RecD2 helicase limits replication fork stress in *Bacillus subtilis*

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ABSTRACT

DNA helicases have important roles in genome maintenance. The RecD helicase has been well-studied as a component of the heterotrimeric RecBCD helicase-nuclease enzyme important for double-strand break repair in Escherichia coli. Interestingly, many bacteria lack RecBC and instead contain a RecD2 helicase, which is not known to function as part of a larger complex. Depending on the organism studied, RecD2 has been shown to provide resistance to a broad range of DNA damaging agents while also contributing to mismatch repair (MMR). Here we investigated the importance of Bacillus subtilis RecD2 helicase to genome integrity. We show that deletion of recD2 confers a modest increase in spontaneous mutation rate, and that the mutational signature in ΔrecD2 cells is not consistent with a MMR 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 DNA damaging agents. We found that loss of RecD2 in B. subtilis sensitized cells to several DNA damaging agents that can block or impair replication fork movement. Measurement of replication fork progression in vivo shows that fork movement is slowed in ΔrecD2 cells, supporting the hypothesis that RecD2 is important for normal replication fork progression. Biochemical characterization of B. subtilis RecD2 showed it is a 5´-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 when forks encounter 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 (1, 2)]. 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 [(3) for review (2, 4-7)]. In accordance with their many roles \textit{in vivo}, helicases show tremendous diversity and have been classified into “superfamilies” based on their sequence motifs (8). Superfamily 1 (SF1) is comprised of helicases that often function in DNA repair [for review (2, 9)]. Prominent members of this family include \textit{E. coli} UvrD, Rep and RecD (10-12). The SF1 family is further sub-divided into SF1A and SF1B. SF1A helicases translocate along DNA in the 3´-5´ direction, whereas SF1B helicases translocate in the 5´-3´ direction [for review (2, 9)].

The SF1B helicases have members present in systems from bacteriophages to human (13-16). SF1B helicases have clear roles in genome maintenance although members of this family have not been nearly as well studied \textit{in vitro} or \textit{in vivo} as compared with their SF1A helicase counterparts. Examples of the SF1B helicases include bacteriophage T4 Dda (DNA-dependent ATPase), a DNA replication enzyme; \textit{E. coli} RecD, a critical component of the RecBCD helicase-nuclease 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 (14, 17, 18). 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 (15, 19). RecD2 helicases are typically found in bacteria that lack the RecBC proteins (4, 15, 19, 20). 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 (15, 19, 20) (Figure 1). The RecD2 protein from Deinococcus radiodurans has been shown to be important for resistance to gamma irradiation, hydrogen peroxide, and UV irradiation in vivo (21) and it is able to unwind short hairpin and forked DNA structures in a 5'-3' direction (22-24). A recent genome-wide screen for genes associated with a colony papillation phenotype in Bacillus anthracis identified the gene BAS4289 encoding a RecD2 homolog (20, 25). Transposon-insertion mutagenesis of recD2 led to an increased frequency of spontaneous mutation in vivo, which produced a mutation spectrum consistent with a defect in MMR (20). Unlike D. radiodurans, loss of RecD2 function in B. anthracis had no effect on UV sensitivity (20). Therefore, RecD2 in B. anthracis appears to function as a MMR helicase and has yet to be shown to contribute to cellular resistance to damage caused by exogenous sources (20). Taken together, 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 (20).

To better understand the cellular roles of RecD2 helicases, we examined RecD2 from the Gram-positive bacterium Bacillus subtilis. We found that deletion of recD2 conferred a modest increase in spontaneous mutation rate, although the mutation spectrum is inconsistent with a role in MMR. We also found that loss of recD2 function in B. subtilis sensitized cells to mitomycin C, methyl methanesulfonate, UV and the DNA break inducing peptide phleomycin. RecD2 was further shown to be important for normal replication fork progression in vivo. Biochemical
analysis of purified RecD2 showed that the helicase forms a complex with single-stranded DNA-binding protein (SSB) and that it unwinds DNA with a 5'-3' directionality. Our combined data support a model where *B. subtilis* RecD2 is recruited by SSB to help maintain replication forks during normal growth and when replication forks encounter sites of DNA damage.

**MATERIALS AND METHODS**

*Bacteriology.* All strains used in this study are derivative of PY79 and are listed in Table 1. Unless stated otherwise, isopropylthio-β-galactoside (IPTG) and antibiotics were used at the following final concentrations: 10 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 S1. The in-frame markerless deletion of *recD2* (BWW150) was created using the procedure as described (26). 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 22°C and diluted back 30 fold in LB, then grown for another 8 hours at 22°C 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 (ΔrecD2, recA-gfpA206Kmut2) was constructed by transformation of BWW150 with LAS40 (recA-gfpA206Kmut2) chromosomal DNA (27) followed by selection for spectinomycin resistance.

**Fluorescence microscopy.** Fluorescence microscopy was performed essentially as described (27). Briefly, LAS40 and BWW264 were grown in S750 minimal media + 2% glucose at 37°C with shaking to OD₆₀₀ ~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 TMA-DPH (1:1000 dilution) followed by imaging cells using an Olympus BX61 microscope as described (28-31). 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 OD₆₀₀ 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 OD₆₀₀ 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 (32). 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 S750, 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 (33) 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, followed by scoring.

**Mutation spectrum.** The mutation spectrum was generated essentially as described (33). Briefly, at least 50 independent cultures were grown in LB+ 200 µM thymidine and grown to OD₆₀₀ 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 BL21DE3 recA⁻ E. coli to generate BWW102 for overexpression of 6xHis-RecD2. 6xHis-RecD2 was overexpressed using standard procedures (31). 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. The lysate was prepared by centrifugation at 15,000 RPM for 60 min at 4°C. 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 Tris-HCl 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).
DTT) overnight at 4°C. The 6xHis tag was cleaved by addition of 250 ng Prescission protease to cleavage buffer and overnight dialysis at 4°C. Prescission protease and intact 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 -80°C.

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 (34). 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).

*Protein dot blots.* Blots were performed essentially as described (35). For probing with SSB as prey RecD2 and DnaG were applied to the membrane in the range of 12.5 to 50 pmols, while BSA was from 62.5 to 250 pmols. The membrane was then incubated with 40 pM SSB before washing and exposing to film as described (35). For the reciprocal blot, 6.25 to 25 ng of
SSB and BSA were applied to the membrane before incubating with 5 ng/ml RecD2 in binding solution as described (35). The membrane was subsequently processed and imaged as described (35).

**DNA helicase assay.** 3' and 5' overhang substrates were made as follows: oSW079 was phosphorylated by T4 polynucleotide kinase with [γ-32P]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 MgCl2. DNA substrates (~1 nM) were incubated in 50 mM HEPES-HCl, pH 7.5, 2 mM DTT, 2 mM ATP, 4 mM MgOAc2, 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 (27, 36). Cells were grown in S750 minimal media to an OD600~0.4, 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 (37) using bwa v0.5.9-r16 with the default parameters, except when running bwa samse, when we set the –n parameter to 1, as described (38). Subsequent analysis was performed using the statistical package R. Alignment data were binned into 500 base wide non-overlapping windows and log2 (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 \( -c \) 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 MMR in *B. anthracis* (20). To understand if *B. subtilis* recD2 also functioned in MMR, we constructed a clean deletion of the *recD2* (*yrrC*) coding region as previously described [“Materials and Methods” (26, 31)]. The mutation rate of the \( \Delta \text{recD2} \) strain was measured and compared to wild type *B. subtilis* and a strain deleted for the MMR genes *mutS* and *mutL* (\( \Delta \text{mutSL} \)). In *B. anthracis* loss of *recD2* increased mutation frequency ~40-fold in an assay that measures formation of rifampin resistant colonies (20). In contrast to these results, we found that the \( \Delta \text{recD2} \) *B. subtilis* had a far more modest (2.8-fold) increase in mutation rate as measured by scoring for rifampin resistant colonies (Figure 2A). Because rifampin resistance measures base-pair substitutions in the *rpoB* gene and does not measure insertions or deletions (33, 39, 40), we developed an assay that would detect
additional types of mutations in *B. subtilis* by scoring for trimethoprim resistance (32, 41). Trimethoprim inhibits dihydrofolate reductase thereby decreasing concentrations of tetrahydrofolate, a critical cofactor for cellular metabolism (41). The levels of tetrahydrofolate are further depleted by thymidylate synthase (*thyA*), which requires tetrahydrofolate for activity (42). Therefore, base-pair substitution, insertion or deletion mutations that inactive *thyA* provide enough tetrahydrofolate to allow for trimethoprim resistance and growth in the presence of thymidine (42). *B. subtilis* has two thymidylate synthetase genes, *thyA* and *thyB*, both of which would need to be inactivated to cause trimethoprim resistance (43). 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) (43). We found that ∆mutSL increased mutation rate 20-fold over wild type whereas the ∆recD2 conferred a 3.6-fold increase in mutation rate (Figure 2B). These results show that loss of *recD2* leads to a modest increase in mutation rate, although it is unclear if the increase in mutation rate is due to a decrease in MMR efficiency or by effects on other cellular pathways.

To determine if the modest trimethoprim resistance in the ∆recD2 strain resulted from impaired efficiency of MMR, we determined the mutation spectrum for trimethoprim resistance in the *thyA* gene from at least 50 independent colonies for each strain examined. We found that ∆recD2 and the ∆mutSL strains showed very different mutation spectra (Figure 3 and Table S2). The ∆mutSL spectrum consisted of transitions, insertions, and deletions, but not transversions, which is a spectrum indicative of a MMR defect (20, 33, 44, 45). In contrast the ∆recD2 mutation spectrum showed an increase in transversion mutations in addition to transitions, insertions and deletions (Figure 3 and Table S2). Considering the ~4-fold increase in mutation
rate and the thyA spectrum of ΔrecD2 strain providing a mutational signature differing from the ΔmutSL strain, we conclude that loss of RecD2 causes a mild increase in mutagenesis through a MMR-independent mechanism or multiple mechanisms.

A possible explanation for the increase in transversion mutations in the ΔrecD2 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 (46-49). Therefore, we tested the idea that the mutagenesis observed in the ΔrecD2 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* (46-50). Since DnaE is essential (51, 52), we cannot test a *dnaE* deletion to determine if loss of *dnaE* relieves the mutagenesis observed in ΔrecD2 cells. Instead, we tested alleles deficient for *polY1* and *polY2* in the ΔrecD2 background to determine if the ΔrecD2 mutagenesis was dependent on either Y-family polymerase. We found that loss of *polY1*, *polY2* or both led to mutation rates that were within error of the ΔrecD2 strain (Figure 2C). Therefore, because disruption of the Y-family polymerases did not reduce the ΔrecD2 conferred mutagenesis we speculate that DnaE may be responsible for the moderate mutagenesis observed in the absence of RecD2 helicase.

DnaE-GFP foci are elevated in ΔrecD2 cells.

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* (30, 53). Since the Y-family polymerases were not involved in the observed mutagenesis and do not localize as foci (data not shown), we asked if the percentage of cells with DnaE-GFP foci was elevated in cells deleted for
ΔrecD2. Indeed, we found that the percentage of cells with DnaE-GFP foci were increased in cells deleted for recD2 (Table 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 increase in transversion mutations observed in ΔrecD2 cells (Figure 3 and Table 2).

It was 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 MMR (20). Our results, in consideration with the data from B. anthracis (20), shows that RecD2 functions differently in B. subtilis as compared with B. anthracis. Because B. subtilis RecD2 does not appear to have a role in MMR, the experiments described below were performed to understand if RecD2 is important for replication or repair in B. subtilis. We found that B. subtilis RecD2 is important for fork maintenance during normal growth and in response to DNA damage. RecD2 is important for resistance to mitomycin C, methyl methanesulfonate, and phleomycin. D. radiodurans RecD2 is important for resistance to DNA damage (17, 21).

Therefore, we asked if ΔrecD2 B. subtilis cells were also sensitive to DNA damage. We performed spot plate assays where an equal amount of cells were plated on LB agar containing methyl methanesulfonate, mitomycin C, phleomycin, or hydrogen peroxide (H₂O₂) (Figure 4). In addition, we performed a spot plate analysis where cells were serial diluted followed by challenge with UV-irradiation (Figure 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 (NER) and have
known sensitivities to DNA damaging agents [for review (4)]. Consistent with earlier work, the
uvrA::spc strain was sensitive to UV and mitomycin C treatments, but not methyl
methanesulfonate (54) (Figure 4A). The \( \Delta \text{recD2} \) strain was not sensitive to H\(_2\)O\(_2\), but showed a
sensitivity to UV and methyl methanesulfonate (Figure 4A). Treatment with H\(_2\)O\(_2\) generates
small 8-oxo-G lesions following exposure, while UV exposure generates pyrimidine dimers,
which can cause replication stress [for review (55)]. Methyl methanesulfonate primarily adds
methyl groups to guanine and adenine bases (forming 7-methylguanine and 3-methyladenine),
which damages DNA and can cause severe blocks to replication (56). In \( B. \text{subtilis} \), we found
that the \( \Delta \text{recD2} \) strain was also sensitive to mitomycin C. The crosslinks that form as a result of
mitomycin C treatment are repaired by NER and homologous recombination [for review (57)]
and are known to block replication in \( B. \text{subtilis} \) cells (58).

We challenged cells with phleomycin to generate single and double stranded breaks and
found that the \( \Delta \text{recD2} \) allele conferred sensitivity. As a control we show \( \text{recD2} \) ectopically
expressed from an IPTG regulated promoter complements the phleomycin sensitivity of \( \Delta \text{recD2} \)
cells (Figure 4B). These results show that \( \Delta \text{recD2} \) cells are sensitive to 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 \( B. \text{subtilis} \) \( \Delta \text{recD2} \) strain is
sensitive to a wide range of damaging agents that can impose blocks to replication. Furthermore,
the \( \text{recD2} \) deletion in \( B. \text{subtilis} \) shows very little overlap in function to a \( \text{recD2} \) deficiency in
either \( D. \text{radiodurans} \) or \( B. \text{anthracis} \). We discuss these differences in greater detail below.

Cells deleted for \( \text{recD2} \) show replication fork stress. The results presented above suggest that
\( B. \text{subtilis} \) \( \text{recD2} \) has a role in genome maintenance when cells experience DNA damage, which
is strikingly similar to the role of human helicase B (hDHB) (17). Prior work reported that ectopic expression of YFP-RecD2 (YrrC) colocalized with the replisome in \textit{B. subtilis} [(59) and for review (4)]. This result suggests that RecD2 may be present at the fork during normal growth.

To determine the subcellular localization of RecD2 we fused \textit{recD2} to several different C-terminal fluorescent protein fusions followed by imaging in cells damaged or left untreated. RecD2 with a functional C-terminal fusion expressed from its native promoters did not form distinct foci that we could observe by standard epifluorescence microscopy (Figure 4B and 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 or absence of RecD2.

We have previously shown that RecA-GFP foci formation is an \textit{in vivo} marker for replicative stress in \textit{B. subtilis} (27, 30, 60, 61). We define replicative stress as any perturbation to the replication fork that increases exposed ssDNA. Thus, we imaged RecA-GFP in the \textit{ΔrecD2} 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\%) (\textit{p}<0.001) (Table 3). Following DNA damage with phleomycin, an agent that the \textit{ΔrecD2} 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 3). These results suggest that in the absence of \textit{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 \textit{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 (see “Material and Methods”). Exponential-phase \textit{B. subtilis} cells display
a linear decrease in log2-transformed sequencing coverage with a peak at the origin of replication in whole genome sequencing experiments (Figure 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 (Figures 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 log2-transformed coverage data to a quadratic model, we found that the $\Delta$recD2 data yielded a more extreme fork collapse factor (FCF) than the control, indicating that replication forks collapsed more frequently in the strain lacking RecD2 (Figures 5D and 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′-3′ helicase.** Because we found that RecD2 is important for fork progression and *B. subtilis* RecD2 helicase was originally identified as an interaction partner of SSB (59) we asked if RecD2 directly binds SSB. To this end, 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 (62)) and BSA (negative control) in increasing amounts on a nitrocellulose membrane and incubated with SSB; SSB was detected using an SSB antiserum as described (30, 31, 35) (Figure 6A right panel). 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. We suggest that SSB interaction may mediate recruitment of RecD2 to replication forks in *B. subtilis* where it functions. Interestingly, our results are similar to results found in human cells where several agents that cause replication fork stress cause SF1B helicase DHB to localize to chromatin through interaction with replication protein A (RPA) the eukaryotic analog to bacterial SSB (17). 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’-3’ direction, meaning that they preferentially unwind 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’- 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 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 6B lanes 1-6, Figure 6C). A RecD2 K373A variant, which is predicted to be defective in ATP hydrolysis, has no detectable helicase activity (Figure 6B, lane 8). Our data indicate that RecD2 is an ATP hydrolysis-dependent 5’-3’ helicase.

**Plasticity of RecD2 helicases in bacterial organisms.** RecD2 helicases are commonly found in bacteria that lack the RecBCD helicase-nuclease pathway. 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 MMR and
resistance to oxidative damage, which is different from our findings with *B. subtilis* RecD2 (20, 21). Taking these studies into consideration, it appears that RecD2 helicases are important for genome integrity but that the precise roles for RecD2 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* (15). If so, evolutionarily diverse RecD2 enzymes may have adapted to function in different replication or repair functions where 5’-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 Δrep 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 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 with 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.*
This work was supported by National Science Foundation grant MCB1050948 to L.A.S., National Institutes of Health grant GM098885 to J.L.K., and a National Science Foundation REU supplement to S.A.B. S.R.W. was supported in part by an NIH training grant in Molecular Biosciences (GM07215). J.W.S. was supported in part by NIH Genetics Training Grant at the University of Michigan (T32 GM007544). We thank members of the Simmons and Keck laboratories for helpful discussions and comments on the manuscript. In particular, we would like to thank Justin Lenhart for help with the trimethoprim resistance assay and for purified DnaG. J.L.K. is cofounder of Replisoma, Inc.
REFERENCES


Figure 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 (16, 20, 24, 65). *B. subtilis* and *B. anthracis* RecD2 share 57% amino acid identity and 74% homology, whereas *B. subtilis* and *D. radiodurans* RecD2 share 28% amino acid identity and 50% homology.

Figure 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 (31, 66-69). (A) Mutations per generation for the wild type strain PY79, ΔrecD2, and ΔmutSL when plated on rifampin from at least 50 independent cultures; (B) mutations per generation of PY79, ΔrecD2, and ΔmutSL 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, ΔrecD2 and ΔmutSL shown in A are also shown in C.

Figure 3. Trimethoprim mutation spectra of wild type, ΔrecD2 and ΔmutSL *B. subtilis* cells. The DNA sequence of the thyA gene of *B. subtilis* strain PY79 is shown. Wild type (green), ΔmutSL (purple), and ΔrecD2 (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 more detail in Table S2.
Figure 4. Cells deleted for recD2 confer sensitivity to mitomycin C, methyl methanesulfonate, 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 ΔrecD2 with ectopic expression of recD2 from the amyE locus with the IPTG inducible promoter (Pspac). Serial dilutions of the indicated strains were plated on LB agar, LB agar with phleomycin, or LB agar with phleomycin and 10 µM IPTG.

Figure 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. Log2 (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 of two independent experiments. (C) Same as in B, except the genotype is ΔrecD2, 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 6. RecD2 binds SSB and is a 5´-3´ helicase. (A) Immunodot blot of 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 (“Materials and Methods ” (30)). (B) RecD2 unwinding of 5´ or 3´ tail-containing DNA substrates. RecD2 (0-2 nM) or RecD2(K373A) (2 nM) were incubated with 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 total intensity of the lane.
Table 1. List of *B. subtilis* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PY79</td>
<td>SPβ^o</td>
<td>(70)</td>
</tr>
<tr>
<td>LAS4</td>
<td>yqjW::kan</td>
<td>(33, 48)</td>
</tr>
<tr>
<td>LAS5</td>
<td>yqjH::tet</td>
<td>(33, 48)</td>
</tr>
<tr>
<td>LAS24</td>
<td>recA::neo</td>
<td>(71)</td>
</tr>
<tr>
<td>LAS40</td>
<td>recA-gfpA206Kmut2</td>
<td>(27)</td>
</tr>
<tr>
<td>LAS409</td>
<td>uvrA::spe</td>
<td>(54)</td>
</tr>
<tr>
<td>BWW132</td>
<td>ΔmutSL</td>
<td>(72)</td>
</tr>
<tr>
<td>AK74</td>
<td>amyE::P_sac dnaE-gfp</td>
<td>(30)</td>
</tr>
<tr>
<td>BWW150</td>
<td>ΔrecD2</td>
<td></td>
</tr>
<tr>
<td>BWW264</td>
<td>ΔrecD2, recA-gfpA206Kmut2</td>
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</tr>
<tr>
<td>BWW281</td>
<td>ΔrecD2, yqjW::kan</td>
<td></td>
</tr>
<tr>
<td>BWW282</td>
<td>ΔrecD2, yqjH::tet</td>
<td></td>
</tr>
<tr>
<td>BWW283</td>
<td>ΔrecD2, yqjH::tet, yqjW::kan</td>
<td></td>
</tr>
<tr>
<td>BWW307</td>
<td>amyE::P_sac dnaE-GFP, ΔrecD2</td>
<td></td>
</tr>
<tr>
<td>JWS162</td>
<td>dnaB134 zhb83::Tn917 (tet)</td>
<td></td>
</tr>
<tr>
<td>JWS194</td>
<td>ΔrecD2, dnaB134 zhb83::Tn917 (tet)</td>
<td>(5)</td>
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All strains used are derivatives of PY79.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Condition</th>
<th>Percentage of cells with foci+95%CI</th>
<th>One-tailed p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P_{\text{psd}dnaE-gfp}</td>
<td>untreated</td>
<td>42±2.5 (n=2,072)</td>
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</tr>
<tr>
<td>ΔrecD2, P_{\text{psd}dnaE-gfp}</td>
<td>untreated</td>
<td>54±2.4 (n=2,587)</td>
<td>1.7E-12</td>
</tr>
</tbody>
</table>

Cells were grown in S750 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 (31, 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 ΔrecD2, DnaE-GFP cells to wild type DnaE-GFP cells (1.7E-12).
Table 3. RecA-GFP foci are elevated in cells deleted for RecD2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Condition</th>
<th>Total cells scored</th>
<th>Percentage of cells with foci</th>
<th>Two-tailed p-value</th>
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<tr>
<td>recA-gfp</td>
<td>untreated</td>
<td>460</td>
<td>14±3</td>
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<td>∆recD2, recA-gfp</td>
<td>untreated</td>
<td>508</td>
<td>25±4</td>
<td>5.16E-6</td>
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<td>recA-gfp</td>
<td>phleomycin</td>
<td>502</td>
<td>21±4</td>
<td>1.97E-3</td>
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<tr>
<td>∆recD2, recA-gfp</td>
<td>phleomycin</td>
<td>607</td>
<td>39±4</td>
<td>1.86E-19</td>
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Cells were grown in S750 defined minimal medium as described (27, 29, 30, 60, 61). 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 ∆recD2, recA-gfp for the phleomycin challenged samples were significant with p=2.13X10$^{-6}$.
Figure 1.

B. subtilis RecD2 (YrrC) 798 AA
B. anthracis RecD2 778 AA
D. radiodurans RecD2 715 AA
E. coli RecD 608 AA
E. coli UvrD 720 AA

- RecD2 conserved domains
- Pin region
- Oligo binding
- ATP binding and hydrolysis
- Oligo binding and ATP binding and hydrolysis
- SH3 domain protein-protein interaction
- Conformational change
- Conserved region of unknown function
- Motor domain
Figure 2.

Mutation Rate (mutations/generation)

A

B

C
Figure 4.

A

PY79
ΔrecD2
ΔmutSL
uvrA::spc
recA::neo
LB 10 ng/ml MMC
20 ng/ml MMC
50 ng/ml Phleomycin 10 J/m² UV
50 J/m² UV
100 µg/ml MMS

PY79
ΔrecD2
ΔmutSL
uvrA::spc
recA::neo
100 µg/ml MMS
100 ng/ml Phleomycin
20 ng/ml MMC
50 J/m² UV
1 mM H₂O₂

B

PY79
ΔrecD2
ΔmutSL
uvrA::spc
recA::neo
RecD2-GFP
ΔrecD2, amyE::recD2
LB 100 ng/ml phleomycin +10 µM IPTG
100 ng/ml Phleomycin
20 ng/ml MMC
50 J/m² UV
1 mM H₂O₂
Figure 5.

<table>
<thead>
<tr>
<th>Chromosomal Arm</th>
<th>Strain</th>
<th>FCF (x10^{-14})</th>
<th>95% CI (x10^{-14})</th>
<th>Fold change</th>
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<tbody>
<tr>
<td>Right</td>
<td>dnaB134</td>
<td>3.4</td>
<td>2.64-8.1</td>
<td>2.1</td>
</tr>
<tr>
<td>ΔrecD2, dnaB134</td>
<td>7.2</td>
<td>6.4-8.1</td>
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</tr>
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</table>

Wild type, log-phase

$\Delta r^2 = 0.81$

$\Delta r^2 = 0.61$

$\Delta r^2 = 0.75$
Figure 6.

A

SSB; anti-SSB

RecD2
DnaG
BSA

RecD2; anti-RecD2

B

<table>
<thead>
<tr>
<th>[RecD2] nM</th>
<th>0</th>
<th>0.05</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>2 K373A</th>
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<tbody>
<tr>
<td>Substrate</td>
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<td>5'</td>
<td>5'</td>
<td>5'</td>
<td>5'</td>
<td>5'</td>
<td>3'</td>
</tr>
</tbody>
</table>

C

% Substrate Unwound

0.01 0.1 1 10

[RecD2] nM