Chromosome Partitioning in *Bacillus subtilis*

by

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Abstract

Chromosome partitioning is a fundamental process that ensures the stable inheritance of genetic material. I have studied this process in *Bacillus subtilis*, a Gram-positive bacterium that contains a single circular chromosome. The *B. subtilis spo0J* gene product is required for sporulation and for normal chromosome partitioning during vegetative growth. Spo0J is a member of the ParB family of proteins, which are found in several bacterial species.

In this thesis, I describe experiments to characterize the function of Spo0J in chromosome partitioning. I found that Spo0J is a site-specific DNA binding protein, recognizing a 16 bp sequence call *parS*. Spo0J binds to at least eight other *parS* sites, and these eight sites are all located in the origin proximal ~20% of the genome. Insertion of a single *parS* sequence into an unstable plasmid stabilizes that plasmid, indicating that *parS* can function in partitioning.

I also determined the subcellular localization of Spo0J using immunofluorescence and a Spo0J-GFP fusion protein. Spo0J, bound to its eight *parS* sites, typically localizes as two large foci, each near the 1/4 and 3/4 positions of the cell length through most of the cell cycle. This result indicates that the sister origins are separated early, and that the chromosome is in a defined orientation through most of the cell cycle. *spo0J* null mutants appear to have a mild defect in normal origin localization. However, Spo0J does not appear to be involved in simply attaching *parS* DNA near the cell quarter positions. When I inserted multiple *parS* sites into the terminus region of the chromosome, I discovered that the subcellular localization of the terminus was unaffected.

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Chapter 1

Introduction

The proper inheritance of chromosomes is essential for all organisms. In both eukaryotic and prokaryotic organisms, elegant systems have evolved to ensure the orderly duplication and partitioning of the genetic material. Chromosome partitioning encompasses multiple steps in order to set up and move chromosomes to each of the new daughter cells prior to cell division. Improper partitioning of the chromosomes is costly. In bacteria, defects in partitioning leads to the production of chromosomeless (anucleate) cells, a certain selective disadvantage. In yeast, mutations in genes required for chromosome partitioning can be lethal. In humans, Downs syndrome and some types of cancer are associated with improper chromosome partitioning.

All cells accomplish several fundamental tasks in order to partition their chromosomes (reviewed briefly in this chapter. See text below for references). For example, the chromosomes need to be recognized by the partitioning apparatus. This can be accomplished by a *cis*-acting DNA sequence, the centromere, and proteins that recognize this sequence. Also, the duplicated chromosomes need to be oriented so that they move to opposite poles. Centrosomes, kinetochores, and sister-chromatid cohesion proteins contribute to this task in eukaryotic cells. In addition, the duplicated chromosomes must be organized. Sister chromatid cohesion proteins and chromosome condensation proteins are important for this process in eukaryotes, and homologous proteins probably act in prokaryotes. Finally, the chromosomes need to be moved. In eukaryotic cells, microtubules and motor proteins play key roles in the chromosome movement. In prokaryotic cells, the search for the motive force is

underway, and may be due to mitotic-like motor proteins or the action of DNA polymerase.

This thesis focuses on experiments addressing the function of *spo0J*, which is required for normal chromosome partitioning in the Gram positive bacterium *Bacillus subtilis*. Before describing the specific experiments on *spo0J*, I will review aspects of both eukaryotic and prokaryotic chromosome partitioning. In eukaryotes, partitioning of the chromosomes occurs during the M (mitotic) phase of the cell cycle. I will review the eukaryotic cell cycle with special emphasis on factors required for proper chromosome partitioning, and the mechanisms used to set up the mitotic apparatus that drives chromosome partitioning. Next, I will review many of the key experiments that have led to our current understanding of bacterial chromosome partitioning. Special emphasis will be placed upon the partitioning of the origin regions in bacteria, a central subject in this thesis. I will go in depth with studies of proteins related to Spo0J from plasmid partition systems in prokaryotes.

Compared with the study of chromosome partitioning in eukaryotes, the study in prokaryotes is still in its infancy, but growing rapidly. As we begin to learn more about prokaryotic chromosome partitioning, a broad question is whether any of the mechanism involved in this process are similar to mechanisms in eukaryotic mitosis. Lessons and ideas from eukaryotic mitosis can help to frame models for bacterial chromosome partitioning, since the fundamental tasks that need to be accomplished are common to all organisms. However, the precise mechanisms and proteins that accomplish these tasks are

different in prokaryotes and eukaryotes, although there are exceptions. For example, the SMC proteins have been conserved from bacteria to human, and their functions in chromosome partitioning probably overlap. Throughout this thesis, I will make comparisons between eukaryotic and prokaryotic chromosome partitioning. Perhaps the only thing that is clear is that chromosome partitioning in both prokaryotes and eukaryotes is intricate, yet elegant.

OVERVIEW OF EUKARYOTIC MITOSIS

There are four phases in the eukaryotic cell cycle: from the first Gap phase of the cell cycle, called G1, the cell moves to S phase, where DNA replication occurs. This is followed by another gap phase, G2, followed by the mitotic phase, M, where the chromosomes are separated and the cell division occurs. Following the M phase, the two new daughter cells enter the G1 phase. Interphase is a combination of the G1, S, and G2 phases. M phase is further divided into four broad phases; these phases are, in order, prophase, metaphase, anaphase, and telophase. Many of the events, described below, to construct the mitotic apparatus occur during prophase and the beginning of metaphase. By the end of metaphase, the chromosomes are aligned between the centrosomes (described below). During anaphase, the chromosomes move apart, and during telophase, the cell divides. A key difference between eukaryotes and prokaryotes is that in prokaryotes, the S and M phases often overlap, so

partitioning of the chromosome occurs while replication is still proceeding (described below).

The specialized structure that drives chromosome partitioning in eukaryotes is the mitotic apparatus (figure 1-1). The mitotic apparatus contains the bipolar spindle, which is football shaped and is composed of microtubules. The microtubules emanate from the two centrosomes, located at the poles. The duplicated and condensed chromosomes (sister chromatids) attach to the microtubules from opposite poles. The sister chromatids are held together, in part, by sister chromatid cohesion proteins. The microtubules are attached to the kinetochore, a nucleoprotein structure that forms on the centromere. The centromere is the specialized DNA region that is required *in cis* for proper partitioning of the chromosome. During the first step of anaphase, called anaphase A, chromosomes move towards the stationary centrosomes. Microtubule depolymerization at the kinetochores and motor proteins drive the sister chromatids away from each other. During anaphase B, the centrosomes move farther apart, further separating the chromosomes.

All of the components listed above play important roles in the series of steps necessary to partition chromosomes. What follows is a brief review of the properties of these factors and some of what we know of the various stages of mitosis.

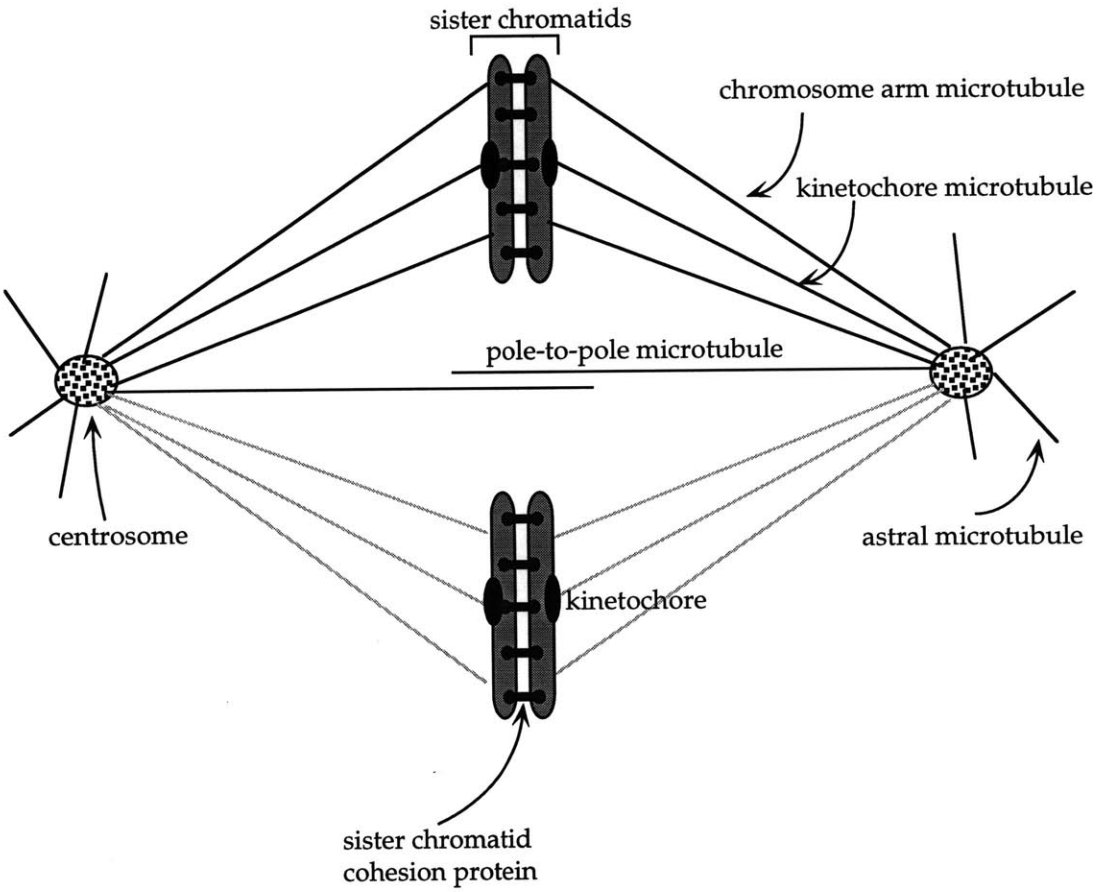


Figure 1-1. Metaphase configuration of the mitotic apparatus.

Microtubules emanate from the centrosome. Microtubules attach to the chromosomes both along the arms and at the kinetochore. The kinetochore is a specialized protein structure that assembles on the centromere. Pole-to-pole microtubules arise from the opposite centrosomes and interdigitate (also called anti-parallel microtubules). Astral microtubules emanating from the centrosome can attach to the cell cortex. The duplicated chromosomes, or sister chromatids, are paired along their length and at the centromeres by sister chromatid cohesion proteins.

Centromeres and kinetochores

A crucial component in eukaryotic mitosis is the centromere. The centromere is a DNA sequence required *in cis* to promote proper chromosome transmission. The centromere is a highly specialized, transcriptionally silent region of the chromosome where the assembly of the kinetochore occurs. In higher eukaryotes, the kinetochore is visualized as a relatively flat, triple-layered proteinaceous structure and sister kinetochores face opposite poles. The centromere and kinetochore serve multiple important function in mitosis. Microtubules emanating from the centrosomes bind to the kinetochore. Some motor proteins act at the kinetochore to generate force for chromosome movement both away and towards the poles. Checkpoint proteins, which function to delay the onset of anaphase if the mitotic spindle is not properly set up, localize to the kinetochore and monitor microtubule attachment and/or tension (Chen et al. 1996; Taylor and McKeon 1997). Some sister chromatid cohesion proteins act at the centromere and may also function to constrain the kinetochore region so the kinetochores face in opposite directions (described below) (Bickel and Orr-Weaver 1996; Kerrebrock et al. 1995; Saitoh et al. 1997).

The centromere and kinetochore proteins have been best characterized in *S. cerevisiae* (reviewed in (Hyman and Sorger 1995)). The budding yeast centromere is ~125 bp and is composed of three regions, CDEI, CDEII, and CDEIII (figure 1-2) (Cottarel et al. 1989). Protein complexes that bind to CDEI and CDEIII have been characterized (Cai and Davis 1989; Espelin et al. 1997;

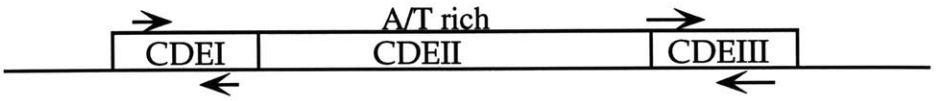
Lechner and Carbon 1991). CDEII and CDEIII are essential for centromere function, and CDEI is important but not essential.

The centromeres of other eukaryotes are more complex. The centromeres from the three *S. pombe* chromosomes are ~40-100 kb in length (Chikashige et al. 1989; Clarke et al. 1986) The *Drosophila melanogaste*r centromere is ~420 kb in length, and the human centromere is several megabases in length (figure 1-2) (Murphy and Karpen 1995 ; Pluta et al. 1995 review; Sun et al. 1997). The *S. pombe* and human centromeres contain large repeat elements, on the order of ~5 kbp. In humans, these large repeats are composed of smaller repeats of a ~200 bp A-T rich region termed alphoid DNA (Harrington et al. 1997; Heller et al. 1996).

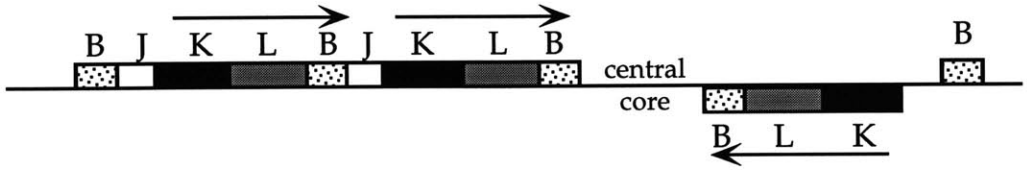
Currently, it is not known precisely what sequence, such as that in budding yeast, defines a centromere in *S. pombe*, *D. melanogaster*, or humans. In fact, in higher eukaryotes, a primary DNA sequence appears to be neither necessary nor sufficient for centromere function. Centromeres and kinetochores of *D. melanogaster* and humans are able to form *de novo* under certain circumstances on previously acentric DNA (du Sart et al. 1997; Williams et al. 1998). Although the specifics are unclear, it appears that an epigenetic mechanism involving heterochromatin is important for this transformation (Karpen and Allshire 1997; Williams, et al. 1998).

The kinetochore components, and their specific functions, are just being described. These proteins mediate several functions, including microtubule binding, signaling to checkpoints, and connecting the chromosome to motor

A. *S. cerevisiae* centromere (~125 bp)



B. *S. pombe* centromere (~40-100 kb)



C. *D. melanogaster* centromere (~420 kb)

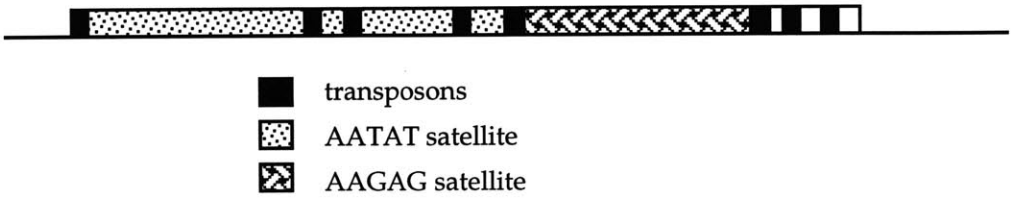


Figure 1-2. Centromeres of *S. cerevisiae*, *S. pombe*, and *D. melanogaster*.

A. The ~125 bp *S. cerevisiae* centromere sequence is composed of CDEI, CDEII, and CDEIII. CDEI and CDEIII both contain inverted repeats (denoted by arrows). The ~90 bp CDEII region contains ~90% A/T base pairs. B. The ~40-100 kb *S. pombe* centromeres from the three *S. pombe* chromosomes contain multiple, different, large repeat elements. Shown is a schematic of the centromere from chromosome 2 (adapted from Clarke *et al.*, 1993). Here, the B, J, K, and L regions (a single repeat is ~2-7 kb) are repeated on either side of the central core (~4-7 kb). Note that the central core is flanked by a large inverted repeat (noted by arrows). C. The *D. melanogaster* centromere was defined to ~420 kb contained within centric heterochromatin. The *D. melanogaster* centromere contains two large regions of repeated satellite DNA sequence (and). Eight transposable elements are also found in the centromere. (Adapted from *Sun et al.*, 1997)

proteins. In *S. cerevisiae* and *S. pombe*, several genes isolated in a screen for mutants defective in chromosome transmission are kinetochore components (Hoyt et al. 1990; Meeks-Wagner et al. 1986; Spencer et al. 1990; Strunnikov et al. 1995; Takahashi et al. 1994). As expected, some of these proteins are DNA binding proteins that specifically recognize the budding yeast *CEN* DNA (Espelin, et al. 1997; Sorger et al. 1994). Some *S. cerevisiae* kinetochore components, such as Cse4p and Mif2p, appear to be homologues to the mammalian kinetochore proteins CENP-A and CENP-C, respectively (Brown 1995; Meluh and Koshland 1995; Meluh et al. 1998; Stoler et al. 1995).

A common requirement in all eukaryotic organisms is that the kinetochore must form once and only once on a chromosome (reviewed in (Wiens and Sorger 1998)). A functionally dicentric chromosome will be broken if it is attached to opposite poles. In *S. cerevisiae*, formation of a single kinetochore is controlled, in part, by the specific *CEN* sequence which is present only once per chromosome. However, in *D. melanogaster* and humans, there is an added level of complexity, since *de novo* formation of a kinetochore must be suppressed.

Sister chromatid cohesion and chromosome condensation factors

Proper sister chromatid cohesion and chromosome condensation are required for efficient chromosome separation. Sister chromatids are attached to each other both along the chromosome arms and at their centromere. Sister

chromatid cohesion proteins presumably function between the two sisters as a molecular "glue."

Sister chromatid cohesion serves multiple functions (reviewed in (Bickel and Orr-Weaver 1996; Biggins and Murray 1998; Miyazaki and Orr-Weaver 1994)). First, through cohesion of identical molecules, i.e. sister chromatids, the cell ensures that they are ultimately segregated apart. In this light, cohesion functions to both let the cell know that two identical molecules need to be segregated, and functions to organize these molecules in a way that they can be segregated from one another. Rather than being two independent entities that need to be partitioned, the sisters are first grouped together prior to partitioning. Next, sister chromatid cohesion proteins may aid in orienting the kinetochore by constraining the centromeric regions so that the kinetochores face opposite directions. Outward facing orientation is important since kinetochores capture microtubules, and each sister kinetochore needs to be attached to microtubules from opposite poles. During meiosis I, a situation arises where the sisters need to face the same pole and homologues face opposite poles. Hence, additional factors may regulate the orientation of kinetochores during meiosis. A third possible function of cohesion proteins is to ensure that the chromatin from the two sisters do not become entangled as they emerge from the replication fork. Cohesion proteins may serve as a wall to keep the DNA from the two sisters organized and separate even while being held together. Another function of sister chromatid cohesion proteins is to counteract the forces on the sister chromatids that attempt to pull the chromosomes towards the centrosomes prior

to anaphase. Finally, the regulated dissolution of sister chromatid cohesion is required at the metaphase-anaphase transition (described below).

In budding yeast, sister chromatid cohesion is established during S phase, and there is speculation that cohesion is coupled with the movement of the replication forks and with chromosome condensation (Skibbens et al. 1999; Toth et al. 1999; Uhlmann and Nasmyth 1998). The *S. cerevisiae scc1* gene (also known as *mcd1*) was discovered in a genetic screen for sister chromatid cohesion mutants (Guacci et al. 1997; Guacci et al. 1993; Michaelis et al. 1997) and Scc1p is synthesized and functions during S phase (Uhlmann and Nasmyth 1998). Timing of cohesion is important; if Scc1p is synthesized after replication, sister chromatids remain separated. It has been proposed that the strict timing of sister chromatid cohesion is important to prevent sister DNA from mixing with one another.

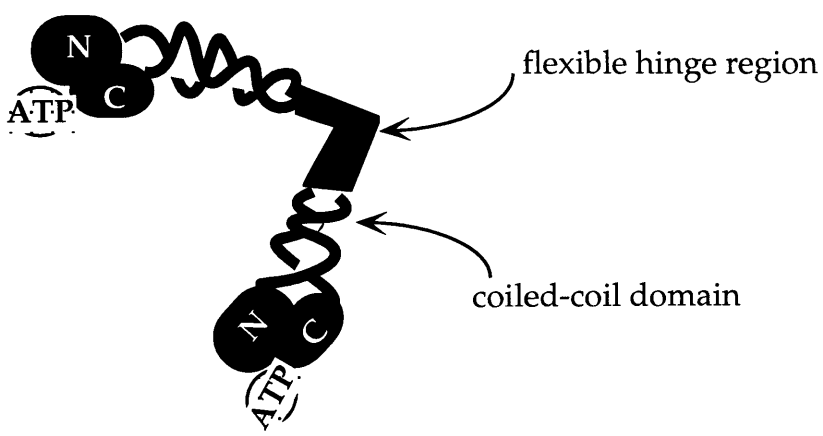
Chromosome condensation occurs during prophase, and in most eukaryotic cells can lead to an approximately 20-100 fold compaction of the chromosomes compared to chromosomes in interphase cells. Without chromosome condensation, the DNA may not be moved efficiently from the division septum. *S. pombe* mutants defective in chromosome condensation genes exhibit a "cut" phenotype, where the DNA is guillotined by the division septum (Saka et al. 1994).

Chromosome condensation has been studied in several other systems, including *S. cerevisiae*, *X. laevis*, and *C. elegans*. Members of the SMC (structural maintenance of chromosomes) family of proteins play a central role in

chromosome condensation and in sister chromatid cohesion (reviewed in (Hirano 1999; Koshland and Strunnikov 1996)). SMC proteins are large polypeptides (1000-1500 a.a.) composed of an N-terminal globular ATPase domain, two coiled-coiled dimerization domains separated by a hinge, and a C-terminal DNA binding domain (figure 1-3). These proteins are conserved from bacteria to human. Most eukaryotic organisms contain multiple SMC proteins that form SMC heterodimers. Eukaryotic SMC proteins are usually found in large complexes with other proteins. In *Xenopus*, the 13S condensin complex is composed of the SMC proteins XCAP-C and XCAP-E and other proteins and functions in chromosome condensation (Hirano et al. 1997). In contrast, the 14S cohesin complex from *Xenopus* , composed of XSMC1 and XSMC-3 and other proteins, is involved in sister chromatid cohesion. XRAD21, a homologue of *S. cerevisiae* Scc1p (described above), is part of the 14S cohesin complex (Losada et al. 1998).

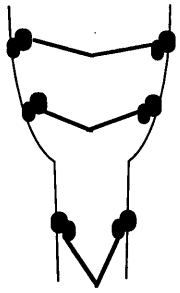
A link between chromosome cohesion and chromosome condensation was revealed when *scc1* mutants (described above) were found to be defective not only in cohesion but also in condensation (Guacci et al. 1997). This led to a model where Scc1p establishes cohesion following S phase, and during prometaphase recruits the condensin complex for chromosome condensation. This model, however, does not appear to be accurate in *Xenopus*, since the

A. SMC dimer



B. Models for SMC in:

Sister chromatid cohesion



Chromosome condensation

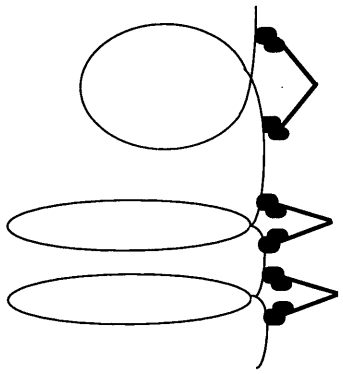


Figure 1-3. SMC proteins involved in both sister chromatid cohesion and chromosome condensation.

A. An anti-parallel SMC homodimer, based on the model of Melby *et al.*, 1998, studying the *B. subtilis* SMC protein. SMC dimers have a flexible hinge region and show ATP-dependent activity *in vitro*. SMC proteins are conserved from bacteria to humans and have been found to function in many aspects of chromosome organization. B. In yeast and *Xenopus*, different SMC proteins function in sister chromatid cohesion and chromosome condensation and function as SMC heterodimers. Simple models for SMC function in sister chromatid cohesion and chromosome condensation are shown. During sister chromatid cohesion, DNA from both sister chromatids can be crosslinked by a single SMC dimer. During chromosome condensation, the DNA is compacted as the flexible hinge allows the two ends to come close toghether. (part B from Hirano, 1998)

cohesin and condensin complexes can act separately (Losada, et al. 1998). Nonetheless, the involvement of SMC in different aspects of chromosome organization has helped in understanding the role of SMC in bacteria.

Microtubules

Microtubules are polymers that are made up of subunits of alpha and beta tubulin heterodimers. Microtubules have inherent polarity. The plus end of the microtubule is defined as the end where both assembly and disassembly of the microtubule preferentially occurs. In mitosis, the minus ends are attached to the centrosome, which is also known as the microtubule organizing center. Gamma-tubulin, a third distinct tubulin family member, localizes to the centrosomes and is required for microtubule nucleation (Moritz et al. 1995; Oakley and Oakley 1989; Stearns et al. 1991; Zheng et al. 1995).

Microtubule assembly occurs with the addition of alpha and beta tubulin heterodimers, and disassembly comes with their removal (figure 1-4). Microtubules display "dynamic instability," a term to describe both the slow growth and rapid depolymerization (called "catastrophe") of individual microtubules (Mitchison and Kirschner 1984). Polymerization and depolymerization of the microtubule is regulated by GTP hydrolysis. In a microtubule, most of the beta subunits have hydrolyzed GTP and are GDP bound. GTP hydrolysis favors depolymerization. The reason why microtubules can grow is because the tip of the microtubule has a GTP cap, where GTP bound

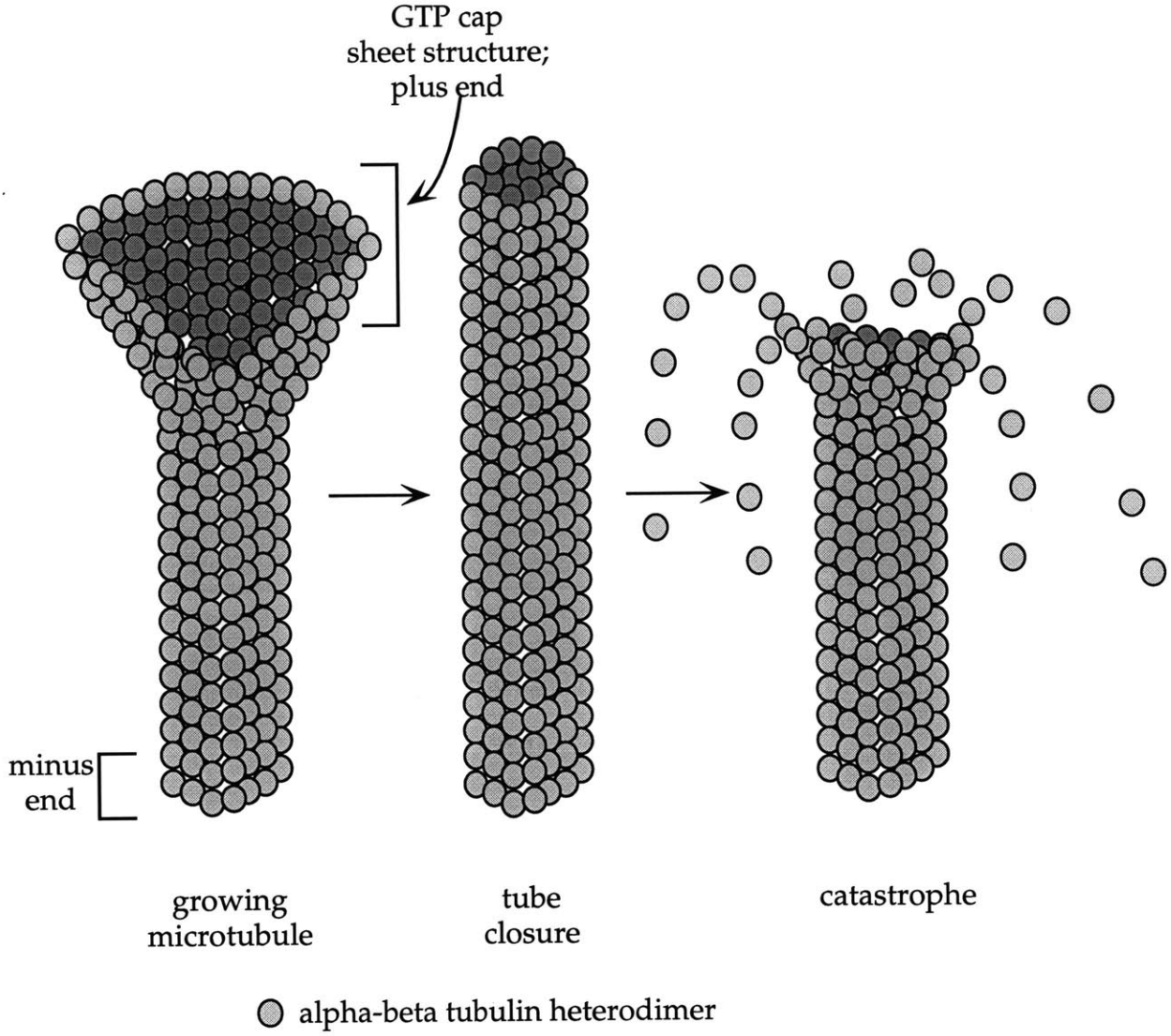


Figure 1-4. Microtubule growth and deploymerization.

Microtubule growth is stabilized by a GTP cap, which forms a sheet structure at the top of the tube. If the sheet closes into a tube, this event is thought to lead to a conformational change in the alpha--beta tubulin in the sheet. This conformational change in the heterodimer leads to hydrolysis of GTP to GDP on the beta tubulin subunit. This leads to catastrophe, where the micotubule rapidly depolymerizes (catastrophe). The built up energy of GTP hydrolysis in the microtubule lattice drives this rapid depolymerization. (Adapted from Hyman and Karsenti, 1996).

to the beta tubulin subunit has not been hydrolyzed (figure 1-4) (Carlier 1989; Drechsel and Kirschner 1994). The GTP cap prevents depolymerization. At the GTP cap, the tube form is opened up into a sheet structure (Chretien et al. 1995; Mandelkow et al. 1991). If this sheet closes up and forms a tube, the current thought is that this leads to a conformational change in the tubulin subunits that triggers GTP hydrolysis. Following tube closure, the free energy of GTP hydrolysis that has built up into the microtubule lattice leads to catastrophe.

Microtubules emanating from the centrosome are divided into four different categories (figure 1-1). Kinetochore microtubules attach to kinetochores during prophase and early metaphase and are responsible for most of the anaphase A movement. Chromosome arm microtubules attach along the length of the chromosome and are responsible for the movement toward the metaphase plate, called congression (described below). Both interdigitating pole-to-pole and astral microtubules are required for centrosome separation (described below). Microtubules, together with motor proteins, are responsible for essentially all the movement in setting up the mitotic apparatus and partitioning chromosomes.

Mitotic motor proteins

Motor proteins move cargo along tracks of microtubules in an ATP-dependent manner (reviewed in (Afshar et al. 1995; Hyman and Karsenti 1996)). Motor proteins move directionally. Some motor proteins, such as kinesin, are

plus end directed. Plus end directed motor proteins carry cargo towards the plus end of a microtubule. Other motor proteins, such as dynein, are minus end directed. Many motors important in mitosis are kinesin-related proteins and are either plus end or minus end directed. Many motor proteins are elongated structures that function as parallel homodimers. Typically, the globular motor "head" domain moves along the microtubules. Dimerization is mediated by a long coiled-coil domain, and the "tail" domain binds to the cargo. Motor proteins sometimes are found as homomultimers of homodimers, and these multimeric motor proteins may be involved in aspects of microtubule bundling.

There are many motor proteins that are important in mitosis. These motors are involved in moving the centrosomes apart, in chromosome movement, and in setting up the football shape of the mitotic spindle.

Centrosomes separate towards opposite poles during S phase to set up the mitotic spindle. Centrosomes also move apart during anaphase B to aid in chromosome partitioning. Centrosome movement is powered, in part, by plus end directed motors (figure 1-5). Members of the BimC family of kinesin-related motor proteins are required for centrosome separation during prophase and during anaphase B (Enos and Morris 1990; Hoyt et al. 1992; Kashina et al. 1996; Roof et al. 1992; Saunders et al. 1995).

How do plus-end directed motors move centrosomes apart? These motors move along interdigitating pole-to-pole microtubules that arise when microtubules from opposite centrosomes overlap one another (figure 1-5). The BimC proteins tether to one microtubule, and use the motor domain to move to

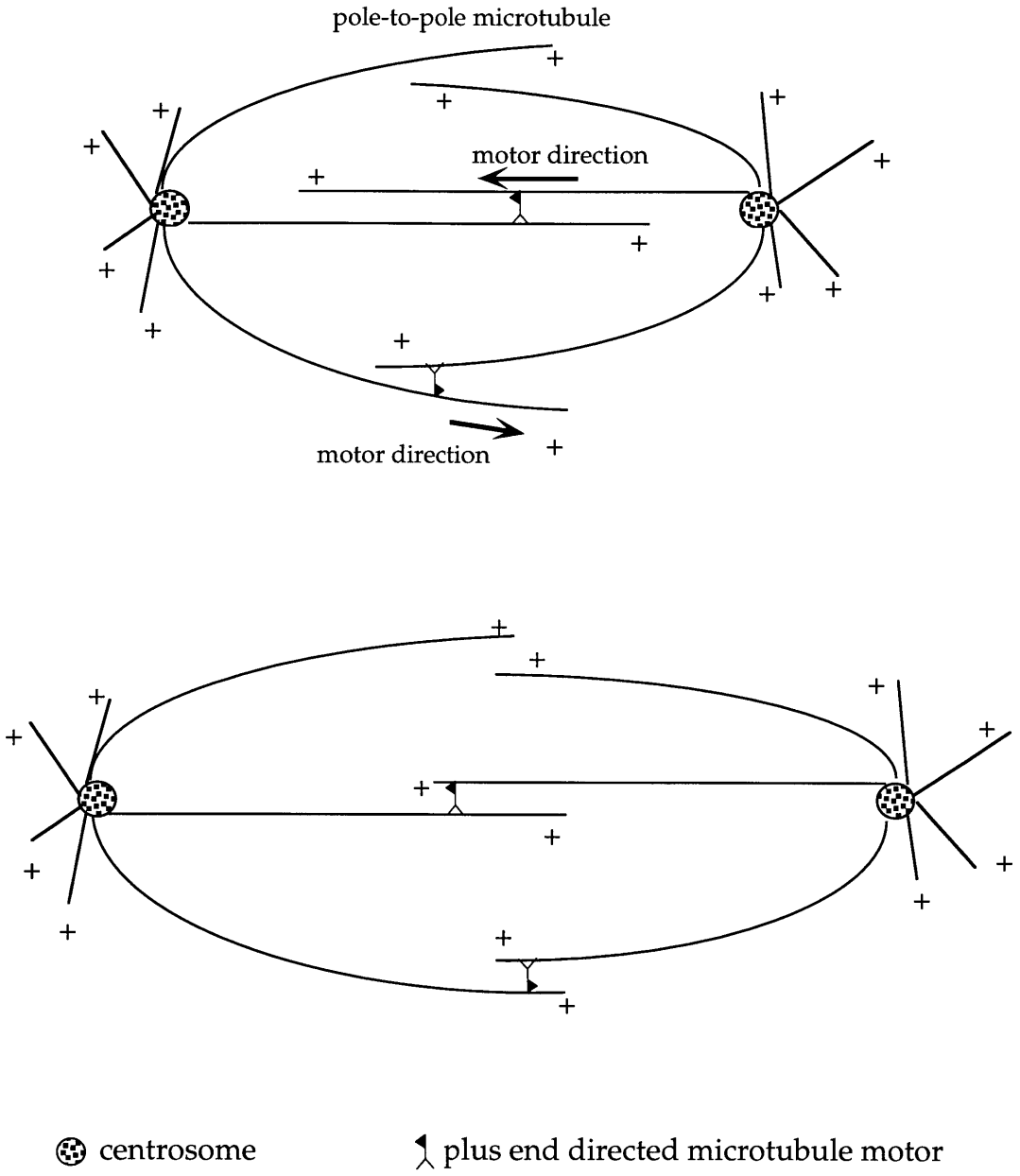


Figure 1-5. Centrosome separation by motors on pole-to-pole microtubules.

A plus end directed motor on interdigitating pole-to-pole microtubules can separate the centrosomes. One possible mechanism is that a motor protein could bind to a microtubule from one pole, and use the motor domain to move to the plus end of a microtubule from an opposite pole.

the plus end of the overlapping microtubule. Centrosome movement is also driven by the minus end directed motor protein dynein (Saunders, et al. 1995; Vaisberg et al. 1993). Dynein tethered to the plasma membrane could pull on the astral microtubules, moving centrosomes further apart.

Microtubules attach to the kinetochores and the chromosome arms

Chromosomes are attached to microtubules both at the kinetochore and along the chromosome arms. Both kinetochore and arm attachments are important for the chromosome movement, called congression, to set up the metaphase plate (Fuller 1995). The metaphase plate refers to the alignment of all the sister chromatids midway between the two centrosomes.

Attachment of the microtubule to the kinetochore occurs at random through a "search and capture" mechanism. Microtubules rapidly grow and shrink (termed "dynamic instability") from the centrosomes during prometaphase. The goal of the microtubule is to attach to a free kinetochore of a sister chromatid. When the side of a microtubule is captured by a chromosome, that microtubule is stabilized (Merdes and De Mey 1990; Rieder and Alexander 1990).

In *S. cerevisiae*, only one microtubule is attached to the kinetochore, but in higher eukaryotes, multiple microtubules attach to the kinetochore. Following the capture of the first microtubule by the kinetochore, the mono-oriented (attached at one kinetochore) sister chromatids move toward the pole, propelled

probably by both microtubule depolymerization and minus end directed motors (Rieder and Alexander 1990). More microtubules are the thought to attach "end-on" (as opposed to the side of the microtubule) to the captured kinetochore, and together these microtubules form a microtubule filament.

Attachment of microtubules to the chromosome arms is also important for chromosome alignment along the metaphase plate and for the football shape of the spindle apparatus. Microtubule capture by the chromosome arms also occurs by stabilization of microtubules emanating from the centrosomes. It has been proposed that microtubule associated proteins (MAPs) and/or motor proteins can stabilize microtubules along the chromosome arms (Afshar, et al. 1995; Mandelkow and Mandelkow 1995; Vernos et al. 1995). The ability of non-kinetochore chromatin factors to stabilize microtubules is well documented. A bipolar spindle can form in a *Xenopus* extract around beads coated with chromatin in the absence of centrosomes (Heald et al. 1996). *In vivo*, meiotic spindle formation in *Xenopus* or female *Drosophila*, and mitotic spindle formation in plants also occurs in the absence of centrosomes and nucleates around chromatin (Gard 1992; Theurkauf and Hawley 1992).

Congression

Once microtubules have attached to the kinetochore during prometaphase, the mono-oriented sister chromatids oscillate both towards and away from the poles. Minus end directed motor proteins and microtubule

depolymerization at the kinetochore govern movement towards the pole. Plus end directed motors and microtubule polymerization, acting both at the kinetochore and the chromosome arms, push the chromosome away from the pole (Hyman and Mitchison 1991; Rieder et al. 1986; Vernos, et al. 1995; Afshar et al, 1995).

The pushing forces acting at the chromosome arms are called the "polar winds" forces and act to push the mono-oriented sister chromatids away from the poles (Skibbens et al. 1993). The pushing force was revealed when the chromosome arm of a mono-oriented sister chromatid was severed, using micromanipulation techniques, to separate it from the kinetochore. The chromosomal fragment with the kinetochore moved towards the pole, but the severed chromosome arm was pushed away from the pole due to the "polar wind" force (Rieder, et al. 1986). Two plus end directed kinesin-related motors, Nod from *D. melangaster* and Xlkp1 from *Xenopus*, contribute to this pushing force (Afshar, et al. 1995; Vernos, et al. 1995). Inhibition of these motors results in improper chromosome positioning.

Equilibrium of tension is the basis for proper chromosome alignment at metaphase, and perhaps is a signal to initiate anaphase (described below) (Nicklas 1997; Skibbens, et al. 1993). In order to congress to the metaphase plate, the remaining unattached kinetochore must attach to a microtubule from the opposite pole. Once attached, a sufficient pulling force from the opposite pole is obtained to drive the sister chromatids to the metaphase plate. At the metaphase plate, the pushing forces on the arms and pulling forces at kinetochore are in

equilibrium. In addition, sister chromatid cohesion proteins function at the kinetochore and along the chromosome arms and act as "protein glue" to counteract the pulling forces towards both poles. Capture of microtubules and equilibrium of tension is so important to the cell that if any error occurs, a checkpoint is activated to keep the cells in mitosis until the proper attachments can be made (Hoyt et al. 1991; Li and Murray 1991). These checkpoint proteins, called Mads and Bubs, are conserved from yeast to humans (Li and Benezra 1996; Taylor and McKeon 1997).

Initiation of anaphase

Equilibrium of tension may be a signal that leads to initiation of anaphase (Li and Nicklas 1995; Nicklas 1997; Rieder et al. 1994). A mono-oriented kinetochore from a praying mantid spermatocyte delays the onset of anaphase. Using micromanipulation techniques, Li and Nicklas pulled on the mono-oriented kinetochore towards the opposite pole, and the cell was able to enter anaphase (Li and Nicklas 1995). This and other experiments led to the hypothesis that the cell senses tension before it initiates anaphase. It is not known how this tension is sensed, but other experiments implicate a putative phosphoprotein of unknown identity as a possible tension sensing factor (Nicklas et al. 1995). Kinetochore proteins may also be putative sensing tension sensing proteins. Checkpoints also appear to be regulated, in part, by these

tension sensing factors. The studies of the spindle assembly checkpoint *mad* and *bub* genes may provide a tool for identifying these tension sensing proteins.

Once tension equilibrium has been achieved on all chromosomes, anaphase initiates. Proteolysis plays a crucial role in the onset of anaphase (Holloway et al. 1993). The anaphase promoting complex (APC) is a multisubunit ubiquitin-ligase that attaches ubiquitin to proteins containing the destruction box (Glotzer et al. 1991; Irniger et al. 1995; King et al. 1995; Sudakin et al. 1995). Proteins that are ubiquitinated are proteolyzed by the proteasome (Ciechanover 1994). Degradation of factors, which may include sister chromatid cohesion proteins and/or anaphase inhibitors, allows the poleward forces at kinetochores to move the chromosomes apart. This has been best characterized in the lower eukaryotes *S. cerevisiae* and *S. pombe*. In *S. cerevisiae*, the Pds1p protein acts as an inhibitor of anaphase (Cohen-Fix et al. 1996; Funabiki et al. 1996; Yamamoto et al. 1996). Pds1p binds to and sequesters Esp1p, an anaphase promoter. Pds1p is a target of the APC, and its degradation by the APC presumably frees Esp1p to promote dissociation of the sister chromatid cohesion proteins, including Scc1p (Ciosk et al. 1998). It is not known exactly how Esp1p promotes dissolution of the sister chromatid cohesion proteins.

Once sister chromatid cohesion is dissolved, the sister chromatids move towards opposite poles. Microtubules attached to kinetochores rapidly depolymerize. *In vitro*, depolymerization of microtubules themselves provides enough force to move chromosomes apart in an ATP-independent matter (Koshland et al. 1988; Lombillo et al. 1995). The function of motor proteins in this

case is to simply keep the chromosome attached to the shrinking microtubule (Lombillo, et al. 1995). However, *in vivo*, minus end directed motor proteins, including dynein are associated with kinetochores and could drive movement (Pfarr et al. 1990; Steuer et al. 1990). However, genetic analysis of dynein mutants in *S. cerevisiae* reveal that dynein does not appear to be involved in anaphase A movement, but is involved in anaphase B movement (Eshel et al. 1993; Saunders, et al. 1995). Most likely, both microtubule depolymerization and motor function are important for chromosome movement *in vivo*.

OVERVIEW OF CHROMOSOME PARTITIONING IN BACTERIA

In prokaryotic cells, recent advances have helped to uncover the series of events that lead to proper duplication and partitioning of the chromosomes to the daughter cells. However, the proteins that coordinate the chromosome partitioning process in prokaryotes are just beginning to be understood.

Bacillus subtilis is a Gram-positive rod-shaped soil bacterium. Both *B. subtilis*, and the intensely studied Gram-negative rod-shaped bacterium, *Escherichia coli*, contain a single, circular chromosome. Replication initiates from a single origin, *oriC*, and proceeds bidirectionally. The proper transmission of genetic material is dependent on a process for partitioning the duplicated chromosomes to either half of the cell prior to cell division. The fidelity of chromosome partitioning in both *E. coli* and *B. subtilis* is high; anucleate cells

accumulate to less than 0.03% percent during vegetative growth (Hiraga et al. 1989; Ireton et al. 1994).

Many of the advances made in the past several years in prokaryotic chromosome partitioning have come with the advent of cell biological techniques to visualize different regions of the chromosome and proteins involved in chromosome partitioning. Experiments using these techniques have painted a picture of the bacterial cell cycle that appears simple, although we are still at an early stage in describing the partitioning process. An overview of our current understanding of the bacterial cell cycle is shown in Figure 1-6 and is described briefly below.

Initiation of replication at *oriC* presumably begins at midcell, where the replicative DNA polymerase localizes (Lemon and Grossman 1998) (Figure 1-6). Soon after duplication of the origin regions, the sister origins move apart to positions near the cell quarters, where they remain for the bulk of the cell cycle (chapter 2,4) (Glaser et al. 1997; Gordon et al. 1997; Lin et al. 1997; Mohl and Gober 1997; Niki and Hiraga 1998; Webb et al. 1997). Replication ends at the terminus of the chromosome, which is diametrically opposed to the *oriC* on the physical map. In contrast to the localization of *oriC*, the terminus region of the chromosome is located near the center of the cell through the bulk of the cell cycle (Gordon, et al. 1997; Niki and Hiraga 1998; Webb, et al. 1997). Unlike in eukaryotic cells, partitioning of the bacterial chromosomes occurs concurrently with DNA replication. Following the completion of DNA replication, site specific recombinases resolve chromosomal dimers that may arise by single

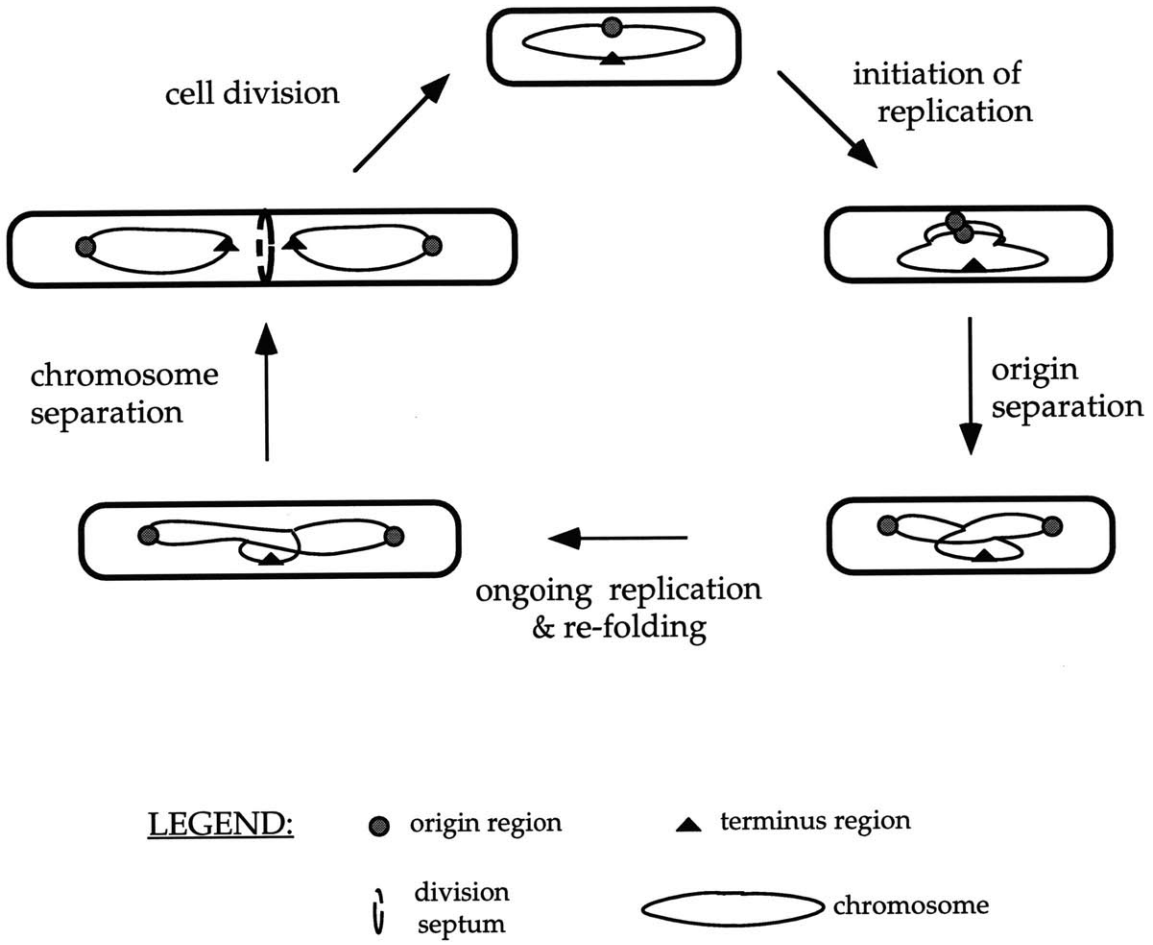


Figure 1-6. Overview of the bacterial cell cycle.

Starting with the cell at the top of the figure: The origin region, the site where DNA replication initiates, is shown as a filled gray circle (●). The replicative polymerase, PolC, localizes at midcell throughout most of the cell cycle and presumably initiation of replication also occurs at midcell. Soon after duplication of the origin region, the sister origins rapidly move apart. The origin regions localize near the cell quarter positions through the bulk of the cell cycle. In contrast, the terminus region (▲), localizes near the center of the cell through most of the cell cycle. While replication continues, the bulk of the nucleoid mass is partitioned towards either cell half. Finally, in the last step of chromosome separation, topoisomerases and recombinases are involved in resolving chromosomal catenanes or chromosomal dimers, respectively. In *B. subtilis* and *E. coli*, during exponential growth, the cell divides at midcell to produce two equally sized daughter cells. Adapted from (Lemon and Grossman, 1998).

crossover events (Blakely et al. 1991; Blakely et al. 1993). Following replication, the sister chromosomes are catenated and must be decatenated by topoisomerases (Adams et al. 1992; Kato et al. 1992; Luttinger et al. 1991).

The distinct localization pattern of the origin region indicates that a mechanism exists to both move and tether the origin regions near the cell poles, and that this is an early step in the bacterial chromosome partitioning process. It is currently unknown how the origin region and the bulk of the chromosome are moved, but the movement might be due to motor proteins or by the extrusion of replicated DNA by DNA polymerase (described below). Proteins from the ParA and ParB family are important for both plasmid and chromosome partitioning. SMC proteins probably function in chromosome organization. The properties of these proteins and the experiments that led to our current understanding of the bacterial chromosome partitioning process are reviewed below.

The origin region

Inheritance of genetic material is dependent not only on the duplication of chromosomes, but also on its partitioning to future daughter cells. This problem was addressed in part by the replicon model, first published by Jacob, Brenner, and Cuzin in 1963 (Jacob et al. 1963). Concerning chromosome replication, the authors proposed that an initiator protein activated DNA replication from a specific region of the chromosome, the "replicator" (or the origin, later called *oriC*). The identification of the origin of replication and the identification and characterization of proteins involved in the initiation of replication supported

these ideas (reviewed in (Kornberg and Baker 1992)). The structure of the origin region and proteins involved in replication are for the most part conserved throughout the prokaryotic kingdom (Ogasawara and Yoshikawa 1992; Salazar et al. 1996).

Concerning partitioning, Jacob, Brenner, and Cuzin proposed that the origin region was attached to the cell membrane at the center of the cell, for two reasons. First, attachment to the cell surface could allow for a direct connection between cell growth and DNA replication. Presumably, DNA replication would proceed in response to some signal related to the cell cycle such as cell size. Secondly, attachment to the center could be a mechanism by which the duplicated chromosomes are partitioned. The authors proposed that growth along the long axis of the cell by preferential insertion of new cell membrane material into the center of the cell would lead to separation of the chromosomes (Jacob, et al. 1963).

The partitioning mechanism proposed by the replicon model was explored in several experiments. It is now clear that insertion of new cell membrane material occurs randomly throughout the cell, inconsistent with cell growth being the driving force proposed in the replicon model (Woldringh et al. 1990; Nanninga et al. 1990). However, we now know that the origin region is associated with the membrane, in part by proteins involved in DNA replication (reviewed below). Not unexpectedly, the replicon model is too simple to account for all we now understand about bacterial chromosome partitioning. However,

the replicon model provided a framework to address aspects of origin localization and origin movement.

Localization of bacterial chromosomal origin and terminus

The cellular localization of the *B. subtilis* and *E. coli* origin and terminus regions was an advance in the understanding of the bacterial chromosome partitioning process. These localization studies show that for most of the cell cycle, the localization of the origin and terminus is defined, not random. The duplicated origin regions are usually located near the cell quarters and the terminus is usually positioned near the center of the cell.

Localization of the origins near the poles was inferred from studies of chromosome orientation during sporulation in *B. subtilis*. Sporulation is a postexponential phase developmental pathway that leads to the production of dormant resistant endospores in response to starvation and crowding (reviewed in (Grossman 1995; Stragier and Losick 1996). During sporulation, the cell divides near a cell pole rather than at midcell, generating a large mother cell and a smaller forespore cell. This specialized asymmetric division initially traps part of the chromosome in the small compartment near the pole, and the remainder of the chromosome is later translocated through (Wu and Errington 1994). Using a genetic mutation that blocked the cells prior to DNA translocation in combination with gene expression studies, it was demonstrated that the origin proximal 30% of the genome is preferentially trapped in the smaller forespore compartment. Hence, it was inferred that the origins are usually localized near

the cell poles in sporulating cells (Sun et al. 1991; Wu and Errington 1994; Wu et al. 1995).

Confirmation of this idea, not only in sporulating cells but also in vegetatively growing cells, came with the visualization of the origin and terminus regions in *B. subtilis* (Webb et al, 1997). The origin and terminus regions were also visualized in the nonsporulating bacterium *E. coli* (Gordon et al, 1997; Niki and Hiraga, 1998). Localization was accomplished either by using FISH (fluorescence *in situ* hybridization) or by inserting multiple tandemly repeated *lac* operator sequences at a desired chromosomal location and visualizing those regions with a GFP-LacI fusion protein (Gordon, et al. 1997; Niki and Hiraga 1998; Webb et al , 1997). In addition, the localization of Spo0J/ParB, a protein that binds to sequences in the origin region (Lin and Grossman 1998), provided independent evidence for the localization of the origin in *B. subtilis* (see below and chapter 2, 4) (Glaser, et al. 1997; Lin, et al. 1997) and in *Caulobacter crescentus*; (Mohl and Gober 1997).

In *B. subtilis*, these results show that in exponentially growing cells, the duplicated origin regions are usually localized near the cell quarters while the terminus is localized at midcell (Webb et al, 1997, Lin et al, 1997, Glaser et al, 1997). During sporulation in *B. subtilis*, the duplicated origins appear much closer to the poles (Webb et al, 1997). In *E. coli*, the duplicated origin regions appear near the poles (~10-15% the length of the cell) and the terminus is at midcell (Niki and Hiraga, 1997; Gordon et al, 1997). In *C. crescentus*, the duplicated origins localize at the poles (Mohl and Gober, 1997). Further

expansion of this work in *B. subtilis* shows that regions intermediate to the origin and terminus on the physical map typically localize to intermediate positions between the origin and terminus in the cell (Teleman et al. 1998).

Are the origins always near the cell quarters in *B. subtilis*? Or, does duplication and separation of the origins occur elsewhere in the cell, such as the center of the cell as proposed by Jacob, Brenner and Cuzin (Jacob, et al. 1963)? The answer to this question is not completely clear. Time-lapse microscopy of *B. subtilis* and *E. coli* cells with the *lacO*-marked origin regions was performed to address this. Origin duplication and separation usually occurs at midcell in *B. subtilis* (after origin movement from near the poles to midcell) (Webb et al. 1998). In *E. coli*, origin duplication usually occurs near the cell pole, and subsquently one sister origin moves to the opposite pole (Gordon, et al. 1997). Visualizing origin localization in germinating spores of *B. subtilis* (germination of spores occurs synchronously) supports a model for midcell duplication (Lewis and Errington 1997). Finally, the replicative DNA polymerase localizes in the center of the cell for most of the cell cycle in *B. subtilis*, favoring a model for duplication of the origins at midcell (Lemon and Grossman 1998). Although *B. subtilis* and *E. coli* may use different mechanisms, the question of where in the cell origin duplication occurs requires more rigorous experiments.

The separation of the origin regions is an active process that cannot be attributed simply to the attachment of the origin regions to the cell membrane and longitudinal growth (Webb et al. 1998; Gordon et al. 1997). Time-lapse microscopy visualizing the origin regions in live cells indicates that the origin

movement is abrupt, with a average maximal velocity of 170 nm/min in *B. subtilis* (Webb, et al. 1998). Similar observation were made in *E. coli* (Gordon, et al. 1997). The velocity of origin movement is within the range of movement that can be attributed to motor proteins or DNA polymerase (Webb et al. 1998). Treatment of the cells with drugs that inhibit cell wall growth or septum formation did not inhibit movement (Gordon et al. 1997; Webb et al. 1998). Together these observations led to the idea that an active mechanism was involved in separation of the origin regions. Two proposals are that the separation of the origin regions and/or the entire nucleoid could be carried out by DNA polymerase (Lemon and Grossman 1998) or by mitotic-like motor proteins (Glaser et al. 1997; Gordon et al. 1997; Lin et al. 1997; Webb et al. 1997).

Membrane association of the origin and terminus regions and replication forks

The attachment of the origin to the membrane occurs in both *E. coli* and *B. subtilis*, but how membrane attachment relates to chromosome partitioning has not been demonstrated conclusively (for review, see (Firshein 1989)). Origin membrane attachment was demonstrated by a number of membrane fractionation techniques (Hendrickson et al. 1982; Laffan and Firshein 1987; Sueoka and Quinn 1968; Winston and Sueoka 1980). In *B. subtilis*, the terminus is also associated with the membrane (Beeson and Sueoka 1979; Sargent and Bennett 1982; Yamaguchi and Yoshikawa 1975). At least two proteins that are

important for origin membrane attachment have been characterized, and in both cases these proteins play roles in DNA replication.

In *E. coli*, origin attachment is detectable in an outermembrane preparation (which may contain innermembrane components) (Hendrickson, et al. 1982) (*E. coli* is has an outermembrane lipid bilayer, a peptidoglycan layer, and an innermembrane lipid bilayer). The attachment of origin DNA is dependent on its methylation state. *E. coli* DNA is methylated at a specific site by the DNA adenine methyltransferase (Dam). It was observed that hemimethylated (methylated on only one strand) origin DNA binds preferentially to an outermembrane preparation (Ogden et al. 1988). Subsequently, the SeqA protein was identified as a factor important for binding hemimethylated origin DNA (Lu et al. 1994; von Freiesleben et al. 1994).

The binding of hemimethylated DNA to SeqA has mainly been studied for its role in sequestering the initiation of replication. Initiation of DNA replication in *E. coli* usually occurs on fully methylated DNA. Following passage of the replication forks, the origin region DNA becomes hemimethylated. The SeqA protein presumably binds to the hemimethylated DNA and functions to prevent reinitiation. *In vitro*, SeqA binds nonspecifically to hemimethylated DNA, calling into question whether origin binding (and attachment) is specific to SeqA (Slater et al. 1995). Nonetheless, an obvious question is if the membrane attachment by SeqA, or some factor in combination with SeqA, also functions in partitioning of the origin regions and/or of the bulk chromosome. Two results suggest that SeqA could function in partitioning of the bulk chromosome. *seqA* mutants

appear to have some chromosome partitioning defect (~1.6% anucleate cells), and the migration of the SeqA protein from midcell to the quarter sites late in the cell cycle suggests it may play some role in partitioning (Bahloul et al. 1996; Hiraga et al. 1998; Onogi et al. 1999). However, this migration of SeqA protein is independent of *oriC*, leading Hiraga *et al* to suggest that SeqA is important for bulk nucleoid partitioning (Hiraga et al. 1998; Onogi et al. 1999).

Origin-membrane association has been best characterized in *B. subtilis* (reviewed in (Firshein 1989; Firshein and Kim 1997)). Dam methylation does not occur in *B. subtilis*. In *B. subtilis*, origin membrane attachment and the initiation of replication are dependent on the gene product of *dnaB* (*B. subtilis* DnaB is distinct from *E. coli* DnaB, the replicative helicase) (Hoshino et al. 1987; Sueoka et al. 1988; Winston and Sueoka 1980). DnaB is a 472 aa protein with two putative ATP binding domains and an N-terminal transmembrane domain (Hoshino, et al. 1987). It is likely that DnaB interacts with origin DNA, directly or indirectly. Preliminary localization studies have shown that DnaB appears to localize as distinct foci in the cell and can localize in the absence of DNA (K. Lemon and AD Grossman, unpublished data). One idea is that DnaB may serve as a membrane anchor for the origin regions and the region of DNA that DnaB contacts could be analogous to a eukaryotic centromere. However, no studies on DnaB have separated its role in DNA replication from a possible role in chromosome partitioning.

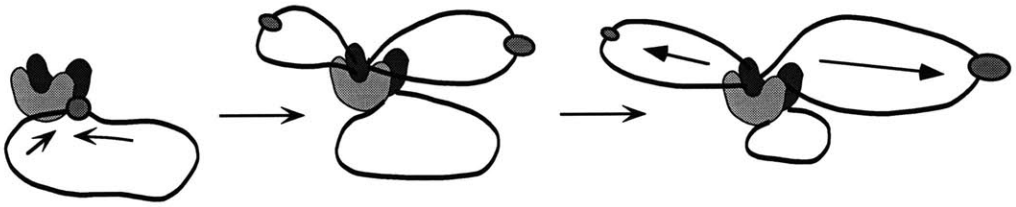
Finally, replication of the entire chromosome is also thought to occur at the membrane, although this has been less well characterized (reviewed in

(Firshein 1989)). Initiation factors, such as *B. subtilis* DnaB and the conserved DnaA protein (Yung and Kornberg 1988) are associated with membranes. Also, DNA Pol III (the replicative polymerase) activity can be detected in purified membrane fractions of *B. subtilis* and *Pneumococcus* (Benjamin et al. 1982; Firshein and Gelman 1981). Replication can proceed *in vitro* from these purified complexes without the addition of other proteins and is inhibited by hydroxyphenylazouracil, a specific inhibitor of DNA PolIII.

Localization of the replicative DNA polymerase

The replicative polymerase was localized in *B. subtilis* by use of a GFP fusion to the C-terminus of PolC. *polC* encodes the gene for the alpha subunit of the PolIII core. The *polC-gfp* fusion is functional, and PolC-GFP is found to be stationary and localizes in the center of the cell (Lemon and Grossman 1998). Localization of PolC-GFP is dependent on ongoing rounds of DNA replication (Lemon and Grossman 1998). These result support a "factory" model of DNA replication, in which the DNA is threaded through the stationary DNA polymerase rather than the polymerase moving along the DNA (Lemon and Grossman 1998). One possibility is that the actions of DNA polymerase may contribute to or be sufficient for the movement of the chromosomes to either half of the cell. In the "extrusion-capture" model, the newly replicated DNA strands are extruded from the stationary polymerase and the origin regions are captured by a protein complex that binds both the origin DNA and regions near the cell poles (figure 1-7) (Lemon and Grossman 1998).

A. Extrusion of DNA by a stationary DNA polymerase



B. Movement of DNA by motor proteins along a polarized track

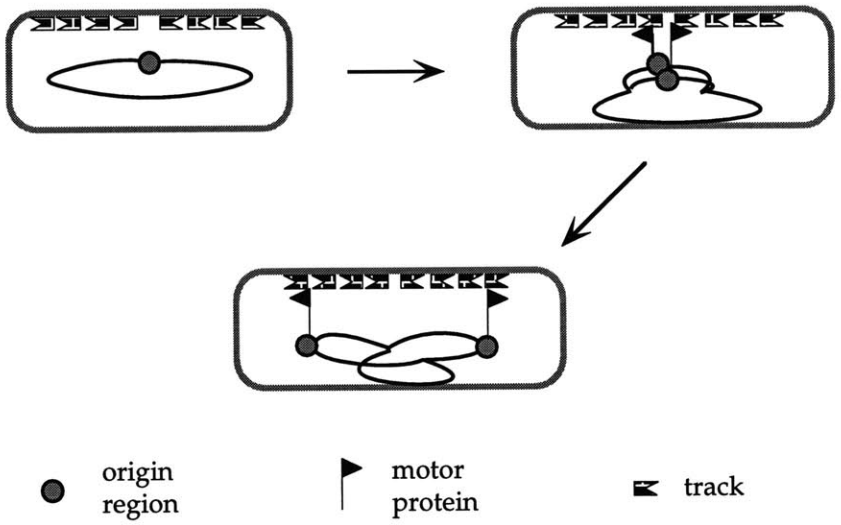


Figure 1-7. Two speculative models to account for the rapid movement of the origin regions.

A. In the extrusion-capture model, the motive force for origin movement is provided by DNA polymerase. DNA polymerase is stationary in the cell. As the chromosome is replicated, the sister chromosomes are pushed away from midcell by the force of polymerization and the origins are captured by an origin anchor near the cell poles. For simplicity, the replisomes are shown as giant U-shaped blobs. (adapted from Lemon and Grossman, 1998). B. Mitotic-like motor proteins may recognize the origin regions and move them along polarized tracks towards opposite poles, analogous to the function of eukaryotic mitotic motor proteins. (Adapted from Hiraga, 1992).

A second possibility is that origin movement and/or chromosome movement is accomplished by the actions of motor proteins (Glaser, et al. 1997; Lin, et al. 1997; Webb, et al. 1998; Webb, et al. 1997), analogous to the motor proteins that move chromosomes during eukaryotic mitosis (figure 1-7). Proteins similar in sequence to kinesins or dyneins have not been identified in genomes of several completely sequenced prokaryotes. It is possible that a novel motor protein functions in bacterial chromosome partitioning. Finally, by structural homology, the best candidates for possible mitotic motor proteins are members of the SMC family, which are conserved from bacteria to human. However, SMC proteins appear to be involved in chromosome organization, as described below.

MukB and SMC proteins

The *mukB* gene was originally identified in *E. coli* in a genetic screen for mutations that led to an increase in the percentage of anucleate ("mukaku" in Japanese) cells (Hiraga, et al. 1989; Niki et al. 1991). The *mukB* gene is in an operon with two other genes, *mukE* and *mukF*, which are also required for proper chromosome partitioning, although the precise functions of these gene products are unknown (Yamanaka et al. 1996). MukE and MukF are not found in *B. subtilis*. The MukB protein encodes a large 1534 amino acid protein with globular N and C terminal regions separated by two central coiled-coil domains that mediate homodimerization (Niki et al. 1992; Niki, et al. 1991).

MukB shares limited amino acid similarity but good structural similarity (see below) with the 1186 amino acid SMC protein of *B. subtilis* (Britton et al. 1998; Moriya et al. 1998; Oguro et al. 1996). *B. subtilis* SMC also has a structure, composed of two globular domains separated by two elongated coiled-coil domains (figure 1-3) (Melby et al, 1998). Currently, all but two completely sequenced bacterial genomes contain an SMC homologue, and the two that do not have SMC do contain MukB. Both proteins contain a nucleotide binding domain and the C-terminus has been implicated in DNA binding (Akhmedov et al. 1998; Niki, et al. 1991; Oguro, et al. 1996). *B. subtilis* SMC is capable of making homodimers (Melby et al. 1998), and null mutations in *smc* and *mukB* cause similar phenotypes (figure 1-3 part A) (Britton et al. 1998; Hirano and Hirano 1998; Moriya et al. 1998; Oguro, et al. 1996). Deletion of *smc* or *mukB* leads to the production of ~ 10% or ~ 5% anucleate cells in a growing culture, respectively (*mukB* experiments were done at 22°C in enriched media, *smc* experiments done at 30°C in minimal media) (Britton et al. 1998; Niki et al. 1991). In addition, both *smc* and *mukB* null cells are temperature sensitive for growth (Britton et al. 1998; Moriya et al. 1998; Niki et al. 1991). *B. subtilis* SMC and *E. coli* MukB probably play similar roles in chromosome partitioning.

The evidence suggests that MukB and SMC probably function in some aspect of chromosome organization. However, the structural similarity (both proteins have been visualized by electron microscopy) of these proteins to mitotic motor proteins initially led to the speculation that these proteins may function as motors, moving along some as yet unidentified track, to partition

chromosomes (figure 1-7) (Hiraga 1992). Both the SMC and MukB protein have been purified and studied *in vitro*. MukB is capable of interacting with DNA, although only nonspecific interactions have been detected *in vitro* (Niki, et al. 1992). Purified *B. subtilis* SMC binds preferentially to single-stranded DNA and can aggregate ssDNA in an ATP-dependent manner (Hirano and Hirano 1998). Also, *B. subtilis* SMC can reanneal ssDNA in an ATP-stimulated manner (Hirano and Hirano 1998). These biochemical results, in addition to the homology to eukaryotic SMC proteins involved in chromosome condensation (see below), suggest that *B. subtilis* SMC may have some role in chromosome compaction or organization, rather than in chromosome movement activity (Hirano and Hirano 1998).

By electron microscopy, the flexible hinge region clearly allows the two globular domains of MukB or SMC homodimer to interact. The homodimer appears to be anti-parallel, although more conclusive experiments need to be done to clarify this result (figure 1-3A) (Melby, et al. 1998; Niki, et al. 1992). A parallel homodimer would be suggestive of a motor protein, with a chromosome binding domain at one end and another end that binds to a track. An antiparallel homodimer would be more suggestive of a role in chromosome organization, with DNA binding domains at both ends. The chromosome organization model is also supported by studies of the SMC proteins from eukaryotes.

As mentioned above, the SMC genes were originally identified in yeast and *Xenopus* as genes required for chromosome condensation during mitosis (reviewed in (Koshland and Strunnikov 1996)). Mutations in *S. cerevisiae smc2*

and *S. pombe smc* genes *cut-3* and *cut-14* lead to defects in chromosome condensation and defects in mitosis (Saka, et al. 1994; Strunnikov et al. 1995). Other members of this family are also involved in processes that affect chromosome structure. The *C. elegans* DPY-27 protein, an SMC homologue, is required for dosage compensation, whereby the levels of X chromosome gene expression are reduced two-fold (Chuang et al. 1994). In addition, other *smc* genes are also important for sister chromatid cohesion during eukaryotic mitosis (see above) (Guacci, et al. 1997; Guacci, et al. 1993; Michaelis, et al. 1997). In sum, these studies indicate the importance of SMC in chromosome organization.

Null mutations in *B. subtilis smc* appear to cause defects in chromosome organization. *smc* cells are defective in the formation of the Spo0J foci (Britton, et al. 1998; Moriya, et al. 1998). As described below, Spo0J localizes as large foci near the poles of the cell (similar to origin localization, chapter 2,4) and Spo0J binds to at least eight sites in the origin region of the chromosome. The Spo0J focus is probably composed of multiple Spo0J proteins bound to its eight sites, and the distal most sites are ~775 kb apart (Lin and Grossman 1998). Presumably, interaction of Spo0J at all these sites leads to the formation of a specialized nucleoprotein structure important for partitioning (see below and chapters 2,3). The number of Spo0J foci per cell (in cells with DNA) is reduced in an *smc* null, although Spo0J protein levels are unchanged (Britton, et al. 1998; Moriya, et al. 1998). The defect in Spo0J localization in *smc* null cells may reflect defects in the chromosome organization that perturb the formation of the Spo0J focus. We favor the model that SMC and MukB proteins are involved in aspects

of chromosome organization rather than movement. These proteins may function in chromosome partitioning by refolding the newly replicated DNA and organizing it so that the sister chromosomes are bundled away from one another, so that they can be properly segregated.

Partitioning of the F and P1 plasmids

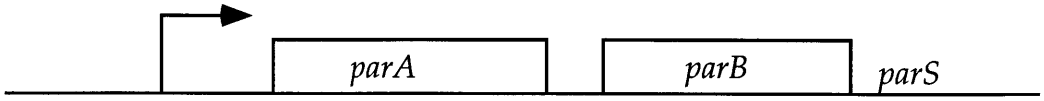
Many of the advances made in bacterial chromosome partitioning came from the characterization of the *par* operon from the P1 prophage and the F plasmid found in *E. coli.* P1 exists as a plasmid in its lysogenic state. The ~100kb genomes P1 and the F plasmid exist in approximately unit copy with respect to the chromosome (Collins and Pritchard 1973; Ikeda and Tomizawa 1968). Despite their low copy number, the plasmids are partitioned faithfully, with plasmid loss measured to be ~ 0.001-0.02% per generation, depending on the specific assay conditions (Austin et al. 1981; Lane et al. 1987). The plasmid encoded locus responsible for the high fidelity of plasmid partitioning was identified as the *par* (partition) operon in P1 and the *sop* (stability of plasmid) operon in F (Austin and Abeles 1983; Abeles and Austin 1985; Ogura and Hiraga 1983). Chromosomally encoded homologues are also involved in bacterial chromosome partitioning (described below).

The *par* and *sop* operons each encode two proteins, ParA and ParB in P1 and SopA and SopB in F. The ParA/SopA proteins are ~25% identical to one another in sequence as are the ParB/SopB proteins. Members of the ParA and

ParB families have since been isolated in a number of plasmid and chromosomal systems and in many cases have been shown to be important for partitioning (figure 1-8). In the better studied P1 and F systems, mutations of the entire operon, the *parA* locus, or the *parB* locus cause the same phenotype (Abeles et al. 1985; Ogura and Hiraga 1983). Each results in a ~100 fold increase in the levels of plasmid loss, with a loss rate of ~4 % per generation (Lane, et al. 1987).

The ParB family of proteins function in partitioning as site-specific DNA binding proteins. These proteins bind to a site referred to as a centromere-like site, called *parS* in P1 and *sopC* in the F plasmid. The site is located immediately downstream of *parB* (*sopB*) (Funnell 1991; Hayakawa et al. 1985; Martin et al. 1987 Davis, 1988; Mori et al. 1989). *parS* and *sopC* are required *in cis* for proper plasmid partitioning, and mutation of these sites phenocopy mutations in the *par* operon (Austin and Abeles 1983; Ogura and Hiraga 1983). *parS* alone on an unstable plasmid can stabilize that plasmid, provided that the ParA and ParB genes are supplied *in trans* (Martin et al. 1987).

parS is composed of binding sites for ParB and for the DNA binding and bending protein IHF (figure 1-9) (Funnell 1988). IHF stimulates ParB binding to *parS*, and mutations in a subunit of IHF perturbs plasmid stability, but not as greatly as deletion of the *par* operon (Davis and Austin 1988; Funnell 1988; Funnell and Gagnier 1993). *sopC* is composed a 43 bp sequence that is tandemly repeated 12 times (figure 1-9). Each *sopC* repeat contains a 7 bp inverted repeat to which SopB binds, and a single 43 bp repeat is sufficient for partitioning (Biek and Shi 1994; Hayakawa et al. 1985; Mori et al. 1986; Mori et al. 1989).



PLASMID		
P1 prophage	ParA (398aa)	ParB (334aa)
P7 prophage	ParA (401aa)	ParB (322aa)
F plasmid	SopA (388aa)	SopB (323aa)
CHROMOSOMAL		
Bacillus subtilis	Soj (253aa)	Spo0J (282aa)
Caulobacter crescentus	ParA (266aa)	ParB (293aa)
Pseudomonas putida	Orf263	Orf290
Mycobacterium leprae	Orf278	Orf333
Coxiella burnetti	Orf256	Orf288
Sreptococcus pneomoniae		Orf253
Streptomyces coelicolor	Orf255	Orf345
Helicobacter pylori	Orf265	Orf291
Chlamydia pneumoniae	Orf255	Orf286
Mycobacterium tuberculosis	Orf347	Orf344
Treponema pallidum	Orf253	Orf324
Borrelia burgdorferi	Orf250	Orf260
Chlamydia trachomatis	Orf255	Orf281
Rickettsia prowazekii	Orf255	Orf286

Fig. 1-8 Plasmid and Chromosomally encoded ParA and ParB homologues.

ParA and ParB proteins are conserved and found in several bacterial species. Some are encoded in plasmids, and some are chromosomally encoded. In the cases where the function of the proteins have not be experimentally determined, these proteins are called "Orf" followed by the predicted size of the protein product in amino acids (aa).

The ParB binding site, usually called *parS*, is typically located immediately downstream of the *parB* gene. One binding site for the *B. subtilis* Spo0J protein is located internal to the *spo0J* gene, and eight others are located in the origin proximal region of the chromosome (chapter 3) (Lin and Grossman, 1998).

P1 ParB recognizes two sets of sequences, called the A and B boxes (figure 1-9). The A box is composed primarily of a heptad sequence and the B box is a hexamer sequence; a 22 bp fragment containing an inverted repeat of two A boxes appears to be the most important for *parS* function (Davis et al. 1990; Funnell and Gagnier 1993; Martin et al. 1991). The A and B boxes flank both sides of the IHF binding site. The stoichiometry of P1 ParB binding to *parS* is unknown, but P1 ParB proteins are capable of dimerizing in solution (Funnell 1991). P1 ParB is capable of nucleating the assembly of an extended ParB protofilament from *parS* and that can lead to gene silencing >5 kb away from a *parS* site (Rodionov et al. 1999). This large ParB nucleoprotein structure may be important for partitioning.

The ParA proteins contain an ATPase domain known as the Walker A box (Koonin 1993; Motallebi-Veshareh et al. 1990). Both P1 ParA and F SopA have been shown *in vitro* to have ATPase activity (Davis et al. 1992; Watanabe et al. 1992). The ParA proteins have two roles: 1. Autoregulation of *par* operon transcription and 2. In partitioning. Both P1 ParA and F SopA act as repressors of *par* operon transcription by binding to operator sequences located upstream of the *parA* gene (Davey and Funnell 1994; Davis et al. 1992; Friedman and Austin 1988; Hirano et al. 1998). The repressor function is important for partition because excess amounts of ParA or ParB or both proteins debilitates partitioning (Abeles, et al. 1985; Funnell 1988). *In vitro*, P1 ParA bound to ADP binds specifically to the *par* operator (Bouet and Funnell 1999; Hayes et al. 1994).

A. P1 *par* site, *parS* (~90bp)

B. F plasmid *par* site, *sopC* (~500 bp; 12 direct repeats of a 43 bp sequence)

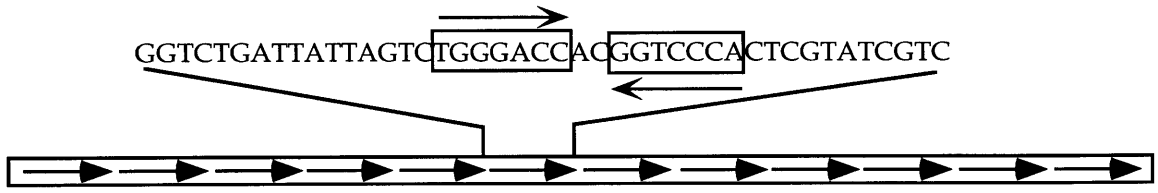


Figure 1-9. *par* sites from P1 prophage and F plasmid.

A. The *parS* site from P1 is composed of A and B boxes, both of which are contacted by ParB. The A and B boxes flank the IHF binding site. Binding of IHF to this site bends the DNA. A fragment containg an inverted repeat composed of the 7 bp A boxes, designated with arrows, retains some *par* function *in vivo*. B. The F plasmid *sopC* region is composed of twelve direct repeats of a 43 bp sequence. Within each repeat, purified SopB protects a region containing a 7 bp inverted repeat, designated with arrows.

ParA also has a direct role in partitioning in addition to its role in autoregulation. This was inferred from mutations in *parA* that do not affect its autoregulatory role but do affect partitioning. In a separate experiment, the ATPase domain of ParA was mutated by site-directed mutagenesis and the mutant *parA* expressed in a system that bypasses autoregulation. This mutant is also defective in partitioning (Davis et al. 1996). These results show that some aspect of ATP binding or hydrolysis is important for partitioning. P1 ParA *in vitro* can interact with the IHF-ParB-*parS* complex in the presence of ATP. It is unclear how ATP hydrolysis affects partition, since complex formation also occurs in the presence of the non-hydrolyzable analog ATPγS (Bouet and Funnell 1999).

Localization of the P1 and F plasmids and SopA and SopB

The subcellular localization of the low-copy *E. coli* mini- P1 and F plasmids was also accomplished by FISH and the visualization of GFP-LacI bound to *lacO* repeats inserted into the plasmids (Gordon, et al. 1997; Niki and Hiraga 1997). A "mini"-P1 or F plasmid is created by insertion of the *par* operon into an unstable vector, resulting in stability of that plasmid that is similar to that of the parental P1 and F plasmids (Austin and Abeles 1983; Ogura and Hiraga 1983).

The subcellular localization studies of the mini-P1 and F plasmids demonstrated that these plasmids localize at midcell early in the cell cycle. Later the duplicated sister genomes localize to positions 1/4 and 3/4 along the length

of the cell (Gordon, et al. 1997; Niki and Hiraga 1997). Importantly, the vector that lacks the F partitioning locus appears to localize randomly in nucleoid-free regions of the cell, indicating the importance of the *par* genes in the positioning of the plasmid genomes (Niki and Hiraga 1997). Interestingly, treatment of the cells with the drug cephalexin, which blocks septum formation, leads to mislocalization of the mini-P1 but does not affect F plasmid or *E. coli* origin localization. This indicates that distinct mechanisms govern partitioning of the P1, F, and the *E. coli* chromosomal origin region (Gordon, et al. 1997).

The localization of the F SopA and SopB proteins is somewhat controversial since the proteins could not be visualized when expressed from their own wild type promoters, presumably due to low abundance. Also, the two reported localization results are not similar to each other (Hirano, et al. 1998; Kim and Wang 1998). In our laboratory, localization artifacts have arisen from fixation, protein overexpression, and/or centrifugation steps (K. Lemon, P. Levin, D. Lin, J. Lindow, A Grossman, unpublished observations). A SopB-GFP fusion protein localizes near, but not at, the poles of live cells and is only visible in cells overexpressing the protein (Kim and Wang 1998). Interestingly, deletion of the C-terminal region abolishes DNA binding but does not abolish localization. This led Kim and Wang to propose that the localization reflects a mechanism to tether plasmids near poles, rather than being a reflection of the partitioning mechanism that actually moves the plasmids from the cell center to near the cell poles (Kim and Wang 1998). In a separate study, the SopA and SopB proteins were localized by immunofluorescence and could only be

visualized when the operator sequences in the *sopA-sopB* operon were deleted, leading to ~25-fold overexpression of the proteins (Hirano, et al. 1998). Under these conditions, both proteins appear to localize throughout the cell (Hirano, et al. 1998). Interestingly, when *sopB* is deleted in this system, SopA appears to localize on the nucleoid. This switch in localization is similar to the localization of *B. subtilis* Soj, a ParA homologue, which changes localization in the absence of Spo0J, a ParB homologue (described below) (J. Quisel, D. Lin, and A Grossman, manuscript in preparation).

Models for ParA and ParB function in plasmid partitioning

What is the mechanism by which the Par proteins promote faithful partitioning? Two proposals are that the Par proteins are involved either in plasmid pairing or in plasmid positioning (reviewed in (Austin and Nordstrom 1990; Austin 1988; Hiraga 1992; Williams and Thomas 1992)).

Clearly, positioning is inherent to partitioning, since a copy of each of the duplicated sisters must be in either half of the cell before the cell divides. Experimentally, it has been shown that the localization patterns of the P1 and F plasmids are not random, but that they localize at the quarter points of the cell prior to cell division (Gordon et al. 1997; Niki and Hiraga 1997). It has been proposed that the ParA and ParB proteins tether the plasmids (through the *parS* site) to some receptor located at these positions. In addition, one would also have to propose that different receptors exist for the P1 and F plasmids, since the

two can coexist in the same cell (Austin and Nordstrom 1990; Austin 1984). Invoking different receptors is consistent with the differential effects of cephalexin, the cell division inhibitor, on P1 and F partitioning (Gordon et al. 1997). However, another expectation of this model is that there would be two and only two functional molecules of a receptor in the cell (one at each quarter point). If there were multiple receptors, then both sisters plasmids could attach the same half of cell. Alternatively, one could invoke a mechanism whereby attachment of the other sister plasmid is blocked in one daughter cell once one is attached.

The pairing model hypothesizes that at some point in the cell cycle the two different plasmids pair through the partitioning complex, recognize one another, and then are segregated apart. Pairing is somewhat similar to the alignment of sister chromatids during metaphase in eukaryotes. While it is clear that the positioning of the plasmids is altered in vector without the *par* locus (Niki and Hiraga 1997), this could arise if pairing is a prerequisite for positioning. Pairing has been shown in the analogous (but not homologous) plasmid R1 system (Jensen et al. 1998), and is also explored for a chromosomally encoded ParB homologue, Spo0J, in this thesis (appendix 1).

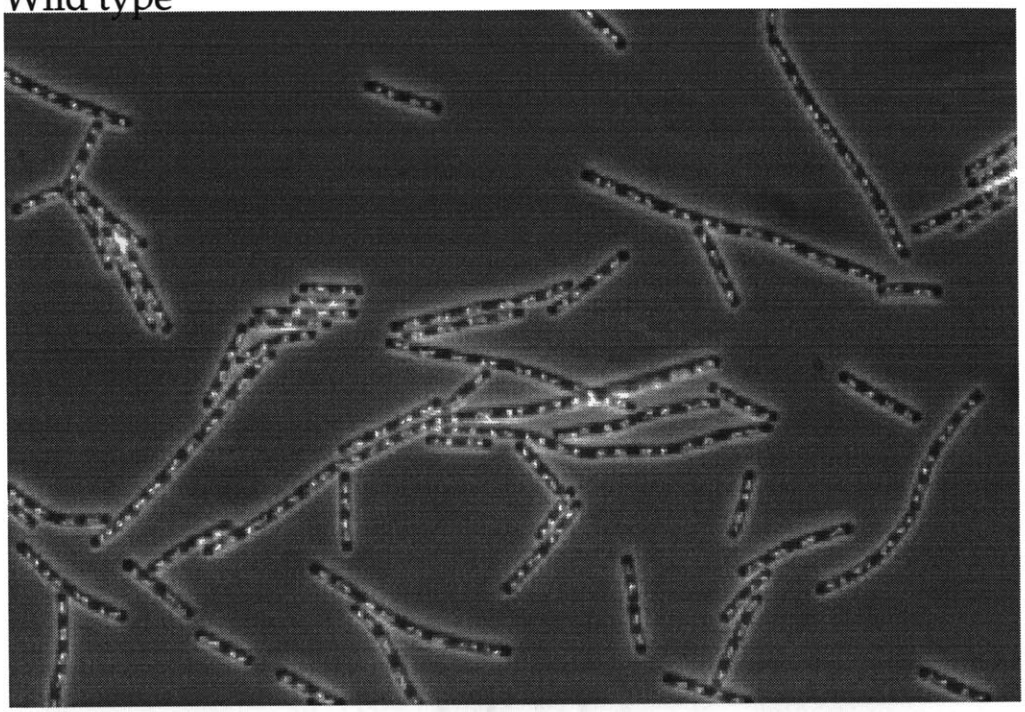
Proteins homologous to those involved in P1 and F plasmid partitioning may have a role in partitioning of the chromosomes of several bacteria. The study of the *par* operons of P1 and F may also provide insight into chromosome partitioning in bacteria.

Chromosomally encoded ParA and ParB homologues

Chromosomally encoded ParA and ParB homologues have been identified several different prokaryotic organisms, including: *Bacillus subtilis*, *Pseudomonas putida*, *Caulobacter crescentus*, *Mycobacterium leprae*, *Deinococcus radiodurans*, *Borrelia burgdorferi*, *Mycobacterium tuberculosis*, and *Helicobacter pylori* (figure 1-8). In nearly every case, the operon is located near the origin of replication. Curiously, *E. coli* does not encode a *parAB* operon, although P1 and F use *E. coli* as a host.

In two organisms, *B. subtilis* and *C. crescentus*, the chromosomal *parAB* operon has been shown to be important for chromosome partitioning. Spo0J, the ParB homologue in *B. subtilis*, was originally identified because it was required for the initiation of sporulation. Null mutations in *B. subtilis spo0J*, in addition to their defect in sporulation, also leads to the accumulation of approximately ~1.5% anucleate cells during exponential phase, a ~20-100 fold higher than that observed in wild type cells (figure 1-10) (Ireton, et al. 1994). Surprisingly, null mutations in the *B. subtilis* ParA homologue, *soj*, do not appear to lead to a chromosome partition defect, but do suppress the *spo0J* sporulation defect (Ireton, et al. 1994). These results indicate that Soj is an inhibitor of sporulation, and that Spo0J normally functions in sporulation by antagonizing Soj. However, Soj also seems to play some role in partitioning, as described below. In *C. crescentus*, a null mutation in either *parA* or *parB* is lethal, and overexpression of

Wild type



Δspo0J

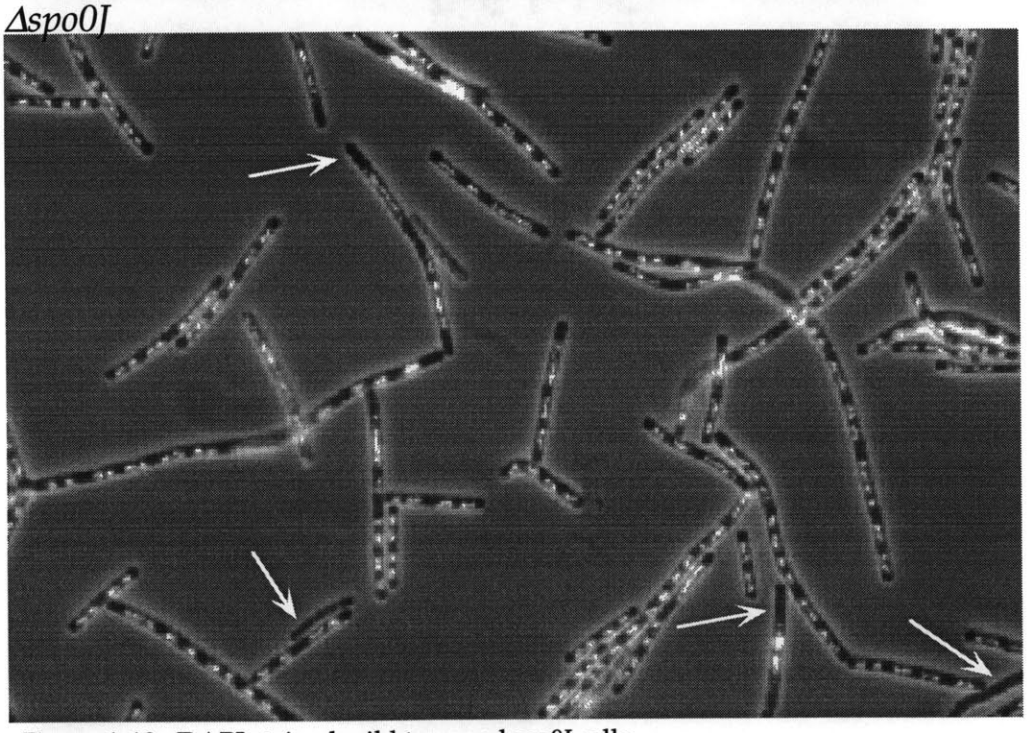


Figure 1-10. DAPI stained wild type and *spo0J* cells.

Vegetatively growing *B. subtilis* wild type and *Δspo0J* cells were fixed and stained with the DNA dye DAPI. Shown is a combined phase-fluorescence photomicrograph. Cells are phase dark and the DNA are the white bodies inside the cell. Top panel shows wild type cells, and bottom panel shows *Δspo0J* cells. Arrows indicate some anucleate cells.

the proteins leads to the accumulation of ~5% anucleate cells (Mohl and Gober 1997).

Spo0J is a site specific DNA binding protein, similar to the plasmid ParB proteins. Spo0J recognizes a 16 bp sequence called *parS* (chapter 3) (Lin and Grossman 1998). The first *B. subtilis parS* sequence identified was located internal to the *spo0J* gene, similar to the plasmid systems, in which the binding site is near the *parB* gene (see above). *parS* functions in partitioning, since insertion of this 16 bp sequence onto an unstable plasmid stabilizes that plasmid. *parS*-mediated plasmid stability is dependent on *spo0J* and *soj*, demonstrating a role for *soj* in partitioning (chapter 3) (Lin and Grossman 1998). By searching the completely sequenced *B. subtilis* genome for sequences identical or similar to the binding site internal to *spo0J*, a total of 8 binding sites were identified. These eight sites are all occupied *in vivo* by Spo0J as shown by a crosslinking technique. Interestingly, all eight sites are located in the origin proximal ~20% of the chromosome, and six of the eight sites were located in the origin proximal ~10% of the genome (chapter 3) (Lin and Grossman 1998).

The localization of the Spo0J/ParB proteins from *B. subtilis* and *C. crescentus*, in addition to the direct localization of the origin regions (described above) provided the first glimpse of the spatial localization of the origin regions in the cell. *B. subtilis* Spo0J localizes near the cell quarters and *C. crescentus* ParB localizes near the cell poles (chapter 2) (Glaser, et al. 1997; Lin, et al. 1997; Mohl and Gober 1997). These results, in parallel with the localization of the origin regions, indicate that the origin regions of the cell are separated early to either

daughter cell through most of the cell cycle. As expected, Spo0J co-localizes with the origin region (Teleman, et al. 1998 ; Lewis and Errington, 1997).

These results suggested that the chromosomal Spo0J/ParB proteins, via their association with *parS*, may be involved in tethering origin DNA to regions near the cell quarters (Sharpe and Errington 1996; Lin, et al. 1997; Mohl and Gober 1997). The tethering model was tested by inserting multiple *parS* sites into other regions of the chromosome (chapter 4). If the tethering model was correct, then these regions of the chromosome with the introduced *parS* site should localize near the cell poles (chapter 4). However, insertion of multiple *parS* sites into the terminus region does not influence the localization of the terminus, indicating that Spo0J probably has a role other than functioning as an origin tether (chapter 4). One hypothesis is that the *B. subtilis* membrane protein DnaB, required for origin-membrane attachment, may serve as an origin tethering factor (discussed in chapter 5 and above) (Winston and Sueoka 1980) (K. Lemon and A. Grossman, unpublished).

The function of the chromosomally encoded Spo0J/ParB proteins is still a mystery. It is possible that these proteins are also involved in pairing of sister origin regions prior to their separation, similar to that proposed for the plasmid encoded ParB proteins (see above, chapter 4, and appendix 1). Pairing could serve to signal that two sister origins exist, and should be partitioned. The pairing model, as well as other models for Spo0J/ParB function, are discussed in detail in Chapter 5.

Equally unclear is the function of the chromosomally encoded Soj/ParA proteins. *C. crescentus* ParA also localizes in a pattern similar to that of ParB. One proposal from work in the plasmid systems is that the ParA proteins act as "unpairing" proteins, dissociating the paired sister genomes (Bouet and Funnell 1999). *B. subtilis* Soj does not localize in a pattern similar to Spo0J. Instead, Soj localizes at the extreme poles of wild type cells as a band, while in cells deleted for *spo0J*, Soj localizes on the nucleoid where it associates and represses sporulation promoters (J. Quisel, D. Lin, A. Grossman, manuscript in preparation). Also, in wild type cells during sporulation, Soj appears to oscillate in the cell from one pole to another over the course of several minutes (J. Quisel, D. Lin, and A. Grossman, manuscript in preparation). How these observations relate to partitioning is unclear. Biochemical characterization of these proteins may begin to shed light upon their function.

Bacterial Chromosome Dynamics

Scientists have only taken a first step toward understanding the process of bacterial chromosome partitioning. The observations of the spatial organization of the chromosome and the identification and characterization of factors involved in partitioning have been, and will continue to be, exciting. The full understanding of the underlying mechanisms of prokaryotic chromosome partitioning simply awaits further identification of the factors involved, dissection of the their functions, and coalescence of their roles.

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Chapter 2

Bipolar localization of a chromosome partition protein in *Bacillus subtilis*

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Abstract

We have determined the sub-cellular localization of the chromosome partition protein Spo0J of *Bacillus subtilis* by immunofluorescence microscopy and visualizing fluorescence of a Spo0J-GFP fusion protein. Spo0J was associated with a region of the nucleoid proximal to the cell pole, both in growing cells dividing symmetrically, and in sporulating cells dividing asymmetrically. Additional experiments indicated that Spo0J was bound to sites in the origin-proximal third of the chromosome. These results show that the replicating chromosomes are oriented in a specific manner during the division cycle, with the Spo0J binding region positioned toward the cell poles. Experiments characterizing cells at different stages of the cell cycle showed that chromosome orientation is established prior to the initiation of cell division. Our results indicate that there is a mechanism for orienting the chromosomes and that the chromosome partition protein Spo0J might be part of a bacterial mitotic-like apparatus.

Introduction

Chromosome segregation is a fundamental process necessary for propagation of all organisms. Although not fully understood, many components of the segregation machinery have been identified and characterized in eukaryotes, including, centromeres, centromere-binding proteins, and the mitotic apparatus (Hyman and Sorger 1995; Murray and Hunt 1993). In contrast, these components, or their analogues, have not been identified in bacteria.

The study of bacterial chromosome segregation has focused mostly on *Escherichia coli* and *Bacillus subtilis* (Hiraga 1992; Rothfield 1994; Wake and Errington 1995). Both organisms have a circular chromosome with a single origin of replication. Segregation is divided into two steps: physical separation of replicated chromosomes and partitioning of these chromosomes to daughter cells. Topoisomerases and site specific recombinases are involved in the decatenation and separation of the replicated chromosomes. Several gene products have been identified that are involved in the partitioning of chromosomes to dividing cells (e.g. *mukA, mukB*), but the molecular mechanisms by which these proteins function are not yet known (Hiraga 1992; Wake and Errington 1995). While it is clear that the chromosomes are associated with the cell membrane, at least during part of the cell cycle, it is not known if or how this association is related to partitioning. In addition, it is not clear if the circular chromosome has a defined orientation in the cell (Hiraga 1992; Rothfield 1994; Wake and Errington 1995).

In *B. subtilis*, the *spo0J* gene product is needed for the initiation of sporulation and for proper chromosome partitioning during vegetative growth (Ireton et al. 1994) and sporulation (Sharpe and Errington 1996). *soj*, the gene immediately upstream from and co-transcribed with *spo0J*, is a negative regulator of sporulation (Ireton et al. 1994), but does not seem to be required for partitioning. Null mutations in *soj* bypass the need for *spo0J* in sporulation, but not partitioning (Ireton et al. 1994; Sharpe and Errington 1996)

Soj and Spo0J are similar to a family of proteins involved in plasmid partitioning, including ParA/ParB of prophage P1 and SopA/SopB of F (Hiraga 1992; Ogasawara and Yoshikawa 1992; Wake and Errington 1995 review). ParB (SopB) binds to a centromere-like site, *parS*, located near the plasmid origin of replication, and by a mechanism that is not clear, mediates partitioning. Null mutations in *parB* (or *sopB*) or deletion of the binding site (*parS* or *sopC*) lead to a rate of plasmid loss ~100-fold greater than that of wild type (Lane et al. 1987; Austin and Abeles 1983; Ogura and Hiraga 1983). The plasmid encoded ParA proteins are also required for partitioning. ParA is an ATPase that binds DNA, regulates transcription, and interacts with ParB (Davey and Funnell 1994; Davis et al. 1992; Hiraga 1992). Soj is a DNA binding protein (DL & ADG, unpublished results), and, by analogy to ParA and ParB, is probably an ATPase that interacts with Spo0J.

In *B. subtilis*, null mutations in *spo0J* cause a defect in chromosome partitioning during vegetative growth. Approximately 1.5% of cells in a growing culture of a *spo0J* mutant are anucleate, a frequency ~100-fold higher than that

observed in wild type cells (Ireton et al. 1994). *spo0J* homologues have been found in several bacterial species, including *Pseudomonas putida* (Ogasawara and Yoshikawa 1992), *Coxiella burnetii*, *Mycobacterium leprae*, and *Caulobacter crescentus* (J. Gober, personal communication), and it is likely that these homologues are also involved in chromosome partitioning.

Spo0J is also involved in chromosome partitioning during sporulation (Sharpe and Errington 1996). Sporulating cells divide asymmetrically to produce two cell types, the larger mother cell and the smaller forespore, each with an intact chromosome and a distinct pattern of gene expression. Immediately following asymmetric division, ~30% of the chromosome, centered around the origin of replication, is positioned in the forespore (Wu and Errington 1994). The remainder of the chromosome is translocated into the forespore in a process that requires the *spoIIIE* gene product (Wu and Errington 1994; Wu et al. 1995). In the absence of SpoIIIE, the *oriC*-proximal 30% of the chromosome is "trapped" in the forespore while the other 70% remains in the mother cell. Spo0J is involved in positioning the origin-proximal part of the chromosome in the forespore (Sharpe and Errington 1996).

We have determined the sub-cellular location of Spo0J using immunofluorescence microscopy with anti-Spo0J antibodies, and using a fusion of Spo0J to the *Aequorea victoria* green fluorescent protein (GFP) (Chalfie et al. 1994). Spo0J was associated with the regions of the nucleoid located near the cell poles, both during symmetric division in growing cells, and asymmetric division in sporulating cells. Experiments with a *spoIIIE* mutant indicated that Spo0J co-

localized with the 30% of the origin-proximal part of the chromosome that is trapped in the forespore. These results demonstrate that the chromosome is oriented in a specific manner such that the Spo0J binding region is placed toward a cell pole, and suggest that Spo0J is directly involved in chromosome partitioning.

Materials and Methods

Plasmids

spo0J-(*his*)$_6$ was constructed in pET21 (Novagen). An XhoI site was introduced by site directed mutagenesis (Ausubel et al. 1990) at the 3' end of *spo0J*, in place of the stop codon. An EcoRI to XhoI fragment containing *spo0J* was cloned into pET21 to generate pDL3, containing *spo0J*-(*his*)$_6$.

pDL50B contains the in-frame *spo0J-gfp* fusion in the vector pGEM*cat*. Briefly, *spo0J*-(*his*)$_6$, with the XhoI site at the junction between *spo0J* and the (*his*)$_6$ tag, was cloned into pGEM*cat* (Youngman et al. 1989) to give pDL8. *gfp* was PCR amplified from plasmid pJK19-1 {a gift from J. Kahana (Kahana and Silver 1996)} using PCR primers LIN13 (5'-GGAGATCTCGAGATGGCTAGCAAAGGAG-3') and LIN14 (5'-GATCATGGCATGCACACCCGTCCTGTG-3'). The PCR product was digested with XhoI and SphI (introduced in the primers, underlined above), and ligated into pDL8 that had been digested with XhoI and SphI to produce plasmid pDL50B.

Strains

Wild type *B. subtilis* strains were JH642 {*trp, phe*} (Perego et al. 1988) and PY79 {prototroph} (Youngman et al. 1984). The *spoIIIE* mutant was RL1259 {*spoIIIE36 amyE*::(*sspE*(2G)-*lacZ tet*)}. The *spoIIIE36* mutation (Wu, et al. 1995) and *sspE*(2G)-*lacZ* fusion (Sun et al. 1991) have been described. The *spo0J-gfp* fusion was introduced into *B. subtilis* strain AG1468 {*Δspo0J*::*spc*} (Ireton et al. 1994) by

single crossover at *spo0J*, selecting for chloramphenicol-resistance, to give strain DCL233. BL21 lambda DE3 (Novagen) was the *E. coli* strain used to overexpress Spo0J-(his)$_6$.

Antibodies.

Antibodies against Spo0J-(his)$_6$ were raised in rabbits (BabCo) and antibodies against FtsZ were raised in chickens (Immuno-Dynamics). Antibodies were affinity-purified essentially as described (Pringle et al. 1991). We estimate that there are ~200-400 molecules of Spo0J per cell during growth in minimal medium, and ~500-1,000 molecules per cell during sporulation, based on immuno-blot analysis and comparison to known amounts of purified Spo0J protein (DL & ADG, unpublished results).

Immunofluorescence microscopy.

Cells were grown at 37°C in S7$_{50}$ minimal medium (Jaacks et al. 1989) with 0.1% glucose, 0.1% glutamate, and required amino acids (40 µg/ml), and samples were taken during exponential growth. Cells were induced to sporulate by the resuspension method (Nicholson and Setlow 1990; Sterlini and Mandelstam 1969). Samples were taken at 0, 90, and 180 min. after the onset of sporulation (the time of resuspension).

Cells were prepared for immunofluorescence microsocopy essentially as described (Harry et al. 1995; Pogliano et al. 1995). Secondary antibodies coupled to fluorophores FITC or Cy3 (Jackson ImmunoResearch) were used as indicated.

Two different microscopes were used and the fluorescence of the Cy3 fluorophore appeared orange with one (Figure 2-1B, F, Q) and red with the other (Figure 2-1M, O). A *spo0J* null mutant had no detectable immuno-staining with the anti-Spo0J antibodies (data not shown).

Visualization of Spo0J-GFP

Cells containing the *spo0J-gfp* fusion (DCL233) were grown to confluence on an LB agar plate, scraped from the plate, resuspended in minimal salts (Spizizen 1958), and adhered to a poly-L-lysine coated slide. DNA was visualized by staining with 7-amino-actinomycin D (7-AAD; Molecular Probes, Eugene, OR). GFP was visualized essentially as described (Webb et al. 1995).

Results

Localization of Spo0J during vegetative growth

To identify the sub-cellular location of Spo0J, we performed immunofluorescence microscopy using antibodies against Spo0J. During vegetative growth in defined minimal medium (doubling time ~45 min.), Spo0J was associated with the nucleoid. Figure 2-1A-J shows the staining pattern from two different fields of cells (A - D, and I representing one field and E - H and J the other). Nucleoids were visualized with DAPI and appear as bright blue bodies, often in chains as *B. subtilis* tends to grow as septated filaments of cells (Figure 2-1A, E). The majority of nucleoid bodies (≥75%) had discrete immuno-staining of Spo0J, with at least one defined site of Spo0J staining per nucleoid (Figure 2-1A, B, I; E, F, J). Many of the nucleoids had two defined sites of Spo0J staining, usually with each site on opposite ends of the nucleoid and towards the cell poles (Figure 2-1A, B, I; E, F, J).

To determine the location of Spo0J at different times during the cell cycle and relative to the mid-cell, we compared the localization of Spo0J to that of the cell division protein FtsZ. FtsZ forms a ring structure at the mid-cell, with a nucleoid on each side, and marks the site of septation (Addinall et al. 1996; Bi and Lutkenhaus 1991; Levin and Losick 1996; Wang and Lutkenhaus 1993). In cells without an FtsZ ring, the nucleoid is usually in the middle of the cell. As the cell grows and the chromosome replicates, the nucleoid begins to separate into two discrete chromosomal bodies and the FtsZ ring forms at mid-cell. The

FtsZ ring contracts and disappears during septation, then reforms prior to the next division (reviewed in (Erickson 1995; Lutkenhaus 1993)).

Several different patterns of staining were observed when comparing the localization of Spo0J (Figure 2-1B, F), FtsZ (Figure 2-1C, G), and the nucleoids (Figure 2-1A, I; E, J). A total of 421 cells that had discrete sites of Spo0J staining were counted and divided into four classes, based on the presence or absence of the FtsZ ring, and the number of sites of Spo0J staining per nucleoid (Figure 2-2A). Classes 1 and 2 consist of cells with no FtsZ ring and a single nucleoid body with either a single site (class 1) or two sites (class 2) of Spo0J staining. Classes 3 and 4 consist of cells with an FtsZ ring and two nucleoid bodies, one on each side of the FtsZ ring and a single site (class 3) or two sites (class 4) of Spo0J staining per nucleoid. The four classes are described in more detail below.

A small proportion of cells (4%) had a single nucleoid with one site of Spo0J staining and no FtsZ ring (Figure 2-2A, class 1). (There are no examples of these in Figure 2-1A-J). These are probably cells that have recently divided (no FtsZ ring), but that have not replicated enough of the chromosome to duplicate the Spo0J binding site.

Of the 165 cells with no FtsZ ring (18 class 1 + 147 class 2), the majority (87%) had two distinct sites of Spo0J staining on a single nucleoid (Figure 2-2A, class 2; Figure 2-1A-D, I). In these cells, the sites of Spo0J localization were near the ends of the nucleoid, toward the cell poles. These are probably cells that have replicated the Spo0J binding region and have recently divided (no FtsZ ring). The two distinct sites of Spo0J localization at the ends of the nucleoid

indicate that chromosome orientation has already been established and that the newly replicated regions have been separated. Furthermore, the Spo0J binding region is likely to be in the part of the chromosome that replicates early (the origin region).

(The formal possibility exists that there are two Spo0J binding regions per chromosome. This seems highly unlikely based on the relatively large proportion of cells (see below) that have distinct nucleoids on each side of the FtsZ ring with a single site of Spo0J localization per nucleoid. In addition, if there were two distinct binding regions per chromosome, we would expect to see a significant number of nucleoids with three sites of Spo0J staining. This has not been observed.)

Approximately 60% of the cells had an FtsZ ring at mid-cell. (In contrast, >90% of cells growing in rich medium, with a rapid doubling time, have an FtsZ ring (Addinall, et al. 1996; Levin and Losick 1996). The cells that had FtsZ rings and discrete sites of Spo0J staining fell into two classes (Figure 2-2A, classes 3, 4), one class with a single site (class 3) and the other with two sites (class 4) of Spo0J staining per nucleoid. Figure 2-1D & H shows an overlay of the Spo0J (Figure 2-1B, F) and FtsZ (Figure 2-1C, G) staining and a sketch of several of the cells next to the image. Figure 2-1I & J shows an overlay of the DNA (A, E) and Spo0J (B, F) staining. Nucleoids with single sites and those with two sites of Spo0J staining are clearly visible and are illustrative of the way in which other cells were classified.

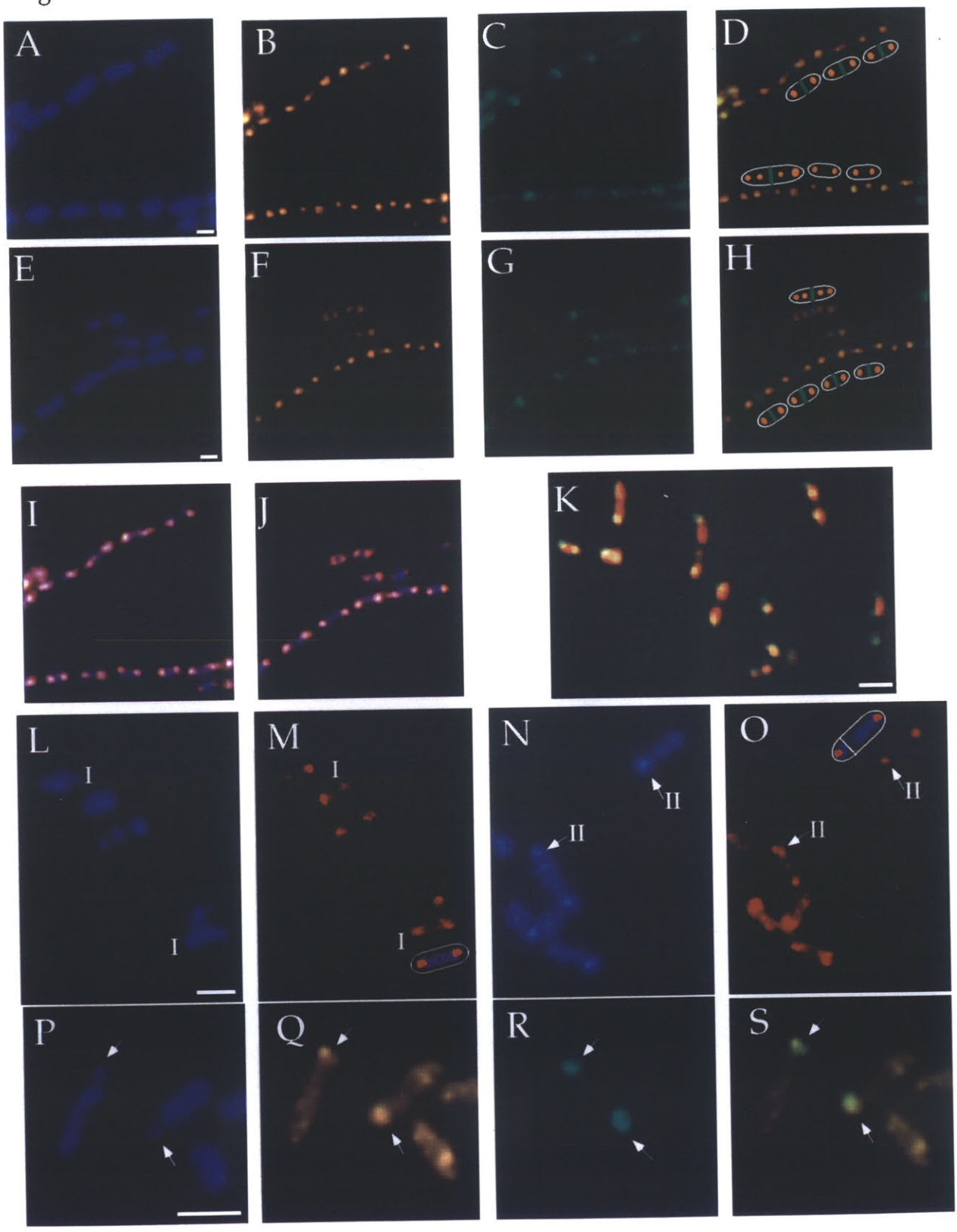
Of the 256 cells with an FtsZ ring (139 class 3 + 117 class 4), 54% had a single site of Spo0J per nucleoid (Figure 2-2A, class 3) while 46% had two sites of Spo0J per nucleoid (Figure 2-2A, class 4). In the cells with a single site of Spo0J staining per nucleoid, Spo0J often appeared oriented toward the cell pole, away from the FtsZ ring at mid-cell.

A significant fraction (46%) of the cells with an FtsZ ring had two sites of Spo0J staining per nucleoid (Figure 2-2A, class 4; Figure 2-1A-J). Each nucleoid had a site of Spo0J localization toward the cell pole and a second site of Spo0J localization closer to the FtsZ ring at midcell. We infer that these chromosomes have already begun a new round of DNA replication and that the Spo0J binding region has been duplicated, indicating again that the Spo0J binding region is probably near the origin of replication. In addition, since the FtsZ ring is still present and Spo0J is localized in a bipolar manner on each nucleoid, before cell division, the defined orientation and polarity of the chromosome is established for cell division in the next generation, before the current division has been completed. The machinery that separates the replicating chromosomes and establishes this polarity must be assembled and functional in this predivisional cell. After division, the young cells do not have an FtsZ ring and most have two sites of Spo0J (Figure 2-2A, class 2) localized in the bipolar manner that was established before division.

Figure 2-1. Localization of Spo0J during growth and sporulation. The bar in the lower corner of the first panel of each set represents ~1 μm.

A-J. Growth in minimal medium. Panels A-D, & I represent one field of cells, and panels E-H, & J a different field of cells.
A, E. DAPI staining to visualize nucleoids (blue).
B, F. Immuno-staining of Spo0J with affinity-purified rabbit antibodies and secondary antibodies coupled to the fluorophore Cy-3 (orange).
C, G. Immuno-staining of FtsZ with affinity-purified chicken antibodies and secondary antibodies coupled to the fluorophore FITC (green).
D. Overlay of exposures of Spo0J (B) and FtsZ (C). Cartoons of six cells are indicated. The three cells at the top each have an FtsZ ring with a single site of Spo0J staining per nucleoid (class 3 of Figure 2-2). Of the three cells at the bottom, the large cell to the left has an FtsZ ring with a nucleoid on each side and two sites of Spo0J per nucleoid (class 4 of Figure 2-2). The other two cells have no FtsZ ring and a single nucleoid with two sites of Spo0J (class 2 of Figure 2).
H. Overlay of exposures of Spo0J (F) and FtsZ (G). Cartoons of five cells are indicated; all have FtsZ rings. The large cell at the top has two sites of Spo0J per nucleoid (class 4 of Figure 2-2), while all of the cells at the bottom have one site of Spo0J per nucleoid (class 3 of Figure 2-2).
I. Overlay of exposures of DAPI (A) and Spo0J (B).
J. Overlay of exposures of DAPI (D) and Spo0J (E).
K. Spo0J-GFP localizes to the nucleoids in a bipolar manner. Living cells were stained with 7-AAD to visualize the DNA (red). Endogenous fluorescence from Spo0J-GFP appears yellow when it completely overlaps with the red from the DNA.
L-O. Localization of Spo0J during early stages of sporulation in wild type cells. Cells were stained with DAPI (blue) to visualize DNA (L, N), and immuno-stained (red) to visualize Spo0J (M, O).
L, M. Three cells are at stage I, indicated by "I", and a sketch of one of these is shown in panel M.
N, O. The arrows point to the condensed forespore nucleoids from two different stage II sporangia (II). A sketch is shown of one of these at the top of panel O.
P-S. Spo0J co-localizes with the origin-proximal 30% of the chromosome in the forespore of a *spoIIIE* mutant. Two sporangia of the *spoIIIE* mutant stained with DAPI (blue) to visualize DNA (P), and immuno-stained for Spo0J (orange) (Q), and ß-galactosidase (green) (R). ß-galactosidase is produced from the *sspE(2G)-lacZ* fusion that is expressed only in the forespore. The fusion is in the origin-proximal part of the chromosome that gets trapped in the forespore in the *spoIIIE* mutant. Panel S shows an overlay of the Spo0J and ß-galactosidase staining. Arrows indicate the forespore. Note that in contrast to wild type sporangia (N) that have highly condensed forespore nucleoids, the *spoIIIE* mutant forespore (P) has much less staining, reflecting the absence of a complete chromosome.

Figure 2-1



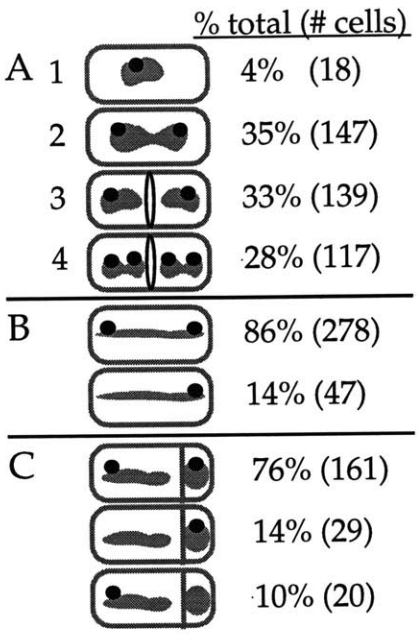


Figure 2-2. Analysis of Spo0J localization. Shaded blobs represent the nucleoids and black balls indicate Spo0J on the nucleoid.

(A). Localization of Spo0J during vegetative growth (as in Figure 2-1A-J). In several different experiments, 75-90% of the cells had discrete sites of Spo0J staining and ~60% of all cells had visible, well-defined FtsZ rings. The proportion of cells with discrete sites of Spo0J staining was similar in cells with and without FtsZ rings indicating that the appearance of sites of Spo0J staining does not correlate with the presence or absence of FtsZ rings.

Classes 1 and 2; cells with no FtsZ ring and a single nucleoid body with either a single site (class 1) or two sites (class 2) of Spo0J staining. Classes 3 and 4; cells with an FtsZ ring (indicated by the oval at mid-cell) and two nucleoid bodies, one on each side of the FtsZ ring and a single site (class 3) or two sites (class 4) of Spo0J staining per nucleoid. Of the 117 cells with an FtsZ ring and two sites of Spo0J per nucleoid, 7 cells actually had two sites on one nucleoid and only one site on the other nucleoid. We infer that in a small fraction of cells, reinitiation of replication or subsequent elongation did not occur synchronously on each chromosome. Alternatively, there were two Spo0J sites, but we could not resolve them. If these cells divide before the appearance of the second site of Spo0J staining, they would produce a few cells with a single nucleoid with a single site of Spo0J staining (class 1).

For simplicity, cells in all four classes are drawn the same length. In fact, there is a rough correlation with the indicated class and cell length, with class 4 cells the longest and class 1 & 2 cells the shortest (see Figure 2-1).

(B). Localization of Spo0J during stage I of sporulation.

(C). Localization of Spo0J during stage II of sporulation.

Visualization of Spo0J-GFP

To visualize the subcellular localization of Spo0J in living cells, in the absence of fixation, we constructed a Spo0J-GFP fusion and determined the localization based on endogenous fluorescence of GFP. The *spo0J-gfp* fusion produced a functional gene product as judged by the ability to complement a *spo0J* null mutation (data not shown). Spo0J-GFP was associated with the nucleoid and most cells had Spo0J clearly localized toward the poles of the nucleoid (Figure 2-1K). These results are consistent with those from immunofluorescence and indicate that the bipolar localization of Spo0J was not caused by potential artifacts from fixation, permeablization, or any other immuno-staining procedures.

Localization of Spo0J during sporulation

Because Spo0J is involved in chromosome partitioning during asymmetric division in sporulating cells (Sharpe and Errington 1996) we determined the sub-cellular location of Spo0J during the early stages of sporulation. Cells entering stage I of sporulation have an axial filament, a single nucleoid body (containing at least two chromosomes) that stretches the length of the predivisional cell. Figure 2-1L shows the DAPI staining of several cells, three of which are at stage I of sporulation (I). Figure 2-1M is the same field of cells, but with Spo0J staining shown, and a sketch of one of the sporangia below the image. During sporulation, over 90% of the cells that had an axial filament had discrete staining of Spo0J. In 86% of these cells, Spo0J was associated with the both poles of the

axial filament (as seen in Figure 2-1L, M; Figure 2-2B), while in the remaining 14%, Spo0J was localized to one end of the axial filament (Figure 2-2B).

At the beginning of stage II of sporulation, a polar septum is formed creating two cells of unequal size; the larger mother cell and the smaller forespore. The stage II sporangia (mother cell plus forespore) are distinguishable by DAPI staining as the forespore nucleoid is highly condensed and brightly staining while the mother cell nucleoid is more diffuse. Figure 2-1N shows the DAPI staining of several stage II sporangia, with arrows pointing to the condensed forespore nucleoid in two of these. Over 90% of the sporangia with a condensed forespore nucleoid (examples in Figure 2-1N, O) had discrete immuno-staining of Spo0J. In 76% of these, Spo0J was still associated with the nucleoid in a bipolar manner (Figure 2-1N, O; Figure 2C). That is, both the mother cell and forespore nucleoids had Spo0J staining, and in the mother cell, this staining was toward the cell pole away from the forespore. In the much smaller forespore, it is difficult to determine if Spo0J staining is to a localized part of the highly condensed nucleoid. At later times during sporulation, the chromosomes have already segregated and fewer sporangia had discrete localization of Spo0J (data not shown).

Spo0J binds to the region of the chromosome around the origin of replication

When the polar septum forms at stage II to generate the two cell types, it bisects one end of the axial filament, trapping ~30% of one chromosome in the

smaller forespore and leaving ~70% of the forespore chromosome in the larger mother cell. Mutations in *spoIIIE* prevent the translocation of the chromosome into the forespore, resulting in a forespore that contains only the origin-proximal 30% of the chromosome (Wu and Errington 1994; Wu et al. 1995).

We found that Spo0J was localized to the 30% of the chromosome that gets trapped in the forespore in a *spoIIIE* mutant. Figure 2-1P-S shows two sporangia from the *spoIIIE* mutant. The arrows point to the two forespore cells, each with a bit of the nucleoid (Figure 2-1P). We visualized the forespore by immuno-staining ß-galactosidase (Figure 2-1R, green) that was produced from a gene fusion (*sspE(2G)-lacZ*) that is in the origin-proximal 30% of the chromosome and that is expressed only in the forespore. In 53 sporangia examined that had ß-galactosidase staining in the forespore, 48 sporangia had Spo0J staining (Figure 2-1Q, S) that co-localized with ß-galactosidase staining (Figure 2-1R, S). This indicates that Spo0J binds to a region of the origin-proximal 30% of the chromosome that is trapped in the forespore in the *spoIIIE* mutant, consistent with our results from growing cells that also indicated the Spo0J binding region is in the origin-proximal part of the chromosome.

Discussion

We favor a model in which Spo0J is a direct participant in chromosome partitioning, during both symmetric division in vegetatively growing cells and asymmetric division in sporulating cells. Evidence supporting this model includes the bipolar localization of Spo0J on the nucleoids, the chromosome partition defect during growth (Ireton et al. 1994) and sporulation (Sharpe and Errington 1996) caused by *spo0J* null mutations, and the homology of Spo0J to partition proteins of the ParB family (Ogasawara and Yoshikawa 1992). We propose that Spo0J assembles on a centromere-like site, probably in the origin region, and that this assembly interacts with as yet unidentified cellular machinery that drives the partitioning of the nucleoids to the daughter cells. While a specific binding site for Spo0J has not yet been identified, *in vitro* experiments indicate that Spo0J is a DNA binding protein with a high degree of cooperativity (DL & AG, unpublished results). We suspect that *in vivo*, Spo0J binding to specific sites helps to nucleate assembly of a large complex that contains many molecules of Spo0J.

The machinery that orients Spo0J in a bipolar manner on the nucleoid is established in the predivisional cell. Based on our results, it is clear that replicating chromosomes are in a defined orientation in the cell, with the Spo0J binding region of each chromosome oriented toward the cell poles during most of the cell cycle. Similar results have been obtained independently by Errington and co-workers (J. Errington, personal communication).

Webb et al. (Webb et al. 1997) used a different approach to visualize the orientation of the chromosome. The *oriC* region was visualized using a LacI-GFP fusion bound to an array of *lac* operators that had been integrated into the chromosome near *oriC*. Fluorescence from LacI-GFP was observed toward the cell poles (Webb et al. 1997), in a manner similar to the localization of Spo0J (Figure 2-1). In contrast, in cells with the *lac* operator array near the terminus of replication, the fluorescence was observed closer to mid-cell (Webb, et al. 1997). The results with LacI-GFP indicate that both the origin and terminus of replication can be oriented in a specific manner, at least during part of the *B. subtilis* cell cycle.

We envision the following sequence of events during the bacterial cell cycle (Figure 2-3), keeping in mind that in rapidly growing cells (doubling time ≤60 min.) there is more than one replication fork per chromosome to compensate for the fact that chromosomal replication takes longer than cell division. 1) Immediately after cell division, there is a single, partly replicated nucleoid located at approximately midcell. Most of these cells have two sites of Spo0J localization, one at each end of the nucleoid, oriented toward a cell pole. 2) During cell growth and continued DNA replication, the nucleoid begins to separate into two distinct bodies that move in opposite directions toward the cell poles. During this time, the FtsZ ring forms at midcell and marks the site of the next division event (Bi and Lutkenhaus 1991; Erickson 1995; Lutkenhaus 1993). As the nucleoids become separated, localization of Spo0J at the end of each nucleoid toward the cell poles and away from the FtsZ ring becomes even

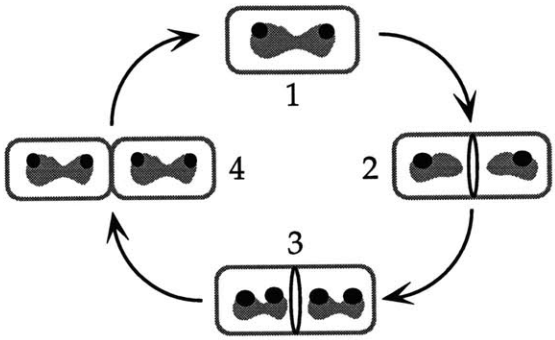


Figure 2-3. Model for chromosome segregation and Spo0J localization during the cell cycle.

1. Immediately after cell division, there is no FtsZ ring and a partly replicated chromosome with two discrete sites of Spo0J staining oriented toward the cell poles. 2. As replication and cell growth continue, the FtsZ ring forms and the chromosomes continue to separate. Spo0J localization becomes markedly polar, with one site on each nucleoid oriented toward the cell pole and away from the FtsZ ring at midcell. 3. DNA replication reinitiates, most of the time on both chromosomes, and progresses far enough so that the Spo0J binding region is replicated. This is visualized as two discrete sites of Spo0J staining on each of the two nucleoids. One site on each nucleoid is toward a cell pole while the other site is closer to the FtsZ ring at midcell. 4. The FtsZ ring contracts and disassembles during septation and division, generating two cells with partly replicated chromosomes and two distinct sites of Spo0J staining.

more pronounced. 3) Before cell division, another round of DNA replication initiates in most cells and proceeds far enough such that the Spo0J binding region is replicated. This appears in our experiments as a nucleoid with two distinct sites of Spo0J, one toward the cell pole and the other closer to the FtsZ ring at midcell. This observation indicates that the newly replicated origin-proximal regions (including the Spo0J binding regions) are separated before the cell divides. 4) Finally, cell division occurs, and the FtsZ ring contracts and disassembles. In a small percentage of the cells (Figure 2-2A, class 1), division occurs before replication has reinitiated, or at least before a second Spo0J binding region is visible.

Spo0J is an important component contributing to the fidelity of chromosome partitioning. The frequency of anucleate cells caused by a *spo0J* null mutation is ~100-fold higher than that of wild type cells (Ireton, et al. 1994). Spo0J (ParB) homologues have been found, based on chromosomal DNA sequence, in several other organisms, including *P. putida* (Ogasawara and Yoshikawa 1992), *C. burnetii*, and *M. leprae*. The Spo0J homologue of *C. crescentus* is involved in chromosome partitioning (J. Gober, personal communication), and it seems likely that the ParB/Spo0J proteins from other organisms will be found to have similar roles in partitioning. These organisms do not undergo sporulation, indicating that Spo0J first evolved to help ensure the proper transmission of chromosomes (or plasmids) to dividing cells.

We suspect that during the course of evolution, *B. subtilis* adapted part of the chromosome segregation machinery (Spo0J) to create a checkpoint that

couples the initiation of sporulation to cell cycle events. While Spo0J plays a functional role in chromosome segregation during vegetative growth and sporulation, it also plays a regulatory role during the initiation of sporulation. Spo0J acts to inhibit the function of the regulatory protein encoded by *soj*. Soj is a negative regulator of sporulation, functioning to inhibit or antagonize the function of the transcription factor Spo0A (Ireton, et al. 1994). The Spo0A transcription factor activates many of the early sporulation genes and is required for formation of the axial filament and to activate the switch from symmetric to asymmetric division (Grossman 1995; Hoch 1993; Levin and Losick 1996). We suspect that at a particular step in the partitioning pathway, Spo0J acts to antagonize Soj, signaling that the chromosome segregation process is functioning normally.

The components of the bacterial chromosome segregation machinery have remained elusive. However, Spo0J of *B. subtilis* should now provide access to these components, including a centromere-like site, factors that assemble on this site, and the machinery that drives segregation of the chromosomes to dividing cells. It will be interesting to use Spo0J to identify these components and to determine how chromosomes are accurately segregated, how cell polarity is used to generate chromosomal orientation, how the various cell cycle processes are regulated and coordinated, and how they have been adapted to regulate developmental processes.

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Chapter 3

Identification and Characterization of a Bacterial Chromosome Partitioning Site

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Abstract

We have identified a DNA site involved in chromosome partitioning in *Bacillus subtilis*. This site was identified *in vivo* as the binding site for the chromosome partitioning protein Spo0J, a member of the ParB family of partitioning proteins. Spo0J is a site-specific DNA binding protein that recognizes a 16 bp sequence found in *spo0J*. Allowing two mismatches, this sequence occurs 10 times in the entire *B. subtilis* chromosome, all in the origin-proximal ~20%. Eight of the 10 sequences are bound to Spo0J *in vivo*. The presence of a site on an otherwise unstable plasmid stabilized the plasmid in a Spo0J-dependent manner, demonstrating that this site, called *parS*, can function as a partitioning site. This site and Spo0J are conserved in a wide range of bacterial species.

Introduction

Efficient chromosome partitioning ensures the stable inheritance of genetic material to progeny cells. In eukaryotes, the spindle pole body, the bipolar mitotic spindle, motor proteins, the centromere, kinetochore and cohesion proteins are parts of the mitotic apparatus that function in concert to ensure faithful segregation of chromosomes. In contrast to the situation in eukaryotes, the underlying components and mechanisms governing chromosome partitioning in prokaryotes have not been well defined {reviewed in (Hiraga, 1992; Wake and Errington, 1995)}. Whereas several bacterial genes have been identified that are involved in chromosome partitioning, *cis*-acting DNA sequences have yet to be defined. By determining the binding site for the chromosome partition protein Spo0J, we have identified a DNA sequence in the *B. subtilis* chromosome that functions as a partitioning, or centromere-like, site.

The *B. subtilis spo0J* gene product is required for efficient chromosome partitioning during vegetative growth and sporulation. Approximately 1.5% of the cells in a growing culture of a *spo0J* mutant are anucleate, a frequency ~100-fold greater than that of wild type cells (Ireton et al., 1994). Spo0J and Soj (encoded by the gene upstream from and cotranscribed with *spo0J*) are similar to the ParB (SopB) and ParA (SopA) family of plasmid-encoded partition proteins, respectively (Ogasawara and Yoshikawa, 1992). Chromosomally encoded homologues of ParB have been found in a wide range of bacterial species, including *Pseudomonas putida, Caulobacter crescentus, Streptomyces coelicolor,*

Streptococcus pneumoniae, *Mycobacterium leprae*, *Helicobacter pylori*, and *Streptococcus pyogenes*. *C. crescentus* ParB is involved in chromosome partitioning (Mohl and Gober, 1997) and it is likely that the basic mechanism by which ParB homologues function in partitioning is conserved.

Most of what is known about the biochemical function of the ParB family of proteins comes from work with ParB from the P1 prophage and SopB from the F plasmid of *E. coli* {reviewed in (Nordström and Austin, 1989; Austin and Nordström, 1990; Hiraga, 1992; Williams and Thomas, 1992)}. ParB (SopB) binds to a centromere-like sequence, *parS* (*sopC*), located immediately downstream of the *parB* gene. *parA* (*sopA*), immediately upstream of *parB* (*sopB*), encodes an ATPase that interacts with ParB. All three components, ParA, ParB, and *parS*, are required for plasmid partitioning. One predominant model for plasmid partitioning proposes a pairing function for ParB proteins (Nordström and Austin, 1989; Austin and Nordström, 1990; Williams and Thomas, 1992). It is thought that plasmids are paired via interaction between ParB-*parS* complexes from two plasmids. Concurrent with or subsequent to pairing, positioning occurs such that each daughter cell receives a plasmid. Recent experiments have shown that the Par system is required for proper subcellular localization (positioning) of the plasmid. During the course of the *E. coli* cell cycle, the P1 and F plasmids move from midcell to the 1/4 and 3/4 positions along the length of the cell (Gordon et al., 1997; Niki and Hiraga, 1997). Loss of *sopABC* results in improper positioning of F plasmids (Niki and Hiraga, 1997).

Recent work has indicated that the origin of replication (*oriC*) of the *B. subtilis* (and *E. coli*) chromosome is in a defined orientation for most of the bacterial cell cycle (Glaser et al., 1997; Gordon et al., 1997; Lin et al., 1997; Webb et al., 1997). In new-born cells, the origin region is positioned near the pole of the nucleoid body, oriented toward a cell pole. After replication of this region, one of the two origins rapidly moves towards the opposite pole of the nucleoid. This movement indicates the function of a mitotic-like apparatus for separating sister origin regions (Gordon et al., 1997; Webb et al., 1997).

Studies with *C. crescentus* and *B. subtilis* have shown that the chromosomally-encoded ParB/Spo0J proteins are needed for proper chromosome partitioning (Ireton et al., 1994; Glaser et al., 1997; Lewis and Errington, 1997; Lin et al., 1997; Mohl and Gober, 1997). The existence of a partitioning site(s) bound by Spo0J has been inferred from the similarity to the family of plasmid ParB proteins and the subcellular localization of Spo0J (Glaser et al., 1997; Lewis and Errington, 1997; Lin et al., 1997; Mohl and Gober, 1997). The subcellular localization of Spo0J is similar to that of the origin region and Spo0J appears to co-localize with the origin-proximal 30% of the *B. subtilis* chromosome (Glaser et al., 1997; Lewis and Errington, 1997; Lin et al., 1997). These localization experiments led to the idea that Spo0J associates with a site(s) in the origin-proximal region of the chromosome that functions in chromosome partitioning.

In this paper, we describe the identification and characterization of the binding site for Spo0J. This site, named *parS*, is a 16 bp sequence containing an

imperfect 8 bp inverted repeat and is found in the *spo0J* gene. Cloning a single *parS* into an otherwise unstable plasmid stabilizes the plasmid in a Spo0J-dependent manner. A search of the recently completed *B. subtilis* genome (Kunst et al., 1997), allowing for 2 mismatches from the site in *spo0J*, revealed the existence of ten potential binding sites which are all located in the origin proximal ~20% of the chromosome. Eight of these sites are bound to Spo0J *in vivo*. We propose that the binding of Spo0J to these multiple *par* sites is involved in pairing newly replicated origin regions before the rapid separation mediated by a mitotic-like apparatus.

Results

Identification of the Spo0J binding site *in vivo*

By analogy to the plasmid partition systems, we suspected that the Spo0J binding site would be near the *spo0J* gene, approximately 10 kb from the *B. subtilis* origin of replication. To identify DNA associated with Spo0J *in vivo*, we used a chromatin immunoprecipitation assay (Solomon and Varshavsky, 1985; Hecht et al., 1996; Strahl-Bolsinger et al., 1997) (see Experimental procedures). Briefly, formaldehyde was added to cells during exponential growth to crosslink protein and DNA, cells were lysed and the DNA sheared to an average size of approximately 500 to 1,000 bp. The Spo0J-DNA complexes were then immunoprecipitated using affinity-purified polyclonal antibodies against Spo0J, the crosslinks reversed, and the precipitated DNA analyzed by PCR.

Four sets of primers were used in the PCR assay to test for the presence of different chromosomal regions in the immunoprecipitate. Each primer set specifically amplified a different sized fragment, ranging from ~200 to ~620 bp. DNA from the *spo0J* region was specifically immunoprecipitated, while little or no DNA was detected from three other chromosomal regions (Figure 3-1). Furthermore, in parallel experiments, no DNA was detected from a *spo0J* null mutant (Figure 3-1, lane 3). These results indicate that Spo0J, or protein closely associated with Spo0J, binds to a site(s) in or near the *spo0J* gene.

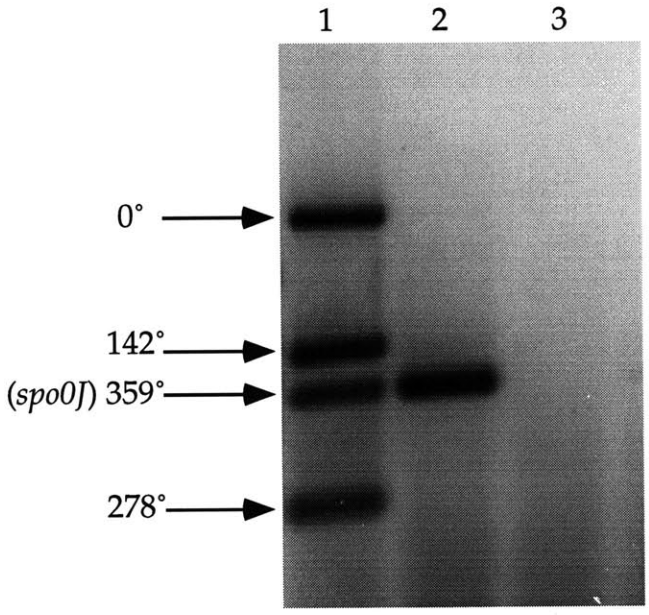


Figure 3-1. *In vivo* association of Spo0J with DNA from near the *spo0J* gene.

Affinity-purified antibody was used to immunoprecipitate Spo0J from cell extracts after formaldehyde crosslinking *in vivo* (see Experimental procedures). After reversal of the crosslinks, DNA in the immunoprecipitate was amplified by PCR using four sets of primer pairs from four different regions of the chromosome: 0° (~620 bp), 142° (~380 bp), 278° (~200 bp), and 359° (~330 bp, in *spo0J*). (The ~4,200 kbp *B. subtilis* chromosome is 360° and the origin of replication is at 0°/360°. One degree is ~11.7 kbp). The PCR products were separated on an agarose gel. The four primer pairs were used together in PCR with total chromosomal DNA (lane 1); DNA from the immunoprecipitate from wild type cells (lane 2); and DNA from the immunoprecipitate from a *spo0J* null mutant (lane 3). The chromosomal location of the PCR products are indicated to the left.

The Spo0J binding site was defined more precisely by cloning DNA fragments into a multicopy plasmid and testing *in vivo* for binding to Spo0J using the same approach. Plasmid pIK219 contains an ~760 bp restriction fragment that includes the 3' end of *spo0J* and extends ~540 bp downstream (Figure 3-2A). Spo0J was able to bind to this plasmid *in vivo* (Figure 3-2A), but not to the parent vector (Figure 3-2C), indicating that the insert contains a Spo0J binding site(s). Subclones of pIK219 were constructed to further define the binding site (Figure 3-2A). A 55 bp fragment, contained in pDL90A, was sufficient to confer binding to Spo0J (Figure 3-2). This 55 bp fragment is internal to *spo0J*, indicating that the Spo0J binding site was located in *spo0J*.

Further analysis defined a 16 bp sequence in pDL90A, composed of an imperfect 8 bp inverted repeat, that is able to bind Spo0J *in vivo*. Several derivatives of pDL90A were constructed and tested for binding to Spo0J (Figure 3-2B, C). Deletion of the inverted repeat (pDL105) or base changes in 7 positions of the inverted repeat (pDL106) greatly reduced or eliminated the ability of Spo0J protein to crosslink to the plasmid *in vivo* (Figure 3-2C). In contrast, pDL104, which contains only a 16 bp insert with the 8 bp imperfect inverted repeat could be crosslinked to Spo0J (Figure 3-2C). Thus, the 16 bp sequence, 5'-TGTTCCACGTGAAACA-3', interacts with Spo0J *in vivo*, either directly or indirectly.

Figure 3-2. *In vivo* identification of the Spo0J binding site located in the *spo0J* gene.

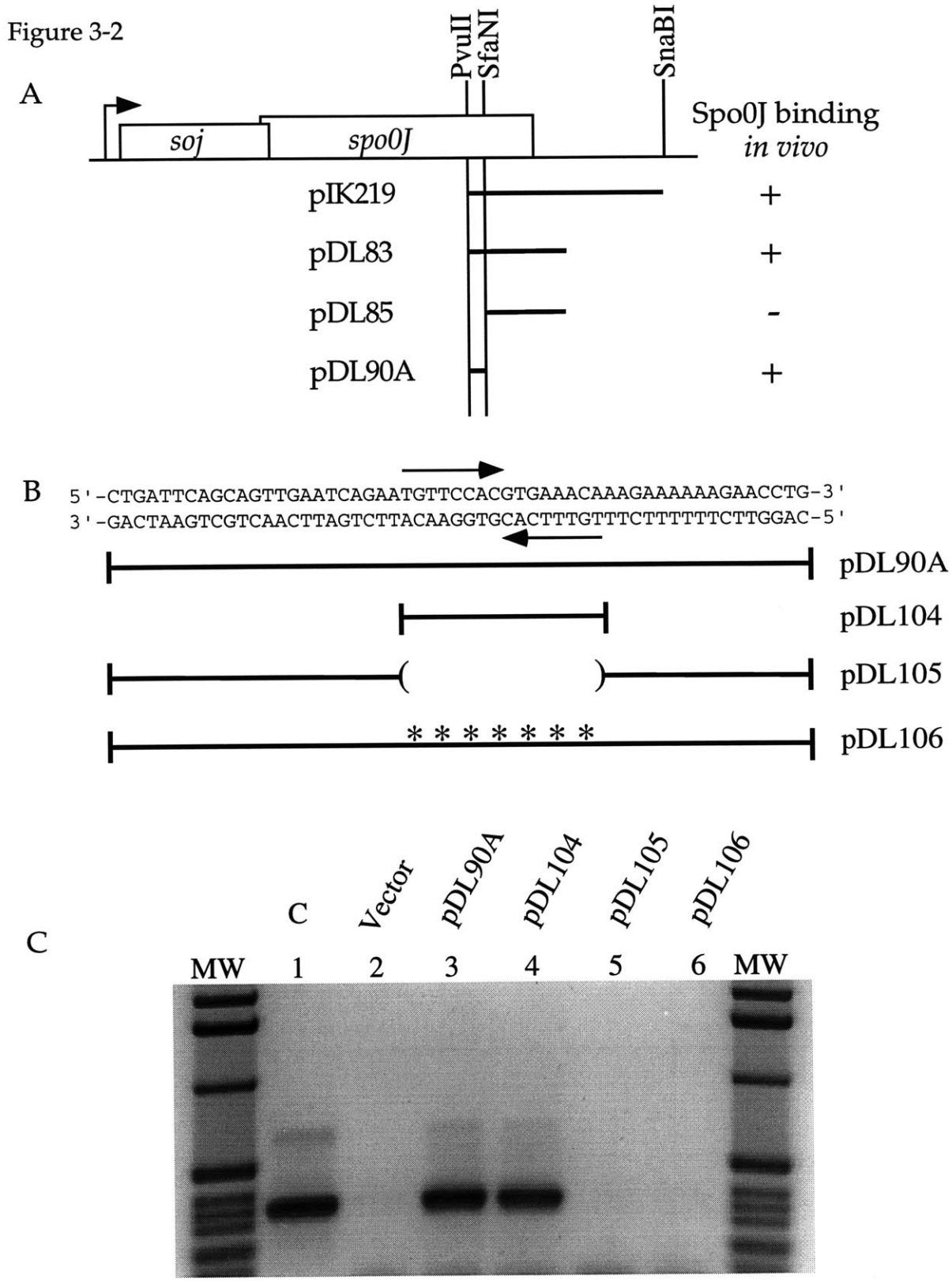
Plasmids containing different inserts from the *spo0J* region were tested *in vivo* for binding to Spo0J using formaldehyde protein-DNA crosslinking and immunoprecipitation. Immunoprecipitated DNA was analyzed by PCR using plasmid-specific primers.

(A) The *soj-spo0J* operon is drawn schematically and the inserts contained in different plasmids are indicated below. The presence (+) or absence (-) of the four different plasmids in the immunoprecipitate is indicated. The Spo0J binding site was contained in the 55 bp PvuII to SfaNI fragment in plasmid pDL90A.

(B) The 16 bp sequence containing an 8 bp imperfect inverted repeat is indicated by arrows above and below the sequence of the 55 bp insert in pDL90A. The inserts contained in plasmids pDL104, pDL105, and pDL106 are drawn schematically. pDL106 contains 7 changes from the wild type (5'-CGTGCCCAGGGAGACC-3'; underlined bases are mutant).

(C) The 16 bp sequence is the Spo0J binding site. PCR reactions with plasmid-specific primers and the indicated DNA. lane 1: control with purified vector DNA. lanes 2-6: immunoprecipitated DNA from strains containing the indicated plasmid. MW = molecular weight markers.

Figure 3-2


A
PvuII
SfaNI
SnaBI
soj
spo0J
Spo0J binding
in vivo
pIK219
+
pDL83
+
pDL85
-
pDL90A
+
B
5'-CTGATTCAGCAGTTGAATCAGAATGTTCCACGTGAAACAAAGAAAAAAGAACCTG-3'
3'-GACTAAGTCGTCAACTTAGTCTTACAAGGTGCACTTTGTTTCTTTTTTCTTGGAC-5'
pDL90A
pDL104
pDL105
pDL106
C
C
Vector
pDL90A
pDL104
pDL105
pDL106
MW
1
2
3
4
5
6
MW

Spo0J is a site specific DNA binding protein

The *in vivo* crosslinking-immunoprecipitation results did not address whether the specificity of Spo0J for the DNA site was due to Spo0J or another factor. Since formaldehyde is capable of crosslinking protein to protein and protein to DNA, the possibility remained that Spo0J was interacting with another protein, which provided the specificity of interaction with the 16 bp site. To address whether Spo0J itself binds specifically to the site, we performed gel mobility shift assays with Spo0J protein.

Purified Spo0J protein was able to bind, *in vitro*, to a DNA fragment containing the 16 bp site identified in the *in vivo* experiments. Hexa-histidine-tagged Spo0J, which functions *in vivo*, was purified (Experimental procedures) and tested for binding to a radiolabeled 24 bp DNA fragment containing the wild type site from within the *spo0J* gene (Figure 3-3A). Half-maximal DNA binding was observed at a Spo0J concentration of ~300 nM, and there was one major shifted band (Figure 3-3A, B). Use of a larger DNA fragment as a probe resulted in multiple slower-migrating bands, indicating that several molecules of Spo0J were binding per DNA fragment (data not shown). Formation of these larger shifted species appeared to be cooperative (data not shown).

The specificity of Spo0J binding to the 16 bp site was demonstrated by competition experiments with different unlabeled DNA fragments. A 24 bp fragment containing 7 changes in the 16 bp site (mutant) was not an efficient

Figure 3-3. Site-specific binding of Spo0J to DNA *in vitro*.

Gel mobility shift assays were used to measure binding of purified Spo0J-his$_6$ protein to DNA. In all cases, a radiolabeled 24 bp DNA fragment, containing the 16 bp Spo0J binding site (as determined *in vivo*) was used as a probe.

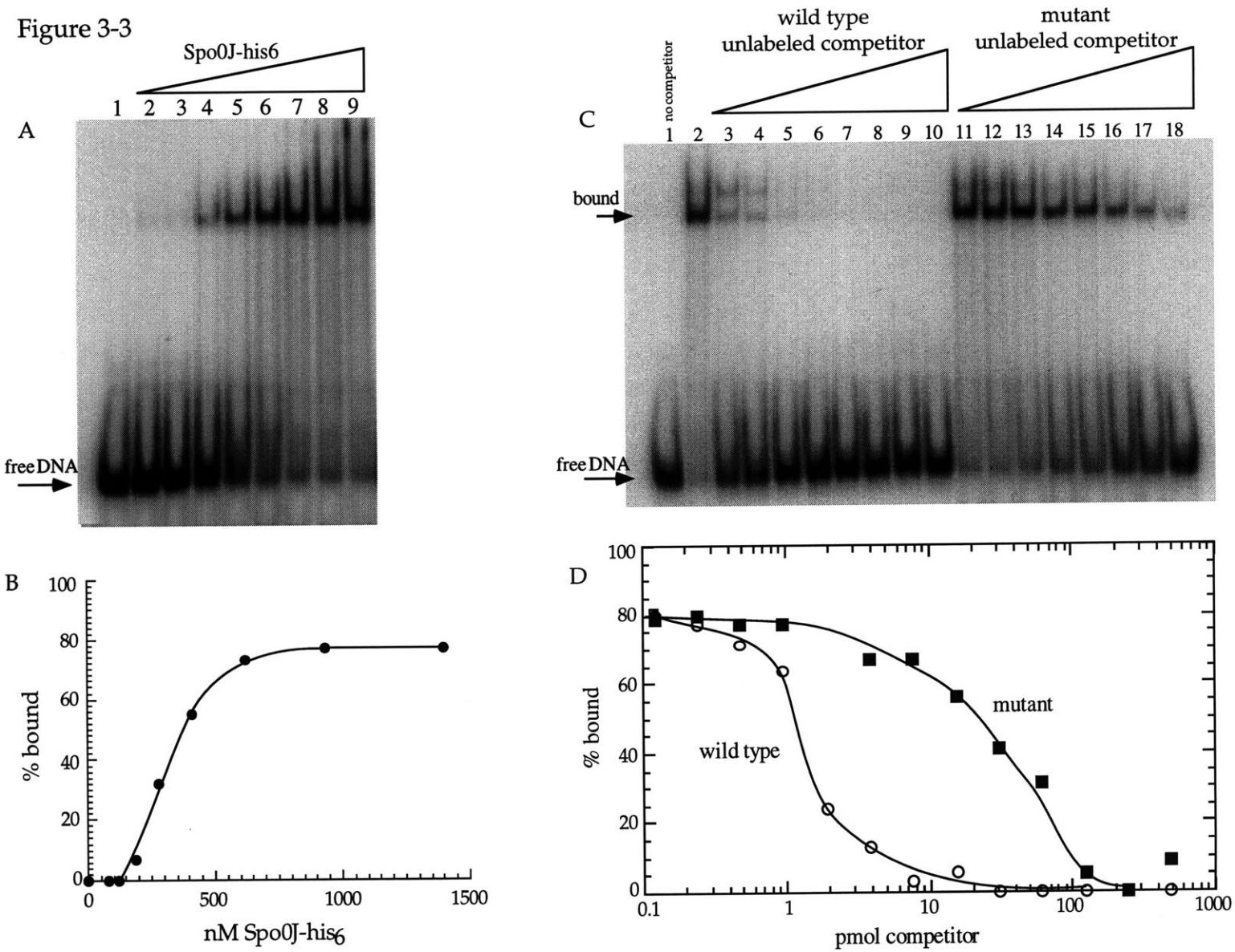
A) Gel shift assays were performed with ~1.5 fmol radiolabeled DNA mixed with various concentrations of purified Spo0J protein in a reaction volume of 15 µl: no protein (lane 1), 80 nM (lane 2), 120 nM (lane 3), 190 nM (lane 4), 280 nM (lane 5), 410 nM (lane 6), 620 nM (lane 7), 930 nM (lane 8), and 1,400 nM (lane 9).

(B) Percent of radiolabeled DNA bound (100 - % free) is plotted as a function of the concentration of Spo0J protein. At the highest protein concentrations, ~80% of the probe DNA was bound. Half-maximal binding was at a protein concentration of ~300 nM. Data are from the exeriment in panel A. Similar results were obtained in several experiments.

C) Competition experiment with mutant and wild type sites. ~1.5 fmol of the radiolabeled 24 bp DNA fragment was incubated in 15 µl reactions with either no protein (lane 1) or 710 nM Spo0J (lanes 2 - 18). Competition assays were performed with increasing amounts of unlabeled DNA fragments of 24 bp containing either the wild type (lanes 3 - 10) or mutant (lanes 11 - 18) Spo0J binding sites. The mutant contained the 7 bp changes indicated in Figure 3-2. Amounts of competitor DNA were: none (lane 2), 1.93 pmol (lanes 3, 11), 3.85 pmol (lanes 4, 12), 7.7 pmol (lanes 5, 13), 15.4 pmol (lanes 6, 14), 30.8 pmol (lanes 7, 15), 61.6 pmol (lanes 8, 16), 123 pmol (lanes 9, 17), and 246 pmol (lanes 10, 18).

(D) Percent of radiolabeled DNA bound (100 - %free) is plotted as a function of the concentration of unlabeled wild type competitor (○) or unlabeled mutant competitor (■). Data are from an experiment similar to that shown in Figure 3-3C.

Figure 3-3


Spo0J-his6
1 2 3 4 5 6 7 8 9
A
free DNA
B
% bound
nM Spo0J-his6
0 500 1000 1500
0 20 40 60 80 100
no competitor
wild type
unlabeled competitor
mutant
unlabeled competitor
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
bound
free DNA
D
% bound
pmol competitor
0.1 1 10 100 1000
mutant
wild type

competitor compared to the 24 bp fragment with the wild type site (Figure 3-3C, D). Approximately 45-fold more of the mutant fragment was needed to compete to the same extent as the wild type (Figure 3-3D). Similar results were obtained with a different competitor with completely unrelated sequence (data not shown). Taken together, these results demonstrate that Spo0J binds directly to DNA in a site specific manner.

Spo0J binds to multiple sites in the origin proximal region of the chromosome

We identified a total of ten potential Spo0J binding sites (including the one in *spo0J*) in the entire *B. subtilis* chromosome by inspection of the published genomic sequence (Kunst et al., 1997). The genome was searched with the sequence in *spo0J*, and up to two mismatches were allowed. All 10 sites are located in the origin proximal ~20% of the chromosome. Using PCR with primers specific for each region, eight of the ten potential binding sites were detected in the Spo0J-immunoprecipitate, although at varying levels (Figure 3-4A). The map location of each of these 8 sites is indicated in Figure 3-4B.

To test the relative affinity or occupancy of Spo0J binding to these sites, we compared the relative amounts of each site in the immunoprecipitate. This was accomplished by comparing the amount of PCR amplified DNA from serial dilutions of the Spo0J-immunoprecipitate to that from dilutions of the total input DNA before the immunoprecipitation reactions. Six of the sites, located at 4°,

359° (in *spo0J*), 356°, 355°, 354°, and 330°, were most abundant in the immunoprecipitate (Figure 3-4A) and therefore are designated as "strong" sites. The amount of DNA from each of these sites was approximately 5-25-fold greater than that for the sites located at 15° and 40° (Figure 3-4A). Potential sites at 31° and 347° were not detected in the Spo0J-immunoprecipitate (data not shown).

A consensus Spo0J binding sequence, 5'-TGTTNCACGTGAAACA-3', was derived from alignment of the 8 sites (Figure 3-4A). Four of the strong sites, 4°, 359° (in *spo0J*), 354°, and 330°, contained perfect matches to the consensus. Two strong sites, 356° and 355°, differ from consensus in a single position, one weak site, 15°, differs from consensus in one position, whereas the other weak site, 40°, differs in two positions (Figure 3-4A). The two potential sites that are not bound detectably *in vivo* both differ in 2 positions (Figure 3-4). Additional potential binding sites, some outside of the *oriC* region, can be found in the *B. subtilis* genome by allowing for more mismatches. We have not tested for Spo0J binding to these other sequences.

Figure 3-4. There are multiple Spo0J binding sites in the *B. subtilis* chromosome.

Ten potential sites were identified from the complete *B. subtilis* genomic sequence. Eight of these sites were associated with Spo0J *in vivo* and their sequence (A) and approximate map position are indicated (A, B). The consensus sequence derived from comparison of the 8 sites is shown. In each site, differences from consensus are underlined.

(A) Serial 5-fold dilutions of the immunoprecipitated DNA (lanes 1 - 5) or the total input DNA (lanes 6 - 11) were amplified by PCR with primers specific to sequences flanking each site. Separate PCR reactions were done for each primer pair. Dilutions of 1/5 (lane 1), 1/25 (lane 2), 1/125 (lane 3), 1/625 (lane 4), and 1/3125 (lane 5) of the immunoprecipitated DNA and dilutions of 1/5 (lane 6), 1/25 (lane 7), 1/125 (lane 8), 1/625 (lane 9), 1/3125 (lane 10) and 1/15625 (lane 11) of the total DNA before immunoprecipitation were used. Potential sites at 31° (5'-TGATCCTCGTGAAACA) and 347° (5'-TGTTCCGAGTGAAACA) differ from consensus in 2 positions (underlined) and were not detected in the immunoprecipitates (data not shown).

Figure 3-4 A.

site at:	sequence (5'→3')
4°	TGTTACACGTGAAACA
359°	TGTTCCACGTGAAACA
356°	TGTTTCATGTGAAACA
355°	CGTTTCACGTGAAACA
354°	TGTTACACGTGAAACA
330°	TGTTACACGTGAAACA
40°	CGTTCCATGTGAAACA
15°	TGTTACACGTGTAACA
consensus	TGTTNCACGTGAAACA

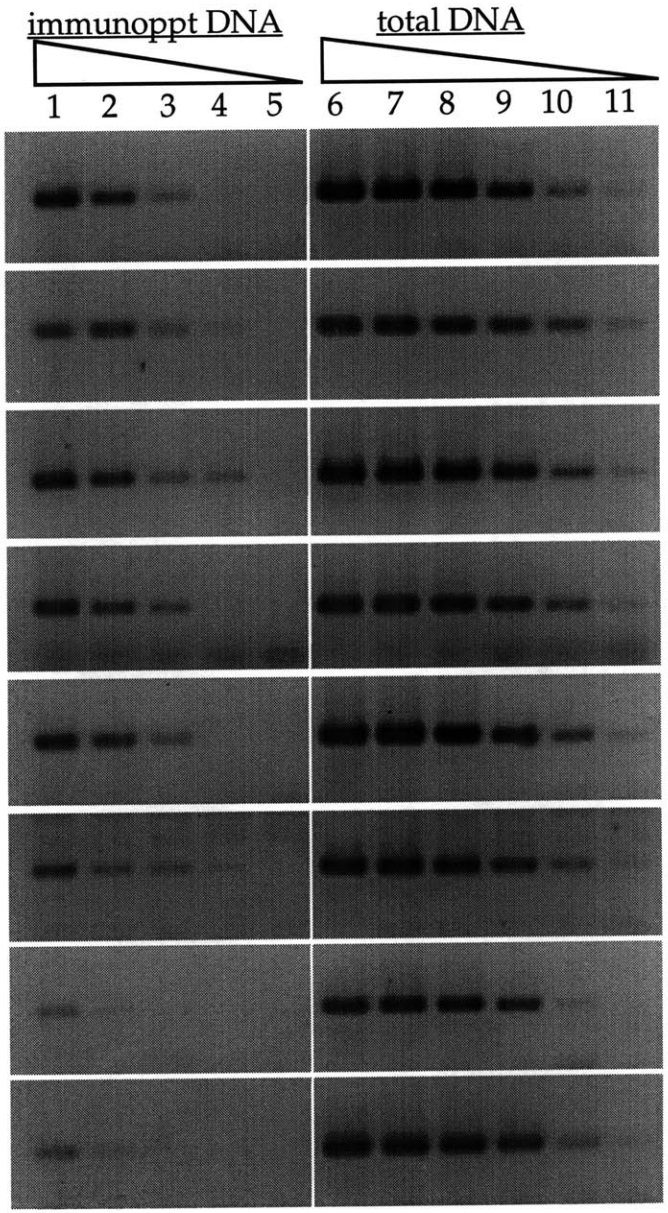
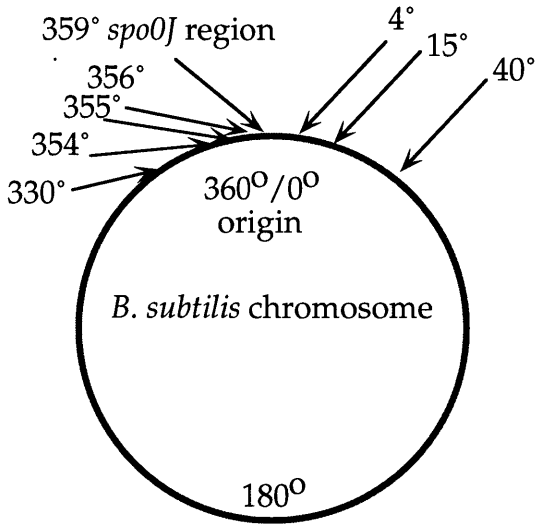


Figure 3-4 B.


359° spo0J region
4°
15°
40°
356°
355°
354°
330°
360°/0°
origin
B. subtilis chromosome
180°

Mutations in six sites cause increased binding to a weak site

Because a null mutation in *spo0J* causes an ~100-fold increase in the frequency of anucleate cells (Ireton et al., 1994), we reasoned that elimination of most (or all) of the Spo0J binding sites might also cause an increase in the frequency of anucleate cells. To test this, we deleted five of the sites (4°, 40°, 330°, 354°, and 356°) that are not located in open reading frames. Each site was replaced in the chromosome with a different drug resistance cassette (Experimental procedures). In addition, 7 bp (of 16) in the site in *spo0J* were changed without affecting the amino acid sequence of the gene product (see Experimental procedures). This is the same 7 bp mutation that does not bind Spo0J *in vivo* (Figure 3-2) and that does not compete well in the *in vitro* binding assay (Figure 3-3C, D).

The strain with six sites inactivated, DCL484, had only a small increase (~5-fold) in the frequency of anucleate cells compared to wild type, far less than that of a *spo0J* null mutant (data not shown). This lack of a strong partitioning defect appears to be due to compensation by other Spo0J binding sites. DNA from the Spo0J binding site at 15° was 10-20-fold more abundant in immunoprecipitates from the multiple-site mutant compared to that from wild type (Figure 3-5). These results indicate that at least some of the Spo0J binding sites are occupied more often in the absence of other sites. We suspect that this increased occupancy compensates for loss of multiple binding sites.

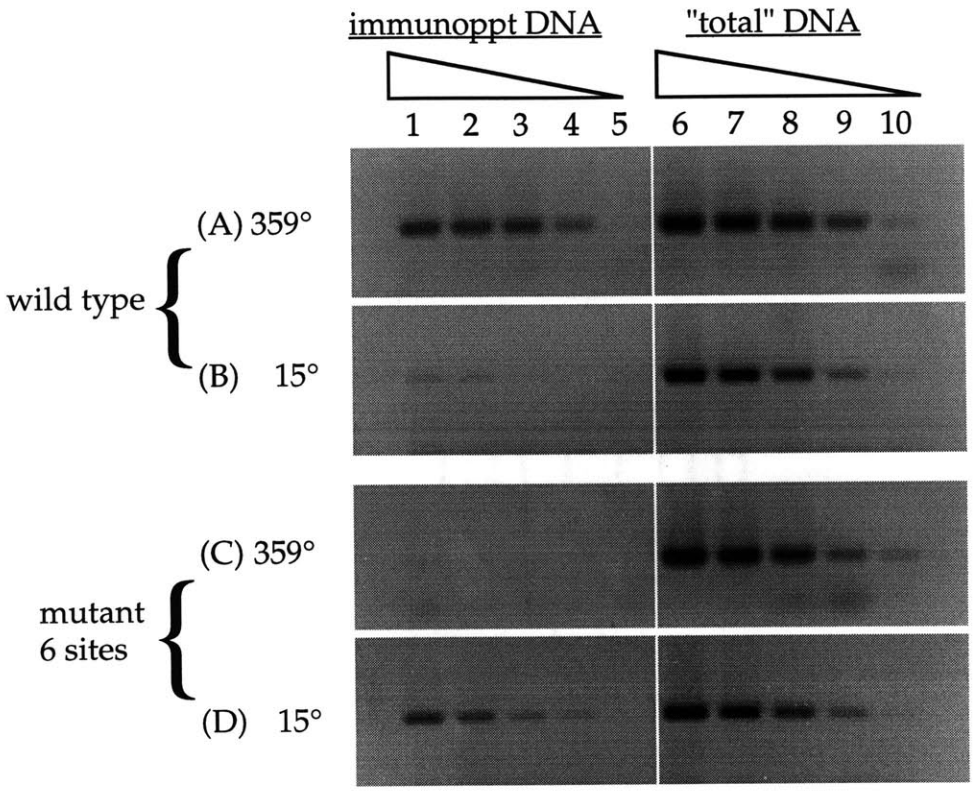


Figure 3-5. Increased occupancy of the Spo0J binding site at 15° in the mutant with 6 sites inactivated.

Binding of Spo0J to the sites at 359° (in *spo0J*) and 15° in wild type cells (A, B) and in the strain (DCL484) with six sites inactivated (C, D) was measured as above (Figure 3-4). Dilutions of 1/5 (lane 1), 1/20 (lane 2), 1/80 (lane 3), 1/320 (lane 4) and 1/1280 (lane 5) of the immunoprecipitated DNA and dilutions of 1/25 (lane 6), 1/125 (lane 7), 1/625 (lane 8), 1/3125 (lane 9) and 1/15625 (lane 10) of the total DNA before immunoprecipitation were used in PCR.

The Spo0J binding site is a partitioning site

A single Spo0J binding site was able to confer a partition function to a heterologous replicon. We cloned the binding site into an unstable, low copy *B. subtilis* plasmid and tested for effects on plasmid stability. pDL110 contains a chloramphenicol resistance marker for selection in *B. subtilis*, and the origin of replication and the gene encoding the replication initiation protein from pLS32, a plasmid originally isolated from *B. natto* (Hassan et al., 1997). To test for plasmid stability, we measured the fraction of cells containing a plasmid after several generations of growth in the absence of selection (Experimental procedures).

A plasmid with a functional Spo0J binding site was much more stable than plasmids without the binding site. When grown in the presence of chloramphenicol to select for the presence of the plasmid, the percentage of cells containing the vector without a Spo0J binding site (pDL110) was only 10 - 15%, compared to ~60% for the plasmid (pDL125) with a Spo0J binding site (Table 3-1; Figure 3-6A). During growth without selection, the vector was rapidly lost; after ~20 generations <0.1% of the cells contained a plasmid (Figure 3-6A). In contrast, the plasmid with a single Spo0J binding site (pDL125) was much more stable; after ~20 generations ~20% of the cells still had a plasmid (Figure 3-6A). A plasmid (pDL126) that has the same insert but with the 7 bp mutation in the Spo0J binding site was not stabilized (Figure 3-6A), demonstrating that the increased stability of pDL125 was due to the Spo0J binding site. Quantitative Southern blot experiments indicated that the Spo0J binding site did not affect the

copy number of the plasmid (Table 3-1). Together, these results indicate that the Spo0J binding site acts as a partitioning site, and we call each chromosome partition site *parS*.

The increased stability of the plasmid containing a single *parS* (pDL125) was dependent on *spo0J*. pDL125 was no longer stable in cells containing a null mutation in *spo0J* (Figure 3-6B). Unexpectedly, *soj*, the gene immediately upstream from *spo0J*, was also required for plasmid stability: a non-polar null mutation in *soj* (Ireton et al., 1994) prevented stabilization of pDL125 (Figure 3-6B). The instability of pDL125 was similar in cells containing a mutation in either *soj* or *spo0J*, and was no worse in cells containing mutations in both *soj* and *spo0J* (Figure 3-6B). The *soj* gene product is a member of the ParA family of partition proteins, a putative ATPase, and an inhibitor of sporulation (Ireton et al., 1994; Grossman, 1995). An *soj* null mutation has relatively little effect on chromosome partitioning (Ireton et al., 1994). In contrast, in the P1 and F plasmid systems, the ParA (SopA) protein is required for partitioning. Although an *soj* null mutant does not have an obvious chromosome partitioning defect, our results indicate that Soj plays some role in partitioning and this function may be redundant in the context of chromosome partitioning.

Table 3-1. Copy number comparison of plasmids with and without a Spo0J binding site.

plasmid	fraction of cells with plasmid[a]	plasmid/chromosome in total population of cells[b]	plasmid/chromosome per plasmid-containing cell[c]
pDL110 (vector)	0.13	0.21	1.6
pDL110 (vector)	0.14	0.27	1.9
pDL125 (*parS*)	0.74	1.56	2.1
pDL125 (*parS*)	0.59	1.09	1.8

[a]Data are shown from two experiments with each of two plasmids, pDL110 (the vector without a Spo0J binding site), and pDL125 (pDL110 with a Spo0J binding site). Cells were grown in chloramphenicol to select for the presence of the plasmid. Even with selection, only a fraction of the cells actually contained a plasmid as judged by plating efficiency in the presence and absence of chloramphenicol.

[b]DNA was prepared from exponentially growing cells containing the indicated plasmid. Numbers are the ratio of the intensity of the plasmid band to the chromosomal fragment, as determined by phosphorimager analysis of a Southern blot. Since two different DNA probes were used, one for the plasmid and one for the chromosomal fragment, these ratios are not an absolute indication of copy number.

[c]The plasmid/chromosome ratio was divided by the fraction of cells containing a plasmid.

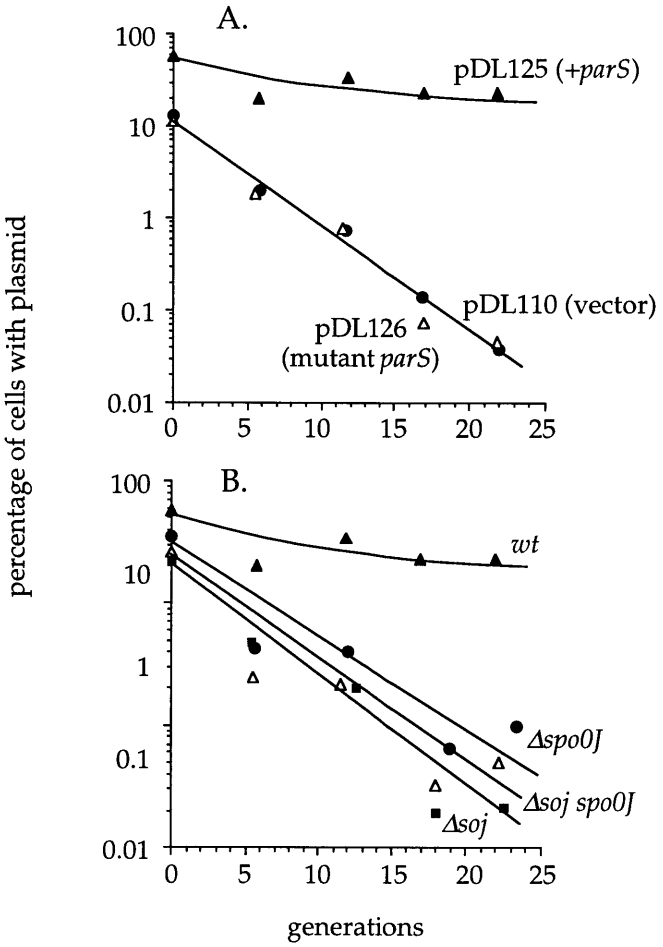


Figure 3-6. The Spo0J binding site can stabilize a plasmid.

Cells were grown for several generations in the absence of selection and tested for the presence of the plasmid. Zero (0) generations is the time at which selection for the plasmid was removed. For wild type strains with pDL125, 58% of the cells grown with selection actually contained a plasmid, as judged by the fraction of colonies resistant to chloramphenicol. For strains containing pDL110 or pDL126, ~10-15% of the cells grown with selection contained the plasmid.

(A) Wild type cells containing the parental plasmid (pDL110,•) and a plasmid containing a mutated Spo0J binding site (pDL126,▵) are rapidly lost in the absence of selection. In contrast, a plasmid with a wild type Spo0J binding site (pDL125,▲) is stabilized.

(B) Stabilization of pDL125 depends on *spo0J* and *soj*. pDL125 was stabilized in wild type cells (▲) (same data as in Figure 4-6A), but not in cells containing a mutation in *spo0J* (•) or *soj* (▪). The instability was no worse in the *soj spo0J* double mutant (▵).

Possible chromosome partition sites in other organisms

Chromosomally encoded homologues of Spo0J/ParB are widespread. Database searches reveal that homologues are found in at least 15 different bacterial species and we suspect that a DNA binding site for many of these will be located in or near the structural gene. In fact, 10 organisms that have a Spo0J/ParB homologue also have a potential binding site that matches the consensus sequence of *parS* of *B. subtilis*. In five of these organisms, this sequence is in or near the structural gene (Table 3-2). In others, including *Deinococcus radiodurans*, *Pseudomonas aueruginosa*, *Vibrio cholerae*, *Treponema pallidum*, and *Neisseriae gonorrhoeae*, the location of these sites with respect to the *parB/spo0J* gene is not clear from the available sequence information. We postulate that, in at least several of these organisms, these sites are chromosome partition sites and that the role of ParB/Spo0J in chromosome partitioning is conserved.

Table 3-2. Possible Spo0J/ParB binding sites in other bacteria that are similar to the *B. subtilis* consensus.

organism	sequence	distance from *spo0J/parB* gene
Bacillus subtilis	TGTTNCACGTGAAACA	consensus[a]
Mycobacterium leprae	TGTTTCATGTGAAACA	~0.9 kb
	TGTTTCACGTGAAACA	~1.8 kb
Mycobacterium tuberculosis	TGTTTCACGTGAAACA	~2 kb
	TGTTTCACGTGAAACA	~1.1 kb
Streptomyces coelicolor	CGTTTCACGTGAAACA	~1 kb
	GGTTTCACGTGAAACA	internal
Borrelia burgdorferi	TGTTCCACGTGGAACA	~0.1 kb
Streptococcus pyogenes	TGATTCACGTGAAACA	~7 kb

[a]One *parS* is found internal to *spo0J*, seven others are in the origin proximal 20% of the chromosome (Figure 3-4B).

Discussion

We have identified a family of chromosome partitioning sites (*parS*) from *B. subtilis*. Each *parS* is the binding site for the chromosome partition protein Spo0J, and is a 16 bp sequence composed of an imperfect 8 bp inverted repeat. The presence of a single site, on an otherwise unstable plasmid, stabilizes the plasmid, indicating that the sequence functions as a partition site. There are at least 8 *parS* sites in the *B. subtilis* genome that are occupied *in vivo*. All are located in the origin-proximal 20% of the chromosome (Figure 3-4B). The subcellular localization of Spo0J to the poles of the bacterial nucleoid (Glaser et al., 1997; Lin et al., 1997) is probably a direct reflection of the coordinate binding of Spo0J to these sites.

Spo0J and *parS* contribute to the efficiency of chromosome partitioning. The multiple sites appear to be redundant and cells compensate for loss of several sites with increased binding of Spo0J to other sites. Null mutations in *spo0J* cause a 100-fold increase in the frequency of anucleate cells (Ireton et al., 1994). This is a significant increase that would probably be lethal in nature in competition with wild type organisms. That ~98% of the cells of a *spo0J* mutant manage to get an intact genome indicates that other mechanisms contribute to efficient partitioning.

One additional component required for efficient chromosome partitioning in *B. subtilis*, and probably in most other organisms, is the *smc* gene product. A homologue of the eukaryotic SMC proteins (structural maintenance of

chromosomes) has been identified in *B. subtilis* (Oguro et al., 1996). Like those in eukaryotes {(Peterson, 1994; Hirano et al., 1995; Koshland and Strunnikov, 1996; Heck, 1997), and references therein}, *B. subtilis* SMC is required for efficient chromosome partitioning and condensation (R. Britton, DCHL, & ADG, submitted). An *smc* null mutant has nucleoids that appear less condensed than those in wild type cells. In addition, ~10% of the cells in a growing culture of the *smc* mutant are anucleate. Most strikingly, an *smc spo0J* double mutant has a synthetic phenotype; there is a severe growth defect and ~25% of the cells in a culture are anucleate (R. Britton, DCHL, ADG, submitted). This phenotype is discussed below in the context of models for Spo0J function.

The role of Spo0J in chromosome partitioning

One possible function of Spo0J bound to multiple *parS* sites might be to help position the origin region of the chromosome. The partition system of the *E. coli* F plasmid is required for proper plasmid positioning. A plasmid containing the *sopABC* system is localized at midcell in newborn cells and at the 1/4 and 3/4 positions in older cells preparing to divide (Gordon et al., 1997; Niki and Hiraga, 1997). In contrast, a plasmid missing the *sop* system is localized randomly in the cytosolic space (Niki and Hiraga, 1997).

During most of the cell cycle in *B. subtilis*, the *oriC* region is positioned near the pole of the nucleoid, oriented toward a cell pole (Glaser et al., 1997; Lin et al., 1997). A null mutation in *spo0J* causes the *oriC* region to be mislocalized in a small fraction of cells, but in the majority of mutant cells the origin region

appears properly positioned (DCHL, ADG, unpublished results). Preliminary experiments indicate that proteins required for DNA replication may be involved in establishing the position of the *oriC* region (KP Lemon, ADG, unpublished results). Thus, it appears that Spo0J is not required to establish, but might be involved in maintaining chromosome orientation.

In growing cells, sister origins are rapidly separated and become positioned at opposite ends of the nucleoid, oriented toward opposite cell poles (Glaser et al., 1997; Gordon et al., 1997; Lin et al., 1997; Webb et al., 1997). We speculate that the function of Spo0J bound to the multiple *parS* sites is to pair sister origin regions for recognition by components involved in separation and movement (Figure 3-7). This origin-pairing model for Spo0J is an extension of models for sister chromatid pairing or cohesion in eukaryotes (Miyazaki and Orr-Weaver, 1994; Bickel and Orr-Weaver, 1996; Guacci et al., 1997; Heck, 1997; Michaelis et al., 1997) and plasmid pairing in prokaryotes (Nordström and Austin, 1989; Austin and Nordström, 1990; Williams and Thomas, 1992).

We propose that after the origin region is duplicated, a Spo0J-*parS* complex on one chromosome contacts a Spo0J-*parS* complex on the other chromosome, pairing the sister origins for part of the cell cycle. We suspect that this pairing function may serve to indicate that two sister origins exist and are ready to be partitioned, and may help to distinguish sister origins from non-sisters during rapid growth when there are several overlapping rounds of replication. Pairing may also help to orient the origin regions such that one is "selected" to be moved toward the opposite pole. The Spo0J-*parS* complex seems

to persist during most (all?) of the cell cycle (Glaser et al., 1997; Lewis and Errington, 1997; Lin et al., 1997), indicating that disruption of sister origin pairing is not mediated by degradation of Spo0J. The putative ATPase Soj (ParA) might be involved in disruption of sister origin pairing, but if so, its function appears to be redundant.

The postulated pairing of sister origin regions by Spo0J-*parS* complexes is somewhat analogous to the function of sister-chromatid cohesion proteins in eukaryotes { (Miyazaki and Orr-Weaver, 1994; Bickel and Orr-Weaver, 1996; Heck, 1997) , and references therein}. These proteins are involved in pairing the sister chromatids at centromeric regions and along the length of the chromosomes until the metaphase-anaphase transition, ensuring that the sister chromatids are not separated precociously.

The pairing model for Spo0J-*parS* function helps to explain the synthetic phenotype of a *spo0J smc* double mutant. In wild type cells, with highly condensed, compact nucleoids, it seem likely that newly replicated origin regions might remain near each other. In the *smc* mutant, defective in chromosome condensation, the pairing function of Spo0J becomes much more important to maintain proximity of the newly replicated origins before they are actively separated. Whereas other models are also consistent with the phenotype of the *spo0J smc* double mutant, we currently favor the pairing model, especially in light of recent findings that SMC and SMC-associated proteins are involved in chromosome cohesion (pairing) in yeast (Guacci et al., 1997; Michaelis et al., 1997).

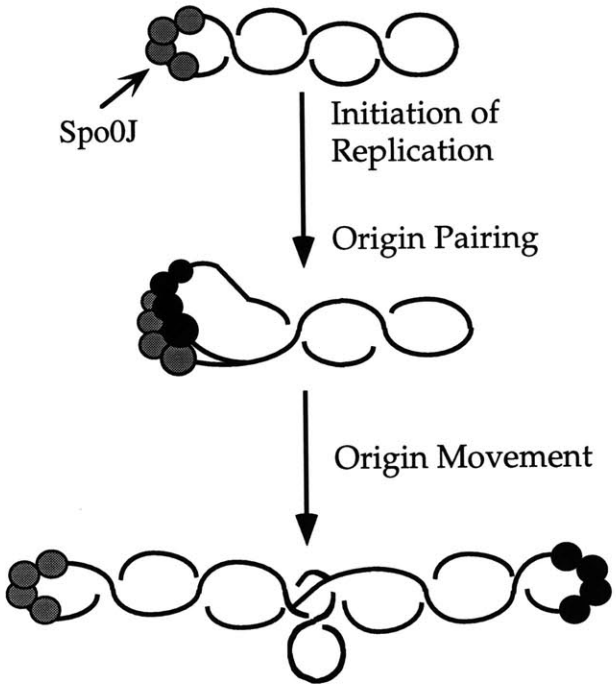


Figure 3-7. Spo0J may be involved in pairing newly replicated origin regions.

Gray and black circles represent Spo0J binding to *par* sites in the origin region of the chromosome (not drawn to scale). We postulate that Spo0J is involved in pairing the newly replicated origins. After origin pairing, separation of origins is governed by as yet uncharacterized proteins. Spo0J may also be involved in maintaining the polar localization of the origin region, perhaps by interacting with proteins near the poles of the cell.

Chromosome dynamics

Our current view of chromosome partitioning in bacteria involves orientation and active movement of the origin region, and continuous condensation and compaction of the entire chromosome. Regions of the chromosome near and including the origin of replication are positioned at an end of the nucleoid toward a cell pole. We propose that newly replicated origins are paired by Spo0J until the segregation machinery separates them and re-positions one origin toward each pole. Condensation, partly by SMC, facilitates pairing. Condensation also is likely to provide a mechanism to move the bulk of the chromosome mass away from mid-cell and toward the position where the origin has been established. Division at midcell creates two cells, each with an intact genome. The continuing challenge is to identify the remaining components involved in chromosome partitioning and to determine their mechanisms of action.

Experimental Procedures

Strains and plasmids

B. subtilis strains are all derivatives of AG174 (JH642) and contain the *trpC* and *pheA* mutations. Standard procedures (Harwood and Cutting, 1990) were used for transformations and strain constructions. Strains and relevant genotypes are listed in Table 3-3. Plasmids are described in Table 3-4, or in the text below.

Construction of strain DCL484

Strain DCL484 contains mutations in six of the 8 known Spo0J binding sites. Five of the sites were deleted and a drug-resistance cassette inserted. For each mutation, DNA (~400 bp) from upstream and downstream of the Spo0J binding site was amplified by PCR and cloned upstream and downstream of a drug-resistance cassette. A different drug-resistance marker was used for each mutation. Sequences of all oligonucleotides used in the PCR are available upon request.

Each mutation was introduced by transformation into the *B. subtilis* chromosome by double crossover, selecting for resistance to the specific marker. Each mutation was confirmed by PCR analysis. The following plasmids were used: pDL112 replaces 32 bp, removing the Spo0J binding site at 330°, with a phleomycin-resistance cassette; pDL113 replaces ~140 bp, removing the Spo0J binding site at 356°, with a erythromycin-resistance cassette; pDL114 replaces 19 bp, removing the Spo0J binding site at 4°, with a kanamycin-resistance cassette;

pDL116A replaces 117 bp, removing the Spo0J binding site at 354°, with a tetracycline-resistance cassette; pDL122 replaces ~60 bp, removing the Spo0J binding site at 40°, with a spectinomycin-resistance cassette.

Seven of the 16 bp in the Spo0J binding site in *spo0J* were changed so as not to alter the gene product. In order to create a strain with the 7 bp changes in *spo0J*, strain AG1468 (*Δspo0J::spc*) (Ireton et al., 1994) was transformed with pDL107 (which contains *spo0J* with the 7 bp site mutation, Table 3-4) and chloramphenicol-resistant transformants, which arise by single crossover at the *spo0J* locus, were selected. As expected, two classes of transformants were obtained, Spo+ for crossovers upstream, and Spo- for crossovers downstream of the *spc* insertion in *spo0J*. A Spo- transformant was chosen (strain DCL440). Excision of pDL107 from DCL440 by a single crossover created a strain (DCL468) that is Spo+, chloramphenicol- and spectinomycin-sensitive, and has the 7 bp mutation in the Spo0J binding site. The presence of the mutation was confirmed by PCR and DNA sequencing.

Formaldehyde crosslinking and immunoprecipitations

Cells were grown at 37° for several generations in defined minimal medium (Vasantha and Freese, 1980; Jaacks et al., 1989) containing 1% glucose, 0.1% glutamate, 40μg/ml tryptophan, 40μg/ml phenylalanine, trace metals, and appropriate antibiotics when necessary, and samples were taken during exponential growth (OD600 ~0.6). Crosslinking and sample preparation were based on chromatin immunoprecipitation assays (Solomon and Varshavsky,

1985; Hecht et al., 1996; Strahl-Bolsinger et al., 1997). Samples were treated with NaPO4 (final concentration 10 mM) and formaldehyde (final concentration 1%) for 10 minutes at room temperature followed by 30 minutes at 4°C. Cells (10 ml) were pelleted and washed twice with 10 ml of 1X phosphate buffered saline pH 7.3 (Ausubel et al., 1990). Cells were resuspended in 500 µl of solution A (10 mM Tris pH 8, 20% sucrose, 50 mM NaCl, 10 mM EDTA) containing 20 mg/ml lysozyme and incubated at 37°C for 30 minutes. 500 µl of 2X IP buffer (100 mM Tris pH 7, 300 mM NaCl, 2% Triton X-100) and PMSF (final concentration 1 mM) was added and the cell extract was incubated an additional 10 min at 37°. The DNA was sheared by sonication to an average size of ~500 - 1,000 bp. Insoluble cellular debris was removed by centrifugation and the supernatant was transferred to a fresh microfuge tube. In order to determine the relative amount of DNA immunoprecipitated to the total DNA before immunoprecipitation, 75 µl of supernatant ("total" DNA control) was removed and saved for later analysis.

Protein and protein-DNA complexes were immunoprecipitated (1 hr, room temperature) with affinity purified polyclonal anti-Spo0J antibodies (Lin et al., 1997) followed by incubation with 30 µl of a 50% Protein A-Sepharose slurry (1 hr room temperature). Complexes were collected by centrifugation, and washed five times with 1X IP buffer and twice with 1 ml TE (10 mM Tris pH 8, 0.1 mM EDTA). The slurry was resuspended in 50 µl of TE. The 75 µl "total" DNA control was treated with *S. griseus* protease (final concentration 0.1 mg/ml) for 10 minutes at 37°C and SDS was added to 0.67%. Formaldehyde crosslinks of

both the total DNA and the immunoprecipitate were reversed by incubation at 65°C for six hours and samples were used in PCR without further treatment.

PCR was performed with Taq DNA polymerase using serial dilutions of the immunoprecipitate and the total DNA control as the template. Oligonucleotide primers were typically 20-25 bases in length and amplified a ~300-450 bp product. Sequences of all primers are available upon request. PCR products were separated on agarose gels and stained with ethidium bromide. Relative affinities of Spo0J to different *par* sites were determined by comparing the intensity of bands in the linear range of the PCR from both the immunoprecipitate and "total" DNA control. Gels were photographed onto Polaroid 665 film and the negatives were scanned using Adobe Photoshop software.

Spo0J-his6

Spo0J with a hexa-histidine tag at the C-terminus is functional in *B. subtilis*, both in sporulation and chromosome partitioning (Lin et al., 1997). Spo0J-his6 was purified from *E. coli* strain DCL128, a BL21 (lambda DE3) strain carrying a plasmid, pDL3, with *spo0J-his6* under the control of the T7 promoter in pET21(+) (Lin et al., 1997). An extract from the over-producing strain was loaded onto a metal chelating column (Pharmacia) that had been charged with NiSO4, according to instructions from the manufacturer. Spo0J-his6 was eluted with a linear gradient of imidazole (60 mM to 1 M) in buffer containing 20 mM Tris pH 8, 500 mM NaCl. Fractions containing Spo0J-his6 were pooled and dialyzed into

buffer containing 20 mM Tris pH 8, 250 mM NaCl, 1 mM EDTA, 1 mM DTT. Following dialysis, glycerol was added to 10% and protein concentration was determined with BioRad protein assay kit using BSA as a standard. Spo0J-his6 was ~90% pure as judged by SDS polyacrylamide gel electrophoresis and staining with Coomassie blue.

DNA binding assays

A 24 bp DNA fragment containing the Spo0J binding site was used as the probe in gel mobility shift assays. Two oligonucleotides, 5'-AGAATGTTCCACGTGAAACAAAGA-3' (LIN-71), and its complement 5'-TCTTTGTTTCACGTGGAACATTCT-3' (LIN-72), were annealed and radiolabeled using polynucleotide kinase and gamma-32P-ATP. The radiolabeled 24 bp fragment was gel-purified and resuspended in TE. Binding reactions (15 µl) were performed in 20 mM Hepes pH 7.6, 292 mM NaCl, 5% glycerol, 1 mM DTT and contained approximately 1.5 fmol of DNA. Reactions were incubated for 15 minutes at 32°C, and then loaded onto a prerun 8% polyacrylamide (29:1) gel in 0.5X TBE. Gels were run at 4°C at 150V, dried, and exposed to a Phosphorimager cassette (Molecular Dynamics). Bands were quantitated using ImageQuant software.

Competition assays were performed with both the unlabeled 24 bp fragment containing a wild type Spo0J binding site, and a 24 bp fragment containing seven base pair changes in the Spo0J binding site. The mutant fragment was made by annealing the oligomers 5'-

AGAACGTGCCCAGGGAGACCAAGA-3' (LIN-120) and its complement 5'-TCTTGGTCTCCCTGGGCACGTTCT-3' (LIN-121).

Plasmid Stability Assays

Cells containing the indicated plasmids were grown in defined minimal medium containing 1% sodium succinate, 0.1% glutamate, 40 μg/ml tryptophan, 40 μg/ml phenylalanine, 100 μg/ml threonine (when needed), and trace metals. Cells were grown first for several generations with chloramphenicol to select for the plasmid. At generation time zero, cells were removed from chloramphenicol-containing medium by centrifugation, resuspended and used to inoculate fresh medium in the absence of antibiotic. Cells were maintained in exponential growth by dilution into fresh medium when the culture reached mid to late exponential phase. The percentage of cells containing a plasmid was determined by measuring the fraction of cells that were resistant to chloramphenicol, as determined by colony forming ability on LB plates with and without antibiotic.

Determination of relative plasmid copy number

The relative copy number of plasmids with and without *parS* was determined by quantitative Southern blots using probes specific to plasmid and chromosomal sequences. The plasmid specific probe was an ~1,500 bp EcoRI-AlwNI fragment from pDL110. The chromosomal specific probe was a ~1,200 bp EcoRI-XhoI fragment from pDL20 that extends from the 3' end of *dnaA* into the 5' end of *dnaN* (immediately downstream of *dnaA*). The *dnaA - dnaN* fragment in

pDL20 was cloned from PCR products amplified from chromosomal DNA. Probes were labeled with α-32P-dATP using random priming with a hexanucleotide mix (Pharmacia) according to the manufacturer's instructions.

Total DNA was prepared from cells in exponential growth in defined minimal medium, as for the plasmid stability assays. DNA was digested with EcoRI, separated on a 0.8% agarose gel in 1X TAE buffer, and transferred to a nitrocellulose membrane. Hybridization was done essentially as described (Ausubel et al., 1990) using both plasmid and chromosome specific probes simultaneously. Results were visualized with a phosphorimager and band intensity was quantitated used ImageQuant software. The ratio of the plasmid specific band to the chromosome specific band was determined.

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Table 3-3. *B. subtilis* strains used.

strain	relevant genotype
AG174 (JH642)	*trpC pheA*
AG1468	*Δspo0J::spc* (Ireton et al., 1994)
AG1505	*Δ(soj spo0J)::spc* (Ireton et al., 1994)
KI1944	*Δ(soj spo0J)::spc thr::(Δsoj spo0J+)* (Ireton et al., 1994)
DCL108	pHP13
DCL352	pIK219
DCL365	pDL83
DCL367	pDL85
DCL381	pDL90A
DCL430	pDL104
DCL431	pDL105
DCL432	pDL106
DCL438	pDL110
DCL484	sextuple *parS* mutant, *parS-6* (see Experimental procedures)
DCL490	pDL125
DCL491	pDL126
DCL492	*Δ(soj spo0J)::spc*; pDL125
DCL494	*Δspo0J::spc*; pDL125
DCL497	*Δ(soj spo0J)::spc thr::(Δsoj spo0J+)*; pDL125

Table 3-4. Plasmids used

plasmid	description
vectors	
pHP13	Cm, MLS; *B. subtilis* and *E. coli* shuttle vector (Harwood and Cutting, 1990)
pBPA23	Contains the replicon from pLS32 from *B. natto* (Hassan et al., 1997) .
pGEM*cat*	Ap, Cm; integrative vector (Harwood and Cutting,1990)
pJH101	Ap, Tet, Cm; integrative vector (Harwood and Cutting, 1990)
other plasmids	
pIK219	Contains an ~760 bp fragment, extending ~540 bp downstream of *spo0J* (Figure 3-2A), cloned into pHP13. Used to define the Spo0J binding site *in vivo*.
pDL83	Contains an ~310 bp fragment, extending ~100 bp downstream of *spo0J* (Figure 3-2A), cloned into pHP13. Used to define the Spo0J binding site *in vivo*.
pDL85	Contains an ~255 bp fragment (Figure 3-2A) cloned into pHP13. Used to define the Spo0J binding site *in vivo*.
pDL90A	Contains the 55 bp fragment from PvuII to SfaNI in *spo0J* (Figure 3-2A) cloned into pHP13. Used to define the Spo0J binding site *in vivo*.
pDL104	Contains the 16 bp Spo0J binding site (Figure 3-2B) cloned into the SmaI site of pHP13. Single stranded oligomers 5'-TGTTCCACGTGAAACA-3' (LIN-73) and its complement 5'-TGTTTCACGTGGAACA-3' (LIN-74) were annealed, phosphorylated with polynucleotide kinase, and cloned into pHP13. The plasmid was verified by DNA sequencing.
pDL105	Contains a 38 bp fragment, missing the Spo0J binding site (Figure 3-2B), cloned into the SmaI site of pHP13. Single stranded oligomers 5'-CTGATTCAGCAGTTGAATCAGAAAAGAAAAAAGAACCTG-3' (LIN-75) and its complement 5'-CAGGTTCTTTTTTCTTTTCTGATTCAACTGCTGAATCAG-3' (LIN-76) were annealed, phosphorylated with polynucleotide kinase, and cloned into pHP13. DNA sequencing revealed that the plasmid is essentially pDL90A with a 17 bp deletion removing the Spo0J binding site.

pDL106	Contains a 55 bp fragment with 7 bp changes in the Spo0J binding site, cloned into pHP13. Single strand oligomers 5'-CTGATTCAGCAGTTGAATCAGAACGTGCCCAGGGAGACCA AGAAAAAAGAACCTG-3' (LIN-77) and its complement 5'-CAGGTTCTTTTTTCTTGGTCTCCCTGGGCACGTTCTGATTCAA CTGCTGAATCAG-3' (LIN-78) were annealed, phosphorylated with polynucleotide kinase, and cloned into pHP13. The annealed oligomers contain the 55 bp insert in pDL90A except that 7 bp in *parS* have been changed (underlined above). The plasmid was verified by sequencing.
pDL107	Contains all of *spo0J*, with the 7 bp mutation in *parS*, cloned into pGEM*cat*. Used to construct the multiple *parS* mutant strain, DCL484.
pDL110	Contains the ~1.5 kb EcoRI-XbaI fragment from pBPA23 (containing the replicon of pLS32) cloned between the EcoRI-NheI sites in pJH101. Used in the plasmid stability experiments.
pDL125	Contains the 55 bp fragment of *spo0J* from pDL90A, with *parS*, cloned into pDL110. The ~60 bp EcoRI-HindIII fragment from pDL90A was cloned between the EcoRI-HindIII sites of pDL110. Used in the plasmid stability experiments.
pDL126	Contains the 55 bp fragment of *spo0J* from pDL106, with the mutant *parS*, cloned into pDL110. The ~60 bp EcoRI-HindIII fragment from pDL106 was cloned between the EcoRI-HindIII sites of pDL110. Used in the plasmid stability experiments.

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Chapter 4

Origin localization in wild type and *spo0J* cells, and the effects of inserting multiple *parS* partitioning sites into the terminus region of *Bacillus subtilis*

RATIONALE

Origin localization in *E. coli* has been accomplished by both tagging the origin with *lacO*/GFP-LacI (Gordon et al. 1997) and with FISH (fluorescence *in situ* hybridization) (Niki and Hiraga 1998). In *E. coli*, the duplicated sister origin regions are near positions ~10-15% of the cell length (Gordon et al. 1997, Niki and Hiraga 1998). Both the F and P1 plasmids localize at midcell in young cells, and at the quarter points in older cells as visualized with either the *lacO*/GFP-LacI technique or with FISH (Gordon et al. 1997; Niki and Hiraga 1997).

In *B. subtilis*, the origin regions have been visualized by using both the *lacO*/GFP-LacI chromosome tagging technique (Webb et al. 1997) and by visualization of the Spo0J protein that binds to multiple sites in the origin regions (Lin et al, 1997; Glaser et al, 1997; Lin and Grossman, 1998). I repeated these experiments using an improved LacI-GFP fusion protein that allowed me to measure origin localization in exponentially growing cells, rather than from a culture where part of the cells may be lagging following dilution from a stationary phase culture (Teleman et al. 1998; Webb et al. 1998). I was interested in measuring, in a large population of cells, whether the duplicated origins were near the cell quarters, as the P1 and F plasmids in *E. coli*, or ~10-15% of the cell length, as the *E. coli* origin, or elsewhere. Similar results to those described in this chapter have also been made (Webb et al. 1997, 1998; Sharpe and Errington 1998). Also, Sharpe and Errington reported that interfocal distance of Spo0J-GFP foci is constant with respect to cell length (Sharpe and Errington 1998). I

repeated these experiments with Spo0J-GFP (and with the *lacO*/LacI-GFP tagged origin region), and found that the interfocal distance increases with increasing cell length.

I was also interested in testing the effects of a *spo0J* null mutation on origin localization. Webb et al reported that origin movement appeared to be normal in most *spo0J* cells, although origin localization was aberrant in a subpopulation of cells (Webb et al. 1998). In sporulating cells, there were multiple origin foci in a *spo0J* null background (Webb et al. 1997). Using the improved LacI-GFP fusion protein, I was interested in characterizing origin localization in further detail in a *spo0J* null.

Another question that I explored was whether Spo0J determines the cellular localization of its binding site *parS*. All of the eight known *parS* sites are in the origin region of the chromosome (chapter 3) (Lin and Grossman 1998), and Spo0J colocalizes with the origin region on the poles of the nucleoid (chapter 2) (Glaser et al. 1997; Lewis and Errington 1997; Lin et al. 1997; Teleman et al. 1998). I tested whether placing an array of *parS* sites in the terminus would affect the localization of the terminus, or would affect the localization of Spo0J. I also tested whether the *parS* array at the terminus could function in chromosome partitioning, and whether the *parS* array would affect sporulation. Inserting the *parS* array at the terminus also allowed me to address the validity of the "tethering" model for Spo0J function (described below).

RESULTS

Origin localization in wild type and *spo0J* mutant cells

To measure origin localization in vegetatively growing cells, I took advantage of an improved LacI-GFP fusion protein. As done previously, an array of ~256 *lacO* sequences was inserted at ~359° (0°/360° is *oriC*) (Webb et al. 1997). This region of the chromosome was visualized with a C-terminal LacI-GFP fusion protein (courtesy of KP Lemon and AD Grossman, unpublished data), rather than an N-terminal GFP fusion as done previously (Webb et al. 1997). As previously reported, the N-terminal GFP-LacI fusion was easily detected in stationary phase cells, but was very dim and visible in only a portion of exponentially growing cells, (Teleman et al. 1998; Webb et al. 1998). With the new C-terminal LacI-GFP fusion, visualization of fluorescence was greatly improved and could be easily detected in virtually all vegetatively growing cells. The C-terminal LacI-GFP fusion, like the N-terminal fusion, lacks the final 11 amino acids of LacI, rendering it defective in tetramerization (Robinett et al. 1996). Using this C-terminal GFP fusion, I measured origin number and position relative to the cell poles in ~250 live wild type and *spo0J* cells. The cell outline was visualized with the membrane dye FM4-64 (Pogliano et al. 1999).

In a wild type background, the majority of the cells (~82.5%) contained two foci and these foci were located near the cell quarters (table 4-1, figure 4-1). The distances of the foci were measured from the same pole, and the first focus was defined as the focus closest to a pole. Measurements were made to the

center of the focus. Most of the first foci (~71.3%) were located between 15-30% of the cell length, peaking around 25%, and most of the second foci (66.8%) were located between 60-75% of the cell length, peaking around 70% (figure 4-1). Both foci probably migrated to the quarter positions following duplication at midcell (Lemon and Grossman 1998; Webb et al. 1998). Approximately 7.4% of the cells contained a single focus, and this focus was typically located near midcell (table 4-1, figure 4-1). These results are consistent with those previously reported (Webb et al, 1997, 1998).

The striking difference between wild type and *spo0J* cells was the frequency of cells with respect to the number of foci per cell (table 4-1) (Webb et al, 1997). Approximately 45.8% of *spo0J* cells had two foci, compared with ~82.5% of wild type cells (table 4-1). The decrease in 2 foci cells in the *spo0J* mutant came with an increase in both the percentage of cells with one focus and cells with three or more foci. Approximately 27.5% of *spo0J* cells had one focus, compared with ~7.4% for wild type. It is likely that many of the cells scored as having a single focus actually contained two unresolvable foci. Also, ~26% of *spo0J* cells had three or more foci, compared with ~10% for wild type. Finally, as previously noted (Webb et al. 1997), the *spo0J* cells were approximately 10% longer than wild type cells.

The positions of the foci within each class were similar in *spo0J* and wild type cells and were not random, consistent with previously reported results (Webb et al, 1997, 1998). Of the single focus *spo0J* cells, the LacI-GFP marked origin was located near midcell, similar to wild type cells. Of the two foci *spo0J*

Table 4-1. Characterization of strains with *lacO*/LacI-GFP marked origins.

strain	genotype	% 1 focus	%2 foci	%3 foci	%4 foci	%5 foci	# cells counted	avg cell length (μm)
DCL696	*wild type*	7.4	82.5	4.8	5.2		229	2.79
DCL705	*Δspo0J*	27.5	45.8	16.8	9.2	0.8	262	3.06
DCL711	*ΔparS5; ter::parS16*	17.4	70.8	6.3	5.6		144	2.86

Cells were grown in defined S7 minimal media at 30°C and cells were examined during mid-exponential phase. The *lacO* plasmid was amplified using high concentrations (25μg/ml) of chloramphenicol prior to the experiment. All distance measurements were performed using Openlabs 2.0 software (see Materials and Methods).

Figure 4-1. Comparison of LacI-GFP marked origin localization in wild type, *spo0J*, and *ΔparS5; ter::parS16* strains.

A. The positions of the foci as a percentage of cell length are plotted against the percent of one or 2 foci cells. The graphs on the left show the positions of the single focus in wild type, *spo0J*, and *ΔparS5; ter::parS16* cells with only a single origin dot. The graphs on the right show the positions of the two foci in cells with 2 dots of LacI-GFP.

B. In wild type, *spo0J*, and *ΔparS5; ter::parS16* cells with two foci, the interfocal length was measured and plotted against total cell length. The interfocal length increases with increasing cell length.

Figure 4-1 A. Position of origins marked with LacI-GFP

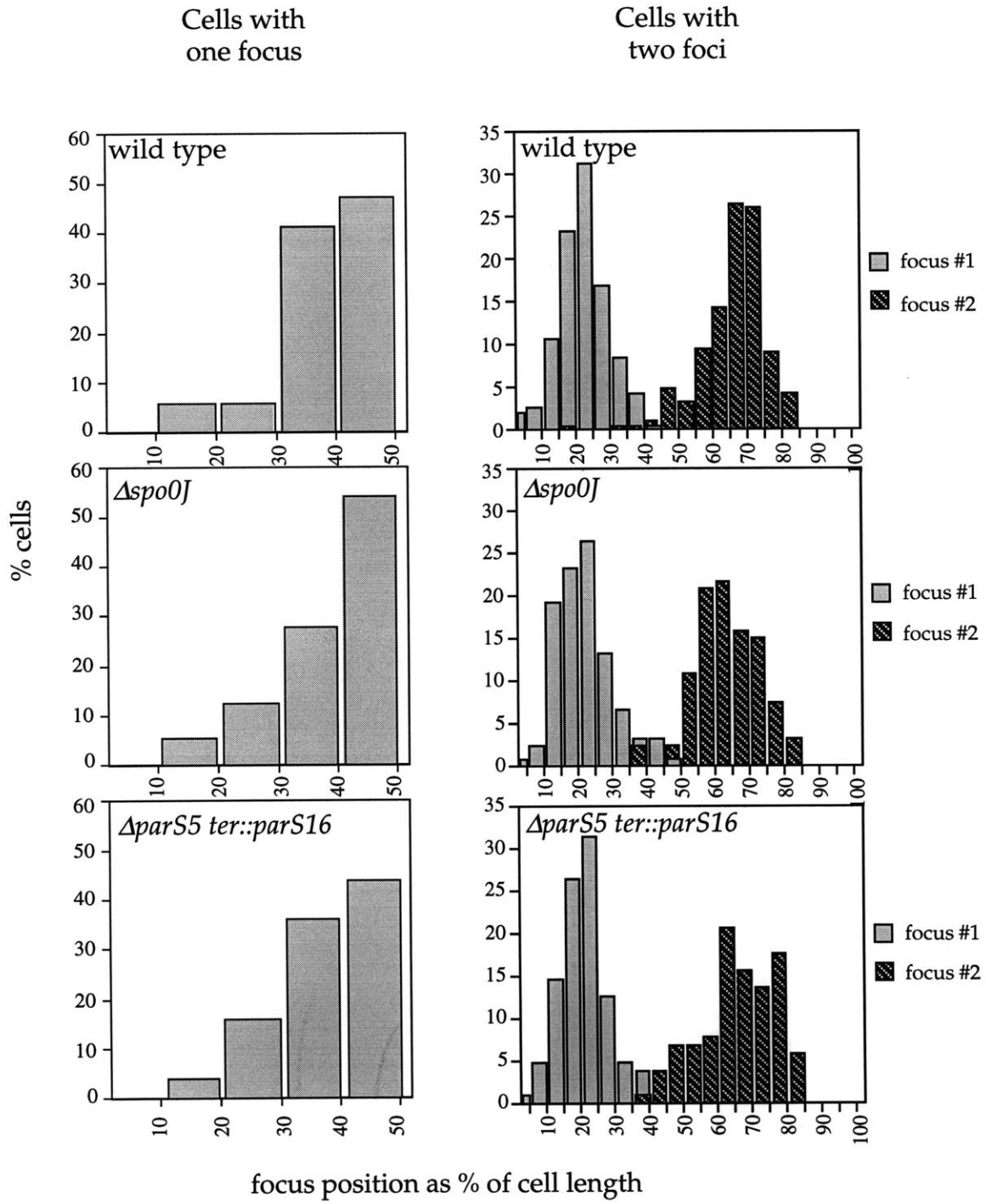
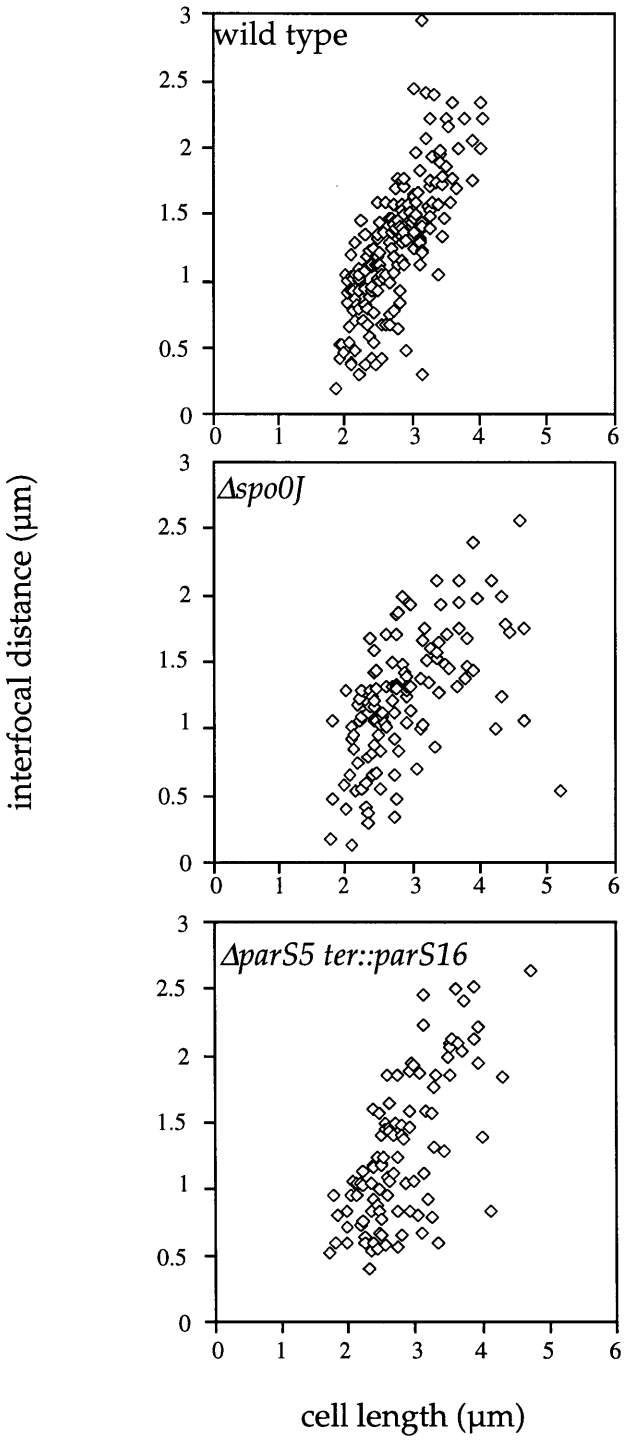


Figure 4-1 B. Interfocal Distance as a function of cell length in cells with 2 LacI-GFP origin foci



cells, most of the first foci (63%) were located between 15-30% of the cell length most of the second foci (73.3%) were between 55-75% of the cell length (figure 4-1). The positions of the foci in 3 and 4 foci cells were also similar between the wild type and *spo0J* cells (data not shown). In the Discussion section, I describe possible models to explain the differences between wild type and *spo0J* cells.

Although most of the *spo0J* cells with two separated foci appeared normal, some cells showed an impairment in the separation of the origin foci in a *spo0J* background (see also Webb et al, 1998). This is most easily seen when the interfocal distance in cells with two foci was measured and plotted as a function of cell length (figure 4-1B). In wild type cells, the distance between the origin foci increased with increasing cell length. My results, and those previously reported (Webb et al, 1998), clearly indicate that the interfocal origin distance is not fixed. This is inconsistent with the results of Sharpe and Errington (Sharpe and Errington 1998). In general, most *spo0J* 2 foci cells followed a similar pattern, although many cells do not appear to separate the origins as well (figure 4-1B). For example, there are many long cells (≥4μm) in a *spo0J* background with closely spaced origins, and these cells were not seen in a wild type background, (figure 4-1B).

Description of strains containing the *parS16* array

Another question that I explored was whether Spo0J determines the cellular localization of its binding site *parS*. I tested whether placing an array of *parS* sites in the terminus would affect the localization of the terminus, or would

affect the localization of Spo0J. The terminus, in contrast to the origin region, typically localizes near the center of the cell and is usually distinct from the origin region localization (Gordon et al. 1997; Niki and Hiraga 1998; Teleman et al. 1998; Webb et al. 1997). Also, I was interested in determining whether an array of *parS* sites in the terminus would affect normal chromosome partitioning.

This experiment also allowed me to test one model that has been proposed for Spo0J and plasmid ParB function. In the tethering model, Spo0J, bound to *parS*, is involved in tethering the *parS* DNA (and hence the origin regions) near the cell quarter positions (Austin and Nordstrom 1990; Glaser et al. 1997; Lin et al. 1997; Sharpe and Errington 1996). Tethering may occur by interaction of Spo0J with a protein that localizes near the quarter positions. If the tethering model was correct, then the *parS* array at the terminus should bring the terminus near the cell quarter positions.

An array of 16 tandemly repeated *parS* sites (called *parS16*) was constructed using a strategy similar to that used to make the multiple *lacO* array (figure 4-2) (Robinett et al. 1996). The 16 *parS* sites on the *parS* array are each separated by approximately 60 bp, and correspond to the *parS* sequence and flanking DNA from the "strong" *parS* site found in *spo0J* (Lin and Grossman 1998). The *parS16* array functioned as well as a single *parS* site in stabilizing an unstable plasmid (figure 4-2). The plasmid containing the *parS16* array and terminus region DNA was integrated into the terminus region by homologous recombination.

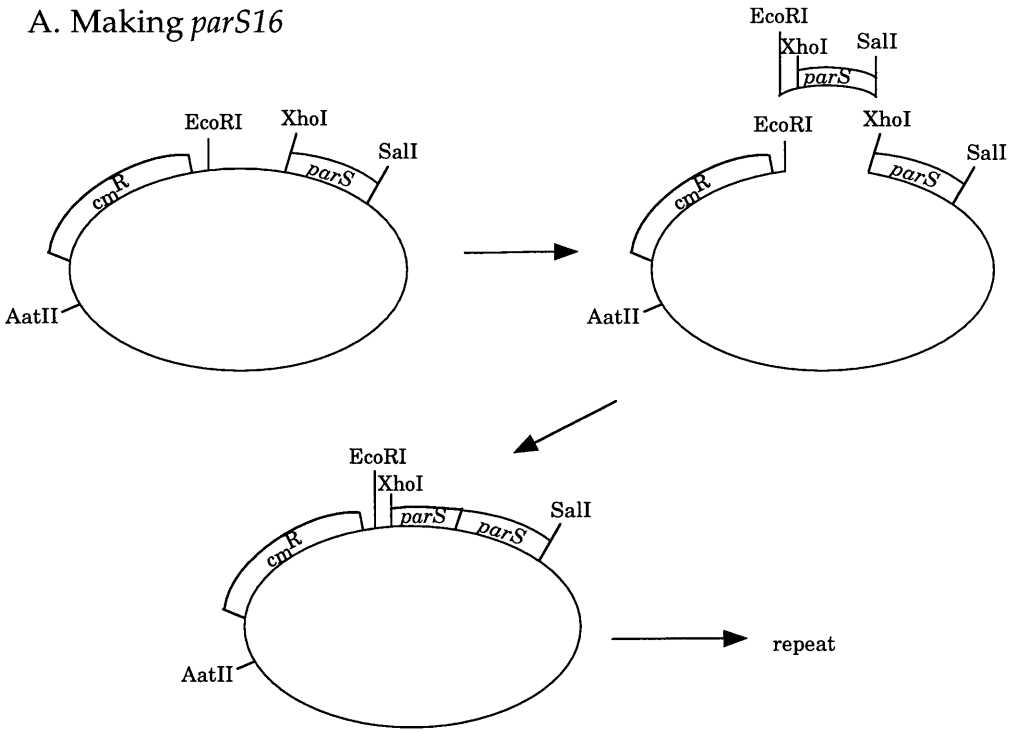
Figure 4-2. Construction of the *parS16* array and plasmid stability assay with the *parS16* array.

A. The *parS16* array was constructed using a strategy similar to that used to make the 256 repeat *lacO* array (Robinett et al, 1996). Restriction endonucleases SalI and XhoI leave compatible cohesive ends that, when ligated, are no longer recognized by either enzyme. During each round of ligation, a parental plasmid is digested with EcoRI and XhoI, and the insert is the fragment derived from an EcoRI and SalI digest. Following each round of ligation, the number of *parS* repeats increases two fold.

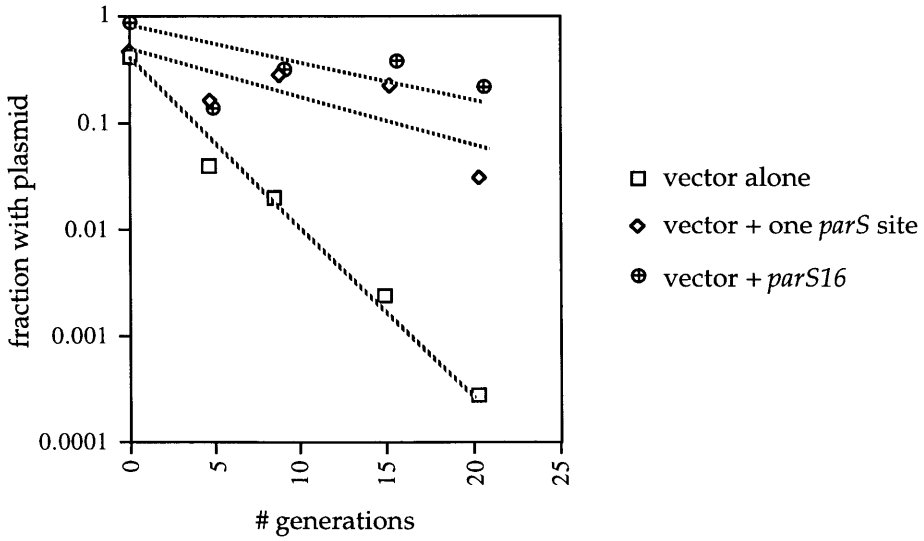
B. The *parS16* array was cloned into an unstable vector and tested for plasmid stability as described previously (Lin and Grossman, 1998). The *parS16* array stabilized the plasmid as well as a single *parS* site.

Figure 4-2.

A. Making *parS16*



B. Plasmid Stability Assay



It was formally possible that Spo0J or other putative "tethering" factors to which Spo0J binds near the cell poles were limiting, which could lead to the wild type *parS* sites in the origin being "preferred" over the *ter::parS16* array. In order to increase the occupancy of Spo0J at the *ter::parS16* array, I also mutated six of the eight known origin proximal *parS* sites. Previously, I showed that mutations of six *parS* sites led to increased occupancy of Spo0J at the remaining sites (Chapter 3) (Lin and Grossman 1998). I therefore postulated that I could increase the occupancy of Spo0J to the *ter::parS16* array by mutating six of the eight known Spo0J binding sites (*ΔparS6*).

A Spo0J-GFP fusion protein, expressed from the endogenous *spo0J* locus, was used to determine the localization of Spo0J in these cells and was the only functional copy of *spo0J* in the cell (Lin, et al. 1997). Strain DCL616 contains *spo0J-gfp* with a mutation in *parS* in the *spo0J-gfp* gene and is otherwise wild type (called the "control" strain). Strain DCL631 contains *spo0J-gfp* with the *parS* mutation in *spo0J-gfp*, and deletion of 5 other *parS* sites, and the *ter::parS16* array (called "*ΔparS6, ter::parS16*"). I mutated the *parS* site in the *spo0J-gfp* gene in both strains in order to equalize Spo0J-GFP protein levels, since mutation of *parS* in *spo0J* appears to decrease the levels of the Spo0J protein ~4 fold in the cell (S. Venkatasubrahmanyam, D. Lin, and A. Grossman, unpublished data). The *parS* mutation in *spo0J* eliminates Spo0J binding but does not affect the amino acid sequence of Spo0J or have a chromosome partitioning defect (Lin and Grossman 1998).

Localization of Spo0J in a *ter::parS16* strain

Using fluorescence microscopy, I localized Spo0J-GFP in both the control strain and *ΔparS6, ter::parS16* strain. Both the localization of Spo0J-GFP and the percentage of cells with respect to number of foci were strikingly different between the two strains (table 4-2, figure 4-3). The number and position of Spo0J-GFP foci in the control strain were similar to origin regions (Figure 4-1), but the pattern of Spo0J-GFP localization in the*ΔparS6, ter::parS16* strain was similar to the localization reported for the terminus region (Webb et al. 1997; Gordon et al. 1997). Approximately 77.1% of control cells contained two foci, compared with ~27.1% of *ΔparS6, ter::parS16* cells. In contrast, ~18.2% of the control cells contained one focus, compared with ~71% of *ΔparS6, ter::parS16* cells (table 4-2). The single focus in both strains was located near midcell. However, the single focus *ΔparS6, ter::parS16* cells were ~38% longer than the single focus control cells (table 4-2, figure 4-3). The longer cell length indicates that although these cells are farther along in the cell cycle (by which time the origins should separate), there is only one focus, which most likely represents an unduplicated terminus rather than an unduplicated origin region.

Of the cells with two foci, the localization of Spo0J-GFP in the control strain were located near the cell quarter positions, but was shifted closer to midcell in the *ΔparS6, ter::parS16* strain. As expected, Spo0J-GFP localization in the control strain was similar to the origin localization described above. Of the

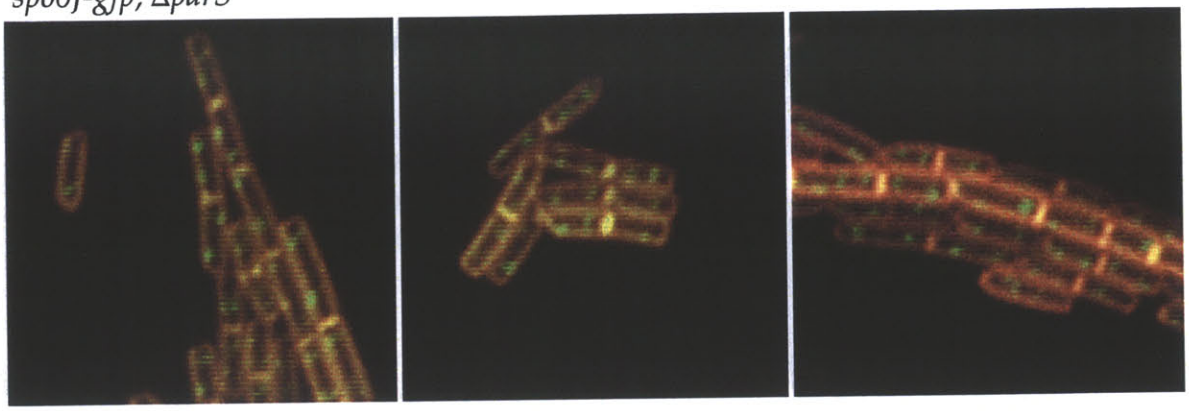
Table 4-2. Characterization of Spo0J-GFP in DCL616 and DCL631 (*ΔparS6; ter::parS16*) cells

strain	genotype	% 1 focus	% 2 foci	% 3 foci	% 4 foci	total cells	avg cell length 1 focus cells (μm)	avg cell length 2 foci cells (μm)	avg cell length (μm)
DCL616	*spo0J-gfp* , *ΔparS* in *spo0J*	18.2	77.1	2.5	2.2	236	2.01	2.77	2.66
DCL631	*spo0J-gfp, ΔparS6 ter::parS16*	71.0	27.4	1.5	<0.004	259	2.79	3.77	3.08

Cells were grown in S7 minimal media at 30°C and examined during mid-exponential phase. Scoring was done from printed photomicrographs with a standard ruler (See Materials and Methods).

A.

spo0J-gfp, ΔparS



spo0J-gfp, ΔparS6 ter::parS16

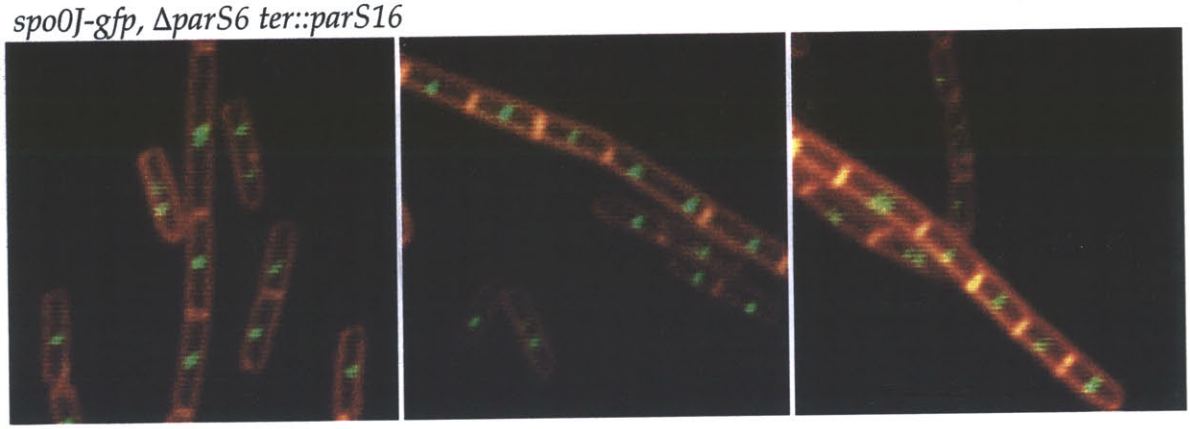


Figure 4-3. Localization of Spo0J-GFP in Δ*parS* and Δ*parS6; ter::parS16* strains.

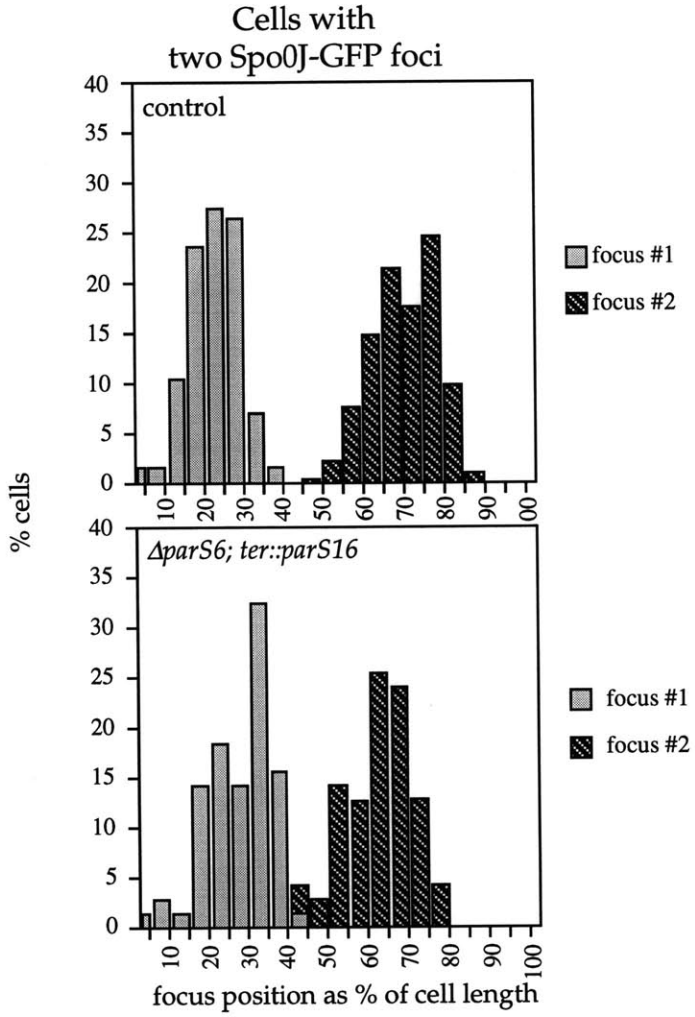
A. Three panels of Spo0J-GFP from each strain are shown. The top three panels are from the control strain, and the bottom three panels are from the Δ*parS6; ter::parS16* strain. The membrane is stained with the red-orange dye FM4-64 and the green foci inside the cell are Spo0J-GFP.

B. (next page) Distribution of Spo0J-GFP foci in cells with two foci. The positions of the foci as a percentage of cell length are plotted against the percent of 2 foci cells.

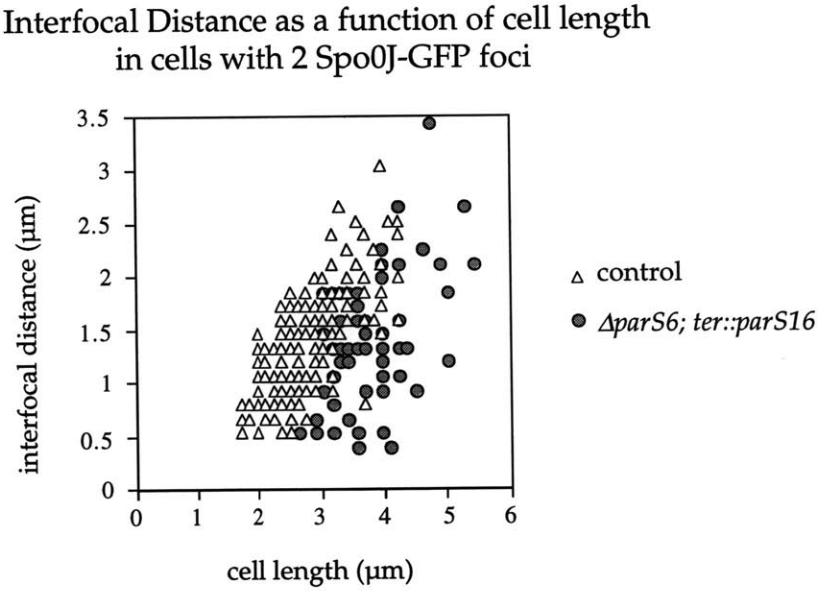
C. (next page) Interfocal distance plotted against cell length in control and Δ*parS6; ter::parS16* cells with 2 Spo0J-GFP foci. Note that two foci Δ*parS6; ter::parS16* cells are much longer than the 2 foci control cells (see also table 4-2).

Figure 4-3

B.


Cells with
two Spo0J-GFP foci
control
ΔparS6; ter::parS16
% cells
focus #1
focus #2
focus position as % of cell length

C.


Interfocal Distance as a function of cell length
in cells with 2 Spo0J-GFP foci
interfocal distance (μm)
cell length (μm)
control
ΔparS6; ter::parS16

control cells with two foci, most of the first foci (~77.5%), defined as the focus closest to a cell pole) were typically located between 15-30% of the cell length, peaking around 25%. Most of the second foci (~78.5%) were located between 60-80% of the cell length, peaking around 75% (figure 4-3). These foci represent origin regions that have been duplicated and separated apart. In contrast to the control strain, the two foci in *ΔparS6, ter::parS16* were shifted closer to midcell and probably represent duplicated terminus regions (figure 4-3B). The average cell length of the 2 foci *ΔparS6, ter::parS16* cells was ~35% longer than that in control strain, consistent with the 2 foci *ΔparS6, ter::parS16* cells being further along in the cell cycle and containing two duplicated terminus regions (Table 4-2). This was most apparent by plotting the interfocal distance as a function of cell length (figure 4-3 C). The two foci control cells separate early, similar to origin regions (figure 4-3C, 4-1 B), but the two foci *ΔparS6, ter::parS16* cells separate late, similar to what has been described for terminus regions (figure 4-3C) (Gordon, et al. 1997; Niki and Hiraga 1998; Webb, et al. 1998). In sum, these results indicate that insertion of *parS16* at the terminus alters the localization of Spo0J and does not affect the localization of the terminus in the cell.

ter::parS16 does not affect localization of the origin regions

The single central Spo0J-GFP focus in the DCL631 *ter::parS16* strain does not represent mislocalization of the origin regions to the center of the cell through most of the cell cycle. Using the *lacO*/LacI-GFP chromosome tagging technique, I visualized the origins in the *ter::parS16* strain. Because I was limited

by the number of drug selection markers, I was able to mutate only 5 of the 8 endogenous *parS* sites in a strain carrying LacI-GFP and the *lacO* cassette (strain DCL711).

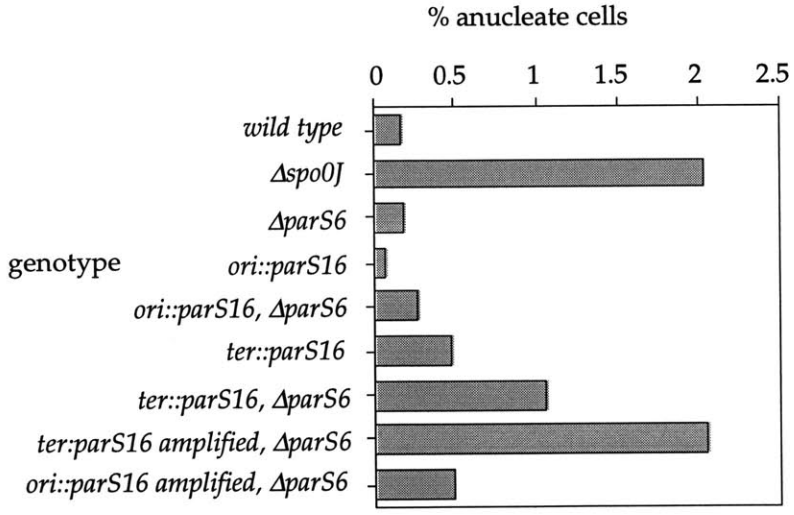
The localization of the origin regions in the *ΔparS5, ter::parS16* strain was similar to that in wild type cells. Of the 144 *ΔparS5, ter::parS16* cells counted, ~70.8% of the cells had two origin foci (Table 4-1). The positions of these foci in the cell were similar to that in wild type and *ΔspoOJ* cells (figure 4-1) and clearly were not in the center of the cell. These results suggest that the single SpoOJ-GFP focus in the *ter::parS16* cells represents only the *ter::parS16* array, rather than the *ter::parS16* array and the origin regions.

Anucleate cell production in *ter::parS16* and *ori::parS16* strains

The *ter::parS16* strain had a mild chromosome partitioning defect in a strain where 6 of the 8 endogenous *parS* sites were mutated (~1.05% anucleate cells, Table 4-3). The chromosome partitioning defect of *ter::parS16* was decreased when all of the wild type *parS* sites were present (~0.47% anucleate cells) but was worse in a strain when the *ter::parS16* array was amplified and also contained the 6 *parS* mutations (~2.05% anucleate cells, see Materials and Methods). In contrast, wild type cells and a strain deleted for 6 of 8 *parS* (*ΔparS6*) sites accumulated ~0.17% anucleate cells. In contrast, strains containing the *parS* array inserted near the origin, either in the presence of all the wild type sites and in the absence of six of the endogenous *parS* sites, accumulated only 0.067% and 0.26%

Table 4-3 Anucleate cell production in cells carrying *parS16* at the terminus or the origin

strain	genotype	# anucleate cells	total cells	% anucleate
AG174	*wild type*	18	11534	0.16
AG1468	*ΔspoOJ*	141	6977	2.02
DCL484	*ΔparS6*	11	6299	0.17
DCL665	*ori::parS16*	4	5996	0.067
DCL668	*ori::parS16, ΔparS6*	22	8508	0.26
DCL559	*ter::parS16*	53	11210	0.47
DCL602	*ter::parS16, ΔparS6*	164	15648	1.05
DCL602	*ter:parS16 amplified, ΔparS6*	45	2194	2.05
DCL668	*ori::parS16 amplified, ΔparS6*	10	2021	0.49



Cells were grown in 2XSG sporulation media at 37°C. Cells (500µl) were taken at mid-exponential phase, and fixed with 100µl of 16% paraformaldehyde/0.05% glutaraldehyde for ~15 minutes at room temperature and ~45 minutes on ice. The fixed cells were washed twice with 1X Phosphate Buffered Saline (PBS) pH 7.4, and adhered to poly-L-lysine coated slides. Cells were stained overnight with 1µg/ml DAPI in 1X PBS. Combined phase and fluorescence microscopy was used to detect the DNA and the cell outline.

anucleate cells, respectively. The anucleate phenotype of strains bearing the *ter::parS16* array is probably caused by titration of Spo0J from the remaining wild type *parS* sites in the origin region to the *parS16* array. The *ter::parS16* array caused a more severe partitioning defect than the *ori::parS16* array, suggesting that Spo0J bound to *parS* sites in the terminus does not function in partitioning and that *parS* needs to be in the origin region to function in partitioning. Future experiments will test whether the *parS16* array at intermediate chromosomal positions can function in partitioning.

Although the *ΔparS6, ter::parS16* strain has a chromosome partitioning defect, the cells sporulate normally. The *parS16* array inserted at either the terminus or in the origin region did not have an appreciable sporulation defect in rich or minimal sporulation medium (data not shown). Amplification of the *parS16* array did not cause a sporulation defect and the timing of sporulation was also unaffected (data not shown). This indicates that the sporulation and chromosome partitioning roles of Spo0J can be separated. These results may also indicate that localization of Spo0J near the poles is not required for sporulation, since the *ter::parS16* targets Spo0J to midcell in vegetatively growing cells (figure 4-3). Localization of Spo0J-GFP in the *ΔparS6; ter::parS16* should be examined during sporulation.

DISCUSSION

Localization of the origin regions

I have presented results in this paper measuring the localization of the *B. subtilis* origin regions to positions near the cell quarters. This was accomplished by using both an improved LacI-GFP fusion protein (table 4-1, figure 4-1) and by localization of Spo0J-GFP (Figure 4-3). The distance between two origin foci increases with increasing cell length, staying near the cell quarter positions (figure, 4-1B, 4-3C).

The localization of the *B. subtilis* origin region is similar to the localization of the *E. coli* P1 and F plasmids, which localize at the cell quarters in older cells (Gordon, et al. 1997; Niki and Hiraga 1997). Members of the ParA and ParB family contribute to partitioning of P1, F, and *B. subtilis*, but not for the *E. coli* chromosome. A major difference is that loss of the *sop* system leads to complete mislocalization of F (Niki and Hiraga 1997), but origin localization is not so drastically affected by null mutations in *spo0J* (see also Webb et al., 1997, 1998). The replicative polymerase also localizes at the cell quarters late in the cell cycle, which become midcell following cell division (Lemon and Grossman 1998). Perhaps the origins are sequestered at the quarter positions, and await the migration of the polymerase from the midcell to the quarters in order to initiate another round of replication.

Localization of the origin regions in a *spo0J* null

The major difference of origin localization in wild type and *spo0J* cells is the number of foci per cell. *spo0J* cells have a lower percentage of cells with two foci, and a larger percentage of cells with either one focus or three or more foci (table 4-1). In addition, the average cell length was about 10% longer in *spo0J* cells compared with wild type cells (table 4-1) (Webb et al, 1997). The localization of the origins was similar in the two strains when comparing cells with equal amounts of foci (figure 4-1) (Webb et al, 1998).

The model that I favor that is consistent with the data is for a role of Spo0J in chromosome organization. I propose that defects in Spo0J mediated chromosome organization lead to entanglement of some sister origins and a delay in cell division. When sister origins become entangled, the LacI-GFP marked origin regions may not be resolvable, leading to an increase in the cells scored as having one focus. The entangled foci can eventually untangle, since *spo0J* mutant cells do not exhibit an obvious growth defect. In addition, most sister origins do not become entangled (but a fraction are entangled) and the separated foci localize properly near the cell quarters. Finally, loss of *spo0J* leads to a cell division delay without inhibition of the initiation of replication. Replication reinitiates much earlier than cell division in a *spo0J* null, leading to an increase in the percentage of cells with 3 or more origin foci.

How is Spo0J involved in chromosome organization of the origin regions? Chromosome organization may be important to ensure that only the DNA from one sister origin migrates to a single quarter position, rather than attempting to

go towards either quarter position. One possible mechanism of chromosome organization is that Spo0J forms a specialized nucleoprotein complex through interaction of Spo0J bound to its eight *parS* sites in the origin proximal region. Another mechanism of chromosome organization is that Spo0J, bound to *parS*, pairs two sister origins together. These mechanisms are not mutually exclusive.

I do not believe that the function of Spo0J is to tether *parS* and the origin regions near the cell quarters, consistent with the conclusion previously made (Webb et al, 1997, 1998). The strongest results against this model are from experiments with *ter::parS16*. It is clear that in *ter::parS16* strain, the terminus does not localize near the cell quarters, instead Spo0J localizes to the terminus. In addition, the origin regions are not mislocalized, although the bulk of the Spo0J protein is at the terminus. Most likely a Spo0J independent mechanism is important for tethering of the origin regions. There are of course many different models that are consistent with the data presented here. The precise determination of the function of Spo0J awaits further experimentation.

Examination of *spo0J* and *ΔparS6, ter::parS16* cells

It is interesting that in *ΔparS6, ter::parS16* cells, origin localization and number of foci per cell is similar to that in wild type cells, but anucleate cells form, similar to the phenotype of *spo0J* mutant cells. There are two possible reasons for this. One explanation is that the phenotype of the *ΔparS6, ter::parS16* strain is intermediate to that of wild type and *spo0J* mutant cells and behave as a weak *spo0J* allele. This weak phenotype may be attributable to the residual

function of the remaining 2 *parS* sites in the origin region. Using *in vivo* formaldehyde cross-linking (Lin and Grossman, 1998), I found that Spo0J is still bound to the remaining 2 *parS* sites, but the binding is reduced compared to cells without the *ter::parS16* array (D Lin and AD Grossman, unpublished data). The *ΔparS6,ter::parS16* has a phenotype of a possible weak *spo0J* allele: The percentage of cells with respect to number of foci, the average cell length, and the anucleate phenotype appear to be intermediate between wild type and *spo0J* mutant cells. In DAPI-stained *spo0J* mutant cells, there are also aberrant nucleoid structures, which is not apparent with the *ter::parS16* cells

Another interpretation of the difference between *ter::parS16* and *spo0J* mutant cells is that different mechanisms contribute to the defects seen in the two strains. To be consistent with the data, this mechanism would have to affect *ter::parS16* without affecting *ori::parS16* cells, since *ori::parS16* cells appear *par+* . The terminus region of the *B. subtilis* chromosome is membrane associated, and the sister termini partition abruptly, perhaps underlying a terminus specific partition apparatus (Beeson and Sueoka 1979; Webb, et al. 1998). One possibility is that the *ter::parS16* array could perturb this process, leading to the production of anucleate cells. Perhaps moving the *ter::parS16* array ~100-200 hundred kilobases (~10-20°) away would alleviate this effect.

Table 4-4

***B. subtilis* strains used**

strain	genotype	ref. or comment
AG174	wild type *trp phe* (aka JH642)	lab strain
AG1468	*Δspo0J::spec*	(Ireton et al. 1994)
DCL484	*ΔparS6::spec kan phleo tet MLS*, unmarked in *spo0J*	(Lin and Grossman 1998)
DCL559	*ter::pDL141 (parS16)* cm	
DCL602	*ΔparS6::spec kan phleo tet MLS*, unmarked in *spo0J* *ter::pDL141 (parS16) cm*	
DCL616	*spo0J::pDL152 spo0J-gfp MLS (ΔparS* in *spo0J)*	
DCL631	*spo0J::pDL152 spo0J-gfp MLS (ΔparS*in *spo0J)* *ΔparS* at 5 other loci *spec kan phleo tet MLS* *ter::pDL141 (parS16)cm*	
DCL665	*ori::pDL168A (parS16) cm*	
DCL668	*ΔparS6::spec kan phleo tet MLS*, unmarked in *spo0J* *ori::pDL168A (parS16) cm*	
DCL693	downstream of *spo0J::pDL175 lacO* array *cm* *Δthr::Ppen-lacIΔ11gfpmut2 MLS*	
DCL696	isogenic to DCL693 but *lacO* array amplified on cm25	
DCL697	*Δspo0J::spec* downstream of *spo0J::pDL175 lacO* array *cm* *Δthr::Ppen-lacIΔ11gfpmut2 MLS*	
DCL705	isogenic to DCL697 but *lacO* array amplified on cm25	
DCL710	downstream of *spo0J::pDL175 lacO* array *cm* *ΔparS5::spec phleo tet MLS*, unmarked in *spo0J* *ter::pDL178 (parS16) kan* *Δthr::Ppen-lacIΔ11gfpmut2 MLS*	
DCL711	isogenic to DCL710 but *lacO* array amplified on cm25	
KPL471	*Δthr::Ppen-lacIΔ11gfpmut2 MLS (pKL160)*	(K.Lemon & A Grossman, unpublished

Plasmid construction

Sequences of all oligonucleotides are available upon request.

The *parS16* array was constructed using a strategy similar to that used to construct the 256 *lacO* repeats (figure 4-2) (Robinett, et al. 1996). In brief, this strategy takes advantage of the compatible cohesive ends of SalI and XhoI to repeat a unit multiple times. The repeated unit is increased two fold after every round of ligation (figure 4-2). Plasmid pGem-cat (Harwood and Cutting 1990) was used as the parental plasmid in the construction of *parS16*. Plasmid pDL135 contains a single ~60 bp insert which contains a single *parS* site. The insert contains oligos LIN-116 and LIN-117 annealed and inserted into the SmaI and SalI sites of pGem-cat. The insert was verified by DNA sequencing. Plasmid pDL136 contains 2 *parS* sites, pDL137 contains 4 *parS* sites, pDL138 contains 8 *parS* sites, and pDL139 contains 16 tandemly repeated *parS* sites (*parS16*).

The *parS16* array was targeted to either the origin or the terminus on a plasmid containing sufficient homology for integration and a chloramphenicol resistance gene. A chloramphenicol resistance gene for use in *B. subtilis* was excised from pMI1101 and inserted into the SphI site of the cloning vector pET-21(+) (Novagen). This created plasmid pET-21(+)-cat. To target this plasmid to the terminus region by homologous recombination, oligos LIN-118 and LIN-119, which both contain engineered AatII sites, were used to amplify an ~490 bp fragment from the *cgeD* gene (181°) using PCR. The amplified product was

digested with AatII and inserted into the AatII site in pET-21(+)-cat to create pDL124. The *parS16* array was excised from pDL139 by digestion with EcoRI and HindIII, and this fragment was ligated into EcoRI-HindIII digested pDL124 to create plasmid pDL141. To construct a *parS16* plasmid that would integrate in the origin region, oligos LIN-145 and LIN-146, which both contain engineered AatII sites, were used to amplify a ~660 bp fragment corresponding to DNA downstream of *spo0J* (359°). This PCR fragment was digested with AatII and replaced the *ter* (*cgeD*) region DNA in AatII digested pDL141 to create pDL168A. Plasmid pDL141 and pDL168A integrated into *B. subtilis* by single crossover and could be amplified using high concentrations of chloramphenicol (25μg/ml)

Plasmid pDL152 was constructed to create a strain (DCL616) which had *spo0J-gfp* expressed from the *spo0J* locus but contained a mutation in *parS* in *spo0J*. The 3′ end of *spo0J* with the *parS* mutation was amplified from pDL107 (Lin and Grossman 1998) using PCR and inserted in frame to *gfp*. The parental plasmid for pDL152 is pDG647, which contains an *MLS* marker.

Plasmid pDL175 was used to insert the *lacO* cassette downstream of *spo0J*. Oligos LIN-145 and LIN-146 were used to amplify a ~660 bp fragment downstream of *spo0J*. The PCR product was digested with AatII and BglII, and the resulting ~380 bp fragment was inserted into the *lacO* containing plasmid pAT12 (Webb, et al. 1997).

Plasmid pDL178 was used to insert *parS16* near the terminus (in the *cgeD* operon at 181°) and is marked with the kanamycin resistance gene. First, the kanamycin resistance gene cassette from pDG782 (obtained from the *Bacillus* Genetic Stock Center) was amplified by PCR and inserted into pGem-3Zf(+) (Promega) to create pGK71 (also known as pGem-kan; courtesy of Iren Kurtser, unpublished data). pGK71 was digested with AatII, and a ~480 bp AatII fragment containing DNA from the *cgeD* operon from pDL124 was inserted there. This created plasmid pDL170. Finally, the *parS16* cassette from pDL139 was excised with EcoRI and SalI and inserted into pDL170 that had been digested with EcoRI and SalI to create pDL178.

Microscopy

For strains DCL616 and DCL631, cells were grown at 30°C in S7 minimal media containing 1% glucose, 0.1% glutamate, trace metals, and ~50 µg/ml tryptophan and phenylalanine (Jaacks et al. 1989). Cells were grown in the presence of 0.1µg/ml FM4-64 and centrifuged briefly (~10-20sec) before observation. Cells were collected during mid-exponential growth and examined. For DCL616 and DCL631, microscopy was performed essentially as described (Lemon and Grossman 1998). Scoring was done off of printed photomicrographs using a ruler. Measurements were taken in relation to a single pole. The pole that was chosen was the one closest to a focus. Measurement from the poles was made to the center of a focus (of LacI-GFP or Spo0J-GFP).

For strains DCL696, 705, and 711, S7 media was also supplemented with ~100µg/ml threonine and cells were grown in the presence of 0.5µg/ml FM4-64. For DCL696, 705 and 711, microscopy was performed with a Nikon E800 Microscope equipped with a Hamamatsu Digital Camera controlled by Openlabs 2.0 software. Red and green images were captured separately and merged using Openlabs 2.0 software. Scoring was done using the measurement function in Openlabs 2.0.

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Chapter 5

Discussion

Summary of thesis

This thesis has described experiments aimed towards understanding the role of the chromosome partitioning protein Spo0J. In summary, Spo0J is a site-specific DNA binding protein that recognizes a 16 bp sequence, *parS* (chapter 3) (Lin and Grossman 1998). Plasmid stability assays reveal that *parS* can contribute to partitioning (chapter 3). There are at least eight *parS* sequences in the completely sequenced *B. subtilis* genome; all eight *parS* sites are in the origin proximal region (chapter 3). Preliminary results indicate that *parS* needs to be in the origin to contribute to partitioning; insertion of multiple *parS* sites into the terminus led to a *par* defect, whereas insertion of multiple *parS* sites into the origin did not (chapter 4). Immunofluorescence microscopy experiments demonstrate that Spo0J localizes in the cell typically as two large foci near the cell quarters (chapter 2,4) (Glaser et al. 1997; Lin et al. 1997). As expected, Spo0J co-localizes with the origin region (Lewis and Errington 1997; Teleman et al. 1998). Null mutations in *spo0J* affect normal origin localization in vegetatively growing cells, but this does not appear to be due to a role for Spo0J in tethering *parS* to the cell quarter positions (chapter 4).

Exploring models for bacterial chromosome partitioning and Spo0J

Both prokaryotic and eukaryotic cells must accomplish several fundamental tasks in order to partition their chromosomes. First, the

chromosomes need to be recognized and oriented by the partitioning apparatus in order to move them apart. The duplicated chromosomes must also be organized in a way such that the entire DNA from a single chromosome moves towards the same pole. Finally, a mechanism must exist to move the chromosomes.

As described in the introductory chapter, some of the key components that accomplish this task in eukaryotic cells are centromeres, kinetochores, sister chromatid cohesion proteins, centrosomes, chromosome condensation proteins, motor proteins, and microtubules. These components make up and coordinate the movements of the mitotic apparatus in order to partition chromosomes.

We are just beginning to learn about the components and the mechanisms that govern chromosome partitioning in bacteria. Below, I will discuss some of the aspects of bacterial chromosome partitioning. One of the key questions that I will discuss revolves around the motive force for bacterial chromosome partitioning. One possibility is that mitotic-like motor proteins drive chromosome partitioning, and another possibility is that DNA polymerase provides the motive force. A second aspect that I will discuss is the role of Spo0J and what step in the partitioning process it may be involved in. I favor a chromosome organization model for Spo0J. I will describe why I believe chromosome organization is important and the data that supports a role for Spo0J in chromosome organization. I will also discuss what a possible centromere sequence in prokaryotes could be, and describe why I think *parS* does

not fill the role of centromere. Finally, I will discuss other aspects of Spo0J that were not highlighted in this thesis, in particular the role of Spo0J in sporulation.

What provides the force to move the bacterial chromosome?

One unanswered question in prokaryotic cell biology is the nature of the mechanism that drives the origins and/or the bulk chromosomes apart. Eukaryotic mitosis was first observed over 100 years ago. No structures similar to the mitotic spindle have been observed in prokaryotic cells, raising the question whether prokaryotic cells use a conceptually similar or different mechanism than eukaryotic cells to move chromosomes.

As reviewed in the introductory chapter, time-lapse images of origin movement in both *B. subtilis* and *E. coli* show that origin separation is rapid and occurs soon after the origin regions are duplicated (Gordon et al. 1997; Sharpe and Errington 1998; Webb et al. 1998). Measurements of the movement of the entire nucleoid have differed as to whether bulk nucleoid movement is a gradual process (Sharpe et al. 1998; van Helvoort and Woldringh 1994), or rapid (Donachie and Begg 1989; Hiraga et al. 1990). Distinct mechanisms could govern origin and bulk chromosome partitioning. There are two proposed mechanisms for the motive force for bacterial chromosome movement. These models are not mutually exclusive. In the first model, motor proteins, similar in nature to eukaryotic mitotic motors, bind to and move the origin regions (and/or the entire nucleoid) (Gordon et al. 1997; Hiraga 1992; Webb et al. 1997). In the

second model, the extrusion of DNA by the stationary DNA polymerase provides the motive force for movement (figure 1-7) (Lemon and Grossman 1998).

It is possible that a novel bacterial protein can carry out a function similar to the motors that move eukaryotic chromosomes. Many bacterial genomes, including those of *B. subtilis* (Kunst et al. 1997) and *E. coli* (Blattner et al. 1997) have been completely sequenced. Proteins homologous to kinesin or dynein are not present. The SMC family of proteins, also conserved in bacteria, are structurally similar to mitotic motor proteins, although these proteins seem to play a role in chromosome organization rather than movement (see chapter 1) (Britton et al. 1998; Hirano 1999; Moriya et al. 1998). It may be possible to identify this mitotic-like motor in a genetic screen for colonies with an increase in anucleate cells, similar to that done for *E. coli muk* mutants (Hiraga et al. 1989). I would suspect that a loss-of-function mutation in a partitioning motor gene would not be lethal because, on a simple level, if replication is unaffected and partitioning occurred at random, then ~25% of the cells would be anucleate.

Could DNA replication provide the motive force for origin separation and/or bulk chromosome movement (see Chapter 1, figure 1-7) (Lemon and Grossman 1998)? The rate of replication by DNA polymerase could account for the rate measured for origin separation (Webb et al. 1998). Two observations of Spo0J/ParB localization are consistent with DNA polymerization possibly driving separation. In both cases, DNA polymerization was halted, and the Spo0J/ParB focus remained in the center of the cell. Treatment of *C. crescentus*

cells with hydroxyurea, which inhibits deoxyribonucleotide synthesis and hence DNA elongation, leads to a single ParB focus (which could contain 2 duplicated but unresolvable foci) localizing in the center of the cell (Mohl and Gober 1997). Similarly, when *B. subtilis* thymine auxotrophs are starved for thymine to inhibit DNA elongation, a single Spo0J focus or two partially separated Spo0J foci are located in the center of the cell (Glaser et al. 1997). These results indicate that replication is required for attaching the origin to positions near the poles, although it does not prove that replication is the driving force.

Both the motor model and the replication model could account for origin movement and movement of the entire bacterial nucleoid. In another model, Hiraga *et al* have also proposed that partitioning of the bulk of the nucleoid could occur by chromosome refolding (Niki and Hiraga 1998; Onogi et al. 1999). Hiraga *et al* propose the existence of a "refolding centers" located at the cell quarters (Niki and Hiraga 1998). Perhaps the refolding centers reorganize the newly replicated DNA coming from the polymerase into loops, or compact the newly replicated DNA, and thus contribute to partitioning.

In any model for chromosome partitioning, there must be a way to orient the chromosomes so they move in opposite directions. If the mitotic motor protein model is correct, this raises the question of the track on which it moves, as the track may provide the directionality for movement. In eukaryotic cells, mitotic motors move directionally along a track of microtubules (chapter 1). One idea is that the track for the putative prokaryotic chromosome partitioning motor protein could be laid upon the membrane (see figure 1-7) (Hiraga 1992; Rothfield

1994). A track structure has not been identified in bacteria. The closest homologue to tubulin in prokaryotes is the cell division protein FtsZ (Erickson 1995). However, this protein localizes as a ring at mid-cell, and temperature sensitive *ftsZ* mutants, at the restrictive temperature, appear to partition their chromosomes accurately (Dai and Lutkenhaus 1991). If DNA polymerase provides the motive force, then perhaps the orientation of the replisome influences the direction of movement.

Chromosome Organization model for Spo0J

What step in the partitioning process does Spo0J function? The chromosome organization model that I will describe below proposes that Spo0J functions prior to the attachment of the origins at the cell quarter positions. I believe that the function of Spo0J is important, but not essential, for proper origin separation.

I propose a role for Spo0J in chromosome organization and compaction of the origin region. In this model, I imagine that Spo0J, bound to a *parS* site, could nucleate the assembly of a Spo0J filament, similar to that described for the P1 ParB protein (Rodionov et al, 1999). This Spo0J-*parS* filament would interact with a Spo0J-*parS* filament at another *parS* site on the same chromosome. Interaction of Spo0J filaments at all *parS* sites on the same chromosome would lead to the formation of a specialized nucleoprotein structure that would organize the origin region in a manner that contributes to partitioning.

How would this Spo0J organized origin structure contribute to chromosome partitioning? One possibility is that this structure could help to present the origin regions to a putative partitioning apparatus so that the origin regions can be efficiently moved towards and tethered at the cell quarter positions. Another possibility is that this structure, by essentially compacting the origin region, could help to ensure that all of the origin DNA efficiently moves towards the same daughter cell. The localization of the origin regions is only mildly perturbed in a *spo0J* null mutant (chapter 4). Therefore, the organization of the origin region by Spo0J contributes to, but is not absolutely required for, proper origin movement and tethering at the cell quarter points. I propose that the defects observed in origin localization in *spo0J* mutants are secondary to defects in origin region organization.

How do the observed phenotypes of origin localization in a *spo0J* null mutant (chapter 4) fit with the Spo0J origin region organization model? In *spo0J* mutant cells, compared with wild type cells, there is an increase in the percent of cells with one origin focus or three or more origin foci, but a decrease in the percent with two origin foci (origins are marked with LacI-GFP, chapter 4) (Webb et al, 1997. Assuming that Spo0J does not affect replication, many of the cells with one visible focus may actually contain two origin foci. Perhaps in the absence of Spo0J mediated origin organization, the duplicated sister origin regions become noncovalently entangled, and are visualized as only a single origin focus. Cell division is also delayed in a *spo0J* null mutant (chapter 4; Webb

et al, 1997), so replication may reinitiate much earlier than cell division, leading to an increase in the percent of cells with three or more origin foci.

The chromosome organization model predicts that all the *parS* sites are located near one another in the cell. This is consistent with the localization of Spo0J, where Spo0J is visualized as a single focus rather than eight (or possibly more) separate foci. One could test this prediction more rigorously with FISH using differentially labeled probes specific to the distal most *parS* sites. In wild type cells, I would predict that these probes should colocalize, whereas in *spo0J* mutant cells, I would predict that these probes would be separable in a higher percentage of cells.

Interestingly, *spo0J* null mutations have a synthetic phenotype when combined with *smc* null mutations (Britton et al, 1998). In minimal media, *spo0J* null mutants accumulate ~0.6% anucleate cells, *smc* null mutants accumulate ~10.4% anucleate cells, and *spo0J smc* double mutants accumulate ~26.4% anucleate cells (Britton et al, 1999). SMC, as described in the first chapter, probably has a role in organization and compaction of the entire chromosome (Britton et al, 1998; Hirano, 1999). How does the observed phenotype of the double mutant fit with the origin organization model for Spo0J? In the absence of Spo0J, SMC may still contribute a significant level of organization to the origin region. In the absence of SMC, the origin regions can be organized by Spo0J. In the absence of both proteins, the origin region as well as the rest of the chromosome becomes disorganized, leading to the synthetic partitioning phenotype (Britton et al, 1999).

If Spo0J organizes the origin region by bringing together multiple *parS* sites, this raises the question of how a single *parS* site on a plasmid can stabilize the plasmid. A plasmid with 16 *parS* sites is no more stable than a plasmid with a single *parS* site (chapter 4). One possibility is that the Spo0J filament, nucleated from the single *parS* site, can spread through most of the plasmid, making multiple sites unnecessary. A single Spo0J filament may contribute to compaction of the plasmid. It would be interesting to test whether a larger plasmid requires more *parS* sites for stabilization.

Another possibility is that *B. subtilis parS* stabilizes the plasmid by a mechanism other than simply organizing a single plasmid. For example, in chapter 3, we introduced a cohesion (then called pairing) model for Spo0J, based on the pairing models proposed for the F, P1, and R1 plasmid systems (Austin, 1988 ; Austin and Nordstrom, 1990; Jensen et al, 1998). Cohesion of the duplicated *B. subtilis parS* plasmids, through Spo0J-Spo0J interactions, may help to orient the *parS* containing plasmids prior to movements. It is also possible that this mechanism contributes to chromosome partitioning. Cohesion of chromosomal origins, through interaction of the large Spo0J complex on each sister chromosome (described above), may contribute to yet another level of chromosome organization prior to origin separation (also discussed in chapter 3).

What is the bacterial centromere?

The eukaryotic centromere is multifunctional (reviewed in chapter 1). The eukaryotic centromere is the DNA site where the kinetochore proteins assemble,

where some sister chromatid cohesion proteins function, and where attachment to the microtubules occurs. What is a bacterial centromere sequence? The term centromere is used loosely, since prokaryotes do not employ a mitotic apparatus (described in chapter 1) identical to that in eukaryotic cells. I would define a prokaryotic centromere to be a *cis*-acting sequence, most likely near the origin region, which is required for proper movement and tethering of the origin region to the cell quarter position. I would suspect that insertion of a bacterial centromere into another region of the chromosome would force that region to localize near the cell quarters. Also, deletion of this bacterial centromere would lead to mislocalization of the origin region. I believe that a bacterial centromere sequence does exist because of the ordered localization of the origin region.

The *B. subtilis parS* sequence is probably not a centromere-like site. This idea is in contrast to the model proposed in chapter 2. The evidence that argues against *parS* being a centromere sequence is from the experiments described in chapter 4. When multiple *parS* sites were inserted into the terminus region of the chromosome, Spo0J moved to the center of the cell, rather than the terminus localizing near the cell quarters. This results indicates that Spo0J is not involved in simply tethering *parS* DNA at the cell quarter positions (Webb et al, 1997, 1998). However, in the chromosome organization model, Spo0J does contribute to normal origin movement and attachment.

The mechanism of action of *B. subtilis* Spo0J and *parS* may not be entirely identical to that of the plasmid ParB proteins and *parS* sites. For example, the F plasmid *sopC* (*parS*) site appears to behave as a centromere-like site. Plasmids

without the partitioning locus are localized in the nucleoid free regions, but vectors with the partitioning locus are stable and localize near the cell quarter positions (Niki and Hiraga, 1997). Interestingly, F plasmid SopB (when overexpressed) can localize to the cell quarter positions independent of DNA, and this localization depends on the N-terminal ~70 amino acids of SopB (Kim and Wang, 1998). Most of these 70 amino acids are missing in Spo0J (Spo0J is composed of 282 amino acids and F SopB is composed of 323 amino acids) (Hanai et al. 1996) indicating that Spo0J may not be capable of this particular tethering function.

The plasmid ParB proteins may have two functions that promote partitioning: 1. Sister plasmid cohesion (also called pairing) at midcell (Austin 1988) followed by 2. Plasmid tethering at the cell quarter positions (mediated by the N-terminus of the ParB proteins) (Kim and Wang, 1998). Perhaps Spo0J has evolved to act only in a modification of the first function, in organizing origin regions through interactions of all the *parS* sites. One important experiment would be to test localization of a plasmid with a *B. subtilis parS* site. I would suspect that the *B. subtilis parS* plasmid localizes differently from the chromosomal origin regions.

If the *B. subtilis parS* sequence is not a centromere-like sequence, this raises the question of what, if anything, is the centromere-like site. In both *E. coli* and *B. subtilis*, the origin regions are attached to the cell membrane (chapter 1) (reviewed in (Firshein 1989; Firshein and Kim 1997)). Although it is not known if this membrane attachment is required for partitioning, it is tempting to speculate

that membrane attachment is involved in origin movement and origin tethering near the cell quarter positions. If this were so, the likely candidates for the roles of centromere site and centromere binding proteins would be those factors involved in membrane attachment of the origin region. However, in both *E. coli* and *B. subtilis*, *oriC* plasmids are not stably maintained without selection, indicating that *oriC* is probably not a bacterial centromere site (Moriya et al. 1992; Ogura and Hiraga 1983). It is possible that a region adjacent to *oriC* is a centromere site.

In *B. subtilis*, two origin membrane complexes are detected, called type I and type II (Firshein 1989). The type I membrane complex is salt-resistant, while the type II membrane complex is salt-sensitive. The membrane replication protein DnaB is required for the type I membrane complex, but not type II (chapter 1) (Winston and Sueoka 1980). One strategy for mapping the DnaB binding site, and a potential centromere site, would be to identify the DNA in this complex definitively. Interestingly, the DNA in the type II membrane complex was mapped to ~45 kb counterclockwise to *oriC*, and deletion of this region does not lead to a growth phenotype (it is not clear if the authors examined anucleate cell production) (Itaya et al. 1992). A cluster of three strong *parS* sites is also located ~44-66 kb counterclockwise to *oriC* (Lin and Grossman 1998). It would be interesting to test whether Spo0J is required for type II membrane complex formation

Testing Spo0J for ability to bring together two *parS* sites *in vitro*

In the chromosome organization model described above, I imagine that *parS* sites on the same chromosome, although separated by several kilobases, are brought together by Spo0J-Spo0J interactions while bound to *parS*. One way I have begun to test this *in vitro* is through the use of a ligation-mediated interaction assay. In this assay, if Spo0J can interact with itself while bound to *parS*, as proposed by the chromosome organization model, then Spo0J should increase the rate of intermolecular ligation of two separate DNA fragments that contain *parS*.

Using the *in vitro* ligation-mediated interaction assay, I have found that Spo0J can interact with itself while bound to DNA and can stimulate the rate of intermolecular ligation (appendix 1). However, there was no difference in the Spo0J stimulated rate of intermolecular ligation of *parS*+ or *parS*- DNA, calling into question whether the *in vitro* results are relevant to an *in vivo* function of Spo0J (appendix 1). It is possible that a specificity factor is required. One candidate for this factor is Soj, a ParA homologue, which is required for *B. subtilis* *parS* mediated plasmid partitioning (chapter 3) (Lin and Grossman 1998). Although high concentrations of Soj appeared to decrease the rate of intermolecular ligation (appendix 1), I did not test whether low concentrations of Soj increased the specificity of Spo0J stimulated ligation of *parS*. Soj is a putative ATPase. In the analogous plasmid R1 system, the ATPase ParM stimulates ParR interaction when ParR is bound to the R1 partitioning site *parC* (Jensen et al. 1998). Jensen and Gerdes proposed that this result supports a pairing model for

R1 sister plasmids, which would occur prior to sister plasmid separation. Jensen and Gerdes also demonstrated pairing of R1 plasmids using electron microscopy (Jensen et al. 1998); electron microscopy of Spo0J and *parS* should also be examined.

The stimulation of intermolecular ligation that was observed for Spo0J occurred at concentrations of protein that were lower than the amount used to detect a gel shift (appendix 1). As measured by gel shift assays, the K_d for Spo0J binding is ~300nM (chapter 3), whereas the concentration needed for maximum stimulation of intermolecular ligation is ~70nM (appendix 1, figure A1-2). A gel shift, if any, is barely detectable at ~70nM, but the DNA probe is gradually shifted to slower and slower mobility forms as higher concentrations of Spo0J are used (figure A1-2). An explanation for this difference is that the Spo0J-DNA complex may be easily disrupted during electrophoresis in the gel shift assay, and requires higher concentrations of protein to be detected. However, it is also possible that high concentrations of Spo0J inhibit the ligation reaction because Spo0J can nucleate the assembly of a Spo0J polymer that essentially coats the DNA at high concentrations of DNA. The ability of P1 ParB to act in gene silencing is a reflection of the nucleation of a large ParB complex that can spread >5 kb *in vivo* (Rodionov et al. 1999).

Is there a connection between Spo0J and cell division?

Cell division may be slightly delayed in *Δspo0J* cells, as the mutant cells are ~10% longer than wild type cells (chapter 4) (Webb et al. 1997). The delay in

cell division in a *spo0J* null may be the result of an activated checkpoint function that attempts to briefly stall septation. This delay in cell division may allow other mechanisms to attempt to correct the partitioning defects. However, *Δspo0J* cells still have a partitioning defect since anucleate cells arise and origin localization is not completely wild type (chapter 4) (Ireton et al. 1994) (Webb et al, 1998). Therefore, this checkpoint is only partially capable of correcting the defects. One might expect that a double mutation of *spo0J* with this checkpoint gene would have a synthetic effect.

It is possible that the delay in septation is due to Soj, but the cell size of a *soj-spo0J* double mutant has not been carefully measured. Soj localizes at the extreme poles of cells in wild type cells, but in a *spo0J* null localizes predominantly on the nucleoid. The poles of the cell are the site of the previous cell divisions, and the midcell is a future cell pole. Soj and ParA proteins show similarity to the MinD protein, which is involved in blocking potential division sites near the poles (de Boer et al. 1989; Motallebi-Veshareh et al. 1990). Perhaps in a *spo0J* null, a small portion of the Soj, separate from that localized on the nucleoid, could be activated to delay septation.

How does Spo0J function in sporulation?

Spo0J clearly has two functions, one in chromosome partitioning during exponential growth and the other as an inhibitor of a repressor of sporulation, Soj (Ireton, et al. 1994). Soj inhibits sporulation by repressing expression of genes

required for sporulation (J. Quisel, D. Lin, A. Grossman, manuscript in preparation) (Cervin et al. 1998). As described above, the localization of Soj is different in wild type and *spo0J* null mutant cells. In wild type cells, Soj is localized at the extreme poles of the cell as a ring or band. In *spo0J* null mutant cells, Soj localizes on the nucleoid, where it represses sporulation promoters. We favor the model that Soj is regulated by some aspect of chromosome organization or partitioning that involves Spo0J, rather than the two functions of Spo0J acting independently. This would serve to couple some information about partitioning or chromosome organization to sporulation.

Soj and Spo0J probably interact, based on analogy to the P1 ParA and ParB system (Bouet and Funnell 1999; Davis et al. 1992). Because the proteins do not co-localize, Spo0J probably does not simply sequester Soj from repressing sporulation promoters. Rather, Spo0J probably regulates Soj activity by regulating a stable change (such as ATP or ADP binding) in the Soj protein. The question remains, under what conditions do Spo0J and Soj interact *in vivo*? How does Spo0J regulate Soj, and how might this be important for sporulation? I will discuss two of the many possible models. The first model is that Spo0J, when localized near the cell quarters, regulates Soj. The second model is the Spo0J focus, regardless of location in the cell, regulates Soj. In the first model, location of the focus is important for sporulation. In the second model, the formation of the focus itself is important for sporulation. Both models could explain mechanisms to relay some information about the chromosome partitioning process to sporulation. For example, in the first model, localization of Spo0J near

the cell quarter positions may signal that two chromosome are about to be formed (rather than a single unduplicated Spo0J focus, which is at midcell). In the second model, the formation of a Spo0J focus may relay some information about chromosome organization or chromosome integrity.

The limited available data make it hard to favor either model, but at least one result is inconsistent with the first model. When an array of 16 *parS* sites was placed into the terminus region, Spo0J localized in the center of the cell, but the cells sporulated at wild type levels (chapter 4). However, the localization of Spo0J was done in vegetative cells, so needs to be examined for sporulating cells. Also, Soj should be localized in these cells, to test whether Soj remains at the poles. If, during sporulation, Spo0J is at midcell in the *ter::parS16* strain and Soj is at the poles, then this result would be inconsistent with the first model.

Results consistent with the second model, with Soj active as a repressor in the absence of a Spo0J focus, comes from studies of the *spo0J93* allele. *spo0J93* cells are defective for sporulation (Hranueli et al. 1974) and chromosome partitioning (K. Ireton and AD Grossman, unpublished) and contain a missense mutation in a conserved amino acid (appendix 2). By immunofluorescence microscopy and visualization of a Spo0J93-GFP fusion protein, I observed that the mutant protein does not form a focus but localizes throughout the cell (appendix 2). Since the Spo0J focus is clearly not formed, it is consistent with the idea that Soj responds to Spo0J focus formation. *spo0J93* is probably not a simple loss-of-function allele: Spo0J93 is capable of binding to *parS in vivo* as shown by formaldehyde cross-linking (D. Lin and A.D. Grossman, data not shown). *In*

vitro, purified Spo0J93 can increase the rate of intermolecular ligation of two DNA molecules (as measured by the protocol in appendix 1), indicating that Spo0J93 can interact with itself and with DNA (D. Lin and A.D. Grossman, data not shown).

To test the second model further, it would be interesting to find conditions where the Spo0J focus was perturbed without affecting the Spo0J protein. The size of the Spo0J focus can be reduced without affecting sporulation. The Spo0J focus was ~2-3 fold smaller, as judged by eye, in cells missing six *parS* sites compared with wild type (D. Lin and AD Grossman, unpublished data). However, these cells were wild type for sporulation. It would be interesting to see if deletion of all eight known *parS* sites leads to mislocalization of wild type Spo0J and a defect in sporulation.

Our understanding of how Spo0J influences sporulation and what Soj is responding to is limited. We only know of a few conditions under which Soj represses sporulation, namely in the absence of Spo0J (an artificial situation), and from a limited characterization of the *spo0J93* allele. Interestingly, *soj* null mutations do partially suppress the sporulation defect of an *ftsA* allele (Ireton, et al. 1994). *ftsA* is normally involved in cell division (Sánchez et al. 1994), and it is not known if *B. subtilis* FtsA is involved in some aspect of chromosome partitioning or how this may relate to Spo0J . It would be a breakthrough to be able to define other conditions where Soj is activated to repress sporulation, and to see how these conditions affect Spo0J. In the future, it will be important to begin analyzing Spo0J by making site directed mutations or by alanine scanning

mutagenesis. These mutant proteins can be tested for sporulation, localization, partitioning, and DNA binding.

Future Directions in Bacterial Chromosome Partitioning

Our current understanding of bacterial chromosome partitioning is limited since only a small handful of genes in this process have been identified in bacteria. Further, their functions have not been absolutely defined. The molecular understanding of bacterial mitosis awaits the identification of other partitioning factors. If DNA replication initiation and elongation proteins contribute to partitioning, then characterization of their partitioning activities is crucial. Perhaps a genetic screen, such as that done for *E. coli muk* mutants (Hiraga et al. 1989), will prove fruitful in identifying other partitioning proteins.

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Appendix 1

Testing whether Spo0J-*parS* can interact with Spo0J-*parS* through *in vitro* ligation assays

I have tested whether Spo0J bound to *parS* can interact with Spo0J bound to *parS*on a separate molecule. I explored this *in vitro* mainly by using a ligation assay (figure A1-2 to A1-4). I also tested Spo0J-*parS*/ Spo0J-*parS* interactions with a gel shift assay (figure A1-1), although I did not pursue this route because of success with the ligation assay.

The ligation mediated assay was also used by Jensen *et al* to show interactions of the plasmid R1 partition protein ParR boundto the R1 centromere site *parC* (Jensen et al. 1998). In this assay for Spo0J, I used a labeled DNA probe with restriction enzyme produced sticky ends. If the Spo0J bound to *parS* can interact with another Spo0J-*parS* complex, then then this may increase the rate of intermolecular ligation of the DNA probe. The ligation reactions are quenched with a Proteinase K/EDTA stop buffer and the products are separated on a native acrylamide gel.

The main conclusions from this work are that Spo0J can interact with itself when bound to DNA, although Spo0J shows low specificity for wild type *parS* DNA. Also, preliminary results show that addition of high concentrations of Soj inhibited the Spo0J mediated stimulation of intermolecular ligation, although this could be due to a number of non-specific reasons.

Below is a brief protocol of the ligation assays shown in Figure A1-2 to A1-4. The rationale of the experiments and discussion of the results are in the figure legends.

Ligation Assay to explore Spo0J-*parS*/ Spo0J-*parS* interactions:

Materials

Spo0J-his6: Purified from DCL128 (chapter 2) (Lin et al. 1997) using a Ni-NTA resin according to manufacturer's protocol (Qiagen Corporation). Spo0J stored in : 20mM Tris pH8, 250mM KCl, 1mM DTT and 10% glycerol. (purified from 8/29/98-9/9/98 in my laboratory notebooks). Use of KCl (instead of NaCl (Lin and Grossman 1998)) is important since Na2+ inhibits DNA ligase.

T4 DNA Ligase: 400 NEB U/µl (New England Biolabs)

Reaction conditions: 20mM Hepes pH 7.6, 1mM DTT, 3.5mM MgCl2, 1mM ATP, and ~67mM KCl (supplied from Spo0J-his6 buffer).

Stop Buffer: 150mM EDTA, 4 mg/ml Proteinase K, 30% glycerol, bromphenol blue and xylene cyanol dyes (a tiny pinch was added when 10ml of Stop buffer was made). Add 4µl of Stop solution/ 15µl aliquot

Plasmids used to make probes: pDL135 (chapter 4), pDL169 (same as pDL135 but contains 7 mutations in *parS* (*parS*-).

Protocol

1. Assemble a master reaction of DNA and Spo0J-his6 protein and incubate at 32°C for 15 minutes before addition of ligase

2. Take reactions out of 32°C, place at room temperature

3. Take a 0′ timepoint (15µl aliquot) prior to addition of ligase and add 4µl proteinase K/EDTA stop solution to 0′ timepoint

4. Add DNA ligase to final concentration of 16 NEB U/µl to remaining master reaction

5. Ligate at room temperature

6. At desired timepoints (e.g. 3′, 9′, 27′), take a 15µl aliquot from the master reaction and quench with the addition of 4µl of Proteinase K/EDTA stop solution

7. Incubate all samples at 37°C for ~10 minutes prior to loading on a gel to allow Proteinase K to digest polypeptides

8. Load sample onto a acrylamide gel. I usually poured a 8% (37.5:1) acrylamide gel in 0.5X TBE buffer.

9. Dry gel and expose to phosphorimager cassette

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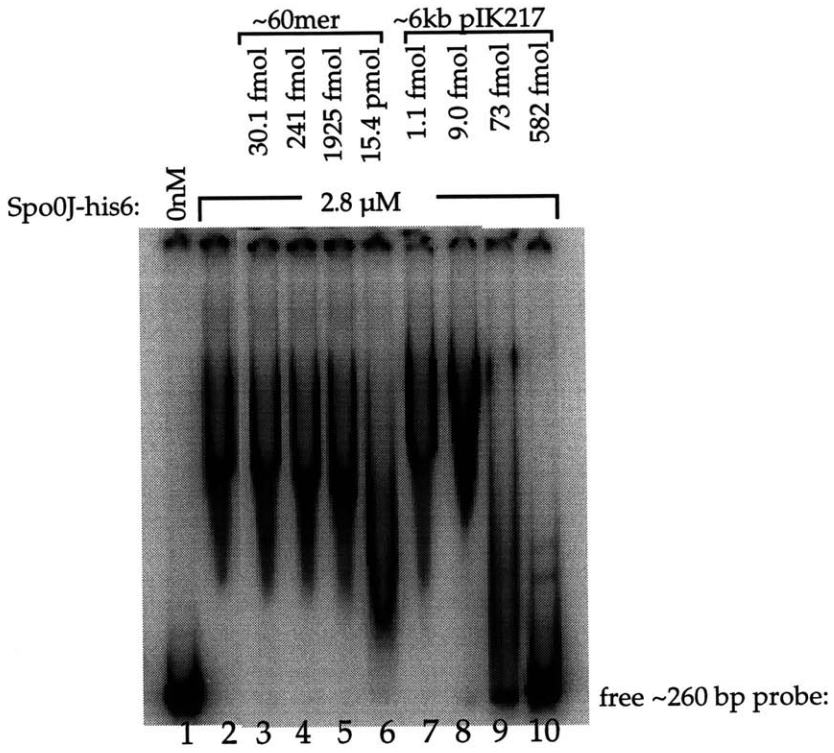


Figure A1-1. Gel Shift Spo0J-*parS*/ Spo0J-*parS* Interaction Assay
Probe: ~260 bp *parS*+ probe with XbaI sticky ends

The rationale behind the Gel Shift Interaction Assay is that if a Spo0J-*parS*/ Spo0J-*parS* complex exists, I may be able to detect a mixed complex, where the DNA molecules in the complex are of two different sizes. Assuming that a mixed complex migrates differently from a complex in which the DNA molecules in the complex were identical, then I should be able to detect the mixed complex on a native gel.

In this assay, I compared the effects of two unlabeled *parS*+ DNA molecules of very different sizes (~60 bp vs. ~6 kb plasmid) on the mobility shift of a labeled ~260 bp *parS*+ DNA probe. If a mixed complex exists, I expected to see different species when I added the ~60 bp DNA versus the 6 kb pIK217 plasmid. Addition of 2.8 µM Spo0J-his6 produced a gel shift (lane 2). I next added increasing amounts of a ~60 mer unlabeled DNA (annealed oligos LIN-116 and LIN-117, lanes 3-6). Higher amounts of competitor led to a increased mobility species, which could represent a mixed complex or less Spo0J-his6 bound to the DNA due to competition (lane 6). When increasing amounts of unlabeled plasmid pIK217 (pIK217 contains *parS*+) were added, the band supershifted before being competed away (lanes 7,8 supershift; lanes 9,10 competition). If a stable plasmid+260mer complex exists, I expect that this species would not be able to enter the gel. I speculate that the supershifted species seen in the presence of small amounts of pIK217 represents a transiently paired complex that entered the gel more slowly. I did not pursue this further since the ligation-mediated interaction assays were promising.

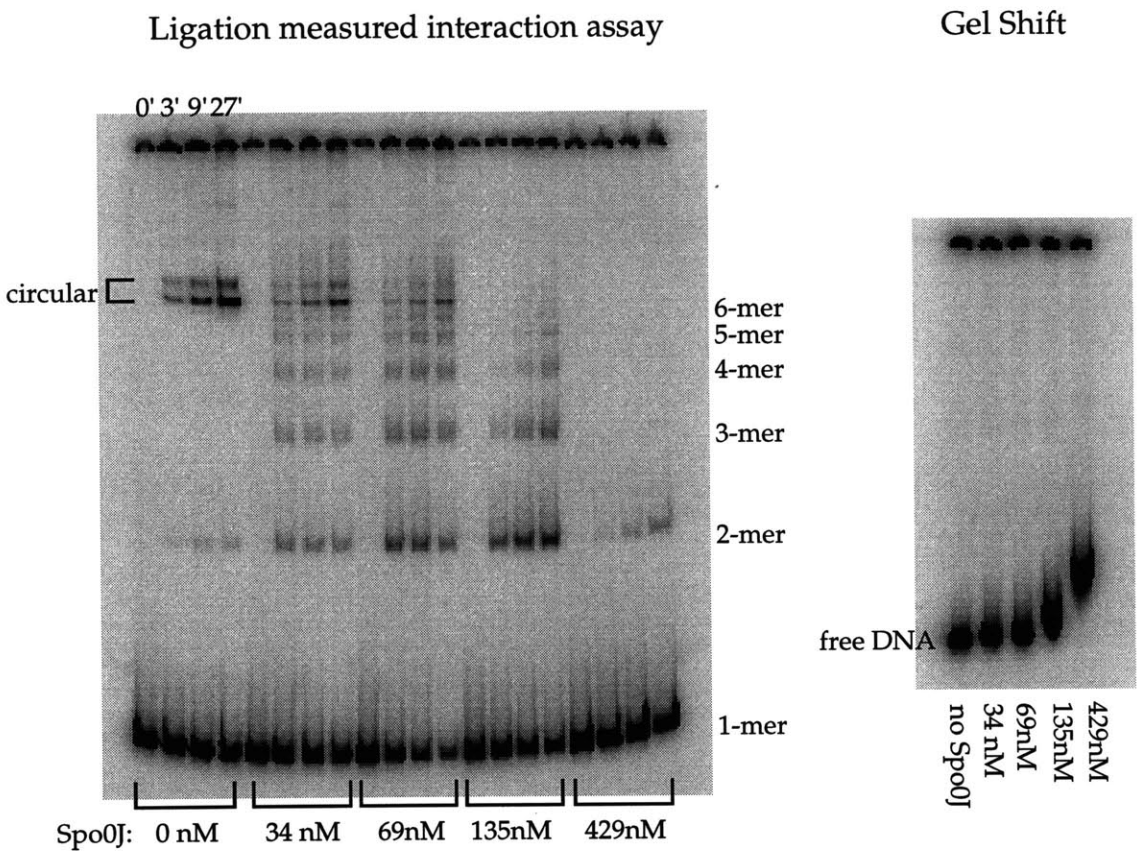
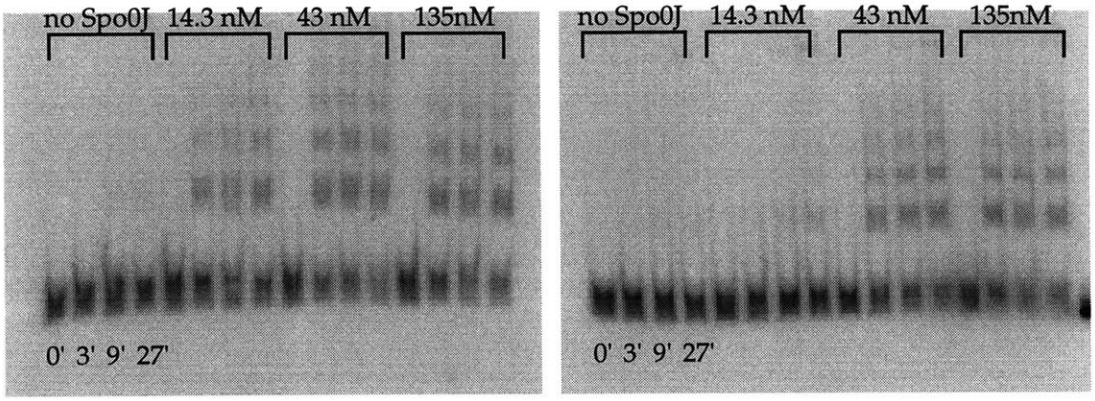


Figure A1-2. Ligation measured Spo0J-*parS*/ Spo0J-*parS* interaction assays

DNA probe: ~260 bp with XbaI sticky ends

Spo0J-*parS*/ Spo0J-*parS* Interaction Assay measured with DNA ligase: Ligase was added at time=0'. A time course was performed with 4 different concentrations of purified Spo0J-his6, and a control with no Spo0J-his6 protein. Aliquots were taken at 0', 3', 9', and 27' and quenched with a proteinase K/EDTA stop solution. The addition of Spo0J stimulated intermolecular ligation. In a separate experiment, similar amounts of BSA or lysozyme did not stimulate intermolecular ligation, indicating that the effect of Spo0J-his6 was specific and not due to macromolecular crowding (data not shown). In another experiment, the circular species were determined by its insensitivity to Exonuclease III treatment (data not shown).

Gel Shift: Before ligase was added to initiate the assay, an aliquot was taken from each reaction and loaded directly onto a native polyacrylamide gel (see Chapter 3 Experimental Procedures). Note that at high concentrations of Spo0J-his6 (429 nM), the inter- and intra-molecular ligation is decreased, but the probe is still bound by Spo0J-his6 and migrates more slowly. This is probably due to Spo0J-his6 coating the DNA.



parS+ *parS*-

Figure A1-3. Spo0J-DNA/ Spo0J-DNA interaction shows low preference for *parS*+ DNA.

Ligation measured interaction assays were performed similarly to those described in Figures A1-2.

Several probes were made containing either the *parS*+ or *parS*- sequence. The *parS*+ sequence, in all ligation assays, contains the *parS* sequence internal to *spo0J* (chapter 3). The *parS*- sequence contains the 7 bp mutations that is not recognized by Spo0J *in vivo* (chapter 3). A ligation measured interaction assay with an ~100 bp probe with EcoRI sticky ends is shown. Different concentrations of Spo0J-his6 were tested for the *parS*+ probe (left) and the *parS*- probe (right). Addition of 14.3 nM Spo0J-his6 stimulated Spo0J-*parS*+/ Spo0J-*parS*+ interactions, but not Spo0J-*parS*-/ Spo0J-*parS*- interactions. However, addition of 43nM Spo0J-his6 stimulated Spo0J-*parS*- / Spo0J-*parS*- interactions to the same extent as addition of 14.3nM did to Spo0J-*parS*+/ Spo0J-*parS*+ interactions. Thus, Spo0J-DNA interacting with Spo0J-DNA was ~2-3 more efficient with *parS*+ DNA over *parS*- DNA. Similar results were obtained with ~60 bp probes with XbaI sticky ends.

Specificity and ability to interact is sensitive to both DNA concentration and size. When the DNA concentration was increased 20X over what is shown above, then no specificity for *parS*+ was detected. Also, Spo0J-his6 showed no specificity for ~260 bp probe over a wide concentration range of DNA. Finally, Spo0J could not stimulate intermolecular ligation of a ~24 bp probe with XbaI sticky ends, probably due to steric hindrance of DNA ligase by Spo0J-his6 bound to DNA (data not shown in all cases).

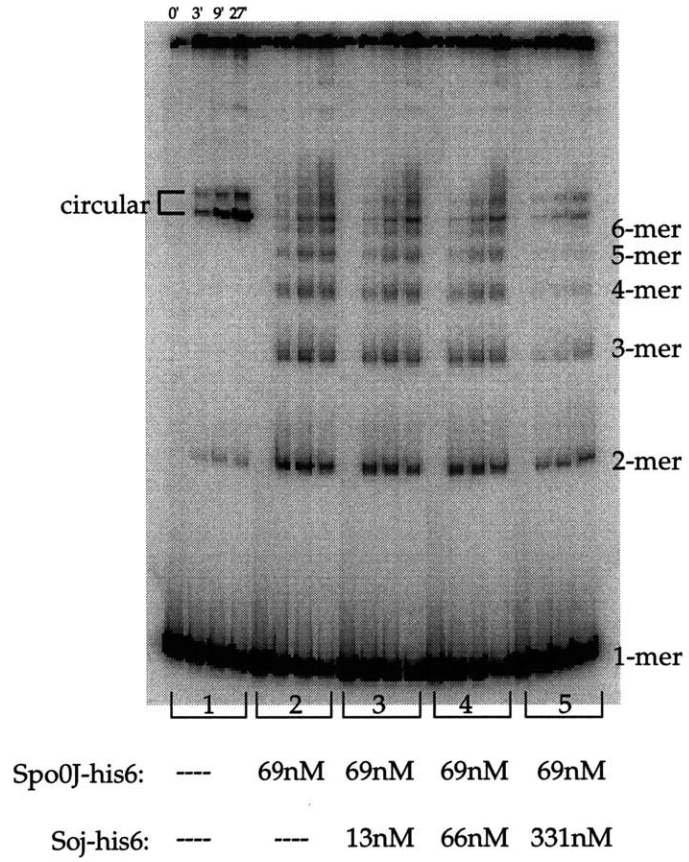


Figure A1-4. Effect of Soj on Spo0J-*parS*/ Spo0J-*parS* interactions

Ligation measured interaction assays were performed as in Figure A1-2 with either no (first set) or 69nM Spo0J-his6 (last 4 sets). To test the effect of Soj-his6 in this reaction, increasing amounts of Soj-his6 were added (last three sets). Adding approximately 5X molar excess of Soj (331nM) led to a decrease in Spo0J-mediated intermolecular ligation. Note that the intramolecular circular products do not appear to be affected as strongly as the intermolecular products (compare the 2nd set to the 5th set).

In the future, several experiments can be done to test if this is a specific effect of Soj on Spo0J-*parS*/ Spo0J-*parS* interactions. First, reactions should be done with Soj alone, to test whether Soj affects the natural intermolecular ligation rate. Secondly, it would be interesting if Soj ATPase mutants had differential effects on Spo0J-*parS*/ Spo0J-*parS* interactions. Finally, gel shift reactions should be done in parallel, to see if Soj-his6 is stably binding to the complex.

Appendix 2

Characterization of the *spo0J93* allele

Spo0J93

The *spo0J93* allele was originally identified in a screen for sporulation mutants (Errington 1993; Hranueli et al. 1974). The sporulation and chromosome partitioning phenotype of *spo0J93* cells is similar to that in *spo0J* null mutant cells (Ireton et al. 1994) (K. Ireton and AD Grossman, unpublished data).

I sequenced the *spo0J93* allele. Spo0J93 contains a glycine to serine missense mutation at position 77 (Figure A2 part A). This glycine residue is conserved in many of the plasmid and chromosomal ParB homologues (Hanai et al. 1996). I also localized Spo0J93 by both immunofluorescence (data not shown) and a Spo0J93-GFP fusion protein (Figure A2 parts B-E). Whereas wild type Spo0J localizes predominantly as two large foci in the cell, Spo0J93 localizes throughout most of the cell, perhaps predominantly on the nucleoid. This localization pattern could also be interpreted as localization in both the cytosolic space and on the nucleoid.

The Spo0J93 Gly77Ser mutation probably does not cause global misfolding of the protein. Formaldehyde crosslinking immunoprecipitation experiments reveal that Spo0J93 can interact with *parS* DNA *in vivo*. In addition, Spo0J93-his6 can interact with itself; purified Spo0J93-his6 can stimulate intermolecular ligation of DNA (described in appendix 1, data not shown). One possibility, consistent with the subcellular localization data, is that Spo0J93 interacts nonspecifically with DNA. This can be easily tested with formaldehyde crosslinking IPs (protocol described in chapter 3) using PCR primers to chromosomal regions distal to *parS* DNA, or with gel shift assays (chapter 3).

A more extensive discussion of Spo0J93 is presented in chapter 5.

Plasmids used:

pDL70A and pDL70B: These plasmids are identical but were isolated from different colonies when constructed. These plasmids carry *spo0J93* in pGem-cat (Harwood and Cutting 1990) and were used as a source of template in the sequencing reactions. These plasmids were constructed by amplifying *spo0J93* from AG146 chromosomal DNA using primers 0J-2 and 0J-3 (Ireton et al. 1994), that flank *spo0J*. The resulting PCR product was digested with EcoRI and BamHI (the primers contain EcoRI and BamHI sites) and inserted into pGem-cat that had been digested with EcoRI and BamHI. Two separate isolates (pDL70A and pDL70B) were sequenced, and both contain the same mutation in *spo0J93*. Sequencing of pDL70A and pDL70B was performed with the Sequanase kit (Stratagene) using primer 0J-2.

pDL78: This plasmid contains *spo0J93-his6* under the control of the T7 promoter in pET21 (+) (Novagen) and was used to overexpress Spo0J93-his6 for protein purification. This plasmid was constructed by amplifying *spo0J93* from pDL70A using PCR with primers 0J-2 (Ireton, et al. 1994) and LIN-57. LIN-57 contains an engineered XhoI site which allows *spo0J93* to be inserted in frame at the 3′ end to the hexa-histidine tag in pET21 (+). The resulting PCR product was digested with EcoRI and XhoI and inserted into pET21 (+) that had been digested with EcoRI and XhoI.

pDL148: This plasmid contains the 3′ end of *spo0J* fused in frame to *gfp*. pDL148 was recombined into wild type (AG174) and *spo0J93* (AG146) cells by single crossover to create Spo0J-GFP and Spo0J93-GFP fusion proteins, respectively. pDL148 was constructed by excising all but the 3′ end of *spo0J* from pDL50B (pDL50B contains full length *spo0J-gfp*; chapter 2) by digesting pDL50B with EcoRI and XcmI, blunting the ends with T4 DNA polymerase, and recircularizing the plasmid.

B. subtilis and *E. coli* strains used:

strain name	genotype
AG146	*spo0J93 trp phe* (JH642 background)
DCL580	*spo0J-gfp (pDL148) trp phe*
DCL581	*spo0J93-gfp (pDL148) trp phe*
DCL349	BL21λDE3 (*E. coli*) with pDL178 (*spo0J93-his6*)

References:

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Hanai, R., R. Liu, P. Benedetti, P.R. Caron, A.S. Lynch and J.C. Wang. 1996. Molecular dissection of a protein SopB essential for *Escherichia coli* F plasmid partition. *J Biol Chem.* **271:** 17469-75.

Harwood, C.R. and Cutting, S.M. 1990. Molecular Biological Methods for *Bacillus*. (Chichester, England: John Wiley & Sons).

Hranueli, D., P.J. Piggot and J. Mandelstam. 1974. Statistical estimate of the total number of operons specific for *Bacillus subtilis* sporulation. *J Bacteriol.* **119:** 684-90.

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

A.

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amino acid position  ...74  75  76  77  78  79...

                         ATT GTT GCG GGT GAA CGG
wild type Spo0J          Ile Leu Ala Gly Glu Arg

                         ATT GTT GCG AGT GAA CGG
Spo0J93                  Ile Leu Ala Ser Glu Arg
```

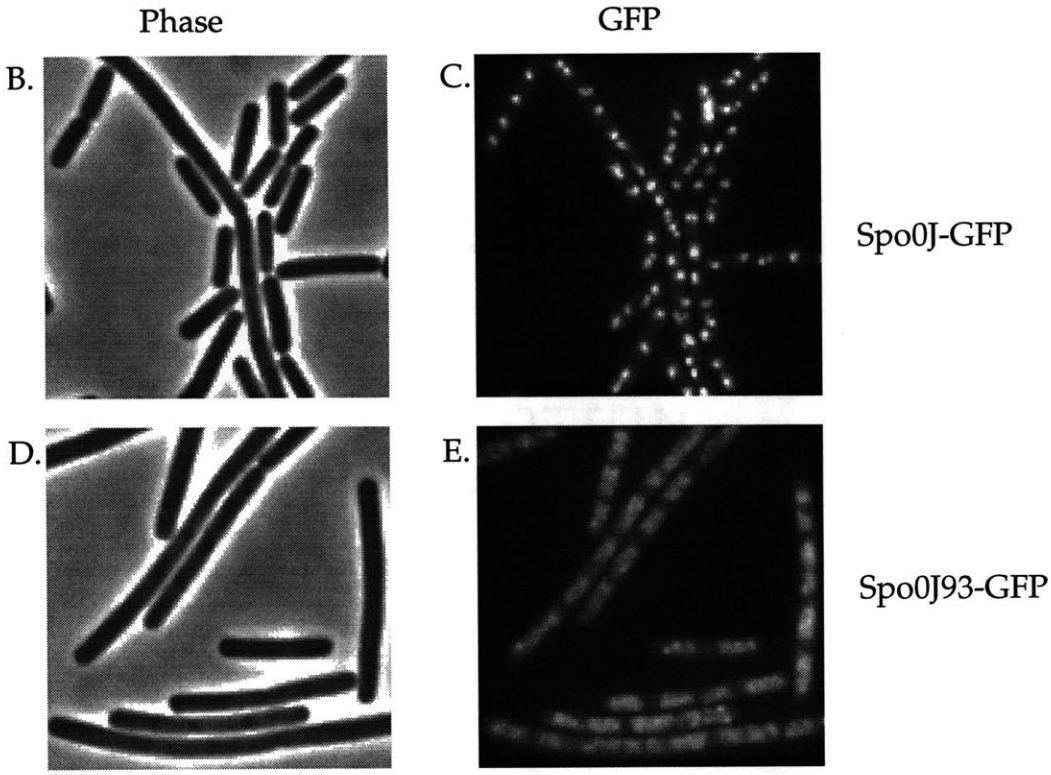

Figure A2. Sequence of the *spo0J93* allele and localization of Spo0J93-GFP.

A. DNA and protein sequence of wild type Spo0J and Spo0J93. *spo0J93* contains a G to A change in a glycine codon that results in a glycine to serine missense mutation at amino acid residue 77.

B-D. Localization of Spo0J-GFP (B, C; strain DCL580) and Spo0J93-GFP (D, E; strain DCL581). Cells were grown in S750 media with 1% glucose as the carbon source at 30°C. Cells were examined near mid-exponential growth. Both phase contrast (B, D) and fluorescence (C, E) photomicrographs were taken.

THE END