ParA-mediated plasmid partition driven by protein pattern self-organization

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DNA segregation ensures the stable inheritance of genetic material prior to cell division. Many bacterial chromosomes and low-copy plasmids, such as the plasmids P1 and F, employ a three-component system to partition replicated genomes: a partition site on the DNA target, typically called *parS*, a partition site binding protein, typically called ParB, and a Walker-type ATPase, typically called ParA, which also binds non-specific DNA. *In vivo*, the ParA family of ATPases forms dynamic patterns over the nucleoid, but how ATP-driven patterning is involved in partition is unknown. We reconstituted and visualized ParA-mediated plasmid partition inside a DNA-carpeted flowcell, which acts as an artificial nucleoid. ParA and ParB transiently bridged plasmid to the DNA carpet. ParB-stimulated ATP hydrolysis by ParA resulted in ParA disassembly from the bridging complex and from the surrounding DNA carpet, which led to plasmid detachment. Our results support a diffusion-ratchet model, where ParB on the plasmid chases and redistributes the ParA gradient on the nucleoid, which in turn mobilizes the plasmid.

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Introduction

How is energy used to transport and spatially organize large objects, such as DNA, in a cell? In eukaryotes, a mechanically driven mitotic spindle apparatus separates chromosomes. In bacteria, the most common DNA segregation mechanism employed by chromosomes and plasmids is still unclear. All bacterial chromosomes and most naturally occurring

plasmids are of low-copy number, and many employ active segregation (or partition) systems to ensure inheritance. Partition systems in bacteria are minimalistic, involving only three principal components: an NTPase that drives partition, a partition site on the DNA target, and a partition site binding protein that forms a large partition complex on the DNA target. The most prevalent class of partition systems in the microbial world use ParA-type ATPases that carry a deviant Walker-type active site, but the mechanism remains elusive.

The plasmids P1 and F are stably maintained in *Escherichia coli* and their partition systems are paradigms for studying ParA-mediated DNA segregation. The three essential plasmid-encoded components are ParA (or F SopA)—the ATPase, ParB (or F SopB)—the partition site binding protein, and *parS* (or F *sopC*)—the partition site on the plasmid. ParBs load onto and around their cognate partition site to form partition complexes, which have been observed as punctate foci *in vivo* by fluorescence microscopy (Hirano *et al*, 1998; Erdmann *et al*, 1999; Lim *et al*, 2005; Adachi *et al*, 2006; Sengupta *et al*, 2010). ParA ATPase activity is critical to the partition process (Ebersbach and Gerdes, 2001; Fung *et al*, 2001; Barilla *et al*, 2005; Pratto *et al*, 2008). ParA alone has weak ATPase activity that is mildly stimulated by non-specific DNA (nsDNA) or ParB (Davis *et al*, 1992; Watanabe *et al*, 1992). Together, ParB and DNA synergistically stimulate ParA ATPase activity. But how ATP hydrolysis generates the force for plasmid partition has been unclear.

Fluorescent fusions of P1 ParA have been shown to colocalize with the nucleoid (Hatano and Niki, 2010; Sengupta *et al*, 2010). ParA also colocalizes with plasmids bearing *parS* in the presence of ParB, but only when the plasmids are relatively immobile. ParA localization *in vivo* fits well with *in vitro* studies showing that, like other ParAs, P1 ParA-ATP binds nsDNA (Leonard *et al*, 2005; Hester and Lutkenhaus, 2007; Castaing *et al*, 2008; Vecchiarelli *et al*, 2010) and the ParB-*parS* partition complex (Bouet and Funnell, 1999; Havey *et al*, 2012). Covisualization of other plasmids, such as F plasmid, with their cognate ParA in the presence of ParB in living cells has shown that different ParAs can form different patterns, yet all result in uniform distribution of plasmid copies over the nucleoid region of the cell (Ebersbach and Gerdes, 2005; Adachi *et al*, 2006; Hatano *et al*, 2007; Ringgaard *et al*, 2009; Sengupta *et al*, 2010). The observations suggest that the continual redistribution of nucleoid-bound ParA could move plasmid DNA. Several ParAs form filaments *in vitro*, and *in vivo* images of fluorescently labelled ParAs have been interpreted as long continuous helical filaments (reviewed in Vecchiarelli *et al*, 2012). The resulting ‘filament-pulling model’ proposes that the polymerization/depolymerization cycles of plasmid-associated ParA filaments result in the continuous relocation of plasmids over the nucleoid (Ringgaard *et al*, 2009).

We have previously searched for and failed to observe P1 ParA filaments on DNA, therefore proposing a fundamentally

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different model for plasmid movement that does not invoke a mechanical filament (Vecchiarelli *et al*, 2010). In this diffusion-ratchet model, the plasmid is transported within the narrow cytosolic space between the nucleoid and the cell membrane. ParA-ATP dynamically interacts with the nucleoid via nsDNA binding, and ParB loads onto the plasmid via *parS* to form the partition complex. Plasmid-bound ParB stimulates the ATPase activity of nucleoid-bound ParA, resulting in the local release of ParA from the nucleoid. We have shown previously that ParA must then undergo a series of slow, ATP-dependent transitions before regaining nsDNA binding activity. This time delay prevents ParA from preferentially rebinding the nucleoid in the vicinity of the plasmid from where it dissociated. A critical outcome of this delay is the generation of a ParA clearing on the nucleoid around the partition complex. Plasmid drift towards a stochastically chosen direction would then be reinforced by the higher concentration of ParA at the movement front, relative to the lower concentration of ParA in the wake of plasmid movement. Thus, the model proposes that redistribution of nucleoid-bound ParA drives plasmid motion.

Our previous study of ParA interaction with nsDNA highlighted the role of a time delay in the diffusion-ratchet mechanism; but we did not study the interactions of all system components. Here, we examined the interplay among all system components by reconstituting the P1 plasmid partition system *in vitro*. Fluorescently labelled ParA, ParB, and plasmid (encoding *parS*) were introduced into a DNA-carpeted flowcell, which acts as a biomimetic of the nucleoid surface. The system dynamics were visualized by total internal reflection fluorescence microscopy (TIRFM). TIRFM is ideally suited for studying surface-mediated patterning systems as it only illuminates fluorescent molecules associated with the DNA carpet. We also reconstituted the Sop partition system of F plasmid and observed very similar system dynamics. Our cell-free system faithfully recapitulates key steps of ParA-mediated plasmid partition as observed *in vivo* and predicted by the diffusion-ratchet model. In particular, we successfully reproduced the generation of a depletion zone of the ParA ATPase on the DNA carpet surrounding the partition complex prior to its movement.

Results

Interactions among ParA, ParB, and nucleotide

We previously proposed the bacterial nucleoid plays a key role in the ParA-mediated partition reaction as a matrix along which the plasmid travels (Vecchiarelli *et al*, 2010). Therefore, reconstitution of a ParA-mediated partition system in a cell-free set-up required an artificial nucleoid surface within our reaction vessel. The inner surface of a 25- μ m-thick flowcell was coated with sonicated nsDNA at high density, and two-colour TIRFM was used to visualize the system dynamics on the DNA carpet (Figure 1A).

We first studied P1 ParA-GFP interactions with the DNA carpet. ParA undergoes a slow, ATP-specific conformational change that activates its nsDNA binding activity (Vecchiarelli *et al*, 2010). When ParA-GFP was preincubated with ATP and infused into the flowcell, it evenly accumulated on the DNA carpet over time (Figure 1B). We estimated the saturation density of ParA-GFP on the DNA carpet to be $\sim 7.5 \times 10^4$ dimers/ μm^2 . When ParA-GFP and ATP were

preincubated with ParB prior to infusion, the steady-state binding level of ParA-GFP on the DNA carpet decreased as the ParB concentration increased (Figure 1B). The data suggest that ParB can inhibit the nsDNA-binding activity of ParA-ATP.

In the absence of DNA, ATP γ S does not support the conformational change in P1 ParA necessary for nsDNA binding (Vecchiarelli *et al*, 2010). In the presence of DNA, ATP γ S supports the ParA conformational change, albeit less efficiently than with ATP (Supplementary Figure S1). Here, we found that ATP γ S supported moderate nsDNA binding (Figure 1C). DNA binding with ATP γ S was highly dependent on ParA concentration; at 50 nM ParA, DNA binding was barely detectable. The conformational change in ParA-ATP necessary for DNA binding is also ParA concentration dependent and accelerated by nsDNA (Vecchiarelli *et al*, 2010). However, unlike the reaction with ATP, ParB enhanced ParA-GFP binding to the DNA carpet with ATP γ S (Figure 1C). ParB may interact with ParA-ATP γ S to accelerate and stabilize its transition to the DNA-bound state. The findings are consistent with the observation that ParA-ATP γ S and ParB form stable protein-DNA complexes (Havey *et al*, 2012). ADP did not support ParA binding to the DNA carpet.

ParA and ParB FRAP analysis

We used fluorescence recovery after photobleaching (FRAP) experiments to estimate the exchange time of carpet-bound ParA-GFP (Figure 1D). At physiologically relevant carpet densities of ParA-GFP (less than a few % saturation), fluorescence recovery took place with two time constants, ~ 4 s (30% of total fraction) and ~ 34 s (53% of total fraction) (Figure 1E; Supplementary Table S1). The data suggest that there are two distinct states of ParA bound to the DNA carpet. The slower rate is consistent with the previously estimated dissociation rate constant (1.5–3/min), which is much faster than the rate of ATP hydrolysis by ParA in the presence of DNA (0.025/min; Vecchiarelli *et al*, 2010). The difference in rates indicates that protein exchange is not tightly coupled to ATP hydrolysis. Previous measurements did not detect the faster event perhaps for technical reasons. Recovery did not change dramatically when ATP was replaced with ATP γ S (~ 35 s; Supplementary Table S1); again demonstrating that ParA dissociation from nsDNA does not depend on ATP hydrolysis. Together, the data show that ParA-ATP can dynamically bind and dissociate from nsDNA without cycling through ATP hydrolysis, and suggest that ParA-ATP can diffuse on the DNA carpet prior to dissociation.

In the presence of an equimolar input concentration of ParB, much less ParB was bound to the DNA carpet compared to ParA (see below). However, the dominant ParA-GFP recovery time constant increased from 34 to 91 s (Figure 1F). A similar recovery time constant (108 s) was observed with ATP γ S at low carpet-bound ParA-GFP density (Supplementary Table S1). Thus, ParB appears to stabilize ParA-ATP interactions on the DNA carpet, but without forming a stable equimolar complex. ParB exchange on the DNA carpet was much faster than ParA with a time constant of ~ 5 s (Figure 1G). This rate was not noticeably affected by the presence of ParA (Figures 1H and I). The finding provides further support that a persistent ParA-ParB interaction does not occur on the DNA. The data also indicate that ParB may induce a conformational change in DNA-bound ParA-ATP that temporarily stabilizes the ParA-DNA interaction. Ultimately,

Figure 1 Binding and exchange kinetics of ParA-GFP and ParB on the DNA carpet. **(A)** The inner surface of the flowcell was coated with sonicated nsDNA to mimic the nucleoid surface (DNA carpet). Fluorescently labelled Par system components were infused into the flowcell and system dynamics were visualized using two-colour prism-type TIRFM. **(B)** ParA-GFP binding to the DNA carpet is suppressed by ParB. ParA-GFP (500 nM, all concentrations represent the final value in the sample upon infusion into the flowcell) was preincubated with ATP (1 mM) and mixed with different concentrations of ParB at the indicated ratios. Samples were infused into the DNA-carpeted flowcell at 20 μ l/min and flow was stopped at the time marked by arrows. **(C)** Nucleotide effects on ParA binding to the DNA carpet. As in **(B)**, except ATP γ S or ADP (1 mM) replaced ATP, with or without ParB (500 nM). **(D)** Time-lapse images of ParA-GFP FRAP on the DNA carpet. In all, 500 nM ParA-GFP (preincubated at 10 μ M with 1 mM ATP) was infused until ParA-GFP on the DNA carpet reached 2% saturation. Flow was stopped and a circular region of the DNA carpet was photobleached at $t = 0$ s. **(E)** Typical FRAP curve of ParA-GFP was fitted to a double exponential function (red curve). The average fast recovery time was 4.2 ± 1.3 s (30% of total bleached fraction) and the average slow recovery time was 34 ± 2 s (53% of total bleached fraction). The background was subtracted and the signal was normalized relative to the prebleach intensity. Residuals are plotted above. **(F)** As in **(E)**, except in the presence of ParB (500 nM). Typical FRAP curve was fitted to a single exponential function (red curve), recovery time 91 ± 7 s with a minor fraction at < 1 s. **(G)** Typical FRAP curve of the carpet-bound ParB-Alexa647 was fitted to a single exponential function (red curve). Average recovery time was 4.7 ± 1.0 s. **(H)** Time-lapse images of carpet-bound ParB-Alexa647 (10% labelled, 500 nM total concentration) in an FRAP experiment in the presence of 500 nM ParA. **(I)** As in **(G)**, except in the presence of 500 nM ParA. FRAP curve was fitted to a single exponential function (red curve). Average recovery time was 5.5 ± 0.5 s.

this conformational change in ParA leads to accelerated ATP hydrolysis and dissociation from DNA, which we assume to take place more efficiently in the vicinity of the partition complex where local ParB concentration is higher. The slower

ParA exchange rate in the presence of ParB indicates that ParB also inhibits DNA binding by ParA from solution. This means that ParA would remain in solution for a longer time before re-binding DNA after ATP hydrolysis.

Figure 2 P1 partition complex dynamics on the DNA carpet. ParA-GFP (1 μ M, green) was mixed with an equal volume of ParB (1 μ M), *parS*-Alexa647 (0.2 nM, red), and ATP (2 mM) during infusion into the DNA-carpeted flowcell. Flow was stopped after 2 min and observation continued. (A) Time-lapse images of a typical partition complex from the time of binding to the DNA carpet to the time of release. Scale bar, 2 μ m. (B) ParA-GFP (top) and corresponding *parS* plasmid (middle) intensities in early arriving partition complexes. Six areas that contained an anchored partition complex were monitored and the fluorescence time traces in different colours were superimposed. Average ParA-GFP intensity on the DNA carpet or *parS* background intensity was subtracted. Arbitrary intensity is shown on the left and the corresponding estimated number of ParA monomers or plasmid copies is on the right. Partition complexes continued to anchor and dissociate from the DNA carpet after flow stoppage (bottom panel). (C) Typical time-lapse images of a partition complex formed with ATP γ S. Partition complexes irreversibly attached to the DNA carpet with greater amounts of ParA-GFP and plasmid. (D) ParA-GFP (top) and *parS*-Alexa647 (bottom) fluorescence time traces of (C).

Par proteins mediate *parS* plasmid dynamics

We next set out to observe ParA, ParB, and plasmid interactions on the DNA carpet. A mixing tee was installed at the inlet of the flowcell to combine two solutions; one containing ParA-GFP, and the other containing ParB, *parS* plasmid labelled with Alexa647 (*parS*-Alexa647) and ATP. The samples were mixed during infusion into the flowcell for 2 min. Flow was stopped, and observation of the system dynamics was continued. With equimolar concentrations (500 nM after mixing) of ParA-GFP and ParB, and 0.1 nM *parS*-Alexa647, colocalized foci of ParA-GFP and plasmids were found to anchor to the DNA carpet as the sample was infused (Supplementary Movie S1, left panel). We believe that ParB binds *parS* plasmid to form a partition complex that also accumulates ParA to acquire carpet-anchoring capability (see below). Both ParA-GFP and plasmid copy number within these foci decreased over time (Figures 2A and B, top and middle). When ParA-GFP content became hard to distinguish from that on the DNA carpet, the partition complex detached. Upon anchoring, the complexes contained 5–20 plasmid copies and 500–1500 ParA-GFP monomers (Figure 2B, top and middle). Partition complexes that anchored after flow stoppage had fewer plasmid copies and less ParA-GFP, which was difficult to detect over the ParA-GFP intensity on the carpet (Figure 2B, bottom). The findings indicate that ParA-ATP can associate with the partition complex to form a dynamic tether between plasmid and nucleoid.

With an excess input concentration of ParA-GFP over ParB, many partition complexes accumulated on the DNA carpet, and each complex stayed longer on the DNA carpet (Supplementary Movie S2, top panel). With less input ParA-GFP compared to ParB, fewer partition complexes were tethered to the carpet. ParB could not anchor *parS*-Alexa647

to the DNA carpet in the absence of ParA. Together, the data show that the input ParA:ParB ratio influences the steady-state level of ParA binding to nsDNA, and ParA density on the DNA directly correlates with the stability of bridging between partition complexes and the nucleoid.

When ATP was replaced with ATP γ S, ParA-GFP accumulated on the DNA carpet and the partition complexes became irreversibly anchored (Figures 2C and D). ParA-GFP did not disassemble from these complexes. We conclude that ParB-stimulated ATP hydrolysis by ParA is required for ParA disassembly from the partition complex as well as the subsequent detachment of the partition complex from the DNA carpet.

P1 partition complex dynamics on the DNA carpet

In the above experiments, the partition complexes that bound the DNA carpet were relatively small. As a result, the details of their dynamic behaviour were difficult to study. By pre-incubating *parS* plasmid with ParA and ParB, the resulting partition complexes that anchored to the DNA carpet were much larger, with some containing >1000 plasmid copies (Supplementary Figure S2). Despite their size, they behaved in a qualitatively similar manner to the smaller partition complexes that were assembled without preincubation. These partition complex clusters bound the DNA carpet, remained tethered for several minutes, and detached (Figure 3A; Supplementary Movie S3). A few partition complexes jumped several microns, and were repositioned on the DNA carpet with ParA-GFP replenished (Figure 3A, Complexes 2 and 3). For some partition complex clusters, the plasmid content remained relatively stable (Figure 3B, Complex 2), while others lost a significant number of plasmid copies while anchored (Figure 3B, Complex 4). ParA-GFP

Figure 3 Dynamics of large P1 partition complex clusters. ParA-GFP (500 nM, green), ParB (500 nM), and *parS*-Alexa647 (0.5 nM, red) were preincubated for 80 min before infusion into a flowcell for 2 min. Flow was stopped and observation continued. (A) Time-lapse images of partition complexes including Complexes 1–4 that generated a ParA-depletion zone on the DNA carpet prior to release. Complexes 2 and 3 jumped and rebound to the carpet (dotted arrows). Scale bar, 2 μm. Time zero was set arbitrarily. (B) ParA-GFP and *parS*-Alexa647 fluorescence time traces from circular regions containing the Complexes 2 and 4 in (A). (C) Time-lapse images of Complexes 2, 3, and 4 from (A).

content, on the other hand, was consistently higher upon initial anchoring (5–22 ParA monomers/plasmid) and decreased at a relatively steady rate over the lifetime of the complex on the DNA carpet. When the amount of complex-bound ParA-GFP became low, the complexes detached, again identifying ParA as the limiting factor for plasmid bridging to the DNA carpet.

As ParA-GFP disassembled, many partition complexes engaged in tethered Brownian motion just prior to release from the DNA carpet (Supplementary Movie S3). At this point, ParA-GFP not only disassembled from the complex itself, but also from the surrounding DNA carpet (Figures 3B and C). The resulting ParA-GFP depletion zones were most obvious for the larger clusters (Figure 3C; Supplementary Movie S4). Once the partition complex detached, the remaining ParA-GFP depletion zone recovered within several minutes to match the ParA-GFP density on the surrounding DNA carpet area that was unperturbed. ParA depletion on the DNA carpet is strikingly similar to nucleoid patterning observed *in vivo* for several ParA-mediated systems.

Intriguingly, some partition complexes ‘rolled’ to the edge of the ParA-depletion zone prior to full detachment from the

DNA carpet (Figure 3C, Complex 3; Supplementary Figure S3A; Supplementary Movie S4). Even more striking, some large clusters underwent bidirectional splitting. For example, Complex 4 (Figure 3C) splits into two separate clusters, each of which remained tethered to the DNA carpet. ParA content also divided with no obvious structures remaining in between the daughter complexes. These complexes broke up further into smaller clusters and eventually dissociated from the DNA carpet (Supplementary Figure S3B; Supplementary Movie S4). The results suggest how ParA gradients on the nucleoid can be utilized to directionally transport partition complexes in the cell, and to bidirectionally split a single partition complex with multiple plasmid copies via a diffusion-ratchet mechanism (see Discussion).

Local stimulation of ParA ATPase by ParB governs partition complex dynamics

ParB also disassembled from the partition complexes, but more slowly than ParA-GFP (Figure 4; Supplementary Movie S5). The partition complex shown in Figure 4A bidirectionally splits into two daughter clusters. The daughter complex with a higher ParB content, relative to ParA, released

Figure 4 ParA and ParB content in partition complex clusters on the DNA carpet. (A) Disassembly of ParA and ParB from a typical large partition complex cluster is shown in time-lapse sequence. ParA-GFP (500 nM, green), ParB (total 666 nM ParB, including 3 % ParB-Alexa647, red), and unlabelled *parS* plasmid (0.5 nM) were preincubated for 30 min with ATP (1 mM) before infusion into the flowcell. (B) ParA-GFP and ParB-Alexa647 fluorescence time traces of the partition complex in (A). (C) Change in ParA:ParB ratio from the time of complex binding to dissociation in (A). ParA-GFP content is underestimated just prior to complex release due to ParA-GFP depletion from the underlying carpet. The red line represents a linear fit from $t = 118\text{--}287$ s. ParA:ParB ratio at the time of release was also separately estimated based on the fluorescence intensity drop when the complex detached (red dot). (D) A typical depletion zone of ParA-GFP (green) and ParB-Alexa647 (red) on the DNA carpet created by a partition complex. Images were time averaged over 20 s. The left panel set corresponds to 20 s leading to complex release, the middle panel set corresponds to 20 s immediately following complex release, and the right panel set represents the 20-s period starting 1 min after the middle panels. Scale bar, 2 μm. (E) Fluorescence intensity profiles of the ParA-GFP and ParB-Alexa647 depletion zones from the white box in (D).

from the DNA carpet much faster, suggesting that ParA:ParB ratio of the complex controls its dynamics on the carpet (see also Supplementary Figures S4E–J). The starting ParA:ParB

ratio of anchored partition complexes that exhibited dynamic behaviour was $\sim 2\text{--}3$ (Figure 4C; Supplementary Figure S4H–J). This ratio typically decreased to $\sim 0.7\text{--}2$ before complex detachment. The carpet-bound ParA:ParB ratio was $\sim 3\text{--}4$ (calculated from Figure 4B). When the complexes detached, a faint ParB-depletion zone was also detected on the DNA carpet, which shared a similar radius as the ParA-GFP depletion zone (Figures 4D and E; Supplementary Movie S6). It remains to be determined how the protein dynamics within and around a partition complex lead to ParB depletion.

When ATP γ S was used instead of ATP, large partition complex clusters accumulated on the DNA carpet with a very high ParA-GFP content (Figures 5A and B; Supplementary Movie S7). ParA-GFP did not disassemble, nor did the complexes dissociate from the carpet. The data again show that ATP hydrolysis is a prerequisite for ParA disassembly and plasmid release from the DNA carpet.

Similar observations were made when *parS* was not present on the plasmid substrate. The tethered complexes were formed with ATP, but they did not display dynamic behaviour and remained carpet-bound (Figures 5C and D; Supplementary Movie S7). The evidence suggests that *parS*-specific binding by ParB increases the local ParB concentration at the partition complex. This results in a localized and efficient stimulation of ParA ATPase activity and the subsequent release of ParA in the vicinity of the partition complex, allowing the partition complex to move.

Sop system dynamics of F plasmid in a cell-free system

The Sop system of F plasmid is one of several ParA-mediated partition systems studied *in vivo*, where the plasmid appears to dynamically redistribute its cognate ParA (SopA) on the nucleoid (reviewed in Vecchiarelli *et al*, 2012). The P1 Par system, on the other hand, has not been observed to form ParA-depletion zones on the nucleoid (Hatano and Niki, 2010). Nevertheless, we believe that the P1 ParA-depletion zone we observe here is equivalent to those observed *in vivo* for other ParAs. To determine whether the underlying mechanisms are indeed fundamentally similar, we also studied the F Sop system in our cell-free set-up.

As with the P1 system, we first used a mixing tee to mix SopA-GFP with a sample containing ATP, SopB, and pBR322::*sopC* plasmid labelled with Alexa647 (*sopC*-Alexa647) during infusion into the flowcell. Flow was stopped, and the system dynamics were visualized over time (Figure 6A; Supplementary Movie S1, right panel). The F Sop system dynamics mirrored that found for the P1 Par system. SopA-GFP initially produced a spike in carpet binding, which rapidly dropped to a steady-state density of $\sim 1\%$ saturation after flow stoppage (Figure 6B). The plasmids transiently bound the DNA carpet as small foci, many with a detectable level of colocalized SopA-GFP intensity above that on the carpet. These foci lost SopA-GFP over the course of several minutes and then released, indicating that the plasmid was tethered to the carpet via SopA-GFP, and its disassembly was a prerequisite to plasmid release. For the F Sop system, ATP γ S or ADP could not support plasmid bridging to the DNA carpet.

The number of *sopC*-Alexa647 plasmid copies present in each focus was estimated at several time points after flow stoppage (Figure 6C). The foci that first bound the DNA carpet had roughly one to five plasmid copies. After longer incubation in the flowcell, the plasmid foci arriving on the

Figure 5 Partition complex cluster dynamics requires ATP hydrolysis and *parS* on the plasmid. (A) Time-lapse images of partition complex clusters irreversibly anchoring to the DNA carpet in the presence of ATP γ S (1 mM) with 500 nM ParA-GFP (green), 500 nM ParB, and 0.5 nM *parS*-Alexa647 (red). (B) Typical fluorescence time traces of ParA-GFP (green, top) and *parS*-Alexa647 (red, bottom) of a region on the DNA carpet with a bound partition complex cluster. (C) Time-lapse images of complex clusters containing plasmid without *parS* irreversibly anchoring to the DNA carpet. Scale bar, 2 μ m. (D) Typical fluorescence time traces of ParA-GFP (green, top) and non-*parS*-Alexa647 (red, bottom) of a region on the DNA carpet with a bound partition complex cluster. Flow stopped at 130 s.

carpet grew larger. After 1 h, some foci had >20 plasmid copies associated. Both Sop proteins were required for the bridging of plasmid to the DNA carpet. With either protein omitted, plasmid dynamics were similar to that observed for plasmid alone, which rapidly diffused in and out of the illumination volume (Figure 6D; Supplementary Movie S8). Together, the data suggest that the partition complex recruits the partition ATPase and this interaction is required for plasmid clustering as well as plasmid bridging to the DNA carpet.

Notably, SopA-GFP could tether plasmids to the DNA carpet without SopB, but only at very high carpet densities of SopA-GFP (Figure 6D, >20% saturation). Similarly, with high input concentrations of SopA-GFP over SopB, the plasmids irreversibly stuck as small foci as SopA-GFP accumulated on the DNA carpet (Supplementary Movie S2, bottom panel). With more SopB than SopA-GFP, on the other hand, fewer plasmid foci bound the carpet, and those that did exhibited short dwell times. We conclude that when the ParB-stimulated rate of ATPase release from the partition complex is faster than the rate of ParA replenishment, the carpet-plasmid bridges disassemble and the plasmid is released.

When all Sop components were preincubated prior to infusion, large preassembled *sopC*-plasmid clusters anchored to the DNA carpet and produced SopA-GFP depletion zones (Figure 7A; Supplementary Movie S9). The depletion zones on the DNA carpet are strikingly similar to the depletion of nucleoid-bound SopA in the vicinity of SopB-bound plasmid *in vivo* (Hatano *et al*, 2007). Despite the removal of SopA-GFP on the carpet, a small quantity remained associated with the plasmid focus. The plasmid clusters eventually dissociated and the SopA-GFP depletion zone was refilled (Figure 7B; Supplementary Movie S9). Unlike the P1 system, we did not observe SopB-depletion zones. Together, the data show that the partition complex can actively remove the partition ATPase, not only from the plasmid, but also from the surrounding DNA carpet at a rate that is faster than the rebinding of new ATPase from solution.

A P1 ParA mutant that exhibits a propagation defective (*Par^{PD}*) phenotype *in vivo* blocks the dynamic behaviour of partition complexes

There is a class of ParA mutants that produce an *in vivo* phenotype termed as *par^{PD}* (propagation defective), where the plasmid is lost from a bacterial cell population more rapidly than those without any partition system (Youngren and Austin, 1997; Fung *et al*, 2001). Many *par^{PD}* mutants arise from mutating the ATP-binding pocket of ParAs, suggesting that ATP binding and/or hydrolysis is perturbed (reviewed in Funnell and Slavcev, 2004). P1 ParA[K122R] is a *par^{PD}* mutant and when it is coexpressed with wild-type P1 ParA, the mutant phenotype manifests, demonstrating the mutation is dominant (BE Funnell, unpublished observations). *In vitro*, the K122R mutation damages ParA ATPase activity so that basal ATPase is reduced and is only slightly stimulated by ParB (BE Funnell, unpublished observations).

When P1 partition complexes were assembled using ParA[K122R]-GFP instead of wild-type ParA, they behaved similarly to those assembled in the presence of ATP γ S (Figure 8A; Supplementary Movie S10). The data indicate that ParB-stimulated release of ParA from the partition

complex is blocked for the mutant protein. We then tested the dominance of ParA[K122R] by mixing the mutant protein with wild-type ParA at a 1:5 ratio. When ParA[K122R] was labelled with GFP to detect the mutant protein level in the partition complex, it remained stable (Figure 8B). On the other hand, when GFP was fused to wild-type ParA, ParA-GFP was observed to disassemble from the partition complex until reaching a low but stable steady state (Figure 8C). No ParA-GFP depletion zones were formed and the partition complexes remained stably tethered to the DNA carpet, presumably due to the persistent anchoring provided by

ParA[K122R]. The findings strongly suggest that the inability of ParB to stimulate the release of ParA[K122R] on and around the partition complex is the cause of the *in vivo* *par*^{PD} phenotype, and explains the mechanism of dominance.

Discussion

ParA ATPases supply energy to transport and position a growing number of large cargoes in bacteria including plasmids, chromosomal domains, and protein assemblages. But how this energy is coupled to the cargo dynamics observed *in vivo* has been unclear. For plasmid partition, ParB demarcates plasmid DNA as ‘cargo’ by binding onto and around *parS* (Rodionov and Yarmolinsky, 2004; Vecchiarelli *et al*, 2007). DNA also participates in another capacity by binding ParA-ATP. The vast majority of ‘nsDNA’ *in vivo* is organized as the bacterial nucleoid and we have argued that its surface acts as the matrix for cargo transport (Vecchiarelli *et al*, 2010).

Our cell-free reconstitution of ParA-mediated plasmid partition has provided observations that are consistent with, and further elucidate the details of the diffusion-ratchet mechanism of transport and positioning. Here, and in a study of the F Sop system (Vecchiarelli *et al*, 2013, in press), we find that both ParA and SopA bind the DNA carpet distributively and with fast exchange kinetics that are not coupled to ATP hydrolysis. Considering the *in vivo* concentrations of ParA and chromosomal DNA, our data suggest that ParA family members dynamically bind the nucleoid as dimers or relatively small oligomers at an average density of <1% saturation.

ParA-mediated partition system dynamics on a DNA carpet

We directly visualized ParA-ATP and ParB-mediated bridging between *parS* plasmid and the DNA carpet. This bridging activity was also observed for the F Sop system. Under typical reaction conditions, both proteins and ATP were required, which is consistent with the DNA-bridging complexes that have been observed previously, and proposed to play a role in bridging plasmid to the nucleoid surface (Havey *et al*, 2012), and also for partition complex clustering (Soberón *et al*,

Figure 6 F plasmid dynamics in the presence of the Sop system. (A) Covisualization of SopA-GFP (green) and *sopC*-Alexa647 (red). SopA-GFP (1 μ M) was mixed with an equal volume of SopB (1 μ M), *sopC*-Alexa647 (0.2 nM), and ATP (2 mM) during infusion into the flowcell. Flow was stopped after 2 min and observation continued. Time-lapse images are shown. Scale bar, 5 μ m. (B) Typical fluorescence time traces of SopA-GFP and *sopC*-Alexa647 of a partition complex. The average SopA-GFP intensity on the carpet was subtracted from an area with a partition complex (top panel) to obtain the plasmid-associated SopA-GFP time trace (middle panel). *sopC*-Alexa647 intensity was plotted to show the time of plasmid binding to the DNA carpet to the time of release (bottom panel). Time-lapse images show the decreasing intensity of SopA-GFP colocalized with the tethered plasmid focus. Scale bar, 2 μ m. (C) Estimate of plasmid copy number within a partition complex. Intensities of individual *sopC*-Alexa647 plasmids were quantified and fitted to a Gaussian distribution (inset, $n = 80$) to obtain an average intensity for single plasmids. This value was then used to estimate the copy number in plasmid clusters formed with SopA and SopB at the time points indicated ($n = 40$ clusters/time point). (D) SopA and SopB effects on plasmid dynamics. *sopC*-Alexa647 (0.1 nM) was infused alone, with 0.5 μ M SopB, or with 0.5 μ M SopA-GFP up to 10 or 20% of saturation of the DNA carpet. Scale bar, 5 μ m.

Figure 7 F partition complex effects on the surrounding DNA carpet. (A) Wiggling plasmid clusters form SopA-GFP depletion zones on the DNA carpet. In all, 0.5 μ M SopA-GFP (green), 1 μ M SopB (mixed 9:1 with SopB-Alexa647), and 0.1 nM *sopC* were preincubated together with 1 mM ATP for 15 min at 23°C and infused into the flowcell at 20 μ l/min for 2 min before flow stoppage and data acquisition. SopA-GFP and SopB-Alexa647 intensities were averaged over 500 frames and merged (left column). SopA-GFP was also covisualized with *sopC*-Alexa647 (0.1 nM, red) and merged (right column). Scale bar, 1 μ m. (B) SopA-GFP refilling following plasmid release. Time-lapse images show SopA-GFP and SopB-Alexa647 intensities over 1.5 min (top). Fluorescence time traces of SopA-GFP and SopB-Alexa647 associated with the plasmid cluster (On Focus) relative to the intensities on the DNA carpet (On Carpet; bottom). Scale bar, 2 μ m.

Figure 8 The *par^{PD}* mutant ParA[K122R] irreversibly anchors partition complexes to the DNA carpet. (A) Time-lapse images of partition complex clusters preassembled with ParA[K122R]-GFP (500 nM, green), unlabelled ParB (500 nM), and *parS*-Alexa647 (0.5 nM, red) irreversibly anchoring to the DNA carpet (top). Typical time traces of ParA[K122R]-GFP (green) and *parS*-Alexa647 (red) of a DNA carpet area with a bound partition complex cluster (bottom). The sharp rise of *parS*-Alexa647 intensity marks the time of the partition complex binding to the carpet. (B) As in (A), except ParA[K122R]-GFP was mixed with wild-type ParA at 1:5 ratio (combined concentration 500 nM). (C) As in (B), except GFP was fused to wild-type ParA, instead of the mutant protein. Flow stopped at 180 s. Scale bar, 2 μ m.

2011). Considering that a high concentration of ParA on the DNA carpet (and the plasmid) was sufficient for anchoring plasmid without ParB, the bridge might be solely composed of ParA in a specific state, and ParB may act to locally populate this state of ParA.

The partition complexes initially anchored to the DNA carpet with limited mobility, due to the large number of protein-mediated anchor points between the partition complex and the DNA carpet (Figure 9A). ParB-stimulated ATP hydrolysis by ParA was necessary for the net decrease in ParA

from tethered partition complexes, which led to a decrease in the ParA:ParB ratio of the complex. ParB also disassembled but at a slower rate. It is likely that the decrease in ParB content was in part due to the release of ParB-bound plasmids. As ParA disassembly decreased the number of anchor points, the complexes acquired freedom for a wider range of motion (Figure 9B, top). This tethered Brownian motion appeared to contribute to the development of a ParA-depletion zone on the surrounding DNA carpet, either by direct contact with ParB in the partition complex, or by diffusional

(inter-segmental) transfer from the DNA carpet to the partition complex. Ultimately, the remaining copies of plasmid within a tethered complex dissociated as a cluster from the DNA carpet, and the ParA-depletion zone quickly refilled

(Figure 9B, bottom). The dissociated clusters still had a high ParB content and a detectable level of ParA that maintained inter-plasmid bridging.

Is spatial confinement a key parameter for ParA-mediated partition?

It was generally not possible to follow a partition complex once it was released from the carpet. This was in part due to our current flowcell geometry, which has a depth of 25 μm . A partition complex that was freed from all anchor points diffuses away from the DNA carpet, rather than moving laterally along the carpet (Figure 9B). As discussed earlier, we found no evidence of a stable ParA-ParB interaction on the DNA carpet, suggesting that the interaction is highly transient at the individual level. This would explain why the ParA-ParB interaction alone was insufficient in keeping a mobile partition complex associated with the DNA carpet. Inside a bacterial cell, on the other hand, confinement of the partition complex within the narrow space between the nucleoid and membrane would facilitate a continued interaction between the partition complex and nucleoid (Figure 9C).

Under spatial confinement, our model predicts that once all anchor points disassemble, individually transient ParA-ParB associations would allow the partition complex, containing many ParB molecules, to quickly survey its contact density within the ParA gradient on the nucleoid. Because ParA does not dissociate from DNA immediately upon transient interaction with ParB, the local ParA distribution does not change instantaneously. The delay in ParA release would stabilize the time-averaged direction of the partition complex within the depletion zone. The partition complex would move along the path of the steepest ParA concentration gradient away from the centre of the depletion zone in a perpetual search for an increasing number of ParA-ParB contacts. A partition complex would only be obliged to turn around at a nucleoid pole, or when its motion stalls at another ParA-depletion zone

Figure 9 A diffusion-ratchet model for ParA-mediated partition. (A) ParB loads onto the plasmid at *parS* to form the partition complex. ParA-ATP dynamically binds the DNA carpet. ParA is converted to a specific state (ParA-AXP) on the DNA carpet (and plasmid) upon ParB interaction, which tethers the partition complex to the DNA carpet. ParA-AXP converts to ParA-ADP and releases from the DNA carpet in the vicinity of the partition complex. ADP to ATP nucleotide exchange and a series of ATP-dependent transitions (black arrows) delay ParA reactivation for DNA binding, thus allowing ParA to diffuse before rebinding the DNA carpet. (B) ParB-stimulated release of ParA decreases the anchor points between plasmid and DNA carpet, and the plasmid begins to 'wobble'. This tethered Brownian motion accelerates the removal of ParA from the surrounding DNA carpet, creating a ParA-depletion zone. In the absence of spatial confinement, the partition complex releases from the DNA carpet when the last anchor point disassembles, and the ParA-depletion zone refills. (C) In a bacterial cell, the small gap between the inner membrane and nucleoid surface provides spatial confinement. Once a partition complex loses all anchor points and forms a ParA-depletion zone, its stochastic drift towards one direction would be reinforced by the high concentration of ParA contacts at its movement front. A reversal in direction would be suppressed by the ParA-depletion zone in the wake of movement. (D) A loss of inter-plasmid bridging within a partition complex leads to splitting of the complex. The split complexes would chase opposite slopes of the ParA gradient and separate. When two partition complexes approach each other, the converging motion would be stalled by the depletion of ParA between the two complexes and their directions of motion would reverse.

surrounding a second partition complex. This spatial interference would encourage multiple partition complexes on one nucleoid to segregate into equidistant positions (Figure 9D).

P1 Par and F Sop comparisons of *in vivo* plasmid dynamics

P1 ParB forms punctate foci *in vivo* that remain colocalized with plasmids bearing *parS* (Erdmann *et al*, 1999; Sengupta *et al*, 2010). ParA, on the other hand, uniformly distributes over the nucleoid region of the cell, and also forms foci that colocalize with relatively immobile plasmids bearing *parS* (Hatano and Niki, 2010). But during periods of plasmid movement, the colocalized ParA foci disappear and only reappear once the plasmid has been repositioned.

The P1 Par system dynamics observed in our cell-free set-up recapitulated many aspects of the *in vivo* behaviour. The small partition complexes that assembled and anchored to the DNA carpet resembled the immobile P1 partition complexes observed *in vivo*. Loss of ParA from these stationary partition complexes led to plasmid release from the DNA carpet. This behaviour mimics the immobile to mobile transition of the partition complex, coupled to the disappearance of colocalized ParA foci *in vivo*. The local repositioning and splitting of large partition complexes that were occasionally observed in this study also resembled the *in vivo* dynamics. However, we suspect these motions observed here may not fully mirror the *in vivo* dynamics due to the flowcell geometry issues discussed above.

As with small partition complexes anchored to the DNA carpet, robust ParA-depletion zones have not been observed for the P1 system *in vivo* (Hatano and Niki, 2010; Sengupta *et al*, 2010). However, based on our observation of ParA depletion around large partition complex clusters, we believe that a small depletion zone develops transiently at the time of plasmid movement *in vivo*. If the depletion zone is not large enough or perhaps fails to keep up with the moving partition complex, then the partition complex might outrun the edge of the depletion zone, re-anchor to the nucleoid, and stall. This could explain the 'stick and move' dynamics of the P1 partition system *in vivo*.

If the depletion zone follows the motion of the partition complex with an appropriate delay, then a perpetual directed motion would result. This mode of plasmid movement has been observed *in vivo* for many ParA-mediated systems, such as F plasmid or pB171 (Hatano *et al*, 2007; Ringgaard *et al*, 2009). Plasmids encoding these systems continually 'roll' close to the receding edge of a dynamic ParA (or SopA) depletion zone on the nucleoid. We believe that the P1 ParA- and F SopA-depletion zones we observed on the DNA carpet are equivalent to the ATPase-depletion zones observed *in vivo* (Hatano *et al*, 2007; Ringgaard *et al*, 2009). We consider both modes of motion as variations of the same diffusion-ratchet mechanism. The similar behaviours observed for Par and Sop in our cell-free set-up indicate that despite the different *in vivo* dynamics, the underlying mechanisms of ParA-mediated segregation are highly similar.

Materials and methods

Plasmids and proteins

See Supplementary data for the plasmids used, and for the procedure for fluorescence labelling. Non-fluorescent ParAs,

both wild type and mutant, were purified by two ion-exchange chromatography steps as previously described (Davey and Funnell, 1994; Fung *et al*, 2001). GFP fusions of ParA proteins were purified as described (Vecchiarelli *et al*, 2010) with modifications as detailed in Supplementary data. Wild-type ParB and ParB[S80C, C214S] were purified essentially as described (Bouet and Funnell, 1999), and fluorescently labelled with Alexa647 maleimide. The labelling efficiency of the preparation used here was 10%. For the F Sop system, construction of strains and plasmids, and protein purification and labelling are described elsewhere (Vecchiarelli *et al*, 2013, in press).

Flowcell preparation

The lipid bilayer-coated and Neutravidin-bound flowcells were prepared as described previously (Han and Mizuuchi, 2010). Sonicated and biotinylated salmon sperm DNA was attached to the flowcell surface at high density as described in Supplementary data.

TIRF microscopy

The details of TIRF microscopy are described in Supplementary data.

P1 partition complex dynamics

For assembly of P1 partition complexes in the DNA-carpeted flowcell, samples were loaded in two separate syringes, (A) 1 μ M ParA-GFP (or other specified concentrations) in 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1.8 mg/ml ascorbic acid, 10% Glycerol and (B) 1 μ M ParB (or other specified concentrations) and 200 pM *parS*-Alexa647 in the same buffer plus 2 mM ATP. Samples (A) and (B) at 23°C were mixed through a Micro Static Mixing Tee (Upchurch Scientific) at the inlet of the flowcell at 40 μ l/min combined rate as they entered the flowcell. Flow was stopped at 2 min and observation continued. For experiments with preincubation of partition complexes, samples (A) 10 μ M ParA-GFP in 10 μ l 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1 mM ATP, 1.8 mg/ml ascorbic acid, 10% Glycerol and (B) 5 μ M ParB and 5 nM *parS*-Alexa647 in 20 μ l of the same buffer were incubated separately for 15 min, combined and diluted with 170 μ l of the same buffer, and incubated for variable periods (5–80 min) at room temperature. Portions of this sample were infused into the DNA-carpeted flowcell at 20 μ l/min for 2 min (unless indicated otherwise), flow was stopped and observation continued. Experiments with both ParA[K122R] (or ParA[K122R-GFP]) and wild-type ParA (or ParA-GFP) contained the two proteins at a ratio of 1:5 at a combined concentration of 500 nM. For experiments that contained fluorescently labelled ParB, the labelled protein preparation described above was mixed with wild-type ParB at a 1:2.3 molar ratio and used at a combined concentration of 666 nM. Reactions that involved ParB-Alexa647 included 0.1 mg/ml casein in the buffer. Carpet-bound protein concentration, and protein and plasmid copy numbers in carpet-bound complexes were estimated as described in Supplementary data.

F partition complex dynamics

Unless stated otherwise, all Sop experiments were performed in Sop buffer: 50 mM HEPES pH 7.5, 100 mM KCl, 10% Glycerol, 5 mM MgCl₂, 2 mM DTT, 0.1 mg/ml α -casein, and 0.6 mg/ml ascorbic acid. In all, 2 mM phosphoenolpyruvate (Sigma) and 10 μ g/ml Pyruvate kinase (Sigma) were also added for ATP regeneration. When components were mixed upstream of the flowcell, the two samples (as specified) were loaded into separate syringes and incubated at 23°C for 15 min. The connected tubing was filled and attached to the static mixing tee. The tubing downstream of the mixing tee (dead volume = 10 μ l) was connected to the flowcell inlet. For most experiments, samples were mixed and infused at a combined rate of 40 μ l/min for 2 min and flow was stopped for data acquisition. When components were preincubated prior to infusion, the preincubated sample was typically infused into the flowcell at 20 μ l/min for 2 min and flow was stopped for data acquisition.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.