ANALYSIS OF THE NUCLEOPROTEIN COMPLEXES ESSENTIAL FOR P1 PLASMID PARTITION

by

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ABSTRACT

For all organisms, segregation and proper intracellular localization of DNA are essential processes in ensuring faithful inheritance of genetic material. In prokaryotes, several different mechanisms have developed for efficiently moving chromosomal DNA to proper cellular locations prior to cell division, and the same holds true for bacterial plasmids. Low-copy-number plasmids and bacterial chromosomes encode active partition systems to ensure their inheritance within a bacterial cell population. One of the well-studied models of partition is that of the P1 plasmid in E. coli. The partition system encoded by the P1 plasmid is known as parABS - ParA is the partition ATPase, ParB is the partition site binding protein and parS is the partition site. The goal of this thesis was to investigate the nucleoprotein complexes essential in the P1 plasmid partition reaction. First, I examined how a single ParB dimer can bind its complicated arrangement of recognition motifs in parS to initiate the partition reaction. I then characterized a novel ParA interaction with the host nucleoid that is critical for proper P1 plasmid dynamics in vivo. Finally, I demonstrate how ParA can act as an adaptor between the nucleoid and the partition complex; effectively allowing the plasmid to use the nucleoid as a track for its intracellular movement and localization. My thesis work provides evidence towards a model that explains the P1 plasmid partition mechanism.
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LIST OF ABBREVIATIONS

ADP-BeFx – Adenosine 5’-diphosphate-beryllium fluoride
ADP-AlF₄ – Adenosine 5’-diphosphate-aluminum fluoride
ADP-VO₄ – Adenosine 5’-diphosphate-vanadate
AMP-PNP – Adenylyl-imidodiphosphate
ATPγS – Adenosine 5’-(γ-thiotriphosphate)
BAD – ParB-ParA-DNA
bp – base pair (s)
CD – Circular dichroism
ChIP – Chromatin Immunoprecipitation
DLS – Dynamic Light-Scattering
DTT – Dithiothreitol
DNA – Deoxyribonucleic acid
EDTA – Ethylenediaminetetraacetic acid
EM – Electron Microscopy
F5M – Fluorescein-5-maleimide
FISH – Fluorescent in situ Hybridization
H-NS – Histone-like nucleoid structuring protein
GFP – Green fluorescent protein
IPTG – Isopropylthiogalactoside
IHF – Integration host factor
NEM – N-Ethylmaleimide
nsDNA – non-specific DNA
PAGE – Polyacrylamide gel electrophoresis
Pi – Inorganic phosphate
TMR – Tetramethylrhodamine-5-maleimide
SDS – Sodium dodecyl sulfate
SEC-LS – Size Exclusion Chromatography resolved – Light-Scattering
SMC – Structural Maintenance of Chromosomes
CHAPTER 1

CHROMOSOME AND PLASMID DYNAMICS IN BACTERIA
For all organisms, segregation and proper intracellular localization of DNA are essential processes in ensuring faithful inheritance of genetic material. In eukaryotic cells, condensed chromosomal DNA is aligned at mid-cell, and sister chromatids are pulled apart by microtubule fibers anchored via the kinetochore to the centromere (Scholey et al., 2003). In prokaryotes, several different mechanisms have developed for efficiently moving chromosomal DNA to defined cellular locations prior to cell division, and the same holds true for bacterial plasmids. While high-copy number plasmids can propagate stably by random segregation, low-copy-number genomes, including chromosomes and plasmids, cannot rely on random distribution to ensure their inheritance within a bacterial cell population. For plasmids, proper maintenance occurs through various strategies such as replication and recombination control systems, post-segregational killing mechanisms, and active partitioning mechanisms.

In this introductory chapter, I will first describe some of the general aspects of plasmid and chromosome segregation in bacteria, as well as summarize the other influences on plasmid maintenance. I then highlight the similarities and differences among DNA partition systems in the microbial world with emphasis on the nucleoprotein complexes that are necessary for the partition reaction. This will be followed by a more detailed description of P1 plasmid partition in *E. coli*, which is one of the well-studied models of the DNA segregation process in bacteria. Finally, I explore the consideration that the P1 partition system uses a plasmid localization mechanism that is similar to the means in which *Escherichia coli* (*E.coli*) localizes its cell division machinery.

**GENERAL BIOLOGY OF Par SYSTEMS**

Classically, the loci encoding partition determinants were defined by their requirement for plasmid stability but not for plasmid replication. Deletion of these regions resulted in plasmid destabilization without a significant change in the plasmid copy-number, suggesting the two processes were independent of one another. This was inferred also because several partition loci were shown to promote plasmid stability when coupled to different replicons.

Plasmid segregation (or as it is more commonly referred to for prokaryotes - partition) in bacteria relies on a family of partition systems (or par systems) that encode three critical components: a *cis*-acting partition site, which is the functional equivalent of a eukaryotic centromere, and two *trans*-acting proteins (Fig. 1-1). In the generic *par* system, the partition site,
often containing one or more inverted repeat(s) as recognition motifs, acts as the loading site for the rest of the partition machinery. One of the Par proteins is the key partition site binding protein, and the other is an ATPase that uses ATP-hydrolysis to drive the segregation reaction. Also, one or both Par protein(s) often act in the repression of their own genes.

Analysis of the rapidly growing library of plasmid and bacterial chromosome sequences suggests that partition systems are almost ubiquitous in the microbial world. For plasmids, partition systems are the only segregation machinery and are thus essential in maintaining stability in a bacterial cell population. For bacterial chromosomes, the par systems are just one of several processes that contribute to proper chromosome stability. Regardless, the conservation of par systems in the prokaryotic world argues that many of the steps in plasmid partition will be common for bacterial chromosome segregation.

In prokaryotes the molecular mechanisms involved in dynamically arranging chromosomes and plasmids in bacteria have proven to be elusive. Cell biology techniques have illustrated that plasmids and chromosomes are indeed specifically positioned and oriented inside bacterial cells (Berlatzky et al., 2008; Ebersbach and Gerdes, 2004; Gordon et al., 1997; Gordon et al., 2004; Ho et al., 2002; Jensen and Gerdes, 1999; Li and Austin, 2002; Niki and Hiraga, 1997, 1998; Webb et al., 1998; Webb et al., 1997). The first indication that partition systems are positioning systems came from intracellular localization studies on several low- and medium-copy-number plasmids. The plasmids were visualized using FISH (fluorescence in situ hybridization), and/or by tagging them with LacI-GFP fusions bound to tandem lac operator arrays inserted into their genomes (Gordon et al., 1997; Ho et al., 2002; Jensen and Gerdes, 1999; Pogliano and Helinski, 2001; Weitao et al., 2000). These studies revealed that plasmid copies are actively separated from each other and positioned in the cell, in a cell-cycle dependent fashion, before cell division. Par protein localization has also been examined by immunofluorescence and by the use of fluorescent fusions (Bignell and Thomas, 2001; Erdmann et al., 1999; Hirano et al., 1998; Jensen and Gerdes, 1999; Kim and Wang, 1998; Moller-Jensen et al., 2002). Together, the findings revealed several critical aspects of plasmid localization. First, plasmids are restricted to preferred intracellular locations, and this positioning is dependent on their partition system (Bignell and Thomas, 2001; Erdmann et al., 1999; Gordon et al., 1997; Ho et al., 2002; Jensen and Gerdes, 1999; Niki and Hiraga, 1997; Pogliano and Helinski, 2001). With a few exceptions that will be discussed later, most plasmid-species appear to move from the
cell center in newborn cells, to localize at the ¼ and ¾ positions in more mature cells. Second, the number of fluorescent foci was restricted and lower than the expected copy-number of the plasmids, suggesting that plasmids are grouped or paired together within a limited number of complexes (Bignell and Thomas, 2001; Gordon et al., 1997; Weitao et al., 2000). Finally, although different plasmid-species localize to similar intracellular vicinities, they did not colocalize (Ho et al., 2002). This observation was originally interpreted as each plasmid-species having a distinct host-encoded tethering signal. The ensuing general model for plasmid positioning required a tethering signal at mid-cell, which replicates and relocates to the quarter positions sometime after cell division. Following septum formation, these sites become the mid-cell of the new daughter cells, and thus the cycle continues.

One well-studied model of partition is that of the P1 plasmid in *E. coli*. The Par system of P1 is known as *parABS* - ParA is the partition ATPase, ParB is the partition site binding protein and *parS* is the partition site. The goal of this thesis was to investigate the nucleoprotein complexes essential in the P1 plasmid partition reaction. First, I examined how a single ParB dimer can bind its complicated arrangement of recognition motifs in *parS* to initiate the partition reaction. I then characterize a novel ParA interaction with the host nucleoid that is critical for proper P1 plasmid dynamics *in vivo*. Finally, there has been a considerable amount of effort in attempting to identity the host-encoded tethering signal (see below). To conclude my thesis, I demonstrate how ParA can act as an adaptor between the nucleoid and the ParB-*parS* partition complex; effectively allowing the plasmid to use the nucleoid, not necessarily as a tether, but as a track for its intracellular movement and localization.

**BACTERIAL CHROMOSOME ORGANIZATION AND MOVEMENT**

The *par* loci encoded by bacterial chromosomes are similar to those encoded by plasmids. All partition systems that have been identified from chromosomes are part of the same family of partition systems as that of P1 (Gerdes et al., 2000). However, unlike plasmid based partition systems, which are the primary segregation machinery, chromosomal partition systems are one of several processes that regulate their segregation. It is important to understand the similarities and differences found in the DNA segregation processes for chromosomes and plasmids. Here I summarize the chromosome segregation process in bacteria, and describe its various influences.
Chromosome Segregation

Bacterial chromosomes have a well-defined intracellular address for the replication origin (oriC) and the terminus (terC) (Ivy et al., 2003; Sherratt, 2003), and in all bacteria investigated, movement of newly replicated oriC and terC copies occurs in a highly reproducible and directed manner (Fig. 1-2) (Thanbichler and Shapiro, 2006). In slow growing E. coli cells, oriC and terC are located proximal to the old and new cell-poles, respectively (Niki and Hiraga, 1998). They both migrate to the mid-cell region, where bidirectional DNA replication occurs starting at oriC (Draper and Gober, 2002; Gordon and Wright, 2000). The oriC copies move away from each other, toward opposite poles, while terC remains centrally positioned. After completion of replication, DNA segregation and cell division, the termini are once again situated close to the new poles. Under fast growing conditions, the frequency of oriC firing increases and chromosome dynamics are adjusted accordingly. The difference in localization patterns for both plasmids and chromosomes is a reflection of the different types of partition and chromosomal maintenance systems that have evolved. The chromosomal maintenance factors that I will highlight are chromosome condensation, the bacterial cytoskeleton, and chromosome-based partition systems, as they are relevant to the plasmid partition process.

Chromosome Compaction

A bacterium faces the problem of compacting a large genome ~1000-fold so it can fit into the cell body. A variety of mechanisms have been identified in nucleoid organization suggesting that it is an intricate process.

DNA Supercoiling: An important contribution to both plasmid and chromosome compaction in prokaryotes is made by DNA supercoiling. Many cellular processes involving DNA in bacteria require topoisomerases to maintain the DNA in a defined under-wound state (Wang, 1996). To relieve torsional tension, different regions of the chromosome coil around each other, generating plectonemic helical structures. Although negative supercoiling has a crucial role in the compaction of the chromosome into a nucleoid, it is not sufficient to give the observed compaction (Zimmerman, 2006).

Nucleoid Binding Proteins: Only ~3% of the nucleoid is DNA by mass (Reyes-Lamothe et al., 2008). Two major groups of proteins function in chromosome compaction and organization: small nucleoid-associated proteins, and structural maintenance of chromosomes
(SMC) complexes.

Small nucleoid-associated proteins like HU (Histone-like protein from *E. coli* strain U93), H-NS (histone-like nucleoid-structuring protein), and IHF (Integration Host Factor) have both regulatory and structural roles, as do histones in eukaryotes (Nasmyth and Haering, 2005; Zimmerman, 2006). Many of these proteins have DNA-bending activity, and thus potentially contribute to chromosome structure, but their precise functions are often unknown. By assembling into nucleoprotein complexes, these proteins dramatically change the conformation of supercoiled DNA. All prefer intrinsically bent DNA.

SMC proteins are the central components of eukaryotic condensin and cohesion complexes (Losada and Hirano, 2005; Nasmyth and Haering, 2005). Unlike eukaryotes, bacteria usually possess a single SMC protein such as MukB. MukB is the active core of the MukBEF complex of *E. coli*. Mutant SMC proteins in bacteria result in poorly condensed chromosomes that segregate abnormally (Britton et al., 1998; Holmes and Cozzarelli, 2000; Jensen and Gerdes, 1999; Niki et al., 1991). The segregation defect caused by the lack of *mukB* function in *E. coli* can be suppressed by increasing the negative superhelical density of the chromosome, implying shared roles for plectonemic supercoiling and DNA condensation in promoting bacterial chromosome segregation (Holmes and Cozzarelli, 2000; Niki et al., 1991). The observation that *mukB* mutants are hypersensitive to inhibitors of gyrase supports this argument. The “extrusion-capture” model for replication and segregation postulates that MukB protein may pull newly replicated DNA poleward by supercoiling it into a more compacted form.

**Nucleoid Organization:** Little insight has been gained into how a bacterial cell maintains nucleoid organization, while at the same time allowing DNA replication, transcription and segregation to occur. Recent reports indicate that the *E. coli* nucleoid is organized into ~400 independent supercoiled ~10 kb domains *in vivo* (Postow et al., 2004; Shuang et al., 2005), and an even higher level of organization into macrodomains has been proposed (Espéli and Boccard, 2006; Niki and Hiraga, 1998). During replication and segregation, these topological domains must break and reform to allow the transit of the replisome.

In *E. coli*, sequential layering of the newly replicated and segregated DNA on both sides of the origin leads to two sister oriC’s being positioned close to the nucleoid quarters before cell division. Therefore, oriC localization to the mid-cell in newborn cells could simply be a consequence of the replication-segregation process. The mechanism seems to be independent of
the precise chromosomal position of oriC, but instead acts on a broader ori-proximal region (Gordon et al., 2002). MukBEF is most likely the key organizing factor that maintains ori positioning close to the cell quarters because in its absence, sister ori’s move to the outer nucleoid edge (Danilova et al., 2007). Overall, the processes of replication, segregation and compaction alone may lead to the observed ori positioning at mid-cell in newborn cells. In other words, there may be no need for cellular positioning markers for the organization of any genetic locus. Falling in line with this implication, the model I propose in this thesis for plasmid positioning also does not rely on a host-encoded cellular tether.

The Bacterial Cytoskeleton

We know now that like eukaryotic cells, prokaryotes have a cytoskeleton (Graumann, 2007). The actin-like Mre/Mbl proteins were identified as cytoskeletal elements that mediate rod-shaped cell morphology through positioning of the peptidoglycan-synthesizing machinery (Errington, 2003; Figge et al., 2004; Jones et al., 2001; Kruse and Gerdes, 2005). These proteins are found in a variety of bacteria, forming helical structures below the surface of the cytoplasmic membrane. A central role in chromosome segregation has been proposed for these bacterial actin homologues, particularly MreB.

The inactivation or overexpression of MreB was found to severely affect proper localization of oriC during DNA segregation (Gitai et al., 2004; Kruse et al., 2003; Soufo and Graumann, 2003). The interpretation of this result is complicated by the fact that the alteration in MreB expression also results in cell shape abnormalities, therefore it is difficult to determine if MreB plays a direct role in the segregation process or globally alters cell polarity. Notably, an mreB deletion in B. subtilis can be complemented by osmoprotective substances, suggesting its sole function may be maintaining cell morphology. However, B. subtilis encodes two additional cytoskeletal proteins, Mbl and MreBH, which have also been implicated in chromosome segregation, suggesting there may be some redundancy (Soufo and Graumann, 2003).

More definitive proof showing that the cytoskeleton mediates DNA segregation in at least a subgroup of bacteria has come from studies in Caulobacter crescentus using a small-molecule inhibitor of MreB called A22 (Gitai, 2005; Iwai et al., 2002). A22 rapidly disassembles the helical MreB filaments in vivo. Following treatment with A22, DNA replication progresses normally, but newly duplicated oriC’s do not separate. Upon the removal of A22, MreB spirals
reformed and the origins rapidly moved to opposite cell poles. The data suggest the cytoskeleton of *C. crescentus* is specifically required for the initial stages of DNA segregation. Supporting this conclusion, chromatin immunoprecipitation (ChIP) assays in *C. crescentus* and *E. coli* have both shown that MreB interacts with loci in the vicinity of *oriC* (Gitai, 2005; Kruse et al., 2006). MreB affects on plasmid dynamics have not been studied.

**PLASMID MAINTENANCE**

This thesis focuses on how active partitioning systems maintain plasmids. However, it is important to note that the stable inheritance of plasmids in a bacterial population is ensured by several types of maintenance systems, the components of which are encoded either by the plasmid itself or by the host chromosome (Williams and Thomas, 1992). Here, I briefly discuss some of the well-studied maintenance systems with emphasis on the systems used by P1.

**Copy-number control**

Plasmid maintenance must be balanced with any selective disadvantage the plasmid may impose upon the host. For example, high-copy-number plasmids are generally smaller than low-copy-number plasmids; a correlation that may help to minimize the metabolic burden imposed by the plasmids on their host cell. Consequently, efficient copy-number control of such plasmids is crucial.

The P1 prophage is relatively a large plasmid (91.6 kb) and is maintained at a very low copy number; approximately one copy per host chromosome (Prentki et al., 1977). Tight regulation of the P1 copy-number is essential for both plasmid maintenance and optimal cell growth. Changes in copy-number are strictly regulated by two mechanisms: plasmid replication control and multimer resolution.

**Replication Control:** RepA is the replication initiator protein encoded by P1 (Abeles, 1986). The replication region of P1 contains a copy-number-control locus, *incA*, which consists of nine repeats of a DNA sequence that is specifically recognized by RepA (Pal et al., 1986). Five copies of these ‘iterons’ are found within the plasmid origin, *oriR* (Abeles, 1986; Chatteraj et al., 1985). RepA binding to both regions bridges or ‘handcuffs’ *incA* and *oriR* via RepA-RepA interaction (Pal and Chatorraj, 1988). This handcuffing inhibits DNA replication, probably by steric hindrance. As replication increases the copy-number, the number of sites
available for RepA to bind also increases. This increases the number of possible handcuffing interactions, thereby inhibiting further replication.

**Multimer resolution:** Plasmids within a bacterial cell are prone to host-encoded homologous recombination, which can result in plasmid multimerization. Plasmid oligomerization reduces the number of segregating plasmid units in a cell and leads to plasmid loss. Plasmids such as P1, whose copy number is already very low, use a site-specific recombination system - Cre-loxP. This system is used extensively to promote site-specific recombination in eukaryotic molecular biology, and in P1, its primary function is to aid in plasmid stability. P1 encodes the Cre recombinase that mediates site-specific recombination between two loxP sites, each 34 bp in length (Austin et al., 1981). Other plasmids use host-recombinases. One example of this is the ColE1 plasmid that directs the *E. coli* Xer recombinase to its resolution site, cer (Clerget, 1991; Colloms et al., 1990; Tolmasky et al., 2000).

**Post-segregational killing**

Another way plasmids ensure their stability in a population is through the use of Toxin-Antitoxin (TA) systems. TA systems increase the plasmid prevalence (number of plasmid-containing cells/total number of cells) in growing bacterial populations by selectively eliminating daughter cells that did not inherit a plasmid copy at cell division (Gerdes et al., 1986; Jaffe et al., 1985). This post-segregational killing mechanism relies on the differential stability of the toxin and antitoxin (Tsuchimoto et al., 1992; Van Melderen et al., 1994). If the plasmid encoding the TA system is lost, the antitoxin decays but the toxin persists, killing the cell (Yarmolinsky, 1995). In theory, this should eliminate all plasmid-free cells from the population, irrespective of the manner by which the plasmid was lost, thus ensuring plasmid maintenance. In practice however, the majority of toxin-antitoxin systems have proven to be inefficient on their own (Jensen and Gerdes, 1995).

The TA system of P1 consists of two proteins: the toxin, Doc (death on curing), and the antidote Phd (prevent host death) (Lehn herr et al., 1993). Doc is resistant to proteolysis and considered to be stable (Lehn herr and Yarmolinsky, 1995). Phd, on the other hand, is easily degraded by the ClpXP-protease system, and therefore continual expression is needed to prevent toxicity by Doc. Doc both directly and indirectly targets protein synthesis. Doc directly inhibits translation elongation by associating with the 30S ribosomal subunit (Liu et al., 2008). Doc
indirectly inhibits translation by activating the *relBE* TA system encoded by the host (Garcia-Pino et al., 2008). RelE induces cleavage of mRNAs that are in association with the translating ribosome. Phd is thought to inhibit toxicity by forming a complex with Doc (Gazit and Sauer, 1999).

Several different types of TA systems are found in a variety of bacterial plasmids and chromosomes (Pandey and Gerdes, 2005). The TA system of F plasmid is called *ccd*. CcdB is the toxic protein that targets DNA gyrase and CcdA is the antitoxin protein (Hiraga et al., 1986). CcdB indirectly causes double strand breaks in the chromosome by inhibiting the religation step of the gyrase reaction (Bernard et al., 1993). CcdA binds CcdB to inactivate it (Maki et al., 1992). The TA system of R1 plasmid encodes a protein toxin (Hok) and a small RNA antitoxin (Sok). The Hok protein kills cells by damaging the cell membrane (Gerdes et al., 1986). Sok-RNA represses *hok* translation by binding its translation initiation region (Thisted et al., 1994).

**PARTITION SYSTEM CLASSES**

Most naturally occurring plasmids encode partition loci with three essential components, for their specific intracellular positioning, and thus stable propagation: a *cis*-acting partition site, a partition site binding protein and a partition ATPase. Individual partition components vary considerably from system to system, so a useful classification scheme for all *par* loci was established (Fig. 1-1) (Gerdes et al., 2000). The classification method organizes the systems according to the type of partition ATPase encoded. Type I partition systems encode Walker-type ATPases, Type II partition systems encode actin-like ATPases, and Type III partition systems encode tubulin-like GTPases (Bork et al., 1992; Koonin, 1993; Larsen et al., 2007). In contrast to the Type II system, the partition mechanism behind the more efficient Type I system is not well understood (Ebersbach et al., 2005; Hayes and Barilla, 2006). In this section, I will first introduce the Type II and Type III loci as the former has a well understood mechanism (Garner et al., 2004; Gerdes et al., 2004; Møller-Jensen et al., 2003) and the latter has only been recently identified (Larsen et al., 2007). This will be followed by a detailed description of the Type I class to which P1 and all chromosomal *par* systems are categorized.
Partition Systems Encoding Actin-like (Type II) and Tubulin-like ATPases (Type III)

**R1 plasmid (Type II):** The R1 plasmid has a Type II class partition system, which is the most well characterized bacterial plasmid segregation system (Fig. 1-1). Using the lacO/LacI-GFP system, it has been shown that the plasmid is dynamically localized within an *E. coli* cell (Jensen and Gerdes, 1999). In cells with a single focus, plasmid positioning was for the most part random, with a slight bias for mid-cell and pole positions. In the vast majority of cells with two foci, the plasmids were found at opposite poles, suggesting that the R1 plasmid is actively segregated to the poles.

The partition site, parC, contains two sets of five 11 bp direct repeats (iterons) separated by a region containing the par promoter (Dam and Gerdes, 1994; Jensen et al., 1994). ParR is the partition site binding protein and is a dimer in solution. A partition complex forms when several ParR dimers bind parC cooperatively through site-specific recognition of the iterons on either side of the par promoter (Breuner et al., 1996; Jensen et al., 1994). Therefore, formation of the partition complex also acts to repress par gene expression; a feature shared with Type Ib systems described below (Dam and Gerdes, 1994; Jensen et al., 1994).

ParM is the partition ATPase and is structurally and biochemically related to mammalian actin. *In vitro*, ParM polymerizes in an ATP-dependent manner, into double helical protofilaments that are reminiscent of F-actin (Moller-Jensen et al., 2002; van den Ent et al., 2002). Despite this similarity, they differ in several distinct features. ParM and actin share <15% sequence similarity with a notable lack of conservation in the regions involved in the subunit-subunit interface within the filament (Löwe and Amos, 2009). Structurally, the monomers have a very similar fold, but the filaments form with opposite handedness; ParM forms a left-handed helix and F-actin forms a right-handed helix. Strikingly, the modes of assembly also differ. The nucleation of ParM is a rapid and spontaneous process (Garner et al., 2004; Moller-Jensen et al., 2002), with filaments elongating in a symmetrical bidirectional fashion that is contrary to the polarized growth of actin. In addition, ParM filaments never reach equilibrium between association and dissociation, as is typical for treadmilling actin polymers. Instead, ParM polymers continuously cycle between phases of rapid growth and complete disassembly (Garner et al., 2004; Garner et al., 2007). This behavior, which is known as dynamic instability, has previously only been observed for eukaryotic tubulin.

Using ParM-GFP fusions, localization studies have revealed that ParM can form axial
filaments (Moller-Jensen et al., 2002). The filaments interact with the ParR-parC partition complex, which stabilizes the filament ends and stimulates ParM ATPase activity (Jensen and Gerdes, 1997; Moller-Jensen et al., 2002). Each filament end is bordered by a copy of the plasmid, which are pushed apart towards the cell poles by the polymerization of ParM between them (Møller-Jensen et al., 2003). The biochemical mechanism of this segregation process has been recently unveiled by reconstituting the R1 partitioning system in vitro (Garner et al., 2007).

DNA containing parC was attached to beads and mixed with ParR and ParM. The addition of ATP induced the formation of numerous short filaments that extended from the bead, which then appeared to shrink and regrow, owing to their dynamic instability. When two beads came into close proximity, the filaments joined and started to grow, pushing the beads apart. Elongation occurred exclusively at the interface between ParM and the ParR-parC complex on the beads. The interpretation was that the ParR-parC complex stabilized the filament ends, and prevented the spontaneous disassembly of the polymer. These data suggest that the three-component partition system of R1 is sufficient to place plasmid copies at opposite cell poles without the help of additional host factors.

The current and most accepted model for R1 plasmid partition uses the concept of “search and capture” (Fig. 1-3). In this model, ParM filaments continuously assemble throughout the cytoplasm but rapidly decay in the absence of stabilizing interactions with the ParR-parC complex on the plasmids (Fig. 1-3A). Filaments stabilized at one end will search the cytoplasm and, upon capture of a second plasmid, extend into a pole to pole spindle (Fig. 1-3B-C). This is analogous to microtubules extending from the eukaryotic spindle-pole-body in the search for chromosomes during mitotic prometaphase. Although bipolar stabilization of ParM filaments is favoured when two plasmid copies are in close proximity, plasmid pairing itself is not required. In other words, ParM filaments could separate paired sister plasmids and any plasmids that come into proximity by diffusion. At a high-copy number of R1, it has been shown recently that the latter situation may occur quite often (Campbell and Mullins, 2007).

Overall it has been shown that R1 plasmid partition is autonomous; initiating from anywhere in the cytoplasm and in any direction relative to the rod-shaped cell. It has no requirement for host cell factors (apart from ATP) and can take place repeatedly and at any stage in the host cell cycle (Campbell and Mullins, 2007).
**pBtoxis plasmid (Type III):** Type III partition systems encode GTPases that show homology with tubulin. The plasmid pBtoxis from *Bacillus thuringiensis* encodes a protein essential for its stability, TubZ (Larsen et al., 2007; Tang et al., 2006). TubZ shows sequence similarity to tubulin, and assembles into highly dynamic filaments that translocate rapidly through the cell (Larsen et al., 2007). FRAP analyses revealed that filament migration is achieved by an actin-like treadmilling mechanism. It is still unclear how the translocation of TubZ filaments relates to plasmid stabilization.

**Partition Systems encoding Walker-type ATPases (Type I)**

Type I systems are most commonly found in the prokaryotic world, and encode ATPases that have a Walker-A box (also called P-loop) motif (Koonin, 1993). This family includes P1 ParA, the partition ATPases of all chromosomal par loci studied to date, and MinD, a protein involved in site-selection for cell-division in *E.coli* (Koonin, 1993; Motallebi-Veshareh et al., 1990). All Type I ATPases most likely function in partition in the same manner, but the mechanism is still unclear. All have a weak intrinsic Mg$^{2+}$-dependent ATPase activity that is significantly stimulated by their cognate partition complex (Barillà et al., 2005; Davis et al., 1992; Pratto et al., 2008; Watanabe et al., 1992). Walker-box mutations have revealed a correlation between the ability of these proteins to hydrolyze ATP and to mediate plasmid stability, indicating that ATP-hydrolysis plays a role in the plasmid partition process (Barillà et al., 2005; Ebersbach and Gerdes, 2001; Fung et al., 2001; Pratto et al., 2008).

The Walker-type ATPases involved in plasmid partitioning have been further sub-divided into Type Ia and Type Ib for a variety of reasons that are summarized in Table 1-1 and illustrated in Fig. 1-1 (Gerdes et al., 2000). The Type Ia subgroup is represented by two of the best studied partition systems; *parABS* from P1 plasmid and *sopABC* from F plasmid. The Type Ib subgroup includes *parFGH* from *Salmonella Newport* plasmid pTP228, and *parABC* from *E. coli* virulence plasmid pB171. More recently, members of this family of ATPases have been referred to as WACAs (Walker A cytoskeletal ATPases) because several can form polymers (Michie, 2006), although the action of these ATPases has not been established as cytoskeletal elements.

**Type Ia systems:** The *E. coli* F factor episome and the P1 prophage of bacteriophage P1 contain two of the first plasmid partition systems to be identified (Austin and Abeles, 1983; Ogura and Hiraga, 1983), and are classified as Type Ia model systems (Fig 1-1). The partition
system of F plasmid is called _sop_ (stability of plasmid). SopA is the partition ATPase, SopB is the partition site binding protein and _sopC_ is the _cis_-acting partition site. _parABS_ (described in detail later) and _sopABC_ have several similarities in their loci arrangement and gene product functions. However, F _sopC_ is much simpler in sequence than P1 _parS_, consisting of 12 copies of a 43 bp sequence repeated in tandem (Helsberg and Eichenlaub, 1986; Mori et al., 1986). Each 43 bp sequence contains a short inverted repeat to which SopB binds as a dimer (Hayashi et al., 2001; Mori et al., 1986; Mori et al., 1989). Like P1 ParB, a HTH motif in the center of SopB binds the inverted repeat in _sopC_ (Hanai et al., 1996; Ravin et al., 2003). But unlike P1 ParB, the C-terminal dimer domain of SopB is not required for DNA-binding (Ravin et al., 2003). Binding of SopB to _sopC_ _in vivo_ increases the linking number of the plasmid indicating the DNA is wrapped around the protein core (Biek and Shi, 1994; Biek and Strings, 1995; Lynch and Wang, 1994). Increasing SopB concentration increases the linking number even further, even when only a single inverted repeat from _sopC_ is present. This suggests that a dimer of SopB site-specifically bound to DNA can recruit several SopB dimers around the _sopC_ site, and promote wrapping of the flanking non-specific DNA, resulting in a large partition complex. Overall, even though the partition sites are different, the evidence suggests that the architecture of the ParB- _parS_ and SopB- _sopC_ partition complexes are similar.

The partition ATPases, F SopA and P1 ParA, are also similar. Over-expression of SopA destabilizes F plasmid (Lemonnier et al., 2000). Further investigation revealed that excess SopA counteracts the increase in plasmid linking-number normally induced by the SopB- _sopC_ interaction, suggesting that SopA disrupts the partition complex. The SopA effect required ATP-binding and/or hydrolysis, and was mediated by direct interaction of SopA with SopB (Ravin et al., 2003).

ParA and SopA are also required for plasmid positioning _in vivo_. In the _sop_ system, SopB forms large foci that colocalize with F plasmid either at mid-cell or the quarter positions, in a _sopC_ and SopA dependent manner (Adachi et al., 2006; Hirano et al., 1998). Recent real-time microscopy using fluorescent fusions of SopA and SopB has shown that the SopB- _sopC_ partition complex continually chases after a cloud of nucleoid-associated SopA. The result suggests that these plasmids are always moving, and never fixed to a specific intracellular address for a long period of time (Hatano et al., 2007). The average location of F plasmid in these studies was at mid-cell when only a single-copy was present and at the quarters when two
copies were present.

Like many Walker-type ATPases, SopA can form small, bundled filaments in vitro, but the requirements for polymerization vary from system to system. How these polymers are involved at the molecular level, and whether all Type I partition ATPases behave in a similar fashion is unclear.

**Type Ib systems:** The general arrangement of Type Ib loci is similar to that of the Type II arrangement where the partition site likely overlaps the promoter for the par genes, and just downstream are the genes for the ATPase and a very small ParB (Fig. 1-1). The partition ATPases of this class lack the N-terminal region present in Type Ia ATPases that is required for site-specific binding to their cognate promoter/operator region and autoregulation of their operon (see below). This is consistent with the observation that Type Ib partition ATPases do not have repressor function (Ebersbach et al., 2005). The partition site binding proteins of this class are less than 100 amino acids in length, bear no sequence similarity to each other or to Type Ia ParBs, and do not contain HTH motifs for their DNA-binding activities (Barillà et al., 2005; Fothergill et al., 2005; Gerdes et al., 2000).

The structures of two partition site binding proteins from this class have been solved: ParG from plasmid TP228 (Golovanov et al., 2003), and ω from pSM19035 (Weihofen et al., 2006). Both form dimers consisting of a folded domain containing intertwined C-terminal regions of each monomer subunit and a flexible domain consisting of the unstructured N-terminal regions. The folded C-terminal part of the dimers has a ribbon-helix-helix (RHH) architecture. This motif is responsible for DNA-binding activity as shown for the ω structure bound to its specific sequence (Pratto et al., 2008; Weihofen et al., 2006). However, how these proteins assemble and whether they form large partition complexes is not yet known.

Two of the better characterized Type Ib ATPases, ParA of pB171 and ParF of TP228, form bundled polymers in vitro (Barillà et al., 2005; Ebersbach et al., 2006). These filament bundles look identical to those formed by SopA of F plasmid. Both pB171 ParA and ParF polymerization is supported by ATP, but not ADP. Non-hydrolyzable ATP analogues also supported polymer formation showing that ATP-binding but not hydrolysis is required. The polymers were shown to be regulated by their cognate partition complex (Barilla et al., 2007; Barillà et al., 2005; Machón et al., 2007). For example, ParG, which stimulates the ATPase activity of ParF, was shown to associate with ParF filaments and control polymerization: At low
ParG:ParF ratios, ParG appeared to enhance ParF polymerization and filament bundling, whereas high ParG concentrations resulted in lesser polymerization. These observations suggest that ParG mediates its effect on ParF polymerization through stimulation of the ATPase activity of ParF. Consistent with this idea, Walker-A box mutants of ParF showed defects in polymerization activity (Barillà et al., 2005). In Chapter 4, I investigate P1 ParA polymerization.

**Chromosome-encoded Partition Systems**

Many bacterial chromosomes encode partition systems that fall into the Type I class. Type II systems have not been found in bacterial chromosomes as of yet (Gerdes et al., 2000). Similar to plasmid-encoded Type I loci, chromosomal par loci encode two trans-acting proteins that act on a number of cis-acting sites on the chromosome. However, the number and distribution of the cis-acting sites vary among species, and they are not obligatorily linked to the par operon. In general, the cis-acting sites are clustered around the origin-proximal region of the chromosome (Bartosik et al., 2004; Godfrin-Estvenon et al., 2002; Jakimowicz et al., 2007).

Some of the better studied examples of chromosomal Type I loci are from *Bacillus subtilis*, *Caulobacter crescentus*, *Streptomyces coelicolor*, *Vibrio cholera*, *Pseudomonas putida* and *Pseudomonas aeruginosa*. Only *C. crescentus* encodes a partition system that is essential (Mohl and Gober, 1997). *E. coli* and several close relatives do not contain Type I partition systems (Yamaichi and Niki, 2004). Below I draw attention to one of the better characterized chromosomal partition systems related to parABS - SpoOJ/Soj from *B. subtilis*.

**B. subtilis**: The chromosomal par locus of *B. subtilis* encodes Soj, an analogue of ParA, and SpoOJ, an analogue of ParB. Like Type Ia partition site binding proteins, SpoOJ encodes a HTH-motif, which mediates specific binding of SpoOJ to 8 cis-acting parS sites (Leonard et al., 2004). This initial complex nucleates the formation of a nucleoprotein complex where SpoOJ spreads and covers the origin-proximal region (~20-25% of chromosome) (Gruber and Errington, 2009; Lin and Grossman, 1998; Sullivan et al., 2009). Fluorescence microscopy has shown that binding of SpoOJ to parS creates small nucleoprotein complexes that, in the presence of Soj, condense into a discrete focus at each origin region (Glaser et al., 1997; Marston and Errington, 1999). During vegetative growth, SpoOJ foci follow the replicated origin regions as they move apart and segregate to positions near the cell quarters. Deletion of SpoOJ leads to abnormal nucleoid morphology in elongated cells, and an increase in the frequency of anucleate cells.
Together, these findings led to the suggestion that SpoOJ and Soj are involved in origin organization and/or segregation (Autret and Errington, 2001; Ireton et al., 1994).

Recently, two reports have shown that SpoOJ recruits SMC complexes to the origin region to facilitate proper segregation and organization of chromosomal DNA (Gruber and Errington, 2009; Sullivan et al., 2009). SMC was specifically localized to the ori region of the *B. subtilis* chromosome, using SpoOJ-parS nucleoprotein filaments as loading zones. In spoOJ or parS mutants, SMC localization was disrupted, and a large region (~1Mbp) surrounding the origin of replication was severely disorganized. Introducing ectopic parS sites created aberrant “hot-spots” for SMC binding, and resulted in dramatic chromosome disorganization and segregation defects.

Soj is a Walker-type ATPase that interacts with SpoOJ and is required for synchronous DNA replication, proper separation of sister origins and the regulation of sporulation (Ireton et al., 1994; Lee and Grossman, 2006; Leonard et al., 2005a). Curiously, deletion of Soj does not seem to affect cell- or nucleoid-morphology (Ireton et al., 1994). This is consistent with the finding that mutations in SpoOJ disrupts the organization of about one quarter of the *B. subtilis* chromosome, while Soj inactivation only affects the positioning of a discrete region located next to the replication origin (Sullivan et al., 2009). Soj is therefore likely to cooperate with SpoOJ to actively partition the origin regions.

Previous studies describing the localization of wild-type Soj have shown that the protein colocalizes with the nucleoid, dynamically oscillates, and also displays a “nucleoid jumping” activity where fluorescence is restricted to only a subset of nucleoids in the cell (Marston and Errington, 1999). Soj-GFP was overexpressed in these assays. A more recent study that expressed GFP-Soj at physiological concentrations, showed it colocalizing with SpoOJ as punctate foci (Murray and Errington, 2008). In the absence of SpoOJ, GFP-Soj was uniformly dispersed over the nucleoid region of the cell. Together the results suggest that the dynamic localization patterns of Soj, and possibly all Type I partition ATPases, are not only dependent on interaction with their cognate partition complex, but are also influenced by their expression level.

Biochemical and structural analysis of Soj has shown that the protein is a dynamic molecular switch that is capable of forming an ATP-dependent “sandwich” dimer (Leonard et al., 2005a). The ATP-bound dimer binds cooperatively to non-specific DNA and has ATPase activity (Hester and Lutkenhaus, 2007; Leonard et al., 2005a; McLeod and Spiegelman, 2005).
Mutational analysis lead to the isolation of Soj intermediates and a dissection of the in vivo localization patterns of these Soj variants (Murray and Errington, 2008). Soj variants deficient in ATP-binding could not colocalize with the nucleoid or SpoOJ. Alternatively, an ATP-binding proficient and ATPase deficient Soj mutant could still interact with SpoOJ and had a stronger affinity to towards DNA than wild-type Soj. Together, the evidence suggests Soj in its ATP-bound form interacts with DNA non-specifically and, upon contact with SpoOJ, its ATPase activity is stimulated, resulting in Soj disassembly from DNA.

Notably, Soj directly interacts with the replication initiator protein DnaA, acting as a spatially regulated molecular switch that is capable of either inhibiting or activating replication (Murray and Errington, 2008). It remains to be tested if other Type I partition ATPases can also regulate their cognate DNA replication systems.

**P1 PLASMID PARTITION**

P1 is a temperate phage that can lysogenize E. coli. Following infection, P1 DNA does not insert into the chromosome, but rather circularizes and is repressed in the lysogenic mode. The P1 plasmid is the resulting prophage of the bacteriophage P1, and it exists as a stable, autonomously replicating, low-copy-number episome in E. coli (Prentki et al., 1977). The P1 partition system has served as a paradigm for homologous systems found in many naturally occurring bacterial plasmids as well as several bacterial chromosomes. Ultimately, partition systems are positioning systems. The exact details of positioning are not completely understood and vary slightly from system to system. For P1, the positioning reaction is coordinated with the E. coli cell-cycle (Fig. 1-4). First, in newborn cells, the plasmid is located at the mid-cell. The plasmid replicates and the sister copies are thought to pair or group together. Next, an unknown signal allows for partition, and the plasmids localize to the ¼ and ¾ positions of the cell. Following cell division, there is a faithful inheritance of a single copy of the P1 plasmid to the daughter cells. It has been suggested that there may be a host encoded tethering mechanism, which aids in directing partition and localization. In this thesis, I will propose that the bacterial nucleoid can act, not as a tether per se, but rather a track for plasmid movement and localization.

The P1 plasmid is lost less than once in every $10^5$ cell divisions (Austin et al., 1981). This stable maintenance is absolutely dependent upon its partition system - parABS (Fig. 1-5A). A 2.5 kb region of the P1 plasmid contains the par operon encoding the genes for ParA and
ParB, and immediately downstream is the cis-acting partition site, parS (Austin and Abeles, 1983). All three elements are essential for partition. ParA and ParB perform two major functions in P1 partition: (1) autoregulating the par operon and (2) physically segregating the plasmids (Abeles et al., 1985; Davis et al., 1996; Friedman and Austin, 1988; Hayes and Austin, 1994). ParA directly regulates parA and parB expression by binding to parOP (Fig. 1-5B) and acting as a transcriptional repressor (Davey and Funnell, 1997). ParB acts as a corepressor by stimulating ParA repressor activity.

In partition, an intricate series of DNA-protein and protein-protein interactions ultimately leads to P1 plasmid localization within an E. coli cell. The reaction starts with ParB, the key partition site binding protein, which loads onto and around parS (Fig. 1-5C), and forms a large nucleoprotein complex called the partition complex (Davis and Austin, 1988; Funnell, 1988b). Following plasmid replication, ParB-ParB interactions are proposed to mediate plasmid pairing (Bouet et al., 2000; Edgar et al., 2001; Erdmann et al., 1999). ParA interacts with this complex and, through an unknown process, mediates the specific localization of the plasmids (Davis et al., 1996; Erdmann et al., 1999; Li and Austin, 2002). The only host factor known to participate in P1 partition is E. coli integration host factor (IHF), which assists ParB in the initial DNA binding step by greatly increasing the affinity of ParB for parS (Funnell, 1988b).

**P1 ParB and the Partition Complex**

**parS - The P1 partition site:** parS is the cis-acting partition site of the P1 plasmid (Fig. 1-5C). It contains four copies of a heptameric sequence called BoxA (ATTTCAA/C), and two copies of a hexameric sequence called BoxB (TCGCCA). ParB can recognize all motifs, but not all copies are necessary for partition (Davis and Austin, 1988; Davis et al., 1990; Funnell and Gagnier, 1993, 1994; Martin et al., 1991). The A- and B-boxes are asymmetrically arranged around an IHF binding site. The spacing and orientation of these motifs are critical for complex formation *in vitro* as well as *parS* activity *in vivo* (Funnell and Gagnier, 1993; Hayes and Austin, 1994).

The wild-type parS site is 84 bp long and can be divided into three main sections: left, right and central (Fig. 1-5C). The left section contains boxes B1 and A1, the right is composed of boxes A2, A3, B2 and A4, and the center contains an IHF binding site (Davis et al., 1990; Funnell, 1988b, 1991). IHF bends parS, which allows ParB to contact its specific binding motifs
flanking the bend, resulting in a high-affinity protein-DNA complex (Funnell, 1991; Funnell and Gagnier, 1993). IHF can be partially replaced by intrinsically bent DNA, showing that its serves as an architectural protein - bending the DNA and allowing ParB to interact with both the left and right arms of parS simultaneously (Hayes and Austin, 1994).

IHF is not essential for P1 partition (Funnell, 1988b), but it does increase plasmid stability. Examining the ParB-parS interaction in the presence and absence of IHF has provided significant insight on the nature of the P1 partition complex. Without IHF, ParB binding to parS is weak, but still specific for the right-half of parS. However at high enough concentration, ParB can interact with both arms of parS simultaneously independent of IHF. In vivo, a 22 bp sequence on the right side of parS (boxes A2-A3-B2, called parS-small in Fig. 2-1) is sufficient but not optimal for partition (Martin et al., 1991). Therefore ParB can associate with parS in two ways: the “IHF independent”, low-affinity interaction, where the left arm of parS is dispensable, and the “IHF dependent”, high-affinity interaction, where ParB binds the left and right arms of parS across an IHF-directed bend.

in vivo partition-incompatibility assays suggest that IHF is always a component of the P1 partition complex (Funnell, 1988b). Partition-mediated incompatibility is the ability of two different plasmids to displace each other as a result of being maintained by the same partition system. The phenomenon is believed to be a consequence of competition during an event or events in the partition reaction, and is thus considered a function of partition activity (Austin and Nordstrom, 1990). In wild-type cells, plasmids that are partitioned by an IHF-independent partition complex (e.g. using a parS-small site) cannot displace a plasmid partitioned by an IHF-dependent complex (e.g. using a wild-type parS site). In IHF mutants, these two plasmid-species are incompatible. Therefore, in wild-type cells, the P1 partition complex is formed with ParB and IHF.

DNaseI footprinting and methylation protection and interference experiments have shown that ParB contacts multiple A- and B-boxes when binding parS (Fig. 1-5C) (Davis and Austin, 1988; Davis et al., 1990; Funnell and Gagnier, 1993). In these studies, specific regions of parS were mutated to determine which boxes were essential for ParB binding. In vitro, Boxes A1 and A4 were the only motifs that were dispensable. Consistent with these in vitro results, all motifs except Boxes A1 and A4 are necessary for wild-type parS activity in vivo (Davis et al., 1990; Funnell and Gagnier, 1993; Hayes and Austin, 1994). Interestingly however, the position and
orientation of Boxes A1 and A4 have been conserved in a variety of related *parS* sites (Funnell and Slavcev, 2004). The optimal *parS* site extends from Box B1 to B2 and requires IHF (called *parS* in Fig. 2-1). In Chapter 2, I find ParB prefers to bind certain A- and B-box combinations within *parS*, and I also identify a role for boxes A1 and A4 in instances when plasmid supercoiling is altered.

**Physical Properties of ParB:** The ParB protein is 333 amino acids in length, and has a molecular mass of 38 KDa. With 55 lysine and arginine residues, ParB is considered extremely basic (pI=9.1) and runs anomalously as a 45 KDa species on SDS-polyacrylamide gels. When comparing ParBs from plasmids and bacterial chromosomes, the sequence similarities are modest, with little sequence similarity in the entire C-terminal half of the proteins (Hanai et al., 1996). Despite this, two regions of ParB are noticeably conserved: a helix-turn-helix (HTH) motif and another called the B-motif (Fig. 1-6A). Residues 166-187 of P1 ParB correspond to the HTH motif and are found in most ParB-like proteins (Dodd and Egan, 1990; Hanai et al., 1996; Lobocka and Yarmolinsky, 1996). The ‘B motif’, which resides roughly between residues 78 and 116, has no known function and it corresponds to no known structural motifs.

Biochemical characterization of ParB has identified domains necessary for self-association and ParA interaction (Fig. 1-6A). As suggested by cross-linking, gel-filtration assays, and size-exclusion chromatography, the crystal structure of the C-terminal half of ParB confirmed that it is an asymmetric dimer in solution (Funnell, 1991; Schumacher and Funnell, 2005). The dimerization domain was first narrowed to between residues 275 and 325 by deletion fragment analysis and cross-linking experiments (Funnell, 1991; Schumacher and Funnell, 2005; Surtees, 2001; Surtees and Funnell, 1999). The ParB crystal structure further resolved this domain and its architecture (see below). A second self-association domain was also functionally identified in the N-terminal half of the protein. Interestingly, oligomerization via this second domain was inhibited in the context of full-length ParB (Surtees and Funnell, 1999), functioning only when a significant portion of the C-terminus of ParB was removed. The N-terminus of ParB (residues 1-142) is relatively flexible, and readily degraded in proteolysis experiments. The C-terminal half of ParB (residues 142-333), on the other hand, forms a stable proteolytic fragment. It has been suggested that the N-terminal multimerization domain is primarily responsible in forming the higher-order partition complex, but only becomes active once ParB
dimerizes and binds parS via its C-terminal half.

In addition to the ParB oligomerization domain, yeast-two-hybrid and domain-swapping experiments identified a ParA interaction domain at the extreme N-terminus of ParB (Radnedge et al., 1998; Surtees and Funnell, 1999). The data are consistent with ParA and ParB competing for their respective interfaces at the N-termini of ParB dimers already associated with the partition complex. It is possible that this competition plays a key role in the spatial and temporal switch from plasmid pairing via ParB-ParB interaction to plasmid partition via ParA-ParB interaction.

Domain swapping, mutagenesis, and protein fragment analysis identified the C-terminal half of ParB (residues 142-333) as the region containing all the information required to assemble the dimeric, high-affinity ParB complex at parS in the presence of IHF (Fig. 1-6A) (Surtees and Funnell, 2001). ParB is an unusual site-specific DNA-binding protein in that it recognizes two distinct sequence motifs in parS (Davis et al., 1990; Funnell and Gagnier, 1993). This feature is uncommon among ParB-like proteins as the majority of partition sites in other systems consist of only one type of inverted repeat sequence. Despite this difference, there is a subfamily of PI ParB-like proteins, and all have a HTH motif responsible for binding their respective partition site. Mutagenesis of the two DNA binding domains showed that the HTH motif at the center of ParB binds A-boxes and the dimer domain at the extreme C-terminus binds B-boxes (Surtees, 2001). Overall, the biochemical studies proposed a reasonable architecture for the PI partition complex where a dimer of ParB containing a total of four DNA-binding domains is wrapped by the parS site, thus allowing the dimer to bind all essential A- and B-boxes (B1, A2, A3 and B2) simultaneously over an IHF directed bend. The crystal structure of ParB confirmed most but not all features suggested from the biochemistry.

The Crystal Structure of ParB(142-333): Attempts to crystallize a dimer of wild-type ParB bound to a full parS site in the presence of IHF have been unsuccessful. However, the biochemical analyses of parS and ParB described above provided clues that allowed for the intelligent design of a stable ParB fragment, with a DNA substrate that crystallized to a resolution of 2.93Å (Schumacher and Funnell, 2005). Schumacher and Funnell (2005) determined crystal structures of ParB(142-333) bound to DNA duplexes encoding parS-small. Each crystallographic asymmetric unit (ASU) contained one ParB(142-333) dimer simultaneously bound to three parS-small sites – one bound to Box-A2, one bound to Box-A3
and one bound to Box-B2 (Fig. 1-6B). The ParB(142-333) dimer could be broken down into two DNA binding modules: the N-terminal all-helical domain (residues 147-270) containing the HTH motif, which bound the A-boxes, and the C-terminal dimer domain (residues 275-333), which bound the B-boxes. No stabilizing interactions were found between the two domains, suggesting they function as completely independent DNA-binding modules. The two domains were connected via a four residue pivot point linker. The flexibility of this linker allows for essentially free rotation of the two DNA-binding modules relative to each other (Fig. 1-6C). In Chapter 2, I find that this free rotation allows the DNA-binding modules of a ParB dimer to contact a variety of distinct A- and B-box arrangements when binding the full parS site.

Several unexpected features of ParB were identified from the crystal structure. First, unlike most canonical HTH motifs that bind inverted repeats immediately adjacent to one another, the ParB structure has its HTH domains on opposite sides of the dimer pointing away from each other (Fig. 1-6B). The DNA-binding interface in the dimer domain had sequence-specific contributions from both monomers, explaining why studies have shown dimerization to be important for DNA-binding (Lobocka and Yarmolinsky, 1996; Surtees and Funnell, 2001). Most surprising was the finding that the individual DNA-binding modules of a given ParB(142-333) dimer could not simultaneously contact all A- and B-boxes located on the same parS-small site, as this would require a translation that is too far for the 4-residue pivot-point linker to extend (Fig. 1-6D). This result was unexpected because DNaseI footprinting assays have suggested that a single dimer of ParB can contact all essential A- and B-box motifs simultaneously. This led me to reassess the nature of the ParB-parS interaction. How can a single dimer of ParB bind multiple A- and B-boxes in high-affinity complex assembly? In this thesis, I present evidence that P1 partition complex assembly involves several modes of protein-DNA recognition.

**The ParB-IHF-parS Partition Complex:** The P1 partition reaction initiates with a dimer of ParB binding the parS site over an IHF directed bend, forming a high-affinity core complex. This nucleates the formation of a higher-order complex where several dimers of ParB load onto and around the parS site presumably through ParB-ParB as well as ParB-DNA interactions. The final complex size is unknown, however in vivo immunofluorescence microscopy of ParB suggests the complex is very large (Erdmann et al., 1999). When parS is present, ParB forms large, punctate foci with little background fluorescence. This suggests that
many, if not most, of the ParB dimers within the cell converge onto the plasmid from a ParB-parS nucleation point. Quantitative westerns have estimated that there are ~7000 molecules of ParB within the cell (Funnell, 1991; Funnell and Gagnier, 1994). The minimum number of ParB molecules that must join the complex for partition to occur is unknown.

ParB has been shown to silence genes up to several kilobases away from parS (Rodionov et al., 1999). This gene silencing phenotype has also been shown for the related SopB protein of F plasmid (Lynch and Wang, 1995). ChIP assays showed that P1 ParB can spread for several kilobases from a parS site, providing a mechanism for gene silencing by preventing access of RNA polymerase to promoters (Rodionov et al., 1999). ParB was overexpressed in these assays so the biological significance of ParB silencing is still up for debate. When insulators or “roadblocks” were used to limit the extent of ParB spreading from parS to 300 bp, plasmid stability lessened although it was not eliminated (Rodionov et al., 1999). By moving the roadblocks such that ParB could spread across ~450 bp, partition activity was fully restored. The interpretation of these findings was that the minimal length of DNA required for the partition complex to spread and become fully active is ~400 bp. It is attractive to speculate that this large partition complex establishes a cellular landmark for the partition ATPase, ParA.

P1 ParA

**Physical properties of ParA:** ParA is 398 amino acid polypeptide, with a molecular mass of 44 KDa. There are four main regions of conservation in ParA (Fig. 1-7A) (Koonin, 1993; Motallebi-Veshareh et al., 1990). Three of the motifs are involved in adenine nucleotide-Mg\(^{2+}\) binding: Walker A (residues 116-124), Walker A’ (residues 147-155), and Walker B (residues 246-251). Consequently, ParA is characterized as a Walker-type partition ATPase. Walker-box mutations abrogate nucleotide-binding and ATP-cycling activities of ParA (Davis et al., 1996; Fung et al., 2001). ParA has weak ATPase activity that is stimulated by ParB ~3-fold, and by DNA of no particular length, sequence or topology ~1.5-fold (Davis et al., 1992). Together, ParB and DNA stimulate ParA ATPase activity cooperatively ~10-15-fold. The function of the final region of similarity, the ‘ParA-specific’ motif (343-350), is thought to provide adenine nucleotide specificity (Dunham et al., 2009a).
Domain swapping experiments identified regions of ParA involved in ParB interaction (Hayes and Austin, 1994). The C-terminal half of ParA was found to be responsible for interaction with ParB (Fig. 1-7A). The region of ParA, and more importantly, the specific residues responsible for ParB interaction must be delineated further.

The oligomeric state of ParA is controlled by adenine nucleotide. Glycerol gradient sedimentation of ParA has shown that it can exist in monomer-dimer equilibrium, and the addition of ATP or ADP shifts this equilibrium to dimer form (Davey and Funnell, 1994). Recently, the crystal structures of apoParA and ParA-ADP have been solved. Both crystal forms were dimers (Dunham et al., 2009a). Crystals were obtained at concentrations between 70-100 μM ParA; however, the physiological concentration of ParA has been measured to be ~1 μM (Funnell, unpublished data). Using size-exclusion chromatography resolved-light scattering (SEC-LS) assays, the majority of apoParA was still shown to be dimeric at concentrations as low as 5 μM. Given that adenine nucleotide binding lowers the $K_d$ for dimerization even further, it is presently difficult to suggest any in vivo relevance to the monomer form of ParA. Adding ATP to crystallographic concentrations of ParA led to the formation of string-like precipitates (Dunham et al., 2009a), and therefore a structure of P1 ParA-ATP has yet to be solved.

The apoParA structure was conformationally flexible, and could be broken down into three main regions: an extended N-terminal α-helix (residues 1-43), a winged-HTH motif (residues 44-104) and a large C-terminal domain (residues 105-398) (Fig. 1-7B) (Dunham et al., 2009a). The dimer interface is quite large in the apo form, with contributions from both the N-terminal and C-terminal domains. The C-terminal domain contains all Walker-boxes, and is structurally similar to the chromosomal ParA homologue Soj from T. thermophilus, a gram-positive bacteria (Leonard et al., 2005a). The conservation of these structures in gram-negative and gram-positive bacteria argues that perhaps all related partition ATPases respond to nucleotide in a similar manner.

Adenine nucleotide modulates ParA structure and function: ParA-ADP is the repressor form and ParA-ATP is the form directly involved in the partition reaction (Bouet and Funnell, 1999). The ParA-ADP structure provided insight into the molecular mechanisms involved in the ATP-ADP switch that controls ParA function. ADP binding to ParA had significant structural consequences (Fig. 1-7C). Overall, ParA-ADP was more conformationally rigid than apoParA (Dunham et al., 2009a). ADP-binding locked ParA into one specific dimer state, and elicited a
large-scale ordering of regions that either interact with ADP or are near residues that interact with ADP. This drastic conformational transition is consistent with circular dichroism (CD) data showing that ParA helicity changes upon binding adenine nucleotide (Davey and Funnell, 1994). The structural transition allows the side-chain of conserved Lysine-122, a critical residue in adenine nucleotide binding, to interact with the β-phosphate of ADP (Dunham et al., 2009a). K122Q and K122E mutants of ParA show little to no ATPase activity, and have altered repressor and partition functions (Fung et al., 2001). The ‘ParA-specific’ motif also becomes ordered on ADP-binding, with several of its residues contacting the adenine moiety of ADP; explaining ParA’s discrimination against guanine nucleotides (Davey and Funnell, 1994).

ParA-ADP acts as a transcriptional repressor of parA and parB by site-specifically binding to a ~40 bp imperfect inverted repeat in the promoter/operator region of the par operon (Davey and Funnell, 1994; Davis et al., 1992). By modeling a 40-bp DNA duplex onto the ParA-ADP dimer structure, its DNA-binding interface emerged (Dunham et al., 2009a). A single ParA-ADP dimer has four DNA-binding elements: two N-terminal winged-HTH motifs (residues 22-66), and two C-terminal basic regions called motif 1 (residues 351-354) and motif 2 (residues 365-380) (Fig. 1-7). The two HTH motifs, essential for operator-specific DNA binding (Radnedge et al., 1998), dock into successive major grooves on the DNA substrate and the adjacent wings fit into the minor grooves (Dunham et al., 2009a) (Fig. 1-7C). The two C-terminal basic regions of ParA form a non-specific interaction with the phosphate backbone in the center of the DNA substrate. Mutating these motifs led to defects in parOP binding.

There are several other crystal structures from related Walker-type ATPases available; including NifH (or Fe protein), ArsA, and MinD (Georgiadis et al., 1992; Hayashi et al., 2001; Sakai et al., 2001; Schindelin et al., 1997; Zhou et al., 2000). In these proteins, their ATP-binding pocket resembles those in G-proteins that are involved in signal transduction in eukaryotes. The equivalent Walker A’ and Walker B motifs have been called “switch I” and “switch II”, respectively, because they are thought to communicate signals from ATP-binding and hydrolysis to other regions of the protein and to other protein partners (Hayashi et al., 2001; Jang et al., 2000). It is possible that these motifs in ParA function in a similar manner, perhaps by transmitting signals to the ParB interaction interface of ParA.
Regulation of ParA and ParB: Altering the concentrations of ParA and ParB relative to each other or to the number of parS sites in the cell can disrupt partition (Abeles et al., 1985; Funnell, 1988a; Hayes and Austin, 1994). The par promoter is relatively strong when not repressed, and therefore, the repressor function of ParA is considered essential for partition. When parAB is expressed from parOP, DNaseI footprinting has shown that several dimers of ParA cooperatively spread across ~150 bp of DNA, emanating from an initial site-specific interaction with an imperfect inverted repeat within parOP (Fig. 1-5B) (Davey and Funnell, 1994; Davis et al., 1992). ParA-ADP presumably acts as a transcriptional repressor, interfering with RNA polymerase binding to the par promoter.

ATP can also promote site-specific DNA binding by ParA (5-10 fold), but to a lesser extent than ADP (an additional 5-10 fold increase) (Davey and Funnell, 1997). Non-hydrolyzable analogues of ATP stimulate parOP binding to a level equal to that of ADP, suggesting that ParA ATP-cycling is somehow inhibitory. CD has shown that ParA bound to ATP has a slightly greater helicity than when bound to ADP or ATPγS, indicating that ParA conformation is exquisitely sensitive to the type of adenine nucleotide bound. In Chapter 3, I explore how this ATP-specific conformational change in ParA influences its DNA-binding activity.

Mutation of the Walker-boxes in ParA has provided insight into the activities of ParA. ParA variants that cannot bind or hydrolyze ATP also have significant defects in both repressor and partition functions (Davis et al., 1996; Fung et al., 2001). Other mutations have been isolated that halt ParA ATP-cycling activity, but are still competent in ATP-binding at physiological concentrations. These are defective for partition, but behave as “super-repressors” of the par operon; in other words, they repress transcription much more strongly than wild-type ParA (Fung et al., 2001). In vitro, these ParA variants can still bind DNA site-specifically in a nucleotide dependent manner, but the distinction between ATP and ADP is lost. Therefore, I propose that these mutants are locked in the ‘ADP-bound’ form, regardless of the nucleotide bound.

In vivo, ParB stimulates the repressor activity of ParA (Friedman and Austin, 1988). In vitro, ParB stimulates ParA binding to parOP, but only in the presence of ATP. This suggests that ATP-hydrolysis is required for ParB stimulation and also shows that ParB can prevent or counteract the inhibitory effects of ATP-cycling on parOP-binding activity by ParA (Davey and
ParB has no repressor activity on its own, and is therefore considered a co-repressor (Friedman and Austin, 1988). The level of ParB-stimulated repression was similar to that found for ParA in the presence of ADP (Davey and Funnell, 1997). Together, the results indicate that ParB acts as a co-repressor by altering ParA ATP-cycling activity in two possible ways: (i) stimulating ATP-hydrolysis, and therefore increasing the amount of ParA-ADP available for site-specific parOP binding, and/or (ii) stimulating a conformational reversal of ParA-ATP back to its site-specific DNA-binding form.

The parS site was also found to play a role in par operon repression (Hao and Yarmolinsky, 2002). The presence of the partition site reduced parA and parB expression in vivo even when placed in trans from the par operon. Thus, the added repression is not a result of ParB spreading from the partition complex at parS over parOP. However, the effect did require ParA and ParB. An explanation for these findings is that the ParB-parS partition complex influences par regulation via the act of partition. The partition activities of ParA require an interaction with ParB-parS partition complex and a stimulation of its hydrolytic activity. A consequence of these events is an increase in the concentration of ParA-ADP - the repressor form (Bouet and Funnell, 1999; Hao and Yarmolinsky, 2002). Similar results have been shown in the F plasmid system (Watanabe et al., 1992; Yates et al., 1999).

**Roles of ParA in partition:** The par promoter can be substituted for a low-level constitutive promoter without significantly affecting P1 plasmid stability. Therefore ParA repressor activity on parOP is dispensable for partition. However, ParA is still required for plasmid stability showing that, apart from repression, ParA is a direct player in the partition reaction. In this section, I describe the genetic studies and fluorescence microscopy that have shown ParA’s dual role in the partition reaction; namely, the dissociation of paired plasmids, and their subsequent positioning.

One particular type of ParA mutant, called “propagation defective” or Par\(^{PD}\), has yielded a wealth of information into the dissociative role of ParA (Fung et al., 2001). The mutant phenotype is considered to be “worse-than-null”, as plasmids that carry parA null mutations can still be established, and are slowly lost from a cell population by random diffusion. As the phenotypic classification suggests, stability assays show that P1 plasmids encoding Par\(^{PD}\) mutants cannot be established or maintained in a cell (Fig. 1-8). This phenotype is dependent on both ParB and parS, indicating that the mutation alters how ParA interacts with the partition
complex. It is hypothesized that ParA Par\textsuperscript{PD} mutants retain the ability to associate with the ParB-par\textsuperscript{S} partition complex, and promote further complex assembly, but have lost their plasmid dissociation activity. Consequently, following each round of replication, the plasmids remain grouped together, and are unable to separate from one another. In Chapter 4, I employ a light-scattering approach to examine how ATP-cycling by ParA modulates the assembly and disassembly of the ParA-ParB-par\textsuperscript{S} complex.

Following the physical segregation of the plasmids, ParA acts to position the segregants. Immunofluorescence visualization of ParB and the ParB-par\textsuperscript{S} partition complex identified the requirement for ParA in plasmid positioning (Erdmann et al., 1999). As mentioned, partition complexes appear as large punctate foci, and their positions correlate with the positions of P1 plasmids, as measured by tagging with GFP-LacI or by FISH (Gordon et al., 1997; Ho et al., 2002). In the presence of ParA, complexes properly localize: if a cell had a single focus, it was localized at mid-cell, and if a cell had two foci, they were localized at the quarter-positions (Erdmann et al., 1999). Some of the longest cells contained four foci arranged equidistant to each other. In all cases, the foci were localized over the nucleoid region of the cell. In the absence of ParA, the focus size, number and positioning were altered. There were fewer foci per cell, the number of foci did not correlate with cell length, and they were mislocalized. Intriguingly, the foci did not mislocalize randomly. In cells with a single nucleoid, they were primarily found at the cell poles, and in cells with multiple nucleoids they were also found in the cytosolic space in between nucleoids. Two possible mechanisms could account for this localization defect: cytosol sequestration or nucleoid occlusion. Since the plasmids seem to be randomly mislocalized in the cytosol and not confined to a particular intracellular address, I find it more likely that, in the absence of ParA, the partition complexes are occluded from the nucleoid region of the cell. ParA could possibly alleviate this exclusion by indirectly stimulating a physical interaction between ParB and the nucleoid. Alternatively, ParA could act directly in tethering the ParB-par\textsuperscript{S} partition complex to a nucleoid-associated protein or to the bacterial chromosome itself. How the partition complex distinguishes these sites is not understood.

The nucleoid is a region within a bacterial cell comprised of protein and the bacterial chromosome. While the P1 partition proteins have not been shown to interact with any nucleoid associated proteins, ParA could interact with the nucleoid through a non-specific interaction with the bacterial chromosome. \textit{In vitro}, ParA can bind DNA of no particular topology, length or
sequence, and this activity increases in the presence of ATP (Funnell, unpublished data). Moreover, DNA-binding stimulates ParA ATPase activity, and when in the presence of ParB, stimulation is synergistic. Similar findings were obtained with the partition ATPase of F plasmid, SopA (Bouet et al., 2007a). Recently, a SopA mutant deficient in non-specific DNA-binding activity, but functional for all other known biochemical properties, displayed a severe partition defect in vivo (Castaing et al., 2008). Furthermore, a conserved set of arginines within the chromosomal ParA homologue, Soj, were shown to be essential in non-specific DNA-binding in vitro, nucleoid colocalization in vivo, and maintaining plasmid stability (Hester and Lutkenhaus, 2007). Thus, a ParA-non-specific DNA interaction is presumed to be important for the partition mechanism but how it is involved at the molecular level is still unclear. I favor the hypothesis that a ParA-nucleoid interaction is needed to localize plasmids in areas of the cell normally occluded by the bacterial chromosome. In Chapter 3, I provide evidence arguing that ParA can interact with the nucleoid through a non-specific interaction with the chromosome, and I propose a role for this interaction in directing plasmid movement.

**Steps in the Plasmid Partition Reaction:**

**Plasmid Pairing:** Once partition complexes are assembled and functional, it is thought that these complexes can pair or cluster plasmids through ParB-ParB interaction. Several arguments have been put forth supporting the idea that plasmids can pair following replication. (1) The earliest suggestion of pairing came from a model explaining partition-mediated incompatibility between plasmids that are partitioned by similar partition sites (Austin and Nordstrom, 1990). In this model, the formation of mixed pairs of plasmids would result in the randomization of plasmid positioning (Fig. 1-9). (2) Expression of high levels of ParB caused plasmids containing parS to be less stable than if they were segregated randomly without an active partition system (Funnell, 1988a). Over-expression of ParB even destabilized high-copy plasmids without reducing their copy number suggesting that plasmids were segregating as clumps, rather than individual molecules. (3) As mentioned previously, the identification of the ParPD is consistent with the idea that plasmid pairing is normally mediated by wild-type ParB and ParA (Fung et al., 2001; Youngren et al., 2000). (4) An in vivo topological assay that used DNA looping as a measure for interaction between two parS sites on the same plasmid showed that ParB could pair the two sites intramolecularly (Edgar et al., 2001). (5) In vivo fluorescence
visualization of ParB foci and plasmid foci imply that multiple plasmids are contained with one focus (Erdmann et al., 1999; Ho et al., 2002). (6) So far the only direct *in vitro* evidence for a pairing event comes from electron microscopy (EM) studies on the plasmids R1 and pB171 (Jensen et al., 1998; Ringgaard et al., 2007). In these cases, plasmid pairing was shown to be dependent upon the cognate partition complex. While it is convincing that pairing can occur, recent real-time fluorescence microscopy of R1 plasmid *in vivo* has shown that it is not essential for the partition reaction (Campbell and Mullins, 2007). The R1 partition system falls into a different class of partition system than that of P1 and pB171 (see below), suggesting that a pairing event, essential or not, is likely to be a common feature of all partition systems.

The way in which P1 ParB can pair plasmids is unknown. Previous models have suggested that contacts between ParB and its recognition motifs from the two *parS* sites would likely be important in the formation of a solenoid-like partition complex, and would also likely play a role in the ability of ParB to spread onto DNA to form the large nucleoprotein complexes observed *in vivo* (Surtees, 2001). Is pairing mediated solely through ParB-ParB contacts, or can individual dimers of ParB contact two different DNA molecules simultaneously? A notable feature of the ParB structure is its unique bridging function that not only explains intramolecular bridging between the two arms of *parS*, but could also direct intermolecular plasmid pairing by bridging two independent *parS* sites simultaneously (Schumacher and Funnell, 2005). In Chapter 2, I propose a model in which ParB dimers can use motifs in *parS* to bridge between the two plasmid copies.

**Plasmid Dissociation:** The plasmid dissociation step in the partition mechanism is poorly understood. But several clues suggest that the answer lies in understanding how ParA interacts with the ParB-*parS* partition complex. *In vitro*, ParA can interact with the ParB-*parS* complex as measured in gel-mobility shift assays (Bouet and Funnell, 1999). At a high ParB:ParA ratio, ParA promoted the formation of a complex that was larger than the initial ParB-*parS* complex. It remains to be determined whether ParA physically joins the complex, helps to recruit more ParB dimers, or both. At a low ParB:ParA ratio, ParA interferes with ParB oligomerization and the ParB-*parS* interaction. I speculate that these observations are a reflection of the interplay between ParA and ParB observed *in vivo*. At relatively high concentrations of ParB, ParA cannot dissociate paired or clustered plasmids, but can still play a role in complex assembly and/or stability. At relatively low concentrations of ParB, the
dissociative role of ParA predominates.

In the gel-mobility shift assays, the ParA-ParB-parS interaction could only be supported by ATP if it was supplied in all media - reaction buffer, gel matrix, and running buffer (Bouet and Funnell, 1999). Although this assay has been successful in identifying the protein-DNA and protein-protein interactions essential for partition, the biologically relevant nature and stoichiometry of these complexes is still in question. Several other separative methods have proven unsuccessful in isolating the ParA-ParB-parS complex. For these reasons, I propose that the ParA-ParB and ParB-ParB interactions required to form the large partition complex at parS are transient, highly dynamic, and dependent upon ParA ATP-cycling. The capability of ParA to first associate with the partition complex and subsequently dissociate the plasmids seems to stem from its ability to modulate the size of the partition complex in response to an unknown spatial and/or temporal signal. In Chapter 4, I use a solution based light-scattering approach to further study the ParA-ParB-parS complex in real-time, without the use of separative steps.

**Plasmid Localization:** Following dissociation, why and how does the partition system localize plasmids to the quarter positions? The answer lies in identifying what constitutes the intracellular locations of a plasmid. The fact that there are a limited number of partition complexes in a cell does suggest that partition systems can count. In a typical cell, the magic number seems to be one for new born cells, and two for more mature cells. It is easy to imagine that partition systems limit the number of complexes by tethering themselves to a finite number of sites within the host. In this scenario, the number of tethers present in a cell would dictate the number of sites a partition complex could localize to, and any extra copies of the plasmid would be forced to join a group of plasmids at these pre-determined sites.

The earliest tethering model suggested the inner membrane as the attachment point responsible for the localization of the replication and segregation machinery for both plasmid and chromosomal DNA (Jacob et al., 1963). It proposes that the outwards growth of the membrane would move apart newly replicated DNA. Several studies refute this model showing that newly replicated chromosomal loci, and actively partitioned plasmids segregate at a rate that is much faster than the average rate of cell elongation (Fiebig et al., 2006).

Currently, there is also no direct evidence of a host-protein responsible for tethering plasmids; although, several plausible candidates have been tested. P1 plasmids with functional partition systems were found to be stable in *E. coli mukB* mutants, therefore showing that MukB
is not essential in P1 partition (Funnell and Gagnier, 1995). The cell division proteins, FtsZ and FtsI, have also been ruled out (Erdmann et al., 1999; Pogliano et al., 1997). Inhibition of FtsI using cephalaxin treatment or the use of temperature sensitive mutants of FtsZ both create long filamentous E. coli cells. In both treatments, P1 was distributed properly in the vast majority of filamentous cells as measured by FISH and immunofluorescence of ParB. Similar results were found for F and RK2 plasmids (Ho et al., 2002). Together, the current evidence argues against a role for MukB, the cell division machinery, and the act of cell division itself in plasmid partition.

The replication apparatus has also been an appealing candidate for plasmid tethering and/or localization. It seems intuitive for a plasmid to ‘hang around’ the replisome to coordinate its replication and segregation processes. It was previously thought that the replication machinery of E. coli and B. subtilis reside as stationary factories, which DNA spools through during its replication (Lemon and Grossman, 1998, 2001). Coincidentally, the location of the replication machinery matched the intracellular addresses of the plasmids – mid-cell and the quarter positions. However, there is evidence that questions this idea. First, inhibiting replication does not affect P1 partition (Treptow et al., 1994). Also, given the speed of DNA polymerization (~1 Kb/s) plasmid positioning as a result of replication would be transient. In principle, the replication time for the P1 plasmid (91.6 Kb) is ~1.5 minutes.

While the act of replication is not a likely candidate for plasmid localization, the physical attachment to a replisome subunit has not been ruled out. If plasmids were permanently attached to a fixed replication factory, then plasmids with different partition systems should colocalize in a cell. This is not the case. P1, RK2 and F plasmids localize in the same vicinity of mid-cell or the ¼ and ¾ positions, but they do not colocalize (Pogliano, 2002). In addition, RK2 plasmid can localize to these same positions in several different bacteria, implying that if there is a specific host-protein tether, the association is conserved in a wide variety of bacteria and plasmids.

With improvements in cell biology techniques it has most recently been shown that replisomes can actually assemble anywhere in a cell, and their positions are determined, not by a tether, but by the location of the replication origins with which they are associated (Reyes-Lamothe et al., 2008). This coincides with the finding that plasmids without a partition site from E. coli and B. subtilis can replicate far from mid-cell, and when at high-copy, they promote the formation of several replisomes (Niki and Hiraga, 1997; Onogi et al., 2002). Overall, there has
been no record of any replisome component physically interacting with a plasmid partition component. Even though the possibility of plasmids being tethered to their own unique replisome inside the cell still exists, in light of the above findings, I find it more likely that this association results in the plasmid localizing the replisome and not vice versa.

**ParA DYNAMICS & THE TYPE I PARTITION MECHANISM**

**The Controversy on the Dynamics of Type I Partition ATPases**

Immunofluorescence visualization of P1 ParA and the use of a ParA-GFP fusion have provided limited information as to how P1 ParA positions plasmids. Both methods show ParA distributed diffusely throughout the cell; possibly being more concentrated over the nucleoids (Erdmann, 1999; Zhang, unpublished results). However, several fluorescent variants of partition ATPases related to P1 ParA (SopA from F plasmid, ParA from the *E. coli* virulence plasmid pB171, ParAI from chromosome I of *V. cholera*, δ of pSM19035 and Soj from *B. subtilis*) have been observed to colocalize with the nucleoid (Ebersbach et al., 2005; Lim et al., 2005; Marston and Errington, 1999; Pratto et al., 2008; Quisel et al., 1999; Raskin and deBoer, 1999). With these partition ATPases, nucleoid colocalization was dynamic, exhibiting oscillatory behaviors. A cloud of fluorescence would amass at one end of the nucleoid, diffuse, and then re-associate with the opposite end of the nucleoid. ATPase activity and the ability to interact with their cognate partition complex were shown to be necessary for oscillation (Ebersbach et al., 2005; Lim et al., 2005; Marston and Errington, 1999; Pratto et al., 2008; Quisel et al., 1999; Raskin and deBoer, 1999). With either activity removed, the ATPases were static, and uniformly coated the nucleoid. Since oscillation and plasmid partition share the same requirements, it is possible that this dynamic movement is important for plasmid partition and chromosome segregation processes. But, whether oscillation plays a direct role in partition, or is solely a byproduct of the reaction, remains to be determined. Although the bulk of P1 ParA-GFP in a cell has not been observed to oscillate, we cannot rule out the possibility that a subpopulation of the protein also exhibits this dynamic behavior.

Deconvolution microscopy resolved these dynamic ‘clouds’ to look like helical structures (Ebersbach and Gerdes, 2004). Since several Type I partition ATPases also form filaments *in vitro*, it was inevitable for these intracellular helical structures to be eventually called ‘filaments’. As a result, several models arose where helical, self-supporting ParA filaments physically push
or pull plasmids via their polymerization-depolymerization cycles (Bouet et al., 2007b; Ebersbach and Gerdes, 2004; Hatano et al., 2007); following the lead of eukaryotic mitosis and the Type II partition mechanism, exemplified by R1 plasmid partition. Several other bacterial proteins involved in a variety of other cellular processes, besides DNA segregation, have been shown to form this spiral-like pattern. The nature and biological relevance of this in vivo pattern shown for partition ATPases is still up for debate.

The Min Division-Site Selection System:

A key feature that defines the partition activity of a Walker-type ATPase is its oscillatory movement in vivo. Yet, there has been little biochemical data that relates the dynamics to a molecular mechanism for partition. A similar dynamic system behavior has been studied extensively for more than a decade, and that is the cell division system of E. coli called MinCDE.

The MinCDE system selects the cell-division site in E. coli (Lutkenhaus, 2007). Inactivating the Min system leads to the formation of mini-cells as a result of the aberrant placement of the cell-division machinery near the cell poles. MinD, like P1 ParA, is a Walker-type ATPase (deBoer et al., 1991; Koonin, 1993; Michie, 2006). A unique feature that separates MinD from Type I partition ATPases is its ability to associate with the membrane when bound to ATP (Hu et al., 2002). In the presence of ATP (or ATPγS) and phospholipids, MinD polymerizes into small bundled filaments (Suefuji et al., 2002). As it will become more apparent in Chapter 3, I believe a firm understanding of the MinCDE system is crucial in solving the localization mechanism employed by parABS, and potentially all Type I partition systems.

The weak ATPase activity of MinD and its ability to be stimulated are remarkably similar functions to that observed with Type I partition ATPases (deBoer et al., 1991). ATP-hydrolysis by MinD is cooperatively stimulated (~10-fold) by MinE and phospholipid vesicles (Hu et al., 2002). By stimulating the ATPase activity of membrane-bound MinD, MinE converts MinD to a form no longer competent for membrane binding; effectively disassembling MinD polymers from the membrane (Hu et al., 2002).

MinC is the effector of the Min system responsible for inhibiting cell division by directly antagonizing the initiator of septum formation, FtsZ (Boer et al., 1990; de Boer et al., 1992). When away from MinC, FtsZ has the potential to polymerize on the membrane into a “Z-ring” – the structure required to initiate cell-division (Lutkenhaus, 1993). On its own, MinC inhibits
FtsZ poorly as it does not have membrane binding activity (de Boer et al., 1992). MinD forms a complex with MinC (MinCD) and it is the membrane binding activity of MinD that recruits MinC to the membrane, allowing for strong FtsZ inhibition.

The remarkable aspect of the Min system is its ability to inhibit division everywhere but at mid-cell. It was real-time microscopy of MinD-GFP that demonstrated its astonishing oscillatory activity (Raskin and deBoer, 1999). During a single oscillatory period, MinD formed a polar zone on the membrane, extending toward mid-cell, which then started to shrink toward the pole. As this polar zone disappeared, a new polar zone would establish at the other end, and then the cycle repeated. The oscillation of MinD was dependent upon MinE, and in its absence, MinD uniformly bound the inner membrane surface (Raskin and deBoer, 1999). MinE also oscillated as an E-ring, which was continually associated with the disassembling edge of the MinD polar-zone (Fu et al., 2001; Shih and Rothfield, 2006). Deletion of MinC has no effect on MinD oscillation proving it is only along for the ride with MinD. It is widely accepted now that E. coli identifies its mid-cell through this oscillatory model (Fig. 1-10). A wave of membrane-bound MinCD is continually chased by MinE, such that the spatiotemporal average of MinC concentration will always be lowest at mid-cell; thus allowing FtsZ to localize and initiate cell division at mid-cell.

Deconvolution microscopy further resolved the MinD waves to look like membrane-associated helical structures (Shih et al., 2003). As mentioned previously, the cytoskeletal element, MreB, also forms an identical helical-structure at the inner membrane surface. Despite this similarity, Min oscillation is maintained in ΔmreB cells, indicating that the MreB cytoskeleton is not the basis for the MinD helical array (Shih et al., 2003). Unlike MinD spirals that traverse the entire cell, the spirals formed by Type I ATPases are confined to the nucleoid region of the cell.

Once MinD is released from one pole, how does it find the opposite pole for reassembly? In filamentous cells, MinD oscillates in a striped pattern, indicating that a pole-localized nucleation point is not necessary (Raskin and deBoer, 1999). Oscillation can also setup in spherical cells (Shih et al., 2005) suggesting that dynamic pattern formation does not require a positional marker. Consequently, the Min system is referred to as ‘self-organizing’ because its ordered behavior is solely a result of the interactions among the system components and a membrane-matrix. A key feature of self-organization is energy consumption, such as nucleotide
hydrolysis, which allows for dynamic behavior at equilibrium (Nédélec et al., 2003).

The most recent model that explains the biochemical activities of the individual Min system components in the context of their oscillatory behavior in vivo is as follows (Lutkenhaus, 2007) (Fig. 1-10): Upon MinD-MinE interaction at the membrane, MinD ATPase activity is stimulated and both proteins are released into the cytoplasm (Fig. 1-10A). MinE can immediately rebind to nearby MinD that is still membrane-bound in the occupied cell half, whereas the disassembled MinD-ADP subunit must undergo nucleotide exchange (Fig. 1-10B). Cytoplasmic MinD-ATP prefers to bind membrane already bound by MinD, therefore the occupied pole acts as a vacuum, lowering the cytoplasmic concentration of MinD-ATP. At the same time, MinD-ATP that diffuses to the opposite pole binds the membrane less efficiently as it must undergo de novo interactions with the new pole. But, as the cytoplasmic concentration of MinD-ATP at the new pole rises, rebinding will eventually occur (Fig. 1-10C), leading to the formation of a new polar zone (Fig. 1-10D).

THESIS RATIONALE

The goal of my thesis work was to elucidate the mechanism of P1 plasmid partition by characterizing nucleoprotein complexes involved in the reaction: The ParB-parS partition complex, the ParA-nucleoid complex, and finally the ParB-ParA-DNA complex.

In Chapter 2, I describe how a single dimer of ParB recognizes its complicated arrangement of recognition motifs when it loads onto the full parS site in the presence of IHF. Together, my results show that a ParB dimer can load onto parS in a variety of ways, so that the initial ParB-IHF-parS complex consists of a mixture of different orientations of ParB bound to parS.

In Chapter 3, I present a series of experiments performed in collaboration with the Mizuuchi group that characterizes the non-specific DNA binding activity of ParA. We find that an ATP-specific conformational change licenses ParA to bind non-specific DNA, and the kinetics of binding provides a time-delay switch that allows the ParB cluster bound around parS to generate an uneven distribution of the nucleoid-associated ParA, which we propose provides the motive force for plasmid segregation.

In Chapter 4, I use a light-scattering approach to study the interaction of ParA with the ParB-parS partition complex. I found that ParA forms a large, but dynamic nucleoprotein
complex with ParB and DNA. The kinetics of the complex were modulated by ParA ATP-cycling. From the results, I propose that ParA-ATP can polymerize along DNA from a ParB-parS nucleation core and ATP-hydrolysis leads to disassembly. In this light, I present a model where ParA filaments act as dynamic adaptors between the nucleoid and the plasmid.

In Chapter 5, I summarize my thesis and provide a general discussion of the importance of my work and its contribution to the field of DNA segregation in bacteria. In addition, I suggest some future investigations that will further address some of the key questions that remain in the field of plasmid partition.

In the Appendix, I explain the construction, purification and characterization of a spectrum of single-cysteine ParB variants. I found that several of these variants were functional both in vivo and in vitro, and could be labeled with a variety of thiol-reactive probes without altering activity. I describe in detail how these modifiable ParB variants will be useful tools in dissecting the protein-protein and protein-DNA interactions required for P1 plasmid partition.
<table>
<thead>
<tr>
<th></th>
<th>Type Ia</th>
<th>Type Ib</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Partition ATPase</strong></td>
<td>Size: 321-420 aa N-terminal HTH-motif Repressor of <em>par</em> operon</td>
<td>Size: 192-308 aa no HTH-motif present</td>
</tr>
<tr>
<td><strong>Partition Site Binding protein</strong></td>
<td>Size: 312-342 aa Moderate homology</td>
<td>Size: 46-131 aa Few Homologies Repressor of <em>par</em> operon</td>
</tr>
<tr>
<td><strong>Partition site</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Located downstream of operon</td>
<td>Located upstream of operon</td>
</tr>
<tr>
<td><strong>Model Systems Included</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td><em>parABS</em> of P1 <em>sopABC</em> of F</td>
<td><em>par2</em> of pB171 <em>parFGH</em> of pTP228</td>
</tr>
</tbody>
</table>

<sup>a</sup> Some *par* loci contain more than one *cis*-acting region (e.g. *par2* of pB171 has two *cis*-acting sites) (see fig.1-1) (Ebersbach and Gerdes, 2001; Gerdes et al., 2000).

<sup>b</sup> The model systems highlighted are examples of those discussed in this thesis. The number of Type Ia and Ib systems is an extensive list.
Figure 1-1. Genetic organization of representative Type Ia (plasmids P1 and F), Type Ib (plasmids pTP228 and pB171) and Type II (plasmid R1) partition loci. Genes encoding the ATPase (blue arrow) and partition site binding protein (red arrow) are indicated. The partition site and promoter regions are indicated as black and white rectangles, respectively. Green arrows indicate the DNA-binding properties of the gene product in forming the partition complex. Pink arcs indicate the gene products role in regulation of promoter activity. Type III partition loci are not illustrated as the genetic organization has not been characterized thoroughly.
Figure 1-2. *ori* and *ter* positioning during *E.coli* chromosome segregation. In slow growing *E. coli* cells, *ori* and *ter* are located proximal to the old and new cell-poles, respectively (top). They both migrate to the mid-cell region, where bidirectional DNA replication occurs starting at *ori* (right). The *ori* copies move away from each other, toward opposite poles, while *ter* remains centrally positioned (bottom). After completion of replication, DNA segregation and cell division, the termini are once again situated close to the new poles (left).
Figure 1-3. Molecular model of plasmid partition by the R1 par operon. (A) Nucleation of new filaments occurs throughout the cell. Filaments attached to one plasmid will search for a second plasmid. (B) When two plasmids encounter each other, filaments are bound at each end by a plasmid and form a spindle. This prevents the filaments from undergoing catastrophe. (C) As these stabilized filaments polymerize, the two plasmids are forced to opposite poles. (D) After reaching a pole, pushing against both ends of the cell causes the filament to dissociate from the plasmid at one end and quickly depolymerize (adapted from Campbell, 2007).
Figure 1-4. Schematic of the general steps of P1 partition over an *E.coli* cell-cycle. In new-born cells, plasmids are located at mid-cell (top). They replicate and are thought to group together (Right). An unknown signal then triggers partition and the plasmids localize to the ¼ and ¾ positions (Bottom). Following resolution of the replicated host chromosome (yellow bodies), division occurs (Left), and there is a faithful inheritance of a single copy of the P1 plasmid to each of the daughter cells. Red circles represent the ParB-parS partition complex on the plasmid.
Figure 1-5. The P1 par operon. (A) parA (blue arrow) and parB (red arrow) genes are downstream of the par promoter/operator region, parOP (white box) and upstream of the partition site, parS (black box). (B) parOP contains the transcription signals (-35 and -10), the ribosome binding site (RBS), and start codon for ParA (ATG). The inverted repeat (black arrows) between -10 and RBS are recognized by ParA. (C) parS contains four copies of BoxA (light blue), and two copies of BoxB (orange) asymmetrically arranged around an IHF binding site (black rectangle). Arrows above the A- and B-boxes represent their sequence direction. Boxes A2 and A3 make up an inverted repeat. A detailed illustration of parS is described in Fig. 2-1.
Figure 1-6. P1 ParB. (A) Functional domains and conserved sequences. The scale shows residue length and positioning. ParB is represented as a white rectangle and the positions of conserved motifs are drawn in. The DNA-binding motifs are highlighted with color: The HTH motif in blue and the dimer-domain in orange. The bars below ParB show the regions for which a function has been defined. Black bars correspond to a protein interaction interface, and the white bar corresponds to the sequence required for parS binding: The HTH-motif binds A-boxes (Blue) and the dimer domain binds B-boxes (Orange). (B) Structure of the ParB-parS-small complex. ParB(142-333) subunits are coloured cyan and pink, and secondary-structural elements are labelled for one subunit. On parS-small, boxes A2, A3 and B2 are coloured yellow, green and blue respectively. (C) Representation of the ParB dimer with the HTH domains attached by flexible linkers to the dimer domain. (D) Model of individual DNA-binding domains bound to the same parS-small duplex, showing that one ParB dimer cannot bind all motifs simultaneously on one parS-small site. (B and D) reprinted by permission from Macmillian Publishers Ltd: Schumacher and Funnell (Nature), © 2005.
Figure 1-7. P1 ParA. (A) Functional domains and conserved sequences. The scale shows residue length and positioning. ParA is represented as a white rectangle and the positions of conserved motifs are drawn in. The DNA-binding and Walker-box motifs are highlighted in red and blue, respectively. The bars below ParA show the regions for which a function has been defined. Red bars correspond to DNA-binding regions, the yellow bar corresponds to a critical dimerization interface, and the black bar corresponds to the ParB interaction interface. (B) Ribbon diagram of an apoParA dimer. The right subunit has its secondary-structural elements labelled, and is color-coded as in (A). (C) A ParA-ADP dimer modelled onto a 40-bp DNA substrate. After ParA binds ADP, refolds and stabilizes, modelling predicts a role for motif 1 and 2 in non-specific DNA-binding. (B and C) reprinted by permission from Macmillian Publishers Ltd: Dunham et al. (EMBO), © 2009.
**Figure 1-8. The Par\textsuperscript{PD} phenotype.** In the presence a functional partition system (\textit{par}\textsuperscript{+}), plasmids are properly localized and therefore stable in an \textit{E. coli} cell population. In the absence of a functional partition system (\textit{par}⁻), the plasmids are mislocalized and segregate by random diffusion, leading to a slow loss of the plasmid in the cell population. Plasmids encoding a ‘propagation defective’ mutant (\textit{par}\textsuperscript{PD}) cannot be established or maintained in a cell population. A possible mechanism for this ‘worse-than-random’ phenotype is that the plasmids remain glued following replication, and therefore segregate as clumps instead of individual molecules. Red circles represent the partition complex on the plasmid.
Figure 1-9. The mixed pairing model of incompatibility. (Top) When plasmids encode sufficiently different partition systems (represented as different colour complexes), both plasmid sets are compatible and properly localize following replication. (Bottom) When two otherwise different plasmids (represented as red and black plasmids) share the same partition determinants, random orientation of heterologous pairs leads to frequent missegregation, producing the incompatibility phenotype.
Oscillation of the Min proteins in *E. coli*. (A) MinD-ATP binds to the membrane and recruits MinC. MinE displaces MinC and stimulates MinD ATPase activity, causing release of the proteins from the membrane. (B) Released MinE can immediately rebind to MinD on the membrane, but released MinD must undergo a currently unknown time-delay mechanism to regenerate MinD-ATP in the opposite half of the cell to initiate assembly at the opposite pole. (C) Once MinD-ATP concentrations are sufficiently high, MinD-ATP forms a new polar zone. (D) As MinE is released from the old pole, it binds to the end of the MinD polar zone and the process repeats.
CHAPTER 2

P1 PARTITION COMPLEX ASSEMBLY INVOLVES SEVERAL MODES OF PROTEIN-DNA RECOGNITION

A version of this chapter has been published as:


Adapted with permission from the American Society for Biochemistry and Molecular Biology (Vecchiarelli et al., 2007).

Barbara Funnell constructed the parS variants and performed the incompatibility assays. I performed all other experiments in this chapter.
The partition complex assembly at parS is nucleated by a dimer of P1 ParB and IHF. This initial complex promotes the loading of more ParB dimers and the pairing of plasmids during the cell cycle. In this chapter I characterized how a dimer of ParB recognizes its complicated arrangement of recognition motifs when it loads onto the full parS site in the presence of IHF. I addressed this question by examining ParB binding activities to parS mutants containing different combinations and subsets of the A- and B-box motifs in parS. ParB binding to linear and supercoiled DNA was measured in electrophoretic and filter binding assays, respectively.

Biochemical assays have shown that the C-terminal half of ParB (residues 142 to 333) contains all the information necessary to form the dimeric, high-affinity (IHF-stimulated) ParB complex at parS (Surtees and Funnell, 2001). The crystal structure of ParB[142-333] bound to a 25 bp DNA duplex containing the parS-small sequence has been determined (Schumacher and Funnell, 2005) (Fig. 1-6B). The crystal structure revealed that ParB contains two essentially independent DNA-binding domains separated by a short (four-residue) flexible linker. This arrangement permits ParB to contact a variety of A- and B-box combinations within parS. As functional studies predicted, the crystal structure of ParB[142-333] confirms that the helix-turn-helix (HTH) motif found near the center of ParB binds the A-boxes within parS and the dimerization domain at the extreme C-terminus binds the B-boxes (Hayes et al., 1993; Radnedge et al., 1996; Schumacher and Funnell, 2005; Surtees and Funnell, 2001). The dimerization interaction creates a novel DNA binding motif that requires contributions from each monomer to bind a Box B sequence. The arrangement of the HTH motifs is unusual in that they are on opposite sides of the dimer pointing away from each other so they cannot simultaneously contact a single inverted repeat. In fact, one ParB dimer contacts its different motifs on separate copies of the same 25-mer parS-small oligonucleotide, effectively bridging different DNA molecules. The bridging activity illustrates how ParB might contact separate motifs intramolecularly across an IHF-directed bend, but also suggests that it may participate in pairing plasmids intermolecularly across parS sites (Schumacher and Funnell, 2005).

Here I am interested in how ParB contacts the parS site, particularly across the IHF-directed bend and potentially across adjacent DNA molecules. Chemical footprinting experiments showed that all A- and B-boxes within parS were protected by a dimer of ParB (Bouet et al., 2000), but the crystal structure implied that one dimer could not contact all motifs...
simultaneously (Schumacher and Funnell, 2005). In this study I examine how a dimer of ParB recognizes the parS site. Do the different A- and B-boxes work together in providing a high affinity binding site for ParB or is there a distinct set of ParB-IHF-parS complexes that use specific A- and B-box combinations? Does ParB have a binding preference for specific A- or B-boxes within parS? To address these questions, variants of parS were created with A- and B-boxes systematically mutated individually or in combination with each other. I find that ParB prefers different subsets of motifs when it initially binds to parS. ParB shows preferences for certain motifs that are dependent on position and on plasmid topology. In the simplest arrangement, one motif on either side of the IHF-directed bend is sufficient to form a complex, although affinity differs depending on the specific motifs. Therefore a ParB dimer can load onto parS in different ways so that the initial ParB-IHF-parS complex that assembles consists of a mixture of different orientations of ParB. This arrangement supports a complex in which parS motifs are available and utilized for inter- as well as intramolecular parS recognition. I propose a model where the arrangement of recognition motifs in parS is necessary to build the proper architecture of partition complexes at and across partition sites.
EXPERIMENTAL PROCEDURES

**Bacterial Strains and Plasmids** - *E. coli* strain DH5 (F<sup>−</sup> endA1 hsdR17 (rK<sup>−</sup> mK<sup>+</sup>) supE44 thi-1 gyrA96 recA1) was used to construct and maintain plasmids. The plasmid pBend5, a derivative of pBend2 (Kim et al., 1989), was used as the vector to create plasmids containing the *parS* variants, which were the DNA substrates for biochemical experiments. All *parS* variants used in this study were constructed by cloning complementary synthetic oligonucleotides (80- to 88-mers, depending on the variant; Table 2-1) into the *Hpa*I site of pBend5. The plasmid *parS* sequences were confirmed by DNA sequencing at York University (Toronto) and/or Macrogen Inc. (South Korea).

**Reagents** – Synthetic oligodeoxynucleotides were purchased from Invitrogen Corp. Sources for other reagents were as follows: [α-<sup>32</sup>P]dCTP and [<sup>3</sup>H] S-adenosyl-L-methionine, Perkin Elmer; bovine serum albumin (BSA) and salmon sperm DNA, Sigma; restriction enzymes, Klenow DNA polymerase and *Hae*III methylase, New England Biolabs.

**DNA and Proteins** - For gel mobility shift assays, the DNA substrates were total *Xho*I restriction digests of plasmid DNA. DNA fragments were labeled at their 3'-ends with [α-<sup>32</sup>P]-dCTP and DNA polymerase I (Klenow fragment), and then purified by phenol-chloroform extraction and ethanol precipitation steps. For nitrocellulose filter binding assays, supercoiled plasmid DNAs were purified in cesium chloride gradients, and labeled with [<sup>3</sup>H] S-adenosyl-L-methionine and *Hae*III methylase as described (Funnell, 1988b).

ParB (Fraction V) was purified as described previously (Bouet and Funnell, 1999). IHF was purified essentially as described (Nash et al., 1987), except that the phosphocellulose step was followed by MonoS chromatography in 15 mM phosphate buffer, pH 6.4, 5% glycerol, and protein was eluted with a 50 mM to 1 M NaCl gradient in the same buffer.

**Gel Mobility Shift Assays** - The standard reaction mixture (10 μl) contained 1 nM <sup>32</sup>P-labelled DNA in 50 mM Hepes-KOH (pH 7.5), 150 mM KCl, 10% glycerol, 80 μg of BSA/ml and 250 μg of sonicated salmon sperm DNA/ml. IHF, when present, was included at 500 nM. The mixtures were assembled on ice, incubated for 20 min at 30°C, and analyzed by electrophoresis in 5% polyacrylamide gels in 90 mM Tris-borate, 1 mM EDTA. Electrophoresis was performed at 150 V for 3.5 h at 4°C. The gels were dried onto Whatman DE81 paper and exposed to a phosphor screen for imaging by a PhosphorImager. Data were quantified using ImageQuant software (Molecular Dynamics). For each lane in the gels, the radioactivity (as phosphorimager
counts) in the area corresponding to a dimeric ParB-IHF-parS complex was measured and expressed as a fraction of the value corresponding to the parS DNA fragment in the absence of ParB.

**Analysis of the data** – All variants were tested typically three to six times for binding by ParB over a range of ParB concentrations, and the results from these titrations were averaged. When a dimer of ParB binds parS, the interaction occurs in an equimolar fashion. Therefore, the averaged data set from a particular variant was then fit to the Langmuir binding equation (Larsson and Axelsson, 1991; Ma and Lieber, 2001; Reichheld and Davidson, 2006) using SigmaPlot 2000 software:

\[ I = I_0 + \left( I_o \frac{[B]}{K_d} \right) / \left( 1 + \frac{[B]}{K_d} \right) \]

The band intensity, I, is a function of the free ParB concentration [B] and the dissociation constant (K_d), and [B] is defined by the following equation:


where [S] is the total concentration of parS substrate and [B_T] is the total concentration of ParB.
RESULTS:

Not all A and B boxes are required for a dimer of ParB to recognize and bind to parS.

The first step in partition complex assembly is the binding of one ParB dimer to parS across the IHF-directed bend (Bouet et al., 2000). I was interested in the nature of this protein-DNA interaction as well as the role of different motifs in partition complex function. The crystal structure of ParB[142-333] complexed with parS-small has raised questions about how ParB recognizes a full parS site (Schumacher and Funnell, 2005). The Funnell lab has typically studied the interaction by gel mobility shift assays in order to examine specific complexes and monitor the stoichiometry of assembly (Bouet et al., 2000). In situ OP-Cu footprinting of the dimeric ParB-IHF-parS complex showed protection of all A- and B-boxes within parS, suggesting two possible mechanisms of ParB binding to parS: 1) a single dimer of ParB binds all or most of its recognition sequences on both the left and right arms of parS simultaneously or 2) a dimer of ParB can bind various combinations of motifs across an IHF-directed bend, so that the initial complex actually represents a mixture of complexes with different modes of ParB binding to parS. To examine these possibilities, Barbara Funnell and I created a spectrum of mutant sites in which A- or B-boxes were removed by substitution with a restriction site (Fig. 2-2). Substitution allowed for the removal of specific base pair contacts with ParB while maintaining the sequence, spacing, and orientation of the remaining boxes. In my nomenclature, mutations are designated by the box name in lower case (for example, parS[a2] lacks the Box A2 specific sequence; Fig. 2-2). The use of different restriction sites also marked each parS variant differently. We constructed a negative control, called parS[Δab], which lacks all A- and B-box sequences but retains the IHF binding site (Fig. 2-2). I defined parS+ as all sequences between and including Box B1 to B2 (Fig. 2-1) because Box A4 is dispensable for partition in vivo (Funnell and Gagnier, 1994; Hayes and Austin, 1994), and its omission allowed us to reduce the number and complexity of variants that we created. I did subsequently confirm that Box A4 was dispensable in my assays; I observed no quantitative differences in ParB binding affinity between parS+ and the parS+-A4 site (see below and Table 2-2).

I first tested the effect of mutating individual motifs on the formation of the partition complex. The parS substrates were incubated with ParB and IHF and the resulting complexes were separated by gel electrophoresis (Fig. 2-3). An apparent dissociation constant (K_{dapp}) was then determined through quantitative analysis of ParB-IHF-parS complex formation (Table 2-2).
The data were evaluated using the Langmuir formula (Experimental Procedures), a common way of describing specific, high-affinity binding of two molecules (Larsson and Axelsson, 1991; Ma and Lieber, 2001; Reichheld and Davidson, 2006). The half-interval method was then used on the fitted curves to obtain the $K_{dapp}$ where $K_{dapp}$ is defined as the amount of ParB necessary for 50% maximal binding. The theoretical Langmuir curves and experimental binding curves produced $K_{dapp}$ values that were very similar.

The results showed that not all motifs are necessary for ParB’s initial recognition of $parS$, and that some motifs are more important than others (Figs. 2-3 & 2-4; Table 2-2). Mutation of Box B1 or Box A2 had no significant effect on complex formation compared to that with $parS^+$. However, removal of the other three motifs did reduce ParB affinity for $parS$. Mutation of Box A3 or Box B2 each reduced complex formation approximately 3 to 4 fold. The removal of Box A1 produced the greatest reduction in complex formation. The $K_{dapp}$ was 30-fold greater than when using $parS^+$. Therefore in this assay Box A1 is a stronger recognition sequence than Box B1 for ParB to bridge over the IHF directed bend. Comparison of $parS[a3]$ to $parS[a2]$ indicates that Box A3 is the preferred A-box on the right arm of $parS$. Finally, the data indicate that this initial ParB-IHF-$parS$ complex is only a precursor of the functional partition complex since not all motifs necessary for $parS$ function are necessary to form this first complex. I also noted that ParB bound to all $parS$ variants with the same stoichiometry as to $parS^+$ since the mobility of the ParB-IHF-$parS$ complexes were identical in all gels that I examined (Fig. 2-3).

**Motif use is influenced by substrate topology.**

The effect of mutation of Box A1 on complex formation was unexpected because genetic assays had shown that it is dispensable for $parS$ activity in vivo (Davis et al., 1990). Barbara Funnell confirmed that the single motif mutations I used in this study behaved identically in vivo to mutations previously published (Funnell and Gagnier, 1993, 1994; Hayes and Austin, 1994) using incompatibility assays, which measure the ability of $parS$ to destabilize a miniP1 plasmid partitioned by $parS^+$. The $parS[b1]$, $parS[a2]$, $parS[a3]$, and $parS[b2]$ substitutions destroyed $parS$ activity (were Inc-), compared to $parS[a1]$, which behaved as $parS^+$ (Inc+; data not shown). I considered the possibility that DNA topology was important because plasmid DNA would normally be negatively supercoiled in vivo. I used nitrocellulose filter binding assays with $^3$H-labeled plasmid DNA in vitro to test the influence of mutations in each motif on ParB binding to
supercoiled substrates (Fig. 2-5). This assay is effective at examining ParB binding directly because under these conditions IHF binds DNA to nitrocellulose poorly (Funnell, 1988b). In contrast to the assays on linear DNA, ParB bound \( \text{parS}^\ast, \text{parS}[a1] \) and \( \text{parS}[b1] \) to similar extents in this assay (Fig. 2-5A). The mutations in the right side of \( \text{parS} \) however had similar effects on supercoiled DNA as they manifested on linear DNA. Mutation of Box A2 did not affect ParB binding activity, whereas mutation of Box A3 or Box B2 resulted in weaker \( \text{parS} \) sites.

Since removing either one of the left-side boxes did not significantly affect binding, I removed both Boxes B1 and A1 (Fig. 2-5A). The ParB binding activity to \( \text{parS}[b1,a1] \) was greatly reduced; therefore either Box B1 or Box A1 on the left arm of \( \text{parS} \) is necessary for this complex but either one will suffice in this assay. This result confirms that the complexes measured in these filter-binding assays are using recognition motifs on both the left and right arms of \( \text{parS} \) over an IHF directed bend and thus ParB’s use of Box B1 is strongly influenced by the topological context of the \( \text{parS} \) site. Therefore the ability to use Box B1 is dependent on negative supercoiling, but in plasmids with this topology, either Box A1 or B1 is sufficient to bridge across the IHF-directed bend.

**The role of right-side motifs in ParB recognition.**

I next examined \( \text{parS} \) variants lacking two motifs (Fig. 2-2). First, each right-side motif was mutated in combination with either Box A1 or Box B1 (Fig. 2-6A and Table 2-2). All double mutants lacking A1 were poor substrates for ParB. \( \text{parS} \) sites lacking Box B1 and one right-side motif were similar to or slightly weaker \( \text{parS} \) sites than the corresponding \( \text{parS} \) sites with single right-side mutations. Therefore two motifs on the right side are sufficient to mediate a relatively strong interaction with ParB as long as there is a specific left-side interaction. I concluded that Box B1 could not contribute significantly because the substrates were linear. I confirmed this conclusion by testing two of these right-side mutations (\( a2 \) or \( a3 \)) in the presence of either \( a1 \) or \( b1 \) substitutions in \( \text{parS} \) sites on supercoiled plasmids in nitrocellulose filter binding assays. In this experiment, \( \text{parS}[a1, a2] \) behaved as \( \text{parS}[b1,a2] \), and \( \text{parS}[a1,a3] \) behaved as \( \text{parS}[b1,a3] \) (Fig. 2-5B). Therefore, as seen with the single mutants, either Box B1 or A1 is sufficient for a specific interaction of ParB with the left-side of \( \text{parS} \) on supercoiled substrates.

Since, with the exception of Box A1, the gel-shifts and filter binding assays showed similar
motif preferences for ParB, I continued the analysis using gel electrophoresis as it allowed me to identify and quantify the dimeric ParB-IHF-parS complexes. I next examined the behavior of parS sites with double mutations in the right side of parS (Fig. 2-6B) to see if any of the three right-side motifs were sufficient to mediate a specific interaction with ParB, and if so, whether they were equivalent. The answer was yes, all could interact specifically with ParB, but no, they were not equivalent. parS sites with only Box A3 or Box B2 were weaker but specific sites (parS[a2,b2] and parS[a2,a3] were better substrates than parS[Δab]; Fig. 2-6B). However the parS site with only Box A2 (parS[a3,b2]; Fig. 2-6B) was similar to parS sites lacking only one motif on the right side of parS (Fig. 2-6A; Table 2-2). The simplest interpretation is that in the presence of IHF and a specific left-side interaction, Box A2 alone is sufficient to mediate a relatively strong interaction on the right side of parS. However in the absence of Box A2, both Boxes A3 and B2 must be present for highest binding activity of the right side of parS (Fig. 2-6B and Table 2-2).

Finally, I tested parS variants that contained only motifs A1 and A2 (parS[b1,a3,b2]), only A1 and A3 (parS[b1,a2,b2]) and only A1 and B2 (parS[b1,a3,b2]) to confirm that only one motif on each side of parS was sufficient to mediate a specific interaction. Within experimental error, the affinity of ParB for these parS variants was similar to that when Box B1 was present (Fig. 2-6B; Table 2-2).

My results indicating a role for Box A1 on linear DNA prompted me to reassess the involvement of Box A4 in complex formation, even though the presence of Box A4 did not affect the activity of parS+ (Figs 2-3 & 2-4, Table 2-2). I asked whether Box A4 could substitute as a single motif on the right side of parS. ParB bound weakly to parS with only Boxes A1 and A4 (Fig 2-6B; Table 2-2), and not to parS with Boxes B1 and A4. Note that Box A4 is 22 bp, or two turns of the helix, to the right of Box A2, so I interpret the A1-A4 activity as ParB bridging across the bend in a specific and similar, but weaker fashion as between A1 and A2.

In summary, the parS variants that were constructed for this analysis fell into three general categories: strong binding sites (with Kdapp similar to or only a few fold higher than parS+), weak but specific binding sites (such as parS[a2,b2]), and sites with no detectable specific binding (equivalent to parS[Δab]). Several representative parS sites are graphed over a large titration range of ParB to illustrate these groups in Fig. 2-6C. All parS sites with intact A1 boxes fell into the former two categories (with linear DNA), indicating that bridging across the IHF-
directed bend could occur with a minimum of one motif on each side of parS.

**ParB interactions across the bend in parS.**

All of the previous assays included IHF, so I have not directly measured the role of the IHF-directed bend. To ask how ParB binds to parS in the absence of the bend, I measured ParB interactions with parS sites lacking one or more motifs in the absence of IHF (Fig. 2-7A). Consistent with previous observations that the right side of parS, or parS-small (Fig. 2-1), is the minimal partition site, removal of motifs on the left side of parS did not affect ParB binding in the absence of IHF. In other words, without the bend, ParB does not interact productively with the left side of parS. Examination of the right side mutants showed that Box A2 mutations did not affect minimal binding activity, but mutations in Box A3 or Box B2 essentially destroyed specific ParB binding activity as measured in this assay.

I directly compared ParB binding with and without IHF for several of these parS derivatives (Fig. 2-7B-E). As seen previously, ParB binding to parS+ is strongly stimulated by IHF (>50-fold; Fig. 2-7B). IHF also strongly stimulated ParB binding to parS variants with one right side mutation (Figs 2-7D & E). In fact, since the binding of ParB was very weak to parS[a3] or parS[b2] without IHF, ParB binding to these sites was essentially IHF dependent (Fig. 2-7E and data not shown). ParB binding to sites lacking both left-side motifs (parS[b1,a1]; Fig. 2-7C) was similar with and without IHF. These data further confirm that a dimer of ParB normally sits on parS so that it bridges motifs across the IHF-directed bend. Barbara Funnell constructed one other parS variant to emphasize the importance of the direction of the IHF-directed bend. She took advantage of my observation that Boxes A1 and A2 were sufficient to form a high-affinity complex and constructed an A1-A2 parS site in which the orientation of A1 and A2 were reversed but their positions in parS were maintained (parS[RevA1A2]; Fig. 2-2). This arrangement changed the orientation of these Box A motifs with respect to the bend but not with respect to each other. This parS variant showed no specific DNA binding activity by ParB in this assay (Fig. 2-6C; Table 2-2), further confirming that the direction of the IHF bend is important for ParB-DNA contacts.
DISCUSSION:

ParB recognizes different subsets of its motifs when it binds to parS – A central question that arose from the crystal structure of P1 ParB protein bound to parS-small was how ParB would bind and assemble on the complete parS site across an IHF-directed bend. In this study, I have addressed the initial recognition step by ParB using a number of parS sites with different combinations of its Box A and Box B motifs. My results reveal several modes of ParB loading onto parS and that ParB has a motif preference that is dependent on motif position and on supercoiling of the plasmid substrate. High affinity interactions do not require all Box A and Box B motifs. In the simplest arrangement, only one motif is necessary on either side of the bend. On linear DNA molecules, the main ParB contact on the left side of parS is with Box A1, whereas on supercoiled substrates, ParB can use either Box A1 or Box B1. On the right side of parS, the data indicate that the highest affinity interactions are either with Box A2 or a combination of Box A3 and Box B2. Since previous chemical footprinting of the dimeric ParB complex at parS (done in the gel) showed protection of all motifs in parS (Bouet et al., 2000), I conclude that a dimer of ParB can load onto parS+ in several different ways, leading to a mixture of different complexes of ParB making contacts with specific subsets of motifs.

Motif use depends on plasmid topology - The ability to form a stable ParB complex using only the Box B1 motif on the left side of parS required negative superhelicity in the plasmid substrate. At low concentrations of ParB, ParB bound well to parS[a1] only on supercoiled plasmid DNA (Figs 2-4 and 2-5A). On supercoiled DNA, either Box A1 or Box B1 was necessary and sufficient for ParB to interact with the left-arm of parS. It is worth noting that differences in affinity with K_d’s below 5 nM would not have been detected in the filter binding assay because the DNA concentration was approximately 5 nM. It is therefore possible that differences in affinity between Box B1 and A1 were below my limit of sensitivity. The motif preference on the right-side of parS (either A2 or A3+B2) was similar on both linear and supercoiled DNA (Figs. 2-4 and 2-5A). These findings suggest supercoiling is most important in stimulating tighter interactions with the left-arm of parS, specifically Box B1, although it may improve affinity for all motifs. How does supercoiling make Box B1 a strong binding site for ParB? Negatively supercoiled plasmid DNA exists primarily in branched plectonemic conformations, and strong bends in the DNA, such as the one in parS imposed by IHF, are typically positioned at the superhelix ends (reviewed in Higgins and Vologodskii, 2004; Kanaar
The diameter of the superhelix axis is inversely dependent on the superhelical density, and negative supercoiling should favor tighter wrapping of parS around ParB. Therefore I expect that negative superhelicity affects the orientation and position of Box B1 so it is closer to the protein than when the DNA is relaxed.

ParB recognition of the right side of parS - The data indicate that a dimer of ParB can bind the right-arm of parS in several ways in the presence of IHF, but two combinations yield the strongest complexes. The general picture is that ParB interacts best with either Box A2 or a combination of Boxes A3 and B2. On linear DNA, the best substrates with the fewest motifs are parS sites with only Boxes A1 and A2 (parS[b1,a3,b2]) or with Boxes A1, A3 and B2 (parS[b1,a2]). The presence of either only Box A3 or Box B2 on the right side of parS (in combination with Box A1) supported weaker but specific complex assembly. The ability of ParB to assemble a complex using only one motif on the right side of parS was absolutely dependent on the IHF bend and on a specific interaction with the left side of parS.

ParB geometry on parS with IHF - Taken together, the results show that a dimer of ParB can span the IHF-directed bend in a variety of combinations, particularly on a supercoiled substrate. A dimer can span the IHF-directed bend by binding to one motif on each side of parS. What does this mean for the geometry of the protein in these complexes? The crystal structure of ParB[142-333] shows two essentially independent DNA binding domains that can rotate with respect to each other (Schumacher and Funnell, 2005), so the flexibility of ParB and also potentially the DNA allows ParB to contact parS across the bend in a variety of conformations. The biochemical data here support this prediction. The arrangement of HTH motifs on opposite sides of the protein is consistent with each HTH motif binding an A Box spanning the bend, and the simplest arrangement would be with Boxes A1 and A2. Both contacts are necessary since Box A2 is not sufficient to bind ParB in the absence of IHF (Fig. 2-7). The observation that the affinity of ParB for the A1+A3+B2 combination is significantly higher than either A1+A3 or A1+B2 alone indicates cooperativity between Boxes A3 and B2 (Fig. 2-6C; Table 2-2). However the flexible linker between the HTH domain and the dimerization DNA binding interface is too short for the protein to fold back and contact two adjacent motifs (Schumacher and Funnell, 2005). I think that the best explanation for these observations is that the ParB complexes are dynamic; that Box A3 and Box B2 motifs in close proximity increase the affinity compared to each alone because the protein comes on and off between these motifs rapidly and during
migration of the complexes in the gel. Indeed, I think that the data support that ParB interactions may always be dynamic in the presence of multiple motifs, especially in the wild-type site. For example, although the two best subsites (on linear DNA) are the A1+A2 or A1+A3+B2 combinations, they are still weaker sites than \( parS^+ \) (Table 2-2). The motifs help each other, even though the HTH domains cannot contact more than two Box A motifs simultaneously, consistent with rapid shuttling among these different combinations of complexes in solution. Finally, the reason that Box B2 helps A3 and not A2 is likely that some rotations of ParB are more favorable, such as the one that contacts Boxes A1 and A2. Because Boxes A2 and A3 are inverted repeats, binding of ParB to Boxes A1 and A3 would require one HTH domain to rotate 180° with respect to the protein conformation when ParB contacts Boxes A1 and A2. I speculate that this rotation is also stabilized by the Box B2-dimer domain interaction.

Why does \( parS \) contain multiple Box A and Box B motifs? - On a supercoiled plasmid in my assays, either Box A1 or B1 was sufficient to bind ParB, but \( in vivo \), Box B1 is the required motif for activity of the full \( parS^+ \) site. Why is Box A1 not sufficient for \( parS^+ \) activity \( in vivo \), and are there conditions in which it is necessary? One explanation for the former question is that the architecture of a complex that loads solely onto Box A1 is different than one loaded via Box B1, and that architecture as well as protein affinity is important for the next steps in the partition reaction. For example, perhaps the pairing reaction requires that the HTH interactions are intermolecular across A Boxes on the right side of \( parS \). The conservation of Box A1 among a number of related plasmid partition sites (Funnell and Slavcev, 2004) implies that Box A1 is utilized or required under certain lifestyles of the plasmid, for example ones that involve altered plasmid topology. Box A1 may expedite partition complex assembly under conditions when plasmid DNA is not negatively supercoiled (or less negatively supercoiled), such as immediately following passage of a replication fork, or during initial transfer of the plasmid into a bacterial cell (by infection of phage such as P1 or by conjugation of other plasmids, for example). The presence of multiple copies of recognition sequences for partition site binding proteins is a general feature of many different partition systems, such as in F plasmid \( sopC \) site, even though all copies may not be necessary for plasmid stability in the laboratory (Biek and Shi, 1994).

\( parS \) is the loading site for the segregation machinery, mediated by ParB protein. Following recognition and binding of \( parS \) by a dimer of ParB, multiple dimers load onto \( parS \) to form large complexes visualized \( in vivo \) as large foci using immunofluorescence or fluorescent forms
of ParB (Erdmann et al., 1999; Li and Austin, 2002). In vivo, ParB has been observed to spread along the DNA away from parS (Rodionov et al., 1999) and to pair parS sites (Edgar et al., 2001). Pairing between P1 parS sites has not been seen in vitro (Bouet and Funnell, 1999; Bouet et al., 2000), however in the type of gel shift experiments performed here, I predict that stable pairing requires many ParB molecules. Evidence also suggests that ParB must spread onto several hundred base pairs around parS for efficient partition (Rodionov and Yarmolinsky, 2004), consistent with the idea that many ParBs must assemble and act together during the partition reaction. The specific interactions of ParB require the parS+ site, even though a dimer can load relatively efficiently on sites with subsets of its recognition motifs. I propose that the requirement for multiple motifs reflects specific higher-order interactions with ParB. These could reflect the sequence requirements to load additional dimers of ParB onto parS, or to bridge adjacent parS sites in a pairing reaction. The latter is particularly attractive since the crystal structure of ParB[142-333] shows a three-way bridging activity of the protein; that is, the potential to bridge across parS sites as well as across the IHF-directed bend is supported by structural data. The interactions of ParB with all four essential motifs (B1, A2, A3 and B2) would supply the specificity for parS recognition both intramolecularly, whereas other protein-protein interactions, likely mediated by the oligomerization domains in the N-terminus of ParB (Surtees and Funnell, 1999), would supply the strength to keep pairs together.
Table 2-1. Sequences of *parS* DNA inserts in plasmids used in this study.

Changes to the wild-type sequence are indicated in bold text for each variant. Complementary synthetic oligonucleotides corresponding to these sequences were used to construct each plasmid.

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th><em>parS</em> Variant</th>
<th><em>parS</em> sequenceb</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBEF283</td>
<td>Δαβ</td>
<td>CTTCGGCAT TCAAATTTCG CAATTACTG ACGTCTTATA AAGTAAATTA CTCTAAAAATT TCAAGGTGAA ATGCGCAOGA</td>
</tr>
<tr>
<td>pBEF313</td>
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</tr>
<tr>
<td>pBEF301</td>
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</tr>
<tr>
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</tr>
<tr>
<td>pBEF285</td>
<td>a3 (SpeI)</td>
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</tr>
<tr>
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<td>a3 (BglII)</td>
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<tr>
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<tr>
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</tr>
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<td>pBEF317</td>
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<tr>
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<tr>
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<tr>
<td>pBEF292</td>
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<tr>
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<td>pBEF327</td>
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</tbody>
</table>

* All plasmids were constructed by Barbara Funnell.

b Only the sequence of the upper strand (as in Fig. 2-1) is shown.
### Table 2-2. Summary of ParB binding activity to parS site variants in the presence of IHF.

<table>
<thead>
<tr>
<th>parS Variant</th>
<th>Boxes Remaining&lt;sup&gt;a&lt;/sup&gt;</th>
<th>K&lt;sub&gt;d,app&lt;/sub&gt; &lt;sup&gt;b&lt;/sup&gt; (nM)</th>
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<tr>
<td>parS&lt;sup&gt;*&lt;/sup&gt;</td>
<td>B1 A1 A2 A3 B2</td>
<td>17 ± 4</td>
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<tr>
<td>Δa b</td>
<td>- - - - -</td>
<td>NB</td>
</tr>
<tr>
<td>b1</td>
<td>- A1 A2 A3 B2</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>a1</td>
<td>B1 - A2 A3 B2</td>
<td>580 ± 40</td>
</tr>
<tr>
<td>a2</td>
<td>B1 A1 - A3 B2</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>a3</td>
<td>B1 A1 A2 - B2</td>
<td>61 ± 25</td>
</tr>
<tr>
<td>b2</td>
<td>B1 A1 A2 A3 -</td>
<td>58 ± 23</td>
</tr>
<tr>
<td>b1, a1</td>
<td>- - A2 A3 B2</td>
<td>690 ± 210</td>
</tr>
<tr>
<td>b1, a2</td>
<td>- A1 - A3 B2</td>
<td>49 ± 20</td>
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<tr>
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<td>- A1 A2 - B2</td>
<td>119 ± 59</td>
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<td>b1, b2</td>
<td>- A1 A2 A3 -</td>
<td>56 ± 28</td>
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<td>a1, a2</td>
<td>B1 - - A3 B2</td>
<td>&gt;1 µM</td>
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<td>a1, a3</td>
<td>B1 - A2 - B2</td>
<td>NB</td>
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<tr>
<td>a1, b2</td>
<td>B1 - A2 A3 -</td>
<td>NB</td>
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<td>59 ± 35</td>
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<td>B1 A1 - A3 -</td>
<td>400 ± 160</td>
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<td>a2, a3</td>
<td>B1 A1 - - B2</td>
<td>450 ± 150</td>
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<tr>
<td>b1, a2, b2</td>
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<td>550 ± 180</td>
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<tr>
<td>b1, a2, a3</td>
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<td>RevA1A2</td>
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</table>

<sup>a</sup> Dashes indicate the position of boxes that have been mutated (with respect to parS<sup>*</sup>).

<sup>b</sup> K<sub>d,app</sub> was calculated as described under Experimental Procedures.

NB, no measurable binding activity.

>1 µM, substrate binding below 50% at 1 µM ParB.
**Figure 2-1. The sequence of the P1 parS site.** Blue rectangles and orange boxes outline the Box A and Box B recognition motifs, respectively. The thick black line shows the IHF binding site. \( \text{parS}^+ \) is defined as all the sequences between and including Box B1 to B2. The 22 bp sequence encoding boxes A2, A3 and B2 is the \( \text{parS-small} \).
Figure 2-2. *parS* site variants used in this analysis. Diagrams of *parS* variants show wild-type A- and B-boxes colored as in Fig. 2-1. A- or B-boxes that have been substituted with a restriction site are black. The restriction site substitutions are labeled for each variant, and their designations are: *E*, *Eco*RI; *S*, *Spe*I; *B*, *Bgl*II; *A*, *Aat*II; *As*, *Asel*I; *F*, *Fsp*I. In several variants, more than one substitution was created (Table 2-2), indicated by more than one site separated by a slash. The different substitutions behaved identically so all were used for data analysis.
Figure 2-3. DNA binding activity of ParB to parS variants lacking one motif. The DNA substrates were $^{32}$P-labeled restriction digests of plasmid DNA. DNA fragments were incubated with increasing amounts of ParB in the presence of IHF and analyzed by electrophoresis. The positions of free DNA (parS, 213 bp), the parS-IHF complexes and the parS-IHF-ParB complexes are indicated. Note that the large vector fragment (3 kb) contains an IHF binding site. ParB dimer concentration is shown in nM above each lane. A, parS*-A4. B, parS*. C, parS[$\Delta$ab]. D, parS[b1]. E, parS[a1]. F, parS[a2]. G, parS[a3]. H, parS[b2].
Figure 2-4. DNA binding activity of ParB to parS variants lacking one motif. ParB binding activity was examined as illustrated in Figure 2-3, and was quantified as described in Experimental Procedures. The data points are an average of at least three independent determinations and the binding curves were calculated as described in experimental procedures. ParB concentration is reported as the concentration of the dimer. parS-large (●), parS+ (+), Δab (▲), b1 (■), a1 (□), a2 (○), a3 (△), b2 (●), b1,a1 (◇).
Figure 2-5. ParB binding activity to supercoiled plasmids containing parS variants. ParB binding activity to ^3^H-labeled supercoiled plasmids was measured using nitrocellulose filter binding assays. The reaction mixture (20 μL) contained 5 nM ^3^H-labeled plasmid DNA with the indicated parS site, 100 nM IHF and the indicated amounts of ParB. Assays were performed as described previously (Funnell, 1988b). The data points are an average of at least three independent determinations for each variant and the binding curves were calculated as described in experimental procedures. A, DNA binding activity of ParB to parS site variants lacking one motif. parS^+ (+), Vector (▲), b1 (■), a1 (□), a2 (○), a3 (∆), b2 (●), b1,a1 (). B, DNA binding activity of ParB to parS site variants with a single motif mutated from either side of parS. parS^+ (+), Vector (▲), b1,a2 (●), b1,a3 (■), a1,a2 (○), a1,a3 (□). Vector was the plasmid pBend5.
Figure 2-6. DNA binding activity of ParB to parS variants lacking multiple motifs. ParB binding was examined by electrophoretic mobility shift assays as in Fig. 2-3. The data points are an average of at least three independent determinations and the binding curves were calculated as described in Experimental Procedures. A, DNA binding activity of ParB to parS site variants with a single motif mutated from either side of parS. parS$^+$ (+), Δab (▲), b1,a2 (■), b1,a3 (●), b1,b2 (♦), a1,a2 (□), a1,a3 (○), a1,b2 (◊). B, DNA binding activity of ParB to parS site variants with two motifs mutated from the right side of parS (filled symbols) and parS site variants with a single motif remaining on either side of parS (empty symbols). parS$^+$ (+), Δab (▽), a2,a3 (■), a2,b2 (●), a3,b2 (▲), b1,a2,a3 (□), b1,a2,b2 (○), b1,a3,b2 (△), A1$^+$,A4$^+$ (∇), B1$^+$,A4$^+$ (◊). C, DNA binding activity of ParB to parS site variants, shown to high concentrations of ParB. parS$^+$ (+), Δab (▲), b1,a2 (■), b1,a3,b2 (▽), b1,a2,b2 (○), b1,a2,a3 (□), a1,b2 (●), a2,b2 (♦), a2,a3 (◊), Rev A1A2 (◇).
Figure 2-7. Comparison of ParB DNA binding activity with and without IHF. Electrophoretic mobility shift assays were performed as described in Experimental Procedures. A, DNA binding activity of ParB in the absence of IHF to parS site variants with a single motif mutated (empty symbols) or parS site variants missing the left arm (filled symbols). The data points are an average of at least three independent determinations. parS⁺ (+), Δab (▲), b1 (□), a1 (○), a2 (△), a3 (▽), b2 (◇), b1,a1 (■), b1,a1,a2 (●). B-E, Increasing amounts of ParB were incubated with each of the parS substrates in the presence and absence of IHF and analyzed by electrophoresis. The positions of free DNA (parS), parS-IHF complexes, parS-ParB complexes and parS-IHF-ParB complexes are indicated. ParB dimer concentration is shown in nM above each lane. B, parS⁺. C, parS[b1,a1]. D, parS[a2]. E, parS[a3].
CHAPTER 3

ATP CONTROL OF DYNAMIC P1 ParA-DNA INTERACTIONS SUPPORTS A CRITICAL ROLE FOR THE NUCLEOID IN PLASMID PARTITION

A version of this chapter has been submitted for publication as:


Tan X and Han YW performed the TIRF microscopy and analysis (Figure 3-2). Mizuuchi K undertook the stopped-flow DNA-binding experiments (Figure 3-6). I performed all other experiments in this chapter.
Several Walker-type partition ATPases such as P1 ParA have been shown to interact with DNA non-specifically in vitro and colocalize with the bacterial nucleoid in vivo (see Chapter 1). However, the role of this interaction in the partition reaction has not been elucidated. In this chapter, I characterize the ParA-DNA interaction and propose a novel model for plasmid partition that incorporates the bacterial nucleoid.

P1 ParA has two known biological functions, which are modulated by the type of adenine nucleotide bound (Davey and Funnell, 1997). The ADP-form of ParA regulates the expression of parA and parB through site-specific DNA binding to the par operator. However, the operator binding activity of ParA is not directly required for the partition reaction itself (Davis et al., 1996). ParA-ATP is thought to act in the physical segregation of the plasmids. In vivo, hydrolysis deficient ParA mutants are defective for partition (Davis et al., 1996; Fung et al., 2001) and in vitro a ParA interaction with the partition complex is ATP dependent (Bouet and Funnell, 1999). The role and mechanism of nucleotide hydrolysis by ParA in plasmid localization are unknown.

A striking difference between Walker-type ATPases and the ATPases encoded by the other classes of partition systems is their ability to bind non-specific DNA (see Chapter 1). Several have been shown to directly bind DNA in an ATP-dependent fashion. In those tested, the hydrolytic activity of all Walker-type ATPases is stimulated by DNA. Non-specific DNA-binding in vitro is consistent with the in vivo finding that several fluorescent fusions of Walker-type partition ATPases colocalize with the bacterial nucleoid. A set of conserved arginines within the chromosomal ParA homologue of B. Subtilis (Soj) were shown to be essential in both nucleoid binding and plasmid stability in vivo, suggesting that the DNA binding activity of Walker-type ATPases plays an essential role in partition (Hester and Lutkenhaus, 2007). Also, an F SopA mutant deficient in DNA binding but functional for all other known biochemical properties displays a severe in vivo partition defect (Castaing et al., 2008). Thus, a ParA-non-specific DNA interaction is presumed to be important for the partition reaction but mechanistic insight is lacking. Dynamic oscillatory behaviours similar to that exhibited by MinD-GFP have been observed with several fluorescent variants of Walker-type partition ATPases (Ebersbach and Gerdes, 2004; Fogel and Waldor, 2006; Hatano et al., 2007; Marston and Errington, 1999; Pratto et al., 2008; Quisel et al., 1999; Raskin and deBoer, 1999). Unlike MinD oscillations that traverse the entire cell length, the oscillation of partition ATPases is restricted to the nucleoid.
region. Although the bulk of P1 ParA-GFP in a cell has not been observed to oscillate, we cannot rule out the possibility that a subpopulation of the protein also exhibits this dynamic behavior.

To investigate the role of DNA binding by Walker-type ATPases in partition, I, in collaboration with Kiyoshi Mizuuchi’s lab, used total internal reflection fluorescence microscopy (TIRFM) to visualize the interaction between DNA and a fluorescent variant of P1 ParA. We find that ParA non-specific DNA binding activity is highly dynamic and specific for ATP. Kiyoshi Mizuuchi and I performed a series of kinetic analyses to identify the form of ParA responsible for DNA binding. We found that ParA becomes competent for non-specific DNA binding after an ATP-dependent multi-step conformational change that precedes hydrolysis. I propose that P1 plasmid localization requires a critical ATP-dependent interaction between ParA and the host bacterial chromosome. A novel model that uses a reaction/diffusion type mechanism to explain par mediated plasmid movement and positioning is presented.
EXPERIMENTAL PROCEDURES

Escherichia coli strains, media and plasmids. Plasmids were maintained in the E.coli K12 strain DH5 [F⁻ endA1 hsdR17 (rk'mk') supE44 thi-1 recA1 gyrA96 relA1]. All bacterial cells were grown in LB medium or on LB plates. When used, the concentrations of ampicillin and chloramphenicol were 100 and 25 mg/ml, respectively. Plasmid pEF4 contains the parA gene under control of a modified β-lactamase promoter (blaP₁) (Fung et al, 2001). The parA-gfp gene fusion was constructed by Natalie Erdmann, a former student in the Funnell lab. The gfp(mut3.1) gene was inserted 3’ of the parA gene of pEF4, and included the linker sequence 5’AAAGAGGAGAAATTAAGC (which removed the C-terminal Asn residue of ParA and inserted the 6 residue linker KEEKLS between ParA and GFP in the protein), creating pNE34. I created a plasmid used for ParA-GFP protein production (pAV12), which contains the parA-gfp gene under the control of a bacteriophage T7 promoter in the vector pET15b (Novagen). The miniP1 plasmid used for stability analyses, pBEF240, contains a 4 bp insertion in the parA gene of pLG49 (Funnell and Gagnier, 1994). The insertion was created by filling in the overhangs created by Xho I digestion with Klenow polymerase followed by religation (constructed by Barbara Funnell).

Plasmid stability assays. A colony of E.coli DH5Δlac cells containing a miniP1 plasmid (pBEF240 – parS⁺ parB⁺ parA⁻) and one of the plasmids coding for a parA allele was picked from an LB agar plate containing ampicillin and chloramphenical, diluted into LB with ampicillin, and grown overnight at 37°C. Samples were taken from the beginning and the end of the growth period and plated for single colonies on LB agar plates with ampicillin. Resulting colonies were transferred using toothpicks onto LB plates with chloramphenicol to monitor the presence of the miniP1 plasmid.

Protein Purification. Wild-type ParA was purified as described previously (Davey and Funnell, 1994). ParA-GFP was purified essentially as described for wild-type ParA with modifications: Protein expression was induced by IPTG at 20°C for 6-8 hours. Following the S-sepharose chromatography step, a 1 ml HiTrap Q HP (GE Healthcare) column was equilibrated with Q buffer containing 140 mM KCl, and bound protein was eluted with a linear 140 mM to 1 M KCl gradient. The following buffers were used for ParA-GFP purification: sonication buffer, 50 mM Tris-HCl, pH 7.5, 10% (v/w) sucrose, 1 mM EDTA, and 2 mM 4-dithiothreitol (DTT); S Buffer,
25 mM imidazole pH 6.5, 0.1 mM EDTA, 10% (v/v) glycerol; Q Buffer, 25 mM HEPES, pH 7.5, 0.1 mM EDTA, 10% (v/v) glycerol.

**Assay buffers.** Buffer A: 100 mM NaCl, 10 mM MgCl₂, 50 mM Tris-HCl pH 7.5. Buffer B: 100 mM NaCl, 5 mM MgCl₂, 50 mM Tris-HCl pH 7.5. Nucleotide cofactor (From Roche and Sigma in the form of Na²⁺-salt) or other additives were included as indicated for each experiment.

**TIRF Microscopy.** The fluorescence microscopy was carried out essentially as described (Greene and Mizuuchi, 2002) with modifications as described in Han and Mizuuchi (2009). The flow cells used were as described (Tan et al., 2007) except there were two inlet port flow cells. The treatments of the flow cells prior to experiments were essentially as described in Han and Mizuuchi (2009). The reaction was in buffer B supplemented with 0.1 mg α-casein/ml and the nucleotide cofactor as specified for each experiment.

**ATPase assays.** [γ-³²P]ATP (Perkin Elmer) was purified from contaminating ²⁵P₃ prior to use with a 1 ml gel filtration column (P-2 fine resin, BioRad). Protein/DNA mixture and [γ-³²P]ATP, both pre-equilibrated in buffer A at 23°C, were rapidly mixed and at intervals, 30 µl samples were quenched by the addition of 30 µl 1% SDS and 20 mM Na₂EDTA. Samples were spotted onto PEI-TLC (polyethyleneimine cellulose-thin layer chromatography) plates and developed in 0.5 M LiCl in 1 M formic acid. Dried plates were exposed to Phosphor Imaging plates and quantified by a Typhoon TRIO Variable Mode Imager (GE Healthcare).

**Nucleotide binding and release assays.** Nucleotide binding by ParA was determined as the amount of ATP that co-eluted with ParA on a spin column (G-50 resin, GE Healthcare). The 1 ml column was first equilibrated with buffer A. Mixtures (30µl) containing [α-³²P]ATP (Perkin Elmer), ParA, and where indicated, unlabelled ATP competitor were incubated at 23°C, then loaded onto the column and centrifuged at 2000xg for 2 minutes. The entire reaction volume was spotted onto glass fibre filters, dried and quantified by liquid scintillation counting.

**Tryptophan Fluorescence assays.** Measurements were performed in a Photon Technology International (PTI) fluorescence system maintained at 23°C. The excitation wavelength was 295 ± 1.25 nm, and the emission was monitored at 320 ± 1.25 nm. Reaction components were manually mixed in buffer A 15 minutes prior to the measurements. For pre-steady state measurements, changes in tryptophan fluorescence were monitored as a function of time at a
wavelength of 320 ± 1.24 nm immediately following the addition of 1mM ATP. All results are an average of at least 3 independent experiments.

**DNA Binding Assay.** The DNA was labelled with Alexa-Fluor-514 (Molecular Probes) at every 11bp. Fluorescence quenching by ParA was measured in stopped-flow experiments as described (Han & Mizuuchi, 2009).
RESULTS

*ParA-GFP is functional both in vivo and in vitro*

To assess the biological function of ParA-GFP *in vivo*, the stability of a *parA* miniP1 plasmid (pBEF240 - *parB*<sup>+</sup>, *parS*<sup>+</sup>) was assayed with ParA-GFP expressed *in trans* near physiological levels (Fig. 3-1A). The fusion protein maintained the miniP1 plasmid in host cells essentially as well as wild-type ParA. Therefore, ParA-GFP retains functional activity to participate in active plasmid partitioning in the presence of ParB and *parS*.

I next used ATPase assays to determine whether the purified ParA-GFP fusion protein retained wild-type function *in vitro*. ParA-GFP hydrolyzes ATP at levels comparable to that of wild-type ParA (Fig. 3-1B). In the presence of ParB stimulation, wild-type ParA is stimulated ~5-fold. ParB stimulates ParA-GFP ATPase activity ~3-fold. Overall, ParA-GFP exhibits ParB stimulated ATPase activity indicating that most of the structural and biochemical properties of wild-type ParA are retained in this fluorescent derivative.

*ParA binds DNA non-specifically in the presence of ATP.*

ParA-GFP was used to study the interaction dynamics between P1 ParA and non-specific DNA in real time. Xin Tan and Yong-Woon Han visualized ParA-DNA binding by total internal reflection fluorescence microscopy (TIRFM) with λ-DNA molecules tethered at one end to the flow cell surface (Greene and Mizuuchi, 2002). With TIRFM, only ParA-GFP molecules located within the first 100-150 nm of the flow-cell surface are sufficiently excited by the evanescent wave to allow detection of the fluorescence signal with very low background. Buffer flow extended and confined the DNA molecules, along with the bound ParA-GFP, within the evanescent illumination.

When a buffer containing ParA-GFP was infused into the flow cell with ATP and Mg<sup>2+</sup>, ParA-GFP assembled onto DNA rapidly (Fig. 3-2A). In the absence of ATP, no DNA binding was detected. At different concentrations of ParA-GFP, the apparent steady state level of ParA-GFP bound to DNA changed accordingly. Above 100 nM, the DNA was quickly saturated. At 10 to 50 nM, partial covering of DNA by ParA-GFP was observed with rapid flickering of the fluorescence signal along each DNA molecule, indicating ongoing fast association and dissociation cycles of ParA-GFP molecules. The fluorescence pattern along the DNA indicated that the ParA unit responsible for DNA binding was relatively small oligomers; the newly
formed foci on the DNA were estimated to be composed of less than 10 GFP units, and long continuous coverage of the DNA was only observed at near saturating concentrations of ParA-GFP. Below 50 nM ParA-GFP, the fluorescence signal at steady state was less than proportional to the protein concentration, becoming undetectable below 10 nM in some experiments, indicating the involvement of a cooperative process (data not shown). In the experiments above, ATP was incubated with the final concentration of ParA for several minutes before DNA binding was initiated. We tested the effect of pre-incubating different concentrations of ParA with ATP followed by dilution to 25 nM ParA immediately prior into infusion to the flow cell (Fig. 3-2B). Pre-incubation at higher ParA concentrations greatly enhanced DNA binding, indicating the existence of higher-order ParA concentration dependent step prior to DNA binding.

The dissociation kinetics of ParA from DNA was investigated using the TIRF approach. After ParA-GFP binding to DNA reached apparent steady state, the solution was switched to a “disassembly buffer” (without ParA), and then the fluorescence intensity integrated over individual DNA molecules was monitored as a function of time (Fig. 3-2C). The fluorescence decrease fit a single exponential decay function and displayed an off-rate of 0.8-1.8 min$^{-1}$ (Fig. 3-2D). The off-rate was slightly slower when ATP was in the disassembly buffer suggesting that loss of bound ATP by ParA could be a small contributing factor for the dissociation (Fig. 3-2E). Addition of competitor DNA in the disassembly buffer significantly accelerated the apparent dissociation rate (Fig. 3-2E), indicating that ATP-bound ParA engages in inter-segmental transfer between DNA sites at higher local DNA concentrations.

Next, Xin Tan and Yong-Woon Han investigated the nucleotide cofactor specificity for ParA-DNA binding. ATP in the above experiments was replaced with AMP, ADP, ADP+Pi, AMP-PNP, ATP$\gamma$S, ADP-AlF$_4$, ADP-BeFx, or ADP-VO$_4$. None of the nucleotide cofactors tested except ATP supported ParA-DNA binding (data not shown). However, all adenosine di- and tri-phosphates examined inhibited DNA binding in the presence of ATP, indicating that ParA could bind these nucleotides (data not shown). Thus, the ParA conformation necessary for DNA binding is highly specific for ATP. What might be the reason for this stringent nucleotide specificity?
ATP hydrolysis is not a prerequisite for DNA binding and DNA association/dissociation is uncoupled from the ATPase cycle.

We considered a possibility that the conformation of ParA competent for DNA binding is reachable only through the ATP hydrolysis step, and not when the reaction is started with the hydrolytic products (ADP with or without Pi). This explains why non-hydrolysable ATP analogs would not function. Since the steady state rate of ATP turnover by ParA is slow (0.017-0.025 min⁻¹ at 23°C, see below), this proposal raises the possibility that the hydrolysis step is fast and the product release step is slow, resulting in accumulation of a post-hydrolysis state such as a special ADP-bound form of ParA. In this case, pre-steady state kinetics of ATP hydrolysis should exhibit an initial burst phase. However, when I tested ParA ATPase activity under pre-steady state conditions, there was no sign of an initial burst (Fig. 3-3). Moreover, no significant deviation from the steady state rate of hydrolysis was observed in the single turn over period in the presence or absence of DNA (Fig. 3-3). Thus, the product release step is not rate-limiting in the ATPase cycle, and ParA bound to its hydrolytic products must be a minor fraction of the enzyme population at steady state.

ParA binds ATP in multiple steps.

The rate that ParA dissociates from DNA in the presence of ATP (Fig. 3-2E) is much faster than the steady state rate of ATP hydrolysis, which indicates that the dissociation step of ParA from DNA is unlikely to be coupled to any of the ATPase cycle steps. This observation indicates that an event upstream of hydrolysis is responsible for licensing ParA to bind DNA. Therefore, I next focused on the ATP binding process. Kiyoshi Mizuuchi and I first monitored nucleotide binding by ParA by making use of the fluorescent analogs MANT-ATP and MANT-ADP. The fluorescence quantum yield of MANT-labeled nucleotides frequently increases upon enzyme binding, allowing for kinetic measurements (Cremo and Yount, 1987). Although MANT-ATP did not support ParA-DNA binding, MANT-ADP bound ParA with similar affinity as ADP with an apparent K_d of approximately 30 nM (Kiyoshi Mizuuchi, unpublished data), and the change in fluorescence of MANT was a useful tool to detect initial nucleotide interactions with ParA. MANT-nucleotide binding kinetics took 20 seconds to reach apparent steady state (Kiyoshi Mizuuchi, unpublished data). The observed binding kinetics is slower than expected for simple nucleotide docking and suggests the involvement of a protein conformational step.
I then measured ATP binding to ParA using spin columns to separate ParA-bound ATP from free ATP. This method specifically detects stable ParA-ATP complexes, which elute in the void volume (Fig. 3-4A). The amount of the stably bound ATP was proportional to and stoichiometric with ParA concentration (0.8 ATP:1 ParA) after ~10 minutes of incubation. The pseudo-first-order association rate constant was $0.16 \pm 0.02$ min$^{-1}$. To obtain the off-rate, [$^{32}$P]-ATP was first incubated with ParA and then chased with an excess of unlabeled ATP. The amount of [$^{32}$P]-ATP that remained bound to ParA was quantified as a function of time (Fig. 3-4B). The observed off-rate constant was $0.02 \pm 0.01$ min$^{-1}$ and the half-life of the stable ParA-$^{32}$P-ATP complexes was ~14 min. This observation is similar to that reported for F SopA (Bouet et al., 2007a). From the results reported below, I expected the kinetics of formation of the stably ATP-bound form of ParA to be faster in the presence of DNA. Spin-column experiments in the presence of 100 µg/ml DNA agreed with this prediction (Fig. 3-4A inset). Formation of the stable, nucleotide-bound ParA species was unique to ATP, as it was not detectable using ADP (data not shown). Together the nucleotide binding assays suggest that ParA binds ATP in multiple slow steps to reach the stably ATP-bound form. Therefore, I next sought to detect a nucleotide-induced conformational change in ParA.

**ParA undergoes an ATP-specific conformational change detectable by tryptophan fluorescence.**

The structural effects of adenosine nucleotide binding by ParA has previously been detected by circular dichroism (Davey and Funnell, 1997). ParA displayed a slightly greater helicity when bound to ATP than when bound to ADP or ATPγS. To further investigate the structural change of ParA elicited by nucleotide binding, I measured tryptophan fluorescence changes. ParA contains only one tryptophan at position 216, which is located away from the ATP binding site, but relatively close to the ADP-bound dimer interface (Dunham et al., 2009a) (Fig. 1-7). I first measured tryptophan fluorescence of ParA at steady-state to identify which cofactors promoted a conformational transition that can be monitored by this method (Fig. 3-5A). In the presence of Mg$^{2+}$ and ATP, ParA tryptophan fluorescence decreased approximately 20% relative to ParA without nucleotide. ADP and ATPγS induced a small but reproducible increase in fluorescence. In the presence of AMP, no significant change was observed. Tryptophan fluorescence measurements from all controls lacking Mg$^{2+}$ or nucleotide were
similar to ParA alone. These results confirm that ParA undergoes an ATP-specific conformational change, and I call this final conformation ParA*-ATP.

I next tested whether DNA binding elicited any further conformational changes. The addition of DNA along with ATP did not noticeably alter fluorescence compared to ATP alone (Fig. 3-5A). Therefore after the ATP-dependent transition, ParA does not undergo additional structural transitions upon binding DNA that are detectable by this method. I hypothesized that the stably ATP-bound state, the lower tryptophan fluorescence state (ParA*-ATP), and the DNA binding competent state are one and the same, and transition to this ParA form is slow due to a high activation energy barrier, which the non-hydrolysable ATP analogs are unable to overcome.

**Changes in ParA conformation induced by ATP are slow.**

If the above hypothesis is correct, the kinetics of forming ParA*-ATP upon addition of ATP should match the kinetics of forming the stable, ATP-bound state reported in the previous section. To determine the rate of this conformational transition, I performed pre-steady state kinetic measurements of ParA tryptophan fluorescence change in the presence of ATP. When ATP was added to ParA, the tryptophan fluorescence signal displayed a decrease in fluorescence that was very slow, taking ~10 minutes to reach an apparent steady state at the ParA concentration range tested here (Fig. 3-5B). Based on the equally slow kinetics and the nucleotide specificity, I propose that this transition generates the stable ATP-bound state of ParA (Fig. 3-4A).

While the presence of DNA together with ATP did not greatly alter the extent of ParA tryptophan fluorescence at steady-state, DNA might influence the reaction kinetics. I tested this possibility and found that non-specific DNA increased the rate of transition ~10-fold, allowing ParA conformation to reach an apparent steady state level within ~1 min at all ParA concentrations tested (Fig. 3-5C). This parallels the effect of DNA on formation of the stable ATP-bound form of ParA (Fig. 3-4A, inset). Again, the extent of the fluorescence change was not dramatically different with or without DNA (Fig. 3-5B-C), as already shown at steady state (Fig. 3-5A).

Although the time scales of the conformational change are similar at ParA concentrations between 1.25 and 10 μM, the extent of the change exhibited higher-order protein concentration dependency. In the presence of DNA, this dependency became even more pronounced. The
acceleration of the transition by DNA was dependent on DNA concentration (or rather the protein/DNA ratio) (Fig. 3-5D). If as proposed this ATP-bound state, ParA*-ATP, represents the DNA-binding form of ParA, the fact that DNA accelerates this transition indicates that the transition state for this conformational change must be able to interact with DNA at least transiently. The ATP hydrolysis kinetics showed the steady state rate from the beginning (Fig. 3-3), indicating that the conformational change does not strongly affect the hydrolysis rate.

**ParA*-ATP is the DNA binding competent state.**

Is this ATP-specific form of ParA, ParA*-ATP, the non-specific DNA-binding form of ParA? Kiyoshi Mizuuchi measured the kinetics of DNA binding by ParA using fluorescently labelled DNA. ParA bound to labelled and unlabelled DNA equally well as judged by competition experiments (data not shown). ParA binding to the labelled DNA resulted in significant quenching of the fluorescence signal. Therefore, fluorescence quenching was used as the readout for DNA binding by ParA (Fig. 3-6).

First, DNA binding by preformed ParA*-ATP was very fast. When ParA was preincubated with ATP for 15 min, binding to labelled DNA occurred in approximately 20 msec (Fig. 3-6A). When ATP and DNA were simultaneously mixed with ParA, the DNA binding by ParA was much slower (Fig. 3-6B). This time course is very similar to that of the formation of ParA*-ATP in the presence of DNA (see Fig. 3-5C). No difference in binding kinetics was observed if DNA was first pre-incubated either with ParA or with ATP (data not shown). DNA binding kinetics was measured at different ParA concentrations (Fig. 3-6B). Each time course exhibited sigmoidal kinetics, and above 1 μM ParA the reaction approached apparent steady state 20-50 sec after ATP addition. Varying ParA concentrations at constant DNA concentration showed that the DNA was saturated at around 3 μM ParA, which corresponds to ~6 bp/ParA monomer (see Fig. 3-6C). If the fraction of ParA that binds to DNA were constant, every two-fold reduction of the ParA concentration should have halved the extent of the observed fluorescence quenching. Instead, every two-fold reduction of ParA concentration resulted in significantly more than a two-fold reduction in DNA binding (Fig. 3-6B), indicating higher-order ParA concentration dependency of the extent of conversion to the DNA binding form. The implications of these results regarding the oligomeric species of ParA that binds DNA will be discussed later.
Next, the kinetics of generation of the DNA binding form of ParA were examined when ParA was preincubated with ATP in the absence of DNA. ParA and ATP were mixed and, at intervals, added to the labeled DNA in a stopped-flow apparatus. The amount of DNA binding was measured for the first 0.25 sec after mixing to determine the amount of ParA in the DNA-binding competent form that was generated during the pre-incubation. The results (Fig. 3-6C) demonstrate that the kinetics of this conformational change is slow, taking more than 5 minutes as predicted by the tryptophan fluorescence experiments. Thus, the kinetics of the DNA binding capacity of ParA agree very well with those of the tryptophan fluorescence experiments. We conclude that the ParA conformation with stably bound ATP and reduced tryptophan fluorescence represents the DNA binding competent state. Therefore ParA*-ATP is the form of ParA that binds non-specifically to DNA as observed by TIRF microscopy (Fig. 3-1).
DISCUSSION

ParA belongs to a family of Walker-type ATPases that have important roles in chromosome and plasmid partition, but the underlying mechanism has been elusive. Possibilities of self-supporting filament formation have been suggested for this class of partition ATPase following the lead of the actin-type partition ATPases and also based on the observations of ATP-dependent aggregation of some of the members of this ATPase class (Barillà et al., 2005; Bouet et al., 2007b; Ebersbach et al., 2006; Lim et al., 2005; Suefuji et al., 2002). However, I propose a model where self-supporting filament formation is not involved in the P1 partition mechanism. Indeed, concentration dependency was observed in forming ParA*-ATP (Fig. 3-5B-C) and for ParA-DNA binding activity (Fig. 3-6B), but further analysis argues that the primary unit of ParA that binds DNA is no greater than a dimer (Dunham, 2009; Mizuuchi, unpublished data). In addition, as I show in the next chapter, I have not observed any evidence for self-supporting filament formation by P1 ParA alone or in the presence of nucleotide at protein concentrations near the estimated in vivo concentration. When DNA-bound, ParA still did not produce long contiguous oligomers until near saturation.

In this chapter, I examined ATP-dependent interaction dynamics between ParA and DNA, an abundant material inside bacterial cells in the form of the bacterial chromosome. Based on these findings, I consider a variation of the reaction-diffusion type mechanism that describes septum localization in bacterial cell division with the principal players MinD and MinE, of which MinD belongs to the Walker-type partition ATPase family (Koonin, 1993). Dynamic oscillation of membrane-bound MinD from cell-pole to cell-pole generates a time-averaged MinD distribution minimum at mid-cell. The initiation of cell division by FtsZ polymerization is limited to the mid-cell because MinC, an inhibitor of FtsZ polymerization, binds MinD, and thus the time-averaged MinC concentration is also lowest at mid-cell. Considering the fact that some of the Walker-type partition ATPases have also been observed to engage in intracellular oscillation (see Chapter 1), it is attractive to consider the mechanisms of these systems under the same light. In contrast to the ATP-dependent membrane binding activity of MinD, ParA has ATP-dependent DNA binding activity. I propose that the time-delay mechanism found here for ParA-DNA binding after ATP-binding is one of the key elements in the partition mechanism.
The three critical aspects of the mechanisms considered here are: (1) slow diffusion media such as a membrane surface or nucleoids (I will call this entity a matrix for this discussion), (2) an ATPase whose binding to the matrix is controlled through nucleotide-induced conformational changes, and (3) a second protein component that controls the ATPase activity. Together, with possible participation of additional players (such as parS for P1 partition), the system generates dynamic instability in the local concentrations of the ATPase bound to the matrix and also that of the ATPase stimulator. This instability drives the oscillation, which could be used for a cargo-carrying function, such as plasmid DNA for segregation or MinC for septum localization.

At first glance, Min oscillation looks like a relatively simple system - MinE chases MinD back and forth over a membrane matrix. As a result, a number of reaction-diffusion type models have been proposed to explain MinD oscillation, whose ATPase activity is controlled by MinE (Drew et al., 2005; Howard et al., 2001; Huang et al., 2003; Kruse, 2002; Meinhardt and deBoer, 2001). Reaction-diffusion systems are mathematical models that describe how the concentration of one or more substances distributed in space changes under the influence of two processes: local chemical reactions and diffusion, which causes the reaction products to spread out in space. Several models with subtle variations of the same theme were successful in simulating the oscillatory behaviour of the Min system. All approaches involve a set of coupled reaction-diffusion equations that represent the concentrations of MinD and MinE on the membrane and in the cytoplasm as a function of cell length and time (Howard and Kruse, 2005). A critical feature for the success of these models is that diffusion of a protein on the membrane must be slower than in the cytoplasm. In other words, unrestricted random-diffusion can only occur when the proteins are off the membrane matrix. An equally important feature in most of these models is that MinD cooperatively binds to the membrane and recruits MinE, which ultimately leads to the detachment of both proteins. Both of these assumptions have been experimentally validated (Lackner et al., 2003; Mileykovskaya et al., 2003).

The downfall to these simulations are the false assumptions instated to prevent MinD from binding the same polar zone from which it dissociated, and ensuring that cytoplasmic MinD reassembles at the opposite pole. To put it differently, if MinE and MinD diffuse at the same rate, then relocalization would not be possible because MinD concentration would be highest where it came off the membrane, and would likely rebind close to its release position. Therefore,
a mechanism must be at play which allows MinD to lose its positional memory. The first reaction-diffusion model to successfully model Min oscillation assumed MinD subunits are inactive following disassembly from the membrane. The loss of protein molecules was compensated for by the synthesis of new MinD. This allowed new MinD molecules to enter the cytoplasm as randomly diffuse. However, MinD oscillation does not require ongoing protein synthesis (Raskin and deBoer, 1999), and thus alternative models followed (Drew et al., 2005; Howard et al., 2001; Huang et al., 2003; Kruse, 2002). A more promising model invoked a somewhat slow nucleotide exchange step by MinD to help achieve oscillation (Huang et al., 2003). This time-delay mechanism would allow MinD to randomly diffuse throughout the cytoplasm before reassociating with the membrane. Contrary to this assumption, a recent study revealed that MinD nucleotide exchange is sufficiently fast (Loose et al., 2008). What then could be the source of this crucial delay mechanism required for oscillation? I believe our experimental evidence is the first to clearly describe the time-delayed activation of an ATPase for matrix binding.

Figure 3-7 shows a simplified diffusion ratchet-type model of plasmid motion that incorporates the time-delay for DNA rebinding of ParA. ParA exists in essentially two states - “active” (ParA*-ATP: DNA binding competent) and “inactive” (ParA-ATP and ParA-ADP: non-DNA binding). On regions of the nucleoid away from the partition complex, practically all the “active” ParA would indeed be DNA bound considering the off-rate is two to three orders of magnitude slower than the on-rate. Therefore, “active” ParA binding to the nucleoid would restrict ParA diffusion (Fig. 3-7A). What does this mean for the P1 plasmid partition complex? This complex contains many ParB molecules centered around the parS site on the plasmid as has been shown both in vivo and in vitro (Erdmann et al., 1999; Rodionov and Yarmolinsky, 2004). The partition complex would have higher affinity for the nucleoid area with a higher density of bound ParA where a larger number of ParA-ParB contacts would be made (Fig. 3-7B). But as ParB associates with the nucleoid-bound ParA, ATP hydrolysis is quickly induced and ParA dissociates from the nucleoid. Because the dissociated ParA cannot rebind DNA immediately, it loses the positional memory of the site from which it dissociated. When ParA DNA-binding activity is regained, the new binding site would be essentially random across the nucleoid, instead of being narrowly centered around the site of dissociation (Fig. 3-7C). This generates a low-density area of nucleoid-bound ParA in the vicinity of the partition complex, and this area of
ParA clearance would expand with Brownian motion of the partition complex. Now the partition complex is located in a higher relative potential energy area with reduced ParA contacts and would therefore drift away to establish new ParA contacts at a neighbouring area where the nucleoid-bound ParA concentration is higher (Fig. 3-7D). This starts the movement of the partition complex along the nucleoid. The initial drift toward one stochastically chosen direction enforces the continued movement towards the same direction, as a result of the lower concentration of the nucleoid-bound ParA in its wake. Upon reaching a nucleoid end, the complex would be forced to turn around and move toward the other end; explaining the oscillatory patterns observed with a number of partition systems (Ebersbach and Gerdes, 2004; Fogel and Waldor, 2006; Hatano et al., 2007; Marston and Errington, 1999; Pratto et al., 2008; Quisel et al., 1999).

When the plasmid replicates and an additional complex is formed, each partition complex would generate a low density ParA distribution region around itself (Fig. 3-7E). Thus, when the two partition complexes approach each other, their movements would be suppressed to avoid the low ParA density area occupied by the other complex. In other words, when multiple partition complexes come into close proximity, there would be a repulsive “force” that develops, which results in the entities turning away from each other. Thus, multiple partition complexes would develop a trend to position themselves equidistant to each other along the nucleoid (Fig. 3-7F), which agrees with patterns seen by in vivo fluorescence microscopy (Erdmann et al., 1999). This could be considered as a form of “interference” phenomena, and explains many aspects of the partition behaviour of the replicated copies of the plasmid.

A large number of ParB molecules bound to the plasmid makes it easy to imagine how the plasmid might glide along the nucleoid surface covered sparsely by ParA molecules without losing all the ParA-mediated contacts with the nucleoid at any given time. Could this be the only major impact of the ParA-ParB interaction? I believe that the partition complex plays a more intimate role in controlling ParA. In the next chapter, I investigate the ParA-ParB-parS complex.
A

<table>
<thead>
<tr>
<th>Plasmid Providing ParA (parA allele)</th>
<th>miniP1 plasmid (pBEF240) retention after overnight growth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322 (no ParA)</td>
<td>30 ± 6</td>
</tr>
<tr>
<td>pEF4 (parA)</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>pNE34 (parA-gfp)</td>
<td>96 ± 3</td>
</tr>
</tbody>
</table>

Figure 3-1. ParA-GFP function. (A) Stability of miniP1 plasmid (pBEF240) with ParA-GFP. ‘Retention’ represents the ratio of the frequency of cells with miniP1 after overnight growth divided by the frequency of cells with miniP1 at the start, expressed as a percentage. The data represent the average of at least three independent experiments. (B) ATPase activity of ParA-GFP. At the concentrations indicated, ParA or ParA-GFP was incubated with 0.1mg of sonicated-DNA/ml in the absence and presence of ParB (1 μg) for 1.5 hours at 23°C. $^{32}$P$_i$ production was quantified as described in Experimental Procedures.
Figure 3-2. ParA-DNA interaction dynamics observed by TIRF microscopy.
(A) Representative image of ParA assembly. Scale bar indicates 10 µm. (B) ParA was pre-incubated at the indicated concentrations with 1 mM ATP for 15 min and diluted to 25 nM before infusion. Total fluorescence signal from single tethered DNA molecules was plotted as a function of time. (C) Measurement of ParA dissociation rate constant for individual DNA molecules. Time-lapsed fluorescence images of an individual lambda DNA molecule. (D) Corresponding ParA dissociation curve based on the fluorescent intensity in (C), with a single-exponential fit. (E) Rate constant distribution histograms of ParA disassembly in plain buffer (green), buffer containing 1 mM ATP (red) and buffer containing 0.5 mg/mL sonicated-DNA (yellow) with Gaussian fit.
Figure 3-3. Pre-steady state kinetics of ATP hydrolysis by ParA. ParA and [γ-32P]ATP (10 μM and 1 mM final concentrations, respectively) were rapidly mixed in buffer A in the absence (filled circles) or presence (open circles) of 100 μg of sonicated-DNA/ml. The hydrolysis product was measured after the indicated reaction time at 23°C as described in experimental procedures.
Figure 3-4. Kinetics of stable ParA-ATP complex formation and dissociation. (A) 2.5 μM ParA (75 pmoles) and 1 mM [α-32P]ATP were mixed in 30 μl buffer A and after the indicated incubation time at 23 °C, separated by a spin column. Stably ParA-bound ATP excluded by the column was measured. The same experiment was carried out in the presence of 100 μg of sonicated-DNA/ml (inset). (B) 2.5 μM ParA and 0.1 mM [α-32P]ATP were mixed in 30 μl buffer A and after 15 min at 23°C, 2 mM unlabeled ATP was added. The sample was applied to a spin column after the indicated period and [α-32P]ATP that remained stably bound to ParA was measured.
Figure 3-5. Changes in ParA conformation as detected by tryptophan fluorescence. For all panels, ‘relative tryptophan fluorescence’ is on the y-axis. (A) Steady state tryptophan fluorescence change of ParA (5 μM) caused by the additives as indicated (1 mM nucleotide). (B) ATP induces a slow ParA tryptophan fluorescence change. (C) The slow, ATP-specific change in ParA tryptophan fluorescence is accelerated by the presence of sonicated-DNA (100 μg/ml). (D) The acceleration of tryptophan fluorescence change in ParA (5 μM) is DNA concentration dependent. ‘Relative tryptophan fluorescence’ represents the tryptophan fluorescence intensity normalized to ParA tryptophan fluorescence prior to nucleotilde addition.
Figure 3-6. DNA binding kinetics of ParA. (A) 1.25 μM ParA was preincubated with 1 mM ATP (or ADP) for 15 min and rapidly mixed with 12.5 μg/ml fluorescence labelled DNA, and fluorescence quenching by the bound protein was monitored. (B) ParA, at the indicated final concentrations, was rapidly mixed with 12.5 μg/ml fluorescence labelled DNA together with ATP. Concentrations are final in the reaction. (C) Kinetics of acquisition of DNA binding capacity by ParA upon addition of ATP in the absence of DNA.
Figure 3-7. A mechanism for *par*-mediated partition. (A) On the nucleoid, ParA*-ATP is activated to dynamically bind DNA. ParB loads onto the plasmid (black squiggle) at *parS* forming the partition complex. (B) ParB stimulates ParA ATPase activity, which clears ParA from the nucleoid in the vicinity of the partition complex. Drift of the partition complex to one direction commits its movement to continue in the same direction because of the low ParA concentration in its wake. (C) ParA exchanges ADP for ATP and undergoes a slow conformational change. This time delay allows ParA to randomly diffuse and rebind the nucleoid after ParA*-ATP reforms. (D) At the nucleoid pole, the partition complex changes direction. The uneven distribution of ParA on the nucleoid generates plasmid movement. (E) After plasmid replication, the partition complexes develop repulsive interactions as they remove ParA in opposite directions. (F) Multiple partition complexes develop a trend to position themselves equidistant to each other along the nucleoid.
CHAPTER 4

ParB, ParA, AND DNA INTERACTIONS GENERATE A LARGE NUCLEOPROTEIN COMPLEX

A version of this chapter is being prepared for publication.

I performed all experiments in this chapter.
ParA-ATP interacts with the ParB-parS partition complex (Bouet and Funnell, 1999), but how this interaction relates to its role in partition is unknown. In this chapter, I use a light-scattering approach to study how ATP-cycling by ParA modulates the assembly and disassembly of a ParB-ParA-DNA complex, which I refer to as the BAD complex.

Several partition ATPases have been shown to polymerize and models where self-supporting filaments physically push or pull plasmids to specific intracellular addresses in a bacterial cell have been proposed (Barillà et al., 2005; Bouet et al., 2007b; Ebersbach et al., 2006; Pratto et al., 2008). The clearest example is the dynamic instability exhibited by the actin-like ATPase, ParM, from R1 plasmid (Fig. 1-3) (Moller-Jensen et al., 2002; Garner et al., 2004). Binding of ATP allows ParM to polymerize to form protofilaments, which appear to be capped and stabilized by ParR-parC partition complexes and then lengthened by successive insertion of ParM-ATP subunits at the filament–partition complex interface. Attached plasmids are thus pushed towards the cell poles.

The functional significance of protein polymerization in the partition mechanism is still unknown. It remains to be elucidated whether bacterial DNA segregation mediated by Walker- and actin-type ATPases is mechanistically distinct. I favour the idea that the partition mechanisms are distinct as several pieces of evidence suggest Walker-type polymerization does not result in the formation of a mitotic-spindle-apparatus analogous to that formed by ParM. The requirements for polymerization of Walker-type ATPases differ widely from ParM and from each other. For example, SopA can polymerize into small filament bundles in an ATP dependent manner (ATPγS cannot substitute), stimulated by SopB, and inhibited by non-specific DNA (Bouet et al., 2007a). ParA from pB171 and ParF from TP228 also polymerize into small filament bundles with ATP or ATPγS (Machón et al., 2007), and ParF’s cognate ParB (ParG) was found to stabilize ParF filaments (filament bundles were less frayed) (Barillà et al., 2005). ParF also has a propensity to oligomerize without nucleotide. For Soj and MinD, polymerization occurs across a DNA matrix, and a membrane matrix respectively (Leonard et al., 2005b; Suefuji et al., 2002). In several of these cases, the dual actions of ATP-binding and ATP-hydrolysis can be considered as the means by which a polymerization–depolymerization cycle is set up to bring about partition. P1 ParA can form a filamentous precipitate at high concentration with ATP but it is a dimer at physiological concentrations (Dunham, 2009; Kiyoshi Mizuuchi, unpublished data). Polymerization of ParA into filaments has not been studied extensively.
In the previous chapter, I showed how a ParA-nucleoid interaction can explain *in vivo* ParA dynamics. The next important issue is the effect of the ParB-\(parS\) partition complex on ParA binding to non-specific DNA. Walker-type partition ATPases that oscillate *in vivo* lose this dynamic behaviour and uniformly coat the nucleoid in the absence of their cognate partition complex or their hydrolytic activity (Ebersbach and Gerdes, 2001; Marston and Errington, 1999). Moreover, DNA drastically enhances ParB stimulation of ParA ATPase activity regardless of its sequence, length or topology. The nature of the BAD complex - a pre-requisite to plasmid partition and movement - has yet to be defined. Here I report that in the presence of ATP or ATP\(_\gamma\)S, ParA can associate with ParB and DNA to form large dynamic complexes that are detectable *in vitro* by an increase in light-scatter. While ParA ATP-binding is required for complex assembly, I find that ATP-hydrolysis and ATP to ADP nucleotide exchange leads to complex disassembly. I propose that a ParA-ATP interaction with the ParB-\(parS\) partition complex stabilizes the ParA-nucleoid interaction, effectively allowing ParA to lock onto DNA and form bundled filaments, which act as dynamic adaptors between the nucleoid and the plasmid. The subsequent stimulation of ParA ATPase activity by the ParB-\(parS\) partition complex generates ParA polymer instability and plasmid movement over the nucleoid track.
EXPERIMENTAL PROCEDURES:

Reagents and buffers - Sources for reagents were as follows: [γ-32P]ATP were from PerkinElmer; nucleotides, sonicated salmon sperm DNA, DNaseI and α-casein were from Sigma. All experiments were performed in Buffer A: 50 mM Tris-HCl, pH 7.5 at 25°C, 100 mM NaCl, 10 mM MgCl₂ and 0.1 mg of Casein/ml.

DNA – The 80 bp DNA fragment was made by hybridizing complementary 80 bp oligos (Upper strand – CTTTTCAAAACTAGTTTATTAACGTGTTTTTAAAGTAAAT TACTCTAAA AAGATCTATCGA, Lower strand - GAAAAGTTTGAATCAAATTTGAC TGACAAAA ATTTCATTTAATGAGATTTTTCTAGATAAGCT). 100 pmoles of each oligo were mixed and hybridized by ramping the temperature of the sample down from 95°C to 45°C; dropping 10°C at 5 min intervals. The fragment was isolated through gel-extraction. 48 Kb DNA preparation was λ DNA from New England Biolabs. Sonicated-salmon-sperm DNA was used to generate DNA-size spectrum. The sonicated-DNA was run on an agarose gel and DNA-size ranges were extracted. DNA-size range was estimated using the DNA size-ladder. Non-specific plasmid DNA used was pBend5 (Kim et al., 1989) and the parS⁺ containing plasmid was pBEF165 (Funnell, 1991). Plasmids were purified in cesium chloride gradients.

Protein purification – ParB (Bouet and Funnell, 1999), ParA (Davey and Funnell, 1994), ParA[K122R] (Funnell, unpublished data), ParA[K122Q] and ParA[K122E] (Fung et al., 2001) were purified as described.

ATPase assays – Assays were performed as described in Chapter 3, except the reactions were quenched after a 90 minute incubation and the amount of ATP hydrolyzed was quantified by a PhosphorImager (Amersham Biosciences).

Light-Scattering – Measurements were performed in a Photon Technology International (PTI) fluorescence system (Birmingham, NJ) at 23°C. The 60 μl sample was illuminated with 467nm-light, and the scattered-light at the same wavelength was collected at a 90° angle. For time-based measurements, a stable baseline was obtained, the cuvette was removed from its holder to add a complex component or nucleotide, and the sample contents were mixed rapidly before repositioning the cuvette (dead time = ~5 sec). For time-based competition assays, the scan was paused, competitor was added, the cuvette was repositioned, and monitoring the changes in light-scatter was resumed. For steady-state measurements, samples were incubated at 23°C for 30 min prior to scanning. Unless indicated, all intensity measurements are given in arbitrary units.
(A.U.); the light scattered prior to component addition was subtracted from the data presented. For the competition assays, the light scattered prior to competitor addition was subtracted from the data presented. Light-scattering data from the competition assays were fit to a multi-parameter exponential decay function using the software package, SigmaPlot V10.0 (Systat Software Inc.).

**Dynamic Light-Scattering** – Reactions mixtures were assembled as in the light-scattering experiments, except that the final volume was 200 µl and the dead-time needed to assemble the sample and start the scan was ~5 minutes. Samples were exposed to a laser-light at 514nm and data were collected at 90° using a Brookhaven Instruments BI-200SM goniometer. Autocorrelation functions were collected every 5 minutes at 23°C with a Brookhaven Instruments BI-9000 AT autocorrelator with sampling times (τ) of 1 µsec to 1sec. The autocorrelation function was imported into the BI-ISDAW-Size Distribution Software, which calculates the intensity as being proportional to the size and concentration of the scattering particles in the following way:

Time-dependent fluctuations in the scattered light arise from the particles undergoing Brownian motion and therefore the distance in between is constantly varying. Fluctuations are quantified via a second order correlation function given by,

\[ g^{(2)}(\tau) = \frac{[I(t)I(t+\tau)]}{[I(t)]^2} \]  \hspace{1cm} (1)

where \( I(t) \) is the intensity of the scattered light at time \( t \), and the brackets indicate averaging over all \( t \). The correlation function depends on the delay \( \tau \), that is, the amount that a duplicate intensity trace is shifted from the original before the averaging is performed.

The correlation function for a monodisperse sample can be analyzed by the equation:

\[ g^{(2)}(\tau) = B + \beta \exp(-2\Gamma \tau) \]  \hspace{1cm} (2)

where \( B \) is the baseline of the correlation function at infinite delay, \( \beta \) is the correlation function amplitude at zero delay, and \( \Gamma \) is the decay rate.

A nonlinear least squares fitting algorithm was used to fit the measured correlation function to equation 2 to retrieve the correlation function decay rate \( \Gamma \). \( \Gamma \) is then converted to the diffusion constant \( D \) for the particle via the relation:

\[ D = \frac{\Gamma}{q^2} \]  \hspace{1cm} (3)
Here, \( q \) is the magnitude of the scattering vector, and is given by,

\[
q = \frac{4\pi n_0 \sin (\theta/2)}{\lambda_0}
\]  

(4)

where \( n_0 \) is the solvent index of refraction, \( \lambda_0 \) is the vacuum wavelength of the incident light, and \( \theta \) is the scattering angle.

Finally, the diffusion constant is interpreted as the hydrodynamic diameter (\( d_h \)) of a diffusing sphere via the Stokes-Einstein equation:

\[
d_h = \frac{kT}{3\pi\eta D}
\]  

(5)

where \( k \) is Boltzmann's constant, \( T \) is the temperature in Kelvin, and \( \eta \) is the solvent viscosity.
RESULTS:

*ParA-ATP, ParB and DNA form a large nucleoprotein complex in solution.*

How does an interaction between ParA and ParB-parS complex lead to plasmid partition? *In vitro*, gel-shift assays have suggested that ParA can interact with the ParB-parS partition complex with ATP and ATPγS (Bouet and Funnell, 1999). This interaction is thought to be a prerequisite to the partition reaction and therefore a detailed understanding of this complex is critical to unveiling the mechanism. The transient nature of this complex is thought to be a result of ParB stimulating ParA ATPase activity, which leads to ParA-ADP disassembly from the complex. Since the positive interactions between ParA and ParB ultimately lead to disassembly, the study of this complex at equilibrium using separative techniques, such as gel-shift assays, has been difficult.

In this chapter, I use a solution-based approach called 90° offline light-scattering to study the BAD complex without the use of separative steps. The method is a measure of turbidity that tests whether a large proportion of the components in solution can oligomerize into large structures. Changes in light-scatter were measured after incubating all sample components for 30 min (steady-state), or in real-time, immediately after mixing the reaction components (time-based). The samples were manually mixed in a cuvette, positioned in the fluorometer, and scanned to monitor the changes in light-scatter at a wavelength of 467nm. In the time-based assay, I was able to start, pause, and stop the scan to add additional components or competitors. The “dead-time” required for mixing the sample, inserting the cuvette, and starting or resuming the scan was 5 ± 2 seconds.

I first wanted to identify the partition components that were necessary to support an increase in light-scatter intensity, as this signal change would represent the formation of large structures in solution. ParA, ParB and non-specific DNA (nsDNA), were incubated in different combinations with a saturating concentration of ATP, and then changes in light-scatter were measured at steady-state (Fig. 4-1). I first used sonicated salmon-sperm DNA as a nsDNA substrate in order to determine if assembly could occur in the absence of a parS site. Several Walker-type partition ATPases have been shown to polymerize but the DNA and protein requirements vary from system to system (Barillà et al., 2005; Bouet et al., 2007b; Ebersbach et al., 2006; Pratto et al., 2008). Here, a significant increase in light-scatter was observed only when ATP was added to ParA, ParB and DNA together (Fig. 4-1). No changes in light-scatter
were observed in the absence of any one component. ParA-ATP and ParB have nsDNA binding activities alone; however these nucleoprotein complexes were not detectable under the conditions tested here. It is possible that these complexes are too dynamic, too small in size and/or too few in number to elicit a change in light-scatter. Together, the results show ParA-ATP interacts with ParB and DNA to form a large nucleoprotein complex.

Gel shifts show that ParA can interact with the ParB-parS complex with ATP or ATPγS (Bouet and Funnell, 1999); however, ParA binding to nsDNA is ATP-dependent (see Chapter 3). To identify the nucleotide requirements for BAD complex assembly, a variety of adenine nucleotides were incubated with ParA, ParB and nsDNA and changes in light-scatter were measured at steady-state (Fig. 4-1, ParA+ParB+DNA group). An increase in light-scatter was observed in the presence of ATP or ATPγS. The maximum change in light-scatter was greater with ATPγS showing that ParA-ATP binding is required for complex assembly and suggests that ATP-hydrolysis may lead to disassembly. ADP and AMP did not elicit an increase in light scatter. Overall, ATP-binding is required for ParA to interact with ParB and DNA to form the BAD complex, which produces a significant increase in light-scatter. The finding that ATPγS can support the formation of the BAD complex, but cannot support ParA’s nsDNA-binding activity (see Chapter 3) suggests that there are at least two distinct forms of ParA-ATP: one that stably associates with ParB and DNA, and another that can dynamically associate with DNA non-specifically.

**A conformational change in ParA limits the rate of BAD complex formation.**

The BAD complex is made up of multiple components. I wanted to order the steps of the assembly mechanism using order-of-addition experiments. In these time-based experiments, ParA was always in premix 1, and the other components (ParB, DNA and ATP) were either added at the same time, or after a 30 minute interval (added as premix 2), in all possible combinations (seven in total) (Fig. 4-2A). As I show later, complex assembly was influenced by DNA length, therefore for these experiments I switched to a nsDNA substrate of uniform length (3 Kb plasmid). After mixing the premixes, I monitored the changes in light-scatter over time (Fig. 4-2B). Although there were seven different combinations (in addition to measurements of each premix separately), two patterns emerged, which I summarize in Fig. 4-2C. The actual reaction curves are shown in Supplementary Figure S4-1 at the end of the chapter. These
patterns were a hyperbolic curve (blue curve) or a sigmoidal curve (red curve). The difference in pattern depended on whether ParA was preincubated with or without ATP. When ParA and ATP were preincubated together, a hyperbolic curve was produced (Figure 4-2C, reactions 1-3). The initial time point for these reactions showed a light-scattering signal ~3-fold greater than the signal produced by the premixes (Fig.4-2C, blue line vs black line at t=0). This result indicates that complex assembly initiated during the dead-time (prior to starting the scan), and therefore, the hyperbolic decrease in light-scatter is considered as a rapid disassembly of the BAD complex to possibly a more stable state.

When ATP was added in premix 2 (ie. immediately before measurement by light-scattering), a sigmoidal curve was produced (Fig. 4-2C, reactions 4-7). The sigmoidal increase in light-scatter shows that this method of complex assembly requires a long lag-phase, which lasts several seconds (~15 sec). The lag-phase was followed by an exponential increase in light-scatter that peaked at an intensity that was two-fold greater than the light-scatter produced prior to mixing all components (Fig. 4-2C). This maximal change in light-scatter was transient and dropped to a more stable state. Intriguingly, the hyperbolic and sigmoidal curves aligned after ~75 seconds, suggesting that both methods of complex assembly eventually equilibrate to the same complex-size distribution (Fig. 4-2C, blue vs red line at t ≥ 75 sec). All premixes showed no significant changes in light-scatter over a 10 min time course (Fig. 4-2C, black line and Fig. S4-1).

Together, the results show that ParA-ATP binding is a prerequisite event in forming the BAD complex. Interestingly, we know ATP-binding by ParA is too fast to be responsible for the slow sigmoidal kinetics of complex assembly (see Chapter 3). Therefore, I propose that the slow rate-limiting step in BAD complex formation shown here is a result of a slow conformational change in ParA that follows stable ATP-binding, and is essential for ParA to associate with ParB and DNA. Furthermore, ATPγS also supports the BAD complex, showing that this conformational change occurs upstream of hydrolysis. It is attractive to speculate that the rate-limiting step identified here for BAD complex formation represents the same slow conformational change in ParA that activates its nsDNA-binding activity (see Chapter 3). I was unable to order the assembly mechanism in more detail because all other events were too rapid to be detected with this approach.
**BAD Complex size is modulated by the ParA:ParB:DNA ratio and DNA length.**

I took advantage of my finding that the ParA-ATP association is the rate-limiting step in BAD complex assembly. For all subsequent experiments, I started all time-based assays by adding ATP last to a preincubated solution containing all other components. This order of addition produces the sigmoidal curve, which allows me to monitor the start of complex assembly, providing the lag-phase is considerably longer than the dead-time (5 sec).

Using this approach, I investigated the protein and DNA requirements further by titrating ParA, ParB and DNA individually, while keeping all other component concentrations fixed (See Supplementary Figure S4-2 for raw data). All phases of the sigmoidal curve showed concentration dependencies for ParA, ParB and DNA. I first examined how varying the protein or DNA concentration influenced the lag-phase of the curve (Fig. 4-3A). Increasing ParA concentration decreased the lag-phase, showing that assembly initiates more rapidly at higher ParA concentrations (Fig. 4-3B). At 1 µM ParA (1:2 ratio of ParA:ParB), the lag-phase lasted approximately 35 seconds. Above 4 µM ParA (2:1 ratio of ParA:ParB), the light-scatter increased almost immediately, with little or no lag-phase. Like ParA, increasing the ParB concentration decreased the lag-phase, but it did not fall below 40 sec even at 8 µM ParB (1:8 ratio of ParA:ParB) (Fig. 4-3C). Notably, 40 sec is the approximate lag-time when using 1 µM ParA (Fig. 4-3B), suggesting the ability of ParB to stimulate BAD complex assembly is limited by the amount of ParA present. I conclude that ParA is the major protein determinant in the rate at which the complex assembles. Unlike ParA and ParB, increasing the DNA concentration increased the lag-time almost linearly (Fig. 4-3D). These findings suggest that a high protein:DNA ratio initiates assembly more rapidly, while a low protein:DNA ratio prolongs the start of assembly. Together with the sigmoidal kinetics, I suggest that the BAD complex initiates by way of a nucleation event on the DNA. In this light, a low protein:DNA ratio would require a long lag-time to reach the critical concentration necessary to nucleate assembly. At a high protein:DNA ratio, the critical local concentration required to form the nucleation core occurs more readily.

I also compared the component concentration dependencies on the extent of light-scatter at steady-state (Fig. 4-4A). The extent of light-scatter is defined as the change in light-scatter at a given time. For example, the extent of light-scatter at steady state is the change in light-scatter following a 30 minute incubation of all components at 23°C. A ParA titration showed that at
least 0.5 μM ParA (1:4 ratio of ParA:ParB) was necessary to detect a significant increase in light-scatter (Fig. 4-4B). Above this concentration, the extent of complex assembly increased almost linearly without reaching a plateau up to 10 μM ParA. A ParB titration showed that the extent of light-scatter was greatest with 1 μM ParB (1:1 ratio of ParA:ParB) (Fig. 4-4C). Unlike ParA, ParB concentrations > 2 μM began to inhibit the increase in light-scatter, and at 10 μM ParB (1:10 ratio of ParA:ParB) no change in light-scatter was observed. This trend was also found when varying the DNA concentration (Fig. 4-4D). The greatest increase in light-scatter was observed between 10 and 20 μg/ml DNA, and deviations from this concentration in either direction decreased the extent of light-scatter. Above 50 μg/ml DNA, no change in light-scatter was observed.

Overall, the ratio that provided the greatest increase in the extent of light-scatter was ≥1 ParA dimer(s): 2 ParB dimers: 120 bp of DNA. Any deviations from this ratio by altering the relative DNA or ParB concentration decreased the extent of light-scatter. However, increasing the relative ParA concentration continued to increase the amount of light-scatter almost linearly. These results suggest that while only a limited amount of DNA and ParB can associate with the BAD complex, ParA can be continually added, and is therefore likely to be the major protein subunit of the BAD complex. Other Walker-type partition ATPases similar to P1 ParA have been shown to associate with nsDNA and polymerize (Batt et al., 2009; Bouet et al., 2007a; Leonard et al., 2005a). In line with these findings, I tentatively interpret the increase in light-scatter as P1 ParA polymerizing across DNA from a ParB-DNA nucleation core.

ParA polymerization could occur via DNA-spreading, intramolecular DNA-bridging, or both. In this case, the extent of BAD complex assembly should also depend on the size of the DNA substrate. In the experiments above, a 3 Kb plasmid was used as the DNA substrate. I next tested the effect of DNA length. ParA and ParB were preincubated with DNA substrates of various lengths (Fig. 4-5A). The smallest fragment (80 bp) was made by hybridizing complementary oligos, and the largest (48 Kb) was λ DNA. All other DNA preparations were sonicated salmon-sperm DNA isolated by gel extraction (see experimental procedures). Changes in light-scatter were monitored immediately after adding ATP. A small but reproducible increase in light-scatter was supported with a DNA fragment range of 300-500 bp (Fig. 4-5B). Larger fragments supported greater changes in light-scatter. The extent of light-scatter at steady state increased linearly with increasing DNA substrate length, and seemed to plateau at DNA
lengths greater than 10 kb (Fig. 4-5C). The data show that DNA length influences the size-distribution of the BAD complex, and suggests that the mechanism of assembly at least partially requires an intramolecular interaction such as DNA-spreading and/or bridging.

I used both supercoiled plasmid and linear DNA molecules to study DNA effects on complex assembly. When using DNA substrates of the same length, the kinetics of the complex were identical regardless of the DNA substrate topology (Fig. 4-5D). This shows that DNA topology does not have a significant effect on BAD complex assembly as detected by this method.

**ATP-hydrolysis by ParA leads to BAD complex disassembly.**

ATPγS supported a greater increase in light-scatter compared to ATP at steady-state (Fig. 4-1). This initial result lead me to monitor the changes in light-scatter immediately following the addition of different ATP-analogues to shed light on how the steps in ATP-cycling by ParA influence the kinetics of the BAD complex. Both ATP and ATPγS supported identical sigmoidal kinetics, except the extent of assembly, as shown previously, was greater with ATPγS (Fig. 4-6A). ADP and AMPPNP, a non-hydrolyzable analogue of ATP, could not support complex formation. The result that ATPγS, and not AMPPNP, could support assembly further demonstrates how ParA function is exquisitely sensitive to the type of adenine nucleotide bound.

By extending the time-course and reducing the amount of adenine nucleotide in solution, I was able to further study the effects of ATP-hydrolysis on complex stability (Fig. 4-6B). Following the addition of ATP, the light-scatter signal dropped to initial values within 1 hour. After adding ATPγS, the maximum extent of light-scatter was higher and the rate of decrease was significantly slower than compared to the kinetics supported by ATP. The very slow linear decrease in light-scatter observed with this analogue could be a result of several possibilities. For example, ParA may be able to hydrolyze ATPγS at a very slow rate, or it could represent the breakdown of the nucleotide over a long time period at 23°C. The decrease could also reflect a change in the shape or size of the complex. In the presence of ADP, there were no significant changes in light-scatter over one hour. Together, the results indicate that ATP-binding by ParA is a prerequisite in forming the BAD complex and ATP-hydrolysis by ParA leads to disassembly.

The decrease in light-scatter supported by ParA-ATP may represent protein aggregation rather than an orderly disassembly of the BAD complex due to the depletion of ATP. To test this
possibility, I formed the BAD complex with ATP, allowed it to disassemble, and then replenished the ATP supply (Fig. 4-6C). Following the second ATP addition, the signal immediately increased to the previous maximal extent of light-scatter and then dropped back to initial values with kinetics that were similar to the first round of disassembly. The result shows that the disassembled ParA subunits in solution can rebind ATP and can be recycled in BAD complex assembly.

**ATP to ADP nucleotide exchange by ParA leads to BAD complex disassembly.**

I have shown that ATP-hydrolysis by ParA is one process that leads to BAD complex disassembly. However, the disassembly kinetics do not fit a single-exponential equation (Fig. 4-6B-C) suggesting that there are additional processes, aside from ATP-hydrolysis, that contribute to the decrease in light-scatter. I reasoned that ATP to ADP nucleotide exchange may also play a role in disassembly, since ADP could not support the BAD complex (Fig. 4-1). To test this possibility, I used an excess of ADP as competitor against assembled BAD complexes, thus rendering its disassembly irreversible and unidirectional. The BAD complex was formed with 100 μM ATP or ATPγS, and once the maximal extent of light-scatter was reached, a saturating concentration of ADP was added (Fig. 4-6D). When the samples were assembled with ParA-ATP, ADP addition resulted in a rapid decrease in light-scatter with a rate of 2.6/min. The light-scatter signal returned to values prior to complex assembly within two minutes of ADP addition. AMPPNP was also successful as a competitor showing that although this ATP-analogue cannot support complex formation, it still binds ParA. When complexes were preformed with ParA-ATPγS, ADP competition produced a disassembly rate of 0.54/min, a rate 5-times slower than when the samples were assembled with ATP. It is possible that the differences in disassembly rates shown here represent the differences in relative affinity of ParA for these adenine nucleotides. The data show that the ParA subunits in complex with ParB and DNA can undergo rapid nucleotide exchange, suggesting that the BAD complex is dynamic. Overall, in addition to ATP-hydrolysis, the ATP to ADP nucleotide exchange by ParA is an additional process that leads to BAD complex disassembly.
The parS site stabilizes the BAD complex.

Up until this point, I have studied BAD complex assembly using DNA substrates that do not encode parS, thus showing that nsDNA interactions with ParA and/or ParB are sufficient. I next studied the influence of parS on the kinetics of the BAD complex in real-time using plasmid DNA with or without a parS site. In both cases, the lag-phase and rate of assembly were identical (Fig. 4-7A, inset). However, the plasmid with parS displayed a greater extent of light-scatter that was relatively stable even 30-minutes after ATP-addition (Fig. 4-7A). Without parS, disassembly occurred much more rapidly. The comparison was performed with different DNA concentrations, and parS always supported a more stable BAD complex between 10 to 100 μg/ml DNA (data not shown). Together, the data suggest that site-specific interactions between ParB and parS stabilize the BAD complex while those formed on nsDNA are less stable.

One interpretation of these data is that ParA and ParB assemble onto DNA via a spreading or bridging mechanism that uses the parS site as a nucleation point. If ParA and ParB are stably bound to DNA, then they should protect DNA from DNaseI digestion to some extent. To test this, I formed the BAD complex by adding ATP to a mixture of ParA, ParB and plasmid-DNA encoding parS. Once the maximal extent of light scatter was reached, DNaseI was added (Fig. 4-7B). Following the addition of DNaseI, the light-scatter signal fell at rate of 0.13/min, taking ~30 minutes to reach initial values. The slow decrease in light-scatter suggests that the DNA is being degraded, but very slowly. When DNaseI was preincubated with ParA, ParB and DNA for five minutes prior to ATP addition, no change in light-scatter signal was observed. Together these data show that the complex significantly protects the DNA from digestion. The slow degradation of DNA could be due to competition for accessible DNA between DNaseI and protein subunits undergoing exchange.

ParA Walker-box mutants are altered in BAD complex assembly.

I have shown that ATP-binding, hydrolysis and ATP to ADP nucleotide exchange all influence the kinetics of the BAD complex in different ways. Therefore, ATP-cycling by ParA is a critical modulator of the BAD complex. Following the mutation of the catalytic lysine conserved in the nucleotide-binding site of ParA (K122) three phenotypic classes of mutants were identified (Fung et al., 2001). In the first class, ParA[K122E] showed weak repressor activity and was defective for partition and ATPase activities. It was concluded that the ability
of this mutant to bind ATP has been damaged (Fung et al., 2001). In the second class, ParA[K122Q] was also deficient for partition and ATPase activity, but was classified as a ‘‘super-repressor’’ because its repressor activity alone was comparable to that of wild-type ParA in the presence of the corepressor, ParB. Although K122Q could not hydrolyze ATP, it could still bind ATP. Finally, ParA[K122R] had weak repressor activity, and showed a partition defect that was worse than a parA null mutant. This phenotype is known as ParPD, or propagation defective (Fung et al., 2001; Youngren et al., 2000). Functional studies on the repressor activity and ATPase activity of these mutants suggested that all three variants are insensitive to ParB stimulation; however, the in vivo ParPD effect of ParA[K122R] requires ParB. The current hypothesis is that ParPD ParA mutants cause P1 plasmids to segregate in clumps rather than as individual units, but how this is accomplished is unknown. I used this spectrum of ParA mutants to further dissect how ATP-cycling modulates BAD complex formation, and to identify the molecular mechanism behind the ParPD phenotype.

First, I determined which of these mutants could support the BAD complex. Each ParA variant was incubated with ParB, sonicated-DNA and adenine nucleotide for 30-minutes and the steady-state change in light scatter was measured (Fig. 4-8A). Of the three mutants, only ParA[K122R] supported complex formation. When comparing wild-type ParA to ParA[K122R], it was apparent that ParA[K122R] supported a signal roughly 2-times greater than that of wild-type ParA in the presence of ATP or ATPγS. ParA[K122Q] and ParA[K122E] could not support complex formation under any of the conditions tested here. As mentioned, while both of these mutants are deficient in their ATPase activity, ParA[K122Q] can still bind ATP. This result shows that ATP-binding by ParA is necessary but not sufficient for assembly of the BAD complex. A conformational change in ParA, not supported by ParA[K122Q], must occur subsequent to ATP-binding for it to form the BAD complex.

I further studied the protein and DNA requirements for an increase in light-scatter with ParA[K122R]. I incubated ParA[K122R] alone or in combination with ParB and/or sonicated-DNA, and measured changes in light-scatter at steady-state (Fig. 4-8B). Although, ParA[K122R] supports a greater change in light-scatter compared to wild-type ParA, both share the same requirements for complex assembly. Together, the data show that the ParPD mutant, ParA[K122R], supports the formation of a BAD complex that is greater in size, number and/or stability.
As shown previously, DNA length influences the kinetics of BAD complex formation. I wanted to determine if complexes formed with ParA[K122R] showed a similar dependence. ParA or ParA[K122R] was incubated with ATP, ParB and DNA ranging from 80 bp to 48 kb in length, and the steady state change in light-scatter was measured. At all DNA lengths tested, the extent of light-scatter was significantly greater with ParA[K122R] (Fig. 4-8C). From this I conclude that while both show a correlation between complex size and the length of DNA substrate, ParA[K122R] supports greater assembly of the BAD complex relative to that formed with wild-type ParA.

**ParA[K122R] forms a Super-BAD complex.**

Under the buffer conditions used in these assays, ParA[K122R] shows a weak ATPase activity that is comparable to that of wild-type ParA (Fig. 4-9). But unlike wild-type ParA, ATP-hydrolysis by ParA[K122R] cannot be stimulated by ParB. If ATP-hydrolysis leads to complex instability, it is reasonable to suggest that the greater extent of light-scatter supported by ParA[K122R]-ATP should also be more stable, and not decrease as rapidly as it does with wild-type ParA. To test this, the BAD complex was formed with either ParA or ParA[K122R] and the kinetics were monitored in real-time (Fig. 4-10A). Both assembly and disassembly kinetics were altered with the ParA mutant. During assembly, the lag-phase was approximately 3-times longer with ParA[K122R] (Fig. 4-10A, inset). This difference can be attributed to ParA[K122R] having a weaker affinity for ATP and/or the number of active sites in the mutant ParA population are fewer than that of the wild-type ParA population. Nevertheless, once the maximum extent of light-scatter was achieved, the BAD complex supported by ParA[K122R] proved to be profoundly stable (Fig. 4-10A). After one hour, the light-scatter signal supported by ParA[K122R] had decreased less than 15%. The data suggest that although ParB cannot stimulate the ATPase activity of ParA[K122R], they can still associate to form a very large and very stable nucleoprotein complex, which I call the Super-BAD complex.

I wanted to further examine how ParA[K122R] could support such a profoundly stable complex. Is it solely the result of having an ATPase activity that cannot be stimulated by ParB or is nucleotide-exchange also altered in this mutant? To test this possibility, the Super-BAD complex was assembled with ATP and an excess of ADP was used as competitor (Fig. 4-10B). As shown previously, BAD complexes formed with wild-type ParA rapidly disassembled upon
ADP competition. The Super-BAD complex formed with ParA[K122R], on the other hand, was insensitive to ADP competition. Overall, the data suggest that the increased stability of the Super-BAD complex supported by ParA[K122R] is a result of two altered processes in its ATP-cycling activity: (1) its ATPase activity cannot be stimulated by ParB, and (2) in the context of an assembled complex, it also shows little to no nucleotide-exchange. The combinatorial effect results in a Super-BAD complex that is even more stable than the one supported by ParA-ATPγS (Fig. 4-10B), which still undergoes nucleotide-exchange even though hydrolysis is considerably lessened.

Size-distribution of complexes becomes uniform over time.

The 90° offline light-scattering measurements provided a population-averaged physical measure of the relative amount of complex at any given time. This method however does not provide an absolute measure of complex size nor the size-distribution in solution. I performed dynamic light-scattering (DLS) experiments to resolve the size-distribution of the complexes formed with ParA-ATP, ParA-ATPγS and ParA[K122R]-ATP. ParA or ParA[K122R] was pre-incubated with ParB and plasmid DNA, and assembly was initiated by the addition of a high concentration of adenine nucleotide. The mean diameter was then measured over a time-course (Fig. 4-11A). The DLS results were consistent with those found using 90° offline light-scattering. ParA-ATPγS supported larger complexes than ParA-ATP, and ParA[K122R]-ATP formed the largest complexes.

The size-distribution of the complexes formed over the time-course allowed me to differentiate whether the complexes were growing in size or number (Fig. 4-11B). At 15 min, the complexes supported by ParA-ATP, ParA-ATPγS and ParA[K122R]-ATP had a very wide distribution range. The distribution ranges were 100-600 nm for complexes supported by ParA-ATP, 200-1200 nm for complexes supported by ParA-ATPγS and 600-1100 nm for complexes supported by ParA[K122R]-ATP. Over time, this distribution shifted up in size and narrowed significantly to a discrete size range: 450-650 nm for complexes supported by ParA-ATP, 650-825 nm for complexes supported by ParA-ATPγS and 1000-1100 nm for complexes supported by ParA[K122R]-ATP. Together, the DLS results suggest that the increase in light-scatter observed in the 90° offline light-scattering assays translates primarily into a growth in complex size, not in number.
DISCUSSION:

Here I show that ParA can form a large nucleoprotein complex with ParB and DNA in the presence of ATP or ATPγS. The nature of this BAD complex remains to be determined. However, I favour the possibility that the increase in light-scatter measured here is predominantly a result of ParA polymerization from a ParB-DNA nucleation core for several reasons. The first and strongest piece of evidence that supports this explanation comes from the component titration data (Fig. 4-4 and Fig. S4-2). Increasing the ParA concentration continued to linearly increase the extent of assembly, while too much ParB or DNA lead to inhibition of complex assembly. These findings argue that ParA subunits can be continually added to the complex and should therefore be considered as the major protein subunit of the complex.

Second, the kinetics of complex assembly and disassembly were directly influenced by ParA ATP-cycling. Finally, given that several Walker-type ATPases have been confirmed to filament by EM and light-scattering assays (Barillà et al., 2005; Bouet et al., 2007b; Ebersbach et al., 2006; Pratto et al., 2008), it is likely that PI ParA shares this activity. Therefore, I interpret the increase in light-scatter and the formation of the BAD complex as ParA polymerization from a ParB-DNA nucleation core.

I found that ParA polymers are dynamic. The transient signal observed after ATP-addition shows that ParA ATPase activity regulates filament formation (Fig. 4-6B). Furthermore, addition of ATPγS stabilized the filaments. Together, these results strongly support that ATP binding and hydrolysis are involved in the regulation of filament dynamics. ATP-binding was found to promote complex assembly and ATP-hydrolysis by ParA lead to disassembly. Moreover, ADP was found to have an active role in antagonizing polymerization (Fig.4-6D), revealing that the ATP- and ADP-bound forms of ParA are proficient and suppressed, respectively, in polymerization. Thus, the stimulation of ParA polymerization by ATP and its inhibition by ADP might be a fundamental regulatory mechanism in vivo.

ParA polymerization depended on the presence of both ParB and DNA. The strict dependency could in principle be due to filament stabilization by ParB via stoichiometric interaction between ParA and ParB along the filament. However, I find here that ParA polymerization is triggered by the presence of ParB and DNA in substoichiometric amounts (Fig. 4-4B). Order-of-addition experiments showed that the complex would only assemble following a slow ParA-ATP association step (Fig. 4-2). I suspect this lag is caused by the same slow
conformational change identified in Chapter 3, which renders ParA competent for nsDNA-binding. Intriguingly, ParA-ATPγS can support the BAD complex but, in the absence of ParB, cannot bind nsDNA. ParB stimulation may provide the activation energy needed for ParA-ATPγS to transition into the nsDNA-binding form. Overall, it is possible that the ParB-parS complex nucleates ParA polymerization and promotes ParA spreading along nsDNA.

The stimulation of ParA ATPase activity by ParB is likely to be an important regulatory factor for the ParA assembly-disassembly cycle in vivo. ParB exerts a profound effect on ParA polymerization in vitro. At high ParB:ParA ratios, ParB antagonizes ParA filamentation (Fig. 4-4C). At low ParB:ParA ratios, ParB promotes rapid and extensive ParA filamentation. This dichotomy mirrors the ratio-dependent effects produced by MinE on MinD polymers (Suefuji et al., 2002) and ParG on ParF polymers (Barillà et al., 2005). In these cases, the accessory protein appears to stabilize the filaments by bundling them together. Similarly, I find that ParB bound to parS promotes the assembly and/or stability of ParA polymers (Fig. 4-7A). Polymer stabilization could be accomplished by lateral crosslinking of adjacent ParA protofilaments analogously to microtubule-associated proteins (MAPs) that promote microtubule stability often by crosslinking neighbouring tubulin monomers. One mode of operation of MAPs is through oligomerization (Bray, 2001). Perhaps, the oligomeric ParB complex at parS mediates the joining of ParA monomers that reside in adjacent protofilaments thereby promoting polymer bundling on the nucleoid in the vicinity of the plasmid. Upon stimulation of ParA ATPase activity, the protofilaments disassemble. This does not result in the plasmid losing its association with the nucleoid, because the partition complex connections are transferred to laterally associated ParA polymers.

The dynamic properties of ParA are fundamental to the plasmid partition process. It has long been speculated that ATP binding and hydrolysis by Walker-type partition ATPases provide the motive force for plasmid dissociation and localization. I show that ParA polymerization is regulated by (at least) two mechanisms, ATP-cycling and through interaction with the ParB-parS complex. In light of these findings, I propose an explanation for the elusive link between ATP-cycling and plasmid partition by Walker-type partition ATPases: reversible and dynamic polymerization of these proteins is crucial for proper DNA segregation. The results presented in this chapter allow me to add more detail to the ratchet-type mechanism presented in Chapter 3.

In the model (Fig. 4-12A), ParA-ATP dynamically samples the nucleoid. Upon interaction with
the partition complex, ParA is locked onto the nucleoid and forms short, laterally associated polymers. But as the partition complex associates with the nucleoid-bound ParA clusters, ATP hydrolysis is quickly induced and ParA-ADP dissociates from the nucleoid. Because ParA-ADP cannot rebind DNA or re-associate with ParA polymers, it loses the positional memory of the site of dissociation. This generates a low-density area of nucleoid-bound ParA in the wake of the partition complex. New ParA contacts are made where the nucleoid-bound ParA concentration is higher, namely the movement front. A large number of ParB molecules bound to the plasmid makes it easy to imagine how the plasmid might glide along the nucleoid surface using these laterally associated ParA polymers without losing all the ParA-mediated contacts with the nucleoid at any given time. As a result of the lower concentration of the nucleoid-bound ParA in its wake, the initial drift toward one stochastically chosen direction enforces the continued movement towards the same direction. When the plasmid replicates and an additional complex is formed, the plasmids pair for an indeterminate amount of time (Fig. 4-12B). While in physical proximity to one another, a low density ParA distribution is generated in the region between the paired complexes, therefore producing a repulsive force between the two plasmids (Fig. 4-12C). The model allows for the two partition complexes to approach each other and rejoin. However, their movements would be suppressed to avoid the low ParA density area occupied by the other complex.

The phenotypes conferred by mutation in the Walker A motif of ParA corroborate the importance of nucleotide binding and hydrolysis, and establish a functional coupling between polymerization and plasmid partition. Both the super-repressor mutant, ParA[K122Q], and the weak-repressor, ParA[K122E], are inactive in partition and have no ATPase activity (Fung et al., 2001). Here I show they both lack polymerization activity even though ParA[K122Q] can still bind ATP. In the presence of ATP or ADP, ParA[K122Q] has strong par repressor activity and parOP binding activity, but is insensitive to ParB stimulation (Fung et al., 2001). This argues that following ATP-binding, the conformational change required for extensive ParA polymerization is not required for ParA repressor function.

The ParPD mutant, ParA[K122R], exhibited extensive polymerization with ParB, DNA and ATP or ATPγS, but the polymers were no longer dynamic. Unlike wild-type ParA polymers, these hyperpolymers were insensitive to ADP competition (Fig. 4-10B), even though ParA[K122R] can bind ADP (Fung and Funnell, unpublished data). This raises the possibility
that ParA[K122R] has abrogated nucleotide-exchange activity and/or the subunits within the mutant polymer no longer exchange with those in solution. Strikingly, the hydrolytic activity of this mutant is insensitive to stimulation by ParB. This shows that the stimulatory activities of ParB in ATP hydrolysis and polymerization are separable. Extensive polymerization by ParA[K122R] might translate into filaments that are intrinsically more crosslinked, because the intermonomer and/or interprotofilament interactions are cemented without the ParB stimulated action of hydrolysis to disassemble the contacts. In vivo, it is possible that static ParA[K122R] polymers bundle on the nucleoid after associating with plasmids through the ParB-parS complex, thus irreversibly clumping or gluing the plasmids together. The biochemical data presented here are the first to provide a molecular mechanism for the ParPD phenotype. This study has provided a glimpse of the composition and dynamics of the partition complex, but in order to fully understand the overall system behaviour, it is important to observe the interplay between ParA, ParB, a plasmid bearing parS and the nucleoid simultaneously.

Plasmid partitioning has mainly been studied in species of the γ Proteobacteria phylum. Among the Firmicutes phylum, the partition ATPases studied thus far are δ from Streptococcus pyogenes pSM19035 and Soj from the Bacillus subtilis chromosome. The evolutionary distance between Firmicutes and γ Proteobacteria exceed that between plants and animals, and this raises the question whether bacteria of these two phyla share the same mechanism of DNA partitioning. Soj polymerization requires a DNA matrix, and δ polymerization requires a DNA matrix and its cognate ParB (ω) (Hester and Lutkenhaus, 2007; Leonard et al., 2005a; Pratto et al., 2008). This matrix driven polymerization was thought to be unique to partition systems in Firmicutes (Pratto et al., 2008). The data presented here suggests otherwise. Unlike all of other filament-forming Walker-type partition ATPases that are encoded by γ proteobacterial plasmids, the data presented here are the first to show a dependence on ParB and a DNA matrix for P1 ParA polymerization. Together, the evidence reveals the remarkable diversity among partition ATPases and suggests that, although similarities exist, the mechanisms of plasmid partition may be equally diverse.
Figure 4-1. BAD complex assembly as measured by steady-state light-scattering. The mixtures contained 1 μM ParA, 2 μM ParB and 0.1 mg of sonicated-DNA/ml were preincubated with 1 mM adenine nucleotide as indicated for 30 minutes at 23°C, and changes in light-scatter were measured at 467nm. The light-scatter prior to nucleotide addition was subtracted from the raw data to obtain ‘Relative Light-scatter’.
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**Figure 4-2. Order-of-addition effects on the kinetics of BAD complex assembly.**

(A) All preincubation combinations of 1 μM ParA, 2 μM ParB, 20 μg/ml plasmid DNA and 1 mM ATP (Reactions 1 to 7). Reactions 1 to 3 have ParA preincubated with ATP, and reactions 4 to 7 have ParA and ATP in separate premixes. (B) Preincubations were performed for 30 min at 23°C. Each reaction was started by mixing premix 1 and 2 together, and changes in light-scatter were measured immediately in real-time. (C) The blue line is representative of the light-scatter curves obtained for reactions 1 to 3 and the red line is representative of the curves obtained for reactions 4 to 7. Before mixing, all premixes showed no changes in light-scatter (black line). ‘Light Scatter’ represents raw light-scattering intensities. The curves for all reactions are provided in Supplementary Figure S4-1.
Figure 4-3. ParA, ParB and DNA concentration effects on initiation of BAD complex assembly. (A) Immediately following ATP-addition (1mM), changes in light-scatter were monitored over time. The lag-time was obtained by extrapolating the slope of the exponential phase of the curve to the x-intercept. (B) ParA, (C) ParB, and (D) DNA concentration effects on the lag-time of assembly. When fixed, the concentrations of ParA, ParB and plasmid DNA were 1 μM, 2 μM and 20 μg/ml respectively. The curves for all titrations are provided in Supplementary Figure S4-2.
Figure 4-4. ParA, ParB and DNA concentration effects the extent of BAD complex assembly. (A) Immediately following ATP-addition (1mM), changes in light-scatter were monitored over time. The extent of light-scatter was obtained 30 minutes after the ATP-addition (steady state). (B) ParA, (C) ParB and (D) DNA concentration effects on the extent of light-scatter at steady-state. When fixed, the concentrations of ParA, ParB and plasmid DNA were 1 µM, 2 µM and 20 µg/ml respectively. The curves for all titrations are provided in Supplementary Figure S4-2.
Figure 4-5. DNA effects on BAD complex assembly. (A) Agarose gel showing the size ranges of DNA-substrates. DNA substrates were gel-extracted sonicated-DNA fragments; with the exception of the 80 bp and 48 Kb substrates which were hybridized oligos and λ DNA respectively. (B) DNA length effects on the kinetics of complex assembly. 20 µg/ml DNA (length range indicated) was preincubated with 1 µM ParA and 2 µM ParB for 30 minutes at 23°C. 1 mM ATP was added and changes in light-scatter were monitored over time. (C) DNA length effects on the extent of light-scatter at steady state. (D) DNA topology effects. Complexes were formed as in (B), except the DNA substrates were supercoiled or linearized plasmid-DNA of the same length.
Figure 4-6. Adenine nucleotide effects on BAD complex assembly. (A) Samples containing 1 μM ParA, 2 μM ParB and 0.1 mg/ml sonicated-DNA were preincubated for 30 min at 23°C. Adenine nucleotide was added to 1 mM and changes in light-scatter were monitored over time. (B) The samples were assembled as in (A) except the adenine nucleotide concentration was 100 μM, and the time-course was extended to one hour. (C) Complexes were formed as in (B) with 100 μM ATP. At 1 hour post-ATP addition, the scan was paused, an additional 100 μM ATP was added, and monitoring was immediately resumed. (D) ADP and AMPPNP competition on assembled complexes. Complexes were assembled with ParA-ATP (green) or ParA-ATPγS (red) as in (B) except with 100 μM adenine nucleotide. At t=0, 2 mM ADP or AMPPNP was added where indicated, and monitoring was resumed. The curves were normalized to the light-scatter of the sample just prior to competition.
Figure 4-7. *parS* effects on BAD complex assembly. (A) *parS* stabilizes the BAD complex. Samples comprised of 1 μM ParA, 2 μM ParB and 50 μg/ml plasmid DNA, with or without *parS*, were preincubated for 30 minutes at 23°C. 1 mM ATP was added and changes in light-scatter were monitored over time. Inset is a magnification of the time-course from 0 to 150 sec. (B) ParA and/or ParB protects DNA from DNase I degradation. Samples were assembled with 1 μM ParA, 2 μM ParB and 50 μg/ml plasmid DNA containing *parS* and preincubated for 30 minutes at 23°C. Following the addition of 1 mM ATP, the light-scatter signal was allowed to reach a plateau. At t = 0, the stability of the light-scatter signal was monitored following the addition of 14 μg/ml DNase I (red) or reaction buffer (green). When DNase was added 5 min prior to ATP addition, no change in light-scatter was detected (black).
Figure 4-8. BAD complex assembly with Walker-box mutants of ParA. (A) 2 μM ParB and 0.1 of mg sonicated-DNA/ml were incubated with 1 μM wild-type or mutant ParA and 1 mM adenine nucleotide for 30 minutes at 23°C. Changes in light-scatter were then monitored at steady state. (B) 1 μM ParA[K122R] and 1 mM adenine nucleotide was incubated alone or in the presence of 2 μM ParB and/or 0.1mg/ml sonicated-DNA and changes in light-scatter were monitored at steady-state. (C) DNA length effects on the extent of light-scatter with ParA or ParA[K122R]. 20 μg/ml DNA (DNA length indicated) was preincubated with 1 μM ParA or ParA[K122R], 1mM ATP and 2 μM ParB for 30 minutes at 23°C and then changes in light-scatter were measured at steady-state.
Figure 4-9. **ParA[K122R] ATPase activity.** In the presence or absence of 2 μM ParB, ParA or ParA[K122R] was incubated with 2 mM [γ-32P]-ATP and 20 μg/ml sonicated-DNA for 90 min at 23°C. 32P_i production was quantified as described in Chapter 3.
Figure 4-10. The kinetics of Super-BAD assembly and disassembly with ParA[K122R]. (A) Samples assembled with 1 μM ParA or ParA[K122R], 2 μM ParB and 0.1 mg/ml sonicated-DNA were pre-incubated for 30 minutes at 23°C. ATP (to 500 μM) was then added, and changes in light-scatter were monitored over time. Inset is a magnification of the time-course from 0 to 150 seconds. (B) Complexes were formed as in (A) with ATP or ATPγS. At t=0, 2 mM ADP or AMP was added where indicated, and changes in light-scatter were monitored over time. The curves were normalized to the light-scatter of the sample just prior to competition.
Figure 4-11. Quantification of complex size using dynamic light-scattering. (A) ParA or ParA[K122R] (at 1 μM) was preincubated with 2 μM ParB and 20 μg/ml plasmid DNA. ATP or ATPγS (to 1mM) was added and changes in light-scatter were monitored at 5 min intervals. (B) Size-distribution of complexes supported by ParA-ATP (left), ParA-ATPγS (middle) or ParA[K122R]-ATP (right) at the time points indicated. Data analysis is described in experimental procedures.
Figure 4-12. Ratchet-type model describing the motion of a plasmid along the bacterial nucleoid. (A) Prior to replication and partition. ParA-ATP dynamically associates with the nucleoid in regions away from the partition complex. Upon contact with the partition complex, ParA-ATP polymers are stabilized by ParB on the nucleoid. The partition complex tracks across the nucleoid by forming new ParA contacts in its front and disassembling ParA-ADP from the nucleoid in its wake. (B) Following replication, the sister-plasmids develop repulsive interactions and dissociate as they track along ParA polymers in opposite directions. (C) The wakes of the partition complexes form a region of the nucleoid devoid of ParA-ATP polymers, which enforces the bidirectional movement of the plasmids.
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**A**

**B**

**C**

**D**

**E**

**F**

**G**

**H**
Figure S4-1. Order-of-addition effects on the kinetics of complex assembly. (A) The preincubation combinations of 1 μM ParA, 2 μM ParB, 0.1 mg/ml sonicated-DNA and 1 mM ATP (Reactions 1 to 7). Preincubations were performed for 30 min at 23°C. Reactions 1 to 3 have ParA preincubated with ATP, and reactions 4 to 7 have ParA and ATP in separate premixes. Reactions were started by mixing premixes 1 and 2 together, and changes in light-scatter were measured immediately in real-time. (B-D) Reaction curves when ParA and ATP are in the same premix prior to mixing. (E-H) Reaction curves when ParA and ATP are in separate premixes prior to mixing. Before mixing, premixes from all reactions showed no changes in light-scatter (black line).
Figure S4-2. ParA, ParB and DNA concentration effects on complex assembly. (A) ParA, (B) ParB and (C) DNA were incubated at the concentrations indicated while all other component concentrations were fixed. Immediately following ATP-addition (1mM), changes in light-scatter were monitored over time. When fixed, the concentrations of ParA, ParB and plasmid DNA were 1 μM, 2 μM and 20 μg/ml respectively.
CHAPTER 5

GENERAL DISCUSSION & FUTURE DIRECTIONS

I performed all experiments in this chapter.
GENERAL DISCUSSION

*ParB is a flexible intra- and potentially inter-molecular bridging protein*

In chapter 2, I show that ParB can bind multiple arrangements of A- and B-box elements in the *parS* site. These findings highlight the importance of the flexible linker of ParB, which permits the rotation of the HTH domain relative to the dimerized dimer domain, and supports a model for ParB-*parS* partition complex assembly.

The BoxB sequences within *parS* are contacted by the dimer domain of ParB, which represents a new type of DNA-binding motif (Schumacher and Funnell, 2005). The dimer domain of ParB must dimerize to be functional as a DNA-binding module (Schumacher and Funnell, 2005; Surtees, 2001). The manner in which the dimer domain interacts with *parS* is unique and presents a mechanism for how ParB may mediate DNA condensation. When ParB binds to a full *parS* site, each face of the dimer domain interacts with the B-boxes arranged as direct repeats on a single looped DNA molecule. This interaction leads to the juxtaposition of the two *parS* arms in close proximity and at sharp angles relative to each other. It can be argued that ParB-BoxB interactions are perfectly suited to function in the formation of a wrapped solenoid-type nucleoprotein complex; an ultrastructure predicted to be key in partition complex assembly for R1 plasmid (Salje and Lowe, 2008). Sequence comparison of the dimer domains of other P1 ParB-like proteins shows that the dimer domain mediates critical species-specific DNA-binding interactions (Davis et al., 1992; Hayes et al., 1993; Radnedge et al., 1996; Radnedge et al., 1998). However, structural homology searches to date suggest that this motif is not found in ParBs outside the P1 ParB family.

The BoxA sequences within *parS* and the DNA flanking the *parS* site are extremely A/T rich. A/T tracts are known to narrow the minor grooves of DNA and therefore support an indirect readout mechanism for ParB-BoxA binding (Schumacher et al., 2007a). In the indirect readout mechanism, a local sequence-dependent DNA structure is recognized through protein contacts with the sugar–phosphate backbone and/or non-specific parts of the DNA bases (Vlieghe et al., 1999). In this way, DNA features such as minor groove width add another dimension to the recognition event. It is possible that the HTH domain of ParB may play a role in non-specific spreading onto the A/T-rich DNA surrounding *parS* and thus provides a mechanism for ParB spreading and the formation of the higher order ParB-*parS* partition complex.
An important prediction from my results is that additional ParB molecules could load via both specific and non-specific contacts to the DNA. Specific contacts would be mediated by the HTH domain and dimer domain interacting with the remaining BoxA and BoxB motifs respectively. Finally, non-specific contacts would permit ParB spreading and DNA condensation. At physiological concentrations, ParB spreads ~400 bp on either side of parS (Rodionov et al., 1999; Rodionov and Yarmolinsky, 2004). Non-specific DNA-binding alone would be of relatively low affinity compared with the parS-specific interactions; however, they are presumably aided by N-terminal ParB linkages, which have been shown to form transient higher order oligomers (Surtees and Funnell, 1999). These weak ParB-ParB and ParB-DNA interactions would act together in anchoring several ParB dimers onto and around parS. It is attractive to speculate that the flexible attachment of the two DNA-binding motifs would be essential to further link DNA elements on the same or different P1 plasmids.

Following my study of ParB-parS binding, structural studies showed that a ParB dimer can bridge up to four potentially distant DNA sites (Schumacher et al., 2007b). These findings support my model of ParB binding to a single parS site, but also support the possibility of intermolecular pairing. Since the regions surrounding parS are notably A/T-rich, the HTH domains of ParB would likely function in long distance DNA spreading and condensation by binding and bridging the A/T-rich sites dispersed within and around parS. The dimer domain on the other hand would contact the BoxB motifs bordering the parS site, aiding in DNA condensation and partition complex colocalization.

Could nucleotide binding control ParA function by modulating its DNA-binding specificity?

The nucleotide specificity for the different DNA-binding forms of ParA shows that these activities are controlled by the same ATP-ADP switch that controls ParA function in repression or partition: ParA-ADP binds site-specifically to parOP and functions in repression, and ParA-ATP binds DNA non-specifically and functions in partition. Unlike Type Ib ATPases, Type Ia ATPases (like P1 ParA) have an N-terminal regulatory fragment that is required for site-specific DNA-binding. The fact that partition ATPases from both subclasses exhibit non-specific DNA binding activity supports the idea that the non-specific DNA binding interface is separate and distinct from the N-terminal regulatory fragment.
Could the non-specific DNA-binding interface found at the C-terminus of the ParA-ADP crystal structure (motif 1 and motif 2 in Fig. 1-7) also form when ParA is ATP-bound? Although a ParA-ATP structure is not available, the structure of Soj bound to ATP supports this theory (Leonard et al., 2005a). ATP binding to Soj elicited a structural change that forms a basic DNA-binding interface, which was shown to be critical in non-specific DNA-binding and partition functions (Hester and Lutkenhaus, 2007). The residues composing this interface are conserved among other chromosomal homologues, including the ParAs from Caulobacter crescentus, Pseudomonas aeruginosa, Pseudomonas putida, Streptomyces coelicolor, and chromosome I of Vibrio cholera, indicating that DNA binding is a common feature of members of this family. Mutating this basic interface in SopA of F plasmid also led to a loss of non-specific DNA-binding activity and caused severe partition defects in vivo (Castaing et al., 2008). An alignment shows that motif 1 and motif 2 of ParA corresponds to the regions in SopA and Soj required for non-specific DNA-binding (Castaing et al., 2008). Therefore, it is likely that motif 1 and motif 2 are critical elements in the non-specific DNA binding activity of ParA-ATP. Nevertheless, it remains to be tested if mutagenesis of these motifs compromises the non-specific DNA-binding activity of ParA and P1 plasmid stability.

A role for the nucleoid in P1 plasmid partition

In Chapter 3, I suggest that the non-specific DNA-binding activity of ParA translates to an affinity for the bacterial chromosome and not the plasmid in vivo. Several observations support this model. First, several fluorescent fusions of Type I partition ATPases colocalize with the nucleoid (see Chapter 1). Second, the P1 plasmid is 91.6 kb while the E. coli genome is 4,600 kb. Therefore the percentage of available DNA inside the cell contributed by a single plasmid is less than 2%. Finally, the evidence suggests that ParB can spread over the plasmid from a ParB-parS nucleation core, suggesting that even if ParA were to bind the plasmid, ParB interaction would stimulate its ATPase activity and immediately render ParA incompetent for DNA binding. Overall, I think it is likely that the non-specific DNA interaction with ParA we observed in vitro represents an association with the nucleoid in vivo.

One of the first experiments that investigated a potential role of the nucleoid in plasmid partition was through the use of mukB mutants, which produce anucleate cells at a relatively high frequency (Hiraga et al., 1989). Both P1 and F plasmid have been shown to segregate into
anucleate cells (Funnel and Gagnier, 1995; Hatano et al., 2007), suggesting that the bacterial chromosome itself is not necessary for partition. In these studies it is not clear how many anucleate cells received the plasmid, and there are several alternative interpretations to the findings. First, it is unclear how anucleate cells form. *mukB* mutants have a decondensed chromosome that can be guillotined upon cell division. In this case, it is possible that the plasmids piggybacked onto a portion of fragmented nucleoid that was later degraded. Another possibility is that these plasmids entered anucleate cells by random diffusion. This explanation is not unreasonable as all *mukB* mutants have chromosomes that are severely perturbed. Therefore, partition could be defective in all cells of this population; not just anucleate ones. Finally, the previous studies oppose several pieces of evidence highlighted throughout this thesis that designates the nucleoid as the only host-factor (excluding ATP) that is critical for the partition reaction.

**Min and Par Localization Systems**

In chapters 3 and 4, I propose the *parABS* system of P1, and most likely all Type I partition systems, segregate and localize DNA using a mechanism that is comparable to that used by the MinCDE system in localizing the cell division machinery in *E. coli*. In using this analogy to explain the P1 partition reaction, I substitute ParA for MinD, the ParB-ParS partition complex for MinE, a nucleoid matrix in place of the membrane, and finally, instead of septum localization, the *par* system acts to localize the plasmid(s).

MinD shares a number of biophysical and dynamic characteristics with Type I partition ATPases, including P1 ParA. First, all show weak ATPase activity alone, and are cooperatively stimulated by an accessory protein and a defined matrix. For MinD, MinE is the accessory protein and a lipid membrane is the matrix. For partition ATPases, the accessory protein is the cognate ParB and the matrix is DNA (in the form of a nucleoid). Second, the structures of MinD and all solved Type I ATPase structures (Soj, P1 ParA, δ of pSM19035) are homologous and respond to adenine-nucleotide binding in a similar manner (Dunham et al., 2009b; Hu et al., 2002; Leonard et al., 2005b; Pratto et al., 2008). Thirdly, MinD and many Type I partition ATPases form ATP-dependent filament bundles that look similar to each other (Barillà et al., 2005; Bouet et al., 2007b; Ebersbach et al., 2006; Shih et al., 2003). While MinD requires a lipid matrix to polymerize, Type I partition ATPases show a variety of polymer requirements; but
some do need a matrix. For example, Soj and δ polymerization is DNA-dependent (Hester and Lutkenhaus, 2007; Leonard et al., 2005b; Pratto et al., 2008). Finally, like MinD, several Type I ATPases (pB171 ParA, SopA of F plasmid, δ of pSM19035, ParAI of V. cholera, Soj of B. subtilis) have also been observed to oscillate (Ebersbach and Gerdes, 2004; Hatano et al., 2007; Lim et al., 2005; Marston and Errington, 1999; Pratto et al., 2008). But unlike MinD oscillation that rapidly travels the membrane from pole-to-pole, the oscillation of Type I partition ATPases is restricted to the nucleoid.

Therefore the matrix association is an important distinction between the localization mechanisms of Min and Par. MinD has an ATP-specific affinity for the membrane via phospholipids (Hu et al., 2002; Raskin and deBoer, 1999), and ParAs show an ATP-dependent affinity for the nucleoid via non-specific DNA binding activity (Bouet et al., 2007a; Leonard et al., 2005b). Structural and biochemical analyses support this difference because MinD has an amphipathic helix necessary for membrane association (Mileykovskaya et al., 2003; Zhou and Lutkenhaus, 2003), while several partition ATPases have been shown to utilize the C-terminal, positively-charged interface for non-specific DNA-binding as discussed previously (Castaing et al., 2008; Dunham et al., 2009b; Hester and Lutkenhaus, 2007).

Revisiting the Controversy on the Dynamics of Type I Partition ATPases

It remains to be elucidated whether bacterial DNA segregation mediated by Walker- and actin-type ATPases is mechanistically distinct. I favor the idea that the partition mechanisms are distinct as several pieces of evidence suggest that Walker-type ATPases do not form a mitotic-spindle-apparatus analogous to that formed by actin-like ParM from R1 plasmid. (1) In vitro, ParM filaments are long (several microns) and discrete; consisting of two protofilaments that have little or no tendency to bundle or branch, even at micromolar concentrations (van den Ent et al., 2002). Electron Microscopy of Walker-type filaments, on the other hand, are significantly shorter (<100nm), and show a strong tendency to bundle together with branched or frayed ends (Barillà et al., 2005; Bouet et al., 2007b; Suefuji et al., 2002). (2) In vivo fluorescence microscopy clearly shows ParM forming long, linear filaments that traverse the longitudinal axis of a bacterial cell (Moller-Jensen et al., 2002). Although several Walker-type ATPases form spiral-like structures in vivo, I hesitate in calling them self-supporting filaments as they bear no similarities to the discrete and linear filaments formed by ParM (Ebersbach and Gerdes, 2001;
Garner et al., 2004; Marston and Errington, 1999; Quisel et al., 1999). (3) In addition to nucleoid colocalization, several Walker-type partition ATPases show an affinity to non-specific DNA in vitro (Bouet et al., 2007b; Castaing et al., 2008; Leonard et al., 2005a), supporting a role for the nucleoid in the partition process. ParM shows no such association, and can partition plasmids in the absence of a nucleoid (Campbell and Mullins, 2007). (4) ParM polymerization is self-supporting in the presence of ATP or ATPγS, and is stimulated by the partition complex (Garner et al., 2004). For Walker-type ATPases, the component requirements for polymerization vary widely from ParM and from each other. Together, I find it unlikely that the filaments formed by Walker-type partition ATPases are self-supporting cables that physically and bidirectionally push plasmids apart. In Chapter 4, I have provided an alternative model that describes how dynamic filament bundles of ParA can associate with the ParB-parS partition complex to control its movement and localization over a nucleoid track.

A New Model for Incompatibility

Copies of the same plasmid are partitioned and stabilized as a result of the actions of their partition system. If an otherwise different plasmid with the same partition system is introduced, the plasmids destabilize each other and are termed incompatible (Fig. 1-9). Plasmid partition and incompatibility are consequences of the same mechanism; therefore, any model that describes one should explain the other.

It has generally been assumed that sister plasmids are paired via their partition complexes, and segregation begins with active splitting of the pair by ParA. A paired configuration orients each plasmid copy relative to the other; helping to ensure that they part bidirectionally. Perhaps the most robust model to explain incompatibility is based on the idea that partition complexes of a given specificity pair with each other, regardless of the replicon on which they are situated (Fig. 1-9). This would allow the formation of mixed as well as sister pairs. Mixed pairs are oriented in either direction with equal probability so that the partition apparatus segregates their plasmids randomly and therefore plasmid loss ensues.

As noted previously, there is good evidence that partition complexes can pair plasmids (see Chapter 1). This event has maintained its status as the necessary step for the ‘mixed-pairs’ model of incompatibility (Funnell, 2005). However, several questions are left unanswered by this model. For example, how is the proximity of sister copies overcome to allow access to a
competing partition complex? Another possible difficulty with this model is the observation that a plasmid carrying two identical par loci is stable (Austin, 1984). Multiple partition sites in a single plasmid might be expected to pair more readily in cis than with a copy on another molecule, resulting in a block to inter-plasmid pairing and destabilization of the plasmid. Moreover, several naturally occurring and highly stable plasmids have dispersed partition sites, such as N15 and RK2. If forming mixed pairs is the only mechanism behind incompatibility, then there is an unknown feature of the partition mechanism that prefers inter-plasmid pairs rather than intra-plasmid pairs as substrates for partition.

In an attempt to visualize incompatibility, Ebersbach et al. (2005) used fluorescence tagging to visualize competing plasmids and found that these plasmids did not form mixed pairs, but were uniformly localized over the entire length of the nucleoid region. This result fits with my ‘repulsion’ model where partition complexes interact to repel each other rather than to cohere (Fig. 5-1). In this case, incompatibility results from randomized positioning over the nucleoid rather than from random pairing. As a consequence, the repulsion would eventually result in cells having all copies of a given plasmid in one cell-half at the moment of division so that the other half becomes a negative segregant for this plasmid. This feature of my model has also been supported by fluorescence microscopy studies, as a substantial fraction of the population was seen to display such asymmetric distributions (Ebersbach et al., 2005).

My ‘repulsion’ model in no way weakens the assertion that partition-site-based incompatibility is a direct reflection of the partition mechanism. However, it does shift the focus away from the partition site itself as a component of partition complexes which pair plasmids, and towards the active segregation event mediated by the dynamic interplay between ParA and the ParB-parS partition complex. In other words, incompatibility is based not on direct interactions between parS-bound ParB dimers, but on the influence of ParA on the entire partition complex. Implicit in my model is the idea that plasmid copies are continually repositioning themselves in response to the presence of any other plasmids with the same partition complex (Fig. 5-1). A significant difference from the mixed-pairs model is that in my repulsion model, non-central positioning of incompatible plasmids can cause their replicates to segregate entirely within a cell-half without ever having to cross mid-cell (Fig. 5-1). Therefore, a prerequisite assumption is that plasmid replication can occur anywhere in the cell (Niki and
Hiraga, 1997), as partition is expected to follow it closely. The repulsion model does not entirely negate mixed pairing, and the two should not be considered mutually exclusive.

**Impact on the Field of Plasmid Partition**

The plasmid partition field has been focused on identifying the host-factor(s) required to hold bacterial plasmids in position. My thesis has been able to address this question in addition to another that has received little attention: Are partition systems required merely to deliver the plasmid to their positions, or must they also hold them in place without external influences once partitioned? One study suggested that P1 plasmids are maintained at mid-cell only temporarily, and then slowly 'drift' in the cytoplasm (Li and Austin, 2002). If drift does occur, it must be restricted in some manner, or else positional information would be lost. My thesis suggests plasmid positioning is not a function of the number of host-encoded tethers inside the cell, but rather a function of the relative concentrations of ParA on the nucleoid and ParB at the partition complex, and ultimately the interplay between these two nucleoprotein complexes. The model I propose is one where the P1 plasmid tracks along the nucleoid, and is corralled into intracellular regions that are dictated by continuous interaction between nucleoid-associated ParA and the ParB-parS partition complex. This interaction sets up a dynamic equilibrium between ParA and ParB that drives plasmid movement, and the equidistant positioning of plasmids over the nucleoid. When ParB concentrations are elevated relative to ParA, plasmids are forced to rejoin with the same partition complex more regularly. When ParA concentrations are elevated relative to ParB, plasmids are repulsed from one another more efficiently. Ultimately, my work and ensuing model will promote several changes to the way the field thinks about plasmid partition. First, plasmids are always moving, and should not be considered as ‘fixed’ to specific intracellular locations in the cell. Second, the partition reaction is not a one shot deal. Rather ParA and ParB continually work throughout the cell-cycle to ensure plasmid copies are localized over the bacterial nucleoid, but at the same time away from each other. Finally, although plasmids can occasionally bump into each other and transiently pair, the main focus of the partition system is to continuously repulse plasmids encoding the same partition system.
In conclusion, my thesis work provides evidence towards a model that negates the requirement of a host-protein tether, or a plasmid-pairing step, and provides a more parsimonious explanation to the phenomenon of incompatibility and P1 plasmid partition.
FUTURE DIRECTIONS

Forming the higher-order ParB-parS partition complex

I have been able to characterize in detail how a dimer of ParB binds parS. However, it is unclear how additional ParB dimers join to form the functional partition complex. Can more than one ParB dimer site-specifically contact the parS site? With a wild-type parS site, ParB multimers can be resolved and quantified using gel-shifts. By altering the experimental conditions I used in studying the initial ParB dimer interaction with parS, it is possible to use the mutant parS sites in gel-shift assays to determine if unbound A- and B-boxes contribute to the assembly of the higher-order partition complex (Bouet et al., 2000). Only the parS mutants that were bound by ParB at high-affinity would be useful in this study: parS[b1], parS[a2], parS[a3], parS[b2], parS[b1,a2], parS[b1,b2], parS[a3,b2], parS[b1,a3,b2] (refer to Table 2-2). This is to be sure that any defects in higher-order complex assembly are not an indirect consequence of defects in the initial ParB-parS interaction. Higher-order complex formation with parS[b1,a3,b2] would be a particularly interesting result because it has only two motifs for site-specific contact. If a tetramer of ParB were to bind this site it would suggest that a single dimer binding to parS is sufficient in nucleating the formation of a higher-order partition complex and subsequent spreading from this nucleation core can be achieved through non-specific interactions with flanking DNA and/or ParB-ParB interaction. Alternatively, if all parS variants do not support a higher-order complex it would indicate that additional site-specific contacts within parS are needed to load additional ParB dimers onto and around parS.

ParB effects on conformation and DNA-binding activity of ParA

A question that still remains concerns the effect of ParB on ParA*-ATP, the ATP-induced conformation of ParA identified in Chapter 3. ParB greatly stimulates ParA ATPase activity in the presence of DNA (Davis et al., 1992). Thus, it is easy to imagine that ParB-stimulated ATP hydrolysis by ParA leads to ParA dissociation from DNA. Could this be the only major impact of ParB? I believe that ParB plays a more intimate role in controlling ParA conformational changes. Preliminary ParA tryptophan fluorescence assays support the idea that ParB not only accelerates ParA ATP hydrolysis and conformational change back to the non-DNA binding state, but it also helps in the conformational change toward the DNA binding state (Vecchiarelli and Mizuuchi, unpublished data). However, changes in tryptophan fluorescence
signal by ParA are difficult to quantify as they are convoluted by the tryptophan fluorescence of ParB. Fortunately, ParB only has one tryptophan residue, which is non-conserved. I have constructed, characterized and purified a fully functional, tryptophan-free ParB variant (ParB[W282Y]) (Fig. 5-2).

With these additional considerations, it is likely that the model presented in this thesis will only form a basis to the intricate interactions at play. I believe the reaction mechanism is almost certainly more complex than a simple reaction-diffusion model describable by a small set of equations, as exemplified by the complex behavior of the MinD/E system recently observed in a cell free system (Ivanov & Mizuuchi, 2009).

An in vitro ‘systems biology’ approach to studying plasmid partition

This thesis has contributed to identifying and characterizing the nucleoprotein complexes involved in the P1 partition reaction and has ultimately provided clues to dissecting the mechanism behind DNA segregation in bacteria. Now that these complexes have been defined, it is important to study them simultaneously to fully understand how the system behavior generates plasmid movement and localization. In other words, an in vitro “systems-biology” approach using TIRFM would be powerful in dissecting the partition mechanism. From the model I propose in this thesis, the system would consist of a static DNA-matrix that would represent the nucleoid, and freely diffusible ATP, ParA, ParB and plasmid DNA (parS\(^{+}\)). To form a DNA-matrix, the flow cell surface will be coated with a DNA blanket. Preliminary evidence shows this is possible at a variety of DNA densities (Han, and Mizuuchi unpublished data). The buffer that passes through the flow cell would contain ATP, plasmid DNA (parS\(^{+}\)), ParA-GFP, and a single-cysteine ParB variant covalently modified with a thiol-reactive fluor, such as TMR (refer to Appendix). Prior to this thesis work, a functional and fluorescent version of ParB was not available (Li and Austin, 2002). The plasmid DNA could also be fluorescently labeled in these experiments (Han and Mizuuchi, 2009). In theory, the interplay of the fluorescently labeled system components over a DNA-coated flow cell can be visualized using TIRFM. According to my model, I would expect ParA-GFP to coat the DNA-matrix in a dynamic and ATP-dependent manner. ParB would cluster over parS forming foci. These foci would only appear if the complexes were tethered to the DNA-blanket via a ParA-GFP interaction, trapping them within the evanescent illumination of the microscopy setup. It is my
hope that visualizing the ParB-parS complexes on a DNA-blanket coated with dynamically associated ParA-GFP will yield insight into how the entire system can pair plasmids, dissociate them, and move them along a nucleoid track.
Figure 5-1. Repulsion model that explains incompatibility. (A) A snapshot of competing plasmids (black and purple squiggles) tracking along the nucleoid bidirectionally as a result of the low ParA density at mid-cell. On average, the plasmids would be localized at the quarter positions. (B) The plasmids replicate, and the sisters dissociate as a result of their repulsive interactions. (C) A snapshot of how plasmid repulsion limits plasmid movement as a result of small changes in ParA flux. Repulsion limits the movement of the competing plasmids to their respective cell-halves, thus leading to a loss of stability for both plasmids. Arrows inside the cell illustrate ParA flux and how it is influenced by ParA ATP-cycling. Arrows outside the cell illustrate plasmid movement.
### A

<table>
<thead>
<tr>
<th>Plasmid Providing ParB (parB allele)</th>
<th>miniP1 plasmid (pBEF246) retention after overnight growth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322 (no ParB)</td>
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</tr>
<tr>
<td>pEF5 (parB⁺)</td>
<td>95 ± 6</td>
</tr>
<tr>
<td>pAV13 (parB[W282Y])</td>
<td>94 ± 5</td>
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</table>

### B

![Graph showing the intensity (A.U.) of ParB and ParB[W282Y] over wavelength (nm)]

### C

![Graph showing ATP hydrolyzed (pmoles) against ParB (µM)]
**Figure 5-2. Properties of ParB[W282Y].** (A) Stability of miniP1 plasmid (pBEF246) with ParB[W282Y]. ‘Retention’ represents the ratio of the frequency of cells with miniP1 after overnight growth divided by the frequency of cells with miniP1 at the start, expressed as a percentage. The data represent the average of at least three independent experiments. (B) ParB[W282Y] does not have tryptophan fluorescence. 1 μM ParB[W282Y] (red) was excited at 295nm and fluorescence emission was measured across the wavelength range indicated. (C) Stimulatory activity of ParB[W282Y] compared to wild-type ParB. ParA (2 μM), 1mM [$\gamma$-32P]-ATP and 0.1 mg of sonicated-DNA/ml was incubated with wild-type ParB or ParB[W282Y] at the concentrations indicated for 1.5 hours at 23°C. 32P i production was quantified as described in Chapter 3.
APPENDIX

CONSTRUCTION, PURIFICATION AND MODIFICATION OF SINGLE-CYSTEINE ParB VARIANTS

I performed all experiments in this chapter.
In this chapter, I create a spectrum of single-cysteine ParB variants that can be modified with thiol-reactive probes and used in future study of the ParA-ParB and ParB-ParB interactions essential for the partition reaction.

There is only structural data for the C-terminal half of ParB. N-terminal organization and function is still unclear, however domain analyses and bioinformatics have provided clues. The extreme N-terminus of ParB interacts with ParA, although the limits of this domain have not been established. Much of the N-terminal half of ParB has been implicated in its oligomerization activity (Radnedge et al., 1998; Surtees and Funnell, 1999). It is unclear how ParB physically interacts with ParA and with itself. Does the ParB self-association domain overlap with the interaction interface for ParA? If so, could a competition for binding provide a mechanistic switch from ParB-ParB mediated pairing and ParA-ParB mediated partition? Here, I performed site-specific mutagenesis on the parB gene, inserting single cysteine residues along the N-terminal half of ParB that can be covalently modified with an assortment of thiol-reactive interfering reagents and cross-linkers. These probes will be useful in mapping the protein interaction interfaces in ParB using a variety of in vitro assays that are described in the discussion of this chapter.

Similar strategies have already been successful in mapping the interaction between MutH and MutL (Toedt et al., 2003) and the sonic hedgehog-receptor interaction (Pepinsky and Garber, 2000). It has advantages over other mapping strategies, such as alanine scanning mutagenesis, not relying on the exact identification of hotspots in binding interfaces (DeLano, 2002) and should be applicable for the mapping the ParB and ParA interaction interfaces in the N-terminal half of ParB.

ParB is an excellent candidate for this approach as it has only one cysteine, which is not essential and can be substituted (see below). Twelve single-cysteine ParB variants were created, nine of which support P1 plasmid stability at levels comparable to that supported by wild-type ParB. Three single-cysteine ParB mutants, shown to be functional in vivo were purified, and were also functional in vitro even after covalent modification with several fluorescent and non-fluorescent probes. The modifiable variants created here will be useful tools for a variety of in vitro assays that will aid in mapping and characterizing ParB interactions with DNA, other ParB molecules, and ParA.
EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids - The plasmids were constructed in *E. coli* strain DH5 (F\(^{-}\) endA1 hsdR17 (rK\(^{-}\) mK\(^{+}\)) supE44 thi-1 gyrA96 recA1). When the *parB* gene was under the P\(_{L}\) promoter from \(\lambda\), plasmids were maintained in MC1061 (\(\Delta(araA-leu)7697\ araD139\Delta(codB-lac)3=\Delta lac74\ galK16\ galE15\ mcrA0\ relA1\ rpsL150\ mcrB9999\ hsdR2\ \lambda^{-}\ F\)) and transformed into M5219 (F\(\lambda\)lacZam trpAam rpsL \(\lambda\)bio252, cl857, \(\Delta\)H1) for ParB over-expression.

Reagents – Synthetic oligodeoxynucleotides were purchased from Invitrogen Corp. Sources for other reagents were as follows: [\(\gamma\)-\(^{32}\)P]-ATP, Perkin Elmer; Ellman’s Reagent (DNTB), bovine serum albumin (BSA) and salmon sperm DNA, Sigma; restriction enzymes, T4 polynucleotide kinase, New England Biolabs; T4 polynucleotide kinase; N-ethyl maleimide (NEM), Fluorescein-5-maleimide (F5M), Tetramethylrhodamine-5-maleimide (TMR), Molecular Probes.

ParB Purification – ParB (Fraction V) was purified as described previously (Bouet and Funnell, 1999), except the cells were lysed by adding 0.2 mg/ml lysozyme in lysis buffer (29.1 \(\mu\)g/ml (NH\(_4\))\(^2\)SO\(_4\), 20 \(\mu\)M Spermidine, 10\(\mu\)M EDTA in Tris-sucrose) and incubated on ice for 45 minutes. The cells were then warmed at 37\(^{\circ}\)C with continuous swirling for 20 seconds and returned to ice for cooling; this was repeated five times and then spun at 15,000 rpm for 1 hour at 4\(^{\circ}\)C. Also, during FPLC purification, MonoS chromatography was replaced by HiTrap SPHP chromatography and the protein was eluted with a 150 mM to 1 M KCl gradient prior to gel filtration.

IHF was purified as described in Chapter 2.

Site-directed Mutagenesis - Site-directed mutagenesis was carried out using the Quickchange protocol (Stratagene) (Kirsch and Joly, 1998) first using the plasmid pEF5 (encodes *parB*) as a template to create pAV10; a plasmid encoding a ParB variant that has the native cysteine at position-214 substituted out for a serine. pAV10 was then used as a template for the introduction of single-cysteine residues in ParB. The primers used for PCR and their corresponding mutation in ParB are shown in Table A-1. *Escherichia coli* XL1-blue supercompetent cells were transformed with the PCR product. Marker positive clones were inoculated in LB medium containing ampicillin for overnight growth. Plasmid DNA was isolated using the QIAprap Spin Miniprep (Qiagen). BglII-XcmI fragments containing the mutant *parB* genes were sub-cloned back into pEF5 creating the plasmids listed in Table A-1. The plasmid *parB* sequences were
confirmed before and after sub-cloning by DNA sequencing at York University (Toronto) and/or Macrogen Inc. (South Korea).

**Plasmid Stability Assays** – A plasmid encoding wild-type ParA along with a ParB variant (Table A-1) was introduced into *E.coli* DH5 cells containing a miniP1 plasmid (pBEF246 - ΔparAB, parS†). A colony was picked from an LB agar plate containing ampicillin and chloramphenicol (miniP1 marker), diluted into LB with ampicillin, and grown for approximately 15 generations (overnight at 37 °C). Samples were taken from the beginning and the end of the growth period and plated for single colonies on LB agar plates with ampicillin. Resulting colonies were transferred using toothpicks onto LB plates with chloramphenicol to monitor the presence of the miniP1 plasmid.

**Thiol Quantification** – Dialysis using gel-filtration buffer (from ParB purification) free of DTT was used to remove DTT from the ParB preps. In a 0.7 ml reaction volume, 5μM of each ParB variant was mixed with 140 μL pH 8 Buffer (100mM Boric Acid, 0.2mM EDTA and NaOH used to pH) and 0.2 mM Ellman’s reagent. The reaction was incubated at 23°C for 5 min and the A<sub>412</sub> was measured. The molar ratio of thiol groups to ParB monomers was calculated using the equation below:

\[
\text{mol SH} = \frac{\text{Reaction Volume} \times (A_{412(\text{reaction})} - A_{412(\text{no protein blank})} - A_{412(\text{Ellman’s reagent blank})})}{(\Delta \varepsilon_{412} \times l)W}
\]

Where \(\Delta \varepsilon_{412}\) is the change in the molar extinction coefficient of Ellman’s reagent (14150/M*cm) and l is the cuvette path length.

**ParB labeling** - Dialysis using gel-filtration buffer (from ParB purification) free of DTT was used to remove DTT from the ParB preps. When fixed in concentration, 250 μM NEM, F5M or TMR was incubated with 50 μM ParB variant in a final reaction volume of 40 μL for 30 min at 23°C. The labeling reaction was quenched with 10 mM DTT, free-label was removed using a G50 spin-column, and the labeled protein was concentrated using a YM-30 microcon concentration column. Labeling efficiency was monitored by running a portion of the mixture on SDS-PAGE, and visualizing the ratio of fluorescence associated with the ParB-sized band to free-label. Fluorescence was visualized by scanning the gel with a Typhoon 9400 –Variable mode image. For F5M labeling, the gels were exposed to a 488nm laser and fluorescence emission was visualized using a 520 nm band-pass filter. For TMR labeling, the gels were exposed to a 532 nm laser and fluorescence emission was visualized using a 610 nm band-pass
filter.

**Gel Mobility Shift Assays** - As described in Chapter 2 except the DNA substrate was created by hybridizing complementary 80 bp oligos encoding the parS site (Upper strand – CTTTCGCCA TTCAATTTTCACCTATTAACTGACTGTTTTTAAAGTAAATTACTCTAAAAATTCAAGGT GAAATCGCCACGA; Lower strand - GAAAGCGGTA AGTTTAAAGTGATAATTGAC TGACAAAAAT TTCATTTAAT GAGATTTTAA AGTTCCACTT TAGCGGTGCT). 100 pmoles of each oligo were mixed and hybridized by ramping the temperature of the sample down from 95°C to 45°C; dropping 10°C at 5 min intervals. DNA-duplexes were then labeled at their 5’-ends with [$\gamma$-32P]-ATP and T4 polynucleotide kinase, and purified from contaminants over a G50 spin column.

**ATPase Assays** – As described in Chapter 4.
RESULTS

Strategy for site-specific ParB mutagenesis

First, I generated a cysteine-free variant of ParB by changing the single non-conserved Cysteine-214 to Serine. I tested the biological activity of each mutant, including ParB[C214S], in vivo using a plasmid retention assay. In this assay, a plasmid encoding wild-type ParA along with a ParB variant was introduced into E. coli cells containing a \( \Delta \)parAB, parS\(^{+}\) miniP1 plasmid. Cells were grown in the absence of selection for miniP1 for approximately 15 generations and then tested for the presence of miniP1 (see experimental directions). ParB[C214S] was able to substitute for wild-type ParB in vivo without a loss in P1 plasmid stability (Table A-2). Next, I strategically identified serine and threonine residues that were potentially surface exposed, and could therefore act as targets for modification. The following bioinformatic tools were used in choosing the residue positions: (1) an alignment of closely related plasmid-ParB sequences (Fig. A-1A), (2) an alignment of P1 ParB with more distantly related plasmid-SopB and chromosomal-ParB sequences (Fig. A-1B), and finally (3) the structural information from a chromosomal ParB, SpoOJ of *Thermus thermophilus* (Leonard et al., 2005a) (Fig. A-1C). Twelve strategically positioned sites were selected in the cysteine-free ParB background. In an effort to preserve wild-type function, none of the residues chosen were conserved in plasmid- or chromosomal-ParBs, with one exception (Fig. A-1B). A semi-conserved Serine (Ser-109) within the conserved B-motif was also substituted as a previous mutation to Alanine in this position was shown to be functional in vivo (Guerin & Funnell, unpublished data). The residues replaced by cysteine are indicated on the primary sequence of P1 ParB in Figure A-1A and B. Together the mutated positions cover the entire N-terminal half of ParB. Because there is no crystal structure of the N-terminal half of P1 ParB, the substituted residue positions were superimposed on the SpoOJ structure from *T. thermophilus* (Leonard et al., 2005a) to show that the residues are potentially surface exposed (Figure A-1C). To maintain wild-type DNA-binding activity, the C-terminal half of ParB, which is critical for site-specific binding to parS, was not altered (Schumacher and Funnell, 2005).
**Purified ParB variants are functional both in vivo and in vitro, and have solvent accessible cysteine residues**

A prerequisite to purifying these ParB variants for modification is that the mutation will not affect the function of the protein. I first tested their activity *in vivo* using the plasmid retention assay described above. Of the twelve single-cysteine ParB variants, nine were fully functional, and three (ParB[T39C,C214S], ParB[S72C,C214S], and ParB[S109C,C214S]) showed only small reductions in activity (Table A-2). In general, all ParB variants were at least somewhat functional *in vivo* (compare to Δpar, Table A-2). All ParB variants must therefore be able to perform the functions necessary for the partition reaction, albeit with reduced efficiency in a few cases.

For these ParB variants to be useful in biochemical studies, they must be functional following purification and their thiol groups must be solvent accessible for modification. In addition to the cysteine-free ParB variant (ParB[C214S]), three of the twelve single-cysteine ParB variants were chosen for purification: ParB[S19C,C214S], ParB[S32C,C214S] and ParB[S80C,C214S]. These mutant proteins were chosen because they were fully functional *in vivo*, and together, they span a large region of the N-terminus of ParB. I followed the standard purification protocol for wild-type ParB (Bouet and Funnell, 1999). All four ParB variants behaved identically to wild-type ParB throughout the purification process and were purified to over 95% homogeneity, as judged by Coomassie Blue-stained SDS-polyacrylamide gels (data not shown). When incubated with ParA, all variants stimulated ParA ATPase activity to a similar extent as wild-type ParB (Fig. A-2, unmodified). All purified ParB variants alone had negligible contaminating ATPase activity (Fig. A-2, no ParA).

Next I tested the accessibility of the thiol groups for modification. Ellman’s reagent is used for quantification of thiol-accessibility. The reagent reacts with thiol-containing compounds to produce a yellow color that can be quantified spectrophotometrically (Haugland, 2002). The molar ratio of thiol groups to ParB monomers was calculated to be approximately 1:1 for all single-cysteine ParB variants (Table A-3), meaning that the thiol group of each mutant protein is accessible to solvent. As expected, ParB[C214S] showed negligible reactivity. The results show that the purified single-cysteine ParB variants have accessible thiol groups that can be site-specifically and quantifiably modified.
Site-specific modification of single-cysteine ParB variants

The above results predict that the ParB variants should label efficiently with thiol reactive probes. I modified the ParB variants with thiol-specific (maleimide-derivatized) reagents ranging in radius of action, which is the contact distance in all directions from its point of covalent attachment (Fig. A-3). N-ethylmaleimide (NEM) has a radius of less than 0.5 nm, and Fluorescein-5-maleimide (F5M) and Tetramethylrhodamine-5-maleimide (TMR) both have a radius of action of ~1 nm. The extent of modification with varying excess of F5M and TMR was monitored by SDS-PAGE and fluorescence imaging (see experimental directions) (Fig A-4A). When quantifying the fluorescence intensity it was found that a 5-fold excess of dye was sufficient for maximal labeling of the single-cysteine ParB variants while at the same time keeping the non-specific labeling of ParB[C214S] to a minimum (Fig. A-4B). The modified proteins were purified from excess label using G50 spin columns. Free F5M was removed more efficiently than free TMR, but in both cases > 90% of the fluorescence signal intensity was from the labeled ParB-variant (Fig. A-4C). NEM is not fluorescent, therefore to show efficient NEM modification, I competed NEM modified ParB-variants with a 10-fold excess of F5M to show that it could not be modified further (data not shown). Overall, I developed a ParB labeling protocol that allowed for thiol-specific modification, low levels of non-specific labeling and efficient removal of free label.

Modified ParB variants are fully functional in vitro

The final requirement for these ParB-variants to be considered useful tools is that they must maintain wild-type function following the modification. Using ATPase assays, I determined if these probes of variable size and charge affected ParB function in stimulating the ATPase activity of ParA (Fig. A-2). All ParB-variants stimulated ParA ATPase activity at levels identical to wild-type ParB when unmodified, or modified with NEM, F5M or TMR. Interestingly, when wild-type ParB was modified at cysteine-214 with TMR or F5M, its stimulatory activity fell dramatically. NEM modification of wild-type ParB did not have the same effect suggesting that the greater radius of action of F5M and TMR was responsible for abrogating ParB function.

The TMR- and F5M-modified ParB-variants were also tested for site-specific DNA-binding to parS. 80-bp DNA-duplexes encoding parS were radiolabelled and incubated with
IHF and increasing concentration of modified ParB variant. The resulting complexes were separated using gel electrophoresis (Fig. A-5). The single-cysteine substitution and the subsequent modification with F5M (Fig. A-5A) or TMR (Fig. A-5B) had no effect on complex formation with any of the ParB-variants when compared to unmodified, wild-type ParB. This step-by-step characterization has provided good evidence that these ParB variants are excellent candidates for modification with a variety of different probes without altering ParB function.
DISCUSSION

Guided by the structure of SpoOJ, and sequence alignments of plasmid- and chromosomal-ParB proteins, I generated 12 variants of ParB containing a single cysteine residue located at strategic positions. Nine variants were fully functional in vivo, and three displayed subtle defects in partition function (Table A-2). ParB[S109C,C214S] showed the most significant decrease in activity. The substitution is located in the conserved B-motif, further showing the importance of this motif for ParB function. Three ParB-variants (ParB[S19C,C214S], ParB[S38C,C214S] and ParB[S80C,C214S]) that were functional in vivo were purified, and their cysteine residues were shown to be accessible for modification. All three variants stimulated ParA ATPase activity, bound parS site-specifically, and oligomerized at parS before and after modification with all thiol-reactive probes tested (Fig. A-2, A-5). Overall, the amino acid exchanges and modifications do not significantly interfere with the partition functions of ParB, and therefore my selection of positions to be modified was reasonable. The modified ParB variants will be vital in mapping the interaction domains of the N-terminal half of ParB using cross-linking reagents and interfering probes. Also, fluorescent probes will be useful in dissecting the protein-protein and protein-DNA interactions required by ParB for partition. The rationale of these future experiments is described below.

Mapping the ParA- and ParB-binding interfaces on ParB - The partition complex forms presumably by ParB spreading across DNA flanking parS (Rodionov and Yarmolinsky, 2004) and by ParB multimerization through its N-terminus (Surtees and Funnell, 1999). Domain swapping experiments suggest that ParA also interacts with the partition complex through interactions with the N-terminus of ParB; although the domain limits have not been established (Radnedge et al., 1998). What is the biochemical nature of the ParA-ParB and ParB-ParB interaction? How does nucleotide binding and hydrolysis modulate these interactions?

Identification of the specific N-terminal ParB residues involved in ParA-ParB and/or ParB-ParB interaction is fundamental to understanding the temporal and spatial switch that controls plasmid pairing and dissociation. The functional, single-cysteine ParB mutants will be valuable in mapping ParA-ParB interaction sites and ParB self-association sites using an interference strategy (Creighton and Freedman, 1993). For example, by introducing more or less bulky modifications at the single-cysteine positions, it is possible to determine their interference with ParB stimulation of ParA ATPase activity and ParB oligomerization. Domain analysis of ParB
suggests that ParB variants modified near the extreme N-terminus would disrupt interaction with ParA. If the interfering probe is in a region necessary for ParB-ParB interaction, oligomerization would be prevented, and gel-shifts would show a decrease, or absence of, higher-order ParB-parS complexes. The biochemical assays suggest that all three purified ParB variants modified with NEM (radius of action <0.5nm) or the larger probes, F5M or TMR (radius of action ~1nm), do not alter ParA-ParB or ParB-ParB interaction (Fig. A-2 and A-5). However, it must be appreciated that a number of these ParB variants must be tested and even if interference is not achieved with a particular variant, it does not mean that the entire region is not involved in the particular interaction being studied. Alternatively, if interference is found with particular modified variant, further structural studies such as limited tryptic digestion or circular dichroism must be performed to confirm that the loss of activity is not due to a general loss in protein folding and stability.

**Identifying the requirements for ParA-ParB Interaction** - Can ParA and ParB only interact on DNA when ParA is ATP-bound or can they interact off the DNA-matrix? A direct physical association between ParA and ParB has never been identified in vitro. As a parallel approach to interference, a variety of thiol reactive cross-linking reagents can be used to capture protein-protein complexes by covalently attaching the modified single-cysteine ParB variants to ParA. The rapid reactivity of a variety of heterobifunctional cross-linkers allows even transient interactions to be frozen in a complex (Pierce, 2004). For example, it is possible to examine the requirements for ParA-ParB interaction by incubating modified ParB (bait) and ParA (prey) proteins in the presence and absence of parS DNA and/or adenine nucleotide. Following cross-linking, the complexes can be resolved on an SDS-polyacrylamide gel. If DNA is a requirement, more ParA-ParB complexes will form in the presence of DNA than in its absence. Similarly, if ATP is a required cofactor, then the presence of ATP in samples containing modified ParB and wild-type ParA should increase ParA-ParB cross-linking. Based on the data presented here, I predict that DNA, along with ATP or ATPγS, would facilitate cross-linking and ADP or no nucleotide would show little or no ParA-ParB cross-linking.
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<tr>
<td>pAV19</td>
<td>GCAAATAGGTGTTAG</td>
<td>ACA to TGC</td>
<td>ParB[T100C,C214S]</td>
</tr>
<tr>
<td></td>
<td>AGGGGAAATTGGATAGTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CATCCTACAATTTCCTTGCCGGAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CATCCTACAATTTCCTTGCCGGAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAV20</td>
<td>ATTGAATTTTGGCTGTTG</td>
<td>TCC to TGC</td>
<td>ParB[S109C,C214S]</td>
</tr>
<tr>
<td></td>
<td>CCCGGCGAGCTGTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AGCTCAGCTGACGCGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAV21</td>
<td>GCGATTTTGGCAAT</td>
<td>TCT to TGT</td>
<td>ParB[S38C,C214S]</td>
</tr>
<tr>
<td></td>
<td>GCTGCTGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AATACCTGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAV22</td>
<td>GCGGATTTGATAGT</td>
<td>TCT to TGT</td>
<td>ParB[S57C,C214S]</td>
</tr>
<tr>
<td></td>
<td>ACTGTCGACGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AGCTGCTGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AATACCTGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAV23</td>
<td>TGTGCACTCTGGGAAA</td>
<td>ACT to TGT</td>
<td>ParB[S80C,C214S]</td>
</tr>
<tr>
<td></td>
<td>CATGCTGAATTGGCAATTGAATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCTCAGCTGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCTCAGCTGAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The bold bases highlight the codon substituted into the ParB sequence.
The plasmid pEF5 encodes the parB alleles

\[ \text{miniP1} = \text{pBEF246}; \parS^+, \parA^+, \parB^- \]

Cysteineless-ParB variant
ParB variants chosen for purification are highlighted in bold.

Table A-2. Stability of miniP1 plasmids with ParB variants

<table>
<thead>
<tr>
<th>ParB Variant(^a)</th>
<th>% cells that have miniP1(^b) after 15 generations</th>
</tr>
</thead>
<tbody>
<tr>
<td>ParB (wild-type)</td>
<td>&gt;99</td>
</tr>
<tr>
<td>ParB[C214S](^c)</td>
<td>&gt;99</td>
</tr>
<tr>
<td>ParB[S19C, C214S]</td>
<td>&gt;99</td>
</tr>
<tr>
<td>ParB[S24C, C214S]</td>
<td>&gt;99</td>
</tr>
<tr>
<td>ParB[S27C, C214S]</td>
<td>&gt;99</td>
</tr>
<tr>
<td>ParB[S38C, C214S]</td>
<td>&gt;99</td>
</tr>
<tr>
<td>ParB[T39C, C214S]</td>
<td>93</td>
</tr>
<tr>
<td>ParB[S57C, C214S]</td>
<td>&gt;99</td>
</tr>
<tr>
<td>ParB[S72C, C214S]</td>
<td>88</td>
</tr>
<tr>
<td>ParB[S80C, C214S]</td>
<td>&gt;99</td>
</tr>
<tr>
<td>ParB[T100C, C214S]</td>
<td>&gt;99</td>
</tr>
<tr>
<td>ParB[S109C, C214S]</td>
<td>75</td>
</tr>
<tr>
<td>ParB[S114C, C214S]</td>
<td>&gt;99</td>
</tr>
<tr>
<td>ParB[S132C, C214S]</td>
<td>97</td>
</tr>
<tr>
<td>No ParB</td>
<td>30 (par(^-))</td>
</tr>
</tbody>
</table>

\(^a\) The plasmid pEF5 encodes the parB alleles
\(^b\) miniP1 = pBEF246; \parS^+, \parA^+, \parB^- 
\(^c\) Cysteineless-ParB variant

ParB variants chosen for purification are highlighted in bold.
Table A-3. Quantification of thiol-group accessibility using Ellman’s Reagent

<table>
<thead>
<tr>
<th>ParB Variant</th>
<th>mol SH/mol ParB*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ParB (wild-type)</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>ParB[C214S]</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>ParB[S19C,C214S]</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>ParB[S38C,C214S]</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>ParB[S80C,C214S]</td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>

* The molar ratio of accessible thiol-groups per monomer of ParB.
Figure A-1. Bioinformatic tools used in ParB mutagenesis strategy. (A) ClustalW sequence alignment of the N-terminus of plasmid-encoded ParBs with high sequence identity to P1 ParB (40 to 60% identity). Residues highlighted in red with grey background are absolutely conserved among all aligned plasmid-ParBs. Red arrows identify residues substituted for cysteine in P1 ParB. The black square highlights the conserved B-motif. (B) ClustalW sequence alignment of the N-terminus of plasmid- and chromosome-ParBs/SopBs. SopB proteins have sequence identities between 30 and 35% to P1 ParB. Chromosomally encoded ParBs have sequence identities below 15% with P1 ParB. Residues highlighted in red with yellow background are absolutely conserved among all aligned plasmid- and chromosomal-ParBs. (C) Phylogram of aligned ParB homologues. Homologues are grouped into three categories: Plasmid ParBs, Plasmid SopBs, and Chromosomal ParBs. (D) Structure of the SpoI1 dimer (residues 1-222) from T. thermophilus with the putative locations of the residues selected for cysteine substitution shown in grey. Mutations are only labeled on one monomer. Colored ribbons show the relative domain positions of each monomer.
Figure A-2. ParA ATPase stimulation by modified and unmodified single-cysteine ParB variants. 0.5 μg of each ParB-variant was incubated with 1mM [γ-32P]ATP in the absence (empty bars) or presence (coloured bars) of 1μg ParA. The black bar shows the ATPase activity of 1μg ParA alone.
Figure A-3. Thiol-specific reagents used in this study. (A) The smallest compound N-ethylmaleimide (NEM; MW = 125, Net charge = 0) has a radius of action below 0.5 nm, (B) followed by Fluorescein-5-maleimide (F5M; MW = 427, Net charge = -2) and (C) TetramethylRhodamine-5-maleimide (TMR; MW = 481, Net charge = 0) with a radius of action of approximately 1 nm.
Figure A-4. Efficiency of ParB labelling and label removal. (A) The extent of ParB-variant modification with varying excess of F5M (green) and TMR (red). Labelling was monitored by SDS-PAGE followed by fluorescence imaging. (B) Quantification of labelling efficiency with F5M (top graph) and TMR (bottom graph). (C) Fluorescence imaging of an SDS-PAGE gel before and after free label removal. ParB[S38C,C214S] was labelled with a 5-fold molar excess of F5M or TMR, and free-label was removed using a G50 spin-column. All other ParB variants behaved similarly.
Figure A-5. DNA-binding activity of modified ParB-variants to *parS*. The DNA substrate was a $^{32}$P-labelled 80-bp DNA-duplex encoding *parS*. The DNA was incubated with increasing amounts of F5M-modified (A) or TMR-modified (B) ParB-variant in the presence of IHF and analyzed by electrophoresis. The positions of free *parS*, the *parS*-IHF complex, the *parS*-IHF-ParB complex, and the *parS*-IHF-ParB$^2$ complex are indicated. Dimer concentration (in nM) of each ParB-variant is shown above each lane. Wild-type ParB was unmodified.
REFERENCES


Li, Y., and Austin, S. (2002). The P1 plasmid is segregated to daughter cells by a 'capture and ejection' mechanism coordinated with *Escherichia coli* cell division. Mol. Microbiol. 46, 63-74.


