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Dynamic Control of the DNA Replication Initiation Protein DnaA by Soj/ParA

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SUMMARY

Regulation of DNA replication and segregation is essential for all cells. Orthologs of the plasmid partitioning genes *parA, parB,* and *parS* are present in bacterial genomes throughout the prokaryotic evolutionary tree and are required for accurate chromosome segregation. However, the mechanism(s) by which *parABS* genes ensure proper DNA segregation have remained unclear. Here we report that the ParA ortholog in *B. subtilis* (Soj) controls the activity of the DNA replication initiator protein DnaA. Subcellular localization of several Soj mutants indicates that Soj acts as a spatially regulated molecular switch, capable of either inhibiting or activating DnaA. We show that the classical effect of Soj inhibiting sporulation is an indirect consequence of its action on DnaA through activation of the Sda DNA replication checkpoint. These results suggest that the pleiotropy manifested by chromosomal *parABS* mutations could be the indirect effects of a primary activity regulating DNA replication initiation.

INTRODUCTION

Proper transmission of genetic material is essential for the viability of all organisms. Nucleic acid replication and segregation must be precisely coordinated to ensure accurate genome inheritance. Eubacterial chromosomes contain a single origin of replication (*oriC*) that is recognized by the initiator protein DnaA. DnaA bound at *oriC* forms a homo-oligomer that mediates open complex formation and allows assembly of an initiation complex that loads the replicative helicase. Production of the initiation complex is followed by recruitment of the remaining replisome components, leading to replication of the bacterial chromosome (Kornberg and Baker, 1992; Messer et al., 2001; Mott and Berger, 2007). After duplication, daughter chromosomes are rapidly segregated toward opposite poles of the cell as part of a coordinated regulatory network to ensure accurate chromosome inheritance (Errington et al., 2005; Thanbichler and Shapiro, 2006a).

Orthologs of the plasmid partitioning proteins ParA and ParB are present on the chromosomes of bacteria found throughout all branches of the prokaryotic evolutionary tree and are most often located proximal to *oriC* (Gerdes et al., 2000; Livny et al., 2007). They have been shown to affect accurate chromosome segregation in several species, suggesting that they play an important and active role required for proper inheritance of bacterial genomes. However, in spite of being the focus of study for several decades, the molecular mechanism(s) underlying the activities of bacterial chromosomal partitioning genes are poorly understood.

The *par* operons derive their name from homology to partitioning systems of low copy number plasmids that ensure faithful pDNA segregation into daughter cells (Austin and Abeles, 1983; Gerdes et al., 1985; Ogura and Hiraga, 1983). The bacterial orthologs are required for chromosome replication and segregation, chromosome origin localization and separation, cell division, and developmental gene regulation in *Bacillus subtilis* (Hranueli et al., 1974; Ireton et al., 1994; Lee and Grossman, 2006; Lee et al., 2003; Ogura et al., 2003; Sharpe and Errington, 1996; Wu and Errington, 2003); cell-cycle progression and cell division in *Caulobacter crescentus* (Mohl et al., 2001); chromosome segregation and cell growth in *Mycobacterium smegmatis* (Jakimowicz et al., 2007a); chromosome organization and segregation, cell growth, and motility in *Pseudomonas aeruginosa* (Bartosik et al., 2004; Lasocki et al., 2007); chromosome segregation and cell morphology in *Pseudomonas putida* (Godfrin-Estevenon et al., 2002; Lewis et al., 2002); chromosome segregation and cell division in *Streptomyces coelicolor* (Jakimowicz et al., 2007b; Kim et al., 2000); and chromosome origin localization in *Vibrio cholerae* (Fogel and Waldor, 2006; Saint-Dic et al., 2006).

One of the best studied chromosomal partitioning systems is the *B. subtilis par* operon (referred to as *soj(parA), spo0J(parB),* and *parS*). *parS* is a specific DNA sequence motif that acts as the binding site for the DNA-binding protein Spo0J (Leonard et al., 2004; Lin and Grossman, 1998). In *B. subtilis* eight of the ten *parS* sites are located close to the origin of DNA replication, and the sites closest to *oriC* are most frequently bound by Spo0J (Breier and Grossman, 2007; Lin and Grossman, 1998). *parS* nucleates the spreading of Spo0J into flanking regions of DNA to create large nucleoprotein structures that extend for several kbp around a *parS* site (Breier and Grossman, 2007; Murray et al., 2006).

Soj is a Walker-type ATPase that interacts with Spo0J and is required for proper separation of sister origins and synchronous DNA replication, as well as for the regulation of sporulation

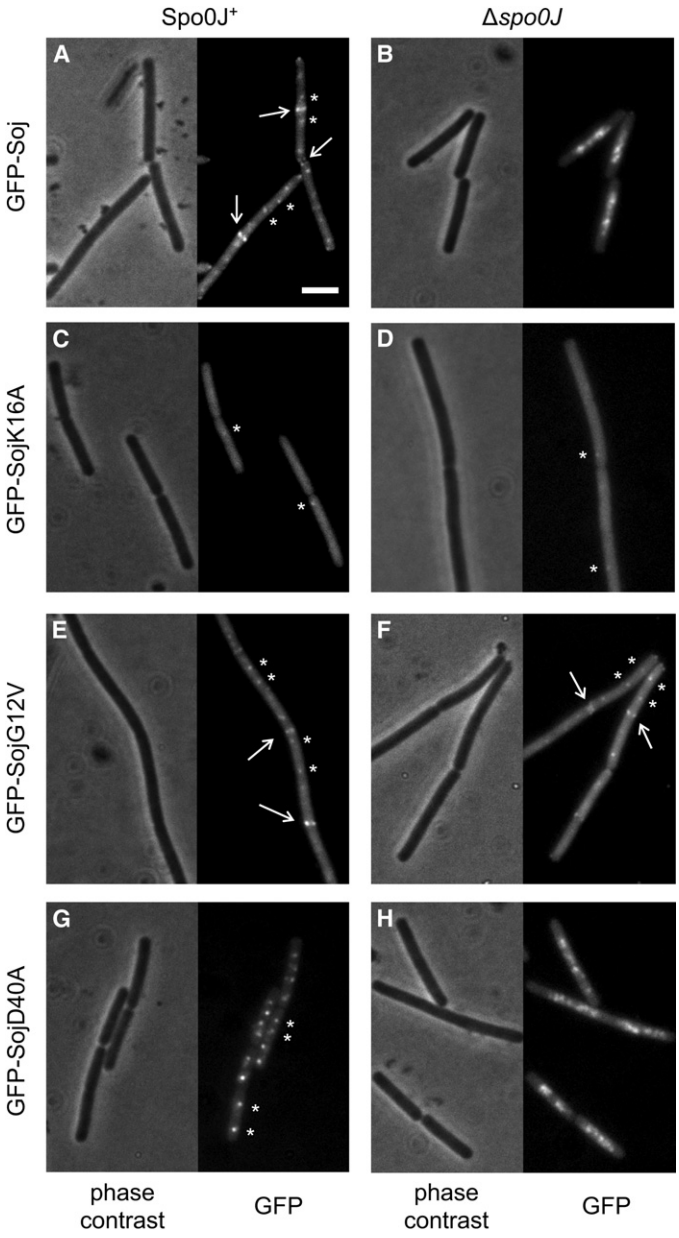

Figure 1. Localization of Wild-Type Soj and Soj Mutants in the Presence and Absence of Spo0J

The localization of GFP-Soj variants was observed using epifluorescence microscopy. Cells were grown in CH medium at 30°C. The left column shows results using a wild-type strain and the right column shows results using a *Δspo0J* mutant. An asterisk (*) denotes localization as a focus and an arrow (→) indicates localization at a septum. (A) HM4, (B) HM13, (C) HM7, (D) HM24, (E) HM14, (F) HM25, (G) HM5, (H) HM23. Scale bar: 3 μm.

(Ireton et al., 1994; Lee and Grossman, 2006; Leonard et al., 2005; Ogura et al., 2003). Biochemical and structural analysis of *Thermus thermophilis* Soj has shown that the protein is a dynamic molecular switch that is capable of forming an ATP-dependent "sandwich" dimer (Leonard et al., 2005). The ATP-bound dimer binds cooperatively to nonspecific DNA and contains ATP-hydrolysis activity (Hester and Lutkenhaus, 2007; Leonard et al., 2005; McLeod and Spiegelman, 2005). ATP hydrolysis by Soj leads to dissociation from DNA and resets the cycle.

We have investigated the activities of *B. subtilis* Soj in vivo by studying the effects of three mutations that alter the functions of Soj proteins in vitro. These mutations inhibit specific activities of Soj (ATP binding, cooperative DNA binding, or ATP hydrolysis) and lead to accumulation of different protein intermediates. We find that the Soj variants have distinct intracellular localization patterns and that they differentially regulate initiation of DNA replication. Both Soj localization and regulation of DNA replication initiation require the DNA replication initiator protein DnaA. Additionally, we show that Soj regulates sporulation by activating the DNA replication initiation checkpoint protein Sda.

RESULTS

Localization of Mutant Soj Proteins in Living Cells

Previous work describing the localization of wild-type Soj suggested that the protein either (1) localized mostly to cell poles or (2) localized dynamically to a subset of nucleoids within the cell (Marston and Errington, 1999; Quisel et al., 1999). To reconcile the differences between these observations, *soj* was replaced with *gfp-soj* (expressed from its native transcriptional and translational expression system at its endogenous location in the chromosome) and GFP-Soj localization was determined using epifluorescence microscopy. In this strain, GFP-Soj was observed to localize to septa (Figure 1A, arrows) and as relatively faint punctate foci within the cytoplasm (Figure 1A, asterisks) (similar results were obtained with a Soj-GFP fusion; data not shown). The localization pattern of GFP-Soj was dependent on Spo0J, and in a *Δspo0J* mutant GFP-Soj colocalized with the nucleoid as previously shown (Figure 1B) (Marston and Errington, 1999; Quisel et al., 1999).

The differences in the localization patterns reported for Soj could be due to the expression level of the GFP fusion. To test this the *gfp-soj* chimera was placed at an ectopic locus under the control of an inducible expression system (Figure S1A available online). At low expression levels GFP-Soj was observed to localize to septa and as foci within the cytoplasm. However, at higher expression levels the protein formed bright patches that colocalized with a subset of nucleoids similar to patterns previously reported. Thus, the localization of GFP-Soj depends on its expression level and the correct pattern is probably that observed at low expression levels. For the remainder of this work (unless noted) all *soj* alleles and fusions were expressed under native control (western blot analysis showed that all Soj proteins were expressed to approximately the same level as wild-type Soj; Figure S2).

To begin exploring the consequences of conformational changes in Soj, the subcellular distributions of various Soj mutants were compared with that of the wild-type. (N.B., for *B. subtilis:* SojK16A = ATP binding deficient, cooperative DNA

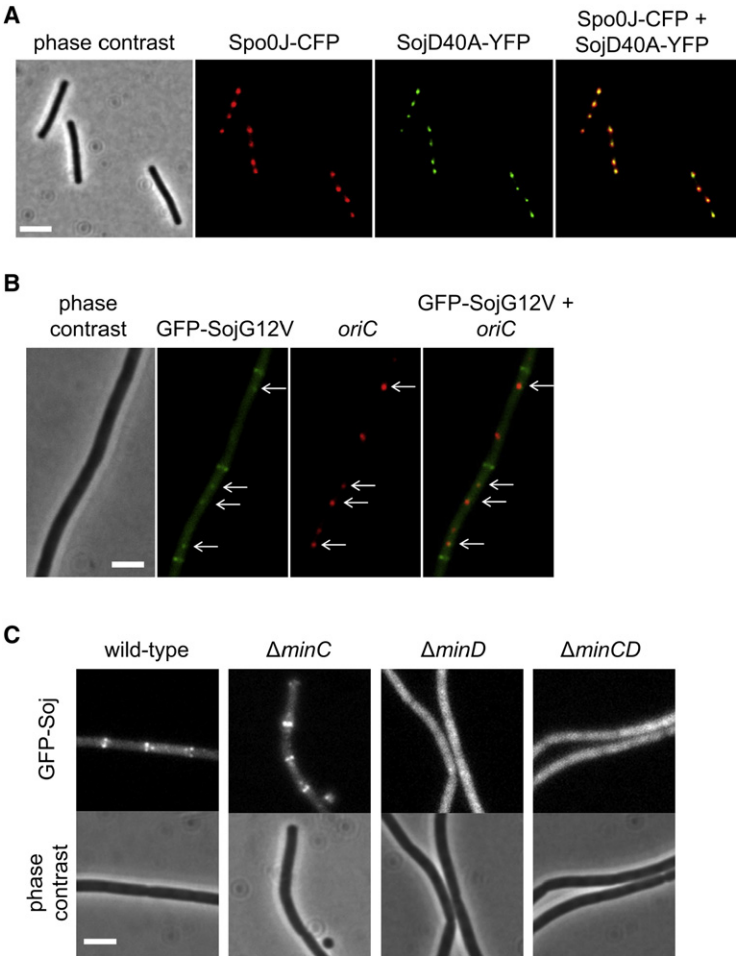

Figure 2. Localization Determinants of Soj

(A) SojD40A colocalizes with Spo0J. The localization of SojD40A-YFP and Spo0J-CFP within single cells was determined using epifluorescence microscopy. Strain HM85 was grown in S7-minimal medium at 30°C. The phase-contrast image shows the outline of each cell. Scale bar: 4 μm.

(B) SojG12V colocalizes with *oriC*. The localization of GFP-SojG12V and the *oriC* region of the chromosome within single cells was determined using epifluorescence microscopy. The *oriC* region was labeled with an array of *tetO* operators bound by TetR-mCherry. Arrows (→) indicate GFP-SojG12V foci. Strain HM355 was grown in CH-minimal medium at 30°C. The phase-contrast image shows the outline of each cell. Scale bar: 3 μm.

(C) Polar localization of Soj requires MinD. Localization of GFP-Soj was determined in *min* mutant strains. Cells were grown in CH medium at 30°C. The phase-contrast image shows the outline of each cell. Wild-type (HM69), *ΔminC* (HM70), *ΔminD* (HM71), *ΔminCD* (HM72). Scale bar: 3 μm.

binding deficient; SojG12V = ATP binding proficient, cooperative DNA binding deficient; SojD40A = ATP_2-bound dimer, cooperative DNA binding proficient, ATP hydrolysis deficient (see Figure 6). These biochemical properties were reported for the *T. thermophilus* Soj mutant proteins (Leonard et al., 2005) and have been confirmed for the corresponding *B. subtilis* mutant proteins (B. McLeod and G. Spiegelman, personal communication; H. Ferreira and J.E., unpublished).

The GFP-SojK16A mutant was predominantly distributed throughout the cytoplasm, although some faint foci could be discerned (Figure 1C). This pattern was unchanged in the absence of *spo0J* (Figure 1D). In contrast, the GFP-SojD40A mutant localized exclusively as bright foci within the cell (Figure 1G), with no hint of polar fluorescence. This pattern was reminiscent of the localization of Spo0J, which associates with sites near *oriC* (Glaser et al., 1997; Lin et al., 1997). To test whether these foci had a basis in interaction with Spo0J, we constructed a strain to look simultaneously at SojD40A (fused to YFP) and Spo0J (fused to CFP). As shown in Figure 2A, almost complete colocalization was observed. This strongly suggested that SojD40A interacts with Spo0J. Such an interaction would be consistent with in vitro data showing that Spo0J stimulates the ATP-hydrolysis activity of the Soj dimer (Leonard et al., 2005). To further test this idea, we examined the localization of GFP-SojD40A in the absence of Spo0J. Now, GFP-SojD40A localized in a dispersed nucleoid associated pattern, similar to that of wild-type GFP-Soj in the absence of Spo0J (Figure 1H). We interpret this to indicate that the ATP-dimer form of Soj (in which state SojD40A is thought to be trapped) binds to DNA in a manner that is relatively nonspecific in the absence of Spo0J but that is strongly influenced by Spo0J when present.

Finally, we examined the distribution of the GFP-SojG12V mutant. This protein localized at septa and formed faint foci within the cell (Figure 1E). Remarkably, however, this pattern was unchanged in the absence of Spo0J (Figure 1F). Therefore, these foci are different from those of GFP-SojD40A, even though their frequency and localization strongly suggested an association with the *oriC* region of the chromosome. To determine if GFP-SojG12V foci were localized near *oriC,* we constructed an additional strain harboring an array of *tetO* operators near *oriC* that can be visualized using a red fluorescent TetR reporter protein (TetR-mCherry). As shown in Figure 2B, all GFP-SojG12V foci colocalized with the *oriC* region of the chromosome.

The septal bands formed by GFP-SojG12V have previously been shown to depend on the cell-division inhibitor MinD (Autret and Errington, 2003). As for wild-type GFP-Soj, the polar localization was also dependent on MinD and in *ΔminD* mutants GFP-Soj was observed throughout the cytoplasm (Figure 2C). Septal localization of GFP-Soj was not affected in a *ΔminC* mutant (Figure 2C). Since both *ΔminC* and *ΔminD* mutants form minicells, loss of GFP-Soj septal localization was specific to the *ΔminD* mutant. Furthermore, it appeared that GFP-Soj was enriched at septa in a *ΔminC* mutant, suggesting that MinC and Soj might compete for binding with MinD. GFP-Soj did associate with the nucleoid in a *ΔminD Δspo0J* double mutant (data not shown), indicating that Spo0J remains competent to inhibit Soj DNA binding in the absence of MinD.

To place the SojG12V mutant protein within the Soj activity pathway we determined the localization of double mutants that inhibited either ATP binding or ATP hydrolysis of the

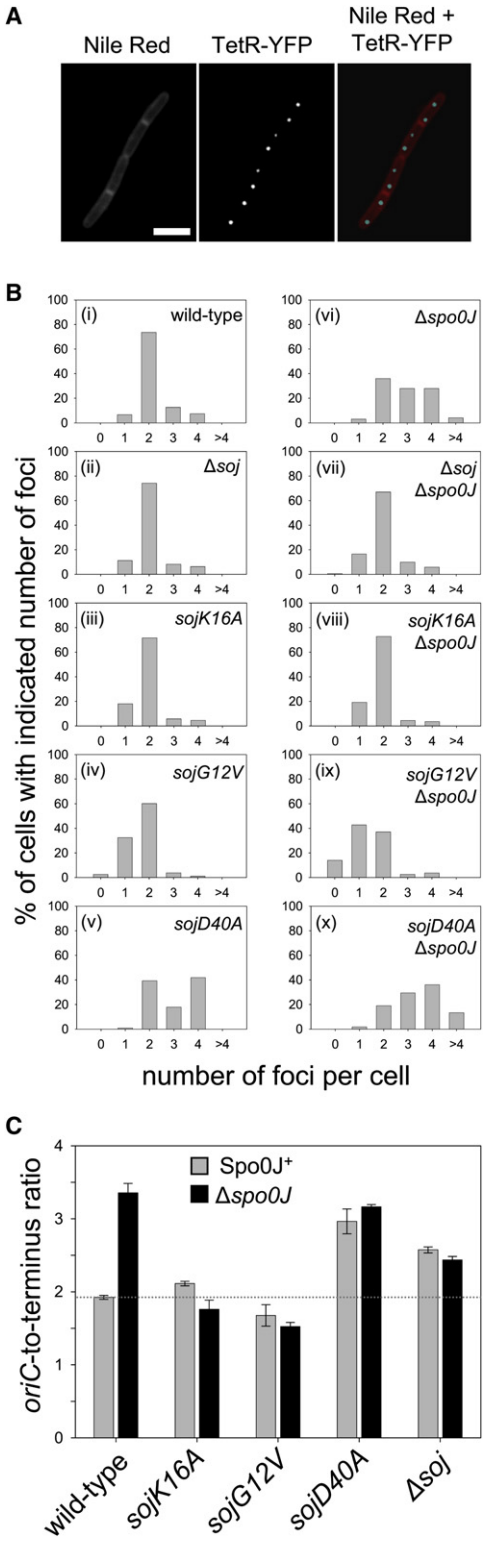

Figure 3. Soj Mutants Alter the Number of Chromosome Origins per Cell

(A) An example of origin localization in wild-type *B. subtilis* (HM130). The *oriC* region was labeled with an array of *tetO* operators bound by TetR-YFP, and the number of fluorescent foci per cell was determined using epifluorescence microscopy. Cells were grown in S7-minimal medium at 30°C. Cell membranes were stained with Nile Red to distinguish individual cells. Scale bar: 3 μm.

(B) Bar charts showing the results from chromosome origin counts. The relevant strain information is shown in the upper right corner of each panel. At least 200 cells were counted for each strain. (i) HM130, (ii) HM171, (iii) HM133, (iv) HM131, (v) HM135, (vi) HM168, (vii) HM194, (viii) HM196, (ix) HM197, (x) HM195.

(C) The *oriC*-to-terminus ratio of each Soj mutant in a wild-type strain (gray bars) and in a *Δspo0J* mutant (black bars) was determined using marker frequency analysis. Cells were grown in CH medium at 30°C. The steady-state growth rates for all strains were indistinguishable from each other (although we note that strains encoding the SojG12V mutant protein reproducibly showed a longer lag phase following dilution of stationary phase cultures). The results are shown as the average ± standard deviation (n = 3). The dashed line represents the average ratio for the wild-type strain. Wild-type (HM34), *Δspo0J* (HM42), *sojK16A* (HM38), *sojK16A Δspo0J* (HM46), *sojG12V* (HM40), *sojG12V Δspo0J* (HM48), *sojD40A* (HM36), *sojD40A Δspo0J* (HM44), *Δsoj* (HM161), *Δsoj Δspo0J* (HM183).

GFP-SojG12V mutant protein. As shown in Figure S4A, the GFP-Soj(G12V,K16A) mutant protein was dispersed throughout the cytoplasm, while the GFP-Soj(G12V,D40A) mutant protein remained localized as foci and at septa. These results are consistent with the SojG12V mutant protein being bound by ATP, placing it between the SojK16A mutant (nucleotide free) and the SojD40A mutant (DNA-binding-proficient ATP-bound dimer). We conclude that SojG12V associates with septa and also with a factor or site located in or near *oriC*, but that it is not capable of cooperatively binding to the nucleoid and that it has little or no association with Spo0J.

soj Mutations Affect the Control of DNA Replication Initiation

soj null mutants do not have an obvious cellular phenotype (Ireton et al., 1994), and fluorescence microscopy on *sojK16A* and *sojD40A* mutants revealed that growth, division, and chromosome segregation were within normal limits (Table S1). It was surprising, then, that the *sojG12V* mutant displayed a strikingly abnormal phenotype with a high frequency of cells lacking DNA (2.6% compared to <0.1% for wild-type; Figure S3 and Table S1). To address the basis for the *sojG12V* defect we examined the replication and segregation of the *oriC* region marked with an array of *tetO* operators to which a fluorescent TetR reporter protein (TetR-YFP) binds (Lau et al., 2003). Strains were grown in a medium supporting a relatively slow growth rate, and samples were taken to determine the number of TetR-YFP foci per cell. Cell membranes were stained with the fluorescent dye Nile Red to distinguish individual cells (Figure 3A).

In the wild-type strain a majority of cells contained two foci (Figure 3Bi). A minority of cells contained more than two foci (13% with three foci, 7% with four foci), and only 7% contained a single focus. The *Δsoj* and *sojK16A* mutant distributions were similar to those of the wild-type strain (Figures 3Bii and 3Biii). In contrast, the *sojD40A* mutant had more foci per cell (Figure 3Bv), while the *sojG12V* mutant had fewer foci per cell (Figure 3Biv). The *sojG12V* mutant also produced cells that lacked a TetR-YFP focus, consistent with our finding of cells lacking DNA (Figure S3).

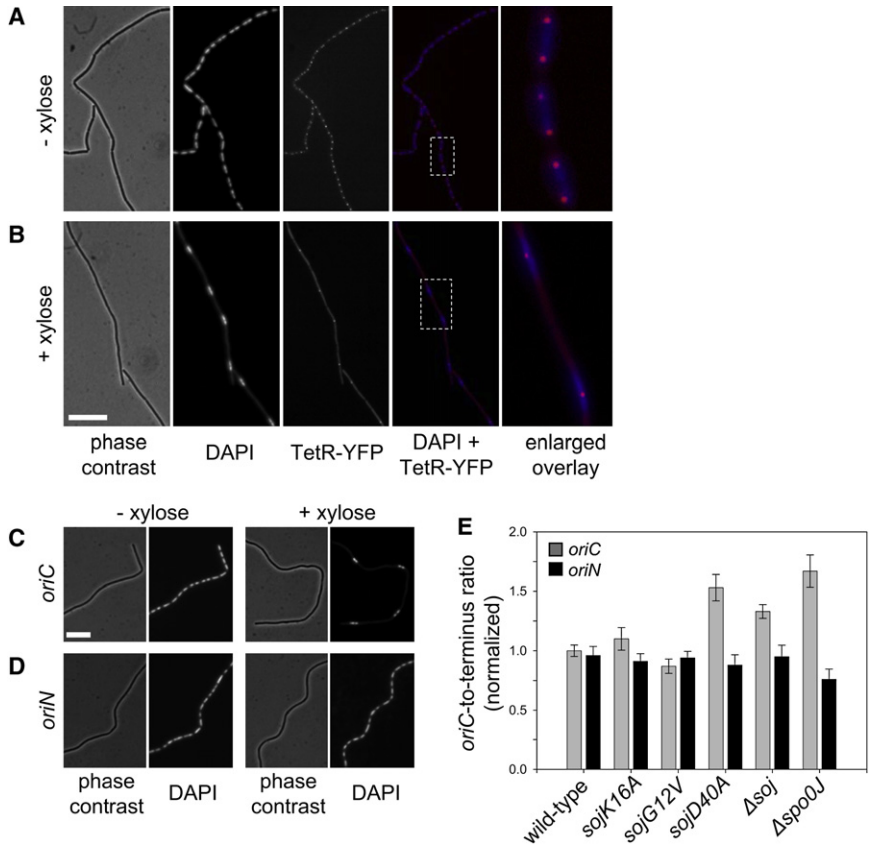

Figure 4. Regulation of DNA Replication Initiation by Soj Requires DnaA at OriC

Localization of the chromosome origin and bulk DNA in the absence (A) or presence (B) of SojG12V expression from a xylose-inducible promoter. The *oriC* region was labeled with an array of *tetO* operators bound by TetR-YFP. Strain HM247 was grown in S7-minimal medium at 30°C either in the absence or presence of 0.1% xylose. The DNA was labeled with DAPI. The phase-contrast image shows the outline of each cell. Scale bar: 20 μm. The dashed box indicates the region that is shown enlarged.

Localization of bulk DNA in the presence or absence of SojG12V expression in either a DnaA-dependent *oriC* strain (C) (HM207) or a DnaA-independent *oriN* strain (D) (HM208). Cells were grown in S7-minimal medium at 30°C in the absence or presence of 0.1% xylose. Scale bar: 9 μm.

(E) The *oriC*-to-terminus ratio of each Soj mutant in a wild-type strain (gray bars) compared to a DnaA-independent *oriN* strain (black bars). Cells were grown in CH medium at 30°C. Values were normalized to the wild-type *oriC*-to-terminus ratio, and the results are shown as the average ± standard deviation (n = 3). *soj oriC* (HM222), *soj oriN* (HM228), *sojK16A oriC* (HM223), *sojK16A oriN* (HM229), *sojG12V oriC* (HM224), *sojG12V oriN* (HM230), *sojD40A oriC* (HM225), *sojD40A oriN* (HM231), *Δsoj oriC* (HM227), *Δsoj oriN* (HM233), *soj Δspo0J oriC* (HM226), *soj Δspo0J oriN* (HM232).

The effects of these *soj* alleles on origin copy number was then tested for dependence on *spo0J*. For wild-type Soj the number of foci per cell was greatly increased in the *Δspo0J* mutant, consistent with a previous report (Figures 3Bi and 3Bvi; Lee et al., 2003). Deletion of *spo0J* appeared to exacerbate the phenotypes of the *sojG12V* and *sojD40A* mutants causing a further decrease or increase in the number of foci per cell, respectively (Figures 3Biv, 3Bv, 3Bix, and 3Bx and Table S1). These results indicate that Spo0J strongly regulates the activity of wild-type Soj, but that it has only minor effects on the activity of the *sojG12V* and *sojD40A* mutant proteins.

To test more directly and unambiguously for effects on origin copy number, DNA sites near the origin and terminus of replication were measured by quantitative PCR. This marker frequency analysis supported the idea of early initiation of DNA replication in the *sojD40A* mutant and in the *Δspo0J* mutant (Figure 3C; Lee et al., 2003). Appreciable premature initiation was also observed in the *Δsoj* mutant and in the *Δsoj Δspo0J* double mutant, although to a lesser degree than for the *sojD40A* or *Δspo0J* mutants (Figure 3C). In contrast, the *sojG12V* and *sojK16A* mutants exhibited a significant decrease in origin copy number compared to the *Δsoj* mutant (Figure 3C), consistent with inhibition of DNA replication initiation (similar to wild-type) and in accordance with flow cytometry data (Ogura et al., 2003).

It was surprising that different Soj mutant proteins had opposing activities on the rate of DNA replication initiation. To examine whether wild-type Soj could act as both a repressor and an activator of DNA replication initiation, we titrated Soj levels in cells with an inducible expression system. Compared to the control strain, at low expression levels of Soj there was a decrease in the origin copy number (Figure S1B). However, further induction of Soj reversed this effect and dramatically increased the origin copy number (Figure S1B), consistent with a previous report (Ogura et al., 2003). Thus, wild-type Soj appears to be capable of either negative or positive regulation of DNA replication initiation.

Inhibition of Chromosome Replication Initiation by Soj Mutant Proteins

If the SojG12V and SojK16A mutant proteins are negative regulators of DNA replication initiation, overproduction of these proteins should cause a more severe inhibition of replication. Examination of cells overexpressing these proteins lent support to this idea (similar results were obtained with SojG12V and K16A; only those of the former mutant are shown). As shown in Figure 4A, without SojG12V overexpression the nucleoids were located adjacent to one another and were present in all of the exponentially growing cells. A pair of origins (tagged with TetR-YFP foci) were usually associated with each nucleoid, one at each outer edge. Induction of SojG12V led to a dramatic change in DNA distribution; many cells lacked DNA and the rare nucleoids were separated from one another by large spaces (Figure 4B; the same effect was observed when a Soj(G12V,D40A) ATPase-deficient double mutant was overexpressed; Figure S4B). Furthermore, almost every nucleoid only had a single *oriC* focus, located approximately in the middle of the small bilobed structure

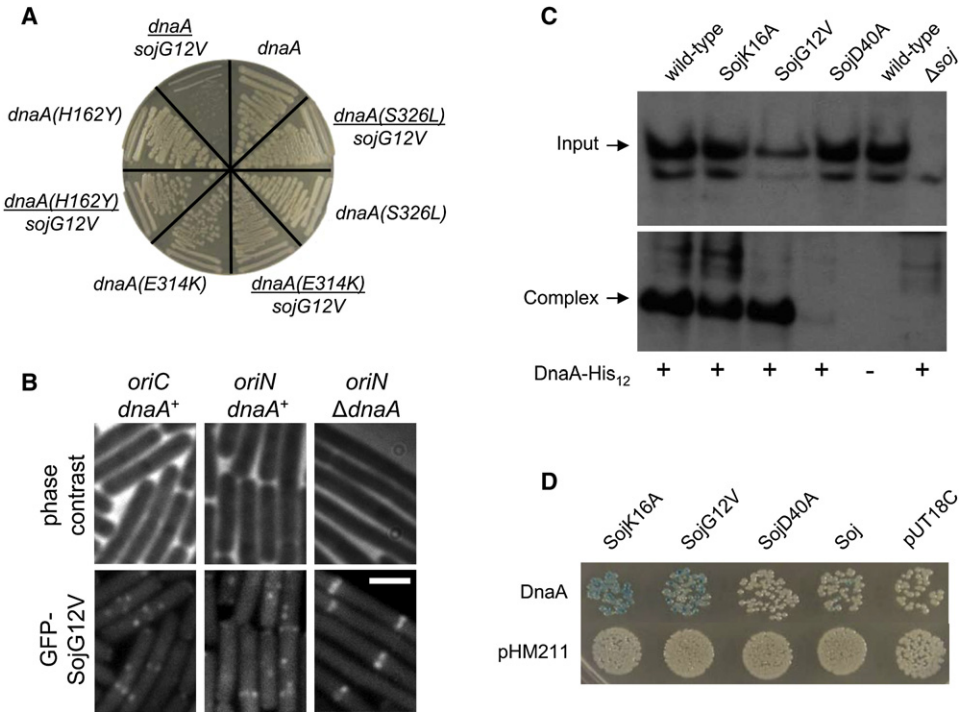

Figure 5. Genetic and Physical Interactions between SojG12V and DnaA

(A) Inhibition of DNA replication initiation by SojG12V is suppressed by mutations in DnaA. Strains were grown on nutrient agar medium supplemented with 1% xylose and 0.5 mM IPTG. *dnaA* (HM299), *dnaA/sojG12V* (HM295), *dnaA(H162Y)* (HM300), *dnaA(H162Y)/sojG12V* (HM296), *dnaA(E314K)* (HM301), *dnaA(E314K)/sojG12V* (HM297), *dnaA(S326L)* (HM302), *dnaA(S326L)/sojG12V* (HM298).

(B) Localization of GFP-SojG12V requires DnaA. Cells were grown in CH medium at 30°C in the presence of 0.025% xylose. *oriC dnaA*$^+$ (HM276), *oriN dnaA*$^+$ (HM268), *oriN ΔdnaA* (HM272). Scale bar: 2 μm.

(C) Soj forms a complex with DnaA-His$_{12}$. Soj proteins were detected by western blot analysis. The top panel shows the amount of each Soj protein in the lysate following crosslinking and cell disruption. The bottom panel shows the amount of Soj isolated from DnaA-His$_{12}$ complexes following purification. Wild-type/*dnaA-his$_{12}$* (HM330), *sojK16A/dnaA-his$_{12}$* (HM332), *sojG12V/dnaA-his$_{12}$* (HM333), *sojD40A/dnaA-his$_{12}$* (HM331), *Δsoj/dnaA-his$_{12}$* (HM334), wild-type (168ca).

(D) Bacterial two-hybrid analysis of Soj variants and DnaA (see Experimental Procedures). Colonies were analyzed on nutrient agar plates supplemented with X-gal. The appearance of blue pigment within colonies indicates a positive interaction.

(Figure 4B). Membrane staining showed that septa were present between the separated nucleoids (data not shown), suggesting that the DNA-damage checkpoint was not induced (Kawai et al., 2003; Love and Yasbin, 1984). This striking phenotype was very reminiscent of that generated by mutations affecting the initiation of DNA replication (Imai et al., 2000). Marker frequency analysis confirmed that the origin-to-terminus ratio was decreased by ~50% when SojG12V was overexpressed (data not shown).

Repression and Activation of DNA Replication Initiation by Soj Mutants Requires DnaA

To test whether the inhibition of DNA replication initiation by SojG12V required DnaA activity at *oriC*, an ectopic copy of *sojG12V* was overexpressed in a strain that bypasses the requirement for DnaA at *oriC* by replicating from an ectopic origin (*oriN*), which utilizes its cognate replication initiator protein RepN (Hassan et al., 1997; Moriya et al., 1997). Overexpression of SojG12V in the *oriN* strain had no effect on nucleoid distribution, indicating that the inhibition of DNA replication by SojG12V requires the activity of DnaA at *oriC* (Figures 4C and 4D).

We speculated that the activation of DNA replication initiation by SojD40A (and by wild-type Soj in a *Δspo0J* mutant; Figure 3C) might also act through DnaA at *oriC*. Marker frequency analysis was used to determine the effect of each Soj variant in an *oriN* strain background and in all cases the effects on replication initiation were lost (Figure 4E), indicating that both negative and positive regulation of DNA replication initiation by Soj requires DnaA at *oriC*.

Point Mutations in *dnaA* Bypass the Inhibition of DNA Replication by SojG12V

To test whether SojG12V might regulate DnaA directly, we selected for mutations near *oriC* that suppressed the inhibition of DNA replication initiation caused by SojG12V overexpression (see Experimental Procedures). Three mutants were found in which the overexpression phenotype was suppressed (Figure 5A). DNA sequencing revealed that each of these mutants contained unique changes within *dnaA* that created single amino acid substitutions: H162Y, E314K, and S326L. Marker frequency analysis showed that each of the *dnaA* mutants overinitiated DNA replication (data not shown), thereby bypassing the

inhibitory activity of SojG12V. The interpretation of these results is consistent with data from similar mutants previously described in *E. coli.* H162 lies in the alpha helix of the AAA+ domain that contains residues of the Walker A motif required for ATP binding (Mott and Berger, 2007). In *E. coli* mutation of an adjacent residue (*E.c*.A184V) leads to an ATP-binding defect in vitro and asynchronous replication initiation in vivo (Carr and Kaguni, 1996; Skarstad et al., 1988). E314 is located adjacent to the invariant sensor II arginine residue (R313) that makes contact with the γ phosphate of ATP in the DnaA oligomer. In *E. coli* mutation of the homologous arginine residue to alanine (*E.c.* R334A) inhibits ATP hydrolysis in vitro and leads to overinitiation in vivo (Nishida et al., 2002).

Interaction of Soj with DnaA

The observation that several different mutations in *dnaA* suppressed the inhibition of DNA replication initiation by SojG12V strongly suggested that DnaA is the target of SojG12V action. To begin testing this hypothesis we examined the localization of GFP-SojG12V in the absence of *dnaA* by taking advantage of an *oriN* strain. We found that GFP-SojG12V no longer formed foci within the cytoplasm of a *ΔdnaA* mutant (Figure 5B), indicating that this localization is DnaA dependent.

To determine if any of the Soj proteins form a complex with DnaA, we used an in vivo crosslinking method (Ishikawa et al., 2006, 2007). Briefly, the endogenous *dnaA* gene was replaced by an allele encoding a C-terminal His tag, and this fusion protein was used to purify protein complexes from *B. subtilis* cells. The results showed that SojG12V, SojK16A, and wild-type Soj (but not SojD40A) are associated in a complex with DnaA (Figure 5C). These complexes were resistant to DNase treatment (data not shown), and control strains that did not contain the *dnaA-his*$_{12}$ allele or that carried a *Δsoj* mutation confirmed that the observed DnaA-Soj interactions were specific (Figure 5C). Moreover, SojG12V remained in a complex with DnaA in a *Δspo0J* mutant strain (data not shown), indicating that Spo0J is not required for this interaction.

A bacterial two-hybrid system (Karimova et al., 1998) was used to test for a direct interaction between Soj and DnaA. As shown in Figure 5D both SojK16A and SojG12V interacted strongly with DnaA. Wild-type Soj also interacted with DnaA, albeit less than the SojK16A and SojG12V mutants, but no interaction was detected with SojD40A (Figure 5D). Taken together these results indicate that Soj physically interacts with DnaA at a step upstream of its cooperative DNA-binding activity.

Inhibition of Sporulation by Soj Requires ATP-Dependent Dimerization and Acts through the Sda-Dependent DNA Replication Initiation Checkpoint

Soj blocks an early step of spore development in a *Δspo0J* mutant (Ireton et al., 1994). Based on transcriptional reporter assays and chromatin immunoprecipitation it was proposed that Soj interacts with several genes required for sporulation (*spoIIA, spoIIE, spoIIG)* and directly represses their expression (Cervin et al., 1998; Ireton et al., 1994; Quisel and Grossman, 2000). However, in vitro studies using purified components have failed to recapitulate specific transcriptional repression of these sporulation genes by Soj (McLeod and Spiegelman, 2005).

Table 1. Inhibition of Sporulation by Soj Requires the Chromosome Replication Initiation Checkpoint Protein Sda

Strain	Genotype	# Colonies/ml	# Spores/ml	% Sporulation
JH642	Wild-type	3.2×10^8	1.4×10^8	44
HM248	*sojD40A*	4.8×10^8	1.6×10^5	0.033
HM249	*Δspo0J*	3.0×10^8	6.0×10^2	0.00020
BB668	*Δsda*	2.4×10^8	1.2×10^8	50
HM251	*sojD40A Δsda*	3.0×10^8	1.0×10^8	33
HM252	*Δspo0J Δsda*	2.6×10^8	8.0×10^7	31

Each *soj* mutant was tested for its ability to repress sporulation in the absence of *spo0J* and only *sojD40A* was found to have this activity (Table S2). Indeed, this allele gave a Spo$^-$ phenotype even in the presence of *spo0J*. This result indicates that it is the ATP-bound form of Soj that inhibits sporulation. The observation that *sojD40A* inhibits sporulation in the presence of *spo0J* is consistent with the model that in wild-type cells Spo0J counteracts Soj inhibition of sporulation by activating ATP hydrolysis to drive dissociation from DNA.

In light of the evidence for regulation of DnaA by Soj (Figures 4 and 5), we asked whether inhibition of sporulation by Soj requires the DNA replication checkpoint protein Sda. In *B. subtilis* Sda represses sporulation in response to altered DNA replication initiation caused by mutations in *dnaA* (Burkholder et al., 2001). Table 1 shows that deletion of *sda* suppresses the sporulation block imposed by Soj and SojD40A, indicating that Soj acts upstream of Sda in the DNA replication checkpoint pathway. Taken together with the marker frequency analysis (Figure 3C), these results show that activation of DnaA by Soj causes over-replication and indirectly inhibits sporulation by triggering the Sda checkpoint. These results also indicate that the mild over-replication observed in a *Δsoj* mutant is insufficient to elicit the Sda checkpoint, suggesting that either a high level of over-replication is required to activate Sda or Soj itself is required to activate Sda.

DISCUSSION

Soj Mutant Proteins Have Distinct Localization Patterns

In this report we have elucidated several distinct elements of the *B. subtilis* Soj localization cycle. Examination of the GFP-SojG12V mutant (ATP-bound but unable to bind DNA cooperatively) showed that localization is DnaA dependent (cytoplasmic foci) and MinD dependent (septal), but that its behavior is not affected by the presence or absence of Spo0J. In contrast, localization of the GFP-SojD40A mutant (ATP-bound dimer) was Spo0J dependent and in the absence of Spo0J was mainly concentrated over the nucleoid. This is consistent with the idea that ATP binding by Soj is needed for cooperative DNA binding and that this is the form of Soj that interacts with Spo0J. Presumably, interaction of Spo0J with wild-type Soj leads to stimulation of the ATP-hydrolysis activity of Soj, leading to release from the DNA. Finally, the majority of the GFP-SojK16A mutant (nucleotide free) was localized throughout the cytoplasm with only rare foci observable, indicating that ATP binding promotes focus formation and is required for septal localization.

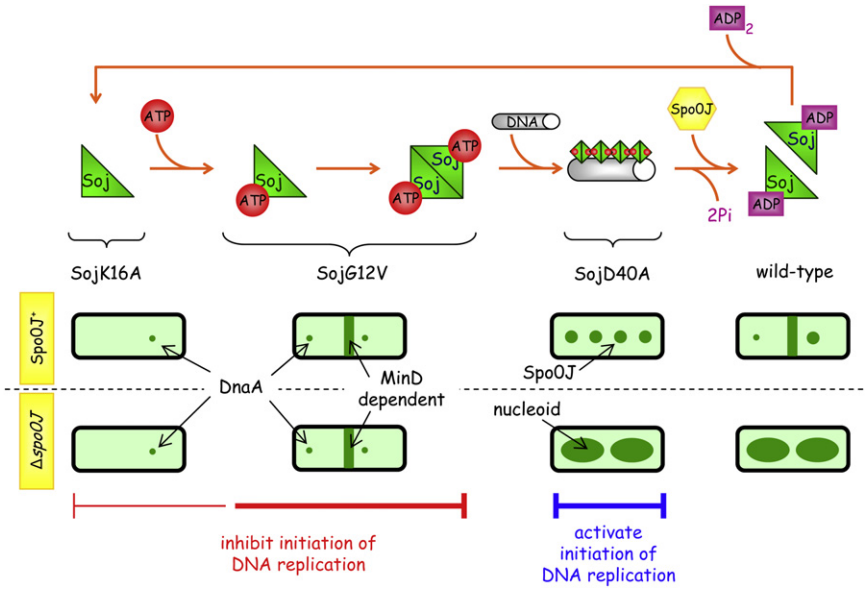

Figure 6. Summary of Soj Localization Patterns and Regulatory Activities

(Top) Pathway describing Soj ATP binding, dimerization, cooperative DNA binding, and ATP hydrolysis (adapted from Leonard et al., 2005). (Bottom) Summary of localization data and affects on DNA replication initiation for each Soj mutant protein in either a wild-type strain or a *Δspo0J* mutant strain.

In the presence of Spo0J, wild-type GFP-Soj localizes to septa and as punctate foci within the cytoplasm. In the absence of Spo0J, GFP-Soj appears to localize nonspecifically with the nucleoid, consistent with GFP-Soj accumulating as an ATP-bound dimer under this condition. Previous studies suggested that GFP-Soj accumulates at a subset of nucleoids and cooperatively relocalizes from the bound nucleoid to an adjacent unbound nucleoid (Marston and Errington, 1999; Quisel et al., 1999). Although we have not observed this dynamic "nucleoid jumping" during the current study in which GFP-Soj was expressed at lower, more physiological levels, it nonetheless suggests that Soj has the ability to form nucleoprotein complexes cooperatively in vivo, as has been observed in vitro (Leonard et al., 2005).

Soj Regulates the DNA Replication Initiation Protein DnaA

In addition to their distinct localization patterns, we report that the Soj mutant proteins exert opposing effects on DNA replication initiation by regulating DnaA action. SojG12V and SojK16A inhibit DnaA-dependent DNA replication initiation, mutations in *dnaA* can specifically suppress this inhibition, and the GFP-SojG12V foci colocalize with *oriC* in a DnaA-dependent manner. Moreover, we have found that SojG12V, SojK16A, and wild-type Soj form a complex with DnaA in vivo, and that they interact with DnaA as judged by two-hybrid analysis. Taken together these results indicate that Soj directly inhibits DnaA activity.

In contrast to SojG12V, SojD40A (and wild-type Soj in a *Δspo0J* mutant) stimulated the initiation of DNA replication. The degree of activation was significantly higher in these mutant strains than in a *Δsoj* mutant, and this difference correlated with the ability to induce Sda-dependent repression of sporulation, indicating that cooperative DNA binding by Soj positively regulates DnaA. Interaction of SojD40A with DnaA was not detected by two-hybrid analysis and was only weakly detected by cross-linking in vivo, suggesting that it may activate DnaA indirectly, perhaps by altering DNA topology near *oriC*.

Dynamic Localization of Soj and the Control of Initiation

The results summarized above are consistent with a model in which Soj is an important regulator of the initiation of DNA replication that can advance or delay initiation, depending on its quaternary state (Figure 6). To our knowledge this is the first bacterial regulatory system shown to act as both a positive and a negative regulator of DnaA activity.

We propose that during the majority of cell growth Soj is in the inhibitory state, interacting with DnaA at *oriC*. This is in line with the data showing that SojG12V localizes similarly to wild-type Soj (Figures 1A and 1E) and that cells initiate DNA replication early when Soj is not present (Figure 3C). The dramatic switch in the state of Soj in the presence and absence of Spo0J (see Figures 1A and 1B) suggests that Spo0J plays an important role in maintaining Soj in the inhibitory state. Interaction with Spo0J presumably takes place in the active, DNA-bound, ATP-dimer form, as judged by the apparent affinity of D40A for Spo0J (Figures 1G, 1H, and 2A). We suggest that in wild-type cells concentration of Soj at DnaA-*oriC* results in dimerization and cooperative polymerization on the chromosome at or near *oriC*. Propagation along the chromosome then leads to interaction with Spo0J at nearby *parS* domains, which triggers nucleotide hydrolysis and release of Soj from the DNA. The resultant equilibrium is normally in favor of the inhibitory state of Soj. At some point in the cell cycle this equilibrium is broken and Soj accumulates in the activator state, thus helping to trigger the initiation of DNA replication (Figure 6). We do not yet understand the nature of the signal that leads to switching of Soj activity (nor how the D40A state of Soj stimulates initiation). However, given the interactions of Soj with Spo0J, DnaA, and MinD, we think that the signal is likely to involve the relative localization of origin regions and cell poles. Since there are high concentrations of the inhibitory form of Soj at cell poles, via the MinD-dependent interaction (Figure 2C), proximity to the cell pole, potentially indicating that the cell is small and has a high cellular DNA content, could be used to delay initiation until further cell growth has occurred. Inhibition by proximity to MinD at the cell poles might be especially important during the early stages of sporulation in *B. subtilis*. At this time *oriC* regions move to the extreme cell poles in preparation for asymmetrical division, an event that needs to be coordinated with a block in further rounds of DNA replication (see Errington, 2003).

Might the Pleiotropic Phenotypes of Other Bacterial *parAB* Mutants Be due to Effects on DNA Replication?

Bioinformatic and experimental analyses have shown that the chromosomal *parABS* genes are most often located close to origins of replication and play important roles in bacterial chromosome biology (see Introduction). However, the pleiotropic nature of mutations affecting *parAB* genes has made it difficult to identify the primary target leading to the diverse phenotypic effects. Our results show that the prominent inhibition of sporulation by Soj is an indirect effect of its action in regulating DnaA (Table 1). We close by speculating that many, perhaps all, ParA proteins also regulate their cognate DNA replication systems and that many of the diverse phenotypes attributed to *parAB* mutations in other bacteria could be secondary to altered regulation of DNA replication.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

The *B. subtilis* strains used are listed in Table S3.

Media

Nutrient agar (Oxoid) was used for routine selection and maintenance of both *B. subtilis* and *E. coli* strains. M9 minimal media containing 15 g/l agar, 0.5% glycerol, and required amino acids were used for maintenance of *oriN*-dependent strains. For *B. subtilis,* cells were grown in casein hydrolysate (CH) medium, Schaeffer's broth, or S7-defined minimal medium (using 50 mM MOPS buffer instead of 100 mM) containing 0.5% glycerol and 0.1% glutamate. For *E. coli,* cells were grown in Luria-Bertani (LB) medium. Supplements were added as required: 20 μg/ml tryptophan, 40 μg/ml phenylalanine, 30 μg/ml (for single copy plasmids) or 100 μg/ml ampicillan, 5 μg/ml chloramphenicol, 2 μg/ml kanamycin, 50 μg/ml spectinomycin, and 15 μg/ml tetracycline.

Microscopy

To visualize cells during exponential growth, starter cultures were grown overnight then diluted 1:100 into fresh medium and allowed to achieve at least three doublings before observation. Where S7-minimal medium was used, the overnight culture was supplemented with 0.02% casamino acids to inhibit sporulation. Cells were mounted on ~1.2% agar pads (containing the same medium used for growth) immobilized within a Gene Frame (ABgene) using a 0.13–0.17 mm glass coverslip (VWR). To visualize nucleoids the DNA was stained with 2 μg/ml 4′-6-diamidino-2-phenylindole (DAPI) (Sigma). To visualize individual cells the cell membrane was stained with 2 μg/ml Nile Red (Sigma). Microscopy was performed on an inverted epifluorescence microscope (Zeiss Axiovert 200M) fitted with a Plan-Neofluar objective (Zeiss 100×/1.30 Oil Ph3), a 300W xenon arc-lamp transmitted through a liquid light guide (Sutter Instruments), and a Sony CoolSnap HQ cooled CCD camera (Roper Scientific). All filters were Modified Magnetron ET Sets from Chroma and details are available upon request. Digital images were acquired and analyzed with METAMORPH software (version V.6.2r6).

Marker Frequency Analysis

Cells were grown in CH medium at 30°C as described for microscopy. Sodium azide (0.5%; Sigma) was added to exponentially growing cells (A_{600} = 0.1–0.3) to prevent further growth. Chromosomal DNA was isolated using a DNeasy Blood and Tissue Kit (QIAGEN). Power SYBR Green PCR Master Mix was used for PCR reactions (Applied Biosystems). Q-PCR was performed in a LightCycler 480 Instrument (Roche, Inc.). By use of crossing points and PCR efficiency a relative quantification analysis was performed using LightCycler Software version 4.0 (Roche, Inc.) to determine the *ori/ter* ratio of each sample. These results were normalized to the *ori/ter* ratio of a DNA sample from *B. subtilis* spores in which the *ori/ter* ratio is 1.

Sporulation Assays

Sporulation frequencies were determined as the ratio of heat-resistant (80°C for 20 min) colony-forming units to total colony-forming units. Cells were grown in Schaeffers broth and assayed ~24 hr after inoculation (A_{600} ~0.02).

Mutagenesis and Selection of DnaA Mutants

Strain HM259 bearing the *erm* antibiotic cassette at *oriC* was treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) as previously described (Errington and Mandelstam, 1983). Mutagenized chromosomal DNA was isolated, transformed into HM240, and plated in the presence of 1% xylose to induce overexpression of *sojG12V*. Large colonies were selected (260 from a starting pool of ~188,000 clones) and patched to confirm the presence of correct antibiotic markers. Chromosomal DNA from 41 candidates was isolated and backcrossed into HM240, and transformants were selected in the absence or presence of *sojG12V* overexpression. Seven candidates that were tightly linked to *erm* and grew well in both the absence and presence of SojG12V overexpression were selected and the *dnaA* gene was sequenced (one allele *dnaA(c484t)*; four alleles *dnaA(g940a);* two independent alleles of *dnaA(c977t)*). Each *dnaA* mutant was amplified by PCR and cloned into pMUTIN4 (Vagner et al., 1998), resequenced, and integrated into HM240 by single crossover to test for suppression of SojG12V overexpression (the start codon of each *dnaA* allele was changed to a stop codon so that only the mutant copy of *dnaA* was expressed).

Purification of In Vivo Protein-Protein Complexes

DnaA-His_{12} protein complexes were purified from *B. subtilis* as described (Ishikawa et al., 2006, 2007) with the following modifications. Strains were grown at 30°C in 40 ml of LB medium until the A_{600} reached 0.4–0.5. After crosslinking cells were washed with phosphate-buffered saline prior to brief storage in liquid nitrogen. Cell pellets were resuspended in buffer UT and disrupted by sonication (20 min at level 6 using a Sonics Vibracell). The eluate was passed through a Microcon-10 filter (Millipore) to concentrate the sample. Crosslinks were dissociated by heating at 90°C for 60 min and half of the sample was used for SDS-PAGE (4%–12% NuPAGE Novex Bis-Tris Gel; Invitrogen) followed by western blot analysis using α-Soj polyclonal antibodies.

Bacterial Two-Hybrid Assay

E. coli strain BTH101 was transformed using each combination of complimentary plasmids. A 10 μl aliquot from each transformation reaction was spotted onto a nutrient agar plate containing 100 μg/ml ampicillan, 50 μg/ml kanamycin, and 0.008% X-gal. Plates were incubated at 37°C overnight and then shifted to room temperature (~22°C) for an additional 24 hr. Images were collected using a standard digital camera.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, three tables, and four figures and can be found with this article online at http://www.cell.com/cgi/content/full/135/1/74/DC1/.

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