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Recruitment of SMC by ParB-*parS* Organizes the Origin Region and Promotes Efficient Chromosome Segregation

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SUMMARY

Organization and segregation of replicated chromosomes are essential processes during cell division in all organisms. Similar to eukaryotes, bacteria possess centromere-like DNA sequences (*parS*) that cluster at the origin of replication and the structural maintenance of chromosomes (SMC) complexes for faithful chromosome segregation. In *Bacillus subtilis*, *parS* sites are bound by the partitioning protein Spo0J (ParB), and we show here that Spo0J recruits the SMC complex to the origin. We demonstrate that the SMC complex colocalizes with Spo0J at the origin and that insertion of *parS* sites near the replication terminus targets SMC to this position leading to defects in chromosome organization and segregation. Consistent with these findings, the subcellular localization of the SMC complex is disrupted in the absence of Spo0J or the *parS* sites. We propose a model in which recruitment of SMC to the origin by Spo0J-*parS* organizes the origin region and promotes efficient chromosome segregation.

INTRODUCTION

A fundamental unsolved problem in the biology of bacteria is how chromosomes are organized and faithfully segregated during the cell cycle. Insights into these processes have emerged from cytological methods to visualize specific positions on the chromosome and their movement during growth and division. In *Bacillus subtilis*, the newly replicated origins move from mid-cell toward opposite cell poles (Webb et al., 1998). Moreover, the location of a particular region of the chromosome inside the cell correlates with its position in the genome (Nielsen et al., 2006; Niki et al., 2000; Teleman et al., 1998; Viollier et al., 2004; Wu and Errington, 1998). How this organization is achieved and how the factors responsible for its maintenance participate in chromosome segregation are still poorly understood.

Two of the most highly conserved factors implicated in both the organization and segregation of bacterial chromosomes are the structural maintenance of chromosomes (SMC) condensation complex and the chromosomally encoded plasmid partitioning system (Britton et al., 1998; Livny et al., 2007). SMC complexes are present in all eukaryotes and in most bacteria (Hirano, 2006; Nasmyth and Haering, 2005). In eukaryotes, they participate in mitotic chromosome condensation, sister chromatid cohesion, recombination, and X chromosome dosage compensation. In *B. subtilis*, the SMC complex (composed of SMC, ScpA [the kleisin subunit], and ScpB) is required for chromosome compaction and faithful DNA segregation (Britton et al., 1998; Hirano and Hirano, 2004; Mascarenhas et al., 2002; Soppa et al., 2002). In chromatin immunoprecipitation (ChIP) experiments, *B. subtilis* SMC can be crosslinked to all regions of the genome, suggesting that it acts throughout the chromosome (Lindow et al., 2002). However, subcellular localization of SMC indicates that it is also concentrated in discrete foci (Britton et al., 1998; Mascarenhas et al., 2002). The function of these foci remains unclear (Lindow et al., 2002; Volkov et al., 2003). The loss of chromosome condensation in the absence of the SMC complex suggests that bacterial SMC is most similar to eukaryotic condensin (Hirano, 2006; Nasmyth and Haering, 2005). How SMC complexes function to compact bacterial and eukaryotic chromosomes is not known.

The plasmid-encoded *par* locus consists of two genes often called *parA* and *parB* and a centromere-like sequence referred to as *parS*. All three elements are essential for faithful plasmid inheritance (Ebersbach and Gerdes, 2005). ParB binds to its cognate *parS* site and spreads along the DNA forming a nucleoprotein complex. ParA proteins are Walker-box ATPases that act on the ParB-*parS* complex to partition the plasmids toward opposite cell poles. Chromosomally encoded orthologs of ParA, ParB, and *parS* have been identified in >65% of all sequenced bacterial genomes (Livny et al., 2007). In almost all cases, the *parS* site is located in close proximity to the origin of replication. Moreover, most genomes have more than one origin-proximal *parS* (Livny et al., 2007). Work in several model organisms indicates that the chromosomal partitioning system performs a similar function to its plasmid counterpart. However, instead of segregating entire chromosomes, the chromosomal partitioning system participates in repositioning of the replicated origins toward opposite cell poles (Fogel and Waldor, 2006; Lee and Grossman, 2006; Toro et al., 2008; Wu and Errington, 2002, 2003).

In *Bacillus subtilis*, the ParA protein is called Soj and the ParB protein is referred to as Spo0J (Ireton et al., 1994). Ten *parS* sites

have been identified in the *B. subtilis* chromosome. Eight of these sites (with the highest affinity for Spo0J) are located in the origin-proximal 20% of the chromosome (Breier and Grossman, 2007; Lin and Grossman, 1998; Murray et al., 2006). Both Soj and Spo0J are required to maintain an unstable plasmid in which a *parS* site has been inserted (Lin and Grossman, 1998; Yamaichi and Niki, 2000). Moreover, both proteins are necessary for efficient repositioning of chromosomal origins (Lee and Grossman, 2006). Interestingly, a Soj (ParA) mutant has virtually no defect in chromosome segregation as assayed by the production of anucleate cells (Ireton et al., 1994). This result suggests that functionally redundant mechanisms ensure faithful chromosome segregation in the absence of efficient origin repositioning. Consistent with this idea, cells lacking Soj and the chromosome condensation protein SMC have a synthetic chromosome segregation defect (Lee and Grossman, 2006). Paradoxically, unlike Soj mutants, cells lacking Spo0J (ParB) are defective in chromosome segregation. In a Spo0J mutant, 1%–2% of the cells are anucleate, a frequency ~100-fold higher than wild-type (Ireton et al., 1994). It is unclear why Spo0J plays a more central role than Soj in faithful chromosome segregation. One possible explanation is that, in addition to its role in origin segregation, Spo0J has been implicated in chromosome organization.

Using assays to study chromosome organization during sporulation, it was observed that the origin region of the chromosome is disorganized in cells lacking both Soj and Spo0J (Lee et al., 2003; Sharpe and Errington, 1996; Wu and Errington, 2002). Spo0J mutants cannot enter sporulation but are suppressed by a mutation in *soj* (Ireton et al., 1994). Importantly, chromosome organization appears normal in the absence of Soj, suggesting that Spo0J alone is responsible for organizing the origin region. ChIP experiments indicate that Spo0J binds all eight origin-proximal *parS* sites in vivo (Breier and Grossman, 2007; Lin and Grossman, 1998; Murray et al., 2006), and fluorescence microscopy suggests that Spo0J localizes as a single focus per origin (Glaser et al., 1997; Lewis and Errington, 1997; Lin et al., 1997). These results have led to the current view that Spo0J organizes the origin region by gathering the dispersed origin-proximal *parS* sites into a single nucleoprotein complex.

Here, we investigate how Spo0J bound to *parS* organizes the origin region. Using a single-cell-based assay to quantitatively assess chromosome organization and deletions of the origin-proximal *parS* sites, we show that gathering dispersed *parS* sites is not the mechanism by which Spo0J organizes the origin region. These findings led us to the discovery that Spo0J bound to *parS* recruits the SMC condensation complex to the origin. We show that SMC foci are lost in the absence of Spo0J or the eight origin-proximal *parS* sites. Moreover, insertion of *parS* sites near the terminus targets the SMC complex to this ectopic position and causes gross perturbations to chromosome organization and segregation. Finally, we show that purified SMC binds Spo0J-coated DNA with higher affinity than naked DNA or DNA coated with an unrelated DNA-binding protein. All together, our data support a model in which recruitment of the SMC complex to the origin by Spo0J-*parS* organizes the origin region and promotes efficient chromosome segregation. These data link two of the most highly conserved factors in chromosome dynamics and suggest that targeting SMC complexes to the origin by ParB bound to *parS* is likely to be a feature of chromosome organization and segregation in many bacteria. In addition, interesting parallels exist between the recruitment of the *B. subtilis* SMC complex to the origin and the targeting of the SMC dosage compensation complex to the X chromosomes in *C. elegans*. Finally, these data highlight fundamental similarities and important differences in how chromosomes are faithfully segregated in bacteria and eukaryotes.

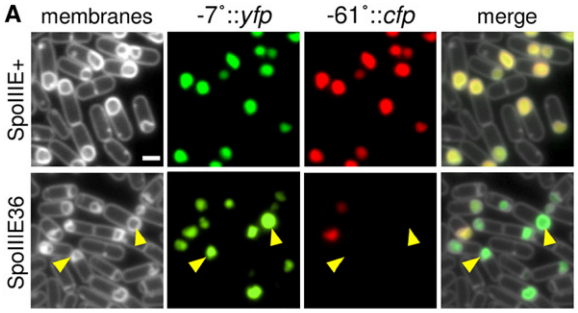
RESULTS

A Quantitative Single-Cell Assay to Analyze Chromosome Organization

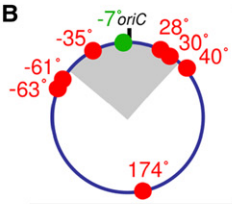
To quantitatively assess the roles of Spo0J and the *parS* sites in organizing the chromosome, we modified an assay originally described by Wu and Errington (Wu and Errington, 1998) to monitor the organization of the replicated chromosomes during sporulation. Sporulating *B. subtilis* cells divide asymmetrically generating a large mother cell and a small forespore. Prior to polar division, the replicated chromosomes adopt an elongated structure that extends from one cell pole to the other (known as the axial filament). The origins reside at the extreme poles and the termini at mid-cell. As a result of axial filament formation, the polar division plane traps approximately one-third of the forespore chromosome in the small spore compartment. The rest of the chromosome is then pumped into the forespore by a DNA translocase called SpoIIIE (Wu and Errington, 1994). The original assay and our modified version take advantage of a mutant in the SpoIIIE translocase (*spoIIIE36*) that engages the forespore chromosome after polar division but is blocked in DNA transport. Using this mutant, the organization of the axial filament at the time of division can be assessed by monitoring which regions of DNA are trapped in the spore compartment by the polar septum. To do this, we fused *cfp* and *yfp* to a promoter (P_{spoIIQ}) that is recognized by a forespore-specific transcription factor. These two reporters were inserted at different positions on the *B. subtilis* chromosome (Figure 1B). Accordingly, depending on their location in the axial filament, the spore compartment contained one, both, or neither of the fluorescent reporters (Figure 1A). The original assay was a population-based assay using a *lacZ* reporter inserted at different chromosomal positions (Wu and Errington, 1998). The assay described here monitors every cell in the field and provides greater sensitivity allowing us to detect and quantify more subtle perturbations in chromosome organization.

Synchronous sporulation was induced and CFP and YFP fluorescence were analyzed 30–45 min after polar division was complete to allow for synthesis and maturation of the fluorescent proteins. Because DNA transport is blocked, the results provide a "snapshot" of the organization of the axial filament at the time of polar division. Assisted by imaging software, we assessed chromosome organization in 400–1000 sporulating cells per field (Figure S1 available online). Only small variations were observed in six independent experiments (Figure S2).

For our experiments, we placed one promoter fusion (*yfp*) at a site (−7°) close to the origin of replication. This chromosomal position is located near the cell pole during sporulation and is trapped in the forespore in 97%–99% of the cells (the sum of the first two classes in Figure 1B). This reporter served as our baseline site

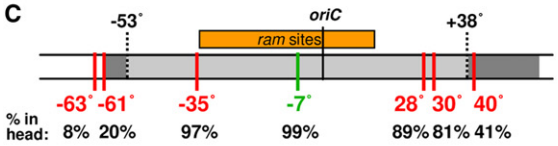


B

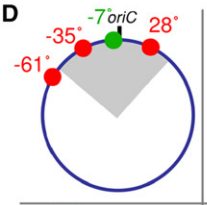


	reporter in body	reporter in head	origin in body
-35°	3%	94%	3%
+28°	11%	88%	1%
+30°	19%	79%	2%
+40°	59%	40%	1%
-61°	80%	20%	0%
-63°	92%	8%	0%
+174°	100%	0%	0%

C



D



		reporter in body	reporter in head	origin in body	both loci in body
+28°	WT	12%	86%	2%	0%
	Δsoj	9%	72%	16%	2%
	Δ(soj spo0J)	41%	32%	14%	13%
-35°	WT	4%	95%	1%	0%
	Δsoj	2%	77%	21%	0%
	Δ(soj spo0J)	8%	61%	11%	20%
-61°	WT	82%	18%	0%	0%
	Δsoj	57%	16%	1%	26%
	Δ(soj spo0J)	48%	36%	1%	15%

Figure 1. Quantitative Chromosome Organization Assay

(A) Fluorescent images of sporulating cells containing the YFP forespore reporter (false-colored green) at −7° and the CFP forespore reporter (false-colored red) at −61°. Cells harboring the wild-type DNA translocase (SpoIIIE+) efficiently pump the chromosome and all forespores contain YFP and CFP fluorescence. In the pumping-deficient mutant (SpoIIIE36), the fluorescent image provides a "snapshot" of the organization of the chromosome at the time of polar division. In many cells the CFP reporter at −61° is not polarly localized and these forespores only contain the −7° YFP reporter (arrowheads). Scale bar, 1 μm.

(B) Quantitative analysis of the CFP reporter inserted at positions in the chromosome (red circles) relative to the −7° YFP reporter (green circle). Schematics of the three possible outcomes are shown. For simplicity, only the forespore chromosome is diagramed. Two fields of >400 sporangia each were scored for each strain.

(C) Schematic representation of the results in (B). The closer a reporter is to the origin, the more likely it is present in the head of the axial filament. The dark gray bar marks the "forespore region" identified by Wu and Errington (1998) and the light gray bar and the gray pie wedges in (B) and (D) show the region present in the forespore in >50% of sporulating cells based on the data presented here. This region is off-centered from the *oriC* and symmetrical around the RacA-binding sites (*ram* sites).

(D) Chromosome organization in the absence of Soj and Spo0J. The CFP reporter inserted at three positions (−61°, −35°, +28°) was analyzed relative to the −7° YFP reporter. A fourth class of cells was included in the analysis: forespores that fail to trap either reporter. This class is defined as 0% in wild-type cells (see Experimental Procedures).

to which we compared a second reporter (*cfp*) inserted at different locations around the chromosome (Figure 1B). Similar to the original study (Wu and Errington, 1998), we found that genomic positions close to the origin of replication were more frequently present in the forespore at the time of septation (for simplicity, we refer to this region as the "head" of the axial filament) while sites further from the origin were usually present in the mother cell (the "body" of the axial filament) (Figure 1B). A chromosomal position near the terminus (+174°) was never found in the forespore. Interpolating from our data, we estimate that the region of the chromosome from −53° to +38° is trapped in the forespore in at least 50% of the sporulating cells (Figure 1C). This region represents one-quarter of the forespore chromosome (~1 Mb). Strikingly, it is almost perfectly centered on the 25 binding sites (*ram* sites) for the RacA protein that anchors this region at the cell poles during sporulation (Ben-Yehuda et al., 2005).

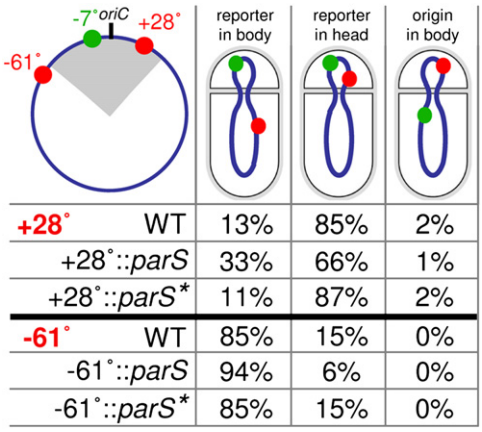
It was previously reported that the forespore chromosome was less organized in a strain lacking Soj and Spo0J (Lee et al., 2003; Sharpe and Errington, 1996; Wu and Errington, 2002). To validate our single-cell-based assay, we analyzed three chromosomal positions relative to the −7° baseline site in this double mutant. Strikingly, the organization of the chromosome was dramatically altered (Figure 1D); sites that were normally excluded from the forespore were now more frequently present in the head of the axial filament. Moreover, regions that would normally be anchored by RacA at the poles were frequently excluded from the forespore in this mutant. We refer to this phenotype as chromosome disorganization. Because of the extent of this disorganization, we included a fourth class in our analysis: those cells that lacked both reporters (Figure 1D). Our analysis indicates that the disorganization of the chromosome in the absence of Soj and Spo0J is far greater than was previously appreciated, and this likely reflects the sensitivity of the single-cell-based assay. These results support the idea that Spo0J and Soj play an important role in organizing the chromosome. Furthermore, they are consistent with the prevailing model that Spo0J bound to the origin-proximal *parS* sites organizes this region of the chromosome by recruiting these loci into a large nucleoprotein complex (Autret

et al., 2001; Breier and Grossman, 2007; Lin and Grossman, 1998; Murray et al., 2006; Wu and Errington, 2002).

Next, we analyzed a Soj mutant. In previous work using the population-based (*lacZ*) assay, the chromosome appeared properly organized in the absence of Soj although a synthetic chromosome organization defect was observed in a strain lacking both Soj and RacA (Wu and Errington, 2003). In our assay, the Soj mutant had a subtle but reproducible phenotype. Specifically, the −7° reporter next to the origin of replication was excluded from the forespore in 18%–27% of the sporulating cells (the sum of classes 3 and 4 in Figure 1D). Importantly, other chromosomal positions were not significantly affected by the absence of Soj. We interpret the exclusion of the −7° site in the Soj mutant as a defect in origin repositioning rather than chromosome organization. Soj/ParA has been similarly implicated in origin segregation in vegetatively growing *B. subtilis*, *V. cholerae*, and *C. crescentus* (Fogel and Waldor, 2006; Lee and Grossman, 2006; Toro et al., 2008). Furthermore, since the Soj mutant did not significantly impact the organization of chromosomal positions outside of the origin, the defect in chromosome organization in the Δ(*soj spo0J*) double mutant is likely due to the absence of Spo0J (Autret et al., 2001; Lee et al., 2003; Wu and Errington, 2003). In support of this idea, analysis of chromosome organization in a Δ*spo0J,* Δ*sda* double mutant (the absence of the Sda checkpoint protein can also suppress the sporulation defect of the Spo0J mutant; Murray and Errington, 2008) revealed a disorganization phenotype similar to that in the Δ(*soj spo0J*) mutant (data not shown). Finally, the disorganization of the chromosome in the absence of Spo0J is not due to overreplication (a phenotype associated with mutants in Spo0J and Soj; Lee and Grossman, 2006; Lee et al., 2003) because a Soj mutant does not have a strong organization defect. Moreover, analysis of a mutant (Δ*yabA*) that causes overreplication (Hayashi et al., 2005) also did not have a significant effect on chromosome organization (data not shown).

Ectopic *parS* Sites Bound by Spo0J Are Excluded from the Cell Pole

The current view is that Spo0J organizes the origin region by gathering the origin-proximal *parS* sites into a single polar nucleoprotein complex. This model predicts that insertion of a *parS* site at an ectopic chromosomal location will result in recruitment of this position into the nucleoprotein complex and therefore increase its polar localization. To test this, we introduced a consensus *parS* site at +28°, adjacent to the *cfp* reporter, and monitored the frequency of inclusion of this position in the head of the axial filament (Figure 2). Surprisingly, the addition of a *parS* site *reduced* the frequency of polar localization. Normally, 10%–13% of the cells fail to trap the +28° position in the forespore. Insertion of the *parS* site increased this frequency almost 3-fold to 33%. This "exclusion phenomenon" was reproducible from field to field and in six independent experiments. Moreover, similar results were observed when a consensus *parS* was inserted at −61° or +30° (Figure 2 and data not shown). Consistent with the idea that Spo0J bound to the ectopic *parS* was responsible for excluding this position from the forespore, replacement of the consensus *parS* site with a *parS* mutant (*parS**) that could not be bound by Spo0J (Lin and Grossman, 1998) restored normal chromosome organization (Figure 2).



	reporter in body	reporter in head	origin in body
+28° WT	13%	85%	2%
+28°::*parS*	33%	66%	1%
+28°::*parS**	11%	87%	2%
-61° WT	85%	15%	0%
-61°::*parS*	94%	6%	0%
-61°::*parS**	85%	15%	0%

Figure 2. An Ectopic Spo0J-Binding Site Disrupts Chromosome Organization

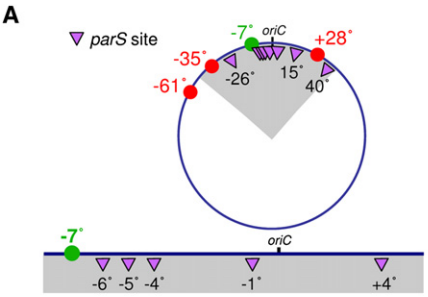
Analysis of chromosome organization in strains harboring a consensus *parS* site or a mutated *parS* site (*parS**) inserted adjacent to the CFP reporter at +28° and −61°.

Analysis of the +28° position in a strain that contained a *parS* site 20 kb away (inserted at +30°) caused a similar exclusion of the +28° position (Figure S3A). This result indicates that the exclusion is not a result of Spo0J bound to *parS* silencing the adjacent fluorescent reporter. In support of this conclusion, Spo0J spreading in vivo does not effect the expression of the genes in the nucleoprotein complex (Breier and Grossman, 2007; Murray et al., 2006). Finally, this chromosome disorganization phenotype appears specific for Spo0J bound to *parS* because an array of tet operators ($(tetO)_{120}$) (Lau et al., 2003) bound by the tet repressor (TetR) did not alter the organization of a neighboring reporter (Figure S3B). We conclude that ectopic *parS* sites bound by Spo0J promote *exclusion* of chromosomal regions from the forespore. These surprising results are not consistent with the model that Spo0J organizes the chromosome by gathering the *parS* sites into a polar complex.

A Single *parS* Site Is Largely Sufficient for Chromosome Organization

There are eight origin-proximal *parS* sites in *B. subtilis* (Lin and Grossman, 1998). Five are tightly clustered around the origin of replication and three are more dispersed (Figure 3A). To more directly investigate whether Spo0J organizes the origin region by gathering *parS* sites into a polar complex, we deleted the three dispersed sites (−26°, +15°, and +40°) by allelic replacement and monitored chromosome organization using our single-cell-based assay. Surprisingly, the organization of the chromosome in the mutant was indistinguishable from wild-type (Figure 3B).

To confirm that the *parS* sites are indeed important for chromosome organization and origin positioning, we systematically deleted all eight *parS* sites. As expected, in the absence of the Spo0J-binding sites, GFP-Spo0J failed to form fluorescent foci and instead localized as a diffuse haze (Figure 3C). Immunoblot analysis indicates that this localization pattern is not due to release of free GFP by proteolysis (Figure 3D). Analysis of different chromosomal positions using our single-cell-based



B

	reporter in body	reporter in head	origin in body	both loci in body
+28° WT	12%	87%	1%	0%
Δ3 *parS*	13%	85%	0%	2%
Δ8 *parS*	49%	27%	10%	13%
Δ8, -7°::*parS*	24%	76%	0%	0%
Δ8, -7°::*parS**	36%	33%	12%	19%
-35° WT	2%	96%	2%	0%
Δ3 *parS*	3%	95%	2%	0%
Δ8 *parS*	5%	52%	12%	30%
Δ8, -7°::*parS*	2%	95%	2%	1%
Δ8, -7°::*parS**	6%	54%	12%	28%
-61° WT	88%	11%	0%	0%
Δ3 *parS*	85%	14%	0%	1%
Δ8 *parS*	40%	31%	2%	27%
Δ8, -7°::*parS*	71%	29%	0%	0%
Δ8, -7°::*parS**	43%	25%	2%	30%

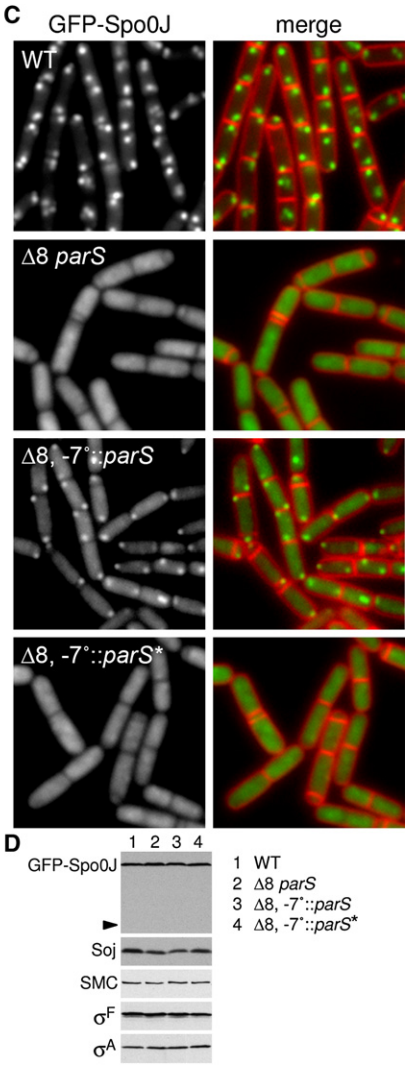


Figure 3. Chromosome Organization in the Absence of *parS* Sites

(A) Schematic diagram of the eight origin-proximal *parS* sites (purple triangles). The five *parS* sites tightly clustered around the origin are depicted below the schematic.

(B) Quantitative analysis of chromosome organization in strains lacking the three dispersed *parS* sites at −26°, +15°, and +40° (Δ3 *parS*); lacking all eight *parS* sites (Δ8 *parS*); or with a single consensus *parS* or *parS** site at −7°. CFP reporters inserted at +28°, −35°, and −61° were analyzed relative to the −7° YFP reporter.

(C) Localization of GFP-Spo0J at an early stage of sporulation in wild-type, or strains lacking all eight *parS* sites (Δ8 *parS*), containing a single consensus *parS* site at −7° (−7°::*parS*) or a mutated *parS* site (*parS**) at the same position. Membranes (false-colored red) were stained with the dye TMA-DPH.

(D) Immunoblot analysis of the strains shown in (C). In the absence of the eight *parS* sites, GFP-Spo0J remained intact and the levels of Soj and SMC were similar to wild-type. GFP-Spo0J was analyzed using anti-GFP antibodies and the arrowhead identifies the predicted size of free GFP. All strains efficiently entered sporulation as judged by the levels of the sporulation transcription factor σ^F. σ^A was used to control for loading.

assay demonstrated that removing the eight origin-proximal *parS* sites disrupts origin positioning and organization of the chromosome (Figure 3B). The loss of organization in the Δ8 *parS* mutant was qualitatively similar to the defect observed in the strain lacking Spo0J and Soj. We do not understand the quantitative difference but suspect that nonspecific DNA binding of Spo0J in the absence of *parS* sites (Breier and Grossman, 2007) impacts chromosome organization.

We wondered whether a single origin-proximal *parS* site might be sufficient for wild-type chromosome organization. To test this, we inserted a consensus *parS* site at the −7° position in the Δ8 *parS* strain. This single *parS* site restored polar GFP-Spo0J foci (Figure 3C) to the mutant. The foci were weaker than in wild-type, consistent with the decrease in number of Spo0J-binding sites. Analysis of chromosome organization in this strain revealed that a single origin-proximal *parS* site restored origin repositioning to the Δ8 *parS* strain and was largely sufficient for chromosome organization (Figure 3B). Fluorescence microscopy and chromatin immunoprecipitation data are consistent with the idea that *parS* sites bound by Spo0J cluster; however, the results of Figures 2 and 3 challenge the model that clustering is the mechanism by which Spo0J organizes the origin region.

Spo0J and *parS* Are Required for the Subcellular Localization of the SMC Complex

Based on the results described above, we hypothesized that Spo0J bound to *parS* organizes the origin region by recruiting a protein (or protein complex) involved in global chromosome organization. To identify this factor, we took a candidate approach. One factor we considered was the chromosome condensation complex composed of SMC/ScpA(kleisin)/ScpB. SMC can be crosslinked to DNA throughout the chromosome (Lindow et al., 2002) but has also been shown to localize as discrete foci (Figure 4A) (Britton et al., 1998; Mascarenhas et al., 2002). We wondered whether these foci were organizing centers and whether Spo0J bound to *parS* was required for their formation. To investigate this, we examined the localization of ScpB-YFP in a Spo0J mutant. In the absence of Spo0J, ScpB-YFP failed to form discrete foci (Figures 4A and S4). Instead, the protein appeared diffuse in the cytoplasm and in faint puncta. Immunoblot analysis indicates that the diffuse signal is not due to cleavage of ScpB-YFP and release of free YFP (Figure 4B). Similar results were obtained with GFP-SMC and ScpA-YFP (Figure S4B and data not shown). In support of the idea that *parS* sites are also required for the discrete foci, ScpB-YFP localization was disrupted in the Δ8 *parS* mutant (Figure 4B). Furthermore, the insertion of a single *parS* site partially restored the foci.

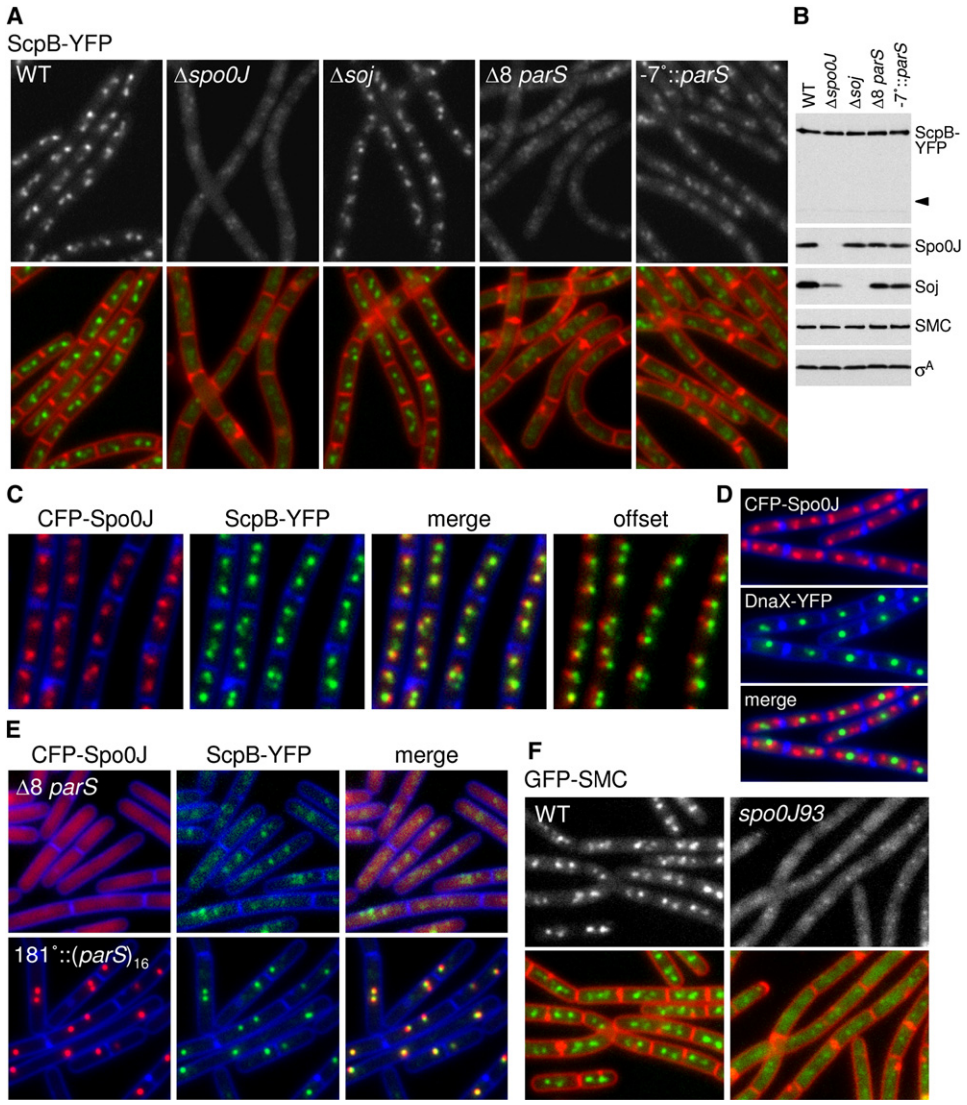


Figure 4. Spo0J Bound to *parS* Recruits the Structural Maintenance of Chromosomes Complex

(A) Localization of ScpB-YFP was visualized in wild-type and strains lacking Spo0J (Δ*spo0J*), Soj (Δ*soj*), the eight origin-proximal *parS* sites (Δ8 *parS*), and a strain carrying a single consensus *parS* site at −7° (−7°::*parS*). The signal intensities in all five images were normalized for direct comparison.

(B) Immunoblot analysis of strains in (A). ScpB-YFP remained intact in all strains analyzed. ScpB-YFP was analyzed using anti-GFP antibodies and the arrowhead identifies the predicted size of free YFP. SMC, Spo0J, Soj levels were also analyzed for comparison. σ^A was used to control for loading.

(C) Colocalization of CFP-Spo0J (false-colored red) and ScpB-YFP (false-colored green) in wild-type cells during vegetative growth. CFP and YFP fluorescence are also shown slightly offset to facilitate visualization.

(D) As a control for the resolution of fluorescent foci, CFP-Spo0J (red) and DnaX-YFP (green) were visualized in wild-type cells grown in minimal medium.

(E) Localization of CFP-Spo0J (red) and ScpB-YFP (green) in cells lacking all eight origin-proximal *parS* sites (Δ8 *parS*) and in the same strain with 16 *parS* sites inserted near the terminus.

(F) The localization of GFP-SMC in wild-type and a Spo0J93 mutant. The signal intensities in the two images were normalized for direct comparison.

Importantly, in the absence of Soj, a condition that does not significantly disrupt chromosome organization, the ScpB-YFP foci were still detectable (Figure 4B). The mislocalization of the SMC complex in the absence of Spo0J or the *parS* sites was more qualitative than quantitative. There were always a subset of cells in the Δ*spo0J* and Δ8 *parS* strains that had weak ScpB-YFP, ScpA-YFP, or GFP-SMC foci and perhaps this explains why this phenotype was not previously observed (Mascarenhas et al., 2002; Volkov et al., 2003). However, the loss of and/or reduction in discrete polar foci were unambiguous when comparing large fields of cells (Figure S4). The results presented in Figure 4A are from vegetatively growing cells. Similar results were obtained during the early stages of sporulation; however, the signal was weaker and the ScpB-YFP signal became diffuse upon polar division (data not shown). In summary, these results are consistent with the idea that Spo0J

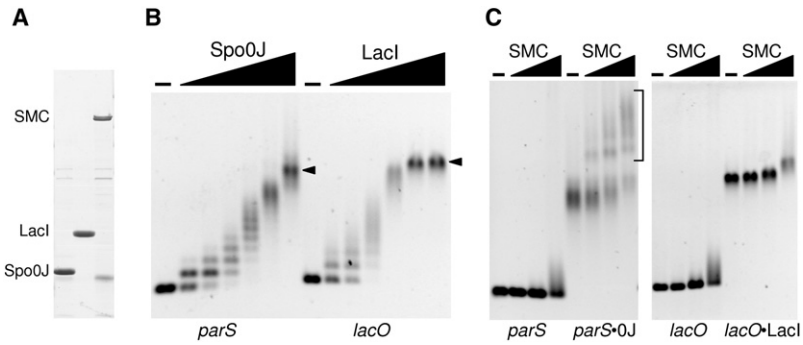


Figure 5. SMC Binds Spo0J-Coated DNA with Higher Affinity than Free DNA or LacI-Coated DNA

Electrophoretic mobility-shift analysis of purified Spo0J, *lac* repressor (LacI), and SMC. The DNA substrates were a 461 bp fragment containing a *parS* site and a 574 bp fragment containing an array of 15 *lacO* sites.

(A) Coomassie-stained gel of the purified proteins.

(B) Spo0J and LacI coat their respective DNA substrates. The protein concentrations ranged from 56 nM to 1.8 μM with two-fold step increases. Fully saturated DNA substrates are indicated (arrowheads).

(C) SMC has the highest affinity for the Spo0J nucleoprotein complex. The concentration of Spo0J and LacI used to generate the filament substrates were 1.8 μM. The concentrations of SMC were 33 nM, 100 nM, and 300 nM. The super-shifted species containing SMC and Spo0J-*parS* are indicated (bracket).

bound to *parS* recruits the SMC chromosome condensation complex to the origin.

SMC Foci Require Nucleoprotein Complexes of Spo0J

Spo0J and other ParB proteins bind their cognate *parS* sites and spread along the DNA, generating a nucleoprotein complex that has been hypothesized to be a filament (Breier and Grossman, 2007; Murray et al., 2006; Rodionov et al., 1999). To investigate whether the localization of SMC requires Spo0J-coated DNA, we used a mutant (*spo0J93*) that binds to *parS* but is impaired in spreading (Breier and Grossman, 2007). Strikingly, GFP-SMC and ScpB-YFP failed to form discrete foci in the Spo0J93 mutant (Figure 4F and data not shown). Thus, the formation of SMC foci appears to require a nucleoprotein filament of Spo0J. This finding prompted us to investigate whether Spo0J spreading was also required for proper organization of the chromosome. To test this, we subjected the Spo0J93 mutant to our quantitative organization assay. The mutant displayed a similar disorganization phenotype to the *spo0J* null (Figure S5). Thus, these results suggest that formation of SMC foci correlates with organization of the origin.

Spo0J and the SMC Complex Colocalize

To investigate whether the SMC foci colocalize with Spo0J bound to the origin-proximal *parS* sites, we performed a double-labeling experiment. Visualization of CFP-Spo0J and ScpB-YFP by fluorescence microscopy revealed that Spo0J and SMC foci indeed colocalize (Figure 4C). Although not all foci were perfectly superimposable, every focus of CFP-Spo0J overlapped with or was immediately adjacent to a focus of ScpB-YFP (Figure 4C). To determine whether the apparent colocalization of ScpB and Spo0J was real and not due to our inability to resolve these relatively large fluorescent foci, we visualized CFP-Spo0J and a YFP fusion to the tau subunit of DNA polymerase (DnaX-YFP). In most cells (61%), the CFP-Spo0J foci were present close to the cell quarters while one or two DnaX-YFP foci were located at mid-cell (Figure 4D). Importantly, in these cells, the Spo0J foci and the replisome foci did not colocalize and were easily resolved. It has been reported previously that the majority of cells lacking SMC retain Spo0J foci (Britton et al., 1998). Thus, this result and the data in Figures 4A and 4C support the idea that Spo0J-*parS* recruits the SMC complex to the origin region.

Spo0J Bound to an Array of *parS* Sites near the Terminus Recruits the SMC Complex

To test whether Spo0J bound to *parS* can recruit the SMC complex, we inserted an array of 16 *parS* sites near the terminus (+181°) (Lee et al., 2003) in a strain lacking the eight origin-proximal *parS* sites. In this strain, the CFP-Spo0J fusion localized to one or two foci near mid-cell (Figure 4E). Strikingly, an ScpB-YFP focus colocalized with every focus of CFP-Spo0J. Importantly, in a strain lacking the *parS* array, CFP-Spo0J localized as a diffuse haze and ScpB-YFP localization was diffuse with faint puncta (Figure 4E) as seen in Figures 3C and 4A, respectively. These results demonstrate that Spo0J bound to *parS* directly or indirectly recruits the SMC complex.

SMC Binds Spo0J-Coated DNA with Higher Affinity than Naked DNA

To investigate whether a Spo0J nucleoprotein complex directly recruits SMC, we compared SMC binding to naked DNA and Spo0J-coated DNA in vitro. For these experiments, we used purified Spo0J (Figure 5A) and a 461 bp DNA fragment that contains the −1° *parS* site. At low concentrations (25–50 nM), Spo0J bound this fragment resulting in a shift in mobility on an agarose gel (Murray et al., 2006). At concentrations above 1.5 μM, Spo0J saturated the DNA forming a nucleoprotein complex. Using this gel-mobility shift assay, we compared SMC binding to free DNA and the Spo0J-coated substrate. SMC is capable of binding DNA in the absence of its partner proteins ScpA and ScpB (Hirano and Hirano, 1998, 2004). Consistent with what has been reported previously, SMC bound naked DNA at 300 nM protein but little binding was detected below this concentration (Figure 5C). By contrast, SMC bound the Spo0J nucleoprotein complex at concentrations as low as 20 nM, forming a discrete super-shifted complex (Figure 5C and data not shown). At higher SMC concentrations additional super-shifted complexes were detected (Figure 5C). Since the SMC cohesin complex is thought to topologically embrace DNA (Nasmyth and Haering, 2005), it was formally possible that Spo0J increased SMC binding by nonspecifically shielding the DNA phosphate backbone. Accordingly, we

tested SMC binding to DNA coated with an unrelated DNA-binding protein: the *lac* repressor (LacI). For these experiments we used a DNA fragment containing 15 *lacO* operators. To ensure that LacI would coat the DNA and not form DNA loops, we used a mutant that lacks the last 11 amino acids required for tetramerization. LacIΔ11 efficiently saturated the *lacO* array at concentrations above 1 μM (Figure 5B). Importantly, SMC bound the naked *lacO* array and the LacIΔ11-coated DNA with affinities similar to the naked *parS* DNA fragment (Figure 5C). In all three cases, an SMC complex was only detectable at concentrations of SMC above 250 nM. Collectively, these results support the idea that Spo0J bound to *parS* directly recruits SMC to the origin.

Recruitment of the SMC Complex to an Ectopic Chromosomal Site Impairs Chromosome Segregation

Collectively, our data suggest that recruitment of SMC to the origin by Spo0J bound to *pars* organizes the origin region. This model predicts that recruitment of SMC to an ectopic position should impact global chromosome organization and perhaps DNA segregation. To test this, we monitored chromosome morphology in cells lacking the eight origin-proximal *parS* sites and harboring 16 *parS* sites inserted at −150°. Vegetatively growing wild-type cells have a condensed DNA mass (called the nucleoid) that adopts a bilobed structure during DNA replication that frequently segregates prior to cell division (Figure 6). In the absence of the eight *parS* sites, the chromosome appeared less condensed. Moreover, in this mutant, 0.8% of the cells failed to inherit a chromosome resulting in anucleate cells. This frequency of anucleate cells was similar to that of a Spo0J null mutant (Ireton et al., 1994) and was 40- to 100-fold higher than that of wild-type. Analysis of the strain harboring the *parS* array at −150° revealed gross defects in nucleoid morphology (Figures 6 and S6). In addition, 8.5% of the cells lacked DNA and 7.3% had chromosomes bisected by a cell division septum. Similar results were obtained with the *parS* array inserted at +181° (data not shown). Importantly, the defects in nucleoid morphology and chromosome segregation could be suppressed by a Spo0J mutant but not a Soj mutant (data not shown), indicating that these phenotypes were not due to Soj acting on the Spo0J-*parS* complex. We cannot rule out the possibility that Spo0J itself is responsible for these phenotypes; however, the colocalization of the SMC condensation complex to these ectopic *parS* sites (Figure 4E) and the role of SMC in chromosome compaction support the idea that inappropriate recruitment of SMC by Spo0J-*parS* is responsible for the defects. Moreover, these results suggest that the recruitment of SMC to the origin in wild-type cells has functional consequences for chromosome organization and segregation.

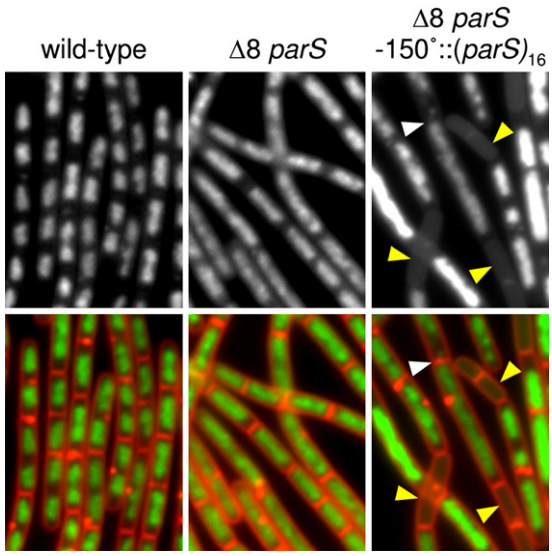


Figure 6. Recruitment of SMC to an Ectopic Site Impairs Chromosome Organization and DNA Segregation

DNA was visualized by DAPI (false-colored green) and membranes were visualized with FM4-64 (red). Wild-type cells have compact nucleoids that form bilobed structures during DNA replication and often segregate prior to cell division. In the absence of the eight *parS* sites (Δ*parS*), the nucleoids are less compact. Insertion of an array of 16 *parS* sites in a strain lacking the origin-proximal *parS* sites causes an increase in production of anucleate cells (yellow arrowheads), cell divisions on top of the DNA (white arrowheads), and aberrant nucleoid morphology.

DISCUSSION

We have shown that Spo0J (ParB) bound to *parS* recruits the SMC condensation complex to the origin. Efficient recruitment of SMC appears to require a nucleoprotein filament of Spo0J seeded by binding to *parS*. Collectively, our data are most consistent with the idea that Spo0J participates in chromosome organization not by gathering dispersed *parS* sites but rather by targeting the SMC condensation complex to the origin. In support of this idea, recruitment of SMC to ectopic positions results in perturbations to nucleoid morphology and defects in chromosome segregation. Furthermore, we have found that depletion of SMC as cells enter sporulation results in disorganization of the axial filament (Figure S7). This model provides an explanation for the surprising "exclusion phenomenon" we observed in Figure 2 in which insertion of a consensus *parS* site at +28° or −61° resulted in *loss* of polar localization. We suspect that recruitment of SMC caused inappropriate condensation of these regions of the chromosome thereby altering their cellular positions. We hypothesize that SMC present at the origin acts as an "organization center" interacting with multiple regions of the chromosome that are many hundreds of kilobases away.

Our data and previously published findings are most consistent with a model for chromosome segregation (Figure 7) in which ParA acts on the ParB-*parS* complex to reposition the newly replicated origins toward the cell poles. Recruitment of SMC to the origins by ParB-*parS* then organizes the origin region and helps drive efficient chromosome segregation by compacting the DNA as it emerges from the replisome located at mid-cell (Lemon and Grossman, 1998). In this model, ParB bound to *parS* functions in both origin repositioning and recruitment of SMC to the origin region. Since the absence of Soj has almost no impact on chromosome segregation, we hypothesize that the defect in chromosome segregation in the Spo0J mutant is principally due to the inability to recruit SMC to the origin. Consistent with this model, the synthetic chromosome segregation defect in a Δ*soj*, Δ*smc* double mutant is indistinguishable from that in a Δ*spo0J,* Δ*smc*

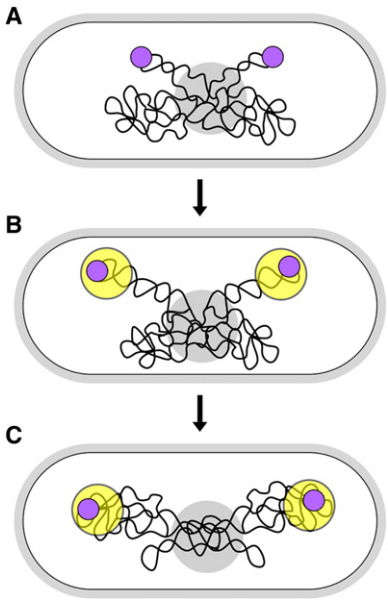


Figure 7. Proposed Model for Chromosome Segregation

(A) Upon replication of the origin region, Soj/ParA (not shown) helps reposition the origins by acting on Spo0J/ParB (purple circle) bound to the *parS* sites.

(B) ParB-*parS* recruits the SMC condensation complex (yellow circle) to the repositioned origins.

(C) SMC organizes this region and promotes efficient chromosome segregation through compaction of the DNA as it emerges from the replisome (gray circle) located at mid-cell.

double mutant (Lee and Grossman, 2006). Finally, we note that an SMC null mutant has a much more severe chromosome segregation defect than a Spo0J null (Britton et al., 1998). This result indicates that the recruitment of SMC to the repositioned origins is not essential for SMC function. However, our data suggest that the condensation complex functions most efficiently in chromosome segregation when it is recruited to these polar sites.

The targeting of SMC complexes to the origin by ParB bound to *parS* is likely to be a conserved feature of chromosome organization and segregation in many bacteria. Most bacteria that have a partitioning locus also encode the proteins that comprise the SMC complex. Those bacteria that lack SMC/ScpA/ScpB usually have its functional analog MukBEF. Furthermore, most *parS* sites reside adjacent to the origin. Importantly, our data suggest that even a single *parS* site (or a small cluster of sites) is sufficient to recruit the condensation complex and participate in chromosome organization. Interestingly, in *C. crescentus*, SMC localizes to several discrete foci during the cell cycle (Jensen and Shapiro, 2003). Prior to cytokinesis, two bright foci of SMC are present at or near the cell poles where ParB and the *parS* sites are located (Mohl and Gober, 1997; Thanbichler and Shapiro, 2006; Viollier et al., 2004). Based on the data presented here, we hypothesize that ParB bound to *parS* recruits SMC to these polar positions. It is noteworthy that *E. coli* and most γ-proteobacteria lack the partitioning locus but have MukBEF. Despite the absence of ParB and *parS*, Sherratt and colleagues have recently reported that *E. coli* MukB colocalizes with the origin region of the chromosome (Danilova et al., 2007). We hypothesize that a system analogous to ParB-*parS* is responsible for recruiting the condensation complex to this site.

Recruitment of Eukaryotic and Bacterial SMC Complexes

The role of Spo0J-*parS* in recruiting SMC to the origin has interesting parallels to the mechanisms by which eukaryotic SMC complexes are targeted to chromosomes. In *Caenorhabditis elegans*, a specialized SMC complex is specifically targeted to the X chromosomes in hermaphrodites (Meyer, 2005). This complex (called the dosage compensation complex) downregulates X-linked gene expression by half to a level equivalent to the expression from the single X chromosome in males. The dosage compensation complex is targeted to the X chromosomes by a hermaphrodite-specific protein called SDC-2 (Dawes et al., 1999). SDC-2 associates with sequence elements on the X chromosome (called *rex*) and can localize to these sites in the absence of the SMC complex. It is not known whether SDC-2 normally recognizes these elements before or after its association with the complex. By analogy to Spo0J, we hypothesize that SDC-2 first localizes to the *rex* sites and then recruits the dosage compensation complex. Interestingly, Meyer and colleagues have shown that clusters of two sequence motifs within the *rex* elements are necessary for efficient targeting of the complex (McDonel et al., 2006). Although a single *parS* site bound by Spo0J can recruit SMC in *B. subtilis*, our data suggest that a cluster of origin-proximal *parS* sites is more efficient at recruiting SMC and, in turn, organizing the origin region (Figures 3 and 4). The potency of clustered sites could reflect cooperative interactions between SMC complexes. Intriguingly, most bacterial chromosomes have at least two origin-proximal *parS* sites (Livny et al., 2007), and those with only one frequently do not encode SMC.

Work from Meyer and colleagues also indicates that after X chromosome targeting by SDC-2, the SMC dosage compensation complexes can spread to sites adjacent to the *rex* elements (Meyer, 2005). Our colocalization data are consistent with the idea that, after recruitment by Spo0J-*parS*, SMC can also spread to neighboring sites. In wild-type cells, the polar foci of CFP-Spo0J and ScpB-YFP were frequently adjacent to each other or even interdigitated rather than superimposable (Figure 4C). Moreover, the SMC foci were generally larger and more diffuse than the Spo0J foci. In the case of dosage compensation, this spreading appears to be critical to downregulate X-linked gene expression. In *B. subtilis*, we hypothesize that spreading from the origin allows SMC to organize and compact a larger region of the chromosome.

Similarities and Differences in Chromosome Segregation in Bacteria and Eukaryotes

In eukaryotes, the SMC condensin complex plays a central role in resolving the tangle of replicated chromosomes into morphologically distinct rods during the transition from interphase to metaphase (Hirano, 2006; Nasmyth and Haering, 2005). By the time the sister chromatids are compacted and aligned at the metaphase plate, the vast majority of chromosome segregation has already occurred. All that remains is the repositioning of the highly organized and condensed chromatids to opposite cell halves through the action of motor proteins and microtubules.

In bacteria, the SMC complex likely plays a similar role in driving chromosome segregation through compaction and resolution of the replicated chromosomes. The results presented here suggest that SMC normally performs these functions after the replicated origins are repositioned toward the poles and ParB bound to *parS* recruits the complex to these polar sites. Thus, although SMC complexes play fundamentally similar roles in chromosome segregation in bacteria and eukaryotes they appear to act at distinct steps in the process. In eukaryotes, resolution of sister chromatids, mediated in part by condensin, *precedes* the extrinsic forces exerted by microtubules and motors that physically move the sisters apart. By contrast, in bacteria, efficient chromosome segregation initiates with extrinsic forces exerted by the partitioning system on the replicated origins and is then followed by intrinsic forces mediated by SMC complexes present at the repositioned origins. Thus, despite the apparent differences in this essential biological process, at the core bacteria and eukaryotes use remarkably similar strategies to segregate their chromosomes.

EXPERIMENTAL PROCEDURES

General Methods

All *B. subtilis* strains were derived from the prototrophic strain PY79 (Youngman et al., 1983). Cells were grown in CH medium at 37°C. Sporulation was induced by resuspension according to the method of Sterlini-Mandelstam (Harwood and Cutting, 1990). Recombinant proteins were expressed in *E. coli* and purified by affinity chromatography using Ni^{2+}-agarose as described (Doan and Rudner, 2007). Fluorescence microscopy and immunoblot analysis were performed as previously described (Doan and Rudner, 2007). The Supplemental Data contain a description of all the plasmids used in this study and tables of strains (Table S1), plasmids (Table S2), and oligonucleotide primers (Tables S3 and S4).

Quantitative Forespore-Trapping Assay

Using color thresholding and integrated morphometry analysis followed by visual inspection, forespores with YFP and CFP fluorescence were scored as "on" or "off" after correction for background fluorescence. Forespores containing neither CFP nor YFP fluorescence were scored manually and the frequency of this class in the mutant strains was adjusted based on the percent of this class in wild-type. In ~15% of the wild-type sporulating cells synthesis of the fluorescent reporters had not yet reached detectable levels at the time of image acquisition.

Gel-Mobility Shift Analysis

Protein DNA complexes were analyzed as described previously (Hirano and Hirano, 1998; Murray et al., 2006) with minor modifications. The DNA substrates were a 461 bp PCR product from the *spo0J* gene containing the −1° *parS* site and a 574 bp PCR product containing 15 *lac* operators. Fifty nanograms of each DNA substrate was incubated with purified Spo0J or LacIΔ11 in 10 μl binding buffer (20 mM HEPES KOH [pH7.6], 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 5% glycerol, 1 mM ATP) for 10 min at room temperature. The protein DNA complexes were resolved on a pre-run 0.7% TBE agarose gel at 2.8V/cm for 8 hr at 4°C. DNA was visualized with ethidium bromide. For SMC-binding experiments, the DNA substrates were incubated with 1.8 μM Spo0J, 1.8 μM LacIΔ11, or binding buffer alone for 10 min at room temperature followed by the addition of SMC and incubation for an additional 10 min.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00505-4.

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