THESIS APPROVAL

The abstract and thesis of Katherine Ona for the Master of Science in Biology were presented October 31, 2008, and accepted by the thesis committee and the department.

COMMITTEE APPROVALS:

_____
Justin Courcelle, Chair

_____
Suzanne Estes

_____
Mike Bartlett

DEPARTMENT APPROVAL:

_____
Michael Murphy, Chair
Department of Biology

ABSTRACT

An abstract of the thesis of Katherine Ona for the Master of Science in Biology presented on October 31, 2008.

Title: Inhibition and Replication Recovery Following Nitrofurazone or Formaldehyde Treatment, Two Agents that Induce Structurally Distinct DNA Lesions in *Escherichia coli*

Replication forks encounter a variety of impediments that can prevent the faithful duplication of the genetic material. To deal with these challenges, cells contain a variety of mechanisms to allow replication to complete its task. The mechanism that operates in each situation is likely to vary depending on the form of DNA damage or block that is encountered. UV-induced DNA damage has served as a useful model to dissect the general question of how replication deals with blocks to progression and has been fairly well characterized. Following UV-induced arrest of replication in *Escherichia coli*, several *recF* pathway gene products have been shown to process and protect the arrested replication fork until either repair enzymes or alternative polymerases are recruited to remove or bypass the impediment. Far less is known about how the cell deals with other challenges *in vivo*. In this study, we characterized how *E. coli* replicate in the presence of nitrofurazone and formaldehyde, two agents that form lesions that are structurally distinct from those produced by UV-irradiation. Similar to UV-induced damage, we found that survival and recovery following

treatment with nitrofurazone requires *recA,* nucleotide excision repair, the *recBC* and *recF* pathway proteins, and also to a lesser extent, translesion synthesis. By contrast, survival after formaldehyde treatment was distinct from UV-induced damage in that it depended primarily on *recA* and the *recBC* pathway but not these other processes. These results suggest that UV- and nitrofurazone-induced damage are processed similarly by the cell. Both agents primarily induce smaller DNA adducts or lesions. By contrast, the protein-DNA adducts formed by formaldehyde pose a distinct challenge to the cell and are processed through a significantly different pathway.

INHIBITION AND REPLICATION RECOVERY FROM NITROFURAZONE AND FORMALDEHYDE, AGENTS THAT INDUCE STRUCTURALLY DISTINCT DNA LESIONS IN *ESCHERICHIA COLI*

by

KATHERINE LOUISE REYES ONA

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CHAPTER I

INTRODUCTION

DNA damage present when cells are replicating poses an especially potent threat. If damaged DNA is replicated, there is a high potential for mutations, rearrangements or lethality to occur (10). Numerous DNA damaging agents have been shown to inhibit replication, but with the exception of a couple of model lesions, the molecular events that allow cells to deal with these challenges are not well characterized. Considering the potentially severe consequences that can result from the improper processing of DNA damage, it is important to characterize how replication is faithfully restored in the face of a variety of challenges. One of the main objectives of this thesis is to examine whether the structure of the replication-arresting lesion determines how the lesion is processed and replication is restored.

Escherichia coli has proven to be a useful model organism to examine how replication occurs on damaged templates and to identify the genes that are involved in repairing the DNA. The high level of conservation among replication and repair proteins throughout evolutionarily diverse organisms allows one to extend observations made in the genetically amenable system of *E. coli* to higher organisms such as humans.

While a number of impediments may block replication, experiments characterizing UV-induced damage have been the most extensively characterized. UV irradiation causes the formation of cyclobutane pyrimidine dimers (CPD) and 6-4

photoproducts on the DNA (32, 33). Both of these lesions block DNA polymerases and can arrest the progression of the replication fork (22, 43). Following UV-irradiation in *Escherichia coli,* RecA, RecF, RecO and RecR are required to protect and maintain the blocked replication fork until the UV-induced lesion is removed or bypassed so that replication can resume (4, 8, 9, 11, 12, 14). RecF, O and R function together to load and stabilize a RecA nucleoprotein filament onto the DNA at the site of the arrest (7). In UV-irradiated cells lacking any of these gene products, replication forks are not maintained, extensive DNA degradation occurs at the arrested site and replication fails to recover (4, 7, 9, 12, 13). At times prior to the resumption of DNA synthesis, the RecJ nuclease and RecQ helicase partially unwind and degrade the nascent lagging strand at the arrested site (9, 36). This processing is thought to effectively move the branch point of the replication fork back to allow repair enzymes access to the lesion (9). In addition to *recF* pathway mutants, cells lacking RecBCD are also hypersensitive to UV-irradiation, although precisely why remains unclear (17). RecBCD is an exonuclease that is required for conjugational and transductional recombination (27). It binds and exonucleolytically processes double strand ends *in vitro* and *in vivo* (27). To explain the hypersensitivity of *recBC* mutants, some researchers have speculated that replication forks may collapse when they arrest at DNA damage, forming a double strand DNA end for the RecBCD enzyme (28, 29, 31). However, this model has not been supported by studies *in vivo* showing that the nuclease activity of RecJ and RecQ, but not RecBCD, process the nascent DNA at the forks arrested by UV damage (5, 12, 25). Thus, the substrate that requires RecBCD-processing following UV-irradiation *in vivo* remains to be identified.

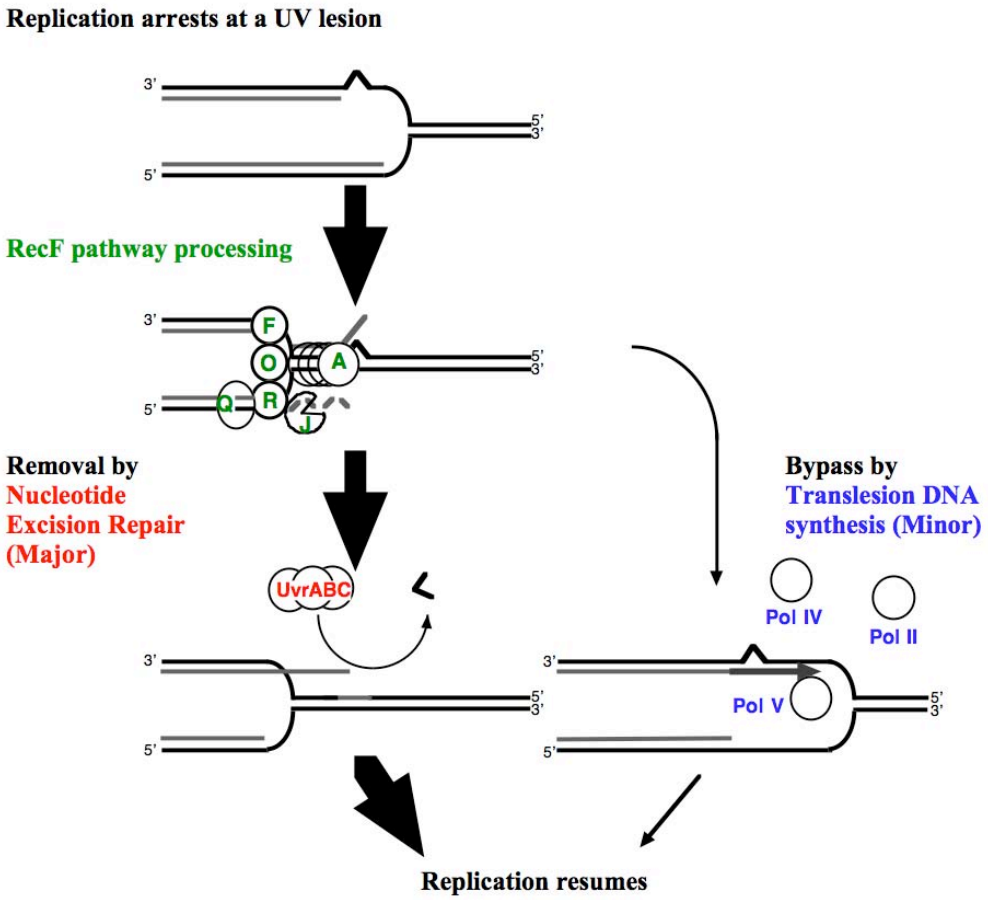

Figure 1.1 Current model of the mechanism for replication recovery following exposure to UV-induced DNA damage

In conjunction with the proteins that process the DNA fork, other enzymes are required to either repair the lesion or bypass it such that replication can resume. In *E. coli,* there are two primary pathways for removing damaged nucleotides in an error-free manner: base excision repair and nucleotide excision repair (19, 38). The initial recognition step of base excision repair is accomplished by one of at least nine different glycosylases (reviewed in (19)). Following base recognition and removal of the damaged base, apurinic/apyrimidinic (AP) endonucleases make an incision which removes the remaining nucleoside, leaving a single nucleotide gap. DNA polymerase

and ligase then fill in the AP site with the correct nucleotide. The lesions recognized by base excision repair are generally small modified bases.

Bulky adducts or base modifications that distort the DNA helix are removed by nucleotide excision repair (reviewed in (38)). This repair pathway removes adducts by making dual incisions 12-14 bp around the damaged nucleotide followed by displacement and resynthesis of the region using the complementary strand as a template (37, 40, 41). Unlike BER, which can utilize any of several distinct glycosylases, nucleotide excision repair is accomplished by only one recognition complex, comprised of UvrA, UvrB and UvrC. Mutants lacking any one of these gene products are hypersensitive to a long list of DNA damaging agents, demonstrating the diversity of lesions recognized by this excinuclease complex, including cyclobutane pyrimidine dimers and 6-4 photoproducts produced by UV, bulky adducts formed by carcinogens like benzo(a)pyrene and acetylaminofluorine, and chemicals like cisplatin and psoralens (Figure 1.2) (38).

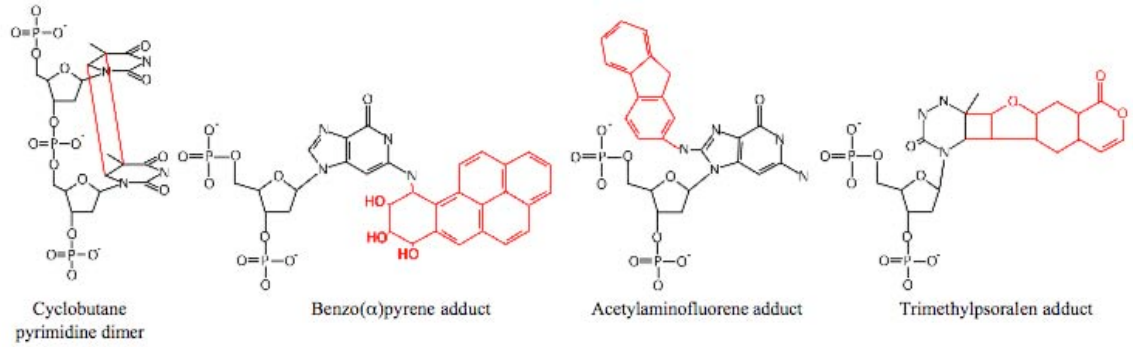

Figure 1.2 Nucleotide excision repair catalyzes the excision of an array of bulky adducts. Structures in red denote abnormal covalent modifications (modified from (18)).

In addition to lesion removal, there are tolerance mechanisms that allow the lesion to persist. One of these mechanisms, termed translesion DNA synthesis, involves specialized polymerases. Pol II, Pol IV and Pol V, encoded by *polB, dinB* and *umuDC,* that are able to synthesize DNA through damaged templates whereas the replicative polymerase, Pol III, cannot (23). Transcription of these polymerases is upregulated in the presence of DNA damage as part of the 'SOS response' (16). The ability of these polymerases to bypass a lesion is due, in part, to a modified active site within the polymerase that allows altered bases to be accommodated (30, 44). This form of synthesis is often associated with a higher error rate, leading to a higher mutation frequency (23). Characterizations *in vitro* and *in vivo* have shown that the mutation frequency depends on both the polymerase and the type of lesion. For example, Pol V is the only polymerase capable of synthesis through pyrimidine dimers, however this occurs with an elevated error frequency relative to the replicative polymerase, Pol III (45). In contrast, Pol IV can synthesize through bulky lesions such as N^2-dG adducts produced by benzo(a)pyrene, 4-NQO, nitrofurazone and methylglyoxal, and base methylation induced by alkylating agents (3, 24, 47). In each of these cases, the polymerase preferentially incorporates the correct base, although the error rate is still higher than Pol III on the undamaged template. Mutants lacking the specific polymerase required for bypass are hypersensitive to these DNA damaging agents, suggesting that lesion bypass can contribute to survival *in vivo* (3, 24, 47). Synthesis by Pol II results in a -2 frameshift mutation when the template contains an AAF-lesion, but *polB* mutants are not sensitive to acetylaminofluorine (2).

Recombination is another mechanism that allows lesions to be tolerated rather

than repaired. RecA is required for all recombination in *E. coli* and *recA* mutants are extremely hypersensitive to UV damage (6). While recombinational repair does contribute to survival, cells that depend solely on recombination for processing DNA damage exhibit high rates of lethality and genomic rearrangements (20-22, 39). RecA catalyzes the initial steps of recombination by pairing single stranded DNA with homologous double stranded DNA (26). Early recombination models proposed that following replication, *recA*-mediated strand exchanges with non-damaged homologous regions of the genome allow gaps in DNA opposite lesion-containing regions to be constructed to form complete intact genomes (31). In addition to recombination, RecA binding to DNA also serves to activate the SOS response and, as mentioned earlier to maintain the structural integrity of the replication fork when it is disrupted by UV damage (15).

Although other adducts are known to block replication, the precise mechanism that enables the lesion to be repaired and replication to resume has not been characterized. Therefore, in Chapter II, I examine nitrofurazone, an agent that forms N^2-dG adducts on DNA (Figure 1.3), which are structurally distinct from UV-induced damage, and characterize the requirements for replication recovery by this agent. These requirements are compared to those required for UV.

The repair mechanism recruited to the site of the lesion may also depend on the size of the adduct. DNA-protein crosslinks (DPCs) are very large lesions in which protein become covalently bound to DNA (1, 46). DPCs can be induced by aldehydes, reactive oxygen species, metal compounds, nitrogen mustard, azacytidine, UV and ionizing radiation (1). In comparison to small adducts such as a pyrimidine dimer,

which arrest the polymerase one nucleotide prior to the damaged base, a DPC is unlikely to be able to enter into the active site of the polymerase, and probably would be expected to block synthesis further upstream from the adducted base (Figure 1.3) (1, 34). In this case, how the arrested replication fork is processed is not known. In Chapter III of this thesis, I begin to address this question by identifying the genes that are required for survival and the recovery of replication in the presence of DPCs induced by formaldehyde.

Figure 1.3 Representation of lesions in this study: nucleotide containing an N^2-furfuryl-dG adduct (left) and a DNA-protein crosslink (right). Structures in red denote abnormal covalent modifications.

Whether lesions are repaired or tolerated has significant implications with respect to survival and mutation. In the case of UV, studies support a model in which repair, then translesion synthesis, then recombination operate in this order of priority, frequency and chronology (9, 12). However, it is not known if this order of priority

will extend to other DNA damaging agents. Indeed, some studies prior to this work, have suggested that translesion synthesis may predominate as a mechanism promoting recovery after nitrofurazone, whereas lesions such as DNA-protein crosslinks suggest models that would necessitate alternate pathways, such as recombination (24, 35, 42). Thus, another aim of this thesis is to characterize the relative priorities of the pathways involved in the recovery process following exposure to damaging agents other than UV.

It is reasonable to hypothesize that the structure of the offending lesion plays a role in determining the process recruited to deal with the damage during replication. In this thesis, I investigate the mechanism of replication recovery when challenged by two structurally distinct forms of DNA damage. The requirements for survival and replication recovery from nitrofurazone and formaldehyde were investigated in the following two chapters. In each case, the findings are compared and contrasted with UV-induced DNA damage. Through these studies, we can have a better understanding of how DNA replication is maintained in the face of these diverse challenges.

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CHAPTER II

NITROFURAZONE DISRUPTS REPLICATION THROUGH BOTH DNA DAMAGE-DEPENDENT AND INDEPENDENT MECHANISMS IN *ESCHERICHIA COLI*

ABSTRACT

Nitrofurazone is an antimicrobial agent that, when reduced by cellular nitroreductases, forms metabolites that can damage DNA and inhibit DNA synthesis. In this study, we characterized the mechanism of replication inhibition in *E. coli* treated with nitrofurazone. We found that nitrofurazone exerts its toxicity and affects replication through two distinct mechanisms. When cultures were treated with a prolonged low dose (20 μM) of nitrofurazone, we found that nitrofurazone toxicity was directly associated with DNA damage directly. Based on the genes required for resistance, we infer that nitrofurazone-induced damage encountered during replication is processed similarly to UV-induced damage. Survival following either nitrofurazone or UV-induced damage involves both the RecF and RecBCD pathways, and depends predominantly on nucleotide excision repair. The contribution of translesion synthesis to survival was minor compared to that of nucleotide excision repair and involved Pol IV. By comparison, when high concentrations (200 μM) of nitrofurazone were added to cultures, we observed a rapid inhibition of replication. The high dose inhibition of replication was independent of DNA damage and reversible once the nitrofurazone was removed. We interpret these results to indicate that the antimicrobial activity of

nitrofurazone is mediated through distinct DNA damage-dependent and independent mechanisms.

INTRODUCTION

Replication in the presence of DNA damage is thought to produce most of the mutagenesis, genomic rearrangements, and lethality that occur in all cells. UV-induced photoproducts, x-ray-induced strand breaks, psoralen- or cis platin-interstrand DNA, oxidized bases from reactive oxygen species (ROS), and abasic sites following depurination are just a few of the structurally distinct challenges that the replication machinery must overcome. It seems likely that the mechanisms by which the cell will process these lesions will vary depending on the nature of the impediment.

While a number of the lesions described above are known to block replication, the events associated with UV-induced damage have been the most extensively characterized. UV irradiation causes the formation of cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts on the DNA that block the progression of the replication fork (14, 25, 26, 29). Following the arrest of replication at UV-induced damage, RecA and several RecF pathway proteins are required to process the replication fork such that the blocking lesion is removed or bypassed (2, 5-10). Cells lacking either *recA* or any of several *recF* pathway genes are hypersensitive to UV-induced damage and fail to recover replication following disruption by the lesions (2, 4, 7, 8).

The *recBCD* pathway is also required for resistance to UV-induced damage, although it is not associated with processing or restoring disrupted replication forks directly (1, 7, 16). RecB, -C and -D form an exonuclease/helicase that is involved in

repairing double strand breaks (30). To date however, the lesions or substrates requiring RecBCD-mediated repair after UV irradiation remain unclear.

Survival following UV-induced damage predominantly depends on the removal of the lesions by nucleotide excision repair (27). The ability of nucleotide excision repair to rapidly restore DNA synthesis requires processing of the fork by the RecF-pathway genes, indicating that repair is likely to be coupled with replication (5, 6, 8). In the absence of RecF-pathway processing or repair, the recovery of replication is delayed and survival becomes entirely dependent on translesion synthesis by DNA polymerase V (Pol V) (6). However in repair proficient cells, the contribution of translesion synthesis to the recovery of replication and survival is minor and is only detected following UV doses that exceed the repair capacity of the cell (5, 6).

Less is known about how replication recovers from other forms of DNA damage. We initially chose to characterize the cellular response to nitrofurazone because it forms a structurally distinct DNA lesion that a number of studies have suggested would be processed differently than UV-induced lesions. Nitrofurazone is an antimicrobial agent that has been used to treat skin infections in patients and animals since the 1940s (20). Following activation by cellular nitroreductases, it reacts with the N^2-position of guanine to form an N^2-furfuryl-dG adduct (15, 24). In nitrofurazone-treated *Escherichia coli,* early studies noted that the mutation frequency correlated directly with lethality, leading to the idea that nitrofurazone's toxicity is directly associated with its ability to form lesions in DNA (17, 19, 23, 32). In addition, these studies noted that nitrofurazone inhibits DNA synthesis, an observation consistent with the idea that these lesions disrupt replication.

Whereas nucleotide excision repair is the predominant mechanism promoting survival and recovery of replication after UV-induced damage, several lines of evidence suggest that translesion synthesis might be more important for nitrofurazone-induced DNA damage. *uvrA* mutants, defective in nucleotide excision repair, are reported to be equally resistant to nitrofurazone as wild-type cells (17, 23). Consistent with this observation, the predominant lesions induced by nitrofurans were found to be poor substrates for nucleotide excision repair *in vitro* (34). More recently, cells constitutively expressing the translesion DNA polymerases were shown to be hypersensitive to nitrofurazone when Pol IV was inactivated (15). Furthermore, Pol IV homologs from other organisms have been shown to efficiently replicate over N^2-dG adducts *in vitro* (15, 28, 33).

Taken together, these observations suggest that the cellular response to nitrofurazone will be distinct from its response to UV irradiation; however several aspects remain to be characterized. It is not known whether DNA is the primary target of nitrofurazone or how DNA lesions generated by nitrofurazone are processed during replication, nor is it known whether these lesions are subject to repair. Additionally, no study to date has directly compared the relative contributions of nucleotide excision repair and translesion synthesis in the recovery from nitrofurazone-induced damage. In this study, we characterized the mechanism by which nitrofurazone inhibits DNA replication and identified the genes that contribute to the recovery and survival of *E. coli* treated with nitrofurazone. We found that nitrofurazone inhibits replication through both DNA damage-dependent and independent mechanisms. Similar to UV-induced DNA damage, we found that survival following nitrofurazone-induced

damage predominantly depends on nucleotide excision repair, RecF- and RecBC-pathways. The contribution of translesion polymerases to survival was minor and involved Pol IV rather than Pol V. In addition, we observed that high concentrations of nitrofurazone inhibited DNA replication independent of DNA damage, suggesting that DNA may not be the primary target of its antimicrobial activity.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study were derived from SR108, a *thyA36 deoC2* derivative of W3110 (25). SR108, HL921 [SR108 (srlR-*recA*)306::tn10], HL952 (SR108 *uvrA*::Tn10), CL579 (SR108 *recF6206*::Tet^r), HL924 (SR108 *recJ284*::Tn10), CL575 (SR108 *umuC122*::Tn5), CL634 (SR108 *dinB*::Kan^r), CL636 (SR108 *polB*::Ω Sm-Sp), CL646 (SR108 *polB*:: Ω Sm-Sp *dinB*::Kan^r *umuDC595*::cat) and CL681 (SR108 *polB*:: Ω Sm-Sp *dinB*::Kan^r *umuDC595*::cat *uvrA*::Tn10) have all been reported previously (5, 8-10, 12, 31).

Survival studies. Overnight cultures were grown in Davis media supplemented with 0.4% glucose, 0.2% casamino acids and 10 μg/ml thymine (DGCthy media) (11). Tenfold serial dilutions of these cultures were spotted in triplicate on LB plates containing 10 μg/ml thymine and nitrofurazone (from TCI America) at the indicated concentrations. For UV, plates containing spotted dilution of the cultures were UV-irradiated at the indicated doses using a 15-W, 254-nm Sylvania germicidal lamp at an incident dose of 0.9 J/m^2/s. The plates were incubated at 37°C and the surviving colonies were counted after 48 hours for nitrofurazone treatments or overnight for UV. For acute exposures to nitrofurazone, overnight cultures were sub-cultured 1:100 in

DGCthy and grown in a shaking, 37°C water bath until it reached an OD_{600} of 0.3. At this time, the culture was split. One half received 200 μM nitrofurazone, while the other half was mock-treated and incubation continued for 20 minutes. Cells from each culture were then collected on a Fisherbrand 0.45 μm general membrane filter, washed once with Davis media and resuspended in fresh drug-free DGCthy media. Tenfold serial dilutions of these cultures were spotted in triplicate on LBthy plates and incubated in 37°C overnight. The surviving colonies were counted the following day.

Growth curves. 10^4 and 10^5 cells/ml from fresh overnight cultures were inoculated into a 96-well microtiter plate containing 0, 5 or 10 μM nitrofurazone in DGCthy media. The 96-well microplate was then incubated at 37°C with constant agitation in an incubating plate reader (Bio Whittaker, Model ELX808) and the absorbance at 560 nm was determined at 20-minute intervals.

Rate of replication in the presence of nitrofurazone. Fresh overnight cultures of each strain were sub-cultured 1:100 in DGCthy media supplemented with 0.1 μCi/ml ^{14}C-thymine and grown in a shaking, 37°C water bath to an OD600 of exactly 0.3. The culture was then split equally into four conical tubes, before adding 0, 25, 100 or 200 μM nitrofurazone. Immediately after drug addition and at each time indicated, duplicate 0.5 ml aliquots were pulse-labeled for two minutes with 0.1 μCi/ml ^{3}H-thymidine. The cells were then lysed and the DNA was precipitated by the addition of ice-cold 5% trichloroacetic acid (TCA). The precipitated DNA was collected on Fisherbrand 0.45 μm glass fiber filters, washed twice with ethanol and then dried before the amount of radioactivity on each filter was determined by scintillation using

Biosafe scintillation fluid (RPI Corp.) and a scintillation counter TriCarb 2800 TR, Perkin Elmer.

Recovery of replication assays following removal of nitrofurazone. Fresh overnight cultures were sub-cultured 1:100 in DGCthy media supplemented with 0.1 μCi/ml ^{14}C-thymine and grown in a shaking, 37°C water bath to an OD_{600} of exactly 0.3. The culture was then split equally into two flasks. At this time, half of the sub-culture was treated with 200 μM nitrofurazone for 20 minutes while the other half was mock-treated. The cells in each culture were then collected on-to Fisherbrand 0.45 μm general membrane filters and resuspended in prewarmed DGCthy media containing 0.1 μCi/ml ^{14}C-thymine. At the indicated times, duplicate 0.5ml aliquots were pulse-labeled for two minutes with 0.1 μCi/ml ^{3}H-thymidine. The cells were then lysed and the DNA was precipitated, collected and its radioactivity was determined as described above.

RESULTS AND DISCUSSION

RecF-, RecBC-, and nucleotide excision repair pathways are important for cell survival in the presence of nitrofurazone.

If nitrofurazone toxicity results directly from DNA damage, we would expect to see mutants deficient in processing DNA damage to be hypersensitive to the drug relative to wild- type cells. To examine this question, we compared the survival of various repair mutants to the survival of wild-type cultures when grown in the presence of nitrofurazone. Serial dilutions of cultures were spotted on LB plates containing nitrofurazone at varying concentrations and the number of surviving

colonies at each concentration was determined. For comparison, the survival of each mutant following exposure to UVC irradiation was also determined. We found that similar to the case of UV exposure, cells lacking *recA, recF, recBC* and *recJ* were hypersensitive to nitrofurazone relative to the parental strain (One-tailed Student's T-test, *p*-values < 0.1 for concentrations above 2μM nitrofurazone) (Figure 2.1A). In almost every case, the relative hypersensitivity of each mutant to nitrofurazone was similar to that seen for UV irradiation (Figure 2.1A). The exception to this was *recJ,* which was modestly more sensitive to nitrofurazone than it was to UV irradiation.

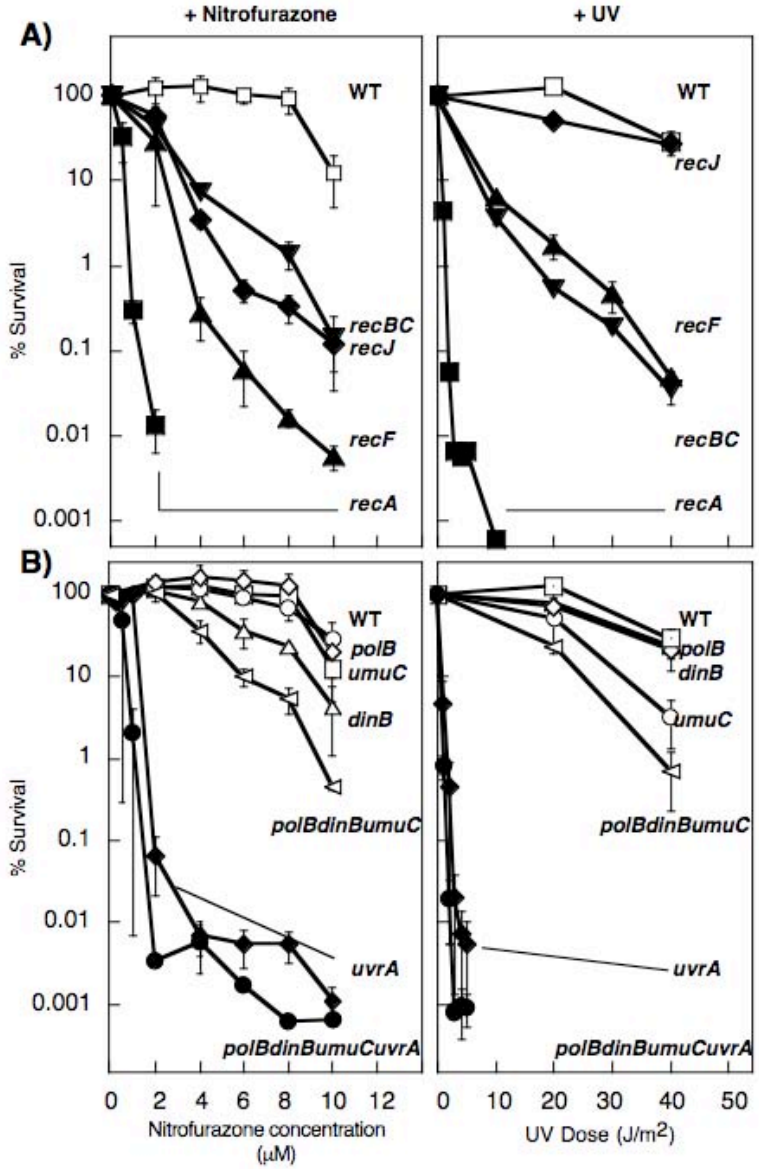

Figure 2.1 The RecBCD, RecF and nucleotide excision repair pathways are required for resistance to nitrofurazone. A.) The survival of wild-type (□); *recA* (■); *recBC* (▼); *recF* (▲) and *recJ* (◆) mutants on plates containing nitrofurazone or exposed to UV irradiation at the indicated dose is plotted. B.) The survival of wild-type (□); *uvrA* (●); *dinB*(◇); *umuC* (△); *polB* (○) and *polBdinBumuC* (◁) mutant. Cells were treated the same way as in (A). Error bars represent the standard error of the mean from at least 3 independent experiments.

Next, we compared the relative contribution that nucleotide excision repair and translesion synthesis have onto survival in the presence of nitrofurazone. In contrast to previous studies, we found that mutants defective in nucleotide excision repair were

hypersensitive to nitrofurazone (Figure 2.1B) (17, 23). The discrepancy between our results and those of earlier studies can be explained by the shorter exposure times used in previous studies. When we treated cultures with nitrofurazone for only 20 minutes and then removed the drug, *uvrA* mutants exhibited no hypersensitivity, consistent with earlier studies (see subsequent sections). We interpret this observation to indicate that significant levels of nitrofurazone-induced DNA damage are not induced within the 20 minute time period. With respect to translesion DNA polymerase mutants, we found that only Pol IV (*dinB* gene product) detectably contributed to survival in the presence of nitrofurazone, consistent with the results of Jarosz *et al* (2006). Mutants lacking either Pol V (*umuDC* gene product) or Pol II (*polB* gene product) were as resistant as wild-type cultures at all nitrofurazone concentrations examined. In the absence of all three polymerases, cells were modestly more sensitive than *dinB* mutants alone, raising the possibility that in the absence of Pol IV, Pol II or Pol V may also participate in processing nitrofurazone-induced damage. Surprisingly, the *uvrA* mutant was significantly more sensitive relative to mutants lacking the translesion DNA polymerases (One-tailed Student's T-test, p-value < 0.1 for all concentrations above 2μM, Figure 2.1B). Previous biochemical data suggested that adducts induced by some nitrofurans were recalcitrant to repair and may be processed primarily by translesion synthesis (34, 15). However, the observed hypersensitivity of *uvrA* mutants compared to translesion synthesis suggests that *in vivo*, repair of nitrofurazone-induced damage by nucleotide excision repair is a prominent mechanism of survival.

As an alternative approach, we also monitored the growth of cultures in media containing different nitrofurazone concentrations. To this end, 10^4 or 10^5 cells from

fresh overnight cultures were inoculated in media containing 0, 5, or 10 μM nitrofurazone. The relative growth of each culture was measured by the change in absorbance of 560 nm light over time. We observed that both cell density and nitrofurazone concentration affected the extent of growth inhibition in culture (Figure 2.2). For example, when media containing 5 or 10 μM nitrofurazone was inoculated with 10^5 wild-type cells/ml, visible growth of the culture was delayed by 1 or 3 hours relative to cultures without nitrofurazone, respectively (Figure 2.2). However, when ten-fold fewer wild-type cells were inoculated into the same media, we observed that the growth of the culture was delayed by 3 and 9 hours relative to unexposed cultures, respectively.

Mutants that were hypersensitive to nitrofurazone on LB agar plates were also more sensitive to nitrofurazone in liquid medium, but again the sensitivity was dependent on both nitrofurazone concentration and cell density. Growth of *uvrA* cultures inoculated with 10^4 cells/ml was completely inhibited by media containing 5 μM nitrofurazone, but cultures inoculated with ten fold more cells were able to grow in this media. By comparison, *recA* mutants failed to grow within the time frame of the assay at all doses and culture dilutions. In the case of all other mutants examined, the extent of the growth delay correlated with their relative sensitivity on LB agar plates containing nitrofurazone. Interestingly, the effect of nitrofurazone on growth in liquid culture was associated with a prolonged lag phase, rather than an overall increase in the doubling time of the growing cultures. Once visible growth had begun, cultures grown in the presence of nitrofurazone grew as quickly as cultures grown in

the absence of nitrofurazone. This delayed onset of exponential growth was observed even in mutants that were hypersensitive to nitrofurazone such as *uvrA* cells.

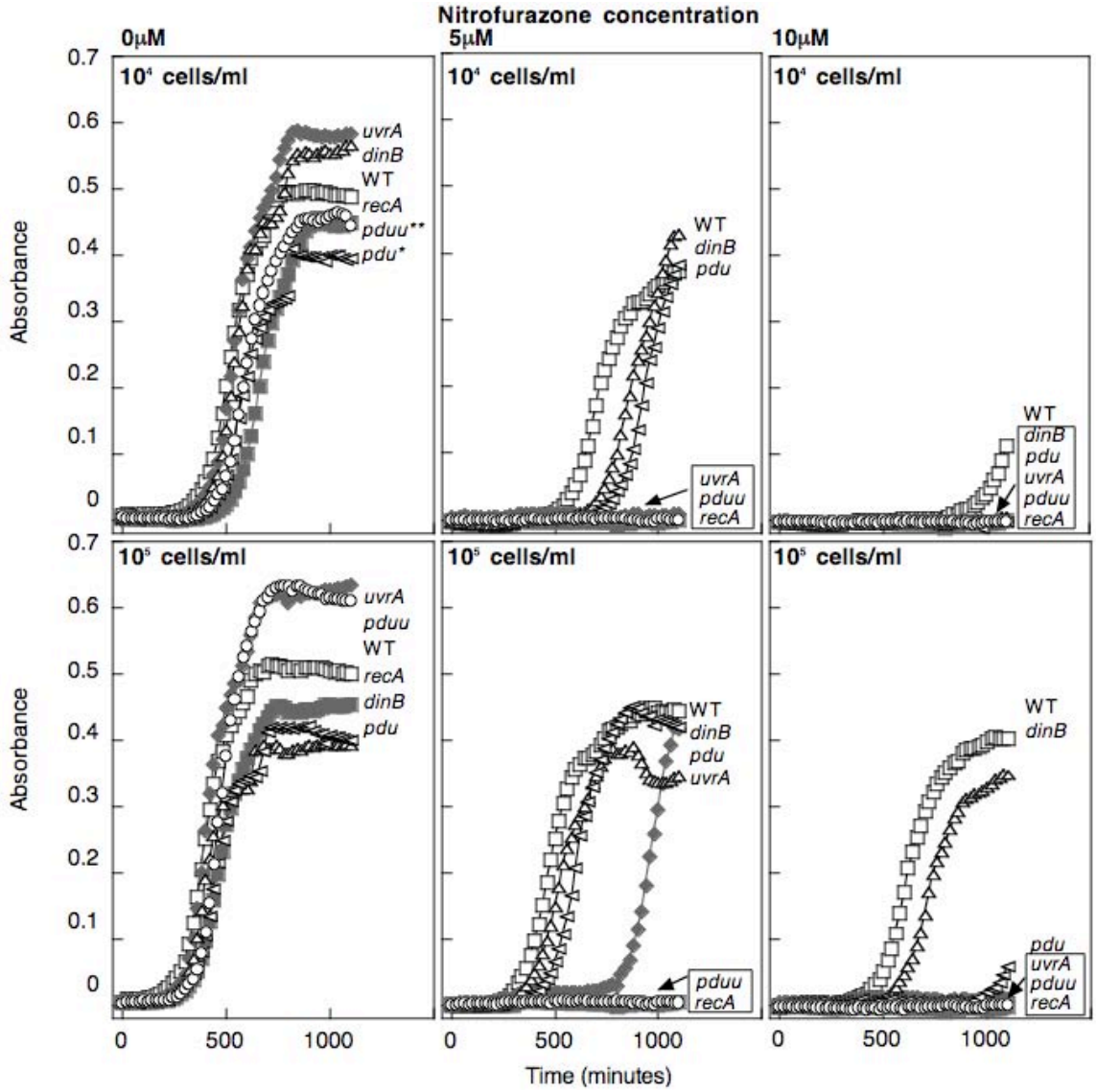

Figure 2.2 Growth inhibition by nitrofurazone depends on both the concentration of the cells and the drug. The growth, as measured by absorbance (560 nm), of wild-type (□), *recA* (■), *uvrA* (●), *dinB*(◇), *polBdinBumuC* (△) and *polBdinBumuCuvrA* (◆) cultures in the presence of nitrofurazone is plotted over time. Cultures were inoculated with 10^4 or 10^5 cells/ml as indicated. Graphs represent one of two individual experiments.

High concentrations of nitrofurazone inhibit DNA synthesis independently from nitrofurazone-induced DNA damage.

The previous results are consistent with the idea that the toxicity of nitrofurazone is associated with the formation of DNA lesions. To examine whether lesions generated by nitrofurazone impede the progress of DNA replication directly, we monitored the rate of DNA synthesis following nitrofurazone addition at different concentrations. To this end, aliquots of ^{14}C-thymine pre-labeled cultures were pulsed with ^{3}H-thymidine for two minutes at various times after the addition of 0, 25, 100 or 200 μM nitrofurazone and the amount of [^{3}H] incorporated into DNA was quantified. If nitrofurazone forms lesions that block DNA replication, we would expect that the amount of ^{3}H incorporation into DNA would decline over time. The ^{14}C-thymine prelabel allowed us to simultaneously monitor total DNA accumulation and served to control for pipetting errors.

In wild-type cultures, the rate of DNA synthesis was unaffected by the addition of 25 μM nitrofurazone. Following the addition of 100 μM nitrofurazone, the rate of DNA synthesis was initially inhibited by ~50% but returned to pretreatment levels within one-hour after addition. Addition of 200 μM nitrofurazone was sufficient to rapidly inhibit DNA synthesis for the duration of the time course (Figure 2.3A). By comparison, the rate of synthesis in *recA* cells was progressively reduced at each of the increasing concentrations examined, consistent with its hypersensitivity to nitrofurazone. DNA synthesis in *recF* mutants was more sensitive to inhibition by nitrofurazone than wild-type cultures, although the effect was less severe than in *recA*

mutants (Figure 2.3A). In *recBC* cultures, DNA synthesis was not substantially more sensitive to inhibition by nitrofurazone than in wild-type cultures (Figure 2.3A).

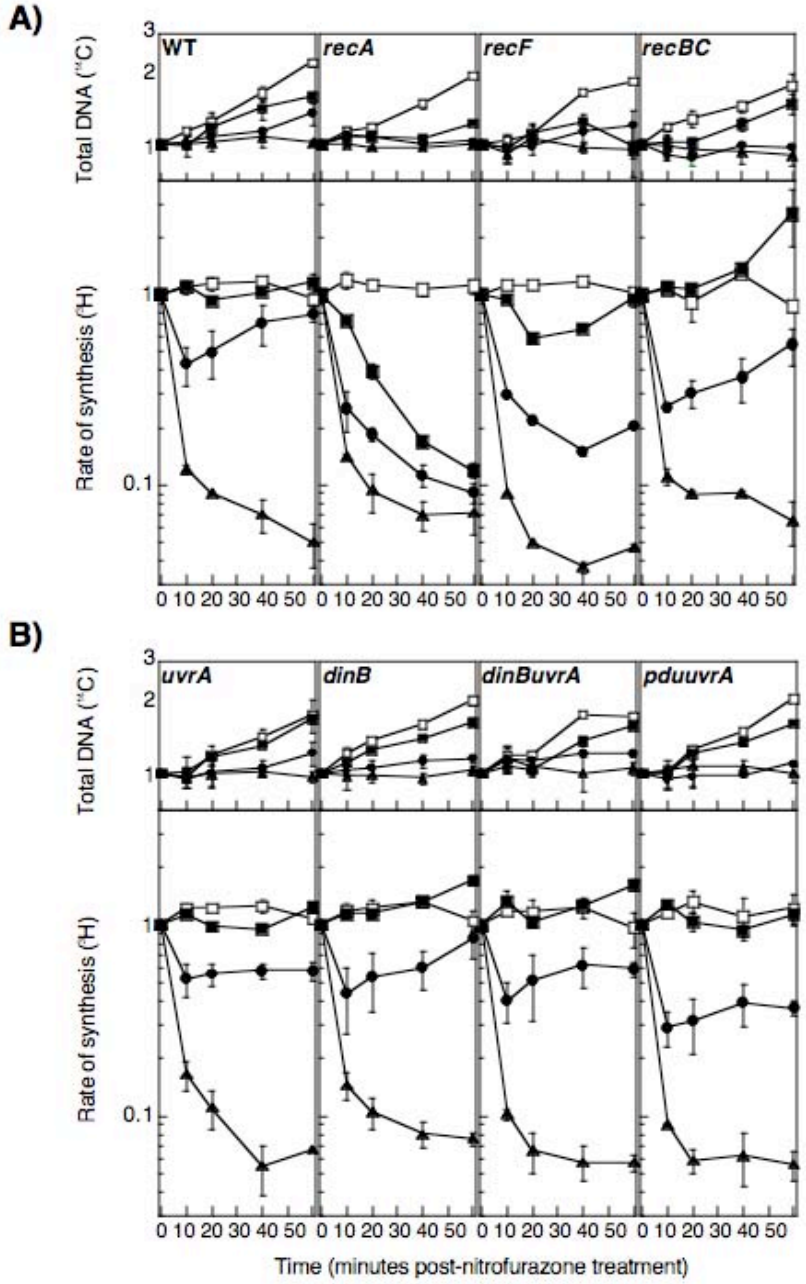

Figure 2.3. *recA* mutants are less able to synthesize DNA in the presence of nitrofurazone than wild-type cells. A.) DNA synthesis of recombination mutants in the presence of nitrofurazone. At the indicated times, aliquots of culture grown in 14C-thymine-containing media were pulse-labeled with [^{3}H]-thymidine for two minutes. The amount of [^{3}H] and [^{14}C] incorporated into the DNA is plotted relative to the amount incorporated just prior to the addition of nitrofurazone. (□) 0μM; (■) 25μM; (●) 100μM; (▲) 200μM nitrofurazone. B.) DNA synthesis of nucleotide excision repair and translesion DNA polymerase mutants in the presence of nitrofurazone. Error bars represent standard error of the mean from at least 2 independent experiments.

We also examined the role that nucleotide excision repair and translesion synthesis in maintaining replication in the presence of nitrofurazone. We found that the rate of DNA synthesis in mutants lacking excision repair, translesion synthesis, or both processes were no more sensitive to nitrofurazone in the media than wild-type cultures (Figure 2.3B). In each of these mutants, complete inhibition of DNA synthesis required the addition of 200 μM nitrofurazone to the media, similar to wild-type cultures. These results were unexpected for two reasons. First, while we did observe a rapid arrest of DNA synthesis in all strains at 200 μM, this dose was an order of magnitude greater than the dose required to inhibit growth on plates or in liquid media (Figure 2.1 and data not shown). The second unexpected observation was that, with the exception of *recA* mutants, DNA synthesis in the presence of nitrofurazone was not inhibited in nitrofurazone-sensitive mutants any more than it was in wild-type cultures. This contrasts with what is observed after UV-irradiation (5-10). In the case of UV-induced damage, mutants deficient in removing the lesions that block replication are inhibited at lower doses than wild-type cultures. We can envision two possibilities to explain these results. One possibility is that there is a threshold concentration below which the cells are able to process or inactivate the nitrofurazone before it can react with the DNA to form lesions. Alternatively, it is possible that induction of DNA lesions occurs relatively slowly and that the inhibition of replication at the 200 μM concentration is due to inactivation of a second cellular target of nitrofurazone, other than the DNA.

To differentiate between the two possibilities described above, we asked which gene products are required for replication to recover once nitrofurazone was removed

from the media. If a threshold concentration of nitrofurazone was required to induce DNA damage and inhibit replication, then mutants defective in processing nitrofurazone-induced damage should fail to recover replication when nitrofurazone is removed. Alternatively, if 200 μM nitrofurazone inhibits replication independently from DNA damage, then we would expect that the hypersensitive strains might recover DNA synthesis once nitrofurazone is removed. To this end, ^{14}C-thymine-prelabeled cultures were either mock treated or exposed to 200 μM nitrofurazone for 20 minutes (a dose and time that inhibits synthesis by more than 90% in all strains, Figure 2.3). The cells were then collected on filters and resuspended in fresh drug-free medium.

In wild-type cultures, we observed that DNA synthesis began to recover within the first 5 minutes after the nitrofurazone was removed, reaching pretreatment levels within 30 minutes of incubation in drug-free medium (Figure 2.4A). The total DNA accumulation, as measured by ^{14}C-thymine incorporation, also recovered once the drug had been removed. Suprisingly, when we examined cultures of *recA* mutants, we observed that they began to recover replication with kinetics similar to that of wild-type cultures upon removal of the drug from the medium. However after 30 minutes, the rate of DNA synthesis slowed, and then began to decline once again (Figure 2.4A).

Curiously, the rate of DNA synthesis recovered similarly to that of wild-type cultures for all other strains examined, including *recF, recBC* and *uvrA,* (Figure 2.4A). The observation that all hypersensitive mutants recover DNA synthesis following removal of the drug suggests that the inhibition of replication in the presence of 200 μM nitrofurazone is not due to lesions blocking or impeding the progress of the

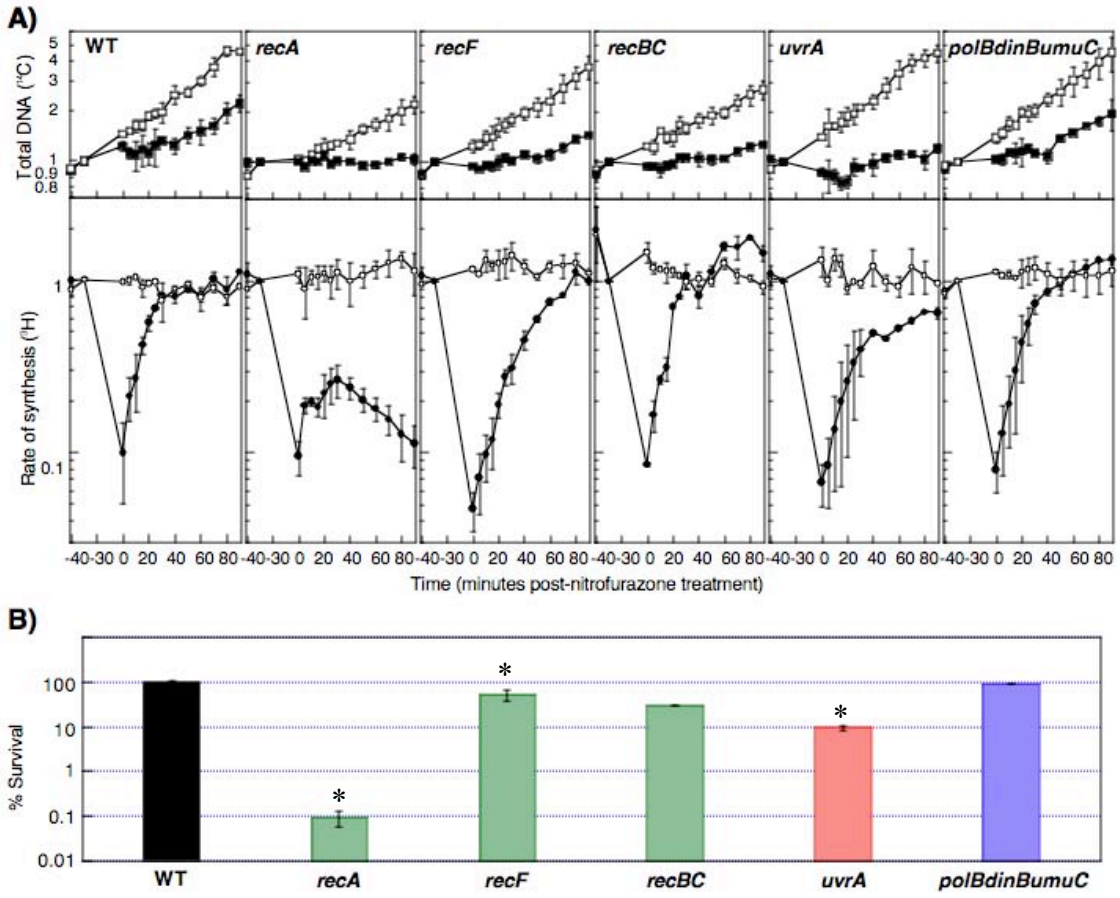

Figure 2.4. Recovery of DNA synthesis after nitrofurazone occurs independently of *recA, recF, recBC uvrA* and the translesion DNA polymerases. A.) Cultures grown in ^{14}C-thymine are treated with 200μM for 20 minutes and then cells were collected on filters and resuspended in fresh drug-free media. At the indicated times, duplicate aliquots of the culture were pulsed with ^{3}H-thymidine for two minutes. The amount of [^{3}H] and [^{14}C] incorporated in the DNA at each time point is plotted relative to the amount incorporated just prior to nitrofurazone addition. (□) 14C, mock-treated; (■)14C, treated; (○) 3H, mock-treated ; (●) 3H, treated. Error bars represent the standard error from two independent experiments**.** B.) Survival of cells following a 20-minute esposure to 200μM nitrofurazone. Error bars represent the standard error of the mean from two independent experiments. * denotes statistical significance relative wild-type cultures (one-tailed Student's T-test, $p<.05$)

replication fork. Consistent with this interpretation, when we measured the survival of each mutant following the 20-minute exposure to 200 μM nitrofurazone we found that in contrast to the results obtained on nitrofurazone plates, the viability of the *uvrA, recF* and *recBC* mutants was not substantially reduced (Figure 2.4B). Thus, we interpret these results to indicate that the inhibition of replication following a high

acute dose of nitrofurazone is not due to the formation of DNA lesions. Rather, it appears to target a second metabolic process required for DNA synthesis to occur. The inhibition is reversible since DNA synthesis recovers immediately upon removal of the drug, even in *recA* mutants. That the rate of synthesis in recovering *recA* cultures begins to decline 30 minutes after the drug is removed is presumably due to DNA damage encountered by replication and is consistent with the idea that the induction of nitrofurazone-induced DNA damage occurs relatively slowly. Previous studies have shown that nitrofurazone negatively affects other aspects of metabolism in *E. coli,* including ATP levels, oxygen consumption, and the conversion of glucose and pyruvate to CO_2 (13, 18). In these studies, the authors were unable to determine whether the inhibition of DNA synthesis occurred directly due to arrest by DNA damage, or indirectly due to decreases in cellular metabolism. The results presented here strongly support the idea that inhibition can occur independently from DNA damage. When we treated cells for 20 minutes with 100 mM sodium azide, which depletes intracellular ATP levels, we observed a similar inhibition of DNA synthesis (data not shown) (3). However, the inhibition that occurred following sodium azide addition occurred less rapidly than following nitrofurazone treatment, suggesting that the cellular target of nitrofurazone affects some aspect of replication more directly.

These results support a model in which the toxicity of nitrofurazone is mediated through two distinct mechanisms. At higher concentrations, nitrofurazone targets an as yet undetermined aspect of metabolism that results in a reversible inhibition of DNA synthesis. The inhibition is not due to DNA lesions that block the replication machinery since mutants lacking the enzymes to remove or tolerate

nitrofurazone-induced lesions are not hypersensitive to acute drug exposure and recover DNA synthesis when the drug is removed. Following a prolonged exposure to lower nitrofurazone doses that are subinhibitory to replication, we observed that the toxicity of nitrofurazone was associated with the formation of DNA damage, as has previously been proposed. Based on the genes that are required for survival during chronic nitrofurazone exposure, we infer that the lesions are processed and repaired similar to UV-induced lesions.

Also similar to UV-induced damage but in contrast to previous studies, we observed that nucleotide excision repair was the predominant mechanism required for surviving nitrofurazone. Previous studies concluding that nucleotide excision repair was not involved in processing these lesions utilized high concentrations of nitrofurazone in cultures with short (20 minute) exposure times (17, 23). In this study, we showed that these conditions inhibit replication but are not sufficient in duration to induce significant levels of DNA damage. In contrast to UV but consistent with previous reports, we found that Pol IV contributed to survival rather that Pol V, although the effect was modest relative to nucleotide excision repair (15, 28).

The dual targets of nitrofurazone toxicity suggest that the antibiotic may have both bacteriostatic and bactericidal activity. While the reversible inhibition of DNA synthesis may be bacteriostatic, nitrofurazone-induced DNA damage would be predicted to be bactericidal. A number of studies report that increased resistance to nitrofurazone can be conferred by mutations that inactivate the cellular nitroreductases (24, 21, 22). However, another study reported that aspects of the toxicity associated with some nitrofuran derivatives do not require cellular activation (18). In light of the

results presented here, it would be of interest to examine whether nitrofurazone resistance is associated with reduced levels of DNA damage, a failure to inhibit replication, or a combination of both these mechanisms.

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CHAPTER III

INHIBITION AND RECOVERY OF DNA REPLICATION FOLLOWING TREATMENT WITH FORMALDEHYDE IN *ESCHERICHIA COLI*

ABSTRACT

DNA-protein crosslinks (DPCs) are large adducts that block DNA polymerases. In this study, we characterized the genetic requirements for survival and the recovery of replication following formaldehyde-induced DPCs. We found that survival in the presence of formaldehyde depends primarily on *recA, recBC,* and to a lesser extent, *uvrA*. *recF* and translesion polymerase mutants were not detectably hypersensitive to formaldehyde. We examined the role that these genes have in the recovery of replication by monitoring the incorporation of radioactive nucleotides into DNA after formaldehyde treatment and observed that recovery was impaired in the absence of *recA, recBC* or *uvrA.* These results are discussed in the context of current models for protein-DNA crosslinks.

INTRODUCTION

DNA-protein crosslinks (DPCs) are large adducts that result from the covalent linkage of protein and DNA. Proteins that are normally associated with DNA, such as those found in the nuclear membrane, nuclear matrix, histones, topoisomerases, DNA repair proteins and DNA polymerases, have all been found to crosslink DNA in the presence of a variety of DNA damaging agents like UV, X-rays, reactive oxygen

species, aldehydes and metals (reviewed in (2)). Our current understanding of the biological significance of these lesions is fragmentary, owing in part to the fact that the agents that generate crosslinks also induce other forms of DNA damage, making it difficult to identify the specific contributions of crosslinks to the phenotypes and toxicity induced by these agents. Nevertheless, the underlying issue for any lesion is how the cell processes the damage such that the DNA can be replicated.

Although several studies in bacteria, yeast and mammals have demonstrated that DNA-protein crosslinks inhibit DNA replication, it is not known how replication processes or recovers from this form of DNA damage (3, 5, 19). Unlike DPCs, the repair of DNA damage induced by UV during DNA replication has been extensively characterized. Near-UV light causes the formation of covalent bond between adjacent pyrimidines and generates cyclobutane pyrimidine dimers or (6-4) photoproducts that distort the double helix (22, 23). When the replication apparatus encounters either of these lesions, it arrests at one nucleotide prior to the damage (24). At this point, the stalled replication fork must be processed before accurate replication can continue. Based on a combination of cellular approaches, it has been shown that the nascent lagging strand at the site of the blocked fork is degraded by the combined action of RecQ helicase and RecJ nuclease, effectively moving the branch point of the replication fork backwards (12, 30, 31). This processing helps target RecA, along with RecF, -O and -R to bind and structurally maintain the replication fork and restores the region surrounding the lesion to a double stranded form, enabling nucleotide excision repair (NER) (6, 8-10, 12).

Several observations suggest protein-DNA crosslinks will be processed and repaired differently from UV-induced damage. The large size of the DPC arrests replication several bases upstream from the adducted base due to steric hindrance (3). In addition, there are conflicting reports about whether nucleotide excision repair participates in the repair of DPCs. Although nucleotide excision repair can recognize bulky adducts that extend over multiple bases, DPC are too large to be substrates for nucleotide excision repair and are recognized poorly *in vitro* (20, 26, 27). *In vivo*, formaldehyde-induced DPC are repaired with similar kinetics in both wild-type and nucleotide excision repair mutants in both yeast and mammalian cells (16, 17, 19, 26, 28).

Other studies, mostly in bacterial systems, have reported that nucleotide excision repair mutants are modestly more sensitive to DPC agents than wild-type cells and have speculated that proteases may be required to partially digest the DPC before nucleotide excision repair can recognize it (16, 19). Consistent with this, Nakano *et al.* demonstrated that nucleotide excision repair is capable of removing DPCs smaller than 14 kDa whereas larger DPCs were removed poorly and repaired through a recombination–dependent mechanism that requires *recA* and *recBC* (25).

Despite the fact that DPCs are produced by a range of DNA damaging agents and disrupt vital processes such as DNA replication and transcription, the mechanism by which DPCs are processed during replication remain poorly characterized. In this study, we characterized several candidate mutants for their ability to survive and replicate in the presence of formaldehyde-induced DPCs. Formaldehyde induces DNA-protein crosslinks by reacting with the amino group of the protein and the

exocyclic amino group of DNA bases, forming a covalent linker between the two macromolecules (2). We found that survival in the presence of formaldehyde requires *recA* and *recBC* but not *recF* or any of the three known translesion DNA polymerases. Mutants lacking *uvrA* are only modestly hypersensitive to formaldehyde but are impaired in recovering replication. In contrast, recovery in *recBC* mutants is delayed but then resume with wild-type kinetics, suggesting that the hypersensitivity of *recBC* mutants is not associated with an inability to recover DNA replication.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study were derived from SR108, a *thyA36 deoC2* derivative of W3110 (25). SR108, HL921 [SR108 (srlR-*recA*)306::tn10], HL952 (SR108 *uvrA*::Tn10), CL2 (SR108 *recB*::argA81 *recC*::Tn10), CL579 (SR108 *recF6206*::Tet^r), HL924 (SR108 *recJ284*::Tn10), CL575 (SR108 *umuC122*::Tn5), CL634 (SR108 *dinB*::Kan^r), CL636 (SR108 *polB*::Ω Sm-Sp), CL646 (SR108 *polB*:: Ω Sm-Sp *dinB*::Kan^r *umuDC595*::cat) and CL681 (SR108 *polB*:: Ω Sm-Sp *dinB*::Kan^r *umuDC595*::cat *uvrA*::Tn10) have all been reported previously (7, 10-12, 15, 32).

Survival studies. Overnight cultures were grown in Davis media supplemented with 0.4% glucose, 0.2% casamino acids and 10 μg/ml thymine (DGCthy media) (14). Tenfold serial dilutions of these cultures were spotted in triplicate on LB plates containing 10 μg/ml thymine and formaldehyde (from Fisher Scientific) at the indicated concentrations. The plates were incubated at 37°C and the surviving colonies were counted overnight. For acute exposures to formaldehyde, overnight cultures were diluted 1:100 in DGCthy and grown in a shaking, 37°C water bath to an OD_{600} of 0.3.

At this time, the culture was split. Half of the culture received 5 mM formaldehyde, while the other half was mock-treated and incubation continued for 15 minutes. Cells from each culture were then collected on a Fisherbrand 0.45 μm general membrane filter, washed once with Davis media and resuspended in fresh drug-free DGCthy media. Tenfold serial dilutions of these cultures were spotted in triplicate on LBthy plates and incubated in 37°C overnight. The surviving colonies were counted the following day.

Recovery of replication assays following removal of formaldehyde. Fresh overnight cultures were sub-cultured 1:100 in DGCthy media supplemented with 0.1 μCi/ml ^{14}C-thymine and grown in a shaking, 37°C water bath to an OD_{600} of exactly 0.3. The culture was then split equally into two flasks. At this time, half of the sub-culture was treated with 5 mM formaldehyde for 15 minutes while the other half was mock-treated. The cells in each culture were then collected on-to Fisherbrand 0.45 μm general membrane filters and resuspended in prewarmed DGCthy media containing 0.1 μCi/ml ^{14}C-thymine. At the indicated times, duplicate 0.5 ml aliquots were pulse-labeled for two minutes with 0.1 μCi/ml ^{3}H-thymidine. The cells were then lysed and the DNA was precipitated by the addition of ice-cold 5% trichloroacetic acid (TCA). The precipitated DNA was collected on Fisherbrand 0.45 μm glass fiber filters, washed twice with ethanol and then dried before the amount of radioactivity on each filter was determined by scintillation using Biosafe scintillation fluid (RPI Corp.) and a scintillation counter TriCarb 2800 TR (Perkin Elmer).

RESULTS

Survival in the presence of formaldehyde requires *recA, recBC,* and to a lesser extent, *uvrA*

To address which genes are required for resistance to DNA-protein crosslinks, we examined the survival of several candidate mutants impaired in recombination, repair, or translesion synthesis functions when grown in the presence of formaldehyde. Serial dilutions of overnight cultures were spotted on plates containing formaldehyde at varying concentrations. The surviving colonies were then counted and compared to the number of colonies on plates without formaldehyde. In all cultures that were examined, we observed that doses below 0.6 mM formaldehyde did not reduce cell survival (Figure 3.1A). At concentrations above 0.6 mM, the survival of all cultures was affected to some extent. In wild-type cultures, survival was reduced by approximately two orders of magnitude at a dose of 1.2 mM formaldehyde.

With respect to recombination, mutants lacking either *recBC* or *recA* were impaired in their survival at doses between 0.6 to 1.2 mM (one-tailed T-test, p-value < 0.1 for 1 mM formaldehyde, Figure 3.1A). In contrast, *recF* or *recJ* mutants were not sensitive to formaldehyde (Figure 3.1A). This is distinct from what is observed after UV-induced DNA damage where both the *recBC* and *recF* pathways contribute to survival. After this study was initiated, Nakano *et al,* reported similar observations regarding the sensitivity of *recF, recBC* and *recA* mutants to formaldehyde (25).

We also examined the role that nucleotide excision repair and translesion synthesis had in survival following formaldehyde exposure. We observed that *uvrA* mutants were modestly more sensitive compared to wild-type cells at the 1 mM dose,

although the effect was not seen at lower doses (Figure 3.1A). Two earlier studies reported that *uvrA* mutants were moderately more sensitive to formaldehyde than wild type cells (16, 29). These studies exposed cells for 30 minutes and did not require cells to grow in the presence of formaldehyde as we did. This may account for the differences observed between our results and those previous studies. Mutants lacking Pol II, Pol IV, Pol V or all three translesion polymerases were not detectably more sensitive to formaldehyde than wild-type cells (Figure 3.1B). A quadruple mutant lacking the translesion polymerases and nucleotide excision repair, *polB dinB umuC uvrA,* was as sensitive as the *uvrA* single mutant. The observation indicates that translesion synthesis does not play a major role in survival from formaldehyde-induced damage.

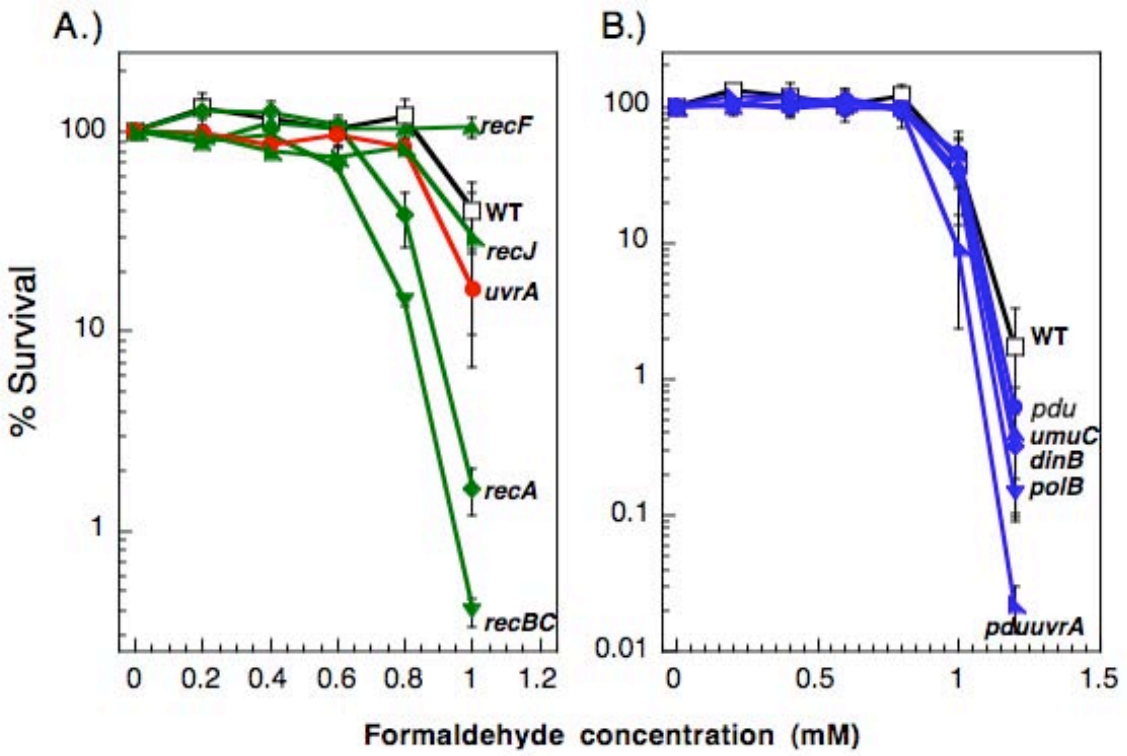

Figure 3.1. Resistance to formaldehyde requires *recA, recBC,* and to a lesser extent, *uvrA*. A.) The survival of wild-type (□); *recA* (■); *recBC* (▼); *recF* (▲) and *recJ* (◆) mutants on plates containing formaldehyde at the indicated dose is plotted. B.) The survival of wild-type (□); *uvrA* (●); *dinB*(◇); *umuC* (△); *polB* (○) and *polBdinBumuC* (◁) mutant. Cells were treated the same way as in (A). Error bars represent the standard error of the mean from at least 2 independent experiments.

Replication recovery from formaldehyde-induced DNA damage requires *recA* while recovery in *recBC* and *uvrA* mutants are delayed

DNA-protein crosslinks are predicted to block the progression of the replication machinery. However, we would predict that the arrested fork and mechanism of recovery would differ from that of UV-induced pyrimidine dimers since the large size of DPCs are predicted to block the DNA polymerase several bases upstream from the adducted nucleotide. To examine the effect of formaldehyde-induced DPCs on replication *in vivo* and to determine the requirements for replication to recover, we monitored the rate of synthesis and accumulation of total genomic DNA in cultures treated with formaldehyde. In this assay, cultures pre-labeled with ^{14}C-thymine were treated with 5 mM formaldehyde for 15 minutes, a dose that reduces survival by 90% in wild-type cultures (Figure 3.2B). At different time points before and after formaldehyde treatment, duplicate aliquots of the culture were pulse-labeled with ^{3}H-thymidine for two minutes. The amount of [^{14}C] and [^{3}H] incorporated into DNA at each time provides a measure of the total genomic DNA accumulation and rate of DNA synthesis, respectively.

After formaldehyde treatment, we observed that the rate of replication was initially inhibited by about 97% relative to untreated cultures (Figure 3.2A). Robust synthesis started to recover in wild-type cultures at about 50 to 60 minutes after treatment, as measured by [^{3}H] incorporation (Figure 3.2A). This coincides with the time that the total genomic DNA also began to increase as measured by [^{14}C] incorporation. In contrast, the rate of synthesis in *recA* mutants did not recover within the 90-minute time course (Figure 3.2A). This lack of synthesis corresponded with a

notable amount of degradation that occurred in the total genomic DNA. At this dose, the survival of *recA* mutants was reduced by four orders of magnitude (Figure 3.2B).

As expected from the survival studies, the recovery of replication in *recF* mutants appeared to be nearly identical to that observed in wild-type cultures. This is distinct from what is observed after UV-induced damage where *recF* mutants are hypersensitive and fail to recover replication (Figure 3.2B). Unexpectedly, *recBC* mutants, which are hypersensitive to formaldehyde, also appeared to resume DNA synthesis and accumulate DNA 60 minutes after formaldehyde treatment, similar to the time seen in wild-type cultures (Figure 3.2B). Although the rate of synthesis and accumulation was less than wild-type cultures, the time at which recovery occurred was identical. The reduced rate of synthesis during recovery can be explained by the observation that *recBC* mutants grow more slowly than wild-type cells even in the absence of DNA damage (13). Consistent with this, we observed that untreated *recBC* mutants accumulated DNA more slowly that wild-type cells, doubling in about 70 minutes rather than 40 minutes as in wild-type cells (Figure 3.2A)

To determine the effect that nucleotide excision repair has on the recovery of DNA synthesis, we examined *uvrA* mutants. The resumption of DNA synthesis in *uvrA* cultures also began to occur at 60 minutes post-treatment, but the amount of synthesis that ensued at this time was small compared to wild-type, *recF* or *recBC* mutants (Figure 3.2B). The impaired recovery of *uvrA* mutants would be consistent with the modest hypersensitivity to formaldehyde as observed here and in previous studies (Figure 3.2B) (16, 17, 26, 28).

These results clearly distinguish the mechanisms that repair formaldehyde-induced damage from that of UV. The finding that *recBC* cells are hypersensitive yet able to recover replication suggests that the lethality in these cells is due to reasons other than the inability to recover DNA synthesis. In this aspect, formaldehyde-induced damage is similar to UV-induced damage. *recBC* mutants are hypersensitive to UV-irradiation but still remain able to recover synthesis and continue replication similar to wild-type cells (1). The similar phenotype could suggest a common function of *recBC* after both UV and formaldehyde, however what that function is remains unclear.

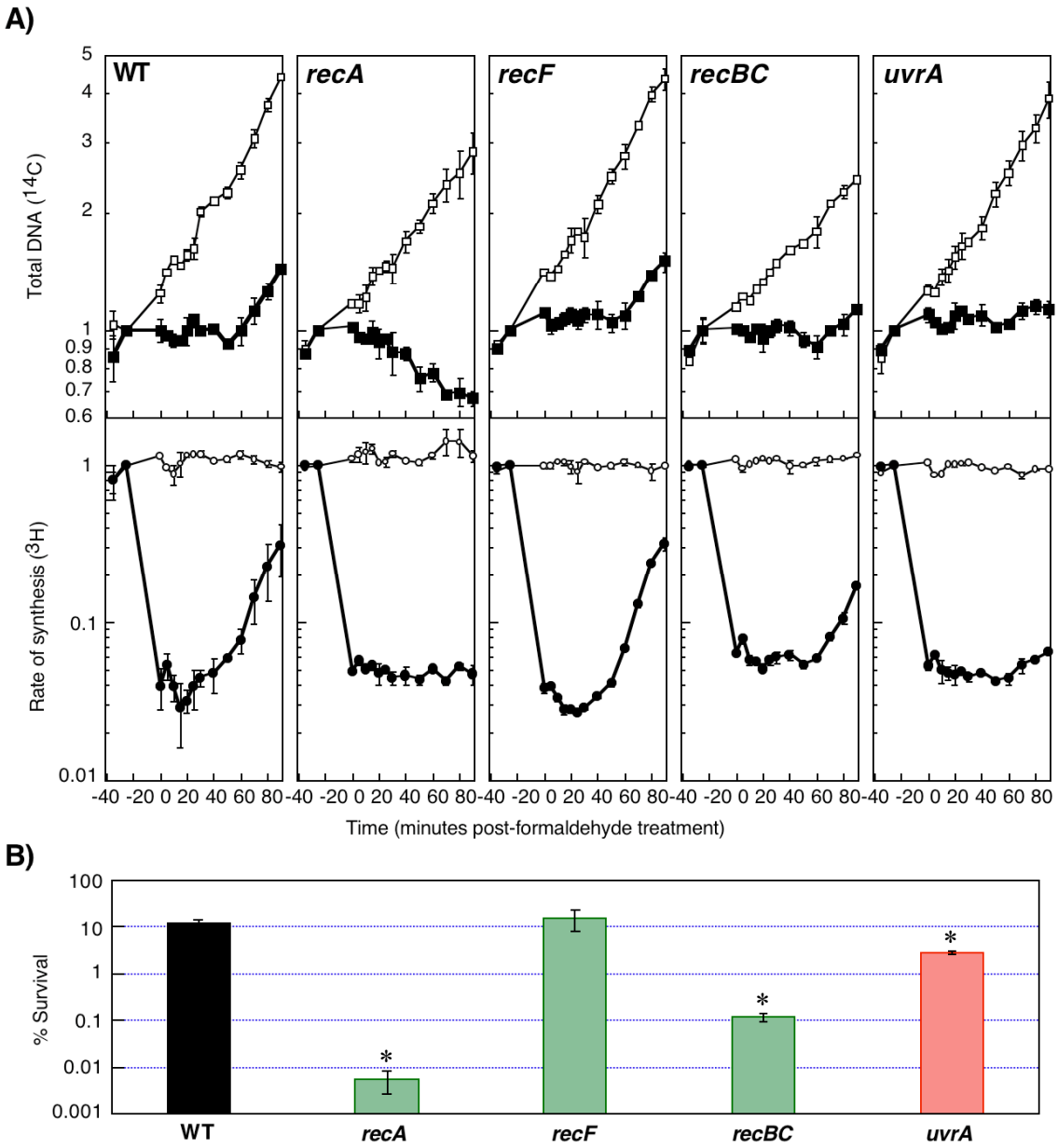

Figure 3.2. Replication recovery from formaldehyde-induced DNA damage requires *recA* while recovery in *recBC* and *uvrA* mutants are delayed A.) Cultures grown in 14C-thymine are treated with 5mM M formaldehyde for 15 minutes and then cells were collected on filters and resuspended in fresh drug-free media. At the indicated times, duplicate aliquots of the culture were pulsed with 3H-thymidine for two minutes. The amount of [3H] and [14C] incorporated in the DNA at each time point is plotted relative to the amount incorporated just prior to formaldehyde addition. (□) 14C, mock-treated; (■)14C, treated; (○) 3H, mock-treated ; (●) 3H, treated. Error bars represent the standard error from two independent experiments**.** B.) Survival of cells following a 15-minute exposure to 5mM formaldehyde. Error bars represent the standard error of the mean from two independent experiments. * denotes statistical significance relative to wild-type cultures (one-tailed Student's T-test, $p<.05$)

DISCUSSION

Using assays to monitor survival and the recovery of DNA replication at times after formaldehyde treatment, we observed that the requirements for both survival and recovery are distinct from that of UV-induced damage. Our results would suggest that DPC-induced cell death does not correlate directly to the disruption of replication as is seen with UV-induced damage. RecF is required to restore replication following disruption by UV-induced damage (6, 8-12). The observation that *recF* is not required for survival or recovery after formaldehyde implies that DPCs do not disrupt replication in the same manner as UV-induced lesions, but instead may block replication progression without disrupting the machinery. Previous studies have shown that DNA-binding proteins form natural pause sites for replication, but do not affect the overall integrity of the replisome (18, 21). Analogously, protein crosslinks to DNA may similarly pause, rather than disrupt the replication machinery. Consistent with this interpretation, we observe an inhibition of DNA synthesis following formaldehyde treatment, but find that it is able to resume without RecF, suggesting that the replication apparatus pauses at formaldehyde-induced lesions in a manner that allows replication to simply resume once the DPC is removed (or processed in some way).

The modest sensitivity of *uvrA* mutants to formaldehyde is consistent with previous studies in *E. coli,* yeast and human cells that found nucleotide excision repair modestly contributed to survival (16, 17, 26, 28). In this study we found that replication begins to recover at a time similar to wild-type cells, but that the extent of recovery is limited. Earlier studies speculated that the lack of a more severe effect in

NER mutants may be due to the spontaneous release of DPCs that occurs even in the absence of excision repair (25, 26). Alternatively, we would also not rule out the possibility that translesion synthesis may account for some of the recovery observed in the absence of NER. By analogy, a similar impaired recovery is observed in *uvr* mutants after UV-induced damage. In this case, the limited recovery that does occur has been attributed to translesion synthesis (7, 8). Yet a third possibility for the modest effect of *uvrA* is that formaldehyde may induce two forms of lesions – one that does not depend on NER, and one that does. Irrespective of these three possibilities, we can conclude that formaldehyde sensitivity in *uvrA* mutants corresponds with an impaired ability to resume replication after treatment. This is consistent with the interpretation that the lethality observed in *uvrA* mutants may be due to the persistence of protein-bound blocks that prevent replication from proceeding, even if the block does not disrupt it.

The cause of lethality in *recBC* mutants is clearly distinct from that in *uvrA* mutants. *recBC* mutants were capable of recovering replication similar to wild-type cells yet their survival was more than an order of magnitude lower than *uvrA* mutants. We interpret this to indicate that the lethality in these mutants is not due to a defect in being able to recover replication directly. However, at this time we are unable to identify the precise substrate or lesion that requires RecBCD for survival. Nevertheless, the pronounced hypersensitivity of *recBC* mutants makes it clear that RecBCD is involved in a primary mechanism by which formaldehyde-induced damage is repaired (4, 25, 32).

We initiated this study to determine if the mechanisms operating to repair and

restore replication after UV-induced damage could be extended to other structurally distinct forms of damage. The findings presented here show that DPCs are processed and repaired differently than UV-induced damage. Our results support that idea that although DNA synthesis is blocked, replication is not disrupted by formaldehyde-induced damage as it is by UV-induced damage. Further we show that replication disruption may not be the primary mechanism that contributes to the lethality after formaldehyde treatment. The unique hypersensitivity of *recBC* mutants may provide a platform for future studies to begin to dissect the molecular mechanism by which these adducts are processed and repaired.

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CHAPTER IV

CONCLUDING REMARKS

Decades of observations in *E. coli* have provided the foundation for our understanding of how DNA replication is restored following DNA damage. Much of this work has focused on using UV-induced lesions as a model. A remaining challenge has been to determine whether the same processes operate in the presence of other DNA damaging agents and whether observations in *E. coli* cells can be extended to other organisms. The main objective of this thesis was to characterize the survival and recovery of DNA replication in *E. coli* cells following exposure to nitrofurazone and formaldehyde, two agents that form structurally distinct lesions from those induced by UV.

Following a modest UV dose, mutants lacking *recA, uvrA* or *recF* are hypersensitive and are unable to recover replication (3-5). These observations led to our current model in which the *recF* pathway gene products function to process the replication fork following arrest such that lesions can be repaired and replication can resume (1-8). *recBC* mutants are also hyerpsensitive to UV. However, these mutants recover replication normally and the cause of their hypersensitivity remains unclear (2).

When we treated cultures with nitrofurazone, an agent that induces a small, but structurally distinct lesion from that of UV, we found that the requirements for survival were almost identical to those after UV-irradiation. The exception to this is

that we observed Pol IV, rather than Pol V, contributed to survival after nitrofurazone treatment. However, similar to UV-induced damage, the contribution of translesion synthesis overall was minor relative to that of nucleotide excision repair. Our ability to measure the recovery of replication disruption following nitrofurazone exposure was hampered by the apparent inhibition of replication by a mechanism that is independent from DNA damage. Thus the conclusion that nitrofurazone-induced damage is processed similarly to UV is based predominantly on the genetic requirements for survival.

In contrast to nitrofurazone-induced damage, the requirements for survival and recovery after formaldehyde were distinct from UV. Formaldehyde-induced DNA protein crosslinks are unique DNA lesions that are extraordinarily large compared to either UV or nitrofurazone-induced lesions. Therefore, to some extent, it was not surprising that the genes required for survival differed in this case. Most notably, *recF* was not required for survival or the recovery of replication when DPCs block DNA synthesis, in stark contrast to UV. *recBC* mutants were the most hypersensitive mutant examined to formaldehyde but were able to recover replication. This suggests that the RecBCD pathway is a primary mechanism by which DPCs are processed and repaired, although this molecular mechanism remains to be elucidated.

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APPENDIX A

PUBLISHED PAPERS IN WHICH AUTHOR OF THESIS PERFORMED MUTAGENESIS EXPERIMENTS

RecA433 CELLS ARE DEFECTIVE IN *recF*-MEDIATED PROCESSING OF DISRUPTED REPLICATION FORKS BUT RETAIN *recBCD*-MEDIATED FUNCTIONS

Qais Al-Hadid, Katherine Ona, Charmain Courcelle, Justin Courcelle

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ABSTRACT

RecA is required for recombinational processes and cell survival following UV- induced DNA damage. *recA433* is a historically important mutant allele that separates the recombination and survival functions of RecA. *recA433* mutants remain proficient in recombination as measured by conjugation or transduction, but are hypersensitive to UV- induced DNA damage. The cellular functions carried out by RecA require either *recF* pathway proteins or *recBC* pathway proteins to initiate RecA-loading onto the appropriate DNA substrates. In this study, we characterized the ability of *recA433* to carry out functions associated with either the *recF* pathway or *recBC* pathway. We show that several phenotypic deficiencies exhibited by *recA433* mutants are similar to *recF* mutants but distinct from *recBC* mutants. In contrast to *recBC* mutants, *recA433* and *recF* mutants fail to process or resume replication following disruption by UV-induced DNA damage. However, *recA433* and *recF* mutants remain proficient in conjugational recombination and are resistant to formaldehyde protein-DNA crosslinks, functions that are impaired in *recBC* mutants. The results are consistent with a model in which the *recA433* mutation selectively impairs RecA functions associated with the RecF pathway, while retaining the ability

to carry out RecBCD pathway-mediated functions. These results are discussed in the context of the *recF* and *recBC* pathways and the potential substrates utilized in each case.

INTRODUCTION

recA was originally identified as a gene required for the formation of recombinant DNA molecules in conjugating bacteria [1]. In addition to this role, it was subsequently shown to be required for survival in the presence of DNA damage [2, 3]. Purified RecA monomers bind cooperatively to single-strand DNA and then pair it with homologous duplex DNA in vitro. The product of this reaction yields a RecA protein filament bound in a three-stranded DNA structure [4-7]. During recombinational processes or in the presence of double strand breaks, this strand pairing activity is thought to be required to bring together separate DNA molecules and initiate exchange or rejoining. This same biochemical activity is also required in the presence of DNA damage to maintain and process replication forks that are blocked by DNA lesions [8-11]. The binding of RecA to the strands of the blocked replication fork allows repair enzymes and translesion polymerases to gain access to the offending lesion and allow replication to recover [8, 12- 14]. RecA bound to DNA becomes conformationally active and promotes the autocatalytic cleavage of the LexA repressor, resulting in the upregulation of more than forty genes that function to repair or allow synthesis through DNA lesions, delaying cell division, and restoring replication and the integrity of the DNA (reviewed in [15]). The activated form of RecA also promotes the autocatalytic cleavage of UmuD through a similar mechanism

to that occurring with LexA and is required for translesion synthesis and UV-induced mutagenesis to occur [16-18].

In *Escherichia coli*, RecA functions are thought to initiate through either the *recBC* pathway or the *recFOR* pathway [19, 20]. Each pathway recognizes distinct DNA substrates and loads RecA at these sites to initiate recombination or repair [21-24]. *recB or recC* mutants exhibit a 102- to 103-fold reduction in their recombination efficiency, are hypersensitive to UV and X-rays, and have a low plating efficiency [25, 26]. Biochemically, RecB and RecC form a complex with RecD, that binds double strand DNA ends and serves to unwind and partially degrade the DNA before recruiting RecA to these sites to initiate strand pairing and exchange during recombination or repair [20]. While RecBCD is generally considered to initiate repair at double strand breaks, several aspects of *recBC* mutant phenotypes remain enigmatic. For instance, it is not clear why *recBC* is required for survival following UV irradiation or other agents that generally do not induce double strand-breaks [27]. Curiously, *recBC* mutants grow poorly compared to wild type cells or *recA* mutants in the absence of any exogenous DNA damage [25]. Further, in the absence of RecBCD, plasmids are not stably maintained [28-30]. *recF*, *recO*, or *recR* mutants are proficient in conjugational transductional recombination, but are hypersensitive to UV-induced DNA damage (but not X-ray- or oxidative-induced DNA damage), and are required for replication to resume when it is blocked or disrupted [9, 19, 31]. Purified RecF, RecO, and RecR form a complex on DNA that recognizes single to double strand junctions such as those found on gapped DNA or at replication forks and promote RecA-filament formation at these sites [23, 24, 32]. In vivo, RecFOR binding is

required for maintaining the integrity of the replication fork DNA, translesion synthesis, and repair of the blocking lesion [9, 12-14, 33]. Thus, while both RecBC and RecF pathways promote RecA loading onto DNA, they each recognize unique DNA substrates and are involved in distinct functions of cellular metabolism.

In the genetic characterization of RecA, several mutant *recA* alleles have been isolated that have altered phenotypes. Alleles have been identified that are constitutively active, act dominantly over the wild type allele, or selectively impair a specific function such as UV resistance or mutagenesis [34-38]. *recA433* was originally isolated as a mutant that reduced the mutation frequency following DNA damage and contains a single point mutation that changes amino acid 243 from arginine to histidine [18, 37].

Subsequently, it was found to retain its ability to catalyze conjugational and transductional recombination, despite remaining hypersensitive to UV-induced DNA damage [36]. Further characterization of *recA433* has demonstrated that it remains functional for cleavage of the LexA repressor as measured by upregulation of the SOS- regulated *sulA* gene, the plasmid encoded MucA protein as measured by mutagenesis, and the lambda CI repressor as measured by induction of phage lambda [36, 37, 39]. However, RecA433 is curiously defective in cleavage of UmuD, as measured by mutagenesis and direct western analysis of the cleavage product [36, 37]. The differential retention of protein function led investigators to propose that the RecA433 defect may relate to a specific inability of the protein to interact with specific protein partners [37]. While RecA433 has been found to retain a subset of its cellular functions, the mechanism by which this occurs remains uncharacterized.

We noted that aspects of the *recA433* phenotype, namely recombination proficiency and UV hypersensitivity, paralleled those of a *recF* mutant. We postulated that both mutations may be impaired at a common molecular step. To address this possibility and further characterize the nature of the *recA433* defect we examined its ability to recover replication following UV-induced DNA damage and directly compared it to that of *recF* and *recBC* mutants. We find that the *recA433* mutation is similar to *recF* but distinct from *recBC* mutants in that it renders cells unable to recover DNA synthesis following UV-induced DNA damage.

MATERIALS AND METHODS

Bacterial strains. All bacterial strains used in this study, except those used for the conjugation assay, are derived from SR108, a *thyA36 deoC2* derivative of W3110 [8, 49]. SR108, CL579 (SR108 *recF6206*::*tet857*), and HL1034 (SR108 D*(srlR-recA)306*::Tn*10*) have been reported previously [8, 49]. CL856 (SR108 *srlC300*::Tn*10 recA433)* was constructed by P1 transduction of *srlC300*::Tn*10 recA433* genes from DE190 (gift from Don G Ennis) into SR108, selecting for tetracycline resistance. CL1056 (SR108 *recC ptrA recBD*::*cam*) was made by P1 transduction of *recCptrA recBrecD*::*cam* from KM78 (gift from Kenan C. Murphy) into SR108, selecting for chloramphenicol resistance. The presence of *recA433* and *recC ptrA recBrecD* was confirmed by the UV hypersensitive phenotype of recipient strains.

For conjugation, the Hfr strain PK3 (*xyl thr leu thi lac*).was utilized as a donor [50, 51], and recipients were all derived from AB1157 (*thr-1, ara-14, leuB6, D(gpt-*

proA)62, lacY1, tsx-33, supE44, galK2, lambda-, rac-, hisG4(Oc), rfbD1, mgl-51, rpsL31, kdgK51, xyl-5, mtl-1, argE3, thi-1) [52]. CL1215 (AB1157 *srlC300*::Tn*10 recA433)* was constructed by P1 transduction of *srlC300*::Tn*10 recA433* genes from DE190 into AB1157. CL1216 (AB1157 *recF6206*::*tet857*) was made by P1 transduction of *recF6206*::*tet857* from CL579 [8] into AB1157. CL1230 (AB1157 *recC ptrA recBD*::*cam*) was made by P1 transduction of *recC ptrA recBD*::*cam* from KM78 into AB1157. CL1237 was made by P1 transduction of D*(srlR-recA)306*::Tn*10*) from HL921 [9] into AB1157. The presence of *recA433, recF6206, recC ptrA recBD,* and D*(srlR- recA)306* was confirmed by the UV hypersensitive phenotype of recipient strains.

UV survival. A Sylvania 15-watt germicidal lamp (254 nm) at an incident dose of 0.9 J/m2/s (0.2 J/m2/s for doses of 5 J/m2 or less) was used for irradiations. Cells were grown in Davis medium [53] supplemented with 0.4% glucose, 0.2% casamino acids, and 10 μg/ml thymine (DGCthy media). Cultures were inoculated from fresh overnight cultures and grown to an OD600 between 0.4 and 0.5. Serial dilutions of each culture were plated in triplicate on Luria-Bertani plates supplemented with 10 μg/ml thymine (LBthy) and UV irradiated at the indicated doses. Plates were incubated overnight at 37°C, and colonies were counted the next day.

Conjugational recombination. Fresh overnight cultures of the *arg*+ StrS donor (PK3), and the *arg*- StrR recipient (AB1157) were diluted 1:25 in 5ml of LBthy media and both strains were grown for 3 hours in a 37°C water bath. 1 ml of the donor and

recipient cultures were then mixed together along with 1 ml of LB broth and the cells were collected on a 25mm Fisherbrand 0.45 μm general filtration membrane. The filter was placed on an LB-thy plate for 1 hour and then the cells were resuspended in 5ml of 1X Davis and serial dilutions were plated on DGCthy media supplemented with 50 μg/ml streptomycin to select for transconjugates. The number of donor and recipient cells were determined by plating serial dilutions of PK3 and AB1157 cultures on LB-thy media. Plates were incubated overnight at 37°C, and colonies were counted the next day.

Recovery of DNA synthesis. This approach was modified from Khidhir et al. [11]. Fresh overnight cultures were diluted 1:100 and grown in DGCthy media supplemented with 0.1 μCi/ml of [14C]thymine to an OD600 of precisely 0.3, at which point half of the culture received an incident dose of 0.9 J/m2 for 30 s, and the other half of the culture was mock irradiated. At the times indicated, duplicate 0.5-ml aliquots of culture were pulse labeled with 1 μCi/ml [3H]thymidine for 2 min at 37°C. The cells were then lysed and the DNA was precipitated in cold 5% trichloroacetic acid (TCA), filtered onto Millipore glass fiber filters, and the amount of 3H and 14C in each sample determined by liquid scintillation counting.

Nascent DNA degradation. A 0.1 ml aliquot of each strain was taken from an overnight culture and transferred to 10-ml of DGCthy (0.4% glucose, 0.2% Casamino acid,10 μg/ml thymine and 1X Davis) containing 0.1 μCi of [14C] thymine/ml. The cultures were then grown to an O.D. of 0.4, pulsed for 5 seconds with 1μCi of [3H]

thymidine/ml, filtered with a 0.45 μm membrane and then rinsed twice wit 3 ml of NET buffer (100 mM NaCl, 10mM Tris, pH 8.0, 10mM EDTA, pH 8.0. The cells were then resuspended in pre-warmed unlabeled DGCthy medium and irradiated with a UV dose of 30 J/m2. At the times indicated, duplicate 0.2-ml aliquots (triplicate for the 0 tome-point) of the culture were precipitated in cold 5 % TCA and filtered onto Millipore glass fiber filters. The amounts of 3H and 14C were determined with a scintillation counter.

2-D agarose gel analysis. Fresh overnight cultures of cells that contain the plasmid pBR322 were grown in the presence of 100 μg/ml ampicillin. The overnight cultures were diluted 1:100 and grown without ampicillin selection in a shaking incubator at 37° C to an OD600 of 0.5(~ 5 x 108cells/ml) and UV-irradiated with 50 J/m2. At the indicated time points, 0.75 ml samples were placed into 0.75 ml cold 2X NET (100 mM NaCl, 10 mM Tris, pH 8.0, 10 mM EDTA). Each sample was pelleted, resuspended in 150 μl of 1 mg/ml lysozyme and 0.2 mg/ml RNaseA in TE (10 mM Tris, pH 8.0, 1 mM EDTA), lysed at 37°C for 30 min. At this time, proteinase K (10 μl, 10 mg/mg) and sarcosyl (10 μl, 20%) was added and incubated at 50°C for 1 hr. Samples were then extracted with 4 volumes of phenol/chloroform/isoamyl alcohol (25/24/1), once with 4 volumes of chloroform/isoamyl alcohol (24/1), and dialysed for 3 hours on 47 mm Whatman 0.05 μm pore disks (Whatman #VMWP04700) floating on a 250 ml beaker of TE. Samples were then digested with PvuII (New England Biolabs) overnight, followed by a one hour digestion with PvuII the next morning and extracted with chloroform/isoamyl alcohol (24/1), and equal volumes (30μl) were

loaded onto the gel. Restricted genomic DNA samples were run in the first dimension in 0.4% agarose, 1X TBE at 1 V/cm. Gel lanes were cut out, recast, and run in the second dimension in 1.0% agarose, 1X TBE at 6.5 V/cm. Gels were transferred to Hybond N+ nylon 25 membranes and probed with pBR322 that had been labeled with 32P by nick translation according to the protocol supplied by Promega using alpha [32-P]dCTP (MP Biomedicals). Radioactivity was visualized and quantitated using a Storm 820 and its associated ImageQuant Software (Molecular Dynamics).

RESULTS

recA433 is similar to *recF* mutants but distinct from *recBCD* mutants with respect to recombination proficiency

Both the *recFOR* pathway and the *recBCD* pathway initiate RecA function by promoting the formation of a RecA filament on DNA. However, each pathway recognizes distinct DNA substrates with separate cellular roles. To examine how the *recA433* phenotypes functionally relate to the *recFOR* and *recBCD* pathway, we compared the UV hypersensitivity and recombination proficiency of *recA433*, *recF*, and *recBC* mutants, directly. We observed that *recA43*3 was more sensitive than either the *recF* or *recBC* mutant, although, consistent with previous observations, it was not as hypersensitive as a *recA* deletion (Fig 1A) [40].

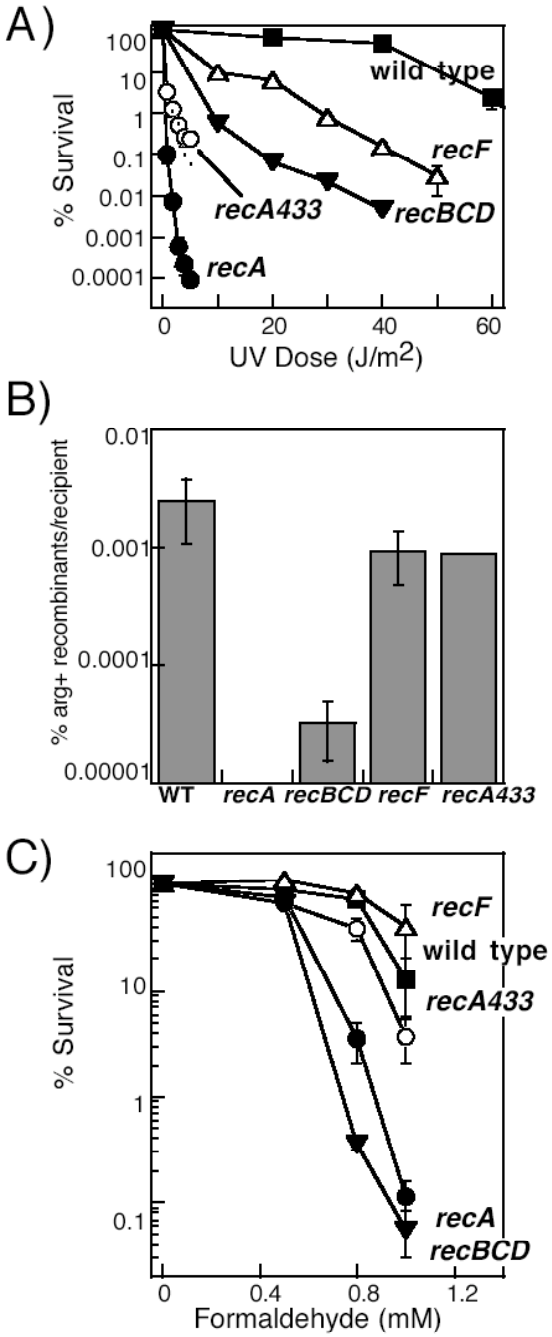

Figure A1. *recF* and *recA433* mutants are hypersensitive to UV, but remain proficient in conjugational recombination and are resistant to formaldehyde. A) The survival of wildtype (squares), *recA* (filled circles), *recBCD* (filled triangles), *recF* (open triangles) and *recA433* (open circles) after UV-irradiation at the indicated dose is plotted. B) The recombination frequency of the arg+ marker following conjugation with the Hfr strain, PK3, is plotted for wildtype, *recA*, *recBCD*, *recF* and *recA433*. Recombination frequency was calculated as the number of arg+ recombinants per recipient cell. Streptomycin was used to counterselect against the donor cells following conjugation. Each strain represents an average of two experiments. Error bars represent the standard error. C) The survival of wildtype (squares), *recA* (filled circles), *recBCD* (filled triangles), *recF* (open triangles) and *recA433* (open circles) grown on plates containing formaldehyde at the indicated dose is plotted.

To compare the frequency of recombination in these strains, we mated an *arg*+ *str*S Hfr donor to *arg*- *str*R recipients of each mutant. The frequency that *arg*+ *str*R recombinants were formed was then quantified in each case. Using this assay, we observed that the recombination frequency in *recA433* mutants was only modestly lower than wild type cells, but was similar to that of *recF* mutants (Fig 1B). By comparison, the frequency of recombination in *recBC* mutants was reduced between 30-80 fold as compared to *recF*, *recA433* or wildtype cultures. The recombination frequency of our

recA deletion mutant was below the limits of detection in our assay.

Thus, we observe that the *recA433* mutation is similar to *recF* mutants but distinct from *recBC* mutants in that it remains proficient for conjugational recombination. One possible explanation for this initial observation could be that the defect associated with the *recA433* allele results in inability to function through the RecF pathway. To further test this possibility, we examined *recA433* mutants using assays that differentiate between functions associated specifically with the *recBCD* pathway and those associated specifically with the *recF* pathway.

recA433 mutants remain resistant to formaldehyde-induced DNA-protein crosslinks

One feature that distinguishes the *recBDC* pathway from the *recF* pathway is their ability to promote survival in the presence of specific forms of DNA damage. *recBC* mutants, but not *recF* mutants, are hypersensitive to nitric oxide [41, 42]. Additionally, *recBC* mutants, but not *recF* mutants, are hypersensitive to

formaldehyde, an agent that induces protein-DNA crosslinks [43]. When we examined the ability of *recA433* to survive in the presence of increasing concentrations of formaldehyde, we observed that it was as resistant to formaldehyde as either wild type or *recF* mutants. By comparison, *recBC* mutants were hypersensitive to formaldehyde as was a *recA* deletion (Figure 1C). Thus, *recA433* mutants are similar to *recF*, but distinct from *recBC* mutants in that they remain resistant to formaldehyde.

recA433 mutants fail to maintain or process replication forks blocked by UV-induced DNA damage

A second phenotype that distinguishes *recBC* mutants from *recF* mutants is their function at replication forks following UV-induced damage. Previous work has shown that RecF-mediated loading of RecA is required to process and maintain replication forks following arrest by UV-induced DNA damage [8, 9]. In the absence of RecF, the nascent DNA at the arrested replication fork undergoes extensive degradation and DNA synthesis fails to recover [8, 9]. By contrast, RecBCD does not appear to be involved in processing the arrested replication fork directly. In UV-irradiated *recBCD* mutants, the replication forks are processed and maintained normally before DNA synthesis resumes [8, 9]. To characterize the defect associated with the *recA433* mutation and compare it to that of *recF* and *recBCD* mutants, we examined whether *recA433* is able to maintain, process, and restore replication forks following arrest by UV-induced DNA damage.

To examine whether replication recovers in *recA433* mutants following arrest,

we followed the total DNA accumulation and rate of DNA synthesis that occurred in UV- irradiated cultures over time. Cultures grown in media containing [14C] thymine were UV irradiated with 27 J/m2 or mock irradiated. To monitor how the rate of DNA synthesis was affected by these treatments, duplicate 0.5-ml aliquots of the 14C-labeled cultures were pulse-labeled with [3H]thymidine for 2 min at periodic intervals before and after irradiation. The rate of DNA synthesis (3H incorporation/min) could then be determined relative to the total amount of DNA present (14C incorporation) at each time.

In UV-irradiated wild type cultures, we observed that the rate of DNA synthesis was initially reduced by greater than 90% immediately following UV irradiation, but the rate of synthesis began to recover within fifteen minutes after irradiation and continued to increase until the end of the assay. Similarly, a transient inhibition of DNA accumulation was observed immediately following irradiation, before recovering at the time robust DNA synthesis was seen to recover (Figure 2). By contrast, in UV-irradiated *recA* deletion mutants, DNA synthesis did not resume following UV irradiation. Consistent with previous observations [8, 9], the lack of recovery in *recA* deletion mutants was associated with the degradation of the cellular DNA, as evidenced by the loss of 14C labeled genomic DNA over the time course.

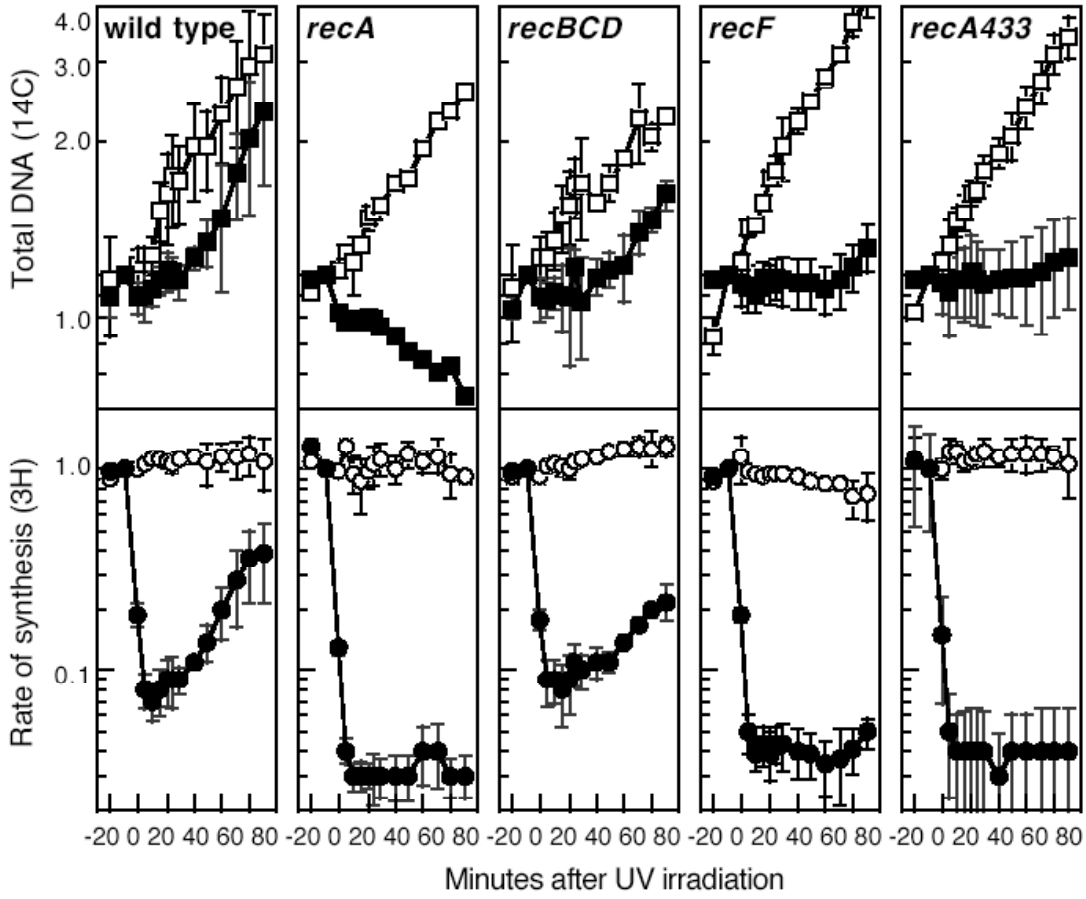

Figure A2. *recF* and *recA433* mutants fail to recover DNA synthesis following disruption by DNA damage. [14C]thymine-labeled cultures were pulsed for 2 minutes with [3H]thymidine at various times after mock-irradiation or UV-irradiation with 27 J/m2. The rate of replication could therefore be compared to the total amount of DNA present at specific times during the recovery period. (open symbols) mock-treated samples; (filled symbols) UV-irradiated; (squares) 14C-labelled total DNA accumulation; (circles) 3H-labelled DNA synthesis per 2 minutes. Each graph represents an average of three independent experiments. Error bars represent one standard deviation. The [14C] and [3H] ranged from 946-5047 cpm and 1822.1-9808 cpm for all experiments, respectively.

Following UV irradiation of *recBCD* mutants, the rate of DNA synthesis began to recover at a similar time as in wild type cultures. By contrast, in UV-irradiated *recF* cultures, DNA synthesis did not resume and no further DNA was observed to accumulate during the time course. When we examined cultures of *recA433*, we observed that DNA synthesis also failed to recover following UV irradiation and

looked similar to that seen in *recF* mutants. Thus, similar to *recF*, the mutation in the RecA433 allele impairs its ability to restore DNA synthesis following arrest.

The lack of recovery in *recF* mutants is associated with the extensive degradation of the nascent DNA at the replication fork. To determine if the defect in *recA433* mutants renders cells unable to protect the nascent DNA from degradation, we examined the fate of the DNA that was made prior to irradiation. To this end, exponentially growing [14C]thymine-labeled cultures were pulsed with [3H]thymidine for 5 seconds to label the DNA at replication forks. The culture was then transferred to nonradioactive medium and immediately UV-irradiated with 27 J/m2. The 14C prelabel allowed us to compare the degradation occurring in the overall genome to that in the 3H-labeled DNA made at replication forks just prior to UV irradiation. Consistent with previous studies, in UV-irradiated wild-type cultures, the overall genomic DNA was protected and only a limited degradation of the nascent DNA was detected at times prior to the recovery of replication (Fig 2). In contrast, in UV-irradiated *recA* deletion cultures, both the DNA at the replication fork and the total genomic DNA were rapidly degraded. Previous work from our lab has shown that the "rec-less" degradation of the overall genome and the nascent DNA is mediated through separate mechanisms. The nascent DNA at the replication fork is degraded by the RecJ nuclease and RecQ helicase, which belong to the *recF* pathway [8, 27, 44]. The genomic DNA is degraded by the RecBCD helicase- nuclease and initiates at an as yet unidentified substrate that is distinct from the arrested replication fork [27, 44]. Consistent with this, we observed that in the absence of *recF*, although the genomic DNA remained primarily intact, extensive degradation occurred on the nascent DNA

that continued throughout the time course. By comparison, in the absence of RecBCD, the nascent degradation ceased after an initial period of degradation at a point that was modestly more than occurred in wild type cells but less than seen in *recF* mutants (Fig. 3). When we examined the degradation pattern in *recA433* mutants, we found that the nascent DNA was extensively degraded, similar to *recF* mutants. In addition, some degradation was also occurred in the overall genomic DNA, though this was less extensive than occurs in the *recA* deletion (Fig. 3).

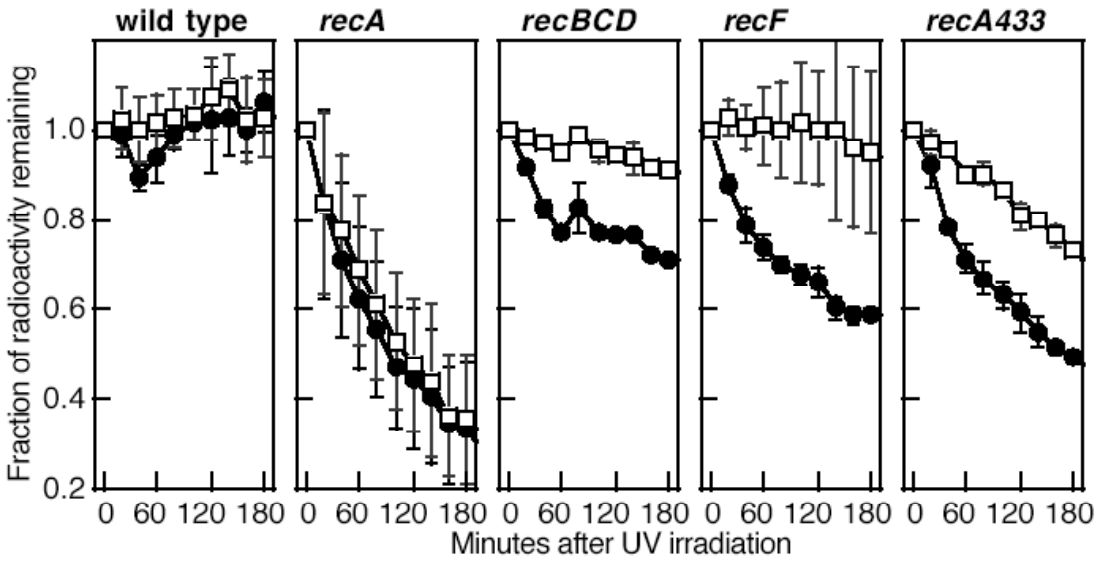

Figure A3. Similar to *recF*, extensive degradation of the nascent DNA occurs at the growing fork after UV-irradiation in *recA433* mutants. [3H]thymidine was added to cells pre-labeled with [14C]thymine for 5 seconds prior to UV-irradiation with 27J/m2 in nonradioactive media. The fraction of [3H]-labeled nascent DNA at the replication fork (closed circles) and [14C]-total DNA (open squares) remaining is plotted over time. Graphs represent an average of 3 independent experiments. Error bars represent one standard deviation. The initial values of 3H and 14C ranged from 1508-7791 cpm and 700-1956 cpm for all experiments, respectively.

The failure to maintain replication forks blocked by DNA damage can also be visualized by examining the replication intermediates on plasmids such as pBR322 following UV irradiation [8]. Previous studies have shown that whereas *recF* mutants fail to maintain UV-induced replication intermediates on plasmids following UV-irradiation, *recBC* mutants maintain and process these UV-induced replication intermediates normally. To examine whether *recA433* mutants are able to maintain UV- induced replication intermediates, we characterized the structural intermediates that occurred on replicating plasmid molecules of pBR322 after 50 J/m2 UV irradiation in *E. coli* cultures using two-dimensional agarose gel analysis [8]. This dose produces 0.5 lesions per plasmid strand and results in approxmately 90% survival of the wild type cells the irradiation to form colonies [8]. Cells containing the plasmid pBR322 were UV- irradiated, and the genomic DNA was purified and digested with Pvu II which linearizes the plasmid at a site downstream from its unidirectional origin of replication. When this DNA is analyzed in a two-dimensional agarose gel, nonreplicating plasmids migrate as linear 4.4-kb fragments and form a prominent spot on the gel. Replicating molecules of pBR322 form Y-shaped structures and migrate more slowly because of their larger size and nonlinear shape. These replicating fragments form an arc that extends out from the linear fragment towards the origin of the gel (Fig. 4). Following UV-irradiation, an increased level of Y-shaped molecules and a transient appearance of intermediates migrating in a cone region beyond the Y-arc are observed. Previous work from our lab has demonstrated that the cone region intermediates consist of molecules with two branch points and are associated with processing DNA damage encountered during replication of the molecule [8, 45]. The

cone region intermediates are maintained and protected from degradation by RecF, RecO, RecR, and RecA until a time that correlates with the repair of the lesion by nucleotide excision repair and the recovery of replication [8]. Consistent with our previous work, when we examined cultures of UV-irradiated cells containing the plasmid, we observed that cone region intermediates appeared in wild type cultures and in *recBCD* cultures, but not in *recF* cultures. When we examined UV-irradiated cultures of *recA433* mutants that contained the plasmid, no cone region intermediates were observed.

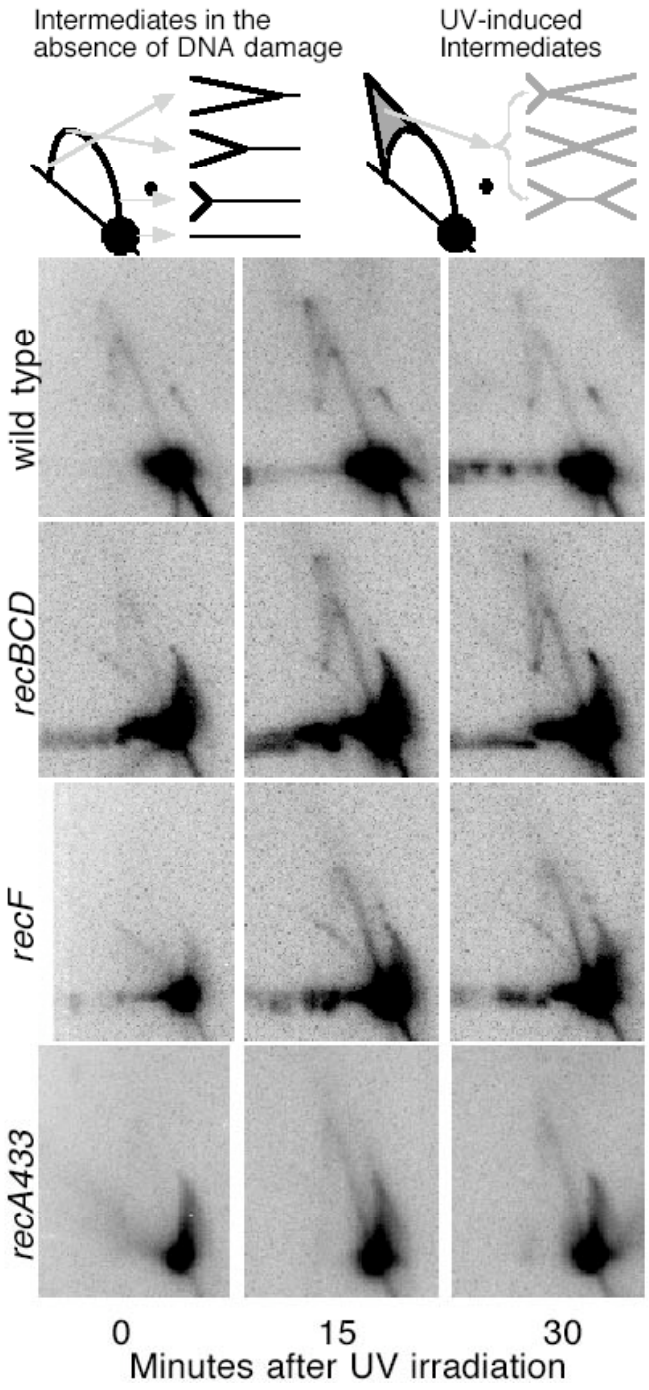

Figure A4. UV-induced replication intermediates are not observed in either *recF* or *recA433* mutants. A) A diagram of the migration pattern of PvuII digested pBR322 plasmid observed by 2D-agarose gel analysis. Nonreplicating linear plasmids run as a linear 4.4-kb fragment. Replicating plasmids form Y-shaped structures that migrate slower than the nonreplicating linear DNA, forming an arc that extends from the linear region. Following UV irradiation, double Y or X-shaped molecules are observed that
migrate in the cone region behind the arc of Y-shaped molecules. i) unirradiated intermediates ii) UV-induced intermediates. B) 2D-agarose gels probed with pBR322 plasmid DNA from cultures of wild type, *recBCD*, *recF*, and *recA433* mutants containing the plasmid at the indicated times following UV

The failure of *recA433* mutants to resume DNA synthesis following UV-irradiation, taken together with the failure to maintain the replication fork following disruption by UV-induced DNA damage as observed by 2-dimensional agarose gel analysis, indicates that the mutation in RecA433 renders cells unable to process replication forks following arrest by UV-induced DNA damage, similar to the defect observed in the absence of RecF.

DISCUSSION

Several of the phenotypes associated with the *recA433* allele mimic that of a cell lacking RecF, but are distinct from cells lacking RecBCD. Both *recA433* and *recF* mutants are proficient in conjugational recombination and remain resistant to formaldehyde treatment. In contrast, *recBCD* mutants are impaired in their ability to carry out conjugational recombination and are hypersensitive to formaldehyde. Using a combination of in vivo labeling together with 2-dimensional agarose-gel analysis, we found that the *recA433* mutation also renders cells unable to process and recover replication forks arrested by UV-induced damage, similar to *recF* mutants. By contrast, *recBCD* mutants remain capable of processing and resuming replication following UV- induced DNA damage. Taken together, these observations suggest that the *recA433* mutation selectively impairs RecA functions associated with the RecF pathway, while retaining RecBCD pathway-mediated functions.

RecF, together with RecO and RecR functions by loading RecA onto DNA

when replication is arrested. Biochemically, the presence of RecF both enhances the nucleation of a RecA filament on DNA and stabilizes it by preventing the filament from disassembling [24, 32, 46]. The impaired loading of RecA filaments in UV-irradiated *recF* mutants results in a failure to process replication forks that are blocked by DNA damage. In cells lacking RecF, the replication fork is not maintained, extensive degradation occurs at the nascent DNA, and replication fails to recover (Fig 5). A similar phenotype is seen in cells containing the *recA433*, even in the presence of RecF. We can imagine two possible mechanisms that may account for the RecA433 defect. The first possibility is that the RecA433 mutation impairs its ability to interact with the RecFOR proteins, but does not affect its ability to interact with RecBCD. This type of interpretation would be consistent with previous Ennis and Woodgate that suggested that the RecA433 mutation may impair specific protein protein interactions. As described in the introduction, RecA433 has been shown to retain its ability to cleave LexA, lambda CI repressor, and MucA in vivo, but is specifically deficient in cleaving UmuD [36, 37, 39].

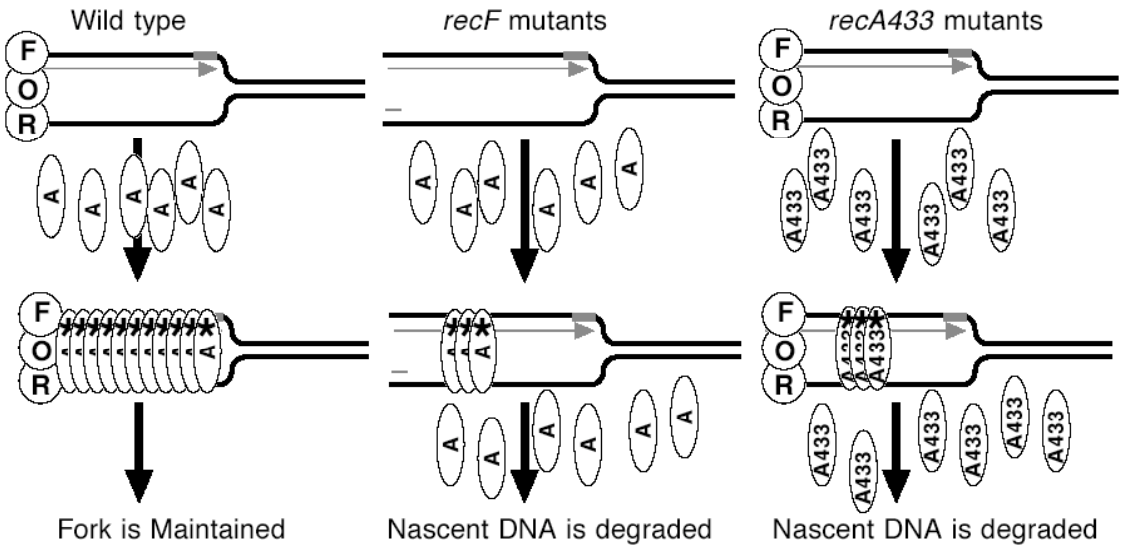

Figure A5. RecA433 allele fails to maintain or protect replication forks follow arrest by DNA damage.

Alternatively, the RecF-like phenotype of RecA433 could also be explained if the mutation renders the protein inherently less able to form an activated or stable filament, irrespective of its ability to interact with RecFOR (Fig 5). This interpretation can be inferred from observations that suggest RecF-mediated functions require higher concentrations of RecA than do RecBCD-mediated functions. The cellular concentration of RecA is severely reduced in *lexA1* mutants due to a non-cleavable repressor that prevents *recA* expression. Despite the limited RecA concentrations, *lexA1* mutants remain proficient in conjugational recombination, but fail to maintain or recover replication following arrest, mimicking the *recF* (and *recA433*) phenotypes in these aspects [11, 47] (and data not shown). If the activation of MucA mutagenesis, SulA expression, and lambda cleavage occur more rapidly than UmuD cleavage following activation, a similar differential retention of these phenotypes could occur if

the RecA433 filaments were less stable (resulting in lower concentrations of activated RecA). Consistent with this type of interpretation, a direct comparison between MucA and UmuD cleavage revealed that MucA was processed more rapidly [39]. Considering that biochemically, RecFOR functions to stabilize RecA filaments and prevent their depolymerization from 5' DNA ends, the two possibilities discussed here are not necessarily mutually exclusive [23, 24, 32]. A number of studies have shown that *recF* mutants also exhibit reduced frequencies of mutagenesis, and recently it was shown that RecFOR is also required for UmuDC-mediated bypass to occur in vitro, demonstrating that the RecFOR proteins are directly associated with the RecA filament and required to maintain the activated form of RecA [33, 48]. Thus, if the RecA433 mutation impairs its ability to interact with itself and form a stable filament, the same mutation may also reduce its ability to interact with RecFOR to stabilize the end of the filament. Differentiating between these possibilities will require biochemical characterization of the purified RecA433 allele. However, the results presented here, demonstrate that the RecA433 mutation specifically impairs several functions associated with the RecF pathway while retaining the ability to carry out RecBCD-mediated functions. In this respect, the RecA433 allele may represent a useful tool for dissecting the specific molecular mechanisms carried out by each pathway in vivo.

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