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Genome-wide hypermutation in a subpopulation of stationary-phase cells underlies recombination-dependent adaptive mutation

Joel Torkelson[1], Reuben S.Harris[1], Mary-Jane Lombardo[1], Jayan Nagendran, Carl Thulin and Susan M.Rosenberg[1,2]

Department of Biochemistry, 4-74 Medical Sciences Building, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

[1]Address after August 1997: Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030-3498, USA

[2]Corresponding author
e-mail: susan.rosenberg@ualberta.ca

Stationary-phase mutation in microbes can produce selected ('adaptive') mutants preferentially. In one system, this occurs via a distinct, recombination-dependent mechanism. Two points of controversy have surrounded these adaptive reversions of an *Escherichia coli lac* mutation. First, are the mutations directed preferentially to the selected gene in a Lamarckian manner? Second, is the adaptive mutation mechanism specific to the F plasmid replicon carrying *lac*? We report that *lac* adaptive mutations are associated with hypermutation in unselected genes, in all replicons in the cell. The associated mutations have a similar sequence spectrum to the adaptive reversions. Thus, the adaptive mutagenesis mechanism is not directed to the *lac* genes, in a Lamarckian manner, nor to the F′ replicon carrying *lac*. Hypermutation was not found in non-revertants exposed to selection. Therefore, the genome-wide hypermutation underlying adaptive mutation occurs in a differentiated subpopulation. The existence of mutable subpopulations in non-growing cells is important in bacterial evolution and could be relevant to the somatic mutations that give rise to cancers in multicellular organisms.

Keywords: adaptive mutation/directed mutation/evolution/recombination/stationary phase

Introduction

'Adaptive' mutations are an unexpected kind of spontaneous mutation that are distinguished from normal spontaneous growth-dependent mutations (e.g. Luria and Delbrück, 1943; Lederberg and Lederberg, 1952) by the criteria that adaptive mutations arise only in the presence of selection for those mutations, and in non-dividing or slowly growing stationary-phase cells (e.g. Ryan, 1955; Cairns *et al.*, 1988; Hall, 1990, 1992; Cairns and Foster, 1991; Jayaraman, 1992; Steele and Jinks-Robertson, 1992; Foster, 1994; reviewed by Drake, 1991; Foster, 1993). Adaptive mutation provoked controversy about whether mutagenesis mechanisms exist that direct mutation preferentially to a selected gene, in a Lamarckian manner (e.g. Cairns *et al.*, 1988; Charlesworth *et al.*, 1988; Cairns, 1993; Lenski and Mittler, 1993a,b). The unorthodoxy of this suggestion led many to argue that adaptive mutation must not exist (reviewed by Foster, 1993; Culotta, 1994). In one experimental system, adaptive mutation has been demonstrated to exist as a process mechanistically distinct from growth-dependent mutation by the following findings. Adaptive reversion of a *lac* frameshift mutation carried on an F′ sex plasmid in *Escherichia coli* (Cairns and Foster, 1991) requires homologous genetic recombination functions (Harris *et al.*, 1994, 1996; Foster *et al.*, 1996), and produces a novel sequence spectrum (Foster and Trimarchi, 1994; Rosenberg *et al.*, 1994). Neither of these is seen in growth-dependent reversions of the same allele. These findings demonstrate a novel mutagenic mechanism for the adaptive *lac* reversions, which includes genetic recombination and has other unique features (reviewed by Rosenberg, 1994; Rosenberg *et al.*, 1995, 1996; and discussed below). However, it has been suggested that this mechanism may represent a peculiarity of sex plasmid biology which would not pertain to mutagenesis in general, and would not operate on replicons other than sex plasmids (Foster and Trimarchi, 1995; Galitski and Roth, 1995; Peters and Benson, 1995; Radicella *et al.*, 1995; and discussed below).

In this report, we address three questions. First, is the mutagenic process that produces recombination-dependent Lac^+ adaptive mutations directed in a Lamarckian manner preferentially to the *lac* gene? Second, is that mutation mechanism specific to the F′ sex plasmid? Third, we test the postulate of Hall (1990) that adaptive mutants arise from a differentiated subpopulation of all the cells exposed to selection which experiences a (random Darwinian) genome-wide hypermutable state (also considered by Ninio, 1991; Harris *et al.*, 1994; Rosenberg, 1994). We find first, that the mutational process that generates the Lac^+ adaptive reversions is not directed to the *lac* genes, and second, that it is not specific to the F′ replicon that carries *lac*. Unselected genes in all replicons in the cell are mutated at a high level in association with Lac adaptive reversion. Third, genome-wide hypermutation underlying adaptive Lac reversion is found to occur in a subpopulation of cells exposed to selection as suggested (Hall, 1990). These findings bear on the molecular mechanism of recombination-dependent adaptive mutation and on its evolutionary significance.

Results

Assay for a hypermutable subpopulation

To test the possibility that random, genome-wide mutation might occur in only a small subpopulation of the cells exposed to selection (Hall, 1990; Ninio, 1991; Harris *et al.*, 1994), a replica-plating screen was used to score

Table I. Plasmid-borne Tet^R mutations are associated with Lac^+ adaptive reversion

Plasmid name[a]	Tet^S mutation	Tet^S mutation nucleotide position[b]	No. of Tet^R mutants among Lac^+ adaptive revertants (Tet^R colonies/ colonies scored)	No. of Tet^R mutants among Lac^- stressed cells (Tet^R colonies/ colonies scored)	No. of Tet^R mutants among Lac^- unstressed cells (Tet^R colonies/ colonies scored)	No. of Tet^R mutants among Lac^+ adaptive revertants re-grown on lactose[c] (Tet^R colonies/colonies scored)
pW17	+1 G	536	93/27 784	0/8894	3/26 097	0/14 513
pW18	−1 G	536	0/2329	0/5687	0/8578	n.t.[d]
pX2	+GC	435	0/3626	0/4407	0/12 554	n.t.[d]
pRDK35	oligonucleotide insertion	23	12/1684	0/4176	n.t.[d]	n.t.[d]

Mutation to tetracycline-resistance (Tet^R) is dominant and the plasmids are multicopy, such that one Tet^R mutation among the multiple Tet gene targets will produce Tet^R, thus providing a sensitive assay.

[a]Derivatives of the *lac* frameshift-bearing strain (Cairns and Foster, 1991) carry pW17, pW18 (Koffel-Schwartz *et al.*, 1984), pX2 (Burnhouf and Fuchs, 1985) or pRDK35 (Doherty *et al.*, 1983), all of which are identical to pBR322 (Sutcliffe, 1979) except for frameshift mutations in the tetracycline resistance (Tet) gene (see Figure 1).

[b]See Figure 1 for sequence position and context information.

[c]This control shows that growth of a Lac^+ cell into a colony on lactose medium is not sufficient to produce associated Tet^R mutations, i.e. that lactose medium is not mutagenic *per se*.

[d]n.t., not tested.

unselected mutations. Unselected mutations were scored amongst Lac^+ adaptive revertant colonies and in two control populations (see Materials and methods): the Lac^- frameshift-bearing cells grown into colonies without exposure to selection (Lac^- unstressed colonies), and the Lac^- frameshift-bearing cells that were exposed to selection, but which did not mutate to Lac^+ (Lac^- stressed cell colonies). To obtain Lac^+ adaptive revertants and Lac^- stressed cells, the *lac* frameshift-bearing cells were plated on lactose minimal medium and incubated for 6–7 days as described (Cairns and Foster, 1991; Harris *et al.*, 1994, 1996). Growth-dependent Lac^+ mutant colonies appear after 2 days incubation and are followed by the appearance of RecBC-dependent adaptive revertants on days 3–7 (Cairns and Foster, 1991; Foster and Trimarchi, 1994; Harris *et al.*, 1994, 1996; Rosenberg *et al.*, 1994; Foster *et al.*, 1996). The Lac^- stressed cells were rescued from the plates after 4–6 days of incubation by resuspending plugs of agar and plating to form colonies on medium containing a utilizable carbon source. The Lac^+ adaptive revertant colonies, and colonies of the Lac^- stressed cells, and of Lac^- unstressed cells were replica-plated to assay mutations in several other genes in three replicons in the cells: a pBR322-derived plasmid, the F′, and the bacterial chromosome. The Lac^- stressed and unstressed cell colonies were grown to be at least as large as the largest Lac^+ adaptive revertant colonies replicated, so that any higher frequencies of unselected mutation detected amongst Lac^+ adaptive revertants could not have arisen trivially because of more cell generations during colony growth. This point is also demonstrated in experiments below.

Unselected mutations in a plasmid

Reversions of mutant tetracycline resistance genes (Tet genes) on pBR322-based plasmids were examined. Four different plasmids were used as mutation targets. Each is identical with pBR322 except for a frameshift mutation that inactivates the Tet gene (Table I). Lac^+ adaptive revertants, and Lac^- unstressed and stressed cells derived from strains carrying each of the plasmids were grown into colonies and replica-plated to rich medium containing tetracycline. The data in Table I show that two of the Tet alleles tested revert frequently enough to score in this assay, and their reversion is 10–100 or more times higher amongst Lac^+ adaptive revertants than in the two Lac^- control populations. The hypermutation is seen amongst adaptive Lac^+ revertants but not amongst Lac^- cells from the same starved cultures. This implies that a subpopulation of cells exposed to starvation on lactose experiences mutability that can affect a gene in another replicon. These data demonstrate a strong correlation between Lac^+ adaptive reversion and reversion of an unselected gene.

A sample of adaptive mutation-associated Tet^R mutations was mapped to the pBR322-based plasmid, and shown not to be associated with the F′ that carries the *lac* genes. Plasmid DNAs prepared from 10 independent Tet^R isolates from the strain carrying plasmid pW17 (Table I) were transformed into a plasmid-free female strain of *E.coli*, the parent of the *lac* frameshift-bearing strain (Cairns and Foster, 1991), and tetracycline resistance was selected. In all cases, tetracycline resistance was transferred to the new cells (as was ampicillin resistance which is also encoded by pBR322), and the female plasmid recipients did not, coincidentally, become male (assayed by resistance to male-specific phage R17). Thus Tet^R transfers with the pBR322-derived plasmid, and the pBR322-derived plasmid has not somehow become associated with the F′. These results indicate that a replicon other than the F′ is mutable during adaptive Lac reversion.

The Tet^R mutations were presumed to occur coincidentally with Lac^+ reversion and not during growth of the Lac^+ colonies, because simple growth of a colony from a single starved or unstarved cell (on non-lactose carbon sources, Table I) was shown to be insufficient to generate high levels of Tet^R mutation. However, if the Lac^+ revertants were heritably mutator, or if lactose medium provoked mutations in Lac^+ revertants, then the comparison with colonies grown on other carbon sources would be insufficient. We have shown that most Lac^+ adaptive revertants do not have a heritable mutator phenotype that could accelerate unselected mutation during

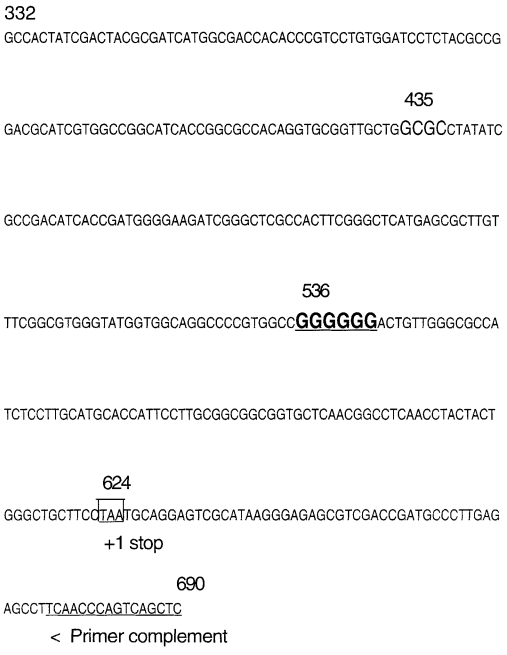

Fig. 1. DNA sequence of a region of the tetracycline resistance (Tet) gene of plasmid pW17. pW17 carries a +1 G mutation at position 536 abolishing function of the Tet gene (Koffel-Schwartz *et al.*, 1984). Tet^+ mutations could restore the reading frame by addition or deletion between the two stop codons spanning the mutation. The downstream +1 stop codon is shown (boxed). The upstream –1 stop codon occurs in a region upstream of the sequenced region shown. Nucleotide position numbers apply to the left-most base immediately below them, and correspond to those used for pBR322 (Sutcliffe, 1979) with the +1 G present in pW17 unnumbered.

colony growth (Longerich *et al.*, 1995; also shown below). We can rule out the unlikely alternative possibility that growth into a colony under lactose selection conditions induces the Tet^R mutations, by showing that Lac^+ revertants isolated, replated and grown into colonies on lactose display low Tet mutation frequencies (Table I). This strengthens the correlation between adaptive Lac^+ reversion and unselected Tet^R mutation.

Sequence similarity to Lac adaptive mutation

To begin to assess whether the Tet^R mutations occur via a mechanism similar to that by which the F′-borne *lac* gene reverts, we examined the sequence specificity of Tet^R reversions (Figure 1). Unlike growth-dependent reversions which are heterogeneous, adaptive reversions of the *lac* +1 frameshift allele are nearly all –1 deletions in small mononucleotide repeats, with a strong hotspot in the 4 C repeat of the original frameshift mutation (Foster and Trimarchi, 1994; Rosenberg *et al.*, 1994). This spectrum is characteristic of DNA polymerase errors that accumulate in the absence of post-synthesis mismatch repair (Longerich *et al.*, 1995). In support of a similar spectrum in the Tet^R mutations, we note that the most active mutagenic target is a +1 frameshift allele in a run of six Gs (pW17, Table I). Two other targets, a –1 G in the same G repeat, and a +GC in a GC dinucleotide repeat, are both less active (Table I, Figure 1), in agreement with previous results regarding the sequence preference of the Lac reversion mechanism (Foster and Trimarchi, 1994; Rosenberg *et al.*, 1994). (A fourth target of unknown sequence is also active.) Second, a region spanning the +1 G Tet mutation (Figure 1) was sequenced (see Materials and methods) in 16 independent Tet^R reversions of the +1 frameshift Tet allele. All 16 reversions are –1 deletions in the six G repeat at position 536, the site of the original +1 frameshift mutation (Figure 1). These results indicate a mutation hotspot for –1 deletions in a six G repeat, consistent with the pattern seen in adaptive Lac reversion.

Mutations in the F′ and the bacterial chromosome

To test whether replicons other than pBR322 experience elevated unselected mutation correlated with Lac^+ adaptive reversion, we screened for mutations in a non-*lac* gene in the F′, *codAB*, and for mutations in multiple genes in the bacterial chromosome. *codAB* encodes cytosine deaminase and transport activities, loss of function of either of which confers resistance to 5-fluorocytosine (5-FC) (De Haan *et al.*, 1972; Lind *et al.*, 1973; Neuhard and Kelln, 1996). *Escherichia coli* can also become 5-FC-resistant ($5\text{-}FC^R$) by loss of function of the chromosomally located *upp* gene, which in addition confers resistance to 5-fluorouracil (5-FU) (Pierard *et al.*, 1972; Neuhard and Kelln, 1996). We obtained high frequencies of $5\text{-}FC^R$ and $5\text{-}FU^R$ mutants associated with Lac^+ adaptive reversion, but not amongst Lac^- stressed and unstressed cells (Table II). The episomal and chromosomal location of the $5\text{-}FC^R$ and $5\text{-}FU^R$ mutations respectively were confirmed by transductional mapping (see Materials and methods). These results demonstrate that unselected genes on the F′ and in the bacterial chromosome are mutable in association with Lac^+ adaptive reversion. The approximate equality of $5\text{-}FU^R$ (chromosomal) with $5\text{-}FC^R$ (F′-located) mutations (Table II) indicates that the *upp* gene in the bacterial chromosome and the *codAB* locus in the F′ are similarly mutable in association with adaptive Lac^+ reversion.

Mutations at multiple chromosomal locations

To generalize the finding that chromosomal loci mutate in association with Lac^+ adaptive reversion, two broad, genome-wide screens were undertaken. First, the ability to ferment the sugars xylose, maltose and fructose was assayed by replica-plating to appropriate indicator media (Table II). All three fermentation pathways are encoded by multigenic regulons and so provide large mutation targets (Berlyn *et al.*, 1996; Böck and Sawyers, 1996). The data in Table II show elevated mutation to Xyl^- and Mal^-, but not $Fruc^-$ phenotypes, amongst Lac^+ adaptive revertants as compared with the two Lac^- control populations. All 15 Xyl^- and 14 Mal^- mutations tested were verified as being located in the bacterial chromosome (see Materials and methods). Thus many, but apparently not all, chromosomal loci hypermutate in association with Lac^+ adaptive reversion. Second, mutants temperature-sensitive (Ts) for growth on minimal medium were also enhanced amongst Lac^+ adaptive revertants compared with Lac^- control populations (Table II). These probably carry auxotrophic Ts mutations.

Timing of unselected mutation

We wished to address directly whether the unselected mutations associated with Lac^+ adaptive reversion arose coincidentally with Lac^+ reversion, and not during subsequent growth of the Lac^+ colony. Thus, we examined the

Table II. F′ and chromosomal unselected mutations are associated with Lac^+ adaptive reversion

Replicon carrying the unselected mutation	Mutant phenotype[a]	Experiment	No. of mutants among Lac^+ adaptive revertants (mutant colonies/ colonies scored)	No. of mutants among Lac^- stressed cells (mutant colonies/ colonies scored)	No. of mutants among Lac^- unstressed cells (mutant colonies/ colonies scored)
F′	5-FC^R	1	121/19 647	1/17 876	2/21 940
	5-FC^R	2	115/41 238	0/66 274	0/12 860
Chromosome	5-FU^R	1	n.t.[b]	n.t.[b]	n.t.[b]
	5-FU^R	2	121/41 238	1/66 274	1/12 860
	Xyl^{-c}		22/42 617	3/69 795	0/26 025
	Mal^{-c}		31/42 617	0/69 795	0/23 471
	$Fruc^{-c}$		2/42 617	1/69 795	1/37 961
	Minimal Ts		10/44 984	1/71 709	1/27 265

[a]A few of the mutants listed among Lac^+ are multiple: two 5-FC^R Mal^-; one 5-FU^R Mal^-; one 5-FU^R Xyl^- Mal^-; one Xyl^- Mal^-; one Mal^- Minimal Ts. Transductional analyses indicate that all of these are separately heritable and thus represent different mutations.
[b]n.t., not tested.
[c]The numbers of fermentation mutants amongst Lac^+ revertants are underestimates because the Lac^- background forms a film of fermentation-positive (red) growth after replica plating. This is not the case for the Lac^- control populations. The underestimation may be most serious for $Fruc^-$ in which only pink (no white) mutants were found.

original (master) colonies from which F′ and chromosomal unselected mutants were identified in the replica-plating screens. The data in Table III show that most of the original 5-FC^R and 5-FU^R Lac^+ colonies are pure with respect to 5-FC^R and 5-FU^R mutations, as assayed by diluting the original colonies, replating on minimal lactose medium and replica-plating to 5-FC medium (on which both 5-FC^R and 5-FU^R cells grow). Thirty-nine of 53 5-FC^R and seven of nine 5-FU^R mutant colonies were pure and not mixed with sensitive cells, as would have been expected if these clones became 5-FC^R and 5-FU^R during growth of the Lac^+ colony. This implies that most of the unselected mutations arose coincidentally with the Lac^+ adaptive reversion event, and agrees with the previous finding that most Lac^+ adaptive revertants do not have a heritable mutator phenotype (Longerich *et al.*, 1995; and below). Of the few mixed colonies obtained, most carried a few per cent of 5-FC^S and 5-FU^S cells, and some carried a small percentage of resistant cells. These probably represent independent Lac^+ revertants that overlapped with the resistant colonies. Likewise, three of four fermentation-defective colonies were shown to be pure and not mixed with fermentation-positive cells. Therefore, the majority of unselected mutations associated with Lac reversion formed coincidentally with Lac reversion. The possibility that unselected mutations formed before Lac reversion can be excluded by their scarcity in the cultures giving rise to Lac revertants (Tables I and II, Lac^- stressed and unstressed cells).

Mutants are not heritably mutator

As found previously for simple Lac^+ adaptive revertants (Longerich *et al.*, 1995), most of the Lac^+ revertants with associated unselected mutations do not possess a heritable mutator phenotype (Table IV). Ten each of the Tet^R, 5-FC^R, Xyl^- and Mal^- mutants, nine 5-FU^R mutants, as well as all six multiple mutants isolated (Table II) were analyzed for mutator phenotype as described previously (Longerich *et al.*, 1995). Only six of the 55 possess a heritable mutator phenotype: two Tet^R, one 5-FU^R, two Mal^- and one 5-FU^R Xyl^- Mal^-.

Discussion

The results presented imply the existence of a mutagenic process at work in the entire genome of a subpopulation of the cells exposed to selection. The mutagenic process that generates RecBC-dependent, adaptive reversions is not directed preferentially to the *lac* genes (as had been suggested by Cairns *et al.*, 1988; Hall, 1988; Davis, 1989; Boe, 1990; Cairns and Foster, 1991; Foster, 1993, 1994), to a region of DNA around the *lac* genes (as was implied in the model of Roth *et al.*, 1996), to the F′ replicon on which the *lac* genes reside (as was predicted by Foster and Trimarchi, 1995; Galitski and Roth, 1995; Peters and Benson, 1995; Radicella *et al.*, 1995; Foster *et al.*, 1996) or to episomes in general. Multiple loci in multiple *E.coli* replicons, including the bacterial chromosome, are hypermutated in association with Lac^+ adaptive reversion.

An alternative interpretation is that a second, correlated but different mutagenic mechanism also operates in cells that mutate adaptively. This is a more complicated hypothesis and is argued against by the similarity of the sequences of the Tet^R adaptive reversion-associated mutations to Lac^+ adaptive reversions (Foster and Trimarchi, 1994; Rosenberg *et al.*, 1994).

A hypermutable subpopulation

Hypermutation of unselected genes is seen amongst adaptive Lac^+ revertants but not amongst Lac^- cells from the same starved cultures. This implies that a subpopulation of cells exposed to starvation on lactose experiences a genome-wide hypermutable state (Rosenberg, 1994; Rosenberg *et al.*, 1995, 1996). This state must be transient because most adaptive revertants are not heritably mutator (Longerich *et al.*, 1995; Table IV). Such a hypermutable subpopulation could be induced by selection, stationary phase or stress (Rosenberg, 1994; Rosenberg *et al.*, 1995, 1996).

A similar conclusion, based on fewer data, was inferred for Trp^+ adaptive reversion, which occurs via a different, recombination gene-independent mechanism (Hall, 1990, 1995). Hall suggested a selection-induced hypermutable

Table III. Lac^+ revertant colonies carrying associated unselected mutations are mostly pure clones[a]

Unselected mutant[b]	No. of Lac^+ master colonies tested[c]	Phenotype[d]
$5\text{-}FC^R$	39	pure
	14	mixed
$5\text{-}FU^R$	7	pure
	2	mixed
Mal^-	2	pure
	1	mixed
Xyl^-	1	pure
Total	66	49 pure
		17 mixed

[a]If the original Lac^+ master colony is pure such that all cells in that colony carry the unselected mutation, then the unselected mutation could not have arisen subsequent to Lac^+, during growth of the Lac^+ cell into a colony.
[b]F′ and chromosomal unselected mutants were analyzed whereas pBR322-borne Tet^R mutants were not because Tet^R is a dominant mutation carried on a multicopy replicon. Therefore, all Lac^+ master colonies with Tet^R mutations should be mixed with respect to the number of cells per colony carrying the Tet^R mutation, even if the Tet^R mutation formed coincidentally with Lac^+.
[c]In order to avoid analyzing colonies contaminated by other nearby colonies, only master colonies that were separated from other colonies on the plate by at least 0.5 cm were examined. This necessitated exclusion of all of the fermentation mutants other than the four reported.
[d]The original Lac^+ master colonies whose replica-plating led to identification of unselected mutants were picked, suspended and replated to minimal lactose medium. The colonies that arose were then replica-plated to 5-FC medium for both $5\text{-}FC^R$ and $5\text{-}FU^R$, and to minimal maltose and minimal xylose media for Mal^- and Xyl^-, respectively. Between 40 and 660 individual colonies were examined per Lac^+ master colony for all except six of the $5\text{-}FC^R$ master colonies in which the numbers tested were 5, 14, 20, 22, 36 and 36. 'Pure' master colonies are those that showed zero derived colonies not carrying the unselected mutation. The percentage of non-mutants per mixed master colony ranged from 1 to 100 (of the derived colonies examined per master colony) and was distributed such that 13 of the 17 mixed colonies contained mostly one phenotype with only a small percentage of the other, whereas four contained between 25 and 51% non-mutant. All of these may represent overlapping Lac^+ master colonies; however, the latter four might also be derived by segregation of a heterozygous unselected mutation that arose close to the same time as Lac^+.

state in which a small subpopulation of the cells experiences genome-wide mutagenesis, but ultimately dies unless a selected (adaptive) mutation is generated (Hall, 1990). This type of model (see also Ninio, 1991; Harris *et al.*, 1994) is supported for RecBC-dependent Lac^+ reversion by the data presented, and we have suggested previously a molecular basis of such death (Harris *et al.*, 1994; Rosenberg 1994; Rosenberg *et al.*, 1995, 1996). However, the data presented here do not require invoking death: Lac^+ revertants represent ~10^{-6} of the population (e.g. see Harris *et al.*, 1994, 1996; also true here). If unselected mutations occurred in 10^{-6} Lac^- starved cells they would not have been detected by our replica-plating screen. Previous failure to detect unselected mutations in the whole population might be because the only unselected mutation target used could not detect frameshift mutations (Foster, 1993).

The size of the subpopulation that gives rise to adaptive Lac revertants can be estimated as follows. If the mutations in unselected genes occurred randomly, then the numbers of (Lac^+) single, double, and triple mutants should fall within a Poisson distribution. The data for single, double, and triple mutants from a single large experiment reported in Table II conform to a Poisson distribution with a mutation rate of 0.5×10^{-2} per day in ~10^{-5} of the whole population. The implication of being able to fit these data to a Poisson distribution is that the mutation rates for unselected genes are similar to that for *lac*.

Additional evolutionary implications

Subpopulations of mutant cells have been found to arise and then overtake stationary-phase bacterial cultures (Zambrano and Kolter, 1996). These so-called GASP mutants (growth advantage in stationary phase) appear to provide variation that allows evolution during the harsh condition of stationary phase. A hypermutable subpopulation of stationary-phase cells such as that reported here could lead to the formation of GASP mutants. The hypermutation could be part of a developmental program for generating subpopulations with GASP ability. This idea, that the environment influences mutation rate, contrasts with the neo-Darwinian tenet of a uniform mutation rate. However, Darwin's formulation of natural selection included this idea (Darwin, 1859).

Molecular mechanism of recombination-dependent stationary-phase mutation

A current list of aspects of the molecular mechanism of recombination-dependent mutation in this system follows. (i) The process of Lac adaptive reversion includes a requirement for recombination genes of the RecBCD system (Harris *et al.*, 1994, 1996; Foster *et al.*, 1996). (ii) RecBCD involvement implicates double-strand DNA breaks (DSBs) as a molecular intermediate, because RecBCD loads onto DNA only at DSBs (Taylor, 1988; Kowalczykowski *et al.*, 1994; Myers and Stahl, 1994). (iii) The adaptive mutation sequences resemble DNA polymerase errors (Foster and Trimarchi, 1994; Rosenberg *et al.*, 1994), probably made by DNA polymerase III (Foster *et al.*, 1995; Harris *et al.*, 1997). (iv) The polymerase errors resemble those that accumulate in the absence of mismatch repair (Longerich *et al.*, 1995). Mismatch repair appears to be diminished during adaptive mutation by a transient deficiency in functional MutL protein (R.S.Harris, G.Feng, K.J.Ross, R.Sidhu, C.Thulin, S.K.Szigety, M.E.Winkler and S.M.Rosenberg, submitted). (v) F-plasmid transfer (Tra) genes are required for Rec-dependent adaptive reversion of *lac* on the F′, and two non-F′ sites tested do not produce Rec-dependent Lac reversions (Foster and Trimarchi, 1995; Galitski and Roth, 1995; Radicella *et al.*, 1995; R.S.Harris and S.M.Rosenberg, unpublished results). However, other loci in other replicons are hypermutable during adaptive mutagenesis (this report).

One way to assemble these pieces follows (see Rosenberg, 1994; Rosenberg *et al.*, 1995, 1996): DSBs generated in a subpopulation of the stressed cells would promote high levels of RecABCD-mediated recombination (Harris *et al.*, 1994). DNA synthesis associated with recombination could include polymerase errors (Harris *et al.*, 1994) which persist as mutations due to down-regulation of mismatch repair (Foster and Trimarchi, 1994;

Table IV. Most unselected mutants associated with Lac reversion are not heritably mutator

Strain[a]	No. of independent isolates tested	No. of independent cultures tested per isolate	No. of spots tested per culture	Mutant colonies per spot[b] [Mean (± SE)[c]]		
				Nalidixic acid^R	Streptomycin^R	Phenotype
Lac$^-$ parent	1	4	25	0.05 (± 0.006)	0.01 (± 0.002)	non-mutator
Lac$^-$ parent[pW17]	1	4	25	0.03 (± 0.004)	<0.01	non-mutator
Lac$^-$ *mutL*	1	4	10	>25 (± 1)[d]	1.1 (± 0.1)	mutator
Lac$^-$ *dam*	1	4	10	4.1 (± 0.2)	0.05 (± 0.02)	mutator
Lac$^+$ Tet^R	8	4	1	<0.03	<0.03	8 non-mutator
Lac$^+$ Tet^R	1	4	1	15 (± 7)	<0.03	mutator
Lac$^+$ Tet^R	1	4	1	11 (± 3)	<0.03	mutator
Lac$^+$ 5-FC^R	10	4	1	0.075 (± 0.02)	0.025 (± 0.01)	10 non-mutator
Lac$^+$ 5-FU^R	8	4	1	<0.03	0.03 (± 0.02)	8 non-mutator
Lac$^+$ 5-FU^R	1	4	1	18 (± 2)	2 (± 0.7)	mutator
Lac$^+$ Mal$^-$	8	4	1	<0.03	<0.03	8 non-mutator
Lac$^+$ Mal$^-$	1	4	1	26 (± 5)	1.5 (± 0.5)	mutator
Lac$^+$ Mal$^-$	1	4	1	6.3 (± 1)	0.75 (± 0.3)	mutator
Lac$^+$ Xyl$^-$	10	4	1	0.075 (± 0.08)	0.075 (± 0.04)	10 non-mutator
Lac$^+$ 5-FC^R Mal$^-$	2	4	1	0.12 (± 0.1)	<0.1	2 non-mutator
Lac$^+$ 5-FU^R Mal$^-$	1	4	1	<0.3	<0.3	non-mutator
Lac$^+$ Mal$^-$Xyl$^-$	1	4	1	<0.3	<0.3	non-mutator
Lac$^+$ Mal$^-$ Ts	1	4	1	<0.3	<0.3	non-mutator
Lac$^+$ 5-FU^R Mal$^-$Xyl$^-$	1	4	1	7.0 (± 2)	0.6 (±0.3)	mutator
Totals	55					49 non-mutator 6 mutator

[a]All are isogenic derivatives of the *lac* frameshift-bearing strain (Cairns and Foster, 1991) designated Lac$^-$ parent here. The *mutL* and *dam* derivatives (Longerich *et al.*, 1995) are positive controls for strong and weak mutator phenotypes respectively. Isolation of the Lac$^+$ revertants with associated unselected mutations is described in Tables I and II.

[b]Spots of 10 μl of culture grown to saturation were made onto solid media containing 100 μg/ml streptomycin or 40 μg/ml nalidixic acid, the plates incubated and the number of mutant colonies per spot counted.

[c]SE, one standard error of the mean.

[d]Greater than 50 colonies per spot causes confluent growth and is uncountable. The number of colonies recorded for such uncountable spots was >50. The calculations were made based on 50 and the final values marked >.

Rosenberg *et al.*, 1994; Longerich *et al.*, 1995; R.S.Harris, G.Feng, K.J.Ross, R.Sidhu, C.Thulin, S.K.Szigety, M.E.Winkler and S.M.Rosenberg, submitted).

Which DNA recombines?

The finding of unselected mutation in replicons other than the F′ bears on the question: 'With which DNA does the double-strand-broken DNA recombine?' The DNA homology used for recombination in recombination-dependent mutation could have been either a whole or partial sister replicon (Harris *et al.*, 1994), a gene duplication (amplification) (Harris *et al.*, 1994) or exogenous DNA taken up into the starving cells. However, if duplications were the homology source, then unselected mutations associated with adaptive reversions might have been confined to sites in the duplicated DNA segment, i.e. next to the adaptive reversion. Thus this gene duplication hypothesis and others (Foster, 1993; Roth *et al.*, 1996) appear less tenable in light of finding genome-wide hypermutation.

Role of F transfer proteins

The role of F transfer proteins may be single-strand nicking of the F transfer origin by the Tra proteins. This nick could be converted to a DSB by, for example, endonuclease activity (Rosenberg *et al.*, 1995), by replication (Harris *et al.*, 1994; Kuzminov, 1995; Foster *et al.*, 1996; Rosenberg *et al.*, 1996) or by DNA repair-like single-strand excision and/or synthesis up to the nick on the nicked strand. Chromosomal and pBR322-located mutable sites could experience DSBs similarly, by two single-strand opposite (but with neither caused by Tra; Rosenberg *et al.*, 1995), or by other mechanisms (Harris *et al.*, 1994; Rosenberg *et al.*, 1996). There is evidence that different chromosomal regions are differently susceptible to DSBs (reviewed by Rosenberg *et al.*, 1995, 1996). This could account for the existence of two sites in the bacterial chromosome that are inactive for recombination-dependent Lac reversion (Foster and Trimarchi, 1995; Galitski and Roth, 1995; Radicella *et al.*, 1995; R.S.Harris and S.M.Rosenberg, unpublished results), and does not exclude the possibility that other chromosomal sites will be active.

Models invoking transfer, transfer synthesis or sex as a precondition to the mutagenesis in this system predict that unselected mutation in association with Lac adaptive reversion would be confined to the F′ (Foster and Trimarchi, 1995; Galitski and Roth, 1995; Peters and Benson, 1995; Radicella *et al.*, 1995; Rosenberg *et al.*, 1995). This prediction appears to be inconsistent with the results reported here.

Generality of the mechanism

The discovery of genome-wide hypermutability underlying this novel recombination-dependent mutagenic mechanism in stressed, non-growing cells re-opens the possibility that this mechanism may apply more broadly. Associations between recombination and either mutations or DNA synthesis have been inferred in other systems. These include mutations associated with double-strand break repair in yeast (Strathern *et al.*, 1995), mutagenesis correlated with recombination, sex or both in bacteria (Demerec,

1962, 1963), yeast (Magni and von Borstel, 1962; Esposito and Bruschi, 1993), and filamentous fungi (Paszewski and Surzycki, 1964), and the association of hyper-recombination or chromosome instability with elevated mutation in the Bloom's and Werner's syndromes in humans (reviewed by German, 1993; Yu *et al.*, 1996). DNA synthesis promoted by recombination is thought to be the source of replication origin-independent 'inducible stable DNA replication' (iSDR) in *E.coli* which has similar, but not identical, genetic requirements to Rec-dependent Lac reversion (Asai and Kogoma, 1994a,b; Foster *et al.*, 1996; Harris *et al.*, 1996).

Thus, the mutagenic mechanism being elucidated in the *lac* system could be relevant in microbial evolution and in other organisms, during normal development and, for example, in the origin of cancer. The abundance of *E.coli* recombination and mutator protein homologs already implicated in such processes (Modrich, 1994; Ellis *et al.*, 1995; Yu *et al.*, 1996) underscores the need to understand all of the ways that these proteins promote genetic change in *E.coli* and in more complicated organisms whose proteins are structurally and functionally similar.

Materials and methods

Obtaining adaptive mutants, Lac⁻ stressed and unstressed cell colonies

Lac^+ adaptive revertants of the *lacI–Z* fusion *lacI* frameshift-bearing strain were obtained after 3–7 days incubation on M9 thiamine 0.1% lactose medium (Cairns and Foster, 1991; Harris *et al.*, 1994, 1996) (+100 μg/ml ampicillin for experiments in Table I). Day 3–7 colonies were marked on the backs of the plates and replica-plated. Lac^- stressed colonies were obtained by resuspending agar taken from between visible Lac^+ adaptive mutant colonies on days 4–6, in M9 salts, and plating on LBH rifampicin plates or on M9 thiamine 0.2% glucose rifampicin plates (LBH, 1% tryptone, 0.5% NaCl, 0.5% yeast extract, 2 μg/ml thymine, pH 7, plate media solidified with 1.5% agar, rifampicin, 100 μg/ml + 100 μg/ml ampicillin for experiments in Table I) on which the *lac*⁻ frameshift-bearing cells form colonies, but the *lac*-deleted scavenger cells plated with them do not. Scavenger cells consume any non-lactose carbon sources (Cairns and Foster, 1991). Lac^- unstressed colonies were grown on M9 thiamine 0.1% glycerol plates (+ 100 μg/ml ampicillin for experiments in Table I).

Sequencing

Tet^R plasmids were isolated, transformed into plasmid-free cells and Tet^R selected to purify the dominant mutant plasmid from Tet^S parental plasmids prior to sequencing. Template DNA prepared by alkaline lysis was purified in QIAprep spin columns (Qiagen). An oligonucleotide primer complementary to the sequence 'Primer complement' in Figure 1 (synthesized on an ABI Model 392 Synthesizer, Applied Biosystems, Foster City, CA) was used for double-stranded sequencing of DNA from the region shown (see Rosenberg *et al.*, 1994).

Identification of unselected mutations

Tet^R mutants were identified on LBH + 20 μg/ml tetracycline plates. 5-FC^R mutants were identified on M9 thiamine glycerol or lactose with 20 μg/ml 5-FC. Those also 5-FU^R were identified by spotting saturated cultures derived from isolated 5-FC^R colonies onto M9 thiamine glycerol or lactose plates with 10 μg/ml 5-FU. Mutants defective in fermentation of xylose (Xyl^-), maltose (Mal^-), and fructose ($Fruc^-$) were identified as white or pink colonies on MacConkey indicator solid medium (Difco) containing 1% of these sugars and rifampicin. The Xyl^- and Mal^- were then confirmed on minimal xylose and maltose media. Mutants Ts for growth on minimal glycerol medium but not on rich medium, were identified by replica-plating the two Lac^- control colony populations to M9 thiamine 0.1% glycerol plates, and to rich LBH plates. Both were incubated at 37 and 43°C. Those that grow at 37 but not 43°C only on minimal medium are designated Minimal Ts, and are probably auxotrophic Ts mutants. (General Ts mutants were too few to quantify in this assay.) None is Ts for proline biosynthesis (all are ts on minimal glycerol proline plates), the only amino acid biosynthetic locus on the F′. For the Lac^+ revertants, phenotypic Lac-ts colonies (presumed *lac*-ts frameshift mutants), which represent a few percent of the total, were screened for other Ts by replica-plating to minimal lactose medium at 37 and 43°C. Those that failed to grow at 43°C were diluted serially by consecutive toothpicking in minimal lactose rifampicin agar (to purify Lac^+ revertants away from non-revertant and rifampicin-sensitive scavenger cells; Cairns and Foster, 1991), incubated at 37°C then replica-plated to minimal glycerol plates at 37 and 43°C. All Minimal Ts mutants isolated were confirmed by picking the original Lac^+ colony, diluting and plating for single colonies on minimal lactose rifampicin at 37°C then replica-plating to minimal glycerol at 37 and 43°C.

Mapping unselected mutations

5-FC^R and 5-FU^R mutations were mapped to the F′ and the chromosome by P1 transductional mapping. The *codAB* locus, whose loss of function confers 5-FC^R, is located next to *lac* on the F′ and is deleted from the bacterial chromosome in this strain (Cairns and Foster, 1991). Phage P1 transductional mapping demonstrated linkage of all 49 independent 5-FC^R mutations tested to the *lac* gene. The *lac*⁻ *codA*$^+$ strain BW7620 (*E.coli* Genetic Stock Center number CGSC6813) was transduced with P1 grown on each 5-FC^R isolate, Lac^+ colonies were selected and co-transduction of 5-FC^R mutant phenotype was observed. The *upp* gene, whose loss of function confers both 5-FC^R and 5-FU^R, is located next to *guaBA* in the bacterial chromosome and is not present on the F′. P1 transductional mapping showed linkage of all 35 independent 5-FU^R mutations tested to the deletion Δ(*gua-xseA*) (Vales *et al.*, 1979). Guanosine-proficient transductants of a Δ(*gua-xseA*) strain were 5-FU^R at the expected frequency. Fifteen Xyl^- and 14 Mal^- mutations tested were confirmed as chromosomal by the ability of the mutants to be transduced to Xyl^+ and Mal^+ phenotypes with phage P1 grown on strain P90C (Cairns and Foster, 1991), the female parent of the *lac* frameshift-bearing strain. P90C carries no F′ and has a chromosomal *lac* deletion identical to that in the frameshift-bearing strain.

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Note added in proof

A paper published after this manuscript's acceptance could seem to suggest that unselected mutation during Lac adaptive mutation is confined to the F′ [Foster,P.L. (1997) Nonadaptive mutations occur on the F′ episome during adaptive mutation conditions in *Escherichia coli*. *J. Bacteriol*., **179**, 1550–1554]. However, as no non-F′ sites were examined, the data are compatible with those reported here.