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Roles of *E. coli* double-strand-break-repair proteins in stress-induced mutation

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Abstract

Special mechanisms of mutation are induced during growth-limiting stress and can generate adaptive mutations that permit growth. These mechanisms may provide improved models for mutagenesis in antibiotic resistance, evolution of pathogens, cancer progression and chemotherapy resistance. Stress-induced reversion of an *Escherichia coli* episomal *lac* frameshift allele specifically requires DNA double-strand-break-repair (DSBR) proteins, the SOS DNA-damage response and its error-prone DNA polymerase, DinB. We distinguished two possible roles for the DSBR proteins. Each might act solely upstream of SOS, to create single-strand DNA that induces SOS. This could upregulate DinB and enhance mutation globally. Or any or all of them might function other than or in addition to SOS promotion, for example, directly in error-prone DSBR. We report that in cells with SOS genes derepressed constitutively, RecA, RuvA, RuvB, RuvC, RecF and TraI remain required for stress-induced mutation, demonstrating that these proteins act other than via SOS induction. RecA and TraI also act by promoting SOS. These and additional results with hyper-mutating *recD* and *recG* mutants support roles for these proteins via error-prone DSBR. Such mechanisms could localize stress-induced mutagenesis to small genomic regions, a potentially important strategy for adaptive evolution, both for reducing additional deleterious mutations in rare adaptive mutants and for concerted evolution of genes.

Keywords

Evolution; Genome instability; Genetic recombination; SOS response; Stress response

1. Introduction

Classical spontaneous mutations occur by a mode that had appeared to be universal [1,2,3, and many subsequent papers]. The mutations occur before cells are exposed to an environment in which the mutation might prove useful, in proliferating cells, and with a definable relationship to cell divisions [4, we call these generation-dependent mutations].

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However, a growing body of work in bacteria and yeast has revealed that mutations also occur in response to stress-inducing, growth-limiting environments, by special mechanisms not seen in rapidly-growing cells [reviewed, Refs. 5,6–8]. These “stress-induced” or “stationary-phase” mutation mechanisms occasionally produce mutations that allow growth in the growth-limiting environment, called “adaptive mutations”. These were controversial initially because of the suggestion that the adaptive mutations might be formed preferentially [9], an idea that was rejected for the most well studied stress-induced mutation mechanism [10–13] and for others [e.g., Ref. 14]. A second round of controversy has concerned the idea of stress-induced increases in genome-wide mutation rate, which, though compatible with Darwinism, appears to challenge conservative neo-Darwinist ideals of constant and gradual evolutionary change [e.g., see Refs. 15,16–19]. In addition to and independently of these issues, the molecular mechanisms of stress-induced mutation are intriguing and important to understand because they may provide superior models for the origins of genetic changes that drive evolution of resistance of pathogens to antibiotics and to our immune systems [20–22], loss of growth control in oncogenesis [23], tumor progression and resistance to chemotherapy [24], aging [25], and perhaps much of evolution generally.

Stress-induced mutation occurs by multiple mutation mechanisms observed with different stress conditions, strains, and organisms, though with some similarities among the mechanisms described so far [6,7,26]. The mutation mechanisms operating in the *Escherichia coli* Lac frameshift reversion assay are the best characterized. In this assay, *E. coli* cells carrying a deletion of the chromosomal *lac* genes and a *lac* +1 frameshift allele on an F' conjugative plasmid are plated onto solid medium with lactose as the sole carbon source [27]. For the next week or so, Lac⁺ revertant colonies accumulate. About half of the colonies observed on the second day of incubation (day two) result from generation-dependent mutations that occurred during growth of the cultures prior to plating; the remaining day-two colonies and colonies that appear on later days are stress-induced adaptive mutants, formed after exposure to the lactose medium [27–30] and consist of two kinds. Most colonies appearing from days 3–6 are Lac⁺ because of compensatory frameshift mutations [31,32] (“adaptive point mutants”). A smaller number carries amplification of the *lac* region as 20–100 tandem repeats, which provide sufficient activity from the leaky *lac* allele to allow growth [29]. The proportion of *lac*-amplified clones increases with time, constituting about 40% by day eight. Although amplification was proposed to be an intermediate in the point-mutation pathway [33], recent data indicate that point mutation and amplification are independent outcomes and mechanisms [19, discussed below], both induced by stress [34]. Also, point mutation can occur in the absence of selected amplification [19,35]. The stress-induced point-mutation mechanism is our main focus here.

Stress-induced point mutation requires *E. coli* double-strand-break-repair (DSBR) and homologous-recombination proteins RecA, RecBC, RecF, and RuvABC [27,36–39]. Conjugal transfer functions, though not actual transfer of the F', are also required [40,41], specifically TraI, an F-encoded single-strand endonuclease and helicase ([35], below, and discussed in Ref. [41]). The SOS DNA damage response and DinB, an error-prone DNA polymerase that it upregulates, are required for point mutation but not *lac*-amplification [39,42]. The RpoS-controlled general/starvation-stress response, which also upregulates DinB [43], is required for both stress-induced point mutation and *lac*-amplification [34]. DNA polymerase I is required only for amplification, not point mutation ([19] and A. Slack, PC Thornton, DB Magner, SM Rosenberg and PJ Hastings, unpublished). Point mutation is increased in mutants lacking RecD DSBR-regulatory protein [36]. In cells lacking RecG DSBR protein, either point mutants, *lac*-amplified clones, or both are increased [37,38].

The involvement of DSB proteins in the Lac system's stress-induced point-mutation mechanism is potentially a feature with some generality, in that DSB proteins are also required for an adaptive mutation mechanism in yeast [44, discussed below]. It is potentially also important both for the mutation mechanism and for its evolutionary consequences. Two equally plausible general models could have accounted for the roles of DSB proteins in stress-induced point mutation [6]. In the first, the DSB proteins play direct roles in mutation, which could occur, for example, via an error-prone-DSB mechanism [Fig. 1A, Ref. 36, and several subsequent papers]. Recent results support such models [35]. Alternatively, because stress-induced point mutation also requires SOS induction [39,42], and some [45–47], or perhaps all [6] of the DSB proteins might participate in SOS induction during stress-induced mutagenesis, the DSB proteins might act indirectly, solely in DNA processing events that lead to SOS induction (e.g., Fig. 1B). This could promote mutation solely by increasing *dinB* expression, which might enhance mutation anywhere that new DNA is synthesized. Any of the proteins might participate via SOS-inducing, SOS-independent, or both mechanisms.

We distinguish these general models and test the roles of several DSB and other proteins here by showing that DSB proteins are required for stress-induced mutagenesis in *E. coli* strains in which the SOS/LexA regulon is derepressed constitutively. Thus they act other than or in addition to via SOS induction. The results support and provide new details for possible error-prone-DSB models for stress-induced point mutation. Error-prone DSB models suggest the evolutionary consequence of preserving the integrity of most of the genome in any given cell while mutating small localized regions extensively.

2. Materials and Methods

2.1 Bacterial strains

E. coli strains (Table 1) were constructed using standard techniques [48]. Relevant genotypes were confirmed prior to each experiment by sensitivities to ultraviolet light, a phage lambda plaque-formation test, and by antibiotic resistances. Additionally, strains carrying the *lexA*(Def) *sulA psiB* mutations were verified as LexA-defective by transformation with plasmid pJP30 [19] carrying the *gfp* gene under the control of the LexA-regulated *sulA* promoter, and were shown to form green colonies (whereas the isogenic *sulA psiB* strains did not). A conjugation assay was used to confirm the presence of the $\Delta traI$ mutation. Antibiotics and other additives were used at the following concentrations ($\mu\text{g/ml}$): chloramphenicol, 25; kanamycin, 50; tetracycline, 10; rifampicin, 100; trimethoprim, 100; X-gal, 40; threonine, 50; tryptophan, 50. The presence of *recG* and *dinB* in strains were confirmed by PCR with primers 5'-GTACCTGCGTGAAAACAGCA-3' and 5'-TAACCTGAAATGGCGGTCTT-3' and 5'-GCATTCTCAAACCCTGAAATC-3' and 5'-AATACCCGCATCCTTATTCCTT-3', respectively. Stress-induced mutation phenotypes of strains were confirmed either by testing independent isolates of the same strain or by rescuing phenotypes with a wild-type copy of the gene via transduction, or both (see Table 3 "rescue" strains).

The $\Delta recF1804::FRT::Kan::FRT$ deletion allele was constructed by Jeanine M. Pennington [SM Rosenberg laboratory per Ref. 49]. This *recF* deletion lacks codons 1–342 of the 357-codon *recF* gene, but leaves the *gyrB* promoter in the terminal portion of *recF* gene intact, so as not to affect expression of the gyrase gene downstream. A derivative with the antibiotic marker removed was made as described [49]. The stress-induced mutation phenotypes (Table 3) of the $\Delta recF1804::FRT::Kan::FRT$ and *recF332::Tn3* alleles in Lex⁺ and in *lexA*(Def) cells are not different ($P=0.571$ and $P=0.482$, respectively, non-parametric Mann-Whitney rank sum test). The *zeb-2233::FRT::ble::FRT* allele was made by Matthew Blankschien [SM Rosenberg laboratory, per 49]. It contains the *Sh ble* gene from

Streptoalloteichus hindustanus [from Ref. 50, strain AR30], which encodes resistance to zeocin. The marker is between chromosomal basepairs 1,937,178 and 1,937,179, between the *pykA* and *msbB* genes, at about 48 minutes in the chromosomal map.

2.2 DNA Sequencing

The *lac* region was amplified with primers 5'-AGGCTATTCTGGTGGCCGGA-3' and 5'-GCCTCTTCGCTATTACGCCAGCT-3' and sequenced (Lone Star Labs, Inc., Houston, TX) with primer 5'-ATATCCC GCCGTTAACCACC-3'.

2.3 Stress-induced mutation assays

Stress-induced mutation assays were as described [38]. All experiments presented had less than two-fold net population change during days 1–3 after plating. For all genotypes, controls were treated the same as the mutant strains during growth prior to plating on lactose. Strains containing *recA*, *recF*, *recD*, *traI*, and *dinB* mutations were grown to saturation at 37 °C for 48 hrs. Strains containing *ruvA*, *ruvB*, *ruvC* and *recG* mutations were grown at 32 °C for 48 hrs to reduce selection for rapidly-growing suppressor mutants [38], and in some experiments were grown at 37 °C and showed similar results. We noticed that incubation temperature prior to plating affected the stress-induced-mutation phenotype in Lex⁺ (“wild-type” control) cells, which produced more Lac⁺ revertants when grown at 32 °C than at 37 °C prior to plating (Table 3, see also [35]), but not in SOS/LexA-constitutive, *lexA*(Def) (Table 3), or SOS-uninducible, *lexA*Δ(*Ind*[−]) cells (Table 3). To control for potential temperature effects, controls and tester strains were grown at the same temperature prior to plating, and in some cases results were repeated with 37 °C pregrowth. For all experiments reported, values shown are mean ± one SEM for multiple independent cultures (usually 6) per strain and graphs are cumulative.

3. Results and Discussion

3.1 Strategies for examining effects on SOS/LexA regulon induction

The LexA transcriptional repressor controls the SOS response to DNA damage [51]. We used two kinds of *lexA* alleles [51,52] to study effects of LexA-regulon induction in vivo: *lexA*(*Ind*[−]) encodes a mutant LexA repressor that cannot undergo proteolytic cleavage making the LexA regulon (SOS genes) “uninducible”; and a *lexA*-defective [“*lexA*(Def)” or “*lexA*”] null allele which causes constitutive regulon derepression and constitutive overproduction of SOS proteins, including Sula, an inhibitor of cell division. *lexA*(Def) cells must therefore also carry a *sulA*-inactivating mutation to grow. Induction of LexA-regulon genes was shown to be required for stress-induced point mutation with the demonstration that *lexA*(*Ind*[−]) cells show reduced point mutation [27,39,42]. However, *lexA*(Def) *sulA* *E. coli* cells also showed reduced mutation, as if the constitutive overproduction of one or more LexA-controlled protein(s) inhibits stress-induced mutation [39]. We identified an F-encoded protein, PsiB, as (one of) the inhibitor(s), by showing that *lexA*(Def) *sulA* *psiB* cells have nearly normal levels of stress-induced point mutation, and have higher levels than seen for *sulA* *psiB* cells, showing that LexA-regulon de-repression promotes stress-induced mutation [39]. PsiB is an inhibitor/negative regulator of RecA [53]. An additional mutation also reactivated mutation in *lexA*(Def) *sulA* cells, suggesting that more than one SOS-upregulated gene might inhibit stress-induced mutation when LexA-controlled genes are overproduced constitutively [54]. We use the *lexA*(Def) *sulA* *psiB* genetic background as our SOS/LexA-constitutive background here and refer to it as “*lexA*(Def)” or “SOS/LexA-constitutive”.

3.2 Stress-induced point mutation in SOS/LexA-constitutive and LexA⁺ strains occurs by a similar mechanism

We wished to establish that stress-induced point mutation in SOS/LexA-constitutive cells occurs by a similar mechanism as in “wild-type” *lexA*⁺ *sulA*⁺ *psiB*⁺ cells (called “Lex⁺” here). First, we asked whether the DinB DNA polymerase is required for stress-induced point mutation in SOS/LexA-constitutive cells. SOS upregulates *dinB* expression [55,56]. If the stress-induced mutation mechanism in SOS/LexA-constitutive cells were similar to the usual mechanism in SOS-inducible cells, DinB should be required. We found that DinB is required similarly in Lex⁺ and SOS/LexA-constitutive cells (Fig. 2A).

Second, we sequenced adaptive point mutations generated in the SOS/LexA-constitutive cells. We compared 43 mutant sequences from SOS/LexA-constitutive cells with previously published mutant sequences for Lex⁺ cells (Fig. 2C, D). As in Lex⁺ cells, most of the Lac⁺ adaptive reversions are –1 deletions at the mononucleotide repeat hotspot at position 1039; the second largest class has –1 deletions mostly at other mononucleotide repeats (both unlike generation-dependent reversions which are heterogeneous [31,32]). Most of the stress-induced mutations in SOS/LexA-constitutive cells are similar to those formed in Lex⁺ cells (Fig. 2C, D).

Third, we find a similar proportion of *lac*-amplified versus point mutant colonies in Lex⁺ and SOS/LexA-constitutive cells (Fig. 2B). This is true even though the SOS/LexA-constitutive cells produced more variable numbers of Lac⁺ colonies in different experiments than we typically see for Lex⁺ cells, with the number of Lac⁺ colonies on average being 2-fold lower than Lex⁺ [Ref. 39, Tables 2 and 3]. The slightly lower number of total Lac⁺ colonies in SOS/LexA-constitutive than Lex⁺ cells could be caused by the *sulA* mutation, which also showed a small decrease relative to Lex⁺ [39], perhaps due to increased cell division in *sulA* mutants causing fewer sister molecules per cell, which might limit opportunities for error-prone DSB [39]. Alternatively, it could be because another gene(s) besides *psiB* inhibits stress-induced mutagenesis somewhat when constitutively overexpressed in *lexA*(Def) cells (discussed, section 3.1). In SOS/LexA-constitutive strains the requirement for DinB, and very similar mutation sequences and proportions of amplified clones to those in Lex⁺ cells, indicate that stress-induced point mutation occurs by a similar or the same mechanism as in Lex⁺.

3.3 RuvABC promote mutation other than or in addition to by promoting SOS

RuvA, RuvB and RuvC homologous recombination proteins function as a resolvase complex that processes Holliday junctions, and all are required for stress-induced mutagenesis in the Lac system [Refs. 37,38, Fig. 3A]. RuvA recognizes and binds the four-stranded DNA junction; RuvB is a branch-migration motor protein (helicase) that moves the structure; and RuvC, is an endonuclease that cleaves Holliday junctions [57]. We find that RuvA, RuvB and RuvC are required for stress-induced mutagenesis in SOS/LexA-constitutive cells (Fig. 3B, Tables 2 and 3). By quantifying mutation rates (per day) derived from multiple independent experiments like those shown singly in Fig. 3 (Tables 2 and 3), we see that loss of RuvA, RuvB, and RuvC reduced stress-induced mutation in SOS/LexA-constitutive cells by about three-fold, five-fold, and three-fold, respectively, and in Lex⁺ cells by 10-fold, 10-fold and 20-fold respectively. (For all genotypes tested, see Table 3 for fold-comparisons and Table 2 for absolute mutation rates used to generate the fold-comparisons.) We conclude that RuvABC play roles in stress-induced mutation in addition to promoting SOS induction, supporting the idea that their well-defined functions in recombination contribute to mutation.

3.4 RecA promotes point mutation both via SOS-induction and SOS-induction-independent step(s)

E. coli RecA is a ssDNA binding protein required for recombination and SOS induction, and is conserved in most studied organisms [57]. In response to DNA damage, RecA becomes activated (RecA*) by loading onto ssDNA. RecA* both induces the SOS response, acting as a co-protease to facilitate the auto-proteolytic cleavage of LexA, and also catalyzes strand exchange with homologous DNA to initiate recombinational repair. We find that RecA is required for stress-induced point mutation in SOS/LexA-constitutive cells (Fig. 3C, Tables 2, 3). However, loss of *recA* caused only a ten-fold decrease in stress-induced mutation in the *lexA*(Def) cells, whereas loss of *recA* in *Lex*⁺ cells caused a 100-fold decrease (Table 3). This implies that in *Lex*⁺ cells RecA is required both for its role in inducing SOS and also for an additional function(s).

3.5 TraI stimulates SOS-induction and an additional step(s)

Previous work showed that one or more conjugative transfer (Tra) function(s), but not actual transfer, is required for stress-induced mutation [40,41]. TraI is an F-encoded endonuclease/helicase that makes single-strand nicks at the origin of transfer, *oriT*, on conjugal plasmids [58]. It has been shown recently to be more strongly required for stress-induced mutation ([35] and see Fig. 3D and Table 3) than other transfer genes [41], and also to be dispensable for stress-induced mutation if a DNA DSB is provided near *lac* by the *I-SceI* double-strand endonuclease [35]; also the *I-SceI* cuts promote mutagenesis more than ten times better than TraI. These data imply that TraI-generated single-strand nicks may become double-strand ends (DSEs) and so promote mutation. Given its role in producing ssDNA and possibly DSEs, TraI might promote mutation solely by promoting SOS induction, or those nicks might become DSEs which could be repaired mutagenically (suggested Refs. [37,59–61]). We find that TraI is required even when SOS is induced constitutively, but less in SOS/LexA-constitutive cells than in *Lex*⁺ (Fig. 3D, Table 3). Whereas $\Delta traI$ decreases mutation about 100-fold in *Lex*⁺ cells, it decreases mutation about five-fold in SOS/LexA-constitutive cells. Therefore, TraI appears to promote mutation in *Lex*⁺ cells via stimulating SOS induction and by an additional route, such as leading to DSEs that are repaired mutagenically.

3.6 RecF promotes mutation other than by promoting SOS

RecF is thought to help RecA load onto ssDNA during recombinational DNA gap repair [62], to function in replication restart [63], and also participates in SOS induction by ssDNA lesions [45]. RecF is partially required for Lac⁺ adaptive mutation and loss of RecF does not decrease mutation further in SOS-uninducible strains carrying an uncleavable LexA repressor [39]. This could mean that the role of RecF is only in promoting SOS induction [39], but might also result from both SOS and RecF-facilitated recombination (or other) function acting in any linear pathway leading to mutation (not just the SOS component of the pathway). Supporting the latter possibility, we find that RecF is required even in SOS/LexA-constitutive cells (Fig. 3E, Table 3). This is true for the *recF332::Tn3* allele used previously and also for two newly-constructed *recF* deletion alleles (Fig. 3E, Tables 2 and 3, Materials and Methods), and all show a similar decrease to that in *Lex*⁺ cells [39, Table 3]. Therefore the role of RecF in stress-induced mutation in *Lex*⁺ cells is other than or in addition to promoting SOS induction.

This suggests the possibility of a recombinational role for RecF in stress-induced mutation. This might seem surprising given the strong RecBC-dependence of stress-induced mutation [36] (implying involvement of DSEs) and the fact that most RecBC-dependent recombination is RecF-independent [57]. However there is a precedent: a form of DNA replication called inducible stable DNA replication (iSDR), which is thought to occur by

priming replication during DSE-repair (similarly to Fig. 1A), strongly requires RecBC and partially requires RecF [64], as also observed for stress-induced mutation ([36,39], Fig. 3E, Table 3). Perhaps RecF facilitates specifically those DSBR reactions that lead to replication, making it appear specific to recombinational processes in which either synthesis [iSDR, see also 63], or errors generated during it (mutation), are the outcome assayed. Though possibly similar, iSDR is not identical to stress-induced mutation in that the former is RuvABC-independent [64].

3.7 RecD inhibits stress-induced mutation independently of effects on SOS induction

RecD is a regulatory subunit of the RecBCD DSBR/recombination enzyme and is required for the exonuclease activity of RecBCD [65–67]. Normally, RecBCD loads only onto DSEs and digests the DNA until it reaches a DNA sequence, Chi, at which the RecD-dependent exonuclease activity is diminished/altered, and ssDNA is produced allowing recombination and SOS induction [57]. In *recD* null mutants, creation of recombinogenic ssDNA appears to occur more efficiently, immediately upon RecBC(D[−]) loading onto DNA ends, and cells show hyperrecombination at DSEs [68,69]. RecB is required for stress-induced mutation and RecD inhibits it (*recD*[−] cells show increased mutation) [36]. The mechanism for this could be either that RecBC-dependent DSBR is required for mutation, or that SOS induction requires RecB [45] and is increased in *recD* mutants, or both. (It was also suggested that *recD* could increase stress-induced mutation by increasing F' copy number relative to the bacterial chromosome [70], however, *recD* also inhibits chromosomal stress-induced mutation making this unlikely [71]). We could not test RecB directly, because we found that SOS/LexA-constitutive *recB* cells are nearly inviable (data not shown). However, we found that, as in Lex⁺ cells, loss of RecD increased stress-induced mutation in SOS/LexA-constitutive cells and in *lexA*(Ind[−]) uninducible [52] cells, in which point mutation is decreased about 85% [42]. In both of these strains, loss of RecD increases stress-induced mutation to a similar extent (Fig. 3F, G, Table 3), and to a similar extent as in Lex⁺ cells [36]. Therefore, RecD inhibits stress-induced mutation other than by inhibiting SOS-induction. This implies that RecBCD functions in stress-induced mutation independently of any additional effects on SOS induction.

3.8 RecD inhibits point mutation

The increased Lac⁺ colonies in *recD* cells ([36], Fig. 3F,G, Table 3) could occur either by an increase in point mutation or *lac*-amplification or both. In Lex⁺ *recD* cells, most Lac⁺ colonies are point mutants [32]. However, given the SOS-independence of the *recD* phenotype (Fig. 3F, G, Table 3), and the SOS-independence of *lac*-amplification [42], it was possible that only *lac*-amplification increased in *lexA*(Def) *recD* and/or *lexA*(Ind[−]) *recD* cells. We determined the fraction of day-6 Lac⁺ colonies with point mutations versus *lac*-amplifications [per Ref. 29] and find that point mutation increased greatly in *recD* cells. In the *lexA*(Def) background, point mutants were ~90% (109/119 and 85/96 from two experiments) and ~99% (141/143 and 99/100) of the total day-6 Lac⁺ colonies in *recD*⁺ and *recD*, respectively. In the *lexA*(Ind[−]) background as well, in which point mutation but not amplification decreases [42], the remaining point mutation also increased in *recD* cells: *recD*⁺ and *recD* derivatives showed ~50% (63/120 and 45/93) and 70–90% (18/26 and 93/100) point mutants among day-6 colonies, respectively. Therefore, we conclude that RecD inhibits point mutation (this section), and that this inhibition occurs independently of effects on SOS induction (previous section).

3.9 RecG inhibits point mutation

RecG is a structure-specific DNA helicase that can branch-migrate Holliday (four-way) junctions and three-way junctions [72], possibly in an opposite direction from RuvAB branch-migration helicase [37,38,73], and has been proposed to regress stalled replication

forks [73]. *recG* mutants are hyper-SOS-induced [46,47], and also show 10–25-fold increased stress-induced mutation ([37,38], Fig. 3H, Tables 2 and 3). First, we show that most or all of that increase is point mutation. As above for *recD* mutants, we see that point mutation is increased by loss of *recG* in the Lex⁺ background. Point mutants were ~50–60% (81/137 and 73/134 from two experiments) and ~97% (126/130 and 118/121) of the total day-8 Lac⁺ colonies in *recG*⁺ and *recG* strains, respectively, indicating that point mutation was greatly stimulated by loss of RecG.

3.10 RecG inhibits SOS induction and an SOS-induction-independent step(s) in point mutation

RecG could inhibit stress-induced point mutation either by unwinding (aborting) a DNA intermediate that otherwise leads to mutation, we suggested a strand-exchange intermediate that primes error-prone synthesis ([38] e.g., Fig. 1A), or by inhibiting SOS induction [16], or both.

Surprisingly, we find that RecG becomes required for mutation in SOS/LexA-constitutive cells (Fig. 3I, Table 3) in contrast to its antimutagenic role in Lex⁺ cells (e.g., Fig. 3H). We verified this result by testing an additional independent construct of the SOS/LexA-constitutive *recG* strain and by rescuing the phenotype with wild-type *recG*⁺ introduced (by P1 transduction) back into these cells to rule out the possibility of accumulation of suppressor mutations in the SOS/LexA-constitutive *recG* background [Fig. 3I, the strain “LexA(Def)(2)” is the rescued strain, Tables 2 and 3]. We hypothesized that RecG might be required in SOS/LexA-constitutive cells to counteract an effect of a LexA-controlled protein(s) that becomes antimutagenic only at abnormally high levels when constitutively overproduced, for example RuvAB (discussed below). In support of this idea, we see that RecG again inhibits stress-induced mutation in SOS/LexA-uninducible *lexA*(Ind[−]) cells (Fig. 3J, Table 3). This is also a strong inhibition of point mutation, rather than amplification, in that in the *lexA*(Ind[−]) background, *recG*⁺ and *recG* derivatives showed ~30% (32/123 and 46/137) and 60–80% (82/105 and 62/112) point mutants among day-8 colonies, respectively. Therefore, RecG inhibits point mutation strongly in Lex⁺ and *lexA*(Ind[−]) cells, but is required in *lexA*(Def) cells. That is, RecG is required for mutation only in the circumstance of constitutive overproduction of LexA-regulated functions.

RecG inhibits stress-induced mutation somewhat less in *lexA*(Ind[−]) than in Lex⁺ cells (Tables 2 and 3), implying that RecG inhibits stress-induced point mutation in Lex⁺ cells both by a small inhibition of SOS-induction, and a larger inhibition of an SOS-independent step. These data support error-prone DSB models for point mutation in which RecG inhibits a mutation intermediate directly (e.g., Fig. 1), and also inhibits SOS induction somewhat.

One model for how RecG inhibits point mutation, but promotes it only during LexA-regulon constitutive overexpression, is that RecG competes with the branch migration activity of RuvAB and branch-migrates Holliday junctions in the opposite polarity [37,38,73]. In Fig. 1A, RuvAB moves the junction leftward and RecG rightward. When RuvAB levels are low (normally), RecG rightward movement would inhibit mutation by unwinding/destabilizing the intermediate that primes the DNA synthesis. When RuvAB levels are higher than in a normal, induced SOS response, as in the SOS/LexA-constitutive strain, RuvAB could inhibit mutation by excessive leftwards migration of the junction, which might impede progression of the replication bubble rightward (because of supercoiling constraints). RecG might then facilitate mutation by branch migration rightward, allowing the replication bubble to progress.

Regardless of the specific model, the fact that one of the ways that RecG inhibits point mutation is independent of action on SOS induction, and that six other DSBR proteins play roles in mutation other than via inducing SOS (above), supports the idea that their roles in homologous recombinational DSBR promote stress-induced point mutation.

4. Further Discussion

We have shown that seven different DNA metabolism proteins that have in common their known roles in DSBR and homologous recombination all affect stress-induced point mutation in the *E. coli* Lac system independently of effects on SOS induction. The proteins are RecA, RecBCD, RuvA, RuvB, RuvC, RecF, and RecG. Whereas any one of these might have an unknown third function that might affect point mutation, in addition to recombination and (known or possible) SOS modulation, it is unlikely that all seven do. This supports error-prone DSBR models for point mutation [e.g., Fig. 1A, Ref. 36, and several subsequent papers]. A recent report also shows directly that DSBs made in cells are mutagenic in stationary phase via a switch to error-prone homologous recombinational repair in stationary phase, and that these induced DSBs promotes Lac⁺ stress-induced point mutation 6000-fold [35]. The results presented here clarify the roles of each of several DSBR and other proteins in stress-induced mutagenesis, showing that all tested act independently of SOS, and that TraI and RecA also act via promoting SOS, as apparently does RecG.

We note that although mutagenic-DSBR models had seemed to be the only possibility when RecBC-dependence of stress-induced Lac⁺ mutation was discovered [36], and for the next few years [37,38], this was because it had been thought initially that induction of genes of the SOS regulon (other than RecA) was not required for point mutation [27]. Later work showed that, contrary to that early report, SOS induction [39] and the SOS-controlled DinB error-prone DNA polymerase [42] were required for stress-induced mutagenesis. This made possible, and even likely, indirect models in which all of the DSBR proteins function solely in steps leading to SOS induction, DinB overproduction, and consequent generalized mutagenesis [Ref. 6, and Fig. 1B]. These models are ruled out by the data presented here, and see [35].

4.1 Regulation of point mutagenesis by SOS and RpoS stress responses restricts mutagenesis in time

In the Lac system, the stress-induced mutations are specific to stationary phase, and are regulated positively by at least two stress responses: the SOS DNA damage response [27,39]; and the RpoS starvation- and general-stress response [34], both of which upregulate the error-prone DNA polymerase, DinB [43,55,56]. Clearly these mutations are stress-induced, and stress-induction is conferred by coupling mutagenesis to known stress responses. Thus mutagenesis is limited in time. One does not know whether stress-induced mutagenesis by this or other mutation mechanisms evolved by selection for mutagenesis per se, or whether increased mutation is an unavoidable byproduct of some other stress-induced process. However, it is possible that increasing mutation rate during stress does confer a selective advantage [14]. Although most mutations are deleterious, failure to change genetically when poorly adapted to an environment could be fatal. With large enough populations of cells, promotion of rare adaptive mutations could be advantageous for a cell clone, even though many members of the clone would be harmed by increasing mutation rate. Coupling to stress-responses restricts the risky experiment of heightened mutagenesis to desperate times. Control of mutation by known stress responses is common to many different stress-induced mutation mechanisms [14,34,74–78].

4.2 Mechanism and regulation: DSBR restricts mutagenesis to localized genomic regions

Coupling of mutagenesis to DSBR (Fig. 1A) could also restrict mutagenesis even further, to small genome segments in any given cell: those segments in which DSBs are being repaired [35]. If the DSBs provoking mutagenesis were spontaneous, they might be relatively infrequent per cell and perhaps randomly distributed (in the genome) in populations. If so, all genomic regions might be mutated in a population [as appears to be the case, 10,11,12], but only small segments in any given cell would undergo mutation (Fig. 1A, bottom). Localizing mutagenesis in the genome (Fig. 1A, bottom) could have two important evolutionary consequences: reducing deleterious mutations in rare adaptive mutant cells; and facilitating concerted evolution of genes. For example, rare adaptive mutants could be crippled by deleterious mutations formed simultaneously and inherited in the same cell as the adaptive mutation. In the Lac system, increased mutation is induced throughout the genome [13], and chromosomal mutations (unlinked to *lac*) occur detectably in Lac⁺ cells [10–12]. If coupling of mutation to DSBR localizes mutagenesis to one or a few genomic regions per cell (Fig. 1A), this could reduce deleterious coincident mutations [35].

Similarly, a possible advantage of stress-induced mutagenesis could be the ability to alter functions of gene products (RNAs and proteins). Even in the Lac system, in which frameshifting deletions are assayed, which inactivate or reactivate genes, this could occur because the DinB DNA polymerase also makes base substitutions [56,79,80]. Evolution of new functions of RNAs, proteins and protein machines, often requires more than one mutation in a relatively small genomic area (a gene or gene cluster) [e.g., 81]. Localization of increased mutagenesis to small genomic regions might accomplish this. Thus, coupling of stress-induced mutagenesis to DSBR may confer at least two selective advantages [35].

4.3 Coupling mutagenesis to DSBR/recombination appears to have evolved more than once

A stress-induced mutation mechanism studied in budding yeast generates adaptive reversions of a *lys* frameshift gene during starvation for lysine, and does so using proteins required for non-homologous end-joining (NHEJ), one of two DSBR mechanisms operative in yeast [44]. NHEJ is a fundamentally different mechanism of DSBR from the homologous recombinational repair discussed here. This implies that this yeast stress-induced mutation mechanism evolved independently of the *E. coli* mechanism studied here. How the yeast NHEJ proteins promote mutagenesis is not yet known, but the convergence of the DSBR-protein dependence of this stress-induced mutation mechanism argues for independent evolution of DSBR-coupled mutagenesis.

Another DSBR-coupled mutation mechanism occurs in yeast. DSBR via homologous recombination targets mutations to the repaired region [82]. Though possibly related to the *E. coli* mechanism, the error-prone DNA polymerase used is not a member of the DinB superfamily [83]. Though not known to be “stress-induced”, this coupling to DSBR might imply stress-responsiveness in that damage responses to DSBs can be considered stress responses. Association of mutagenesis with homologous recombination was noted also previously in yeast [84,85] and in *Salmonella* [86]. Finally, several other bacterial mutation mechanisms are RecA-dependent, and not all use RecA in its capacity for SOS induction [14,26,87]. Some of these could represent examples of coupling of recombinational DNA repair to mutation.

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Fig. 1.

Models for the mechanism of stress-induced point mutation: possible roles of DSBR proteins. (A) Error-prone-DSBR model [modified from Ref. 36]. Mutation is proposed to result from error-prone synthesis during DSBR by homologous recombination. DSBs or ends (DSEs) could result from any of many mechanisms, spontaneously and otherwise [6]. The DSE is processed by RecBCD to reveal single-stranded (ss)DNA used by RecA for strand exchange with homologous DNA. The strand-exchange recombination intermediate is proposed to prime synthesis (dashed lines) and mutations could result from errors (X) made by DinB error-prone DNA polymerase, which is upregulated both by the SOS response and RpoS stationary-phase and general-stress response. Elsewhere, we suggest that DSBs form spontaneously but that their repair switches from high-fidelity to error-prone as part of the RpoS stress response [35]. Error-prone DSBR could lead to localized mutations (Xs) in genomes (discussed in Ref. [35]). (B) Indirect model: DSBR proteins promote SOS induction [modified from Ref. 6]. SOS induction results from accumulation of ssDNA, which is coated with RecA protein, activating RecA as a co-protease (RecA*) that facilitates the autoproteolytic cleavage of the LexA transcriptional repressor, upregulating >40 DNA-damage-response genes [51,88]. In this model, a replication fork stalls and is regressed to form a four-stranded Holliday-junction-like structure (a “chicken foot”). RuvABC could bind and cleave the structure producing DSEs, which RecBCD processes into ssDNA. RecA loads, becomes activated RecA*, inducing SOS and DinB up-regulation leading to DinB-generated errors and so mutations anywhere that new DNA is synthesized. This would disperse mutations (Xs) throughout each cell’s genome [35]. This model illustrates that all

of the DSB α R proteins could be required for point mutation even if DSB α R itself were not. Their sole role could be promoting SOS induction.

Fig. 2. Amounts and kinds of stress-induced mutations are similar in SOS/LexA-constitutive and Lex⁺ cells. (A) DinB is required in both Lex⁺ and SOS/LexA-constitutive cells. Total Lac⁺ colonies (point mutant and *lac*-amplified) are shown. Lex⁺, SMR4562 (◆); *lexA*(Def), SMR5400 (■); *dinB*, SMR5830 (◇); *lexA*(Def) *dinB*, SMR7627 (□) (Table 1). (B) Similar stress-induced point mutation and amplification in Lex⁺ and SOS/LexA-constitutive strains. Point mutants (—); *lac*-amplified colonies (---), distinguished by colony morphology as per [19]. (C, D) Sequences of Lac⁺ adaptive point mutations in the Lex⁺ and SOS/LexA-constitutive strains are mostly –1 deletions at mononucleotide repeats: x, Lex⁺ [31,32]; ●, SOS/LexA constitutive. Base position numbers [32] indicate the base above the right end of the number. (D) Sequence summary. The four “other” mutations (not –1 deletions) in *lexA*(Def) are tandem duplications: of eleven bp (1029–1039); eleven bp (1044–1054); 86bp (1063–1148); and a 55-bp duplication (1081–1135) containing base substitutions at positions 1131 (A to T), 1137 (G to A), and 1145 (G to C). All four of these mutants grow more slowly than normal stress-induced point mutants, taking longer to form visible colonies under exact reconstructions of the selection conditions (data not shown). They are, therefore, likely to be slowly-growing, generation-dependent mutants that were present before lactose selection. They could be more prevalent in the SOS/LexA-constitutive background either because generation-dependent mutation might be altered/enhanced in these cells, or because stress-induced mutation is reduced slightly [Ref. 39, and Tables 2, 3] allowing more generation-dependent mutants in the total on later days.

Fig. 3.

Representative examples of stress-induced mutation data. See Tables 2 and 3 for the mutation rates (per day) and their quantification from multiple experiments. In all graphs: Lex⁺, SMR4562 (◆); *lexA*(Def), SMR5400 (■). (A) RuvABC proteins are required in Lex⁺ cells [37,38]. *ruvA*, SMR1549 (●); *ruvB*, SMR1552 (▲); *ruvC*, SMR789 (—x—). (B) RuvABC proteins are also required in SOS/LexA-constitutive cells. *lexA*(Def) *ruvA*, SMR8542 (○); *lexA*(Def) *ruvB*, SMR8543 (Δ); *lexA*(Def) *ruvC*, SMR8544 (—x—). (C) Two independent constructs of *lexA*(Def) *recA* show decreased stress-induced mutation, though less than in Lex⁺. *recA*, SMR624 (▲); *lexA*(Def) *recA* isolate 1, SMR6488 (—Δ—), isolate 2, SMR8397 (—Δ—). (D) Two independent constructs of *lexA*(Def) *traI* show decreased stress-induced mutation, though less than in Lex⁺. *traI*, SMR6002 (●); *lexA*(Def) *traI* isolate 1, SMR7731 (—○—), isolate 2, SMR8401 (—○—). (E) RecF is required for stress-induced mutation in SOS/LexA-constitutive cells. Two alleles of *recF* show the same level of decrease. *lexA*(Def) Δ*recF*:Kan, SMR8132 (●); *lexA*(Def) *recF*332:Tn3, SMR5711 (▲). (F) Loss of RecD increases stress-induced mutation in SOS/LexA-constitutive cells. *lexA*(Def) *recD*, SMR7727. (G) Loss of RecD in SOS-uninducible cells increases stress-induced mutation. *lexA*(Ind⁻), SMR701; *lexA*(Ind⁻) *recD*, SMR8660. (H) Loss of RecG in Lex⁺ cells increases stress-induced mutants. *recG*, SMR1982 (◇). (I) Loss of RecG in SOS/LexA-constitutive cells decreases stress-induced mutants. *lexA*(Def) *recG*, SMR8129 (○); *lexA*(Def) *recG*⁺ rescue strain (*recG*⁺ transduced into SMR8129) SMR8406 (□). (J) Loss of RecG in SOS-uninducible cells increases in stress-induced mutants. *lexA*(Ind⁻), SMR701 (▲); *lexA*(Ind⁻) *recG*, SMR8546 (▽).

Table 1

Escherichia coli K-12 strains

Strain	Relevant genotype	Reference or source
FC29	$\Delta(lac-proB)_{XIII} ara thi [F' \Delta(lacI-lacZ)]$	[27]
FC40	$\Delta(lac-proB)_{XIII} ara thi Rif^R [F' lacI33\Omega lacZ proAB^+]$	[27]
FS2055	<i>malB::Tn9</i>	FW Stahl (U Oregon)
GY8322	$\Delta(srlR-recA)306::Tn10 [mini-F recA^+]$	S Sommers (Gif sur Yvette)
RDK2655	<i>recG258::Tn10miniKan</i>	R Kolodner (San Diego)
SMR533	FC40 <i>malB::Tn9</i>	FC40 $\times$ P1(FS2055)
SMR624	FC40 $\Delta(srlR-recA)306::Tn10$	[36]
SMR686	FC40 <i>recF332::Tn3</i>	[39]
SMR692	FC40 <i>recD600I::Tet::Kan</i>	[36]
SMR701	FC40 <i>lexA3(Ind⁻)</i>	[39]
SMR789	FC40 <i>ruvC53 eda-5I::Tn10</i>	[38]
SMR868	FC40 <i>lexA3(Ind⁻)</i>	[39]
SMR1549	FC40 <i>ruvA200 eda-5I::Tn10</i>	[38]
SMR1552	FC40 <i>ruvB9 zea-3::Tn10</i>	[38]
SMR1982	FC40 <i>recG258::Tn10miniKan</i>	[38]
SMR4562	$\Delta(lac-proB)_{XIII} ara thi Rif^R [F' lacI33\Omega lacZ proAB^+]$	Independent construction of FC40 [39]
SMR4582	FC40 <i>lexA3(Ind⁻) recG258::Tn10miniKan</i>	SMR868 $\times$ P1(RDK2655)
SMR5400	SMR4562 <i>lexA5I(Def) sulA211 psiB::cat</i>	[39]
SMR5711	SMR5400 <i>recF332::Tn3</i>	SMR5400 $\times$ P1(SMR686)
SMR5830	SMR4562 <i>dinB10 [F' dinB10]</i>	[42]
SMR6002	SMR4562 $\Delta traI::dhfr$	RG Ponder, NC Fonville, SM Rosenberg (unpublished)
SMR6488	SMR5400 $\Delta(srlR-recA)306::Tn10$	SMR5400 $\times$ P1(GY8322)
SMR6575	SMR5400 <i>ruvC53 eda-5I::Tn10</i>	SMR5400 $\times$ P1(SMR789)
SMR7627	SMR5400 <i>dinB10 [F' dinB10]</i>	This work ^a
SMR7727	SMR5400 <i>recD600I::mini-Tet::Kan</i>	SMR5400 $\times$ P1(SMR692)
SMR7731	SMR5400 <i>traI::dhfr</i>	SMR5400 $\times$ P1(SMR6002)
SMR8097	SMR4562 $\Delta recF1804::FRT::Kan::FRT$	This work
SMR8129	SMR5400 <i>recG258::Tn10miniKan</i>	SMR5400 $\times$ P1(SMR1982)
SMR8132	SMR5400 $\Delta recF1804::FRT::Kan::FRT$	SMR5400 $\times$ P1(SMR8097)
SMR8134	MG1655 <i>zeb-2233::FRT::ble::FRT</i>	This work
SMR8142	SMR5400 $\Delta recF1805::FRT$	SMR8132 without Kan
SMR8160	SMR4562 $\Delta recF1805::FRT$	SMR8097 without Kan
SMR8397	SMR5400 $\Delta(srlR-recA)306::Tn10$	SMR5400 $\times$ P1(GY8322)
SMR8401	SMR5400 <i>traI::dhfr</i>	SMR5400 $\times$ P1(SMR6002)
SMR8403	SMR5400 <i>recD600I::mini-Tet::Kan</i>	SMR5400 $\times$ P1(SMR692)
SMR8406	SMR5400 <i>zib-636::Tn10 (recG⁺ rescue strain)</i>	This work ^b
SMR8542	SMR5400 <i>ruvA200 eda-5I::Tn10</i>	SMR5400 $\times$ P1(SMR1549)
SMR8543	SMR5400 <i>ruvB9 zea-3::Tn10</i>	SMR5400 $\times$ P1(SMR1552)

Strain	Relevant genotype	Reference or source
SMR8544	SMR5400 <i>ruvC53 eda-51::Tn10</i>	SMR5400 × P1(SMR789)
SMR8545	FC40 <i>lexA3(Ind⁻) recG258::Tn10</i> miniKan	SMR1982 × P1(SMR533) × P1(SMR701)
SMR8546	FC40 <i>lexA3(Ind⁻) recG258::Tn10</i> miniKan	SMR1982 × P1(SMR533) × P1(SMR701)
SMR8654	SMR5400 <i>zeb-2233::FRT::ble::FRT (ruvA⁺ rescue strain)</i>	SMR8542 × P1(SMR8134)
SMR8656	SMR5400 <i>zeb-2233::FRT::ble::FRT (ruvB⁺ rescue strain)</i>	SMR8543 × P1(SMR8134)
SMR8659	FC40 <i>lexA3(Ind⁻) recD6001::mini-Tet::Kan</i>	SMR701 × P1(SMR692)
SMR8660	FC40 <i>lexA3(Ind⁻) recD6001::mini-Tet::Kan</i>	SMR701 × P1(SMR692)
SMR8665	SMR5400 <i>zeb-2233::FRT::ble::FRT</i>	SMR5400 × P1(SMR8134)
SMR8667	SMR5400 <i>zeb-2233::FRT::ble::FRT</i>	SMR5400 × P1(SMR8134)

^aConstruction made by P1 transduction with *sulA211*, *lexA51(Def)*, and *psiB::cat* alleles derived from DE274 (R. Woodgate, NIH), DE192 (D. Ennis, U. Southwestern Louisiana), and MC1061 (A. Bailone, Gif sur Yvette) strains, respectively.

^bConstruction made by P1 transduction with *recG⁺* linked to *zib-636::Tn10* marker derived from RM4714 (R. Maurer).

Table 2

Stress-induced-mutation rates from multiple experiments

Expt # ^a	Temp ^b	Mutation rates of relevant genotypes compared (mutants per day) ^c		
		Mutant	Isogenic Control	Difference in mutation rate from control ^d
		<i>lexA</i> (Def)	Lex ⁺	
1	37 °C	31	25	1.2
2	37 °C	43	89	0.48
3	37 °C	19	19	0.99
4	37 °C	2.8	16	0.17
5	37 °C	13	38	0.35
6	37 °C	23	28	0.82
7	37 °C	24	36	0.67
8	37 °C	35	23	1.5
9	37 °C	34	29	1.2
10	37 °C	25	29	0.85
11	37 °C	15	19	0.80
12	37 °C	25	31	0.79
13	37 °C	39	60	0.66
14	37 °C	22	52	0.43
15	37 °C	15	34	0.45
16	37 °C	11	28	0.38
		<i>lexA</i> (Def)	Lex ⁺	
17	32° C	40	48	0.85
18	32° C	9.2	37	0.25
19	32° C	16	34	0.47
20	32° C	13	19	0.65
21	32° C	40	28	1.4
22	32° C	14	82	0.17
23	32° C	22	33	0.68
24	32° C	14	56	0.25
25	32° C	15	42	0.37
26	32° C	20	38	0.53
27	32° C	19	63	0.30
28	32° C	16	37	0.43
29	32° C	19	110	0.17
30	32° C	19	110	0.18
		<i>ruvA</i>	Lex ⁺	
18	32° C	2.3	37	0.063
19	32° C	8.3	34	0.25

Expt # ^a	Temp ^b	Mutation rates of relevant genotypes compared (mutants per day) ^c		
		Mutant	Isogenic Control	Difference in mutation rate from control ^d
20	32° C	0.92	19	0.048
26	32° C	4.2	38	0.11
27	32° C	1.6	63	0.026
		<i>ruvB</i>	Lex ⁺	
18	32° C	2.0	37	0.055
19	32° C	6.8	34	0.20
20	32° C	1.3	19	0.066
26	32° C	4.2	38	0.11
27	32° C	1.9	63	0.031
		<i>ruvC</i>	Lex ⁺	
17	32° C	2.1	48	0.045
18	32° C	1.3	37	0.034
19	32° C	4.4	34	0.13
20	32° C	0.94	19	0.049
26	32° C	0.83	38	0.022
27	32° C	0.96	63	0.015
		<i>recA</i>	Lex ⁺	
3	37 °C	0.25	19	0.013
5	37 °C	0.41	38	0.011
6	37 °C	0.99	28	0.035
7	37 °C	0.41	36	0.011
14	37 °C	0.59	52	0.011
15	37 °C	0.46	34	0.013
31	37 °C	0.44	56	0.0078
		<i>traI</i>	Lex ⁺	
5	37 °C	0.38	38	0.0099
6	37 °C	0.51	28	0.018
7	37 °C	0.44	36	0.012
14	37 °C	0.53	52	0.010
15	37 °C	0.40	34	0.012
		<i>recF::Tn3</i>	Lex ⁺	
5	37 °C	4.94	38	0.13
6	37 °C	10	28	0.37
7	37 °C	13	36	0.36
9	37 °C	7.0	29	0.24

Expt # ^a	Temp ^b	Mutation rates of relevant genotypes compared (mutants per day) ^c		
		Mutant	Isogenic Control	Difference in mutation rate from control ^d
10	37 °C	5.2	29	0.18
12	37 °C	13	31	0.43
32	37 °C	3.8	13	0.29
		<i>ΔrecF::Kan</i>	Lex ⁺	
9	37 °C	13	29	0.46
10	37 °C	8.8	29	0.30
12	37 °C	10	31	0.34
14	37 °C	9.8	52	0.19
		<i>ΔrecF</i>	Lex ⁺	
9	37 °C	12	29	0.40
10	37 °C	13	29	0.45
12	37 °C	13	31	0.41
		<i>recG</i>	Lex ⁺	
21	32° C	200	28	7.0
22	32° C	680	82	8.2
23	32° C	940	33	29
24	32° C	450	56	8.0
25	32° C	590	42	14
26	32° C	1010	38	27
28	32° C	760	37	21
33	32° C	740	13	56
34	32° C	620	20	31
35	32° C	520	24	22
36	32° C	1650	76	22
37	32° C	1450	77	19
		<i>lexA(Def) ruvA</i>	<i>lexA(Def)</i>	
27	32° C	1.9	19	0.10
29	32° C	9.2	19	0.48
30	32° C	7.1	19	0.37
38	32° C	1.5	17	0.085
		<i>lexA(Def) ruvA⁺ rescue</i>	<i>lexA(Def)</i>	
39	37 °C	12	19	0.61
40	37 °C	66	90	0.73
41	37 °C	66	57	1.16
		<i>lexA(Def) ruvB</i>	<i>lexA(Def)</i>	

Expt # ^a	Temp ^b	Mutation rates of relevant genotypes compared (mutants per day) ^c		
		Mutant	Isogenic Control	Difference in mutation rate from control ^d
27	32° C	2.5	19	0.13
29	32° C	3.6	19	0.19
30	32° C	5.6	19	0.29
38	32° C	5.3	17	0.31
		<i>lexA(Def) ruvB⁺ rescue</i>	<i>lexA(Def)</i>	
39	37 °C	7.7	19	0.40
40	37 °C	55	90	0.61
41	37 °C	48	57	0.85
		<i>lexA(Def) ruvC(2)</i>	<i>lexA(Def)</i>	
17	32° C	3.9	40	0.097
19	32° C	17	16	1.1
26	32° C	2.1	20	0.11
27	32° C	4.3	19	0.23
27	32° C	5.8	19	0.31
		<i>lexA(Def) recA(2)</i>	<i>lexA(Def)</i>	
1	37 °C	4.0	31	0.13
2	37 °C	3.0	43	0.070
3	37 °C	1.5	19	0.080
5	37 °C	2.3	13	0.17
6	37 °C	7.3	23	0.32
7	37 °C	4.3	24	0.17
14	37 °C	2.7	22	0.12
15	37 °C	1.4	15	0.090
14	37 °C	2.6	22	0.12
15	37 °C	1.6	15	0.10
		<i>lexA(Def) traI(2)</i>	<i>lexA(Def)</i>	
3	37 °C	5.1	19	0.27
5	37 °C	2.1	13	0.15
6	37 °C	3.9	23	0.17
7	37 °C	3.3	24	0.14
14	37 °C	2.7	22	0.12
15	37 °C	2.0	15	0.13
14	37 °C	2.9	22	0.13
15	37 °C	2.5	15	0.16
		<i>lexA(Def) recF::Tn3</i>	<i>lexA(Def)</i>	
3	37 °C	8.8	19	0.45

Expt # ^a	Temp ^b	Mutation rates of relevant genotypes compared (mutants per day) ^c		
		Mutant	Isogenic Control	Difference in mutation rate from control ^d
5	37 °C	5.0	13	0.37
6	37 °C	10	23	0.45
7	37 °C	13	24	0.53
9	37 °C	7.0	34	0.20
10	37 °C	5.2	25	0.21
12	37 °C	13	24	0.55
		<i>lexA(Def) ΔrecF::Kan</i>	<i>lexA(Def)</i>	
9	37 °C	13	34	0.39
10	37 °C	8.8	25	0.35
12	37 °C	10	24	0.43
14	37 °C	9.7	22	0.43
16	37 °C	8.5	11	0.79
42	37 °C	17	32	0.53
43	37 °C	14	25	0.58
		<i>lexA(Def) ΔrecF</i>	<i>lexA(Def)</i>	
9	37 °C	12	34	0.34
10	37 °C	13	25	0.53
12	37 °C	13	25	0.51
		<i>lexA(Def) recD (2)</i>	<i>lexA(Def)</i>	
3	37 °C	53	19	2.7
4	37 °C	7.4	2.8	2.7
6	37 °C	37	23	1.6
7	37 °C	61	24	2.5
8	37 °C	32	35	0.92
11	37 °C	42	15	2.8
14	37 °C	44	22	2.0
15	37 °C	44	15	2.8
14	37 °C	39	22	1.8
15	37 °C	59	15	3.8
		<i>lexA(Def) recG (2)</i>	<i>lexA(Def)</i>	
22	32 °C	1.9	14	0.14
23	32 °C	1.8	22	0.078
24	32 °C	7.0	14	0.50
25	32 °C	1.7	15	0.11
26	32 °C	1.6	20	0.078
27	32 °C	4.1	19	0.22
26	32 °C	1.4	20	0.067

Expt # ^a	Temp ^b	Mutation rates of relevant genotypes compared (mutants per day) ^c		
		Mutant	Isogenic Control	Difference in mutation rate from control ^d
		<i>lexA(Def) recG⁺ rescue</i>	<i>lexA(Def)</i>	
24	32 °C	13	14	0.91
25	32 °C	10	15	0.65
27	32 °C	17	19	0.91
		<i>lexA(Ind⁻)</i>	Lex ⁺	
16	37 °C	8.9	28	0.31
33	37 °C	6.7	13	0.51
34	37 °C	4.7	20	0.23
35	37 °C	9.3	24	0.38
44	37 °C	7.7	33	0.24
45	37 °C	13	51	0.24
46	37 °C	10	52	0.20
47	37 °C	5.4	19	0.29
		<i>lexA(Ind⁻)</i>	Lex ⁺	
28	32 °C	10	37	0.28
36	32 °C	8.4	76	0.11
37	32 °C	8.9	77	0.12
		<i>lexA(Ind⁻) recD(2)</i>	<i>lexA(Ind⁻)</i>	
44	37 °C	48	7.7	6.2
44	37 °C	38	7.7	4.9
45	37 °C	22	13	1.7
45	37 °C	20	13	1.6
		<i>lexA(Ind⁻) recG(3)</i>	<i>lexA(Ind⁻)</i>	
36	32 °C	88	8.4	10
36	32 °C	46	8.4	5.4
36	32 °C	63	8.4	7.5
37	32 °C	110	8.9	12
37	32 °C	112	8.9	13

^aExperiment number of each experiment done. Where the same number appears more than once for a genotype, these are for independent isolates of the same genotype.

^bThe temperature at which each of the experiments were done. See Materials and Methods.

^cStress-induced mutation rates (mutants per day) are the change in colonies from day 4 to 5 [34]. Table 1 for full strain genotypes and information. “*lexA(Def)*” strains also carry *sulA* and *psiB* mutations (Results and Discussion).

^dFold effects are the mutation rate of the mutant strain divided by that of its isogenic control in the same experiment. The means ± SEMs for these are shown in Table 3.

Table 3
Quantification of stress-induced-mutation phenotypes from multiple experiments

Relevant genotypes compared ^a		Temp. ^c	Number of Expts. ^d	Mean difference in mutation rate from control $\pm$ SEM ^e	
Mutant (number of independent isolates) ^b	Isogenic Control				
<i>lexA</i> (Def)	Lex ⁺	37 °C	16	0.7 $\pm$ 0.1	<i>P</i> = 0.065
<i>lexA</i> (Def)	Lex ⁺	32 °C	14	0.5 $\pm$ 0.1	<i>P</i> < 0.001
<i>ruvA</i>	Lex ⁺	32 °C	5	0.1 $\pm$ 0.04	<i>P</i> = 0.009
<i>ruvB</i>	Lex ⁺	32 °C	5	0.09 $\pm$ 0.03	<i>P</i> = 0.009
<i>ruvC</i>	Lex ⁺	32 °C	6	0.05 $\pm$ 0.02	<i>P</i> = 0.004
<i>recA</i>	Lex ⁺	37 °C	7	0.01 $\pm$ 0.003	<i>P</i> = 0.002
<i>trfA</i>	Lex ⁺	37 °C	5	0.01 $\pm$ 0.001	<i>P</i> = 0.009
<i>recF::Tn3</i>	Lex ⁺	37 °C	7	0.3 $\pm$ 0.04	<i>P</i> = 0.003
Δ <i>recF::Kan</i>	Lex ⁺	37 °C	4	0.3 $\pm$ 0.06	<i>P</i> = 0.021
Δ <i>recF</i>	Lex ⁺	37 °C	3	0.4 $\pm$ 0.02	<i>P</i> = 0.050
<i>recG</i>	Lex ⁺	32 °C	12	22 $\pm$ 4	<i>P</i> < 0.001
<i>lexA</i> (Def) <i>ruvA</i>	<i>lexA</i> (Def)	32 °C	4	0.3 $\pm$ 0.1	<i>P</i> = 0.021
<i>lexA</i> (Def) <i>ruvA</i> ⁺ rescue	<i>lexA</i> (Def)	37 °C	3	0.8 $\pm$ 0.2	<i>P</i> = 0.827
<i>lexA</i> (Def) <i>ruvB</i>	<i>lexA</i> (Def)	32 °C	4	0.2 $\pm$ 0.04	<i>P</i> = 0.021
<i>lexA</i> (Def) <i>ruvB</i> ⁺ rescue	<i>lexA</i> (Def)	37 °C	3	0.6 $\pm$ 0.1	<i>P</i> = 0.275
<i>lexA</i> (Def) <i>ruvC</i> (2)	<i>lexA</i> (Def)	32 °C	5	0.4 $\pm$ 0.2	<i>P</i> = 0.016
<i>lexA</i> (Def) <i>recA</i> (2)	<i>lexA</i> (Def)	37 °C	10	0.1 $\pm$ 0.02	<i>P</i> < 0.001
<i>lexA</i> (Def) <i>trfA</i> (2)	<i>lexA</i> (Def)	37 °C	8	0.2 $\pm$ 0.02	<i>P</i> = 0.001
<i>lexA</i> (Def) <i>recF::Tn3</i>	<i>lexA</i> (Def)	37 °C	7	0.4 $\pm$ 0.05	<i>P</i> = 0.003
<i>lexA</i> (Def) Δ <i>recF::Kan</i>	<i>lexA</i> (Def)	37 °C	7	0.5 $\pm$ 0.06	<i>P</i> = 0.006
<i>lexA</i> (Def) Δ <i>recF</i>	<i>lexA</i> (Def)	37 °C	3	0.5 $\pm$ 0.06	<i>P</i> = 0.050
<i>lexA</i> (Def) <i>recD</i> (2)	<i>lexA</i> (Def)	37 °C	10	2 $\pm$ 0.3 ^f	<i>P</i> = 0.002
<i>lexA</i> (Def) <i>recG</i> (2)	<i>lexA</i> (Def)	32 °C	7	0.2 $\pm$ 0.06	<i>P</i> = 0.002
<i>lexA</i> (Def) <i>recG</i> rescue	<i>lexA</i> (Def)	32 °C	3	0.8 $\pm$ 0.09	<i>P</i> = 0.275
<i>lexA</i> (Ind ⁻)	Lex ⁺	37 °C	8	0.3 $\pm$ 0.04	<i>P</i> = 0.001
<i>lexA</i> (Ind ⁻)	Lex ⁺	32 °C	3	0.2 $\pm$ 0.06	<i>P</i> = 0.050

Relevant genotypes compared ^a		Temp. ^c	Number of Expts. ^d	Mean difference in mutation rate from control $\pm$ SEM ^e	
Mutant (number of independent isolates) ^b		Isogenic Control			
<i>lexA</i> (Ind ⁻) <i>recD</i> (2)		37 °C	4	4 $\pm$ 1 ^f	<i>P</i> = 0.019
<i>lexA</i> (Ind ⁻) <i>recG</i> (3)		32 °C	5	10 $\pm$ 1	<i>P</i> = 0.008

^aTable 1 for full strain genotypes and information. “*lexA*(Def)” strains also carry *suIA* and *psiB* mutations (Results and Discussion).

^bThe number of independent isolates of each genotype tested, when >1. For select strains, phenotypes were confirmed as not having been caused by additional mutations in the strains’ genomes (perhaps selected inadvertently as growth-defect-suppressing mutations) by reintroduction of the wild-type gene by transduction. These are designated “rescue”.

^cTemperature at which cultures were grown prior to plating at 37 °C (Materials and Methods).

^dEach independent experiment is one such as those shown in each of Fig. 3A–J.

^eStress-induced mutation rates (mutants per day) are the change in colonies from day 4 to 5 [34]. The mean difference in rate between each mutant and its isogenic control was obtained by pair-wise comparison of the rates from mutant vs. respective control from experiments run in parallel (Table 2). *P* values were obtained by nonparametric Mann-Whitney Rank Sum test using the SYSTAT 11 statistics software by SYSTAT Software, Inc.

^fThe ratio of *lexA*(Def) *recD* and *lexA*(Ind⁻) *recD* to their respective controls is not different, *P* = 0.671