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Exploration of Protein-Protein Interactions Involving *Deinococcus radiodurans* PriA, DnaB and SSB

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Exploration of Protein-Protein Interactions Involving *Deinococcus radiodurans* PriA, DnaB and SSB



Honors Thesis

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Department: Chemistry

Advisor: Matthew E. Lopper, Ph.D.

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Deinococcus radiodurans is a species of bacteria that has sparked a lot of interest since its discovery due to its incredible resistance to ionizing radiation. When exposed to ionizing radiation the genome of *D. radiodurans* will sustain over one hundred double stranded breaks. *D. radiodurans* demonstrates the ability to repair its genome and restart replication after sustaining, typically lethal, DNA damage. This project examined the mechanism of replication restart in *D. radiodurans* by investigating primosome protein PriA interacting with replicative helicase DnaB and single stranded binding protein. Many different types of gel electrophoresis were employed to investigate potential protein complex formations between *D. radiodurans* PriA and DnaB. Native agarose gel electrophoresis successfully revealed an interaction between *D. radiodurans* PriA and DnaB. Results in this work indicate that *D. radiodurans* PriA can interact with DnaB.



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Introduction

When cells are exposed to ionizing radiation their genome becomes shattered (Battista). Depending on the intensity of exposure the DNA sequence of a cell can receive several hundred single stranded breaks, double stranded breaks, and base modifications (Battista). A broken genome with damage in the right places will leave a cell incapable of replicating its DNA sequence and the colony will no longer be able to undergo mitosis (Battista).

Deinococcus radiodurans is a species of bacteria that has shown a rare resistance to ionizing radiation (Makarova). Figure I shown below illustrates the survival power of *D. radiodurans* compared against *Escherichia coli*, a bacterial cell representing normal survival characteristics.

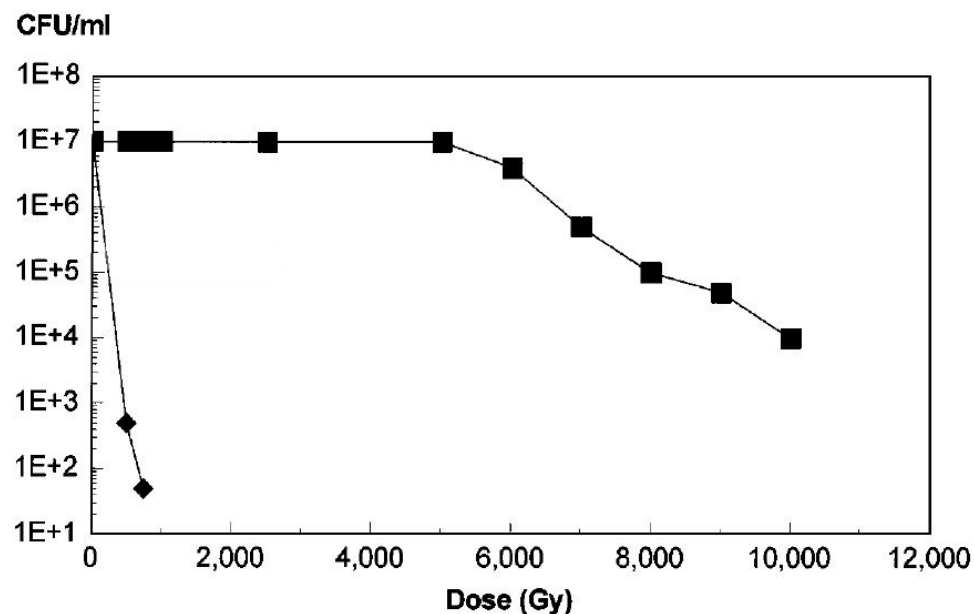


Figure I – *D. radiodurans* viability compared with *E. coli* viability after exposure to ionizing radiation. *D. radiodurans* (represented by squares) is able to remain viable after sustaining much greater amounts of ionizing radiation than *E. coli* (represented by diamonds) (Battista).

D. radiodurans, a member of the *Deinococcaceae* family, was first discovered while attempting to use radiation to sterilize spoiled meat (Blasius). After exposure to 4000 Grays the meat still showed signs of living *D. radiodurans* (Blasius). In addition to ionizing radiation the bacterium has been found to possess unique resistance to ultraviolet radiation and desiccation

(Battista). One study found that *D. radiodurans* could “survive for six years in a desiccator with 10% viability” (Battista). This is not surprising considering desiccation would also have similar, DNA damaging, effects on a cell (Asada). This resistance to desiccation is particularly interesting as it offers a rational explanation for an evolutionary pathway that led to *D. radiodurans* acquiring its resistance to ionizing radiation.

In the years since its discovery it has been found that *D. radiodurans* does not avoid the damaging effects of radiation (Battista). After sustaining damage to the DNA sequence that can be caused by both desiccation and UV radiation the genome will be shattered and many coding genes vital for cell survival will be not be functional (Battista). In order for transcription to occur the DNA comprising each gene must be intact and allow the protein machinery transcribing the DNA to navigate down the double helix (Cox). If the damaged genome is not repaired and replication is not restarted the cell will fail to carry out transcription and translation of essential genes and will no longer be a viable colony forming unit (Battista). *D. radiodurans* demonstrates the ability to both repair its genome and restart replication after sustaining heavy damage (Battista). Currently, very little is clearly understood about the exact DNA repair and replication restart process in *D. radiodurans*. There are several mechanisms that have been proposed based on what is known of other organisms (Blasius). A few examples of this include homologous recombination (the most commonly found repair mechanism), single-strand annealing, and nonhomologous end-joining (Blasius).

When a cell possesses a damaged DNA it will still attempt to transcribe the DNA to mRNA so the cell can continue to function normally (Cox). When the helicase responsible for unwinding DNA ahead of the replisome reaches the site of damage replication will be stopped (Cox). If the helicase remains at the fork then the fork is considered to be stalled (Cox). If all replicative helicases have dissociated from the site then the fork is considered to be collapsed. A stalled replication fork can continue to unwind and replicate as soon as the damage has been repaired (Cox). A collapsed replication fork not only faces the task of fixing the issue that caused

the halt, but also the task of reloading the primosome (the protein complex responsible for loading replicative helicases onto the replication fork).

Based on what is known about replication restart in other organisms it is reasonable to guess that the replication restart mechanism of a collapsed fork in *D. radiodurans* is something like what is illustrated in figure II.

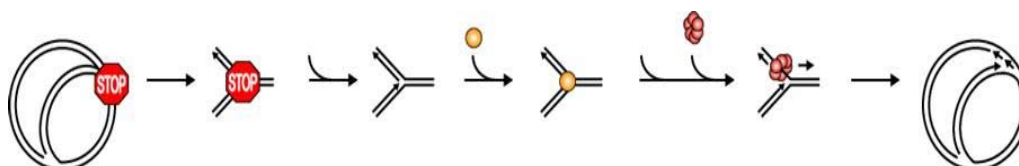


Figure II – A proposed mechanism for the replication restart mechanism of *D. radiodurans* on a collapsed replication fork. Yellow circles represent PriA. Red circles represent DnaB. From what is understood in other species of bacteria we expect that PriA will bind to the replication fork and then assist the hexameric DnaB complex in loading onto the replication fork (Marians, Zhang).

PriA, represented in yellow, is the protein commonly believed to be responsible for initiating the reloading of DNA unwinding helicases (Marians). In the mechanism shown below the primosome attaches itself to the replication fork and then assists the loading of DnaB (a replicative helicase illustrated by the red circles) so that DNA unwinding may continue and replication can resume (Zhang).

Primosomes in different organisms can vary in composition and size. *D. radiodurans* encodes for primosome protein PriA, commonly observed in many species of bacteria. PriA typically demonstrates a small amount of helicase activity altering the geometry of the replication fork and assisting DnaB in loading onto the replication fork (Marians). It is not uncommon to find a PriA in *D. radiodurans*, but the PriA that has been identified shows several remarkably different characteristics that are not normally found in PriA proteins. These characteristic differences make PriA a protein of interest in deciphering the radiation resistance observed in *D. radiodurans*. The first distinct feature of PriA that draws attention is its relative size. Table I

shown below compares the size of a variety of PriA proteins found in organisms from many different phyla. The PriA found in *D. radiodurans* is relatively large. The amino-terminal DNA binding domain of *D. radiodurans* PriA is where most of this extra size is accounted for.

Table I – PriA Size Comparison Over Several Different Phyla. The PriA found in *D. radiodurans* is considerably larger than many other species in different phyla.

Phylum (Class)	Organism	PriA Length (a.a.)
Deinococcus-Thermus	<i>Deinococcus radiodurans</i> R1	925
Proteobacteria (alpha)	<i>Rickettsia felis</i> URRWXCal2	648
Proteobacteria (epsilon)	<i>Helicobacter pylori</i> J99	619
Firmicutes	<i>Clostridium acetobutylicum</i> ATCC 824	733
Chlamydiae	<i>Chlamydomophila pneumoniae</i> TW-183	749
Cyanobacteria	<i>Thermosynechococcus elongatus</i> BP-1	850
Planctomycetes	<i>Isosphaera pallida</i> ATCC 43644	855
Chloroflexi	<i>Dehalococcoides ethenogenes</i> 195	815

A second interesting of feature is illustrated in figure III. Figure three shown below is an amino acid sequence logo of the Walker A motif found in PriA representing 100 different organisms with the amino acid sequence of *D. radiodurans* PriA listed below the logo. Comparing the logo and the *D. radiodurans* PriA sequence it is apparent that there are many differences in sequence.

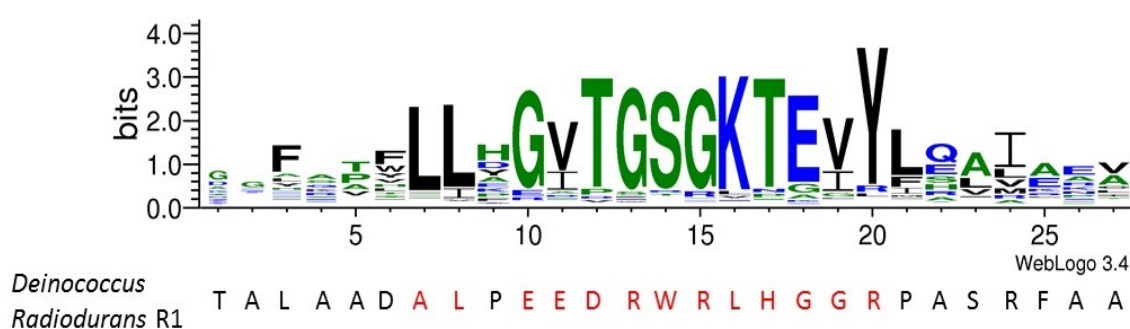


Figure III – Logo comparing the PriA Walker A motif amino acid sequence of 100 different organisms. *D. radiodurans* clearly shows a great number of differences in this particular area of its amino acids sequence (Crooks, Schneider).

PriA in *D. radiodurans* conserves enough of its amino acid sequence to still be considered a PriA protein, but fails to conserve many of the essential motifs that lead to PriA function.

In addition to PriA and DnaB, another protein of interest is the single stranded binding protein (SSB) of *D. radiodurans* (Eggington). When replication forks are unwound the DNA will be temporarily single stranded (Eggington). SSB is typically found on single stranded DNA placing it in close proximity to where DnaB and PriA are suspected to be found and functional (Eggington). It is believed that SSB's main task is to stabilize single stranded DNA while it is separated from its partner strand, but it may also assist PriA in attaching to the replication fork (Eggington). If PriA and SSB demonstrate some level of physical interaction it would improve confidence in the idea that PriA is functioning as a replication restart protein in *D. radiodurans*.

If *D. radiodurans* restarts replication similar to other organisms its PriA would attach at the replication fork and assist with loading the replicative helicase DnaB on to the fork. Once DnaB has been loaded the helicase will be able to continue moving down the parental DNA and opening up the double stranded DNA. For this mechanism to be valid PriA and DnaB would be expected to physically interact. This project was focused on identifying the presence of possible protein complex formations involving *D. radiodurans* PriA, DnaB, and SSB. If the proteins cannot form some kind of protein complex then the replication restart mechanism displayed in *D. radiodurans* may be very different from the commonly observed model of replication restart in bacteria.

Experimental Methods

Protein Preparation - Deinococcus Radiodurans PriA was prepared by growing *Escherichia coli* BL21(DE3) harboring plasmid pET28b:(Drad)priA in 50 µg/mL kanamycin and 50 µg/ml chloramphenicol in 6.2 L of Luria-Bertani (LB) medium prepared with: 70 g tryptone, 31 g yeast extract, and 65 g NaCl until OD₆₀₀ 0.497 was reached. 0.5 mM IPTG was introduced to the cultures to begin overexpression of the *priA* gene. After four additional hours of growth the cells were collected by centrifugation at 2643 x g. The cells were immersed in a lysis buffer containing: 10 mM Tris-HCl pH 8.5, 10% glycerol, 0.5 M NaCl, 10 mM imidazole, 1 mM β-

mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed by sonication and the lysates were clarified in a high speed centrifuge at 18000 x g for 20 minutes at 19 °C. The clarified solution was incubated with nickel-NTA agarose beads for one hour at 19 °C, washed with lysis buffer and bound proteins were eluted with: 10 mM Tris-HCl pH 8.5, 10% glycerol, 100 mM NaCl, 250 mM imidazole, and 1 mM β-mercaptoethanol. The protein solution was run through a QFF ion exchange column beginning at 0.5 mL/min 7.5 °C with QFF Buffer A containing: 10 mM Tris-HCl pH 8.5, 10% glycerol, 100 mM NaCl, 1 mM β-mercaptoethanol. After the protein bound to the column QFF Buffer B containing: 10 mM Tris-HCl pH 8.5, 10% glycerol, 1 M NaCl, 1 mM β-mercaptoethanol was slowly added into the column creating a linear charge gradient. The fractionated protein was collected and dialyzed in a dialysis buffer containing: 10 mM Tris-HCl pH 8.5, 10% glycerol, 100 mM NaCl, 1 mM β-mercaptoethanol. Thrombin was added to the PriA solution while it dialyzed overnight at 6.7 °C. The protein was run through a second QFF ion exchange column similar to the first run through the ion exchange column. The fractionated protein was collected and then concentrated by centrifugation at in a Centriprep YM-3 concentrator at 2643 x g until the volume was below 2 mL. The PriA was run at 0.5 mL/min 7.5 °C through a HiPrep HR 16/10 sephacryl S300 column, concentrated by centrifugation at 5000 x g down to a total approximate volume of 1.5 mL and then aliquoted and frozen at -80 °C.

Deinococcus Radiodurans DnaB was prepared by growing *Escherichia coli* BL21(DE3) harboring plasmid pET28b:(Drad)priA under conditions similar to the preparation of PriA. The only difference in the preparation of DnaB was that the first QFF ion exchange column run in the PriA preparation was omitted. The purified DnaB was concentrated down to an approximate volume of 1.2 mL and was stored at -80 °C.

The *Deinococcus Radiodurans* SSB used for this project was prepared previously.

Size Exclusion Chromatography – 2L of High Prep 16/10 S300 Buffer (S300 buffer) was prepared containing: 10 mM Tris-HCl, 10% glycerol, 150 mM NaCl, 1 mM – β -mercaptoethanol. For the first experiment 1.3 μ mol *Deinococcus Radiodurans* SSB was loaded into the FPLC and run at 0.5 mL/min and 7.5 $^{\circ}$ C with S300 buffer. A second run through the S300 column was run similar to the first experiment, this time with 12 μ mol SSB. Next 0.2 μ mol PriA was diluted to 300 μ L and run through the column. A mixture of 0.4 μ mol PriA and 9.5 μ mol SSB was run through the S300 column at 0.5 mL/min and 7.5 $^{\circ}$ C. The output from this experiment was fractionated. SDS PAGE was performed on aliquots 14-24 on a 12.5% acrylamide gel.

Native PAGE – 200 mL of buffer solution containing 0.25 M tris base and 0.125 M boric acid was prepared pH 8.57. All continuous native gels were composed of: 1.5 mL acrylamide:bis-acrylamide 29:1 (40% solution), 2 mL tris-boric acid pH 8.57, 6.55 mL water, and 50 μ L of APS followed by 10 μ L of TEMED. 1 mL of sample buffer for the native PAGE gels was prepared with: 0.1 M tris-boric acid, 41 μ M glycerol, 580 μ L water, 20 μ L bromophenol blue. The running buffer used in all native PAGE experiments consisted of: 25 mM Tris, 192 mM glycine and 0.1% SDS pH 8.3. 1/10 SSB was prepared by diluting the stock SSB 1:10 with water. The contents of each gel are discussed in the data analysis section of this report. The first gel was run for 30 minutes at 100 V and then stained in 3.5 mM coomassie brilliant blue R-250 prepared with a solution of 25% (v/v) methanol and 7.5% (v/v) acetic acid for 1 hour and then destained in the 25% (v/v) methanol and 7.5% (v/v) acetic acid solution. A second gel was run with the same contents as the first gel, but for 3 hours at 100 V. The gel was stained similar to how the first gel was stained.

Certain gels in this project were silver stained using the following procedure. First the gel was soaked in a 30% ethanol and 10% acetic acid solution for 30 minutes. Then the gel was soaked in 20% ethanol for 10 minutes and then again in fresh 20% ethanol for another ten minutes. Following the ethanol solutions the gel was soaked in water for 10 minutes twice. The gel was then placed in a 0.8 mM $\text{Na}_2\text{S}_2\text{O}_3$ sensitizing solution for 1 minute, rinsed in water twice

for 1 minute each time and then left to soak in 12 mM AgNO₃ for 1 hour. After the silver solution soak, the gel was rinsed with water and then placed in a developer solution consisting of: 96 mL H₂O, 250 µL formaldehyde, 125 µL 10% Na₂S₂O₃ and 3 g K₂CO₃. The gel was left in the developer solution for approximately thirty seconds. To stop developing the gel was put in a solution of 4 g tris base, 2 mL acetic acid and 100 mL H₂O for 1 hour.

Cross-Linking PAGE – Fisher Scientific 8-20% bis-acrylamide SDS PAGE gels were used for all cross-linking PAGE experiments. Sample buffer (4x SB+) was prepared containing: 200 mM Tris-HCl, 8% SDS, 40% glycerol, 400 mM β-mercaptoethanol, 0.4% bromophenol blue. A gel was run at 200 V for 1 hour.

The gel was stained in 0.25% coomassie brilliant blue R-250 for 1 hour and then destained in 25% (v/v) methanol and 7.5% (v/v) acetic acid. After full destaining the gel was placed in 30% ethanol and 10% acetic acid for 16 hours. The gel was soaked in 20% ethanol for 10 minutes, fresh 20% ethanol for 10 minutes, water for 10 minutes and then fresh water for another 10 minutes. 0.8 mM Na₂S₂O₃ was prepared and the gel was placed in the solution for 1 minute, water for one minute, fresh water again for 1 minute, and 12mM AgNO₃ for 1 hour and 30 minutes. The gel was rinsed for 10 seconds in water and then placed in a developer solution for 4 minutes composed of: 96 mL water, 250 µL formaldehyde and 125 µL 10% Na₂S₂O₃. After developing the gel was placed in a stop solution for 1 hour composed of: 4g tris base, 2 mL acetic acid, 100mL H₂O. The gel was rinsed in water for 30 minutes twice. A second cross-linking gel was run under conditions similar to the first cross-linking gel.

Stacking Native PAGE – Two discontinuous native PAGE experiments were performed. The acrylamide gels were made with two layers. To prepare the gel 20 mL of resolving gel was made with 3 mL acrylamide:bis-acrylamide 29:1 (40% solution), 5 mL Tris-HCl pH 8.8, 12 mL H₂O, 100 µL APS and 20 µL TEMED. 5 mL of the upper stacking layer was prepared with 750 µL acrylamide, 1250 µL Tris-HCl pH 6.8, 3 mL H₂O, 25 µL APS and 5 µL TEMED. 50 mL of running buffer was composed with 1.515 g tris base, 7.2 g glycine and 50 mL H₂O. All solutions

that were loaded in the discontinuous native PAGE experiments were run with a solution buffer. 1 mL of the solution buffer was prepared with 125 μ L tris HCl pH 6.8, 300 μ L glycerol, 20 μ L bromophenol blue and 550 μ L H₂O. One gel was run using this procedure for 3 hours at 70 V. The gel was stained using standard coomassie brilliant blue R-250 staining procedure described earlier in this report.

Blue Native PAGE – Several different variations of blue native PAGE were performed. This PAGE required two different kinds of running buffers. One buffer was used on the side of the cathode and the other on the side of the anode. The cathode buffer prepared was 50 mM tricine, 7.5 mM imidazole and 0.24 mM coomassie brilliant blue R-250. The anode buffer was composed of 0.025 M imidazole. The sample buffer for the blue native PAGE experiments was simply 5% glycerol in H₂O. The gels contained varying amounts (3.5%-14%) of acrylamide but were always constructed to be 0.03 mM imidazole. To prepare 5 mL of gel 50 μ L APS and 5 μ L TEMED were added to induce polymerization.

The first gel run under blue native gel conditions was 3.5% acrylamide and was electrophoresed for 3 hours at 500 V at 4 °C. For the second gel the coomassie brilliant blue R-250 was taken out of the cathode buffer and the sample buffer was modified to be 5% glycerol (v/v) and 5% coomassie brilliant blue R-250 (weight/vol). The gel was run with double the protein used in the first gel for 20 minutes at 350 V. For the third experiment original cathode, anode and solution buffers were used and two gels were prepared. One gel was 10.5% acrylamide and the second was discontinuous with 3.5%, 7%, 10% and 14% acrylamide layers. The third set of gels also contained samples with the detergent triton X-100. These gels were both run at 100 V for 1 hour and 15 minutes. For the fourth blue native PAGE experiment a 10.5% gel was constructed and run at 100 V for 1 hour. No triton X-100 was used for the fourth experiment and before loading the wells the samples were given a 10 minute incubation period on ice. The fifth blue native PAGE experiment was similar to the previous experiment but a 9% gel was used and

the gel was run at 100 V for 1 hour. The final two gels run under blue native PAGE conditions were run for 1 hour and 34 minutes at 100 V with varying amounts of triton X-100.

Native Agarose Gel Electrophoresis – Many native agarose gel experiments were performed during this project. The running buffer for all experiments was constant containing: 25 mM Tris-HCl and 19.2 mM glycine. The gels were made of varying concentrations of 0.8% agarose prepared in the running buffer. Sample buffer for all native agarose gels included: 20% glycerol, 0.2% bromophenol blue, 0.12 M tris base. All gels were stained and destained using standard coomassie brilliant blue R-250 procedure previously described in this report. The first gel was a 0.8% agarose gel run for 1 hour at 50 V with all three proteins of interest and all mixture combinations present. The second gel run was adjusted to 0.7% agarose and allowed to run for 3 hours and 5 minutes at the same voltage with similar protein mixture lanes. A third 0.6% gel was run at 50 V for 7 hours and 52 minutes. The next 0.7% gel was electrophoresed for 4 hours at 50 V with fresh protein from the -80 °C. This gel contained mixtures of PriA and DnaB and mixtures of PriA and SSB. The same gel was repeated with 0.6% agarose and run under similar time and voltage conditions. The final two native agarose gels were 0.6% agarose and run for 3 hours and 45 minutes at 50 V. The protein mixtures for the final gels were given a 10 minute incubation period on ice before being loaded into the wells of the agarose gel for analysis.

Several successful native agarose gels were selected to be further analyzed by extracting protein bands of interest and running them through SDS PAGE. Bands were sliced out of the successful gels using a micro scoopula and spun down at 2643 x g. The resulting pieces of gel were mixed with small amounts of 4x SB+ and then melted in a 95 °C heating block. The running buffer used for the SDS PAGE experiments of the extracted bands is the same running buffer that was used in the cross-linking SDS PAGE experiments earlier described. The sample buffer was 4x SB+ (also previously described). All SDS PAGE gels in this portion of the project were run at 200 V for 45 minutes. For increased resolution all of these gels were silver stained using the silver staining procedure described previously in this report.

Results and Discussion

Protein Preparation – The *D. radiodurans* PriA was prepared first. The PriA was grown in *E. coli* strains that contained previously transformed plasmids containing the *D. radiodurans* PriA gene. Cultures were grown to overexpress the PriA gene using IPTG, sonicated to lyse the cells open and then purified through several different chromatographic purification techniques and centrifugation. The DnaB was prepared very similar to how the PriA was prepared. Exact details are listed in the methods and procedures section so I won't list much detail here. The quality control gels for both preparations are shown below in figure IV. The final concentration of purified PriA was found to be 1.12 g/L and the purified DnaB was found to be 1.72 g/L. The PriA and DnaB preparations both contained some high molecular weight contaminants, but appear to be have a relatively high purity of around 85-90%. The SSB that was previously prepared had a stock concentration of 49.7 g/L.

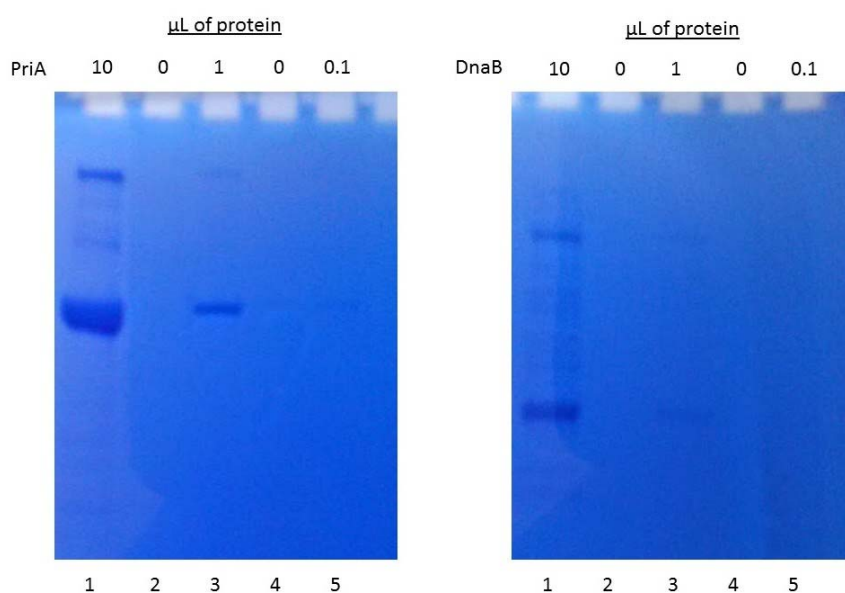


Figure IV – PriA and DnaB Quality Control Gels. For each protein purified an SDS PAGE analysis was performed to identify purity of the product. One tenth serial dilutions were run with gap lanes to give an idea of relative purity. Both proteins were identified to be roughly 85-90% pure.

Size Exclusion Chromatography – Size exclusion chromatography was used to test for formation of the hypothesized protein complexes formed between the three proteins investigated in this paper. The first run performed with 1.3 μmol of SSB served as a baseline for later protein mixing experiments. The chromatogram obtained did not show any strong peaks so a second experiment was set up using 12 μmol of SSB in hopes that the signal on the chromatogram would be stronger. The second experiment showed a distinct peak at a retention volume of 74.13 mL which was assumed to be the SSB. To obtain a control chromatogram for the *Deinococcus radiodurans* PriA 0.2 μmol of PriA was sent through the column. This run generated a strong signal peak at a retention volume of 63.54 mL believed to be the PriA, but generated a strange baseline with higher absorbance following the peak – not shown. This was believed to be due to some kind of error in the UV spectrometer monitoring absorbance of the analyte exiting the column.

0.4 μmol of PriA was then added to 9.5 μmol of SSB and then loaded for another trial in the column. Due to stock concentrations being quite different the SSB was present in vast excess. The baselines previously generated showed that the amounts of protein used above would be sufficient to generate a signal in the chromatogram. The chromatogram obtained from the first (shown in figure V) run showed one very clear peak at a retention volume of 74.55 mL and then two smaller peaks that appeared to be integrated into the larger peak with retention volumes of 48.82 mL and 62.82 mL.

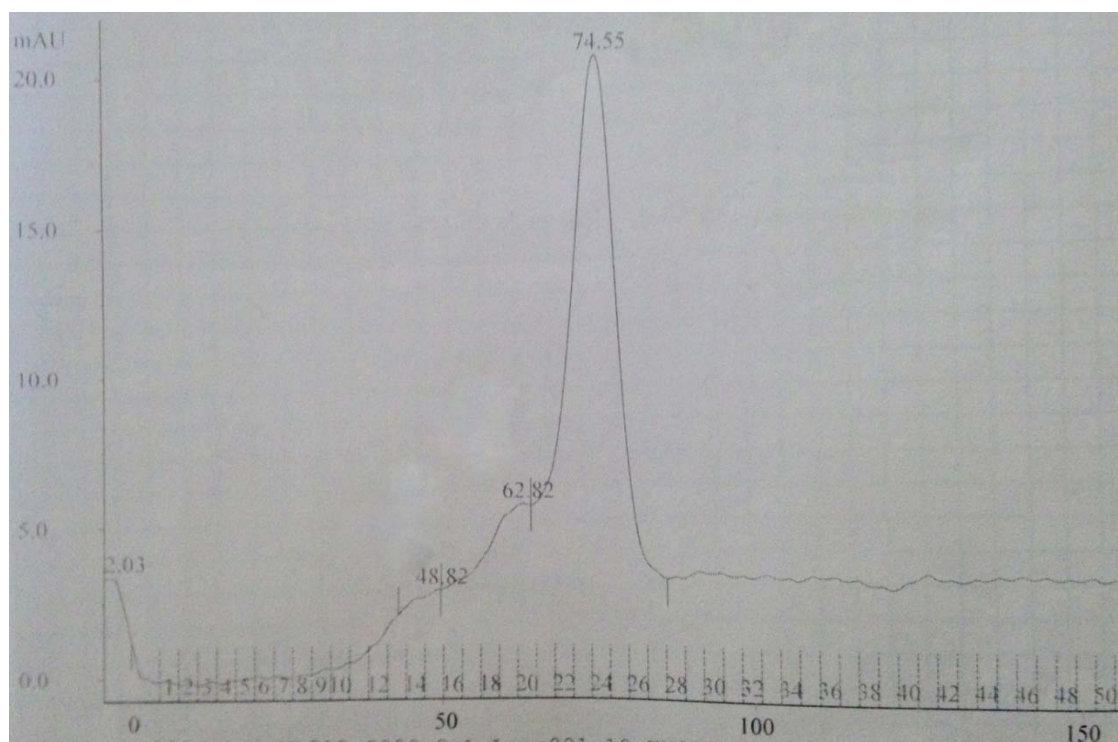


Figure V - Chromatogram containing a mixture of PriA and SSB. The chromatogram shows three tentative peaks. However, it is difficult to say for sure whether or not the two smaller peaks are individual proteins migrating alone since the peaks overlap.

This is somewhat consistent with what would be expected from this experiment. The large peak at 74.55 mL was likely an excess of SSB alone, the peak at 62.82 mL was probably the PriA by itself and then the peak at 48.82 mL was thought to be the hypothesized protein complex. The problem shown by this chromatogram was that the intensity of the signals received were very weak and close to one another. It was hard to say whether or not the peaks we observed were in fact what we speculated. To get better results it would require using a much larger amount of protein stock and even then may not be successful. Size exclusion chromatography was set aside and many different forms of PAGE were used to analyze the protein mixtures.

Native PAGE – The first kind of gel electrophoresis attempted was continuous native PAGE. Based on computer generated titration curves at pH 8.5 all three proteins explored in this report should have a negative charge lower than -10. Therefore all native PAGE experiments were run at pH 8.5. The first experiments were performed before the DnaB preparation was

completed so no DnaB was used in these preliminary assays. The first gel run at 100 V for 30 minutes yielded a relatively strong signal indicating the protein had entered the gel as anticipated. However, the bands obtained were not compact and had not entered the gel more than a few centimeters. A second identical experiment was set up except the duration of electrophoresis was extended to 3 hours. This second gel showed very weak and dispersed signals. The protein had entered the gel, but the signal was not strong enough to be useful for future assays when DnaB was ready.

Cross-Linking PAGE – Native PAGE was not very promising based on initial experimentation so several SDS PAGE cross-linking experiments were performed. The protein mixtures were incubated with glutaraldehyde to covalently link any interactions holding the protein complexes together. In the first gel run under these experimental parameters lanes with glutaraldehyde were run next to identical lanes lacking glutaraldehyde to act as control lanes. The samples were then heated and electrophoresed through the polyacrylamide gel. Initially the gels were stained with standard coomassie brilliant blue R-250 staining procedure but the gels yielded extremely faint bands of protein. The gels were fully destained and then stained again using a silver staining technique in hopes of getting better resolution to analyze the results of the cross-linked gels. The silver staining gave much stronger signals, but the lanes containing the cross-linked proteins showed much weaker signals than the lanes lacking glutaraldehyde. The cross-linking SDS PAGE experiments were not clear enough to provide us with any insight or confidence in my hypothesis that PriA and DnaB will interact.

Stacking Native PAGE – We returned to native PAGE for further experimentation, but switched from continuous native PAGE to discontinuous native PAGE. The problem found with continuous native PAGE was that when the protein was able to enter the gel the bands were not compact and distinct. By generating a gradient of acrylamide concentration I hoped to slow migration of the small proteins running through the gel and increase resolution on the bands as the protein lagging behind caught up –moving quicker through the lower concentration of

acrylamide. The resulting gel produced from this experiment showed tighter bands that had migrated into the gel, but still extremely weak signals. The lanes all behaved as expected, but again did not provide a high degree of confidence in my hypothesis.

Blue Native PAGE – This led me to perform several blue native PAGE experiments in hopes of getting clearer answers. Blue native PAGE is very similar to native PAGE with just one major difference. In Blue native PAGE a separate anode and cathode buffer must be prepared. The cathode buffer contains a high concentration of coomassie brilliant blue R-250 giving it a strong blue color. The advantage of using blue native PAGE is that, ideally, you can see the protein as it migrates through the gel. The first attempt using this technique yielded a dark blue gel that would not destain. In addition, no protein was clearly observed migrating through the gel. There were several dark spots in the very top of the wells which could possibly indicate precipitated protein. The cathode buffer was thought to contain too much coomassie brilliant blue R-250 – masking all protein signals.

For the second experiment I took the coomassie brilliant blue R-250 out of the cathode buffer and put a small amount in the sample buffer. The main purpose of coomassie brilliant blue R-250 in this kind of PAGE is to bind to the proteins of interest and assist them in running through the gel. By putting the coomassie brilliant blue R-250 in the sample buffer and giving them a short incubation period the coomassie brilliant blue R-250 should still be able to bind to the protein before entering the gel. This second gel had a much clearer solvent front, but still not distinct protein bands migrating through. The dark spots in the tops of the wells were also much more prevalent, again, leading us to think that the protein was precipitating before entering the gel. Several papers that I read going through blue native PAGE indicated that protein solubility is an issue commonly experienced.

Detergents are known to increase solubility of proteins. Using a light microscopic I was able to determine that the detergent triton X-100 had a strong impact on solubility of the proteins I was studying in solution. The only issue that arises when using detergent is that using too much

will alter the natural characteristics of the proteins and can eventually denature them if enough is present. However, many papers had all reported success with detergent in blue native PAGE protein mixing experiments so I attempted more blue native PAGE experimentation with detergent. For my first trial with triton X-100 I ran two gels with lanes in pairs. A lane with detergent neighbored a lane without detergent to act as a control. Also, for these experiments I returned to using the cathode buffer with coomassie brilliant blue R-250 and one gel was made with a polyacrylamide concentration gradient. This was done in four layers: 3.5%, 7%, 10.5% and 14% polyacrylamide. Surprisingly, these gels yielded primarily clear distinct protein signals in lanes containing no detergent. The continuous gel was virtually identical in construction to the first blue native PAGE described in this report, but yielded a beautifully destained gel with clear signals for DnaB. The problem found with this technique was that the PriA and SSB did display a strong signal and in the protein mixture lanes the DnaB by itself masked any potential protein complex signal obtained.

A continuous 9% polyacrylamide gel was run with no detergent in hopes of replicating the results from the previous gel and getting better results, but showed no clear bands at all. The next gel attempted used varying amounts of detergent to determine what the optimum concentration of detergent might be for the highest resolution gel. Several variations were performed changing the levels of detergent and glycerol. These gels showed improvements in seeing SSB in the gel, but no conditions were ever identified that worked well with all three proteins being studied.

Native Agarose Gel Electrophoresis – Blue native PAGE did not turn out to be successful so several agarose gel electrophoresis experiments were performed. Most of the problems noticed in previous experimentation seemed to revolve around PriA not entering the gel. This resistance may likely be due PriA's relatively large size of roughly 99,000 kDa. Traditionally agarose gel electrophoresis is used for separating fragments of DNA, but since PriA is a large protein I attempted to run my protein mixing experiments in agarose gels. Agarose gels are slightly more

porous making it easier for PriA to enter. The first gel was 0.8% agarose and showed strong, clear and distinct signals for PriA, DnaB and SSB. The only correction for the second gel was the run time. The first gel showed bands for all proteins but had not migrated very far into the gel. The second gel was therefore run for three times the length of the first gel and yielded clear results well spaced apart. It should also be noted that casting the lanes in the middle of the gel seemed to give clearer and stronger signals. The reasoning behind this is still a mystery, but as it seemed to help all gels near the end of this study were cast in the middle of the gel.

After establishing a methodology for testing the protein interactions that worked well with all three proteins of interest I then refined my work even further to produce gels with biologically relevant stoichiometric ratios to establish the degree to which the protein complexes form. The final gels run tested interactions between PriA and DnaB as well as PriA and SSB. The interaction between DnaB and SSB may be important, but was not the main focus of this project and due to size of the gels I focused on just the interactions of PriA with SSB and PriA with DnaB. Another restriction that dictated the stoichiometry of my mixing experiments came from the stock concentrations of the proteins I worked with. The SSB had a concentration of 1.12 g/L which is quite significantly larger than the concentration of the other two proteins worked with. In almost all experiments run with SSB it was present in vast excess. The PriA and DnaB in contrast had very similar stock concentrations of 1.12 g/L (PriA) and 1.72 g/L (DnaB) making it much easier to get realistic stoichiometric ratios. The first gel run with predetermined stoichiometric ratios is shown below along with an SDS PAGE experiment run on selected extracted bands from the original agarose gel. It is also important to recall that DnaB exists predominantly as a hexamer and SSB commonly forms a dimer with itself. These complex quaternary structures may be important in understanding the complex formations observed through these proteins interacting.

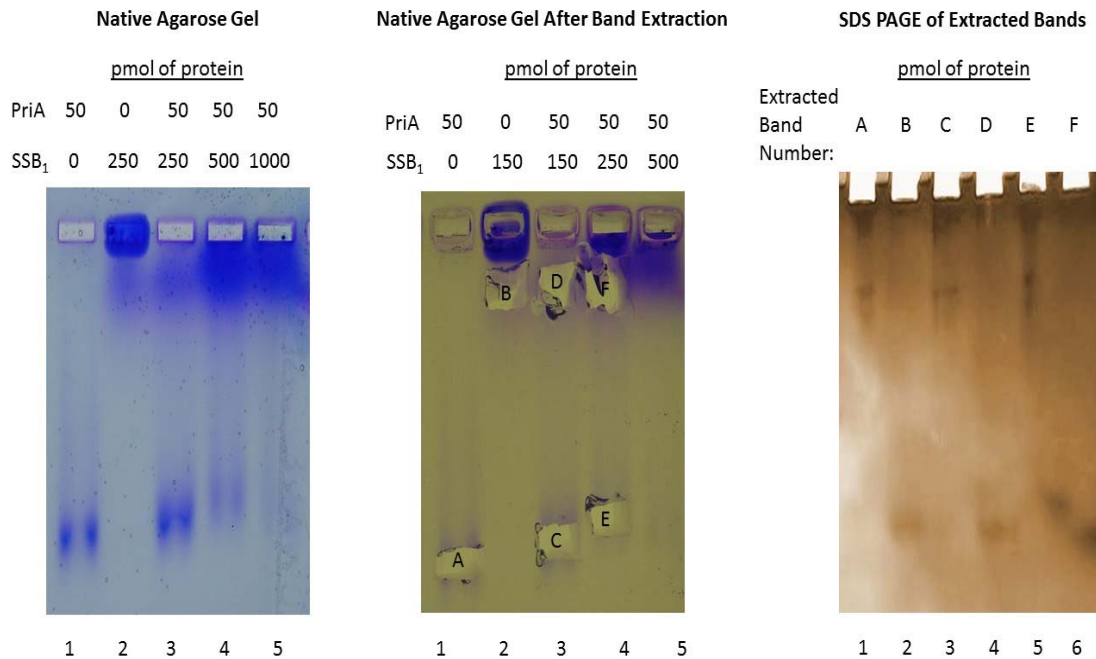


Figure VI - Native agarose gel illustrating the potential complex formed between PriA and SSB (left) next to a gel showing extraction sites for protein bands (middle) then run through SDS PAGE (right) for further analysis. The gel on the left displays a mixture of PriA and SSB that is the strongest argument obtained for the presence of a physical interaction between PriA and SSB. The gel in the middle is the gel on the left after extracting several bands of interest that were run through SDS PAGE analysis in the gel on the right. The SDS PAGE gel on the right was supposed to improve the confidence by verifying that the proteins suspected to be in certain bands were actually present where I would expect to find them.

Figure VI illustrates the mixing of PriA and SSB. In my experiments I titrated SSB with PriA giving me several different stoichiometric ratios to observe. Based on the gel on the far left it is apparent that as SSB is present in greater excess PriA is able to complex more and more. The PriA band observed in the lane 1 is hardly visible at all in lane 5. In the SDS PAGE analysis of the native agarose gel experiment lanes containing D and F should have small amounts of PriA. Based on migration patterns of PriA from other lanes it is hard to argue that there is a significant amount of PriA in these two bands. This prompts the question as to where the PriA is. The PriA did not migrate by itself in the native agarose gel, but does not clearly appear to be in the bands I would expect to find it in based on SDS PAGE analysis. Based on the evidence obtained in this

study it is difficult to make any decisive conclusions about the nature of the complex formed by PriA and SSB.

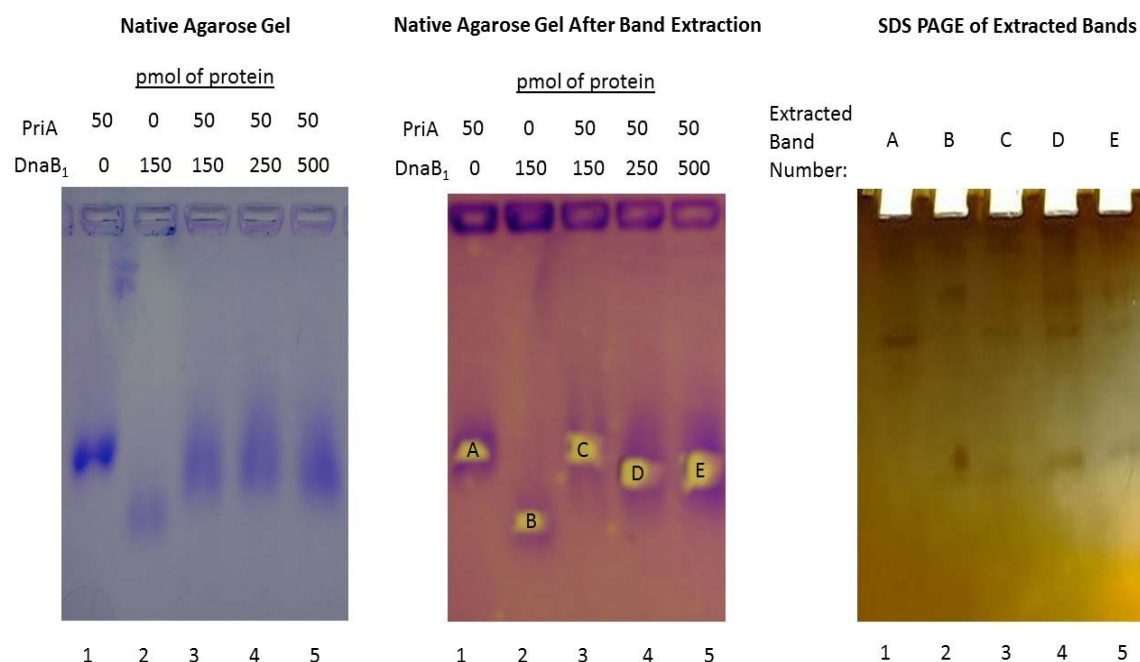


Figure VII - Native agarose gel illustrating the potential complex formed between PriA and DnaB (left) next to a gel showing extraction sites for protein bands (middle) then run through SDS PAGE (right) for further analysis. This figure is very similar to the previous figure, but in this case PriA and DnaB were mixed together. Similar techniques were employed to produce the middle and right images in this figure which were supposed to help improve confidence in my results. Contrary to the previous figure, the SDS PAGE analysis showed what I expected to see. Bands that should be mixtures of PriA and DnaB such as D and E both clearly show the presence of PriA and DnaB in the SDS PAGE analysis.

Figure VII illustrates the mixing of PriA and DnaB. Unfortunately, the two proteins tended to migrate relatively closely to each other. However, it was still enough to see that the migration patterns of the proteins changed when mixed together. Looking at the initial native agarose gel on the left it is clear that the DnaB band by itself, observed in lane 2, is not clearly present in lanes three through five. The SDS PAGE extraction gel confirmed that bands C, D and E all contained both PriA and DnaB. Contrary to PriA and SSB, the evidence for an interaction between PriA and DnaB is much more convincing. This data also suggests that PriA was always

in excess in these mixtures since the DnaB band by itself is not present in lanes 3 through 5. If PriA monomers and DnaB monomers complex 1:1 with each other then it would make sense for there to be not DnaB by itself shown in lane 3, but then there should be some present in lane 5. If we change our guess to 1:6 PriA to DnaB, which biological conditions suggest, then we would expect lane 3 to show an excess of PriA, lane 4 to show almost all protein complexed and lane 5 to show an excess of DnaB. Again, there is no excess of DnaB in lane 5. Based on our data it is

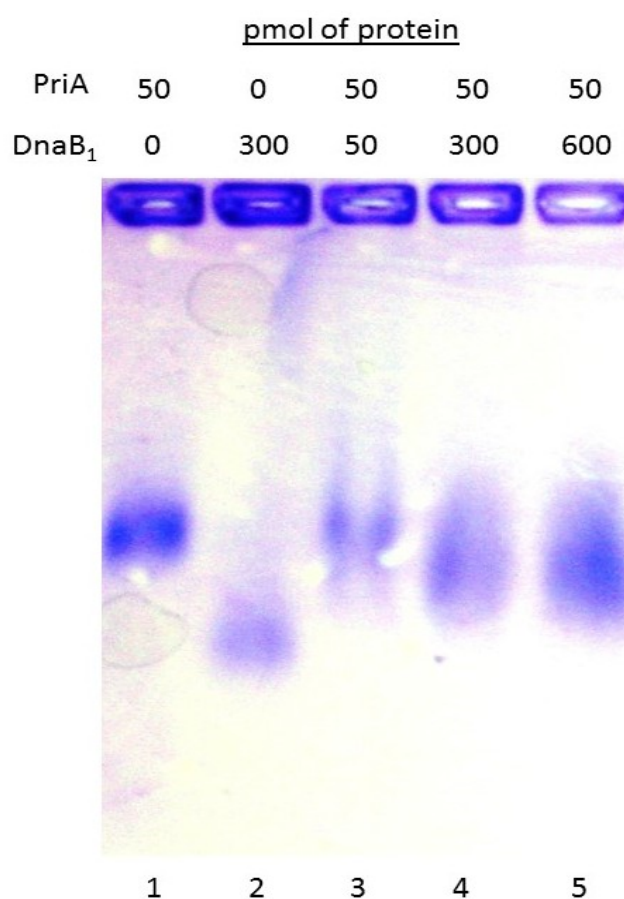


Figure VIII - Native agarose gel illustrating the potential complex formed between PriA and DnaB. This gel was run as a follow up of the native agarose gel illustrated in figure VII.

clear that there is some sort of interaction occurring between PriA and DnaB, but testing has not shown any conclusive arguments for the stoichiometry of the protein complex.

Figure VIII is a native agarose gel containing PriA and DnaB which was modified only by changing the quantities of PriA and DnaB in the mixtures in hopes of gaining further insight on

the complex formation observed. First, it is important to note that these results are consistent with what has previously been observed with native agarose gel electrophoresis. There is still a clear complex formation between PriA and DnaB. The second important aspect of this gel to note is the positioning of the mixture bands in lanes 3 through 5. In this gel it is much easier to see the downward shift of the band as DnaB is present in greater quantities. DnaB never clearly appears to be in vast excess even in the lane with 12:1 DnaB to PriA. It is possible that one PriA monomer can take on several DnaB hexamers. From this gel we can discern that PriA has a large capacity to complex with DnaB. In lane 3 where the two proteins are 1:1 PriA to DnaB there appears to be one strong band that is just PriA and one weaker band shifted downward that is a protein complex mixture. As DnaB is titrated the mixture band becomes stronger, but never displays an excess of DnaB which would be indicated by a DnaB band alone and an absence of the PriA alone band.

PriA and DnaB show a clear capacity to interact and form protein complexes with each other. PriA and SSB also indicate that they may be able to interact. However, the evidence generated in this project was not conclusive enough to claim that a complex formation occurs when these two are close enough to each other. While PriA and DnaB interacting does not conclusively tell us that PriA acts as a primosome protein, findings in this project tell us that the possibility of PriA behaving as a primosome protein is still valid. The results of this project support my hypothesis that PriA is involved in replication restart in *D. radiodurans*.

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