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Lack of Replication Disruption Following H2O2-induced Damage in *Escherichia coli*

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Abstract

Hydrogen peroxide (H_2O_2) toxicity has long been thought to be predominantly due to oxidative DNA damage that can disrupt DNA replication and result in lethality. Curiously and contrary to this view, it is also well established that the glycosylases responsible for repairing oxidized-base damage are as resistant as wild-type cells when treated with H_2O_2. The observation raises the possibility that H_2O_2-induced DNA damage does not disrupt or prevent replication. To examine this possibility, I examined the sensitivity of *recF* mutants to H_2O_2. RecF is known to be required to maintain and restore replication forks after disruption by DNA damage. Survival curves of mutants treated with either UV irradiation or H_2O_2 were generated and, as expected, *recF* mutants were shown to die off quicker after UV exposure, relative to wild-type cells. However, *recF* mutants were not hypersensitive to H_2O_2. The results would be consistent with the idea that DNA damage induced by H_2O_2 does not disrupt DNA replication and may not factor significantly into its lethality.

Introduction

The most fundamental element of all cellular life, DNA, is under a seemingly constant barrage of damaging elements, particularly of interest are those agents which cause lesions in the DNA Two major forms of lesion-causing damage, UV irradiation and oxidative damage, are known to increase genomic instability, as a result of the lesions they generate. The sorts of lesions which these types of damage create are unique and distinct from each other, UV only creating two while oxidation has several different forms of damage. The major forms of oxidative base damage are thymine glycols and 8-oxoguanine while the major forms of UV damage are 6,4 photoproducts and cyclobutene pyrimidine dimers (see Fig. 1).[4][25]

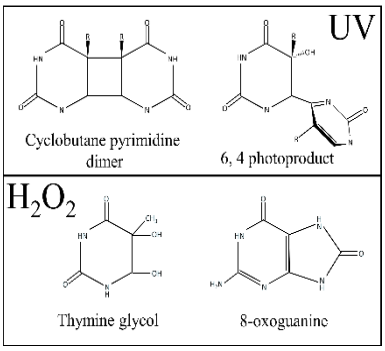

Fig 1. The major forms of both UV and oxidative DNA damage.

One difference immediately evident between these two types of damage is the size of these lesions, UV-lesions are characteristically bulky. This bulkiness can cause significant structural distortions to the DNA. 6,4 photoproducts are known for causing adjacent bases to move into a perpendicular position, contrary to normal parallel conformation.[5] While oxidative-lesions are less bulky, they may fail to base pair properly or create a structure that physically blocks the DNA polymerases.[22] Apart from the physical changes to the DNA structure, these lesions also have the capacity to act as mutagenizing agents when polymerases incorporate the incorrect base during replication, and both UV irradiation and H_2O_2 have been used for mutagenesis for some time.[2][23] These taken together can explain some of the lethality of these agents to bacterial cells. Additionally, both types of lesions have been shown to block DNA polymerases *in vitro* within the *Escherichia coli* model[5][22] and are thought to disrupt replication *in vivo* as well (see Fig. 2). This stoppage of replication, would almost certainly prove fatal to the cell, if replication cannot be restored to complete the duplication of the chromosome before the cell divides.[10]

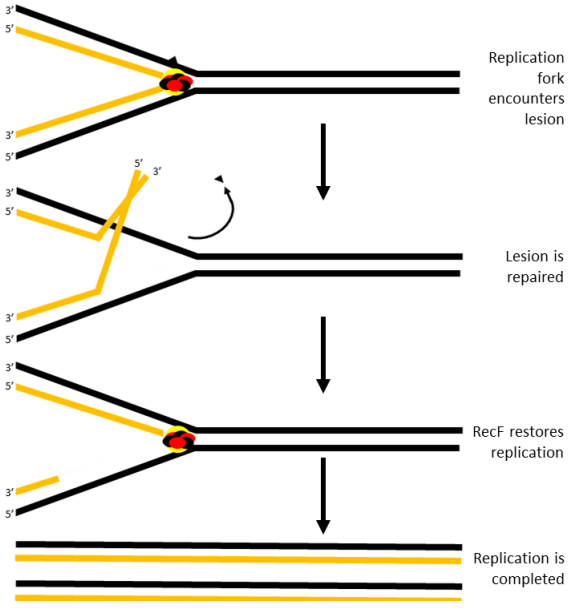

Fig 2. The propose operation of the replication fork upon encountering a UV lesion, followed by resolution of damage and restoration of replication. Adapted from Courcelle et al. 1999, *J Bacteriol,* 181:916.

Given the great threat to cells from these types of damage, it follows that cells have certain repair pathways which can be used to restore DNA to its proper state, and have, in fact, evolved many systems to deal with the different types of damage.[10] Two of these pathways, nucleotide and base excision repair, account for the repair of UV and oxidative-lesions, and act very differently on the enzymatic level. In the base excision repair pathway, the incorrect base is rotated out from the DNA helix and excised by a DNA glycosylase, followed by subsequent incisions of the sugar-phosphate backbone by either an associated AP-lyase activity or AP-endonuclease. [24] During nucleotide excision repair, the dual incisions surrounding the altered base(s) fully excise the damaged region. In both pathways, the excised region is then resynthesized by polymerase I and ligated by DNA ligase [18]. The nucleotide excision repair exonuclease, which is required to remove UV-lesions, is made up of UvrA, UvrB and UvrC.[27] Mutants lacking any one of these

three proteins cannot remove DNA lesions after UV and are hypersensitive.[3][16][30] In contrast, although the Endo III and Fapy glycosylases, encoded by *nth* and *fpg*, are similarly required to remove H_2O_2-induced lesions, mutants lacking these proteins remain as resistant as wild-type cells when treated with H_2O_2.[30] Lethality from H_2O_2 is often thought to result from DNA lesions that prevent the replication of the genome. However, this latter observation raises the possibility that the presence of oxidative-lesions do not prevent genome replication and imply that H_2O_2-induced lethality may result from an alternate mechanism or impediment.

When replication is disrupted by DNA damage, restoring DNA synthesis requires RecF, along with several of the proteins associated with the *recFOR* pathway; RecF, RecO, RecR, and RecA are needed to maintain and protect the DNA at the replication fork until the blocking lesion can be repaired.[8][9][10][11] Other recF pathway-associated proteins, RecJ, a 5 –3 single-stranded exonuclease, RecQ, a 3 –5 DNA helicase, process or partially degrade the nascent DNA at the fork at times prior to the resumption of DNA synthesis.[10][11] This nascent DNA processing enhances RecF, -O, and -Rs ability to maintain the arrested fork and restore the lesion-containing region to a form that is accessible to repair enzymes.[8][9][10][11] Under conditions where either the nascent DNA processing or repair cannot occur, the recovery of DNA synthesis is impaired and higher levels of lethality, rearrangements, and mutagenesis are observed.[7][12][21][28]

Understanding how DNA damage is processed by the cell is vastly important to human health. Mutations in human repair pathways homologous to those in *E. coli* can lead to serious health issues, including cancers.[1][6][14] Notably, the crystal structure of RecF has been identified as strikingly similar to a human protein, Rad50. The structure of RecF has been shown to have a strong similarity to the head domain of the Rad50, despite the addition of a long coiled coil structure in Rad50. In spite of this difference, they have been shown to have similar subdomains in RecF's ATPase subdomain and the so-called Lobe II of Rad50.[24] Additionally, Rad50 has a very similar function as a part of a repair pathway.[18] Eukaryotic cells and *E.* coli are separated by vast evolutionary time, and yet this mechanism is seemingly conserved across domains, and so understanding the bacterial system can lead to greater understanding of the human system.[24] Furthermore, this eukaryotic protein Rad50 has been directly shown to be correlated to certain cancers when it is mutated. When it is not present in cells, DNA damage cannot be repaired and chromosomal instability follows.[18] Given the further understanding of this specific function in relation to human health could allow, at some point, the development of a more targeted therapeutic.

Considering the unique lack of sensitivity of glycosylase mutants that fail to remove oxidative DNA damage, I considered the possibility that these lesions were not disrupting replication. To test this idea, *recF* mutants, which are required to restore replication after disruption, were exposed to UV- and H_2O_2-damage,. If replication were disrupted by oxidative DNA damage, then I would expect to observe that recF mutants are hypersensitive when treated with H_2O_2.

Methods

UV Survivals. In examine survival of the various mutants and wild type cells; *E. coli* SR108 wild type, *xth*, *recF*, and *uvrA* mutants were struck out on LB plates enriched with 10µg/ml thymine (LBthy) and left to incubate at 37°C overnight. Single colonies were then picked and grown in 2 mL of DGCthy medium overnight at 37°C. These cultures were then diluted 1:100 in 5 mL of DGCthy, and were grown for 4-4.5 hours, at which point the cultures were serially diluted in six ten fold increments and spotted on LBthy plates in triplicate. The wild type, *xth*, and *recF* plates were then UV irradiated using a 15W germicidal lamp with 20, 40, 60, 80 and 100 J/m^2 on a rotating platform, keeping behind an unirradiated control. *uvrA* was irradiated at 1, 2, 3, 4 and 5 J/m^2 due to the hypersensitive phenotype expected of this control, as with the other strains, an unirradiated control was set aside. The plates were then incubated at 37°C overnight. The colonies were counted and the fraction surviving, and able to form colonies, were determined relative to the unirradiated control.[10]

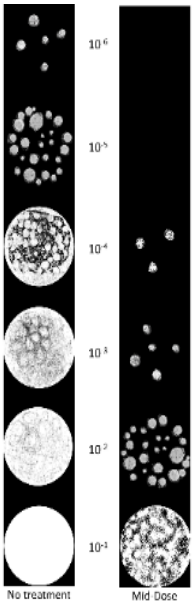

Fig 3: Depiction of a typical row of dilutions on a spot plate.

H_2O_2 **Survivals.** *E. coli* SR108 wild type, *xth*, *recF*, and *uvrA* mutants were struck out on LBthy plates and left to incubate at 37°C overnight, and were then grown in 2 mL of DGCthy medium overnight, also incubated at 37°C. These cultures were diluted 1:100 in 5 mL of DGCthy, and were grown for 3.5-4 hours. Five sets of dilutions were set up for each strain kept on ice, one set for each was serially diluted with the subculture to act as the untreated control. 10 mM H_2O_2 was then added to the subculture. 0.1-ml samples were taken from wild type, *recF*, and *uvrA* at 5, 10, 20 and 30 min post-H_2O_2 addition and serially diluted as before. The same procedure was completed for *xth*, except aliquots were taken at 2.5, 5, 7.5, and 10 minutes to account for the predicted hypersensitivity. The dilutions were then spotted in triplicate onto LBthy plates, incubated at 37°C overnight, and the surviving fraction was determined as described above.

Results

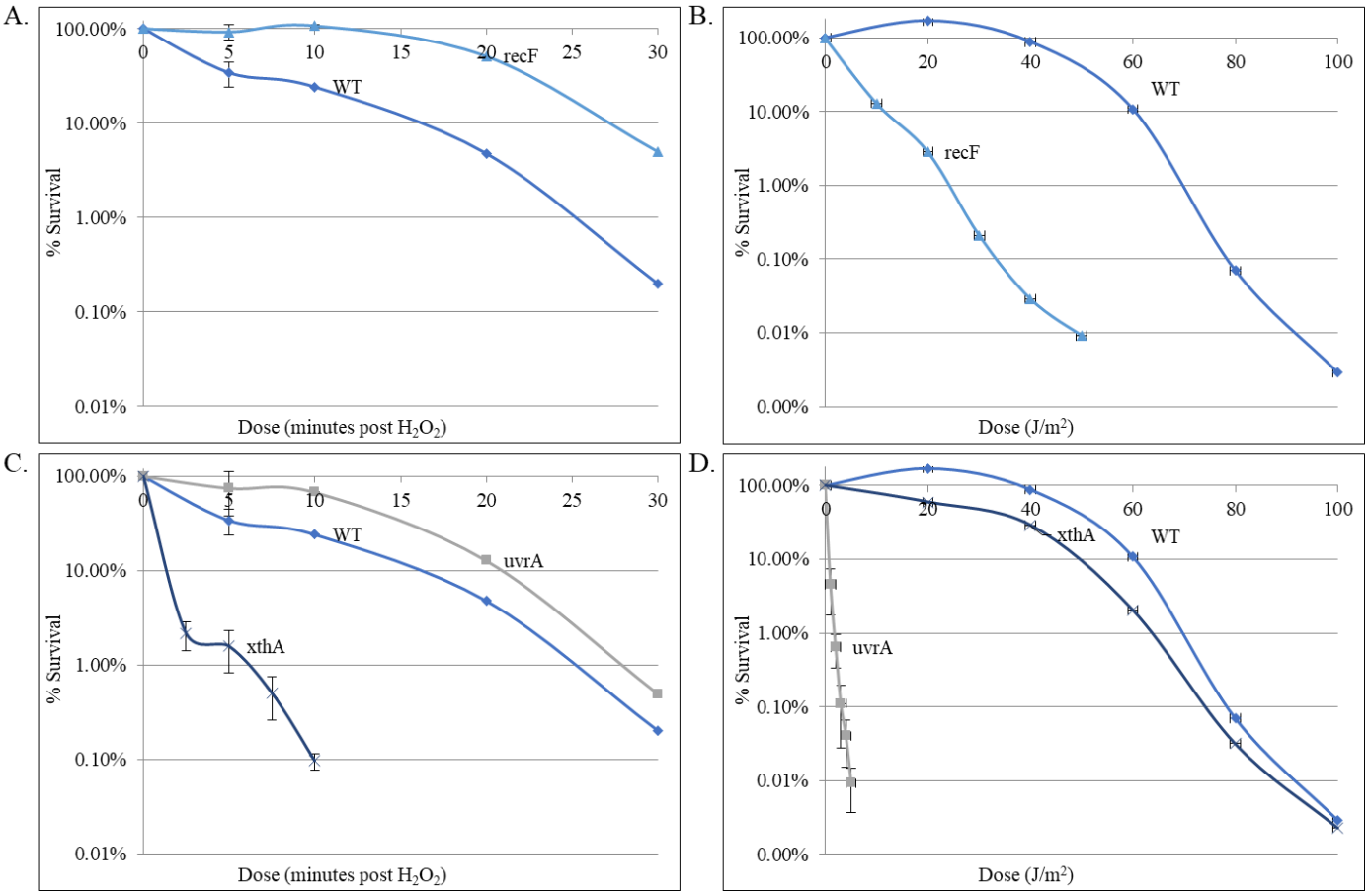

Fig 4. A. Logarithmically scaled percent survival of E. coli SR108 and mutant strain *recF* in increasing doses of H_2O_2 (minutes post H_2O_2). B. Logarithmically scaled percent survival of E. coli SR108 and *recF* in increasing doses of UV irradiation (J/m^2). C. Logarithmically scaled percent survival of E. coli SR108, *uvrA*, and *xthA* in increasing doses of H_2O_2 (minutes post H_2O_2); acting as controls. D. Logarithmically scaled percent survival of E. coli SR108, *uvrA*, and *xthA* in increasing doses of UV irradiation (J/m^2); acting as controls.

To determine if RecF is required for resistance following H_2O_2 treatment, cultures of *recF* and its wild type parental strain were treated with 10mm H_2O_2 for various times and percent survival of each dose was calculated relative to the control plate. These survival rates were then plotted against dose. As shown in Figure 4A, *recF* remained as resistant as wild type at all doses examined. In fact, it was modestly more resistant than the wild type strain at each dose. As a control, I also exposed these strains to UV irradiation. Previous studies have shown that following the disruption of replication in the absence of *recF*, cells are hypersensitive to UV.[9][19]

As expected, the *recF* mutant showed hypersensitivity, as compared to wild type *E. coli*, under increasing doses of UV irradiation (Fig. 4B). Together, the observed UV sensitivity of *recF* verified the mutation in this strain and implies that RecF is not hypersensitive in the presence of H_2O_2-induced DNA damage.

To control for the trivial explanation that our H_2O_2 preparation was ineffective or our concentrations were miscalculated, I also examined the sensitivities of both *uvrA* and *xth* mutants to UV and H_2O_2 treatment. *uvrA* mutants are known to be sensitive to UV but not H_2O_2.[21][23] Conversely, xth encoding the primary AP-endonuclease of *E. coli*, has been shown to contribute to survival in the presence of H_2O_2.[13][22] As shown in Figures 4C and 4D, *uvrA* and *xthA* mutants behaved as expected, with *uvrA* being hypersensitive

to UV damage, even at drastically lower doses than the rest of the mutants, and *xthA* surviving well when compared to wild type. This converse is true, when exposed to increasing doses of hydrogen peroxide.

uvrA showed no sensitivity, as compared to wild type, when exposed to increasing doses of hydrogen peroxide but showed a very drastic hypersensitivity to UV irradiation. This is expected due to the involvement of *uvrA* in UV lesion nucleotide excision repair.[23][27] Opposed to this trend, *xthA* acted as expected; showing little sensitivity to UV while showing sensitivity to H_2O_2 The results demonstrate the potency of the H_2O_2 preparation and show the unexpected results that recF does not contribute to survival in the presence of H_2O_2-induced DNA damage.

Discussion

The results listed are consistent with the idea that, in contrast to UV-induced DNA damage, DNA damage induced by H_2O_2 may not disrupt DNA replication. This interpretation is supported by the observations that mutants lacking glycosylases required to remove these lesions are not hypersensitive to DNA damage, continuing to grow and form colonies.[27] They are also supported by the results presented here, showing that the absence of RecF[10], which is required to restore replication disrupted by DNA damage, is not hyposensitive to H_2O_2. Taken together, it would seem that this repair is not necessary for the replication to proceed. In fact, it is possible that replication fork simply goes past these oxidative-lesions, and replication can be completed without implementation of the *recFOR* pathway. This is quite peculiar, as previous work had shown that some oxidative-lesions represent blocks to DNA polymerase I in vitro on oligo substrates, leading the authors to suggest that these lesions are likely to disrupt replication.[22] Further, transformation of phi X 174 phage containing oxidative-lesions was shown to impair phage survival.[5][26] Both of these observations support the idea that oxidative-lesions should disrupt replication. However, the former study used polymerase I, not the replicative polymerase III. Thus, it is possible that the replicative polymerase would not be blocked at these lesions as was observed for Pol I. The second study employed the single strand phage phi X 174 phage as a mechanism to prevent repair, so that polymerization could be examined specifically. Replication of single stranded DNA may not require or utilize the entire assembly of replisomal proteins in vivo, and thus, again, may yield significant differences to lesions encountered by the replisome in double stranded DNA on the chromosome.

It is also possible that our although recF is not hypersensitive, that replication is disrupted and that an alternative, undiscovered mechanism prevents lethality and restores replication under these conditions. Pursuit of this possibility would require further studies, perhaps screening for mutants in a recF background that render cells hypersensitive to H_2O_2.

The biochemistry and introduction of these lesions into DNA is dependent on the reactivity of oxygen, and byproducts of enzymatic processes which result in harmful forms of oxygen, such as free radicals or hydrogen peroxide. Oxygen is intrinsic to the life processes of cells and is also, mostly, readily available in the atmosphere.[4] UV-irradiation is also strongly relevant in the environment, most notably the sun. Given this information, it may, then, seem counterintuitive that the cell would have a system in place to stop at UV-lesions for repair, but not for oxidative-lesions, when both of these types of damage are readily taken on by the environment. Additionally, it may seem counter-intuitive based on how many types of oxidative-lesions exist. However, there are two highly speculative possibilities which could be interesting to explore in a future study. One of the major differences between oxidative- and UV-lesions is the size, UV-lesions tend to be quite bulky. In this sense, it is possible that the UV-lesions simply pose a greater

threat to genomic stability and the ability for the DNA to function as intended. There may also potentially be some evolutionary explanation in that *E. coli* species are commonly found to be living within the intestinal tracts of mammals, wherein they would experience little UV irradiation, but would experience higher levels of oxidative stress. It could be some adaptation to otherwise survive oxidative-lesions in order to expend less energy removing damage which constantly befalls the bacteria, particularly taken with the previous idea that oxidative-lesions are not nearly as bulky as UV-lesions and may not compromise the structure nor function of the DNA. Since this is highly speculative, it is very clear that neither of these options may be true, and otherwise a novel mechanism exists which does not utilize RecF as does replication of DNA containing UV-lesion.

Regardless, this research stands as a starting point for many other research questions in the future. There are many avenues which could be explored in regard to oxidative damage and its interaction within the cell. Of interest, many chemotherapeutics appear to cause oxidative damage, and approaching the interaction of these chemicals within the cell could lead to interesting discoveries.

Acknowledgements

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