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Mutation in *Escherichia coli* under starvation conditions: a new pathway leading to small deletions in strains defective in mismatch correction

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Strains of *Escherichia coli* carrying the *mutY* mutation lack a mismatch correction glycosylase that removes adenines from various mismatch situations. In growing bacteria, 8-oxoguanine–adenine mispairs persist and can give rise to G→T transversions during subsequent replication cycles. We now show that when *trpA23 mutY* bacteria are held under tryptophan starvation conditions the tryptophan-independent mutants that arise include small in-frame deletions in addition to transversions. The *trpA23* reversion system appears to be unusual in that small in-frame deletions occurring in a particular region of the gene can lead to the production of a functional protein. We suggest that this is a consequence of the deletion causing the polar group on the arginine at the *trpA23* site to be pulled away from the active site of the enzyme. Such deletions are also found with starved bacteria defective in methyl-directed mismatch correction activity (*mutH*, *mutL* or *mutS*), and deletion mutations are also found among the much lower number of mutants that arise in bacteria wild-type for mismatch correction. There is thus a pathway, hitherto undetected, leading to deletions probably from mismatches under conditions of growth restraint. RecA, UmuC, UvrA, MutH,L,S, SbcC and SbcD proteins are not required for the operation of the deletion pathway. A possible explanation is that the deletion pathway is not dependent upon further replication and that it fails to be discernible in growing cells because it is relatively slow acting and mismatches are likely to encounter a DNA replication fork before the initial step of the deletion pathway.

Keywords: deletions/8-hydroxyguanine/mismatch correction/starvation mutagenesis/tryptophan synthetase

Introduction

The spontaneously occurring oxidation product of guanine in DNA, 7,8-dihydro-8-oxoguanine (8-oxoG), gives rise to G→T transversions when it encounters the bacterial chromosomal replication complex because its ability to pair with adenine is comparable with its ability to pair with cytosine (Maki and Sekiguchi, 1992; Tajiri *et al.*, 1995). Transversions of this type comprise the vast majority of the mutations arising in growing cells of *mutY* mutants which lack a mismatch-correction glycosylase capable of removing adenine from the DNA when it is mispaired with 8-oxoG (Urios *et al.*, 1994). 8-OxoG also occurs in the DNA of starved bacteria, for example in an auxotrophic strain deprived of its required amino acid, and can lead to prototrophic revertants where these are capable of being generated by G→T transversions. In a *mutY trpA23* strain of *Escherichia coli*, prototrophic mutants with G→T transversions at the *trpA23* site arose at an elevated rate when the bacteria were incubated under starvation conditions, but they were accompanied by slower growing prototrophic mutants that were not seen in growing cells (Bridges *et al.*, 1996). We have investigated the nature of these slow-growing mutants and find that many of them are small in-frame deletions in the *trpA* gene that result in a functional gene product. They also arise under starvation conditions in bacteria carrying mutations (*mutL*, *mutH* or *mutS*) which block the methyl-directed mismatch repair system. We suggest that persisting mismatches are prone to give rise to small deletions under starvation conditions.

Results

Deletions in mutY and mutY$^+$ bacteria

Phenotypic characterization studies indicated that prototrophic revertants isolated from growing cultures of the *trpA23 mutY* strain IC3742 all contained, as predicted, transversions from G to T at nucleotide position 632 (Urios *et al.*, 1994; Bridges *et al.*, 1996). The revertants arising under conditions of tryptophan starvation, however, included some more complex changes that were not readily identified with the phenotypic classification of Yanofsky *et al.* (1966b). Initial sequencing determinations showed that some of these were small in-frame deletions and others showed no detectable change in the sequenced window. The latter were presumably extragenic or remote intragenic suppressors. In this study, we have concentrated solely on the small in-frame deletions. It quickly became apparent that these grew more slowly than the transversion mutations, and growth rate was used subsequently to screen out the latter prior to sequencing. A rare fast-growing deletion mutant could, therefore, have been missed.

The results of two experiments with tryptophan-starved IC3742 are shown in Table I. Forty revertants were isolated, of which seven (17.5%) were deletions, three were not changed within the sequenced window and two were C→A transversions at position 698. The remaining 28 were G632T transversions as determined by phenotypic characterization (Yanofsky *et al.*, 1966a); in 18 of these that were sequenced the transversion was confirmed.

We characterized 149 mutants of IC3126, the *mutY*$^+$ parent of IC3742, and found that 18 of them were slow growers, of which three showed no change within the sequencing window, one was a transition mutation known to confer a slow-growing phenotype and 14 (9.4%) were

Table I. Nature of deletions found among Trp$^+$ revertants of the *trpA23* strain IC3126 and its *mutY* derivative IC3742 arising during tryptophan starvation

Strain	Experiment	Sequencing windows	Revertants screened	Bases deleted	Amino acids deleted	Times found
IC3742	1	550–682	20	657–668 or 658–669	220–223	1
				644–646 or 645–647 or 646–648	216	2
				650–667 or 651–668 or 652–669	218–223	1
				646–654	216–218	1
				660–662 or 661–663	221	1
	2	565–715	20	664–675 or 665–676 or 666–677 or 667–678 or 668–679	222–225 or 223–226	1
IC3126	1	564–703	65	644–646 or 645–647 or 646–648	216	6
				672–677	225–226 I(ATT)224 I(ATA)[a]	1
				673–678 or 674–679	225–226	2
	2	564–719	84	644–646 or 645–647 or 646–648	216	4
				672–677	225–226 I(ATT)224 I(ATA)[a]	1

[a]Also involved a change of codon for the indicated amino acid.

Table II. Nature of deletions found among Trp$^+$ revertants of the *trpA23 mutY recA* strain CM1343 and the *uvr*$^+$ strain CM1349 arising during tryptophan starvation

Strain	Experiment	Sequencing windows	Revertants screened	Bases deleted	Amino acids deleted	Times found
CM1343	1	530–678	40	644–646 or 645–647 or 646–648	216	2
		565–710		646–663 or 647–664 or 648–665	216–221	1
	2	565–711	20	646–654	216–218	1
CM1349	1	565–702	64	644–646 or 645–647 or 646–648	216	1
				646–654	216–218	1

deletion mutants. The number of mutations to Trp$^+$ appearing in IC3126 under comparable conditions was no more than 15% of the number in IC3742. Thus the number of deletion mutants in IC3126 was 1.4% (i.e. 9.4/100×15) of the total number of mutants in IC3742 under the same conditions and, since 17.5% of those in IC3742 are deletion mutants, it follows that the majority (>90%) of the deletion mutants seen in IC3742 are attributable to the presence of the *mutY* mutation.

Although we had found no slow-growing deletion mutants among revertants arising in growing *mutY* bacteria (our unpublished data), we had used the plate method of Newcombe (1948) in which bacteria grow to form a lawn using a limiting amount of the required amino acid, and revertants arising during this growth form colonies that are counted after 2 or 3 days. It was thought possible that slow-growing deletion mutants might have been missed. Sixty mutants appearing on limiting nutrient plates from days 5 to 8 were therefore picked off. One was an unknown suppressor but none were deletion mutants. We also tested 19 mutants arising during a Luria–Delbruck-type experiment in L-broth where slow-growing revertants should not have experienced serious adverse selection; none contained deletions. Our results lead us to conclude that 8-oxoG–adenine mispairs do not give rise to deletion mutations in actively growing *mutY* bacteria within the limits of our method, i.e. they constitute probably <1% of the auxotrophs that arise.

Effect of DNA repair deficiencies

The basic genotype of the strains used in this study includes two DNA repair deficiencies, one in nucleotide excision repair (*uvrA155*) and one in error-prone repair (*umuCD* deletion). These repair systems are therefore not essential for the process that leads to deletions. In an experiment in which 64 mutants from a *uvr*$^+$ strain were isolated, there were 22 slow growers among which were two deletion mutants (Table II). While neither *uvr*$^+$ nor *uvr*$^-$ genotypes are essential for the occurrence of deletion mutants, the result suggests the possibility that nucleotide excision repair might remove some of the configurations involving mismatches that are prone to lead to deletions.

To examine a possible requirement for RecA protein and the other inducible gene products dependent upon it, a deletion of *recA* was transduced into IC3742 to form strain CM1343. When incubated under conditions of tryptophan starvation, prototrophic mutants arose at a slower rate than in IC3742 (Figure 1). We considered that this could well reflect a reduced number of viable *recA* bacteria on the plate. It is difficult to determine viability under the conditions obtaining on the plate because of the presence of prototrophic bacteria growing up among the auxotrophs. A limited study is, however, possible if the number of cells plated is reduced to eliminate pre-existing revertants and if the period of study is restricted to the first 24 h. Three experiments were carried out in which 2×10^6 bacteria (as determined by direct microscopic count) were plated on to the surface of minimal agar plates. When washed off after 24 h, the viable count of IC3742 as determined on nutrient agar had increased by a factor of 36, whereas that of the *recA* strain CM1343 had increased by a factor of only 6.4. Observation of the lawns that appeared on such plates in the course of incubation for a week suggested that there were similar numbers of cells on plates of IC3742 and CM1343. We presume that lethal sectoring was occurring in the *recA*

strain at a rate sufficient to give a low viable count and that this is the most probable explanation for the lower rate of appearance of prototrophic mutants.

A total of 60 revertants from two starvation experiments with CM1343 were examined. Four revertants carried small in-frame deletions and there were three slow growers with no change in the sequenced window (Table II). Clearly, RecA protein is not essential for the formation of small in-frame deletions, although the uncertainty associated with inviability on the plate means that we cannot say with confidence that *recA* has no effect on the yield of deletions.

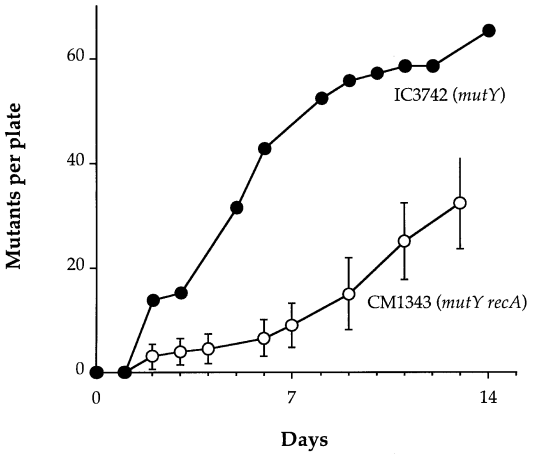

Fig. 1. Appearance of mutants on minimal plates for the *mutY recA* strain CM1343 (3×10^8 bacteria per plate, mean of three experiments). The result of an experiment with IC3742 (*mutY*) run concurrently is shown for comparison; an average of 21.7 mutants per plate which appeared on day 1, presumably carried over from the inoculum, was subtracted from the subsequent counts.

Deletions in bacteria defective in mismatch correction

The major mismatch correction pathway in *E.coli* is the methyl-directed pathway specified by *mutH*, *mutL* and *mutS*. To see whether this pathway is necessary for the formation of deletions in *mutY* bacteria, two experiments were carried out with the *mutL mutY* strain CM1331. Among 40 revertants arising under starvation conditions there were two deletion revertants (Table III), showing that MutL (one of the methyl-directed mismatch correction proteins) is also not essential for the deletion process in *mutY* bacteria.

A possible explanation of the result with *mutY* bacteria is that a mismatch involving adenine and a damaged base such as 8-oxoG can lead to the occurrence of deletions under certain conditions. We may ask whether mismatches between normal bases have the same property. Such mismatches formed during DNA replication may persist in (*mutY*$^+$) strains defective in methyl-directed mismatch correction. Figure 2 shows the rate at which mutations arise under conditions of tryptophan starvation in a *mutL* strain in comparison with the *mutY* and parental *mut*$^+$ strains. The *mutL* mutation results in a greatly elevated rate of mutation under these conditions, and the even greater response in a *mutL mutY* double mutant is consistent with the *mutL* and *mutY* mutator effects under starvation conditions being essentially independent. (The absolute numbers of mutants per plate for IC3742 in these experiments should not be compared with those published previously as a different make of agar was employed. We have observed that the rate of appearance of mutations under starvation conditions differs between makes of agar and sometimes between different batches of the same make. We suspect that this may reflect the influence of metal ions on the rate of generation of active oxygen

Table III. Nature of deletions found among prototrophic revertants of the *trpA23* strains CM1330 (*mutL*), CM1331 (*mutY mutL*), CM1359 (*mutS*) and CM1362 (*mutH*)

Strain	Experiment	Sequencing windows	Revertants screened	Bases deleted	Amino acids deleted	Times found
CM1330	1	554–680	20	624–632 or 625–633 or 626–634 or 627–635	209–211	1
				646–657 or 647–658	216–219	1
				646–663 or 647–664 or 648–665	216–221	1
	2	565–715	20	646–654	216–218	1
				644–646 or 645–647 or 646–648	216	1
				651–659	218–220 P(CCG) 217 P(CCA)[a]	1
CM1331	1	554–680	20	646–654	216–218	1
				646–663 or 647–664 or 648–665	216–221	1
	2	565–711	20	none found		
CM1359		570–718	86	644–646 or 645–647 or 646–648	216	4
				646–654	216–218	1
				646–657 or 647–658	216–219	1
				657–659	220 Q(CAG) 219 Q(CAA)[a]	1
				658–666 or 659–667	220–222	1
CM1362		565–711	88	666–680 or 667–681 or 668–682 or 669–683	224–228 A(GCG) 223 A(GCT)[a]	2
				644–646 or 645–647 or 646–648	216	5
				643–645	215	1
				646–663 or 647–664 or 648–665	216–221	1

[a]Also involved a change of codon for the indicated amino acid.

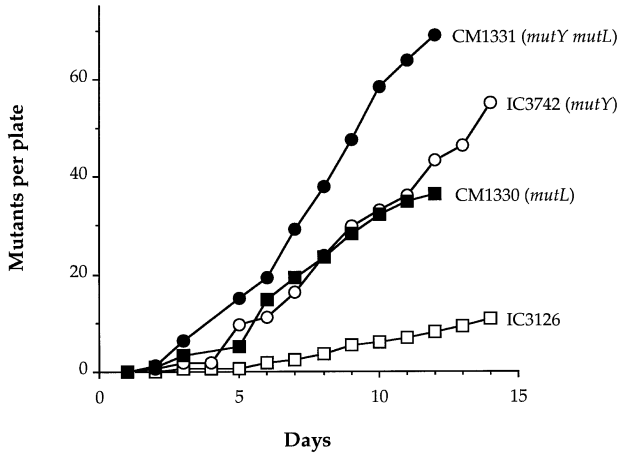

Fig. 2. Appearance of tryptophan-independent mutants with time of incubation on minimal plates for *E.coli* IC3126 and derivatives carrying *mutY* and *mutL* (10^7 bacteria per plate, means of two experiments).

species, but we have not attempted to do a systematic study.)

Two sequencing experiments were carried out with the *mutL* strain CM1330 (Table II). Out of a total of 40 revertants, there were 11 slow growers. Of these, six carried small in-frame deletions, three had no change within the sequenced window and two had base pair changes (A631G and C698T). No deletions were found among 20 revertants arising under growth conditions. In an experiment with the *mutS* strain CM1359, there were 40 slow growers among 86 revertants. Of these, eight carried small in-frame deletions, three had no change within the sequenced window and the remainder were base pair substitution mutations. In the *mutH* strain CM1362, 37 out of 88 tested were slow growers, of which nine carried small deletions. The proportion of the deletions that can be attributed to the defect in mismatch correction is 90% for *mutL* and ~83% for *mutS* and *mutH*. These results show that under conditions of tryptophan starvation, deletions can arise from DNA mismatches persisting because of inactivity of the MutH, MutS or MutL proteins.

Nature of the deletions

All the deletions that we have found lie in the same region of the *trpA* gene, between positions 624–627 and 680–683, corresponding to amino acid positions 209–228 (Figure 3). Only in-frame deletions are seen, because out-of-frame deletions in this region would certainly have resulted in a non-functional protein. Presumably, deletions resulting in the loss of more than a few amino acids would not restore function to the protein. Figure 3 also shows the sequences flanking the deletions, and it can be seen that in three cases there are three or four base repeated sequences that could conceivably have given rise to the deletion by a recombinational mechanism involving loss of one of the repeats. Of the remaining 11 types of deletion, three are flanked by a two base direct repeat and four by a single base repeat.

Deletions in an sbcC,D strain

Since *sbcC* and *sbcD* gene functions are required for a deletion pathway in phage λ (Leach, 1996), three experiments were performed with CM1407, a *mutL* strain which carries a deletion through *sbcC,D* (Table IV). The rate of appearance of mutants on starvation plates was similar to that observed with the *mutL* parent. In the first experiment, 71 mutants arising under starvation conditions were studied. There were 10 deletion mutants and five slow growers, with no change in the *trpA* sequence window. In the second experiment, 104 were screened of which eight were deletion mutants and six had no change in the sequence window; in the third experiment 109 mutants were screened of which seven were deletion mutants and 17 had no change in the sequence window. Thus 8.8% of the mutants carried deletions in *trpA* compared with 10.7% in the *mutH,L,S* strains combined. We conclude that the pathway that leads to deletion mutations under starvation conditions is not significantly affected by *sbcC,D*.

Discussion

Small in-frame deletions

There have been several reports that spontaneous mutational processes in starved cells are different from those in growing cells and lead to a different spectrum of mutations (Cairns and Foster, 1991; Prival and Cebula, 1992; Bridges, 1993; Mackay *et al.*, 1994; Taddei *et al.*, 1995). The present results with prototrophic revertants of *trpA23* bacteria show that the same is true in certain mutator strains. Both *mutY* and *mutL* bacteria are characterized by the persistence of mismatched base pairs which would be expected to give rise solely to an excess of base pair substitution mutations (any one or two base frameshifts would not be detected in this system). While this appeared to be true in growing cells, when the excess of mutants that arise during amino acid starvation was examined, a substantial proportion of small in-frame deletions was found together with other as yet unidentified changes. A mean value for the proportion of deletions in all the strains combined is close to 9%. Obviously only in-frame deletions are compatible with functional activity and, of these, presumably only relatively short ones (our longest deletion is 27 bp long). Within the same region, therefore, it may be assumed that twice as many out-of-frame deletions are likely to be formed but do not lead to functional protein. The deletions resulting in restoration of activity are confined to a relatively short region; all of our deletions lie between positions 622 and 683. A further unknown proportion of deletions may have occurred that extended beyond this region but did not result in a functional protein; these also would not have been selected.

As an example, we may take the *mutY* strain where 17.5% of the mutants carried in-frame deletions and 75% carried transversions. If we assume (i) that double the number of out-of frame deletions were probably formed but escaped detection, (ii) that the deletions originated from mismatches situated within the deleted sequence and (iii) that the average probability of transversion occurring at each of the 30 G–C base pairs within the region is similar to that for transversion at the *trpA23* site, it is readily calculated that mismatches in this region of some 60 bases are as likely to lead to deletions as to transversions. We may also note that the proportion of auxotrophs that are deletions is going to be underestimated because their slower rate of growth means their rate of appearance

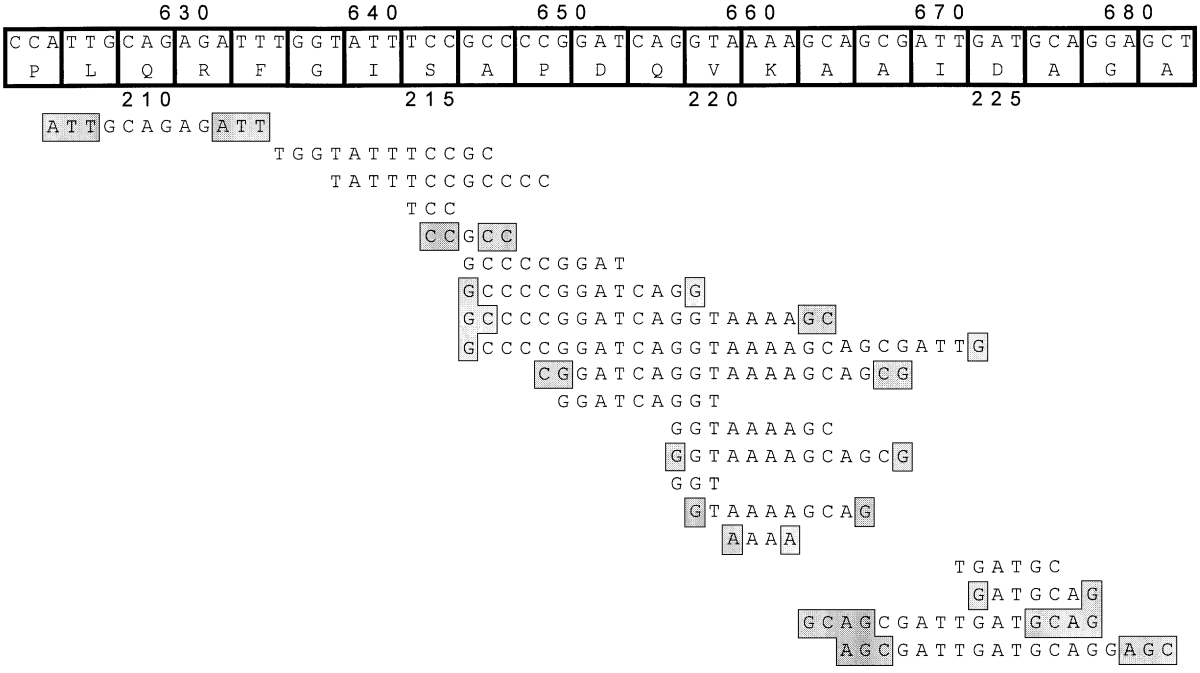

Fig. 3. Small deletions found in the *trpA23* gene capable of restoring function to the TrpA protein. The shaded bases represent possible direct repeats, one set of which might have been lost during the formation of the deletion. In these cases, it is not possible to distinguish which of the repeated bases mark the ends of the deletion.

Table IV. Nature of deletions found among Trp$^+$ revertants of the *mutL sbcDC* strain CM1407 arising during tryptophan starvation

Experiment	Sequencing windows	Revertants screened	Bases deleted	Amino acids deleted	Times found
1	565–712	71	624–632 or 625–633 or 626–634 or 627–635	209–211	1
			636–647	213–216 F(TTT) 212 F(TTC)[a]	1
			644–646 or 645–647 or 646–648	216	1
			646–654	216–218	2
			646–663 or 647–664 or 648–665	216–221	2
			657–665	220–222 Q(CAG) 219 Q(CAA)	1
			657–659	220 Q(CAG) 219 Q(CAA)	1
			646–672 or 647–673	216–224	1
			639–650	214–217 G(GGT) 213 G(GGG)	1
2	565–700	104	644–646 or 645–647 or 646–648	216	2
			646–654	216–218	1
			646–657 or 647–658	216–219	2
			646–672 or 647–673	216–224	1
			646–663 or 647–664 or 648–665	216–221	1
3	565–698	109	644–646 or 645–647 or 646–648	216	3
			646–654	216–218	1
			646–657 or 647–658	216–219	1
			657–659	220 Q(CAG) 219 Q(CAA)[a]	1
			660–662 or 661–663	221	1

[a]Also involved a change of codon for the indicated amino acid.

on the plates will lag behind that of transversion auxotrophs arising at the same time.

The high frequency of small deletions seems to arise from a conjunction of at least two circumstances. The first is the lower frequency of chromosomal replication cycles associated with starvation for a required amino acid. It should not be thought that such bacteria are totally devoid of DNA synthesis. There is recent evidence from *mutT* bacteria that the amount of DNA synthesized per day is equivalent to several genomes, even with relatively tight auxotrophies such as ochre mutations (Bridges, 1996). In the case of the missense *trpA23*, however, the auxotrophy is somewhat leaky and a lawn of residual growth becomes visible on tryptophan-deficient plates after a week or so. The second circumstance needed for deletion formation is, we suggest, the persistence of certain mismatched regions of DNA. This is consistent with the fact that deletions are enhanced over the wild-type level in bacteria defective at *mutY, mutH*, *mutS* and *mutL*, all of which loci are defective in the ability to correct mismatched regions of DNA. The mismatches could be with damaged bases (e.g. 8-oxoG), with natural but uncomplementary bases or where a short region of one strand has become looped out; both of the latter types would be expected to persist in *mutH*, *L* or *S* bacteria. While there is no evidence for MutH,L,S activity on loops involving between six and 27

bases, it is possible that a very weak activity could become significant in starvation conditions where replication is infrequent.

Protein function

If the tryptophan independence of our mutants were due to extragenic second site mutations and if the deletions were irrelevant, then we would have found out-of-frame as well as in-frame deletions. The fact that all deletions detected were in-frame argues that the *trpA* genes containing the deletions are responsible for conferring the observed prototrophy. We therefore need to consider how these deletions might restore activity to the defective gene product.

The three-dimensional structure of the tryptophan synthetase complex from *Salmonella typhimurium* has been determined by X-ray crystallography (Hyde *et al.*, 1988). A comparison of the amino acid sequences of the *E.coli* and S.*typhimurium* TrpA proteins indicates that they are similar in the regions coding for β-sheet 7 and α-helix 7.

The *trpA23* mutation in *E.coli* codes for an arginine residue at amino acid position 211 in the α subunit where the wild-type residue is glycine. This amino acid is situated in β-sheet 7 which is close to the active site of the tryptophan synthetase A protein. Any of nine amino acids at this site, namely glycine, serine, threonine, valine, alanine, cysteine, asparagine, leucine and isoleucine (partially active) results in an active protein (Yanofsky *et al.*, 1966b; Murgola, 1985). These vary considerably in size, but all except one lack a polar terminal group, the exception being the small asparagine residue. Eight others, however, glutamine, arginine, lysine, tryptophan, tyrosine, glutamic acid, histidine and aspartic acid, are known to result in an inactive protein, and all of them possess a polar terminal group and/or a bulky ring moiety. We think it likely that the inactivity they confer is due to steric or ionic interference in the region of the active site. We suggest that some small in-frame deletions may restore activity to TrpA23 protein by lessening the proximity of the polar group to the active site. Examination of the protein structure (see Figure 3 of Hyde *et al.*, 1988) shows that this is not unreasonable. With one exception, the small in-frame deletions lie remote from the active site and are all situated in α-helix 7, which is directly connected to β-sheet 7, or in the adjacent part of β-sheet 7. The effect of these deletions will be to shorten the α-helix–β-sheet region, one consequence of which might be to pull the β-sheet, and thus the interfering residue, away from the active site. The exceptional deletion results in the loss of the *trpA23* arginine itself. Such internally suppressed proteins might not be expected to function as well as the wild-type, and this is borne out by the slower rate of growth of the bacteria. As little as 2% of wild-type tryptophan synthetase α subunit activity is known to confer virtually the wild-type growth rate (see Murgola, 1985).

Mechanism of formation

An enhanced rate of formation of deletions is a feature of the mutator activity of the *polA1* mutation of DNA polymerase I, and the deletions are almost always associated with a 5′-GTGG-3′ sequence which is thought to be a pause site for this and possibly other polymerases (Fix *et al.*, 1987; Jankovic *et al.*, 1990). This nucleotide sequence is not, however, found in the region of *trpA23* susceptible to deletion. Examination of the sequences at the ends of the types of deletion in Figure 3 shows that most of them have direct repeats of at least one base, of which one repeat has become lost during the formation of the deletion. Three of them have direct repeats of three or four bases, a length which might, with assistance, be sufficient to establish homologous pairing. *A priori*, therefore, one might argue that the deletions we see could have arisen in two distinct ways. One could involve a recombination event between 3–4 base direct repeats, the other would not. We have observed *trpA23* deletions of the second sort in a *mutY* strain carrying a deletion of the *recA* gene, showing that RecA protein is not required for their formation. The involvement of RecA protein in recombination events leading to deletions at 3–4 base direct repeats, however, cannot at present be excluded.

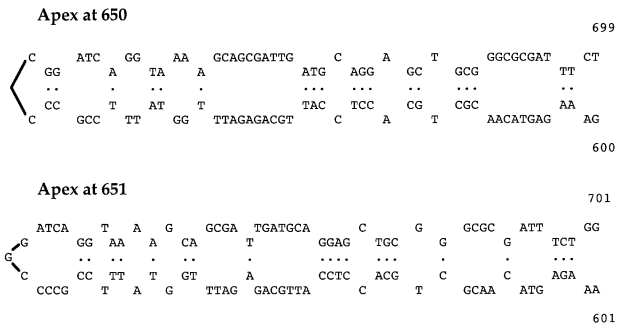

Fig. 4. Possible secondary structures of DNA in the region of *trpA* where deletions are found capable of restoring function to the TrpA23 protein.

We have examined the secondary structure of the DNA in the region susceptible to deletions to see whether there are any features that might contribute to their formation. As can be seen from Figure 4, there are at least two possible weak stem–loop structures that could exist, and their putative loops are sited close to the centre of the region in which the deletions are found. It is known that deletions can arise at stem–loop structures (Sinden *et al.*, 1991; Balbinder *et al.*, 1993). Although spontaneous deletion of such structures in pBR325-based plasmids is greatly enhanced when the SOS system is fully induced (*recA730 lexA71 umu*$^+$), there remains in *umuC* bacteria a pathway that is not blocked by the *recA* mutation (Balbinder *et al.*, 1993), and that could be the same as that operating on the chromosome in our system.

On the basis of work with rather larger palindromes, a RecA-independent deletion pathway has been characterized that requires *sbcC* and *sbcD* gene functions (Leach, 1996). The SbcC and SbcD polypetides have been purified and shown to have double-strand exonuclease and hairpin cleavage activities (Leach, 1996). Although the stem–loop structures in *trpA* are weak, they would presumably be more likely to form under the conditions of low overall transcription associated with starvation. We found, however, that a *mutL* strain carrying a deletion through *sbcC* and *D* showed a similar level of deletion mutations to other strains defective in methyl-directed mismatch correction. If there is a deletion pathway involving

Table V. Bacterial strains used

Strain	Relevant genotype	Source
TT101	*mutM*::*mini* Tn*10*	M.Michaels, via B.Sedgwick
WU3610 *mutL*	*mutL*::Tn*10*	R.Bockrath
WU3610 *mutH*	*mutH471*::Tn*5*	R.Bockrath
WU3610 *mutS*	*mutS201*::*kan*^{R}	R.Bockrath
CC101T	*mutT*::*kan*^{R}	H.Maki
DL733	del(*sbcDC*::*Kan*^{R})	D.R.F.Leach
RW202	del(*srlR-recA*)*306*::Tn*10*	R.Woodgate
IC3126	*trpA23 uvrA155* del(*umuDC*)::*cat*	M.Blanco
IC3742	as IC3126 but *mutY68*::*kan*^{R}	M Blanco
CM1330	as IC3126 but *mutL*::Tn*10*	this work P1 (WU3610 *mutL*)×IC3126
CM1331	as IC3742 but *mutL*::Tn*10*	this work P1 (WU3610 *mutL*)×IC3742
CM1343	as IC3742 but del(*srlR-recA*)*306*::Tn*10*	this work P1 (RW202)×IC3742
CM1348	*uvr*$^+$ *zjb1*::Tn*10*	laboratory stock, derivative of WU3610
CM1349	as IC3742 but *uvr*$^+$ *zjb1*::Tn*10*	this work P1 (CM1348)×IC3742
CM1353	as IC3126 but *mutM*::*mini*Tn*10*	this work P1 (TT101)×IC3126
CM1358	as CM1353 but *mutT*::*kan*^{R}	this work P1 (CC101T)×CM1353
CM1359	as IC3126 but *mutS201*::*kan*^{R}	this work P1 (WU3610 *mutS*)×IC3126
CM1362	as IC3126 but *mutH471*::Tn*5*	this work P1 (WU3610*mutH*)×IC3126
CM1406	as IC3126 but del(*sbcDC*::*Kan*^{R})	this work P1 (DL733)×IC3126
CM1407	as CM1406 but *mutL*::Tn*10*	this work P1 (WU3610 *mutL*)×CM1406

recombination it must be independent of *sbcCD* as well as *recA*.

The simplest explanation of the data is that there is a pathway in starved bacteria by which persisting mismatches give rise not to point mutations but to deletions. The formation of deletions is not dependent upon the RecA, UvrA, UmuC, MutH,L,S, SbcC or SbcD proteins and thus appears to reflect the operation of a novel pathway. While there is no obvious reason why mismatches should result in deletions, one might consider the possibility of specific endonucleolytic attack that may or may not be influenced by the structural peculiarities of the region. The apparent absence of deletions among bacteria that are growing normally may be explained if the deletion pathway is activated relatively infrequently and does not depend on further DNA replication so that the mismatches are likely to encounter a replication fork and give rise to a base substitution before the deletion pathway is activated.

Wider implications

The only deletions detectable by the system reported here are small in-frame deletions, but it is reasonable to assume that larger deletions and out-of-frame deletions also occur as a result of oxidative damage to DNA. In mammals, where detection of deletions tends to be restricted to large deletions, it has been found that such deletions accumulate with age in mitochondrial DNA (Cortopassi *et al.*, 1992). Mitochondria are responsible for oxidative phosphorylation, and the steady-state level of oxidized bases such as 8-oxoG in mitochondrial DNA is 16 times higher than in nuclear DNA (Richter, 1988; Hruszkewycz and Bergtold, 1990). Deletions in mitochondrial DNA are generally associated with direct repeats, as are some of those reported in the present study.

Materials and methods

Bacterial strains

The bacterial strains used are shown in Table V. Tryptophan auxotrophy was conferred by the *trpA23* mutation, originally isolated and characterized by Yanofsky and his colleagues (Yanofsky *et al.*, 1966b). This had been moved into a derivative of the B/r strain WP2, to replace the *trpE65* mutation normally present there, by Urios *et al.* (1994) who also made the *mutY* derivative. *trpA23* bacteria have an arginine instead of a glycine residue at position 211 of the tryptophan synthetase A protein, resulting from a replacement of a G–C base pair by an A–T base pair in the *trpA* gene. Several types of prototrophic mutations can occur at this codon, including G→T transversions which occur at a high rate in the *mutY* derivative (Urios *et al.*, 1994).

Selection of mutants

Bacteria were grown overnight in nutrient broth, centrifuged and resuspended in phage buffer (Boyle and Symonds, 1969). Aliquots of 0.1 ml were spread on to the surface of minimal (Davis and Mingioli, 1950) plates containing 0.4% glucose and solidified with Difco Bacto agar; the number of bacteria per aliquot was varied according to the mutator activity of the strain. Plates were incubated at 37°C and scored at intervals for mutant colonies. Mutants appearing after day 4 were arbitrarily deemed to have arisen largely from mutations that occurred on the plate. Such mutants were picked off on day 9, streaked on to minimal plates and subsequently characterized as described below.

Amplification and sequencing of slow growing Trp$^+$ mutants

Trp$^+$ mutants were grown overnight in 10 ml of L-broth and a 1 ml aliquot was centrifuged and washed with sterile water and finally resuspended in 50 µl of water. The suspension was heated at 99.9°C for 10 min and then centrifuged. Five µl of the cleared lysate was used to seed a PCR containing 100 pmol of primers TRPA1 (GCCCGCCAAATGCCGATGACGA) and TRPA2 (CGGGGTAAGCGAAACGGTAAAAAGATA) to amplify a 452 bp region of the *trpA* gene encompassing the site of the *trpA23* mutation in codon 211. The PCR product, produced after 30 amplification cycles (94°C 1 min, 65°C 1 min and 72°C for 1 min) was cleaned using the Wizard DNA cleanup kit (Promega Corp.) according to the manufacturer's instructions. Primer TRPA1 was biotinylated at the 5′ end to allow solid phase sequencing of the PCR product, with primer TRPA3 (AGCATTTTCTCTGGCTCAT), after attachment to Dynabeads–M280 streptavidin (Dynal, UK) and denaturation of the duplex DNA.

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