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Honors Thesis

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Abstract
The bacteria *Deinococcus radiodurans (D. rad)* is able to survive multiple double stranded breaks to its DNA with no detriment to its health as its genome can be fully repaired in a matter of hours. During bacterial DNA replication, points of DNA damage can cause replication to cease, requiring an origin independent replisome loading pathway to resume the replication. This pathway is called replication restart , a highly conserved pathway in bacteria.

In the *E. coli* replication restart pathway primosome proteins function by binding to the DNA fork and facilitating the loading of the DnaB helicase onto the replication fork. The *E. coli* primosome protein A (PriA) acts as a helicase to unwind a portion of the double stranded DNA at forks without leading strand gaps, while primosome protein C helps facilitate DnaB loading at replication forks with a leading strand gap. Previous experiments have shown that the *D. rad* PriA does not seem to have any activity as a helicase. Additionally, the *D. rad* PriA is much larger than other bacterial PriAs indicating that it may have a different functionality. *D. rad* does not have a PriC protein and because replication restart pathways are crucial to the health of bacterial cells it is possible that *D. rad* PriA might have additional functionality to compensate for the lack of PriC.

The purpose of this project was to test the ability of the *D. rad* replication restart proteins to load *D. rad* DnaB onto DNA forks with a leading strand gap, an activity that PriC would accomplish in *E. coli*. The required proteins were cultured in specialized *E. coli* bacteria and then extracted and purified. Helicase assays were then used to test the ability of PriA to load DnaB onto DNA forks with a leading strand gap with the results indicating that no DnaB-mediated unwinding occurred.

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Introduction

DNA is copied through a process known as replication. Successful replication is imperative to the success of an organism because without a way of successfully copying a cell's DNA the cell would have no way of passing its genome on to a future generation. The initiation of bacterial DNA replication is origin dependent; meaning replication can only be initiated at specific places along the chromosome called origins of replication. If some of the template DNA is damaged the replication fork can become inactivated (Cox et al., 2000). The replication enzymes (replisome) must then be reloaded for replication to continue. This requires an origin-independent method of replication initiation which is performed in a pathway known as replication restart. Replication restart is used often by cells as the stalling of replication forks is not an irregular event, but rather one that happens quite regularly under normal aerobic conditions (Cox et al., 2000).

E. coli has two major replisome loading systems. The first system involves PriA-PriB-DnaT system, and the second involves PriC and Rep proteins (Lopper et al. 2007; Heller and Marians, 2005). Both allow the replicative helicase, DnaB, to bind to the DNA and continue replication. DnaB is a replicative helicase that is responsible for unwinding chromosomal DNA and allows the DNA synthesis enzymes to access the information contained in each template strand. The PriC and PriA-PriB-DnaT system accomplish the same task of loading DnaB onto DNA, but differ in the state of the starting DNA. The PriA-PriB-DnaT system acts on a DNA fork that contains exclusively double stranded DNA; while the PriC system acts on a DNA fork with a portion of single stranded DNA, which is known as a DNA fork with a leading strand gap (Heller and Marians, 2005). A leading strand gap is a section of single stranded DNA that has yet to be replicated near

the replication fork. These two systems allow for *E. coli* to replicate damaged DNA that would otherwise lead to failed replication and eventually cell death. The two systems are shown below in figure 1.
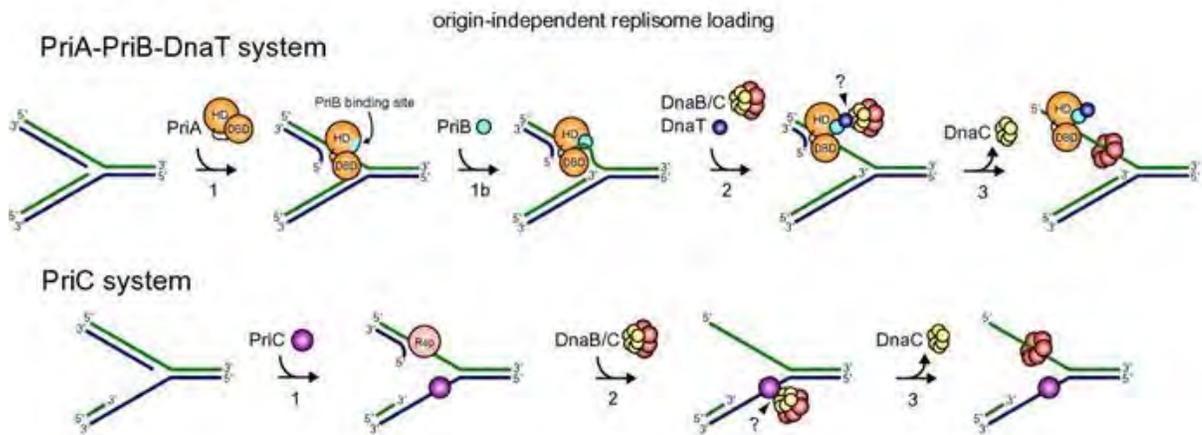

Figure 1: The two modes of origin-independent replisome loading in bacteria are demonstrated above. The top demonstrates a DNA fork without a leading strand gap (PriA-PriB-DnaT system). The bottom demonstrates origin-independent loading on a fork with a leading strand gap (PriC system).

The organism of research in this study is *Deinococcus radiodurans*: an extremophile that is capable of surviving under extremely high levels of ionizing radiation (Battista et al. 1997). Because this type of radiation is capable of doing tremendous damage to DNA molecules it is thought that the *D. rad* bacteria must have a very sophisticated way of repairing damaged DNA(Battista et al. 1997). Interestingly, the *D. rad* bacteria contains only two of the proteins involved in the typical origin-independent replisome binding demonstrated in figure 1: PriA and DnaB (Cox and Battista, 2005). Interestingly, the *D. rad* PriA is unique from other bacterial PriA and is about 200 amino acids longer than the typical length of about 732 amino acids (Cox and

Battista, 2005). Because the *D. rad* bacteria do not have all of the additional proteins that

other bacteria need to have a functioning origin-independent replisome loading system, it

is possible that *D. rad* PriA may have an additional functionality to help compensate for

these missing proteins. Additionally it appears that the *D. rad* PriA does not have the

same helicase properties that are essential to the function of PriA (M. Lopper,

unpublished data).

The focus of this project is to see if *D. rad* PriA alone can load the *D. rad* DnaB

onto DNA forks with a leading strand gap. In some bacteria this processes is performed

by the PriC protein as show in figure 1(Heller and Marians, 2005). Because *D. rad* does

not have this protein it is hypothesized that the *D. rad* PriA can bind DnaB to the DNA

forks that have a leading strand gap. Because the PriC system works on a DNA fork with

a leading strand gap (a portion of single stranded DNA), SSB will also be included in

these assays as it is a protein that works to regulate protein activity in single stranded

portions of *D. rad* DNA (Eggington et al. 2004; Cox, et al. 2010). The inclusion of SSB

will help create an environment that better reflects cellular conditions. The results will

give some insight into how the *D. rad* bacterium replication restart pathway functions.

To test this hypothesis the three proteins to be tested must all be purified: *D. rad*

PriA, *D. rad* SSB, and *D. rad* DnaB. The recombinant protein will be used to conduct

experiments to determine the ability of *D. rad* PriA to load DnaB onto DNA with a

leading strand gap. DNA substrates will be constructed to mimic a replication fork with a

leading strand gap. The DNA fork will be a series of 25 complementary nucleotides

followed by a stretch of 25 noncomplementary nucleotides resulting in a stretch of single

stranded DNA. One of the strands is also labeled with fluorescein at its 3' end. The described DNA fork is shown below in figure 2
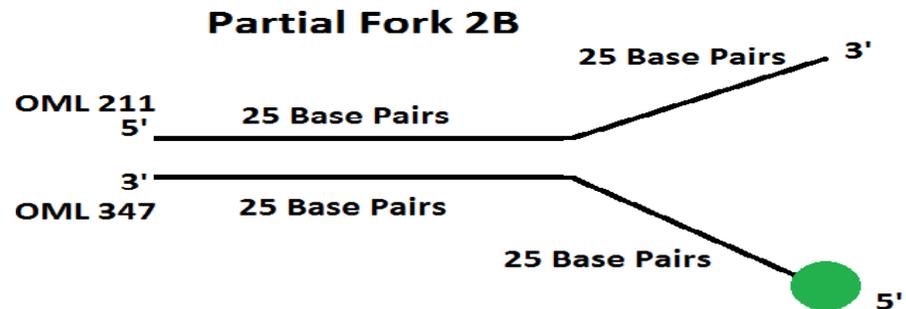
Partial Fork 2B

OML 211
5' _____ 25 Base Pairs _____ 25 Base Pairs — 3'

3' _____ 25 Base Pairs
OML 347

25 Base Pairs
5'

Figure 2: The above image represents the partial DNA fork that was used for our helicase assays. The fork consists of two 50 nucleotide strands with one being labeled with a fluorescein tag represented above as the green circle.

The proteins and DNA substrates will be used in helicase assays to test the helicase activity of DnaB. If the partial fork is unwound by helicase activity it will be split into the two single stranded portions of the fork. This change from double stranded to single stranded DNA can be measured with fluorescence spectroscopy, or visualized with the use of gel electrophoresis. Successful loading of the DnaB will be indicated by unwound DNA substrates. In a helicase assay where SSB is not present DnaB should be able to unwind a DNA fork with a leading strand gap, and the presence of SSB bound to the single stranded portion of the DNA fork should prevent the DnaB from being able to load. PriA will then be added to an assay with both SSB and DnaB, and if unwinding occurs this will support the hypothesis that *D. rad* PriA can load DnaB onto replication forks with a leading strand gap.

Materials and Methods:

Purifying *D. rad* PriA. The first step in purifying *D. rad* PriA was growing the protein in BL21(DE3) CodonPlus RP *E. coli.* The specialized cells have the gene for *D. rad* PriA regulated by the *lac* operon, enabling control of the cell's expression of the protein of interest. The protein of interest also included a His-tag to facilitate purification by nickel affinity chromatography. 6 liters of LB medium (10 g/L Tryptone, 5 g/L yeast extract, and 10 g/L NaCl) were made by placing 1 liter of LB solution into 6 two-liter baffled Erlenmeyer flasks and inoculating them with equal portions of overnight cell culture. The resulting solutions were cultivated in an incubator at 37°C until they reached an absorbance within an ideal range which was 0.4-0.6 at 600nm. When this critical absorbance was reached the cultures were induced with 0.5mM isopropyl β-D-thiogalactopyranoside (IPTG) to induce production of the protein of interest. After incubating for 4 hours after induction the flasks were removed from the incubator and the solution decanted into containers to be centrifuged for 20 minutes with a force of 3716xg at 4°C into a cell pellet. The resulting cell pellets were stored in a -80°C freezer until needed. To start the purification process of *D. rad* PriA the cells were suspended in a lysis buffer (10 mM Tris-HCl pH 8, 10% glycerol v/v, 0.5 M NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride) using the ratio of 1 gram of cell pellet to 5 mL of lysis buffer.

The appropriate amount of lysis buffer was formulated and added to the cell pellets. The cell pellet had to be stirred in order to break up large chunks of cell material in order to achieve a higher yield of protein of interest. The solution was then sonicated while on ice using five 30 second pulse bursts. The lysate was then clarified by

centrifugation at 40,000 x g for 20 minutes at 4° C. An aliquot was taken of the clarified lysate.

The clarified lysate was incubated with Ni^{2+}-NTA agarose beads for 1 hour at 4° C with gentle rocking. This was performed by taking two mL of the Ni^{2+}-NTA agarose beads and adding three mL of lysis buffer and the resulting 5mL was divided amongst five 50mL conical tubes in which the clarified lysate was also divided. The five conical tubes were then placed on a refrigerated mixer and incubated for 1 hour.

The solution was transferred in small quantities to a 20mL plastic column to catch the Ni^{2+}-NTA agarose beads and drain off the supernatant which was collected in a 250mL Erlenmeyer flask. An aliquot of the flow through was taken. The column was then washed with an additional 10ml of lysis buffer and additional aliquots were taken of the first and last few drops of the wash. All of the aliquots were stored in a -20°C freezer.

To elute the protein of interest from the Ni^{2+}-NTA agarose beads we prepared 15mL of elution buffer (10 mM Tris-HCL pH 8, 10% glycerol, 100 mM NaCl, 250 mM imidazole, and 1 mM β-mercaptoethanol) and washed the Ni column with this buffer. An Aliquot of the effluent was taken.

The effluent was then place in 3.5 kDa molecular weight cut off dialysis tubing and suspended in 1 liter of dialysis buffer (10 mM Tris-HCl pH 8, 10% glycerol, 100 mM NaCl, 1 mM β-mercaptoethanol, and 250 mM MgCl) and allowed to sit overnight at 4°C. This step removed the His-tag from the protein of interest. The dialysis tubing was then removed and the protein solution poured into a 15 mL conical tube and centrifuged for 20min with a force of 4000 x g at 4°C in order to clarify the elutant. This solution was then diluted from 12.5mL to 40 mL using QFF buffer A (10 mM Tris-HCl pH 8, 10%

glycerol, 100 mM NaCl, and 1 mM β-mercaptoethanol). This solution was then loaded onto the QFF column and the column was run at a rate of 0.5 mL per minute and a 10 column-volume salt gradient with a final concentration of 50% buffer B (10 mM Tris-HCl pH 8, 10% glycerol, 1M NaCl, and 1 mM β-mercaptoethanol).

The UV absorbance of the effluent indicated that the protein did not bind to the column. The fluid that passed through the column during the loading of the protein as well as the first few collection tubes were combined to be concentrated. A concentrator with a 10,000 amu filter was used to condense the dilute protein containing solution with a force of 2,643 x g at 4°C from 15mL down to 7mL. The volume of the protein solution was checked every half hour, and filtrate discarded until the final volume of the protein solution was 7mL

The protein solution was then run through an S-300 sizing column. The graph of the S-300 was analyzed and the appropriate collection tubes were pooled and concentrated from 12 mL to 400 μL using a 3,000 molecular weight cut off concentrator. The concentrator was placed in a centrifuge set at a force of 2,643 x g at 4°C for 3 hours.

The protein was then quantified using a Thermo Scientific Evolution 220 Spectrometer set to 280nm and a micro cuvette. A blank was prepared with 145mL of 8M guanidine and 5 mL of S-300 buffer. The sample contained 145 mL of 8M guanidine and 5mL of concentrated protein. The remaining protein solution was then aliquoted into 10μL portions in microcenterfuge tubes and stored in a -80°C freezer.

An SDS page gel was then run with all of the aliquots of the purification process as well as a portion of the purified protein and dilutions to determine purity. After

running the gel-electrophoresis for 40 minutes the SDS page gel was soaked in coomassie stain and then destained over a course of several days.

SSB Purification: The procedure for purifying the *D. rad* SSB was the same as for purifying *D. rad* PriA as previously demonstrated apart from a few subtle changes. The QFF column was successful and an additional chromatography was not needed in purifying the protein. The protein containing fractions were then pooled and concentrated using a 3,000 molecular weight cut off concentrator at 4° and 2643 x g. The concentrated protein was then quantified.

DnaB Purification: The procedure for purifying the *D. rad* SSB was the same as for purifying *D. rad* PriA as previously demonstrated apart from a few subtle changes. 12 liters of LB were made. Like the PriA the QFF was not very successful, but the flow though was sufficiently purified that additional column chromatography was not necessary. The protein containing fractions were then pooled and concentrated using a 3,000 molecular weight cut off concentrator at 4° and 2643 x g until the volume of concentrated protein was 300μL. The concentrated protein was then quantified.

Construction of Forked DNA substrate: To prepare the DNA fork there are four steps that were followed: 1) add components, 2) heat the components followed by a slow cooling, 3) purify the resulting forks with a gel, 4) elute to 500 μL. The following components were added to a micro centrifuge tube in preparing the partial DNA fork: 1 μL 10x STE Buffer (10 mM Tris HCl pH 8, 50mM NaCl, and 1mM EDTA), 6 μL milliQ water, 1 μL OML 347, and 2 μL OML 211.

The heating and cooling process was performed by heating the micro centrifuge tube with the necessary components at 95°C for 5 minutes before slowly cooling to 70°C

and incubating at 70°C for an additional hour. This was performed by programming a PCR machine to heat the samples at 95°C for 5 minutes before cooling 0.3°C per minute until at 70°C. At 70°C the machine incubated for 1 hour before cooling 0.5°C per minute until the sample reaches 25°C upon which the sample will be kept refrigerated at 4°C.

The resulting fork was purified by running the sample through a 6% acrylamide gel using electrophoresis in Tris-borate EDTA buffer prepared from a 5x buffer stock containing: 54g/L Tris base, 27.5g/L boric acid, and 10mM EDTA. The DNA fork was observed as it migrated down the gel (it was visible due to the fluorescein tag) and stopped the electrophoresis when the fork had run half way down the gel. The portion of the gel containing our fork was then cut out and placed in 3.5 kDa molecular weight cut off dialysis tubing with 500µL of TBE buffer. The resulting section of tubing was floated in an electrophoresis tank. A current of 30 volts was applied for 80 minutes before raising the voltage to 40 volts for an additional 100 minutes in order to remove the DNA fork from the cut out piece of gel.

Helicase Assays: The various components of the assays included: DNA fork, buffer, SSB (if necessary), PriA (if necessary), DnaB (if necessary), an incubation period to allow proteins to bind onto the labeled DNA fork, complementary DNA, ATP, longer incubation period to allow potential helicase activity, heat (for the unwound standard only), 10% SDS, and DNA gel buffer.

After the necessary components were added and incubations completed the solutions were resolved on a 10% polyacrylamide gel using a voltage of 80 volts. The resulting gels were then analyzed and imaged.

Results and Discussion

Protein Purification: The concentration of total protein in the sample was determined for our *D. rad* PriA protein by measuring absorbance with a spectrometer. The absorbance was measured by diluting the concentrated PriA with an 8M guanidine solution using a 30 fold dilution to create a final volume of 150μL. A window cuvette made of quartz was used to take 6 absorbance readings at 280nm which averaged to 0.061. From literature the molar extinction coefficient was calculated to be 184,370 M^{-1}cm^{-1}. From Beer Lambert's Law the total protein concentration is then 9.93 μM.

The purity of the sample was determined using gel electrophoresis. A gel was run with the pure sample as well as a set of serial dilutions to provide a standard against which the impurities could be compared against to provide an approximate measure of purity for the sample. The resulting gel is shown below in figure 3.

Figure 3: Lane 1 refers to the protein ladder of standard lengths, lane 3 refers to the aliquot taken before the S-300 column was run, lane 5 refers to the 1/100 dilution and is not easily visible, lane 7 refers to the 1/10 dilution, and lane 9 refers to the protein sample. The contaminants are very distinct, which is not ideal.
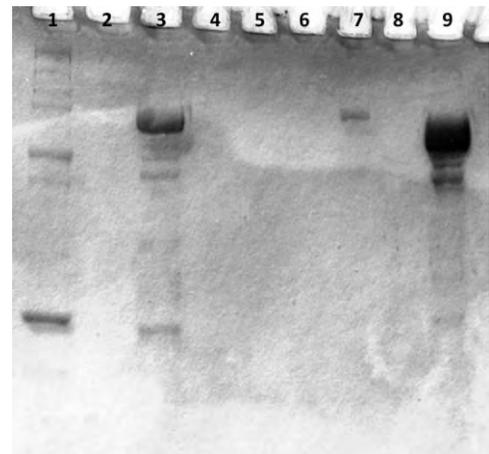
From the results in figure 3 the purity of the *D. rad* PriA was determined to be between 75-85%. The poor purity is likely due in part to the failed attempt to bind the

protein to the QFF column. The protein never seemed to bind to the column indicating that the salt concentration was too high for the salt gradient.

A similar set of gels were run for the *D. rad* SSB purification. The first has all of the aliquots of the purification process, and the second has the serial dilutions to determine protein purity.. The resulting gels are shown in figures 4 and 5.
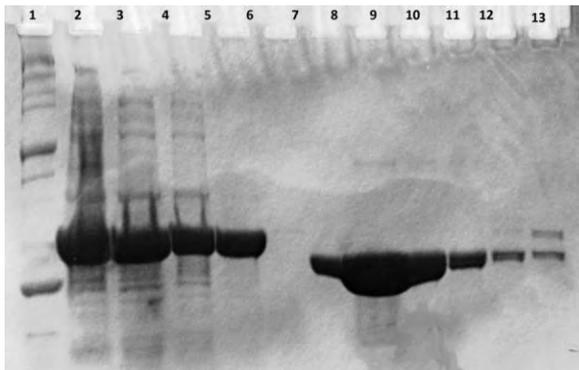

Figure 4: This image demonstrates several aliquots as well several of the fractions we pulled from ionizing column showing the progress of the protein of interest. Lane 1 corresponds to the protein ladder. Lane 2 corresponds to the lysate. Lane 3 corresponds to the flow through of the Ni column. Lane 4 corresponds to first part of the wash. Land 5 corresponds to the late wash. Lane 6 corresponds to the effluent. The remaining lanes correspond to fractions from the QFF column.
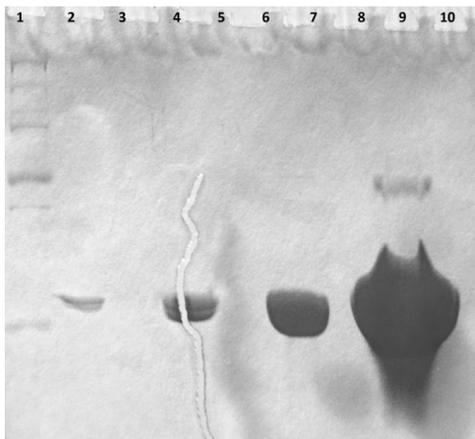

Figure 5: This image demonstrates the purity of the purified *D. rad* SSB protein. Lane 1 refers to our standard protein ladder, lane 2 is a 1/1000 dilution of purified SSB protein, lane 4 is a 1/100 dilution of the SSB protein, lane 6 is a 1/10 dilution, and lane 8 is our pure protein with some visible contaminants. By comparing the size of these contaminating bands from the diluted protein samples the

overall purity of the sample can be estimated. The contaminating band is smaller than the 1/100 dilution but larger than the 1/1000 dilution, therefore the purity of this protein sample is greater than 99%. The white lines are due to cracks in the gel which were caused during the drying process.

The concentration of total protein in the sample was determined with spectroscopy. The absorbance was measured by diluting the concentrated SSB with an 8M guanidine solution. Typically a 30 fold dilution would be used, but the SSB was so concentrated a 100 fold dilution was used instead with a final solution volume of 150μL. A window cuvette made of quartz was used to take 6 absorbance readings at 280nm which averaged to 0.62317. From literature the molar extinction coefficient is $4.01\pm0.2\times10^4M^{-1}cm^{-1}$ (Eggington et al. 2004). Through using Beer Lambert's Law the total protein concentration was determined to be 1.5579mM. The molecular weight of *D. rad* SSB is 33 kDa, therefore the concentration of SSB was 51 g/L. As a whole the SSB purification went very smoothly giving very high yields and purity.

The results for the *D. rad* DnaB indicated protein purity around 90%. Like the *D. rad* PriA purification, the QFF column chromatography was not very successful as the initial salt concentration was likely too high. Another issue was the low yield. Due to the low yield additional column chromatography was not attempted. Based off of the aliquots taken throughout the purification process, it is likely that much of the protein was lost in the process of clarifying the cell lysate as aliquots taken after that step show a marked decrease in the presence of *D. rad* DnaB protein.

During the quantification process the concentration of total protein in the sample was also determined. From literature the molar extinction coefficient was calculated to be

12,920M^{-1}cm^{-1}. The average absorbance of the 6 readings from the 15 fold dilution sample was 0.02012, by Beer Lambert's Law the total protein concentration was 23.413μM.

Helicase Assays: Initially attempts were made at using fluorescence polarization spectroscopy to test the helicase assays. However all of the results were too inconsistent to be reliable. As a result the helicase assays were tested using polyacrylamide gel electrophoresis.

With this method there are a couple concerns. First in order to visualize the bands of DNA requires a much higher concentration of DNA, making the chance that DNA strands that had already been separated could anneal with other complementary strands is much more likely with a high concentration of DNA. To counter this effect an even higher concentration of a strand complementary to the unlabeled strand to serve as a "DNA trap" and out-compete the labeled strand should any annealling occur.

The series of helicase assays indicated that the DnaB was unable to mediate unwinding of partial fork 2B even without the presence of SSB. This conclusion was reached over a series of experiments as the focus of the project shifted away from *D. rad* PriA loading *D. rad* DnaB onto a SSB bound DNA fork with a leading strand gap, towards testing *D. rad* DnaB's ability to act as a helicase. The first gel that was run was designed to test all of the different combinations of *D.rad* proteins. All of the components are shown below in figure 6, with the resulting gel shown in figure 7.

Master Table for first gel - Additions performed from left to right										
Well #	OML 347	DNA Fork	Buffer	DNA Trap	SSB	PriA	DnaB	ATP	95° Heat	Incubation	DNA Buffer
1	Yes	-	-	-	-	-	-	-	-	1 Hour	5μL
2	-	6.7μM	9μL	166.7μM	-	-	-	10μL	-	1 Hour	5μL
3	-	6.7μM	9μL	166.7μM	-	-	-	10μL	Yes	1 Hour	5μL
4	-	6.7μM	5.15μL	166.7μM	200μM	-	-	10μL	-	1 Hour	5μL
5	-	6.7μM	7.48μL	166.7μM	-	500nM	-	10μL	-	1 Hour	5μL
6	-	6.7μM	7.72μL	166.7μM	-	-	500nM	10μL	-	1 Hour	5μL
7	-	6.7μM	3.86μL	166.7μM	200μM	-	500nM	10μL	-	1 Hour	5μL
8	-	6.7μM	6.21μL	166.7μM	-	500nM	500nM	10μL	-	1 Hour	5μL
9	-	6.7μM	2.35μL	166.7μM	200μM	500nM	500nM	10μL	-	1 Hour	5μL

Figure 6: As the nucleotides are not easily visible, lane 1 was designated to be our visual marker while running the gel. Lanes 2 and 3 were our standards corresponding to wound and unwound DNA respectively. The remaining lanes each tested a combination of PriA, DnaB, and SSB proteins
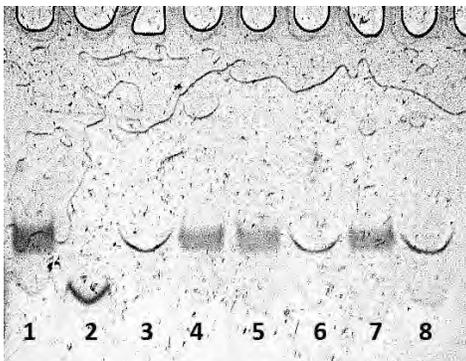

Figure 7: This gel is oriented from left to right, and lane 2 clearly shows a faster migrating product indicating the smaller unwound DNA standard. Lane 1 served as the unwound DNA standard. All other lanes migrated at the same speed, indicating that no helicase activity had occurred.

This result was unexpected. Without the presence of SSB, DnaB is expected to bind and unwind a DNA fork with a leading strand gap such as partial fork 2B (Heller and Marians, 2005). Because the DnaB did not demonstrate helicase activity prevented any conclusion s being drawn about PriA's ability to load DnaB. After viewing the results of the first gel a second gel was designed to test the order of addition of the DNA

trap. Also the DnaB was titrated to test the effect of a higher concentration of DnaB. The table demonstrating the order of addition is below with the corresponding gel in figures 8 and 9 respectively.

Master Table for second gel - Additions performed from left to right											
Well #	DNA Fork	Trap	DnaB	Incubate	Trap	Δ	ATP	Incubate	10%SDS	Δ	DNA Buffer
1	6.7μM	166.7μM	-	-	-	-	10μL	1 Hour	5μL	-	7μL
2	6.7μM	166.7μM	-	-	-	2min	10μL	1 Hour	7μL	2min	7μL
3	6.7μM	166.7μM	500nM	-	-	-	10μL	1 Hour	7μL	-	7μL
4	6.7μM	-	500nM	10 min	166.7μM	-	10μL	1 Hour	7μL	-	7μL
5	6.7μM	166.7μM	1000nM	-	-	-	10μL	1 Hour	7μL	-	7μL
6	6.7μM	-	1000nM	10min	166.7μM	-	10μL	1 Hour	7μL	-	7μL
7	6.7μM	166.7μM	2000nM	-	-	-	10μL	1 Hour	7μL	-	7μL
8	6.7μM	-	2000nM	10min	166.7μM	-	10μL	1 Hour	7μL	-	7μL

Figure 8: Wells 1 and 2 correspond to the DNA fork standards of bound and unbound respectively as the heat (Δ) will disassociate the DNA strands. The other 6 wells tested three different concentrations of DnaB and the order of addition of the complementary DNA ("Trap"). Buffer was also added to ensure equal volumes.
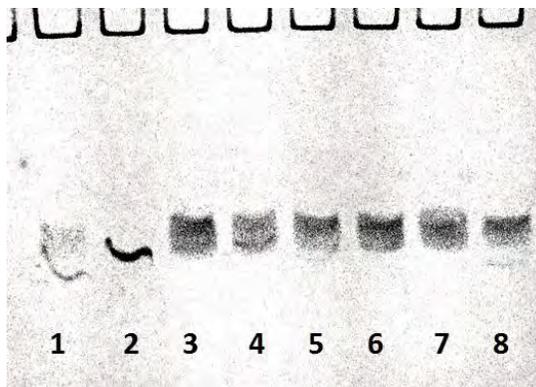

Figure 9: This is the gel run with the components depicted in figure 14. Lanes 1 and 2 correspond to the standards, and again like figure 13 there is nothing in this figure to indicate any helicase activity.

While there did not seem to be any additional success due to higher concentrations of DnaB, the highest concentrations of DnaB with high concentrations of PriA were tested to see if perhaps higher concentrations would lead to unwinding. The components used for this new round of experiments and the resulting gel are shown below in figures 10 and 11 respectively.

Master Table for Third gel - Additions performed from left to right										
Well #	DNA Fork	DnaB	PriA	Trap	ATP	Δ	Incubate	Δ	10%SDS	DNA Buffer
1	6.7μM	-	-	167μM	10μL	-	1 Hour	-	5μL	8μL
2	6.7μM	-	-	167μM	10μL	2min	1 Hour	2min	5μL	8μL
3	6.7μM	2μM	-	167μM	10μL	-	1 Hour	-	5μL	8μL
4	6.7μM	2μM	2μM	167μM	10μL	-	1 Hour	-	5μL	8μL
5	6.7μM	-	2μM	167μM	10μL	-	1 Hour	-	5μL	8μL

Figure 10: Again the first two wells were designated for the DNA standards. The remaining three wells tested higher concentrations of DnaB and PriA together.
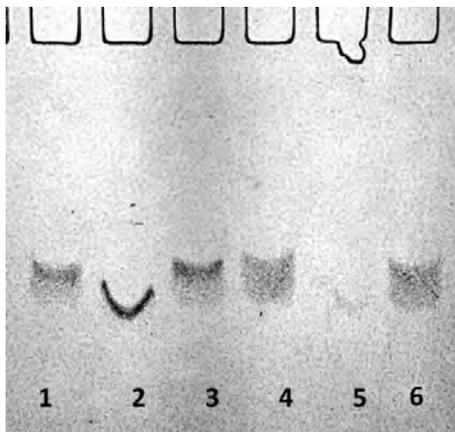

Figure 11: This figure shows the results of the gel demonstrated in figure 10. Again apart from the unwound standard there is nothing to indicate any helicase activity. The gel was loaded from left to right, but the fifth well was skipped due to the air bubble that had formed.

Again the *D. rad* DnaB failed to unwind the DNA fork. However one final helicase assay was attempted with long incubation times to confirm that no helicase activity is occurring. The resulting gel and table containing the components are shown below in figures 12 and 13 respectively.

				Master Table for Fourth gel - Additions performed from left to right							
Well #	DNA Fork	DnaB	PriA	Incubate	Trap	ATP	Δ	Incubate	Δ	10%SDS	DNA Buffer
1	6.7μM	-	-	30 min	167μM	10μL	-	2 Hours	-	5μL	8μL
2	6.7μM	-	-	30 min	167μM	10μL	2min	2 Hours	2min	5μL	8μL
3	6.7μM	2μM	-	30 min	167μM	10μL	-	2 Hours	-	5μL	8μL
4	6.7μM	2μM	2μM	30 min	167μM	10μL	-	2 Hours	-	5μL	8μL
5	6.7μM	-	2μM	30 min	167μM	10μL	-	2 Hours	-	5μL	8μL

Figure 12: The above figure demonstrates the components that were included in the fourth set of helicase assays. This set of experiments tested the same protein combinations as before, but with longer incubation times.
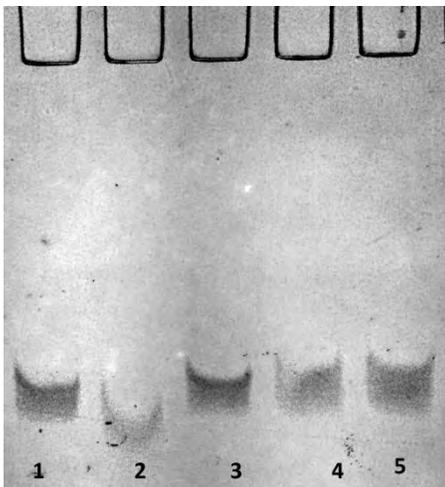

Figure 13: This gel was loaded left to right, and again the only lane to indicate any potential unwinding is lane 2 which is the denatured DNA standard. This indicates that the conditions presented are not suitable for helicase activity to occur.

The results from the helicase assays performed consistently indicated that the DNA fork remains intact when exposed to *D. rad* PriA, DnaB, and SSB. indicating that the DnaB being tested did not unwind the partial fork as it should. Therefore no further conclusions can be drawn about *D. rad* PriA's ability to load *D. rad* DnaB onto a replication fork with a leading strand gap. However further testing of the *D. rad* DnaB

should be conducted to either confirm the results that it does not have the expected helicase activity, or that these results are due to faulty protein preparation.

These results do not come as a total surprise as it should be expected that a helicase that could unwind small pieces of DNA should be highly regulated in a cell that is capable of surviving hundreds of double stranded breaks. While the experiment was conducted to test to see if *D. rad* DnaB and *D. rad* PriA have additional functionality, it is also possible that other required proteins could serve as accessory factors for DnaB helicase activity that have yet to be discovered. Regardless, continuing to develop a greater understanding of *D. rad's* origin independent loading mechanisms remains of great interest as the state of the replication restart pathway in *D. rad* is a mystery.

Acknowledgements

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