RecD2 helicase limits replication fork stress in *Bacillus subtilis*

By

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To my wife, my sister, and my parents.
Thank you for your unwavering love and support.

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I came back to academics by a rather strange route. After spending stints at a couple different biotech companies I found myself on the website of a professor named Lyle Simmons. I thought to myself, “His research seems really cool… I should apply to tech in his lab!” Little did I know that I would be his first full-time hire, his 3rd graduate student, and still be there 7 years later.

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Chapter 1: Introduction

Overview

Helicases separate duplex DNA and RNA substrates into single stranded (ss) DNA or RNA segments. Due to this activity, helicases provide important roles in virtually all nucleic acid transactions from DNA replication and repair to RNA processing and ribosome maturation. There are 26 predicted or identified helicases in the *B. subtilis* genome, many of which have no known function (Table 1.1). Helicases have a number of conserved biochemical features, ATP binding and hydrolysis are among the most conserved since helicases harness the fuel of ATP to unwind and remodel nucleic acid polymers. In this work, I will provide an overview of DNA helicases with an emphasis on RecD2, the subject of this dissertation.

Replicative DNA helicases

Helicases are classified into a number of superfamilies (SF), numbered SF 1-6, and further subdivided by directionality of translocation along ssDNA, either 3′-5′ (A) or 5′-3′ (B), but are most simply divided by their status as replicative or accessory (for review [1]). Two of the most widely studied replicative helicases, responsible for the separation of DNA at the replication fork, are *E. coli* DnaB (homologous to *B. subtilis* DnaC) and the eukaryotic mini chromosome maintenance (MCM) complex, comprised of MCM 2-7 [2-4]. These helicases fall into superfamilies 4 and 6, respectively, but

share a similar architecture in that they are both hexameric, though DnaB self associates into a homohexamer and translocates 5′-3′ along the DNA (as seen in Figure 1.1a showing *B. subtilis* DnaC) as opposed to the MCM complex forming a heterohexamer which translocates 3′-5′ [4].

Accessory DNA helicases

Accessory helicases have a wide array of functions in DNA replication and repair. These include roles in DNA mismatch repair (*E. coli* UvrD, *B. anthracis* RecD2) and the repair of damaged DNA (*E. coli* UvrD, RecB and RecD, and *B. subtilis* AddA), as well as the removal of replication blocks (*E. coli* Rep, *B. subtilis* PcrA) [1, 5-9].

The role of an accessory helicase in methylation dependent DNA mismatch repair (MMR) has been well studied in *E. coli* by examining UvrD [10-12]. Briefly, following nicking of the nascent strand by MutH, UvrD is loaded at the nick by MutL, and translocates 3′-5′ in the direction of the mismatch displacing the mismatched nucleotide (for review [13]). A role for a helicase in methylation independent MMR has only recently been established with RecD2 of *B. anthracis* [5]. There is little data however to suggest a mechanism of RecD2 recruitment or method for strand discrimination. Response to endogenous and exogenous DNA damaging agents can involve several different pathways. In *E. coli* a primary pathway for dealing with small lesions is the nucleotide excision repair pathway, which relies on the UvrA,B,C, and D proteins. UvrAB scans the DNA for lesions and upon recognition, UvrA departs and UvrC is recruited. The UvrBC complex cleaves upstream and downstream of the lesion and UvrD is recruited to displace the resulting segment (for review [14-16]).

When it comes to accessory helicases removing blocks to replication there are two particularly well studied bacterial helicases, *E. coli* Rep and *B. subtilis* PcrA. It is known that *E. coli* replication forks progress more slowly in the absence of the Rep helicase, which is in large part because Rep acts at the replication fork, interacting with the replicative polymerase, to assist in removal of nucleo-protein barriers [17]. PcrA of *B. subtilis* performs an analogous role to Rep in that in its absence, the rate of DNA synthesis is decreased [18]. Additionally, expression of *B. subtilis* PcrA in *E. coli* complements the UV-sensitivity of an *uvrD* mutant [18]. Interestingly, expression of PcrA in *E. coli* also allows for the creation of an *uvrD rep* double mutant, which is otherwise not viable [18]. The aforementioned helicases all belong to SF1, though the other helicase superfamilies all have members participating in accessory functions as well [1].

RecD Helicases

RecD represents a subset of helicases composed of RecD1 and RecD2. RecD1, referred to here as RecD, has been studied primarily in *E. coli* where it functions as a 5′-3′ helicase in the RecBCD helicase nuclease complex required for end-resection during DNA double strand break (DSB) repair (for review, [8]). Briefly, at a double strand break the RecBCD complex is recruited and RecB translocates along one strand in the 3′-5′ direction while RecD translocates along the opposing strand in the 5′-3′ direction, while RecB coincidentally degrades both strands [8, 19]. Upon reaching a specific nucleotide sequence, called a chi site, the nuclease activity of RecB is modulated to

provide a 3′ overhang for RecA loading, followed by dissociation of the RecBCD complex [8].

The biochemical activity of *E. coli* RecD has been well studied, and the RecBCD helicase-nuclease complex has been crystalized [20]. While it was initially thought that RecB was the sole responsible helicase for the translocation of the RecBCD complex, it was later shown that RecD also had helicase activity in the opposite polarity to RecB [19]. Biochemical characterization of RecD outside of the complex had shown RecD to be an ATPase whose activity is substantially stimulated by the presence of ssDNA [19, 21]. A major breakthrough in the understanding of the kinetics of unwinding and processivity of the RecBCD complex came when RecD was shown to be a 5′-3′ helicase, and when in complex with RecBC increased the rate of unwinding over RecBC alone [19]. This discovery helped explain how the RecBCD complex could maintain extremely high processivity and speed of unwinding, uncharacteristic of any SF1 enzyme on its own [19].

RecD2 helicases

Bioinformatic searches and comparisons of sequenced bacterial genomes has revealed that *Bacillus subtilis* and many other organisms have a protein with high amino acid sequence similarity to the *E. coli* RecD (*B. subtilis* RecD2 is 27% identical to *E. coli* RecD), but lack RecB and RecC [22, 23]. In the studied organisms that maintain a RecD, but lack RecBC, this novel RecD has been designated RecD2, and I will occasionally refer to the *E. coli* RecD as RecD1 for comparison [19-21]. Beyond the lack of RecB and RecC as binding partners, RecD2 differs from RecD in a number of ways.

In sequence and structure, RecD2 is found to be unique from RecD1 by the addition of a well-conserved N-terminal domain of unknown function [24-26]. Because of the addition of this sizeable N-terminal domain, the RecD2 proteins are considerably larger than RecD1 proteins and have been characterized as either being fewer than 655 amino acids (RecD1) or greater than 710 amino acids (RecD2) [24]. RecD2 helicases are also found in a considerably greater number of sequenced bacterial genomes than the much better studied RecD. Interestingly, more than 270 sequenced bacterial genomes contain a RecD2 gene while a only 22 fully sequenced bacterial genomes contain the *E. coli*-type RecD [5, 24]. Genetic studies testing deletions of *recD2* in *Deinococcus radiodurans*, *Bacillus anthracis*, and *Bacillus subtilis* resulted in phenotypes with different sensitivities to DNA damaging agents as well as differing mutation rates and spectrums [5, 26-28]. Therefore, it seems that the *in vivo* roles of RecD2 are specific for the organisms in which it has been studied. Below, I discuss what is currently known about RecD2 helicases.

RecD2 was initially characterized in *D. radiodurans* under the hypothesis that it may be contributing to resistance to gamma irradiation in the absence of a known DSB repair complex such as RecBCD or the analogous complex AddAB [25]. As the name would suggest, *D. radiodurans* is radiation resistant and it was therefore thought that RecD2 may play a critical role in survival of high doses of ionizing radiation. Most initial studies of *D. radiodurans* RecD2 were strictly biochemical. Similar to *E. coli* RecD, ATP hydrolysis activity of *D. radiodurans* RecD2 was dependent on the presence of DNA [25]. RecD2 also showed a distinct increase in ATPase activity in the presence of ssDNA when compared to dsDNA [25]. Examination of directionality of RecD2 showed

that in accordance with its sequence similarity to RecD1, it is a 5′-3′ DNA helicase capable of unwinding a 5′ overhang substrate. In contrast it was incapable of unwind a 3′- overhang substrate [25]. It was also found that RecD2 is not particularly efficient at unwinding long (52+ nt) substrates, suggesting that RecD2 has low processivity, although the unwinding could be stimulated by the addition of SSB [25]. The means by which addition of SSB enhanced RecD2 substrate unwinding was not clear, however, leaving open the possibility that rather than SSB stimulating unwinding, it merely kept unwound substrates single stranded rather than actually stimulating unwinding by RecD2. More biochemical studies are necessary to understand the mechanism by which SSB enhances RecD2 activity.

Following the first biochemical characterization of *D. radiodurans* RecD2, separate studies attempted to assign a role to RecD2 *in vivo* [27, 28]. The first group found that in the absence of RecD2, *D. radiodurans* was sensitive to hydrogen peroxide [27]. After testing cell free extracts of *D. radiodurans* with or without RecD2, *E. coli* RecD, or various domains of RecD, they concluded that *D. radiodurans* RecD2 was involved in regulating catalase activity which allows for the scavenging of reactive oxygen species [27]. While this is a possibility, they were quick to note that the 50% decrease in free radical scavenging does not explain the 1000-fold increase in sensitivity to hydrogen peroxide that they noticed in survival assays [27]. Additionally, Zhou *et al.* concluded that *D. radiodurans* lacking RecD2 were hardly sensitive to either gamma or UV radiation, showing no significant difference in survival after exposure to up to 8 kGy of ionizing radiation, though at a UV fluence of 600 Jm^{-2} there was roughly

50% decreased in survival, which was statistically different from wild type [27]. In considering these studies, the role of RecD2 in *D. radiodurans* remained unclear.

Servinsky and Julin concluded that in the absence of RecD2, *D. radiodurans* was sensitive to both gamma and UV radiation, in addition to hydrogen peroxide [28]. Taking the *in vivo* work a few steps further, Servinsky and Julin challenged their *D. radiodurans* *recD*::*kan* strain with two other DNA damaging agents, Mitomycin C (MMC) and Methyl methanesulfonate (MMS) [28]. These two agents cause different types of damage. Methyl methanesulfonate is an alkylating agent which modifies guanines and adenines forming 7-methylguanine and 3-methyladenine, respectively [29]. Treatment with MMC results in the addition of bulky adducts to sequence specific guanines, which are then capable of forming intrastrand crosslinks [30]. Challenge by either agent prior to plating made no significant difference when comparing wild type to the *recD2* mutant [28]. Transformation efficiency was also examined, as *D. radiodurans* is naturally competent and able to integrate DNA into its chromosome by homologous recombination [31]. It was found that the transformation efficiency of the *recD*::*kan* mutant was 3- to 7-fold or 30- to 100-fold greater than wild type depending on the method of transformation tested [28]. The insensitivity of the *recD*::*kan* allele to MMC and MMS, combined with the sensitivity to hydrogen peroxide and both gamma and UV radiation, led to the conclusion that RecD2 was likely involved in survival of oxidative damage, rather than repair of double-strand breaks [28]. It is curious to note that while the results of both radiation treatments were different in both groups, they came to similar conclusions regarding the role of RecD2 in oxidative damage [27, 28]. The conflicts in these reports could be due to varied methods of treatment by both groups, or that the groups used

different strains of *D. radiodurans* for their radiation studies [27, 28]. Another possibility is that the *recD2* gene appears to be located in an operon with several downstream ORFs of unknown function, which could be compromised in the *recD*::*kan* background, as the effects of *recD*::*kan* were only able to be partially complemented by plasmid-born *recD2* [28].

Subsequent studies of RecD2 in *D. radiodurans* have focused on crystalizing the protein and further understanding the mechanism underlying ATPase and helicase activity of RecD proteins [32-35]. Saikrishnan *et al.* crystalized an N-terminal truncation of RecD2 and found that the structure was similar to the structure published for *E. coli* RecD. In previous work, SF1 helicases were separated into two primary domains (1 and 2), each made up of two subdomains (A and B) [36]. The conserved helicase motifs were determined to fall within subdomains 1A and 2A in the PcrA structure, but left questions as to the functions of the 1B and 2B domains [36]. The new *D. radiodurans* RecD2 structures better resolved these two domains of RecD2, which were disordered in the *E. coli* RecBCD structure, bolstering the knowledge of RecD structures and helping to elucidate the roles of the these domains in RecD proteins [20, 33]. Using the structure of RecD2, Saikrishnan *et al.* were able to determine that domain 1B forms a pin structure, necessary for separation of duplex DNA [33]. This was confirmed biochemically, as a pin-less mutant was incapable of separating DNA strands, though still retained a majority of DNA binding and ATPase activity [33]. Structures of another N-terminal truncation of RecD2 bound to ssDNA and a ternary complex of RecD2, ssDNA, and ADPNP resolved that domain 2B adopts a SRC homology 3 (SH3) fold [33]. SH3 folds are common in eukaryotes and are known to be primarily involved in

protein-protein interactions, specifically favoring peptides rich in prolines [37]. Other structures in eukaryotes and archaea have found that DNA-binding domains can also form SH3-like folds, though previously only shown to bind dsDNA [38-41]. Intriguingly, in the *D. radiodurans* RecD2 structure the SH3 fold binds ssDNA, and interactions of the SH3 fold with ssDNA are critical for helicase activity [33]. However, this interaction occurs on a face of the protein distinct from the known interface of dsDNA or peptide interactions from other organisms, inviting the possibility that the SH3 fold may still be available for peptide binding while RecD2 is DNA bound [32].

The RecD2 crystal structures also allowed Saikrishnan *et al.* to propose a mechanism for the 5´-3´ translocation of RecD2 along single stranded DNA [32]. Briefly, ssDNA is bound across the 1A and 2A domains until ATP is bound, at which point those domains undergo a shift translocating domains 2A and 2B along the ssDNA and forcing them to have tighter interaction with the bound ssDNA [32]. Upon ATP hydrolysis domain 1A changes conformations and opens slightly, allowing the DNA to slide along domain 1A as it is pulled back by domains 2A and 2B resulting in a hypothesized single base translocation per ATP hydrolysis event [32].Translocation rate along ssDNA was determined to occur at 95 +/- 5 nt/sec and ATP hydrolysis activity was found to be 98 +/- 12 hydrolysis reactions for a single molecule of RecD2 per second [32].

DNA binding and unwinding as well as the oligomerization state of *D. radiodurans* RecD2 were further examined biochemically [34]. It was found that the kinetic step size of RecD2 unwinding of duplex DNA was roughly 3-4 bp, with the rate constant of unwinding being 5.5 steps per second, or 15-20 bp/s corroborating the low processivity previously established [34]. While this may seem contradictory to the much

higher rate of translocation established earlier (95 nt/sec), it has previously been shown that translocation along ssDNA is not necessarily indicative of the separation rate for dsDNA [32, 34, 42, 43]. It is also important to note that the kinetic step size of DNA unwinding is significantly longer than the step size of DNA translocation, which has been shown to be common in both monomeric and dimeric helicases [32, 34, 42, 43]. RecD2 was also found to exist as a monomer in solution by gel filtration but was shown to self associate by glutaraldehyde crosslinking [34]. This was a potentially interesting finding because other SF1 helicases have been shown to bind and translocate along ssDNA while monomeric, but fail to unwind duplex DNA unless dimerized [43]. Shadrick and Julin were able to conclude that the functional form of RecD2 was monomeric, however, as the dimeric, crosslinked species observed in their experiment was absent when DNA was included [34].

RecD2 is toxic in a heterologous organism

Searching for a possible role in replication fork progression, *D. radiodurans* RecD2 and T4 Dda, *E. coli* Rep, and UvrD were all tested to see if expression in *E. coli* promoted replication fork progression through protein-DNA complexes [17]. Both Dda and RecD2 are 5′-3′ SF1 helicases, and Dda is known to remove transcription complexes ahead of replication, as well as independently of replication [44, 45]. *E. coli* Rep and UvrD, which both translocate 3′-5′, were shown to promote replisome progression through protein impediments [17]. Conversely, the 5′-3′ helicases T4 Dda and *D. radiodurans* RecD2 were shown to be of no assistance or actually detrimental, respectively, to fork progress measured biochemically [17]. The presence of RecD2

increased the degree of blockage that these cells experienced and reduced polymerase readthrough at nucleoprotein complexes [17]. These results suggest that the 5′-3′ directionality of accessory helicases (co-incident with the replicative helicase progression) is detrimental, whereas the 3′-5′ helicase activity of Rep and UvrD is beneficial to the progress of replication forks [17]. One conclusion the authors made is that Rep and UvrD are able to proactively remove impediments as they are seated on the opposite strand to the replicative helicase DnaB, but translocating in the same net direction of replication [17]. This suggests that helicases translocating in the opposite direction to the replication fork pose a strong barrier to the continuation of replication [17]. Interestingly, while both RecD2 and Dda failed to promote replication through a blockage in Δ*rep*Δ*uvrD* cells, and neither RecD2 nor Dda were able to complement a Δ*rep*Δ*uvrD* mutant, Dda expression was found to inhibit growth in otherwise wild-type *E. coli* cells [17].

The decline in replication fork progression caused by the presence of RecD2 in *E. coli* was examined further to determine whether this effect was specific to paused replication complexes or was more broadly applicable, and whether the decline was caused specifically by helicase activity [7]. The ability of helicases to promote replication after fork blockage was examined. Plasmid replication was blocked at a long set of tandem *lac* operators by addition of LacI, followed shortly by the addition of a helicase, and then allowed to resume by addition of IPTG [7]. Addition of *E. coli* Rep, UvrD, or *B. stearothermophilus* PcrA, allowed for replication similar to when no helicase was added, but addition of *D. radiodurans* RecD2 severely impaired the ability of replication complexes to resume after IPTG addition [7]. To test whether the failure to restart was a

result of the helicase activity of RecD2 a pin-less variant was created which was still able to bind but unable to separate duplex DNA [7, 33]. Addition of the pin-less RecD2 variant to paused replication forks had little effect on the ability of replication forks to restart, similar to that of UvrD, Rep, and PcrA, specifically implicating the helicase activity of RecD2 in preventing replication restart after removal of the block [7]. To elicit more insight into RecD2-dependent inactivation of replisomes, elongating replisomes or those stalled by supercoiling induced topological strain were examined with or without RecD2 or the pin-less variant. Again, the pin-less RecD2 had no effect on DNA synthesis following release of topological strain by DNA gyrase, compared to no helicase, when added to both elongating and paused replisomes [7]. Curiously, RecD2 only negatively affected DNA synthesis following replication stalling, not during elongation, indicating that replication forks must be paused for RecD2 to inactivate them [7]. Having tested the effect of RecD2 on forks paused by both a LacO array and supercoiling induced topological strain, Gupta *et al*. tested whether forks stalled at a DNA lesion could be inactivated by RecD2. As with the nucleoprotein blockages, RecD2 inactivated forks paused at cyclobutane pyrimidine dimers, yet the pin-less mutant did not, indicating that helicase activity or strand separation was required for inactivation [7].

To assess the consequences of RecD2 expression *in vivo* viability and DNA content were examined. Expression of RecD2 in a variety of strain backgrounds from recombination deficient to nucleotide excision repair deficient had little consequence on cell viability except in a Δ*rep* mutant, where viability was decreased roughly 1000-fold compared to wild type [7]. Concordant with the findings on replisome inactivation, the expression of the pin-less mutant was inconsequential [7]. The ability of Rep to mitigate

RecD2 toxicity is also dependent upon Rep's own helicase activity as an ATP hydrolysis deficient mutant was sensitive to RecD2 overexpression [7]. To separate Rep's two roles in *E. coli,* RecD2 was expressed in a strain where Rep is unable to bind DnaB, limiting its access to the replication fork. In the strain with the DnaB-binding deficient Rep protein RecD2 was toxic, indicating that it is the nucleoprotein removal function of Rep that is crucial for survival following RecD2 overexpression [7]. Because fork inactivation could be extremely detrimental to replication processes encountering endogenous DNA damage, chromosomal DNA content was examined and it was found that cells expressing RecD2 were inhibited for chromosomal duplication, which again required helicase activity [7].

These studies were interesting and indicate that a helicase operating out of context could be detrimental to a paused replication fork. While we know RecD2 can inactivate paused replication forks, there is no data available to conclude how RecD2 inactivates the forks. Figure 1.2 provides a schematic for two potential ways that RecD2 could inactivate *E. coli* forks. First, RecD2 translocation could cause the removal of the replicative helicase on the lagging strand while paused at a crosslink (Figure 1.2a). While binding of *E. coli* SSB to RecD2 has not been examined, I have shown that *B. subtilis* SSB and RecD2 interact, so RecD2 may be loaded onto ssDNA by SSB [26]. Alternatively, and perhaps more likely, disassembly of the polymerase complex on the leading strand could be achieved by loading at a nick, made during recombination or topoisomerase mediated supercoil relaxation, near a stalled fork and progressing 5'-3' along the template, removing the polymerase, and separating newly replicated dsDNA strands (Figure 1.2b).

B. anthracis RecD2 is involved in DNA mismatch repair

In a separate study, the role of RecD2 was examined in *B. anthracis*, this time within its native context. During separate transposon-insertion papillation assays, a relatively simple screen for increased mutagenesis, two previously unidentified loci were implicated for roles in DNA mismatch repair [5, 46]. The first locus, BAS5315 or *yycJ*, was determined to have a strong mutator phenotype upon disruption and have a mutation spectrum similar to that of a mismatch repair deletion, consisting of overwhelmingly transition mutations or insertions and deletions [46]. The second locus, BAS4289 or *recD2*, was also found to cause a spontaneous mutator phenotype upon interruption [5]. *B. anthracis* RecD2 shares high sequence similarity/identity to *D. radiodurans* RecD2 (30% identity) allowed for the intriguing possibility that the *B. anthracis* RecD2 may be the first helicase identified outside of *E. coli* and its close relatives to participate in DNA mismatch repair (MMR) [46].

To assess the potential involvement of *B. anthracis* RecD2 in MMR, the BAS4289 locus which codes for RecD2 was disrupted using a suicide vector gene disruption and the resulting strain was assessed for spontaneous mutation frequency and mutation spectrum [5]. *B. anthracis recD2* deficient cells resulted in a spontaneous mutation frequency of 538 X 10^{-9}, while a MMR deficient mutant, *mutS*, resulted in a frequency of 863 X 10^{-9} [5]. Though the *recD2* deficient strain had a mutation frequency only 62% of a mismatch repair null, it should be noted that the spontaneous mutation frequency of an *uvrD* mutant in *E. coli* has a mutation frequency of roughly half of a *mutSL* mutant, and UvrD is the helicase involved in MutH-dependent MMR [10-12].

This initial observation suggested that *B. anthracis* RecD2 functioned in MMR. Interestingly, the *B. anthracis* genome also encodes a UvrD ortholog. *B. anthracis*, *uvrD* is predicted to encode a protein with 31% identity with *E. coli* UvrD. Disruption of *B. anthracis uvrD* resulted in a spontaneous mutation frequency nearly identical to that of wild type demonstrating that *B. anthracis uvrD* does not function in MMR [5]. Further testing revealed that *B. anthracis recD2* is epistatic to *mutS*, as a *recD2 mutS* mutant has a mutation frequency within the error of *mutS* alone [5]. A hallmark of MMR deficiency is the strict occurrence of transition mutations and insertions and deletions in the mutation spectrum. To further test a role for RecD2 in MMR, the mutation spectrum within the *rpoB* gene was analyzed by sequencing *rpoB* from rifampin resistant colonies in wild type, *mutS* and *recD2* deficient strains. Of the 40 *mutS* colonies examined, mutations occurred in only three nucleotides and they were all transitions [5]. Of the 47 *recD2* colonies sequenced mutations occurred in only 2 nucleotides, which were also 39 of the 40 for *mutS*, and they too were exclusively transitions [5]. Assayed in a more comprehensive way using the *nprR* gene, which encodes a transcriptional regulator for an extracellular protease and whose disruption causes a papillation phenotype, mutational specificity was examined again and it was found that both *recD2* and *mutS* mutants had substantial increases in short insertions and deletions in *nprR* compared to wild type, but also a surprising lack of large deletions which was the mutation most frequently seen in wild type cells [5, 46]. These data, taken together, strongly implicate RecD2 in the DNA mismatch repair pathway of *B. anthracis*.

RecD2 N-terminal region

To investigate the importance of the N-terminal region of RecD2, which has remained unstudied, *recD2* deletion were complemented ectopically with RecD2 mutants containing single amino acid substitutions: G112T, F208A, D212A, and K368Q [5]. The RecD2(K368Q) mutant has a change in the invariant lysine in the ATPase domain, rendering it nonfunctional, while the remaining mutants tested changes in residues that are conserved across known RecD2 proteins [5]. These strains were then assayed for spontaneous mutagenesis frequency on rifampin plates [5]. While none of the mutants were able to completely rescue the mutator phenotype, *recD2*(F208A) and *recD2*(K368Q) remained most impaired at roughly 50% of the mutation frequency of the *recD2* disruption [5]. Since these RecD2 single amino acid substitution mutants were unable to complement the *recD2* disruption, it is clear that the N-terminus is important for RecD2 function although the contribution of this region to RecD2 biology is not understood.

RecD2 binds single stranded binding protein (SSB)

In a pull-down assay of SSB binding partners in *B. subtilis*, a novel protein YrrC (since renamed RecD2 [26]) was identified as part of the SSB interactome [47]. Following the pull-down, RecD2 was ectopically expressed with a N-terminal GFP tag from a xylose inducible promoter and examined for localization in cells expressing wild type SSB or a C-terminal truncation of SSB which is impaired for protein-protein interactions [47]. It was found that GFP-RecD2 was recruited to the nucleoid in the vast majority of cells, with an average of ~2 GFP-RecD2 foci/nucleoid [47]. The authors did not verify this interaction *in vitro* however, so it remained unknown if SSB binds RecD2 directly, or

whether it is mediated through other binding partners [47]. This question will be addressed in Chapter 2. The potential interaction of SSB with RecD2 provides a possible platform for RecD2 recruitment to its site of action. The molecular details of RecD2-SSB interaction are unknown.

RecD2 warrants significant further study

RecD2 represents an important and yet poorly characterized helicase in bacteria with several orthologs present in mammalian cells. Previous RecD2 investigations have left many fundamental questions unanswered regarding its function and activity in individual organisms. The studies of RecD2 from *Deinococcus* radiodurans, while most extensive, have concentrated primarily on characterizing the biochemical activity of RecD2 and using structural data to provide mechanistic insight into translocation along ssDNA and ATP hydrolysis activity of SF1B helicases [25, 27, 32-35]. The RecD2 characterization in *D. radiodurans* has illuminated a role for RecD2 in the response to oxidative damage and both UV and IR damage, though surprisingly not to alkylating agents [27, 28]. These results illustrate the complexity of DNA repair functions and, unfortunately, the enzymatic pathways governing many different types of DNA repair are not well understood. It is also not clear what specific type of lesion/or repair process RecD2 might be recruited to act upon. Thus the role of *D. radiodurans* RecD2 in DNA damage repair remains unclear, and warrants further investigation. Additionally, knockouts of *D. radiodurans* RecD2 were never examined for a spontaneous mutator

phenotype and should be tested, considering the function of *B. anthracis* RecD2 in mismatch repair [5].

The importance of the RecD2 helicase in *B. anthracis'* mismatch repair pathway is clear, though the mechanistic details are not understood. The data regarding the function of RecD2 in *B. anthracis* place its role firmly within the MMR pathway, but also in the mitigation of oxidative damage [5]. While this seems to agree with RecD2's function in *D. radiodurans*, cells disrupted for *recD2* in *B. anthracis* were not sensitive to UV [5]. Because no other DNA damaging agents were tested, there are few conclusions that can be made about the role of RecD2 in other DNA repair pathways in *B. anthracis* aside from its role in MMR. There is also no information available about the enzymatic activity of RecD2 in *B. anthracis* such as ATPase activity, processivity, or translocase or helicase activity, which may help to elucidate other possible functions.

The only data suggesting potential binding partners for RecD2, or a means by which it could localize to its site of action, came from a Tap-tag pull-down followed by MALDI-TOF to identify RecD2 as a potential binding partner of SSB [47]. Because SSB is known to coordinate the activities of many proteins at the replication fork, SSB is a particularly attractive option for direct recruitment of RecD2 to the replication fork [47]. I address the hypothesis that SSB is a direct binding partner of RecD2 in Chapters 2 and 4. It is possible that SSB binding takes place in the SH3 domain of RecD2, highlighted in a model of RecD2 based on and aligned to the *D. radiodurans* RecD2 structure bound to ssDNA and ADPNP (Figure 1.3b) [48]. The SH3 domain is required for ssDNA binding in *D. radiodurans*, but the domain is quite large, much of which is surface

exposed as shown in Figure 1.3b, inviting the possibility that it is involved in both protein-protein and protein-ssDNA interactions.

One of the most curious aspects of RecD2 function is that the *D. radiodurans* protein inactivates paused replication forks in *E. coli* [7]. This leads to the intriguing possibility that *recD2* dysregulation or overexpression may result in aberrant fork collapse or toxicity in the native organism. To date, increased RecD2 expression has not been examined in a native context. I investigate the effects of RecD2 expression and toxicity in *B. subtilis* in Chapter 3.

Taken together, the work described above provides the foundation for exploration of the biological role of RecD2 in *B. subtilis*. There are an abundance of questions to be answered about RecD2, some of which will be answered in the upcoming chapters. A major question is whether RecD2 is involved in MMR in organisms outside of *B. anthracis.* This question is answered for *B. subtilis* in Chapter 2 and published in Walsh *et al.,* 2014, where I demonstrate that RecD2 is not involved in MMR in *B. subtilis* and in its absence there is a modest increase in mutation rate which appears to be SOS dependent. Another critical question is whether RecD2 alleviates sensitivities to a variety of DNA damaging agents. While some DNA damaging agents were tested with *D. radiodurans,* fewer were tested with *B. anthracis*, and available data do not provide a clear picture concerning the involvement of RecD2 in cellular response to a wide range of DNA damage [5, 27, 28]. There were also different sensitivities across organisms with *recD2* deficiency [5, 27, 28]. Additionally, there was no examination of *recD2* mutants' sensitivities to chemically induced double strand breaks, or replication fork stalling, which would be helpful in illuminating a more specific role for RecD2 in the

repair of DNA damage or perhaps fork elongation. *B. subtilis* deleted for *recD2* is examined for sensitivity to a broad spectrum of DNA damaging agents in Chapter 2, and published [26], and I show that Δ*recD2* is sensitive to a variety of DNA damaging agents.

In the prior research there was also little indication of the role of RecD2 at the replication fork, aside from the knowledge that expression of *D. radiodurans* RecD2 in *E. coli* caused stalled forks to fail to restart [7]. Examination of the role of RecD2 at the replication fork in the native organism will be critical to our understanding RecD2 function *in vivo*. I show in Chapter 2 that *B. subtilis* RecD2 helps mitigate replication fork stress, and that in its absence there is an increase in fork collapse using genomic approaches to track replication fork collapse *in vivo* [26]. Using RecA-GFP foci as a proxy for increased ssDNA at the replication I also show an increase in replication fork stress in the Δ*recD2* mutant, which is a hallmark of replication fork stress [26]. It is also known that RecD2 is not regulated by the SOS response in *B. subtilis*, but given the RecA-GFP focus formation data it will be interesting to examine the transcriptional regulation of cells lacking *recD2*, and those overexpressing it as well. It will also be interesting to determine how much RecD2 is present in the cell at any given time, as the amount of RecD2 may provide insight into how tightly regulated RecD2 expression is. These questions will be addressed in Chapter 3.

Another important question lies in understanding the function of the N-terminus of RecD2. The N-terminal region is highlighted in orange in Figure 1.3c, but it is important to note that this is a model generated by PHYRE2, based on and aligned to the structure of *D. radiodurans* RecD2 bound to ssDNA and ADPNP [33, 48]. This model

predicts shows the layout of the N-terminus and conserved residues. The two studies regarding the function of the N-terminus only note that it is critical for RecD2 catalase activity and hydrogen peroxide survival in *D. radiodurans* or MMR in *B. anthracis* [5, 27]. In Chapter 3, I investigate conserved residues in the N-terminal region of RecD2 and show that F209 (highlighted in purple) (Figure 1.3d) is critical for RecD2 function *in vivo*.

Understanding the binding partners and the kinetic activity of RecD2 will be critical for understanding its function *in vivo*, as will determining the function of the N-terminus, and these studies will pave the path to further understand the roles RecD2 is playing in DNA replication and repair.

Figures

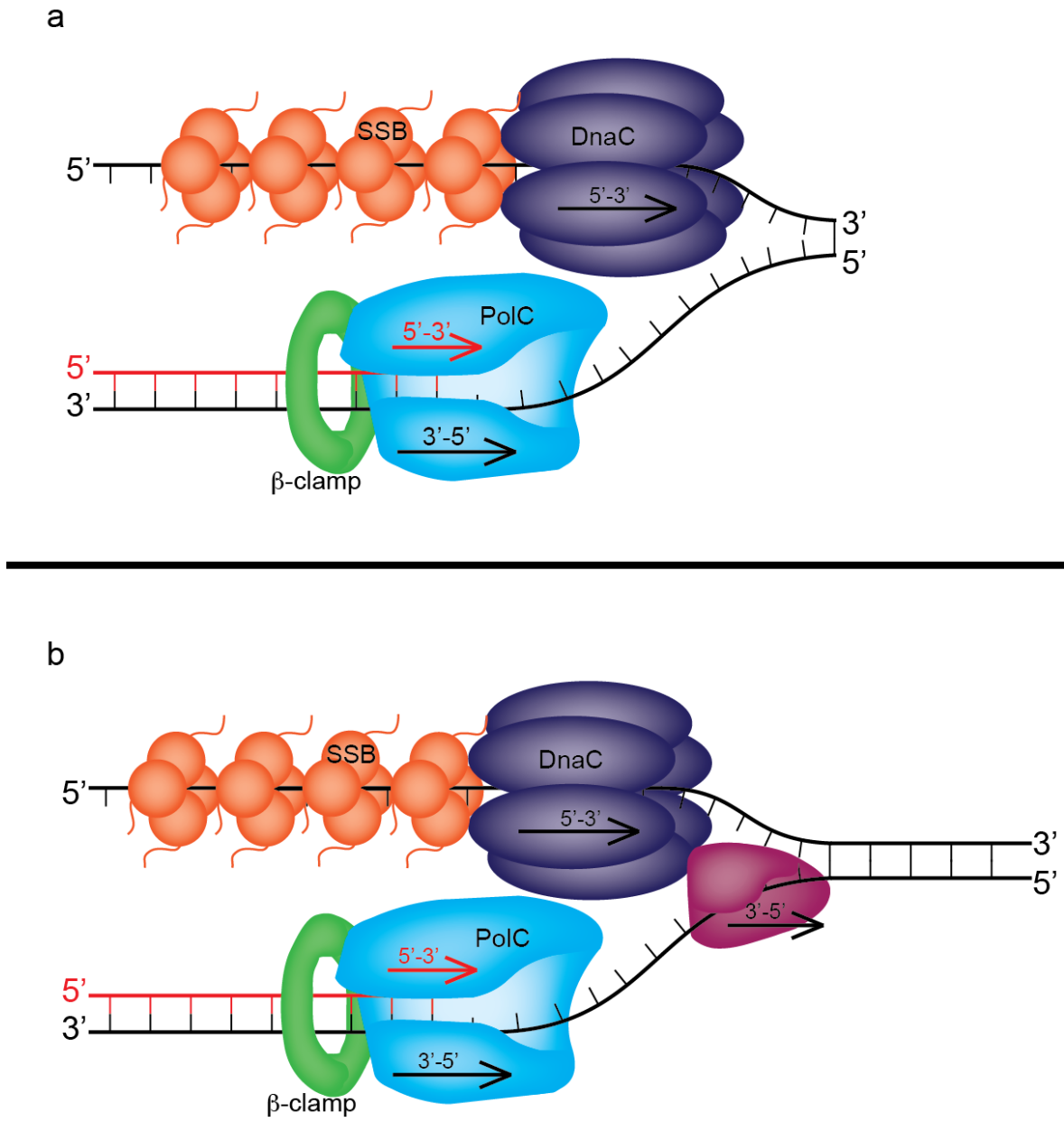

Figure 1.1. Roles for helicases at the replication fork
1a: *B. subtilis* replicative helicase (DnaC) proceeds 5′-3′ separating duplex DNA while the replicative polymerase (PolC) translocates 3′-5′ (black), incorporating nucleotides into the nascent strand 5′-3′ (red). 1b: An accessory helicase may travel ahead of the fork, removing impediments, translocating 3′-5′. This figure is partially based on information from the following sources: [49, 50]

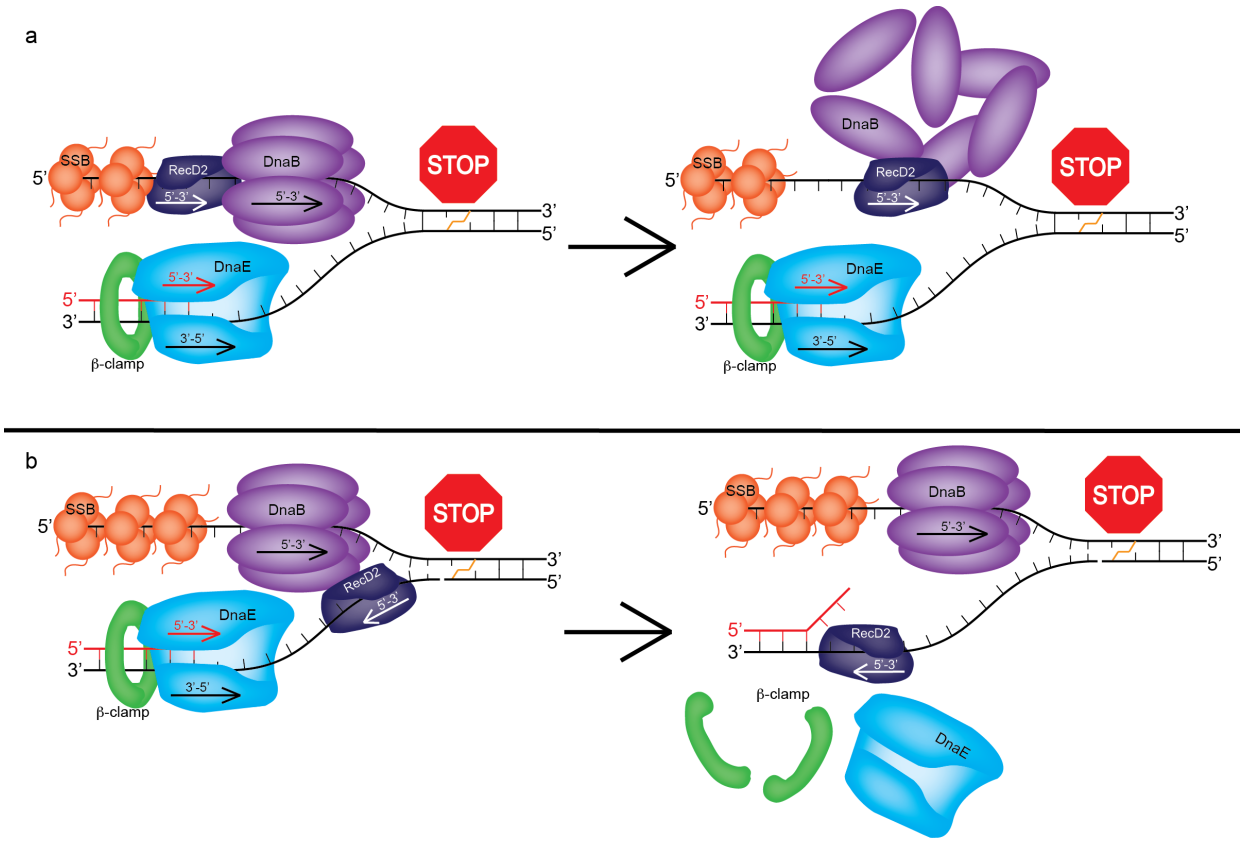

Figure 1.2. Proposed mechanisms of replication fork inactivation by RecD2. 2a: Upon replication fork pausing, RecD2 may be inappropriately recruited by SSB and translocate 5´-3´ into the *E. coli* replicative helicase, DnaB, dislodging it from the lagging strand. 2b: Upon replication fork pausing, RecD2 may be loaded at a nick and translocate 5´-3´ along the leading strand displacing the replicative polymerase and processivity factor. This figure is partially based on information from the following sources: [7, 50]

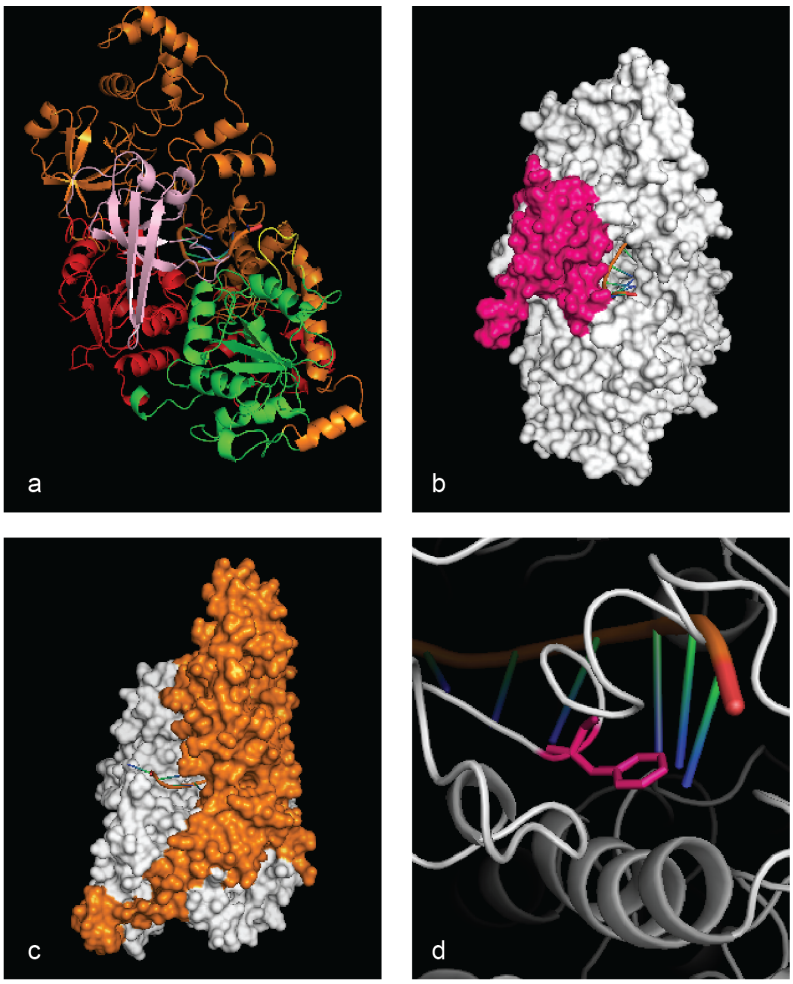

Figure 1.3. Domains and important residues of RecD2
3a: Model of the structure of *B. subtilis* RecD2 with the individual domains highlighted (N-terminal: orange. 1a: green. 1b: yellow. 2a: red. 2b: pink.). 3b: Model of RecD2, with ssDNA, with the SH3 domain highlighted in pink. 3c: Model of RecD2 with the N-terminal domain highlighted in orange. 3d: Model of RecD2 with residue F209 in magenta to show predicted proximity to ssDNA. Phyre2-generated model is predicted from PDB numbers 1IXR, 4GLX, 3GP8, 1HJP, 1DGS, 3E1S [32, 33, 48, 51-54].

Table 1.1. Known and putative helicases of *B. subtilis*.

Helicase	Gene	Protein length (AA)	Function
DnaC	*dnaC*	454	ATP-dependent replicative DNA helicase (essential)
PcrA	*pcrA*	739	ATP-dependent accessory helicase (essential)
PriA	*priA*	805	ATP-dependent primosome helicase (essential)
AddA	*addA*	1232	DNA end resectioning
AddB	*addB*	1166	DNA end resectioning
ComFA	*comFA*	463	Competence DNA uptake
DinG	*dinG*	931	ATP-dependent DNA helicase, damage inducible
HelD	*yvgS*	774	ATP-dependent, stimulates RNA transcription
RecD2	***yrrC***	**798**	**ATP-dependent DNA helicase, replication fork progression**
RecG	*recG*	682	ATP-dependent DNA helicase, Holiday Junction branch migration
RecQ	*yocI*	591	ATP-dependent DNA helicase, DNA repair
RecS	*recQ*	496	ATP-dependent DNA helicase, DNA repair
RuvA	*ruvA*	201	ATP-dependent DNA helicase, Holiday junction helicase
RuvB	*ruvB*	334	ATP-dependent DNA helicase, Holiday junction helicase
YeeB	*yeeB*	598	Unknown function, similar to ATP-dependent DEAD box helicase
YjcD	*yjcD*	759	Unknown function, similar to ATP-dependent DNA helicase
YorI	*yorI*	504	Unknown function, similar to ATP-dependent replicative DNA helicase
YprA	*yprA*	749	Unknown function, similar to ATP-dependent DNA helicase
YpvA	*ypvA*	641	Unknown function, similar to XerD
YqhH	*yqhH*	557	Unknown function, similar to SNF2 helicase
YwqA	*ywqA*	922	Unknown function, similar to SNF2 helicase
Rho	*rho*	427	Transcription termination
CshA	*ydbR*	511	DEAD-box RNA helicase, ribosome biogenesis
CshB	*yqfR*	438	DEAD-box RNA helicase, ribosome biogenesis
DeaD	*deaD*	479	DEAD-box RNA helicase, ribosome biogenesis
YfmL	*yfmL*	376	DEAD-box RNA helicase, ribosome biogenesis

Compiled from the list of annotated helicases on SubtiList [55] based on conserved amino acid motifs and other assorted publications [9, 26, 56-61].

References

1. Singleton, M.R., M.S. Dillingham, and D.B. Wigley, *Structure and mechanism of helicases and nucleic acid translocases.* Annual review of biochemistry, 2007. 76: p. 23-50.
2. Barcena, M., et al., *The DnaB.DnaC complex: a structure based on dimers assembled around an occluded channel.* The EMBO journal, 2001. 20(6): p. 1462-8.
3. Pape, T., et al., *Hexameric ring structure of the full-length archaeal MCM protein complex.* EMBO reports, 2003. 4(11): p. 1079-83.
4. Patel, S.S. and K.M. Picha, *Structure and function of hexameric helicases.* Annual review of biochemistry, 2000. 69: p. 651-97.
5. Yang, H., et al., *The role of Bacillus anthracis RecD2 helicase in DNA mismatch repair.* DNA repair, 2011. 10(11): p. 1121-30.
6. Matson, S.W. and A.B. Robertson, *The UvrD helicase and its modulation by the mismatch repair protein MutL.* Nucleic acids research, 2006. 34(15): p. 4089-97.
7. Gupta, M.K., et al., *Protein-DNA complexes are the primary sources of replication fork pausing in Escherichia coli.* Proceedings of the National Academy of Sciences of the United States of America, 2013. 110(18): p. 7252-7.
8. Wigley, D.B., *Bacterial DNA repair: recent insights into the mechanism of RecBCD, AddAB and AdnAB.* Nature reviews. Microbiology, 2013. 11(1): p. 9-13.
9. Lenhart, J.S., et al., *DNA repair and genome maintenance in Bacillus subtilis.* Microbiology and molecular biology reviews : MMBR, 2012. 76(3): p. 530-64.
10. Brosh, R.M., Jr. and S.W. Matson, *Mutations in motif II of Escherichia coli DNA helicase II render the enzyme nonfunctional in both mismatch repair and excision repair with differential effects on the unwinding reaction.* Journal of bacteriology, 1995. 177(19): p. 5612-21.
11. Brosh, R.M., Jr. and S.W. Matson, *A point mutation in Escherichia coli DNA helicase II renders the enzyme nonfunctional in two DNA repair pathways. Evidence for initiation of unwinding from a nick in vivo.* The Journal of biological chemistry, 1997. 272(1): p. 572-9.
12. Schaaper, R.M., *Base selection, proofreading, and mismatch repair during DNA replication in Escherichia coli.* The Journal of biological chemistry, 1993. 268(32): p. 23762-5.
13. Kunkel, T.A. and D.A. Erie, *DNA mismatch repair.* Annual review of biochemistry, 2005. 74: p. 681-710.
14. Sancar, A., *DNA excision repair.* Annual review of biochemistry, 1996. 65: p. 43-81.
15. Sancar, A., *Mechanisms of DNA excision repair.* Science, 1994. 266(5193): p. 1954-6.
16. Sancar, A. and G.B. Sancar, *DNA repair enzymes.* Annual review of biochemistry, 1988. 57: p. 29-67.
17. Guy, C.P., et al., *Rep provides a second motor at the replisome to promote duplication of protein-bound DNA.* Molecular cell, 2009. 36(4): p. 654-66.

18. Petit, M.A., et al., *PcrA is an essential DNA helicase of Bacillus subtilis fulfilling functions both in repair and rolling-circle replication.* Molecular microbiology, 1998. 29(1): p. 261-73.
19. Dillingham, M.S., M. Spies, and S.C. Kowalczykowski, *RecBCD enzyme is a bipolar DNA helicase.* Nature, 2003. 423(6942): p. 893-7.
20. Singleton, M.R., et al., *Crystal structure of RecBCD enzyme reveals a machine for processing DNA breaks.* Nature, 2004. 432(7014): p. 187-93.
21. Chen, H.W., et al., *The RecD subunit of the RecBCD enzyme from Escherichia coli is a single-stranded DNA-dependent ATPase.* The Journal of biological chemistry, 1997. 272(15): p. 10072-9.
22. Altschul, S.F., et al., *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.* Nucleic acids research, 1997. 25(17): p. 3389-402.
23. Altschul, S.F., et al., *Protein database searches using compositionally adjusted substitution matrices.* The FEBS journal, 2005. 272(20): p. 5101-9.
24. Montague, M., et al., *The evolution of RecD outside of the RecBCD complex.* Journal of molecular evolution, 2009. 69(4): p. 360-71.
25. Wang, J. and D.A. Julin, *DNA helicase activity of the RecD protein from Deinococcus radiodurans.* The Journal of biological chemistry, 2004. 279(50): p. 52024-32.
26. Walsh, B.W., et al., *RecD2 helicase limits replication fork stress in Bacillus subtilis.* Journal of bacteriology, 2014. 196(7): p. 1359-68.
27. Zhou, Q., et al., *A new role of Deinococcus radiodurans RecD in antioxidant pathway.* FEMS microbiology letters, 2007. 271(1): p. 118-25.
28. Servinsky, M.D. and D.A. Julin, *Effect of a recD mutation on DNA damage resistance and transformation in Deinococcus radiodurans.* Journal of bacteriology, 2007. 189(14): p. 5101-7.
29. Beranek, D.T., *Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents.* Mutation research, 1990. 231(1): p. 11-30.
30. Dronkert, M.L. and R. Kanaar, *Repair of DNA interstrand cross-links.* Mutation research, 2001. 486(4): p. 217-47.
31. Moseley, B.E. and J.K. Setlow, *Transformation in Micrococcus radiodurans and the ultraviolet sensitivity of its transforming DNA.* Proceedings of the National Academy of Sciences of the United States of America, 1968. 61(1): p. 176-83.
32. Saikrishnan, K., et al., *Mechanistic basis of 5'-3' translocation in SF1B helicases.* Cell, 2009. 137(5): p. 849-59.
33. Saikrishnan, K., et al., *DNA binding to RecD: role of the 1B domain in SF1B helicase activity.* The EMBO journal, 2008. 27(16): p. 2222-9.
34. Shadrick, W.R. and D.A. Julin, *Kinetics of DNA unwinding by the RecD2 helicase from Deinococcus radiodurans.* The Journal of biological chemistry, 2010. 285(23): p. 17292-300.
35. Toseland, C.P. and M.R. Webb, *ATPase mechanism of the 5'-3' DNA helicase, RecD2: evidence for a pre-hydrolysis conformation change.* The Journal of biological chemistry, 2013. 288(35): p. 25183-93.

36. Bird, L.E., et al., *Characterisation of Bacillus stearothermophilus PcrA helicase: evidence against an active rolling mechanism.* Nucleic acids research, 1998. 26(11): p. 2686-93.
37. Zarrinpar, A., R.P. Bhattacharyya, and W.A. Lim, *The structure and function of proline recognition domains.* Science's STKE : signal transduction knowledge environment, 2003. 2003(179): p. RE8.
38. Eijkelenboom, A.P., et al., *The DNA-binding domain of HIV-1 integrase has an SH3-like fold.* Nature structural biology, 1995. 2(9): p. 807-10.
39. Lodi, P.J., et al., *Solution structure of the DNA binding domain of HIV-1 integrase.* Biochemistry, 1995. 34(31): p. 9826-33.
40. Gao, Y.G., et al., *The crystal structure of the hyperthermophile chromosomal protein Sso7d bound to DNA.* Nature structural biology, 1998. 5(9): p. 782-6.
41. Robinson, H., et al., *The hyperthermophile chromosomal protein Sac7d sharply kinks DNA.* Nature, 1998. 392(6672): p. 202-5.
42. Serebrov, V., R.K. Beran, and A.M. Pyle, *Establishing a mechanistic basis for the large kinetic steps of the NS3 helicase.* The Journal of biological chemistry, 2009. 284(4): p. 2512-21.
43. Yang, Y., et al., *Evidence for a functional dimeric form of the PcrA helicase in DNA unwinding.* Nucleic acids research, 2008. 36(6): p. 1976-89.
44. Bedinger, P., et al., *Properties of the T4 bacteriophage DNA replication apparatus: the T4 dda DNA helicase is required to pass a bound RNA polymerase molecule.* Cell, 1983. 34(1): p. 115-23.
45. Liu, B., M.L. Wong, and B. Alberts, *A transcribing RNA polymerase molecule survives DNA replication without aborting its growing RNA chain.* Proceedings of the National Academy of Sciences of the United States of America, 1994. 91(22): p. 10660-4.
46. Yang, H., et al., *Papillation in Bacillus anthracis colonies: a tool for finding new mutators.* Molecular microbiology, 2011. 79(5): p. 1276-93.
47. Costes, A., et al., *The C-terminal domain of the bacterial SSB protein acts as a DNA maintenance hub at active chromosome replication forks.* PLoS genetics, 2010. 6(12): p. e1001238.
48. Kelley, L.A., et al., *The Phyre2 web portal for protein modeling, prediction and analysis.* Nature protocols, 2015. 10(6): p. 845-58.
49. Sanders, G.M., H.G. Dallmann, and C.S. McHenry, *Reconstitution of the B. subtilis replisome with 13 proteins including two distinct replicases.* Molecular cell, 2010. 37(2): p. 273-81.
50. Yao, N.Y. and M. O'Donnell, *SnapShot: The replisome.* Cell, 2010. 141(6): p. 1088, 1088 e1.
51. Yamada, K., et al., *Crystal structure of the RuvA-RuvB complex: a structural basis for the Holliday junction migrating motor machinery.* Molecular cell, 2002. 10(3): p. 671-81.
52. Surivet, J.-P., et al., *Structure-guided design, synthesis and biological evaluation of novel DNA ligase inhibitors with in vitro and in vivo anti-staphylococcal activity.* Bioorganic & medicinal chemistry letters, 2012. 22(21): p. 6705-6711.
53. Nishino, T., et al., *Functional analyses of the domain structure in the Holliday junction binding protein RuvA.* Structure, 1998. 6(1): p. 11-21.

54. Lee, J.Y., et al., *Crystal structure of NAD+-dependent DNA ligase: modular architecture and functional implications.* The EMBO journal, 2000. 19(5): p. 1119-1129.
55. Moszer, I., et al., *SubtiList: the reference database for the Bacillus subtilis genome.* Nucleic acids research, 2002. 30(1): p. 62-5.
56. Hwang, J.Y. and R.H. Doi, *Transcription-termination factor Rho from Bacills subtilis.* European journal of biochemistry / FEBS, 1980. 104(1): p. 313-20.
57. Carrasco, B., et al., *Genetic recombination in Bacillus subtilis 168: effect of DeltahelD on DNA repair and homologous recombination.* Journal of bacteriology, 2001. 183(19): p. 5772-7.
58. Wiedermannova, J., et al., *Characterization of HelD, an interacting partner of RNA polymerase from Bacillus subtilis.* Nucleic acids research, 2014. 42(8): p. 5151-63.
59. Nakanishi, A., et al., *Identification of DNA gyrase inhibitor (GyrI) in Escherichia coli.* The Journal of biological chemistry, 1998. 273(4): p. 1933-8.
60. Hunger, K., et al., *Cold-induced putative DEAD box RNA helicases CshA and CshB are essential for cold adaptation and interact with cold shock protein B in Bacillus subtilis.* Journal of bacteriology, 2006. 188(1): p. 240-8.
61. Lehnik-Habrink, M., et al., *DEAD-Box RNA helicases in Bacillus subtilis have multiple functions and act independently from each other.* Journal of bacteriology, 2013. 195(3): p. 534-44.

Chapter 2

RecD2 helicase limits replication fork stress in *Bacillus subtilis*

Abstract

DNA helicases have important roles in replication, recombination, and repair. The RecD helicase has been well-studied as a component of the RecBCD helicase-nuclease enzyme important for double-strand break repair *Escherichia coli.* Interestingly*,* many bacteria lack RecB and RecC and instead contain a RecD2 helicase, which is not known to function as part of a larger complex. Depending on the organism studied, RecD2 helicases have been shown to provide resistance to a broad range of DNA damaging agents while also contributing to the mismatch repair pathway. Here we investigated the importance of *Bacillus subtilis* RecD2 helicase (*yrrC*) to genome integrity. We show that deletion of *recD2* confers a modest increase in spontaneous mutagenesis, and the mutational signature in Δ*recD2* cells is not consistent with a mismatch repair defect, indicating a new function for RecD2 in *B. subtilis*. To further characterize the role of RecD2, we tested the deletion strain for sensitivity to several DNA damaging agents. We found that loss of RecD2 in *B. subtilis* sensitized cells to mitomycin C and the DNA break-inducing peptide phleomycin. Measurement of replication fork progression *in vivo* shows that fork movement is slowed in Δ*recD2* cells, supporting the hypothesis that RecD2 is important for replication fork

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progression. Biochemical characterization of *B. subtilis* RecD2 indicates it is a 5´ to 3´ helicase and that it directly binds single-stranded DNA binding protein. Together, our results highlight novel roles for RecD2 in DNA replication, which help to maintain replication fork integrity during normal growth and following DNA damage.

Introduction

Helicases are ATP-fueled molecular machines that remodel nucleic acid polymers in systems ranging from viruses and bacteria to eukaryotic cells [for review [2, 3]]. DNA helicases are responsible for separating duplex DNA into ssDNA segments. This activity is essential for DNA replication and helicases have critical roles in many repair processes including, nucleotide excision repair (NER), homologous recombination and replication fork restart [[4] for review [3, 5-8]]. In accordance with their many roles *in vivo*, helicases show tremendous diversity and have been classified into “superfamilies” based on their sequence motifs [9]. Superfamily 1 (SF1) is comprised of helicases that often function in DNA repair [for review [3, 10]]. Prominent members of this family include *E. coli* UvrD, Rep and RecD [11-13]. The SF1 family is further sub-divided into SF1A and SF1B. SF1A helicases translocate along DNA in the 3´ to 5´ direction, whereas SF1B helicases translocate in the 5´ to 3´ direction [for review [3, 10]].

The SF1B helicases have members present in systems from bacteriophages to human [14-17]. SF1B helicases have clear roles in genome maintenance although members of this family have not been nearly as well studied *in vitro* or *in vivo* as compared with studies of SF1A helicases. Examples of the SF1B helicases include bacteriophage T4 Dda (DNA-dependent ATPase), a DNA replication enzyme; *E. coli* RecD, a critical component of the RecBCD helicase-nuclease enzyme required for end processing during double-strand break repair; and human DNA helicase B (HELB/hDHB), which is recruited into repair foci and enriched on chromatin following challenge with agents that cause replication stress [15, 18, 19]. From these studies, it is

clear that SF1B helicases are important for replication and repair, however many bacterial SF1B helicases have not been studied and their roles in genome maintenance remain unknown.

The bacterial RecD family helicases are represented by RecD and RecD2 [16, 20]. As mentioned above, RecD acts as a subunit in the RecBCD helicase-nuclease complex [6, 21], whereas RecD2 helicases are typically found in bacteria that lack the RecBC proteins [5, 16, 20, 22]. RecD2 is homologous to RecD in the C-terminal domain, however RecD2 is distinct in that it contains a long N-terminal extension that is not present in RecD [16, 20, 22] (Figure 2.1). The RecD2 protein from *Deinococcus radiodurans* has been shown to be important in resistance to gamma irradiation, hydrogen peroxide, and UV irradiation *in vivo* [23] and it is able to unwind short hairpin and forked DNA structures in a 5´ to 3´ direction [24-26].

A genome-wide screen for a colony papillation phenotype in *Bacillus anthracis* identified the gene BAS4289 encoding a RecD2 homolog [22, 27]. Transposon-insertion mutagenesis of *recD2* increased the frequency of spontaneous mutation *in vivo*, which produced a mutation spectrum consistent with a defect in mismatch repair [22]. Unlike *D. radiodurans*, loss of RecD2 function in *B. anthracis* had no effect on UV sensitivity [22]. Therefore, RecD2 in *B. anthracis* appears to function as a mismatch repair helicase and has yet to be shown to contribute to cellular resistance to damage caused by endogenous or exogenous sources [22]. Thus, RecD2 helicases represent a group of highly conserved bacterial helicases that have important roles in diverse DNA repair pathways, however this group has been largely unstudied even though RecD2 is present in over 200 different bacterial species [22].

To better understand the cellular roles of RecD2 proteins, we examined the RecD2 from the Gram-positive bacterium *Bacillus subtilis*. We found that deletion of *recD2* conferred a modest increase in spontaneous mutation rate however the mutation spectrum is inconsistent with a role in mismatch repair. We also found that loss of *recD2* function in *B. subtilis* sensitized cells to mitomycin C, methyl methanesulfonate and the DNA break inducing peptide phleomycin. Further analysis of RecD2 shows that it directly binds single-stranded DNA binding protein (SSB), is a 5´ to 3´ helicase and is important for normal replication fork progression *in vivo*. Together, our data presented here, supports a role for *B. subtilis* RecD2 in limiting replication fork stress during normal growth and in response to DNA damage.

Materials and Methods

Bacteriology

All strains used in this study are derivative of PY79 and are listed in Table 2.1. Unless stated otherwise, isopropylthio-β-galactoside (IPTG) and antibiotics were used at the following final concentrations: 500 μM IPTG, 100 μg/ml spectinomycin (Spc), 5 μg/ml tetracyclin (Tet), 5 μg/ml chloramphenicol (Cat) and 150 μg/ml rifampin (Rif).

All primers used in this study are listed in supporting Table 2.4. The in-frame markerless deletion of *recD2* (BWW150) was created using the procedure as described [28]. Briefly, the upstream and downstream regions of *recD2* were amplified using: oBWW233 and oBWW234; oBWW235 and oBWW236. The upstream region was digested with SalI and BamHI while the downstream region was digested with BamHI and EcoRI. Both regions were ligated into pMiniMAD2 to make pBW98. pBW98 was used to transform MC1061 *E. coli* for propagation using ampicillin for selection generating BWW141. Plasmid pBW98 was subsequently used to transform PY79 at the restrictive temperature to favor a single crossover integration followed by selection for MLS resistance. To evict the plasmid, the strain was incubated in 3 ml LB for 18 hours at 22ºC and diluted back 30 fold in LB, then grown for another 8 hours at 22ºC and diluted back 30 fold. Dilution and subsequent growth was repeated 3 times. Cultures were serial diluted and plated onto LB. Individual colonies were then struck onto LB and LB+MLS to ensure the plasmid had been evicted. The absence of *recD2* was confirmed by diagnostic PCR and the resulting strain designated as BWW150. BWW264 (Δ*recD2*, *recA-gfpA206Kmut2*) was constructed by transformation of BWW150 with of LAS40

(*recA-gfpA206Kmut2)* chromosomal DNA [29] followed by selection for spectinomycin resistance.

Fluorescence microscopy

Fluorescence microscopy was performed essentially as described [29]. Briefly, LAS40 and BWW264 were grown in $S7_{50}$ minimal media + 2% glucose at 37ºC with shaking to OD_{600} ~0.5. Cultures were split and phleomycin was added to 50 ng/ml. Split cultures were allowed to incubate another 30 minutes prior to imaging. Membranes were stained with TMADPH (1:1000 dilution). Cells were imaged on using an Olympus BX61 microscope as described [30-33]. RecA-GFP foci were visualized by exposure for 200 ms and membranes were imaged for 25 ms. RecA-GFP foci were scored using Adobe Photoshop.

Mutation rate analysis

A single colony was used to inoculate 3 ml of LB and grown at 37˚C until it reached an OD_{600} of 1.0. The culture was then diluted 1:1000 in LB and multiple 3 ml tubes of the newly inoculated dilute culture were grown to an OD_{600} of 0.8-1.2. At this step, 1 ml of cells was harvested by centrifugation for 3 minutes at 10,000 RPM. The supernatant was aspirated and cells were resuspended in 0.85% saline. From the initial saline resuspension, a portion was plated onto LB plates containing 100 µg/ml rifampin and the same volume of cells from the 10^{-6} dilution was plated onto LB. The plates were incubated overnight, with LB-rifampin plates incubated at 37˚C and LB plates incubated at 30˚C, and scored the following morning.

The trimethoprim resistance assay was performed similarly to the rifampin assay as described above except cells were grown in LB supplemented with 200 μM thymidine and plated at different dilutions [34]. A portion of the 10^{-6} dilution was plated on LB +200 μM thymidine, while an equal portion of the 10^{-1} dilution was plated on minimal media plates [1% glucose 50%, 1X $S7_{50}$, 0.1% glutamate, 0.2% casamino acids, 0.1 μM tryptophan, 0.1 μM phenylalanine, 0.2 μM thymidine, 34 μM trimethoprim and 1X metals] similar to [35] with addition of trimethoprim. Trimethoprim containing plates were incubated for about 20 hours, LB+thymidine at 30˚C and trimethoprim minimal media at 46˚C, and counted.

Mutation spectrum

The mutation spectrum was generated essentially as described [35]. Briefly, 50 independent cultures were grown in LB+ 200 μM thymidine and grown to OD_{600} of about 0.8, followed by plating and growth on minimal agar containing trimethoprim. A single colony was then removed and colony purified followed by PCR amplification of the *thyA* gene using the following primers oSAB14, oSAB15, oSAB17, and oSAB18. The sequencing results were analyzed using Sequencher.

Purification of *B. subtilis* RecD2 and SSB

The *recD2* gene was amplified using oBWW204 and oBWW205 followed by digestion with BamHI and XhoI and ligation into pET28T resulting in plasmid pBW98. pBW98 was used to transform $BL21_{DE3}^{recA-}$ *E. coli* to generate BWW102 for overexpression of 6xHis-RecD2. 6xHis-RecD2 was overexpressed using standard

procedures [33]. Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% sucrose, 20 mM spermidine trihydrochloride). Lysozyme was added to 0.4 mg/ml and lysis was allowed to proceed for 2 hours on ice. Lysate was prepared by centrifugation at 15,000 RPM for 60 min at 4ºC. Supernatants were collected and applied to a HisTrap FF Crude column equilibrated with buffer A (20 mM Tris-HCl pH 7.6, 500 mM NaCl, 20 mM imidazole, 10% glycerol, 1 mM DTT). RecD2 was purified using a 20 column volume gradient from buffer A to buffer B (20 mM TrisHCl pH 7.6, 500 mM NaCl, 500 mM imidazole, 10% glycerol, 1 mM DTT). Fractions containing RecD2 protein were determined by SDS-PAGE and dialyzed with cleavage buffer (20 mM Tris-HCl pH 7.6, 150 mM NaCl, 10% glycerol, 1 mM DTT) overnight at 4ºC. The 6xHis tag was cleaved by addition of 250 ng Prescission protease to cleavage buffer and overnight dialysis at 4ºC. Prescission protease and uncleaved 6xHis-RecD2 was removed by gravity drip chromatography over Ni-NTA agarose beads and reduced glutathione agarose beads. RecD2 was further purified by anion exchange chromatography using a HiTrap Q HP equilibrated with buffer QA (20 mM Tris-HCl, pH 7.6, 20 mM NaCl, 1 mM DTT, 1 mM EDTA, and 10% glycerol) and eluted over a gradient from buffer QA to QB (20 mM Tris-HCl, pH 7.6, 1 M NaCl, 1 mM DTT, 1 mM EDTA, and 10% glycerol). Fractions containing pure protein were identified by SDS-PAGE followed by dialysis into storage buffer (20 mM Tris HCl, pH7.6, 150 mM NaCl, 1 mM DTT, 50% glycerol), frozen in liquid nitrogen and stored at -80ºC.

RecD2(K373A) plasmid was generated by site directed mutagenesis using pBW98 as a template and oligos oBWW237 and oBWW238. Overexpression and purification for RecD2(K373A) was as described above for wild type RecD2.

The *B. subtilis ssbA* gene (referred to here as *ssb*) was amplified using oBWW63 and oBWW64. This fragment was digested with BamHI and XhoI and ligated into pET28T to generate pBW18 as described [36]. Plasmid pBW18 was used to transform *E. coli* $BL21_{DE3}^{recA-}$ cells resulting in strain BWW18. The 6xHis-SSB was overexpressed the tag cleaved and the protein purified in the same manner as RecD2 described above. For both SSB and RecD2 PreScission protease cleavage of the histidine tag leaves the sequence GPGS on the N-terminus of the protein (GE Healthcare Life Sciences).

DNA helicase assay

3′ and 5′ overhang substrates were made as follows: oSW079 was phosphorylated by T4 polynucleotide kinase with [γ-^{32}P]ATP, annealed to oSW080 (for 3′ overhang) or oSW081 (for 5′ overhang), resolved by 10% native PAGE, and purified by electroelution. DNA substrates were subsequently dialyzed against 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$. DNA substrates (~1 nM) were incubated in 50 mM HEPES-HCl, pH 7.5, 2 mM DTT, 2 mM ATP, 4 mM $MgOAc_2$, 40 g/l bovine serum albumin, and 1% glycerol with 0-2 nM RecD2 at 37°C for 25 min. Reactions were terminated by the addition of 20 mM EDTA, 0.5% SDS, 0.2 mg/ml proteinase K, and 2.5 ng/μl of oSW079. Samples were resolved on a 10% native polyacrylamide gel. The gel was then fixed in 10% methanol, 7% acetic acid, and 5% glycerol, dried and exposed to a phosphorimager screen and imaged on a Typhoon FLA 9000. Band intensities were quantified using ImageQuant (GE Healthcare) and percent unwinding was determined by dividing the intensity of the single strand product band by the total intensity in the lane.

In vivo replication fork impediment assay

Replication initiation and replication fork restart were halted using a temperature sensitive allele of the helicase loader (*dnaB134*) as previously described [29, 37]. Cells were grown in $S7_{50}$ minimal medium to an OD_{600}~0.4, at which time they were shifted to the nonpermissive temperature (45°C). After 45 minutes, 25 ml ice-cold methanol was added to 25 ml of culture and the cells were collected by centrifugation and genomic DNA purified. DNA was submitted to the University of Michigan DNA Sequencing Core for library preparation and 50-base single-end Illumina sequencing. Reads were aligned to our laboratory PY79 reference genome using bwa v0.5.9-r16 with the default parameters, except when running bwa samse, when we set the –n parameter to 1. Subsequent analysis was performed using the statistical package R. Alignment data were binned into 500 base wide non-overlapping windows and $\log_2$(read depth) at each window was calculated. In order to assess replication fork stress, the data from each arm of the PY79 chromosome were separately fit to a quadratic model ($y = a + bx + cx^2$) where a is the y-intercept, b relates to the initial rate of replication fork progression, and negative c is the fork collapse factor. The fork collapse factor provides a quantitative measure of replication fork collapse along the genome under conditions in which collapsed replication forks will not re-initiate DNA replication.

Results and discussion

B. subtilis RecD2 has a role in limiting spontaneous mutagenesis

The RecD2 helicase has been shown to function in mismatch repair in *B. anthracis* [22]. To understand if *B. subtilis recD2* also functioned in mismatch repair, we constructed a clean deletion of the *recD2* (*yrrC*) coding region as previously described (Materials and Methods, [28, 33]. The mutation rate of the Δ*recD2* strain was measured and compared to wild type *B. subtilis* and a strain deleted for the MMR genes *mutS* and *mutL* (Δ*mutSL*). In *B. anthracis* loss of *recD2* increased mutation frequency ~40-fold in an assay that measures formation of rifampin resistant colonies [22]. In contrast to these results, we found that the Δ*recD2 B. subtilis* had a far more modest (2.8-fold) increase in mutation rate as measured by formation of rifampin resistant colonies (Figure 2.2A).

Because rifampin resistance measures base-pair substitutions in the *rpoB* gene and does not measure insertions or deletions [35, 38, 39], we developed an assay that was sensitive to additional types of mutations to measure spontaneous mutagenesis in *B. subtilis* by scoring for trimethoprim resistance [34, 40]. Trimethoprim inhibits dihydrofolate reductase thereby decreasing concentrations of tetrahydrofolate, a critical cofactor for cellular metabolism [40]. The levels of tetrahydrofolate are further depleted by the enzyme thymidylate synthase (*thyA*), which requires tetrahydrofolate for activity [41]. Therefore, base-pair substitutions, insertions or deletion mutations that inactive *thyA* provide enough tetrahydrofolate to allow for trimethoprim resistance and growth in the presence of thymidine [41]. *B. subtilis* has two thymidylate synthetase genes, *thyA* and *thyB,* both of which need to be nonfunctional to cause trimethoprim resistance [42].

We took advantage of the observation that the *thyB* gene in *B. subtilis* encodes a naturally temperature sensitive protein allowing for an assay to identify mutations in the *thyA* that cause trimethoprim resistance at elevated temperatures when ThyB is inactive (see Materials and Methods). We found that Δ*mutSL* increased mutation rate 20-fold over wild type and the Δ*recD2* conferred a 3.6-fold increase in mutation rate (Figure 2.2B). These results show that loss of *recD2* causes a modest increase in mutation rate, although it is unclear if the increase in mutation rate is due to a decrease in mismatch repair efficiency or by effects on other cellular pathways.

To examine whether the mutations resulting in trimethoprim resistance in the Δ*recD2* strain result from impaired efficiency of mismatch repair, we determined the mutation spectrum for trimethoprim resistance in the *thyA* gene from at least 50 independent colonies for each strain tested. We found that Δ*recD2* and the Δ*mutSL* strains showed very different mutation spectra (Figure 2.3; see Table 2.5 in the supplemental material). The Δ*mutSL* spectrum consisted of transitions, insertions, and deletions, but not transversions, which is a spectrum indicative of an MMR [22, 35, 43, 44]. In contrast the Δ*recD2* mutation spectrum showed an increase in transversion mutations in addition to transitions, insertions and deletions (Figure 2.3; see table 2.5 in the supplemental material). Considering the ~4-fold increase in mutation rate and the fact that the *thyA* spectrum of the Δ*recD2* strain provided a mutational signature different from that for the Δ*mutSL* strain, we conclude that the loss of RecD2 causes a mild increase in mutagenesis through an MMR-independent mechanism or multiple mechanisms.

A possible explanation for the increase in transversion mutations in the Δ*recD2* strain is through activation of "error prone" DNA polymerases. DNA polymerases specialized in lesion bypass, including the Y-family DNA polymerases, are prone to insertion of transversion mutations [45-48]. Therefore, we tested the idea that the mutagenesis observed in the Δ*recD2* strain is caused by increased usage of lesion bypass DNA polymerases in the absence of RecD2. In *B. subtilis* polymerases Pol Y1 (YqjH), PolY2 (YqjW) and essential replicative polymerase DnaE have been shown to be involved in lesion bypass *in vitro* or *in vivo* [45-49]. Since DnaE is essential [50, 51], we cannot test a *dnaE* deletion to determine if loss of *dnaE* relieves the mutagenesis observed in Δ*recD2* cells. We did however test alleles deficient for *polY1* and *polY2* in the Δ*recD2* background. We found that loss of *polY1*, *polY2* or both led to mutation rates that were within error of the Δ*recD2* strain (Figure 2.2C). Therefore, because disruption of the Y-family polymerases did not reduce the Δ*recD2* conferred mutagenesis we speculate that DnaE may be responsible for the moderate mutagenesis observed in the absence of RecD2 helicase.

To test the involvement of DnaE, we and others have shown that an ectopically expressed DnaE-GFP fusion protein forms foci at replication centers *in vivo* [32, 52]. Since the Y-family polymerases were not involved in the mutagenesis, we asked if the percentage of cells with DnaE-GFP foci were elevated in cells deleted for Δ*recD2*. Indeed, we found that the percentage of cells with DnaE-GFP foci were increased in cells deleted for *recD2* (Table 2.2). Although we are unable to assay DnaE directly for a role in the mutagenesis caused by loss of RecD2 the increase in the percentage of cells with DnaE-GFP foci supports the hypothesis that DnaE could be used to bypass lesions

encountered *in vivo* possibly contributing to the mutation spectrum observed in the absence of RecD2 (Figure 2.3 and Table 2.2).

It has been shown previously that loss of *B. anthracis recD2* increases mutagenesis ~40-fold and that the mutation spectrum consists of transitions supporting a role for *B. anthracis* RecD2 in mismatch repair [22]. Our results, in consideration with the data from *B. anthracis* [22], shows that RecD2 functions differently in *B. subtilis* as compared with *B. anthracis*. Because *B. subtilis* RecD2 does not have a role in mismatch repair, the experiments described below were performed to understand if RecD2 is important for replication or repair in *B. subtilis*.

RecD2 is important for survival after DNA damage

Because *D. radiodurans* RecD2 and human DNA Helicase B are important for resistance to agents that perturb replication [18, 23], we asked if Δ*recD2 B. subtilis* cells were sensitive to DNA damage. We performed spot plate assays where an equal amount of cells were plated on LB agar containing methanesulfonate, mitomycin C, phleomycin, or hydrogen peroxide (H_2O_2) (Figure 2.4A). In addition, we also performed a spot plate analysis where cells were serial diluted followed by challenge with UV-irradiation (Figure 2.4A).

As controls for the assay, we used strains with the *recA::neo* or *uvrA::spc* alleles, which cause defects in homologous recombination and nucleotide excision repair [for review [5]]. Consistent with earlier work, and the established role of UvrA in nucleotide excision repair (NER), the *uvrA*::*spc* strain was sensitive to UV and mitomycin C treatments, but not methyl methanesulfonate [53] (Figure 2.4A). Treatment

with H_2O_2 generates small 8-oxo-G lesions following exposure, while UV exposure generates pyrimidine dimers, which can cause replication stress (for review [54]). Methyl methanesulfonate primarily adds methyl groups to guanine and adenine bases (forming 7-methylguanine and 3-methyladenine, respectively), which damages DNA and can cause severe blocks to replication [55]. In *B. subtilis*, we found that the Δ*recD2* strain was also sensitive to mitomycin C. The cross-links that form as a result of mitomycin C treatment are repaired by NER and homologous recombination (for a review, see reference [56]) and are known to block replication in *B. subtilis* cells [57].

We challenged cells with phleomycin to generate single- and double-stranded breaks and found that the Δ*recD2* allele conferred sensitivity. As a control, we showed that *recD2* ectopically expressed from an IPTG-regulated promoter complements the phleomycin sensitivity of Δ*recD2* cells (Figure 2.4B). These results show that Δ*recD2* cells are sensitive to the bulky N^2 adducts generated by mitomycin C, single- and double-stranded breaks generated by phleomycin, and replication-blocking alkylation and UV damage. We conclude that the *B. subtilis* ∆*recD2* strain is sensitive to a wide range of damaging agents that can impose blocks to replication. Furthermore, the *B. subtilis* strain with the *recD2* deletion showed very little overlap in function to that of a strain of either *D. radiodurans* or*B. anthracis* with *recD2* deficiency. We discuss these differences in greater detail below.

Cells with the *recD2* deletion show replication fork stress

The results presented above suggest that *B. subtilis recD2* has some role in genome maintenance when cells experience DNA damage. Prior work reported that

ectopic expression of YFP-RecD2 (YrrC) colocalized with the replisome in *B. subtilis* [[58] and for review [5]]. This result suggests that RecD2 may be present at the fork during normal growth. To determine the subcellular localization of RecD2 we fused *recD2* to several different C-terminal fluorescent protein fusions followed by imaging in cells damaged or left untreated. RecD2 with a C-terminal fusion expressed from its native promoters did not form distinct foci that we could observe by standard epifluorescence microscopy (data not shown). Therefore, we tested whether the ability of RecA to form foci in response to endogenous and exogenous sources of DNA damage was influenced by the presence of absence of RecD2.

We have previously shown that RecA-GFP foci formation is an *in vivo* marker for replicative stress in *B. subtilis* [29, 32, 59, 60]. We define replicative stress as any perturbation to the replication fork that increases exposed ssDNA. Thus, we imaged RecA-GFP in the Δ*recD2* strain and found that the percentage of cells with RecA-GFP foci were elevated nearly 2-fold relative to the wild type strain (14% to 25%) ($p<0.001$) (Table 2.3). Following DNA damage with phleomycin, an agent that the Δ*recD2* strain was sensitive to, we again observed a nearly 2-fold elevation in the percentage of cells with RecA-GFP foci (20% and 38% of cells respectively) (Table 2.3). These results suggest that in the absence of *recD2* more single-stranded DNA is generated at the replication fork causing an increase in the RecA-GFP localization response. This result is indicative of DNA replication fork stress in *B. subtilis*.

As a direct means of observing replication stress, we measured the rate at which replication forks collapse using whole-genome sequencing under conditions preventing re-initiation of replication. Exponential-phase *B. subtilis* cells display a linear decrease in

log_2-transformed sequencing coverage with a peak at the origin of replication in whole genome sequencing experiments (Figure 2.5A). However, we arrested DNA replication initiation for 45 minutes prior to harvesting genomic DNA for sequencing. This causes the decrease in coverage moving away from the origin of replication no longer to be linear, but rather to be quadratic in nature (Figures 2.5B-D), curving increasingly downward from the origin. The coefficient describing the severity of the curve determined by the rate of replication fork collapse is defined here as the fork collapse factor (FCF) (see "Materials and Methods"). Upon fitting the log_2-transformed coverage data to a quadratic model, we found that the Δ*recD2,* data yielded a more extreme fork collapse factor (FCF) than did the control, indicating that replication forks collapsed more frequently in the strain lacking RecD2 (Figures 2.5D and 2.5E). We conclude that this novel method of observing replication fork collapse strongly suggests that RecD2 stabilizes or aids in normal replication fork progression in *B. subtilis*.

RecD2 binds SSB and is a 5´ to 3´ helicase

The *B. subtilis* RecD2 helicase was originally identified as an interaction partner of the single-stranded DNA-binding protein (SSB) [58]. To determine if RecD2 directly binds SSB, we overexpressed and purified RecD2 and SSB and performed an immunodot blot to probe for interaction between RecD2 and SSB. We spotted RecD2, DnaG (positive control as a known SSB-interacting protein [61]) and BSA (negative control) in increasing amounts on a nitrocellulose membrane and incubated with SSB; SSB was detected using an SSB antiserum as described [32, 33, 62] (Figure 2.6A). We found that RecD2 and DnaG both retained SSB on the membrane, whereas BSA did

not. We performed the reciprocal experiment and spotted SSB while probing with RecD2 and affinity-purified antibodies against RecD2. We found that SSB retained RecD2, whereas BSA did not. Thus the RecD2/SSB interaction appears to be direct. Interestingly, our results are similar to results found in human cells, where several agents that cause replication fork stress cause the SF1B helicase DHB to localize to chromatin through interaction with replication protein A (RPA), the eukaryotic analog to bacterial SSB [18]. This work did not find that hDHB was important for normal fork progression; however, our work does show that *B. subtilis* RecD2 is indeed important for fork progression in untreated cells.

RecD2 helicases in other organisms have been shown to unwind DNA in the 5′ to 3′ direction, meaning that they preferentially unwinding partial duplex DNA structures with a 5′ single-stranded tail (55, 67). Using an *in vitro* helicase assay, we found that *B. subtilis* RecD2 was also a 5′ to 3′ helicase. *B. subtilis* RecD2 unwound a 5′ tail substrate in an enzyme-concentration-dependent manner but was not active on a 3′ tail substrate (Figure 2.6B, compare lanes 6 and 7). DNA unwinding increases as the RecD2 concentration is increased from 0 to 2 nM, with 60% of substrate unwound at the highest concentration tested (Figure 2.6B lanes 1-6, Figure 2.6C). A RecD2 K373A variant, which is predicted to be defective in ATP hydrolysis, has no detectable helicase activity (Figure 2.6B, lane 8). Our data indicate that RecD2 is an ATP hydrolysis-dependent 5′ to 3′ helicase.

Plasticity of RecD2 helicases in bacterial organisms

RecD2 helicases are commonly found in bacteria that lack the RecBCD enzyme.

Our study of *B. subtilis* RecD2 has shown that RecD2 is important for resistance to several DNA damaging agents that can result in replication fork stress including bulky adducts, alkylation damage and strand breaks. Studies of RecD2 helicases from *B. anthracis* and *D. radiodurans* identified roles for RecD2 in mismatch repair and resistance to oxidative and UV damage [22, 23]. Taking these studies into consideration, it appears that RecD2 helicases are important for genome integrity but that the precise roles for RecD2 enzymes vary considerably between bacterial species. These observations underscore the impressive plasticity of RecD2 helicases across different organisms. Evolutionary studies suggest that *recD2* is the ancestor to *recD* [16]. If so, RecD2 may have adapted to function in different repair pathways where 5´ to 3´ helicase activity was most advantageous. We suggest that even though RecD2 is highly conserved and present in hundreds of bacterial species, the specific role of RecD2 to genome integrity will differ considerably between organisms as the helicase has diverged and become specialized based on the challenges each organism encounters during their replication cycle.

It was recently shown that *D. radiodurans* RecD2 causes collapsed *E. coli*replication forks to fail to reactivate *in vitro* and *in vivo* [63]. Interestingly, *D. radiodurans* RecD2 overexpressed in *E. coli* was lethal to Δ*rep* helicase mutants. Our work shows that *B. subtilis* RecD2 is important for normal fork progression and that forks collapse more frequently in the absence of RecD2. We also show that the loss of *recD2* sensitizes cells to DNA damage and increases replication fork stress. Both studies show that RecD2 in the native or in a heterologous system functions at replication forks. Taking these results into consideration along with those of our study,

we suggest that RecD2 is important for fork maintenance at native levels and RecD2 may become deleterious for fork reactivation when expression is elevated, perhaps bypassing factors that regulate RecD2 activity or access to 5′ DNA substrates *in vivo*.

Figures

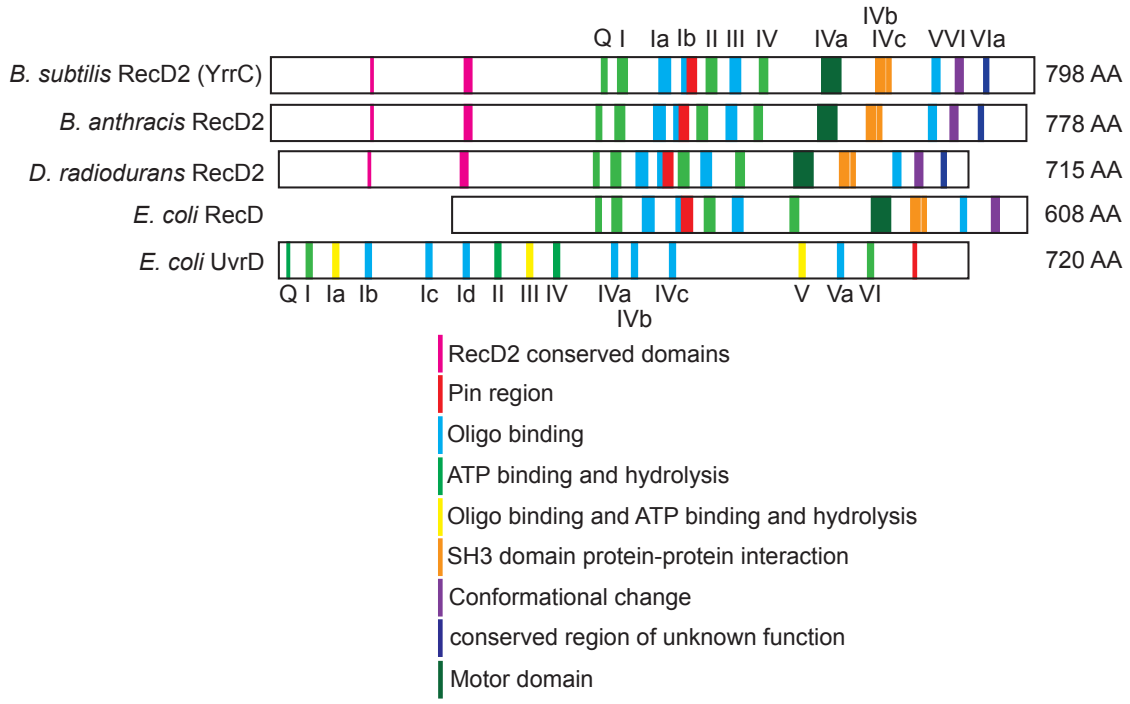


Figure 2.1. Functional domain alignment of RecD, RecD2 and UvrD. Sequences were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) as described [64]. Shown is a schematic representation of the indicated helicase domains. Conserved helicase domains and functions were assigned accordingly [17, 22, 26, 65]. *B. subtilis* and *B. anthracis* RecD2 share 57% amino acid identify and 74% homology, whereas *B. subtilis* and *D. radiodurans* RecD2 share 28% amino acid identity and 50% homology.

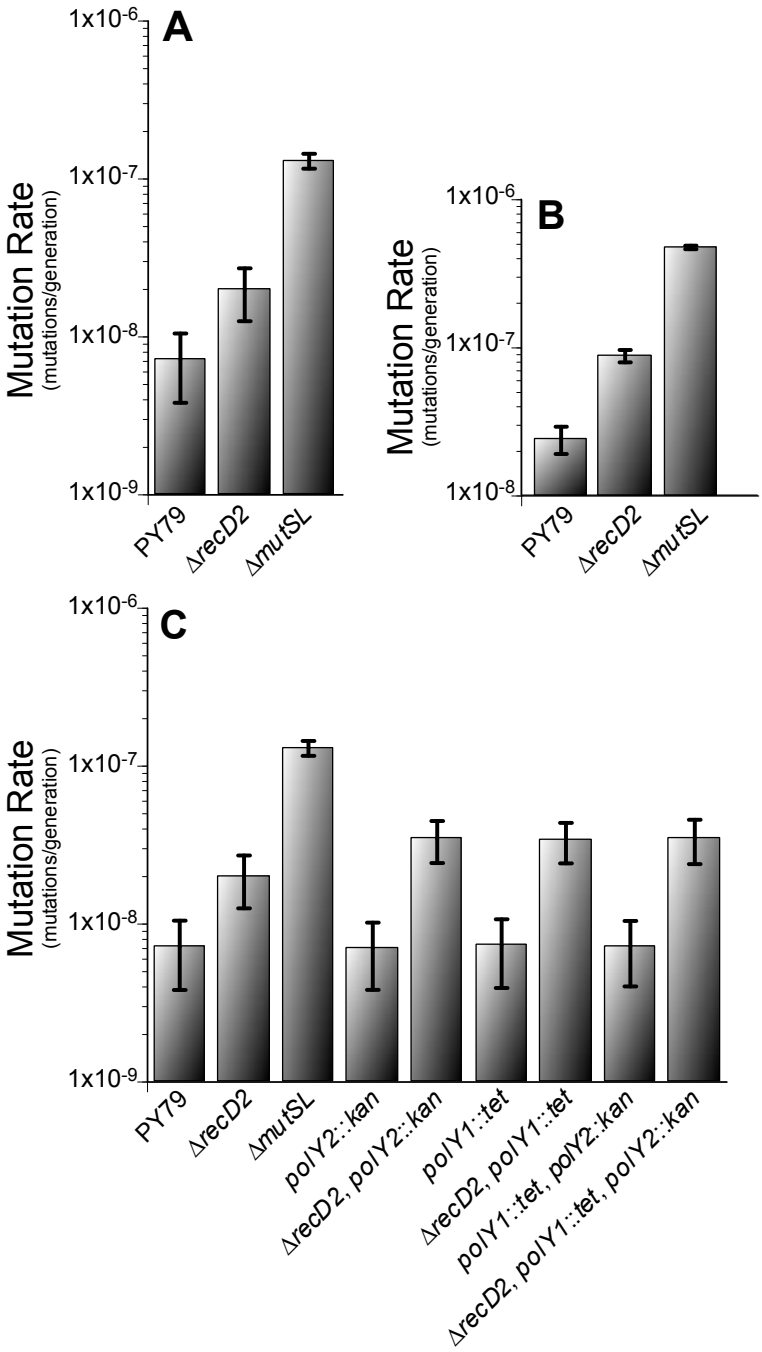


Figure 2.2. Deletion of *recD2* increase spontaneous mutagenesis in *B. subtilis*. Shown are bar graphs representing spontaneous mutation rate (10^{-9} mutations per generation± 95% confidence intervals) using the MSS Maximum Likelihood Method as described [33, 66-69]. **(**A) Mutations per generation for the wild type strain PY79, Δ*recD2*, and Δ*mutSL* when plated on rifampin from at least 50 independent cultures; (B) mutations per generation of PY79, Δ*recD2*, and Δ*mutSL* strains when plated on trimethoprim from at least 20 independent cultures; (C) mutations per generation of indicated strains when plated on rifampin. The data for PY79, D*recD2* and Δ*mutSL* shown in A are also shown in C.

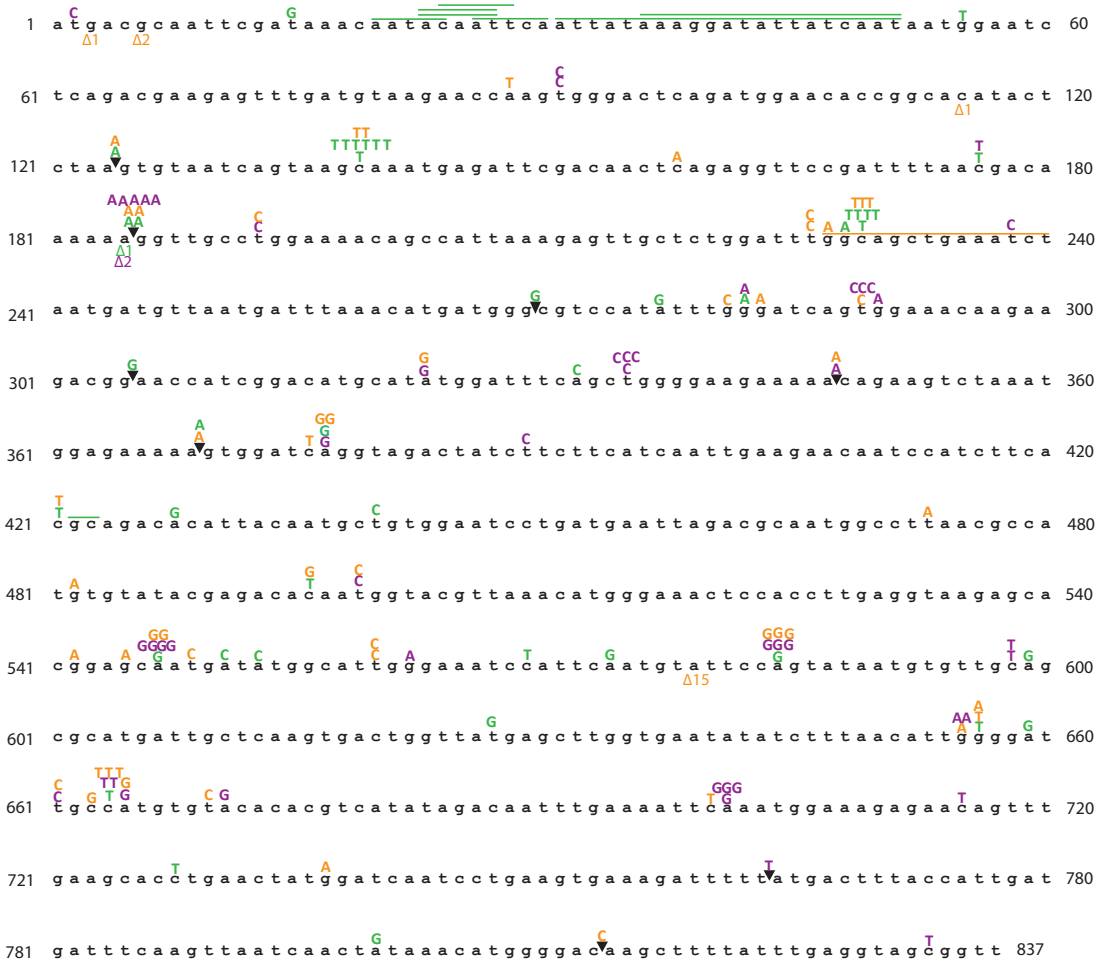


Figure 3.

Figure 2.3. Trimethoprim mutation spectra of *B. subtilis* wild type, Δ*recD2* and Δ*mutSL* cells. The DNA sequence of the *thyA* gene of *B. subtilis* strain PY79 is shown. Wild type (green), Δ*mutSL* (purple), and Δ*recD2* (orange) spectra are shown above the sequence. Filled triangles correspond to insertion events, open triangles correspond to deletions and a solid line demarks duplications. The data shown here are also presented in Table 2.5 in the supplemental material.

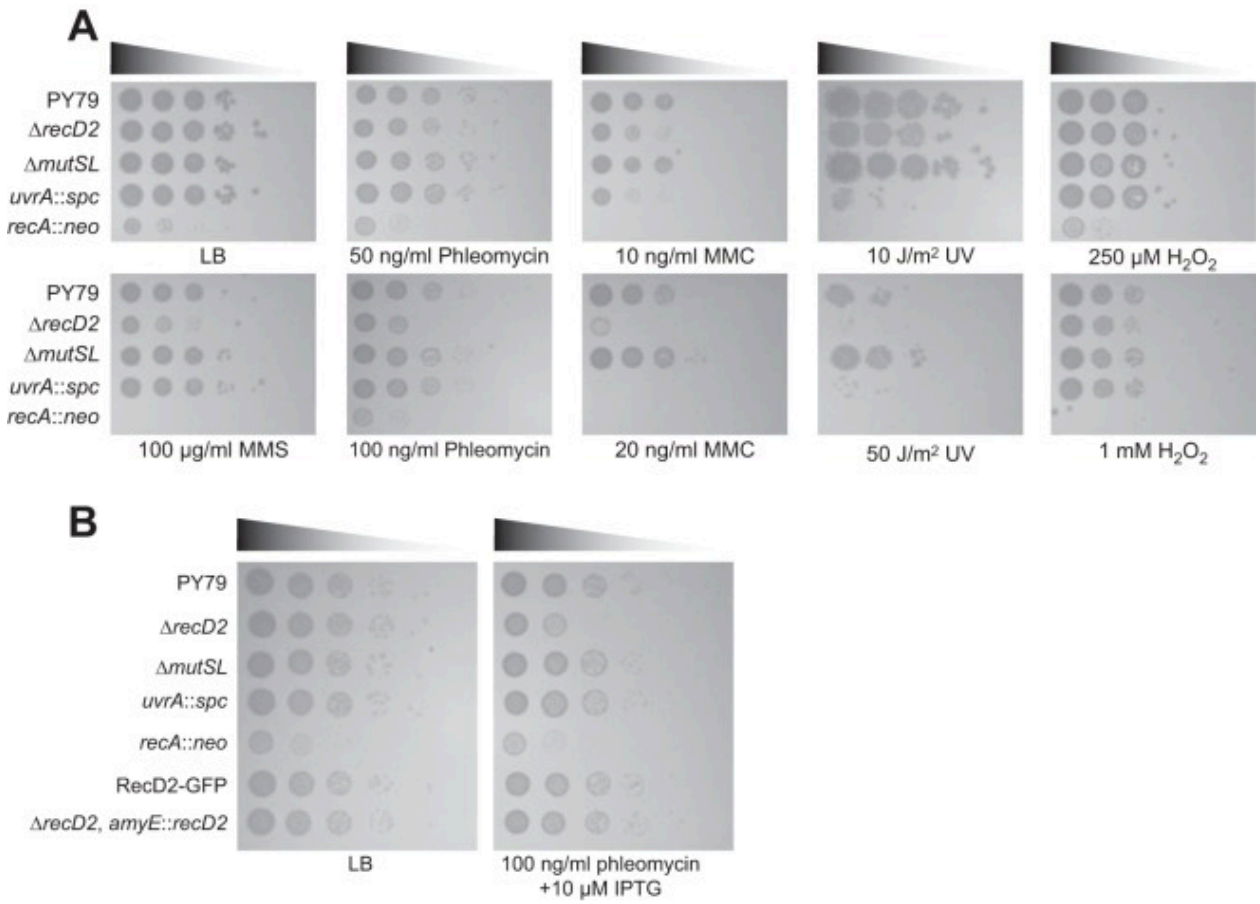
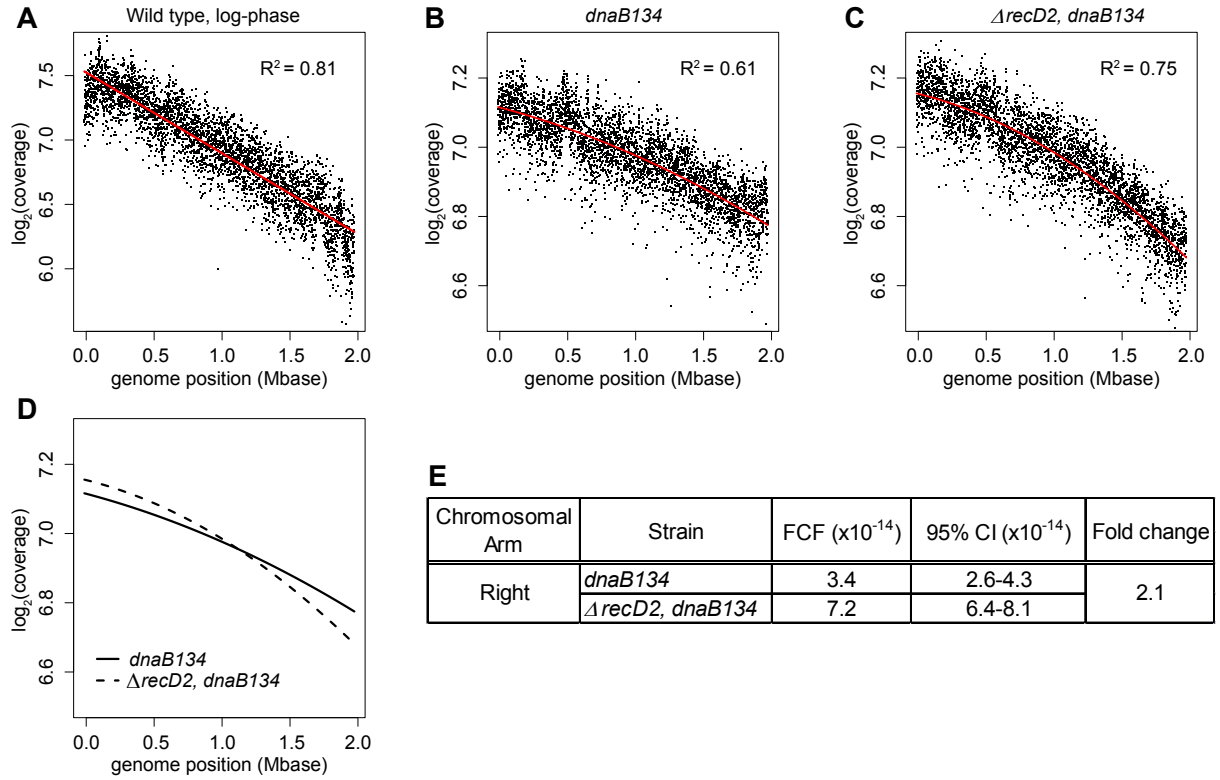


Figure 2.4. The *recD2* deletion confers sensitivity to mitomycin C (MMC), methyl methanesulfonate (MMS), **phleomycin and UV. (**A) Serial dilutions of the indicated strains were plated on LB agar or LB agar with the indicated DNA damaging agent. (B) Complementation of Δ*recD2* with ectopic expression of *recD2* from the *amyE* locus with the IPTG inducible promoter (P_{spank}). Serial dilutions of the indicated strains were plated on LB agar, LB agar with phleomycin, or LB agar with phleomycin and 10 μM IPTG.



E

Chromosomal Arm	Strain	FCF ($x10^{-14}$)	95% CI ($x10^{-14}$)	Fold change
Right	*dnaB134*	3.4	2.6-4.3	2.1
	ΔrecD2, dnaB134	7.2	6.4-8.1	

Figure 2.5. RecD2 stabilizes ongoing replication. (A) Coverage data from the right arm of the chromosome of an exponential-phase wild type culture is plotted with the red line denoting a linear fit to the data. (B) Replication initiation was halted in *dnaB134* cells for 45 minutes followed by sequencing of genomic DNA. Log_2(coverage) of the right arm is plotted (black dots) and the red line shows a quadratic fit to the data. The data are the result to two independent experiments. (C) Same as in B, except the genotype is Δ*recD2, dnaB134.* The data are the result of two independent experiments. (D) The quadratic fits from B (solid line) and C (dashed line) are plotted together for comparison. (E) A table showing the fork collapse factor (FCF) for the right arm of the chromosome in each strain tested.

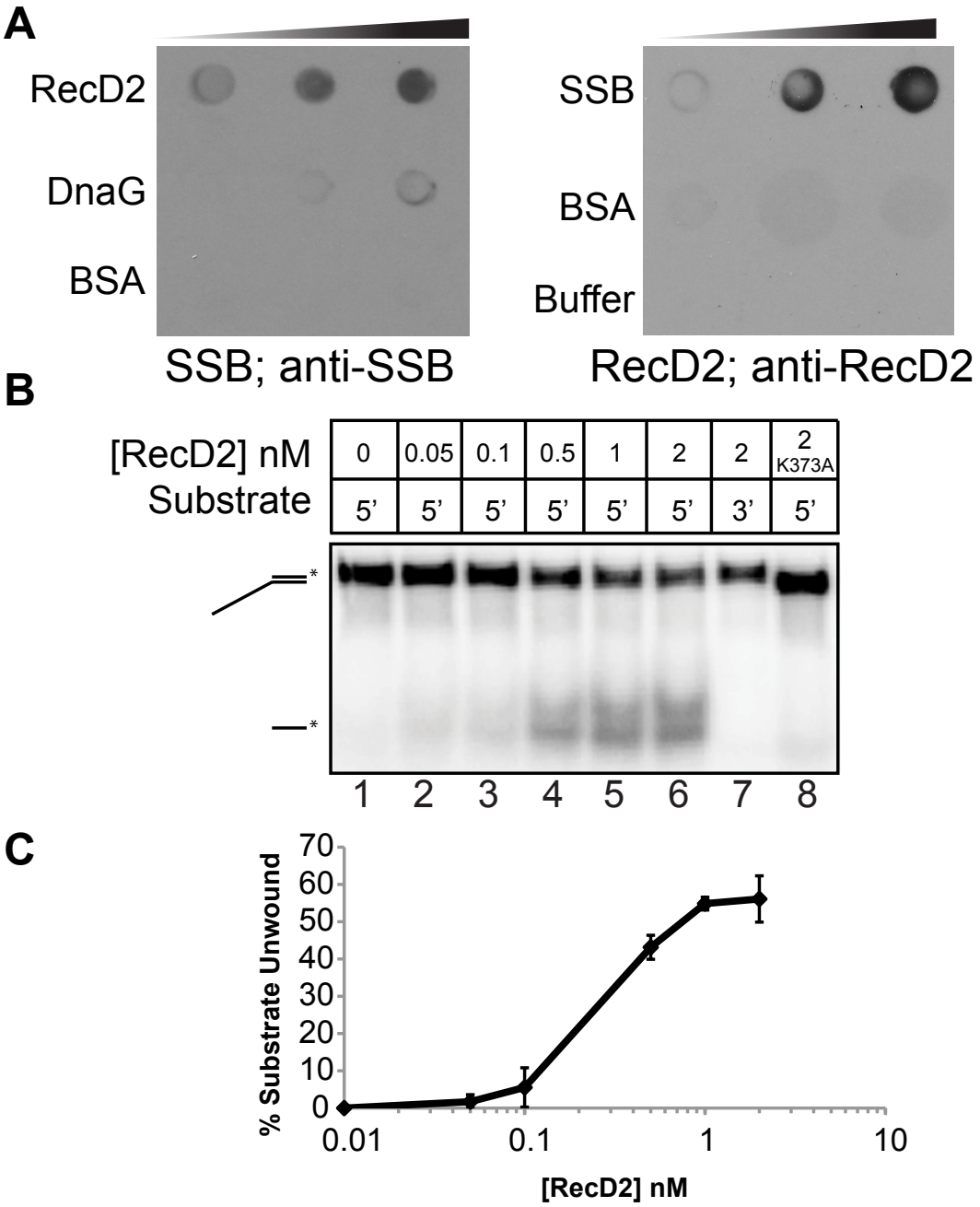


Figure 2.6. RecD2 binds SSB and is a 5´ to 3´ helicase. (A) Immuno-dot blot of the RecD2 interaction with SSB. Each protein was serially diluted onto a nitrocellulose membrane and then incubated with either SSB (left) or RecD2 (right). The membrane was subsequently probed with polyclonal affinity-purified anti-SSB or anti-RecD2 antibodies, as described in Materials and Methods [32]. (B) RecD2 unwinding of 5′ or 3′ tail-containing DNA substrates. RecD2 (0 to 2 nM) or RecD2(K373A) (2 nM) was incubated with the indicated DNA substrate for 25 min. (C) Quantification of substrate unwinding by RecD2. Percent unwinding by RecD2 was determined by dividing the intensity of the single-strand product band by the total intensity of the lane.

Table 2.1. List of *B. subtilis* strains

Strain	Relevant Genotype	Reference(s)
PY79	SPβ^{o}	[70]
LAS4	*yqjW*::*kan*	[35, 47]
LAS5	*yqjH*::*tet*	[35, 47]
LAS24	*recA*::*neo*	[71]
LAS40	*recA-gfpA206Kmut2*	[29]
LAS409	*uvrA*::*spc*	[53]
BWW132	Δ*mutSL*	[72]
AK74	*amyE::P$_{spac}$dnaE-gfp*	[32]
BWW150	Δ*recD2*	
BWW264	Δ*recD2*, *recA-gfpA206Kmut2*	
BWW281	Δ*recD2, yqjW*::*kan*	
BWW282	Δ*recD2, yqjH*::*tet*	
BWW283	Δ*recD2, yqjH*::*tet, yqjW*::*kan*	
BWW307	*amyE*::P$_{spac}$*dnaE*-GFP, Δ*recD2*	
JWS162	*dnaB134 zhb83::Tn917 (tet)*	[5]
JWS194	Δ*recD2, dnaB134 zhb83::Tn917 (tet)*	

All strains used are derivatives of PY79.

Table 2.2. The percentage of cells with DnaE-GFP foci is elevated in cells lacking RecD2 helicase.

Strain	Condition	Percentage of cells with foci+95%CI	One-tailed p-value
P_{spac}*dnaE-gfp*	untreated	42±2.5 (n=2,072)	
Δ*recD2,* P_{spac}*dnaE-gfp*	untreated	54±2.4 (n=2,587)	$1.7E^{-12}$

Cells were grown in $S7_{50}$ defined minimal medium to an OD_{600} of 0.4-0.6 with 1% arabinose and 0.125% xylose prior to treatment. Cultures were left untreated prior to imaging. Cell membranes were stained with TMA-DPH as described [33, 69]. Above, we present the percentage of cells with foci ± the 95% confidence interval. The number *n* in parenthesis represents the total number of cells scored from at least six independent experiments. The one-tailed p-value represents a comparison of Δ*recD2,* DnaE-GFP cells to wild type DnaE-GFP cells ($1.7E^{-12}$)

Table 2.3. RecA-GFP foci are elevated in cells with a *recD2* deletion

Strain	Condition	Total cells scored	Percentage of cells with foci	Two-tailed p-value
recA-gfp	untreated	460	14±3	
Δ*recD2, recA-gfp*	untreated	508	25±4	$5.16E^{-6}$
recA-gfp	phleomycin	502	21±4	$1.97E^{-3}$
Δ*recD2, recA-gfp*	phleomycin	607	39±4	$1.86E^{-19}$

Cells were grown in $S7_{50}$ defined minimal medium as described [29, 31, 32, 59, 60]. After cells reached an OD_{600} of 0.4, cultures were slit with 50 ng/ml phleomycin added to one culture while the other was left untreated. Cultures were allowed to continue growth for 30 minutes followed by imaging. The data shown above represents cells from at least two independent cultures on separate days.

Two-tailed p-values represent the difference between the indicated strain and the *recA-gfp* untreated control. In addition the difference between *recA-gfp* and Δ*recD2*, *recA-gfp* for the phleomycin challenged samples were significant with $p=2.13X10^{-6}$.

Supplemental Information

Results

It has been previously shown *D. radiodurans* cells deleted for *recD2* have an increase in transformation efficiency, suggesting that *D. radiodurans* RecD2 may unwind crossover species during transformation [53]. To investigate a possible role for *recD2* in decreasing transformation of competent *B. subtilis* cells, we tested Δ*recD2* for effects on integration of a plasmid into the *amyE* locus by transformation (data not shown). As a positive control we used a strain deficient in the repressor of *comK* (*rok*$^-$) [73], which increases transformation frequency and we deleted *comK*, as a negative control, which prevents transformation [74, 75]. We found that cells deleted for *recD2* were wild type for transformation indicating that *recD2* does not affect genetic transformation in *B. subtilis* (data not shown).

Table 2.4 Primer sequences used

Primer	Sequence
oBWW63	cgcggatccatgcttaaccgagttgtattagtcggaaga
oBWW64	ccgctcgagctagaatggaagatcatcatccgagatgtcaat
oBWW204	cgcggatccgtgcagcagcatccggatcagcttaaactg
oBWW205	ccgctcgagttattgctgttcttctttcataaaatcgaatgg
oBWW233	acgcgtcgacaagcgcttgaattagacagtagcgc
oBWW234	cgcggatccgagctgtccctcctcctgtgcc
oBWW235	cgcggatccaatgctcccgtgcaagcgggag
oBWW236	ccggaattcgaaaagaaaccgtccgagagttc
oBWW237	gaacaggagctacgacggtaatcagaggg
oBWW238	ccgtcgtagctcctgttcccgggc
oBWW246	cgcgtcgactaaggaggtatacatgtgcagcagcatccggatcagcttaaactg
oBWW247	ccggcatgcctattgctgttcttctttcataaaatcgaatggtgtaatgcc
oSAB14	gaagaacaatccatcttctt
oSAB15	cattgtgtctcgtatacaca
oSAB17	gcttagtatttgcgataatattgcattcgt
oSAB18	catcttccagattgcttaatgaaattaggatacc
oSW079	gacgctcggttcgtctaggaccgtcattagtatgttgatatacatagaccttaccgcagtg attcgcttgtcagtccattgaagcacaattacccacgc
oSW080	ctaatgacggtcctagacgaaccgagcgtc
oSW081	gcgtgggtaattgtgcttcaatggactgac

Table 2.5. Mutation spectrum in the *thyA* gene

Location	Base change	Wild type	Δ*mutSL*	Δ*recD2*
2	T-->C	0	1	0
3,6	Δ1, Δ2	0	0	1
15	T-->G	1	0	0
20	duplication AATAC	1	0	0
23	duplication ACAAT	2	0	0
24	duplicationCAATT	1	0	0
26	duplication ATTCA	1	0	0
31	duplication ATTATAAAGGATATTATCAAT	1	0	0
36	duplication AAAGGATATTATCAAT	1	0	0
55	G-->T	1	0	0
88	A-->T	0	0	1
91	T-->C	0	2	0
115	Δ1	0	0	1
139	C-->T	7	0	2
158	C-->A	0	0	1
176	C-->T	1	1	0
185	insertion A	2	5	2
185	Δ1	1	2	0
193	T-->C	0	1	1
226	T-->C	0	0	2
227	duplication GGCAGCTGAAATCT	0	0	1
227	G-->A	0	0	1
228	G-->A	1	0	0
229	C-->T	5	0	3
238	T-->C	0	1	0
after 269	insertion G	1	0	0
280	T-->C	0	0	1
281	G-->A	1	1	0
282	G-->A	0	0	1
289	T-->C	0	3	1
290	G-->A	0	1	0
305	insertion G	1	0	0
323	A-->G	0	1	1
332	A-->C	1	0	0
335	T-->C	0	4	0
after 347	insertion A	0	1	1
369	insertion A	1	0	1
376	C-->T	0	0	1
377	A-->G	1	1	2
389	T-->C	0	1	0
421	C-->T	1	0	1
422	Duplication GC	1	0	0
428	A-->G	1	0	0
440	T-->C	1	0	0

Table 2.5 Continued

Location	Base change	Wild type	Δ*mutSL*	Δ*recD2*
473	T-->A	0	0	1
482	G-->A	0	0	1
496	C-->T	1	0	0
496	C-->G	0	0	1
499	T-->C	0	1	1
542	G-->A	0	0	1
545	G-->A	0	0	1
547	A-->G	1	4	2
551	A-->C	1	0	0
553	A-->C	1	0	0
560	T-->C	0	0	2
562	G-->A	0	1	0
569	C-->T	1	0	0
574	A-->G	1	0	0
579-593	Δ15	0	0	1
584	A-->G	1	3	3
598	C-->T	0	2	0
599	A-->G	1	0	0
627	T-->G	1	0	0
655	G-->A	0	2	1
656	G-->T	1	0	1
656	G-->A	0	0	1
659	A-->G	1	0	0
661	T-->C	0	1	1
663	C-->G	0	0	1
664	C-->T	1	2	3
665	A-->G	0	1	1
670	T-->C	0	0	1
671	A-->G	0	1	0
700	C-->T	0	0	1
701	A-->G	0	4	0
715	C-->T	0	1	0
728	C-->T	1	0	0
737	G-->A	0	0	1
after 763	insertion T	0	1	0
800	A-->G	1	0	0
813	insertion C	0	0	1
833	C-->T	0	1	0
Transitions		29	42	38
Transversions		7	0	6
Insertions/deletions		6	9	8
Duplications		8	0	1
Total		50	51	53

The mutation spectrum was determined by sequencing the entire *thyA* gene from isolates on plates with trimethoprim as described in “Materials and Methods.” All isolates sequenced were from independent cultures. The data shown here are also presented and summarized in Figure 2.3.

References

1. Walsh, B.W., et al., *RecD2 helicase limits replication fork stress in Bacillus subtilis.* Journal of bacteriology, 2014. 196(7): p. 1359-68.
2. Pyle, A.M., *Translocation and unwinding mechanisms of RNA and DNA helicases.* Annual review of biophysics, 2008. 37: p. 317-36.
3. Manthei, K.A. and J.L. Keck, *The BLM dissolvasome in DNA replication and repair.* Cellular and molecular life sciences : CMLS, 2013.
4. Lo, Y.H., et al., *The crystal structure of a replicative hexameric helicase DnaC and its complex with single-stranded DNA.* Nucleic acids research, 2009. 37(3): p. 804-14.
5. Lenhart, J.S., et al., *DNA repair and genome maintenance in Bacillus subtilis.* Microbiology and molecular biology reviews : MMBR, 2012. 76(3): p. 530-64.
6. Dillingham, M.S. and S.C. Kowalczykowski, *RecBCD enzyme and the repair of double-stranded DNA breaks.* Microbiol Mol Biol Rev, 2008. 72(4): p. 642-71, Table of Contents.
7. Yeeles, J.T. and M.S. Dillingham, *The processing of double-stranded DNA breaks for recombinational repair by helicase-nuclease complexes.* DNA Repair (Amst), 2010. 9(3): p. 276-85.
8. Bell, S.P. and J.M. Kaguni, *Helicase loading at chromosomal origins of replication.* Cold Spring Harbor perspectives in biology, 2013. 5(6).
9. Gorbalenya, A.E. and E.V. Koonin, *Helicases: amino acid sequence comparisons and structure-function relationships.* Curr Opin Struct Biol, 1993. 3: p. 419-429.
10. Dillingham, M.S., *Superfamily I helicases as modular components of DNA-processing machines.* Biochemical Society transactions, 2011. 39(2): p. 413-23.
11. Hall, M.C., J.R. Jordan, and S.W. Matson, *Evidence for a physical interaction between the Escherichia coli methyl-directed mismatch repair proteins MutL and UvrD.* Embo J, 1998. 17(5): p. 1535-41.
12. Hall, M.C. and S.W. Matson, *The Escherichia coli MutL protein physically interacts with MutH and stimulates the MutH-associated endonuclease activity.* J Biol Chem, 1999. 274(3): p. 1306-12.
13. Matson, S.W., *Escherichia coli DNA helicase II (uvrD gene product) catalyzes the unwinding of DNA.RNA hybrids in vitro.* Proceedings of the National Academy of Sciences of the United States of America, 1989. 86(12): p. 4430-4.
14. Bae, S.H. and Y.S. Seo, *Characterization of the enzymatic properties of the yeast dna2 Helicase/endonuclease suggests a new model for Okazaki fragment processing.* The Journal of biological chemistry, 2000. 275(48): p. 38022-31.
15. Gu, J., et al., *Cell cycle-dependent regulation of a human DNA helicase that localizes in DNA damage foci.* Molecular biology of the cell, 2004. 15(7): p. 3320-32.
16. Montague, M., et al., *The evolution of RecD outside of the RecBCD complex.* Journal of molecular evolution, 2009. 69(4): p. 360-71.
17. Saikrishnan, K., et al., *Mechanistic basis of 5'-3' translocation in SF1B helicases.* Cell, 2009. 137(5): p. 849-59.

18. Guler, G.D., et al., *Human DNA helicase B (HDHB) binds to replication protein A and facilitates cellular recovery from replication stress.* The Journal of biological chemistry, 2012. 287(9): p. 6469-81.
19. Taneja, P., et al., *A dominant-negative mutant of human DNA helicase B blocks the onset of chromosomal DNA replication.* The Journal of Biological Chemistry, 2002. 277(43): p. 40853-61.
20. Rocha, E.P., E. Cornet, and B. Michel, *Comparative and evolutionary analysis of the bacterial homologous recombination systems.* PLoS genetics, 2005. 1(2): p. e15.
21. Dillingham, M.S., M. Spies, and S.C. Kowalczykowski, *RecBCD enzyme is a bipolar DNA helicase.* Nature, 2003. 423(6942): p. 893-7.
22. Yang, H., et al., *The role of Bacillus anthracis RecD2 helicase in DNA mismatch repair.* DNA Repair (Amst), 2011.
23. Servinsky, M.D. and D.A. Julin, *Effect of a recD mutation on DNA damage resistance and transformation in Deinococcus radiodurans.* Journal of bacteriology, 2007. 189(14): p. 5101-7.
24. Shadrick, W.R. and D.A. Julin, *Kinetics of DNA unwinding by the RecD2 helicase from Deinococcus radiodurans.* The Journal of biological chemistry, 2010. 285(23): p. 17292-300.
25. Wang, J. and D.A. Julin, *DNA helicase activity of the RecD protein from Deinococcus radiodurans.* The Journal of biological chemistry, 2004. 279(50): p. 52024-32.
26. Saikrishnan, K., et al., *DNA binding to RecD: role of the 1B domain in SF1B helicase activity.* The EMBO journal, 2008. 27(16): p. 2222-9.
27. Yang, H., et al., *Papillation in Bacillus anthracis colonies: a tool for finding new mutators.* Mol Microbiol, 2011. 79(5): p. 1276-93.
28. Patrick, J.E. and D.B. Kearns, *MinJ (YvjD) is a topological determinant of cell division in Bacillus subtilis.* Mol Microbiol, 2008. 70(5): p. 1166-79.
29. Simmons, L.A., A.D. Grossman, and G.C. Walker, *Replication is required for the RecA localization response to DNA damage in Bacillus subtilis.* Proc Natl Acad Sci U S A, 2007. 104(4): p. 1360-5.
30. Klauck, E., J. Bohringer, and R. Hengge-Aronis, *The LysR-like regulator LeuO in Escherichia coli is involved in the translational regulation of rpoS by affecting the expression of the small regulatory DsrA-RNA.* Mol Microbiol, 1997. 25(3): p. 559-69.
31. Klocko, A.D., et al., *Imaging mismatch repair and cellular responses to DNA damage in Bacillus subtilis.* J Vis Exp, 2010. 36(36): p. 1-4.
32. Klocko, A.D., et al., *Mismatch repair causes the dynamic release of an essential DNA polymerase from the replication fork.* Mol Microbiol, 2011. 82(3): p. 648-63.
33. Lenhart, J.S., et al., *DnaN clamp zones provide a platform for spatiotemporal coupling of mismatch detection to DNA replication.* Molecular microbiology, 2013. 87(3): p. 553-68.
34. Davies, B.W., et al., *DNA damage and reactive nitrogen species are barriers to Vibrio cholerae colonization of the infant mouse intestine.* PLoS Pathog, 2011. 7(2): p. e1001295.

35. Dupes, N.M., et al., *Mutations in the Bacillus subtilis beta clamp that separate its roles in DNA replication from mismatch repair.* J Bacteriol, 2010. 192(13): p. 3452-63.
36. Simmons, L.A., et al., *Beta clamp directs localization of mismatch repair in Bacillus subtilis.* Mol Cell, 2008. 29(3): p. 291-301.
37. Berkmen, M.B. and A.D. Grossman, *Spatial and temporal organization of the Bacillus subtilis replication cycle.* Mol Microbiol, 2006. 62(1): p. 57-71.
38. Maughan, H., B. Galeano, and W.L. Nicholson, *Novel rpoB mutations conferring rifampin resistance on Bacillus subtilis: global effects on growth, competence, sporulation, and germination.* Journal of Bacteriology, 2004. 186(8): p. 2481-6.
39. Nicholson, W.L. and H. Maughan, *The spectrum of spontaneous rifampin resistance mutations in the rpoB gene of Bacillus subtilis 168 spores differs from that of vegetative cells and resembles that of Mycobacterium tuberculosis.* Journal of Bacteriology, 2002. 184(17): p. 4936-40.
40. Belfort, M. and J. Pedersen-Lane, *Genetic system for analyzing Escherichia coli thymidylate synthase.* Journal of Bacteriology, 1984. 160(1): p. 371-8.
41. Dutra, B.E. and S.T. Lovett, *Cis and trans-acting effects on a mutational hotspot involving a replication template switch.* Journal of Molecular Biology, 2006. 356(2): p. 300-11.
42. Neuhard, J., et al., *Two thymidylate synthetases in Bacillus subtilis.* Proceedings of the National Academy of Sciences of the United States of America, 1978. 75(3): p. 1194-8.
43. Calmann, M.A. and M.G. Marinus, *Differential effects of cisplatin and MNNG on dna mutants of Escherichia coli.* Mutation Research, 2005. 578(1-2): p. 406-16.
44. Garibyan, L., et al., *Use of the rpoB gene to determine the specificity of base substitution mutations on the Escherichia coli chromosome.* DNA repair, 2003. 2(5): p. 593-608.
45. Duigou, S., et al., *Distinctive genetic features exhibited by the Y-family DNA polymerases in Bacillus subtilis.* Mol Microbiol, 2004. 54(2): p. 439-51.
46. Sung, H.M. and R.E. Yasbin, *Adaptive, or stationary-phase, mutagenesis, a component of bacterial differentiation in Bacillus subtilis.* J Bacteriol, 2002. 184(20): p. 5641-53.
47. Sung, H.M., et al., *Roles of YqjH and YqjW, homologs of the Escherichia coli UmuC/DinB or Y superfamily of DNA polymerases, in stationary-phase mutagenesis and UV-induced mutagenesis of Bacillus subtilis.* J Bacteriol, 2003. 185(7): p. 2153-60.
48. Le Chatelier, E., et al., *Involvement of DnaE, the second replicative DNA polymerase from Bacillus subtilis, in DNA mutagenesis.* J Biol Chem, 2004. 279(3): p. 1757-67.
49. Rivas-Castillo, A.M., et al., *Role of the Y-family DNA polymerases YqjH and YqjW in protecting sporulating Bacillus subtilis cells from DNA damage.* Current microbiology, 2010. 60(4): p. 263-7.
50. Dervyn, E., et al., *Two essential DNA polymerases at the bacterial replication fork.* Science, 2001. 294(5547): p. 1716-1719.
51. Kobayashi, K., et al., *Essential Bacillus subtilis genes.* Proc Natl Acad Sci U S A, 2003. 100(8): p. 4678-83.

52. Dervyn, E., et al., *The bacterial condensin/cohesin-like protein complex acts in DNA repair and regulation of gene expression.* Mol Microbiol, 2004. 51(6): p. 1629-40.
53. Smith, B.T., A.D. Grossman, and G.C. Walker, *Localization of UvrA and effect of DNA damage on the chromosome of Bacillus subtilis.* J. Bacteriol., 2002. 184(2): p. 488-493.
54. Friedberg, E.C., et al., *DNA Repair and Mutagenesis: Second Edition*2006, Washington, DC: American Society for Microbiology.
55. Beranek, D.T., *Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents.* Mutation research, 1990. 231(1): p. 11-30.
56. Dronkert, M.L. and R. Kanaar, *Repair of DNA interstrand cross-links.* Mutat Res, 2001. 486(4): p. 217-47.
57. Goranov, A.I., et al., *Characterization of the global transcriptional responses to different types of DNA damage and disruption of replication in Bacillus subtilis.* Journal of bacteriology, 2006. 188(15): p. 5595-605.
58. Costes, A., et al., *The C-terminal domain of the bacterial SSB protein acts as a DNA maintenance hub at active chromosome replication forks.* PLoS Genet, 2010. 6(12): p. e1001238.
59. Simmons, L.A., et al., *Comparison of responses to double-strand breaks between Escherichia coli and Bacillus subtilis reveals different requirements for SOS induction.* J Bacteriol, 2009. 191(4): p. 1152-61.
60. Marceau, A.H., et al., *Protein interactions in genome maintenance as novel antibacterial targets.* PLoS One, 2013. 8(3): p. e58765.
61. Naue, N., et al., *The helicase-binding domain of Escherichia coli DnaG primase interacts with the highly conserved C-terminal region of single-stranded DNA-binding protein.* Nucleic acids research, 2013. 41(8): p. 4507-17.
62. Walsh, B.W., et al., *Far Western blotting as a rapid and efficient method for detecting interactions between DNA replication and DNA repair proteins.* Methods in molecular biology, 2012. 922: p. 161-8.
63. Gupta, M.K., et al., *Protein-DNA complexes are the primary sources of replication fork pausing in Escherichia coli.* Proceedings of the National Academy of Sciences of the United States of America, 2013. 110(18): p. 7252-7.
64. Sievers, F., et al., *Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega.* Molecular systems biology, 2011. 7: p. 539.
65. Lee, J.Y. and W. Yang, *UvrD helicase unwinds DNA one base pair at a time by a two-part power stroke.* Cell, 2006. 127(7): p. 1349-60.
66. Bolz, N.J., et al., *Residues in the N-terminal domain of MutL required for mismatch repair in Bacillus subtilis.* Journal of Bacteriology, 2012. 194(19): p. 5361-7.
67. Foster, P.L., *Methods for determining spontaneous mutation rates.* Methods Enzymol, 2006. 409: p. 195-213.
68. Hall, B.M., et al., *Fluctuation analysis CalculatOR: a web tool for the determination of mutation rate using Luria-Delbruck fluctuation analysis.* Bioinformatics, 2009. 25(12): p. 1564-5.

69. Lenhart, J.S., et al., *Trapping and visualizing intermediate steps in the mismatch repair pathway in vivo.* Molecular microbiology, 2013.
70. Youngman, P., J.B. Perkins, and R. Losick, *Construction of a cloning site near one end of Tn917 into which foreign DNA may be inserted without affecting transposition in Bacillus subtilis or expression of the transposon-borne erm gene.* Plasmid, 1984. 12(1): p. 1-9.
71. Sciochetti, S.A., G.W. Blakely, and P.J. Piggot, *Growth phase variation in cell and nucleoid morphology in a Bacillus subtilis recA mutant.* Journal of Bacteriology, 2001. 183(9): p. 2963-8.
72. Yao, N.Y., et al., *Cost of rNTP/dNTP pool imbalance at the replication fork.* Proceedings of the National Academy of Sciences of the United States of America, 2013. 110(32): p. 12942-7.
73. Hoa, T.T., et al., *Rok (YkuW) regulates genetic competence in Bacillus subtilis by directly repressing comK.* Molecular microbiology, 2002. 43(1): p. 15-26.
74. van Sinderen, D., et al., *Molecular cloning and sequence of comK, a gene required for genetic competence in Bacillus subtilis.* Molecular microbiology, 1994. 11(4): p. 695-703.
75. van Sinderen, D., et al., *comK encodes the competence transcription factor, the key regulatory protein for competence development in Bacillus subtilis.* Molecular microbiology, 1995. 15(3): p. 455-62.

Chapter 3

Conserved amino acids in the N-terminus of RecD2 are critical for its function.

Abstract

DNA helicases are critical for the majority of DNA replication and repair processes. RecD2 proteins have been shown to function in a variety of processes, depending upon the organism within which it has been studied. For example, in *Bacillus anthracis*, RecD2 functions in DNA mismatch repair, while in *Deinococcus radiodurans* RecD2 functions in the repair of DNA damage. In *Bacillus subtilis*, RecD2 contributes to replication fork stabilization and genome integrity although the overall mechanism is still unclear. Herein we report that RecD2 is expressed at a level of roughly 100 copies per cell during exponential growth and that dysregulation of RecD2 expression has a negative impact on *B. subtilis* growth. We show that ectopic expression of *recD2* or a *recD2* deletion increases mutation rate and induces the SOS response. Furthermore, ectopic expression leads to a block in replication fork progression and toxicity. We identify specific variants of RecD2 that mitigate ectopic expression-induced toxicity while also conferring sensitivity to DNA damaging agents at lower levels of expression. Taken together, these results show that the dysregulation of RecD2 is toxic to *Bacillus subtilis.*

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Introduction

Helicases are present in all organisms from bacteriophages and viruses to eukaryotes, and their ability to separate nucleic acid polymers is dependent upon ATP hydrolysis (for reviews, [1-3]). Helicases are responsible for the separation of duplex DNA strands into single-stranded DNA (ssDNA) and are required for the majority of DNA replication and repair transactions. The replicative helicase in *Bacillus subtilis*, DnaC, is an essential protein and is required for the separation of double-stranded DNA (dsDNA) at the replication fork, allowing for the use of both strands as template DNA during replication [4]. Helicases also have critical roles in a number of DNA repair processes including DNA MisMatch Repair (MMR) Nucleotide Excision Repair (NER), Homologous Recombination (HR), and replication fork restart, all of which are critical processes for survival following exposure to exogenous DNA damage (for review, [5-8]. The diverse functions of helicases within DNA replication, repair, and transcription processes has contributed to their classification into several Super Families (SF#) which are further distinguished by directionality of translocation (3′-5′ [A] or 5′-3′ [B]), with the RecD proteins belonging to the SF1B family [9-11].

SF1B helicases are present in all types of organisms and have historically been well studied in bacteriophage T4 (Dda), *E. coli* (RecD), and humans (DNA helicase B) [9, 12, 13]. RecD proteins have since been subdivided into two separate types, a canonical RecD, and RecD2. RecD is distinguished by a shorter length (<655 amino acids) and traditionally associated with RecB and RecC proteins, as examined in *Escherichia coli* and its close relatives [9]. RecD is a 5′-3′ helicase which interacts with RecBC forming the RecBCD helicase-nuclease complex whose primary function is to

catalyze DNA end-resection during DNA double strand break (DSB) repair in *E. coli* (for review, [14]). In contrast to RecD, RecD2 helicases differ in several respects. RecD2 helicases are present in organisms which lack RecB and RecC, and RecD2 contains an extended N-terminal extension which is absent from RecD [9, 15, 16]. The C-terminal region of RecD2 proteins are otherwise very similar to RecD in sequence and domain architecture [9, 10, 16, 17]. The function of the RecD2 N-terminal region has yet to be determined in any RecD2 homolog.

RecD2 proteins have only recently begun to be studied, but have been shown to have remarkably different functions in DNA repair across the organisms examined [15-18]. In *Deinococcus radiodurans* RecD2 has been shown to translocate in the 5′-3′ direction to separate dsDNA, and function in cellular resistance to gamma and UV irradiation, as well as oxidative damage by hydrogen peroxide [15, 18-20]. An N-terminal truncation of *D. radiodurans* RecD2 has also been crystalized and has been integral in understanding the structure-function relationship of RecD proteins [20, 21]. Interestingly, heterologous expression of *D. radiodurans* RecD2 in *E. coli* was shown to inactivate paused replication forks that arise due to conflicts with transcription [22]. In *Bacillus anthracis*, a mutation in *recD2* conferred by transposon-insertion mutagenesis resulted in a spontaneous mutation frequency and spectrum placing *recD2* within the DNA mismatch repair (MMR) pathway [17]. In addition to its role in MMR, the *recD2* mutant was found to be sensitive to hydrogen peroxide, indicating a role in resistance to oxidative DNA damage [17]. Contrary to the *D. radiodurans* protein however, a *recD2* mutant in *B. anthracis* did not render cells sensitive to UV, illustrating the varied functions of RecD2 proteins in bacterial species [17].

Our prior work in *Bacillus subtilis*, (Chapter 2), has shown that RecD2 is a 5′-3′ helicase [16]. Additionally, we have shown that RecD2 contributes to survival following challenge to a wide variety of DNA damaging agents from alkylating agents to DSB inducing agents, but does not appear to contribute to MMR [16]. We found that deletion of *recD2* did cause a mild increase in mutation rate, although the underlying mechanism remained unclear. Beyond the contribution to DNA damage repair, we have shown that replication forks collapse more frequently in the absence of RecD2 [16]. We have also shown that RecD2 binds SSB directly, and it is possible that this interaction is responsible for its localization to the site of action [16].

While our prior work in Chapter 2 established a new function for RecD2 within *B. subtilis*, there are more mechanistic and structure-function based questions that have yet to be answered. A primary question will be to address how dysregulation of RecD2 affects *B. subtilis* cells. We have found that there are ~100 copies of the RecD2 in *B. subtilis* during exponential growth, and we show that both deletion and overexpression of RecD2 results in induction of the SOS response, though significant overexpression results in a much stronger activation. Cells with a Δ*recD2* can be complemented by low-level expression from an ectopic locus, however stronger ectopic expression of RecD2 is toxic. Here we show that mutation of conserved residues in the N-terminus are able to partially or fully mitigate the toxicity caused by ectopic expression, and one mutant in particular (*F209A*) also renders the cells sensitive to Mitomycin C (MMC) challenge indicating a defect *in vivo*. This provides insight into the function of the N-terminus of the protein. In this Chapter we continue our study of RecD2 to further understand its role in maintaining replication fork integrity

Results and Discussion

RecD2 concentration is ~100 molecules per cell

DNA helicases function in a variety of DNA transactions and, in accordance with their varied functions, have a substantial range of cellular concentrations. For instance, the primary replicative helicase of *E. coli*, DnaB, has a relatively low cellular concentration of between 10-20 hexameric helicase complexes or roughly 100 hexameric helicase complexes, depending on the group estimating the concentration [23-25]. For comparison, accessory DNA helicases can be present in high intracellular concentrations. For instance, the *E. coli* DNA repair helicase UvrD is present in roughly 5000-8000 monomers per cell at basal levels of expression [25]. Under SOS inducing conditions, the amount per cell increases to between 25,000 and 65,000 monomers per cell [26, 27]. To gain insight into the function of RecD2, we examined the amount of RecD2 present per cell by quantitative western blotting. First, a linear gradient of RecD2 concentrations was established (Figure 3.1A). Then, three separate cultures of wild type *B. subtilis* strain PY79 were grown to mid-exponential phase (OD_{600} ~ 0.5) and harvested. Cell lysates were then probed with affinity purified anti-RecD2 antibodies, along with the gradient of purified RecD2 as shown (Figure 3.1A), and quantified (see "Material and Methods"). Using this approach we found that exponentially growing cultures express 99 $\pm$ 15 molecules of RecD2 per cell.

In *E. coli* and *B. subtilis* several different helicase are under control of the SOS response, including *E. coli* UvrD and *B. subtilis* PcrA. Even though RecD2 was not shown to be regulated by LexA, we asked if the protein levels were damage-inducible

[28]. We found that RecD2 protein levels were unchanged following challenge with MMC (Figure 3.1C). With this data, we conclude that there are approximately 100 RecD2 molecules per cell and that RecD2 is not DNA damage inducible.

Dysregulation of RecD2 induces the SOS response

Given the effect of *D. radiodurans* RecD2 expression in *E. coli*, we asked what the effect was when *recD2* is ectopically expressed in its native organism [22]. To address this, we ectopically expressed RecD2 followed by analysis of genome-wide gene expression using RNA-seq. We examined the differential expression of the transcriptome for both a *recD2* deletion and a strain with ectopic *recD2* overexpression followed by comparison with wild type, and a strain with constitutive SOS induction (Δ*lexA)* as a control. Given that there is an increase in RecA-GFP foci in a Δ*recD2* strain it is expected that the SOS response would be activated [16]. Interestingly, for both the Δ*recD2* and the *recD2* ectopic expression strain, we primarily observed significant differential expression in genes that comprise the SOS-regulon (Figure 3.2) [28]. Thirty eight of the genes known to be regulated by LexA binding, as well as *recD2*, can be observed in the heat map (Figure 3.2) [28]. While *recD2* is not part of the SOS response (Figure 3.1 and [28]), it is clear that the deletion or ectopic expression of *recD2* causes SOS induction (Figure 3.2). Furthermore, we show that ectopic expression of RecD2 causes a significantly stronger induction of the SOS response than does the Δ*recD2* allele. With these results we conclude that dysregulation of RecD2 causes induction of the SOS response, a mutagenic process, presumably due to increased replication fork stress.

RecD2 ectopic expression is toxic to *B. subtilis* cells.

We have previously explored the consequences of a Δ*recD2* deletion in *B. subtilis*, and shown an increase in spontaneous mutation rate, greater susceptibility to a variety of DNA damaging agents, and an increase in the rate at which replication forks collapse [16]. It is clear that Δ*recD2* causes an increase in the SOS response, as shown in the RNA-seq experiment (Figure 3.2). It has also been shown that heterologous expression of *D. radiodurans* RecD2 in *E. coli* inactivates paused, but not active, replication forks [22]. This could be due to incompatibilities between orthologs. With these results in mind, we examined the consequences of RecD2 overexpression in the *B. subtilis*. To investigate this question we used a strain which ectopically expresses RecD2 from the *amyE* locus under IPTG control and completely complements Δ*recD2* at 10 μM IPTG [16]. To quantitatively assess the degree of sensitivity to RecD2 expression we performed a growth curve of *B. subtilis* cultures during expression of RecD2 by 1 mM IPTG (Figure 3.3a). The culture maintained normal exponential growth until ~1.5 hours after treatment with 1 mM IPTG, at which point growth tapered off (Figure 3.3a). To determine whether the effect of RecD2 overexpression was merely a cessation of growth or actual resulted in cell death, survival on LB plates after overexpression of RecD2 for 1.5 hours was examined and compared to untreated cells. We found that there was a roughly 70-fold decrease in survival when we compared the RecD2 ectopic expression strain (mean 3.7 X 10^6 CFU/mL) to the wild type (2.65 X 10^8 CFU/mL) with the same OD/mL of cells plated (Figure 3.3b).

In addition to examining the effect of RecD2 overexpression on growth, we sought to determine whether the increase in RecD2 concentration also increased

mutation rate. After growing the overexpression strain from a starting OD_{600} of 0.05 with 100 μM IPTG for 90 minutes to induce overexpression of RecD2 but prevent killing associated with expression at 1 mM IPTG, the strain was plated on Rif and LB plates in order to establish a mutation rate. The mutation rate of the RecD2 expression strain with 100 μM IPTG, as measured on Rif plates as 1.50×10^{-9} mutations per generation, as compared to the wild type control which was measured to have a mutation rate of 7.30×10^{-10} as can be seen in Figure 3.3c [16, 29-32]. While the mutation rate of the strain overexpressing RecD2 is 2-fold higher than wild-type, there is no statistical difference in mutation rate (Figure 3.3c).

Since we have previously found that replication forks collapse more frequently in the absence of RecD2, and we know that overexpression of RecD2 causes cell death, and it has also been shown that expression of *D. radiodurans* RecD2 in a heterologous organism results in the inactivation of stalled replication forks, we investigated whether the overexpression of RecD2 causes replication forks to collapse more frequently [16, 22]. Using whole genome sequence coverage as a proxy for replication fork progression as before [16], we found that there is decreased sequence coverage further from the origin in the RecD2 overexpression strain (Figure 3.4. Blue line) than there is in the otherwise wildtype *dnaB134* strain (red line), indicating an increase in replication fork stalling or collapse. As a positive control, treatment with 20 ng/mL MMC was also included, as it is known to block DNA replication in *B. subtilis* (Figure 3.4) [33]. As expected, sequence coverage decreased significantly between the origin and terminus with MMC treatment. Together, these data indicate that the overexpression of RecD2

causes an increase in replication fork collapse, and that overexpression of RecD2 is toxic in *B. subtilis.*

RecD2 overexpression toxicity can be mitigated by N-terminal missense mutations

RecD2 orthologs have a conserved N-terminal region of unknown function, which distinguishes it from the more well studied *E. coli*-type RecD protein. There are two highly conserved motifs that occur at amino acids 109-112, and 206-213 in the *B. subtilis* RecD2 protein (Figure 3.5 and [16, 17, 20, 34]). The first motif is comprised of GIG(X) in which the X is actually a lysine in *B. subtilis*, *D. radiodurans*, and *B. anthracis*, and the second motif is comprised of GIGFX_3D in which the X_3 is GKA in *B. subtilis* as can be seen in the highlighted areas of Figure 3.5. There is also a highly conserved arginine at amino acid 296 in *B. subtilis* (Figure 3.5 and [16, 17, 20, 34]). To determine the relative importance of these amino acids (GK111-112, F209, and R296) in *B. subtilis in vivo* each was substituted individually for different amino acids generating GK111-112RA, F209A, and R296A, respectively, and each of these *recD2* mutants were inserted for ectopic expression into the Δ*recD2* strain. We studied a catalytic dead mutant, K373A, in parallel as a control. We previously determined that the RecD2 K373A variant is defective for helicase activity *in vitro* [16]. Each of these strains was plated on LB agar containing either Mitomycin C (MMC) or 1 mM IPTG to examine the sensitivity to DNA damaging agents and the potential toxicity by RecD2 overexpression. As seen in Figure 3.6a (row 3), expression of RecD2 at 1 mM IPTG is toxic, but this toxicity is either partially (in the case of GK111-112RA and R296A) or fully (F209A)

mitigated by genes carrying each of these missense mutations. Interestingly, however, the catalytic dead mutant (K373A) was still toxic, indicating that helicase activity is not required for the toxicity we observe. One possibility is that the RecD2 variants misfold and do not accumulate to wild type levels *in vivo* resulting in the observed phenotype. We performed Western blots and found (Figure 3.6b), that each of these mutant proteins do indeed accumulate *in vivo* to the same level as the wild type RecD2 following ectopic expression. When challenged with MMC and expressed to levels which rescue Δ*recD2* function (10 μM IPTG), RecD2(F209A) is sensitive to MMC and, as expected, so is the catalytically dead mutant, RecD2(K373A) (Figure 3.6a).

It is clear that overexpression of RecD2 is toxic, but the mechanism by which RecD2 toxicity occurs is still unknown. Oddly, the catalytic dead mutant (K373A) remains toxic when overexpressed, which suggests two possible modes of toxicity. First, RecD2 and RecD2(K373A) could be binding DNA aberrantly and either acting to separate DNA strands inappropriately in the case of RecD2, or binding and causing a road block on the DNA. This hypothesis would make sense in light of the fact that *D. radiodurans* RecD2 inactivates paused replication forks when ectopically expressed in *E. coli* [22]. This hypothesis is bolstered by the predicted model of *B. subtilis* RecD2 which suggests that amino acid F209 may be exposed to DNA and function in DNA binding (Chapter 1, Figure 1.3d), a possibility also suggested for the analogous residue in *B. anthracis* by Yang *et al.* [17]. Both K373A and F209A should be tested in DNA binding assays to determine whether they are capable of binding DNA, and F209A should be tested to examine its helicase activity, since the F209A mutant appears to have some ability to process MMC lesions (Figure 3.6). The other hypothesis which

might explain the toxicity exhibited by RecD2 overexpression is that RecD2 may be sequestering SSB away from other DNA transactions and leaving ssDNA available to attack or preventing other proteins from binding SSB. Initially, RecD2 was identified as a member of the SSB interactome in a pull-down assay, and we have since corroborated this result and found that RecD2 binds SSB *in vitro* by glutaraldehyde crosslinking (Chapter 4 Figure 4.1), as well as *in vivo* by far Western blotting [16, 35]. SSB is important for the recruitment of several proteins known to function at replication forks and in their absence forks may pause or collapse [35]. Additionally, SSB has been shown to bind the essential DNA polymerase DnaE [35]. If these proteins are not able access their substrates, replication could pause or forks may collapse. Addressing these two possibilities for toxicity will help bring some great insight into the structure and function of RecD2. This could be done by creating SSB-binding deficient mutants of RecD2, perhaps within the SH3 domain as mentioned in chapter 1, or also by utilizing the SSBΔ35 mutant which lacks 2 of the 3 PF domains on the C-terminus of SSB which are required for its binding interactions [35]. If SSBΔ35 were able to rescue the overexpression toxicity phenotype, it would indicate that SSB is the limiting factor in overexpression toxicity. Interestingly, the overexpression toxicity phenotype of RecD2 provides a means by which we can assess the importance of amino acids within the protein, and can be exploited to further understand the function of the N-terminus of RecD2.

Materials and Methods

Bacteriology

All strains used are derivatives of PY79 and listed in Table 3.1. Antibiotics were used in the following concentrations: 100 μg/mL spectinomycin (Spc), 150 μg/mL rifampicin (Rif), 5 μg/mL tetracycline. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at 10 μM to induce RecD2 expression unless otherwise noted. To generate the ectopic expression strains of RecD2, the *recD2* gene was amplified via PCR from PY79 with SalI and SphI overhangs. The resulting fragment was digested with SalI and SphI restriction enzymes, ligated into pDR110 generating the plasmid pBW100, and transformed into *E. coli* MC1061 for propagation. pDR110 harbors an IPTG inducible P_{spac} promoter upstream of its multiple cloning site and allows for insertion into the *amyE* locus of *B. subtilis*. This plasmid was transformed into PY79 and selected for by resistance to spectinomycin generating BWW223. Site directed mutagenesis was performed on pBW100 using oligonucleotides specified in Table 3.3 to generate plasmids, pBW101 (RecD2(GK111-112RA)), pBW102 (RecD2(F209A)), and pBW103 (RecD2(R296A)). The individual mutants were sequenced to verify the presence of the desired mutation. These plasmids were each transformed into PY79 and selected for resistance to spectinomycin and then sequenced to confirm insertion and desired sequences and stored as BWW358, BWW359, and BWW360. Temperature sensitive *dnaB134* mutants were created by transforming the RecD2 overexpression strain (BWW223) with the *dnaB134* allele and selecting for tetracycline and spectinomycin resistance [16, 36, 37]. Tet resistant colonies were then streaked out again onto two

separate plates and one was incubated at 42˚C to determine whether *dnaB134* was present. This strain was stored as BWW478 (Δ*recD2*, *amyE*::P_{spac}-*recD2*, *dnab134*).

Growth curves were performed by inoculating a single colony into 2 mL LB and growing to OD_{600} = 0.5. Cultures were then diluted to an OD_{600} of 0.05 into either LB or LB + 1 mM IPTG. Cultures were grown and optical densities were measured half hour to an OD_{600} of 0.5 and back diluted to an OD_{600} of 0.05, to maintain logarithmic growth, at which point the optical density was measured every 30 minutes and recorded.

Mutation rate analysis

Single colonies were selected to inoculate 2 ml cultures of LB broth and grown at 37°C until reaching an OD_{600} of 0.5. The culture was then diluted to an OD_{600} of 0.05 in LB or LB + 100 µg/mL Spc + 100µM IPTG, and the freshly inoculated, dilute cultures were grown to an OD_{600} of 0.8 to 1.2. At this step, 1 ml of cells was harvested by centrifugation for 5 min at 10,000 rpm. The supernatant was aspirated, and cells were resuspended in 100 µl of 0.85% saline and plated onto LB-Rif. Additionally, 100 µL of the new culture was diluted to 10^{-6} and plated onto LB. The plates were incubated overnight, with LB-rifampin plates being incubated at 37°C and LB plates being incubated at 30°C, and scored on the following morning. Mutation rate was calculated as previously [16, 29-32].

Quantitation of RecD2 and western blotting

RecD2 was purified as described [16]. RecD2 was quantified by Bradford and UV spectroscopy (for review [38]). A linear gradient of purified RecD2 concentrations was

established and imaged by quantitative western blotting using the LiCOR system and the IRDye 800CW secondary antibody. Three cultures of PY79 were grown to mid-exponential phase (OD_{600} ~0.5). 20 mL of cells was harvested, lysed, and used for western blotting while a small portion of the culture was diluted to determine CFU. Cell lysates were then probed with affinity purified anti-RecD2 antibodies, along with the gradient of purified RecD2 as shown (Figure 3.1A), and quantified using the LiCOR imaging software. The values were plotted against the standard curve of RecD2 concentrations to give a quantity of protein per lane in ng and then converted to molarity, which was further divided by number of cells to give number of molecules of RecD2 per cell. Cultures of N-terminal amino acid substitution mutants were probed as above.

To determine whether RecD2 was damage inducible, PY79 cells were grown to OD_{600} = 0.2 and treated with 20 ng/mL Mitomycin C for 1 hour to induce the SOS response. Cells were harvested and equal amounts of treated and untreated culture, along with lysates of the Δ*recD2* strain, were probed with affinity purified antibodies against RecD2, LiCOR IRdye800CW secondary antibodies, and then imaged with the LiCOR imaging system.

RNA-seq analysis

Strains were plated on LB overnight and grown at 30°C. Cultures were inoculated into LB from single colonies and grown with constant shaking at 30°C to an OD_{600} between 0.5-0.7. Following addition of one volume of ice cold methanol cells were harvested by centrifugation. RNA was purified using the RiboPure RNA Purification Kit

(bacteria) according to the manufacturer's recommendations (Life Technologies). Ribosomal RNA was depleted using the Ribo-Zero Magnetic Kit (Bacteria) according to the manufacturer's instructions (Epicentre). cDNA and subsequent library preparation, followed by sequencing, was performed by the University of Michigan Sequencing Core. Fifty-base single end reads were sequenced on the Illumina HiSeq 2000. Sequence alignment was performed using the Burrows-Wheeler Aligner [39], bwa version 0.7.8-r455, to the *B. subtilis* PY79 reference genome [40]. Subsequent analysis was performed using the limma package in R [41].

In vivo replication fork collapse assay

Sample prep and sequencing for replication fork collapse assay was performed essentially as before [16]. Briefly, cells were grown in LB to an OD_{600} of ~0.5 and then shifted to 45˚C, the nonpermissive temperature of the *dnaB134* allele. After 45 minutes at the nonpermissive temperature, 20 ml of methanol was added to 20 ml of culture, and the cells were collected by centrifugation and the genomic DNA was purified. DNA was submitted to the University of Michigan DNA Sequencing Core for library preparation and 50-base single-end Illumina sequencing. Reads were aligned to the PY79 reference genome of our laboratory, accession number CP006881.1 [40]. Subsequent analyses were performed in R. Reads overlapping 1 kb bins along the reference genome were counted, and the values were normalized for sequencing coverage. Trends in coverage (counts in a given bin per million reads mapped) along the genome length were smoothed using loess, and the smoothed values were shifted to have identical coverage

at position zero so that they could be directly compared.

Figures

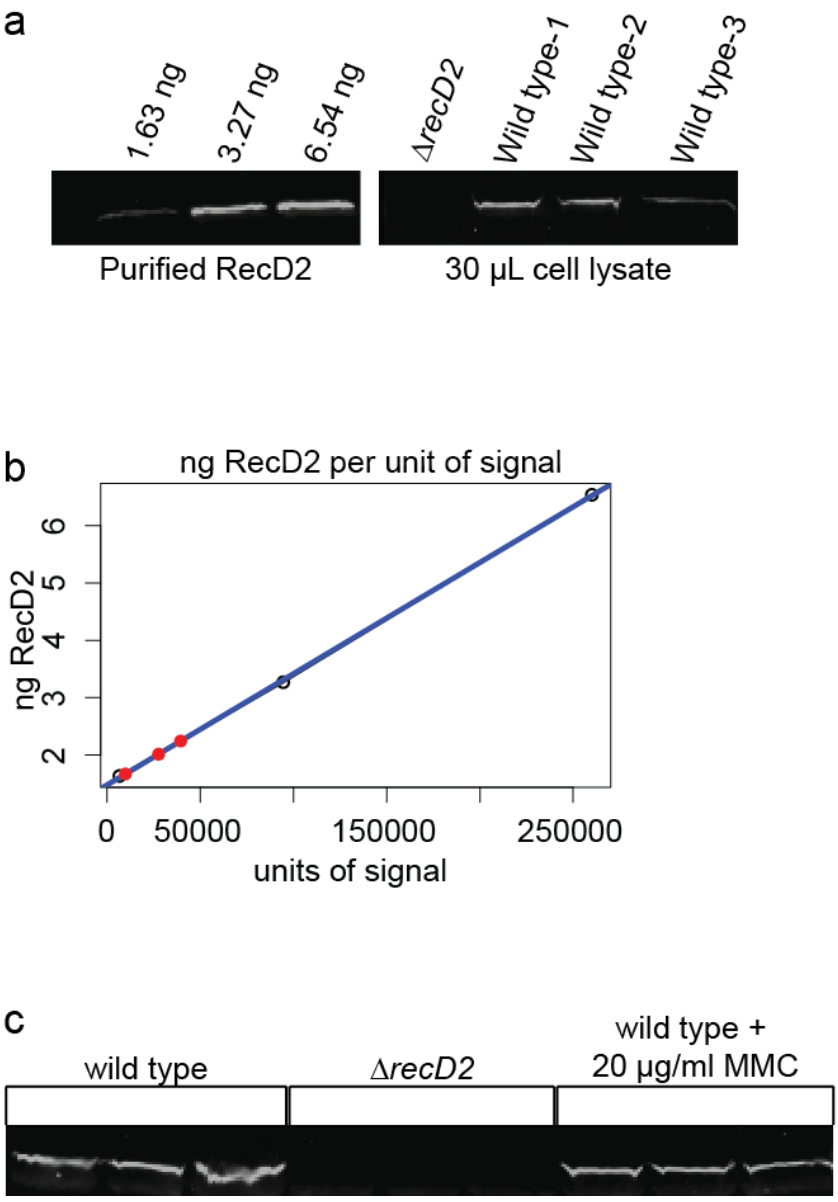

Figure 3.1. RecD2 is present at 99.6 ± 15.0 molecules per cell
a. Western blot of increasing concentrations of RecD2 protein compared to 30 µL of cell lysate from either Δ*recD2* or 3 independent cultures of wild type. b. LiCOR signal totals plotted for known concentration of RecD2 (black circles) with linear fit (blue line). Signal of RecD2 from wild type lysates is plotted along the line to give amount of RecD2 per 30 µL lysate. c. Western blot of three independent cultures each of wild type (PY79), Δ*recD2*, and wild type treated with 20 µg/ml MMC for 1 hour.

Figure 3.2. Δ*recD2* and RecD2 overexpression induce the SOS response. Each gene listed on the right, except RecD2, is known to be regulated by LexA and is thus under transcriptional control of the SOS response. Yellow indicates an increase in expression of the same gene compared to wild type, while blue indicates a decrease. In the far right column, Δ*lexA*, the SOS response is constitutively active and genes highly upregulated compared to wild type are bright yellow, while *lexA* is blue since it has been deleted. Both the Δ*recD2* and the RecD2 overexpression heat maps display increased expression of the genes involved in SOS, while *recD2* is blue in the Δ*recD2* lane as it is absent.

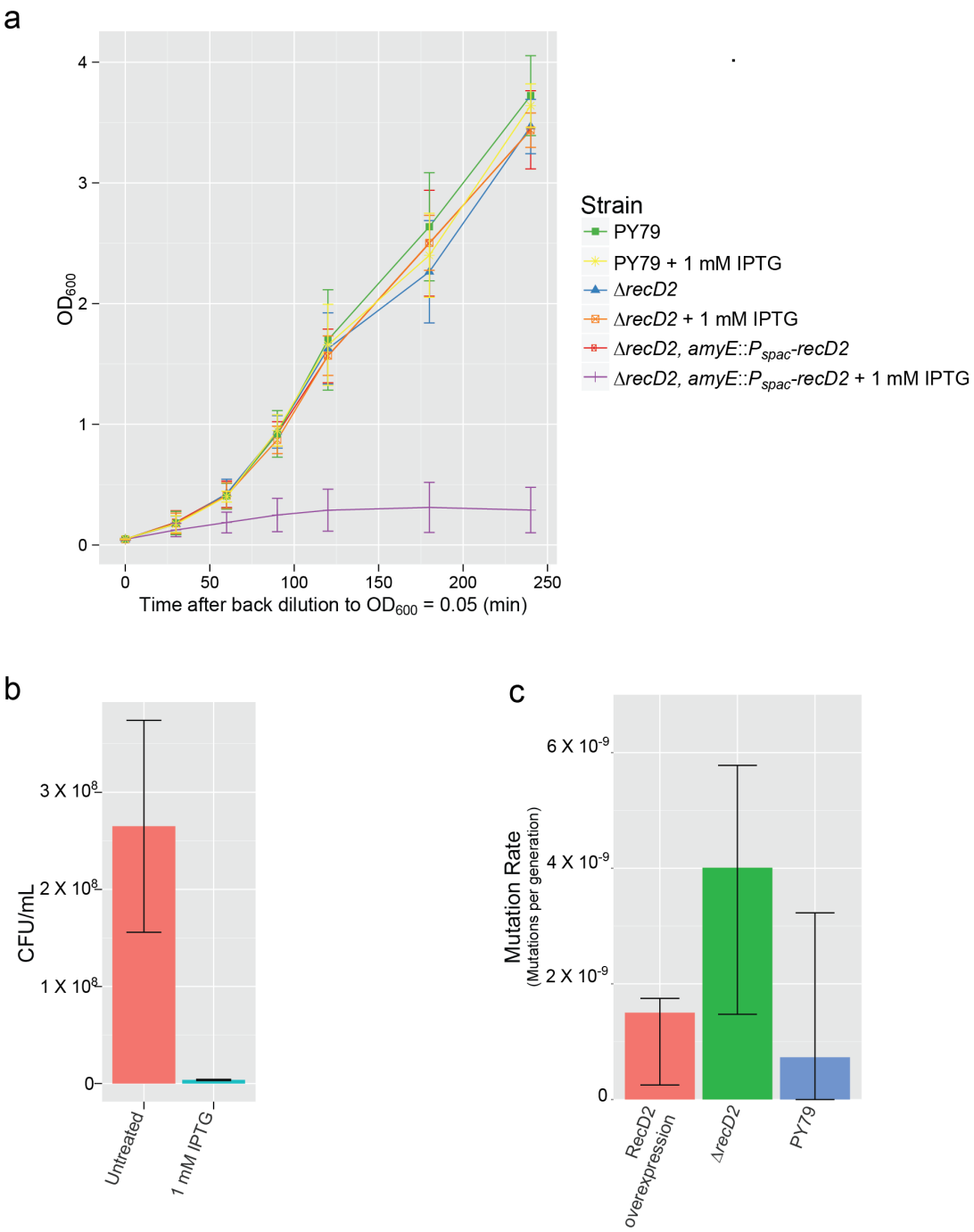

Figure 3.3. Overexpression of RecD2 is toxic to *B. subtilis*
3a. Growth curve of PY79 (wild type), Δ*recD2*, and Δ*recD2* with RecD2 expressed ectopically, either untreated or with the addition of 1 mM IPTG. 3b. Bar graph representing the CFU/mL (± 95% confidence interval) of BWW223 (Δ*recD2*, *amyE*::P_{spac}-*recD2*) with or without 1 mM IPTG treatment. 3c. Bar graph representing the spontaneous mutation rate ± 95% confidence intervals of at least 20 independent cultures each of BWW223 + 100 µM IPTG, BWW150, and wild type generated as described previously [16, 29-32].

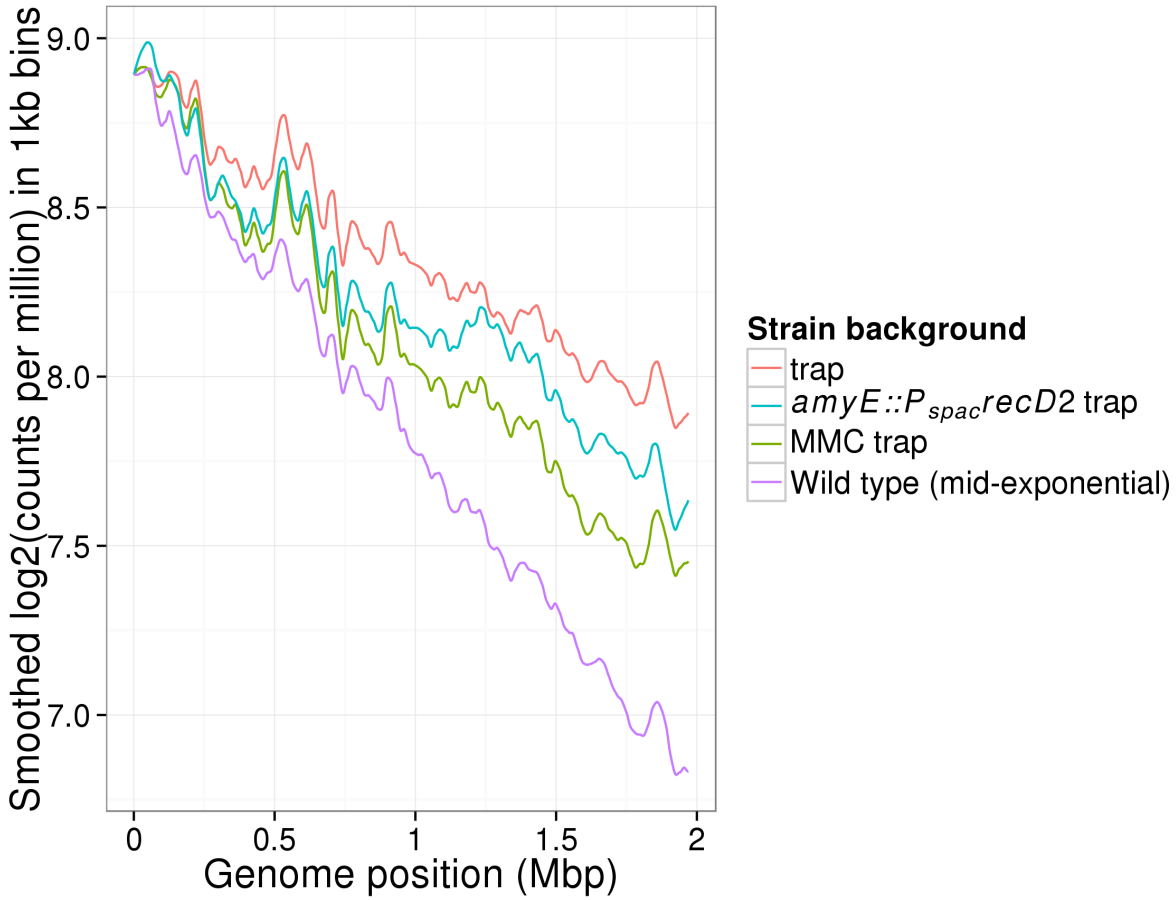

Figure 3.4. Replication forks collapse more frequently during replication stress. Whole genome sequencing coverage of the right arm of the chromosome is shown for strains listed with or without treatment. Wild type (indigo line) was grown to mid-exponential before genomic DNA isolation and sequencing. The remaining strains all harbor the *dnaB134* allele and halted replication initiation during mid-exponential phase and were either treated with 100 μM IPTG (*amyE*::P_{spac}-*recd2*) or 20 ng/mL MMC for 1 hour prior to switching to the non-permissive temperature.

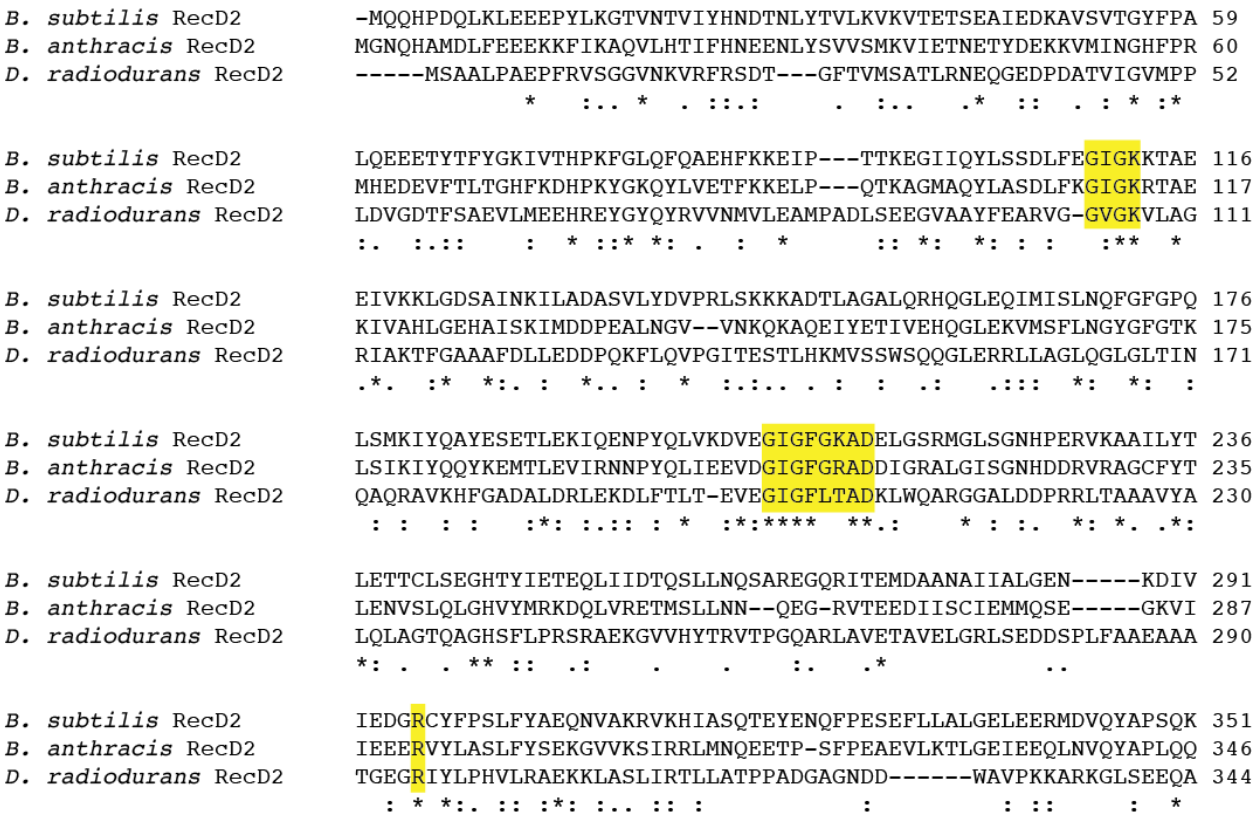

Figure 3.5. Alignment of RecD2 N-terminus regions.
The amino acid sequences of the RecD2 protein from *B. subtilis*, *B. anthracis*, and *D. radiodurans* were compared using ClustalW2 [42, 43]. Blocks highlighted in yellow indicate sequence motifs noted for high conservation and targeted for mutagenesis [16, 17, 20, 34].

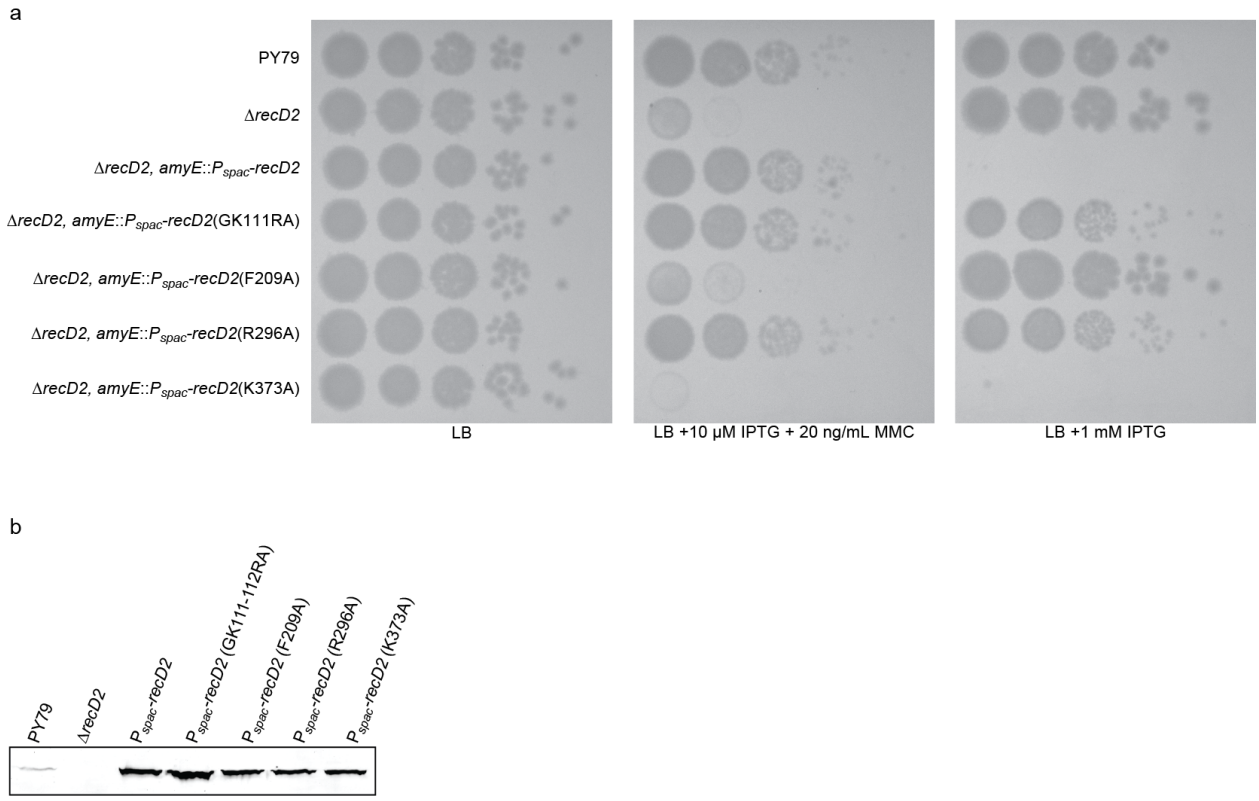

Figure 3.6. RecD2 overexpression toxicity can be mitigated by amino acid substitutions in the N-terminus.

5a. Indicated strains were grown to an OD_{600} of 0.5 and serial diluted in 10-fold increments. Each was plated on either LB, LB + 10 μM IPTG + 20 ng/mL MMC, or LB + 1 mM IPTG. 5b. Western blot of the indicated strains each treated with 100 μM IPTG to induce overexpression of RecD2 (where possible) to show accumulation of protein.

Tables

Table 3.1. List of *B. subtilis* strains

Strain	Relevant Genotype	Reference(s)
PY79	SPβ^{o}	[44]
BWW150	Δ*recD2*	[16]
BWW	Δ*recD2*, *amyE*::P_{spac}-*recD2*	
BWW	Δ*recD2*, *amyE*::P_{spac}-*recD2*(K373A)	
BWW358	Δ*recD2*, *amyE*::P_{spac}-*recD2*(GK111-112RA)	
BWW359	Δ*recD2*, *amyE*::P_{spac}-*recD2*(F209A)	
BWW360	Δ*recD2*, *amyE*::P_{spac}-*recD2*(R296A)	
BWW478	Δ*recD2*, *amyE*::P_{spac}-*recD2, dnaB134 zhb83::Tn917 (tet)*	

All strains are derived from PY79.

Table 3.2. RNA-seq analysis of Δ*recD2* VS PY79

Gene	Log2 Fold Change	Adjusted P value	Gene product
recD2	-7.292	8.60E-06	RecD2 DNA helicase
pyrD	-3.275	4.98E-02	Dihydroorotate dehydrogenase B (NAD(+)), catalytic subunit
yfjA	-1.485	2.64E-02	Uncharacterized protein yfjA
yfjB	-1.325	3.65E-02	Uncharacterized protein yfjB
pdaC	-1.262	1.30E-02	Uncharacterized protein yjeA
yfjC	-1.122	4.55E-02	Uncharacterized protein yfjC
cydA	-1.063	3.81E-02	Cytochrome d ubiquinol oxidase subunit 1
ykuN	1.005	4.92E-03	Probable flavodoxin-1
srfAD	1.011	4.32E-02	Surfactin synthase thioesterase subunit
yjdG	1.028	3.65E-02	Uncharacterized N-acetyltransferase YjdG
srfAC	1.038	3.65E-02	Surfactin synthase subunit 3
ykuP	1.039	4.92E-03	Probable flavodoxin-2
yhaO	1.103	2.24E-03	Uncharacterized metallophosphoesterase yhaO
ykuO	1.113	2.40E-03	Uncharacterized protein ykuO
lexA	1.240	2.89E-03	LexA repressor
U712_06680	1.291	4.32E-02	Hypothetical Protein
yhaZ	1.326	1.45E-02	Uncharacterized protein yhaZ
manP	1.333	2.64E-02	PTS system mannose-specific EIIBCA component
yneB	1.459	3.56E-03	Resolvase-like protein yneB
yjdF	1.483	2.22E-02	Uncharacterized protein yjdF
yneA	1.522	1.59E-02	Cell division suppressor protein yneA
yhjD	1.543	3.14E-02	Uncharacterized protein yhjD
yxkC	1.557	3.87E-02	Uncharacterized protein yxkC
manA	1.576	2.07E-02	Mannose-6-phosphate isomerase manA
dinB	1.752	1.10E-02	Protein dinB
ymzE	2.182	3.65E-02	Hypothetical Protein
glnM	2.464	4.61E-02	Probable glutamine ABC transporter permease protein glnM
ybfG	2.975	4.24E-02	Uncharacterized protein ybfG
yrzQ	3.246	9.00E-03	Uncharacterized protein yrzQ
xkdD	3.359	4.87E-02	Phage-like element PBSX protein xkdD
yrzR	3.391	2.64E-03	Uncharacterized protein yrzR
yrrD	3.573	2.40E-03	Uncharacterized protein yrrD
xlyB	3.770	4.37E-02	N-acetylmuramoyl-L-alanine amidase XlyB
U712_06510	3.804	4.79E-02	Hypothetical Protein
xlyA	4.169	3.01E-02	N-acetylmuramoyl-L-alanine amidase XlyA
xkdQ	4.245	3.65E-02	Phage-like element PBSX protein xkdQ
xhlA	4.260	4.22E-02	Protein xhlA

Table 3.2. continued

Gene	Log2 Fold Change	Adjusted P value	Gene product
xkdO	4.275	2.64E-02	Phage-like element PBSX protein xkdO
xkdT	4.277	3.66E-02	Phage-like element PBSX protein XkdT
xkdX	4.281	4.87E-02	Phage-like element PBSX protein xkdX
xkdP	4.307	3.65E-02	Phage-like element PBSX protein xkdP
xtmB	4.351	2.64E-02	PBSX phage terminase large subunit
xkdR	4.421	4.02E-02	Phage-like element PBSX protein xkdR
xkdW	4.459	4.41E-02	Phage-like element PBSX protein xkdW
xhlB	4.470	4.12E-02	Holin
xkdU	4.474	3.65E-02	Phage-like element PBSX protein xkdU
xkdV	4.486	2.64E-02	Phage-like element PBSX protein xkdV
xkdG	4.489	2.64E-02	Phage-like element PBSX protein xkdG
xepA	4.687	3.65E-02	Phage-like element PBSX protein xepA
xkdH	4.766	3.65E-02	Phage-like element PBSX protein xkdH
xkdM	4.767	2.71E-02	Phage-like element PBSX protein xkdM
xkdE	4.797	2.64E-02	Phage-like element PBSX protein xkdE
ykzM	4.814	3.65E-02	Uncharacterized protein ykzM
xkdK	4.824	2.22E-02	Phage-like element PBSX protein xkdK
ykzL	4.846	3.65E-02	Uncharacterized protein ykzL
xkdI	4.857	3.65E-02	Phage-like element PBSX protein xkdI
xkdS	4.883	3.79E-02	Phage-like element PBSX protein xkdS
xtmA	4.891	2.64E-02	PBSX phage terminase small subunit
xkdF	4.904	2.64E-02	Phage-like element PBSX protein xkdF
xkdJ	4.935	3.43E-02	Phage-like element PBSX protein xkdJ

RNA-seq analysis was performed as described in the materials and methods. The above data reflect the difference in gene expression between a Δ*recD2* strain VS PY79 sorted by adjusted P-value selecting only values above adj. P-value <0.05, and subsequently selected for Log2-fold change of less than -1 or greater than 1. Gene products are taken from NCBI annotations of the genes listed.

Table 3.3. RNA-seq analysis of RecD2 overexpression VS PY79

Gene	Log2 Fold Change	Adjusted P value	Gene product
yqjX	3.918	1.23E-03	Uncharacterized protein yqjX
yrrD	3.776	2.92E-07	Uncharacterized protein yrrD
ymzE	3.607	2.18E-04	Hypothetical Protein
yrzR	3.565	8.29E-07	Uncharacterized protein yrzR
yrzQ	3.537	6.54E-06	Uncharacterized protein yrzQ
pstA	3.459	2.20E-04	Probable ABC transporter permease protein yqgI
recD2	3.452	1.49E-09	Uncharacterized protein yrrC
polYB	3.366	8.22E-04	DNA polymerase IV 2
glnH	3.363	1.28E-04	ABC transporter glutamine-binding protein glnH
glnM	3.338	1.07E-04	Probable glutamine ABC transporter permease protein glnM
pstC	3.323	1.81E-04	Probable ABC transporter permease protein yqgH
yneA	3.251	5.32E-07	Cell division suppressor protein yneA
yneB	3.133	1.13E-07	Resolvase-like protein yneB
dinB	3.074	3.80E-06	Protein dinB
yhjD	3.046	4.10E-05	Uncharacterized protein yhjD
pstS	3.029	1.33E-04	Phosphate-binding protein pstS
pstBB	2.982	1.14E-03	Phosphate import ATP-binding protein PstB 1
U712_06510	2.981	8.61E-03	Hypothetical Protein
pstBA	2.972	8.36E-04	Phosphate import ATP-binding protein PstB 2
U712_01570	2.871	4.40E-04	Hypothetical Protein
glnP	2.864	1.49E-04	Probable glutamine ABC transporter permease protein glnP
xkdC	2.774	8.72E-03	Phage-like element PBSX protein xkdC
xkdF	2.588	4.09E-03	Phage-like element PBSX protein xkdF
amyE	2.547	3.25E-05	Alpha-amylase
xtmA	2.479	6.05E-03	PBSX phage terminase small subunit
xkdK	2.415	2.99E-03	Phage-like element PBSX protein xkdK
tagC	2.414	2.97E-04	Putative major teichoic acid biosynthesis protein C
xkdM	2.371	6.65E-03	Phage-like element PBSX protein xkdM
xkdE	2.318	7.06E-03	Phage-like element PBSX protein xkdE
uvrB	2.257	3.32E-07	UvrABC system protein B
uvrA	2.227	3.19E-07	UvrABC system protein A
yhaZ	2.141	3.19E-07	Uncharacterized protein yhaZ
yvzB	2.030	8.83E-06	Putative flagellin yvzB
xkdG	2.024	9.03E-03	Phage-like element PBSX protein xkdG
spoIIID	2.015	9.58E-03	Stage III sporulation protein D
xtmB	1.932	9.49E-03	PBSX phage terminase large subunit
yxkC	1.880	2.60E-04	Uncharacterized protein yxkC

Table 3.3. continued

Gene	Log2 Fold Change	Adjusted P value	Gene product
yybL	1.673	2.05E-04	Uncharacterized protein yybL
bglH	1.559	1.86E-03	Aryl-phospho-beta-D-glucosidase BglH
yybM	1.537	1.07E-04	Uncharacterized protein yybM
proJ	1.522	2.04E-04	Glutamate 5-kinase 2
yhaO	1.482	1.13E-07	Uncharacterized metallophosphoesterase yhaO
bglP	1.474	1.14E-03	PTS system beta-glucoside-specific EIIBCA component
proH	1.469	2.29E-04	Pyrroline-5-carboxylate reductase 1
yoaO	1.454	2.82E-03	Uncharacterized protein yoaO
yjdF	1.445	6.22E-05	Uncharacterized protein yjdF
manA	1.438	1.50E-04	Mannose-6-phosphate isomerase manA
cwlD	1.433	1.07E-04	Germination-specific N-acetylmuramoyl-L-alanine amidase
recA	1.423	1.59E-08	RecA recombinase
ybaK	1.418	3.52E-04	Uncharacterized protein ybaK
yybK	1.406	3.90E-04	Uncharacterized protein yybK
phoB	1.404	2.26E-03	Alkaline phosphatase 3
xkdA	1.387	5.66E-03	Phage-like element PBSX protein xkdA
yybN	1.386	1.08E-03	Uncharacterized protein yybN
sspF	1.373	5.96E-03	Protein sspF
yxzC	1.372	1.33E-04	Hypothetical Protein
yxiE	1.356	1.45E-03	Universal stress protein YxiE
yxiM	1.350	3.21E-04	Uncharacterized esterase yxiM
yjdG	1.339	1.70E-04	Uncharacterized N-acetyltransferase YjdG
U712_19770	1.335	9.78E-04	Hypothetical Protein
epsB	1.334	4.45E-03	Putative tyrosine-protein kinase YveL
yxzJ	1.301	3.40E-04	Uncharacterized protein yxzJ
sbcE	1.265	1.33E-07	Uncharacterized protein yhaN
comEA	1.262	4.92E-03	ComE operon protein 1
lexA	1.260	8.83E-06	LexA repressor
srfAD	1.246	3.68E-03	Surfactin synthase thioesterase subunit
cccA	1.242	8.28E-04	Cytochrome c-550
yxiK	1.237	3.38E-04	Uncharacterized protein yxiK
uvrC	1.233	3.19E-07	UvrABC system protein C
csfB	1.231	1.34E-03	Hypothetical Protein
yxeC	1.211	3.09E-05	Uncharacterized protein yxeC
yxiG	1.200	5.27E-04	Uncharacterized protein yxiG
pspA	1.198	1.93E-04	Phage shock protein A-like
ldh	1.190	1.03E-04	L-lactate dehydrogenase
trnE	1.181	6.87E-04	Uncharacterized protein yjdI

Table 3.3. continued

Gene	Log2 Fold Change	Adjusted P value	Gene product
yxiI	1.173	6.57E-04	Uncharacterized protein yxiI
yydI	1.171	9.80E-04	Probable peptide export ATP-binding protein YydI
yydH	1.166	1.93E-03	Putative peptide zinc metalloprotease protein yydH
yyzJ	1.164	3.94E-03	Hypothetical Protein
opuBA	1.155	1.70E-04	Choline transport ATP-binding protein OpuBA
yydF	1.152	8.58E-03	Putative exported peptide yydF
spoVG	1.151	9.72E-04	Putative septation protein spoVG
yxiF	1.144	6.62E-04	Uncharacterized protein yxiF
srfAC	1.143	3.15E-03	Surfactin synthase subunit 3
yvzJ	1.117	5.26E-05	Uncharacterized lipoprotein yvzJ
yxeD	1.116	2.52E-04	Uncharacterized protein yxeD
appD	1.114	8.54E-03	Oligopeptide transport ATP-binding protein AppD
yydJ	1.103	3.03E-03	Probable peptide export permease protein yydJ
U712_00255	1.093	2.87E-03	Hypothetical Protein
ruvA	1.093	5.72E-06	Holliday junction ATP-dependent DNA helicase ruvA
epsH	1.092	2.81E-03	Putative glycosyltransferase epsH
veg	1.085	3.66E-04	Protein veg
yydC	1.083	7.88E-05	Uncharacterized protein yydC
asnO	1.080	3.20E-03	Asparagine synthetase [glutamine-hydrolyzing] 3
yisN	1.080	4.07E-03	Uncharacterized protein yisN
yxzG	1.076	1.50E-03	Uncharacterized protein yxzG
ymaC	1.061	8.37E-05	UPF0714 protein ymaC
yxiH	1.041	9.80E-04	Uncharacterized protein yxiH
ynzC	1.041	1.77E-06	UPF0291 protein ynzC
yppF	1.038	1.59E-03	Uncharacterized protein yppF
yheI	1.029	5.36E-03	Probable multidrug resistance ABC transporter ATP-binding/permease protein YheI
srfAB	1.022	4.98E-03	Surfactin synthase subunit 2
polYA	1.022	2.30E-05	DNA polymerase IV 1
yxxG	1.015	9.97E-05	Uncharacterized protein yxxG
yprB	1.002	5.72E-06	Uncharacterized protein yprB
yhdX	-1.002	6.26E-03	Uncharacterized protein yhdX
yukD	-1.036	2.04E-04	Uncharacterized ubiquitin-like protein yukD
guaC	-1.042	1.35E-03	GMP reductase
ykuT	-1.045	3.94E-03	Uncharacterized mscS family protein ykuT
xpt	-1.055	5.36E-04	Xanthine phosphoribosyltransferase
yfhD	-1.065	1.39E-03	Uncharacterized protein yfhD
bscR	-1.080	9.01E-04	HTH-type transcriptional repressor BscR
purA	-1.087	6.23E-05	Adenylosuccinate synthetase

Table 3.3. continued

Gene	Log2 Fold Change	Adjusted P value	Gene product
U712_07735	-1.090	4.21E-03	Hypothetical Protein
yfkD	-1.099	8.46E-03	Uncharacterized protein yfkD
ycdA	-1.137	1.28E-04	Uncharacterized lipoprotein ycdA
pdaC	-1.144	4.10E-05	Uncharacterized protein yjeA
sigB	-1.153	9.51E-03	RNA polymerase sigma-B factor
ykbA	-1.158	5.23E-05	Serine/threonine exchanger SteT
cysC	-1.166	8.22E-04	Probable adenylyl-sulfate kinase
yjlB	-1.211	4.21E-03	Uncharacterized protein yjlB
yrkN	-1.219	6.39E-03	Uncharacterized protein yrkN
pbuX	-1.222	4.58E-04	Xanthine permease
cysP	-1.223	2.99E-04	Sulfate permease CysP
pbuO	-1.224	4.10E-05	Hypothetical Protein
cysH	-1.227	1.49E-04	Phosphoadenosine phosphosulfate reductase
yjhB	-1.243	2.05E-04	Putative ADP-ribose pyrophosphatase yjhB
yxaI	-1.284	1.09E-05	Uncharacterized protein yxaI
ykgA	-1.310	8.72E-03	Uncharacterized protein ykgA
yfjF	-1.329	1.09E-05	UPF0060 membrane protein yfjF
yvcA	-1.377	2.05E-04	Putative lipoprotein yvcA
yyzE	-1.381	9.51E-03	Putative phosphotransferase enzyme IIA component yyzE
pbuG	-1.463	5.26E-05	Guanine/hypoxanthine permease pbuG
yflS	-1.463	4.04E-04	Putative malate transporter yflS
sat	-1.506	1.21E-04	Sulfate adenylyltransferase
purK	-1.560	1.33E-04	N5-carboxyaminoimidazole ribonucleotide synthase
U712_04015	-1.564	6.83E-03	Hypothetical Protein
yfkE	-1.580	7.46E-03	Putative cation exchanger yfkE
sacB	-1.585	1.23E-03	Levansucrase
purB	-1.589	3.54E-05	Adenylosuccinate lyase
purE	-1.604	2.05E-04	N5-carboxyaminoimidazole ribonucleotide mutase
yfjD	-1.611	7.59E-06	Uncharacterized lipoprotein yfjD
yfjE	-1.641	7.90E-06	Uncharacterized protein yfjE
yrhD	-1.709	9.78E-04	Uncharacterized protein yrhD
ykzI	-1.725	6.46E-03	Uncharacterized protein ykzI
yhdN	-1.886	7.12E-03	General stress protein 69
yhcO	-1.893	2.03E-03	Uncharacterized protein yhcO
yrhE	-2.000	1.06E-03	Putative formate dehydrogenase yrhE
yfjC	-2.149	1.26E-05	Uncharacterized protein yfjC
purQ	-2.169	3.00E-05	Phosphoribosylformylglycinamidine synthase 1
purS	-2.185	5.12E-05	UPF0062 protein yexA

Table 3.3. continued

Gene	Log2 Fold Change	Adjusted P value	Gene product
purC	-2.212	6.96E-05	Phosphoribosylaminoimidazole-succinocarboxamide synthase
purD	-2.235	3.77E-06	Phosphoribosylamine--glycine ligase
purN	-2.301	5.08E-06	Phosphoribosylglycinamide formyltransferase
purH	-2.411	1.03E-06	Bifunctional purine biosynthesis protein purH
purL	-2.465	5.08E-06	Phosphoribosylformylglycinamidine synthase 2
yfjB	-2.582	8.84E-06	Uncharacterized protein yfjB
purF	-2.612	6.16E-06	Amidophosphoribosyltransferase
purM	-2.637	1.77E-06	Phosphoribosylformylglycinamidine cyclo-ligase
pyrAB	-2.849	2.13E-03	Carbamoyl-phosphate synthase pyrimidine-specific large chain
yfjA	-2.903	2.94E-06	Uncharacterized protein yfjA
pyrK	-2.996	6.57E-04	Dihydroorotate dehydrogenase B (NAD(+)), electron transfer subunit
pyrF	-3.136	4.76E-04	Orotidine 5'-phosphate decarboxylase
pyrD	-3.259	2.65E-04	Dihydroorotate dehydrogenase B (NAD(+)), catalytic subunit
pyrE	-3.427	3.57E-04	Orotate phosphoribosyltransferase

RNA-seq analysis was performed as described in the materials and methods. The above data reflect the difference in gene expression between the RecD2 overexpression strain VS PY79 sorted by adjusted P-value selecting only values above adj. P-value <0.01, and subsequently selected for Log2-fold change of less than -1 or greater than 1. Gene products are taken from NCBI annotations of the genes listed.

References

1. Singleton, M.R., M.S. Dillingham, and D.B. Wigley, *Structure and mechanism of helicases and nucleic acid translocases.* Annual review of biochemistry, 2007. 76: p. 23-50.
2. Pyle, A.M., *Translocation and unwinding mechanisms of RNA and DNA helicases.* Annual review of biophysics, 2008. 37: p. 317-36.
3. Manthei, K.A. and J.L. Keck, *The BLM dissolvasome in DNA replication and repair.* Cellular and molecular life sciences : CMLS, 2013. 70(21): p. 4067-84.
4. Lo, Y.H., et al., *The crystal structure of a replicative hexameric helicase DnaC and its complex with single-stranded DNA.* Nucleic acids research, 2009. 37(3): p. 804-14.
5. Lenhart, J.S., et al., *DNA repair and genome maintenance in Bacillus subtilis.* Microbiology and molecular biology reviews : MMBR, 2012. 76(3): p. 530-64.
6. Dillingham, M.S. and S.C. Kowalczykowski, *RecBCD enzyme and the repair of double-stranded DNA breaks.* Microbiology and molecular biology reviews : MMBR, 2008. 72(4): p. 642-71, Table of Contents.
7. Yeeles, J.T. and M.S. Dillingham, *The processing of double-stranded DNA breaks for recombinational repair by helicase-nuclease complexes.* DNA repair, 2010. 9(3): p. 276-85.
8. Bell, S.P. and J.M. Kaguni, *Helicase loading at chromosomal origins of replication.* Cold Spring Harbor perspectives in biology, 2013. 5(6).
9. Montague, M., et al., *The evolution of RecD outside of the RecBCD complex.* Journal of molecular evolution, 2009. 69(4): p. 360-71.
10. Rocha, E.P., E. Cornet, and B. Michel, *Comparative and evolutionary analysis of the bacterial homologous recombination systems.* PLoS genetics, 2005. 1(2): p. e15.
11. Dillingham, M.S., *Superfamily I helicases as modular components of DNA-processing machines.* Biochemical Society transactions, 2011. 39(2): p. 413-23.
12. Hacker, K.J. and B.M. Alberts, *Overexpression, purification, sequence analysis, and characterization of the T4 bacteriophage dda DNA helicase.* The Journal of biological chemistry, 1992. 267(29): p. 20674-81.
13. Gu, J., et al., *Cell cycle-dependent regulation of a human DNA helicase that localizes in DNA damage foci.* Molecular biology of the cell, 2004. 15(7): p. 3320-32.
14. Wigley, D.B., *Bacterial DNA repair: recent insights into the mechanism of RecBCD, AddAB and AdnAB.* Nature reviews. Microbiology, 2013. 11(1): p. 9-13.
15. Wang, J. and D.A. Julin, *DNA helicase activity of the RecD protein from Deinococcus radiodurans.* The Journal of biological chemistry, 2004. 279(50): p. 52024-32.
16. Walsh, B.W., et al., *RecD2 helicase limits replication fork stress in Bacillus subtilis.* Journal of bacteriology, 2014. 196(7): p. 1359-68.
17. Yang, H., et al., *The role of Bacillus anthracis RecD2 helicase in DNA mismatch repair.* DNA repair, 2011. 10(11): p. 1121-30.

18. Servinsky, M.D. and D.A. Julin, *Effect of a recD mutation on DNA damage resistance and transformation in Deinococcus radiodurans.* Journal of bacteriology, 2007. 189(14): p. 5101-7.
19. Shadrick, W.R. and D.A. Julin, *Kinetics of DNA unwinding by the RecD2 helicase from Deinococcus radiodurans.* The Journal of biological chemistry, 2010. 285(23): p. 17292-300.
20. Saikrishnan, K., et al., *DNA binding to RecD: role of the 1B domain in SF1B helicase activity.* The EMBO journal, 2008. 27(16): p. 2222-9.
21. Toseland, C.P. and M.R. Webb, *ATPase mechanism of the 5'-3' DNA helicase, RecD2: evidence for a pre-hydrolysis conformation change.* The Journal of biological chemistry, 2013. 288(35): p. 25183-93.
22. Gupta, M.K., et al., *Protein-DNA complexes are the primary sources of replication fork pausing in Escherichia coli.* Proceedings of the National Academy of Sciences of the United States of America, 2013. 110(18): p. 7252-7.
23. Wickner, S., M. Wright, and J. Hurwitz, *Association of DNA-dependent and -independent ribonucleoside triphosphatase activities with dnaB gene product of Escherichia coli.* Proceedings of the National Academy of Sciences of the United States of America, 1974. 71(3): p. 783-7.
24. Ueda, K., R. McMacken, and A. Kornberg, *dnaB protein of Escherichia coli. Purification and role in the replication of phiX174 DNA.* The Journal of biological chemistry, 1978. 253(1): p. 261-9.
25. Klinkert, M.Q., A. Klein, and M. Abdel-Monem, *Studies on the functions of DNA helicase I and DNA helicase II of Escherichia coli.* The Journal of biological chemistry, 1980. 255(20): p. 9746-52.
26. Kumura, K. and M. Sekiguchi, *Identification of the uvrD gene product of Escherichia coli as DNA helicase II and its induction by DNA-damaging agents.* The Journal of biological chemistry, 1984. 259(3): p. 1560-5.
27. Kuzminov, A., *Recombinational repair of DNA damage in Escherichia coli and bacteriophage lambda.* Microbiology and molecular biology reviews : MMBR, 1999. 63(4): p. 751-813, table of contents.
28. Au, N., et al., *Genetic composition of the Bacillus subtilis SOS system.* Journal of bacteriology, 2005. 187(22): p. 7655-66.
29. Bolz, N.J., et al., *Residues in the N-terminal domain of MutL required for mismatch repair in Bacillus subtilis.* Journal of bacteriology, 2012. 194(19): p. 5361-7.
30. Foster, P.L., *Methods for determining spontaneous mutation rates.* Methods in enzymology, 2006. 409: p. 195-213.
31. Hall, B.M., et al., *Fluctuation analysis CalculatOR: a web tool for the determination of mutation rate using Luria-Delbruck fluctuation analysis.* Bioinformatics, 2009. 25(12): p. 1564-5.
32. Lenhart, J.S., et al., *DnaN clamp zones provide a platform for spatiotemporal coupling of mismatch detection to DNA replication.* Molecular microbiology, 2013. 87(3): p. 553-68.
33. Goranov, A.I., et al., *Characterization of the global transcriptional responses to different types of DNA damage and disruption of replication in Bacillus subtilis.* Journal of bacteriology, 2006. 188(15): p. 5595-605.

34. Saikrishnan, K., et al., *Mechanistic basis of 5'-3' translocation in SF1B helicases.* Cell, 2009. 137(5): p. 849-59.
35. Costes, A., et al., *The C-terminal domain of the bacterial SSB protein acts as a DNA maintenance hub at active chromosome replication forks.* PLoS genetics, 2010. 6(12): p. e1001238.
36. Simmons, L.A., A.D. Grossman, and G.C. Walker, *Replication is required for the RecA localization response to DNA damage in Bacillus subtilis.* Proceedings of the National Academy of Sciences of the United States of America, 2007. 104(4): p. 1360-5.
37. Berkmen, M.B. and A.D. Grossman, *Spatial and temporal organization of the Bacillus subtilis replication cycle.* Molecular microbiology, 2006. 62(1): p. 57-71.
38. Noble, J.E. and M.J. Bailey, *Quantitation of protein.* Methods in enzymology, 2009. 463: p. 73-95.
39. Li, H. and R. Durbin, *Fast and accurate short read alignment with Burrows-Wheeler transform.* Bioinformatics, 2009. 25(14): p. 1754-60.
40. Schroeder, J.W. and L.A. Simmons, *Complete Genome Sequence of Bacillus subtilis Strain PY79.* Genome announcements, 2013. 1(6).
41. Law, C.W., et al., *voom: Precision weights unlock linear model analysis tools for RNA-seq read counts.* Genome biology, 2014. 15(2): p. R29.
42. Larkin, M.A., et al., *Clustal W and Clustal X version 2.0.* Bioinformatics, 2007. 23(21): p. 2947-2948.
43. Goujon, M., et al., *A new bioinformatics analysis tools framework at EMBL-EBI.* Nucleic acids research, 2010. 38(Web Server): p. W695-W699.
44. Youngman, P., J.B. Perkins, and R. Losick, *Construction of a cloning site near one end of Tn917 into which foreign DNA may be inserted without affecting transposition in Bacillus subtilis or expression of the transposon-borne erm gene.* Plasmid, 1984. 12(1): p. 1-9.

Chapter 4

Discussion and Future Directions

Introduction

Helicases are an integral part of nucleic acid transactions including DNA replication and repair, as well as RNA transcription, among other roles, and are critical in all organisms from bacteriophages and viruses up to humans and other eukaryotes. DNA helicases are essential for the separation of DNA strands at the replication fork during ongoing genome replication, as well as central to many DNA repair processes (for review [1]). In *Bacillus subtilis* there are 26 predicted or identified helicases, some of which have undergone no experimentation (Chapter 1, Table 1.1). Prior to the work in Chapters 2 and 3, RecD2 of *B. subtilis* was almost entirely unstudied; it was found to bind Single Stranded DNA Binding protein (SSB) in a pull-down assay [2]. In the prior chapters, I have shown that RecD2, when present at its native level (~100 copies per cell), is important for replication fork stability and survival when challenged with a number of DNA damaging agents [3]. Further, overexpression of RecD2 is toxic in *B. subtilis*, and dysregulation of RecD2, whether by deletion or overexpression, induces the SOS DNA damage response.

I want to thank Lindsay Matthews for performing the glutaraldehyde cross-linking in Figure 4.1 and contributing the image.

Identification and characterization of RecD2

Historically, RecD2 helicases have been largely underexplored, with attention only given to them over the last decade [3-7]. Following the studies performed in *Deinococcus radiodurans* and *Bacillus anthracis*, we were curious whether the *B. subtilis* genome contains a RecD2 homolog, and if so, what processes it may be involved in. The studies in *D. radiodurans* showed strains lacking *recD2* are sensitive to hydrogen peroxide, UV, and gamma radiation, and had an increased transformation efficiency [5, 7]. Prior to our work in *B. subtilis*, the only biochemical characterization of RecD2 had been done using the *D. radiodurans* protein [5-11]. RecD2 was identified in *B. anthracis* and found to contribute to DNA mismatch repair, though Δ*recD2* cells were not found to be sensitive to UV [12]. These differences indicate that the function of RecD2 differs by organism, even with the high sequence identity seen between the two organisms (30%) [12].

RecD2 helicases demonstrate high sequence similarity at a primary structure level. *B. subtilis* RecD2 is quite similar to both *D. radiodurans* RecD2 (28% identical, 50% homologous) and *B. anthracis* RecD2 (57% identical, 74% homologous) (Chapter 2)[3], but given the differences in their roles *in vivo*, it was important to examine the functions of RecD2 in *B. subtilis*. The examination of RecD2 in *B. subtilis* in the prior chapters has illustrated that RecD2 plays a role in limiting spontaneous mutagenesis (Chapter 2), and that the increase in mutagenesis seen in Δ*recD2* is probably caused by the induction of the SOS response (Chapter 3) [3]. Deletion of *recD2* also renders cells sensitive to a wide variety of DNA damaging agents (Chapter 2) [3]. Importantly, it was shown that in the absence of RecD2 replication forks collapse more frequently than in

an a wild type strain (Δ*recD2, dnaB134* VS *dnaB134* alone), and in the absence of *recD2* cells show induction of the SOS response (Chapters 2, 3) [3]. In light of the knowledge that heterologous expression of *D. radiodurans* RecD2 prevented the restart of stalled replication forks in *E. coli*, overexpression of RecD2 was examined in Chapter 3 [13]. It was found that overexpression of *B. subtilis* RecD2 is toxic in the native organism, causes an increase in replication fork collapse and induction of the SOS response (Chapter 3) [3]. Both of these facts illustrate the need for RecD2 to be present at native levels (~100 copies per cell) to properly perform its function (Chapter 3). It was also demonstrated that conserved residues in the N-terminus of RecD2 are critical for its function *in vivo* (Chapter 3).

Determining the function of the RecD2 N-terminus

Moving forward, it will be interesting to address the question of the function of the N-terminus. As stated in Chapter 1, and suggested by Yang *et al.,* the N-terminus may play a role in DNA binding. This can be tested utilizing a double filter binding assay to determine whether RecD2 or mutant proteins, such as those examined in Chapter 3, are able to bind ss- or dsDNA, and can provide a quantitative measure of affinity [14]. Because RecD2(F209A) is not toxic upon overexpression while RecD2(K373A) is (Chapter 3), it would be telling if the F209A mutant were deficient for DNA binding as we would begin to be able to assign a mechanism to the overexpression toxicity. We also know that the F209A mutant is unable to mitigate sensitivity to Mitomycin C, rendering the protein non-functional in one of its best-established roles (Chapter 3). I hypothesize

that a primary function of the N-terminus is in DNA binding and it will be important to determine if this is indeed the case.

RecD2 DNA substrate specificity

Some of the eukaryotic helicases most similar to RecD2 have been shown to assist in replication fork progression through areas of high GC-content [15-17]. The DNA double filter binding assay could also be used to determine the substrate specificity of RecD2 and perhaps elucidate a clearer role for RecD2 *in vivo*. I hypothesize that based on its similarity to Rrm3p and Pif1 that RecD2 will have a preference for high GC-content DNA. Additionally, it is possible that RecD2 plays a role at very specific sites *in vivo*, as its known that Rrm3p and Pif1 of *Saccharomyces cerevisiae* tend to localize to the telomeres, but also have a primary role in preventing replication fork stalling, similar to what we show for *B. subtilis* RecD2 [3, 15-20]. ChIP-seq should be performed to determine whether RecD2 is enriched at specific genomic loci, perhaps at areas of high transcription, GC-rich or perhaps RecD2 is constitutively enriched at the replication fork *in vivo*.

Elucidating the RecD2-SSB binding site

The only binding partner to have been identified for RecD2 is single stranded DNA binding protein (SSB) [2, 3]. It is known that the C-terminus of SSB is critical for interaction with other proteins, and that it tends to bind hydrophobic pockets of its binding partners [2, 21-24]. One of the questions that remains unanswered is, where does SSB bind RecD2? There are several possible ways of addressing this. It may be

possible to identify the binding site by crosslinking SSB and RecD2 together and then digesting the protein and visualizing by mass spectroscopy. As a proof of concept, Lindsay Matthews crosslinked RecD2, SSB, and RecD2+SSB using glutaraldehyde as a crosslinking agent (Figure 4.1). Future crosslinking experiments would need to take place with a zero-length crosslinker, such as EDC to provide the highest specificity for a mass spectroscopy experiment.

An alternative method to determine the SSB binding site would be to test a peptide array of RecD2 to determine possible SSB binding sites. If SSB was found preferentially bound to specific peptides of RecD2, these amino acids could be changed by site directed mutagenesis and the disruption of RecD2-SSB binding could be confirmed by far Western blotting [3, 25, 26].

If the above methods fail, site directed mutagenesis could be performed on regions that are modeled to have hydrophobic pockets, or in the SH3 domain of the protein [8]. The SH3 domain is an attractive target as SH3 domains are known to bind regions rich in prolines, and the SSB C-terminus has 3 PF motifs [27]. Mutating the SH3 domain of RecD2 and testing SSB binding via far Western could elucidate whether that the SH3 is the SSB binding site.

Elucidating the SSB binding site of RecD2 will be beneficial in understanding how and why RecD2 is localized and may also provide an explanation of RecD2 overexpression toxicity. Were toxicity to be mitigated in a SSB-binding deficient mutant, or in an SSB overexpression mutant, it would offer credence to the idea that RecD2 is sequestering SSB and preventing it from functioning properly, allowing for endogenous DNA damage on the naked ssDNA. Though it is possible that mitigation of RecD2

overexpression toxicity by failure to bind SSB would merely impede its localization to the DNA.

Concluding Remarks

RecD2 is an accessory DNA helicase whose functions have yet to be completely explored. While its clear that RecD2 plays important roles in DNA replication and repair, we know little about substrate preference or binding partners which may regulate its activity. It is also clear that we need greater diversity of study of accessory helicases. For instance, while RecD2 helicases have been identified in over 270 different sequenced bacterial genomes, the traditional RecD helicases appear to be present in only about 20 [4, 12]. This conservation seems rather telling as RecD2 helicases have eukaryotic orthologs such as human DNA helicase B (23% identical), and *S. cerevisiae* Rrm3p (26% identical) and Pif1 (24% identical) [28, 29].

For comparison, RecD2 is one of 26 identified or predicted helicases in *B. subtilis* (Chapter 1, Table 1.1). Among them 7 have no known function. By contrast, humans encode 95 non-redundant helicases, 31 of which are DNA helicases [30]. Of the 31 identified human DNA helicases, 7 of them have variants identified with a variety of disease states such as Xeroderma pigmentosum, Cockayne's syndrome, and Bloom syndrome, among others (for review, [31]). It also seems likely that some helicases which are not yet associated with disease may acquire mutations which will contribute to genomic instability and predisposition to cancer [32]. For instance, human DNA helicase B is involved in recovery from replication stress but has no diseases associated with it [33]. It is also tempting to speculate that telomere and mitochondrial

DNA associate helicases may be associated with human disease as the absence of Pif1 in *Saccharomyces cerevisiae* and mice causes loss of mitochondrial DNA, which is correlated with aging and disease in humans [34, 35]. Pif1 family helicases are also negative regulators of telomerase; shown to bind long telomeres and prevent binding of telomerase so that shorter telomeres might be lengthened preferentially [36].

RecD2 and other accessory helicases have only begun to be explored in many organisms and there is still much to learn about their roles in DNA replication and repair.

Materials and Methods

Glutaraldehyde cross-linking

Cross-linking reactions (10 µL total volume) were performed by incubating 4 µM of RecD2 (assuming a dimer) and 4 µM of SSB (assuming a tetramer) in reaction buffer (20 mM HEPES pH 7.7, 150 mM NaCl, 1 mM DTT, and 1 mM EDTA) for 30 minutes at room temperature. Glutaraldehyde was prepared from a 50% stock using 20 mM HEPES pH 7.7 and added to the reactions at a final concentration of 0.0008%, 0.0016% and 0.0032%. The reactions were left at room temperature for 15 minutes before quenching with 1 µL of 1 M TRIS for 10 minutes. The reactions were then mixed with 2 µL of 6X SDS-loading dye and run on a 6% SDS polyacrylamide gel.

Figure

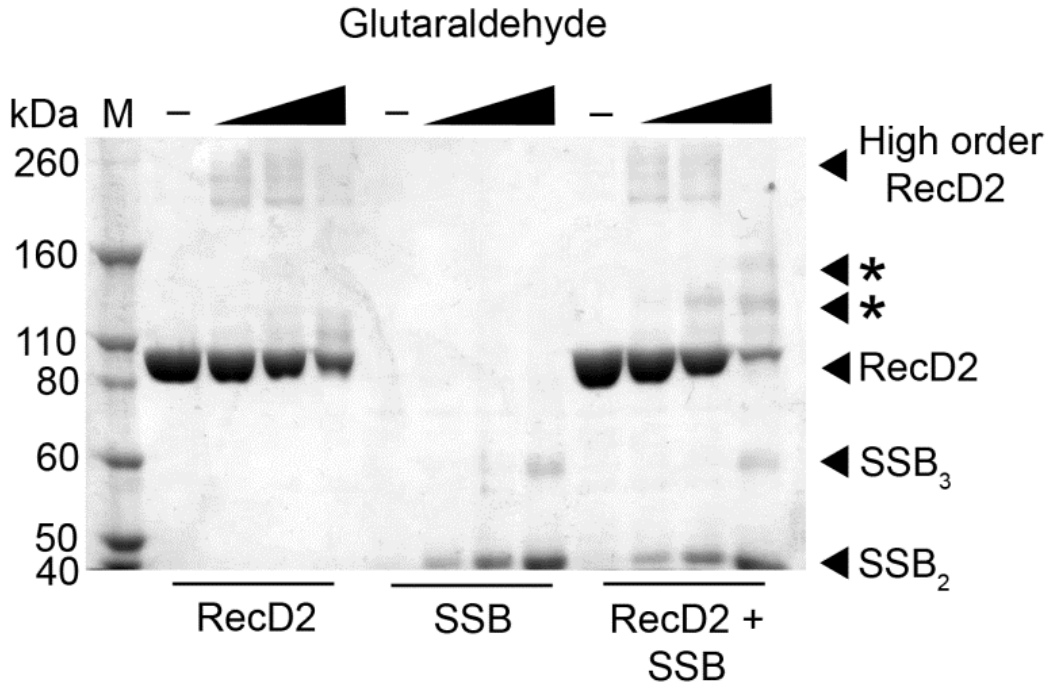

Figure 4.1. Glutaraldehyde crosslinking of RecD2, SSB, or RecD2+SSB
Glutaraldehyde was added in increasing amounts to the indicated proteins and allowed to react for 30 minutes before running on SDS-PAGE. The protein or number of crosslinked subunits is on the right. *= RecD2 crosslinked to SSB dimer or trimer.

References

1. Singleton, M.R., M.S. Dillingham, and D.B. Wigley, *Structure and mechanism of helicases and nucleic acid translocases.* Annual review of biochemistry, 2007. 76: p. 23-50.
2. Costes, A., et al., *The C-terminal domain of the bacterial SSB protein acts as a DNA maintenance hub at active chromosome replication forks.* PLoS genetics, 2010. 6(12): p. e1001238.
3. Walsh, B.W., et al., *RecD2 helicase limits replication fork stress in Bacillus subtilis.* Journal of bacteriology, 2014. 196(7): p. 1359-68.
4. Montague, M., et al., *The evolution of RecD outside of the RecBCD complex.* Journal of molecular evolution, 2009. 69(4): p. 360-71.
5. Servinsky, M.D. and D.A. Julin, *Effect of a recD mutation on DNA damage resistance and transformation in Deinococcus radiodurans.* Journal of bacteriology, 2007. 189(14): p. 5101-7.
6. Wang, J. and D.A. Julin, *DNA helicase activity of the RecD protein from Deinococcus radiodurans.* The Journal of biological chemistry, 2004. 279(50): p. 52024-32.
7. Zhou, Q., et al., *A new role of Deinococcus radiodurans RecD in antioxidant pathway.* FEMS microbiology letters, 2007. 271(1): p. 118-25.
8. Saikrishnan, K., et al., *DNA binding to RecD: role of the 1B domain in SF1B helicase activity.* The EMBO journal, 2008. 27(16): p. 2222-9.
9. Saikrishnan, K., et al., *Mechanistic basis of 5'-3' translocation in SF1B helicases.* Cell, 2009. 137(5): p. 849-59.
10. Shadrick, W.R. and D.A. Julin, *Kinetics of DNA unwinding by the RecD2 helicase from Deinococcus radiodurans.* The Journal of biological chemistry, 2010. 285(23): p. 17292-300.
11. Toseland, C.P. and M.R. Webb, *ATPase mechanism of the 5'-3' DNA helicase, RecD2: evidence for a pre-hydrolysis conformation change.* The Journal of biological chemistry, 2013. 288(35): p. 25183-93.
12. Yang, H., et al., *The role of Bacillus anthracis RecD2 helicase in DNA mismatch repair.* DNA repair, 2011. 10(11): p. 1121-30.
13. Gupta, M.K., et al., *Protein-DNA complexes are the primary sources of replication fork pausing in Escherichia coli.* Proceedings of the National Academy of Sciences of the United States of America, 2013. 110(18): p. 7252-7.
14. Wong, I. and T.M. Lohman, *A double-filter method for nitrocellulose-filter binding: application to protein-nucleic acid interactions.* Proceedings of the National Academy of Sciences of the United States of America, 1993. 90(12): p. 5428-32.
15. Ivessa, A.S., et al., *The Saccharomyces cerevisiae helicase Rrm3p facilitates replication past nonhistone protein-DNA complexes.* Molecular cell, 2003. 12(6): p. 1525-36.
16. Ivessa, A.S., et al., *Saccharomyces Rrm3p, a 5' to 3' DNA helicase that promotes replication fork progression through telomeric and subtelomeric DNA.* Genes & development, 2002. 16(11): p. 1383-96.

17. Ivessa, A.S., J.Q. Zhou, and V.A. Zakian, *The Saccharomyces Pif1p DNA helicase and the highly related Rrm3p have opposite effects on replication fork progression in ribosomal DNA.* Cell, 2000. 100(4): p. 479-89.
18. Azvolinsky, A., et al., *The S. cerevisiae Rrm3p DNA helicase moves with the replication fork and affects replication of all yeast chromosomes.* Genes & development, 2006. 20(22): p. 3104-16.
19. Bessler, J.B. and V.A. Zakian, *The amino terminus of the Saccharomyces cerevisiae DNA helicase Rrm3p modulates protein function altering replication and checkpoint activity.* Genetics, 2004. 168(3): p. 1205-18.
20. Torres, J.Z., S.L. Schnakenberg, and V.A. Zakian, *Saccharomyces cerevisiae Rrm3p DNA helicase promotes genome integrity by preventing replication fork stalling: viability of rrm3 cells requires the intra-S-phase checkpoint and fork restart activities.* Molecular and cellular biology, 2004. 24(8): p. 3198-212.
21. Marceau, A.H., et al., *Structure of the SSB-DNA polymerase III interface and its role in DNA replication.* The EMBO journal, 2011. 30(20): p. 4236-47.
22. Petzold, C., et al., *Interaction with Single-stranded DNA-binding Protein Stimulates Escherichia coli Ribonuclease HI Enzymatic Activity.* The Journal of biological chemistry, 2015. 290(23): p. 14626-36.
23. Naue, N., et al., *The helicase-binding domain of Escherichia coli DnaG primase interacts with the highly conserved C-terminal region of single-stranded DNA-binding protein.* Nucleic acids research, 2013. 41(8): p. 4507-17.
24. Naue, N. and U. Curth, *Investigation of protein-protein interactions of single-stranded DNA-binding proteins by analytical ultracentrifugation.* Methods in molecular biology, 2012. 922: p. 133-49.
25. Lenhart, J.S., et al., *Trapping and visualizing intermediate steps in the mismatch repair pathway in vivo.* Molecular microbiology, 2013. 90(4): p. 680-98.
26. Walsh, B.W., et al., *Far Western blotting as a rapid and efficient method for detecting interactions between DNA replication and DNA repair proteins.* Methods in molecular biology, 2012. 922: p. 161-8.
27. Zarrinpar, A., R.P. Bhattacharyya, and W.A. Lim, *The structure and function of proline recognition domains.* Science's STKE : signal transduction knowledge environment, 2003. 2003(179): p. RE8.
28. Altschul, S.F., et al., *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.* Nucleic acids research, 1997. 25(17): p. 3389-402.
29. Altschul, S.F., et al., *Protein database searches using compositionally adjusted substitution matrices.* The FEBS journal, 2005. 272(20): p. 5101-9.
30. Umate, P., N. Tuteja, and R. Tuteja, *Genome-wide comprehensive analysis of human helicases.* Communicative & integrative biology, 2011. 4(1): p. 118-37.
31. Suhasini, A.N. and R.M. Brosh, *Disease-causing missense mutations in human DNA helicase disorders.* Mutation Research/Reviews in Mutation Research, 2013. 752(2): p. 138-152.
32. Sharma, S., K.M. Doherty, and R.M. Brosh, Jr., *Mechanisms of RecQ helicases in pathways of DNA metabolism and maintenance of genomic stability.* The Biochemical journal, 2006. 398(3): p. 319-37.

33. Guler, G.D., et al., *Human DNA helicase B (HDHB) binds to replication protein A and facilitates cellular recovery from replication stress.* The Journal of biological chemistry, 2012. 287(9): p. 6469-81.
34. Schulz, V.P. and V.A. Zakian, *The saccharomyces PIF1 DNA helicase inhibits telomere elongation and de novo telomere formation.* Cell, 1994. 76(1): p. 145-55.
35. Bochman, M.L., N. Sabouri, and V.A. Zakian, *Unwinding the functions of the Pif1 family helicases.* DNA repair, 2010. 9(3): p. 237-49.
36. Phillips, J.A., et al., *The pif1 helicase, a negative regulator of telomerase, acts preferentially at long telomeres.* PLoS genetics, 2015. 11(4): p. e1005186.