Molecular analysis of mutS expression and mutation in natural isolates of pathogenic Escherichia coli

Baoguang Li,1 Ho-Ching T. Tsui,2† J. Eugene LeClerc,1 Manashi Dey,1 Malcolm E. Winkler2† and Thomas A. Cebula1

Correspondence
Thomas A. Cebula
tac@cfsan.fda.gov

1Center for Food Safety and Applied Nutrition, Food and Drug Administration, Laurel, MD 20708, USA
2Department of Microbiology and Molecular Genetics, University of Texas Houston Medical School, Houston, TX 77030, USA

Deficiencies in the MutS protein disrupt methyl-directed mismatch repair (MMR), generating a mutator phenotype typified by high mutation rates and promiscuous recombination. How such deficiencies might arise in the natural environment was determined by analysing pathogenic strains of Escherichia coli. Quantitative Western immunoblotting showed that the amount of MutS in a wild-type strain of the enterohaemorrhagic pathogen E. coli O157 : H7 decreased about 26-fold in stationary-phase cells as compared with the amount present during exponential-phase growth. The depletion of MutS in O157 : H7 is significantly greater than that observed for a laboratory-attenuated E. coli K-12 strain. In the case of stable mutators, mutS defects in strains identified among natural isolates were analysed, including two E. coli O157 : H7 strains, a diarrhoeagenic E. coli O55 : H7 strain, and a uropathogenic strain from the E. coli reference (ECOR) collection. No MutS could be detected in the four strains by Western immunoblot analyses. RNase T2 protection assays showed that the strains were either deficient in mutS transcripts or produced transcripts truncated at the 3' end. Nucleotide sequence analysis revealed extensive deletions in the mutS region of three strains, ranging from 7 to 17 kb relative to E. coli K-12 sequence, while the ECOR mutator contained a premature stop codon in addition to other nucleotide changes in the mutS coding sequence. These results provide insights into the status of the mutS gene and its product in pathogenic strains of E. coli.

INTRODUCTION

Mutators arise in bacterial populations at frequencies of $10^{-5}$ to $10^{-6}$, as shown in studies using laboratory strains of Escherichia coli (Mao et al., 1997; Boe et al., 2000) and Salmonella typhimurium (LeClerc et al., 1998). Surveys of natural isolates of E. coli and Salmonella showed that mutators occur in the environment at much higher frequencies of around 1–5% (LeClerc et al., 1996). This rise of mutator alleles in natural populations implies an important role for mutators in the evolution of microbes, such as a source of genetic variants that adapt to unstable environments (Cox, 1976). While the hypermutable phenotype may produce the beneficial mutations that increase the frequency of linked mutator alleles in populations, we called attention to the role that promiscuous recombination could play in evolution (LeClerc et al., 1996; LeClerc & Cebula, 1997).

Notably, although spontaneous mutation rates are enhanced by mutation in at least 25 separate genetic loci (Miller, 1998; Horst et al., 1999), the overwhelming majority of mutators found thus far among natural strains are due to defects in the mutS, mutH, mutL or uvrD gene (LeClerc et al., 1996; Matic et al., 1997). These genes define the methyl-directed mismatch repair (MMR) pathway, a DNA repair system that not only corrects base mismatches in newly replicated DNA but is also the main barrier for recombination of mismatched heteroduplexes in DNA (i.e. homeologous recombination) (Modrich & Lahue, 1996). That is, MMR defects relax the recombination barrier, allowing DNA exchange between species that normally do not mate. This promiscuous property, unique to MMR mutants, may explain the emergence and persistence of MMR mutants in natural populations. Most importantly, it implicates a role for horizontal gene transfer in gaining the benefits for survival and growth. Since we reported the high incidence of

1Present address: Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285, USA.

Abbreviations: DEC, diarrhoeagenic Escherichia coli; ECOR, Escherichia coli reference; MMR, methyl-directed mismatch repair.

Received 31 December 2002
Accepted 22 January 2003

0002-6213 © 2003 SGM Printed in Great Britain

DOI 10.1099/mic.0.26213-0
MMR-defective mutators among *E. coli* and *Salmonella* pathogens (LeClerc et al., 1996), the accounts of mutator subpopulations among pathogenic isolates of *E. coli* (Matic et al., 1997; Denamur et al., 2002), *Pseudomonas aeruginosa* (Oliver et al., 2000), *Neisseria meningitidis* (Richardson et al., 2002) and *Streptococcus pneumoniae* (Negri et al., 2002) bolster the view that these mutators are positively selected in nature, most likely because of the beneficial mutations they carry.

Since the earliest observations of mutators in natural populations (Jysum, 1960; Gross & Siegal, 1981), several features of their incidence in the feral setting have been puzzling. That is, although laboratory experiments have shown that a high mutation rate can be advantageous, as evidenced by the successful competition of mutators against their non-mutator counterparts in chemostat cultures (Gibson et al., 1970; Nestman & Hill, 1973; Cox & Gibson, 1974; Tröbner & Piechocki, 1981; Chao & Cox, 1983) and the invasion of non-mutator clones by mutators in continuous growth experiments (Sniegowski et al., 1997), mutators have not overtaken natural populations of bacteria. Moreover, most organisms appear to have evolved a seemingly constant mutation rate (Drake, 1991; Ochman et al., 1999). Finally, since deleterious mutations arise at rates roughly $10^4$ times those of beneficial mutations (Cebula & LeClerc, 2000), a heightened mutation rate should merely hasten the mutator to extinction in a stable environment. Yet, our studies demonstrated that MMR mutator alleles persist among natural clones at frequencies 10- to 1000-fold greater than expected for deleterious mutations (LeClerc et al., 1996). Our results suggested, therefore, that the mutator state must be a temporary situation; i.e. a mutator allele rises to prominence with a beneficial trait that it spawned (i.e. hitch-hikes) and then falls when selection again sweeps the population (see Shaver et al., 2002). Haploidy dictates, after all, that if the trait is to be maintained in perpetuity, the bacterium must ‘lose’ (by reversion or recombination) or otherwise quiet (by suppression) the mutator allele.

A transient mutator state might also result, however, because of an altered expression of MMR proteins. As downregulation of MutS, and to a lesser extent MutH, is known to occur in stationary phase and in starved bacteria (Feng et al., 1996), and the amount of MutS in *E. coli* K-12 is already near limiting for MMR in exponentially growing cells, the decreases of MMR proteins observed in stationary-phase cells and in nutrient-deprived cells are of especial interest. If similar downregulation occurs in natural isolates, as observed in laboratory cultures of *E. coli* K-12, the decreased amounts of MutS and MutH could contribute significantly to increased mutagenesis and homeologous recombination as cells enter and leave the stationary phase or otherwise stressed environments.

To evaluate mechanisms that may be involved in the emergence of a mutator phenotype, we determined the molecular basis for MMR deficiencies in natural isolates. To do this, we analysed a wild-type isolate of enterohaemorrhagic *E. coli* O157:H7 for levels of MutS, MutH and MutL proteins in growing and stationary-phase cultures. We also determined the molecular defects in stable mutators identified among pathogenic isolates of *E. coli*: two *E. coli* O157:H7 isolates, EC503 and EC535; a diarrhoeagenic *E. coli* of the O55:H7 serotype, DEC5A; and a uropathogenic *E. coli* from the *E. coli* reference (ECOR) strain collection, ECOR48. Genetic complementation studies showed that normal mutability could be restored in each of these mutator strains by plasmid copies of a wild-type *mutS* gene (LeClerc et al., 1996), hence molecular analyses were carried out to define the nature of their MutS defects.

**METHODS**

**Bacterial strains.** *E. coli* MG1655, used for *E. coli* genome sequence analysis (Blattner et al., 1997), and *E. coli* W3110 are K-12 strains described in Bachman (1987). *E. coli* EC536, EC535 and EC503 are O157:H7 strains from the Food and Drug Administration (FDA) bacterial pathogen collection and have been described previously (LeClerc et al., 1996). *E. coli* DEC5A is an O55:H7 strain from the reference collection of diarrhoeagenic *E. coli* (DEC) described by Whittam et al. (1993). ECOR48 is a uropathogenic *E. coli* strain from the ECOR collection described by Ochman & Selander (1984).

**Growth conditions.** For routine growth, cells from frozen stocks of each bacterial strain were grown in LB medium (Miller, 1972) with shaking at 37 °C. For RNase protection assays and Western blotting experiments, bacterial stocks were added to 5 ml enriched minimal salts/glucose [EMMG; Vogel-Bonner (1:1)] minimal salts, 0-01 mM FeSO$_4$, 0-4% (v/v) glucose and 0-5% (w/v) vitamin-assay Casamino acids (Difco Laboratories)]. Cultures were grown overnight with shaking (300 r.p.m.) at 37 °C, and the overnight cultures were diluted 200-fold with fresh EMMG and grown with shaking. Samples for RNase T2 protection assays were collected during exponential growth at a turbidity of 50 Klett (660 nm) units ($5 \times 10^8$ cells per ml determined for MG1655). Samples for Western analysis were collected from cultures grown to mid-exponential phase (a turbidity of 50–65 Klett units) or transitional phase (200 Klett units) and stationary phase (24 and 48 h after inoculation).

**Western blotting.** Samples for Western blotting were prepared as described previously (Feng et al., 1996) or with the following modifications. Portions of cultures (20, 7 and 3 ml) of strains MG1655 and EC536 were removed at culture turbidity values of 50–65 Klett units and 200 Klett units, or at 24 and 48 h after inoculation. After centrifugation at 5000 g for 7 min at room temperature, pellets were washed once with 20 ml of 1x E salts and washed a second time in 1 ml of 1x E salts in microfuge tubes. The final pellets were resuspended in 300 µl buffer containing 0-125 M Tris/HCl, pH 6-8, and 4% SDS and boiled for 10 min. Total protein concentrations were determined by using the Bio-Rad D$_2$ protein assay kit on samples diluted 20-fold in water and on BSA standards in the same diluted buffer. For analysis on SDS-PAGE gels, samples were diluted in a solution containing 2 M DTT, 40% (v/v) glycerol and 0-1% bromophenol blue, so that final protein samples were resuspended in Laemmli buffer (Laemmli, 1970; 2% SDS, 100 mM DTT, 10% glycerol, 62.5 mM Tris/HCl, pH 6.8).

Quantitative Western blotting assays to detect MutS, MutL and MutH were carried out as described previously (Tsui et al., 1997) or with the changes described below. Antiserum against hexahistidine (His-6)-tagged *E. coli* MutS, MutL and MutH proteins were produced as described.
previously (Feng & Winkler, 1995) and affinity-purified with CNBr-activated Sepharose 4B (Pharmacia Biotech) coupled to His-6-tagged *E. coli* MutS, His-6-tagged *E. coli* MutL or His-6-tagged *E. coli* MutH as described previously (Harris et al., 1997). In gel analysis, the His-6 tag (about 2 kDa) did not appreciably change the migration of the tagged proteins. To further reduce background, the antibodies were pre-adsorbed to lysates prepared from *mutS*, *mutL* or *mutH* insertion mutants (Feng et al., 1996). Alkaline-phosphatase-conjugated secondary antibodies or ECL Western blotting detection reagents (Amersham) were used for detection.

**RNase T2 protection assays of chromosomal transcripts.** RNA was prepared by adding portions of bacterial cultures directly to lysis solutions without intervening steps as described previously (Feng et al., 1996). RNase T2 protection assays of transcripts from the bacterial chromosome were conducted as described by Tsui et al. (1994). RNA probes for detecting *mutS* transcripts (see Fig. 2) were synthesized using the following phage RNA polymerases and linearized plasmid templates: SP6 and EcoRI-treated pTX414 (5' end of *mutS* transcripts); T7 and HincII-treated pTX541 (3' end of *mutS* transcripts); and SP6 and EcoRI-treated pTX541 (antisense transcripts at the 3' end of *mutS*) (Tsui et al., 1997). A series of labelled, undigested probes of known length were used as size standards to determine the lengths of the protected fragments (standard errors of 5–10%) (Tsui et al., 1994). Each hybridization reaction contained 25 μg total RNA from cells grown in EMMG. Radioactivity in bands was quantified by using an InstantImager (Packard).

**PCR.** Standard PCRs and long PCRs were performed according to the manufacturer's directions using the AmpliTaq DNA Polymerase Kit and the GeneAmp XL PCR Kit (Perkin Elmer), respectively. Primers used to produce amplification products for sequencing the *mutS* region of *E. coli* strains are listed in Table 1. In the experiment shown in Fig. 4, amplification products of 17–8 and 14–1 kb are expected to be produced on templates prepared from wild-type strains of *E. coli* K-12 (W3110) and *E. coli* O157:H7 (EC536), respectively, using primers F21 and BL260 (Table 1).

**Cloning the *mutS* gene of ECOR48.** Chromosomal DNA was prepared from an overnight culture of ECOR48 using the Purigene DNA Isolation Kit (Gentra) according to the manufacturer's directions.
Table 1. Primers for DNA amplification by PCR

<table>
<thead>
<tr>
<th>Strain</th>
<th>Primer pair</th>
<th>Sequence</th>
<th>Location*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC503</td>
<td>BL117</td>
<td>5’-TTGGCGCTGATGGCCCTACA</td>
<td>31 285</td>
</tr>
<tr>
<td></td>
<td>RPOS5</td>
<td>5’-CTGAAACATACGCGAAGCTTG</td>
<td>39 431</td>
</tr>
<tr>
<td>DEC5A</td>
<td>F1</td>
<td>5’-ACCGCTTCCCGTATATGC</td>
<td>26 546</td>
</tr>
<tr>
<td></td>
<td>BL259</td>
<td>5’-TTGCAGTGGACGACGTCTC</td>
<td>41 500</td>
</tr>
<tr>
<td>EC535</td>
<td>F21</td>
<td>5’-ACCGCGTGCAGCTGCTGCC</td>
<td>24 780</td>
</tr>
<tr>
<td></td>
<td>BL260</td>
<td>5’-GTACATGCACTCGTGTTAC</td>
<td>42 549</td>
</tr>
<tr>
<td>EC536, EC503, EC535 and DEC5A</td>
<td>F8†</td>
<td>5’-CAGTAAATTTGAGCATCTGC</td>
<td>24 481</td>
</tr>
<tr>
<td></td>
<td>BL260†</td>
<td>5’-GTACATGCACTGTGTATAC</td>
<td>42 549</td>
</tr>
<tr>
<td>ECOR48</td>
<td>BL95</td>
<td>5’-CTGAAACAGCGACCCTGC</td>
<td>28 655</td>
</tr>
<tr>
<td></td>
<td>BL190</td>
<td>5’-CACGGCAGTGAGGTAGCTA</td>
<td>28 834</td>
</tr>
</tbody>
</table>

*Sequence location based on E. coli K-12 genome, approximately 61–62 min (GenBank accession no. U29579).
†Served as a common primer pair for PCR amplification of EC536, EC503, EC535 and DEC5A.

DNA, digested with Sall and BglII, produced a 3-9 kb fragment containing an intact mutS gene and flanking regions as determined by restriction digestion of PCR product from the mutS region of ECOR48. DNA, purified from the 3-9 kb region of an agarose gel, was ligated with Sall- and BglII-digested vector pTZ72 (Promega) and electroporated into ElectroMax DH10B cells (Gibco-BRL). A 32P-end-labelled oligonucleotide, specific for the mutS region of ECOR48 (5’-GCCGGAGAGCCATACTCTCC), was used to probe transformed cells by colony filter hybridization as described previously (Cebula & Koch, 1990). Plasmid DNA was prepared from probe-positive clones, and the presence of the mutS gene from ECOR48 was confirmed by PCR and restriction digestion analysis.

For sequencing the mutS gene of strain ECOR48, the ECOR48 mutS gene clone was grown in LB medium at 37 °C overnight and subjected to a Wizard Plus Midiprep DNA Purification System (Promega), according to the manufacturer’s instructions. Plasmid DNA was further purified by phenol extraction and ethanol precipitation. The 3-9 kb DNA fragment, covering the mutS gene and its flanking sequences, was sequenced from plasmid DNA using a commercial service (Lark Technologies).

Nucleotide sequencing and analysis. To determine the exact end points of mutS deletions in strains EC503, EC535 and DE5A, PCR products containing the junction fragments were sequenced. DNA sequencing was performed using the T7 Sequenase Version 2.0 DNA Sequencing Kit (Amersham) according to the manufacturer’s instructions. Programs from the Wisconsin Sequence Analysis Package (Genetics Computer Group) were used for sequence analyses.

RESULTS

Detection of MutS in wild-type and mutant strains

Western analysis of the MutS proteins from E. coli K-12 strains and pathogenic E. coli strains is shown in Fig. 1. MutS was detected in the wild-type E. coli O157:H7 strain EC536; the intensity of the MutS-specific band in EC536 is similar to that of the K-12 prototrophic strains MG1655 and W3110. It should be noted that protein bands on gels often appeared blurred and/or shifted upward in the case of extracts from the natural pathogen EC536, possibly because these extracts contain considerably more outer-membrane components, such as lipopolysaccharides (data not shown).

Quantification of the MutS, MutH and MutL proteins in wild-type strains

We have previously reported that the amounts of MutS and MutH are reduced in K-12 strain MG1655 as cells enter stationary phase (Feng et al., 1996). To investigate whether similar downregulation occurs in the pathogenic E. coli O157:H7 strain, we performed Western analysis of the MutS, MutL and MutH proteins using protein samples obtained from EC536 and MG1655 strains that were grown to mid-exponential, transitional or stationary phase. Similar to results reported previously (Feng et al., 1996), MutS amounts in MG1655 decreased 4-5-fold as cells entered stationary phase (24 and 48 h after inoculation, lanes 3 and 4, Fig. 2) when compared to cells grown to mid-exponential phase (lane 1) or transitional phase (lane 2). As shown in Fig. 2, MutS amounts decreased more in EC536 than in MG1655 after cells entered stationary phase. While MutS amounts in the exponential phase and transitional phase were similar in the two strains, MutS amounts were almost non-detectable in the stationary-phase EC536 strain (lanes 3 and 4). From a curve generated from the quantification standards with different amounts of purified MutS spiked into extracts of null strains (Fig. 2), we estimated that the amount of MutS in EC536 was decreased about 26-fold in stationary-phase cells compared to that in exponential-phase cells. MutH amounts decreased about twofold in EC536 as cells entered stationary phase (Fig. 2), similar to that observed with MG1655 (Fig. 2, and Feng et al., 1996). MutL amounts remained relatively constant in the different growth phases in strains EC536 and MG1655 (Fig. 2, and Feng et al., 1996).
Mapping and measurement of mutS transcripts

To determine whether the transcriptional organization of the mutS gene in E. coli O157:H7 is similar to that in MG1655, we mapped the transcripts by RNase T2 protection assays using RNA probes specific for the 5' or 3' end of the mutS gene (Fig. 3a). EC536, the wild-type O157:H7 strain, showed similar patterns of 5' and 3' ends of mutS transcripts as in MG1655 (Fig. 3b). Three major protected transcripts of 520, 480 and 428 nt were seen using the 5'-end mutS probe on RNA from MG1655 and EC536. The 5' ends of the 520 and 480 nt protected species were mapped to positions approximately 74 and 34 nt, respectively, upstream from the mutS start codon of MG1655 (Tsui et al., 1997). The 428 nt protected species is a partial mutS transcript (Tsui et al., 1997). Two major protected transcripts of 990 and 920 nt were seen using the 3'-end mutS probe on RNA from MG1655 and EC536. These bands correspond to mutS transcripts with 3' ends at approximately 97 and 29 nt downstream from the mutS stop codon. Together, these results show that mutS in EC536 (O157:H7) is in a single-gene operon, as was reported previously for MG1655 (K-12) (Tsui et al., 1997), and that the transcription starts, stops and possible processing sites appear similar for the enterohaemorrhagic and laboratory strains of E. coli.

Fig. 3. Mapping of mutS transcripts in E. coli strains. (a) Structure (drawn to scale) and transcription of the mutS genomic region in E. coli K-12 (Tsui et al., 1997). Putative promoters, P_{mutS} and P_{o218b}, and terminators, Term1 and Term2, were described by Tsui et al. (1997). Δ, start of deletion in the 3' end of mutS in strain EC503. Lines indicate RNA probes used in RNase T2 assays. (b) mutS transcripts in E. coli strains. RNase T2 protection assays (see Methods) were performed using RNA probes specific to the 5' or 3' ends of mutS, or antisense transcripts at the 3' end of mutS.
Mapping of *mutS* transcripts was carried out on the mutator strains EC503, EC535, DEC5A and ECOR48 to examine the nature of their *mutS* defects (Fig. 3b). No protected band, corresponding to the 5’ end of *mutS* transcripts, was observed in strains EC535 and DEC5A, indicating an absence of *mutS* mRNA in these strains. In strain EC503, hybridization of the 5’ probe showed patterns and amounts of 5’ *mutS* transcript similar to RNA from the non-mutator EC536. However, the protected transcripts of EC503 at the 3’ end were much less abundant and shorter than those of EC536. The 620 nt protected band from EC503 corresponds to a transcript with a 3’ end about 270 nt upstream from the *mutS* stop codon. In strain ECOR48, *mutS* transcript was detected using the 5’-end probe, but not with the 3’-end probe. The results from analyses of EC503 and ECOR48 transcripts suggested that mutations in the *mutS* structural genes of these strains affected transcript length or stability. The presence of antisense transcripts was also investigated in order to test for DNA contamination in RNA preparations and to determine if antisense transcription was identical in wild-type and mutant strains. It is notable that protected antisense transcripts that correspond to the 3’ end of the *mutS* gene were detected in EC503 (Fig. 3b), but not in EC536 (Fig. 3b) or MG1655 (data not shown).

### PCR and sequence analysis of EC503, EC535 and DEC5A mutant DNAs

In EC536, *mutS* is adjacent on its 5’ side to *fhlA* and on its 3’ side to the o218–yclD–yclC–yclB–slyA–rpoS gene cluster (cf. Fig. 3). Attempts to PCR-amplify an intact *mutS* gene from the EC503, EC535 and DEC5A mutators failed, suggesting that primer-annealing sites were disrupted by deletions or rearrangements of the gene in these strains. To determine the extent of possible *mutS* deletions, the mutants were probed under low stringency conditions using a series of 32P-end-labelled oligonucleotides designed to anneal at 1.5 kb intervals throughout a 12 kb region surrounding *mutS* from either *E. coli* K-12 or O157:H7. The results of colony hybridization indicated that EC503 contained a deletion of the 3’ end of the *mutS* gene and open reading frames (ORFs) between the *mutS* and *rpoS* genes; EC535 and DEC5A carried extensive deletions encompassing *mutS* and neighbouring 5’-*fhlA* gene and extending at least 3 kb to the *rpoS* gene (data not shown).

Analysis of the EC503 mutant by PCR amplification of DNA from the 5’ end of the *mutS* gene to the middle of the *rpoS* gene followed by sequencing of the PCR product confirmed that the 3’ end of the *mutS* gene and ORFs between the *mutS* and *rpoS* genes were deleted in this mutant. Initial PCR analysis of the more extensive deletions in EC535 and DEC5A involved amplification of short products on each side of the deletions in order to determine their approximate extent. Primers, established for productive PCR, were then used in long PCRs for amplification of products containing the deletion junctions. Sequence analysis of these PCR products defined the deletions in mutant DNAs. Table 2 gives the sizes and positions of the deletions and shows wild-type sequence present at the deletion end points based on the K-12 genome. Sequences of 6–8 base pairs are repeated at the end points of the deletions, leaving one intact copy in the cases of EC503 and EC535 or deleting both copies in DEC5A. Fig. 4 shows the PCR products amplified from primers at fixed sites in the three mutant DNAs. In the experiment of Fig. 4, an amplification product of 14 kb is expected to be produced on template prepared from a wild-type strain of *E. coli* O157:H7 (EC536), while products of

### Table 2. Deletion end points in *mutS* mutants of *E. coli*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Start point*</th>
<th>Sequence</th>
<th>End point*</th>
<th>Sequence</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC503</td>
<td>31 746</td>
<td>GAGGT</td>
<td>39 195</td>
<td>GAGGT</td>
<td>7 449</td>
</tr>
<tr>
<td>DEC5A</td>
<td>27 146</td>
<td>AGCCGC†</td>
<td>41 019</td>
<td>AGCCGC†</td>
<td>13 873</td>
</tr>
<tr>
<td>EC535</td>
<td>25 189</td>
<td>AGAGAAT</td>
<td>42 445</td>
<td>AGAGAAT</td>
<td>17 257</td>
</tr>
</tbody>
</table>

*Sequence location based on *E. coli* K-12 genome, approximately 61–62 min (GenBank accession no. U29579).
†Sequence immediately inside the deletion location based on *E. coli* K-12 genome, approximately 61–62 min (GenBank accession no. U29579).
We report here that the amount of MutS in a natural E. coli pathogen is depleted to barely detectable levels when cells are in the stationary phase. As compared with levels in exponentially growing cells, the MutS protein in enterohaemorrhagic E. coli O157:H7 in stationary phase was decreased about 26-fold. While the cellular amounts of MutS during exponential- and transitional-phase growth were similar in O157:H7 and a laboratory-attenuated K-12 strain, the decline in MutS levels in O157:H7 was nearly sixfold greater than in the K-12 strain. Comparison of protein levels in the two strains is useful here, because calculation of MutS binding to DNA mismatches in exponentially growing K-12 cells showed the amount of MutS to be nearly limiting for mismatch repair (Feng et al., 1996). These data suggest that stationary phase depletion of MutS in cells in the natural habitat could have a greater impact than recognized from studies using laboratory-attenuated strains. Such a comparison with the non-pathogenic K-12 strain suggests, for instance, that the pathogen may be predisposed to an advantageous transient mutator phenotype in the stationary-phase condition.

No antisense transcript to mutS was detected in EC536 during exponential or early stationary phase (data not shown). Curiously, an antisense transcript was observed in the EC503 deletion mutant. Although antisense transcription from rpoS and other downstream genes from mutS could provide an exquisite mechanism for effecting a transient mutator phenotype, there is no evidence that an antisense mechanism operates to mediate MutS amount under the conditions tested so far.

To date, studies have investigated increased mutagenesis caused by decreases of MutS in stationary-phase cells (Schaaper & Radman, 1989; Zhao & Winkler, 2000), though increased homeologous recombination caused by MutS depletion may be the more significant occurrence in resting or non-dividing cells in nature. Examining recombination in ‘starved’ bacteria may be especially important not only because MutS expression is compromised severely under nutrient-deprived conditions (Feng et al., 1996) but also because cells in the natural habitat constantly experience a ‘feast or famine’ existence. Such conditions may signal the need for a transient hypermutable and/or hyper-recombinagenic phenotype.

Analysis of molecular defects in the stable E. coli mutators EC503, EC535 and DEC5A revealed large deletions incapable of direct reversion to the wild-type allele. This outcome is compatible with findings on defects in natural P. aeruginosa mutators, which were frameshift, insertion,
deletion or multiple substitution mutations (Oliver et al., 2002). While the nonsense mutation in the mutS gene of the ECOR48 mutator is susceptible to simple reversion, other base changes in the gene – the expected consequence for an inactive gene in a mutator background – also likely lead to its inactivation. The question then remains how an adaptive mutant can break its linkage with the mutator locus and thereby escape the deleterious effects of a heightened mutation rate. Besides reversion at the mutator locus, the mutator phenotype may be suppressed by mutation at other loci, as evidenced by results from long-term growth experiments using a mutT strain of E. coli (Tröbner & Piechocki, 1984). In these studies, isolates taken after 2200 generations of chemostat growth had lower mutation frequencies than the starting culture, but transduction analyses showed that the mutT allele itself had not changed. The lower mutation rates of the isolates were likely due to secondary mutations that suppressed the mutator phenotype (Tröbner & Piechocki, 1984).

Another option, one particularly feasible in promiscuous MMR mutators in feral settings, is repair of the mutator defect by homeologous recombination. An intact mut locus could be inherited from a Mut+ cell after horizontal transfer of sequence from either closely or distantly related species. Phylogenetic analysis of mutS-positive alleles from naturally occurring E. coli strains (including pathogens and strains of the ECOR collection) showed that mutS alleles indeed have been recombined, evidenced by a phylogeny of mutS alleles different from that of the E. coli strains in which they reside (Denamur et al., 2000; Brown et al., 2001a, b). Such differences between gene evolution and strain evolution are indicative of horizontal exchange, a conclusion also supported by evidence for specific transfer events in the mutS region of the E. coli chromosome (LeClerc et al., 1999; Culham & Wood, 2000; Reid et al., 2000). The identification of mutH among a class of genes inherited from horizontal transfer (Médigue et al., 1991) suggests that defective mutH alleles may also have been repaired by homeologous recombination.

The occurrence of strains carrying deletions that encompass multiple loci, as many as 15 genes and ORFs in the EC355 mutant, is surprising in view of the environmental challenges of natural conditions. We have argued that the preponderance of mutS mutants among mutators in nature is indicative of the benefits of adaptive change generated by increased recombination and mutagenesis (LeClerc et al., 1996). The deletion mutants analysed here also affect the rpoS gene, however, and many genes needed for survival during environmental stress are regulated by the RpoS sigma factor. There are rpoS mutants of E. coli that have a selective advantage over wild-type cells as they emerge from stationary- to exponential-phase growth (Zambrino et al., 1993) and rpoS mutants are common in aged cultures (Sutton et al., 2000). It is possible that other, compensatory changes in these mutator strains overcome the limitations of growth in the absence of RpoS regulation.

**ACKNOWLEDGEMENTS**

We gratefully acknowledge Mr William L. Payne for his dedicated and diligent efforts in the laboratory. In addition to research carried out in FDA laboratories, this work was in part supported by NIH grant RO1-CA77103 to M. W. and by resources at the Lilly Research Laboratories.

**REFERENCES**


