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Cairnsian mutagenesis in *Escherichia coli*: Genetic evidence for two pathways regulated by *mut*S and *mut*L genes

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Abstract. The phenomenon of Cairnsian mutagenesis was studied in *Escherichia coli* mutants bearing mutations in *mut*S, *mut*L, *rec*A and *lex*A genes. It is shown that development of resistance to exogenous valine could be used as an example of Cairnsian response. Strains defective in *mut*S and *mut*L show a high frequency of Cairnsian mutagenesis to valine resistance. The response in *mut*S mutants is dependent upon cleavability of the LexA protein whereas that in *mut*L is not. The latter is independent of *rec*A also. The need for LexA protein cleavage in *mut*S mutants can be bypassed by over-production of the RecA protein due to a *rec*A operator constitutive mutation. Genetic evidence is presented to show that the products of *mut*S and *mut*L genes negatively control two pathways of Cairnsian mutagenesis. Cairnsian response is also elicited when *mut*S or *mut*L strains are grown under conditions wherein a required nutrient is present in sub-optimal concentrations. Random, unselected mutagenic events are likely to occur during or after Cairnsian mutagenesis provided the cells are SOS inducible.

Keywords. Mutagenesis; Cairnsian response; *mut*S; *mut*L; SOS response; adaptive mutagenesis.

1. Introduction

Mutation and natural selection are the two most important facets of evolution. While mutation generates genetic variation, selection operates by influencing the distribution of mutants in a population. The classic experiments of Luria and Delbruck (1943), Lederberg and Lederberg (1952) and Cavalli-Sforza and Lederberg (1956) have generated an axiom that mutation and selection are independent of each other and that mutations arise regardless of selection pressure. There is no gainsaying that this concept has been amply vindicated by experiments. It has been shown that in growing cells of *E. coli* processes such as wrong base selection during replication (Sevestopoulos and Glaser 1977), faulty proof reading by the DNA polymerase III-associated $3' \rightarrow 5'$ exonuclease (Di Francesco *et al.* 1984) and defects in the methyl-directed mismatch correction systems (reviewed by Radman and Wagner 1986 and Modrich 1987) lead to elevated mutation frequency. This implies that spontaneous mutation in *E. coli* could be the result of uncorrected replication errors although such a conclusion has been disputed (J. Cairns, personal communication). It should be noted that the current ideas on the origin of spontaneous mutations are based on experiments done with actively growing cells. In such cells replication errors which might manifest as mutations if left uncorrected will involve any base pair in any locus and therefore could be expected to be by and large random with respect to the time and locus of occurrence and not dictated by need or environment. However, some bias with respect to transcription and

Dedicated to the memory of S. E. Luria

DNA secondary structure have been reported (Davis 1989; Mellon and Hanawalt 1989; Trinh and Sinden 1991).

There have also been a number of reports on the occurrence of selection-specified mutations in *E. coli*. Mutations in the *ebg* operon which allow lactose utilization by a Δ *lac* strain exposed to lactose (Hall and Clarke 1977), mutations to citrate utilization during prolonged exposure to citrate in the absence of other carbon sources (Hall 1982), excision of Mu phage to allow growth in presence of lactose and arabinose (Shapiro 1984), mutations in the *bgl* operon to allow utilization of β-glucosides (Hall 1988), reversion of nonsense and frameshift mutations in *lac*Z on exposure to lactose (Cairns *et al.* 1988; Cairns and Foster 1991, and personal communication), reversion of *trp*A and *trp*B mutations during tryptophan starvation (Hall 1990, 1991a), excision of Tn*3* to restore prototrophy during isoleucine–valine starvation (Boe 1990) etc., could be mentioned among such reports. In all the above cases, the mutations probably did not exist prior to exposure to the selective environment and occurred only after exposure. Moreover, it was also shown, at least in some cases, that the experimental conditions did not result in a generalised increase in mutation rates (Cairns *et al.* 1988; Hall 1988, 1990). Thus, the occurrence of such mutations seem to violate the dictum of randomness and appear to be selection-dependent, "directed" events. Explicit statement of this idea by Cairns *et al.* (1988) has generated considerable debate (see Hall 1990 and Keller 1992, for references) and has brought to the forefront a very basic question: whether spontaneous mutations occur in total disregard of the environment as well as usefulness or are dictated by the environment and utility or both. Mutations which arise at a higher probability when useful than when neutral were called "Cairnsian" (Hall 1990). They have also been called 'directed mutations", "adaptive mutations", "selection specified mutations" etc. I shall stick to the usage "Cairnsian mutations". In this communication I present genetic evidence to show that there could be two pathways of Cairnsian mutagenesis in *E. coli*, one under the control of the *mut*S gene and the other under the control of the *mut*L gene.

1.1 *Models of Cairnsian mutagenesis*

Foster (1992) has critically reviewed the experimental evidence for directed mutations and assessed various hypotheses proposed to explain the same. I shall confine myself to a brief description of two models which are relevant to the present report. Stahl (1988) proposed that in non-growing cells random segments of the genome could be rendered single stranded due to nicking followed by helicase action and exonucleolytic degradation. Error-prone repair of such gaps would generate mismatched heteroduplexes. If such a lesion happens to be located on the coding strand of the DNA and if its transcription and translation allow cell growth, the mutation gets fixed. An important element in the above model is the life span of the mismatches which have a high probability of getting repaired by the methyl-directed mismatch repair (MMR) system. Boe (1990) showed that a *mut*S mutant which is defective in MMR exhibited significant DNA turnover in the stationary phase and a higher frequency of Cairnsian (directed) mutation (excision of a transposon). Since the above model invokes the generation of single-stranded DNA which has been shown to be the inducing signal for the SOS response (Sassanfar and Roberts 1990) it is not unreasonable to expect an interrelationship between the two processes. This was one of the main objectives when

this work started. I wished to examine the effect(s) of mutations in the genes which constitute the SOS network and also the ones involved in MMR (mainly *mut*S and *mut*L) on Cairnsian response.

Another hypothesis, proposed by Hall (1990), invokes the generation of a "hypermutable" state in some cells of a population under stress for growth. Such cells were postulated to undergo random mutagenesis. If one or more such mutation(s) allow(s) cell growth those cells which suffered them will exit the hypermutable state and survive, while others will eventually die. Any mutation(s) which occurred along with the favoured one(s) will "hitch hike" as the cells grow (Hall 1990, 1991b). Therefore I wished to see whether unselected mutations could also arise during Cairnsian mutagenesis.

In the present report, I have attempted to answer the above questions using the development of resistance to exogenous valine and reversion of amino acid auxotrophy as the experimental systems.

2. Materials and methods

2.1 *Bacterial strains*

All experiments reported herein were done with the *E. coli* K12 strain AB1157 and its derivatives whose relevant markers are: (*his*G4 *pro*A2 *leu*B6 *thr*1 *arg*E3 *rps*L). GW3722 and GW3724 have Tn*5* insertions in *mut*S and *mut*L, respectively, and were obtained from G. C. Walker via J. Das. Tn*10* insertion mutants in *mut*S and *mut*L were obtained from H. Echols. Whenever required these mutations were introduced into other strains by P1 transduction, selecting for kanamycin- or tetracyline-resistance as the case may be. The mutator phenotype of the transductants was verified by checking for increased frequency of spontaneous mutation to rifampicin resistance.

A *rec*A430 derivative of AB1157 was constructed by P1 transduction using DM2453 (*rec*A430 *srl*::Tn*10*; obtained from D. Mount via J. W. Little) as donor, selecting for tetracycline-resistant transductants and scoring for hypersensitivity to UV.

MV1138 is a derivative of AB1157 carrying the *rec*A281 mutation and obtained from T. Kogoma. JC11867 is isogenic to MV1138 but carries in addition the *lex*A3 (Ind^-) mutation; also obtained from T. Kogoma.

DM49 is a *lex*A3 (Ind^-) derivative of AB1157, obtained from D. Mount via K. Dharmalingam.

Media: Conventional LB and M9 minimal salts media were used (Miller 1972). Minimal medium was supplemented with the required amino acids at 30 μg/ml (referred to as complete minimal medium).

2.2 *Measurement of mutation frequency*

2.2a *Valine resistance*: Cells were grown overnight (approximately $3–5 \times 10^9$ cells/ml) in complete minimal medium. Appropriate dilutions were spread on complete minimal plates to obtain total cell titre and on similar plates containing in addition valine (30, 60, 70 or 100 μg/ml). Occasionally traces (30–50 ng/ml) of isoleucine were also included. Colonies were counted every day for upto 6–8 days.

2.2b *Reversion of auxotrophic markers*: Cells were grown overnight (approximately $3–5 \times 10^9$ cells/ml) in complete minimal medium, centrifuged and washed free of medium. Appropriate dilutions were plated on complete minimal medium, medium lacking an amino acid and having the same amino acid at sub-optimal concentration (see § 3·6). The number of colonies were scored after 48–72 h.

All frequencies were calculated with respect to the number of viable cells plated. Limited growth or death of cells on the plates during prolonged incubation could alter the actual number of cells and hence the actual frequencies. The authenticity of mutants was always checked by testing colonies on appropriate plates.

3. Results

3.1 *Development of valine resistance as an example of Cairnsian response*

Mutations which occur at a higher probability when useful than when neutral have been called Cairnsian mutations (Hall 1990). For example, when Lac$^-$ *E. coli* is plated on a medium providing lactose as the sole source of carbon, the Lac$^+$ revertants present at the time of plating would give rise to colonies in approximately 48 h after plating. If colonies appear and continue to appear even several days after plating such colonies qualify to be called Cairnsian mutants provided certain conditions are fulfilled (see below and Foster 1992). I have observed that development of resistance to exogenous valine could be used as an example of Cairnsian mutagenesis. Growth of *E. coli* K12 is sensitive to exogenous valine which inhibits the acetohydroxy acid synthases I and III. This creates artificial auxotrophy for isoleucine. Inhibition of growth by valine could be overcome by the addition of isoleucine or by mutations at several loci (see Vinopal 1987, Umbarger 1987). When cells from Val^s *E. coli* are plated on minimal medium supplemented with valine the preexisting Val^r mutants would show up as colonies by 36–48 h post plating. The colonies appearing later than 2 days after plating could be called Cairnsian mutants if it is shown that their late appearance is due to their late generation rather than slow growth rate.

Figure 1 shows the rate of appearance of Val^r colonies when approximately 10^8 cells of *thr*$^+$ AB1157 (see § 4) are plated on minimal medium supplemented with 30 and 60 μg/ml valine. It can be seen that (i) the number of Val^r colonies keeps increasing with time, and (ii) the numbers are higher on the 30 μg/ml valine plates. Interestingly, all the colonies from the 30 μg/ml medium, irrespective of the time of appearance, grew equally well and without any apparent lag when tested on 60 μg/ml medium. The continuous appearance of post-plating Val^r colonies and the absence of any apparent growth defect in them suggest that they could be Cairnsian mutants. While the *per diem* increase in the number of Val^r colonies is independent of the concentration of valine in the medium, the large difference in the initial number (on day 2) is difficult to explain at the moment. While it is important to show that Val^r colonies arise in response to valine stress, it is equally important to show that they do not arise in the absence of such stress or in response to any other stress. I shall do so in a subsequent section of this report. For the present, I shall use the development of valine resistance to examine other questions. In the experiments presented below I have taken the frequency of Val^r at 48 h after plating as "basal" and that at 8 days after plating as "Cairnsian". This is purely arbitrary. It is

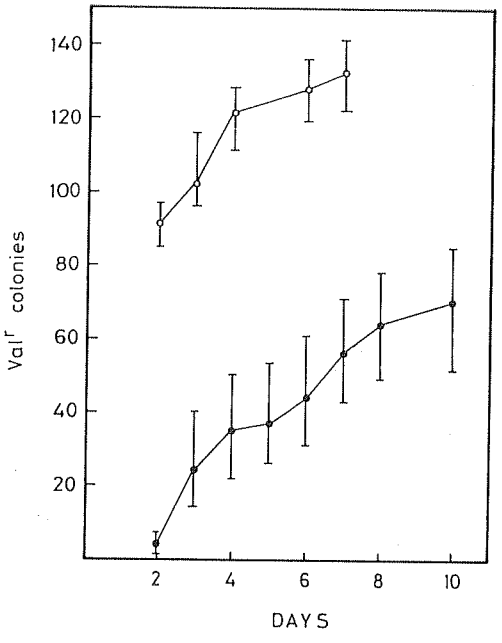

Figure 1. 3×10^7 cells of *thr*$^+$ AB1157 were spread on minimal plates supplemented with 30 and 60 μg/ml valine (4 plates each). The colony numbers were counted for upto 10 days. The points on the curves are the mean number of colonies. The vertical bars represent the range. Open circles: 30 μg/ml valine, filled circles: 60 μg/ml valine.

possible that some of the Val^r colonies at early intervals could have actually arisen in response to valine stress and some at late intervals could be slow growers present initially. I have therefore used high concentrations of valine (60–100 μg/ml) in order to delay the onset of appearance of Cairnsian mutants as far as possible while allowing time for the preexisting mutants to form colonies.

3.2 *Cairnsian mutagenesis to valine resistance in mutS and mutL derivatives of AB1157 and DM49*

Methyl-directed mismatch repair in *E. coli* is under the control of several genes such as *mut*S, *mut*L, *mut*H, *mut*U, *uvr*D etc. (see § 1 for references). AB1157 and DM49 carry the wild type alleles of *mut*S and *mut*L genes. While AB1157 is proficient in the induction of the SOS response, DM49 is deficient in the same since it harbours the *lex*A3 (Ind$^-$) mutation which leads to the synthesis of a non-cleavable LexA repressor (Markham *et al.* 1981). The two strains do show Cairnsian mutagenesis to valine resistance but the frequency is low (of the order of 10^{-6}). This is generally true of strains which are *mut*S$^+$ and *mut*L$^+$ (see § 4). As pointed out earlier, Boe (1990) showed that the frequency of transposon excision under selection pressure is higher in a *mut*S strain than in a wild-type strain. However, it has also been reported (Mittler and Lenski 1990) that increased frequency of transposon excision under selection pressure could be a nonspecific stress response rather than a directed mutational event. Therefore it was of interest to see how the development of valine resistance would be influenced by *mut*S and *mut*L lesions. It would also be of interest to see the interrelationship, if any, between the Cairnsian response and the SOS response. Therefore the effect of the *lex*A3 (Ind$^-$) mutation in

Table 1. Valine resistance in *mut*S and *mut*L strains.

Strain	Relevant genotype[a]	Val (μg/ml)	Frequency of valine resistance[b]	
			Basal	Cairnsian
GW3732	*mut*S::Tn*5*	60	1×10^{-5}	2×10^{-4}
GW3734	*mut*L::Tn*5*	60	$0{\cdot}8 \times 10^{-5}$	$2{\cdot}4 \times 10^{-4}$
		100	1×10^{-5}	$3{\cdot}4 \times 10^{-4}$
DM49/S	*lex*A3 (Ind$^-$)	60	$2{\cdot}3 \times 10^{-5}$	Nil (only basal)
	*mut*S::Tn*5*	100	$1{\cdot}6 \times 10^{-5}$	Nil (only basal)
DM49/L	*lex*A3 (Ind$^-$)	60	3×10^{-5}	$8{\cdot}6 \times 10^{-4}$
	*mut*L::Tn*5*	100	1×10^{-5}	$9{\cdot}4 \times 10^{-4}$
1157/430/S	*rec*A430 *mut*S::Tn*5*	60	4×10^{-6}	9×10^{-6}
		100	3×10^{-6}	7×10^{-6}
1157/430/L	*rec*A430 *mut*L::Tn*5*	60	2×10^{-5}	6×10^{-4}

a: All strains are derivatives of AB1157 whose genotype is given in §2. Only the characteristic genotypes are mentioned here.

b: One-tenth of a millilitre of a 100-fold dilution of overnight cultures of the respective strains in minimal medium, corresponding to $3 - 5 \times 10^6$ cells, were spread on minimal medium plates containing the indicated concentration of valine. Cell titres were determined simultaneously on minimal medium plates lacking valine. Basal frequencies were calculated from the number of colonies present 2 days after plating and Cairnsian frequencies were calculated from the number of additional colonies appearing after 2 days and upto 6–8 days. The values are the means of three independent cultures plated in duplicate.

combination with *mut*S and *mut*L mutations was also studied. It can be seen from table 1 that the basal frequency of valine resistance is comparable in *mut*S and *mut*L strains as well as their *lex*A3 (Ind$^-$) derivatives. These strains also displayed elevated frequencies of rifampicin resistance (data not shown). Thus the expression of the conventional mutator phenotype in *mut*S and *mut*L genetic backgrounds is independent of LexA protein cleavage. In addition, the *mut*S and *mut*L mutants also showed very pronounced Cairnsian mutagenesis to valine resistance. However, the *lex*A3 (Ind$^-$) *mut*S strain failed to show Cairnsian mutagenesis unlike the *lex*A3 (Ind$^-$) *mut*L strain. This suggests that cleavage of the LexA repressor is necessary for the *mut*S mutant to elicit Cairnsian response, but not for the *mut*L mutant.

The need for LexA protein cleavage for the expression of Cairnsian response in *mut*S mutants was also shown by another experiment. Strains harbouring the *rec*A430 mutation are known to be recombination proficient but defective in inducible DNA repair, UV mutagenesis, prophage induction etc. (Morand *et al.* 1977). Sassanfar and Roberts (1990) have shown that LexA protein cleavage in a *rec*A430 mutant is not as efficient as in a *rec*A$^+$ strain upon SOS induction. Data presented in table 1 show that in a *rec*A430 *mut*S mutant the frequency of Cairnsian mutagenesis is very low. The above experiments show that the expression of Cairnsian response in *mut*S strains depends upon cleavability of the LexA protein. Mutations in *lex*A which block the process also block Cairnsian mutagenesis. If the occurrence of Cairnsian mutations were dependent solely on the life span of mismatched heteroduplexes, one should have

observed similar responses in *lex*A3 (Ind$^-$) derivatives of *mut*S as well as *mut*L mutants especially since the expression of their conventional (replication-associated) mutator phenotype is independent of the allelic status of the *lex*A gene. That this is not so suggests that there could be two modes of Cairnsian mutagenesis, one dependent on LexA protein cleavability (*mut*S) and the other independent of the same (*mut*L). Since the viability of *mut* strains is very sensitive to starvation stress (Boe 1990; Foster and Cairns, personal communication) the lack of Cairnsian response in *mut*S *lex*A3 (Ind$^-$) strain could be simply due to loss of viability. However, I have observed that the loss of viability is not very significant upon amino acid starvation (data not shown). Perhaps the *mut* strains are much more sensitive to carbon starvation than amino acid deprivation. The data presented by Boe (1990) and the experiments of Foster and Cairns (personal communication) involve carbon starvation whereas valine sensitivity is due to isoleucine limitation.

3.3 *Elevated levels of RecA protein restores Cairnsian mutagenesis in a mutS lexA3 (Ind$^-$) strain*

The immediate consequence of LexA protein cleavage is the enhanced expression of the SOS genes which are normally kept under partial repression by the LexA protein (Walker 1984, 1987), *rec*A being one among them. If Cairnsian response requires only elevated levels of the RecA protein but none of the other proteins of the SOS system, it should be possible to elicit the response in mutants in which the RecA protein is synthesized at elevated levels but the SOS system is nevertheless inoperative. The strain JC11867 harbours the *rec*A281 mutation which is an operator constitutive mutation in *rec*A (Clark 1982) and the *lex*A3 (Ind$^-$) mutation. MV1138 is isogenic with JC11867 but *lex*A$^+$. The *rec*A281 mutation leads to the synthesis of elevated (derepressed) levels of the RecA protein (Clark 1982). Cairnsian mutagenesis to valine resistance was scored in the *mut*S and *mut*L derivatives of MV1138 and JC11867. Table 2 shows that the

Table 2. Effect of *rec*A281 mutation on Cairnsian mutagenesis.

Strain	Relevant genotype	Frequency of Cairnsian mutation to Val^r (60 μg/ml)
MV1138/S	*rec*A281 *mut*S::Tn*5*	$2{\cdot}2 \times 10^{-4}$
MV1138/L	*rec*A281 *mut*L::Tn*5*	$2{\cdot}5 \times 10^{-4}$
JC11867/S	*rec*A281 *lex*A3 (Ind$^-$) *mut*S::Tn*5*	2×10^{-4}
JC11867/L	*rec*A281 *lex*A3 (Ind$^-$) *mut*L::Tn*5*	$2{\cdot}7 \times 10^{-4}$
GW3734/ΔR	*mut*L::Tn*5* Δ*rec*A *srl*::Tn*10*	$7{\cdot}5 \times 10^{-4}$

Experimental conditions as given under table 1.

response in JC11867 *mut*S is as good as in MV1138 *mut*S. This shows that elevated levels of the RecA protein alleviate the inhibition of Cairnsian response due to the *lex*A3 (Ind$^-$) mutation. It should be noted that in the *lex*A3 (Ind$^-$) *rec*A281 mutant all the SOS genes other than *rec*A are still under repression by the LexA protein. Therefore LexA protein cleavability in Cairnsian response seems to be required only to increase the levels of the RecA protein. Derepression of other SOS genes does not seem to be obligatory.

3.4 *Cairnsian response in mutL strains is recA independent*

The positive manifestation of Cairnsian response in the *lex*A3 (Ind$^-$) *mut*L genetic background suggests that in such cells either the basal levels of RecA protein are sufficient or the process does not need RecA protein at all. In order to distinguish between these two alternatives the *rec*A gene of GW3734 (*mut*L::Tn*5*) was deleted by conjugation with MCL30 (Hfr Δ *rec*A *srl*::Tn*10*), selecting for tetracycline resistant recombinants and screening for hypersensitivity to UV and recombination deficiency. Data presented in table 2 show that the *mut*L Δ *rec*A strain is as proficient in Cairnsian mutagenesis as its *rec*A$^+$ counterpart. This shows that expression of Cairnsian response in *mut*L strains is independent of *rec*A.

3.5 *The possible nature of involvement of mutS and mutL genes in Cairnsian mutagenesis*

It is possible to imagine two ways by which the *mut*S-*mut*L genes could be involved in Cairnsian mutagenesis, besides their postulated (Boe 1990) role in the process allowing longer survival of mismatches. There could be two pathways of Cairnsian mutagenesis, one under the control of *mut*S and the other under that of *mut*L. The respective gene products could be positively required or they could function as repressors, negatively regulating the process. In the model invoking positive roles for the MutS and MutL proteins, the low frequencies observed in wild-type (*mut*S$^+$ *mut*L$^+$) cells could be explained by additionally postulating mutual antagonism between the two proteins so as to keep Cairnsian mutagenesis as low as possible. Since *mut*S and *mut*L mutants are *mut*L$^+$ and *mut*S$^+$ respectively, the response observed in *mut*S and *mut*L strains could be due to MutL$^+$ and MutS$^+$ products. In the negative control model, the response observed in *mut*S and *mut*L strains could be due to derepression of the respective pathways. Here too, the response would be kept minimal in wild-type cells by the repressor action of the two proteins. In either model one of the pathways could be postulated to require high levels of RecA protein brought about by LexA protein cleavage and consequent derepression of *rec*A.

The two models predict different outcomes when both *mut*S and *mut*L genes are inactivated. According to the positive control model *mut*S–*mut*L double mutants would be expected to be incapable of Cairnsian mutagenesis. On the other hand, the negative control model predicts enhanced Cairnsian mutagenesis in the double mutants relative to the singles. The availability of Tn*5* and Tn*10* insertions in *mut*S and *mut*L allows easy construction of double mutants by appropriate crosses. Such double mutants were constructed in *rec*A$^+$ and *lex*A3 (Ind$^-$) genetic backgrounds and their proficiency in eliciting Cairnsian response was examined. The data are presented in

Table 3. Cairnsian mutagenesis in *mut*S–*mut*L double mutants.

Strain	Relevant genotype	Frequency of Cairnsian mutation to Val^r (100 μg/ml)
1157/S	*rec*A$^+$ *mut*S::Tn*10*	5×10^{-4}
1157/SL	*rec*A$^+$ *mut*S::Tn*10* *mut*L::Tn*5*	$3{\cdot}8 \times 10^{-4}$
DM49/L	*lex*A3 (Ind$^-$) *mut*L::Tn*5*	1×10^{-3}
DM49/SL	*lex*A3 (Ind$^-$) *mut*L::Tn*5* *mut*S::Tn*10*	$1{\cdot}1 \times 10^{-3}$

Experimental conditions as given under table 1.

table 3. It can be seen that in a *lex*A$^+$ *mut*S strain, additional inactivation of *mut*L does not affect Cairnsian mutagenesis. Similarly in a *lex*A3 (Ind$^-$) *mut*L strain additional inactivation of *mut*S is inconsequential. These results favour the negative control model. It should be noted that the frequency of Cairnsian mutagenesis to valine resistance in *mut*S–*mut*L double mutants is not appreciably different from the single mutants. This implies the operation of additional control mechanisms.

The notion of negative control by *mut*S and *mut*L could be substantiated further by showing that the respective wild-type alleles would complement the mutant alleles and thereby reduce the magnitude of Cairnsian response. Therefore F′711 and F′728 spanning *mut*S and *mut*L, respectively, were introduced into desired recipients. (In order to allow easy selection, the F's had Tn*10* transposed on to them by introducing *pps*::Tn*10* marker by P1 transduction and growing the *tet*^{r} transductants for 3 cycles in LB-tet medium. The F′ donors were crossed with *mut*S::Tn*5* and *mut*L::Tn*5* and transconjugants were selected on plates containing tetracycline and kanamycin). The data presented in table 4 show that F′711 greatly reduced the Cairnsian response in the *mut*S strain but not in the *mut*L strain. Likewise F′728 reduced the response in *mut*L but not in the *mut*S strain. Thus there seem to be two pathways of Cairnsian mutagenesis which are negatively regulated by the products of *mut*S and *mut*L genes. Inactivation of either or both of these genes leads to derepression of one or both pathways. The *mut*S controlled pathway seems to be dependent on LexA protein cleavability while the other is not.

Table 4. Reduction of Cairnsian response in *mut*S and *mut*L mutants by the respective wild-type alleles.

		Frequency of valine resistance (70 μg/ml)	
Strain	Chromosome/F′	Basal	Cairnsian
GW3732/711	*mut*S::Tn*5*/*mut*S$^+$	$0{\cdot}5 \times 10^{-5}$	2×10^{-5}
GW3732/728	*mut*S::Tn*5*/*mut*L$^+$	$1{\cdot}0 \times 10^{-5}$	2×10^{-4}
GW3734/711	*mut*L::Tn*5*/*mut*S$^+$	$3{\cdot}3 \times 10^{-5}$	1×10^{-3}
GW3734/728	*mut*L::Tn*5*/*mut*L$^+$	$0{\cdot}2 \times 10^{-5}$	$0{\cdot}6 \times 10^{-5}$

Experimental conditions as given under table 1.

3.6 *Generality of the Cairnsian response*

Cairnsian mutation to valine resistance can be viewed as a stress response to partial starvation for isoleucine. Inhibition of isoleucine biosynthesis by valine may limit the availability of the former and thereby impose a state of semi-starvation. A question that can be asked is whether partial starvation for any other amino acid will elicit a similar response. In order to answer this, I plated the strains harbouring *mut*S and *mut*L mutations in *rec*A$^+$ and *rec*A430 backgrounds on two sets of minimal plates, one lacking an amino acid and the other supplemented with the same in sub-optimal concentrations (1·2 μg/ml as against the usual 30 μg/ml). The colonies appearing on the former kind of plates, 36–48 h after plating, will be the preexisting revertants present at the time of plating while those appearing on the latter kind of plates will also include Cairnsian mutants, if they arise. Data presented in table 5 show that the number of "revertants" are higher on plates containing sub-optimal concentration of the nutrient than on plates lacking the same. It could be argued that sub-optimal concentration of a required nutrient would allow limited growth and hence the higher reversion frequencies could be due to higher cell titre rather than Cairnsian mutagenesis. That this might not be so is shown by the data obtained with *rec*A430 *mut*S and *rec*A430 *mut*L strains (table 5). While the medium would have allowed approximately the same extent of growth for both strains only one (*rec*A430 *mut*L) showed the response. As was shown above for the development of valine resistance, here too LexA protein cleavability seems essential for the *mut*S dependent response but not for the *mut*L dependent one. Thus Cairnsian response appears to be a general response to (starvation) stress.

3.7 *Are the post plating Arg$^+$, Leu$^+$ and Val^r colonies Cairnsian?*

Since the experiments described herein were done with mutator strains, it is imperative to show that what was measured is indeed the Cairnsian response and not generalized mutagenesis. Foster (1992) has outlined three criteria to be fulfilled for directed (Cairnsian) mutagenesis, as follows: (i) the mutants do not preexist, (ii) the culture under selection does not grow, and (iii) the mutants would not have arisen had they not been

Table 5. Cairnsian reversion of *leu*B6 and *arg*E3 under partial starvation.

Strain	Relevant genotype	Revertant selected	Reversion frequency		Fold increase
			Total starvation	Partial starvation	
GW3732	*mut*S::Tn*5*	Leu$^+$	2×10^{-6}	2×10^{-4}	100
		Arg$^+$	3×10^{-5}	7×10^{-4}	23
GW3734	*mut*L::Tn*5*	Leu$^+$	5×10^{-6}	3×10^{-4}	60
		Arg$^+$	2×10^{-5}	9×10^{-4}	45
1157/S/430	*rec*A430	Leu$^+$	2×10^{-6}	1×10^{-5}	5
	*mut*S::Tn*5*	Arg$^+$	8×10^{-6}	4×10^{-5}	5
1157/L/430	*rec*A430	Leu$^+$	3×10^{-6}	3×10^{-4}	100
	*mut*L::Tn*5*	Arg$^+$	4×10^{-6}	8×10^{-4}	200

Experimental conditions as given under table 1.

selected. The last is perhaps the crucial and most difficult to show experimentally. I attempted to show this using a simple experimental strategy as outlined below.

Minimal medium plates containing histidine, proline and threonine but lacking arginine and leucine were prepared and dried well. Two troughs were dug out from the left and right ends of the plates. To one of the troughs, say the left one, 50 μl of a 10 mg/ml solution of arginine was added and to the other a similar amount of leucine was added. The troughs were filled with saline and the plates left at 37°C overnight. Diffusion of the amino acids generates an arginine gradient from left to right and a leucine gradient from right to left. Aliquots of *mut*S or *mut*L cultures were spread on the plates and incubation continued for 3–4 days. The cells deposited close to the arginine and leucine troughs will encounter sub-optimal concentrations of leucine and arginine, respectively, while those at the centre will have enough of both. Figures 2A and 2B show that there is a streak of confluent growth at the centre and single colonies over a faint lawn on either side of the streak. The number of cells plated ($2–4 \times 10^6$) ensures that the colonies are not preexisting Arg^+ or Leu^+ revertants, which even if present in small numbers, would show up 24 h after plating. All the colonies (120/120, *mut*S and 90/90, *mut*L) on the leucine-supplemented side of the plate were Arg^+ Leu^- while all (75/75 *mut*S and *mut*L each) on the arginine-supplemented side were Arg^- Leu^+. However, a small fraction (2–5%) of the Arg^+ or Leu^+ colonies had a higher than normal fraction of Leu^+ or Arg^+ revertants, respectively, as was seen by the appearance of isolated single colonies when gridded on appropriate selection plates. These could be the hitch hikers (Hall 1991b; also see below) which might have arisen during or after the primary Cairnsian event.

These experiments show that the type of Cairnsian mutants that arises depends upon the selection pressure the organism is subjected to. However, they do not show that a given class of mutants will not arise if there were no selection pressure. This was shown by another experiment described below. Two troughs were dug out at the left and right ends of minimal plates lacking arginine. Both the troughs were filled with saline providing just 10 μg of arginine. The right trough received in addition 0·5 mg of valine such that the selection pressure at this end was for arginine prototrophy and valine resistance, while the pressure on the other end was only for arginine prototrophy. After allowing diffusion and equilibration, aliquots of *mut*S and *mut*L cultures were spread on to the plates and incubated for 3 days at 37°C. Figure 3A shows a typical pattern of growth usually observed, colonies appearing on one end of the plate (the one which received only limiting arginine) and not on the other. After marking and counting the colonies (initial), 0·5 mg each of arginine and valine were added to the left trough and 0·5 mg of arginine alone was added to the right trough. Incubation was continued for another 36 h and additional colonies appearing on both sides were counted (final). Figure 3B shows a typical pattern. Data presented in table 6 shows that Val^r mutants arose only if there was a selection pressure for the same. If the selection pressure was only for arginine prototrophy, no (or very few) Val^r mutants came up. (The small colonies barely visible at the left side of figure 3B could be the Val^r mutants induced after exposure to valine.)

3.8 *Non-specific mutagenesis and Cairnsian response*

The results presented in the previous section show that the type of Cairnsian mutants obtained is dependent upon the selection pressure exerted on the population. However,

Table 6. Valine stress is necessary for the generation of Val^r mutants.

Strain	Colony numbers			
	± Arg end		Val ± Arg end	
	Initial	Final	Initial	Final
GW3722 (*mut*S::Tn5)	125	132	0	131
	195	195	17	87
	79	87	4	60
GW3724 (*mut*L::Tn5)	40	40	0	60
	60	68	0	75
	104	109	3	54

For experimental details see text.

the proportion of unselected mutants among the colonies of selected ones was found to be higher than normal. The Hall model described at the outset readily explains the occurrence of unselected mutants as the outcome of chance events accompanying (or following?) the selected event. In order to understand this aspect in greater detail, I examined the *mut*S Val^r and *mut*L Val^r colonies arising by the Cairnsian mode (very late appearance) for the presence of Arg$^+$ revertants. Ten randomly chosen colonies from each class were grown in complete minimal medium and 10 μl aliquots of a 100-fold dilution were spotted on arginine selective plates. Figure 4B shows that many of the *mut*S Val^r and *mut*L Val^r colonies had varying numbers of Arg$^+$ cells. Table 7 presents quantitative data on the proportion of Arg$^+$, Leu$^+$ and His$^+$ "revertants" among a few *mut*S Val^r and *mut*L Val^r colonies. It is interesting to note that the proportions of the three classes of unselected mutants is not uniform in all the colonies examined. Moreover, while all the His$^+$ were also Arg$^+$, many of the Arg$^+$ were His$^-$. I have made some observations on this aspect which will be published elsewhere.

An unexpected result was obtained when Val^r derivatives of *rec*A281 *lex*A3 (Ind$^-$) *mut*S and *rec*A281 *lex*A3 (Ind$^-$) *mut*L were screened for the proportion of *arg*E$^+$. Out of 65 colonies of each class screened none had Arg$^+$ revertants. This shows that the occurrence of unselected mutants is dependent on cleavage of LexA protein and thus an SOS function. Moreover the uncoupling of Cairnsian mutagenesis and the associated non-specific mutagenesis shows that the two may not be interrelated and that the underlying mechanisms could be different (see § 4).

4. Discussion

In this communication I have shown that *mut*S and *mut*L mutants of *E. coli* (AB1157) have a high propensity to undergo Cairnsian mutagenesis when subjected to stress. Addition of valine inhibits isoleucine biosynthesis and creates artificial auxotrophy for the latter. Provision of leucine or arginine at sub-optimal concentrations also creates a state of semi-starvation. Under these conditions, occurrence of appropriate mutation(s) would help to overcome the stress. This process, an instance of need-specified (Cairnsian) mutagenesis, occurs at high frequencies in *mut*S and *mut*L mutants. A required condition for this process to occur seems to be a state of partial starvation

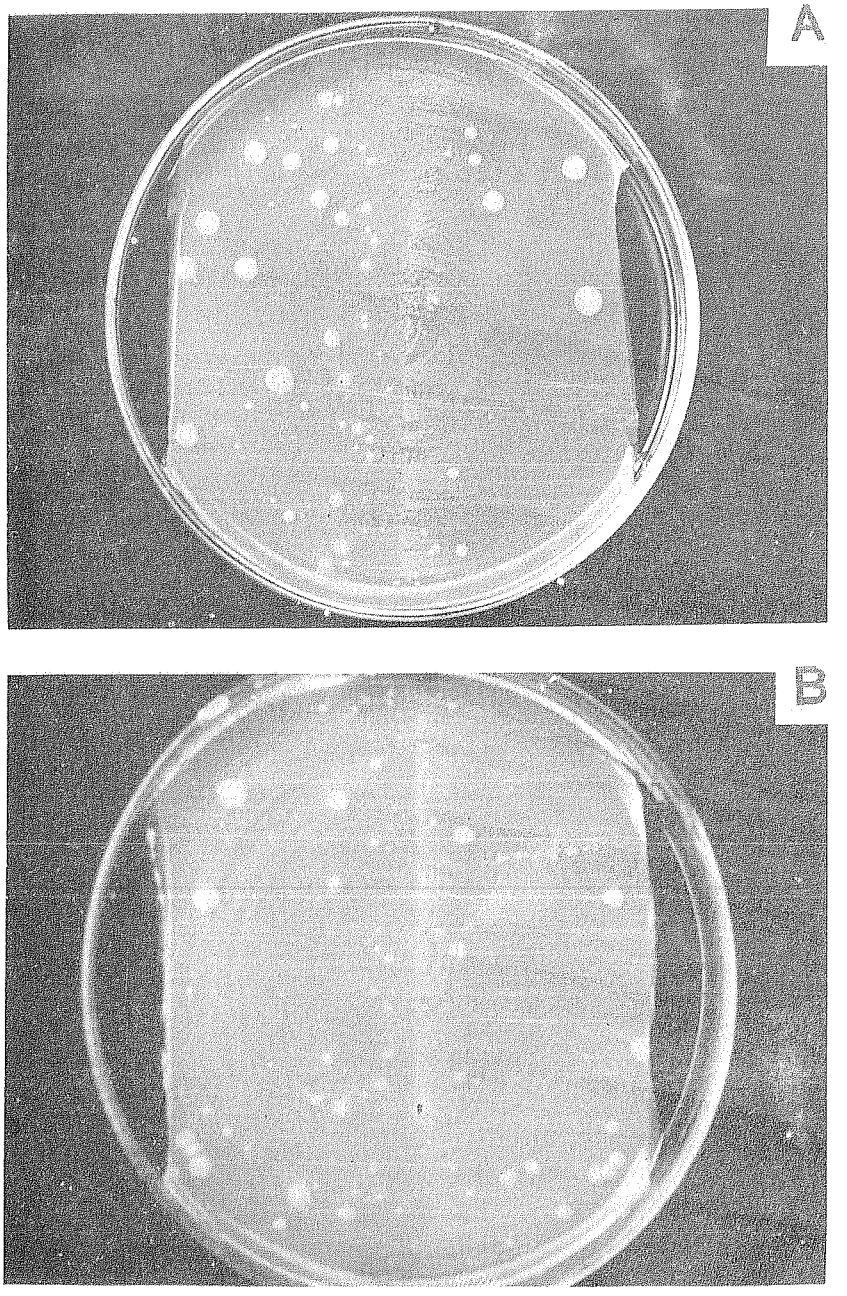

Figure 2. Cairnsian reversion to Arg^+ and Leu^+ in *mut*S and *mut*L strains. For details see text. The left troughs received arginine and the right troughs received leucine. A: *mut*S; B: *mut*L.

rather than total starvation. When the *mut*S and *mut*L mutants are spread on plates devoid of leucine or arginine, only the preexisting revertants show up as colonies. The colony numbers on such plates do not increase, or increase only marginally, on

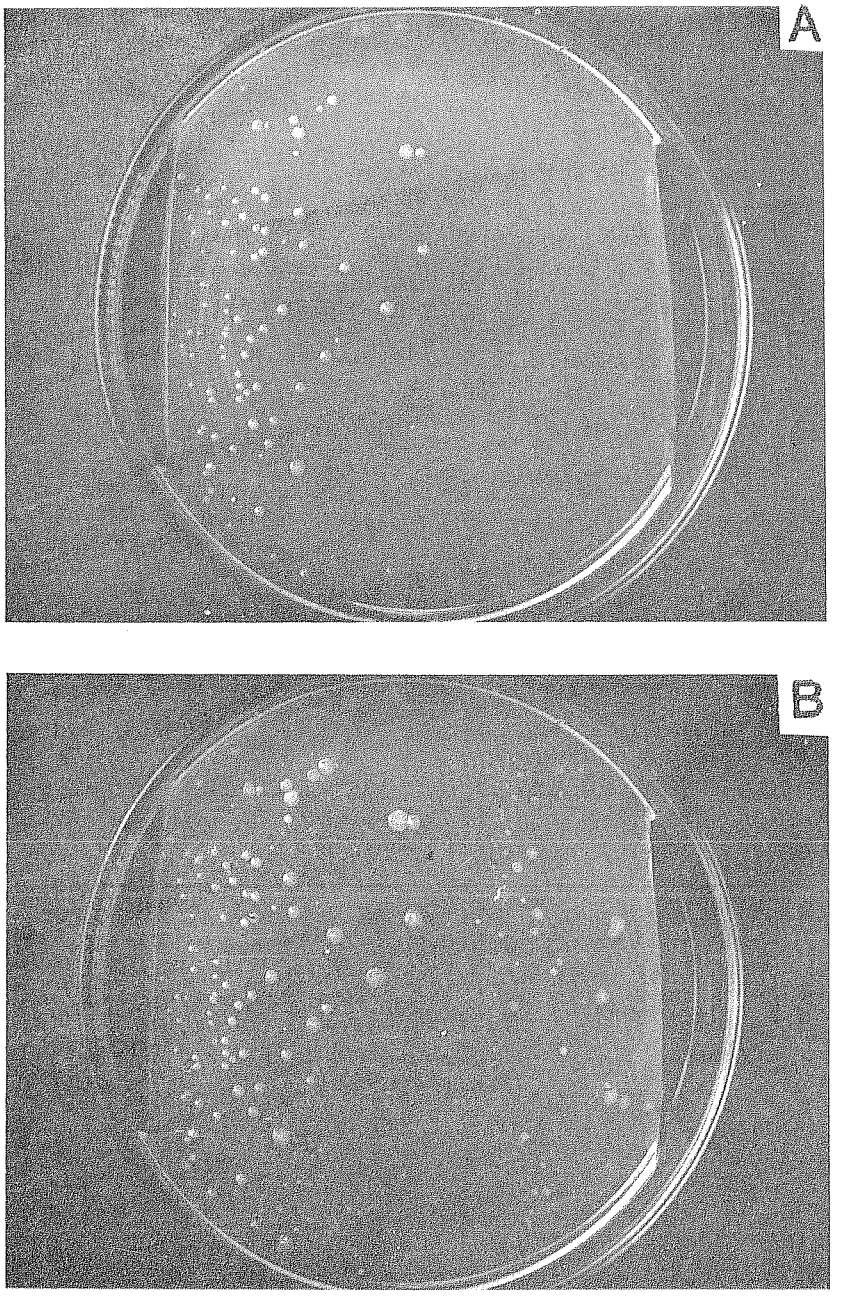

Figure 3. Valine stress is necessary for generation of Val^r mutants by Cairnsian mode. For details see text. The right trough received 10 μg arginine and 500 μg valine. The left trough received only 10 μg arginine. The strain used was *mut*L. A: initial, B: final.

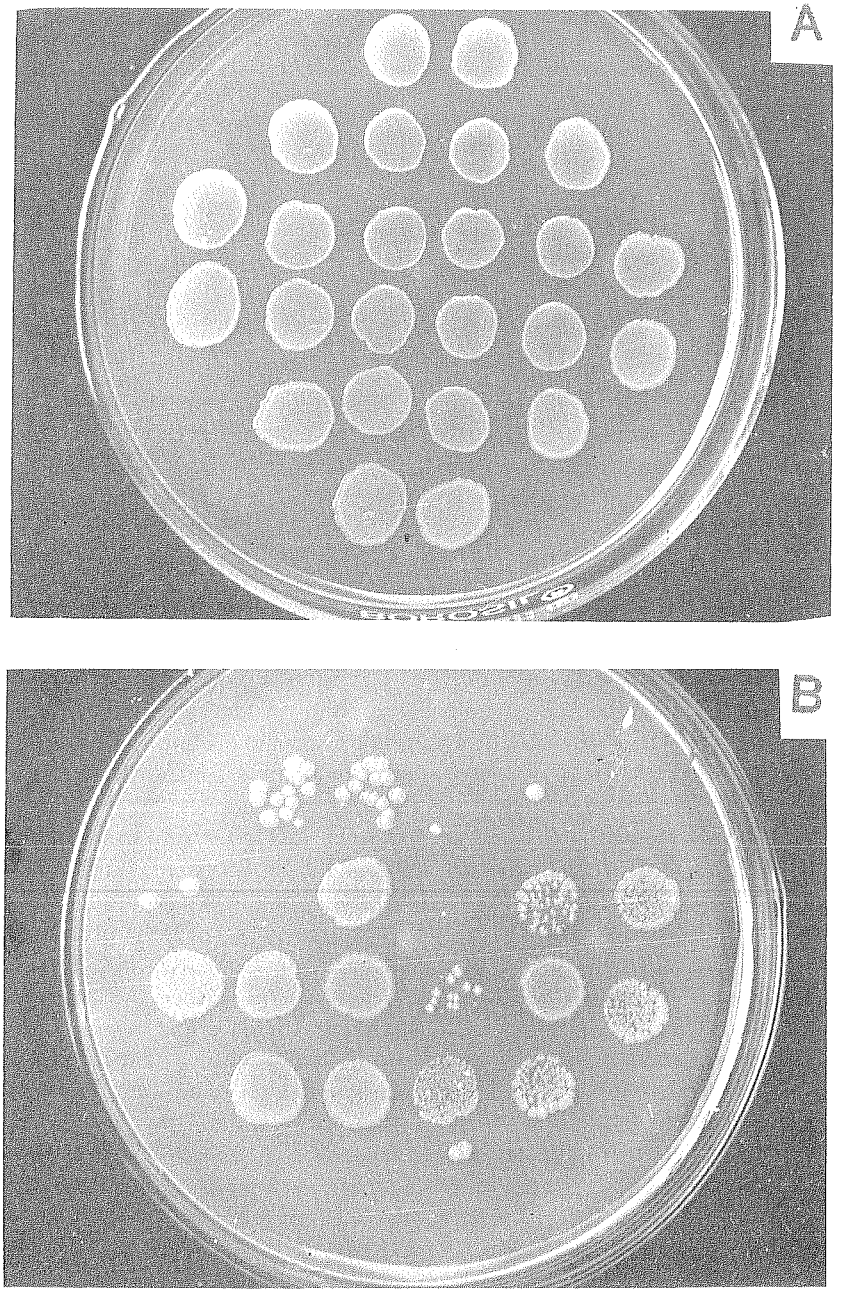

Figure 4. Non-specific mutagenesis in *mut*S Val^r and *mut*L Val^r. For details see text. A: complete medium B: Arg selective medium. Spots 1 and 2, *mut*S control; 3–12, *mut*S Val^r; 13–22, *mut*L Val^r; 23 and 24, *mut*L control. Numberings are from left to right.

Table 7. Non-specific mutagenesis in Val^r *mut*S and Val^r *mut*L colonies.

Strain	Revertant frequency ($\times 10^6$)		
	Arg$^+$	Leu$^+$	His$^+$
*mut*S	< 1	< 1	< 1
*mut*S Val^r(7)	2×10^4	< 1	3×10^2
*mut*S Val^r(9)	4×10^2	< 1	2
*mut*S Val^r(10)	2×10^3	< 1	1
*mut*L	3	< 1	3
*mut*L Val^r(1)	4×10^3	1	4×10^3
*mut*L Val^r(2)	2×10^4	< 1	1×10^3
*mut*L Val^r(8)	1×10^4	< 1	$2{\cdot}5 \times 10^2$

The *mut*S and *mut*L are GW3722 and GW3724 respectively. The numbers in parentheses are colony numbers of figure 4.

prolonged incubation. On the other hand, presence of leucine or arginine at sub-optimal concentration which allows limited growth results in Cairnsian mutagenesis. Similarly addition of valine also restricts the extent of growth and leads to Cairnsian mutagenesis. Total starvation for carbon, nitrogen or phosphorus has been shown to result in elevated expression of a number of genes (reviewed by Matin 1991). I have observed that such starved cells are no better than unstarved cells in eliciting the Cairnsian response. Either the extent of starvation specific protein synthesis or, more likely, the starvation specific proteins *per se* seem inadequate for Cairnsian response to occur.

Cairnsian response in *mut*S mutants seems to require higher than basal levels of the RecA protein and none of the other proteins of the SOS system. Inhibition of the response by the *lex*A3 (Ind$^-$) and *rec*A430 mutations and alleviation of such inhibition by the *rec*A281 mutation strongly point to this. Amplification of RecA protein levels is usually brought about by cleavage of the LexA repressor (see Walker 1984, 1987) an act which will also trigger the SOS response. I tend to think that the occurrence of unselected mutations among the Cairnsian mutants could be due to SOS induction. Interestingly unselected mutations do not occur if the requirement for high RecA protein levels is met by the *rec*A281 mutation in a *mut*S *lex*A3 (Ind$^-$) strain since in this case the SOS system will still be under repression. On the other hand, Cairnsian response in *mut*L mutants appears to be independent of *rec*A. Even then unselected mutations arise among the selected Cairnsian mutants in *mut*L but not in *mut*L *lex*A3 (Ind$^-$) genetic backgrounds. Therefore, it appears that the stress itself leads to LexA protein cleavage and SOS induction, whether required or not.

The requirements for Cairnsian mutagenesis in *mut*S background are very similar to those described for induced stable DNA replication (iSDR; Kogoma 1986; Magee and Kogoma 1990). It would be interesting to see the interrelationship, if any, between the two processes.

An intriguing question is the poor occurrence of Cairnsian response in the wild-type (*mut*S$^+$ *mut*L$^+$) background. It is only with difficulty that one could demonstrate Cairnsian mutagenesis in wild-type cells. According to the model presented in this communication, repression of the two pathways would help to maintain Cairnsian mutagenesis at a low level. However, a low potential to undergo Cairnsian mutagenesis

would also minimize the adaptive value of the process. There could be mechanisms, not understood as yet, that trigger Cairnsian response in wild-type cells.

Foster (1992) has discussed some of the possible mechanisms of control of directed mutagenesis. It is perhaps worth mentioning at this point that the results presented in figure 1 of this report were obtained with a spontaneous *thr*$^+$ revertant of AB1157. When the 'wild type' (*thr*$^-$) AB1157 was used, the response was very poor. Moreover, in the *thr*$^+$ derivative, presence or absence of threonine in the medium was inconsequential, ruling out the possibility that excess threonine could be inhibitory. The reason for this phenomenon is clear at the moment. It is somewhat analogous to the positive effect of the *uvr-bio* deletion reported by Cairns *et al.* (1988; see also Foster 1992) except the effect of *thr*-1 mutation seems to be negative.

The process may also depend upon the particular mutational event that is scored. I have observed Cairnsian type "reversion" of AB1157 to *thr*$^+$ and CSH57 (Miller 1972) to Ilv$^+$ in *mut*S$^+$ *mut*L$^+$ backgrounds. In the mutator backgrounds the frequency of reversion for these markers is so high that the entire culture, or a substantial fraction thereof, appears to be Thr$^+$ or Ilv$^+$. It is interesting to note that markers such as *thr*1 of AB1157 or *ilv* of CSH57 are the ones that could be described as "leaky". AB1157 grows slowly in the absence of threonine and Thr$^+$ colonies keep appearing as incubation continues. As could be expected, a *thr*::Tn*10* derivative of AB1157 neither grows nor gives rise to Thr$^+$ colonies in the absence of threonine. Thus there seems to be a relationship between the leakiness of mutant alleles and the generation of Cairnsian mutants. Perhaps the *mut*S/*mut*L mutations increase this intrinsic leakiness (through mechanisms not clear at the moment) and thereby facilitate Cairnsian mutagenesis. The degree of enhanced leakiness may vary with the marker. In the case of *thr*1 it may be quite high. In the case of other markers such as *leu*B6 or *arg*E3 it may not be high enough to trigger the response. Hence these markers need suboptimal supplementation with leucine or arginine.

Thus, there seem to be two ways by which *E. coli* can mutate. The classical, replication-associated mode, characterized by its randomness, and the Cairnsian mode, which occurs in response to specific needs and confers selective advantage.

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Note added in proof:

I have observed that a *dam*13 (*dam*::Tn*9*) derivative of AB1157 is no better than *dam*$^+$ AB1157 in eliciting Cairnsian response. If Cairnsian response is the result of uncorrected mismatches, increasing the life span of mismatches (*mut*S/*mut*L) or correction of the wrong (parental) strand of mismatches (*dam*) will have the same consequence. That this is not so in Cairnsian response supports the notion outlined in this report that the role of the MutS and MutL products in Cairnsian mutagenesis is not simply to prolong the life span of mismatches.