RecO Couples the Assembly of RecA-Mediated DNA Repair Complexes to Replication Forks In *Bacillus subtilis*

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Undergraduate Honors Thesis in Biology
December 1, 2011

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Abstract

All organisms have evolved efficient DNA repair pathways to cope with constant endogenous and exogenous stresses that create nicks, gaps and other lesions in their DNA. Double strand breaks (DSBs) are one of the most lethal forms of DNA damage and are repaired by a form of homologous recombination known as double strand break repair. RecA is a recombinase that is required for homologous recombination and is a positive regulator of the DNA damage response. In addition to being highly conserved amongst bacterial systems, Rad51 and Dcm1 are RecA homologs that are critical for genome maintenance in eukaryotic systems. The mechanism responsible for RecA loading during double strand break repair is well understood in the Gram-negative bacterium *Escherichia coli*. However, the pathway responsible for RecA loading in *B. subtilis* is unclear and the recombination mediator proteins involved are not well understood.

In this work, we used a RecA-GFP fusion to monitor the localization of RecA-GFP into foci in response to DNA damage in actively replicating cells. Using this approach as an *in vivo* assay for RecA loading, we found that the protein RecO is obligatory for recruitment of RecA-GFP into foci in replicating cells. Furthermore, we tested several mutant forms of RecO and quantified their effect on RecA-GFP localization *in vivo.* Taken together, our results show that RecO is required for RecA-GFP localization at the replication fork, suggesting

that RecO couples homologous recombination to DNA replication fork status in live cells.

Introduction

DNA repair pathways are critical for maintaining genomic integrity in all domains of life [for review [1]]. Many different DNA repair pathways exist, and each are activated upon recognition of a specific form of DNA damage [for review [2]]. DNA double strand breaks (DSBs) are the most detrimental type of DNA damage to the cell and a single, unrepaired DSB is lethal [e.g. [3, 4]]. In addition to being cytotoxic, DSBs can also lead to deletions and rearrangements. In eukaryotic systems, genome rearrangements have the potential to cause tumor formation [5, 6], and in prokaryotic systems, rearrangements can reduce cell fitness or cause cell death [6, 7]. DSBs are caused by a variety of DNA damaging agents such as gamma radiation, and toxic chemicals including phleomycin [8, 9]. For example, when the DNA replication complex (replisome) encounters a nick or a gap in the template strand, a DSB is formed and the replication fork is inactivated [for review [10]]. In order maintain genome integrity, it is imperative that DSBs are repaired in order to reactivate the replication fork and allow for the resumption and completion of chromosomal DNA replication [7].

Non-homologous end joining (NHEJ) and homologous recombination are two different pathways that are used to repair DSBs *in vivo* [11]. While NHEJ is a less complicated form of DSB repair [for review [12, 13]], it is also error-prone and limited to phases when bacterial and eukaryotic cells are either quiescent or have one genome copy [5]. In contrast to NHEJ, homologous recombination is very accurate, and this pathway repairs DSBs during S phase and G2, when

cells are either actively duplicating their genomic DNA or contain a second copy [5, 11, 14].

RecA is a highly conserved recombinase that is critical for several DNA repair pathways as well as the SOS response, the transcriptional response to DNA damage in many bacterial organisms [15, 16]. Bacterial RecA is homologous to Rad51 and Dcm1 in eukaryotes which, when mutated, are embryonic lethal in mammals [17]. In all organisms, RecA has three main functions: 1) facilitating strand exchange during homologous recombination 2) stabilizing stalled replication forks and 3) regulating the SOS-transcriptional response via inactivation of the LexA repressor. By promoting its self-cleavage, LexA controlled genes become de-repressed [1]. As a critical protein to cell survival following DNA damage or replication fork perturbations, RecA is structurally and mechanistically well conserved between prokaryotic and eukaryotic systems and it is the founding member of the RecA/Rad51/Dcm1/ RadA superfamily of proteins [18].

During homologous recombination, the 5´ ends on each side of the break are digested by an exonuclease, leaving two exposed 3´ overhangs of single stranded DNA (ssDNA) [1]. Single stranded binding protein (SSB) subsequently binds to and stabilizes the ssDNA regions [19]. Recombination mediator proteins (RMPs) [20] are then recruited to the site of the DSB through a largely unknown mechanism [21-25]. With the help of RMPs through another unknown mechanism [20, 22], RecA is then recruited to the site of the DSB, where it displaces SSB in complex with ssDNA [21-25]. RecA facilitates strand invasion of

the 3´ end into an undamaged homologous region of an intact double stranded DNA (dsDNA), eventually resulting in two undamaged molecules of dsDNA following resolution of the crossover species [for review [1], [26]], (see Fig. 1 for model).

There are two pathways that operate via homologous recombination: double strand break repair and daughter strand gap repair [for review [15, 27, 28]]. In *Escherichia coli,* daughter strand gap repair mends breaks or gaps opposite lesions that occur in one of the strands of DNA [29, 30]. Due to SSB's high affinity for ssDNA, it immediately binds to the ssDNA opposite from the gap, presenting an obstacle to RecA filament formation on ssDNA that must be overcome in order for strand pairing, and ultimately repair, to occur [31]. To aid in this process, *E. coli* RecA loading is facilitated by the RecFOR proteins *in vitro* [20, 32]. In *E. coli*, RecO is known to interact with RecR and also binds the C-terminal domain of SSB, suggesting that RecOR complex could be recruited to daughter strand gaps *in vivo* by interaction between RecO and SSB [33]. Biochemically, RecOR binds to SSB protein, but does not remove it from ssDNA, to form a RecOR/SSB/ssDNA substrate that RecA can more efficiently nucleate. This allows RecA to polymerize and form a nucleoprotein filament on ssDNA that displaces SSB [33].

In *E. coli* DSB repair, RecBCD translocase is used. This is a protein complex that is not present in Gram-positive bacteria, including *B. subtilis* [for review [34-36]]. The RecBCD enzyme is active in the presence of double stranded DNA ends [22, 37, 38]. As a complex, RecBCD possesses helicase and

exonuclease activity to process blunt double stranded ends and to recruit and load RecA onto RecBCD generated ssDNA [22, 37, 38]. In the absence of RecBCD, the RecF pathway can, under very rare circumstances, facilitate DSB repair in a mechanism that uses recombination mediator proteins similar to those used in Gram-positive prokaryotic DSB repair [39]. However, the RecBCD pathway predominates 99% of the time in the repair of DSBs and does not participate in daughter strand gap repair [22, 37, 38].

Though these two pathways are well studied biochemically in the Gram-negative model bacterium *E. coli* [for review [1]], the roles that the RecBCD and RecFOR pathways have in DNA repair are not well conserved or understood outside of *E. coli* and its close relatives [35, 36]. *B. subtilis* is a Gram-positive bacterium that serves as a model organism to study DNA repair pathways that are conserved with those of more complex organisms and less experimentally tractable Gram-positive bacterial systems [40]. The recombination mediator proteins that facilitate DSB repair in *B. subtilis* are homologous to those used in daughter strand gap repair in *E. coli*, a distinctly different pathway used to repair a different form of DNA damage [35, 36].

In *B. subtilis*, upon the occurrence of a DSB, AddAB complex, the analogue of RecBCD in *E. coli* [41-45], is recruited to the DSB through an unknown mechanism, where it digests the 5´ ends, leaving an exposed 3´ end of ssDNA for RecA loading [46-48]. In *E. coli*, RecBCD is known to interact and physically recruit RecA to the site of the DSB [49]. In *B. subtilis*, there is no evidence that AddAB binds RecA or functions to load it, directly or indirectly [35,

36, 46]. Thus, it is currently unknown how RecA is recruited to sites of DSBs *in vivo* and what proteins facilitate RecA loading onto ssDNA in Gram-positive systems *in vivo* or *in vitro*.

Recent work in *B.* subtilis has shown a connection between DNA replication status and RecA-GFP localization *in vivo*, suggesting that DNA replication may have a role. It has been shown that DNA replication is required to elicit RecA-GFP focus formation in response to DNA damaging agents, including a site-specific DSB generated from the homing endonuclease I-SceI [8]. This work suggests that the movement of a replication fork to the site of a DSB is required for RecA to organize into a repair focus, and that a protein component present at the replication fork could function in RecA loading and nucleate formation of RecA-GFP foci in live cells [8]. This mechanism is different from *E. coli* in that replication fork movement does not appear to be required for RecA-GFP focus formation *in vivo* [50]. The mechanism and protein components responsible for the DNA replication-dependent localization of RecA-GFP in response to DSBs have remained unknown.

Some current evidence suggests that RecO and/or SSB could mediate the RecA-GFP localization response to DSBs in *B. subtilis* [21]. Unlike in *E. coli* where both RecO and RecR cooperate to load RecA onto SSB coated ssDNA *in vitro,* in *B. subtilis*, RecO can operate in the absence of RecR [23, 24, 32]. It has also been shown in *Saccharomyces cerevisiae* that Rad51 localization is blocked in a *rad52* (eukaryotic RecO analog) mutant, suggesting that the eukaryotic RecO analog can couple its cognate recombinase to DNA replication fork status

[51]. In addition, RecO has been shown to interact with the C-terminal domain of SSB proteins suggesting that SSB could mediate localization of RecO, and thus RecA, to ssDNA at DSB sites *in vivo* [21]. The observations described above suggest that RecO may help recruit RecA to SSB coated ssDNA *in vivo,* mediating the formation of RecA-GFP foci in response to DSBs. However, current published data show that RecA-GFP localization in unaffected in a *recO* mutant and thus, the mechanism coupling RecA-GFP to DNA replication is unknown in *B. subtilis* [6].

Here we investigated the mechanism(s) that signals RecA-GFP foci to form in response to DSBs in live *B. subtilis* cells. We took a candidate approach and hypothesized that SSB and/or RecO could be required to couple RecA-GFP focus formation to DNA replication status in *B. subtilis*. Since the gene encoding SSB is essential for DNA synthesis, we asked if *recO* was necessary to observe the organization of RecA-GFP into foci following DNA damage *in vivo*. We show that cells challenged with DNA damaging agents show an increase in the percentage of cells with RecA-GFP foci, confirming previously published data [6, 8, 52, 53]. In striking contrast, we found that RecA-GFP focus formation was almost completely blocked in untreated cells and cells challenged with exogenous DNA damage that are deficient for *recO*. When the *recO::cat* deficiency was complimented by expressing *recO*$^{+}$ from an ectopic locus, we observed the complete restoration in the percentage of cells with RecA-GFP foci in response to two different types of DNA damage. Furthermore, we investigated two other proteins, RecF in the RecFOR pathway, and RecN, a DSB repair

protein. We found that while RecA-GFP foci were slightly abnormal in *recN* deficient cells and there was a synthetic growth effect in *recF* deficient cells, RecA-GFP was still able to localize. This shows that loss of RecA-GFP foci in *recO* deficient cells is specific to RecO and not simply caused by a deficiency of any protein in the RecFOR or recombination pathway. To identify the region of RecO that is important for targeting RecA-GFP foci to replication forks, we constructed and examined several *recO* mutant alleles for supporting RecA-GFP foci. We made three different mutations in *B. subtilis* RecO based on the published crystal structure of RecO in *D. radiodurans* [54]. These mutations were hypothesized to change different biochemical activities of RecO in an effort to understand the functional activities required for RecA-GFP recruitment. Residues that seemed important for DNA binding and protein interactions in other prokaryotic systems were shown to not be required in *B. subtilis* RecO for RecA-GFP recruitment. Together, our results demonstrate that RecO is required to couple RecA-GFP foci to DNA synthesis and DSB repair in *B. subtilis.*

Materials and Methods

Bacteriological methods

The bacterial plasmids and strains used in this project are listed in Table 1 and Table 2 respectively. Where indicated, the following antibiotic concentrations were used: 5 µg/mL chloramphenicol (cat), 100 µg/mL spectinomycin (spc), 12.5 µg/mL tetracycline (tet), 0.5 µg/mL erythromycin (erm), 100 ng/mL MMC, 3 µM phleomycin and 5 µM IPTG [40, 55].

Live Cell Microscopy

Microscopy of live cells was done essentially as described [56]. Briefly, frozen stocked cells were struck on plates containing the appropriate antibiotics and incubated overnight at 30 °C. A starting culture was inoculated in 1X defined $S7_{50}$ minimal media supplemented with 0.2% glucose and grown to an optical density 600 nm (OD_{600}) of 0.35-0.50 as described [57, 58]. Cultures were split and 100 ng/mL mitomycin C (MMC) or 3 µM phleomycin was added to one culture, while the other was left untreated as a control. Both cultures were then grown for an additional hour, resulting in an OD_{600} between 0.6 and 0.7. Following incubation, 300 µL aliquots of cells were stained with 0.3 µL of the vital membrane stain, FM4-64, and placed onto 1% agarose pads made with 1X spizizen salts. Cells were imaged with an Olympus BX61 microscope using an Olympus 100X oil immersion 1.45 NA TIRFM objective lens. Membrane images were taken using TRITC at a gain of 40 and an exposure length of 70 ms. GFP foci were imaged with a gain of 20 and exposure length of 175 ms using FITC

excitation. Imaging for each strain was performed at least three independent times on different days.

Plasmid construction

pEB1 (P_{spac}-*recO)* was constructed by PCR amplification of the 765 nucleotide *recO* coding sequence using the following primers: forward, 5´-GGGCCAAGCTTAAGGAGGTATACATATGACAAAATGTGAGGGATCGTTCTT; reverse, 5´-GGGACATGCATGCTTAACTTTTGTTTTCACCCATAAGATGTTT. The PCR product was digested with SphI and HindIII, the same enzymes used to digest pDR66 [59]. Plasmid pEB1 was then constructed by ligation of the *recO* coding region with double digested pDR66. The resulting plasmid allows for integration and expression of *recO* from a P_{spac} promoter at the *amyE* locus.

pEB2 (P_{spac}-*recON13E*) was constructed by PCR amplification of pEB1 using primers to create an *N13E* missense mutation using the following primers: forward, 5´- CGCACAGAAGATTACGGAGAGACGAATAAAATCG ; reverse, 5´-GTAATCTTCTGTGCGAAGAACGATCCCTTC .

pEB3 (P_{spac}-*recOR37E*) was constructed by PCR amplification of pEB1 using primers to create an *R37E* missense mutation using the following primers: forward, 5´- GTTATGGCAGAAGGCGCCAAAAAACCG ; reverse, 5´-CGGTTTTTTGGCGCCTTCTGCCATAACACCTATTTTTCC .

pEB4 (P_{spac}-*recOΔ24* in pDR110) was constructed by PCR amplification of the 693 base pair *recO* coding sequence removing the sequence coding for the last 24 amino acids of RecO using the following primers: forward 5´-GGGCCCAAGCTTAAGGAGGTATACATATGCTGACAAAATGTGAAGGGA

TCGTTCTT ; reverse, 5´- GGGACATGCATGCTTATCCCGAATATTCTT CGTAATATAAATCAAT. The PCR product was digested with SphI and HindIII, the same enzymes used to digest pDR66. Plasmid pEB4 was then constructed by ligation of *recO* coding region with double digested pDR110. The resulting plasmid allows for integration and expression of *recO* from a P_{spac} promoter at the *amyE* locus using a spectinomycin antibiotic marker.

pEB5 (P_{spac}-*recOΔ24* in pDR66*)* was constructed by the double digestion of *recOΔ24* in pEB4 and pJS2 (P_{spac}-*mutL* in pDR66) using SphI and HindIII followed by gel purification and ligation of double digested *recOΔ24* and pDR66. The resulting plasmid allows for integration and expression of *recOΔ24* from a P_{spac} promoter at the *amyE* locus using a chloramphenicol antibiotic marker.

pEB6 was constructed by Sequence Ligation-Independent Cloning (SLIC) of *recO* into pET28aPB [60]. The following primers were used for PCR amplification of the entire *recO* coding region. pET28PB: forward, 5´- GGATCCGGGCCCCTGG ; reverse, 5´- GAAAACAAAAGTTAATAA GCACCACCACCACCACC. *recO*: forward, 5´- CCAGGGGCCCGGATCCATG CTGACAAAATGTGAAGGGAT ; reverse, 5´- GGTGGTGGTGGTGCTCGAGTTA TTAACTTTTGTTTTCACCCATAAGATGTTTC. Briefly, 1 µg of linear pET28aPB (PCR amplified) and 1 µg of *recO* were separately treated with 0.5 U of T4 DNA polymerase, 2 µL 10X NEBuffer2 and 0.2 µL 100X BSA, and incubated at room temperature for 30 minutes. The reaction was stopped by adding 2 µL of 10 mM dCTP to each reaction. For the annealing reaction, a 1:1 insert :vector (0.074 pmol of vector DNA and 266 ng *recO*) in NEBuffer2 were combined, brought to

10 µL with ddH_2O and left to incubate for 30 minutes at 37 ºC. Subsequently, 5 µL of the annealing reaction was added to 150 µL of $CaCl_2$ chemically competent *E. coli* cells (MC1061) for transformation.

All constructed clones were sequenced prior to use by the University of Michigan core sequencing facility (http://seqcore.brcf.med.umich.edu/). The following primers were used for sequencing verification of plasmid construction and the *recO* coding region. pDR66: forward, 5´- CTACATCCA GAACAACCTCTGCTAAAATT ; reverse, 5´- GACTCAAACATCAAATCTTACAAA TGTAGTCTTTG. pDR110: forward, 5´- GCTCGAGGGTAAATGTGAGCA; reverse, 5´- CGACTCAAACATCAAATCTTACAAATGTAGTCTTTG. pET28aPB: currently unsequenced.

Construction of *B. subtilis* strains with *recO* ectopic expression

Restriction enzymes were used according to the manufacturer's recommendations (New England Bio labs, Ipswich MA). The gene was amplified via PCR and purified using a commercially available DNA purification kit (Qiagen). Wild type competent *B. subtilis* cells (PY79) were made by inoculating 2 mL LM broth (2 mL LB, 6 mM $MgSO_4$) with a single colony for 3 hours and taking 0.5 mL of that culture and inoculating 10 mL of MD medium (9.12 mL 1.1X PC buffer, 0.8 mL 25% glucose, 100 µL 5mg/mL tryptophan, 50 µL 2.2 mg/mL ferric ammonium citrate, 250 µL 100 mg/mL potassium aspartate, 30 µL 1M $MgSO_4$, 50 µL10 mg/mL phenylalanine) followed by continued growth at 37 ºC for 5 hours. 150 ng of plasmid DNA was used to transform competent *B. subtilis* cells for 1 hour at 37 ºC and plated on Luria Burtani (LB)+agar plates containing

the appropriate antibiotic for selection. Following colony purification, single isolates were plated on starch plates followed by treatment with iodine (actual solution) to confirm disruption of the amylase gene (*amyE*) by double crossover as described [40]. PY79 was used as a negative control and LAS47 (*mutS::mutS-820-gfp* ; *amyE::*P_{spac}*-mutL*) was used as a positive control in a screen for double crossover integration at *amyE*. Two mL iodine was pipetted onto each plate to determine which colonies had integrated *recO* by double crossover into the chromosome. Due to matching antibiotic resistance cassettes (chloramphenicol) in both the *recO::cat* ; *recA-gfp* and *amyE::*P_{spac}*-recO*, the chloramphenicol antibiotic resistance cassette was changed *in vivo* with the tetracycline antibiotic resistance cassette (*pCm::Tet)* in the *amyE::*P_{spac}*-recO* strain, creating ERB2. The *recO::cat* genomic DNA was isolated and used to transform ERB2 generating ERB3. ERB4 was generated by subsequently transforming ERB3 with genomic DNA containing the r*ecA-gfp* allele (spc). All strains that were constructed using a mutant form of *recO* expressed from *amyE* were built similarly.

Site-directed mutagenesis of *recO*

Point mutations were made in pEB1 (*recO* in pDR66) and amplified via PCR as described [61]. Briefly, 0.5 µL Dpn1 and 2.5 µL NEBuffer4 were added to the PCR product, followed by incubation at 37 ºC for 5 hours. The plasmid was then purified via PCR cleanup (Qiagen), and used to transform chemically competent *E. coli* cells (MC1061). The plasmids were isolated (Qiagen) and the missense mutation of interest was verified by sequence analysis. The *recO* alleles were

used to transform wild type (PY79) *B. subtilis* followed by integration at the *amyE* locus. Each *recO* allele was verified to integrate by double crossover at the *amyE* locus as described above.

Chromosomal DNA Purification

The desired strain was struck out onto an LB+agar plate with appropriate antibiotic selection and grown at 30 °C overnight. One colony was used to inoculate 25 mL of LB medium and grown at 37 °C to an OD_{600} of 1.0. Cells were concentrated by centrifugation for 5 minutes at 6,000 revolutions per minute (rpm) and re-suspended in 4 mL NE Buffer (0.1 M NaCl, 0.05 M EDTA) and placed in a 37 °C incubator for 5 minutes. 0.02 mL of 40 mg/mL lysozyme in NE Buffer (2.8 mL 0.35 M EDTA, 0.4 mL 5M NaCl, 16.8 mL ddH_2O) was added to the sample and placed at 37 °C for 15 minutes. 0.5 mL 10% dodium lauroyl sarcosinate (Sarkosyl) was added and put on ice for 5 minutes. 4 mL tris-buffered phenol and 4 mL 24:1 chloroform:isoamyl alcohol was added and centrifuged at 5,000 rpm for 15 minutes. The phenol/chloroform extraction was repeated until the top layer was clarified (usually 3-4 times). The top layer was placed in a test tube using an inverted pipette to avoid DNA shearing, 0.4 mL 3 M sodium oxaloacetate (NaOAc) was added and gently overlaid with 4 mL ice-cold 100% ethanol. DNA was spooled (extracted) using a flame sealed Pasteur pipette, air-dried, gently washed in 70% ethanol, and air-dried again. DNA was re-suspended in 500 µL TE buffer (10 mM Tris brought to a pH 8.0 with HCl, 1 mM EDTA) and stored at 4 ºC.

Purification of RecA

The *recA* overproduction plasmid was previously constructed and is a kind gift from Dr. C. Lee and Dr. Alan Grossman (MIT) and stored in strain LAS539. Cells were struck out onto ampicillin containing LB+agar and grown in a 37 ºC incubator overnight. One colony was inoculated into 25 mL LB medium with a 1:1000 dilution 100 µM ampicillin and grown at 37 °C overnight. 10 mL culture were inoculated into 1 L LB and grown to an OD_{600} of 0.8 at 37 °C. Over expression was induced using 1:1000 dilution 1M IPTG and grown overnight at 18 °C. Cells were harvested by centrifugation for 30 minutes at 4,000 rpm. The pellet was re-suspended in 25 mL lysis buffer (20 mM Tris pH 7.6, 300 mM NaCl, 20 mM imidazole, 10% sucrose). 20 mM spermadine trihydrachloride and 0.5 U deoxyribonuclease were added following re-suspension. The cells were lysed twice using a French press with a cell pressure of 14,000 psi. The lysate was clarified by centrifugation at 15,000 rpm for 30 minutes. The supernatant was frozen in LN_2 and stored for further purification.

Ammonium Sulfate Precipitation

RecA was initially purified via ammonium sulfate precipitation. Based on previous purifications of native RecA from *B. subtilis* [62], two ammonium sulfate precipitations were made: 0-40% (4.61 g in 19 mL protein) and 40-70% (3.56 g). After each cut, proteins were concentrated by centrifugation at 10,000 rpm for 30 minutes. Pellets were saved and additional ammonium sulfate cuts were done in the supernatant. Each precipitation was electrophoresed on a 10% SDS-poly-acrylamide gradient (SDS-PAGE) gel and stained with Coomassie Blue (450 mL

MeOH, 90 mL acetic acid, 1 g Coomassie Brilliant Blue) to determine where maximum protein precipitation occurred. RecA was found in the 70% ammonium sulfate precipitation.

Anion Exchange Chromatography

RecA has an isoelectric point of 4.8 and is predicted to have an overall negative charge under neutral conditions (http://www.scripps.edu/~cdputnam/protcalc.html). Thus, RecA was purified using an anion exchange column. RecA protein was dissolved in back buffer (30 mM Tris-HCl pH 7.0, 5% glycerol w/v, 0.5 mM EDTA (Ethylenediaminetetraacetic acid), 1 mM (dithiothreitol) DTT) in order to achieve a similar conductivity as the start buffer (30 mM Tris-HCl pH 7.6, 10% glycerol w/v, 0.5 mM EDTA, 1 mM DTT, 100 mM NaCl). The final conductivity was 9.68 mS for the protein/buffer solution and 10.64 mS for the start buffer. A perfusion chromatography (BioCad) instrument was washed with elution buffer (10% glycerol w/v, 30 mM Tris-HCL pH 7.5, 1M NaCl, 1 mM DTT, 0.5 mM EDTA) and then equilibrated with start buffer. The protein/buffer solution was subsequently applied to a Q column. RecA was eluted from the matrix using a NaCl gradient from 0 M to 1 M. The column was then washed with 100% elution buffer to elute any remaining protein.

A second 70% ammonium sulfate cut was performed. For 6 mL RecA protein, 2.83 g ammonium sulfate was added. The protein was concentrated by centrifugation at 10,000 rpm for 30 minutes. The pellet was then dissolved in 2 mL 1X PBS and 10% glycerol w/v. Protein was centrifuged again at 5,000 rpm for 10 minutes to pellet out the aggregates.

Two 5 mL desalting columns were placed in series and equilibrated with 1X PBS and 10% glycerol w/v (also used as the elution buffer). For 2 mL protein, 1 mL elution buffer was used. Protein was eluted into 0.5 mL aliquots.

The Bradford Assay was performed to determine which aliquots contained protein. A few drops of BioRad protein assay were spotted onto parafilm and 1-2 drops of protein were added. A color change from red to blue indicated the presence of protein. Aliquots containing protein were combined. A 1:10 dilution in elution buffer was done to take an absorbance reading.

Due to a high amount of DNA bound within the protein (seen by a prominent peak at 260 nm), RecA was subsequently purified using a hydroxy apatite column. The hydroxyl apatite column was prepared with ddH_2O to replace the 20% ethanol (storage liquid). The column was equilibrated with start buffer (5 mM phosphate (3.576 mL K_2HPO_4 and 1.424 mL KH_2PO_4), 400 mM NaCl, 10% w/v glycerol). RecA was eluted from the column using an elution buffer (3M phosphate, 400 mM NaCl, 10% glycerol w/v). To place RecA into a more stable buffer, protein was eluted through a desalting column using 1X PBS and 10% glycerol w/v. A Bradford Assay was conducted to determine which fractions contained protein. Fractions containing protein was electrophoresed on a 10% SDS-PAGE gel to determine purity.

The concentration of RecA was determined by electrophoresing various amounts of protein next to a gradient of 10 mg/mL lysozyme in PBS on a 10% SDS-PAGE gel followed by staining with Coomassie Blue. The gel was scanned and a standard curve of lysozyme was compared to RecA to determine its

concentration using ImageJ. Purified RecA had a concentration of 1.5 µg/µL. 900 µL of protein in 1X PBS was sent to Covance (Denver, PA) for antiserum generation. MI1289 and MI1290 terminal bleeds have been received following RecA inoculation.

Western Blot Analysis

Strains were grown in 10 mL 1X defined $S7_{50}$ minimal media, treated accordingly at mid exponential phase, and concentrated by centrifugation. Cells were lysed using 300 µL lysis buffer (0.5 M EDTA, 0.1M $MgCl_2$, 0.1mM 4-(2-aminoethyl)-benzenesulfonyl Fluroide (AEBSF), 40 mg/mL lysozyme in NE buffer, 1 mg/µL DNase I and ddH_2O) as previously described [63]. 10% SDS (33 µL) was diluted to 1% in lysis buffer. Proteins were loaded with 1 part SDS sample dye: 3 part DTT and separated on a 10% SDS-PAGE gel using Bio-Rad equipment. Proteins were then transferred to a nitrocellulose membrane (Whatman) using 1X transfer buffer (24 mM Tris, 192 mM glycine, pH 8.2) for 1 hour at 60 volts as described [64]. Membranes were probed with the appropriate primary antibody (rabbit) at a 1:1000 dilution in 2% milk, 1X Tris buffered saline and 0.02% TBS-Tween. 15 mL milk was incubated with a nitrocellulose membrane and placed on an orbital shaker at room temperature for 2 hours. 1:1000 primary antibody was added to fresh blocking solution was incubated with the membrane overnight on an orbital shaker at 4 °C. The nitrocellulose membrane was washed 3X in 0.02% TBS-tween followed by 1:1000 dilution of goat-anti-rabbit-HRP conjugated secondary antibody in 2% milk/TBS-Tween and placed on an orbital shaker at room temperature for 2 hours. The nitrocellulose membrane was washed again

3X in 0.02% TBS-tween followed by incubation with 2 mL SuperSignal West Pico Luminol/ Enhancer Solution and 2 mL SuperSignal West Pico Stable Peroxide Solution (Thermo Scientific). Blots were exposed to film (BioExpress) for 3 minutes prior to developing.

Homology Modeling

The RecO homology model was generated as previously described using SWISS-MODEL (http://swissmodel.expasy.org/) [65], but the RecO structure was based on that of *Deinococcus radiodurans* (Protein Data Bank [PDB] accession number 1u5kA; http://www.rcsb.org) and the *B. subtilis* RecO deep sequence [66-70]. Each mutation and truncation made was highlighted on the *B. subtilis* RecO homology model using PyMOL (http://pymol.sourceforge.net/).

Results

RecA-GFP organizes into foci in response to phleomycin generated double strand breaks.

A functional RecA-GFP fusion using a monomeric green fluorescent protein tag was constructed to visualize the localization of RecA-GFP *in vivo* [8]. In agreement with previously published data, when left untreated, RecA-GFP localizes into foci in ~10% of cells (n=3346) (Fig. 2A) [8]. Upon challenge with 100 ng/mL of mitomycin C (MMC), we found that the percentage of cells with foci increased to ~79% (n=2482) (Fig. 2B) as has been previously described [52, 71]. Since MMC is capable of causing inter and intra-crosslinks as well as monoadducts [72], we examined the localization of RecA-GFP foci following challenge with a double strand break inducing agent. To this end, we challenged cells with 3 μM phleomycin to induce DSBs. We found that RecA-GFP organized into foci in ~81% of cells (n=995) (Fig. 2C), demonstrating that RecA-GFP localizes in response to phleomycin generated DSBs.

To control that RecA protein was causing localization and not the GFP moiety, we imaged a strain in which a fluorescent moiety, *yfp,* was placed at an ectopic locus under an IPTG inducible promoter. We found that YFP, unattached to a protein, showed diffuse localization, and did not form foci under any condition tested (Fig. 3). We conclude that localization of RecA-GFP in response to DSBs is due to the recruitment of RecA and not the fluorescent protein appendage, GFP.

RecA-GFP focus formation is dependent on RecO.

It was previously shown that DNA replication is required for RecA-GFP foci to form in response to MMC challenge and in response to a site-specific double strand break [8]. The protein(s) that couple the RecA-GFP focus formation response to DNA synthesis are unknown. To identify proteins that may function to target RecA-GFP foci to ongoing DNA synthesis, we took a candidate approach. We hypothesized that since the RecFOR pathway is important in homologous recombination in *B. subtilis*, and these proteins are responsible for nucleating RecA onto SSB coated ssDNA during daughter strand gap repair in *E. coli*, that a component of the RecFOR pathway could couple RecA-GFP foci to DNA synthesis.

To this end, we imaged and quantified RecA-GFP foci in *B. subtilis* cells bearing a *recO* deficiency (*recO::cat*). This strain was grown under identical conditions as the *recA-gfp* strain described above. In the absence of *recO*, RecA-GFP foci localized in only ~4% of untreated cells (n=2291) (Fig. 4A), cells challenged with MMC (n=1028) (Fig. 4B) and cells challenged with phleomycin (n=801) (Fig.4C). Furthermore, the few "foci" that were quantified, were not positioned at midcell, the subcellular position indicative of localization at the replisome [73], but instead formed near the cell poles. We hypothesize that these "foci" are accumulated RecA that are improperly targeted in the absence of *recO*. Furthermore, the percentage of RecA-GFP foci that localized near midcell in the absence of *recO* function in response to DNA damage is <1% (n=2291).

These results indicate that RecA-GFP is broken for localization in the absence of RecO.

Complementation of *recO* at an ectopic locus restores RecA-GFP focus formation in response to DNA damage.

We asked if RecA-GFP focus formation could be restored to wild type levels following ectopic expression of *recO* in cells deficient for the native *recO* allele. All cells were grown in the presence of isopropyl-beta-D-thiogalactopyranosid (IPTG) to induce expression of *recO*. We tested several different concentrations of IPTG to determine the lowest amount of inducer necessary to restore RecA-GFP foci formation to that observed in cells containing the wild type *recO* allele. In performing an IPTG titration, we found that 5 μM was the lowest amount of IPTG that would restore RecA-GFP foci to wild type levels. We found that, when untreated, RecA-GFP organized into foci in ~11% of cells (n=1558) (Fig.5A). The percentage of cells with RecA-GFP foci following ectopic *recO* expression was statistically identical to cells with *recO* at its native locus. Upon challenge with MMC and phleomycin, the number of cells with RecA-GFP foci increased to ~84% in MMC challenged cells (n=1197) (Fig. 5B) and ~77% in phleomycin challenged cells (n=1026) (Fig. 5C). These quantifications are statistically identical to cells with *recO* expressed from its native locus. With these results, we conclude that RecO protein is necessary for RecA-GFP localization. It should be noted that our results are in contrast to a previously published study, which showed very little effect of a *recO* deletion on the ability of RecA-GFP to form foci [6] [see discussion].

RecA-GFP focus formation is not dependent on RecN.

The RecFOR pathway is composed of RecF, RecO, RecR [74] and the DSB repair protein, RecN, which is hypothesized to function before RecA binding [6]**.** RecN is an SMC-like protein that is involved in the early steps of recombination, and is hypothesized to precede RecA binding to 3' ssDNA regions [6, 75]. To determine if proteins in addition to RecO are critical for the DNA damage-dependent localization of RecA-GFP, we examined RecA-GFP focus formation in cells deficient for *recN*. We found that in *recN* deficient cells, RecA-GFP foci formed in ~10% of untreated cells (n=1086) (Fig. 6A), a similar percentage as untreated wild type *recN* ; *recA-gfp* cells. Following challenge with MMC, RecA-GFP formed foci in ~75% of *recN* deficient cells (n=1256) (Fig. 6B), which was statistically equivalent to wild type *recN ; recA-gfp* cells challenged with MMC (see Fig. 7 for quantification). Though the percentage of cells with RecA-GFP foci is unchanged, RecA-GFP foci are aberrantly shaped in the absence of *recN* (Fig. 6 and Fig. 7). With these results we conclude that RecN may aid in the organization of RecA-GFP into complexes, however, *recN* is not required for RecA to respond to DNA damage encountered by the replication fork.

RecA-GFP protein levels remain stable under all conditions.

Because we found that *recO* was required for RecA-GFP to organize into foci, we were concerned that proteolytic release of GFP from RecA in *recO* deficient cells would yield the same result, providing the appearance that *recO* was required for RecA-GFP to form foci. We prepared extracts from cells with RecA-GFP in the *recO::cat* genetic background. GFP is a 27 kDa protein [76]; the arrow shows

the expected position that GFP protein would migrate if it had been proteolytically released from RecA using antibodies against the GFP moiety. As shown in Fig. 8, GFP is not proteolytically released from RecA, indicating that RecA-GFP remains intact in *recO* deficient cells. Thus, the loss of RecA-GFP focal localization in the *recO* deficient cells is not due to loss of the GFP appendage from RecA, but instead due to a failed organization of RecA-GFP into foci.

When cells are challenged with high levels of DNA damaging agents, they begin to induce the SOS response [77]. More RecA can be produced in response to increasing amounts of single-stranded DNA. However, when RecA-GFP was detected by western blotting using polyclonal anti-GFP antibodies, protein levels were fairly close to being equal, whether the extracts had been challenged with MMC or not (Fig. 8). This result shows that the increase in the percentage of cells with foci in response to treatment with MMC is not due to the induced expression of RecA-GFP, but merely the re-distribution of pre-existing protein, confirming previous observations [8].

RecA-GFP forms foci in the absence of RecF despite an apparent synthetic effect on growth.

RecF is another recombination protein that binds single stranded DNA and helps facilitate RecA loading [32]. As an additional candidate, we asked if RecF, like RecO, was necessary for RecA localization. Cells lacking *recF* have wild type morphology (data not shown). When the *recA-gfp* allele was used to transform *recF* deficient cells, a synthetic effect occurred, causing the cells to become extremely filamented and wider than normal (Fig. 9). However, it is evident that

RecA-GFP is still able to form foci even in *recF* deficient cells (Fig. 9). Due to the inability to stain the membranes, corresponding DIC images are shown. Upon challenge of cells with MMC for 1h, it appears that there is an increase in RecA-GFP foci (Fig. 9B). However, this percentage cannot be quantitatively determined due to the inability to quantify the number of individual cells. This is necessary since *B. subtilis* can grow in chains, thus a membrane stain is required to view septa for the scoring of individual cells. We conclude that, despite an apparent synthetic effect created by the *recF::spc* allele, RecA-GFP can still localize in the absence of *recF* [see discussion].

Structure function analysis of RecO mutants and their affect on RecA-GFP focus formation

The function of RecO in the RecFOR pathway in *Escherichia coli* is well understood and structural studies in *Deinococcus radiodurans* have led to further understanding of RecO function. However, RecO is a poorly conserved protein and its structure and function in *B. subtilis* are not understood. To our knowledge, not a single *recO* point mutation has been published in *B. subtilis*. Since we have demonstrated that RecO is required for RecA-GFP focus formation, we would like to know which residues are important for targeting RecA-GFP to replication forks. Based on the published crystal structure of *D. radiodurans* RecO, we identified somewhat conserved residues in *B. subtilis* that may have an important role in the RecA recruitment pathway. We targeted three surface exposed residues in *B. subtilis* RecO that could be required for RecA-GFP to localize into foci (Fig. 10). If these residues were critical for DNA binding

or interaction with RecA, we would expect to see a significant reduction or loss of focus formation for RecA-GFP in the presence of the mutant *recO.* We made two separate missense mutations in *recO*: *N13E* and *R37E*. In both strains, RecA-GFP formed foci at a statistically equivalent percentage as the *recO*$^{+}$ strain. When untreated, RecA-GFP localized in the same percentage as it does in *recO*$^{+}$, ~11% in *recON13E* (n=1076) (Fig. 11A) and ~14% in *recOR37E* (n=1048) (Fig. 12A). When challenged with MMC, the percentage of RecA-GFP foci was slightly less than in *recO*$^{+}$ cells, ~69% in *recON13E* (n=998) (Fig. 11B) and ~67% in *recOR37E* (n=936) (Fig. 12B). This difference from *recO*$^{+}$ cells is not statistically significant.

The third mutation we made was a 24 amino acid truncation of the C-terminal end of RecO. This region of RecO is surface exposed and poorly conserved between bacterial systems and thus we could not identify single amino acid changes that we could hypothesize would alter RecO function. In untreated cells, we found that RecA-GFP localizes in ~11% of untreated cells (n= 1048) (Fig. 13A) and in ~73% of cells challenged with MMC (n= 950) (Fig. 13B). These results are equivalent to those of the *recO*$^{+}$ strain (see Fig. 14 for quantification of mutations). Thus, we conclude that the C-terminal domain of RecO is not important for the localization of RecA-GFP *in vivo.* Although this result is negative, the C-terminal region of RecO has been hypothesized to possibly have a role in protein-protein interactions. However, we find that the

last 24 amino acids in the C-terminal are not important for RecA recruitment *in vivo.*

Discussion

We show that RecO is critical for the assembly of RecA-mediated DNA repair complexes in actively replicating *Bacillus subtilis* cells. When *recO* was disrupted from the chromosome, RecA-GFP focus formation was almost entirely eliminated (foci were seen in ~4% of the cells). Upon challenge with mitomycin C, lack of focus formation persisted (foci were seen in ~4% of cells). MMC induces crosslinks in dsDNA, generating adducts between the second and seventh nitrogen in guanine molecules. Homologous recombination and nucleotide excision repair (NER) work in tandem to incise the interstrand crosslinks (ICL), which can subsequently lead to double strand breaks [72]. Since MMC does not directly cause DSBs, we sought to confirm that RecA localization was indeed induced by DSBs and not some other form of DNA damage. When cells were challenged with phleomycin, a peptide known to cause DSBs [9], RecA-GFP formed foci in a nearly identical percentage of cells as compared to cells challenged with MMC. Upon complementation of *recO*$^{+}$ from an ectopic locus, RecA-GFP formed foci in an equivalent percentage of cells as wild type *recA-gfp* cells unchallenged and challenged by DNA damaging agents MMC and phleomycin (~84% and ~77% respectively). Our findings strikingly contrast what is currently known about the importance of RecO for RecA localization.

It has been published that when RecA-GFP is moved into a *recO* mutant background, that RecA-GFP is able to form foci in 25% of cells in response to being challenged with 50 ng/mL of MMC [6]. It was concluded that focus formation and localization of RecA-GFP was mostly unaffected in the absence of

recO [6]. How do we explain this discrepancy? In our experiments, *recA-gfp* is expressed from its native locus as the only source of *recA* in the cell. In the prior published work, RecA-GFP was expressed ectopically from an inducible promoter with the native *recA* allele intact. We propose that cells saturated with RecA-GFP were able to bypass the need for RecO for recruitment to ssDNA, thus making it appear that RecO is not important for coupling RecA-GFP to DNA replication status.

Based on our initial findings, we investigated other DSB repair proteins for an effect on RecA localization. We found that, although *recN* and *recF* deficiencies cause a qualitative change in the appearance of RecA-GFP foci, the percentage of cells with localization is largely unchanged. These results show that RecO is primarily responsible for coupling RecA-GFP foci to ongoing DNA replication. RecO protein is poorly conserved at the amino acid sequence level and because of this, it has been unclear, in any system, the region of RecO that is important for either RecA interaction or function in RecA loading. We thus used the crystal structure of *D. radiodurans* RecO to guide a mutagenesis study in an effort to identify a region that is important for RecA interaction or at least functional activities of RecO that are important for focus formation *in vivo* and presumably RecA loading. Based on the published crystal structure of *D. radiodurans* RecO, we identified somewhat conserved residues in *B. subtilis* that might have an important role in the RecA recruitment pathway. We targeted three areas in *B. subtilis* RecO that could be required for RecA-GFP to localize into foci. Two of these residues are critical for DNA binding in *D. radiodurans*

and the C-terminus is hypothesized to be important for protein-protein interactions. If these areas of RecO are important for RecA recruitment in *B. subtilis*, we would have expected to see a significant reduction or loss of RecA-GFP focus formation.

The surface exposed *recON13E* and *recOR37E* mutations are predicted to affect DNA binding by RecO [78]. Upon changing the N13E and R37E amino acids in two separate strains, we found virtually no effect on the coupling of RecA-GFP foci to DNA replication. Though the percentage of foci in MMC treated cells is lower than that in wild type cells, it is not significantly lower. We suggest that this decrease is due to a difference in RecO levels of the mutant proteins or perhaps a slight decrease in their ability to recruit RecA. Based on our data, we conclude that neither residue is important for RecA recruitment in *B. subtilis*, suggesting that either DNA binding activity by RecO is not important for recruitment of RecA to replication forks and that the SSB interaction is more critical in this process, or that these substitutions did not significantly alter DNA binding. The latter will need to be tested biochemically and the former idea is plausible since SSB is bound to DNA and RecO interacts with SSB [21]. It is possible that when RecO is bound to SSB, it is this interaction that is critical for RecA recruitment and not DNA binding by RecO (see Fig. 15 for working model). We are currently exploring experiments that discriminate between these two possibilities.

The C-terminus of *B. subtilis* RecO has negligible conservation with RecO homologues in other prokaryotic systems. Thus, it was difficult to choose a

specific residue in the C-terminus to mutate in order to disrupt RecO function and perhaps RecA interaction. Therefore, we truncated the 24 amino acids of the C-terminal domain of RecO, which are surface exposed, and visualized its effect on RecA-GFP localization in response to DNA damage. We found that in both untreated and MMC challenged cells, RecA-GFP localized in the same percentage as *recO*$^{+}$ cells. Based on these results, none of the targeted residues were found to be necessary for RecA-GFP localization. Further mutagenesis studies are being conducted to determine sites of importance for RecA interaction and/or DNA binding. It should be noted that the site on RecO where RecA may bind has not been found in any organism.

We constructed RecO-GFP at its native locus and under control of its native promoter to quantify and qualify focus formation to determine if RecO was naturally localizing at the replisome or solely in response to DSBs (data not shown). Cells were identically treated to those in all other experiments. We found that RecO-GFP failed to organize into foci in both untreated and MMC challenged cells. We assume that this is most like due to the relatively low levels of RecO in *B. subtilis* [79]. Other published work shows that RecO-GFP does form foci at the replisome in live cells [6]. In these experiments, RecO-GFP was overexpressed with the native *recO* allele intact [6]. Based on our finding that RecO-GFP did not form foci when expressed from its native locus as the only source of RecO *in vivo*, we are concerned that prior work examining RecO-GFP localization may be caused by artifacts from overexpression. Other experiments will be necessary to determine if RecO is present at replication forks, which is

perhaps a level that is not high enough to visualize using fluorescence microscopy.

In addition to RecO, we sought to determine if there were other recombination proteins that are critical for the DNA damage dependent localization of RecA. In *recN* deficient cells, we show that, quantitatively, RecA-GFP forms foci at an equivalent percentage as in *recA-gfp* cells with *recN*$^+$ at its native locus. Qualitatively, the foci were aberrant in the absence of RecN. This suggests that RecN plays a role early on in RecA-GFP localization, but this role is not necessary for initial assembly. We also attempted to examine RecA-GFP localization in a *recF* deficient strain. Cells were extremely filamented, but did contain distinct RecA-GFP foci. It appeared that the percentage of foci increased after cells were challenged with MMC, however we were unable to quantify the percentage of cells with RecA-GFP foci due to our inability to obtain clear membrane stains, which is common in filamenting cells. Upon imaging *recF* mutants with *recA*$^+$ using DIC, we found that cells had wild type morphologies. We suggest that filamentation is due to a synthetic effect upon addition of *recA-gfp* to the *recF* deficient cells. Based on our preliminary evidence, we show that RecA-GFP can form foci in the absence of RecF, suggesting that RecF is not necessary for RecA-GFP recruitment. The *recF* allele used in this study is a disruption and not a deletion [80, 81]. The *recF* gene is in an operon with other genes involved in DNA metabolism, including an essential topoisomerase, *gyrB*, located downstream of *recF.* Surprisingly, this particular *recF* allele has been used in almost all the prior work characterizing *recF* gene function in *B. subtilis*

[80]. We cannot ignore the possibility that cell filamentation and RecA-GFP focus formation is not due to a polar effect on *gyrB*. We plan to visualize RecA-GFP in a background where *recF* has been cleanly deleted to avoid disrupting other important genes that lie downstream of *recF* in the operon.

In conclusion, we show that, in striking contrast to what has been previously published, RecO is critical for RecA-GFP focus formation. Our work identifies a critical role for RecO in coupling RecA to replication status. Such a role for RecO *in vivo* has been unknown or at least unappreciated. RecN and RecF, though important in this pathway, are not critical, as RecA-GFP can still localize in their absence.

Future Directions

We hope to determine how RecO is mediating RecA's recruitment to the replisome. We have purified RecA and now have antiserum against it (Fig. 16). We have also cloned *recO* into an expression vector with a cleavable His_6-Tag for purification. We plan to use far western blotting and pull down assays to determine if RecO interacts directly with RecA. We also have SSB purified to determine if RecA or RecO bind SSB, and if this interaction is mediated by the C-terminal tail of SSB.

Table 1. List of Plasmids

Plasmids	Genotype	Source
pEB1	*recO* in pDR66	This work
pEB2	*recON13E* in pDR66	This work
pEB3	*recOR37E* in pDR66	This work
pEB4	*recOΔ24* in pDR110	This work
pEB5	*recOΔ24* in pDR66	This work
pEB6	*recO* in pET28aPB	This work
pEB7	*recOΔ44* in pDR110	This Work
pJS2	*mutL* in pDR66	Gift from Jeremy Schroeder
pJL10	*dnaN* in pDR110	Gift from Justin Lenhart
pCm::Tet	*pCM::Tet*	BGCS
pCM::Er	*pCM::Er*	BGCS

Plasmids obtained from the bacillus genetic stock center (BGCS) are indicated.

Table 2. List of Strains

Strains	Genotype	Source
LAS508	PY79 (wild type)	[82]
ERB1	*amyE::P_{spac}-recO*	This work
ERB2	*amyE::P_{spac}-recO*	This work
ERB3	*amyE::P_{spac}-recO; recO :: cat*	This work
ERB4	*∆smc* ; *recA-mgfp*	This work
ERB5	*amyE::P_{spac}-recO ; recO::cat ; recA-mgfp*	This work
ERB6	*recF::neo*	This work
ERB7	*recF::neo ; recA-gfp*	This work
ERB8	*recO* in pDR66 (*E. coli)*	This work
ERB9	*recO* in pet28aPB (*E. coli)*	This work
ERB10	*recON13E* in pDR66 (*E. coli)*	This work
ERB11	*recOR37E* in pDR66 (*E. coli)*	This work
ERB12	*amyE::P_{spac}-recON13E*	This work
ERB13	*amyE::P_{spac}-recOR37E*	This work
ERB14	*amyE::P_{spac}-recON13E*	This work
ERB15	*amyE::P_{spac}-recOR37E*	This work
ERB16	*recOΔ24* in pDR110 (*E. coli)*	This work
ERB17	*amyE::P_{spac}-recON13E ; recO::cat*	This work
ERB18	*amyE::P_{spac}-recOR37E ; recO::cat*	This work
ERB19	*amyE::P_{spac}-recOΔ24*	This work
ERB20	*recO-gfp*	This work
ERB21	*amyE::Pspac-recON13E ; recO:: cat ; recA-mgfp*	This work
ERB22	*amyE::P_{spac}-recOR37E ; recO::cat ; recA-mgfp*	This work
ERB23	*recOΔ24* in pDR66 (*E. coli*)	This work
ERB24	*amyE::P_{spac}-recOΔ24*	This work
ERB25	*amyE::P_{spac}-recOΔ24*	This work
ERB26	*amyE::P_{spac}-recOΔ24*	This work
ERB27	*amyE::P_{spac}-recOΔ24 ; recO::cat*	This work
ERB28	*amyE::P_{spac}-recOΔ24 ; recO::cat*	This work
ERB29	*amyE::P_{spac}-recOΔ24 ; recO::cat ; recA-gfp*	This work
ERB30	*recOΔ44* in pDR110	This Work
LAS40	*recA-23mgfp*	**[**8]
LAS47	*mutS::mutS-820-gfp ; amyE::P_{spac}-mutL*	Laboratory Stock
LAS153	*recF::spc*	[80, 81]
LAS172	*recO-18mgfp*	Laboratory Stock

Table 2 continued. List of Strains

LAS197	*recO::cat ; recA-gfp*	Laboratory Stock
LAS198	*recN::cat ; recA-gfp*	Laboratory Stock
LAS290	*amyE::*P_{spac}*-YFP*	[65]
LAS539	*recA* in pET11T	Laboratory Stock

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Acknowledgements

I would like to thank Dr. Lyle Simmons for his encouragement and help with my research and writing my thesis over the past year. His support allowed me to simultaneously pursue and accomplish my academic and athletic goals, a feat I never thought was possible.

I would also like to thank the Ph.D. students in the lab: Jeremy Schroeder, Justin Lenhart and Brian Walsh for initially walking me through experimental techniques and helping me critically analyze the progress and results of my research.

In addition, thank you to my fellow undergraduate students Nick Bolz and Gabriella Szewczyk for helping me keep my experiments going while I was traveling for athletics and helping me with various techniques when I first joined the lab.

Lastly, I would like to thank the Honors Summer Fellowship for giving me the opportunity to stay in Ann Arbor over the summer and continue my research. Without the support of the fellow students in the cohort and funding, I would not have been able to produce this work.

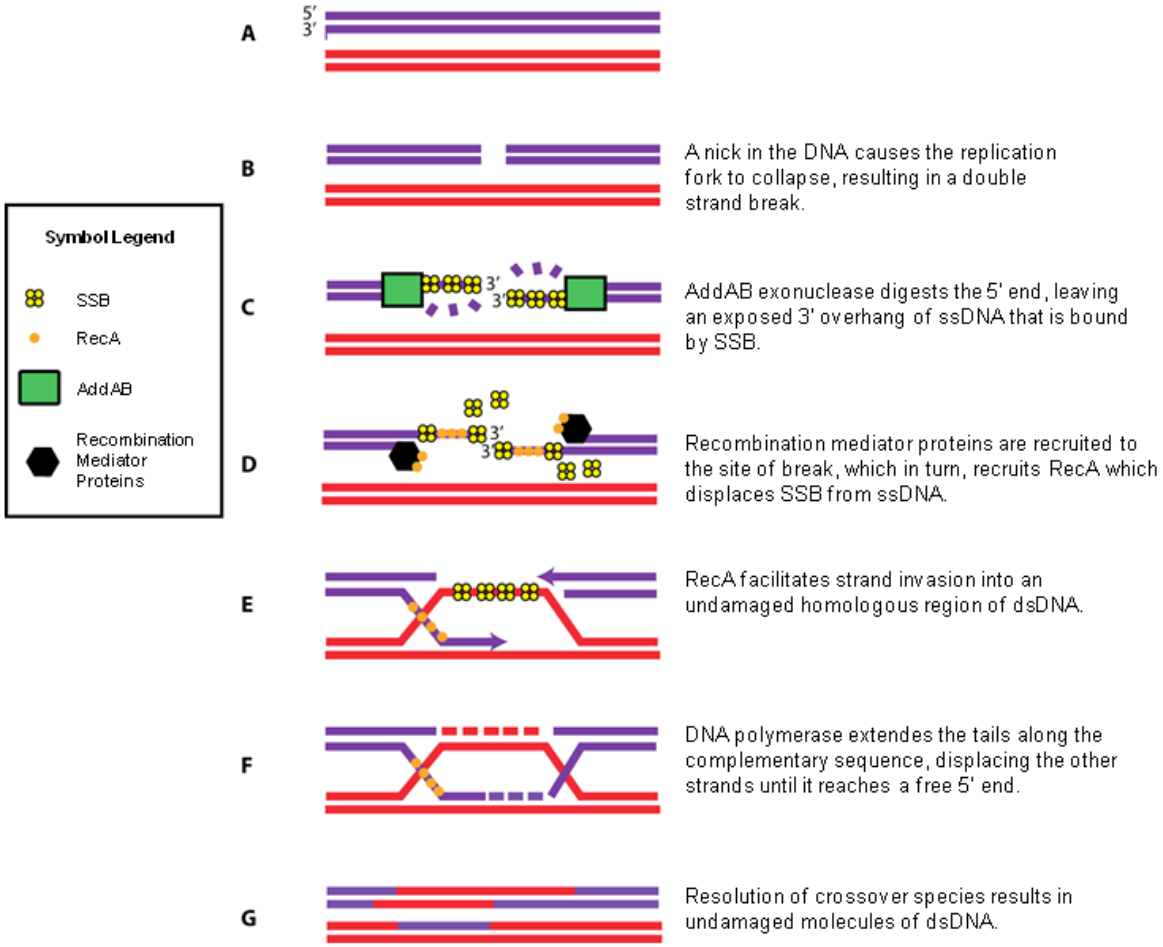

Figure 1. Molecular mechanism of DNA double strand break repair [1].

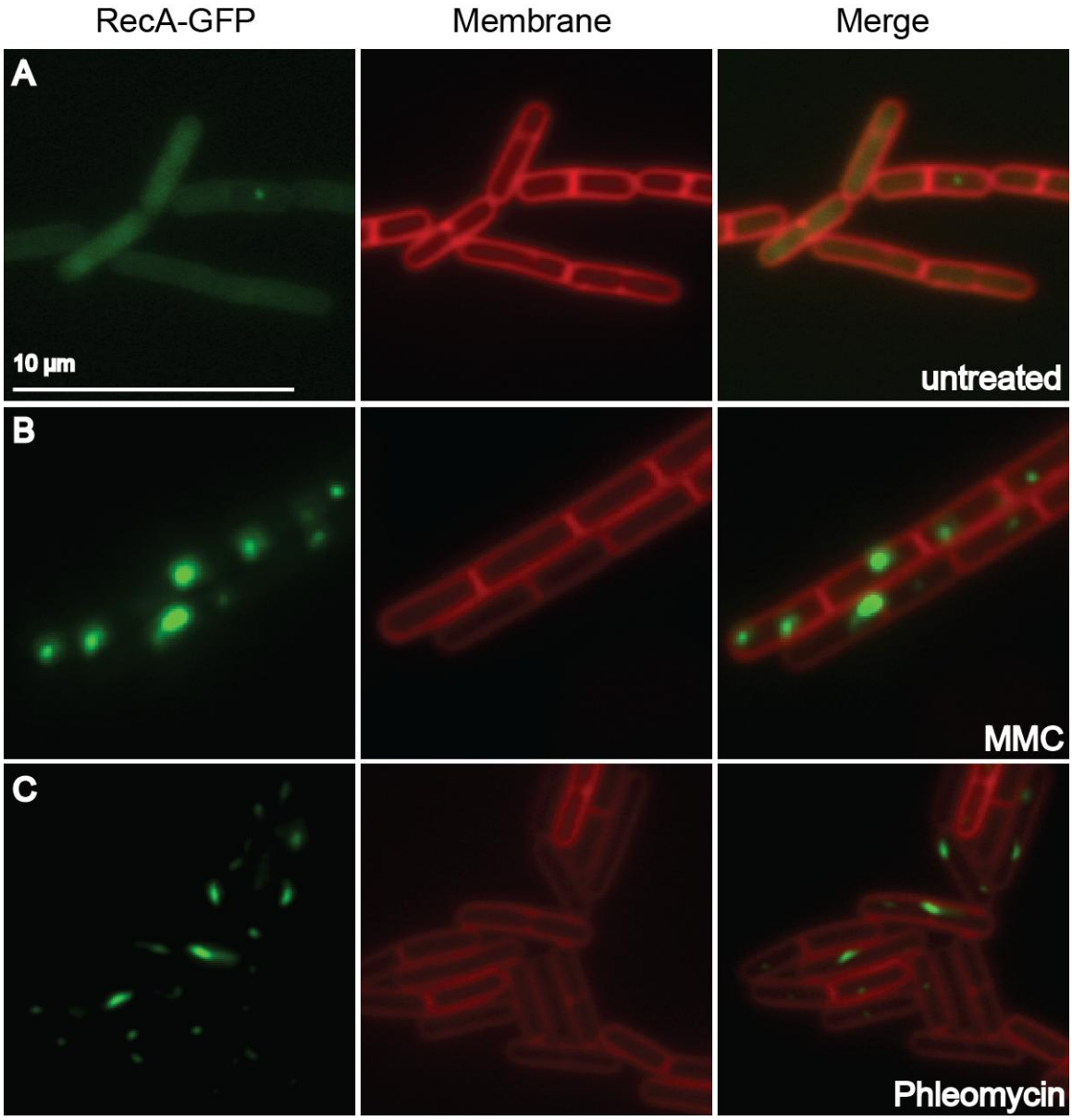

Figure 2. RecA-GFP organizes into foci in response to DNA damage. Shown are representative images of RecA-GFP foci in live *B. subtilis* cells. The membrane was stained with the vital membrane dye FM4-64 and is shown in red. Shown are cells with the *recA-gfp* allele that were **(A)** untreated **(B)** challenged with mitomycin C (MMC) (100 ng/mL) and **(C)** challenged with the double strand break inducing agent, phleomycin (3 µM).

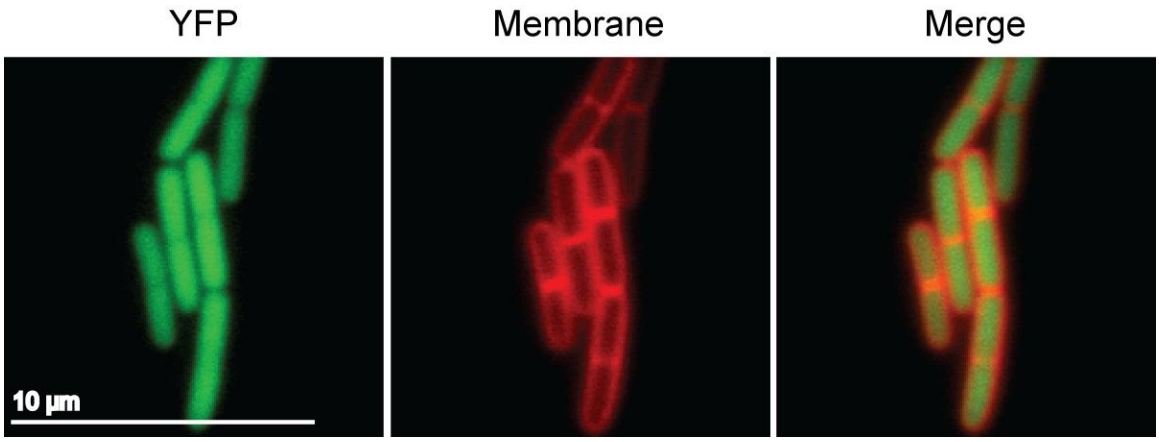

Figure 3. YFP does not localize into foci under any treatment. YFP was expressed from an IPTG regulated promoter at the *amyE* locus and visualized using microscopy.

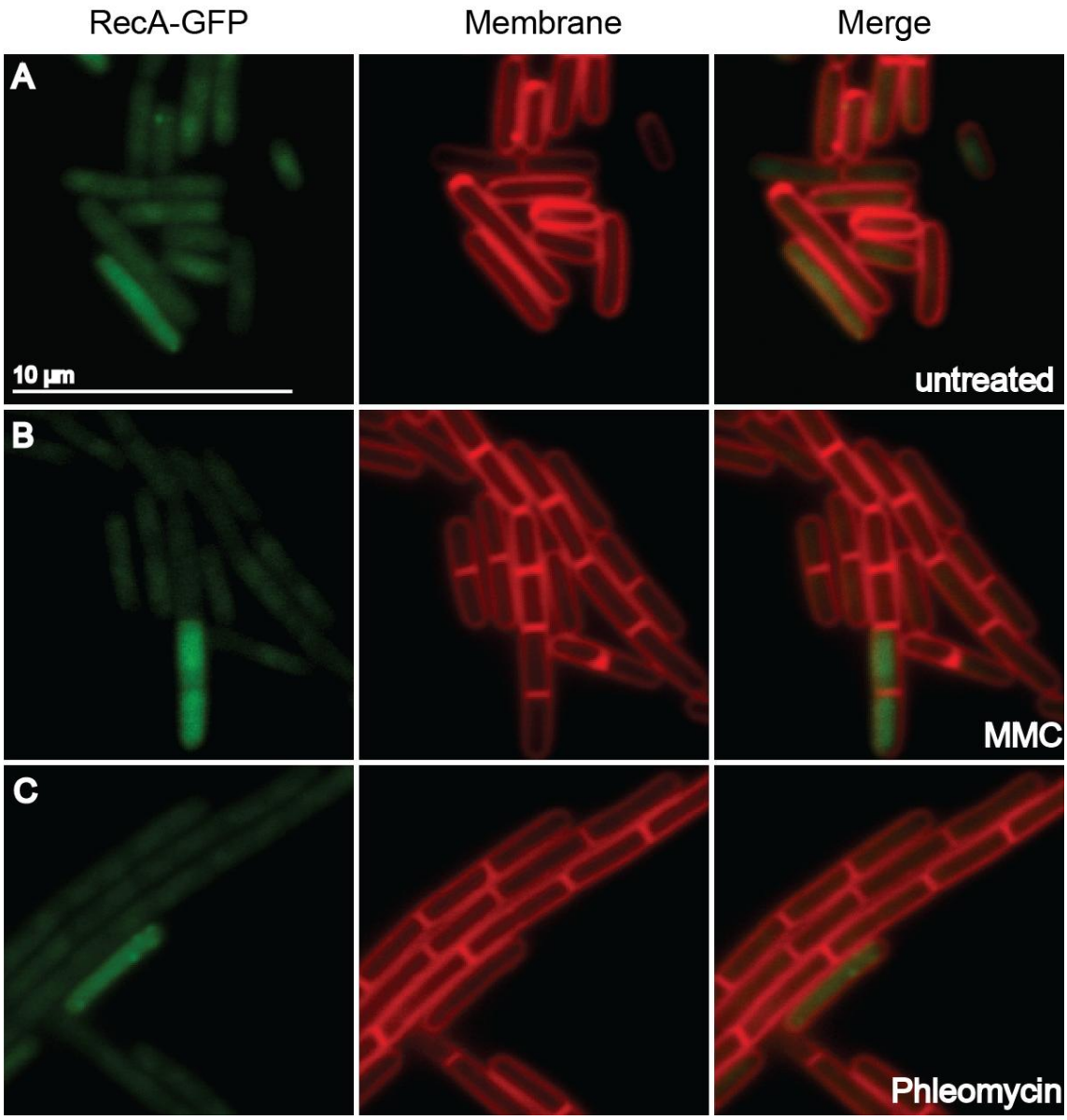

Figure 4. RecA-GFP focus formation is dependent on RecO. Shown are cells with *recA-gfp* and a *recO::cat* allele that were **(A)** untreated **(B)** challenged with MMC and **(C)** challenged with phleomycin.

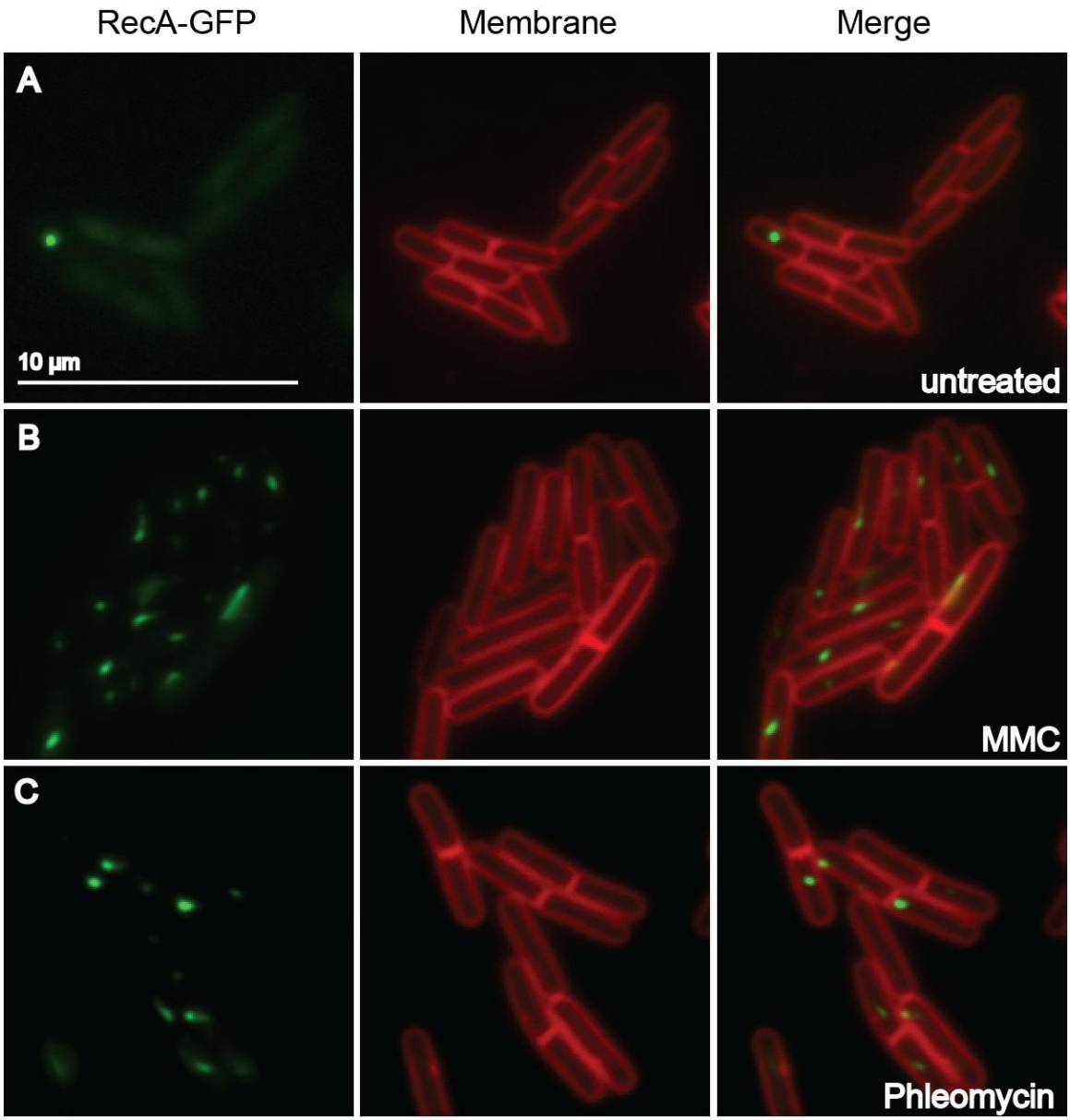

Figure 5. Complementation of *recO*$^+$ from *amyE* restores RecA-GFP focus formation in response to DNA damage. Shown are cells with *amyE::P*$_{spac}$ *recO*$^+$; *recO::cat* ; *recA-gfp* that were **(A)** untreated **(B)** MMC treated or **(C)** phleomycin treated. Expression of ectopically expressed *recO*$^+$ was with 5 µM IPTG.

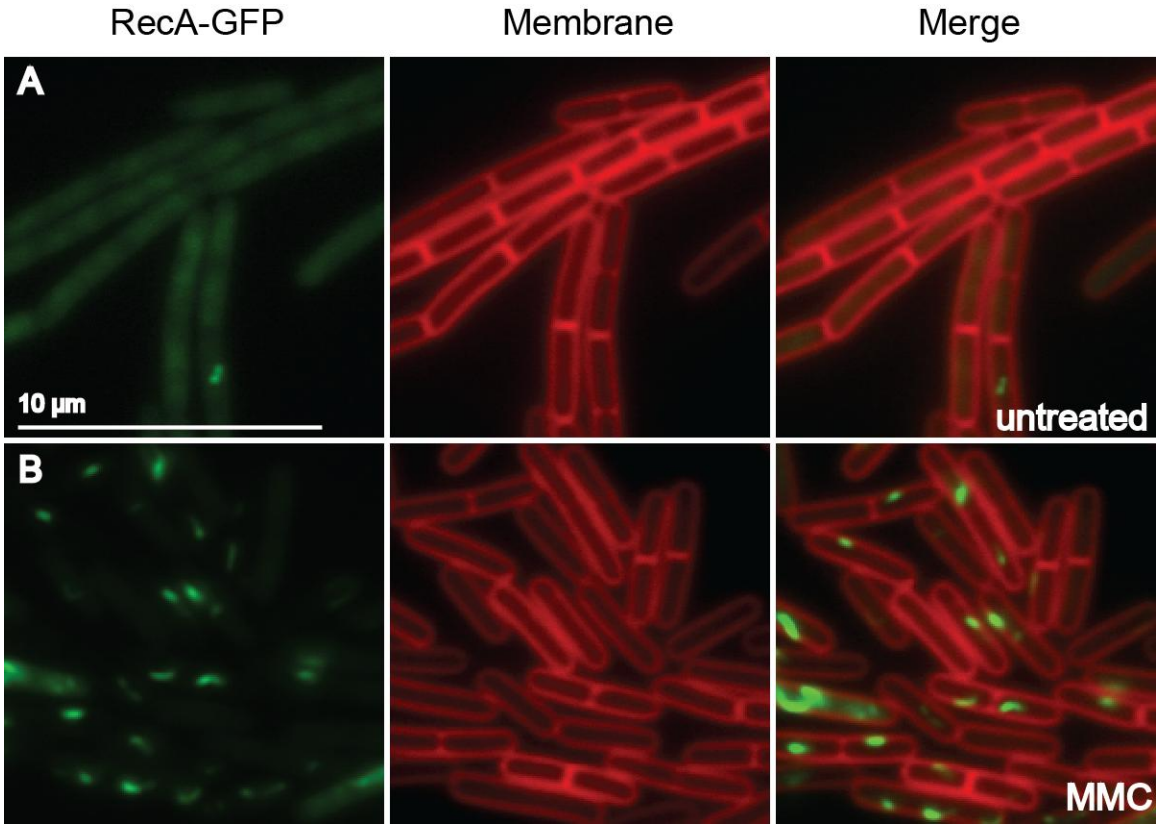

Figure 6. The *recN* gene aids in RecA-GFP focus formation, but is not required. Shown are cells with *recA-gfp* and a *recN::cat* allele that were **(A)** Untreated **(B)** MMC treated.

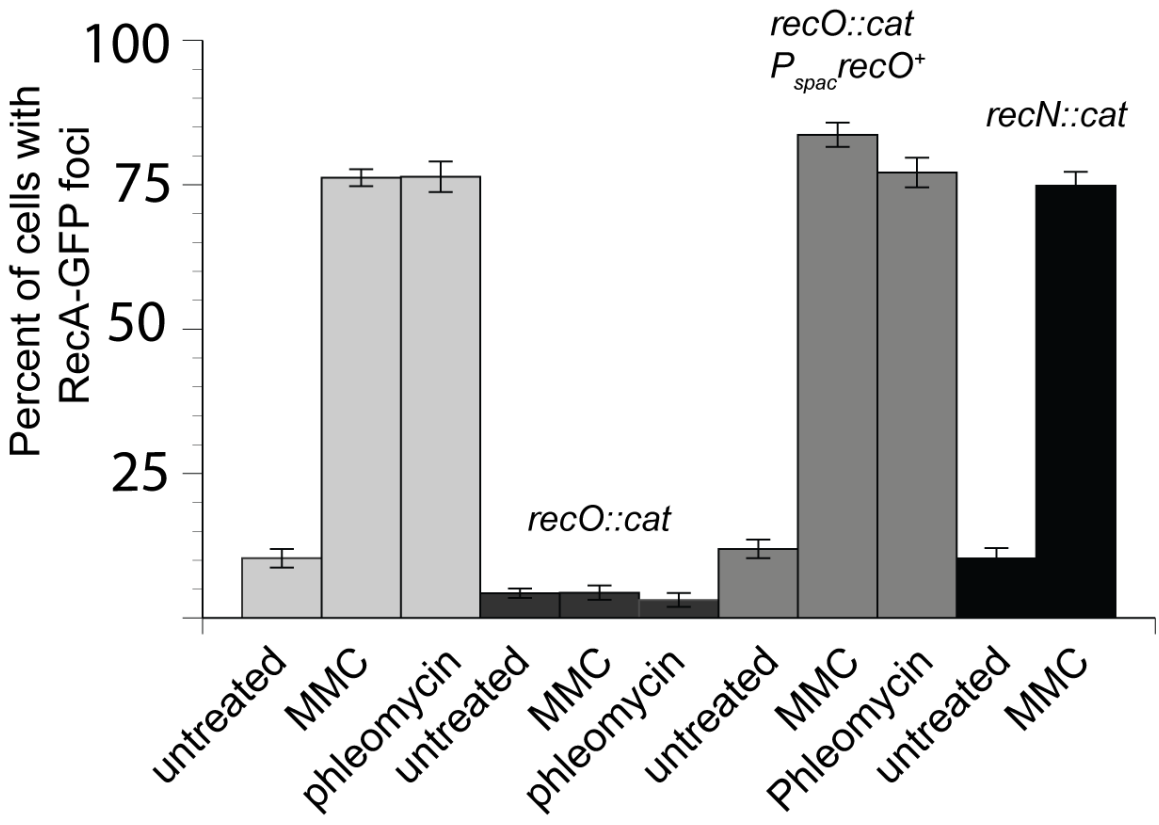

Figure 7. The *recO* gene is critical for RecA-GFP foci to form in untreated cells and in response to DNA damage. Shown is the quantification of the percentage of cells with RecA-GFP foci in four different strains in the order from left to right: (1) *recA-gfp* [light grey] (2) *recO::cat ; recA-gfp* (3) $P_{spac}recO^{+}$ *; recO::cat ; recA-gfp* (4) *recN::cat ; recA-gfp.* Cells were untreated and challenged in liquid culture with mitomycin C and phleomycin for 1 hour. The error bars reflect the 95% confidence interval. The number of cells scored in all populations is greater than 800.

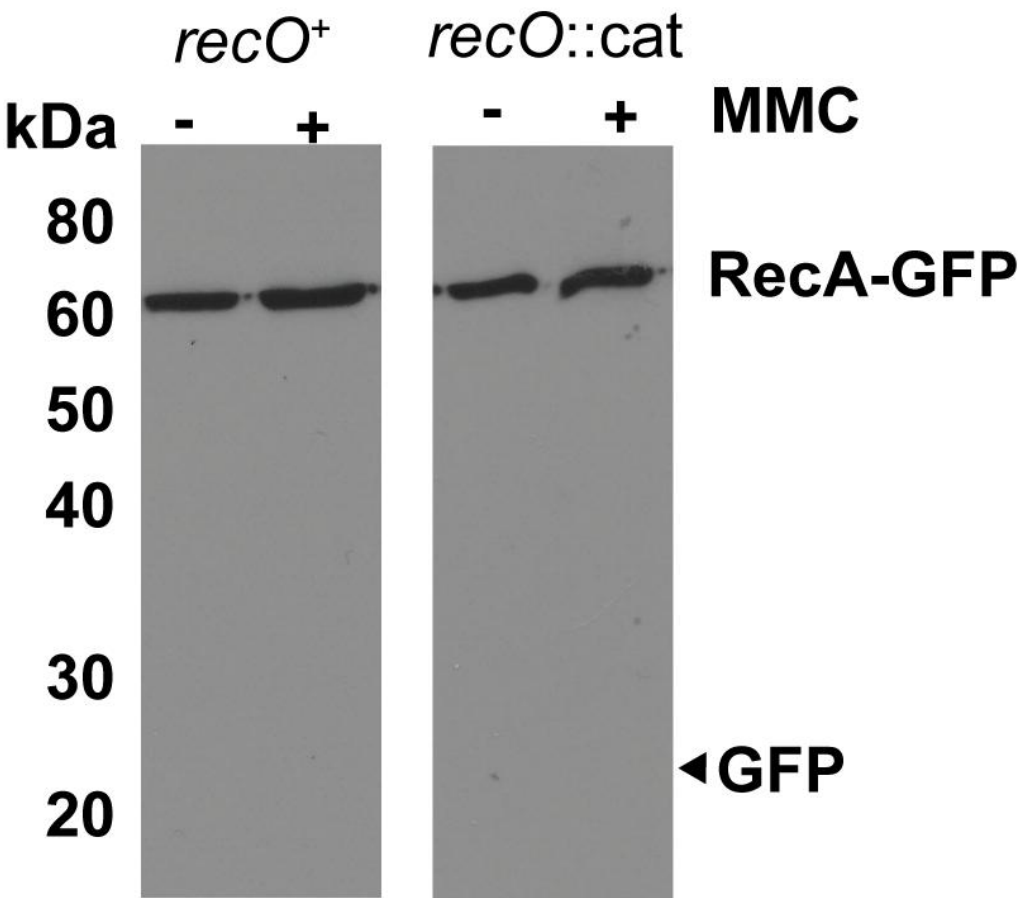

Figure 8. RecA-GFP protein levels do not substantially increase following low levels of MMC challenge. Cells were challenged with 100 ng/mL MMC for 1 hour followed by detection of RecA-GFP in whole cell extracts using polyclonal anti-GFP antibodies. The expected position of proteolytically released GFP is shown.

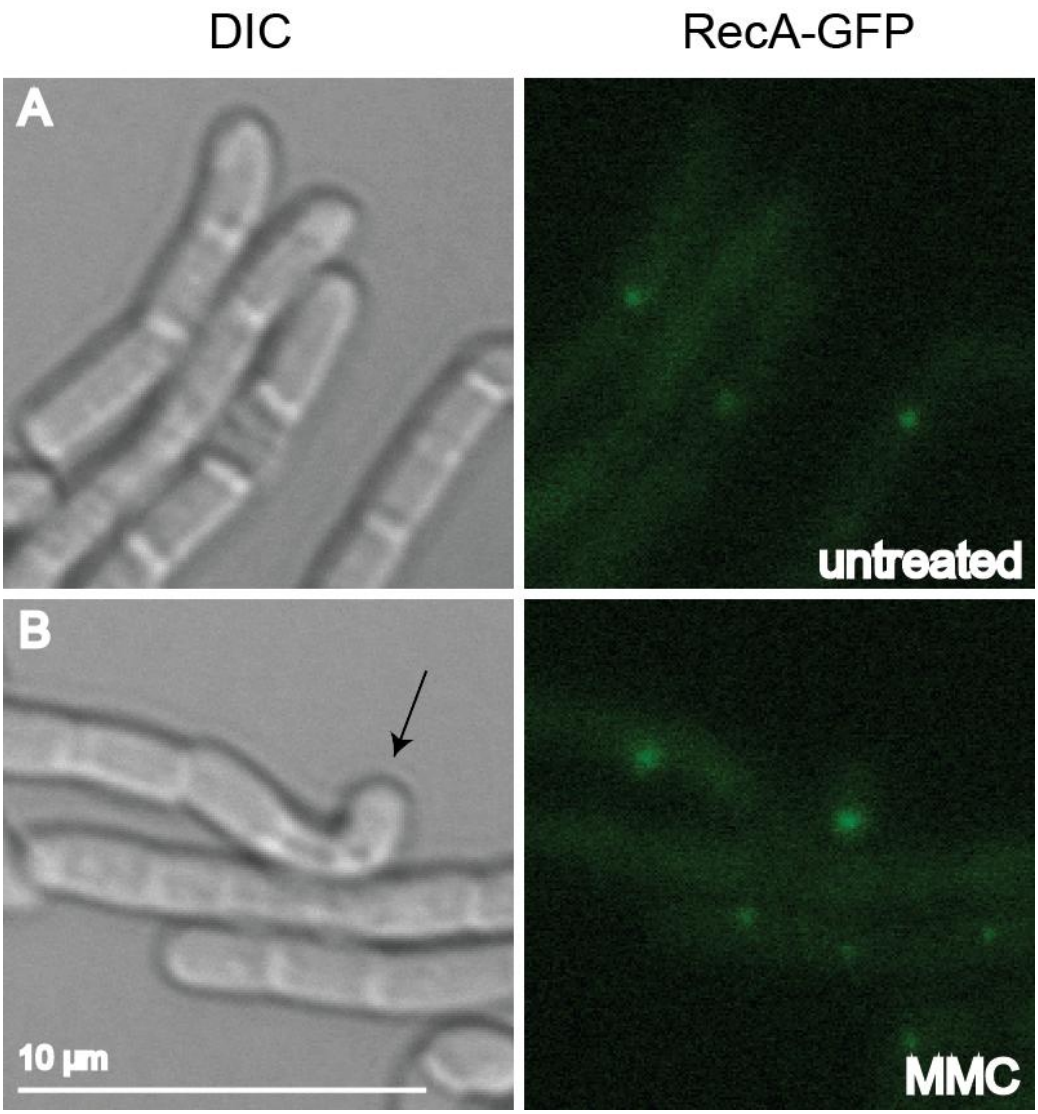

Figure 9. RecA-GFP is able to form foci in *recF* deficient cells despite an apparent synthetic effect. When *recA-gfp* was integrated into a strain with the *recF::spc* allele, cells became engorged and elongated. Despite a decrease in viability, RecA-GFP is able to form foci in the absence of RecF. **(A)** DIC of and RecA-GFP in untreated cells. **(B)** DIC of and RecA-GFP in MMC challenged cells. The arrow shows gross cell shape effects.

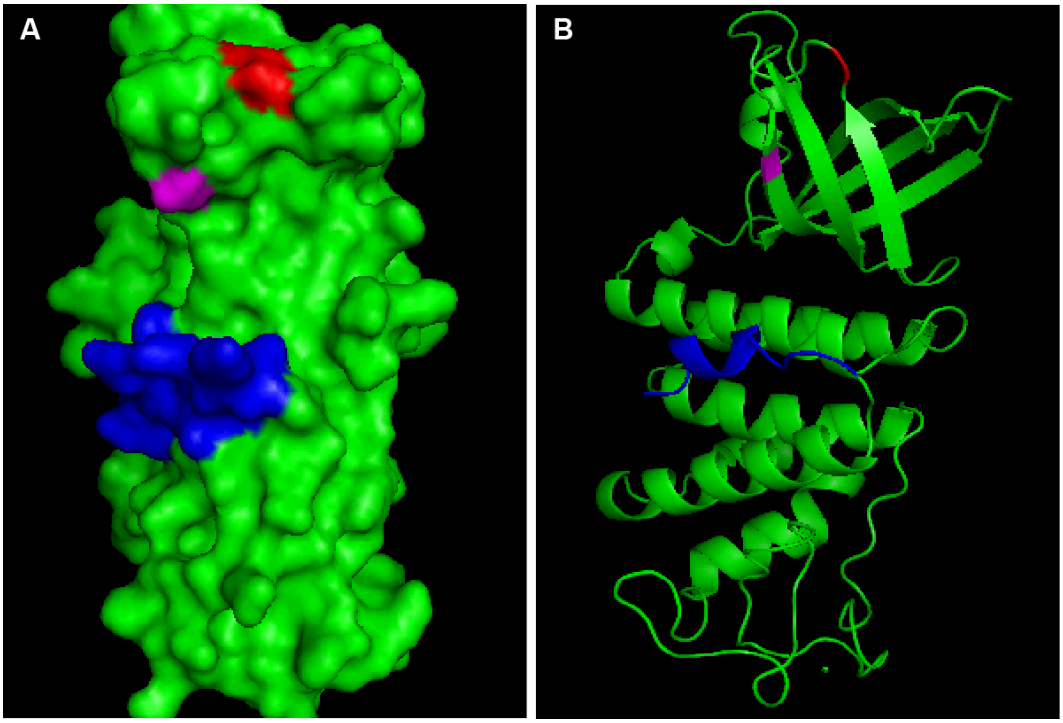

Figure 10. Model of *B. subtilis* RecO. A model of RecO was generated based on the *D. radiodurans* structure of RecO using SWISS-MODEL. Mutations are highlighted in color. Pink: N13E. Red: R37E. Blue: 24 amino acid C-terminal truncation. Two models of the protein are shown: **(A)** surface model **(B)** ribbon diagram showing secondary and tertiary structure.

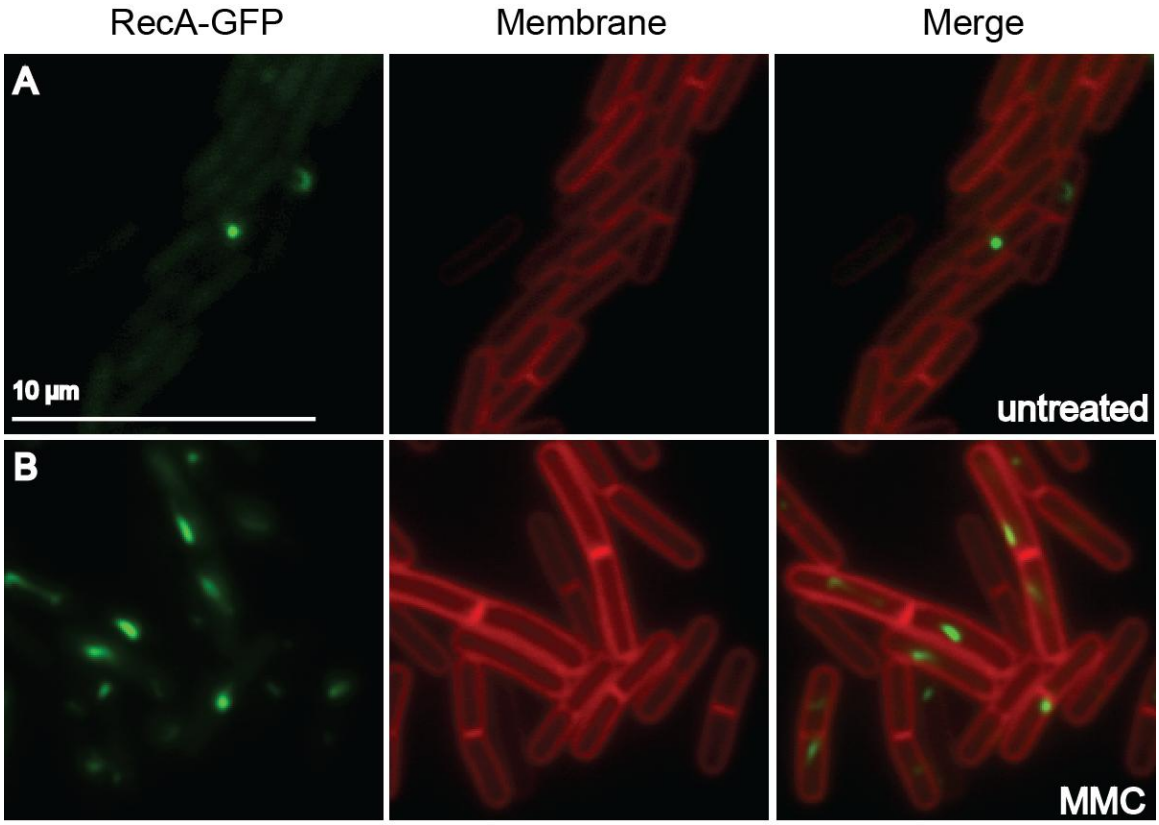

Figure 11. RecON13E in *B. subtilis* is not required for RecA-GFP localization. The *recON13E* allele was expressed from an ectopic locus under an IPTG inducible promoter. In a strain bearing the *recO::cat* allele, 5 µM IPTG was added to induce mutant RecO production. Cells shown are **(A)** untreated and **(B)** challenged with 100 ng/mL MMC for 1 hour.

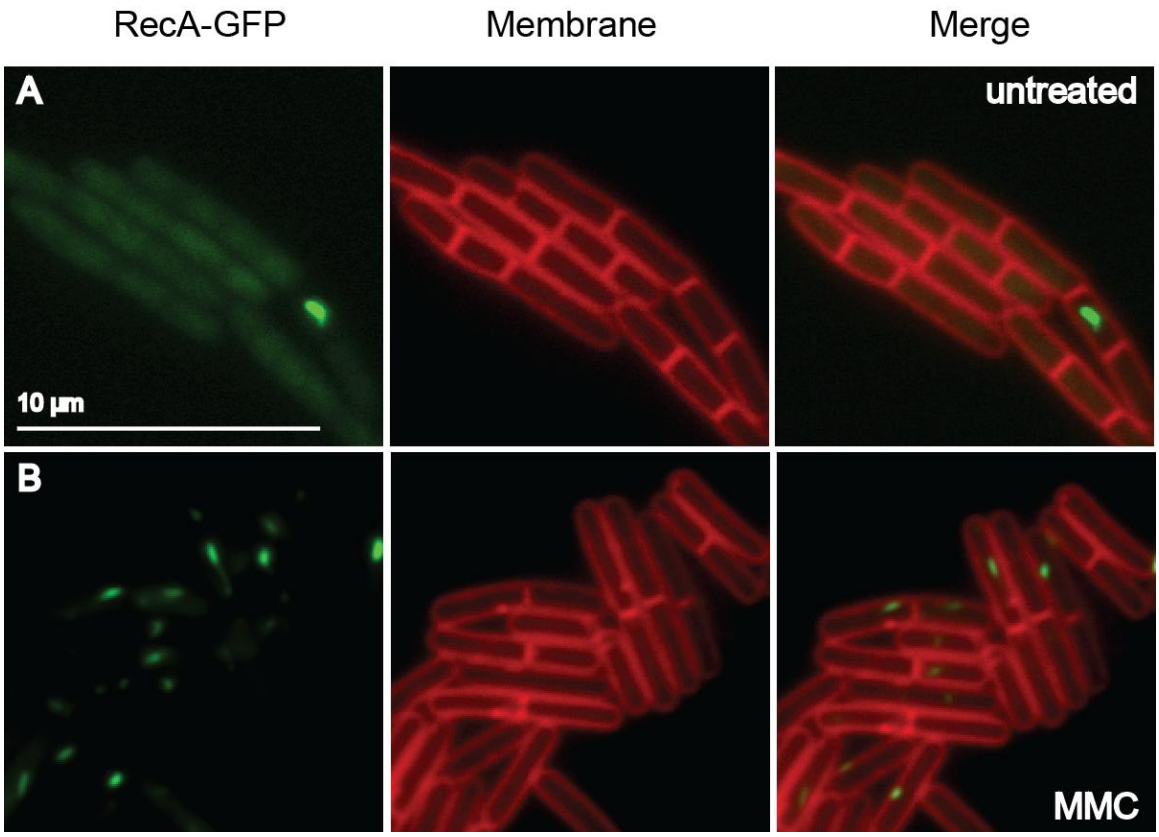

Figure 12. RecOR37E does not play a critical role in recruitment of RecA-GFP foci. The *recOR37E* allele was expressed ectopically under an IPTG inducible promoter. The native *recO* allele was disrupted, ensuring that the mutant form of RecO was the only source of RecO in the cell. The *recOR37E gene* was expressed using 5 µM of IPTG. Cells were **(A)** untreated and **(B)** challenged with MMC.

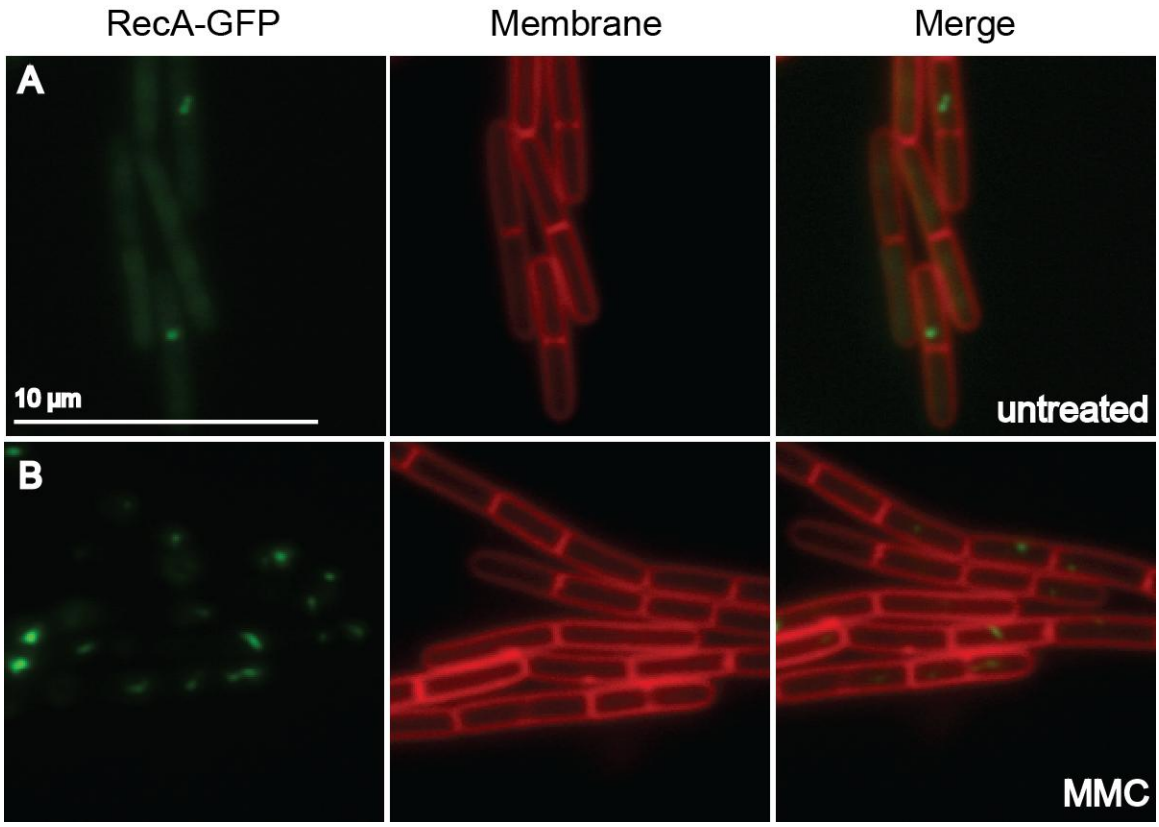

Figure 13. The variable C-terminal tail in *B. subtilis* RecO does not have a role the localization of RecA-GFP. Shown is RecA-GFP localization in a P_{spac}*recOΔ24 ; recO::cat* background. Cells were **(A)** untreated and **(B)** challenged with MMC for 1 hour.

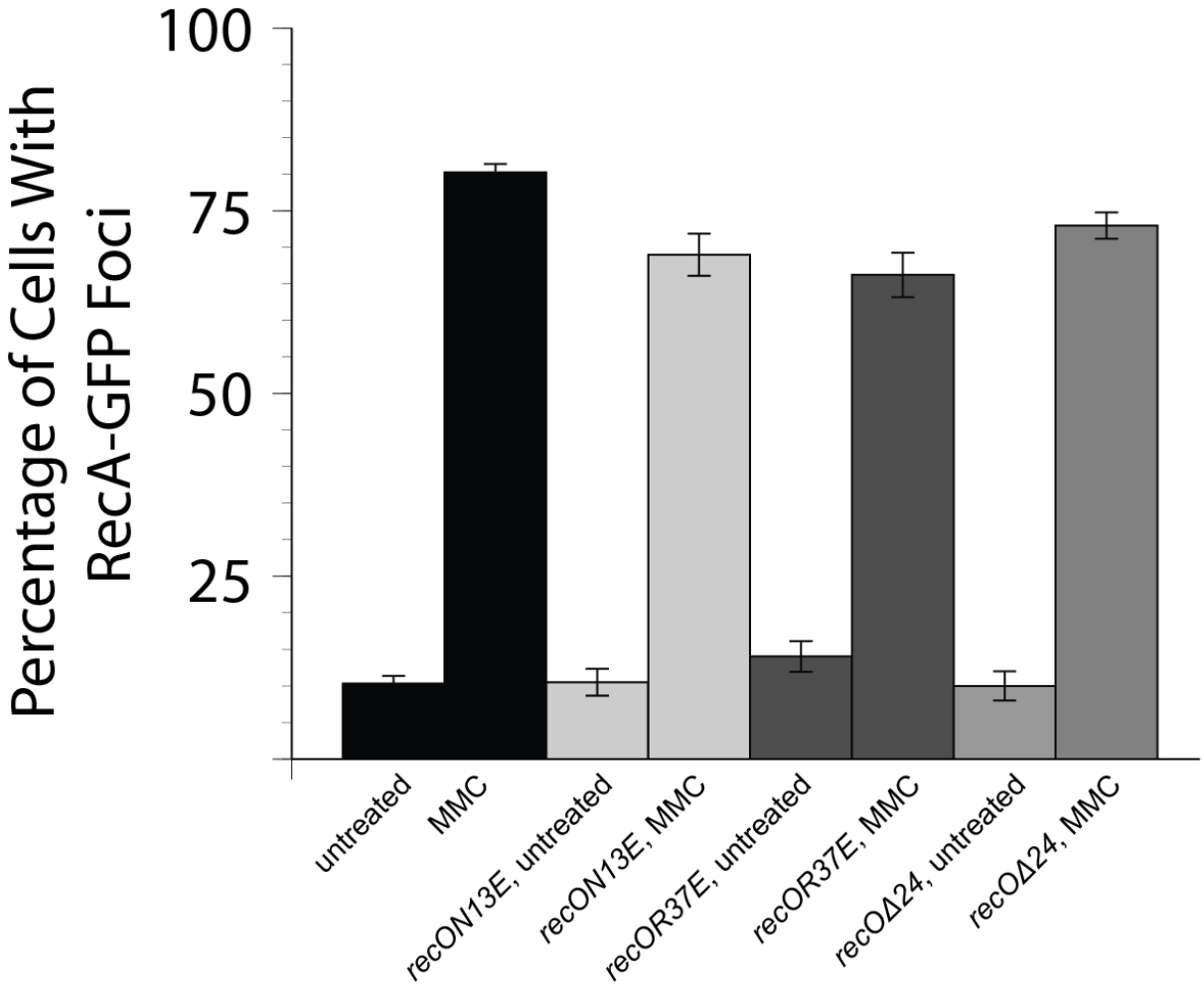

Figure 14. Quantification of mutant RecO on the recruitment of RecA-GFP into foci. Shown is the quantification of RecA-GFP foci in *recO*$^{+}$ and 3 different mutant *recO* strains: (1) *recA-gfp* (2) P_{spac}*recON13E ; recO::cat ; recA-gfp* (3) P_{spac}*recOR37E ; recO::cat ; recA-gfp* and (4) P_{spac}*recOΔ24 ; recO::cat ; recA-gfp*. Cells were untreated and challenged in liquid culture with MMC for 1 hour. The error bars reflect a 95% confidence interval. The number of cells scored in all populations is greater than 975.

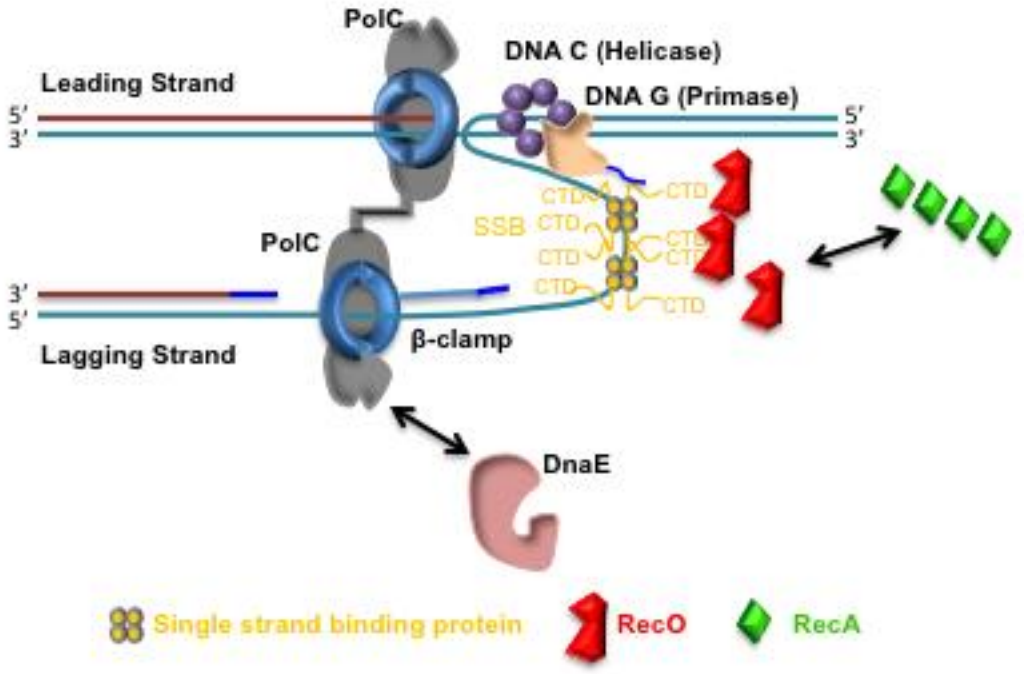

Figure 15. Working model for SSB and RecO-dependent recruitment of RecA to replication forks in *B. subtilis.* The C-terminal domain (CTD) of SSB binds to several proteins in *B. subtilis* including RecO. We propose that once a replication fork encounters a site of DNA damage, RecO binds the CTD of SSB. The RecO/SSB complex recruits RecA, which displaces SSB. This allows RecA to polymerize on ssDNA to aid in stabilizing stalled replication forks, function in recombinational repair, and induce the SOS response.

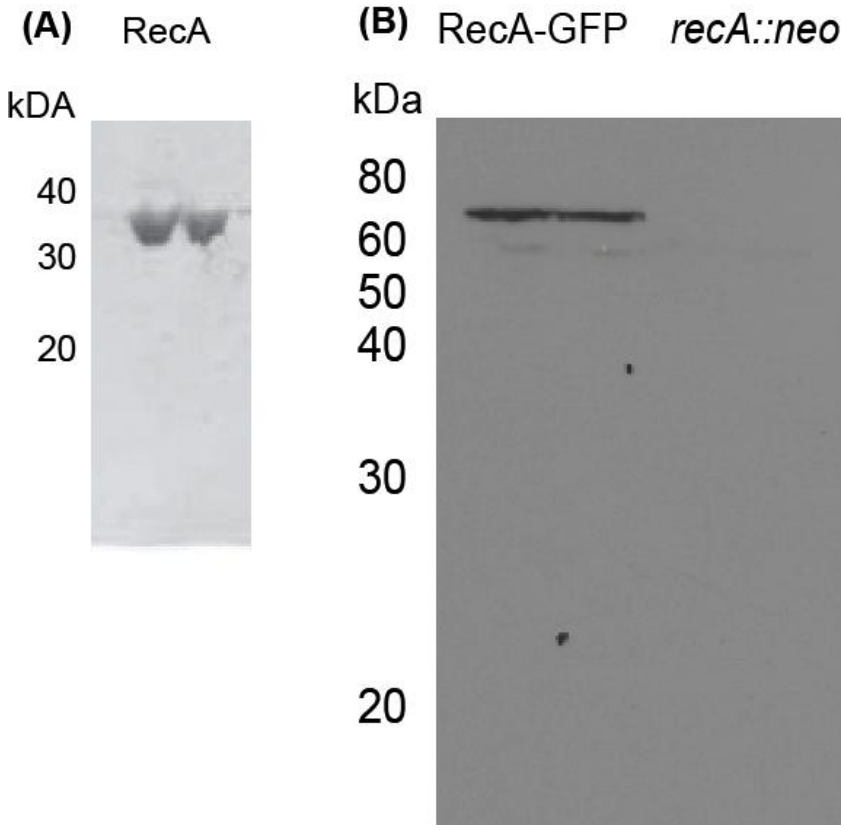

Figure 16. Purification and test of anti-serum against RecA. RecA (37933.6 Da) was purified in three different steps (materials and methods) and antibodies were generated in a rabbit (Covance). **(A)** An SDS-PAGE of purified RecA stained with Coomassie Blue. **(B)** An immunoblot of two different strains: *recA-gfp* and *recA::neo*. Proteins were probed with a 1:500 dilution of anti-RecA in a 2% milk/ 0.02% TBS-Tween (pH = 7.5) blocking buffer and exposed for 45 seconds.