Structure, function, and therapeutic targeting of SSB protein interactions

By

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Structure, function, and therapeutic targeting of SSB protein interactions

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ABSTRACT

DNA unwinding creates single-stranded (ss) DNA intermediates that serve as templates for diverse cellular functions. Exposed ssDNA results in two specific problems for the cell; first, ssDNA is thermodynamically less stable than dsDNA, which leads to spontaneous formation of duplex secondary structures that impede genome maintenance processes. Second, relative to dsDNA, ssDNA is hypersensitive to chemical and nucleolytic attacks that can cause damage to the genome. These potential problems are solved by encoding specialized ssDNA-binding proteins (SSBs) that bind to and stabilize ssDNA structures required for essential genomic processes. The SSB protein in bacteria contains two functionally distinct regions; the N-terminal oligonucleotide/oligosaccharide binding (OB) fold is responsible for oligomerization and ssDNA
binding, while the flexible amphipathic C-terminal tail (SSB-Ct) is the site of its essential interactions with many proteins involved in DNA processing and genome maintenance.

SSB is directly involved with the replication process in bacteria. In *Escherichia coli*, SSB’s association with the χ subunit of the DNA polymerase III holoenzyme had been proposed to confer stability to the replisome and to aid delivery of primers to the lagging-strand DNA polymerase. In this work I crystallographically identified the SSB-binding site on χ. This enabled me to create a series of χ variants that destabilized the χ/SSB interface and conduct biochemical and cellular studies to delineate the role of the interaction in replication. Sequence changes in χ that block complex formation with SSB lead to salt-dependent replication defects *in vitro*, highlighting the roles of the χ/SSB complex in maintaining the replisome. Destabilization of the χ/SSB complex *in vivo* produced temperature-dependent cell cycle defects likely arising from replisome instability.

In addition to its role in DNA replication, SSB interacts with many heterologous proteins involved in nucleic acid processing and these interactions are essential to bacteria. I have shown that compounds that disrupt SSB interactions *in vitro* act as potent antibiotics *in vivo*. In addition, I have shown that essential protein/protein interactions in bacteria are a strong target for development of novel antibacterial therapeutics.
CITATIONS

This dissertation contains the following primary research articles and book chapters:


Additional contents of this dissertation have been submitted for publication in Molecular Microbiology:

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Chapter 1

Functions of single-strand DNA-binding proteins in DNA

Replication, Recombination, and Repair

This work was written by Aimee H. Marceau has been published as a book chapter in “Single-Stranded DNA Binding Proteins: Methods and Protocols” (James L. Keck, volume editor) Methods in Molecular Biology, volume 922 (Humana Press, John Walker, series editor).
1.1 Single-stranded DNA-binding proteins

Double-stranded (ds) DNA contains all of the necessary genetic information, although practical use of this information requires unwinding of the duplex DNA. DNA unwinding creates single-stranded (ss) DNA intermediates that serve as templates for myriad cellular functions. Exposure of ssDNA presents several problems to the cell. First, ssDNA is thermodynamically less stable than dsDNA, which leads to spontaneous formation of duplex secondary structures that impede genome maintenance processes. Second, relative to dsDNA, ssDNA is hypersensitive to chemical and nucleolytic attacks that can cause damage to the genome. Cells deal with these potential problems by encoding specialized ssDNA-binding proteins (SSBs) that bind to and stabilize ssDNA structures required for essential genomic processes.

SSBs are essential proteins found in all domains of life. SSBs bind ssDNA with high affinity and in a sequence-independent manner and, in doing so, help to form the central nucleoprotein complex substrate for DNA replication, recombination, and repair processes. While SSBs are found in every organism, the proteins themselves share surprisingly little sequence similarity, subunit composition, and oligomerization states. All SSB proteins contain at least one DNA-binding oligonucleotide/oligosaccharide binding (OB) fold, which consists minimally of a five stranded beta-sheet arranged as a beta barrel capped by a single alpha helix. The OB fold is responsible for both ssDNA binding and oligomerization (for SSBs that operate as oligomers). The overall organization of OB folds varies between bacteria, eukaryotes, and archaea.

As part of SSB/ssDNA cellular structures, SSBs play direct roles in the DNA replication, recombination, and repair. In many cases, SSBs have been found to form specific complexes with diverse genome maintenance proteins, often helping to recruit SSB/ssDNA-processing
enzymes to their proper cellular sites of action. This clustering of genome maintenance factors can help to stimulate and coordinate the activities of individual enzymes and is also important for dislodging SSB from ssDNA. These features support a model in which DNA metabolic processes have evolved to work on ssDNA/SSB nucleoprotein filaments rather than on naked ssDNA.

1.2 Structural diversity among SSB proteins.

The structural organization of ssDNA-binding proteins (SSBs) enables simultaneous ssDNA and heterologous protein binding. Outside of the observations that most SSBs contain at least one conserved DNA-binding OB domain, SSBs from different kingdoms of life are strikingly structurally divergent (Flynn and Zou, 2010; Theobald et al., 2003). The OB fold is responsible for SSB’s ssDNA binding and, in cases where the SSB has a functional quaternary structure, for oligomerization. OB folds can vary in length from 70 to 150 amino acids, each forming, minimally, a beta barrel consisting of five antiparallel beta strands capped by an alpha helix. The primary sequence of the SSB OB fold is not well conserved, and the loops connecting the β strands can vary in length and organization (Murzin, 1993).

The majority of SSBs can be characterized based on their phylogenetic distribution as being “bacterial” or “eukaryotic.” The major eukaryotic SSB proteins are also called Replication Protein A, or RPA. Archaeal SSBs have representatives from both divisions. For example Sulfolobus solfataricus (Wadsworth and White, 2001) has a bacterial-like SSB, while Methanococcus jannaschii (Kelly et al., 1998) has an SSB that is more similar to RPA. Most
SSBs appear to function as oligomers. For example, most bacterial SSBs function in an oligomeric state in which each protomer contains a single OB fold per polypeptide resulting in the formation of a homotetramer (Raghunathan et al., 2000). Interestingly, the presence of a small number of SSBs that encode two OB folds per polypeptide and function as homodimers indicates that there is some diversity within the bacterial protein class (Bernstein et al., 2004). In contrast to bacterial SSBs, eukaryotic RPAs generally function as heterotrimers with multiple OB folds distributed throughout the protomers (Bochkarev et al., 1997; Bochkareva et al., 2001). While bacterial SSBs function as either homotetramers (e.g., *Escherichia coli* SSB, ecSSB) (Fig. 1.1a) or more rarely as homodimers (e.g., *Deinococcus radiodurans* SSB, drSSB), both subtypes contain a total of four OB folds. The homodimers contain two OB folds per monomer and have been found thus far only in the *Deinococcus-thermus* genera (Bernstein et al., 2004; Dabrowski et al., 2002; Shereda et al., 2008). Each bacterial SSB OB fold is capable of binding to ssDNA in an arrangement where the DNA wraps around the outside of the oligomeric protein (Figure 1A) (Raghunathan et al., 2000). EcSSB is the best studied bacterial SSB and its DNA binding has been shown to be surprisingly complex. Depending on the in vitro conditions and protein:ssDNA ratio, ecSSB tetramers can utilize either two or four OB folds to bind ssDNA. The interaction of SSB with ssDNA involves both hydrophobic stacking interactions as well as ionic phosphate backbone contacts (Bujalowski et al., 1988; Casas-Finet et al., 1987; Lohman and Ferrari, 1994). In addition to their OB fold, bacterial SSBs contain a highly conserved amphipathic unstructured C-terminus (SSB-Ct) that interacts with heterologous proteins (described in more detail below) (Costes et al., 2010; Shereda et al., 2008).
Eukaryotic RPAs generally function as heterotrimers and are typified by the human protein, which comprises 70, 32, and 14 kDa subunits, RPA70, RPA34, and RPA14, respectively (Figure 1D). RPA contains a total of six OB folds among its three subunits each of which is referred to as a “DNA-binding domain” (DBD-A through DBD-F). This nomenclature is somewhat misleading however, as only four of the OB folds appear to be responsible for binding to ssDNA. Three of these four (DBD-A through C) are located within RPA70, while the fourth (DBD-D) is located within RPA32 (Bochkarev et al., 1997; Bochkareva et al., 2002; Fanning et al., 2006; Richard et al., 2009; Wold, 1997). In total, RPA70 contains four OB folds, three of which are involved in binding DNA, whereas RPA32 and RPA14 each contain a single OB fold. The three proteins interact with one another via OB domains (DBD-C, DBD-D, and DBD-E) and an additional three-helix bundle (Figure 1D) (Bochkareva et al., 2002). As with bacterial SSBs, RPA binds to ssDNA via stacking interactions of aromatic residues with individual DNA bases and interactions between the side chains of RPA and both the phosphate backbone and individual bases of the ssDNA (Figure 1B) (Bochkarev et al., 1997; Flynn and Zou, 2010; Murzin, 1993).

RPA lacks the SSB-Ct element found in bacterial SSBs but retains the ability to interact with genome maintenance proteins through multiple sites on RPA70 and RPA32. Additionally RPA32 contains a large N-terminal region that is phosphorylated in response to DNA damage and appears to play a role in regulating protein-protein interactions. RPA14 contains DBD-E and plays an essential role in stabilizing the structure of the complex (Richard et al., 2009). Human RPA (hsRPA) also contains a zinc finger motif on RPA70 that regulates ssDNA binding in a reduction/oxidation-sensitive manner (Bochkareva et al., 2000; Park et al., 1999). ssDNA binding by RPA is enhanced over 10-fold in reducing conditions compared to oxidizing
conditions (Park et al., 1999). The cytoplasm is generally more of a reducing environment than the nucleus. It is possible that increased DNA binding by RPA under reducing conditions serves as a defense mechanism against foreign DNA, specifically viral DNA.

The archaeal SSBs are particularly interesting because they share qualities with both bacterial SSB and eukaryotic RPA. For example, the crenarchaeal *S. solfataricus* SSB (ssSSB) contains an N-terminal OB fold and a C-terminal tail similar to bacterial SSB, but unlike bacterial SSB, it binds ssDNA as a monomer. The ssSSB OB fold structurally resembles the eukaryotic RPA70 DBD-B more closely than the bacterial OB folds; however the C-terminal tail is thought to function in protein/protein interactions like ecSSB (Haseltine and Kowalczykowski, 2002; Kerr et al., 2003; Wadsworth and White, 2001). In contrast, the SSB of the euryarchaeal *M. jannaschii* (mjSSB) is more similar to that of full-length hsRPA70 than its crenarchaeal relatives. The sequence contains four tandem predicted OB folds and a putative zinc finger motif. MjSSB functions as a monomer in solution (Kelly et al., 1998). Unlike ssSSB and mjSSB, the RPA from *Pyrococcus furiosus* (pfRPA) is composed of three subunits, RPA41, RPA32, and RPA14. RPA41 has some similarity to the eukaryotic RPA70, whereas the other two subunits lack homology to known proteins (Komori and Ishino, 2001). Overall, SSBs found in archaea have diverse subunit composition and potentially divergent structures.

Bacteriophage SSBs can function either as monomers (e.g., T4 gp32) or as dimers (e.g., T7 gp2.5) (Hollis et al., 2001; Shamoo et al., 1995). T4 pg32 consists of three domains: the amino-terminal 17 residues are essential for cooperativity in its ssDNA binding functions, the core domain directly binds ssDNA, and the C-terminal 46 residues contain the protein interaction site (Figure 1F). The core domain contains a zinc-stabilized OB fold (Shamoo et al., 1995; Shereda...
et al., 2008). Each T7 gp2.5 monomer is composed of two domains: an N-terminal OB fold and a C-terminal tail. Both the OB fold and C-terminus are required for dimer formation and ssDNA binding. The C-terminal tail also mediates protein interactions (Figure 1E) (Hollis et al., 2001; Kim and Richardson, 1994; Shereda et al., 2008).

In summary, while sharing a common structural fold, SSBs from all domains of life exhibit diverse subunit arrangements. Despite this diversity, all SSBs possess common ssDNA- and protein-binding functions in the cell. It is possible that the differences among eukaryotic, prokaryotic, and archaeal SSBs are a result of the divergent evolutionary steps taken to satisfy the common ssDNA- and heterologous protein-binding needs during genome maintenance.

### 1.3 Strategies for binding ssDNA in SSBs.

SSBs bind to ssDNA with such high affinity that, barring SSB depletion, any exposed ssDNA in the cell is almost certain to be coated by SSB. Despite this common high-affinity binding, SSBs from different domains of life interact with ssDNA using surprisingly distinct mechanisms.

The most extensively studied bacterial SSB is the protein from *E. coli*. The four OB folds present in the ecSSB structure allow the protein to bind ssDNA in two distinct modes (Figure 1A). These modes are named by the number of nucleotides (nt) occluded by the ecSSB tetramer: (SSB)$_{65}$, and (SSB)$_{35}$. In the limited-cooperativity (SSB)$_{65}$ mode, all four OB domains in each tetramer interact with ssDNA (Figure 1A) (Lohman and Ferrari, 1994; Raghunathan et al., 2000; Shereda et al., 2008). In the unlimited-cooperativity (SSB)$_{35}$ mode, two OB folds per SSB tetramer interact with the ssDNA. The DNA binding in the (SSB)$_{35}$ mode is highly cooperative,
stimulating SSB to form long nucleoprotein filaments that coat ssDNA (Ferrari et al., 1994; Lohman and Ferrari, 1994). The stability of each binding mode is influenced \( \text{in vitro} \) by the concentration of monovalent salts, \( \text{Mg}^{2+} \), spermidine, and spermine. Under high ionic conditions (>200 mM NaCl) and low SSB:ssDNA ratios, the \((\text{SSB})_{65}\) mode is favored, whereas the \((\text{SSB})_{35}\) mode is more stable under lower ionic strengths and high SSB:ssDNA conditions (Bujalowski et al., 1988; Lohman and Ferrari, 1994). The \((\text{SSB})_{35}\) binding mode has been proposed to function in DNA replication, whereas the \((\text{SSB})_{65}\) mode may function under conditions favorable for homologous recombination (Lohman and Bujalowski, 1994).

The published structure of ecSSB bound to ssDNA provided the first structural models explaining how DNA wraps around the protein (Figure 1A). The DNA makes several direct contacts with Trp40, Trp54, and Phe60 in the crystal structure (Raghunathan et al., 2000). These results are consistent with Trp quenching studies and mutational analysis (Casas-Finet et al., 1987; Ferrari et al., 1997; Merrill et al., 1984). In addition, electrostatic interactions utilize several Lys residues (43, 62, 73, and 87) and the N-terminus of SSB to bind the ssDNA backbone (Raghunathan et al., 2000). The hydrophobic and ionic interactions together allow SSB to recognize and bind ssDNA independent of the sequence.

RPA binds to ssDNA using the same ssDNA-binding pockets in the OB domain but aligns along the ssDNA in a very different manner from the preformed tetrameric bacterial SSB DNA-binding core. RPA’s four DNA-binding OB folds bind ssDNA in a sequential manner with 5’ to 3’ polarity, resulting in RPA70 being localized 5’ to RPA32 on the ssDNA. First, DBD-A of RPA70 initiates binding to 8-10 ssDNA nucleotides. This allows for DBD-B ssDNA binding, occluding a total of \(~10\) nucleotides (Figure 1B). The ssDNA binding of the RPA70 A and B
domains, which are separated by a short flexible linker, is coordinated with a conformational change in RPA70. Two loops within domains A and B enclose the ssDNA, which next allows DBD-C to bind, occluding an overall site of 12-23 nucleotides. Lastly, DBD-D from RPA32 binds to ssDNA leading to full binding that occludes 28-30 nucleotides (Arunkumar et al., 2003; Bochkarev et al., 1997; Bochkareva et al., 2001; Richard et al., 2008; Wold, 1997; Wyka et al., 2003). Each of these different binding intermediate states is detectible in vitro, and have been termed globular, elongated contracted, and elongated extended (Wold, 1997). In yeast RPA, the transition from lower to higher binding site size is salt dependent (Broderick et al., 2010; Oakley and Patrick, 2010; Richard et al., 2009). Unlike bacterial SSBs, which wrap ssDNA around the four relatively static OB folds, the conformation of RPA changes upon binding resulting in an elongated ssDNA substrate (Figure 1B). Additionally, RPA can bind to multiple ssDNA substrates concurrently, allowing it to function as an ssDNA bridge in the process of DNA repair (Bochkareva et al., 2002; Pestryakov et al., 2004). When RPA binds to ssDNA the conformational change allows the N-terminus of RPA32 to be more readily phosphorylated (Wold, 1997) (discussed in a later section).

Compared to bacterial and eukaryotic SSBs, far less is known about archaeal SSB DNA binding. These SSBs are similar in structure to the ssDNA binding domains from eukaryotic RPA, with the structure of the archaeal ssSSB being most similar to RPA70 DBD-B. The current model for ssSSB-ssDNA binding is based on this similarity. The model suggests that when ssSSB binds to ssDNA it undergoes a conformational change from an open to a closed state. Because the binding site consists of only 4-5 nucleotides per monomer (Haseltine and Kowalczykowski, 2002; Wadsworth and White, 2001), it is highly unlikely that ssDNA wraps around the protein.
The binding site size and the similarity to RPA suggest that ssSSB binds to ssDNA with SSB monomers multimerized along the substrate (Kerr et al., 2003). MjSSB is similar to RPA70 in that it contains four predicted OB folds in tandem. DNA binding by mjSSB occludes between 15-20 nucleotides, which is slightly smaller than RPA (Kelly et al., 1998). These are the only two archaeal SSBs with published ssDNA binding information. Without ssDNA bound protein structural information, it is difficult to compare these proteins to bacterial SSB and eukaryotic RPA.

Bacteriophage T7 gp2.5 binds to ssDNA in an auto-regulated manner, with the C-terminus of the protein competing with ssDNA for the binding site. The exact manner of its ssDNA binding is unknown but the C-terminus is proposed to act as a switch that prevents the DNA binding cleft from interacting with improper negatively charged surfaces. The OB fold contains aromatic and positively charged residues important for ssDNA interaction. T4 gp32 binds cooperatively to ssDNA via its N-terminal domain (gp32-gp32 interaction) and its central core (ssDNA binding). Both gp2.5 and gp32 have two clear binding modes (Hollis et al., 2001; Kim and Richardson, 1994). The proteins first weakly interact nonelectrostatically with dsDNA backbone. Then, the proteins slide along dsDNA, which increases the association rate of the second binding mode in which the proteins bind to ssDNA (Shamoo et al., 1995).

All SSBs bind ssDNA with high affinity, thereby protecting any exposed ssDNA in the cell. In general, SSBs associate with ssDNA via both hydrophobic stacking ionic backbone interactions. Bacterial SSBs and RPA have multiple binding modes that are detectible in vitro that may have in vivo significance. Bacterial SSBs bind to ssDNA by wrapping the DNA around the outside of the homotetramer/homodimer utilizing two to four OB folds per SSB (Figure 1A). RPA binds to
ssDNA in a sequential 5’ to 3’ manner, resulting in an extended arrangement of the protein along the ssDNA (Figure 1B). The differences in ssDNA binding by SSBs from various domains of life may reflect the distinct genomic needs for ssDNA protection and utilization as a substrate.

1.4 Protein interactions with SSBs.

In addition to their core DNA-binding activities, SSBs have a second essential role in the organization, localization, and stimulation of genome maintenance proteins through direct physical interactions. Bacterial SSBs interact with heterologous proteins through their evolutionarily conserved SSB-Ct elements, which include both acidic and hydrophobic residues (Asp-Asp-Asp-Ile-Pro-Phe in *E. coli* SSB). Select bacterial SSBs from *E. coli* and *Bacillus subtilis* have well-characterized interaction networks, which highlight how integrated SSBs are in genome biological processes. Eukaryotic RPAs similarly interact with a large number of proteins but, unlike their bacterial counterparts, RPAs appear to have multiple protein-interaction sites on RPA70 and RPA32. Very little is known about the protein interactions of archaeal SSBs. The few known protein partners reveal potentially interesting connections between transcription and genome maintenance.

Bacterial SSBs have been shown to interact with over a dozen different proteins in *E. coli* and *B. subtilis*. These binding partners are involved in a wide range of functions in DNA metabolism. In all cases characterized to date, SSB’s binding partners bind to the SSB-Ct. Unlike the well-ordered OB domains of bacterial SSBs, the C-terminus is structurally dynamic and can be readily proteolyzed. Interestingly, removal of the SSB-Ct in ecSSB alters its ssDNA binding properties.
(Shereda et al., 2008; Williams et al., 1983; Zhou et al., 2011), which could indicate that protein occupancy at the SSB-Ct could similarly affect DNA binding. Mutations in the SSB-Ct are detrimental to cell survival and, at least in *E. coli*, removal of the SSB-Ct is lethal. One well-characterized SSB-Ct mutation (*ssb113*) encodes for a variant in which the penultimate Pro is changed to a Ser. This change leads to temperature-sensitive lethality in *ssb113*-mutant *E. coli* (Chase et al., 1984). The SSB113 protein itself retains apparent wild-type ssDNA-binding functions, but at nonpermissive temperatures DNA replication is no longer supported. In addition, this strain exhibits DNA damage hypersensitivity at all temperatures (Chase et al., 1984; Meyer et al., 1980; Wang and Smith, 1982). DNA damage hypersensitivity and temperature-dependent replication are the direct results of loss of SSB interaction with partner proteins involved in DNA repair and replication (Greenberg and Donch, 1974; Kelman et al., 1998; Yuzhakov et al., 1999).

**1.4a Bacterial SSB/DNA replication interfaces.** During *E. coli* DNA replication, SSB interacts directly with at least two key proteins: the χ subunit of the replicative DNA polymerase III holoenzyme and primase (Breier, 2005; Glover and McHenry, 1998, 2001; Gulbis et al., 2004; Kelman and O'Donnell, 1995; Kelman et al., 1998; Simonetta et al., 2009; Yuzhakov et al., 1999). Variants of χ that fail to interact with SSB have been incorporated into the holoenzyme and assayed both *in vivo* and *in vitro* (Marceau et al., 2011). *In vitro* the loss of the interaction results in a salt-dependent decoupling of leading- and lagging-strand DNA synthesis. *In vivo* the cells exhibit slower replication rates and temperature-sensitive growth. The interaction between χ and SSB appears to be primarily important for stabilization of the replisome but is not
absolutely required for replication (Glover and McHenry, 1998; Kelman et al., 1998; Marceau et al., 2011). During replication, the replisome cannot initiate DNA synthesis de novo -- instead it extends preformed RNA primers generated by primase (DnaG). Primase interacts with both DnaB (replicative helicase) and SSB (Wu et al., 1992; Yuzhakov et al., 1999). The precise role of the SSB/primase interaction is not well understood. It is possible that the interaction stabilizes primase on the RNA-DNA duplex, to aid in primase protection of the hybrid from degradation or dissociation prior to Okazaki fragment synthesis initiation (Rowen and Kornberg, 1978; Shereda et al., 2008; Yuzhakov et al., 1999). In *B. subtilis*, one of the essential DNA polymerases (DnaE) also directly interacts with the SSB C-terminus, although the role of this interaction has not been investigated (Costes et al., 2010).

1.4b Bacterial SSB/DNA replication restart interfaces. The process of DNA replication does not always proceed smoothly, and the replisome can stall and/or dissociate from the fork when it encounters DNA damage or bound proteins that block progression on the DNA. In instances where the replisome dissociates, it must be reloaded by proteins referred to collectively as the DNA replication restart primosome. SSB is intimately involved in this process through its interaction with PriA, a 3’-to-5’ DNA helicase that initiates the major bacterial restart processes (Cadman and McGlynn, 2004; Costes et al., 2010; Kozlov et al., 2010; Lecointe et al., 2007). PriA appears to be constitutively bound to the replication fork in both *E. coli* and *B. subtilis* via its interaction with the SSB-Ct of the respective SSBs. Upon recognition of a stalled DNA replication fork structure, PriA nucleates assembly of the primosomal complex. Interaction with SSB stimulates PriA helicase activity, and PriA can displace SSB from ssDNA allowing
primosome assembly, and ultimately DNA replication proteins, onto the collapsed fork (Arai et al., 1981; Cadman and McGlynn, 2004; Kozlov et al., 2010).

1.4c Bacterial SSB/DNA recombination interfaces.

SSBs from both *E. coli* and *B. subtilis* interact with several proteins involved in DNA recombinational repair via the SSB-Ct, localizing the proteins to the site of repair and in many cases stimulating their activity. Two key recombination initiation proteins from the RecF recombination pathway, RecQ DNA helicase and RecJ nuclease, both bind to and are stimulated by SSB. SSB-stimulated RecQ helicase activity enhances unwinding efficiency (Shereda et al., 2007), whereas SSB-stimulated RecJ 5’ to 3’ exonuclease activity creates an ssDNA template for downstream RecA-mediated recombination (Courcelle et al., 1997; Courcelle et al., 1999; Han et al., 2006; Lovett and Kolodner, 1989).

One common challenge in RecA-mediated recombination is that RecA nucleation onto ssDNA/SSB substrates is impeded by SSB. Specialized proteins called RecA mediators catalyze RecA nucleation by displacing SSB. One such mediator, RecO, plays an important role in this process by directly binding to SSB (Hobbs et al., 2007; Inoue et al., 2008; Ryzhikov et al., 2011). In addition to its RecA supporting activity, RecO binds ssDNA and dsDNA and has a DNA annealing activity that is also stimulated by SSB (Luisi-DeLuca, 1995; Luisi-DeLuca and Kolodner, 1994). In addition to RecO, MgsA has recently been shown to interact with SSB and to facilitate RecA loading at stalled replication forks (Costes et al., 2010; Page et al., 2011). RecG is a monomeric DNA helicase important in remodeling stalled replication forks and
unusual DNA structures (McGlynn and Lloyd, 2002; McGlynn et al., 2000). RecG’s interaction with SSB stabilizes its binding to various DNA structures and stimulates its ATPase activity (Lecointe et al., 2007; Slocum et al., 2007). RecS, a putative helicase found in *B. subtilis* interacts with SSB in complex with YpbB. Its exact function in the cell is unknown. In *B. subtilis*, these proteins have been shown to localize to the replication forks in a manner dependent on the SSB-Ct (Costes et al., 2010).

1.4d Bacterial SSB/DNA repair interfaces. DNA repair is essential for maintaining the fidelity of genomes. Not surprisingly, SSB interacts with several enzymes involved in repair and stimulates their activities as well as localizing them to the site of repair. Exonuclease I (ExoI) processively degrades ssDNA in a 3’-to-5’ direction. This activity is stimulated by binding to SSB, specifically the SSB-Ct, and is important for methyl-directed DNA mismatch repair (Genschel et al., 2000; Lu and Keck, 2008; Shereda et al., 2008). SSB also interacts directly with uracil DNA glycosylase (UDG), which catalyzes the first step in base excision repair. This interaction between UDG and SSB is widely conserved from eukaryotes to viruses (Costes et al., 2010; Dianov and Lindahl, 1994; Purnapatre et al., 1999; Shereda et al., 2008). This interaction localizes UDG to the replication fork potentially to aid in removal of dUTP misincorporation. DNA polymerase II (Pol II) is a DNA repair polymerase that is induced early in the SOS response (Iwasaki et al., 1990). This polymerase is involved in synthesis over lesions and repair of damaged DNA (Pages et al., 2005; Shereda et al., 2008). SSB supports binding of Pol II to ssDNA and stimulates its polymerase activity (Molineux and Gefter, 1974; Shereda et al., 2008; Weiner et al., 1975). Pol II has poor processivity, but in complex with SSB and processivity
factors (clamp loader complex and the β subunit), the processivity is greatly increased, which is required for bypass of abasic sites (Bonner et al., 1992; Dalrymple et al., 2001). DNA polymerase V (Pol V) is another mutagenic polymerase that interacts with SSB via its SSB-Ct (Arad et al., 2008). Pol V conducts translesion synthesis on damaged DNA (Goodman, 2000). SSB allows Pol V access to the 3’ end of a DNA gap flanked by RecA filaments and thus facilitates its activity on damaged DNA (Arad et al., 2008). SbcC is an ssDNA nuclease that is specific for ssDNA palindromic structures; it is localized to the replication fork via its interaction with SSB (Costes et al., 2010). SSB plays a key role in DNA repair by recruiting, localizing, and stimulating the activity of proteins involved in a variety of repair pathways.

1.4e Other bacterial SSB/protein interfaces. *E. coli* SSB also interacts with several other proteins with roles outside of canonical genome maintenance pathways. Exonuclease IX, misnamed since it is not an exonuclease and has no known cellular function (Hodskinsson et al., 2007; Shafritz et al., 1998), binds to SSB. Bacteriophage N4 viron RNA polymerase (vRNAP) requires SSB for early transcription (Glucksmann-Kuis et al., 1996). N4 phage relies on ecSSB to support vRNAP in displacing nascent RNA from the ssDNA template. By binding to both the DNA template and the RNA product, SSB prevents the formation of a RNA-DNA hybrid and this results in increased template recycling (Davydova and Rothman-Denes, 2003). In *B. subtilis* SSB also interacts with XseA and YrrC, localizing them to the replication fork. Neither protein has a well-defined function in the cell (Costes et al., 2010).
1.4f RPA/DNA replication interfaces. As with its bacterial SSB counterparts, RPA interacts with numerous proteins involved in a wide variety of genome maintenance processes: chromosomal and viral DNA replication, DNA repair, DNA recombination, cell division, checkpoint control, DNA damage response, and transcription. RPA/replication protein interactions have been described in a wide variety of species (Fanning et al., 2006; Richard et al., 2009). DNA polymerase α-DNA primase interacts with RPA from *Homo sapiens*, and *Saccharomyces cerevisiae*. The SV40 large tumor antigen, Bovine papillomavirus E2, and Epstein-Barr virus EBNA-1 all interact with RPA from *H. sapiens*. In fact, RPA was initially identified because it is essential for SV40 replication (Borowiec et al., 1990). The large T-antigen of SV-40 directly interacts with RPA, DNA polymerase α/primase, and topoisomerase to coordinate replisome assembly and function (Borowiec et al., 1990; Hurwitz et al., 1990). RTHI nuclease has been shown to interact with RPA from *S. cerevisiae* (Fanning et al., 2006; Wold, 1997). In many cases the exact functions of the interaction between RPA and heterologous proteins are not well understood.

RPA is essential for replication in eukaryotes and is involved in both replication initiation and elongation of the replication fork. RPA localizes to origins of replication independent of the origin recognition complex (Richard et al., 2008). It is thought that RPA binds the small amount of ssDNA exposed at the origin, after which cdc45 unwinds more of the origin and more RPA can bind to the ssDNA (Mimura et al., 2000). After the recognition of the origin by the pre-initiation complex, DNA polymerase α/primase is recruited and bound to the ssDNA via an interaction with RPA. The interaction stabilizes the DNA polymerase α/primase on ssDNA and RPA acts like a fidelity clamp, reducing the rate of nucleotide missincorporation (Frick and
Richardson, 2001; Maga et al., 2001). The primase subunit synthesizes ~12 nt RNA primers which are elongated to 20 nt by polymerase α; this protein initiates all of the Okazaki fragments on the lagging strand (Conaway and Lehman, 1982a, b). After initiation, the more processive DNA polymerases ε (leading strand) and δ (lagging strand) replace polymerase α in a replication factor C (RFC) organized switch. RFC competes with polymerase α for binding to RPA causing the polymerase to dissociate (Maga and Hubscher, 1996). RPA helps coordinate the removal of the RNA primers of the Okazaki fragments. RPA recruits Dna2 (helicase/nuclease) to the DNA polymerase δ displaced RNA-DNA flap and stimulates cleavage of the flap resulting in shorter fragments. The short fragment is processed by Fen1 and the resulting nicked duplex DNA is fixed by DNA ligase (Bae et al., 2001; Bae and Seo, 2000).

1.4g RPA/DNA repair interfaces. RPA functions in DNA repair via protein-protein interactions by recruiting and organizing repair proteins at the site of damage. Homology-directed repair (HDR) is employed to repair double strand DNA breaks, ssDNA gaps, interstrand crosslinks and in recovery of collapsed or stalled replication forks (Michel et al., 2004; Paques and Haber, 1999). At the site of a double-strand DNA break, the DNA is resected 5’ to 3’ and RPA binds to the ssDNA. RPA is then removed by RAD51, likely via its interaction with RPA70 (Stauffer and Chazin, 2004). RAD51 then mediates strand exchange, aided by RPA, and by a second SSB, hSSB1, in humans (Richard et al., 2008; Richard et al., 2009). The loading of RAD51 is facilitated by RAD52, which binds to both RPA70 and RPA32 (Butland et al., 2005; Mer et al., 2000). Additionally, RPA displacement may be facilitated by BRCA2 via a direct
interaction. In fact, a common cancer-predisposing mutant of BCRA2 does not interact with RPA (Richard et al., 2009; Wong et al., 2003).

At stalled replication forks, RPA appears to function by exchanging proteins required during different parts of the repair process. RPA interacts with members of the RecQ family of repair helicases, including Bloom syndrome protein (BLM) and Werner syndrome protein (WRN) (Constantinou et al., 2000; Doherty et al., 2005; Shen et al., 2003). The interaction recruits the proteins to stalled replication forks and may limit RAD51 activity. RPA stimulates the helicase activity of both BLM and WRN (Bugreev et al., 2007; Wu, 2008), which could facilitate their activity in fork repair. A number of the Fanconi anemia (FA) proteins, which are involved in replication fork stabilization and recovery, also associate with RPA (Wang et al., 2008). One of the FA proteins, FANCD2, also functions in concert with BLM (Pichierri et al., 2004). After a replication fork has stalled RPA interacts with FANCJ to increase its processivity and helicase activity (Gupta et al., 2007).

Translesion synthesis (TLS) utilizes polymerases with more open active sites that lack proofreading activity, enabling them to synthesize DNA over lesions (Kunkel et al., 2003). In eukaryotes, the ubiquitination of PCNA likely regulates the switch from HDR to TLS. If PCNA is polyubiquitinated, the lesion is repaired by HDR but if it is monoubiquitinated then it is repaired by TLS (Kannouche and Lehmann, 2004; Kannouche et al., 2004). RPA interacts with Rad18/Rad6 which catalyses the monoubiquitination of PCNA, thus RPA is involved in regulating the switch between TLS and HDR (Davies et al., 2008). RPA may also interact with the mutagenic DNA polymerase λ (Crespan et al., 2007; Krasikova et al., 2008). PCNA in
conjunction with RPA likely has a role in recruiting and regulating the action of the TLS polymerases (Crespan et al., 2007; Krasikova et al., 2008).

Since DNA damage can occur anywhere in the genome other mechanisms that operate independently of replication structures exist to deal with these fidelity problems and RPA is involved. The nucleotide excision repair (NER) pathway removes a variety of different DNA lesions that occur in response to environmental or endogenous genotoxic stressors throughout the entire genome. RPA plays an essential role in NER, stabilizing ssDNA intermediates and recruiting specific proteins to the site of repair (Coverley et al., 1992; Coverley et al., 1991; Guzder et al., 1996). As mentioned previously, RPA interacts with UDG, which removes uracil from DNA (Nagelhus et al., 1997). The RPA34 subunit interacts with XPA nucleases and the RPA-XPA complex fully opens the DNA around the lesion site, with RPA bound to the undamaged strand (He et al., 1995). While the 5’ end of RPA interacts with XPG to open the DNA, the 3’ end recruits and interacts with the nuclease ERCCI-XPF determining the orientation with which the nucleases bind while simultaneously protecting the undamaged DNA from nuclease attack (He et al., 1995; Matsunaga et al., 1996).

RPA also functions in the DNA damage checkpoint response pathways, which delays cell cycle entry after DNA damage. RPA interacts with the 9-1-1 complex, specifically directing its loading onto DNA (Kumagai et al., 2006). The 9-1-1 complex recruits a variety of proteins that bind to and stimulate the ATR-ATRIP kinase activity (Zou et al., 2003; Zou et al., 2006). ATR is a PI3 family kinase that functions to regulate replication stress caused by replication inhibitors or DNA damage (Zou et al., 2003). ssDNA bound by RPA may help localize the ATR-ATRIP complex to the site of damaged DNA. ATRIP interacts with RPA70 and then recruits ATR.
When the complex binds to RPA-coated ssDNA (Zou et al., 2006), ATR becomes activated and is able to phosphorylate additional targets in the checkpoint response.

1.4h Archaeal SSB/protein interactions. Archaeal SSB interaction discovery is in its beginning stages. *S. solfataricus* SSB interacts with RNA polymerase (RNA pol) via its C-terminal tail, stimulating its activity under limiting conditions (Richard et al., 2004). Additionally, SSB can melt AT-rich promoter sequences allowing RNA pol access to those genes. Finally, SSB may aid in the formation of the pre-initiation complex at promoter site of archaeal chromatin (Richard et al., 2004). This highlights a potentially important role of SSB in transcription.

*Methanothermobacter thermautotrophicus* RPA interacts with the DNA repair helicase Hel308 via its C-terminus resulting in a very modest stimulation of its helicase activity (Woodman et al., 2011). The interaction aids localization and loading of the helicase to its site of activity at blocked replication forks. It is not clear if the interaction is critical for helicase activity. The heterotrimERIC pfRPA co-precipitates with RadA, which functions to resolve Holliday Junction intermediates (Komori and Ishino, 2001). The interaction between pfRPA and RadA stimulates its strand exchange activity *in vitro*. In addition, pfRPA may interact with Hjc, a recombination protein, as well as with DNA polymerases and primase (Komori and Ishino, 2001). The exact functions and consequences of pfRPA’s interactions have yet to be determined.

In summary, SSBs from all organisms mediate important genome maintenance processes via protein-protein interactions. Both SSB and RPA are capable of stimulating the activity of
enzymes as well as localizing proteins to their sites of action (SSB/ssDNA substrates). Bacterial SSB protein-protein interactions all appear to be mediated by the conserved SSB-Ct. RPA’s interactions can involve several different domains in the protein. Archeael RPA/SSB protein interaction studies have not advanced enough to determine a common binding domain, but the known interactions utilize the C-terminus. Protein-protein interactions allow SSB to function as a central organizer in replication, recombination, and repair.

1.5 Posttranslational modifications

Posttranslational modifications are a well-studied biological strategy for altering the function of a protein after it is synthesized by the addition or removal of modifying groups. It has been known for over ten years that RPA is phosphorylated, mostly on the N-terminus of RPA32, but the exact function remains unclear (Fanning et al., 2006; Richard et al., 2009). Identification of the roles of SSB posttranslational modifications in bacteria lags behind eukaryotes. Recently phosphorylation of bsSSB has been described; the modification may result in stronger ssDNA binding (Mijakovic et al., 2006). A deeper appreciation of the effects of posttranslational modifications of SSB and RPA promises to enhance our understanding of their roles in DNA maintenance.

Phosphorylation of bacterial SSBs has thus far been described in B. subtilis (Mijakovic et al., 2006). The phosphorylation of bsSSB does not correlate with any specific cellular process. BsSSB appears to be phosphorylated by the YwqD kinase and dephosphorylated by the YwqE phosphatase. BsSSB phosphorylation reportedly enhances ssDNA binding ~200-fold over that of
unphosphorylated bsSSB. In addition, the level of phosphorylated bsSSB decreases in response to DNA damage, possibly resulting from the protein being removed from damaged ssDNA by proteins involved in DNA repair.

The phosphorylation of RPA appears to be critical for its role in DNA replication and repair. The N-terminus of RPA32 is phosphorylated during the S and G2/M phase of the cell cycle by Cdk2 family kinases (Din et al., 1990; Dutta et al., 1991; Dutta and Stillman, 1992). During mitosis, RPA32 is inactivated by hyperphosphorylation, resulting in RPA disassembly from chromatin, a potentially essential step (Richard et al., 2009). In addition RPA is hyperphosphorylated in response to DNA damage by the kinases ATM, ATR, and DNA-PK; the exact role of this phosphorylation event remains under debate. Some studies have shown that modification inhibits DNA replication *in vivo* and *in vitro* whereas others show no effect on ssDNA binding or *in vitro* SV40 DNA replication assays (Richard et al., 2009). Clearly, a significant amount of work is needed to better define the role of RPA phosphorylation in replication and DNA repair.

### 1.6 Organisms with multiple SSBs

SSBs have many important roles in the cell, and in most cases these appear to be dealt with by a single, general protein. However, there are also some situations in which specialized SSBs may be needed and, as a result, alternative SSBs have evolved. Frequently the alternative SSBs are nonessential, have different domain arrangements, or lack certain domains found in the primary cellular SSB. In bacteria, two alternative SSBs have recently been described. Eukaryotes also
have a second SSB that is found in the mitochondria and supports its DNA replication and human cells have two additional SSBs, hSSB1 and hSSB2, which have specialized functions in DNA repair.

*Bacillus subtilis* (and many other naturally competent bacteria) possesses two SSBs. One is similar to the *E. coli* SSB and contains an N-terminal OB fold and a C-terminal tail that mediates its interactions with other proteins. This protein (SsbA) is primarily expressed in log phase in actively replicating cells, with about four-fold lower expression in stationary phase (Lindner et al., 2004). In contrast the second SSB (SsbB), encoded by the *ywpH* gene, contains an OB fold but lacks the C-terminus found in the primary SSB. The single OB fold forms a stable tetramer capable of binding ssDNA (Lindner et al., 2004). SsbB is expressed primarily in stationary phase and aids *B. subtilis* in its natural competence. Transcriptional regulation of *ywpH* is controlled by ComK and its pattern of expression is consistent with late competence genes (Lindner et al., 2004). While SsbB is not essential for cell survival, mutants that lack *ywpH* are 50-fold less competent than wild-type cells (Lindner et al., 2004). The precise function of SsbB in natural competence is not yet known. Many bacteria that are naturally competent possess homologues to SsbB (Lindner et al., 2004).

*D. radiodurans* also contains at least two SSBs. As described earlier, the primary SSB contains two OB folds per monomer for binding DNA and dimerization and a C-terminal tail that directs the interactions with other proteins (Bernstein et al., 2004). Its second SSB, DdrB, is not expressed under normal conditions but is induced to high levels in response to DNA damage caused by ionizing radiation. Similar to the primary SSB, DdrB contains two domains, an N-terminal domain with a unique fold responsible for oligomerization and ssDNA binding and a
dynamic C-terminus that may play a role in protein-protein interactions similar to SSB. The C-terminus has a strong similarity to that of the canonical SSB, implying that the two proteins may bind to similar groups of interaction partners. Unlike previously characterized SSBs, DdrB forms a stable pentamer arranged in a ring structure (Norais et al., 2009; Sugiman-Marangos and Junop, 2010). DdrB has been shown to bind ~5nt per monomer (Sugiman-Marangos and Junop, 2010). The exact function of DdrB in the cell is not clear; there is some evidence that it may affect RecA binding to ssDNA (Norais et al., 2009).

All eukaryotic cells have at least two SSBs: RPA, which is found in the nucleus, and mtSSB (Figure 1C), which is located in the mitochondria. RPA and mtSSB are not evolutionarily related; mtSSB is more structurally similar to tetrameric bacterial SSBs (Figures 1A and C). The structure and organization of mtSSB are slightly different from ecSSB however; mtSSB has an extended N-terminal domain, followed by an OB fold domain and it lacks the amphipathic C-terminus. Deletion of mtSSB from the mitochondria results in a loss of mitochondrial DNA replication and eventual mitochondrial loss. mtSSB interacts with, and promotes the activity of, two proteins’ function in mtDNA replication: DNA polymerase γ and mtDNA helicase (Oliveira and Kaguni, 2010). The N- and C-terminal regions of mtSSB may play a role in modulating the protein-protein interactions (Oliveira and Kaguni, 2010).

All eukaryotes have mtSSB; however humans have at least two additional SSB proteins. The structures of hSSB1 and hSSB2 appear to be more closely related to archaeal and bacterial SSBs than to RPA. The domains of the protein are organized into an N-terminal OB fold and a C-terminal tail that may be involved in protein-protein interactions. hSSB2 appears to be expressed preferentially in lymphocytes and testes, hinting that this protein may play a role in meiotic
recombination and class switching (Richard et al., 2009). Unlike hSSB2, hSSB1 is expressed in many cell types and has an important role in the repair of double-strand DNA breaks. hSSB1-depleted cells have increased sensitivity to DNA-damaging agents, greater genomic instability, faulty checkpoint activation, and defective DNA repair (Richard et al., 2008).

1.7 Summary

SSBs are remarkably well-adapted structural and organizational proteins that are central factors in genome biological processes in all cells. In addition to their eponymous DNA-binding functions, SSBs play essential roles in establishing critical protein organizational units through multivalent protein complex assembly. From eukaryotes to prokaryotes, SSBs are components in every nucleic acid transaction that requires single-stranded intermediates. This volume assembles the methods that have led to critical discoveries in the SSB field for the past 40 years and that will continue to pave the way to new findings that better define the biological functions of SSBs.
Figure 1.1

Structures of SSB and RPA from all domains of life (a) The structure of E. coli SSB bound to single-strand DNA (ssDNA) (red) (pdb 1EYG) (Raghunathan et al., 2000). The structure is shown as each monomer colored green or blue. (B) RPA70 DNA binding domain A and DNA binding domain B are shown in green bound to ssDNA (red) (pdb 1JMC) (Bochkarev et al., 1997). (C) The structure of human mitochondria SSB (pdb 2DUD). The structure is shown as each monomer colored green or blue. (D) The trimerization core RPA (pdb 1L1O). RPA70 is shown in red, RPA32 in green and RPA14 in blue. The three proteins interact primarily through a three-helical bundle (Bochkareva et al., 2002). (E) The structure of the gp2.5 dimer, each monomer is shown in either green or blue (pdb 1JE5) (Hollis et al., 2001). (F) The structure of gp32 core protein (pdb 1GPC) (Shamoo et al., 1995). (G) The structure of Sulfolobus solfataricus SSB (pdb 1O7I). The structurally dynamic C-terminus was not ordered in the crystal structure. (Kerr et al., 2003).
1.8 References


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Chapter 2

Structure of the SSB-DNA polymerase III interface and its role in DNA replication

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The majority of the experiments were designed and conducted by Aimee H. Marceau. Soon Bahng and Kenneth J. Marians designed and executed the purified pol III assays. Shawn C. Massoni and Steven J. Sandler designed and executed the microscopy experiments.
2.1 Summary

Interactions between single-stranded DNA-binding proteins (SSBs) and the DNA replication machinery are found in all organisms, but the roles of these contacts remain poorly defined. In *Escherichia coli*, SSB’s association with the χ subunit of the DNA polymerase III holoenzyme has been proposed to confer stability to the replisome and to aid delivery of primers to the lagging-strand DNA polymerase. Here, the SSB-binding site on χ is identified crystallographically and biochemical and cellular studies delineate the consequences of destabilizing the χ/SSB interface. An essential role for the χ/SSB interaction in lagging-strand primer utilization is not supported. However, sequence changes in χ that block complex formation with SSB lead to salt-dependent uncoupling of leading- and lagging-strand DNA synthesis and to a surprising obstruction of the leading-strand DNA polymerase *in vitro*, pointing to roles for the χ/SSB complex in replisome establishment and maintenance. Destabilization of the χ/SSB complex *in vivo* produces cells with temperature-dependent cell cycle defects that appear to arise from replisome instability.

2.2 Introduction

In *Escherichia coli*, DNA replication is initiated at a single locus, oriC, at which two divergent replication forks are generated (Johnson and O'Donnell, 2005; van Oijen and Loparo, 2010). Multi-protein complexes called replisomes assemble on each fork to carry out replication (Figure 2.1A). Within this complex the genome is unwound to produce single-stranded DNA (ssDNA) templates used by the DNA Polymerase III holoenzyme (Pol III HE) for DNA synthesis. Due to the anti-parallel structure of duplex DNA and the unidirectional activity of DNA polymerases,
DNA replication is semi-discontinuous -- one DNA strand (leading) is replicated continuously whereas the other strand (lagging) is synthesized discontinuously as Okazaki fragments, each of which requires a separate priming and DNA polymerase loading event. An important consequence of this arrangement is that the lagging-strand template is transiently exposed during replication as an extended loop of ssDNA that can be several thousand bases in length (Alberts et al., 1983).

To prevent formation of inhibitory secondary structures in the exposed lagging-strand DNA during replication, tetrameric ssDNA-binding proteins (SSBs) coat the ssDNA template. As such, SSB/ssDNA nucleoprotein complexes are the bona fide substrates used in lagging-strand DNA synthesis. To engage this substrate, the *E. coli* Pol III HE binds directly to SSB forming an interface that has been proposed to be important for stabilization of the replication machinery and for facilitating Okazaki fragment replication initiation (Downey and McHenry, 2010; Glover and McHenry, 1998; Kelman et al., 1998; Wu et al., 1992b; Yuzhakov et al., 1999). Interactions between SSBs and the replication machinery have been found in eukaryotes as well (Oakley and Patrick, 2010), suggesting that such interfaces are broadly utilized in cellular DNA replication pathways.

The *E. coli* Pol III HE is composed of two DNA polymerases (α, ε and θ subunits), one processivity clamp (β2) for each polymerase, and a central complex called the DnaX or γ complex ((τ/γ)3, δ, δ', χ and ψ subunits). DnaX catalyzes loading of β2 onto DNA and integrates replisome function through protein-protein interactions (Johnson and O'Donnell, 2005) (Figure 2.1A). Each DNA polymerase interacts with β2 to achieve the processivity necessary for extended DNA synthesis during genome replication (Stukenberg et al., 1991). This arrangement
necessitates $\beta_2$ loading during initiation of the leading strand and at the start of each new Okazaki fragment. The ATP-dependent $\beta_2$ loading reaction is driven by $(\tau/\gamma)_3/\delta/\delta'$ from the DnaX complex (Bloom, 2006); the $\chi/\psi$ subcomplex stimulates this function (Olson et al., 1995; Simonetta et al., 2009). The $\chi$ subunit interacts directly with SSB, forming an interface that has multiple proposed functions (Downey and McHenry, 2010; Glover and McHenry, 1998; Kelman et al., 1998; Wu et al., 1992b; Yuzhakov et al., 1999).

One model for the function of the $\chi/SSB$ interaction is an architectural/stabilization role in which $\chi/\psi$ links the clamp loader machinery to the lagging strand through direct physical interactions (Figure 2.1A). In this arrangement, $\psi$ binds to the $\tau/\gamma$ subunits in the $(\tau/\gamma)_3/\delta/\delta'$ subcomplex while $\chi$ binds to SSB associated with the lagging-strand template DNA (Glover and McHenry, 1998, 2001; Kelman et al., 1998; Simonetta et al., 2009). Previous studies have mapped the $\psi$-binding sites on $\tau/\gamma$, showing that $\psi$ binding augments the stability of the DnaX complex and stimulates its activity (Olson et al., 1995; Simonetta et al., 2009). $\chi$ appears to stimulate DnaX-complex activities in vitro only when SSB is present, forming a $\chi/SSB$ complex that could position the lagging-strand template for clamp loading (Glover and McHenry, 1998; Kelman et al., 1998). In this function, $\chi$ binds to SSB’s conserved C-terminus (SSB-Ct, -Asp-Asp-Asp-Ile-Pro-Phe in *E. coli* SSB) (Glover and McHenry, 1998; Kelman et al., 1998; Yuan and McHenry, 2009). An *E. coli* SSB variant in which the SSB-Ct Pro is changed to Ser (SSB113) has a temperature- and salt-dependent interaction with $\chi$ and destabilizes the replisome in vitro (Kelman et al., 1998). In addition, DNA replication in *E. coli ssb113* cells is temperature-dependent (Greenberg and Donch, 1974; Meyer et al., 1979), suggesting that the $\chi/SSB$ interaction is important for replication in vivo. However, since the SSB-Ct acts as a docking site
for more than a dozen genome maintenance proteins in *E. coli* (Shereda et al., 2008), the *ssb113* phenotype could be the result of multiple lost interactions rather than unique disruption of the χ/SSB complex. The χ encoding *holC* gene is not essential in *E. coli*, but *holC* mutants have temperature-sensitive viability and have higher rates of deletions in repetitive genomic sequences (Kelman and O'Donnell, 1995; Saveson and Lovett, 1997), consistent with its proposed role in replisome stability. Genetic interactions between χ/ψ and genes involved in DNA replication initiation (Nordman et al., 2007), recombinational repair (Flores et al., 2001; Kelman and O'Donnell, 1995), DNA replication restart, and SOS (Flores et al., 2002; Viguera et al., 2003) pathways suggest that the χ/ψ subcomplex may have functions outside of the elongation phase of DNA replication.

A second model for χ/ψ subcomplex function implicates the χ/SSB interaction as a step in the initiation of Okazaki fragment synthesis (Yuzhakov et al., 1999). This model arises from *in vitro* observations in which lagging-strand replication is blocked when χ is excluded from the Pol III HE or when SSB113 is substituted for SSB. Under either condition, primase binds stably to the primer/template and β2 loading is disallowed. This behavior is explained by invoking a hand-off mechanism in which χ destabilizes the primase/primer/template/SSB complex by competing with primase for binding to SSB (Yuzhakov et al., 1999). In this model, as primase is released from the primer/template, β2 and DNA polymerase are loaded onto the primed lagging strand. It is unclear, however, whether this model illustrates a required mechanism for lagging-strand polymerase loading, given the viability of *holC*-mutant *E. coli* (Kelman and O'Donnell, 1995; Saveson and Lovett, 1997). Moreover, since ψ activity is affected by the absence of χ (Kelman et al., 1998; Yuan and McHenry, 2009), indirect effects stemming from χ-deficient replication
are possible. Structural information on the χ/SSB interface would foster directed studies that define the function of the interaction in replication.

In this paper, structural, biochemical and genetic studies are combined to define the roles of the χ/SSB interface in DNA replication. The X-ray crystal structure of the *E. coli* χ/ψ subcomplex bound to a peptide comprising a portion of the SSB-Ct identifies the SSB binding site on χ. Based on the structure, χ variants with impaired SSB binding were created and tested for function. In contrast to the proposed essential role of the χ/SSB complex in handing off primers to the lagging-strand DNA polymerase, χ variants with destabilized SSB interactions *in vitro* support lagging-strand DNA synthesis in reconstituted *in vitro* replication assays. There are, however, three pronounced replication defects associated with the variants in elevated salt concentrations: leading- and lagging-strand replication is uncoupled, leading-strand polymerase primer engagement is blocked, and Okazaki fragments are shorter compared to wild-type (wt) Pol III HE. These findings are consistent with a role of χ/SSB complex formation in replisome stability and point to an unexpected function for the complex in establishing and maintaining coupled leading- and lagging-strand DNA replication. *E. coli* with SSB-binding-deficient *holC* mutations display temperature-sensitive growth, cell filamentation, chromosome partition defects, and SOS induction, which are consistent with significantly destabilized replication processes. Together, these studies define major roles for the χ/SSB interface in stabilizing the replisome and in coupling leading- and lagging-strand DNA synthesis at replication forks.
2.3 Results

2.3a X-ray crystal structure of the $\chi/\psi$/SSB-Ct ternary complex. Investigations into the role of the $\chi$/SSB complex in DNA replication have been limited by a lack of structural data defining the molecular basis of the interaction. A crystallographic effort was therefore undertaken to determine the structure of the *E. coli* $\chi/\psi$/SSB-Ct ternary complex. Crystallization attempts with *E. coli* $\chi/\psi$ bound to a peptide that includes the entire conserved SSB-Ct failed to produce crystals. However, crystals of $\chi/\psi$ bound to a shortened SSB-Ct peptide comprising the C-terminal-most four residues of *E. coli* SSB (with an N-terminal Trp residue added for quantification) formed readily and its structure was determined to 1.85-Å resolution (Figure 2.1B and Table 2.1). $F_o-F_c$ electron density maps revealed the position of a single SSB-Ct peptide associated with the $\chi$ surface (Figure 2.1 C).

The SSB-Ct-binding surface on $\chi$ is composed of conserved hydrophobic and charged residues (Figure 2.1 D-E). F177, the C-terminal-most SSB-Ct residue, appears to serve as an anchor in the interaction. Its side chain is bound in a hydrophobic pocket on $\chi$ while its $\alpha$-carboxyl group forms ionic bonds with the R128 side chain from $\chi$ (Figures 2.1 C-D). The more N-terminal residues on the SSB-Ct peptide are adjacent to a pair of basic residues in $\chi$ (K132 and R135), which could be important for ionic interactions with full-length SSB. A similar binding arrangement involving a hydrophobic pocket and ionic interactions has been observed in a complex formed between *E. coli* Exonuclease I and the SSB-Ct and has been proposed for *E. coli* RecQ (Lu and Keck, 2008; Shereda et al., 2009). Residues that form the SSB-binding site on *E.
*coli* \(\chi\) are well conserved among the \(\alpha\)-, \(\beta\)-, and \(\gamma\)-proteobacteria (Figure 2.1E), suggesting that the interaction scheme described in the structure is utilized in other bacterial species.

2.3b The \(\chi/SSB\) interface is critical for association in vitro. \(\chi\) variant proteins in which residues with apparent roles in SSB-Ct binding were substituted with alanine were purified and tested in SSB-binding experiments. Each of the \(\chi\) variants (R128A, K132A, and R135A) retained the ability to bind \(\psi\) in size-exclusion chromatographic experiments, indicating that overall folding was not dramatically altered by the sequence changes.

Changes in \(\chi/\psi\) variant binding to SSB were measured using isothermal titration calorimetry. In these experiments, heat generated by SSB binding to \(\chi/\psi\) was fitted using a single site model to determine the stability and stoichiometry of the interaction. At 25 °C, SSB binds \(\chi/\psi\) in a \(~1:1\) (SSB monomer: \(\chi/\psi\) heterodimer) complex with an apparent dissociation constant (K\(_d\)) of 8.9 +/- 0.7 µM (Figure 2.2); since SSB is a tetramer, this means that 4 \(\chi/\psi\) heterodimers can bind to each tetramer. These results are consistent with a previous calorimetry experiment performed under slightly different solution conditions (6.4 µM K\(_d\) and 1:1 \(\chi/SSB\) monomer stoichiometry (Kozlov et al., 2010b)). In contrast, R128A \(\chi/\psi\) failed to bind SSB (Figure 2.2). K132A and R135A \(\chi/\psi\) variants retained the ability to bind SSB but with reduced affinities (23.3 +/- 2.3 and 20.9 +/- 1.7 µM K\(_d\), respectively). These results match the structural model well; R128 is critical for binding SSB *in vitro* whereas K132 and R135 play more minor roles. Similar results were also observed in a qualitative co-precipitation experiment (Figure 2.3). These results are consistent with those from a recent study that predicted the SSB-binding site on \(\chi\) (Naue et al., 2010). The binding
affinities of wt and variant \( \chi/\psi \) complexes to SSB were essentially temperature independent between 25 and 40 °C (Figure 2.2). In contrast, wt \( \chi/\psi \) binding to SSB113 (an SSB variant with the penultimate proline substituted for serine) was temperature dependent as observed previously (Yuzhakov et al., 1999) (Figure 2.2). \( \chi \) variants that were destabilized for SSB interaction were used to assess the function of the interaction in replication.

2.3c \( \chi \) variants with SSB-binding deficiencies affect DNA replication in vitro. To measure the impact of destabilizing the \( \chi/\text{SSB} \) interface on DNA replication, \textit{E. coli} Pol III HE complexes were reconstituted by combining Pol III HE lacking \( \beta_2 \) (referred to as Pol III* (Wickner et al., 1973)) that included either wt \( \chi \) (wt Pol III*), R128A variant \( \chi \) (R128A \( \chi \) Pol III*), or R128E/K132E double charge reversal variant \( \chi \) (R128E/K132E \( \chi \) Pol III*) with \( \beta_2 \). R128E/K132E \( \chi \) Pol III* was included to test a more dramatically destabilized \( \chi \) variant than the R128A \( \chi \) protein, which may have some residual affinity for SSB in the context of the actively replicating replisome. Interestingly, SSB co-purified with wt Pol III*, but failed to co-purify with R128A \( \chi \) Pol III* or R128E/K132E \( \chi \) Pol III* (Figure 2.4), confirming the identification of the SSB binding site on \( \chi \). The specific primer extension activities of the three Pol III* preparations were nearly identical (Figure 2.4), indicating that each was functional.

Based on the proposed role of \( \chi \) in mediating the handoff of primers to the lagging-strand DNA polymerase (Yuzhakov et al., 1999), one prediction is that lagging-strand replication would be severely impaired or, perhaps, non-existent with R128A \( \chi \) Pol III* or R128E/K132E \( \chi \) Pol III*. Rolling circle DNA replication assays using a 7 kbp tailed form II (TFII) DNA template (Xu and Marians, 2000) were used to test this prediction. In these assays, replisomes formed on the TFII
DNA produce long leading-strand products and populations of shorter Okazaki fragments (Figure 2.5A). Okazaki fragment size is set by the frequency of primer synthesis by primase (DnaG) and the efficiency with which these primers are used to initiate lagging-strand synthesis. Therefore fragment size is inversely related to the concentration of DnaG in the reaction mixture (Zechner et al., 1992). In contrast to the predicted effect of the $\chi$ sequence change on lagging-strand DNA synthesis, all three Pol III* preparations supported leading- and lagging-strand DNA replication on these substrates and produced Okazaki fragments of roughly the same sizes (Figures 3B-D). Some reduction in leading-strand DNA synthesis was observed with R128E/K132E $\chi$ Pol III* (Figure 2.5B and D), but the complex was clearly function under the conditions tested.

The proposed function of the $\chi$/SSB interaction in stabilizing the replisome (Glover and McHenry, 1998; Kelman et al., 1998) was tested by carrying out rolling-circle DNA replication assays at a moderately elevated salt concentration (70 mM KCl) (Figures 3E-G). Under these conditions, both R128A $\chi$ Pol III* and R128E/K132E $\chi$ Pol III* were less active than wt Pol III* in DNA synthesis (Figure 2.5G). Surprisingly, the most dramatic effect was a reduction in the abundance of leading-strand products (Figure 2.5E). A more subtle effect on lagging-strand synthesis was also observed in which $\chi$-variant-supported DNA replication generated shorter Okazaki fragments than wt Pol III* (Figures 3E and 3F). The salt sensitivity of R128A $\chi$ Pol III* and R128E/K132E $\chi$ Pol III* strongly supports a role for the $\chi$/SSB interaction in replisome stability. In general, the observed replication defects are more pronounced in assays using R128E/K132E $\chi$ Pol III* than R128A $\chi$ Pol III*, consistent with the idea that the R128A $\chi$ may
have some residual affinity for SSB within the replisome that is further destabilized in the double charge-reversal R128E/K132E χ variant.

The impaired activities of R128A χ Pol III* and R128E/K132E χ Pol III* point to a previously unidentified function for the χ/SSB interaction in stabilizing leading-strand polymerase/primer/template complexes. The novel role was confirmed in a second assay in which oriC-dependent plasmid replication was measured in vitro (Figure 2.6). As with rolling-circle replication, leading-strand synthesis by R128A χ Pol III* and R128E/K132E χ Pol III* is impaired relative to wt Pol III* in a salt-dependent manner. Therefore, in two independent in vitro assays, the χ/SSB interaction appears to be important for replisome stability and the consequence of destabilizing the interface is impaired leading-strand DNA synthesis. When the replisome is reconstituted in the absence of χ or χ/ψ, the protein complex also exhibits salt sensitive replication (Glover and McHenry, 1998; Kelman et al., 1998), consistent with the observations reported here.

The activities of R128A χ Pol III* and R128E/K132E χ Pol III* appeared to be most diminished at low primase concentrations (Figure 2.5 and Figure 2.6). Earlier observations of leading-strand priming by primase (Heller and Marians, 2006) led to the postulation that interactions between primase and the replication machinery could help stabilize leading-strand polymerase/primer/template binding. Under primase-deficient conditions with moderate salt, rolling-circle replication by R128A χ Pol III* and R128E/K132E χ Pol III* is greatly diminished (Figure 2.5), reinforcing the notion that the χ/SSB interaction is needed for replisome integrity. However, high concentrations of primase appeared to stabilize leading-strand DNA synthesis in
moderate salt, possibly because primase/DnaB interactions stabilize binding of the leading-strand DNA polymerase to the replication machinery.

The appearance of Okazaki fragments without concomitant leading-strand synthesis implies that the leading-strand polymerase has either disengaged from the 3'-OH of the nascent leading strand or has become non-processive under the modest-salt conditions with the R128A and R128E/K132E χ variants. However, concerted unwinding of the duplex template with priming and synthesis of the lagging strand still occurs. The possibility that uncoupled unwinding of the template could be followed by a general priming-type reaction on the resulting ssDNA is unlikely because SSB, whose presence blocks general priming (Arai and Kornberg, 1979), would coat the ssDNA thereby preventing DNA synthesis. Whereas it is possible that DnaC810 (a DnaC protein variant that loads/reloads DnaB in the absence of DnaA and an origin of replication which are normally required for replication initiation) used in the rolling-circle replication assay could overcome the inhibitory effect of SSB (Figure 2.5), this cannot happen in the oriC reaction where wt DnaC is present (Figure 2.6). We therefore conclude that within the context of a replisome, the χ/SSB interaction is essential for sustained leading-strand synthesis under conditions of moderate salt concentrations.

2.3d Destabilizing the χ/SSB interface results in temperature-dependent cellular effects. To test the consequences of destabilizing the χ/SSB interaction in vivo, E. coli strains in which the χ structural gene (holC) was mutated to encode R128A, K132A, or R135A χ variant proteins (AHM102, AHM103, or AHM104, respectively) were constructed along with an isogenic wt χ
strain (AHM101). A second set of charge-reversal mutations was made to holC since the effects of charge reversal on DNA replication in vitro were somewhat more dramatic than charge neutralization (R128E χ (AHM105), K132E χ (AHM106), and R128E/K132E χ (AHM107)). As a first test, strain viability was measured by plating serial dilutions of saturated cultures on rich (Luria Broth) or minimal (MOPS) agar and grown at several temperatures (Figure 2.7 and Figure 2.8A). Surprisingly, each the holC E. coli strains encoding an Ala-substituted χ protein, including R128A χ, grew nearly as well as the isogenic wt strain. One possible explanation was the R128A sequence change is insufficient to block χ binding to SSB in vivo. Consistent with this model, holC E. coli strains encoding Glu-substituted χ proteins, which should repulse SSB more strongly than the Ala-substituted variants, manifested measurable defects. AHM107 growth was strongly impaired, with significantly smaller and/or fewer colonies formed at nearly all temperatures tested in either rich or minimal media (Figure 2.7 and Figure 2.8A). Consistent, but more modest, effects were also observed with AHM105. To ensure that the effects observed with AHM107 were not due to misfolding of the R128E/K132E χ variant, the protein was purified and tested for proper folding. The R128E/K132E χ variant maintains the ability to form a complex with ψ and is degraded in limited proteolysis experiments at the same rate as wt χ (Figure 2.9), indicating that it is properly folded. Additionally, the defects in AHM107 could be complemented by transformation with a plasmid encoding wt holC (Figure 2.10), indicating that the phenotype was not due to unanticipated problems in the strain outside of the R128E/K132E χ sequence change.

To examine how sequence changes in χ alter cellular function, the growth kinetics of AHM102 (R128A χ), AHM105 (R128E χ), AHM107 (R128E/K132E χ), and AHM101 (isogenic wt χ
control), were measured at 30 and 37 °C (Figure 2.7B and Figure 2.11). At 30 °C, AHM102 and AHM105 growth curves were indistinguishable from AHM101. AHM107 grew with a 1.4-fold slower generation time relative to the control strain. In contrast, at 37 °C, severe differences between AHM107 and the control strain became apparent. Unlike the wt cells, which had a brief lag phase, AHM107 took nearly 15 hours to transition to log phase. At either temperature, once AHM107 cultures reached log phase the generation time was 1.4-fold lower than the control strain. Thus, the majority of the temperature-dependence of the AHM107 growth characteristics arises from an extended lag phase. One possibility was that the holC mutation in AHM107 entirely blocks growth at 37 °C and that a suppressor mutation must be generated to allow entrance into lag phase. This possibility was excluded by re-diluting a culture of AHM107 cells that had been grown for 31 hours at 37 °C into fresh media and examining the culture’s growth kinetics. The culture had the same extended lag phase as AHM107 cells, indicating that heritable generic suppressors were not responsible for its eventual transition to log phase.

2.3e Destabilization of the χ/SSB interface modestly affects DNA replication rates in E. coli.

To test the effects of the χ mutants on DNA replication directly, in vivo DNA replication rates were measured in early log phase E. coli holC mutant cultures using a [3H] thymidine incorporation assay. AHM101 incorporation rates were ~2-fold higher than AHM107 at both 30 and 37 °C, consistent with reduced replication activity in the AHM107 χ variant (Figure 2.8C). This result parallels the 1.4-fold longer doubling time observed for AHM107 in log phase relative to the control strain (Figure 2.8B). A more modest defect was observed with AHM105 whereas AHM102 was indistinguishable from AHM101.
2.3f SOS induction and partitioning defects in holC mutant strains. Many temperature sensitive DNA replication mutants show increased levels of SOS induction and filamentation (Saveson and Lovett, 1997; Viguera et al., 2003). Defects in DNA replication may also lead to aberrant nucleoid structures and/or chromosome partitioning problems. To determine if holC mutations imparted increased SOS induction and defects in nucleoid structure and/or partitioning, their effects were assessed in an E. coli strain containing a psulA-gfp transcriptional fusion at the lambda attachment site (McCool et al., 2004; McCool and Sandler, 2001) and a hupA::mcherry translational fusion. In these strains, psulA-gfp expression is dependent upon SOS induction while hupA::mcherry expresses a mCherry-tagged version of the histone-like HU-2 protein to label genomic DNA. The strains were grown to mid-log phase in minimal media at 30, 37, or 42 °C; cell morphology, psulA-gfp expression (SOS induction), and mCherry staining (nucleoid structure) were then measured. Representative images are shown in Figure 2.12 with quantitative data from many images provided in Table 2.2. Cells encoding R128E/K132E χ exhibited temperature-dependent SOS induction and cell filamentation: at 30 °C, SOS was induced in 2.9% of cells and 1.0% of cells were filamentous, whereas at 37 and 42 °C, SOS levels increased (6.5 and 17.3%, respectively) as did filamentation (4.0 and 6.0%, respectively). The same cells also displayed apparent chromosome partitioning defects as evidenced by the diffuse nucleoid structure observed in filamented cells. The observed partition defect was dependent on RecA, as deletion of recA produced cells with more distinct nucleoids (Figure 2.12). This type of behavior has been seen previously with the Par- phenotype of priA mutants (McCool and Sandler, 2001). E. coli cells encoding the single-site variant R128E χ also exhibited temperature-dependent induction of SOS and cell filamentation, although the levels were reduced relative to the R128E/K132E χ double-mutant (Table 2.2).
isogenic wt holC, R128A, and K132E strains were all indistinguishable from each other, with no measurable increase in SOS or filamentation over the conditions tested. These results, in conjunction with the growth defects noted earlier, indicate that the interaction between χ and SSB is important for proper and efficient genome replication.

2.4 Discussion

The interaction between SSB and the cellular DNA replication machinery has been proposed to be important for replisome stability and for delivery of primers to the lagging-strand DNA polymerase throughout replication. These models have been tested using a combination of structural, biochemical and genetic experiments. The crystal structure of an SSB-Ct peptide bound to the χ/ψ replisomal subcomplex from E. coli was determined, allowing χ variant proteins with destabilized SSB binding sites to be created. In contrast to its proposed role in primer delivery, reconstituted Pol III HE that included χ variants with destabilized SSB binding sites supported robust lagging-strand DNA synthesis \textit{in vitro}. However, effects that were consistent with conditional destabilization of the replisome were observed with the variant Pol III HEs in modest salt conditions. These included uncoupled leading- and lagging-strand replication, obstruction of primer engagement by the leading-strand polymerase, and a reduction in the lengths of Okazaki fragments. In parallel with these observations, destabilization of the χ/SSB complex \textit{in vivo} produces cells with temperature-dependent SOS induction and cell filamentation along with growth and chromosome partitioning defects that are consistent with replisome instability and aberrant replication. These findings define a role for the χ/SSB complex
formation in replisome stability and point to its unexpected importance in establishing and maintaining coupled leading- and lagging-strand DNA replication.

2.4a Role of the χ/SSB interaction in DNA replication complexes. Previous experiments examining the role of the χ/SSB interface have been limited to studies in which χ or χ/ψ are omitted, or where SSB variant proteins with altered SSB-Ct sequences were used (Saveson and Lovett, 1997; Yuan and McHenry, 2009; Yuzhakov et al., 1999). Each of these approaches has limitations that arise from the multifunctional nature of the protein complexes involved. For example, omission of χ not only eliminates the interaction between the DnaX complex and SSB but it also has effects on ψ, which has limited solubility in the absence of χ (Xiao et al., 1993). Moreover, alteration of the SSB-Ct has effects on its interactions both with other proteins and with ssDNA (Downey and McHenry, 2010; Kozlov et al., 2010a; Kozlov et al., 2010b). These complications led us to take a structural approach to identify the SSB-Ct binding site on χ, which showed that χ relies on an electrostatic surface for interaction with SSB that is similar to sites found in other SSB-interacting proteins (Lu and Keck, 2008; Shereda et al., 2009). This information was used in subsequent biochemical and genetic experiments to test the effects of χ variants that can no longer bind SSB, but that retain the ability to form a complex with ψ.

Several biochemical and cellular defects were associated with sequence changes in χ that destabilized its interaction with SSB. These effects cumulatively point to a role for the χ/SSB interaction in stabilizing the DNA replication machinery in *E. coli*. One unexpected effect was the particularly strong impact of χ/SSB complex destabilization on leading-strand replication. In
both rolling-circle and oriC-dependent replication, extension of the leading strand by DNA polymerase was notably impaired under moderately elevated salt conditions when the χ/SSB interface was weakened (Figure 2.5 and Figure 2.6). This effect may be related to a previous observation in which SSB bound to the lagging-strand template stimulates strand displacement by DNA polymerase on the leading strand; this action is due to a network of interactions important for leading-strand polymerase processivity (Yuan and McHenry, 2009). Our results suggest that under modestly stringent conditions (elevated salt concentrations in vitro or high temperature in vivo), a similar network may be important for leading-strand replication in the context of the full replisome. The protein interactions in this arrangement could stabilize the replication machinery through both favorable thermodynamic interactions in the complex and architectural effects on the replication fork structure. Overall, this scheme would help coordinate the activities of the replisome on the leading- and lagging-strands.

2.4b Cellular importance of the χ/SSB interaction. Mutations in E. coli holC that destabilize the χ/SSB interface in vivo had severe temperature-dependent effects on growth rate, induction of SOS and filamentation, and chromosomal partitioning that appear to arise from conditionally impaired DNA replication. The growth kinetic defects are largely due to a temperature-dependent extension of the lag phase of cultures – the most impaired holC mutant strain had a 15-hour lag phase (~7-fold longer than the isogeneic wt strain) but only a ~2-fold reduction in DNA replication rates once log phase was reached. Several factors could potentially explain the extension of the lag phase. First, the differences could point to a defect in the ability to initiate DNA replication. Replication in vitro under restrictive conditions was characterized by
significant uncoupling of the leading- and lagging-strands that appeared to be primarily due to blockage of leading-strand primer extension (Figure 2.5 and Figure 2.6). Impacts of the destabilized χ/SSB interaction on leading-strand synthesis were apparent with both pre-primed templates (TFII DNA, Figure 2.5) and templates that had to be primed de novo (oriC, Figure 2.6). These results highlight the role of the interaction in leading strand synthesis. A molecular role for the χ/SSB interface in establishment of leading-strand synthesis is consistent with earlier genetic experiments that indicated χ could play an important role in DNA replication initiation (Nordman et al., 2007).

A second effect, which may contribute to the extended lag phase, stems from the observation that the mutant cells had strongly induced SOS, and cell filamentation and partitioning defects that reflect serious genome structural defects. The replisome, weakened by the loss of the χ/SSB interaction, may copy DNA with significantly less efficiency than wt cells, which could produce damaged chromosomal products that require time to be repaired. Similar DNA-damage-dependent delays in entry to log phase in E. coli have been described as a possible bacterial equivalent of a checkpoint that regulates the eukaryotic cell cycle (Murli et al., 2000).

Taken together, our observations have defined the structure of the χ/SSB interface and shown that this molecular contact is critical for replisomal stability and for productive leading-strand DNA synthesis. These studies help define the roles of interaction between the cellular DNA replication machinery and SSB bound to the lagging strand template. Similar interactions are likely to be important for replication in all organisms.
2.5 Experimental Procedures

Bacterial strains and primers:

Information on bacterial strains and primers used in this study is provided in Tables 2.3 and 2.4.

Protein overexpression and purification:

*E. coli* χ/ψ. The open reading frames of *holC* (encodes χ) and *holD* (encodes ψ) from *E. coli* K12 strain MG1655 were PCR amplified and subcloned into either pET15b (creating pET15-χ) or pET28b (creating pET28-ψ) using primers listed in Table 2.4. pET15-χ expresses an N-terminal hexahistidine tagged χ; pET28-ψ encodes untagged ψ with an inserted glycine after the start codon. Site-directed mutations of pET15-χ were generated by the QuikChange scheme (Stratagene). DNA sequencing confirmed the fidelity of the coding region of all plasmids.

BL21 DE3 cells transformed with pLysS (Novagen) and pET15-χ (or a pET15-χ derivative plasmid encoding a χ variant) were grown at 37 °C in LB medium supplemented with 100 µg/mL ampicillin (Ap) and 25 µg/mL chloramphenicol (Cm). One mM isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture at midlog phase (OD_{600nm} ~0.6) and growth was continued for 3 h to induce χ over-expression. Cells were centrifuged, suspended in χ-lysis buffer (50 mM Tris-HCl, pH 8.8, 0.5 M NaCl, 10 mM imidazole, 20% glycerol, 2 mM
phenylmethanesulfonyl fluoride (PMSF), 0.2 mM benzamidine) and lysed by sonication on ice. The lysate was centrifuged and the supernatant was incubated with nickel-sepharose resin (GE Healthcare) equilibrated in lysis buffer for 1 h. The resin was then packed in a column, washed with lysis buffer, and χ was eluted with high imidazole buffer (50 mM Tris-HCl, pH 8.8, 0.5 M NaCl, 300 mM imidazole, 20% glycerol). The eluent was dialyzed against 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.0, 150 mM NaCl, 10 mM 2-mercaptoethanol, 20% glycerol, diluted 1:1 with low-salt buffer (20 mM MES, pH 6.0, 50 mM NaCl, 10% glycerol), and loaded onto a S-Fastflow ion-exchange column (GE Healthcare) equilibrated in low-salt buffer. χ-enriched fractions were eluted with a NaCl gradient, pooled, concentrated, and dialyzed against 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol. The histidine tag was cleaved by thrombin and χ was dialyzed against storage buffer (50% glycerol, 15 mM dithiothreitol (DTT), 500 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA)).

E. coli ψ was purified as described previously (Xiao et al., 1993), with minor exceptions. BL21 DE3 cells transformed with pLysS and pET28-ψ were grown in LB medium supplemented with 50 µg/mL kanamycin (Km) and 25 µg/mL Cm. IPTG (1 mM) was added to the culture at midlog phase (OD600 nm ~0.6) and growth was continued for 3 h to induce ψ over-expression. Cells were centrifuged, suspended in ψ-lysis buffer (50 mM Tris-HCl, pH 7.5, 10% sucrose, 10 mM EDTA, 1 mM PMSF) and lysed by sonication on ice. The lysate was clarified by centrifugation and the insoluble fraction was resuspended in low-salt ψ buffer (20 mM Tris-HCl, pH7.5, 20% glycerol, 0.5 mM EDTA, 2 mM DTT, 10 mM NaCl), sonicated on ice and centrifuged. The pellet was resuspended in high-salt ψ buffer (20 mM Tris-HCl, pH 7.5, 20% glycerol, 0.5 mM EDTA, 2 mM DTT, 1 M NaCl), centrifuged, then the pellet resuspended with
low-salt $\psi$ buffer and centrifuged. $\psi$ was solubilized from the pellet in urea buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 2 mM DTT, 6 M Urea), clarified by centrifugation and the supernatant was loaded onto Q-Fastflow column (GE Healthcare) equilibrated in QFF buffer A (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 2 mM DTT, 6 M Urea). $\psi$ was eluted over by a salt gradient from 0 to 750 mM NaCl.

The $\chi/\psi$ complex was made by mixing $\psi$ and $\chi$ in a 1:1 molar ratio and resolving the complex on a Sephacryl S-100 column in 5% MPD, 10 mM Tris-HCl pH 8.8, 10 mM imidazole, and 100 mM NaCl. $\chi/\psi$-containing fractions were pooled, concentrated, and stored at 4 °C.

**E. coli SSB.** The plasmids for over-expression of *E. coli* SSB and SSB113 were gifts from Michael Cox. SSB and SSB113 protein was purified as in (Lohman et al., 1986).

**SSB-Ct peptide.** SSB-Ct peptide (Trp-Asp-Ile-Pro-Phe) was synthesized and purified by the University of Wisconsin Biotechnology Center.

**Pol III*.** Approximately 900 ml cell suspension (about 450 g of cells) of BLR(pHOC 2.6.1) (a gift of C. McHenry, University of Colorado) or variants in which the *holC* open reading frame was mutated to encode R128A or R128E/K132E $\chi$ was thawed at 4 °C. The density of the suspension was adjusted to OD$_{595}$ of 200 by the addition of 50 mM Tris-HCl, pH 8.0 at 4 °C,
10% sucrose. The suspension was brought to 20 mM EDTA, 150 mM NaCl, 20 mM spermidine, and 5 mM DTT, followed by the addition of solid Tris base to adjust the pH to 8.5. Cells were lysed by the addition of lysozyme (200 µg/ml) and incubated for 50 min at 4 °C, 4 min at 37 °C, then another 10 min at 4 °C. After centrifugation for 90 min at 11,000 rpm, the supernatant (Fraction 1) was made 0.07% in Polymin P by the dropwise addition of a 1% solution. The mixture was cleared by centrifugation for 30 min at 14,500 rpm. The protein was precipitated by the addition of 0.226 g/ml of solid ammonium sulfate, and the pellet was collected by centrifugation for 45 min. The protein was back extracted with 1/10 volume Buffer A (50 mM Tris-HCl, pH 7.5 at 4 °C, 5 mM DTT, 1 mM EDTA, 20% glycerol) + 100 mM NaCl containing 0.2 g/ml ammonium sulfate, centrifuged for 30 min at 20,000 rpm, then back extracted again with 1/40 volume Buffer A + 100 mM NaCl containing 0.17 g/ml ammonium sulfate. The pellet was resuspended in 1/80 volume 25 mM Tris-HCl, pH 7.5 at 4 °C, 5 mM DTT, 100 mM NaCl, 5% glycerol to give fraction 2. The activity was determined by general priming assay as described (Tougu et al., 1994) (Figure 2.4). A portion of fraction 2 (27.5%) was diluted with buffer A to adjust the conductivity to match Buffer A + 30 mM NaCl, and applied to a 10 ml Heparin-agarose column that had been equilibrated previously with Buffer A + 30 mM NaCl. The column was then washed with 25 ml of equilibration buffer, and protein eluted with a 100 ml gradient of 30 to 400 mM NaCl in Buffer A. Active fractions (eluting at approximately 200 mM NaCl) were pooled (fraction 3) and precipitated by the addition of an equal volume of 100% saturated ammonium sulfate. The pellet was resuspended in 400 µl Buffer A + 100 mM NaCl and filtered through a 24 ml Superose 6 column (Amersham) equilibrated and developed with Buffer A + 100 mM NaCl. Active fractions were pooled (fraction 3), and 100% glycerol was
added to increase the final concentration to 38% and stored at -80 °C. Other replication enzymes (E. coli DnaA, DnaB, DnaC, β₂, and gyrase) were purified as described in (Wu et al., 1992a).

**Crystallization and structure determination of χ/ψ/SSB-Ct:**

E. coli χ/ψ (7.5 mg/ml in 1.5% MPD, 3 mM Tris-HCl, pH 8.8, 3 mM imidazole, and 30 mM NaCl) was mixed 1:1 (vol) with well solution (25% PEG 4000, 0.2 M ammonium sulfate, 0.1 M sodium acetate, pH 4.6) in hanging-drop vapor-diffusion crystallization trials to form apo χ/ψ crystals used for seeds in generating χ/ψ/SSB-Ct crystals. χ/ψ (7.5 mg/ml) combined with SSB-Ct peptide (1:2 molar ratio of χ/ψ:SSB-Ct) was mixed with an equal volume of well solution (13-20% PEG 4000, 0.2 M ammonium sulfate, 5% glycerol, 0.1M sodium acetate pH 4.4) in hanging-drop vapor-diffusion crystallization trails. Apo χ/ψ crystals were streak seeded into the χ/ψ/SSB-Ct drops to generate ternary complex crystals. χ/ψ/SSB-Ct crystals were transferred into cryoprotectant (13-20% PEG 4000, 0.2 M ammonium sulfate, 25% glycerol, 0.1 M sodium acetate, pH 4.4) and flash frozen in liquid nitrogen. Diffraction data were indexed and scaled using HKL2000 (Otwinowski and Minor, 1997). The χ/ψ/SSB-Ct structure was determined by molecular replacement, using Phaser (McCoy et al., 2007) with the previously determined χ/ψ structure (Gulbis et al., 2004) as a search model. Iterative model building and refinement with Coot (Emsley and Cowtan, 2004) and REFMAC (Murshudov et al., 1997), produced the final model. Model coordinates and structure factors have been deposited in the Protein Data Bank (3SXU accession code).
Isothermal Titration Calorimetry:

*E. coli* χ/ψ (or a χ/ψ subcomplex with a χ variant) (30 µM) and SSB (or SSB113) (600 µM as monomer) were dialyzed against 20 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol. χ/ψ was thermostatted in a stirred sample cell (2 ml) at either 25 °C or 40 °C and SSB or SSB113 was titrated with the following regime: 1 injection (1 µl for 2 seconds), 4 injections (4 µl for 8 seconds each), then 32 injections (8 µl for 16 seconds each). Data were fitted to a single-site model (1 SSB monomer binding to 1 χ/ψ subcomplex) using a nonlinear iterative least squares algorithm (MicroCal ORIGIN). Since SSB is a tetramer, the binding arrangement in solution is 4 χ/ψ subcomplexes per SSB tetramer.

**In vitro DNA replication assays:**

TFII DNA was prepared and purified by sucrose gradient centrifugation as described (Wu et al., 1992a) using the primer TFII annealed to M13mp18 phage DNA. The first 35 nucleotides of the TFII primer forms the tail and the other 35 nucleotides anneal to the DNA. Rolling circle DNA replication reaction mixtures (10 µl) containing 50 mM Hepes-KOH (pH 8.0), 12 mM Mg(OAc)₂, 10 mM DTT, 40 µM [α-³²P]dATP (1000-3000 cpm/pmol), 40 µM dGTP, dCTP, and TTP, 200 µM GTP, CTP, and UTP, 1 mM ATP, 4 nM TFII DNA, 400 nM DnaC810, 360 nM DnaB, 20 nM Pol III* (or a Pol III* variant), 30 nM β, 250 nM SSB (tetramer), and the indicated concentrations of KCl and DnaG were incubated at 37 °C for 5 min as described (Wu et al., 1992a). Reactions were terminated by the addition of EDTA to 20 mM and analyzed by electrophoresis through 0.6% denaturing alkaline agarose gels using 30 mM NaOH-2 mM EDTA.
as the electrophoresis buffer. OriC replication reaction mixtures (10 µl) containing 80 mM Hepes-KOH pH 8.0, 10 mM Mg(OAc)$_2$, 10 mM DTT, 100 µg/ml BSA, 40 µM [$\alpha$-$^{32}$P]dATP (1000-3000 cpm/pmol), 40 µM dGTP, dCTP, and TTP, 200 µM GTP, CTP, and UTP, 2 mM ATP, 2 nM oriC plasmid DNA, 500 nM DnaA, 200 nM DnaC, 200 nM DnaB, 20 nM Pol III*, 30 nM $\beta_2$, 250 nM SSB (as tetramer), 20 nM DNA gyrase, and the indicated concentrations of KCl (0 or 70 mM) and DnaG (0 to 1980 nM) were incubated at 37 °C for 10 min as described in (Hiasa & Marians, 1994). Reactions were terminated and analyzed as for the rolling circle reaction.

**Construction of holC mutant E. coli:**

All bacterial strains used in this work are derivatives of *E. coli* K12 and are described in Table 2.3. All P1 transductions were selected on 2% agar plates containing minimal media + 0.1% glucose. Where appropriate, plates also contained the following antibiotics at these final concentrations: tetracycline 10 µg/ml, Cm 25 µg/ml, or Km 50 µg/ml. All transductants were purified on the same type of media on which they were selected, at 30°C for AHM derivatives.

*E. coli* strain AHM101 was constructed by PCR amplification of holC (including 120 bp 5’ of the open reading frame) and subcloned into pET15b. The Cm-resistance (Cm$^R$) cassette from pKD13 was subcloned 5’ of the holC to create pET15b-AHM101. Site-directed mutagenesis of pET15b-AHM101 was used to create pET15b-AHM102-107. The Cm$^R$-holC element of each
plasmid were independently electroporated into *E. coli* strain MG1655 with pKD46 via the Wanner method, and recombinants were selected by plating on Cm-containing media (Datsenko, 2000). Genomic insertions were verified by colony PCR and confirmed by sequencing.

The *hupA::mcherry* linear DNA fragment was generated by amplifying genomic DNA by PCR using primers listed in Table 2.4 for the Km-resistance cassette containing FRT sites from the same strain. Both linear fragments were then purified using the QIAEX II kit (Qiagen). Purified DNA fragments were then combined in a primerless PCR followed by a third PCR using primers extending into a single linear fragment containing *mcherry* and Km resistance. The linear *hupA100::mcherry::kan* fragment was then transferred to the chromosome by transformation of SS4414 competent cells containing plasmid pKD46 (Datsenko, 2000; Datsenko and Wanner, 2000). Km-resistant colonies were purified and screened for Ap sensitivity, indicating loss of pKD46. Cells were then screened microscopically for red nucleoids and *sulAp-gfp* expression.

To test for wild type *hupA* activity, this mutation was combined *del(hupB)::Tn10*. *hupA hupB* double mutants have diffuse nucleoids, chromosome partitioning defects and are highly filamented where single mutants appear essentially wild type (Dri et al 1991). The *hupA100::mcherry::kan del(hupB)::Tn10* double mutants appeared similar to wild type (data not shown). Plasmid copies of *hupA::gfp* have been shown to give the same pattern of nucleoids as cells stains with DAPI (Wery et al, 2001). The resulting strain with *hupA100::mcherry::kan* was called SS6279.
Phenotypic analysis of holC mutant E. coli:

Saturated cultures of AHM101-AHM107 strains were serially diluted (10-fold) in 145 mM NaCl and 10 µl from each dilution was spot plated onto LB or minimal media Cm agar and incubated at 25, 30, 37, or 42 °C to test for changes in colony morphology and viability. For experiments with minimal media agar, the strains were washed once in MOPS media prior to dilution.

Growth curves were performed starting with saturated cultures grown at 37 °C in LB Cm from a single colony from freshly streaked cultures. Saturated cultures (5 µl) were added to 50 ml of LB or minimal media (5 µl) with Cm. The cultures were then placed at 30 or 37 °C, with shaking. Growth was monitored with colony counts using 10-fold serial dilutions in 145 mM NaCl followed by plating on LB cat and growth at 37 °C. Data are the mean of three independent replicates with error bars representing one standard deviation of the mean. For minimal media growth curves, the saturated cultures were washed with MOPS prior to being resuspended in MOPS. Generation time was calculated using the colony counts during the log phase of cell growth with the equation $t_{\text{gen}} = 1/k$; $k = (3.32 \times \log_{10} \left[ N_{t2} / N_{t1} \right]) / (t_2 - t_1)$ where k is the growth rate constant, $t_2$ is the time when the population is $N_{t2}$ and $t_1$ is the time when the population is $N_{t1}$.
DNA replication rate determination:

Strains were grown at 37 °C in minimal media as described above until the OD$_{600}$ reached 0.3, at which time DNA synthesis was measured by measuring [$^3$H] thymidine incorporation as described in (Courcelle and Courcelle, 2006) except that the label solution was composed of MOPS media with 0.1% glucose and 5 µCi/ml of [$^3$H] thymidine. Data are the mean of three independent replicates of each strain with error bars representing one standard deviation of the mean.

Microscopy:

Cultures were grown in MOPS minimal medium until mid-log phase (OD$_{600}$ = 0.3-0.4). Cells were concentrated 10-fold in MOPS minimal and approximately 2µl of this mixture was loaded onto fresh MOPS minimal 1% agarose pads and a cover slip was applied. Agarose pads were prepared using a protocol from P. Levin. Microscopy was carried out using an epifluorescent Nikon E600 microscope. An ORCA-ER-cooled charge-coupled device (CCD) camera (Hammamatsu) and Openlabs software (Improvision) were used for all image acquisition. The exposure time was 300 ms. Approximately nine fields (three on three different days) containing calibration beads were photographed. A phase-contrast image and two fluorescent images (green and red) of each field were taken. Openlab 5.0 and Volocity 3.5 (Improvision, Inc.) were used to measure the amount of fluorescence and cell size in individual live cells. Calibration of the fluorescence intensity was set by calibration beads (InSpeck Green (505/515) microscope image intensity calibration kit 2.5 µm I-7219 from Molecular Probes). The relative fluorescence
intensity value of an individual cell is calculated by dividing the average calibrated pixel value of a particular cell by the average calibrated pixel value of a strain containing Δattλ::sulAp Ωgfp-mut2 (SS8343).
Figure 2.1
Figure 2.1. Structure of the *E. coli* χψ/SSB-Ct complex.  (A) Model of the *E. coli* replisome. SSB tetramers are depicted as light blue spheres; SSB-Ct elements (thin black lines) are omitted from all but one tetramer for clarity. (Inset) Close up of the lagging-strand/replisome interface; SSB-Ct elements from one SSB tetramer are illustrated with the sequence of the χ-bound tail provided.  (B) Ribbon diagram of the crystal structure of *E. coli* χ/ψ (green/red) in complex with the SSB-Ct peptide (light blue).  (C) Ribbon diagram depicting the SSB-binding site on χ. Key residues are labeled and represented as sticks.  F₀-Fc omit electron density for the SSB-Ct is shown.  (D and E) Surface representations of the χ/SSB interface depicting χ electrostatics [D, electropositive (blue) and electronegative (red)] or evolutionary conservation [E, invariant (red) and highly conserved (salmon) residues shared among 50 identifiable χ homologs].
Table 2.1. Diffraction data and crystal structure solution

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<td>$I/\sigma$ (last shell)</td>
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<td>rms deviation bond angles, °</td>
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<tr>
<td>Ramachandran statistics (% most favored/allowed/additionally allowed/disallowed)</td>
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† $R_{\text{sym}} = \Sigma \Sigma |I_j| - <|I|> |\Sigma I_j$, where $I_j$ is the intensity measurement for reflection $j$ and $<|I|>$ is the mean intensity for multiply recorded reflections.

‡ $R_{\text{work}}/R_{\text{free}} = \Sigma ||F_{\text{obs}}| - |F_{\text{calc}}||/|F_{\text{obs}}|$, where the working and free R factors are calculated by using the working and free reflection sets, respectively. The free R reflections (5% of the total) were held aside throughout refinement.
Figure 2.2.
Figure 2.2. Identification of \( \chi \) variants with compromised SSB binding. (A) Calorimetric analysis of the \( \chi/\psi/\text{SSB} \) interaction for wt \( \chi/\psi \) (left) and R128A \( \chi/\psi \) (right). Heats evolved from titration of SSB into \( \chi/\psi \) solutions are shown in the top panels; derived binding isotherms are shown in the bottom panels. (B) Summary of calorimetry experiments. A single site model was used to fit the data. “N” is the number of SSB monomer sites per \( \chi/\psi \) subcomplex; “nd” denotes when no binding was detected. Since SSB is a tetramer, 4 \( \chi/\psi \) subcomplexes bind to each SSB tetramer when \( N = 1 \).
Figure 2.3

<table>
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<tr>
<th>χψ variant</th>
<th>WT</th>
<th>WT</th>
<th>R128A</th>
<th>K132A</th>
<th>R135A</th>
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<tbody>
<tr>
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<td>+</td>
<td>-</td>
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<td>+</td>
<td>+</td>
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<td>3</td>
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<tr>
<td>Lane 2</td>
<td>3</td>
<td>4</td>
<td>5</td>
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</table>
Figure 2.3. Identification of χ variants with compromised SSB binding via co-precipitation. *E. coli* SSB precipitates in low concentrations of ammonium sulfate that are insufficient to precipitate most other proteins; however, SSB-associated proteins, such as χ/ψ, coprecipitate with SSB in these conditions (Genschel et al, 2000; Shereda et al, 2007). *E. coli* SSB (20 μM tetramers), χ/ψ (or a χ/ψ subcomplex with a χ variant) (20 μM dimers), or a mixture of the two were mixed in 10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 10% v/v glycerol on ice for 15 minutes. Reactions were adjusted to 150 g/L ammonium sulfate, incubated on ice for an additional 15 minutes, then centrifuged for 1 minute at 13,000 RPM. Supernatant was removed and pellets were washed three times with 50 μl of co-precipitation buffer plus 150 g/liter ammonium sulfate. Pellet fractions were resolved by SDS-PAGE. Consistent with other SSB interacting proteins, the wild type *E. coli* χ/ψ subcomplex precipitates in this assay in an SSB dependent manner (compare lanes 2 and 3). Among the χ variants, the R128A variant had only background levels of χ/ψ co-precipitated with SSB (lane 4). In contrast, the K132A, and R135A χ sequence changes had little or no effect on SSB co-precipitation. These results suggested that Arg128 in χ plays a critical role in stabilizing the χ/SSB interface.
Figure 2.4

A

B

[\text{\textsuperscript{32}P} \text{dAMP incorp.} (pmol)]

[\text{[Pol III\textsuperscript{*}]} (nM)]

\begin{itemize}
  \item Pol III\textsuperscript{*}
  \item Pol III\textsuperscript{*} R128A\scriptstyle\chi
  \item Pol III\textsuperscript{*} R128E/K132E\scriptstyle\chi
\end{itemize}
Figure 2.4. Purified Pol III* preparations and activity. (A) Five µg of fraction 3 wt Pol III*, R128Aχ Pol III* and R128E/K132E χ Pol III* were analyzed by SDS-PAGE through 8%-20% gradient gels. Gels were stained with Coomassie Brilliant Blue and images were obtained using a Kodak 4000 rPro Image Station. Note the presence of SSB in the wt Pol III* but not in the R128Aχ or R128E/K132E Pol III* preparation. (B) The activity of the Pol III* preparations was compared in general priming reactions as described under Experimental Procedures.
Figure 2.5

(A) 

$5'$ -> 7 kb

TFII

$\rightarrow$ Pol III*, $\beta$, SSB, DnaB/C, DnaG (0-1890 nM)

[\(\alpha^{32}\text{P}]dATP, \text{other dNTPs and all NTPs}\)

(B) No Salt

<table>
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<tr>
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<th>WT</th>
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<th>R128E/K132E$x$</th>
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<tbody>
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<td>1890</td>
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<tr>
<td>[DnaG] (nM)</td>
<td>118</td>
<td>1890</td>
<td>118</td>
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</table>

(C) Okazaki fragment size (nt)

(D) [\(\alpha^{32}\text{P}]dAMP incorp. (pmol)

(E) 70 mM KCl

<table>
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<tr>
<td>[DnaG] (nM)</td>
<td>118</td>
<td>1890</td>
<td>118</td>
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(F) Okazaki fragment size (nt)

(G) [\(\alpha^{32}\text{P}]dAMP incorp. (pmol)
Figure 2.5. **Conditional rolling-circle DNA replication by the R128A χ Pol III HE.** Rolling circle DNA replication reactions (schematically outlined in (A)) were catalyzed by wt, R128A χ, or R128E/K132E Pol III HE in the absence of added salt (B-D) or with 70 mM KCl added (E-G). Primase (DnaG) was titrated in separate reactions from 0 to 1890 nM. (B and E) Alkaline agarose gel electrophoretic analysis of the replication products. Template (temp) that becomes labeled in the reaction but does not support rolling circle replication is indicated, as are leading-strand (lead) and lagging-strand (lag) DNA products. (C and F) Average Okazaki fragment size as a function of primase concentration. (D and G) Total DNA synthesis as a function of primase concentration. Equal amounts of radioactivity were applied in each lane of the gels shown in panels B and E.
Figure 2.6

A

No Salt

<table>
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<th>R128E/K132E&lt;sub&gt;x&lt;/sub&gt;</th>
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<tr>
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<td>0.16</td>
<td>0.16</td>
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<tr>
<td>2.54</td>
<td>2.54</td>
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[Graph showing DNA gel electrophoresis with markers labeled as lead and lag.]

B

70 mM KCl

<table>
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<tr>
<td>2.54</td>
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[Graph showing DNA gel electrophoresis with markers labeled as lead and lag.]
**Figure 2.6.** R128Aχ and R128E/K132E χ Pol III* exhibit uncoupled leading- and lagging-strand synthesis at moderately elevated salt concentrations during oriC-dependent DNA replication. DNA replication reactions supported by an oriC plasmid DNA template in the presence of wild-type, R128A χ, or R128E/K132E χ Pol III* and either in the absence of added salt (A) or in the presence of 70 mM KCl (B) were performed and analyzed as indicated under Experimental Procedures.
Figure 2.7.
Figure 2.7. *E. coli holC* variant growth at various temperatures. *E. coli holC* strains spot plated on to rich (left) or minimal (right) media were incubated at the indicated temperatures.
Figure 2.8.
Figure 2.8. Growth and DNA replication in *E. coli holC* mutant strains.  (A) Saturated cultures of *E. coli holC* strains spot plated on minimal media were incubated at 30 or 37 °C for 48 or 24 hours respectively.  (B) Strains were diluted from a saturated culture and grown in minimal media at 30 °C (top) or 37 °C (bottom) and the number of colony forming units (cfu) per ml of culture were counted over time. Error bars represent one standard deviation from the mean. (C) Relative incorporation of [³H] thymidine by strains (each at OD₆₀₀ = 0.3) in minimal media at either 30 or 37 °C. The cells were grown in MOPS minimal medium to OD₆₀₀ ~0.3, individual aliquots of culture were removed at the time indicated and incubated with a labeling solution containing [³H] thymidine. The [³H] thymidine incorporation was stop after 2 minutes and the counts per minute (cpm) were measured in a scintillation counter. The cpm were normalized based on the amount of incorporation in the wt holC strain at each temperature. Error bars represent one standard deviation from the mean.
Figure 2.9
Figure 2.9. χ R128E/K132E variant is properly folded. (top) χ/ψ subcomplexes with either wildtype or R128E/K132E χ were resolved on a size exclusion column. Both complexes elute with the same retention volume. (bottom) Both complex are degraded by proteases with similar kinetics and breakdown products.
Figure 2.10.
Figure 2.10. A plasmid-born copy of wild-type *holC* complements the slow-growth phenotype of AHM107 cells. AHM107 was transformed with a plasmid containing wild-type *holC*, spot plated onto rich media and grown at the indicated temperatures. AHM101 (isogenic wild-type *holC* cells) and AHM107 controls were included for comparison.
Figure 2.11
Figure 2.11. Growth curves of *holC* strains. *E. coli holC* strains AHM101 (wild-type isogenic control), AHM102 (R128Aχ), AHM105 (R128E), and AHM107 (R128E/K132E χ) were grown in minimal media at 30 °C (top) or 37 °C (bottom) and the number of cfu per ml of culture were counted over time in triplicate. Error bars represent one standard deviation of the mean.
Figure 2.12

(A) Phase Contrast  hupA::mcherry  psulA-gfp  Merge

pepA::cat

pepA::cat holC R128E K132E

30 °C

pepA::cat holC R128E K132E

pepA::cat holC R128E K132E del(recA)100

37 °C

(B) pepA::cat holC R128EK132E  pepA::cat holC R128EK132E del(recA)100

37 °C
Figure 2.12. Filamentation and SOS induction in *E. coli* holC mutant strains. (A) Cells were grown at 30 or 37 °C as indicated in MOPS minimal medium. Fields of cells were imaged to show cell morphology (phase contrast), nucleoid structure (*hupA::mcherry*) or SOS induction (*sulAp::gfp*). Bright green spots are beads used for quantitation. (Inset) Close-up images of cells. (B) Close-up of *hupA::mcherry* of the strains indicated grown at 37 °C.
Table 2.2 Temperature-dependent cellular effects of *holC* mutations in *E. coli*

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Strain</th>
<th><em>holC</em></th>
<th><em>pepA</em></th>
<th>Relative fluorescence intensity</th>
<th>% SOS induced</th>
<th>% filamented</th>
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<tbody>
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<td><strong>30 °C</strong></td>
<td>SS8343</td>
<td>+</td>
<td>+</td>
<td>1.0 ± 0.0</td>
<td>0.4 ± 0.6</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>SS8383</td>
<td>+</td>
<td><em>cat</em></td>
<td>1.9 ± 0.8</td>
<td>1.4 ± 1.0</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>SS8384</td>
<td><em>R128A</em></td>
<td><em>cat</em></td>
<td>1.7 ± 0.7</td>
<td>1.9 ± 1.2</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>SS8385</td>
<td><em>R128E</em></td>
<td><em>cat</em></td>
<td>1.4 ± 0.5</td>
<td>1.0 ± 0.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>SS8386</td>
<td><em>K132E</em></td>
<td><em>cat</em></td>
<td>1.3 ± 0.9</td>
<td>1.0 ± 0.7</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>SS8387</td>
<td><em>R128E</em></td>
<td><em>K132E</em></td>
<td>2.0 ± 0.7</td>
<td>2.9 ± 2.2</td>
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<td><strong>37 °C</strong></td>
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<tr>
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<td>+</td>
<td><em>cat</em></td>
<td>1.0 ± 0.3</td>
<td>0.6 ± 0.2</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>SS8384</td>
<td><em>R128A</em></td>
<td><em>cat</em></td>
<td>1.1 ± 0.7</td>
<td>1.0 ± 0.8</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
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<td><em>R128E</em></td>
<td><em>cat</em></td>
<td>1.4 ± 0.6</td>
<td>2.3 ± 1.6</td>
<td>1.4</td>
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<td><em>K132E</em></td>
<td><em>cat</em></td>
<td>1.1 ± 0.1</td>
<td>1.0 ± 0.7</td>
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<tr>
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<td>SS8383</td>
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<td><em>cat</em></td>
<td>1.1 ± 0.6</td>
<td>0.3 ± 0.4</td>
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<td>0.5 ± 0.1</td>
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<td>4.2 ± 1.5</td>
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<td></td>
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<td><em>K132E</em></td>
<td><em>cat</em></td>
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<td>0.6 ± 0.3</td>
<td>0.3</td>
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<tr>
<td></td>
<td>SS8387</td>
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Table 2.3 Strains used in this study

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<th>Strain</th>
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<th>Other relevant genotype</th>
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<tr>
<td>MG1655</td>
<td>+</td>
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<td>Lab stock</td>
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<tr>
<td>AHM101</td>
<td>+</td>
<td>Cm</td>
<td>This study(^a)</td>
</tr>
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<td>AHM102</td>
<td>R128A</td>
<td>Cm</td>
<td>This study(^a)</td>
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<tr>
<td>AHM103</td>
<td>K132A</td>
<td>Cm</td>
<td>This study(^a)</td>
</tr>
<tr>
<td>AHM104</td>
<td>R135A</td>
<td>Cm</td>
<td>This study(^a)</td>
</tr>
<tr>
<td>AHM105</td>
<td>R128E</td>
<td>Cm</td>
<td>This study(^a)</td>
</tr>
<tr>
<td>AHM106</td>
<td>K132E</td>
<td>Cm</td>
<td>This study(^a)</td>
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<td>AHM107</td>
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<td>Cm</td>
<td>This study(^a)</td>
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<tr>
<td>SS1465</td>
<td>+</td>
<td>gal76::Tn10 del(attB)::psulA-gfp</td>
<td>Centore et al (2009)</td>
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<tr>
<td>SS4223</td>
<td>+</td>
<td>del(pyrB)10b(^b)::kan</td>
<td>Datsenko and Wanner (2000)</td>
</tr>
<tr>
<td>SS4414</td>
<td>+</td>
<td>lexA3 malE::Tn10-9</td>
<td>Centore et al (2009)</td>
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<td>hupA100::gfp-901</td>
<td>Lab stock</td>
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<td>SS6268</td>
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<td>SS6279</td>
<td>+</td>
<td>hupA100::mcherry</td>
<td>Lab stock(^a)</td>
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<td>SS7073</td>
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<td>SS1465 → MG1655(^c)</td>
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<tr>
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<tr>
<td>SS7117</td>
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<tr>
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<tr>
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<td>+</td>
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<td>SS4223 →</td>
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<tr>
<td>Strain</td>
<td>Mutation</td>
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<td>gal76::Tn10 del(attB)::psulA-gfp pepA::cat</td>
<td>AHM107 → SS8377e</td>
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a See Materials and Methods for details on strain construction.
b The 100 allele (from the Keio collection (Datsenko and Wanner 2000)) essentially replaces the gene in question with a kan gene between two FRT sites. The 200 allele is a result of removing the kan gene with FLP recombinase, induced by shift to 42°C and expressed from the chloramphenicol/ampicillin resistance plasmid pCP20 (Datsenko and Wanner 2000). The strain was then grown at 37°C and Kanstr Cam/Amps strains were screened.
c Select for tetracycline resistance. Screen for GFP.
d Select for kanamycin resistance. Screen by PCR if necessary.
e Select pyrB+ on minimal media plates in the absence of uracil. Screen chloramphenicol resistance, PCR if necessary.
Table 2.4 Primers used in this study.

<table>
<thead>
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<th>Primer name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>χ F <em>(holC</em> for overexpression)</td>
<td>GCCAATCATATGAAAAACGCACGTTCTACCTT</td>
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<tr>
<td>χ R <em>(holC</em> for overexpression)</td>
<td>CGCGGATCCTTTATTTCCAGGTGTTGCCGTATTACAG</td>
</tr>
<tr>
<td>ψ F <em>(holD</em> for overexpression)</td>
<td>GCGCCATGGGCACTCCCGAGAGACTGGCAGTGA</td>
</tr>
<tr>
<td>ψ R <em>(holD</em> for overexpression)</td>
<td>GCGCTCGAGTCAGTCTTCTGAGGGAAGAAATC</td>
</tr>
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<td>TF II</td>
<td>TTAGAGAGTTACCGATAGCTAGCTGCAATGATGTCAATAA CCGTGTATAGCTATTTTCATTTGGG</td>
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<td>P-69 F <em>HolC R128A SDM</em></td>
<td>CTCTGAAAACACTGCGGACAAGCCTATAAAGCCTA</td>
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<tr>
<td>P-70 R <em>holC R128A SDM</em></td>
<td>TAGGCTTTATAGCTTCTGAGCAGCTGATTTGCTAGAG</td>
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<tr>
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<td>P-74 R <em>holC K132A SDM</em></td>
<td>CCAGCCACCAGGTAGCTCGATAGCGTTTCCGCGCCCA</td>
</tr>
<tr>
<td>P-75 F <em>holC R135A SDM</em></td>
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<td>P-76 R <em>holC R135A SDM</em></td>
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<tr>
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<td>p-123 R <em>holC sdm R128E</em></td>
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<td>p-124 F <em>holC sdm K132E</em></td>
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<td>P-135 F CAM from pKD32</td>
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<td>p-136 R cam XhoI from pKD32</td>
<td>ACGTGCTCACATCGAGGTGTTGAGGCTGAGCTGTTTC</td>
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<td>promoter</td>
<td>PCR primers for holC screening (Wanner)</td>
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<tr>
<td>p-140 F colony screening of holC</td>
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<tr>
<td>p-129 R holC colony PCR</td>
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<tr>
<td>p-139 F for holC Wanner</td>
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<td>p-86 (holC for Wanner)</td>
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<td>prSJS908 (hupA::mcherry linear DNA)</td>
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2.6 References


Chapter 3

Protein interactions in genome maintenance as novel antibacterial targets

The work highlighted in this chapter was designed and executed primarily by Aimee H. Marceau. Douglas A. Bernstein discovered the compounds described in this chapter. Brian W. Walsh and Lyle A. Simmons designed and executed the microscopy experiments and Walker Shapiro conducted the replication experiments with *E. coli*. Thank you Adel Talaat, Sarah Marcus, David Andes, Joe Dillard, and Kathleen Hackett for growing various bacterial strains, Elyse Wagner and John Denu for performing human cell growth experiments,
3.1 Summary

Antibacterial compounds typically act by directly inhibiting essential bacterial enzymes. Although this general mechanism of action has fueled traditional antibiotic discovery efforts for decades, new antibiotic development has not kept pace with the emergence of drug resistant bacterial strains, severely limiting the therapeutic tools available for treating bacterial infections. Here we test an alternative antibacterial lead-compound identification strategy in which essential protein-protein interactions are targeted rather than enzymatic activities. Bacterial single-stranded DNA-binding proteins (SSBs) interact with over a dozen different DNA replication, recombination, and repair proteins. Three small molecules that block SSB interactions with its protein partners are shown to have antibacterial activity against diverse bacterial species. Consistent with a model in which the compounds target SSB/protein interactions, treatment of *Bacillus subtilis* cultures with the compounds leads to rapid inhibition of DNA replication and recombination, ultimately leading to cell death. Similar DNA replication blockage by the SSB/protein interaction inhibitors is observed in *Escherichia coli*, although inhibition is only observed in a strain with increased membrane permeability, suggesting that antibacterial activity is limited by cytoplasmic accessibility. Our results highlight the utility of targeting protein-protein interactions, particularly those that mediate genome maintenance, as a powerful approach for identifying new antibacterial compounds.

3.2 Introduction

Antibiotic-resistant bacterial infections present a major threat to public health, making the development of new antibiotics a high priority (D'Costa et al., 2011; Nikaido, 2009; Talbot et al.,
In spite of the need for new treatment options, many pharmaceutical companies have abandoned their antibacterial development efforts and those continued have found that traditional targeting of enzyme active sites yields few new drugs (Fischbach and Walsh, 2009; Fox, 2006; Morel and Mossialos, 2010; Silver, 2011; Talbot et al., 2006; Wright, 2012). These constraints have limited the number of therapeutics available to fight infections (particularly those caused by multi-drug resistant pathogens) and highlight the need for new approaches to identify antibacterial lead compounds.

An emerging alternative lead-discovery approach investigated here takes advantage of small-molecule inhibitors that block essential protein-protein interactions (PPIs) as antibacterial agents. PPIs range in complexity from simple dimeric complexes formed between two proteins to intricate networks in which “hub” proteins bind simultaneously to many protein partners. PPIs are essential for nearly every cellular process and successes in developing therapeutic PPI inhibitors against eukaryotic targets (reviewed in (Wells and McClendon, 2007)) suggest that such sites could be fruitful for antibacterial drug discovery. Indeed, compounds that disrupt homooligomeric or heterooligomeric protein complexes formed by the FtsZ bacterial cell division protein have been found to have antibacterial activity (Beuria et al., 2005; Margalit et al., 2004; Stokes et al., 2005; Wang et al., 2003). These observations support the idea that small molecules that block essential PPIs found uniquely in bacteria could provide novel broad-spectrum therapeutic tools to fight the growing number of drug resistant bacterial infections.

Bacterial single-stranded (ss) DNA-binding proteins (SSBs) are homotetrameric proteins that bind and protect ssDNA formed during cellular genome maintenance processes such as DNA replication and homologous recombination (Lohman and Ferrari, 1994). SSBs also function as
hub proteins by binding and recruiting over a dozen different genome maintenance enzymes to their cellular sites of action through direct protein interactions that are mediated by SSB’s evolutionarily-conserved C-terminus (SSB-Ct, Asp-Asp-Asp-Ile-Pro-Phe in *Escherichia coli*) (Shereda et al., 2008). Disruption of SSB/protein interactions by sequence changes in the SSB-Ct can be lethal to bacteria; in particular, the C-terminal-most Pro-Phe dipeptide appears to be critical for cell survival in *E. coli*. Deletion of the SSB-Ct Phe or substitution with a Cys is lethal, whereas altering the SSB-Ct Pro to a Ser causes temperature-sensitive lethality (Chase et al., 1984; Genschel et al., 2000; Meyer et al., 1979; Wang and Smith, 1982). Structural studies of several different proteins in complex with synthetic SSB-Ct peptides have shown the SSB-Ct is bound by sites that share a common electrostatic arrangement. SSB-Ct binding sites include basic residues that coordinate the \( \alpha \)-carboxyl group of the Phe and the side chains of the acidic Asp residues as well as a hydrophobic pocket that accommodates the hydrophobic side chains of the Pro-Phe dipeptide (Lu and Keck, 2008; Marceau et al., 2011; Page et al., 2011; Ryzhikov et al., 2011; Shereda et al., 2008; Shereda et al., 2009). Although eukaryotic SSBs (Replication Protein A) also interact with a wide variety of protein partners (Fanning et al., 2006; Oakley and Patrick, 2010), they do so with distinct mechanisms since they lack an SSB-Ct sequence. Given that SSB-Ct/protein interactions are essential and conserved only in bacteria, it has been hypothesized that small molecules that compete with SSB for binding to its protein partners could serve as excellent antibacterial lead compounds (Lu et al., 2010).

Small molecules that block protein interactions between *E. coli* SSB and one of its binding partners (Exonuclease I) have been identified (Lu et al., 2010) (Figure 3.1A). Of these compounds, MPTA is a structural mimic of the SSB-Ct Pro-Phe dipeptide and it broadly
inhibits SSB/protein interaction, blocking complex formation with both Exonuclease I and additional binding partners (RecQ and PriA DNA helicases) (Lu et al., 2010). In contrast, BCBP and CFAM are not obvious structural mimics of the SSB-Ct and each exhibits more specific inhibition of the SSB/Exonuclease I interface, with less activity against either the SSB/RecQ or SSB/PriA complexes. Structural and mechanistic studies further showed that that each of the inhibitors directly competes with the SSB-Ct for binding to Exonuclease I (Lu et al., 2010).

Here, we use these SSB PPI inhibitors to test the hypothesis that blocking formation of essential SSB/protein interactions in vivo is lethal to bacteria. Each of the SSB PPI inhibitors has antibacterial activity against a diverse panel of bacterial species, with the general SSB PPI inhibitor, MPTA, having the most potent activity. Treatment with each of the three compounds leads to rapid cessation of DNA replication and recombination, and ultimately to cell death in a manner that requires cytoplasmic accessibility. These results support a model in which the compounds act by disrupting SSB/protein interactions in vivo, and suggest that targeting PPIs, particularly those that mediate genome maintenance, is a powerful approach for developing novel antibacterial compounds.

3.3 Results

3.3a Small molecule inhibitors that block SSB/protein interactions have antibacterial activity. Bacterial SSBs directly bind over a dozen different proteins with roles in DNA replication, recombination, repair and replication restart to form essential genome maintenance complexes (Shereda et al., 2008). To test the hypothesis that chemical blockage of SSB/protein interactions would hinder bacterial growth, several diverse bacterial strains were incubated on
solid media required for their growth, as described in *Experimental Procedures*, that either omitted or included the inhibitors. Consistent with the hypothesis, *Bacillus subtilis, Neisseria gonorrhoeae, N. meningitidis, N. lactamica*, and *Listeria monocytogenes* failed to form colonies in the presence of MPTA (50 µM), BCBP (100 µM), or CFAM (100 µM) (Figure 3.1 and Table 3.1). Several other bacterial species were resistant to the compounds, including *E. coli, Mycobacterium smegmatis, M. avium paratuberculosis, M. bovis, M. tuberculosis*, and *Staphylococcus aureus* (Figure 3.1 and Table 3.1). Thus the compounds inhibited the growth of many, but not all, bacterial species tested.

One possible explanation for the differential SSB PPI inhibitor sensitivity of bacterial species is that membrane impermeability limits cytoplasmic accessibility for the compounds in the resistant bacterial species. To test this idea, colony formation of an *E. coli* strain carrying a mutation that confers increased membrane permeability (*imp-4213* (Braun and Silhavy, 2002; Sampson et al., 1989)) was tested on plates containing the SSB PPI inhibitors. Unlike the compound-resistant wildtype *E. coli*, the *E. coli imp-4213* strain was sensitive to each of the inhibitors (Figure 3.1B and Table 3.1). This observation is consistent with the idea that resistance to the SSB PPI inhibitors in wildtype *E. coli* is due to poor cell membrane penetration.

The increased membrane permeability of *E. coli imp-4213* cells renders them unable to grow on MacConkey’s agar due to the toxicity of cytoplasmic accumulation of bile salts present in the media (Ruiz et al., 2005; Sampson et al., 1989). Suppressors of the *imp-4213* mutation that restrict membrane permeability are readily detected by their ability to form colonies on MacConkey’s agar (Ruiz et al., 2005; Sampson et al., 1989). To further explore the connection between membrane permeability and SSB PPI inhibitor sensitivity, we generated spontaneous
suppressor mutants of *E. coli* imp-4213 that allowed growth on the compounds and tested whether they had also regained the ability to grow on MacConkey’s agar. Out of 455 *E. coli* imp-4213 colonies that were able to grow in the presence of the compounds, 448 were also able to grow on MacConkey’s agar. The seven colonies that were sensitive to bile salts but resistant to the compounds exhibited mucoid phenotypes, indicating changes to capsule (Beiser and Davis, 1957) that likely exclude the SSB PPI inhibitors but not bile salts. Thus, resistance to the SSB PPI inhibitors appears to require exclusion of the compounds from the cell cytoplasm.

A second possible explanation for the differential SSB PPI inhibitor sensitivity of bacterial species is that media components in some of the plate assays may sequester the hydrophobic compounds, reducing their free concentrations to levels that are insufficient for growth inhibition. Consistent with this model, *S. aureus*, which was resistant to the compounds when grown on Mueller-Hinton agar, was found to be sensitive to the compounds when grown in lysogeny broth (LB) (Tables 3.1 and 3.2). The major difference between LB and Mueller-Hinton media is the presence of beef heart infusion in the Mueller-Hinton media, which could change the availability of the PPI inhibitors. To confirm this idea, *B. subtilis*, which was sensitive to the compounds in LB, was more resistant when grown on Mueller-Hinton agar (Table 3.1). These data show that the apparent resistance of *S. aureus* and perhaps other strains shown in Figure 3.1 could be due to compound sequestration by the growth media. These restrictions limit the utility of these particular compounds in therapeutic settings. Nonetheless these data support the hypothesis that inhibitors of SSB PPIs can serve as antibacterial compounds against a broad spectrum of benign and pathogenic bacterial species.
SSB PPI inhibitors have low minimal inhibitory concentrations and are bactericidal.

The potency of each of the PPI inhibitors was assessed in model Gram-positive (*B. subtilis*) and Gram-negative (*E. coli imp-4213*) bacterial strains. With *B. subtilis*, minimal inhibitory concentration (MIC) values for MPTA, BCBP, and CFAM were 16, 20, and 32 µM, respectively. As a comparison, the MIC for the antibiotic kanamycin measured under the same experimental conditions was 5 µM. With *E. coli imp-4213*, the MIC values for MPTA, BCBP, and CFAM were 10, 60, and 36 µM, respectively, while the MIC for kanamycin was 3 µM. Thus MTPA, which blocks the greatest number of SSB PPIs (Lu et al., 2010), is the most potent of the three compounds and has similar MIC values compared to an established antibiotic.

Time-kill experiments were carried out with *B. subtilis* to determine whether the PPI inhibitors act by a bactericidal mechanism, killing over 99.9% of the cells in 24 hours, or by a bacteriostatic mechanism, killing or suppressing growth in less than 99.9% of cells over 24 hours (National Committee for Clinical Laboratory and Wikler). Survival of *B. subtilis* after the addition of 0.5×, 1×, 2× or 4× MIC levels of the compounds to a growing culture was measured by counting viable colony-forming units (CFUs) over time (Figure 3.2A-D). In control experiments, the addition of DMSO carrier (negative control) or antibacterial compounds known to damage DNA (nalidixic acid and mitomycin C, positive controls) were also measured. The addition of the SSB PPI inhibitors or the positive controls led to significant decreases in *B. subtilis* CFUs in a concentration-dependent manner whereas the DMSO negative control did not (Figure 3.2A-D). MPTA was bactericidal at 2× and 4× MIC levels, with a time to bactericidal killing of less than 1 hour (Figure 3.2B). Similar results were observed for BCBP at 2× or 4× MIC levels, with bactericidal killing also observed in 4 hours at 1× MIC and in 24 hours at 0.5×
MIC (Figure 3.2C). Additionally, cells treated with CFAM exhibited killing in 1 hour at 4× MIC and in 4 hours at 2× MIC (Figure 3.2D).

To assess the kinetics of cell recovery after acute exposure to the compounds, the post-antibiotic effect (PAE) of treatment with each compound was assessed. A long PAE is expected if bacterial growth continues to be suppressed after a short exposure to the antibiotic, which is considered a characteristic of a good antibiotic. In these experiments, *B. subtilis* cultures at $10^6$ CFU/mL were exposed to 1×, 2× or 4× MIC levels of each compound (or a DMSO control) for 60 minutes. Cells were washed to remove the compound and transferred to media lacking the inhibitors, and recovery was assessed by measuring CFUs over time. The PAE for MPTA was concentration dependent whereas the compound concentration did not affect the PAE of either CFAM or BCBP (Figure 3.3A-C). These results show that MPTA rapidly and efficiently kills bacteria and that treated cultures need extended recovery time after treatment. In contrast, the PAE for BCBP and CFAM did not change with increasing concentration, indicating that these compounds have a less rapid effect on cells when compared to MPTA. These differential results could be linked to the ability of MPTA to inhibit multiple SSB interactions, while both BCBP and CFAM have more specific inhibitory effects (Lu et al., 2010).

### 3.3c SSB PPI inhibitors disrupt DNA replication *in vivo*

Because SSB PPIs are critical for DNA replication (Shereda et al., 2008), we hypothesized that the inhibitors could disrupt DNA replication *in vivo*. This hypothesis was tested first by measuring the effects of each PPI inhibitor on the rate of DNA replication in exponential phase *B. subtilis* and *E. coli imp-4213*
cultures. Pre- and post-inhibitor treatment replication rates were measured in *B. subtilis* by pulse labeling aliquots of bacterial culture with[^3]H thymidine and quantifying incorporation of the label into DNA (Courcelle and Courcelle, 2006). In control experiments, the addition of DMSO had no effect on[^3]H thymidine incorporation rates (Figure 3.4A). In contrast, the addition of nalidixic acid, which impairs replication by inhibiting gyrase (Bradbury and Pucci, 2008), led to an immediate reduction in the replication rate that was further reduced over the course of the experiment (Figure 3.4A). The effects of the SSB PPI inhibitors were markedly similar to that of the nalidixic acid positive control; addition of MPTA, BCBP, or CFAM at 2× MIC levels led to an immediate inhibition of replication that was further reduced over time (Figure 3.4A). To test whether this reduction was an indirect result of rapid cell death rather than inhibition of the DNA replication processes itself, the number of CFUs in identically treated cultures over the same time frame were counted. These results showed that DNA replication inhibition precedes cell death after treatment with the compounds (Figure 3.4B). Parallel experiments performed with *E. coli imp-4213* confirm that the compounds also rapidly block DNA replication in this strain as well, indicating that the compounds block replication in two very distantly related bacterial species (Figure 3.5).

### 3.3d SSB PPI inhibitors disrupt RecA focus formation in vivo

We next asked whether the compounds could affect additional genome maintenance processes that depend on protein interactions with SSB by assessing their effects on recombination initiation in cells. Activity of the bacterial recombinase, RecA, is limited by its ability to access to ssDNA, which is typically sequestered by SSB. RecA assembly in cells is therefore highly dependent on mediator proteins
that bind directly to SSB and modify the SSB/DNA structure to provide RecA access to ssDNA (Hobbs et al., 2007; Inoue et al., 2008). Assembly of RecA in vivo can be quantified by following formation of foci of RecA-GFP fusion proteins (Kidane and Graumann, 2005; Simmons et al., 2007). Formation of RecA foci is linked to DNA replication and to the initiation of recombinational DNA repair of stalled replication forks in B. subtilis (Kidane and Graumann, 2005; Simmons et al., 2007).

RecA-GFP foci were scored in DMSO-control and PPI inhibitor-treated B. subtilis cultures. Within 5 minutes, a decrease in the number of foci associated with nucleoids was observed in cells treated with ~1× MIC levels of the compounds but not in the DMSO controls (Figure 3.4C, Table 3.3). Foci in cells treated with MPTA decreased by ~30% relative to the DMSO control, whereas BCBP- and CFAM-treated cells decreased by over 50% and 70% respectively (Table 3.3). Cell survival was measured over the same time course by incubating the cells with BacLight reagents immediately after compound treatment (Figure 3.6). In all cases between 77% and 90% of cells survived the treatment (Table 3.4 and 3.5), indicating that the compounds do not immediately kill cells. We also determined viability by microscopy using a different procedure (Lamsa et al., 2012) and obtained consistent cell survival results (data not shown).

3.4 Discussion

Infections by antibiotic-resistant bacterial strains are a major worldwide public health problem. New strategies for identifying novel antibacterial targets and compounds are needed to help stem
this crisis. Here we have examined the question of whether protein interfaces that mediate physical interactions between SSB and its protein partners could provide novel targets for antibacterial development. Because, nearly every genome maintenance pathway in bacteria depends on interactions with SSB to engage SSB/DNA structures (Shereda et al., 2008), we hypothesized that compounds blocking SSB/protein interactions could simultaneously impair multiple genomic processes (Figure 3.7), ultimately leading to cell death.

Consistent with this model, we found that small molecule SSB PPI inhibitors impair growth in a wide variety of bacterial species, including disease causing bacteria such as species of Neisseria and L. monocytogenes. Antibacterial activity depended on cytoplasmic accessibility (Figure 3.1B, Table 3.1, and 3.3-3.5), which was consistent with the predicted cytoplasmic mechanism of action of the inhibitors. Each of the compounds was bactericidal and MPTA had a very strong PAE on the growth of B. subtilis, indicating that it is able to continue inhibiting bacterial growth even after the free concentration has dropped to sub-MIC levels. Interestingly, of the compounds tested, MPTA had previously been found to be able to disrupt the largest number SSB/protein complexes (Lu et al., 2010), which may in part explain its potent PAE properties.

Consistent with the predicted mechanism of action (Figure 3.7), each of the compounds inhibited DNA replication and recombination initiation in vivo. These observations align well with the previously published in vitro biochemical activities of the compounds in disrupting SSB/protein interactions, specifically ExoI, PriA, and RecQ (Lu et al., 2010). The ability of the compounds to rapidly disrupt and halt replication is most likely attributable to their abrogation of SSB interactions with the key components of the DNA replication machinery, such as primase or the replicative DNA polymerase DnaE (Costes et al., 2010; Yuzhakov et al., 1999; Zechner et al.,
1992). Additionally, since the requisite DNA replication restart pathways rely heavily on proteins that interact with SSB to restart stalled replication forks (Shereda et al., 2008), the normal cellular mechanisms aiding in repair of failed replication processes may also be impaired by the SSB PPI inhibitors. In support of this notion, the inhibitors reduce the recombinational repair capacity of the cells, as evidenced by the reduction of RecA foci upon treatment. We propose that the compounds’ abilities to inhibit several genome maintenance pathways simultaneously lead to an impassable problem for bacteria that ultimately causes cell death.

Antibiotics that directly target genome maintenance, particularly SSB/protein interactions as described here, could provide excellent lead compounds for future broad-spectrum therapeutics. First, such inhibitors could act exclusively against bacterial DNA replication, recombination, and repair processes since these pathways are catalyzed by functionally similar but structurally disparate protein complexes in eukaryotes and prokaryotes. To date these distinctions have only been minimally exploited for the development of antibacterial compounds (Robinson et al., 2012). Unfortunately, the compounds described herein are toxic to human cells in culture (data not shown). It is possible that chemical modification of the compounds could alleviate these negative effects, allowing derivative compounds to be selectively active against bacteria. A second appealing feature of these compounds is that, since hub proteins such as SSB form complexes with a large number of proteins, resistance based on sequence changes in multiple SSB-interaction partners would seem nearly impossible to select for within a single cell. Our results show that cytoplasmic accessibility, however, would remain a challenge, leaving open a possibility for resistance based on efflux changes.
Taken together, we have validated the idea that direct targeting of PPIs, particularly those that involve SSB, is an effective strategy for the development of new antibiotic lead compounds. We suggest that similar strategies targeting different essential PPIs or that target the central genome processes of bacteria could prove important for future development of novel antibacterial compounds that will help alleviate the problem of antibiotic resistance bacterial infections and significantly diversify our antibacterial arsenal.

3.5 Experimental Procedures

**Plate-based antibacterial sensitivity experiments.** Plates of LB (Sambrook and Russell, 2001), Middlebrook 7H10 (Fisher Scientific), Mueller-Hinton (Sigma-Aldrich) and GC (Fisher (Kellogg et al., 1963)) agar were made with 50 µM MPTA, 100 µM BCBP, or 100 µM CFAM. *B. subtilis* (strain PY79), *E. coli* (strain MG1655), *E. coli imp-4213*, and *L. monocytogenes* (strain 10403S) were grown on LB agar. *N. gonorrhoeae* (strain MS11) and *N. meningitides* (strain 13102) were grown on GC agar. *M. smegmatis* (strain mc²155), *M. avium paratuberculosis* (strain K10), *M. bovis* (strain BCG), and *M. tuberculosis* (strain H37Rv) were grown on 7H10 medium. *S. aureus* (strain 33591) was grown on Mueller Hinton. The individual species were streaked from either a frozen stock or a growing culture onto the appropriate medium, and then grown at the required temperature overnight (or for several weeks with the *Mycobacterial* strains).
**MIC measurements.** Minimum inhibitory concentrations were determined for each specific media employed. \(10^6\) CFU/mL of either *B. subtilis* or *E. coli imp-4213* were inoculated into either liquid media or onto solid plates containing concentrations of the compounds ranging from 1 to 100 \(\mu\)M. Plates or liquid culture without visible growth were determined to be the MIC. These numbers were determined over the course of 3-5 independent experiments. This method was adapted from the NCCLS (National Committee for Clinical Laboratory Standards., 1999).

**Time-kill experiments.** Triplicate cultures of *B. subtilis* PY79 (\(1 \times 10^6\) CFU/ml) at 37 °C were grown in media supplemented with 0.5\(\times\), 1\(\times\), 2\(\times\), or 4\(\times\) the MIC of MPTA, BCBP or CFAM, or with DMSO, 1 \(\mu\)g/mL mitomycin C, or 25 \(\mu\)g/mL nalidixic acid as controls. The number of CFU/mL of culture was measured from aliquots (0, 1, 2, 4, 8, and 24 hours) that were serially diluted in sterile saline, plated on LB agar, and incubated at 37 °C (Miles et al., 1938). The lower limit of detection for colony counts was 100 CFU/mL. The time dependent killing curves were constructed by plotting mean colony counts over time; error bars represent one standard deviation of the mean.

**Post-antibiotic effect experiments.** The PAE of each compound was determined by the equation \(\text{PAE} = T - C\), where \(T\) is the time required for CFU levels to increase 1-log above the count observed immediately after drug removal, and \(C\) is the time required for the untreated control CFU to increase 1-log above the count observed after completing the wash procedure used to remove the compounds in the test cultures. The compounds were added to the indicated
amounts of 1×, 2×, and 4× MIC after the zero time point. The cells were treated for 60 min followed by thorough washing and transfer to fresh medium. After transfer to fresh medium, aliquots were removed, serially diluted and plated on LB at each time point from 0-8 hours and 24 hours.

**DNA replication rate measurements.** *B. subtilis* (PY79) was grown in defined S750 minimal media. (Berkmen and Grossman, 2006) supplemented with 1% glucose at 37 °C to an OD600 of 0.3 to 0.4. At each time point, 0.5 ml of the culture was removed and added to the pulse-label solution containing 10 μCi/ml [3H] thymidine. Cells were grown for 5 minutes at 37 °C with shaking to allow label incorporation and replication was stopped by the addition of 5 mL of cold 10% TCA. Immediately prior to the 20 min time point the cells were treated by the addition of DMSO (150 µL), MPTA (20 µM), BCBP (16 µM), CFAM (48 µM), or 25 µg/mL nalidixic acid. All samples were processed and cell survival was accessed as previously described (Courcelle and Courcelle, 2006; Marceau et al., 2011). Identical experiments were conducted with *E. coli imp-4213*.

**RecA-GFP microscopy.** Strain LAS40 (*recA-mgfp*) was grown in defined S750 minimal media supplemented with 2% glucose at 30 °C to an OD600 of 0.4. The culture was split, a portion of cells were left untreated while the other portion was treated with MPTA (10 µM), BCBP (8 µM), or CFAM (24 µM) for 5 min. Cells were visualized by fluorescence microscopy (Klocko et al., 2010; Simmons et al., 2009; Simmons et al., 2007) and the percentage of nucleoids with RecA-
GFP foci was scored. A small percentage of cells treated with each of the compounds exhibited punctate RecA-GFP localization and/or membrane-associated RecA-GFP, which is not consistent with discrete, nucleoid associated foci. These localizations were scored, but excluded from the calculation.
Table 3.1 Survival of diverse bacterial species in the presence of antibacterial compounds.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No treatment</th>
<th>MPTA (50 µM)</th>
<th>BCBP (100 µM)</th>
<th>CFAM (100 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>E. coli imp-4213</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>+</td>
<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
</tr>
<tr>
<td><em>N. gonorrhoeae</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>N. meningitidis</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>+</td>
<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>N. lactamica</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>M. avium paratuberculosis</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>+</td>
<td>+</td>
<td>IG</td>
<td>+</td>
</tr>
</tbody>
</table>

+ indicates visible growth, - indicates no visible growth. -/+ indicate media differences with compound sensitivity on LB and less sensitivity on Mueller-Hinton. IG indicates that growth of the species was inhibited at that compound concentration, specifically there were very few colonies compared to the untreated control.
### Table 3.2 Minimum inhibitory concentrations and IC50 values

<table>
<thead>
<tr>
<th>Strains</th>
<th>MPTA</th>
<th>BCBP</th>
<th>CFAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli imp-4213</td>
<td>10 µM</td>
<td>62 µM</td>
<td>36 µM</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>12-16 µM</td>
<td>8-11 µM</td>
<td>24-40 µM</td>
</tr>
<tr>
<td>B. subtilis on Mueller Hinton</td>
<td>&gt; 50 µM</td>
<td>&gt; 100 µM</td>
<td>40-100 µM</td>
</tr>
<tr>
<td>S. aureus on Mueller Hinton</td>
<td>&gt; 50 µM</td>
<td>&gt; 100 µM</td>
<td>&gt; 100 µM</td>
</tr>
<tr>
<td>E. coli imp-4213 (IC50)</td>
<td>11 µM</td>
<td>6 µM</td>
<td>30 µM</td>
</tr>
<tr>
<td>S. aureus (IC50)</td>
<td>29 µM</td>
<td>5.5 µM</td>
<td>18 µM</td>
</tr>
</tbody>
</table>

The ranges listed for B. subtilis and E. coli imp-4213 in the first two rows are the results of MIC tests in both liquid and solid LB. The next row of B. subtilis on Mueller Hinton reflects results of growth on solid media. The MIC values of S. aureus on Mueller Hinton are from growth on solid media. The IC50 results are from a 96 well plate assay based on optical density.
Table 3.3 RecA-GFP foci are reduced following treatment of cells with small molecules that inhibit interaction with SSB

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nucleoids with foci(^a)</th>
<th>Total nucleoids scored</th>
<th>Percentage of nucleoids with foci ± 95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>2250</td>
<td>26,860</td>
<td>8.38 ± 0.331</td>
<td>--</td>
</tr>
<tr>
<td>10 µM MPTA</td>
<td>366</td>
<td>6189</td>
<td>5.91 ± 0.588</td>
<td>4.89 × 10(^{-11})</td>
</tr>
<tr>
<td>8 µM BCBP</td>
<td>286</td>
<td>7595</td>
<td>3.77 ± 0.428</td>
<td>2.36 × 10(^{-42})</td>
</tr>
<tr>
<td>24 µM CFAM</td>
<td>206</td>
<td>7995</td>
<td>2.58 ± 0.347</td>
<td>4.29 × 10(^{-71})</td>
</tr>
</tbody>
</table>

Strain LAS40 (*recA-mgfp*) was grown in defined S7\(_{50}\) minimal media supplemented with 2% glucose to an OD\(_{600}\) of 0.4. The culture was split, a portion of cells were left untreated while the other portion subjected to challenge with MPTA, BCBP, or CFAM as shown. Cells were visualized by microscopy after 5 minutes, and the percentage of nucleoids with RecA-GFP foci were scored.

\(^a\)A small percentage of cells treated with each of the compounds exhibited RecA-GFP localization which was punctate and/or membrane associated, which is not consistent with discrete, nucleoid associated foci. These localizations were scored, but excluded from the calculation shown above. The number of cells with mislocalized RecA-GFP foci are as follows for each compound: MPTA, 28; BCBP, 30; CFAM, 9.
Table 3.4 Percent killing of LAS40 (*recA-mgfp*) cells following challenge with small molecules that inhibit interaction with SSB

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells alive</th>
<th>Total cells</th>
<th>Percentage of cells living ± 95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>3662</td>
<td>3860</td>
<td>94.9 ± 0.7</td>
<td>--</td>
</tr>
<tr>
<td>10 µM MPTA</td>
<td>1067</td>
<td>1384</td>
<td>77.1 ± 2.2</td>
<td>2.51 × 10⁻⁸¹</td>
</tr>
<tr>
<td>8 µM BCBP</td>
<td>2087</td>
<td>2323</td>
<td>89.9 ± 1.2</td>
<td>3.25 × 10⁻¹⁴</td>
</tr>
<tr>
<td>24 µM CFAM</td>
<td>1270</td>
<td>1616</td>
<td>78.6 ± 2.0</td>
<td>1.13 × 10⁻⁻⁷⁵</td>
</tr>
</tbody>
</table>

Strain LAS40 (*recA-mgfp*) was grown in defined S750 minimal media supplemented with 2% glucose to an OD₆₀₀ of 0.4. In exponential phase, the culture was split and a portion of cells were left untreated while the other portion was challenged with MPTA, BCBP, or CFAM as indicated for 1 minute. Immediately following challenge, cells were incubated with the BacLight reagents (Invitrogen). The cells were then visualized by microscopy on 1% agarose pads after 5 minutes, and the number of dead cells was scored relative to the number of live cells in each culture. The 95% confidence interval is reported and the p-value indicating statistical significance.
Table 3.5 Percent killing of LAS508 (PY79) cells following challenged with small molecules that inhibit interaction with SSB

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells alive</th>
<th>Total cells</th>
<th>Percentage of cells living ± 95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>8086</td>
<td>8250</td>
<td>98.0 ± 0.3</td>
<td>--</td>
</tr>
<tr>
<td>10 µM MPTA</td>
<td>1834</td>
<td>2404</td>
<td>76.3 ± 1.7</td>
<td>4.79 × 10^{-300}</td>
</tr>
<tr>
<td>8 µM BCBP</td>
<td>2670</td>
<td>2731</td>
<td>97.8 ± 0.5</td>
<td>0.216</td>
</tr>
<tr>
<td>24 µM CFAM</td>
<td>3208</td>
<td>3652</td>
<td>87.8 ± 1.1</td>
<td>8.92 × 10^{-120}</td>
</tr>
</tbody>
</table>

Strain LAS508 (PY79) was grown in defined S750 minimal media supplemented with 2% glucose to an OD$_{600}$ of 0.4. During exponential phase, the culture was split and a portion of culture was untreated while the other portion was challenged with MPTA, BCBP, or CFAM as indicated for 1 minute. Immediately following challenge, cells were incubated with the BacLight reagents (Invitrogen). The cells were then visualized by microscopy after 5 minutes, and the number of dead cells were scored relative to the number of live cells in each culture. The 95% confidence interval (CI) is indicated as well as the p-value for statistical significance.
Figure 3.1

A

MPTA

BCBP

CFAM

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Figure 3.1 MPTA, CFAM and BCBP inhibit the growth of multiple prokaryotic species.  

A. Structures of MPTA, BCBP and CFAM (Lu et al., 2010).  B. Colony formation of several bacterial strains in the absence (left) or presence (right) of 50 µM MPTA.  

*E. coli* (*Ec*), *E. coli imp-4213* (*Ec 4213*), *B. subtilis* (*Bs*), *Neisseria gonorrhoeae* (*Ng*), *Neisseria meningitidis* (*Nm*), *Neisseria lactamica* (*Nl*), *Listeria monocytogenes* (*Lm*), *Mycobacterium smegmatis* (*Ms*) were plated without (left) or with MPTA (right). Identical experiments were performed with BCBP and CFAM, with the results summarized in Table 3.1.
Figure 3.2

A. Controls

B. MPTA

C. BCBP

D. CFAM

Legend:
- DMSO
- MMC
- NA
- 0.5X MIC
- 1X MIC
- 2X MIC
- 4X MIC
Figure 3.2 MPTA, BCBP, and CFAM are bactericidal. Time kill curves of *B. subtilis* PY79 treated with (A) controls, (B) MPTA, (C) BCBP, or (D) CFAM at 0.5×, 1×, 2×, or 4× MIC levels. Dimethyl sulfoxide (DMSO), mitomycin c (MMC), and nalidixic acid (NA) were used as controls. Data points are the mean from three independent experiments with error bars representing one standard deviation of the mean.
Figure 3.3

A. MPTA

B. BCBP

C. CFAM

Graphs showing growth curves with different concentrations of MIC.
Figure 3.3 MPTA, BCBP, and CFAM suppress growth after compound removal. Post antibiotic effect of *B. subtilis* PY79 treated with (A) MPTA, (B) BCBP, or (B) CFAM, after 1 hour of treatment with 1×, 2×, or 4× MIC levels. CFUs were measured at time zero (just prior to treatment) and at the times indicated after treatment. Data points are the mean from three independent experiments with error bars representing one standard deviation of the mean.
Figure 3.4.
Figure 3.4 The SSB PPI inhibitors block DNA replication and recombination in *B. subtilis* prior to cell death. (A) Incorporation of [³H] thymidine over time is measured in the absence or presence of MPTA (18 µM), BCBP (16 µM), CFAM (48 µM), or Nalidixic acid (NA, 25 µg/mL). Compounds were added to the culture at the 20 min time point. Duplicate samples were measured for each time point and each experiment was repeated in triplicate. All samples were normalized to the time zero reading with the data point as the mean and error bars are one standard deviation from the mean. (B) CFU/ml of *B. subtilis* under the same conditions as in A. Data points are the mean from all experiments with error bars representing one standard deviation of the mean. (C) Cultures of *B. subtilis* LAS40 (*recA-mgfp*) were split; one portion was left untreated and the others were challenged with MPTA (10 µM), BCBP (8 µM), or CFAM (24 µM) for 5 min prior to imaging.
Figure 3.5

E. coli imp-4213 [\(^3\)H] thymidine incorporation

- DMSO
- Nalidixic Acid (25 µg/mL)
- MPTA 2X MIC
- BCBP 2X MIC
- CFAM 2X MIC

Normalized CPM vs. Time (min)
Figure 3.5 The SSB PPI inhibitors block DNA replication and recombination in *E. coli* \textit{imp4213}. Incorporation of \[^{3}H\] thymidine over time is measured in the absence or presence of MPTA (20 \(\mu\)M), BCBP (120 \(\mu\)M), CFAM (72 \(\mu\)M), or Nalidixic acid (NA, 25 \(\mu\)g/mL) added to the culture at the 20 min time point. Duplicate sample were measured for each time point, samples taken every 5 minutes and each experiment was conducted in triplicate. All samples were normalized to the time zero reading, data points are the mean of all three experiments with error bars representing one standard deviation from the mean.
Figure 3.6

<table>
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<th>RecA-GFP</th>
<th>PY79</th>
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<td>3 µm</td>
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</table>

<table>
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<th>10 µM MPTA</th>
<th>RecA-GFP</th>
<th>PY79</th>
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<table>
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<th>10 µM BCBP</th>
<th>RecA-GFP</th>
<th>PY79</th>
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<td>3 µm</td>
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</table>

<table>
<thead>
<tr>
<th>24 µM CFAM</th>
<th>RecA-GFP</th>
<th>PY79</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 µm</td>
<td></td>
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</tbody>
</table>
Figure 3.6 Treatment of *B. subtilis* cells with SSB PPI inhibiting small molecules. *B. subtilis* strains LAS40 (recA-mgfp) (left) was grown in defined S750 minimal media supplemented with 2% glucose to an OD$_{600}$ of 0.4. In exponential phase, the cultures were split and a portion of cells were left untreated while the other portion was challenged with MPTA, BCBP, or CFAM as shown for 1 minute. Immediately following challenge, cells were incubated with the BacLight reagents (Invitrogen). Cells were then visualized by microscopy after 5 minutes. Shown are combined images of live (green) and dead (red) cells after treatment with each of the compounds. Treatment with SSB interaction inhibiting small molecules causes only modest killing in wild type cells. Strain PY79 (right) was grown in defined S750 minimal media as in all other experiments. In mid-exponential phase, the culture was split and a portion of the culture was untreated while the other portion was challenged with each of the indicated compounds for 1 minute. Cells were incubated with the BacLight reagents (Invitrogen) immediately following compound challenge. Cells were then visualized by microscopy after 5 minutes.
Figure 3.7
**Figure 3.7. Model of SSB PPI inhibitor disruption of essential SSB interactions.**

SSB/protein interactions are essential for multiple genome maintenance processes (left) but can be blocked by SSB PPI inhibitors (MPTA (red circle), shown as an example) competing with SSB for binding. In the presented model, this activity blocks multiple genome maintenance which ultimately leads to cell death.
References


National Committee for Clinical Laboratory, S., and Wikler, M.A. Performance standards for antimicrobial susceptibility testing: fourteenth informational supplement (Wayne, Pa., NCCLS).


Chapter 4

Conclusions and Future Directions
Bacterial single-stranded (ss) DNA binding protein (SSB), unlike its name implies is more than an inert DNA binding protein. Through its interactions with heterologous proteins SSB serves as a genome maintenance hub; organizing and localizing enzymes and proteins to their sites of action and maintaining the ssDNA/SSB substrate upon which they work. SSBs diverse roles in DNA replication, recombination and repair are highlighted in Chapter 1.

**SSBs interaction with the χ of the E. coli replisome serves to stabilize the replication machinery.**

Prior to publication of Chapter 2 of this work, the primer hand-off hypothesis was strongly supported by peer reviewed evidence (Kelman et al., 1998; Yuzhakov et al., 1999). Obtaining the crystal structure defining the molecular interface between the E. coli replisome and SSB facilitated targeted mutational studies that enabled abrogation of only the χ/SSB interaction. In this way the role of the interaction in the process of replication was directly test and while we cannot state there is no credence to the primer hand-off hypothesis we can concluded that the interaction is not essential for lagging strand replication. Rather our observations support the idea that this molecular contact is critical for replisomal stability and important for productive leading-strand DNA synthesis, with only minor observable effects on lagging strand replication. The studies described in Chapter 2 help define the roles of interaction between the cellular DNA replication machinery and SSB bound to the lagging strand template. The conservation SSB’s role in replication make it highly likely that similar interactions are important for replication in all organisms.
Essential SSB interactions are an excellent target for the development of new antibiotics. Chapter 2 of this work highlighted the importance of the interaction between SSB and the *E. coli* replisome. In Chapter 3 the potential for SSB interactions in particular and essential protein/protein interactions in general, to be used as a target for novel antimicrobial therapeutics is strongly supported. The work described in Chapter 3 has validated the idea that direct targeting of SSB’s essential protein/protein interactions is a valuable strategy for the development of new antibiotics. Similar strategies that target different essential protein/protein interactions or that target the central genome processes of bacteria have the potential to yield new class of antibiotics that will help alleviate the problem of antibiotic resistance bacterial infections and significantly enhance the diversity our antibacterial therapeutics. Further work in the lab will focus on finding compounds that block SSB interactions with a broad range of targets, are antibacterial, and have minor effects on human cells.

Finding novel SSB interaction partners and expanding knowledge of SSB’s role as a hub protein.

Through the use of N-terminal tandem affinity purification tags I was able to identify and confirm three novel SSB interaction partners (appendix I). The SSB interaction with MgsA is fully characterized in appendix II (Page et al., 2011). Employing the potential interaction partners in a yeast-two hybrid assay, in tandem with SSB, enabled me to test the validity of many possible interactions in a simple experiment. From this experiment I was able to conclude that both PriC and RNase HI interact with SSB. Further experimentation with RNase HI confirmed the affinity of the interaction with SSB is within in the range of other binding proteins. The PriC interaction project has become part of Sarah Wessel thesis and the RNase HI interaction has
become Christine Petzold thesis project. I spent a large amount of time attempting to determine if FtsZ and SSB interact, currently the results are inconclusive. In addition and I am pursuing the potential interaction between DnaE, DnaB, RecF, and SSB through a variety of methods. I will purify each of the proteins and test for an interaction by ammonium sulfate precipitation (appendix III), fluorescence polarization and isothermal titration calorimetry. Expanding our knowledge of the SSB interactome will facilitate and improve our comprehension of genome maintenance processes in bacteria.

References


Appendix I

Novel SSB protein interactions

All work in this section was performed by Aimee Marceau with one exception. The *E. coli* SSB N-terminal TAP tag was cloned into an expression vector by Dr. Nicholas George.
A1.1 SUMMARY

Bacterial single-stranded DNA binding proteins (SSBs) interact with many important proteins involved in genome maintenance. The methods employed to discover these interactions generally involved finding a functional interaction or using a tagged protein (Arifuzzaman et al., 2006; Butland et al., 2005; Costes et al., 2010). In the majority of large scale identification experiments the affinity tags were placed on the C-terminus of SSB (SSB-Ct); however, the SSB-Ct is the site of its interactions with heterologous proteins. In this study, I employed an N-terminal tandem affinity tagged SSB and was able to find interaction partners missed by previous experiments.

A1.2 INTRODUCTION

Single-stranded (ss) DNA binding proteins (SSBs) have two known functional roles in the cell. First SSBs binds to any ssDNA exposed in the processes of DNA replication, recombination and repair. In doing so, SSB prevents the ssDNA from forming secondary hairpin structures, and protects the exposed DNA from damage and attack from nucleases (Lohman and Ferrari, 1994; Raghunathan et al., 2000; Shereda et al., 2007). Second SSB interacts with a large number of proteins involved in processing nucleic acids via its highly conserved amphipathic C-terminus (SSB-Ct) (Shereda et al., 2008).

Several large scale protein interaction studies have been conducted in *E. coli*; these studies are the source of the majority of SSB’s known interaction partners (Arifuzzaman et al., 2006; Butland et al., 2005). While the tandem affinity purification (TAP) tag study identified the majority of proteins that are now known to interact with SSB (Table A1.1), they used a C-
terminally TAP tagged SSB construct. Placing a large tag on the SSB-Ct, the site of its interaction with heterologous proteins, may prevent identification of the part of SSB interactome. In this study we employed an N-terminal TAP tag SSB (N-TAP SSB) construct in an effort of identify novel interaction partners of SSB in E. coil and Bacillus subtilis.

A1.3 RESULTS

Tandem affinity purification of SSB identifies possible new interaction partners. Large scale protein interaction studies in E. coli have identified and confirmed many of the proteins that are known to interact with SSB. However, these studies were limited by the use of C-terminal tags, which may occlude some proteins that normally interact with SSB by preventing access to the SSB-Ct. To this end I employed an N-TAP SSB in an IPTG inducible over-expression vector. After affinity purification and mass-spectroscopy peptide identification a list of potential SSB partners was generated (Table A1.2, A1.3). This method was validated by the identification of multiple known SSB interacting proteins, PriA, Topo III, Exo I, RecQ, and RecJ. In addition several new proteins were identified as potential interaction partners of SSB: MgsA, RNase HI, DinG, FtsZ, PriC, PriB, DnaB, Ribonuclease E, MinD, MinE, TopoI, SecA, RecA, YbjN, and RNA polymerase. The listed potential partners were selected from a much more extensive list likely containing many contaminating proteins. The interaction of MgsA and SSB was identified by this study and is fully described in appendix two (Costes et al., 2010; Page et al., 2011).

Tandem affinity purification of BsSSBa and BsSSBb identifies possible new interaction partners. No large scale protein interaction studies have been conducted in B. subtilis, however, a recent study identified several proteins in B. subtilis that interact with SSBA. This study was limited by the use of a C-terminal TAP tag, which as stated above may occlude some proteins
that normally interact with SSB by preventing access to the SSB-Ct. To this end I employed an
N-TAP SSBa and N-TAP SSBb in an IPTG inducible over-expression system that integrates into
the amyE locus of the *B. subtilis* genome. After affinity purification and mass-spectroscopy
peptide identification a list of potential SSB partners was generated (Table A1.3). This method
was validated by the identification of multiple known SSB interacting proteins, PriA, RecQ,
RecJ, and RecG. In addition several new proteins were identified as potential interaction
The listed potential partners were selected from a much more extensive list likely containing
many contaminating proteins (Table A1.3).

**Yeast-two hybrids confirm two new SSB interaction partners.**

Interaction between two proteins in a yeast-two hybrid assay is indicated by growth of the
transformed strain on selective media (James et al., 1996). Each vector containing a possible
interaction partner fused to one of the Gal4 domains was co-transformed into PJ69-4a with either
the opposite domain fused to SSB or with the opposite domain alone as a negative control. From
these experiments I determined that SSB interacts with PriC and RNase HI, two previously
unknown binding partners (Figure A1.1). The PriC/SSB interaction project is being pursued by
Sarah Wessel and the RNase HI/SSB interaction project is being pursued by Christine Petzold.
The yeast-two hybrid experiment provides a method to test protein interactions without purifying
the punitive binding partner. However, a negative result does not definitively show there is no
interaction *in vivo.*
Isothermal titration calorimetry identifies a one to one interaction of SSB and RNase HI.

Heat generated by SSB binding to RNase HI was fitted using a single site model to determine the stability and stoichiometry of the interaction. At 25 °C, SSB binds RNase HI in a ~1:1 (SSB monomer: RNase HI) complex with an apparent dissociation constant (Kd) of 6.2 +/- 0.2 µM (Figure A1.2); since SSB is a tetramer, this means that four RNase HI monomers can bind to each tetramer.

A1.4 EXPERIMENTAL PROCEDURES

Tandem affinity purification.

The open reading frame of *E. coli* SSB was amplified by PCR and subcloned in frame into pBS1761, SSB N-terminally fused to the tag was then cut out of the vector using NcoI/XhoI and subcloned into pET28b to produce an expression vector encoding SSB with an N-terminal dual affinity tag (includes Protein A and calmodulin peptide binding domains separated by a TEV protease cleavage site). The open reading frame of the resulting plasmid (pNTAP-SSB) was sequenced to confirm the sequence integrity. *E. coli* DE3 plysS transformed with pNTAP-SSB was grown at 37 °C in 8 L of Lysogeny Broth (LB) medium supplemented with 50 µg/ml kanamycin and 25 µg/ml chloramphenicol to midlog phase (OD$_{600\ nm}$ of ~0.6), induced by the addition of 2 µM IPTG (150 µM IPTG for SOS induced cells) and grown for 3 additional hours. Cells were harvested by centrifugation and suspended in 50 ml NP-40 buffer (25 mM dibasic sodium phosphate, 25 mM monobasic sodium phosphate, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 4 mg/l leupeptin, 0.1 mM sodium vanadate, 19.5 mg/l benzamidine, 8.7 mg/l
phenylmethylsulfonyl fluoride (PMSF), 1% Nonidet P-40 substitute (NP-40)) and lysed by sonication. Soluble lysate was incubated for 1 hour at 4 °C with IgG-Sepharose beads (preequilibrated in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40); beads were then washed with 3 volumes of equilibration buffer. TEV cleavage buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1 mM DTT) with 15 μl of 15 μM TEV protease was incubated with the lysate/bead mixture with shaking for ~12 hours at 16 °C. The eluent was incubated with 300 μl calmodulin affinity resin (Stratagene) and 3 μl 1 M CaCl₂ in calmodulin binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 10 mM magnesium acetate, 1 mM imidazole, 10 mM 2-mercaptoethanol) for 1 hour with shaking. Resin was washed with calmodulin binding buffer and TAP-SSB was eluted first with a high salt buffer (calmodulin binding buffer adjusted to 1.5 M NaCl) and then with EGTA Elution Buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% NP-40, 1 mM magnesium acetate, 10 mM imidazole, 10 mM 2-mercaptoethanol, 20 mM ethyleneglycolbisaminoethytltetraacetic acid (EGTA)). High-salt and EGTA-eluted protein samples were precipitated with trichloroacetic acid (25% w/v) on ice for 30 minutes, pelleted by centrifugation, washed twice with ice-cold acetone. The entire acetone pellet was digested with trypsin, and subjected to MALDI-TOF mass spectrometry for identification of peptides (University of Wisconsin Mass Spectrometry facility). To induce cells for SOS 10 μg/mL nalidixic acid was added 2 hours after induction by 150 μM IPTG. All other purification steps were performed as noted above.

**Cloning and tandem affinity purification of B. subtilis (Bs)SSBa and SSBBb.** The open reading frame of SSBa (ssb) and SSBB (ywpH) from B. subtilis were subcloned in frame into N-terminal TAP vector. The N-TAP SSBa and N-TAP SSBB were then subcloned in to pDR111
(amyE::Phyper-spank) with a ribosome binding site. The resulting vector was sequenced to insure the integrity of the construct. The vectors were independently transformed into B. subtilis (PY79). Transformed B. subtilis cells incorporate the N-TAP SSBa or N-TAP SSBb into the amyE locus (suicide vector), expression is inducible by IPTG. 8 liters of B. subtilis transformed with N-TAP SSBa or N-TAP SSBb at grown at 30 °C in LB supplemented with 60 µg/mL spectinomycin and 1 mM IPTG. Cells were grown to OD_{600nm} ~0.7 and harvested by centrifugation. The pellets were resuspended in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2 mM EDTA, 0.5 mM DDT, suspended cells were mixed with 0.5 mg/mL lysozyme, incubated on ice for 20 minutes, at 37 °C for 20 minutes and then on ice for 10 minutes. All additional steps were performed at 4 °C. Benzamidine and phenylmethylsulfonyl fluoride were added to 2 mM respectively. Cells were sonicated on ice for 3 rounds of 40 seconds at 80 % power. Soluble lysate was incubated for 1 hour at 4 °C with IgG-Sepharose beads (preequilibrated in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2 mM EDTA, 0.5 mM DDT). All additional steps and buffers were performed at indicated in the above section.

Yeast-two hybrid.

The open reading frames of E. coli SSB, rna, holC, zipA, priC, dnaB, minD, dnaK, dnaJ, rne, parC, recF, iadA, polB, deaD, dinG, secA, yecE, yfiC, fisA, dnaE were individually amplified by PCR and subcloned in frame into pGAD-C3 and pGBD-C3. This created an N-terminal fusion with either the GAL4 activation domain (AD) or binding domain (BD). The identity of the genes was confirmed by sequencing. The vectors were transformed into pJK69-4A in various combinations of AD and BD to test for interaction with SSB. Cultures of pJK69-4a were grown in YPD at 30 °C with shaking at 270 rpm. 1 mL of overnight night culture was transferred to an
eppendorf tube and centrifuged for 1 minute at 5,000 x g. The supernatant was removed and 125 µL of solution A (80% polyethylene glycol 3350, 20% 1 M lithium acetate), 5 µL of carrier DNA (herring testes DNA) and 3 µL of each plasmid was added to each tube. The cell pellet was resuspended and then vortexed. The cells were heat shocked at 42 °C for 1 hour, with vortexing every 10 minutes. The cells were spun at 5,000 x g for 1 minute and the supernatant was removed. The cell pellets were resuspended in 200 µL of 2% glucose and plated on Leu⁻Trp⁻ SD medium. The transformed cells were grown at 30 °C for several days. After the formation of visible colonies, cultures of the transformed cells were grown overnight in liquid Leu⁻Trp⁻ SD medium at 30 °C with shaking at 270 rpm. The saturated cultures were then back diluted to an OD₆₀₀ of ~0.1 in 2% glucose and then 10-fold serially diluted in a 96 well plate with 2% glucose. The dilutions were spot plated onto Ade⁻His⁻ SD medium and grown at 30 °C. A positive protein/protein interaction is accessed by growth of the experimental transformants and no growth of the negative controls.

**RNase HI Purification.**

*E. coli* BL21 (DE3) pLysS transformed with pJK502 (expression plasmids with *r*na open reading frame) were grown in Lysogeny Broth (LB) medium with 100 µg/mL ampicillin and 25 µg/mL chloramphenicol at 37 °C. Protein expression was induced by the addition of 1 mM isopropyl-β-thiogalactoside at O.D.₆₀₀ nm ~0.6 and were harvested by centrifugation after 3 hours. Cell pellets were resuspended in 50 mM Tris-HCL, pH 8, 20 mM NaCl, 20 mM MgCl₂, 0.5 mM EDTA and lysed by sonication on wet ice. All subsequent steps were performed at 4 °C. The soluble lysate was loaded onto a Heparin chromatography column, washed with buffer until UV drops to background levels and then developed from 0 to 220 mM KCl. Fractions were run on SDS-
PAGE and significantly purified fractions were pooled, concentrated and dialyzed against 50 mM Tris-HCl, pH 7.0, 1 mM EDTA and subjects to ion-exchange chromatography over SPFF FPLC column with a gradient of 0 to 300 mM NaCl, with RNase HI elution at 200 mM NaCl. Pure fractions were determined by polyacrylamide gel electrophoresis and concentrated to 2 mL and loaded onto Sephadex-100 size-exclusion FPLC column equilibrated in 50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 300 mM NaCl. Pure fractions were determined by polyacrylamide gel electrophoresis and protein concentration was determined using O.D. 280nm in 6.0 M guanidine HCl.

**Isothermal Titration Calorimetry.**

SSB (547µM) (purification described in Chapter 2) and RNase HI (30.9µM) were dialyzed against 20 mM Tris HCl, pH 8.0, 150 mM NaCl, 5% glycerol, 1 mM EDTA, 1 mM β-2-mercaptoethanol. RNase HI was thermostatted in a stirred sample cell (2 ml) at 25 °C and SSB was titrated with the following regime: 2 injections (4 µl for 5 seconds), 2 injections (4 µl for 5 seconds each), then 32 injections (8 µl for 16 seconds each). Data were fitted to a single-site model (1 SSB monomer binding to 1 RNase HI) using a nonlinear iterative least squares algorithm (MicroCal ORIGIN). Since SSB is a tetramer, the binding arrangement in solution is 4 RNase HI monomers per SSB tetramer.

**A1.5 DISCUSSION**

SSB functions as a central organizational protein in genome maintenance through its myriad interactions with protein involved in DNA processing. The majority of our knowledge about SSB interactions results from large scale protein interaction studies as well as studies focused on
specific interactions (Arad et al., 2008; Arifuzzaman et al., 2006; Bonner et al., 1992; Butland et al., 2005; Cadman and McGlynn, 2004; Costes et al., 2010; Davydova and Rothman-Denes, 2003; Genschel et al., 2000; Glover and McHenry, 1998; Han et al., 2006; Hobbs et al., 2007; Hodkinson et al., 2007; Inoue et al., 2008; Kelman et al., 1998; Kozlov et al., 2010; Lu and Keck, 2008; Marceau et al., 2011; Page et al., 2011; Ryzhikov et al., 2011; Shereda et al., 2007; Shereda et al., 2008; Shereda et al., 2009; Slocum et al., 2007; Suski and Marians, 2008; Umezu and Nakayama, 1993; Yuan and McHenry, 2009; Yuzhakov et al., 1999). However, it remains likely that the list of known SSB binding partners is incomplete. The main goal of this project was to expand our knowledge of SSBs interactome, by identifying previously uncharacterized protein interactions.

Ideally the use of N-terminal tagged SSB will allow for the identification of proteins that do not have high affinity binding to SSB or that may be affected by the presence of a C-terminal tag. Indeed I was able to identify several new potential interaction partners of SSB. I validated the interaction of SSB with PriC and RNase HI I using a yeast-two hybrid experiment (Figure A1.1) and SSB’s interaction with MgsA has been characterized in appendix II of this work (Page et al., 2011). In addition, RNase HI was shown by ITC to have a strong affinity and a stoichiometry of 1:1 for SSB. Several other interesting potential interactions were uncovered in this study. I have attempted to characterize an interaction between *E. coli* SSB and FtsZ however I have been unable to conclusively validate the TAP tag findings. Several other interesting findings are highlighted by the different protein pulled down in the stationary phase TAP versus the TAP from cells induced for SOS (by the addition of the DNA damaging agent nalidixic acid) (Table A1.3). The TAP results from *B. subtilis* have yet to be validated but a previously published work
has shown that PriA, RecQ, RecJ, and RecO interact with BsSSBa. Future experiments are needed to increase the interactome of SSB in *B. subtilis*.
Table A1.1 SSB interaction partners

<table>
<thead>
<tr>
<th>Protein</th>
<th>E. coli</th>
<th>B. subtilis</th>
<th>Large scale Identification method</th>
</tr>
</thead>
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<tr>
<td>PriA</td>
<td>+</td>
<td>+</td>
<td>Ec: C-tap; Bs: in vitro studies, in vivo foci formation</td>
</tr>
<tr>
<td>DnaE (Pol III)</td>
<td>+</td>
<td>+</td>
<td>Ec: C-tap; Bs: in vitro studies, in vivo foci formation</td>
</tr>
<tr>
<td>HolC (χ)</td>
<td>+</td>
<td>NA</td>
<td>Ec: C-tap</td>
</tr>
<tr>
<td>DnaG (Primase)</td>
<td>+</td>
<td>U</td>
<td></td>
</tr>
<tr>
<td>RecQ</td>
<td>+</td>
<td>+</td>
<td>Ec: C-tap; Bs: C-tap</td>
</tr>
<tr>
<td>RecJ</td>
<td>+</td>
<td>+</td>
<td>Ec: C-tap; Bs: C-tap</td>
</tr>
<tr>
<td>RecG</td>
<td>+</td>
<td>+</td>
<td>Ec: C-tap; Bs: C-tap</td>
</tr>
<tr>
<td>RecO</td>
<td>+</td>
<td>+</td>
<td>Bs: C-tap</td>
</tr>
<tr>
<td>UDG</td>
<td>+</td>
<td>+</td>
<td>Ec: his-tag; Bs: C-tap</td>
</tr>
<tr>
<td>YpbB/RecS</td>
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<td>+</td>
<td>Bs: C-tap</td>
</tr>
<tr>
<td>YrrC</td>
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<td>+</td>
<td>Bs: C-tap</td>
</tr>
<tr>
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</tr>
<tr>
<td>MgsA</td>
<td>+</td>
<td>+</td>
<td>Bs: C-tap; (Ec: also N-TAP SSB this study)</td>
</tr>
<tr>
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<td>U</td>
<td>Ec: C-tap</td>
</tr>
<tr>
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<td>U</td>
<td>Ec: C-tap</td>
</tr>
<tr>
<td>TopoII</td>
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<td>U</td>
<td>Ec: C-tap</td>
</tr>
<tr>
<td>DNA Pol II</td>
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<tr>
<td>DNA Pol V</td>
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</tr>
<tr>
<td><strong>Total</strong></td>
<td>15</td>
<td>11</td>
<td></td>
</tr>
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</table>

(Arifuzzaman et al., 2006; Butland et al., 2005; Shereda et al., 2008) (Cadman and McGlynn, 2004; Page et al., 2011) (Glover and McHenry, 1998; Handa et al., 2001; Kelman et al., 1998; Lecointe et al., 2007; Molineux and Gefter, 1975; Shereda et al., 2007; Sigal et al., 1972; Suski and Marians, 2008; Umezu and Kolodner, 1994; Umezu and Nakayama, 1993; Yuzhakov et al., 1999)

The papers that describe the interactions in this table are cited above. Not applicable (NA), Unknown (U) – the interaction has not been studied.
Table A1.2 N-TAP SSB interaction partners

<table>
<thead>
<tr>
<th>Ec. Stationary phase</th>
<th>Ec. SOS cells</th>
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</thead>
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<tr>
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<td>PriA</td>
</tr>
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<td>TopoIII</td>
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<td>ExoI</td>
</tr>
<tr>
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<td>RecQ</td>
</tr>
<tr>
<td>RecJ</td>
<td>RecJ</td>
</tr>
<tr>
<td></td>
<td>HolC</td>
</tr>
<tr>
<td></td>
<td>PolB</td>
</tr>
<tr>
<td></td>
<td>RecG</td>
</tr>
<tr>
<td>MgsA*</td>
<td>MgsA*</td>
</tr>
<tr>
<td>RNase HI*</td>
<td>RNase HI*</td>
</tr>
<tr>
<td>DinG*</td>
<td>DinG*</td>
</tr>
<tr>
<td>FtsZ</td>
<td>FtsZ</td>
</tr>
<tr>
<td>PriC*</td>
<td>DnaE</td>
</tr>
<tr>
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<td>DnaQ</td>
</tr>
<tr>
<td>DnaB</td>
<td>DnaX</td>
</tr>
<tr>
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</tr>
<tr>
<td>MinD</td>
<td>HolB</td>
</tr>
<tr>
<td>MinE</td>
<td>HolD</td>
</tr>
<tr>
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<td>FabZ</td>
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<td>SecA</td>
<td>ParC</td>
</tr>
<tr>
<td>RecA</td>
<td></td>
</tr>
<tr>
<td>YbjN</td>
<td></td>
</tr>
<tr>
<td>RNA polymerase</td>
<td></td>
</tr>
</tbody>
</table>

Bold indicates proteins that have been previously shown to interact with SSB. * Indicates interesting potential interactions.
Figure A1.1. Yeast two hybrid positive interactions with SSB

- SSB AD/ pGBD
- pGAD/ SSB BD
- SSB AD/ SSB BD
- holC AD/ SSB BD
- SSB AD/ holC BD
- rna AD/ SSB BD
- SSB AD/ PriC BD
Calorimetric analysis of the RNase HI/SSB interaction for full length SSB and RNase HI. Heats evolved from titration of SSB into a RNase HI solution is shown in the top panels; the derived binding isotherms is shown in the bottom panel.
Table A1.3 Complete list of all proteins pulled down in the TAP experiments

<table>
<thead>
<tr>
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<th>Ec. SOS cells</th>
<th>BsSSBa</th>
<th>BsSSBb</th>
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</thead>
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<td>ParA</td>
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<td>TopoIII</td>
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<td>MutS</td>
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<td>TopA</td>
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<td>RecJ</td>
<td>RecG</td>
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<td>MutSB</td>
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<td>RNase HI</td>
<td>PolB</td>
<td>DnaB</td>
<td>RecJ</td>
</tr>
<tr>
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<td>RecG</td>
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</tr>
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<td>PolC</td>
<td>MreB</td>
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<td>PriC</td>
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<td>HolD</td>
<td>MreB</td>
<td>RuvB</td>
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<td>FabZ</td>
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<td>RNA polymerase</td>
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<td>RecF</td>
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<td>OmpF</td>
<td>HfdD</td>
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<td>Ribosomal proteins</td>
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<tr>
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A1.6 References


Appendix II

Structure and biochemical activities of *Escherichia coli* MgsA

The work shown in this appendix was conducted by Asher N. Page, Nicholas P. George. Aimee Marceau discovered the interaction between MgsA and SSB as shown in appendix one. The authors thank Elizabeth A. Wood for construction of the MgsA expressing plasmid pEAW354, and Darryl R. McCaslin for assistance with the execution and analysis of the analytical ultracentrifugation experiments. The work was published: Page, AN, George, NP, Marceau, AH, Cox, MM & Keck, JL (2011) “Structure and biochemical activities of *Escherichia coli* MgsA” *Journal of Biological Chemistry* **286**, 12075-12085.
A2.1 Summary

Bacterial “Maintenance of Genome Stability Protein A” (MgsA) and related eukaryotic enzymes play important roles in cellular responses to stalled DNA replication processes. Sequence information identifies MgsA enzymes as members of the clamp-loader clade of AAA\(^+\) proteins, but structural information defining the family has been limited. Here, the X-ray crystal structure of \textit{E. coli} MgsA is described, revealing a homotetrameric arrangement for the protein that distinguishes it from other clamp-loader-clade AAA\(^+\) proteins. Each MgsA protomer is comprised of three elements: ATP-binding and helical lid domains (conserved among AAA\(^+\) proteins) and a tetramerization domain. Although the tetramerization domains bury the greatest amount of surface area in the MgsA oligomer, each of the domains participates in oligomerization to form a highly intertwined quaternary structure. Phosphate is bound at each AAA\(^+\) ATP-binding site but the active sites do not appear to be in a catalytically competent conformation due to displacement of Arg finger residues. \textit{E. coli} MgsA is also shown to form a complex with the single-stranded DNA-binding protein (SSB) through co-purification and biochemical studies. MgsA DNA-dependent ATPase activity is inhibited by SSB. Together, these structural and biochemical observations provide insights into the mechanisms of MgsA-family AAA\(^+\) proteins.

A2.2 Introduction

In bacteria, DNA replication forks can stall when they encounter DNA lesions, template strand breaks, DNA-bound proteins or other impediments. Stalled replication forks occur as often as once per cell generation during normal cell growth and must be repaired (Cox, 2002; Cox et al.,
There are multiple causes of replication fork stalling and/or collapse, and a range of recovery mechanisms that likely reflect the variety of DNA/protein structures that are encountered when these events occur. Most of the repair pathways involve recombinational DNA repair and the RecA recombinase (Cox, 2002; Cox et al., 2000; Kowalczykowski, 2000; Kuzminov, 1999; Marians, 2000; Michel et al., 2004).

MgsA (also called RarA; bacteria), Mgs1 (yeast), and Wrin1 (mammals) constitute a family of evolutionarily conserved proteins with roles in the recovery of stalled replication processes. *E. coli* MgsA and its *Saccharomyces cerevisiae* and *Homo sapiens* homologues share ~40% sequence identity and over 55% similarity (Barre et al., 2001) and are members of the AAA⁺ (ATPases associated with a variety of cellular activities) family of proteins (Neuwald et al., 1999). As implied by their name, AAA⁺ proteins are involved in diverse cellular pathways including protein degradation, DNA metabolism, and membrane fusion (Brunger and DeLaBarre, 2003; Davey et al., 2002; Duderstadt and Berger, 2008; Erzberger and Berger, 2006; Erzberger et al., 2006; Hanson and Whiteheart, 2005). AAA⁺ proteins contain a conserved 200-250 residue nucleoside triphosphate (NTP)-binding/hydrolysis domain and generally function as oligomeric molecular machines. The NTP binding sites of AAA⁺ proteins are typically positioned at the interface of adjacent subunits allowing for intersubunit coupling of NTP binding and hydrolysis events to remodeling of target macromolecules (Gai et al., 2004; Wang et al., 2001).

AAA⁺ proteins have been classified into various clades based on their sequences and structures (Ammelburg et al., 2006; Erzberger and Berger, 2006; Iyer et al., 2004). Based on these studies, the MgsA/Mgs1/Wrin1 family is grouped with bacterial and eukaryotic clamp loader proteins in the “clamp loader” AAA⁺ clade. The clamp loader clade is characterized by a
minimal AAA+ fold (Erzberger and Berger, 2006) and a SR[CAT] motif that contains a conserved “Arg finger” residue that is important for NTP hydrolysis (Iyer et al., 2004). The clamp loader proteins load processivity clamps onto primer/template junctions during DNA replication (Bowman et al., 2005; O'Donnell, 2006; O'Donnell and Kuriyan, 2006; Turner et al., 1999; Yao et al., 2006). Representative clamp loader structures reveal the complexes to be heteropentamers possessing an oligomerization domain C-terminal to the AAA\(^+\) module (Bowman et al., 2004a; Jeruzalmi et al., 2001). However, insights into the structures of the MgsA-family proteins have thus far been lacking.

While its precise function has remained elusive, genetic experiments suggest *E. coli* MgsA is important in facilitating the recovery of stalled replication forks (Hishida et al., 2001; Lestini and Michel, 2007; Shibata et al., 2005). MgsA function is a prerequisite to RecA protein loading at stalled forks in a temperature-sensitive *dnaE* mutant (encodes the catalytic subunit of the replicative DNA Polymerase III holoenzyme), but not in a temperature-sensitive *dnaN* mutant (encodes the processivity β clamp) (Lestini and Michel, 2007). This implies that MgsA is involved in the processing of some stalled replication forks but not others and that this processing may allow, or perhaps promote, RecA loading. Inasmuch as the replisome usually remains intact at the site of a fork when fork stalling occurs in the *dnaEts* mutant, but not at stalled forks in the *dnaNts* mutant, it is possible that MgsA somehow facilitates replisome disassembly prior to fork repair. Effects of the *dnaE486* mutant, another temperature sensitive mutant of the catalytic subunit of DNA Polymerase III, also suggest a role for MgsA in the processing of stalled forks and maintaining genome stability (Shibata et al., 2005). The *dnaE486* mutant has a growth defect and forms filamented cells, indicative of SOS induction, at a semi-restrictive temperature (Kelman and O'Donnell, 1995; Vandewiele et al., 2002). Interestingly, an
mgsA deletion suppresses the growth defect of dnaE486 cells but produces a high proportion of anucleate cells, suggesting that MgsA may prevent aberrant DNA replication and allow for RecA filament formation and SOS induction. Similar results have been seen with recA and recQ mutants (Hishida et al., 2004; Shibata et al., 2005); RecQ helicase is thought to unwind DNA at stalled replication forks generating ssDNA onto which RecA can be loaded. These data indicate that MgsA promotes replication fork repair in some E. coli DNA replisome mutants, possibly through a RecA-dependent pathway. Related to its proposed functions in replication, fluorescently-tagged MgsA localizes to the replisome (Lau et al., 2003; Sherratt et al., 2004), although how this localization is achieved is not known.

Studies of the S. cerevisiae homologue of MgsA, Mgs1, also yield insights into functions of the larger protein family in DNA metabolism. Post-replication repair (PRR) encompasses the error-prone and error-free pathways of repairing stalled replication forks in S. cerevisiae. The Mgs1 protein maintains genome stability by suppressing the error-prone RAD6 PRR pathway in the absence of exogenous DNA damage (Hishida et al., 2002; Hishida et al., 2006). The RAD6 and RAD52 pathways define two of the major PRR processes in S. cerevisiae. The RAD52 pathway utilizes homologous recombination in an error-free mechanism. Interestingly, when the RAD6 pathway is inhibited by rad6 or rad18 mutations, deleting mgs1 is lethal, however activation of the RAD52 pathway by overexpressing Rad52 or deleting srs2 suppresses this lethality (Hishida et al., 2002; Hishida et al., 2006). This suggests that Mgs1 modulates the response to stalled replication forks and may be involved in a novel PRR pathway. Mgs1 also physically interacts with the processivity factor PCNA in yeast (Hishida et al., 2006), which plays a pivotal role in the channeling of stalled forks into the various repair pathways (Hoege et
Formation of the Mgs1-PCNA interaction could be important in PRR pathway selection.

Data implying roles in replication fork repair for the MgsA protein family extends to humans. *H. sapiens* Wrinp1, the human MgsA homolog, possesses an ubiquitin-binding zinc finger (UBZ) domain that is involved in its localization to the replisome (Bish and Myers, 2007; Crosetto et al., 2008). Treatment of cells with fork stalling agents causes an increase in the number of Wrinp1 foci suggesting that Wrinp1 is prevalent at stalled replication forks (Crosetto et al., 2008). The translesion synthesis DNA polymerases η and κ (Bienko et al., 2005; Guo et al., 2006) and ubiquitin ligase RAD18 (Crosetto et al., 2008; Nakajima et al., 2006; Watanabe et al., 2004), which are heavily involved in the recovery of stalled replication forks, also possess UBZ domains and localize to stalled replication forks in a UBZ-dependent manner. Post-translational modifications, such as ubiquitination and sumoylation of replisome machinery, are known to be important regulatory pathways for DNA replication, recombination and repair (Haracska et al., 2004; Watts, 2006). The UBZ domain of Wrinp1 controls its localization to stalled replication forks with post-translational modifications to replisome components likely functioning as the signal.

To better define the structure and function of the MgsA/Mgs1/Wrinp1 protein family, we have determined the X-ray crystal structure of *E. coli* MgsA and characterized its biochemical activities. MgsA is distinct among clamp-loader-clade AAA^{+} proteins characterized to date in that it assembles as a homotetramer. MgsA protomers are comprised of AAA^{+} and tetramerization domains, each of which participates in oligomerization to form a highly intertwined quaternary structure. Phosphate is bound at each AAA^{+} ATP-binding site but the active sites are in a catalytically incompetent conformation due to displacement of Arg finger
residues. MgsA is furthermore shown to form a complex with SSB through co-purification and in vitro binding studies. This interaction may help target MgsA to the replication fork in vivo. Together, these studies help define the structural and biochemical mechanisms that underpin MgsA activity.

A2.3 Experimental Procedures

DNA Substrates. Circular single-stranded DNA was prepared from bacteriophage M13mp18 using previously described methods (Haruta et al., 2003; Neuendorf and Cox, 1986). The concentrations of bacteriophage ssDNA was determined by absorbance at 260 nm, using 36 μg/ml•A_{260 nm} as conversion factor. All DNA concentrations are reported as total nucleotides (μM).

Protein Purification. The open reading frame of E. coli mgsA (strain MG1655) was amplified by PCR and subcloned in frame into NdeI/BamHI-digested pET21A (Novagen). The open reading frame of the resulting plasmid (pEAW354) was sequenced to confirm the sequence integrity. The nuclease-deficient E. coli K12 strain STL2669 [Δ(recA-srlR)306::Tn10 xonA2(sbcB^-)], a gift from Susan T. Lovett (Brandeis University), was transformed with pEAW354 and pT7 pol26 (Mertens et al., 1995). Cells were grown in Luria-Bertani medium containing 100 μg/ml ampicillin and 40 μg/ml kanamycin at 37 °C to an A_{600 nm} of ~0.5. MgsA protein over-expression was induced by the addition of 0.8 mM isopropyl 1-thio-β-d-galactopyranoside (IPTG) and growth at 37 °C for 4 hours. All subsequent steps were performed at 4°C. Pelleted cells were resuspended in sucrose solution (25% (w/v) sucrose, 250 mM Tris-HCl (80% cation), pH 7.7, 7 mM ethylenediaminetetraacetic acid (EDTA), 1 μM Pepstatin, 1
µM Leupeptin, 1 µM E-64) and lysed by addition of lysozyme to a final concentration of 1.6 g/l and sonication. Insoluble material was removed by centrifugation. MgsA was precipitated from the cell lysate supernatant by the addition of solid NH₄(SO₄)₂ to 35% saturation. In all subsequent steps, 1 mM dithiothreitol (DTT) was added to buffers. The NH₄(SO₄)₂ pellet was resuspended in 20 mM Tris-Cl (80% cation), pH 7.7, 1 mM EDTA, 10% glycerol (R buffer) containing 450 mM NH₄(SO₄)₂ and applied to a butyl-sepharose (Amersham Biosciences) column. MgsA was eluted using a NH₄(SO₄)₂ linear gradient from 600 mM to 0 mM in R buffer. MgsA fractions were pooled, dialyzed into P buffer (20 mM phosphate, pH 7.0, 1 mM EDTA and 10% glycerol), and flowed through a ceramic hydroxyapatite (Bio-Rad) column. MgsA in the flow through was precipitated by adding an equal volume of R buffer (no glycerol) containing 90% saturated NH₄(SO₄)₂, then resuspended in R buffer (<5 mL) and applied onto a HiPrep 16/60 Sephacryl S-300 (Amersham Biosciences) column equilibrated in R buffer supplemented with 1.2 M NaCl. MgsA eluted in three peaks; the third peak contained the most MgsA protein and was pooled. The resulting MgsA protein was >95% pure by gel and was dialyzed into R buffer, aliquoted, and flash frozen in liquid nitrogen and stored at -80°C. The purified protein was free of any detectable nuclease activity. The concentration of the MgsA protein was calculated using the native extinction coefficient 5.44x10⁴ M⁻¹cm⁻¹. The native extinction coefficient was determined as described previously (Edelhoch, 1967).

To express an Arg156Ala MgsA variant, pEAW354 was used as a PCR template with the same upstream primer used in constructing pEAW354, and a downstream primer consisting of bases 521-453 of the mgsA gene. Bases 521-516 include a ScaI restriction enzyme site. The bases ACG at 468-466 coding for the reverse complement of Arg were changed to CGC to code for the reverse complement of Ala. The PCR product was digested with NdeI and ScaI and
substituted for the equivalent fragment in pEAW354 to create pEAW662. The presence of the Arg156Ala codon change was confirmed by sequencing. The MgsA Arg156Ala protein was purified identically to wild-type MgsA.

*E. coli* SSB (Shan et al., 1996), SSBΔC8 (Hobbs et al., 2007), SSBΔC1 (Shereda et al., 2009) and SSB-mixed (Lu and Keck, 2008) proteins were purified as described. The concentration of all SSB protein variants was determined using the extinction coefficient of 2.83x10^4 M^{-1}cm^{-1} (Lohman and Overman, 1985).

**MgsA Crystallization and Structure Determination.** *E. coli* MgsA (4 g/l in R buffer) was crystallized by suspending 1 µl protein mixed with 1 µl mother liquor solution (10 mM MES, pH 6.6, 38% Ethylene Glycol, 16% Glycerol) over 1 ml of mother liquor solution in a hanging-drop vapor-diffusion experiment. Crystals formed after several weeks at room temperature and were frozen in liquid nitrogen directly from the drop.

Diffraction data were indexed and scaled using HKL2000 (Otwinowski and Minor, 1997) and molecular replacement was carried out with Phaser (McCoy et al., 2007) using a structure of the tetramerization domain from *H. influenza* MgsA as a partial model (PDB 3BGE). Phase estimates from molecular replacement were sufficient to produce 2F_o-F_c and F_o-F_c electron density maps that permitted model building of the AAA^+ domain through repetitive cycles of manual structure building using COOT (Emsley and Cowtan, 2004) and refinement by REFMAC5 (Winn et al., 2001). Coordinate and structure factor files have been deposited at the Protein Data Bank (PDB ID 3PVS)

**Analytical Ultracentrifugation.** A sample of MgsA was dialyzed overnight against 20 mM Tris-Cl (80% cation), pH 7.7, 150 mM NaCl, 0.1 mM EDTA, then diluted with dialysis
buffer to create samples at 3.8, 8.3 and 15 µM. Centrifugation was performed using a Beckman XLA analytical ultracentrifuge, with measurements carried out at 20 °C. Equilibrium data were collected at speeds of 3,600, 4,800, 5,800, 7,800 and 10,000 rpm followed by a final experiment at 5,800 rpm to test for non-reversible aggregation during the experiment. The presence of non-sedimenting baseline was established by a high-speed spin to deplete all protein material. Samples were assumed to be at equilibrium when gradients collected 3 or more hours apart were superimposable. The density of the buffer was computed as 1.0039 g/ml using the density increment approach (Laue and Shah, 1992). The partial specific volume of the protein was computed from the amino acid sequence to be 0.732 ml/g. All data were processed and analyzed using Beckman provided software or programs written for Igo Pro (Wavemetrics, Inc, Lake Oswego, OR). Semi-log absorbance data as a function of squared radial distance were globally fitted using models containing one or two molecular species (Laue, 1995). Inclusion of a second species slightly improved the fits. The best fit was generated using a model where the dominant species is tetrameric, with a smaller amount of octamer present.

**ATPase Assay.** A coupled spectrophotometric enzyme assay (Lindsley and Cox, 1990; Morrical et al., 1986) was used to measure the ATPase activities of MgsA. The assays were carried out in a Varian Cary 300 dual beam spectrophotometer equipped with a temperature controller and a 12-position cell changer. The cell path lengths are 1 cm and band pass was 2 nm. The reaction were carried out at 37 °C in 25 mM Tris-Acetate (80% cation), 1 mM DTT, 3 mM potassium glutamate, 10 mM Mg(OAc)$_2$, 5% (w/v) glycerol, an ATP regeneration system (10 units/ml pyruvate kinase, 2.2 mM phosphoenolpyruvate), a coupling system (3 mM NADH and 10 units/ml lactate dehydrogenase) and the indicated concentrations of MgsA, MgsA Arg156Ala, SSB, SSBΔC8 and DNA.
**Tandem Affinity Purification.** The open reading frame of *E. coli mgsA* was amplified by PCR and subcloned in frame into a specialized cloning vector pCN70 (C. Norais and M. M. Cox, unpublished data) to produce an expression vector encoding MgsA with an N-terminal dual affinity tag (includes Protein A and calmodulin peptide binding domains separated by a TEV protease cleavage site). The open reading frame of the resulting plasmid (pTAP-MgsA) was sequenced to confirm the sequence integrity. *E. coli* K12 strain MG1655 (DE3) transformed with pTAP-MgsA was grown at 37 °C in 4 L of Luria-Bertani medium supplemented with 50 µg/ml ampicillin to midlog phase (OD₆₀₀ of ~0.5), induced by the addition of 1 µM IPTG and grown for 3 additional hours. Cells were harvested by centrifugation and suspended in 50 ml NP-40 buffer (6 mM dibasic sodium phosphate, 4 mM monobasic sodium phosphate, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 4 mg/l leupeptin, 0.1 mM sodium vanadate, 19.5 mg/l benzamidine, 8.7 mg/l phenylmethylsulfonyl fluoride (PMSF), 1% Nonidet P-40 substitute (NP-40)) and lysed by sonication. Soluble lysate was incubated for 1 hour at 4 °C with IgG-Sepharose beads (preequilibrated in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40); beads were then washed with 3 volumes of equilibration buffer. TEV cleavage buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1 mM DTT) with 15 µl of 15 µM TEV protease was incubated with the lysate/bead mixture with shaking for ~12 hours at 16 °C. The eluent was incubated with 300 µl calmodulin affinity resin (Stratagene) and 3 µl 1 M CaCl₂ in calmodulin binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 10 mM magnesium acetate, 1 mM imidazole, 10 mM 2-mercaptoethanol) for 1 hour with shaking. Resin was washed with calmodulin binding buffer and TAP-MgsA was eluted first with a high salt buffer (calmodulin binding buffer adjusted to 1 M NaCl) and then with EGTA Elution Buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% NP-40, 1 mM magnesium acetate, 10 mM imidazole, 10
mM 2-mercaptoethanol, 20 mM ethyleneglycolbisaminoethyltetraacetic acid (EGTA)). High-salt and EGTA-eluted protein samples were precipitated with trichloroacetic acid (25% w/v) on ice for 30 minutes, pelleted by centrifugation, washed twice with ice-cold acetone, and suspended in 30 µl gel buffer. After SDS-PAGE, individual bands were excised, digested with trypsin, and subjected to MALDI-TOF mass spectrometry for identification of peptides (University of Wisconsin Mass Spectrometry facility).

**Surface Plasmon Resonance (SPR).** All SPR measurements were performed on a Biacore 2000 equipped with a sensor chip SA (GE Healthcare). The sensor chip SA was pretreated with three 1 min pulses of 1 M NaCl at 100 µl/min, after which 0.25 pmol (~300 response units (RU)) of biotinylated-d(T)$_{35}$ was flowed onto the cell at 5 µl/min in TBS200 (200 mM NaCl, 20 mM Tris, pH 7.5 0.01% Tween 20, 3 mM EDTA and 1 mM DTT). The chip was further rinsed with TBS200 at 100 µl/min for 5 minutes, then flushed with TBS50 (50 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.01% Tween 20, 3 mM EDTA and 1 mM DTT.) SSB diluted into TBS50 was subsequently loaded to the chip surface at 200 nM (tetramer) until saturated (~1500 RU) then rinsed with TBS50 until stable. For SPR measurements with MgsA, various concentrations (50 nM to 1500 nM) of MgsA in TBS50 were flowed through the flow cell for 90 seconds at 30 µl/min then MgsA was allowed to dissociate for 12 minutes. Raw RU were background-subtracted against an empty flow cell (~100 RU at the highest MgsA concentration used) and the maximum RU for each run were plotted against concentration of MgsA and fit to obtain equilibrium binding data using the GraphPad Prism program (Version 4.02, SmartDrawNet).
A2.4 Results

X-ray crystal structure of *E. coli* MgsA. Together with the bacterial and eukaryotic processivity clamp loader proteins, the MgsA family of proteins forms the “clamp loader clade” of AAA$^+$ proteins [Reviewed in (Erzberger and Berger, 2006; Iyer et al., 2004)]. Whereas the structures and direct functions of the clamp loader proteins are well established (Bowman et al., 2004b; Jeruzalmi et al., 2001), similar information defining the MgsA family is lacking. A crystallographic approach was therefore taken to better understand how MgsA proteins function.

*E. coli* MgsA formed crystals that diffracted X-rays to 2.5 Å resolution (Table 1). Experimental phasing experiments with selenomethionine-incorporated protein were not successful due to poor solubility of the derivative proteins. However, the structure of a domain from the *Haemophilus influenzae* MgsA protein was available for use as a molecular replacement model (PDB code: 3BGE; unpublished result from the New York SGX Research Center for Structural Genomics available from the RCSB Protein Data Bank). This domain comprised the C-terminal domain of *H. influenzae* MgsA and shared 82% identity with the same domain from *E. coli* MgsA. Molecular replacement produced difference electron density maps that permitted building of the AAA$^+$ domain of *E. coli* MgsA through several rounds of manual model building and structure refinement.

The crystal structure of *E. coli* MgsA revealed three apparent structural domains in the protein: N-terminal ATP-binding (residues ~22-165) and adjacent helical lid domains (residues ~166-247 and a short N-terminal helix (14-21)) that are conserved among AAA$^+$ proteins and a C-terminal tetramerization domain (residues ~251-446) (Figure 1A). MgsA assembled as a highly intertwined pseudosymmetric tetramer in the crystal in which each protomer physically
contacts each of the three remaining subunits in the tetramer (Figure 1B). The tetramerization domains contribute the bulk of the buried surface area in the tetramer (burying 2069-2168 Å² of surface area between adjacent molecules) with more modest contributions from the AAA\(^+\) domain (413-457 Å²). Overall, an average of 2573 Å² is buried between pairs of MgsA proteins within the tetramer. This is more buried surface area than in the \textit{E. coli} clamp loader (1JR3) (Jeruzalmi et al., 2001), in which 470-1133 Å² and 383-1450 Å² are buried between the oligomerization and AAA\(^+\) domains, respectively. Sedimentation equilibrium analytical ultracentrifugation experiments confirmed that MgsA forms primarily a tetramer in solution. Previous gradient centrifugation studies of the human MgsA homologue, Wmip1, identified it as a homo-octamer (Tsurimoto et al., 2005). Whether the differences between the apparent quaternary structures of MgsA and Wmip1 are a result of sequence differences or of differences in the approaches used to examine higher order assemblies is not clear.

The conservation of each residue in \textit{E. coli} MgsA was analyzed across 157 MgsA homologs using the Consurf program (Ashkenazy et al.). The active site pocket and the surrounding area are extremely conserved, as are the Arginine finger and several neighboring residues (Figure 1C and 1D). The final region that is highly conserved is the surface within the tetramerization domains that forms the interface between neighboring subunits. Collectively, the conservation of residues in the active site and the protomer-protomer interface suggest a common mode of ATP hydrolysis and oligomerization throughout MgsA homologues. The interior cavity of the MgsA tetramer is relatively poorly conserved and is generally neutral to modestly positively charged.

\textit{AAA\(^+\) and tetramerization domains of E. coli MgsA.} The N-terminal AAA\(^+\) domain includes both the ATP-binding core and a conserved helical lid element that are commonly found
among AAA⁺ proteins. The ATP-binding core domain forms a RecA-like fold in which a central 5-stranded parallel β-sheet is sandwiched by α-helices (Figure 1A). The helical lid element is C-terminal to the ATP-binding core in the AAA⁺ domain. These first two domains are responsible for NTP binding and hydrolysis in AAA⁺ proteins (Duderstadt and Berger, 2008; Erzberger and Berger, 2006; Hanson and Whiteheart, 2005). The ATP-binding and helical lid domains of MgsA overlay with the related domains from the *E. coli* clamp loader with root mean square deviations (RMSDs) of 2.9 Å over 151 residues and 1.7 Å over 75 residues, respectively, demonstrating the considerable structural conservation within the AAA⁺ family.

The C-terminal tetramerization domain is comprised of several α-helices that pack alongside neighboring subunits to mediate MgsA oligomerization. Overall, the oligomerization domains form a tightly bound collar and the N-terminal AAA⁺ domains extend out from the collar in a splayed out arrangement, similar to the architecture seen in the inactive state of the *E. coli* clamp loader (Kazmirski et al., 2004). The most striking differences between the structure of MgsA and the clamp-loader complexes are that MgsA assembles as a tetramer as opposed to the pentameric arrangement observed for the clamp loaders and that the collar domain of MgsA forms a closed oligomer rather than the open arrangement of the clamp loader complex. Comparison of the MgsA structure to that of the *E. coli* clamp loader reveals an additional ~100 residues on the C-terminus of MgsA, not present in the clamp loader, that make extensive contact with the neighboring protomer and drive formation of a tetramer (Figure 2).

*MgsA ATPase active site architecture.* The ATPase active sites in AAA⁺ proteins are formed at the interface between neighboring subunits and the elements that contribute to nucleotide binding and hydrolysis are both well conserved and well characterized for several family members (reviewed in (Hanson and Whiteheart, 2005; Iyer et al., 2004; Ogura et al., 2004).
In the MgsA structure, phosphate ions were found in proximity to all four of the ATP-binding sites (Figure 1B), suggesting that the protein most likely catalyzes ATP hydrolysis in a manner that is similar to that observed with other AAA$^+$ proteins. The binding sites are essentially identical in all four subunits and are in open positions.

The P loop (or Walker-A motif), located on the loop following $\beta$1, is a key ATP binding and hydrolysis element of AAA$^+$ and related proteins (Walker et al., 1982). The consensus sequence of the P loop includes a conserved lysine residue that contacts the $\gamma$-phosphate of ATP and is essential for ATP hydrolysis in AAA$^+$ proteins (Duderstadt and Berger, 2008; Erzberger and Berger, 2006; Hanson and Whiteheart, 2005). The conserved Lys63 residue in MgsA appears to perform this function, as demonstrated by mutational analysis of the yeast Mgs1 homologue (Hishida et al., 2001). Consistent with this role in ATP binding/hydrolysis, the nitrogen of Lys63 is positioned ~4 Å from the phosphate ions in each of the MgsA protomers (Figure 3). Additionally, backbone amide groups from other residues within the MgsA P loop (Gly60, Thr61, Gly62 and Lys63) are directed towards the phosphate ion, creating a basic pocket that we predict would accommodate the triphosphate of ATP.

The metal-binding Walker-B motif is a second integral and well characterized element of ATPases (Walker et al., 1982). Located at the apex of $\beta$3, the MgsA Walker-B motif contains two conserved acidic residues, Asp113 and Glu114 (Figure 3). The R-group of Asp113 is located ~6 Å from the active site phosphate, consistent with the placement of the analogous residue in the *S. cerevisiae* clamp loader structure (Bowman et al., 2004a). This distal positioning would allow it to coordinate Mg$^{2+}$ situated next to the $\beta$- and $\gamma$-phosphates of ATP (Iyer et al., 2004). The acidic R-group of Glu114 is also directed towards the phosphate ion and the active site, putting it in position to prime a water molecule for nucleophilic attack (Story and Steitz, 1992).
The sensor 1 and 2 elements are additional conserved elements in AAA\textsuperscript{+} proteins that contact the nucleotide and mediate structural changes throughout the protein in response to hydrolysis (Guenther et al., 1997). The sensor 1 region, located at the apex of β4, sits between the P loop and the Walker-B motif (Figure 3). Conserved polar residues in this region have been implicated in ATP hydrolysis in AAA\textsuperscript{+} proteins (Karata et al., 1999; Steel et al., 2000). There are two Thr residues in the sensor 1 region of MgsA; in the crystal structure, the R group of Thr142 is directed towards the active site whereas the neighboring Thr141 is directed away from the active site. The sensor 2 region is located in the α-helical bundle and packs against the P loop (Figure 3). An Arg in the region, Arg209 (conserved in clamp loaders (Guenther et al., 1997)), binds the phosphate ion, indicating that it is in a good location for sensing nucleotide binding/hydrolysis and transmitting conformational changes. Sequence changes in this Arg are known to lead to diminished ATP binding (Lew and Gralla, 2002; Rombel et al., 1999) in the AAA\textsuperscript{+} proteins NtrC and PspF and ATP hydrolysis in the AAA\textsuperscript{+} protein DnaA (Nishida et al., 2002). The effects of mutating the Thr141 and Arg209 residues in MgsA have not yet been studied, but the presented structure suggests roles for the residues in ATP binding and/or hydrolysis.

The Arg finger is a highly conserved residue in AAA\textsuperscript{+} proteins that makes contact with the γ-phosphate of ATP in the neighboring subunit and is an essential element of the active site (Ogura et al., 2004). Interestingly, the Arg finger of MgsA, Arg156, is positioned ~40 Å from the active site of the neighboring protomer, suggesting that the presented MgsA structure represents a form of the molecule that is not competent for ATPase activity (Figure 2C). To confirm that Arg156 is in fact required for activity, an MgsA variant protein in which the residue was changed to an Ala was purified and tested. The Arg156Ala variant is ATPase deficient.
(described further below), consistent with the prediction that Arg156 functions as the Arg finger in MgsA. We hypothesize that ATP binding induces structural changes that positions the Arg finger near the nucleotide of the adjacent protomer and generates a functional active site. Based on results presented below, we further predict that DNA binding assists this conformational rearrangement. This is similar to what has been observed with *S. cerevisiae* clamp loader protein (Bowman et al., 2004a). In that case, the AAA⁺ modules form tighter interfacial contacts when bound to its cognate processivity clamp and non-hydrolyzable nucleotide, and the active site residues are hypothesized to be optimally positioned for ATP hydrolysis. The two subdomains of AAA⁺ proteins, the ATP-binding and lid domains, tend to be further apart in the NTP-free state and closer together in the nucleotide bound state (Hanson and Whiteheart, 2005). In the *S. cerevisiae* clamp loader structure, the two subdomains are rotated close together to create a tight nucleotide binding site. In MgsA, a rotation of the ATP-binding core towards the lid would tighten the interface between the AAA⁺ modules and may trigger positioning of the Arg finger of one protomer near the active site of the adjacent protomer (Figure 2C). A similar ATP-dependent interdomain pivoting has been described for the SV40 T-antigen AAA⁺ protein, (Gai et al., 2004).

*MgsA physically interacts with SSB.* Cellular binding partners that are thought to target eukaryotic Mgs1 and Wrnip1 proteins to sites of DNA replication are known (Bish and Myers, 2007; Crosetto et al., 2008; Hishida et al., 2006), but similar studies have not been carried out with bacterial MgsA. To better understand the functions of MgsA, tandem-affinity purification (TAP) (Rigaut et al., 1999) was used to identify cellular interaction partners of *E. coli* MgsA. An N-terminal TAP-tagged MgsA protein was expressed in *E. coli* to facilitate complex formation with its cellular protein partners. A complex including TAP-MgsA and associated proteins was
purified by TAP and the purified components were resolved via SDS-PAGE (Figure 4). Distinct bands were apparent on the gel and each was excised and identified by mass spectrometry. Two different proteins were present in the preparations: MgsA and the single-strand DNA-binding protein (SSB). The other bands are degradation products of MgsA or SSB. MgsA was also identified in a reciprocal experiment with an N-terminal TAP-tagged SSB but, interestingly, was not identified in an earlier study using a C-terminal TAP-tagged SSB (Butland et al., 2005). These results show that MgsA and SSB proteins interact in a manner that could require the native SSB C-terminal structure.

*E. coli* SSB is composed of two functional domains: an N-terminal DNA-binding domain and a structurally dynamic C-terminal tail ending in a highly conserved amphipathic sequence (SSB-Ct) that mediates interactions with diverse genome maintenance proteins (Shereda et al., 2008). A biochemical approach using SPR was used to examine the interaction between MgsA and SSB interaction and to determine whether this interaction depended upon the SSB-Ct. SSB was flowed over a biotinylated dT₃₅ ssDNA bound to an SPR sensor chip. Under the conditions tested, SSB binding to the ssDNA appears irreversible as evidenced by the stable signal when buffer alone is flowed over the chip. MgsA was subsequently flowed over the SSB/ssDNA substrate and binding was monitored by the change in response units. Analysis of binding experiments with different MgsA concentration confirmed its interaction with SSB and showed that the MgsA-SSB/ssDNA complex has an apparent dissociation constant (Kₐ) of 0.36 ± 0.02 μM (Figure 5). This complex stability is consistent with the Kₐ range expected from other SSB/protein interactions (Shereda et al., 2008).

SSB variants with altered SSB-Ct sequences were substituted in the SPR assay to test the predicted importance of the SSB-Ct for the MgsA–SSB interaction. The *E. coli* SSB-Ct sequence
(Asp-Phe-Asp-Asp-Ile-Pro-Phe) is highly conserved among bacteria with the C-terminal-most Phe being invariant (Shereda et al., 2008). This conservation reflects the importance of the residues in the SSB-Ct in protein interactions. The variants tested in the MgsA interaction assay were SSBΔC8 (lacks the entire SSB-Ct), SSBΔC1 (lacks the C-terminal-most SSB-Ct Phe), and SSB-mixed (final 8 residues are rearranged). The most severe binding defect was seen with the SSBΔC8 protein. Substituting SSBΔC8 for wild-type SSB weakened the affinity of MgsA for SSB by ~20-fold while the overall change in response units at the highest MgsA concentration was about one-fourth of that observed with wild-type SSB. More modest binding defects were seen with SSBΔC1 and SSB-mixed. The SSBΔC1 variant had ~4-fold weaker binding affinity for MgsA indicating that the C-terminal-most residue is important in the MgsA-SSB interaction. Similarly, substituting the SSB-mixed protein weakened the MgsA-SSB interaction by ~7-fold. Collectively, these data demonstrate the importance of the SSB-Ct for the MgsA-SSB interaction.

**MgsA ATPase activity.** The yeast homologue of MgsA, Mgs1, was previously shown to be a DNA-dependent ATPase (Hishida et al., 2001). *E. coli* MgsA was therefore tested for ATPase activity. In the absence of DNA, MgsA hydrolyzed ATP at a very low level (3.39 +/- 0.29 min⁻¹) (Figure 6A). Addition of M13mp18 circular ssDNA (cssDNA) to the reactions strongly stimulated MgsA ATPase activity in a DNA-concentration-dependent manner (up to 70.6 +/- 4.4 min⁻¹ with 20 µM cssDNA) (Figure 6B). This stimulation of ATPase appeared to level out at a ratio of approximately 600-1000 nucleotides of DNA per MgsA tetramer.

The effect of SSB on MgsA DNA-dependent ATP hydrolysis was investigated. When SSB was pre-incubated with the DNA prior to MgsA addition, low concentrations of SSB appeared to have little effect on ATP hydrolysis whereas higher SSB concentrations
(approaching the levels needed to saturate the cssDNA) inhibited ATP hydrolysis (Figure 6C). Supersaturating levels of SSB had little additional inhibitory effects, suggesting that the protein was indirectly inhibiting MgsA by competing for binding to the DNA substrate. Interestingly, when SSBΔC8 was used in the assay, ATP hydrolysis inhibition was apparent at lower SSB concentrations than observed with wild-type SSB (Figure 6D). This could be due to the higher affinity binding by SSBΔC8 compared to full-length SSB (Kozlov et al.) and/or due to the impaired interaction between MgsA and SSBΔC8.

A2.5 Discussion

Bacterial MgsA and its eukaryotic homologs, Mgs1 and Wnpi1, comprise a family of AAA\(^+\) enzymes that function in cellular responses to replication fork stalling. How these proteins function and the identity of their substrates are not clear, but their importance in genome maintenance is well documented (Hishida et al., 2001; Hishida et al., 2002; Hishida et al., 2006; Shibata et al., 2005; Yoshimura et al., 2009). To better understand the structure and function of MgsA/Mgs1/Wnpi1-family enzymes, we have determined the 2.5-Å resolution X-ray crystal structure of \textit{E. coli} MgsA. The structure reveals a homotetrameric structure for MgsA that is distinct from other clamp-loader-clade AAA\(^+\) proteins, which function as heteropentamers. The arrangement of the MgsA AAA\(^+\) domains does not appear to be competent for catalysis, since the conserved Arg finger elements are not adjacent to the ATPase active sites. This implies that a significant conformational rearrangement takes place upon substrate binding to facilitate formation of an active ATPase site between AAA\(^+\) domains. MgsA is shown to have DNA-stimulated ATPase activity and to physically associate with SSB. Interactions between MgsA
and SSB appear to be mediated predominantly by the C-terminus of SSB, consistent with observations with other SSB-interacting proteins (Shereda et al., 2008). Interaction between MgsA and SSB could help localize MgsA to replication forks, as has been observed in *E. coli* (Lau et al., 2003; Sherratt et al., 2004).

The domain architecture of MgsA shares similarity with the clamp loaders proteins from bacteria and eukaryotes. In each of these proteins, C-terminal oligomerization domains mediate formation of a collar domain that is decorated with AAA\(^+\) domains extending away from the collar. However, the two groups of proteins differ in their oligomeric state (tetramer for MgsA versus pentamer the clamp loaders) in a manner that appears to be dictated by the oligomerization domain. Comparing the structures of MgsA and the *E. coli* clamp loader shows that more surface area is buried between the subunits in the MgsA than in the clamp loader. Much of this additional area is supplied by a \(~100\) residue C-terminal shelf that is present in MgsA but lacking from the clamp loader (Figure 2A). Nonetheless the overall similarity among the clamp-loader-clade proteins suggests that MgsA could possibly perform a clamp-loader-like function, loading and/or unloading proteins onto DNA. The precise substrate(s) upon which MgsA acts remains unclear. Efforts to identify an interaction between MgsA and \(\beta\), the processivity clamp substrate for the bacterial clamp loader, have thus far not identified an interaction (data not shown). Future studies will be required to determine whether MgsA acts upon protein substrates.

AAA\(^+\) proteins couple the chemical energy of ATP hydrolysis to the mechanical energy necessary to drive conformational changes within the enzyme that remodel the structures of macromolecular targets (Hanson and Whiteheart, 2005). A complete understanding of the structural mechanisms governing AAA\(^+\) enzymes function therefore requires determination of
multiple structural states. Comparing the structure of MgsA presented here to other AAA$^+$ proteins, the arrangement of the AAA$^+$ domains suggests that an inactive conformation of MgsA was captured under our crystallization conditions. However, the local arrangement of the Walker A, Walker B, Sensor 1, and Sensor 2 motifs in MgsA are all in reasonable positions for ATP binding. This suggests that rearrangement of the AAA$^+$ domains to properly localize the Arg finger elements to the ATP binding site could be a trigger for ATP hydrolysis. The presented structure provides a physical model for comparison with other AAA$^+$ proteins that could help define the conformational changes that must accompany catalytic activation and defines a structure with which to compare future substrate-bound structures of MgsA. The DNA-stimulated nature of MgsA ATPase activity suggests that association with DNA could be an important molecular cue that induces formation of an active arrangement of AAA$^+$ domains within the MgsA tetramer.

The interaction between MgsA and SSB provides a possible mechanism by which MgsA localizes to sites of DNA replication in cells. Several other genome maintenance proteins utilize interactions with SSB for proper localization and/or biochemical activation (Shereda et al., 2008). Given MgsA’s noted cellular localization to sites of replication (Lau et al., 2003; Sherratt et al., 2004), identification of an interaction between the two proteins is consistent with a similar role for SSB with MgsA. It is not clear whether this interaction functions solely to localize MgsA. Interaction could facilitate MgsA activity or, alternatively, SSB/DNA structures could possibly be substrates upon which MgsA acts. Future experiments will be required to address the importance of the MgsA/SSB interaction on MgsA biochemical activities.
Figure A2.1
Figure A2.1 Structure of the *E. coli* MgsA protein. *A*, Ribbon diagram of a single MgsA protomer with ATP-binding (orange), lid (cyan), and tetramerization (green) colored to reflect the domain architecture. Phosphate is modeled in red. *B*, The crystallographic asymmetric unit is shown with one protomer in ribbon form and the three remaining protomers in surface form. *C*, Conservation of sequences in 157 MgsA homologues. Invariant (pink) and highly conserved (magenta) residues are color coded. The four MgsA protomers of the asymmetric (and biological) unit are shown in four shades of grey. Areas of high conservation are apparent in the active site pocket and as the interface between subunits in the oligomerization domains. *D*, Conservation around the Arg finger (circled in yellow), view is looking down on ATP-binding domain. *E*, View of a portion of the 2Fo-Fc electron density map contoured to 1.5 σ superimposed with the refined MgsA structure.
Figure A2.2
Figure A2.2  Comparison of MgsA to the bacterial clamp loader.  

A, Overlay of the oligomerization domain of the gamma protein of the *E. coli* clamp loader (PDB 1JR3, chain B) (Grey) aligned with the oligomerization domain of MgsA demonstrates that MgsA possesses an additional ~100 C-terminal residues not present in the clamp loader.  

B, The additional ~100 residues on the C-terminus of MgsA (colored in black) wrap around the neighboring protomer to form an extensive interface.  

C, The Arg finger residues (Arg156) of neighboring protomers are shown as grey sticks demonstrating the positioning relative to the neighboring ATP binding site (identified by the phosphate ions, shown as red spheres).
Figure A2.3 MgsA active site architecture. Conserved elements of the MgsA active site are labeled and key residues are shown as sticks. The distances between the phosphate ion and Lys63 residue (Walker A) or Arg209 (Sensor II) are shown. W-A, Walker-A; W-B, Walker-B; S-I, Sensor-I; S-II, Sensor-II
Figure A2.4 MgsA physically interacts with *E. coli* SSB *in vivo*. SDS-PAGE of TAP-MgsA and co-purifying proteins. SSB and MgsA were confirmed by mass spectrometry. Proteins were eluted by a high salt elution followed by an EGTA elution (see Experimental Procedures).
Figure A2.5

The MgsA-SSB interaction is largely dependent on the SSB C-terminal tail. Surface Plasmon resonance was used to study the MgsA-SSB interaction. Equilibrium response units were plotted versus MgsA concentrations and fitted to hyperbolic binding curves.
Figure A2.6

Graphs showing ATP hydrolyzed in μM over time (min) for different conditions:

- **A** (MgsA): Various concentrations labeled 0, 0.125, 0.25, 1, and 2.
- **B** (ssDNA): Concentrations labeled 0, 30, 20, 10, and 5.
- **C** (SSB): Concentrations labeled 0, 0.25, 0.5, 1, 2, and 3.
- **D** (SSBΔC8): Concentrations labeled 0, 0.25, 0.5, 1, 2, and 3.
Figure A2.6 MgsA DNA-dependent ATP hydrolysis. A, The DNA-independent ATPase activity of MgsA was measured at various MgsA concentrations indicated in the panel. B, MgsA or MgsA Arg156Ala (125 nM) was added to reactions with the indicated circular single-stranded DNA concentration. C, D, The indicated concentration of *E. coli* SSB or SSBΔC8 was added to reactions containing 20 μM nt DNA prior to adding 125 nM MgsA.
Table A2.1  X-ray crystallographic data collection and structure refinement statistics

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**Refinement**

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\(^a\) R\(_{\text{sym}}\) = \Sigma j|I_j| - \langle I \rangle |\Sigma j|/\Sigma j|, where I\(_j\) is the intensity measurement for reflection j and \langle I \rangle is the mean intensity for multiply recorded reflections.

\(^b\) R\(_{\text{work}}\)/R\(_{\text{free}}\) = \Sigma |F\(_{\text{obs}}\)| - |F\(_{\text{calc}}\)|/|F\(_{\text{obs}}\)|, where the working and free R factors are calculated by using the working and free reflection sets, respectively. The free R reflections (5% of the total) were held aside throughout refinement.
A 2.6 References


Appendix III

Ammonium sulfate co-precipitation of SSB and interacting proteins

A3.1 Summary

Bacterial single strand DNA binding protein (SSB) interacts with many proteins involved in the diverse process of genome maintenance. The interactions are mediated by the essential and conserved amphipathic C-terminus (SSB-Ct). SSB plays a critical role in localizing and stimulating the activity of a wide variety of DNA processing proteins. The interaction partners have been identified and studied using a variety of methods, one of which, ammonium sulfate co-precipitation is described here.

**Keywords:** SSB, SSB-Ct, interaction, co-precipitation, ammonium sulfate

A3.2 Introduction

The process of genome maintenance in the cell is complex, SSB functions as an organizational hub for the many proteins involved in DNA replication, recombination and repair (Shereda et al., 2008) SSB binds to the single strand DNA (ssDNA) exposed during replication and in the process of repair (Lohman and Overman, 1985). While the N-terminal domain of SSB binds to ssDNA protecting it from damage and preventing secondary structure formation, the SSB-Ct interacts with the proteins involved in processing the ssDNA (Shereda et al., 2008). Proteins that interact with SSB were discovered through tandem affinity purification, His-tag pulldowns and
co-purification (Butland et al., 2005; Purnapatre et al., 1999; Shereda et al., 2008). These interactions were further validated by several quantitative and qualitative methods. One of the simplest qualitative methods takes advantage of SSB precipitation in a low concentration of ammonium sulfate (Genschel et al., 2000; Marceau et al., 2011; Shereda et al., 2007). If an interaction partner of SSB is added to the solution when SSB precipitates it will pull the protein into the pellet. Ammonium sulfate co-precipitation provides a quick a qualitative method to access the interaction of SSB with other proteins.

A3.3 Materials

1. 450 g/L Ammonium sulfate
2. 1M Tris, pH 8.0
3. Glycerol
4. 5M NaCl
5. Centrifuge (1.5mL to 0.6mL tubes) at 4 °C
6. Purified *E. coli* SSB (Note 1 and 4)
7. Purified protein(s) of interest
8. SDS running buffer: 0.1% SDS,
9. 10-15% gradient SDS PAGE gel
10. Coomassie Brilliant Blue

Buffers: (all buffers at 4 °C)

Coprecipitation buffer: 10 mM Tris-HCl, pH 7.0-8.0, 10% glycerol, 150 mM NaCl

450 g/L ammonium sulfate.
**A3.4 Methods**

SSB Co-precipitation:

3.1. Mix *E. coli* SSB (20 µM/monomer) with 20 µM of its binding partner in co-precipitation buffer. Prepare two additional tubes containing SSB at 20 µM or protein partner at 20 µM respectively as controls. (Notes 2-4)

3.2 Incubate together for 15 minutes on ice for 15 minutes.

3.3 Add ammonium sulfate from a 450 g/L stock to a final concentration of 150 g/L for a final volume of 20 µL, and mix the solution well.

3.4 Incubate the proteins for 15 minutes on ice and collected the precipitate by centrifugation in a microcentrifuge.

3.5 Wash the pellet three times with co-precipitation buffer plus 150 g/L ammonium sulfate.

3.6 Resuspend the pellet in SDS running buffer and resolve the proteins by SDS-PAGE.

**A3.5 Notes**

1. SSB co-precipitation experiments have only been published using *E. coli* SSB and the binding partners (Genschel et al., 2000; Marceau et al., 2011; Shereda et al., 2007). Attempts have been made using *B. subtilis* SSB these have thus far been unsuccessful.

2. It is important to establish that the binding partner does not precipitate at the concentration of ammonium sulfate used in the assay. SSB will precipitate at somewhat lower ammonium sulfate concentrations as well, so this may be reduced.

3. If a different buffer is used it is helpful to run a panel of SSB precipitation experiments from 100 to 200 g/L of ammonium sulfate. This panel allows you to establish the best concentration to
use under your conditions. This same ammonium sulfate panel can be run with the binding partner to establish if it precipitates under similar conditions without SSB.

4. Purified SSB lacking the SSB-Ct or with mutations in the C-terminus can also be used in the assay to verify it is the site of the interaction.

A3.6 References


