Published in final edited form as:
Mol Microbiol. 2015 March ; 95(5): 754–768. doi:10.1111/mmi.12885.

Escherichia coli radD (yejH) gene: a novel function involved in radiation resistance and double-strand break repair

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Summary

A transposon insertion screen implicated the *yejH* gene in the repair of ionizing radiation-induced damage. The *yejH* gene, which exhibits significant homology to the human transcription-coupled DNA repair gene XPB, is involved in the repair of double strand DNA breaks. Deletion of *yejH* significantly sensitized cells to agents that cause double strand breaks (ionizing radiation, UV radiation, ciprofloxacin). In addition, deletion of both *yejH* and *radA* hypersensitized the cells to ionizing radiation, UV, and ciprofloxacin damage, indicating that these two genes have complementary repair functions. The Δ*yejH* Δ*radA* double deletion also showed a substantial decline in viability following an induced double-strand DNA break, of a magnitude comparable to the defect measured when the *recA*, *recB*, *recG*, or *priA* genes are deleted. The ATPase activity and C-terminal zinc finger motif of *yejH* play an important role in its repair function, as targeted mutant alleles of *yejH* did not rescue sensitivity. We propose that *yejH* be re-named *radD*, reflecting its role in the DNA repair of radiation damage.

Keywords

yejH; radD; radA; ionizing radiation; DSBR

Introduction

Cellular DNA is routinely subjected to environmental, chemical, and metabolic damage. DNA backbone breakage can lead to double-strand breaks, which must be repaired in order for the genome to be replicated. There are several common sources of strand breaks. Ionizing radiation (IR) can generate breaks primarily via the generation of reactive oxygen species such as hydroxyl radicals (Bresler *et al*., 1979, Swarts *et al*., 2007, Ward, 1988). The reactive oxygen byproducts of aerobic metabolism can similarly give rise to strand breaks (Collins *et al*., 2005, Mikkelsen & Wardman, 2003). UV irradiation causes base pair lesions that can lead to transient strand breakage during nucleotide excision repair (Sinha & Hader, 2002). Protein-DNA adducts, caused by chemicals such as the gyrase-inhibiting quinolones, also lead to strand breaks following transcription, replication, or proteolysis (Drlica *et al*., 2008).

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In bacteria, double-strand break repair (DSBR) is mediated through the recombinational DNA repair pathway catalyzed by the RecBCD helicase/exonuclease (Anderson & Kowalczykowski, 1997, Dillingham & Kowalczykowski, 2008, Spies & Kowalczykowski, 2006, Taylor & Smith, 2003), RecA recombinase (Cox, 2000, Cox *et al*., 2000, Cox, 2007, Kowalczykowski & Eggleston, 1994, Lusetti & Cox, 2002), and RuvABC resolvase (Kuzminov, 1999). While this process is relatively well understood, it is possible that additional *in vivo* components have not yet been identified. In addition, the function of some proteins already implicated in DSBR is poorly understood. For example, loss of the *radA* gene function clearly sensitizes cells to ionizing radiation (Diver *et al*., 1982, Byrne *et al*., 2014a). The *radA* gene product appears to play a role in processing branched DNA recombination intermediates, similar to *recG*, although this role has not been clearly defined (Beam *et al*., 2002).

The current recognized repertoire of *E. coli* DNA repair genes has been compiled in screens carried out over a period of nearly four decades (Konrad, 1977, Mahdi & Lloyd, 1989, Volkert & Nguyen, 1984, Kolodner *et al*., 1985, Ohta *et al*., 1999, Clark & Margulies, 1965, Howard-Flanders, 1968, Modrich, 1987). Screens to identify genes involved in radiation resistance were part of these efforts. The *recN* and *recG* genes have a demonstrated role in radiation resistance as well as DNA double strand break repair, and they were originally assigned a “rad” nomenclature (*radB* and *radC*, respectively) until their functions were further explored (Lombardo & Rosenberg, 2000, Sargentini & Smith, 1986).

Modern screening technologies provide ever more robust pathways to identify previously overlooked genes playing a role in almost any pathway or process of interest. We can more readily carry out saturating screens using disrupting, traceable inserts in every non-essential gene (van Opijnen *et al*., 2009). Using a transposon insertion library, we were able to identify all non-essential genes in *Escherichia coli* that are involved in responding to ionizing radiation damage (Byrne *et al*., 2014a). While the identified genes covered a range of DNA repair and protein metabolism factors, one that caught our attention was the previously uncharacterized gene *yejH*. Although *yejH* and the *uvr* proteins probably address different types of radiation-induced DNA damage, deleting *yejH* from the founder strain yielded a radiation sensitivity phenotype similar to that seen for *uvrA/B* deletions (Byrne *et al*., 2014a).

In this report, we investigate the function of *yejH*. We establish a role for the *yejH* gene product in the repair of double strand breaks that largely overlaps that of the genes *radA* and *recG*. The results justify a replacement of the generic and functionally uninformative *yejH* gene name with the more appropriately descriptive designation *radD*.

Results

Identification of yejH as a potential radiation repair gene

The *yejH* gene was identified during a genome-wide transposon-insertion screen for all non-essential genes with a role in recovery from ionizing radiation (IR) (Byrne *et al*., 2014a). BLAST searching revealed that the closest homolog to YejH/RadD is the archaeal or human XPB, a superfamily 2 helicase important for transcription initiation and transcription-

coupled nucleotide excision repair (Fuss & Tainer, 2011). YejH/RadD contains all seven of the superfamily 2 helicase motifs (I, Ia, and II-VI), including the Walker A motif associated with ATP hydrolysis (I), indicating a possible helicase function (Fig. 1). Although YejH does not contain the N-terminal DNA recognition domain (DRD) found in XPB, it does contain a cluster of cysteines in the C-terminus. Utilizing the motif prediction program SVMProt (http://jing.cz3.nus.edu.sg/cgibin/svmprot.cgi; (Cai *et al*., 2003)), the structure of this cluster correlates most closely with a zinc binding motif (99% correlation with Zn, relative to 68% with Fe). This structural feature may assist with DNA binding.

The effect of *yejH/radD* gene inactivation on IR survival was confirmed by deleting the *yejH* gene and observing increased radiation sensitivity ((Byrne *et al*., 2014a), and Fig. 2). The D_{37} for the Δ*yejH/radD* strain was 602 Gy (Fig. S1). This may be compared to a D_{37} of 1015 Gy for the founder strain used as the control strain in this study (Fig. S1), which includes a deletion of the cryptic e14 prophage that is lost rapidly in trials to generate radiation resistance by directed evolution (Harris *et al*., 2009, Byrne *et al*., 2014b). Deletion of e14 has a small but significant positive effect on IR sensitivity (Harris *et al*., 2009, Byrne *et al*., 2014b). It is deleted in all strains used in the present study to eliminate any effects its spontaneous (and unobserved) loss might cause in experiments involving irradiation. For the remainder of this report, we simply refer to the *yejH* gene as *radD* and the founder Δe14 strain as wildtype.

The putative ATP hydrolytic function of radD contributes to radiation damage repair

A mutation was made in the conserved lysine of the Walker A motif (K37R), a change classically associated with the elimination of ATPase function (Moarefi *et al*., 2000). This mutant was inserted onto the genome in its normal chromosomal location, as well as on a plasmid for protein expression (Tables 1&2). Irradiated *E. coli* containing the *radD* K37R mutant in place of wildtype *radD* on the chromosome showed an intermediate level of survival between that of wildtype and the Δ*radD* strain (Fig. 2A), suggesting that the putative ATPase deficient mutant can perform some but not all of the functions of *radD* in responding to radiation damage.

To confirm that the IR sensitivity phenotype was indeed due to lack of the *radD* gene, an expression plasmid containing wildtype *radD* was transformed into the Δ*radD* strain. This plasmid was able to rescue the phenotype of irradiated Δ*radD* cells to nearly wildtype levels (Fig. 2A). Expression of the gene was not induced with IPTG, indicating that a low background level of protein expression is sufficient to rescue the phenotype. Survival of irradiated Δ*radD* cells with a plasmid containing the *radD* K37R mutant was less, albeit very similar to the genomic *radD* K37R mutant. An empty vector control produced similar levels of resistance to that of the plasmid expressing *radD* K37R (but less than one expressing the wild type *radD*), suggesting that vector-mediated expression of the *radD* K37R mutant protein did not confer any significant increase in IR resistance. Overall, the effects of the presence or absence of the wild type *radD* gene indicates that elimination of *radD* function is responsible for the observed IR sensitivity phenotype. A possible effect of the RadD K37R mutant protein on IR survival is not confirmed by these results.

radD and radA have complementary functions in radiation damage repair

To further explore *radD* functions, the *radD* gene was deleted in combination with several other genes. A Δ*radD* mutation increased the effects of Δ*uvrA* or Δ*uvrB* (Fig. 2B). The increase in sensitivity is substantial, approximately additive (Fig. S2). The *uvrA* and *uvrB* gene products are involved in nucleotide excision repair and crosslink repair (Sancar & Rupp, 1983, Sladek *et al*., 1989). In contrast, as shown below, the *radD* gene product is involved in some aspect of DNA double strand break repair, and the relationship with *uvrAB* was not further explored. The *radD* deletion was also combined with a deletion of the *uup* gene, as the loss of *uup* confers sensitivity to ionizing radiation at levels similar to that seen when the *radD* function is lost (Byrne *et al*., 2014a). A Δ*radD* Δ*uup* strain was no more sensitive to ionizing radiation than Δ*radD* alone (Fig. 2C). This may indicate that *uup* and *radD* participate in a joint pathway, but this has not been further explored. A Δ*radD* Δ*recG* strain grew very slowly and accumulated suppressor mutations rapidly. A Δ*radD recG*$^{-}$ strain behaved similarly. We isolated multiple examples of the suppressors from both double mutant strains. Similar to suppressors of *recG* deficiency that were previously isolated by the Lloyd (Al Deib *et al*., 1996) and Kogoma (Kogoma *et al*., 1996) laboratories, all but one of the sequenced suppressors appeared in the gene *priA* and are listed in Table 3. One of these, *priA* A520P, appeared twice (once in the set obtained from each of the double mutant strains) and is identical to a *priA* suppressor of *recG* deficiency isolated previously (Al Deib *et al*., 1996). We presume that the *priA* changes eliminate the PriA helicase activity without eliminating primosome assembly as observed in the earlier studies. One of our *priA* alleles (*priA* IN W689 (RW); insertion of codons encoding RW after codon 689) suppressed the UV sensitivity of a Δ*recG* strain (Fig. S3). However, the same *priA* allele increased the UV sensitivity of a Δ*radD* strain (Fig. S3). We conclude that the suppressors work primarily by suppressing the effects of the *recG* deficiency rather than mitigating the effects of the *radD* deletion. The one suppressor not found in *priA* has not yet been identified.

The combination of Δ*radD* and Δ*radA* produced a significant and nearly additive decrease in survival post-irradiation (Fig. 2C and Fig. S2). The function of *radA* is poorly understood, but a link with RecA protein and DNA double strand break repair has been evident (Beam *et al*., 2002). This suggests the *radD* and *radA* genes have complementary functions in the cellular response to radiation damage. The results of combining Δ*radD* with Δ*radA* appeared the most immediately informative and formed much of the basis of the continued work described below.

radD and radA also respond to UV irradiation damage

We continued to investigate the effects of the Δ*radD* and Δ*radD*Δ*radA* genotypes by exploring UV irradiation. In contrast to a previous report (Beam *et al*., 2002), we were able to consistently demonstrate UV sensitivity (albeit quite modest) in the Δ*radA* strain (Fig. 3). This is likely due to the higher doses of UV irradiation used in the current study.

As with IR, the Δ*radD* and Δ*radA* strains both exhibited only small defects in viability as single mutants when exposed to high doses of UV. However, the Δ*radD*Δ*radA* strain displayed a greatly enhanced, and in this case slightly synergistic, sensitivity (Fig. 3A and S2). The effects of the two deleted genes together are somewhat greater here than observed

in the accompanying article (Deani Cooper, Daniel C. Boyle and Susan T. Lovett, accompanying paper), most likely due to the higher doses of UV irradiation used in our study. In contrast to the IR results, the Δ*radD* strain showed a somewhat less severe effect than the Δ*radA* strain, indicating that the two enzymes may target different types of damage. The UV dose levels utilized in Fig. 3 were directly validated (Fig. S4).

To complement the UV sensitivity phenotype, we provided the wildtype *radD* gene on an expression plasmid (Table 2). Due to the modest difference in UV sensitivity observed between founder and Δ*radD* strains in response to UV, we chose to complement the Δ*radD*Δ*radA* strain to produce an effect that was potentially more readily measurable. Indeed, the Δ*radD*Δ*radA* strain containing *radD* on a plasmid restores UV viability to a level that is within error of that observed with the Δ*radA* strain (Fig. 3A). Strikingly, adding back the Walker A mutant *radD* K37R to the Δ*radD*Δ*radA* strain resulted in an increased sensitivity to UV irradiation. This suggests that RadD K37R may be binding to but not processing a DNA intermediate or protein-DNA complex, blocking its processing by alternative pathways. These results were confirmed by adding the *radD* K37R plasmid into the wild type strain and observing a dominant negative effect following UV irradiation (Fig 3B). The addition of wildtype *radD* into the wild type strain also had a somewhat negative effect at the highest dose of UV, suggesting that increased levels of RadD may interfere with some DNA repair events.

Based on the sequence of RadD (Fig. 1), the helicase domain is likely conserved in the core of the protein, while the C-terminus may be involved in protein-DNA or protein-protein interactions. To determine the importance of these regions, two additional *radD* alleles were generated and inserted into the expression plasmid to be used in complementation tests (Table 2). A RadD core enzyme was generated by truncation shortly after helicase motif VI, removing all C-terminal residues after amino acid 355. A RadD C437A mutant changed one of the four cysteines of the putative zinc finger to alanine. These two mutants, along with the Walker A mutant RadD K37R, were tested for their ability to complement the UV sensitivity of the Δ*radD*Δ*radA* strain, using spot plating to observe qualitative viability defects. Although the constructs were designed to eliminate a distinct domain or motif, we cannot rule out that these mutations could have affected proper protein folding, Unlike the wildtype *radD* gene, none of the three mutant alleles were able to complement the viability defect (Fig. 3C), suggesting that ATP hydrolysis, the C-terminus, and the zinc finger motif are all important for responding to UV irradiation damage. An empty vector control also exhibited no complementation (Fig. 3D). As seen in Figure 3A&C, complementation with the K37R mutation again made the cells somewhat more sensitive to UV than the Δ*radD*Δ*radA* strain. The other two variants did not produce this effect (Fig. 3C). The latter result may reflect a general loss of structural integrity due to the mutations, or a targeted loss of a DNA binding activity.

radD and radA are synergistic in their response to ciprofloxacin treatment

To further confirm the type of damage to which Δ*radD* mutants are susceptible, we implemented a radiation-free method that is known to induce double-strand breaks. We

chose ciprofloxacin, an inhibitor of gyrase that traps covalent protein-DNA adducts, leading to double-strand breaks during replication, transcription, or proteolysis.

Wildtype and mutant strains were grown and spot plated on LB plates containing increasing concentrations of ciprofloxacin. Unlike the irradiation experiments, colonies grown on ciprofloxacin plates were of widely varying sizes, making colony counting impractical. Therefore, only qualitative results are shown. At the lower dose of ciprofloxacin (0.005 μg/mL), the founder, Δ*radD*, and Δ*radA* strains exhibit no defect. In contrast, the Δ*radD*Δ*radA* strain exhibits a dramatic decrease in viability (Fig. 4A). At the higher dose (0.01 μg/mL ciprofloxacin), the wildtype strain begins to show a growth defect, indicated by the smaller size of the colonies. This is expected, as this dose exceeds the reported minimal inhibitory concentration (MIC) of wildtype *E. coli* (0.004 μg/mL) (Andrews, 2001). However, the Δ*radD* and Δ*radA* strains clearly exhibit a viability defect compared to wild type, similar to that seen for the double mutant at the lower dose (Fig. 4A). At the higher dose, the Δ*radD*Δ*radA* strain is completely inviable. These results indicate that *radD* and *radA* are also important for repairing enzymatically-induced, as opposed to radiation-induced, DNA strand breaks. These results have been corroborated (Deani Cooper, Daniel C. Boyle and Susan T. Lovett, accompanying paper).

As with radiation-induced damage, plasmids containing wildtype and mutant *radD* were transformed into the Δ*radD*Δ*radA* strain to test for complementation. Only the wildtype *radD* could rescue ciprofloxacin sensitivity (Fig. 4B), indicating that full-length, wildtype *radD* gene is needed to repair ciprofloxacin-induced damage. As seen in the UV sensitivity tests in Figure 3, an attempt at complementation with the K37R variant appeared to slightly increase sensitivity to ciprofloxacin. The empty vector control provided no measurable complementation (Fig. 4C)

radD and radA are important for responding to an induced double-strand break

Because the *radD* and *radA* deletion strains appear to be susceptible to types of damage that are known to cause double-strand DNA breaks, we utilized a system that induces a single and site-specific DNA double-strand break in the genome. Due to a palindrome artificially inserted into the *lacZ* gene and an arabinose-induced promoter in front of the *sbcCD* genes, cells plated on arabinose will incur a double-strand break during replication (Eykelenboom *et al*., 2008). Viability will be compromised if the break is not efficiently repaired. The single deletions of *radD* or *radA* do not produce a substantial decline in viability, although both exhibit a slight growth defect manifested by smaller colony size. Following the pattern established in earlier experiments, the Δ*radD*Δ*radA* strain exhibits an obvious viability defect (Fig. 5), comparable to that previously seen for deletions of *recA*, *recG*, *ruvAB*, or *priA* (Eykelenboom *et al*., 2008). As in previous cases, wildtype *radD* introduced on a plasmid rescues this phenotype, while complementation with any of the mutant variants (or the empty vector) does not (Fig. 5).

The ΔradDΔradA strain exhibits elevated levels of SOS response

In bacteria, DNA strand breaks lead to the induction of a number of repair genes in a process known as the SOS response (Walker *et al*., 2000, Michel, 2005). To test for an increased

induction of the SOS response due to persistent strand breaks, a reporter plasmid in which the GFP protein is expressed from the *recN* promoter (pEAW 915, Table 2) was transformed into each of the single and double mutant strains. The *recN* gene is strongly induced at an early stage of the bacterial SOS response (Finch *et al*., 1985). In the absence of exogenous damage, a small but reproducible induction of GFP was detectable in the mutant strains (Fig. 6B). These strains exhibited very little difference in overall growth rate (Fig. 6A), but showed increased SOS beginning around mid-log phase (at 200 min, OD_{600} ~0.5). This may correspond to the increase in cells entering stationary phase. However, if this is the case, the cause is not yet evident. The effect is exacerbated in the double mutant Δ*radD*Δ*radA* strain. The levels of SOS induction in the double mutant Δ*radD*Δ*radA* strain are significant but very modest. Induction of the SOS response by antagonists such as ciprofloxacin produces an SOS response that is more than an order of magnitude greater on this scale and with the same assay (Fig. S5).

Unlike radA, deletion of radD does not significantly change levels of conjugational recombination

It was previously reported that deletion of *radA* decreases levels of RecA-mediated conjugational recombination (Beam *et al*., 2002). To determine the effect of a *radD* deletion on conjugational recombination, HFR recipient strains were constructed with deletions for *radA*, *radD*, both *radA* and *radD*, *recG*, and *ruvB* (Table 1), with the *recG* and *ruvB* mutants serving as negative controls. Following conjugation and plating, the resulting colonies were counted and normalized to the level of our HFR recipient control strain (EAW 174, Table 1). As previously reported, the *radA* single deletion displayed slightly less recombination than the control strain. However, the Δ*radD* and Δ*radD*Δ*radA* strains had recombination levels that were within error of the control strain (Fig. 7). This represents the first phenotype for which *radD* mutants differ from the *radA* deletion strain, and indicates that *radD* does not participate in pathways that directly contribute to conjugational recombination.

Discussion

The work in this report establishes a role for *radD (*formerly known as *yejH)* in the cellular systems that repair DNA double strand breaks. The elimination of RadD function sensitizes the cells to a series of agents or conditions that cause DNA double strand breaks and modestly promotes induction of the SOS response. Deletions of *radD* exhibit synergistic effects with deletions of *radA*, and we hypothesize that the RadD protein plays a direct role in DNA double strand break repair. This would entail a specialized function for the protein that does not contribute to conjugational recombination, where *radD* function has no observable effect. Given their approximately additive sensitivity to DNA damaging agents (even synergistic with respect to UV), it appears that *radD* and *radA* serve a previously overlooked, overlapping or complementary function in the repair of double strand breaks in DNA. That function likely also complements that of several other enzymes as described below.

Although little is known about the role of *radA*, it has been proposed that the enzyme is involved in processing recombination intermediates (Beam *et al*., 2002). It appears that *radA*

works in the *recA* repair pathway, as the phenotypes of *radA* mutants are *recA*-dependent (Diver *et al*., 1982). As recombinational repair is the main pathway of DSB repair in bacteria, it would follow that enzymes involved in DSB repair are part of the *recA* pathway. The *radA* and *radD* genes are not homologous. However, they share several features that are likely important in DNA repair, including ATP hydrolysis motifs (Walker A and B) and putative Zn fingers (Fig. 1 and (Beam *et al*., 2002)). The original identifying mutation in *radA* was a cysteine-to-tyrosine mutation in the putative zinc finger that caused an increased sensitivity to radiation (Diver *et al*., 1982). We have shown that the Zn finger and Walker A motifs of *radD* are important for surviving radiation exposure.

Interestingly, strains lacking *radA* or *radD* gene function respond differently to IR and UV irradiation, especially with respect to complementation with *radD* K37R. After exposure to IR, the Δ*radD* strain is more sensitive than Δ*radA*. The *radD* K37R provides a partial rescue of this sensitivity when present on the chromosome. However, this effect could not be confirmed, since the same mutant protein expressed from a plasmid exhibits the same degree of apparent rescue as does an empty vector control. This suggests the role of RadD in DNA double strand break repair is largely (if not entirely) dependent on its ATPase activity. In contrast, after exposure to UV, the Δ*radD* strain is somewhat less sensitive than Δ*radA*. Unlike its effects in the IR experiments, complementation attempts with *radD* K37R actually increase UV sensitivity, exhibiting a dominant negative effect. Thus, the ATPase-deficient RadD may interfere with some repair function after UV irradiation. Whereas some studies have suggested that the effects of UV and IR are interchangeable (Arrage *et al*., 1993), the two sources do cause different direct effects. IR causes DNA strand breaks via ionization and the creation of reactive oxygen species. UV largely promotes the formation of pyrimidine dimers that may indirectly lead to some strand breaks when replication forks encounter the template breaks or other barriers created transiently during repair (Khan & Kuzminov, 2012). These differences may help explain the failure to detect the effects of *radD* function in earlier genetic screens for DNA repair genes.

Evidence increasingly suggests the presence of a complex system that supports the work of RecA protein in DNA double strand break repair, processing the branched DNA intermediates that are generated by RecA. Significant components include the RecG, RuvABC, RecQ/Topoisomerase III, UvrD, and RadA proteins. Additional proteins and enzymes, notably RecBCD and RecFOR, prepare DNA substrates prior to RecA action and promote RecA loading. With this study and an accompanying report (Cooper et al, accompanying paper), RadD now joins this system. The RecG, RuvABC, and RecQ/Topoisomerase III proteins appear to have specific DNA structures as their reaction targets (Adams *et al*., 1994, Asai & Kogoma, 1994, Baharoglu *et al*., 2006, Bennett & West, 1995, Fonville *et al*., 2010, Lloyd *et al*., 1988, Mahdi *et al*., 2003, Harmon & Kowalczykowski, 1998). In DNA double strand break repair, UvrD helicase functions in removing RecA filaments from the DNA (Centore & Sandler, 2007, Centore *et al*., 2009, Veaute *et al*., 2005). The RadA and RadD proteins could function on DNA substrates, or alternatively might be involved in the remodeling of protein complexes bound to DNA as DNA double strand break repair progresses. Genetic evidence for involvement of these proteins in an interconnected network continues to accumulate, although enough distinctions are present to

indicate specialized roles for each. A Δ*radA*Δ*recG*Δ*ruvA* (or Δ*radA*Δ*recG*Δ*ruvC)* triple mutant displays a deficiency in conjugational recombination comparable to a Δ*recA* mutant (Beam *et al*., 2002). We have found that Δ*radD*, when combined with a *recG* insertion mutant, is slow growing and quickly accumulates suppressors even though Δ*radD* Δ*ruvB* mutants grow normally (S.H. Chen & E.A. Wood, unpublished results). In addition, several of the phenotypes seen for the Δ*radD*Δ*radA* mutants are similar to those seen for Δ*recG* and Δ*ruvAB* mutants, including radiation sensitivity and viability defects following an induced double-strand break (Eykelenboom *et al*., 2008).

The major phenotype of mutants lacking *radD* function is a deficiency in recovery from the effects of ionizing radiation. However, this sheds only limited light on the precise molecular function of RadD. Additional observations may provide a clue. The homology of RadD to archaeal and human XPB proteins, with their demonstrated involvement in transcription and transcription-coupled DNA repair, among other functions (Fuss & Tainer, 2011, Schaeffer *et al*., 1993) may suggest a role at the interface of DNA double strand break repair and transcription. Recent results from *Vibrio cholerae* potentially provide a more direct link between RadD and transcription complexes (Baharoglu *et al*., 2014). In *V. cholerae*, Δ*yejH/radD*, Δ*rnhA*, and Δ*mfd* mutant strains grow slowly in the presence of tobramycin (which stalls RNA polymerases among other effects). More intriguing, overexpression of *V. cholerae yejH/radD* in *E. coli* suppresses the UV sensitivity of an *E. coli* Δ*mfd* mutation (Baharoglu *et al*., 2014), suggesting that *yejH/radD* can replace the RNA polymerase displacement activity of the Mfd protein (Haines *et al*., 2014, Mahdi *et al*., 2003, Park *et al*., 2002, Sancar & Reardon, 2004, Savery, 2007, Smith *et al*., 2012, Trautinger *et al*., 2005). The *V. cholerae* and *E. coli* YejH/RadD proteins are 58% identical, including the helicase motifs and the C-terminal cysteine residues, and 65% similar. What need would arise for RNA polymerase displacement in DNA double strand break repair? In a bacterial genome, double strand breaks introduced stochastically during irradiation or by any mechanism have a significant chance of occurring within a gene that is being actively transcribed. The fate of RNA polymerase complexes when they encounter a double strand break has not been carefully explored. Limited stability of RNA polymerases stalled at DNA strand breaks was observed in one study (Nudler *et al*., 1996). However, the chloride containing buffers used in the study are known to destabilize RNA polymerase and are not a good mimic of *in vivo* conditions (Leirmo *et al*., 1987, Record *et al*., 1985, Shaner *et al*., 1983). If RNA polymerases remain stably bound to DNA at the sites of double strand breaks, their removal may be a prerequisite to repair. The degree to which RNA polymerases stalled at DNA ends represent a barrier to DNA double strand break repair is currently unknown and requires further exploration.

Experimental procedures

Strain construction

A modification of the procedure devised by Datsenko and Wanner (Datsenko & Wanner, 2000) was used to make chromosomal gene knockouts. The plasmid pEAW507 was used as a template in a PCR. pEAW507 consists of a pJFS42 mutant FRT-Kan^{R}-wt FRT cassette in an Ampicillin resistant backbone (Senecoff *et al*., 1988). pEAW507 was used because, after

eliminating the Kan^R, the mutant FRT remaining on the chromosome cannot react with any other FRTs used in subsequent gene modifications. The PCR primers were about 50 bases before the start of the gene of interest with the 21 bases before the start of the FRT-Kan^R-FRT cassette, and about 50 bases after the stop of the gene of interest with 21 bases after the end of the FRT-Kan^R-FRT cassette. The gel purified PCR product was electroporated into the bacterial strain previously transformed with plasmid pKD46. L-arabinose was added to express λ Red recombinase from the pKD46. Kanamycin resistant colonies were screened for Ampicillin sensitivity, and used as a template in confirmation PCR with primers located in the chromosomal regions both upstream and downstream of the gene of interest. The PCR product was sequenced to confirm the chromosomal deletion. In the case of double deletions, the kan cassette of the first deletion was first removed (as per Datsenko and Wanner (Datsenko & Wanner, 2000)), followed by insertion of the second deletion.

EAW9 is a *recG*$^-$ strain. To construct EAW9, a plasmid containing the *recG* gene was digested with PmeI which cuts at base 1618 of *recG*. A Kanamycin gene flanked by HincII sites was excised from plasmid pKan and ligated into the PmeI site of the *recG* gene. The *recG* gene interrupted by the Kanamycin gene was excised from the plasmid by restriction digestion, and electroporated into a strain (MG1655 Δ*recA*) containing the plasmid pKD46 (Datsenko & Wanner, 2000). A Kanamycin resistant colony was chosen. To confirm the *recG* chromosomal disruption, a PCR product was generated with primers for the chromosomal region upstream and downstream of the *recG* gene and sequenced.

EAW368 is founderΔe14Δ*radD recG*$^-$. It was made by transduction of EAW232 to *recG*$^-$ with P1 grown on EAW9.

Plasmid construction

pEAW724 is the wt *radD* gene in the overproduction vector pET21a(Novagen). *E. coli* MG1655 genomic DNA was used as a template in a PCR with a primer consisting of a NdeI site followed by the first 27 bases of the *radD* gene. The ATG in the NdeI site is also the start codon for *radD*. The other primer consisted of a BamHI site followed by the last 25 bases of the *radD* gene. Changes were made for better codon usage in codons 4, 5, and 584. The PCR product was digested with NdeI and BamHI and inserted into pET21a digested with the same enzymes. The resulting plasmid, designated pEAW724, was directly sequenced to confirm the presence of the wt *radD* gene.

pEAW752 is the first 355 amino acids of *radD* in the overproduction vector pET21a(Novagen). pEAW724 was used as a template in a PCR with a primer consisting of a NdeI site followed by the first 27 bases of the *radD* gene. The ATG in the NdeI site is also the start codon for *radD*. The other primer consisted of a BamHI site followed by a stop codon, and bases 1065-1048 of the *radD* gene. Changes were made for better codon usage in codons 4, and 5. The PCR product was digested with NdeI and BamHI and inserted into pET21a digested with the same enzymes. The resulting plasmid, designated pEAW752, was directly sequenced to confirm the presence of *radD* aa 1-355.

pEAW755 is *radD* K37R mutant in the overproduction vector pET21a(Novagen). It was made by QuikChange site-directed mutagenesis (Agilent Technologies) of pEAW724

template using primers consisting of the *radD* gene bases 98-125, and their complement. The AAA bases coding for the Lys at aa 37 were changed to CGT to code for an Arg. The resulting plasmid, designated pEAW755, was directly sequenced to confirm the presence of the *radD* K37R mutant. pEAW977 is *radD* C437A mutant in the overproduction vector pET21a(Novagen). It was constructed in a fashion similar to pEAW755 except the QuikChange mutagenesis primers had the TGT bases coding for Cys at aa 437 changed to GCA to code for Ala.

pEAW915 is SuperGlo GFP(Qbiogene) under the control of the *E. coli recN* promoter, in the plasmid pACYC184. To clone the *recN* promoter, *E. coli* MG1655 genomic DNA was used as a template in a PCR with a primer consisting of a BglII site followed by bases 200-180 upstream of the start of the *recN* gene. The other primer consisted of a NheI site followed by a NdeI site and bases 1-21 upstream of the *recN* gene. The PCR product was digested with NheI and BglII, and ligated to Qbiogene's pQBI63 plasmid cut with the same enzymes. The *recN* promoter and SuperGlo GFP were excised from the resulting plasmid with BglII and HindIII, which cuts downstream of the end of SuperGlo GFP, and ligated to pACYC184 cut with BamHI and HindIII. The resulting plasmid was designated pEAW915.

Ionizing radiation resistance assays

Using an overnight culture, strains were inoculated into LB with appropriate antibiotic to an OD_{600} of approximately 0.02 and grown at 37°C to an OD_{600} of approximately 0.4. Cultures were then incubated on ice 10 min; 15 mL of culture was spun down in a tabletop centrifuge at 4°C, and cells were resuspended in 800 μl LB.

For the 0 Gy timepoint, 100 μl was removed prior to irradiation. Cells were irradiated in a Shepherd Mark I Model 30 irradiator with a Cesium 137 source at a rate of 662.37 rad/min to 1000 Gy and 2000 Gy, with 100 μl of sample removed at each point.

Irradiated and non-irradiated cells were serially diluted (100 μl into 900 μl) into M9 media, and 100 μl of appropriate dilutions were spread onto LB plates. Plates were incubated at 37°C overnight, and colonies counted the following morning.

Plating for UV, ciprofloxacin, induced double-strand break

For UV irradiation, cells were grown and serially diluted as above, and 100 μl of appropriate dilutions were spread onto LB plates. For the complementation experiments, 10 μl of each dilution (10^{-2} through 10^{-6}) was spotted onto an LB plate. The plates were then exposed to UV in a Spectrolinker XL-1000 UV crosslinker (Spectronics Corp). Pictures or colony counts were taken after incubating at 37°C overnight.

For ciprofloxacin experiments, plates were poured with LB agar containing the ciprofloxacin (0.005 or 0.01 μg/mL). Cells were grown and serially diluted as above, and spot plated (10 μl, 10^{-2} through 10^{-6}) on the ciprofloxacin-containing plates. Pictures were taken after growing overnight at 37°C.

For the induced double-strand break assay, strains with or without the palindrome sequence (Table 1) were grown as above, serially diluted, and spot plated (10 μl, 10^{-2} through 10^{-6})

on LB plates containing either 0.5% glucose or 0.2% arabinose. Pictures were taken after growing overnight at 37°C.

Complementation assays

To test for complementation of the *radD* gene, cells lacking the *radD* gene (EAW 232 & EAW 370, Table 1) were made chemically competent. Plasmids containing the *radD* gene (wildtype or mutant; pEAW 724, 752, 755, 977) were individually transformed into the strains, and selected for on ampicillin plates. Strains were grown and plated as above, with the addition of ampicillin in the growth media.

SOS response assay

Overnight cultures were diluted 1:100 in fresh LB, and 200 μl was added to the wells of a black-walled, clear-bottom 96 well plate (Corning). For each sample, three overnights were grown from separate colonies, and each overnight filled three wells in the plate (three biological and three technical replicates, for 9 total wells per sample). The plate was inserted into a Tecan infinite M1000 Pro plate reader. A program was used to incubate the plate at 37°C with orbital shaking. Every 10 min, the plate was briefly shaken linearly, and the OD_{600} and 509 nm emission (with 474 nm excitation) were read.

Conjugational recombination assays

Donor EAW175 and recipient EAW174 strains were constructed by P1 transductions from several strains which were kindly provided from Steve Sandler. EAW175 was made by a consecutive P1 transduction of (1) the Δ*(metA)::kan* allele from SS6311 into CAG5052 (*KL227 btuB3191::Tn10 metB1 relA1 89′→6′)* to obtain an intermediate strain EAW173, checked by Tet^r and Kan^r phenotypes, then followed by flipping out the *kan* cassette; and (2) the *ilvO::kan* allele from SS4761 into the intermediate EAW173 strain, checking for both Tet^r and Kan^r phenotypes.

To make the recipient strain, the *kan* cassette was flipped out first from the SS338 (Δ*(attB)::psulA-gfp* δ*(metE)100::kan*) strain and strain EAW174 was made by P1 transduction of the Δ*(aroB)::kan* allele from SS2495 to SS3388. The Δ*recA::kan* allele from EAW20 was then transferred to EAW174 by P1 transduction to make recipient EAW188. Additional recipient strains were constructed by using P1 transduction to delete the *radD*, *radA*, *recG*, and *ruvB* genes individually, as well as the combination of *radD radA*, from the EAW 174 strain.

Conjugation was carried out essentially as described previously (Miller, 1972, Experiments in molecular genetics, CSHL) with the following exceptions. Donor strain was grown at 37°C in Luria-Bertani (LB) broth with Tetracycline until an optical density (OD_{600}) of 0.7 was reached. The recipient strains were grown with Chloramphenicol, Kanamycin and Streptomycin until an optical density (OD_{600}) of 0.5 was reached. All strains were spun down and gently resuspended in the initial volume of fresh LB broth twice to remove antibiotics. Mating was carried out by mixing 200 μl of donor cells with 1800 μl of recipient cells and incubating 100 min at 37°C. For ease of colony counting, the mating mixture was diluted 1:100 in LB broth, and 100 μl (500 μl for the *ruvB* strain) was mixed with 3 mL of

pre-warmed 0.7% Bacto agar solution to prevent additional mating and immediately poured onto a minimal media plate. The plate was rested for a few minutes at room temperature to allow the agar to set before being turned upside down and incubated for 40 hours at 37°C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health Grant GM32335 (to MMC). We thank Dr. David Leach for the kind gift of the double strand break-inducing strains, Dr. Steve Sandler for the original HFR strains, Dr. Bénédicte Michel for helpful discussion of results and for helpful comments on the manuscript, and Dr. Susan Lovett for sharing results prior to publication and commenting on early drafts of this paper.

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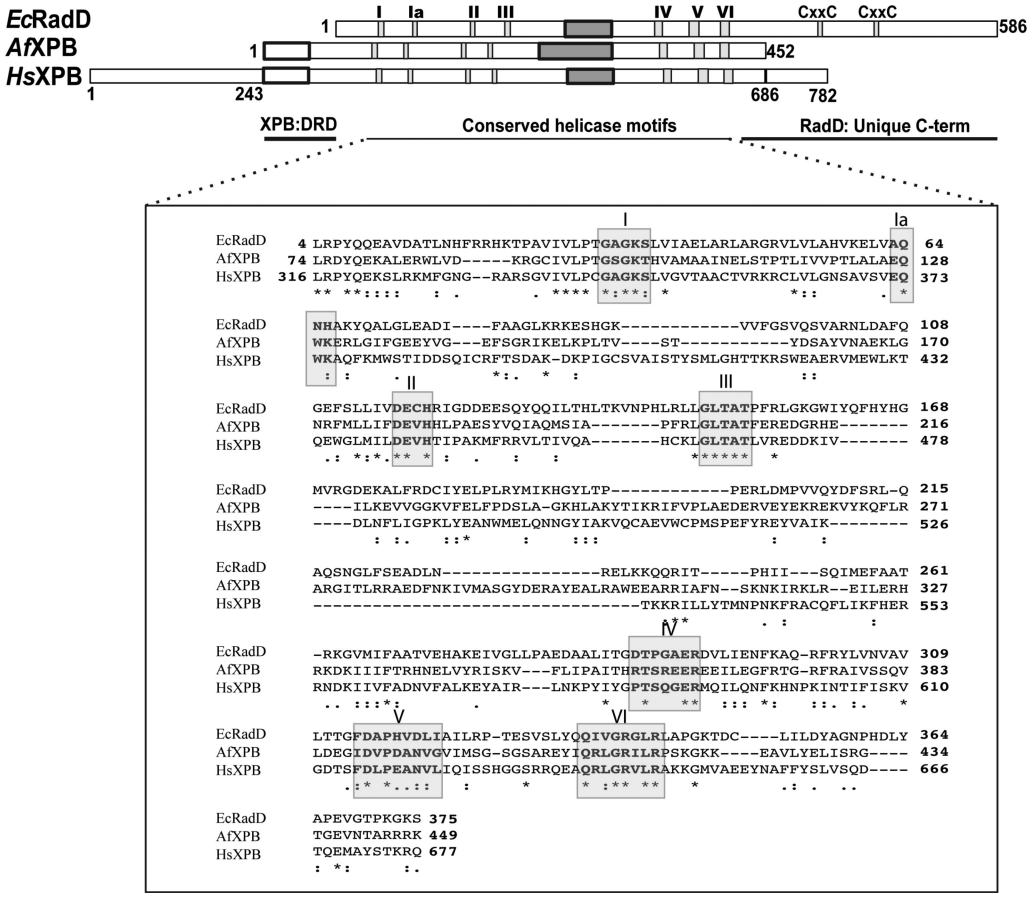

Figure 1. Alignment of RadD and XPB

The closest homologue to RadD is the human or archaeal XPB protein, which is involved in transcription initiation and transcription-coupled nucleotide excision repair. RadD contains all seven helicase motifs typical of the superfamily 2 helicases. Although RadD lacks the N-terminal “DNA recognition domain” found in XPB, it contains a C-terminal putative zinc finger motif.

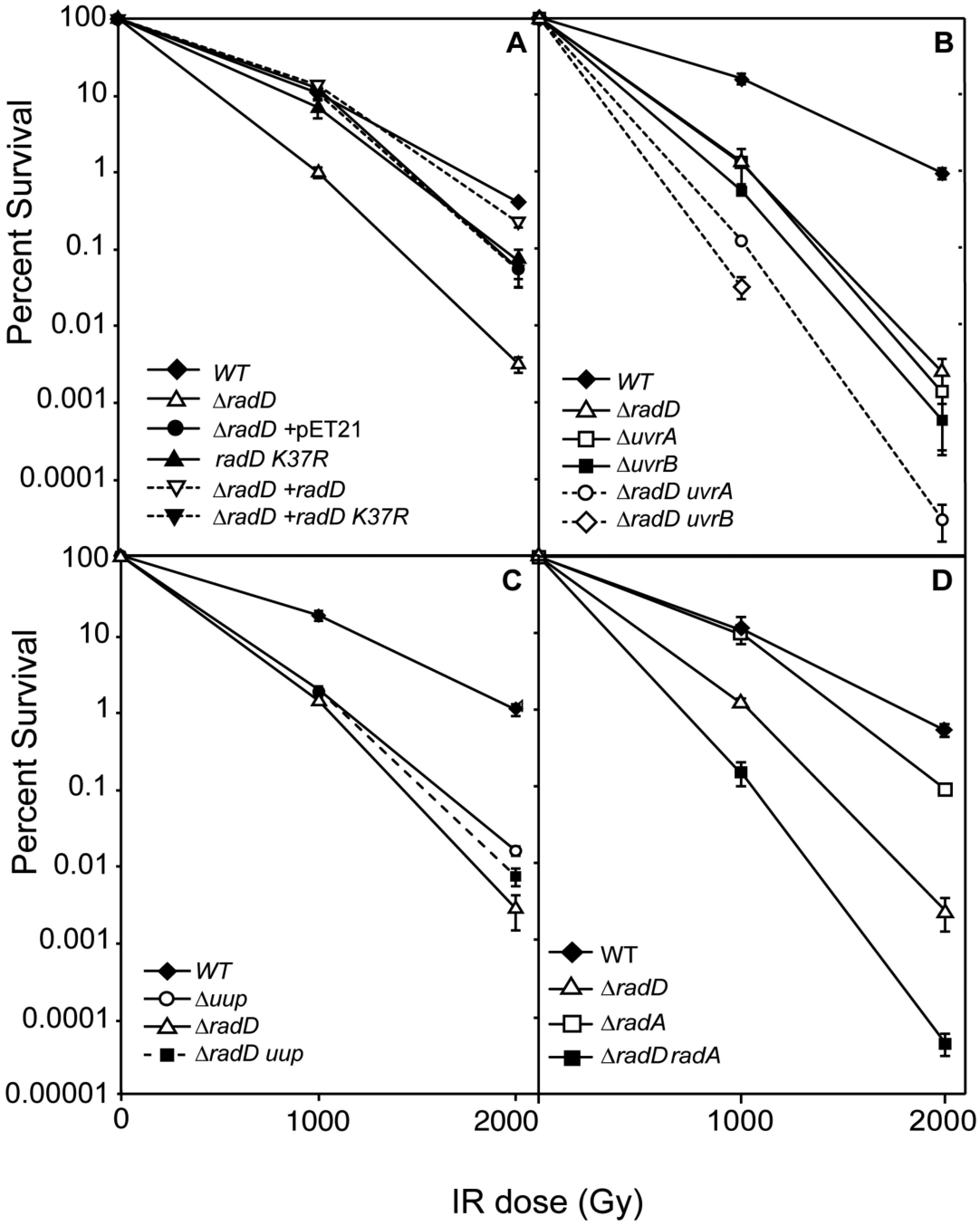

Figure 2. The function of radD is needed after exposure to ionizing radiation (IR)

Wildtype (founder Δe14) and deletion strains (Table 1) were exposed to high doses of ionizing radiation. Cell viability was determined after each dose and used to calculate percent survival. **A.** The effects of IR on strains lacking the function of the *radD* gene or with a putative ATPase mutation (K37R) are shown. The "+" indicates complementation with the indicated *radD* gene variant expressed at background levels on the plasmid pET21 without induction, or empty vector control. **B.** The effects of IR on strains lacking the function of the genes *radD uvrA or B*, or both *radD* and one of the *uvr* genes. The effects of

the loss of *radD* and the *uvrA/B* genes together are further assessed in Fig. S2. **C.** The effects of IR on strains lacking the function of the genes *radD uup*, or both are shown. **D**. The effects of IR on strains lacking the function of the genes *radD radA*, or both *radD* and *radA* are shown. The effects of the loss of *radD* and the *radA* genes together are further assessed in Fig. S2.

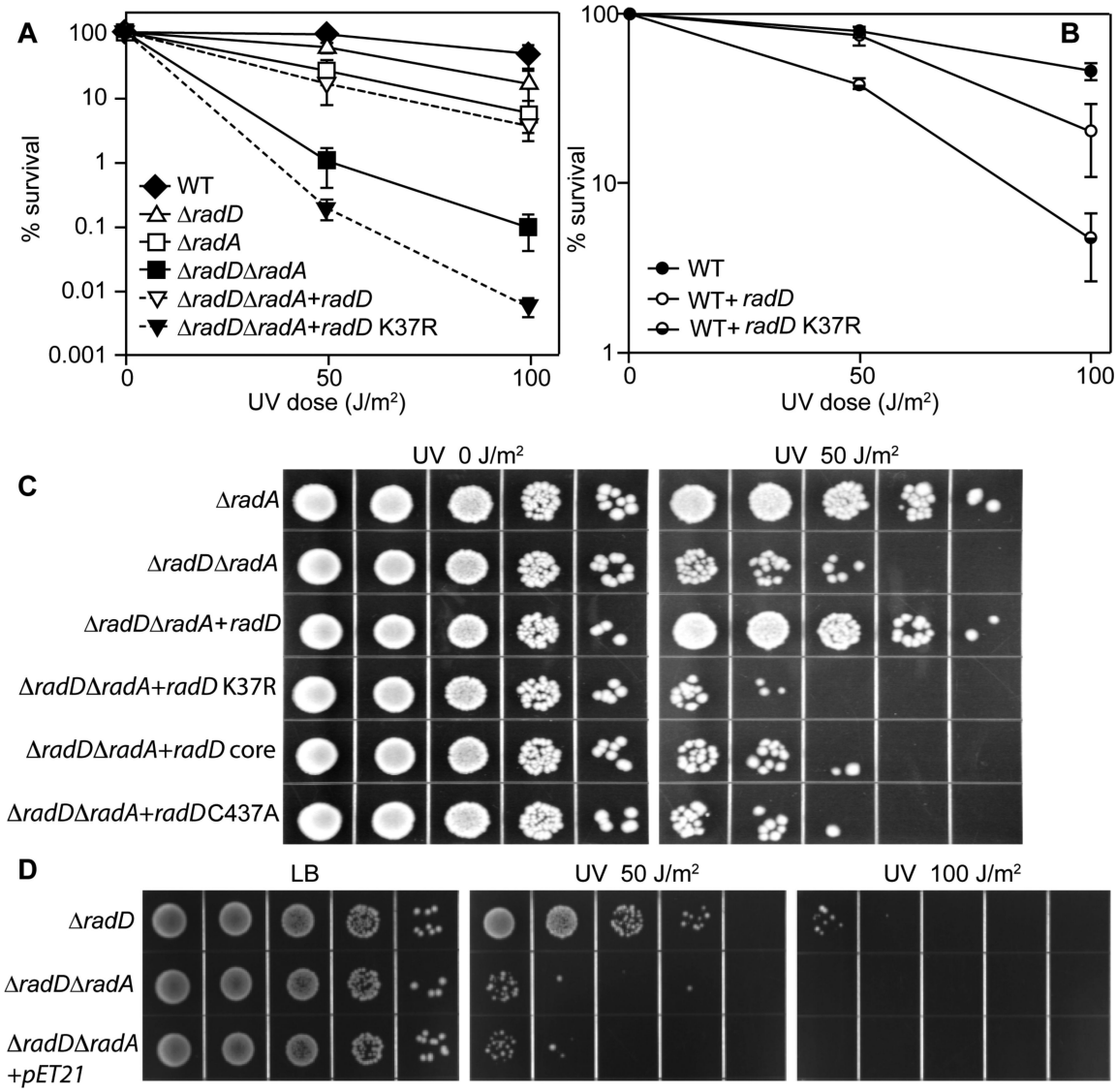

Figure 3. The function of radD is needed to respond to UV radiation

The *radD* and *radA* mutant strains (Table 1) were exposed to UV radiation. UV dosage is validated in Fig S3. **A.** Strains were plated, exposed to UV radiation, and colonies counted to obtain viability data, which was normalized against the zero dose point to obtain percent survival. **B.** The effects of UV irradiation on strains with elevated levels of the RadD or RadD K37R protein are shown. The RadD proteins were expressed at background levels from pET21. **C** and **D.** Strains were spot plated on LB prior to UV exposure to show a qualitative viability defect. Spots (left to right in each series of five) represent a serial dilution of 1:10, ending in 10^{-6}. The "+" indicates complementation with the indicated *radD* gene variant expressed at background levels on the plasmid pET21 without induction, or empty vector control.

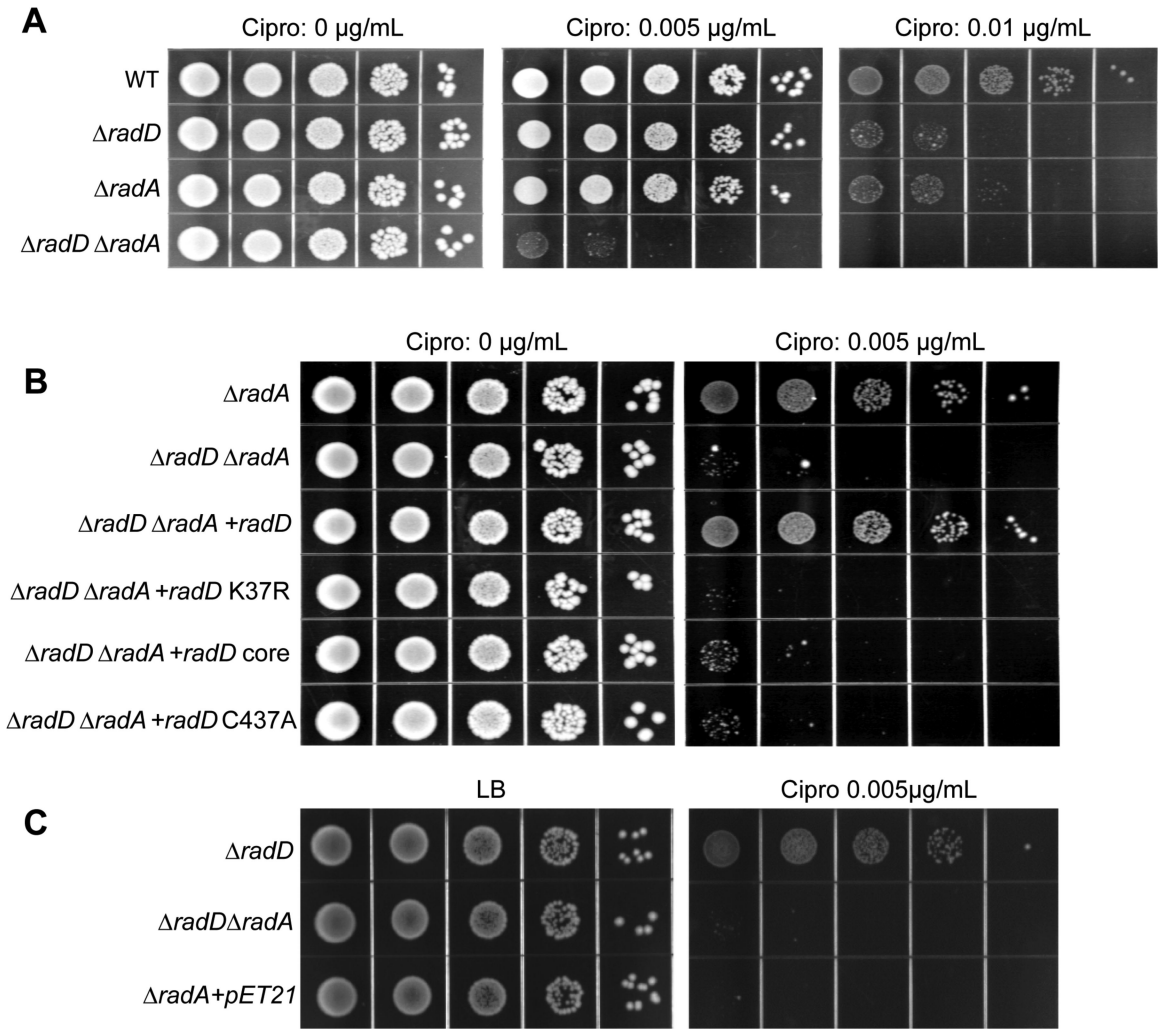

Figure 4. Deletion of radD renders cells sensitive to ciprofloxacin

Cells were grown to log phase, serially diluted 1:10, and spot plated on LB plates containing varying amounts of ciprofloxacin. **A.** The effects of ciprofloxacin on cells lacking the function of *radD*, *radA*, or both are shown. **B** and **C.** Complementation of a strain lacking both *radD* and *radA* function by either RadD or RadD variants expressed at background levels on pET21. An empty vector control is provided in panel **C**. The "+" indicates complementation with the indicated *radD* gene variant expressed at background levels on the plasmid pET21 without induction, or empty vector control.

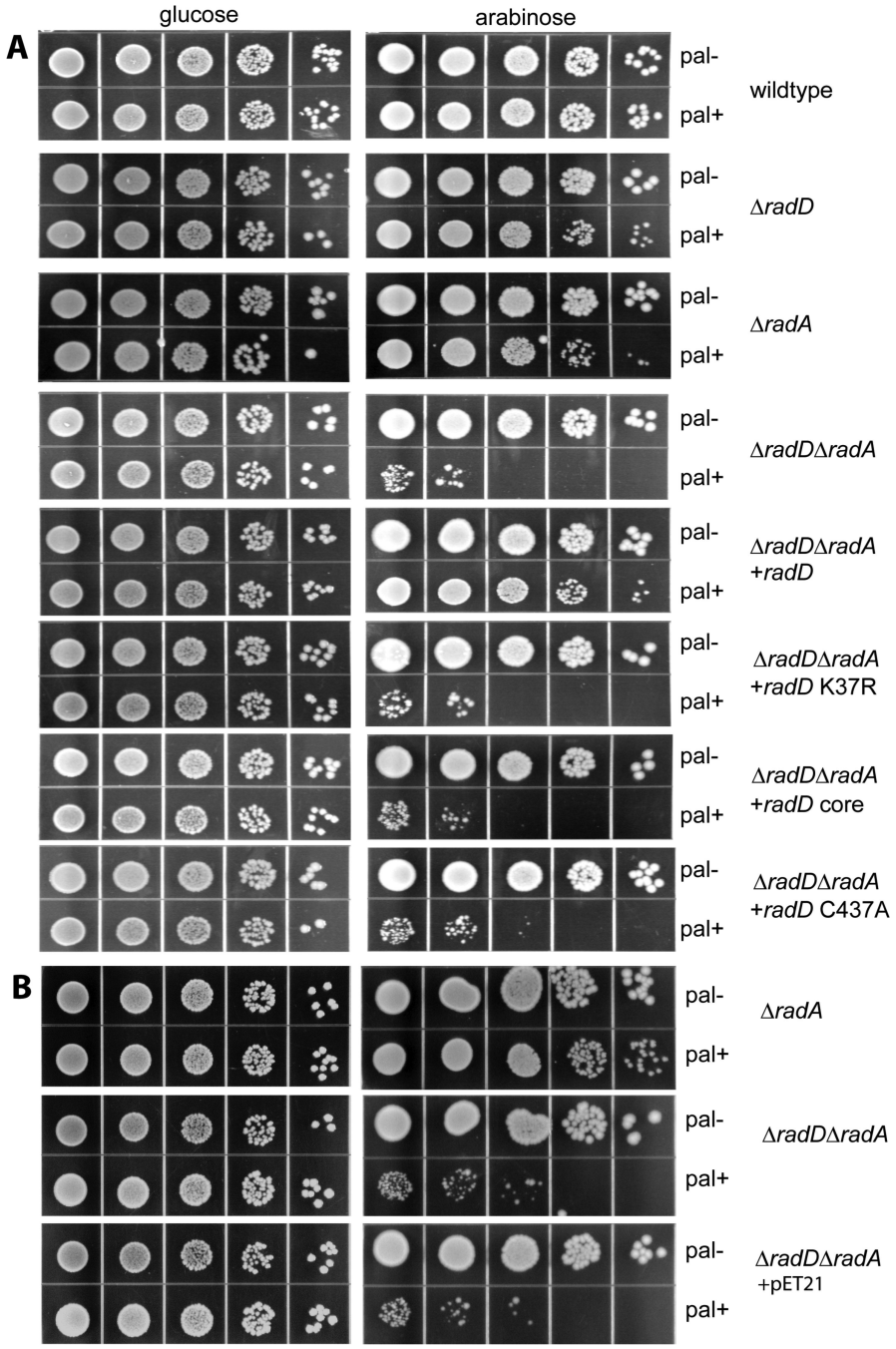

Figure 5. The functions of radD and radA are needed for repairing an induced double-strand break

The presence of a palindrome sequence (pal+) and arabinose will induce a targeted double-strand break in the *lacZ* gene (Eykelenboom *et al*., 2008). If not efficiently repaired, this break will lead to a low viability phenotype. The effects of this targeted double strand break on strains lacking the function of *radD*, *radA*, or both are shown. Complementation of a strain lacking both *radD* and *radA* function by either RadD or RadD variants expressed at background levels on pET21 is also shown. An empty vector control is provided in panel **B**. The "+" preceding a gene or plasmid name indicates complementation with the indicated

radD gene variant expressed at background levels on the plasmid pET21 without induction, or empty vector control.

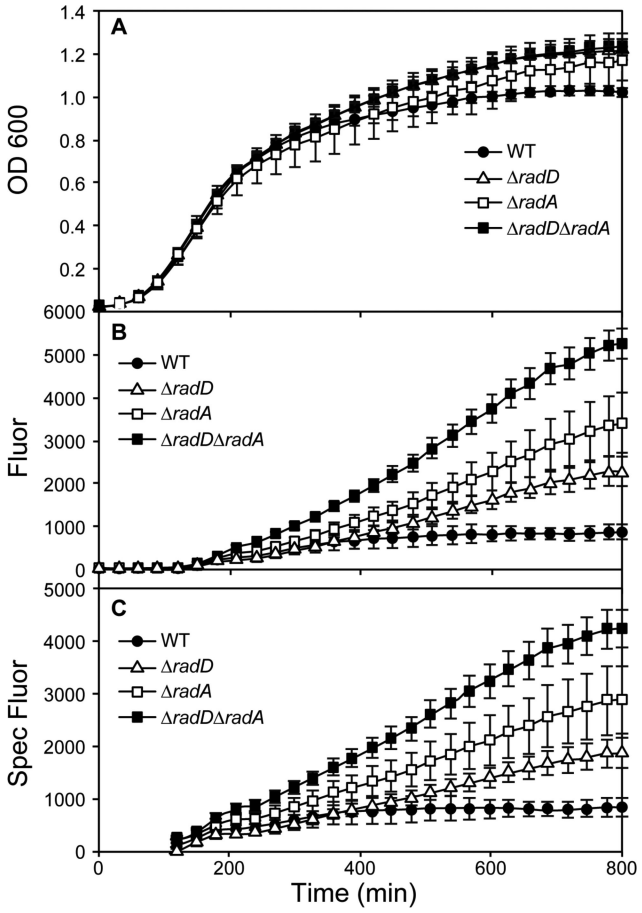

Figure 6. A ΔradD ΔradA double mutant shows low levels of constitutive SOS response

Single and double mutant strains containing a plasmid expressing GFP under SOS control were grown in LB. **A.** Growth was monitored at 600 nm. **B.** SOS gene induction was measured with excitation at 474 nm and emission at 509 nm. **C.** Specific fluorescence, defined as measured fluorescence from panel B divided by the OD_{600} taken from panel A is shown. Due to the error inherent in dividing very small numbers, specific fluorescence is not shown for times prior to 100 min.

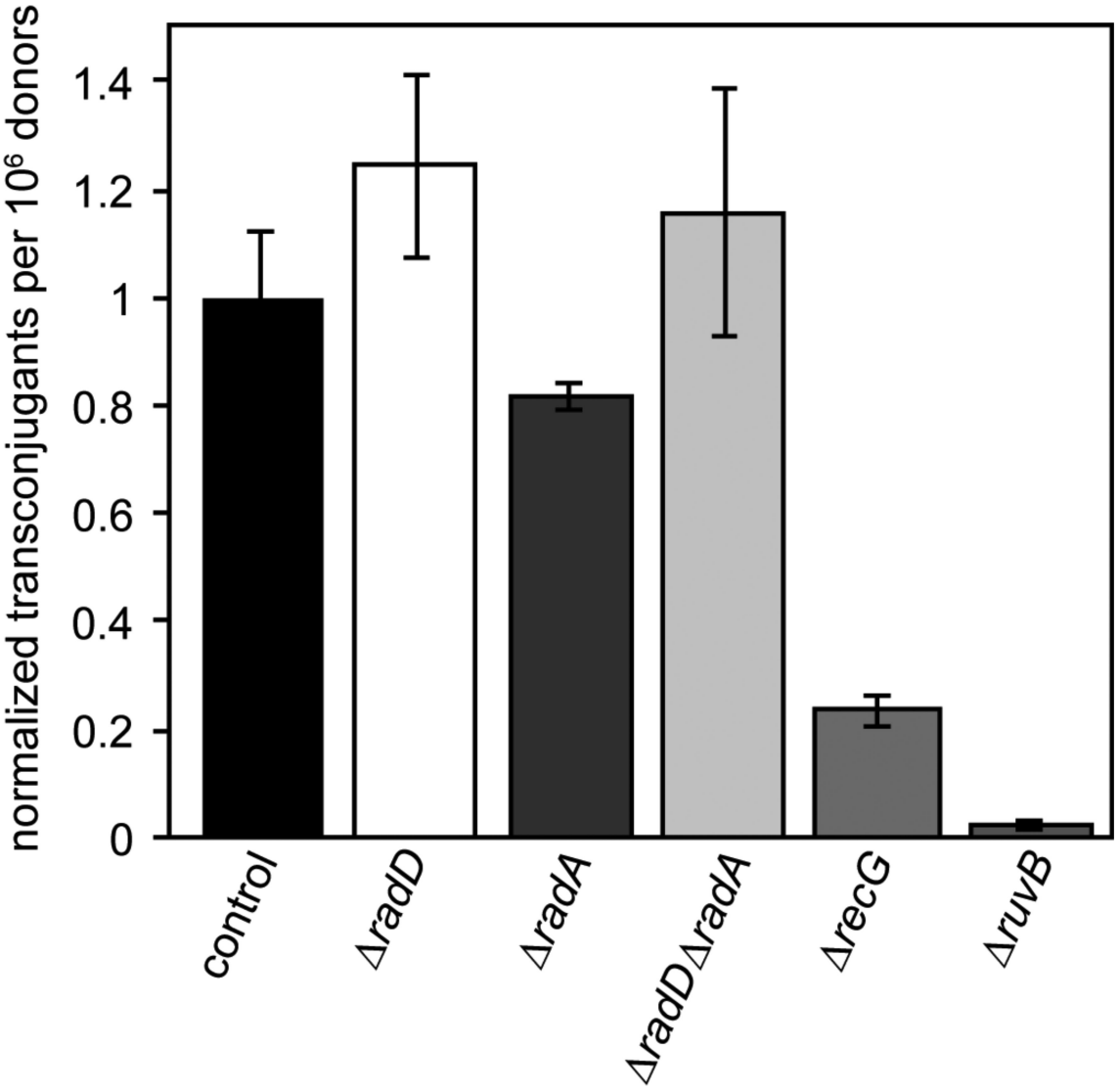

Figure 7. The radD deletion does not significantly inhibit conjugational recombination

The HFR recipient strains were constructed with deletions of known or predicted recombination genes (*radD, radA, radD/radA, recG, ruvB* see Table 1). See Methods for details.

Table 1

Table of strains used

Strain name	Genotype	Reference
EAW9	MG1655 Δ*recA recG*$^{-}$	This study
EAW 7704	Founder Δe14	(Byrne *et al.*, 2014a)
EAW 232	Founder Δe14 Δ*radD*	(Byrne *et al.*, 2014a)
EAW 252	Founder Δe14 Δ*radA*	(Byrne *et al.*, 2014a)
EAW 278	Founder Δe14 *radA K37R*	This study
EAW 368	FounderΔe14 Δ*radD recG*$^{-}$.	This study
EAW 370	Founder Δe14 Δ*radD* Δ*radA*	This study
EAW 404	DL2006 Δ*radD*	This study
EAW 406	DL2006 Δ*radA*	This study
EAW 416	DL2573 Δ*radD*	This study
EAW 418	DL2573 Δ*radA*	This study
EAW 424	DL2006 Δ*radD* Δ*radA*	This study
EAW 425	DL2573 Δ*radD* Δ*radA*	This study
EAW 522	Founder Δe14 Δ*radD* Δ*recG*	This study
DL2006	BW27784 ΔP_{sbcDC} P_{BAD}-*sbcDC lacZ::pal246 cynX*::Gm^{R}	Eykelenboom et al, 2008
DL2573	BW2+7784 ΔP_{sbcDC} P_{BAD}-*sbcDC lacZ*$^{+}$ *cynX*::Gm^{R}	Eykelenboom et al, 2008
EAW 175	CAG5052 Δ*metA* Δ*ilvO*	This study
EAW 174	SS3388 Δ*aroB*	This study
EAW 477	EAW 174 *recG*$^{-}$	This study
EAW 478	EAW 174 Δ*radD*	This study
EAW 479	EAW 174 Δ*radA*	This study
EAW 480	EAW 174 Δ*ruvB*	This study
EAW 482	EAW 174 Δ*radD* Δ*radA*	This study
CAG5052	KL227. *btuB3191*::Tn10 metB1 relA1 9′ ->6′	(Singer *et al.*, 1989)
SS3388	JC13509 Δ*attB*::psulA-GFP Δ*metE 100*::Kan	gift from Steven Sandler

Table 2

Table of plasmids used

Strain name	Description	Reference
pEAW 724	pET21a RadD	This study
pEAW 752	pET21a RadD 355aa truncation	This study
pEAW 755	pET21a RadD K37R	This study
pEAW 977	pET21a RadD C437A	This study
pEAW 915	pACYC184 with a recN promoter in front of Super-Glo GFP	This study

Table 3

Suppressor mutations in the *priA* gene, arising in Δ*radD* Δ*recG* (EAW 522) or Δ*radD recG*$^{-}$ (EAW 368) strains.

Mutation	PriA substitution
Suppressors of Δ*radD recG*	
832 T→G	S278A
1136 G→C	R379P
2182 G→T	D728Y
1558 G→C	A520P**
1470 A→G	T491A
CGCTGG	IN W689(RW)*
Suppressors of Δ*radD* Δ*recG*	
1286 G→C	G429A
1861 G→C	A621P
904 G→A	G302F
1480 C→T	L494F
1558 G→C	A520P**

*Insertion of two new codons encoding RW, after codon 689. Note that this is a new repetition of codons 686-687 and 688-689, which encode a tandem repeat of the sequence RW (*i.e.*, RWRW→RWRWRW).

**Mutation also found previously in response to *recG* deficiency (Al Deib *et al.*, 1996).