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Stalled replication fork repair and misrepair during thymineless death in *Escherichia coli*

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Abstract

Starvation for DNA precursor dTTP, known as ‘thymineless death’ (TLD), kills bacterial and eukaryotic cells alike. Despite numerous investigations, toxic mechanisms behind TLD remain unknown, although wrong nucleotide incorporation with subsequent excision dominates the explanations. We show that kinetics of TLD in *Escherichia coli* is not affected by mutations in DNA repair, ruling out excision after massive misincorporation as the cause of TLD. We found that the rate of DNA synthesis in thymine-starved cells decreases exponentially, indicating replication fork stalling. Processing of stalled replication forks by recombinational repair is known to fragment the chromosome, and we detect significant chromosomal fragmentation during TLD. Moreover, we report that, out of major recombinational repair functions, only inactivation of *recF* and *recO* relieves TLD, identifying the poisoning mechanism. Inactivation of *recJ* and *rep* has slight effect, while the *recA*, *recBC*, *ruvABC*, *recG* and *uvrD* mutations all accelerate TLD, identifying the protection mechanisms. Our epistatic analysis argues for two distinct pathways protecting against TLD: RecABCD/Ruv repairs the double-strand breaks, whereas UvrD counteracts RecAFO-catalyzed toxic single-strand gap processing.

Introduction

Interference with the biosynthesis of DNA precursor dTTP by inhibiting the key enzyme thymidylate synthase kills cells of any type; thus, thymidylate synthase inhibitors are used to treat a variety of conditions, from bacterial infections to cancer (Van Triest & Peters 1999; Chernyshev *et al.* 2007). The unique toxicity of dTTP starvation was discovered 55 years ago in thymidylate synthase-deficient *Escherichia coli* and called ‘thymineless death’ (TLD) (Cohen & Barner 1954). Early on it was shown that only cells with replicating chromosomes are susceptible to TLD, implicating replication in the dTTP starvation-caused chromosomal damage (Maaloe & Hanawalt 1961). Yet, the toxic mechanisms behind dTTP starvation are still debated, reflecting both their complicated nature and disagreement among results from different experimental systems and laboratories (Kunz & Haynes 1982; Ahmad *et al.* 1998; Ladner 2001; Nakayama 2005). Especially intriguing are the roles various recombinational repair activities play during TLD, as some of them help, others are clearly toxic, while the

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Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

Figure S1 TLD kinetics in the *uvrD recBCD* double mutant.

Table S1 Strains

Data S1 Discussion.

Additional Supporting Information may be found in the online version of this article.

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most central players, RecA in bacteria and Rad51 in eukaryotes, appear to have no role at all (Kunz & Haynes 1982; Ahmad *et al.* 1998). Recombinational repair has two branches—one mending blocked single-strand gaps, the other mending double-strand breaks (Kuzminov 1999). Interestingly, inactivation of the single-strand gap-mending branch of recombinational repair increases resistance to TLD, while inactivation of the double-strand break-mending branch makes cells sensitive to it (Kunz & Haynes 1982; Ahmad *et al.* 1998). However, the lack of effect of the *recA* defect (Cummings & Mondale 1967; Anderson & Barbour 1973; Nakayama *et al.* 1982), which inactivates the critical step in recombinational repair, questions the involvement of homologous strand exchange in TLD, which still leaves a possibility that other functions of Rec proteins are involved, like linear DNA degradation by RecBCD or single-strand DNA binding by RecFOR.

This variety of confusing and frequently contradictory results leaves TLD without an established mechanism; instead, several possibilities are suggested, which have common elements. A consensus that fits experimental data best starts with incorporation of wrong nucleotides into DNA in place of missing thymine, with subsequent futile excision cycles (with the decreasing DNA synthesis capacity) causing enlargement of single-strand gaps in both DNA strands (Breitman *et al.* 1972; Kunz & Haynes 1982; Ahmad *et al.* 1998; Ladner 2001; Nakayama 2005) (Fig. 1A – D). At this point, individual proposals go their own ways. Some postulate that futile excision cycles kill by themselves (Ladner 2001); some other propose that persistent single-strand gaps trigger formation of branched DNA species, which are the ultimate toxic products of recombination (Nakayama *et al.* 1994; Ahmad *et al.* 1998; Nakayama 2005) (Fig. 1E). The third type of proposals notes that the widening single-strand gaps in opposite DNA strands may eventually overlap, leading to double-strand breaks, which are lethal (Kunz & Haynes 1982; Ahmad *et al.* 1998) (Fig. 1F). However, it should be stressed that experimental support for any of the three ideas is either weak or nonexistent, as exemplified by the conflicting data on TLD-induced double-strand breaks in *E. coli* (Yoshinaga 1973; Nakayama *et al.* 1994; Guarino *et al.* 2007).

A recent conceptual advance—the finding that inhibition of DNA synthesis triggers processing of stalled replication forks by the recombinational repair enzymes, sometimes to the detriment of the cell (Michel *et al.* 2007)—argues for a different scenario of what could be happening in cells with inadequate dTTP supply. Because processing of stalled replication forks by recombinational repair functions fragments the chromosome, we would like to propose that TLD is a direct result of replication fork stalling, followed by improper processing by recombinational repair leading to irreparable chromosomal lesions (Fig. 1G – I). The difference from the previous explanations is that no incorporation of wrong nucleotides and no futile excision cycles with widening single-strand gaps away from replication forks are required to explain TLD; rather, the toxic action happens at the inhibited replication forks themselves. To test these ideas, we characterized TLD both physically and genetically in *E. coli* deleted for the thymidylate synthase gene, *thyA*. The *thyA* mutant strains can be conveniently propagated in the presence of exogenous thymine [or more frequently thymidine, which is the next step in the *in vivo* conversion of thymine into dTTP (Neuhard & Kelln 1996)], and then studied after thymidine removal (Cohen & Barner 1954; Ahmad *et al.* 1998). Thymidylate synthase-negative mutant cells of higher eukaryotes also undergo TLD, with similar chromosomal problems (Seno *et al.* 1985). In this report, we are addressing the nature of the connection between recombinational repair and TLD.

Results

Three phases of thymine starvation

To bring order to the diversity of contradictory observations surrounding TLD, especially concerning the influence of various mutants in the DNA metabolism (Ahmad *et al.* 1998), we standardized our TLD analysis by using precise deletion alleles of the genes in question and by implementing four modifications of a typical format. Cells undergo TLD with different kinetics in different conditions, dying sooner in conditions promoting faster growth—for instance, at 37 °C when compared to 28 °C or in richer media (Ahmad 1980; Kuong & Kuzminov 2009). As the first modification, we grew our cells at 28 °C in M9CAA or MOPS minimal media, because these conditions enhanced the survival differences between the mutants and the wild-type cells. As the second modification, we extended the standard time course of thymine starvation to 6 h, which allowed us to detect significant differences in strain survival. The third modification was a genetic one: when supplemented with thymidine, *E. coli thyA* mutants tend to accumulate low thymidine requiring suppressors by inactivating the *deoCADB* operon (Neuhard & Kelln 1996). To avoid this complication, we deleted all four *deo* genes, so that our ‘wild-type’ strain was a $\Delta\textit{deoCADB}$ mutant, while our experimental strain was the double deletion $\Delta\textit{thyA} \Delta\textit{deoCADB}$ mutant. The fourth adjustment was a requirement for multiple repetitions: the number of independent experiments to build a TLD curve in this work varies between three and 37, with the median number around seven. These modifications enabled us, in some cases, to detect unseen before differences between wild type and mutant curves, and in some other cases to be sure that a mutant is not different from wild type.

Using a novel approach to label and quantify DNA in thymineless conditions, we recently documented a gradual yet severe inhibition of DNA synthesis in thymine-starved cells (Kuong & Kuzminov 2009), indicating replication fork stalling and / or disintegration. Here, we show that removal of thymidine from the growth medium leads to exponential drop in rates of DNA synthesis by our *thyA deoCADB* mutant, comparable to the inhibition of the *dnaB(Ts)* mutant at the nonpermissive temperature (Fig. 2A). Continued replication with such a dramatic DNA synthesis rate inhibition indicates replication fork stalling. Perhaps, even more importantly, in contrast to the general understanding that, once thymidine is removed, DNA replication in *thyA* mutants stalls immediately, we observe that, even after one hour after thymidine removal, the rate of DNA replication is still approximately 10% of the original one (Fig. 2A). In other words, although replication forks are slowing down dramatically, they keep making limited progress.

By monitoring the titer of thymine-starved cultures in our experimental conditions, we distinguished the following phases of TLD (Fig. 2B): (i) the resistance phase (no loss of viability) of 1.5 h; (ii) the rapid exponential death (RED) phase, with approximately 500-fold loss of titer that lasts approximately the next 3 h; and (iii) the survival phase, with the remaining cell titer being stable for at least 24 h (not shown).

To test whether the RED phase is caused by massive incorporation of wrong bases (uracils, mismatches, modified nucleotides) into DNA with their subsequent excision, we determined TLD kinetics in the *ung*, *mutS* and *uvrA* mutants, blocked, correspondingly, for uracil excision, for mismatch removal and for nucleotide excision repair. We found no significant difference in the TLD kinetics between any of the three mutants and the wild-type cells (Fig. 2C), indicating that excision of wrong bases from DNA is not a major part of the TLD mechanism. A recent report also shows no influence of the *uvrA* defect on kinetics of TLD (Morganroth & Hanawalt 2006).

SOS induction reveals non-repairable chromosomal lesions

Because thymine starvation does not kill via incorporation and excision of wrong bases, but is concomitant with replication fork stalling (Fig. 2), we hypothesized that TLD is caused by failure to repair chromosomal lesions at replication forks—complex DNA lesions, normally mended by recombinational repair. We decided to test this two-part notion (both the formation of chromosomal lesions and the failure to repair them) by following the kinetics of induction of the SOS response during thymine starvation. SOS response is the increased expression of genes of DNA repair and DNA damage tolerance and is induced by formation of chromosomal lesions (Kuzminov 1999). SOS response is readily induced by thymine starvation (Huisman & D'Ari 1981), but the kinetics is typically followed during the first hour only. Extending the SOS measurements beyond two hours, when DNA synthesis almost ceases, should indicate reparability of the chromosomal lesions and whether or not they continue to form. If chromosomal lesions are formed and then repaired, SOS response will be induced and then will either plateau or will go away. However, if the chromosomal lesions are not repaired, or if the lesions form faster than they are repaired, this will result in ever increasing SOS levels.

What we in fact observed was a gradual increase in the SOS expression during thymine starvation, which seemed to plateau by two hours, apparently reflecting the resistance phase, and then continued climbing again, reflecting the RED phase (Fig. 2D). At the same time, cells treated with a DNA cross-linking agent mitomycin C for one hour showed plateaued SOS induction, consistent with the initial heavy DNA damage which later on was mostly repaired (Fig. 2D). Together, these results suggest that (i) chromosomal damage occurs right from the start of thymine starvation, but this initial damage is repairable; (ii) later on something triggers continuous formation of irreparable chromosomal lesions or repairable damage in initially unaffected cells.

Thymine starvation induces chromosome fragmentation

One way stalled replication forks are processed is through their reversal with subsequent breakage, which generates double-strand DNA breaks if not prevented by RecBCD (Michel *et al.* 2007). Breakage of both forks of the replication bubble should lead to formation of subchromosomal fragments, detectable as chromosomal fragmentation. Chromosomal fragmentation during TLD was reported in one study (Yoshinaga 1973), but a subsequent study failed to confirm the original observation and suggested it was a prophage induction artifact (Nakayama *et al.* 1994). Recently, however, chromosomal fragmentation in *E. coli* was reported after a mere 30-min thymine starvation (Guarino *et al.* 2007). Using our sensitive and quantitative protocol of chromosomal fragmentation detection in *recBCD* mutants (Kouzminova *et al.* 2004), we found substantial chromosomal fragmentation during TLD (Fig. 3A,B). Significant chromosomal fragmentation during TLD was even detectable in the *recBCD*⁺ cells (Fig. 3A,B), suggesting formation of irreparable double-strand breaks. The observed initial spike in the wild-type cells, consistent with the result of others (Guarino *et al.* 2007), might be because of a different fragmentation mechanism and needs furthermore investigation. It should be noted that, in contrast to previous results of others (Handa & Kobayashi 2003; Guarino *et al.* 2007), the levels of spontaneous chromosomal fragmentation in our $\Delta rec\text{-}BCD$ mutants is unusually low and is not statistically different from the spontaneous fragmentation in the wild-type cells, most likely because of our use of the MOPS-minimal phosphate medium. We conclude that at least one type of irreparable chromosomal lesions formed during TLD is represented by double-strand DNA breaks. We propose that these breaks are formed at stalled replication forks, as a result of their improper processing by recombinational repair functions.

Effect of recombinational repair mutants on the levels of SOS induction

Recombinational repair in wild-type *E. coli* comprises the two early path-ways starting -with two types of chromosomal lesions that license the central homologous strand exchange reaction catalyzed by RecA, followed finally by the two late path-ways providing alternative means to resolve recombination intermediates (Fig. 3C). The function of activities of the early pathways is to recognize the chromosomal lesion, to prepare it for RecA polymerization and to stimulate ('license') RecA polymerization on it. If the chromosomal lesion is a double-strand end, then these three steps (recognition, preparation and licensing) are catalyzed by RecBCD; if the chromosomal lesion is a blocked single-strand gap, the same three steps are performed by RecFOR (Kuzminov 1999) (Fig. 3C). After RecA filament homologously pairs and exchanges strands between the damaged DNA and its intact sister duplex, the resulting recombinational repair intermediate is eliminated by either RuvABC resolvase (through resolution of Holliday junctions) or, alternatively, by RecG, via a still unknown mechanism (Fig. 3C). It could be that RuvABC preferentially resolves individual Holliday junctions, while RecG preferentially handles pairs of close DNA junctions (Kuzminov 1999), but there is no strong evidence either for or against this hypothetical distinction.

In order to test whether thymine starvation induces any specific type of chromosomal lesions and / or recombinational repair intermediates, we tested the levels of SOS induction during TLD in single mutants in recombinational repair. The *recA* mutant served as a negative control, as RecA cleaves the LexA repressor to induce the SOS regulon. We measured SOS at the 4-h time point (viability is about 1%). The level of SOS induction of the *recBCD*, *recF*, *recG* and *ruvABC* mutants during thymine starvation turned out to be surprisingly uniform (Fig. 3D): all these recombinational repair mutants showed lower SOS induction than wild-type cells, although only the *recBCD* mutant results were significantly different from the wild type. Importantly, none of these single mutants was either grossly deficient in SOS, nor induced higher than the wild type, suggesting that both types of chromosomal lesions (double-strand breaks, single-strand gaps) and both types of recombinational repair intermediates (individual Holliday junctions, pairs of close junctions) form during thymine starvation and contribute to SOS induction. In contrast, the double *recBCD recF* mutant did not induce much SOS (Fig. 3D), indicating no additional types of chromosomal lesions besides double-strand breaks and single-strand gaps. The SOS induction in the *recG ruvABC* double mutant was not different from the one in the *ruvABC* single mutant.

We also tested how different levels of DNA repair and DNA damage tolerance influence either the resistance or the RED phases of TLD, by employing mutants either unable to induce the SOS response (*lexA3*) or expressing SOS genes constitutively (*lexA71*). The former mutant is known to have no effect on TLD kinetics (Howe & Mount 1975; Morganroth & Hanawalt 2006), while the latter one was never tested. We found that constitutive SOS induction has a modest effect on the kinetics of TLD (Fig. 3E), while the inability to induce SOS eliminates the resistance phase and at the same time slows down RED, translating into a 'hybrid' TLD curve of *lexA3* mutant (Fig. 3E), which contradicts the aforementioned literature data (Howe & Mount 1975; Morganroth & Hanawalt 2006). Among the recombinational genes that we tested in this work, *recA* and *ruvAB* are induced by SOS, so next we tested the effect of inactivation of individual recombinational repair genes on TLD kinetics.

Role of major recombinational repair activities during thymine starvation

As already mentioned, RecBC-initiated recombinational repair mends double-strand breaks, whereas the RecFOR-initiated repair mends single-strand gaps (Fig. 3C). Previously, three independent studies reported that the *recBC* mutants are hypersensitive to TLD (Cummings

& Mondale 1967; Ahmad 1980; Nakayama *et al.* 1982). In our hands, the *recBCD* mutants began to lose viability almost immediately after the thymidine supplementation is withdrawn from the growth medium, although the rate of death is not faster than that in the wild-type cells (Fig. 4A). This sensitivity of the *recBCD* mutants indicates the importance of double-strand break repair for the resistance to thymine starvation. In the wild-type cells, the RecBC recombinational repair helicase forms a part of the RecBCD enzyme, the fastest exonuclease known and a potent linear DNA degradation enzyme (Dillingham & Kowalczykowski 2008), while in the *recD* mutants DNA degradation is reduced twofold without affecting recombination (Rinken *et al.* 1992). A single *recD* mutation in our hands has no influence on the TLD kinetics (Fig. 4A), suggesting that the wild-type rates of linear DNA degradation are not needed for TLD resistance.

Confirming the single earlier reports, we found that the *recF* (Nakayama *et al.* 1982) and the *recO* (Nakayama *et al.* 1988) defects protect from TLD by slowing death, while leaving the length of the resistance phase unchanged (Fig. 4B). Another defect that was reported to protect against TLD was *recJ* (Nakayama *et al.* 1988); however, our *recJ* allele showed almost no protection (Fig. 4B). The slower death in the *recF* and *recO* mutants indicates poisoning of the cells by the RecFO-initiated repair of unfillable single-strand gaps. Overall, the opposing effects on TLD of the defects in the two early pathways of recombinational repair indicate that: (i) double-strand breaks happen early on during thymine starvation, but are successfully repaired by RecBC (or subchromosomal fragments degraded by RecBCD) during the resistance phase; and (ii) during the RED phase, unfillable single-strand gaps accumulate, and initiation of their repair by RecF poisons the cells, for example by making some double-strand breaks non-repairable.

There is a disagreement in the literature about the effect of the *recA* mutations on TLD: two papers report that the *recA* defect makes *E. coli* more resistant to TLD (Inouye 1971; Ahmad 1980), whereas three others report that the *recA* defect has no effect on TLD (Cummings & Mondale 1967; Anderson & Barbour 1973; Nakayama *et al.* 1982). In our conditions, the Δ *recA* mutants show a hybrid TLD curve similar to the *lexA3* mutant: they are hypersensitive to TLD early on after thymidine removal, but eventually stabilize at a higher level of survival than the wild-type cells (Fig. 4C). The *recA* mutant's result argues that the *lexA3* result could be because of inability to amplify the RecA amount in response to massive chromosomal damage during TLD. The shape of the hybrid curve suggests dual role for RecA during thymine starvation: (i) at first RecA helps by participating in the RecBC-catalyzed repair of double-strand breaks during the resistance phase; and (ii) later on RecA becomes detrimental by participating in the RecF-catalyzed poisonous repair of single-strand gaps, and its participation in double-strand break repair can no longer help. Finally, the effect of RecA inactivation on the resistance phase is noticeably less than the effect of RecBCD inactivation (Fig. 4, compare A with C), suggesting that RecBCD has a role in TLD resistance beyond its participation in RecA-catalyzed recombinational repair of double-strand breaks (Fig. 3C). This role may be prevention of double-strand breaks via duplex DNA degradation [augmented by ssDNA-specific exonucleases in the *recD* mutants (Miranda & Kuzminov 2003)].

In contrast to the several reports on the role of *recA*, *recBC*, and *recF* mutants in TLD, the behavior of mutants in the late pathways of recombinational repair during thymine starvation received much less attention, as there was only a single report on *ruv* (Nakayama *et al.* 1994). Confirming this report, we found that the *ruvABC* mutant cells are hypersensitive to TLD, just like the *recBCD* mutants, but after a short resistance period (Fig. 4D). We interpret this result to mean that (i) RuvABC is critical for repair of double-strand DNA breaks that accumulate early on during thymine starvation; (ii) the critical participation of RuvABC in this repair starts approximately 30 min later than the

participation of RecBC; and (iii) unlike RecA, RuvABC does not have a big role in the RecF-catalyzed poisonous repair of single-strand gaps later on. In contrast, we found that the *recG* mutant cells not only survive TLD slightly better than WT cells, but display the 'hybrid curve' (Fig. 4D), suggesting that RecG also participates both in the RecBC-catalyzed double-strand break repair and in the RecF-catalyzed poisoning by single-strand gaps. Thus, this poisoning is not limited to the formation of recombination intermediates, but also appears to include their resolution.

Epistatic analysis of recombinational repair mutants during thymine starvation

It was formally possible that the various effects of mutations in recombinational repair on TLD are because of operation of several independent pathways, rather than a single postulated pathway with the two early and two late branches (Fig. 3C). Epistatic analysis, during which individual mutations are combined pairwise, is a standard test of whether the two genes work in the same pathway (effect of one mutation hides the effect of the other one) or in independent pathways (the double mutant shows an enhancement of effects of single mutants, usually a multiplicative effect) (Haynes & Kunz 1981).

For example, because *recA* is the master gene of recombinational repair, we thought that the double *recBCD recF* mutant, in which both early branches of recombinational repair are blocked, or the double *ruvABC recG* mutant, in which both late branches of recombinational repair are blocked, would produce the *recA*-like ('hybrid') TLD kinetics. Instead, we found that both double mutants were more sensitive to thymine starvation than the *recA* single mutant, but with some differences. The kinetics of the *recBCD recF* double mutant coincided with the one of the *recBCD* single mutants (Fig. 5A), showing that the RecF-catalyzed events are not the reason the *recBCD* mutant dies faster. In contrast, the *ruvABC recG* double mutant effect on the TLD curve could be described as addition of individual effects of the single *recG* and *ruvABC* mutants, showing that both functions contribute uniquely to the resistance phase (Fig. 5B). Overall, the TLD kinetics of the *recBC recF* and *ruv recG* double mutants confirm that the repairable chromosomal lesions, specifically double-strand breaks, form early on during thymidine starvation, and their RecBC- and RuvABC/RecG-catalyzed repair is critical for survival during the resistance phase, before the later formation of the single-strand gaps and their toxic repair by RecFO (finished by RecG?) initiates the RED phase.

To test whether the faster RED phase in the *recBCD* mutant depends on the RecA-catalyzed reaction, we compared TLD kinetics of the *recA recBCD* double mutant with those of the corresponding single mutants (Fig. 5C). We found that the double mutant kinetics mostly parallels the one of the *recBCD* single mutant (*recBCD* is epistatic to *recA*), ruling out early poisoning of the *recBCD* mutants by the RecA-promoted processes. Likewise, we also tested whether the resistance to thymine starvation of the *recF* single mutant is independent of RecA (Fig. 5D). We found that the *recA recF* double mutant behaves like the *recA* single mutant (*recA* is epistatic to *recF*), showing that the *recF* defect cannot slow RED without RecA.

TLD kinetics of the *recBCD ruvABC* double mutant follows the kinetics of the single *ruvABC* mutant (Fig. 5E), which shows that (i) RecBCD and RuvABC work in the same pathway of resistance to TLD, most likely repair of double-strand breaks; and (ii) the *ruvABC* defect is epistatic to the *recBCD* one, meaning that a small fraction of these early double-strand breaks are produced by RuvABC. On the other hand, the TLD curve of the *recF ruvABC* mutant runs between the curves of the individual *recF* and *ruvABC* mutants, suggesting that formation of some late double-strand breaks depends on RecF (Fig. 5F). In summary, because in our analysis of the double mutants we found no enhancement of the effects of single mutants (with the exception of *recG ruv*), we conclude that there is a single

type of chromosomal lesions requiring RecA-dependent repair during the resistance phase of thymine starvation (most likely double-strand breaks) and another single type of chromosomal lesions whose RecA-dependent repair is toxic during the RED phase of thymine starvation (most likely single-strand gaps, either translating into, or blocking the repair of, double-strand breaks, which then kill).

Role of the UvrD helicase during thymine starvation

To test this reasoning, we needed an activity that would affect RecA-promoted pairing at stalled replication forks. Two related helicases: Rep and UvrD—are suspected to act on or around stalled replication forks. Rep is required for the nicking-initiated DNA replication in small bacteriophages (Denhardt *et al.* 1967); in addition, the *rep* mutants replicate their chromosomal DNA slower, implicating Rep as an auxiliary function at the *E. coli* replication fork (Lane & Denhardt 1975). Finally, the original *rep* mutant also exhibited faster TLD (Denhardt *et al.* 1967). However, under our conditions, the TLD kinetics of the Δrep mutant turned out to be at least no different from the wild-type kinetics, if not slower (Fig. 6A), indicating that the Rep helicase participates in neither resistance to TLD, nor in the TLD poisoning. The conflict between our results and the previous results could be because of the presence of other TLD-modifying mutations in the original *rep* mutant isolate, as well as because of the difference in growth conditions, to which TLD is extremely sensitive.

The other helicase, UvrD, is responsible for the quality control over the RecA-promoted strand exchange intermediates, dissociating intermediates of insufficient length [reviewed in (Kuzminov 1999)]. In addition, it was recently demonstrated that UvrD inhibits RecA FOR binding at stalled replication forks, replication fork reversal and replication restart (Lestini & Michel 2007). The *uvrD* mutants were found long ago to be hypersensitive to TLD (Siegel 1973), but the reason was unknown. We confirmed that the *uvrD* mutants are hypersensitive to TLD, with kinetics superficially similar to the one of *ruvABC* mutant (compare Fig. 6B versus Fig. 5D). At face value, this could mean UvrD participation in the repair of double-strand breaks. Alternatively, UvrD could have nothing to do with the double-strand break (DSB) repair, but single-strand gaps, whose poisonous repair UvrD was supposed to supervise, could start forming earlier than the beginning of the RED phase. In this case, UvrD would be successful in disrupting improper RecA FOR-promoted pairing during the second half of the resistance phase, whereas the RED phase would signify UvrD's failure to continue doing so.

We distinguished between the two explanations by determining the TLD kinetics of the *recBCD uvrD* versus *recA uvrD* and *recF uvrD* double mutants. If UvrD disrupts the improper RecA-catalyzed pairing at single-strand gaps, the *recA* and the *recF* defects should be epistatic to UvrD, and this was what we indeed observed. In particular, the *uvrD recA* double mutant duplicated the TLD kinetics of the *recA* single mutant (Fig. 6C), while the *uvrD recF* double mutant behaved essentially as the *recF* single mutant (Fig. 6D). If UvrD's role in TLD is indeed to counteract the toxic effects of RecF and RecA, rather than participating in RecBCD-promoted double-strand break repair, then its effect on TLD should be synergistic with the one of *recBC* defect, and, again, this was found to be the case (Figs 6E and S1 in Supporting Information). The synergy of the *recBC* and *uvrD* effects clearly shows that RecBCD's role in TLD is separate from the UvrD's role.

In fact, this synergy is expressed even at the viability level in *Thy*⁺ cells, as the viability of the *uvrD* mutant in our hands is close to 50%, the viability of the *recBCD* mutant (in this study) is close to 40%, whereas the viability of the double *uvrD recBCD* mutant is 11% (even less than the product of the viability indices of the single mutants) (Fig. 6F). This low viability of the *uvrD recBCD* double mutants (without any thymine-starvation) suggests that the RecAFO-poisoning also happens in normally growing cells. However, the viability of

the double *recA uvrD* and *recF uvrD* mutants turned out to be also the product of viabilities of the corresponding single mutants (Fig. 6F), arguing against the RecAF-poisoning in growing cells. Unfortunately, we could not construct the *ruvABC uvrD* double mutant, because of its inviability (Lestini & Michel 2007).

Overall, we conclude that: (i) both types of chromosomal lesions are formed throughout thymine starvation, first double-strand breaks, shortly followed by single-strand gaps; (ii) during the initial resistance phase, DSBs are all successfully repaired (by RecA+RecBC +RuvABC), whereas single-strand gaps (SSGs) are successfully prevented from being turned into toxic recombination intermediates (by UvrD); and (iii) *something* eventually triggers the RED phase, the proximal cause of which is the RecAFO-catalyzed toxic repair of single-strand gaps at stalled replication forks. The same repair is apparently nontoxic in growing cells. Thus, the trigger may be the decrease of the DNA synthesis rate below a certain level [for example, below 10%, the rate at one hour of thymine starvation (Fig. 2A)], critical for maintenance of replication forks.

Discussion

The enigma of cell death in the absence of thymidine entices experimental inquiries and at the same time eludes them for over half a century. Although the deep rooting of TLD in DNA replication and repair is quite obvious (see the Introduction), there were recent attempts to link TLD to either RNA synthesis (Morganroth & Hanawalt 2006) or RNA degradation (Sat *et al.* 2003). Especially intriguing was a possible involvement of the toxin–antitoxin pair ChpAR (MazEF) in TLD (Sat *et al.* 2003), but neither the magnitude of the TLD effect (Godoy *et al.* 2006), nor the cell death from ChpA/MazF overexpression (Christensen *et al.* 2003) were subsequently confirmed, and the effect itself contradicted earlier observations with RNA synthesis inhibitors [as discussed in (Morganroth & Hanawalt 2006)].

The consensus of several extant models posits that wrong DNA base incorporation and excision is at the root of TLD. After finding that thymine-starved cells shut down DNA replication, and that TLD kinetics is not influenced by inactivation of excision of wrong DNA nucleotides, we hypothesized that TLD is because of irreparable *chromosomal* lesions, generated during processing of stalled replication forks by recombinational repair. We tested these ideas in the *thyA E. coli* mutants and found that: (1) thymine-starved cultures go through three distinct phases: (i) resistance, (ii) RED and (iii) survival; (2) continuous SOS induction during TLD indicates formation of irreparable chromosomal lesions, and there is indeed a significant chromosomal fragmentation in thymine-starved cells; (3) making the SOS response uninducible decreases the resistance, but also increases the survival of thymine starvation; (4) the *recA*, *recBC*, *ruvABC*, *recG* and *uvrD* defects accelerate TLD by shortening the resistance phase, while the *recF* and *recO* mutations alleviate TLD by slowing the RED phase; (5) defects in the double-strand break repair are epistatic to defects in the single-strand gap repair, indicating that, at least in the beginning, double-strand breaks form independently of the (toxic) single-strand gap processing; and (6) UvrD controls the toxic single-strand gap repair by RecAFO, and the breakdown of this control is the proximal trigger of the RED phase of TLD. We invite the reader to visit the Data S1 in Supporting Information for detailed discussion of the first four points; the points #5 and #6 are discussed in the following paragraphs.

Some double-strand breaks form independently of the (toxic) single-strand gap processing

Our finding that *recA*, *recBC* and *ruvABC* mutants accelerate TLD indicates early formation of repairable double-strand breaks during thymine starvation and raises the question about

the mechanism of their formation. Stalled replication forks are known to reverse and turn into Holliday junctions, which are then cut by RuvABC, breaking the replication forks and fragmenting the chromosome (Michel *et al.* 2007). However, this cannot be the major mechanism of chromosomal fragmentation during TLD—otherwise *ruvABC* mutants would have suppressed TLD instead of accelerating it. The fact that the *uvrD* defect also shortens the resistance phase suggests early formation of blocked single-strand gaps, making it possible that some double-strand breaks form as a consequence of the single-strand gap processing. The TLD kinetics in the *recF ruv* double mutant, which is intermediate between the kinetics of *recF* and *ruv* single mutants, suggests that at least some double-strand breaks during TLD are the result of RecF-dependent processes. However, the TLD kinetics in the *recBCD recF* double mutants, which matches the one of the single *recBCD* mutant, rules out the possibility that the RecF-processing of single-strand gaps is the only way to generate double-strand breaks during thymine starvation. Apparently, thymine starvation triggers formation of the early double-strand breaks directly. The mechanisms of both direct and RecF-dependent chromosomal fragmentation during TLD remain a mystery to be investigated.

UvrD failure may be one of the proximal triggers of the RED phase of TLD

UvrD is known to counteract improper RecFOR-licensed RecA filament assembly at stalled replication forks in other systems (Lestini & Michel 2007), and now we show that UvrD also exercises quality control over the RecA filament formation during TLD. This conclusion follows from three facts: (i) the *uvrD* defect accelerates TLD; (ii) the *recF* defect alleviates TLD; and (iii) the *recF* defect is epistatic to the *uvrD* defect: in the *recF uvrD* double mutant TLD remains alleviated. The interesting twist is the earlier formation of these RecA_{FOR}-catalyzed improper recombination intermediates, as suggested by the shorter resistance phase in the *uvrD* mutants. This result provides an insight into what triggers the RED phase of thymine starvation: it is the eventual failure of UvrD to disrupt the continuous RecFOR-licensed toxic RecA-promoted pairing of single-strand gaps at stalled replication forks, rather than the pairing itself. The out-of-control RecA filamentation and formation of recombination intermediates may then block the access to double-strand ends and prevent their critical repair by RecBC.

Broader implications of our findings

The TLD phenomenon offers a unique opportunity to gain insights into the intricate interplay between DNA replication and recombination without exogenous poisoning of the system. We showed that two distinct protection mechanisms, by RecABCD/Ruv on the one hand and by UvrD on the other, are required for the initial protection against TLD. Interestingly, the two mechanisms also operate during normal growth, which is reflected in the poor viability of the *recBCD uvrD* mutants (this study) and the co-lethality of *ruvABC uvrD* mutants (Lestini & Michel 2007). The real puzzle is the RecA_{FOR} pathway for repair of blocked single-strand DNA gaps, beneficial against exogenous DNA damage (like UV), yet toxic during the nucleotide imbalance scenario of TLD. Even more confusing, RecA is both the central player in the RecBC-dependent protection pathway and in the RecFO-dependent toxic pathway. One more unclear relationship is epistasis of the *recA* defect to the *recF*, *ruvABC* and to the *uvrD* defects, but not to the *recBC* defect. Perhaps, this reflects the situation when RecBCD not only *repairs* double-strand breaks, but also *prevents* formation of more double-strand breaks, for example via DNA degradation at reversed replication forks (Miranda & Kuzminov 2003). How RecA, the primary savior from chromosomal damage, turns into a contributor to the toxicity during TLD, should be one of the central questions of future TLD investigations.

Conclusion

TLD is an interesting phenomenon, about which numerous studies were carried out, but results were frequently conflicting, and no single mechanism of the mysterious death was ever agreed upon. In light of our study, we narrowed it down to the formation of toxic chromosomal lesions by recombination proteins acting on either (or both) abruptly stalled replication forks or gapped chromosomes generated by polymerases trying to replicate DNA in the absence of dTTP (Fig. 7). In other words, TLD is the result of inappropriate recombination in the attempt to restore replication, a situation similar to the one with mutationally-destabilized replisomes (Lestini & Michel 2007; Michel *et al.* 2007). We showed that when the RecFOR activities involved with generation of these toxic lesions are removed, cells have much slower dying phase as long as the RecBCD, RecA and RuvABC activities are intact. We speculate that one type of the irreparable chromosomal lesions is the so-called ‘exploded’ replication fork, while some other, yet to be specified types, can be generated from forks engaged in futile replication (Fig. 7). Future studies will be directed at the nature of the toxic lesions generated, the mechanism of the repair/recovery of stalled replication forks, and how nucleotide imbalance brought by thymidine starvation, stalls replication in the first hand. Specifically, an enigma of hydroxyurea, which also stalls replication forks, but does not cause the TLD-like RED phase (Kuong & Kuzminov 2009), needs to be explained.

Experimental procedures

Growth conditions

All strains (see the Table S1 in Supporting Information for mutation confirmation and the Table of strains) were propagated on LB-agar plates with appropriate antibiotics, supplemented with 10 µg/mL thymidine in the case of all *thyA* mutants. LB broth per 1 L contains: 10 g tryptone, 5 g Yeast extract, 5 g NaCl, pH 7.4 with 250 µL 4 M NaOH; LB agar contained 15 g agar per 1 L of LB broth; M9CAA medium contain 1xM9 salts (Difco), 2 mM MgSO₄, 0.1 mM CaCl₂, 10 mg/L thiamine (B1), 0.2% glucose and 0.2% Casamino acids (Difco). MOPS-minimal phosphate medium was as described (Neidhardt *et al.* 1974). Antibiotics were used in the following concentrations: kanamycin: 50 µg/mL, ampicillin: 100 µg/mL, tetracycline: 10 µg/mL, chloramphenicol: 12.5 µg/mL.

To measure TLD kinetics, cells were grown at 28 °C in M9CAA or MOPS-minimal phosphate medium with 10 µg/mL thymidine until mid-logarithmic phase (OD₆₀₀ approximately 0.15), supplemented thymidine was removed by collecting cells on nitrocellulose membrane filters (type 0.22 µm GS; Millipore), followed by washes with 3 volumes of 1% NaCl and final resuspension in the original medium, but this time without thymidine. Viability of cultures was measured by spotting 10 µL of serial dilutions in 1% NaCl on LB plates supplemented with 10 µg/mL thymidine. While still small (after 1–3 days incubation of the plates at 22 °C), colonies at each spot were counted under the stereomicroscope. All titers were then normalized to the titer at time 0 (= thymidine removal).

Measuring SOS induction

To determine the level of SOS induction, P1 transduction was used to introduce the MuΔX: *cat*-derived construct with the *lacZ* gene fused under the *sulA* promoter (Ossanna & Mount 1989) into the KKW58 *thyA* and other *thyA rec* strains. The *thyA deoCABD* mutant cultures were switched to the same medium without thymidine, and SOS induction was measured at various time points. Assays were performed as before (Kouzminova *et al.* 2004) with the following modifications: experiments were performed in M9CAA at 30 °C without antibiotics and 500 µL of culture were measured per assay. As a positive control, KJK106

thyA sulA::lacZ supplemented with thymidine was treated with 100 ng/mL mitomycin C, a cross-linking agent (not shown). As a control for no SOS induction, we used $\Delta recA$ cells. Because certain *rec* mutants may slightly induce SOS even during *thy*⁺ growth, Miller Units (MU) readings from the same set of cultures but supplemented with thymidine were subtracted as background. For Fig. 3D, values from KJK106 were taken as 1 and all strains were normalized to KJK106.

Measuring the rate of DNA synthesis by ³²P-orthophosphate incorporation

Experiments were performed as previously described (Kuong & Kuzminov 2009) except the following modifications: cells were grown in MOPS with thymidine supplemented for *thyA* until OD₆₀₀ approximately 0.15, then supplemented thymidine was removed by collecting cells on nitrocellulose membrane filters (type 0.22 μ m GS, Millipore), followed by washes with 3 volumes of 1% NaCl and final resuspension in MOPS, but this time without thymidine; *thyA* cultures were grown at 30 °C throughout the experiment; *dnaB22(Ts)* were grown at 30 °C until OD₆₀₀ approximately 0.15 and were switched to 42 °C when thymidine was removed from *thyA* cultures. At each indicated time point, 1.8 mL of cultures was aliquotted for OD₆₀₀ measurement, then ³²P-orthophosphate was added to the aliquot and the aliquot was put back into shaking for 10 min for ³²P-orthophosphate incorporation before cells were collected for making agarose plugs. The same volume of cells was used in each plug regardless of the OD₆₀₀ increase, which is mostly because of cell filamentation instead of the actual increase of chromosomal content. DNA-seI treatment of the plug confirmed that everything showing up at the top of the gel is DNA species and the signal from both the well and the lane were taken into account. As positive controls, *thyA* cultures grown with thymidine and *dnaB22(Ts)* grown at 30 °C throughout the experiment were used.

Measuring DSBs by pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis and quantification of chromosomal fragmentation were performed as before (Kouzminova *et al.* 2004; Kuong & Kuzminov 2009) with some minor modifications: all strains were grown in the MOPS-minimal phosphate medium; overnight cultures were diluted and grown with 5 μ Ci of ³²P-orthophosphoric acid per ml of culture for 2.5 h at 30 °C before supplemental thymidine was removed by centrifugation, then the same amount of ³²P-orthophosphoric acid was added back to the cultures resuspended in the MOPS-minimal phosphate medium without thymidine. OD₆₀₀ was taken to determine the volume of aliquots to be used so that, within the experiment, the cell mass per lane was the same for all samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Ideas about the possible trigger of thymineless death. Filled lines, template DNA strands; open lines, newly synthesized DNA strands; X, wrong DNA base. The 'white death' signs mark scenarios based on incorporation of wrong bases. The 'black death' sign marks the scenario based on replication fork stalling. (A) normal DNA duplex; (B) during dTTP starvation, wrong bases accumulate in DNA; (C) excision of wrong bases; (D) because of the lack of dTTP, excision repair is futile, leading to widening of excision gaps (lethal situation #1); (E) persistent single-strand gaps trigger recombination and formation of abnormal branched DNA species (lethal situation #2); (F) further widening of excision gaps leads to their overlapping and double-strand breaks (lethal situation #3); (G) normal replication fork; (H) replication fork stalling because of dTTP starvation, with unfinished Okazaki fragments accumulating; (I) improper recombination processing of a stalled replication fork (lethal situation #4).

Figure 2.

Thymine starvation blocks DNA synthesis, induces SOS response and kills, but not through excision of wrong DNA bases. (A) Thymine starvation inhibits DNA synthesis. Normalization was to the rates at time 0.5 h without treatment. The values are means of three-to-six independent measurements $\pm$ SEM. Strains: *thyA*, KKW58; *dnaB*(Ts), KJK148. (B) Parameters of thymineless death (TLD) in a *thyA* mutant (KKW58) that have no other defects. The values are means of 37 independent measurements $\pm$ SEM (sometimes obscured by the symbols). (C) The kinetics of TLD in mutants, deficient in uracil excision, methyl-directed mismatch removal or in nucleotide-excision repair. The wild-type TLD

curve from panel B is shown for comparison. The values are means of three independent measurements $\pm$ SEM. Strains are: *thyA ung*, KJK78; *thyA mutS*, KJK179; *thyA uvrA*, KJK185. (D) Kinetics of SOS induction during TLD. The values are means of four independent measurements $\pm$ SEM. The strain is KJK106. As a control (white crosses), the same strain is treated with 100 ng/mL mitomycin C in the growth medium + thymidine for 1 h. After MC is removed, incubation is continued in the same medium.

Figure 3. Chromosomal fragmentation and genetics of SOS induction during thymine starvation. (A) A representative pulsed-field gel of chromosomal DNA of *thyA* and *thyA recBCD* mutants during thymine starvation. The strains are: wild type, KKW58; $\Delta recBCD$, KJK63. (B) Quantification of chromosomal fragmentation during thymineless death (TLD). The wild-type TLD curve from Fig. 2B is shown for comparison. The values are means of three independent measurements from gels like in 'A' $\pm$ SEM. (C) A scheme of the recombinational repair pathways. The SOS response is induced by RecA filament polymerization on the damaged chromosomes, licensed either by RecBC (at double-strand

ends) or by RecFOR (at single-strand gaps). (D) The level of SOS induction in recombinational repair mutants. The measurement was taken four hours after thymidine removal. The data are means of four-to-seven independent measurements $\pm$ SEM. The strains are: Wild type, KJK106; *recA*, KJK125; *recBCD*, KJK129; *recF*, KJK127; *recG*, KJK135; *ruvABC*, KJK137; *recBCD recF*, KJK216; *recG ruvABC*, KJK217. (E) The kinetics of TLD in two types of *lexA* mutants. The *lexA3* mutant produces an uncleavable SOS repressor and cannot be induced (IND–), while the *lexA7I* mutant does not produce the repressor and is constitutively induced for SOS. Sula is the SOS-induced cell division inhibitor and needs to be inactivated for *lexA7I* mutant to be viable. The wild-type TLD curve from Fig. 2B is shown for comparison. The values are means of four independent measurements $\pm$ SEM. Strains are: *thyA*, KKW58; *thyA lexA3*, KJK116; *thyA lexA7I*, KJK123.

Figure 4.

The roles of recombinational repair in thymineless death (TLD). In all panels, the wild-type curve from Fig. 2B is shown for comparison. (A) TLD kinetics in the *recBCD* and *recD* mutants. The values are means of five-to-eight independent measurements $\pm$ SEM. The strains are: *recBCD*, KJK63; *recD*, KJK99. (B) TLD kinetics in the *recF*, *recO* and *recJ* mutants. The values are means of 4–13 independent measurements $\pm$ SEM. The strains are: *recF*, KJK67; *recO*, KJK194; *recJ*, KJK192. (C) TLD kinetics in the *recA* mutant. The values are means of 24 independent measurements $\pm$ SEM. The strain is KJK61. (D) TLD kinetics in the *ruvABC* and *recG* mutants. The values are means of 7–11 independent measurements $\pm$ SEM. The strains are: *ruvABC*, KJK65; *recG*, KJK68.

Figure 5. Epistatic analysis of recombinational repair defects in thymineless death (TLD). In all panels, the single mutant TLD kinetics from Fig. 4 is shown for comparison. (A) TLD kinetics in the *recBCD recF* double mutant. The values are means of eight independent measurements $\pm$ SEM. The strain is KJK72. (B) TLD kinetics in the *recG ruvABC* double mutant. The values are means of seven independent measurements $\pm$ SEM. The strain is KJK73. (C) TLD kinetics in the *recA recBCD* double mutant. The values are means of five independent measurements $\pm$ SEM. The strain is KJK102. (D) TLD kinetics in the *recA recF* double mutant. The values are means of six independent measurements $\pm$ SEM. The

strain is KJK104. (E) TLD kinetics in the *recBCD ruvABC* double mutant. The values are means of four independent measurements $\pm$ SEM. The strain is KJK212. (F) TLD kinetics in the *recF ruvABC* double mutant. The values are means of four to five independent measurements $\pm$ SEM. The strain is KJK211.

Figure 6. Thymineless death (TLD) in the *rep* and *uvrD* mutants. The wild-type and single mutant TLD kinetics from previous figures are shown for comparison. (A) TLD kinetics in the Δrep mutant. The values are means of three independent measurements $\pm$ SEM. The strain is KJK202. (B) TLD kinetics in the $\Delta uvrD$ mutant. The values are means of three independent measurements $\pm$ SEM. The strain is KJK112. (C) TLD kinetics in the $\Delta uvrD \Delta recA$ double mutant. The values are means of three independent measurements $\pm$ SEM. The strain is KJK140. (D) TLD kinetics in the $\Delta uvrD \Delta recF$ double mutant. The values are means of three independent measurements $\pm$ SEM. The strain is KJK144. (E) TLD kinetics in the $\Delta uvrD$

recBCD double mutant. In contrast to all other TLD kinetics, for which strains were pre-grown in thymidine-supplemented medium at 28 °C, this set of strains was grown in the presence of thymidine at 42 °C to accelerate the otherwise impractically slow growth of the *uvrD recBCD* double mutant. The experiment itself was at 28 °C, as in all other cases. The values are means of three independent measurements $\pm$ SEM. The strain is KJK158. (F) Viability of *recA*, *recBCD*, *recF* and *ruvABC* mutants in the UvrD+ and *uvrD* mutant backgrounds. All strains are Thy+ and grown in LB. No value for the *ruvABC uvrD* double mutant reflects its known co-lethality.

Figure 7.
A model of chromosomal lesion prevention, repair and misrepair during thymineless death. Old DNA strands are shown by filled lines, newly-synthesized strands are shown as open lines. Question mark, a hypothetical transition with an unclear mechanism.