary-phase mutations are also mechanistically distinct from growth-dependent reversions of the same allele. Unlike growth-dependent Lac reversion, the stationary-phase mutation mechanism (1) requires homologous recombination (Harris et al. 1994, 1996; Foster et al. 1996); (2) includes DNA double-strand breaks that are implicated as a molecular intermediate in the mutagenesis (Harris et al. 1994); and (3) produces mutations that are nearly all single-base deletions in small mononucleotide repeats, whereas growth-dependent Lac reversions are heterogeneous (Foster and Trimarchi 1994; Rosenberg et al. 1994). Furthermore, (4) this unique sequence spectrum is identical to that observed in mismatch repair-defective cells, implying failure of mismatch repair in stationary-phase mutation (Longerich et al. 1995); and (5) although this question has not been addressed for the growth-dependent mutations, the stationary-phase mutations have been shown to occur genome-wide, in a hypermutable subpopulation of the stressed cells (Torkelson et al. 1997). This specific mutation mechanism is called recombination-dependent stationary-phase mutation. It is proposed to occur by DNA polymerase errors occurring during DNA synthesis primed by recombinational strand-exchange intermediates, which form at DNA double-strand breaks, in cells with attenuated mismatch repair (for review, see Rosenberg 1994, 1997; Rosenberg et al. 1995, 1996) or in DNA substrates that are refractory to mismatch repair (Kuzminov 1995; Longerich et al. 1995).

Key features of recombination-dependent mutation are seen in some (Taddei et al. 1995) but not all other stationary-phase mutation systems assayed (e.g., Foster and Trimarchi 1995; Galitski and Roth 1995, 1996; Hall 1995; Radicella et al. 1995). For those systems with no known features distinct from growth-dependent mutation (e.g., Foster and Trimarchi 1995; Galitski and Roth 1995, 1996; Radicella et al. 1995), the stationary-phase mutations may occur by the same mechanism as growth-dependent mutation and not be a distinct phenomenon. For example, the cells may be growing or replicating DNA cryptically.

We tested whether mismatch repair proteins MutS, MutL, or both become limiting during recombination-dependent stationary-phase reversion of a *lac* frameshift mutation. If the mismatch repair proteins become lim-

iting transiently during stationary-phase mutation, then overproduction of those proteins from plasmids might be expected to restore mismatch repair function and inhibit formation of stationary-phase Lac⁺ revertants. To measure stationary-phase reversions of the *lac* frameshift mutation, the frameshift-bearing cells are spread on minimal lactose plates (see Materials and Methods; Cairns and Foster 1991; Harris et al. 1994, 1996). At about 2 days of incubation, growth-dependent revertant colonies appear. These are followed by stationary-phase revertant colonies that accumulate during the next several days (Cairns and Foster 1991; Foster 1993) and form via the different, recombination-dependent molecular mechanism (for review, see Rosenberg 1994, 1997; Rosenberg et al. 1995, 1996). Cells carrying a null mutation in the recombination gene *recA* form the initial growth-dependent mutations but then fail to accumulate any recombination-dependent Lac⁺ reversions over the next several days (Harris et al. 1994; see also Fig. 1A, below).

Overproduction of MutL inhibits stationary-phase Lac⁺ mutation

Data in Figure 1 show that overproduction of MutL mismatch repair protein from a multicopy plasmid depresses stationary-phase Lac reversion by about fourfold relative to that seen with a strain carrying a control plasmid. The results of multiple experiments of this type are compiled in Table 1. The depression is seen when MutL is over-

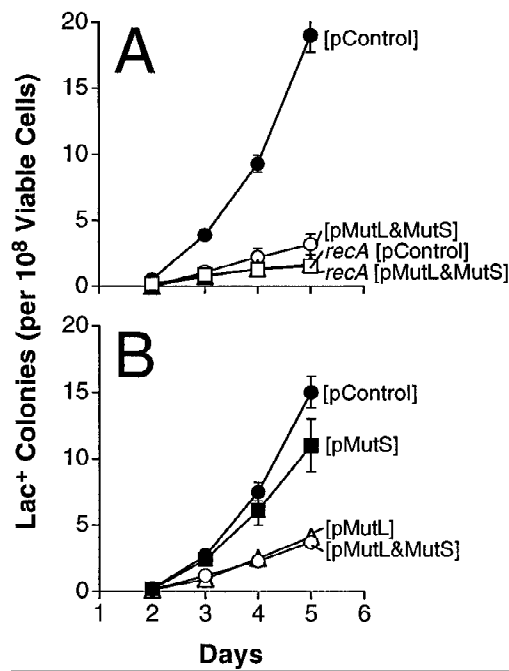


Figure 1. Overproduction of MutL alone, or with MutS, depresses stationary-phase Lac⁺ reversion. Plasmids [pControl], [pMutL&MutS], [pMutS], and [pMutL] are pSL4, pSL7, pSL6, and pSL5, respectively (Materials and Methods). Error bars represent one standard error of the mean (S.E.M.) and are smaller than the data point where not visible.

produced either alone or in combination with MutS and is not observed when only MutS is overproduced (Fig. 1B; Table 1). This indicates that MutL is limiting, or stabilizes another protein that is limiting, during stationary-phase mutation. The plasmid producing both MutL and MutS was used in many of the experiments reported here. However, the results in Figure 1B and Table 1 demonstrate that overproduction of MutL alone is sufficient to depress mutation.

Overproduction of MutL does not inhibit growth-dependent Lac⁺ mutation

To assess whether the effect of MutL overproduction on mutation is specific to stationary-phase mutation, the effects of MutL (and MutS) overproduction on growth-dependent reversion of the same *lac* frameshift allele were assessed in two ways.

First, the mechanism of stationary-phase, but not growth-dependent, Lac⁺ reversion in these strains requires recombination genes (Harris et al. 1994, 1996; Foster et al. 1996), including functional *recA*. Thus, one may examine growth-dependent Lac⁺ reversion in the absence of any contribution of the recombination-dependent mutation mechanism by using a *recA* null mutant strain. Data in Figure 1A show that the *recA* cells overproducing MutL (and MutS) display no decrease in *recA*-independent Lac⁺ mutation relative to cells carrying the control plasmid.

Second, data in Table 2 (experiments 1–3) show that growth-dependent Lac reversion rates are unaffected by overproducing MutL (and MutS), relative to the mutation rates in strains bearing the control plasmid.

These data appear to contrast with results from a previous study in which co-overproduction of MutS and MutL appeared to inhibit growth-dependent Lac reversion relative to a control plasmid-bearing strain (Foster et al. 1996). However, in that study all growth-dependent mutants were scored after 2 days. We found that cells carrying the MutS and MutL-overproducing plasmid take longer than 2 days to form colonies (Table 2). Therefore we scored the growth-dependent mutants of each strain after an experimentally determined incubation time specific for that strain (Table 2). When controlled for speed of colony formation in this way, no difference in growth-dependent reversion rates is detected between control and overproducing strains (Table 2). These results allow us to infer that mismatch repair protein MutL is limiting specifically during stationary-phase and not growth-dependent Lac reversion.

Mutants that display stationary-phase hypermutation show greater MutL-promoted depression of stationary-phase mutation

The apparent deficiency of functional MutL during stationary-phase Lac reversion (Fig. 1B; Table 1) might be partial rather than absolute. If so, then strains that are hypermutable for stationary-phase Lac mutation by vir-

Table 1. *MutL overproduction diminishes stationary-phase mutation*

Mismatch repair protein overproduced from plasmid	Experiment no.	Cumulative no. of Lac ⁺ colonies by day 5 per 10 ⁸ viable cells ^a (mean ± S.E.M.) ^b	Decrease in stationary-phase mutation relative to control plasmid-bearing strain	
			within each experiment	average (mean ± S.E.M.)
None (control plasmid)	1	3.3 ± 0.2	1	1
	2	11 ± 2.1	1	
	3	19 ± 1.3	1	
	4	5.8 ± 1.9	1	
	5	89 ± 8.6	1	
	6	15 ± 1.2	1	
	7	23 ± 2.6	1	
	8	18 ± 2.2	1	
	9	37 ± 3.6	1	
MutL	5	14 ± 2.0	6.4	4.0 ± 0.7
	6	4.1 ± 0.4	3.7	
	7	7.5 ± 0.7	3.1	
	8	8.0 ± 0.7	2.3	
	9	7.9 ± 0.8	4.7	
MutS	5	56 ± 5.2	1.6	1.3 ± 0.2
	6	11 ± 2.0	1.4	
	7	20 ± 2.4	1.2	
	8	23 ± 2.3	0.8	
	9	22 ± 5.8	1.7	
MutL & MutS	1	1.1 ± 0.2	3.0	3.9 ± 0.7
	2	1.8 ± 0.4	6.1	
	3	3.2 ± 0.8	5.9	
	4	1.8 ± 0.2	3.2	
	5	15 ± 1.7	5.9	
	6	3.7 ± 0.5	4.1	
	7	19 ± 1.9	1.2	
	8	19 ± 1.9	1.2	
	9	20 ± 2.2	1.9	

^aIn each experiment the mean number of Lac⁺ colonies was determined from 8 to 12 independent cultures of each strain. Jackpots of growth-dependent mutants were excluded from the calculations. Jackpots were defined as cultures containing a number of mutants greater than two standard deviations above the mean number of mutants calculated without that culture.

^b(S.E.M.) One standard error of the mean.

tue of creating more DNA polymerase errors might reduce mismatch repair protein levels further by titrating away the limiting protein. Because stationary-phase Lac reversion uses recombination functions, whereas growth-dependent reversion does not (Harris et al. 1994, 1996; Foster et al. 1996), this idea can be tested using *rec* mutants that are hypermutable specifically in stationary-phase Lac reversion. A hyper-recombinogenic and stationary-phase hypermutable *recD* mutant strain (Harris et al. 1994) was used. The data in Figure 2 show that overexpression of MutL (with MutS) causes a dramatic 15-fold reduction of stationary-phase Lac reversion in this strain, whereas growth-dependent Lac reversion is unaffected (Fig. 2B, *recA recD* results; Table 2, experiments 4–7).

A stationary-phase hypermutable *recG* strain (Foster et al. 1996; Harris et al. 1996) was affected similarly by overproduction of MutS plus MutL (Foster et al. 1996; data not shown). Foster et al. (1996) postulated a direct protein–protein interaction between RecG and the MutS and MutL proteins. Our results showing a similarly large

depression of stationary-phase Lac reversion by overproducing MutL and MutS in a *recD* strain argue against this interpretation because RecD and RecG act at different stages in recombination, on different DNA intermediates (Rosenberg and Hastings 1991; Kowalczykowski et al. 1994; Myers and Stahl 1994; West 1992, 1994). The data support the idea that the hypermutable *recD* and *recG* strains simply provide more recombination intermediates that are hypothesized to prime the DNA synthesis with polymerase errors, which leads to stationary-phase Lac reversion (Harris et al. 1994, 1996; Foster et al. 1996). The increased errors would reduce effective MutL levels further by titration.

This latter interpretation, that MutL mismatch repair protein is only partially limiting during stationary-phase mutation, is consistent with the observation that a *mutL*-defective strain shows *recA*-dependent stationary-phase hypermutation (Harris et al. 1997).

An alternative explanation for the observation that overproduction of MutL inhibits stationary-phase but not growth-dependent Lac⁺ mutation could be that MutL

Table 2. Mismatch repair proteins are not limiting during growth-dependent Lac⁺ mutation

Relevant genotype ^a	Number of hours to form a colony ^b	Experiment	Number of independent cultures ^c	Mutation rate (Lac ⁺ /cell per generation) × 10 ⁻¹⁰ ^c	Mean ± S.D.
<i>rec</i> ⁺ [pControl]	52	1	40	3.0	8.0 ± 5.6
	56	2	40	7.0	
	67	3	40	14	
<i>rec</i> ⁺ [pMutSL]	71	1	40	11	8.9 ± 1.9
	58	2	39	7.2	
	67	3	40	8.6	
Δ <i>recA</i> [pControl]	64	1	40	3.0	2.5 ± 1.4 ^d
	55	2	40	0.98	
	67	3	40	3.6	
Δ <i>recA</i> [pMutSL]	72	1	32	5.8	5.4 ± 0.6 ^d
	58	2	40	5.8	
	67	3	40	4.7	
<i>recD</i> [pControl]	54	4	40	16	19 ± 11 ^e
	62	5	40	11	
	52	6	40	34	
	52	7	40	13	
<i>recD</i> [pMutSL]	N.D.	4	N.D.	N.D.	13 ± 2.5 ^e
	70	5	40	9.6	
	61	6	31	14	
	77	7	40	14	
<i>recD</i> Δ <i>recA</i> [pControl]	54	4	40	6.2	5.3 ± 0.7 ^e
	58	5	40	4.5	
	53	6	40	5.1	
	69	7	40	5.3	
<i>recD</i> Δ <i>recA</i> [pMutSL]	60	4	40	5.7	9.6 ± 7.1 ^e
	61	5	40	2.8	
	58	6	38	11	
	79	7	40	19	

(N.D.) Not done.

^aSee Materials and Methods for names and constructions of plasmids.^bDetermined with 10–12 different Lac⁺ revertants of each genotype (except in experiment 4 in which six different revertants were used) as a *t*₅₀, the time at which half of the colony-forming units have produced visible colonies under experimental conditions.^cGrowth-dependent Lac⁺ reversion rates were measured (Harris et al. 1996) and mutation rates calculated by the method of the median (Lea and Coulson 1949; von Borstel 1978).^dThe apparent decrease in growth-dependent mutation in *recA* compared with *rec*⁺ is not statistically significant (see standard deviations on data from isogenic *recA* and *rec*⁺ strain pairs). Even with the large variance on these numbers, however, it is clear that there is no mutation-depressing effect of MutL overproduction in these growth-dependent reversion events (compare overproducing versus control plasmid in each otherwise isogenic pair) as there is in stationary-phase reversion (Table 1).^eThe *recD* strain displays stationary-phase Lac⁺ hypermutation (Harris et al. 1994) and also appears hypermutable in growth-dependent Lac⁺ reversion here. The apparent elevation of growth-dependent mutation in the stationary-phase hypermutable *recD* strain appears to be attributable to spillover of postplating, RecA-dependent stationary-phase revertants into growth-dependent revertant colony counts. This is indicated by the finding that the increase in *recD* is entirely *recA*⁺-dependent (experiments 4–7).

somehow inhibits homologous recombination, which is required for the stationary-phase mutation only (Harris et al. 1994, 1996; Foster et al. 1996). Measurements of the efficiencies of phage P1 transductional recombination showed no such inhibition by the MutL-overproducing plasmid (data not shown), thereby discouraging this alternative.

MutL overproduction does not inhibit mutation by preventing *MutS* or *MutH* decline during stationary phase and starvation

We wished to address the possibility that MutL overproduction might inhibit stationary-phase mutation by pre-

venting the reported declines in either MutS or MutH proteins in starving, stationary-phase cells (Feng et al. 1996). Therefore, we used quantitative Western blots to determine the levels of MutL, MutS, and MutH proteins in cells overproducing MutL, MutS, and MutL plus MutS during stationary-phase starvation on lactose medium and during growth.

The Western blots were as performed previously (Feng et al. 1996) with modifications (Materials and Methods; see Fig. 3 for representative blots). Figure 4 summarizes quantification of the amounts of MutL, MutS, and MutH proteins in growing and starved stationary-phase cells carrying the control and overproducing plasmids. The data are from three to five independent experiments for

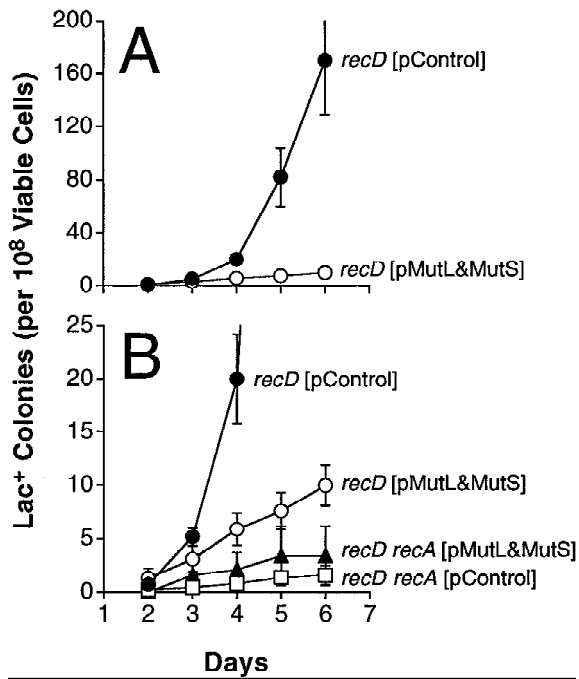


Figure 2. Inhibition of stationary-phase Lac⁺ reversion in a hypermutable *recD* strain. Plasmids [pControl], and [pMutL&MutS] are pSL4, and pSL7, respectively (Materials and Methods). Error bars as in Fig. 1.

each determination. The results can be summarized as follows.

First, we see that the plasmids constructed and used to overproduce MutL do so by ~20- to 30-fold (Fig. 4A, open bars and shaded bars). Those overproducing MutS do so by 60- to 130-fold (Fig. 4B, hatched bars and shaded bars). Less than 10 percent breakdown products were observed in the overproducing strain (data not shown). This demonstration that MutS is overproduced allows us to rule out the possibility that the MutS plasmid did not inhibit stationary-phase mutation (Fig. 1B; Table 1) because of a failure to overproduce MutS protein. We conclude that MutS overproduction does not inhibit stationary-phase Lac reversion.

Second, in strains carrying the control plasmid, we observe declines in MutS and MutH proteins early in stationary phase and on prolonged exposure of the *lac*⁻ cells to starvation on lactose minimal medium (Fig. 4B-D, solid bars). This is similar to results reported previously with plasmid-free cells (Feng et al. 1996).

Third, we note that overproduction of MutS plus MutL may have a small stabilizing effect on MutH, preventing MutH decline during prolonged starvation (Fig. 4C). This can be assessed only in the experiment measuring MutH as nanograms per micrograms of total cellular protein (Fig. 4C) and not by measuring MutH monomers per cell, because we observe that cells overproducing MutS plus MutL are at least twice as long as normal cells (data not shown). The mechanism of this enlargement is unknown. However, it may suggest an interaction between MutS and MutL and prokaryotic cell cycle control simi-

lar to that observed with eukaryotic mismatch repair and eukaryotic cell cycle regulation (Hawn et al. 1995; Anthony et al. 1996). If the increase in MutH seen in Figure 4C is significant, then MutS plus MutL might make direct contact with MutH such that their overabundance could prevent MutH loss. If, for example, MutH were a target of a stationary phase-specific protease (Gottesman and Maurizi 1992; Miller 1996), contact with MutS plus MutL might protect against such proteolysis. Alternatively, overproduction of MutS and MutL might titrate such a protease directly. Other explanations are possible.

Finally, neither stabilization of MutS, nor significant stabilization of MutH is seen when MutL is overproduced alone (Figs. 4B-D, open bars). For MutH, two different sets of experiments are shown (Fig. 4C,D). The first, measured in nanograms of MutH protein per 150 μ g of total cellular protein (Fig. 4C), shows a slight but statistically insignificant trend in prevention of MutH decline by overproducing MutL. The second set is measured as numbers of MutH monomers per cell. This is may be a more relevant measure, as the protein composition of cells changes dramatically in stationary phase, mostly because of loss of ribosomes (Davis et al. 1986; Bremer and Dennis 1987). These data show no significant prevention of MutH loss by MutL overproduction (Fig. 4D, open bars). We conclude that stabilization of MutH does not correlate with the depression of station-

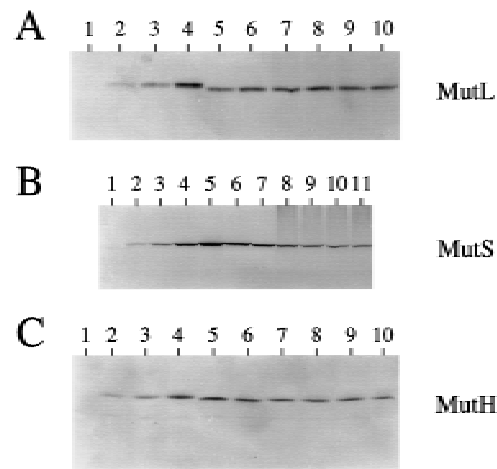


Figure 3. Representative quantitative Western immunoblots. Data from multiple experiments of this type are summarized and described in Fig. 4. (A) MutL levels. (Lanes 1-4) Quantification standards (Feng et al. 1996; see Materials and Methods; legend to Fig. 4): 0, 4.5, 9, and 18 ng of His₆-MutL protein, respectively; (lanes 5-10) MutL in cultures grown exponentially (9.9 ng) and at days 0 (12.9 ng), 2 (12.5 ng), 4 (12.1 ng), 6 (9.6 ng), and 8 (9.9 ng), respectively. (B) MutS levels. (Lanes 1-5) Quantification standards: 0, 2.5, 5, 10, and 20 ng of His₆-MutS protein, respectively; (lanes 6-11) MutS in cultures grown exponentially (15.4 ng) and at days 0 (10.8 ng), 2 (7.7 ng), 4 (6.2 ng), 6 (4.8 ng), and 8 (4.3 ng), respectively. (C) MutH levels. (Lanes 1-4) Quantification standards: 0, 0.25, 0.5, and 1.0 ng of His₆-MutH protein, respectively; (lanes 5-10) MutH in cultures grown exponentially (0.78 ng) and at days 0 (0.54 ng), 2 (0.52 ng), 4 (0.44 ng), 6 (0.47 ng), and 8 (0.36 ng), respectively.

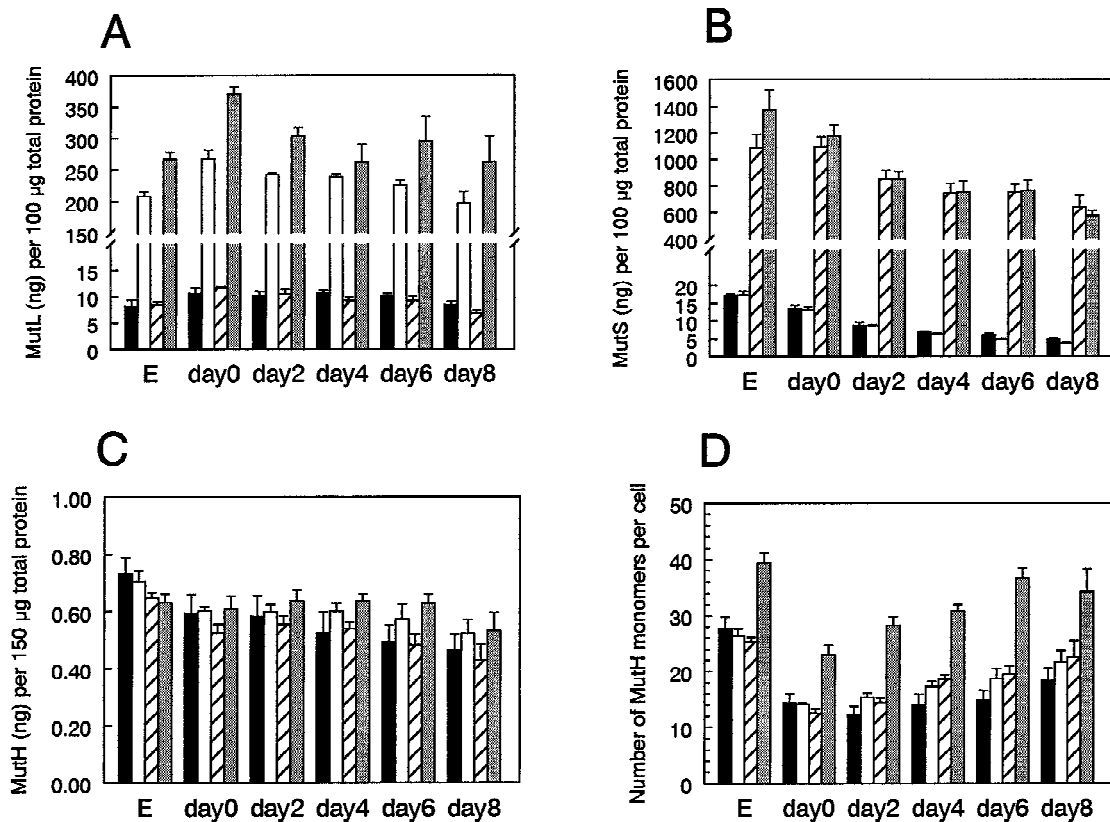


Figure 4. Amounts of MutL, MutS, and MutH proteins in growing, stationary-phase, and starved cells carrying MutL- and MutS-overproducing plasmids. Data are summaries of quantifications from Western blots performed as in Feng et al. (1996; see Materials and Methods). Days 0–8 indicate days after plating the stationary-phase *lac*⁻ cells on lactose medium (Materials and Methods). (E) Exponential cultures. At least three experiments were performed. Each histogram bar represents the mean (error bars, S.E.M.). (Solid bars) pControl; (open bars) pMutL; (hatched bars) pMutS; (shaded bars) pMutL and pMutS. (A) MutL levels. (B) MutS levels. For MutH, two different sets of experiments are shown. (C) The first was measured in nanograms of MutH protein per 150 μg of total cellular protein. (D) The second set was measured as numbers of MutH monomers per cell [see Materials and Methods for the recalibration of the number of MutH monomers per cell with respect to previous results (Feng et al. 1996)]. This is discussed further in the text.

ary-phase Lac reversion which is seen in cells producing MutL alone (Fig. 1B; Table 1). Thus, the depressing effect of MutL on stationary-phase mutation cannot be explained by stabilization of either MutS or MutH levels during starvation.

Discussion

The results reported here imply that during recombination-dependent, stationary-phase mutation, mismatch repair activity is diminished by a decrease in the level of functional MutL protein. The data also imply that the observed declines in MutS and MutH proteins (Fig. 4B–D; Feng et al. 1996) are proportional to decreased replication during starvation and stationary phase and do not cause a loss of mismatch repair function.

Significance

As far as we know, the results reported here represent the first natural circumstance in which mismatch repair

activity has been shown to be modulated, not constitutive. This is significant because of the powerful effect of the mismatch repair system on maintenance of genetic and genomic stability in organisms from bacteria to humans. Cells that lack mismatch repair have 1000-fold higher spontaneous mutation rates (Modrich 1991), recombine sequences of only partial identity (Rayssiguier et al. 1989; Worth et al. 1994; Baker et al. 1995, 1996; de Wind et al. 1995; Matic et al. 1995, 1996; Chambers et al. 1996; Zahrt and Maloy 1997) causing genome rearrangements (Petit et al. 1991), and manifest microsatellite instability and cancer (for review, see Modrich 1994, 1995; Radman et al. 1995).

Mismatch repair deficiency is associated with successful bacterial pathogenesis and with nonpathogenic commensal bacteria of the human gut (LeClerc et al. 1996; Matic et al. 1997), implying that a mutator phenotype is selected in the war between pathogens and the host immune system and in nonpathogenic commensalism. But because the majority of the successful pathogens, natural isolates, and survivors of selection in general (Mao et al. 1997; Sniegowski et al. 1997) were not heritably mis-

mismatch repair-defective, these may have succeeded by a transient mismatch repair deficiency.

Mismatch repair also prevents interspecies recombination (Rayssiguier et al. 1989; Matic et al. 1995, 1996; Hunter et al. 1996; Zahrt and Maloy 1997), and its component proteins participate in transcription-coupled repair of damaged DNA (Mellon and Champe 1996) and in very-short-patch repair (Jones et al. 1987a,b; Lieb 1987; Raposa and Fox 1987; Zell and Fritz 1987). Diminished mismatch repair in response to environmental signals would mean that mutation, improper recombination, genome rearrangements, and genetic instability might vary in response to the environment of a cell. This would have profound consequences for evolution, development, microbial pathogenesis, cancer formation, tumor progression, and acquisition of drug resistance in tumors and pathogens.

Mechanism

The lack of functional MutL implied by the data reported here cannot be attributed simply to a decrease in the amount of MutL protein in stationary phase, as MutL levels do not appear to change during stationary phase and starvation (Fig. 4A; Feng et al. 1996). Several explanations for functional MutL deficiency are possible.

First, MutL protein might be modified or processed in stationary phase to a nonfunctional form.

Second, though present in abundant quantity, MutL might become limiting by means of titration by a DNA substrate formed during stationary phase. Creation of such a substrate could be a regulation mechanism. If it occurs, it is demonstrably a natural part of starvation and not artificially induced in this system.

Evidence for reduction of mismatch repair activity by titration was found first in the Hex mismatch repair system of *Streptococcus*, which can be titrated by DNA substrates during natural transformation (Guild and Shoemaker 1974; Humbert et al. 1995; Tiraby et al. 1975). Mismatch repair in *E. coli* can also be saturated artificially: by excess polymerase errors of a proofreading-defective mutant DNA polymerase (Schaaper 1988; Damagnez et al. 1989; Schaaper and Radman 1989); by mutagens thought to increase polymerase error (Cupples et al. 1990); or by overproduction of single-stranded DNA with regions of secondary structure containing mismatched bases (Maas et al. 1994, 1996). Thus, DNA substrates can titrate out mismatch repair proteins in *E. coli* in growing cells. Overproduction of Vsr protein, which interacts with MutL and MutS in very-short-patch DNA repair, can also titrate out mismatch repair activity (Doiron et al. 1996; Macintyre et al. 1997). The limiting proteins titrated out were MutL or MutH (Schaaper and Radman 1989; Macintyre et al. 1997) or MutS alone (Maas et al. 1996). Reduction of MutL by titration is compatible with the results of Schaaper and Radman (1989) and those of Macintyre et al. (1997) but is not obviously so with those of Maas et al. (1996). The titration hypothesis need not conflict with the apparent abundance of MutL and scarcity of MutS and MutH in

stationary phase, for MutL may be used as an expendable rather than a catalytic component of the reaction (Schaaper and Radman 1989). If so, spent MutL protein might be visible on Western blots though useless to the cell for mismatch repair. Along these lines, the down-regulation of a protein that rejuvenates used MutL would also be compatible with our results. Finally, although MutS is the known DNA-binding component, its interaction with MutL could allow MutL to be titrated out by DNA substrates.

Third, functional MutL could be reduced by stationary-phase up-regulation of a protein such as Vsr, whose overproduction has been shown to diminish methyl-directed mismatch repair and might titrate MutL directly, rather than via a DNA intermediate (Macintyre et al. 1997).

Fourth, overproduction of MutL might stabilize a mismatch repair protein, other than MutS or MutH, which normally declines in stationary phase.

Fifth, recent results indicate that stationary-phase Lac reversion in the system used here occurs as part of genome-wide hypermutation in a subpopulation of the cells exposed to starvation (Torkelson et al. 1997). The size of the mutagenic subpopulation was estimated to be between 10^{-4} and 10^{-5} of all of the cells starved on lactose. Thus, MutL levels might decline only in cells of the subpopulation, which would be undetectable in Western analyses of the whole population. Any of the four previous mechanisms might also occur in the subpopulation or the whole population. Work to distinguish these possible mechanisms is in progress.

Why MutL? MutL is thought to coordinate the mismatch-bound MutS with the MutH endonuclease in a complex of all three proteins (for review, see Modrich 1991). Others have reported saturated mismatch repair that could be restored by MutL or MutH (discussed above; Schaaper and Radman 1989; Macintyre et al. 1997). As we were unable to overexpress MutH in our experiments (data not shown), this is possible in our assay system too. One possibility is that MutH might be a reusable endonuclease but that each reuse might require interaction with a fresh MutL, and MutL might be unable to recycle at all. If so, then overexpression of either protein would restore mismatch repair. Thus, we suggest that MutL is not only a coordinator of MutS with MutH but also a sensitive step at which regulation of the system could occur.

Regulation or random breakdown of mismatch repair in stationary phase?

Is the mechanism of MutL dysfunction a regulated response or, in the case of the fifth hypothesis, might it represent random loss of MutL protein in the subpopulation? One nonrandom aspect of the results is that only MutL (not other mismatch repair proteins) appears to become limiting (though it is possible that MutL or MutH might be limiting as discussed above). Ninio (1991) suggested a random model in which the normal error rates of replication, transcription, and translation

should lead to transient and heritable mutator subpopulations, for example, by faulty synthesis of (MutL or) any protein involved in replication fidelity. Ninio's model predicts frequencies of heritable mutator mutants to be found among cells carrying mutations that are far greater than those observed in this system (Torkelson et al. 1997), suggesting the possibility that the mutator state is not randomly achieved but, rather, is programmed. However, the error rates used in Ninio's calculations may not apply during starvation.

Whether the loss of MutL function is accidental or programmed, it occurs in response to environmental conditions. Environmental influence over genetic stability could be important for reasons discussed above.

Implications for stationary-phase mutation

Bacteria differentiate and execute specific developmental programs to deal with stationary phase and starvation (Siegele and Kolter 1992; Kolter et al. 1993) during which they generate special mutants with the ability to prevail under limiting conditions (Zambrano and Kolter 1996). The stationary-phase mutation-specific loss of mismatch repair function reported here could be part of a developmental program for generating such mutants.

Stationary-phase reversions of the *lac* frameshift mutation in the system used here have been shown to result from a genome-wide hypermutable state in a subpopulation of the starved cells (Torkelson et al. 1997). MutL deficiency might or might not be the special feature that makes the subpopulation different. For example, the whole population might be MutL-deficient but only the subpopulation might, for example, perform the recombination necessary for Rec-dependent stationary-phase mutation. The subpopulation might be differentiated by possessing the DNA double-strand breaks that initiate recombination (Harris et al. 1994; Rosenberg 1994, 1997; Rosenberg et al. 1995, 1996).

Also (Torkelson et al. 1997), stationary-phase mutations are found not to be directed, in a Lamarckian manner, to the gene under selection (*lac*) but, rather, to occur in multiple unselected genes in all replicons in the cell (for evidence of unselected mutation, see also Foster 1997). This supports Darwinian models for stationary-phase mutation that include random mutation followed by selection for the adaptive mutation. However, the implication of the findings reported here—that mutation rates could be altered significantly by decreasing mismatch repair in response to environmental cues—suggests that the cells may generate the variation on which selection acts more vigorously when they “need” to evolve. Where such a mutagenic mode fits in the continuum between Lamarckian and Darwinian mutation models will probably be a subject of continuing discussion. Down-regulation of mismatch repair could provide a molecular mechanism for achieving rapid genetic change when selection is present. On the microscopic scale of changes in bacterial genotype, this could contribute to mechanisms producing the punctuations in punctuated equilibria (Eldredge and Gould 1972).

Materials and methods

Plasmids

Plasmids were modified from pBR322 (Bolivar et al. 1977) to carry kanamycin resistance (Kan^R) (pSL4) and the following *E. coli* genes regulated by their natural promoters: *mutL* (pSL5); *mutS* (pSL6); and *mutS* and *mutL* (pSL7). These genes are overexpressed because of the high copy number of pBR322. In pSL4 the *HindIII*–*Bam*HI fragment of pBR322 is replaced by the Kan^R -conferring *HindIII*–*Bam*HI fragment of pKC31 (R.N. Rao, described by, e.g., Rosenberg 1988). pSL5 contains a *PstI*–*HindIII* fragment of pAL51 carrying *E. coli mutL* (Lu et al. 1984) and the *HindIII*–*Bam*HI fragment of pKC31 replacing the *PstI*–*Bam*HI fragment of pBR322. In pSL6 the pBR322 *Clal*–*Bam*HI fragment is replaced by the *mutS*-containing *Clal*–*HindIII* fragment of pMS312 (Su and Modrich 1986) and the *HindIII*–*Bam*HI fragment of pKC31. In pSL7 the *Clal*–*PstI* fragment of pBR322 is replaced by the *Clal*–*Bgl*III fragment of pSL6, the *Bam*HI–*HindIII* fragment of pKC31, and the *HindIII*–*PstI* fragment of pAL51. All plasmid genotypes were confirmed by restriction mapping and by complementation of the mutator phenotype (assayed as by Longerich et al. 1995; Torkelson et al. 1997) of *mutS* and/or *mutL*-defective *E. coli* strains.

Lac⁺ mutation assays

For *Lac*⁺ frameshift reversion studies, derivatives of a strain carrying the *lacI33* allele (Cairns and Foster 1991) and carrying the plasmids described above were used. This strain is deleted for the chromosomal *lac* operon and bears an F' episome carrying a *lacI*–*lacZ* fusion gene with a +1 frameshift mutation in *lacI* that is polar on *lacZ* (Cairns and Foster 1991). Procedures for measurement of stationary-phase *Lac*⁺ mutation and for measurements of growth-dependent *Lac*⁺ mutation rates were modified from Harris et al. (1996) as follows: Kanamycin (at 50 $\mu\text{g}/\text{ml}$) was included in the minimal glycerol broth, and 5 $\mu\text{g}/\text{ml}$ of kanamycin in the minimal lactose plates and top agar. The scavenger cell strain is as before except that it carries the Kan^R -conferring control plasmid pSL4.

Western analyses

For measuring protein levels during starvation on lactose medium, it is important that *Lac*⁺ revertants do not accumulate in the population assayed. Thus, the strain background analyzed was FC29 (Cairns and Foster 1991), which is isogenic to the *lac* frameshift-bearing strain used except for the absence of the *lac* operon. The absence of this operon makes it nonrevertible. Derivatives of this strain carrying the plasmids described here were used. Western analyses were as described (Feng et al. 1996) with the following changes. (1) Bacteria were grown in media as described above for *Lac* reversion studies, washed, and spread on minimal lactose plates with 5 $\mu\text{g}/\text{ml}$ of kanamycin plates as described above, but with no top agar. Plates were incubated for 8 days at 37°C. Every second day cells were washed off 10 plates with M9 salts and protein samples were prepared. Exponential and day 0 samples were prepared using liquid cultures at a density of about 30 Klett units, and the saturated culture, respectively. (2) MutS and MutH antisera were affinity-purified: One-milliliter columns containing ~2 mg of His₆–MutS or His₆–MutH (Feng and Winkler 1995) coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden), prepared according to instructions (Pharmacia), were washed sequentially with 15 ml of 6 M guanidine HCl, 25 ml of buffer A (50 mM Tris-HCl at pH 7.4), 25 ml of Buffer B (buffer A + 4.5 M MgCl₂ and 1.0 mg/ml of BSA), and equilibrated with 50 ml of buffer A.

Ten milliliters of antisera were run through the columns, which were washed with 40 ml of 1 M guanidine-HCl and 20 ml of buffer A and eluted with 10 ml of buffer B. Elutions were dialyzed against 3 liters of PBS, and 3 liters PBS plus 35% glycerol. MutL antiserum was a gift from P. Modrich (Duke University, Durham, NC).

The absolute amount of MutH per cell has been recalibrated with respect to previous experiments (Feng et al. 1996) based on our finding that the standard MutH protein preparation to which calibration was performed previously had aggregated when the His₆ affinity tag was cleaved off MutH. This aggregation was not detected until later experiments in which large amounts of standard were prepared. The new estimate for MutH in cells growing exponentially in enriched minimal salts glucose (EMMG) medium (Feng et al. 1996) is 34 ± 7 monomers per cell.

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