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Review Article

Connecting the dots: key insights on ParB for chromosome segregation from single-molecule studies

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Abstract

Bacterial cells require DNA segregation machinery to properly distribute a genome to both daughter cells upon division. The most common system involved in chromosome and plasmid segregation in bacteria is the ParABS system. A core protein of this system - partition protein B (ParB) - regulates chromosome organization and chromosome segregation during the bacterial cell cycle. Over the past decades, research has greatly advanced our knowledge of the ParABS system. However, many intricate details of the mechanism of ParB proteins were only recently uncovered using *in vitro* single-molecule techniques. These approaches allowed the exploration of ParB proteins in precisely controlled environments, free from the complexities of the cellular milieu. This review covers the early developments of this field but emphasizes recent advances in our knowledge of the mechanistic understanding of ParB proteins as revealed by *in vitro* single-molecule methods. Furthermore, we provide an outlook on future endeavors in investigating ParB, ParB-like proteins, and their interaction partners.

Keywords: single-molecule studies; ParB; ParABS system; magnetic tweezers; optical tweezers; atomic force microscopy

Introduction

To ensure that both chromosomal and plasmid DNA content is equally distributed to offspring, bacteria utilize partition systems (Par) that maintain the fidelity and precision of DNA segregation. Initially identified in plasmids nearly 40 years ago, partition systems have been shown to be crucial in securing the proper segregation of plasmids during cell division, maintaining their integrity and genes within the bacterial population (Austin and Abeles 1983a, b). With the rise of DNA sequencing and comparative genomics, it has become apparent that certain chromosomal regions carry genes with high homology to the plasmid partition systems (Ogasawara and Yoshikawa 1992). The fact that these genes were shown to affect the chromosome dynamics (Mysliwiec et al. 1991, Ireton et al. 1994) suggested that a system once thought to be unique to plasmids could also be integral to segregating chromosomes, primary carriers of genetic information. Phylogenetic lineages of plasmid and chromosomal Par systems showed that these systems likely originated from plasmids via horizontal gene transfer (Gerdes et al. 2000), and were later repurposed for chromosomal segregation. Concluding evidence of the similarity between plasmid and chromosomal Par systems came as an essential partition site—*parS* site, was found on the chromosomal DNA (Lin and Grossman 1998), which is necessary for loading Par proteins to the DNA. Today, we recognize that the ParABS system is essential for proper functioning of many bacteria. Deletion of this system leads to severe defects and decreased fitness in many species (reviewed by Kawalek et al. 2020).

The chromosomal ParABS system consists of three key players, ParA and ParB proteins, and a ParB loading site - *parS*. The *parS* sites are typically short (~16 bp), inverted repeats located proximal to the origin of replication (Livny et al. 2007). Most bacterial species have between one and four repeats of putative *parS* sites, although this number can rise to 20 or more in some species (Jakimowicz et al. 2002, Tran et al. 2018, Jung et al. 2019).

ParB proteins are relatively small proteins with three distinct domains (Fig. 1A). This domain organization is conserved across species. The C-terminal domain (CTD) contains a dimerization surface, whereby two ParB monomers are brought together and reside in a dimeric state in the cells (Fig. 1B) (Leonard et al. 2004, Fisher et al. 2017). The CTD also plays a crucial role in nonspecific DNA-binding activities, facilitating ParB spreading and the formation of a nucleoprotein complex (Hayes and Barillà 2006, Chen et al. 2015, Fisher et al. 2017). This domain is connected via a disordered linker to the Helix-Turn-Helix motif (HTH) that allows for flexibility between the CTD domain and the rest of the protein and that provides for a DNA-storing lumen (Fig. 1B). The HTH domain enables ParB to bind specifically to the *parS* sequence (Jalal et al. 2020a). Finally, the N-terminal part of the protein (NTD) is the most highly conserved amongst ParB and ParB-like pro-

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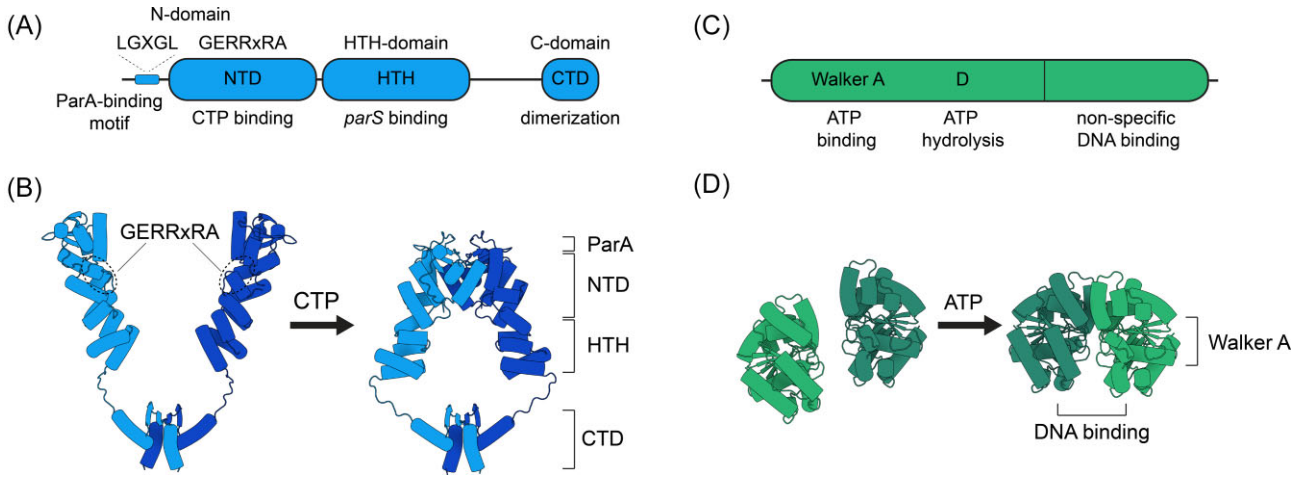

Figure 1. Domain architecture and conformational states of ParB and ParA proteins. (A) The ParB protein is depicted with its three distinct domains: the NTD, the domain containing the HTH motif, and the CTD. Functions associated with each domain are noted. The disordered ParA-binding motif containing LGXGL consensus sequence is represented as a smaller blue box. (B) ParB monomers dimerize through their CTDs, forming an open clamp configuration. Dashed region represents the CTP-binding pocket containing conserved GERRxRA residues. Upon binding to CTP and *parS* (not shown), the ParB clamp adopts a closed conformation. Corresponding domains are marked. (C) Representation of the ParA protein highlighting its characteristic Walker A motif and a conserved aspartate residue essential for ATP hydrolysis. (D) Representation of two ParA monomers that dimerize in the presence of ATP, highlighting the specific motifs involved in this process. The protein part responsible for DNA binding is marked. All structures were obtained using AlphaFold2 (Jumper et al. 2021, Mirdita et al. 2021) on UniProt entries P26497 (Spo0J/ParB) and P37522 (Soj/ParA).

teins. The N-terminal domain (NTD) has a highly conserved ParA-binding motif (LG-R/K-GL) (Leonard et al. 2005). Positively charged residues in this motif are crucial for interaction with ParA and stimulation of ParA ATPase activity (Leonard et al. 2005, Barillà et al. 2007). Additionally, the NTD features an arginine-rich motif (GERRxRA) (Fig. 1A and B). Mutations in this motif significantly impair the protein's function and the overall survival of bacterial cells (Leonard et al. 2004, Schumacher and Funnell 2005, Graham et al. 2014, Song et al. 2017, Tran et al. 2018, Jalal et al. 2020b).

It was the recent integration of both *in vitro* and *in vivo* research that brought a surprising and transformative insight to light, namely that ParB (Soh et al. 2019) and ParB-like (Osorio-Valeriano et al. 2019) proteins are CTP hydrolases (unlike the common ATPases and GTPases). These groundbreaking studies demonstrated that the amino acids in the GERRxRA motif directly interact with the CTP nucleotide, which turns out to be a crucial cofactor of ParB protein (Osorio-Valeriano et al. 2019, 2021, Soh et al. 2019, Jalal et al. 2020a, 2021a, Antar et al. 2021). In fact, the importance of CTP binding has been shown to be a vital functional characteristic across all investigated ParB proteins to date. Most importantly, CTP-bound NTDs of two ParB monomers will undergo dimerization, resulting in a clamp formation (Fig. 1B) (Soh et al. 2019, Antar et al. 2021, Jalal et al. 2021b, Osorio-Valeriano et al. 2021). Once attached, the clamp enables ParB to be rapidly released from the *parS* site but not disengage from the DNA. Instead, ParB spreads to the neighboring DNA through one-dimensional diffusion while remaining topologically trapped by embracing DNA within the lumen of the clamp. Disengaging from the *parS* site frees this loading site up for new ParB dimers to load, resulting in concentrated ParB localization near the *parS* site. Another important feature of the NTD is a target interface that allows interactions with the SMC (Structural Maintenance of Chromosomes) proteins (Bock et al. 2022). This interaction is essential for recruiting SMCs to the *ori* region (Gruber and Errington 2009, Sullivan et al. 2009), where they initiate the process of macroscale chromosome organization and segregation (Wang et al. 2017).

ParA proteins are members of the ParA/MinD family of ATPases, as reviewed in Vecchiarelli et al. (2012). They are identified by a distinct Walker A motif essential for ATP binding. Importantly, ParA proteins often carry basic residues at their C-terminal end, which are pivotal for nonspecific DNA binding (Hester and Lutkenhaus 2007) (Fig. 1C). In the presence of ATP, ParA dimerizes, resulting in the entrapment of two ATP molecules at the dimer interface (Leonard et al. 2005). This dimerization results in the formation of a composite interface for DNA contact, which allows ParA dimers to bind across the entire chromosome nonspecifically (Fig. 1D). ParA dimer formation and ParA-DNA interaction are modulated by ATP hydrolysis, with the hydrolyzed state causing ParA to revert to a monomeric form and dissociate from DNA into the cytoplasm (Scholefield et al. 2011). While ParA proteins have a basal ATPase activity, ATP hydrolysis is strongly increased via direct interaction with their partners - ParB proteins (Davis et al. 1992), which is essential for efficient origin segregation to the new cell pole. In some bacteria, ParA proteins are essential for the proper segregation of DNA during cell division, while in others, they may be nonessential while still playing a role in various cellular processes. For instance, in *Bacillus subtilis*, they also affect sporulation (Wu and Errington 2003), DNA replication (Murray and Errington 2008, Scholefield et al. 2012), and the organization of DNA through interactions with SMC proteins (Roberts et al. 2022). These additional roles of ParA are dependent on its ATP/ADP state, indicating that the ParB–ParA interaction orchestrates a broader spectrum of cellular processes than previously understood.

In cells, the core function of ParB protein centers around binding to *parS* sites and achieving a high local concentration (~10 mM; Guilhas et al. 2019), which culminates in the formation of a dense nucleoprotein complex - known as the "partition complex" or "segrosome" (Fig. 2) (Lin and Grossman 1998, Hayes and Barillà 2006, Graham et al. 2014, Jalal and Le 2020, McLean and Le 2023). Recent insights from *in vitro* and *in vivo* studies have

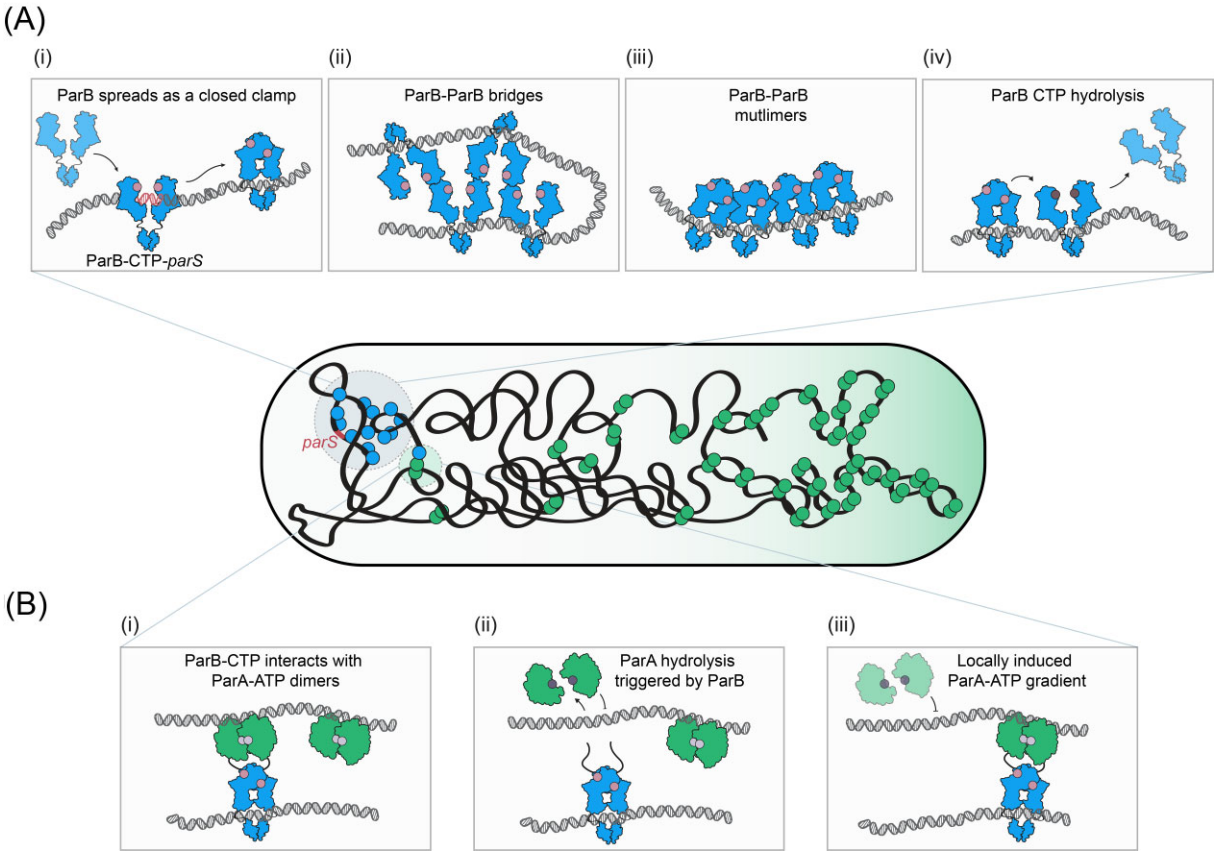

Figure 2. Overview of the ParABS system mechanism in chromosome segregation. (A) Interaction of ParB protein with the *parS* sequence and formation of the partitioning complex. (i) ParB binds CTP in an "open clamp" configuration and recognizes the *parS* sequence. A conformational change causes the clamp to close around the DNA upon binding. Once closed, ParB detaches from *parS* and spreads along the DNA. (ii) Adjacent ParB-CTP molecules bridge using their NTDs, enabling interactions between these DNA-bound ParB dimers. (iii) ParB-CTP further enhance DNA compaction through multimerization. (iv) ParB eventually hydrolyzes CTP to CDP. This action prompts the clamp to revert to its "open" state, leading to ParB's release from the DNA. (B) Dynamics of the ParB-*parS* complex's intracellular movement mediated by the ParA gradient. (i) ParA-ATP dimers bind to the DNA in a nonspecific manner. The ParB clamp interacts with the ParA-ATP dimers through its N-terminus. (ii) ParB induces the ATPase activity of ParA-ATP, leading to the hydrolysis of ATP to ADP. Post ATP hydrolysis, the ParA-ADP monomers release from the DNA. (iii) Following the detachment of some ParA dimers, a local gradient of ParA on the DNA is established. This causes the ParB clamp to move on the DNA, interacting with the next available ParA-ATP dimer.

underscored the critical role of the CTPase activity of ParB in the formation of this complex (Soh et al. 2019, Jalal et al. 2020a, 2021a, Antar et al. 2021, Osorio-Valeriano et al. 2021, Tišma et al. 2022). Aside from promoting more efficient binding to the *parS* site and spreading to adjacent DNA (Fig. 2A-i), CTP is also involved in ParB multimerization and ParB bridging (Fig. 2A-ii and iii), which are essential for the partition complex formation. Several recent studies even postulate that ParB undergoes liquid-liquid phase separation (LLPS), where CTP binding acts as a regulating step for the formation of phase-separated droplets (Guilhas et al. 2019, Babl et al. 2022). Further, CTP hydrolysis favors the dissociation of ParB from the DNA. This step is important as it recycles ParB proteins, allowing them to repeatedly bind to *parS* sites. This continuous cycle of binding and release maintains a steady presence of ParB near *parS* sites, not allowing the ParB clamps to diffuse too far away from the loading site, which is essential for effective chromosome segregation (Soh et al. 2019, Jalal et al. 2020b, Osorio-Valeriano et al. 2021) (Fig. 2A-iv).

The partition complex initially forms near the origin of replication. When the origin is duplicated during DNA replication, partition complexes assemble on both origins of the nascent chromosomes. The bidirectional movement required for this separation is driven by the interaction between the ParB-*parS* partition complex and a gradient of ParA proteins (Fig. 2) (Hu et al. 2017). The ParB-*parS* partition complex interacts with adjacent ParA-ATP, stimulating ParA's ATPase activity (Fig. 2B-i). This catalytic activity leads to the hydrolysis of ATP to ADP, causing ParA-ADP monomers to detach from the DNA (Fig. 2B-ii) (Bouet and Funnell 1999, Zhang and Schumacher 2017, Chu et al. 2019). The ParA-ADP monomers are temporarily inhibited from DNA binding due to a kinetic time delay, providing a time window before they can rebind ATP and, subsequently bind the DNA (Vecchiarelli et al. 2010, 2014a). Together with ParB-stimulated ATP hydrolysis, this delay establishes a local gradient of ATP-bound ParA, with regions of higher concentration distant from the ParB-*parS* complex. ParB-*parS* complex starts directionally moving toward areas of higher ParA concentration (Fig. 2B-iii). This ParB directional movement has been explained by several models: diffusion-ratchet model (Hu et al. 2017), DNA relay model (Lim et al. 2014), and the hitch-hiking model (Le Gall et al. 2016). In all models, as the complex moves, it continues to engage with ParA, ensuring consistent ATP hydrolysis and the maintenance of local gradient ParA-ATP (Hwang et al. 2013, Vecchiarelli et al. 2013, Lim et al. 2014). This intricate mechanism drives the replicated chromosome directionally across the cell, ensuring effective chromosome segregation before the cell divides.

Single-molecule techniques to study ParB–DNA interactions

The *in vivo* studies provided a foundational understanding of the ParABS system and its effects on chromosome integrity in various bacterial species. Research on ParB in living cells spotlighted critical steps of its action: binding to a specific DNA sequence (*parS*), forming a partition complex (seen as a bright foci under the microscope), and a directional movement toward the new cell pole following replication (Glaser et al. 1997, Lin et al. 1997, Webb et al. 1997). Subsequent efforts from biochemical studies further increased our understanding of ParB mechanism by unveiling its CTPase activity (Osorio-Valeriano et al. 2019, Soh et al. 2019, Jalal et al. 2020b) and the detailed mechanics of clamp closure upon interaction with the *parS* site (Antar et al. 2021, Jalal et al. 2021b, Osorio-Valeriano et al. 2021). These studies posed new questions on the mechanistic details of these proteins in the presence of their indispensable cofactors.

In this context, *in vitro* single-molecule studies have emerged as a vital complementary approach to address mechanistic questions on the ParABS system, offering highly controlled conditions that can shed light onto the ParB behavior and its interaction with associated proteins. The primary objective of this review is to cover the new insights into the ParB mode of action that was recently obtained from single-molecule *in vitro* studies. We discuss the results on the ParABS system from a range of methodologies, paving the way for future experiments that may address open questions in ParB research.

Magnetic tweezers: DNA condensation

Initial observations of ParB proteins localizing at a very small area of the chromosome raised hypotheses of the protein inducing local condensation of the DNA (Murray et al. 2006). Magnetic tweezers (MT) proved instrumental in investigating these phenomena due to their real-time ability to observe DNA condensation. In a typical MT experiment, a DNA molecule of interest is tethered between a glass surface with one of its ends and a micron-size magnetic bead at its other end (Fig. 3A). Using magnets positioned above the bead, one applies a constant force on the molecule, and thus MT function as a force clamp where DNA is stretched to a certain length. If a DNA-binding protein affects the conformation of the DNA, the length change can be precisely measured (Fig. 3A). This is done by observing the size and the pattern of the diffraction rings around the magnetic bead as it moves downwards, out of the z-plane. These patterns can be converted very precisely (with a resolution of a few nm) to the position of the bead (van Loenhout et al. 2012). MT is a label-free technique that offers a way to study protein-DNA interaction devoid of any extra modifications on the protein, which potentially might cause a change in its physiological behavior. MT can be highly parallelized, allowing measurements of thousands of beads in parallel (De Vlaminck et al. 2011, De Vlaminck and Dekker 2012). While direct visualization of protein binding or clustering is challenging due to the absence of protein labels, it is possible to visualize protein in MT by combining fluorescence microscopy and MT, but this involves rather complex setups (Madariaga-Marcos et al. 2019).

First studies of ParB proteins in MT (Taylor et al. 2015), done in the absence of CTP, showed that very high concentrations (1 μM) of ParB proteins were required to promote DNA condensation both in the presence and absence of a *parS* site on the DNA (Fig. 3B). Further studies with truncated and mutated proteins showed that some *B. subtilis* ParB proteins employ specific Lys residues at the C-terminal dimerization domain of the protein, which are necessary for nonspecific DNA binding and condensation (Fisher et al. 2017). Mutations of these residues caused severe defects in partition complex formation *in vivo*, just as proposed by the MT results, underscoring the potent synergy achieved when combining *in vivo* and *in vitro* approaches in understanding complex biological processes (Fisher et al. 2017).

Following the discovery of the CTP binding and hydrolase activity of ParB proteins (Osorio-Valeriano et al. 2019, Soh et al. 2019), MT were again used for studying the real-time DNA condensation by ParB proteins at single-molecule level. These experiments showed that DNA condensation can occur at much lower concentrations of ParB (10 nM) in the presence of CTP nucleotide, and they also showed a strong dependence on the presence of a *parS* site (Balaguer et al. 2021, Taylor et al. 2021) (Fig. 3C). The stark contrast to the previous findings (Taylor et al. 2015, Fisher et al. 2017) likely was due to the larger loading rate of ParB proteins onto the DNA in the presence of CTP and *parS* (Osorio-Valeriano et al. 2019, Soh et al. 2019, Jalal et al. 2020a). MT also allowed fast screening of different buffer conditions, which showed the necessity of the presence of Mg^{2+} ions and a high specificity for DNA condensation only in the presence of CTP and not other nucleotides (GTP, ATP, and UTP) (Balaguer et al. 2021).

With a precise measurement of DNA length and forces in the MT setup, it was shown that ParB proteins could effectively condense DNA molecules only when the forces on the DNA were reduced to the range of 0.2-0.6 pN (Balaguer et al. 2021, Tišma et al. 2023). This is a low force range compared to the forces exerted by single motor proteins such as RNA polymerases (25 pN; Wang et al. 1998), DNA translocases (29 pN; Saleh et al. 2004) or helicases (50 pN; Liu et al. 2018). This shows that the ParB-DNA cluster involves weak interactions that are easily disrupted by external forces. Indeed, subjecting the condensed ParB-DNA cluster to external forces of 5 pN resulted in gradual decondensation of the cluster over the course of 1-1.5 min (Taylor et al. 2021). Interestingly, decondensation was also shown to be highly dependent on the presence of CTP, whereby in the absence of the nucleotide, ParB-DNA clusters decondensed even faster, within 5 s (Taylor et al. 2021). Similar effects were also observed in bulk assays, whereby the presence of CTP or nonhydrolyzable CTP analog (CTPγS) significantly extended the release time of ParB proteins from the DNA (Antar et al. 2021, Osorio-Valeriano et al. 2021).

Further MT experiments showed that a single *parS* site is sufficient for ParB molecules to condense the DNA (Tišma et al. 2023). Conformational changes in ParB proteins were found to be necessary to condense the DNA efficiently: by using cross-linking mutants that prevent clamp opening (such as Fig. 2A-iv), it was shown that ParB proteins cannot condense DNA efficiently when NTDs are cross-linked after loading onto the DNA (Tišma et al. 2023). Conversely, when the C-terminus of the ParB protein was cross-linked, there was no effect on the condensation dynamics. Using nonhydrolyzable CTPγS or a nonhydrolyzing mutant protein ($ParB^{E111Q}$ in *B. subtilis* that forces the ParB clamp into a prolonged closed state; Antar et al. 2021) failed to promote an efficient DNA condensation (akin to Fig. 2A-ii) (Tišma et al. 2023).

A modified version of MT where the magnetic bead is not directly attached to the DNA, but rather to RNAP, which was loaded onto the DNA (Janissen et al. 2018), was used to test the effect of a ParB:DNA cluster on a transcribing RNAP (Tišma et al. 2023). Interestingly, the presence of the ParB condensate did not affect the RNAP processivity and only had a slightly reducing effect on the average transcription speed. This suggests that the gene repression effects of ParB that were reported previously (Lynch and Wang 1995, Rodionov et al. 1999, Jakimowicz et al. 2002, Bartosik

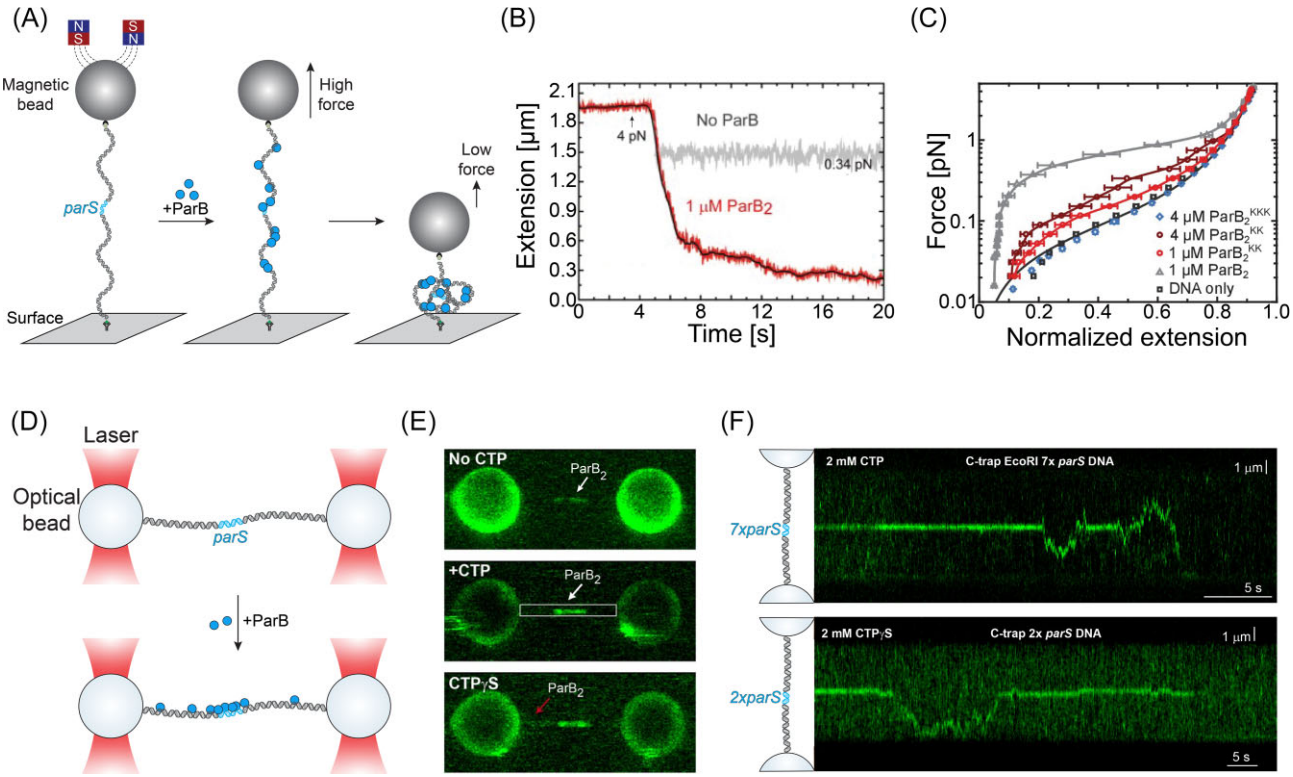

Figure 3. Force spectroscopy tweezers techniques for investigating ParB proteins. (A) MT setup. Schematics of a typical experiment where the DNA is held stretched at high force (~5 pN) during the addition of ParB proteins, and then released to low forces, whereupon condensation occurs. (B) A MT setup was used measure the DNA-condensation in the presence of ParB in real-time. Adapted from Taylor et al. (2015). (C) Force extension measurements of wild type $ParB_{Bsub}$ in comparison to different Lys mutants at the CTD interface. Adapter from Fisher et al. (2017). (D) Optical tweezers setup. Two optical beads are trapped using focused laser beams, with a DNA molecule stretched between them. Upon moving these beads to another channel with ParB proteins, coverage of the loading site and adjacent regions will occur. (E) OT was used for visualization of ParB binding to *parS* proximal region in the absence and presence of CTP/CTPgS nucleotides. (F) Single-particle tracking of one-dimensional diffusion by ParB proteins. E and F adapted from Balaguer et al. (2021).

et al. 2004, Kusiak et al. 2011, Venkova-Canova et al. 2013, Kawalek et al. 2017) likely act on access of the RNAP to the promotor, rather than being due to halting or blocking a transcribing RNAP.

Taken together, MT provided valuable knowledge of the underlying mechanism and requirements for DNA condensation by ParB proteins, which were inaccessible (or laborious to test) in *vivo*. There are many options to expand this type of experiment. Its capacity for swiftly testing various conditions (like nucleotides, salts, and loading site numbers) makes it an easily accessible and useful technique. MT can for example be utilized to test the interaction of ParB with supercoiled DNA molecules (Taylor et al. 2021), or multiple DNA molecules can be tethered to the bead. Cross-testing chromosomal or plasmid ParB proteins from different species and ParB-like proteins would be the next step in learning about the generality of the DNA-condensation process in chromosome segregation and plasmid partitioning.

Optical tweezers: *parS* binding and spreading

Optical tweezers (OT) also stand out as a useful tool, offering a complementary approach to MT for studying ParB proteins, as they enable direct visualization of ParB protein on the DNA while also allowing for adjustable forces to be applied to the DNA. In OT, functionalized micron-size beads are trapped using a highly focused laser, thus providing a position clamp. A typical OT experiment starts by trapping two beads within a microfluidic channel and ensuring the attachment of a single DNA molecule between them (Fig. 3D). These beads connected by DNA are moved within the microfluidic channels where, through laminar flow and multiple wells, a constant supply of various buffers and conditions can be maintained. Individual beads can be moved independently allowing the application of a stretching force to the tethered DNA molecule. Importantly both the DNA and the proteins can be labeled using fluorescent dyes, which allows visualization of both components using confocal microscopy. This enables direct, single-molecule observation and timelapse tracking of all phenomena exhibited by the investigated proteins (Fig. 3E). While OT is beneficial for direct visualization, it also faces challenges. Notably, its low throughput poses a significant constraint for testing many molecules. A typical experiment involves recording one molecule at a time under distinct conditions. Furthermore, extended exposure to laser illumination can induce DNA breakage, hindering extended data acquisition. Moreover, OTs typically operate at higher forces (1-100 pN) and will have a low signal-to-noise ratio for conditions under which ParB proteins can efficiently condense the DNA (0.01-0.5 pN).

Yet, OT has been used to study the binding and spreading of ParB proteins in the presence of CTP and *parS* sites. In fact, it allowed the first single-molecule traces of diffusing ParB proteins to be visualized (Balaguer et al. 2021) (Fig. 3F). Due to CTP hydrolysis and dissociation of ParB proteins from the DNA, many traces could be acquired using a single DNA molecule. This allowed the quantification of the diffusion coefficient for *B. subtilis* ParB protein (0.41 ± 0.02 µm^2/s), which showed that these proteins, experience similar dynamics as common DNA-binding proteins during their target search phase when they are nonspecifically attached

to the DNA (Stracy et al. 2015, 2021). Guo et al. (2022) reported that ParB proteins can connect and traverse as multimers - a previously undescribed mode of movement, especially in the presence of CTP. Additionally, OT experiments allowed the quantification of ParB spreading on the DNA in real time. By incorporating multiple *parS* sites in a linear DNA construct, Balaguer et al. (2021) observed a substantial increase in the signal near the *parS* sites and the neighboring regions, offering one of the first visualizations of ParB spreading. Moreover, they reported that ParB diffusion on stretched DNA molecules can be blocked by a strongly attached DNA-binding protein (an inactive $EcoRI^{E111Q}$).

Several recent studies have postulated that plasmid and chromosomal ParB proteins undergo LLPS on DNA (Guilhas et al. 2019, Babl et al. 2022), whereby the *parS* site would initiate the binding by ParB proteins, which would attract multiple new ParB proteins to that position and locally induce droplet formation. OT represents a suitable system for testing this, as it potentially may distinguish between the two most prominent types of phase separation - LLPS and bridging-induced phase separation. With the ability to stretch the DNA and thus vary the force, the necessity for a DNA scaffold in the phase-separated droplet can be tested and quantified.

Single-molecule stretching assays: ParB spreading, recruitment, and DNA condensation

Single-molecule stretching assays allow scientists to directly visualize interactions and dynamics of ParB proteins on DNA. These techniques rely on DNA molecules that are stretched along a PEGylated or lipid-coated surface, where they covalently attach either with one (Greene et al. 2010) or with both DNA ends (Ganji et al. 2016) to the surface (Fig. 4). In single-end tethering with typically many DNA molecules in parallel ("DNA curtains;" Granéli et al. 2006, or "PIFE" - protein-induced fluorescence enhancement; Song et al. 2016), molecules have one loose end and are maintained in the stretched state by a continuous flow (Fig. 4A). When the DNA is tethered with both ends to the surface (Ganji et al. 2016), the effect of proteins on the DNA can be observed without an applied flow. In these setups, DNA and proteins can simultaneously be labeled and tracked with a high signal-to-noise ratio using TIRF or HILO microscopy. A strong advantage of these techniques is that many DNA molecules can be imaged simultaneously. A disadvantage, however, is low spatial resolution (~300 nm) and the inability to control the force on the DNA with a knob, such as in MT or OT.

Early experiments with single-end tethered DNA molecules in OTs provided direct confirmations of DNA condensation by ParB proteins (Graham et al. 2014). These data showed the intriguing propensity of ParB proteins to progressively condense DNA molecules across a spectrum of chromosomal (*B. subtilis*, *S. pneumoniae*, *P. aeruginosa*, and *V. cholerae*) and plasmid ParB proteins (Graham et al. 2014) (Fig. 4B and C). Interestingly, the condensation occurred only from the free DNA end, where the stretching force approximated zero (or very low values), rather than continuously over the DNA molecule. This raised hypotheses that ParB proteins condense the DNA by stabilizing large DNA loops since the regions stretched at higher forces, close to the tether point, were condensed last. Subsequently, the same assay was used for detailed screening of the amino-acid residues involved in bridging interactions and condensation (Song et al. 2017). Many residues present in the NTD of ParB from *B. subtilis* were shown to be crucial for DNA condensation as when mutated (R39A, H57E, L60E, R79A, R80A, R82A, and R105E), this ability was entirely lost even at very high protein concentrations (300 nM). The lack of *in vitro* condensation was consistent with *in vivo* data that showed a complete absence of a fluorescent focus or diffuse protein signal in most cases (Song et al. 2017). While these results were obtained before discovering a CTP requirement for the clamping mode of DNA loading by ParB proteins (Osorio-Valeriano et al. 2019, Soh et al. 2019), they were helpful to identify residues that may play a role in bridging interactions in the posthydrolysis open protein state. Following the discovery of the CTP hydrolase activity of ParB and increased interest in single-molecule studies, the same DNA curtains assay was used to test the effects of common protein tags on the ParB function by screening a high number of conditions and mutants (Molina et al. 2023).

A different type of DNA stretching assay features single DNA molecules that are tethered to the surface at both ends (Ganji et al. 2016) (Fig. 4D). This assay can use torsionally constrained DNA molecules that contain supercoiling within the molecule, thus allowing the study of ParB proteins on supercoiled DNA, although most experiments are done on torsionally relaxed (i.e. nicked) DNA. This assay was initially used to demonstrate the ParB loading and spreading from *parS* sites in the presence of CTP molecules (Soh et al. 2019). Adding roadblocks ($EcoRI^{E111Q}$) flanking the *parS* site was shown to efficiently block the diffusion of ParB proteins and restrict the localization to within the region between the two roadblocks. A similar assay, but with a longer (42 kbp) DNA molecule, was used to show single diffusing ParB molecules and quantify the diffusion coefficient (0.06 ± 0.01 $\mu m^2/s$ for $ParB_{Bsub}$; Tišma et al. 2022), which matched the *in vivo* single particle tracking of ParB proteins within the partition complex (Guilhas et al. 2019). This assay enabled measuring the residence times of ParB molecules (~76 s) (Tišma et al. 2022). Interestingly, the residence times showed a nonexponential distribution, unlike standard protein–DNA interactions, which pointed to the hypothesis that a loaded ParB dimer needs to hydrolyze both CTP molecules before dissociating from the DNA (Tišma et al. 2022). This result is an example of the power of the single-molecule technique to reveal intricate mechanistic details of the ParB mechanism. Using direct visualization, Tišma et al. (2022) observed that ParB dimers loaded on *parS* can additionally recruit new ParB dimers to a genomic location distant from *parS* by *in-trans* recruitment (Fig. 4E), which was an unexpected finding that went beyond the common wisdom that ParB only loads at the *parS* site. This allowed ParB proteins to efficiently spread on the linear DNA molecules even in the presence of firmly bound DNA roadblocks, since, at low forces, spatial fluctuations in the DNA allow ParB to recruit new ParB at a faraway genomic location beyond the roadblock.

The double-tethered DNA molecules also provided a robust framework for probing the DNA condensation process (Fig. 4F). Weakly stretched molecules allowed observation of the high dynamics of DNA condensation whereby the ParB–DNA cluster showed continuous fluctuations in the amount of DNA contained within it (Tišma et al. 2023). These data also allowed quantification of the stalling force of the growing ParB-DNA cluster (0.2 pN) (Tišma et al. 2023).

Summing up, single-molecule stretching assays have emerged as a powerful tool for visualizing the behavior of a single (or low number of) ParB protein(s) on a single DNA molecule. It allows observing behaviors that cannot be measured *in vivo* or in bulk biochemical assays. Looking forward, these assays can for example be used for a variety of ParB-related proteins on DNA, or to study the interaction of ParB with supercoiled DNA, which is highly abundant in *in vivo* settings both near the chromosome origin and plasmid DNA.

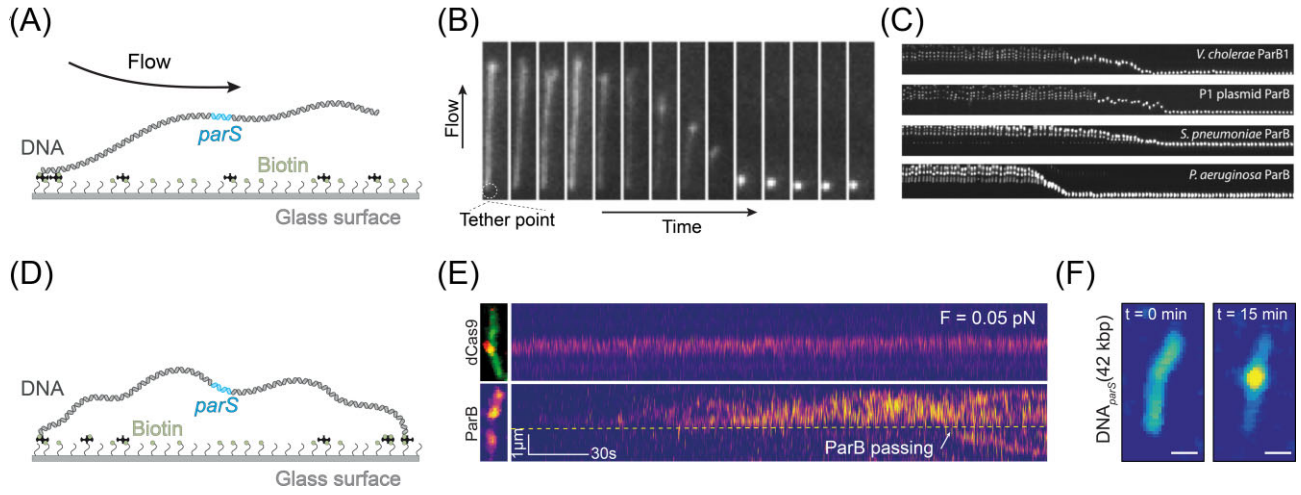

Figure 4. Single-molecule stretching assays for real-time observation of DNA condensation by ParB. (A) DNA stretching assay using single-tethered molecules with high flow. (B) and (C) This assay was used for initial real-time imaging of DNA condensation in multiple ParB variants. Adapted from Graham et al. (2014). (D) DNA stretching assay using double-tethered molecules without flow. (E) This assay was used to show ParB-ParB recruitment over a DNA-roadblock in real-time (Tišma et al. 2022) and (F) DNA condensation in the presence of CTP (Tišma et al. 2023).

Atomic force microscopy: ParB-ParB bridging

Atomic force microscopy (AFM) is a single-molecule technique that offers the highest spatial resolution for studying ParB-DNA interactions. The method deploys a cantilever with a pointed tip to scan the surface where the tip deflections are translated into a 3D image of the surface at a nanometer resolution (Fig. 5A). In addition to nanometer resolution, AFM allows studying ParB and DNA interactions without additional labeling of the molecules. AFM can either capture static images of the molecules on the surface (dry AFM) or dynamic behavior in liquid where the molecules are weakly attached to the surface (high-speed AFM-hsAFM). Interpreting AFM images demands caution due to the potential impact of surface interactions on the process of DNA binding by ParB proteins. While hsAFM, with its swiftly scanning tip, can compile high-resolution images, the tip interactions with the molecules of interest can also influence the dynamics. Finally, in the case of dry AFM, deposited molecules can adopt artificial conformations during surface attachment and sample drying.

In exploring the structure of the ParB:DNA condensate, Balaguer et al. (2021) used AFM to show that the *parS* site and CTP significantly increased ParB binding to the DNA (Fig. 5B). ParB-DNA structures showed condensed conformations with a high density of proteins around the plasmid DNA (Balaguer et al. 2021). This was confirmed in the subsequent study (Tišma et al. 2023) and extended to study the initiation of the ParB–DNA condensate formation using hsAFM. ParB loading and bridging dynamics were imaged at high spatial and temporal resolution. It was shown that ParB proteins initiate complex formation by forming transient loops which persist for ∼50 s, slightly less than the proteins' residence time on the DNA (Tišma et al. 2022) (Fig. 5C).

Further insight into the early and late stages of ParB:DNA condensate formation would benefit from using high-resolution techniques. HsAFM has the spatial and temporal resolving power for studying highly dynamic DNA–protein interactions, capturing transient and short-lived states that conventional techniques might miss. This is especially important when investigating processes like DNA binding, looping, and condensation mediated by ParB. Observing the condensation process in its entirety, from ParB loading to fully condensed ParB–DNA cluster could further shed light on the mechanics of this process.

Cargo transport assay: ParB-ParA interactions

While the previously described techniques offered great progress in studying ParB proteins, the ParABS system in its entirety has been less explored. However, visualization of all the components of the system has been done using a "cargo transport assay" (Hwang et al. 2013, Vecchiarelli et al. 2013, 2014b). Here, a standard TIRF setup images a glass surface that is covered by a "DNA carpet," where, in the presence of ATP, ParA proteins efficiently bind nonspecifically to the DNA-carpet (Fig. 6A) (Hwang et al. 2013). This ParA-DNA surface can now be used as a mimic of the bacterial nucleoid to study the interaction of ParB–plasmid complexes (Hwang et al. 2013, Vecchiarelli et al. 2013). A caveat of such systems is that the plasmids or beads will only briefly interact with the surface before diffusing away. Also, when investigating ParB-plasmid complexes, inadvertently large plasmid clusters form, which are dissimilar to a controlled single molecule setup or *in vivo* scenario. In an attempt to circumvent these issues, magnetic beads were used which could be attracted to the surface for a long time at very low force (∼5 fN) and do not show large aggregates seen when using a plasmid substrate (Vecchiarelli et al. 2014b).

This assay was initially used to test the interactions between plasmid ParA and ParB proteins (from P1 and F-plasmid) (Hwang et al. 2013). The dynamics of these proteins were obtained using fluorescent recovery after photobleaching, whereby ParA proteins were shown to dynamically exchange on the DNA much faster than their ATP hydrolysis rate (hydrolysis rate - $r_{ATP} = 0.2$ min^{-1}, dissociation rate - $r_{SopA} = 8$ min^{-1}; Hwang et al. 2013). Upon addition of ParB proteins DNA-bound ParA-ATP was stabilized from dissociating from the DNA, but its ATP hydrolysis rate increased. This resulted in the formation of a ParA-depletion zone in the local vicinity of the ParB-plasmid complex. This depletion zone caused a persistent and directional movement of both ParB-plasmid complexes (Fig. 6B) (Hwang et al. 2013, Vecchiarelli et al. 2013) and ParB-covered beads (Fig. 6C and D) (Vecchiarelli et al. 2014a). In some cases, large plasmid complexes would split and directionally move away from each other, akin to plasmid segregation in cells (Hwang et al. 2013). Measurements of the directed superdiffusive behavior of particles (Vecchiarelli et al. 2014b), and further insights from *in silico* simulations of the same system (Hu et al.

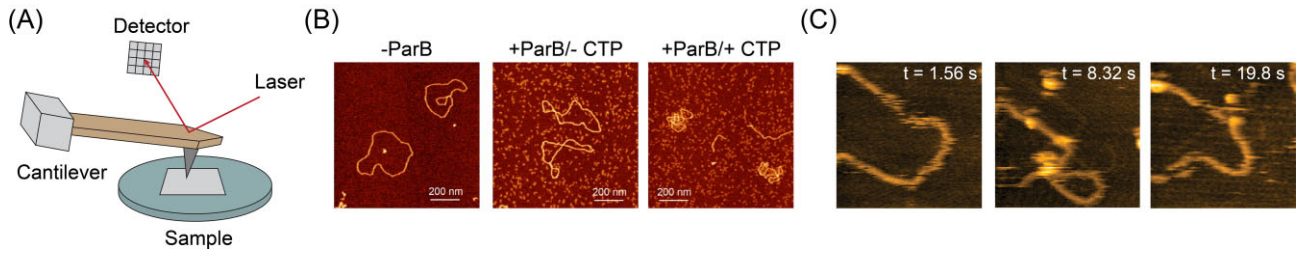

Figure 5. AFM allows high-resolution imaging of ParB–DNA complexes. (A) AFM setup. (B) AFM was used to obtain high-resolution images of the ParB–DNA complex in the presence of the *parS* site and CTP nucleotide. Adapted from Balaguer et al. (2021). (C) High-speed liquid-cell AFM was used for real-time visualization of the transient loop formation by DNA-bound ParB proteins (Tišma et al. 2023).

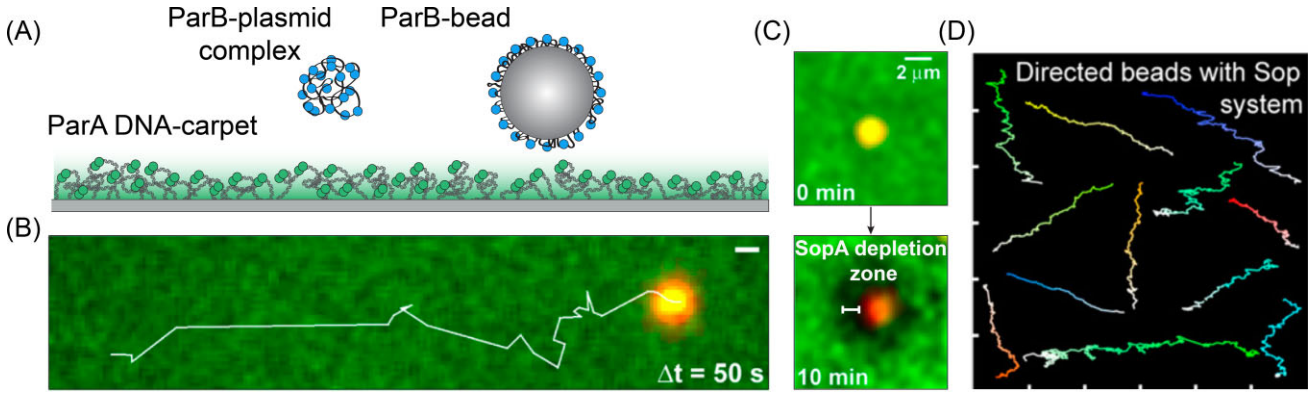

Figure 6. Cargo transport assay achieves studying of all components of ParABS system in real-time. (A) Schematic representation of cargo transport assay. The surface is covered with DNA molecules, which are bound by ParA-ATP proteins. Condensed plasmids containing a *parS* site, or magnetic beads carrying *parS*-DNA are covered with ParB proteins and observed via fluorescent TIRF microscopy. (B) ParB-plasmid complex (from P1 plasmid) directionally moving on the ParA-DNA carpet. Adapted from Vecchiarelli et al. (2013). (C) ParB-covered magnetic bead held at the surface via weak magnetic force (~5 fN), creates a ParA depletion zone around it. Adapted from Vecchiarelli et al. (2014a). (D) Multiple trajectories of the ParB-covered beads in the presence of ParA on the DNA carpet. ParA induces directional movement of the ParB-covered beads. Adapted from Vecchiarelli et al. (2014b).

2015) pointed to a general mechanism of the segregation of ParB-DNA complex based on a diffusion-ratchet model.

The cargo transport assay provided an important step toward a single-molecule investigation of the complete ParABS system. While dating back to the days before the CTPase activity of ParB proteins was discovered, these cargo transport experiments provided important insight into the mechanisms of plasmid segregation (Hu et al. 2017) and a baseline for further investigations on chromosomal segregation systems using a slightly modified diffusion-ratchet model (Lim et al. 2014). Looking forward, this system could be applied to study the dynamics of chromosomal ParA and ParB proteins, in the presence of both essential nucleotides (ATP/CTP), as well as to explore differences between different ParABS systems (as initial studies already showed differences between P1- and F-plasmid) (Hwang et al. 2013, Vecchiarelli et al. 2013).

Future perspectives: ParB interaction partners, ParB variants, and ParB-like proteins

While our understanding of the mechanism of ParB has deepened, it represents just a piece of the larger puzzle. Its function and interactions within the broader ParABS system, especially with proteins like ParA, still faces questions. Despite its significant role in chromosome or plasmid segregation, the ParA protein remains relatively underexplored in single-molecule experiments (Vecchiarelli et al. 2013). Understanding how ParA protein coats the DNA and promotes the directional movement of ParB-CTP-*parS* will aid in creating a comprehensive picture of the individual factors in the ParABS system. The interaction between a CTPase ParB and the ATPase ParA may also change the respective dynamics in the presence of both actors (Taylor et al. 2021). More single-molecule experiments with both ParA and ParB could bring us closer to resolving the ParABS system. Furthermore, ParA proteins can transport cargo different from the ParB-*parS* complex using a diffusion ratchet mechanism (Pulianmackal et al. 2023). Therefore, studying chromosomal ParA in single-molecule studies will open up a range of possibilities that can be applied to different systems.

A second prominent interaction partner of ParB is the SMC protein complex (Gruber and Errington 2009, Sullivan et al. 2009, Bock et al. 2022), which is essential in organizing the bacterial chromosome in a distinct overall shape where both chromosomal arms are juxtaposed (Le et al. 2013, Wang et al. 2015, 2017, Tran et al. 2017). ParB proteins seem to be the sole recruiter of SMC proteins near the origin of replication, presumably at *parS* (Bock et al. 2022). In fact, Antar et al. (2021) reported that ParB mutants ($ParB^{E78Q}$/$ParB^{E111Q}$ in *B. subtilis*), which are rendered nonfunctional for partition complex formation in cells, can still efficiently recruit SMC proteins to the origin of replication. How this interaction occurs at a single-molecule level remains entirely unclear. While *B. subtilis* SMC (at very high concentrations) was shown to condense the DNA in a DNA curtains assay (Kim and Loparo 2016), no single-molecule work has explored ParB and SMC in tandem, despite their known vital interaction within cells. The single-molecule stretching assay covered in this

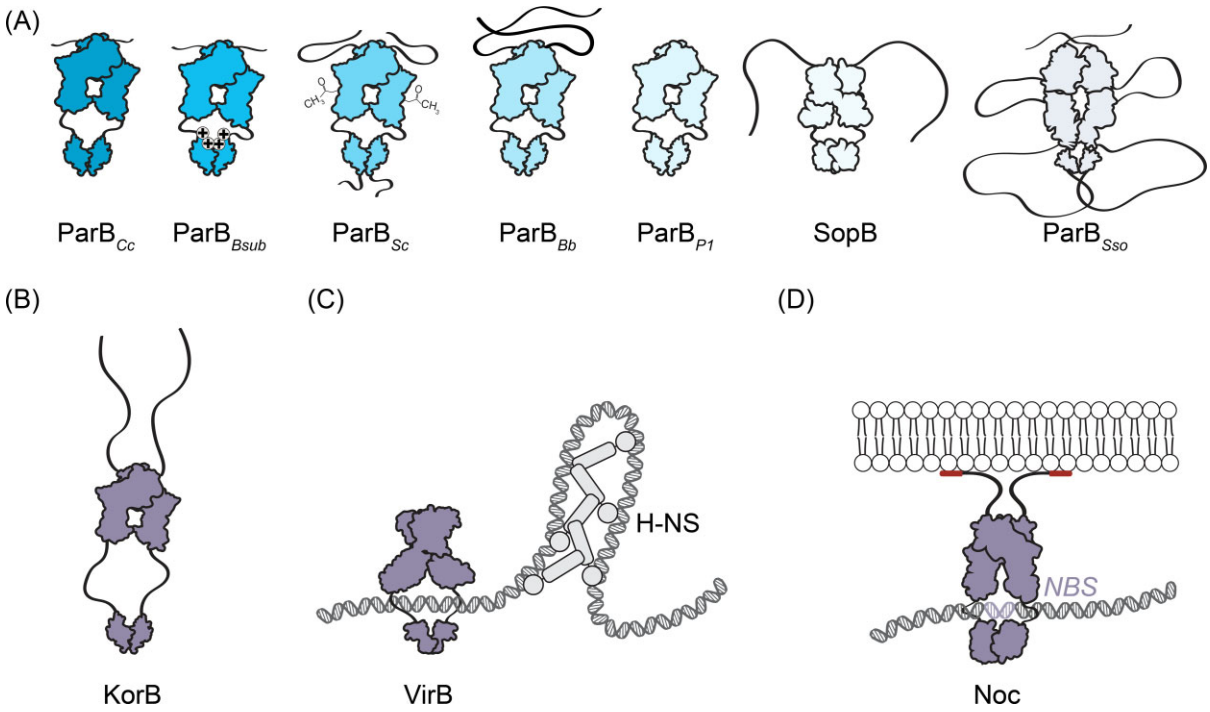

Figure 7. Schematic representations of various ParB and ParB-like proteins. (A) From left to right (Cc, *Caulobacter crescentus*; Bsub, *Bacillus subtilis*; Sc, *Streptomyces coelicolor*; Bb, *Bdellovibrio bacteriovorus*; P1, plasmid P1; SopB, plasmid F; and Sso, *Sulfolobus solfataricus*). ParB$_{Cc}$ lacks the positively charged lysines at its C-terminus in contrast to ParB$_{Bsub}$, which is important for nonspecific DNA contact during spreading and bridging (Fisher et al. 2017). ParB$_{Sc}$ is characterized by HTH-domain acetylation that impacts its parS binding (Li et al. 2020). ParB$_{Bb}$ binds parS in a growth-phase-dependent manner (Kaljević et al. 2023). ParB$_{P1}$ and SopB (UniProt: P62558) are plasmid-borne and show potential differences in interaction with ParA (Vecchiarelli et al. 2013), which could be compared to chromosomal ParB proteins. ParB$_{Sso}$ originating from archaea (Schumacher et al. 2015), showcases long disordered domains between the NTD, HTH, and CTD domains (UniProt: O93707). (B) ParB-like proteins that harbor unique roles beyond chromosome or plasmid segregation. From left to right: KorB, which functions as a transcriptional regulator for plasmid genes (Thomas and Hussain, 1984, Kornacki et al. 1990) (UniProt: P07674). Noc binds CTP and DNA but also to the cell membrane, orchestrating the cell division process (Jalal et al. 2021a) (UniProt: P37524). VirB is a ParB-like protein with CTP binding, that modulates the transcription of virulence genes in Shigella (Antar and Gruber 2023, Gerson et al. 2023, Jakob et al. 2023) (UniProt: P0A247). All schematic representations are based on AlphaFold2 predictions of the dimeric state (Jumper et al. 2021, Mirdita et al. 2021).

review poses an ideal platform (Ganji et al. 2018, Davidson et al. 2019, Pradhan et al. 2023) for such a study of DNA condensation (Ryu et al. 2021) by SMCs. However, so far, *in vitro* reconstitution of loop extrusion by bacterial SMCs has not been successful.

In many organisms, chromosomal ParB proteins interact with species-specific protein factors such as DivIVA (Perry and Edwards 2006), MipZ (Thanbichler and Shapiro 2006), PopZ (Bowman et al. 2008, Ebersbach et al. 2008), FtsZ (Donovan et al. 2010), and many others - signaling the prominent role of ParB proteins. These interactions can significantly change the ParB behavior (binding, spreading, DNA condensation, and dynamics). These interactions remain largely unexplored in single-molecule studies, presenting an intriguing avenue for future research.

ParB proteins manifest subtle species-specific variations shaped by evolutionary pressures and specific requirements of their host (Fig. 7A). Current single-molecule studies have so far explored ParB proteins from a limited number of species, i.e. mostly *B. subtilis* and *Caulobacter crescentus*. Expanding single-molecule investigations to ParABS systems from other organisms may provide a comprehensive picture of the spectrum of ParB behaviors (Jalal et al. 2020a, Kaljević et al. 2021, 2023) and interactions (Kawalek et al. 2020).

Finally, there is also a broad range of ParB-like proteins. These are related in structure (often exhibiting CTPase activity; Osorio-Valeriano et al. 2019, Jalal et al. 2021a, Antar and Gruber 2023), but vary significantly in their functional roles within the cell (Fig. 7B). An intriguing example is the KorB protein found in the RK2 plasmid of *Pseudomonas*, which maintains a partitioning role but also modulates transcriptional regulation with its interaction partner, KorA (Kolatka et al. 2010). A single-molecule study on the KorAB system, their binding, potential spreading, and dynamics, would provide a powerful complement to current *in vivo* or biochemical studies. Other interesting ParB-like candidates are VirB (Antar and Gruber 2023, Gerson et al. 2023, Jakob et al. 2023), a key transcriptional factor of *Shigella* virulence genes, and Noc (Nucleoid occlusion protein; Jalal et al. 2021b) from *Firmicutes*, which is involved in cell division and interacts with the cell membrane.

Concluding remarks

Single-molecule techniques have illuminated mechanistic intricacies of ParB proteins, revealing nuanced details of their role in initiating and maintaining the formation of the partition complex. Single-molecule techniques have equipped us with the capacity to measure ParB binding to *parS* quantitatively (Balaguer et al. 2021, Tišma et al. 2022), observe real-time DNA condensation (Graham et al. 2014, Song et al. 2017, Tišma et al. 2023), and even ParA-ParB interactions (Vecchiarelli et al. 2014a), thus enlightening our understanding of the assembly of the partition complex. Further, new phenomena such as ParB-ParB recruitment (Tišma et al. 2022) and ParB interaction with an RNA polymerase (Tišma et al. 2023) could be resolved on a single protein level. Moving forward, a ma-

jor frontier lies in reconciling *in vitro* data with *in vivo* contexts, to ensure that mechanistic insights garnered from single-molecule experiments are validated within the multifaceted environment of the living cell. Expanding single-molecule techniques to other ParB and ParB-like proteins will not only provide insights into their mechanism but also address foundational questions: How are the dynamics affected in the presence of different interaction partners? How do evolutionary differences affect the binding, spreading, or DNA condensation by ParB/ParB-like proteins? What are common behaviors of ParB proteins?

Expanding the pool of single-molecule techniques to study the dynamic behavior of ParB and ParB-like proteins can further add to our understanding of these systems. Fluorescence Correlation Spectroscopy and Förster Resonance Energy Transfer studies could further inform us on the precise dynamics of two monomers assembling into a typical "open" dimer state, as well as the detailed dynamics of clamp closure, which is essential in all ParB proteins to date (Jalal et al. 2020a, Antar et al. 2021, Osorio-Valeriano et al. 2021). TIRF microscopy, in combination with surface-bound Noc proteins (akin to microtubule motility assays; Howard et al. 1989), could be useful for studying the dynamics of Noc binding to the chromosomal DNA.

Finally, in addition to experimental work, *in silico* studies have greatly contributed to the insights into the ParB mechanism of action on a single molecule level (Tišma et al. 2022, Connolley et al. 2023) and within the cellular context (Broedersz et al. 2014, Walter et al. 2020, 2021, Osorio-Valeriano et al. 2021, Connolley et al. 2023). A holistic approach, starting from detailed insights from single-molecule studies and testing these insights in an *in vivo* context, is essential for a robust and complete understanding of molecular mechanisms like that of ParB proteins in their natural cellular environments. By unraveling the complexities and subtle variations of ParB proteins across different organisms, we further inch closer to fully decoding the mechanisms that underpin bacterial chromosome segregation.

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