Modulation of allele leakiness and adaptive mutability in *Escherichia coli*

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Abstract

It is shown that partial phenotypic suppression of two ochre mutations (*argE3* and *lacZU118*) and an amber mutation (in *argE*) by sublethal concentrations of streptomycin in an *rpsL*$^+$ (streptomycin-sensitive) derivative of the *Escherichia coli* strain AB1157 greatly enhances their adaptive mutability under selection. Streptomycin also increases adaptive mutability brought about by the *ppm* mutation described earlier. Inactivation of *recA* affects neither phenotypic suppression by streptomycin nor replication-associated mutagenesis but abolishes adaptive mutagenesis. These results indicate a causal relationship between allele leakiness and adaptive mutability.

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Introduction

Mutagenesis is a very fundamental biological process. Traditionally, experiments on bacterial mutagenesis have been done using growing bacterial cultures because of the belief that only growing cells (containing replicating DNA) are mutable. However, it has been shown lately that bacteria are prone to mutagenesis not only during growth but also when subjected to such selection pressures that prevent growth but are not lethal. Mutagenesis under nonlethal selection has been loosely called 'adaptive' since the mutations that are usually recovered enable the cells to counter the pressure and resume growth. However, many workers prefer to use the term 'stationary phase' mutagenesis instead. This process has been extensively debated since 1988 and many reviews on the subject are available (Foster 1993, 1998; Lenski and Mittler 1993; MacPhee 1993; Sniegowski 1995; Benson 1997; Cairns 1998; Rosenberg *et al*. 1998; for additional references see Jayaraman 1995) . It was proposed by Hall (1990) that when cells are subjected to nonlethal selection stress a minority subpopulation enters into a transient, hypermutable state during which mutations (adaptive and nonadaptive) could occur. A cell suffering an adaptive mutation manages to grow because of favourable selection while others do not grow or may even perish eventually; any nonadaptive mutation which happens to exist at the time of occurrence of the adaptive one has a good chance of 'hitch-hiking' with the latter. A recent report has shown that such hypermutable cells comprise approximately 0.06% of the population (Rosche *et al*. 1999). Work in my laboratory (Jayaraman 1995, 1999) and that of Galistsky and Roth (1996) has shown that 'leakiness' of mutant alleles could be an important parameter of adaptive mutability. The *ppm* (postplating mutagenesis) and mutagenic ochre suppressor mutations described by me in *Escherichia coli* have been shown to enhance adaptive mutability by enhancing the intrinsic leakiness of mutant alleles (Jayaraman 1995, 1999). It was suggested that limited growth under selection, facilitated by allele leakiness, could be the trigger that pushes cells into a hypermutable state resulting in random, genome-wide mutagenesis (Jayaraman 1999). The importance of limited cell growth in DNA turnover and adaptive mutability has also been shown by Bridges (1998) and Bridges and Eriera (1998). In this report I provide additional experimental evidence to show the causal role of allele leakiness in adaptive mutagenesis. It is shown that phenotypic modulation of allele leakiness influences adaptive mutagenesis in the same way that genetic modulation by the *ppm* and ochre suppressor mutations described earlier does.

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Experimental methods

All the experiments described here were done with derivatives of the *E. coli* strain AB1157 whose relevant genotype is *thr1 leuB6 lacY1 proA2 hisG4 argE3 rpsL31*. Other mutational markers were added to AB1157 or existing ones deleted from it, as described below.

(a) The region spanning *gpt* and *lac* was deleted as described earlier (Jayaraman 1999). This allows the introduction of marked *lacZ* alleles through F′*lacZ* $proA^+B^+$ elements.
(b) The *rpsL31* mutation conferring streptomycin resistance was replaced by the $rpsL^+$ allele by cotransduction with linked Tn*10* from JW375 (*zhc511*::Tn*10*; earlier linkage map) and screening the Tet^r transductants for streptomycin sensitivity.
(c) The above Sm^s derivative was cured of the Tn*10* element by plating on Bochner's medium (Bochner *et al*. 1980). This yields a Tet^s, Sm^s, *lac–pro* deleted derivative of AB1157.
(d) Whenever required the *ppm* or *mutS* mutations were introduced by cotransduction with linked Tn*10* from appropriate donors.
(e) The *recA* gene was inactivated by transducing a *recA*::*cam* insertion as described earlier (Jayaraman 1995).
(f) Adaptive mutation to Lac^+ was scored on minimal medium supplemented with glycerol (0.002%) and lactose (0.4%) besides the required nutritional supplements. As a routine practice, IPTG (10^{-4} M) was always included, even if not required. Sometimes, X-gal (20 µg/ml) was also included for better visualization of Lac^+ colonies. Adaptive mutation to arginine independence was scored on minimal medium with glucose as the carbon source and lacking arginine.

Overnight cultures in Luria broth were diluted to A_{600} 0.25 (10^8 cfu/ml), 0.1 ml aliquots were spread on selective media, and incubated at 37°C. Colonies were scored after 3 and 8–10 days.

Results and discussion

Modulation of allele leakiness by streptomycin: rationale and design of experiments

The leaky phenotype exhibited by a mutant could result from one or both of the following. The mutant protein could have considerable residual biological activity which might satisfy the need of the cell to a limited extent and this could result in a leaky phenotype. The intrinsic leakiness of the *thr1* allele of AB1157 could be an example of this. Alternatively, occasional mistranslation of mutant codons as normal ones could result in the synthesis of small amounts of normal proteins which could lead to a leaky phenotype. The *ppm* and ochre suppressor mutations described earlier have been suggested to act by this mechanism (Jayaraman 1995, 1999). During the early years of attempts to decipher the genetic code Kaji and Kaji (1965) and Pestka *et al*. (1965) observed that the antibiotic streptomycin caused occasional mistranslation of codons in *in vitro* amino acid incorporation systems. That such mistranslation of codons could also occur *in vivo* was first demonstrated by Gorini and Kataja (1965), who showed that a streptomycin-sensitive, arginine auxotroph of *E. coli* could grow in the absence of the required amino acid if sublethal amounts of streptomycin were provided, a phenomenon called 'phenotypic suppression'. Phenotypic suppression by streptomycin has been demonstrated with nonsense and missense mutations in bacteria and bacteriophages. (For detailed information on streptomycin-induced mistranslation of codons *in vitro* and phenotypic suppression of mutations by streptomycin *in vivo*, the reader is referred to the early monographs (Gale *et al.* 1972 and Lewin 1974).) In effect, phenotypic suppression is the ultimate result of increasing the leakiness of mutant alleles to such an extent that sufficient amounts of wild-type (or functionally proficient) gene product is made available to satisfy cellular requirements. It could, therefore, be expected that concentrations of streptomycin that are not high enough for full manifestation of phenotypic suppression but are sufficient to marginally increase allele leakiness would stimulate adaptive mutagenesis under selection, by permitting restricted growth. To test this notion, I first determined the colony forming ability of derivatives of AB1157 in the absence of arginine but in the presence of varying concentrations of streptomycin. The observations are presented in table 1. It can be seen that in the $rpsL^+$ (streptomycin-sensitive) genetic background, AB1157 is unable to form colonies on arginine-less medium. Addition of streptomycin at sublethal concentrations (up to 3 µg/ml) progressively increases colony forming ability in the absence of arginine (section A of table 1). However, in the *rpsL31* (streptomycin-resistant) genetic background, phenotypic suppression is totally abolished even at high concentrations of the antibiotic (section B). The *ppm* mutation described earlier (Jayaraman 1995) was suggested to increase the leakiness of the *argE3* mutation and thereby favour adaptive mutagenesis to arginine independence under selection. In agreement with such a notion the $rpsL^+$ *ppm* mutant is able to form colonies in arginine-less medium even in the absence of the antibiotic, albeit poorly; addition of streptomycin at very small concentrations (0.2 and 0.5 µg/ml), which are ineffective in the ppm^+ strain, fully restores colony forming ability (section C). In the *rpsL31 ppm* gnetic background phenotypic suppression of *argE3* was very poor even at high (150 µg/ml) concentrations of streptomycin (section D). The behaviour of $recA^0$ derivatives was identical to that of their $recA^+$ counterparts with respect to phenotypic suppression of *argE3* (sections E and F).

Table 1. Phenotypic suppression of *argE3* by streptomycin in different genetic backgrounds.

Genetic background	Concentration of streptomycin (μg/ml)	Colony formation arginine-less medium
A. *rpsL*$^+$ *ppm*$^+$	0	Negative
	0.2	Negative
	0.5	Very poor
	1.0	Poor
	2.0	Normal
	3.0	Normal
B. *rpsL31 ppm*$^+$	0	Negative
	2.0	Negative
	150.0	Negative
C. *rpsL*$^+$ *ppm*	0	Positive but poor
	0.2	Normal
	0.5	Normal
	2.0	Normal
D. *rpsL31 ppm*	0	Negative
	2.0	Negative
	150.0	Negative
E. *rpsL*$^+$ *ppm*$^+$ *recA*^{0}	0	Negative
	2.0	Normal
F. *rpsL*$^+$ *ppm recA*^{0}	0.2	Normal
	2.0	Normal

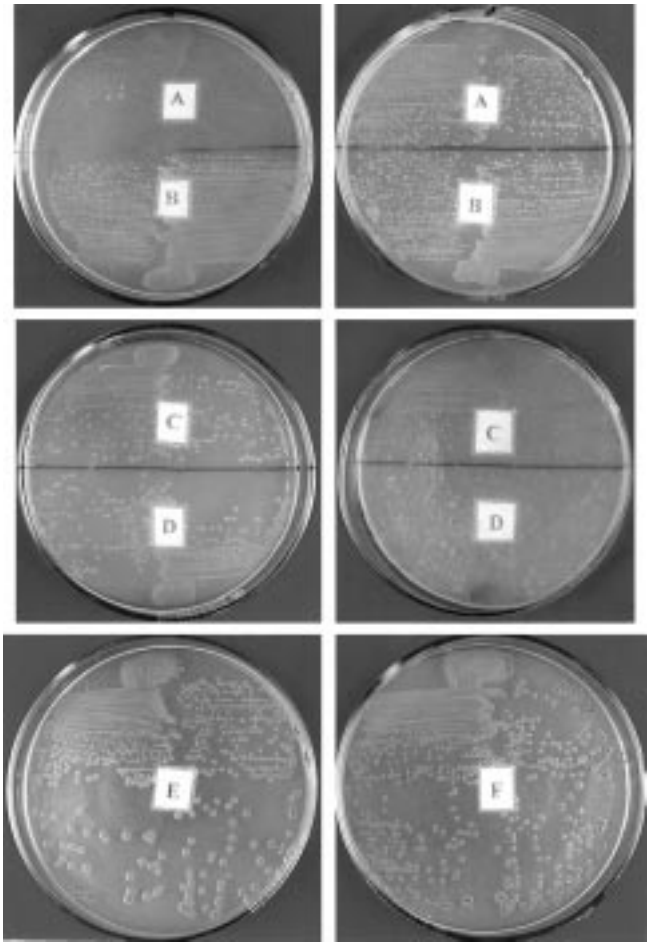

Figure 1. Phenotypic suppression of *argE3* and *lacZU118* mutations by streptomycin in some derivatives of AB1157. Top row: AB1157 *rpsL*$^+$ (A) and AB1157 *ppm rpsL*$^+$ (B) on arginine-less medium supplemented with 0.5 μg/ml (left) and 2.0 μg/ml (right) of streptomycin. Middle row: AB1157 *rpsL*$^+$/F'*lacZU118* (C) and AB1157 *mutS*::Tn*10 rpsL*$^+$/F'*lacZU118* (D) on glycerol mimimal medium + IPTG (10^{-4} M) + X-gal (20 μg/ml) in absence (left) and presence (right) of streptomycin (2 μg/ml). Bottom row: AB1157 *ppm rpsL*$^+$ in glycerol minimal medium + IPTG (10^{-4} M) + X-gal (20 μg/ml) in absence (E) and presence (F) of streptomycin (2 μg/ml).

The *argE3* mutation is an ochre (UAA) nonsense mutation (Meinnel *et al.* 1992). The observations described above indicate that in presence of streptomycin UAA is read as a sense codon. If this were true other ochre mutations also should respond similarly. To test this the F'*lacZU118* (ochre) element was introduced into the Δ(*lac–pro*) *rpsL*$^+$ AB1157 and Pro$^+$ exconjugants were selected. One of them was streaked for single colonies on glycerol minimal medium supplemented with IPTG (10^{-4} M) and X-gal (20 μg/ml). In absence of streptomycin the colonies were white but in its presence (2 μg/ml) the colonies were pale blue. A similar construct in the *ppm* genetic background gave blue colonies irrespective of the presence of streptomycin. These observation are shown in figure 1.

Stimulation of adaptive mutagenesis to arginine independence by streptomycin in some derivatives of AB1157

It can be seen from table 1 and figure 1 that 2–3 μg/ml streptomycin is necessary for full phenotypic suppression of *argE3* in *rpsL*$^+$ AB1157. Lower concentrations might still enhance leakiness but not to an extent sufficient for colony formation in the absence of arginine. As pointed out earlier such concentrations could be expected to promote adaptive mutagenesis to arginine independence by allowing leaky, even imperceptible, growth in the absence of arginine. To test this notion, *rpsL*$^+$ AB1157 was plated on arginine-less medium containing 0, 0.1, 0.2 and 0.5 μg/ml streptomycin. It can be seen from table 2 that even at 0.1 and 0.2 μg/ml, streptomycin stimulated adaptive mutagenesis to arginine independence (section A of table 2). At 0.5 μg/ml, although there was no colony formation, a lawn consisting of very very small colonies resulted when a large number of cells ($\sim 10^{-7}$) were plated. Larger, arginine-independent colonies could be clearly seen embedded in the lawn, but accurate counting was difficult. Inactivtation of one of the genes involved in methyl-directed mismatch repair (*mutS*) was found to enhance the response to streptomycin 30-fold (section B), whereas inactivation of *recA* abolished the response (section C).

As shown in table 1, the *ppm* mutation by itself causes phenotypic suppression of *argE3* in the *rpsL*$^+$ background, although the colonies formed are very small in size. In

Table 2. Stimulation of adaptive mutagenesis to arginine independence by streptomycin in AB1157 derivatives.

Relevant genotype	Concentration of streptomycin (μg/ml)	Arg^+ colonies per 10^7 cfu	
		Day 3	Day 10
A. *rpsL*$^+$ *ppm*$^+$	0	1	1
	0.1	0	80 ± 3 (4)
	0.2	1	235 ± 6 (4)
	0.5	Faint lawn	Dense lawn
B. *rpsL*$^+$ *ppm*$^+$ *mutS*::Tn*10*	0	7 (4)	8 ± 3 (4)*
	0.1	10 (4)	255 ± 18 (4)*
	0.2	8 (4)	630 ± 11 (4)*
C. *rpsL*$^+$ *ppm*$^+$ *mutS*::Tn*10* recA^0	0.2	10 (5)	11 (5)*
D. *rpsL*$^+$ *ppm*	0	2 (4)	152 ± 9 (7)
	0.5	Dense lawn	Dense lawn
E. *rpsL31 ppm*$^+$	0	0	1
	150	0	1
F. *rpsL31 ppm*	0	3 (4)	3 (4)
	150	2 (4)	252 ± 28 (4)

*10^6 cfu were used in these experiments.

agreement with this observation, the *ppm rpsL*$^+$ strain adapts to arginine independence very well in the absence of streptomycin (table 2, section D); addition of streptomycin increases the leakiness of *argE3* enough to result in a dense lawn of cells.

Strigini and Gorini (1970) showed that some mutations in the *rpsL* locus, conferring resistance to streptomycin, drastically reduced phenotypic suppression by the antibiotic. As shown above (table 1, section B) the *rpsL31* mutation present in AB1157 belongs to this class; a *ppm*$^+$ *rpsL31* strain does not show phenotypic suppression of *argE3* even at very high concentrations of streptomycin. Moreover the *rpsL31* mutation also counters the ability of the *ppm* mutation to elicit phenotypic suppression of *argE3* (table 1, section D). In complete agreement with these observations, it was found that the *ppm*$^+$ *rpsL31* mutant does not mutate to arginine independence in the presence or absence of streptomycin (table 2, secion E). However, the *ppm rpsL31* mutant does so, but only in the presence of streptomycin (table 2, section F). As noted earlier (Jayaraman 1995), the emergence of Arg^+ colonies was preceded by the appearance of a faint lawn, indicative of an increase in the leakiness of *argE3* to an extent sufficient for adaptive mutagenesis but not for overt colony formation (full phenotypic suppression).

Phenotypic suppression of lacZU118 mutation and adaptation to lactose utilization

It can be seen from figure 1 that streptomycin increased the leakiness of the *lacZU118* (ochre) mutation in the *rpsL*$^+$ genetic background. Therefore it could be expected that adaptive mutagenesis to Lac^+ will be stimulated by streptomycin in *rpsL*$^+$ strains, harbouring the F′*lacZU118* episome. When this was tested in the *rpsL*$^+$/F′*lacZU118* strain, for reasons not known the response was somewhat poor and inconsistent. On some occasions adaptation was observed (only in the presence of 2 μg/ml streptomycin) after prolonged periods (10–15 days) of incubation. On other occasions there was no response with or without streptomycin. However, in the *rpsL*$^+$ *mutS*::Tn*10*/F′*lacZU118* strain, consistently good adaptation to Lac^+ was seen in the presence of streptomycin (table 3, section A). A good number of Lac^+ colonies were seen even in the absence of streptomycin but this was significantly lower than that observed in its presence. These could be Lac^+ variants arising from replication-associated mutagenic processes, before and after imposition of selection, since the strain harboured a mutator mutation (*mutS*::Tn*10*) and the selection medium contained 0.002% glycerol which would permit limited growth. That they are indeed so was shown by the following experiment. When a *recA*^{0} derivative of the *rpsL*$^+$ *mutS*::Tn*10*/F′*lacZU118* was used, the streptomycin response was abolished while the background (presumably replication-associated) mutagenesis was unaffected (table 3, section B).

It can be seen from table 1 and figure 1 that the *ppm* mutation increases the leakiness of *argE3* and *lacZU118* mutations even in the absence of streptomycin. It was also shown above (table 2) that the *rpsL*$^+$ *ppm* mutant adapts to arginine independence in the absence of streptomycin. In complete agreement with these observations, it was found that the *rpsL*$^+$ *ppm*/F′*lacZU118* strain adapts to Lac^+ in the absence of streptomycin, which caused a four-fold increase in the frequency (table 3, section C). The stimulation of adaptive mutagenesis to arginine independence by streptomycin seen in the *ppm rpsL31* stain (table 2, section F) was also observed in the case of lactose adaptation (table 3, section D).

Table 3. Emergence of Lac$^+$ variants in *rpsL*$^+$ AB1157 derivatives harbouring the F′*lacZU118* episome.

Relevant genotype	Lac$^+$ colonies per 10^6 cfu after 8 days	
	− streptomycin	+ streptomycin (2 μg/ml)
A. *rpsL*$^+$ *mutS*::Tn*10*/F′*lacZU118*	126 ± 9 (6)	483 ± 39 (6)
B. *rpsL*$^+$ *mutS*::Tn*10 recA*^{0} F′*lacZU118*	79 ± 8 (4)	94 ± 11 (4)
C. *rpsL*$^+$ *ppm*/F′*lacZU118*	60 ± 5 (4)*	228 ± 39 (4)*
D. *rpsL31 ppm mutS*::Tn*10* F′*lacZU118*	119 ± 9 (4)	236 ± 13 (4)

*10^7 cfu were used for these experiments.

Table 4. Adaptation to arginine independence in CSH108.

Relevant markers	Concentration of streptomycin (μg/ml)	Arg$^+$ colonies per 10^8 cfu plated	
		Day 3	Day 10
argE (amber)	0	4 ± 1 (4)	27 ± 4 (4)
	0.25	9 ± 2 (4)	153 ± 4
argE(amber) *recA*^{0}	0.25	1 (4)	6 (4)

The genotype of CSH108 is: *ara* Δ(*gpt–lac*) *gyrA argE* (amber) *rpoB*/F′*lacI lacZ* (amber) *proA*$^+$*B*$^+$.

Phenotypic suppression of amber mutations in argE and lacZ and its effect on adaptive mutagenesis

The *argE* and *lacZU118* mutations described above are ochre (UAA) nonsense mutations in the respective genes. The *E. coli* strain CSH108 carries amber (UAG) lesions in *argE* (chromosomal) and *lacZ* (episomal). It was of interest to see whether these would also be sensitive to phenotypic suppression by streptomycin. It was observed that colonies of CSH108 were white on glycerol–IPTG–X-gal medium but pale blue if the medium contained streptomycin (2 μg/ml) also, indicating phenotypic suppression. However, the *lacZ* amber mutation in CSH108 reverted to Lac$^+$ at a high frequency, even in nonmutator genetic backgrounds, and hence could not be used for adaptation experiments. On the other hand, the *argE* amber mutation presented no such difficulty. Therefore the effect of streptomycin on adaptation to arginine independence was studied in CSH108. The data are presented in table 4. It can be seen that the *argE* (amber) mutant mutates to arginine independence even in the absence of streptomycin, suggesting that the intrinsic leakiness of *argE* (amber) could be more than that of *argE* (ochre). Addition of streptomycin at low concentration (0.25 μg/ml) greatly enhances the response. Adaptation is totally abolished in the *recA*^{0} derivative of CSH108. Identical results were obtained when a *mutS*::Tn*10* derivative of CSH108 was used, except that the background (number of Arg$^+$ colonies on day 3) was considerably higher because of the mutator background (data not shown). The *recA*^{0} mutation abolished the adaptation response but not the background (replication-associated mutagenesis). Thus, these results are in complete agreement with those obtained with ochre mutants in *argE* and *lacZ*.

Concluding remarks

In this communication I have shown that partial phenotypic suppression of two ochre mutations (*argE3* and *lacZU118*) and an amber mutation in *argE* by sublethal concentrations of streptomycin in *rpsL*$^+$ (streptomycin-sensitive) genetic background greatly accentuates mutagenesis under selection for Arg$^+$ or Lac$^+$. In addition, streptomycin also enhances such mutagenesis brought about by the *ppm* mutation. The present results support the notion, discussed in detail earlier (Jayaraman 1995, 1999), that allele leakiness is a crucial parameter of adaptive mutagenesis. Limited growth facilitated by allele leakiness could push the cells under selection stress into a state of random hypermutability. Selection would favour the survival and growth of 'adapted' cells while others may not grow or may die out. A direct and testable prediction stemming from this and earlier work is that leaky growth is more mutation-prone than normal gorwth. Results of preliminary experiments to test this hypothesis have been very encouraging. The data will be published elsewhere. Mutagenesis during leaky growth could be of biological significance. Since under natural conditions bacteria suffer from severe nutrient deprivation and survive mostly in the nondividing state, any mechanism, genetic or phenotypic, that would permit leaky growth could be a source of generation of genetic variability. This idea can also be extended to the onset of malignancy from quiescent cells. Inactivation of *recA* does not affect phenotypic suppression by streptomycin but abolishes the consequent adaptive mutagenesis. Replication-associated mutagenesis is not affected by the inactivation of *recA*.

Note added in proof: I have observed that the *lacZ* mutation present in F′CC105 (GAG → GTG, leading to E_{461} to V_{461}) reverts a lot more when *rpsL*$^+$ AB1157 carrying it is grown in the absence of arginine but presence of streptomycin (2–3 μg/ml, leaky growth) than in the presence of arginine, with or without streptomycin (normal growth).

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