Cytidine triphosphate promotes efficient ParB-dependent DNA condensation by facilitating one-dimensional spreading from parS

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SUMMARY

Faithful segregation of bacterial chromosomes relies on the ParABS partitioning system and the SMC complex. In this work, we used single molecule techniques to investigate the role of cytidine triphosphate (CTP) binding and hydrolysis in the critical interaction between centromere-like parS DNA sequences and the ParB CTPase. Using a combined dual optical tweezers confocal microscope, we observe the specific interaction of ParB with parS directly. Binding around parS is enhanced 4-fold by the presence of CTP or the non-hydrolysable analogue CTPγS. However, ParB proteins are also detected at a lower density in distal non-specific regions of DNA. This requires the presence of a parS loading site and is prevented by roadblocks on DNA, consistent with one dimensional diffusion by a sliding clamp. Magnetic tweezers experiments show that the spreading activity, which has an absolute requirement for CTP binding but not hydrolysis, results in the condensation of parS-containing DNA molecules at low nanomolar protein concentrations. We propose a model in which ParB-CTP-Mg$^{2+}$ complexes move along DNA following loading at parS sites and protein:protein interactions result in the localised condensation of DNA within ParB networks.
INTRODUCTION

In bacterial cells, the separation of sister chromosomes is performed by the ParABS system and the SMC complex (Marbouty et al., 2015; Song and Loparo, 2015; Wang et al., 2014). The ParABS system consists of the ATPase protein ParA, the DNA-binding protein ParB, and a centromere-like palindromic DNA sequence named parS (Funnell, 2016; Lin and Grossman, 1998). In vivo imaging experiments in Bacillus subtilis showed that multiple ParB proteins co-localize with SMC complexes at a given parS site forming distinctive clusters in the cell (Gruber and Errington, 2009; Sullivan et al., 2009). Notably, chromatin immuno-precipitation (ChiP) experiments indicate that ParB covers regions of up to 18 kilobase pairs (kbp) of DNA surrounding parS (Breier and Grossman, 2007; Graham et al., 2014; Minnen et al., 2016; Murray et al., 2006; Rodionov et al., 1999), a phenomenon named spreading. Originally, this spreading was interpreted as the formation of a nucleoprotein filament extending from a parS nucleation site (Murray et al., 2006; Rodionov et al., 1999). However, it later became clear that ParB foci contained far too few proteins to coat tens of kbp-long DNA segments (Graham et al., 2014). Instead, we and others have shown that ParB can self-associate to form networks which include specific binding to parS sequences but also non-specific binding to distal DNA segments. Overall, this results in the condensation and bridging of DNA at low forces (below 1 pN) (Fisher et al., 2017; Graham et al., 2014; Madariaga-Marcos et al., 2019, 2018; Taylor et al., 2015), and could explain how distant regions of DNA are bound by limited numbers of ParB proteins as shown in ChiP experiments. However, DNA condensation has only been observed in vitro at high ParB concentrations, in the low micromolar range. Moreover, and unexpectedly, parS sequences did not affect DNA condensation under these conditions (Taylor et al., 2015). Therefore the mechanism of ParB spreading and condensation, and in particular the molecular basis for the specific localisation around parS, has remained unclear despite extensive investigation in vivo, in vitro and in silico (Broedersz et al., 2014; Guilhas et al., 2020; Sanchez et al., 2015; Walter et al., 2020).

ParB proteins comprise three distinct domains (Figure 1A and 1B). The N-terminal domain (NTD) binds ParA (Bouet and Funnell, 1999; Davis et al., 1992; Radnedge et al., 1998; Vecchiarelli et al., 2010), and was recently appreciated to contain a CTP binding pocket that serves as a CTP-dependent dimerization interface (Osorio-Valeriano et al., 2019; Soh et al., 2019). Mutation R80A in the CTP binding pocket has been shown to impair nucleoid segregation and ParB spreading (Autret et al., 2001; Breier and Grossman, 2007). A central DNA binding domain (CDBD) binds specifically to the palindromic parS sequence and may also facilitate dimerization (Leonard et al., 2004; Schumacher and Funnell, 2005). In this central region, the mutation R149G within a helix-turn-helix motif impedes parS binding (Autret et al., 2001; Fisher et al., 2017; Gruber and Errington, 2009). Finally, the C-terminal domain (CTD) forms dimers with a lysine-rich surface that binds to DNA non-specifically (Fisher et al., 2017). Interactions between CTDs are essential for condensation in vitro and for the formation of ParB foci in vivo (Fisher et al., 2017). Based upon these results we proposed a model for DNA condensation dependent on ParB CTD dimerization and non-specific DNA (nsDNA) binding (Fisher et al., 2017). Further work using lateral pulling of long DNA molecules in a magnetic tweezers (MT) setup combined with total internal reflection fluorescence microscopy (TIRFM) demonstrated that ParB networks are highly dynamic and display a continual exchange of protein-protein and protein-DNA interfaces (Madariaga-Marcos et al., 2019).
Recently, two laboratories demonstrated independent that *B. subtilis* ParB and the *Myxococcus xanthus* ParB hydrolyse cytidine triphosphate (CTP) to cytidine diphosphate (CDP) (Osorio-Valeriano et al., 2019; Soh et al., 2019) and require CTP for partition complex formation *in vivo* (Osorio-Valeriano et al., 2019). Importantly, parS DNA stimulates the binding and hydrolysis of CTP (Osorio-Valeriano et al., 2019; Soh et al., 2019), CTP has been shown to be necessary for ParB spreading *in vitro* (Jalal et al., 2020) and a model has been proposed in which centromeres assemble via the loading of ParB-DNA sliding clamps at parS sequences (Jalal et al., 2020; Soh et al., 2019). These observations fundamentally change our understanding of how ParB can become engaged with non-specific DNA surrounding parS, but the significance of CTP-dependent spreading in the formation of the ParB networks that cause DNA bridging and condensation has not been addressed.

Here, we investigated the role of CTP binding and hydrolysis in the binding of ParB to parS sequences and non-specific DNA at the single-molecule level. We present the first visualisation of the specific binding of ParB to parS. ParB proteins were also detected at non-specific DNA far from parS, albeit at a much lower density. Importantly, this only occurred in parS-containing DNA, suggesting spreading from parS sites. The placement of tight-binding protein roadblocks on DNA constrained the spreading, suggesting it arises from one-dimensional movement along the contour of DNA, consistent with a sliding clamp model. CTP binding mediated by a Mg²⁺ cofactor, but not CTP hydrolysis, was critical for spreading from parS to non-parS sites which triggered DNA condensation at nanomolar protein concentration. We propose a model where ParB-CTP-Mg²⁺ loads to parS, diffuses to non-parS sites and then self-associates, resulting in the condensation of kilobase-pair long DNA molecules.

**RESULTS**

**Direct visualisation of the specific binding of ParB to parS sequences**

In our previous work, performed in the absence of CTP, we used a combination of TIRFM and DNA stretching (by either flow or magnetic tweezers) to visualise binding of ParB to long DNA molecules (Madariaga-Marcos et al., 2019). Although specific ParB-parS interactions have been detected in electrophoretic mobility shift assays (EMSA) (Taylor et al., 2015), they have not yet been observed directly. Instead, regardless of the presence or absence of parS sequences, we and others observe a uniform non-specific coating of DNA with ParB accompanied by rapid DNA bridging and condensation (Graham et al., 2014; Madariaga-Marcos et al., 2019).

In this study, we investigated the effect of CTP on the specific and non-specific binding of ParB to parS using a fluorophore-conjugated ParB<sup>AF488</sup>, which retains specific and non-specific DNA binding activity (Madariaga-Marcos et al., 2019), and an experimental setup that combines confocal fluorescence microscopy with dual optical tweezers (C-trap, Lumicks) (Candelli et al., 2011; Newton et al., 2019) (see Methods, Figure 1 and Figure 1 –figure supplement 1). In this approach, DNA molecules are perpendicular to the optical axis of the microscope providing a homogeneous illumination along the molecule and confocal imaging provides a very high signal-to-noise ratio (albeit with a limited spatial resolution of around 250 nm). Additionally, the force applied to DNA is better defined and more uniform than in flow-stretch experiments.
Single DNA molecules were immobilized between two polystyrene beads and extended to almost their contour length by a force of ~20 pN. To amplify the potential signal from specific binding to parS sites, we used a DNA substrate that contains 39 copies of the partially degenerated parS sequence (5’-TGTTCCAGTGAACAA) (Breier and Grossman, 2007; Taylor et al., 2015) arranged in two clusters (Figure 1C and Figure 1 – figure supplement 2 and 3). Then, we incubated the DNA with 20 nM ParB488 and took confocal images of the region of interest, including the beads as a reference, in the presence and absence of CTP-Mg2+ (Figure 1D). The images clearly showed two bright regions, one larger than the other, corresponding to the two parS clusters separated by 1905 bp. Note that, due to the design of the molecule (see Methods), double-length substrates can also be generated and trapped between the beads. In this case, the number of bright clusters were doubled, as expected (Figure 1D). Fluorescence intensity profiles of ParB correlated with the position of the parS clusters (Figure 1E). Importantly, ParB binding to parS did not require CTP in agreement with previous EMSA assays (Taylor et al., 2015) and bio-layer interferometric analysis (Jalal et al., 2020). However, the presence of CTP enhanced the fluorescence intensity within the parS clusters by 3-4 fold compared to in the absence of CTP (Figure 1F). Previously, we showed that the ParB binding equilibrium is established rapidly (within tens of seconds, (Madariaga-Marcos et al., 2019)) compared to our incubation time before confocal imaging. Therefore, these images represent the steady-state occupancy of ParB on DNA and the higher fluorescence intensity measured in the CTP case reflects a greater number of ParB molecules bound at or around the parS sequences compared to the no CTP condition. It is formally possible that the higher fluorescence measured in the presence of CTP could reflect a fluorescence enhancement effect, but this is unlikely since the ParB labelling site (S68C) is on a surface-exposed loop that is distant from the buried CTP molecules (Soh et al., 2019). In some images, a faint fluorescence signal was also observed outside the parS region (see red arrow in Figure 1D) in the CTP and CTPγS conditions and we will return to this point later. Control experiments with non-parS DNA did not show any protein binding at this ParB concentration (see below). This is the first direct visualization of ParB association specifically and precisely at parS sequences, and shows that this interaction is mediated by CTP binding in agreement with recently published works (Jalal et al., 2020; Osorio-Valeriano et al., 2019; Soh et al., 2019).

Next, we investigated the dynamics of the ParB-DNA interaction by taking kymographs with CTP/CTPγS or in the absence of nucleotide (see methods). The fluorescence intensity at the parS sites decayed with time but ParB remained visible for 30 s in both CTP/CTPγS conditions (Figure 1-figure supplement 4A and 4B). This helped to reveal the positioning of ParB relative to the parS sites throughout the 30 s kymograph (Figure 1-figure supplement 4C). The 39xparS DNA substrate includes two sequence clusters separated by 1905 bp, the smaller of which contains two groups of closely-spaced parS sequences, and the larger of which contains four such groups (Figure 1-figure supplement 4C). The 30 s average intensity profile clearly distinguished 6 foci corresponding to groups of ParB molecules precisely at their expected positions. The gradual decay of the fluorescence over tens of seconds (Figure 1-figure supplement 4D) indicates that the photobleaching kinetics are faster than the rates of ParB binding and unbinding. If the opposite were true then efficient protein turnover would result in a constant fluorescence level as was observed in our previous experiments performed at much higher ParB concentration (Madariaga-Marcos et al., 2019). Interestingly, the fluorescence at
the parS clusters decayed marginally more slowly in the presence of CTPγS compared to CTP (Figure 1-figure supplement 4D).

ParB spreading from parS sites occurs by sliding and requires CTP binding but not hydrolysis

With the aim of exploring ParB spreading from parS sites to distal DNA sites, we performed experiments in which we incubated the ParB protein with the DNA for 2 minutes before illuminating the sample (Figure 2A). By doing this, we prevented photobleaching of the proteins in the process of loading and spreading. We then compared the fluorescence intensity profiles of the first images obtained after incubation under different experimental conditions (Figure 2B). Importantly, ParB proteins were now more clearly identified outside of the parS region (compared to Figure 1D), but only under CTP or CTPγS conditions (Figure 2B). Indeed, ParB proteins were sparsely distributed along the entire non-specific region (i.e., they did not only accumulate at or near the parS cluster) (red arrow, Figure 2B and 2C). The intensity in this non-parS region decayed within 1-2 seconds; an apparently shorter timescale than in the parS regions (Video 1), re-enforcing the idea that protein turnover is slow compared to photobleaching. Crucially, a control experiment using a non-parS DNA showed no protein bound at all in the presence of CTP (Figure 2B and 2C), supporting the notion that proteins located outside parS reached that location through the parS entry site.

Previous in vitro and in vivo experiments have shown that ParB spreading is hindered by DNA binding protein “roadblocks” engineered close to parS (Murray et al., 2006; Rodionov and Yarmolinsky, 2004; Soh et al., 2019). Therefore, to investigate the mechanism by which ParB spreads to non-specific sites we fabricated a 17-kb DNA molecule that contains the same 39x parS cluster flanked by two groups of 5xEcoRI sites (Figure 2D). These sequences would act as roadblocks after binding of EcoRI[E111G], a catalytically inactive variant of the EcoRI restriction enzyme which has been used as a model protein roadblock (Figure 2E) (Brüning et al., 2018; King et al., 1989). Note that, if movement of ParB occurs along the contour of DNA (i.e., by sliding from parS), then we expect ~5-kbp and ~1-kbp DNA segments of the molecule to remain free of ParB proteins. Additionally, the molecule contains a ~3.6-kbp non-parS area between the last parS sequence of the 39x parS cluster and one of the 5xEcoRI sites which we would expect to become populated with ParB via a sliding mechanism (Figure 2D). The imaging experiments were performed as described in Figure 2A but included an additional incubation step with 100 nM EcoRI[E111G] prior to incubation with ParB[E488]. The confocal laser was turned on after the incubations with EcoRI[E111G] and ParB[E488], and confocal images were obtained on a tandem EcoRI 39x parS DNA (Figure 2F). As expected, brighter regions correlated very well with the parS clusters. Importantly, a faint region also appeared flanking the parS cluster and bordered by the EcoRI[E111G] roadblocks, which appeared to have constrained ParB spreading (see red arrows, Figure 2F). Indeed, fluorescence profiles showed high intensity associated with the parS region, lower intensity signals produced by ParB spreading from parS, (see red arrows, Figure 2F and Figure 2G), and no signal associated with regions protected by EcoRI sites (see blue arrows, Figure 2F and Figure 2G). Subsequent confocal images reflected the photobleaching of this region in contrast with the brighter parS area, confirming the low exchange of proteins outside parS (Video 2). Altogether, these experiments show that CTP binding promotes movement of ParB over kilobase-pair distances away from parS sites to non-specific regions of DNA. The fact
that this movement can be constrained by protein roadblocks suggests that it occurs by sliding from parS.

**CTP binding dramatically enhances the parS sequence-specificity of ParB-dependent DNA condensation**

We have previously shown that ParB condenses DNA and that the CTD plays an important role in this function (Fisher et al., 2017; Taylor et al., 2015). However, condensation occurred at micromolar protein concentration and was not apparently specific to parS-containing DNA. Now, we aimed to revisit these experiments in the light of the discovery of CTP as an important mediator of ParB-DNA interactions (Osorio-Valeriano et al., 2019; Soh et al., 2019). Note that the optical trap experiments described above were necessarily performed at forces which are non-permissive for condensation, to keep the DNA extended for optimal fluorescence visualization. We therefore switched to a magnetic tweezers setup which is more appropriate for low-force experiments (Taylor et al., 2015) (Figure 3A). Single DNA molecules containing a set of 13x parS sequences were immobilized between a glass surface and super-paramagnetic beads (Figure 3B). A pair of magnets were then employed to stretch the DNA and apply forces in the 0.1-5 pN range. The DNA was incubated with ParB at the higher force level for two minutes, and then the force was lowered to 0.33 pN, which is permissive for DNA condensation. The extension of the tether was monitored in real time leading to condensation time-course plots.

DNAs containing parS rapidly condensed in assays with 50 nM ParB, CTP and Mg\(^{2+}\) (Figure 3C). In fact, DNA condensation was observed at even lower concentrations of 5-10 nM, but the rate was markedly slower (Figure 3- figure supplement 1A). In the presence of CTP-Mg\(^{2+}\), DNA was condensed by ParB at forces of up to 1 pN using only 10 nM protein (Figure 3D). This maximum condensation force was similar to that described in non-parS DNA using micromolar ParB concentrations (Taylor et al., 2015), suggesting a similar mechanism of condensation. Experiments using ATP, UTP or GTP did not produce any DNA condensation, confirming the specificity of ParB for CTP and linking CTP binding to condensation (Figure 3E).

The recent crystal structure of the *M. xanthus* ParB-like protein PadC showed that Mg\(^{2+}\) is a cofactor of CTP at the CTP binding site (Osorio-Valeriano et al., 2019). Therefore, we explored the role of Mg\(^{2+}\) in the ParB condensation function. ParB did not induce any condensation in a buffer containing 1 mM EDTA and 200 nM ParB\(_2\) (Figure 3F). Mutation of the R80 residue of ParB to alanine leads to loss of function in *B. subtilis* and impairs subcellular localization of ParB (Autret et al., 2001; Graham et al., 2014). Additionally, CTP binding is also abolished in BsparB\(^{R80A}\) (Soh et al., 2019). We therefore ask if this mutation might affect the condensation function of ParB. Indeed, magnetic tweezers experiments showed no DNA condensation by BsparB\(^{R80A}\) under CTP-Mg\(^{2+}\) conditions (Figure 3G) supporting the idea that CTP binding is required for condensation at nanomolar ParB concentrations. In the absence of CTP, ParB was unable to condense parS-containing DNA unless its concentration was raised to the micromolar range, as reported previously (Figure 3- figure supplements 1B and 1C) (Taylor et al., 2015).

We next asked whether CTP hydrolysis is required for DNA condensation by exploiting the non-hydrolysable analogue CTP\(_\gamma\)S. We took time-courses in the presence of 2 mM CTP\(_\gamma\)S and obtained force-extension curves at different ParB concentrations (Figure 4A). We did not
observe any significant difference in force-extension measurements compared to the CTP case (Figure 3D). The DNA was still condensed at nanomolar ParB and condensation was abolished by EDTA (Figure 4B). Altogether, we conclude that binding to both CTP and the Mg$^{2+}$ cofactor are required for DNA condensation by nanomolar ParB, but that CTP hydrolysis is not.

Finally, we investigated whether the dramatic stimulation of DNA condensation afforded by CTP was specific to parS-containing DNA and explored how condensation was affected by the number of parS sequences present in the substrate. Experiments using a DNA substrate with scrambled parS sequences did not show condensation even at ParB$_2$ concentrations of 200 nM (Figure 5A). Moreover, to confirm that the DNA condensation was mediated by parS binding we used the mutant ParB$^{R149G}$ that cannot bind parS (Autret et al., 2001; Fisher et al., 2017; Gruber and Errington, 2009). As expected, no condensation was observed using ParB$^{R149G}$ under conditions proficient for condensation with wild type ParB (Figure 5B). We next obtained force-extension curves using substrates containing different numbers of parS sequences. DNA molecules containing from 1 to 26 parS sequences and similar overall length were fabricated (Figure 5C and Figure 5E, see methods). DNA condensation was observed in substrates with 26, 13, and 7 parS sequences with a clear correlation between the number of parS and the maximum force permissive for condensation (Figure 5D). Experiments with substrates containing 1, 2, or 4 copies of parS did not result in condensation even at a relatively high ParB$_2$ concentration of 200 nM (Figure 5E and Figure 5F). These experiments indicated a requirement for a minimal number of parS sites (between 5 and 7) for condensation under these conditions.

Together, these data show that CTP binding dramatically enhances DNA condensation by ParB such that it occurs efficiently at low nanomolar ParB concentration. Importantly, this stimulatory effect is completely specific for parS-containing DNA molecules as CTP does not improve the condensation of parS-free molecules that can be observed at high concentration of ParB. This presumably reflects the CTP- and parS-dependent recruitment of ParB sliding clamps that subsequently multimerise to effect bridging interactions between distal DNA segments.

**DISCUSSION**

We report here the first visualisation of ParB binding to parS sequences. Previous observation of the specific binding to parS was hindered by the fact that ParB binding to DNA induces condensation and bridging (Graham et al., 2014; Taylor et al., 2015). To overcome this issue, we and others stretched the DNA molecules using flow or magnetic pulling combined with TIRF microscopy (Graham et al., 2014; Madariaga-Marcos et al., 2019). However, these experiments were performed in the absence of CTP and required high concentrations of ParB, conditions which allow ParB to interact directly (i.e., from free solution rather than via parS loading sites) with non-specific DNA. The recent discovery that ParB is a parS-dependent CTPase has led to the proposal of radically new models for ParB-DNA interactions in which parS acts as a loading site for ParB-DNA sliding clamps (Osorio-Valeriano et al., 2019; Soh et al., 2019). Together with bulk bio-layer interferometric analysis (Jalal et al., 2020), this work suggests that ParB binds to parS in the apo state and CTP-binding induces a conformational change that liberates the ParB dimer from parS allowing spreading. Motivated by these important new observations, we have revisited our earlier single molecule experiments using much lower concentrations of ParB in
the presence of CTP. Our results confirm many aspects of the published models but also extend them, by addressing how CTP facilitates localised DNA condensation around parS sites.

In an attempt to visualise the specific binding of ParB to parS in the presence of CTP, Soh et al. observed stretched DNA bound to a glass surface using TIRF microscopy and reported accumulation of ParB around parS (Soh et al., 2019). Here, we used a combination of optical tweezers and confocal microscopy that allowed us not only to observe more precisely the direct binding of ParB to single DNA molecules, but also facilitated the quantification of fluorescence intensity around parS sites and distal non-specific sites. In our assay we found very good correlation between the position of ParB in time-averaged intensity profiles and the position of the groups of parS sites engineered into our substrate. These profiles easily allowed us to distinguish between the different orientations of substrate molecules (Figure 1). In these experiments, confocal illumination was initiated after a long ParB incubation with the substrate DNA such that the initial images should reflect equilibrium binding conditions (Madariaga-Marcos et al., 2019). In the absence of CTP, we observed a clear fluorescence intensity at parS sequences, and a zero intensity (within error) in distal regions of non-specific DNA. We interpret this as reflecting the highly specific binding of ParB in an open clamp conformation directly to the parS sequences via the HtH motifs in the CDBD domain (Figure 6, step 1).

When we next added either CTP or the non-hydrolysable analogue CTP\(\gamma\)S, we saw a 4-fold increased fluorescence intensity around the parS sequences and a lower (but clearly non-zero) intensity in distal non-specific regions of the DNA. Note that, because this is a single molecule experiment, the increased intensity associated with the parS clusters strongly suggests that there is a higher density of ParB dimers on the DNA at or near (i.e., within the spatial resolution of the imaging; \(\sim 250\) nm) parS sequences. We interpret this as the CTP binding-dependent conversion of parS-bound ParB dimers into sliding clamps that move into neighbouring regions of the DNA to allow additional ParB open clamps to be recruited to the DNA (Figure 6, step 2). It is interesting to note that the presence of CTP and CTP\(\gamma\)S does not prevent the initial binding of ParB to DNA by favouring a closed clamp structure before DNA association. This might suggest either that the DNA entry gate for ParB is a different interface (e.g., transient opening of the CTD) or simply that the CTP cannot bind efficiently until after DNA enters ParB though the NTD entry gate. In either case, following the formation of closed ParB clamps on DNA, we imagine that they spread but remain largely restrained to the region of non-specific DNA immediately surrounding parS sequences as a result of protein:protein interactions (Figure 6, step 2, interlaced circles). In our C-trap experiment, the ends of the DNA substrate are held at the beads, meaning that the DNA cannot begin to condense by sliding into the ParB networks that are forming around parS. We will return to this point in our interpretation of the magnetic tweezers experiments below.

The weaker fluorescence intensity in distal non-specific regions of the substrate is observed exclusively in CTP or CTP\(\gamma\)S conditions and is interpreted as ParB sliding clamps which have escaped from the ParB network at parS (Figure 6, step 2). Evidence that these ParB molecules are indeed involved in one-dimensional sliding interactions with the DNA is provided by roadblock experiments with a catalytically-inactive EcoRI mutant. The weaker fluorescence intensity associated with sparsely distributed ParB molecules in non-specific DNA regions was
only observed in segments of DNA containing parS sequences and was corralled by EcoRI roadblocks placed either side of the centromere sequences (Figure 6, step 2).

Despite the fact that free ParB is always present in the imaging channel in our experiments, we observed a rapid decay of the fluorescence intensity upon illumination. This suggests that the photobleaching rate is faster than the binding and unbinding kinetics of ParB. Interestingly, we observed an apparently faster fluorescence loss at distal regions of non-specific DNA when compared to the regions close to parS (Video 1 and 2). This may reflect the much higher density of ParB that our model anticipates in parS regions compared to distal non-specific DNA. Alternatively, continuous loading at parS may counteract the fluorescence decay caused by photobleaching to a greater extent than at distal DNA regions (where ParB cannot bind from free solution). We also found that the fluorescence decay was marginally slower in experiments performed with CTPγS compared to CTP. This is consistent with the idea that the role of CTP hydrolysis is to open the ParB clamp to allow release from DNA and recycling.

In order to investigate the effects of CTP on ParB-induced DNA condensation we employed a magnetic tweezers apparatus. In the presence of CTP-Mg2+, ParB condensed parS-containing DNA at nanomolar concentrations, much lower than those reported before (Fisher et al., 2017; Taylor et al., 2015) (Figure 3). Importantly, this strong stimulatory effect of CTP on DNA condensation, which is completely specific for parS-containing DNA, helps to resolve the question of how ParB networks can form uniquely at parS loci within the huge bacterial chromosome. This had not been readily apparent from previous experiments performed without CTP (see Graham et al., 2014; Madariaga-Marcos et al., 2019; Taylor et al., 2015) for discussion). Control experiments showed that efficient condensation required both the parS sequence and either CTP or CTPγS, suggesting that it is CTP binding but not its hydrolysis that is important for promoting condensation. Interestingly, we also found a parS sequence dose-dependence for condensation. The condensation force increased with the number of parS sites (Figure 5) and a minimum value of between 5 and 7 sites was required to observe condensation under the conditions used here.

We can interpret the behaviour observed in the magnetic tweezers using a simple extension of the model described above for the confocal microscopy experiments. In the magnetic tweezers experiment, the DNA ends are held apart by a very low force and the DNA is able to condense. Therefore, the ParB dimers which load around the parS sequence (Figure 6, step 2), or those which escape from the ParB network, can self-associate and draw DNA into the complex (Figure 6, step 3). This creates a dynamic network of ParB molecules constraining DNA loops. The greater the number of parS sequences present in the DNA, the greater will be the loading rate of ParB clamps into non-specific regions of the DNA, and the more stable will be the condensation against weak restraining forces. In vivo experiments have shown that a single parS is enough to promote chromosome segregation (Broedersz et al., 2014; Jecz et al., 2015; Wang et al., 2017). This might simply reflect the difference in restraining forces, ParB concentrations, or solution conditions that are found in vivo compared to our magnetic tweezers experiments. Alternatively, a failure to condense DNA over large distance scales does not necessarily imply the lack of formation of a ParB-parS complex which is sufficient for chromosome segregation.
The molecular basis for the protein-protein interactions that hold the ParB network together in our model remain unclear and are an important subject for future study. ParB contains multiple non-exclusive interfaces within all three domains that could be relevant to this activity (Song et al., 2017). For example, we have previously provided direct evidence that disruption of CTD-CTD interactions decondenses DNA in vitro and prevents the formation of ParB networks in vivo, so these protein-protein interactions may contribute to network formation (Figure 6, step 3). However, we note that our results can also be re-interpreted in the light of the new concept of topological engagement between DNA and a ParB toroidal clamp. If CTD-CTD interactions are important for closing the sliding clamp around DNA as has been suggested (Jalal et al., 2020; Soh et al., 2019), then disruption of these interactions would dissolve ParB networks, not by breaking protein-protein interactions between ParB molecules, but rather by releasing the constrained DNA loops and promoting ParB dissociation into free solution. Other possible interfaces that may establish ParB networks include those that have been observed between the CDBD or NTD (Chen et al., 2015; Leonard et al., 2004; Schumacher and Funnell, 2005). A final open question concerns the loading of the SMC complexes at bacterial centromeres which leads to the individualisation of bacterial chromosomes following DNA replication (Hayes and Barillà, 2006; Schumacher, 2008). Despite the intimate functional relationship between the ParABS partitioning system and SMC complexes, the nature of the physical interactions between these systems and their regulation are not understood.

MATERIALS AND METHODS

Protein preparation

WT-ParB, R149G ParB and AF488-ParB were prepared as described (Fisher et al., 2017; Madariaga-Marcos et al., 2019). An expression construct for R80A ParB was generated by site-directed mutagenesis of the wild type expression plasmid (QuikChangeII XL, Agilent Technologies). The mutant protein was expressed and purified in the same manner as wild type. The EcoRI E111G variant was a gift from Michelle Hawkins (University of York) and was prepared as described previously (King et al., 1989).

Fabrication of DNA plasmids with multiple copies of parS

DNA plasmids containing multiples parS sequences (optimal sequence of B. subtilis parS = 5’-TGTCCACGTAAACA) were produced by modification of the plasmids described in (Taylor et al., 2015), where the cloning of a plasmid containing a ‘scrambled’ parS site (scrambled parS: 5’-CGTGCCCAAGGAGACA; bold represents mutated nucleotides) was also reported. Plasmids with increasing number of parS sequences were produced as follows. First, we annealed two long oligonucleotides (Table S1) containing 2 parS sites separated by a single XbaI restriction site. The oligonucleotides were hybridized by heating at 95°C for 5 min, and cooled down to 20°C at a -1°C min\(^{-1}\) rate in hybridization buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 200 mM NaCl and 5 mM MgCl\(_2\)). These oligonucleotides were designed to create an incomplete XbaI site at both ends after ligation, so that once ligated to a cloning plasmid they cannot be cut again by XbaI. The single bona-fide XbaI site located in the middle of the oligonucleotide insert
allows repetition of the ligation process in the cloning plasmid as many times as desired to add new pairs of parS sequences. Plasmids containing 1x parS, 2x parS, 4x parS, and 7x parS were obtained following this procedure. Inserts for subsequent cloning of plasmids containing 13x parS, 26x parS, and 39x parS were produced by PCR using a high-fidelity polymerase (Phusion Polymerase, NEB) (see Table S1 for primer sequences). Plasmids were cloned in DH5α Competent cells (ThermoFisher Scientific) and purified from cultures using a QIAprep Spin Miniprep Kit (QIAGEN). All plasmids were checked by DNA sequence analysis. A detailed description of these procedures is included in Supplementary Methods and in Figure 1 – figure supplement 2.

Fabrication of large plasmids (> 17 kbp) for C-trap experiments

Long fragments of DNA (> 17 kbp) containing a custom sequence for C-trap experiments were fabricated as follows. A large plasmid was formed by ligation of three pieces. Two of them correspond to two PCR-fabricated DNAs (C1 and C2) derived from lambda DNA (NEB) as template and including suitable restriction sites in the designed primers (Table S1). The third part (C3) containing the sequence of interest (e.g., multiple copies of the parS sequence) was produced either by PCR or plasmid digestion. The three fragments were then ligated and DH5α competent cells transformed by regular heat shock procedure. Large plasmids were purified from cultures using QIAprep Spin Miniprep Kit and checked by DNA sequence analysis. A detailed description of these procedures is included in Supplementary Methods and in Figure 1 – figure supplement 3.

Plasmids containing the parS region flanked by two clusters of 5x EcoRI restriction sites were produced following the same procedure but replacing parts C1 and C2 with fragments C1-EcoRI and C2-EcoRI, each one including a cluster of 5x EcoRI restriction sites at the desired position (Table S1).

Magnetic tweezers DNA substrates

MT DNA substrates were produced as described in (Taylor et al., 2015) and essentially consist of a central part (~6 kbp) containing different number of parS sequences or a non-specific scrambled parS site, flanked by two smaller fragments (~1 kbp) labelled with biotins or digoxigenins used as immobilization handles. Handles for MT substrates were prepared by PCR (see Table S1 for primers) including 200 μM final concentration of each dNTP (G,C,T,A) and 10 μM Bio-16-dUTP or Dig-11-dUTP (all from Roche). Labelled handles specifically bind either to a glass surface covered with anti-digoxigenins or to superparamagnetic beads covered with streptavidin. About 40% of molecules fabricated using this procedure were torsionally constrained in MT experiments. Sequences of the central part of magnetic tweezers substrate are included in Supplementary Methods.

C-trap DNA substrates

C-trap DNA substrates consisted of a large central part of ~17 kbp (EcoRI 39x parS DNA) or ~25 kbp (39x parS DNA) containing 39 copies of the parS sequence, flanked or not by 2 clusters of 5x EcoRI restriction sites, respectively, were produced by linearization of large plasmids with NotI (NEB). Without further purification, the fragment was ligated to highly biotinylated handles of
~1 kbp ending in NotI. Handles for C-trap substrates were prepared by PCR (see Table S1 for primers) including 200 μM final concentration of each dNTP (G,C,A), 140 μM dTTP, and 66 μM Bio-16-dUTP. These handles were highly biotinylated to facilitate the capture of DNA molecules in C-trap experiments. As both sides of the DNA fragment end in NotI, it is possible to generate tandem (double-length) tethers of ~34 kbp or ~50 kbp (tandem EcoRI 39x parS DNA or 39x parS DNA, respectively) flanked by the labelled handles. Sequences of the central part of C-trap substrates are included in Supplementary Methods.

A control C-trap DNA substrate based on lambda DNA was prepared according to a previously described protocol (Wasserman et al., 2020) with slight modifications. 10 nM lambda DNA was incubated with 33 μM each of dGTP, dATP, biotin-16-dUTP and biotin-14-dCTP (Thermo Fisher), and 5 units of DNA polymerase (Klenow Fragment (3'→5' exo-), NEB) in 1X NEB2 buffer for 1 hour at 37°C. Reaction was followed by heat inactivation of the enzyme for 20 min at 75°C. Sample was ready to use in C-trap experiments without further purification. DNAs were never exposed to intercalanting dyes or UV radiation during their production and were stored at 4°C.

Measurement of NTP hydrolysis by Malachite Green colorimetric detection

A pair of oligonucleotides containing a Scrambled parS site, 1x parS or 2x parS sites (see Table S1 for sequences) were hybridised by heating at 95°C for 5 min, and cooled down to 20°C at a -1°C min⁻¹ rate in hybridization buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 200 mM NaCl and 5 mM MgCl₂). Mixtures of NTP (2x) and DNA (2x) in reaction buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 4 mM MgCl₂, 1 mM DTT and 0.1% Tween-20) were prepared on ice. Protein solutions (2x) containing either wild type ParB or ParB(AF488) in reaction buffer were also prepared on ice. NTP/DNA pre-mix (5 μl) was added to protein solution (5 μl) and mixed on ice. Phosphate standards and blanks were prepared in parallel for each experiment. After mixing, samples containing 1 mM NTP, 0.5 μM DNA, 8 μM ParB were placed in a PCR machine set to 25 °C for 30 min. Additionally, different concentrations (0.25 μM, 0.75 μM, and 1 μM) of a DNA with 2 parS sequences were tested in presence of 1 mM CTP. Samples were diluted by the addition of 70 μL water, then mixed with 20 μL working reagent (WR) (Sigma, Ref MAK 307) and transferred to a flat bottom 96 well plate. The plate was incubated for 30 minutes at 25 °C and the absorbance was measured at a wavelength of 620 nm in a SpectraMax® iD3 (VERTEX Technics) plate reader that uses the SoftMax® Pro7 software. Readings were performed in rounds to preserve the same 30 min WR incubation time for all samples. Absorbance values from the phosphate standard samples were corrected with the absorbance for 0 μM phosphate. Absorbance values from the ParB samples were corrected by the reaction buffer absorbance (blank). Absorbance values from the phosphate standard samples were used to plot an OD₆₂₀ nm versus pmol phosphate standard curve. All samples were tested in duplicate. ParB(AF488) retains parS-stimulated CTPase activity within 2-fold levels of wild type protein (Figure 1 –figure supplement 1B and 1C).

Magnetic tweezers experiments

Magnetic tweezers assays were performed using a home-made setup similar to an apparatus that has been described previously (Carrasco et al., 2013; Kemmerich et al., 2016; Pastrana et al., 2016; Strick et al., 1998). Briefly, optical images of micrometer-sized superparamagnetic beads tethered to a glass surface by DNA constructs are acquired with a 100x oil-immersion objective.
objective and a CCD camera. Real-time image videomicroscopy analysis determines the spatial coordinates of the beads with nm accuracy in the x, y and z directions (Pastrana et al., 2016). A step-motor located above the sample moves a pair of magnets allowing the application of stretching forces to the bead-DNA system. Applied forces can be quantified from the Brownian excursions of the bead and the extension of the DNA tether (Strick et al., 1998). Data were acquired at 120 Hz to minimize sampling artefacts in force determination. We used vertically aligned magnets coupled to an iron holder to achieve a force of up to 5 pN.

DNA was diluted and mixed in ParB-Mg²⁺ buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.2 mg/ml BSA, 0.1 % Tween-20, 1 mM DTT, 4 mM MgCl₂) or ParB-EDTA buffer (by replacing the 4 mM MgCl₂ with 1 mM EDTA) and then incubated with 1 µm diameter magnetic beads (Dynabeads, Myone streptavidin, Invitrogen) for 10 min. Magnetic beads were previously washed three times and diluted in PBS. DNA:beads ratios were adjusted for each substrate to obtain single tethered beads. Following DNA-beads incubation, then sample was injected in a double parafilm® (Sigma)-layer flow cell. After 10-min adsorption of the beads to the surface we applied a force of 4 pN to remove the non-attached beads and washed with buffer to clean the chamber. Torsionally-constrained molecules and beads with more than a single DNA molecule were identified from its distinct rotation-extension curves (Gutierrez-Escribano et al., 2020) and discarded for further analysis.

Time-course data was obtained by recording the extension of the tether at a low force of 0.33 pN, after a 2-min incubation of DNA tethers and ParB at 4 pN. To obtain force-extension curves, we measured the extension of the tethers at decreasing forces from 5 pN to 0.01 pN for a total measuring time of around 15 min. Force-extension curves were first measured on naked DNA (no ParB data) and always from high to low force. Then, the experiment was repeated on the same molecule but at quoted ParB concentrations. This method allowed us to obtain force-extension curves in the absence and presence of protein for each tethered-DNA molecule. No ParB DNA curves were fitted to the worm-like chain model using Origin Software. Molecules with a discrepancy of contour length of ±15% from the crystallographic length were discarded for the analysis.

C-trap fluorescence experiments

We used a dual-optical tweezers setup combined with confocal microscopy and microfluidics (C-trap) from Lumicks (Lumicks B.V). Our system has three laser lines (488 nm, 532 nm 535 nm) for confocal microscopy and provides a force resolution of <0.1 pN at 100 Hz, distance resolution of <0.3 nm at 100 Hz, and confocal scanning with < 1 nm spot positioning accuracy (Lumicks). In this work we used a 488 nm laser for illumination and a 500-525 nm filter for its fluorescence. We used a 5 channel microfluidic chamber (Figure 1 –figure supplement 1A). Channel 1 contained 4.38 µm SPHERO Streptavidin coated polystyrene beads diluted at 0.005% w/v in fishing buffer (FB, 10 mM Tris-HCl pH 8.0, and 50 mM NaCl). Channel 2 included the 39x parS DNA in FB and channel 3 only FB. First, two beads were trapped using the dual optical tweezers in channel 1 and moved to channel 2 to attach DNA molecules to the beads. The capture of DNA was detected by performing a preliminary force-extension curve in channel 2. Then, the bead-DNA system was moved to channel 3, where further force-extension curves were performed to check for single-molecule captures, and to define the zero force point. Finally, a stretching force of 19-23 pN was set, and the bead-DNA system moved to the protein channel 4 that contains...
ParB$_2^{AF488}$ at quoted concentrations in ParB-Mg$^{2+}$ buffer supplemented with an oxygen scavenger system (1 mM Trolox, 20 mM glucose, 8 µg/ml glucose oxidase and 20 µg/ml catalase). Confocal images (scans) and kymographs were performed in the protein channel 4 (Figure 1 – figure supplement 1A), which was previously passivated with BSA (0.1% w/v in PBS) and Pluronic F128 (0.5% w/v in PBS).

Spreading experiments include a 2 min incubation time in channel 4 before turning on the confocal laser. Spreading-blocking experiments also used four channels but in this case we inject 100 nM EcoRI$^{E111G}$ in ParB buffer in channel 3. Following a 2 min incubation in channel 3 to allow binding of the blocking protein, the bead-DNA system was moved to channel 4, which in this case contained 100 nM EcoRI$^{E111G}$ and 20 nM ParB$_2^{AF488}$ in ParB buffer supplemented with the oxygen scavenger (see above). An additional 2-min incubation was performed before confocal imaging.

Confocal images of a defined area were taken using a pixel size of 100 nm and a scan velocity of 1 µm/ms. With these parameters, typical images of experiments using single or tandem 39x parS DNA were obtained every 2 s and 2.7 s, respectively. Confocal laser intensity at the sample was 3.4 µW.

Kymographs were obtained by single-line scans between the two beads using a pixel size of 100 nm and a pixel time of 0.1 ms. Typical temporal resolution of kymographs taken on single or tandem 39x parS DNA were 25 ms and 32 ms, respectively.

C-trap data analysis

Data acquisition was carried out using Bluelake, the commercial software included in the Lumicks C-trap. This software provides HDF5 files, which can be processed using Lumicks’ Pylake Python package. We used homemade Python scripts to export the confocal scans or kymographs in ASCII matrix files or in TIFF format. Python scripts can be found at https://github.com/Moreno-HerreroLab/C-TrapDataProcessing. Profiles were obtained from ASCII files using a home-written LabView program. Images of scans or kymographs were produced using the WSxM freeware (Horcas et al., 2007). Animated GIFs were produced using Image J from individual scans saved in TIFF.

SUPPLEMENTAL INFORMATION

This paper contains supplemental information.

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AUTHOR CONTRIBUTIONS

F. de A. B. carried out all C-trap and MT experiments and performed data analysis. C. A.-R. produced all DNA substrates and carried out NTPase assays. C. A.-R. and G. L. M. F. produced all ParB proteins. S. de B. produced Python scripts for analysis of C-trap data. C. L. P. developed methods for magnetic tweezers measurements. M.S.D. and F.M.-H. conceived the project, wrote, reviewed and edited the manuscript. F. M. –H. wrote the first draft of the manuscript and supervised the project. All authors critically reviewed the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.
FIGURE CAPTIONS

Figure 1. Direct visualisation of ParB specific binding to parS sites. (A) Domains and functional motifs of ParB as reported previously (Bartosik et al., 2004; Kusiak et al., 2011). Mutations R80A, defective for CTP binding, and R149G, defective for parS binding, are indicated. (B) ParB dimer cartoon showing dimerization through the central and C-terminal domains. The nucleotide binding site at the NTD is also indicated. (C) Schematic representation of the single-length 39x parS DNA used for C-trap experiments. The DNA contains 39 parS sequences distributed in 6 groups forming two clusters separated by 1905 bp (39x parS DNA). The positions of the parS sites in the DNA cartoon are represented to scale. (D) Schematic of the C-trap experiment where single and tandem (double-length) tethers are immobilised between two beads and scanned with a confocal microscope using 488 nm illumination (upper part). Representative confocal images of the experiment under no CTP, 2 mM CTP, or 2 mM CTPγS conditions (lower part) and 20 nM ParB2AF488. Dark to bright regions correspond to a scale of 0-30 photon counts for single-length tethers and 0-50 counts for tandem tethers. (E) Representative profiles (500 nm width) of the fluorescence intensity along the DNA axis of the confocal images depicted in D (only single-length tether data). Positions of the parS sequences are included to scale in the background. Brighter regions between the beads correlate with the position of the parS clusters. ParB proteins are also observed outside the parS region (red arrow) and in general the fluorescence intensity outside the parS region is always above the background and larger in CTPγS compared to CTP experiments. (F) Quantification of fluorescence intensity at the parS-containing region under no CTP, CTP and CTPγS conditions.

Figure 1 – figure supplement 1. C-trap layout and NTP hydrolysis experiments. (A) Cartoon of the fluid chamber employed for the experiments performed in this work. We used four laminar flows, containing streptavidin-coated beads, biotinylated DNA, buffer, or fluorescently labelled proteins. Confocal images and kymographs were obtained in channel 4 after a 2 min protein incubation. (B) NTP hydrolysis by wild type ParB was measured using a colorimetric assay. Mean values are shown from four repeat experiments alongside the standard error of the mean. ParB hydrolyses CTP in the absence of DNA at ~0.2 CTP molecules per dimer, per minute. CTP hydrolysis increases 3-4-fold in the presence of parS DNA. Nucleotides ATP, CTPγS, GTP, or UTP cannot be hydrolysed by ParB. The CTP hydrolysis rate increases linearly with the concentration of parS sequences. (C) NTP hydrolysis by wild type ParB or ParB2AF488 was measured in the absence or presence of DNA. Mean values are shown from two repeat experiments alongside the standard error of the mean. ParB2AF488 retains parS-stimulated CTPase activity within 2-fold levels of wild type protein.

Figure 1 – figure supplement 2. Fabrication of small DNA plasmids. Schematic representation of the steps followed to fabricate DNA plasmids containing multiple copies of parS which were used to prepare the magnetic tweezers DNA substrates.

Figure 1 – figure supplement 3. Fabrication of large DNA plasmids. Schematic representation of the steps followed to fabricate the large plasmids used to make the C-trap DNA substrates. This scheme specifically represents the cloning of the large 39x parS plasmid. Further details are described in the Supplementary Methods section.
Figure 1 – figure supplement 4. (A) Fluorescence kymograph of a single 39x parS DNA molecule (Figure 1C) obtained with the C-trap including 20 nM ParB, and 2 mM CTP. A cartoon of the experiment with a schematic of the DNA showing the positions of the parS sequences plotted to scale is included on the left. (B) Same experiment as in A but using 2 mM CTPγS. Regions corresponding to the position of the parS groups could be clearly identified. (C) Average intensity profile (30 s) obtained along the DNA molecule. Positions of the parS sequences are included to scale in the background. The fluorescence intensity peaks correlate very well with the position of the 6 groups of parS sites. (D) Time-evolution of the fluorescence intensity at the parS region obtained from kymographs under no CTP, CTP, and CTPγS conditions.

Figure 2. CTP binding promotes ParB spreading from parS. (A) Cartoon of the experiment. First, a tandem 39x parS DNA molecule is incubated with 20 nM ParB2 and 2 mM CTP-Mg2+. Then, following a 2 min incubation, the confocal laser is turned on and confocal images are taken. (B) Representative confocal images taken after 2 min ParB incubation in the dark using tandem 39x parS DNA under no CTP, CTP, or CTPγS conditions, as well as parS-free DNA (lambda DNA) and 2 mM CTP-Mg2+. ParB appears in non-parS regions only when using parS DNA and under CTP or CTPγS conditions (red arrows). Dark to bright regions correspond to a scale of 0-50 photon counts for parS DNA tethers and 0-25 counts for lambda DNA. (C) Corresponding average profiles (500 nm width) of the fluorescence intensity taken along the DNA axis of the confocal images. Positions of the parS sequences are included to scale in the background. (D) Schematic representation of the single-length EcoRI 39x parS DNA used for C-trap roadblock experiments. The DNA contains 39 parS sequences arranged as in Figure 1C, but also includes two groups of 5xEcoRI sites flanking the parS region. Note that one of the 5xEcoRI groups is located 3613 bp away from the last parS sequence, potentially allowing spreading from the parS region. The positions of the parS sites in the DNA cartoon are represented to scale. (E) Cartoon of the roadblock experiment designed to limit ParB spreading using the EcoRI\textsuperscript{E111G} mutant as a roadblock. The experiment is identical to that described in A, but first includes a 2 min pre-incubation with 100 nM EcoRI\textsuperscript{E111G}, which is capable of DNA binding to EcoRI sites but unable to cleave the DNA, thus acting as a roadblock. (F) Confocal image showing limited spreading due to EcoRI\textsuperscript{E111G} blocking in tandem EcoRI 39x parS DNA. Brighter regions correspond to parS binding and the two dimmed regions correspond to limited spreading up to the EcoRI sites (red arrows). Regions inaccessible to ParB spreading are indicated with blue arrows. (G) Corresponding average profile (500 nm width) of the fluorescence intensity taken along the DNA axis of the confocal image. Positions of the parS sequences and EcoRI sites are included to scale in the background. Red arrows indicate the limited spreading of ParB up to EcoRI sites. Blue arrows indicate inaccessible regions to ParB.

Figure 3. DNA condensation is induced by ParB at nanomolar concentrations in the presence of CTP. (A) Cartoon of the basic magnetic tweezers components and the layout of the experiment. (B) Schematic representation of the 13x parS DNA used for MT experiments. The positions of the parS sites in the DNA cartoon are represented to scale. (C) Condensation assay. DNA is held at 4 pN while 50 nM ParB\textsubscript{D} is injected into the fluid cell in the presence of 2 mM CTP and 4 mM
MgCl₂. Following a 2-min incubation, the force is lowered to 0.3 pN and the extension recorded (red data). The extension in the absence of protein is shown in black. DNA could not recover the original extension by force after condensation at low force. (D) Average force-extension curves of 13x parS DNA molecules in the presence of 2 mM CTP, 4 mM MgCl₂ and increasing concentrations of ParB₂. A concentration of only 10 nM ParB₂ was able to condense the 13x parS DNA. (E) Average force-extension curves of 13x parS DNA taken under the stated conditions and in the presence of different nucleotides or with no nucleotide. Only CTP produces condensation of parS DNA. Solid lines in the condensed data are guides for the eye. Errors are standard error of the mean for measurements taken on different molecules (N ≥ 7). (F) Condensation assay of 13x parS DNA under 2 mM CTP and 1 mM EDTA conditions. DNA condensation by ParB and CTP requires Mg²⁺. (G) CTP-binding mutant, ParB<sub>R80A</sub>, does not condense 13x parS DNA under standard CTP-Mg²⁺ conditions. No ParB data represent force-extension curves of DNA taken in the absence of protein and are fitted to the worm-like chain model. Errors are standard error of the mean for measurements taken on different molecules (N = 7).

**Figure 3** – figure supplement 1. Low nanomolar concentrations of ParB condense parS DNA in the presence of CTP-Mg²⁺. (A) Condensation time-courses showing that a minimum of 5 nM ParB₂ condenses 13x parS DNA within a minute under 2 mM CTP, 4 mM MgCl₂ conditions. (B) Control experiment showing no condensation in the absence of CTP. (C) A protein concentration above 500 nM condenses DNA via non-specific interactions, as previously reported (Taylor et al., 2015). No ParB data represent force-extension curves of DNA taken in the absence of protein and are fitted to the worm-like chain model. Errors are standard error of the mean for measurements taken on different molecules (N ≥ 7).

**Figure 4.** DNA condensation by nanomolar ParB requires CTP binding but not hydrolysis. (A) Average force-extension curves of 13x parS DNA molecules in the presence of 2 mM CTPγS, 4 mM MgCl₂ and increasing concentrations of ParB. Results obtained with CTP (Figure 3D) and CTPγS were very similar. No ParB data represent force-extension curves of DNA taken in the absence of protein and are fitted to the worm-like chain model. Solid lines in condensed data are guides for the eye. Errors are standard error of the mean for measurements taken on different molecules (N ≥ 7). (B) Condensation assay of 13x parS DNA under 2 mM CTPγS and 1 mM EDTA conditions. DNA condensation by ParB and CTPγS requires Mg²⁺.

**Figure 5.** DNA condensation by nanomolar ParB is parS dependent. (A) ParB does not condense scrambled parS DNA under standard CTP-Mg²⁺ conditions. Errors are standard error of the mean of measurements on different molecules (N = 5). (B) The ParS-binding mutant, ParB<sub>R149G</sub>, does not condense 13x parS DNA under standard CTP-Mg²⁺ conditions. Errors are standard error of the mean of measurements on different molecules (N = 14). (C) Schematic representation of DNA substrates containing 7, 13, and 26 copies of parS. The positions of the parS sites in the DNA cartoon are represented to scale. (D) Average force-extension curves of 7x parS DNA, 13x parS DNA, and 26x parS DNA obtained under standard CTP-Mg²⁺ conditions. The condensation force correlates with increasing number of parS sequences. Solid lines in condensed data are guides for the eye. Errors are standard error of the mean of measurements on different
molecules \((N \geq 7)\). (E) Schematic representation of DNA substrates containing 1, 2, and 4 copies of \(parS\). The positions of the \(parS\) sites in the DNA cartoon are represented to scale. (F) Average force-extension curves of 1x \(parS\) DNA, 2x \(parS\) DNA, and 4x \(parS\) DNA obtained under standard CTP-Mg\(^{2+}\) conditions. No condensation was observed for these three experiments. Errors are standard error of the mean of measurements on different molecules \((N \geq 7)\). No ParB data represent force-extension curves of DNA taken in the absence of protein and are fitted to the worm-like chain model.

**Figure 6.** Model for ParB-dependent DNA condensation around \(parS\) sequences. (Step 1) ParB binding to \(parS\) does not require CTP as observed from C-trap experiments. \(parS\)-bound apo-ParB does not spread from \(parS\). (Step 2) CTP binding to ParB induces a conformational change to a sliding clamp which then escapes from \(parS\) to neighbouring non-specific DNA. Potential interactions between the ParB proteins around \(parS\) are represented by interlaced blue circles. Some ParB proteins are able to slide/diffuse long distances. (Step 3) ParB spreading and diffusion promotes the interaction with other CTP-ParB dimers through the CTD of ParB (Fisher et al., 2017), resulting in DNA condensation by forming large DNA loops. Alternatively, other protein-protein interaction such those mediated by the NTD (shown in figure) or the CDBD of ParB could result in DNA condensation. CTP hydrolysis might be a means to recover ParB dimers from the DNA (black arrow). Protein roadblocks constrain diffusion of ParB proteins (red arrow).
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Figure 1

A

|   | NTD | 96  | 102 | CDBD | 216 | 233 | 282 |
|---|-----|-----|-----|------|-----|-----|-----|
|   | N   |     |     |      |     |     | C   |

ParA interaction & CTP binding

parS specific interaction & dimerisation interface

nsDNA binding & dimerisation interface

B

C

NTD

CDBD

CTD

C-trap 39x parS DNA

24974 bp

1656 bp

1905 bp

3595 bp

Confocal scanner

Optical trap 1

Optical trap 2

Optical trap 1

Optical trap 2

C-trap 39x parS DNA

2X C-trap 39x parS DNA

No CTP

+CTP

CTPγS

No CTP

+CTP

CTPγS

E

F

Intensity (photons)

Intensity at parS (photons)
Figure 1 - figure supplement 1

A

C-trap fluidic cell

1 Beads
2 DNA
3 Buffer
4 ParB2

B

NTP hydrolysis rate (ParB dimer-1, min-1)

No DNA
0.5 µM scrambled parS
0.5 µM 1x parS
0.5 µM 2x parS
ATP + 0.5 µM 2x parS
GTP + 0.5 µM 2x parS
UTP + 0.5 µM 2x parS
No DNA
0.5 µM scrambled parS
0.5 µM 1x parS
0.5 µM 2x parS
0.5 µM 2x parS
0.5 µM 2x parS
0.5 µM 2x parS
0.75 µM 2x parS
1 µM 2x parS

0.0
0.2
0.4
0.6
0.8
1.0

1 mM CTP
1 mM NTP
1 mM CTPγS
1 mM CTP

C

NTP hydrolysis rate (ParB dimer-1, min-1)

No DNA
0.5 µM scrambled parS
0.5 µM 2x parS
0.5 µM 2x parS
0.5 µM 2x parS

1 mM CTP

wtParB
AF488-ParB
Figure 1 - figure supplement 2

**4x parS plasmid**

Step 1) Annealing of two long oligonucleotides

Step 2) Ligation into 2x parS linearised plasmid

**7x parS plasmid**

Step 1) Annealing of two long oligonucleotides

Step 2) Ligation into 4x parS linearised plasmid

**13x parS plasmid**

Step 1) PCR amplification of 6x parS fragment

Step 2) Ligation into 7x parS linearised plasmid

**26x parS and 39x parS plasmids**

Step 1) PCR amplification of 13x parS fragment

Step 2) Ligation into 13x parS linearised plasmid
Step 1) PCR of Lambda DNA fragments, digestion and purification (C1 & C2)

C1
NotI 5018 bp  Sall 5041 bp
BamHI
C2 9006 bp NotI 9051 bp

Step 2) Digestion of 39x parS plasmid, dephosphorylation and purification (C3)

Step 3) Triple ligation of fragments C1, C2 & C3
1. Ligase reaction. Ratio 1:1:1 (0.04 pmol/each)
2. Transformation of DH5α cells by heat shock
3. Colony PCR and pick possible positive colonies
4. Screening of isolated plasmids
5. Sequencing of positive plasmid
Figure 1 - figure supplement 4

A. 20 nM ParB2 and 2 mM CTP

B. 20 nM ParB2 and 2 mM CTP

C. 20 nM ParB2 and 2 mM CTP

D. 20 nM ParB2 and 2 mM CTP

- Figure 1A: Shows the position of 20 nM ParB2 and 2 mM CTP.
- Figure 1B: Shows the position of 20 nM ParB2 and 2 mM CTP.
- Figure 1C: Shows the intensity at parS (photons) vs. position (µm).
- Figure 1D: Shows the intensity at parS (photons) vs. time (s).
Figure 2

A
ParB incubation (laser off)
↓ 2 mins
Confocal imaging

B
Trap 1
No CTP
Trap 2

C
Trap 1
ParB incubation (laser off)
Confocal imaging

D
C-trap EcoRI 39x parS DNA

E
EcoRIE111G incubation (laser off)
↓ 2 min
ParB incubation (laser off)
↓ 2 min
Confocal imaging

F
Trap 1
Trap 2

G
Graph showing intensity (photons) vs. position (µm)
Figure 3

A

B

C

D

E

F

G
Figure 3 - figure supplement 1

A

| 2 mM CTP | 4 mM MgCl₂ |
|----------|------------|
| No ParB  | 2 nM ParB₂ |
| 5 nM ParB₂ |
| 10 nM ParB₂ |

B

13x parS DNA

| 0 mM CTP | 4 mM MgCl₂ |
|----------|------------|
| No ParB  | 200 nM ParB₂ |

C

13x parS DNA

| 0 mM CTP | 4 mM MgCl₂ |

Normalised extension vs. Force (pN)
Figure 4

A 13x parS DNA
2 mM CTPγS 4 mM MgCl₂

B 13x parS DNA
2 mM CTPγS 1 mM EDTA

Force (pN)

Normalised extension

Extension (µm)

Time (s)

Force (pN)

No ParB
10 nM ParB₂
50 nM ParB₂
200 nM ParB₂

No ParB
200 nM ParB₂
Figure 5

A  Scrambled parS DNA
2 mM CTP  4 mM MgCl₂

B  13x parS DNA
2 mM CTP  4 mM MgCl₂

C  7x parS DNA

D  200 nM ParB₂
2 mM CTP  4 mM MgCl₂

E  1x parS DNA

F  200 nM ParB₂
2 mM CTP  4 mM MgCl₂
1. ParB binding to \textit{parS} does not require CTP

2. CTP binding promotes escape from \textit{parS}

3. Spreading promotes DNA condensation
Cytidine triphosphate promotes efficient ParB-dependent DNA condensation by facilitating one-dimensional spreading from parS

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SUPPLEMENTARY METHODS
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Fabrication of DNA plasmids with multiple copies of parS

All constructs were cloned into the vector pET28a(+) (Novagen). Plasmids were cloned in DH5α Competent cells (ThermoFisher Scientific), and after selection of possible positive colonies by colony PCR, plasmids were purified from cultures using QIAprep Spin Miniprep Kit (QIAGEN), analyzed by restriction digestion and finally checked by DNA sequence analysis. These plasmids were employed to prepare different magnetic tweezers substrates which sequences are included below.

'Scrambled' parS DNA

The cloning of a plasmid containing a ‘scrambled’ parS site (scrambled parS: 5’-CGTGCCCAAGGGAGACA) was described in (Taylor et al., 2015).

1x parS DNA

The cloning of a plasmid containing a 1x parS sequence (optimal sequence of B. subtilis parS = 5’-TGGTACGTGAAACA) was also described in (Taylor et al., 2015).

2x parS DNA

The plasmid containing 2x parS sequences was derived from the 1 x parS plasmid by ligation of annealed synthetic oligonucleotides containing the parS sequence with appropriate overhangs into the cut NcoI site.

4x parS DNA

Two long 5’-phosphorylated oligonucleotides (Table S1) containing 2x parS sites separated by an XbaI restriction site and ending in a modified XbaI restriction site, were hybridised as described in Materials and Methods section. The plasmid containing 2x parS sequences was digested with XbaI, dephosphorylated with rSAP (NEB) and ligated with this pair of hybridized oligonucleotides rendering a plasmid with 4x parS sites.

The oligonucleotides were designed to create an incomplete XbaI site at both ends after ligation in such a way that once ligated, XbaI cannot cut again in those positions. In addition, in the middle of the annealed oligonucleotides a bona-fide XbaI site was included, allowing the repetition of the ligation process to insert the annealed oligonucleotides as many times as desired to increase the number of copies of the parS site (Figure 1-figure supplement 2).

7x parS DNA

The plasmid containing 4x parS sequences was digested with XbaI, dephosphorylated and ligated again with the pair of hybridized oligonucleotides rendering a plasmid with 6x parS sites (not employed in this paper). However, in this step of cloning and by chance, the pair of hybridized oligonucleotides entered two times during the ligation process. Although that ligation should render a plasmid with 8x parS sites, notice that one of the parS sites was incomplete (TTTCACGTGAAACA) probably because an error during the synthesis of one of the oligonucleotides, and therefore that incomplete sequence was not considered as a parS site and the final construct is said to contain 7x parS sequences.

13x parS DNA
The plasmid containing 7x parS sequences was used as a template to amplify by PCR (Phusion Polymerase, NEB) the 6 parS fragment by using primers 32.F pET28 PCR NdeI and 33.R pET28 PCR NdeI (Table S1). The PCR fragment was digested with NdeI, and ligated into the plasmid with 7x parS sequences previously digested with NdeI and dephosphorylated, rendering a plasmid with 13x parS sites.

26x parS DNA and 39x parS DNA

The plasmid containing 13x parS sequences was used as a template to amplify by PCR the 13 parS fragment by using 50.F pET28 37 Sphl-BglII and 51.R pET28 BglII-Sphl (Table S1). The PCR fragment was digested with BglII, and ligated into the plasmid with 13x parS sequences previously digested with BglII and dephosphorylated, rendering a plasmid with 26x parS sites. In addition, in this step of cloning and by chance, in a different plasmid the PCR fragment entered two times during the ligation process, and therefore we also obtained a plasmid with 39x parS sites. This last one was employed to prepare large plasmids described below.

Fabrication of large plasmids (>17 kb) for C-trap experiments

39x parS DNA

The large plasmid containing 39x parS sites was obtained by ligation of three fragments of DNA (Figure 1 – figure supplement 3). Two of them correspond to two PCR-fabricated DNAs (C1 and C2) derived from Lambda DNA (NEB) as template and including suitable restriction sites in the designed primers (Table S1). Fragment C1 (green), that corresponds to positions 33464-38474 bp of Lambda DNA, was amplified with oligos 20Lambda_F_NotI and 21Lambda_R_SalI, purified and digested with NotI and SalI. Fragment C2 (black), that corresponds to positions 5475-14509 bp of Lambda DNA, was amplified with oligos 22Lambda_F_BamHI and 23Lambda_R_NotI, purified and digested with NotI and BamHI. The fragment containing the 39x parS sites (C3, brown in the scheme) was obtained by digestion of the plasmid containing 39x parS sites described above with BamHI and SalI, dephosphorylation and purification by gel extraction with Gel extraction Kit (QIAGEN). The three fragments were then ligated at a ratio 1:1:1 and DH5α Competent cells were transformed by regular heat shock procedure. After selection of possible good colonies, large plasmids were purified from cultures using QIAprep Spin Miniprep Kit and checked by restriction digestion followed by DNA sequence analysis.

EcoRI 39x parS DNA

The large plasmid containing the 39x parS region flanked by two regions of 5xEcoRI restriction sites (named as EcoRI 39x parS) was produced following the same procedure but replacing parts C1 and C2 with fragments C1-EcoRI and C2-EcoRI, each one including 5 EcoRI restriction sites at the desired position. However, during the PCR amplification of Fragment C2-EcoRI with oligos 177_Lambda_F_5Eco BamHI and 23Lambda_R_NotI, the forward oligo was annealed in a different position of the Lambda DNA due to a similar sequence, and therefore the amplified fragment was shorter and corresponds to positions 13254-14509 bp of lambda DNA.
| Fragment | Oligonucleotide | Sequence |
|----------|----------------|----------|
| **Annealed oligonucleotides with 2 parS sites** | | |
| 30.P-up 2ParS Xbal-BsrGI | [Pho]CTAGCTGTACATTAAATCGAATGTCCACGTGAAACAGGAAAAAGAACCCTCTTAGCTAGCCAGTCCAGTGGTCCACG TGGAAACAAGTTCGAGTCCAAATATGG | |
| 31.P-down 2ParS Xbal-BsrGI | [Pho]CTAGCCATATTTGACCTCGGACCCTTTGCCAGTGGGACACT GAGCCCTGGACTTAGTCTAGAGAAGGTTTTTTTTCTTTGTTCAC GGGACATTTCTGATTAATGTCAG | |
| **6 parS PCR fragment** | | |
| 32.F pET28 PCR NdeI | GCCTAAGTCTATGCGGAAACAAGCGCCTCAT | |
| 33.R pET28 PCR NdeI | GCCTAAGTCTATGCGGAAACAAGCGCCTCAT | |
| **13 parS PCR fragment** | | |
| 50.F pET28 37 Sphi-BgII | GCCTAAGTCTATGCGGAAACAAGCGCCTCAT | |
| 51.R pET28 BgII-Sphi | GCCTAAGTCTATGCGGAAACAAGCGCCTCAT | |
| **Lambda C1 fragment** | | |
| 20Lambda_F_NotI | GCCTAAGTCTATGCGGAAACAAGCGCCTCAT | |
| 21Lambda_R_SalI | GCCTAAGTCTATGCGGAAACAAGCGCCTCAT | |
| **Lambda C2 fragment** | | |
| 22Lambda_F_BamHI | GCCTAAGTCTATGCGGAAACAAGCGCCTCAT | |
| 23Lambda_R_NotI | GCCTAAGTCTATGCGGAAACAAGCGCCTCAT | |
| **Lambda C1-EcoRI fragment** | | |
| 20Lambda_F_NotI | GCCTAAGTCTATGCGGAAACAAGCGCCTCAT | |
| 176.Lambda_R_SalEco | TAAGTGCGACGAATTCTATCGGAATTTCCGATAGGAATTCCGACG GCTCCTATTATATATACATATAACG | |
| **Lambda C2-EcoRI fragment** | | |
| 177.Lambda_F_SalEcoBamHI | TAAGTGCGACGAATTCTATCGGAATTTCCGATAGGAATTCCGACG GCTCCTATTATATATACATATAACG | |
| 23Lambda_R_NotI | GCCTAAGTCTATGCGGAAACAAGCGCCTCAT | |
| **For amplification of MT central part** | | |
| FMH_F_NotI_pET28-SpoOJ | GCCTAAGTCTATGCGGAAACAAGCGCCTCAT | |
| FMH_R_Xhol_pET28-SpoOJ | GCCTAAGTCTATGCGGAAACAAGCGCCTCAT | |
| **For amplification of MT handles** | | |
| FMH_F2_NotI | GCCTAAGTCTATGCGGAAACAAGCGCCTCAT | |
| JOE_R1 | AGTAAACGCGTCAGAACC | |
Sequences of DNA fragments used in this work.

DNA sequences corresponding to the central part (not including the handles) of Magnetic Tweezers and C-trap DNA substrates employed in this work. Scrambled parS sequence is highlighted in pink. ParS sequences in inverted (TGTTTCACGTGGAACA) or direct (TGTTCACGTGGAACA) orientation are highlighted in yellow. EcoRI restriction sites present in the central part of the C-trap substrate EcoRI 39x parS DNA are highlighted in red.

**MT 'Scrambled' parS DNA (5971 bp)**

| Fragment | Sequence |
|----------|----------|
| parS-Scrambled-1 | CAGCAGTTGAATCAGAAGTCCAGCTGCATGCATGTTCCACGTGAAACAAGAAAAAAGAA CCTGT |
| parS-Scrambled-2 | ACAGGCTTTTTTTTGTCTGATGATCAAC TGCTG |
| parS-1 | CAGCAGTTGAATCAGAAGTCCAGCTGCATGCATGTTCCACGTGAAACAAGAAAAAAGAA CCTGT |
| parS-2 | ACAGGCTTTTTTTTGTCTGATGATCAAC TGCTG |
| 1x parS for NTP hydrolysis assay | CTAGGCTTGGTTAAGGGCGGTTTTTGCAGCAGTGGTTCTGAGAAAAAAGAAAAAAGAA CCTGT |
| 2x parS for NTP hydrolysis assay | CTAGGCTTGGTTAAGGGCGGTTTTTGCAGCAGTGGTTCTGAGAAAAAAGAAAAAAGAA CCTGT |

**Scrambled parS for NTP hydrolysis assay**

| Fragment | Sequence |
|----------|----------|
| parS-Scrambled-1 | CAGCAGTTGAATCAGAAGTCCAGCTGCATGCATGTTCCACGTGAAACAAGAAAAAAGAA CCTGT |
| parS-Scrambled-2 | ACAGGCTTTTTTTTGTCTGATGATCAAC TGCTG |

**28.2ParS XbaI up**

| Fragment | Sequence |
|----------|----------|
| 28.2ParS XbaI up | CTAGGCTTGGTTAAGGGCGGTTTTTGCAGCAGTGGTTCTGAGAAAAAAGAAAAAAGAA CCTGT |

**29.2ParS XbaI down**

| Fragment | Sequence |
|----------|----------|
| 29.2ParS XbaI down | CTAGGCTTGGTTAAGGGCGGTTTTTGCAGCAGTGGTTCTGAGAAAAAAGAAAAAAGAA CCTGT |
TTTGAACTGAATGGCAAAGGCACCAGTACGCGCCCCACGCTGACGGTTTCTAACCTGTACGGTATGGTCACCGGGATGGCGGAAGATA
TGCAGAGCTCGGTGGGCAGGCAGTGGTTCCGGCCTAAGGGTTTACGCCCGTCTTCTGGGTAGGCGGTGAACCTTCGACGGAAACCTG
CCGACGGAAACGGATGGCGCTGCTCTTTCTGGGGACGTATCATGCTGGCCAACACCTGCACCTGGACCTATCGCGGTGACGAGTGCG
GTTATAGCGGCTCCTGGCTGCAACTGAGCGCGGTGAGTGCCTCCTTTGTACTGTCCACGCCGACGGAAACGGATGGCGCTGTTTTTCC
GGGACGTATCATGCTGGCCAACACCTGCACCTGGACCTATCGCGGTGACGAGTGCGGTTATAGCGGTCCGGCTGTCGCGGATGAATAT
GCCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGC
CGAGCGAATCTCGGGCGTTTTCTCCGCCCTCGCCTCCGGCGAGGGGGAAAGATATTTCCCCTGCGTGAATATCTCCGGTGAGCCGGAGG
CTATTTCCGTATGTCGCCGGAAGACTGGCTGCAGGCAGAAATGCA
GGGTGAGATTGTGGCGCGTTGGTCCACAGCCACCGGTGGTCTGCCCTGGCTGAGTGAGGCCGACCGGCGGCTGCAGGTGCAGAGTGATTT
GCCGTGGTGGCTGGTCTGCCGGGGGACGATTCATAAGTTCCGCTGTGTGCCGCATCTCACCCGGGCGGCTTTGAGCACGGTGAGCGA
CTGGTACACACTGTTCCGGGC