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Thymineless death is inhibited by CsrA in *Escherichia coli* lacking the SOS response

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Abstract

Thymineless death (TLD) is the rapid loss of colony-forming ability in bacterial, yeast and human cells starved for thymine, and is the mechanism of action of common chemotherapeutic drugs. In *Escherichia coli*, significant loss of viability during TLD requires the SOS replication-stress/ DNA-damage response, specifically its role in inducing the inhibitor of cell division, SulA. An independent RecQ- and RecJ-dependent TLD pathway accounts for a similarly large additional component of TLD, and a third SOS- and RecQ/J-independent TLD pathway has also been observed. Although two groups have implicated the SOS-response in TLD, an SOS-deficient mutant strain from an earlier study was found to be sensitive to thymine deprivation. We performed whole-genome resequencing on that SOS-deficient strain and find that, compared with the SOS-proficient control strain, it contains five mutations in addition to the SOS-blocking *lexA*(Ind[−]) mutation. One of the additional mutations, *csrA*, confers TLD sensitivity specifically in SOS-defective strains. We find that CsrA, a carbon storage regulator, reduces TLD in SOS- or SulA-defective cells, and that the increased TLD that occurs in *csrA*[−] SOS-defective cells is dependent on RecQ. We consider a hypothesis in which the modulation of nucleotide pools by CsrA might inhibit TLD specifically in SOS-deficient (SulA-deficient) cells.

Keywords

csrA; DNA damage response; DNA repair; RecQ; SOS response; thymineless death

1. Introduction

Thymineless death (TLD) is the rapid loss of colony-forming ability of cells deprived of thymine. TLD occurs in *Escherichia coli*, yeast, and human cells [1] and is the mechanism of action of commonly used cancer chemotherapeutic drugs such as 5-fluorouracil and methotrexate [2, 3]. Understanding the mechanism(s) of TLD and the identities of proteins that promote and protect against TLD is important to both fundamental aspects of DNA

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Conflict of interest

There are no conflicts of interest.

metabolism and repair, and to cancer treatment strategies. Past and recent work identifying bacterial mutants with diminished or enhanced TLD suggests that human tumors with mutations in homologous and analogous genes may respond differently to TLD-inducing therapies, implying that finding these genes may allow personalization of treatment strategies. Although several DNA repair proteins were shown early on to be required for TLD, and special DNA structures were noted during TLD, the molecular mechanisms of TLD remained largely obscure until recently.

Cohen and Barner first described thymineless death as lethal “unbalanced” growth caused by thymine deprivation [4]. Inhibition of transcription or translation inhibits TLD, indicating that new protein synthesis and/or DNA replication, which requires new protein synthesis at each initiation, is required for TLD [5]. At the protein level, several “Rec” proteins, which function in DNA repair by homologous recombination (HR) and other aspects of DNA metabolism, promote thymineless death. These include RecA [6, 7], RecF [6–8], RecO [9], RecJ [9], and RecQ, the last of which was discovered in a screen for mutants resistant to TLD [10]. Conversely RecBCD prevents TLD [7, 8, 11]. At the DNA level, during thymine starvation Nakayama and Hanawalt observed DNA breaks that could be repaired after thymine addition [12]. In cells starved for thymine, Nakayama *et al.* observed special DNA structures, referred to as non-migrating DNA, including X-structures and Y-structures, dependent on presynaptic HR proteins RecA, RecF, RecJ, RecO, and RecQ, leading the authors to hypothesize that the non-migrating DNA might be HR intermediates [13]. In addition to DNA breaks, DNA degradation occurs during TLD [6, 14], and is most severe near the origin of replication [6, 14], then later occurs at the replication terminus [6]. The origin-specific degradation requires RecF, RecJ, RecO [14], RecA and RecBCD [15], and shows a slight reduction in mutant lacking RecQ [14], suggesting that either recombination or the SOS DNA-damage/replication-stress response, or both, promote origin-specific DNA degradation. (All of these proteins but RecQ promote SOS induction with some kinds of DNA damage.) Whereas the RecA, RecF, RecJ, and RecO promotion of both death [6–9] and origin-specific DNA degradation [14, 15] appear congruent (*i.e.*, one expects reduced survival with origin-DNA loss), the RecBCD properties appear paradoxical; RecBCD promotes destruction of origin-proximal DNA [15], but its net effect is to *prevent* TLD [7, 8, 11]. This suggests that origin-proximal DNA destruction is not the predominant role of RecBCD in TLD—that another survival-enhancing role of RecBCD dominates. What this role might be is not understood.

At least three independent pathways of TLD occur in *E. coli*: an SOS DNA-damage-response-dependent pathway underlies a large component of TLD; a separate RecQ/RecJ-dependent pathway contributes another similarly large component, and a third component of TLD occurs independently of the SOS response or RecQ [6]. Three studies implicated a role for the SOS response in TLD in *E. coli*. The SOS response is activated by RecA protein binding persistent single-stranded DNA, then facilitating the auto-proteolytic cleavage of the LexA transcriptional repressor, which causes upregulation of the SOS genes [16]. We [6] and others [7] found that mutants unable to mount an SOS response because of a *lexA*(Ind[−]) mutation, encoding an uncleavable LexA repressor, suffer about 10-fold less TLD than SOS-proficient cells, indicating that one pathway of TLD requires activation of the SOS response. Cleavage of LexA during an SOS response upregulates the transcription of about 40 DNA damage-response genes [17]. Fonville *et al.* [6] found that the function of only one of them—the cell division inhibitor Sula, which is expressed only during SOS—need be knocked out to block the SOS-dependent component of TLD [6]. Thus, one component or pathway (component requiring a discreet set of genes or epistasis group) of TLD was suggested to result from a thymine-starvation-induced irreversible block to cell division mediated by Sula, and cells that lack Sula or a functional SOS response survive this pathway [6]. Using transcriptional profiling with microarrays, Sangurdekar *et al.* showed

that during TLD, the SOS response genes are strongly and consistently upregulated [14], which would be expected given the causal role of SOS in TLD [6, 7], and might additionally be a consequence of TLD. A second independent pathway of TLD in *E. coli* requires RecQ [6, 10] and RecJ [6] recombinational DNA repair proteins, such that cells that lack RecQ or RecJ and RecA (or that carry the *lexA*-uncleavable mutation) have even less TLD than cells blocked for SOS or RecQ/J singly [6]. Although both RecA and RecQ/J function in HR, at least part of their roles in TLD were independent and additive indicating separate pathways, implying that the RecQ/J route is not solely recombinational. Because there was still some TLD in cells that lack RecA and RecQ, it must be the case that at least one more route(s) to TLD occurs that is independent of both RecQ/J and the SOS response (RecA, LexA and Sula) [6]. The third component of TLD has unknown genetic requirements. We used whole-genome sequencing to examine a strain with mutations that cause TLD sensitivity with the possibility of identifying a new gene(s) with a role in TLD.

Although the recent work demonstrates that the SOS response underlies one of (at least) three largely independent mechanisms of TLD, an earlier report [18] contained an apparently conflicting finding. Morganroth and Hanawalt [18] reported that a *lexA*(Ind⁻) (SOS-off) mutant strain was no more resistant to TLD than SOS-proficient cells, even though they used *lexA3*(Ind⁻), the same *lexA*(Ind⁻) mutation that conferred TLD resistance in the two subsequent studies discussed above [6, 7]. Sequencing of the *lexA* gene in the Morganroth [18] *lexA*⁺ and *lexA3*(Ind⁻) mutant, and the Fonville *et al.* [6] *lexA*⁺ parent and *lexA3*(Ind⁻) mutant derivative, showed that all strains had the correct *lexA* alleles [6]. However replacing the *lexA*⁺ gene of the Morganroth *lexA*⁺ control strain with *lexA3*(Ind⁻), then conferred TLD resistance, thus indicating that the problem lay in the genetic background (the rest of the genome) of the *lexA3*(Ind⁻) strain used by Morganroth and Hanawalt [6]. That is, the data implied that the Morganroth *lexA3*(Ind⁻) strain must contain a mutation(s) somewhere in its genome that masked the TLD-resistance caused by the *lexA3*(Ind⁻) SOS-defective mutation [6]. This unknown mutation would be expected to cause TLD-sensitivity.

Here, we use whole-genome sequencing to identify additional mutation(s) underlying the unexpected TLD sensitivity of the Morganroth *lexA3*(Ind⁻) SOS-deficient strain. We find that it has five genetic differences from the SOS-proficient strain with which it was compared. We find that one of these, the *csrA* mutation, causes sensitivity specifically in SOS-deficient *lexA3*(Ind⁻) strains, or strains that lack the SOS-induced cell-division inhibitor, Sula. CsrA is an RNA binding protein that regulates diguanylate cyclases [19], motility [20], and glycogen biosynthesis [21] and was not previously implicated in TLD. We consider a hypothesis in which, the CsrA role in inhibiting diguanylate cyclases might promote TLD-resistance specifically in SOS/SulA-deficient cells.

2. Materials and Methods

2.1. Strains

Bacterial strains used in this study are listed in Table 1. P1 transductions were performed as described [23]. Missense mutations in *yliF* and *csrA* were linked with selectable markers using Red-mediated short-homology recombination as described [24]. Antibiotic-resistance cassettes were inserted downstream of the *csrA16* and *yliF147* mutations external to their respective open reading frames, between nucleotides 2,816,985 / 2,816,986 and 875,844 / 875,923. The following primers were used to generate PCR products for the insertion of selectable markers downstream of *csrA* and *yliF*; *csrA*-pKD-F2 (ctactatctgtcagacctccattcttctgtgataaaacacatatgaatcctccttag), *csrA*-pKD-R2 (cacttcacgttcaaattagctgtgtaaaacgtcaaaaagaggtgtagctggagctgcttc), *yliF*-pKD-F2 (agtaatcattattcgcaggccggacaaatgatttgcgccgcatatgaatcctccttag), and *yliF*-pKD-R2

(**agta****cagattt****gtggg****tagccgggtttaattcaggc****gtgtaggctggagctgcttc**), (bases in bold indicate identity with the chromosome (used in short-homology recombination) and 20 remaining bases amplify the antibiotic-resistance cassette). Strains used in this study were verified with PCR, UV sensitivity, and sequencing.

2.2. Library preparation, whole-genome sequencing, read mapping and detection of genomic variants

Genomic (g) DNA was prepared from bacterial cultures using the Qiagen whole-genome prep kit. *E. coli* genomic DNA (3µg) was fragmented to an average size of 165bp using a Covaris S2 sonicator (duty cycle 10%; intensity 5; 100cycles/burst; 5°C; 6min). Barcoded SOLiD 5500xl-compatible libraries were made from the fragmented gDNA using a SOLiD Fragment Library kit (Applied Biosystems) using standard manufacturer's protocols. Completed libraries were quantified by qPCR using a KAPA library quantification kit for Applied Biosystems SOLiD platform and pooled in equimolar quantities. The pooled libraries were sequenced on a SOLiD 4 to give single fragment 50bp colorspace reads.

After determining the SOLiD read quality score metrics from each sequenced library, read alignment and variant calling with read data were conducted using the BioScope 1.3.1 genomic analysis software (Life Technologies) genome resequencing pipeline for single fragment reads. Briefly, reads were first processed using the SOLiD Accuracy Enhancement Tool (SAET) calibrated to the genome size of *E. coli*. Reads were then mapped to the *E. coli* reference genome (NCBI RefSeq: NC_000913.2) using the gapped aligner and the subsequent alignments recorded in BAM format. Duplicate reads were then identified within the resulting BAM files and marked for filtering from downstream analysis.

Putative small insertion/deletion (InDel) variants (up to 11bp for deletions and 3bp for insertions) were identified using the BioScope small InDel algorithm by processing gapped read alignment data. Putative single nucleotide variants (SNVs) were identified from the alignment data using BioScope diBayes algorithm with parameters selected for low false-positive variant calls.

From primary read alignments a minimum base quality value of 20 was required for individual read bases to be considered in determining SNVs. Additionally, the SNV had to be identified from reads mapped to both strands of the reference sequence. For SNVs identified within the genomic coordinates of coding genes (based upon RefSeq genome annotation), potential changes to the amino acid translation of the gene were assessed by incorporating each variant into the gene nucleotide sequence and comparing the translated product to that of the unchanged gene.

2.3. Thymineless death assays

TLD was assayed per [6]. Cultures were grown from single colonies to saturation in 5 ml of thy+ medium (M9 medium with 0.1% glucose, 0.5% casamino acids, and 50µg/ml thymine), then diluted 20-fold in triplicate into 5 ml thy+ medium, and shaken for 70 minutes at 37°C. One ml of each diluted culture was pelleted, and the cells washed twice in thy- medium (M9 with 0.1% glucose, and 0.5% casamino acids), then resuspended in a final volume of 2 ml thy- medium, at which cultures had OD₆₀₀ of ~0.2. The cultures were incubated shaking at 37°C and sampled every 30 minutes for five hours by dilution into 1X M9 salts, then plated for cfu on LBH solid medium supplemented with thymine. Colonies were counted using a Microbiology International Colony Counter after 24 hours of incubation at 37°C per [6]. Curves displayed are the averages of 3–5 independent experiments of 3 cultures each (error bars ± SEM).

2.4. Statistical analyses

Statistical significance was determined by two-way ANOVA with repeated measures on three or more independent experiments using a Tukey post-hoc test. Significance was determined as a P value ≤ 0.05 . IBM SPSS Statistics software was used to perform statistical tests.

3. Results

3.1. Additional mutations in the Morganroth *lexA3*(Ind⁻) strain

We determined the whole-genome sequences of the following five strains: the Fonville *et al.* *lexA*⁺ parent and its *lexA3*(Ind⁻) mutant derivative [6], the Morganroth *lexA*⁺ and *lexA3*(Ind⁻) strains, HL353 and HL354, respectively [18], and the *lexA3*(Ind⁻) derivative of HL353 constructed by Fonville *et al.* [6] (Table 2). The Fonville *et al.* and Morganroth strain backgrounds, respectively, had 33 (Table S1) and 44 (Table S2) unique genetic differences from the *E. coli* K12 reference genome (strain MG1655) (differences that the other strain background did not possess) and also had 6 genetic differences from the reference genome in common (Table S3). None of these is relevant to the failure of HL354 to show TLD resistance, because they are also present in the *lexA3*(Ind⁻) derivative of HL353, which did show resistance [6].

In addition to those “background” mutations, the differences between the five strains are summarized in Table 2. The deletions listed in Table 2 were verified by PCR and conventional sequencing across the junctions, and missense mutations were verified by PCR and conventional sequencing. The identities of these five mutations are summarized as follows. First, the Fonville *et al.* *lexA3*(Ind⁻) strain [6] and the *lexA3*(Ind⁻) derivative of HL353 [6] both carry the *malB*::Tn9 selectable marker which is linked with *lexA3*(Ind⁻), which allowed us to construct *lexA3*(Ind⁻) derivatives by selecting for the chloramphenicol resistance conferred by the linked Tn9 in phage P1-mediated transductions [6]. Second, HL353 carries an *rpoD* mutation not shared by HL354. Third, HL354 carries two missense mutations and two deletions not shared by HL353 (Table 2). The missense mutations are in the *csrA* and *yliF* genes, encoding a carbon storage regulator and a predicted diguanylate cyclase, respectively. The two deletions are Δ *rihA-gltLKJI* (a 4,213 bp deletion from bp 682,859 to 687,072) and Δ *fimEAICD::IS1* (a 4,153 bp deletion from bp 4,540,073 to 4,544,226 with an IS1 mobile element insertion). The Δ *rihA-gltLKJI* deletion includes *rihA*, a gene encoding ribonucleotide hydrolase 1, and glutamate transport genes (*gltL*, *gltK*, *gltJ*, and *gltI*) [25]. The Δ *fimEAICD* deletion includes a cluster of *fim* genes required for type I pilus formation [26]. The four mutations unique to the HL354 Morganroth *lexA3*(Ind⁻) strain are the most likely candidates for genes that may play a role in TLD. Of the four mutations, we focused on *csrA* and *yliF* because they are functionally linked--CsrA inhibits *yliF* translation [19]--and discovered that CsrA protects cells from TLD.

3.2. *csrA* mutation causes TLD sensitivity in SOS-deficient cells

First, we show that the selectable marker *malB*::Tn9 is not responsible for the TLD-resistance of the Fonville *et al.* *lexA3*(Ind⁻) strain. We compared the kinetics of TLD in the Fonville *et al.* *lexA*⁺ parent, its *lexA3*(Ind⁻) *malB*::Tn9 derivative, and a *lexA3*(Ind⁻) derivative that is *malB*⁺. The data in Figure 1A show that TLD resistance is caused by the *lexA3*(Ind⁻) mutation, regardless of whether or not the strain also carries the linked transposon insertion. Both the *lexA3*(Ind⁻) *malB*::Tn9 and the *lexA3*(Ind⁻) *malB*⁺ strains are more TLD resistant than their *lexA*⁺ parent ($P = 0.013$ and 0.045 , respectively, at time points 180 to 300 minutes, two-way ANOVA with repeated measures). We conclude that *lexA3*(Ind⁻), encoding a uncleavable LexA repressor [27], causes TLD-resistance irrespective of its linkage with the transposon selectable marker.

Second, we can infer that the *rpoD* mutation in *lexA*⁺ HL353 did not mask its TLD sensitivity, and thus prevent its utility as a TLD-sensitive control strain, because the *lexA3*(Ind⁻) derivative of HL353 made by Fonville *et al.* was more TLD resistant [6], and because both HL353 and its *lexA3*(Ind⁻) derivative had TLD sensitivities similar to those of *lexA*⁺ and *lexA3*(Ind⁻) strains, respectively, in a different, *rpoD*⁺ strain background [6].

Third, we find that the *csrA* missense mutation causes TLD sensitivity of *lexA3*(Ind⁻) strains as follows. Because *csrA* and *yliF* might plausibly interact functionally, we moved each missense mutation singly and together into the Fonville *et al.* *lexA3*(Ind⁻) strain and assayed TLD. The *yliF* mutation caused no increase in TLD-sensitivity, either singly or in combination with the *csrA* mutation (Fig. 1B). By contrast, the *csrA* mutation caused TLD-sensitivity in the Fonville *et al.* *lexA3*(Ind⁻) strain, making it as TLD sensitive as its isogenic *lexA*⁺ parent (Fig. 1B). Thus, we identify CsrA as an anti-TLD protein. We focus on its mode of action as follows.

3.4. *csrA* mutation increases TLD specifically in SOS-defective cells, dependently on RecQ

First, the data in Figure 2A show that *csrA16* (the allele found in HL354, named here) does not increase TLD-sensitivity in the Fonville *et al.* [6] *lexA*⁺ parent strain. Because the only difference between this strain and the Fonville *et al.* *lexA3*(Ind⁻) strain relevant to TLD is the functional *lexA* gene, we conclude that *csrA* mutation increases TLD specifically in SOS-defective cells. In addition, the TLD sensitivity caused by *csrA16* does not require a SOS response.

Second, we find that the increased TLD caused by *csrA16* in *lexA3*(Ind⁻) cells requires RecQ. The RecQ- and SOS-dependent TLD mechanisms are mostly independent, such that a *recQ lexA3*(Ind⁻) mutant is more resistant to TLD than either single mutant [6] (Fig. 2B). We compared the TLD-sensitivity of the *lexA3*(Ind⁻) *recQ* strain with that of the *csrA lexA3*(Ind⁻) *recQ* triple mutant and observe no difference in TLD (Fig. 2B). That is, *csrA* mutation increases sensitivity of *lexA3*(Ind⁻) cells ($P = 0.48$ at time points 240 to 300 minutes, two-way ANOVA with repeated measures), but not *lexA3*(Ind⁻) *recQ* cells ($P = 0.11$ at time points 240 to 300 minutes, two-way ANOVA with repeated measures) (Fig. 2B). Therefore, we conclude that RecQ is required for the increased TLD caused by *csrA* mutation in SOS-deficient cells.

3.5. *csrA* mutation causes TLD sensitivity in Sula-defective cells

Our data imply that CsrA protects SOS-deficient (Fig. 1B), but not SOS-proficient cells from TLD (Fig. 2A). We tested whether the lack of Sula expression in SOS-deficient cells could account for the sensitivity of SOS-deficient cells to CsrA status, as it does for the TLD resistance of SOS-deficient cells observed previously [6]. We compared a *sulA csrA* double mutant with the *sulA* single mutant. As observed previously [6], the *sulA* single mutant is more resistant to TLD than the SOS-proficient *lexA*⁺ parent strain (Fig. 2C, $P = 0.037$, t 180 min, two-way ANOVA with repeated measures). Importantly, the *csrA sulA* double mutant is as sensitive to TLD as the Sula⁺ parent—that is, the *csrA* mutation made the *sulA* strain TLD sensitive (Fig. 2C). The *csrA sulA* double mutant is also as sensitive as the *csrA* single mutant, which is similar to the parent (Fig. 2C). The data show that *sulA*-defective cells are sensitized to TLD by the *csrA* mutation and suggest that failure to express *sulA* may underlie why SOS-deficient cells uniquely require CsrA⁺ function for their enhanced TLD resistance.

4. Discussion

We identified *csrA* as a gene required for TLD resistance in SOS-defective cells and cells that lack Sula, the SOS-induced inhibitor of cell division. We show that lack of Sula can account for why SOS-defective cells require CsrA⁺ function for their enhanced TLD resistance (Fig. 2C). In Fig. 3A we suggest one possible mechanism by which loss of CsrA function might cause increased sensitivity to TLD. CsrA inhibits diguanylate cyclases [19], leading to an increase in nucleotide precursors including deoxy-ribose-1-phosphate (d-ribose-1-P), in the cell [22] (Fig. 3A). Although *E. coli* can synthesize d-ribose-1-P *de novo* by other pathways from glucose, a recent study shows that even with unlimited glucose and functional *de-novo* synthesis pathways, thymine is not used efficiently by cells that lack the pathway that scavenges d-ribose-1-P from dGTP [22], and dGTP is made from GTP (see Fig. 3A) which diguanylate cyclases remove. Thus, cells with less d-ribose-1-P scavenged from dGTP require more thymine [22]. In the absence of functional CsrA, diguanylate cyclases are not inhibited and more GTP would be converted into cyclic-di-GMP resulting in less dGTP, less d-ribose-1-P and more TLD sensitivity. Further work is needed to test the hypothesis that CsrA confers TLD resistance by indirectly increasing d-ribose-1-P levels, which enhance thymine utilization (per [22]).

One possible reason that cells lacking Sula specifically may require the extra d-ribose-1-P suggested to result from CsrA action could be that failure to arrest cell division when DNA is damaged might decrease the chance of constructing a functional chromosome from scavenged nucleotides (Fig. 3B). During DNA damage, SOS-proficient cells halt cell division using Sula [28] (Fig. 3B right). These division-arrested cells can continue to replicate DNA resulting in an accumulation of chromosomes [28], which might be recycled, such that division blocked cells might potentially possess more DNA from which nucleotide precursors could be scavenged. One consequence of this is that, first, SOS- or Sula-defective strains have more cells per culture after thymine starvation than SOS-/Sula-proficient strains, in which cell division is blocked. Because the SOS/Sula-defective strains have *more* cells, but yet show greater death with the *csrA* mutation than SOS-Sula-proficient strains, this implies that the greater death caused by *csrA* may be under-estimated; it could be even greater, per cell, than is detected in our assays. Second, in division-arrested cells with many chromosomes, a functional chromosome might ultimately be cobbled together from multiple damaged and recycling chromosomes, and so Sula-mediated division arrest might make cells less dependent on CsrA-enhanced dTTP levels. By contrast SOS-defective or Sula⁻ cells might continue to divide during thymine deprivation despite accumulating DNA damage, and so might have fewer chromosomes per cell than Sula⁺ division-arrested cells (Fig. 3B left). According to this hypothesis, dividing cells (Sula⁻) might therefore have greater dependence on CsrA-promoted d-ribose-1-P, and so show increased TLD when CsrA function is lost. Further work is needed to test this hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The *csrA* missense mutation confers TLD-sensitivity that masks TLD resistance bestowed by the *lexA3*(Ind⁻) “SOS-off” mutation. (A) The *malB::Tn9* marker used to construct *lexA3*(Ind⁻) derivatives of the Fonville *et al.* parent and HL353, the Morganroth *lexA⁺* (by Fonville *et al.* [6]) does not affect TLD resistance. *lexA3*(Ind⁻) *malB::Tn9* (SMR10669) and *lexA3*(Ind⁻) (SMR15895) are both significantly more resistant to TLD than their isogenic parent (AB2497) at $t = 180$ min. ($P = 0.045$ and 0.013 , respectively, two-way ANOVA with repeated measures). Mean $\pm$ SEM of 3 independent experiments of 3 cultures each. (B) The *csrA* and not the *ylf* missense mutation confers TLD sensitivity to a *lexA3*(Ind⁻) strain, making it as TLD sensitive as its *lexA3⁺* parent. The parent (AB2497), *lexA3*(Ind⁻) *csrA* (SMR17409), and *lexA3*(Ind⁻) *ylf csrA* (SMR17410) are significantly more sensitive to TLD than *lexA3*(Ind⁻) (SMR15895) ($P = 0.012$, 0.027 , and 0.038) and than *lexA3*(Ind⁻) *ylf* (SMR17407) ($P = 0.007$, 0.015 , and 0.021 at $t = 180$ min, two-way ANOVA with repeated measures). Mean $\pm$ SEM of 5 independent experiments, 3 cultures each.

Figure 2.

csrA⁻-enhanced TLD is specific to SOS- or Sula-deficient cells and requires RecQ. (A) *csrA*-defect (SMR17504) does not confer TLD sensitivity to the SOS-proficient parent (AB2497). At t = 180 min, $P = 1.000$ (two-way ANOVA with repeated measures). Mean $\pm$ SEM of 3 independent experiments of 3 cultures each. (B) The increased TLD in *csrA*-deficient SOS-deficient cells requires RecQ. *recQ lexA(Ind⁻) csrA* (SMR17619) cells are not more sensitive than *recQ lexA(Ind⁻)* (SMR17505) cells at t = 240 min. Also, *recQ lexA(Ind⁻) csrA* (SMR17619) are more resistant than *lexA(Ind⁻)* (SMR15895) and *lexA(Ind⁻) csrA* (SMR17409) at t = 240 min ($P = 0.017$ and 0.0002 , two-way ANOVA with repeated measures). Mean $\pm$ SEM of 4 independent experiments of 3 cultures each. For comparison, the *lexA(Ind⁻)* single mutant strain is roughly equally TLD resistant to an otherwise isogenic $\Delta recQ$ single-mutant strains [6]. (C) *csrA* mutation increases TLD sensitivity of otherwise SOS-proficient *sulA*-defective cells. Whereas, as shown previously [6], *sulA* (SMR10674) cells are more resistant to TLD than the isogenic Sula⁺ parent (AB2497, $P = 0.037$ at t = 180 min, two-way ANOVA with repeated measures), *sulA csrA* (SMR17733) cells are more sensitive than the *sulA* single mutant at t = 240 ($P = 0.012$, two-way ANOVA with repeated measures) and are as sensitive as the Sula⁺ parent or *csrA* single mutant. Mean $\pm$ SEM of 5 independent experiments of 3 cultures each.

Figure 3.

One way that CsrA might promote TLD-resistance specifically in SOS⁻ or SulA-defective cells. (A) CsrA inhibits diguanylate cyclases [19] which convert GTP into cyclic-di-GMP. GTP can be converted to GDP by GTPases [29], reduced to dGDP by nucleoside diphosphate reductase 1 [30], phosphorylated to dGTP by nucleoside diphosphate kinase [31, 32], then broken down to nucleotide precursors including deoxy-guanosine, deoxy-ribose-1-phosphate (d-ribose-1-P), which is needed to make thymine into thymidine (deoxy-ribothymine) [22]. Thymidine is converted to dTMP by thymidine kinase [22], and dTMP (thymidylate) allows survival of thymine starvation [1]. One way that CsrA might promote TLD-resistance in SulA⁻ or SOS-off cells might be by increasing d-ribose-1-P pools that enhance thymine utilization even in cells capable of de novo d-ribose-1-P synthesis (per [22]). (B) In the absence of the SOS-induced inhibitor of cell division, SulA, cells with DNA damage continue to divide [28] and so have fewer chromosomes per cell than non-dividing (SulA⁺) cells [28]. CsrA might promote TLD resistance specifically in *sulA*-defective and SOS-defective cells, because their fewer chromosomes may lead to lower levels of d-ribose-1-P obtained which we suggest might be obtained by recycling damaged chromosomes in the same cell. In division-arrested SulA⁺ SOS-proficient cells, d-ribose-1-P might be salvageable from multiple damaged chromosomes and such salvage might sometimes permit a functional chromosome to be constructed from the parts of other nonfunctional (recycling) chromosomes, thus reducing dependence of CsrA-promoted d-ribose-1-P production. Further work is needed to test this hypothesis.

Table 1

Escherichia coli strains and plasmids used in this study

Plasmid/strain	Relevant genotype	Reference or source
pKD46	Red recombinase vector	[24]
pKD3	Source of FRTcatFRT cassette	[24]
pKD4	Source of FRTKanFRT cassette	[24]
AB2497 ^a	AB1157 <i>thyA12 deoB6</i> ^b	[33] CGSC2497 ^c
HL353 ^d	<i>thyA lac rpoD deo</i> ^e	[18]
HL354 ^f	HL353 <i>lexA3</i> (Ind ⁻) <i>deo</i> ^e <i>csrA16 yliF147 ΔrihA-gltLKJI ΔfimEAICD:IS1</i>	[18]
SMR10669 ^a	AB2497 <i>lexA3</i> (Ind ⁻) <i>malB:Tn9</i>	[6]
SMR10674	AB2497 <i>sulA100:Tn5</i>	[6]
SMR10675 ^d	HL353 <i>lexA3</i> (Ind ⁻) <i>malB:Tn9</i>	[6]
SMR10683	AB2497 <i>ΔrecQ1906:FRT lexA3</i> (Ind ⁻) <i>malB:Tn9</i>	[6]
SMR15727	HL354 [pKD46]	HL354 × pKD46
SMR15895	AB2497 <i>lexA3</i> (Ind ⁻)	SMR10669 spontaneous Mal ⁺ (precise excision)
SMR16225	AB2497 [pKD46]	AB2497 × pKD46
SMR16305	<i>yliF147 zbh:FRTKanFRT</i>	SMR15727 × <i>zbh:FRTKanFRT</i> (Red-mediated recombineering)
SMR17082	AB2497 <i>zga:FRTcatFRT</i>	SMR16225 × <i>zga:FRTcatFRT</i> (Red-mediated recombineering)
SMR17083	<i>csrA16 zga:FRTcatFRT</i>	SMR15727 × <i>zga:FRTcatFRT</i> (Red-mediated recombineering)
SMR17407	AB2497 <i>lexA3</i> (Ind ⁻) <i>yliF147 zbh:FRTKanFRT</i>	SMR15895 × P1(SMR16305)
SMR17409	AB2497 <i>lexA3</i> (Ind ⁻) <i>csrA16 zga:FRTcatFRT</i>	SMR15895 × P1(SMR17083)
SMR17410	AB2497 <i>lexA3</i> (Ind ⁻) <i>yliF147 zbh:FRTKanFRT csrA16 zga:FRTcatFRT</i>	SMR17407 × P1(SMR17083)
SMR17504	AB2497 <i>csrA16 zga:FRTcatFRT</i>	AB2497 × P1(17083)
SMR17505	AB2497 <i>lexA3</i> (Ind ⁻) <i>ΔrecQ1906::FRT</i>	SMR10683 spontaneous Mal ⁺ (precise excision)
SMR17619	AB2497 <i>lexA3</i> (Ind ⁻) <i>ΔrecQ1906::FRT csrA16 zga:FRTcatFRT</i>	SMR17505 × P1(17083)
SMR17733	AB2497 <i>sulA100:Tn5 csrA16 zga:FRTcatFRT</i>	SMR10674 × P1(17083)

^aWhole-genome sequencing revealed additional mutations listed in Tables S1 and S3.^bThe *deoB6* mutation was reported to be present in this strain (CGSC2497^c) but was not detected by whole-genome resequencing done here.^cCGSC is the *E. coli* Genetic Stock Center (Yale University).^dThe *rpoD* mutation (Table 2) was not known previously to be present and was found here by whole-genome resequencing. Whole-genome resequencing also revealed additional mutations listed in Tables S2 and S3.^eThe *deo* mutation is reported to be present in this strain [18] but was not detected by whole-genome sequencing.^fThe *csrA*, *yliF*, *ΔrihA-gltLKJI* and *ΔfimEAICD:IS1* (Table 2) are new alleles found here by whole-genome resequencing and were not known previously to be present. Whole-genome resequencing also revealed additional mutations listed in Tables S2 and S3.

Table 2

Additional genetic elements in strains used in previous studies of thymineless death

Source of strain sequenced (strain name) [reference]	TLD phenotype	Additional mutations ^a
Fonville <i>et al.</i> parent (AB2497) [6]	S	
Fonville <i>et al.</i> <i>lexA3</i> (Ind ⁻) (SMR10669) [6]	R	<i>malB</i> :Tn9 ^b
Morganroth <i>lexA</i> ⁺ (HL353) [18]	S	<i>rpoD</i> ^c
Morganroth <i>lexA3</i> (Ind ⁻) (HL354) [18]	S	<i>csrA</i> 16 ^d , <i>yliF</i> 147 ^e , Δ <i>rhlA-gltLKJI</i> ^f , Δ <i>fimEAICD:IS1</i> ^g
HL353 <i>lexA3</i> (Ind ⁻) constructed by Fonville <i>et al.</i> (SMR10675) [6]	R	<i>rpoD</i> ^c <i>malB</i> :Tn9 ^b

^a Mutations other than *lexA3*(Ind⁻) that differ between each *lexA*⁺ the *lexA3*(Ind⁻) strain to which it was compared by [6, 18] or between the two strain backgrounds used. Apart from *malB*:Tn9, which was used by design as a selectable marker for constructing *lexA3*(Ind⁻) isogenic derivatives of AB2497 and HL353 (to make SMR10669 and SMR10675, respectively), the remaining mutations were discovered here by whole-genome resequencing. The whole genome resequencing results show that the *lexA*⁺ and *lexA3*(Ind⁻) strains of Morganroth and Hanawalt differed by five additional mutations: an *rpoD* allele unique to the *lexA*⁺ HL353 and new mutations *csrA*, *yliF*, Δ *rhlA-gltLKJI* and Δ *fimEAICD* mutations found in the HL354 *lexA3*(Ind⁻) mutant.

^b Selectable maker near *lexA* used to select transductants some of which also received the *lexA3*(Ind⁻) allele.

^c G to C, missense: alanine 500 to proline

^d G to A, missense: aspartic acid 16 to asparagine

^e A to T, missense: asparagine 147 to isoleucine

^f 4.2 kb deletion of genes indicated (basepairs 682,859 to 687,072).

^g 4.0 kb deletion of genes indicated (basepairs 4,540,073 to 4,544,226) with an *IS1* mobile element insertion.