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The bacterial chromosome segregation protein Spo0J spreads along DNA from *parS* nucleation sites

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Summary

Regulation of chromosome inheritance is essential to ensure proper transmission of genetic information. To accomplish accurate genome segregation, cells organize their chromosomes and actively separate them prior to cytokinesis. In *Bacillus subtilis* the Spo0J protein is required for accurate chromosome segregation and it regulates the developmental switch from vegetative growth to sporulation. Spo0J is a DNA-binding protein that recognizes at least eight identified *parS* sites located near the origin of replication. As judged by fluorescence microscopy, Spo0J forms discrete foci associated with the *oriC* region of the chromosome throughout the cell cycle. In an attempt to determine the mechanisms utilized by Spo0J to facilitate productive chromosome segregation, we have investigated the DNA binding activity of Spo0J. *In vivo* we find Spo0J associates with several kilobases of DNA flanking its specific binding sites (*parS*) through a *parS*-dependent nucleation event that promotes lateral spreading of Spo0J along the chromosome. Using purified components we find that Spo0J has the ability to coat non-specific DNA substrates. These 'Spo0J domains' provide large structures near *oriC* that could potentially demark, organize or localize the origin region of the chromosome.

Introduction

Chromosome organization and segregation are critical for cell cycle progression. In eukaryotes, mitosis delineates these activities into well-defined steps: sister chromosome condensation, formation of the mitotic spindle and active segregation of replicated chromosomes via microtubules anchored to kinetochores. Progression through mitosis is tightly regulated to ensure proper chromosome segregation and cytokinesis. Although no such overt cycle is present in bacterial cells, it has become clear that the genomes in these unicellular organisms are segregated in an organized process. Bacterial chromosomes are assembled into domains, which are arranged into higher-order structures that adopt a specific orientation within the cell (Wu and Errington, 1994; 1998; Niki and Hiraga, 1998; Teleman *et al*., 1998; Niki *et al*., 2000; Roos *et al*., 2001; Viollier *et al*., 2004; Stein *et al*., 2005). Evidence from several microorganisms suggests that bacterial chromosomes are actively segregated towards opposite poles within the cell as part of a co-ordinated regulatory network which increases accurate chromosome inheritance (Glaser *et al*., 1997; Webb *et al*., 1997; Niki and Hiraga, 1998; Jensen and Shapiro, 1999; Li *et al*., 2002; Lau *et al*., 2003; Viollier *et al*., 2004; Bates and Kleckner, 2005).

With the structure and the dynamic behaviour of bacterial chromosomes emerging, our need now is to understand the mechanisms responsible for these activities. The *parA*, *parB* and *parS* genes are factors that specifically participate in bacterial chromosome segregation. This family of genes was first identified as segregation stability determinants harboured on low-copy-number plasmids (reviewed by Gerdes *et al*., 2000). *parS* is a *cis*-acting DNA binding site that interacts directly with the *trans*-acting ParA and ParB proteins where they form various nucleoprotein complexes (dependent on the particular plasmid). *parAB* are expressed from an autoregulated operon; ParA is an ATPase and ParB is a DNA-binding protein. Homologous genes are present in the genomes of almost all bacteria and are grouped into a distinct phylogeny separate from their plasmid-born relatives (Gerdes *et al*., 2000; Yamaichi and Niki, 2000).

In this report we have focused on characterizing the interaction of a chromosomally encoded ParB protein with its binding site (*parS*). Work on several chromosomal ParB proteins suggests they play important roles in chromosome biology. In *Bacillus subtilis*, *Pseudomonas putida* and *Streptomyces coelicolor* ParB is required for accurate chromosome segregation (Ireton *et al*., 1994; Kim *et al*., 2000; Lewis *et al*., 2002), in *Pseudomonas aeruginosa* overexpression of ParB affects chromosome organization

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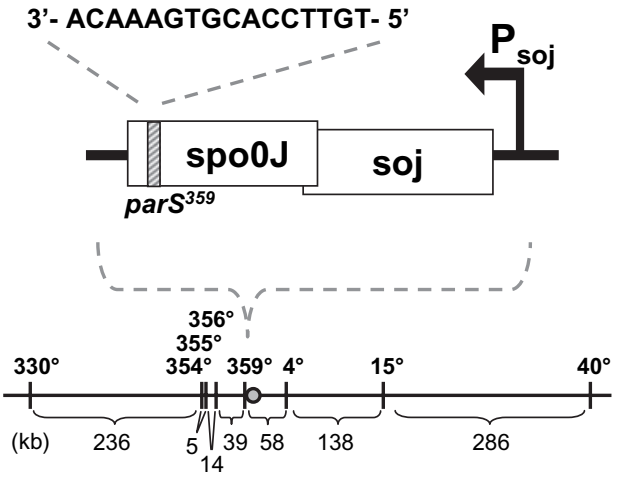

Fig. 1. The *oriC* region of the *B. subtilis* chromosome. *parS* sites are designated by their location on the genetic map. The hatched box within *spo0J* represents *parS*^{359} (with the DNA sequence shown above).

and inhibits growth (Bartosik *et al*., 2004), and in *Caulobacter crescentus* ParB is required for proper cell cycle progression (Mohl *et al*., 2001).

The *parB* homologue in *B. subtilis* is *spo0J*, initially identified as a factor required for efficient sporulation (Hranueli *et al*., 1974). Spo0J was later shown to be a DNA-binding protein required for accurate chromosome segregation, organization and localization (Ireton *et al*., 1994; Sharpe and Errington, 1996; Lin and Grossman, 1998; Marston and Errington, 1999; Lee *et al*., 2003; Lee and Grossman, 2006). Chromatin immunoprecipitation (ChIP) has demonstrated Spo0J associates with at least eight binding sites (*parS*, a 16 bp inverted repeat sequence) that are scattered around the *oriC*-proximal region of the chromosome (Fig. 1; Lin and Grossman, 1998). Fluorescence microscopy revealed that Spo0J forms single discrete foci that colocalize with the *oriC* region of the chromosome throughout the cell cycle (Glaser *et al*., 1997; Lewis and Errington, 1997; Lin *et al*., 1997; Teleman *et al*., 1998). The foci presumably represent Spo0J bound to each of the dispersed *parS* sites, although it has been estimated that there are ~1000 molecules of Spo0J in the cell, in great excess over the eight known *parS* binding sites (Glaser *et al*., 1997; Lin *et al*., 1997).

We have investigated the interactions of Spo0J with DNA *in vivo* and *in vitro* to begin dissecting the roles of ParB proteins in bacterial chromosome segregation. The results suggest that Spo0J associates with several kilobases (kb) of DNA flanking *parS* binding sites through a mechanism involving nucleation at *parS* followed by lateral spreading of Spo0J along the DNA. We have termed these extensive structures 'Spo0J domains' and hypothesize that they may affect chromosome segregation directly by organizing or localizing the *oriC* region, as well as indirectly through demarcation of the *oriC* region for the regulator Soj.

Results

Spo0J associates with DNA flanking parS *sites* in vivo

To determine the DNA-binding pattern of Spo0J *in vivo* we performed formaldehyde-mediated DNA–protein cross-linking followed by immunoprecipitation of Spo0J complexes. The isolated DNA was analysed by polymerase chain reaction (PCR) utilizing several primer pairs that surround a *parS* site. Figure 2A shows there is an enrichment of DNA recovered at *parS*^{359} (consistent with previous results; Lin and Grossman, 1998) as well as from the flanking DNA. The enrichment of DNA distal to *parS* decreases as a function of distance. In a *spo0J* null strain (but *parS*$^{+}$), no enrichment was observed (Fig. 2A).

To ensure the observed enrichment pattern was not due to incomplete shearing of the DNA, we utilized the replication terminus protein (RTP) which binds specifically to *ter* sites located near the terminus of replication as a control. A culture was exposed to cross-linking agent, the DNA was sheared and the sample was split, followed by ChIP using either α-Spo0J or α-RTP antibodies. After normalizing for the amount of DNA enriched at each protein's specific binding site, enrichment of DNA flanking *terI/II* was not observed when RTP was immunoprecipitated, whereas there was an enrichment of DNA extending for ~5 bp in either direction away from *parS*^{359} when Spo0J was immunoprecipitated (Fig. 2B). In the absence of Soj, we observed no change in the pattern of DNA immunoprecipitated by Spo0J (data not shown). These results support the hypothesis that Spo0J associates with DNA, directly or indirectly, surrounding a *parS* binding site.

The *parS*^{359} site investigated above is located within the *spo0J* gene (Fig. 1). To determine whether Spo0J associates with DNA flanking other known *parS* sites we investigated *parS*^{4} and *parS*^{356}. Figure 2C shows that after ChIP there is an enrichment of DNA surrounding both of these *parS* sites. We note that the enrichment of recovered DNA in the *parS*^{356} region extended counterclockwise over the whole of the 7 kb probed, suggesting that this region of the chromosome contains a high density of Spo0J. This may be due to the presence of two additional *parS* sites located 14 and 19 kb away in that direction.

Lateral spreading of Spo0J in vivo

Although the experiments described above indicated that Spo0J interacts with DNA flanking *parS* sites, they did not illuminate the mechanism by which Spo0J contacts these regions. Based on the observation that the frequency of

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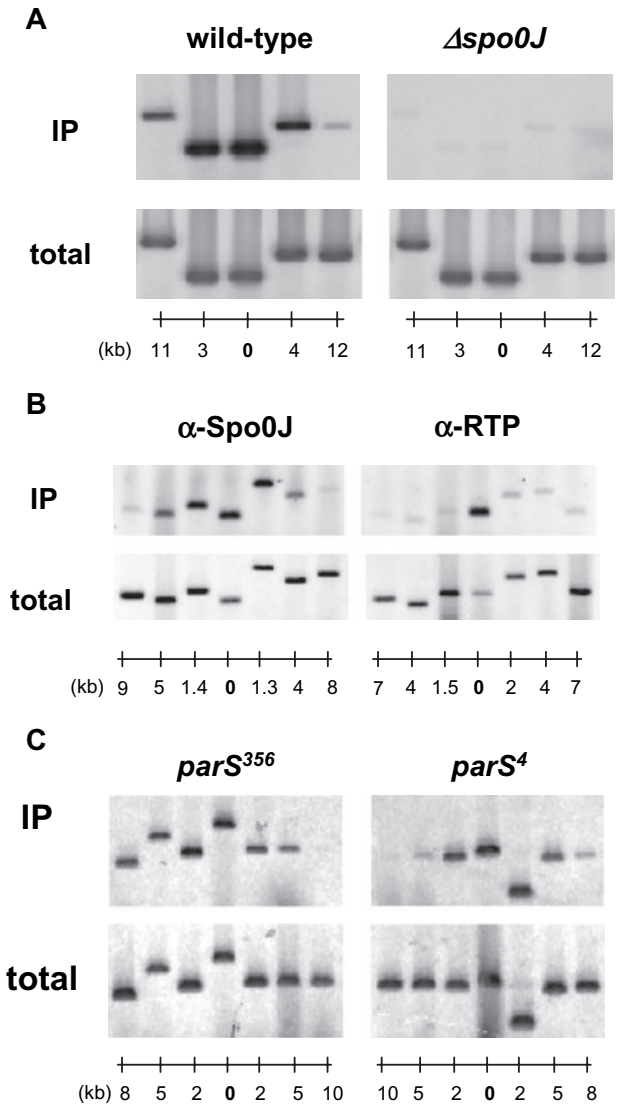

Fig. 2. Spo0J interacts with DNA flanking a *parS* binding site *in vivo*.
A. Enrichment of DNA at *parS*^{359} by PCR after cross-linking and immunoprecipitation (IP) of Spo0J from either wild-type *B. subtilis* 168 or a Δ*spo0J* mutant (HM2). Detection of total input DNA for each primer pair is shown below the corresponding immunoprecipitated DNA. The distance and orientation of each primer pair relative to *parS* is shown in kilobases.
B. Enrichment of DNA after ChIP utilizing either Spo0J or RTP-GFP from strain HM3, followed by PCR of sites flanking *parS*^{359} or *terI/II* respectively. To normalize the amount of DNA enriched by RTP-GFP and Spo0J at their specific binding sites a dilution series using the immunoprecipitated DNA from each reaction was used as a substrate for PCR reactions to find a level of input that produced equal signals. Because the signal from the RTP-GFP reaction was less than from Spo0J, the Spo0J sample was diluted until the enrichment at *parS* equalled the enrichment at *terI/II*.
C. Enrichment of DNA after ChIP of Spo0J from *parS*^{4} and *parS*^{356} in wild-type *B. subtilis* 168.

cross-linking decreased as a function of distance away from *parS*, we hypothesized that Spo0J would initially bind at a *parS* site and subsequently spread laterally along the DNA. To test this model, we began by replacing the *parS*^{4} site with an antibiotic resistance cassette to determine whether the binding site was necessary to promote Spo0J binding several kilobases away. None of the probed DNA sequences surrounding the *parS*^{4} site were enriched after ChIP when *parS*^{4} was replaced, although using the same sample Spo0J did enrich DNA flanking the *parS*^{359} site (Fig. 3A). This result suggests that a *parS* site is required for Spo0J to contact the surrounding region of the chromosome.

We next constructed an inducible 'roadblock' by introducing a binding site (*xylO*) for the xylose repressor, XylR (Gartner *et al.*, 1988; 1992), adjacent to *parS*^{359}. If Spo0J associates with DNA by spreading away from *parS*^{359}, then this roadblock should inhibit the enrichment of DNA bound to Spo0J distal to *parS*. Alternatively, if Spo0J associates with *parS*-flanking DNA by looping or hopping,

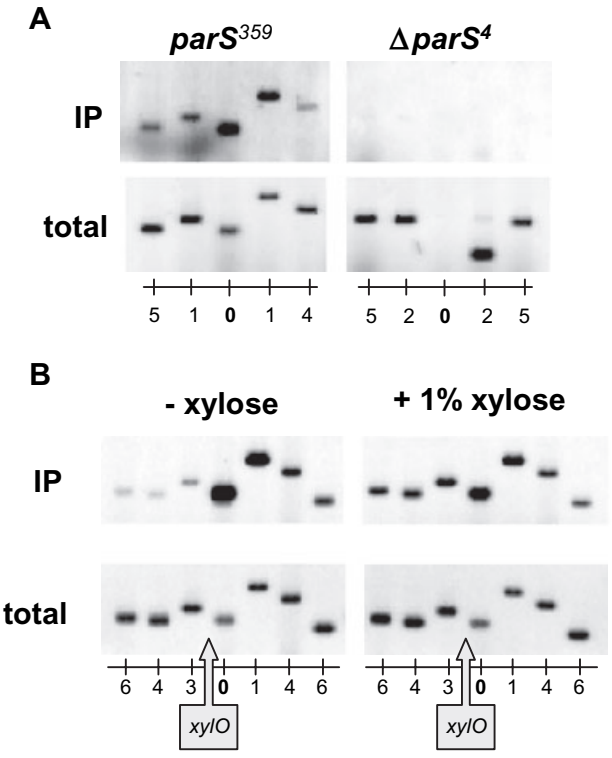

Fig. 3. Spo0J spreads from a *parS*-dependent nucleation site.
A. A *parS* site is required to nucleate the spreading of Spo0J *in vivo*. Spo0J–DNA complexes were immunoprecipitated from strain HM28 (Δ*parS*^{4}).
B. Spreading of Spo0J from *parS*^{359} can be blocked by a DNA-binding protein. Prior to ChIP analysis, strain HM22 (containing the promoter-operator region from the *xyl* operon downstream of *spo0J*) was grown overnight in the absence of xylose and was then subcultured into fresh medium with and without 1% xylose.

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then the XylR roadblock would not be expected to alter the binding pattern of Spo0J. Figure 3B shows that under growth conditions in which XylR binds *xlyO* tightly (without xylose) there was a decrease in the recovery of DNA beyond the roadblock, although binding of Spo0J to *parS*^{359} and to the DNA located in the opposite direction to the roadblock was not perturbed. Under growth conditions in which XylR binds only weakly to DNA (i.e. in the presence of 1% xylose), Spo0J was able to associate with flanking sequences beyond *xylO* (Fig. 3B). This result supports a model in which Spo0J spreads along DNA laterally from a *parS* nucleation site.

Site-specific Spo0J DNA binding activity in vitro

To directly analyse the DNA-binding properties of Spo0J we purified the protein utilizing a C-terminal His_6-epitope (Spo0J-His_6 is functional in *B. subtilis*; Lin *et al*., 1997). The DNA binding activity of our Spo0J-His_6 preparation was evaluated using both an electrophoretic mobility shift assay (as previously described by Lin and Grossman, 1998) and a DNase I protection assay. Using a 24 bp DNA fragment containing the *parS* site, addition of Spo0J-His_6 produced a single shifted protein–DNA complex (Fig. 4A) with a dissociation constant ~250 nM (data not shown). To confirm that the binding of Spo0J-His_6 to *parS* is specific, a competition experiment was performed using unlabelled substrates that contained either a wild-type *parS* site or a mutated *parS* site (containing seven changes within the 16 bp site). Figure 4A shows that the competitor containing the wild-type *parS* site was able to liberate the labelled DNA probes, while the competitor containing the mutant *parS* site was not.

Several attempts to detect the footprint of Spo0J-His_6 on short linear *parS*-containing substrates were unsuccessful (data not shown), therefore we attempted to footprint Spo0J-His_6 using an indirect end-labelling approach to examine the DNase I cleavage pattern of Spo0J-His_6 on a *parS*-containing plasmid. Spo0J-His_6 was incubated with the *parS*-containing plasmid at concentrations leading to either partial or saturated binding of the substrate (Fig. 4B, lanes 2 and 1 respectively). A clear footprint was obtained over the *parS* site on both the top and bottom strands of the DNA protecting ~30 bp of DNA (Fig. 4B), which is consistent with the prediction that Spo0J binds DNA as a dimer making contact with two successive major grooves of the double helix (Leonard *et al*., 2004). Using gel filtration we found that Spo0J-His_6 exists as a dimer in solution, eluting in the range of 96–55 kDa (the theoretical molecular weight of the Spo0J monomer is 32.2 kDa) (Fig. 4C). A Spo0J variant deleted for the 20 C-terminal amino acids, Spo0JΔ20-His_6 (theoretical molecular weight of 29.8 kDa), eluted in the range of 48–30 kDa (Fig. 4D), consistent with it being a monomer. The requirement of the C-terminal domain for efficient dimerization appears to be conserved among ParB homologues (Lobocka and Yarmolinsky, 1996; Lukaszewicz *et al*., 2002; Figge *et al*., 2003; Bartosik *et al*., 2004; Leonard *et al*., 2004). These results confirm that Spo0J-His_6 binds specifically to *parS* as a dimer.

Coating of longer DNA substrates by Spo0J

To investigate Spo0J-His_6 binding to more physiologically relevant DNA substrates we utilized a ~700 bp fragment containing a *parS* site near one end. The reactions were resolved using agarose to allow the entry of large-molecular-weight complexes into the gel matrix. Figure 5A shows that at low concentrations of Spo0J-His_6 a single shifted complex appears. As the concentration of Spo0J-His_6 was increased, first several distinct complexes were discernible, followed by the labelled DNA running as a smear (suggesting some variation in the amount of protein present on each substrate molecule), and then resolving back into what appeared to be a single species. The appearance of distinct nucleoprotein complexes at high concentrations of Spo0J-His_6 suggested that the DNA substrate had been saturated.

To characterize the stoichiometry of Spo0J-His_6 to DNA within the supershifted complexes, Spo0J-His_6 was allowed to bind and shift two DNA fragments of different sizes (Fig. 5B). Each of the shifted bands was then excised from an agarose gel, boiled in the presence of SDS, and the proteins contained in them were separated by SDS-PAGE. The proteins were stained and quantified by comparison with a dilution series of purified Spo0J-His_6 (Fig. 5C). The amounts of Spo0J present in the shifted bands were estimated to be 730 ng (23 pmol) associated with 200 ng (0.12 pmol) of the 2.7 kb fragment; and 350 ng (11 pmol) with 100 ng (0.20 pmol) of the 0.8 kb fragment. Thus, in both cases Spo0J appears to bind to DNA in proportion to the length of the substrate, with each Spo0J dimer occupying ~30 bp. These results are consistent with Spo0J-His_6 spreading along the labelled DNA until saturated.

Discussion

We have shown that the chromosome segregation protein Spo0J of *B. subtilis* contacts several thousand base pairs of DNA flanking its specific binding site, *parS*, creating multiple domains of Spo0J near the origin of chromosome replication. The results suggest that Spo0J interacts with DNA through a mechanism of lateral spreading from a *parS* nucleation site. In keeping with these observations, it has been suggested that overexpression of the chromosomal ParB homologue from *P. aeruginosa* inhibits the transcription of genes flanking its *parS* site as a conse-

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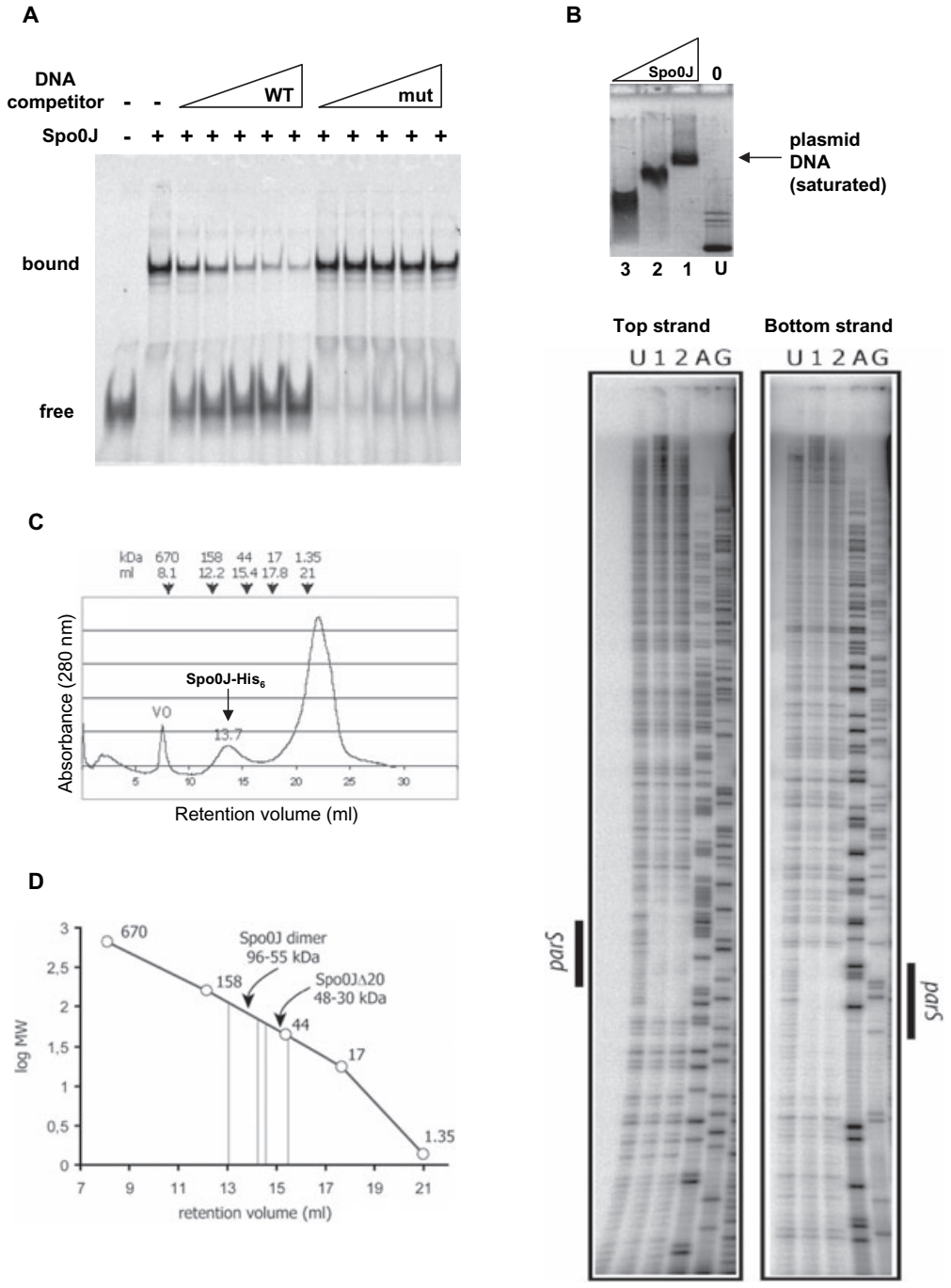

Fig. 4. Spo0J binds specifically to *parS* as a dimer.
A. To demonstrate specific binding at a *parS* site, Spo0J-His_6 was incubated with a 24 bp fluorescently labelled DNA fragment containing the wild-type *parS* sequence in the presence of increasing competitor DNA containing either a wild-type *parS* site or a mutant *parS* site. Where indicated, competitor DNA was added in excess of the fluorescently labelled DNA substrate using twofold steps ranging from a 1.25-fold excess to a 20-fold excess.
B. DNase I footprint of Spo0J bound to *parS*. Supercoiled pHM57 was bound by Spo0J-His_6 and subsequently treated with DNase I, followed by the analysis of the protection patterns generated by using primer extension and denaturing sequencing gels. An aliquot of the reaction was loaded onto an agarose gel (shown above the footprint) to confirm binding of Spo0J to the supercoiled plasmid. U, unbound DNA. A and G, chain termination sequencing reactions using ddATP and ddGTP respectively.
C. Purified Spo0J-His_6 was applied to a Superdex200 column and eluted protein was detected by UV absorbance (280 nm). VO is the void volume. The peak at ~21 ml represents low-molecular-weight components. The molecular weight standards used to build the standard curve are indicated.
D. Gel filtration of Spo0J-His_6 and Spo0JΔ20-His_6. Proteins were found to be eluted in the molecular weight (MW) ranges indicated by the vertical lines.

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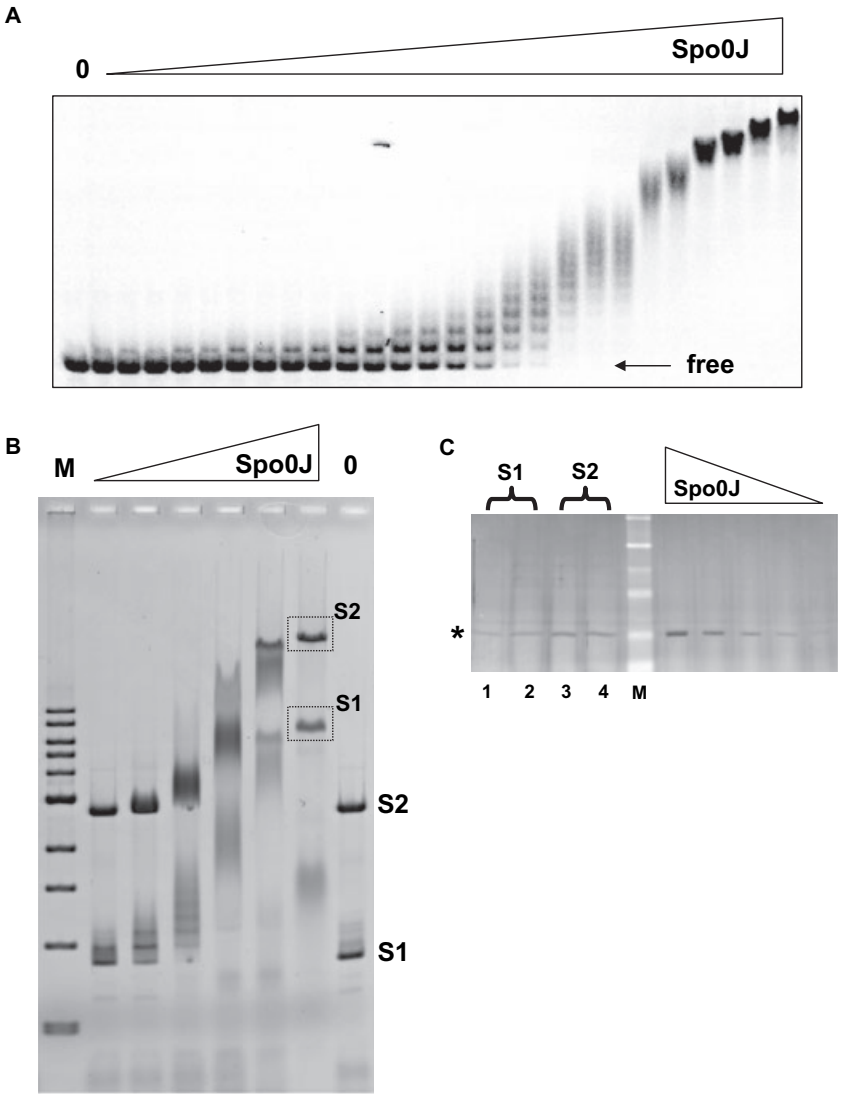

Fig. 5. Spo0J coats long DNA substrates.
A. A fluorescently labelled 686 bp DNA fragment, containing *parS* near one end, was incubated with increasing amounts of Spo0J-His_6 (in 50% increments from 0.7 nM to 4000 nM).
B. DNA substrates of different size were incubated with increasing amounts of purified Spo0J-His_6 (in twofold increments from 120 nM to 3840 nM). The larger fragment was generated by digestion of pUC18 with HindIII (200 ng). The smaller fragment was a PCR product of the *spo0J* gene (100 ng) containing a $parS^{359}$ site.
C. Determination of the amount of Spo0J-His_6 present in the S1 and S2 complexes. Agarose blocks containing the retarded complexes were extracted from the gel shown in (B) (lane 2) and the Spo0J-His_6 contained in them was resolved by 10% SDS-PAGE (lanes 1–2 and 3–4 correspond to Spo0J-His_6 extracted from the complexes S1 and S2 respectively) alongside a serial dilution of purified Spo0J-His_6 as a concentration standard.
M, molecular weight standards.

quence of ParB spreading along the DNA (Bartosik *et al*., 2004). In addition, the plasmid-encoded homologue of ParB from the P1 plasmid has been shown to spread along DNA (Rodionov *et al*., 1999).

We have also demonstrated specific binding of Spo0J to *parS* and non-specific coating of large DNA substrates by Spo0J *in vitro*. The calculated value of one Spo0J dimer per 30 bp of DNA on supershifted substrates (Fig. 5B and C) is close to the size of the footprint (Fig. 4B), suggesting that the dimers are tightly packed and potentially touching, which may facilitate the lateral spreading. However, we note that we were unable to demonstrate spreading of Spo0J from a *parS* nucleation site *in vitro*. That is, we did not observe protection of DNA flanking *parS* using DNase I footprinting (Fig. 4), and we did not observe preferential coating of large DNA substrates carrying a *parS* site during competition experiments (compare pUC18/HindIII and the PCR fragment containing *spo0J* in Fig. 5B; data not shown). Thus, we may be missing a factor that is required for the nucleation process at *parS*, or perhaps the conditions of our *in vitro* reactions need to be amended.

The Spo0J domains may be involved in organizing the *oriC* region of the chromosome. It has been previously reported that the discreet Spo0J-GFP foci observed in wild-type strains become fragmented in the absence of Soj (Marston and Errington, 1999), suggesting that Soj may cause the Spo0J domains to coalesce. Supporting this hypothesis, related ParA homologues have been implicated in modulating their respective ParB–*parS* nucleoprotein complexes (Mohl and Gober, 1997; Bouet and Funnell, 1999; Figge *et al*., 2003).

Recent work using the Spo0J protein of *Thermus thermophilus* has shown that Spo0J stimulates the ATPase activity of Soj *in vitro* (Leonard *et al*., 2005). We propose that the Spo0J domains will provide multiple large binding

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Table 1. Strains and plasmids used in this study.

Strain/plasmid	Relevant genotype	Construction or reference
Strain		
168	*trpC2*	Laboratory stock
DCL484	*trpC2 pheA parS*-6 (sextuple *parS* mutant)	Lin & Grossman (1998)
HM2	*trpC2* Δ*spo0J*::*spc*	AG1505 (chr) → 168 (Spc^r)
HM3	*trpC2 cat rtp-gfp*	pHM56 → 168 (Cat^r)
HM22	*trpC2 spo0J xylO neo yyaC*	pHM39 → 168 (Kan^r)
HM28	*trpC2* Δ*parS*^{4}::*kan*	DCL484 (chr) → 168 (Kan^r)
Plasmid		
pBEST501	*bla neo*	Itaya *et al.* (1989)
pHM1	*bla cat*	This work
pHM2	*bla cat* P_{soj} *soj spo0J*	This work
pHM8	*bla neo yyaC*	This work
pHM16	*bla cat* P_{soj} *soj spo0J neo yyaC*	This work
pHM39	*bla cat* P_{soj} *soj spo0J xylO neo yyaC*	This work
pHM56	*bla cat 'rtp-gfpmut1*	This work
pHM57	*bla parS* (contains C-terminal 282 bp of *spo0J*)	This work
pSG1151	*bla cat gfpmut1*	Lewis and Marston (1999)
pSG1301	*bla cat*	Stevens *et al.* (1992)
pSG4650	*bla spo0J-his*$_6$	Autret *et al.* (2001)
pSG4902	*bla cat* P_{xyl} *-gfpmut1*	Wu and Errington (2003)
pTrc99A	*bla lacI*^{q}*lacO* P_{tac}	Pharmacia

targets for Soj to increase the probability that Soj will contact the origin region of the chromosome as it cycles through its various subcellular localizations (Marston and Errington, 1999; Quisel *et al.*, 1999).

The Spo0J domains may also play a direct role in partitioning the origin region of the chromosome towards the cell poles. It has been reported that separation of sister *oriC* regions is impaired in the absence of Spo0J, and that sister regions near the terminus of replication with *parS* sites integrated nearby are further apart in the presence of Spo0J (Lee *et al.*, 2003; Lee and Grossman, 2006). Akin to the mechanism of eukaryotic chromosome segregation, the Spo0J domains could act as kinetochore-like scaffolds and interact with motor proteins to either push or pull chromosomes apart.

Experimental procedures

Bacterial strains and general methods

Bacillus subtilis 168 and derivatives are shown in Table 1. *Escherichia coli* ER2566 (derivative of BL21, New England Biolabs) was used for overexpressing Spo0J-His$_6$ from pSG4650. Transformation of competent *B. subtilis* cells was performed using an optimized two-step starvation procedure as previously described (Anagnostopoulos and Spizizen, 1961; Hamoen *et al.*, 2002). Ampicillin was utilized at 100 μg ml^{-1}, chloramphenicol at 5 μg ml^{-1} and kanamycin at 5 μg ml^{-1}.

Plasmid construction

General manipulation of DNA was performed as described (Sambrook *et al.*, 1989). All constructs were verified by sequencing. Sequences of oligonucleotides used in this study are available upon request. pHM1 was constructed by cutting pSG1301 with AflIII, filling the overhangs using Klenow fragment (New England Biolabs), then ligating the blunt ends together to destroy the AflIII site. pHM2 was constructed by amplifying the operon containing *soj* and *spo0J* (including DNA sequences upstream containing the promoter, P_{soj}) from *B. subtilis* 168 using oHM10 and oHM11, then cloning the PCR fragment into pHM2 using ClaI and EcoRI. pHM8 was constructed by amplifying *yyaC* using oHM81 and oHM82, then cloning the PCR fragment into pBEST501 using NotI and SacI. pHM16 was constructed by subcloning the SacI to XbaI fragment from pHM8 (containing *yyaC* and *neo*) into pHM2. pHM39 was constructed by subcloning the BglII to XbaI fragment from pSG4902 (containing *xlyO*) into pHM16 cut with BamHI and XbaI. pHM56 was constructed by amplifying the C-terminal fragment of *rtp* using the primers rtp-F and rtp-R, then cloning the PCR fragment into pSG1151 using XhoI and EcoRI. To construct pHM57, *spo0J* was PCR-amplified using T7 primers (Novagen 69348-3 and Novagen 69337-3). The PCR product was gel purified, digested with HindIII (which cuts inside *spo0J*) and BamHI, then cloned into pUC18 digested with the same enzymes.

In vivo *protein–DNA cross-linking and immunoprecipitation*

In vivo cross-linking and immunoprecipitation was performed as described by Lin and Grossman (1998), with the following modifications. Cultures were grown in S-medium supplemented with hydrolysed casein as described (Marston *et al.*, 1998) to an A_{600} of ~0.5, and RNase A (100 μg ml^{-1}) was added to the lysis buffer. The collected Protein A–antibody complexes were washed for 5 min with each of the following solutions: (i) 0.5× IP buffer, (ii) 0.5× IP buffer containing 500 mM NaCl, (iii) Stringent buffer: 250 mM LiCl, Tris-Cl

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(pH 8) (10 mM), EDTA (10 mM), sodium deoxycholate (0.5%), Nonidet P40 (0.5%), and (iv) TE.

Protein expression and purification

Escherichia coli ER2566 harbouring plasmid pSG4650 was grown at 37°C until the OD_{600} reached 0.5. Proteins were induced for 2 h at 30°C following addition of 1 mM IPTG. Cells were harvested and broken by use of a French press. Spo0J-His_6 was purified to near homogeneity (> 98% estimated by SDS-PAGE) by a two-step procedure. The lysate was first passed through a HiTrap Chelating column (Amersham) charged with Ni^{2+} [loaded and washed with 300 mM NaCl, 30 mM HEPES-KOH (pH 7.4), plus 10% glycerol], and eluted by a step gradient with 0.2 M imidazole. The nickel-purified Spo0J-His_6 was further purified by passage through a HiTrap Heparin column (Amersham) and eluted by a linear gradient of NaCl. Fractions containing Spo0J-His_6 were pooled and subjected to buffer exchange using a PD-10 desalting column (Amersham) with 300 mM NaCl, 30 mM HEPES-KOH (pH 7.4), 1 mM DTT and 10% glycerol. Samples were concentrated using an Ultrafree-15 centrifugal filter device (Millipore) and stored at either –20°C or –70°C.

In vitro *DNA binding assays*

Spo0J-His_6 binding specificity was assayed essentially as reported by Lin and Grossman (1998). A 24 bp DNA fragment containing *parS* was constructed by annealing two oligonucleotides, 5′-AGAATGTTCCACGTGAAACAAAGA-3′ (oHM3) and its compliment 5′-TCTTTGTTTCACGTGG AACATTCT-3′ (oHM4), that had been labelled at their 5′-termini with a Cy5 fluorophore (Sigma-Genosys). The 24 bp unlabelled competitor DNA contained either a wild-type *parS* site (oHM3+oHM4) or a mutant site [5′-AGAATGT TCCACGTGAAACAAAGA-3′ (oHM7) and its compliment 5′-TCTTTGTTTCACGTGGAACATTCT-3′ (oHM8)]. Binding reactions (10 μl) were performed in 250 mM NaCl, 50 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM DTT, 10% glycerol, 100 μg ml^{-1} BSA, 10 μg ml^{-1} non-specific competitor DNA (annealed oHM7+oHM8; see below), and contained 25 nM labelled DNA probe. The reactions were assembled on ice and then incubated for 10 min at 30°C before being loaded directly onto an pre-run 8% polyacrylamide gel (0.5× TBE) and electrophoresed at 120 V for 45 min at 4°C. Gels were imaged directly using a Fuji FLA-5000 fluorescent imager and bands were quantified using AIDA software.

To observe Spo0J-His_6 binding to larger DNA fragments (686 bp), a region within *spo0J* was amplified using primers oHM27 (conjugated to Cy5) and oHM74. Binding reactions (10 μl) were performed in 250 mM NaCl, 50 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM DTT, 10% glycerol, 100 μg ml^{-1} BSA, 25 μg ml^{-1} sheared salmon sperm DNA and contained 2.5 μg ml^{-1} labelled DNA probe. The reactions were assembled on ice, incubated for 10 min at 30°C, then returned to ice for 5 min before being loaded directly onto an pre-run 0.7% agarose gel (0.5× TBE) and electrophoresed at 50 V for 5 h at 4°C. Gels were imaged directly using a Fuji FLA-5000 fluorescent imager and bands were quantified using AIDA software.

To determine the stoichiometry of Spo0J-His_6 to DNA, increasing amounts of purified protein were incubated with a mixture of *parS*- and non-*parS*-carrying DNAs (*spo0J* gene and pUC18/HindIII respectively) in 10 μl of reactions containing 100 mM KCl, 30 mM HEPES-KOH [pH 7.4], and 10 mM $MgCl_2$; reactions were performed at ~25°C for 15 min. Free DNA and complexes were separated by electrophoresis through 1% agarose gel in 0.5× TBE, stained with ethidium bromide and detected using the Fuji FLA-5000 fluorescent imager.

DNase I footprinting

Modified from Gralla (1985). Supercoiled pHM57 (400 ng) was incubated for 15 min at room temperature with different concentrations of Spo0J-His_6 in 20 μl of reactions containing 100 mM KCl, 30 mM HEPES-KOH (pH 7.4) and 10 mM $MgCl_2$. Following incubation, the reaction was split into two aliquots; one was treated with DNase I and the other was loaded onto an agarose gel to verify that the binding reaction was complete. DNase I was added to the reactions to a concentration sufficient to produce nicks on the DNA (estimated by preliminary experiments testing for relaxation of supercoiled pHM57). Nicking was carried out for 20 min at room temperature and stopped by phenol extraction. DNA was purified by gel filtration using G-25 spin columns (Amersham), followed by primer extension using AmpliTaq DNA Polymerase CS (Applied Biosystems) with ^{32}P-end-labelled primers designed to anneal at ~80 bases upstream or downstream of the *parS* site in pHM57 (forward: 5′-CAAGCTTGAACC GCTGGTAC; reverse: 5′-CTGTTGTTCCAAAATAATTTTG). A and G chain termination sequencing reactions of the region of interest were produced using the same primers and the PCR-based kit AmpliCycle (Applied Biosystems). Extended products were resolved by denaturing 7% PAGE.

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