EVOLUTIONARY COVARIANCE AMONG DNA REPLICATION RESTART PRIMOSOME GENES

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Linda Berg

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EVOLUTIONARY COVARIANCE AMONG DNA REPLICATION RESTART
PRIMOSOME GENES

Name: Berg, Linda

APPROVED BY:

Matthew E. Lopper, Ph.D.
Faculty Advisor
Assistant Professor
Department of Chemistry
University of Dayton

Vladimir Benin, Ph.D.
Committee Member
Associate Professor
Department of Chemistry
University of Dayton

R. Gerald Keil, Ph.D.
Committee Member
Professor
Department of Chemistry
University of Dayton

Angela Mammana, Ph.D.
Committee Member
Assistant Professor
Department of Chemistry
University of Dayton
ABSTRACT

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Name: Berg, Linda
University of Dayton

Advisor: Dr. Matthew E. Lopper

The process of DNA replication can be hindered by DNA damage, and disruptions in replication ultimately lead to cell death if not corrected. Bacteria solve this problem with a mechanism called “DNA replication restart,” which is catalyzed by primosome proteins. However, not all bacteria encode the full complement of primosome proteins found in the well-studied bacterium, *E. coli*. This suggests that differences might exist in DNA replication restart pathways among diverse bacteria. *N. gonorrhoeae*, for example, lacks a DnaT homolog. DnaT is thought to affect interactions between primosome protein PriB and single-stranded DNA. Since *N. gonorrhoeae* PriB has a weak interaction with single-stranded DNA, as opposed to the strong interaction between PriB and single-stranded DNA seen in *E. coli* (which encodes a DnaT homolog), we hypothesized that the presence of a DnaT homolog could be used to predict the affinity with which a bacterial PriB binds single-stranded DNA. Binary interactions between PriB and single-stranded DNA of two bacteria, *K. pneumoniae* (which encodes a DnaT homolog) and *Y. enterocolitica* (which lacks a DnaT homolog) were analyzed.
Both *K. pneumoniae* and *Y. enterocolitica* have a high affinity PriB:ssDNA interaction with dissociation constants of 62 ± 14 nM and 84 ± 8 nM, respectively. Thus, the presence of DnaT cannot be used to predict affinities of binary interactions between PriB and single-stranded DNA. However, the experimentally measured binding constants combined with amino acid sequence alignments of the PriB homologs have led to the definition of parameters for high affinity binary interactions between PriB and single-stranded DNA.

A recent report describes the identification of a novel PriB protein in *K. pneumoniae* that is significantly shorter than most sequenced PriB homologs (H.C. Hsieh, and C.Y. Huang, Identification of a novel protein, PriB, in *Klebsiella pneumoniae*. *Biochem Biophys Res Commun* **404** (2011) 546-51). The *K. pneumoniae* PriB protein is proposed to comprise 55 amino acid residues, in contrast to *E. coli* PriB which comprises 104 amino acid residues and has a length that is typical of most sequenced PriB homologs. Our investigations suggest that the *priB* gene of *K. pneumoniae* encodes a 104-amino acid PriB protein, akin to its *E. coli* counterpart. We have cloned the *K. pneumoniae priB* gene and purified the 104-amino acid *K. pneumoniae* PriB protein. Gel filtration experiments reveal that the *K. pneumoniae* PriB protein is a dimer, and equilibrium DNA binding experiments demonstrate that *K. pneumoniae* PriB’s single-stranded DNA-binding activity is similar to that of *E. coli* PriB. These results indicate that the PriB homolog of *K. pneumoniae* is similar in structure and in function to that of *E. coli.*
Dedicated to my parents, Gunther and Ina Berg, and my siblings, Larsen, Wiebke, Joshua, and Niels
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>$K_{d,app}$</td>
<td>Apparent dissociation constant</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnical Information</td>
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CHAPTER I
BACKGROUND

The survival of cellular organisms depends on the complete and faithful duplication of their genetic material, deoxyribonucleic acid (DNA). DNA encodes the genetic information that ultimately generates proteins which are crucial components in all cellular metabolic processes. DNA replication is a major housekeeping function that over billions of years has provided cellular organisms with the ability to reproduce with high fidelity. During the process of replication two identical DNA molecules are produced via the DNA replication machinery also referred to as replisome. (Figure 1).\(^1\)

Throughout the life of a cell, the process of DNA replication is challenged by environmental and chemical factors that can bring about damage to the DNA. DNA damage can occur due to ionizing radiation, oxidative reactions, or spontaneous depurination and deamination reactions of DNA bases. Unrepaired lesions can lead to the generation of gaps, single-stranded nucleic acid breaks, or double-stranded chromosomal breaks which can disrupt the DNA replication machinery and cause the replication fork to stall or collapse.\(^2,3\)
Figure 1. DNA replication mechanism of bacteria. DNA replication is initiated at a sequence-specific location called the origin of replication, oriC. Following the initiation phase, two strands are synthesized in opposite directions until the replisome encounters a terminal sequence (ter) which signals the end of replication. DNA damage can cause a halt in DNA replication and, if not corrected, cell death.

Since failure to replicate the genome can result in cell death, microorganisms have adapted to these challenges by developing various mechanisms to recognize and repair DNA damage and ensure complete replication of their genetic information. But how does the cell resume DNA replication when the process has been disrupted due to encounters with DNA damage? How does the replication machinery reconnect with the DNA once it has been separated?

*Escherichia coli* has proved to be an excellent model organism to investigate these questions. The loading of the DNA replication machinery can proceed via two different mechanisms (Figure 2). Origin-dependent replisome loading is initiated by DnaA, which binds in a sequence-specific manner to the origin of replication and opens
the DNA double-strand (Figure 2A, step 1). Then, the DnaB is loaded on to single-stranded DNA (ssDNA) by the way of the DnaB/C complex (Figure 2A, step 2), where DnaB acts as helicase and further unwinds the DNA (Figure 2A, step 3). Once the replication fork stalls or collapses, DnaA cannot bind to the DNA to reinitiate replication because it cannot recognize specific structures, it only binds DNA in a sequence-specific manner. Therefore, origin-dependent replisome loading cannot facilitate the loading of the DNA replication machinery after a stalled replication fork has been repaired.

In order to reinitiate DNA replication, bacteria have developed a highly specific DNA replication reactivation mechanism called “DNA replication restart.” DNA replication restart pathways make use of groups of proteins collectively referred to as primosomes to bind to DNA after a stalled fork has been repaired and facilitate the reloading of the replication machinery in order for replication to continue.²

In E. coli, DNA replication restart is catalyzed by primosome proteins, including PriA, PriB, DnaT, and DnaG, that collectively facilitate reloading of the replisome to allow DNA replication to continue (Figure 2B).⁶ The function of these primosome proteins involves coordinated protein and nucleic acid binding within a large nucleoprotein complex called the DNA replication restart primosome. PriA helicase is the initiator protein that binds to the repaired DNA replication fork and unwinds double-stranded DNA at the fork, converting it into ssDNA (Figure 2B, step 1).⁷,⁸,⁹ PriB binds to PriA, stabilizes PriA on the DNA, and stimulates its helicase-activity (Figure 2B, step 1b).⁸,¹⁰ The PriA:PriB:DNA ternary complex recruits DnaT to the DNA, which could lead to release of ssDNA from PriB.⁸,¹¹ The replicative helicase, DnaB/C, is recruited to the fork where it unwinds the parental duplex DNA to stimulate priming by DnaG
(Figure 2B, step 2 and 3). The last step entails the loading of DNA polymerase III, which allows DNA replication to resume.

![Diagram of replisome loading systems](Figure 2)

**Figure 2. Replisome loading systems.** A) DnaA binds to specific DNA sequences at the origin of replication. It unwinds the duplex DNA and facilitates loading of the DnaB/C complex. B) PriA helicase binds to the repaired DNA replication fork and unwinds double-stranded DNA at the fork (step 1). PriB binds to PriA and ssDNA (step 1b). The PriA:PriB:DNA ternary complex recruits DnaT to the DNA (step 2). The replicative helicase, DnaB, and its accessory protein, DnaC, are recruited to the fork, and DnaB facilitates unwinding of the parental duplex DNA (step 3).

Although studies with *E. coli* have revealed a tremendous amount of information about the mechanism of DNA replication restart, it cannot be uniformly applied to all prokaryotes because some organisms do not encode the full complement of primosome protein genes. Genome sequencing projects have revealed that *priA* genes are highly conserved among sequenced bacterial genomes, but *priB*, *priC*, and *dnaT* genes are not. The absence of one or more of these primosome genes from bacterial genomes suggests that there might be mechanistic differences in DNA replication restart pathways across diverse bacterial species. *Neisseria gonorrhoeae*, for example, lacks a DnaT homolog. A *dnaT* gene is encoded by *E. coli* and the DnaT protein is thought to affect interactions between primosome protein PriB and ssDNA. This raises the question of how a bacterium might compensate for lack of a primosome protein, such as DnaT, that provides otherwise important functions in some bacteria.
Therefore, we investigated binary interactions within the DNA replication restart primosomes of two prokaryotes, *Klebsiella pneumoniae* (which encodes a DnaT homolog) and *Yersinia enterocolitica* (which lacks a DnaT homolog) in order to analyze what effects the absence of DnaT has on the overall interaction of the other primosome proteins with one another and on the overall function of the DNA replication restart primosome.

This comparative study involved cloning the primosome protein genes from diverse bacteria and purifying their primosome proteins. In the course of this study, a research report was published by others that describes the identification of a novel PriB protein in *K. pneumoniae* that is significantly shorter than most sequenced PriB homologs. The *K. pneumoniae* PriB protein is proposed to comprise 55 amino acid residues, in contrast to *E. coli* PriB which comprises 104 amino acid residues and has a length that is typical of most sequenced PriB homologs. Despite this published research report, our investigations show that the PriB homolog of *K. pneumoniae* is similar in structure and in function to that of *E. coli*.
CHAPTER II
INVESTIGATING THE STRUCTURE AND FUNCTION OF *K. PNEUMONIAE* PRIB

2.1. INTRODUCTION

Previous studies revealed that *E. coli* PriB is a homodimer that consists of a single structural domain. Each monomer comprises two oligonucleotide/oligosaccharide binding (OB) folds that have the characteristic shape of a β-barrel (Figure 3). The core of the β-barrel is surrounded from both surfaces by two antiparallel β sheets. The β2 and β3 strands of each monomer contribute to the formation of a six-stranded β sheet, which is dominated primarily by negatively charged amino acids. L23 loops connect the β2 and β3 strands. The β3 and β4 strands are connected by α helices. The opposite surface is also comprised by two antiparallel β sheets, β4 and β5 strands, which are connected via L45 loops. This surface of the β-barrel contains a large number of positively charged amino acids residues.

![Figure 3. Ribbon diagram of the crystal structure of *E. coli* PriB. PriB forms a homodimeric β-barrel. Monomers are colored in red and blue and individual β strands are numbered.](image-url)

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Most of what is known today about primosome protein PriB and its function in DNA replication restart is because of studies done with *E. coli*. *E. coli* belongs to the \(\gamma\)-proteobacteria class and is a member of the enterobacteriaceae family. Huang *et al.* recently reported the identification of a novel *priB* gene in *K. pneumonia*, a bacterial organism that belongs to the same family as *E. coli*\(^\text{12}\). According to their study, the *K. pneumoniae* PriB protein is only 55 amino acids in length, which is considerably shorter than *E. coli* PriB. The sequence that *K. pneumoniae* PriB appears to be missing corresponds to the amino terminal half of *E. coli* PriB and includes amino acid residues important for dimerization and a few that are involved in binding DNA. The authors also report that PriB proteins from *Pectobacterium carotovorum*, *Yersinia ruckeri*, and *Salmonella enterica* have considerably shorter amino acid sequences compared to *E. coli* PriB. Therefore, Huang *et al.* conclude that the structure and function of PriB in those bacterial species must be different from the structure and function of *E. coli* PriB\(^\text{12}\).

Our investigations show that the PriB protein of *K. pneumoniae* is a full-length PriB homolog whose sequence is the same length as *E. coli* PriB. The full-length *priB* gene from *K. pneumonia* was cloned. The recombinant *K. pneumoniae* PriB protein was overexpressed and purified, and its quaternary structure and DNA binding activity were examined. Our results indicate that the structure and function of *K. pneumoniae* PriB are highly similar to that of *E. coli* PriB. Thus, *K. pneumoniae* PriB does not likely represent a novel PriB homolog. The sequence analysis of other bacterial *priB* genes that have been proposed to be missing amino-terminal sequences reveals that they, too, are full-length *priB* genes that encode proteins whose lengths are comparable to *E. coli* PriB.
2.2. MATERIALS AND METHODS

2.2.1. Cloning K. pneumoniae priB and E. coli priB

The priB gene of K. pneumoniae was amplified from strain MGH78578 genomic DNA by polymerase chain reaction (PCR) using primers oML292 (5′-GCG TAT TCC ATA TGA CCA ACC GTC TGG AGC TG) and oML293 (5′-GTC ACG GAT CCC TAG TCT CCA GAA TCT ATC AAT TC). The PCR-amplified product was cloned into the pET28b expression vector (Novagen) using NdeI and BamHI restriction sites. The resulting plasmid contains a six-Histidine tag upstream of the complete coding sequence of the K. pneumoniae priB gene, which is under the control of a T7 promoter for overexpression in hosts harboring T7 polymerase controlled by the lacUV5 promoter. The cloning of the priB gene of E. coli was described previously.13 The fidelity of priB gene was confirmed by DNA sequencing. All plasmids were individually transformed into BL21(DE3) E. coli to allow recombinant protein overexpression following induction with isopropyl-β-D-thiogalactopyranoside (IPTG). The fidelity of priB genes was confirmed by DNA sequencing.

2.2.2. Purification of K. pneumoniae PriB

K. pneumoniae PriB protein was purified from BL21(DE3) E. coli harboring the pET28b:Kpn-priB plasmid. Cells were grown in Luria-Bertani (LB) medium containing 50 µg/ml kanamycin and 50 µg/ml chloramphenicol at 37°C until an OD_{600} of 0.4 was reached. Expression of PriB was induced with 0.5 mM IPTG for 3 hr and cells were harvested by centrifugation at 5,000 × g. Cells were lysed in 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.5 M NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF by
sonication on ice. The lysate was clarified by centrifugation at 40,000×g. His-tagged PriB was bound to nickel-NTA agarose (Qiagen) and eluted in 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.5 M NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol. The nickel-NTA agarose eluate was dialyzed against 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.3 M NaCl, 1 mM β-mercaptoethanol, concentrated, and resolved through a HiPrep HR 16/10 sephacryl S-100 size-exclusion chromatography column (GE Healthcare) in 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.5 M NaCl, 1 mM β-mercaptoethanol. PriB fractions were pooled, concentrated, and stored at –80°C.

2.2.3. Purification of *E. coli* PriB

*E. coli* PriB protein was purified from BL21(DE3) *E. coli* harboring the pET28b:Ec-priB plasmid as describes previously.13 Briefly, cells were grown in LB medium containing 50 µg/ml kanamycin and 50 µg/ml chloramphenicol at 37°C until an OD₆₀₀ of 0.4 was reached. Expression of PriB was induced with 1 mM IPTG for 3 hr and cells were harvested by centrifugation at 5,000 × g. Cells were lysed in 10 mM Hepes pH 7, 10% (v/v) glycerol, 1 M NaCl, 10 mM imidazole, 0.1 M glucose, 1 mM β-mercaptoethanol, 1 mM PMSF by sonication on ice. The lysate was clarified by centrifugation at 40,000×g. His-tagged PriB was bound to nickel-NTA agarose (Qiagen) and eluted in 10 mM Hepes pH 7, 10% (v/v) glycerol, 1 M NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol. The nickel-NTA agarose eluate was concentrated and purified through a HiPrep HR 16/10 sephacryl S-100 size-exclusion chromatography column (GE Healthcare) in 10 mM Hepes pH 7, 10% (v/v) glycerol, 1 M NaCl, 1 mM β-mercaptoethanol. PriB fractions were pooled, concentrated, and stored at –80°C.
2.2.4. Gel filtration

Purified *K. pneumoniae* PriB and *E. coli* PriB were individually applied to a HiPrep HR 16/10 sephacryl S-100 size-exclusion chromatography column (GE Healthcare) and resolved at 0.35 ml/min with 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.5 M NaCl, 1 mM β-mercaptoethanol. The column was calibrated under identical conditions with protein standards of known molecular weight: thyroglobulin (670,000 Da), bovine gamma-globulin (158,000 Da), chicken ovalbumin (44,000 Da), equine myoglobin (17,000 Da), and vitamin B12 (1,350 Da) (BioRad). Protein was detected in the column eluate by measuring the absorbance at 280 nm.

2.2.5. Equilibrium DNA binding assays

Fluorescence polarization (FP) spectroscopy was used to determine physical interaction between PriB and fluorescein-labeled ssDNA. It was performed at 25°C with a Beacon 2000 fluorescence polarization system (Invitrogen) (Figure 4).

![Figure 4. Detection of fluorescence polarization](image-url)

*Figure 4. Detection of fluorescence polarization.* Monochromatic light travels from a light source through a vertical polarizer. Vertical polarized light excites molecules in the sample tube. The light emitted by excited molecules passes through vertical as well as horizontal polarizers and is analyzed accordingly.
FP is based on the theory that small molecules tumble at a faster rate than large molecules. Therefore, if small fluorescent-labeled molecules are excited, the light they emit is depolarized. On the other hand, larger molecules emit light polarized light and subsequently have a high polarization value (Figure 4). FP is a powerful technique to quantitatively measure the interaction between fluorescein-labeled ssDNA and primosome protein PriB. If PriB and fluorescein-labeled ssDNA form a complex, the complex will have a greater fluorescence anisotropy than the unbound DNA and this difference can be readily detected by fluorescence polarization spectroscopy (Figure 5).

PriB proteins were diluted serially from 10,000 nM to 0.01 nM into 20 mM Tris–HCl pH 8, 50 mM NaCl, 4% (v/v) glycerol, 1 mM MgCl₂, 1 mM β-mercaptoethanol, 0.1 mg/ml bovine serum albumin (BSA) and incubated with 1 nM 3′-fluorescein-labeled ssDNA oligonucleotides of varying lengths: 15-mer (5′-TAG CAA TGT AAT CGT), 30-mer (5′-GCG TGG GTA ATT GTG CTT CAA TGG ACT GAC), 45-mer (5′-GCC GTG
ATC ACC AAT GCA GAT TGA CGA ACC TTT GCT CCA GTA ACC) in a total volume of 100 μl. Apparent dissociation constants ($K_{d,app}$) were calculated by determining the concentration of titrant required to bind 50% of the fluorescein-labeled ssDNA. The unbound state is reported by the fluorescence polarization of the fluorescein-labeled ssDNA in the absence of PriB. The fully-bound state is reported by the fluorescence polarization of the fluorescein-labeled ssDNA in the presence of a sufficient concentration of PriB to saturate the fluorescence polarization signal. Data are reported in triplicate and associated uncertainties are one standard deviation of the mean.

2.3. RESULTS AND DISCUSSION

2.3.1. Sequence analysis

According to the genetic sequence database at the National Center for Biotechnical Information (NCBI), *K. pneumoniae* PriB protein (GenBank ID:YP_001338213) consists of 55 amino acids as predicted *ab initio* by Genemark 2.0. Given that the vast majority of PriB proteins have a sequence of approximately 104 amino acids, the genome of *K. pneumoniae* in the region upstream of the *priB* gene was examined and it was noticed that the start codon of the *priB* gene reported in the database is preceded by a sequence that would code for a stretch of amino acids that is highly similar to the amino-terminal region of *E. coli* PriB. By including this additional upstream sequence, along with the annotated *K. pneumoniae priB* sequence, an open reading frame in the *K. pneumoniae* genome was identified that codes for a 104-amino acid protein whose amino acid sequence is 95% identical to that of *E. coli* PriB (Figure 6). Given that the annotated *K. pneumoniae* PriB sequence was predicted *ab initio*, it was
proposed that the priB gene of *K. pneumoniae* encodes a 104-amino acid protein whose sequence is highly similar to that of *E. coli* PriB.

![Figure 6. Multiple amino acid sequence alignment of PriB homologs.](image)

The sequences of the other PriB homologs reported by Hsieh and Huang to be shorter than would be expected based on the sequence of a typical PriB homolog were also examined. The amino acid sequence of *Pectobacterium carotovorum* PriB reported in the NCBI database is 106 amino acids in length, and the amino acid sequence of *Salmonella enterica* PriB is 104 amino acids in length. The amino acid sequence of *Yersinia ruckeri* PriB, as reported in the NCBI database, is 55 amino acids in length. The genome of *Yersinia ruckeri* in the region upstream of the priB gene was analyzed in the same manner as for *K. pneumoniae* priB and additional sequence upstream of the annotated priB start codon that could code for the missing amino-terminal region of *Y. ruckeri* PriB was found. Based on this sequence analysis, it was proposed that the priB genes of *K. pneumoniae*, *P. carotovorum*, *Y. ruckeri*, and *S. enterica* all encode proteins of comparable length to *E. coli* PriB (Figure 6).

### 2.3.2. Quaternary structure of *K. pneumoniae* PriB

A previous report by Hsieh and Huang describes a 55-amino acid variant of *K. pneumoniae* PriB as a monomeric protein, while *E. coli* PriB exists as a
In *E. coli*, the dimerization interface of PriB is extensive and involves a large number of contacts between individual monomers. Among the interactions are hydrogen bonds that form between the amino-terminal β1 strand of one monomer and the amino-terminal β1 strand of the other monomer. Since these β strands include amino acids 1-11, it is possible that a variant of PriB that lacks a portion of its amino-terminus could exist as a monomeric protein. This appears to be the case for the 55-amino acid variant of *K. pneumoniae* PriB that is missing residues analogous to amino acid residues 1–49 of *E. coli* PriB.

Since sequence comparison of *K. pneumoniae* PriB and *E. coli* PriB reveals that the two PriB proteins are highly similar to one another at the level of primary structure, the recombinant *E. coli* PriB and *K. pneumoniae* PriB proteins were purified and their quaternary structure was analyzed using gel filtration. *E. coli* PriB and *K. pneumoniae* PriB each migrate through a sephacryl S-100 size-exclusion chromatography column as a single peak with retention volumes of 62.61 ml and 62.86 ml, respectively (Figure 7).

**Figure 7.** Gel filtration of PriB proteins from *K. pneumoniae* and *E. coli*. Equivalent amounts of (a) *K. pneumoniae* PriB and (b) *E. coli* PriB, each at approximately 3.4 g/l, were individually resolved through a sephacryl S-100 size-exclusion chromatography column under identical experimental conditions as described in Materials and Methods.
Based on a calibration of the column using proteins of known molecular weight, it was determined that *E. coli* PriB migrates as a dimer with a molecular weight of approximately 22,519 Da and *K. pneumoniae* PriB migrates as a dimer with a molecular weight of approximately 22,416 Da. These results indicate that *E. coli* PriB and *K. pneumoniae* PriB have highly similar quaternary structures under these experimental conditions.

### 2.3.3. DNA binding activity of *K. pneumoniae* PriB

Due to the high degree of similarity between *K. pneumoniae* PriB and *E. coli* PriB at the level of primary and quaternary structure, it was hypothesized that the mechanism of ssDNA binding is similar between the two PriB homologs. To test this hypothesis, fluorescence polarization spectroscopy was used to measure the DNA binding activity of *K. pneumoniae* PriB to compare it with that of *E. coli* PriB. For these experiments, the apparent equilibrium binding constant for the interaction between *K. pneumoniae* PriB and fluorescein-labeled ssDNA oligonucleotides was determined. The fluorescein tag on the ssDNAs allowed to measure PriB binding to the ssDNA due to the increase in fluorescence polarization of the PriB:ssDNA complex relative to the unbound ssDNA. *K. pneumoniae* PriB protein was serially diluted and incubated with 1 nM fluorescein-labeled ssDNA and the fluorescence polarization was measured. Apparent dissociation constants were obtained by determining the concentration of PriB needed to achieve 50% binding to each of the various ssDNA substrates.
When *K. pneumoniae* PriB was incubated with each of the fluorescein-labeled ssDNA oligonucleotides, a PriB-dependent increase in fluorescence polarization was observed, indicating that *K. pneumoniae* PriB binds to the ssDNAs (Figure 8).

The apparent dissociation constants for 15-base, 30-base, and 45-base fluorescein-labeled ssDNAs are $50 \pm 3$ nM, $45 \pm 7$ nM, and $62 \pm 14$ nM, respectively. As a comparison, *E. coli* PriB’s apparent dissociation constant for the same 30-base fluorescein-labeled ssDNA, measured using the same instrument and under similar experimental conditions, is $34.6 \pm 7.7$ nM. These results indicate that the affinity of *K. pneumoniae* PriB for ssDNA is highly similar to that of *E. coli* PriB. Overall, these results support the hypothesis that *K. pneumoniae* PriB is a 104-amino acid ssDNA-binding protein whose structure and function mirrors that of the *E. coli* PriB homolog.

![Figure 8. Single-stranded DNA-binding activity of *K. pneumoniae* PriB. PriB protein was diluted and incubated with fluorescein-labeled 15-base (blue circles), 30-base (red squares), or 45-base (green triangles) ssDNA oligonucleotides as described in Materials and Methods. Measurements are reported in triplicate and error bars represent one standard deviation of the mean.](image-url)
CHAPTER III
INVESTIGATING INTERACTIONS BETWEEN PRIMOSOME PROTEINS AND DNA

3.1. INTRODUCTION

During the origin-independent replisome loading system of *E. coli*, PriA helicase binds to the repaired DNA replication fork and unwinds double-stranded DNA at the fork. PriB binds to PriA and ssDNA and the PriA:PriB:DNA ternary complex recruits DnaT to the DNA (Figure 2). Studies of individual binary interactions among *E. coli*’s primosome components have shown that the affinity of PriA for PriB is rather low and that PriA’s association and effective binding with PriB depends on the presence of DNA. These previous studies also showed that PriB has a rather high affinity for ssDNA and it was suggested that DnaT might function to facilitate the release of ssDNA from PriB to make that ssDNA available for loading the DnaB replicative helicase. Presumably this function would be required because PriB binds ssDNA very strongly (Figure 9).

While most of what is known about the mechanism of the DNA replication restart pathways has arisen from studies using *E. coli*, recent studies using *N. gonorrhoeae* revealed that the DNA replication restart mechanism proposed for *E. coli* cannot be uniformly applied to all prokaryotes. Investigations of individual binary interactions of primosome proteins and DNA in *N. gonorrhoeae* revealed that *N. gonorrhoeae* PriA has
a high affinity for its cognate PriB (in stark contrast to the low affinity interaction that is observed in *E. coli*). It was also shown that the affinity of *N. gonorrhoeae* PriB for ssDNA is rather low (again, in stark contrast to what is observed in *E. coli*) (Figure 9).\(^{15}\) Since *N. gonorrhoeae* lacks a DnaT homolog it was suggested that a low affinity interaction between its PriB and ssDNA has evolved so that ssDNA can be released from PriB without assistance.

![Figure 9. Binary interactions among primosome proteins in *E. coli* and *N. gonorrhoeae*. A) In *E. coli*, strong binding interactions between PriA and ssDNA and PriB and ssDNA are observed, which are balanced by weak binding interactions between PriA and PriB. B) In *N. gonorrhoeae*, strong binding interactions between PriA and ssDNA and PriA and PriB are observed, which are balance by weak binding interactions between PriB and ssDNA.](image)

Work using *E. coli* and *N. gonorrhoeae* model organism raises the question of how a bacterium might compensate for lack of a primosome protein, such as DnaT, that provides otherwise important functions in some bacteria. Since *N. gonorrhoeae* PriB has a weak interaction with ssDNA, as opposed to the strong interaction between PriB and ssDNA seen for *E. coli*, it was hypothesized that the presence of a DnaT homolog could be used to predict the affinity with which a bacterial PriB would bind ssDNA. To test this hypothesis, binary interactions within the DNA replication restart primosomes of two prokaryotes, *K. pneumoniae* (which encodes a DnaT homolog) and *Y. enterocolitica* (which lacks a DnaT homolog) were investigated in order to analyze what effects the
absence of DnaT has on the overall interaction of the other primosome proteins with one another and on the overall function of the DNA replication restart primosome.

3.2. MATERIALS AND METHODS

3.2.1. Cloning Y. enterocolitica priB

The priB gene of Y. enterocolitica was amplified from strain 8081 genomic DNA by PCR using primers oML294 (5’-GCG TAT TCG CTA GCA TGG TGA CCA CTA ATC GTC) and oML295 (5’-GTC ACG GAT CCC TAG TCT CCA GAA TCT ATA AAT TC). The PCR-amplified product was cloned into the pET28b expression vector (Novagen) using NdeI and BamHI restriction sites. The resulting plasmid contains a six-Histidine tag upstream of the complete coding sequence of the Y. enterocolitica priB gene, which is under the control of a T7 promoter for overexpression in hosts harboring T7 polymerase controlled by the lacUV5 promoter. The fidelity of priB gene was confirmed by DNA sequencing. All plasmids were individually transformed into BL21(DE3) E. coli to allow recombinant protein overexpression following induction with IPTG. The fidelity of priB genes was confirmed by DNA sequencing.

3.2.2. Site-directed mutagenesis

The N. gonorrhoeae PriB Q44F and Q49W variants were constructed using QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Mutant Q44F was generated with primers oML 327 (5’-CAG GAG GAA
AAT GGG TTC CAA TGC CTT GTC C) and oML 328 (5’-GGA CAA GGC ATT GGA ACC CAT TTT CCT CCT G). Variant Q49W was generated with primers oML 329 (5’-CAA TGC CTT GTC TGG TTG GAA ATC CCC GC) and oML 330 (5’-GCG GGG ATT TCC AAC CAG ACA AGG CAT TG) using the wild-type plasmid pET28b:Ngon-priB as a template. Double-variant Q44F,Q49W was constructed with primers oML 329 and oML 330 using plasmid pET28b:NgonPriB Q44F as a template. The recombinant priB genes are under the control of a T7 promoter for overexpression in hosts harboring T7 polymerase controlled by the lacUV5 promoter. All plasmids were individually transformed into BL21(DE3) *E. coli* to allow recombinant protein overexpression following induction with IPTG. The fidelity of priB genes was confirmed by DNA sequencing.

3.2.3. Purification of *K. pneumoniae* PriB

*K. pneumoniae* PriB protein was purified from BL21(DE3) *E. coli* harboring the pET28b:Kpn-priB plasmid. Cells were grown in LB medium containing 50 µg/ml kanamycin and 50 µg/ml chloramphenicol at 37°C until an OD_{600} of 0.4 was reached. Expression of PriB was induced with 0.5 mM IPTG for 3 hours and cells were harvested by centrifugation at 5,000 × g. Cells were lysed in 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.5 M NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF by sonication on ice. The lysate was clarified by centrifugation at 40,000×g. His-tagged PriB was bound to nickel-NTA agarose (Qiagen) and eluted in 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.5 M NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol. The nickel-NTA agarose eluate was dialyzed against 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.3
M NaCl, 1 mM β-mercaptoethanol, concentrated, and resolved through a HiPrep HR 16/10 sephacryl S-100 size-exclusion chromatography column (GE Healthcare) in 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.5 M NaCl, 1 mM β-mercaptoethanol. PriB fractions were pooled, concentrated, and stored at –80°C.

3.2.4. Purification of *Y. enterocolitica* PriB

*Y. enterocolitica* PriB protein was purified from BL21(DE3) *E. coli* harboring the pET28b:Yent-priB plasmid. Cells were grown in LB medium containing 50 µg/ml kanamycin and 50 µg/ml chloramphenicol at 37°C until an OD₆₀₀ of 0.4 was reached. Expression of PriB was induced with 0.5 mM IPTG for 3 hours and cells were harvested by centrifugation at 5,000 × g. Cells were lysed in 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.5 M NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF by sonication on ice. The lysate was clarified by centrifugation at 40,000×g. His-tagged PriB was bound to nickel-NTA agarose (Qiagen) and eluted in 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.5 M NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol. The nickel-NTA agarose eluate was dialyzed against 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.3 M NaCl, 1 mM β-mercaptoethanol, concentrated, and resolved through a HiPrep HR 16/10 sephacryl S-100 size-exclusion chromatography column (GE Healthcare) in 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.5 M NaCl, 1 mM β-mercaptoethanol. PriB fractions were pooled, concentrated, and stored at –80°C.
3.2.5. Purification of *N. gonorrhoeae* PriB variants

*N. gonorrhoeae* PriB variants were purified from BL21(DE3) *E. coli* harboring the pET28b:Ngon-priB plasmid. Cells were grown in LB medium containing 50 µg/ml kanamycin and 50 µg/ml chloramphenicol at 37°C until an OD₆₀₀ of 0.4 was reached. Expression of PriB was induced with 0.5 mM IPTG for 3 hours and cells were harvested by centrifugation at 5,000 × g. Cells were lysed in 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.5 M NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF by sonication on ice. The lysate was clarified by centrifugation at 40,000×g. His-tagged PriB variants were bound to nickel-NTA agarose (Qiagen) and eluted in 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.5 M NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol. The nickel-NTA agarose eluate was dialyzed against 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.3 M NaCl, 1 mM β-mercaptoethanol, concentrated, and resolved through a HiPrep HR 16/10 sephacryl S-100 size-exclusion chromatography column (GE Healthcare) in 10 mM Tris–HCl pH 8, 10% (v/v) glycerol, 0.5 M NaCl, 1 mM β-mercaptoethanol. PriB variants fractions were pooled, concentrated, and stored at −80°C.

3.2.6. Equilibrium DNA binding assays

Fluorescence polarization spectroscopy was performed at 25°C with a Beacon 2000 fluorescence polarization system (Invitrogen). PriB proteins were diluted serially from 10,000 nM to 0.01 nM into 20 mM Tris–HCl pH 8, 50 mM NaCl, 4% (v/v) glycerol, 1 mM MgCl₂, 1 mM β-mercaptoethanol, 0.1 mg/ml bovine serum albumin (BSA) and incubated with 1 nM 3′-fluorescein-labeled ssDNA oligonucleotides of varying lengths: 15-mer (5′-TAG CAA TGT AAT CGT), 30-mer (5′-GCG TGG GTA
ATT GTG CTT CAA TGG ACT GAC), 45-mer (5'-GCC GTG ATC ACC AAT GCA GAT TGA CGA ACC TTT GCT CCA GTA ACC) in a total volume of 100 μl. Apparent dissociation constants (K_{d,app}) were calculated by determining the concentration of titrant required to bind 50% of the fluorescein-labeled ssDNA. The unbound state is reported by the fluorescence anisotropy of the fluorescein-labeled ssDNA in the absence of PriB. The fully-bound state is reported by the fluorescence anisotropy of the fluorescein-labeled ssDNA in the presence of a sufficient concentration of PriB to saturate the fluorescence anisotropy signal. Data are reported in triplicate and associated uncertainties are one standard deviation of the mean.

3.2.7. DNA unwinding assay

DNA substrate, Fork 3, was constructed as described previously (Figure 10). DNA substrate was diluted to 1 nM in 20 mM Tris-HCl pH8, 50 nM NaCl, 3 mM MgCl₂, 1 mM β-mercaptoethanol, 1 mM ATP.

![Figure 10. Structure of DNA substrate Fork 3.](image)

Concentrations of *N. gonorrhoeae* PriB:Q44F,Q49W variant ranging from 0 nM to 25 nM were added to the DNA and incubated at 37°C for 5 min. Constant concentrations of 2 nM PriA were added to the reaction mixtures and incubated at 37°C for 10 min to facilitate duplex DNA unwinding. Sodium dodecyl sulfate (SDS) was
added to the mixture in order to stop the reactions. Fluorescence polarization spectroscopy was used to determine the amount of duplex DNA unwound by measuring fluorescence anisotropy (Figure 11). Reaction mixtures were heated to 95°C and then rapidly cooled to 25°C in order to completely denature the DNA. Fluorescence anisotropy values of intact DNA substrate were compared to fluorescence anisotropy values of fully denatured DNA to calculate the fraction of unwound DNA. Data are reported in triplicate and associated uncertainties are one standard deviation of the mean.

![Figure 11. DNA unwinding assay. A) Intact fluorescein-labeled DNA fork is incubated with ATP, PriA, and PriB. PriA, whose helicase activity is stimulated by PriB, unwinds the DNA substrate. B) As DNA substrate is unwound, a decrease in fluorescence anisotropy can be observed.](image)

3.3. RESULTS AND DISCUSSION

3.3.1. DNA binding activity of *K. pneumoniae* PriB and *Y. enterocolitica* PriB

It was predicted that in organisms that encode a *dnaT*, primosome protein PriB will show a high affinity interaction with ssDNA. Due to the presence of DnaT homolog in *K. pneumoniae* PriB, it was hypothesized that the affinity PriB for ssDNA would be high (similar to that of *E. coli* PriB). To test this hypothesis, the DNA binding activity of *K. pneumoniae* PriB was measured using fluorescence polarization spectroscopy. The apparent equilibrium binding constant for the interaction between *K. pneumoniae* PriB and fluorescein-labeled ssDNA oligonucleotides was determined and compared to that of *E. coli* PriB. When *K. pneumoniae* PriB was incubated with each of the fluorescein-
labeled ssDNA oligonucleotides, a PriB-dependent increase in fluorescence polarization was observed, indicating that *K. pneumoniae* PriB binds to the ssDNAs (Figure 12). The affinity of *K. pneumoniae* PriB for ssDNA, is $45 \pm 7$ nM, which is highly similar to that of *E. coli* PriB. Therefore, *K. pneumoniae* PriB shows high affinity interactions with ssDNAs as predicted (Table 1).

![Figure 12. Single-stranded DNA-binding activity of *K. pneumoniae* PriB and *Y. enterocolitica* PriB. PriB proteins were diluted serially and incubated with fluorescein-labeled 15-base (blue circles), 30-base (red squares), or 45-base (green triangles) ssDNA oligonucleotides as described in Material and Methods. Measurements are reported in triplicate and error bars represent one standard deviation of the mean.](image)

Recent studies using *N. gonorrhoeae* revealed that the affinity of its PriB for ssDNA is rather low (in stark contrast to what is observed in *E. coli*). Therefore, it was predicted that in organisms that lack a *dnaT*, PriB shows a low affinity interaction with ssDNA. Due to the absence of a DnaT homolog in *Y. enterocolitica* PriB, it was hypothesized that the affinities of PriB for ssDNA are similar to that of *N. gonorrhoeae* PriB. To test this hypothesis, the DNA binding activity of *Y. enterocolitica* PriB was measured using fluorescence polarization spectroscopy (Figure 12). The apparent equilibrium binding constant for the interaction between *Y. enterocolitica* PriB and fluorescein-labeled ssDNA oligonucleotides was determined and compared to that of *N. gonorrhoeae* PriB.
The apparent dissociation constants for 15-base, 30-base, and 45-base fluorescein-labeled ssDNAs are 100 ± 22 nM, 84 ± 8 nM, and 59 ± 7, respectively (Table 1). As a comparison, *N. gonorrhoeae* PriB’s apparent dissociation constant for the same 30-base fluorescein-labeled ssDNA, measured using the same instrument and under similar experimental conditions, is 574 ± 66 nM. Thus, *Y. enterocolitica* PriB has an approximately 6-fold higher binding affinity for ssDNA than *N. gonorrhoeae* PriB. These results are in stark contrast to what was predicted and do not support the hypothesis. The binding affinities reported for *Y. enterocolitica* PriB are similar to *E. coli* PriB and *K. pneumoniae* PriB. Therefore, it was concluded that primosome protein DnaT cannot be used as an indicator to predict binding affinities between PriB and ssDNA.

**Table 1. Apparent dissociation constants for PriB:ssDNA complexes.**

<table>
<thead>
<tr>
<th></th>
<th>Apparent K_d, nM 15-mer</th>
<th>Apparent K_d, nM 30-mer</th>
<th>Apparent K_d, nM 45-mer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em></td>
<td>50 ± 3</td>
<td>45 ± 7</td>
<td>62 ± 14</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em></td>
<td>100 ± 22</td>
<td>84 ± 8</td>
<td>59 ± 7</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>N/A</td>
<td>34.7 ± 7.7</td>
<td>N/A</td>
</tr>
<tr>
<td><em>N. gonorrhoeae</em></td>
<td>N/A</td>
<td>574 ± 66</td>
<td>1018 ± 34</td>
</tr>
<tr>
<td><em>N. gonorrhoeae</em> Q44F,Q49W variant</td>
<td>N/A</td>
<td>145 ± 24</td>
<td>N/A</td>
</tr>
</tbody>
</table>

This conclusion led to the investigation of the amino acid sequence of PriB homologs of *E. coli, K. pneumoniae, Y. enterocolitica, and N. gonorrhoeae* in order to identify which elements of the primary amino acid sequence of a PriB would provide the greatest predictive power for the strength of the PriB:ssDNA interaction.
3.3.2. Primary amino acid sequence analysis

In previous research done with *E. coli* it had been determined that amino acid residues F42 and W47 are important for PriB and ssDNA binding.\(^{11,13}\) These aromatic amino acid residues facilitate binding through DNA-base stacking interactions. It had also been identified that positively charged residues located along the L\(_{45}\) loops contribute to DNA binding through ionic interactions with the negatively charged DNA backbone such as lysine residues at position 82, 84, and 89 (Figure 13). These results had been supported by equilibrium DNA binding assays of *E. coli* PriB variants and ssDNA. PriB variants F42A, W47A, K82A, and double variant W47A/K82A had a dissociation constant of 72.8 ± 11.5 nM, 112.3 ± 7.2 nM, 218.2 ± 9.4 nM, and 426.6 ± 52.3 nM, respectively.\(^{11}\)

![Figure 13. Ribbon diagram of *E. coli* PriB. A) *E. coli* PriB binds ssDNA. B) Aromatic amino acid residues F42 and W47 (orange) facilitate base-stacking interactions between PriB and ssDNA. Positively charged amino acid residues K82, K84, and K89 are responsible for ionic interactions with the negatively charged DNA backbone.](image)

The primary amino acid sequences of PriB homologs of *E. coli*, *K. pneumoniae*, *Y. enterocolitica*, and *N. gonorrhoeae* were aligned and evaluated according to the
presence of previous identified amino acids facilitating the binding of PriB and ssDNA (Figure 14).

![Figure 14. Multiple amino acid sequence alignment of PriB homologs. Amino acid sequences of Escherichia coli PriB (GenBank ID:NP_418622), Klebsiella pneumoniae PriB (GenBank ID:YP_001338213), Yersinia enterocolitica PriB (GenBank ID: YP_001004766), and Neisseria gonorrhoeae PriB (GenBank ID:YP_207725) were aligned using the program ClustalX. Well-conserved amino acid residues are highlighted in yellow. Substitution through glutamine and leucine residues are highlighted in red and green, respectively.](image)

The amino acid sequence of *E. coli* PriB and *K. pneumoniae* reported in the NCBI database is 104 amino acids in length. According to the sequence alignment, F42 and W47 are well-conserved in *E. coli* PriB and *K. pneumoniae* PriB. The amino acid sequence of *Y. enterocolitica* PriB reported in the NCBI database is 106 amino acids in length. It is likely that the F44 and W49 residues are analogous to the F42 and W47 residues of *E. coli* PriB and *K. pneumoniae* PriB, respectively. The amino acid sequence of *N. gonorrhoeae* PriB reported in the NCBI database is 100 amino acids in length. In *N. gonorrhoeae* PriB, it appears that at positions 44 and 49 two glutamine residues have been substituted for the aromatic amino acid residues found in *E. coli* PriB, *K. pneumoniae* PriB, and *Y. enterocolitica* PriB. The positively charged amino acid residues along the L45 loops are well conserved in *E. coli* PriB and *K. pneumoniae* PriB. In *N. gonorrhoeae* PriB, arginine residues at positions 81 and 91 are analogous to K82 and K89 in *E. coli* PriB and *K. pneumoniae* PriB, respectively. However, at position 86 a positively charged amino acid is replaced by a nonpolar leucine residue. Although in *Y. enterocolitica* PriB positively charged amino acid residues can be found at position 86
and 91, a positively charged amino acid at position 81 has been substituted by a glutamine residue.

From this analysis, it was concluded that the main amino acids important for ssDNA binding are phenylalanine and tryptophan located on the surface of PriB that forms an extended six-stranded β sheet as previously described in chapter 2. Their presence in *E. coli* PriB, *K. pneumoniae* PriB, and *Y. enterocolitica* PriB explains the high affinity interactions observed between ssDNA and PriB. Their absence in *N. gonorrhoeae* PriB supports the low affinity PriB:ssDNA interaction. Secondary amino acids important in the interaction between PriB and ssDNA are positively charged amino acid residues in the L45 loops. In *Y. enterocolitica* PriB and *N. gonorrhoeae* PriB, positively charged amino acid residues are only partially conserved. Since phenylalanine and tryptophan are present in *Y. enterocolitica* PriB, it can be speculated that these aromatic amino acids might be able to overcome slight weaknesses in ionic interactions between positively charged amino acid residues and ssDNA. However, our results also show that the presence of positively charged amino acids in *N. gonorrhoeae* PriB cannot facilitate strong binding between ssDNA and PriB in the absence of phenylalanine and tryptophan.

This conclusion can also be supported by analysis of temperature factors (Figure 15). The temperature factor is used in assessing the motion of atoms within a crystal structure. Aromatic amino acids phenylalanine and tryptophan are located within an area of the protein that is under rigid constraints (green). Whereas, positively charged lysine and arginine residues located along the L45 loop are more flexible (orange). *Y. enterocolitica* PriB might overcome slight weaknesses in ionic interactions due to the
absence of a positively charged amino acid at position 84 because the loop can adjust its position and thereby accommodate optimal binding with ssDNA. On the other hand, the aromatic amino acids such as F42 and W47 in *E. coli* PriB are located in a region that is rather rigid. Therefore, we propose that the protein cannot structurally conform to compensate for the absence of phenylalanine and tryptophan.

![Ribbon diagram of *E. coli* PriB based on temperature factors.](image)

**Figure 15. Ribbon diagram of *E. coli* PriB based on temperature factors.** Regions of the crystal structure can be classified as high disorder (red) to low disorder (violet). Aromatic amino acid residues are located within an area of the protein that is under rigid constraints (green). Positively charged amino acid residues can be found in a region of the protein that is more flexible (orange).

Additionally, multiple amino acid sequence alignment of PriB homologs of a variety of different bacterial organisms were categorized according to their family and the presence or absence of a DnaT homolog (Figure 16). *E. coli, K. pneumoniae, Y. enterocolitica,* and *N. gonorrhoeae* are members of a major group of bacteria called the proteobacteria. Although *E. coli, K. pneumoniae, Y. enterocolitica,* and *N. gonorrhoeae* all share a common ancestor, *E. coli, K. pneumoniae,* and *Y. enterocolitica* belong to the γ-proteobacteria class whereas *N. gonorrhoeae* is part of the β-proteobacteria class.
Different families such as vibronales, pasteurellales, and enterobacteriales have evolved within the γ-proteobacteria class. Genome sequence projects have shown that family members of vibronales and pasteurellales do not encode a dnaT. However, within the enterobacteriales family there are some members which encode a dnaT and some that lack dnaT (Figure 16).

![Figure 16](image)

Figure 16. Multiple amino acid sequence alignment of PriB homologs of bacterial organisms belonging to the γ-proteobacteria class. Amino acid sequences of Vibrio cholera PriB (GenBank ID:YP_004938769), Pasteurella dagmatis PriB (GenBank ID:ZP_05919241), Haemophilus influenzae PriB (GenBank ID:YP_248255), Pectobacterium atrosepticum PriB (GenBank ID:YP_051700), Photorhabus asymbiotica PriB (GenBank ID:YP_003042900), Erwinia tasmaniensis PriB (GenBank ID:YP_001908869), Proteus mirabilis PriB (GenBank ID:YP_002153059), Yersinia enterocolitica PriB (GenBank ID:YP_001004766), Escherichia coli PriB (GenBank ID:NP_418622), Klebsiella pneumoniae PriB (GenBank ID:YP_001338213), Salmonella enterica PriB (GenBank ID:YP_005245358), Citrobacter koseri PriB (GenBank ID:YP_001454225) and Chronobacter sakazakii PriB (GenBank ID:ATMM77) were aligned using the program ClustalX. Well-conserved amino acid residues are highlighted in yellow. Substitution through glutamine residues are highlighted in red. Substitution through leucine, isoleucine, and phenylalanine are highlighted in green.

It appears that bacterial organisms within the vibronales and pasteurellales family show much variety in their amino acid sequence within the region of PriB that contributes to interactions with ssDNA. Within the enterobacteriales family two different trends are observed. In bacterial organisms that lack a dnaT, tryptophan as well as two of the positively charged amino acids along the L45 loops are well conserved. However, the amino acid sequences of PriB of microorganisms that encode dnaT are almost identical to
one another. According to the phylogenic study, bacterial organisms within the enterobacteriales family that encode or lack dnaT show strong affinities of PriB for ssDNA.

Lopper et al. had proposed a hand-off mechanism for primosome assembly in E. coli, which also addressed the possible function of DnaT. Upon binding of PriA helicase to the repaired DNA replication fork, it undergoes a change in conformation and opens up the PriB binding site. PriB binds to PriA forming a PriA:PriB:DNA complex and thereby stabilizes PriA on the DNA and stimulates its helicase-activity. The PriA:PriB:DNA ternary complex recruits DnaT to the DNA. The binding of DnaT was proposed to proceed in two different steps. In the first step, DnaT competes with DNA for binding to PriB by interacting with E39, R44, and through interactions with DNA bound by PriB. Thereby, the affinity for PriB is decreased for DNA. During the second step, the complex undergoes a rearrangement that allows DnaT to interact with residues W47 and K82, which leads to the release of ssDNA from PriB. It was suggested that DnaT strips PriB off the DNA.\textsuperscript{11}

Due to the absence of a DnaT homolog in Y. enterocolitica but the presence of phenylalanine and tryptophan in the amino acid sequence of PriB and the high binding affinity of PriB and ssDNA, the question remains of what DnaT contributes to primosome assembly and DNA replication restart.

3.3.3. DNA binding activity of N. gonorrhoeae PriB variant

In N. gonorrhoeae because it lacks aromatic amino acid residues at the DNA binding site of the PriB β-barrel the affinity of PriB for ssDNA is rather low. Thus, it was
proposed that *N. gonorrhoeae* PriB could be converted into a high-affinity ssDNA binder by substituting amino acid residues Q44 and Q49 for phenylalanine and tryptophan, respectively. To test this hypothesis, site-directed mutagenesis was used to construct a *N. gonorrhoeae* PriB:Q44F,Q49W variant. The DNA binding activity of the variant was measured using fluorescence polarization spectroscopy (Figure 17). The apparent equilibrium binding constant for the interaction between *N. gonorrhoeae* PriB variant and fluorescein-labeled ssDNA oligonucleotides was determined and compared to that of wild-type of *N. gonorrhoeae* PriB.

![Figure 17. Single-stranded DNA-binding activity of *N. gonorrhoeae* PriB:Q44F,Q49W variant.](image)

Figure 17. Single-stranded DNA-binding activity of *N. gonorrhoeae* PriB:Q44F,Q49W variant. PriB proteins were diluted serially and incubated with fluorescein-labeled ssDNA oligonucleotides of varying lengths as described in Materials and Methods. Measurements are reported in triplicate and error bars represent one standard deviation of the mean.

The apparent dissociation constant for 30-base fluorescein-labeled ssDNAs is 145 ± 24 nM (Table 1). As a comparison, wild-type *N. gonorrhoeae* PriB’s apparent dissociation constant for the same 30-base fluorescein-labeled ssDNA, measured using the same instrument and under similar experimental conditions, is 574 ± 66 nM. This is an almost 4-fold increase in binding affinity and supports our conclusion that
phenylalanine and tryptophan are the main contributors of the high affinity binding between PriB and ssDNA. Although *N. gonorrhoeae* PriB variant shows a higher binding interaction with ssDNA, it was of interest to examine if its ability to stimulate the helicase activity of its cognate PriA had been compromised by the site-directed mutations.

### 3.3.4. *N. gonorrhoeae* PriB variant stimulates the helicase activity of PriA

Recent studies revealed that although wild-type *N. gonorrhoeae* PriB has a weak binding affinity for ssDNA, it stimulates the helicase activity of PriA. It has been reported that in the presence of the *N. gonorrhoeae* PriB, a 2.4-fold increase in its cognate PriA’s helicase activity was observed. Furthermore, PriB variant K34A, which renders ssDNA binding defective, shows nearly identical stimulatory function as wild-type PriB. Therefore, it was of interest to examine whether an increase in the affinity of PriB for ssDNA would affect the helicase activity of its cognate PriA.\(^{20}\) Fluorescence polarization spectroscopy was used in order to determine if the variant stimulates the helicase activity of PriA (Figure 18).
Figure 18. *N. gonorrhoeae* PriB:Q44F,Q49W variant stimulates the helicase activity of PriA. 2 nM PriA unwinds 1 nM Fork 3 in the presence of *N. gonorrhoeae* PriB variant (blue circles) and *N. gonorrhoeae* PriB wild-type (red squares). Measurements are reported in triplicate and error bars represent one standard deviation of the mean.

Despite the higher affinity of *N. gonorrhoeae* PriB variant for ssDNA, a 1.2-fold decrease in the unwinding activity of its cognate PriA was observed. This represents a relatively minor change in activity. These results are consistent with previous findings that the binding of PriB with ssDNA might not be a major contributor in the stimulation of the helicase activity of PriA. In previous studies, it has been proposed that the lower affinity of *N. gonorrhoeae* PriB for ssDNA might be due to the decrease in positive electrostatic surface charge potential relative to the surface of *E. coli* PriB. Additionally, amino acid sequence analysis reveals that aromatic residues important for ssDNA binding are not well conserved in *N. gonorrhoeae* PriB. Therefore, it was contemplated that *N. gonorrhoeae* PriB’s surface has been adapted throughout evolution in order to enhance PriA’s interaction with PriB while sacrificing PriB affinity for ssDNA. Thus, it can be speculated that glutamine residues that have been substituted for phenylalanine and tryptophan might be involved in PriA:PriB binding and therefore, a slight decrease in
helicase stimulation is observed because the PriA: PriB interaction has been compromised to a light degree. To test this hypothesis, an equilibrium protein-protein binding assay between *N. gonorrhoeae* PriA and PriB:Q44F,Q49W variant could be performed. Fluorescence polarization spectroscopy could be used to determine whether PriA and the PriB:Q44F,Q49W variant form a stable complex.

Additionally, previous studies using *N. gonorrhoeae* proposed that due to the weak interaction between PriB and ssDNA, PriA might be activated through an allosteric activation mechanism.20 It was suggested that as PriB binds to PriA, PriA undergoes conformational changes which might enhance its ATP hydrolysis activity and consequently enhances the overall helicase activity of PriA. This hypothesis is supported by the generation of *N. gonorrhoeae* PriB variants that show even lower affinity for ssDNA than the wild-type PriB, while retaining the ability to stimulate the DNA unwinding activity of PriA. Here, we constructed a PriB variant that shows a higher affinity for ssDNA, and reveals only minor decrease in the unwinding activity of its cognate PriA. Therefore, it can be speculated that the interaction between PriB and ssDNA might not be as significant in the stimulation of PriA’s unwinding activity as the interaction between PriA and PriB.
Chapter IV
Conclusions

We analyzed binary interactions within the DNA replication restart primosomes of *K. pneumoniae* (which encode a DnaT homolog) and *Y. enterocolitica* (which lacks DnaT homolog) in order to analyze what effects the absence of DnaT has on the overall interaction of the other primosome proteins with one another. Our investigations show that high affinity interactions of PriB and ssDNA are observed for *K. pneumoniae* as well as *Y. enterocolitica*. Therefore, we conclude that the presence or absence of DnaT cannot be used to predict affinities of binary interactions between PriB and ssDNA.

Furthermore, we confirm that aromatic acid residues tryptophan and phenylalanine, residing along the surface of PriB that forms an extended six-stranded sheet, are important in binding interactions between PriB and ssDNA. We also show that positively charged amino acids along the L45 loops contribute to the PriB:ssDNA binding activity.

However, *Y. enterocolitica* is a unique bacterial organism since it does not encode a *dnaT*, but shows high affinity interactions between PriB and ssDNA (perhaps due to the presence of amino acid residues F44 and W49). Therefore, it can be questioned how bacteria might compensate for lack of a primosome protein, such as DnaT. If *E. coli* PriB requires DnaT to release ssDNA, by what mechanism does *Y. enterocolitica* PriB release ssDNA?
Future studies could shed important light on how *Y. enterocolitica* solves the common problem of releasing PriB from ssDNA. Molecular interactions assays could be perform to study binary affinities between *Y. enterocolitica* PriB and its cognate PriA in order to determine whether they are similar to affinities observed in *E. coli* or *N. gonorrhoeae*. It would be also interesting to perform a binary interaction between PriB and ssDNA of *Y. enterocolitica* while incubating DnaT from *E. coli* to investigate whether ssDNA and DnaT will compete with one another for binding to PriB.
REFERENCES


