Effects of the conserved proteins, Soj (ParA) and Spo0J (ParB) on chromosome partitioning in *Bacillus subtilis*

by

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Submitted to the Department of Biology on April 14, 2004, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology

ABSTRACT

The Par system contributes to stable inheritance of plasmids and chromosomes in diverse bacterial species. Faithful chromosome partitioning is important to dividing cells because anucleate, or chromosomeless, cells are not viable. Since the discovery of the Par system in low-copy number plasmids over 20 years ago, research has focused on characterizing its components: ParA, a DNA-binding ATPase, ParB, a DNA-binding protein that interacts with ParA, and *parS*, the site bound by ParB. How these proteins function to stabilize *parS*-containing plasmids or chromosomes remains to be elucidated.

Less is known about the chromosomally-encoded Par systems. Intriguingly, in several organisms including *Bacillus subtilis*, chromosomally-encoded Par systems play important roles in cellular processes besides chromosome partitioning.

This thesis focuses on characterizing the roles of Soj (ParA) and Spo0J (ParB) of *B. subtilis* in chromosome partitioning. These proteins were first identified by their effects on sporulation. My results indicate that Soj and Spo0J contribute to timely separation of replicated chromosomal origins, and argue against the model that Spo0J anchors replicated origins to putative receptors in opposite halves of the cell. Additionally, I show that Soj and Spo0J regulate replication of initiation, and propose that this regulation may occur by a similar genetic hierarchy through which they regulate initiation of sporulation.

Thesis supervisor: Alan D. Grossman
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Chapter 1

Introduction

"I then most always saw, with great wonder, that in the said matter there were many very little living animalcules, very prettily a-moving."

- Anton Von Leeuwenhoek, describing the appearance of plaque from his mouth under a compound microscope (1683)
All dividing cells must accurately replicate and partition their chromosomes in order to ensure that each daughter cell receives a complete copy of the genome. Transmission of an incomplete genome perturbs cellular functions, such as the mechanisms that control cell division, and results in cell death if essential genes are lost. Extensive studies have yielded a rich understanding of chromosome partitioning in eukaryotic cells (Nasmyth, 2001). In contrast, our understanding of chromosome partitioning in prokaryotes is less advanced, due mainly to the fact that technology for visualizing subcellular details within tiny bacterial cells was not available until relatively recently. Bacteria, ranging from one to a few microns in length (~two orders of magnitude smaller than eukaryotic cells), were not amenable to the early cytological experiments that began the field of eukaryotic chromosome segregation.

How do bacteria partition their chromosomes? Genetic, biochemical and cytological approaches have led to the identification and characterization of several players that contribute to this process. To learn more about bacterial chromosome partitioning, I have studied members of a well-conserved family of proteins involved in plasmid and chromosome partitioning in diverse bacterial species. The partition proteins Soj (ParA) and Spo0J (ParB) are encoded in the chromosome of the Gram-positive rod-shaped soil bacterium *Bacillus subtilis*. The central focus of this thesis is the characterization of how Soj and Spo0J affect certain steps in chromosome partitioning. My results demonstrate that Soj and Spo0J exert several effects on the origin region of the chromosome: they affect replication initiation (Lee et al., 2003; Ogura et al., 2003) and facilitate timely separation of replicated origins (Chapters 2 and 3), and Spo0J contributes to positioning of replicated origins at the cell quarters (Lee et al., 2003).
In the first half of this chapter, I review the process of bacterial chromosome partitioning and describe the roles of several factors involved. In the second half, I focus on the Par system in *B. subtilis* and other organisms.

**I. BACTERIAL CHROMOSOME PARTITIONING**

**Bacterial chromosomes**

Many bacteria have a single circular chromosome. *B. subtilis* possesses a 4.2 Mbase circular chromosome with a unique origin of replication, *oriC*, located at 0°/360° on the circle, and the terminus region located near the 180° region of the circle (Figure 1-1).

**The problem of partitioning**

The purpose of chromosome partitioning is to move each of the sister chromosomes apart such that cell division occurs between the separated copies, producing two cells that each contain a complete genetic complement. In order to move them apart, physical connections between replicated sister chromosomes must be resolved to produce two separable molecules. Because production of anucleate (chromosomeless) cells is a fatal event, there is tremendous selective pressure for chromosome partitioning to occur with high fidelity: in *B. subtilis*, only ~one in $10^4$ cell division events produces an anucleate cell (Ireton et al., 1994).

**Bacterial chromosome partitioning in the context of the cell cycle**

I describe chromosome partitioning in the context of a simplified model of a bacterial cell cycle, and how the chromosome has a specific subcellular orientation for the majority of the cell cycle. Then, I discuss two related controversial issues: whether replicated regions of the chromosome separate soon after they are replicated or remain cohesed until the end of the replication cycle, and where in the cell replication initiation is thought to occur.
Figure 1-1. Model for chromosome partitioning during a simplified bacterial cell cycle. *B. subtilis* contains a circular chromosome with the origin (O) at $0^\circ/360^\circ$ and the terminus region (T) near $180^\circ$ on the circle. Replication initiates from the origin (top) and proceeds bidirectionally (right and bottom). Sister origins separate and become positioned near the cell quarters while the replisome (grey triangle) continues to replicate the remainder of the chromosome (bottom). The division septum (dotted line) forms at midcell (left), and the cycle begins anew.

**Simplified model of a bacterial cell cycle.** This model is “simplified” because of the assumptions that newborn cells have a single copy of the chromosome, and that only one round of replication occurs at any given time. These assumptions are true for slowly growing cells. Under rapid growth conditions, cells can perform multiple rounds of replication simultaneously by initiating the next round of replication before the first round finishes (a phenomenon known as multi-fork, or dichotomous, replication). Multi-fork replication enables bacteria to divide
with a doubling time shorter than the amount of time required to complete a single round of chromosome replication.

At the beginning of the cell cycle, newborn cells possess a single chromosome (Figure 1-1). Replication initiates from the chromosomal origin, oriC, and proceeds bidirectionally around the circle. Shortly after the origin is replicated, sister origins separate towards opposite cell halves and become captured there even as the remainder of the chromosome is being replicated (Lee et al., 2003; Li et al., 2002; Lin et al., 1997; Niki et al., 2000; Sharpe and Errington, 1998; Webb et al., 1998; Webb et al., 1997). In B. subtilis, the origins are maintained at the cell quarters for the majority of the cell cycle (Lee et al., 2003).

The chromosome is replicated by the replisome, a protein complex containing DNA polymerase and associated factors (Baker and Bell, 1998; Benkovic et al., 2001; Kornberg and Baker, 1992). The replisome is positioned predominantly at or near midcell or future midcell positions, and remains there while the DNA being replicated moves through it (Lemon and Grossman, 1998; Lemon and Grossman, 2000). When replication finishes, specific mechanisms act at the terminus region to resolve the replicated chromosomes into two physically separable molecules. The sister molecules are generally moved out of the way by the time the division septum forms at midcell. Otherwise, mechanisms for post-septational chromosome partitioning can clear trapped DNA from the septum. The cycle begins anew.

**Chromosomes have a specific orientation for the majority of the cell cycle.** For the majority of the cell cycle, chromosomes have a specific subcellular orientation, visualized using fluorescent in situ hybridization (FISH) or fluorescent DNA-binding proteins: replicated origins are positioned in opposite halves of the cell, the terminus region is located at or near midcell, and intervening regions occupy average positions between the origins and the terminus (Gordon et
al., 1997; Lee et al., 2003; Li et al., 2002; Lin et al., 1997; Niki et al., 2000; Sharpe and Errington, 1998; Teleman et al., 1998; Webb et al., 1998; Webb et al., 1997). Several mutations that disrupt chromosome positioning compromise partitioning, such as Δsmc, which perturbs overall chromosome orientation (Graumann, 2000), and Δspo0J, which perturbs positioning of replicated origins such that they are closer together than the cell quarters (Lee et al., 2003).

**Separation soon after replication, or sister chromosome cohesion?** Controversy exists over whether chromosomal regions separate soon after they are replicated (Lemon and Grossman, 2001; Li et al., 2002), or whether sister chromosomes remain cohesed until the end of the replication cycle (Sunako et al., 2001; Bates and Kleckner, unpublished data).

The observation that replicated origins separate before the terminus region in *E. coli* and *B. subtilis*, described above, argues against the sister chromosome cohesion model. However, two studies in synchronized cells of *E. coli* indicate that, under certain conditions, replicated sister regions may remain cohesed for the majority of the replication cycle: experiments visualizing several different regions of the chromosome using FISH indicate that under certain conditions, sister regions appear to remain associated until late in the replication cycle, when they separate into two spatially resolvable foci (Sunako et al., 2001). In addition, experiments comparing the number of origins per cell by flow cytometry to the number of spatially resolvable origins by FISH indicate that origins can remain associated long after they are replicated (Bates and Kleckner, unpublished data). It is possible that under certain growth conditions, sister chromosomes remain cohesed for most of the cell cycle, whereas under other conditions, replicated regions could separate soon after they are replicated. The latter must be true at least during multi-fork replication conditions. I suspect that the disparate results could reflect different modes of chromosome separation under different growth conditions.
Finally, even under growth conditions in which replicated origins separate long before the terminus separates, it is possible that specific regions of the chromosome, such as the origin region, might be paired for a transient portion of the cell cycle (Chapter 3).

**Where in the cell does replication initiation occur?** Where replication initiates is a controversial matter. I favor the model that initiation occurs at midcell (or at future midcell sites) (Lemon and Grossman, 2000), for several reasons: 1) the origin is positioned at or near midcell in newborn cells of *B. subtilis* and *E. coli* (Niki et al., 2000; Roos et al., 1999; Sharpe and Errington, 1998; Teleman et al., 1998), 2) the replisome is positioned at or near midcell in *B. subtilis* and *E. coli* (Brendler et al., 2000; Lemon and Grossman, 1998), and 3) germinating spores of *B. subtilis* that have just initiated replication incorporate BrdU near midcell (Lewis and Errington, 1997).

Contrasting results are obtained in cells of *B. subtilis* synchronized using a temperature-sensitive allele of *dnaA*. In this case, the origin is located near a pole at the beginning of the replication cycle, visualized by Spo0J-GFP, suggesting that replication may initiate near a pole (Imai et al., 2000). The discrepancy could be due to the use of a temperature-sensitive initiation mutant.

**Several factors contribute to chromosome partitioning**

Bacterial chromosome partitioning can be broken down into several processes: oriented movement of replicated chromosomal regions towards opposite halves of the cell, separation and capture of replicated origins, chromosome compaction/organization, physical resolution of replication products, and post-septational partitioning where necessary.

Several factors are involved in these processes. Some act all over the chromosome, while some act on specific regions of the chromosome. I describe how several of these factors work in
greater detail (Figure 1-2). i. The replisome could potentially contribute motive force to separate replicated sister regions in an oriented way. ii. Separation and capture of replicated origins at the cell quarters could involve interactions with the cell membrane and recognition of cis-acting sites by putative cellular anchors. iii. SMC-mediated chromosome compaction and organization could contribute to separation of replicated regions. iv. XerCD and TopoIV resolve dimeric or catenated (interlinked) chromosomes, respectively. v. The DNA translocase SpoIIIIE provides a backup system in case chromosome partitioning is not completed prior to cell division.

Figure 1-2. Several factors contribute to chromosome partitioning. Some act all over the chromosome, while others act on specific regions. Small boxes flanking the origin represent the known parS sites bound by Spo0J.

i. What moves chromosomes apart?

An early model for moving chromosomes apart. The replicon model proposed that replicated sister origins are attached to the cell membrane by receptors at midcell, and that sister chromosomes are moved apart by preferential insertion of new membrane material in between the origins (Jacob et al., 1963). This model was invalidated by two discoveries: 1) insertion of new membrane material occurs throughout the cell (Hiemstra et al., 1987; Wientjes and
Nanninga, 1989), and 2) in many bacteria, replicated origins can separate with speeds that far exceed the rate of cell elongation (Gordon et al., 1997; Jensen and Shapiro, 1999; Webb et al., 1998). The hunt was on for factors that could separate chromosomes quickly and in an oriented way.

**Spatially-oriented movement from the replisome: the extrusion-capture model.**

The “extrusion-capture” model proposes that the replisome may contribute to chromosome partitioning by extruding replicated sister regions in opposite directions, hence conferring orientation and motive force for partitioning replicated sister chromosomes (Lemon and Grossman, 2001). Consistent with this model, the replisome occupies a characteristic subcellular position at or near midcell or future midcell positions (Lemon and Grossman, 1998), DNA undergoing replication appears to move through the replisome, and replicated sister regions end up in opposite halves of the cell (Lemon and Grossman, 2000). The “capture” part of the model proposes that replicated origins become captured at the cell quarters by putative cellular receptors, discussed next. As the rest of the chromosome is replicated, sister regions are partitioned by continued replisome-mediated extrusion. Although the replisome has ~1/3000th the mass of a chromosome, it could plausibly move entire chromosomes apart by constantly extruding sister regions as they are replicated, analogous to moving an immense rope, one coil at a time. Energy for moving chromosomes could potentially be provided by components of the replisome that hydrolyze nucleotide triphosphates: 1) DNA Polymerase, which adds nucleotides to growing DNA strands, and/or 2) helicase, an ATPase that unwinds the parent strands using energy from ATP hydrolysis.

In *B. subtilis*’ evolutionarily distant relative, *E. coli*, the bulk of chromosome replication also appears to occur at midcell or at sites that become midcell upon cell division. Consistent with
this model, the replisome and SeqA, a protein thought to bind newly-replicated DNA (Brendler et al., 2000; Slater et al., 1995), occupy characteristic subcellular positions near midcell or the cell quarters in *E. coli* (Kongsuwan et al., 2002; Niki et al., 2000; Onogi et al., 1999). The fact that chromosome replication appears to occur near midcell in *B. subtilis* and *E. coli* suggests that many bacteria may use central replication factories to partition their chromosomes.

### ii. Separation and capture of replicated origins

Separation and capture of replicated origins may involve interactions with factors associated with the cell membrane. Replicated origins could become captured at the cell quarters by putative anchors that recognize *cis*-acting sites near the origin region. Capture of replicated origins at the cell quarters could keep them separated and establish the direction for each sister chromosome to move toward as it is replicated.

**Association of origins with the cell membrane.** The origin region is preferentially associated with the cell membrane for at least part of the cell cycle of *B. subtilis* (Firshein, 1989; Sueoka, 1998). Membrane attachment of replicated origins could potentially contribute to chromosome partitioning by simplifying the spatial orientation problem from three dimensions to two dimensions. Replicated origins could move along the membrane as they separate, and become captured and maintained by putative membrane-associated anchors at the cell quarters. Further studies will be needed to identify these anchors.

**Cis-acting sites may contribute to positioning of the origin region.** Recent experiments identified *cis*-acting sites in *B. subtilis* and *E. coli* that appear to contribute to positioning of replicated origins at opposite cell halves (Kadoya et al., 2002; Yamaichi and Niki, 2004). In *B. subtilis*, the *cis*-acting site is located within *oriC*. When the endogenous *oriC* is inactivated, insertion of this site along with a new origin at an ectopic position in the chromosome is
sufficient to recruit this region to the cell quarters (Kadoya et al., 2002). However, the site does not appear to be required for proper positioning of the endogenous oriC (Barker and Grossman, unpublished results), suggesting that there are probably additional factors that contribute to origin positioning.

In *E. coli*, an origin-proximal site called *migS* is sufficient to confer bipolar positioning when inserted into ectopic chromosomal regions that are normally closer together (Yamaichi and Niki, 2004), and deleting *migS* causes replicated origins to mislocalize closer together. Putative DNA-binding protein(s) might recognize these cis-acting sites and help to position them either by tethering them to membrane-bound anchors at the cell quarters, or by facilitating their bipolar migration.

iii. Chromosome compaction and organization by SMC

As newly-replicated regions are extruded from the replisome, ongoing chromosome compaction and organization could contribute to orderly separation of replicated sister regions by: 1) drawing them towards their respective origins that are anchored in opposite cell halves, 2) preventing tangling, and/or 3) making chromosomes easier to move by making them more rigid and condensed. Chromosomes are compacted and organized by several abundant, nonspecific DNA-binding proteins such as SMC and HBsu of *B. subtilis*, and MukB and HU of *E. coli*, as well as topoisomerases that mediate supercoiling (Drlica, 1990; Trun and Marko, 1998). I focus on SMC, one of the best-characterized chromosome compaction proteins of *B. subtilis*. *soj*, *spo0J*, and *smc* exhibit genetic interactions, described next and in Chapter 3.

Structural Maintenance of Chromosomes proteins (SMC) are conserved in all kingdoms of life and are involved in many chromosome-organizing processes, including: chromosome compaction, sister chromatid cohesion, and dosage compensation (Hirano, 2002). Most bacteria
possess a single smc gene or a functional analog called mukB. B. subtilis SMC (BsSMC) is a
moderately abundant, nonspecific DNA-binding protein with ATPase activity (Hirano, 1998;
Hirano, 2002; Lindow et al., 2002b). It appears to compact chromosomes by binding all over the
DNA and constraining negative supercoils (Lindow et al., 2002a). An smc null mutant has
severe defects in nucleoid condensation, chromosome orientation, and partitioning (Britton et al.,
1998; Graumann et al., 1998; Moriya et al., 1998). Inactivating spo0J causes a synthetic
partitioning defect with Δsmc, suggesting that they may perform partially overlapping functions
in chromosome organization (Britton et al., 1998).

SMC proteins have N- and C- terminal “head” domains containing Walker A and B
nucleotide binding motifs, respectively (Hirano, 2002). In between the head domains are two
long coiled-coil domains separated by a flexible hinge (Fig 1-3A). Each SMC molecule is bent
at the hinge and forms an intramolecular coiled coil, bringing together the head domains to form
an intact ATPase (Losada and Hirano, 2001; Lowe et al., 2001; Melby et al., 1998). The
molecules dimerize via interactions between hinge domains (Figure 1-3B).

By analogy to cohesin, an SMC-containing complex from Saccharomyces cerevisiae,
BsSMC is thought to form a functional ring-shaped complex with two other proteins: ScpA, a
well-conserved kleisin family member (from the Greek kleismo for “closure”), thought to bridge
the SMC head domains, and ScpB, a protein found in many prokaryotes (Figure 1-3B) (Gruber et
al., 2003; Lindow et al., 2002b; Schleiffer et al., 2003; Soppa et al., 2002; Volkov et al., 2003).
Ring-shaped SMC complexes could compact chromosomes by holding distant regions of the
DNA together in close proximity (Fig 1-3C) (Gruber et al., 2003). SMC, ScpA, and ScpB have a
similar subcellular localization in B. subtilis: they form foci near or coincident with foci of the
Figure 1-3. SMC molecules and SMC-containing complexes.  

A. Each SMC molecule has globular N- and C-terminal domains containing Walker A and Walker B nucleotide-binding domains, respectively, and a central flexible hinge domain flanked by long coiled-coils. B. SMC molecules dimerize via associations between the hinge domains, and are thought to form functional ring-shaped complexes with ScpA and ScpB (black curves). C. SMC-containing rings could compact chromosomes by constraining DNA loops.
replisome (Lindow et al., 2002b). These results lead to a model in which a portion of the intracellular SMC-containing complexes are concentrated into a pool at or near the replisome, and load onto newly-replicated DNA (Lindow et al., 2002b). Ongoing SMC-mediated compaction could facilitate orderly movement of newly-replicated DNA towards opposite halves of the cell.

iv. Physical resolution of replicated sister chromosomes

**XerCD-mediated recombination at the terminus resolves chromosome dimers.**

Homologous recombination between circular molecules can generate chromosome dimers that must be resolved into monomers prior to partitioning into separate daughter cells. A conserved family of site-specific recombinases called XerC and XerD resolve dimers by stimulating recombination between sister copies of a terminus-proximal site called dif (Hill, 1996). In *E. coli*, a protein called FtsK, located at the septal membrane, appears to couple dimer resolution to cell division by sensing chromosome dimers that are unable to clear the septum and triggering Xer-mediated recombination between sister dif sites (Aussel et al., 2002; Sherratt et al., 2001). While *B. subtilis* has two proteins homologous to FtsK, SpoIIIIE (discussed later) (Begg et al., 1995) and YtpT, neither appears to be required for dimer resolution (Sciochetti et al., 2001). The role of terminus attachment to the membrane is less characterized in *B. subtilis*.

**TopoIV separates catenated chromosomes.** Another topological barrier to separation is twisting together, or catenation, of replicated sister chromosomes. Decatenation is achieved by TopoIV, a type II topoisomerase encoded by *parC* and *parE*, genes that are essential for viability in *E. coli* and *B. subtilis* (Adams et al., 1992; Huang et al., 1998; Kato et al., 1990; Peng and Marians, 1993; Ullsperger and Cozzarelli, 1996). TopoIV is thought to act near replicated
termini, unlinking sister molecules just prior to cell division (Espeli et al., 2003a; Espeli et al., 2003b; Hojgaard et al., 1999).

v. Post-septational chromosome partitioning by SpoIIIIE

*B. subtilis* SpoIIIIE is a septally-located, ATP-dependent DNA translocase that is thought to facilitate post-septational chromosome partitioning in cells where the division septum has trapped the chromosome (called “guillotined” or “cut” cells) (Bath et al., 2000; Wu and Errington, 1997). SpoIIIIE is thought to act as a backup partitioning system, reducing the proportion of guillotined cells by pumping the trapped DNA out of the way of the division septum (Britton and Grossman, 1999; Sharpe and Errington, 1995). A specialized role for SpoIIIIE occurs during sporulation: septum formation traps the chromosome such that the forespore initially receives only the origin-proximal 30% of the chromosome, and SpoIIIIE translocates the remainder of the chromosome into the forespore (Wu and Errington, 1994; Wu et al., 1995).

Chromosome partitioning involves a division of labor into several areas including chromosome movement, compaction, positioning, resolution, and backup mechanisms that make chromosome partitioning remarkably robust. I have described several factors that specialize in these areas. For the remainder of this chapter, I focus on describing the Par system, involved in chromosome and plasmid partitioning in many bacteria.

II. INTRODUCTION TO THE PAR SYSTEM

A brief introduction to Soj (ParA) and Spo0J (ParB)

Soj (ParA) and Spo0J (ParB) of *B. subtilis* belong to the well-conserved Par family of partitioning proteins. Soj and Spo0J were first discovered due to their effects on sporulation (discussed later), and their similarity to Par proteins led to experiments investigating their effects
on chromosome partitioning (Ireton et al., 1994). Normally, chromosome partitioning occurs with high fidelity such that <0.02% of cells are anucleate (Ireton et al., 1994). Inactivating spo0J or soj-spo0J causes an ~100-fold increase in the production of anucleate cells, such that 1-2% of cells are anucleate (Ireton et al., 1994) (Figure 1-4). In addition, the mutants are ~20% longer than wild type cells (Ch. 2 and 3) and have irregular nucleoid sizes and guillotined cells (Figure 1-4). Unlike the Δspo0J and Δ(soj-spo0J) mutants, the Δsoj mutant has normal cell lengths and does not have a significant chromosome partitioning defect, producing ~0.06% anucleate cells (Ireton et al., 1994). However, inactivating soj destabilizes a parS plasmid (Lin and Grossman, 1998), demonstrating a partitioning role for Soj and suggesting that factor(s) that are redundant to Soj may contribute to chromosome partitioning. I found that Δsoj enhances the chromosome partitioning defect of an Δsmc mutation as strongly as Δspo0J, indicating that Soj and Spo0J probably function in the same chromosome partitioning pathway (Chapter 3). Results of this thesis indicate that Soj and Spo0J contribute to separation of replicated sister origins, origin positioning, and also regulate replication initiation (Lee et al., 2003) (and Chapter 3).

I start this section by introducing two types of plasmid-encoded Par systems, and chromosomally-encoded Par systems.
Figure 1-4. Chromosome partitioning defects of a *spo0J* null.  

**A.** Wild-type cells. All cells in the field have a nucleoid.  

**B.** *spo0J* null cells. A *spo0J* null has cells ~20% longer than wild type cells (Lee 2003), guillotined chromosomes (yellow arrows) and 1-2% anucleates (Ireton 1994). Live cells with membranes stained red using FM-4-64 and DNA stained blue with DAPI.
Two types of plasmid-encoded Par systems

There are two known, nonhomologous types of plasmid-encoded partitioning systems that consist of two trans-acting partitioning proteins that stabilize a plasmid bearing a cis-acting site. Other mechanisms that favor propagation of plasmid-containing cells include plasmid multimer resolution systems and post-segregational killing systems, which I will not discuss.

My thesis focuses on the widely-conserved ParA/ParB/parS system, found in phages and plasmids involved in spreading antibiotic resistance and virulence factors (Yamaichi, 2000; Youngren et al., 2000), as well as many bacterial chromosomes (discussed later). ParA is a DNA-binding protein and an ATPase of the Walker-type superfamily, ParB is a DNA-binding protein that interacts with ParA, and parS is the DNA site bound by ParB (Figure 1-5). ParA and ParB are encoded by the parAB operon, and a parS site is usually located downstream of or within the operon. A second, less common type of plasmid partitioning system is the ParM/ParR/parC system found in plasmid R1. ParM is an actin-like ATPase, ParR is a DNA-binding protein, and parC is the site bound by ParR (Gerdes et al., 2000; Moller-Jensen et al., 2002).

![Diagram of ParA and ParB]

Figure 1-5. The par operon. ParA is a Walker-type ATPase that binds DNA. ParB is a DNA-binding protein that binds parS sites and interacts with ParA. A parS site is usually located downstream of the par operon or within the coding region for ParB.
One obvious distinguishing feature between these systems is that ParA and ParM are different types of ATPases: ParA belongs to a subgroup within the Walker-type ATPase superfamily, defined by a deviant form of the Walker A purine binding motif, a conserved Box IV ATPase motif, and a Walker B purine binding motif (Koonin, 1993; Motallebi-Veshareh et al., 1990; Yamaichi, 2000). ParM belongs to a superfamily of actin-like ATPases that share a characteristic structure known as the “actin fold”, consisting of two alpha/beta domains flanking an ATP binding domain (Bork et al., 1992; Gerdes et al., 2000; Hurley, 1996; Kabsch and Holmes, 1995).

The ParM/ParR/parC system of plasmid R1 is better understood than that of the ParA/ParB/parS system described thus far. Although the two systems are not homologous, they could potentially perform similar functions that are general to plasmid partitioning systems. I briefly describe a model for how plasmid R1 is partitioned.

**Partitioning mechanism of the ParM/ParR/parC system.** Replicated R1 plasmids appear to be partitioned by ParR-mediated pairing followed by ParM polymerization into a filament that pushes the plasmids towards opposite cell poles (Moller-Jensen et al., 2002). ParR can pair plasmids by bringing together parC sites in trans: paired complexes can be visualized using electron microscopy, and ParR can stimulate the rate of intermolecular ligation between parC-containing DNA molecules (Jensen et al., 1998). ParR and parC stimulate ParM polymerization, suggesting that the paired complex may act as a nucleation point (Moller-Jensen et al., 2002).

It is conceivable that the ParA/ParB/parS system could also function by pairing and separating plasmids (discussed later), although not necessarily by the same molecular mechanism, since ParA is not known to polymerize like ParM. For the remainder of the introduction, I focus on the ParA/ParB/parS system (and refer to it as the Par system).
**Discovery of the ParA/ParB/parS partitioning system.** The Par system was first discovered in the P1 prophage and F plasmid of *E. coli*. (In the F plasmid, the components are called SopA, SopB, and sopC, for “stability of plasmid”). Since these extrachromosomal elements are present in only one to two copies per cell, they would be rapidly lost if segregation were random. Initial studies identified ~3.0 kb regions on the plasmids, found to encode two protein products, ParA and ParB. When supplied in *trans*, these proteins were sufficient to stabilize a plasmid carrying the *cis*-acting site, *parS* (Austin and Abeles, 1983a; Austin and Abeles, 1983b; Ogura and Hiraga, 1983). Plasmid loss in the absence of the Par system was not due to insufficient replication or defects in monomer resolution, indicating that the Par system performed some other, genuine partitioning function (Austin and Abeles, 1983a; Austin and Abeles, 1983b; Ogura and Hiraga, 1983; Som et al., 1981).

**Chromosomally-encoded Par systems**

Chromosomally-encoded Par systems were identified by homology to the plasmid-encoded *parA* and *parB* genes and continue to be discovered as sequencing of bacterial genomes progresses. More than 50 members of the Par family have been identified in plasmids and chromosomes of diverse Gram-positive and Gram-negative bacteria (Niki et al., 2000). Several human pathogens have chromosomally-encoded Par homologs, including *Bacillus anthracis*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, and *Vibrio cholerae*. While most bacteria with chromosomally-encoded Par systems have a single circular chromosome, some interesting exceptions include *Vibrio cholerae* and *Deinococcus radiodurans*, which have multiple chromosomes encoding multiple Par systems, and *Streptomyces coelicolor*, which has a linear chromosome (Yamaichi, 2000). Chromosomally-encoded Par systems appear to be less
prevailing in enteric bacteria, although some of these, like *E. coli*, can harbor extrachromosomal elements with a Par system (Yamaichi, 2000).

**Plasmid and chromosome partitioning by the Par system**

*The importance of being par*: loss of plasmids and chromosomes in the absence of the Par system. Inactivating plasmid-encoded or chromosomally-encoded Par systems causes a similar (within 10-fold) probability of missegregation during each cell division event (Austin and Abeles, 1983a; Austin and Abeles, 1983b; Biek and Shi, 1994; Ireton et al., 1994; Ogura and Hiraga, 1983; Yates et al., 1999). However, the rates of loss differ: plasmids are rapidly lost from the population because plasmidless cells continue to grow and divide, assuming the plasmids do not provide an essential function. In contrast, because anucleate cells are not viable, the proportion of anucleates remains constant. Assuming that wild type cells missegregate plasmids with a probability of 0.01% per generation (1/10 000 cell divisions produces a plasmidless cell), and the loss rate in the absence of the Par system is 1% per generation, then after 100 divisions, 64% of cells in the population would be plasmidless. In contrast, if the same loss rates are applied to chromosome partitioning, then the proportion of anucleates remains constant at 1%, regardless of the number of cell divisions.

Although inactivating chromosomally-encoded par genes causes production of a small proportion of anucleate cells, this is enough to cause a significant competitive disadvantage. In *B. subtilis*, a spo0J null produces ~1-2% anucleates (Ireton et al., 1994). If we assume that wild type and Δspo0J mutant strains have a chromosome loss rate of 0.01% and 1% per generation, respectively, then after 100 generations, a mixed population that started with equal numbers of wild type and Δspo0J mutant cells would contain only ~27% of Δspo0J cells, and after 500 generations, the Δspo0J mutants would make up less than 1% of the population.
**Par systems are functionally interchangeable.** Par systems, where tested, appear to be functionally interchangeable. Plasmid Par systems can be exchanged, chromosomally-encoded Par proteins can stabilize plasmids containing their cognate parS site, and Par systems can even function in different organisms (Austin and Abeles, 1983a; Niki and Hiraga, 1999; Yamaichi, 2000; Youngren et al., 2000). These results indicate that plasmid and chromosomally-encoded Par systems can probably perform similar functions and do not require their cognate origin to function. In addition, if these Par systems interact with specific cellular factors, then these factors must be well-conserved.

**Studies of Par systems in B. subtilis and beyond**

Studies of chromosomally-encoded and plasmid-encoded Par systems lead to several models for Par system function. One model for Spo0J function proposes that Spo0J organizes the origin region into a compacted nucleoprotein structure that facilitates separation of replicated origins. Plasmid studies have led to two prevalent models for Par system function: 1) plasmid pairing, and 2) attachment of plasmids to cellular anchors. Some of these models seem more applicable to chromosomes and some seem more applicable to plasmids. One prevalent question in the field is whether Par systems perform similar or different functions on plasmids and chromosomes, an issue I revisit throughout the thesis. In this section, I describe studies of Par systems in *B. subtilis* and other organisms that lead to these models for Par system function.

**The partition complex has a higher-order architecture**

**Plasmid partition complexes.** ParB binding to parS results in formation of a partition complex in which many molecules of ParB are thought to bind and share higher-order interactions. In P1 and F plasmid, many ParB/SopB dimers join the partition complex and spread to sites adjacent to parS, forming a structure in which DNA around the partition site may
be bent or wrapped around the bound proteins (Biek and Shi, 1994; Bouet and Funnell, 1999; Bouet, 2000; Kubo et al., 2002; Lynch and Wang, 1995; Rodionov, 1999). In P1, ParB dimers bound to the DNA may have higher-order interactions with each other (Surtees and Funnell, 1999), which could potentially bring together parS sites from different plasmids (discussed later).

**Spo0J may form a higher-order partition complex at the origin region.** Spo0J of *B. subtilis* also appears to form a higher-order partition complex. Spo0J binds to at least eight parS sites located within the origin-proximal 20% of the chromosome (Lin and Grossman, 1998), and may bring them together via higher-order interactions (Figure 1-6). Consistent with this model, Spo0J-GFP forms one distinct focus on the origin region even though the parS sites are spread over nearly 800 kb (Lee et al., 2003; Lin and Grossman, 1998; Sharpe and Errington, 1998; Teleman et al., 1998). In addition, inactivating spo0J strongly enhances the chromosome partitioning defect of an smc null, consistent with the model that Spo0J may provide small but significant structure to the origin region (Britton et al., 1998). Inactivating six of the eight known parS sites increases anucleate production, though not as severely as inactivating spo0J (Lin D, unpublished data), indicating that proper formation of a higher-order Spo0J nucleoprotein structure at the origin region may contribute to chromosome partitioning.

**The nosecone model, or how a Spo0J nucleoprotein structure at the origin could contribute to partitioning.** These results lead to a model that Spo0J may bring the parS sites together, organizing the origin region into a compact nucleoprotein structure that could act like the nosecone of a plane. Just as a nosecone’s aerodynamic shape minimizes air resistance
Figure 1-6. Spo0J binding sites and subcellular localization. A. Spo0J binds at least eight known sites, called parS, spread over nearly 800 kb on either side of the origin [Lin DC 1998]. B. Spo0J-GFP forms a focus on the origin region. For the majority of the cell cycle, there are two replicated foci of Spo0J-GFP residing at the cell quarters (Lee P 2003; Lin DC 1997; Errington J 1998). Live cells with Spo0J-GFP foci in green and cell membranes stained red by the vital membrane dye FM 4-64.
against the front of a plane, compaction of replicated origins could decrease the resistance against them as they migrate through the cytoplasm, and could thus contribute to origin separation. Nosecone formation could also prevent tangling of replicated sister origins and stiffen them, making them easier to push apart. Consistent with this model, inactivating spo0J causes replicated sister origins to be abnormally close together in a proportion of cells (Lee et al., 2003) (and Chapter 3). The nosecone model seems less applicable to plasmids. It seems less plausible that plasmids, already ~50 times smaller than the chromosome, require further compaction or organization to facilitate partitioning.

![Diagram](image)

Figure 1-7. Model for how formation of a Spo0J nucleoprotein structure could contribute to separation of replicated origins. Spo0J could bind and bring together parS sites, forming a nucleoprotein structure that compacts the origin region. Compaction could decrease resistance against the origins as they move apart, hence facilitating separation.

**ParA interacts with the partition complex**

ParA interacts with and affects the partition complex. This interaction may affect the nucleotide-bound state of ParA. ParA-mediated ATP hydrolysis appears to be important for a yet uncharacterized step in the partitioning reaction.

**ParA affects the partition complex.** ParA-ATP interacts with and modulates the size of partition complexes. The ATP-bound form of P1 ParA interacts with the partition complex and
can either supershift the complex, suggesting that ParA-ATP may recruit additional ParB molecules or join the complex itself, or disassemble the complex, depending on the concentration of ParB present (Bouet and Funnell, 1999). ParA of F plasmid or C. crescentus can also disassemble ParB nucleoprotein complexes (Figge et al., 2003; Lemonnier et al., 2000; Mohl and Gober, 1997). In B. subtilis, inactivating soj can cause foci of Spo0J-GFP to dissociate into many smaller foci (Marston and Errington, 1999), indicating that Soj affects the Spo0J nucleoprotein complex.

**ParB may affect the nucleotide-bound state of ParA.** ParB appears to affect the nucleotide-bound state of ParA. ParA’s activities in partitioning and other cellular functions is regulated by the bound nucleotide (discussed later). Studies in C. crescentus indicate that ParB may be a nucleotide exchange factor for ParA (Easter and Gober, 2002; Figge et al., 2003). Also, P1 ParB and DNA increase the overall rate of ParA-mediated ATP hydrolysis (Davis et al., 1992). This experiment did not distinguish whether interaction with ParB and DNA stimulates ATP hydrolysis directly or facilitates nucleotide exchange. Further experiments will be needed to test the model that interaction with the partition complex stimulates ParA-mediated ATP hydrolysis directly.

**ParA-mediated ATP hydrolysis may be important for partitioning.** ParA-mediated ATP hydrolysis may be important for partitioning, since mutations that impair ATP hydrolysis cause P1 plasmid loss (Fung et al., 2001). Energy from ATP hydrolysis could presumably be coupled to a step in the partition reaction, such as separation of paired plasmids or dissociation of plasmids from a cellular anchor. Alternatively, ATP binding and hydrolysis could regulate interaction and dissociation of ParA from the partition complex.
ParB may pair $parS$-containing DNA

One model proposes that the Par system might pair $parS$-containing DNA prior to separation, similar to the partitioning system of plasmid R1. Plasmid studies suggest that higher-order interactions between ParB dimers bound to different $parS$ sites could pair plasmids by bringing together $parS$ sites from different molecules. Two-hybrid analysis identified a putative P1 ParB oligomerization domain (Surtees and Funnell, 1999). In addition, it appears that P1 ParB can at least bring together two $parS$ sites that are located on opposite ends of the same plasmid (Edgar et al., 2001). Finally, ParB overproduction causes faster-than-random loss of P1 plasmids without lowering the copy number, suggesting that excess ParB can group plasmids together, thereby decreasing the number of freely segregable units (Funnell, 1988).

A similar phenotype was observed in mutants of P1 ParA called $par^{PD}$ (propagation-defective), that cause faster-than-random plasmid loss (Youngren and Austin, 1997). The $par^{PD}$ mutants appear to be defective in releasing ParB-mediated pairing. Consistent with this, the accelerated loss rate requires ParB. The $par^{PD}$ mutants also appear to promote ParB-mediated pairing, since the loss rate is faster than for plasmids lacking $parA$.

**Pairing model for Par system function.** These results lead to a model that the Par system pairs plasmids prior to directed separation (Austin and Abeles, 1983b; Nordström and Austin, 1989; Surtees and Funnell, 2003). Pairing could contribute to plasmid partitioning in several ways: 1) by selecting or orienting plasmids for partitioning in opposite directions, 2) by keeping replicated plasmids organized and untangled, 3) by forming a complex that signals that there are two sisters ready to be separated.

If Spo0J pairs chromosomal origins in *B. subtilis*, then pairing must last for only a short portion of the replication cycle, since replicated origins separate early in the cell cycle, long
before the 270° and 180° regions of the chromosome separate (Lee et al., 2003; Teleman et al., 1998) (and Chapter 3).

Subcellular location of P1 and F plasmids and their Par proteins

Subcellular positioning of P1 and F plasmids. The Par system is necessary for proper plasmid positioning. P1 and F plasmids occupy characteristic subcellular positions, seen using FISH or the lacO/LacI-GFP system: the plasmids initially localize near midcell, then move to the cell quarters following replication (Gordon et al., 1997; Gordon et al., 2004; Ho et al., 2002; Niki and Hiraga, 1997). In the absence of an intact Par system, positioning at the cell quarters is perturbed such that replicated plasmids are often found at a pole and midcell position, at opposite poles, or close together at midcell (Erdmann, 1999; Gordon et al., 2004; Niki and Hiraga, 1997; Yamaichi and Niki, 2004).

Subcellular location of the P1 and F plasmid Par proteins. ParB and SopB form foci, detectable by IFM, that most likely reflect the subcellular location of P1 and F plasmids (Erdmann, 1999; Gordon et al., 1997; Hirano et al., 1998; Ho et al., 2002). ParB focus formation depends on the presence of parS, and proper subcellular localization of the foci requires ParA (Erdmann, 1999).

In P1 and F, ParA/SopA subcellular localization is distinct from ParB localization, suggesting that ParA interactions with the partition complex may be transient and/or involve only a small fraction of ParA molecules in the cell. (Erdmann, 1999; Hirano et al., 1998).

Anchor model for Par system function. The observation that proper subcellular positioning of P1 and F plasmids requires the Par system led to a popular model for Par function, proposing that the Par system tethers plasmids to cellular anchors located at the cell quarters (Kim and Wang, 1998; Ogura and Hiraga, 1983; Rodionov, 1999; Watanabe et al., 1989;
Yamaichi and Niki, 2004). Positioning of replicated plasmids at the cell quarters would ensure that each daughter cell would receive plasmid(s), since cell division occurs at midcell. ParA could regulate attachment and detachment from these anchors (Erdmann, 1999; Surtees and Funnell, 2003), and ATP hydrolysis might be important for detachment (Fung et al., 2001).

What conserved cellular factors could serve as anchors or positional guides? Possible candidates include components of the replication machinery (Ho et al., 2002; Surtees and Funnell, 2003), factors involved in specifying cell shape (Soufo and Graumann, 2003), or specific zones in the cell membrane (Surtees and Funnell, 2003).

**Molecular explanations for plasmid compatibility: selective pairing or distinct anchors?**

Plasmids with different Par systems are compatible, that is, they can be stably maintained in the same cell in the presence of their cognate Par proteins (Austin and Abeles, 1983a; Niki and Hiraga, 1999; Niki et al., 2000; Younghen et al., 2000). In contrast, plasmids with the same Par system are incompatible. The plasmid pairing and anchoring models for Par system function rationalize these observations in distinct ways.

The plasmid pairing model provides an attractive explanation for these observations: compatibility could be due to selective pairing between plasmids with the same parS site (Figure 1-7Ai). Incompatibility could be due to formation of mixed pairs between plasmids with identical parS sites (Figure 1-7Aii).

Alternatively, if the Par system tethers plasmids to host anchors, then compatibility could be due to the existence of distinct cellular anchors for plasmids with different Par systems (Figure 1-7Bi). Incompatibility could be due to competition for a saturable anchor (Figure 1-7Bii). The main turn-off of this model is that in order to account for the observation that different Par systems from various organisms can function in the *E. coli* (Austin and Abeles, 1983a; Niki and
Hiraga, 1999; Yamaichi, 2000; Youngren et al., 2000), one must invoke that *E. coli* possesses several sets of well-conserved anchors at the cell quarters (Figure 1-7Biii). In my opinion, the pairing model seems more likely because it does not require the existence of distinct anchors.

**Par systems can control other cellular processes**

Par systems can control cellular processes in addition to chromosome partitioning: plasmid-encoded Par proteins regulate transcription from the *par* operon (Friedman and Austin, 1988; Hayes et al., 1994; Hirano et al., 1998; Mori et al., 1989), *spo0J* is required for efficient sporulation in *B. subtilis* (Ireton et al., 1994), and *parA* and *parB* of *C. crescentus* are required for FtsZ ring formation, an early step in cell division (Mohl et al., 2001). *B. subtilis* and *C. crescentus* may have adapted the Par system to monitor the state of chromosome partitioning and couple correct chromosome partitioning to the onset of subsequent cellular processes. *soj* and *spo0J* are also involved in regulation of replication initiation (Lee et al., 2003; Ogura et al., 2003) (and Chapter 3).

**A nucleotide switch regulates ParA’s activities in transcription and partitioning.** The Par systems of P1, F, and P7 plasmids regulate expression from their own respective operons. ParA/SopA is a transcriptional repressor of the *par* promoter (Friedman and Austin, 1988; Hayes et al., 1994; Hirano et al., 1998; Mori et al., 1989), and maximal repression requires ParB (Davey and Funnell, 1997; Friedman and Austin, 1988). Proper overall and relative levels of ParA and ParB are critical for partitioning, shown in *C. crescentus* (Mohl and Gober, 1997), and P1 prophage (Abeles et al., 1985; Funnell, 1988; Hayes et al., 1994).

A nucleotide switch toggles P1 ParA between partition mode and repression mode: ParA-ATP but not ParA-ADP can interact with the partition complex *in vitro* (Bouet and Funnell, 1999), and ParA-ADP binds to the *par* promoter with greater affinity than ParA-ATP (Davey
and Funnell, 1994). As described previously, ParB is thought to affect the nucleotide-bound state of ParA, either by stimulating ATP hydrolysis directly or promoting nucleotide exchange.

A checkpoint coupling cell division to chromosome partitioning in *C. crescentus*: the ParB focus has landed. In *C. crescentus*, *parA* and *parB* are essential because they are required for FtsZ ring formation, an early step in cell division (Mohl et al., 2001). One model proposes that the Par system delays cell division until replicated origins arrive at opposite cell poles. ParA-ADP appears to inhibit cell division (Easter and Gober, 2002; Mohl and Gober, 1997), and could do so by repressing transcription of a yet unidentified cell division protein, by analogy to other ParA-ADP’s that function as transcriptional repressors. ParB forms a nucleoprotein structure at the origin region (Figge et al., 2003; Mohl and Gober, 1997). Successful origin partitioning, presumably signaled by landing of ParB nucleoprotein complexes at opposite cell poles, could stimulate relocation of ParA from the DNA to the cell poles, relieving the block to cell division (Figge et al., 2003; Mohl et al., 2001).

Where the par gene got its “spo”. Soj and Spo0J were first identified as factors involved in the decision to sporulate. This regulation acts primarily through transcription, rather than partitioning of chromosomes into spores.

*spo0J* was discovered as a gene required for efficient sporulation (Sandman et al., 1987). Inactivating *spo0J* causes a ~300-fold decrease in spore production, a defect that is suppressed by simultaneously deleting *soj* (*suppressor of spo0J*). Spo0J is required for sporulation because it inhibits Soj, a repressor of early sporulation genes (Figure 1-8). Targets of Soj include *spo0A*, a gene that encodes the primary sporulation transcription factor, as well as several direct targets of Spo0A (*spoIIA, spoIIE, spoIIG*) (Cervin et al., 1998; Quisel and Grossman, 2000).
Figure 1-8. Soj and Spo0J regulate initiation of sporulation. Spo0J relieves Soj-mediated repression of early sporulation genes (Quisel 1999). Inactivating spo0J causes a sporulation defect that is suppressed by simultaneously inactivating soj.

Spo0J relieves Soj-mediated repression by regulating its subcellular location. During exponential growth, GFP-Soj normally concentrates into bands at the cell poles, with weaker staining throughout the cell (Quisel et al., 1999) (Figure 1-9A, B). Upon entry into stationary phase, when cells are poised to initiate sporulation, GFP-Soj bands widen into foci that oscillate from pole to pole on a timescale of minutes. Inactivating spo0J perturbs Soj localization such that it appears to be bound all over the nucleoid in exponential and stationary phase (Figure 1-9C, D). This mislocalization is correlated with increased association of Soj with sporulation promoters (Quisel et al., 1999). Spo0J-mediated displacement of Soj from the nucleoid to the cell poles could normally serve to prevent a stable association of Soj with sporulation promoters, thereby antagonizing Soj’s repressor activity. The significance of the Soj
Figure 1-9. Subcellular localization of GFP-tagged Soj. DIC (A) and GFP-Soj (B) in wild type cells. Soj localizes as concentrated bands at the cell septa, with fainter staining throughout the cell. Soj-GFP (C) and nucleoids (D) in Δspo0J mutant cells. In the absence of spo0J, Soj localizes all over the nucleoid. A GFP fusion to a predicted ATPase-defective Soj mutant localizes to the cell septa in the presence (E) or absence (F) of spo0J. All micrographs adapted from (Quisel 1999).
oscillations is unclear, although it has been proposed that they may allow Soj to sample the nucleoid repeatedly and inhibit initiation of sporulation until chromosome partitioning has succeeded (Quisel et al., 1999).

Spo0J is thought to regulate Soj’s subcellular location by affecting the nucleotide-bound state of Soj. Spo0J could potentially act as a nucleotide exchange factor for Soj, like ParB of C. crescentus. A form of Soj with a mutation in the Walker A box, predicted to compromise ATP hydrolysis, localizes as bands at the cell poles, even in the absence of spo0J (Figure 1-9E, F) (Quisel et al., 1999). This result indicates that the ATP-bound form of Soj is probably the form that localizes to the poles. Taken together, these results lead to a model that Soj-ADP is the DNA-bound repressor form, similar to what is seen for other ParA proteins, that Soj-ATP is sequestered at the cell poles, and that Spo0J may relieve Soj-mediated repression by stimulating nucleotide exchange, converting the repressor form into Soj-ATP (Figure 1-10).

![Diagram](image-url)

Figure 1-10. Model for how Soj and Spo0J regulate initiation of sporulation. Soj-ADP localizes all over the nucleoid and represses sporulation gene expression. Soj-ATP is sequestered to the cell poles. Spo0J relieves Soj-mediated repression by acting as a nucleotide exchange factor, increasing the proportion of ATP-bound Soj.
Summary of chapters

While the precise molecular function of the Par system remains to be elucidated, experiments in this thesis characterize several new effects of the chromosomally-encoded Par proteins Soj and Spo0J on origin partitioning in *B. subtilis*. My results indicate that Soj and Spo0J function somehow contributes to timely separation of replicated sister origins, and that the primary role of these proteins is probably not to facilitate attachment to anchors at the cell quarters. Results presented in Chapter 2 show that, while Spo0J contributes to positioning of replicated origins at the cell quarters, Spo0J is not sufficient to position *parS* sites inserted far from the origin region at the cell quarters. This result renders Spo0J-mediated anchoring model less likely. Results described in Chapter 3 indicate that inactivating soj and/or spo0J causes a defect in separating replicated origins, but does not appreciably affect separation of a region outside the origin. I describe development of a system for simultaneously visualizing any two regions of the chromosome in live cells of *B. subtilis*, which was used in these last experiments. Results in Chapters 2 and 3 indicate that Soj and Spo0J also regulate replication initiation.

Finally, in Chapter 3 and 4, I summarize the phenotypic effects of Δsoj, Δspo0J, and Δ(soj-spo0J) mutations. This comparison leads me to propose that: 1) Soj and Spo0J may regulate initiation of sporulation as well as replication initiation via a similar order of interactions, by which Spo0J acts through Soj. 2) In contrast, Soj and Spo0J may contribute to chromosome partitioning, origin separation and positioning via a distinct order of interactions, by which Soj acts through Spo0J. Further study will be required to elucidate the molecular mechanisms by which Soj and Spo0J affect these processes.
References


Chapter 2

Effects of the chromosome partitioning protein Spo0J (ParB) on oriC positioning and replication initiation in *Bacillus subtilis*

Philina S. Lee, Daniel Chi-Hong Lin, Shigeki Moriya, and Alan D. Grossman

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Daniel Lin began the experiments studying the effects of Δspo0J on origin position. He also constructed the parS array and performed preliminary experiments indicating that Spo0J cannot recruit a parS array inserted near the terminus to the cell quarters.

Shigeki Moriya performed flow cytometry analysis on wild type and Δspo0J mutants.
Abstract

Spo0J (ParB) of *Bacillus subtilis* is a DNA binding protein that belongs to a conserved family of proteins required for efficient plasmid and chromosome partitioning in many bacterial species. We found that Spo0J contributes to the positioning of the chromosomal *oriC* region, but probably not by recruiting the origin regions to specific subcellular locations. In wild type cells during exponential growth, duplicated origin regions were generally positioned around the cell quarters. In a *spo0J* null mutant, sister origin regions were often closer together, nearer to mid-cell. We found that the subcellular location of Spo0J (Spo0J-GFP) was a consequence of the chromosomal position of the Spo0J binding sites. When an array of binding sites (*parS* sites) was inserted at various chromosomal locations, in the absence of six of the eight known *parS* sites, Spo0J-GFP was no longer found predominantly at the cell quarters, indicating that Spo0J is not sufficient to recruit chromosomal *parS* sites to the cell quarters. *spo0J* also affected chromosome positioning during sporulation. A *spo0J* null mutant had an increase in the number of cells with some origin-distal regions located in the forespore. In addition, a *spo0J* null mutation caused an increase in the number of foci per cell of LacI-GFP bound to arrays of *lac* operators inserted in various positions in the chromosome, including the origin region, an increase in the DNA/protein ratio, and an increase in origins per cell as determined by flow cytometry. These results indicate that the *spo0J* mutant produced a significant proportion of cells with increased chromosome content, probably due to increased and asynchronous initiation of DNA replication.
Introduction

All dividing cells are faced with the challenges of accurately replicating and partitioning their chromosomes so that each daughter cell receives one complete copy of the genome. Spo0J is a DNA binding protein that contributes to the fidelity of chromosome segregation in Bacillus subtilis. A spo0J null mutant produces 1 - 2% anucleate cells, a frequency approximately 100-fold greater than that of wild type cells (Ireton et al., 1994). In B. subtilis, Spo0J is also required for sporulation, and this requirement is alleviated by a null mutation in soj (suppressor of spo0J). That is, a soj spo0J double mutant is able to sporulate, although the double mutant still has a defect in chromosome partitioning (Ireton et al., 1994). Soj (ParA) and Spo0J (ParB) homologs are found in many non-sporulating bacteria, and where tested, one or both are involved in accurate chromosome partitioning (Godfrin-Estevenon et al., 2002; Kim et al., 2000; Lewis et al., 2002; Mohl and Gober, 1997).

Soj (ParA) and Spo0J (ParB) belong to the well-conserved Par family of partitioning proteins. Members of this family are involved in both plasmid and chromosome segregation. When placed on a plasmid, the chromosomal systems can stabilize the plasmid (Godfrin-Estevenon et al., 2002; Lin and Grossman, 1998; Yamaichi, 2000). Our understanding of this system comes largely from studies of partitioning of the low-copy P1 prophage and F plasmid of Escherichia coli: ParA is an ATPase and ParB is a DNA-binding protein that interacts with ParA and parS, the binding site for ParB {(Bignell and Thomas, 2001; Bouet and Funnell, 1999; Davey and Funnell, 1997; Draper and Gober, 2002; Easter and Gober, 2002; Gordon and Wright, 2000; Hiraga, 2000) and references therein}.

One model for the function of the Par system proposes that Par proteins recruit parS-containing DNA to specific intracellular locations. This model is supported by the observations that a variety of plasmids are positioned at the cell quarters (Bignell et al., 1999; Erdmann, 1999; Gordon et al., 1997; Ho et al., 2002; Kim and Wang, 1998; Niki and Hiraga, 1997; Niki and Hiraga, 1999; Pogliano et al., 2001) and that the cognate Par system is required for this localization (Bignell et al., 1999; Erdmann, 1999; Kim and Wang, 1998; Niki and Hiraga, 1997). Par systems
are required for stable inheritance of these plasmids (reviewed in (Bignell and Thomas, 2001; Draper and Gober, 2002; Gordon and Wright, 2000; Hiraga, 2000)), leading to the suggestion that positioning at the cell quarters is important for plasmid segregation (Erdmann, 1999; Gordon et al., 1997; Kim and Wang, 1998; Niki and Hiraga, 1997). In further support of the model that Par systems are involved in positioning, inserting a sopC site in an oriC plasmid (that is otherwise not positioned at the cell quarters) is sufficient to cause positioning of this plasmid at the cell quarters in the presence of SopA and SopB (Niki and Hiraga, 1999). Remarkably, insertion of the chromosomally encoded soj, spo0J, and parS from B. subtilis into a plasmid in E. coli causes positioning of sister copies of the plasmid at the cell quarters (Yamaichi, 2000).

We were interested in determining whether Spo0J contributes positional information to the chromosomal origin region in B. subtilis. Spo0J binds at least eight parS sites located within the origin-proximal 20% of the chromosome, spread over a distance of ~800 kbp (Lin and Grossman, 1998). The subcellular position of Spo0J is indicative of origin position (Glaser et al., 1997; Lewis and Errington, 1997; Lin and Grossman, 1998; Lin et al., 1997; Sharpe and Errington, 1998; Teleman et al., 1998; Webb et al., 1998; Webb et al., 1997). In both E. coli and B. subtilis, following duplication, sister origins are separated to opposite halves of the cell, where they remain for the majority of the cell cycle (Glaser et al., 1997; Gordon et al., 1997; Lewis and Errington, 1997; Lin and Grossman, 1998; Lin et al., 1997; Niki and Hiraga, 1998; Roos et al., 1999; Sharpe and Errington, 1998; Teleman et al., 1998; Webb et al., 1998; Webb et al., 1997).

Spo0J is thought to contribute to chromosome orientation in sporulating cells (Sharpe and Errington, 1996). Early during sporulation, replicated origins become positioned at the extreme poles of the cell (Glaser et al., 1997; Graumann and Losick, 2001; Lin et al., 1997; Sharpe and Errington, 1996; Webb et al., 1997). Cell division occurs very close to one of the poles, producing a forespore and a larger mother cell. The polar septum closes around the nucleoid such that the origin-proximal 30% of a chromosome becomes trapped in the forespore (Wu and Errington, 1994). The remainder of that chromosome is pumped from the mother cell into the forespore by SpoIIIE, a DNA translocase located in the septum (Wu and Errington, 1997; Wu et al., 1995).
There is some disagreement over the contribution (or lack thereof) of spoOJ to faithful origin trapping in the forespore (Frandsen et al., 1999; Sharpe and Errington, 1996). Furthermore, inactivation of spoOJ appears to cause increased chromosomal copy numbers in cells that have been induced to sporulate, suggesting that trapping of genes outside the origin-proximal 30% of the chromosome could be due to random trapping of regions from extra chromosomes, rather than a direct involvement of spoOJ in establishing chromosome orientation (Webb et al., 1997).

To examine the contributions of SpoOJ to origin positioning, we measured origin position in wild type and spoOJ mutant cells during vegetative growth and sporulation. Our results indicate that SpoOJ contributes to the normal positioning of sister origins at the cell quarters during vegetative growth. In addition, SpoOJ appeared to affect chromosome positioning during sporulation, but probably indirectly. We also found that the subcellular position of SpoOJ was dependent on the chromosomal location of its binding sites. That is, an array of parS sites inserted at various locations in the chromosome, in the absence of six of the eight known binding sites, recruited SpoOJ away from the cell quarters. Finally, we found that spoOJ mutant cells were longer than wild type and had increased DNA content, most likely due to increased and asynchronous initiation of DNA replication.

**Materials and Methods**

**Media and growth conditions.** For all microscopy experiments, cells were grown at 30°C in S7 defined minimal medium (with MOPS buffer at 50 mM rather than 100 mM) supplemented with 1% glucose and 0.1% glutamate (Jaacks et al., 1989; Vasantha and Freese, 1980) and required amino acids (tryptophan, 40μg/ml; phenylalanine, 40 μg/ml; threonine, 120 μg/ml) as needed. Sporulation was induced by the addition of mycophenolic acid to 30 μg/ml (Freese et al., 1981). Cultures for flow cytometry were grown at 30°C in antibiotic medium 3 (Difco Laboratories, Detroit, MI) supplemented with adenine and guanosine (each at 20 μg/ml). Routine growth and strain constructions were done on LB plates and antibiotics were used at standard concentrations.

**Strains, alleles, and plasmids.** *B. subtilis* strains used in this study are listed in Table 1. Most are derivatives of AG174 (JH642), contain the trpC and pheA mutations, unless indicated
otherwise. Standard procedures were used for strain constructions (Harwood and Cutting, 1990).

**spo0J and rtp.** *spo0J-gfp* (Lin et al., 1997) and *rtp-yfp* (Lemon et al., 2001) fusions were described previously. In each case, the fusion is driven from the endogenous promoter, is the only expressed copy of the gene, and is mostly functional. *pDL152* contains *mls* (encoding erythromycin and lincomycin resistance) and the 3' end of *spo0J* with the *parS* site inactivated (Lin and Grossman, 1998) fused to *gfp*. It was integrated into the chromosomal *spo0J* to generate the *spo0J-gfp* fusion.

Δ*s*po0J::*spc* and Δ(val-spo0J)::*spc* are deletion-insertion mutations from strains AG1468 and AG1505, respectively (Ireton et al., 1994). *spo0J::ermC* is a null allele with *ermC* inserted into the *BglII* site in *spo0J*.

ΔspoIII::*tet*. A *spoIII* null allele was constructed by replacing all of the *spoIII* coding region with a tetracycline-resistance cassette (*tet*). An ~1000 bp fragment immediately upstream of *spoIII* was amplified by PCR using oligos LEE-19 and LEE-20, which added NheI and XhoI restriction sites at the ends of the fragment. An ~1000 bp fragment immediately downstream of *spoIII* was amplified using oligos LEE-21 and LEE-22, which added PstI and BamHI sites at the ends of the fragment. These fragments were cloned into the corresponding restriction sites on either side of the *tet* gene in pDG1513 (Guerout-Fleury et al., 1995), creating the *spoIII* knockout plasmid pPSL5. ΔspoIII::*tet* was introduced by transformation into the *B. subtilis* chromosome selecting for resistance to tetracycline and checking for a defect in sporulation. PCR was used to confirm that the ΔspoIII::*tet* allele was integrated by double crossover.

**The lac operator system.** LacI-GFP or LacI-CFP was used to visualize regions of the chromosome marked with arrays of *lac* operators. The *lacI-gfp* and *lacI-cfp* fusions are driven by a constitutive promoter and are integrated into *thrc* and have been described in detail (Lemon and Grossman, 2000; Lemon et al., 2001). Briefly, *lacI* is missing the C-terminal 11 codons (to inhibit tetramerization) and is fused to either *gfpmut2* (*lacI-gfp*) or to *cfpw7* (*lacI-cfp*).
Table 1. *Bacillus subtilis* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype or characteristics (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCL629</td>
<td><em>spo0J-gfp</em> (<em>parS</em> in <em>spo0J</em>) Δ<em>parS6</em> (see Materials and Methods)</td>
</tr>
<tr>
<td>DCL631</td>
<td><em>cgeD</em> (181°)::pDL141 (<em>parS16</em> cat); <em>spo0J-gfp</em> (<em>parS</em> in <em>spo0J</em>) Δ<em>parS6</em></td>
</tr>
<tr>
<td>DCL696</td>
<td><em>yyaC</em> (359°)::pDL175 (<em>lacO</em> cassette, cat); thrC::(lacI-gfp, mls)</td>
</tr>
<tr>
<td>DCL705</td>
<td>Δ<em>spo0J::spc yyaC</em> (359°)::pDL175 (<em>lacO</em> cassette, cat) thrC::(lacI-gfp mls)</td>
</tr>
<tr>
<td>IRN424</td>
<td>rtp::pGK97 (rtp-yfp spc) (Lemon et al., 2001)</td>
</tr>
<tr>
<td>KPL686</td>
<td><em>cgeD</em> (181°)::pAT14 (<em>lacO</em> cassette cat); thrC::(lacI-cfp mls) (Lemon et al., 2001)</td>
</tr>
<tr>
<td>KPL716</td>
<td><em>cotS</em> (270°)::pAT25 (<em>lacO</em> cassette cat); thrC::(lacI-cfp mls) (Lemon and Grossman, 2000)</td>
</tr>
<tr>
<td>KPL718</td>
<td><em>yheH</em> (90°)::pAT26 (<em>lacO</em> cassette cat); thrC::(lacI-cfp mls)</td>
</tr>
<tr>
<td>PSL10</td>
<td><em>spo0J::pDL50B</em> (<em>spo0J-gfp</em> cat)</td>
</tr>
<tr>
<td>PSL23</td>
<td><em>cotS</em> (270°)::pPSL1A (<em>parS16</em> cat); <em>spo0J-gfp</em> (<em>parS</em> in <em>spo0J</em>) Δ<em>parS6</em></td>
</tr>
<tr>
<td>PSL25</td>
<td><em>yheH</em> (90°)::pPSL2A (<em>parS16</em> cat); <em>spo0J-gfp</em> (<em>parS</em> in <em>spo0J</em>) Δ<em>parS6</em></td>
</tr>
<tr>
<td>PSL27</td>
<td><em>yqkF</em> (210°)::pPSL3A (<em>parS16</em> cat), <em>spo0J-gfp</em> (<em>parS</em> in <em>spo0J</em>) Δ<em>parS6</em></td>
</tr>
<tr>
<td>PSL62</td>
<td><em>yyaC</em> (359°)::pDL175 (<em>lacO</em> cassette, cat) thrC::(lacI-gfp mls) Δ<em>spoIIIIE::tet</em></td>
</tr>
<tr>
<td>PSL64</td>
<td><em>cgeD</em> (181°)::pAT14 (<em>lacO</em> cassette cat); thrC::(lacI-cfp mls); Δ<em>spoIIIIE::tet</em></td>
</tr>
<tr>
<td>PSL66</td>
<td><em>cotS</em> (270°)::pAT25 (<em>lacO</em> cassette cat); thrC::(lacI-cfp mls); Δ<em>spoIIIIE::tet</em></td>
</tr>
<tr>
<td>PSL73</td>
<td>Δ(<em>soj-spo0J)::spc <em>yyaC</em> (359°)::pDL175 (<em>lacO</em> cassette cat); thrC::(lacI-gfp mls); Δ</em>spoIIIIE::tet*</td>
</tr>
<tr>
<td>PSL74</td>
<td>Δ(<em>soj-spo0J)::spc; <em>cgeD</em>(181°)::pAT14 (<em>lacO</em> cassette cat); thrC::(lacI-cfp mls); Δ</em>spoIIIIE::tet*</td>
</tr>
<tr>
<td>PSL76</td>
<td>Δ(<em>soj-spo0J)::spc; <em>cotS</em> (270°)::pAT25 (<em>lacO</em> cassette cat); thrC::(lacI-cfp mls); Δ</em>spoIIIIE::tet*</td>
</tr>
<tr>
<td>PSL101</td>
<td>Δ<em>spo0J::spc cotS</em> (270°)::pAT25 (<em>lacO</em> cassette cat); thrC::(lacI-cfp mls)</td>
</tr>
<tr>
<td>PSL110</td>
<td>Δ<em>spo0J::spc cgeD</em> (181°)::pAT14 (<em>lacO</em> cassette cat); thrC::(lacI-cfp mls)</td>
</tr>
<tr>
<td>CRK6000</td>
<td>purA16 metB5 hisA3 guaB (Moriya et al., 1990)</td>
</tr>
<tr>
<td>NIS6074</td>
<td>CRK6000 <em>spo0J::ermC</em></td>
</tr>
</tbody>
</table>
The lacO cassette was inserted into several regions of the chromosome (90°, 181°, 270°, and 359°) by homologous recombination. To insert the lacO cassette into the 359° region of the chromosome, oligos LIN-145 and LIN-146 were used to amplify a ~660 bp fragment from yyaC (immediately downstream from spo0J). The PCR product was digested with AatII and BgIII, and the resulting ~380 bp fragment was inserted into the lacO cassette-containing plasmid pAT12 (Webb et al., 1997), creating pDL175. Plasmid pDL175 (which can not replicate in B. subtilis) was integrated into the B. subtilis chromosome by single crossover selecting for resistance to chloramphenicol. Chromosomal DNA from a transformant was used to introduce the array into additional strains. The lacO cassettes at 90°, 181°, and 270° were transformed into appropriate strains using chromosomal DNA from AT53, AT54, and AT52 (Teleman et al., 1998). The cassettes were amplified by selecting for resistance to 25 μg/ml chloramphenicol as described previously (Teleman et al., 1998; Webb et al., 1997).

parS arrays. pDL139 contains 16 tandemly repeated parS sites (parS16), and was used as the parent plasmid for targeting the parS array into various chromosomal sites. It was constructed by a strategy similar to that used to construct the lacO cassette (Robinett et al., 1996), taking advantage of the compatible cohesive ends of SalI and XhoI and loss of both sites upon ligation. First, a plasmid (pDL135) was constructed with an approximately 60 bp fragment from spo0J that contains the 16 bp parS site internal to spo0J. Single stranded oligonucleotides LIN116 (65 nucleotides) and LIN117 (61 nucleotides) were annealed, giving a double stranded oligo with a single parS site approximately in the middle, one blunt end with an XhoI site, and a SalI overhang at the other end. This largely double stranded oligo was ligated into pGEMcat (Harwood and Cutting, 1990) that had been digested with SmaI and SalI to generate pDL135. An EcoRI to SalI fragment from this plasmid was then cloned back into pDL135 that had been digested with EcoRI and XhoI, generating pDL136, containing two parS sites. This cycle was repeated 3 more times, each time using the new plasmid as source of insert (EcoRI to SalI) and vector (cloning between EcoRI and XhoI) to generate pDL137 (4 parS sites), pDL138 (8 parS sites), and finally pDL139 (parS16). The
parS16 array was able to stabilize an otherwise unstable low copy plasmid (data not shown) to an extent indistinguishable from that previously determined for a single parS site (Lin and Grossman, 1998), and plasmid stability was dependent on both soj and spo0J (data not shown).

The parS16 array was targeted to specific chromosomal sites by cloning fragments from the location of interest into a plasmid that had the parS16 array. Plasmids were then integrated into the chromosome by single crossover-homologous recombination into the targeted location. The positions targeted were identical to or within few hundred base pairs of the regions in which the lac operator arrays had been inserted.

For the terminus region, the parS16 array was targeted downstream of cgeD at 181° using pDL141, a derivative of pET21(+) (Novagen) that contains a selectable marker for B. subtilis (cat), a fragment from cgeD, and the parS16 array. The cat gene was excised from pMI1101 (Youngman et al., 1984) and inserted into the SphI site of pET21(+), generating pET21cat. An approximately 490 bp fragment from cgeD was amplified by PCR (using oligos LIN118 and LIN119, containing AatII sites) and cloned into the AatII site of pET21cat, generating pDL124. The parS16 array was excised from pDL139 by digestion with EcoRI and HindIII and ligated into EcoRI-HindIII digested pDL124 to create plasmid pDL141.

Derivatives of pDL139 were made to target the parS16 array to 90° (pPSL2A), 210° (pPSL3A), and 270° (pPSL1A). In each case a fragment of chromosomal DNA was amplified by PCR, using primers containing EcoRI sites near the ends, and cloned into the EcoRI site of pDL139. pPSL1A (270°) contains a 994 bp fragment from the 3' end of cotS (generated by PCR using primers LEE-8 and LEE-9). pPSL2A (90°) contains a 673 bp fragment from the 3' end of yheH, (generated by PCR using primers LEE-10 and LEE-11). pPSL3A (210°) contains a 510 bp fragment from the 3' end of yqkF (generated by PCR using primers LEE-12 and LEE-13).

The parS6 mutant. Plasmids containing the parS16 array were introduced into strain DCL629 that contains a spo0J-gfp fusion, created by integrating pDL152 into the spo0J locus, and has six of the eight known endogenous parS sites inactivated, referred to as ΔparS6 (Table 1). DCL629 was constructed in a manner similar to that described previously for strain DCL484 with six of the
eight known Spo0J binding sites inactivated (Lin and Grossman, 1998). Five of the parS mutations are deletion-insertions and are marked with antibiotic resistance cassettes, spc, kan, phl, tet, and mls. The parS site in spo0J was inactivated by changing 7 bp (of the 16 bp binding site) without altering the gene product (Lin and Grossman, 1998).

**Fluorescence microscopy.** Where indicated, cells were stained with the vital dye FM4-64 (200 ng/ml, Molecular Probes) to visualize membranes, and DAPI to visualize nucleoids (40-80 ng/ml). Microscopy was performed essentially as described previously (Lemon and Grossman, 1998; Lemon and Grossman, 2000). Briefly, cells were immobilized on pads of 1% agarose in 1X T’base, 1 mM MgSO4 (Harwood and Cutting, 1990) and images were captured with a Nikon E800 Microscope equipped with a Hamamatsu Digital Camera and filters TRITC for FM4-64, Chroma filter set #31044 for CFP, and #41012 for GFP. Improvision OpenLabs 2.0 software was used to process images.

**Measurement of focus position in cells with two foci.** Focus positions in cells with two foci were measured using a strategy designed to eliminate any unintentional visual bias in scoring. For each cell, the focus closest to a pole was designated as focus A, and the other focus was designated as focus B. Three measurements were made: 1) $a$, the distance from focus A to the closest pole, 2) $b$, the distance from that same pole to focus B, and 3) $l$, the cell length. This created a systematic bias in scoring such that in every cell, focus A was closer to a pole than focus B. In order to remove this bias, a random number generator was used to assign each focus an approximately 50% chance of being counted as closest to a pole. The corresponding distance of each focus from the same cell pole (the $a$ and $b$ measurements) was then recalculated.

The focus position as a percentage of cell length was determined using the measurements described above. We determined the distance of each focus from the nearest cell pole ($a$ for focus A; $l$ minus $b$ for focus B) and divided by cell length, times 100 percent, to give the focus position as a percentage of cell length. These numbers were averaged for all the cells in a sample. The 95% confidence intervals for the mean were calculated using the approximation that, for large samples ($n>30$) that can not be assumed to be normally distributed, the 95% confidence interval is

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approximately equal to the sample mean ± 1.96 times the standard error of the mean (Hogg and Tanis, 1997). This is an indication of confidence in the mean and not an indication of the breadth of the distribution.

Interfocal distance was calculated by subtracting $a$ from $b$. The correlation coefficient between cell length and interfocal distance was calculated using the least squares method of linear regression (Hogg and Tanis, 1997).

**Flow cytometry.** Cells were grown at 30°C in antibiotic medium 3 (Difco Laboratories, Detroit, MI) supplemented with adenine and guanosine (both at 20 μg/ml). Flow cytometry was performed essentially as described previously (Ogura et al., 2001). Briefly, chloramphenicol was added (200 μg/ml) to cells to inhibit protein synthesis and block initiation of new rounds of replication. Cells were incubated for 5 hours to allow ongoing rounds of replication to finish. Next, cells were collected, fixed with ethanol, and treated with a mixture of fluorescent dyes, mithramycin A and ethidium bromide. The amount of DNA per cell was measured using a Bryte HS flow cytometer (Bio-Rad Laboratories).

**Measurement of DNA/protein ratios.** The DNA to protein ratio was determined essentially as described previously (Kadoya et al., 2002). Briefly, 30 ml of exponentially growing cells were collected by centrifugation, resuspended, lysed, and separated into nucleic acid and protein fractions. DNA and protein concentrations in the fractions were measured using colorimetric methods as described (Burton, 1956; Lowry, 1951). Reported values are averages from 3 - 4 experiments, followed by the standard deviations from the mean.
Results and Discussion

Replicated origins are positioned at or near the cell quarters. We measured the intracellular position of origin regions (0°/360° on the circular chromosome) along the length of cells during exponential growth. The origin region was visualized using either Spo0J-GFP bound to endogenous origin-proximal parS sites (Fig. 1A), or LacI-GFP (or LacI-CFP) bound to an array of lac operators inserted at 359°. Under the growth conditions used, the majority of cells (~84%) had two spatially resolved foci of the origin region as visualized with LacI-GFP bound to lac operators at 359° (Table 2). When origin position was plotted against cell length for cells with two foci, origins appeared to be distributed around the cell quarters using both Spo0J-GFP and the lacO/LacI-GFP systems (Fig. 2A and 2B; Table 3). Average origin positions, relative to the nearest pole (± the 95% confidence interval for the mean) were at or near the cell quarters: 27.9 ± 1.4% and 26.1 ± 1.0% of cell length for foci of Spo0J-GFP and LacI-GFP, respectively (Table 3). There was no significant difference in the average positions determined by the two methods, consistent with the previous finding that Spo0J usually colocalizes with LacI-GFP bound to lac operators inserted at 359° (Teleman et al., 1998).

We also analyzed the distance between foci (interfocal distance) and compared the interfocal distance to cell length. Using either Spo0J-GFP (Fig. 2A) or LacI-GFP bound to lac operators at 359° (Fig. 2B) to visualize origin regions, the interfocal distance between replicated origins increased as cell length increased (Fig. 3A, 3B), consistent with the notion that sister origin regions are positioned relative to cell length. This indicates that origins are not positioned by a segregation apparatus of fixed length and contrasts with a previous report indicating that the distance between Spo0J-GFP foci is fixed in B. subtilis (Sharpe and Errington, 1998). We do not understand the reasons for this discrepancy, but it could be due to differences in the Spo0J-GFP fusions, strain backgrounds, or growth conditions.
**Figure 1.** (A) The *Bacillus subtilis* chromosome with parS sites shown. *B. subtilis* has a circular chromosome with the origin of replication at 0°/360° and the terminus region at 180°. Spo0J binds eight known parS sites located in the origin-proximal 20% of the chromosome. The parS sites are located at 330°, 354°, 355°, 356°, 359°, 4°, 15°, and 40° and are indicated by flags. (B) Strains were constructed, each with an array of 16 parS sites inserted in a different region of the chromosome (90°, 181°, 210°, and 270°), indicated by the shaded rectangles. In each strain six of the eight known endogenous parS sites were inactivated. The remaining parS sites were located at 355° and 15°, indicated by flags.
Table 2. Number of foci per cell of various chromosomal regions during exponential growth in wild type and spo0J mutant cells.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Region</th>
<th>spo0J</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>&gt;4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCL696</td>
<td>359°</td>
<td>+</td>
<td>1.2</td>
<td>1.9</td>
<td>83.8</td>
<td>2.9</td>
<td>10.0</td>
<td>0.2</td>
<td>421</td>
</tr>
<tr>
<td>DCL705</td>
<td>359°</td>
<td>-</td>
<td>4.4</td>
<td>15.3</td>
<td>44.9</td>
<td>20.1</td>
<td>11.2</td>
<td>4.1</td>
<td>517</td>
</tr>
<tr>
<td>KPL716</td>
<td>270°</td>
<td>+</td>
<td>0.2</td>
<td>31.8</td>
<td>64.5</td>
<td>2.1</td>
<td>1.2</td>
<td>0.2</td>
<td>515</td>
</tr>
<tr>
<td>PSL101</td>
<td>270°</td>
<td>-</td>
<td>3.5</td>
<td>20.4</td>
<td>60.5</td>
<td>9.0</td>
<td>6.1</td>
<td>0.6</td>
<td>491</td>
</tr>
<tr>
<td>KPL686</td>
<td>181°</td>
<td>+</td>
<td>0.6</td>
<td>74.2</td>
<td>24.9</td>
<td>0.4</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>511</td>
</tr>
<tr>
<td>PSL110</td>
<td>181°</td>
<td>-</td>
<td>1.4</td>
<td>50.0</td>
<td>46.4</td>
<td>0.9</td>
<td>1.4</td>
<td>&lt;0.5</td>
<td>222</td>
</tr>
</tbody>
</table>

^a^Indicated strains were grown in defined minimal medium and samples were taken during exponential growth. The number of foci per cell of LacI-GFP (or LacI-CFP) was determined and the percentage of cells with the indicated number of foci was calculated.

^b^An array of lac operators was inserted in the indicated region of the chromosome and visualized with LacI-GFP or LacI-CFP.

^c^"+" indicates wild type spo0J and "-" indicates a spo0J null mutant.

^d^The total number of cells analyzed for each strain.
Figure 2. Location of sister copies of various regions of the chromosome. Strains producing Spo0J-GFP, LacI-GFP, LacI-CFP, or Rtp-YFP were grown at 30° in defined minimal medium. Only cells with two foci of the region of interest were analyzed. The distance from each focus to the same cell pole was measured from images of live cells in exponential growth (Materials and Methods) and is plotted on the x-axis and cell length is plotted on the y-axis. Cell length and the midcell positions are indicated by solid lines. Cell quarter positions are indicated by dotted lines. The number of cells analyzed (n) is indicated in the lower right corner of each panel. One focus is indicated with red open circles and the other with blue crosses.

A. Position of Spo0J-GFP bound to endogenous parS sites (strain PSL10).

B, C. Position of sister origin regions (359°) visualized with LacI-GFP bound to lac operator arrays in spo0J* (strain DCL696) and spo0J' (strain DCL705) cells.

D, E. Position of Spo0J-GFP bound to a parS array (D, strain PSL25) or LacI-CFP bound to a lac operator array (E, strain KPL718) inserted at 90°.

F. Position of Spo0J-GFP bound to a parS array inserted at 210° (strain PSL27).

G, H. Position of Spo0J-GFP bound to a parS array (G, strain DCL631) or LacI-CFP bound to a lac operator array (H, strain KPL686) inserted at 181°.

I. Position of Rtp-YFP bound to endogenous ter sites (strain IRN424).

J, K. Position of Spo0J-GFP bound to a parS array (J, strain PSL23) or LacI-CFP bound to a lac operator array (K, strain KPL716) inserted at 270°.
Table 3. Subcellular positions of sister copies of various regions of the chromosome.

<table>
<thead>
<tr>
<th>strain</th>
<th>region/genotype</th>
<th>focus position</th>
<th>interfocal distance</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSL10</td>
<td>spo0J-gfp</td>
<td>27.9 ± 1.4</td>
<td>44.3 ± 1.5</td>
<td>236</td>
</tr>
<tr>
<td>DCL696</td>
<td>359°::lacO</td>
<td>26.1 ± 1.0</td>
<td>47.8 ± 1.2</td>
<td>335</td>
</tr>
<tr>
<td>DCL705</td>
<td>359°::lacO; Δspo0J</td>
<td>30.1 ± 1.5</td>
<td>39.7 ± 1.8</td>
<td>222</td>
</tr>
<tr>
<td>PSL23</td>
<td>270°::parS</td>
<td>33.5 ± 1.6</td>
<td>33.0 ± 1.6</td>
<td>273</td>
</tr>
<tr>
<td>KPL716</td>
<td>270°::lacO</td>
<td>32.6 ± 1.3</td>
<td>34.8 ± 1.6</td>
<td>232</td>
</tr>
<tr>
<td>PSL25</td>
<td>90°::parS</td>
<td>34.5 ± 2.5</td>
<td>31.1 ± 2.4</td>
<td>123</td>
</tr>
<tr>
<td>KPL718</td>
<td>90°::lacO</td>
<td>34.9 ± 1.9</td>
<td>30.2 ± 2.0</td>
<td>123</td>
</tr>
<tr>
<td>PSL27</td>
<td>210°::parS</td>
<td>34.5 ± 1.4</td>
<td>31.0 ± 1.7</td>
<td>214</td>
</tr>
<tr>
<td>DCL631</td>
<td>181°::parS</td>
<td>31.5 ± 1.5</td>
<td>37.0 ± 1.6</td>
<td>276</td>
</tr>
<tr>
<td>KPL686</td>
<td>181°::lacO</td>
<td>38.6 ± 1.4</td>
<td>22.8 ± 1.7</td>
<td>193</td>
</tr>
<tr>
<td>IRN424</td>
<td>rtp-yfp</td>
<td>38.3 ± 1.5</td>
<td>23.4 ± 1.7</td>
<td>180</td>
</tr>
</tbody>
</table>

\(^a\) Indicated strains were grown in defined minimal medium and samples taken during exponential growth. Spo0J-GFP was used to visualize the subcellular location of parS arrays and Lacl-GFP (or Lacl-CFP) was used to visualize lac operator arrays. The arrays were integrated into the chromosome in the indicated regions. For strains PSL10 (spo0J-gfp) and IRN424 (rtp-yfp), there were no ectopic arrays and Spo0J-GFP was bound to the endogenous parS sites in the origin region (Lin and Grossman, 1998; Lin et al., 1997) and Rtp-YFP was bound to endogenous ter sites in the terminus region (Lemon et al., 2001).

\(^b\) The distance of each focus from the nearest cell pole was measured in cells with two foci, and is presented as a percentage of cell length ± the 95% confidence intervals.

\(^c\) The distance between foci (interfocal distance) was determined as a percentage of cell length in cells with two foci.

\(^d\) The total number of cells with two foci that were analyzed.
A  
spo0J-gfp  
r = 0.625  
n = 236

B  
359°::lacO, spo0J+  
r = 0.730  
n = 335

C  
359°::lacO, spo0J-  
r = 0.375  
n = 222
Figure 3. Relationship between cell length and interfocal distance.

Data from Fig. 2A-C were used to determine the distance between the two foci in each cell (the interfocal distance). This is plotted as a function of cell length. (A) Spo0J-GFP from Fig. 2A. (B) LacI-GFP in spo0J cells from Fig 2B. (C) LacI-GFP from spo0J mutant cells from Fig 2C. The correlation coefficient, r, which measures how strongly the interfocal distance and cell length are correlated, is shown for each plot. (r = 1 for two perfectly, positively correlated variables.)

An ellipse was drawn around the distribution of wild type cells (B), and this ellipse was superimposed on the corresponding plots in panels A and C. The interfocal distances in most of the spo0J mutant cells were within this ellipse, a subset (~15%) fell below and to the right of the wild type distribution. These cells had replicated origins that were closer together than origins in wild type cells of similar length.
Replicated origins are closer together in spo0J null cells. We compared the position of replicated origins in a large number of spo0J mutant cells to that of wild type cells. Origin regions were visualized using LacI-GFP bound to a lacO cassette inserted at 359°. The average position of the origin regions in spo0J null mutants was 30.1 ± 1.5% of cell length from the nearest pole (Fig. 2C; Table 3). This gives an average interfocal distance of 39.7 ± 1.8% of cell length in the spo0J mutant (Table 3). In contrast, the average distance between sister origin regions in wild type cells was 47.8 ± 1.2% of cell length (Table 3).

The interfocal distance was not as tightly correlated with cell length in the spo0J mutant (Fig. 3C) as in wild type cells (Fig. 3B). A subset of spo0J null cells had replicated origins that were closer together than origins in wild type cells of similar length. The smaller interfocal distance in this subset of cells probably accounts for the smaller average interfocal distance in the spo0J mutant. The subset of cells having aberrantly closely-spaced origins was approximately 15% of the spo0J null cells, estimated from the number of cells falling below the wild type distribution (Fig. 3B, C). This is greater than the proportion (1 - 2%) of anucleate cells produced by a spo0J null mutant, indicating that most of the cells with mispositioned origin regions can still successfully partition sister chromosomes. In fact, the vast majority of spo0J mutant cells still manage to separate sister origins to opposite halves of the cell (Fig. 2C).

Following duplication, origin regions move toward opposite halves of the cell in both B. subtilis and E. coli (Glaser et al., 1997; Gordon et al., 1997; Lewis and Errington, 1997; Lin and Grossman, 1998; Lin et al., 1997; Niki and Hiraga, 1998; Roos et al., 1999; Sharpe and Errington, 1998; Teleman et al., 1998; Webb et al., 1998; Webb et al., 1997). Our data indicate that in B. subtilis the sister origin regions are then positioned at or near the cell quarters. The defect in positioning in the spo0J null mutant could reflect a defect in: 1) recruiting the origins to the quarters, 2) separating newly duplicated sister origins, or 3) maintaining origin regions at the cell quarters after they are initially positioned there. These possibilities are not mutually exclusive. Results presented below indicate that Spo0J is not sufficient to recruit chromosomal binding sites to the cell quarters, leading
us to favor a role for Spo0J in separation of sister origin regions or in maintenance of sister origins at the cell quarters.

**parS arrays at 90°, 181°, 210°, or 270° are not recruited to the cell quarters.** A simple model for the function of ParB proteins is that they recruit their cognate binding sites to a characteristic subcellular position (for example, (Kim and Wang, 1998)). This model can account for many observations regarding the plasmid Par systems and the fact that chromosomal systems can function to stabilize a plasmid (Godfrin-Estvenon et al., 2002; Lin and Grossman, 1998; Yamaichi, 2000) and position plasmids at the cell quarters (Yamaichi, 2000). In this view, Spo0J could be positioned at the cell quarters and recruit or tether its binding sites there. To test this model, we measured the subcellular position of Spo0J-GFP bound to an array of 16 parS (parS16) sites inserted into different regions of the chromosome (90°, 181°, 210°, and 270°), in cells with six of the eight known endogenous parS sites inactivated (Fig. 1B) (Lin and Grossman, 1998).

In cells with the parS16 array inserted in the 270° region of the chromosome, the subcellular position of Spo0J-GFP was different from that of the origin region (Fig. 2A, B, and J; Table 3). Spo0J-GFP foci in cells with the parS16 array at the 270° region showed a broader distribution than origin foci and were positioned 33.5 ± 1.6 % of cell length from the nearest pole (Table 3). The results indicate that Spo0J is not sufficient to recruit parS sites at the 270° region to the cell quarters.

We also measured the position of Spo0J-GFP bound to ectopic parS sites inserted at three other regions in the chromosome, 90°, 181°, and 210° (Fig. 1B). None of these regions were recruited to the cell quarters (Fig 2D, G, F; Table 3) and in each case, the subcellular position of Spo0J-GFP was statistically distinguishable from that of the origin at the cell quarters. Again, these results indicate that Spo0J is not sufficient to recruit its binding sites to the cell quarters.

**Subcellular position of Spo0J-GFP bound to parS16 arrays at 90° and 270° is a reflection of the normal position of each chromosomal region.** For the 270°, 90°, and 181° regions, we compared the positioning of Spo0J-GFP bound to the parS16 array to that of the normal position of these regions as visualized with LacI-CFP bound to an array of lac operators
inserted at the same chromosomal region (see Materials and Methods).

The subcellular position of Spo0J-GFP bound to parS sites at 90° and 270° appeared to be a reflection of the normal subcellular position of those regions. The average position of LacI-CFP bound to an array of lac operators inserted at 90° was 34.9 ± 1.9% of cell length (Fig. 2E; Table 3). This was statistically indistinguishable from the average position of Spo0J-GFP bound to the parS16 array inserted at 90° (Fig. 2D; Table 3). Similarly, the average position of LacI-CFP bound to an array of lac operators at 270° was 32.6 ± 1.3% of cell length (Fig. 2K; Table 3), statistically indistinguishable from the average position of Spo0J-GFP bound to the parS16 array at 270° (Fig. 2J; Table 3). These results indicate that not only is Spo0J insufficient to recruit its binding sites to the cell quarters, but that the subcellular position of Spo0J is largely determined by the subcellular position of its binding sites, at least for the 90° and 270° regions.

In contrast to our findings with the 90° and 270° regions, the subcellular position of Spo0J-GFP bound to the parS array at 181° was significantly different from the subcellular position of LacI-CFP bound to lac operators at that position. In cells with two foci, the foci of Spo0J-GFP bound to the parS16 array inserted at 181° were farther apart than LacI-CFP bound to lac operators inserted at the same position (Fig. 2G, H; Table 3). The average focus positions were 31.5 ± 1.5% vs. 38.6 ± 1.4% of cell length, for Spo0J-GFP and LacI-CFP, respectively. We also measured the position of the terminus region using a fusion of YFP to the replication termination protein Rtp. Rtp binds to multiple sites in the terminus region (Lewis et al., 1990; Lewis et al., 1989) and has been used to visualize the subcellular position of this region (Lemon et al., 2001). We found that the subcellular position of Rtp-YFP was similar to that determined for the 181° region determined using the lac system (Fig. 2H, I; Table 3), consistent with previous findings (Lemon et al., 2001). These results indicate that the position of the terminus region is perturbed in cells with Spo0J bound to an array of parS sites at 181°. The terminus and origin regions are different from other regions of the chromosome in that they both appear to interact, either directly or indirectly, with factors associated with the bacterial membrane (Firshein, 1989; Hoshino et al., 1987; Winston and Sueoka, 1980; Wu and Errington, 1997). It is possible that Spo0J bound to the parS array inserted
at 181° interferes with endogenous positional information, or that the parS array titrates Spo0J away from other sites that affect, either directly or indirectly, positioning of the terminus region.

**Effects of spo0J on positioning various regions of the chromosome in the forespore.**

During sporulation, the origin proximal 30% of one chromosome is initially positioned in the forespore with the remaining ~70% in the larger mother cell (Wu and Errington, 1994). The mother cell also contains an intact chromosome which is essential for sporulation. Loss of function mutations in spoIIIIE trap the sporangium (forespore plus mother cell) in this situation by preventing the translocation of the remainder of the chromosome into the forespore (Wu and Errington, 1997; Wu et al., 1995). Previous findings were interpreted to indicate that Spo0J might provide a "centromere-like" function and play a role in positioning origin regions in the forespore during the early stages of sporulation (Sharpe and Errington, 1996). The experiments involved monitoring expression of genes that are induced only in the forespore and placing the reporter gene in different chromosomal locations. The findings were interpreted to indicate that a spo0J null mutation causes a defect in orienting the duplicated chromosomes such that origin regions were sometimes excluded from the forespore and other regions were inappropriately positioned in the forespore (Sharpe and Errington, 1996). These results and interpretations were called into question by a somewhat different type of analysis investigating the effects on sporulation of the transient genetic asymmetry between the forespore and the mother cell (Frandsen et al., 1999). These results indicated that the 174° and 192° regions of the chromosome were not positioned in the forespore in a spo0J mutant, apparently contradicting the earlier report (Sharpe and Errington, 1996). Finally, it has been suggested that during sporulation some spo0J mutant cells have an increased number of chromosomes, and parts of these might be randomly trapped in the forespore and not reflect a role for Spo0J in positioning origin regions to the pole (Webb et al., 1997).

In order to measure the effects of Spo0J on positioning of the origin region in the forespore, we visualized the 359° (Fig. 4A-E) and the 181° and 270° regions of the chromosome in sporulating cells. Regions of the chromosome were visualized using LacI-GFP or LacI-CFP bound to an array of lac operators inserted in the region of interest. Deleting spo0J causes a sporulation defect that is
Figure 4. Visualization of chromosomal regions during sporulation and growth.

A - E. Cells were grown in defined minimal medium and induced to sporulate by the addition of mycophenolic acid. Samples were taken four hours after the initiation of sporulation and membranes were stained with FM4-64. The position of origin regions was visualized with LacI-GFP bound to an array of lac operators inserted at 359°. Note that for cells with two or more foci, there was no detectable effect of spo0J on the frequency of positioning an origin region in the forespore (see text). Images shown are to illustrate some of the different types of sporangia observed. (A) Typical origin position in spo0J+ cells (strain PSL62 spo0J+ ΔspoIII+::tet).

(B - E) spo0J mutant cells (strain PSL73 Δ(soji-spo0J)::spc ΔspoIII::tet) with two origin foci located in the mother cell (B) and the chromosomal DNA visualized with DAPI (C) indicating that there is DNA in the forespore. (D) A spo0J mutant cell with a single focus of the origin region that is excluded from the forespore. (E) A spo0J mutant cell with four copies of the origin region, one of which is in the forespore.

F. Increased copies of the terminus region, visualized with LacI-CFP bound to an array of lac operators inserted at 181°, in a spo0J mutant (strain PSL110) during exponential growth.
suppressed by deleting soj (Ireton et al., 1994). Therefore, as in previous reports (Frandsen et al., 1999; Sharpe and Errington, 1996; Webb et al., 1997), comparisons were made between wild type and soj spo0J double mutants. In addition, we utilized strains that contained a null mutation in spoIIIe, the gene necessary for transport of the origin-distal 70% of the chromosome into the forespore (Wu and Errington, 1997; Wu et al., 1995). A spoIIIe null mutation prevents translocation of the entire chromosome into the forespore, and allows visualization of the region that is trapped. Images of live cells were captured four hours after the induction of sporulation and cells that had undergone polar septation were analyzed. The number of foci located in the forespore and mother cell was determined for each chromosomal region of interest (Table 4).

The origin (359°) region. Our results indicate that parS and Spo0J do not provide a "centromere-like" function for positioning origin regions to opposite poles during sporulation. In cells that had undergone asymmetric septation and had two foci of LacI-GFP bound to arrays of lac operators in the origin (359°) region (Fig. 4A-C), there was no noticeable affect of spo0J on positioning of foci in the forespore. Ninety-three percent (297 of 319) of the sporangia from spo0J+ cells and 92% (224 of 243) of the sporangia from soj spo0J mutant cells had one focus of LacI-GFP in the forespore and one in the mother cell (Fig. 4A). This finding is not consistent with the notion that Spo0J functions as an anchor to hold or recruit sister origins to opposite cell poles during sporulation.

There was an effect of spo0J on trapping the origin region in the forespore (Table 4), but only in cells with one focus of the origin region. There were only seven sporangia (1.9% of the total) from the spo0J+ cells that had a single focus of the origin region, and in five of those the focus was in the forespore. In striking contrast, there were 43 sporangia (12% of the total) from the soj spo0J mutant that had a single focus of the origin region (Table 5). Only seven of these had the focus in the forespore and the remaining 36 had the focus in the mother cell (Fig. 4D). Most of the soj-spo0J null cells that failed to trap origins still had chromosomal DNA trapped in the forespore, as visualized by DAPI staining (Fig 4C and data not shown). soj single mutants trapped the origin at a
Table 4. Effect of *spo0J* on positioning chromosomal regions in the forespore.

<table>
<thead>
<tr>
<th>strain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>region</th>
<th>(soj-spo0J)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% of sporangia with region in forespore&lt;sup&gt;c&lt;/sup&gt;</th>
<th>total&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSL66</td>
<td>270°</td>
<td>+</td>
<td>&lt;0.28 (0)</td>
<td>361</td>
</tr>
<tr>
<td>PSL76</td>
<td>270°</td>
<td>-</td>
<td>3.8 (21)</td>
<td>551</td>
</tr>
<tr>
<td>PSL64</td>
<td>181°</td>
<td>+</td>
<td>&lt;0.24 (0)</td>
<td>420</td>
</tr>
<tr>
<td>PSL74</td>
<td>181°</td>
<td>-</td>
<td>0.25 (1)</td>
<td>402</td>
</tr>
<tr>
<td>PSL62</td>
<td>359°</td>
<td>+</td>
<td>92 (331)</td>
<td>360</td>
</tr>
<tr>
<td>PSL73</td>
<td>359°</td>
<td>-</td>
<td>83 (287)</td>
<td>346</td>
</tr>
</tbody>
</table>

<sup>a</sup>Indicated strains (all containing a null mutation in *spoIII*E) have an array of lac operators inserted at the indicated region of the chromosome. Growth was in defined minimal medium and sporulation was induced by the addition of mycophenolic acid. Samples were taken four hours after the initiation of sporulation and stained with the membrane dye FM4-64 to allow identification of cells that had an asymmetric septum.

<sup>b</sup>"+" indicates wild type soj and spo0J; "-" indicates a soj-spo0J double mutant.

<sup>c</sup>Percentage (and total number, in parenthesis) of sporangia (mother cell plus forespore) with a focus of LacI-GFP (or LacI-CFP) positioned entirely in the forespore.

<sup>d</sup>The total number of sporangia analyzed for each strain.
Table 5. Effect of spoOJ on chromosome content during sporulation.

<table>
<thead>
<tr>
<th>strain</th>
<th>Region</th>
<th>(soj-spoOJ)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>&gt;4</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSL62</td>
<td>359°</td>
<td>+</td>
<td>0.28</td>
<td>1.9</td>
<td>89</td>
<td>7.2</td>
<td>1.9</td>
<td>&lt;0.28</td>
<td>360</td>
</tr>
<tr>
<td>PSL73</td>
<td>359°</td>
<td>-</td>
<td>0.87</td>
<td>12</td>
<td>70</td>
<td>10</td>
<td>6.1</td>
<td>&lt;0.29</td>
<td>346</td>
</tr>
<tr>
<td>PSL66</td>
<td>270°</td>
<td>+</td>
<td>0.55</td>
<td>1.7</td>
<td>94</td>
<td>2.8</td>
<td>1.4</td>
<td>&lt;0.28</td>
<td>361</td>
</tr>
<tr>
<td>PSL76</td>
<td>270°</td>
<td>-</td>
<td>1.6</td>
<td>6.5</td>
<td>62</td>
<td>17</td>
<td>12</td>
<td>1.6</td>
<td>551</td>
</tr>
<tr>
<td>PSL64</td>
<td>181°</td>
<td>+</td>
<td>0.71</td>
<td>32</td>
<td>63</td>
<td>1.7</td>
<td>0.9</td>
<td>&lt;0.24</td>
<td>420</td>
</tr>
<tr>
<td>PSL74</td>
<td>181°</td>
<td>-</td>
<td>1.0</td>
<td>21</td>
<td>65</td>
<td>6.0</td>
<td>5.7</td>
<td>0.7</td>
<td>402</td>
</tr>
</tbody>
</table>

\(^a\)Indicated strains (all containing a null mutation in spo\textit{III}E) were grown in defined minimal medium and induced to sporulate by the addition of mycophenolic acid. Samples were taken four hours after the initiation of sporulation and stained with the membrane dye FM4-64 to allow identification of cells that had divided asymmetrically. The number of foci per sporangium (mother cell plus forespore) of LacI-GFP (or LacI-CFP) was determined for cells that had undergone an asymmetric septation. The percentage of sporangia with the indicated number of foci for each strain is presented.

\(^b\)An array of lac operators was inserted in the indicated region of the chromosome and visualized with LacI-GFP or LacI-CFP.

\(^c\) "+" indicates wild type soj and spoOJ; "-" indicates a soj-spoOJ double mutant.

\(^d\)The total number of sporangia analyzed for each strain is indicated.
frequency similar to wild type cells, indicating that the trapping defect was due to the absence of spo0J (data not shown). These results indicate that Spo0J does influence positioning of the origin region in the forespore, but probably because it affects the number of sporangia that have only a single focus of the origin region. The spo0J null mutant also had an increase in the number of sporangia that had more than two foci of the origin region (Table 5), although almost all of these had a focus positioned in the forespore (Fig. 4E). These effects of spo0J on the number of origin regions per sporangium are probably due to asynchronous replication and defects in coordinating cell division with replication (see below).

The 270° and 181° (terminus) regions. We also examined the effects of loss of spo0J on trapping the 270° and 181° regions in the forespore. In spo0J+ cells that had undergone asymmetric septation, we never (of 361 sporangia) observed positioning of the 270° region in the forespore (Table 4). However, in the spo0J mutant 3.8% of the sporangia (21 of 551) had the 270° region positioned in the forespore (Table 4). Of these, ten had one focus in the mother cell and one focus in the forespore, and one sporangium had a focus only in the forespore. The remaining ten sporangia had one focus in the forespore and multiple foci in the mother cell. These observations indicate that approximately half the time mispositioning of the 270° region in the forespore is probably due to cells with extra copies of this region. Many of the sporangia that did not have the 270° region trapped in the forespore had extra foci of the region in the mother cell (see below), consistent with the previous observation that some cells in a population of spo0J mutants have extra copies of the chromosome (Webb et al., 1997).

In contrast to the 270° region, only 0.25% of the spo0J mutant sporangia (1 of 402) had the 181° region positioned in the forespore, virtually indistinguishable from the spo0J+ sporangia (0 of 420) (Table 4). Thus, it appears that even in the absence of spo0J, the terminus region (181°) is rarely trapped in the forespore. These findings probably explain part of the apparent discrepancy between previous reports (Frandsen et al., 1999; Sharpe and Errington, 1996). If the terminus region is not significantly positioned in the forespore in the spo0J mutant, then as reported (Frandsen et al., 1999), experiments to measure expression in the forespore of genes in the

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terminus region would indicate little or no expression.

**spo0J null mutants have more origin foci and increased chromosomal copy number.** Cells containing the *spo0J* null mutation had phenotypes in addition to the origin positioning defect. Inactivating *spo0J* affected the number of origin foci per cell: there were fewer cells with two foci and more cells with one, three or more foci relative to wild type, visualized with LacI-GFP bound to *lac* operators at 359° (Table 2). Additionally, the mutant cells had more foci of the 270° and 181° regions of the chromosome, visualized by LacI-CFP bound to *lac* operators inserted in these regions (Table 2; Fig. 4F). These results indicate that a subset of the *spo0J* mutant cells has an increase in chromosomal copy number. Similar results were observed in sporulating cells (Table 5), consistent with a previous observation (Webb et al., 1997).

The increased number of chromosomes in some cells could be due to a defect in the timing of replication initiation: if origins fired more often, this could increase the chromosomal copy number, and if origins fired asynchronously within the same cell, this could create cells with three foci of the origin region. The increase in cells with one focus of the origin could be due, in part, to asynchronous initiation (for example, a cell with three foci of the origin could divide to produce a one-focus cell and a two-focus cell). We would also expect that the *spo0J* mutant would have an increase in the percentage of cells with a single focus of other regions. However, this is not the case (Table 2), indicating that the increase in the percentage of cells with a single focus of the origin region might be due to something other than or in addition to a asynchronous replication. A defect in separation of sister origins could also contribute to an increase in cells that appear to have a single focus of the origin. The finding the sister origins are on average closer together in the *spo0J* mutant is also consistent with the notion that Spo0J is somehow facilitating separation.

Flow cytometry results confirmed that inactivating *spo0J* caused increased chromosome content as well as asynchronous initiation of replication. Replication run-off assays were performed to count the number of origins per cell. Ongoing rounds of replication were allowed to finish while re-initiation and cell division were inhibited. Under the growth conditions used (see Materials and Methods), the majority of wild type cells had four origins per cell, and origin numbers reflected
Figure 5. Early and asynchronous initiation of replication in spo0J mutant cells.

spo0J+ (A, strain CRK6000) and spo0J mutant (B, strain NIK6074) cells were grown in Difco antibiotic medium 3 and samples taken during exponential growth and treated for analysis by flow cytometry (see Materials and Methods). Histograms show the number of cells with a given amount of DNA per cell after completion of rounds of replication as determined by flow cytometry. The numbers on the x-axis represent the number of origins per cell at the time the sample was taken. More than 20,000 cells were analyzed in each experiment.
synchronous replication initiation in most of the population (Fig. 5A). Inactivating spoOJ caused a shift towards six to eight origins per cell (Fig. 5B), consistent with the observation that a spoOJ null caused increased numbers of foci per cell of several chromosomal regions visualized by the lac system. These results could be due to: 1) replication initiation occurring asynchronously and at a smaller cell mass in the absence of spoOJ, and/or 2) effects of spoOJ on growth rate or cell division. Under the growth conditions used, inactivating spoOJ did not cause a detectable change in doubling time (54 ± 2 minutes for the wild type and the spoOJ mutant). Although spoOJ mutant cells were found to be 15 to 20 percent larger (1.17-fold) than wild type cells by light scattering, the origin/mass ratio was 1.6 times higher in a spoOJ null mutant than in wild type, indicating that the increased chromosomal content was due to early initiation rather than a cell division defect alone. Furthermore, the DNA/protein ratio was higher in a spoOJ null mutant (0.060 ± 0.001) than in wild type (0.041 ± 0.002), indicating that there is overreplication in the spoOJ mutant.

In addition to the increased chromosome content in some of the spoOJ mutant cells, the average length of spoOJ null mutant cells measured from micrographs of exponentially growing cells was ~20% greater than that of wild type, consistent with the light-scattering results obtained by flow cytometry. The average length of wild type cells was 2.45 ± 0.07 μm, whereas the spoOJ null mutant had an average length of 2.93 ± 0.15 μm (expressed as the 95% confidence intervals for the means). This difference is indicative of a defect or delay in cell division in the spoOJ mutants.

Interestingly, depletion of ParB in Caulobacter crescentus causes a severe defect in cell division (Mohl et al., 2001), perhaps indicating a role for many of the ParB members in regulating cytokinesis. We suspect that the defect in cell division in the spoOJ mutants could partly contribute to the increased chromosome copy number. However, this alone cannot account for the presence of cells with asynchronous numbers of origin foci or the increased origin-mass and DNA-protein ratios observed in the spoOJ mutant. Taken together, the results indicate that spoOJ is a negative regulator, either directly or indirectly, of replication initiation in B. subtilis.

Phenotypes of spoOJ mutants. Experiments presented here indicate that duplicated origin
regions are typically found approximately at the cell quarters and that Spo0J contributes to this positioning. Sister origins were closer together in a subpopulation of spo0J mutant cells. However, binding of Spo0J to an array of parS sites was not sufficient to recruit these sites to the cell quarters. It seems likely that Spo0J indirectly contributes to positioning duplicated origins at the cell quarters.

Rather than providing positional information per se, it is possible that Spo0J contributes to separation of sister origin regions. If Spo0J is helping to compact the origin region, as previously suggested (Autret et al., 2001; Britton and Grossman, 1999), then perhaps this compaction facilitates movement of the sister origin regions away from each other. Compaction could also enhance the function of other proteins that might bind to the origin region and contribute to positioning. A recent report identified a site in the origin region that appears to be the main determinant of origin positioning (Kadoya et al., 2002). Whereas Spo0J does not appear to bind to this site, perhaps Spo0J bound to its sites in the origin region contributes to the function of this positioning site. A broad region of the chromosome that appears to contribute to positioning of the origin region in the forespore has also been identified (Wu and Errington, 2002). However, Spo0J function appears to override the effects of this region and the interactions between Spo0J function and this large partitioning region are not understood.

In addition to the alteration in positioning of sister origin regions, a subpopulation of the spo0J null mutant cells had extra copies of the chromosome and appeared to have asynchronous replication. These phenotypes are somewhat similar to those caused by seqA mutations of E. coli ((Hiraga, 2000; Lu et al., 1994) and references therein) and may indicate that binding of Spo0J to endogenous parS sites sequesters origins and prevents inappropriate initiation of replication.

**Apparent discrepancy between plasmid and chromosomal par systems.** Our results highlight an apparent discrepancy between plasmid and chromosomal Par systems. Spo0J was not sufficient to position ectopic chromosomal parS sites at the cell quarters. However, plasmid Par proteins are required, and appear to be sufficient for positioning duplicated plasmids at the cell quarters. In fact, when placed on a derivative of the E. coli F plasmid, the B. subtilis Par system (soj,
spo0J, and parS) confers positional information such that duplicated plasmids are now found predominantly at the cell quarters and the plasmid is significantly stabilized, in *E. coli* (Yamaichi, 2000). In *B. subtilis*, both *soj* and *spo0J* are necessary for plasmid stability (Lin and Grossman, 1998).

ParA/ParB/parS partitioning systems are widespread. First characterized in the low copy plasmid F and the prophage P1, homologous systems have been found in numerous plasmids and on many bacterial chromosomes (reviewed in (Bignell and Thomas, 2001; Draper and Gober, 2002; Gordon and Wright, 2000; Hiraga, 2000)). It would be comforting to think that the mechanisms by which the plasmid and chromosomal systems contribute to partitioning are similar, and the fact that a chromosomal system can function on a plasmid are consistent with this view. However, there appear to be significant differences between the plasmid and chromosomal systems. First, as indicated above, the Par system from *B. subtilis* does not appear to be sufficient to provide positional information to ectopic sites in the chromosome. Furthermore, while loss of the *B. subtilis* Par system causes some perturbation in the positioning of sister origin regions, there still appears to be significant positioning. In addition, in the plasmid systems, and for chromosomal systems to function on a plasmid, both the *parA* and *parB* products are required (Erdmann, 1999; Lin and Grossman, 1998; Niki and Hiraga, 1997). In contrast, in *B. subtilis*, loss of *parB* has much more severe an effect on chromosome partitioning than loss of *parA* (Ireton et al., 1994).

We do not understand the nature of these differences between plasmids and chromosomes. They could reflect differences in the mechanisms by which the Par systems function on plasmids and chromosomes. However, we think it more likely that they reflect differences between plasmids and chromosomes (size, time it takes for replication, proximity of *parS* sites to the origin), and that the biochemical functions of the homologous Par systems are quite similar. For example, if the primary positional information comes from another site and a protein that binds to that site, as appears to be the case for the chromosome (Kadoya et al., 2002), and could be the case for plasmids, then the Par system could facilitate function of the primary system, but not be providing positional information per se. For plasmids (and the chromosome too), the system providing
positional information could be related to the origin of replication and replication initiation proteins. Further analysis of and comparisons between plasmid and chromosomal Par systems should help determine the mechanisms by which they act.

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References


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Chapter 3

Effects of the chromosome partitioning proteins Soj (ParA) and Spo0J (ParB) on origin separation and replication initiation in *Bacillus subtilis*

(Manuscript in preparation)
Abstract

Soj (ParA) and Spo0J (ParB) of *Bacillus subtilis* belong a conserved family of proteins required for efficient plasmid and chromosome partitioning in many bacterial species. Although a soj null mutation does not cause an appreciable chromosome partitioning defect on its own, we show that inactivating soj caused a synthetic chromosome partitioning defect in combination with a null mutation in smc, the gene encoding Structural Maintenance of Chromosomes (SMC) protein. To characterize the Δsoj mutant further, we measured effects on replication initiation and origin positioning in comparison with Δspo0J and Δ(soj-spo0J) mutants. Δsoj, Δspo0J, and Δ(soj-spo0J) mutations caused overinitiation of replication and affected origin positioning within cells. Finally, we demonstrated that inactivating soj, spo0J, or both caused a defect in separating replicated origins, although these mutations did not significantly affect separation of regions outside the origin. We propose that Soj and Spo0J contribute to timely separation of replicated origins, and that the origin positioning and chromosome partitioning phenotypes of Δsoj, Δspo0J and Δ(soj-spo0J) mutants may be a secondary consequence of a defect in separating replicated origins.
Introduction

Dividing cells must partition their chromosomes accurately in order for daughter cells to receive a complete copy of the genome. Successful completion of chromosome partitioning is critical because loss of genetic material can perturb cellular functions and reduce viability. Bacteria possess several known chromosome partitioning factors that minimize the likelihood that a cell division event produces an anucleate, or chromosomeless cell.

The well-conserved Par system contributes to accurate partitioning of low copy-number plasmids and chromosomes in diverse bacterial species (Bignell and Thomas, 2001; Hiraga, 2000; Surtees and Funnell, 2003). The Par system is defined by two trans-acting factors and a cis-acting site: ParA, a Walker-type ATPase that binds DNA, ParB, a DNA-binding protein that interacts with ParA, and parS, the site bound by ParB. The molecular mechanism of how the Par system functions remains unknown, although studies of the plasmid Par systems lead to two prevalent models: 1) that the Par system may attach parS-containing DNA to putative anchors at the cell quarters (Kim and Wang, 1998; Ogura and Hiraga, 1983; Rodionov, 1999; Watanabe et al., 1989; Yamaichi and Niki, 2004), or 2) that the Par system may pair plasmids prior to partitioning them in opposite directions (Austin and Abeles, 1983b; Edgar et al., 2001; Funnell, 1988; Nordström and Austin, 1989; Surtees and Funnell, 2003; Youngren and Austin, 1997).

Less is known about the function of chromosomally-encoded Par systems. It appears likely that the Par system helps to partition replicated origins, given that known or putative ParB binding sites are located near the origin region (Kim et al., 2000; Lin and Grossman, 1998; Mohl and Gober, 1997). Partitioning of chromosomal origins probably involves a mechanism that separates them, as well as a mechanism that maintains their characteristic subcellular positions. In many bacteria, replicated origins separate soon after initiation of replication, and move to
opposite halves of the cell even as replication of distal regions continues (Jensen and Shapiro, 1999; Lemon and Grossman, 2001; Li et al., 2002). For the majority of the cell cycle, replicated origins occupy characteristic positions: at the cell quarters in *Bacillus subtilis* (Lee et al., 2003; Lin et al., 1997; Sharpe and Errington, 1998; Webb et al., 1998; Webb et al., 1997), at the cell poles in *Caulobacter crescentus* (Figge et al., 2003; Mohl and Gober, 1997), and near the cell quarters or poles in *Escherichia coli* (Gordon et al., 1997; Lau et al., 2003; Li et al., 2002; Niki et al., 2000). In this study, we present data indicating that the Par system of *B. subtilis* contributes to separation of replicated origins. *E. coli* is not known to have a chromosomally-encoded Par system, hence other factors must contribute to chromosome partitioning.

*B. subtilis* possesses chromosomally-encoded homologs of ParA and ParB called Soj and Spo0J, respectively. Spo0J binds to at least eight parS sites spread over nearly 800 kb in the origin-proximal 20% of the chromosome (Lin and Grossman, 1998). Spo0J binds the parS sites and probably brings them together, forming a nucleoprotein complex that can be visualized as a focus using immunofluorescence microscopy or a GFP fusion to Spo0J (Lin et al., 1997; Sharpe and Errington, 1998; Teleman et al., 1998). The subcellular location of foci of Spo0J reflects the position of chromosomal origins (Lin et al., 1997; Sharpe and Errington, 1998; Teleman et al., 1998). In the Δspo0J mutant, replicated origins are mispositioned such that they are often closer together than the cell quarters (Lee et al., 2003; Ogura et al., 2003), leading to the hypothesis that Spo0J may contribute to separation of replicated origins and/or maintenance of replicated origins at the cell quarters. In addition to its effects on origin positioning and chromosome partitioning, the Δspo0J mutant has overinitiation and asynchronous replication (Lee et al., 2003; Ogura et al., 2003), and a ~300-fold decrease in spore formation (Ireton et al., 1994). The mechanism by which Spo0J regulates initiation of replication remains to be elucidated.
Inactivating soj alone does not cause an appreciable chromosome partitioning defect, but inactivating spo0J or (soj-spo0J) causes an ~hundredfold increase in the production of anucleate cells such that ~1% of cells are anucleate (Ireton et al., 1994). In other Par systems, ΔparA mutants have defects in plasmid and chromosome partitioning to a similar extent as ΔparB mutants (Austin and Abeles, 1983a; Austin and Abeles, 1983b; Easter and Gober, 2002; Figge et al., 2003; Mohl and Gober, 1997; Ogura and Hiraga, 1983). Either Soj does not function like other ParA proteins, or Soj could contribute to chromosome partitioning.

We found that a Δsoj null mutation, like a Δspo0J mutation, caused a synthetic chromosome partitioning defect in combination with a null mutation in smc, the gene encoding structural maintenance of chromosomes protein. We analyzed phenotypes of a Δsoj mutant in comparison with Δspo0J and Δ(soj-spo0J) mutants. Δsoj, Δspo0J, and Δ(soj-spo0J) mutants had overinitiation of replication, seen as an increased DNA to protein ratio and an increased number of foci of several chromosomal regions visualized using LacI-GFP bound to arrays of lac operators inserted in the chromosome. In cells with two duplicated foci of the origin, the foci were located at the normal, cell quarter positions in the Δsoj mutant, but were closer together in a subset of Δ(soj-spo0J) cells, like the Δspo0J mutant. In cells that appeared to have a single origin, the focus was shifted away from the central, wild-type position in Δsoj, Δspo0J, and Δ(soj-spo0J) mutants. We found that a significant fraction of these mutant cells actually contained replicated origins that had not separated enough to be spatially resolved. These mutations did not significantly affect separation of a region outside the origin. The results indicate that Δsoj, Δspo0J, and Δ(soj-spo0J) mutations caused defects in separating replicated origins, and that the separation defect was specific to the origin region.
Materials and Methods

**Media and growth conditions.** For all experiments, cultures were grown at 30°C in S7 defined minimal medium with MOPS (morpholinepropanesulfonic acid) buffer at 50 mM rather than 100 mM) supplemented with 0.1% glutamate, required amino acids (tryptophan, 40 µg/mL; phenylalanine, 40 µg/mL; and threonine, 120 µg/mL), and either 1% glucose or 1% succinate (Jaacks et al., 1989; Vasantha and Freese, 1980). Unless otherwise noted, cells were inoculated into liquid cultures from light lawns on Luria-Bertani (LB) plates. Strains were constructed and maintained on LB plates at 30°C, with the exception of strains containing Δsmc, ΔscpA and ΔscpB mutations (see below). Antibiotics were used at standard concentrations.

**Media and growth conditions for Δsmc, ΔscpA and ΔscpB strains.** The chromosome partitioning defect of Δsmc, ΔscpA, and ΔscpB mutants is more severe in LB than in minimal medium (Britton et al., 1998; Soppa et al., 2002). We found that inoculating cultures from light lawns on LB plates caused a more severe chromosome partitioning defect than minimal plates, even after three generations of growth in minimal medium: 13.3% of cells were anucleate from LB plates, similar to the previously reported partitioning defect of an smc null (Britton et al., 1998; Moriya et al., 1998), compared to 2-3% from minimal plates. For all experiments involving Δsmc, ΔscpA and ΔscpB strains, we used Spizizen’s minimal plates (Harwood and Cutting, 1990) supplemented with 1% glucose, 0.1% glutamate and required amino acids for preparing light lawns, strain construction and routine maintenance. 1 mM IPTG was added to induce expression of downstream genes in ΔscpA and ΔscpB strains.

**Strains, alleles, and plasmids.** *B. subtilis* strains (Table 1) are derived from JH642 (*trpC2 pheA1*) and were constructed using standard procedures (Harwood and Cutting, 1990). Plasmids used in this study are described (Table 2).
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<th>Strain</th>
<th>Relevant genotype or characteristics (reference)</th>
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<tr>
<td>AG174</td>
<td>trpC pheA (Hoch and Mathews, 1973)</td>
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<tr>
<td>AG1468</td>
<td>Δ(spo0J)::spc (Ireton et al., 1994)</td>
</tr>
<tr>
<td>AG1505</td>
<td>Δ(soj-spo0J)::spc (Ireton et al., 1994)</td>
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<td>DCL696</td>
<td>yyaC (359º):pDL175 (lacO cassette, cat); thrC::(lacI-gfp, erm) (Lee et al., 2003)</td>
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<td>DCL705</td>
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<td>PSL107</td>
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<td>PSL521</td>
<td>Δsoj::spc; Δsmc::kan</td>
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<tr>
<td>PSL568</td>
<td>Δ(soj-spo0J)::spc; Δsmc::kan</td>
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PSL576  $\Delta$(soj-spo0J)::spc
alsD(316°)::pPSL27 (lacO cassette, cat); thrC::(lacI-gfp, erm)
yvfl(300°)::pPSL18 (tetO cassette); cgeD(181°)::Ppen(mut TAGG)-tetR-yfp (tet)

PSL578  $\Delta$soj in-frame;
alsD(316°)::pPSL27 (lacO cassette, cat); thrC::(lacI-gfp, erm)
yvfl(300°)::pPSL18 (tetO cassette); cgeD(181°)::Ppen(mut TAGG)-tetR-yfp (tet)

PSL580  $\Delta$spo0J::(spc);
alsD(316°)::pPSL27 (lacO cassette, cat); thrC::(lacI-gfp, erm)
yvfl(300°)::pPSL18 (tetO cassette); cgeD(181°)::Ppen(mut TAGG)-tetR-yfp (tet)

PSL582  alsD(316°)::pPSL27 (lacO cassette, cat); thrC::(lacI-gfp, erm)
yvfl(300°)::pPSL18 (tetO cassette); cgeD(181°)::Ppen(mut TAGG)-tetR-yfp (tet)

PSL640  scpA::pMUTIN$\Delta$scpA (erm)

PSL642  scpB::pMUTIN$\Delta$scpB (erm)

PSL654  $\Delta$soj in-frame; scpB::pMUTIN$\Delta$scpB

PSL696  $\Delta$(soj-spo0J); thrC::(sojG12V, erm)

RB35  $\Delta$mc::kan (Britton et al., 1998)

RB41  $\Delta$spo0J::spc; $\Delta$mc::kan (Britton et al., 1998)

SV132  $\Delta$soj in-frame deletion, no drug resistance
Table 2. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Description</th>
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<tr>
<td>pAT14</td>
<td>To integrate lacO cassette at cgeD ((181^\circ)) (Teleman et al., 1998)</td>
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<td>pAT25</td>
<td>To integrate lacO cassette at cotS ((270^\circ)) (Teleman et al., 1998)</td>
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<td>pBW3</td>
<td>tetR-gfp plasmid (Dworkin and Losick, 2002)</td>
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<td>pDL175</td>
<td>To integrate lacO cassette at yycC ((359^\circ)) (Lee et al., 2003)</td>
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<td>pIK185</td>
<td>soj, spo0J plasmid (Ireton et al., 1994)</td>
</tr>
<tr>
<td>pIK207</td>
<td>sojG12V, spo0J plasmid</td>
</tr>
<tr>
<td>pIK208</td>
<td>Δsoj, spo0J plasmid (Ireton et al., 1994)</td>
</tr>
<tr>
<td>pIK210</td>
<td>To integrate ((sojG12V, spo0J)) at thrC</td>
</tr>
<tr>
<td>pLAU44</td>
<td>Contains ~9kb array of 240 tetO2 sites (Lau et al., 2003)</td>
</tr>
<tr>
<td>pMUTINΔscpA</td>
<td>To disrupt scpA while permitting expression of downstream genes (Soppa et al., 2002)</td>
</tr>
<tr>
<td>pMUTINΔscpB</td>
<td>To disrupt scpB while permitting expression of downstream genes (Soppa et al., 2002)</td>
</tr>
<tr>
<td>pPSL6</td>
<td>Contains ~9kb array of 240 tetO2 sites cloned into pDG792 (Guerout-Fleury et al., 1995)</td>
</tr>
<tr>
<td>pPSL10</td>
<td>To integrate tetO cassette at hutM ((345^\circ))</td>
</tr>
<tr>
<td>pPSL18</td>
<td>To integrate tetO cassette at yycl ((300^\circ))</td>
</tr>
<tr>
<td>pPSL27</td>
<td>To integrate lacO cassette at alsD ((316^\circ))</td>
</tr>
<tr>
<td>pPSL36</td>
<td>Contains Ppen-tetR-yfp flanked by upstream and downstream regions of cgeD ((181^\circ)), used to make pPSL38</td>
</tr>
<tr>
<td>pPSL38</td>
<td>To integrate Ppen*(TATG (\rightarrow) TAGG)-tetR-yfp at cgeD ((181^\circ))</td>
</tr>
<tr>
<td>pPSL50</td>
<td>To integrate sojG12V at thrC</td>
</tr>
<tr>
<td>pRV11</td>
<td>Used to create in-frame Δsoj deletion in the chromosome</td>
</tr>
</tbody>
</table>
(i.) **Δsoj in-frame deletion.** The Δsoj in-frame deletion was constructed by Shivkumar Venkatasubramanyam. pIK208 contains an in-frame Δsoj deletion removing codons 6-235 of 253, inclusive, from the soj coding region, followed by the full-length spo0J gene (Ireton et al., 1994). A HindIII fragment was removed from pIK208, removing the C-terminal 278 nucleotides from spo0J, creating pRV11. pRV11 was integrated into the *B. subtilis* chromosome by single crossover, selecting for chloramphenicol-resistant sporulation-defective transformants. SV132, a spontaneous derivative that retained the in-frame Δsoj mutation but had lost plasmid-derived sequences by homologous recombination, was obtained by selecting for sporulation-competent, chloramphenical-sensitive derivatives. The Δsoj mutation was confirmed by PCR and restriction digests. Western blotting confirmed that Spo0J protein was expressed at a level indistinguishable from wild type (data not shown).

(ii.) **ΔscpA and ΔscpB null mutants.** scpA or scpB were disrupted while permitting expression of downstream genes from a Pspac promoter as described (Soppa et al., 2002). The ΔscpA and ΔscpB mutations were introduced into the JH642 background using chromosomal DNA from NIS6031 and NIS6042 (Lindow et al., 2002b), selecting for erythromycin-lincomycin resistance and sensitivity to spectinomycin. This created PSL640 and PSL642, respectively.

(iii.) **soj ATPase mutant.** Site-directed mutagenesis (Ausubel et al., 1990) of plasmid pIK185 (Ireton et al., 1994) was used to introduce the sojG12V mutation predicted to compromise ATP hydrolysis, generating pIK207. A BamHI/EcoRI fragment of pIK207, containing sojG12V followed by the intact spo0J gene, was subcloned into BamHI/EcoRI-cut pDG795 (provided by P. Stragier) (Ireton et al., 1994), creating pIK210. pIK210 was used to integrate (sojG12V-spo0J) at the thrC locus of AG174 competent cells by double crossover, and
chromosomal DNA from this strain was used to move *(sojG12V- spo0J)* into competent cells of AG1505, creating K11936. pPSL50 was used to insert *sojG12V* alone at the *thrC* locus. To construct pPSL50, a 1549 bp EcoRI/HindIII fragment from pIK210 containing the intact *soj* gene and a 5' fragment of *spo0J* was subcloned into EcoRI/HindIII-cut pDG795. pPSL50 was transformed into competent cells of AG174, and chromosomal DNA from this strain was used to move *sojG12V* into competent cells of AG1505, creating PSL696.

**(iv.) lac operator system.** LacI-green fluorescent protein (GFP) and LacI-cyan fluorescent protein (CFP) were used to visualize regions of the chromosome marked with arrays of *lac* operators. The *lacI-gfp* and *lacI-cfp* fusions have been described extensively (Lemon and Grossman, 2000; Lemon et al., 2001). Briefly, *lacI* is missing the C-terminal 11 codons (to inhibit tetramerization), and is fused to either *gfpmut2* or *cfpw7*.

The *lacO* cassette was inserted into several regions of the chromosome (181°, 270°, 316°, and 359°) by homologous recombination. The *lacO* cassettes at the 181° and 270° regions, inserted at the sporulation genes *cgeD* and *cotS*, respectively, were introduced using chromosomal DNA from AT54 and AT52, respectively (Teleman et al., 1998). The *lacO* cassette at the 359° region was introduced using chromosomal DNA from a strain that had been transformed with the plasmid pDL175 (Lee et al., 2003). To insert the *lacO* cassette at the 316° region of the chromosome, oligonucleotides LEE-106 and LEE-82 were used to amplify a 3' fragment extending past the stop codon of *alsD*, so that integration into the chromosome would not disrupt the coding region. The PCR product was digested with BamHI and KpnI and subcloned into the *lacO*-cassette containing plasmid pAT12 (Teleman et al., 1998), creating pPSL27. The *lacO* cassettes were amplified by selecting for resistance to chloramphenicol (25 µg/mL) as described previously (Teleman et al., 1998; Webb et al., 1997).
(v.) tet operator system. The tetO/TetR-yellow fluorescent protein (YFP) system was used in conjunction with the lacO/LacI-CFP system so that two different regions of the chromosome could be visualized simultaneously. pPSL38 contains the tetR-yfp fusion under control of Ppen*, a mutagenized version of the Ppen promoter that contains a TATG→TAGG mutation in the extended -10 region, flanked by upstream and downstream regions of the cgeD gene for integration into the chromosome by double crossover. To construct this vector, tetR-gfp from pBW3 (Dworkin and Losick, 2002) was converted to tetR-yfp using site-directed mutagenesis to convert threonine 203 to tyrosine (Ormo et al., 1996). pPSL36 contains tetR-yfp under control of the Ppen promoter, flanked by upstream and downstream regions of cgeD. Mutagenic oligopeptides with the sequence TANN in the extended -10 region (N = random nucleotide) were used to mutagenize pPSL36 to decrease expression of TetR-YFP. One of the mutagenized products, named pPSL38, was selected that had a bright signal and low background in the presence of tet operators inserted at the 359° region of the chromosome, but did not detectably perturb cellular physiology. Sequencing revealed a TATG→TAGG mutation in the extended -10 region.

To visualize the 300° or 345° regions of the chromosome, a tetO cassette was inserted there by homologous recombination. pLAU44 contains an ~9kb array of 240 tetO2 sites with randomized sequences between the sites to minimize recombination (Lau et al., 2003). The tetO2 cassette was subcloned NheI/SalI into the kanamycin-resistant vector pDG792 (Guerout-Fleury et al., 1995), to create pPSL6. tetO integration vectors were designed to integrate downstream of chromosomal genes without disrupting them. To insert the tetO cassette at the 300° region of the chromosome, oligonucleotides LEE-85 and LEE-87 were used to amplify a 3’ fragment extending past the stop codon of the yvfl gene. The PCR product was digested with
PstI and Kpn and subcloned into pPSL6, creating pPSL18. pPSL18 was transformed into cells of the wild type strain AG174, and chromosomal DNA from this strain was used to introduce the tetO cassette at the 300° region. To insert the tetO cassette at the 345° region of the chromosome, oligonucleotides LEE-58 and LEE-59 were used to amplify a 3’ fragment extending past the stop codon of the hutM gene. The PCR product was digested with PstI and Kpn and subcloned into pPSL6, creating pPSL10. pPSL10 was transformed into cells of the wild type strain AG174, and chromosomal DNA from this strain was used to introduce the tetO cassette at the 345° region.

**Measurement of DNA to protein ratios.** Cultures were inoculated from light lawns on Spizizen’s minimal 1% glucose plates with required amino acids, and grown at 30°C in S7 minimal media with 1% glucose and required amino acids. 5 OD ml’s of cells in mid-exponential growth (OD₆₀₀ = 0.4-0.6) were collected by centrifugation. Nucleic acid and protein fractions were extracted (Kadoya et al., 2002). The cell pellet was resuspended in 0.5 ml of ice-cold 0.25 N perchloric acid and incubated for 30 minutes on ice. Insoluble materials were collected by centrifugation and the supernatant was discarded. To extract nucleic acids, the pellet was resuspended in 0.2 ml of 0.5 N perchloric acid and incubated at 70°C for 15 minutes. The soluble fraction was collected and the extraction step was repeated. To extract proteins, the remaining pellet was resuspended in 0.25 ml of 1 M NaOH and incubated at 95°C for 10 minutes, and the soluble fraction was collected. DNA and protein concentrations were quantified using the diphenylamine reaction and the Lowry BioRad DC Protein Assay Kit, respectively, with appropriate standards.

**Fluorescence microscopy.** Live cells were stained with 4’, 6’-diamidino-2-phenylindole (DAPI) (40 to 80 ng/mL) to visualize nucleoids, and the vital dye FM4-64 (10-200ng/mL;
Molecular Probes) to visualize cell membranes. The lower concentration of FM4-64 was used for strains containing \textit{tetR-yfp} fusions to minimize the signal from the dye under the YFP filter. Microscopy was performed as described previously (Lemon and Grossman, 1998; Lemon and Grossman, 2000). Briefly, cells were immobilized on pads of 1% agarose in 1X T'base-1mM MgSO$_4$ (Harwood and Cutting, 1990), and images were captured with a Nikon E800 microscope equipped with a Hamamatsu digital camera. The filters used were: tetramethylrhodamine isothiocyanate for FM4-64, chroma filter set 31044 for CFP, 41012 for GFP, and 41029 for YFP. Improvision OpenLabs software was used to process images.

**Measurement of focus position in cells with two foci of the origin region.** Focus position in cells with two foci was measured as described previously, using a strategy designed to eliminate any unintentional visual bias in scoring (Lee et al., 2003). Briefly, for each cell, the focus closest to a pole was designated as focus $A$, and the other focus was designated as focus $B$. Three measurements were made: 1) $a$, the distance from focus $A$ to the closest pole, 2) $b$, the distance from that same pole to focus $B$, and 3) $l$, the cell length. This created a systematic bias in scoring such that in every cell, focus $A$ was closer to a pole than focus $B$. In order to remove this bias, a random number generator was used to assign each focus an approximately 50% chance of being counted as closest to a pole. The corresponding distance of each focus from the same cell pole (the $a$ and $b$ measurements) was then recalculated. To construct graphs of focus positions in cells with two foci, the $a$ and $b$ measurements were plotted against cell length for each cell scored.

Focus position as a percentage of cell length was determined by dividing the distance of each focus from the nearest cell pole ($a$ for focus $A$, $l$ minus $b$ for focus $B$) by the cell length and multiplying by 100. These numbers were averaged for all cells scored, and were reported
followed by the 95% confidence interval for the mean. Interfocal distance as a proportion of cell length was calculated as \((b-a)\) divided by \(l\).

**Measurement of focus position in cells with one focus of the origin region.** Focus positions in cells with one focus were measured as follows. For each cell, two measurements were made: 1) \(a\), the distance from a focus to the nearest pole, and 2) \(l\), the cell length. This created a systematic bias in scoring such that in every cell, the distance \(a\) was smaller than half of the cell length. In order to remove this bias, a random number generator was used to assign each focus an \(~50\%\) chance of remaining as scored, and a \(~50\%\) chance of recalculating \(a\) as the distance from the focus to the far pole. To construct graphs of focus positions in cells with one focus, the recalculated \(a\) measurements were plotted against cell length for each cell scored. Also, focus position as a percentage of cell length \((a/l)\) was calculated for each cell.

**Results/Discussion**

**Inactivating soj enhances the chromosome partitioning defect of an smc null mutant**

*B. subtilis* SMC (BsSMC) is a DNA-binding protein with ATPase activity that contributes to chromosome compaction and organization (Hirano, 1998; Hirano, 2002; Lindow et al., 2002b). BsSMC appears to compact the chromosome by binding all over the nucleoid and constraining supercoils (Lindow et al., 2002a; Lindow et al., 2002b). An *smc* mutant has irregularly-shaped nucleoids and a chromosome partitioning defect such that there is increased production of anucleate and “cut” cells in which the division septum bisects the nucleoid (Britton et al., 1998; Graumann et al., 1998; Moriya et al., 1998). In addition, inactivating *smc* causes perturbed subcellular positioning of the chromosomal origin and terminus regions (Britton et al., 1998; Graumann, 2000). An *smc* mutant background can sensitize cells to mutations that affect any of
these aspects of chromosome compaction, organization, partitioning or positioning. For example, an Δsmc mutation is synthetically lethal with a null mutation in spoIIE, a DNA translocase that moves cut chromosomes out of the division septum (Britton and Grossman, 1999; Britton et al., 1998). In addition, inactivating smc causes a synthetic chromosome partitioning phenotype when combined with a spo0J null, consistent with the model that Spo0J and SMC have partially overlapping roles in chromosome organization (Britton and Grossman, 1999; Britton et al., 1998).

We found that inactivating soj caused a synthetic chromosome partitioning phenotype in combination with an smc null mutation. During exponential growth at 30°C in defined minimal medium with glucose as a carbon source, a Δsoj mutant did not have a detectable chromosome partitioning defect on its own, but a spo0J null mutant did (0 out of 2411 cells were anucleate or cut in Δsoj cells, compared to 9 out of 2001 in Δspo0J cells and 0 out of 1556 in wild type cells) (Table 3). Previous results in 2XSG medium at 37°C showed that Δsoj does not cause an appreciable chromosome partitioning defect, whereas Δspo0J and Δ(soj-spo0J) mutants produce 1-2% anucleate cells (Ireton et al., 1994).

We found that the Δsmc mutant had 2.1% anucleate and 2.5% cut cells, while the Δsoj Δsmc, Δspo0J Δsmc, or Δ(soj-spo0J) Δsmc mutants had 18-19% anucleates and 8-12% cut cells, respectively (Table 3). The chromosome partitioning defect of the Δsmc mutant was lower than previously reported (Britton et al., 1998; Moriya et al., 1998), due to the growth conditions used (see Materials and Methods).
Table 3. Chromosome partitioning defects of various mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>% Anucleate</th>
<th>% Cut</th>
<th>% Anucleate + Cut</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG174</td>
<td>Wild type</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
<td>1556</td>
</tr>
<tr>
<td>SV132</td>
<td>Δsoj</td>
<td>&lt; 0.04</td>
<td>&lt; 0.04</td>
<td>&lt; 0.04</td>
<td>2411</td>
</tr>
<tr>
<td>AG1468</td>
<td>Δspo0J</td>
<td>0.3</td>
<td>0.2</td>
<td>0.5</td>
<td>2001</td>
</tr>
<tr>
<td>RB35</td>
<td>Δsmc</td>
<td>2.1</td>
<td>2.5</td>
<td>4.6</td>
<td>1685</td>
</tr>
<tr>
<td>PSL521</td>
<td>Δsoj Δsmc</td>
<td>18.3</td>
<td>12.0</td>
<td>30.3</td>
<td>717</td>
</tr>
<tr>
<td>RB41</td>
<td>Δspo0J Δsmc</td>
<td>19.0</td>
<td>9.0</td>
<td>28.0</td>
<td>290</td>
</tr>
<tr>
<td>PSL568</td>
<td>Δ(soj-spo0J) Δsmc</td>
<td>19.1</td>
<td>7.5</td>
<td>26.6</td>
<td>225</td>
</tr>
<tr>
<td>PSL640</td>
<td>ΔscpA</td>
<td>0.9</td>
<td>1.3</td>
<td>2.2</td>
<td>534</td>
</tr>
<tr>
<td>PSL652</td>
<td>Δsoj ΔscpA</td>
<td>8.1</td>
<td>6.9</td>
<td>15.0</td>
<td>334</td>
</tr>
<tr>
<td>PSL642</td>
<td>ΔscpB</td>
<td>2.7</td>
<td>1.8</td>
<td>4.5</td>
<td>552</td>
</tr>
<tr>
<td>PSL654</td>
<td>Δsoj ΔscpB</td>
<td>21.1</td>
<td>12.7</td>
<td>33.8</td>
<td>346</td>
</tr>
</tbody>
</table>

aIndicated strains were inoculated from light lawns on minimal plates into defined minimal medium with 1% glucose and grown at 30°C. Samples were taken for microscopy during exponential growth. The cell membrane was stained with FM4-64 and the nucleoid was stained with DAPI.

bCells devoid of any visible DAPI staining were scored as anucleate.

cCells containing a nucleoid bisected by the division septum were scored as cut.

dThe total number of cells analyzed.

BsSMC can form a complex with two other proteins, ScpA and ScpB (Lindow et al., 2002b; Soppa et al., 2002; Volkov et al., 2003). Inactivating soj enhanced anucleate production of the ΔscpA or ΔscpB mutants by nearly 10-fold, similar to the fold enhancement of the Δsmc mutant (Table 3). The difference between the ΔscpA and ΔscpB mutant phenotypes could be due to the strain background or growth conditions used, since previous experiments did not show a two- to three-fold worse chromosome partitioning defect in the ΔscpB mutant than the ΔscpA mutant (Soppa et al., 2002). Our results are consistent with the model that BsSMC, in a complex with ScpA and ScpB, contributes to chromosome partitioning. In addition, the results indicate that the synthetic chromosome partitioning defect of a Δsoj Δsmc double mutant is due to loss of Δsmc, rather than the gene that is downstream of smc, called srb.
The Δsmc mutant background uncovers a role for Soj in chromosome partitioning. The finding that Δspo0J, Δsoj, and Δ(soj-spo0J) mutations enhanced the chromosome partitioning defect of an smc null mutation to a similar extent indicates that Soj and Spo0J probably perform chromosome partitioning functions in the same pathway, analogous to ParA and ParB from other organisms (Austin and Abeles, 1983a; Austin and Abeles, 1983b; Easter and Gober, 2002; Figge et al., 2003; Mohl and Gober, 1997; Ogura and Hiraga, 1983). A simple model is that Soj may help Spo0J bring the parS sites together, forming an organized nucleoprotein complex that compacts the origin region. Consistent with this model, inactivating soj can cause foci of Spo0J-GFP to mislocalize as many smaller, fragmented foci (Marston and Errington, 1999). ParA from other organisms appears to modulate the size of ParB nucleoprotein complexes on parS-containing DNA (Bouet and Funnell, 1999; Figge et al., 2003; Lemonnier et al., 2000; Mohl and Gober, 1997), indicating that ParA proteins may generally regulate ParB binding and/or higher-order interactions between ParB molecules bound at the partition complex.

If Soj contributes to Spo0J function by helping Spo0J bring parS sites together, then we would expect a Δsoj mutant to have a chromosome partitioning defect. However, a Δsoj mutation alone does not affect chromosome partitioning appreciably. The fact that a Δsoj mutation strongly enhances the chromosome partitioning defect of an Δsmc mutant indicates that SMC may compensate for the absence of soj. In a Δsoj single mutant, SMC could help Spo0J bring together the parS sites. SMC-mediated chromosome compaction could bring parS sites closer together, which could presumably facilitate higher-order interactions between Spo0J molecules bound to parS sites. Consistent with this model, inactivating both soj and smc caused a severe chromosome partitioning defect like a Δspo0J Δsmc double mutant.
Although a Δsoj mutation alone does not cause an appreciable chromosome partitioning defect, we found effects of Δsoj on other phenotypes that are also perturbed in a Δspo0J mutant. Below, we describe more detailed analyses of phenotypic effects of a soj null mutation.

**Replication overinitiates in Δsoj and Δ(soj-spo0J) null mutants**

We found that Δsoj and Δ(soj-spo0J) mutants had increased chromosome content like Δspo0J mutants (Lee et al., 2003). We used LacI-GFP bound to an array of lac operators inserted in the chromosome (Gordon et al., 1997; Webb et al., 1997) to visualize several chromosomal regions: the origin-proximal 359° region, the 270° region, and the terminus-proximal 181° region.

Inactivating soj or soj and spo0J increased the proportion of cells with higher numbers of origin foci during exponential growth at 30°C in defined minimal medium, with glucose as a carbon source. The majority of wild type cells (80.9%) had two foci of the origin region (Table 4) (Lee et al., 2003). The Δsoj and Δ(soj-spo0J) mutants had fewer cells with two foci (63.6% in Δsoj, 63.5% in Δ(soj-spo0J)) and a corresponding increase in cells with three or more foci of the origin region (Table 4). The spo0J null mutant has even fewer two-focus cells (44.9%), and a significant increase in cells with one and three or more foci (Table 4) (Lee et al., 2003). All of the mutants had more cells with three foci of the origin region, indicating that replication may have been initiating asynchronously. Δsoj, Δspo0J, and Δ(soj-spo0J) mutants also had increased numbers of foci of the 270° and 181° regions of the chromosome, indicating that there were more chromosomes per cell (Table 4).

An increase in chromosome content could be due to: 1) overinitiation of replication, and/or 2) a delay in cell division such that an extra round of replication starts before the cell divides. A hallmark of overinitiation of replication is an increased DNA to cell mass ratio, due to increased
Table 4. Number of foci per cell of several chromosomal regions during exponential growth in wild type and mutant cells.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Insertion region</th>
<th>Genotype</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>&gt;4</th>
<th>No. cells analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCL696d</td>
<td>359°</td>
<td>wild type</td>
<td>0.7</td>
<td>3.1</td>
<td>80.9</td>
<td>4.9</td>
<td>10.3</td>
<td>0.1</td>
<td>881</td>
</tr>
<tr>
<td>PSL41</td>
<td>Δsoj</td>
<td></td>
<td>&lt;0.2</td>
<td>6.3</td>
<td>63.6</td>
<td>13.3</td>
<td>15.8</td>
<td>1.0</td>
<td>602</td>
</tr>
<tr>
<td>DCL705e</td>
<td>Δspo0J</td>
<td></td>
<td>4.4</td>
<td>15.3</td>
<td>44.9</td>
<td>20.1</td>
<td>11.2</td>
<td>4.1</td>
<td>517</td>
</tr>
<tr>
<td>PSL37</td>
<td>Δ(soj-spo0J)</td>
<td></td>
<td>0.8</td>
<td>8.7</td>
<td>63.5</td>
<td>13.9</td>
<td>13.1</td>
<td>&lt;0.4</td>
<td>252</td>
</tr>
<tr>
<td>KPL716d</td>
<td>270°</td>
<td>wild type</td>
<td>0.3</td>
<td>33.6</td>
<td>63.6</td>
<td>1.6</td>
<td>0.8</td>
<td>0.1</td>
<td>761</td>
</tr>
<tr>
<td>PSL119</td>
<td>Δsoj</td>
<td></td>
<td>&lt;0.3</td>
<td>18.2</td>
<td>68.5</td>
<td>7.1</td>
<td>6.2</td>
<td>&lt;0.3</td>
<td>324</td>
</tr>
<tr>
<td>PSL101e</td>
<td>Δspo0J</td>
<td></td>
<td>3.5</td>
<td>20.4</td>
<td>60.5</td>
<td>9.0</td>
<td>6.1</td>
<td>0.6</td>
<td>491</td>
</tr>
<tr>
<td>PSL100</td>
<td>Δ(soj-spo0J)</td>
<td></td>
<td>1.2</td>
<td>18.9</td>
<td>61.5</td>
<td>10.7</td>
<td>7.4</td>
<td>0.4</td>
<td>244</td>
</tr>
<tr>
<td>KPL686d</td>
<td>181°</td>
<td>wild type</td>
<td>0.8</td>
<td>79.3</td>
<td>19.6</td>
<td>0.3</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>1004</td>
</tr>
<tr>
<td>PSL118</td>
<td>Δsoj</td>
<td></td>
<td>0.6</td>
<td>70.6</td>
<td>27.7</td>
<td>1.1</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>469</td>
</tr>
<tr>
<td>PSL110e</td>
<td>Δspo0J</td>
<td></td>
<td>1.4</td>
<td>50.0</td>
<td>46.4</td>
<td>0.9</td>
<td>1.4</td>
<td>&lt;0.5</td>
<td>222</td>
</tr>
<tr>
<td>PSL107</td>
<td>Δ(soj-spo0J)</td>
<td></td>
<td>2.5</td>
<td>47.5</td>
<td>43.6</td>
<td>3.7</td>
<td>2.1</td>
<td>0.6</td>
<td>518</td>
</tr>
</tbody>
</table>

a Indicated strains were grown in defined S7 minimal media with 1% glucose and required amino acids at 30°C. Samples were taken during exponential growth. The number of foci per cell of LacI-GFP (or LacI-CFP) was determined and the percentage of cells with the indicated number of foci was calculated.

b An array of lac operators was inserted in the indicated region of the chromosome and visualized with LacI-GFP or LacI-CFP.

c The total number of cells analyzed for each strain.

d Combined data from newly repeated and previously published results (Lee et al., 2003).

e Previously published results (Lee et al., 2003).
frequency of replication initiation events. Conditions that perturb cell division, such as yneA-induced filamentation in *B. subtilis* (Kawai 2003 Mol Micro), or inactivation of *ftsZ* in *E. coli* (Dai and Lutkenhaus, 1991; Huls et al., 1999) do not obviously affect the DNA to cell mass ratio, since cell growth continues and the filaments appear to contain normally-spaced nucleoids. To determine whether the Δsoj, Δspo0J and Δ(soj-spo0J) mutants had overinitiation of replication, we measured the DNA to protein ratio. The DNA to protein ratio is a standard assay used to measure perturbations to DNA replication. The DNA to protein ratio is not affected in an Δsmc mutant, which produces anucleate cells and a significant fraction of elongated cells (Moriya et al., 1998).

The DNA to protein ratio of the Δsoj, Δspo0J and Δ(soj-spo0J) mutants was significantly higher than the wild type ratio, indicating that the mutations cause overinitiation of replication (Table 5). Wild type cells had a DNA to protein ratio of 3.0 ± 0.4, compared to 3.9 ± 0.6 in Δsoj, 4.3 ± 0.6 in Δspo0J, and 3.7 ± 0.6 in Δ(soj-spo0J) mutants (± 95% confidence interval for the mean) (Table 4). The results are consistent with experiments showing that Δspo0J causes overinitiation of replication (Lee et al., 2003). The increased DNA to protein ratio of the Δ(soj-spo0J) double mutant relative to wild type cells was more pronounced than previously reported (Ogura et al., 2003), which could reflect differences in the strain backgrounds or growth media used (see Materials and methods). The Δsoj and Δ(soj-spo0J) mutations did not appear to increase the DNA to protein ratio as severely as Δspo0J (Table 5). Flow cytometry demonstrates that inactivating soj significantly suppresses the amount of overinitiation in a spo0J null mutant,
Table 5. DNA to protein ratio of wild type and mutant cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>DNA to protein ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG174</td>
<td>wild type</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>SV132</td>
<td>Δsoj</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>AG1468 Δspo0J</td>
<td></td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>AG1505 Δ(soj-spo0J)</td>
<td></td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>KI1937 Δ(soj-spo0J); thr-(soj, spo0J)</td>
<td></td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>KI1913 Δ(soj-spo0J); thr-(soj)</td>
<td></td>
<td>3.8 ± 0.7</td>
</tr>
<tr>
<td>KI1936 Δ(soj-spo0J); thr-(sojG12V, spo0J)</td>
<td></td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>PSL696 Δ(soj-spo0J); thr-(sojG12V)</td>
<td></td>
<td>2.7 ± 0.7</td>
</tr>
</tbody>
</table>

*a* Indicated strains were inoculated from light lawns on Spizizen's minimal 1% glucose plates into defined minimal medium with 1% glucose as a carbon source and grown at 30°C. Samples were collected during exponential growth at an OD600 of 0.4-0.6. Total nucleic acids and protein were extracted and assayed for DNA and protein content as described in Materials and Methods. *b* Experimentally determined ratios are from 9-10 independent experiments for the first four strains, and from 4 experiments for the last four strains. Ratios are followed by the 95% confidence interval for the mean.
although suppression is not complete (Ogura et al., 2003), consistent with our result.

The finding that the Δsoj-spo0J double mutant had overinitiation of replication to a similar
degree as the Δsoj mutant indicates that soj is epistatic for replication initiation control. Spo0J
probably regulates initiation of replication through its effects on Soj (Figure 1), through the same
order of interactions by which Soj and Spo0J regulate sporulation (Quisel et al., 1999). Spo0J
appears to affect the nucleotide-bound state of Soj, increasing the proportion of ATP-bound Soj
which is unable to repress sporulation (Quisel et al., 1999). We wanted to test whether Spo0J
affects replication initiation by affecting the nucleotide-bound state of Soj. By this model,
inactivating spo0J causes overinitiation of replication by affecting the ratio of Soj-ATP to Soj-
ADP in the cell. In order to test the model, we used a mutant form of Soj that contains a glycine
12 to valine (G12V) substitution in a conserved Walker-box residue predicted to impair
nucleotide hydrolysis (Quisel et al., 1999). The SojG12V protein probably exists predominantly
in an ATP-bound state in the cell. If Spo0J regulates replication initiation by affecting the
nucleotide-bound state of Soj, then the level of replication initiation in the hydrolysis-defective
sojG12V mutant should not be affected by the presence or absence of spo0J.

We found that the DNA to protein ratio of the sojG12V mutant was less sensitive to the
presence or absence of spo0J, consistent with the model that Spo0J regulates replication
initiation by affecting the nucleotide-bound state of Soj. Strains were constructed with sojG12V
and spo0J genes inserted at the thrC locus, driven by the endogenous promoter, in a background
with the endogenous (soj-spo0J) locus inactivated. The DNA to protein ratio of the sojG12V
mutant was 2.4 ± 0.4 in the presence of spo0J, compared to 2.7 ± 0.7 in the absence of spo0J.
<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Wild type</th>
<th>Δsoj</th>
<th>Δspo0J</th>
<th>Δ(soj-spo0J)</th>
<th>Proposed order of interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporulation</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Spo0J → Soj-ADP → Sporulation</td>
</tr>
<tr>
<td>Controlled initiation of replication</td>
<td>+</td>
<td>-</td>
<td>--</td>
<td>-</td>
<td>Spo0J → Soj-ADP → Replication</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Spo0J → Soj-ATP → initiation</td>
</tr>
<tr>
<td>Origin separation</td>
<td>+</td>
<td>-</td>
<td>--</td>
<td>--</td>
<td>Soj → Spo0J → Origin separation ↓</td>
</tr>
<tr>
<td>Chromosome partitioning</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Chromosome partitioning</td>
</tr>
<tr>
<td>Positioning two foci of the origin</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Origin positioning</td>
</tr>
<tr>
<td>Timely cell division</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Cell division</td>
</tr>
</tbody>
</table>

Figure 1. Proposed orders of interactions by which Soj and Spo0J regulate different cellular processes. Soj and Spo0J appear to regulate replication initiation through the same order of interactions by which they regulate sporulation. For both phenotypes, soj is epistatic, indicating that Spo0J may regulate these processes through its effects on Soj. We propose that Spo0J affects these processes by affecting the nucleotide-bound state of Soj.

Soj and Spo0J probably affect chromosome partitioning, separation and positioning of replicated origins, and cell division through a different order of interactions than the order by which they affect sporulation and replication initiation. For these phenotypes, spo0J is epistatic, indicating that Soj may regulate these processes through its effects on Spo0J. We propose that Soj contributes to Spo0J function. The effects of Δspo0J and Δ(soj-spo0J) mutations on chromosome partitioning, positioning of replicated foci of the origin, and cell division may be consequences of a primary defect in origin positioning. smc may partly compensate for the absence of Δsoj, so that a Δsoj mutation alone has a less severe effect on origin separation than Δspo0J and Δ(soj-spo0J) mutations, and does not have detectable effects on chromosome partitioning, positioning of two foci of the origin, or cell division.
(Table 5). In contrast, in strains constructed the same way, inactivating spo0J had a significant effect on replication initiation in the presence of wild type soj. The DNA to protein ratio of a strain containing wild type soj at the thrC locus was 2.7 ± 0.4 in the presence of spo0J, compared to 3.8 ± 0.6 in the absence of spo0J (Table 5). Flow cytometry experiments show that the sojG12V mutant has decreased initiation of replication (Ogura et al., 2003). Consistent with this, we found that the DNA to protein ratio of the sojG12V single mutant appeared slightly lower than in the corresponding wild type cells (2.4 ± 0.4 in sojG12V compared to 2.7 ± 0.4 in wild type cells) (Table 5). These results indicate that Soj-ATP may inhibit replication initiation.

We propose a model that Soj-ATP inhibits replication initiation and that Spo0J exerts effects on replication initiation by acting as a nucleotide exchange factor for Soj (Figure 2). Several lines of evidence are consistent with this model. Spo0J appears to increase the proportion of ATP-bound Soj in the cell (Quisel et al., 1999), and ParB of C. crescentus appears to act as a nucleotide exchange factor for ParA (Easter and Gober, 2002; Figge et al., 2003). Where tested, ParA-ATP and ParA-ADP have distinct activities, indicating that ParA activity is regulated by the bound nucleotide (Bouet and Funnell, 1999; Easter and Gober, 2002).

Our model also proposes that Soj-ADP promotes initiation (Figure 2). Overexpressing Soj causes overinitiation (Ogura et al., 2003), indicating that some form of Soj may promote initiation. Presumably, overexpression of Soj would lead to the accumulation of Soj-ADP by increasing the intracellular ratio of Soj to the nucleotide exchange factor Spo0J. Consistent with this hypothesis, overexpression of Spo0J together with Soj suppresses the overinitiation phenotype (Ogura et al., 2003). This model predicts that inactivating Δspo0J would cause a
Figure 2. Model for regulation of replication initiation by Soj and Spo0J. We propose a model in which Soj-ATP inhibits initiation and Soj-ADP promotes initiation. Spo0J may affect replication initiation by acting as a nucleotide exchange factor Soj. By our model, inactivating Δspo0J causes overinitiation of replication due to an increase in the cellular proportion of Soj-ADP and a decrease in the proportion of Soj-ATP. Because inactivating soj caused net overinitiation of replication, we postulate that Soj-ATP represses initiation more strongly than Soj-ADP promotes it. The effects of either form of Soj on replication initiation are not necessarily direct.
more severe overinitiation defect than Δsoj or Δ(soj-spo0J) mutants, due to the accumulation of Soj-ADP, which promotes initiation of replication, and the depletion of Soj-ATP, which inhibits initiation of replication. Our results agree with this prediction (Table 5).

How could Soj regulate replication initiation? There are several potential mechanisms. Soj-ADP could act directly at the origin and stimulate assembly or activity of the initiation complex. Alternatively, Soj could affect the activity of another protein that regulates replication initiation, by affecting its transcription or its subcellular location. Soj-ATP appears to localize to the cell poles or the edges of the nucleoid (Quisel and Errington references), while Soj-ADP appears to bind all over the nucleoid and act as a transcriptional repressor of sporulation genes (Quisel reference). Further experiments are needed to elucidate the mechanism by which Soj regulates replication initiation.

**Effects of soj and spo0J on cell division**

Cell length was not affected in the Δsoj mutant, but was ~20% longer in Δ(soj-spo0J) cells, similar to Δspo0J mutants (Lee et al., 2003). Average cell lengths in wild type, Δsoj and Δ(soj-spo0J) cells were 2.78 ± 0.10 μm, 2.82 ± 0.09 μm, and 3.29 ± 0.14 μm, respectively (results ± 95% confidence intervals, >200 cells scored). The increased cell length of Δspo0J and Δ(soj-spo0J) mutants is not sufficient to account for the increased DNA to protein ratio, since a cell division delay alone is not expected to affect this ratio. Hence, these mutations probably delay cell division in addition to causing overinitiation of replication. Furthermore, Soj and Spo0J appear to affect cell division through a different order of interactions than the order by which they affect initiation of replication. *soj* was epistatic for the overinitiation of replication.
phenotype, whereas spo0J was epistatic for the cell division delay phenotype, as was seen for the chromosome partitioning phenotype (Figure 1).

Our results differ from a report that the length of Δ(soj-spo0J) cells is similar to wild type cells (Ogura et al., 2003), probably due to differences in the growth conditions or the strain backgrounds used. The fact that Δspo0J and Δ(soj-spo0J) cells both had more foci of the 181° region relative to wild type and Δsoj cells (Table 4) could be due to the delay in cell division, which could give replicated termini more time to separate and become spatially resolvable.

Effects of Δsoj and Δ(soj-spo0J) on origin positioning

Cells with two foci of the origin. Duplicated foci of the origin were positioned normally at the cell quarters in the Δsoj mutant, but were closer together in Δ(soj-spo0J) cells, similar to the Δspo0J mutant (Lee et al., 2003) (Figure 3, Table 6). Cells were grown in defined minimal medium at 30°C with glucose as a carbon source and harvested during exponential growth. Origins were visualized as above using LacI-GFP bound to an array of lac operators inserted at 359°. Average distance from a focus to the nearest pole was 26.2 ± 1.3% of cell length in the Δsoj mutant, statistically indistinguishable from positioning at 26.1 ± 1.0% of cell length in wild type cells (± 95% confidence interval for the mean) (Table 6). The Δ(soj-spo0J) mutant had foci positioned at 31.2 ± 1.8% of cell length, similar to 30.1 ± 1.5% in the Δspo0J mutant (Table 6).

In Δspo0J and Δ(soj-spo0J) cells, the foci of the origins were positioned closer together than in wild type cells (Figure 4, Table 6): average interfocal distance was 39.7 ± 1.8% of cell length in Δspo0J (Lee et al., 2003) and 37.6 ± 1.9% of cell length in Δ(soj-spo0J), compared to 47.8 ± 1.2% in wild type cells (Table 6). Average interfocal distance in the Δsoj mutant was 47.5 ± 1.3%, indistinguishable from wild type cells (Table 6). Our results indicate that the detection of
Figure 3. Subcellular locations of duplicated foci of the origin. The origin region was visualized using LacI-GFP bound to an array of lac operator integrated at the 359° region of the chromosome. Strains were grown at 30°C in defined minimal medium with 1% glucose. Only cells with two foci of the origin were analyzed. The distance from each focus to the same cell pole was measured from images of live cells in exponential growth (Materials and Methods) and is plotted on the x axis, and cell length is plotted on the y axis. Cell length and the midcell positions are indicated by solid lines. Cell quarter positions are indicated by dotted lines. The number of cells analyzed (n) is indicated in the lower right-hand corner of each panel. One focus is indicated with red open circles and the other with blue crosses. Position of duplicated foci of the origin in wild type (A), Δsoj mutant (B), Δspo0J mutant (C), and Δ(soj-spo0J) mutant strains. Panels A and C were previously published (Lee et al., 2003) and are included for comparison.
Table 6. Subcellular positioning of replicated sister origins in cells with two foci of the origin region.

<table>
<thead>
<tr>
<th>strain</th>
<th>genotype</th>
<th>focus position$^b$</th>
<th>interfocal distance$^c$</th>
<th>total$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCL696e</td>
<td>wt</td>
<td>26.1 ± 1.0</td>
<td>47.8 ± 1.2</td>
<td>335</td>
</tr>
<tr>
<td>PSL41</td>
<td>Δsoj</td>
<td>26.2 ± 1.3</td>
<td>47.5 ± 1.3</td>
<td>392</td>
</tr>
<tr>
<td>DCL705e</td>
<td>Δspo0J</td>
<td>30.1 ± 1.5</td>
<td>39.7 ± 1.8</td>
<td>222</td>
</tr>
<tr>
<td>PSL37</td>
<td>Δ(soj-spo0J)</td>
<td>31.2 ± 1.8</td>
<td>37.6 ± 1.9</td>
<td>174</td>
</tr>
</tbody>
</table>

$^a$Indicated strains were grown at 30°C in minimal medium supplemented with 1% glucose and required amino acids, and samples were taken for microscopy during exponential growth. LacI-GFP was used to visualize lac operator arrays integrated at the 359° region of the chromosome.

$^b$The distance from each focus to the nearest cell pole was measured in cells with two foci, and is presented as a percentage of cell length ± the 95% confidence intervals.

$^c$The distance between each focus was measured in cells with two foci, and is presented as a percentage of cell length ± the 95% confidence intervals.

$^d$The total number of cells with one focus that were analyzed.

$^e$Previously published results (Lee et al., 2003).
Figure 4. Relationship between cell length and interfocal distance. Data from Figure 1A to 1D were used to determine the distance between the two foci in each cell (interfocal distance). This is plotted as a function of cell length for wild type (A), Δsoj mutant (B), Δspo0J mutant (C), and Δ(soj-spo0J) mutant strains. An ellipse was drawn around the distribution of wild type cells (A) and superimposed on the corresponding plots in panels B, C, and D. The interfocal distances in most of the Δspo0J and Δ(soj-spo0J) mutant cells were within this ellipse; a subset (~15%) fell below and to the right of the wild type distribution. These cells had replicated origins that were closer together than the origins in wild type cells of similar length. Panels A and C were previously published (Lee et al., 2003) and are included for comparison.
defects in positioning duplicated, spatially resolved origins correlates with defects in chromosome partitioning. \( \Delta \text{spo}0J \) and \( \Delta (\text{soj}\text{-}\text{spo}0J) \) mutants had defects in positioning replicated origins as well as defects in chromosome partitioning. The \( \Delta \text{soj} \) mutant did not have detectable defects in positioning of spatially resolved origins or in chromosome partitioning. The effects of \( \Delta \text{spo}0J \) and \( \Delta (\text{soj}\text{-}\text{spo}0J) \) mutants on positioning of duplicated foci of the origin could be due to a defect in separating replicated origins and/or a defect in maintaining the position of replicated origins at the cell quarters. Below, we present results indicating that the \( \Delta \text{spo}0J \) and \( \Delta (\text{soj}\text{-}\text{spo}0J) \) mutants, and, to a lesser extent, the \( \Delta \text{soj} \) mutant, have defects in separating replicated origins.

**Cells with one focus of the origin.**

The \( \Delta \text{soj} \), \( \Delta \text{spo}0J \), and \( \Delta (\text{soj}\text{-}\text{spo}0J) \) mutations perturbed focus positioning in cells with a single focus of the origin (Figure 5). During exponential growth at 30°C in defined minimal media with succinate as a carbon source, the majority of wild type cells had a focus positioned between 35% and 65% of cell length, and only 12.6% of cells had a focus outside this region (Figure 5). In the \( \Delta \text{soj} \), \( \Delta \text{spo}0J \), and \( \Delta (\text{soj}\text{-}\text{spo}0J) \) mutants, 41.9%, 26.6%, and 25.0% of cells had a focus outside this region (Figure 5).

Two possible causes for mispositioning of the origin region in these mutants are: 1) Soj and Spo0J could normally contribute to origin positioning at midcell, and 2) some of the mutant cells that appear to have a single origin might actually contain two replicated sister origins that remained close together and moved away from midcell. Consistent with the latter hypothesis, we found that \( \Delta \text{soj} \), \( \Delta \text{spo}0J \), and \( \Delta (\text{soj}\text{-}\text{spo}0J) \) mutations caused a defect in separating replicated
Figure 5. Subcellular location of a single focus of the origin. The origin region was visualized using LacI-GFP bound to an array of lac operator integrated at the 359° region of the chromosome. Strains were grown at 30°C in defined minimal medium with 1% succinate. Only cells with a single focus of the origin were analyzed. For panels A, C, E, and G, focus position as a percentage of cell length was measured from images of live cells in exponential growth. For each panel, the percentage of cells with a focus in each 5% increment of cell length (0 to 5%, 5 to 10%, ... 95 to 100% of cell length) was calculated and plotted as a histogram. The length increments from 0 to 35% and 65-100% are highlighted in grey, and the proportion of cells with a focus in these ranges is indicated in the upper right-hand corner of each histogram. For panels B, D, F, and H, the same data sets as in panels A, C, E, and G are used. The distance from the focus to the cell pole is plotted on the x axis, and cell length is plotted on the y axis. Cell length and the midcell positions are indicated by solid lines. Cell quarter positions are indicated by dotted lines. Data are shown for wild type (A) and (B), Δsoj mutant (C) and (D), Δspo0J mutant (E) and (F), and Δ(soj-spo0J) mutant strains (G) and (H).
origins such that a significant proportion of cells that appeared to have a single origin actually did contain replicated origins that had not separated. However, the results indicate that other factors in addition to an origin separation defect probably contribute to mispositioning in cells with one focus of the origin.

Inactivating soj and/or spo0J impairs separation of replicated DNA in the origin region

To determine whether cells of the Δsoj, Δspo0J, and Δ(soj-spo0J) mutants contained replicated origins that were too close together to be spatially resolved, we visualized the origin and a nearby distal region simultaneously, using LacI-CFP bound to an array of lac operators inserted at the 359° region of the chromosome, and TetR-YFP bound to an array of tet operators inserted at the 345° region of the chromosome (located ~164 kb distal to the 359° region) (Figure 6A). Cells that have two foci of the distal region must have initiated replication whether or not the replicated origins are spatially resolvable. In general, cells with fewer foci of the origin region than the distal region must contain replicated origins that have not separated.

We found that inactivating soj, spo0J, or both caused a defect in origin separation. During exponential growth at 30°C in defined minimal medium with succinate as a carbon source, 3.2% of wild type cells had fewer foci of the origin region than the distal region, compared with 13.7% in Δsoj, 20.3% in Δspo0J, and 21.6% in Δ(soj-spo0J) mutants, respectively (Table 7). In addition, each of the mutants had an increase in the proportion of cells with more foci of the origin region than the distal region (Table 7). Overinitiation of replication in these mutants could potentially increase the relative copy number of the origin region to the 345° region.
Figure 6. *B. subtilis* strains with *lacO* and *tetO* arrays inserted in the chromosome. (A) Strain with a *lacO* array at 359° and a *tetO* array 345° for measuring separation of replicated sister origins. (B) Strain with a *lacO* array at 316° and a *tetO* array at 300° for measuring separation of replicated sister 316° regions. *B. subtilis* has a circular chromosome with the origin of replication, O, at 360°/0° and the terminus region, T, near 180°. In each panel, the positions of the known *parS* sites, at 334°, 354°, 355°, 356°, 359°, 4°, 15°, and 40° are indicated by boxes. The origin-proximal region containing the known *parS* sites is shaded in grey.
Table 7. Comparision of numbers of foci of the origin (359°) and 345° regions of the chromosome

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>%359°&gt;345</th>
<th>%359°=345</th>
<th>%359°&lt;345</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSL438</td>
<td>Wild type</td>
<td>4.3</td>
<td>92.4</td>
<td>3.2</td>
<td>277</td>
</tr>
<tr>
<td>PSL392</td>
<td>Δsoj</td>
<td>9.8</td>
<td>76.5</td>
<td>13.7</td>
<td>285</td>
</tr>
<tr>
<td>PSL440</td>
<td>Δspo0J</td>
<td>7.3</td>
<td>72.4</td>
<td>20.3</td>
<td>246</td>
</tr>
<tr>
<td>PSL390</td>
<td>Δ(soj-spo0J)</td>
<td>11.4</td>
<td>67.0</td>
<td>21.6</td>
<td>273</td>
</tr>
</tbody>
</table>

Table 8. Comparison of numbers of foci of the 316° and 300° regions of the chromosome

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>%316°&gt;300</th>
<th>%316°=300</th>
<th>%316°&lt;300</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSL582</td>
<td>Wild type</td>
<td>2.5</td>
<td>96.0</td>
<td>1.4</td>
<td>281</td>
</tr>
<tr>
<td>PSL578</td>
<td>Δsoj</td>
<td>3.9</td>
<td>95.4</td>
<td>0.7</td>
<td>285</td>
</tr>
<tr>
<td>PSL580</td>
<td>Δspo0J</td>
<td>5.1</td>
<td>91.7</td>
<td>3.1</td>
<td>292</td>
</tr>
<tr>
<td>PSL576</td>
<td>Δ(soj-spo0J)</td>
<td>5.1</td>
<td>92.2</td>
<td>2.8</td>
<td>217</td>
</tr>
</tbody>
</table>

a,b,c,d: Indicated strains were grown at 30°C in S750 minimal medium with 1% succinate supplemented with required amino acids. Cells were harvested for microscopy during exponential growth (see Materials and Methods).

Each of the mutants caused a defect in origin separation, which may have contributed to the defect in positioning the focus of the origin in cells that appeared to have a single origin. However, there are probably additional factors that can affect origin positioning in these mutants. The Δsoj mutant had the most severe defect in positioning the single focus of the origin (Figure 5), but had a less severe defect in separating replicated origins than the Δspo0J and Δ(soj-spo0J) mutants (Table 7). Hence, the defect in positioning the single focus of the origin in the Δsoj mutant cannot be due solely to the defect in separating replicated origins. Further experiments will be needed to determine the basis for origin mispositioning in these cells.

Although Δsoj, Δspo0J, and Δ(soj-spo0J) mutants have defects in separating replicated origins, the origins eventually separate in most of the cells, since the proportion of cells with an
origin separation defect far outweighs the proportion of anucleate cells produced. This delay in origin separation could be due to several possible primary defects, including: 1) a defect that slows migration of replicated origins, 2) a defect in releasing origins from the constraints of putative factors that could pair them or anchor them in close proximity. Further experiments will be needed to distinguish between these possibilities.

**Inactivating soj and/or spo0J has little effect on separation of replicated DNA outside the origin region**

To determine whether Δsoj, Δspo0J, or Δ(soj-spo0J) mutations affected separation of chromosomal regions outside the origin region, we simultaneously visualized the 316° region, using LacI-CFP bound to an array of lac operators, and the 300° region (located ~187 kb distal), using TetR-YFP bound to an array of tet operators (Figure 6B). Both regions are located outside the origin-proximal 20% that contains the known parS sites. The 316° region is replicated first. Cells that failed to separate the 316° region before the 300° region would have fewer foci of the 316° region than the 300° region.

Inactivating soj, spo0J, or both did not substantially affect separation of the 316° region (Table 8). In wild type cells, 1.4% had fewer foci of the 316° region than the 300° region, compared to 0.7% in Δsoj, 3.1% in Δspo0J, and 2.8% in Δ(soj-spo0J) mutants (Table 8). The lack of an appreciable separation defect was not due to the fact that the 316° and 300° regions are slightly further apart than 359° and 345°, since a significant defect in origin separation was also seen when 359° and 329° were visualized simultaneously (data not shown). Taken together, our results demonstrate that soj and spo0J are required for efficient separation of replicated DNA in the origin region, and that these effects are specific to the origin region.
Soj and Spo0J may contribute to chromosome partitioning by helping to separate replicated origins

The most significant finding was that Δsoj, Δspo0J, and Δ(soj-spo0J) mutants had a defect in separating replicated origins. Δspo0J and Δ(soj-spo0J) mutants had the most severe defect in separating replicated origins, correlated with a detectable defect in chromosome partitioning, a phenotype in which replicated origins were mispositioned closer together than the cell quarters even after they had separated, and a delay in cell division (Figure 1). We propose that these phenotypes may be the consequence of a primary defect in separating replicated origins. A severe defect in separating replicated origins could potentially delay cell division until replicated origins separate, and could lead to production of anucleate cells in cases where the origins did not separate at all. The origin separation defect of the Δsoj mutant was not as severe as the Δspo0J and Δ(soj-spo0J) mutants (13.7% compared to 20.3% and 21.6% of cells had replicated origins that were not separated, respectively), and could be too weak to cause detectable effects on these phenotypes. For all of these phenotypes, spo0J was epistatic, consistent with the model that Soj may affect these processes through its effects on Spo0J (Figure 1).

I favor a model in which Spo0J-mediated compaction and organization of the origin region contributes to timely separation of replicated origins. Soj could contribute to Spo0J function by helping Spo0J bring the origin-proximal parS sites together. A Δsoj mutation does not cause a detectable chromosome partitioning defect on its own, but we found that a Δsoj mutation severely enhanced the chromosome partitioning defect of an Δsmc mutant to a similar extent as a Δspo0J mutation. These results are consistent with a model that SMC-mediated compaction may also help Spo0J bring parS sites together, compensating for Soj. This could explain why a Δsoj
mutation alone does not affect chromosome partitioning, origin separation, positioning of
duplicated foci of the origin, and cell division as severely as \( \Delta \text{spo0J} \) and \( \Delta(\text{soj-spo0J}) \) mutations.

**Effects of the Par system on plasmids vs. chromosomes**

The Par system has different phenotypic effects on plasmid and chromosome positioning. Spo0J is not sufficient to recruit \( \text{parS} \) sites inserted at ectopic positions in the chromosome to the cell quarters (Lee et al., 2003), arguing against the model that Spo0J’s primary function is to facilitate attachment of replicated origins to putative anchors at the cell quarters. In contrast, plasmid-encoded Par systems are sufficient to recruit \( \text{parS} \) plasmids to the cell quarters, consistent with the anchoring model (Erdmann, 1999; Niki and Hiraga, 1997; Niki and Hiraga, 1999). Soj and Spo0J can also recruit \( \text{parS} \) plasmids to the cell quarters (Yamaichi, 2000). In addition, chromosomally-encoded Par systems can stabilize plasmids containing the cognate \( \text{parS} \) site (Godfrin-Estevenon et al., 2002; Lin and Grossman, 1998). One interpretation of these results is that the same Par system can perform different functions on plasmids and chromosomes. However, a more likely interpretation is that the Par system performs the same function on plasmids and chromosomes, and that plasmid recruitment to the cell quarters is a secondary effect of a Par-dependent function other than anchoring. The difference in phenotypic effects could reflect innate differences between plasmids and chromosomes, such as size.

What similar biochemical function could the Par system perform on plasmids and chromosomes? In both cases, ParB could bind and bring together \( \text{parS} \) sites, forming a nucleoprotein structure that facilitates partitioning. ParB appears to mediate plasmid pairing by bringing together \( \text{parS} \) sites on sister molecules (Edgar et al., 2001; Funnell, 1988; Youngren and Austin, 1997). This could potentially allow for coordinated plasmid separation in opposite
directions. In the case of chromosomes, similar types of higher-order interactions might also bring together parS sites that are located far apart on the same chromosome, organizing the origin region into a compacted structure that facilitates separation of replicated origins. Compaction could help replicated origins move apart by minimizing resistance against them as they move through the cytoplasm.

The finding that replicated origins, but not other regions of the chromosome, remain closely associated in the absence of soj and/or spo0J indicates that there may be barriers to origin separation. Replicated origins might be paired and/or anchored at nearby sites by an unidentified factor, and Soj and Spo0J function could overcome this barrier to separation. Alternatively, other regions of the chromosome might also have their own specialized partitioning mechanisms. We have described an assay that can be used to test candidate factors for effects on separation of a chromosomal region by simultaneous visualization of that region and a distal region.

Soj and Spo0J affect initiation of replication. Our results are consistent with the model that Spo0J regulates initiation of replication by affecting the nucleotide-bound state of Soj (Figure 2), through the same order of interactions by which Spo0J and Soj regulate sporulation (Figure 1). This is distinct from the order of interactions by which they affect chromosome partitioning, separation and positioning of replicated origins, and cell division. Further experiments will be needed to determine how Soj regulates replication initiation. It would also be interesting to test whether the hydrolysis-defective sojG12V mutation affects other phenotypes, such as chromosome partitioning in an Δsnc null background. In addition, it would be interesting to test whether other chromosomally-encoded Par systems regulate initiation of replication. Where tested, plasmid-encoded Par systems do not affect plasmid copy number substantially, and plasmid loss in the absence of the par system is not due to insufficient replication (Austin et al.,
1982; Ogura and Hiraga, 1983). However, it is formally possible that the Par system may have subtle effects on plasmid replication.

The Par system is a widely-conserved prokaryotic partitioning mechanism. By contributing to faithful chromosome and plasmid partitioning, Par systems confer a significant survival advantage and affect the spread of plasmid-borne antibiotic resistance and virulence genes (Yamauchi, 2000; Youngren et al., 2000). It will be interesting to determine whether Par systems perform similar functions on plasmids and chromosomes. Understanding the mechanism of Par system function could also provide important insights into related questions, such as how subcellular positions of plasmids and chromosomal regions are specified. Finally, further studies will elucidate how chromosomally-encoded Par systems regulate cellular processes like replication initiation, sporulation, and cell division (Cervin et al., 1998; Lee et al., 2003; Mohl et al., 2001; Ogura et al., 2003; Quisel et al., 1999).
References


Chapter 4

Concluding Remarks
Concluding Remarks

Partitioning of replicated chromosomal origins is thought to involve a separation mechanism and a capture mechanism whereby the origins move apart and become attached to anchors at the cell quarters. My results indicate that Soj and Spo0J of B. subtilis probably contribute primarily to separation of replicated origins, rather than anchoring of origins at the cell quarters. Inactivating soj and/or spo0J caused a defect in separating replicated origins, such that the origins remained close together even after distal regions separated. In addition, Spo0J was not sufficient to recruit an array of chromosomal parS sites to the cell quarters, arguing against the notion that Spo0J’s primary role is to facilitate anchoring. Finally, results described in Chapter 3 indicate that Soj probably functions in the same chromosome partitioning pathway as Spo0J, analogous to other Par systems.

I postulate that Spo0J binding to the origin-proximal parS sites results in formation of a higher-order nucleoprotein complex that somehow contributes to origin separation. Spo0J could bind and bring together parS sites in cis, compacting and organizing the origin region. Soj could help Spo0J to bring the parS sites together. Compaction of the origin region could minimize resistance against replicated origins as they move through the cytoplasm, facilitating separation of replicated origins.

Further studies should focus on characterizing the partitioning complex in greater detail. A top priority is to determine whether Spo0J can truly bring parS sites together. An assay could be used that measures pairing-induced effects on plasmid supercoiling when two sites on opposite ends of the same plasmid are held together in vivo (Edgar et
al., 2001). Alternatively, one could use cytological methods to ask whether Spo0J can bring the origin-proximal parS sites in close proximity with a parS array inserted far from the origin region of the chromosome.

In addition, to learn more about the partition complex, it would be interesting to determine whether Spo0J binds to chromosomal sites in addition to the eight known parS sites, and whether Spo0J binding spreads to regions adjacent to the parS sites, like P1 ParB (Rodionov, 1999). Unlike ParB, Spo0J does not appear to silence transcription of genes adjacent to parS sites (Kuester-Schock, unpublished data). However, Spo0J can form higher-order complexes on parS-containing DNA (Lin and Grossman, 1998), indicating that several molecules of Spo0J can bind to DNA containing a single parS site. Chromosomal regions bound by Spo0J in vivo could be identified by performing chromosome immunoprecipitation using anti-Spo0J antibodies, followed by microarray analysis to identify the bound DNA sites.

Finally, I believe that Soj contributes to Spo0J function. Perhaps Soj affects higher-order interactions between Spo0J dimers bound to parS sites, as speculated by Errington et al. upon observing that inactivating soj caused foci of Spo0J-GFP to dissipate into many smaller foci (Marston and Errington, 1999). Soj might also affect the extent of Spo0J binding to chromosomal regions. It would be interesting to test whether a Δsoj mutation affects Spo0J-mediated pairing of parS sites and/or the extent of Spo0J binding using the in vivo assays and experiments described above.

In the last few remarks, I discuss some issues that arise directly from this work. Could the Par system perform similar partitioning functions on plasmids and chromosomes? Could the function of Soj and SMC proteins have similar effects that
could account for the genetic interactions between *soj*, *spo0J*, and *smc*? How are localized factors maintained at specific subcellular positions?

**Could the Par system perform similar partitioning functions on plasmids and chromosomes?**

In the discussion section of Chapter 3, I described how Par systems can have different phenotypic effects on plasmid and chromosome partitioning. I reiterate these differences briefly and discuss in greater detail how they could be accounted for. *Spo0J* is not sufficient to recruit *parS* sites inserted at ectopic positions in the chromosome to the cell quarters (Lee et al., 2003), arguing against a primary function in anchoring. In contrast, *Soj* and *Spo0J*, as well as plasmid-encoded Par systems, are sufficient to recruit plasmids to the cell quarters (Niki and Hiraga, 1999; Niki et al., 2000). In addition, Par systems from chromosomes function on plasmids (Godfrin-Estevenon et al., 2002; Lin and Grossman, 1998). Could the same Par system perform different functions on plasmids and chromosomes? This seems unlikely. Instead, I believe that plasmid-encoded and chromosomally-encoded Par systems probably perform similar functions on plasmids and chromosomes, and that different phenotypic effects are due to inherent differences between plasmids and chromosomes.

Plasmids and chromosomes differ in many ways. One obvious difference is size. The chromosome is nearly 50 times larger than P1 and F plasmids. Another potential difference is that chromosome partitioning may be assisted by factors that do not act on plasmids. When the Par system is inactivated, the majority of plasmids mislocalize to internucleoid spaces (Erdmann, 1999; Niki and Hiraga, 1999; Niki et al., 2000), whereas the majority of replicated chromosomal origins can still reach the cell quarters (Lee et al.,
2003). One interpretation is that additional positioning factors could act on the chromosomal origins that do not act on plasmids. It is also possible that plasmids, being smaller, might be more freely diffusible than chromosomal regions if they are not anchored properly. Another implication of the small size of plasmids is that it seems less likely that replisome-mediated extrusion could drive plasmid migration from midcell to the cell quarters. Plasmid replication is not required for the P1 Par system to mediate plasmid partitioning (Treptow et al., 1994), consistent with the notion that an additional factor may be providing the motive force. P1 and F plasmids migrate with speeds ~40-50 times faster than the rate of cell elongation, arguing against diffusion alone (Gordon et al., 2004). Possible candidates for factors that could drive plasmid migration include components of the replisome or replication initiation factors with which plasmids could hitch a ride when they relocate from midcell to the cell quarters to set up the next round of replication (Pogliano et al., 2001). Alternatively, plasmids could be partitioned by a yet-undiscovered partitioning apparatus.

What similar function could the Par system perform on plasmids and chromosomes? In both cases, ParB could bind and bring together parS sites, forming a nucleoprotein structure that facilitates separation. In the case of plasmids or chromosomal origins, bringing together parS sites on sister molecules could facilitate pairing, which could potentially allow for coordinated separation in opposite directions. Alternatively, similar types of higher-order interactions might also bring together parS sites that are located far apart on the same chromosome, organizing the origin region into a nosecone-like structure that facilitates origin movement, as described above.
Could Spo0J pair replicated sister origins? If so, then inactivating spo0J should decrease the proportion of cells with paired origins. However, a spo0J null mutant has more cells in which replicated origins have not separated (Chapter 3), indicating that there may be more cells with paired origins. One potential explanation is that an additional, unidentified factor could keep origins in close proximity by pairing them or anchoring them to nearby sites, and that Soj and Spo0J function overcomes this factor. If replicated origins are paired, then pairing must last for a short portion of the cell cycle, since origins separate early in the replication cycle, long before the 270° and 181° regions of the chromosome (Lee et al., 2003; Lin et al., 1997; Sharpe and Errington, 1998; Teleman et al., 1998; Webb et al., 1998; Webb et al., 1997). Additionally, the finding that only 3.2% of wild type cells contain replicated origins that have not separated even after a nearby distal region has separated (Chapter 3) indicates that if the origins are paired, then this must only last for a very short portion of the cell cycle. Alternatively, the origins might not be paired, and this small proportion could indicate that origins almost always separate before distal regions, although at a low frequency (3.2%), origins fail to separate before distal regions.

Further speculation: could Soj and SMC both contribute to interactions in the partition complex?

I have presented a general model for Par system function in which ParB proteins form a higher-order partition complex on parS-containing DNA, and ParA proteins modulate interactions within this complex. In light this model, I speculate on how Soj and SMC proteins might have similar effects that could account for the genetic interactions between soj, spo0J, and smc, observed in Chapter 3.
In other Par systems, ΔparA mutants have plasmid or chromosome partitioning defects as severe as ΔparB mutants (Abeles et al., 1985; Friedman and Austin, 1988; Lewis et al., 2002; Ogura and Hiraga, 1983). In contrast, a Δsoj mutation alone does not significantly affect chromosome partitioning unless Δsme is also inactivated. One way to account for this is that the functions of Soj and SMC might both help Spo0J to form a complex in which the parS sites are brought together. For example, SMC-mediated compaction of DNA in the origin region could help by bringing parS sites closer together. This effect of SMC could compensate for Soj's role in contributing to Spo0J function, so that a Δsoj mutation alone does not compromise chromosome partitioning appreciably. It would be interesting to test whether Δsme affects the partition complex, using the experiments described at the beginning of the concluding remarks.

Navigating the cell: how do localized factors find their place?

Under various growth conditions, origins in B. subtilis can be positioned at midcell, the cell quarters, the cell eighths, and even at the cell poles, indicating that each of these positions, and probably even finer divisions, can be measured by the cell. Several other chromosome replication and partitioning factors inhabit specific subcellular positions, including the replisome, SMC, Soj, and SpoIIE. Of course, a host of additional factors beyond the scope of this thesis are positioned in specific locations within the cell. Attachment to anchors in the cell membrane or to cytoskeletal structures could contribute to positioning of these factors by restricting their movement. Ultimately, some subcellular positioning mechanism must enable a cell to position factors at specific locations along its length. One example of such a mechanism is the Min system of E. coli.
Attachment to the cell membrane or cytoskeletal elements could restrict movement. Attachment of intracellular factors to protein complexes anchored in the cell membrane could restrict their movement. In addition, cytoskeletal filaments formed by the actin-like proteins MreB and Mbl (van den Ent et al., 2001; Young, 2003) could provide stable structures to which intracellular factors could be anchored and maintained. Consistent with this, mreB and mbl appear to influence chromosome orientation (Soufo and Graumann, 2003) as well as subcellular positioning of peptidoglycan synthesis machinery (Scheffers et al., 2004).

The Min system of E. coli helps the cell find its middle. How does a cell position factors at specific locations along its length? Lessons can be learned from the well-characterized Min system of E. coli, which specifies FtsZ ring formation at midcell (Lutkenhaus and Sundaramoorthy, 2003; RayChaudhuri et al., 2001). FtsZ ring formation is an early step in cell division. In the absence of the min system, FtsZ rings often form near a pole instead of at midcell, producing a viable daughter and an anucleate minicell upon cell division. The Min system consists of MinC, a protein that inhibits FtsZ ring formation, and two proteins that regulate its subcellular location, MinD and MinE. MinD is an ATPase that is highly similar to ParA, and both proteins belong to the same subgroup of the Walker ATPase superfamily (Koonin, 1993; Motallebi-Veshareh et al., 1990; Yamaichi, 2000). Like Soj, MinD oscillates from pole to pole, and oscillations involve ATP hydrolysis. The Min system works by establishing intracellular protein gradients that regulate where the FtsZ ring can form. MinD recruits MinC to a cell pole, and MinE stimulates the proteins to detach and relocate to the opposite cell pole, such that they oscillate between poles on a timescale of minutes. MinE concentrates into a
ring near midcell that also oscillates (Fu et al., 2001). When the MinE ring reaches the pole harboring MinCD complexes, it destabilizes them from that pole, setting the oscillations in motion. MinE is thought to destabilize the polar MinCD complexes by stimulating MinD-mediated ATP hydrolysis. The net result of these oscillations is the establishment of a time-averaged subcellular gradient with MinCD concentration being highest at the poles and lowest at midcell, permitting FtsZ ring formation at midcell.

In *B. subtilis*, which does not have a MinE homolog, MinC and MinD do not oscillate. They are retained at the poles by an unrelated polar protein called DivIVA (Edwards and Errington, 1997), and inhibit polar cell division. *B. subtilis* probably has additional position-sensing mechanisms that allow fine discrimination of the midcell, quarters, eighths, and beyond. Further studies will be needed to identify the factors involved.

**Fertile ground**

There are enough open questions related to the Par system and broader aspects of bacterial chromosome and plasmid partitioning to feed many more generations of scientists. What is the primary function of the Par system? Does the Par system perform similar functions on plasmids and chromosomes? Is pairing a prerequisite to plasmid or chromosome partitioning? What factors recognize and position *cis*-acting sites within the origin region and other regions of the chromosome? Are there additional subcellular positioning systems and how do they work? Is there a yet undiscovered partitioning apparatus? Further experiments will expand our knowledge of these processes and identify new factors involved.


Appendix A:

Tips for using the $\text{lacO/lacI-cfp; tetO/tetR-yfp}$ system
(a.k.a. double-labeled strains) for visualizing two regions of the chromosome simultaneously
Summary

This appendix is for all you folks interested in using the lacO/lacI-cfp; tetO/tetR-yfp system to visualize two regions of the chromosome simultaneously. I have included tips on how to build and use double-labeled strains, and describe some idiosyncrasies I encountered working with this system so that you do not have to reinvent the wheel. The following useful parts for constructing double-labeled strains are tabulated: bacterial strains containing the lacI-cfp and/or tetR-yfp (Table 1), plasmids for inserting the tetO cassette in the chromosome (Table 2), and plasmids for inserting lacO in the chromosome (Table 3). The construction of the lacO/lacI-cfp system is described extensively in (Lemon and Grossman, 2000; Lemon et al., 2001; Teleman et al., 1998), and the tetO/tetR-yfp system is described in Chapter 3. Good luck with your experiments!

The “EVE” strain

PSL457 is a B. subtilis strain that contains both lacI-cfp and tetR-yfp integrated into the chromosome for your strain-building pleasure. This strain, nicknamed “EVE”, is ready to receive lacO and tetO cassettes wherever you desire.

Tetracycline concentration for building and maintaining TetR-YFP strains

I found that 6.25 ng/mL tetracycline was the best concentration for transforming in tetR-yfp and maintaining strains containing the fusion.

Chromosomal DNA from PSL445 can be used to introduce tetR-yfp into your strain. PSL445 contains tetR-yfp already integrated by double crossover at the cgeD locus (181°). The double crossover was confirmed by PCR, but even so, tetR-yfp can still recombine out of the chromosome albeit at low frequency. To reduce the probability of this heinous event, maintain strains on 6.25 ug/mL tetracycline. If you are transforming additional components into a strain
already containing \textit{tetR-yfp}, make sure you confirm that the chosen transformants are tetracycline resistant after each step.

Finally, if you find that the \textit{lacO} cassette you are using is unstable without selection, you may want to experiment with double-drug selection (6.25 \text{ug/mL} tetracycline to maintain \textit{tetR-yfp}, plus 5 \text{ug/mL} chloramphenicol to maintain \textit{lacO} cassette).

\textbf{The level of TetR-YFP expression is crucial for healthy cells}

The \textit{tetO/tetR-yfp} system has the potential to make cells visibly sick if \textit{tetR-yfp} is expressed at high levels. Sick cells are misshapen, can appear swollen at one end, have abnormal nucleoid morphology, and often contain a single bright focus of TetR-YFP. In order to reduce expression of \textit{tetR-yfp}, I mutagenized the \textit{Ppen} promoter driving \textit{tetR-yfp} and selected for mutants that gave proper origin localization and healthy-looking cells (described in Chapter 3), creating plasmid \textit{pPSL38} in \textit{E. coli} strain PSL354 (Table 1).

\textbf{What if my cells look sick?}

Although the \textit{tetO/tetR-yfp} system was optimized (described above) to keep cells healthy, it is still possible that using this system in combination with other mutant backgrounds could perturb the cells. If this happens, try adding \textasciitilde30 \text{ng/mL} tetracycline to the growth media. This decreases the proportion of TetR-YFP bound to the \textit{tetO} cassette. I found that this improved cell and nucleoid morphology and could restore proper localization in strains expressing high levels of TetR-YFP, without compromising the signal too much. You can experiment with the tetracycline concentration to tailor it to the particular strain you are working with (I did a titration using 6 – 1250 \text{ng/mL}), but note that adding too much will decrease the signal to noise ratio significantly.
Which lacO cassette should I use?

We have two types of lacO cassettes in plasmids pAT12 and pLAU43, respectively (Table 3). If you are building a new integration vector, I strongly recommend use of the lacO cassette from pLAU43 because it is less recombinogenic (the cassettes have random sequences between each lacO). That means your strains will have bright foci that are stable. With pAT12, the arrays can shrink (so that after streaking a couple times, the foci get dimmer and the background goes up).

Taking pictures of double-labeled strains

In double-labeled cells, foci of LacI-CFP give a fairly robust signal. Foci of TetR-YFP can be quite faint (although on its own, it is also fairly robust). The signal from the red membrane dye FM4-64 can also interfere with detection of TetR-YFP. In order to maximize your ability to see TetR-YFP, try titrating the FM4-64 concentration down. Take the membrane and TetR-YFP exposures prior to LacI-CFP. Also, exposing the cells to the YFP excitation wavelength for a few seconds before taking the picture can sometimes give brighter foci. Try different exposure times (I used up to 3 seconds), gain and offset settings.

Table 1. Useful bacterial strains for lacO/lacI-cfp; tetO/tetR-yfp system.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype or characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>KPL682</td>
<td>thrC::Ppen-lacI-cfpw7 (erm) (made by Katherine Lemon)</td>
</tr>
<tr>
<td>PSL354</td>
<td>AG104 E. coli containing pPSL38, plasmid to integrate Ppen(mutTATG-&gt;TAGG)-tetR-yfp (tet) at 181°(cgeD) in the chromosome by double crossover</td>
</tr>
<tr>
<td>PSL445</td>
<td>181°(cgeD)::Ppen(mutTATG-&gt;TAGG)-tetR-yfp (tet), double crossover</td>
</tr>
<tr>
<td>PSL457</td>
<td>“EVE” strain: thrC::Ppen-lacI-cfpw7 (erm); 181°(cgeD)::Ppen(mutTATG-&gt;TAGG)-tetR-yfp (tet)</td>
</tr>
<tr>
<td>PSL478</td>
<td>359°(yyac)::tetO (kan)</td>
</tr>
<tr>
<td></td>
<td>181°(cgeD)::Ppen(mutTATG-&gt;TAGG)-tetR-yfp (tet)</td>
</tr>
</tbody>
</table>
Table 2. Useful *tetO2* cassette plasmids.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDG364</td>
<td>To integrate <em>tetO(112)</em> at <em>amyE</em> (from Jonathan Dworkin)</td>
</tr>
<tr>
<td>pLAU44</td>
<td><em>tetO2</em> array cassette plasmid cloned NheI/XbaI in pUC+ (from David Sherratt’s lab)</td>
</tr>
<tr>
<td>pPSL6</td>
<td><em>tetO2</em> array cassette cloned NheI/Sall into pDG792 <em>(kan)</em>, clone in chromosomal fragments PstI/KpnI to Campbell <em>tetO2</em> into the chromosome</td>
</tr>
<tr>
<td>pPSL9</td>
<td>To integrate <em>tetO2</em> cassette at 90°(<em>yheH</em>) <em>(kan)</em></td>
</tr>
<tr>
<td>pPSL10</td>
<td>To integrate <em>tetO2</em> cassette at 345°(<em>hutM</em>) <em>(kan)</em></td>
</tr>
<tr>
<td>pPSL11</td>
<td>To integrate <em>tetO2</em> cassette at 181°(<em>cgeD</em>) <em>(kan)</em> Note: can’t use with <em>tetR-yfp</em> at <em>cgeD!</em></td>
</tr>
<tr>
<td>pPSL12</td>
<td>To integrate <em>tetO2</em> cassette at 359°(<em>yyaC</em>) <em>(kan)</em></td>
</tr>
<tr>
<td>pPSL13</td>
<td>To integrate <em>tetO2</em> cassette at 270°(<em>cotS</em>) <em>(kan)</em></td>
</tr>
<tr>
<td>pPSL18</td>
<td>To integrate <em>tetO2</em> cassette at 300°(<em>ywf1</em>) <em>(kan)</em></td>
</tr>
<tr>
<td>pPSL19</td>
<td>To integrate <em>tetO2</em> cassette at 316°(<em>ailD</em>) <em>(kan)</em></td>
</tr>
<tr>
<td>pPSL20</td>
<td>To integrate <em>tetO2</em> cassette at 329°(<em>speB</em>) <em>(kan)</em></td>
</tr>
<tr>
<td>pPSL45</td>
<td>To integrate <em>tetO2</em> cassette at 334° <em>(kan)</em></td>
</tr>
<tr>
<td>pPSL46</td>
<td>To integrate <em>tetO2</em> cassette at 354° <em>(kan)</em></td>
</tr>
<tr>
<td>pPSL47</td>
<td>To integrate <em>tetO2</em> cassette at 280° <em>(kan)</em></td>
</tr>
</tbody>
</table>

***Time-saving note:** See my strain book to find out which of these plasmids have already been integrated into the *B. subtilis* chromosome, to see the cloning strategies I used, and to find out the primers I used to amplify each chromosomal regions. (Look up the strain using the vector names, the cloning strategies and primers are listed in the comments for each strain).

Table 3. Useful *lacO* cassette plasmids.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLAU43</td>
<td><em>lacO</em> cassette cloned NheI/XhoI into pUC+ plasmid <em>(kan, amp)</em>. Random sequences between each <em>lacO</em> to minimize recombination. Clone through <em>recA- E. coli</em> (from David Sherratt)</td>
</tr>
<tr>
<td>pAT12</td>
<td><em>lacO</em> cassette plasmid <em>(cm, amp)</em> (from Losick lab)</td>
</tr>
<tr>
<td>pPSL26</td>
<td>To integrate <em>lacO</em> cassette from pAT12 at 300° <em>(cm)</em></td>
</tr>
<tr>
<td>pPSL27</td>
<td>To integrate <em>lacO</em> cassette from pAT12 at 316° <em>(cm)</em></td>
</tr>
<tr>
<td>pPSL44a</td>
<td><em>lacO</em> cassette XhoI fragment from pLAU43 cloned into XhoI-cut pGemcat</td>
</tr>
<tr>
<td>pPSL48</td>
<td>To integrate <em>lacO</em> cassette from pLAU43 at 4° <em>(cm)</em></td>
</tr>
</tbody>
</table>

***Time-saving note:** Katherine Lemon and Daniel Lin also used constructs to insert *lacO* arrays at 359°, 270°, 181°.
References


Appendix B:

Effects of *soj* on subcellular localization of Spo0J-GFP
Summary of results

I analyzed the effects of inactivating soj on subcellular localization of Spo0J-GFP. I found that during exponential growth in defined minimal medium at 30°C with glucose or succinate as a carbon source, discrete foci of Spo0J-GFP formed whether or not soj was present (Figure 1). I measured subcellular positioning of foci of Spo0J-GFP and found that inactivating soj affected positioning of the focus of Spo0J-GFP in cells with a single focus, but positioning of duplicated foci of Spo0J-GFP was not affected. These results agree with the results showing effects of soj on positioning of the origin region, measured using the lacO/lacI-gfp system (Chapter 3).

The strains used were PSL10 (spo0J-gfp) and PSL5a (Δsoj in-frame, spo0J-gfp). Growth conditions, data analysis methods, and the Δsoj in-frame deletion are the same as those described in Chapter 3.

Inactivating soj perturbs positioning of the focus in cells with one focus of Spo0J-GFP

During exponential growth in minimal medium with succinate as a carbon source, inactivating soj affected focus positioning in cells with a single focus of Spo0J-GFP (Figure 2). In wild type cells, the focus of Spo0J-GFP was positioned predominantly at or near midcell, whereas in the Δsoj mutant, there were more cells with the focus of Spo0J-GFP close to a pole (Figure 2). 38.2% of wild type cells contained a focus away from midcell in the 0-35% and 65-100% of cell length range, compared to 70.0% in the Δsoj mutant (Figure 2A and 2C). A similar trend was observed when I measured origin positioning in the presence or absence of soj using the lacO/lacI-gfp system to visualize origins (Chapter 3). Mispositioning of the origin region, visualized using Spo0J-GFP or the lacO/lacI-gfp system, is probably due in part to the
Figure 1. Spo0J-GFP localization. Spo0J-GFP in the presence (A) and absence (B) of soj. In both cases, Spo0J-GFP forms discrete foci. White arrows indicate cells that have a single focus of Spo0J-GFP. In wild type cells with a single focus of Spo0J-GFP (A), the focus is positioned at or near midcell, whereas in the Δsoj mutant (B), many of the cells have a focus of Spo0J-GFP positioned near a cell pole.
A. Wild type

Focus position as % of cell length

% Cells

B. Cell length (µm)

Pole to focus distance (µm)

n = 68

C. Δsoj

Focus position as % of cell length

% Cells

D. Cell length (µm)

Pole to focus distance (µm)

n = 90
Figure 2. Positioning of Spo0J-GFP in cells with a single focus. Strains were grown at 30°C in defined minimal medium with 1% succinate. Only cells with a single focus of the origin were analyzed. For panels A and C, focus position as a percentage of cell length was measured from images of live cells during exponential growth. For each panel, the percentage of cells with a focus in each 5% increment of cell length (0 to 5%, 5 to 10%, … 95 to 100% of cell length) was calculated and plotted as a histogram. The length increments from 0 to 35% and 65-100% are highlighted in grey, and the proportion of cells with a focus in these ranges is indicated in the upper right-hand corner of each histogram. For panels B and D, the same data sets as in panels A and C are used. The distance from the focus to the cell pole is plotted on the x axis, and cell length is plotted on the y axis. Cell length and the midcell positions are indicated by solid lines. Cell quarter positions are indicated by dotted lines. Data are shown for wild type (A) and (B), and Δsoj mutant cells (C) and (D).
fact that some of these one-focus cells actually have replicated origins that have not separated (Chapter 3). Consistent with this, the Δsoj null had a significantly higher percentage of cells with one focus of Spo0J-GFP compared to wild type cells (Table 1).

Table 1. Number of foci of Spo0J-GFP per cell in minimal succinate medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Percentage of cells with indicated number of foci^a</th>
<th>n cells^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSL10</td>
<td>spo0J-gfp</td>
<td>&lt;0.1 9.3 71.2 10.3 8.2 1.0</td>
<td>670</td>
</tr>
<tr>
<td>PSL5a</td>
<td>Δsoj, spo0J-gfp</td>
<td>0.4 36.9 59.6 1.1 2.1 &lt;0.4</td>
<td>282</td>
</tr>
</tbody>
</table>

^a Indicated strains were grown in defined S7 minimal media with 1% succinate and required amino acids at 30°C. Samples were taken during exponential growth. The number of foci per cell of Spo0J-GFP was determined and the percentage of cells with the indicated number of foci was calculated.

^b The total number of cells analyzed for each strain.

Duplicated foci of Spo0J-GFP are positioned normally whether or not soj is present

During exponential growth in minimal medium with glucose as a carbon source, inactivating soj did not affect focus positioning in cells with two duplicated foci of Spo0J-GFP (Figure 3). The average distance from a focus to the nearest pole was 27.3 ± 1.6% of cell length in the Δsoj mutant, statistically indistinguishable from 27.9 ± 1.4% of cell length in wild type cells (± 95% confidence interval for the mean). In addition, the average interfocal distances as a percentage of cell length were also indistinguishable: 45.4 ± 2.1% in the Δsoj mutant, compared with 44.3 ± 1.5% in wild type cells. These results agree with origin localization using the lacO/lacI-gfp system, described in Chapter 3. Data and figures for Spo0J-GFP localization in otherwise wild type cells were previously published (Lee et al., 2003). The number of foci of Spo0J-GFP per cell are tabulated (Table 2).
Table 2. Number of foci of Spo0J-GFP per cell in minimal glucose medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>&gt;4</th>
<th>n cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSL10</td>
<td>spo0J-gfp</td>
<td>&lt;0.1</td>
<td>4.6</td>
<td>55.2</td>
<td>18.4</td>
<td>20.7</td>
<td>1.1</td>
<td>1169</td>
</tr>
<tr>
<td>PSL5a</td>
<td>Δsoj, spo0J-gfp</td>
<td>&lt;0.1</td>
<td>9.1</td>
<td>63.6</td>
<td>12.5</td>
<td>13.6</td>
<td>1.2</td>
<td>870</td>
</tr>
</tbody>
</table>

aIndicated strains were grown in defined S7 minimal media with 1% glucose and required amino acids at 30°C. Samples were taken during exponential growth. The number of foci per cell of Spo0J-GFP was determined and the percentage of cells with the indicated number of foci was calculated.

bThe total number of cells analyzed for each strain.

Comparing these results to a previous report

Another group reported that inactivating soj causes discrete foci of Spo0J-GFP to dissociate into many smaller, fragmented foci (Marston and Errington, 1999) (Figure 4). This contrasts from my findings that discrete foci of Spo0J-GFP still form in the absence of soj. The discrepancy could be due to differences in the way the strains were constructed. PSL10 and PSL5a contain spo0J-gfp at the endogenous locus, driven by the endogenous promoter, and this is the sole copy of spo0J in the chromosome. In PSL5a, soj is inactivated using an in-frame deletion (described in Chapter 3) that allows expression of the downstream gene, spo0J-gfp, to a level within 2-fold of wild type. In contrast, the strains used by Errington et al. contain spo0J-gfp driven by the PxyI promoter at the amyE locus, and the endogenous copy of spo0J is left intact. The discrepancy in results may be due to differences in the levels of Spo0J protein in the strains used, or could also be due to different strain backgrounds or growth conditions used.

Although my results differed from those of Errington et al., I believe that their results provide a valid demonstration of in vivo conditions under which soj appears to affect Spo0J nucleoprotein complexes, visualized using Spo0J-GFP.
Figure 3. Subcellular locations of duplicated foci of Spo0J-GFP. Position of duplicated foci of the origin in wild type (A and C) and Δsoj mutant (B and D) strains. Strains were grown at 30°C in defined minimal medium with 1% glucose. Only cells with two foci of Spo0J-GFP were analyzed. In panels A and C, the distance from each focus to the same cell pole was measured from images of live cells in exponential growth and is plotted on the x axis, and cell length is plotted on the y axis. Cell length and the midcell positions are indicated by solid lines. Cell quarter positions are indicated by dotted lines. The number of cells analyzed (n) is indicated in the lower right-hand corner of each panel. One focus is indicated with red open circles and the other with blue crosses. In panels B and D, the interfocal distance is plotted against cell length. An ellipse was drawn around the distribution of wild type cells (A) and superimposed on the corresponding plots in panel B.
Figure 4. Inactivating soj can perturb localization of Spo0J-GFP foci. Cells were grown at 30°C in S medium containing 1% xylose to induce expression of Spo0J-GFP. (A) Spo0J-GFP forms distinct foci in otherwise wild type cells. (B) Spo0J-GFP mislocalizes as several smaller, fragmented foci in the absence of soj. Micrographs adapted from (Marston and Errington, 1999).
References
