BACILLUS SUBTILIS SOJ (PARA): THE INVOLVEMENT OF DNA BINDING AND DYNAMIC LOCALIZATION IN PLASMID PARTITIONING

BY

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Table of Contents

List of Figures

List of Tables

Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
DAPI	4'-6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GFP	green fluorescent protein
His-	hexahistidine affinity tag
HTH	helix turn helix
IPTG	isopropyl-β-thiogalactopyranoside
LB	Luria-Bertani medium
OD	optical density

Abstract

The ParA family of ATPases encompasses proteins with a wide variety of functions. The prototype of this family is the ParA ATPase from the P1 prophage plasmid, which, along with ParB and the *parS* binding site, is required for the faithful maintenance of this prophage in the episomal state. Many other low copy plasmids as well as bacterial chromosomes encode homologues of ParA. The function of these proteins in plasmid and chromosome segregation is still a mystery, yet their involvement in the process at some level is evident. Here, we have characterized a chromosomal ParA homologue, Soj from *Bacillus subtilis*, as it behaves and functions in plasmid partitioning in *Escherichia coli* and in the regulation of sporulation in *B. subtilis*.

We have studied the localization and behavior of this protein in the heterologous host, *E. coli*. In *B. subtilis*, GFP-fusions to Soj localize to the nucleoids and poles and undergo movement from one end of the cell to the other on the DNA. We have observed this movement in *E. coli* in the absence of any other *B. subtilis* elements besides Spo0J (the *B. subtilis* ParB homologue) and the *parS* binding site, and we have determined that Spo0J and *parS* are required for this movement. These requirements are the same as those for the maintenance of a low copy plasmid by Soj and Spo0J in *E. coli*, supporting the possibility that Soj movement is required for its function in plasmid partitioning.

We have also found that Soj binds non-specifically to DNA through conserved arginine residues that map to the surface of the structure of the Soj dimer. We identified these residues by alignment of Soj with other chromosomal ParA homologues and identification of conserved basic residues that mapped to the surface of the structure of

Thermus thermophilus Soj. We were able to identify two key arginines that are important for the interaction of Soj with DNA. By mutating these residues, we were able to assess the importance of DNA binding for two of the known functions of Soj: for its role in plasmid maintenance in *E. coli* and for its role in regulation of sporulation in *B. subtilis*. We find that Soj can not function in either of these activities if it can not bind to DNA. Our findings allowed us to generate a model for how the Soj dimer is oriented on the DNA, enabling us to envision how the binding of dimers is propagated along the length of the DNA.

We have extended the ParA/Soj DNA binding work by identifying conserved positively charged residues in ParA from the plasmid pB171 that may be important for its DNA binding. The mutation of these residues prevents ParA from binding to DNA *in vivo* or reduces its affinity for DNA in *in vitro* assays.

This work has drawn attention to the importance of a characteristic of ParA proteins that has previously been overlooked: non-specific DNA binding. As we have determined that DNA binding is essential for Soj function, models for plasmid partitioning can now include the DNA binding property of ParA, and the role of DNA binding in the functions of these proteins can be further explored.

Chapter I: Introduction

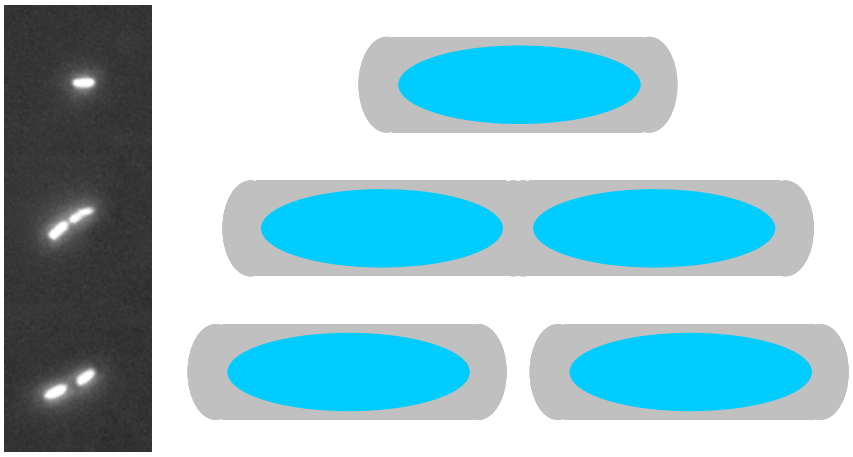
Chromosome and plasmid segregation

Bacterial cells must actively segregate their newly replicated chromosomes prior to division to ensure faithful inheritance of their genetic material. In eukaryotic cells, the act of chromosome segregation during mitosis and meiosis can be readily observed microscopically; however, in bacteria, the nature of the process of chromosome and plasmid segregation is not easily resolved by light or even electron microscopy. In rod shaped bacteria, segregating chromosomes start as one oblong, non-descript unit occupying most of the space within the cell and eventually morph into two oblong units, each occupying space in one half of an elongated pre-divisional cell (Figure 1A).

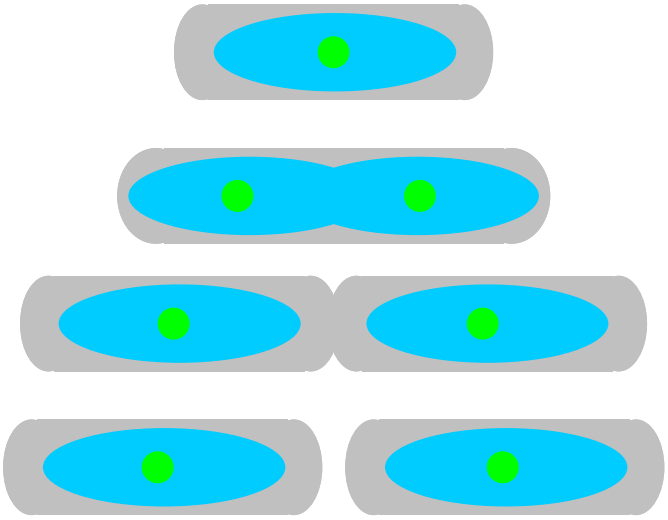
While episomal plasmids that are maintained at a high copy number can seemingly rely on random distribution for their maintenance, low copy number plasmids, like the bacterial chromosome, must be actively partitioned into each daughter cell prior to division. The players involved in and the mechanism driving this active partitioning are the focus of this work. The movement of labeled plasmids can be observed by fluorescence microscopy as segregation is occurring. The apparatus responsible for separation of plasmids by one type of plasmid partitioning system, the Type II partitioning system, has been documented recently and will be discussed briefly below. The Type I plasmid partitioning systems are the most common,

Figure 1. Cartoon of chromosome segregation (A) and plasmid segregation (B) in a rod shaped cell. (A) The chromosome is an oblong mass that occupies most of the space within a cell. As the cell elongates and replication of the chromosome occurs, the nucleoid becomes double lobed prior to separating into two distinct nucleoids. Upon division, each daughter cell receives a complete copy of the chromosome. On the left, *E. coli* cells stained with 4'-6-diamidino-2-phenylindole (DAPI) to label the nucleoid are shown in various steps of segregation as depicted in the cartoon cells on the right. (B) Plasmids are actively partitioned into each half of a cell prior to division in order to ensure that each daughter cell inherits at least one copy of the plasmid. The grey cylinder represents the cell; the blue oval represents the nucleoid; and the green circle represents the plasmid(s) present within the cell.

A



B

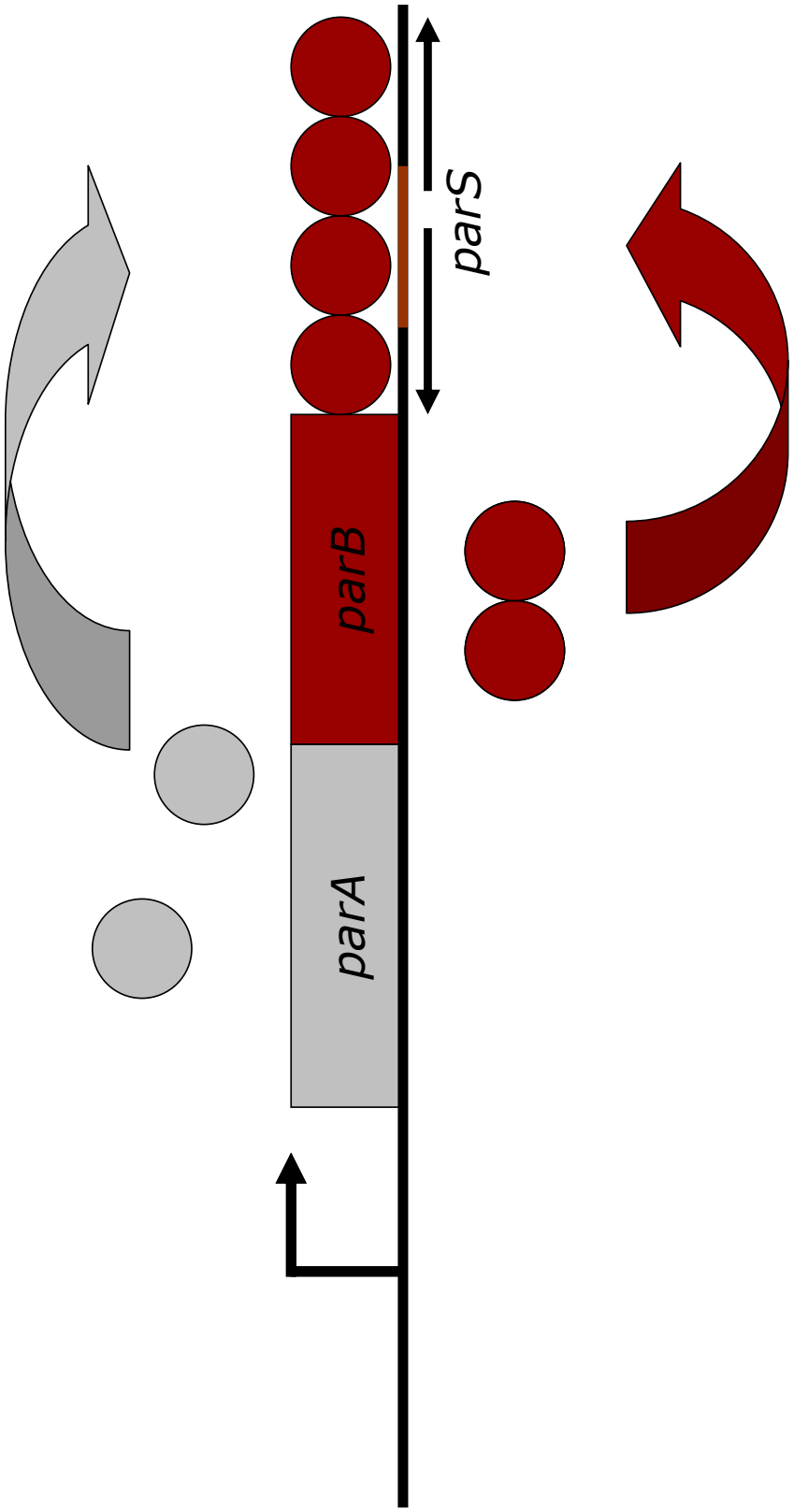


however, and partitioning by these systems occurs as the result of events that have not yet been directly observed. This work will primarily focus on Type I systems.

Plasmids can be localized by fluorescence *in situ* hybridization (FISH) (Niki and Hiraga, 1997) or by observing the position of fluorescent fusion proteins that are bound to specific sequences on the plasmid (LacI/*lacO*; TetR/*tetO*; ParB/*parS*) (Ebersbach *et al.*, 2005; Gordon *et al.*, 1997; Li and Austin, 2002). Labeled plasmids can be followed over time *in vivo*, or the distribution of foci in a population can be assessed in fixed cells by FISH. In either case, plasmids are typically clustered within the cell into a number of foci that is less than the measured copy number of the plasmids. Plasmid clusters are split at some point during the cell cycle prior to division so that each daughter cell receives at least one plasmid cluster (Figure 1B) (Ebersbach and Gerdes, 2005; Ghosh *et al.*, 2006; Pogliano, 2002). The timing of this partitioning event in the cell cycle is the subject of debate as it appears to vary with growth rate, and the visualization techniques currently being used to study plasmid localization may interfere with the timing of the normal partitioning reaction. The timing also may vary depending on the *par* system and plasmid.

While genes essential for chromosome segregation have yet to be identified on any bacterial chromosome, there are loci found on bacterial plasmids which have been shown to be essential for their faithful maintenance. These *par* (for *par*titioning) loci encode two proteins: an ATPase and a DNA binding protein which binds to a *cis*-acting site within or near the locus (Figure 2). All three components are required for

Figure 2. Organization of a typical *par* locus. Each locus encodes an ATPase (ParA; grey spheres) and a DNA binding protein (ParB; dark red spheres) which binds specifically to a *cis*-acting site (*parS*). ParB spreads in both directions from the *parS* site by non-specific interactions with the DNA. All three components are required for plasmid stabilization, and the *parS* site must be present in *cis* on the plasmid being stabilized. Partitioning is thought to occur as the result of interactions between ParA and ParB bound at the *parS* site.


parS
parB
parA

plasmid stabilization. Most plasmids which cannot rely on random distribution for their inheritance encode some type of *par* system and often have other genes which contribute to their stability. *par* loci can be transferred between plasmids with different types of replicons, and they will stabilize the heterologous plasmid as long as all three components of the *par* system are provided (Bartosik *et al.*, 2004; Dubarry *et al.*, 2006; Godfrin-Estevenon *et al.*, 2002; Yamaichi and Niki, 2000).

par loci are encoded by most bacterial genomes, as well, but a direct role for these loci in chromosome segregation has yet to be shown. These loci are designated *par* based on their similarity to plasmid *par* loci. The chromosomal *parAB* locus of *Caulobacter crescentus* is essential for growth (Mohl and Gober, 1997), whereas in *Bacillus subtilis*, *Pseudomonas putida*, *P. aeruginosa*, *Streptomyces coelicolor*, and *Vibrio cholerae* (chromosome I *parAB*), the locus is not essential (Bartosik *et al.*, 2004; Ireton *et al.*, 1994; Kim *et al.*, 2000; Lewis *et al.*, 2002; Saint-Dic *et al.*, 2006). In these organisms, disruption primarily causes segregation defects during developmental shifts such as sporulation or entry into stationary phase. *par* genes are notably absent from the chromosomes of *E. coli* and its close relatives, although low copy plasmids carried by *E. coli* generally bear *par* loci.

Despite the fact that chromosomal *par* loci are typically non-essential, they can be used to improve the stability of an unstable plasmid (Dubarry *et al.*, 2006; Godfrin-Estevenon *et al.*, 2002; Yamaichi and Niki, 2000). This indicates that the chromosomal *par* systems have the potential to function in a partitioning reaction

even though they are not essential for chromosome segregation. Whether or not the chromosomal *par* proteins segregate plasmids by the same mechanism as their plasmid encoded counterparts or whether this mechanism is important for chromosome segregation remains to be seen.

Here, the *par* systems encoded by both chromosomes and plasmids will be discussed.

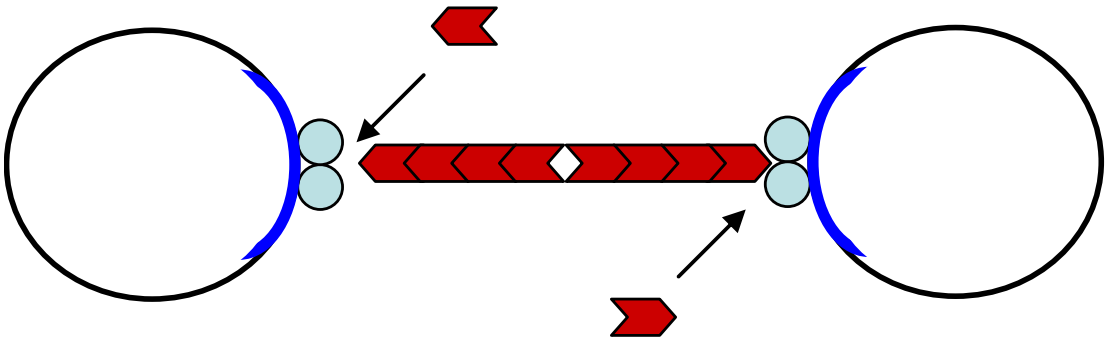
par *loci*

Par loci consist of two genes (*parAB*) and a *cis*-acting DNA binding site (*parS*) (Figure 2). *par* loci have been subdivided into two main groups based on their ATPases. Type I systems encode an ATPase (ParA) with a Walker-type nucleotide binding and hydrolysis motif, while Type II loci encode an ATPase (ParM) with homology to actin (Gerdes *et al.*, 2000; Gerdes *et al.*, 2004). Plasmids may carry one or both types of these loci, but all of the chromosomal *par* loci described to date encode Type I ATPases. Segregation of plasmids by both of these types of systems has been observed microscopically, however, the mechanism behind partitioning by the Type II systems is much more evident than that of Type I systems.

Type II par *systems*

The ATPase of Type II systems is referred to as ParM. Replicated plasmids maintained by these systems are found at both ends of elongating polymers of ParM and are pushed to opposite ends of the cell by the growing ParM filaments (Figure 3) (Moller-Jensen *et al.*, 2002; Moller-Jensen *et al.*, 2003). As a result of this

Figure 3. Plasmid segregation by Type II *par* systems. ParR binds to *parC* on the plasmid. ParM polymerizes between the ParR/*parC* complexes, and the polymers elongate, separating the plasmids from each other. ParM polymer elongation occurs as monomers are added between the ends of the polymers and the ParR/*parC* complex. Plasmid: black circles; ParR: light blue spheres; *parC*: blue line on plasmid; ParM: dark red chevrons.



separation, each daughter cell receives at least one copy of the plasmid upon division. ParM polymerization has been observed to occur *in vivo* and *in vitro*, and the dynamics of this polymerization have been studied in some detail *in vitro* (Campbell and Mullins, 2007b; Garner *et al.*, 2004; Garner *et al.*, 2007; Moller-Jensen *et al.*, 2002; van den Ent *et al.*, 2002). No co-factors are required to nucleate ParM *in vitro*, and the polymers lengthen at equivalent rates from both ends. ParM polymers undergo dynamic instability *in vitro* with rounds of steady lengthening of polymers at both ends followed by rapid breakdown of the polymers from one end (catastrophe). The addition of the partner protein, ParR, and DNA containing its *parC* binding site to the reaction serves to stabilize ParM filaments allowing for growth of the polymer between two ParR/*parC* complexes.

In vivo, ParM polymers are only observed when ParR and *parC* are also present (Moller-Jensen *et al.*, 2002). Based on the studies described above and a recent *in vivo* study, the current model for partitioning by ParM predicts that ParM is continuously polymerizing and depolymerizing *in vivo* (Campbell and Mullins, 2007b; Garner *et al.*, 2004). When the growing polymers encounter ParR/*parC* complexes, they are stabilized. The stabilization of the polymers between two plasmid/ParR complexes along with continued growth of the polymer would allow for separation of the two plasmids within a cell prior to division. This model is supported by experimental evidence. In an *in vivo* study, plasmids were observed to be pushed to opposite ends of the cell by ParM polymers multiple times within one cell cycle (Campbell and Mullins, 2007b). The ParM polymers disassembled after

each separation event, and new polymers arose between plasmids in any subsequent partitioning events. Prior to these *in vivo* observations, Mullins and his colleagues had carried out this separation *in vitro*. ParM polymers form between beads coated with ParR/*parC* and push the beads apart (Garner *et al.*, 2007).

Partitioning by Type II *par* systems has thus been witnessed clearly *in vivo* and recreated *in vitro*. The details of the reaction are not yet completely unveiled, but the machinery responsible for separation of replicated plasmids has been observed in action.

Type I par *systems*

Our primary interest and the focus of this work is the ATPase of the Type I partitioning systems. Type I *par* systems encode an ATPase with a Walker A box nucleotide binding and hydrolysis motif, ParA, and a DNA binding protein, ParB, which binds to a specific site, *parS* (Figure 2). *parS* is generally found within or near the locus, and it can also be found at distal sites, particularly on bacterial chromosomes. As the nomenclature of the Type I ATPases is confusing, the names of the various ParA homologues and their ParB counterparts discussed in this work are outlined in Table 1.

Most of what is known about partitioning by Type I systems comes from studies of plasmid localization and dynamics performed using time-lapse fluorescence microscopy. Plasmids maintained by Type I systems are positioned roughly at

Table 1. ParA and ParB homologue nomenclature.

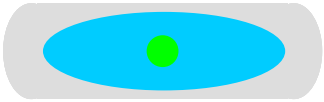
ParA homologue	***ParB homologue***	***Plasmid/Chromosome***
ParA	ParB	P1 plasmid; pB171 plasmid; *C. crescentus, P. aeruginosa, P. putida, S. coelicolor* chromosomes
SopA	SopB	F plasmid
Soj	Spo0J	*B. subtilis* chromosome
ParAI	ParBI	*V. cholerae* chromosome I

midcell or the one-quarter and three-quarters positions, the future division sites of the nascent daughter cells (Figure 4) (Ebersbach and Gerdes, 2004; Gordon *et al.*, 2004; Niki and Hiraga, 1999). In general, they occupy the same space in the cell as the nucleoids and are excluded from the poles or internucleoid region. As in segregation by Type II systems, the plasmid foci divide into two or more foci which move to opposite halves of the cell to yield daughter cells each bearing the plasmid.

While the localization of ParM and ParR provides evidence for their roles in partitioning, the localization of the ParA and ParB proteins in the Type I systems does not provide much insight into the mechanism by which partitioning occurs. Those ParA homologues that have been localized (both chromosomal and plasmid), either by immunofluorescence [P1 ParA (Erdmann *et al.*, 1999) and SopA (Adachi *et al.*, 2006)] or by fluorescent-protein tagged fusions [pB171 ParA (Ebersbach and Gerdes, 2004), Soj (Marston and Errington, 1999; Quisel *et al.*, 1999), Vibrio ParAI (Fogel and Waldor, 2006), SopA (Lim *et al.*, 2005)], have shown a distribution in the cell consistent with co-localization with the nucleoid. In some cases, the distribution of the protein is asymmetric and has been shown to change over time as the bulk of the protein migrates from one end of the cell to the other, apparently on the DNA (Ebersbach and Gerdes, 2001; Fogel and Waldor, 2006; Lim *et al.*, 2005; Marston and Errington, 1999; Quisel *et al.*, 1999). The purpose of this movement, if any, is unknown. Movement is considered likely to be important for the function of these proteins in partitioning because mutations which result in static localization of the protein cause destabilization of plasmids or loss of other known functions of the

Figure 4. Position of plasmids stably maintained by Type I *par* loci. In cells with one focus, the focus is generally found at midcell. Plasmid foci are found at the one-quarter and three-quarters position in cells with two foci.

Midcell



Cell quarters

proteins (Ebersbach and Gerdes, 2004; Marston and Errington, 1999; Quisel *et al.*, 1999). In the cases where the ParB protein has been localized, it generally localizes as a focus which corresponds to the location of the *parS* sequence on the plasmid or chromosomal DNA (Fogel and Waldor, 2006; Glaser *et al.*, 1997; Li and Austin, 2002; Lin *et al.*, 1997; Mohl and Gober, 1997).

Partitioning of plasmids by ParA and ParB has not been directly observed. However, the observation of plasmids by time lapse fluorescence microscopy has revealed that plasmid clusters are split and separated into both halves of an elongated daughter cell prior to division (Gordon *et al.*, 2004; Li and Austin, 2002; Li *et al.*, 2004). Both ParA and ParB are required for this segregation to reliably occur. Based on the current literature, ParA is required for proper localization of plasmid clusters, and likely for appropriate splitting of these clusters prior to division (Ebersbach and Gerdes, 2004; Li *et al.*, 2004). Time-lapse colocalization of the plasmid and ParA has not been performed as it has with ParM, and, as indicated above, the localization of ParA does not provide much evidence for how it is functioning in the partition reaction. Because of this, it has been very difficult to propose a model for partitioning by Type I partitioning systems. Recently, three ParA homologues (SopA from the F plasmid, pB171 ParA and ParF from TP228) have been shown to form filamentous structures *in vitro*. The structures have allowed for the proposal of models for segregation by Type I partitioning systems which include ParM-like segregation mechanisms. The current models for plasmid partitioning will be briefly described later in this Chapter.

ParA and Soj

ParA homologues are ATPases and are a part of a large superfamily of ATPases incorporating protein families with diverse functions. This superfamily includes the partitioning proteins for which it was named as well as proteins such as MinD, which is involved in the regulation of the placement of the division machinery. As with many nucleotide binding proteins, the functions of these proteins often depend on their nucleotide bound state. For example, MinD will only bind to the cytoplasmic membrane when in the ATP-bound dimer form (Hu *et al.*, 2002). Upon hydrolysis of ATP, the ADP-bound MinD releases from the membrane. In *E. coli* and other bacterial species, the cycling of MinD through different nucleotide-bound states allows for the establishment of an oscillation on the cytoplasmic membrane which is required for proper function of the Min system (Figure 5) (Lutkenhaus, 2007; Meinhardt and de Boer, 2001; Rothfield *et al.*, 2005).

ParA homologues, including MinD, share the conserved nucleotide binding motif (highlighted in dark red in Figure 6), but are quite divergent throughout the rest of their length. MinD and the chromosomal ParA/Soj homologues are similar in length, but MinD has a conserved C-terminal amphipathic helix which is not conserved in the plasmid or chromosomal ParA homologues. The Type Ia plasmid ParA homologues are the shortest ParA homologues, and the Type Ib homologues have an amino-terminal DNA binding domain (violet box in Figure 6).

Figure 5. Min oscillation and spatial regulation of Z ring assembly. The Min system consists of a set of three proteins (MinC: the effector; MinD: the carrier; and MinE: the topological specificity factor) which govern the placement of the division septum. MinE stimulates the ATPase of MinD causing it to release from the membrane. MinC is released from the membrane as well. These three proteins undergo a coupled oscillation such that the time-averaged concentration of the cell division inhibitor MinC is lowest at midcell (Lutkenhaus, 2007; Meinhardt and de Boer, 2001; Rothfield *et al.*, 2005).

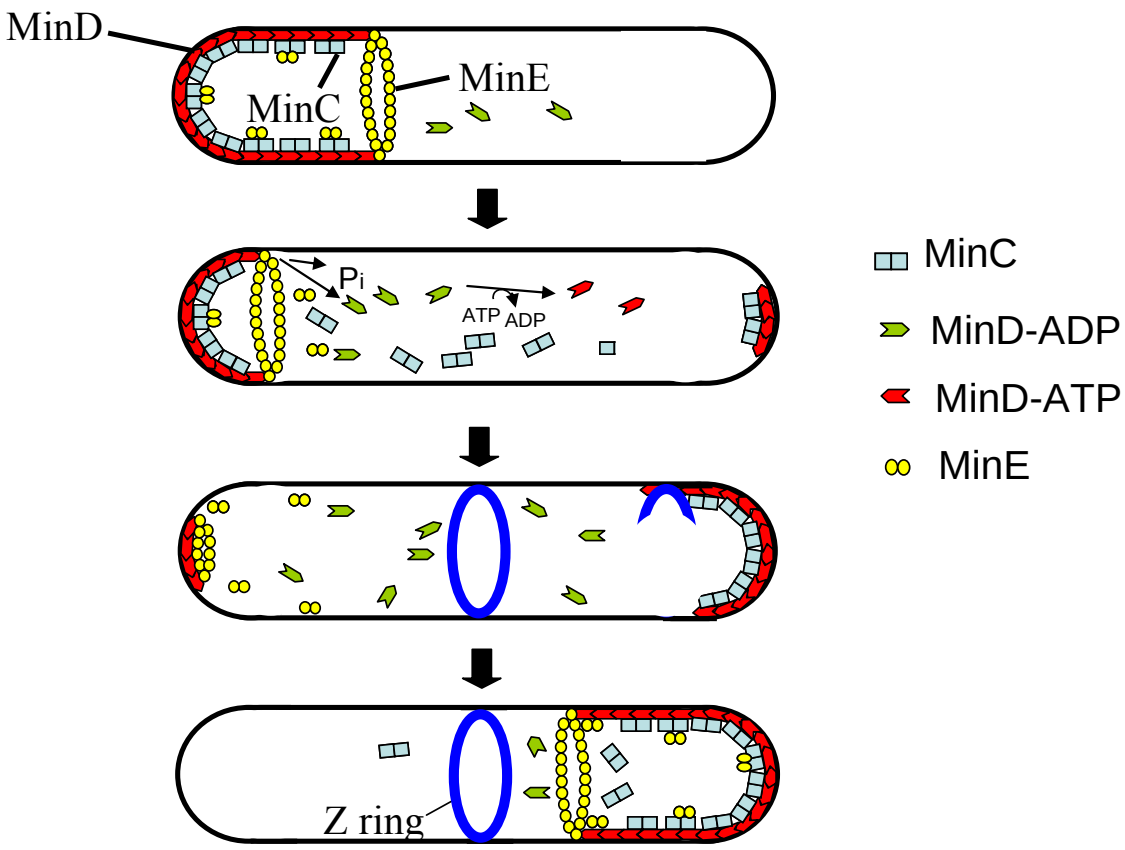
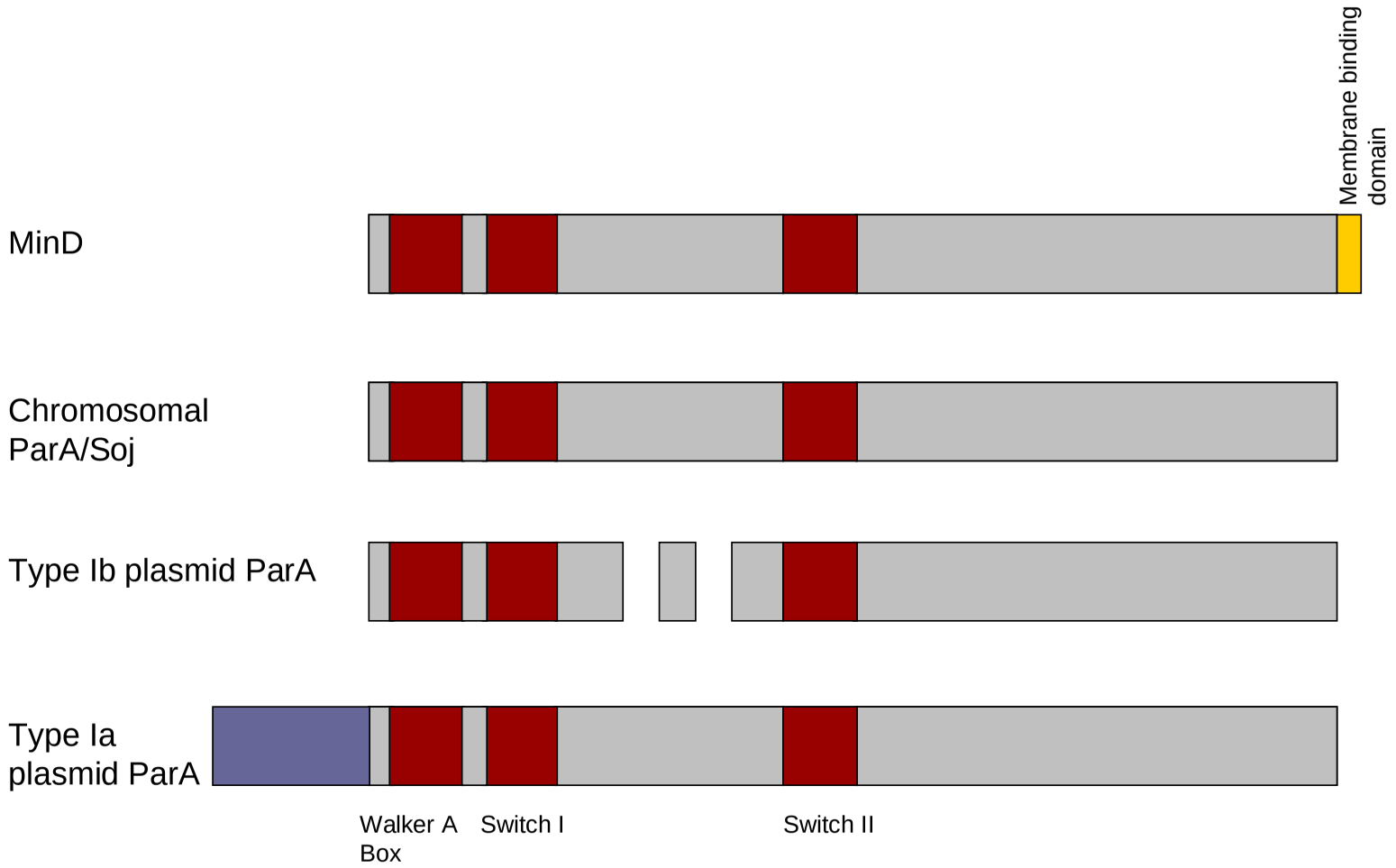

MinD
MinC
MinE
Pi
ATP
ADP
Z ring
MinC
MinD-ADP
MinD-ATP
MinE

Figure 6. Homology between the oscillating ParA ATPases. All ParA family proteins share the nucleotide binding motifs depicted here as dark red boxes labeled Walker A Box, Switch I, and Switch II. Grey regions indicated sequences with low conservation, and the Type Ib plasmid ParAs are shorter than the other ParA homologues and MinD. The C-terminal membrane binding domain is conserved among MinDs, but is not found in the plasmid or chromosomal ParA homologues. Type Ib plasmid ParA homologues have an N-terminal HTH DNA binding domain, and these proteins bind specifically to their own promoters.


Membrane binding domain
MinD
Chromosomal ParA/Soj
Type Ib plasmid ParA
Type Ia plasmid ParA
Walker A Box
Switch I
Switch II

Members of the ParA family are typically dimers when bound to ATP. These proteins have a highly conserved nucleotide binding and hydrolysis motif containing a conserved signature lysine which characterizes the family (Lutkenhaus and Sundaramoorthy, 2003). This lysine reaches across the dimer to interact with the bound nucleotide on the opposite monomer suggesting that the dimer is important for the ATP hydrolysis cycle of these proteins. The nucleotide bound state governs the dimerization of these proteins, modulating their activity. In addition, the ATPase activity of these proteins is generally stimulated by a partner: ParB/Spo0J, for the ParA homologues, and MinE for MinDs. Thus, the function of these proteins is regulated at multiple levels based on their nucleotide bound state, their interaction with partner proteins, and sometimes with other molecules such as the cytoplasmic membrane (MinD) or DNA (ParA).

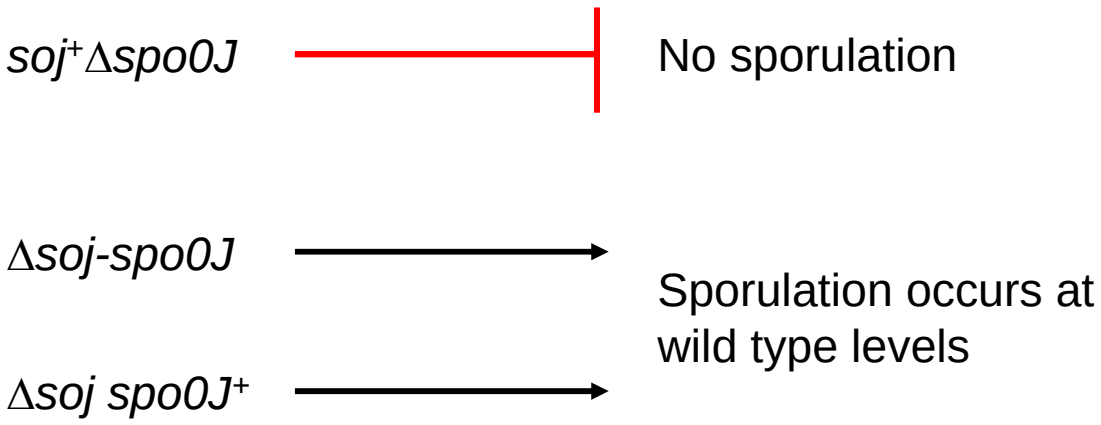
The function and behavior of the ParA homologues involved in plasmid and chromosome segregation is governed by their nucleotide bound state. ParA from pB171, SopA from the F plasmid and ParF from plasmid TP228 all form polymers in the presence of ATP but not ADP (Barilla *et al.*, 2005; Bouet *et al.*, 2007; Ebersbach *et al.*, 2006; Lim *et al.*, 2005). Soj from *T. thermophilus* and *B. subtilis* dimerize and bind to DNA in the ATP-bound form, while remaining monomeric in the presence of ADP (Hester and Lutkenhaus, 2007; Leonard *et al.*, 2005a). Here, we show that ParA from pB171 also dimerizes with ATP but not ADP, and that it binds to DNA with ATP (Chapter V). The activity of ParA from the P1 prophage is also different depending on the nucleotide bound state (Bouet and Funnell, 1999; Davey and

Funnell, 1994, 1997). When bound to ADP, the protein acts as a repressor, binding to the operator region of the par operon. When bound to ATP, however, the protein functions in the partitioning reaction, interacting with ParB bound to *parS*.

The ATPase activity of these proteins is essential for their function in partitioning, as well as for any other roles the chromosomal ParA/Soj homologues may have in their endogenous host. A number of studies have shown that mutation of key residues in the deviant Walker-A motif of several ParA homologues results in diminished activity of the protein (Ebersbach and Gerdes, 2004; Leonard *et al.*, 2005a; Li *et al.*, 2004; Quisel *et al.*, 1999). The requirement for the ATPase in the function of these proteins has led to the reasonable assumption that the partitioning reaction requires energy input, however, it is not clear at what point in the reaction this energy is required.

Chromosomal ParA homologues, while not necessarily essential for chromosome segregation, may have other regulatory roles outside of and perhaps as well as involvement in chromosome segregation. For example, Soj from *B. subtilis* was originally identified for its role in regulation of sporulation (Figure 7) (Ireton *et al.*, 1994). However, in all systems studied, both the ParA and ParB proteins are essential for proper plasmid partitioning. It has also come to light that Soj and Spo0J from *B. subtilis* may be involved in regulation of chromosome replication (Lee *et al.*, 2003; Lee and Grossman, 2006; Ogura *et al.*, 2003).

Figure 7. Soj represses sporulation in *B. subtilis*. In the absence of Spo0J, Soj, represses sporulation in *B. subtilis*. Deletion of *soj* relieves this sporulation block, indicating that Soj is responsible for the block.


soj+Δspo0J
No sporulation
Δsoj-spo0J
Sporulation occurs at wild type levels
Δsoj spo0J+

Plasmid ParA

Several plasmid Type I ParA homologues have been subjected to intense study. These proteins can be further subdivided based on the presence or absence of an N-terminal helix-turn-helix (HTH) DNA binding domain (Figure 6). Those with an N-terminal DNA binding domain, such as P1 ParA and F SopA, have been classified as Type Ia ParA homologues, while those lacking this domain, such as pB171 ParA and TP228 ParA, are referred to as Type Ib ParA homologues (Gerdes *et al.*, 2000). The N-terminal DNA binding domain of Type Ia ParA homologues is used in autoregulation of their operons (Davis *et al.*, 1992). There is no evidence to suggest that the Type Ib ParA homologues are autoregulatory.

ParA from the plasmid pB171, a Type Ib ParA, and SopA from the F plasmid, a Type Ia ParA, have both been shown to display dynamic localization *in vivo* and form ATP dependent polymers *in vitro*. Another Type Ib ParA, ParF from the plasmid TP228, has also been shown to polymerize *in vitro*. So far, *in vivo* localization of Type I plasmid ParA homologues is not clearly consistent with polymers playing an important role in partitioning. However, movement and *in vitro* polymerization are the main clues as to how ParA homologues function in partitioning, as described above in the models for partitioning.

Chromosomal Soj/ParA

Most bacterial chromosomes bear a partitioning locus, often located near the origin of replication. These loci encode Type I ParA ATPases, and the chromosomal ParA homologues are also referred to as Soj due to the nomenclature used in the initial identification of the chromosomal ParA homologue from *B. subtilis* as a suppressor of Spo0J (Ireton *et al.*, 1994). Interestingly, these loci are generally not essential during growth under normal laboratory conditions, although disruption has been shown to have effects on developmental shifts such as sporulation or entry into stationary phase. However, the *par* locus of *C. crescentus* is essential.

Chromosomal ParA homologues do not have the N-terminal extension present in Type Ia ParA homologues (Figure 6) and are not thought to be involved in regulation of their own expression. They are generally slightly longer than the Type Ib ParA homologues. ParA homologues are found on the chromosomes of most bacterial species that have been sequenced to date, but they are notably absent from the genomes of *E. coli* and its close relatives. A direct role for these proteins in chromosome partitioning has not yet been identified, although ParA and ParB of *C. crescentus* are required for growth. Soj and Spo0J of *B. subtilis* have been shown to be important for chromosome segregation in the absence of the structural maintenance of chromosomes (SMC) protein (Lee and Grossman, 2006).

Interestingly, chromosomal *par* loci can be used to maintain an otherwise unstable plasmid (Bartosik *et al.*, 2004; Dubarry *et al.*, 2006; Godfrin-Estevenon *et al.*, 2002; Yamaichi and Niki, 2000). This feature of these systems indicates that they can function in a partitioning reaction even if they are not required for proper chromosome segregation under normal laboratory conditions.

Positioning and partitioning

The *par* loci from P1 (*parABS*) and the F plasmid (*sopABC*) were discovered because they are required for the stable maintenance of their host plasmids (Abeles *et al.*, 1985; Ogura and Hiraga, 1983). Further studies revealed that all three components of the system were required for this stability and that the ATPase activity of the ParA homologue was required for stabilization (Davis *et al.*, 1996). The Type I partitioning systems of other plasmids have been studied, and these also require the ATPase activity of the ParA in addition to ParB and *parS* (Ebersbach and Gerdes, 2001; Libante *et al.*, 2001).

Since the partitioning mechanism for Type I systems has not been identified and cannot be visualized clearly, the localization of plasmids to specific regions of the cell has been used as an indicator of proper segregation. Generally, plasmids maintained by Type I par systems are positioned at approximately midcell or the one-quarter and three-quarters regions. This corresponds roughly to plasmids being confined to the region(s) of the cell occupied by the nucleoid(s). The association between localization and segregation has been demonstrated, at least in the case of the P1

plasmid. In one study, the localization of P1 was studied when the ParA ATPase was mutated (Li *et al.*, 2004). In this study, expression of one ParA mutant, K122E, a mutation to an invariant lysine in the deviant Walker-A nucleotide binding motif which has a decreased ATPase activity and destabilizes plasmid maintenance (Davis *et al.*, 1996), resulted in the plasmids' localization becoming random. With this mutant, the plasmid foci did not always split upon cell division, yielding one daughter cell with a plasmid, and one without. A second mutant, M314I, which causes faster than random loss of the plasmid (Youngren and Austin, 1997), seemed to allow for positioning of the plasmid cluster at midcell, but the focus did not split upon cell division, again resulting in a daughter cell without a plasmid. The disruption of localization observed with these mutants provided insight into the reason for the loss of plasmid stability previously observed and support the correlation between proper plasmid localization and stability.

F plasmid localization and stability have also been shown to correspond (Niki and Hiraga, 1997, 1999). Also, pB171 ParA has been indirectly shown to influence plasmid localization (Ebersbach and Gerdes, 2004). How these ParA homologues influence plasmid positioning and whether or not their dynamic behavior is required for appropriate positioning in unknown. In general, stable plasmids maintained by Type I partitioning systems are localized to midcell in short cells and the cell quarters in longer pre-divisional cells. Because of these studies, however, proper localization (*i.e.*, midcell or quarter cell positions) of plasmids is generally accepted as an indication of plasmid stability.

ParA movement

GFP-fusions to the ParA homologue, MinD, display movement in several bacterial species, and this movement is essential to the role of MinD in the regulation of the placement of the division septum. MinD oscillates from one end of the cell to the other on the membrane with a period of approximately forty seconds at room temperature, and this behavior is governed by the MinD ATPase activity which is stimulated by MinE (Hu and Lutkenhaus, 2001). Mutations that affect the dynamic nature of MinD, such as mutations to the nucleotide binding site, prevent it from functioning to limit division to mid-cell (Zhou *et al.*, 2005). In addition, mutations that prevent MinD from binding to the membrane, such as deletion of the conserved C-terminal tail (Hu and Lutkenhaus, 2003; Szeto *et al.*, 2002) or replacement of hydrophobic residues in this tail with charged residues (Zhou and Lutkenhaus, 2003), prevent the movement of MinD, and, thus, prevent it from performing its role in the regulation of cell division. Therefore, nucleotide binding, appropriate localization, and movement are required for the proper function of MinD in the regulation of septum placement.

Although the localization of ParA and ParB does not clarify their roles in the partitioning reaction, there are possible clues to their function in their behavior. Much like MinD, several ParA homologues have been shown to display dynamic localization *in vivo*. These proteins move on the nucleoid instead of on the

membrane, however, and the movement occurs on a timescale of minutes and does not have a regular periodicity.

Soj from *B. subtilis*, ParA from the *E. coli* plasmid pB171, SopA from the F plasmid, and ParAI from *V. cholera* chromosome I have all been shown to move from one end of the cell to the other, apparently on the nucleoid (Ebersbach and Gerdes, 2001; Fogel and Waldor, 2006; Lim *et al.*, 2005; Marston and Errington, 1999; Quisel *et al.*, 1999). Mutations which prevent this movement, such as mutation of the nucleotide binding site of ParA or deletion of ParB, result in destabilization of plasmids and loss of nucleoid localization. In addition, these mutations in Soj prevent it from repressing sporulation in the absence of Spo0J. In this work, we show that disruption of DNA binding by mutation of conserved residues in Soj prevents movement and also results in the inability of these mutants to function in a plasmid partitioning reaction or in the regulation of sporulation in *B. subtilis* (Hester and Lutkenhaus, 2007).

Plasmid positioning was unaffected when pB171 ParA was statically localized in cephalexin induced filaments (Ebersbach and Gerdes, 2001). The correlation between plasmid positioning and stability has not been demonstrated directly for this plasmid. As the plasmid positioning is unaffected in the absence of ParA movement, these findings call in to question whether or not movement of ParA is required for partitioning. Despite this observation, most models proposed for this system rely on ParA movement in the mechanism.

Assembly

As the machinery responsible for eukaryotic chromosome segregation requires the polymerization of tubulin to separate sister chromatids, speculation about the machinery involved in bacterial chromosome and plasmid partitioning has reasonably included a cytoskeletal element. Although the localization of GFP fusions to ParA and Soj did not seem to support the polymerization of these proteins *in vivo*, the discovery that ParM formed polymers that pushed apart plasmids brought renewed interest to the topic of whether or not the ParA homologues could be functioning by pushing or pulling apart newly replicated plasmids or chromosomal origins. The simplest model for plasmid partitioning would include a polymer exerting force to separate newly replicated plasmids.

The polymerization of three Type I ParA homologues has been documented within the last few years. ParF from the plasmid TP228 was shown to form polymers which can be bundled in the presence of the partner protein, ParG (Barilla *et al.*, 2005). This protein has never been visualized by fluorescence microscopy, so there is no comprehensive model for partitioning of this plasmid by ParF and ParG.

ParA from pB171 has been shown to form polymers in the presence of ATP, as well (Ebersbach *et al.*, 2006). These polymers are included in the models for partitioning put forth by Gerdes and colleagues that were described above.

Finally, SopA from the F plasmid has been reported to polymerize by two independent groups. Lim *et al.* showed that SopA polymerized into filaments which could be observed by fluorescent microscopy when stained with Nile Red (Lim *et al.*, 2005). In addition, they observed that SopA formed radial structures when mixed with plasmid DNA containing a *sopC* site. This group also isolated a mutant form of SopA, SopA1, which appeared to form long filaments *in vivo* and its localization was static, sometimes seeming to cause filamentation of cells expressing the allele. Based on their *in vitro* observations, Lim *et al.* proposed the model described above which involves separation of plasmids by SopA polymers radiating from paired F plasmids, pushing them apart.

SopA was also found to polymerize *in vitro* by Bouet *et al.*(Bouet *et al.*, 2007). In their experiments, SopA polymerized in the presence of ATP. These polymers were detected by electron microscopy. Polymerization of SopA detected by sedimentation was inhibited in the presence of DNA, however. When SopB was present in addition to DNA, the SopA could be sedimented. The reason for this is unclear, although the authors suggest that SopA binds to DNA if it is available, but if SopB is present, it binds the DNA and allows SopA to polymerize and potentially function in the partitioning reaction as a polymer.

DNA binding

The nucleoid localization displayed by ParA and Soj eventually prompted investigation into the DNA binding capabilities of these proteins. Leonard et al.

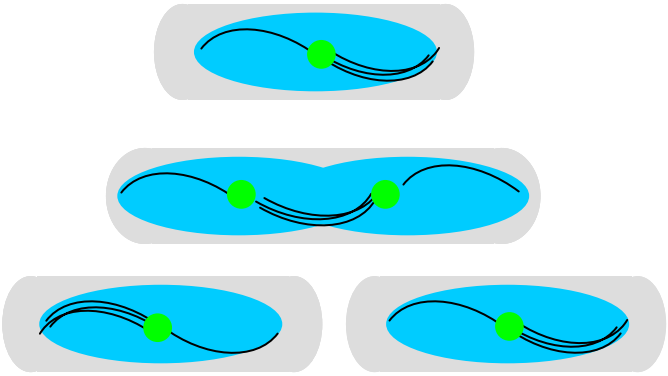
(Leonard *et al.*, 2005a) reported that Soj from *T. thermophilus* bound to DNA *in vitro*. Here, we show that Soj from *B. subtilis* also binds to DNA, and we have identified key residues involved in this binding (Hester and Lutkenhaus, 2007). The non-specific DNA binding characteristics have not been tested for other ParA homologues. We also show here that pB171 ParA can bind to non-specific DNA *in vitro* and that it clearly colocalizes with nucleoid DNA *in vivo* (Chapter V).

Models for partitioning

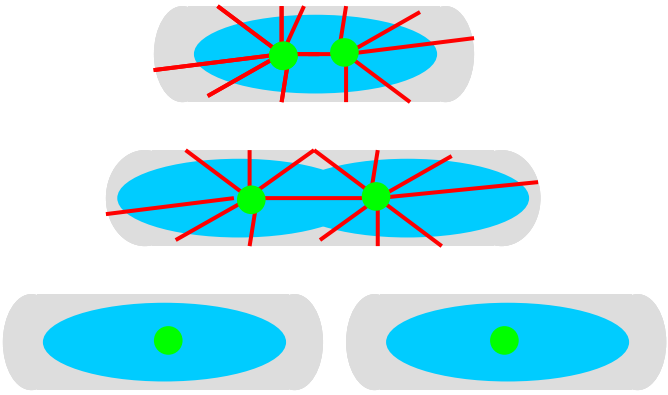
The current models for plasmid partitioning by Type I partition systems include the movement of the ParA in the mechanism as well as polymerization of the ATPase. The first of these that I will discuss was proposed by Kenn Gerdes and colleagues for the partitioning of the plasmid pB171 by its ParAB system and suggests that polymers of ParA continuously form between plasmids and the ends of the nucleoid or between pairs or clusters along the length of the cell (Figure 2A) (Ebersbach and Gerdes, 2005; Ebersbach *et al.*, 2006). These polymers push apart the plasmids, and as the bulk of ParA moves across the cell, the polymers disassemble and reassemble between plasmid clusters or plasmids and the end of the nucleoid, with the bulk of the protein moving from one end of the cell to the other. This redistribution of polymers results in the regular distribution of plasmid foci/clusters along the length of the cell so that plasmids are inherited by each daughter cell at division. This model assumes that the ends of the nucleoid act as toeholds for the ParA polymerization so that if there is only one plasmid focus/cluster in the cell, it is maintained at midcell by the

Figure 8. Representations of partitioning models. (A) Gerdes' model. (B) Model proposed by Lim *et al*. (C) Model for chromosome I partitioning in V. cholerae. Note that some ParA remains at the pole with the static origin. The grey cylinder represents the cell; the blue oval represents the nucleoid; and the green circle represents the plasmid(s) (A and B) or the origin of replication (C). Black curved lines in A represent ParA polymers. Red lines in B represent SopA polymers. Dark blue structures in C represent ParAI polymers.

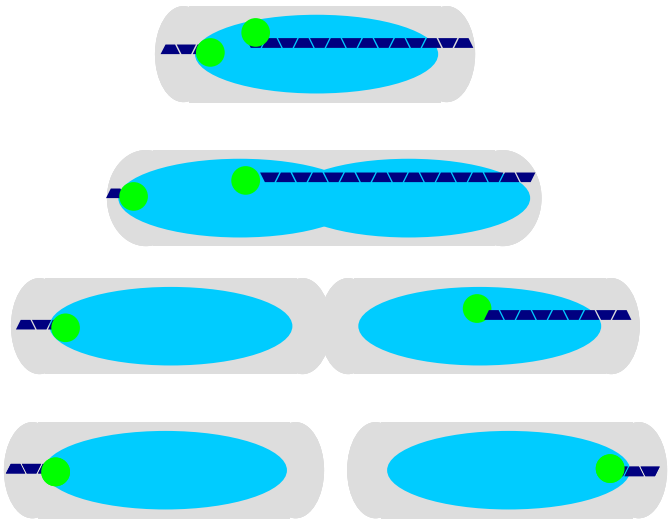
A



B



C



forces generated as polymers form between each end of the nucleoid and the plasmid cluster. Then, as the cell elongates and chromosomes segregate, two foci can be maintained at the quarters as well (Figure 8A). This model assumes that the fluorescence of ParA is more intense where there are more ParA polymers and that the force necessary for movement of plasmids is generated as the ParA polymerizes between plasmids/nucleoid ends. The assumption is that when more ParA polymers are on one side of a plasmid than the other, the plasmid will be pushed in the direction of least resistance, resulting in separation of plasmids when ParA polymers form between them.

Another model has been proposed for plasmid partitioning by SopA of the F plasmid. This model relies on polymerization of the SopA into aster-like structures around plasmid clusters which push the plasmids apart in a process similar to the separation of replicated chromosomes in eukaryotic cells by the mitotic spindle (Figure 8B). This model, proposed by Lim *et al.* (Lim *et al.*, 2005), was developed based on the fact that purified SopA polymerizes into aster-like structures *in vitro* when mixed with SopB and SopC containing DNA. The involvement of SopA polymers in partitioning is supported by the findings of Bouet *et al.*, who documented the polymerization of SopA *in vitro* (Bouet *et al.*, 2007). However, the authors have not taken into account their experimental data which indicates that SopA is often observed to colocalize with only one plasmid focus/cluster within a given cell, even when that cell contains two plasmid foci/clusters as detected by other means. This

information is difficult to reconcile with a model requiring two aster-like SopA/plasmid clusters pushing apart from each other.

Models for chromosome segregation are less detailed. Fogel and Waldor proposed that the ParBI bound origin follows ParAI polymers as they recede towards the opposite pole of the cell (Figure 8C) (Fogel and Waldor, 2006). Their experimental evidence for this model supports the idea that the ParBI bound origin follows the retraction of ParAI structures across the cell. However, as with ParA from pB171 and SopA, the nature of the structures formed by ParAI cannot be resolved by fluorescent microscopy, so the form of the ParAI in these cells is still subject to conjecture. Their model, however, is clearly supported by their published observations.

Currently, there are no in depth models for plasmid or chromosome partitioning by Type I *par* systems because so little can be taken from fluorescence microscopy data in conjunction with genetic data. Dual localization of ParAs and plasmids as well as time-lapse microscopy may help shed some light on the events resulting in plasmid partitioning, although more *in vitro* work will undoubtedly be needed to characterize the interactions between ParA, ParB, and *parS* which result in segregation. The work we have published and present here will also contribute to assembly of a model including another relevant characteristic of ParAs: the requirement for non-specific DNA binding in their function in partitioning. In addition, our work provides some evidence that ParA/Soj movement may be important for plasmid segregation and should continue to be studied for involvement in and included in models of both

plasmid and chromosome segregation. We present a model for plasmid partitioning which incorporates our findings in Chapter VI.

Summary of this work

The goal of the work discussed here is to begin to elucidate the nature of the mechanisms involved in bacterial chromosome and plasmid segregation. We chose to study Soj because it promotes plasmid segregation and moves in *E. coli*, so we could study its function in a partitioning process without the complication of its role in regulating sporulation. When these studies were initiated, the first reports of dynamic movement by Soj and ParA of pB171 had been published and Soj, Spo0J, and *parS* from *B. subtilis* had been shown to be sufficient to support plasmid partitioning with *parS*. Our studies have since shed light on an important function of ParA homologues: the ability of these proteins to bind non-specifically to DNA through arginine residues on the surface of the protein structure. Although the localization of these proteins to the nucleoid has been documented, the importance of DNA binding was not apparent until we showed that disruption of DNA binding by *B. subtilis* Soj prevents it from functioning in plasmid maintenance (Hester and Lutkenhaus, 2007). Furthermore, we have shown that Soj DNA binding is necessary for one of its normal functions in *B. subtilis*: the regulation of sporulation (Chapter IV). More work will be required to determine exactly why DNA binding is important for Soj function, but our results indicate that it is.

We have also shown here that the requirements for Soj movement in the heterologous host, *E. coli*, are the same as those for plasmid maintenance by Soj, Spo0J and *parS* in *E. coli* determined by Yamaichi and Niki (Yamaichi and Niki, 2000). While it is still not clear what role, if any, the movement of Soj plays in its function, our work adds to the findings that circumstances or mutations that disrupt Soj movement disrupt its function in partitioning.

Finally, we have identified candidate residues in the ParA from plasmid pB171 which are likely to be involved in DNA binding by this protein (Chapter V). Mutation of these residues results in cytoplasmic rather than nucleoid localization of GFP-fusions *in vivo*. Unfortunately, we have not been able to well characterize these mutants *in vitro*. However, based on our work with Soj, it seems likely that disruption of DNA binding by any ParA will diminish its ability to participate effectively in plasmid maintenance. We have been able to determine that pB171 ParA displays ATP dependent dimerization and DNA binding, just as Soj does. In addition, we have identified residues which may be important for DNA binding, although further characterization of these mutants will be necessary.

The aim of this work has been to gain an understanding of the role of ParA homologues, in particular *B. subtilis* Soj, in the maintenance of low copy plasmids. I have undertaken three main courses of study to determine how the behavior of Soj could be playing a role in plasmid partitioning: 1) I have characterized the requirements for oscillation of *B. subtilis* Soj in the heterologous *E. coli* host (Chapter

III); 2) I have identified highly conserved arginine residues involved in the non-specific binding of Soj and ParA from pB171 to double stranded DNA (Chapter IV; Chapter V); 3) I have characterized the effects of mutation of these highly conserved arginines on plasmid partitioning in *E. coli* and regulation of sporulation in *B. subtilis* (Chapter IV).

Chapter II: Experimental Procedures

Bacterial Strains and Plasmids

The bacterial strains used in this study are listed in Table 2 along with their relevant genotypes. Plasmids are listed in Table 3. Strains were grown in LB at 37°C unless otherwise indicated. Antibiotics were added as needed at the following concentrations: ampicillin (20 μg/ml for all miniF derivatives, 100μg/ml for all other plasmids) and spectinomycin (50 μg/ml). isopropyl-β-D-thiogalactopyranoside (IPTG) at specified concentrations was used to induce expression from the *lac* promoter. Glucose (0.2%) was used to repress expression of this promoter.

Untagged Soj was expressed from the expression vector pCMN001. This vector was created by cloning a *soj* PCR fragment created with primers 5'EcoRI-soj and 3'HindIII-soj into pJF118EH (Furste *et al.*, 1986).

A plasmid for expressing GFP-Soj (pSEB200; P_{lac}*gfp-soj spo0J*) was created by cloning a *soj* PCR fragment created with primers 5'XbaI-soj and 3'HindIII-soj into pSEB181 (Described in (Zhou and Lutkenhaus, 2004)). pCMN003 (P_{lac}*gfp-soj spo0J*) was created using a PCR product amplified with 5'XbaI-soj and 3'SalI-spo0J and cloned into pSEB181.

For creating the hexahistidine N-terminal fusion to Soj in pCMN011, a PCR product amplified with 5'BamHI-sojfus and 3'HindIII-soj was cloned in frame into pQE80L (Qiagen).

Table 2. Bacterial strains used in this work.

Strain	***Relevant genotype***	***Reference***
MC1061	*araD139 Δ(ara-leu)7696 galU galK16 galE15 Δ(lac)X74 hsdR2($r_k^- m_k^+$) mcrA mcrB1 rpsL*	(Casadaban and Cohen, 1980)
JS219	MC1061 *malPp::lacI^q*	(Cam *et al.*, 1988)
JS238	JS219 *srlC::Tn10 recA1*	(Pichoff *et al.*, 1995)
W3110	Wild type	Laboratory collection
DY380	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lacZ* M15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*) 7649 *gal*U *gal*K *rsp*L *nup*G [λ*cI*857 (*cro-bio*A) <> *tet*]	(Lee *et al.*, 2001)
SW101	DY380 *gal* $^+$	(Warming *et al.*, 2005)
SW102	SW101 *ΔgalK*	(Warming *et al.*, 2005)
MG1655	*rphI ilvG rfb*-50	(Guyer *et al.*, 1981)
TB28	MG1655 *lacIZYA<>frt*	(Bernhardt and de Boer, 2003)
TB104	TB28 cI857 (ts) λP_R::*dnaA*	(Bernhardt and de Boer, 2005)

Mutagenesis of pSEB200, pCMN003, and pCMN011 was performed with the QuikChange Kit from Stratagene using the appropriate primer pairs.

A plasmid for expressing GFP-ParA (pCMN308; P_{lac}*gfp-parA*) was created by cloning a *parA* PCR fragment created with primers 5'XbaI-*parA* pB171 and 3'SalI-*parA* pB171 into pSEB181. For creating the hexahistidine N-terminal fusion to ParA in pCMN310, a PCR product amplified with 5'BamHI-parA pB171 and 3'HindIII-parA pB171 was cloned in frame into pQE80L (Qiagen). Mutagenesis of pCMN308 and pCMN310 was performed with the QuikChange Kit from Stratagene using the appropriate primer pairs.

All primer sequences are listed in Table 4.

Plasmids pXX704, pXX764, and pXX765 were generously provided by Hironori Niki and were previously described (Yamaichi and Niki, 2000).

Site directed mutagenesis was performed on all plasmids using the QuikChange Kit from Stratagene with the appropriate primer pairs. pCMN003 *parS-6* was created in two steps: the first three mutations were made in pCMN003 *parS-3*, then the second set of three mutations was added to this mutated vector. Mutated sequences were verified by sequencing.

Cloning was performed in JS238, while microscopy was performed using W3110 bearing the relevant plasmid, unless otherwise noted.

Table 3. Plasmids used in this work.

Plasmid	*Relevant genotype*	*Reference*
pSEB181	P_{lac}*gfp*	(Zhou and Lutkenhaus, 2004)
pSEB200*	P_{lac}*gfp-soj (B.s)*	This work
pCMN001	P_{tac}*soj (B.s)*	This work
pCMN003*	P_{lac}*gfp-soj spo0J (B.s)*	This work
pCMN011*	P_{lac}*6xhis-soj (B.s)*	This work
pCMN032	pXX765 *soj::galK spo0J parS (B.s)*	This work
pCMN033 R189E	pXX765 *sojR189E spo0J parS (B.s)*	This work
pCMN033 R218A	pXX765 *sojR218A spo0J parS (B.s)*	This work
pXX704	miniF *sopABC*$^+$	(Yamaichi and Niki, 2000)
pXX764	miniF *ΔsopABC parS*$^+$ *(B.s.)*	(Yamaichi and Niki, 2000)
pXX765	miniF *soj spo0J parS (B.s.)*	(Yamaichi and Niki, 2000)
pCMN308*	P_{lac}*gfp-parA (pB171)*	This work
pCMN310*	P_{lac}*6xhis-parA (pB171)*	This work

*Site directed mutagenesis was performed on each of these plasmids using the Stratagene QuikChange kit and the appropriate primers (sequences listed in Table 4). Each of the vectors bearing the mutant alleles of *soj* or *parA* was simply referred to as the parent vector name followed by the amino acid substitution (*i.e.*, pSEB200 R189E). *B.s.* = *B. subtilis.*

Table 4: Sequences of primers used in this work.

Primer Name	***Sequence* (5'-3'; restriction site or mutated bases in bold; *galK* sequence in lowercase italics)**
5'EcoRI-soj	CG**GAATTC**TGAAAGTAGGTGACATCGTGG
3'HindIII-soj	TTG**AAGCTT**TTAGCCATTCGCAGCCACTTCC
5'XbaI-soj	CTTG**TCTAGA**GGAAAAATCATAGCAATTACG
3'SalI-spo0J	CTT**GTCGAC**TTTTTCATTTATGATTCTCG
5'BamHI-sojfus	CG**GGATCC**GGAAAAATCATAGCAATTACG
S GFP-soj R189E	GCTGACAATGCTTGATGCC**GA**GACAAACCTTGGCATTC
AS GFP-soj R189E	GAATGCCAAGGTTTGTC**TC**GGCATCAAGCATTGTCAGC
S GFP-soj R189A	GCTGACAATGCTTGATGCC**GC**GACAAACCTTGGCATTC
AS GFP-soj R189A	GAATGCCAAGGTTTGTC**GC**GGCATCAAGCATTGTCAGC
S GFP-soj K201E	CGGACAAACCTTGGCATTCAAGTGATTGAAGAGGTT**G**AAAAGTATTTTAGGG
AS GFP-soj K201E	CCCTAAAATACTTTT**C**AACCTCTTCAATCACTTGAATGCCAAGGTTTGTCCG
S GFP-soj K201A	CGGACAAACCTTGGCATTCAAGTGATTGAAGAGGTT**GC**AAAGTATTTTAGGG
AS GFP-soj K201A	CCCTAAAATACTTT**GC**AACCTCTTCAATCACTTGAATGCCAAGGTTTGTCCG
S GFP-soj R218E	CAGTTATTCCGCGTAATGTC**GAA**CTGAGTGAGGCACCGAGTC
AS GFP-soj R218E	GACTCGGTGCCTCACTCAG**TTC**GACATTACGCGGAATAACTG
S GFP-soj R218A	CAGTTATTCCGCGTAATGTC**GC**TCTGAGTGAGGCACCGAGTC
AS GFP-soj R218A	GACTCGGTGCCTCACTCAGA**GC**GACATTACGCGGAATAACTG
5'soj-galK	CGCTTTAATCTTTTTTTGATAGAATAGAAGCAAGATAGTACATGTTCATGTGAAAGTAGG*cctgttgacaattaatcatcggca*
3'soj-galK	ATGGGTTTTCCGTGACTCGGTGCCTCACTCAGACGGACATTACGCGGAATAACTGTCTTATATAC*tcagcactgtcctgctccttg*
5'Soj	TGATAGAATAGAAGCAAGATAGTACATGTTCATGTGAAAGTAGGTGACATCGTGGGAAAAATCATAGCAATTACGAACC
3'Soj	TTAGCCATTCGCAGCCACTTCCTTTGCTAAATC
5'XbaI-parA pB171	CCCG**TCTAGA**ATTACTGTAGTTGGTGGGAACAAAGG
3'SalI-parA pB171	CTT**GTCGAC**GTTTCTTCACCATGGGCCAAATACC
5'BamHI-parA pB171	CCCG**GGATCC**ATTACTGTAGTTGGTGGGAACAAAGG
3'HindIII-parA pB171	CTT**AAGCTT**GTTTCTTCACCATGGGCCAAATACC

Mutations in *soj* in the miniF plasmid pXX765 (Yamaichi and Niki, 2000) were created by allelic replacement by recombineering using *galK* selection (Warming *et al.*, 2005). Briefly, *soj* was replaced by a PCR product containing the *galK* gene with its native promoter by recombineering in the strain SW102. Transformants were diluted after electroporation into 25 ml LB supplemented with 0.2% glucose and grown overnight at 30°C to allow for complete segregation of recombinant plasmids from parental plasmids. After washing cells in M9 salts, recombinants were selected for by growth at 30°C on M63 minimal medium supplemented with 0.2% galactose as a carbon source. Plasmids were purified from four gal^+ colonies (phenotype verified on MacConkey galactose agar) and checked by PCR for insertion of *galK* and disruption of *soj*. This *soj::galK* derivative of pXX765 was designated pCMN032 and was electroporated back into SW102. The *galK* cassette was then replaced with the appropriate mutant allele of *soj* by recombineering. Transformants were diluted into 25 ml LB supplemented with 0.2% glucose and grown overnight. Cells were washed in M9 salts, and recombinants lacking the *galK* cassette were selected for by growth at 30°C on M63 minimal medium supplemented with 0.2% glycerol as a carbon source and 0.2% 2-deoxy-galactose for counterselection. Plasmids were purified from four $galK^-$ colonies (phenotype verified by growth on MacConkey galactose agar) for each mutation, the *soj* gene was amplified by PCR, and the PCR products were submitted for sequence analysis. Plasmids carrying only the appropriate mutation were designated pCMN033 R189E and pCMN033 R218A.

These miniF derivatives were transformed into JS238 for use in plasmid stability studies.

B. subtilis strains and plasmids were constructed by standard techniques (Harwood and Cutting, 1990). Essentially, parental strains were made competent and transformed with linearized plasmid DNA bearing the desired allele, and all drug resistances, auxotrophies, and *amyE*- (starch degradation) phenotypes were verified. The sequence of the *soj* allele on the chromosome was verified by sequencing.

Measuring plasmid stability

Plasmid stability assays were performed as described in Yamaichi and Niki (2000) with minor modifications. Briefly, strains bearing the appropriate plasmids were grown to mid-log phase in LB supplemented with ampicillin. Cultures were then diluted to OD_{540} of 0.01 (Time 0) and kept in exponential growth by dilution to 0.01 approximately every 3 hours. At times 0, 6 hours, and 12 hours without selection, appropriate dilutions of each culture were plated on each of five LB plates and five LB plus ampicillin (20μg/ml) plates. The number of colonies on each set of plates was counted and the number of cfu/ml of original culture was extrapolated from these numbers based on the dilution plated. The number of ampicillin resistant colonies was compared to the number of colonies on the LB plates to arrive at the percent of plasmid bearing cells in the original culture at each time point, and the average values from either two (pXX704 and pCMN032) or three (pXX765, pCMN033 R189E, pCMN033 R218A, and pXX764) separate experiments were plotted. The percentage

of plasmids lost per generation (L) was determined using the equation $L = [1-(F_f/F_i)^{1/n}] \times 100$, where F_i is the fraction of cells initially carrying the plasmid and F_f is the fraction of plasmid bearing cells after n generations of nonselective growth (Ravin and Lane, 1999).

Analysis of GFP-Soj and GFP-ParA localization

For analysis of the localization of WT GFP-Soj, W3110 bearing either pSEB200 (P_{lac}*gfp-soj*) or pCMN003 (P_{lac}*gfp-soj spo0J*) or their derivatives were grown overnight in the presence of spectinomycin and glucose, then diluted 1:100 in fresh LB supplemented with only spectinomycin. When the culture had reached an OD_{540} of 0.1, 50 μM (pSEB200) or 100 μM (pCMN003) IPTG was added to the cultures. Cultures were maintained in exponential phase by dilution with pre-warmed LB supplemented with spectinomycin and IPTG or were allowed to enter stationary phase. For the mutant GFP-Soj fusions, W3110 bearing the appropriate plasmids was streaked directly onto an LB plate supplemented with spectinomycin and 500 μM IPTG. Plates were incubated at 37°C for 2-6 hours, and cells were prepared for microscopy and observed as described below. The effects of untagged Soj and His-Soj on nucleoid morphology in JS238 were assessed by the same procedure used to analyze the GFP-Soj mutants.

For analysis of the localization of GFP-ParA, overnight cultures of W3110 bearing either pCMN308 ($P_{lac::}$*gfp-parA*) or a derivative bearing a mutant ParA were streaked

directly onto an LB plate supplemented with spectinomycin and 500 μM IPTG and incubated at 37°C for 2-6 hours. Cells were stained with 4'-6-diamidino-2-phenylindole (DAPI) and mounted on glass slides. Microscopy was performed as described previously (Pichoff and Lutkenhaus, 2005). TB104 strains were grown overnight at 37°C, streaked onto an LB plate supplemented with spectinomycin and 500 μM IPTG and incubated at 37°C for 1 to 2 hours. They were then shifted to 30°C for a minimum of 2 hours prior to microscopy.

For microscopic analysis of the cells expressing GFP-fusions, cells were resuspended in 2 μl of LB containing of DAPI (2 μg/ml) on a glass slide. After the cover slip was put in place, the slide was incubated at room temperature for 10-20 minutes to allow the DAPI to penetrate the cells and stain the DNA. Samples were observed and photographed with a Nikon Eclipse E600 fluorescence microscope equipped with a 100× E Plan oil immersion lens (Nikon Instruments, Melville, NY) and a MagnaFire CCD camera S99802 from OPTRONICS (Goleta, CA). Images were imported to Adobe Photoshop software and assembled.

Western Analysis of GFP-Soj and GFP-ParA fusion protein stability in vivo

W3110 bearing the appropriate GFP-fusion expressing plasmids were grown to OD_{540} of approximately 0.1. GFP-fusion expression was induced by the addition of 1 mM IPTG, and the cultures were allowed to grow for one hour. Cells were harvested and resuspended in SDS PAGE loading dye. The lysates were run on an SDS PAGE gel

after normalizing for OD, and proteins were transferred to a nitrocellulose membrane. GFP and GFP-fusions were detected with a rabbit anti-GFP peptide alkaline phosphatase conjugated antibody (Clonetech), and signal was detected colorimetrically using the Bio-Rad AP-Conjugate Substrate Kit.

Identification of conserved residues and mapping on Soj structure

Soj and chromosomal ParA sequences were obtained from the GenBank database by direct acquisition or upon identification of homologous sequences by BLAST search. Amino acid sequences were aligned in ClustalX (Thompson *et al.*, 1997). Initially, approximately 30 chromosomal ParA homologues that were identified as Soj homologues based on a number of conserved motifs in the primary amino acid sequences (not shown) were aligned. The arginines and lysines that were conserved in the majority of the sequences (including *T. thermophilus* Soj) were located on the *T. thermophilus* Soj dimer structure (2BEK) in PyMol (DeLano Scientific LLC). Six of these mapped to the surface of the dimer. Three of these, R189, K201, and R218 (*B. subtilis* numbering) were selected for further analysis for involvement in DNA binding based on their location at the C terminus which is less highly conserved in the extended ParA family and likely to have a unique function. A fourth conserved arginine, R215, is located near R189 in the dimer structure, but the side chain points inward and is likely involved in interaction with the bound nucleotide.

Alignment of ParA and Soj sequences and identification of candidate basic residues

ParA sequences were retrieved from the GenBank database by BLAST search using the pB171 ParA sequence. In addition, sequences found during other BLAST searches for ParA homologues which aligned closely to ParA were also used in the alignments. When aligning divergent ParA homologues, the identification of conserved basic residues was very difficult. To improve the likelihood of finding good candidate residues, only those ParA sequences that were closely related to pB171 ParA were included in the final alignment against Soj. Here, we have shown the relevant excerpts of an alignment performed with ClustalX. Varying the parameters changed the alignment, however, these residues were always near each other in the alignments.

Protein purification

Wild type and mutant Soj were overexpressed as N-terminal His-tagged fusions in strain JS238 from pCMN011 and the mutant derivatives. For overexpression, an overnight culture grown in the presence of ampicillin and glucose was diluted 1:100 into 1 L of LB supplemented with ampicillin. The culture was grown to OD_{540} 0.6, and 1 mM IPTG was added to the culture. The culture was grown for another 2 hours, and the cells were harvested by centrifugation and washed in 50 ml of 50 mM Tris, pH 7.5. Cell pellets were frozen at -80°C until needed. All subsequent protein purification procedures were performed on ice with pre-chilled buffers or in a 4°C cold box. Frozen cell pellets were thawed on ice and resuspended in 10 ml of Lysis

Buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 10 mM $MgCl_2$). Resuspended cells were then lysed by two passes through a French press. Cell lysates were spun for 30 minutes at 12,500 rpm at 4°C in a Sorvall SS-34 rotor to remove cell debris, and cleared lysates were loaded onto a column containing 5 ml of NiNTA resin (Qiagen) that had been pre-equilibrated in Lysis Buffer. Sample was pulled through the column with a peristaltic pump at 0.5 ml per minute. The column was washed with 25 ml of Wash 1 (Lysis Buffer with 500mM NaCl and 50 mM imidazole), and 25 ml of Wash 2 (Lysis Buffer with 25 mM imidazole). The His-tagged protein was eluted with Elution Buffer (Lysis Buffer with 250 mM imidazole) and collected in 1 ml fractions. Following SDS PAGE analysis, appropriate fractions were pooled and dialyzed against Soj Dialysis Buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol). Dialyzed protein was frozen at -80°C in aliquots until needed.

WT and mutant His-ParA fusions (expressed from pCMN310 in JS238) were purified by the same method as His-Soj and stored in the same buffer.

ATPase Assays

The Soj ATPase assay was carried out using a protocol described previously for detection of free $^{32}P_i$ (Mukherjee *et al.*, 1993). The ATPase activity of wild type His-Soj and the His-Soj mutants (5 μM) was assessed in Soj ATPase Buffer (25mM Tris, pH 7.6, 5 mM $MgCl_2$, 50 mM KCl, 10% glycerol, 1 mM DTT), and specific activity was determined from the amount of released $^{32}P_i$. The reaction was started with the

addition of 1 mM [γ-^{32}P]ATP. Reactions were carried out at 37°C, and released $^{32}P_i$ was measured at 10, 20 and 40 minutes after the addition of [γ-^{32}P]ATP. Specific activity was measured between 10 and 40 minutes.

Size exclusion chromatography

Wild type and mutant His- Soj or His-ParA proteins were analyzed by size-exclusion chromatography on an AKTA-fast protein liquid chromatograph equipped with a Superdex 75HR column at room temperature. Samples (500 μl) of His-Soj or ParA (0.4-0.5 mg) were warmed to room temperature for 20 minutes and loaded on to the column in dialysis buffer, or they were pre-incubated for 20 minutes at room temperature after the addition of nucleotide to 0.2 mM and $MgCl_2$ to 2 mM (to account for 1 mM EDTA in dialysis buffer). After incubation at room temperature, the samples were loaded onto the pre-equilibrated column and eluted at room temperature with FPLC Running Buffer (50 mM Tris, pH 7.5, 100 mM NaCl, and either 1 mM EDTA or 0.2 mM appropriate nucleotide and 2 mM $MgCl_2$).

DNA binding assays

Electrophoretic mobility shift assays were performed as described previously. We used pUC18 plasmid (2.7 kb) at a final concentration of 12.6 nM. Reaction components (except Soj or ParA) were dispensed into individual reaction tubes from a concentrated master mix. Soj Dilution Buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM DTT, 10% glycerol) was added as needed to give a final reaction volume of 10

μl, then Soj or ParA was added to yield the final concentrations indicated. Reactions were incubated for 10 minutes at room temperature prior to being run on a 1% agarose gel as described by Leonard *et al*. (Leonard *et al.*, 2005a).

Sporulation assays

Fresh single colonies of appropriate *B. subtilis* strains were inoculated into 2 ml of DSM (Difco sporulation media) and incubated in a roller at 37°C for approximately 27 hours. Viable cell counts were determined by plating appropriate dilutions on LB agar. Heat-resistant spores were determined by heating samples to 80°C for 20 minutes and then plating appropriate dilutions on LB agar.

Docked model

The model for Soj bound to DNA was created using chains A and B of the Soj dimer structure (2BEK) and the DNA strand from the SRF core complex (1SRS). This DNA was also used to create the manually docked AbrB/DNA complex shown in Bobay *et al*. (Bobay *et al.*, 2005). The isolated chains were manually docked in DeepView/Swiss-PdbViewer (http://www.expasy.org/spdbv/) (Guex and Peitsch, 1997), and a pdb file of the roughly docked structure was created. The roughly docked structure was subjected to five rounds of conjugant gradient minimization in CNS using model_minimize (Brunger *et al.*, 1998). Each round consisted of 200 minimization steps, and a non-bonded cutoff of 13 Å was used. Dielectric constants of 1 and 10 were used, and there were no significant differences in the minimized

models. Figures were created using PyMol (DeLano Scientific; (DeLano, 2002)).

Chapter III: Requirements for dynamic localization of Soj in *E. coli*

Abstract

Soj (ParA) from Bacillus subtilis has been shown to display dynamic localization (Marston and Errington, 1999; Quisel *et al.*, 1999). To better understand the behavior of Soj and the requirements for this behavior, the localization of GFP-Soj was studied in *E. coli*. GFP-Soj colocalized with the nucleoid in *E. coli* and oscillated from one end of the cell to the other on the DNA on a timescale of minutes, as in *B. subtilis*. Localization of Soj to the nucleoid was independent of Spo0J or *parS*, but movement was dependent on both. As we previously reported, expression of GFP-Soj in *E. coli* resulted in disruption of nucleoid morphology and chromosome segregation defects. These problems were not apparent when Spo0J was present, with or without *parS*. Notably, the requirements for oscillation in *E. coli* are the same as the requirements for plasmid stabilization by this system in *E. coli* as reported by Yamaichi and Niki (Yamaichi and Niki, 2000). These results suggest that the movement of Soj may be linked to its role in plasmid and perhaps chromosome segregation.

Introduction

In *B. subtilis*, fusions of GFP to the N- or C-terminus of Soj revealed that the protein was dynamically localized within the cell (Marston and Errington, 1999; Quisel *et al.*, 1999). The fusions were asymmetrically distributed on the nucleoids and poles of the cell. Over time, they could be observed to move from one end of the cell to the other.

Since this behavior was described for Soj, other ParA family members have been shown to undergo similar changes in localization *in vivo* (Ebersbach and Gerdes, 2001; Fogel and Waldor, 2006; Lim *et al.*, 2005). This movement is dependent on the presence of a functional Spo0J (ParB) and occurs on a timescale of minutes without a regular periodicity.

The importance of this behavior in Soj/ParA function is unclear. However, mutations in Soj that disrupt movement also prevent the Soj-mediated block in sporulation that is observed in the absence of Spo0J (Quisel *et al.*, 1999). In addition, mutations in Spo0J that prevent Soj movement result in constitutive repression of sporulation by Soj (Autret *et al.*, 2001), indicating that the dynamic localization of Soj may be important for proper regulation of sporulation. The prevailing hypothesis to explain Soj movement is that Soj and Spo0J work together to sense the state of the replicated and segregated chromosomes prior to sporulation. If chromosome segregation has not occurred properly, Soj remains bound to and represses expression from early sporulation promoters, whereas, if all is well, Spo0J relieves Soj-mediated repression of these promoters, allowing sporulation to occur (Quisel *et al.*, 1999).

Soj, Spo0J and *parS* can be used to stabilize a *ΔsopABC* miniF plasmid in *E. coli* in the absence of any other *B. subtilis* elements (Yamaichi and Niki, 2000). It is not clear how the ability of Soj and Spo0J to maintain a plasmid relates to chromosome segregation or any other roles these proteins may play in *B. subtilis*. It is possible that

the dynamic behavior of Soj is required for plasmid segregation, but the behavior of Soj in *E. coli* has not previously been described.

Soj movement in *B. subtilis* can be compared to that of another member of the ParA family of ATPases: MinD from *E. coli*. The Min system consists of a set of three proteins (MinC: the effector; MinD: the carrier; and MinE: the topological specificity factor) which govern the placement of the division septum. These three proteins undergo a coupled oscillation such that the time-averaged concentration of the cell division inhibitor MinC is lowest at midcell (Figure 5) (Lutkenhaus, 2007; Meinhardt and de Boer, 2001; Rothfield *et al.*, 2005). GFP-fusions of both Soj and MinD are asymmetrically localized within the cell (Soj, mainly on the DNA, and MinD on the membrane), and the movement of both is dependent on functional ATPase activity as well as the presence of a partner protein (Spo0J for Soj, and MinE for MinD) (Hu and Lutkenhaus, 2001; Lutkenhaus and Sundaramoorthy, 2003; Marston and Errington, 1999; Quisel *et al.*, 1999). As Soj can be used to perform plasmid partitioning functions in *E. coli* in the absence of other *B. subtilis* proteins, we sought to determine the behavior of GFP-Soj in *E. coli*.

Results

GFP-Soj oscillates on the nucleoid in E. coli

When GFP-Soj was expressed in W3110 from pCMN003 in the context of the operon (P_{lac}::*gfp-soj spo0J parS*), it was asymmetrically localized on the nucleoids in some

of the cells (Figure 9A). Unlike in *B. subtilis*, GFP-Soj was never detected at the poles of *E. coli* cells. Observation over time revealed that the GFP-Soj in some of these cells was moving from one end of the cell on the DNA to the other over the course of several minutes (Figure 10). As no other *B. subtilis* proteins or genetic material outside of the *soj* and *spo0J* genes were present in the strain, the components required for GFP-Soj oscillation are either conserved in *E. coli* or are contained within the *soj* and *spo0J* coding sequences. This result is consistent with the requirements for plasmid stabilization by Soj, Spo0J, and *parS* (Yamaichi and Niki, 2000). There is a single *parS* sequence in these constructs, and it is located within the *spo0J* gene.

Spo0J is required for GFP-Soj oscillation

To further characterize the requirements for GFP-Soj movement in *E. coli*, the localization of GFP-Soj expressed from pSEB200 (P_{lac}::*gfp-soj*) was assessed. In these cells, the GFP-Soj colocalized with the DNA throughout the entire length of the cell. If movement of GFP-Soj was occurring, it was obscured by the uniform fluorescence on the nucleoids (Figure 11A).

To assess the requirement for Spo0J, GFP-Soj was co-expressed with the Spo0J13 (R80A) mutant (Autret *et al.*, 2001). This mutant of Spo0J does not support oscillation of GFP-Soj in *B. subtilis*. It is thought to be defective in interaction with Soj as it does not prevent the Soj mediated sporulation block. Also, unlike wild type Spo0J-GFP, GFP-fusions to Spo0J13 do not form condensed foci in *B. subtilis* in the

Figure 9. GFP-Soj localization with (A) wild type Spo0J and *parS*, (B) with Spo0J13 and wild type *parS*, and (C) with wild type Spo0J and the mutant *parS*-6 sequence. Note that the asymmetric localization of GFP-Soj is only observed when wild type Spo0J and *parS* are present in A.

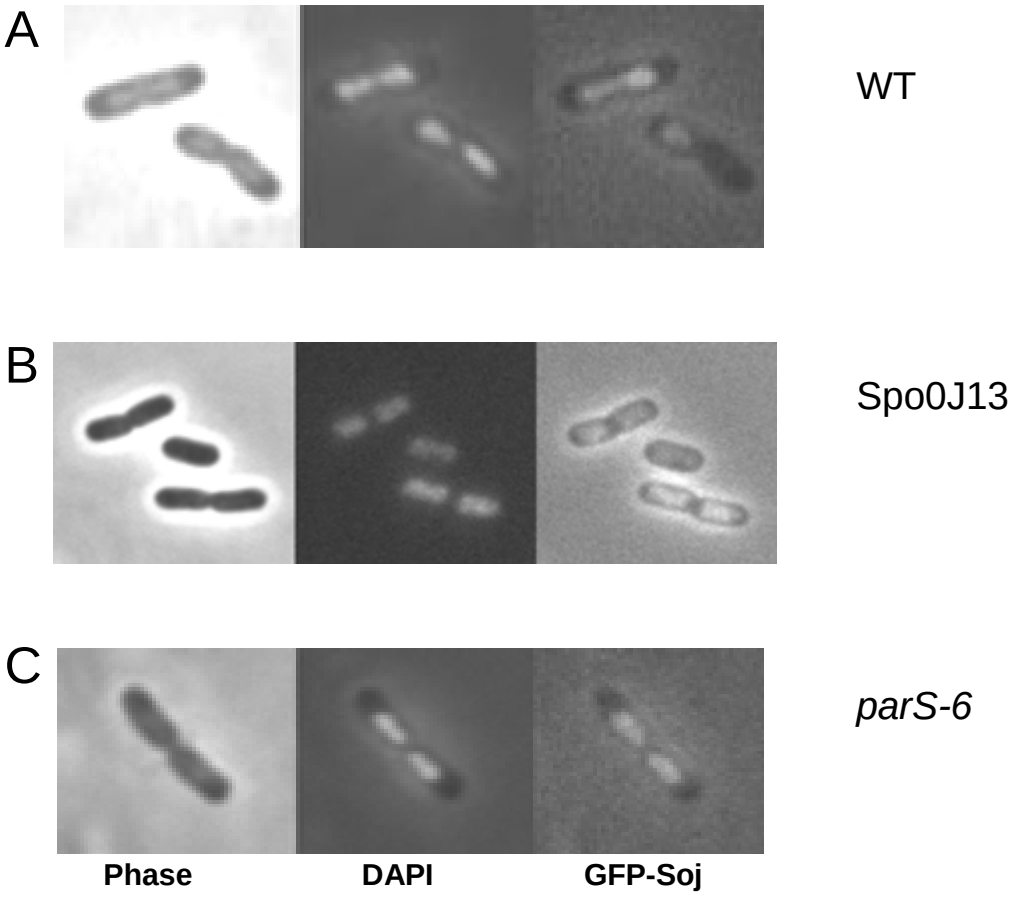

A
WT
B
Spo0J13
C
parS-6
Phase
DAPI
GFP-Soj

Figure 10. GFP-Soj movement on the DNA in *E. coli* cells. Expression of GFP-Soj with Spo0J was induced with 50 μM IPTG from pCMN003 for at least one hour. Images were taken at five minute intervals as indicated.

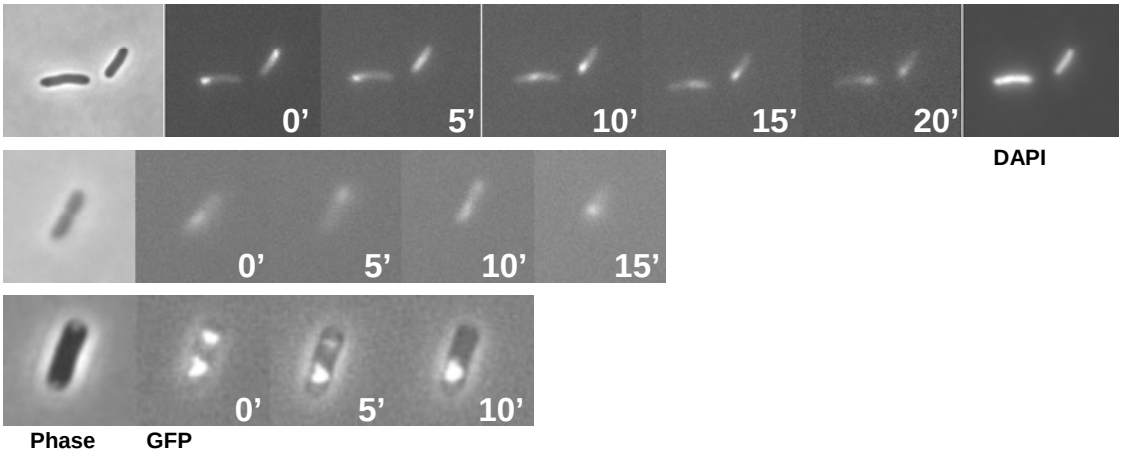
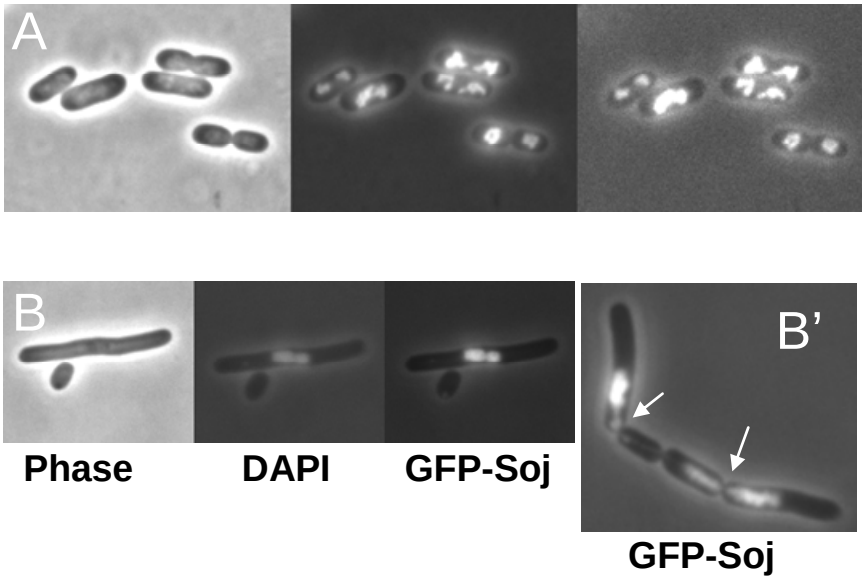

0’
5’
10’
15’
20’
DAPI
0’
5’
10’
15’
0’
5’
10’
Phase
GFP

Figure 11. GFP-Soj statically colocalizes with the DNA in the absence of Spo0J and distorts the nucleoid. (A) GFP-Soj expressed in W3110 from pSEB200. Cells shown are in early exponential phase, approximately 1 h after induction with 50 μM IPTG. (B) GFP-Soj as in *B* except cells are in early stationary phase several hours after induction of GFP-Soj expression. (B') Additional example showing that GFP-Soj induced nucleoid distortion, guillotined nucleoids (arrows), filamentation and anucleate cells. B' is GFP fluorescence, but DAPI staining was identical.


A
B
B'
Phase
DAPI
GFP-Soj
GFP-Soj

presence of Soj. When GFP-Soj was coexpressed with Spo0J13 in JS238 from pCMN003-Spo0J13, GFP-Soj colocalized with the nucleoids and did not display movement (Figure 9B).

An intact parS *sequence is required for GFP-Soj oscillation*

The dynamic behavior of Soj has been examined in *B. subtilis*, but the behavior of GFP-Soj has not been examined in *B. subtilis* lacking more than one of its ten *parS* sites. Our simplified system allowed us to determine whether or not a *parS* site was essential for Soj movement. There is one *parS* sequence within the *spo0J* gene which is the only site present on the pCMN003 plasmid. This *parS* site was mutated by site directed mutagenesis at positions which left the amino acid sequence of Spo0J intact. When GFP-Soj and Spo0J were induced from the resulting plasmid, pCMN003 parS-6, the GFP-Soj signal was symmetrically and evenly distributed on all DNA throughout the cells (Figure 9C). No movement was detectable, revealing that *parS* is required for oscillation and that no sequence on the *E. coli* chromosome was sufficient to support GFP-Soj oscillation.

GFP-Soj causes defects in chromosome segregation in E. coli

Interestingly, after a few generations of growth in the presence of 50 μM IPTG, cells expressing GFP-Soj from pSEB200 began exhibiting chromosome segregation defects apparent because of aberrant nucleoid morphology (Figure 11B and B'). In some cells, the DNA became extremely condensed, while in others, it appeared to be

stretched across the cell. Guillotining of the chromosomes was frequently detected as septa formed and closed over DNA, suggesting that overexpression of GFP-Soj disrupts nucleoid occlusion by interfering with or preventing the activity of SlmA, the *E. col*i protein that prevents division from occurring over the nucleoid (Bernhardt and de Boer, 2005) (Arrows, Figure 11B'). Anucleate cells began to form as well as cells bearing irregularly shaped DNA fragments. These morphological effects were apparent when the culture was kept in exponential growth or allowed to enter into stationary phase, although more of the population exhibited these problems when the culture was allowed to enter into stationary phase. When GFP-Soj was coexpressed with Spo0J, Spo0J13, or Spo0J and the altered *parS* site, the cells did not display the severe phenotype seen with GFP-Soj alone (Figure 9).

DNA binding is required for Soj oscillation

We have identified mutants defective for nucleoid binding by Soj (Hester and Lutkenhaus, 2007). We assessed the behavior of GFP-fusions of these mutants in the presence of Spo0J. GFP-Soj R189E, R189A, and R218E expressed with Spo0J were all uniformly fluorescent in the cytoplasm of the cell, just as they were in the absence of Spo0J (data not shown). R218A, however, was static on the DNA without Spo0J but moved on the DNA in the presence of Spo0J and *parS* (data not shown).

Discussion

The results shown here indicate that Soj, Spo0J and *parS* from *B. subtilis* can support Soj movement in *E. coli* in the absence of any other *B. subtilis* elements. This is significant in light of the fact that this system can be used in *E. coli* to maintain a plasmid. It is possible that this *parABS* system alone is sufficient for the function of plasmid segregation. Alternatively, proteins required for the function of Soj, Spo0J and *parS* in segregation are conserved in *E. coli*. Although movement was not observed in all cells, it is possible that this movement provides a clue as to the nature of the partitioning mechanism employed by Soj and Spo0J in *E. coli*.

As the role of Soj and Spo0J in *B. subtilis* chromosome segregation is not understood, it is possible that there is something to take from their behavior in *E. coli*. This movement was not observed when *parS* was altered or when the Spo0J13 mutant was present, providing evidence that Spo0J and *parS* are both involved in the establishment of Soj movement. As with other *par* systems, it seems likely that Spo0J binds to *parS* forming a functional complex that is important for partitioning and governing the behavior of Soj. From our results, it is also apparent that no sequence on the *E. coli* chromosome is similar enough to *parS* to support Soj oscillation, or perhaps our data raise the possibility that *parS* must be in *cis* to either Soj or Spo0J or to both.

Movement is also not observed unless Soj can bind to the nucleoid, most likely because the cooperative binding of Soj to the substrate of the nucleoid is required for

oscillation to be initiated. This lack of movement has been observed with nucleotide binding mutants of Soj in *B. subtilis*, as well (Quisel *et al.*, 1999). We have verified that GFP-Soj G12V and GFP-Soj K16Q are in the cytoplasm localized and do not oscillate in *E. coli* (data not shown). Both the nucleotide binding mutants and the DNA binding mutants are unable to support plasmid segregation in *E. coli* and cannot block sporulation in *B. subtilis*. This indicates that the dynamic localization of Soj on the DNA involves the ATPase and is important for its function in both processes.

The fact that Spo0J13 could prevent chromosome segregation defects in *E. coli* suggests that it may actually interact with Soj. If so, it indicates that any interaction between GFP-Soj and Spo0J13, while sufficient to prevent chromosome segregation defects, is not sufficient to support oscillation, and that Soj and Spo0J may interact differently for different purposes. Another possibility is that the presence of Spo0J has an effect on the level of GFP-Soj expression that would be the result of binding to *parS* and spreading in either direction from this site, resulting in repressed transcription of the operon. However, recent evidence suggests that transcriptional repression does not occur in *B. subtilis* as the result of Spo0J spreading (Breier and Grossman, 2007). Whether or not transcription is affected by Spo0J spreading in *E. coli* is not known, although Western analysis of GFP-Soj levels in the strains and inducer concentrations used here (50 μM IPTG for pSEB200 and 100 μM IPTG for pCMN003, pCMN003 parS-6 and pCMN003 Spo0J13) revealed that Soj levels were just slightly lower in strains expressing both GFP-Soj and Spo0J (from pCMN003 and derivatives) in comparison to GFP-Soj alone (from pSEB200) (data not shown).

The GFP-Soj levels in all four strains were within two-fold of each other, with the highest and roughly equivalent levels of Soj found in the strains bearing pSEB200 and pCMN003 parS-6. As pSEB200 does not carry Spo0J and Spo0J can not bind to and spread from the mutated *parS* site in pCMN003 parS-6, it is possible that Spo0J has a minor effect on transcription of this operon, thus affecting protein levels in *E. coli*. However, because GFP-Soj was present at approximately the same level when expressed from pSEB200 and pCMN003 parS-6, it seems likely that Spo0J and Spo0J13 can somehow prevent Soj from causing chromosome segregation defects, even in the absence of an intact *parS* site, providing evidence for interaction between Soj and Spo0J13 despite the inability of this mutant to support oscillation or sporulation (Autret *et al.*, 2001).

As movement did not occur in every cell, it is still not clear whether or not movement is necessary for plasmid segregation. Plasmid segregation by the *parABS* system of pB171 occurred in cephalexin induced filamentous cells despite the fact that no movement of the ParA was detected in these cells (Ebersbach and Gerdes, 2004). However, it is also possible that accurate plasmid segregation only occurs in those cells where Soj displays movement. This explanation would account for the reduced efficiency of maintenance observed for the miniF plasmid when maintained by *soj spo0J parS* (Hester and Lutkenhaus, 2007; Yamaichi and Niki, 2000) The work presented here simply clarifies that movement of Soj in *E. coli* requires the same elements as plasmid segregation, Soj (ParA), Spo0J (ParB), and *parS*, and that these requirements are the same as those required to detect movement of Soj in *B. subtilis*.

It has yet to be determined whether or not the movement of Soj/ParA plays a role in the mechanism of plasmid or chromosome partitioning.

Chapter IV: Soj (ParA) DNA binding is mediated by conserved arginines and is essential for plasmid segregation and regulation of sporulation

Abstract

Soj is a member of the ParA family involved in plasmid and chromosomal segregation. It binds nonspecifically and cooperatively to DNA although the function of this binding is unknown. Here, we show that mutation of conserved arginine residues that map to the surface of *Bacillus subtilis* Soj caused only minimal effects on nucleotide dependent dimerization but had dramatic effects on DNA binding. Using a model plasmid partitioning system in *E. coli*, we find that Soj DNA binding mutants are deficient in plasmid segregation. We also find that these mutants do not suppress sporulation of *B. subtilis* in the absence of Spo0J. The location of the arginines on the Soj structure explains why DNA binding is dependent upon dimerization and was used to orient the Soj dimer on the DNA, revealing the axis of Soj polymerization. The arginine residues are conserved among other chromosomal homologues, including the ParA homologues from *Caulobacter crescentus*, *Pseudomonas aeruginosa*, *P. putida*, *Streptomyces coelicolor*, and chromosome I of *Vibrio cholerae* indicating DNA binding is a common feature of members of this family.

Introduction

In bacterial cells, the stable maintenance of low copy plasmids is achieved through the action of partitioning proteins encoded within *par* (for *par*titioning) loci. Bacterial chromosomes also have *par* loci, although they are notably absent from the genome of *Escherichia coli* and its close relatives. These loci are composed of two genes, *parA/F/M* and *parB/G/R*, and one or more *cis*-acting sites, *parS* or *parC*. *parA/F/M* encodes an ATPase (ParA/F/M) and *parB/G/R* encodes a DNA binding protein (ParB/G/R) which specifically binds to *parS* or *parC*. All three components are required for maintenance of low copy plasmids. The chromosomal *parAB* locus of *Caulobacter crescentus* is essential for growth (Mohl and Gober, 1997), whereas in *Bacillus subtilis*, *Pseudomonas putida*, *P. aeruginosa*, *Streptomyces coelicolor*, and *Vibrio cholerae* (chromosome I *parAB*), the locus is not essential. In these organisms, disruption primarily causes segregation defects during developmental shifts such as sporulation or entry into stationary phase (Bartosik *et al.*, 2004; Ireton *et al.*, 1994; Kim *et al.*, 2000; Lewis *et al.*, 2002; Saint-Dic *et al.*, 2006).

Based upon the sequence of the ATPase *par* loci have been divided into two groups (Gerdes *et al.*, 2000; Gerdes *et al.*, 2004). In Type I loci, the ATPase, generally called ParA or ParF, is related to MinD, a protein involved in spatial regulation of cell division. In Type II loci, the ATPase, called ParM, is related to actin and the understanding of how these loci function to mediate plasmid segregation is more advanced. Studies *in vivo* demonstrate that ParM from the R1 plasmid forms dynamic

filaments which can extend across the length of the cell and have plasmids associated with their ends (Campbell and Mullins, 2007b; Moller-Jensen *et al.*, 2002; Moller-Jensen *et al.*, 2003). Studies *in vitro* reveal that ParM filaments undergo dynamic instability in the presence of ATP and that ParM filaments are stabilized by plasmids with ParR bound to the *parC* site (Garner *et al.*, 2004). Subsequent growth of the stabilized filament forces the plasmids to the poles of the cell ensuring inheritance by both daughter cells.

The mechanism of action of Type I systems is less clear, although a mechanism similar to that observed with Type II systems has been suggested and is supported by some evidence. Several ParA homologues have been shown to undergo ATP dependent assembly into polymers *in vitro* raising the possibility that ParA may mediate segregation in a manner similar to the actin-like ParM in that plasmids are either pushed or pulled apart by the dynamic assembly of the ATPase (Barilla *et al.*, 2005; Bouet *et al.*, 2007; Ebersbach *et al.*, 2006; Lim *et al.*, 2005).

GFP fusions to at least four Type I ParA ATPases (Soj, the chromosomal ParA homologue of *B. subtilis*, ParA of the *E. coli* plasmid pB171, SopA from F plasmid and ParAI from chromosome I of *V. cholerae*) have been shown to undergo movement within the cell (Ebersbach and Gerdes, 2001; Fogel and Waldor, 2006; Lim *et al.*, 2005; Marston and Errington, 1999; Quisel *et al.*, 1999). This movement is similar to that observed with MinD which oscillates between the ends of the cell in association with the membrane (Raskin and de Boer, 1999). Soj, SopA and ParA

from pB171 colocalize with the nucleoid and oscillate from one end of the nucleoid to the other or between nucleoids. ParAI from chromosome I of *V. cholerae* migrates across the cell from one end to the other (Ebersbach and Gerdes, 2001; Fogel and Waldor, 2006; Marston and Errington, 1999; Quisel *et al.*, 1999). Where examined, the ParB homologue is required for movement, while both nucleoid localization and oscillation require a functional ATPase (Ebersbach and Gerdes, 2001; Marston and Errington, 1999; Quisel *et al.*, 1999). Since oscillation of ParA homologues and plasmid partitioning requires the same components, it is possible that the oscillation of ParA homologues is important for plasmid partitioning and chromosome segregation processes.

Most plasmids and all known chromosomal loci encode Type I partitioning proteins. Replacing a plasmid *par* locus with one from the chromosome has been shown to stabilize plasmids indicating that the chromosomal *par* systems can function to mediate plasmid maintenance (Bartosik *et al.*, 2004; Dubarry *et al.*, 2006; Godfrin-Estevenon *et al.*, 2002; Yamaichi and Niki, 2000). For example, the *par* locus from *B. subtilis* containing *soj* (*parA*), *spo0J* (*parB*), and *parS* can be used to stabilize a miniF deleted for its *par* locus (*sopABC*) (Yamaichi and Niki, 2000).

Despite the known requirement for par loci on low copy plasmids, a clear role for chromosomal ParA homologues in chromosome segregation has yet to be identified. Null mutants for *parA*/*soj* and *parB*/*spo0J* are generally viable (as discussed above) and display minimal evidence of disruption of chromosome segregation. There is a

clear role for *soj* and *spo0J* of *B. subtilis* in regulation of sporulation, however, as they were initially identified for their involvement in this process. Deletion of *spo0J* results in a block in sporulation which is suppressed by deletion of *soj* (Ireton *et al.*, 1994). Soj mediates this sporulation block by repression of transcription of Spo0A dependent sporulation genes (Cervin *et al.*, 1998; McLeod and Spiegelman, 2005). Deletion of both *soj* and *spo0J* results in a return to wild type levels of sporulation (Ireton *et al.*, 1994).

Recent analysis of the chromosomal ParA homologue, Soj, from *Thermus thermophilus* revealed that it was a DNA binding protein (Leonard *et al.*, 2005a). In the presence of ATP, Soj existed in a monomer-dimer equilibrium. Analysis of the crystal structure of an ATPase deficient mutant of Soj revealed that this mutant (D44A) crystallized as a dimer with ATP, similar to the NifH dimer and to that proposed for MinD (Georgiadis *et al.*, 1992; Lutkenhaus and Sundaramoorthy, 2003). The DNA binding observed in these studies is ATP dependent, suggesting that Soj dimerized and then bound to DNA. Electron microscopy revealed that Soj bound to DNA formed a nucleoprotein filament, raising the possibility that a bound dimer recruited additional dimers to allow the spread of Soj on the DNA. This result raised the possibility that Soj was similar to MinD which is thought to bind to the membrane as a dimer and further associate to undergo surface dependent polymerization. The cooperative membrane binding along with MinE stimulation of the MinD ATPase underlie the mechanism that allows MinD to oscillate in the cell (Hu and Lutkenhaus, 2001; Lackner *et al.*, 2003; Mileykovskaya *et al.*, 2003). To explore the mechanism

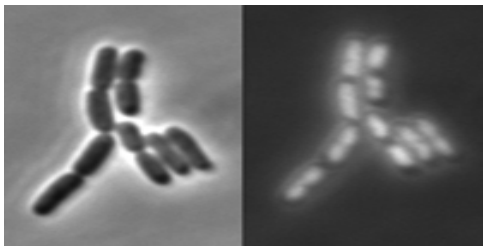
of Soj DNA binding and its role in Soj function, we sought Soj mutants deficient in DNA binding to determine whether this binding is required for Soj promoted plasmid segregation and its role in the regulation of sporulation.

Results

GFP-Soj disrupts nucleoid morphology in E. coli

Expression of GFP-Soj in *E. coli* without Spo0J results in the accumulation of GFP-Soj on the nucleoid(s). Figure 11A shows that GFP-Soj colocalizes with and distorts the nucleoid. Increased expression of GFP-Soj leads to further alterations in nucleoid morphology. After several hours of growth of W3110/pSEB200 (P_{lac}::*gfp-soj*) in the presence of IPTG, the nucleoids were noticeably condensed and the cells were elongated with extended nucleoid free regions (Figure 11B). For comparison, the nucleoid distribution in W3110 carrying pSEB181 (parent vector for pSEB200, encoding only GFP) is shown in Figure 12. In addition to the disruption of nucleoid morphology and segregation caused by overexpression of GFP-Soj, some anucleate cells were formed. Also, guillotining of the nucleoid occurred in a number of cells, indicating that the nucleoid occlusion system is no longer functioning properly (Figure 11B', arrows). The GFP-Soj always colocalized with the DNA, even when there was very little DNA within cells such as those that were the result of a guillotining event. GFP-Soj was not readily detected in the cytoplasm of any cell containing DNA or in any anucleate cells. The same effects on nucleoid morphology

Figure 12. Nucleoid distribution inW3110 cells. Nucleoids are evenly distributed throughout the length of the cell, and there is very little DNA-free space.



Phase **DAPI**

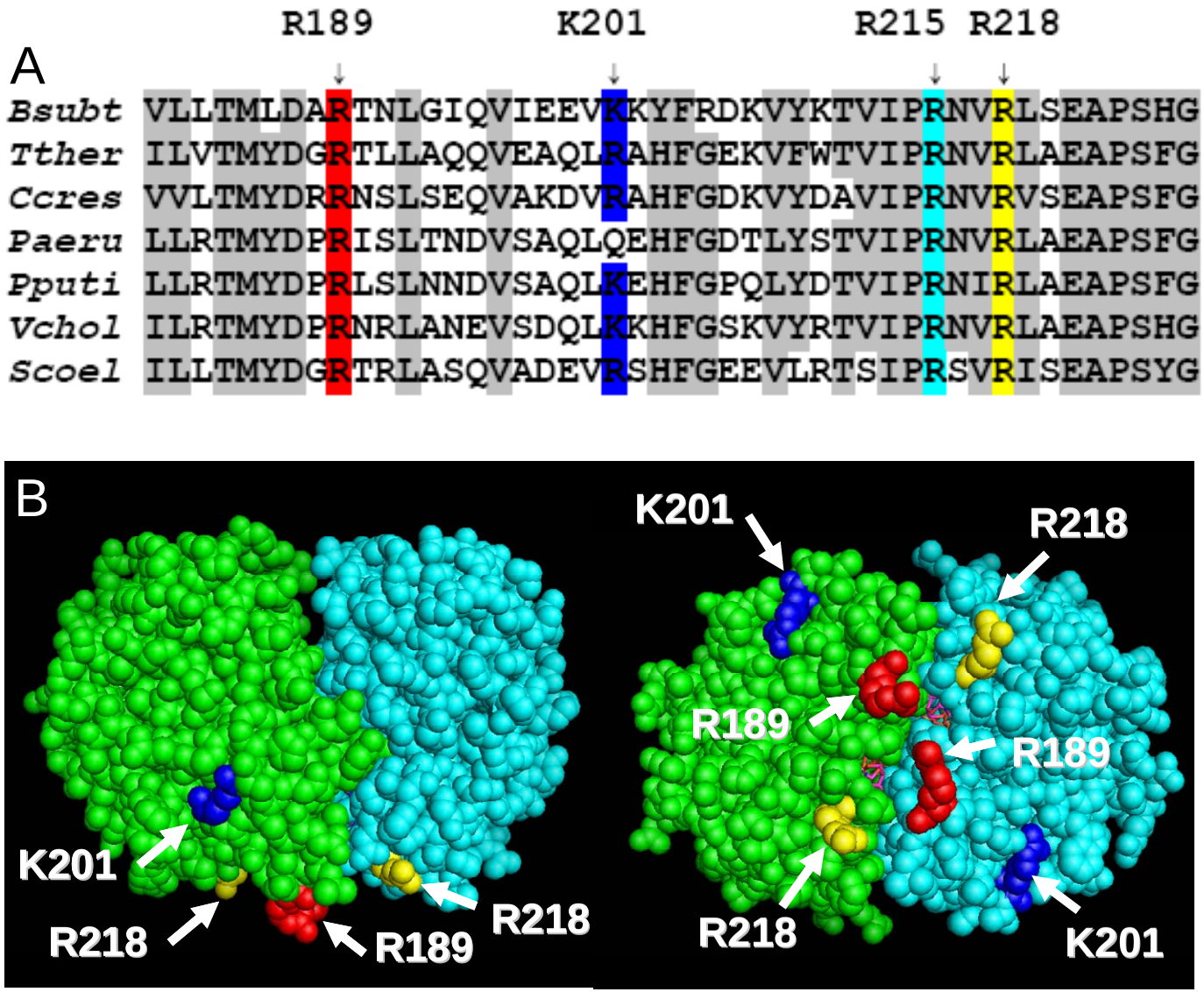
were observed with either His-tagged or untagged Soj indicating it was not peculiar to the GFP fusion (data not shown).

When GFP-Soj was coexpressed with Spo0J in the context of the operon, GFP-Soj was frequently asymmetrically localized on the DNA (Figure 9A) (Chapter III). In some cells, GFP-Soj moved from one end of the cell to the other on the DNA on a timescale of minutes as observed in *B. subtilis* (Figure 10) (Chapter III). The presence of Spo0J and *parS* were required for this movement (Figure 9; Figure 10) (Chapter III). Interestingly, when Spo0J was coexpressed with GFP-Soj, the disruptive effects of Soj on nucleoid and cell morphologies were not observed, despite the fact that GFP-Soj colocalized with the nucleoids in these cells.

Identification of conserved, surface exposed arginine residues

The solution of the *T. thermophilus* Soj crystal structure did not reveal any known structural DNA binding motifs, although Leonard *et al.* showed that the protein bound nonspecifically to DNA in an ATP dependent manner. Since proteins that bind DNA nonspecifically often do so through positively charged residues that interact with the negatively charged phosphate backbone, we aligned Soj/ParA sequences from several bacterial species. We found two essentially invariant arginine residues that mapped to the surface of the *T. thermophilus* Soj structure, R189 and R218 (*B. subtilis* numbering) (Figure 13). These arginines are not conserved in MinD or plasmid ParA sequences. A third basic residue in the same region of the primary sequence, K201 (R194 in *T. thermophilus*), was also found to be surface exposed although it was not

Figure 13. Identification of highly conserved, surface exposed basic residues. (A) A portion of an alignment of chromosomal ParA homologues is shown with conserved basic residues highlighted in color. Other conserved residues are shaded gray. (B) Conserved residues are highlighted on the dimer structure of Soj from *T. thermophilus*. The numbering is according to Soj from *B. subtilis*. R189 and R218 are near the dimer interface, whereas K201 (R194 of *T. thermophilus* Soj) sits back from the interface on the same face of the dimer. *Bsubt*, *B. subtilis*; *Tther*, *T. thermophilus*; *Ccres*, *C. crescentus*; *Paeru*, *P. aeruginosa*; *Pputi*, *P. putida*; *Vchol*, *V. cholera*; *Scoel*, *S. coelicolor*.


A
R189
K201
R215
R218
Bsubt VLLTMLDARTNLGIQVIEEVKKYFRDKVYKTVIPRNVRLSEAPSHG
Tther ILVTMYDGRTLLAQQVEAQLRAHFGEKVFWTVIPRNVRLAEAPSFG
Ccres VVLTMYDRRNSLSEQVAKDVRAHFGDKVYDAVIPRNVRVSEAPSFG
Paeru LLRTMYDPRISLTNDVSAQLQEHFGDTLYSTVIPRNVRLAEAPSFG
Pputi LLRTMYDPRLSLNNDVSAQLKEHFGPQLYDTVIPRNIRLAEAPSFG
Vchol ILRTMYDPRNRLANEVSDQLKKHFGSKVYRTVIPRNVRLAEAPSHG
Scoel ILLTMYDGRTRLASQVADEVRSHFGEEVLRTSIPRSVRISEAPSYG
B
K201
R218
R189
R218
K201
R189
R189
R218
K201
R218

as conserved as R189 and R218. Another highly conserved arginine residue in this region, R215, is not completely exposed and is involved in binding ATP (Leonard *et al.*, 2005a).

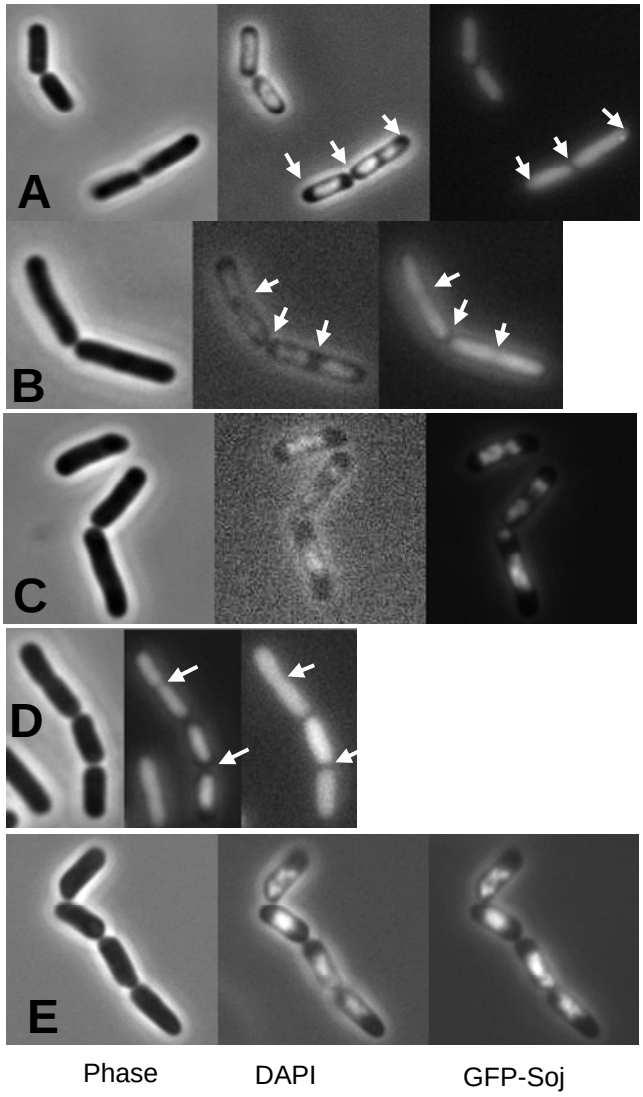
Figure 13B shows the location of these residues on the Soj dimer structure. Both R189 and R218 sit on the same face of the dimer near the dimer interface, while K201 is near this area of the dimer but is farther from the dimer interface.

Mutation of R189 and R218 disrupts DNA binding in vivo

To determine if the basic residues identified above were involved in the association of Soj with DNA, we exploited the phenotype induced by GFP-Soj expression in *E. coli*. GFP-Soj-R189E and GFP-Soj-R218E did not colocalize with the DAPI stained nucleoid (Figure 13A, D). In addition, their expression did not affect nucleoid or cell morphology. In contrast, GFP-Soj-K201E remained associated with the nucleoid and caused the morphological phenotypes observed with wild type Soj (Figure 13C).

The importance of these basic residues for DNA binding was further assessed by mutation to alanine. GFP-Soj-R189A did not colocalize with the nucleoid and both the nucleoid and cells were normal in length and overall morphology. On the other hand, GFP-Soj-R218A associated with the nucleoid and induced morphological defects similar to wild type Soj (Figure 13E). GFP-Soj-R218A was also asymmetrically localized and displayed movement in the presence of Spo0J, similar to the wild type. GFP-Soj-K201A also behaved like wild type GFP-Soj (data not

Figure 14. Effect of mutation of the basic residues on GFP-Soj localization to the nucleoid. W3110 containing pSEB200 derivatives carrying various mutations were analyzed 2 hours after induction with IPTG. (A) R189E. (B) R189A. (C) K201E. (D) R218E. (E) R218A. Arrows indicate DNA-free regions occupied by the GFP-fusions.


A
B
C
D
E
Phase
DAPI
GFP-Soj

shown). All mutant proteins were stable and expressed to approximately the same level as wild type Soj (Figure 15A). Together, these results indicate that residues R189 and R218 are involved in mediating the interaction of Soj with the nucleoid whereas K201 is not involved.

Mutations do not affect ATP- dependent dimerization

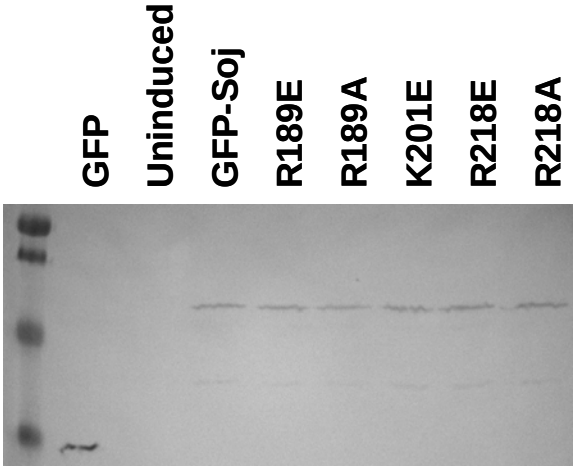
To verify that mutation of the arginine residues had no effect on binding to adenine nucleotide or nucleotide dependent dimerization, we purified the wild type and mutant proteins fused to an N-terminal histidine tag. The purified proteins were all found to have similar basal ATPase activity suggesting they were properly folded (Figure 15B). The wild type and mutant proteins were also analyzed by size-exclusion chromatography. All migrated as monomers when run with ADP or no nucleotide (Figure 16A-D). When run with ATP, the proteins eluted in the position of dimers, indicating that R189 and R218 could be substituted with alanine or glutamate without affecting dimerization. R218A also dimerizes in the presence of ATP, but a larger proportion of this protein was present as a monomer (Figure 16D).

Mutations disrupt DNA binding in vitro

To test for effects on DNA binding, the His-tagged Soj mutant proteins were purified and used in an electrophoretic mobility shift assay using linearized pUC18 DNA (2.7 kb). No shift in the migration of the DNA was detected with wild type Soj or the mutants in the presence of ADP or no nucleotide (data not shown). In the presence of

Figure 15. GFP-Soj mutants are not degraded *in vivo*, and mutations do not disrupt ATPase of His-Soj *in vitro*. (A) GFP fusions of wild type and Soj mutants are stable. Western analysis of the GFP-Soj proteins revealed that they were all stable and expressed to roughly the same level. (B) Purified histidine tagged Soj fusion proteins retained ATPase activity.

A


GFP
Uninduced
GFP-Soj
R189E
R189A
K201E
R218E
R218A

B

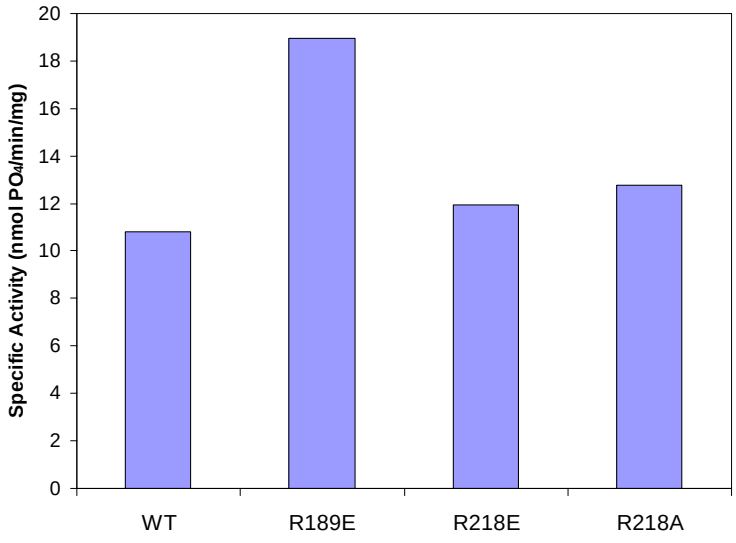
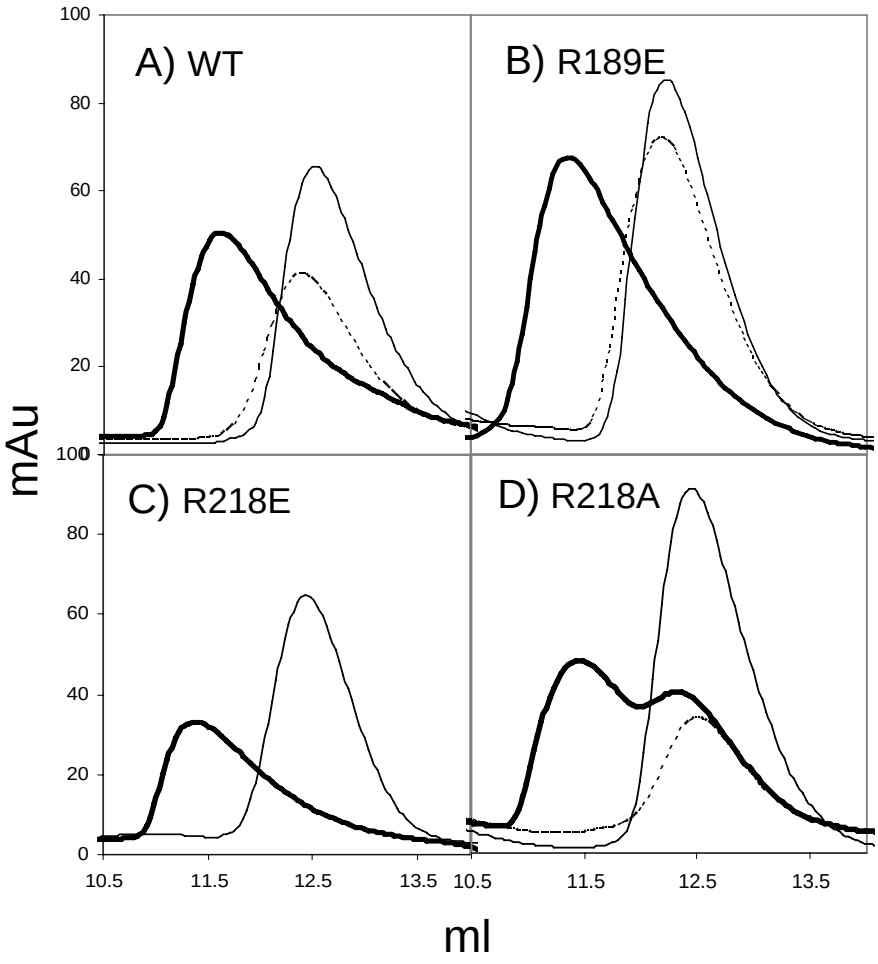

Specific Activity (nmol PO4/min/mg)
0
2
4
6
8
10
12
14
16
18
20
WT
R189E
R218E
R218A

Figure 16. Analysis of mutating basic residues on the dimerization of Soj. Size-exclusion chromatography of purified WT Soj and Soj mutant proteins (0.2 mM ATP). Dashed lines, no nucleotide; thick lines, ATP; thin lines, ADP. Elution of size standards: cytochrome *C* (12.4K), 14.3 ml; carbonic anhydrase (29K), 12.4 ml; and BSA (66K), 10.3 ml. Absorbance was monitored at 280 nm.

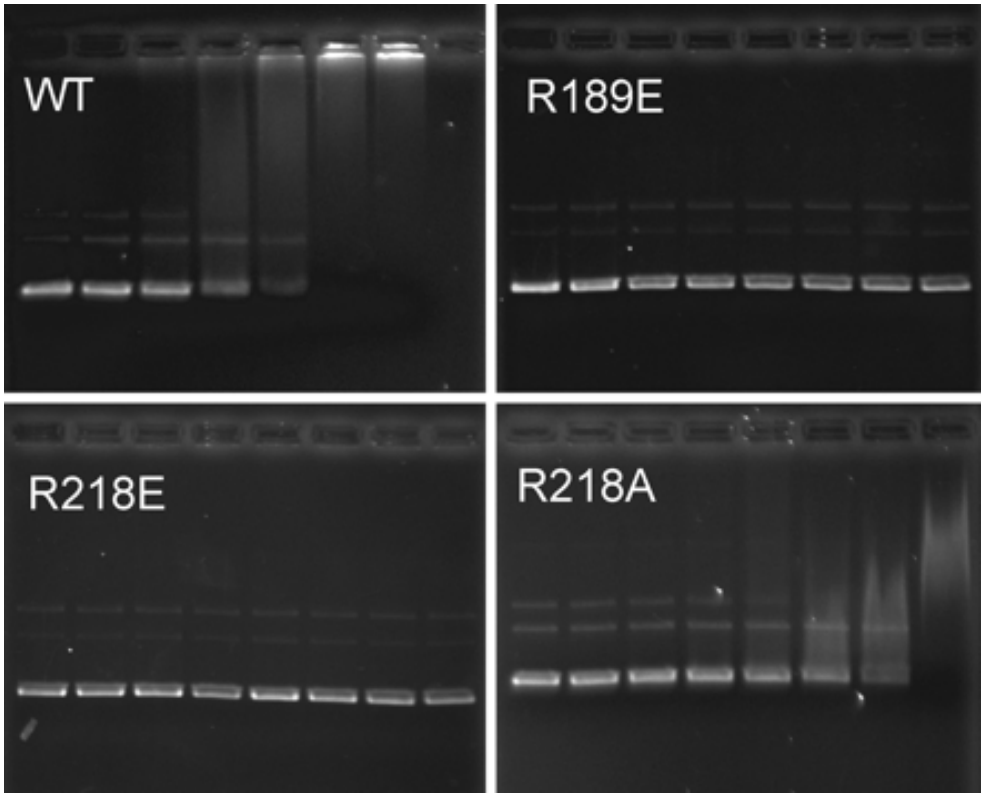

A) WT
B) R189E
C) R218E
D) R218A
mAu
ml
100
80
60
40
20
0
10.5
11.5
12.5
13.5

ATP wild type Soj prevented DNA from entering the gel at concentrations above 5 μM (Figure 17). The abrupt transition suggests cooperativity in the binding of Soj to the DNA. In contrast, the two mutant proteins that do not localize to the nucleoid *in vivo*, R189E and R218E had no effect on the migration of the DNA. Consistent with the *in vivo* localization of R218A, it still bound DNA, but a higher concentration of protein was required to produce a shift. At 7.5 μM of R218A, the DNA starts to shift, but even at 12.5 μM, a complete shift of the DNA is not observed (Figure 17). This result indicates that while the R218A mutant protein binds to DNA, it has a reduced affinity for DNA compared to wild type Soj. This reduced affinity could be the result of the less efficient dimerization observed during the size-exclusion chromatography (Figure 16D). However, it is likely due to loss of the positive charge on the dimer surface as a negative charge at this position (R218E) did not affect dimerization although DNA binding was absent.

Soj DNA binding is required for stabilization of miniF by soj spo0J parS

Yamaichi and Niki (Yamaichi and Niki, 2000) demonstrated that *B. subtilis* Soj and Spo0J along with at least one copy of *parS* could promote the stability of an unstable miniF plasmid. To determine whether or not the nonspecific DNA binding activity of Soj is important for its role in plasmid partitioning, we replaced the wild type *soj* allele in the test plasmid pXX765 with R189E and R218A. This plasmid is a miniF plasmid containing *B. subtilis soj spo0J* as the only functional partitioning locus and was used by Yamaichi and Niki (Yamaichi and Niki, 2000).

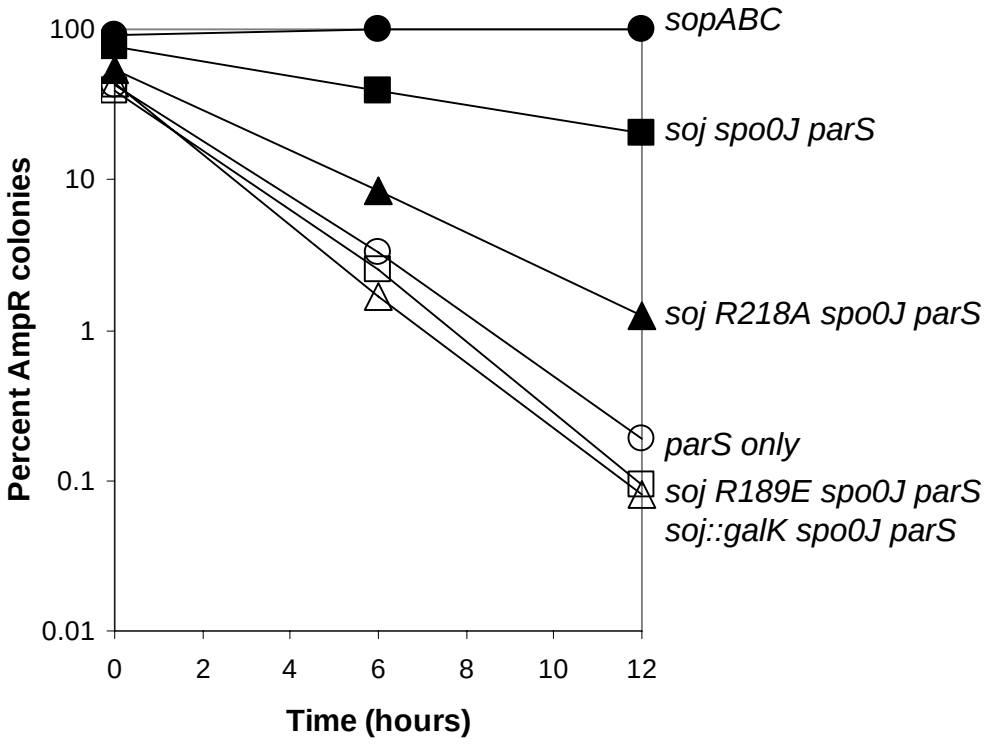
Figure 17. Electrophoretic mobility shift assay of DNA binding by WT Soj and Soj mutant proteins. Increasing amounts of WT or mutant Soj proteins were incubated with pUC18 DNA (12.6 nM) in the presence of either ADP or ATP and run on an agarose gel. Protein concentrations for each set of reactions were as follows: Lane 1, 0 μM Soj; lane 2, 1.25 μM; lane 3, 2.5 μM; lane 4, 5 μM; lane 5, 7.5 μM; lane 6, 10 μM; lane 7, 12.5 μM; and lane 8, 25 μM (not done for WT). Only results with ATP are shown. No shift was observed with ADP.


WT
R189E
R218E
R218A

To test for stability, JS238 carrying the plasmids was grown with selection, diluted into media without antibiotic, and maintained in exponential growth for 12 hours, or approximately 24 generations (Figure 18). After 12 hours, pXX765 (wild type *soj, spo0J* and *parS*) was present in ~20% of the population, consistent with the previously reported stabilization of this plasmid in *E. coli* (Yamaichi and Niki, 2000). In contrast, pCMN033 carrying Soj-R189E substituted for the wild type *soj* allele was essentially lost from the population, as were control plasmids with only *parS* (pXX764) or in which *soj* was disrupted by insertion of *galK* (pCMN032). pCMN033 R218A displayed an intermediate stability and was retained by approximately 1% of the population after 12 hours of unselected growth.

The plasmid carrying wild type *soj* (pXX765) was lost at a rate of 5% per generation, whereas pCMN033 R218A was lost at a rate of 14% per generation. pCMN033 R189E was lost at a rate of 20% per generation, as were the control plasmids pXX764 (*parS* only; 22% per generation) and pCMN032 (*soj::galK spo0J parS*; 23% per generation). It has been shown previously that the *soj* and *spo0J* genes do not affect copy number or multimer resolution of the miniF plasmid (Yamaichi and Niki, 2000). The results obtained with the above mutants indicate that DNA binding by Soj is essential to promote plasmid stability.

Figure 18. Effect of *soj* mutations on plasmid stability. The stability of miniF plasmids bearing WT or mutant Soj alleles was determined. The percentage of plasmid-bearing cells was determined for each strain after dilution into nonselective medium and growth for 0, 6 and 12 h. The average values from three experiments were plotted for pXX765, pCMN033 (R189E), pCMN033 (R218A), and pXX764, whereas the average values from two experiments were plotted for pXX704 and pCMN032.


100
10
1
0.1
0.01
Percent AmpR colonies
0
2
4
6
8
10
12
Time (hours)
sopABC
soj spo0J parS
soj R218A spo0J parS
parS only
soj R189E spo0J parS
soj::galK spo0J parS

Soj DNA binding is required for inhibition of sporulation in B. subtilis

To assess the requirement for Soj DNA binding for its behavior and function in *B. subtilis*, *soj R189E* and *soj R218A* alleles were introduced into strains AG174 (*soj*$^+$ *spo0J*$^+$; wild type *B. subtilis*), AG1505 (*Δsoj Δspo0J*), and AG1468 (*soj*$^+$ *Δspo0J*). Sporulation was assessed, and the results are shown in Table 5 and Table 6. In the absence of Spo0J, WT and R218A behaved similarly, inhibiting sporulation. AMB43 (*soj R189E Δspo0J*), however, sporulated like the wild type parental strain, indicating that DNA binding by Soj is required for its role in the inhibition of sporulation seen in the absence of Spo0J.

To determine whether or not the DNA binding deficiency of Soj R189E would cause it to have a dominant negative effect over WT Soj, sporulation efficiency of a *Δspo0J* strain bearing the native wild type *soj* allele as well as the *soj R189E* or *soj R218A* allele at the *amyE* locus were tested for sporulation efficiency. Consistent with a previous report using wild type *soj* on a multicopy plasmid in a *Δspo0J* strain (Ireton *et al.*, 1994), sporulation was inhibited more in a *Δspo0J* strain when the strain carried *soj* R218A at *amyE* and wild type *soj* at its native locus than in a *soj*$^+$*Δspo0J* strain (Table 6). A heterodiploid *Δspo0J* strain bearing *soj R189E* at *amyE* and wild type *soj* sporulated as efficiently as wild type strain (*soj*$^+$*spo0J*$^+$), however, indicating that *soj R189E* interfered with the function of *soj* in repression of sporulation. Further testing revealed that GFP-Soj (expressed from pSEB200) could be forced off

Table 5. Effects of Soj mutations on regulation of sporulation in *B. subtilis*.

Genotype	Mean % Sporulation
soj^{+} *spo0J*$^{+}$ (Wild type)	29
soj^{+} *Δspo0J*	0.74
Δsoj Δspo0J	39
R189E spo0J^{+}	42
R189E Δspo0J	71
R218A spo0J^{+}	79
R218A Δspo0J	0.76

Table 6. Sporulation in *soj* wild type/mutant heterodiploid strains.

Genotype	Mean % Sporulation
soj^{+} *spo0J*$^{+}$ (Wild type)	42
soj^{+} *Δspo0J*	0.03
Δsoj Δspo0J	39
WT soj^{+}*/R189E spo0J*$^{+}$	49
WT soj^{+}*/R189E Δspo0J*	21
WT soj^{+}*/R218A spo0J*$^{+}$	43
WT soj^{+}*/R218A Δspo0J*	0.00024

of the nucleoid and into the cytoplasm in a small percentage of cells when His-Soj R189E was expressed in *trans* in *E. coli* (data not shown).

Discussion

ParA proteins play a critical role in plasmid and chromosome segregation although the mechanism of Type I ParA homologues is not clear. In this study we have examined a ParA homologue from *B. subtilis*, which along with Spo0J and *parS* can stabilize a plasmid in *E. coli*. We found that DNA binding by Soj is essential for plasmid segregation.

DNA binding by Soj has been described; however, the region(s) of the protein responsible had not been identified. Since nonspecific DNA binding usually involves basic residues we looked for conserved arginine residues and assessed their role by examining the effects of altering these residues on the ability of Soj to condense the nucleoid *in vivo* and bind DNA *in vitro*. Our analysis points to the importance of arginine residues that lie on one face of the Soj dimer. One of these arginines, R189 appears to be essential for binding as changing it to alanine or glutamic acid eliminated DNA binding. Arginine at position 218 also plays a role since changing it to glutamate eliminated binding and changing it to alanine reduced but did not eliminate the ability of Soj to bind DNA.

While it was previously observed that Soj colocalizes with the nucleoid, a role for DNA binding in the function of this protein was unclear. We have shown here that

mutations that prevent Soj from localizing to the nucleoid also prevent it from binding DNA and supporting plasmid segregation. Notably, the degree to which the DNA binding is affected correlates with the severity of the effect on plasmid stabilization. The R189E mutation eliminated DNA binding and plasmid stabilization (~100 fold reduction); whereas the R218A mutation reduced the affinity for DNA and reduced plasmid stability (~10 fold reduction).

Soj condensation of the nucleoid is due to its ability to bind to DNA and is a convenient *in vivo* assay for DNA binding. Nucleoid condensation is also observed upon overexpression of other nonspecific DNA binding proteins (Setlow *et al.*, 1991). Interestingly, the condensation of the DNA by Soj resulted in guillotining of the nucleoid indicating that nucleoid occlusion (NOC), which prevents septation occurring over nucleoids (Bernhardt and de Boer, 2005; Wu and Errington, 2004), was suppressed. This could be the result of Soj competing with effectors of NOC (SlmA in *E. coli*, (32)) for binding to the nucleoid or to the altered nucleoid structure interfering with NOC function. This effect of Soj was ameliorated by the presence of Spo0J suggesting that Spo0J antagonizes this effect of Soj.

Model for Soj binding to DNA

In this and previous work (Leonard *et al.*, 2005a), it was shown that ATP is required for dimerization of Soj and for it to bind double-stranded DNA. Our findings indicate that the DNA binding site on the Soj surface consists of conserved arginines. Based on these findings, it was possible to envision the Soj dimer binding to DNA. The

approximate distances between R189 and R218 are similar to the distances between the phosphate backbone from the inside edge of the major groove to the nearest edge of the minor groove. Using this information and the DNA structure from the Protein Data Bank file 1SRS, a manually docked model of Soj bound to DNA was created in DeepView/Swiss-PdbViewer. This manually docked model was subjected to energy minimization to account for steric hindrances and the model depicted in Figure 18A-C was generated.

In this model the key role of R189 is clear. Upon dimerization, the two R189 residues are appropriately positioned to make contact with the phosphate backbones of the minor groove, potentially stabilizing the interaction of Soj with the DNA. According to this model, R218 interacts with the phosphate backbone of the major groove on either side of the dimer, thus illustrating how this residue could play an important role in binding to the DNA. The interaction of Soj with the DNA is likely stabilized by other undetermined residues as well. Note that K201, which is not required for DNA binding, does not come into contact with the DNA in this model. A bend in the DNA was required for R189 and R218 to be in close proximity to the phosphate backbone. The condensation of the DNA observed within *E. coli* cells overexpressing Soj could be the result of the cumulative effects of Soj-induced bending.

As shown in Figure 18A-C, the dimer interface is parallel to the DNA in our model. Our docked model also illustrates how Soj DNA binding could be cooperative. Binding by one Soj dimer provides the next incoming dimer with two sites for

Figure 19. Model for binding of Soj to DNA. (A) The docked model was created as described in *Experimental Procedures* by using chains A and B from the Soj dimer structure (2BEK) and the DNA from the SRF core complex (1SRS). The model in A was rotated to yield the views in B and C. (D) Model for polymerization of Soj dimers on DNA.

D
C
90°
B
90°
A

interaction: with the first Soj dimer and with the DNA. Also, this orientation of the Soj dimer on the DNA predicts that the dimer interface is parallel to the axis of polymerization (Figure 18D). Importantly, polymerization of Soj would lack polarity, as each side of the dimer along the DNA axis presents an equivalent polymerization interface. It will be interesting to determine if MinD also assembles in a similar manner.

DNA binding by Soj and other ParAs

Although we studied Soj and the role of DNA binding in plasmid segregation, several observations suggest that DNA binding is an important property of all chromosomally encoded ParA proteins that also extends to plasmid ParA homologues. First of all, the two arginine residues we identified here are present in many chromosomally encoded ParA homologues. Such conservation implies that their function is also conserved. The only other chromosomal ParA to be examined for DNA binding is ParA of *C. crescentus* (Easter and Gober, 2002). Although it was observed to bind single stranded DNA, binding to double stranded DNA was not observed. However, the highest concentration of ParA tested was less than1 μM, too low for binding to double stranded DNA to have been observed.

The localization of several plasmid ParA proteins has been examined following fusion to GFP. The most extensively studied is ParA of pB171, which like Soj, oscillates on the nucleoid. Deconvolution of images of the oscillation suggests that the ParA is in spiral structures (Ebersbach and Gerdes, 2004). Mutations in the

Walker A motif prevented spiral formation suggesting that ATP is required for their formation. We have purified this ParA and found that it also binds nonspecifically to DNA *in vitro* in an ATP-dependent manner similar to Soj (data not shown). Another plasmid ParA, SopA of F the plasmid, appears to be nucleoid associated in the absence of SopB and to oscillate on nucleoids when SopB and SopC are present (Lim *et al.*, 2005). It also binds DNA nonspecifically *in vitro* although that aspect of its behavior has not been explored thoroughly (Bouet *et al.*, 2007).

There are substantial differences in the primary amino acid sequences within the ParA superfamily. There is 21% amino acid identity between *B. subtilis* Soj and ParA from pB171, and there is 21% amino acid identity between Soj and SopA. Based on extensive alignments, it has not been possible to identify conserved arginine residues that may be important for DNA binding by SopA or ParA. In the absence of structural data, empirical evidence will be necessary to determine which residues are involved in nonspecific DNA binding, however, it is reasonable to suggest that the binding surface will be generated upon dimerization, as it is for Soj.

DNA binding is essential for the role of Soj in regulating sporulation

Not surprisingly, we find that the ability of Soj to bind to DNA is also necessary for its ability to regulate sporulation in *B. subtilis*. We assessed the sporulation efficiency of *B. subtilis* strains expressing Soj R189E or R218A. In the absence of Spo0J, we determined that the strains bearing the DNA binding mutant sporulated to the same level as the wild type strain, indicating that DNA binding is necessary for

the inhibition of sporulation mediated by Soj. Additionally, we found that Soj R189E acted as a dominant allele over wild type Soj, allowing normal sporulation levels when wild type Soj and Soj R189E were both present, even in the absence of Spo0J. This is most likely due to the inability of the Soj/Soj R189E heterodimer to bind to DNA. Why DNA binding is essential for the function of Soj in regulation of sporulation is not clear, however, it is possible that by disrupting the non-specific DNA binding of Soj, we have disrupted any specific DNA binding that occurs to prevent sporulation in a *Δspo0J* strain.

Assembly of proteins of the ParA family

Several studies have demonstrated that ParA proteins can polymerize *in vitro* independently of DNA (Barilla *et al.*, 2005; Bouet *et al.*, 2007; Ebersbach *et al.*, 2006; Lim *et al.*, 2005). ParF from plasmid TP228, ParA from pB171, and SopA from F have been shown to polymerize by light scattering, sedimentation, and fluorescence and electron microscopy (Barilla *et al.*, 2005; Bouet *et al.*, 2007; Ebersbach *et al.*, 2006; Lim *et al.*, 2005). The electron microscopy reveals small bundles of polymers similar to those reported in one study of MinD (Suefuji *et al.*, 2002). However, several results suggest some caution in interpreting the physiological relevance of these polymers. For instance, in another study of MinD, polymers were only observed to form on the surface of phospholipid vesicles (Hu *et al.*, 2002). In the absence of vesicles, MinD was only observed to dimerize without forming higher order complexes (Hu *et al.*, 2003). Dimerization, but not further

assembly, was observed with Soj from *T. thermophilus* in the absence of DNA (Leonard *et al.*, 2005a). When DNA was present Soj bound cooperatively to generate nucleoprotein filaments.

Bouet *et al* (Bouet *et al.*, 2007) reported that SopA polymerized in an ATP dependent fashion. The addition of double stranded DNA, but not single stranded DNA prevented the formation of polymers. Sequestering the DNA with another DNA binding protein, such as SopB, which also binds DNA nonspecifically, restored SopA polymerization. The authors suggested that SopA is stored on DNA to prevent inappropriate polymerization and that in an area of the cell where the DNA is masked, perhaps by SopB, localized polymerization of SopA occurs and can be used for the partitioning reaction. Our results suggest, at least for Soj, that the DNA binding property is required for the function of this protein in partitioning.

The polymerization of ParA homologues that has been observed in the absence of DNA may simply reflect their tendency to self associate to form dimers and higher order oligomers in the presence of ATP. Perhaps, the filament bundling observed in the absence of DNA, and presumably responsible for the light scattering and sedimentation, does not occur if DNA is present as binding to DNA may mask the bundling surface. In the cell, chromosomal and plasmid DNA are available to serve as substrates for assembly of these proteins, calling in to question whether or not polymers would have an opportunity to form independently of DNA *in vivo*.

Plasmid stabilization

In models of plasmid segregation it has been suggested that ParA is actively involved in plasmid segregation and that polymerization of ParA mediates plasmid movement. Fogel and Waldor (Fogel and Waldor, 2006) observed that following duplication of the origin and the nearby ParB-*parS* focus, one ParB-*parS* focus and associated origin remained near the pole while the other followed a cloud of ParAI as it moved to the other pole resulting in segregation of the associated origin. They proposed that the cloud represented ParAI filaments and that the ParB-*parS* complex induces the depolymerization of these filaments resulting in the movement of the ParB complex by a "Brownian ratchet" mechanism similar to the MinE ring chasing MinD off the membrane. The only change we would suggest is that this cloud of ParAI is ParAI polymerized on the DNA rather than freely polymerized ParAI. Leonard *et al*. have proposed a similar model which is supported by our findings (Leonard *et al.*, 2005b). In summary, we have identified the region of Soj involved in DNA binding, provided a model for this binding and provided evidence that this property of a ParA homologue is critical for proper regulation of sporulation in *B. subtilis* and plasmid stabilization in *E. coli*.

Chapter V: Assessment of the involvement of conserved basic residues in the interaction of pB171 ParA with DNA

Abstract

Several members of the ParA family of partitioning proteins have been shown to bind to DNA *in vitro*, and appear to colocalize with the nucleoids when expressed as GFP-fusions. We have recently shown that Soj from *B. subtilis* interacts with DNA through conserved arginine residues. As ParA from the plasmid pB171 has been observed to form helical filaments over the nucleoid in *E. coli*, we sought to determine the residues involved in the interaction of ParA with DNA. We identified three residues in one region of ParA that influenced localization of GFP-ParA in *E. coli*, although we were unable to further characterize these DNA binding mutants *in vitro*.

Introduction

Bacterial plasmids that are maintained at a low copy number can not depend on random distribution for their faithful maintenance. These plasmids encode partitioning loci which are essential for their active segregation. Partitioning loci encode an ATPase, ParA/F/M, a DNA binding protein, ParB/G/R, and contain one or more *cis*-acting DNA binding sequences, *parS* or *parC*. Most bacterial chromosomes also encode partitioning functions, although the role of these loci, if any, in bacterial chromosome segregation is not clear.

Plasmid partitioning loci are subdivided into two groups based on the nature of the ATPase (Gerdes *et al.*, 2000). Type I partitioning loci encode an ATPase with a deviant Walker A motif (ParA or ParF), while Type II loci encode an actin like ATPase (ParM). Type I loci are further subdivided based on the presence or absence of an N-terminal helix-turn-helix DNA binding domain. Type Ia ParA homologues have this extension for specifically binding to the operator region of their promoters. Type Ib ParA homologues (and ParFs) are shorter and lack this extension.

Partitioning of plasmids by both Type Ia and Ib occurs by an as yet unknown mechanism. Plasmid partitioning by Type II loci is much better understood and has been documented *in vivo* by time-lapse fluorescence microscopy (Campbell and Mullins, 2007a). ParM polymerizes between plasmids paired by ParR, thus pushing the plasmids into opposite halves of the cell. Partitioning by Type I ParA homologues may also occur as the result of polymerization of the ATPase. This mechanism could involve either free polymers or polymers bound to DNA. *In vitro* evidence for free polymers has been observed, as ParA from pB171, SopA from the F plasmid, and ParF from plasmid TP228 have all been shown to polymerize *in vitro* (Barilla *et al.*, 2005; Bouet *et al.*, 2007; Ebersbach *et al.*, 2006; Lim *et al.*, 2005). However, no clear evidence of free polymers *in vivo* has been shown to date.

A number of ParA homologues have been shown to localize to the chromosome, in some cases oscillating from one end of the cell to the other on the DNA. Recently, we determined that Soj from *Bacillus subtilis* binds non-specifically to DNA through

conserved arginine residues on its surface and that this binding was required for Soj to function in plasmid maintenance. As it is likely that other ParA homologues bind non-specifically to DNA and require this binding for their function, we sought to determine which region of pB171 ParA was involved in its non-specific DNA binding.

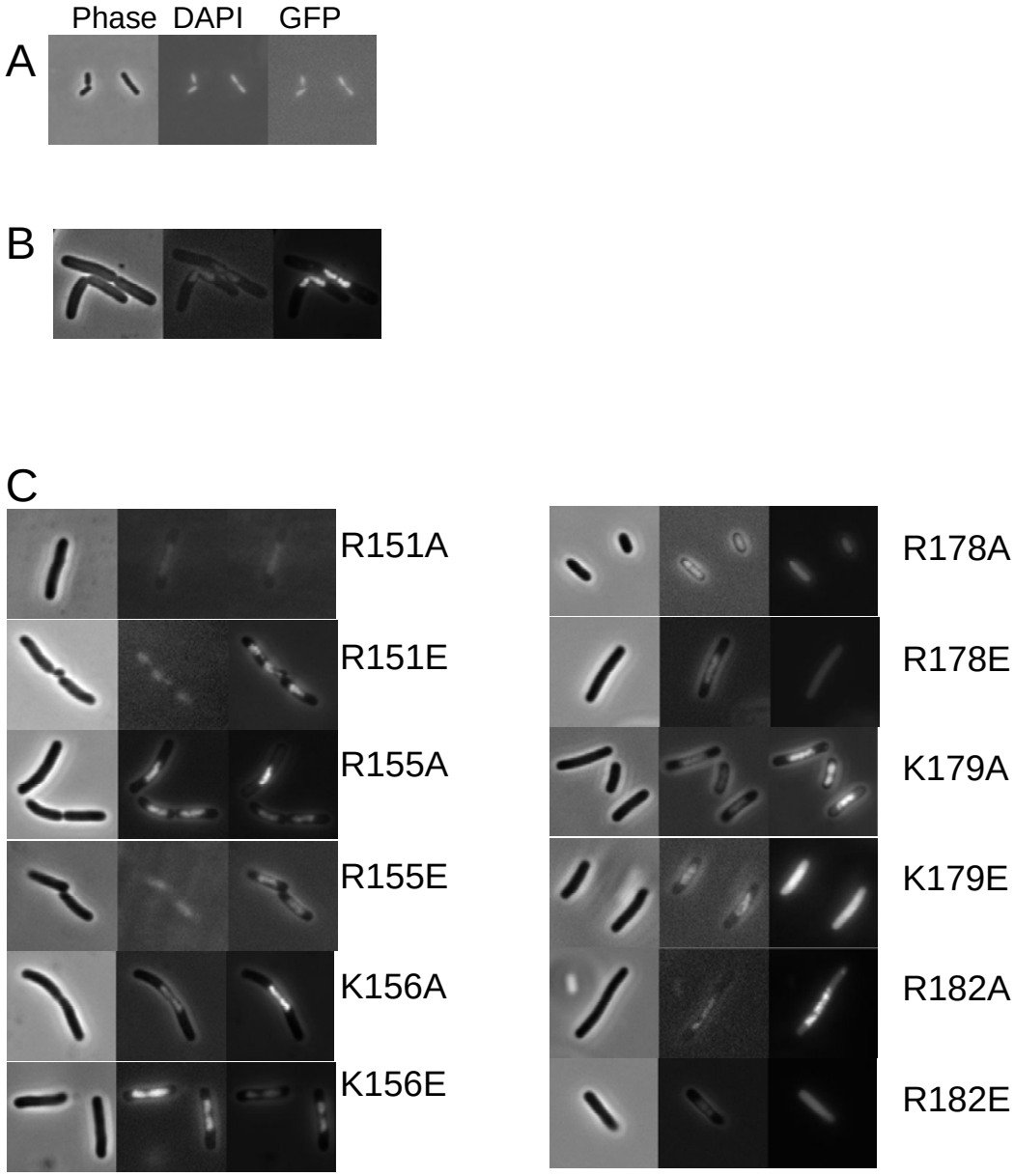
Results

ParA statically associates with DNA in vivo *in the absence of ParB*

When expressed in *E. coli*, a GFP-ParA fusion localizes to the chromosomal DNA. When coexpressed with its partner, ParB, ParA is asymmetrically distributed on the DNA and moves from one end of the cell to the other on a timescale of minutes (Ebersbach and Gerdes, 2001). We observed that in the absence of ParB, the GFP-ParA expressed from pCMN308 is statically associated with all of the DNA in the cell (Figure 20A).

We have previously observed that expression of *B. subtilis* Soj in the absence of Spo0J caused chromosome segregation defects, nucleoid condensation, and distinct morphological abnormalities in *E. coli* (Hester and Lutkenhaus, 2007). We were able to exploit this phenotype to screen for Soj DNA binding deficient mutants. Overexpression of GFP-ParA did not seem to have a dramatic effect on chromosome segregation and did not appear to cause nucleoid condensation (Figure 20A). In small cells, it was sometimes difficult to tell whether the GFP signal was colocalized with

Figure 20. Effect of mutation of the basic residues on GFP-ParA localization to the nucleoid. (A) GFP-ParA expressed from pCMN310 in JS238. ParA is statically colocalized with the nucleoid in the absence of ParB. (B) ParA nucleoid localization is much more apparent when GFP-ParA is expressed from pCMN310 in TB104 cells. (C) TB104 containing pCMN310 derivatives carrying various ParA mutants. Each panel is labeled with the relevant mutation.


Phase
DAPI
GFP
A
B
C
R151A
R151E
R155A
R155E
K156A
K156E
R178A
R178E
K179A
K179E
R182A
R182E

the DNA or was cytoplasmic. To facilitate detection of DNA binding, we overexpressed GFP-ParA fusions in TB104, a strain in which DnaA expression is under the control of the λCI857 promoter (Bernhardt and de Boer, 2005). In these cells, *dnaA* is expressed at 37°C but repressed at 30°C. At 30°C, the eventual depletion of DnaA results in a block in DNA replication and division, but cells continue to elongate. The resulting cells are elongated and have only one or two segregated nucleoids. The increased DNA-free space in these cells enabled us to more clearly determine whether a GFP fusion was bound to DNA or was cytoplasmic. In DnaA depleted TB104 cells, it is clear that the GFP-ParA is colocalized with the chromosomal DNA (Figure 18B).

Identification of residues to test for involvement in DNA binding

As Soj DNA binding is mediated by conserved arginines, we sought to test the involvement of conserved positively charged residues in DNA binding by ParA. In our studies on Soj, we were able to isolate a small number of residues to test as the residues identified in our study are highly conserved among chromosomal ParA homologues but not plasmid ParA homologues or MinDs. In addition, we had the advantage of access to structural data for the Soj homologue from *Thermus thermophilus* (Leonard *et al.*, 2005a) which allowed us to narrow our search to only those residues that are surface exposed. The sequence similarity is not significant between the ParA and Soj homologues, making it difficult to identify good candidate residues. In addition, there are many conserved arginines and lysines throughout the

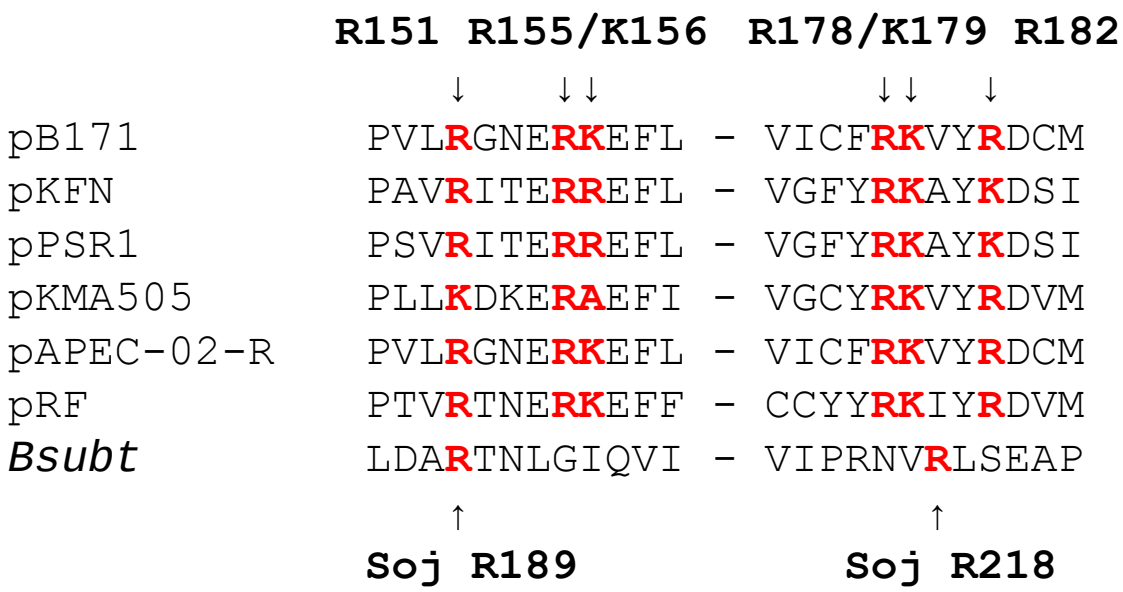
length of the ParA sequence. With the plasmid ParA homologues, no structural information is available; however, we reasoned that regions of ParA which were potentially important for DNA binding could be identified by alignment with Soj.

pB171 ParA homologues were aligned with Soj from *B. subtilis* and *T. thermophilus*. The ParA homologues used in the alignment had roughly 50-60% identity with pB171 ParA and 20-25% identity with Soj. The overall sequence identity among the ParA homologues used here is higher than that among the chromosomal ParA homologues. Because these sequences were all so closely related, it was more difficult to identify candidate residues to test in ParA than it was for the Soj homologues. Without structural information available, all residues had to be considered potentially surface exposed. We narrowed the test pool of arginines and lysines based on alignment in the same region as Soj R189 or R218. Based on the alignment, we selected six residues to test for involvement in DNA binding: R151, R155, and K156 are in the vicinity of R189; and R178, K179, and R182 aligned near R218 (Figure 20).

Mutation of three conserved residues disrupts nucleoid localization in vivo

We used site directed mutagenesis to determine whether or not the above residues were involved in DNA binding. We first mutated each residue to an alanine and looked for localization in TB104. GFP fusions to ParA R151A, R155A, K156A, K179A, and R182A were all colocalized with the chromosomal DNA, although some cytoplasmic fluorescence was also observed with R182A (Figure 20C). The R178A

Figure 21. Identification of highly conserved basic residues in pB171 ParA. A portion of an alignment of plasmid ParA homologues with *B. subtilis* Soj is shown with conserved basic residues highlighted in color. Other conserved residues are shaded gray. Plasmid name is indicated to the left of the alignment. Arrows below the alignment indicate Soj R189 and R218, and the arrows and residues listed above the alignment are those tested here.


R151 R155/K156 R178/K179 R182
pB171 PVLRGNERKEFL - VICFRKVYRDCM
pKFN PAVRITERREFL - VGFYRKAYKDSI
pPSR1 PSVRITERREFL - VGFYRKAYKDSI
pKMA505 PLLKDKERAEFI - VGCYRKVYRDVM
pAPEC-02-R PVLRGNERKEFL - VICFRKVYRDCM
pRF PTVRTNERKEFF - CCYYRKIYRDVM
Bsubt LDARTNLGIQVI - VIPRNVRLSEAP
Soj R189
Soj R218

fusion was cytoplasmic, indicating that this residue may be involved in mediating ParA DNA binding (Figure 20C).

To further assess the involvement of these residues in interaction with DNA, we changed each residue to a glutamate residue. R151E, R155E, and K156E all remained associated with the DNA (Figure 20C), indicating that mutation of these residues does not disrupt the ParA-DNA interaction. R178E, K179E and R182E, however, all displayed cytoplasmic localization (Figure 20C). Although K179E fluorescence was mainly cytoplasmic, some fluorescence overlapping with nucleoid localization was detected with this mutant.

Because degradation of the ParA portion of the fusion could release GFP and lead to the cytoplasmic fluorescence observed upon overexpression of some of the mutant ParA variants, the stability of the GFP-ParA wild type and mutant proteins was assessed by Western blotting (data not shown). Westerns were performed on cell lysates of TB104 bearing the relevant plasmid. GFP fusions were detected using an anti-GFP antibody. Full length fusions were detectable for all constructs. R155E was expressed to a lower level (roughly four-fold less) than the other fusions, but the only detectable band on the blot was the full length protein. Therefore, the fusion proteins were stable, and their localization can be reliably interpreted.

Purification of wild type and mutant ParAs

To confirm the *in vivo* results and further characterize the ParA mutants shown above to be deficient in DNA binding, N-terminal histidine-tagged fusions were purified. Unfortunately, not all of the His-tagged variants could be purified. Most formed inclusion bodies upon overexpression. The wild type and four mutants, ParA R151A, R178E, K179A and R182A, were successfully purified and analyzed for dimerization and DNA binding.

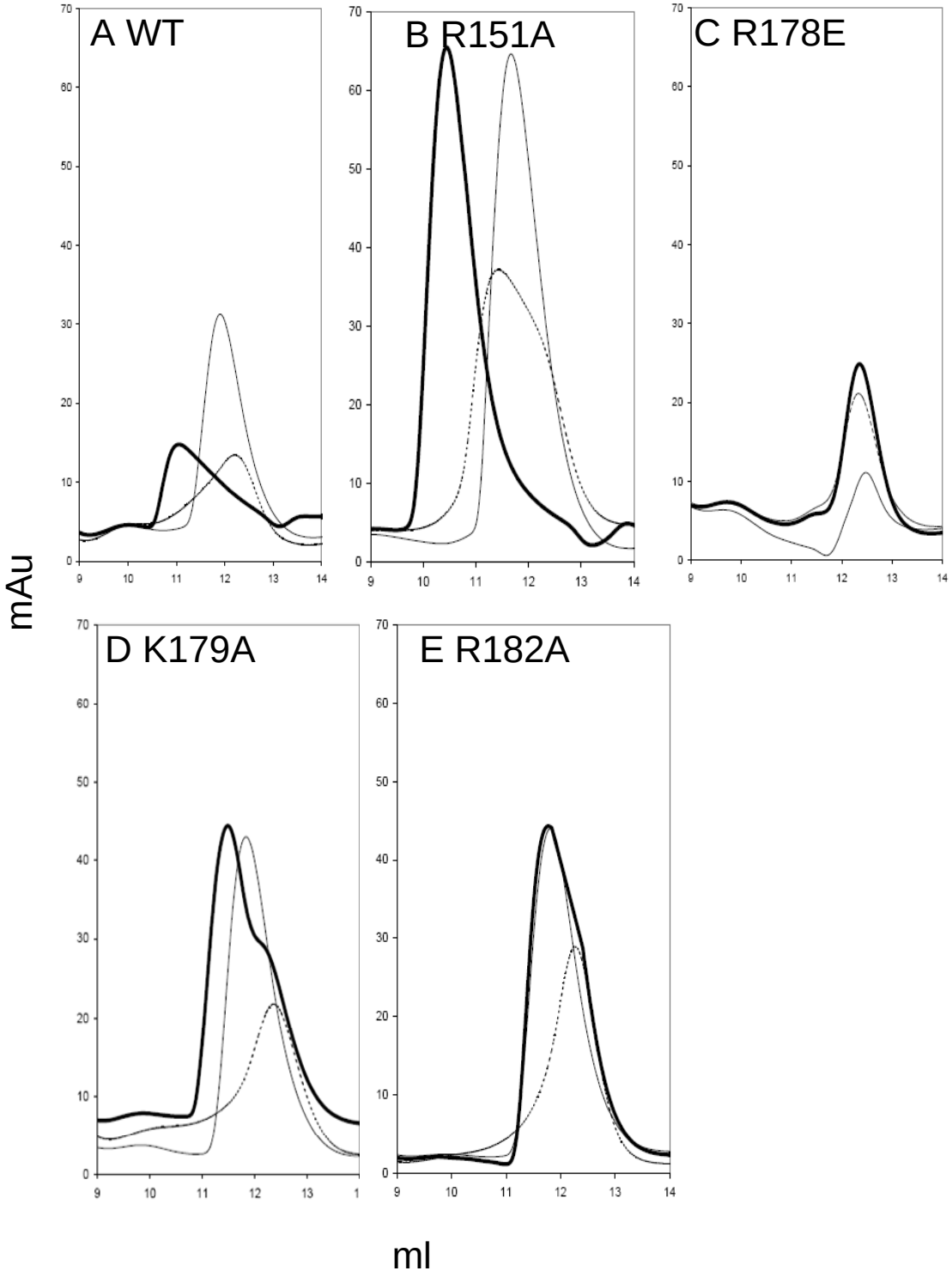
Size exclusion chromatography to assess dimerization of His-tagged ParAs

As shown in Figure 22A, wild type His-ParA ran as a dimer with ATP when analyzed by size exclusion chromatography. It ran as a monomer in the presence of ADP. Of the successfully purified mutants, only R151A and K179A, both of which display nucleoid localization *in vivo*, showed signs of dimerization in the presence of ATP (Figure 22B and D). R151A dimerization is clear as the dimer elutes at an earlier elution volume than the ADP bound monomer. The peak shift with K179A is not as dramatic, but there is a distinct shift. Neither R182A nor R178E showed any sign of dimerization with ATP (Figure 22C and E).

In vitro *DNA binding assays*

These proteins were utilized in electrophoretic mobility shift assays with supercoiled pUC18 DNA to determine whether or not they could bind to DNA *in vitro*. For these assays, protein and DNA were pre-incubated with nucleotide and run on an agarose

Figure 22. Analysis of mutating basic residues on the dimerization of ParA. Size-exclusion chromatography of purified WT ParA and ParA mutant proteins (0.2 mM ATP). Dashed lines, no nucleotide; thick lines, ATP; thin lines, ADP. Elution of size standards: cytochrome *C* (12.4K), 14.3 ml; carbonic anhydrase (29K), 12.4 ml; and BSA (66K), 10.3 ml. Absorbance was monitored at 280 nm.

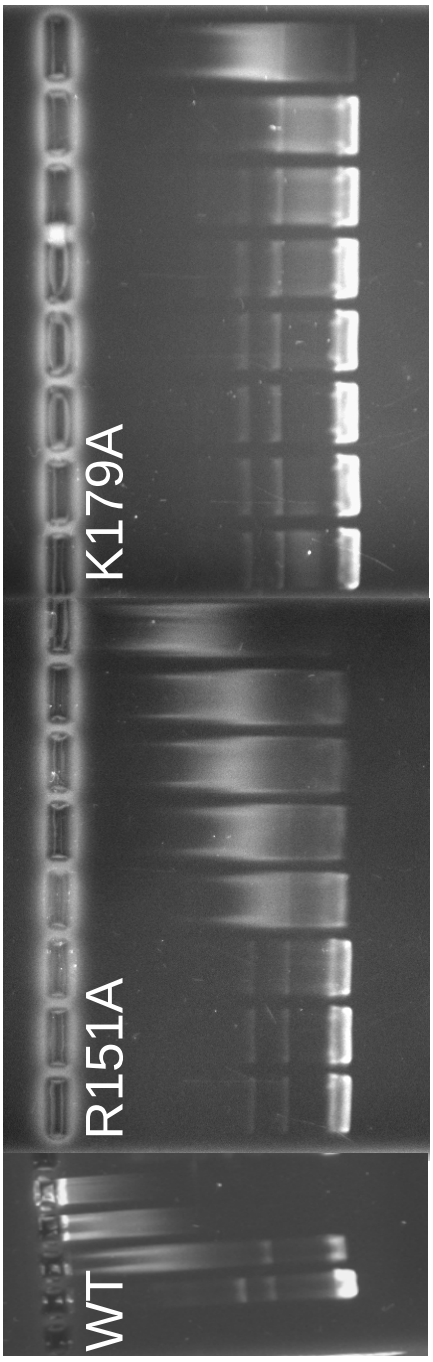

A WT
B R151A
C R178E
D K179A
E R182A
mAu
ml

gel. WT ParA bound to DNA in the presence of ATP and began to affect its migration as low as 2.5 μM, the lowest ParA concentration tested here (Figure 23). As with Soj, some of the shifted DNA began to be retained in the well as early as 5 μM, with a more or less complete shift by 12.5 μM. ParA did not bind to DNA when ADP was in the reaction (data not shown).

ParA R151A and K179A also retained the ability to bind to DNA (Figure 23), supporting their *in vivo* localization. However, both proteins displayed a reduced affinity for DNA indicated by an increase in protein required for detection of a shift. R151A begins to shift the plasmid at 5 μM, and 25 μM ParA R151A is required to see a complete shift of the DNA from its original position in the gel. While this protein clearly binds to DNA, it seems to bind it differently than WT ParA as it never causes the plasmid to be retained in the wells. ParA K179A shifts the DNA at an even higher concentration, requiring 10 μM or more protein for detectable binding. The shift at 25 μM is not as dramatic as the shift at 5 μM with the WT ParA. Neither of these proteins shifted the DNA in the presence of ADP (data not shown).

Neither R178E nor R182A affected the migration of the plasmid with ATP or ADP (data not shown), consistent with the dimerization results that these purified proteins are not in the appropriate conformation to bind DNA.

Figure 23. Electrophoretic mobility shift assay of DNA binding by WT ParA and ParA mutant proteins. Increasing amounts of WT or mutant ParA proteins were incubated with pUC18 DNA (12.6 nM) in the presence of either ADP or ATP and run on an agarose gel. Protein concentrations for the first panel with ParA are Lane 1, 0 μM; Lane 2, 2.5 μM; Lane 3, 5 μM, and Lane 4, 12.5 μM. For the mutant proteins, each set of reactions were as follows: Lane 1, 0 μM; lane 2, 1.25 μM; lane 3, 2.5 μM; lane 4, 5 μM; lane 5, 7.5 μM; lane 6, 10 μM; lane 7, 12.5 μM; and lane 8, 25 μM (not done for WT). Only results with ATP are shown. No shift was observed with ADP.


WT
R151A
K179A

Discussion

The mechanism driving plasmid segregation by Type I ParA ATPases has been elusive. Recent findings have indicated that a polymerized form of these ParA homologues may be involved in partitioning. However, there is little direct evidence for the existence of ParA filaments *in vivo*. As we had determined that Soj DNA binding is necessary for its role in the stabilization of a miniF construct, we have hypothesized that DNA binding by ParA is a direct requirement for the plasmid partitioning mechanism. In this work, we have identified ParA mutants defective in DNA binding which will enable us to test our hypothesis.

Here we have shown that another ParA homologue binds to DNA through basic residues that are highly conserved among closely related proteins. As most of the basic residues throughout the length of ParA are conserved in closely related ParA homologues, candidate residues were identified because they aligned with R189 and R218 of Soj from *B. subtilis*. The residues that seem most likely to be important for ParA DNA binding are those that align with *B. subtilis* Soj R218A in our alignments. As there are gaps in the ParA sequence and the similarity between ParA and Soj is low, it is possible that another region of ParA would contain the residue(s) analogous to Soj R189E.

The K179A mutant dimerizes with ATP but does not shift as much as the wild type or R151A mutant in the size exclusion chromatography analysis. It is possible that the K179A dimer is shorter lived due to reduced affinity of the monomers for each other,

however, this mutant is still capable of binding nucleotide. The R151A mutant, however, dimerizes very well, and binds to DNA. This mutant is predicted to be like Soj R218A since it retains the ability to bind to the nucleoid *in vivo* yet has a reduced affinity for DNA *in vitro*. Like Soj R218A, ParA R151A would be expected to have some defect in stabilization of a plasmid, although it would likely be partially functional for partitioning.

GFP-ParA R182A colocalizes with the nucleoid, indicating that it retains the ability to dimerize and bind ATP *in vivo*, so it would be expected to dimerize. However, there was some cytoplasmic fluorescence with this mutant suggesting that it might not be fully functional *in vivo*, despite the fact that it was stable. The His-tagged version of this mutant does not dimerize with ATP or display DNA binding properties *in vitro* and this may indicate further that this mutant is not properly folded. As R179E is cytoplasmic *in vivo*, its lack of dimerization could indicate that its cytoplasmic localization is the indirect result of loss of nucleotide binding or dimerization rather than loss of interaction with the DNA. As several of the mutants were unable to be purified, it is possible that the proteins that were successfully purified do not behave (*i.e.*, dimerize or bind DNA) because they are not properly folded. The addition of the histidine tag to some of these proteins may adversely affect their conformation and function more than the GFP moiety does.

This work is ongoing, and other ParA residues are being assessed for their involvement in DNA binding. In addition, some of the mutations described here are

being combined to see if an additive loss of DNA binding will further implicate these residues directly in involvement in ParA DNA binding.

Although it has been previously noted that ParA from pB171 is localized to the nucleoid, this property of this protein has not been studied to date. In addition, this protein has been shown to form polymers *in vitro*; however, there is no clear evidence of polymer formation *in vivo*. The work performed here and our recent work on Soj from *B. subtilis* points to a less straightforward mechanism for partitioning. It seems likely that plasmids are segregated by ParA polymerized on the DNA interacting with ParB/*parS* complexes. As suggested in our recent work, ParB/*parS* complexes could be pulled or pushed by ParA polymerizing and depolymerizing on the nucleoid. Plasmid pairs could be separated and pulled or pushed by a Brownian ratchet-type mechanism to separate halves of the cell, although it is still premature to present an in depth model for partitioning by Type I systems.

Chapter VI: Discussion and conclusions

Soj movement in E. coli

It was somewhat surprising that GFP-Soj displayed movement in *E. coli* in the absence of any other *B. subtilis* elements. However, the fact that *B. subtilis* Soj, Spo0J, *parS* system had previously been shown to stabilize a plasmid in *E. coli* suggested that either Soj, Spo0J and *parS* could function autonomously or that any factor that was necessary for their function was conserved in *E. coli*.

We found here that the requirements for oscillation in *E. coli* are the same as those for plasmid partitioning. Because we have studied Soj outside of *B. subtilis* (removing the complications posed by its other roles in regulation of sporulation and possibly chromosome replication and segregation), we were able to show that Soj, Spo0J and *parS* are all that is necessary for the establishment of Soj movement. By assessing the behavior of Soj in *E. coli*, we were able to assess whether or not the *parS* sequence was required for Soj movement. This has not been tested in *B. subtilis* as there are ten sites distributed around the chromosome which would have to be mutated or removed (Breier and Grossman, 2007). In our simplified system, we were able to show that *parS* is required for Soj movement. Our data also indicate that the *E. coli* chromosome either does not contain any sequence that will support the movement of Soj in the absence of *parS* or that *parS* must be present in *cis* to Soj and Spo0J for movement to occur. This work opens doors for more experimental analysis

of the fine details of the requirements for movement and, potentially, the involvement of Soj/ParA movement in the plasmid and chromosome partitioning process.

Our results also provide evidence that the interaction between Soj and Spo0J is more complex than previously thought. Surprisingly, a mutant thought to be defective in interaction with Soj, Spo0J13 (Autret *et al.*, 2001), prevented Soj mediated chromosome segregation defects in *E. coli*. This mutant does not support movement in *B. subtilis* or *E. coli* and does not allow for normal sporulation of *B. subtilis* in the presence of Soj, indicating that its interactions with Soj are modified in some way. It would be interesting to further analyze *B. subtilis* strains bearing wild type Soj and Spo0J13 to gain a better understanding of why Spo0J13 cannot complement the loss of wild type Spo0J.

As the requirements for Soj movement in *E. coli* are the same as those for plasmid partitioning, further analysis of this behavior and mutations in both Soj (ParA) and Spo0J (ParB) which disrupt this movement will be required to fully understand the purpose of movement and the nature of the Soj (ParA) structure as it undergoes movement and participates in the partitioning reaction.

Soj/ParA DNA binding

Despite extensive exploration into the mechanisms behind plasmid and chromosome partitioning, the events resulting in DNA segregation in bacterial cells remain a mystery. Here, we have identified and described a feature of ParA family members

that is likely to be very important in its function in the partitioning process, both in plasmid and chromosome segregation. DNA binding and polymerization of ParA on the DNA are likely to be essential for the function of all ParA homologues.

The most significant finding of this work is that DNA binding by Soj is necessary for its function in plasmid partitioning. None of the models for partitioning have included ParA DNA binding, but we have provided evidence here that this feature of these proteins cannot be overlooked when considering the mechanism of partitioning. We propose a model for plasmid partitioning which includes DNA binding of ParA (Figure 24). As mentioned briefly in Chapter IV, we favor including ParA polymerized on the DNA in the model for chromosome partitioning proposed by Fogel and Waldor (Fogel and Waldor, 2006) in combination with the model put forth by Leonard *et al*. (Leonard *et al.*, 2005b). The plasmids are tethered to the nucleoids by interaction with ParA polymerized on the nucleoids. As the nucleoids are segregated, replicated plasmids would be pulled apart along with the segregating nucleoid. The polymerization of Soj and the depolymerization that occurs as the result of the stimulation of the ATPase by Spo0J/*parS* are likely to be important for the direction of movement of the plasmid. Elaborating this model to include more specific details will require much more work.

Our work allows for the DNA binding of ParA/Soj to be considered when new models for partitioning are generated. Our model for how the Soj dimer binds to DNA (Figure 18) can also be tested and adjusted as experimental evidence becomes

Figure 24. Model for plasmid partitioning by tethering the plasmid to the segregating nucleoids. In this model, ParA polymerized on the nucleoid recruits and tethers the ParB bound plasmid to the nucleoid. As the nucleoids segregate, the plasmids are also segregated. This segregation event will most likely require the stimulation of the ParA ATPase by ParB, and the dynamic polymerization and depolymerization of the ParA.

ParA/Soj
ParB/Spo0J
parS

available. The model in Figure 19 can also be used as a starting point for analysis of the interactions of other ParA homologues with DNA, lipid (in the case of MinD), or other partner proteins.

DNA binding by Soj is also essential for its function in the regulation of sporulation in *B. subtilis*. Further studies are underway to determine why DNA binding is necessary for Soj to prevent sporulation in the absence of Spo0J. It remains to be seen what, if any, role DNA binding plays in chromosome segregation or regulation of replication in *B. subtilis*. It seems likely, however, that Soj DNA binding will be found to be essential for its involvement in these processes as well.

References

Abeles, A.L., Friedman, S.A., and Austin, S.J. (1985) Partition of unit-copy miniplasmids to daughter cells. III. The DNA sequence and functional organization of the P1 partition region. *J Mol Biol* **185**: 261-272.

Adachi, S., Hori, K., and Hiraga, S. (2006) Subcellular positioning of F plasmid mediated by dynamic localization of SopA and SopB. *J Mol Biol* **356**: 850-863.

Autret, S., Nair, R., and Errington, J. (2001) Genetic analysis of the chromosome segregation protein Spo0J of Bacillus subtilis: evidence for separate domains involved in DNA binding and interactions with Soj protein. *Mol Microbiol* **41**: 743-755.

Barilla, D., Rosenberg, M.F., Nobbmann, U., and Hayes, F. (2005) Bacterial DNA segregation dynamics mediated by the polymerizing protein ParF. *Embo J* **24**: 1453-1464.

Bartosik, A.A., Lasocki, K., Mierzejewska, J., Thomas, C.M., and Jagura-Burdzy, G. (2004) ParB of Pseudomonas aeruginosa: interactions with its partner ParA and its target parS and specific effects on bacterial growth. *J Bacteriol* **186**: 6983-6998.

Bernhardt, T.G., and de Boer, P.A. (2003) The Escherichia coli amidase AmiC is a periplasmic septal ring component exported via the twin-arginine transport pathway. *Mol Microbiol* **48**: 1171-1182.

Bernhardt, T.G., and de Boer, P.A.J. (2005) SlmA, a nucleoid-associated, FtsZ-binding protein required for blocking septal ring assmebly over chromosomes in E. coli. *Molecular Cell*.

Bobay, B.G., Andreeva, A., Mueller, G.A., Cavanagh, J., and Murzin, A.G. (2005) Revised structure of the AbrB N-terminal domain unifies a diverse superfamily of putative DNA-binding proteins. *FEBS Lett* **579**: 5669-5674.

Bouet, J.Y., and Funnell, B.E. (1999) P1 ParA interacts with the P1 partition complex at parS and an ATP-ADP switch controls ParA activities. *Embo J* **18**: 1415-1424.

Bouet, J.Y., Ah-Seng, Y., Benmeradi, N., and Lane, D. (2007) Polymerization of SopA partition ATPase: regulation by DNA binding and SopB. *Mol Microbiol* **63**: 468-481.

Breier, A.M., and Grossman, A.D. (2007) Whole-genome analysis of the chromosome partitioning and sporulation protein Spo0J (ParB) reveals spreading and origin-distal sites on the Bacillus subtilis chromosome. *Mol Microbiol* **64**: 703-718.

Brunger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.S., Kuszewski, J., Nilges, M., Pannu, N.S., Read, R.J., Rice, L.M., Simonson, T., and Warren, G.L. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr* **54**: 905-921.

Cam, K., Bejar, S., Gil, D., and Bouche, J.P. (1988) Identification and sequence of gene dicB: translation of the division inhibitor from an in-phase internal start. *Nucleic Acids Res* **16**: 6327-6338.

Campbell, C.S., and Mullins, R.D. (2007a) *In vivo* visualization of type II plasmid segregation: bacterial actin filaments pushing plasmids. *J Cell Biol*.

Campbell, C.S., and Mullins, R.D. (2007b) *In vivo* visualization of type II plasmid segregation: bacterial actin filaments pushing plasmids. *J Cell Biol* **179**: 1059-1066.

Casadaban, M.J., and Cohen, S.N. (1980) Analysis of gene control signals by DNA fusion and cloning in Escherichia coli. *J Mol Biol* **138**: 179-207.

Cervin, M.A., Spiegelman, G.B., Raether, B., Ohlsen, K., Perego, M., and Hoch, J.A. (1998) A negative regulator linking chromosome segregation to developmental transcription in Bacillus subtilis. *Mol Microbiol* **29**: 85-95.

Davey, M.J., and Funnell, B.E. (1994) The P1 plasmid partition protein ParA. A role for ATP in site-specific DNA binding. *J Biol Chem* **269**: 29908-29913.

Davey, M.J., and Funnell, B.E. (1997) Modulation of the P1 plasmid partition protein ParA by ATP, ADP, and P1 ParB. *J Biol Chem* **272**: 15286-15292.

Davis, M.A., Martin, K.A., and Austin, S.J. (1992) Biochemical activities of the parA partition protein of the P1 plasmid. *Mol Microbiol* **6**: 1141-1147.

Davis, M.A., Radnedge, L., Martin, K.A., Hayes, F., Youngren, B., and Austin, S.J. (1996) The P1 ParA protein and its ATPase activity play a direct role in the segregation of plasmid copies to daughter cells. *Mol Microbiol* **21**: 1029-1036.

DeLano, W.L. (2002) The PYMOL Molecular Graphics System. *DeLano Scientific, San Carlos, CA, USA*.

Dubarry, N., Pasta, F., and Lane, D. (2006) ParABS systems of the four replicons of Burkholderia cenocepacia: new chromosome centromeres confer partition specificity. *J Bacteriol* **188**: 1489-1496.

Easter, J., Jr., and Gober, J.W. (2002) ParB-stimulated nucleotide exchange regulates a switch in functionally distinct ParA activities. *Mol Cell* **10**: 427-434.

Ebersbach, G., and Gerdes, K. (2001) The double par locus of virulence factor pB171: DNA segregation is correlated with oscillation of ParA. *Proc Natl Acad Sci U S A* **98**: 15078-15083.

Ebersbach, G., and Gerdes, K. (2004) Bacterial mitosis: partitioning protein ParA oscillates in spiral-shaped structures and positions plasmids at mid-cell. *Mol Microbiol* **52**: 385-398.

Ebersbach, G., and Gerdes, K. (2005) Plasmid segregation mechanisms. *Annu Rev Genet* **39**: 453-479.

Ebersbach, G., Sherratt, D.J., and Gerdes, K. (2005) Partition-associated incompatibility caused by random assortment of pure plasmid clusters. *Mol Microbiol* **56**: 1430-1440.

Ebersbach, G., Ringgaard, S., Moller-Jensen, J., Wang, Q., Sherratt, D.J., and Gerdes, K. (2006) Regular cellular distribution of plasmids by oscillating and filament-forming ParA ATPase of plasmid pB171. *Mol Microbiol* **61**: 1428-1442.

Erdmann, N., Petroff, T., and Funnell, B.E. (1999) Intracellular localization of P1 ParB protein depends on ParA and parS. *Proc Natl Acad Sci U S A* **96**: 14905-14910.

Fogel, M.A., and Waldor, M.K. (2006) A dynamic, mitotic-like mechanism for bacterial chromosome segregation. *Genes Dev* **20**: 3269-3282.

Furste, J.P., Pansegrau, W., Frank, R., Blocker, H., Scholz, P., Bagdasarian, M., and Lanka, E. (1986) Molecular cloning of the plasmid RP4 primase region in a multi-host-range tacP expression vector. *Gene* **48**: 119-131.

Garner, E.C., Campbell, C.S., and Mullins, R.D. (2004) Dynamic instability in a DNA-segregating prokaryotic actin homolog. *Science* **306**: 1021-1025.

Garner, E.C., Campbell, C.S., Weibel, D.B., and Mullins, R.D. (2007) Reconstitution of DNA segregation driven by assembly of a prokaryotic actin homolog. *Science* **315**: 1270-1274.

Georgiadis, M.M., Komiya, H., Chakrabarti, P., Woo, D., Kornuc, J.J., and Rees, D.C. (1992) Crystallographic structure of the nitrogenase iron protein from Azotobacter vinelandii. *Science* **257**: 1653-1659.

Gerdes, K., Moller-Jensen, J., and Bugge Jensen, R. (2000) Plasmid and chromosome partitioning: surprises from phylogeny. *Mol Microbiol* **37**: 455-466.

Gerdes, K., Moller-Jensen, J., Ebersbach, G., Kruse, T., and Nordstrom, K. (2004) Bacterial mitotic machineries. *Cell* **116**: 359-366.

Ghosh, S.K., Hajra, S., Paek, A., and Jayaram, M. (2006) Mechanisms for Chromosome and Plasmid Segregation. *Annu Rev Biochem*.

Glaser, P., Sharpe, M.E., Raether, B., Perego, M., Ohlsen, K., and Errington, J. (1997) Dynamic, mitotic-like behavior of a bacterial protein required for accurate chromosome partitioning. *Genes Dev* **11**: 1160-1168.

Godfrin-Estevenon, A.M., Pasta, F., and Lane, D. (2002) The parAB gene products of Pseudomonas putida exhibit partition activity in both P. putida and Escherichia coli. *Mol Microbiol* **43**: 39-49.

Gordon, G.S., Sitnikov, D., Webb, C.D., Teleman, A., Straight, A., Losick, R., Murray, A.W., and Wright, A. (1997) Chromosome and low copy plasmid segregation in E. coli: visual evidence for distinct mechanisms. *Cell* **90**: 1113-1121.

Gordon, S., Rech, J., Lane, D., and Wright, A. (2004) Kinetics of plasmid segregation in Escherichia coli. *Mol Microbiol* **51**: 461-469.

Guex, N., and Peitsch, M.C. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* **18**: 2714-2723.

Guyer, M.S., Reed, R.R., Steitz, J.A., and Low, K.B. (1981) Identification of a sex-factor-affinity site in E. coli as gamma delta. *Cold Spring Harb Symp Quant Biol* **45 Pt 1**: 135-140.

Harwood, C.R., and Cutting, S.M. (1990) *Molecular biological methods for Bacillus* Chichester, New York John Wiley & Sons.

Hester, C.M., and Lutkenhaus, J. (2007) Soj (ParA) DNA binding is mediated by conserved arginines and is essential for plasmid segregation. *Proc Natl Acad Sci U S A* **104**: 20326-20331.

Hu, Z., and Lutkenhaus, J. (2001) Topological regulation of cell division in E. coli. spatiotemporal oscillation of MinD requires stimulation of its ATPase by MinE and phospholipid. *Mol Cell* **7**: 1337-1343.

Hu, Z., Gogol, E.P., and Lutkenhaus, J. (2002) Dynamic assembly of MinD on phospholipid vesicles regulated by ATP and MinE. *Proc Natl Acad Sci U S A* **99**: 6761-6766.

Hu, Z., and Lutkenhaus, J. (2003) A conserved sequence at the C-terminus of MinD is required for binding to the membrane and targeting MinC to the septum. *Mol Microbiol* **47**: 345-355.

Hu, Z., Saez, C., and Lutkenhaus, J. (2003) Recruitment of MinC, an inhibitor of Z-ring formation, to the membrane in Escherichia coli: role of MinD and MinE. *J Bacteriol* **185**: 196-203.

Ireton, K., Gunther, N.W.t., and Grossman, A.D. (1994) spo0J is required for normal chromosome segregation as well as the initiation of sporulation in Bacillus subtilis. *J Bacteriol* **176**: 5320-5329.

Kim, H.J., Calcutt, M.J., Schmidt, F.J., and Chater, K.F. (2000) Partitioning of the linear chromosome during sporulation of Streptomyces coelicolor A3(2) involves an oriC-linked parAB locus. *J Bacteriol* **182**: 1313-1320.

Lackner, L.L., Raskin, D.M., and de Boer, P.A. (2003) ATP-dependent interactions between Escherichia coli Min proteins and the phospholipid membrane *in vitro*. *J Bacteriol* **185**: 735-749.

Lee, E.C., Yu, D., Martinez de Velasco, J., Tessarollo, L., Swing, D.A., Court, D.L., Jenkins, N.A., and Copeland, N.G. (2001) A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* **73**: 56-65.

Lee, P.S., Lin, D.C., Moriya, S., and Grossman, A.D. (2003) Effects of the chromosome partitioning protein Spo0J (ParB) on oriC positioning and replication initiation in Bacillus subtilis. *J Bacteriol* **185**: 1326-1337.

Lee, P.S., and Grossman, A.D. (2006) The chromosome partitioning proteins Soj (ParA) and Spo0J (ParB) contribute to accurate chromosome partitioning, separation of replicated sister origins, and regulation of replication initiation in Bacillus subtilis. *Mol Microbiol* **60**: 853-869.

Leonard, T.A., Butler, P.J., and Lowe, J. (2005a) Bacterial chromosome segregation: structure and DNA binding of the Soj dimer - a conserved biological switch. *Embo J* **24**: 270-282.

Leonard, T.A., Moller-Jensen, J., and Lowe, J. (2005b) Towards understanding the molecular basis of bacterial DNA segregation. *Philos Trans R Soc Lond B Biol Sci* **360**: 523-535.

Lewis, R.A., Bignell, C.R., Zeng, W., Jones, A.C., and Thomas, C.M. (2002) Chromosome loss from par mutants of Pseudomonas putida depends on growth medium and phase of growth. *Microbiology* **148**: 537-548.

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.

Li, Y., Dabrazhynetskaya, A., Youngren, B., and Austin, S. (2004) The role of Par proteins in the active segregation of the P1 plasmid. *Mol Microbiol* **53**: 93-102.

Libante, V., Thion, L., and Lane, D. (2001) Role of the ATP-binding site of SopA protein in partition of the F plasmid. *J Mol Biol* **314**: 387-399.

Lim, G.E., Derman, A.I., and Pogliano, J. (2005) Bacterial DNA segregation by dynamic SopA polymers. *Proc Natl Acad Sci U S A* **102**: 17658-17663.

Lin, D.C., Levin, P.A., and Grossman, A.D. (1997) Bipolar localization of a chromosome partition protein in Bacillus subtilis. *Proc Natl Acad Sci U S A* **94**: 4721-4726.

Lutkenhaus, J., and Sundaramoorthy, M. (2003) MinD and role of the deviant Walker A motif, dimerization and membrane binding in oscillation. *Mol Microbiol* **48**: 295-303.

Lutkenhaus, J. (2007) Assembly dynamics of the bacterial MinCDE system and spatial regulation of the Z ring. *Annu Rev Biochem* **76**: 539-562.

Marston, A.L., and Errington, J. (1999) Dynamic movement of the ParA-like Soj protein of B. subtilis and its dual role in nucleoid organization and developmental regulation. *Mol Cell* **4**: 673-682.

McLeod, B.N., and Spiegelman, G.B. (2005) Soj antagonizes Spo0A activation of transcription in Bacillus subtilis. *J Bacteriol* **187**: 2532-2536.

Meinhardt, H., and de Boer, P.A. (2001) Pattern formation in Escherichia coli: a model for the pole-to-pole oscillations of Min proteins and the localization of the division site. *Proc Natl Acad Sci U S A* **98**: 14202-14207.

Mileykovskaya, E., Fishov, I., Fu, X., Corbin, B.D., Margolin, W., and Dowhan, W. (2003) Effects of phospholipid composition on MinD-membrane interactions *in vitro* and *in vivo*. *J Biol Chem* **278**: 22193-22198.

Mohl, D.A., and Gober, J.W. (1997) Cell cycle-dependent polar localization of chromosome partitioning proteins in Caulobacter crescentus. *Cell* **88**: 675-684.

Moller-Jensen, J., Jensen, R.B., Lowe, J., and Gerdes, K. (2002) Prokaryotic DNA segregation by an actin-like filament. *Embo J* **21**: 3119-3127.

Moller-Jensen, J., Borch, J., Dam, M., Jensen, R.B., Roepstorff, P., and Gerdes, K. (2003) Bacterial mitosis: ParM of plasmid R1 moves plasmid DNA by an actin-like insertional polymerization mechanism. *Mol Cell* **12**: 1477-1487.

Mukherjee, A., Dai, K., and Lutkenhaus, J. (1993) Escherichia coli cell division protein FtsZ is a guanine nucleotide binding protein. *Proc Natl Acad Sci U S A* **90**: 1053-1057.

Niki, H., and Hiraga, S. (1997) Subcellular distribution of actively partitioning F plasmid during the cell division cycle in E. coli. *Cell* **90**: 951-957.

Niki, H., and Hiraga, S. (1999) Subcellular localization of plasmids containing the oriC region of the Escherichia coli chromosome, with or without the sopABC partitioning system. *Mol Microbiol* **34**: 498-503.

Ogura, T., and Hiraga, S. (1983) Partition mechanism of F plasmid: two plasmid gene-encoded products and a cis-acting region are involved in partition. *Cell* **32**: 351-360.

Ogura, Y., Ogasawara, N., Harry, E.J., and Moriya, S. (2003) Increasing the ratio of Soj to Spo0J promotes replication initiation in Bacillus subtilis. *J Bacteriol* **185**: 6316-6324.

Pichoff, S., Vollrath, B., Touriol, C., and Bouche, J.P. (1995) Deletion analysis of gene minE which encodes the topological specificity factor of cell division in Escherichia coli. *Mol Microbiol* **18**: 321-329.

Pichoff, S., and Lutkenhaus, J. (2005) Tethering the Z ring to the membrane through a conserved membrane targeting sequence in FtsA. *Mol Microbiol* **55**: 1722-1734.

Pogliano, J. (2002) Dynamic cellular location of bacterial plasmids. *Curr Opin Microbiol* **5**: 586-590.

Quisel, J.D., Lin, D.C., and Grossman, A.D. (1999) Control of development by altered localization of a transcription factor in B. subtilis. *Mol Cell* **4**: 665-672.

Raskin, D.M., and de Boer, P.A. (1999) Rapid pole-to-pole oscillation of a protein required for directing division to the middle of Escherichia coli. *Proc Natl Acad Sci U S A* **96**: 4971-4976.

Ravin, N., and Lane, D. (1999) Partition of the linear plasmid N15: interactions of N15 partition functions with the sop locus of the F plasmid. *J Bacteriol* **181**: 6898-6906.

Rothfield, L., Taghbalout, A., and Shih, Y.L. (2005) Spatial control of bacterial division-site placement. *Nat Rev Microbiol* **3**: 959-968.

Saint-Dic, D., Frushour, B.P., Kehrl, J.H., and Kahng, L.S. (2006) A parA Homolog Selectively Influences Positioning of the Large Chromosome Origin in Vibrio cholerae. *J Bacteriol* **188**: 5626-5631.

Setlow, B., Hand, A.R., and Setlow, P. (1991) Synthesis of a Bacillus subtilis small, acid-soluble spore protein in Escherichia coli causes cell DNA to assume some characteristics of spore DNA. *J Bacteriol* **173**: 1642-1653.

Suefuji, K., Valluzzi, R., and RayChaudhuri, D. (2002) Dynamic assembly of MinD into filament bundles modulated by ATP, phospholipids, and MinE. *Proc Natl Acad Sci U S A* **99**: 16776-16781.

Szeto, T.H., Rowland, S.L., Rothfield, L.I., and King, G.F. (2002) Membrane localization of MinD is mediated by a C-terminal motif that is conserved across eubacteria, archaea, and chloroplasts. *Proc Natl Acad Sci U S A* **99**: 15693-15698.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**: 4876-4882.

van den Ent, F., Moller-Jensen, J., Amos, L.A., Gerdes, K., and Lowe, J. (2002) F-actin-like filaments formed by plasmid segregation protein ParM. *Embo J* **21**: 6935-6943.

Warming, S., Costantino, N., Court, D.L., Jenkins, N.A., and Copeland, N.G. (2005) Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res* **33**: e36.

Wu, L.J., and Errington, J. (2004) Coordination of cell division and chromosome segregation by a nucleoid occlusion protein in Bacillus subtilis. *Cell* **117**: 915-925.

Yamaichi, Y., and Niki, H. (2000) Active segregation by the Bacillus subtilis partitioning system in Escherichia coli. *Proc Natl Acad Sci U S A* **97**: 14656-14661.

Youngren, B., and Austin, S. (1997) Altered ParA partition proteins of plasmid P1 act via the partition site to block plasmid propagation. *Mol Microbiol* **25**: 1023-1030.

Zhou, H., and Lutkenhaus, J. (2003) Membrane binding by MinD involves insertion of hydrophobic residues within the C-terminal amphipathic helix into the bilayer. *J Bacteriol* **185**: 4326-4335.

Zhou, H., and Lutkenhaus, J. (2004) The switch I and II regions of MinD are required for binding and activating MinC. *J Bacteriol* **186**: 1546-1555.

Zhou, H., Schulze, R., Cox, S., Saez, C., Hu, Z., and Lutkenhaus, J. (2005) Analysis of MinD mutations reveals residues required for MinE stimulation of the MinD ATPase and residues required for MinC interaction. *J Bacteriol* **187**: 629-638.