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RecA433 cells are defective in *recF*-mediated processing of disrupted replication forks but retain *recBCD*-mediated functions

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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 contains a single amino acid substitution (R243H). This mutation 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-induced 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.

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1. 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 [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 RecA processing of the arrested replication fork restores the lesion-containing region to a double-stranded form that 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 10^2 - to 10^3 -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*

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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 and 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 promotes 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 blot analysis of the cleavage product [36,37]. The differential retention of protein function led investigators to propose that the RecA433 defect may relate to an 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.

2. Materials and methods

2.1. 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::Tn10*) have been reported previously [8,49]. CL856 (SR108 *srlC300::Tn10 recA433*) was constructed by P1 transduction of *srlC300::Tn10 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 *recC ptrA 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::Tn10 recA433*) was constructed by P1 transduction of *srlC300::Tn10 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::Tn10* 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.

2.2. UV survival

A Sylvania 15-W 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.

2.3. Conjugational recombination

Fresh overnight cultures of the *arg* + Str^S donor (PK3), and the *arg*- Str^R recipient (AB1157) were diluted 1:25 in 5 ml of LBthy media and both strains were grown for 3 h 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 25 mm Fisher-brand 0.45 µm general filtration membrane. The filter was placed on an LB-thy plate for 1 h and then the cells were resuspended in 5 ml 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.

2.4. 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/s 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.

2.5. Nascent DNA degradation

A 0.1-ml aliquot of each strain was taken from an overnight culture and transferred to 10-ml of DGcthy medium containing 0.1 µCi of [¹⁴C] thymine/ml. The cultures were then grown to an OD₆₀₀ of 0.4, pulsed for 5 s with 1 µCi of [³H] thymidine/ml, filtered with a 0.45 µm membrane and then rinsed twice with 3 ml of NET buffer (100 mM NaCl, 10 mM Tris, pH 8.0, 10 mM 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 time-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.

2.6. 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 × 10⁸ cells/ml) and UV-irradiated with 50 J/m². At the indicated time-points, 0.75 ml samples were placed into 0.75 ml cold 1X 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 h. 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 h 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 (30 µl) 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 Roche using alpha [32 P]dCTP (MP Biomedicals). Radioactivity was visualized and quantitated using a Storm 820 and its associated ImageQuant Software (Molecular Dynamics).

3. Results

3.1. *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* pathways, we compared the UV hypersensitivity and recombination proficiency of *recA433*, *recF*, and *recBC* mutants, directly. We observed that *recA433* 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].

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 and 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 an 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.

3.2. *recA433* mutants remain resistant to formaldehyde-induced DNA–protein crosslinks

One feature that distinguishes the *recBCD* pathway from the *recF* pathway is its 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 cells or *recF* mutants. By comparison, *recBC* mutants were hypersensitive to formaldehyde as was a *recA* deletion (Fig. 1C). Thus, *recA433* mutants are similar to *recF*, but distinct from *recBC* mutants in that they remain resistant to formaldehyde.

3.3. *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

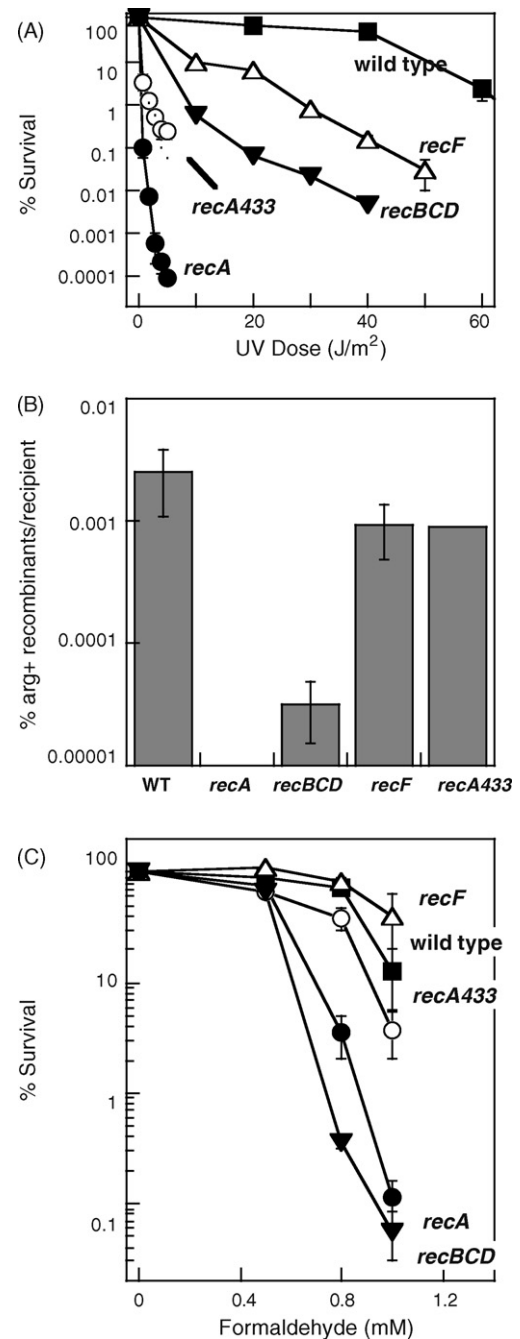


Fig. 1. *RecF* and *recA433* mutants are hypersensitive to UV, but remain proficient in conjugational recombination and are resistant to formaldehyde. (A) The survival of wildtype (squares), *recA* (filled circles), *recBCD* (filled triangles), *recF* (open triangles) and *recA433* (open circles) after UV-irradiation at the indicated doses 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 doses is plotted.

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

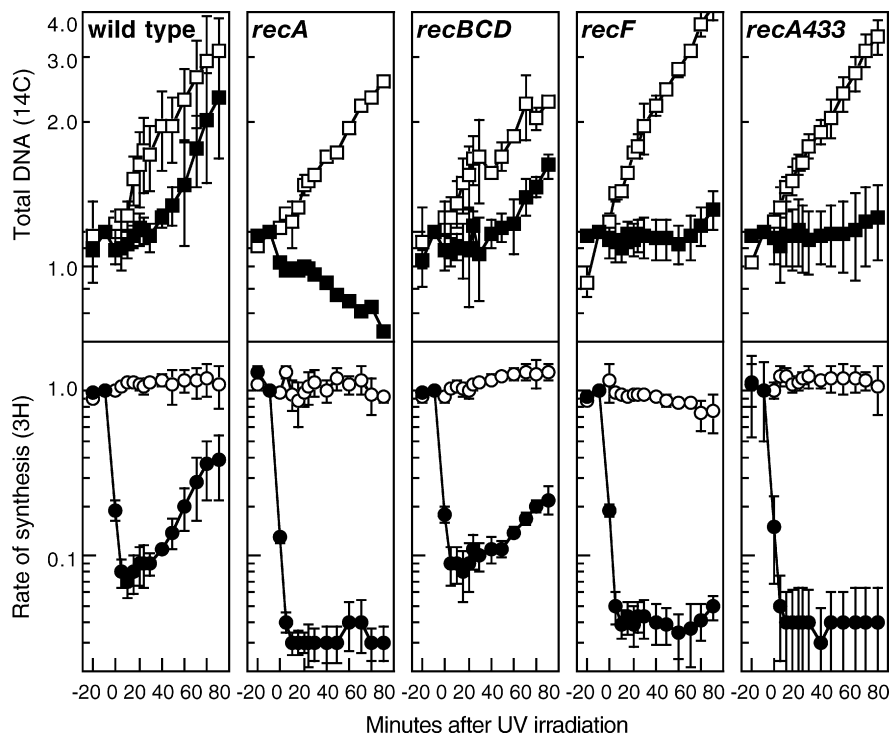


Fig. 2. *recF* and *recA433* mutants fail to recover DNA synthesis following disruption by DNA damage. [^{14}C]thymine-labeled cultures were pulsed for 2 min with [^3H]thymidine at various times after mock-irradiation or UV-irradiation with 27 J/m^2 . The rate of replication could therefore be compared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

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 [^{14}C] thymine were UV-irradiated with 27 J/m^2 or mock irradiated. To monitor how the rate of DNA synthesis was affected by these treatments, aliquots of the ^{14}C -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 (^{14}C 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 (Fig. 2). By contrast, in UV-irradiated *recA* deletion mutants, DNA synthesis did not resume following UV-irradiation.

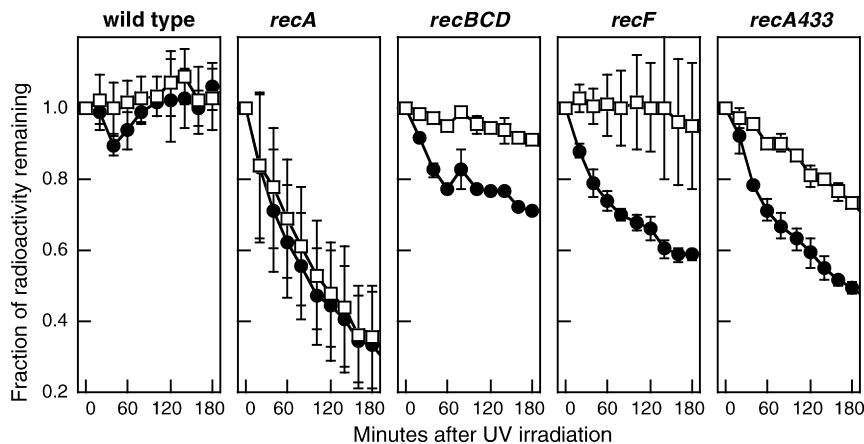


Fig. 3. Similar to *recF*, extensive degradation of the nascent DNA occurs at the growing fork after UV-irradiation in *recA433* mutants. [^3H]thymidine was added to cells pre-labeled with [^{14}C]thymine for 5 s prior to UV-irradiation with 27 J/m^2 in non-radioactive media. The fraction of [^3H]labeled nascent DNA at the replication fork (closed circles) and [^{14}C]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 ^{14}C ranged from 1508 to 7791 cpm and 700 to 1956 cpm for all experiments, respectively.

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 ^{14}C -labeled genomic DNA over the time course.

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 [^{14}C]thymine-labeled cultures were pulsed with [^3H]thymidine for 5 s to label the DNA at replication forks. The culture was then transferred to non-radioactive medium and immediately UV-irradiated with 27 J/m^2 . The ^{14}C pre-label 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. 3). 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 DNA 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 also occurred in the overall genomic DNA, though this was less extensive than occurs in the *recA* deletion (Fig. 3).

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, *recBCD* 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/m^2 UV-irradiation in *E. coli* cultures using two-dimensional agarose gel analysis [8]. This dose produces 0.5 lesions per plasmid strand. Approximately 90% of the wild type cells survive at this dose 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

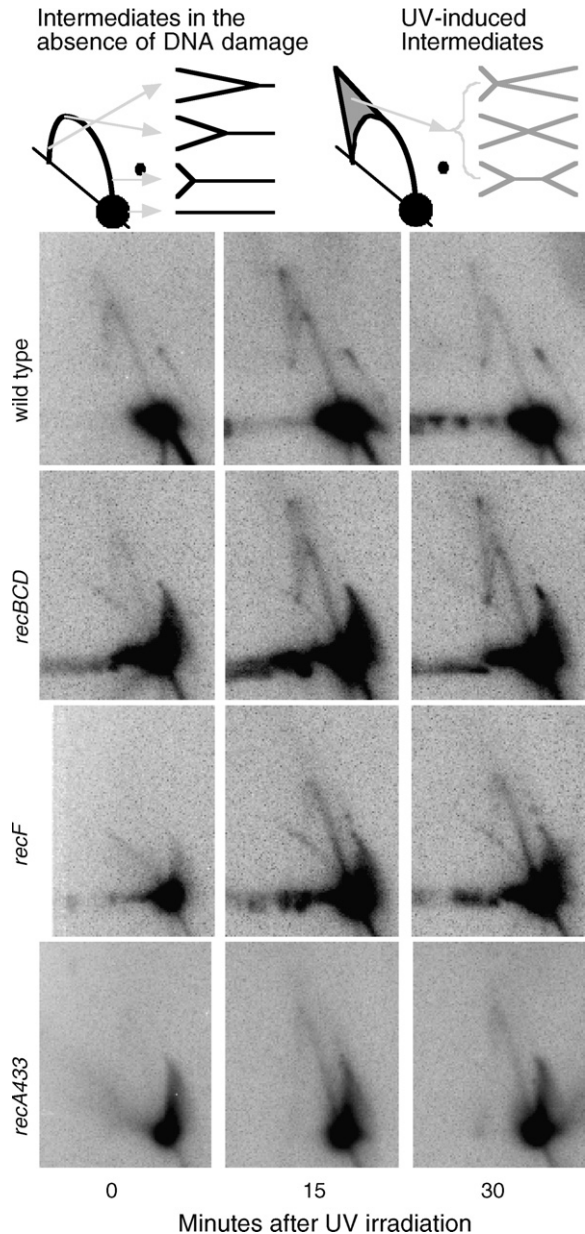


Fig. 4. UV-induced replication intermediates are not observed in either *recF* or *recA433* mutants. The migration pattern of PvuII digested pBR322 plasmid observed by 2D-agarose gel analysis is diagrammed. Non-replicating linear plasmids run as a linear 4.4-kb fragment. Replicating plasmids form Y-shaped structures that migrate slower than the non-replicating 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. 2D-agarose gels were probed with pBR322 plasmid DNA from cultures of wild type, *recBCD*, *recF*, and *recA433* mutants containing the plasmid at the indicated times following UV-irradiation.

a two-dimensional agarose gel, non-replicating 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 non-linear 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

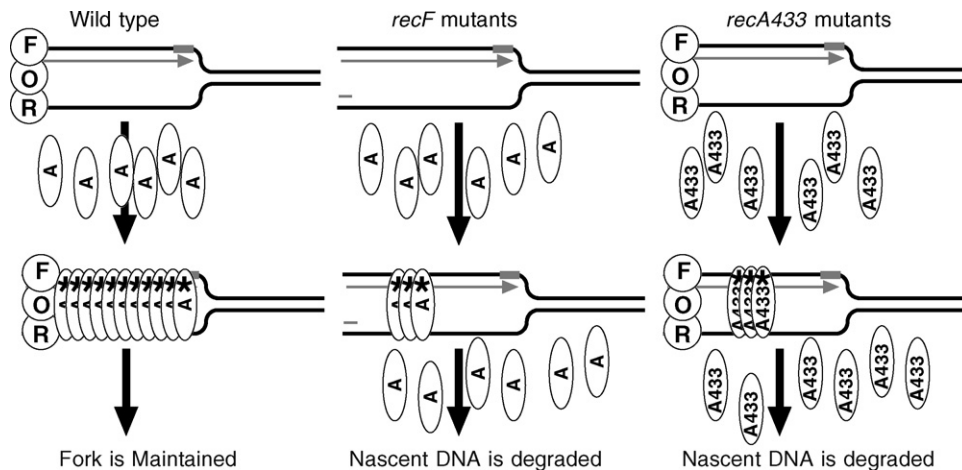


Fig. 5. RecA433 allele fails to maintain or protect replication forks follow arrest by DNA damage.

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.

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 two-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.

4. 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 two-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 *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 work by Ennis and Woodgate that suggested that the RecA433 mutation may impair specific protein–protein interactions [37]. 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].

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.

Conflict of interest

The authors confirm that there is no conflict of interest associated with this work or its submission. No materials were used from other published papers. This manuscript has not been submitted or published elsewhere.

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